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# Horizontal transmission of nervous necrosis virus between turbot *Scophthalmus maximus* and Atlantic cod *Gadus morhua* using cohabitation challenge

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ABSTRACT: Experimental horizontal transmission of nervous necrosis virus (NNV) originating from halibut *Hippoglossus hippoglossus* was studied through cohabitation of intraperitoneally (i.p.) injected fish with uninfected fish for 125 d. The experimental groups consisted of i.p. injected turbot *Scophthalmus maximus* or i.p. injected Atlantic salmon *Salmo salar* with turbot, salmon or Atlantic cod *Gadus morhua* cohabitants. The initial weights were cod 10 g, salmon 40 g and turbot 3 g. NNV was detected in brain, eye and spleen by real-time reverse transcriptase PCR (qRT-PCR) in cod cohabitated with i.p. injected turbot after 90 and 125 d, suggesting NNV infection was transmitted horizontally from the turbot to cod. NNV was not detected in salmon that were cohabitated with i.p. challenged turbot or salmon. This study shows that NNV strains belonging to the Barfin Flounder Nervous Necrosis Virus (BFNNV) clade may be transmitted from halibut to cod via water. Hence there is a potential risk of horizontal transmission of the virus from farmed halibut to farmed and wild cod. The lack of detection of NNV in cohabitant salmon suggests that this fish species is less susceptible than cod, or not susceptible, to horizontal NNV transmission. This result might be influenced by the size of salmon, viral load in i.p. injected cohabitants or insufficient duration of the experiment.

KEY WORDS: Nodavirus · Nervous necrosis virus · Atlantic cod · Atlantic salmon · Cohabitation · Horizontal transmission



## INTRODUCTION

*Betanodavirus*, nervous necrosis virus (NNV), causes the disease viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER). In Norway, this virus has been detected in several fish species including farmed ones such as turbot *Scophthalmus maximus* (Bloch et al. 1991, Johansen et al. 2004), Atlantic halibut *Hippoglossus hippoglossus* (Grotmol et al. 1995, Aspehaug et al. 1999, Hellberg & Dannevig 2002, 2003) and Atlantic cod *Gadus morhua* (Patel et al. 2007). In addition, spotted wolfish *Anarhichas minor* (Johansen et al. 2003, Sommer et al. 2004) and Atlantic salmon *Salmo salar* (Korsnes et al. 2005) were found to be susceptible by intraperitoneal (i.p.) injection with NNV. Since Atlantic salmon is an important farmed fish species in Norway, the potential susceptibility to NNV must be clarified using a more natural route of infection than i.p. injection with the virus. Although there is no report of natural outbreaks of VNN in salmon, encephalitis and mass mortality of farmed salmon smolt have been reported in Ireland (Scullion et al. 1996), and nodavirus-like particles have been detected in the heart tissue of salmon suffering from cardiac myopathy syndrome (Grotmol et al. 1997, Nilsen & Nylund 1998).

Cod farming is expected to increase in volume worldwide, with the largest growth predicted to take

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place in Norway (Kjesbu et al. 2006). Recently NNV has been detected and VNN diagnosed in several commercial cod farms on the west coast and northern part of Norway, and the virus has also been detected in wild Atlantic cod (Nylund et al. 2008). Elsewhere, Johnson et al. (2002) have reported NNV in Atlantic cod in Canada, and Starkey et al. (2001) reported NNV in Atlantic cod hatched from wild stock from the Irish Sea. Hence, nodavirus infections occur in both wild and farmed populations of cod as well as in farmed turbot and halibut, which raises the question of possible transfer of NNV between farmed fish species as well as between wild and farmed fish species.

