Studies on immunostimulants and innate immune gene expression in Atlantic salmon: candidate tools for marker selection

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90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in Biology

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Abstract

The aim of this project was to create tools to select robust fish with better innate immunity for breeding stock. The project was divided into three main stages. The first stage was to study basal expression of antimicrobial peptides (AMP) and inducible nitric oxide synthase (iNOS) of different Atlantic salmon (*Salmo salar*) families and investigate the effect of calcium supplemented β-Hydroxy-β-methyl butyrate (calcium-HMB) and β-Hydroxy-β-methyl butyrate (HMB) in the diet and water, as inductors of expression of these genes. The second stage was to test if increased expression of these genes has a defense role against bacterial and virus infection and the third stage was to initiate a search for single nucleotide polymorphism (SNP) markers in order to use them as selection markers for fish high-tolerance against pathogen infections.

For the first stage, two different experiments were performed. In the first experiment, gill lamella tissue of 1330 fish (133 families and 10 fish per family) were collected in order to study basal expression of Cathelicidin-2 (CATH-2), an important component for the first line of immune defenses. In addition, 14 families were selected, treated with calcium-HMB and, then, gill tissues were collected and analyze in order to study the induction of CATH-2. Different basal expression of CATH-2 was observed and we could see induction of expression in two of the 14 families. Calcium-HMB treatment did not work as expected, possibly due to the presence of calcium; therefore, we decided to change the treatment. In the second experiment, gill and skin tissues of 12 families (12 individuals per family) of Atlantic salmon were collected in order to investigate induction of CATH-2, Hepcidin-1 (Hep-1) and inducible nitric oxide synthase (iNOS), by adding HMB in the diet and water. Up-regulation of CATH-2 in gill samples and CATH-2 and iNOS in skin samples was observed in all the families. Interestingly, Hep-1 expression in skin samples, rather than being enhanced, was in some cases down-regulated. These studies provide us with possible and powerful molecular tools to choose the most robust families.

Once we had established conditions for inducing the expression of these AMPs and iNOS, we conducted a study in which a calculated number of 24-wells plates containing CHSE-214 cells, ASK cells and RTS-11 cells grown to 80% confluency, were pre-treated for 48h with the inducers HMB, PBA and vitaminD3. After this pre-treatment plates with CHSE214 were infected with infectious pancreatic necrosis virus (IPNV) and ASK cells with infections salmon anemia virus (ISAV) at a multiplicity of infection (MOI) of 0.1 (0.1 plaque forming units per cell) in a reference laboratory in Chile. In addition, a CHSE-214 cell plate and a RTS-11 cell plate were infected with *Pisciricketssia salmonis* in a different reference laboratory, also located in Chile. The findings of this study strongly suggest that pre-treating cells with concentrations of HMB known to induce expression of AMPs and iNOS, can lower IPNV and ISAV infectivity by 53% and 26% respectively. Unfortunately dilution of bacterial infection used for *P. salmonis* caused a violent infection and, at the moment of analysis, our internal housekeeping gene control was severely degraded preventing us from drawing any conclusions.

The third stage of the project was approached by analyzing fin samples from the second experiment (144 samples) by ddRAD sequencing. Fin samples were collected from 12 families, 6 containing the major quantitative trait locus (QTL) for IPNV and 6 non-QTLs, all with different basal expression of CATH-2. Our previous experiments allow us to build a phenotype with high and low CATH-2-basal expression families. By performing ddRAD sequencing we hope to find SNPs, which can be used as biomarkers for selection of fish robustness. ddRAD sequencing was successfully performed but data analysis is still underway.

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Abbreviations

AMPs:	Antimicrobial peptides
asCATH-2:	Atlantic salmon CATH-2 primer
ASK:	Atlantic salmon kidney cells
BCAA:	Branched chain amino acids
CAMP:	Human antimicrobial peptide
CATH-1:	Cathelicidin-1
CATH-2:	Cathelicidin-2
CHSE-14:	Chinook salmon embryo cells
csCATH-2:	Chinook salmon CATH-2 primer
Ct:	Cycle Threshold
ddRADSeq:	Double-digested RADSeq
DPI:	Days-post-induction

dsRNA:	Double-stranded RNA
EF1α:	Elongation factor 1α
eNOS:	Endothelial nitric oxide synthase
FBS:	Fetal bovine serum
FD:	Fold Differences
gDNA:	Genomic DNA
HDAC:	Histone deacetylase
HLP-1:	Histone-like-protein 1
HMB:	β-Hydroxy-β-methyl butyrate
hpi:	hours-post-infection
ic:	Infected cells
IFAT:	Indirect fluorescent antibody test
IFNs:	Interferons
IL:	Intereleucin

iNOS:	Inducible nitric oxide synthase
IPNV:	Infectious pancreatic necrosis virus
ISAV:	Infectious salmon anemia virus
ITS:	Internal transcribed spacer primer
KIC:	α-ketoisocaproate
LEAP:	Liver expressed antimicrobial peptide
LPS:	Lipopolysaccharides
MC1:	Complex medium-1
MOI:	Multiplicity of infection
NK cells:	Natural killer cells

nNOS:	Neuronal nitric oxide synthase
NO:	Nitric oxide
NOS:	NO synthase
p:	p value
PBA:	Phenylbutyrate
PD:	Pancreas disease
PFU:	Plaque forming units
PKA:	Potential killing activity
QTL:	Quantitative Trait Locus
RADSeq:	Restriction site-associated DNA sequencing
RBA:	Respiratory burst activity
RQ:	Relative quantity
RTS-11:	Rainbow trout macrophage cells
SE:	Standard error

SHK-1:	Atlantic salmon head kidney cells
SNPs:	Single-nucleotide polymorphisms
SRS:	Salmon rickettsial syndrome
TLRs:	Toll-like receptors
VDR:	Vitamin D Receptor
VitD3:	Vitamin D3
VP1:	RNA-dependent RNA polymerase
VP4:	Viral encoded serine-lysine protease

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1 Introduction

Teleost fish are the earliest class of vertebrates possessing the elements of both innate and adaptive immunity, hence, they play an important role in the development of the immune system and have been useful for further knowledge of the basic functions of immune response components (Whyte 2007).

