Nervous necrosis virus (NNV) in farmed Norwegian fish species

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ACKNOWLEDGEMENTS

LIST OF PAPERS

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

Paper I   **Korsnes**, **K**, Devold, M, Nerland, AH, Nylund, A. 2005. Viral encephalopathy and retinopathy (VER) in Atlantic salmon *Salmo salar* after intraperitoneal challenge with a nodavirus from Atlantic halibut *Hippoglossus hippoglossus*. Diseases of Aquatic Organisms 68:7-15.

Paper II   **Korsnes**, **K**, Karlsbakk, E, Devold, M, Nerland, AH, Nylund, A. (in press) Tissue tropism of nervous necrosis virus NNV in Atlantic cod *Gadus morhua* after intraperitoneal challenge with a virus isolate from diseased Atlantic halibut *Hippoglossus hippoglossus*. Journal of Fish Diseases.

Paper III   **Korsnes**, **K**, Karlsbakk, E, Nylund, A, Nerland, AH. A study of experimental horizontal transmission of nervous necrosis virus NNV to Atlantic salmon *Salmo salar* and Atlantic cod *Gadus morhua* by cohabitation challenge. Manuscript.

Paper IV   Patel, S*, **Korsnes, K***, Bergh, Ø, Vik-Mo, F, Pedersen, J, Nerland, AH. 2007. Nodavirus in farmed Atlantic cod *Gadus morhua* in Norway. Diseases of Aquatic Organisms 77:169-173.

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DEFINITIONS

In the study of transmission of pathogens, a number of different terms are needed to describe the pathogen itself, the infection status of the host and the ways of transmission. Some of the key terms used may need a definition, and this thesis define the terms as:

Isolate: A nervous necrosis virus (NNV) isolate is a virus isolated from one particular source, usually a host like a fish species. I.e. virus originating from an Atlantic cod would be named a cod isolate.

Strain: A number of NNV isolates forming a distinct subgroup sharing genetic characteristics within a clade or genotype that differentiates them from other strains. Strains may consist of isolates from different fish species. The strains are normally based on phylogenetic analyses.

Genotype: NNVs are, at the moment, phylogenetically grouped into 4 distinct clades, and these define the genotypes. Each genotype may consist of several different strains and isolates, which all are closely phylogenetically related.

Persistent infection: A long-lasting infection where the pathogen is not cleared and may replicate and cause clinical signs. This state may be subdivided into latent, chronic or slow infection.

Latent infection: Presence of the pathogen in the host, but with little or no replication of the pathogen and no clinical signs of disease. Disease may develop at a later stage, if the rate of replication is increased. If the host is shedding the pathogen a carrier state appear.

Chronic infection: An infection that last over a long period of time where there is small or slow changes in the development of pathology. Usually the pathogen displays a slow rate of replication and gradually more extensive pathological changes may appear.

Carrier state: A persistent infection in which the pathogen is replicating and eventually shed by the host. If there are no clinical signs, this state could also be named a *covert infection*.

Asymptomatic carrier: The pathogen is replicating without causing any clinical signs or development of disease in the host, and the host sheds the pathogen.

Horizontal transmission: Transmission of a pathogen from an infected individual to a non-infected individual, occurring as direct transmission and/or via a vector.

Vertical transmission: Transmission of a pathogen from brood stock to larvae through contaminated reproductive fluids. In females this is occurring either as transovarian or transovum transmission (pathogen within the egg or on the surface).

CONTENTS

1 INTRODUCTION

During the last four decades, the Norwegian fish farming industry has grown into a high-tech and high-value export industry. Norway is primarily producing Atlantic salmon (*Salmo salar*), and the production reached 626 000 metric tons in 2006. This represents a value of approximately 2.5 billion USD (Directorate of Fisheries, Norway). Fish farming is a growing industry in Norway, and during recent years, the volumes of other fish species such as Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) have increased. The 2006-production was 1185 metric tons of halibut and for 10384 tons of cod, which are fairly small numbers compared to salmon. However, commercial farming of these species is rather new and the growth potential is thought to be substantial. Estimated world production of gadoids in 2010 is 150 – 200 000 t, and the largest growth is predicted to take place in Norway (Kjesbu *et al.* 2006). In 2000, the Norwegian production of cod was only 169 tons, which gives an increase by more than 60 times in six years. By comparison, the production of halibut only doubled in the same period, primarily due to the complexity and lack of survival in the hatcheries. Farming of marine fish species should still be regarded to be at an early stage in Norway.

Fish farming has always involved a risk of disease and spreading of infectious pathogens. This could be exemplified by looking back at the Norwegian salmon-farming industry, where serious disease problems were faced in the mid-eighties and early nineties. Bacterial diseases such as vibriosis, cold water vibriosis, furunculosis and viral diseases such as infectious salmon anaemia (ISA), infectious pancreatic necrosis (IPN) and pancreas disease (PD) caused (and still cause) heavy losses, but also led to an increased awareness of the hazards involved in culturing fish. An industrial collapse was avoided by introduction of efficient bacterial vaccines and improved fish health management. This formed the basis for the rapid growth in salmon production that was seen during the nineties. However, disease problems in salmon-farming are not solved, as new pathogens and diseases are emerging regularly. In addition, viral vaccines have not been as efficacious as the bacterial ones, and viral disease problems are now predominant in salmon farming.

Farming of fish requires adequate growth sites, and the increased volume of produced fish has led to an increase in the number of sites and the total on-site biomass. Introduction of new fish species have also increased the number of sites and the number of potential hosts for pathogens. Fish farms are separated by distance and kept as single-species sites to avoid disease problems. However, pathogens are on the move, and their transportation or

introduction into new areas, with potential new hosts, might be important in spreading diseases. Transmission of pathogens are either occurring horizontally (between hosts), vertically (intraovulary) or as a combination of both. In some cases pathogens are transported long distances in hosts showing no clinical signs of disease. Mortensen *et al.* (2006) define three rough levels of risk involved; 1) low risk, with no long-distance movement and autonomous supply of juveniles, 2) medium risk, with regional movement and regular monitoring of most fatal diseases, and 3) high risk, with free movement and low level of control and surveillance.

With the introductions of new species in aquaculture, new pathogens and diseases have emerged. One of the pathogens causing problems in marine fish farming worldwide is nervous necrosis virus (NNV), which is also known as nodavirus, and this thesis addresses NNV as a pathogen in cold-water fish farming in Norway. The emphasis is on potential transmission of virus and possible consequences for farm operations, and the papers address host susceptibility, virus distribution in host tissue, cohabitant transmission and detection of nodavirus in the most important farmed fish species in the growing Norwegian aquaculture industry.

1.1 Nervous necrosis virus/nodavirus

There are some discrepancies regarding the naming of the virus. NNV belongs in the viral family *Nodaviridae*, but the literature refers to this virus using various names, and it names the disease in several ways as well.

1.1.1 NNV nomenclature

NNV or nodavirus is a small non-enveloped virus of 25-40 nm having a capsid of icosahedral shape (T=3) classified belonging to the *Nodaviridae* family. The name "noda" originates from the Japanese village of Nodamura, where virus particles were isolated from mosquitoes (*Culex tritaeniorhynchus*) in 1956 (Scherer and Hurlbut 1967). The new virus was named Nodamura virus (NoV), and it is regarded as the archetypal species in the family. Nodaviruses were initially only found in insects, but in 1992 Mori *et al.* isolated a virus from fish, in moribund and dead striped jack larvae (*Pseudocaranx dentex*), which was shown to belong to the *Nodaviridae* family. This particular virus was named striped jack nervous necrosis virus (SJNNV) after the fish host. As fish and insect nodaviruses revealed little sequence and protein similarity (Nishizawa *et al.* 1995), Nagai and Nishizawa (1999) suggested the existence of a distinct fish genus within the *Nodaviridae* family. These observations lead to

the division of the *Nodaviridae* in two separate genera; **alphanodavirus** that infect insects and **betanodavirus** infecting fish, with type species NoV and SJNNV respectively. The International Committee of Taxonomy of Viruses (ICTV) has accepted the subdivision in the *Nodaviridae* (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_nodav.htm). Detection of nodaviruses seems not to be restricted to insects and fish, as viruses also have been reported in crustaceans (Jiménez *et al.* 2000, Qian *et al.* 2003, Bonami *et al.* 2005, Gomez *et al.* 2006, Gomez *et al.* 2008).

Piscine nodaviruses (betanodaviruses) cause a serious disease, which has primarily been associated with juvenile stages in several marine fish species (Munday *et al.* 2002). Yoshikoshi and Inoue (1990) were among the first to report the disease. They detected it in hatchery-reared larvae and juveniles of Japanese parrotfish (*Oplegnathus fasciatus),* and due to the clinical signs, they suggested the name *viral nervous necrosis* (VNN). Office International des Epizooties (OIE) is now using both VNN and *viral encephalopathy and retinopathy* (VER) to name the disease caused by the virus (http://www.oie.int/fr/normes/fmanual/a_00024.htm). Other names for the disease have also been used, like "fish encephalitis" (Breuil *et al.* 1991) and "encephalomyelitis" (Bloch *et al.* 1991) without being accepted as formal names. In the literature the terms VNN and VER are now both used, as both reflect a disease from infection of betanodavirus, and this is somewhat confusing. As both VER and VNN, and sometimes even the combination VER/VNN, are commonly used, the OIE adopted both names to avoid mistakes. In this thesis VNN is used when referring to the *disease*.

Betanodavirus strains or genotypes are usually named after the fish species of origin, and the nomenclature applied is also not uniform. These viruses are often referred to as nervous necrosis virus (NNV), and consequently names such as BFNNV (barfin flounder nervous necrosis virus) and SJNNV (striped jack) have arisen. NNV is the term used by the ICTV to name piscine nodavirus. However, some papers in the literature abbreviate nodavirus as NV, and this has resulted in names such as AHNV (Atlantic halibut nodavirus) and TNV (turbot nodavirus). The term encephalitis virus (EV) has also been used, and is the source for abbreviations such as DIEV (*Dicentrarchus labrax* encephalitis virus) and LcEV (*Lates calcarifer*). These terms, other than NNV, name various isolates or strains of the virus. This thesis uses primarily NNV, but also nodavirus and betanodavirus, when referring to the *virus*. The phylogenetic relationship of NNV isolates, strains and genotypes is addressed in section 1.1.3.

1.1.2 NNV – genome architecture

The most extensively studied nodaviruses belong to the alpha genus and among these the Flock House Virus (FHV) and Black Beetle Virus (BBV) have been the systems of choice (Schneemann *et al.* 1998). Since both genera share the basic fundamental genetic architecture and as betanodaviruses have not been widely characterized, alphanodaviruses have often been used as models. However, there are significant structural differences, and these differences are the basis of the division into two separate genera (Nishizawa *et al.* 1995, Nagai and Nishizawa 1999). An increasing number of papers address the characterization of betanodaviruses, and the relationship between the alpha and beta groups is likely to be further investigated.

The nodavirus genome is organized in a simple way, with a bisegmented positive-sense single-stranded RNA, named RNA1 and RNA2, and these molecules are encapsidated in a single virion. A subgenomic segment, RNA3, is not encapsidated in the virion. It is only formed in infected cells. A summarized outline of the genetic architecture is presented in Figure 1.

RNA1 is the largest segment (ca 3.1 kb) encoding the viral part of the RNA-dependent RNA polymerase (RdRp), named protein A. The viral RdRp is important for viral survival not only in the replication, but also in the act of making genome variability. RNA viruses usually have high rates of errors during RdRp-dependent genome replication, and this allows for rapid virus evolution under a selective pressure (Domingo 2000). In alphanodaviruses, both RNA1 and RNA2 segments carry 5' end cap structures, but they do not have poly (A) tails in their 3' ends. Their lacks of poly (A) tails result in blocking of enzymatic and chemical modification (Dasgupta *et al.* 1984, Schneeman *et al.* 1998). In betanodavirus, Delsert *et al.* (1997a) reported that the 3' end of RNA2 in DIEV is undergoing polyadenylation *in vitro*, suggesting there is a difference in the structure from the 3' end compared to alphanodavirus.

The RNA1 segment holds one major open reading frame (ORF) encoding protein A, with molecular weight of approximately 112 kDa (Kaesberg 1987). In striped jack nervous necrosis virus (SJNNV), the ORF is encoding 983 amino acids (aa) and located from nucleotide 65 to 3016. The total length of RNA1 of this virus was 3081 nucleotides (Nagai and Nishizawa 1999). In virus isolated from Atlantic halibut this ORF is located from nucleotide (nt) 79 to 3021, encoding a 981 aa polypeptide (Sommerset and Nerland, 2004). In alphanodaviruses, the ORF encodes a protein of 998 aa (Dasmahapatra *et al.* 1985, Ball 1995). By comparing SJNNV and insect nodavirus (BBV and FHV), Nagai and Nishizawa (1999) found low nucleotide (28.3 %) and amino acid (27.6 %) identities. This resulted in the
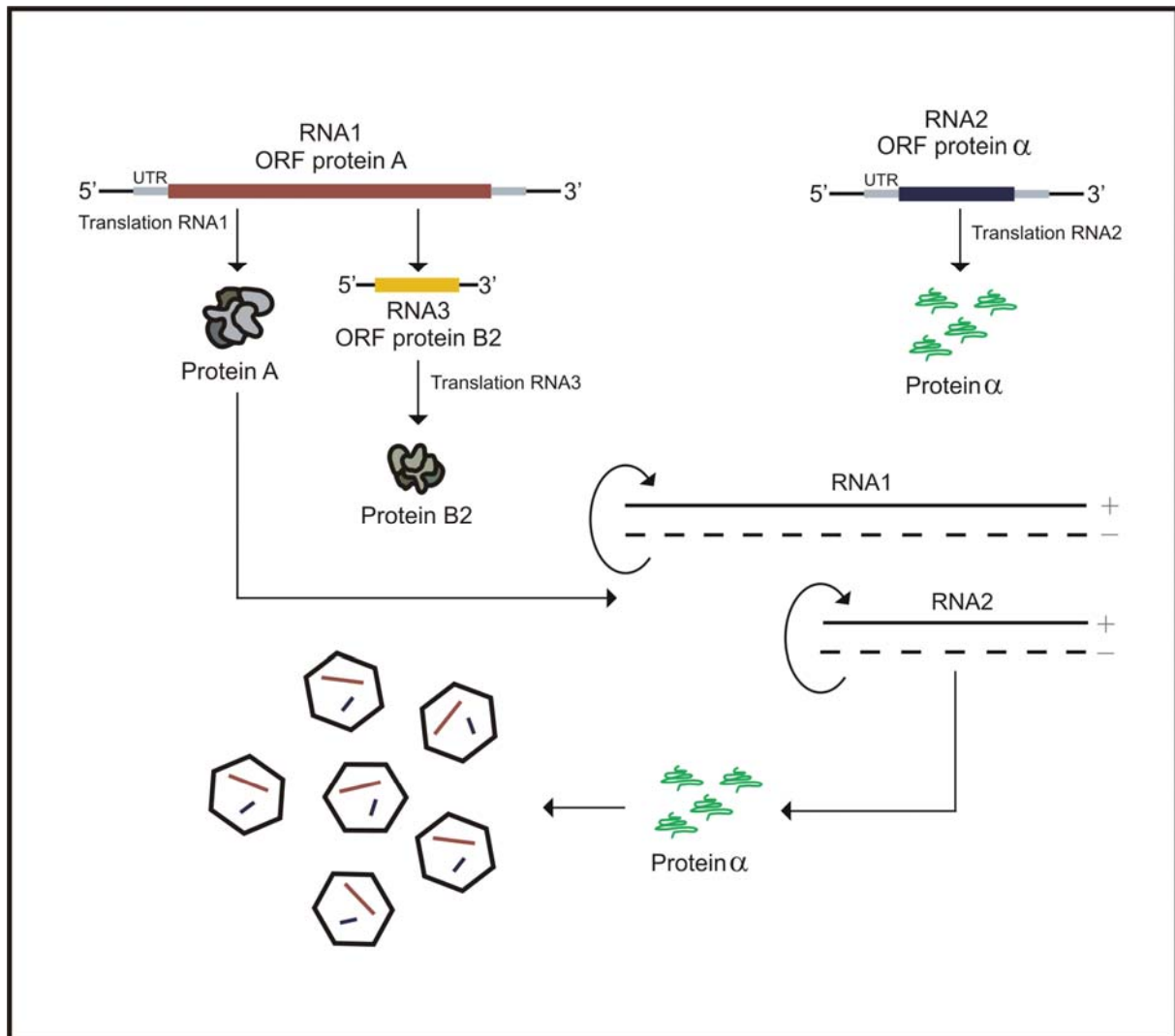
Figure 1. *Outline of replication and the NNV bisegmented genome organization of RNA1 and RNA2 with encoding proteins. RNA1 (ca 3.1 kb) encodes protein A (RdRp) within one open reading frame (ORF) with untranslated conserved regions (UTR) at both 5' and 3' ends. In addition, RNA3 (ca 0.4 kb) is formed during replication as subgenomic RNA, encoding protein B2 in betanodavirus. RNA2 (ca 1.2 kb) encodes protein a (capside protein), with one ORF and UTR at both 5' and 3' ends. See section 1.3.4 for details on replication of NNV. Figure made by Bengt Stangvik.*

suggestion of a separate betanodavirus genus consisting of virus isolates from fish hosts only – *piscinodavirus*. This finding supported the conclusion of Nishizawa *et al.* (1995). Despite these low nucleotide identities between alpha- and betanodaviruses, they share conserved RdRp motifs in the C-terminal residues of the protein (Johnson *et al.* 2001).

RNA2 of alphanodaviruses (ca 1.4 kb) encodes protein α (44 kDa), which is the precursor of viral coat proteins β and γ (Dasgupta and Sgro 1989, Friesen and Rueckert 1981). In alphanodavirus protein α is cleaved autocatalytically into protein β and γ (Gallagher and Rueckert 1988). In betanodaviruses there are little support for the existence of protein γ, and

this suggests that there is a different processing of capsid proteins than in alphanodaviruses (Delsert *et al.* 1997a, Lin *et al.* 2001). In betanodavirus, the number of amino acids in the coat protein ranges from 338-340, with a molecular weight of 39 kDa (Nishizawa *et al.* 1995, Grotmol *et al.* 2000, Tan *et al.* 2001, Guo *et al.* 2003a, Delsert *et al.* 1997a, Chi *et al.* 2001).

The RNA2 segment contains one large ORF, with untranslated regions (UTR) in both 5' and 3' ends. Such UTRs seem to have a function, and in FHV the 3'-end of RNA2 can be folded into a secondary structure containing two hairpin loops (Kaesberg *et al.* 1990). This folding pattern it thought to act as a recognition signal for protein A, and replication is initiated by binding to 3'UTR. Primary and secondary structures of 3'UTR of RNA2 in alphanodaviruses are conserved and might be important for both replication and template recognition by RdRp (Kaesberg *et al.* 1990).

Marshall and Schneemann (2001) have studied properties of the coat protein, including the N-terminal residues of the coat protein of FHV and the effect on packaging of RNA2 into the virion. They demonstrated the importance of N-terminal residues 2-31 in FHV coat protein in formation and packaging of RNA2. Mutants lacking these residues had virions containing little RNA2 but with no effect on packaging of RNA1. In conclusion, these authors suggested the coat protein is containing important determinants for recognition and packaging of RNA2, that the encapsidation of the two genomic RNAs occurs independently and that the coat protein uses different regions for the recognition of RNA1 and RNA2.

Protein α, the precursor of the coat protein, has also been suggested to act as an apoptosis inducer (Guo *et al.* 2003b). These authors used greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses (GGNNV) to study apoptotic activity in culture cells, and such activity was shown to be related to increased activity of caspase-8-like and caspase-3-like proteases in GGNNV infected cells. This study concluded that apoptosis was induced, and that fish caspases are important elements in GGNNV-mediated apoptosis. Guo *et al.* (2003a) have also reported existence of a nucleolus localization signal, aa sequence $_{23}$RRRANNRRR$_{31}$, in the N-terminal region of the coat protein from GGNNV. By fusing protein α with a green fluorescent protein (GFP) from jellyfish, accumulation was observed in both nucleolus and cytoplasm of host SB cells infected with GGNNV. Interestingly, when the nucleolus localization signal was deleted, apoptosis still occurred in transfected SB and Cos-7 cells indicating that the cytoplasm localization might be involved in inducing apoptosis. Chi *et al.* (2001) have reported that the grouper nervous necrosis virus coat protein is a glycoprotein, and is undergoing post-translational gylcosylation. The significance of this protein property, particularly in virus-host immune interactions, has not been studied further.