NNV has been shown to be transmitted vertically in several fish species (Arimoto et al. 1992, Mushiake et al. 1994, Nguyen et al. 1997, Watanabe et al. 2000, Breuil et al. 2002). There are few studies in cold-water fish species, but Grotmol & Totland (2000) showed that washing eggs in ozone-treated seawater increased the rate of survival of Atlantic halibut post hatching. Several studies have reported horizontal transmission of NNV in various fish species (Arimoto et al. 1993, Le Breton et al. 1997, Munday & Nakai 1997, Skliris & Richards 1999, Castric et al. 2001), suggesting that horizontal transmission is playing a role in the spread of the virus. However, few studies have addressed the transmission of cold-water NNV isolates, and these only investigated horizontal transmission by bath challenge in Atlantic halibut (Totland et al. 1999, Dannevig et al. 2000, Húsgard et al. 2001, Grove et al. 2003). NNV has also been detected in high concentrations in rearing units containing Atlantic halibut larvae suffering from VNN (Nerland et al. 2007), showing that the virus may be dispersed via water from aquaculture facilities. Knowledge on the host range of the NNV strains from halibut hatcheries is needed. Hence, the aim of this study was to investigate possible horizontal transfer of nervous necrosis virus (originating from Atlantic halibut) to the farmed fish species Atlantic cod and Atlantic salmon using a cohabitation infectivity model.

## MATERIALS AND METHODS

### Fish

Fish species included in the transmission study were Atlantic salmon, Atlantic cod and turbot obtained from commercial hatcheries in Norway. These fish were 0-yr class and had initial average weights of 40 g, 10 g and 3 g respectively. All fish were not vaccinated, and Atlantic salmon were adapted to seawater as smolt.

Prior to the experiment, the fish were kept for acclimatization to laboratory conditions for 2 wk at 12°C. During the experiment the fish were held in 0.15 $m^3$ tanks with running flow-through seawater (8 l $min^{-1}$) at a constant temperature of 12°C. The fish were monitored daily and fed a commercial feed ad lib. Prior to the experiment, 10 fish from each population of fish species applied were screened for common viral pathogens with real-time RT-PCR. All fish were screened for NNV, and in addition cod were screened for infectious pancreatic necrosis virus (IPNV; Watanabe et al. 2006) and salmon for salmonid alphavirus (SAV; Hodneland et al. 2005) and infectious salmon anaemia virus (ISAV; Plarre et al. 2005).

### Virus preparation

Two different NNV isolates belonging to the BFNNV clade were used, AH95Nor (Grotmol et al. 1995) and AAG01/03 (Korsnes et al. 2005), both originating from natural outbreaks of VNN in juvenile Atlantic halibut. The AAG01/03 isolate was prepared from brain tissue from turbot infected by i.p. injection. A total of 0.4 g brain tissue from 5 turbot showing clinical signs of VNN was homogenized in 3 ml Leibowitz's L15 cell culture medium without glutamine (Cambrex Bio Science Verviers), a further 7 ml cell medium was added and then centrifuged for 5 min at 500 × *g* to remove debris. The supernatant was filtered through 0.45 µm and finally 0.2 µm filters (Whatman), and stored at –80°C. The virus preparation was tested for the presence of NNV by real-time RT-PCR prior to i.p. injection in salmon and turbot. The AH95Nor isolate was propagated using striped snakehead cells (SSN-1) grown according to Dannevig et al. (2000) at 24°C to 60 to 80% confluency. The cell culture was harvested after observation of cytopathic effect (CPE) and prepared as described for the brain tissue. The i.p. dose for both virus isolates was 50 µl in turbot and 200 µl in salmon. The i.p. injected fish were kept alone in the tank for 2 h post injection before the cohabitants were added.