The immune system is divided into two different kinds of response: the innate (non-specific) and the acquired (specific). Although the innate response generally precedes, activates and determines the nature of the acquired response, both interact in order to provide an effective immune response (Fearon Douglas 1996). There are important differences between fish and mammalian immune systems. For example, fish lack both bone narrow and lymph nodes, having the kidney as a major lymphoid organ in addition to the thymus, spleen and mucosa-associated lymphoid tissues (C. McL.Press and Evensen 1999). The acquired immune response of fish has an intrinsic inefficiency, resulting in a limited antibody repertoire, affinity maturation and memory and slow lymphocyte proliferation. In contrast, the innate immune system is a fundamental defense mechanism of fish due to its primary importance in combating infections (Magnadóttir 2006).

The innate system, which can be divided into physical barriers, cellular and humoral components (Magnadóttir 2006), has different defense mechanisms which are constitutive and responsive and provide protection by preventing the attachment, invasion or multiplication of microbes on or in the tissues (Ellis 2001). The mucosa-associated lymphoid tissues (physical barrier) in teleost fish include the gut, skin and gills and are exposed to the external environment and form the initial barrier to pathogens invasion (C. McL.Press and Evensen, 1999; Dalmo et al., 1997). If the invading antigen of the pathogen goes through the first line of defense, it is faced with a repertoire of soluble (complement, transferrins, anti-proteases, haemolysin, lysozyme, interferon, C-reactive protein) and cellular (leukocytes, including macrophages, neutrophils, lymphocytes and scavenger endothelial cells) defenses which interact to initiate the inflammatory response with the help of the principal inducers of the innate immune response, Toll-like receptors (TLRs) (Whyte 2007).

Non-specific humoral defense substances such as antimicrobial peptides, the complement system, natural antibodies containing IgM as immunoglobulin, lectins, cytokines, interferons, interleukins, chemokines, among others have been shown to be involved in immune defenses in teleost fish (Whyte 2007). Moreover, the cellular component of the innate immunity is characterized by its non-specificity, permitting large populations of cells to be mobilized rapidly either at local and/or systemic sites after antigenic stimulation (Whyte 2007). The cellular response starts with an influx of neutrophils followed by arrival of monocytes/macrophages. At the site of inflammation, macrophages may become stimulated with an increase of phagocytic potential and an enhanced antimicrobial activity. Pathogen may be killed after production of these toxic intermediates called antimicrobial peptides (Whyte 2007).

Fish are in contact with high concentrations of bacteria and viruses, present in the environment. Even though fish maintain a healthy state by defending themselves against pathogens using the innate defense mechanisms explained above, viral and bacterial diseases are responsible for many disease outbreaks which currently cause substantial economic losses in aquaculture. Some viruses mainly affect young fish e.g. IPNV in salmonids, while other cause mortality in fish throughout their life e.g. ISAV (Ellis 2001). Comparatively little is known about the host defenses involved in viral infections (Interferon and Mx proteins, anti-viral cytotoxic cells and miscellaneous innate anti-viral defenses such as glucan-induced, complement and genetic resistance) (Ellis 2001) and how viruses overcome them but it is known that for viruses to replicate in fish cells, they first have to attach to the surface of the cell, cross the cell membrane and engage the cell‘s biochemical machinery for nucleic acid and protein production (Ellis 2001). The host defenses of fish against bacterial infections include production of antimicrobial substances and acute phase proteins, non-classical complement activation, release of cytokines, inflammation and phagocytosis. Disease outbreaks and mortalities because of bacterial infections often result from the fish being stressed, leading to compromise of these defense mechanisms (Ellis 2001).

1.1 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are widely distributed in nature. They were described as part of the innate immune system, and are conserved among different species and present in both the animal and plant kingdoms (Zasloff 2002). Fish have evolved to survive in unique aquatic environments, coexisting with diverse microbial communities encountered by different fish species (Masso-Silva & Diamond 2014). AMPs are widely expressed in leukocytes and mucosa epithelial cells, lining the respiratory, gastrointestinal and urogenital systems of the host. More than 1200 different antimicrobial peptides of different origins have been identified or predicted. Most of them share common characteristics e.g. being small (12-50 amino acids), containing positive charge and an amphipathic structure (Lai & Gallo 2009). AMPs can be divided into several categories based on their structures including peptides with α-helix structures, peptides with β-sheet structures stabilized by disulfide bridges or peptides with extended or loop structures (Lai & Gallo 2009).

AMPs are synthesized as inactive pre-proteins and are cleaved to release the active peptide after stimulation (Scocchi et al. 1992). The positive charge of the AMPs causes them to have affinity to negative charge of the microbial membranes, entering into an electrostatic interaction and disrupting the osmotic balance of the microbial membrane (Casadei et al. 2013). This is a powerful immune mechanism due to the difficulty for microbes to change the overall negative charge of their membrane phospholipids, making the development of resistance against AMPs extremely difficult (Yeaman & Yount 2003). Then, peptides migrate through the membrane of the pathogens to the interior and disrupt intracellular targets, ending with the destabilization of the membrane and eventual cell lysis (Broekman 2012).

AMPs belong to four different families: defensins (Nam et al. 2010; Casadei et al. 2009), cathelicidins (Chang et al. 2006; Broekman et al. 2011), piscidins (Zahran & Noga 2010) and liver expressed antimicrobial peptides (LEAP), which include LEAP-1 or Hepcidin-1 (Bao et al. 2005) and LEAP-2 (Zhang et al. 2004).

AMPs exhibit multiple functions relevant for the innate immune system (**Figure 1**). They are known for their capacity to directly kill or inhibit the growth of microbes such as Gram-

positive and Gram-negative bacteria, fungi, protozoa and enveloped virus. AMPs families may react differently to pathogen infections or immune stimulation due to their highly specificity (Lai & Gallo 2009). Besides having antimicrobial and antiviral properties, AMPs have the capacity to suppress inflammation and protect the host from excessive production of pro-inflammatory mediators triggered by microbial products by neutralizing bacterial endotoxins, inhibiting pro-inflammatory cytokine production, inducing anti-inflammatory cytokines and preventing classical and lectin complement cascades (Sunkara 2011; Easton et al. 2009; Groeneveld et al. 2007). AMPs also induce production of various pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 as well as chemokines such as IL-8 and monocyte chemotactic protein-1 from mononuclear phagocytes and epithelial cells (Sunkara 2011; Auvynet & Rosenstein 2009). In addition, AMPs have been shown to promote re-epithelialization angiogenesis and vascularization by inducing proliferation of epithelial cells and vascular endothelial cells and chemo-attracting fibroblast and macrophages (Steinstraesser et al. 2011).

