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Effects of Fat Soluble Vitamins on Viral Infection and Antiviral Responses in Atlantic Salmon (*Salmo salar L.)* Erythrocytes

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Abstract

Viral diseases are vital challenges in the aquaculture industry. In Norway, heart- and skeletal muscle inflammation (HSMI) is one of the major viral diseases affecting Atlantic salmon. In mammals, low vitamin D has been found to increase susceptibility to viral diseases. In this study, the overall aim was to determine if vitamin D_2 and D_3 together with the RXR agonist bexarotene have an effect on infection and antiviral response against *Piscine orthroreovirus* (PRV), the dsRNA virus that causes HSMI. The main target cells for PRV *in vivo* are the erythrocytes (red blood cells), which can also be infected *ex vivo*. To achieve the study objectives, three experiments were performed. Naïve or infected RBCs were isolated from Atlantic salmon, incubated with vitamin D, and naïve cells were either poly I: C stimulated or treated with purified PRV. The virus replication and antiviral gene expression were analyzed by RT-qPCR. The results indicated that vitamin D may potentiate the antiviral response, although results were inconclusive. The study indicates that vitamin D may have an effect on antiviral responses, but this needs to be further investigated.

Table of Contents

1. Introduction

1.1 Aquaculture of Atlantic salmon

Worldwide, aquaculture is the fastest-growing food producing sector, and contributes significantly to the supply of fish for human consumption (FAO, 2010). The expansion in both marine and freshwater aquaculture was made possible due to development in engineering, feed technology (e.g. formulation and processing), breeding and disease control.

Of the marine species being cultured, Atlantic salmon (*Salmo salar* L.) is dominant (MarineHarvest, 2017), mainly because of their high nutritional content, i.e. protein, omega-3 fatty acids and micronutrients (Beveridge et al., 2013; Sprague, Dick, & Tocher, 2016) and growing global market (MarineHarvest, 2017). Likewise, farmed salmon has low feed conversion ratio (FCR) (O. Torrissen et al., 2011) compared to land animals and other fish species. The lower the FCR the more efficient the fish is in retaining the protein and energy from the feed and converting it into nutritious food for human consumption (Ytrestøyl, Aas, & Åsgård, 2015). However, much of the cost pertaining to fish production is due to infectious diseases (Thorarinsson & Powell, 2006) and nutrition. Feed for instance, constitutes about 40% to 50% of the total production cost of farming salmonids in Norway (Moe, 2016).

Globally, the leading salmon producers (Figure 1) are Norway, Chile, United Kingdom and Canada as several natural conditions are adequate in these countries throughout the entire life cycle. Such conditions include cold marine water temperatures (8^0C – 14^0C) and a sheltered coast line (Lart & Green, 2012). Production also takes place in the Faroe Islands, Australia (Tasmania), Ireland, USA and Iceland (Marine Harvest, 2016) with minor production in New Zealand, France and Spain (Lart & Green, 2012).

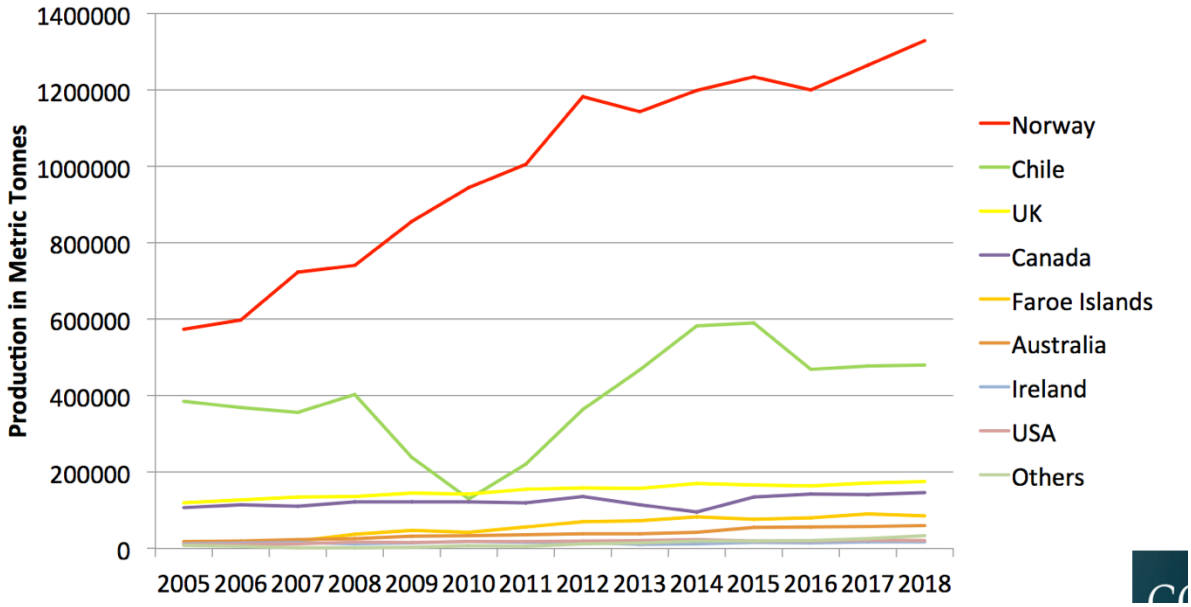

Figure 1: Main producers of farmed Atlantic salmon, 2005 to 2017 (Salmon- World Kontali Analyze: https://www.undercurrentnews.com/2016/09/19/goal-2016-blog-from-guangzhou/)

In Norway, Atlantic salmon farming industry was established in the 1970s and currently it is a predominant species as well as the country's largest fish export item (Taranger et al., 2015). In terms of production and revenue, in 2015 for instance (Figure 2), about 1.3 million tons of salmon were produced, corresponding to about 68% of the world total production and to economic value of NOK 44.3 billion (Statistics Norway, 2016). Despite the large amounts of salmon produced, losses related to viral diseases remain significant (Bornø & Lie Linaker, 2015). And the risk of disease outbreaks mostly increased during the marine phase (Bornø & Lie Linaker, 2015; Taranger et al., 2014).

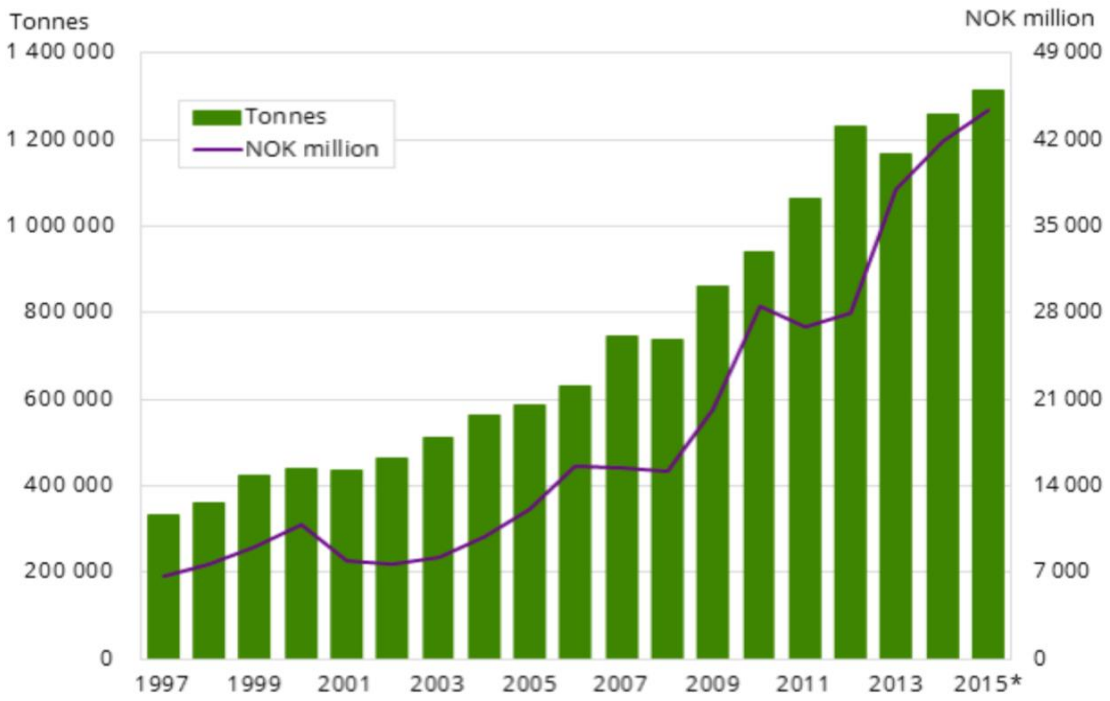

Figure 2**:** Sales of salmon quantity and first-hand value from 1997 to 2015 (Statistics Norway, 2016: www.ssb.no)

1.2 Life cycle of farmed Atlantic salmon and the production challenges

As an anadromous fish, the farmed Atlantic salmon life cycle takes place in both freshwater and seawater (figure 3). In freshwater (FW), the production cycle begins with broodstock where the eggs are stripped from the female and fertilized by being mixed with the milt from the male. Afterwards, the fertilized eggs are disinfected in iodine solution and incubated for hatching (FAO, 2004). On hatching, light manipulation is performed and the alevins (larvae) emerge from the egg, dependent on the contents of their yolk sac for feeding. When the alevins have exhausted their yolk reserves, they develop to fry (Good & Davidson, 2016). As the yolk-sac diminishes, the fry begins feeding on external sources and develop into parr (pre-smolt) (FAO, 2014). Parr are graded, vaccinated against diseases and their health and growth are monitored. The parr develop into smolt. Temperature manipulation and/ or the use of artificial light may speed up the process (Stevenson, 2007).

The seawater (SW) phase starts by transferring the smolts into net pens or floating sea cages after the fish have passed through smoltification or parr–smolt transformation; weight is approximately 60-100 gram (Marine Harvest, 2016). Smoltification in this context, is a series of morphological, physiological and behavioral changes that enable the fish to live in SW

(Stefansson, McCormick, Ebbesson, & Björnsson, 2008). Once in sea cages, the greatest growth phase of salmon continues until salmon reach the desired market weight, i.e. in Norway this is 5-6 kg (FAO, 2014).

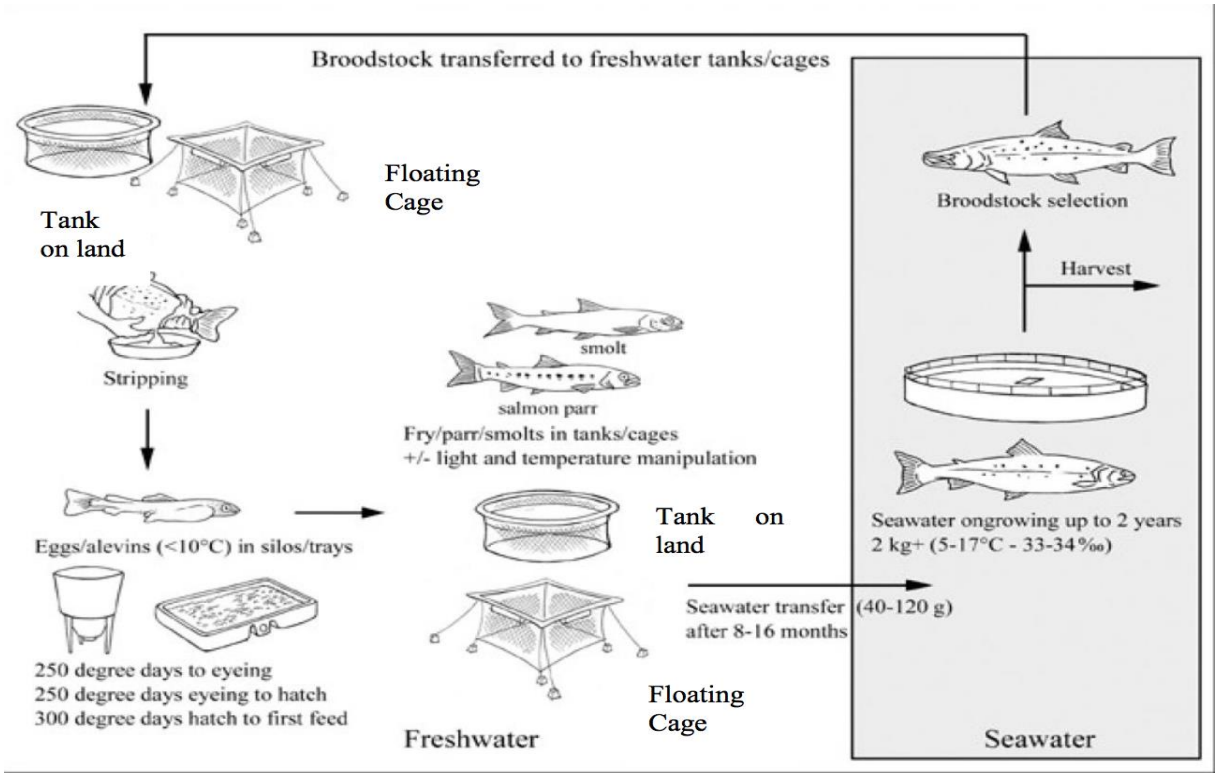

Figure 3: Production cycle for Atlantic salmon including both freshwater and seawater phases (FAO, 2014)

About 300 million smolt are transferred to sea cages annually in Norway. These cages distributed into about 600-1000 coastal sites (Taranger, 2015). Current salmon farming is based on an intensive system. Intensive farming exposes salmon to various stressors. These stressors include poor environmental conditions, confined areas, imbalanced diet distribution and repeated handling and transport (Madaro et al., 2015). These circumstances in turn, favor disease transmission (Kent, 2000; Segner et al., 2012) and have direct or indirect effect on fish immune response and resistance to infectious diseases (Guimarães, Lim, Yildirim-Aksoy, Li, & Klesius, 2014). Mortality and morbidity rates due to infectious diseases are enormous (Robertsen, 2017) and results in huge economic loss. As the SW stage is located in open areas (Robertsen, 2017), viral diseases threaten not only local production but may also impact wild fish populations (Taranger et al., 2014).

1.3 Viral diseases with economic impact on farmed Atlantic salmon

The intense nature of fish farming increases the risk of disease outbreaks including viral diseases (F. S. Kibenge, Godoy, Fast, Workenhe, & Kibenge, 2012). The most important viral diseases in salmon are summarized in Table 1 and the statistics of reported sites with disease outbreaks in Norwegian Atlantic salmon for the years 2004 to 2015 is shown in Figure 4. The main diseases are: infectious salmon anemia (ISA), salmon pancreas disease (PD), infectious pancreatic necrosis (IPN), cardiomyopathy syndrome (CMS) and heart- and skeletal muscle inflammation (HSMI) (Hjeltnes & Walde, 2016). As shown in Table 1, the causative agents for all the aforementioned diseases include are viruses with RNA genomes, with horizontal and vertical route (IPN) of transmission. The diseases are present in SW or in both FW and SW. The extent of disease spread, severity of the disease and economic impact varies for the different viruses (Walker & Winton, 2010).

Table 1: Viral diseases of economic impact on Norwegian farmed salmon

Disease	Causative virus	Virus family	Viral genome	Route of transmission	Present
Infectious pancreatic necrosis (IPN)	IPNV[1]	*Birnaviridae*	dsRNA	Horizontal & vertical	FW & SW
Infectious salmon anaemia (ISA)	ISAV[2]	*Orthomyxoviridae*	ssRNA	Horizontal	FW & SW
Pancreas disease (PD)	SAV[3]	*Togaviridae*	ssRNA	Horizontal	SW*
Cardiomyopathy syndrome (CMS)	PMCV[4]	*Totiviridae*	dsRNA	Horizontal**	SW
Heart and skeletal muscle inflammation (HSMI)	PRV[5]	*Reoviridae*	dsRNA	Horizontal**	FW & SW

1 = Infectious pancreatic necrosis virus; 2 = Infectious salmon anaemia virus; 3 = Salmonid alphavirus; 4 = Piscine myocarditis virus; 5 = Piscine orthroreovirus

*SAV causes disease in fresh water reared rainbow trout in Central Europe.

** The routes of transmission for PMCV and PRV is mostly unknown

ssRNA= single-stranded RNA

dsRNA = double-stranded RNA

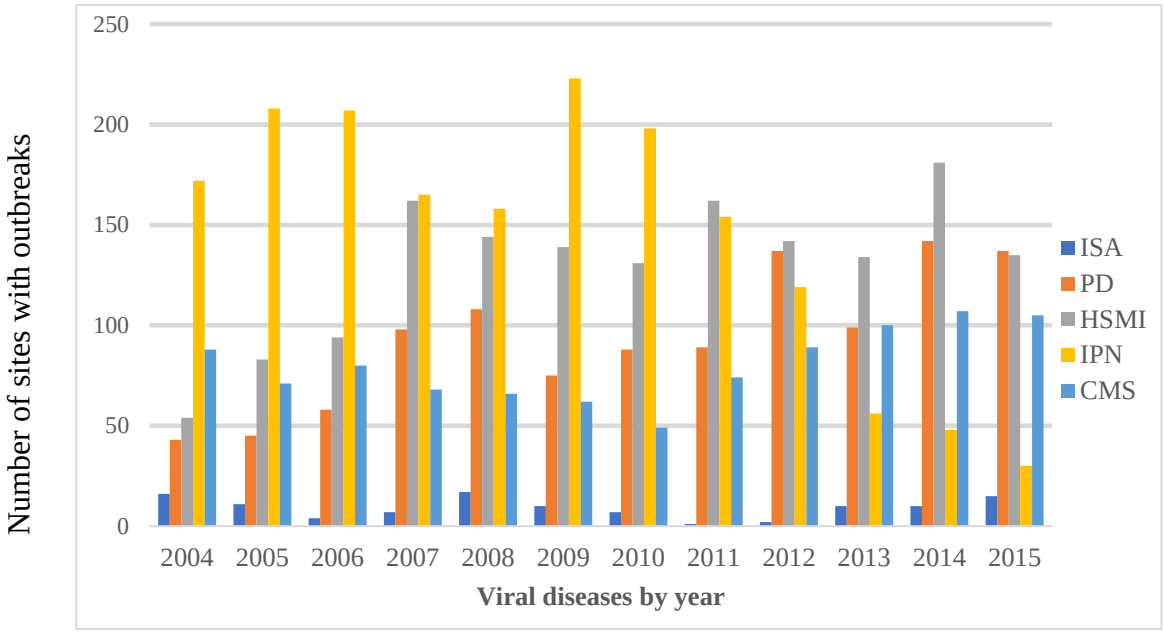

Figure 4: Number of Norwegian salmon farms experiencing outbreaks from different viral diseases from 2004 – 2015 (Hjeltnes & Walde, 2016).

Infectious salmon anaemia **(**ISA) is an disease of marine-farmed Atlantic salmon found in Canada, Chile, Norway, Scotland and Faroe Islands (Godoy et al., 2013). Fish infected by virulent ISAV show anaemia and the virulence varies depending on the virus strain. So far, the worst ISA outbreak in salmon aquaculture occurred in Chile and the Faroe Islands; where the production of stocks was reduced by about 75% and 74% respectively (Asche, Hansen, Tveteras, & Tveterås, 2009). In Norway, ISA was a major disease in the late 1980s (Nylund, Hovland, Hodneland, Nilsen, & Lovik, 1994; Thorud & Djupvik, 1988). However, for the years 2004 to 2015 the highest number of ISA outbreaks (17 sites) was reported in 2008, and then dropped to zero cases in 2011. Afterwards, reported sites with ISA remained low, but in 2015 this number increased to 15 reported sites (Hjeltnes & Walde, 2016).

Pancreas disease **(**PD) was described in farmed Atlantic salmon in Scotland in 1976 (Munro, Ellis, McVicar, McLay, & Needham, 1984). PD is affecting farmed Atlantic salmon in Ireland, Scotland and Norway. While the name suggests that the primary organ damaged is the pancreas, severe cardiac and skeletal myopathies are also key features of this disease. As a result, the infected fish loose appetite and growth rate is reduced. Control is achieved mainly by management and mitigation practices along with vaccination in the areas where PD represents a risk (Harvest, 2014). In Norway, PD affects Atlantic salmon and rainbow trout in

seawater (A. Olsen & Wangel, 1997; Taksdal et al., 2007). The disease is currently among the most important ones in Norway, with 137 reported cases in 2015 (Hjeltnes & Walde, 2016). Currently, there are two separate active epidemics of PD, i.e. SAV subtype 3 (SAV3) and SAV2. While the first causes low to moderate mortality and the epidemic mostly occur in western Norway, the later causes low mortality and the epidemic occurs in mid Norway (Bornø & Lie Linaker, 2015).

The infectious pancreatic necrosis (IPN) virus is prevalent throughout the salmon and trout farms in Europe (Murray, Busby, & Bruno, 2003). The disease can affect Atlantic salmon fry, smolts and larger fish post-transfer (Geoghegan, Cinneide, & Ruane, 2007; Jarp, Taksdal, & Tørud, 1996). In the past, the disease was widely spread in salmon farming industry in Norway. For instance, from 2004 to 2010, IPN caused the highest number of virus outbreak, with about 223 farms affected in 2009. However, this number dropped significantly to about only 30 reported cases in 2015. Selective breeding for genetic resistance is the main reason for the significant reduction of IPN, according to the Norwegian veterinary institute annual report on fish health (Hjeltnes, 2013).

The cardiomyopathy syndrome (CMS) is characterized by severe inflammation and degradation of myocardial tissue (Timmerhaus et al., 2012) and occurs mainly in fish at slaughter weight (Rodger & Turnbull, 2000). In Norway, CMS was diagnosed in in 1985 and then later been identified in Scotland, the Faroe Islands, Denmark and Canada. After PD and HSMI, CMS caused the third highest number of outbreaks in Norwegian salmon farms from 2013 to 2015.

1.4 Heart and skeletal muscle inflammation (HSM1)

1.4.1 History and geographical distributions

Heart and skeletal muscle inflammation (HSMI) is an infectious disease of farmed Atlantic salmon that was initially described in Norway in 1999 (Kongtorp, Kjerstad, Taksdal, Guttvik, & Falk, 2004) but the causative agent remained unknown for many years. A decade later, RNA from infected salmon was sequenced and the findings indicated that a reovirus called Piscine reovirus (PRV), was associated with HSMI (Palacios et al., 2010).

Despite being predominantly reported in fish during the marine phase, HSMI outbreaks in Norway have in recent years also been observed in several hatcheries without seawater intake

(Johansen et al., 2008; Løvoll et al., 2012; Palacios et al., 2010). Moreover, a study has also described a HSMI-like disease with anemia in rainbow trout (*Oncorhynchus mykiss*) in freshwater, which associated with another PRV variant (Hauge et al., 2017; Olsen, Hjortaas, Tengs, Hellberg, & Johansen, 2015). Concerns have also been expressed with regard to the association between PRV and HSMI in wild salmon. Although the virus has been found, HSMI has not yet been detected in the wild populations. This may be because fish raised under farming conditions are less robust against disease development (Garseth, Ekrem, & Biering, 2013).