Host specificity has been reported linked to RNA2 in betanodavirus by Iwamoto *et al.* (2004), and these authors studied this by using reverse-genetics. Sevenband grouper (SG) and striped jack (SJ) larvae were challenged with reassortant viruses. One reassortant contained SJNNV RNA1 and SGNNV RNA2, the other SGNNV RNA1 and SJNNV RNA2. The RNA2 determined development of disease, as only reassortant with SJNNV RNA2 killed SJ and SGNNV RNA2 killed SG (Iwamoto *et al.* 2004). Existence of host specificity is addressed in section 1.3.1.

Partial sequences for betanodavirus RNA2, and corresponding coat protein sequences, have been determined for large numbers of viruses isolated from different fish hosts (Nishizawa *et al.* 1995, Delsert *et al.* 1997a, Aspehaug *et al.* 1999, Tan *et al.* 2001, Lin *et al.* 2001, Grotmol *et al.* 2000, Nishizawa *et al.* 1997, Skliris *et al.* 2001, Dalla Valle *et al.* 2001, Hedge *et al.* 2002, Chi *et al.* 2003, Johansen *et al.* 2004b). RNA2 has been used as a target gene for phylogenetic analyses, and this is discussed in detail below. Nodaviruses have a conserved coat protein sequence close to the N-terminus (Thiéry *et al.* 2004). However, betanodavirus and alphanodavirus display low nucleotide identities between the RNA2 gene fragments (Nishizawa *et al.* 1995).

RNA3 is formed during replication, as a subgenomic RNA of approximately 400 nt, and is transcribed from the 3'end of RNA1 of black beetle virus (Friesen and Rueckert 1982, Guarino *et al.* 1984). In most alphanodaviruses the RNA3 segment contains two ORFs encoding protein B1 and B2 (Schneemann *et al.* 1998). In betanodavirus, Sommerset and Nerland (2004) reported that Atlantic halibut nodavirus (AHNV) subgenomic RNA3 only encode the B2 protein, which was shown to accumulate in large amounts soon after infection. These authors also made alignments of AHNV RNA3 and alphanodavirus RNA3, which showed only marginal nucleotide identities. A study of greasy grouper nervous necrosis virus (GGNNV) suggested existence of both B1 and B2 protein domains at RNA3 (Tan *et al.* 2001). The functions of these proteins have been unknown, but a study of GGNNV by Fenner *et al.* (2006) suggested the B2 protein to antagonize RNA interference. The B2 protein accumulates in the nucleus of the host cell during the late stages of infection and facilitates intracellular viral RNA accumulation. This suppresses cellular RNA interference, which is known to be an antiviral infection defense mechanism (Cullen 2002). Such a function is also known for the B2 protein in *Nodamura virus* (NoV), which infects both insects and rodents (Johnson *et al.* 2004, Sullivan and Ganem 2005), and FHV (Li *et al.* 2002). Iwamoto *et al.* (2005) studied SJNNV RNA3 and protein B2, and showed that the B2 protein was present in the central nervous systems and retinas of infected larvae as well in the cytoplasm of infected

cultured E-11 cells. They also suggested that the RNA3 is synthesized via a premature termination model, and that the SJNNV protein B2 has a potent RNA silencing-suppression activity. In general, the protein B2 encoded by RNA3 seems to be involved in suppression of host cell defense mechanisms and to play a role in the viral infection cycle. The function of protein B1 is unknown, and even its existence might be questionable. However, roles in viral replication (Ball 1995, Harper 1994) or in determining the host range or cytopathology of infected cells (Schneemann *et al.* 1998) have been suggested.

1.1.3 NNV phylogeny

NNV have been characterized into several different groups (Figure 2) based on the phylogenetic relationship. Most of these phylogenetic analyses are based on the partial nucleotide sequences of the RNA2 segment. Initially, Nishizawa *et al.* (1997) suggested the existence of four clusters or genotypes, represented by striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV). These four groups comprise all known betanodaviruses. Although this analysis was based primarily on Japanese virus isolates, the four main clusters have been consistent and supported by other studies (Skliris *et al.* 2001, Chi *et al.* 2003, Dalla Valle *et al.* 2001, Thiéry *et al.* 2004).

Addressing the BFNNV clade, this group shows one particular characteristic; all virus isolates originate from cold-water fish hosts. As the BFNNV group is clustering as a sister group of RGNNV (Nishizawa *et al.* 1997), this may suggest a common ancestry. The BFNNV genotype is named after the barfin flounder (*Verasper moseri*), which is a large flatfish species living primarily in the cold sea basins of the east coast of Hokkaido, Japan (Ando *et al.* 1999). The barfin flounder is farmed commercially, and is regarded as an important aquaculture species in the Northern part of Japan (Mori *et al.* 2006). Several virus isolates from cold-water fish hosts have been reported to cluster within this group. Aspehaug *et al.* (1999) reported that a virus isolate from Atlantic halibut (*Hippoglossus hippoglossus*) showed high nucleotide identity at the RNA2 segment with barfin flounder NNV. Grotmol *et al.* (2000) have also confirmed this finding, and other studies have shown that cold-water NNV isolates from Atlantic cod, winter flounder (*Pseudopleuronectes americanus*) and haddock (*Melanogrammus aeglefinus*) cluster into the BFNNV clade (Johnson *et al.* 2002, Gagné *et al.* 2004, Nylund *et al.* 2008). A new genotype, turbot nodavirus (TNV), has been suggested by Johansen *et al.* (2004b), who isolated a nodavirus from farmed turbot (*Scophthalmus*
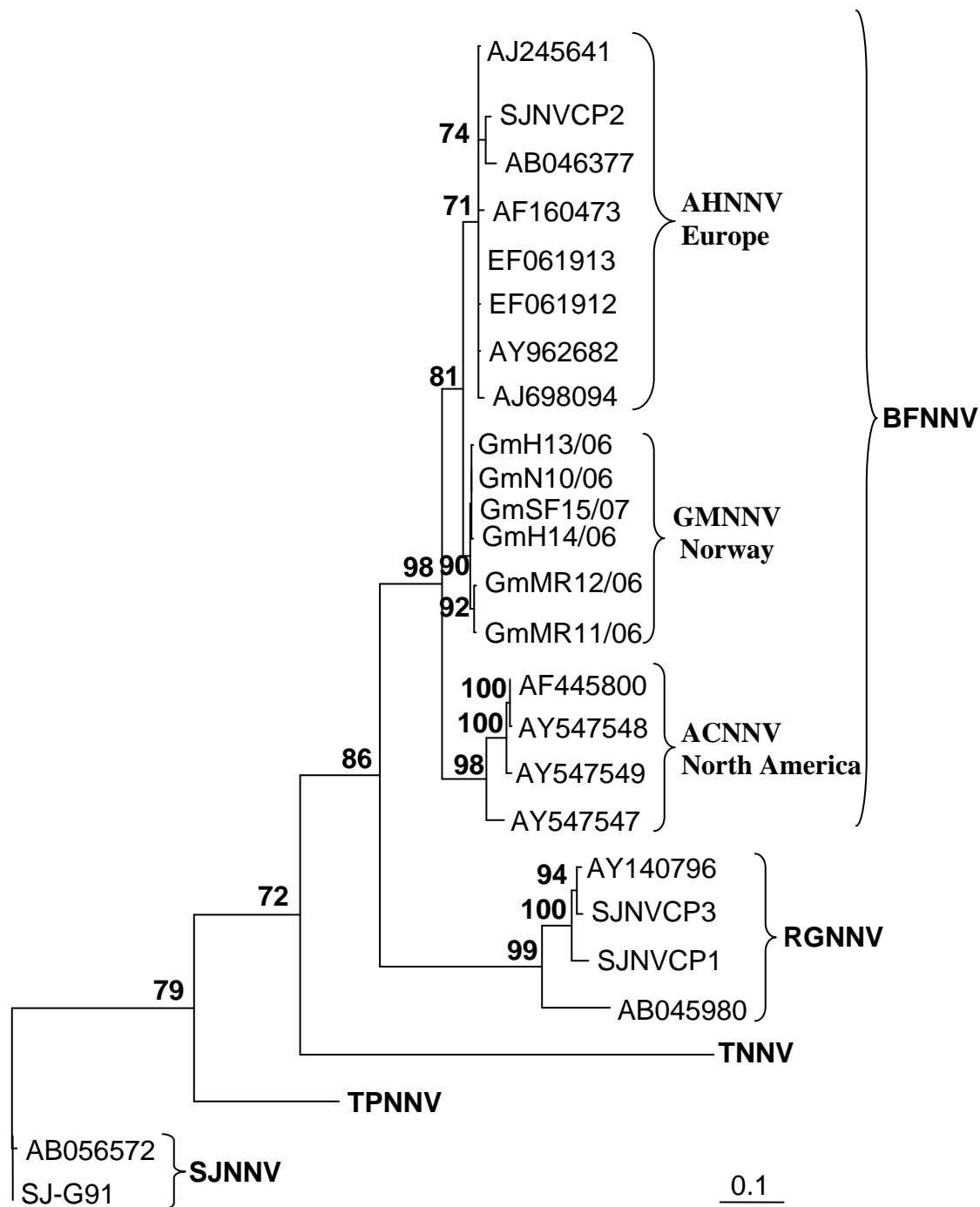
Figure 2. *Phylogenetic position of selected NNV isolates. Phylogram is based on 377 nucleotides from RNA2 segment and applied maximum-likelihood criteria in TREE-PUZZLE (quartet-puzzling steps) to calculate phylogenetic distances. Major clades are BFNNV = Barfin flounder NNV, RGNNV = Red-spotted grouper NNV, TNNV = Turbot NNV, TPNNV = Tiger puffer NNV, SJNNV = Striped jack NNV. Sub-clades within BFNNV are AHNNV = Atlantic halibut NNV, GMNNV =* Gadus morhua *(Atlantic cod) nervous necrosis virus, ACNNV = Atlantic cod NNV. The scale bar shows the number of substitutions as a proportion of branch lengths. The numbers at the nodes are quarter-puzzle support values. Figure extracted from Nylund et al. (2008).*

maximus) in Norway. However, the existence of this genotype has not been verified by other studies, and currently it consists of only one virus isolate.

The grouping of NNV into different genotypes has raised questions of possible host specificity or preference and if/how the virus is transmitted among populations of fish. This is vital information, which will have implications for the aquaculture industry and how introduction of the virus into new areas or hosts should be avoided or addressed. The virus-host interactions, including host specificity and transmission pathways, are discussed in section 1.3, after a description of the disease caused by the virus.

1.2 Viral nervous necrosis (VNN)

1.2.1 Clinical signs and gross pathology

Pathogenesis and clinical signs of VNN are related to the neuroinvasive nature of the virus and the subsequent effect on tissues such as brain and retina. In general, clinical signs of disease are erratic swimming patterns, like looping or spiral swimming, reduced co-ordination and changes in pigmentation. Other non-specific signs include anorexia, lethargy and anaemia. A number of studies have described signs and pathology in primary warm-water fish species, and Munday *et al.* (2002) have reviewed the literature. However, the clinical signs and pathology of cold-water fish species will be presented in more detail.

The first paper on VNN in halibut is Grotmol *et al.* (1995), who reported mass mortality of larval and juvenile hatchery-reared halibut (*Hippoglossus hippoglossus*) in Norway. In this case the initial clinical signs consisted of reduced feeding activity, emptying of the gastro-intestinal tract and lighter skin pigmentation. Occasionally abnormal swimming pattern with belly-up was observed. Most of the mortality occured in the period of early metamorphosis, and mortality rates reached almost 100%. They concluded that the mortality was a result of infection with nodavirus. However, Bloch *et al.* (1991) was the first to report mortality (although not in a cold-water fish spices) in turbot (*Scophthalmus maximus*) in Norway, although they suggested this was caused by a picornavirus, the clinical signs and pathology are consistent with VNN. In the following years outbreak of VNN or clinical signs in halibut have been reported in individuals in different developmental stages from pelagic larvae to metamorphosed juvenile by Grotmol *et al.* (1997a) and adult and sexually mature Atlantic halibut by Aspehaug *et al.* (1999). Presence of NNV in adult fish had been reported previously (Fukunda *et al.* 1996, Arimoto *et al.* 1992, Mushiake *et al.* 1994, Nguyen *et al.* 1997, Le Breton *et al.* 1997), but still the report by Aspehaug *et al.* is the only one of clinical signs of VNN in fish with mean weight of 5.4 kilos. VNN has also been reported in the UK,

in Atlantic cod and Dover sole (*Solea solea*), both species hatched from wild stocks (Starkey *et al.* 2000, 2001). The disease has also been reported in Atlantic cod juveniles, hatched from egg batches obtained from wild brood stock fish, in North America (Johnson *et al.* 2002) and in cultured white seabass (*Atractoscion nobilis*) juveniles (Curtis *et al.* 2001). Recently, the first outbreak of VNN in farmed Atlantic cod in Norway was reported by Paper IV (see section 1.2.4).

In general, observation of pathology and clinical signs of VNN in cold-water fish species are restricted to farmed species only, and to fish in cultivation originating from eggs hatched from wild caught brood stock fish. There are no reports on disease outbreaks in wild fish species, but a number of papers have reported detection of NNV, which is described in section 1.2.3. Mortality seems to be related to size or developmental stages, as larvae and juveniles have higher rates of mortality than adult fish. Temperature may also contribute to disease outbreaks, and some of the described outbreaks of VNN occurred at temperatures above 12 °C. Only marine fish species have been reported to suffer from VNN, but there are some studies on distribution of nodavirus in wild populations (see below). Although there is no report on VNN in salmon (which is an important farmed species in Norway), one paper has described clinical signs and pathology similar to nodavirus infection. Scullion *et al.* (1996) reported encephalitis and mass mortality of farmed salmon smolt in Ireland. Although no causative agent was found, this observation and the finding of nodavirus-like agent in heart tissue of Atlantic salmon suffering from cardiac myopathy syndrome (CMS) (Grotmol *et al.* 1997b, Nilsen and Nylund 1998) may indicate susceptibility in this species. Host specificity is addressed in section 1.3.1 and 4.3.2.

1.2.2 Histopathology

In general, histopathology of VNN includes cellular vacuolation and neuronal degeneration, usually in retina, brain, spinal cord and ganglia in the peripheral nervous system (Munday *et al.* 2002). Typical histopathology is exemplified in Figure 3. These pathological changes are linked to the clinical signs observed during a VNN outbreak, and the CNS is thought to dysfunction. The term "viral nervous necrosis" and "viral encephalopathy and retinopathy" is also related to observed histopathology of the disease.

Histopathology in Atlantic halibut larvae suffering from VNN has been described with vacuolation in cells in all areas of the brain and in all nuclear layers of the retina. The numbers of vacuolated cells may vary, where some individuals only show focal lesions or diffuse degeneration, when others may have widespread and massive vacuolation (Grotmol *et*

al. 1995, Johnson et al 2002). Johansen *et al.* (2002) have shown that persistently infected Atlantic halibut with no clinical signs of disease have focal cell aggregates of virus. These aggregates were seen in all regions of the brain and nuclear cell layers of the retina. The differences observed in various developmental stages, between different fish species and in acute and persistently infected fish demonstrate that further knowledge of the virus-host interactions is required. This has led to discussions of significance of genetic composition of the host, maturity of the host immune system (including the developmental stage) and virulence (Sommerset 2004).

1.2.3 Cold-water species affected

Several fish species from Northern-Europe, from the Atlantic coast of North America and from North Japan have been shown to be susceptible hosts for NNV from the cold-water clade BFNNV (Table 1). In Norway, a recent study detected NNV in wild Atlantic cod (Nylund *et al.* 2008). The detections of NNV in a number of wild fish species on the west coast of Norway are suggesting there is a marine reservoir of virus. However, further sampling has to be done to map the distribution of NNV in wild marine fish species along the Norwegian coast.

Susceptibility has also been shown by experimental challenge with betanodavirus in spotted wolfish (*Anarhichas minor*) (Johansen *et al.* 2003, Sommer *et al.* 2004). A nodavirus-like agent has also been reported in heart tissue of Atlantic salmon (*Salmo salar*) suffering from cardiac myopathy syndrome (CMS) (Grotmol *et al.* 1997b). Although viruses isolated from cold-water hosts have been restricted to the BFNNV group, Thiéry *et al.* (2004) reported that a virus isolate from this clade infected sea bass (*Dicentrarchus labrax*). This virus was isolated during an outbreak of VNN at low temperature, and low host specificity of the virus was suggested. The same conclusion was reached in a study of nodavirus infections in Israeli mariculture (Ucko *et al.* 2004). Other studies have shown susceptibility of fish in freshwater (Hedge *et al.* 2003) or fish reared in freshwater (Skliris and Richards 1999b, Athanassopoulou *et al.* 2003).

1.2.4 VNN in Norway – a brief summary

Bloch *et al.* (1991) were first to report nodavirus in Norway (picornavirus-like agent), in juveniles of turbot (*Scophthalmus maximus*) suffering from encephalomyelitis, later Grotmol *et al.* (1995) reported mortality of larva and juveniles of Atlantic halibut

Table 1. Cold-water fish species host range for nervous necrosis viruses (NNV) from the BFNNV genotype clade. The table list fish species in which NNVs have been detected and/or sequenced. Wild/farmed = whether NNV has been detected in wild or farmed fish.