### Experimental design

Fish were divided into 8 groups held in separate tanks (I to VIII), with 6 groups (I to IV, VII and VIII) consisting of 10 NNV i.p. challenged fish and 30 non-challenged healthy cohabitants in each group. Group V comprised 10 negative control Atlantic salmon injected with L15 cell medium only. Group VI

comprised 40 untreated negative control cod. These were not injected with L15 as no experimental cod were i.p. injected with virus. The experimental groups are outlined in Table 1. Groups III and VIII consisted of AH95Nor i.p. injected Atlantic salmon and turbot, respectively, and the other groups were injected with strain AAG01/03. Groups VII and VIII were used as positive controls for viral strains AAG01/03 and AH95Nor, respectively, since halibut isolates had previously been shown to readily infect turbot. All i.p.-injected fish in each group were tagged with Visible Implant Elastomer (Northwest Marine Technology™), injected in the epidermis (adjacent to the eye) for identification purposes. The duration of the experiment was 125 d.

### Sampling protocol

The infectivity experiment was conducted during the period from May to September. Sampling was performed on Days 25, 55, 65, 90 and 125 post injection, and the samples taken for each treatment are outlined in Table 1. On each sampling day a random selection of 5 cohabitant fish were collected. NNV i.p.-injected fish were only sampled at the end of the experiment on Day 125. The control fish were always sampled first to reduce the risk of cross contamination. The fish subject to sampling were removed from the tank, placed in a separate tank and then killed by an overdose of metacain (Norsk Medisinaldepot). Tissues were sampled aseptically for both real-time RT-PCR analysis and immunohistochemistry (IHC), and consisted of brain (medulla), eye (retina) and spleen. The size of all tissues sampled for PCR analysis was restricted to a maximum weight of 0.03 g, and stored at –80°C until RNA extraction.

NNV was re-sequenced from positive samples from cohabitant fish, and compared to the NNV strains used in this study. Viral RNA was extracted from spleen tissues and sequencing was performed as described below.

At Day 125 the experiment was terminated and 8 fish (5 for PCR and 3 for IHC analysis) were sampled in each group. This sampling did not include the i.p. injected fish. These were sampled separately in all experimental groups for real-time RT-PCR and IHC analysis at the end of the experiment.

### RNA extraction and real-time RT-PCR protocol

RNA was extracted using an RN Easy kit (Qiagen) according to the manufacturer's instructions and stored at –80°C. All tissues sampled were analysed using a real-time RT-PCR assay with nodavirus specific primers and probe as described by Korsnes et al. (2005). This assay targets the RNA2 segment in the viral genome that encodes the capsid protein of NNV. The reference genes, which acted as RNA extraction control, were salmon elongation factor α (Ela α) (Moore et al. 2005) and cod elongation factor 1A (EF1A) (Olsvik et al. 2006). The assays utilised TaqMan® MGB probes (Applied Biosystems) and were performed on an Applied Biosystems 7500 Real-Time PCR System.

Sequencing of viruses from NNV positive tissues was performed using NNV specific primers targeting RNA2 (Korsnes et al. 2005) and utilised the ABI

Table 1. Experimental groups used to test whether 10 fish intraperitoneally (i.p.) injected with nervous necrosis virus (NNV) would transmit the virus to 30 healthy cohabitant (cohab) fish. NNV detected in sampled tissues by real-time RT-PCR shown in **bold**. All samples taken at Day 125 were analysed first, and based on those results, further analyses were performed for other sampling days. In Group I the results were negative and only D55 samples were analysed to verify this. Groups II and III were both negative, Group IV was positive for NNV, and samples from Days 25 and 90 were analysed in addition. Groups V and VI were negative, and this was verified by analysing samples from Days 55 and 90 (cod). Groups VII and VIII were positive for NNV, and no other samples were analysed. x: days post injection on which tissue samples and analysed for each group

