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Genome-wide association study of the genetic basis for resistance to IPNV in rainbow trout

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European master's in animal breeding and genetics (EMABG)

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ABSTRACT

Infectious Pancreatic Necrosis (IPN), caused by the IPN virus, is one of the major threats to the Norwegian rainbow trout (NRT) industry. Despite disinfection, effective management, traditional breeding programs, and efforts to vaccinate the fish, the NRT industry is still prone to IPNV infections, leading to higher mortalities and severe clinical infections in production and breeding stocks, especially at the early life stages. The distinct genetic variations among IPNV isolates and different genetic basis of salmonoids can lead to 10-100% mortalities. The economic losses and welfare concerns called for scientific studies to analyze the molecular pathogenesis and discover the possible solutions to restrict the outbreaks of IPN in aquaculture, which led to the advent of selective breeding as the major step forward. In the present study we have investigated the comparative survival of NRT fry against IPNV challenge and possibilities of reducing this disease problem through selective breeding, and to what extent selection for increased resistance to one pathogenic strain of IPNV will also result in increased resistance to another isolate. It was found that an isolate from Atlantic salmon (IPNV-AS) is more virulent compared to an isolate from rainbow trout (IPNV-RT) which could be attributed to the marked difference in the genetic architecture of the viral genome encoding parts of the viral protein important for host cell interactions and immunogenicity. The estimated heritabilities for survival to both isolates in separate challenge tests were moderate and indicative of potential use in the genomic selection for increased resistance to IPNV in NRT selective breeding programs. The genetic correlation between survival to IPNV-AS and IPNV-RT was very high, meaning that the selection for one of the studied isolates will impart resistance against the other isolate. The GWAS have shown the polygenic nature of the traits under investigation as many QTLs were significantly associated with the traits. This implies that genomic selection is the best way to select against IPNV as it will account for all the genetic variation contributions by the many small QTLs. A detailed understanding of the genetic variation and interactions of the host and the IPNV are crucial in understanding the faith of the disease. Moderate heritability was also found for the viral load to IPNV-AS, and with a moderate negative and thus favorable genetic correlation to survival to IPNV-AS implying that viral load may potentially be used as the preferred selection trait for IPNV as it does not include non-specific mortalities, is more informative than the binary survival trait, and helps to detect clinical infections and carriers in the population. The QTLs should be validated using viral load as a trait, and actual causative mutation should be detected using large-scale data sets.

Table of Contents

List of Tables

List of Figures

1. INTRODUCTION

Aquaculture is the most efficient and rapidly expanding industry to produce sustainable, nutritious, and most importantly healthy seafood for the growing population. The world's marine fish aquaculture increased exponentially over the past few decades and reached 7.4 million tonnes of live weight in 2018 (FAO, 2020). The geographical location of Norway is well suited for marine aquaculture and is the second major producer with an annual production of 1.4 million tonnes of live fish weight (China at the top with 1.5 million tonnes in 2018). The Norwegian aquaculture (NA) consists of Atlantic salmon (AS) with an annual production of 1.4 million tonnes in 2019), rainbow trout (RT) with 83 000 tonnes/annum, and relatively a low production (4000 tonnes/ annum) of a few other marine species (Statistics Norway, 2020).

The Norwegian rainbow trout aquaculture is prone to a wide range of bacterial pathogens (*Flavobacterium branchiophilum, Renibacterium salmoninarum, Aeromonas salmonicida,* etc.) and viral pathogens (*salmonid alphavirus, infectious pancreatic necrosis virus, Piscine myocarditis virus*, etc.), which results in substantial economic losses. The predominant diseases are Infectious Pancreatic Necrosis (IPN), Viral Hemorrhagic Septicaemia (VHS), Furunculosis, Vibriosis, and kidney disease (KD) (FAO, 2009). IPN is caused by the Infectious Pancreatic Necrotic Virus (IPNV) and is one of the major threats to the Norwegian salmon and rainbow trout industry. Over the years 2001-2020 the annual production loss in Norway was 9.5 million Norwegian rainbow trout (NRT) juveniles and 3.6 million grown out (Aquaculture Statistics, 2020). IPNV is one of the major causes of mortality and its outbreaks are major threats to NRT breeding stations and growing farms with 10-100 % mortality (13th EAFP Grado, 2007). Until 2015, the NRT industry faced huge economic losses due to high IPNV prevalence (on average 10 IPNV outbreaks per year, Sommerset *et al.*, 2020).

The outbreak of IPNV was first reported in Canada in 1841 and the first characterization of the virus was done in 1960. In Norway, the first IPNV was isolated from rainbow trout in 1975 and was considered a notifiable disease in 2008 due to higher economic losses and welfare concerns (Håstein & Krogsrud, 1976; Society, 1941; Sommerset *et al.*, 2020; Wolf *et al.*, 1960). The primary target of IPNV is the pancreas of the fish (as the name suggests) followed by the liver and other organs. IPNV outbreak can happen anytime in the production cycle, however, fry, juvenile, and post-smolting stages show higher susceptibility to IPNV.

The infectious IPNV remained prevalent in aquaculture despite disinfection, effective management, selective breeding programs, and efforts to vaccinate the fish. Differential mortalities are attributed to the different genetic basis of salmonoids and distinct genetic variations among IPNV isolates. Different studies were conducted to investigate the molecular basis of virulence. The genetic details are not fully understood but many studies show variation in segment A of the IPNV genome with reference to alteration in virulence (SANO et al., 1992a). A study by Song et al., states that the distinct motifs of the VP2 Capsid Protein (like Thr-217 and Ala-221) are associated with a virulent form of IPNV (Song *et al.,* 2005). However, the molecular analysis of isolates from field outbreaks also shows that substitution of proline instead of threonine at 217 bp in virus protein-2 (VP2) may result in higher mortalities (Bain *et al.,* 2008a).

The molecular epidemiology of IPNV in Norway has shown that genetic variations among IPNV isolates are mostly present at silent positions in the genome. The samples from hatchery and sea farms have shown differences in amino acid motifs. The threonine-alanine pattern was dominating at positions 217and 227 of VP-2 in both study groups but was not found to be significantly linked with fish mortalities. The studies have shown that the horizontal transmission of IPNV which occurs among individuals of the same generation sharing the same space, whereas some other studies have confirmed the vertical transmission i.e., transfer of infectious agent from parents to progeny (13th EAFP Grado, 2007).

One of the remarkable break-through in the history of aquaculture is the utilization of selective breeding to increase the resistance against IPNV. The initial work utilized mortality data from challenge tests to show that resistance is highly heritable ranging from 0.17-0.62 (Kjøglum *et al.,* 2008; Wetten *et al.,* 2007). However, the incredible success was the discovery of QTLs on Chr-26 which are controlling the resistance to IPNV in Atlantic salmon. The advent of selective breeding and the discovery of significant QTLs led to the establishment of IPNV-resistant breeding stocks (Houston et al., 2010; Moen *et al.,* 2009). Considering this, different studies were initiated to analyze the genetic basis of IPNV-resistance in RT and have discovered the QTLs for IPNV-resistance on different chromosomes (AquaGen, accessed 2022-05-09; EAAP Abstracts, 2019; Rodríguez *et al.,* 2019).

IPNV is not a notifiable disease but is still a real concern for the aquaculture industry. Scientific studies have shown that the genetic interactions between the salmonoids and IPNV decides the faith of infections. Until now, the research studies are either focused on the molecular

epidemiology of the virus or the genetic architecture of the salmonoids. The studies conducted so far have their own merits, but detailed genetic studies are missing that can explain the genomic basis of host-pathogen interaction in terms of the severity of infection. The current research was designed with the aim to investigate the comparative survival of NRT fry against IPNV challenge and possibilities of reducing this disease problem through selective breeding, and to what extent selection for increased resistance to one pathogenic strain of IPNV will also result in increased resistance to another isolate.

2. LITERATURE REVIEW

Infectious pancreatic necrosis virus (IPNV) is the causative agent of the major and highly contagious diseases of aquaculture, IPN. It is a member of the Aquabirnavirus genus and Birnaviridae family of viruses. It was first reported in Canada in 1941 in a brook trout outbreak and was first named by M'Gonigle as "catarrhal enteritis" but was later changed by Wood to IPNV after a histopathological study of infected brook trout (Munro & Midtlyng, 2011; Wood *et al.,* 1955; M'Gonigle, 1941).

The IPN viruses were isolated and characterized to analyze the viral properties underlying the molecular basis of host-pathogen interaction and subsequent immune response. Molecular characterization revealed a bi-segmented genome, whereas structural characterization using advanced microscopy revealed an icosahedron capsid structure, the location of sub-viral particle residues, and hypervariable immunogenic regions on it (Coulibaly *et al.,* 2010; Dobos & Roberts, 1983; Santi *et al.,* 2004). This chapter is divided into several sections to extensively describe the viral properties, life cycle, prevalence, distribution, management strategies, QTL mapping, genomic selection, and official statistics of the IPNV and rainbow trout.

2.1 VIRAL GENOME AND STRUCTURE

The IPNV is an icosahedral virus with the RNA genome enclosed by a non-enveloped protein shell. The biochemical analysis of the virus showed a molecular weight of 55 x 10^6 Da, a size range of 57- 74 nm, and is composed of protein (91%) and RNA (9%). IPNV is highly stable against extreme physiochemical conditions like acidic pH, 0- 40 % salinity, and temperatures up to 60 ^oC (Dopazo, 2020; Evensen & Santi, 2008). The purified IPNV showed a sedimentation coefficient of 435-S and a buoyant density of 1.33 g/ml in CsCl (Kim & Leong, 1999). IPNV showed similar biochemical properties to infectious bursal virus (IBDV) and Drosophila X virus (DXV) (Scott *et al.,* 2011a).

Structurally, IPNV contains a very thin protein capsid around genetic material and is non-enveloped. The genome is in the form of double-stranded and bi-segmented RNA (A and B segments) as shown in figure 1 below. The A segment of the dsRNA genome contains ~3100 bp with two open reading frames (ORF) that encode for major capsid proteins and some non-structure proteins, whereas the B segment is composed of ~2784 bp and has one ORF that only

codes for free or genome linked forms of RNA-dependent RNA polymerase (Dobos, 1995; Duncan et al., 1986; Dobos, 1995b)

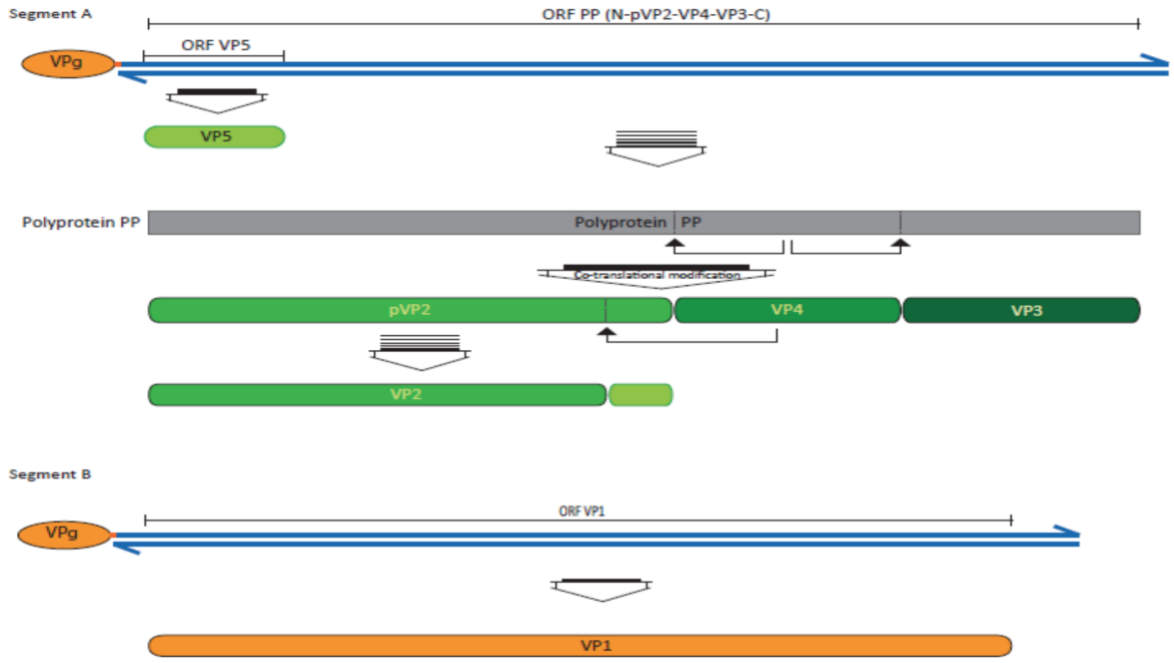

***Figure 1**: Bisegmented IPNV genome* (Dopazo, 2020)

The dsRNA genome of IPN encodes 5 virus proteins (VP). VP1 is a 94 kDa protein encoded by segment B of the genome and it functions as RNA-dependent RNA polymerase that catalyzes the replication of viral RNA. The 54 kDa VP2 is the second viral capsid protein that helps in the attachment of the virus to cell surface receptors. VP2 is encoded together with VP3 by segment A and the VP3 forms ribonucleoprotein by binding with RNA and is present on the inner side of the viral capsid. The VP4 is a protease that cleaves the precursor 106 kDa protein to create major viral proteins. The VP5 is a non-structured protein that is dispensable for in vivo replication of the virus and is not linked with virulence. However, VP5 is not required for the IPN replication *in vitro* (Dobos, 1995; Tapia *et al.*, 2021).