Order	Species	Name	Geographical location	Reference	Wild/farmed
Gadiformes	*Gadus morhua*	Atlantic cod	Norway	Nylund *et al.* (2008)	wild
			Norway	Paper IV Nylund *et al.* (2008)	farmed
			Canada	Johnson *et al.* (2002)	farmed
			UK	Starkey *et al.* (2001)	farmed
	Gadus macrocephalus	Pacific cod	Japan	Nishizawa *et al.* (1997)	unknown
	Melanogrammus aeglefinus	Haddock	Canada	Gagné *et al.* (2004) Johnson *et al.* (2002)	Farmed
	Pollachius virens	Saith	Norway	Nylund *et al.* (2008)	Wild
	Pollachius pollachius	Pollock	Norway	Nylund *et al.* (2008)	Wild
Pleuronectiformes	*Pleuronectes americanus*	Winter flounder	USA	Barker *et al.* (2002)	Wild
	Solea solea	Dover sole / common sole	UK	Starkey *et al.* (2001)	Farmed
	Hippoglossus hippoglossus	Atlantic halibut	UK	Starkey *et al.* (2000)	Farmed
	Hippoglossus hippoglossus	Atlantic halibut	Norway	Grotmol *et al.* (1995) Aspehaug *et al.* (1999)	Farmed
	Pleuronectes platessa	Plaice	Norway	Nylund *et al.* (2008)	Wild
	Verasper moseri	Barfin flounder	Japan	Nishizawa *et al.* (1995)	Farmed
Perciformes	*Scomber scombrus*	Mackerel	Norway	Nylund *et al.* (2008)	wild
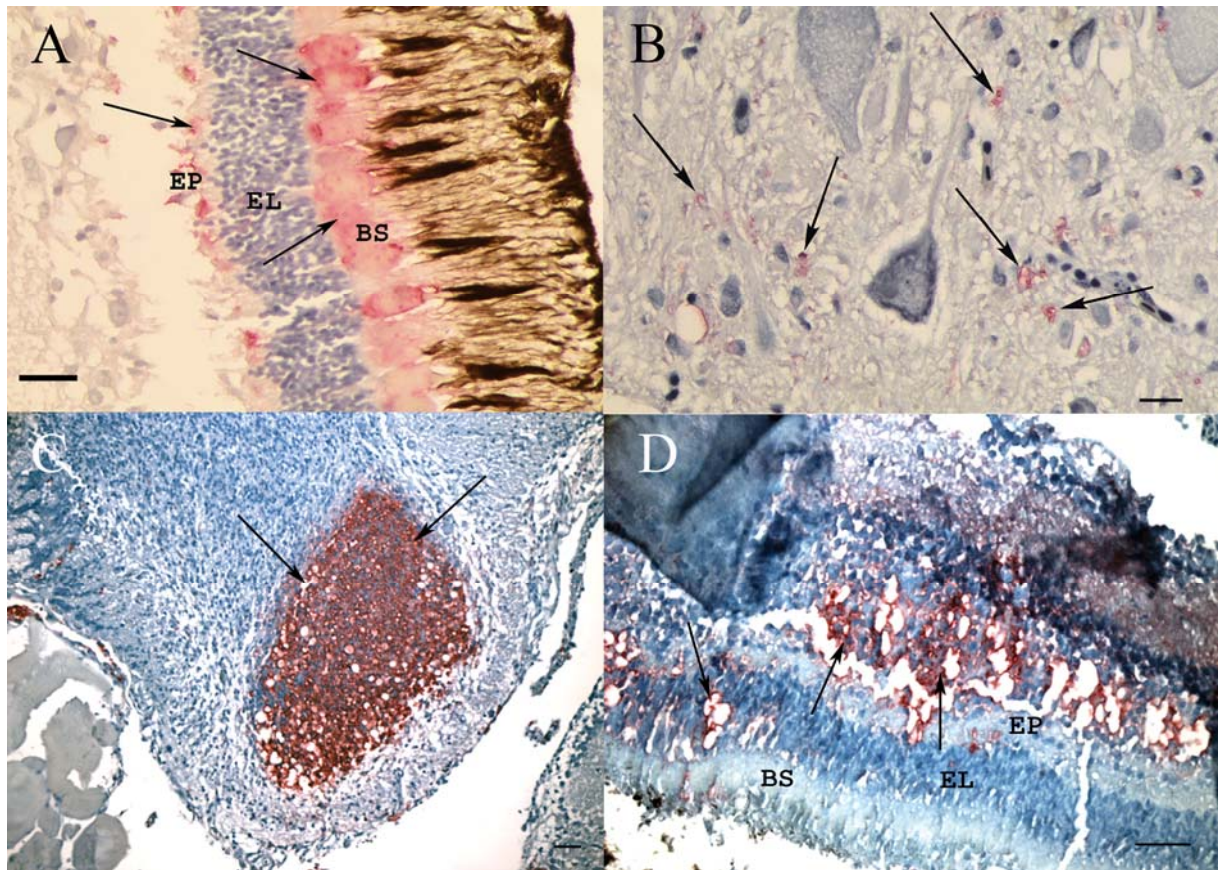
Figure 3. *Histological sections of Atlantic cod, Atlantic salmon and turbot immunolabeled (red colour) with NNV specific antibodies, targeting the viral capside protein.* **A)** *Strong immunolabeling (arrows) in a section of retina in experimentally i.p. challenged Atlantic cod. EP = external plexiform layer, EL = external nuclear layer, BS = basal layer of visual cells.* **B)** *Section of brain in experimentally i.p. challenged Atlantic salmon with immune positive cells (arrows) scattered in the parenchyma.* **C)** *Strong immunolabeling of a large area (indicated by arrows) in a section of brain from experimentally i.p. challenged turbot. In the immunolabeled area, vacuolation of cells are visible.* **D)** *Immune-labeling of a section of retina from experimentally i.p. challenged turbot. Vacuolated cells are visible within EL and EP layers. Scale bars = 20 μm.*

(*Hippoglussus hippoglossus*) associated with nodavirus-like particles in CNS and retina. However, mortality that most likely could be related to nodavirus infection was registered in turbot as early as 1987 (Nylund – unpublished data), based on TEM analysis of sections of brain, observations of clinical signs and absence of other known pathogens. During the time span from 1995 to 1998 several outbreaks of VNN were reported in farmed halibut, and a national surveillance program was established in 1999 in order to monitor the disease. This program relayed on sampling of juveniles from hatcheries producing halibut, turbot and from 2000 Atlantic cod (*Gadus morhua*). The surveillance was running until 2004 when it was terminated due to implementation of EU Directive 91/67. During these years, NNV were

detected at four times; three times in halibut (Hellberg and Dannevig 2002, 2003) and once in turbot (Johansen *et al.* 2004b). From 2004 to mid 2006 there was no official detection of nodavirus, but this changed when VNN was diagnosed in a halibut hatchery in late summer 2006, at a research facility that previously had suffered from the disease in the mid 1990'ties. This outbreak was followed by a new VNN diagnosis in a combined research and commercial growth site for Atlantic cod (Hellberg 2007, Paper IV). This site is also owned and operated by the same institute running the halibut hatchery, and was the first outbreak of VNN in farmed Atlantic cod in Norway. At the end of year 2006 NNV was detected and VNN diagnosed in several commercial cod farms at the west coast and northern part of Norway, suggesting presence of NNV in farmed cod, as well as in farmed turbot and halibut. As nodavirus is appearing in an increasing number of fish farms and in wild fish species (Nylund *et al.* 2008) further knowledge of the virus properties as a pathogen is required. In order to understand some of the nature of NNV infection aetiology, a closer look on the genetic properties, transmission and distribution of virus in farmed and wild fish are essential. First of all, investigation of such properties necessitates sensitive and specific detection tools.

1.2.5 Diagnostic methods and detection

Establishment of specific and sensitive methods for nodavirus detection are important, both as diagnostic tools and for scientific studies of the virus. In general, the most commonly used methods could be divided into molecular, immunological and cell culture.

Initially there were few cell lines in which betanodavirus could be cultivated. Cell lines are important for propagating virus, characterizing and studying viral infectious mechanisms, and the first line reported to support betanodavirus replication were by Frerichs *et al.* (1996). They successfully used a cell line (named SSN-1) derived from striped snakehead (*Ophicephalus striatus*) to isolate nodavirus from diseased sea bass juveniles. The SSN-1 cell line, and the cloned E-11 cell line derived from SSN-1 (Iwamoto *et al.* 2000), was subsequently been used in number of studies. A number of other cell lines have been reported as useful in propagating NNV, including Cos1 (simian) (Delsert *et al.* 1997b), GF-1 derived from grouper (*Epinephelus coioides)* (Chi *et al.* 1999), a tropical marine fish cell line (SF) from Asian sea bass (*Lates calcarifer*) (Chang *et al.* 2001) and TF-line from turbot (*Scophthalmus maximus*) (Aranguren *et al.* 2002b). A concern regarding the SNN-1 and derived cloned types has been reported, as these are persistently infected by retrovirus which might possibly interfere with NNV replication (Lee *et al.* 2002). Another effect on the SSN-1 cell line which has been examined is temperature, suggesting that this cell line to be

suboptimal for proliferating NNV strains isolated from cold-water fish species (Ciulli *et al.* 2004). In Norway, the SNN-1 cell line has been used to propagate cold-water NNV (Dannevig *et al.* 2000, Johansen *et al.* 2003), but the recent published SAF-1 cell line derived from gilthead sea bream (Bandín *et al.* 2006) has been used successfully to proliferate nodavirus isolates from both wild and farmed cold-water fish species in Norway (Nylund *et al.* 2008). The SAF-1 cell line could consequently prove to be an alternative to the SSN-1 type. However, the widespread distribution of NNV has led to development of several other susceptible cell lines that has proven effective to proliferate a number of different NNV strains (Lai *et al.* 2003, Chi *et al.* 2005, Hameed *et al.* 2006, Zhao and Lu 2006, Qin *et al.* 2006).

The unsuccessful attempts to cultivate NNVs in cell lines in the first half of the 90'ties led to a rapid development of alternative methods, including immunologically based assays. These assays could be subdivided into direct and indirect detection of virus particles, and included enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody technique (IFAT) and later immunohistochemistry (IHC) and neutralization tests. The most widely used immunological methods for diagnosing VNN have been ELISA and IHC.

ELISA was one of the first immunological methods to be developed, and one of the first such assays was developed by Arimoto *et al.* (1992) to detect SJNNV. Later, Shieh and Chi (2005) suggested use of an antigen capture ELISA as a more specific and sensitive tool for VNN diagnosis. Several papers have used ELISA to detect virus in various fish species to monitor health status (Mushiake *et al.* 1992, Breuil and Romestand 1999, Watanabe *et al.* 2000, Husgard *et al.* 2001, Huang *et al.* 2001, Breuil *et al.* 2002, Grove *et al.* 2003), but one major problem is the sensitivity, as this method requires an antibody response. In fish displaying persistency or latency, detection of virus by ELISA might prove to be difficult and more sensitive methods such as RT-PCR and real-time RT-PCR have become more commonly used.

Immunohistochemistry (IHC) provides detection of virus particles in histological sections, where both presence and possible pathological changes might be observed. IHC is based on antiserum raised against specific or similar antigens, making studies of possible cross-reactions necessary. Different NNV strains are shown to be serologically distinguishable (Skliris *et al.* 2001, Mori *et al.* 2003), suggesting there are differences in neutralizing epitopes. Consequently, antisera raised against one particular strain of betanodavirus might not detect other serologically distinguishable strains, or they may cross-react with epitopes of non-nodavirus origin. The first detection of NNV from Atlantic halibut

with IHC was performed with an antiserum raised against a striped jack (SJNNV) strain (Grotmol *et al.* 1997a), and this serum was subsequently used by in a number of studies for NNV detection (Grotmol *et al.* 1997b, Grotmol *et al.* 1999, Grotmol *et al.* 2000, Dannevig *et al.* 2000). Later, antisera raised against a NNV isolated from Atlantic halibut (AHNor95) have been frequently used (Johansen *et al.* 2002, 2003, 2004a, Grove *et al.* 2003, Sommerset *et al.* 2005).

Molecular methods for the detection of NNV could be divided into RT-PCR and real-time RT-PCR assays. Molecular methods have played an even more important role in detecting and characterizing virus, and the first ones to develop an RT-PCR assay for betanodavirus were Nishizawa *et al.* (1994), which targeted the RNA2 segment of SJNNV. Subsequently a large number of papers have applied the method to detect various virus strains (i.e. Comps and Raymond 1996, Nguyen *et al.* 1997, Thiéry *et al.* 1999, Péducasse *et al.* 1999, Dalla Valle *et al.* 2000, Iwamoto *et al.* 2001, Huang *et al.* 2001, Gomez *et al.* 2004, Gagné *et al.* 2004, Thiéry *et al.* 2004). In Norway RT-PCR and real-time RT-PCR have been used to detect nodaviruses isolated from Atlantic halibut (Grotmol *et al.* 1997a, Aspehaug *et al.* 1999, Grotmol *et al.* 2000, Johansen *et al.* 2004a), turbot (Johansen *et al.* 2004b) and Atlantic cod (Nylund *et al.* 2008). RT-PCR, followed by sequencing, has also formed the basis for phylogenetic analyses (see section 1.1.3), where strains are grouped according to genetic properties into five distinct clades. The RT-PCR technology has been further developed, and in 1996 the first commercial real-time PCR became available (Heid *et al.* 1996). Real-time PCR is a continuous collection of fluorescent signal from one (or more) PCR over a range of cycles, and the advantages over standard PCR are increased sensitivity and the possibility to be quantitative. The development of real-time PCR has been reviewed by Mackay (2004), with focus on the use in the microbiology laboratory. Real-time RT-PCR methods for NNV detection and quantification have been developed to study the virus, and have vastly improved the sensitivity compared to conventional PCR and nested PCR methodologies (Starkey *et al.* 2004). This has made this method particularly useful to map the distribution of virus in persistently infected fish, where virus particles are present in small numbers. Recently, Starkey *et al.* (2004), Paper I, Grove *et al.* (2006) and Nerland *et al.* (2007) have published real-time RT-PCR assays used to detect NNV in the BFNNV clade.

VNN is no longer listed in section 1.2.3 of the Aquatic Animal Health Code (2008) issued by OIE (http://www.oie.int/eng/normes/fcode/A_summry.htm). Diagnostic methods have been described by OIE, where fish suitable for virological examination include asymptomatic carriers and fish showing clinical signs as described above. The finding of

vacuolation of neural tissue is verified by: 1) cell culture propagation and subsequent virus identification by indirect fluorescent antibody technique (IFAT) and/or reverse-transcription polymerase chain reaction (RT-PCR) or 2) direct detection in fish tissue by IFAT, immunohistochemistry or RT-PCR (http://www.oie.int/eng/normes/fmanual/A_00024.htm).

1.3 Virus-host interactions

The worldwide distribution of NNV does raise some questions of how the virus is spread. Although the mechanisms of the viral infection, including transmission, is not fully understood, the central nervous system (CNS) seems to be the main target organ for replication in all affected fish species. A further examination of possible important virus-host interactions, which might play a role in the viral lifecycle, is necessary. Subsequently, host-specificity, transmission of virus, entry into the host, viral replication and vaccination (immune response) as a possible prophylactic measure are addressed below.

1.3.1 Host specificity

The large number of susceptible NNV hosts, worldwide distribution (see review by Munday *et al.* 2002) and phylogenetic relationships (see section 1.1.3) have resulted in discussions of possible host specificity in strains of NNV. The suggestion of a cold-water genotype clade (BFNNV) has led to discussions on possible host specificity and temperature dependence in betanodavirus strains (Aspehaug *et al.* 1999, Chi *et al.* 1999).

In order to investigate possible transfer to mammalian cells, Delsert *et al.* (1997b) tested a nodavirus (DIEV) in a mammalian cell line (simian Cos1). Replication was observed but virus yield was low. Banu and Nakai (2004) injected nodavirus (in the SJNNV and RGNNV clades) both intra peritoneally and intra muscularly in mice (BALB). The virus was spread to the kidney, but the number of virus particles decreased during the period investigated (72 h). Even more important, the virus strains did not show any neuroinvasive activity, and were not detected in the target organs known from piscine hosts (brain, spinal cord and eye). Consequently, the mouse was not regarded as susceptible to infection with NNV.

The ability of NNV to infect host cells, replicate and infect other host cells are important and could possibly relate to host specificity. When NNV was injected in turbot muscle cells it did replicate, and this might suggest that the virus is able to replicate if forced or helped into host cells (Sommerset *et al.* 2005). There was, in this study, no spreading of virus from infected muscle cells to non-infected cells in close proximity, and this might

suggest that NNV has a high capacity to replicate when the virus has entered into the cell cytoplasm but not necessarily the capacity to enter/spread to a new host cell. Eventually the virus in this study entered into the bloodstream of the host, causing a viremia and a following infection of brain and eye. This study also demonstrates the susceptibility of cells in the central nerve system (CNS). The ability of NNV to be transported from one neuron to another by passing across synaptic clefts has been shown by Ikenaga *et al.* (2002). This may suggest that virus could be transported from the peripheral nerve system or blood to CNS.

Several studies have suggested existence of host specificity when studying susceptibility of various fish species to different NNV strains in challenge experiments (Arimoto *et al.* 1993, Totland *et al.* 1999, Tanaka *et al.* 2003, Iwamoto *et al.* 2004, Thiéry *et al.* 2004). Host specificity has been linked to the RNA2 segment (Iwamoto *et al.* 2004) with suggestion that the variable region (known as the T4 region) is involved in controlling the host specificity (Ito *et al.* 2008). Finding of a NNV isolate in the BFNNV capable to infect sea bass (*Dicentrarchus labrax*) may suggest that different genotypes could infect various fish species and subsequently have low host specificity (Thiéry *et al.* 2004).

In summary, there is support for existence of host specificity of nodavirus in the literature. However, one major concern is the lack of information of possible virulence of isolates and strains of nodavirus, which probably is playing a role in susceptibility and any host specificity. The susceptibility is reported primarily in fish juveniles, but some papers report this in adult fish as well (Aspehaug *et al.* 1999, Skliris and Richards 1999a). This might suggest differences in virulence of isolates used in challenge experiments. The existence of both vertical and horizontal transmission (see below) is suggesting that variation in virulence likely exists. Such variation in virulence of fish pathogenic viruses in cold-water fish farming was suggested by Breuil *et al.* (2001), which used two distinguishable nodavirus isolates to challenge sea bass. In this study there were differences in pathogenicity between the isolates when challenging fertilized eggs and subsequent examination of larvae post hatching. Both isolates were pathogenic to larvae challenged with virus. Variation in virulence has also been demonstrated in infectious pancreatic necrosis virus (IPNV) (Santi *et al.* 2004) and infectious salmon anemia virus (ISAV) (Cunningham *et al.* 2002, Nylund *et al.* 2003), which cause diseases in farming of Atlantic salmon (Salmo salar). These viruses show a distinct difference in host range (and host specificity), but share the property of variation of virulence due to existence of both vertical (Nylund *et al.* 2007) and horizontal transmission. Further studies of virulence in nodavirus isolates, strains and possibly genotypes should consequently be carried out.

1.3.2 Transmission of virus

The first step to study possible transmission of a pathogen usually involves studies of susceptibility by experimental challenges, typically as intra peritoneal (i.p.) or intra muscular (i.m.) injections. Injection of nodavirus does not represent a natural way of transmitting the pathogen, but provide a controlled way of distributing the virus. If the virus does not replicate or spread within the host, susceptibility does not likely exist. On the other hand, if susceptibility is observed, further studies using cohabitation as a model are usually performed in order to examine if the host is susceptible to a water borne transmitted pathogen, known as *horizontal transmission*. The other principal way of transmitting a pathogen is by gametes, known as *vertical transmission*. Pathogens may have one or both ways of transmission, and this will have implications for how the pathogen is spread, both in wild populations of fish and in the aquaculture industry. The mechanisms of how NNV is transmitted are not fully understood, but several studies have addressed transmission of the virus between and within different fish species. Possible routes of transmissions of the virus are given in Figure 4.

Horizontal transmission

Existence of horizontal transmission of NNV has been shown in a number of experimental challenge studies in several susceptible hosts. Such transmission has been demonstrated in farmed fish species such as striped jack (*Pseudocaranx dentex)* (Arimoto *et al.* 1993), sea bass (*Dicentrarchus labrax*) (Le Breton *et al.* 1997, Castric *et al.* 2001, Péducasse *et al.* 1999, Skliris and Richards 1999a) and sea bream (*Sparus aurata*) (Castric *et al.* 2001, Aranguren *et al.* 2002a). One particular observation should be highlighted, that sea bream (*Sparus aurata*) appear to be a potential carrier of NNV, as juvenile sea bass (*Dicentrarchus labrax*) became infected when placed in a tank with experimentally infected sea bream (Castric *et al.* 2001). Existence of horizontal transmission has also been suggested when comparing different NNV isolates in Taiwan, where the study concluded that transmission had occurred as a result of transportation of infected fish, and that virus seemed to be transmitted within aquaculture facilities through use of contaminated equipment and recirculation of water (Castric *et al.* 2003)

There are rather few studies on horizontal transmission in cold-water fish species. The studies have mostly addressed Atlantic halibut that has been i.p. injected or bath challenged with virus (Grotmol *et al.* 1999, Dannevig *et al.* 2000, Grove *et al.* 2003). NNV has also been detected in high concentrations in rearing units with Atlantic halibut larvae suffering from

VNN (Nerland *et al.* 2007), showing that virus may be dispersed via water from aquaculture facilities.

A few studies have addressed the stability of the nodavirus particle, and the effect of chemical disinfectants on inactivation of NNV (Arimoto *et al.* 1996, Frerichs *et al.* 2000). In general, these two studies found that the virus isolates examined showed high resistance to environmental conditions. The ability to withstand degradation is possibly important in horizontal transmission (survival in the environment) and increases the likelihood for such a transmission pathway. One study by Liltved *et al.* (2006), reported that NNV has high resistance to ozone and suggested that fish farms should use considerably higher TRO (total residual oxidant) concentrations and $C T$ value (the product of concentration and contact time) than previously applied for inactivation of virus.

In summary, horizontal transmission of NNV has been documented in several fish species. The published studies may suggest that there is a difference in susceptibility and development of clinical disease, where juveniles are more likely to develop the disease. There are also indications that increased temperature and amount of virus used to challenge influences the development of disease. Horizontal transmission has been shown in adult individuals as well, and may suggest that the virus could possibly be transferred over a prolonged period or even the entire life span, but development of disease are to a lesser degree occurring as the fish is getting older. However, these studies have not addressed possible variation in virulence of nodavirus isolates or strains. Further, clinical disease could perhaps occur if virus in persistently infected adult fish increased amount through viral replication.