To date, HSMI has become among the top three important viral diseases in Norwegian salmon farms based on statistics released by the Veterinary Institute in 2016 (Figure 4). Since 2007 for example, over 130 cases have occurred annually. Later, from 2011 to 2014, outbreaks of HSMI were more prevalent than any other viral disease; with a peak of 181 farm cases in 2014 (Hjeltnes & Walde, 2016). Mid- and northern-Norway (Figure 5) are areas where the disease often occurs (Hjeltnes & Walde, 2016).

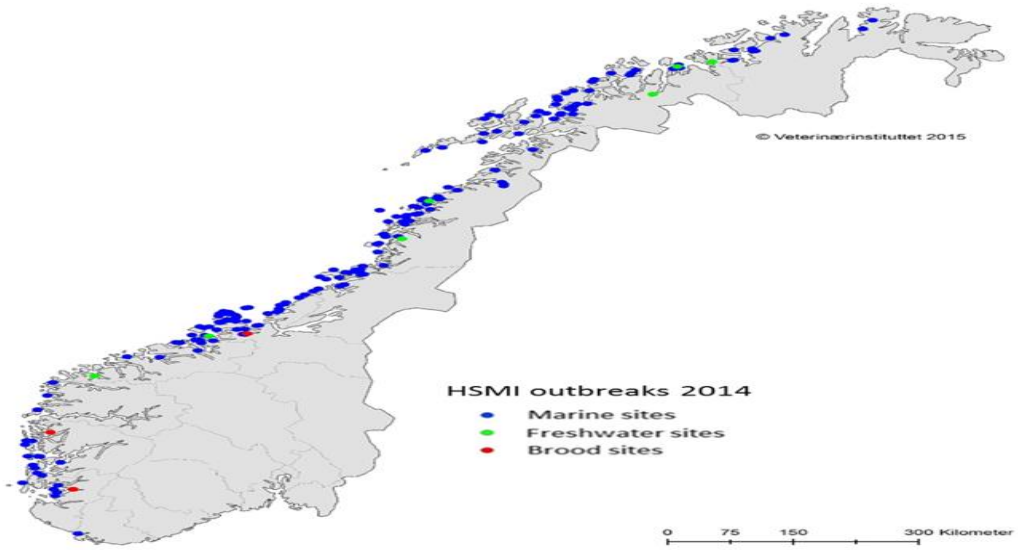

Figure 5: Distribution of HSMI outbreaks in 2014 (Hjeltnes & Walde, 2016)

The rapid spread, coupled with the high morbidity associated with the disease, urged the Norwegian Food Safety Authority (NFSA) to list HSMI as a notifiable disease in 2008. This requires that HSMI diagnosis must be reported to NFSA. In turn, NFSA has the mandate to impose restrictions on farms experiencing disease outbreaks (Kristoffersen, Jensen, & Jansen, 2013). In mid-2014, HSMI was taken off the list and is no longer a notifiable disease (Hjeltnes & Walde, 2016). This may affect the number of registered outbreaks of HSMI in 2015 and thereafter.

An outbreak with similar symptoms and high mortality rates was reported in Scotland 2004, as the first possible incident outside Norway (Ferguson, Kongtorp, Taksdal, Graham, & Falk, 2005). In addition to HSMI diagnosed in Norway and Scotland, the first farm-level diagnosis of HSMI in British Colombia (BC) was recently published (Di Cicco et al., 2017). This study showed inflammatory lesions in heart and skeletal muscle tissue typical of HSMI in a longitudinal study from one Atlantic salmon farm in BC. In Chile, HSMI-like lesions has been described in both Atlantic salmon and Coho salmon (Godoy et al., 2016).

1.4.2 Piscine orthoreovirus (PRV)

PRV is a non-enveloped virus in the genus *Orthoreovirus* of the family *Reoviridae* (Markussen et al., 2013; Palacios et al., 2010). The genome of PRV contains ten segments of dsRNA (Finstad, Falk, Løvoll, Evensen, & Rimstad, 2012; Takano et al., 2016). Like other members of the *Reoviridae*, orthoreovirus particles have a genome enclosed within a double protein capsid. The outer capsid (i.e. σ1, σ3 and μ1 proteins) plays an important role in cell entry (Danthi et al., 2010) and the inner capsid (figure 6) contains enzymes needed to launch virus replication upon cell penetration (Danthi, Holm, Stehle, & Dermody, 2013).

Based on dsRNA size, reovirus segments are divided into three large (L1, L2, L3), three medium (M1, M2, M3) and four small segments (S1, S2, S3, S4). The viral proteins associated with the segments L, M and S are termed with λ (lambda), μ (mu) and σ (sigma) respectively (Day, 2009; Netherton, Moffat, Brooks, & Wileman, 2007).

The ten genome segments contain the coding sequences for the virus proteins (Table 2), eight of which are structural (λ1, λ2, λ3, μ1, μ2, σ1, σ2 and σ3) and present within the virus particle, while two are nonstructural proteins (μNS and σNS). An additional open reading frame (ORF) encoding a 13 kDa protein (homologue of PRV p13) occurs in the same genomic segment as σ3 (Markussen et al., 2013).

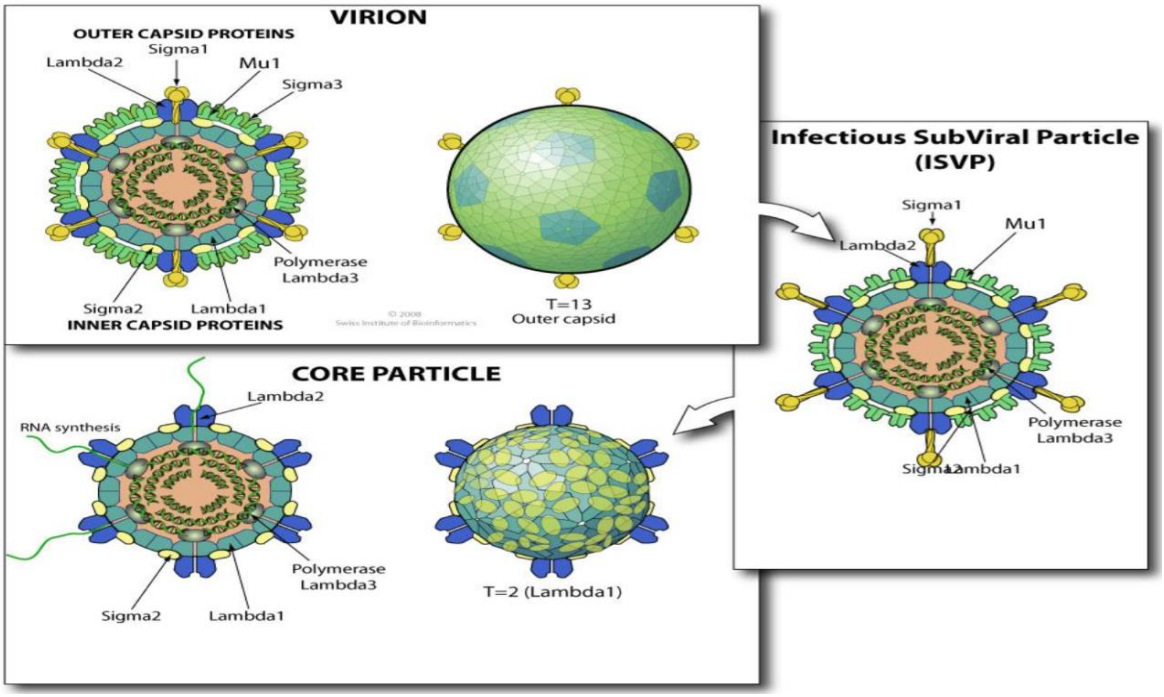

Figure 6: Mammalian reovirus capsid shell with two protein layers and infectious sub-viral particles (http://viralzone.expasy.org/all_by_species/105.html)

Several viruses belonging to the family *Reoviridae* that infect aquatic animals are classified within the genus *Aquareovirus* (AqRV) (Attoui et al., 2002). Previous study of the genome sequences of aquareoviruses C (grass carp reovirus) suggested that they have a common evolutionary origin with genus *Orthoreovirus*, which includes mammalian orthoreoviruses (MRV) and avian orthoreoviruses (ARV) (Attoui et al., 2002; Fang et al., 2000; Kim, Tao, Reinisch, Harrison, & Nibert, 2004). Extensive studies on MRV has provided detailed understanding of replication cycle, protein functions and virion structure which could be used as a model system for investigation of virus-host interactions for PRV (Schiff, Nibert, & Tyler, 2007). Although nucleotide and protein sequence identities between MRV and PRV are low, important structural motifs and key amino acid are conserved in their homologous protein (Markussen et al., 2013). Accordingly, the assumed function and location of PRV proteins have been assigned as depicted in Table 2.

Table 2: PRV-encoded proteins and their putative locations and functions based on the properties of homologous proteins of MRV, ARV and AqRV (Markussen et al., 2013; Takano et al., 2016)

Segment	Protein name	Length (aa)	Theoretical weight (KDa)	Predicted location in virion and functional properties
L1	λ3	1286	144.2	RNA-dependent RNA polymerase
L2	λ2	1290	143.7	Guanylytransferase, methyltransferase
L3	λ1	1282	141.5	Helicase, NTPase, RNA triphosphatase
M1	μ2	760	86.0	NTPase, RNA triphosphatase, RNA binding
M2	μ1	687	74.2	Outer capsid protein: membrane penetration
M3	μNS	752	83.5	Non-structural protein (inclusion formation for replication and assembly)
S1	σ3 p13	330 124	37.0 13.0	outer capsid: zinc metalloprotein p13: cytotoxic, integral membrane protein
S2	σ2	420	45.9	σ2: inner capsid protein, RNA binding
S3	σNS	354	39.1	Non-structural protein (inclusion formation for replication and assembly)
S4	σ1	315	34.6	Cell attachment protein

Initially, PRV was described as being associated with HSMI (Palacios et al., 2010). Through studies on purified PRV from infected blood cells, Wessel and co-authors have proved that PRV is the causative agent of HSMI (Wessel et al., 2017). PRV is ubiquitous amongst Norwegian SW salmon farms even in the absence of clinical disease (Finstad et al., 2012; Løvoll et al., 2012). Although the viral load is significantly elevated during an outbreak (Løvoll et al., 2012; Palacios et al., 2010; Wessel, Olsen, Rimstad, & Dahle, 2015), the virus can be detected at low levels in fish throughout the production cycle (Løvoll et al., 2012; Wiik-Nielsen et al., 2012). During PRV infection, the virus can be detected in heart, skeletal muscle, spleen, head kidney (Finstad et al., 2014; Løvoll et al., 2010) and blood (Finstad et al., 2014; Finstad et al., 2012), i.e. all organs.

For the years 2008 to 2011, a study following Norwegian broodstock and progeny revealed that fertilized eggs were negative for PRV RNA. Therefore, the study concluded that vertical transfer (from the parents to offspring) is not a major route of PRV spread, (Wiik-Nielsen, Ski, Aunsmo, & Løvoll, 2012). Several studies suggest that horizontal transmission (infected to uninfected in the same tank) is a common route of PRV spread (Kongtorp & Taksdal, 2009; Kongtorp, Taksdal, & Lyngøy, 2004).

In Ireland, PRV presence has been detected by PCR at some marine salmon sites, but without an HSMI diagnosis (Rodger, McCleary, & Ruane, 2014). In North-America, PRV has been detected from hatchery Chinook and Coho salmon in Washington State, and in wild Coho salmon from Alaska (M. J. Kibenge et al., 2013; Marty, Morrison, Bidulka, Joseph, & Siah, 2015). In Canada (British Columbia, PRV was found in farmed Atlantic salmon and wild Chinook and Coho salmon.

Most recently, a variant of PRV (PRV-2) was found to be the etiological agent of erythrocytic inclusion body syndrome (EIBS) in farmed Coho salmon in Japan (Takano et al., 2016). Further, a variant similar to the Norwegian strains of PRV from rainbow trout was reported in Chile (Godoy et al., 2016).

1.4.3 Pathology and diagnostic methods of HSMI

HSMI is a disease in farm-raised Atlantic salmon (Kongtorp, Taksdal, et al., 2004). The disease often manifests 5 to 9 months after transfer of smolts from fresh water to seawater but it also has been reported occasionally in freshwater hatcheries (Bornø & Lie, 2015; Johansen et al., 2016; Kongtorp & Taksdal, 2009; Kongtorp, Taksdal, et al., 2004). HSMI in sea cages is often characterized by abnormal swimming behavior and anorexia (Kongtorp, Taksdal, et al., 2004). Distinguishing features of an affected fish include signs of circulatory disturbance with pale heart, fluid accumulation in the abdomen, yellow liver and swollen spleen (Kongtorp, Taksdal, et al., 2004; No, 2012). During infection in sea cages, morbidity can reach up to 100% whereas mortality rate ranges from 0 to 20% (Kongtorp, Kjerstad, et al., 2004). Fish with histopathological changes of HSMI may also appear healthy with no obvious clinical signs (Kongtorp, Halse, Taksdal, & Falk, 2006) while internal gross changes are typical of circulatory disturbance (Mikalsen, Haugland, Rode, Solbakk, & Evensen, 2012).

The most significant histopathological lesions of HSMI are found in heart and red skeletal muscle (Figure 7) and characterized by epi, endo and myocardial inflammation, myositis and necrosis in red skeletal muscle (Finstad et al., 2012; Kongtorp, Taksdal, et al., 2004). Lesions in the skeletal muscle tend to take place mainly during the peak of the outbreak, but with less occurrence in the recovery phase (Kongtorp et al., 2006). Histopathological lesions in other organs include liver necrosis and congestion / hemorrhages in liver, kidney, spleen and gills (Kongtorp, Kjerstad, et al., 2004). So far, the most common methods of PRV detection are real time RT-PCR (Løvoll et al., 2010; Palacios et al., 2010), histology and immunohistochemistry (Finstad et al., 2012).

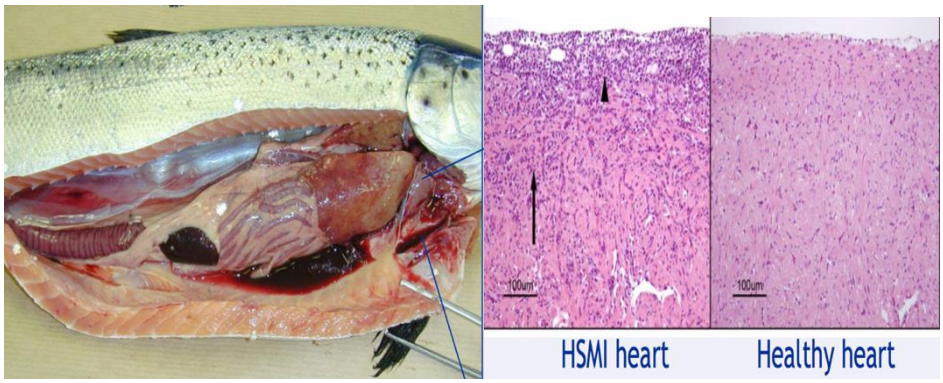

Figure 7: To the left gross pathology with yellow liver, ascites, enlarged and dark spleen, pale heart. To the right infiltration of inflammatory cells is present in the HSMI heart, but not in the healthy heart (Finstad, Dahle, et al., 2014)

1.4.3.1 Erythrocyte infection

The principal function of the erythrocytes in fish is associated with gas exchange and transport (O_2 and CO_2) (Heming, 1984). Other potential functions include, but not limited to, regulating blood flow distribution in skeletal muscle, calcium homeostasis, cell proliferation and antiviral response (Morera et al., 2011). In 1999, Bishlawy and co-authors assumed a relationship between erythrocytes, hemoglobin and the immune system suggesting an active role of erythrocytes in the immune response to pathogens (Bishlawy, 1999).

As for viruses, Workenhe and co-authors were the first to reveal that erythrocytes from Atlantic salmon could play an immunological role in response to virus infection, when they showed IFN-α in response to ISAV (Workenhe et al., 2008). Later, erythrocytes were demonstrated to be an important target cells for the PRV (Finstad, Dahle, et al., 2014).

Most non-mammalian erythrocytes are nucleated and contain the transcriptional and translational machineries to enable virus replication. During the peak phase of PRV infection, more than 50% of all erythrocytes can be infected (Finstad, Dahle, et al., 2014). Consequently, the infection can activate an innate antiviral immune response typical for RNA viruses in the infected erythrocytes (Dahle et al., 2015). PRV RNA and protein has been detected in erythrocytes *in vivo* and *ex vivo* (Finstad, Dahle, et al., 2014; Wessel, Olsen, Rimstad, & Dahle, 2015). It is noteworthy that electron microscopic images of the cytoplasmic inclusions in PRV-infected Atlantic salmon erythrocytes strongly resemble those reported in coho salmon erythrocytes during erythrocytic inclusion body syndrome (EIBS) (Takano et al., 2016).

1.5 Innate immune and virus invasion

1.5.1 Reovirus replication cycle

Viruses are obligate intracellular parasites, i.e. cannot grow or reproduce by themselves (Gelderblom, 1996). Viruses have developed efficient mechanisms for transporting their genetic materials between the host cells that they depend on for replication. Reoviruses and other dsRNA viruses replicate in the cytoplasm of the host cells (Ooms, Jerome, Dermody, & Chappell, 2012), and the complete multiplication cycle is illustrated in figure 8. This cycle can be divided into an early and late phase, separated at the onset of progeny dsRNA formation (Joklik, 1980).

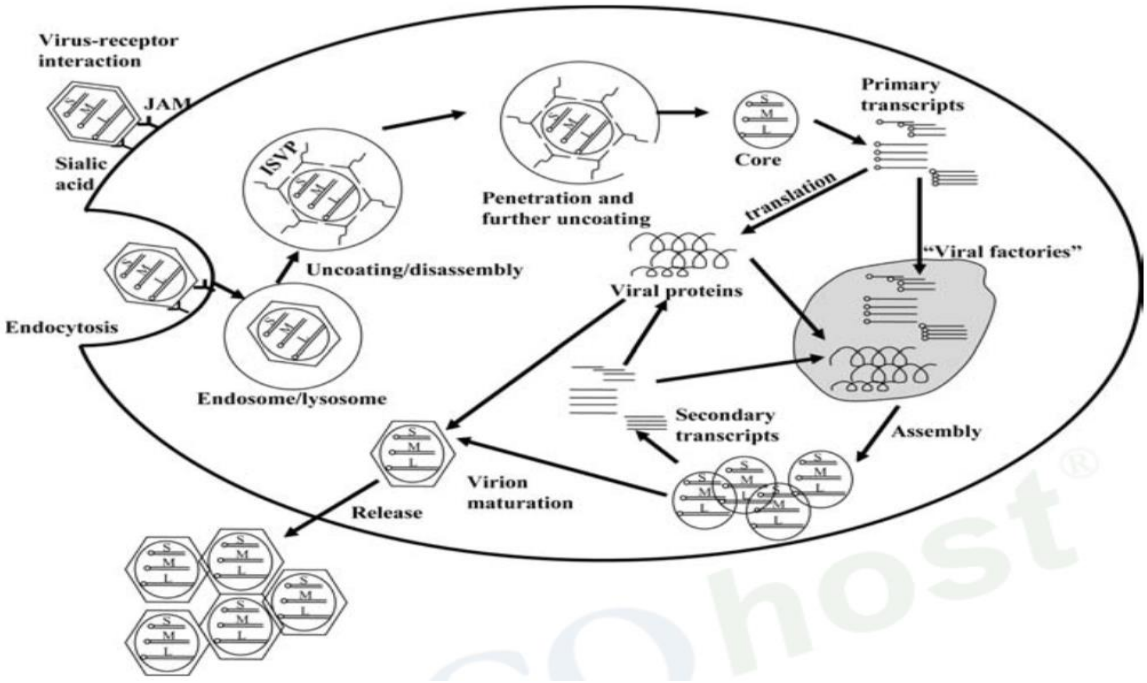

Figure 8: Overview of the reovirus replication cycle (Pan, Marcato, Shmulevitz, & Lee, 2009)

Initially, orthoreoviruses, here represented by the well-studied mammalian orthoreovirus (MRV), attach to the cell surface via the viral σ1 protein (Lee, Hayes, & Joklik, 1981; Weiner, Ault, & Fields, 1980). Attachment of σ1 protein is mediated by two receptors on the cell surface, sialic acid containing carbohydrate and junctional adhesion molecules (JAM) (Barton et al., 2001; Danthi et al., 2013). Carbohydrate binding is believed to attach the virus

to the cell surface prior to high-affinity binding to JAM (i.e. immunoglobulin) (Barton et al., 2003).

Upon binding to the cell membrane, orthoreoviruses penetrate host cells either through receptor-mediated endocytosis, or directly from the plasma membrane, bypassing the endocytic machinery (Boulant, Stanifer, & Lozach, 2015). Beta-integrin takes part in facilitating internalization following attachment, perhaps through interaction with λ3 protein (Maginnis et al., 2006). Once in an acidified endosome (low pH) with lysosome activity, orthoreovirus disassemble (uncoate) the outer capsid shell by the proteolytic activity of cathepsins (Sahin, Egger, McMasters, & Zhou, 2013), generating an infectious subviral particles (ISVPs) (Borsa, Copps, Sargent, Long, & Chapman, 1973; Kirkham et al., 2005).

Following entry, σ1 proteins are released from the particles and transcriptionally active virus cores are formed. Orthoreovirus mRNA (i.e. plus strand RNA) are transcribed in the cores by reovirus RNA – dependent RNA polymerase (λ3) independently of host cell machinery and released into the cytoplasm (primary transcription) (Pan et al., 2009).

Orthoreovirus mRNAs are translated into proteins using the host protein translation machinery. Concordantly, newly produced viral proteins form structures called "viral factories" (Sharpe, Chen, & Fields, 1982) where new core particles will assemble. Non-structural proteins (μNS and σNs), and the structural protein (μ2) form the framework for the viral factories for MRV and are crucial for recruiting viral transcripts and proteins; initiating assembly of virus particles (Kobayashi, Chappell, Danthi, & Dermody, 2006). Viral factories are also demonstrated to be formed by μNS in PRV-infected cells (Haatveit et al., 2016; Haatveit et al., 2017).

Following progeny virus cores formation, secondary transcription begins. Minus strand RNA is synthesized within the assembled cores, using the plus strand RNAs templates. At late phase, outer capsid proteins are added to the cores, and completing the assembly of virus particles and virus release (Oberhaus, Smith, Clayton, Dermody, & Tyler, 1997). The virions of MRV are released by cell lysis after they have self-assembled and have accumulated in the inclusion bodies in the cell's cytoplasm. Such inclusions are also observed in PRV-infected erythrocytes, but no lysis has been observed (Finstad, Storset, et al., 2014; Wessel et al., 2015).