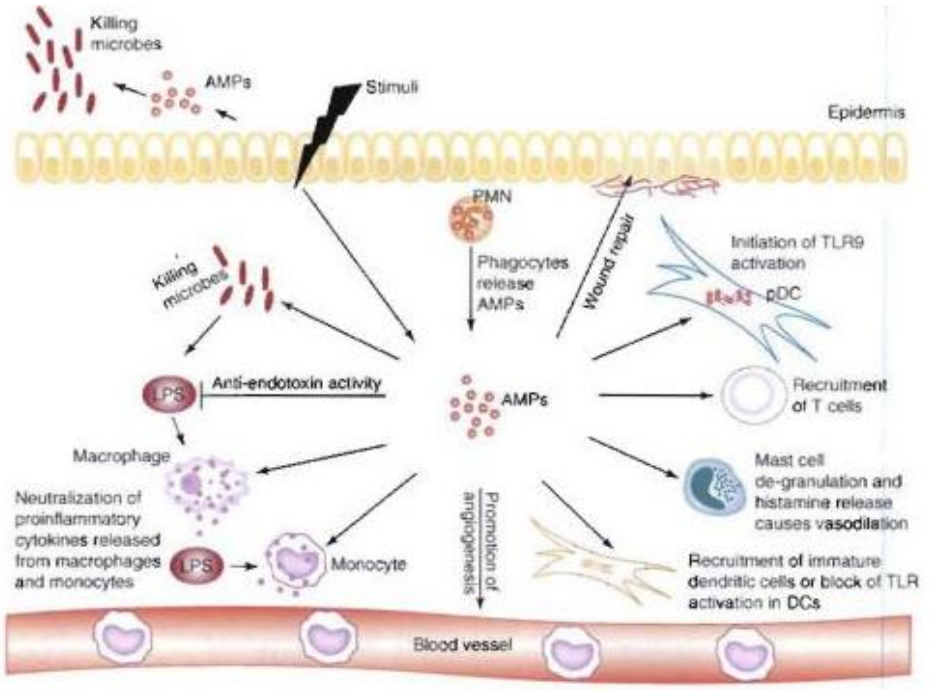

Figure 1. Biological functions of Antimicrobial peptides (AMPs) in host defense. Figure was adopted from (Lai & Gallo 2009)

1.1.1 Cathelicidin

Cathelicidins are a group of peptides that share a highly conserved region (so called "preproregion"), containing a cathelin-like N-terminal domain and a variable C-terminal domain that encodes the mature antimicrobial peptide (**Figure 2**). They are stored in the cytoplasmic granules of neutrophil leukocytes and release the antimicrobial peptides upon leukocyte activation (Zanetti et al. 1995). The activation results in the release of the antimicrobial peptide due to a processing enzyme which, for most of the cathelicidins is elastase (Broekman et al. 2011; Chang et al. 2006). Cathelicidin AMPs are highly heterogeneous and the reason these molecules have been assigned to the same group (cathelicidin proteins) despite showing such a marked structural diversity, is that they share the same structure (Zanetti 2004). This family of cationic peptides is known to exert antimicrobial activity at physiological concentrations and peptides of this class are thought to be an important component of the host immune system (Bridle et al. 2011). In vitro studies showed that mammalian cathelicidins possess broad antimicrobial activities capacitating defense against a range of Gram negative and Gram positive bacteria, fungi, parasites and viruses (Ramanathan et al. 2002). Besides the antimicrobial role, mammalian cathelicidins possess several other biological activities including ability to chemoattract neutrophils, monocytes and T cells and promote angiogenesis and wound healing (Agerberth et al. 2000; Carretero et al. 2007; Bucki et al. 2010).

Cathelicidins were firstly identified in in mammalian myeloid cells (Zanetti et al. 1995; Zanetti 2005). Since then, they have been found in many species such as birds (Xiao et al. 2006; van Dijk et al. 2011), snakes (Zhao et al. 2008) and fish (Uzzell et al. 2003; Chang et al. 2006). Two cathelicidin, named asCATH-1 and asCATH-2, were reported in Atlantic salmon, our organism of interest. Both proteins share common characteristics with mammalian cathelicidin genes. They are transcribed from four exons and possess a highly conserved preproregion and four invariant cysteines clustered in the C-terminal region of a cathelin-like domain (Chang et al. 2006). In contrast to the cathelicidins in mammals, fish cathelicidins have a shorter signal peptide (22 to 26 aa) compared with mammalian (29 to 30 aa) and a longer cathelin-like domain (115 to 127 aa in fish versus 94 to 114 in mammals) (Chang et al. 2006). Interestingly, asCATH-2 showed constitutive expression in a range of organs from healthy Atlantic salmon except the liver, while asCATH-1 was not expressed

in any of the tissues. However, both were induced during infection with *Yersinia ruckeri* and the upregulation occurred in gill and spleen tissues but no bactericidal activity was found against *Y. ruckeri* (Bridle et al. 2011).

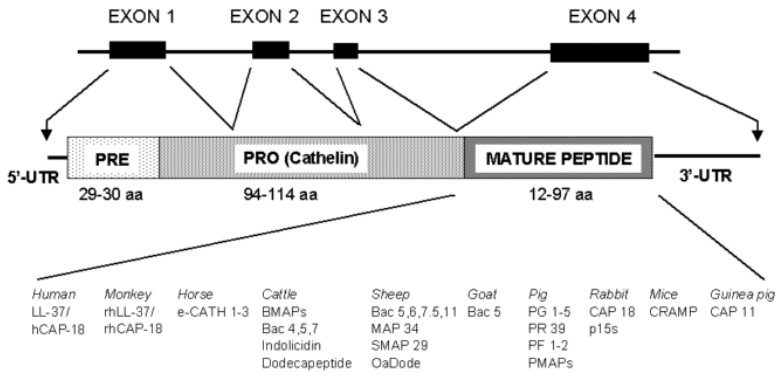

Figure 2. Representation of prepropeptides of Cathelicidin family and a list of encoded precursor peptides (fish encoded peptides are not included). Figure was adopted from (Ramanathan et al. 2002)