The 3' and 5' untranslated regions (UTR) of the IPNV genome have important implications for replication and virulence. One study reported that 5' UTR serves as the attachment site for structural protein VP-1 that helps in the initiation of translation (Dobos, 1995). Whereas other studies reported that 5' UTR together with VP-1 forms the structure to function as an internal ribosome entry site (IRES) to initiate translation and the mutation in this genomic region impacts the infectivity of the virus (Rivas-Aravena *et al.*, 2012).

2.2 LIFE CYCLE AND MOLECULAR PATHOGENESIS

The IPNV infection pathway is relatively well understood. The virus enters the host either through an oral or anal route and then utilizes its VP2 capsid protein for attachment to the ~150-kDa receptor present on the cell membrane of the fish, which led to the internalization of the IPNV. Little literature is available on the uncoating of the capsid as RNA polymerase is functional in both forms; uncoated and encapsulated. The virus hijacks the synthesis machinery of the host cell, utilizing its sources to multiply itself by replication (Scott *et al.,* 2011b).

The infections of IPNV cause a wide range of virulence and pathogenicity in fish species, ranging from overt symptoms to varied mortalities in outbreaks. Different research studies were done to analyze the molecular basis of virulence by comparing amino acid residues of different IPNV isolates from different challenge tests and field outbreaks (Tapia *et al.,* 2021). It was observed that certain amino acid residues at specific locations on segment A of the IPNV genome are responsible for virulence. Initially, (Sano *et al.,* 1992b) studied rainbow trout infected with two IPNV strains; aquabirnavirus (avirulent) and Buhl type (virulent) and demonstrated that segment A is responsible for virulence. (Bruslind *et al.,* 2000) confirmed the study of Sano et al., after experimenting with brook trout infected with different isolates of the same serotype and found the VP2 region of segment A as the main virulence determinant. They suggested threonine residues at position 217 and lysine at position 286 on segment A to distinguish virulent from avirulent strains. The successive studies identified more amino acid residues linked with virulence including 217 and 286 (Julin *et al.,* 2013; Skjesol *et al.,* 2011). (Song *et al.,* 2005) employed reverse genetics and discovered positions 217 and 221 on segment A as major virulence determinants. As a result of these studies, isolates with a specific amino acid motif like I_{64} T_{137} T_{217} A_{221} L_{286} V_{314} are considered virulent (I: isoleucine, T: threonine, A: alanine, L: lysine, V: valine). In addition to this, phylogenetics analysis of Norwegian isolates was done to identify key genetic fingerprints for sub-clinical and clinical infections and the observed motif was like the suggested motif for clinical infections (Mutoloki *et al.,* 2016).

However, the molecular determinants of virulence do not always correspond to IPN outbreaks and mortalities in salmon and trout. (Bain et al., 2008b) discovered that Scottish isolates with low virulence determinants turned out to be highly virulent in one field outbreak analysis. The researchers concluded that other factors together with virulence determinants can impact the rate of mortalities in different IPN outbreaks. Interestingly, it was found that stress plays a

major role in the replication of IPNV and the reversion of avirulent to virulent variants. Altogether, these results verify that the degree of IPNV virulence is dependent on host biology, IPN strain, host-pathogen interaction, and environment (Gadan *et al.,* 2013). Recently, the virulence of the IPNV variants has been evaluated in challenge tests with rainbow trout. The infected stock has shown mortality according to IPNV genotypes, the isolates of Genotype 2 and 5 caused higher mortalities whereas Genotype 6 showed weaker mortalities (Eriksson-Kallio *et al.,* 2020). Moreover, the strains with low virulence determinants in Atlantic salmon have shown higher mortalities in rainbow trout (Ahmadivand *et al.,* 2020; Tamer *et al.,* 2020).

Figure 2*: Clinical hallmarks of IPNV in Rainbow trout (a) abdominal swelling and dark skin (b) abnormal swimming with pseudo sticky feces (c) spleen enlargement* (Büyükekiz et al., 2018)

The virus attacks the host cell and multiplies within the host cell's cytoplasm. The prime targets of the viral are the pancreas, liver, and kidney. The histopathological hallmarks of IPNV infection are the targeted destruction of pancreatic tissue and zymogen granules released from necrotic cells. The pathological and clinical studies have shown that exposure of the fish to

IPNV led to darker skin color, abdominal distension, abnormal swimming, sticky and trailing feces, and stunted growth as shown in figure 2. The virus showed higher variation in mortality, and it was demonstrated that different factors like stress, overcrowding, bad husbandry, and poor environment are the major players in deciding the fate of the IPNV infection and mortality (Kar, 2016a).

The distinctive genetic characteristics and diverse range of mortalities led to the categorization of the IPNV infection into two main groups: clinical and subclinical infections. Different scientific studies have revealed unique genetic fingerprints that code for specified clinical conditions and immunogenicity (Munang'Andu *et al.,* 2013; Mutoloki *et al.,* 2016). The clinical infection is signalized as damage in tissue organs of the host that could lead to higher mortalities in infected stock, whereas subclinical infection is persistent in which infected fish act as carriers of the virus without developing serious symptoms (Johansen & Sommer, 1995; Mangunwiryo & Agius, 1988). The reverse genetics approach highlighted the already studied amino acid motifs at positions 217 and 221 and showed that $T_{217}A_{221}$ codes for clinical and $P_{217}T_{221}$ specify the subclinical form of infection (Santi *et al.,* 2004). This research was supported by (Gadan *et al.,* 2013; Mutoloki *et al.,* 2016; Song *et al.,* 2005) who showed the same genetic codes for IPNV infection categories and the mutation in these residues led to the conversion of the subclinical to the clinical form. The findings have shown the link of pathogenicity and virulence to specific amino acid motifs central to vaccine development.

2.3 CLASSIFICATION OF IPNV

The classification of diversified IPNV strains is based on two approaches. The first classification was marked by (Hill & Way, 1995) into two serogroups: serogroup A with 1-9 serotypes and serogroup B with one serotype B1. The second classification was outlined and extended by (Blake *et al.,* 2001; Nishizawa *et al.,* 2005) and despite diversity, they proposed seven classified genogroups. They found that there is a correlation between strain type, genotype, serotype, and strain origin. The four strains of IPNV (C1, C2, C3, and Ja) were reported from Canada, two strains (Sp and Ab) from Demark, two strains (Te, and TV-1) from the UK, one (WB) strain from the USA, one (He) strain from Germany and one (MaBV) strain was reported from Japan.

2.4 GEOGRAPHICAL DISTRIBUTION

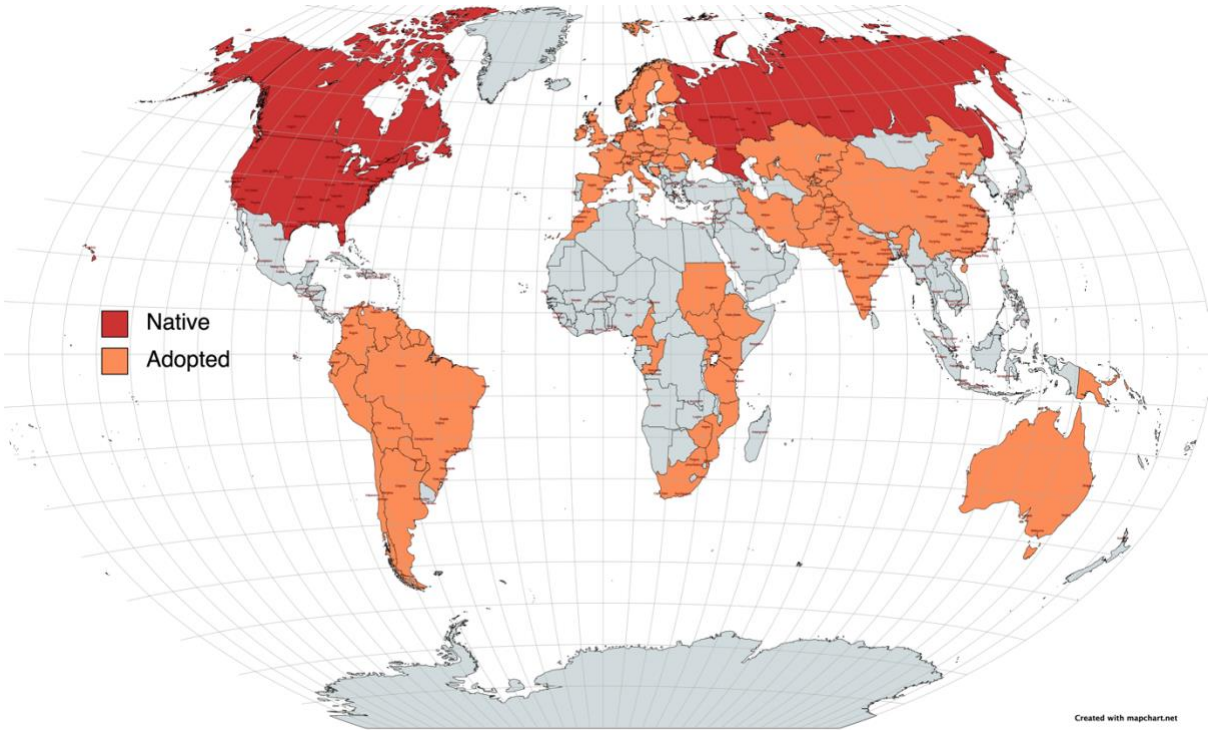

***Figure 3**: Worldwide geographical distribution of Rainbow Trout* (adopted from Muhlfeld *et al.*, 2019; Created with Custom Map | MapChart, 2022.)

The rainbow trout *specie* is native to North America and was distributed throughout the world by the relocation of eyed eggs since 1872 as shown in figure 3 (Fornshell, 2002). The RT has high value because of its simple farming, climate adaptation (can tolerate 0 - 25^{o}C), superior meat quality, and higher compatibility with most conditions (Shamspor, S., H. Khara and H. Golshahi, 2012; support, 2010). That led to the introduction and farming of RT in major parts of the globe such as Europe, the Far East, New Zealand, Central and Southern America, Africa, and East Asia as shown in figure 3 above (Ford, 1984). According to the reports, the eggs were first transported out of America to Japan in 1877, England in 1885, Turkey in the 1970s, and parts of Europe in the 1980s. Finally, the hatcheries in Denmark, England, and Scotland were used to transfer stored eggs of RT throughout Europe including Norway (Okumus, 2002). In Norway, the RT farms are distributed in seven counties and Hordaland County alone is contributing 47 % of the total RT production in Norway (Aquaculture Statistics, 2020). The current distribution of RT farms across Norway is shown in figure 4.