1.5.2 Antiviral immune responses

Pathogen recognition receptors (PRRs)

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) through germline encoded pattern recognition receptors (PRRs) (Akira, Uematsu, & Takeuchi, 2006). Some examples of PAMPs include bacterial lipopolysaccharide and flagellin and viral nucleic acids of both single and double-stranded RNA and DNA (Akira et al., 2006).

Several proteins have been identified to function as PRRs. The main receptors for viruses (e.g. dsRNA and ssRNA) detection are mostly members of Toll-like receptors (TLRs) in the cellular and endosomal membranes and retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) in the cytoplasm as depicted in figure 9 (Aoki, Hikima, Hwang, & Jung, 2013; McCartney & Colonna, 2009). The activation of TLRs and RLRs trigger the production of pro-inflammatory cytokines and interferons (IFNs) as well as activation of cells involved in inflammation and the induction of adaptive immunity.

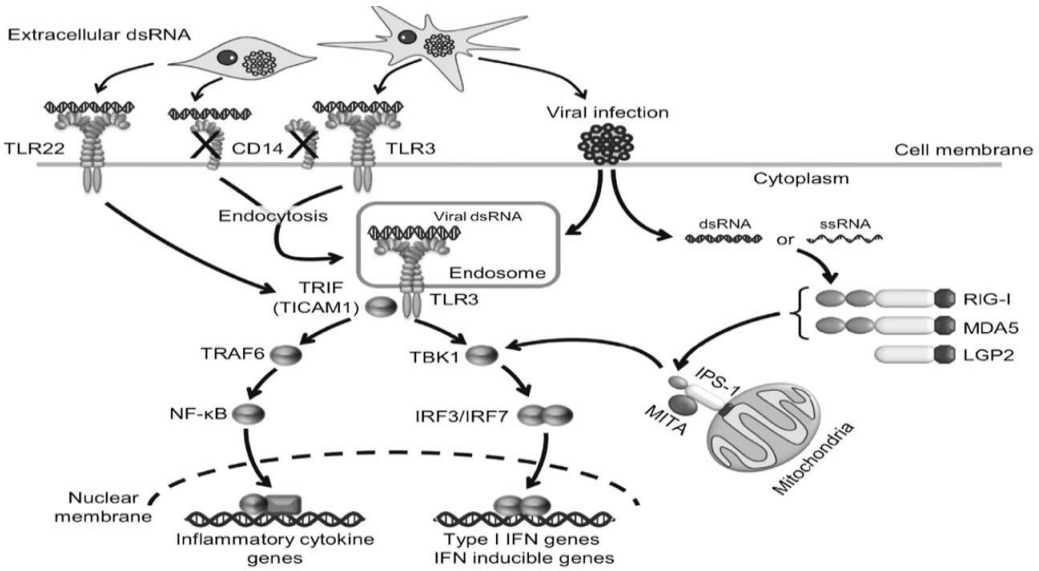

Figure 9: Toll-like receptor (TLR) and retinoic acid – inducible gene I (RIG-I) –like receptor (RLR) mediated viral dsRNA recognition in teleost (Aoki et al., 2013).

Toll-like receptors (TLRs)

In vitro and *in vivo* studies have demonstrated that TLRs recognize various PAMPs by the extracellular domain characterized by leucine-rich repeats (LRRs) and signal through the cytoplasmic domain which is homologous to that of the interleukin 1 (IL-1) receptor (TIR) domain (Akira et al., 2006; Palti, 2011; Rebl, Goldammer, & Seyfert, 2010). Today, 10 (1-10) TLRs have been identified in humans and each TLR has a specific set of ligands that it can sense (Kawai & Akira, 2007). Of these, TLR3, TLR7, TLR 8 and TLR 9 are located in the endosomal compartment and involved in detection of viral RNA or DNA (Kumar, Kawai, & Akira, 2011) as shown in Table 3. Unlike most TLRs which require myeloid differentiation primary response gene 88 (MyD88) for downstream signaling (transmitting signals from TLR and 1L – 1R), TLR3 signaling occurs via TIR – domain containing adapter – inducing IFN-β (TRIF) – dependent pathways (Akira & Takeda, 2004).

Table 3: PAMPs diversity and receptors involved in their recognition (Crozat & Beutler, 2004; Matsuo et al., 2008)

Nucleic acid	PAMP	Pathogen	PRR	Cytosolic	Signal mediators
RNA	ssRNA	Viruses	TLRs 7/ 8	NOD2	MyD88
	dsRNA /poly I: C	Viruses	TLR3	RIG-I/MDA5	TRIF
	dsRNA/ poly I: C	Viruses	TLR22	RIG-I/MDA5	TRIF

In fish, TLR3 is located in the endosomes and has been revealed to recognize viral dsRNA (Samanta, Basu, Swain, Panda, & Jayasankar, 2013). The dsRNA poly I:C has been found to stimulate innate antiviral responses of fish, both *in vitro* and *in vivo* (DeWitte-Orr, Leong, & Bols, 2007). Poly I:C acts as a dsRNA viral mimic (Ichinohe et al., 2005) for the study of the immune response to dsRNA viruses (Huang et al., 2006). A study has revealed that fish injected with poly I:C induced an interferon response (Robertsen, 2006) which in turn provided protection against subsequent viral infection (Fernandez-Trujillo et al., 2008). TLR3 transcripts have a broad tissue distribution in fish. For example, in Atlantic salmon, TLR3 is found expressed in the head kidney, liver, heart, gill, muscle, gut, and spleen (Arnemo, Kavaliauskis, & Gjøen, 2014), and in erythrocytes (Dahle et al., 2015; Morera et al., 2011).

Likewise, for the mammalian TLR7 and TLR8 which recognize viral ssRNA the corresponding genes have also been identified and characterized in salmonids (Palti et al., 2010).

In addition to TLR3, teleost fish express TLR22. This receptor (TLR22) is located in the plasma membrane and can also recognize the synthetic dsRNA poly I: C (Matsuo et al., 2008). Although both TLR3 and TLR22 use the adapter protein TRIF/TICAM-1, their pathways function independently (Matsuo et al., 2008).

RIG – I - like receptors (RLRs)

The RLRs constitute a family of three cytoplasmic RNA helicases (figure 9), such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Aoki et al., 2013) that sense PAMPs (Y.-B. Zhang & Gui, 2012). RIG-I and MDA5 sense dsRNA viruses as well as bind to poly I: C. In addition, they both contain caspase recruitment domain (CARD) that is important for activating downstream signal pathways leading to induced production of type I IFNs in infected cells.

LGP2 contains a RNA binding domain but lack functional CARD and therefore is not considered to be involved in downstream signaling (O. Takeuchi & Akira, 2008; Yoneyama & Fujita, 2007). When dsRNA binds to the repressor domain of RIG-I the CARD domain is exposed allowing interactions with the adaptor molecule, interferon promoter stimulator (IPS-1), which when activated initiates IFN-mediated signaling pathways via IRF3/7 (Jensen & Thomsen, 2012)

MDA5 is another member of the RLR family, which contain the same domain structure as in RIG-I. However, MDA5 differs from RIG-I in that it prefers binding to longer dsRNA, whereas the latter favors binding shorter dsRNA (Jensen & Thomsen, 2012). RIG-I-like proteins have been found in carp and salmon (Biacchesi et al., 2009). MDA5 has been cloned from rainbow trout (Chang et al., 2011). Activated MDA5 induces IFN and ISG transcript expression via the IPS-1, IRF3/7 pathway, which is also relevant for RIG-I (Holland et al., 2008; Loo et al., 2008).

The interferon system

Interferons (IFNs) are autocrine and paracrine cytokines (secreted proteins) that are produced by host cells upon virus infection. Following their secretion, IFNs induce antiviral proteins that inhibit both viral replication in infected cells (Robertsen, 2006; Robertsen, Bergan, Røkenes, Larsen, & Albuquerque, 2003; Samuel, 2001; Zou & Secombes, 2011) and viral

spreading to non-infected cells (Fensterl & Sen, 2009). Based on receptor specificity, protein structure and functional properties, three families of IFNs can be distinguished in mammals including type I (IFN-I), type II (IFN-II) and type III (IFN-III) (Robertsen, 2006; Sadler & Williams, 2008).

The mammalian type I IFNs constitute a multigene family with IFN-α and IFN-β as the predominant members, which function mainly in the antiviral response (Svingerud et al., 2012; Takaoka & Yanai, 2006). Type II (IFN-II) is represented by the single member IFN-γ, that binds a single IFN-γ receptor with two chains, IFNGR1 and IFNGR2 (Young & Bream, 2007). Type III IFN-λ is composed of three members (λ1, λ2, λ3) (Robertsen, 2006; Sadler & Williams, 2008; Sen, 2001) and signal through the IFN-λ receptor (IFN-λR) which is made up of two chains: IL28Rα, a unique subunit, and IL10Rβ, shared with cytokines of the IL10 family (Gad et al., 2009). Both type I IFNs and type III IFNs have antiviral properties and they play a vital role in the innate immune response against viruses.

In contrast, type II IFN-γ is a major product of T-cells and natural killer cells (NK), with key roles in both innate and adaptive immunity against intracellular pathogens (Kotenko et al., 2003; Zou & Secombes, 2016), notably bacteria and parasite (Teles et al., 2013). Apart from stimulating innate antiviral immune responses, IFNs also stimulate adaptive immune responses, and play a role in the cross-talk between innate and adaptive immunity.

So far, only IFN-I and IFN –II have been identified in fish (Robertsen, 2006; Zou & Secombes, 2016). In salmonids, type I IFNs consist of at least six different subtypes (IFNa, IFNb, IFNc, IFNd, IFNe and IFNf) (Robertsen, 2017). Type I IFNs in fish have often been compared to type I mammalian IFNs (Robertsen, 2008). Unlike in mammals, the presence of introns is characteristic of fish IFN, with both Atlantic salmon and Zebrafish having gene structure of four introns and five exons (Lutfalla et al., 2003). Moreover, based on the number of disulfide bridges formed by cysteine residues, IFNα and IFN-β in mammals contain four cysteines (4C - IFNs) and two cysteines (2C - IFNs), respectively. In fish, IFNa, IFNd and IFNe comprise of two cysteines (2C - IFNs) whereas IFNb, IFNc and IFNf contain four cysteine (4C – IFNs) (Hamming, Lutfalla, Levraud, & Hartmann, 2011; Sun, Robertsen, Wang, & Liu, 2009; Zou, Tafalla, Truckle, & Secombes, 2007). In this context, antiviral functions have been demonstrated only for the IFNa, IFNb, IFNc subtypes (Svingerud et al., 2012).

In mammals, the antiviral effect is exerted when type I IFNs bind to IFN-α/IFN-β receptors (IFNAR1 and IFNAR2) (Ivashkiv & Donlin, 2014; Pestka, Krause, & Walter, 2004) on the surface of the same cells or adjacent ones; causing dimerization of the receptor. This receptor association triggers the immune response through the Janus kinase-signal transduction and activator of transcription (JAK-STAT) signaling pathway (Aaronson & Horvath, 2002; O'Shea, Gadina, & Schreiber, 2002) through activating Janus kinases, Jak1 and tyrosine kinase 2 (Tyk2). These tyrosine-kinases phosphorylate STAT1 and STAT2 (Fensterl & Sen, 2009). The activated STAT heterodimers associate with interferon regulatory factor 9 (IRF-9), forming IFN-stimulated gene factor 3 (ISGF3) complex (Fensterl & Sen, 2009; González-Navajas, Lee, David, & Raz, 2012). ISGF3 translocate into the nucleus, bind to the IFN-sensitive response element (ISRE) which in turn activates the interferon stimulated genes (ISGs) (Fensterl & Sen, 2009).

Although little is yet known about the IFN-signaling system of fish, studies have revealed that the signaling pathway (JAK/STAT) functions similarly to the one observed in mammals (Robertsen, 2006). In this regard, Jak1 and Tyk2 have been identified in pufferfish (*T. fluviatilis*) (Leu et al., 2000), and STAT 1 has been cloned from zebrafish and crucian carp (Y. Zhang & Gui, 2004). Additionally, STAT1 is upregulated in rainbow trout following infectious hematopoietic necrosis virus (IHNV) infection (Hansen & La Patra, 2002), and in carp following poly I: C and grass carp haemorrhagic virus (GCHV) infection (Zhang & Gui, 2004).

The interferon stimulated genes (ISGs)

As a consequence of the IFN signaling through the JAK/STAT pathway, induction of IFN-stimulated genes (ISGs) are stimulated (figure 10). In this regard, ISG encoded proteins are diverse group of proteins that inhibit virus infection and replication (Zou & Secombes, 2011). Some proteins upregulated during the innate antiviral immune responses in teleost fish are: virus inhibitory protein, endoplasmic reticulum – associated, interferon-inducible (Viperin), ISG15, dsRNA-dependent protein kinase (PKR) and Myxovirus resistance protein (Mx).

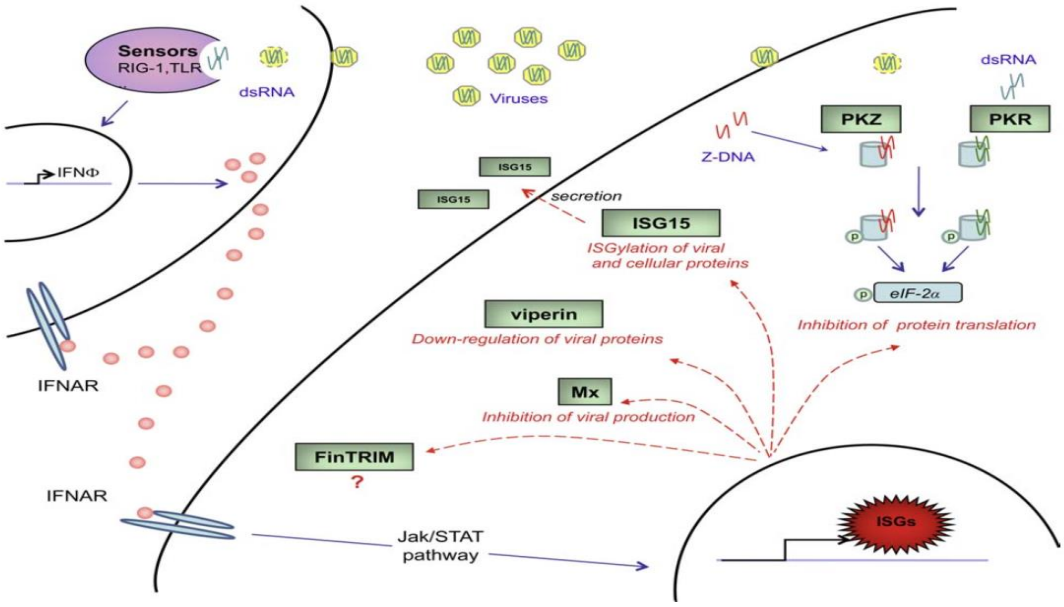

Figure 10: Fish virus induced genes, such as ISG15, PKR, PKZ, Mx, vig1/viperin or finTRIMs.in the context of IFN-signaling (Verrier, Langevin, Benmansour, & Boudinot, 2011)

Interferon stimulated gene 15 kDa (ISG 15)

ISG15 is a ubiquitin-like protein that contain two tandem repeats of ubiquitin-like domains. In mammals, these proteins can conjugate to either cellular or viral target proteins through an ISGylation pathway (Verrier et al., 2011). In teleosts, ISG15 gene transcription has been studied in different species, including Atlantic salmon. In Atlantic salmon, ISG15 was found to be highly expressed after treatment with poly I: C or dsRNA as well as after ISAV infection (Verrier et al., 2011).

PKR

The protein kinase PKR is activated by binding to viral dsRNA. Upon activation, PKR phosphorylates eukaryotic initiation factor 2 (eIF2α), thereby inhibit cellular protein synthesis (Poppers, Mulvey, Khoo, & Mohr, 2000; Sadler & Williams, 2008). PKR is transcribed constitutionally in all tissues, and its expression is upregulated by type I and III IFNs (Ank et al., 2006). PKR-like genes have been characterized in fish, and PKR activity, particularly eIF2α phosphorylation, has been demonstrated in rainbow trout (Garner, Joshi, & Jagus, 2003).

Viperin

Viperin was first discovered in rainbow trout as a VHSV-induced gene 1 (vig-1) (Boudinot, Massin, Blanco, Riffault, & Benmansour, 1999). Recently, viperin has received increasing attention due to its ability to limit spread of viruses as well as its role in modulating innate immune signaling (Helbig & Beard, 2014). Viperin is expressed in most cell types at low basal levels and has been demonstrated to be induced by type I IFN, type II IFN, type III IFN as well as dsDNA and dsRNA viruses (Helbig & Beard, 2014). Its induction through IFN pathways has been demonstrated via stimulation with poly I: C and multiple viruses (Boudinot et al., 2000; Chan, Chang, Liao, & Lin, 2008; Seo, Yaneva, Hinson, & Cresswell, 2011). In Atlantic salmon, transcription data from microarray have confirmed the induction of viperin during macrophages infection with ISAV (Workenhe, Hori, Rise, Kibenge, & Kibenge, 2009) and red blood cells infection with PRV (Dahle et al., 2015; Haatveit et al., 2017).

1.6 Effect of feed on the antiviral immune response

1.6.1 Antiviral and anti-inflammatory effects of feed

To minimize the risk of diseases under rearing conditions, adequate nutrition throughout the production cycle will strengthen the immune system against infections and reduce mortality also during a period when feed intake is limited (Oliva-Teles, 2012; R Waagbø, 1994). In addition to health benefits, adequate availability of micronutrients and their interactions with other dietary nutrients provide palatable and high-quality feed. This in turn, reduces feed wastage (undigested feed) and speed up fish growth. In contrast, accumulated feed waste in the rearing environment can cause eutrophic conditions.

Traditionally, fish nutrition research studies often evaluate how nutrients maximize animal growth, but generally overlook the role of nutrients in protection against disease (Lee, 2015). In farmed fish, dietary nutrients, particularly of marine origins (Craig & Helfrich, 2002), along with vitamins and minerals enhance the immune system function and reduce the susceptibility to disease-causing agents (Lim, Yildirim-Aksoy, & Klesius, 2008). In other words, proper feeding regimes and balanced formulated diets are beneficial for the fish immune system, and hence holds promises to mitigate disease consequences (Pohlenz & Gatlin, 2014).

Nutritional effects on the immune system is a relatively new research area in fish (Waagbø, 1994) and studies on how the fat-soluble vitamins modulate the immune response in fish are few. Nevertheless, considerable research has been undertaken on mechanisms of

vitamin A and D mediated regulation of immune function in mammalian (e.g. human and mice) immune cells. Vitamin A and D deficiency is associated with increased susceptibility to intracellular infections (e.g. viral, mycobacterial, many other bacteria, several protozoan, etc.), and inflammation (Brouwer-Brolsma et al., 2013; Pludowski et al., 2013).

For optimum growth and robust immune system against infectious diseases, Atlantic salmon reared in high density require a balanced diet, which composed of: proteins, lipids, vitamins and minerals as well as a starch (carbohydrate) that binds all the ingredients together in the extrusion manufacture to form feed pellets (Craig & Helfrich, 2009; FAO, 2017).

Fishmeal (FM) and fish oil (FO) obtained from wild caught small bony and oily-fish (Miles & Chapman, 2006) dominated feed formulation for Atlantic salmon (Sørensen et al., 2011; Tacchi et al., 2012), i.e. to mimic their natural feeding habits (Król et al., 2016). FM and FO were used extensively as the major protein and lipid (essential fatty acids) sources respectively because of their excellent nutritional properties (Miles & Chapman, 2006). Further, FO extracted from FM contents fat-soluble vitamins (Craig & Helfrich, 2009; FAO, 2017) and supply about twice the energy compared to proteins and carbohydrates (Craig & Helfrich, 2009).

With reference to energy provision, dietary carbohydrates (e.g. starch) are the most economical and cheap sources (Craig & Helfrich, 2009; Hertrampf & Piedad-Pascual, 2012), yet their utilization among fish species varies (FAO, 2017; NCR, 1993). In tilapia for instance, improved growth rate was observed as they fed on diets with 10- 40% inclusion level of starch (Anderson, Jackson, Matty, & Capper, 1984). Unlike tilapia, Atlantic salmon has low enzyme activity to digest and metabolize complex carbohydrates (HEMRE, Mommsen, & Krogdahl, 2002). This calls for low inclusion rate of carbohydrates (Refstie & Storebakken, 2001), which corresponds to a range of 8 - 11% in Norwegian salmon diet (Ytrestøyl et al., 2015). With low inclusion level of carbohydrates, Atlantic salmon showed improvement in both protein utilization and retention (G. I. Hemre, Sandnes, Lie, Torrissen, & Waagbø, 1995).

Paradoxically, carbohydrates that exceed the metabolic capacity of the fish causes detrimental effect to the natural microbial population in the gut (Sobhana, 2002). In addition, excessive carbohydrate in a diet might result in long-term metabolic disturbances. This is in turn, lower fish ability to resist stress, suppress the immune function and thus Atlantic salmon become susceptible to infectious disease (Lee, 2015).