1.1.2 Hepcidin

Hepcidin, also called LEAP-1 (liver-expressed antimicrobial peptide), is a small cysteine-rich 25-residue antimicrobial peptide synthesized as a prepropeptide and matured by cleavage of a signal peptide (Krause et al. 2000). The Hepcidin gene has three exons and two introns conserved in fish such as catfish (Bao et al. 2005), Atlantic cod (Solstad et al. 2008) and Atlantic salmon (Douglas et al. 2003). It was first isolated from human plasma ultrafiltrate and urine. It was predominantly detected in the liver and, at much lower concentrations, in heart and brain (Park et al. 2001; Krause et al. 2000). Hepcidin has been shown to be widespread in fish. Hepcidin gene expression in catfish was found in most of the tissues except brain and the highest expression was observed in the liver and spleen, followed by gill and intestine. The lowest expression was in muscles, stomach and skin (Bao et al. 2005). Moreover, the catfish Hepcidin gene is expressed early during development and lower expression was found immediately after hatching (Bao et al. 2005). In Atlantic salmon, two kind of Hepcidin exit, Sal1 and Sal2. They differ from each other at four residues in the mature peptide and four residues in the upstream pre-protein portion (Douglas et al. 2003).

Sal1 was found to be expressed at high levels in the liver, blood and muscle and at low levels in gill and skin while Sal2 was barely detectable in the gill and skin (Douglas et al. 2003).

Hepcidin was first acknowledged for its antimicrobial activity (Solstad et al. 2008; Park et al. 2001; Krause et al. 2000), but was later recognized as a key regulator of iron homeostasis (Nicolas et al. 2001) and inhibitor of biofilm formation (Lombardi et al. 2015). Krause et al in 2000 showed the antimicrobial effects of LEAP-1 on Gram-positive bacteria, and on Gram-negative bacteria and the yeast *Saccharomyces cerevisiae*, inhibiting their growth. In addition, antimicrobial activities were found for Atlantic salmon Hepcidin genes, where both Sal1 and Sal2 were upregulated during infection with *Aeromonas salmonicida* but differently dependent on tissue type (Douglas et al. 2003). In catfish, the Hepcidin gene was upregulated after infection with *Edwardsiella ictaluri* in a tissue specific manner as well (Bao et al. 2005).

Hepcidin, in humans, controls levels of extracellular iron, is induced during inflammation, trapping iron in macrophages and decreasing plasma iron concentrations (Ganz & Nemeth 2006; Ganz 2011).Similarly, in fish, Hepcidin increases iron accumulation in macrophages and increases dietary iron absorption in duodenal crypt cells (Douglas et al. 2003).

1.1.3 Inducible nitric oxide synthase (iNOS)

Nitric oxide (NO) is now known to exist in essentially every tissue within the body. NO acts as a regulator and effector molecule in a myriad of biological processes. For instance, it works as inhibitor of cell proliferation, as mediator of antitumor activity, and as neuronal messenger. It also confers nonspecific antimicrobial activity against various pathogens, causes smooth muscle relaxation, alters platelet function (Nathan 1992). The antimicrobial activity of NO as a nonspecific defense mechanism was demonstrated against a variety of pathogens such as extracellular schistosomula of *Schistosoma mansoni* (James & Glaven 1989), extracellular forms of *Trypanosoma brucei* (Vincendeau et al. 1992) and *T. cruzi* (Muñoz-Fernández et al. 1992), and intracellular forms of *Mycobacterium leprae* (Adams et al. 1991). Moreover, NO is produced in various cell types of the immune-system, for instance dendritic cells, NK cells, mast cells and phagocytic cells as well as other cells

including endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts and hepatocytes (Bogdan 2001). NO is formed through the oxidation of l-arginine, by NO synthase (NOS). Three types of NO synthases are known: one inducible type (iNOS) and two constitutive types termed neuronal and endothelial NOS (nNOS and eNOS, respectively) (Saeij et al. 2000; Nathan 1992).

iNOS has been found in macrophages, dendritic cells and natural killer (NK) cells and in cell lines, clones, hybridomas and tumor cells of B or T cell origin (Bogdan 2001). iNOS activity *in vitro* is only detected after exposure of immune cells to cytokines, bacterial lipopolysaccharides (LPS) or parasites (Nathan 1992). Its expression is activated by the binding of NF-κB to the gene promoter and regulated by cytokines such as IL-1β or TNF-α (Bogdan 2001).

High levels of iNOS have been reported in the gills and skin of infected fish, for example after infection of rainbow trout and/or Atlantic salmon with different ectoparasites e.g. *Gyrodactylus derjavini* (Lindenstrøm et al. 2004), *Ichthyophthirius multifilii* (Sigh et al. 2004), Neoparamoeba sp. (Bridle et al. 2006) and *Lepeophtheirus salmonis* (Braden et al. 2012).

1.2 Disease prevention

Global fish production has grown consistently in the last five decades due to increased consumption, from an average of 9.9kg in the 1960s to 19.2kg in 2012 (FAO 2014). According to the latest available statistics collected by FAO, world aquaculture production reached 66.6 million tons, including finfishes, crustaceans, mollusks, amphibians, freshwater turtles and other aquatic animals (such as sea cucumbers, sea urchins, sea squirts and edible jellyfish). Because of this increase in demand, aquaculture companies are currently trying to grow fish in reduced space and as fast as possible under conditions that are not ideal for the physiological functioning of the fish. Some fish may tolerate a certain degree of suboptimal conditions but, beyond a certain level, fish may become stressed and there is evidence that chronic stress can depress AMP levels causing depression of the immune system. Chronic stress has for example been shown to reduce levels of AMPs in channel catfish skin causing a significant decrease in histone-like protein 1 (HLP-1) (Noga

et al. 2011; Robinette & Noga 2001). Downregulation of AMPs, hence, can plausibly cause a decrease in the resistance to disease outbreaks, while close proximity of fish simultaneously increases the likelihood of rapid disease spread.