| Group | Type of fish | | Strain injected | Sampling day analysed | | | | | Mortality | |
|---|---|---|---|---|---|---|---|---|---|---|
| | i.p. (n = 10) | Cohab (n = 30) | | D25 | D55 | D65 | D90 | D125 | i.p. | Cohab |
| I | **Turbot** | Salmon | AAG01/03 | – | x | – | – | x | 6 | 1 |
| II | **Salmon** | Salmon | AAG01/03 | – | – | x | – | x | 2 | 0 |
| III | **Salmon** | Salmon | AH95Nor | – | – | – | – | x | 1 | 4 |
| IV | **Turbot** | **Cod** | AAG01/03 | x | – | – | x | x | 3 | 2 |
| V | Control salmon | | L15 cell medium | – | x | – | – | x | 0 | |
| VI | Control cod | | – | – | x | – | x | x | 0 | |
| VII | **Turbot** | **Turbot** | AAG01/03 | – | – | – | – | x | 3 | 0 |
| VIII | **Turbot** | **Turbot** | AH95Nor | – | – | – | – | x | 5 | 0 |

Prism™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems). Sequenced RNA2 fragments were assembled in Vector NTI™ and aligned with the sequence of AAG01/03 (AY962682) and AH95Nor (AJ245641).

### Immunohistochemistry

Tissues were sampled from all experimental groups at Day 125, including both i.p. injected fish and cohabitants. Samples of brain and retina were fixed in 4% phosphate buffered saline (PBS) formalin, dehydrated in ethanol and embedded in paraffin. Sections of 3 to 5 µm were cut on a microtome (Jung Biocut 2035) and mounted on glass slides. The sections were stained with haematoxylin and eosin, and IHC preparations using primary antibody (Ø233) were made as described by Grotmol et al. (1999), with modifications by Sommerset et al. (2005).

## RESULTS

### Mortality

Mortality was only observed in the NNV exposed groups (Table 1). In total, 27 fish (20 i.p. injected and 7 cohabitants) in the experimental groups died during the experiment. In general there were no visible signs of clinical disease except in the i.p. injected turbot in Groups I, IV, VII and VIII. These moribund turbots, listed as mortality in Table 1, displayed non-specific clinical signs such as inappetence and reduced activity. Examination of the 7 cohabitant fish that died during the experimental period did not reveal any NNV positive tissues.

### Real-time RT-PCR

The virus preparations used for i.p. injection (with AAG01/03 or AH95Nor) were tested and verified as NNV positive by real-time RT-PCR. In Atlantic salmon, brain and heart ventricle tissues screened prior to the infectivity experiment were all negative for IPNV, ISAV, SAV and NNV. In Atlantic cod and turbot, these tissues were examined and found negative for IPNV and NNV. The i.p. injected fish that died during the experiment were not examined for presence of NNV.

Reference genes Ela α in salmon and EF1A in cod were examined for all tissues analysed and used as RNA extraction controls. In salmon, the Ela α was expressed with the following mean (± SD) cycle threshold (Ct) values: brain 18.80 ± 0.72 (n = 44), retina (eye) 18.83 ± 2.88 (n = 40) and spleen 16.29 ± 0.77 (n = 40). In cod, the mean Ct values for EF1A were: brain 16.31 ± 2.50 (n = 31), retina (eye) 16.23 ± 2.97 (n = 26) and spleen 16.83 ± 3.35 (n = 27). Turbot samples were not analysed using a reference gene.

In Group I, with i.p. injected turbot and cohabitant salmon, NNV was not detected in the salmon. Samples of brain, retina and spleen tissues at Days 55 and 125 post injection were all negative (n = 29). The i.p. injected turbot in this group (n = 3) were all positive at Day 125 (Table 2).

In Group II, with i.p. injected Atlantic salmon (strain AAG01/03) and cohabitant Atlantic salmon, NNV was not detected in the cohabitant salmon. Samples of brain, retina and spleen tissues were negative at Days 65 and 125 (n = 28). The i.p. challenged salmon in this group (n = 3) were all positive in individuals examined at Day 125 (Table 2).

In Group III, with i.p. injected salmon (strain AH95Nor) and cohabitant salmon, NNV was not detected in the cohabitant salmon. Samples of brain, retina and spleen tissues were negative at Day 125 (n = 15). The i.p. injected salmon in this group (n = 3) examined at Day 125 were all positive (Table 2).