Vertical transmission

Several studies have suggested that NNV, or more specifically the particular strains which have been studied, to be vertically transmitted. In general, vertical transmission can occur as transovarian transmission where the pathogen is present inside the egg. The other way is if the pathogen only is present on the egg surface (transovum). The result is vertical transmission in both cases. But it is important in cultivation of fish to determine the type of transfer, as disinfection of eggs only would be effective when the pathogen is present on the surface.

Vertical transmission of NNV has been suggested by a number of studies, in striped jack (Arimoto *et al.* 1992, Mushiake *et al.* 1994, Nguyen *et al.* 1997), barfin flounder (*Verasper moseri*) (Watanabe *et al.* 2000) and sea bass (Breuil *et al.* 2002). These studies recognize vertical transmission as an important way of spreading the virus and suggest selection of virus-free sprawers by examination of brood fish. Several different methods have
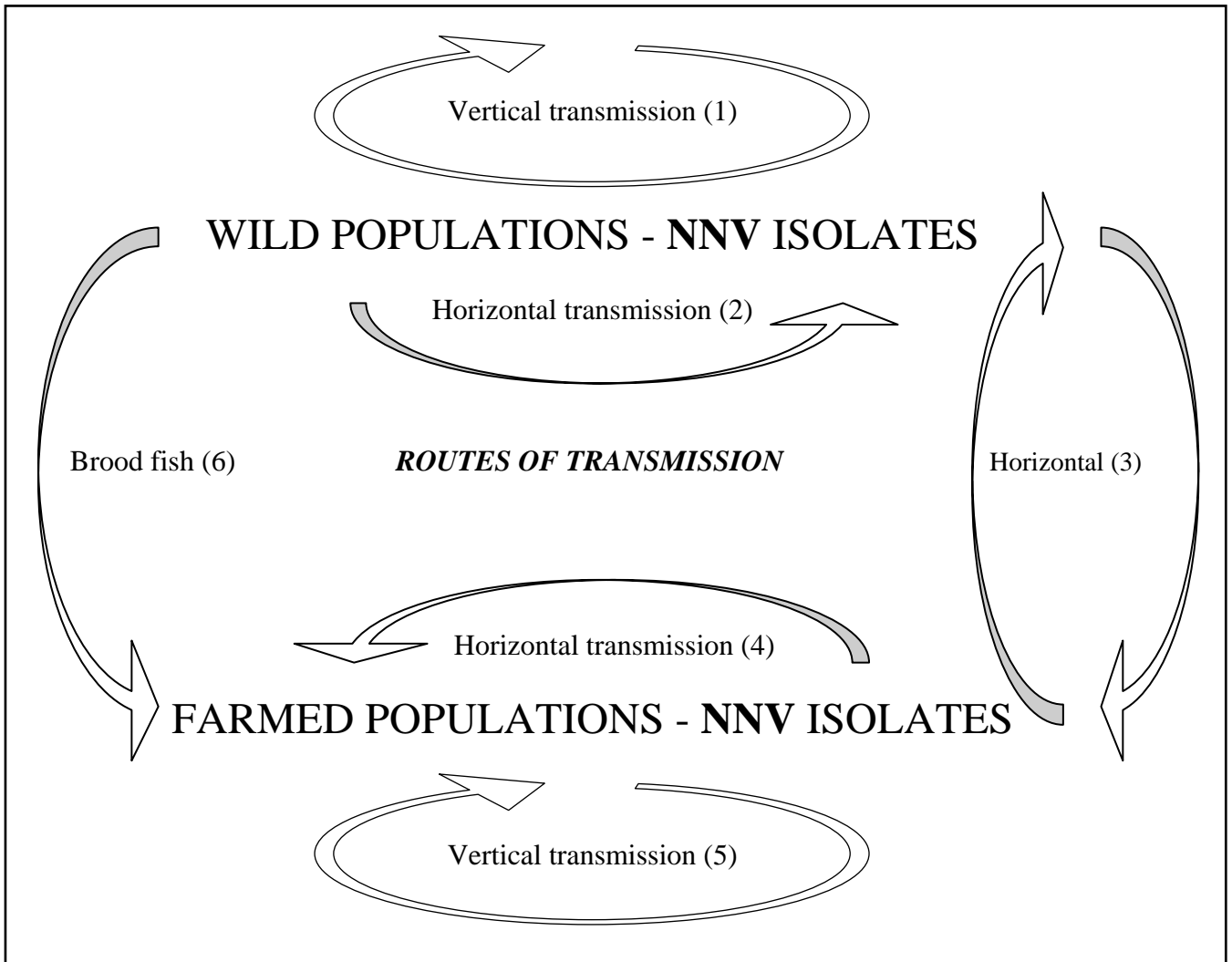
Figure 4. *Suggested transmission routes of nervous necrosis virus (NNV). Different entrances of the virus may be suggested; 1) vertical transmission in wild populations of fish, 2) horizontal transmission between wild fish, 3) horizontal transmission between wild and farmed fish, 4) horizontal transmission between farmed fish, 5) vertical transmission in farmed populations of fish and 6) through use of NNV infected fish utilized as brood fish in farmed fish or stock enhancement of wild populations of fish. The NNV-host interaction may be considered to be different in wild versus farmed populations of fish, as discussed in section 4.3.4.*

been applied to screen fish in these studies, including ELISA (Breuil and Romestrand 1999) and RT-PCR (Nishizawa *et al.* 1994), and application of new methods such as real-time PCR may also be important to identify infected brood fish.

Considering cold-water fish species such as Atlantic cod and halibut, there are few conclusive reports on vertical transmission. However, the report of isolation of NNV from adult Atlantic halibut displaying clinical signs of VNN suggests vertical transmission likely could occur. A study of effect of disinfection with ozone on eggs from Atlantic halibut with increased rate of survival of halibut juveniles post hatching (Grotmol and Totland 2000),

suggests that use of disinfectants might be important to inactivate virus with transovum transmission. Existence of subclinically infected adult fish may also suggest that virus may be transmitted vertically, and in a study which followed a natural outbreak of VNN at a commercial Atlantic halibut farm in Norway such fish were detected after one year (Johansen *et al.* 2004a). During this study, fish were grouped as high- and low-growth groups, where the number of fish positive for virus by PCR and ELISA decreased gradually in the high growth group. However, virus was re-isolated in cell culture from subclinically infected fish suggesting that the virus was still infectious.

In summary, there is strong support for vertical transmission. Studies of striped jack, sea bass and barfin flounder detected NNV in adult individuals and spawners, eggs and larvae post-hatching, and that infected spawners produce offspring which may develop the disease. These studies recommend that selection of virus-free brood fish is essential for controlling the disease. In cold-water species, there is no conclusive study on vertical transfer, but considering the studies on other marine fish species it is likely that vertical transmission is occurring in these species as well. This could be exemplified with the report on adult halibut displaying clinical signs of the disease and subsequent isolation of virus. In addition, disinfection of eggs increased the survival of halibut larvae. The existence of persistently infected halibut after an outbreak of VNN suggests that such fish might become carriers, which could transfer the virus vertically if used as brood fish. In farming of cold-water species (including Atlantic cod), further studies should be carried out to verify existence of vertical transmission and if any such transfer includes transovarian transmission.

1.3.3 Entry of virus into the host

A virus infection involves attachment on the host cell surface, entry into the cytoplasm and replication. In general, viruses replicate either in the cytoplasm or in the nucleus, depending of the type of nucleic acid present in the genome. RNA viruses, in most cases, replicate in the cell's cytoplasm, and carry or encode for their own polymerase. This eliminates the need for entry to the cell nucleus. However, RNA viruses that has a spliced genome or viruses with DNA as intermediate steps during replication, has to enter the cell nucleus. In general, viral mRNAs are synthesised either by viral enzymes or the cell transcriptional machinery. The viral mRNA has to be translated by the cell ribosomes in the cytoplasm, and subsequently the mRNA originating from virus has to be recognised by this system to be expressed as viral proteins.

Mechanisms of how the NNVs enter the host are not known in detail. The literature suggests existence of at least three possible gateways; 1) through intestinal epithelium, 2) via axons and 3) via the bloodstream (viremia) to CNS. The two latter may be considered as pathways when the virus already has entered into the host.

Transportation of betanodavirus in neurons has been studied by Ikenaga *et al.* (2002), who demonstrated that virus is able to move across synaptic clefts and be transported both directions. This study suggests that virus could be transported from the peripheral nerves (vagus) to the CNS. Grotmol *et al.* (1999) suggested the route of infections might be trough the intestinal epithelium, and that spreading to the CNS might be trough axonal transport to brain stem via cranial nerves such as the vagus nerves. Husgard *et al.* (2001) suggested, based on the results in challenge studies using SJNNV, that the virus could have entered the CNS by axonal transport through motor nerves after intramuscular inoculation.

Chi *et al.* (2003) have suggested the intestinal tract as a possible gateway, through feeding with contaminated biological food. Mori *et al.* (2005) have also suggested that the virus may enter into the host by digestion of contaminated food. Studies of horizontal transmission of virus (see above) may also suggest existence of other gateways, but in cohabitation and water borne transmission studies the virus may enter the host via the outer surfaces, including the intestinal tract. Nguyen *et al.* (1996) detected nodavirus in epithelium cells in skin of striped jack larvae in an acute infection, but the role of skin in transmission has not been investigated. The literature also suggests there is difference in susceptibility, and that age or developmental stage is influencing the likelihood of development of disease, where adult fish seems less likely to develop clinical disease. Detection of virus in a number of wild cold-water fish species may suggest that nodavirus infections are common in the marine environment. In addition, the existence of vertical transmission and persistently infected fish may demonstrate that development of clinical disease is dependent on the fate of the virus in the host rather than presence alone. In order to cause disease the virus has to evade the host immune system and replicate in sufficient numbers to cause clinical signs (usually malfunctioning of CNS). This ability, or virulence, may be influenced by the host immunological status (i.e. age/developmental stage) or possible variation in virus virulence.

1.3.4 Viral replication

NNV seem to have some host cell preference, which could be exemplified by the various cell lines used for propagation. Not all cell lines are suitable for cultivation of the virus (see section 1.2.5), suggesting the virus may need certain properties on the surface of the host cell.

Lu *et al.* (2003) demonstrated blocking of CPE in SSN-1 cell line inoculated with nodavirus and a recombinant virus-like particle (VLP). This study may suggest that VLP is occupying receptors on the cell surface and subsequent inhibition of entrance of virus into the host cell. When injecting virus into turbot muscle cells, Sommerset *et al.* (2005) observed replication in infected cells but no spreading to muscle cells in close proximity. Such studies may suggest that NNV is capable to replicate when forced into a cell but not necessarily to spread to other cells. This was also demonstrated by Banu and Nakai (2004), who infected mice with the virus. Although betanodavirus is regarded as neurotropic where CNS is the primary site for replication, the virus has also been detected in non-nervous tissues in fish species such as striped jack (Nguyen *et al.* 1996, Nguyen *et al.* 1997), halibut (Grotmol *et al.* 1999, Grove *et al.* 2003) and grouper *Epinephelus* sp. (Chi *et al.* 2001). However, detection of virus (or virus products) in non-nervous tissues not necessarily demonstrates replication, but could be a result of antigen trapping.

Replication has been most extensively studied in alphanodaviruses, where FHV has been used as a model for investigation of the viral life cycle. In general, all positive stranded RNA viruses replicate in association with intracellular membranes, usually endoplasmic reticulum, lysosomal, mitrochondiral, peroxisomal and other membranes (Ahlquist *et al.* 2003). Mézeth *et al.* (2007) demonstrated that protein A (RdRp) in Atlantic halibut nodavirus strain (AHNV) was localized to cytoplasmic structures resembling mitochondria, and co-localized with mitochondrial proteins. In FHV, protein A acts as a multifunctional RNA replication factor which is encoded by RNA1 (Figure 1 and section 1.1.2 provides an overview of the bipartite genome architecture of nodavirus). Both the RNA1 and RNA2 segments are capped, nonpolyadenylated, and packed together into the capsid (Sommerset and Nerland 2004). The main proteins involved in NNV replication include protein A (encoded by RNA1), protein B1 and B2 (both encoded by RNA3) and coat protein α (encoded by RNA2).

Three steps are involved in the production of positive-sense viral RNA. The first step is translation of parental RNA to provide an early (intermediate) replication protein, an active replicase enzyme. This enzyme is responsible for producing negative-sense strands, which then act as templates for synthesis of positive-sense RNA. These are eventually packed in the capsid. During the replication process, RNA1 and RNA2 are synthesized in approximately equal amounts, while the intermediate RNA3 only is present during the late replication stage (Friesen and Rueckert 1984, Ball 1992). The role of RNA3, which is synthesized during RNA1 replication, may be to prevent that protein A is replicating RNA2 until a minimum amount of RNA1 is present. Such a suppression mechanism of a segment in bipartite genomes

may function to co-ordinate and ensure a balanced replication, giving a sufficient amount of each segment (Zhong and Rueckert 1993, Eckerle and Ball 2002).

Proteins A and B seem to be synthesized at a high rate early in the replication cycle and then slow down, whereas synthesis of protein α peaks late in the cycle (Friesen and Rueckert 1981). Sommerset and Nerland (2004) showed that a hypothetic protein B2 in Atlantic halibut nodavirus accumulate in large amounts soon after infection, and Fenner *et al*. (2006) has suggested that the B2 protein antagonize RNA interference and facilitates intracellular viral RNA accumulation. Iwamoto *et al*. (2005) have suggested that B2 has a potent RNA silencing suppression activity, and that it subsequently is involved in suppression of the host cell defense mechanism.

The main product of the replication cycle is protein α, which also has been suggested to act as an apoptosis inducer (Guo *et al*. 2003b), and the synthesis pattern observed seems to follow the infection cycle of the virus. Protein α (in FHV) is assembled into a provirion and then cleaved autolytically (Gallagher and Rueckert 1988). Assembly of FHV particles is suggested to start with formation of specific nucleoprotein complex (Schneemann *et al*. 1994), which then is propagated into spherically closed particles (Tihova *et al*. 2004).

1.3.5 Vaccination

The principal function of a vaccine is to stimulate both the innate (non-specific) and adaptive (specific) immune system in order to lower the risk of disease development in farmed fish. Generally, the fish is stimulated prior to any pathogen exposure, and vaccinated fish show higher capability to eliminate pathogens after infection than non-stimulated fish. Consequently vaccinated fish are less likely to develop disease if exposed to the pathogen. This capability is linked to a shorter response time to produce protective antibodies, and a stronger response as compared to non-vaccinated fish. Vaccines have been used successfully in Norwegian salmon farming since the beginning of the 90'ties. However, the vast majority of vaccines available are targeting bacterial pathogens, such as *Aeromonas salmonicida* subsp. *salmonicida*, *Listonella (Vibrio) anguillarum* and *Vibrio salmonicida*, which all have caused serious disease problems in salmonide farming. The introduction of vaccines dramatically improved the health of farmed salmonides, and these bacterial pathogens are no longer causing problems in this industry. Vaccines against viral pathogens have also been developed for salmonides, and in Norway there are currently commercially available vaccines against infectious pancreatic necrosis virus (IPNV) and salmonide alphavirus (SAV). However, these

viral vaccines generally provide lower protection (as relative percent survival in challenge experiments) than vaccines targeting the above mentioned bacteria.

Although vaccination has proven to be an efficient prophylactic measure to lower risk in fish farming, a vaccination strategy is depending on the classification of the pathogen (disease) in question. Fish diseases are classified as notifiable in List 1 (exotic), List 2 (non-exotic) or List 3 (national listing) according to OIE and implemented in EU-directive 88/2006. List 1 and 2 diseases are defined as serious within the European Union (EU), while List 3 diseases are defined as serious diseases specific to individual countries in the EU region. In general, a disease is considered as serious based on mortality observed, number of outbreaks and economic losses for the aquaculture industry. Traditionally, such diseases are subject to control measures as removal or stamping-out of diseased populations of fish. This is done in order to eradicate the disease and prevent the disease from being spread to other fish populations. This eradication strategy is the backbone of how disease prevention generally is organized for notifiable diseases, and rule out vaccination as a prophylaxis measure. On the other hand, if the disease is caused by a pathogen difficult or impossible to eradicate (i.e. commonly found in the marine environment); a vaccination strategy is more likely to be an option.

There is currently no commercial vaccine targeting NNV available for farmed cold-water species such as Atlantic cod or halibut. However, several papers target development of possible vaccine against VNN. As the NNV genome is characterized (see section 1.1.2), this open the possibility to produce recombinant vaccines, i.e. express the capsid (coat) protein in bacterial vectors. Tanaka *et al*. (2001) demonstrated that immunization of young sevenband grouper (*Epinephelus septemfasciatus*) by intramuscular injection of recombinant coat protein produced neutralizing antibodies in high titres. They suggested there is a potential for vaccination against VNN in this fish species. Husgard *et al*. (2001) used a recombinant partial capsid protein from SJNNV to immunize (intraperitoneal injection) adult turbot (*Scophthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*). In this study a specific humoral response was found in both species, and a challenge experiment with turbot demonstrated significant protection 10 weeks post vaccination. Yuasa *et al.* (2002) used recombinant coat protein from RGNNV, and detected virus-neutralizing antibodies in previously intramuscularly injected humpback grouper (*Cromileptes altivelis*). In this study, they also found that neutralizing antibodies induced by RGNNV recombinant coat protein were specific for the RGNNV genotype, suggesting that neutralizing epitopes in NNV are different between various genotypes. Thiéry *et al*. (2006) vaccinated sea bass (*Dicentrarchus*

labrax) intramuscularly, using virus-like particles (VPL) made from a single type of NNV coat protein spontaneously assembled in a baculovirus expression system. This study showed that VLPs elicited production of NNV specific antibodies, and that fish vaccinated were protected from challenge with live NNV. The immune response and the protective effect against viral challenge were both dose dependent. A DNA vaccination approach has also been suggested, and Sommerset *et al*. (2003) demonstrated that a DNA vaccine encoding the envelope glycoprotein of VHSV (rhabdo virus causing viral hemorrhagic septicaemia) induced protection against Atlantic halibut nodavirus (AHNV) in turbot (as model fish). They also tested a DNA vaccine carrying the gene encoding the coat protein of AHNV, but no protective properties were detected when challenging vaccinated turbot with this virus strain. Sommerset *et al*. (2005) tested a recombinant AHNV coat protein vaccine against the DNA vaccine, and concluded that only the recombinant vaccine produced protection against AHNV challenge in the turbot model fish.

In summary, the literature suggests the possibility to induce protection against VNN by vaccination, but further work should be done to maximize the immunization effect achieved in the fish species studied. The use of NNV coat protein as neutralizing epitope seems to be promising, but also the DNA vaccination approach should be studied further to develop an effective vaccine against VNN. However, there are at least some considerations regarding use of vaccination as prophylaxis against VNN. Stimulation of the immune system by vaccination require susceptibility to stimulation, but the main VNN problems (high mortality) have been described in the juvenile phase of the life cycle of most affected species when the immune system is not fully developed (section 1.2.1). Consequently, vaccination against VNN is only possible when the fish has reached a specific developmental stage or age.

2 AIMS OF STUDY

The main objective of the present study was to investigate nervous necrosis virus (NNV) as a pathogen in Norwegian aquaculture industry, with emphasis on the fish species Atlantic salmon *Salmo salar* and Atlantic cod *Gadus morhua*. The specific aims could be summarized as follows:

1. To investigate possible susceptibility to NNV of Atlantic salmon, the most important farmed fish species in Norwegian aquaculture.
2. Study possible horizontal transfer of NNV to Atlantic salmon and Atlantic cod.
3. Investigate NNV tissue tropism in Atlantic cod and identify organs possibly suited for non-lethal screening of brood fish.
4. Identify possible consequences for the aquaculture industry, by means of suggestions for prophylactic measures and identification of risk involved in spreading the virus.

The aims were approached in the papers, where Paper IV is addressing the first outbreak of the disease in farmed Atlantic cod in Norway. Paper I investigates NNV susceptibility of Atlantic salmon, Paper II addresses susceptibility and NNV tissue tropism in Atlantic cod with suggestions of possible non-lethal tissues for biopsy. And in Paper III, experimental cohabitant horizontal transmission of the virus to salmon and cod is examined. Finally, the synthesis discusses possible consequences for the aquaculture industry in Norway. By elucidating the results from the present study in the light of the available scientific literature, a possible strategy for how NNV could be managed to minimize the risk of spreading the virus (and subsequent development of clinical disease) is discussed.

