Atlantic halibut reovirus (AHRV): Reservoirs and transmission in culture systems.

A master thesis in Aquaculture Biology by
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Abstract:

The ability to expand within the aquaculture industry is essentially based on reliable production of healthy juvenile fish. In 2013 a new *aquareovirus* was found to be associated with high juvenile mortality at a Norwegian Atlantic halibut production site, but the source of the virus was not identified. Feed and water intake had been tested with negative results. The aim of this study is to test the halibut brood fish as a possible source for the virus. The brood fish are stripped several times during their life time which means that it is necessary to develop non-lethal sampling methods for screening with respect to presence of virus. The Atlantic halibut reovirus (AHRV) is known to replicate in the liver and pancreas tissues of the halibut and large amounts of virus have also been detected in the kidneys. This means that shedding of the virus through faeces or urine could contaminate the eggs during stripping. A method of anal swabbing was developed for testing of faeces and urine for presence of the AHR virus. In addition, eggs and milt from each batch liberated from the brood fish were tested for presence of the virus. Halibut larvae produced during the study period suffered a disease outbreak associated with both AHR virus and Infectious pancreatic necrosis virus (IPNV). The IPN virus is also known to replicate in liver, pancreas and kidney of the halibut, which mean that the same approach for identifying possible virus positive brood fish could be used for both viruses. The ovary fluid from a female halibut tested positive for AHRV and IPNV and the tanks containing larvae hatched from eggs from this female suffered high mortalities. The female brood fish halibut was killed and tissues taken for analyses which revealed presence both AHRV and IPNV in the posterior kidney. Sequencing of segment 10 from AHRV present in both the larvae and the brood fish gave 100% identical sequences which suggest a common origin of the two viruses.

The screening of the broodstock halibut may be an essential part of securing healthy juvenile halibuts in the future.

Content:

1. Introduction:

1.1 The Atlantic halibut (*Hippoglossus hippoglossus*) in aquaculture.

The Atlantic halibut is commercially farmed in Canada, Scotland and Norway (Iceland until 2011). It is an iteropareous fish and each female is able to produce up to 15 batches of eggs each season (Norberg et al., 1991). Eggs from each female are stripped into a jar and externally fertilised with sperm from stripped males. The fertilised eggs are kept in an incubator with upwelling seawater at 6 °C for 10 days (Mangor‐Jensen et al., 1998) and then moved over to a larger upwelling silo (Harboe et al., 1994) where they hatch after 14 days post fertilisation or at the equivalent of 80 day degrees (dd). The mucosal surface of marine fish eggs provides a good substrate and adhesion for the colonisation by bacteria, which makes the eggs vulnerable to infections (Hansen and Olafsen, 1989). *Tenacibaculum ovolyticum* is known to be able to penetrate the eggshell of the Atlantic halibut and thereby induce infection followed by mortality in both eggs and larvae after hatching (Hansen et al., 1992).

From eggs to larvae the halibuts are kept in complete darkness. About 40 days post hatching the larvae are transferred over to an indoor seawater tank with temperature of 12 °C and a 24:0 light regime. The mortality at the larval and juveniles stage is often one of the biggest obstacles in marine aquaculture. In this early life stage the ability of the fish to adjust or compensate for the potential suboptimal conditions is limited or very costly. With the immune system under development and with a high population density, pathogenic organisms like fungi, bacteria, parasites and viruses can rapidly spread causing infection and high mortality.

The larvae are fed on live and enriched *Artemia* nauplii. Addition of nutrients to the artemia might provide an environment suitable for growth of pathogens such as *Vibrio* spp. (Verner-Jeffreys et al., 2003). *Vibrio anguillarum* has been found to cause mortality among the start-feeding larvae (Bergh, 1995). Post metamorphosis the larvae are weaned to dry feed. Malnutrition or suboptimal environmental conditions can result in malpigmentations or incomplete eye migration post metamorphosis.

Several parasites can be found in halibut farming, but they are rarely the primal cause of mortality. In 2003 and 2004, increased mortality of juvenile halibut occurred in an Atlantic

halibut farm in western Norway due to a co-infections with bacteria and the parasite, *Ichthyobodo* (Isaksen et al., 2007). *Ichthyobodo* is an ectoparasite that has been reported to infect farmed Atlantic halibut both in Scotland and Norway (Bruno, 1992, Rødseth, 1995, Bergh et al., 2001, Isaksen et al., 2007). The symptoms are often associated with loss of appetite, change in behaviour and a greyish skin colour (Bergh et al., 2001). This parasitic infection can be treated with formalin followed by improved water flow and reduced density of fish.

When it comes to viral infections in farmed halibut there are no available treatments. The Atlantic halibut nervous necrosis virus (AHNNV) is a single-stranded RNA virus and was detected for the first time in juvenile halibut (*Hippoglossus hippoglossus*) in 1995 (Grotmol et al., 1995, Grotmol et al., 1997). The infected fish typically stopped eating and tended to have a spiral-swimming pattern with looping. Moribund juveniles are often observed lethargic ending up lying upside down on the bottom of the tank (Grotmol et al., 1997). Mortalities from an AHNNV infection can reach up to 100 % (Grotmol et al., 1997). The virus is believed to be spread from infected broodstock to their offspring during the stress caused by spawning (Grotmol, 2000). In brood fish of striped jack, the Striped Jack Nervous Necrosis Virus (SJNNV) was found both on the inside and outside of the newly spawned eggs, giving strong indications of vertical transmission of the virus (Arimoto 1992). In Norway the production of Atlantic halibut was limited by the mortality and problems connected to AHNNV (Grotmol et al., 1995) (Bergh et al., 2001). Due to the presence of AHNNV the Norwegian Atlantic halibut farming was for several years been depended on the import of juveniles from both Canada and Iceland. This may have contributed to a viable production and more diverse genetics, however, transport of live aquatic animals over long distances also poses a risk of an introducing new pathogens (Mortensen et al., 2006).

The infectious pancreatic necrosis virus (IPNV) is a bi-segmented double-stranded RNA virus from the family of *Birnaviridae (*Dobos, 1995). The virus has been annually causing disease in the European salmon industry and restrained the production of juveniles and smolts over several years (Ariel and Olesen, 2002). The number of outbreaks increased until 2009 with a total of 223 aquaculture production sites infected, while in 2014 only 48 infected localities were reported (Olsen.et al 2014). Mortalities from an IPNV infection in a salmon farm can be anything from negligible to almost 100 % (Brun, 2003). There are strong indications that IPNV can be vertical transmitted from brood fish to offspring (Wolf and Quimby, 1969, Reno, 1999). In Atlantic salmon farming hygienic plans were conducted to eradicate “in house strain“ of the IPNV in freshwater sites. The discovery of genetic markers

identified with the IPNV resistance in Atlantic salmon has made the breeding of the IPNV resistant smolt possible (Ozaki et al., 2001). In Atlantic Halibut the IPNV is known to induce mortality during weaning and in early fry. The clinical signs can be distended stomach and uncoordinated swimming (Biering et al., 1994). In 1989 an outbreak of IPN was registered in a halibut farm in Norway (Mortensen et al., 1990) and in 1997 an IPN outbreak in a halibut farm in Scotland caused mortalities up to 90 % (Rodger and Frerichs, 1997). During a challenge experiment using IPNV on Atlantic halibut eggs, one control group turned up weakly positive. This indicate possible vertical transmission of the virus from parent to offspring (Biering and Bergh, 1996).

An indication of a possible new virus disease with high mortalities in halibut farming was first reported in Canada, when juvenile Atlantic halibuts showed signs of multifocal hepatocellular necrosis and as well as acute necrosis of proximal renal tubules (Cusack et al., 2001). The accumulated mortality reached 58 %. Based on morphology from transmission electron microscopy and RT-PCR results, it was suggested that the virus could belong to the family of *Reoviridae* and genus *Aquareovirus.* The broodstock had been captured in the wild and had not been screened for any viral pathogens. Vertical transmission from the broodstock was considered as one possible source of the viral infection (Cusack et al., 2001). In Scotland in 2003, a farm with post weaned halibuts experienced high mortality with symptoms similar to the case of Canada (Ferguson et al., 2003). Fish with clinical signs showed histopathological changes in the liver and transmission electron micrograph revealed large numbers of reovirus-like particles within the hepatocellular cytoplasm. The accumulated mortality reached as high as 98 %. In 2013 samples from a population of farmed Atlantic halibut in Norway showed pathology changes similar to that described from Canada in 2001 and Scotland in 2003. An *aquareovirus* was cultured from the fry and the RNA-depended RNA polymerase gene from the virus showed the highest amino acid sequence identity (80 %) to an isolate belonging to the species *Aquareovirus A* (Blindheim et al., 2014).