With the decline of wild fish stock used in salmon feed together with unpredictable supply and market price (Olsvik, Torstensen, HEMRE, Sanden, & Waagbø, 2011), the need for FM and FO in aquaculture raise both environmental and sustainability challenges (Refstie & Storebakken, 2001; Ytrestøyl et al., 2015). Therefore, considerable research efforts have been directed towards using plant ingredients as potential protein and oil substitutes (NRC, 2011) without compromising growth and health of farm fish (Eslamloo et al., 2017). Consequently, the inclusion rate of plant components has increased while the inclusion level of marine ingredients has decreased. In 1990 for instance, about 90% of Norwegian salmon diets were of marine origin, while in 2013 the inclusion level of marine ingredients was about 30% as depicted in figure 11 (Ytrestøyl, Aas, & Åsgård, 2014), with the balance coming from plant ingredients. The dominating plant ingredients for Norwegian salmon diets are soy protein concentrate (SPC) and rapeseed oil along with considerable amounts of wheat gluten (Figure 12)

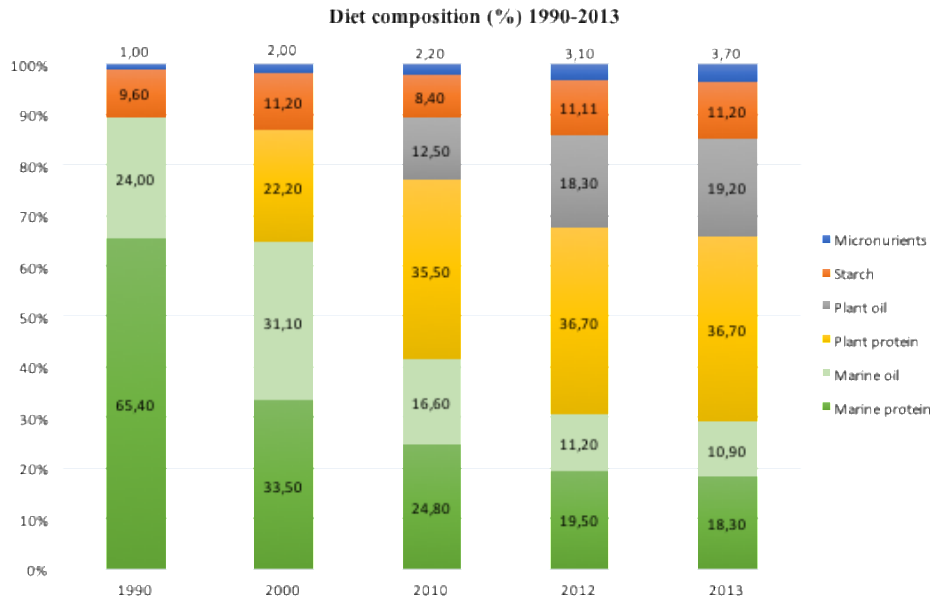

Figure 11: Trends of feed formulation in Norwegian salmon farming from 1990 to 2013. Each ingredient type is depicted as its percentage from the total diet (Ytrestøyl et al., 2014)

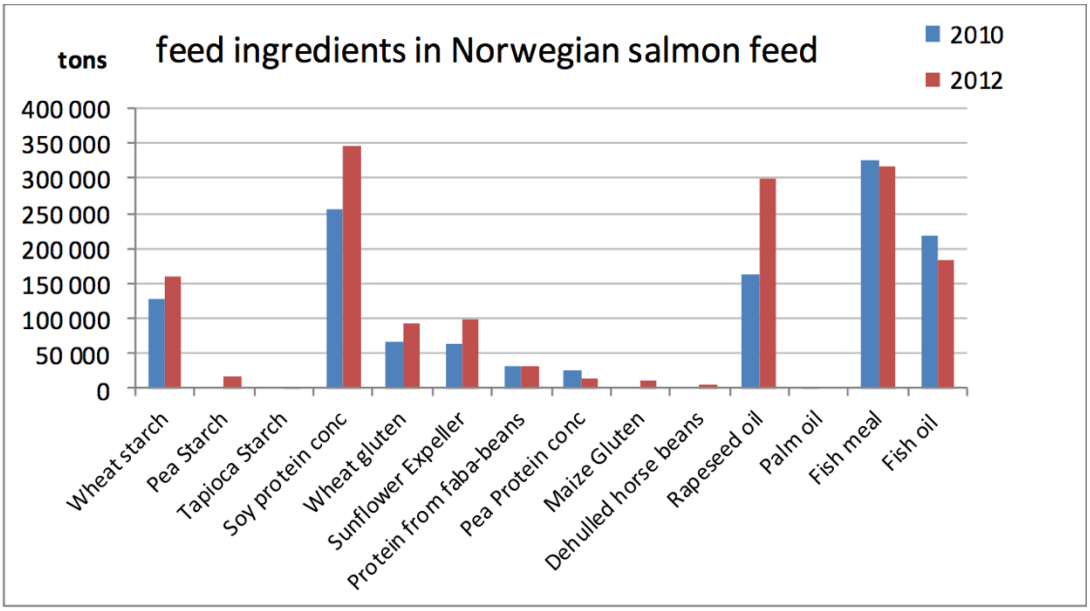

Figure12: Feed ingredients in Norwegian salmon feed in 2010 and 2012 (Ytrestøyl et al., 2014)

As an alternative protein source to FM, soybean is the most common plant protein used in salmonid's feed. Despite the global availability and nutritional characteristics, soybean meal (SBM) has not been incorporated into salmonid diets at high inclusion rates (Dersjant-Li, 2002). Without further processing, the relatively high concentration of carbohydrates and presence of anti-nutritional factors (ANFs) are the major limiting factors (Francis, Makkar, & Becker, 2001; Refstie & Storebakken, 2001).

In general, ANFs such as trypsin inhibitors, lectins, saponins and soy antigens have been shown to induce intestinal enteritis (Drew, Borgeson, & Thiessen, 2007) (A. Krogdahl, Bakke-McKellep, Roed, & Baeverfjord, 2000; Å Krogdahl, Bakke-McKellep, & Baeverfjord, 2003). Moreover, ANFs can lead to metabolic dysfunction in fish liver (Martin et al., 2003), reduced protein deposition (Gómez-Requeni et al., 2003) and impaired immune response (Francis et al., 2001).

A significant proportion of phosphorus (P) in oilseeds (e.g., soybean, rapeseed) on the other hand, is stored in the form of phytic acid and unavailable to the fish (Storebakken, Shearer, & Roem, 2000). This can interfere with the digestive absorption of minerals and amino acids (Deng, Kang, Tao, Rong, & Zhang, 2013), induce inflammation (Åshild Krogdahl, Penn, Thorsen, Refstie, & Bakke, 2010) and inhibit the function of intestinal enzymes that involved in proteolytic activities (Francis et al., 2001).

So far, studies examining responses of Atlantic salmon to plant protein diets reveal that a replacement of FM close to 100% is possible without adverse effects on salmon growth providing the proteins are highly purified and the amino acid profile is well balanced (Espe, Lemme, Petri, & El-Mowafi, 2007). Conversely, a decreased in feed intake and growth rate occurred in post-smolts fed on diets including 80% plant protein and 70% plant oil for an initial period, followed by a period of an increased in growth rate. Consequently, it is concluded that Atlantic salmon requires a longer post smolt acclimatization time when low levels of FM and FO or even their total substitution using plants sources (Torstensen et al., 2008). High inclusion rate of pea protein cause enteritis in the small intestine in salmon (Penn, Bendiksen, Campbell, & Krogdahl, 2011).

Considering replacement of FO with an alternative oil source in Norwegian salmon farming, rapeseed oil (Figure 12) has been the main plant oil used in today´s fish formulated diet (Ytrestøyl et al., 2014). Two mechanisms that are involved in the effects of fatty acids replacement on inflammation and immunity, involve modulation of eicosanoid production and long chain polyunsaturated fatty acids (LCPUFAs) (Calder, 2013).

Vegetable oils contain linoleic acid (LA, 18:2n-6) and monounsaturated fatty acid (MUFA) such as oleic acid (18:1n-9) with the exception of linseed oil which is rich in α-linolenic acid (ALA, 18:3n-3). Unlike dietary ALA (18:3n-3) which can be desaturated and elongated to long-chain highly unsaturated fatty acids (LCHUFAs) in the fish liver (Figure 13) (Sargent, Tocher, & Bell, 2002), Atlantic salmon have limited ability to metabolize LA (Tocher, 2003).

LA is a precursor of arachidonic acid (AA, 20:4n-6), which in turn is the main substrate for eicosanoids (Figure 13). Eicosanoids are metabolized to produce mediators of the immune system such as prostaglandin (PG), thromboxanes or leukotrienes (LT) (Philip Calder, 2001). Of these, prostaglandin E2 (PGE2) have the most important both pro-inflammatory and anti-inflammatory effects and regulate the functions of cells of the immune system (Calder, 1998). In vitro experiments have demonstrated that substitution of FO by plant oils can impair macrophage function (e.g. phagocytic and respiratory burst activity) in rainbow trout (Kiron, Fukuda, Takeuchi, & Watanabe, 1995), gilthead sea bream (Montero et al., 2008), European sea bass (Mourente, Good, & Bell, 2005) and channel catfish (Sheldon Jr & Blazer, 1991).

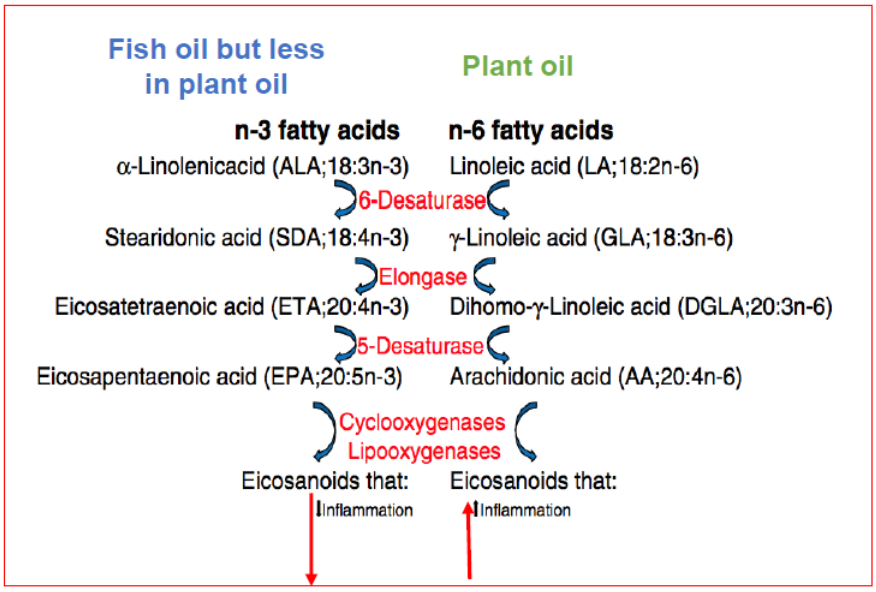

Figure 13: Plant oil contain more n-6 fatty acids and less n-3 fatty acids compared to fish oil. The metabolism of n-3 and n-6 fatty acids is catalyzed by the same enzymes (cyclooxygenase and lipoxygenases) to synthesize eicosanoids with anti-inflammatory (n-3) and pro-inflammatory (n-6) mediators (Calder, 1998; Moldal et al., 2014).

Dietary fish oil which is rich in n-3 polyunsaturated fatty acids (PUFAs) in contrast, increases the amount of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and decreases the amount of AA (20:4n-6) (Moldal et al., 2014). EPA and DHA are precursors of immune mediators such as 3-series PGs, which have anti-inflammatory effects (Figure 13). This anti- inflammatory effect can modulate the severity and outcome of auto-inflammatory diseases (Calder, 2006). Additionally, several studies have demonstrated that high EPA content along with low dietary lipid can reduce the histopathological heart changes of HSMI in Atlantic salmon following challenge (Martinez-Rubio et al., 2014; Martinez-Rubio et al., 2012; Martinez-Rubio et al., 2013). It has also been shown that EPA and DHA can modulate TLR function through interfering with lipid rafts and signaling platforms in cell membranes of immune cells (Calder, 2013; Norris & Dennis, 2012).

Fatty acid requirements in Atlantic salmon depend on the developmental stage. Atlantic salmon parr consumes feed that is rich in linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) with a minimum requirement (1%) of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) combined in the feed (Ruyter, Rosjo, Einen, & Thomassen, 2000). In other words, at parr phase, the fish can grow successfully on diet

containing rapeseed and linseed oil (Bell et al., 1997; Tocher et al., 2000).

Atlantic salmon in SW depends on oil feed rich in EPA and DHA, i.e. feed included marine ingredients. However, previous studies have revealed that salmonids can utilize vegetable oils in SW as long as the diets contain enough α-linolenic acid to satisfy essential fatty acid (EFA) requirements (Bell, Dick, McVicar, Sargent, & Thompson, 1993; Guillou, Soucy, Khalil, & Adambounou, 1995). The effect of dietary deficiencies in fatty acids are particularly evident during stressful phases of the life-cycle of salmon, including smoltification (Sargent et al. 1997).

In summary, fish at young age are more sensitive to nutrient deficiencies than adult (Rune Waagbø, 2010). This is mainly because of gut immaturity, rapid growth and development and less vitamin storage capacity (Hemre et al., 2016). Smolt diets for example, contain high levels of protein (50- 54%) compared to inclusion rate of oil (16-24%). The nutritional value of the protein relates directly to amino acid composition and digestibility (Miles & Chapman, 2006). Here, it is worth mentioning that protein is the most expensive ingredient as well as limiting factor in salmon nutrition (Craig & Helfrich, 2009). As the fish grows, the inclusion levels of protein decreases and the level of oil increases (Craig & Helfrich, 2009; Stead & Laird, 2002) as shown in Table 4.

Table 4: Protein and oil requirements for salmonid at different phases of the life cycle (Huntington, 2004)

Feed type	**% protein**	**% oil**
Salmon starter diets	50 -55	14 – 23
Salmon grower diets	34 - 50	22 - 38
Trout starter diets	50 - 57	14 - 22
Trout grower diets	38 - 50	8 - 33
Other marine species	50 - 60	12 - 24

1.6.2 Effects of fat soluble vitamins

Vitamins are organic compounds needed in small amounts from feed that are vital for different metabolic activities (Blazer, 1992). Fish require vitamins in their diet for optimum health and normal physiological functions (Albahrani & Greaves, 2016; Blazer, 1992). There are two groups of vitamins, water soluble and fat-soluble vitamins. This thesis focuses on those that are fat-soluble.

The fat-soluble vitamins include vitamin A, D, E, and K, and all have been described to enhance disease resistance in many animal species. Due to their hydrophobic nature, fat-soluble vitamins are closely associated with fatty acids. In other words, both are transported together through the intestine and blood (Albahrani & Greaves, 2016; Parker, 1996) and stored in the liver and fat-containing tissue until needed (Ravisankar et al., 2015). Without an adequate amount of fat in the diet, the body can´t effectively absorb the fat-soluble vitamins. Conditions favorable for fat absorption also enhance the absorption of fat-soluble vitamins (Scott, Nesheim, & Young, 1982).

The vitamin requirements of fish depend on life cycle, growth rate and size (Benevenga et al., 1995; Palace & Werner, 2006; Shiau & Lin, 2015) along with physical rearing conditions and sources of other feed ingredients included in the diet (Oliva-Teles, 2012; Palace & Werner, 2006; Shiau & Lin, 2015). Among the fat-soluble vitamins, vitamin A and D have primarily been demonstrated to have a direct modulating activity on the immune system (Klasing, 1998; Mora, Iwata, & Von Andrian, 2008).

1.6.2.1 Vitamin A

Sources and requirements

In diets with high inclusion levels of marine ingredients, vitamin A is mainly derived from retinyl esters or from astaxanthin (Torrissen, 1989). Plant based ingredients in contrast; contain carotenoids such as β-carotene as vitamin A precursors, which can be converted into vitamin A by fish. Cold-water fish can utilize precursors of vitamin A at 12.4 °C to 14 °C, but do not at 9 °C (Poston, Riis, Rumsey, & Ketola, 1977).

The average concentration of vitamin A in fish feed produced in Norway between 2003 and 2008 was about 20 mg retinol/ kg feed and with a maximum concentration of 121 mg retinol (Maage, Julshamn, Hemre, & Lunestad, 2007). Signs of vitamin A toxicity, such as increased

mortality, abnormal vertebral growth, and reduced growth, were found in Atlantic salmon that were fed a diet containing 938 mg retinol / kg. Furthermore, fry given 122 mg retinol/ Kg feed from first feeding for 14 weeks showed symptoms of stress and reduced growth (Ørnsrud, Graff, Høie, Totland, & HEMRE, 2002). However, due to increased inclusion levels of plant ingredients in salmon diet on the expenses of marine ingredients, the old recommendation of vitamin A inclusion level substantially deviate from the real need (Hemre et al., 2016).

Biochemistry and functions

Vitamin A (VA) occurs in three forms, which serve different overlapping function. These structures are: an alcohol (all-trans-retinol), an aldehyde (11-cis-retina and 11-cis-3 dehydro-retinal), and an acid (all-trans-retinoic acid); all together they are termed retinoid (Riccioni, D'Orazio, Menna, & De Lorenzo, 2003).

Vitamin A_1 (all-trans retinol) is found in mammals and marine fish, whereas both vitamins A_1 and vitamin A_2 (3-dehydroretinol) are synthesized and occur in tissues of freshwater fish (Ollilainen, Heinonen, Linkola, Varo, & Koivistoinen, 1989). In freshwater fish, the oxidative conversion of retinol to 3-dehydroretinol occurs (Goswami 1984), as well as the reversible oxidation and reduction reactions of retinol to retinal and of 3-dehydroretinol to 3-dehydroretinal. For example, Nile tilapia (*Oreochromis niloticus*), can convert dietary retinol into 3-dehydroretinol and retinal into 3-dehydroretinal (Katsuyama and Matsuno 1988). In addition, there are provitamin A compounds (the carotenoids) such as ß-carotenoid, which can be converted into retinol. The salmonid intestine appears to be the main organ of provitamin A conversion (Simpson, 1988). An overview of retinoid metabolism and conversion of β-carotenoid into retinol is shown in Figure 14.

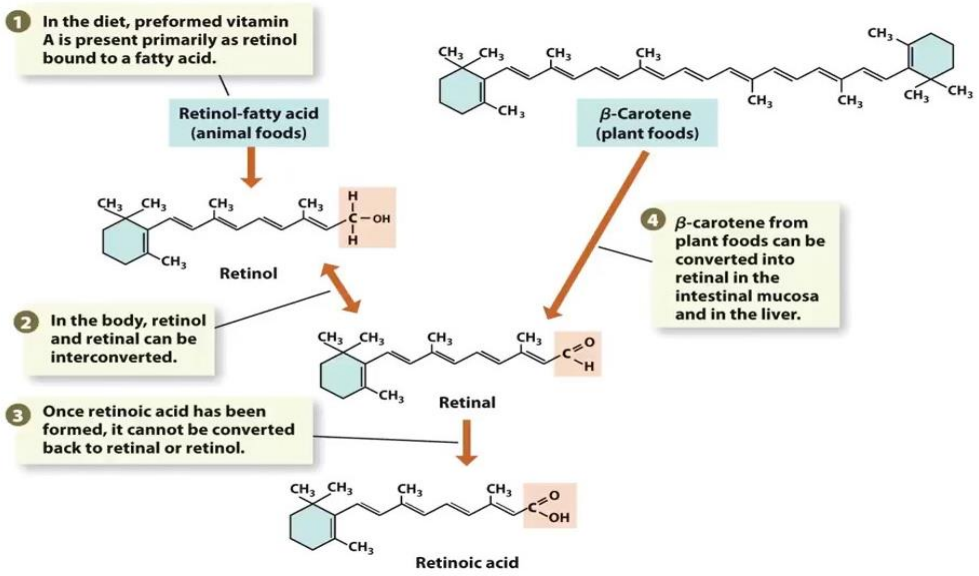

Figure 14: Structure of vitamin A and its metabolites and plant carotenoid (Zempleni, et al. 2013)

Following absorption from the intestine, vitamin A (retinol) is stored mainly in the liver as retinyl esters (Goodman, 1984; Thompson, Fletcher, Houlihan, & Secombes, 1994). Retinoids (VA derivatives or compounds) have diverse biological functions including: vision, immune response, bone mineralization, reproduction, growth and cell differentiation (Defo, Spear, & Couture, 2014). In addition, vitamin A has also antioxidants properties (Tesoriere, D'arpa, Re, & Livrea, 1997). These activities are mediated by interaction of retinoids with nuclear receptors.

From the liver stores, retinol is transported through retinol-binding protein (RBP). As a major carrier form of vitamin A in plasma (Goodman, 1980), RBP delivers retinol to the target cells. Once inside the cell, retinol can be metabolized to all-trans-RA, 9-cis-RA, 3,4-didehydroretinoic acid and to a number of other biologically active retinoids (Thaller & Eichele, 1990). The main active metabolites of vitamin A are all-trans-retinoic acid (RA) and 9-cis-retinoic acid that binds to retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Schräder, Bendik, Becker-Andre, & Carlberg, 1993; R. Zhang, Wang, Li, & Chen, 2015). In combination, RAR and RXR are able to transcribe and regulate hundreds of genes. The synthetic agonist bexarotene is a vitamin A analogue that is designed to optimize binding to retinoid X receptors (RXRs) (Ma et al., 2014; Wu et al., 2016).

RAR and RXR each have three members α, β and γ. However, neither RAR nor RXR receptor proteins are active in their monomeric forms (Zhang et al., 2015). In other words, RAR does not dimerize with itself but forms heterodimeric proteins with RXR (e.g. RAR-RXR). This RAR-RXR heterodimeric protein is the predominant receptor complex for retinoic acid signaling. In addition to forming heterodimers with RAR, RXR can also dimerize with other receptor proteins, such as vitamin D (Bikle, 2014; Ørnsrud, Lock, Glover, & Flik, 2009).

Retinoic acid (RA) binds to the heterodimer RAR-RXR, and afterwards associates with RA response elements in the genome to stimulate target gene expression (Chambon, 1996; Mangelsdorf et al., 1995). Three retinoid isomers, 13-cis-RA, all-trans-RA and 9-cis-RA have been identified as ligands for RAR-RXR, whereas homodimerized RXRs primarily bind to the 9-cis isomer (Allenby et al., 1993). After binding ligands and dimerizing, the receptor ligand complexes associate with specific DNA response elements (RA response elements, RARE) to activate or suppress gene transcription (Ørnsrud et al., 2009).

Vitamin A could influence hematopoiesis of phagocytes and lymphocytes, enhance phagocytosis and intracellular killing, increase production of macrophage activating lymphocytes (Blazer, 1992). Vitamin A also plays a role in improving the host antibody response, as it is required for the maturation of both B cells and T helper cells (Stephensen, 2001).

Examination of the effects of vitamin A supplementation on viral replication and clearance indicates that *in vitro,* retinoids induce type I IFN and directly inhibit replication of measles virus (Trottier et al., 2008; Trottier, Colombo, Mann, Miller, & Ward, 2009). Treatment of human promyelocytic leukemia cells (HL-60) and NB4 cells with combination of RA and IFNα provided a stronger antiviral response against vesicular stomatitis virus (VSV) than with only IFN (Pelicano, Brumpt, Pitha, & Chelbi-Alix, 1999; Pelicano, Li, Schindler, & Chelbi-Alix, 1997). Combined RA-IFN effects have also been associated with the induction of many IFN-stimulated gene (ISG) (Bandyopadhyay, Kumar, Rubin, & Sen, 1992; Lancillotti et al., 1995; Pelicano et al., 1999).

1.6.2.2 Vitamin D

Sources and requirements

In terrestrial vertebrates, vitamin D can be obtained from a diet as well as upon exposure to ultraviolet (UV) radiation from sunlight (Borradale & Kimlin, 2009). Under this context, a mammal can synthesize vitamin D_3 in the skin from 7-dehydrocholesterol (7-DHC) (Holick, 1981). In fish, photosynthesis of vitamin D_3 from 7-DHC was reported in both Mozambique tilapia (*Tilapia mossambicus*) on exposure to artificial UV light (300 nm) as well as in rainbow trout when exposed to blue light at wavelengths between 380 and 480 nm (Pierens & Fraser, 2015). Nevertheless, photosynthesis of vitamin D_3 unlikely plays a significant role in farmed conditions, where fish are not exposed to UV light (SUNITARAO & Raghuramulu, 1997).