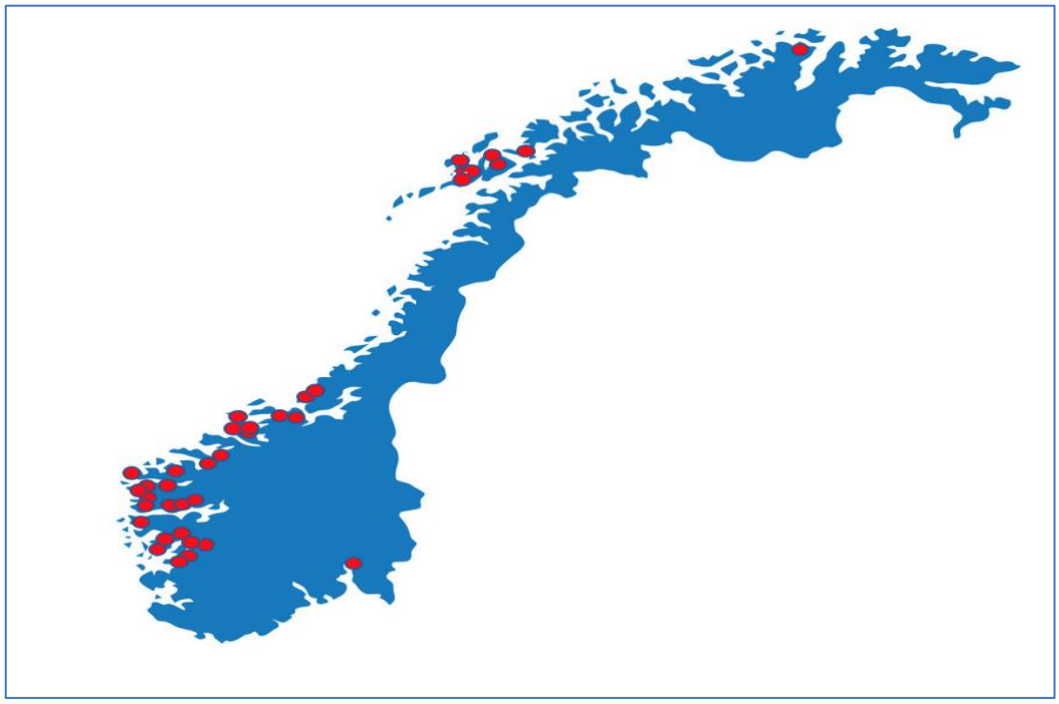

***Figure 4**:Rainbow Trout aquaculture active sites in Norway (adopted from Fish Health report, 2014)*

IPNV outbreak was first reported in North America in 1941 and then later in all parts of the world linked with aquaculture (M'Gonigle, 1941). The IPN virus was first isolated from rainbow trout (RT) in Norway in 1975 and was reported as a notifiable viral infection in aquaculture until 2008(Håstein & Krogsrud, 1976b; Sommerset, 2020). It has been isolated from many aquatic species including bivalve molluscs but major targets of IPNV are found to be Rainbow trout, brook trout, Atlantic salmon, coho salmon, and brown trout (Birnaviridae (chapter of) Virus Taxonomy book, 2012). Different strains of IPNV were reported mostly in different countries of the European, Asian, and American continents (Kar, 2016b).

The IPNV showed a diversified host range and has been isolated from 32 different fish families, 11 molluscs species, and 4 crustaceans species. IPNV is known to cause clinical symptoms of IPN in salmonoids. There are ten serotypes of IPNV, nine of which are virulent, and one is avirulent to salmonoids. The variation of the pathogenicity has been detected between serotypes and even within serotypes (Birnaviridae and Picobirnaviridae (chapter of) Fenner's veterinary virology book, 2017). Different amino acid motifs linked to virulence have been deduced from comparative studies of field outbreaks and verified that T_{217} A_{221} is involved in virulence (Shivappa *et al.*, 2003; Santi *et al.*, 2004).

The IPNV can infect the salmonoids at any developmental stage, but the fry is the most susceptible especially at the early feeding and post smolting phase. As the name suggests, the prime target of IPN is pancreas tissue followed by the liver which is also the target organ of this virus (H. M. Munang'andu et al., 2016). The IPNV is a serious concern for financial losses and animal welfare as the infected stock either shows mortality or acts as a carrier and transmits the infection to naïve stocks.

2.5 MANAGEMENT STRATEGIES

IPN is a highly contagious disease due to its higher transmission ability, severe mortalities, and withstanding nature of the virus. The virus was isolated from a wide range of aquatic species principally salmonoids and it was observed that it can be transmitted from one species to another. One experimental study reveals that blue mussel can serve as the host for IPNV and can be transmitted to fry and smolts. The IPN virus is very tough and can withstand desiccation, different environments, higher temperatures, and exposure to radiation. Therefore, it is a threat as a lifelong infection of farmed fish (Lakshmi *et al.*, 2019).

Different management strategies were derived to effectively control the incidence of IPNV infections. The best way to prevent an IPNV outbreak is to minimize the contact of the virus with the host. This implies that hatchery, breeding facilities, egg sources, and water supply must be virus-free to prevent the spread of the disease. But the resilient nature of the virus led to a wider distribution, recurrent outbreaks, severe mortalities, and carrier survivors in the past. The surviving fish after the viral outbreak can continue to act as a carrier and continuously shed the virus which will lead to either vertical or horizontal transmission of virus to a healthy host. The horizontal transfer of IPNV is between individuals of the same generation living in the same facility, whereas vertical transfer is between generations (from parents to progeny) (Kar, 2016b). Different management strategies are highly recommended to hamper the prevalence and transmission of IPNV. The sterilization treatment of aquaculture facilities with ozone, UV irradiation, chlorine, or iodophor are highly effective against IPNV but higher exposure and concentration are required to effectively control. The IPNV can also be controlled by prolonged exposure to extreme pH, drying, a higher concentration of formalin, and higher temperature. It was found that the virus can be inactivated completely in four days by applying a higher concentration of formalin in a warm environment (Stoskopf, 2015).

Vaccination is the most effective and safe solution to prevent or reduce outbreaks of resistant infectious agents like IPNV. But the major challenge faced by the IPN vaccines is efficacy as most of the vaccines failed to completely stop the post-challenge mortality in aquatic animals (Munang'andu et al., 2014). This results in the urgent need for better comprehension of the immunological basis of IPNV infection, the innate and adaptive immune response of the host against the virus, and the molecular basis of host-pathogen interaction to prevent the recurrence of outbreaks (Gomez-Casado et al., 2011; standardization, 1997). Different immunological studies were initiated to trigger the vaccine development and many scientists studied different cell surface markers and adaptive immune responses in fish using molecular strategies, knockout models, and gene expression studies. They correlated the mechanisms of protective immunity to seek the answers to the questions of IPNV control strategy in salmonoids (Reno *et al.,* 1978).

The vaccines against IPNV have been developed since 1995 and mainly contain VP2 or other fragments like the genomic segment A of IPNV. The developed IPNV vaccines were classified based on antigen: whole virus, viral protein (VP2), segment A of the genome, and provirus. The injections are the preferred administration route for most vaccines, but VP2 antigen can also be administrated orally. Different studies were published over time demonstrating the efficacy evaluation of developed vaccines (Ulrich, 2018). The common parameters used for evaluation were antibody levels, the proportion of post-challenge survival, pathology, and viremia reduction. The detailed immunological studies have revealed that IgM-based humoral immunity is predominant in protection against IPNV but less is known about cellular immunity.

The discovery of the new T-cell activating transcription factors is paving the way for a detailed molecular understanding of cellular immunity. The characterization of T-cell linked cytokines keep up the impression of an effective adaptive immune response in salmonoids in contrast to other animals. Although the discoveries of transcription factors, cytokines, and immune genes have contributed to the detailed information on cellular and humoral immunity, it is still challenging to generate functional tools that explain the effector and protective mechanisms of the salmonoids against IPNV (Munang'andu *et al.,* 2014).

The vaccination trials have shown protection efficacy of more than 80 % but still many outbreaks were reported in vaccinated stocks. The Norwegian aquaculture faced IPNV outbreaks until 2014 which then dropped with the introduction of genomic selection. The high-

yielding output of the genomic selection shows that the QTL-based selection is more effective than vaccines (Ulrich, 2018).

2.6 STATISTICS AND ECONOMIC LOSSES DUE TO IPNV

The Rainbow trout is economically the second most important aquaculture species in Norway after Atlantic salmon. According to official statistics of the Norwegian Directorate of Fisheries, the average sale of slaughtered RT was 75,000 metric tonnes annually over the past 10 years (2011-2020). The annual production is increasing, and Norwegian aquaculture farms harvested the highest ever 96,132 metric tonnes of RT in the year 2020 only. According to the reports, RT farming is economically important, and the value of the slaughtered RT has reached the gross value of 3.77 billion NOK annually. But this highly valuable RT farming in all regions is prone to different challenges like mortalities, fry destruction, escapes, and many others. Figure 5 shown below clearly illustrates that a higher rate of mortalities is the major threat to RT farming and economic losses in Norway (2011-2020) (Aquaculture Statistics, 2020).

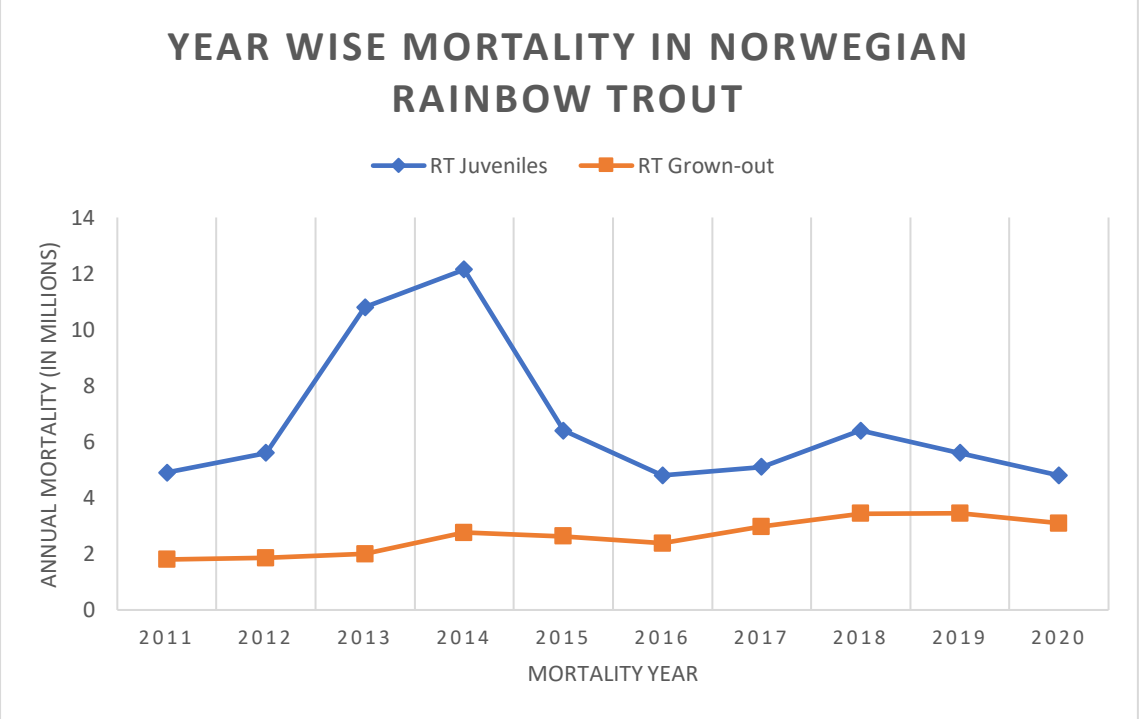

***Figure 5**: Annual mortalities of Rainbow trout (2011-2020) (Inferred from statistics of the Norwegian Directorate of Fisheries, 2021)*

The graph clearly shows that mortality is adding a lot to the production losses. The blue line specifying juvenile mortality shows a sharp decrease in juvenile mortality after 2014, mainly due to the introduction of genomic selection stocks, but still many outbreaks are reported annually. One important thing to remember is that IPNV is not the sole contributor to the shown mortality but is one of the major factors along with pancreas disease (PD), cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation disease (HSMI), and some others. According to the health situation report 2020, IPN was identified in four RT growing sites (two juvenile units and two on growing units). Different research institutions like Nofima and breeding companies are extensively studying the genetic basis of IPNV resistance in RT and commercially available IPNV-resistant QTL stocks and vaccinated fish are widely used to protect the stocks from outbreaks (Aslam et al., 2019). But many hatcheries are still reporting IPN outbreaks that are causing increased mortalities, economic losses, stunted growth, and lower fish welfare.