The presence of AHRV in wild populations of Atlantic halibut is unknown and so far it has only been detected in farmed Atlantic halibuts. Within the family Reoviridae, AHRV is the second virus detected in farmed fish in Norway, the first on being piscine reovirus (PRV) found in farmed Atlantic salmon (Palacios et al., 2010). AHRV is the second member of the genus Aquareovirus that is isolated from a strictly marine fish, the first was from a diseased turbot in China (Ke et al., 2011). During routine inspections the Aquareovirus can be found on seemingly healthy finfish, mollusc and crustaceans, but within the aquaculture industry in

China and East Asia, *aquareovirus* have been the cause of high mortality among the juvenile farmed fishes (Ke et al., 2011, Fang et al., 1989).

1.3 Aim of study.

The main purpose of this study is to determine the source of the AHRV infection at an Atlantic halibut broodstock-farm in Norway with a main focus on the brood stock itself. An important part of the study will be the development of a suitable method for non-lethal testing the large and valuable brood fish.

2. Materials and methods:

2.1 Site description

The halibut broodstock and hatchery site is located in the middle of western Norway in close connection with the Norwegian Sea. The site has a double water intake at 55 and 150 meters respectively. The seawater to the broodstock runs through a sand-filter, while the seawater to the eggs, larvae and juveniles receive an additional treatment in a UV-filter. Temperature and light are adjusted to accommodate the different broodstock groups. Five groups of Atlantic Halibut broodstock are kept in five different tanks and stimulated to spawn at different intervals of the year (Figure 2.1). Two tanks containing 10 male halibut each are kept separate from the female halibuts. Eggs from one female halibut is fertilised with sperm from one male, and is then transferred into an incubator cylinder with upwelling seawater. During the spawning period eggs are periodically released from the female halibut as a batch of eggs. 10 days post fertilisation the batches are moved from the incubators to an upwelling silo, where eggs from several female halibuts are mixed.

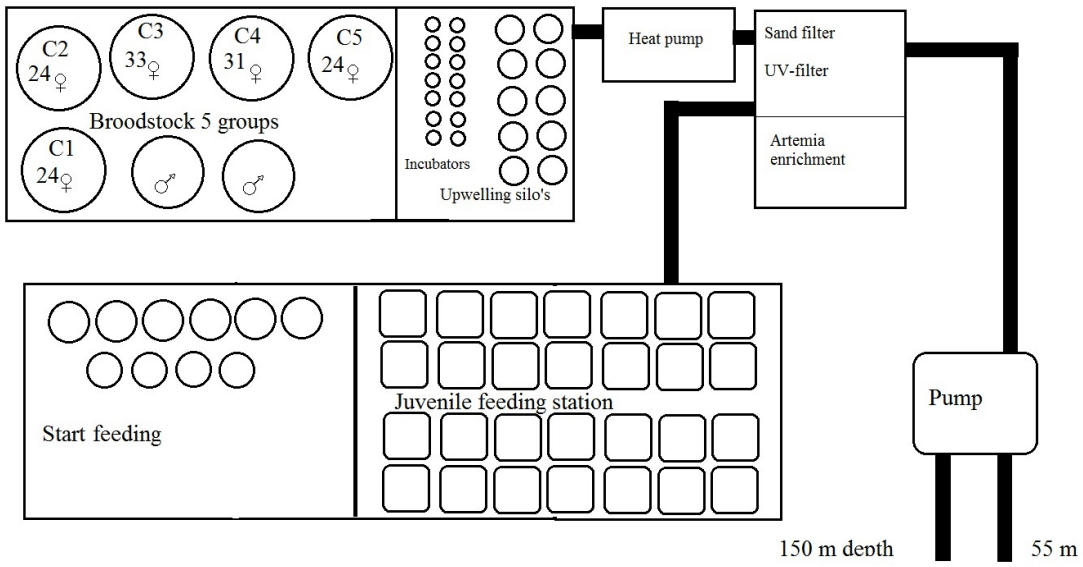

Figure 2.1: Principle outline of the facility. The illustration is a simplified version of the site. Production flow: **1.** Broodstock is stripped of eggs and milt. **2.** Fertilised eggs are transferred to the incubators, and then to the upwelling silo´s. **3.** Hatched larvae´s are transferred to the start feeding tanks. **4.** After weaning the larvae are moved to the Juvenile feeding station. There are 10 male halibuts in each of the male halibut tanks.

The eggs hatch after 14 days past fertilisation and 40 days past hatching the larvae are transferred to the startfeeding tank (Figure 2.2). Enriched *Artemia* nauplii are provided to the halibut larvae as feed. Some mortality is expected during startfeeding. Prolonged or

exponential mortality in startfeeding in combination with a drop in appetite can be indication of bacterial or viral infection. The larvae are weaned to dry feed after metamorphosis. The juveniles are then transferred to the juvenile feeding station. When the juvenile reaches approximate 5 grams they are ready for transport to another facility.

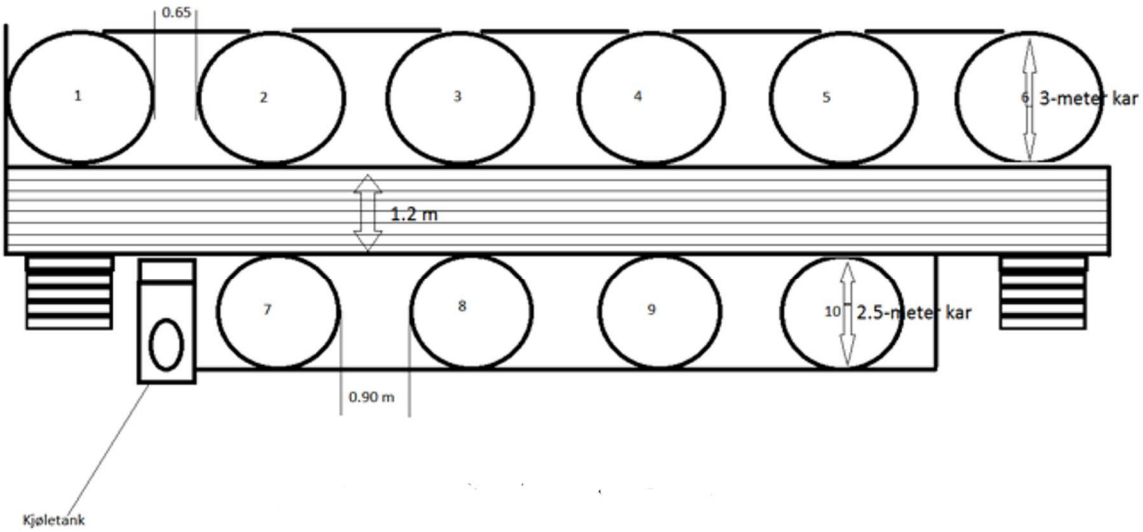

Figure 2.2: The outline of the startfeeding tanks. Tanks 1 to 6 are 3 meters in diameter and tank 7 to 10 is 2.5 meters in diameter.

2.1.1 Outbreak history

Since 2011 there had been a high frequency of unexplainable mortality among the juvenile halibuts. The problem often starts during startfeeding, as larvae suddenly would have a drop in appetite. Larvae viewed under microscope revealed that those not eating tended to have a liver reduced in size. Use of antibiotics or formalin has not had any positive effect on the overall mortalities. In 2013 moribund halibut larvae were sent to the Fish Disease Research Group at the University of Bergen where it was shown that the mortality of the larvae was associated with a new species of Aquareovirus (Blindheim et al 2014). Before this study, the last confirmed AHRV associated mortality was in the beginning of 2014. Other diseases as IPNV had not been observed at site during the last 20 years.

2.2 Sampling

During the outbreak at the site in 2013, the inlet water and live feed *Artemia* naupii were tested and found to be negative for AHRV (pers.com. A.Nylund). A decision was made to search for the presence of AHRV among the broodstock halibuts. Using transmission electron

microscopy (TEM) the virus has been found in the liver, kidney and the pancreas of infected halibut larvae (Cusack et al., 2001, Ferguson et al., 2003, Blindheim et al., 2014). It is possible that the virus may pass from the liver and pancreas through the intestines and into the hindgut. From AHRV present in the kidney, virus can be transmitted out through the urine. Eggs may be contaminated from faeces in gut or urine as they pass the anus. The screening of the broodstock halibut is based eggs and milt, as well as secrete from hindgut. Egg and milt was transferred using a disposable pipette from a collection jug into a 2 ml eppendorf tube, then stored at – 20°C. Several egg samples were collected from each female halibut. Samples from the hindgut were collected with Q-Tips (See section 2.3 Q-Tip method). Ten offspring from each group was collected for future analyses. Biofilm from inlet and outlet water pipe, and the inside of the tank was collected using a Q-Tip as a swab.

A water sample of 1 litre was collected from the production water and analysed for presence of virus. Start feeding tanks with halibut larvae suffering from disease were sampled and analysed. The occurrence of possible new disease, mortality of larvae with white mucus in the intestines, was also sampled. Two selected brood fish halibut were euthanized and several organ tissues were analysed for presence of AHRV and IPNV.