In farmed Atlantic salmon, vitamin D is supplied through feed. The major two dietary sources of vitamin D are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol or calcitriol) from plants and animals (e.g. fishmeal and fish oil) respectively (Del Valle, Yaktine, Taylor, & Ross, 2011). In novel feed ingredients, microalgae contain both vitamin D_3 and provitamin D_3 vitamin whereas fungi and yeasts produce vitamin D_2 by UV-exposure of provitamin D_2 (Rie B Jäpelt & Jette Jakobsen, 2013).

Despite variability in content (Table 5), marine ingredients (fishmeal and fish oil) are considered to be good sources of vitamin D_3. In traditional formulated farm salmon diet (e.g. 45% FM and 30% FO), the vitamin D_3 level is expected to range from 0.01 – 1.25 mg/Kg.

Table 5: Vitamin D_3 content in fish meal and fish oil (Rychen et al., 2017)

Fish meal	**Fish oil**
0.01–0.18 mg/ Kg (Horvli & Lie, 1994)	0.2-3.9 mg/Kg (Opstvedt, Knudsen, & Asbjørnsen, 1997)
0.02–0.15 mg/Kg[b]	0.03– 2.4 mg/Kg[b] (Biomar database)

b = In-house database cited by (Rychen et al., 2017) in the technical dossier Annex II vitamin D supplementing note to the European Food Safety Authority (EFSA)

Today, as salmon feed containing about 80% plant ingredients and the remaining percentage equally divided between fish meal (10%) and fish oil (10%), vitamin D3 levels are expected to range between 0.004 and 0.408 mg/Kg complete feed (Rychen et al., 2017). Consequently, the Norwegian Food Safety and Authority (NFSA) proposed to increase the total level of vitamin D_3 up to 1.5 mg/Kg complete fish feed (Rychen et al., 2017). It is worth mentioning in conventional formulated fish feed dominated by marine ingredients, this requirement is largely met.

All in all, vitamin D_3 is the primary storage form for all fish species and has a higher bioavailability for fish compared with vitamin D_2 (Mattila, Piironen, Haapala, Hirvi, & Uusi-Rauva, 1997; A. Takeuchi, Okano, & Kobayashi, 1991), resulting in a higher uptake from the diet of vitamin D_3 compared with vitamin D2. When Atlantic salmon were fed on diets containing 0.04, 2.21 and 28.68 mg vitamin D_3/Kg feed, no adverse effects on weight gain, survival, plasma level of calcium, red blood cells count or haematocrits were observed (Horvli, Aksnes, & Lie, 1998). Further, fry given diets supplemented with three inclusion rates of vitamin D_3 (i.e. 0.2, 5, and 57 mg/Kg diet) for 14 weeks, showed no differences in weight, length, specific growth rate, mortality, or kidney calcium concentration or any skeleton malformation or histopathological changes from the three dietary groups (Graff, Høie, Totland, & Lie, 2002).

Biochemistry and functions

The main physiological functions of vitamin D in vertebrates, including teleost fish have been thoroughly reviewed by Lock and co-authors (LOCK, Waagbø, Wendelaar Bonga, & Flik, 2010). In general, vitamin D endocrine system plays a vital role in calcium and phosphate homeostasis in mammals as well regulation of hormone secretion, the immune response and cellular proliferation and differentiation in fish.

Vitamin D_2 from plants and from D_3 animals function as prohormones (Tripkovic et al., 2012). They differ structurally in the side chain (Rie Bak Jäpelt & Jette Jakobsen, 2013) as depicted in Figure 15. A common variant is that, the side chain in D_2 has a double bond between carbon-22 (C22) and carbon-23 (C23) and additional methyl group at C-24, whereas D_3 does not (Bikle, 2014; Rie Bak Jäpelt & Jette Jakobsen, 2013; Truswell, 2017). Accordingly, the variation of the side change between D_2 and D_3 affects both their affinity to vitamin D binding

protein (DBP) and their subsequent metabolism (Bikle, 2016).

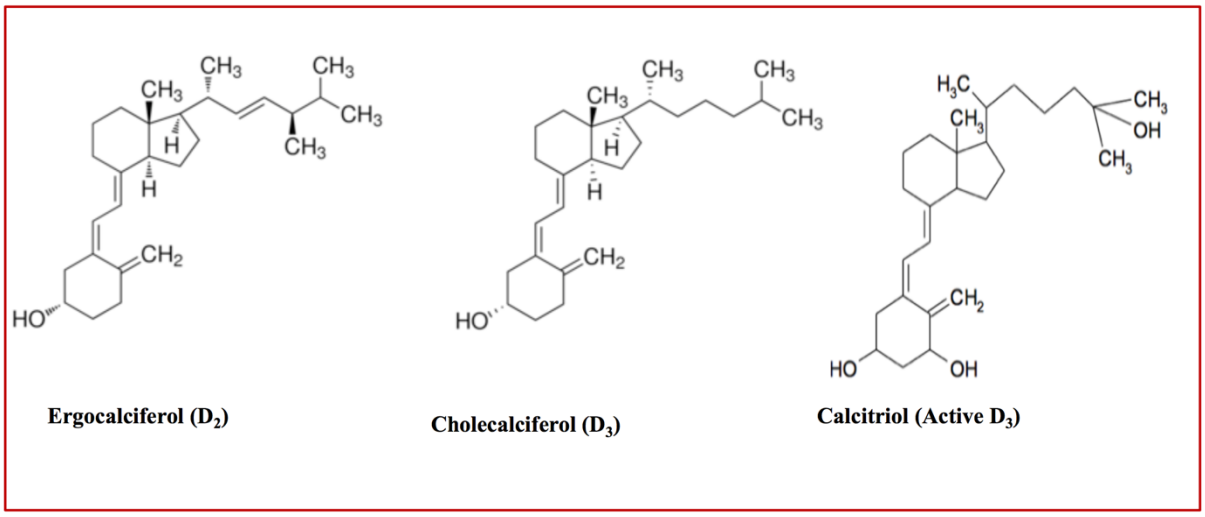

Figure 15: Chemical structures of vitamin D_2 (ergocalciferol), vitamin D_3 (cholecalciferol) and the activated form of vitamin D_3 (calcitriol) (https://www.sigmaaldrich.com)

Both forms of vitamin D, i.e. D_2 and D_3 are biologically inactive. Irrespective of their source (dietary and/or endogenously), activation occurs in two steps (AKSNES, 1999). First, in the liver vitamin D in either D_2 or D_3 form are converted into 25-hydroxyvitamin D calcidiol, i.e. ($25(OH)D_3$ or $25(OH)D_2$) by hydroxylase enzyme cytochrome (CYP 2R). Calcidiol can bind to vitamin D receptor (VDR) but with low affinity (DeLuca, 2004). Then in mammals, the second hydroxylation occurs in the kidney whereby the 1α-hydroxylase enzyme CYP 27B1 converts $25(OH)D_3$ into the high affinity vitamin D receptor (VDR) ligand, calcitriol ($1,25(OH)_2D_3$) (Horvli et al., 1998). D_2 on the other hand, is produced by UV irradiation of the ergosterol in plants and fungi (e.g., mushrooms). Because D_2 differs from D_3 in the side chain, D_2 affinity for vitamin D-binding protein (DBP) is lower than its counterpart. As a result, D_2 has faster clearance from the circulation as well as limited in conversion to 25 hydroxyvitamin D (25OHD) (Houghton & Vieth, 2006).

In fish, further hydroxylation of D_2 and D_3 to the active metabolite $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$, respectively, occur in liver, kidney and several other tissues. Additionally, in an in *vitro* study metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ reported to happen in intestine, muscle and gill of seawater adapted European eel *(Anguilla anguilla)* (Du Bois et al., 1988).

Both calcitriol ($1,25(OH)_2D_3$) and ergocalciferol ($1,25(OH)_2D_2$) (Figure 15) represent the active forms of vitamin D (Del Valle et al., 2011; DeLuca, 2004; Lee, 2015). However, active vitamin D is unstable and can rapidly degrade if not protected or attached to protein carriers. These carriers primarily consist of vitamin D binding proteins (DBPs) and vitamin D receptors (VDRs). (Masterjohn, 2014). In this respect, DBP binds and transports the active vitamin D through blood plasma. Then, the active D dissociates from serum DBP, enters the cell by diffusion and interacts with its intracellular vitamin D receptors (VDRs). The cellular responses to $l,25(OH)_2D_3$ are mediated by the nuclear vitamin D receptor (VDR). The VDR has high affinity and subsequently regulates the expression of several genes (Bikle, 2014; Jurutka et al., 2001). VDR as a heterodimer with RXR binds to specific sites in the genome (VDREs) to activate or in some cases suppress transcription (Bikle, 2014).

Cross-talk between vitamin A and vitamin D

The main active metabolites of VA and VD are retinoic acid (RA) and calcitriol (CTR) respectively. Effects of VA and VD active metabolites are mediated by their nuclear receptors. Genomic actions of CTR are mediated by the VD receptor (VDR), while RA binds to either a RA receptor (RAR) or retinoid X receptor (RXR).

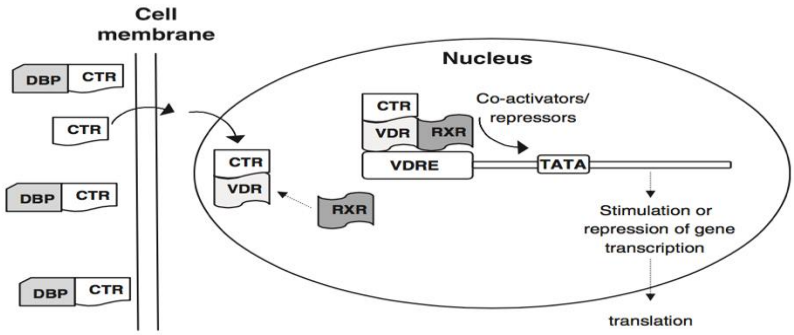

Figure 16: Cross-talk between active metabolites of VA and VD (LOCK et al., 2010).

1.7 Aim of the study

The overall aim of this study was to determine if fat-soluble vitamins have an effect on viral replication and antiviral response in red blood cells (RBCs). In order to achieve the purpose of this study, the following specific objectives were formulated:

- To analyze the effect of vitamin D_2 and D_3 and bexarotene on antiviral responses in RBCs stimulated by Poly I:C
- To investigate the effect of vitamin D_2 and D_3 and bexarotene on PRV infection and replication in RBCs
- To measure the expressions of IFN-I and antiviral proteins in PRV infected RBC

2. Material and methods

2.1 Experimental fish and rearing conditions

Atlantic salmon (AquaGen origin) with an average weight of 60 g were obtained from the aquarium facilities at Norwegian University of Life Sciences (NMBU, Ås) and held in the infection cell in the aquarium research facility at Adamstuen (NMBU/NVI). The fish were reared in two standard (160 L) fiberglass tanks at comparable stocking density of 11–13 g/L, regulated by water level and acclimatized for two weeks prior to experimentation. Flow through water supplied from the municipal water utilities was maintained to provide the required water level for the fish. Before entering the fish tank, the water passed through a carbon filter column and was aerated mechanically in the aquarium facility. Water temperature and oxygen saturation were monitored daily at ranges of $10.5 \pm 1.5\ ^{0}C$ and > 80% O_2-saturation respectively. Additionally, fish were also exposed to a light dark cycle of 12:12 light: darkness (L: D) and fed on commercial diet.

In the above mentioned two tanks, fish were divided into: 21 naïve (control) individuals in one tank whereas 16 virus-injected shedders along with 16 naïve cohabitants were transferred to the other. Shedders were distinguished from the cohabitants by clipping their adipose fin, while the adipose fin in cohabitants remained intact. Three experiments were conducted during the period from the 3rd of April until the 14th of July 2017. In these experiments, blood samples were taken from the naïve fish at week 2 and week 8 after the experimental start, whereas blood samples from the cohabitants were taken at week 6 (6wpc). The remaining fish and samples were intended for another study.

All the conducted experiments were in accordance with the Norwegian Animal Research Authority rules and regulations (NARA) and followed the European Union Directive 2010/ 63 / EU for animal experiments.

2.2 Blood sampling

Initially, fish were anesthetized in water containing benzocaine (80 mg/L, Sigma) and stunned by a blow to the head. Blood samples were then collected from the caudal vein into heparinized vacutainers and kept cold (Figure 17). Subsequently, the collected blood samples were taken to the laboratory (Department of Food Safety and Infection Biology, Norwegian University of Life Sciences (NMBU)) for further processing.

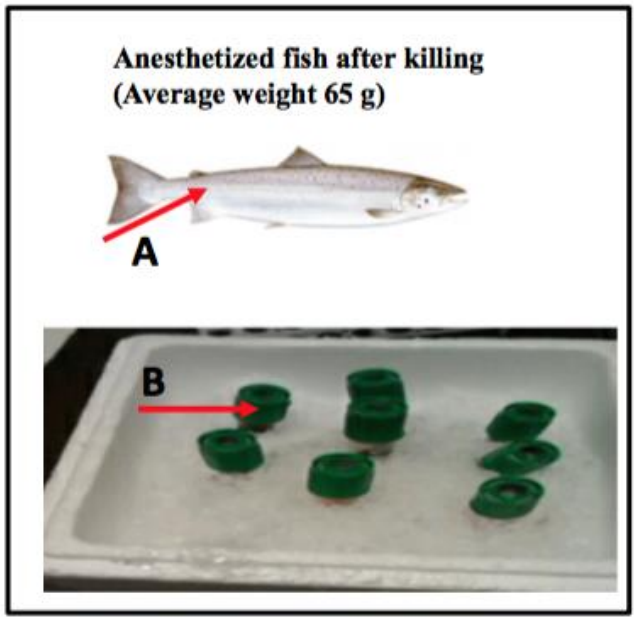

Figure 17: The injection point into the fish caudal vein (A) and collected blood in heparinized vacutainers on ice (B)

2.3 Blood samples processing and isolation of RBCs

At Day 0 of each experiment (sampling day), 100 µl from the heparinized cold blood samples were transferred into 1.5 ml Eppendorf tubes and centrifuged at 3000 rpm for 5 minutes at 4 °C. After spinning, the red blood cells (RBCs) sediment as pellets on the bottom of the tubes whereas the plasma form layers on the top. The supernatant (plasma) were transferred into separate clean tubes and stored at – 80 °C together with the RBCs pellets

The remaining blood samples were diluted in cold distilled phosphate-buffered saline (dPBS) and mixed in a 15-ml tube on ice. In this dilution, the ratio of blood to dPBS was 1:20 respectively (i.e. 0.5 ml blood to 10 ml PBS). Here, it is worth mentioning that the PBS is isotonic and non-toxic to cells and composed of sodium chloride (NaCl), potassium phosphate (K_2PO_4) and potassium chloride (KCl). PBS was used to remove the serum as well as to maintain a stable pH (7.2 – 7.4) when the cells were cultivated.

After the dilution, 15 ml of 49 % Percoll gradient (GE Healthcare, Uppsala, Sweden) was prepared, i.e. for 80 ml gradient, 37.5 ml Percoll, 8 ml 10x PBS and 34.4 ml distilled water (dH_2O) were used. From this gradient, 7.5 ml was transferred into 15 ml tubes. Then, 5ml diluted blood was layered carefully onto the top of the gradient and centrifuged at 500*g* for 20

minutes at 4 °C. The Percoll gradient separated the white blood cells from the red blood cells and plasma protein during centrifugation. Since RBCs have high density, they were separated into the bottom of the gradient, whereas the white blood cells and the remaining supernatant were separated in the middle and top respectively. RBCs were then collected and washed twice in dPBS (15 ml) followed by centrifugation at 500 g for 5 minutes at 4 °C. Subsequently, the resulting supernatant from centrifugation were discarded.

2.4 RBCs counting and culturing conditions

After washing, cell concentrations were required to determine specific seeding densities (cell number per ml in a suspension) for each experiment. To achieve this, cell yield and viability were measured using Countess Automated Cell Counter (Invitrogen, Eugene, Oregon, USA) (figure 18). For the Countess, a 1:1 mixture of cell suspension and trypan blue was prepared. In this regard, 5µl from the cell suspension was mixed with 5µl trypan blue (1:1). Then, this mixture (10µl) was loaded into a hemocytometer chamber that referred to as A in Figure 18.

For ease and accuracy in counting, the hemocytometer was filled with the 10 µl (trypan and cell suspension) mixture (Ott, 2004). The hemocytometer was then inserted into the Countess (B in figure 18) and the image was adjusted for analysis, such that live cells had bright centers and dark edges, whereas dead or damage cells had uniform blue colors throughout the cells (Tran, Puhar, Ngo-Camus, & Ramarao, 2011). This is due to the fact that viable cells possess intact membranes that impermeable to trypan blue, but the dead cells are permeable and take up the dye.

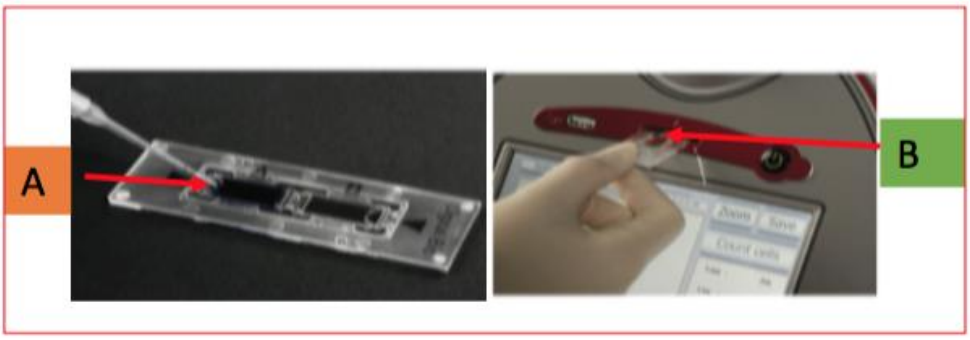

Figure 18: The hemocytometer (left) and the Countess (right)

The total cells counted were then multiplied by the dilution factor giving the total number of cells (n x 10^4 x mL). This value was then multiplied by the total volume of cell suspension from which the count was made. The cells were later diluted to a final concentration (million cells/ml) in Leibovitz's L15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 2% fetal calf serum (FCS) and gentamicin (50 µg/ml). FCS contains the required growth ingredients and protects cells from shear stress. Finally, RBCs were seeded onto 24 well plates and cultured overnight in InFors Ecotr Shaker at 150 rpm and 15 ^{0}C.

After culturing the cells in the Shaker for 24 hours, plates were centrifuged at 500 g for 5 minutes at 15 °C and the media was removed from the wells. Based upon the experimental plate setup, cells were then treated with 1 ml per well of regular media supplemented with gentamicin along with 2 % FBS and different inducing reagents.

2.5 Reagents

Vitamin D_3 or 1,25$(OH)_2D_3$ (calcitriol), vitamin D_2 or 1,25$(OH)_2$ (ercalcitriol) and bexarotene (B) were purchased from TOCRIS Bio-Techne. Vitamin D_2 (ercalcitriol) used in the experiments had purity about 86.6%, whereas vitamin D_3 (calcitriol) and bexarotene had purity of 99.6% and 100% respectively (Table 6). The physical and chemical properties of the above-mentioned reagents are presented in Table 6.

In this study, all the reagents (ercalcitriol, calcitriol and bexarotene) mentioned in table 6 were dissolved in dimethyl sulfoxide (DMSO) on ice, since ethanol is reported to affect intracellular signaling pathways, including those involved in interferon expression**.** Additionally, all reagents were aliquoted into small batches and kept at -20 ^{0}C for long term storage.

Table 6: Physical and chemical properties of the reagents used in the study (TOCRIS Bio-Techne: www.tocris.com)

	Ercalcitriol	**Calcitriol**	**Bexarotene**
Batch molecular formula	$C_{28}H_{44}O_3$	$C_{27}H_{44}O_3$	$C_{24}H_{28}O_2$
Batch molecular weight	428.65	416.64	348.48
Physical appearance	White lyophillised solid	White lyophillised solid	White solid
Solubility	Ethanol	DMSO and ethanol	DMSO to 100mM Ethanol to 20mM with gentle warming
Storage	-20 ^{0}C	-20 ^{0}C	+4 ^{0}C
Concentration used	0.1 µM	0.1 µM	10 µM
Purity	99.3% (used in the study found to be 88.6%)	99.6%	100%

Furthermore, poly I:C (Sigma-Aldrich) acid sodium salt with low molecular weight (LMW) was used as stimulants to mimic virus dsRNA or TLR3 agonist. LMW has a range of 0.2-1kb and comprises short strands of inosine poly (I) homopolymer annealed to strands of cytidine poly(C) homopolymer. Other poly I: C properties are presented in table 7

Table 7: Poly I: C properties

Concentration	50ng/µl
Purity	≥ 99%
Storage	-20 ^{0}C

Media and stock solutions preparations

Leibovitz's L-15 media and vitamin D_2, D_3 and bexarotene stocks were used for the experiments. Before used, L-15 media was prepared with gentamicin and fetus calf serum (FCS). For each 45-ml media, 50 µg/ml gentamicin and 900 ul FCS were added. For the stock preparations of vitamins D_2, and D_3, each 100 µM stock, was dissolved in 2.34 ml DMSO and 1.2 ml DMSO respectively, i.e. 1:100 dilution in DMSO and 1:1000 dilution in media. The final concentration for both D_2 and D_3 was 0.1 µM. As for bexarotene, 100 mM stock was dissolved in 1.66 ml DMSO, i.e. dilution 1:1000 DSMO and 1:1000 in media. Bexarotene final concentration used was 10 µM.

2.6 RBCs harvesting

After incubation, the plates were centrifuged at 500 g for 5 minutes at 4 °C and the media was pipetted off. Afterwards, cells were resuspended by addition of 1 ml of PBS and transferred into 1.5 ml Eppendorf tubes. These tubes, were then spin in micro-centrifuge at 2000 rpm for 5 minutes at 4 °C and PBS (the supernatant) was removed. The remaining pellets on the bottom of the tube (Figure 19) were taken on ice to NVI and stored at -80 °C until RNA isolations were performed.