3 SUMMARY OF PAPERS

Paper I

In this paper homogenate of tissue from juveniles of Atlantic halibut *Hippoglossus hippoglossus* suffering from viral encephalopathy and retinopathy (VER) was used to challenge smolt of Atlantic salmon *Salmo salar* with an initial average weight of 110 g. The nodavirus was administered in the form of an intra peritoneal injection, and the fish were kept for 134 days post challenge. Genotype characterisation of the nodavirus was performed by sequencing the RNA1 and RNA2 segments, and a quantitative real-time PCR (Q-PCR) assay was developed. Tissues from different organs were stained by immunohistochemistry (IHC). Samples were collected at random on days 7, 25, 45, 69, 125 and 134 after challenge. Mortality, clinical signs and pathology of VER were observed only in the challenged group. The Q-PCR detected positive fish only in the challenged group, all of which were positive on all days of sampling. An increase in relative virus concentrations was observed from day 7 to day 25 post challenge. The increased level of virus concentration was maintained in the medulla oblongata throughout the experiment, suggesting persistence or slow elimination of the virus over time. The IHC detected positive cells on three different sampling days; 34, 70 and 74. These results suggest that the nodavirus is transported to the medulla oblongata from the intra peritoneal injection site and is able to replicate in salmon. This nodavirus isolate caused mortality and established a persistent infection in the challenged salmon throughout the experiment. This susceptibility suggests that co-location of salmon and marine species should be avoided until further studies have been carried out.

Paper II

In this paper Atlantic cod *Gadus morhua*, averaging 100 g, were experimentally challenged by i.p. injection of nervous necrosis virus (NNV) originating from Atlantic halibut. Cod tissues, including blood, gill, pectoral fin, barbel, ventricle, atrium, spleen, liver, lateral line (including muscle tissue), eye (retina) and brain, were sampled at Day 25 and 130 and investigated by real-time RT-PCR for presence of NNV. Relative quantifications at Day 130 were calculated using the $2^{-\Delta\Delta Ct}$ method. Immunosuppression by injection of prednisolone-acetate was introduced for a 30 day period, and tissue sampled at Day 180 and relative quantification estimated. No mortality or clinical signs of disease were observed in the challenged group. The challenge resulted in detection of NNV in blood, spleen, kidney, liver, heart atrium, heart ventricle at Day 25, and by the end of the experiment, NNV showed a clear

increase in brain and retina, suggesting these to be the primary tissues for viral replication. There were no increase in relative amount of NNV in blood, atrium, ventricle, spleen, liver and kidney. Corticosteroid implants resulted in a weak increase in virus RNA in spleen, kidney, liver and brain. These findings suggest that Atlantic cod is susceptible to infection with NNV from halibut. The observed tissue tropism patterns suggest an initial viremic phase, followed by neurotrophy. Head-kidney is the best tissue identified for possible NNV detection by non-lethal biopsy, but detection was not possible in all injected fish.

Paper III

In this paper experimental horizontal transmission of nervous necrosis virus NNV originating from halibut *Hippoglossus hippoglossus* was studied through cohabitation of intra-peritoneally (i.p) injected and healthy fish for 125 days. The experimental groups consisted of i.p. injected turbot *Scophthalmus maximus* or i.p. challenged Atlantic salmon *Salmo salar* with salmon or Atlantic cod *Gadus morhua* cohabitants. The initial weight were 10 g for cod, 40 g for salmon and 3 g for turbot. NNV was detected in brain, eye and spleen by real-time RT-PCR in cod cohabitated with i.p. injected turbot after 90 and 125 days. The data are suggesting that NNV has been transferred horizontally from the turbot to cod. There was no detection of NNV in salmon in cohabitation with i.p. challenged turbot or salmon. This study shows that NNV strains from halibut may be transferred to cod via water. Hence there is a potential risk of horizontal transmission of the virus from halibut to cod farms. The lack of detection of NNV in cohabitant salmon suggests that this fish species is less susceptible to horizontal NNV transmission than cod. But this result might be influenced by the size of salmon, viral load in i.p. challenged cohabitants and duration time of the experiment.

Paper IV

This paper reports viral encephalopathy and retinopathy (VER) in 5 to 24 g sized farmed Atlantic cod *Gadus morhua* kept in sea cages at Parisvatn, Hordaland county, at the west coast of Norway. Moderate mortality (10-15 %) was observed along with anorexia and abnormal swimming behaviour, like looping or spiral swimming and reduced coordination. Nodavirus was detected by two different real-time RT-PCR assays, and this was later confirmed by immunohistochemistry. This paper describes the first outbreak of VER in farmed cod in Norway, and the first report that VER affect cod exceeding 5 g in size.

4 SYNOPTIC DISCUSSION

In 2003, when this study was initiated, nervous necrosis viruses had only caused disease problems in farming of Atlantic halibut and turbot. VNN had caused high mortality in juvenile fish during the late 90ties, and was listed by Norwegian Food Safety Authority (Mattilsynet - FSA) as a notifiable disease. As VNN only was reported in farmed marine fish, and limited to a few outbreaks in an industry producing less than 2000 metric tons, little attention was paid to the disease. The halibut and turbot farmers were the only ones who worried as VNN was (and is listed as notifiable in Norway) with possible severe consequences upon detection of the virus, such as stamping-out of the fish population and fallowing of the farm site.

NNV as a fish pathogen can be viewed from three different points of view; scientific, fish farmer and FSA. All three should be regarded as stakeholders with different interests and responsibilities. From the scientific point of view the virus is an internationally well known pathogen, affecting numerous species and causing a disease with distinct features. There are numerous scientific papers describing the virus and disease characteristics, which form the basis for scientific advises to both the farming industry and FSA from national research institutions in Norway. From the farmer's point of view, the virus represents a possible disease problem and consequently economic losses to the company, either because of mortality or as a result of stamping-out of the fish population due to detection of a notifiable disease. From FSAs point of view, the virus represents a potential national disease problem, and based on impact on farmed fish like number of outbreaks, mortality rates and spreading of the disease (geographic distribution of disease outbreaks) it has been listed as notifiable. Status in 2003 was consequently that VNN was a problem affecting halibut and turbot, and the disease was closely monitored by the FSA. However, compared to disease problems in the Atlantic salmon industry, VNN was regarded a marginal problem.

From the scientific point of view NNV was interesting, not only due to the disease problems observed in Norwegian halibut and turbot farming, but also due to the fact that a new promising farmed fish species was starting to increase in volume – the Atlantic cod. At the beginning of the millennium, a lot of research and effort was put into solving problems in the production of cod juveniles. As experienced with halibut, a lack of survival in production of juveniles restricted development of a farming industry around this species. However, in contrast to halibut, cod was regarded as a more promising fish species, principally due to a larger consumer market. Reduction in catches of wild cod and a predicted willingness in the

market to buy farmed cod boosted interest for farming. Consequently the number of cod farming licences increased dramatically during the first half of this decade. The optimistic prospects of cod farming could be exemplified by Kjesbu *et al.* (2006), who estimated the world production to reach 150-200 000 metric tons in 2015. Most of this was predicted to take place in Norway. In other words, cod was considered to become the new "salmon" in the Norwegian aquaculture industry. Establishment of a new farmed fish species led to discussion on possible disease problems, particularly bearing in mind the problems experienced in salmon farming, and one of the diseases in question was VNN. Although this disease was restricted to farmed halibut and turbot in Norway, there were reports on susceptibility in farmed Atlantic cod in the UK (Starkey *et al.* 2001) and Canada (Johnson *et al.* 2002). Another question raised was a possible impact on salmon, the most important farmed fish species in Norway. This was particularly pointed out in discussions on possible poly-culture or co-localization of salmonides and marine fish species. Outbreak of VNN has never been reported in salmon, other than reports of nodavirus-like particles in heart tissue in salmon suffering from cardiac myopathy syndrome (Grotmol *et al.* 1997b, Nilsen and Nylund 1998) and encephalitis in farmed smolt in Scotland (Scullion *et al.* 1996). When all these considerations were taken into account, a study of possible transmission of NNV to and between farmed Norwegian fish species was initiated. In order to evaluate possible consequences for the aquaculture industry, the study aimed to investigate possible susceptibility and horizontal transmission of NNV to salmon and cod. In addition, a study of virus tropism was performed on cod to investigate how the virus is affecting the host in terms of tissue distribution, and subsequent identification of possible organs suitable for non-lethal biopsy for screening purposes.

4.1 Studying possible NNV susceptibility in Atlantic salmon

One of the first tasks was to investigate if Atlantic salmon is a susceptible host to NNV. As there were few and non-conclusive reports on possible NNV infection in salmon, the hypothesis was that NNV would not replicate or cause disease in this species. When designing a challenge study, some choices had to be taken on how to test the hypothesis. The first decisions to make were the type of challenge suitable for studying susceptibility, size of fish and which NNV isolate or strain to apply.

In order to determine if Atlantic salmon could be a susceptible host for NNV, an experimental study should demonstrate that the virus is present in the host, is replicating in nervous tissues and increase in amount during an experimental period. Susceptibility is, in the

simplest form, typically linked to development of disease, where susceptible hosts are those which develop clinical disease and mortality. This is however over-simplifed, and without defining the term disease, it is important to recognise that fish might be susceptible to NNV infection without showing any signs of disease, i.e. as persistently infected or as asymptomatic carriers. The fate of the virus is important, as the pathogen should survive in the susceptible host and be able to multiply in numbers (replicate) to complete the viral lifecycle. In studying viral infections, increased amounts of virus could either result from replication or from accumulation of virus particles in a specific tissue, known as antigen trapping. Consequently, the study should also demonstrate that the viral increase is larger than the dose of virus that the host has been exposed to, and that virus is present in target organ or tissue, for NNVnervous tissues. This is particularly important when using an injection challenge model. Susceptibility is depending of the fate of the virus when entering into the host and various scenarios may be outlined. If the virus is not increasing in amount and eventually cleared, no state of susceptibility exists. This scenario was shown by Banu and Nakai (2004) when injecting NNV in mice (intra peritoneally and intra muscularly), where the virus amount decreased during the experimental period (72 h) and was not detected in central nervous tissues. On the other hand if the virus is replicating, and increasing in amount in central nervous tissues, a state of susceptibility likely exists. There is also a possibility that the virus is neither replicating nor cleared in the host. Then the virus may be maintained in the host for a prolonged period of time. The fate of the virus in the end may define the status, where susceptibility is dependent on replication and presence of virus in central nervous tissues eventually will take place. However, if the virus is cleared, there is likely no state of susceptibility. The most obvious observation of susceptibility is when clinical signs and mortality is present (followed by detection of NNV), but the state of susceptibility is not dependent on development of clinical disease. In summary, when determining if a host may be susceptible to NNV, an increasing amount of virus in central nervous tissue should be demonstrated.

When considering type of challenge there are in general three possible approaches, including cohabitation of healthy salmon with NNV diseased fish, bath challenge of salmon in a suspension of NNV or intra muscular- or intra peritoneal injection of NNV. All approaches have advantages and disadvantages. Cohabitation challenges require a diseased cohabitant fish, either naturally or experimentally infected, which then shed NNV to the healthy fish in the challenge experiment. Bath challenge requires an NNV strain which is propagated in a cell-line, and then re-suspended in water to expose the challenged fish. Injection of NNV

might be regarded as an artificial way of infecting fish, as important parts of the fish immune system (dermis and mucus) is bypassed, but could be regarded as a controlled way of distributing a known amount of virus. The isolate of NNV used for challenging is also influencing the method, i.e. bath challenge requires a cultureable strain to be used. In addition, the size of the fish used is also influencing the method, as juveniles are not suitable for injection due to the small size. When considering the proper challenge method to study NNV susceptibility in salmon, the size of fish was the first parameter discussed.

As NNV has primarily been associated with marine fish species (see Introduction), the hypothesis was that Atlantic salmon could be exposed to the virus in the marine environment, i.e. from wild fish species or farmed fish species such as halibut or cod. Atlantic salmon is hatched and raised in fresh water, and when transferred to marine growth sites the salmon has adapted to the marine environment through a smoltification process. This adaption process is influenced by the size of the fish and light conditions (photoperiod) prior to migration from fresh water to the sea. In commercial hatcheries this is controlled by growth (temperature) and light manipulation (day length) to produce a seawater-adapted smolt. The commercially produced smolts are categorized as 0+ or 1+ smolt, depending on the time spent in the hatcheries. A 0+ smolt is typically 40-60 grams and a 1+ smolt 80-100 grams when transferred to the marine growth site. Only salmon adapted to seawater were considered in order to study any NNV susceptibility.

The next consideration was which NNV isolate to apply. At the time when the study was initiated, only two isolates were available and both originated from Atlantic halibut. The AH95Nor isolate was isolated from juveniles of diseased halibut in a commercial farm that observed high mortality in the fish population. Grotmol *et al*. (1995, 1997a, 2000) characterized this isolate, and it has successfully been propagated in the cell line SSN-1 (Dannevig *et al*. 2000). The other candidate was at the time newly isolated from a VNN outbreak with high mortality in a commercial halibut hatchery. This isolate was isolated from diseased juveniles received by the University of Bergen, and was given the identity code AAG01/03. When considering these two isolates, AAG01/03 was favoured as it was recently isolated from a natural VNN outbreak and thereby confirmed to be pathogenic to Atlantic halibut juveniles. The main problem was, however, that AAG01/03 was not propagating in the SSN-1 cell line which was the only cell line available at the time. AH95Nor was possible to propagate, but this isolate had been passed through several cell passages with possible changes of viral characteristics. By opting for the AAG01/03, due to the recent origin, this eliminated the possibility to produce large amounts of virus for any bath challenge. In order to

use the isolate, the solution was to prepare a homogenate from infected tissues (brain) of diseased juveniles and distribute the NNV challenge as an injection. This is not without problems, although bacteria are eliminated by sterile filtration (0.2 μm), any viral pathogen (and prions and viroids) could possibly be carried forward in the homogenate. It was consequently screened for known fish pathogenic viruses causing problems in salmon farming, i.e. IPNV, SAV and ISAV. The selection of the AAG01/03 isolate and homogenate approach left the susceptibility study with the choice of a cohabitant challenge or as direct injection of NNV in Atlantic salmon.

The use of AAG01/03 isolate from halibut also led to some discussions of relevance due to possible host specificity of NNV. Alternatively an isolate from cod could be used, due to the growing cod farming industry and discussions on possible duo-culture or co-localization of salmon and cod. However, there existed no such isolate of NNV in Norway at the time. The first outbreak of VNN in cod in Norway is in fact described in Paper IV, and importing a foreign strain was not regarded as an option as VNN is regarded a notifiable disease in Norway. As outlined in section 1.3.1 on host specificity of NNV, the literature is not conclusive on this matter. Although host specificity has been suggested to be linked to nucleotide variations in the T4 regions of RNA2 (Ito *et al*. 2008), this has so far not been verified by other studies using different strains and genotypes of NNV. What might be an argument contradicting host specificity is the large variations observed, where NNV strains cluster heterogeneously (see section 1.1.3) into different genotypes and further into subgroups within the clusters. The possibility of host specificity and relevance to the aquaculture industry is discussed in section 4.3. But as a general consideration, a recent observation of an NNV isolate from farmed cod that is genetically similar to halibut isolates (Nylund, unpublished data) suggests there is no definitive host-NNV-isolate link. Although this is just a single observation, it suggests that the use of a halibut NNV may be relevant for studying susceptibility in salmon.

Next consideration was to determine a suitable type of challenge. The use of a homogenate for distributing NNV would anyway result in injection of virus in either an experimentally infected cohabitant fish or directly into the host itself. Use of cohabitation (horizontal transfer) to challenge the salmon would require use of a suitable NNV host, i.e. a experimentally infected halibut. The benefit of this is to mimic a natural transmission way of NNV, a pathway which has been described in several papers (see section 1.3.2). However, as the hypothesis was that salmon probably not was susceptible, an injection of NNV would reveal if the virus eventually would be cleared if forced into the host. This would simplify the

challenge experiment and eliminate the need for an NNV carrier fish (cohabitant) to observe any horizontal transfer of virus.

4.1.1 Experimental intra peritoneal injection of NNV in Atlantic salmon

The challenge of Atlantic salmon with NNV isolate AAG01/03 (Paper I) resulted in mortality in the i.p. challenged group. Also, the challenged fish gradually developed abnormal behaviour, such as hyper-activity and erratic swimming not seen in the control group. Eventually the diseased and moribund fish showed loss of co-ordination and became lethargic. The observation of clinical signs of disease and mortality was unexpected, but the real-time RT-PCR analysis of brain samples revealed presence of NNV. The first detection of NNV in samples was at day 7 post injection of virus, and the samples analysed were positive for the remaining time of the experiment, with samplings at Day 25, 45, 69 and 125. Interestingly, the NNV amount seemed to peak at Day 25, followed by a slow reduction of virus to Day 125. This might suggest that the virus eventually could be cleared, a possibility that could not be examined within the timeframe of the experiment. The detection of NNV in brain was supported by independent detection with immunohistochemistry (IHC). IHC detected specific NNV immunolabeling in histology sections of brain from challenged salmon 34 days post challenge, confirming the real-time RT-PCR results. The immuno-labelled section revealed that NNV was scattered in the brain tissue, in contrast to observations in injected turbot which had focal aggregations in the brain (Paper III). Further examination of the injected salmon with electron microscopy of a brain section at Day 134 confirmed presence of virus particles resembling NNV in shape and size. Consequently, this demonstrates that NNV were transported from the injection site to brain. The state of replication of NNV is supported by the relative increase of virus from 10^3 to 10^4 from Day 7 to 25 post injection of virus and the occurrence of clinical signs of disease, with observed histopathology in CNS. Hence, important observations from this study were presence of NNV in CNS, clinical signs of disease consistent with VNN and mortality. The observations in salmon suggest that a halibut-originating NNV is neuroinvasive, able to replicate and cause disease when distributed as an i.p. injection. These observations suggest susceptibility, but as injection is a by-pass of important immune defence mechanisms such as the physical barrier of mucus and epidermis, salmon with intact "barriers" may still be resistant to infection.

4.1.2 Experimental cohabitation challenge with NNV to Atlantic salmon

As bath challenge with the AGG01/03 NNV isolate was not an option (see section 4.1), a cohabitation model with NNV-injected fish and healthy (non-injected) cohabitants was chosen.

Turbot was selected as one of two fish species used as an i.p. challenged source for waterborne transmission of virus. Turbot has been shown to be a susceptible host to NNV, and has been used in a challenge model for SJNNV (Husgard *et al.* 2001). In addition, turbot has been used successfully in challenge experiments using a halibut isolate of NNV (unpublished data), suggesting this fish species would be suited for a cohabitation challenge study. The second virus carrier was salmon, and it was included to examine any possible transmission of virus from an experimental i.p. challenged salmon to a healthy cohabitant salmon. Adding further complexity to the study, both AAG01/03 and AH95Nor were included. The idea was to compare the two NNV isolates, and examine if there would be any differences in transmission from an i.p. challenged salmon to non-injected fish. Consequently, the experimental groups consisted of one with i.p. challenged NNV (AAG01/03) turbot and healthy non-injected cohabitant salmon, one with i.p. challenged salmon (AAG01/03) and healthy salmon, and one with i.p. challenged salmon (AH95Nor) and healthy cohabitant salmon. A negative control group with i.p. challenged salmon (L15 cell medium) and non-injected salmon was also included. In addition to these experimental groups, two positive control groups with turbot were included to examine horizontal transmission of both NNV isolates. This was important, in order to demonstrate that the challenge model is working and virus is transmitted via water from i.p. challenged to non-injected turbot. Finally, a group with turbot and Atlantic cod and a negative control group with cod were added (see section 4.2).

In order to keep the number of experimental animals to a minimum, 10 fish in each group were i.p. challenged and kept as cohabitants with 30 non-injected fish. Sampling and analysis protocols are described in Paper III. Duration of the experiment was set to 125 days, in order to provide sufficient time for the NNV to be established in the i.p. challenged fish and time to infect cohabitant fish. This was based on observations in i.p. challenged salmon (Paper I) and cod (Paper II). Tissues subject to investigation were CNS (brain and eye) and in addition spleen was included as representative for haematopoietic tissues. The spleen was chosen based on observation in i.p. challenged cod, where NNV was first detected in spleen, kidney and ventricle with subsequent entrance to CNS (Paper II).