Upregulation of AMP expression, contrarily, could represent a powerful tool to enhance the immune system in aquaculture populations (**Figure 3**). Enhanced immune activity, for example enhanced AMP concentrations, could reduce opportunistic infections as AMP responses are typically very rapid and may be protective before stressful events (transportation, grading, tank exchanges, etc.) (Noga et al. 2011). Also, the extremely difficulty for pathogens to evolve resistance to AMPs poses a great advantage. Although some scientist have reported that the effect of immune stimulants on the immune system is minimal and can in some cases be detrimental to animals that are still growing (Bricknell & Dalmo 2005), we hypothesize that substantial enhancement of the innate immune system in aquaculture populations could be accomplished by administering immunostimulants, which are explained bellow. Bao et al. (2005) have shown that the expression of Hepcidin was detected early during embryonic and larval development, and was induced after bacterial infection with *Edwardsiella ictaluri*, making it unlikely that this peptide will be detrimental even to growing fish as long as concentrations are low.

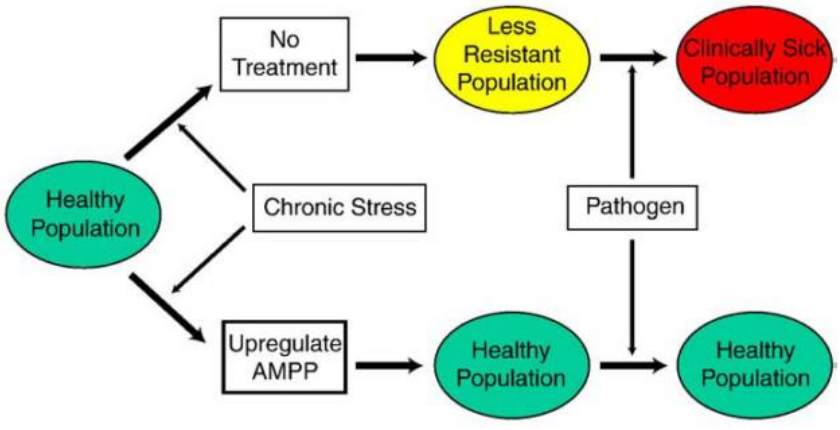

Figure 3.Upregulation of AMPs to anticipate immunosuppressive event. AMPs levels in fish population can be enhanced so that immune defenses are stronger before a stress event, hence, the fish population remains resistant to pathogens present either in the environment or in a latent state in the population. Figure was adopted from (Noga et al. 2011)

1.2.1 B-Hydroxy-β-methyl butyrate (HMB) as immunostimulant

B-Hydroxy-β-methyl butyrate (HMB) is a catabolite of the amino acid leucine. Leucine is an essential amino acid and is also one of the three branched chain amino acids (BCAA), which composes a third of skeletal muscles in the human body, being important in protein synthesis processes. Leucine's catabolism (**Figure 4**) starts with its transamination to α-ketoisocaproate (KIC) in muscle cells, which can be further excreted from muscle, transported to the liver and metabolized in the cytosol of cells to HMB before, being released into circulation. Approximately, 5% of KIC is metabolized to HMB by KIC-dioxygenase (Siwicki et al. 2000; Nissen & Abumrad 1997). Recently, the interest in the role of leucine and leucine catabolites e.g. HMB in disease prevention has increased (Siwicki et al. 2003).

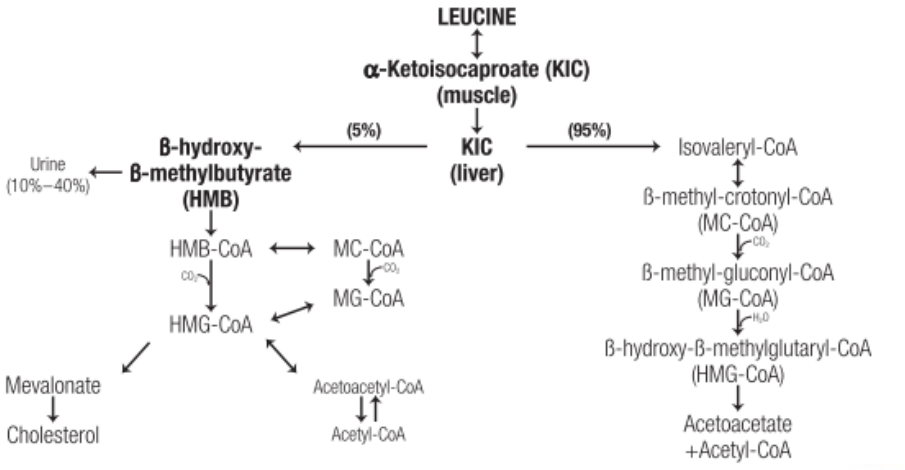

Figure 4.Leucine-HMB metabolic pathway. Figure originally adopted from (Nissen & Abumrad 1997) and modified in a review made by Abbott (Abbot company, 2010)

HMB has been shown to stabilize the muscle cell membrane (Nissen & Abumrad 1997), modulate protein degradation (Eley et al. 2008; Eley et al. 2007) and upregulate protein synthesis (Eley et al. 2008). Several studies have shown that HMB may be able to act as an immunostimulant. For instance, addition of HMB to the medium in both rainbow trout and carp resulted in an increased in respiratory burst activity (RBA), the potential killing activity of neutrophils and monocytes (PKA) and lymphocyte proliferation. This has led to speculations that HMB could improve immunocompetent cell activity in fish (Siwicki et al.

2000). Moreover, in a study on rainbow trout, Siwicki et al in (2003) suggested that HMB acts as an immunomodulator and stimulates the non-specific cellular and humoral immunity. When rainbow trout were fed with HMB and challenged with *A. salmonicida*. HMB showed some protective effects.

In our previous studies performed, we showed that supplementing HMB in food and water stimulates innate immunity, specifically Cathelicidin-2 and iNOS in ATbut, interestingly, the stimulation occurred at different times (Cathelicidin-2 activated in the first days-post-induction, iNOS activated at 13 days-post-induction and Hepcidin-1 expressed at 20 days-post-induction), hence, it may act as some kind of orchestration between innate-immunity-related genes (unpublished results).