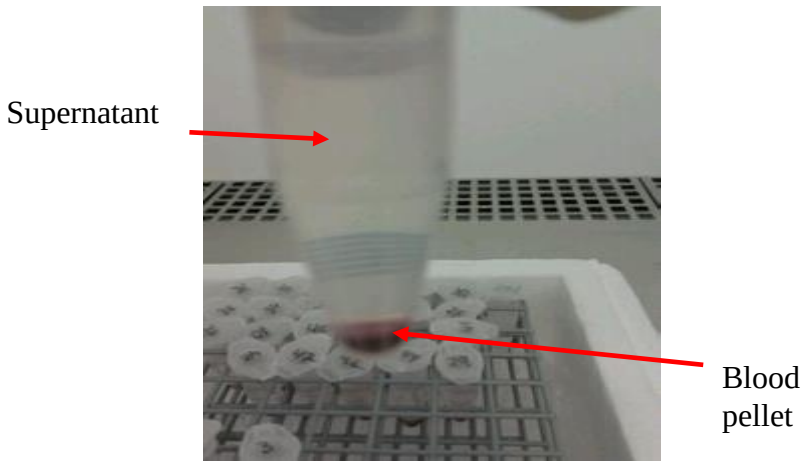

Figure 19: Supernatant and blood pellet after spinning in micro-centrifuge

2.7 RNA isolation

Based on the flowchart depicted in Figure 20, the RNA extraction protocol for all the performed experiments used various QIAGEN RNeasy Plus Mini Kit and underwent addition of different compounds along with centrifugation at NVI laboratories. At the beginning, the frozen pellet samples were mixed with 500 µl QIAzol Lysis Reagent (Qiagen, Hilden, Germany) together with 5 mm stainless steel beads (Qiagen) in 2 ml tube. These samples were then disrupted and homogenized by TissueLyser II (manufactured by Retsch - Qiagen) for 2 × 5 min at 25 Hz followed by incubation for 5 minutes at room temperature. This incubation period permitted a complete dissociation of nucleoprotein complexes (Crump, Chiu, Trudeau, & Kennedy, 2008). During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components (Simms, Cizdziel, & Chomczynski, 1993).

Afterwards, addition of chloroform followed by centrifugation (Biofuge Heraeus Centrifuge), separated the solution into: a lower organic phase, a middle phase that contained denatured proteins and genomic DNA (gDNA) and upper aqueous phases. RNA remained entirely in the supernatant phases. The aqueous (supernatant) phase together with an equal volume of 70% ethanol (200 µl each) were mixed in new Eppendorf tubes. In this context, 70% ethanol addition removed RNAs by precipitation.

Then, a mixture of the supernatant together with the ethanol was transferred into the RNeasy Mini spin columns (Qiagen). This mixture in the spin columns was later centrifuged (Eppendorf centrifuge 5424) at 10000 rpm for 15 seconds. After centrifugation, the RNA remained bound to the column. Subsequently, both RW1 and RPE buffers were added respectively to the columns for washing. These buffers were used to remove biomolecules such as proteins and fatty acids as the target RNA should be free from contaminants (Tan & Yiap, 2009). The columns were then placed in new collection tubes and centrifuged at 13000 rpm for 1 minute to remove traces of buffers.

Finally, the RNA columns were placed in Eppendorf tubes and eluted in 50 µl RNase-free water after centrifugation at 10000 rpm for 1 minute. RNA quantification was determined by using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the optical density at 260 nm (OD_{260})/OD_{280} ratio was used to evaluate the purity of the RNA samples.

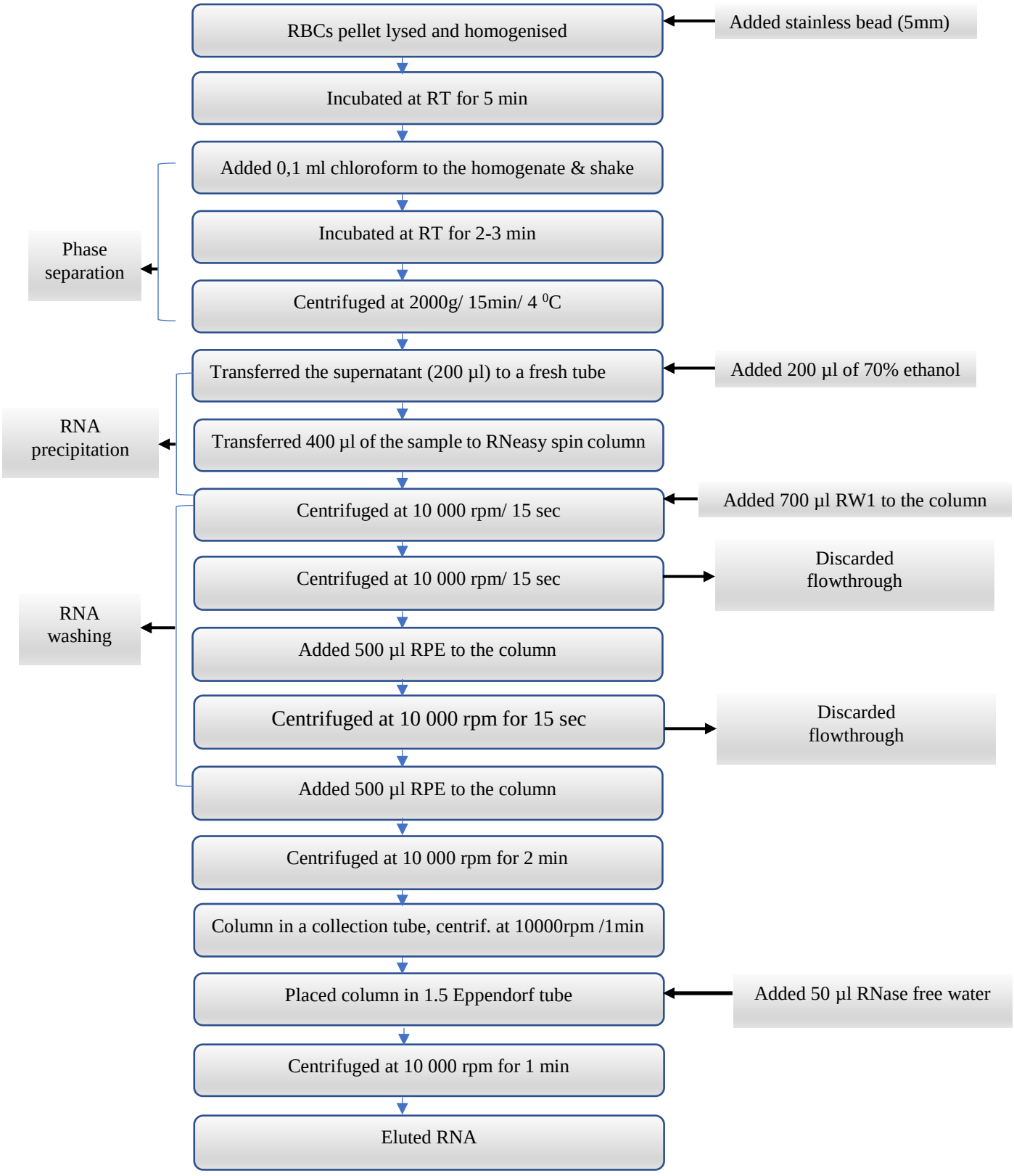

Figure 20: Flow chart describing isolation of RNA

2.8 Complementary DNA (cDNA)

cDNA was produced from RNA template in a process in which the reverse transcriptase (RT) enzyme (a polymerase that synthesizes DNA from RNA), primers, buffers and RNase inhibitor were present. This conversion occurred for the following reasons: First, RNA is considered highly unstable (easily degraded) (Bustin et al., 2009). Second, since RNA cannot serve as template for PCR, therefore the RNA has to be converted into cDNA.

Against this background, high quality RNA is vital for reverse transcription reactions. Thus, all the RNA samples that were used for cDNA synthesis had a ratio of absorbance at 260 nm and 280 nm (260/280) approximately between 1.8. and 2.00 which in turn, indicates RNA purity. Samples within the range of pure RNA were then diluted to 20 ng/µl in RNase free water and RNAse Out (Life Technologies) to a ratio of 1:100 respectively. Later, 10 µl was pipetted from each diluted sample into a new tube. Afterward, the reagents of the QuantiTect reverse transcription kit (Qiagen, Germany) such as: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water were thawed at room temperature.

To avoid particle accumulation in the bottom, each solution was mixed by flicking the tubes, centrifuged briefly and stored on ice. The QuantiTect reverse transcription kit was used to convert RNA into cDNA on three different steps. First, 1x gDNA elimination reaction was prepared as shown in Table 8 below, then mixed and stored on ice.

Table 8: gDNA elimination reaction components

Component	Volume
gDNA Wipeout buffer (7X)	2 µl
RNase-free water	2 µl
Total	4 µl

From this gDNA mixture, 4 µl was added to 10 µl RNA in a new tube to make total volume of 14 µl (Table 9). This volume was then incubated at 42 ^{0}C for 2 minutes to effectively remove contaminating gDNA and then placed immediately on ice. Contaminating gDNA in RNA samples can significantly affect the C_t value of subsequent qPCR reactions by background amplification, especially for genes with low transcription levels.

After gDNA elimination, the RNA sample was made ready for reverse transcription (RT) by adding RT master mix on ice. The RT master mix as presented in table 9 was composed of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer (5X) and RT primer mix. After preparation, 6 µl from the RT master mix was added to the 14 µl mentioned above to make total volume of 20 µl in each tube**.** It is worth mentioning that RT is an enzyme used to make cDNA from RNA template, a process termed reverse transcription. RT buffer provides a suitable chemical environment for optimum activity and stability of enzyme RT

Table 9: Reverse transcription (RT) master mix preparation

Component	Volume
RT master mix	
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer (5X)	4 µl
RT primer mix	1 µl
Template RNA	
Entire genomic DNA elimination reaction	14 µl
Total volume	20 µl

Finally, the cDNA synthesis reaction was placed in the thermocycler. The thermal cycle was set at 42 ^{0}C for 30 minutes and then incubated at 95 ^{0}C for 3 minutes (figure 21) to inactivate the Quantiscript Reverse Transcriptase. The synthesized cDNA was stored at – 20 ^{0}C and later used as template for the quantitative polymerase chain reaction (qPCR).

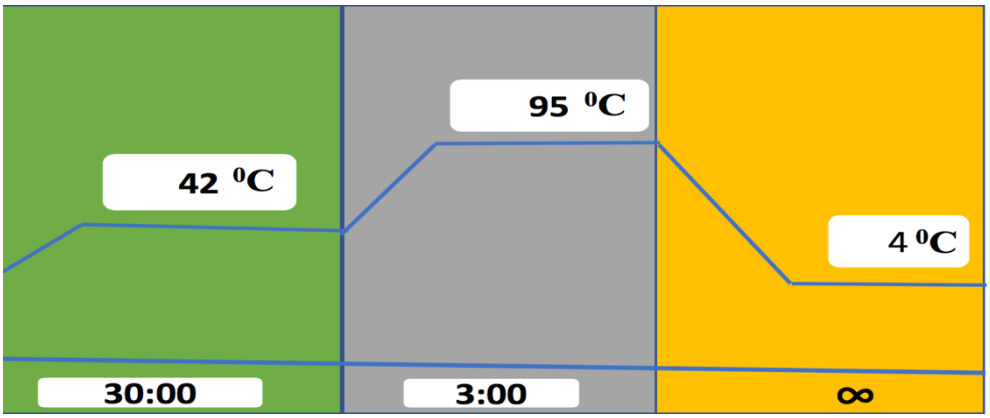

Figure 21: The thermal cycle setting to inactivate RT

2.9 Real- time polymerase chain reaction (PCR)

The quantitative polymerase chain reaction (qPCR), also known as real-time PCR, is a technique widely used in quantifying nucleic acids, because the amplification of the target sequence allows for greater sensitivity of detection (Taylor, 2010; Wong & Medrano, 2005). And when a repeated cycles of heating and cooling (thermocycler) is run, millions of copies can be produced out of a particular DNA sequence (Joshi & Deshpande, 2010).

Real-time quantitation of gene transcripts occurs during the accumulation of the PCR product by detection of the amount of cDNA at each cycle of amplification. Two types of chemistries for detecting PCR products predominantly used are SYBR Green dye and TaqMan probes. Although both assays are potentially rapid and sensitive, their principles of detection and optimization are different.

Fluorogenic probes can be used to detect specific PCR product as it accumulates during PCR cycles. The fluorogenic PCR assay uses the intrinsic 5´ --> 3´ nuclease activity of Taq DNA polymerase to cleave a probe which is labelled with two fluorescent dyes and hybridizes to the amplicon during PCR (Jourdan, Johnson, & Wesley, 2000). When the probe is intact, the two fluorophores interact such that the emission of the reporter dye is quenched. During amplification, the probe is hydrolyzed, relieving the quenching of the reporter and resulting in an increase in its fluorescence intensity. Consequently, an increase in reporter fluorescent emission indicates amplification of target DNA (Livak, Flood, Marmaro, Giusti, & Deetz, 1995).

SYBR Green dye chemistry as the name suggests on the other hand, is a green dye that intercalating only to double- stranded DNA (dsDNA) molecules (Morrison, Weis, & Wittwer, 1998). Upon binding to dsDNA, it emits a strong fluorescence signal that is easily detected. The advantage of SYBR Green dye is cost-effective and easy to use. In contrast, the intercalating SYBR Green dye binds to any dsDNA product and will fluoresce with non-specific products such as primer dimers. Amplified non-specific products affect the efficiency of the amplification of specific products. Given this overview, the investigated study used SYBR Green dye as a method of detection whereas fluorogenic probe was used for PRV analysis.

One of the principles to determine gene expression levels in qPCR is by comparing the expression of the gene of interest in different conditions with reference genes which expressions do not change under the various experimental conditions (Silveira, Alves-Ferreira, Guimarães, da Silva, & de Campos Carneiro, 2009). Accordingly, elongation factor (EF 1α) was used as a reference gene, whereas viperin, PKR, and RIG-I were used as targets. Primers used in the qPCR analysis are presented in Table 10.

Table 10: Primers used in the PCR reactions for the analysis of immune genes

Gene	Primer	Sequence
EFα	EFα (FW)	5'-TGCCCCTCCAGGATGTCTAC-3'
	EFα (R)	5'-CACGGCCCACAGGTACTG-3'
Viperin	Viperin (FW)	5'-AGCAATGGCAGCATGATCAG-3'
	Viperin (R)	5'-TGGTTGGTGTCCTCGTCAAAG-3'
RIG-I	RIG-I (FW)	5'-GCGACCGTCTTACGTCAAAG-3'
	RIG-I (R)	5'-TAGAAACACCTGGGCTGCTG-3'
PKR	PKR (FW)	5'-CAGGATGCAACACCATCATC-3'
	PKR (R)	5'-GGTCTTGACCGGTGACATCT-3'

FW = forward
R = reverse

Additionally, when performing qPCR, one has to decide whether to use a one-step protocol and/ or a two-step protocol. In this study, both procedures were used and the choice of each was made based on the experimental purpose and setup.

2.9.1 The two protocols for qPCR

2.9.1.1 One-step protocol

When detecting or quantifying the presence of dsRNA virus, the use of one-step RT-qPCR protocol is recommended because dsRNA is more challenging to denature for successful cDNA preparation in a separate reaction, and the result from experiment to experiment is too variable using a two-step reaction. One-step RT-qPCR combines the reverse transcription and qPCR reaction in a single reaction (Figure 22). This saves significant time as well as minimizes pipetting errors and cross-contamination. However, all the template in a one-step reaction could be used for one reaction only. Primarily, 5µl from the diluted RNA was denatured at 95 ^{0}C for 5 minutes and placed immediately on ice. Then a reaction mix which contained the reagents presented in Table 11 was prepared on ice.

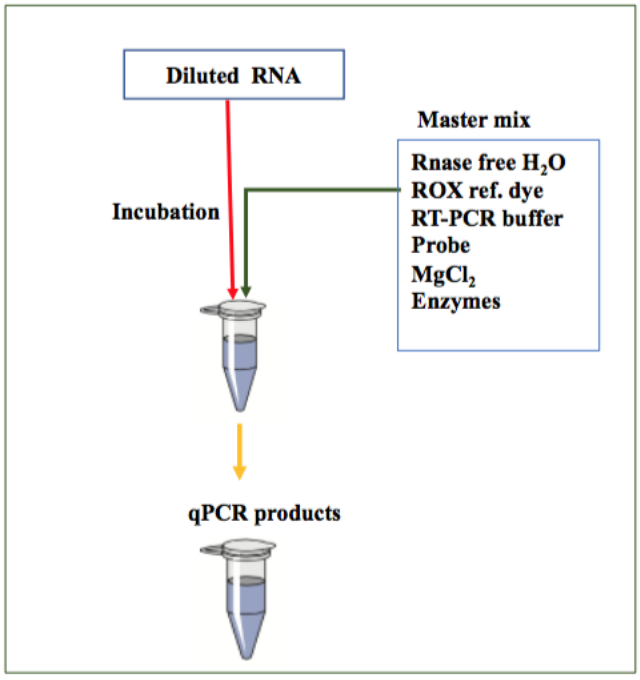

Figure 22: flowchart for one-step qPCR protocol

Table 11: one-step master mix components

QIAGEN One-step protocol	Per sample
QIAGEN One-step RT-PCR buffer (5X)	2.5
RNase free water	1.99
ROX reference dye (20X diluted)	0.5
dNTPs (10mM)	0.5
Primer PRV-F (50 μM)	0.1
Primer PRV-R (50 μM)	0.1
*MGB PRV-probe (30 μM)	0.15
QIAGEN One-step enzyme	1
$MgCl_2$ (25mM)	0.66
Total	7.5

*Minor groove binders

5μl RNA	100 ng	20 ng/μl

After preparation, 7.5 μl from the master mix was added into the denatured RNA. Then, plates were loaded into the PCR machine for DNA amplification. The amplification was performed after setting the thermal cycle parameters (figure 23) with a sense probe. Typical thermal profile for one-step: Segment 1 direct cDNA synthesis by reverse transcription using 30 minutes at 50°C; Segment 2 a 94°C incubation for 10 minutes used for RT inactivation and PCR polymerase activation; Segment 3 direct 40 cycles of qPCR amplification (94°C for15 sec, 54°C for 30 secs and 72°C for 30 sec).

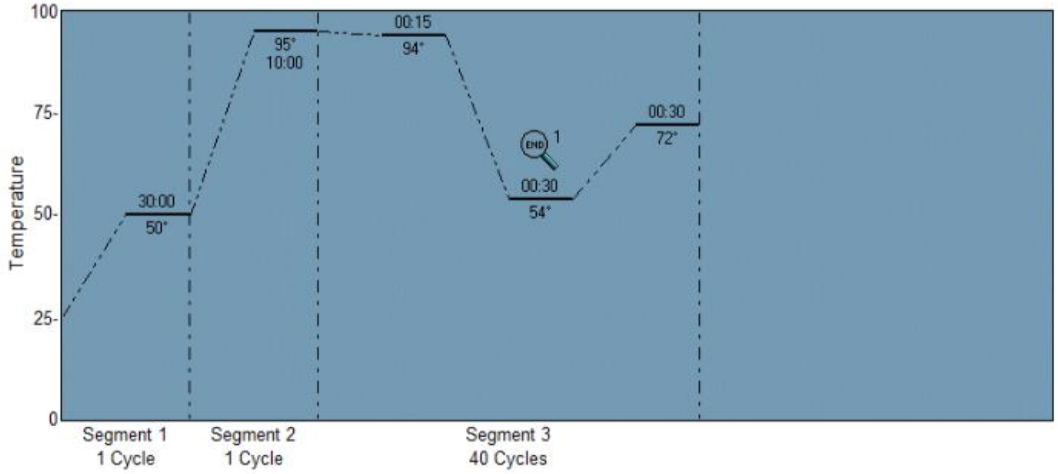

Figure 23: Thermal cycle for one-step qPCR

2.9.1.2 Two-step PCR

With regard to two-step RT-PCR, it involved two separate reactions, i.e. one for cDNA which was described in section 1.7 and the other for qPCR. Before starting, the master mix for SsoAdvanced™ Universal SYBR® Green and other frozen reaction components thawed at room temperature. Thereafter, they were mixed thoroughly and stored on ice protected from light. Then, the reaction mixture included primers, master mix as shown in Table 12 was prepared. The assay master mix was mixed thoroughly to ensure homogeneity and pipetted equally into ninety-six well plates followed by addition of 2 µl cDNA in each well.

Table 12: Real time PCR reaction mix

Component	**Volume per sample (µl)**
2x MMX/ ROX	5
Forward (FW) primer (10 µM)	0,5
Reverse primer (10 µM)	0,5
Nuclease – free water	2
cDNA (10 ng/ well)	2
Total volume/ well	10

Finally, the plates were spin in order to remove the air bubbles and collect any drops of liquid on the tube sides or lid before loading into PCR machine. Upon loading, changes in temperature were used to control the activity of the polymerase and the binding of primers. Consequently, the thermos-cycling profile of the quantitative PCR machine was set-up as shown in figure 24 and reactions were run for 40 cycles. Typical thermal profile for two-step: Segment-1 directs a 95°C for 30 second (s) used for pre-denaturation; Segment-2 a 94°C incubation for 15 s used for denaturation where the hydrogen bonds of DNA broke down giving two separated dsRNA and 60°C for 30 s used for primers annealing.

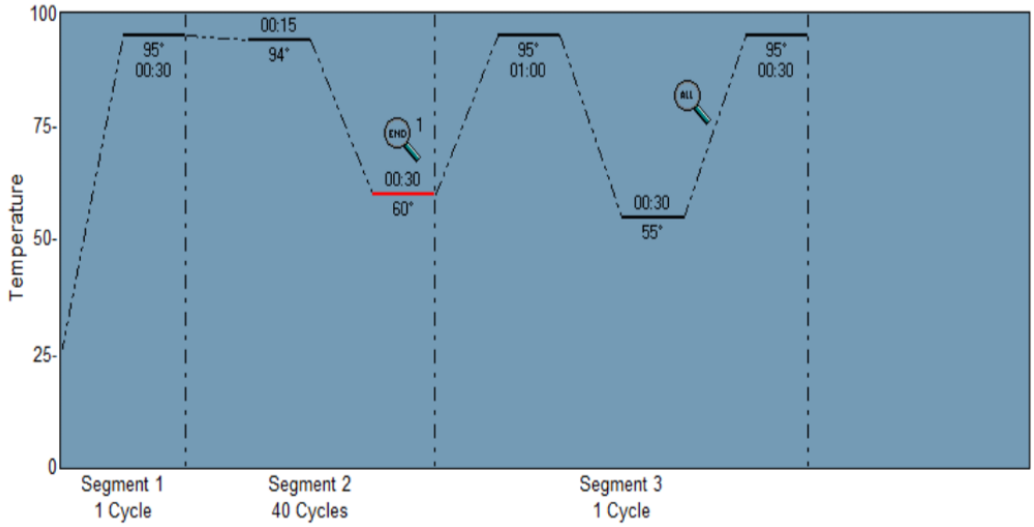

Figure 24: Thermal profile for two-step qPCR

2.9.2 qPCR Data analysis

All data from quantitative RT-PCR were exported into Microsoft Excel. Then, relative quantification method was used to calculate relative increase in gene expression of the studied genes. This is based on the expression levels of a target gene versus a reference gene (EF 1α). To calculate the expression of a target gene in relation to a reference gene, determination of $\Delta\Delta C_T$ was required. Steps in Livak method (Livak & Schmittgen, 2001) followed to calculate $\Delta\Delta C_T$ were:

- Calculation of ΔC_T by subtracting the reference gene from the gene of interest (C_T gene of interest - C_T reference gene).
- Determination of the average ΔC_T for all the replicates at basal level
- Calculation of $\Delta\Delta C_T$ values (ΔC_T for the experiment – ΔC_T control group mean)
- Determination of fold change ($2^{-\Delta\Delta CT}$).