2.3 Q-Tip method

The Atlantic halibut broodstock fish are used for many years, and are considered to be of high value. It is therefore important that the sampling of the fish should be conducted efficient and without stressing or injuring the halibut. During the stripping of eggs, the Atlantic Halibut female is guided up on a rubber-covered table just above water level. After some movement the halibut calms down and it is possible to press out the newly ovulated eggs. The fish should not be out of water more than a couple of minutes, so the time window for sampling is narrow. As the halibut can start moving at any time, any pointy or sharp objects was ruled out as too risky for sampling. The solution became a standard Q-Tip inserted 1.5 cm into the hindgut of the fish, and then cut to fit in an eppendorf Safe-Lock Tube 2.5 ml. The samples were then frozen in at -20° Celsius.

2.4 Optimisation of Q-Tip RNA extraction

The use of Q-Tip for sampling presented some challenges towards the RNA extraction.

i: How much fluid is retained by a Q-tip?

ii: How to extract the sampled material from the Q-Tip and remove the Q-Tip with minimum loss of the fluid from the cotton tip?

i: Eppendorf tubes (size 1.5 ml) was filled with different amounts off water: 50, 100, 150 and 200 µl. The Q-Tip was immersed in the fluid for 10 seconds and the Eppendorf tubes were inspected for residues of water. The largest amount retained by the Q-Tip was then noted as potential maximum amount of sampled material. It also represents the potential amount of solution that could be lost during tissue lysis with Isol as the Q-Tip needs to be removed before adding chloroform.

ii: Four different RNA extraction methods were tested (A-D). All the tested Q-Tips were first added 100 µl supernatant of cell culture media containing AHRV. Each tested method was run in four replicas, where the fourth sample was added the supernatant the day before and kept at -20° C over night.

A. A 1.5 ml Eppendorf tube with the primed Q-Tip was added 1000 µl of Isol and vortex twice for 15 seconds. Then the Q-Tip was moved over in an new Eppendorf tube with a 1000 µl pipette that was cut to fit inside (Figure 2.7.D) Then the Eppendorf tube was centrifuged to force the remaining fluid off the Q-Tip. The Q-Tip and pipette was removed and the retrieved fluid was then transferred to the first Eppendorf tube. 2 µl IPNV-spike was added and the rest of the RNA extraction followed standard procedure (Section 2.6).

B. The primed Q-Tip was added 100 µl of cell culture medium and directly put in a 1000 µl pipette cut to fit inside a 1.5 ml Eppendorf tube (Figure 2.7.D). The tube was then centrifuged for 10 seconds in order to extract fluid and material from the Q-Tip. The pipette and Q-Tip was then removed. Then 1000 µl of Isol and 2 µl IPNV-spike was added. The rest of the RNA extraction followed standard procedure.

C. The primed Q-Tip was added 100 µl of Isol and put directly in a 1000 µl pipette cut to fit inside a 1.5 ml Eppendorf tube (Figure 2.7.D). The tube was then centrifuged for 10

seconds in order to extract fluid and material from the Q-Tip. The pipette and Q-Tip was then removed. Then 900 µl of Isol and 2 µl IPNV-spike was added and the rest of the RNA extraction followed standard procedure.

D. The primed Q-Tip was placed in a 1000 µl pipette and 1000 µl of Isol was pumped up and down the pipette 10 times (Figure 2.1.E). The fluid was then pumped out of the pipette. The pipette and Q-Tip was then removed. 2 µl IPNV-spike was added and the rest of the RNA extraction followed standard procedure.

E. Control group. 100 µl of supernatant AHRV cell culture medium and 2 µl IPNV-spike was added to 1000 µl Isol and followed standard RNA extraction procedure.

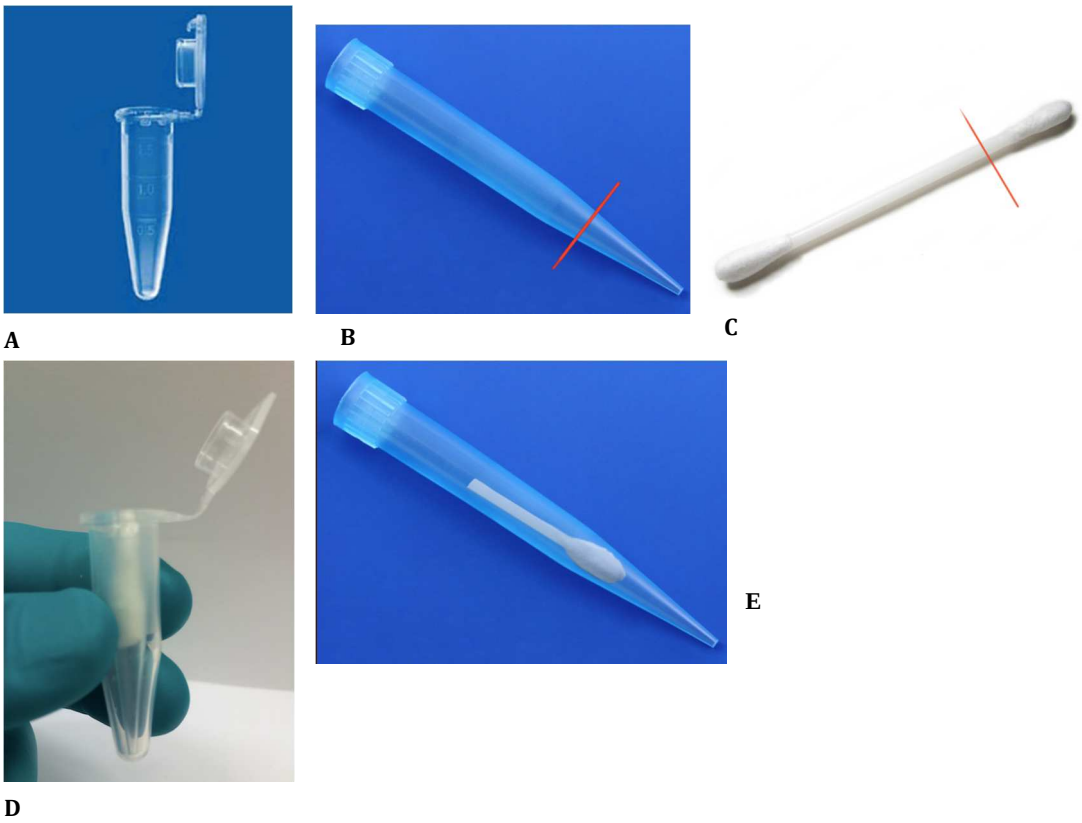

Figure 2.7: A: A standard 1.5 ml Eppendorf tube. **B:** In order to spin off absorbed material from the Q-Tip, the pipette was cut according to the red line to fit inside the Eppendorf tube, creating a funnel. **C:** The Q-Tip was cut according to the red line to fit inside the Eppendorf tube. **D:** The Q-Tip inside the pipette funnel assembled inside the Eppendorf tube. **E:** The Q-Tip inside of a 1000 µl pipette.

2.5 Water filtration

The filtration of water sample was conducted using a vacuum pump (VP 100C VWR®,USA). Before filtration, 20 µL of VHS virus supernatant was added as exogenous control for the real-time RT-PCR. The filtration procedure was a modified VIRADEL method (Andersen et al., 2010). The water sample was filtered through an electro positive Zeta Plus™ 1MDS filter that captures the virus particles in the water. The filter was then transferred to a mini Petri dish and added 1400 µL Lysin buffer and 28 µL β-mercaptoethanol before incubating for 10 minutes with mildly shaking. The lysis buffer was then transferred to 2 eppendorf tubes, 700 µl in each.

Before RNA extraction 7 µl SAV spike was added to act as an extraction control against the filtration control. RNA extraction was conducted with E.Z.N.A-Kit according to the producer's recommendations. The sample was then analysed using real-time RT-PCR with assays for AHRV, IPNV, VHSV and SAV.

2.6 RNA extractions

The RNA extractions was done according to (Devold et al., 2000), with some modifications. Instead of Trizol, 1000 µL Isol-RNA Lysis Reagent (5 PRIME) was added and the tissue sample was homogenized in a TissueLyser LT (Qiagen) for 5 min (50 oscillation). The RNA pellet was washed twice with 75 % and 96 % ethanol. The pellet was then added RNase free water heated to 70 °C. The samples were stored at -20 °C. The purity and concentration of RNA was tested with NanoDrop ND-1000 spectrophometer.

2.7 RT-PCR and Sequencing

In order to identify the AHR virus from selected positive samples, PCR and sequencing was performed. First cDNA was synthesised by running a reverse transcriptase reaction with GeneAmp TM PCR (Applied Biosystem). The first step was run in a 10.0 µl reaction with 1.0 µl AHRV-S10-F1 primer (10 µM), 1.0 µl pd(N)6 (10µM), 4.0 µl RNA and 4.0 µl RNase-free water, in 5 minutes at 70° C. Then this was added to a 16.0 µl reverse transcription-mix containing 5.0 µl (5X) Buffer, 4.0 µl dNTP, 8.0 µl RNase-free water and 0.5 µl 10mM MMLV and incubated at 37° C for 60 minutes. From this 2.0 µl cDNA was used in combination with different primers in a solution containing 2.5 µl dNTP, 1.0 µl forward

primer (10µM), 1.0 µl reverse primer (10µM), 16.0 µl RNase-free water and 0.5 µl Taq DNA polymerase. The PCR program was set to start at 94 °C for 2 minutes and then run 35 cycles with 94 °C in 30 seconds, 56° C in 50 seconds and 72 °C in 2 minutes. The program ends with 72 °C in 5 minutes and then continues stable at 4° C.