2.7 SELECTIVE BREEDING AND QTLs

Selection for disease resistance is an important goal of most breeding programs to produce individuals with inherent capabilities to prevent infection. Selective breeding is the exploitation of the genetic variance for a particular trait, and it provides higher genetic gain with cumulative effects. In selective breeding, the parents are tested against disease challenges and the best performing individuals with specific genetic architecture are selected to produce individuals with desired characteristics. In Rainbow trout (RT), many scientists have reported genetic variance against IPNV and identified putative quantitative trait locus (QTLs) associated with resistance to IPNV. (Ozaki et al., 2007) studied 226 markers for linkage analysis and reported nine QTLs significantly affecting the resistance against IPNV in RT. They have also suggested the polygenic nature of the trait. (Flores-Mara et al., 2017) provided heritability estimates for IPNV-resistance in RT and the reported heritability was 0.39± 0.08. The significantly higher genetic variation and heritability for the studied trait suggested the higher potential for the breeding in RT against IPNV-resistance. All the major selection approaches including pedigree-based selection, marker-assisted selection, and genomic selection can be employed to improve IPNV-resistance in breeding stocks. In all, genetic improvement is one of the most effective, sustainable, and longstanding solutions to improve disease resistance and prevent field outbreaks.

The advancements in SNP genotyping and statistical modeling of genetic and phenotypic information improved selection accuracy, leading to higher genetic gains. The highly polymorphic SNP markers are the most successful markers in quantitative trait studies and QTLs are the genomic regions associated with the studied trait. It is assumed that QTL markers are in linkage disequilibrium (that is non-random association of alleles at one locus with alleles at another locus) with the causative locus (Bush & Moore, 2012; Slatkin, 2008).

2.8 GENOME-WIDE ASSOCIATION STUDIES (GWAS)

The completion of genome sequencing projects made high throughput genotyping possible using dense markers. Therefore, genome-wide association studies (GWAS) became possible to study the genetic basis of the association between studied traits and SNPs that are either causative or maybe in LD with the causative locus (Gutierrez et al., 2015). GWAS are providing higher selection accuracies and genetic gains than traditional or marker-assisted selection (MAS). They are extensively used in land and aquatic animals for selection against a wide range of diseases. The genome-wide studies have revealed that most of the economically important traits are polygenic and individual genes are explaining only a low proportion of genetic variability. Therefore, sophisticated statistical genomics methods have been developed to consider all the genomic markers while estimating the genetic value of the candidate, one such advanced method is genomic selection.

2.9 GENOMIC SELECTION

(Meuwissen et al., 2001) proposed the concept of genomic selection (GS) that incorporate all the available genome-wide SNP markers for the estimation of breeding values. The whole idea is to estimate the genetic merits of the study subject by summing up the small effects of many SNP markers evenly distributed across the genome. The GS method is like MAS, but it requires dense DNA markers and assumes the contribution of many markers in the overall genetic variation. With the advancements in genotyping and sequencing technologies, the GS was adopted by major plant and animal breeding programs for analyzing the genetic merits of economically important traits (Goddard & Hayes, 2007). The aquaculture breeding industries and research institutes are utilizing GS for selecting the breeding individuals for high-performance, disease-resistant, feed efficient, and economically important traits.

3. MATERIAL AND METHODS

The analyzed data were obtained from two parallel challenge tests performed in a large (E-4) and a small (E-5) tank at VESO Vikan, each tank with an experimental group of fry, originated from Osland Stamfisk through crosses of sires and dams using previously known information on QTL(s) previously detected for survival to IPNV-AS (Aslam et al., 2019). Sires and dams of the fry were selected based on QTL genotypes with favorable genotypes termed IPNV-resistant (RR), heterozygous genotype (RS), and homozygous susceptible (SS). The planned mating design was a full factorial design with both sires and dams with the three following genotypes as shown in Table 1: resistant (RR), susceptible (SS), and heterozygous (RS).

***Table 1**: Full-factorial mating design with three different QTL-genotypes*

Sires/Dams (No. of parents)	**RR (4)**	**RS (0)**	**SS (1)**
RR (0)	**RR x RR**	**RR x RS**	**RR x SS**
RS (2)	**RS x RR**	**RS x RS**	**RS x SS**
SS (3)	**RR x SS**	**SS x RS**	**SS x SS**

Unfortunately, due to technical/human error during the production of the different groups, only four of the nine planned crosses were obtained (marked yellow in Table 1) and with a very different contribution from the parents (Figure 6). The mixed crosses resulted in a higher proportion of heterozygotes (RS) in the progeny. It was also found that no parental crossing was done between favorable parents (RRxRR) and only a small proportion of resistant (RR) fry were achieved from the crossing of heterozygote parents.

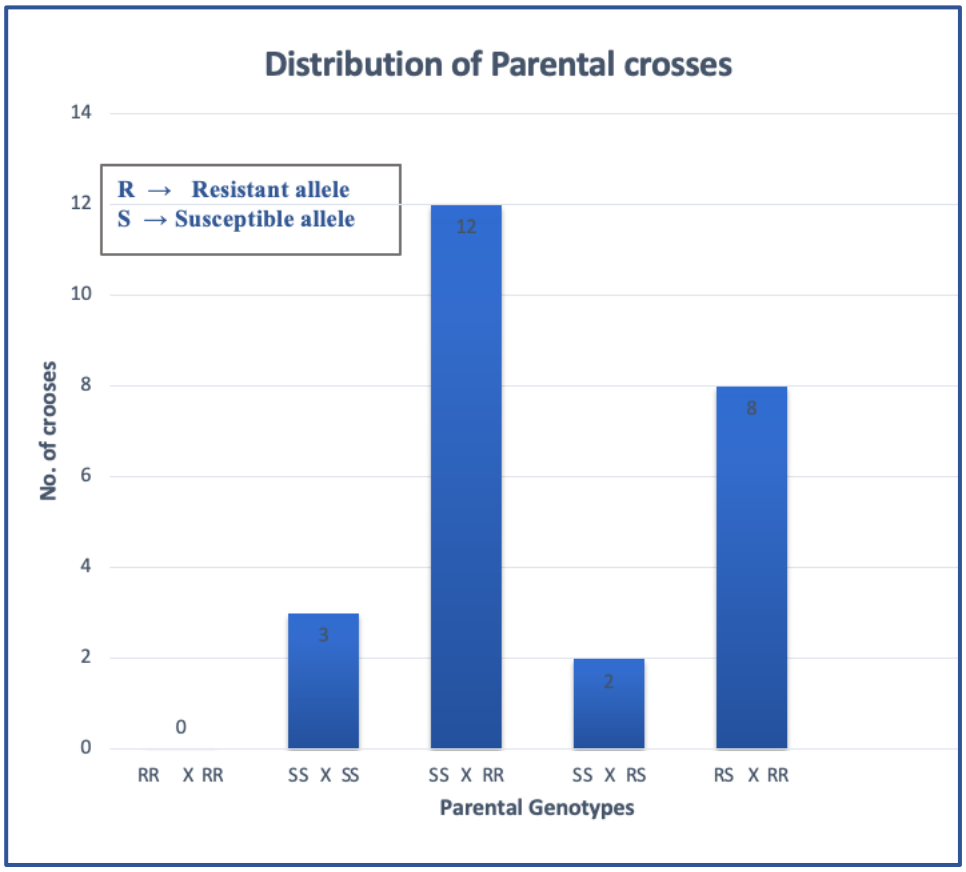

***Figure 6**: The parental crosses information inferred from the genotyped progeny*

The fry were transported from the Osland Stamfisk hatchery to VESO Vikan as ready-to-feed fry. Upon arrival at the Veso Vikan, the fry were nourished for one week by an automatic feeder before the IPNV challenge by bath model. In the bath model, IPNV is added to the study sample tanks up to the concentration of $10^5 – 5 \times 10^5$ $TCID_{50}$ (Median Tissue Culture Infectious Dose) per ml. The study sample tanks were cleaned daily, and the temperature was maintained at 12ºC ± 1ºC. The fry were acclimatized according to the standard protocol of the Veso Vikan. The maximum stocking density was set at 40 kg/m3 and the tanks were monitored closely to observe any unexpected behavior, mortality, or loss of appetite.

3.1 STUDY DESIGN

Figure 7 shows the experimental setup and the number of fry in each tank at the start of the challenge test. The large E-4 tank contained 2259 fry (out of which 475 fry belonged to our studied 25 families and 1784 fry from additional 50 nucleus families), while the smaller E-5 tank only contained 604 fry belonging to the study population.

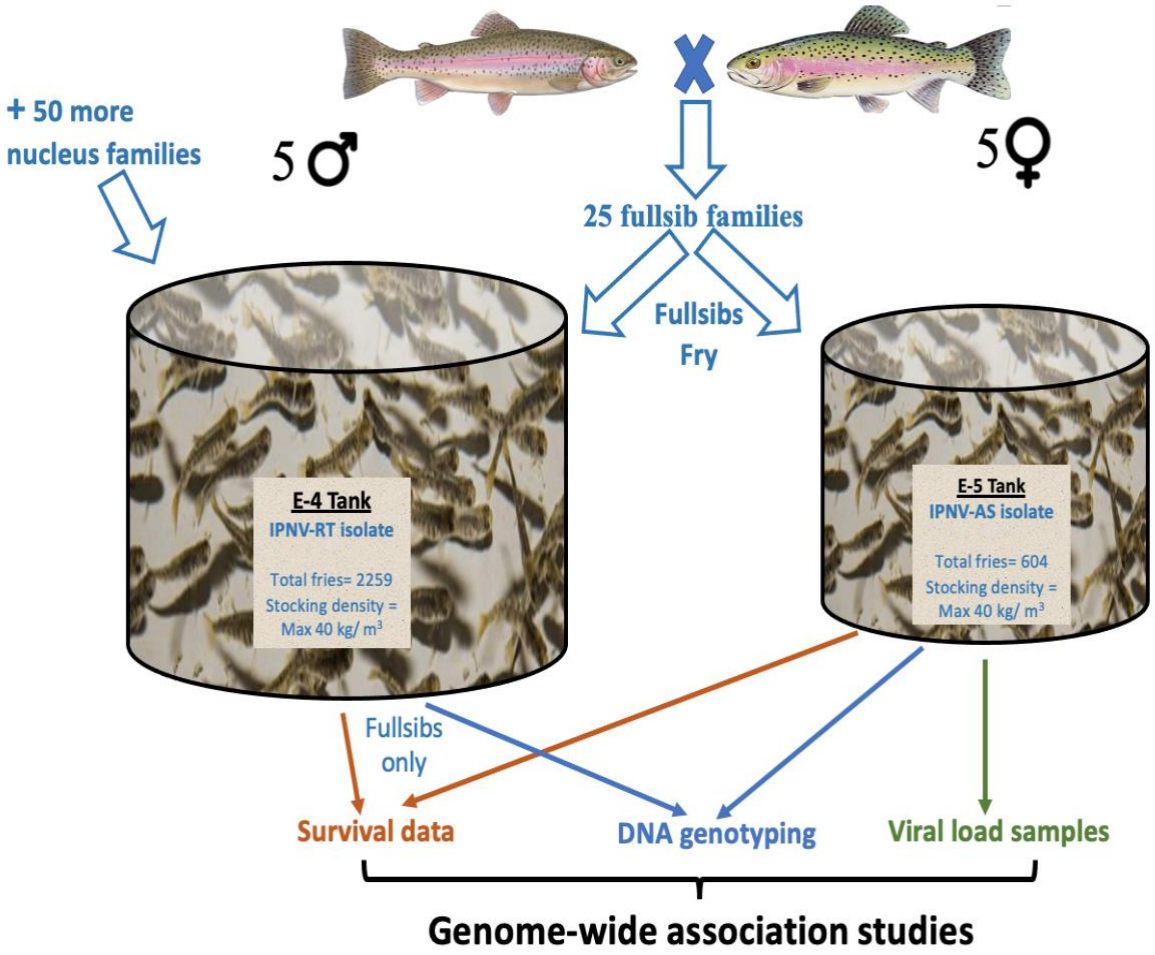

***Figure 7**: Schematic representation of research study design (fish samples in E-4 tank were challenged with IPNV-RT isolate and in E-5 tank with IPNV-AS isolates, and stocking density is same in both tanks).*