NNV was detected in all i.p. challenged turbot and salmon used as source for waterborne transmission of virus, demonstrating that virus was present in all challenged

groups. In the positive control groups with turbot, the cohabitating non-injected turbot were also positive for NNV, demonstrating that both virus isolates could be horizontally transferred under the experimental conditions provided. However, NNV was not detected in any non-injected salmon examined, suggesting there was no transmission or entrance of virus from the NNV i.p. challenged cohabitants. But, in contrast to salmon, NNV was detected in non-injected Atlantic cod kept with NNV i.p. challenged cohabitant cod (see section 4.2.2), which may demonstrate differentiation in susceptibility between these two fish species.

The apparent lack of transmission of NNV to salmon by cohabitation with i.p. challenged fish may relate to the host itself. Studies of transmission of NNV to sea bream (*Sparus aurata*) suggest that this fish species is an asymptomatic carrier of the virus (Castric *et al.* 2001). In challenge experiments with sea bream using i.p., intra muscular (i.m.) and bath exposure only i.m. produced NNV infection and mortality in fish of 2 grams (Aranguren *et al.* 2002a). Although methods to detect NNV in this study were restricted to cell culture and immunoperoxidase assay with less sensitivity than PCR, it may demonstrate that results obtained by i.p. injection may not be reproduced with bath or waterborne transmission of virus. However, Arraguren *et al.* used sea bream with average weight of 2 grams, while Castric *et al.* used fish that was 15 grams in average. These two studies may demonstrate that sea bream is a susceptible host, although development of clinical signs and mortality may be influenced by size of fish and challenge model. The lack of transmission of NNV to cohabitant salmon may also be influenced by the fish size applied, potential little shedding of virus from i.p. infected fish or insufficient time for establishment of detectable levels of NNV in the non-injected cohabitants.

4.1.3 Is Atlantic salmon an NNV susceptible host?

Generally, a susceptible host could be regarded a host in which the virus is able to invade, survive in terms of not being cleared and replicate. The state of susceptibility is subsequently not relying on development of disease in the host, but rather the ability of the virus to proliferate in permissive cells and resist degradation or clearance. The degree of susceptibility may also be linked to virulence; the ability of the virus to invade, survive and proliferate in a susceptible host.

Based on the data obtained from i.p. NNV challenge (Paper I) and cohabitation challenge (Paper III) studies, answering this question requires that some important issues must be addressed. Although these two studies seem to contradict each other, the general consideration should be to regard a seawater adapted smolt as a *potential* susceptible host to

NNV. If NNV enters the smolt, i.e. the blood, the virus seems to have the capacity to replicate in target cells and cause disease. However, salmon may have a natural high degree of protection against NNV infection, possibly through the first line of defence (skin and mucus) which appear to be the most important barrier against infection. As a practical approach in farming of salmon, we may regard NNV susceptibility in smolt as depending on various degrees of risks influencing the likelihood of virus infection. Consequently, there are several factors which may affect this risk that could be suggested, based on general considerations of the host immune system and viral characteristics. These comprise host habitat, size and immune system and virus properties.

First, salmon may be a naïve host in a natural habitat. Geographic separation of host and virus may protect wild salmon, which spawn in fresh water and migrate to the sea as smolt. Although there are reports on NNV in hosts in freshwater (see section 1.2.3) there are so far no reports of virus detection in natural habitats of salmon (lakes and rivers). While likely not exposed to the virus in the juvenile phase, this may occur after migration to the sea. NNV is detected in wild fish species (Table 1), but in order to get infected salmon would have to be in the same habitat as infected wild fish where exsposure to NNV could occur. In the natural life cycle of salmon, a host-pathogen interaction (in terms of co-evolution) may never have evolved, making a natural barrier against NNV infection. However, in farming of salmon exposure to NNV may occur when assembling large populations of salmon at a high number of marine growth sites. This would greatly increase the possibility for contact with virus originating from wild or farmed NNV susceptible fish species.

Larvae and juveniles of salmon may show a higher degree of susceptibility. The size of smolt may provide some protection when transferred to a marine growth site, and as the fish grow larger the likelihood of NNV infection and disease development decreases. However, size alone may not be a protective mechanism, as NNV has been reported in adult brood fish of halibut (Aspehaug *et al.* 1999) and wild adult cod (Nylund *et al.* 2008, own observations).

The apparent inability of the applied NNV isolate to invade healthy cohabiting smolt (Paper III) may also be linked to the host immune system. In particular this may relate to the innate immune system, and first and foremost the physical barrier of skin and mucus. It is possible that salmon is able to clear NNV in contact with the innate immune system, as part of general defence mechanisms against pathogens. This may also be dose dependent, where small amounts of NNV are unable to reach susceptible cells before being cleared. However, if the amount of virus is increased, the immune system suppressed (i.e. stress) or compromised

as a result of by-passing barriers, NNV may be able to invade and eventually reach susceptible cells in the host.

The wide variety of NNV isolates and strains, worldwide distribution and existence of different genotypes (see section 1.1.3) may suggest variations in virus properties. The apparent infection-resistance of smolt challenged with NNV may be linked to the differentiations in virus isolates and the strains ability to invade a host, which again may result in viral host specificity or preference (see section 4.3.2 on host specificity). Several entry routes for NNV into the host have been suggested such as via the bloodstream, axons and intestinal epithelium (see section 1.3.3), and this may require cell recognition and binding to specific cell surface receptors by the virus. As NNV seems to have some host cell preference (see section 1.3.4), this suggests that the virus has limitations in type of cells susceptible to infection. Salmon may be naturally protected against horizontal transmission of NNV if the virus is unable to reach susceptible cells (i.e. cells of CNS). However, the i.p. challenge of smolt demonstrated that there are susceptible cells and that the virus was transported from the injection site and eventually become neuroinvasive.

In salmon farming, there are several ways of in which the risk of NNV infection may increase. If there is a high degree of protection in salmon against horizontal transmission of NNV, this might be compromised. In general this could occur when sub-optimal conditions for salmon arise during farming procedures, resulting in immunosuppression and increased risk of pathogen infections. Such conditions could be exemplified by fish handling (i.e. grading, feeding, high fish density) or changes in environmental conditions (i.e. increasing temperature, oxygen depletion). Entrance to the host may also be provided by parasites, i.e. by salmon lice (*Lepeophtheirus salmonis*) causing erosion and lesions of epidermis and consequently access for NNV directly to the host bloodstream. Skin erosion and possible development of wounds may also be the result of high fish densities (in sea-cages or during transportation), with subsequent risk of invasion of pathogens such as NNV. The observation of NNV in epithelium cells of striped jack (Nguyen *et al.* 1996) also suggests ectoparasites may play a role in transferring virus between hosts. The virus may enter the salmon as a secondary infection, where another pathogen may provide access for NNV through host immunesuppression and/or inducement of disease development. Finally, considering the wide genetic variety of NNV described (see section 1.1.3) possible existence of a strain or isolate (other than AAG01/03) with a better or high capability to invade Atlantic salmon cannot be ruled out.

In summary, Atlantic salmon should be regarded as a potential susceptible host and in farming caution should be taken to avoid increasing any risks of NNV infection. Therefore, salmon farms should not be located in close proximity of farmed fish species known to be susceptible, such as Atlantic halibut or cod.

4.2 Transmission of NNV in Atlantic cod

In 2006 the opportunity to investigate a natural outbreak of VNN in farmed Atlantic cod in Norway arised. Although cod previously had been reported as a susceptible host to NNV (see section 1.2.3), the detection of the virus in this species demonstrated that NNV could be a problem in Norwegian cod farming as well. However, this was also anticipated back in 2003 when this study was initiated, and cod was consequently included. The NNV problems experienced with halibut farming (see section 1.2.4) suggested that the virus could cause high mortality in rearing of juveniles. There was also a concern that NNV could spread from farmed halibut to farmed cod. Consequently, studying susceptibility in cod to virus originating from halibut was regarded as both relevant and important.

The approach of examining susceptibility in cod to a halibut originating NNV faced the same decisions to be made regarding type of challenge and size of fish. As a starting point, this study followed in the same track as the salmon study and applied the AAG01/03 isolate. However, in contrast to salmon, the hypothesis was that cod was most likely susceptible. The focus was on examining the fate of NNV, in terms of tissue tropism, and monitoring any development of disease. Consequently the size of the cod utilised was important to determine.

The study opted for cod in the same size range as the salmon used, and there were a number of reasons for this choice. First, this would be representative for farmed cod that have been transferred from a pre-stocking facility to a sea-cage growth site. Second, the cod would be out of the typical size where VNN had been reported (see section 1.2.1). This was regarded as favourable, as the study aimed to examine the fate of NNV in a cod ready for transportation to an on-growth site and with a fully developed immune system. A link to farmed cod with mimicry of conditions the fish could face was also considered favourable, and consequently influenced the choice of size of both salmon and cod utilised in the study. In cod farming, juveniles are typically transported to a pre-stocking facility at the size of 3-5 gram and kept to a size suitable for transportation to sea-cages for on-growth to marked size of 4-5 kilogram.

As with the study of NNV susceptibility in salmon, i.p. injection of NNV was the chosen challenge model. Although this is not a natural way of transferring the virus to cod, this method provides a controlled way of distributing equal amount of virus to each individual

fish. Monitoring tropism of AAG01/03 in cod would target any susceptibility of cod to this halibut isolate and potential of disease development, but also provide information on possible tissues or organs for none-lethal biopsy useful in brood stock screening for presence of NNV.

4.2.1 NNV tissue tropism in experimental i.p. challenged Atlantic cod

In this experiment two groups of Atlantic cod with average initial weight of ca. 100 grams were used to examine NNV tissue tropism following i.p. challenge (Paper II). The study aimed to investigate tissue tropism and any effect of induced immunosuppression. This would mimic an effect of sexual maturation and spawning or sub-optimal rearing conditions in a cod farm. The hypothesis was that immunosuppression would induce increased replication of virus in tissues and possibly result in development of disease. The effect of this on virus in tissues examined would possibly reveal if any candidates for none-lethal biopsy could be suggested.

A total of 12 different tissues were sampled and real-time RT-PCR analysis of samples from Day 25 revealed that NNV was detected in heart, spleen, liver and kidney in 4 out of 5 fish examined. In contrast tissues as gills, pectoral fin, barbell, lateral line and brain were positive in only 1 (not the same fish) out of 5. These results suggest that NNV was transported from the i.p. injection site and caused an experimental infection in challenged fish. Further, a large relative increase of NNV in brain and retina from Day 25 to 130 suggests the virus (isolated from halibut) is neuroinvasive and able to replicate in Atlantic cod. The tissue tropism observed at Day 25 may indicate a systemic infection, but the results do not support that replication was taking place in tissues examined, as presence of virus could be suggested to be a result of antigen trapping in these tissues. Between Day 25 and 130 there was no increase in relative quantification of virus in heart, spleen, liver and kidney tissues, but a large relative increase was observed in brain and retina (eye) with a 10^5 and 10^3 fold increase respectively. The latter suggests that virus replication was taking place in these tissues, and presence of NNV was verified by IHC. Strong immunolabelling was observed in both brain and in external plexiform layer and the basal segments of the visual cells in retina (see Figure 3A or Paper II). Interestingly, no clinical signs or mortality were observed during the experimental period, suggesting that cod of this size (above 100 grams) is less likely to suffer from VNN (see section 1.3.2). Further studies should be carried out to determine if any development of disease will occur and subsequent viral load necessary to cause mortality in cod of this size.

The use of EF1A as reference gene revealed differences in expression between tissues investigated (see Paper II). Ideally, the expression should be almost identical in all tissues in order to be able to compare samples. However, in the i.p. challenged fish the most stable expression of EF1A was found in gills, eye (retina) and brain. In contrast, atrium, ventricle and lateral line (including muscle) showed a 10-fold less expression when compared to the former, suggesting that comparison between diffenrt tissues in this case should be avoided. Calculation of relative amount of virus should only be done between the same tissue types, i.e. between brain samples at various sampling days when using cod EF1A as reference gene.

In the real-time RT-PCR detection of NNV in tissues, there was no use of a Ct cut-off value. Any signal returned from the PCR which was defined to be above the threshold and not caused by background were included in the data set. This means that there exists a possibility for false positive signals, particularly when the signal was detected at the terminal cycles of the PCR. However, as the study aimed to provide a general overview of how NNV is distributed in the tissues examined, bias could be introduced if some signals were eliminated by a cut-off. Although there might be both false positive and negative detections, the general trend of how NNV is distributed was clear (see Table 1 in Paper II). In this study, each tissue types were analysed from 5 fish and these acted as 5 parallel samples. The Ct signals were divided into different groups, consisting of those above 35 and below 40, those between 25 and 35 and finally those below 25. This way of grouping may seem artificial, but is a way of visualizing the results to evaluate the data set. Ct-values from 35 to 40 are at the terminal end of the cycles and generally have a lower reproducibility and are not suitable for sequencing and verification. Sequencing of NNV is usually only possible in tissues with Ct-values below 35 (own observations). When performing relative quantification, the obtained Ct-values from NNV detections were normalized by deducting the corresponding Ct-value of EF1A expression to produce a ΔCt for all samples.

Immunosuppression was induced by injecting prednisolone-acetate (Johnson and Albright 1992) to investigate the effect of this on NNV load and distribution in the i.p. challenged cod. The hypothesis was that such induction most likely would trigger increased virus production and increase the viral load. In vertical transmission, sexual maturation may result in increased viral replication in persistently infected fish or higher susceptibility to horizontal transmission due to suppression of immune defence mechanisms. The observed effect of the prednisolone-acetate implant was an apparent increased viral replication. Therefore, the effect of immunosuppression should be examined further, as vertical transmission is likely occurring (see section 1.3.2 and 4.3.1).

The detection of VNN in commercially farmed cod in 2006 (see Paper IV) provided an opportunity to compare tissue tropism in the experimentally and naturally infected cod. The examination of tissues in farmed cod suffering from VNN was also regarded as important to validate the experimental i.p. challenge approach. Interestingly, both fish groups showed a similar tissue tropism pattern (see Table 2 in Paper II), where NNV was detected in all tissues examined. This observation in diseased cod suggests that i.p. challenge is producing a similar distribution of NNV. Another interesting observation resulted from relative quantification of NNV in brain and eye (retina) tissues. The diseased cod had a 10^3-times the viral load of NNV seen in these tissues as the i.p. challenged fish at Day 130 (not shown in Paper II), which may explain development of clinical signs and pathology in the farmed fish.

Examination of NNV tissue tropism may also help in determining how to select virus free fish. To help establish NNV-free cod brood stock in farming, tissues suitable for non-lethal biopsy sampling are needed. Since these must be accessible for sampling without any detrimental effects to the fish, organs such as spleen, heart, liver, brain and retina are inaccessible. Tissues examined here were blood, gill, pectoral fin, barbel and head-kidney. Using the relative amount of NNV in brain and retina samples as cross reference, organs suitable for biopsy should reflect these tissues. At Day 25 post injection, only kidney and blood samples showed detectable levels of NNV. Nevertheless, NNV is likely to enter into the bloodstream from the abdominal cavity after i.p. challenge, and upon transportation in blood would result in its establishment in other tissue types. At Day 130, only 1 out of 4 blood samples were positive for NNV compared to 4 out of 5 head-kidney tissues. Apparently, NNV is gradually cleared from the blood following challenge. The tissues from gills, pectoral fin, lateral line and barbel were better suited at this day, but this changed at Day 180 where kidney appeared to be the best tissue (see Table 1 in Paper II). Examination of the commercially farmed cod with VNN diagnosis revealed that almost all tissues examined were positive for NNV (see Table 2 in Paper II), suggesting that the apparent systemic infection observed following i.p. challenge model also occur during a natural VNN outbreak. Based on the observations in the study, head-kidney biopsy samples appear to be best suited for the detection of fish with persistent NNV infection (9 out of 10 detected). The head-kidney in cod is accessible for biopsy sampling, and by targeting the kidney this allows screening for other important viruses and bacteria such as IPNV and *Fransicella piscicida*, the latter a major bacterial problem in cod farming in Norway (Nylund *et al.*, 2006, Olsen *et al.* 2006). The timing of biopsy sampling might also be optimized, since stress or sexual maturation appear to trigger NNV replication and hence may increase detection efficiency. Repeated biopsy

sampling supplemented by screening of reproductive fluids and offspring may eventually lead to NNV-free brood stocks of cod, which would be the best prophylaxis against VNN epizootics among juveniles.

4.2.2 Experimental cohabitation challenge to Atlantic cod

The results from the i.p. challenge of cod suggested that cohabitation challenge should be performed to investigate a more natural way of transmitting the virus. Consequently, both cod and salmon were included in the cohabitation challenge experiment to study horizontal transfer of NNV (Paper III).

In this experiment cohabitant Atlantic cod and i.p. injected turbot were kept in the same tank (see details in Paper III). The NNV isolate applied was the same as used in i.p. challenge of cod (Paper II), originating from Atlantic halibut. Virus was in this experiment detected by real-time RT-PCR in cohabitant cod after 90 and 125 days (see Table 3 in Paper III), where 9 out of 10 cohabitant cod examined tested positive for NNV. These detections strongly suggest that the virus has been transferred horizontally from experimentally infected turbot to the cohabiting cod in the tank.

Interestingly, the virus was detected in both brain and eye (retina) samples, suggesting that the virus originating from halibut is capable of entering the primary organ of replication (CNS). The examination of NNV in i.p. challenged cod has suggested that brain and eye are important for replication of virus in this fish species as well (Paper II). In addition, the detection of NNV in a cohabitant cod may suggest that cod is a susceptible host and is likely to be infected with waterborne NNV. However, although horizontal transmission of NNV has been demonstrated in the literature (see section 1.3.2), the key observation in this study was transmission of an NNV isolate originating from a different fish species. This supports the results in Paper II, where i.p. injection of virus eventually established infection in CNS and further was detectable in most tissues investigated.

However, the cohabitation study did not demonstrate that replication of NNV had occurred in cod. The fate of the virus beyond termination of the experiment at Day 125 is unknown. Presence of the virus in some tissues investigated may be the result of antigen trapping, but since evidence was found for the entrance of the virus to the CNS, this suggests a possibility that replication may eventually occur. Also, according to Nylund *et al.* (2008) the NNV genotype detected by Starkey *et al.* (2001) in cod belong to tha halibut clade of NNV, as does an isolated from farmed cod in Norway (Nylund in Paper II). In both cases sequencing of the virus from host tissues suggests significant virus load and hence suggests NNV replication

had occurred. Triggering of replication could be hypothezied to occur during periods where a state of immunosuppression is present, exemplified with sexual maturation, spawning or stress. A cod with a persistent NNV infection may represent a risk of spreading virus if used as wild-caught brood fish or if cod is infected as farmed fish and carried forward as brood fish. Persistency in farmed cod may also represent a risk of horizontal transmission to cohabitants or wild fish in close proximity of a farming site. In addition, a horizontal transmission of an NNV isolate originating from halibut between two other unrelated fish species is an interesting observation, and raises questions of possible consequences. If NNV strains are capable of being transmitted horizontally across the species barrier, this suggests there is a higher risk of spreading the virus in both farmed and wild populations of fish than might anticipated. The close relationship between halibut and cod isolates of NNV (see section 1.1.3 and 4.3.2) may also suggest that fish species in the Gadiformes and Pleuronectiformes families may be susceptible hosts if exposed to NNV from the BFNNV clade. Further, the observation by Nguyen *et al.* (1996), who detected NNV in epithelium cells in skin of striped jack larvae, raises the question of possible transmission routes. The role that common ectoparasites, like *Caligus elongatus* with low host specificity (Øines and Heuch 2007) may play in transferring NNV between marine fish species is yet to be determined, but could be suggested as being potential vectors in spreading the virus.

4.2.3 VNN in farmed Atlantic cod

In August 2006 there were reports from a combined research and commercial cod farming facility at Parisvatn (in the county of Hordaland) of increased mortality and clinical signs consistent with VNN (see section 1.2.4). This provided an opportunity to investigate a suspected outbreak of VNN in commercially farmed cod in Norway (Paper IV).