The different primer combinations were then run in a gel-electrophoresis. 1 % agerose gel with 2.0 µl GelRed™ (1uL GelRed per 25ml agarose) was immersed in 1x TAE buffer (Appendix). A 2.5 µl smart ladder was added in one well as a molecular weight marker. 5 µl of PCR product from each sample was then mixed with 1 µl loading buffer (6X) and added to designated wells. The gel was connected to 90 V for approximate 30 minutes. The result was visualised using GelLogic 212 pro (Carestream) and Carestream Molecular Imaging Software, Standard Edition, Version 5.0.2.30

To purify the PCR-products an ExoSAP-It (USB®) was used according to protocol. The reaction mix contained 2.5 µl PCR product and 1 µl Single-step ExoSAP-It (USB®), and then run for 37 °C for 15 minutes and 80 °C for 15 minutes.
The sequencing was done using BigDye™ v.3.1 terminator cycle sequencing reaction kit (Applied Biosystems) with the purified PCR product as template. The reaction mix contained 1 µl BigDye®, 1 µl BigDye® 5X buffer, 1 µl of each primer (10µM) (forward and reverse primers in separate tubers) 1 µl template and 6 µl RNase free water. The reaction was run in 35 cycles: 96 °C for 10 seconds and 50 °C for 5 seconds and 60 °C for 4 minutes. After the program was finished 10 µl RNase free water was added each sample and delivered to The Sequencing laboratory at the University of Bergen for prossessing the gene sequences. The VectorNTI software (Invitrogen) was used for assembly and analyse of data. List of primers used in table 2.1.

Name	Sequence 5′ → 3′
AHRV-S10-F1	CGA TCA TCA TGG ACA CCA AGC
AHRV-S10-R1	CAC CTA CAT CAC CGG CTC G
AHRV-S10-F2	ATG GA CAC CAA GCC TCT TC
AHRV-S10-R2	GAT TGT CGT CTT CTG ACC C
AHRV-S10-F3	GCA AAA CTT GGC GAA ACC
AHRV-S10-R3	CGC ATA CCT CTG TTG GAT G
IPNV F1	CGT TAG TGG TAA CCC ACG
IPNV R1	TTG TTA GGG ACA TCA GGC
IPNV F2	ATC CAA AGC TCC ACA CTA CC
IPNV R2	AGC TTG ACC CTG GTG ATC
IPNV F3	TCT CAG CCA AGA TGA CCC AG
IPNV F4	CTG GAG AGA CAT AGT CAG AGG
IPNV R4	CAG AGG GAC CCA TGA TTG
IPNV F5	ACG GGA ACA TAG TAG TCG AG
IPNV R5	GTG CTG ATG AGC TTTC CG
IPNV F6	CAT GGA CCA GAA AGA ACG
IPNV R6	TTC ATC TGT CTT GCG AGC

Table 2.1: List of primers used for sequencing of segment 10 from AHR virus and segment A from the IPN virus.

2.8 Real-time RT-PCR

Real-time RT-PCR was used for detection and quantification of AHRV genome in the samples. The AgPath-ID^{TM}One step RT-PCR kit was utilised in this study according to table 2.2. The real-time RT-PCR analyses were run by the Applied Biosystems 7500 Fast Real-Time PCR System. The list of primers and probes used are in table 2.3

Volume	**Standard reaction**
2X RT-PCR	6.25 µl
10µM forward primer	1.0 µl
10µM reverse primer	1.0 µl
10µM probe	0.22 µl
25X RT-PCR enzyme mix	0.25 µl
RNase-free H_2O	1.75 µl
Template	2.0 µl
Total	12.5 µl

Table 2.2: The Real-time RT-PCR standard reaction volume.

Name	Sequence 5′ → 3′	Refrence
EF1A1 F	CCATGGTTGTGGAGTCCTTCTC	(Øvergård et al., 2010)
EF1A1 R	GATGACACCGACAGCCACTGT	
EF1A1 P	6FAM – CTCCCCTCGGTCGTTTCGCTGTG – BHQ	
AHRV-7F	CCC GTA TTA GCA GTT ATC CTG TAT C	(Blindheim et al., 2014)
AHRV-7R	CCC CAT CCT GCA CAT TCA AG	
AHRV-7P	GAT CCC ATG ATC GGT GAG G	
IPNV-F	ACCCCAGGGTCTCCAGTC	(Nylund et al 2011)
IPNV-R	GGATGGGAGGTCGATCTCGTA	
IPNV-P	TCT TGG CCC CGT TCA TT-	
AHRV-10F	GCTTTATGCGACGCTCTCACT	Present study
AHRV-10R	GCCCCATTGTGATCCAGTTT	
AHRV-10P	ATT TGT ATA TGC CCG G	
VHSV-FO8	TGT CCG TKC TTC TCT CCT ATG TAC T	(Duesund et al., 2010)
VHSV-RO8	GCC CTG RCT GMC TGT GTC A	
VHSV-PO8	CTC ACA GAC ATG GG	
NSP1-F (sav)	CCG GCC CTG AAC CAG TT	(Hodneland and Endresen, 2006)
NSP1-R (sav)	GTA GCC ACC TGG GAG AAA GCT	
NSP1-P (sav)	TCG AAG TGG TGG CCA G	
VNN -F	TTCCAGCGATACGCTGTTGA	Korsnes et al
VNN- R	CACCGCCCGTGTTTGC	
VNN-P	AAA TTC AGC CAA TGT GCC	

Table 2.3: List of primers and probes used for real-time RT-PCR. EF1A1 is the elongation factor for halibut.

2.9 Efficacy test and normalisation of data

The assay AHRV10 is targeting segment 10 that encode the putative outer capsid protein (VP7). To do an efficacy test on assay AHRV10, the primers (forward and reverse) were first optimised using probe, enzyme and buffer concentrations from a Standard AgPath-IDTM One-Step RT-PCR Kit (Life Technologies) in combinations with 10 different forward/reverse primer concentration: 300/300, 300/600, 300/900, 400/400, 600/300, 600/600, 600/900, 900/600 and 900/900 in nM. Then the assay was tested using different concentrations of probe in NM (25, 50, 75, 100, 125, 150, 175, 200 nM and 225 nM). The test of the primers and probe were conducted in triplicates.

To determine the assays ability to detect the target gene, an efficacy test was performed. The target RNA was then diluted in a tenfold series (10^{-1} – 10^{-8}) and run together with the

optimised probe and primers in a real-time RT-PCR. The dilutions series was run in triplicates.

The Ct-values were plotted into an Assay Efficiency Reference standard curve, and the slope and regression values were then calculated. The assay efficacy (E) were calculated using following formula (Pfaffl, 2004):

$$\mathbf{E = (10^{-1/slope})}$$ (Pfaffl, 2004)

The Ct-values were normalised against the elongation factor EF1A1 (Øvergård et al., 2010). The normalised expression (NE) was calculated on the basis of the efficacy (E) and Ct-values of the different assays using the formula by (Simon, 2003).

$$\mathbf{NE = (E_{reference})^{Ct\ reference} / (E_{target})^{Ct\ target}}$$ (Simon, 2003)

Fold increase is normalised expression of a target template divided by the lowest target value.

$$\mathbf{NE_{fold} = NE / NE_{Lowest\ value}}$$

The Efficiencies used in this thesis is listed in table 2.4

ASSAY	EFFICIENCY	REFERENTS
EF1A1	1.96	(Øvergård et al., 2010)
VHSV08	1,98394	(Duesund et al., 2010)
nsP1(SAV)	1.8743	(Andersen et al., 2010)
IPNV	1.944541	Present study
AHRV 10	2.008469	Present study

Table 2.4: The assays and efficiencies values. The IPNV and AHRV 10 were calculated in this study.

3. Results:

3.1 Optimisation and efficacy test

The AHRV 10 assay was optimised, and the applied values are as shown in table 3.1.

Volume	Optimised reaction
2X RT-PCR	6.25 µl
10µM forward primer	1.13 µl
10µM reverse primer	1.13µl
10µM probe	0.28 µl
25X RT-PCR enzyme mix	0.25 µl
RNase-free H_2O	1.46 µl
Template	2.0 µl
Total	12.5 µl

Table 3.1: The Real-time RT-PCR optimised reaction volume for AHRV 10.

The standard curve for the efficacy test of AHRV 10 is shown in figure 3.1.