After one week, the freshwater supply to the tanks was stopped, and finally, the IPNV isolates with the concentration of 5 x 10^5 $TCID_{50}$/mL were used to challenge the experimental fry with IPN virus using the bath model. The fry in tank E-4 was challenged with IPNV-RT isolate acquired from an actual field outbreak at rainbow trout production site, whereas the fry in the E-5 tank were challenged with an IPNV-AS, isolated from an IPNV outbreak in Atlantic salmon. During challenge experiments, mortalities were recorded twice a day and the challenge test was continued until the day-wise mortality reached the minimal limit and terminated on the 40^{th} day. The fry survived till day 40 of the challenge test were considered survivors.

3.2 DNA SAMPLES

A tissue sample from the tails of all the dead fry were collected daily for DNA genotyping and parental identification starting from the day of the challenge until the termination of the

experiment. At the end of the experiment, tissue samples were also obtained from all the survivors. All the collected DNA samples were placed in Nunc 96 well plates filled with 99.5 % absolute alcohol (free of water or fat) and covered with a lid. The 96 well plates are marked 1-12 vertically and A-H horizontally which gave a unique position of each sample. Each of the plates was marked with plate, tank, and experiment number, and samples are electronically marked with the date of sampling and a unique ID. The samples were stored in a refrigerator and all the samples were finally shipped to Nofima AS after the termination of the challenge test.

3.3 GENOTYPING AND QUALITY CONTROL

The DNA genotyping was done using a 57-K Affymetrix axiom SNP array. Quality control was applied at both individual and marker levels; individuals were discarded based on the individual specific genotyping rate of < 98% and poor heterozygosity (± 5sd from mean, possible contaminations), and markers were removed based on minor allele frequency (MAF < 2 %), Hardy Weinberg equilibrium test (HWE p-value < 10^{-10}, might be the outcome of genotype calling error), and the marker specific rate of genotyping rate < 98%. Finally, 1079 individuals (out of 1085) and 31055 SNPs passed the quality check and were retained for further analysis.

3.4 RT-PCR SAMPLES

The 10 cross-sectional tissue samples of the dead fry were collected daily from E-5 tank only. At the end of the challenge test, approximately 200 surviving fries from the E-5 tank were sampled for PCR analysis, whereas all the surviving animals from both tanks were sampled for DNA genotyping. The tissue samples were collected in RNAlater vials. All the sample vials were placed in 96-well plates marked with individual sample positions, plate no, unique study identifier, and sampling tank. All the samples were kept at low temperature (-20 °C) as RNA is unstable at higher temperatures). After the termination of the experiment, all samples were finally shipped to NMBU for RT-PCR analysis of the IPNV viral load.

3.5 POPULATION STRUCTURE ANALYSIS

The PLINK package (PLINK: Whole Genome Data Analysis Toolset, 2022; Purcell et al., 2007) was used to perform a principal component analysis for analyzing population stratification. The obtained eigenvectors and eigenvalues were used to prevent the chances of

false-positive genetic associations by accounting for population sub-structures in GWAS analysis. The first two eigenvectors were plotted to visualize the population structure, and the eigenvalues of each eigenvector were used to compute the variation elucidated by the most important eigenvectors.

3.6 SURVIVAL ANALYSIS

The R studio (R Studio, 2020) was used to analyze and visualize the survival properties of the studied fry samples exposed to the two different IPNV (IPNV-RT and IPNV-AS) isolates. The bar plotting was used to analyze the day-wise survival of the fry in each of the two tanks. Then the genotype file was used for family-based separation and plotting of surviving and dead individuals. The Real-time PCR profiles of the viral load were used to make a boxplot of the viral load inferred from samples collected on different days. Finally, the comparative trend was analyzed in survival and viral throughout the challenge test duration (Therneau & Grambsch, 2000).

3.7 VARIANCE COMPONENT ESTIMATION

The genomic relationship matrix (GRM) was computed using marker data of 31055 SNPs, which was used to estimate variances and heritability. Estimates of variance components for survival traits were computed using the following mixed linear model (MLM) implemented in GCTA package (Yang et al., 2011). The restricted maximum likelihood (REML) approach was used in which the GRM was added as a random polygenic component:

$$y = u + Zg + e$$

where

y = a vector of the binary survival phenotypes (alive=1, dead= 0)
u = the overall mean of the population
Z = incidence matrix
g = vector of genomic breeding values

e = vector of random residual effects

The VanRaden method was used to compute the GRM matrix using the following equation (VanRaden, 2008).

$$G = \frac{ZZ'}{[2\sum_i^l p_i\,(1-p_i)]}$$

where

Z = M - P is the above defined Z matrix, where
M = Genotype X animals matrix with 0,1,2 coding
P = matrix with all elements as 2pi in ith column
p_i = allelic frequency of i^{th} SNP

The following equation was used for the calculation of heritability for survival:

$$h^2_{obs} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

where

h^2_{obs} = heritability

σ_g^2 = genetic variance

σ_e^2 = residual variance

3.8 GENOME-WIDE ASSOCIATION STUDIES (GWAS)

The allele substitution effect of each marker was obtained from a similar statistical model to that above, but that also included the marker effect as a regression variable (Yang et al., 2014)

$$y = u + \sum_{j=1}^{N=2} pca_j + M_i\alpha_i + Zg + e$$

Where;

M_i = Incidence matrix for SNP i containing marker genotypes coded as $0 = AA, 1 = AB|BA, 2 = BB$,

α_i = allele substitution effect of SNP i

GCTA genomic tool was used for trait association studies. The mixed linear model, leaving-one-chromosome-out (--mlma-loco) approach was used for the current study to analyze the genome-wide complex trait for evaluating the allele substitution effect (Yang et al., 2014). The first two PCA were used as covariates in case of inflated λ-value to account for spurious association due to population sub-structures. The output of the association studies was saved in a file and the P-values were presented as $-\log_{10}P$. The files were then imported into the R studio and different packages, including "qqman", were used to make the Q-Q and Manhattan plots from the information provided in the output file of the association studies. The Q-Q plots were used to check if the observed p-values deviated from the expected values under the normal distribution (D. Turner, 2018; Wickham, 2016).

The inflation factor was calculated using the equation $\frac{median\left((a^2/s.e^2)^2\right)}{0.456}$ to observe the inflation of p-values for survival and viral load traits. Ideally, the inflation value (lambda, λ) should be close to 1 which reflects no evidence of inflation in p-values due to unknown reasons (e.g., underlying population structure). The higher values of λ indicate spurious associations due to population sub-structures within the studied population. If the p-values are inflated, then eigenvectors can be used as covariates to adjust the p-values. Manhattan plots were used to visualize the distribution of marker-trait associations and to detect any signal of QTL(s). Two thresholds were calculated to consider significant association of SNPs with survival trait.

The chromosome-wide threshold was calculated using equation $\frac{-\log10(\text{p-value})}{(\text{No.of SNPs/no.of Chromosomes})}$ and Bonferroni threshold was calculated by using the equation $\frac{-\log10(\text{p-value})}{No.of\ SNPs}$. SNPs were considered genome wide significant when they exceeded the Bonferroni threshold for multiple testing of $0.05/31653$, (0.05 = alpha; 31653 = total number of genome-wide distributed SNPs in GWAS analysis) and classified as chromosome-wide significant when Bonferroni threshold for multiple testing surpassed $0.05/1091$, (0.05 = alpha; 1091 = average number of SNPs per chromosome). The chromosome-wide threshold obtained was $-log_{10}(p-value) = 4.34$ and the genome-wide threshold obtained was $-log_{10}(p-value) = 5.80$..

3.9 PROCESSING OF RNA SAMPLES

3.9.1 RNA extraction

The RNA extraction from the fry tissue samples was done using the QIAGEN QIAsymphony RNA kit (QIAsymphony RNA Kit, 2022). The frozen tissue samples were thawed and then a

scalper was used to cut the tissue samples of equal weight (~ 10 milligrams). Eppendorf (2ml) tubes were labeled according to sample numbers and 400 µl of RLT buffer was added to each tube using a multi-step micropipette. The samples were added to each tube and then one stainless bead was added per tube for efficient homogenization. Each sample was then homogenized on a rotor-homogenizer (MP fast-prep 24) at the speed of 4.5 m/s for 30 seconds, a procedure that was repeated three times. The sample tubes were placed on ice for cooling down between the runs.

The homogenized samples were then centrifuged at 13,000 rpm for 3 minutes. The new set of Eppendorf vials compatible with the QIAsmphony Robot was labeled and then finally 400 µl pellet-free supernatant was added to the new vials. The samples were stored overnight and then placed in a QIAsymphony instrument for further processing. The extracted RNA samples were then stored at -80 °C.

3.9.2 RNA quantification

The extracted RNA samples were subjected to quality and quantity assessment. Epoch microplate spectrophotometer was used for the quantification of RNA in our samples. RNase-free water was used as a blank (RNA free) sample for quantification analysis and 2 µl of the samples were placed in duplicates on a cleaned microvolume plate. The spectral scans show the wavelength (nm) of the absorbance (A) ratio at A240-290/280. Purity was accessed using A260/A280 and the final concentration of RNA in the sample was obtained in ng/µL. All the samples showed good purity and concentration of the RNA except four samples which were discarded, and 444 samples proceeded further for RT-PCR analysis.

3.9.3 IPNV primers

The IPNV-specific primers were designed, tested, and ordered from Eurofins Genomics GmbH, Germany. The primers with the following sequences were used in RT-PCR analysis:

***Table 2**: IPNV forward and reverse primer sequences*

Sr. No	Primer	5'- 3' Sequence
1	IPNV-Forward	CAACAGGGTTCGACAAACCATAC
2	IPNV-Reverse	GAAACGCCGACATCGTCAA

The lyophilized primers were diluted with DNases/RNase-free water (100 pmol/µl) to achieve the same concentration. Finally, the primers were diluted to 1:10 factor before use. To be able to compare the relative concentration of amplified viral RNA between samples, Beta-Actin (housekeeping gene) primers were used together with IPNV primers for all samples but in separate PCR reactions. This Beta-Actin gene was used as a reference in all PCR experiments.

3.9.4 Real-time PCR analysis

The BIORAD Universal SYBR Green one-step kit (SYBR® Green | Bio-Rad Laboratories, 2022) was used to run the Real-time PCR analysis of the IPNV challenged RT samples. The kit was one-step as the reverse transcription of RNA into cDNA and then polymerase chain reaction was taking place in a single reaction. The RNA samples and SYBR Green kit reagents were thawed at 4 °C, centrifuged briefly to mix thoroughly, and then placed on ice to protect them. The following reaction components were used to prepare samples for RT-PCR analysis.