Moribund fish were sampled at the site in August and September, and as NNV was the primary suspect in this case brain samples were analysed with real-time RT-PCR. This resulted in a strong positive detection of NNV in the brain samples (low Ct-values), but bacterial sampling also resulted in detection of the fish pathogenic bacterium *Listonella (Vibrio) anguillarum*. At a later stage, immunohistochemistry was performed on brain and retina to confirm the presence of NNV with typical histopathology with vacuoles and focal necrosis (see Figure 3 in Paper IV). The samples were screened for other pathogens, but tested negative for IPNV and *Francisella piscicida*, both known to be pathogenic to cod.

As two known pathogens (NNV and *L. anguillarum* causing vibriosis) were present in the diseased fish, this raised question of which one caused the primary infection or was

responsible for the mortality. Since the sampling first took place when fish were suffering from disease, it proved difficult determining which pathogen causing the primary and secondary infection. But, it is also possible that a synergistic effect of the two pathogens may have existed, triggering development of disease. As a general observation; in later diagnosed outbreaks of VNN in commercial cod farms, other pathogens than NNV have frequently been detected in the diseased fish (own observations).

In determining the role of NNV in the disease outbreak at Parisvatn, a closer look at the anamnesis could provide some useful information. The cod were transferred to sea-cages at the size of 2 grams in June 2006, and were prior to this vaccinated against vibriosis. The mortality rate was estimated to reach 15%, but this probably also included mortality due to cannibalism and other unknown causes, and this rate is lower than in other reports on juveniles suffering from VNN (see section 1.2.1). The conclusion that NNV was contributing to the mortality were eventually based on real-time RT-PCR detection with a 10^3-times increase of NNV in brain compared to experimentally infected cod (see Paper II). Immunohistochemistry verified the presence of NNV with histopathology consistent with VNN. Finally, clinical signs were also consistent with VNN disease. Investigation of NNV tissue tropism of fish from Parisvatn also demonstrated presence of virus in all tissues examined (see Table 2 in Paper II), suggesting a systemic infection in the fish.

Important observations in Paper IV are the size of cod associated with VNN, which is larger than previously reported (see section 1.2.1) and that this outbreak occurred in commercially farmed cod in Norway. The first report raises the question of virulence of NNV and susceptibility in larger cod, and the latter may suggests that VNN could be expected to be a potential disease problem in farming of cod.

4.3 NNV as a pathogen in farming of cold-water fish species

From a fish farming point of view, understanding how NNV is transmitted is important in order to identify effective prophylactic measures to reduce the risk of spreading the virus or development of disease. This thesis aims to see the results from the experiments conducted in a broader context, and in the light of the scientific literature available make some suggestions of how the virus should be controlled in farming of cold-water fish. In order to identify potential risks involved with NNV in farming of salmon, cod and halibut, this involves discussing transmission of NNV, host specificity, wild and farmed fish interactions and examinination of some possible ecological aspects of NNV. First, determination of NNV

transmission modes should be addressed, as this is a cruisial step in indentifying risks of spreading the virus.

4.3.1 Transmission modes of NNV

In nature, dynamics and genetics of interactions between populations of NNV and their host are based on their survival strategies, determined through co-evolution of virus and the host. One crucial stage in the dynamics of interactions is mode of transmission, which could be looked upon as a survival strategy and determines the potential of spreading and persistence of the virus in a host population. In general, transmission is occurring horizontally or vertically and virus may utilize one or both modes. How NNV is transmitted has consequently been a result of the interactions between the virus and susceptible host in *natural* populations of hosts, or wild fish, and a result of the perpetual process of co-evolution. When wild fish is domesticated, the dynamics and genetics of the interactions are likely to be changed, if a shift in the evolutionary direction is introduced i.e. with dramatic changes of the environment or habitat. However, a closer look at the NNV transmission modes is required to unveil the NNV survival strategy.

The literature supports the existence of both vertical and horizontal transmission of NNV (see section 1.3.2), suggesting that there are several gateways for the virus to enter into a potential host (see section 1.3.3). The significance of these two transmission modes may be difficult to evaluate, particularly in wild populations of fish. But, transmission modes have generally been suggested to play a role in determining the virulence of a pathogen (see section 5.2), where horizontal transmission tends to favor higher virulence than vertical transmission which tends to favor mechanisms for long-term persistence and more benign infections (Clayton and Tompkins 1994, Ewald 1994). The outcome of the viral infection may be viewed as a result of the balance between the two transmission processes. Without considering virulence, these modes of transmission represents different risks of spreading the virus in farming of fish, which is outlined in section 4.3.5.

In summary, existence of both horizontal and vertical transmission of NNV should be considered as occurring, and this have consequently some implications in how the virus should be controlled in cold-water aquaculture.

4.3.2 Host specificity in BFNNV?

As viruses of the BFNNV clade have been detected in several different fish species (see Table 1 in section 1.2.3), this raises the question of possible transmission of NNV between fish

species, known as intraspecies transmission. This is an important question to address, as it has implications for the multispecies aquaculture industry and the management and control of NNV.

Since intraspecies transmission of virus require low or no host specificity, this question is equally important to address. This is particularly important in evaluating transmission risks, as any host specificity would have implications for how NNV should be managed in farming of fish. In this study, NNV originating from halibut have been used to investigate susceptibility and transmission to salmon and cod. The results do not seem to support host specificity, though the data could be regarded as insufficient to make a conclusion solely based on the experiments conducted. However, in the literature there is support for existence of host specificity (see section 1.3.1), but a closer look at the isolates in the BFNNV clade may support the findings in this thesis.

The grouping into the original 4 major phylogenetic clades (SJNNV, BFNNV, TPNNV and RGNNV) is apparently related to host specificity. This could be exemplified with the study of Iwamoto *et al.* (2004) and Ito *et al.* (2008) with genetically modified NNV, where the RNA2 segment was suggested to be involved in controlling host specificity in virus from SJNNV and RGNNV clades. The study performed by Totland *et al.* (1999) with halibut challenged with NNV from both SJNNV and BFNNV also suggested host specificity as the SJNNV isolate did not cause disease in halibut. However, the finding of Thiéry *et al.* (2004), who found an NNV isolate from BFNNV capable of infecting a warm-water fish species (sea bass), may suggest that host specificity is not solely linked to grouping in one of the major clades.

Further, although host specificity may be suggested between the major phylogenetic clades, this may not be the case within a single clade. NNV with a high phylogenetic relationship, i.e. those within the BFNNV clade may be more likely to infect cold-water hosts. Although some papers suggests subdivision of BFNNV into species-specific clades, like ACNNV (Gagné *et al.* 2004) or GMNNV (Nylund *et al.* 2008), some NNV isolates are not clustering and linking to the proposed host accordingly. This could be exemplified with the suggested ACNNV and AHNNV sub-clades with more than one host species present.

Another explanation of an apparent host specificity or preference may be related to geographic location rather than the host itself. Geographic clustering of NNV isolates may be the result of how the virus is spread within a susceptible host population. Vertical transmission of virus (see section 1.3.2) would result in an endemic distribution following the geographic borders of the host. Any horizontal transmission from this population would only

Table 2. GenBank accession numbers of NNV isolates included in Figure 5. All available Norwegian cold-water isolates of NNV have been included in the phylogenetic analysis. Table includes fish species in which virus isolate originates, geographic region of host, whether the host was wild or farmed and reference of sequence. Only a few isolates from SJNNV, RGNNV, TPNNV and the only one in TNNV were included to form a complete overview of the major genotypes suggested. GenBank = direct submission in GenBank.

Acssession nos.	Species	Region	Wild/farmed	Reference
EF617326	Atlantic cod	Hordaland	Farmed	GenBank
P10	Atlantic cod	Hordaland	Farmed	unpublished
P9	Atlantic cod	Hordaland	Farmed	unpublished
TK1	Atlantic cod	Troms	Farmed	unpublished
TK2	Atlantic cod	Troms	Farmed	unpublished
EF617327	Atlantic cod	Hordaland	Farmed	GenBank
EF433465	Atlantic cod	Nordland	Farmed	Nylund *et al*. 2008
EF577375	Atlantic cod	Sogn og Fjordane	Farmed	Nylund *et al*. 2008
EF617329	Atlantic cod	Hordaland	Farmed	GenBank
EF617328	Atlantic cod	Hordaland	Farmed	GenBank
AB31305707	Atlantic cod	Møre og Romsdal	Farmed	unpublished
M90	Atlantic cod	Møre og Romsdal	Wild	unpublished
AB31305682	Atlantic cod	Møre og Romsdal	Farmed	unpublished
V1	Atlantic cod	Nordland	Wild	unpublished
EF433469	Atlantic cod	Møre og Romsdal	Wild	Nylund *et al*. 2008
EF433468	Atlantic cod	Møre og Romsdal	Farmed	Nylund *et al*. 2008
AJ245641	Atlantic halibut	Hordaland	Farmed	Grotmol *et al*. 2000
AF160473	Atlantic halibut	Møre og Romsdal	Farmed	Aspehaug *et al*. 1999
K110707	Atlantic cod	Hordaland	Farmed	unpublished
AY962682	Atlantic halibut	Southern Norway	Farmed	Paper I
AJ698094	Sea bass	France	Farmed	Thiéry *et al*. 2004
EU826138	Barfin flounder	Japan	Farmed	GenBank
D38635	Barfin flounder	Japan	Farmed	Nishizawa *et al*. 1995
AF445800	Atlantic cod	North America	Farmed	Johnson et al. 2002
AF547548	Atlantic cod	North America	Farmed	Gagné et al. 2004
D38527	Japanese flounder	Japan	Farmed	Nishizawa *et al*. 1995
D38636	Red-spotted grouper	Japan	Farmed	Nishizawa *et al*. 1995
AB045980	Japanese flounder	Japan	Unknown	GenBank
AJ608266	Turbot	Norway	Farmed	Johansen *et al*. 2004b
D38637	Tiger puffer	Japan	Farmed	Nishizawa *et al*. 1995
D30814	Striped jack	Japan	Farmed	Nishizawa *et al*. 1995
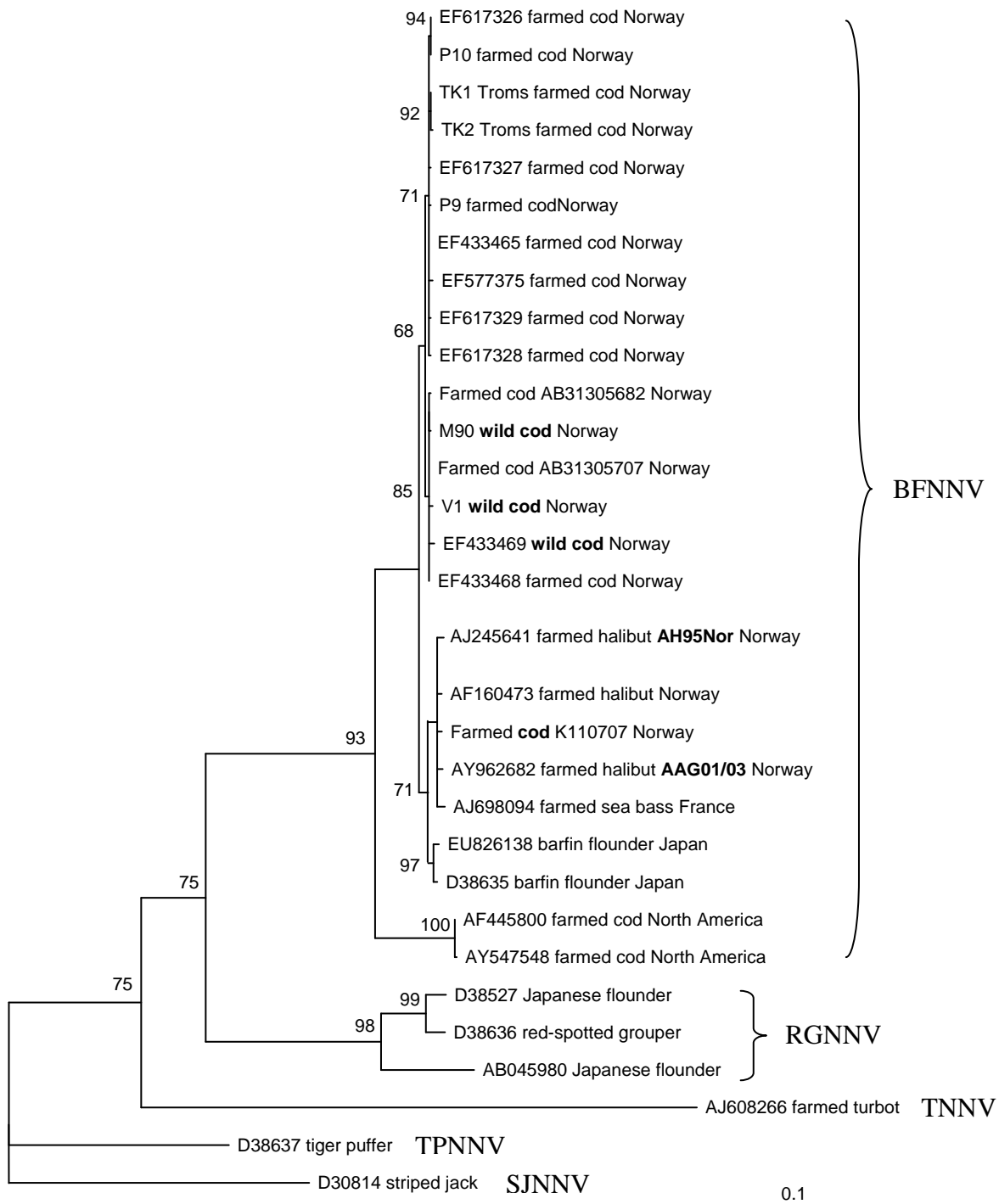
Figure 5. *Phylogenetic position of selected NNV isolates, where all available Norwegian NNV isolates are included. Phylogram is based on 826 nucleotides from RNA2 segment and applied maximum-likelihood criteria in TREE-PUZZLE (quartet-puzzling steps) to calculate phylogenetic distances. Major clades are BFNNV = Barfin flounder NNV, RGNNV = Red-spotted grouper NNV, TNNV = Turbot NNV, TPNNV = Tiger puffer NNV, SJNNV = Striped jack NNV. The scale bar shows the number of substitutions as a proportion of branch lengths. The numbers at the nodes are quarter-puzzle support values.*

occur within the habitat (geographic region) where susceptible host species have contact. This may explain the distinct cold-water BFNNV clade. Long distance migration or introduction of infected hosts could alter the genetic variations of NNV isolates in host populations in a region, and may influence the host-virus interaction. A suggested theory of evolution and ecology of NNV is discussed in section 4.3.4.

Finally, a closer look at the BFNNV clade may provide some more information on phylogenetic relationship useful for determination of possible host specificity. The analysis of Nylund *et al.* (2008) suggests existence of a species specific group consisting of NNV isolates from Atlantic cod. Based on the analysis performed by Nylund *et al.*, all Norwegian isolates available have been added to Figure 5, in order to extend the analysis with even more isolates (Table 2). This new analysis utilised an 826 nt segment of RNA2 and genetic distance calculations and phylogenetic tree estimations under maximum likelihood (ML) criteria were made with Three-Puzzle 5.2 (available at http://www.tree-puzzel.de). As the majority of the NNV sequences included also were utilised by Nylund *et al.*, the new analysis applied identical substitution model and rates; generalised time reversible (GTR + R) with the following nucleotide substitution rates: A-C (1,7523), A-G (3,7202), A-T (1,0720), C-G (0,8776), C-T (5,9839) and G-T (1,0000). The phylogram (Figure 5) was drawn using Tree View (Page 1996). NNV isolate acting as representatives for all the other major clades (SJNNV, RGNNV, TPNNV and the suggested TNV) are included in this analysis for comparison reasons. The phylogram is consistent with the finding of Nylund *et al.*, with an exstended group of NNV isolates from both farmed and wild cod. Interestingly, in the group with NNV isolates from halibut, there is one single isolate from farmed cod present. Generally, the phylogram suggests there is genetic variation within the BFNNV genotype, and this will probably increase as new isolates of NNV are added in the future.

In conclusion, there is little if any support of host specificity in the BFNNV clade based on phylogenetic properties. The cold-water NNV isolates should be regarded as capable of infecting hosts of this clade, at least until other data provide opposite information. Without considering any differences in virulence (see section 5.2), low host specificity represents potential hazard of transmitting NNV horizontally between cold-water fish species known to be susceptible, including the farmed species cod and halibut. This also includes a potential of transmission of NNV between farmed and wild fish.

4.3.3 Interactions between wild and farmed fish?

Interactions between farmed and wild fish (and *vice versa*) may be important in fish farming. In Norway, there are at least 3 concerns regarding the possibility for introduction, horizontal transfer and spreading of NNV: 1) Use of wild-caught Atlantic halibut and cod as brood fish, 2) live storage and on-growth of wild-caught cod and 3) an increasing number of new on-growth sites for farmed cod and halibut along the Norwegian coastline.

There is no hard evidence for NNV transmission between wild and farmed cold-water fish. However, the observation of infected wild fish raises questions of possible interaction. Nylund *et al.* (2008) found NNV in wild Atlantic cod on the west coast of Norway, suggesting that brood fish originating from wild stocks might have been persistently infected when brought in and used as brood stock. Bearing in mind vertical transmission (see section 1.3.2 and 4.3.1), they considered it likely that infected progeny have been spread in cod farming. As the prevalence of infected cod in some areas on the west coast ranged between 20 to 50 % (Nylund - unpublished data), this demonstrates that it is possible to introduce the virus to farmed fish by using wild-caught brood cod. NNVs have also been detected in wild and farmed fish species in Canada and in farmed cod in the UK (see section 1.2.3 and Table 1). In Norway, the finding of nodavirus-positive adult Atlantic halibut used as brood stock (Aspehaug *et al.* 1999) might suggest that there is a hazard in using wild-caught animals as brood fish in farming of this species as well.

There is an increased interest in catching live wild Atlantic cod and keeping the fish for a period to both increase the weight and sell the fish outside traditional fisheries seasons to obtain a better price. Usually the fish is caught during the spawning season, and kept for a few months in sea-cages at sites in close proximity to a processing plant. The benefit of doing this is an added value and improved quality of the fish, and some argue that this is the future for the traditional fisheries in Norway (Midling, personal communication 2007). In 2007 approximately 580 tons of cod were live caught and stored in sea-cages, and in 2008 this is predicted to reach around 1500 t (source: Norsk Råfisklag, 2008). However, assembling large numbers of wild fish with an unknown health status at an on-growth site might represent a potential risk of spreading diseases to farmed and wild fish in the vicinity. Further knowledge should be obtained to determine the risks involved, including those created trough transportation of live fish over long distances. This is likely to increase the risk of spreading and introducing pathogens to new areas (Mortensen *et al.* 2006).

As farming of Atlantic cod has shown a steady increase in Norway during the last years (Directorate of Fisheries, Norway), this trend most likely will require establishment of

numerous new on-growth sites to harbor a continuous increased production. Most of the hatcheries producing cod larvae are at the moment situated along the west coast of Norway (in 2008), and establishment of new on-growth sites involves increased transportation of juveniles into possible new areas suitable for farming. Further, establishment of new on-growth sites in close proximity to existing fish farms and local wild fish populations might also cause horizontal transmission of virus.

4.3.4 Interactions of NNV and host – ecological considerations

With a bird's eye perspective on NNV, a suggested ecology of the virus may provide some insight in dynamics and genetics of interactions of NNV and susceptible hosts. These suggested interactions should, however, be regarded as over-simplified with the sole intention to present a theoretical background in which further discussions on prophylaxis and control of virus in fish farming could be based.