The regression line has a slope of -3.3018 and the Efficiency (E) was calculated to be

AHRV 10 = 2.00846

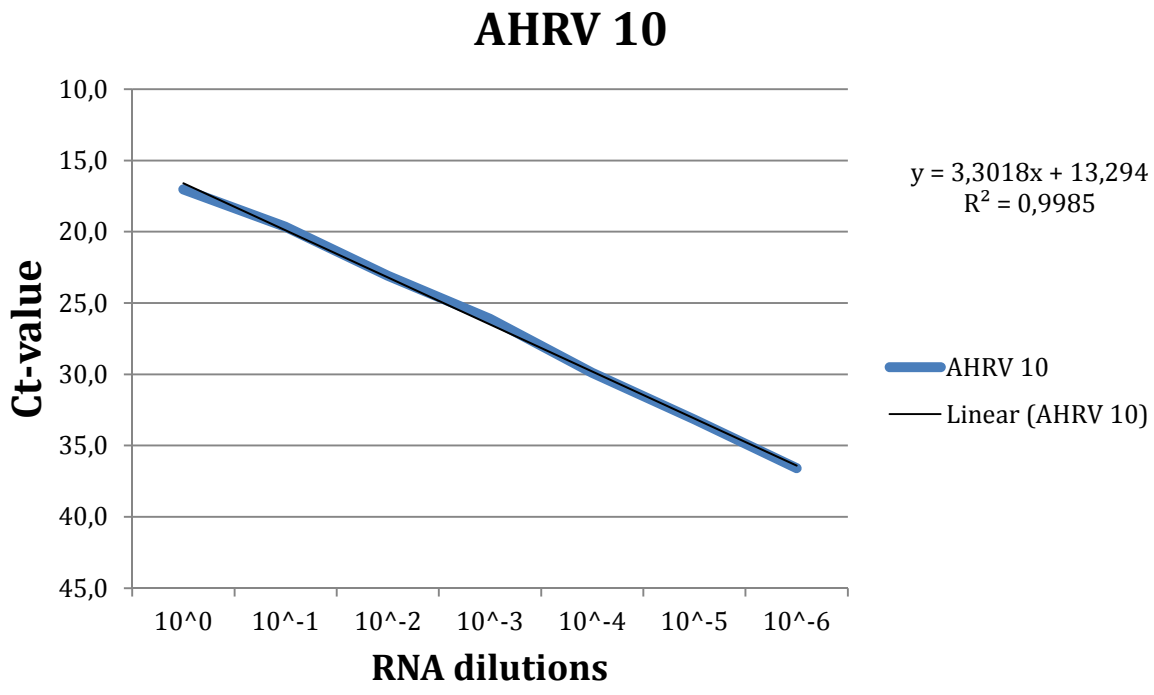

Figure 3.1: The standard curve based on AHRV 10 assay in a 10-fold dilution with triplicates.

The Standard curve for the efficacy test of IPNV shown in figure 3.2.

The regression line has a slope of -3.4624 and the Efficiency (E) was calculated to be

IPNV = 1.944541

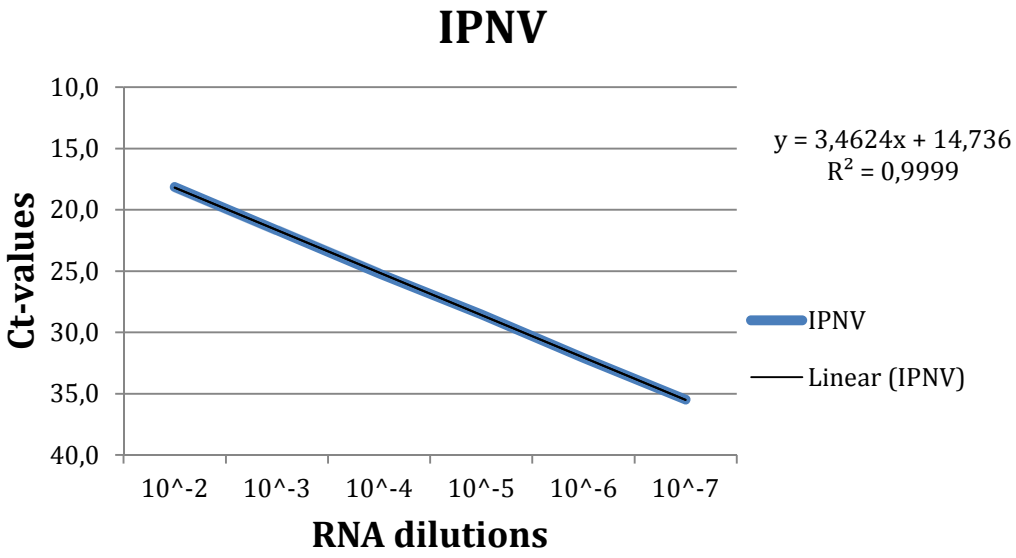

Figure 3.2: The standard curve based on IPNV assay in a 10-fold dilution with triplicates.

3.2 Optimisation of Q-tip method

The results of different methods for RNA-extraction from a Q-Tip are presented in table 3.2. Based on the result, method A was chosen as the most optimal procedure for RNA-extraction from a Q-Tip.

Method	Sample 1	Sample 2	Sample 3	Sample 4 (-20 C)	Mean
A	31.9	31.3	31.3	31.7	31.6
B	33.3	33.8	32.5	33.5	33.2
C	35.4	33.0	32.1	32.6	33.3
D	32.4	32.4	33.6	35.1	33.4
Control	31.9	32.0	32.1		32.0

Table 3.2: Ct-values for AHRV from each method. The procedures of the different methods up until adding of chloroform:
A: (The primed Q-Tip added directly into 1000 µl Isol and votex. Q-Tip then transferred to cut pipette tip inside an Eppendorf tube and spun down, retrieved fluid is then transferred back to the first tube).
B: (The primed Q-Tip was added 100 µl cell culture media first, and then transferred to a cut pipette tip inside an Eppendorf tube and spun down. Retrieved fluid was then added 1000 µl Isol).
C: (The primed Q-Tip was added 100 µl Isol first and then transferred to a cut pipette tip inside an Eppendorf tube and spun down. Retrieved fluid was then added 900 µl Isol).
D: (The primed Q-Tip was placed inside a 1000 µl pipette tip. Then 1000 µl Isol was pumped up and down 10 times with a pipette, and then pumped out into an Eppendorf tube).
Control: (100 µl of AHRV supernatant was rinsed according to standard RNA extraction).

3.3 Screening of eggs, milt, larvae and feces.

Broodstock groups C2, C3, C4, and C5 from June 2014 were analysed. By the end of April 2015 more than 300 samples of Atlantic halibut eggs, ovary fluid, milt, feces and larvae were analysed (Table 3.3). There were no confirmed positives among the analysed samples. In the same period there was no disease connected to AHRV at the site.

Broodstock group	Sample	Numbers	Ct-value AHRV
C2 24 females	Eggs/ovary fluid and milt	-	NT
	Q-Tip from hindgut	-	NT
	Larvae	52	Negative
C3 33 females	Eggs/ovary fluid and milt	72	Negative
	Q-Tip from hindgut	61	Negative
	Larvae	-	NT
C4 31 females	Eggs/ovary fluid and milt	-	NT
	Q-Tip from hindgut	48	Negative
	Larvae	10	Negative
C5 24 females	Eggs/ovary fluid and milt	37	Negative
	Q-Tip from hindgut	37	Negative
	Larvae	-	NT

Table 3.3: An overview of samples analysed. As no test was confirmed positive, and no extraordinary mortality occurred in the facility, no further analyse was conducted within each group. (NT = not tested.)

3.4 Mortality in offspring of broodstock group C1 during startfeeding.

In May 2015 samples from all 10 startfeeding tanks was sent for analyses due to drop in appetite and high mortality in several of the tanks. From previous experience, reduced appetite in combination with higher mortality is typical symptoms of an outbreak of AHRV. What was different in this outbreak was the white thick mucus building up inside of some of the larvae´s intestines. The larvae were tested for AHRV, IPNV and AHNNV. All tests for AHNNV were negative. AHRV and IPNV turned up positive in several of the 10 startfeeding tanks (Table 3.5). The larvae had been transferred into the startfeeding tanks from the 14th to the 27th of May (Table 3.4).

Date in:	14.04.15	15.04.15	16.04.15	17.04.15	21.04.15	23.04.15	27.04.15
Startfeeding tank:	1	2 and 4	3	8	5	9 and 10	7

Table 3.4: The larvae were move into the startfeeding tanks 54 days post fertilisation. The size of the tanks and distance between them is drawn to scale in Figure 2.2. After 4 days in the startfeeding tank the larvae are feed with enriched *artemia*.