2.10 Statistical Analysis

Data from three independent experiments were analyzed with GraphPad Prism 7 software. Statistical significance ($p \leq 0.05$) between individual comparisons was determined using Student t-test. For multiple comparisons, one-way ANOVA was employed.

3. Results

A total of three different experiments were conducted, analyzed by RT-qPCR and their results are presented in three separate sections. Data were normalized for the EF 1α reference gene. Then, fold changes of the target genes as a result of the experimental treatments were plotted., calcitriol and bexarotene

3.1 Experiment 1: Explore the effects of vitamin D_2 (ercalcitriol), D_3 (calcitriol) and the RXR agonist bexarotene on antiviral responses following poly I:C stimulation in RBCs *ex vivo*

Blood samples were taken from the caudal vein of 5 naïve fish (F1- F5) at week two after the fish were transferred to the aquarium. However, only cells from four of these fish were used further in the experiment. The activities performed are summarized in Figure 25.

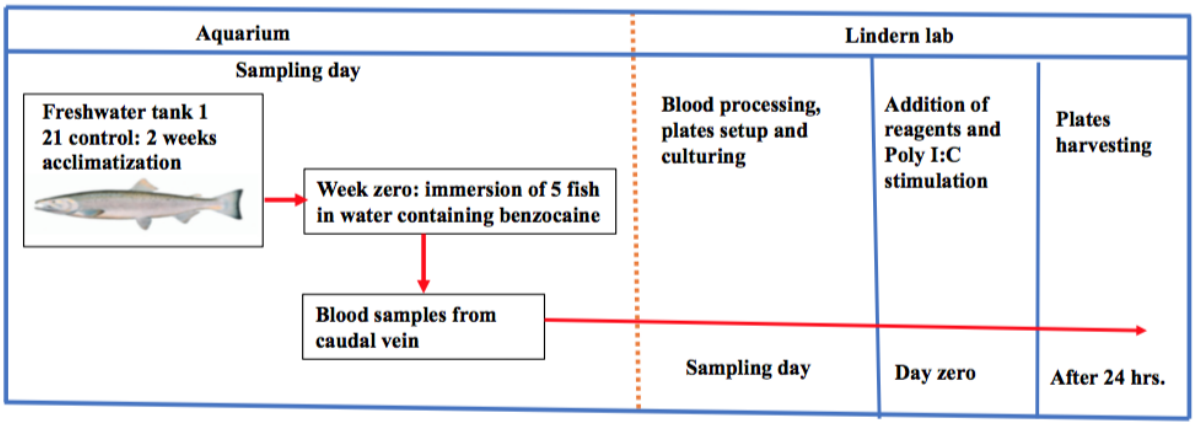

Figure 25: Summary of activities performed in experiment 1

Cell counting

After gradient isolation of RBCs, the total number of cells were counted and the numbers obtained are given in Table 13. The cell viability was measured by trypan blue staining. Following isolation of RBC, the fraction of viable cells was 94-97 % It should be noted that the viability may be higher. RBCs are very small and elongated cells, and therefore the counter sometimes may count live cells as dead ones.

Table 13: Calculated total RBCs

Fish no.	Total cells	Viability
1	600*10^6	98 %
3	420*10^6	97 %
4	480*10^6	94 %
5	480*10^6	95 %

Plates seeding

Following RBC viability assessment, cells were seeded at a density of 60 mill/well in two 24-well plates, one untreated (negative) and one stimulated with poly I:C (50 ng/µl) in all wells. Both stimulated and untreated plates were cultured in a shaker at 150 rpm for 24 h. The untreated cells were used as negative control, which is referred to as basal in Figure 26.

At day zero (reagents addition), incubated plates were centrifuged and then 1 ml/well of either regular L15 with 2% serum and 50 µg/ml gentamicin (L15+) or L15+ combined with the synthetic reagents (ercalcitriol, calcitriol and bexarotene) were added into the corresponding wells. After addition of reagents, plates were incubated for one hour before one plate was stimulated with 50 ng/µl poly I: C. Following incubation for 24 hours (day 1), RBCs were harvested for RNA isolation and consequently cDNA synthesis and qPCR analysis.

Expression of genes in response to stimulation with poly I:C and unstimulating of RBCs

Gene expression analysis was performed based on a two-step RT-qPCR procedure described in section 1.8.1.2. For measuring the effect of vitamin D2, D3 and bexarotene on gene expression, two tests were performed. First, the poly I: C effects on IFN-I, PKR and viperin expression was tested. As presented in Figure 26, the analysis indicated that viperin was upregulated, but not IFN or PKR. Ct values for the reference gene, i.e. EF 1α were relatively

high (mean Ct 26) and with little variations.

Secondly, analysis of the expression of viperin in RBCs treated with the vitamins under questions were carried out (D). The results revealed that poly I: C combined with ercalcitriol give a trend of higher upregulation of viperin than when it was combined with calcitriol or without vitamins. In contrast, ercalcitriol, calcitriol and bexarotene showed no significant viperin regulation without stimulation with poly I:C (D in Figure 26).

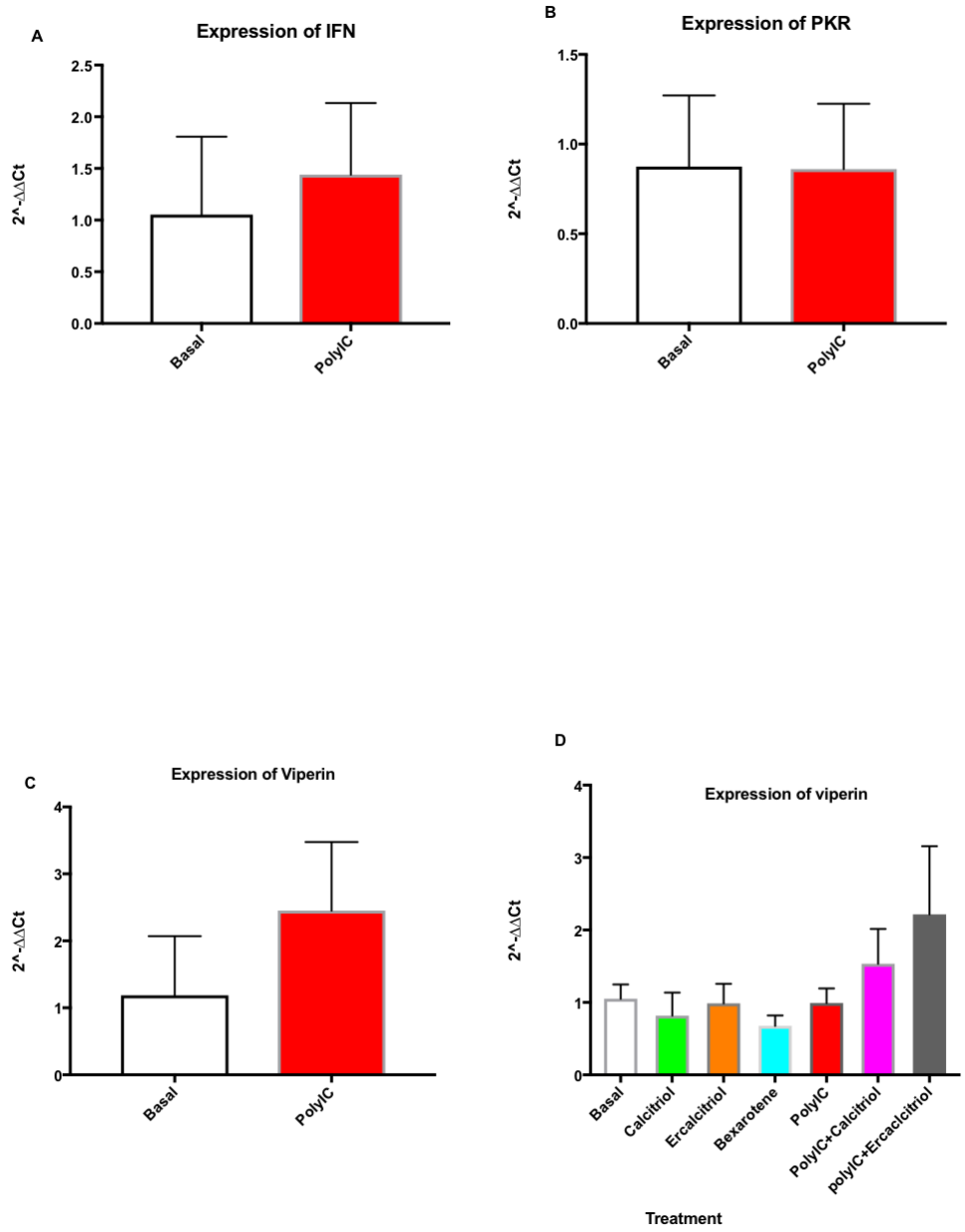

Figure 26: Analysis of expression of antiviral proteins in *ex-vivo* cultured RBCs stimulated with poly I: C (50 ng/µl) (N = 4): A) IFN-I, B) PKR, C) Viperin and D) Expression of viperin in RBCs treated with ercalcitriol, calcitriol and bexarotene at final concentrations of 0.1 µM, 0.1 µM and 10 µM respectively. The bars represent mean values of the fold changes +/- SD.

3.2 Experiment 2: Investigation the effect of vitamin D_2 and D_3 and bexarotene on the antiviral response and PRV replication in RBCs infected by PRV *in vivo*.

This experiment was performed in blood collected from the caudal vein of 3 fish (F1 – F3) that were infected by PRV as they were put in the same tank with PRV shedders, i.e. infection by cohabitation (6 wpc). Two different procedures were used. First, a two-step RT-qPCR was employed to analyze the expression of viperin and RIG-I when infected RBCs were treated or untreated with the above-mentioned vitamins. Secondly, one-step RT-qPCR (described in section 1.8.1.1) was used to analyze the viral load under different treatment conditions. The activities carried out at the different time points are shown in figure 27

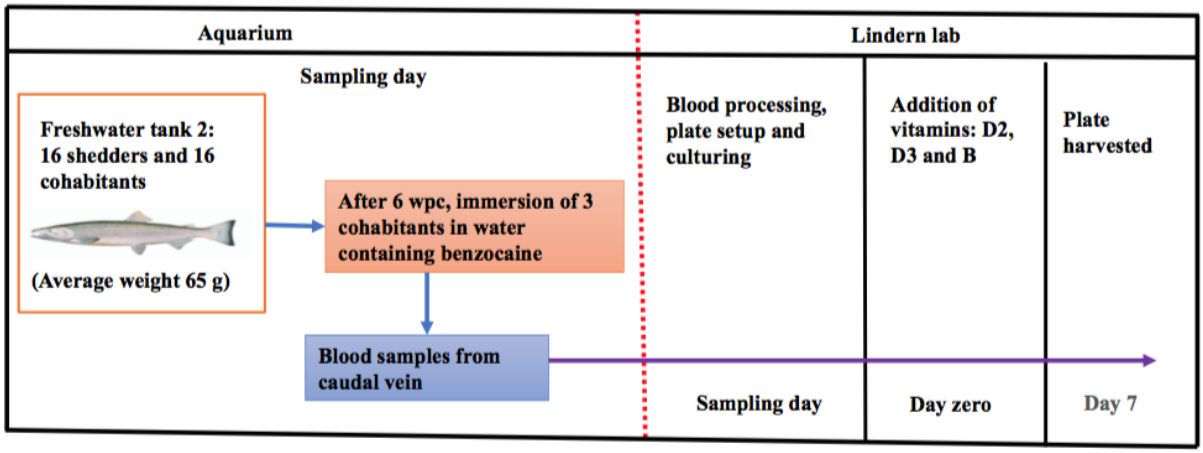

Figure 27: Summary of activities performed in experiment 2

Cell counting

Cell counting was performed as earlier described and the total cells calculated are presented in table 9. After calculation, the percentage of viable cells were found to be highest in fish 1 and lowest in fish 3 (Table 14). The lower viability of cells from two fish in this preparation (<90%) could be because the RBC were infected *in vivo*.

Table 14: Calculated total RBCs in cohabitant fish

Fish no.	Total cells	Viability
1	1.7*10^6	97 %
2	6.6*10^5	86 %
3	1.1*10^6	82 %

Plates seeding

After assessing the viability of the cells and calculating their numbers, plate setup was made. Accordingly, at the sampling day, 1 ml diluted RBCs from the cohabitant fish was cultured in L15+ (supplemented with 2% fetal calf serum (FCS) and gentamicin (50 µg/ml)) at a density of 60 mill/well, and incubated for 24 hours.

At day zero, media and the reagents were prepared as previously described. After preparations, 0.5 ml from each tube with ercalcitriol, calcitriol and bexarotene was added to each well- plate based on the setup to a final concentration of 0.1 µM, 0.1 µM and 10 µM, respectively, and incubated for one week, when the plate was harvested for RNA isolation and qPCR analysis.

PRV analysis

For PRV detection (Figure 28) qPCR analysis was performed in one-step, i.e. with cDNA synthesis and qPCR in the same reaction. The result shows that there were very low levels of virus in the fish at week 6 post challenge (6wpc) and that there was no significant difference in viral levels when RBCs infected by cohabitation were treated with calcitriol and ercalcitriol.

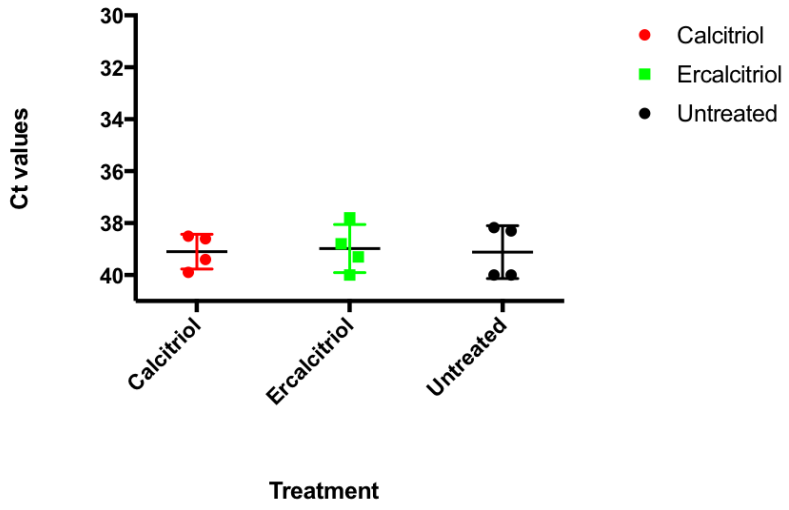

Figure 28: Mean Ct values of PRV levels (n = 3) in fish infected by cohabitation and treated with vitamins D2 and D3 as detected by RT-qPCR in RBCs at 6 wpc ($p \leq 0.05$).

Expression of genes in response to treatment of infected RBCs by cohabitation

After running two-step RT-qPCR to investigate the effect of the vitamins mentioned earlier, mean Ct values for the EF 1α was 30. The expressions of viperin and RIG-I are shown in Figure 29. There is a trend towards lower antiviral responses in RBC treated with calcitriol or ercalcitriol, but no significant difference ($p \leq 0.05$) in viperin and RIG-I expression. When infected cells were treated with calcitriol and bexarotene in combination no regulation was observed.

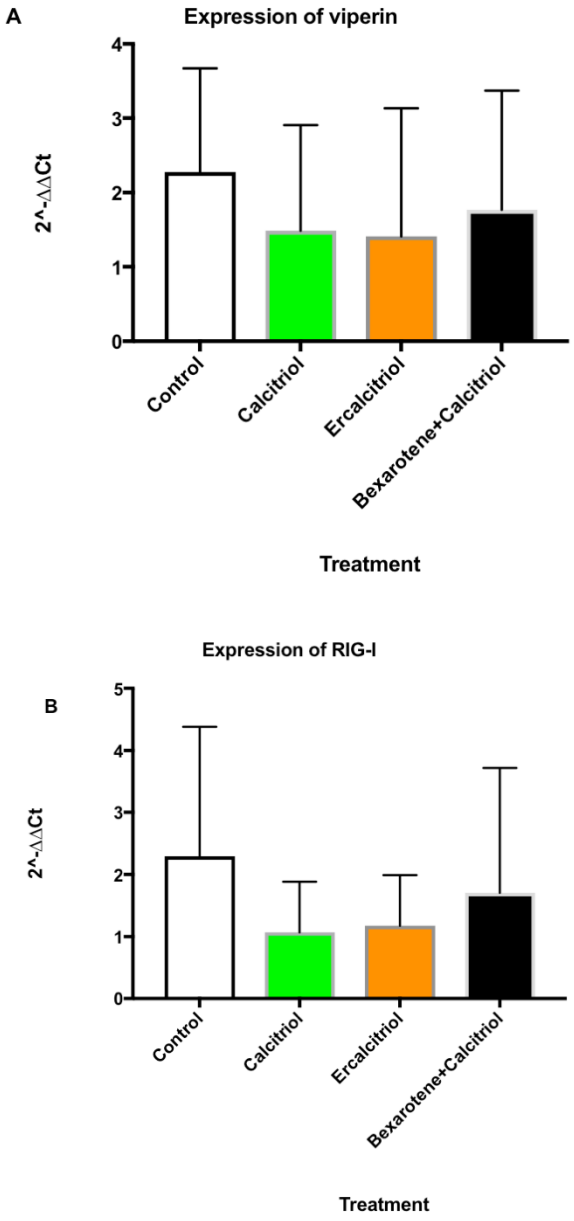

Figure 29: Expression of A) viperin and B) RIG-I in RBCs infected by PRV (infection by cohabitation at 6wpc under vitamin treatment by ercalcitriol (D2), calcitriol (D3) and bexarotene and control conditions (n = 3).

3.3 Experiment 3: Effects of vitamins on the antiviral response and viral load in RBC infected with pure PRV *ex vivo*

In this experiment, blood samples were collected from the caudal vein of 4 naïve fish and a summary of the activities performed is depicted in Figure 30.

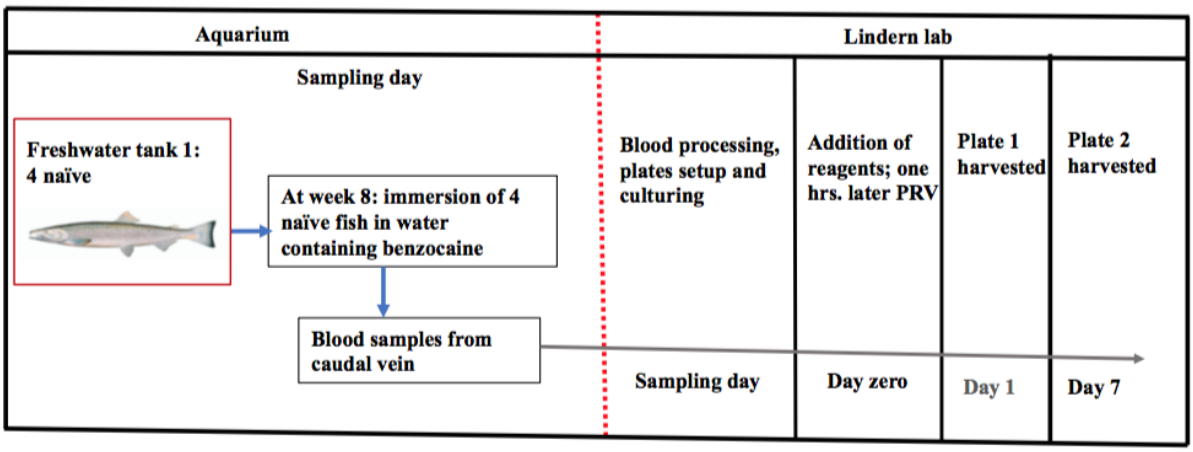

Figure 30: Summary of activities performed in experiment 3

To determine the effect of vitamin D_2, D_3 and bexarotene on the antiviral response and viral load following RBC infection with purified PRV *ex vivo*, the expression of viperin and RIG-I and the viral load were analyzed by two-step and one-step RT-qPCR methods.

Plates seeding

At day zero, media and reagents were prepared. Afterwards, 1.0 ml of L15 mixed with the reagents were added into each well in plate-1 and plate-2 according to the plate setup followed by plates incubation for one hour. Parallel to this, 30 µl from the virus batch B1was diluted in 3 ml L15 which contained 2 % FCS. After preparation, 50 µl from the mixture was added into the relevant wells. Cells were washed thoroughly after 24 hours to get rid of excess virus. After 1 day and 7 days incubation, plate-1 and plate-2 were respectively harvested for RNA isolation and RT-qPCR analysis.

PRV analysis

The levels of PRV are given as Ct values. There were positive PRV levels around Ct 28 in all wells after washing the cells at day 1, but no virus increase after one week, indicating that no replication occurred.

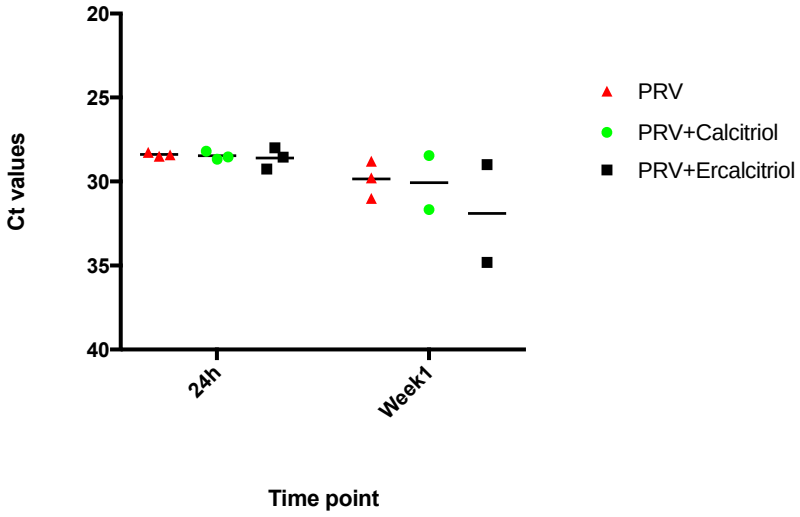

Figure 31: Mean Ct values of PRV levels (n = 2-3) as detected by RT-qPCR in RBCs after 24 hours and one week incubation in RBCs, treated with vitamins and infected *ex vivo*.

Expression of viperin and RIG-I genes

After running two-step RT-qPCR, the expressions of viperin and RIG-I are shown in figure 32. PRV treatment ex vivo tend to upregulate both genes. There is no significant difference ($p \leq 0.05$) when infected RBCs were treated with calcitriol and ercalcitriol in either viperin and RIG-I expressions. However, when infected cells were treated with calcitriol and bexarotene combined, a trend towards upregulation can be observed. The mean Ct value for the EF 1α was Ct 33.