__Table 3__:List of PCR reaction components

Reaction components	Volume for 1 sample per 10 µl reaction
iTaq SYBR Green master mix	5 µl
iScript Reverse transcriptase	0.25 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Nuclease free water	0.875 µl
Template (RNA sample)	3 µl
Total volume	10 µl

For every RT-PCR reaction, a master mix was prepared in an Eppendorf tube for 86 samples that include duplicates of 40 samples, positive control, negative control, and calibrator. The positive and negative controls were used to ensure the experiment was going well, and a calibrator was used to mitigate the between runs difference as multiple runs of samples were performed on two ArixMx Real-Time PCR thermocyclers (one for IPNV and one for reference samples) for efficient comparison.

All the samples were preheated at 70 °C for 5 min and then dispensed into RT-PCR plates using a multichannel clip-lock micropipette. All samples well were covered with a lock and then the 96-well plate was placed in a Labnet mini plate spinner (mps 1000) for 30 seconds to properly mix the sample with reagent. Finally, the sample plates were placed in the ArixMx Real-Time PCR system with the following RT-PCR profile shown in figure 8.

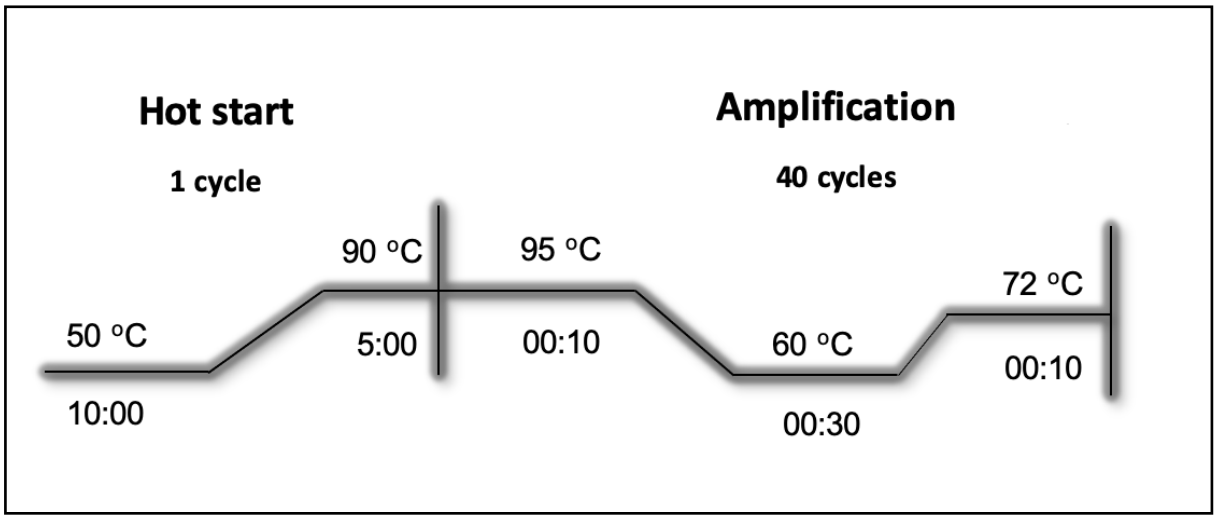

***Figure 8**: Thermal Cycler Profile for RT-PCR amplification of IPNV*

Finally, the average cycle threshold (CT) value was calculated from the PCR output file for all the IPNV positive IPNV and B-Actin tested samples. The combined worksheet was created to merge the CT values from all the PCR reactions for IPNV, B-Actin, calibrator, and positive and negative controls. The following equation was then used to calculate the relative ratio of viral load in studied samples.

$$\text{Ratio} = \left\{ \frac{[(E_{ref})^{CT\,(Sample)} / (E_{target})^{CT\,(Sample)}]}{[(E_{ref})^{CT\,(Calibrator)} / (E_{target})^{CT\,(Calibrator)}]} \right\} \times 1000$$

where.

E= Primer efficiency of reference and target samples
CT= Threshold cycle for the studied samples

4. RESULTS

4.1 CHALLENGE TEST

4.1.1 Experimental population

The three different groups of sires and dams i.e., IPNV-resistant (RR), heterozygous (RS), and homozygous susceptible (SS) genotypes were crossed to produce the studied progeny. Table 4 shows that no crosses were done between IPNV-resistant (RR) sires and dams and only a small fraction of RR fry (196) were obtained from crosses of RR and RS.

Table 4: Genotype-wise distribution of parental crosses and frequency distribution of the progeny fry

Sex	Sire			
	Genotypes	RR	RS	SS
Dam	RR	0 (No. of individuals) (0) (Genotype wise frequency)	219 (120 RS, 99 RR)	120 (120 RS)
	RS	215 (118 RS, 97 RR)	0 (0)	67 (33 SS, 34 RS)
	SS	231 (231 RS)	41 (25 RS, 16 SS)	135 (135 SS)

4.1.2 Survival analysis of full sibs in the E-4 and E-5 tanks

The challenge test with the two different isolates of IPNV showed a significant difference in mortalities with 60% mortality caused by IPNV-AS vs. 27% mortality by IPV-RT (Figure 9). Additionally, IPNV-RT presents a gradual wider phase of mortalities (day 11 to day 17) with no peak mortality while IPNV-AS resulted in a sharp narrower phase of mortalities with peak mortalities at day 8 (Figure 9).

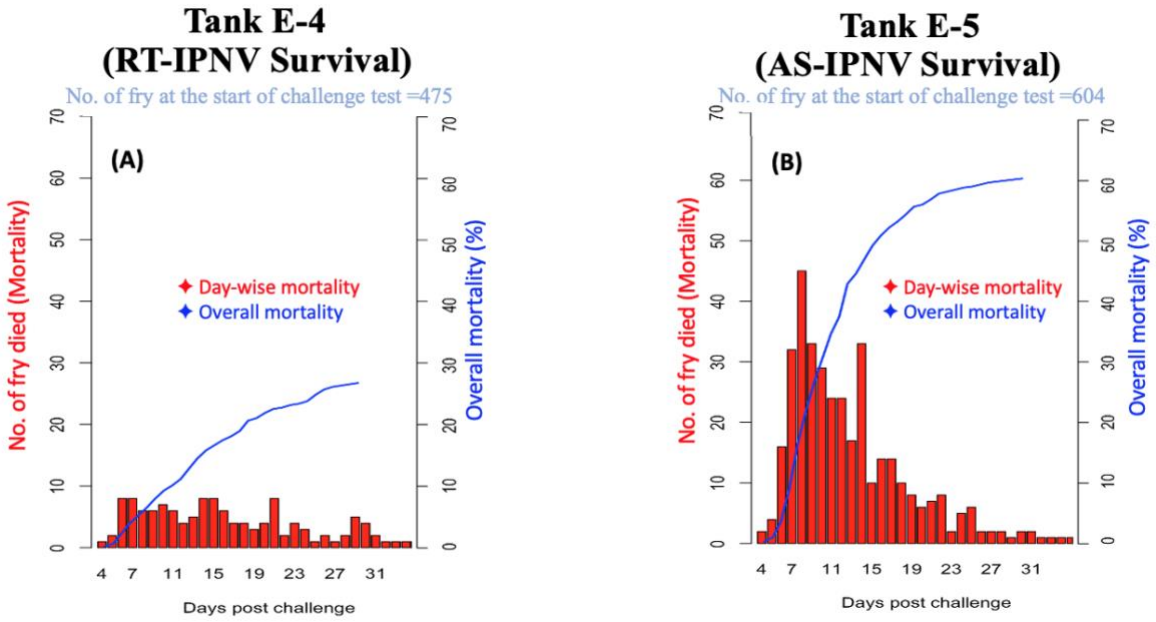

Figure 9: *Day-wise mortality profiles of the IPNV challenge tests with IPNV-RT isolate (A) and IPNV-AS isolate (B).*

4.1.3 Survival distribution across families

The distribution of the 25 full-sib families across the two tanks showed a similar pattern of survival in most of the families, but some families show anomalous behavior (Figure 10).

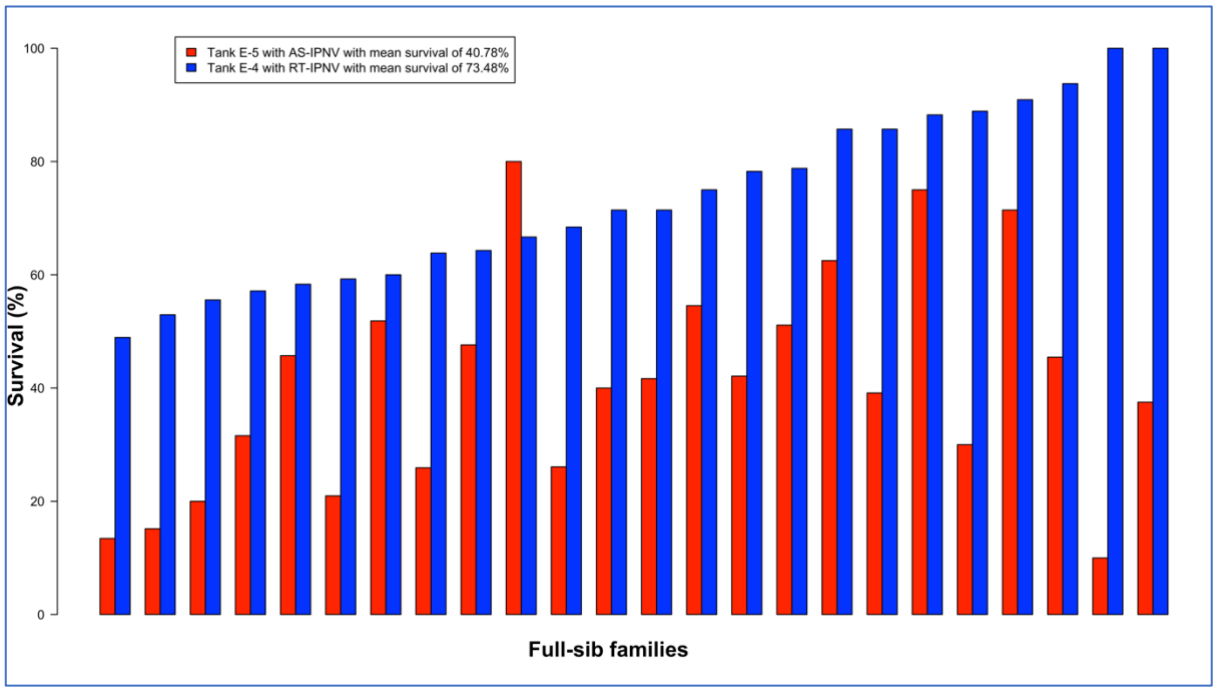

Figure 10*: Survival of 25 full-sibs families challenged with an IPNV-RT isolate and IPNV-AS isolate.*

4.2 ESTIMATION OF GENETIC PARAMETERS

4.2.1 Genetic variances

Genetic and residual variances for survival to IPNV-RT and IPNV-AS were found to be very similar and with moderate heritabilities (Table 5). The estimated heritability for viral load due to IPNV-AS (0.20 ± 0.08) was very similar to the combined estimated heritability for survival (0.21 ± 0.05).

Table 5: *Estimates of genetic variance, residual variance, and heritability for survival to IPNV-RT and IPNV-AS and for the viral load of IPNV-AS*

	Survival			Viral load
	IPNV-RT	**IPNV-AS**	**Combined**	**IPNV-AS**
Genetic variance	0.04 ±0.02	0.05 ± 0.02	0.06 ± 0.01	0.21 ± 0.09
Residual variance	0.19 ± 0.01	0.18 ± 0.01	0.20 ± 0.01	0.86± 0.07
Heritability	0.16 ± 0.06	0.22 ± 0.06	0.21 ± 0.05	0.20 ± 0.08

4.2.2 Genetic correlations

The genetic correlation of unity (1.0 ± 0.11) between survival to IPNV-RT and IPNV-AS shows that the survival against IPNV-RT and IPNV-AS are the same trait and families rank in identical order. The estimated genetic correlation between IPNV-AS survival and IPNV-AS viral load was -0.70 ± 0.20 which shows that selection for increased resistance to viral load will cause a favorable correlated response in IPNV-AS.