Tank	In startfeeding:	Sampled:	N =	Elong.fact. Halibut	AHRV mean Ct	Prevalence	IPNV mean Ct	Prevalence
1	23 days	06.05.15	10	15.3	Negative	0/10	Negative	0/10
2	22 days	06.05.15	10	16.4	**29.2**	10/10	Negative	0/10
3	21 days	06.05.15	10	15.5	**23.2**	10/10	**30.1**	10/10
4	21 days	05.05.15	10	15.0	**19.9**	10/10	**22.0**	10/10
5	15 days	06.05.15	10	15.5	**23.9**	10/10	**27.1**	10/10
6	12 days	06.05.15	10	15.6	**37.2**	2/10	**35.3**	2/10
7	9 days	06.05.15	10	15.4	**36.9**	3/10	**35.9**	7/10
8	19 days	06.05.15	10	14.8	**20.3**	10/10	**27.0**	10/10
9	13 days	06.05.15	10	14.3	**37.0**	5/10	**35.7**	7/10
10	13 days	06.05.15	10	15.3	Negative	0/10	**36.2**	2/10

Table 3.5: Larvae from tank 4 were tested first due to mortality and drop in appetite. The rest of the tanks were sampled the following days. The double infection with both with AHRV and IPNV was unexpected since IPNV had not been present at the facility for more then 20 years.

Tank nr. 1 was not sampled again as no extraordinary mortality was detected. Strict hygiene measures were implemented and the water flow was increased in order to dilute the density of pathogens emitted from moribund larvae. Tanks with high mortality and confirmed positive for AHRV or IPNV were terminated. New sampling was conducted on the 12th of May 6 days after the first sampling. The development of the outbreak is shown in Table 3.5.

Tank	In startfeeding:	Sampled:	Ct-Elong.fact. Halibut	AHRV mean Ct	Prevalence	IPNV mean Ct	Prevalence
1	29 days	NT	-	NT	NT	NT	NT
2	-	Terminated	-	-	-	-	-
3	27 days	12.05.15	13.4	**19.0**	20/20	**18.5**	20/20
4	-	Terminated	-	-	-	-	-
5	-	Terminated	-	-	-	-	-
6	18 days	12.05.15	12.7	Negative	0/20	Negative	0/20
7	16 days	12.05.15	13.7	Negative	0/20	Negative	0/20
8	-	Terminated	-	-	-	-	-
9	19 days	12.05.15	13.8	Negative	0/20	Negative	0/20
10	19 days	12.05.15	15.1	**37.8**	1/20	Negative	0/20

Table 3.5: From the groups not terminated, 20 larvae were taken out from each tank. In tank 3 the Ct-values revealed an increased presence of AHRV and IPNV.

3.5 Water sample.

Water sample from tank 3 was taken short before the tank was terminated (12.05.2015). At the time of sampling the mean Ct-value for AHRV and IPNV in the larvae were 19.0 and 18.5 respectively. The water sample Ct-value for AHRV and IPNV were 25.7 and 26.9, respectively. The high levels of AHRV and IPNV in the production water indicate that moribund larvae are shedding viruses and that the infection pressure on the larvae is high. The control Ct-values for VHSV and SAV were 28.1 and 23.5.

Tank 6 tested positive for AHRV and IPNV in the first round of sampling. On the next sampling 6 days later there was an indication of virus clearance. Then 10 days later some of the larvae´s in the tank was not eating. There were also an increasing numbers of larvae with white thick mucus in the intestines. 15 larvae with white mucus were collected on the 22nd of May. 10 larvae that seemed normal were collected on the 28th of May. On the first of June 40 larvae were taken out, half of them with white mucus in the intestines (Table 3.6)

Tank	In startfeeding:	Sampled:	Elong.fact. Halibut	AHRV mean Ct	Prevalence	IPNV mean Ct	Prevalence
6	12 days	06.05.15	15.6	**37.2**	2/10	**35.3**	2/10
6	18 days	12.05.15	12.7	Negative	0/20	Negative	0/20
6	28 days	22.05.15	19.1	Negative	0/15	**26.2**	11/15
6	36 days	28.05.15	21.5	Negative	0/10	**35.7**	7/10
6	40 days	01.06.15	20.7	Negative	0/20	**18.6**	10/10
6	40 days	01.06.15	17.4	Negative	0/20	**30.5**	8/10

Table 3.6: The mortality and appetite in tank indicated that the larvae were affected. In addition the presence of white mucus in the intestines of some larvae led to further analysing of the tank. A total of five outtakes of samples were carried out from this tank. The elongation factor values marked blue are all from one shipment where the ice had melted and samples were no longer frozen. Larvae with presence of white mucus in the intestine are marked green in the table.

3.7 Female halibuts contributions with eggs to the different startfeeding tanks.

The overview over which female halibut that had contributed to the different tanks is listed in table 3.8.

Halibut:	1	8	10	14	16	21	25	49	54	55	56	57	58	60	61	62	65	66	70	
Tank 1				□	□			□	□					□		□		□		7♀
Tank 2				□		□	□	□	□				□	□		□		□		9♀
Tank 3		□		□	□	□	□	□	□	□		□	□	□		□		□		13♀
Tank 4		□					□	□		□		□	□	□	□					8♀
Tank 5	□				□								□	□						4♀
Tank 6	□				□								□	□						4♀
Tank 7	□				□			□		□					□					5♀
Tank 8	□		□		□		□	□	□	□	□		□	□	□	□				12♀
Tank 9	□		□														□		□	4♀
Tank10			□				□	□		□	□				□	□				7♀

Table 3.8: The contributions of females to the different startfeeding tanks. The top line is the halibut identification number, in general halibut 1, 8, 10 etc. The number at the end of the table reveals how many female halibuts that have contributed to the individual tank. Tanks in red colour turned up positive for AHRV or IPNV and were terminated. Note that female halibut number 58 (marked purple) has contributed only to the terminated tanks.

3.8 Broodstock C1, screening of ovary fluid.

The last batch of eggs from each female halibut contributing with offspring was tested for presence of AHRV and IPNV. The results of the screening are listed in table 3.9.

Female	Elongation fact.	AHRV 10 Ct-value	IPNV Ct-value
Halibut 1	22,7	Negative	Negative
Halibut 2	22,1	Negative	Negative
Halibut 8	22,2	Negative	Negative
Halibut 10	20,2	Negative	Negative
Halibut 16	19,3	Negative	Negative
Halibut 20	22,5	Negative	Negative
Halibut 21	21,1	Negative	Negative
Halibut 25	20,3	Negative	Negative
Halibut 33	22,5	Negative	Negative
Halibut 40	23,4	Negative	Negative
Halibut 41	22,6	Negative	Negative
Halibut 42	21,0	Negative	Negative
Halibut 49	19,5	Negative	Negative
Halibut 54	19,2	Negative	**35.3**
Halibut 55	19,5	Negative	Negative
Halibut 56	19,2	Negative	Negative
Halibut 57	20,0	Negative	Negative
Halibut 58	21,2	**32.6**	**35.6**
Halibut 60	18,3	Negative	Negative
Halibut 61	21,5	Negative	Negative
Halibut 62	20,4	Negative	Negative
Halibut 65	22,6	Negative	Negative
Halibut 66	21,2	Negative	Negative
Halibut 70	20,9	Negative	Negative

Table 3.9: Ovarian fluid from the last batch of each female was tested. Two halibuts tested positive; halibut 54 for IPNV and halibut 58 for AHRV and IPNV.

The materials collected from the broodstock site included sub samples from several of the released batches of eggs. In total 10 samples from female halibut number 58 was sampled in the period 15th of January to 14th of February. In order to determine whether there were any variations of virus in the ovary fluid during this time, all of the 10 samples were analysed (table 3.10). The other egg samples of halibut 54 were negative for AHRV and IPNV. All of the male halibuts tested negative for AHRV or IPNV.

Batch of eggs	Ct-value Elongation factor halibut	Ct-value AHRV	Ct-value IPNV
1.	24,9	Negative	Negative
2.	24,7	Negative	Negative
3.	24,4	**38.4**	**36.4**
4.	24,7	**37.3**	Negative
5.	24,2	**34.5**	**35.6**
6.	25,2	**32.4**	**32.6**
7.	24,3	**33.1**	**32.4**
8.	25,8	**33.4**	**33.9**
9.	25,5	**31.1**	**31.9**
10.	25,8	**30.7**	**29.2**

Table 3.10: Ct-values from halibut number 58 and the different egg-batches. The first batch of eggs was sampled on the 15th of January and the last batch of eggs was sampled the 14th of February. The two first egg batches were negative. From the 3rd batch of eggs, and onwards, there are detectable viral products. The values show an increase in viral products towards the second half of the spawning period.

To further visualise the change of viral load in the egg batches of the halibut 58, the Ct-values were normalised in a Log2 NE-Fold graph in figure 3.4.