NNV could be assumed to be common in wild populations of cold-water fish species (see Table 1), where numerous different isolates (see Figures 2 and 5) suggest that there are large genetic variations of the viruses and numerous susceptible hosts. Using Atlantic cod as an example, the numbers of isolates showing genetic variations are increasing and so far all these isolates cluster phylogenetically close and are suggested to form a distinct sub-clade within BFNNV. Simplified, genetic variations are usually the result of mutations (errors during replication) and genetic recombination, and an observed (suggested) large variation of cod-originating NNV may demonstrate that these viruses are spread and maintained within the cod population. This is best explained with vertical transmission as the primary way of transmitting the virus in cod. Such transmission would facilitate selection of virus isolates with low virulence and high prevalence (persistency) in the host population. If, in contrast, horizontal transmission would be the primary transmission mode, this would facilitate low genetic variation as the isolates showing the best spreading potential would dominate. In this case selection for virus isolates with increasingly higher virulence is the expected result. In cod, NNV could be suggested to be transmitted during maturation and spawning, where the host immune system is suppressed as a result of the physiology involved in the process of reproduction. This would likely trigger increased viral replication in persistently infected cod, as the interplay between virus and immune system is altered. During spawning, large numbers of cod are assembled at the spawning ground, where released NNV particles would have the opportunity to infect hosts with reduced immunological capacity and consequently could be regarded as more susceptible to infection. In this case any susceptible host in the area could be

infected due to horizontal transmission, suggesting that phylogenetically close NNV isolates could be established in new fish species occupying the same habitat as cod. Low host specificity would be facilitated and could explain the suggested geography-specific phylogeny of NNV viruses. The interaction of NNV-host could be regarded as a result of the trade-off (dynamics of selection process of virus isolates) between vertical and horizontal transmission, where low virulence and host specificity in NNV have been facilitated in natural populations of hosts.

In a farming situation this virus-host interaction is likely to be changed, as the evolutionary selection pressure would be influenced by increased density and number of hosts, environmental conditions (sub-optimal), physiological constraints in hosts (stress) and the farming process itself. If NNV is transmitted both vertically and horizontally in farmed fish, the cost of increasing virulence is reduced for NNV as there would be a continuing replacement of new farmed hosts susceptible to infection. Consequently it could be hypothesized that farming would, in the long run, facilitate selection of more virulent virus isolates and thereby possibly increase the significance of horizontal transmission.

4.3.5 NNV in farmed fish - identification of potential risks involved

This section aims to summarize the possible risks and hazards involved with NNV in farming of fish, and form the basis for the prophylaxis and control measures suggested in the next section. Suggested routes of transmission of the virus are summarized in Figure 4.

Implications of vertical transmission of NNV

There are at least two concerns regarding vertical transmission of NNV in farming of marine fish species; use of persistently infected brood fish and spreading of NNV in farmed populations through use of such fish. Although there is no hard evidence that vertical transmission is occurring in cold-water fish species, the literature is strongly supporting this in studies performed in warm-water fish species (see section 1.3.2). Existence of vertical transmission may also be supported by the suggested (theoretical) ecology of NNV as outlined in section 4.3.4.

Any persistently infected brood fish would have the potential to pass the virus on to the offspring. If the health status of brood fish candidates is unknown, in terms of persistency, this involves a risk of introducing the virus to a farmed population of fish. The origin of these candidates may either be from other farmed populations or from wild stocks of fish or a mix of both. Particularly use of wild-caught brood fish could be suggested to represent a high risk of introducing the virus.

If persistently infected brood fish candidates are used, these would be a source of NNV to farmed fish. As long as these candidates are not removed from the brood fish population, the risk of spreading NNV would remain high with a possible continuous supply of infected juveniles.

Implication of horizontal transmission of NNV

Horizontal transmission of NNV represents a risk of waterborne spreading of the virus, either as cohabitation or via potential vectors. The existence of horizontal transmission is well documented in the literature and suggested to play a role in spreading of NNV (see section 1.3.2). In Paper III this was shown when the virus was transferred from turbot to cod by cohabitation. The potential of spreading NNV in cold-water fish farming has also been suggested by Nerland *et al.* (2007), who detected high concentrations of NNV in rearing units with Atlantic halibut larvae suffering from VNN. This observation shows that the virus may be dispersed via water from aquaculture facilities.

Horizontal transmission may occur within a population of farmed fish or between farmed and wild fish. Existence of such transmission routes suggests that there are several ways the virus may enter and infect farmed fish (see Figure 4). Blocking these entrances would be important to reduce the risk of introducing and spreading NNV in farming of fish.

Implications of low host-specificity

Although the number of susceptible host species for viruses of the BFNNV clade is not known, there exists a risk of transferring the virus to new host species including farmed fish. In this study susceptibility in Atlantic salmon was examined, where the conclusion that salmon should be regarded as a potential host was reached. In addition, a halibut-originating NNV isolate were horizontally transmitted from turbot to cod, suggesting NNV could be transmitted from halibut to cod and most likely vice versa. These observations suggest that co-localization of farmed fish species should be avoided.

Implications of NNV in wild fish species and genetic variations

The presence of NNV in a number of wild fish species (Table 1) suggests the existence of a marine reservoir of virus. In farming of marine fish species, this reservoir could potentially be a source of virus, either in wild-caught brood fish or by horizontal transmission from wild to farmed populations. The genetic variations observed in isolates of NNV may also represent a risk in its own rights, as this variation also could reflect variation in virulence or the ability to invade, survive and replicate in new hosts. Interactions between wild and farmed fish could

consequently result in transmission of the isolates in the pool of NNV in wild fish which best adapt to farmed fish.

Implications of transporting live farmed fish

In general transportation of fish could be regarded as involved with risk of spreading the virus in farming of fish. There is at least one concern regarding transportation of fish and risk of spreading NNV. Movement of fish with known or unknown NNV infectious status (persistency) could result in introducing the virus to new areas and hosts with subsequent horizontal transmission. Consequently, any transportation of fish, and particularly fish with unknown health status over long distances, should be considered as potentially hazardous.

4.3.6 Towards a strategy for controlling NNV in farming of fish

The basis for controlling NNV in farming of fish is reducing the risk of introducing and spreading the virus in farmed populations of fish by blocking both vertical and horizontal transmission routes. Achieving this involves application of numerous coordinated preventive measures. Using the current knowledge of NNV, this thesis is suggesting a strategy of how such measures could be applied to minimize the risks suggested to be involved with NNV. The strategy involves screening of fish and eventual development of a vaccine.

Screening of fish

In general screening of fish would provide a survey of infection status in a given population subject to examination. Implementation of real-time PCR technology has vastly improved the sensitivity, and provided a tool to detect small amounts of virus and possibly reveal any persistency. However, as detection of pathogens often occurs at the detection limit of the assay (i.e. carrier state of fish) some care should be taken as there is currently no independent method available for verification of the result. Another consideration is how to interpret the Ct-values returned from an analysis, particularly at the very terminal cycles of the PCR, when sampling in a population of farmed fish at a site. The consequences of such detections may depend on a total evaluation of possible hazards based on experience and knowledge of the pathogen in question. The interpretation of the results should also be done according to the aim of the study, usually by balancing reproducibility against sensitivity. This could be exemplified with use of real-time PCR in diagnostics, where a cut-off Ct-value is applied to discriminate positive and negative samples. Defining the cut-off limit where multiple runs are identical is consequently important and may vary between labs and different real-time PCR assays. When using real-time PCR in mapping distribution of a pathogen in a given

population, sensitivity is more important than reproducibility in order to detect any positive sample. In this case the results should be evaluated against false negative (low reproducibility) and false positive (background, contamination) samples. This inherited problem with real-time PCR should therefore lead to caution in how to interpret the results, which is depending of the aim of the study. The results obtained are influenced by processing of samples, like sampling procedures, storage, temperature and time, and type of assay. When two independent laboratories are analysing identical samples, the analyses may suggest both positive and negative results for single samples and some interesting discussions may arise. However, real-time PCR is a useful tool which has enabled detection of small quantities of pathogens, and in the end it is how the method is applied which determines the power of this technology.

Vaccination

A vaccination strategy should be developed since there most likely is a large reservoir of NNV in wild populations of fish. In farming, this would consequently result in a possibility for exposure to infected wild fish in the vicinity of a farming site. The literature has suggested the possibility of inducing protection against NNV by vaccination (see section 1.3.5), and suggests this could be an important and most promising prophylactic measure for the future. However, there is a major limitation as fish have to reach a specific size or age in order to be vaccinated. This means that this strategy will not be applicable for juveniles, which might be regarded as most susceptible to NNV with following development of VNN. A vaccine would consequently not solve the problem solely, but could be important to provide some protection for adult fish, particularly when young fish are moved from the hatchery or pre-stocking facility to a marine growth site. Another application could be in brood fish, which may obtain higher protection prior to maturation and spawning due to boosting of the immune system. This would most likely increase the resistance to NNV (persistency) with subsequent reduced replication of virus. Although a vaccine would not singlehandedly solve the problems related to NNV, it should be considered as important when applied in concert with other prophylactic methods.

Brood fish

There are two major concerns regarding brood fish and NNV. The first is use of NNV infected brood fish with subsequent vertical (and horizontal) transmission of virus. The best prophylaxis against vertical transmission is NNV-free brood stocks, and this could be achieved by careful screening and monitoring of health status in order to identify infected

(carrier) fish. As brood fish most likely would be persistently infected (if infected), a screening should be performed when the likelihood of detecting the virus is increased. Consequently, this should be performed during sexual maturation and spawning. Methods for screening such fish could be through biopsy (like head-kidney in Atlantic cod) or reproductive fluids. When adding new brood fish to the stock population, the new candidates should be quarantined and screened to reduce the likelihood of introducing NNV. A screening program could be the basis for a selection of an NNV-free brood stock, and screening of the offspring could increase the likelihood of identifying possible infected animals. A screening strategy should consequently consist of both individual examination of the brood stock and multiple screening of offspring to reveal any transmission to egg and larvae.

The second concern is horizontal transmission of NNV to the brood fish stock. The benefit of a selection program for NNV-free brood fish could be jeopardized with exposure to the virus by waterborne transmission of virus. If the brood stock is kept in close proximity of other marine fish farms or wild stocks of known susceptible fish species, this could represent a risk of infecting the brood fish. As a single brood fish should be regarded as valuable, possibly originating from a breeding program and difficult to replace, it would be wise to keep the brood stock in a sheltered aquaculture system to prevent horizontal transmission of NNV.

Larvae and juveniles

In the hatchery, larvae and juveniles would be vulnerable to horizontal transmission of NNV. Their immune system is poorly stimulated with increased susceptibility compared to adult individuals. At this stage, protection against waterborne NNV should be provided by keeping the fish in a sheltered aquaculture system. The source of virus could be from within the aquaculture facility itself or by using untreated (or improperly disinfected) water. The fish should be kept in this closed system until reaching a size suitable for vaccination. A vaccinated fish would be ready for transportation to a marine growth site, where exposure to NNV could occur.

Marine growth-sites

A marine growth site, i.e. where fish are kept in cages at high density, should be regarded to have an increased risk of exposure to NNV. Sources of virus might be other farmed fish at the site (i.e. fish from several hatcheries) or wild stocks of fish in the vicinity. As this is an open system, the prophylactic measures applied should address immune status of the fish in order to avoid immunosuppression and subsequent increased likelihood of infections with

pathogens. Consequently, important general prophylactic measures include only one generation at the site at any time, distance to other farming sites, fallowing of site in-between generations, removal of moribund and dead fish, vaccination when possible and optimal environmental conditions in the cages at the site. Regarding NNV, there is no commercial vaccine available (at the time) and attention should be paid to avoid introduction of NNV (transportation of fish to the site), reduce spreading the virus within the farming site (removal of diseased fish) and reducing likelihood of virus replication in possibly infected fish (reduce stress).

No co-localization of farmed fish species

Co-localization of farmed fish species at a single site (poly culture) is generally involved with increased risk of intraspecies transmission of a number of pathogens. Consequently there are numerous arguments against such practise, and regarding NNV there are some considerations which should be addressed.

The suggested low host specificity of NNV (see section 4.3.2) represents a risk of transmission of virus between any susceptible hosts kept in a fish farm. This thesis has identified Atlantic salmon as a potential host for NNV, and consequently there should be no co-location of salmon and other known susceptible hosts such as Atlantic cod and halibut. In addition, an NNV isolate originating from halibut was successfully transferred from infected turbot to cod. This suggests that virus may be horizontally transmitted from infected halibut (the original species in which the virus was isolated) to cod, and most likely vice versa. In this case co-localization of cod and halibut is also not recommended.

Finally, farmed fish could be infected with NNV through horizontal transmission in a number of possible different ways. Although there is little information available, it might be hypothesized that wild fish could be a source of NNV (see section 4.3.3). It could also be suggested that vectors like bivalves and crustaceans, which are commonly found at marine farming sites, could be a source of NNV as well. However, the role and importance of wild fish stocks and vectors in spreading the virus have yet to be determined.

5 EPILOGUE

5.1 Main conclusions

The main objective of this study was to investigate NNV as a pathogen in Norwegian aquaculture, with emphasis on Atlantic salmon *Salmo salar* and Atlantic cod *Gadus morhua*. The main conclusions could be summarized as follows:

Susceptibility of Atlantic salmon to NNV

Atlantic salmon should be regarded as a potential susceptible host, and caution should be taken to avoid increasing any risks of NNV infection in salmon farming. Salmon farms should not be located in close proximity of farmed fish species known to be susceptible, such as Atlantic halibut *Hippoglossus hippoglossus* or Atlantic cod.

Horizontal transmission of NNV to Atlantic salmon and Atlantic cod

An Atlantic halibut originating NNV isolate was capable to replicate in intraperitoneally (i.p.) injected Atlantic cod, and this isolate was also successfully transferred horizontally to cod. It is likely that NNV could be transported between halibut and cod (both farmed and wild) and most likely vice versa. Halibut and cod farms should not be located in close proximity of each other in order to reduce the risk of horizontal transmission of NNV.

Screening of brood fish of Atlantic cod

The results from i.p. challenged cod suggest that head-kidney is the best organ suited for non-lethal biopsy. Screening of candidates should be performed in fish expected to be immunosuppressed by sexual maturation or sub-optimal rearing conditions (stress) to increase likelihood of detecting NNV.

Prophylaxis and control of NNV

This thesis suggests, based on the experiments conducted and scientific literature studied, that the following prophylactic measures should be considered in farming of marine fish species to control NNV, by reducing the risk of vertical and horizontal transmission of the virus:

1. Fish should be screened prior to introduction as brood fish in order to reduce the likelihood of bringing the virus into aquaculture. This is particularly important when using wild-caught brood fish of marine fish species such as cod (and halibut).

2. Brood fish should be kept in a closed aquaculture system in order to avoid exposure to waterborne NNV (horizontal transmission). Screening of brood fish should be performed during sexual maturation or spawning (state of immunosuppression). This timing would maximize the likelihood of detection of virus.

3. Juveniles should be placed in closed sheltered aquaculture systems, in order to reduce the risk of horizontal transmission of NNV, and kept until size is favourable for vaccination against NNV. Vaccinated fish could be transported to open systems such as cage-based marine farming sites.

4. A vaccination strategy should be developed both for cod and halibut. Vaccination of brood fish should be done to reduce the likelihood of any viral replication during maturation and spawning. Young fish should be vaccinated prior to transportation from a sheltered aquaculture system to an open marine on-growth site where exposure to NNV and subsequent possibility of horizontal transmission may occur.

5.2 Future research

By addressing NNV as a pathogen in farming of fish, it has become clear that there is a lack of information in several areas related to the pathogen-host interaction. One question which tends to pop up when discussing the potential of spreading pathogens in farmed populations of fish is virulence, usually followed by discussions of the possible existence of virulence variation or inducement of increasingly higher virulence of the pathogen. One example of suggested variation of virulence in a fish pathogenic virus is infectious pancreatic necrosis virus (IPNV) (*Birnaviridae*), where virulence has experimentally been linked to a small motif at the VP2 encoding gene (Santi *et al.* 2004). In this study only 4 amino acid positions at the VP2 gene were associated with virulence of IPNV in experimentally challenged fish. The virus has a double-stranded RNA genome, with both horizontal and vertical transmission modes. Potential existence of variation of virulence in NNV may also be speculated, particularly as NNV has similar genetic properties (RNA genome) and transmission modes. However, there is little information on virulence or variations in virulence of NNV isolates, other than anecdotic observations mostly related to mortality rates and possible host specificity (see section 1.2.1 and 1.3.1). But, as any change in virulence would affect the dynamics of a viral infection, some considerations should be addressed and relevance to NNV discussed.

A key question would be if NNV has the ability to show variations in virulence, and in particular to shift (selection) towards higher virulence and consequently induce development

of disease and mortality. A theoretical approach to this would be to examine how evolution of virulence may occur. First, virulence may be defined as the pathogens ability to cause disease or pathogen induced host-mortality, and could i.e. be linked to the ability to invade, survive and replicate in the host. As viruses are depending on the host for replication (as an obligate parasite), there is a close interaction between the virus and the host. Traditionally the view of how the dynamics between the pathogen and host has emerged is as a result of evolutionary equilibria, with a theoretical approach to reach optimality (André and Hochberg 2005). Along this co-evolutionary trajectory it is assumed that there is a fine interplay between transmission, virulence and the cost of host resistance. However, this is probably an over-simplified view, as this predicts that avirulence would be of best interest of both host and pathogen in the long run if virulence is unconnected and independent (May 1995). The interaction is intuitively more complex, and examination of possible roles of pathogen transmission and host resistance may be useful in order to predict the effect on virulence. In the case of NNV, this is unfortunately a bit abstract due to lack of information, but some general ideas could be suggested.

The effect of transmission mode could be exemplified with the hypothesized conflict in selective pressures between horizontal and vertical transmission (Ewald 1994). Generally, horizontal transmission relies on high numbers of pathogens to be released to infect new hosts, which tend to favour high virulence as the pathogen induce increased host exploration. This will increase transmissibility of the pathogen by higher rate of transmission. In contrast, vertical transmission requires a reproducing host with subsequent transfer of pathogen to the offspring. In this case high virulence would reduce the number of hosts (including offspring) by pathogen-induced mortality, and consequently reduce the numbers of both pathogens and hosts. Vertical transmission tends, in this scenario, to favour low virulence, but a too low virulence may result in lost opportunity to infect new hosts. Summarized, the virulence of a pathogen could be looked upon as a result of the pathogen-host interaction, with evolutionary trade-offs between horizontal and vertical transmission (Chen et al. 2006). However, this hypothesis of importance of transmission modes and evolution of virulence has been criticised to be based on verbal arguments and is difficult to evaluate (Day 2001).

The host's ability to resist or recover from an infection may be viewed as an important part of the pathogen-host interaction, and is linked to the host immune system (Anderson and May 1982). This could consequently be regarded as influencing the virulence of a pathogen, where it could be predicted that virulence is increasing if the recovery rate of the host is increasing (van Baalen 1998). The argument is that the benefit of low virulence (persistency)

is reduced with increased risk of immune clearance. However, the clearance may also result from properties of the host immune system and pathogen itself (Restif and Koella 2003). This could be exemplified with the interpretation of Anderson and May (1982) on myxoma virus (affecting rabbits), where field isolates of the virus were tested for clearance in rabbit (Fenner and Ratcliffe 1965). There was variation of clearance between different field isolates with a negative correlation with virulence. The most virulent viruses, in this study, showed better resistance to immunity. Different isolates of a pathogen, i.e. NNV, could consequently have different fates in the host. Again it is difficult to evaluate such interactions, at least as a universal assumption of the interplay of pathogen and host immune system, since there could be great heterogenicity among both pathogens and hosts. Further, the host immune system is likely to show variations due to physiological constraints, i.e. stress response resulting in immunosuppression. This probably affects both susceptibility and immunity of the host and adds further complexity. In conclusion, determination of possible variation of virulence is consequently one interesting field of research of NNV.

Understanding the apparent complex interplay between NNV and the host requires a lot of research, ranging from molecular to evolutionary approaches. Based on the results and conclusions in this thesis, there are at least some obvious fields of research which should be addressed in order to prevent the pathogen from becoming a problem in fish farming. These comprise a determination of the real distribution of NNV in wild populations of fish, possible NNV vectors, examining host and virus specific factors regulating transmission, determining vertical transmission of cold-water NNV isolates, identification of virulence factors in NNV and development of vaccines. Addressing these fields requires a multi-disciplinary approach. Several different scientific approaches may contribute to the understanding of NNV as a pathogen.

Finally, it might be wise to remember the words of Theodosius Dobzhansky when aiming to understand the inner secrets of NNV: *Nothing in biology makes sense except in the light of evolution.*

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