4.3 GENOME-WIDE ASSOCIATION STUDIES (GWAS)

4.3.1 Principal component analysis (PCA)

The PCA plots were used to visualize and adjust for (any) population sub-structures. Figure 11 shows the pattern of stratification of the 25 full-sib families with 11(A) color-coded with dams genetic information and figure 11(B) with the sires information. Figure 11(A) shows that the many clusters belong to the same dams (link Dam f_03 showed four clusters) which then came out as separate genetic identities by genetic combination with sires (figure 11). Of the 15.5 % genetic variation captured by the first two PCAs, PCA-1 captured 8.5 %, and PCA-2 explained 6.96%.

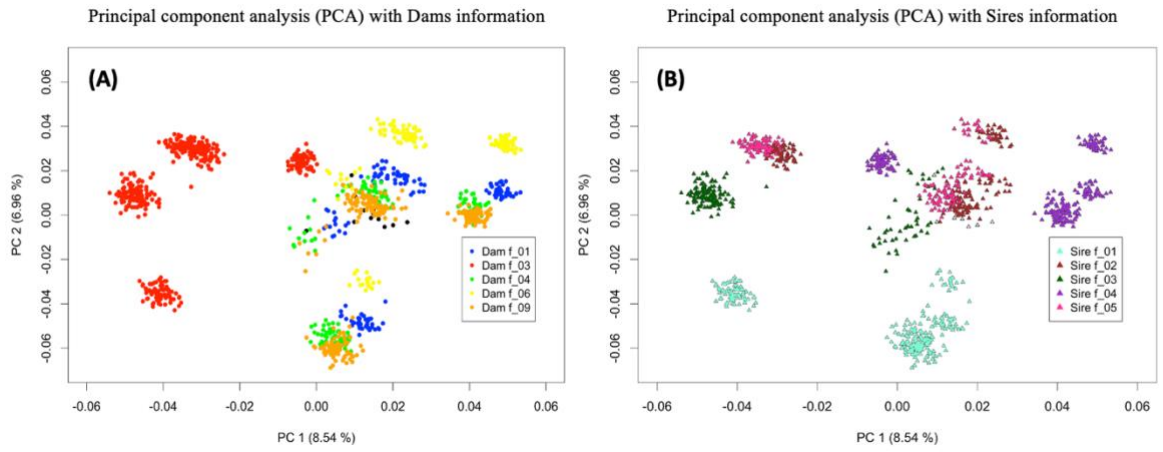

***Figure 11**: Principal component analysis (PCA) for the study samples; individual clusters represent full-sib families and the first two principal components (PCS) explain 15.50 % of the variance. Figure **(A)** highlights clusters of the off-spring colored with dams genetic information and **(B)** shows clusters of the off-spring derived from the sires information. Figure **(B)** depicts subclusters within the dam-based clusters of **(A)** differentiating full and half-sib families*

4.3.2 GWAS for IPNV resistance

The results of the GWAS analyses are depicted via four Manhattan plots, one for each of the four studied traits, survival against 1PNV-RT, IPNV-AS, overall IPNV survival and virus load. Figure 12 (A) shows the overall survival of the fry against the two IPNV isolates and demonstrates that many SNPs on different chromosomes have crossed the chromosome-wide and Bonferroni corrected threshold indicating a polygenic nature of the overall-IPNV survival. Figure 12 (B and C) reveals that many SNPs crossed the chromosome-wide significance

threshold of -$\log_{10}$P =4.34 but not the Bonferroni threshold of -$\log_{10}$P =5.80. It was also observed that for IPNV-RT one SNP on chromosome 1 shows concordance with the highly significant SNPs discovered in the parental generation of the fry (Luqman et al., 2019, whereas all other highly significant SNPs of the parental generation (marked as green dots) are not highly significant in the studied families. However, the Manhattan plot for the viral load of IPNV-AS shows clear peaks on chromosomes 10, 11and 16.

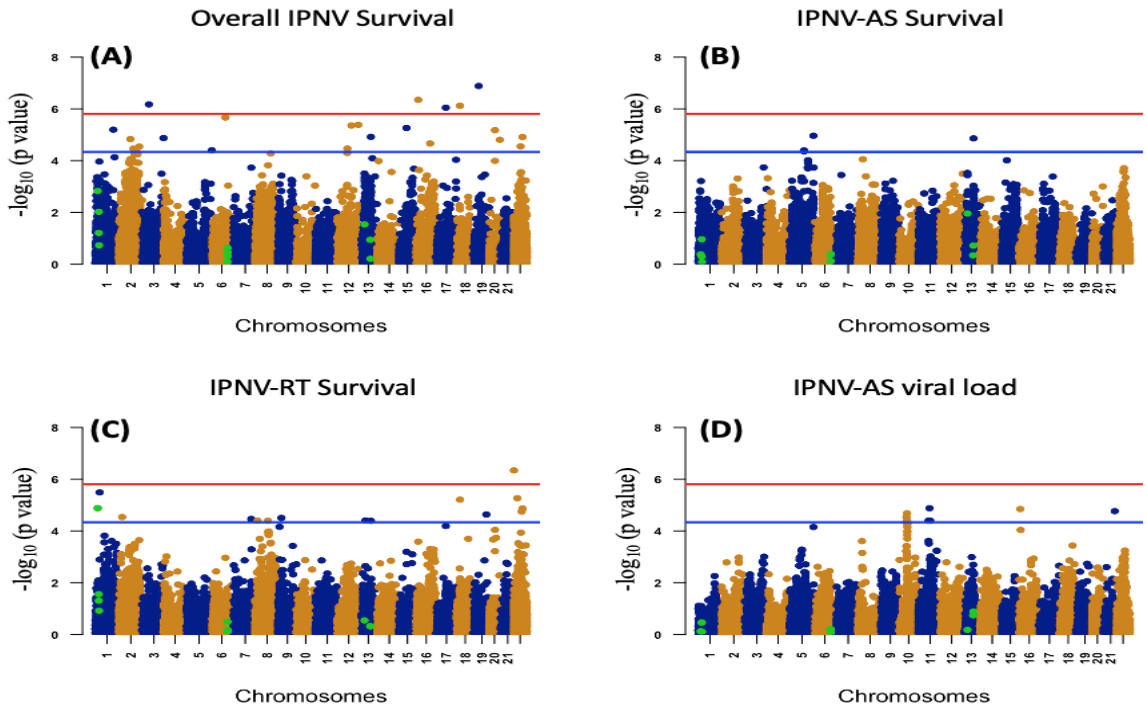

***Figure 12**: Manhattan plot of −log10 P-values derived from genome-wide association studies (GWAS) on overall IPNV survival **(A)**, survival to IPNV-AS **(**B), survival to IPNV-RT **(C),** and viral load for IPNV-AS **(D).** The red and the blue lines represent the genome-wide Bonferroni significance threshold and chromosome-wide threshold (−log10 P values of 5.8 (P=1.8 x 10^{-6}) and 4.3 (P=4.6 x 10^{-5}), respectively. Green dots represent highly significant SNPs in the parental generation*

4.3.3 Quantile-quantile (QQ) plots

Figure 13 demonstrates the concordance between the observed and expected p-values for the studied traits. The inflation factor (lambda; λ) was between 1.05 and 1.15 (except for IPNV-AS survival) which shows that p-values are not inflated due to unknown reasons (e.g., population structure). GWAS results for survival to IPNV-AS showed inflated p-values with

λ-value of 1.21, hence first two eigenvectors were used as covariates to adjust the p-values which resulted λ-value of 1.16.

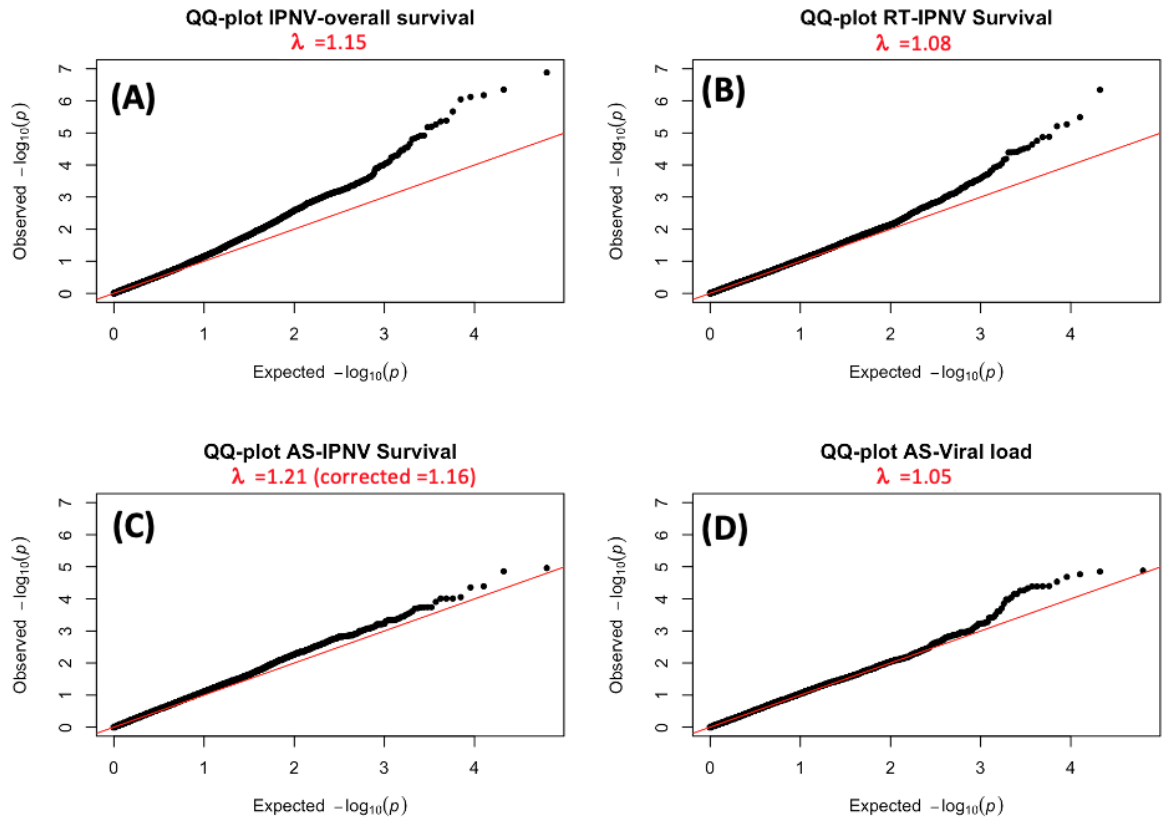

***Figure 13**: Quantile-quantile (QQ-plots) of observed and expected −log10 of the p-value for survival to IPNV-RT and IPNV-AS, and IPNV-AS viral load.*

4.4 COMPARATIVE ANALYSIS

4.4.1 Genotype wise survival analysis

Survival curves across individuals carrying different genotypes (RR, RS, and SS) of known/validated QTL are plotted in Figure 14. As expected, the RR genotype fry showed higher survival throughout the challenge test compared to the RS and SS genotypes (Figure 14).