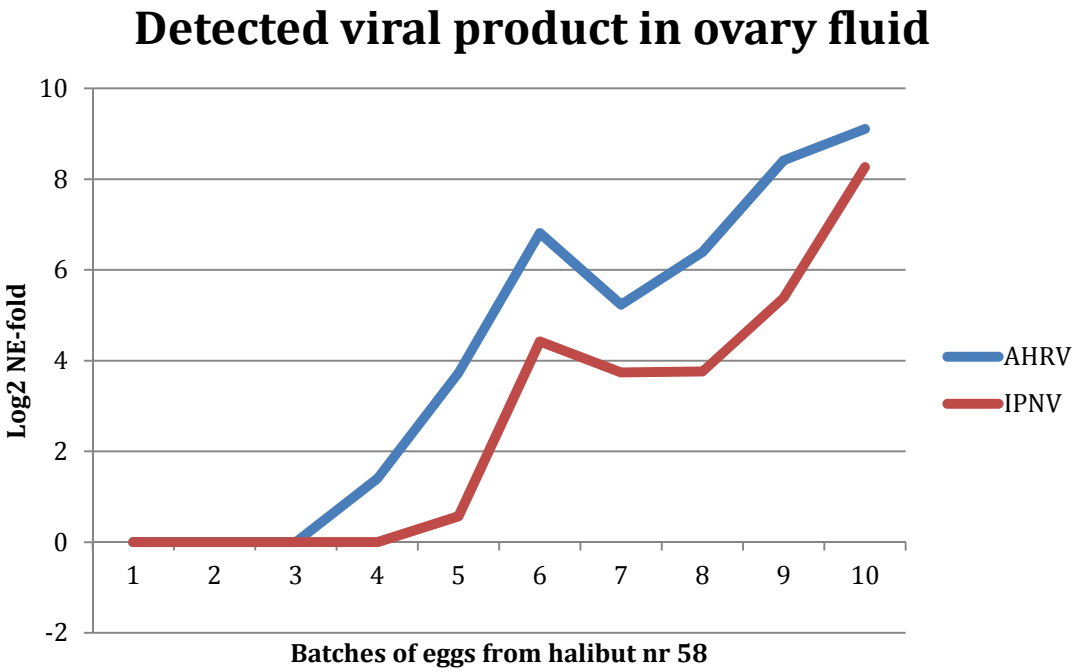

Figure 3.4: The two first batches of eggs were negative for AHRV and IPNV. There is a clear increase of both AHRV and IPNV from the third and fourth batch of spawning toward the last batch. The time period from the first to last sample is one month.

3.9 Tissue screening of halibut 54 and 58.

From the broodstock group C1, two female halibuts (number 54 and 58) were taken out of production and euthanized. Halibut number 54 had tested positive for IPNV in ovarian fluid, while halibut 58 had tested positive for AHRV and IPNV. Different organs were tested and analysed in order to verify presence of AHRV and IPNV (table 3.11).

Organ /tissue	Halibut 58			Halibut 54		
	EF1A1	AHRV 10	IPNV	EF1A1	AHRV 10	IPNV
Gills	17.0	Neg	Neg	21.3	Neg	Neg
Head kidney	20.2	Neg	Neg	22.9	**36.3**	Neg
Anterior kidney	21.4	**36.1**	Neg	22.4	**36.2**	Neg
Posterior kidney	21.4	**34.1**	**37.4**	23.6	**35.7**	Neg
Liver 1	20.4	**34.9**	Neg	23.2	**34.6**	Neg
Liver 2	NT	-	-	22.2	Neg	Neg
Liver 3	NT	-	-	23.0	Neg	Neg
Spleen	23.8	Neg	Neg	25.1	Neg	Neg
Gonad	16.6	**37.0**	Neg	22.4	**38.0**	Neg
Heart Atrium	NT	-	-	16.5	Neg	Neg
Heart Ventricle	15.7	**31.1**	Neg	27.7	Neg	Neg
Bulbus Arteriosus	NT	-	-	18.4	Neg	Neg
Pharynx	NT	-	-	20.0	Neg	Neg
Foregut	20.8	**37.3**	Neg	20.5	Neg	Neg
Hindgut	17.3	Neg	Neg	16.9	Neg	Neg
Anus	17.3	Neg	Neg	17.5	Neg	Neg
Brain	16.6	Neg	Neg	16.6	Neg	Neg
Gallbladder	18.9	Neg	Neg	19.2	Neg	Neg
Stomach	18.0	Neg	Neg	18.0	Neg	Neg
Muscle	17.3	Neg	Neg	NT	-	-
Pseudo Brach	21.7	Neg	Neg	20.2	Neg	Neg
Skin	19.0	Neg	Neg	21.2	Neg	Neg
Langerhans cells	17.4	Neg	Neg	NT	-	-
Faeces	NT	-	-	27.7	Neg	Neg
Blood cells	Neg	Neg	Neg	Neg	Neg	Neg
Plasma	Neg	Neg	Neg	Neg	Neg	Neg
Mucus	NT	-	-	18.3	Neg	Neg

Table 3.11: The two Atlantic broodstock halibuts were dissected the 26th of August, 6 month past spawning. Each tissue was collected with tweezers and scalpel that had been dipped in 70 % alcohol and burned with in an open propane flame. Halibut 58 had tested positive for AHRV and IPNV in the ovary fluid 6 months earlier. Halibut 54 had tested positive for IPNV in the last batch of egg she had released, but did not test positive for IPNV from any of her organ sampled 6 month later. The Halibut 54 had detectible amounts of AHRV in the kidney, liver and gonad, but not in the ovary fluid sampled 6 month earlier.

3.10 Sequencing.

From one moribund larva sampled from tank number 3 the 6th of May, both IPNV and AHRV were detected by real-time RT-PCR. Broodstock halibut number 58 was also confirmed positive for IPNV and AHRV, and was also one of the females contributing with eggs to tank number 3. In order to establish whether there is a relationship between AHRV in the larva and the halibut number 58, sequencing was conducted on the samples. The AHRV segment 10, transcribing the outer surface protein VP7 was chosen as the target (Figure 3.5).

Figure 3.5: The figure shows the alignment of AHRV segment 10 sequenced from both the larva and the brood fish. The larva from tank 3 tagged: MH050515-1. Halibut Brood fish number 58 tagged: MH260815-5.
The alignment shows a 100% identity between the virus from the larva and from the brood fish.

Sequencing of the IPNV found the moribund larvae were conducted in order to determent the type of isolate. The viral region of the VP2 on segment A was chosen for comparison. A search through the GenBank at “The National Center for Biotechnology Information” revealed identity towards two other isolates (Figure 3.6)

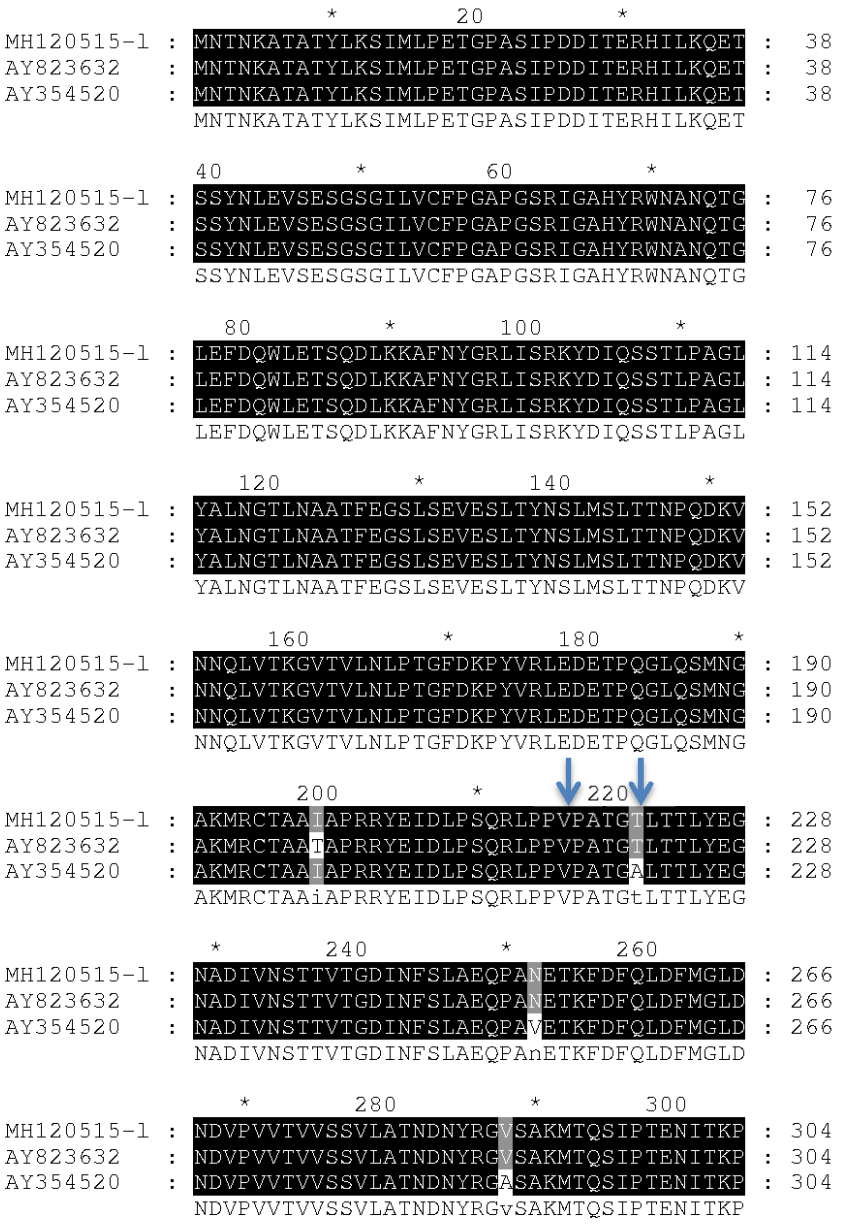

Figure 3.6: The halibut larva from the outbreak marked MH120515-1. AY823632 and AY254520 are isolates from Atlantic salmon Norway (Santi et al., 2005). The isolate found in the moribund halibut larvae is nearly identical to the isolate AY823632 from Atlantic salmon origin. The two blue arrows points at the position 217 and 221. According to (Song et al., 2005) the virulence of the virus is determent by the presence of T(Threonine) in position 217 and an A(Alanine) in the position 221

4 Discussion:

Atlantic halibut reovirus was detected both in larvae suffering from disease and in a brood fish, i.e. the egg-donor to the same group of offspring. The AHR virus from both the larva and the brood fish halibut had 100% matching virus genome of the segment 10 (829 bp), and, hence, the hypothesis of vertical transmission is not contradicted. A co-infection of IPNV was also detected and this virus may have contributed to the mortalities observed.