1.2.2 Phenylbutyrate (PBA) as immunostimulant

Phenylbutyrate (PBA, or 4-phenylbutyrate) is the salt of an aromatic fatty acid, made up of an aromatic ring and butyric acid. It has three main biological effects: ammonia scavenger (Lichter-Konecki et al. 2011), weak histone deacetylase (HDAC) inhibitor (Miller et al. 2011) and endoplasmic reticulum stress inhibitor (Xiao et al. 2011).

Recently studies showed that clinical doses of PBA induce the expression of cathelicidin mRNA in three human cell lines (Steinmann et al. 2009). In addition, these authors reported that PBA acts synergistically with 1,25-dihydroxyvitamin D_3 in the induction of human antimicrobial peptide (CAMP) gene expression at the mRNA level (Steinmann et al. 2009). Even though the mechanism of action for PBA is still unclear Steinmann et al (2009) showed that PBA does not have a direct effect on the chromatin structure at the CAMP gene promoter, but it may increase histone acetylation facilitating expression of other genes, encoding critical factors for CAMP gene expression.

1.2.3 Vitamin D3 as immunostimulant

Vitamin D is an essential nutrient, required for optimal absorption of dietary calcium and phosphate. It can be obtained from diet and by the action of sunlight on the skin. Vitamin D is produced in the skin by the photolytic cleavage of 7-dehydrocholesterol followed by thermal isomerization. Then it is transported to the liver where it is converted to 25-hydroxyvitamin D3. The formation of the steroid hormone 1,25-dihydroxyvitamin D3, also called calcitriol, occurs primarily, but no exclusively, in the kidney and constitutes the final activation step (**Figure 5**) (Dusso et al. 2005). 1,25-dihydroxyvitamin D3 requires, for most of its biological activities, the high-affinity vitamin D receptor (VDR), a transcription factor which is member of the superfamily of nuclear receptors for steroid hormones. This interaction is also necessary for 1,25-dihydroxyvitamin D3 to affect gene expression regulation (Gombart 2011).

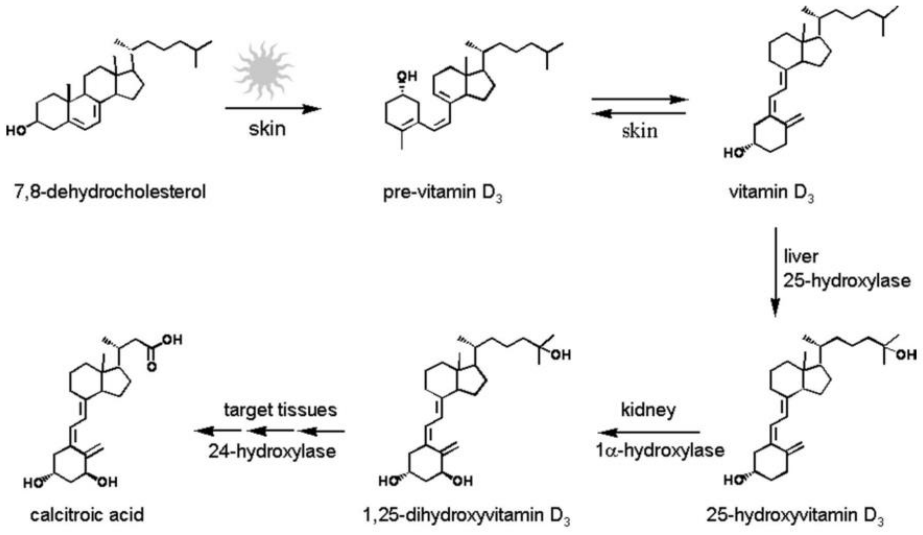

Figure 5. Vitamin D3 synthesis, activation and catabolism . Figure adopted from (Dusso et al. 2005)

Vitamin D is essential for the development and maintenance of a mineralized skeleton, essential to enhance the efficiency of absorption of dietary calcium phosphate, a potent modulator of parathyroid function, among other functions (Dusso et al. 2005). Many studies have suggested diverse functions in preventing cancer, modulating the immune system and controlling various endocrine systems due to the ability of 1,25-dihydroxyvitamin D3 to inhibit growth and promote differentiation of a myriad of cell types (Dusso et al. 2005). In addition, the vitamin D system plays a crucial role in calcium and phosphate handling in Atlantic salmon (Lock et al. 2007). As explained above, 1,25-dihydroxyvitamin D3 regulates the expression of many genes and many of them such as cathelicidin are involved in immunity (Liu et al. 2006).

The importance of 1,25-dihydroxyvitamin D3 for immune function was brought to light by the discovery of VDR expression in activated inflammatory cells (Gombart 2011; Provvedini et al. 1983; Bhalla et al 1983). 1,25-dihydroxyvitamin D3 can boost the innate immune system to combat pathogenic infections *in vitro* (Gombart 2011) and in humans its "antibiotic effect" appears to be mediated in part by induction of genes encoding antimicrobial peptides (Liu et al. 2006; Gombart 2011; Wang et al. 2004). Gombart et al (2011) provided evidence that the CAMP gene is a direct target of VDR that mediates the strong up-regulation of CAMP in response to 1,25-dihydroxyvitamin D3 treatment. There are, however, no published studies in fish in which AMPs are induced by 1,25-dihydroxyvitamin D3 treatment.

1.3 The most prevalent diseases affecting salmonids in aquaculture

1.3.1 Infectious pancreatic necrosis virus (IPNV)

Infectious pancreatic necrosis virus is a bi-segmented double-stranded RNA (dsRNA) virus of the *Birnaviridae* family, encoding five viral proteins. Segment A encodes a polyprotein which is cotranslationally cleaved by the viral encoded serine-lysine protease (VP4) to release the proteins pVP2 and VP3; and segment B encodes the RNA-dependent RNA polymerase VP1 (Dobos 1986; Duncan et al. 1987).

IPNV is responsible for many outbreaks, which occur frequently in farmed salmon fry and post-smolts (Skjesol et al. 2011). However, mortality rates vary between outbreaks because of differences in susceptibility of the host (Guy et al. 2006), due to the influence of environmental stress (Jarp et al. 1994; Taksdal et al. 1998); and as a consequence of different virulence of viral strains (Santi et al. 2005). Although IPNV can be highly destructive in hatchery-reared salmonids, it has been reported to be carried and possibly replicated in hosts for long time periods without causing clinical disease, indicating that the virus is either sequestered and present in low numbers that current detection systems do not detect or is present only as genetic material which would not interact with the immune system (Sadasiv 1995). One study showed that relatively high virus titers (up to 10^5 infectious doses) can be found in healthy fish, but titers of 10^6 to 10^9 infectious doses per gram of tissue would have been determined as lethal (Evensen & Rimstad 1990).