In Group IV, with i.p. injected turbot and cohabitant cod, NNV was detected in the cohabitant cod. Samples of brain, retina or spleen tissues were positive in 9 out of 10 fish examined at Days 90 and 125 (n = 29). The i.p. injected turbot in this group were

Table 2. *Scophthalmus maximus* and *Salmo salar*. Real-time RT-PCR detection of nervous necrosis virus (NNV) positive tissues at Day 125 in intraperitoneally (i.p.) injected fish in experimental Groups I (turbot), II (salmon) and III (salmon). Detection levels of NNV were determined from cycle threshold (Ct) values. x: Ct > 35; xx: Ct = 35–25; xxx: Ct < 25. Virus strains used: AAG01/03 (Group I and II) and AH95Nor (Group III)

| Fish no. | Brain | Retina/eye | Spleen |
|---|---|---|---|
| **Group I** | | | |
| 1 | – | – | x |
| 2 | xx | – | – |
| 3 | xxx | xxx | xx |
| **Group II** | | | |
| 1 | xx | xx | x |
| 2 | xxx | xx | x |
| 3 | xx | xx | xx |
| **Group III** | | | |
| 1 | xx | – | xx |
| 2 | xx | xx | xx |
| 3 | xx | x | xx |

positive for NNV in 2 out of 3 individuals examined at Day 125 (Table 3). There was no detection of NNV in tissues from cohabitant cod examined at Day 26 (n = 15). Sequencing of virus from NNV positive tissues showed it to be identical to the strain used in the i.p. injected turbot in this group (AAG01/03).

In control Group V with salmon, there was no detection of NNV in tissues examined at Days 55 (n = 8) and 125 (n = 15).

Table 3. *Scophthalmus maximus* and *Salmo salar*. Real-time RT-PCR detection of nervous necrosis virus (NNV) positive tissues in experimental Group IV in i.p. challenged turbot and cohabitant Atlantic cod at Day 90 and 125. Detection levels of NNV were determined from cycle threshold (Ct) values. x: Ct > 35; xx: Ct = 35–25; xxx: Ct < 25; n/a: samples not available. Virus strains used: AAG01/03 (Group VII) and AH95Nor (Group VIII)

| Fish no. | Brain | Retina/eye | Spleen |
|---|---|---|---|
| **i.p. challenged Day 125** | | | |
| 1 | – | x | x |
| 2 | – | – | – |
| 3 | xxx | xxx | xx |
| **Cohabitant Day 90** | | | |
| 1 | xx | xx | xx |
| 2 | xx | xx | x |
| 3 | xx | – | – |
| 4 | – | x | – |
| 5 | x | x | – |
| **Cohabitant Day 125** | | | |
| 1 | – | x | – |
| 2 | – | – | – |
| 3 | x | n/a | – |
| 4 | x | x | – |
| 5 | x | x | – |

Table 4. *Scophthalmus maximus*. Real-time RT-PCR detection of nervous necrosis virus (NNV) positive tissues in positive control Group VII in intraperitoneally (i.p.) injected and cohabitant turbot at Day 125. Detection levels of NNV were determined from cycle threshold (Ct) values. x: Ct > 35; xx: Ct = 35–25; xxx: Ct < 25. Fish were challenged with virus strain AAG01/03

| Fish no. | Brain | Retina/eye | Spleen |
|---|---|---|---|
| **i.p. challenged** | | | |
| 1 | – | – | – |
| 2 | – | – | – |
| 3 | – | xxx | x |
| **Cohabitant** | | | |
| 1 | – | – | – |
| 2 | – | – | – |
| 3 | – | – | – |
| 4 | xx | xx | xx |
| 5 | xxx | xxx | xx |

In control Group VI with cod, there was no detection of NNV in any of the tissues examined at Days 55 (n = 9), 90 (n = 9) and 125 (n = 15).