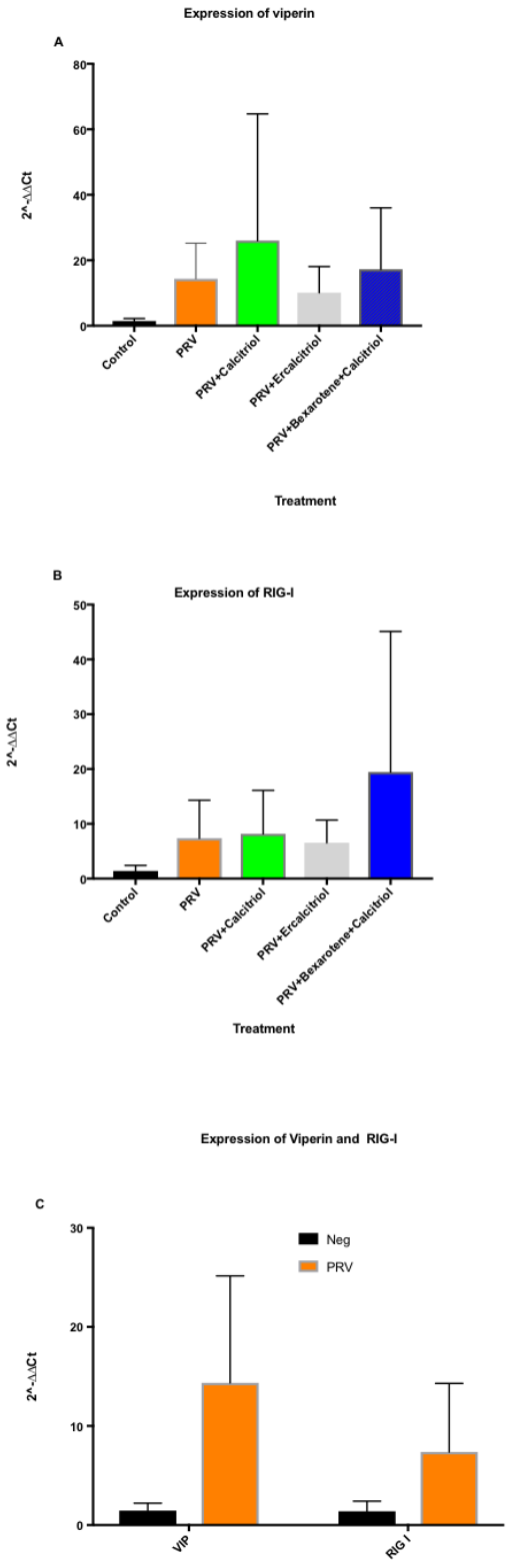

Figure 32**:** Expression of A) viperin, B) RIG-I and C) viperin and RIG-I in RBCs infected by PRV at 8 wpc under vitamin D2, D3 and bexarotene treatment and control conditions (n = 3).

4. Discussion

4.1 Discussion of the methods

Cell culture

Cell culture studies provide a valuable complement to *in vivo* experiments as well as allowing for a more controlled artificial environmental condition. Although primary cells offer many advantages, obtaining optimum viable cells can be a challenge. These challenges entail: avoiding cell mortality during processing, use the correct media for cell growth, distinguish live cells from dead cells during counting, ensure optimal conditions under incubation. Therefore, viability assessment both before and after incubation can help obtain an optimum downstream result.

Reagents used and their properties

Active vitamins, which were used in this experiment are unstable and can easily loose activity. They are not stable for very long in water, and must be stored in organic solvents like ethanol or DMSO. High concentration of ethanol/DMSO may disrupt the natural reaction of the cells. This is especially true for ethanol. To avoid this, DMSO was diluted to 1:1000.

The vitamins need to be stored frozen and dark, and not freeze-thawed more than a couple of times. For that reason, these vitamins need to be aliquoted into small batches prior to storage. In addition, they are toxic in high concentration, and should be handled with gloves. Finally, the active vitamins need to be kept sterile since they are being added to cell cultures.

Optimization of RNA quality

For RT-qPCR analysis special consideration should be given to the pre-PCR steps, since there are many possible sources of errors, especially during pipetting, dilution and RNA isolation. Both RNA purity and integrity play a critical role in the accuracy, reproducibility and relevance of downstream analyses, e.g. gene expression.

RNA Purity

During RNA purification, contaminants such as genomic DNA, DNases, RNases and proteases sometimes end up in the final extract, leading to an impure RNA extract. This is in turn, may lead to some of the following problems:

- **gDNA:** if not eliminated, it can be amplified along with cDNA during qRT-PCR, leading to over-estimation of the actual RNA concentration. However, a control where the reverse transcriptase step is omitted would be a control for this.
- **DNases:** can degrade cDNA for gene expression analysis
- **RNases:** degrade RNA before you analyze it, leading to an unclear set of results
- **Proteases:** can degrade enzymes used in downstream reactions (e.g. reverse transcriptase during cDNA synthesis)

It can be challenging to completely avoid contaminants in the samples, but a variety of post-extraction tools can be used to clean RNA from contaminants. gDNA can be removed by treating the samples with DNase prior to cDNA synthesis.

RNA integrity

This refers to the quality or integrity of the RNA molecules themselves. Total RNA extracts usually contain ribosomal RNA subunits, messenger RNA, tRNA and small RNAs. For gene expression and transcriptome analyses, mRNA is the desired target. RNA is inherently susceptible to RNase degradation and is a chemically unstable molecule which requires a lot more care than DNA. Carrying out experiments using poor RNA quality can have dramatic effects on RT-qPCR results.

Reverse transcription

In the reverse transcription (RT) step the RNA template is reverse transcribed into cDNA, which will later be amplified in the PCR. Therefore, optimization of the RT step is important in favor of PCR optimization. The primer assays used for qPCR have to be specific for the target of interest, sensitive regarding detection of low levels of target, and be designed with the optimal melt temperature for the assay conditions.

Finally, the achievement of reliable RT-qPCR results is only possible after application of an appropriate normalization method to correlate for the different amount of input RNA among samples. This is of course essential because the technique poses problems at various stages of sample preparation and processing (Bustin et al., 2009). The selection and validation of reference gene is vital for RT-qPCR data normalization and the selection of a suitable reference gene therefore remains critical (Kozera & Rapacz, 2013). Reference genes are internal control sequences different from the target. For a gene to be regarded as a reliable reference, it must meet several important criteria, in particular the expression level should be constant, i.e. unaffected by experimental factors. (Chervoneva et al., 2010).

4.2 General discussion

Virus diseases represent serious problems for the farmed fish production globally, including salmon farming industry in Norway. Of these, HSMI is an important disease, and PRV has recently been confirmed to be the causative agent (Wessel et al., 2017). PRV infects RBC prior to the infection of the myocytes of the heart (Finstad, Storset, et al., 2014). In fish, RBCs are nucleated and contain organelles in their cytoplasm, enabling these cells to be transcriptionally active and able to respond to invading pathogens. Upon invasion, cells respond to virus infection mainly through Toll-like receptor including TLR3 and cytosolic retinoic acid-inducible gene I (RIG-I) like receptor (RLR) (Kawai & Akira, 2008). Signaling through these receptors triggers expression of type I IFN (Honda, Takaoka, & Taniguchi, 2006) followed by IFN- stimulated genes (ISGs), in part through the interferon-regulatory factor (IRF) family of transcription factors (Lazear et al., 2013). The ISGs encode a diverse group of antiviral proteins which in turn, limits the virus replication.

During the last years few years, research groups in Norway and elsewhere have put great efforts into developing vaccines. that prepare the immune system of Atlantic salmon to protect against viral diseases in Atlantic salmon. In parallel, the role of feed components in strengthening the fish immune system against infectious diseases is also being studied. The main hypothesis of this study was that vitamin D_2 (ercalcitriol) and D_3 (calcitriol) together with the RXR-agonist bexarotene have an effect on antiviral responses in Atlantic salmon RBCs. This hypothesis was tested using stimulation with synthetic poly I: C *ex vivo* or treatment with PRV *ex vivo*. In addition, it has been hypothesized that these vitamins have an effect on PRV infection *in vivo*. Three experiments were performed. For the poly I: C stimulation experiment, three antiviral immune response genes were measured: IFN-I, PKR, and viperin.

Poly I: C is widely used as synthetic dsRNA analogue *in vivo* and *in vitro* and has also

been demonstrated to induce antiviral responses in Atlantic salmon. Poly I: C is a potential adjuvant candidate however, it has limited clinical applications in mammals due to its potency (DeWitte-Orr, Collins, Bauer, Bowdish, & Mossman, 2010). The toxicity seen in mammals does not seem to be that strong in fish. For example, poly I:C has been used as an effective adjuvant in studies using Japanese flounder (Zhou, Zhang, & Sun, 2014).

In this study, the result of the poly I: C stimulation of RBCs *ex vivo* indicated that there was a tendency towards upregulation of IFN1 and viperin, but the expression of PKR remained stable. One would expect that PKR would increase subsequent to an increase in IFN. This contradiction of result may be due to short time (24 h) of RBCs stimulation with poly I: C. When RBCs were treated with ercalcitriol, calcitriol and bexarotene, the expression of viperin tended to increase when poly I: C was combined with ercalcitriol, and to some extent with calcitriol.

It has been known for many years that Atlantic salmon cell lines secrete factors with antiviral activity after stimulation with poly I: C. Gene expression studies have implicated that IFN and ISGs are the primary factors responsible for this antiviral activity. Poly I: C has been shown to stimulate another type of antiviral defense, apoptosis, in the rainbow trout macrophage-like cell line, RTS11, which appeared to be mediated by PKR. Poly I: C injection in channel catfish (*Ictalurus punctatus*) prior to challenge with a herpesvirus (channel catfish virus or CCV) resulted in the upregulation of Mx mRNA (Plant, Harbottle, & Thune, 2005). Moreover, it was found that poly I:C strongly induced IFNc in head kidney, spleen, gills and heart of Atlantic salmon (Svingerud et al., 2012) whereas Mx1-3 and vig1 are upregulated in response to poly I: C and Chum salmon reovirus (DeWitte-Orr et al., 2007).

The effects of vitamin D_3 dietary administration on the expression of immune-related genes in head-kidney (HK) and gut were investigated in European sea bass (*Dicentrarchus labrax*) (Dioguardi et al., 2017). This study showed a modulation in the activities examined in fish fed with the addition of vitamin D_3. After 2 weeks of administration, diet supplementation with the vitamin resulted in increased phagocytic ability. No significant differences were observed in protease, anti-protease, natural haemolytic complement activities and total IgM level.

At gene level, fucose-binding lectin (fbl) and rhamnose-binding lectin (rbl) transcripts were up-regulated in HK in fish fed with the highest concentration of vitamin D_3-supplemented diets after 4 weeks, while in the gut, an up-regulation of the antimicrobial peptide hepcidine(*hep)* gene was observed in fish fed with vitamin D_3. These results suggest that vitamin D_3 may be of interest for immunostimulatory purpose in fish immune response in fish farms.

With regard to the viral levels, the result shows that there were no significant effects of calcitriol and ercalcitriol treatments. In a previous *ex vivo* erythrocyte study, infected RBC were reported to elicit an antiviral immune response (Wessel et al., 2015). IFN-α was significantly up-regulated at 1 day post infection (dpi). A significant up-regulation at 1 and 7 dpi was observed for Mx, RIG-I and PKR. These latter three genes followed a similar expression pattern with peak expression detected at 7 dpi, before the expression levels returned to baseline (Figure 33). This study demonstrates that PRV replicates in Atlantic salmon erythrocytes *ex vivo*. The *ex vivo* infection model closely reflects the situation in vivo and can be used to study the infection and replication mechanisms of PRV, as well as the antiviral immune responses of salmonid erythrocytes.

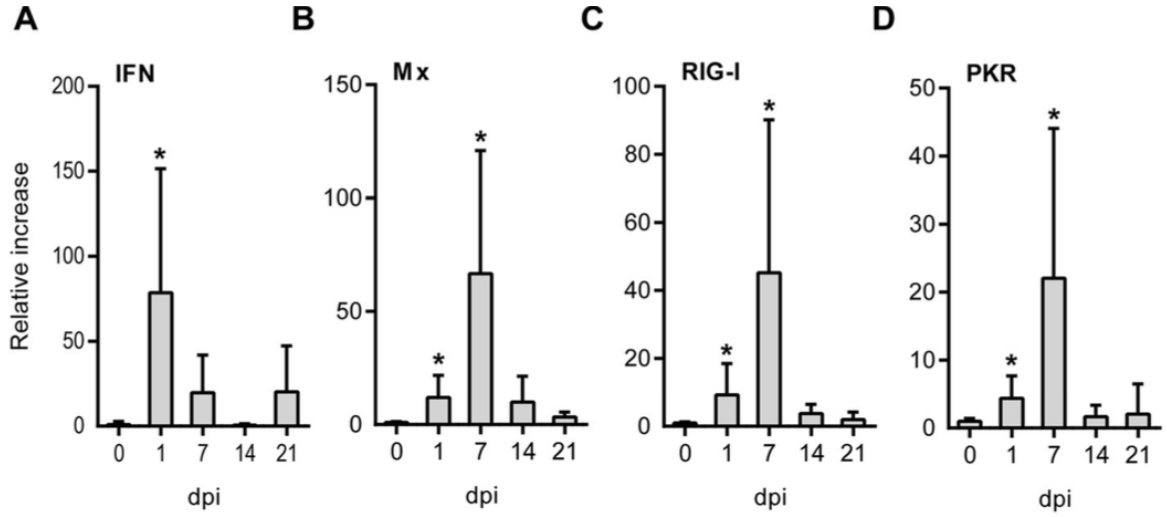

Figure 33: Erythrocytic antiviral responses to PRV infection. Expression of genes involved in antiviral responses was measured by RT-qPCR. The expression levels in infected RBC relative to the paired non-infected controls were calculated for each sample (n = 6) at 1, 7, 12 and 21 dpi. The relative increase (and SD) for IFNα (A), Mx (B), RIG-I (C) and PKR (D) is shown. Data were analyzed using Wilcoxon matched pairs signed rank test. *p < 0.05 (Wessel et al., 2015).

Recently, purified PRV particles were inoculated into naïve Atlantic salmon. The purified virus replicated in inoculated fish and spread to naïve cohabitants, and induced histopathological changes consistent with HSMI. The experiment demonstrated that infective PRV can be purified from blood cells, and that PRV is the etiological agent of HSMI in Atlantic salmon.

The RIG-I-like receptors (RLR) activate the host IFN system upon recognition of viral RNA in the cytoplasm and recently it has been confirmed that it is important to fish antiviral immunity (Chang et al., 2011). In our study, there is a tendency that calcitriol and bexarotene up-regulate RIG-I in RBCs infected by PRV *ex vivo*.

The *in vitro* effects of different innate-immune stimulants on Chinook salmon embryo cell line (CHSE-214), cells were stimulated with various inducers including vitamin D_3 (Estévez Estévez, 2015). The expressions of several immune genes were analyzed using qPCR. However, vitamin D_3 in a concentration of 20 nM (0.2 uM), showed no effect on the expression of RIG1.

Although RIG-I is most noted for its ability to bind dsRNA, recent studies have shown that RIG-I binds the 5'-triphosphate portion of RNA and thus can bind to ssRNA as well (Lu et al., 2010). Therefore, some ssRNA viruses are recognized primarily by RIG-I (influenza, vesicular stomatitis virus.

Influenza viruses infect airway epithelial cells, causing respiratory distress. In the A549 cell line, Influenza A (H1N1) virus was propagated and was used to assess the effect of vitamin D_3. Treatment of these cells with 100 nM and 30 nM of D_3 prior to/ or post-H1N1 exposure respectively did not affect viral clearance but significantly reduces autophagy and restored the apoptosis seen after H1N1 infection back to its constitutive level. However, vitamin D3 significantly decreased the levels of H1N1-induced tumor necrosis factor-alpha (TNF-α), IFN-beta (IFN-β), and ISG15. 1,25[OH]$_2$ D_3 treatment prior to/or post-H1N1 infection significantly down-regulated IL-8 as well as IL-6 RNA levels. These results demonstrate that calcitriol treatment suppresses the HINI-induced transcription of cytokines and chemokines in epithelial cells (Khare et al., 2013). In our study,

Additionally, assessment of the effect of vitamin D on the inflammatory profile of immune cells, using both human-derived immune cell lines and peripheral blood mononuclear cells (PBMCs) suggests that the active metabolite of vitamin D has an anti-inflammatory effect (Calton, Keane, Newsholme, & Soares, 2015) and down-regulates the expression and production of several pro-inflammatory cytokines including TNF- α, IL-1β, IL-6, and IL-8

(Giulietti et al., 2007; Neve, Corrado, & Cantatore, 2014). The two most common concentrations of 1,25$(OH)_2$ that elicited an anti-inflammatory response was 10 nM and 100 nM (Calton et al., 2015). Mechanisms proposed to mediate the anti-inflammatory effect of vitamin D included decreased protein expression of TLR-2 and TLR-4 (Di Rosa et al., 2012; Sadeghi et al., 2006).

While a number of *in vitro* studies have described the general effects of vitamin D metabolites on the function of immune cells and secretion of inflammatory molecules, experiments investigating the specific antiviral effects of vitamin D on virus infection are limited. In contrast to the results above, when rhinovirus infection of epithelial cells results in increased production and secretion of pro-inflammatory cytokines and chemokines, with the secretion of CXCL8 and CXCL10, this was further enhanced following treatment with 1,25$(OH)_2$D. During RSV infection, IκBα expression is reduced, resulting in increased transcription of NF-κB-driven genes. STAT1 is also phosphorylated and able to translocate into the nucleus resulting in increased expression of IRF1 and IRF7. Pre-treatment with 1,25(OH)2D increased IκBα expression and decreased STAT1 phosphorylation, resulting in decreased production of CXCL10, IFN-β, MxA, ISG15, IRF1 and IRF7.

Similarly, influenza A infection causes increased expression of pro-inflammatory cytokines and chemokines, with 1,25$(OH)_2$D treatment causing decreased expression of TNF-α, IFN-β, ISG15, CXCL8, IL-6 and CCL5. Finally, 1,25$(OH)_2$D is also able to increase LL-37 and HBD2 production, which have been shown to have antiviral effects against both RSV and influenza (Greiller & Martineau, 2015).

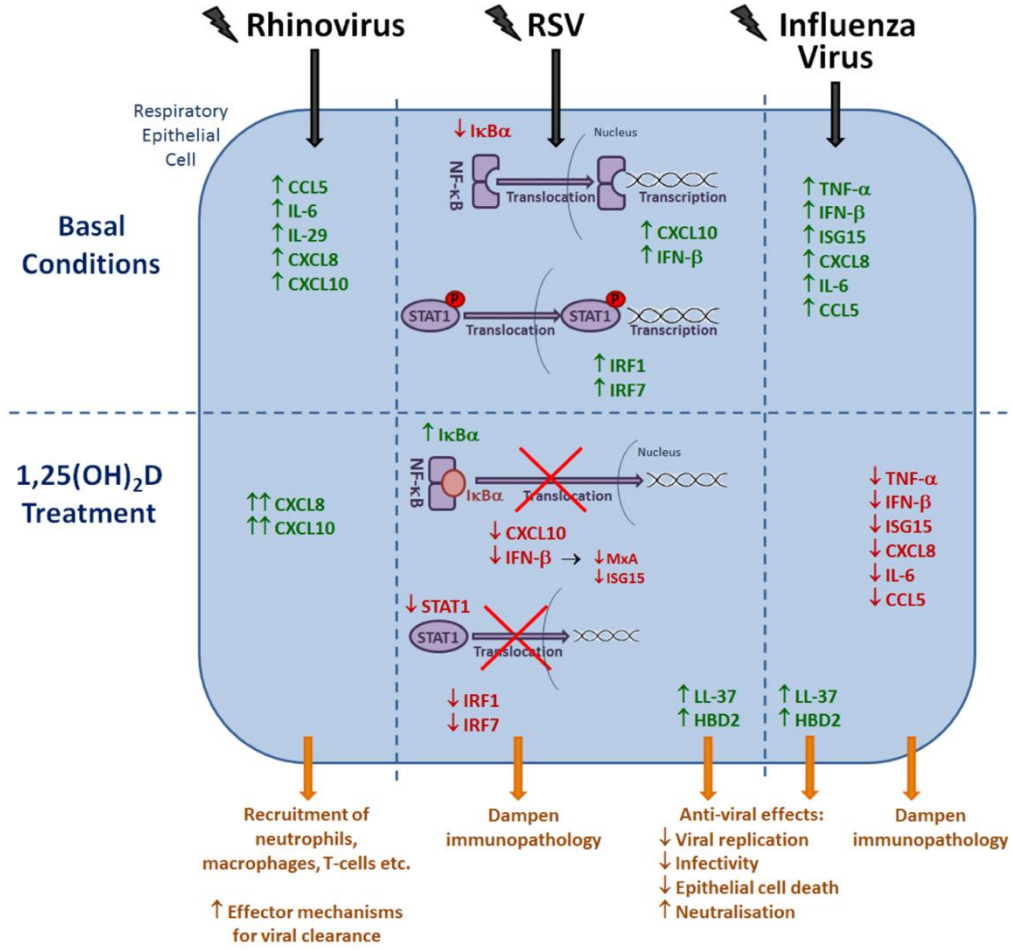

Figure 34: The immunomodulatory actions of 1,25(OH)2D against mammalian respiratory viruses (Greiller & Martineau, 2015).

The studies of the effect of vitamin D on influenza infection indicate that chemokines and pro-inflammatory cytokines are important targets for the vitamin D effect. Further studies on PRV infection and HSMI should focus on these responses.

Chemokines and inflammatory responses most likely play important roles in HSMI development, as HSMI is characterized by inflammation and recruitment of immune cells to the heart and muscle. Even if vitamin D does not affect PRV infection and replication, disease development may depend on the fat-soluble vitamins status of the farmed salmon

5. Conclusion

The present study investigated the effect of vitamin D_2 (ercalcitriol) and D_3 (calcitriol) together with the RXR-agonist bexarotene on antiviral responses in Atlantic salmon RBCs. Although the results are inconclusive, there is a trend that vitamin D can upregulate some antiviral genes in combination with poly I: C or PRV infection. Therefore, further characterization is necessary for understanding the potential for vitamin D to modulate innate immune responses against viruses in RBCs and other cell types. We have only tested one done of vitamins at few time points of stimulation in RBC in our study, and more thorough investigation could reveal more significant effect. Such effects would also be interesting to study *in vivo* by treating with vitamin D in combination with PRV infection

Some of the studies reviewed demonstrated effects of vitamin D on inflammatory responses, i.e. expression and secretion of pro-inflammatory cytokines and chemokines. As HSMI is a disease driven by an inflammation, the benefits of vitamin D on HSMI development should be investigated further.

In conclusion, vitamin D treatment may benefit farmed fish with regards to effect on antiviral responses and viral infection, and inflammation

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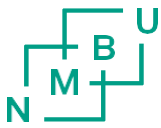

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