IPNV has been isolated from Atlantic salmon (Swanson and Gillespie 1979). Espinoza and Kuznar (2002) found that IPNV can propagate in several lines of cultured cells e.g. chinook salmon embryo cells (CHSE-214). In addition, published studies showed that cytopathic effects or a persistent infection were achieved (Ledo et al. 1990; Sadasiv 1995).

Furthermore, several studies have shown strong activation of immune genes upon challenge with highly virulent IPNV isolates (Ingerslev et al. 2009; Skjesol et al. 2011). Type I interferons (IFNs) and the IFN-inducible Mx gene were among the most highly upregulated genes (Skjesol et al. 2011).

1.3.2 Infectious salmon anemia virus (ISAV)

ISAV is highly infectious in Atlantic salmon. The virus was first found in Norwegian aquaculture facilities (Thorud & Djupvik 1988) but the disease has been described in other countries as well.

Like the influenza virus, ISAV belongs to the family Orthomyxoviridae but it has been classified as a *Isavirus* due to major differences between ISAV and influenza viruses (Kawaoka et al. 2005). ISAV is an enveloped virus, 100–130 nm in diameter, with a genome consisting of eight single-stranded RNA segments with negative polarity (Dannevig et al., 1995). The virus has haemagglutinating, receptor-destroying and fusion activity (Falk et al., 1997; Mjaaland et al., 1997; Rimstad et al., 2011).

Transmission of ISAV is believed to be mediated by sea water, by escaped infected salmonids or wild salmonids, and by boat transport between marine sites. When fish are infected, viral replication occurs within infected fish and the virus can be disseminated throughout most tissues (mid-kidney, head kidney, liver, spleen, intestine, gills, muscle and heart) (Taylor et al. 2011; Rimstad et al. 1999). Clinical signs are usually evident 2-4 weeks post infection and include pale gills, accumulation of ascites, liver congestion, enlarged spleen, congestion of the gut and a severe anemia (Hovland et al. 1994; Evensen et al. 1991).

Several studies showed that the virus can be cultured in Atlantic salmon head kidney cells (SHK-1) (Dannevig et al. 1995) and CHSE-214 cells (Bouchard et al. 1999). However, Bouchard et al. (1999) have claimed that CHSE-214 cells provide a better foundation for a culture-based diagnostic compared to SHK-1 cells, in which ISAV may not produce a definitive cytopathic effect (CPE). Therefore, SHK-1 cells or related (Atlantic salmon kidney cells, also called ASK cells) would be more useful in case we want to see the stimulant effects on cells after infection.

1.3.3 Piscirickettsia salmonis

P. salmonis is the first Gram-negative intracellular bacterial pathogen isolated from fish causing high mortality in salmonids (Bravo & Campos 1989; Fryer & Hedrick 2003). It belongs to *Piscirickettsia* genus and is a coccoid bacterium with a diameter of 0.5-1,5µm, non-motile, highly fastidious and aerobic (Fryer, J.L., & Hedrick 2003). *P. salmonis* replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of susceptible fish hosts or fish cell lines inducing a cytopathic effect (Fryer et al. 1990). It is transmitted horizontally or from fish-to-fish (Fryer, J.L. and Hedrick 2003). This Gram-negative bacterium is widely distributed in salmonids e.g. coho salmon, rainbow trout, cherry salmon, and Atlantic salmon (Garcés et al. 1991; Kent 1992; Bravo 1994). Bravo & Campos (1989) showed that the first signs of the disease in coho salmon began 6-12 weeks after fish were transferred from fresh water to sea water and the mortality range was 30-90%.

Piscirickettsiosis or salmon rickettsial syndrome (SRS) is the name of the disease caused by *P. salmonis*. There is a wide range of symptoms, both external and internal. The bacterium causes a systemic infection that targets the kidneys, liver, spleen, heart, brain, intestine, ovary and gills of salmonids (Cvitanich et al. 1991). Although diverse antimicrobial agents and vaccines have been used to control disease outbreaks, none of these methods seems to provide sufficient control to avoid the disease. Therefore, scientists suggest that the only logical alternative is prevention and control of SRS.

1.4 ddRAD Sequencing

Next-generation sequencing technologies are revolutionizing evolutionary biology because they enable the gathering of information across individuals at a genome-wide scale (Etter et al. 2011). Several papers describe a method called restriction site-associated DNA sequencing (RADSeq) which allows single-nucleotide polymorphisms (SNPs), randomly distributed across the genome, to be identified and scored (Baird et al. 2008; Hohenlohe et al. 2010; Emerson et al. 2010). RADSeq is performed by two simple molecular biology techniques with Illumina sequencing: the use of restriction enzymes to cut DNA into fragments and the use of molecular identifiers (barcodes) to associate sequence reads to particular individuals.

Traditional Restriction-site associated DNA sequencing (RADSeq) uses a single restriction enzyme (RE) to generate reduced representation libraries consisting of all genomic regions adjacent to the RE cut site. On the other hand, double digest RAD sequencing (ddRADSeq) uses a two enzyme double digest followed by precise size selection, excluding regions flanked by either very close or very distant RE recognition sites (**Figure 6**) (Peterson et al. 2012). Costs of library construction by double digest are five to ten fold less than random-shearing methods, which constitutes and advantage, allowing us to construct highly multiplexed libraries due to the ability to decrease read count requirements in sequencing and reduce cost per individual in library construction (Peterson et al. 2012).

RADSeq can be used to detect restriction site presence-absence polymorphisms, SNPs and indels in the sequence flanking the restriction site (Davey & Blaxter 2010). If a reference genome is not available, RAD tags can be analyzed performing *denovo* alignments. In this method, identical reads are aggregated into unique stacks and treated as candidate alleles. The unique sequences with small number of mismatches between them are clustered together for SNPs to be called between alleles at the same locus. On the other hand, if a reference genome is available, raw sequence reads can be aligned to the reference genome using next-generation sequencing bioinformatics tools e.g. Bowtie (Langmead et al. 2009); and SNPs and indels can be identified (Davey & Blaxter 2010).