In Group VII and VIII, i.p. injected turbot and cohabitant turbot, NNV was detected in both the i.p. injected turbot and the cohabitant turbot for both virus strains used (Tables 4 & 5).

### Immunohistochemistry

A total of 33 tissues samples (brain and retina), sampled from all experimental groups, were screened by IHC for presence of NNV. The IHC screening detected NNV in i.p. injected turbot in Groups I, IV and VIII (Fig. 1). There were no NNV positive tissues in the other samples examined. All tissues examined from control fish were negative.

## DISCUSSION

In this infectivity study, 2 strains of NNV were i.p. injected in turbot *Scophthalmus maximus* and Atlantic salmon *Salmo salar*, which were then placed with healthy cohabitant salmon, cod *Gadus morhua* and turbot.

When designing the challenge experiment, turbot was chosen as the infected cohabitant and subsequent source for waterborne transmission of NNV. Turbot has been successfully used in a challenge model for striped jack nervous necrosis virus (SJNNV, Húsgard et al. 2001). Prior to this study, turbot has been used in several challenge experiments

Table 5. *Scophthalmus maximus*. Real-time RT-PCR detection of nervous necrosis virus (NNV) positive tissues in positive control Group VIII in intraperitoneally (i.p.) injected and cohabitant turbot at Day 125. Detection levels of NNV were determined from cycle threshold (Ct) values. x: Ct > 35; xx: Ct = 35–25; xxx: Ct < 25. Fish were challenged with virus strain AH95Nor

| Fish no. | Brain | Retina/eye | Spleen |
|---|---|---|---|
| **i.p. challenged** | | | |
| 1 | – | xx | xx |
| 2 | x | xxx | – |
| 3 | xx | xx | – |
| **Cohabitant** | | | |
| 1 | – | x | – |
| 2 | – | xx | xx |
| 3 | – | x | – |
| 4 | – | xx | – |
| 5 | xx | xx | – |