4.1 The discussion of the methods

The chance of detecting pathogens during sampling depends on presence and amounts of pathogens in the tissues sampled. The screening of the brood fish was limited to non-destructive methods. By not having the possibility to sample the internal organs of the brood fish, the prevalence of potential carrier fish of AHRV or IPNV remains unknown. However the potential risk of vertical transmission should be correlated to the amount of virus detected in eggs, ovary fluid, milt, faeces and urine. In that respect the relevance of these samples are linked more to the active shedders of virus and not so much towards potential subclinical carriers that may not be shedding virus. The faeces samples collected with a Q-Tip did not detect confirmable amounts of positive AHRV or IPNV during the study period. From the development of the method it was shown that material collected on the Q-Tip was retrieved in values close to the control of the test. In retrospect the limited amount of sampled material absorbed in the Q-Tip may have been filled with less valuable material such as production water or mucus before entering the hindgut. Mucus of halibuts also contain a novel 51-mer AMP named hipposin that has strong antibacterial property (Birkemo et al., 2003), this could potentially interfere with the material collected on the Q-Tip. For the future the Q-Tip should maybe equipped with a plastic tube that can be pulled back after the Q-Tip has entered the hindgut.

The tissue samples collected from the brood fish halibut had relative high Ct-values of the elongation factor. High temperature during sampling may have contributed to a degradation of the material. This may have contributed to higher Ct-values or false negative samples in the collected material.

4.2 Discussion

4.2.1 Larvae

The larvae analysed from autumn 2014 to spring 2015 were all negative for AHRV. This correlated also with the health situation at the site as no extraordinary mortalities among the juveniles occurred. However the larvae from generation C1 had a drop in appetite followed by an increased mortality in the beginning of May 2015. A co-infection of AHRV and IPNV were confirmed to be associated with the mortality. The mortality seemed to have originated in two start feeding tanks, number 4 and number 8 placed on opposite side of the aisle separating tanks 1 - 6 from 7 – 10 (cf Figure 2.2). Several of the tanks were terminated as a consequence of the high mortality in combination with confirmed presence of AHRV and IPNV.

Analyses of 1 litre production water from tank 3, collected shortly before termination, revealed that the moribund larvae where shedding viruses of both AHRV and IPNV into the water. The Ct-values for AHRV and IPNV were 25.7 and 26.9 respectively. Hence, spreading of these viruses between the different tanks via aerosols may have contributed to disease outbreaks in neighbouring tanks.

The facility had from the start of the outbreak inflicted strict hygienic measurements, as separate dip nets, changing of gloves and use of disincentives, but spreading of the viruses and development of the disease was not under control. At the end of the start feeding stage, tank number 2, 3, 4, 5, 6 and 8 had to be terminated.

4.2.2 Ovary fluid

After the outbreak in the start feeding tanks, all of the female halibuts contributing with eggs to the C1 generation were screened using ovary fluid from the last egg batch submitted. The presence of viruses in the ovary fluid might increase over time due to the stress of repeated handling and spawning. Two halibuts were found to be positive; female halibut number 58 for AHRV and IPNV, and halibut number 54 for IPNV. A test of every egg batch from female number 58 revealed an increase in virus of both AHRV and IPNV from batch 3 to the last batch (number 10).

The halibut female number 58 had contributed eggs to tanks number 2, 3, 4, 5, 6 and 8, i.e. the same tanks that were terminated. Transmission of viruses from brood fish 54 and 58 could explain the introduction of these viruses to the offspring and explain the disease outbreaks in the start feeding tanks. In farming of sea bass (*Dicentrarchus labrax*) vertical transmission from brood fish via eggs to the offspring is demonstrated using intramuscular injections of live Sb-Atl nodavirus on the brood fish (Breuil et al., 2002). In farming of barfin flounder (*Verasper mosari*) the screening of the brood fish using PCR and antibody detection reduced the number of barfin flounder nervous necrosis virus (BFNNV) outbreak in the juvenile offspring (Watanabe et al., 2000).

4.2.3 Brood fish

The presences of AHRV and IPNV in the ovary fluids lead to total tissues screening of the female halibut number 58 and number 54.

Both IPNV and AHRV were detected by real-time RT-PCR in halibut 58. The presence of IPNV was found in the posterior kidney. The kidney has been shown to be a target organ in a challenge experiment with IPNV on halibut fry (Biering et al., 1994). AHRV was detected in the anterior and posterior kidney, liver, foregut and hearth of halibut 58. From previous outbreak in Canada 2001, Scotland 2003 and Norway 2013 the liver of the moribund halibut larvae´s were affected by severe necrosis and presence of virus (Cusack et al., 2001, Ferguson et al., 2003, Blindheim et al., 2014). From the outbreak in Canada, necrotic kidney tissue was also found in the moribund larvae (Cusack et al., 2001), however this was not detected in Norway 2013, but presence of AHRV in the kidney was detected by real-time RT-PCR (Blindheim et al., 2014). The presence of AHRV in the gut and hearth has previous not been reported, however, during the outbreak in Scotland 2003 syncytial giant cell formation and changes in the mucosal epithelium of two halibuts the were observed (Ferguson et al., 2003).

Halibut 54 had detectible amounts of IPNV in the ovary fluid during spawning 6 month prior to the tissue analyses. Nevertheless detectible amounts of IPNV were not found in the tissues of halibut 54. This might imply that the amount of IPNV in the halibut is greater during spawning then in the rest of the season. As the Ct-values of the halibut elongation factor from the tissue analysed were high (mean Ct-value 20.1) it is possible that a low amount of IPNV

has passed through the analyses undetected. AHRV was detected in the kidney, liver and gonads of the halibut 54, but was not detected 6 month earlier in any of the ovary fluid samples.

4.2.4 Sequencing

AHRV

The sequencing AHRV from brood fish and larvae was based on segment 10, encoding for the outer surface protein VP7. AHRV positive sample from the heart of brood fish halibut number 58 was compered with AHRV positive halibut larvae from the outbreak. The sequenced virus genome revealed 100 % identical sequences in the brood fish and the larva. This means that the virus found in both brood fish and offspring are most likely of the same origin, establishing a connection between the two. This support the theory of the virus being vertical transmitted from brood fish to offspring. However, both the larvae and the brood fish could have received the virus from a common unidentified source.

IPNV

The sequencing of IPNV was conducted to determent the genotype of IPNV found in the moribund halibut larvae. The sequencing revealed that the virus was nearly identical to previous isolates retrieved from Atlantic salmon (isolate: AY823632) (Santi et al., 2005). According to (Song et al., 2005) the virulence of the virus is determent by the presence of T(Threonine) in position 217 and an A(Alanine) in the position 221. The sequence from the halibut larva had a P in position 217 and a T in position 221, hence, it should be of low virulence. The IPN outbreak observed indicated that the virus was highly virulent, despite the absent of an A in position 221. This could be due to the fact that halibut and salmon are two different species, but probably more important is the fact that the mortalities occurred among larvae lacking a completely developed immune system (Patel et al., 2009).

4.3 Conclusion and future perspectives

The results from this study, points in the direction of the brood fish halibuts as the most likely source of the outbreak of both the Atlantic halibut reovirus and the Infectious pancreatic necrosis virus at this site.

Asymptomatic brood fish halibuts can be carriers of AHRV and IPNV and during the stress of spawning they have the potential of shedding viruses that might enter the ovary fluid and hence infect the eggs and offspring.

The future of halibut farming is best secured by establishing a pathogen free broodstock.

By testing the eggs, ovary fluid, milt, urine and feces, active shedders could be found and eliminated from the breeding line.

In order to develop a method for testing the existing and the future broodstock, the target organ for AHRV and IPNV needs to be confirmed. A challenge experiment might determine the target organs, tissue tropism of the viruses and thereby suitable organs for screening.

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Appendix

1 % agarose gel

SeaKem® LA Agarose	4 g
1X TAE buffer	400 mL

Description: Weigh agarose in an Erlend-Meyer, then add 1X TAE buffer and warm in the microwave until the agarose has melted (avoid boiling). Store at 60 °C.