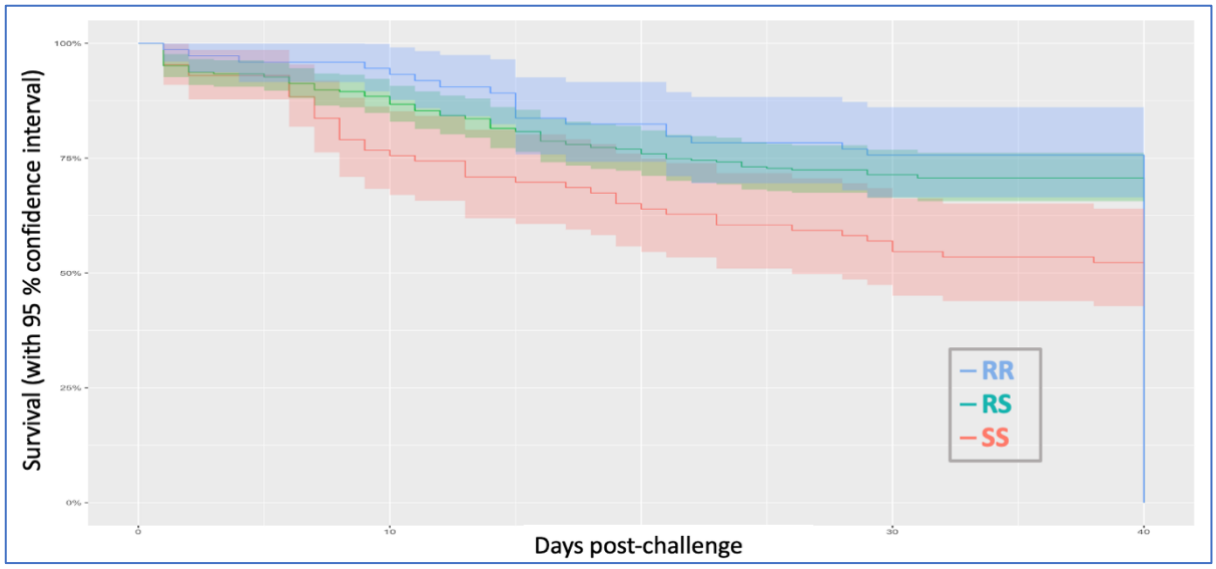

***Figure 14**: Survival curves for survival to IPNV-RT derived from groups of fry bearing RR, RS, or SS QTL genotypes. The color codes represent a 95% confidence interval.*

4.4.2 Survival and viral load analysis

Figure 15 shows concordant dynamic for the survival and relative viral load with higher mortality and virus load at the start of challenge test with decreasing trend through the progression of the challenge test. The different colored blocks, each indicating 10 days intervals, are used to assist in comparing both traits that show concordant behavior over time. The figure clearly shows that the mortality and viral load were high during the first two weeks followed by a decrease in the following weeks.

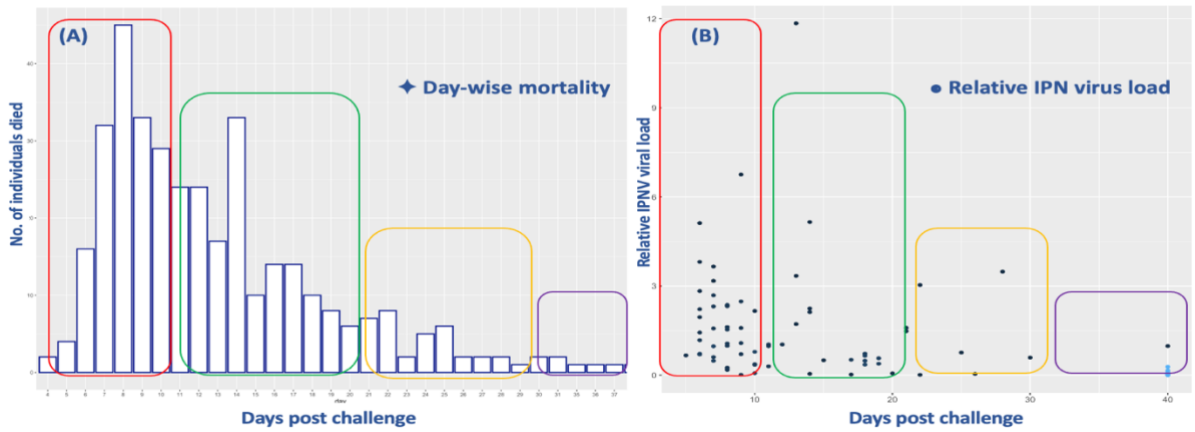

***Figure 15:** Comparative analysis of survival and viral load inferred from tank E-5. Colored rectangular blocks of 10 days intervals are used for comparison over the 40 days challenge test period.*

5. DISCUSSION

The rainbow trout aquaculture is prone to IPNV infections, leading to higher mortalities and severe clinical infections in production and breeding stocks, especially at the early life stages. Despite effective management, careful disinfection, and vaccination, IPNV is still a threat to the aquaculture industry. The economic losses and welfare concerns called for scientific studies to analyze the molecular pathogenesis and discover the possible solutions to restrict the outbreaks of IPN in aquaculture, which led to the advent of selective breeding as the major step forward. In the present study we have investigated the comparative survival against IPNV challenge and possibilities of reducing this disease problem through selective breeding, and to what extent selection for increased resistance to one pathogenic strain of IPNV will also result in increased resistance to another isolate.

The survival of the fry against two different strains of IPNV was quite different with IPNV-AS challenged fry exhibiting two times higher mortalities compared to the IPNV-RT challenged fry over the 40 days span of the experiment. These results indicate the more virulent nature of the IPNV-AS strain compared to the IPNV-RT strain and are consistent with information obtained from a larger data set study of RT fry (Aslam *et al.,* 2019). Both challenge tests have shown peak mortalities during the second and third-week post-challenge followed by low or no mortalities leading to termination of the challenge test. The difference in mortality can be attributed to amino acid substitutions in the VP2 encoding polyprotein of the IPNV genome as shown in table 6 below.

The molecular analysis has revealed 13 amino acid substitutions in the VP2-encoding gene region, particularly in positions located on the surface of the VP2 protein, likely crucial for interaction with receptors in the host cells and immunogenicity (Munang'andu et al. 2012). It is likely that combinations of the mutations (not a single mutation) are playing a crucial role in IPN infection. However, virulence does not correspond always to molecular determinants and the degree of IPNV infection depends on host-pathogen interaction, viral strain, as well as the rearing environment (Song *et al.,* 2015; Mukoloki *et al.,* 2016, Ahmadivand *et al.,* 2020; Tamer *et al.,* 2020). In a recent research publication, (Hillestad *et al.,* 2021) evinced that the substitutions are enhancing the capacity of the virus to escape the defense of the IPNV resistant fish, and a complete understanding of the genetic interaction between host and pathogen is important to deduce the outcome of the infection.

Table 6: List of aminoacids substitutions in VP-2 viral capsid protein

Amino acid position in VP-2 protein of IPNV	IPNV-RT isolate	IPNV-AS isolate
221	A	T
245	S	G
248	E	R
252	V	D
255	K	T
257	D	H
278	V	A
282	N	T
285	Y	H
321	G	D
500	H	Y

The heritability estimates for all the studied traits were moderate and consistent with two different bath challenge test studies with RT that reported an estimate of 0.39 ± 0.08 for fry offspring of 96 sires and 212 Dams (Flores-Mara *et al.*, 2017), and an estimate of 0.32 ± 0.0 for fry offspring of 56 sires and 119 dams (Aslam *et al.*, 2019). However, for 154 days old RT fingerlings of 20 sires and 58 dams Rodriguez *et al.*, (2019) reported a substantial higher heritability for binary survival (0.83 ± 0.03) and a medium-sized heritability for time to death (0.53 ± 0.05) following intraperitoneal injection of the virus (post-challenge days). The marked differences in heritabilities are due to different study populations, different challenge models, likely different viral strains, selective genotyping of the informative samples, and most importantly different growth stages. Overall, the heritability estimates of the current studied traits are moderate and indicative of potential use in the selection for increased resistance to IPNV in RT breeding programs.

The genetic correlation between survival to IPNV-AS and IPNV-RT was very high, meaning that the selection for one of the studied isolates will impart resistance against the other isolate. The genetic correlation between survival and viral load due to IPNV-AS was moderate and

negative and thus favorable, implying that selection for increased resistance to viral load will cause a favorable correlated response in IPNV-AS survival.

The comparative analysis of the survival and viral load in IPNV-AS challenged fry showed peak mortalities and higher IPNV positive samples during the second week followed by a brief increase in the third week and then a decrease in viral loads and related mortalities later. The pattern of survival and viral load indicates two-stage infectivity where the infected fish shed viruses in the water leading to the horizontal transmission of the virus in the same population. In addition to horizontal transmission, scientific studies have also confirmed the vertical transmission of the IPNV to the progeny of infected parents (13th EAFP Grado, 2007).

The outcome of GWAS for QTL(s) is highly influenced by genetics, linkage disequilibrium, traits heritability, QTL detection model, and the number of animals genotyped and phenotyped. In the current study, we did not identify any major QTL that could explain a major proportion of the genetic variations in survival or viral load. However, the QTLs discovered by (Aslam *et al.,* 2019) on chromosomes 1, 6, and 13 in a challenge study with IPNV-AS were also seen in the current study and it was found that only IPNV-RT survival is showing concordance with the previous study whereas all other traits failed to show the similarity, possibly due to the small population size in the present study and thus less statistical power. The highly significant QTLs observed on chromosome 22 for the IPNV-RT survival trait in this study were absent in the study by (Aslam *et al.,* 2019) and need to be evaluated further.

Rodriguez *et al.,* (2019) have reported a significant QTL on chromosome 5 that explains 19 % of the genetic variation for survival against IPNV resistance in a challenge test. The current GWAS study on the IPNV-AS survival trait showed a peak on chromosomes 5 and 13 that are consistent with the Rodriguez findings. Contrary to the QTLs found on Chromosomes 1, 5, 6, 13, and 23 in the above-mentioned studies, the IPNV-AS viral load trait in this study showed clear peaks on chromosomes 10, 11, and 16 that may be due to the quantitative nature of this trait as compared to the less informative binary survival trait. These peaks need to be further evaluated to find the genomic regions associated with IPNV viral load.

The current study has provided significant insight into comparative survival, genetic architecture, and genomic loci associated to survival. However, there were some limitations of the present study. The sample size was small, and the fry were prone to higher mortalities (specific as well as non-specific). The study did not account for the tank effect and at least part

of the different survival of the fry in the two tanks may be due to a tank effect as there was only one tank per IPNV isolate. The qPCR analysis has resulted in very less IPNV positive samples. In addition to this, the technical error in the crossing of parents led to higher heterozygosity in the progeny which led to less power in the statistical analysis.

Overall, the present and the referenced genome-wide association studies have shown the polygenic nature of the studied IPNV traits as many significant QTLs were found to be significantly associated with the traits. The outcomes of the study have applied aspects to the fish breeding industry to restrict the outbreaks of the IPNV. The genomic selection is the best way for fish breeders to select against IPNV as it will account for all the genetic variation contributions by the many small QTLs. A detailed understanding of the genetic variation and interactions of the host and the IPNV are crucial in understanding the faith of the disease. Viral load should be used as a potentially preferred selection trait for IPNV as it does not include non-specific mortalities, is more informative than the binary survival trait, and helps to detect clinical infections and carriers in the population. The QTLs should be validated using viral load as a trait, and actual causative mutation should be detected using large-scale multi-omics (genomics, transcriptomics, proteomics, etc.) data.

6. CONCLUSION

It was found that IPNV-AS is more virulent compared to IPNV-RT which could be attributed to the marked difference in the genetic architecture of the viral genome encoding parts of the viral protein important for host cell interactions and immunogenicity. The indicative estimates of heritabilities for the studied traits were moderate. The GWAS have shown the polygenic nature of the traits under investigation as many QTLs were significantly associated with the traits. This implies that genomic selection is the best strategy to improve the resistance against IPNV in selective breeding programs. The current study can only give indicative estimates due to its limited power, small population size, and more heterozygous samples. The QTLs should be validated using viral load as a trait with a larger data set, and actual causative mutation should be detected using additional omics data.

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