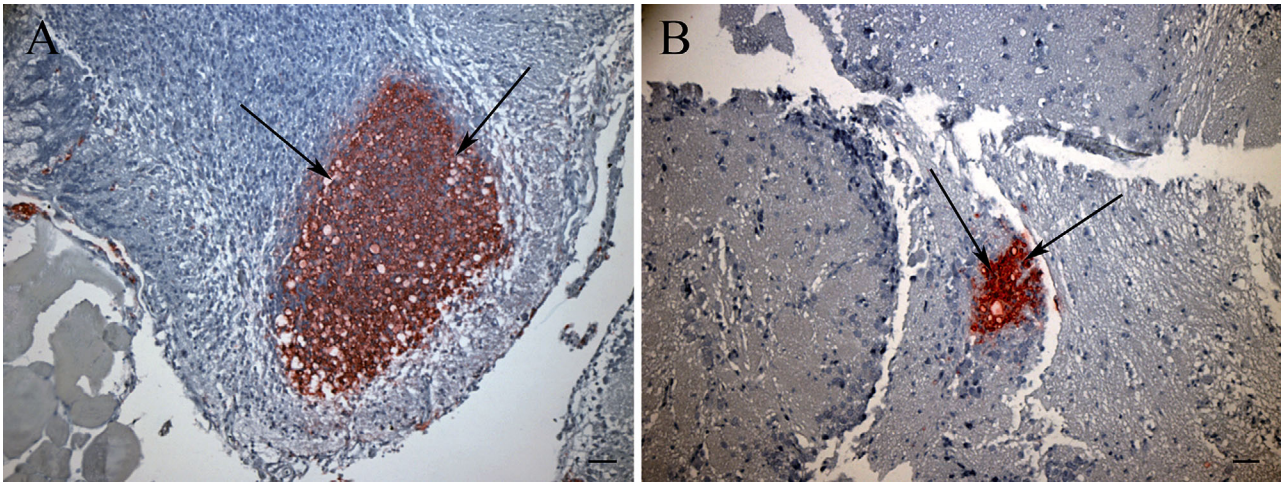


Fig. 1. *Scophthalmus maximus*. Immunohistochemistry (IHC) staining of turbot brain at Day 125 post intraperitoneal injection with nervous necrosis virus (NNV). (A) Large area of NNV IHC-positive brain cells (arrows) in turbot from experimental Group IV. Vacuolated cells are visible within the immunolabelled area. (B) IHC-positive cells (arrow) in brain from turbot in experimental Group I. Scale bar = 20 µm

using halibut strains of NNV (A. H. Nerland unpubl. data), showing this fish species to be suitable for a cohabitation challenge study.

Two strains of halibut NNV were used in the study, to better account for any strain-related differences in transmission and possible disease development in salmon. The use of salmon in this study was motivated by the observation of Korsnes et al. (2005), which showed that salmon i.p. injected with NNV from halibut may develop clinical disease (VNN). The positive controls (Groups VII and VIII) showed that both virus strains caused infections in the i.p. injected turbot and also resulted in infection in turbot cohabitants, thus validating the experimental design. At the time when the study was conducted, no NNV strain from cod was available in Norway. However, phylogenetic analyses of isolates originating from halibut and cod indicate a high degree of similarity. They cluster as closely related groups in the BFNNV clade (Nylund et al. 2008). Moreover, a NNV strain isolated from commercially farmed cod has previously been identified belonging to the halibut cluster (A. Nylund unpubl. data), which supports the use of halibut NNV isolates in interspecies infectivity studies. Although host specificity of NNV has been suggested (Iwamoto et al. 2004, Ito et al. 2008), there are no reports suggesting this for isolates in the BFNNV clade. Interestingly, an isolate closely related to NNV from farmed halibut was found by Thiéry et al. (2004) in sea bass *Dicentrarchus labrax* in France. Existence of commercial halibut growth sites and hatcheries along the coast of Norway, together with reports of VNN outbreaks (Hellberg & Dannevig 2002, 2003) and detection of NNV in water of rearing units of halibut (Nerland et al. 2007), suggest the possibility of horizontal transmission of halibut strains of NNV to susceptible hosts.

The duration of the experiment was set at 125 d, in order to provide sufficient time for NNV to be established in the i.p. injected fish and to infect cohabitant fish. This was based on previous observations in i.p. injected salmon (Korsnes et al. 2005) and cod (Korsnes et al. 2009). Tissues analysed were parts of the central nervous system (CNS) (brain and eye) and spleen as a representative of a haematopoietic tissue. The spleen was chosen based on the observation in i.p. injected cod, where NNV was first detected in spleen, kidney and heart ventricle tissues with subsequent establishment in the CNS (Korsnes et al. 2009).

Cod cohabitated with NNV injected turbot acquired infections as evidenced by NNV positive tissue samples. The virus was also detected in brain and eye (retina) samples, suggesting that the virus is capable of entering the primary organ of replication (CNS). We have previously provided evidence suggesting that the brain and eye are important for replication of NNV in i.p. injected cod (Korsnes et al. 2009). Hence, the i.p. injection, cohabitation and field evidence suggest that cod are susceptible to NNV isolates from halibut, and consequently there appears to be a risk that wild cod in the vicinity of halibut hatcheries and farms may acquire an infection. However, the study did not directly demonstrate that replication of NNV took place in cod. By mechanisms

yet to be identified the virus entered cod tissues, as demonstrated by the presence of viral RNA2 segment sequence. The presence of virus in the spleen may be the result of antigen trapping, but the entrance of the virus into the CNS suggests that replication in known target cells is possible. Cod naturally infected with NNV normally show low levels of virus in CNS samples, and are often referred to as NNV 'carriers' (Nylund et al. 2008). It appears likely that the experimental cod with NNV positive CNS entered such a 'carrier' state.

Important questions remain concerning the factor(s) responsible for virus replication and eventual development of VNN. Korsnes et al. (2009) observed increased virus levels in infected cod receiving prednisolone-acetate, an immunosuppressor used to simulate stress. Hence periods of immunosuppression such as sexual maturation, spawning, handling or suboptimal environmental conditions may promote virus replication. Therefore cod with persistent NNV infections may represent a risk of spreading NNV virus in aquaculture. Horizontal transmission of a halibut isolate of NNV between 2 different fish species raises further questions regarding the actual host range of viruses in the BFNNV clade. The close phylogenetic relationship between halibut and cod isolates of NNV and our observations on interspecies transmission suggest that gadiform and pleuronectiform fishes may be susceptible to NNV strains in the BFNNV clade. Unfortunately, most halibut and cod NNV isolates are from farmed fish, and it has so far not been possible to culture or sequence NNV directly from infections detected in wild fish of other species (Nylund et al. 2008). Interestingly, Nguyen et al. (1996) detected NNV in epithelial cells in the skin of striped jack larvae, but detection was restricted to Days 1 and 2 post-hatch. If infection of the epithelium could occur in older fish as well, the possibility of transmission via common ecto-parasites such as *Caligus* spp. exists. These parasitic copepods feed on the host epidermis and many species show both intra- and inter-specific host transfer. Hence *Caligus* spp. and other caligids are potential vectors of the virus.

I.p. injected turbot and Atlantic salmon became NNV infected (Groups I, II, III), while cohabitant Atlantic salmon did not. As is evident from the other cohabitation groups targeting turbot and cod, the virus is released from infected turbot and consequently Atlantic salmon appear to be refractory when exposed to i.p. injected turbot (Group I) or i.p. injected salmon (Groups II and III). Interestingly, none of the i.p. injected salmon developed clinical signs as described by Korsnes et al. (2005). However, the number of fish injected was small (10 in each group) and this may explain the lack of signs. The lack of transmission of NNV to Atlantic salmon may relate to the host itself. Studies of transmission of NNV to sea bream *Sparus aurata* suggest this species is a sub-clinical carrier of the virus (Castric et al. 2001). In experimental infections with sea bream using i.p., intramuscular (i.m.) and bath exposure, only the i.m. route of inoculation produced NNV infection and mortality in fish of 2 g (Aranguren et al. 2002). Although methods to detect NNV in that study were restricted to cell culture and immunoperoxidase assay, which are less sensitive than PCR, the results support the theory that infection obtained by injection may not be reproduced using bath or waterborne transmission of virus. However, it is also noted that Aranguren et al. (2002) used sea bream with an average weight of 2 g, while Castric et al. (2001) used fish of 15 g on average. These 2 studies demonstrated that sea bream is a susceptible host, but the development of clinical disease may be influenced by fish size and method of exposure. The lack of transmission of NNV to cohabitant Atlantic salmon may also be influenced by the fish size used, potential low shedding of virus from i.p. infected fish or insufficient time for establishment of detectable levels of NNV in the cohabitants. However, the study does demonstrate that under the experimental conditions provided only Atlantic cod became carriers. It is important to emphasize that this study did not demonstrate any disease development in horizontally infected cod.

In conclusion, this study suggests a risk of interspecific NNV transmission to cod kept in close proximity to halibut farms that contain fish with NNV infection. Farmed and wild cod may become carriers of NNV strains released from hatcheries, and contribute to the spread of NNV. A particular risk is posed by introduced strains. The results also suggest that Atlantic salmon is resistant or at least less susceptible to horizontal transmission of NNV than Atlantic cod. However, since many factors may influence susceptibility, we do not exclude the possibility of waterborne transmission to salmon. Further studies are needed on the apparent resistance of salmon to waterborne NNV, particularly using smaller size fish and juveniles.

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