Analyzing population genomic or genetic mapping datasets with millions of raw reads and genetic calls requires an efficient usable set of software tools. Therefore, Catchen et al in (2011) developed Stacks, software that identifies loci, either de novo or from a reference genome, and calls genotypes using maximum likelihood statistical model. They reported the algorithms implemented in Stacks, demonstrated their efficacy through simulation, and tested their ability to reconstruct denovo a zebrafish genetic map (Catchen et al. 2011).

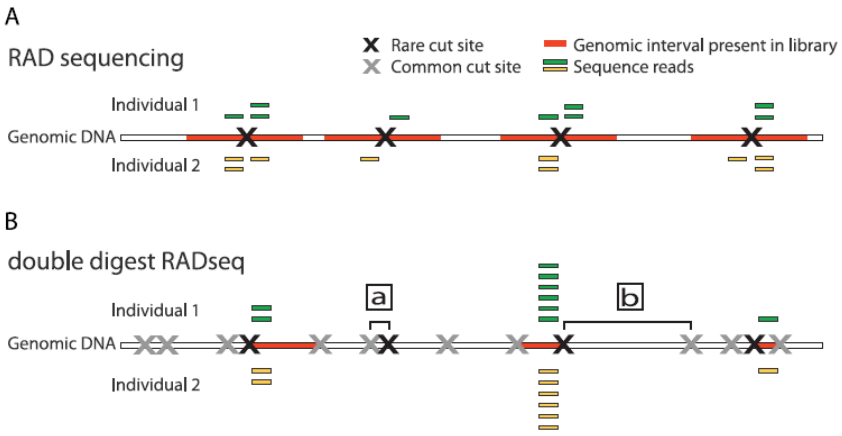

Figure 6. Differences between Traditional Restriction-Site Associated DNA Sequencing (RADSeq) and Double digest RAD sequencing (ddRADSeq). Figure obtained from (Peterson et al. 2012).

2 Project aims

Previously, our group performed experiments in order to investigate the effects of feeding two families of Atlantic salmon with HMB and analyzed the effect on the expression of two AMP genes (CATH-2 and Hepcidin-1) and iNOS. Our previous studies suggested that HMB in food and water acts as an immune-modulator and directly stimulates innate immunity, significantly CATH2 and iNOS expression in Atlantic salmon. The effect was reproducible in both families. Here we extended this study by using a large number of Atlantic salmon families, investigate different tissues (gills and skin) and two immunostimulants (calcium-HMB and HMB). We also include important controls that were missing in the previous study.

In order to find out whether the expression of AMPs could be used as a tool to select the most robust families with better innate immunity for breeding stock, one of the main aims for this study was to **study basal expression of these three genes (CATH-2, Hepcidin-1 and iNOS).**

Another main study aim was **to evaluate the effect of calcium-HMB and HMB, in diet and water, as immunostimulants.** With regard to that, we were interested in assessing whether the effects are different between Atlantic salmon families and between different tissues.

There is limited knowledge of immunostimulant treatments as an alternative and/or complement to antibiotics and vaccines. Therefore, we studied the effect of the two different immunostimulants (calcium-HMB and HMB). To further elucidate if the increased expression of these genes due to the treatments had a defense role against bacterial and virus infections in general or if the effect was specific to the disease, our third main aim was **to test three different treatments (HMB, PBA and vitaminD3) using different cell lines and three different pathogens (the viruses IPNV and ISAV; and the bacterium *P. salmonis*)**.

The last aim of interest was **to provide foundation to identify single SNPs as selection markers for fish highly tolerant or resistant to pathogen infections**, to enable selecting the most robust fish genotypes for salmon farming.

3 Materials and Methods

3.1 Experiment-1: Feeding and fish maintenance

Juvenile Atlantic salmon were obtained from the Stofnfiskur HF, fresh water farm in Kollafjördur. The experiment was performed with a total of 1330 fish from 133 different families, hence, 10 fish per family were collected. The average weight of the fish was approximately 50g. Fish were maintained in one tank at 4-5 °C with constant water flow and fed with fish food (Inicio 3mm, BioMar, Denmark).

Gill lamella samples of the 1330 fish were collected in order to measure basal expression for Cathelicidin-2. Gill lamella were used rather than the whole gill although low RNA measurements were expected due to the fact that fish needed to be alive in order to perform the second part of Experiment-1. Total amount of collected samples varied because some fish died or lost pit-tags.

Three months later, 14 families were carefully chosen to perform the control/treatment experiment. The 14 families were divided into subclasses according to the survival values obtained in two different challenges with Pancreas disease (PD) and IPNV, which were performed by Stofnfiskur. Families 45, 66, 55 and 96 had the highest survival values; families 124, 140, 48 and 60 had medium survival values; families38, 27, 99 and 102 were selected having the lowest survival values; and, finally, families 122 and 85 were selected randomly. Survival values of control and treated fish are shown in **Table 1**.

Table 1. Survival values, control and treated fish numbers of the 14 families selected for Experiment-1					
Family	**PD survival value (%)**	**IPNV survival value (%)**	**N° of Control Fish**	**N° of Treated Fish**	**Total fish number**
45	**19**	**97**	**3**	**7**	**10**
55	**23**	**100**	**5**	**5**	**10**
66	**50**	**97**	**2**	**7**	**9**
96	**42**	**100**	**2**	**7**	**9**
48	**54**	**61**	**2**	**7**	**9**
60	**57**	**64**	**2**	**8**	**10**
124	**46**	**52**	**2**	**8**	**10**
140	**42**	**58**	**2**	**7**	**9**
27	**4**	**0**	**2**	**7**	**9**
38	**3**	**0**	**2**	**7**	**9**
99	**0**	**3**	**2**	**7**	**9**
102	**0**	**3**	**2**	**8**	**10**
85	**64**	**80**	**2**	**8**	**10**
122	**17**	**0**	**2**	**6**	**8**

The average weight of the fish selected for control/treatment experiment was approximately 150g. Fish were divided into two different tanks (control and treated tank). Fish in both tanks were maintained at 4-5°C, control tank with constant water flow while the treated tank contained stagnant water, oxygenated with an air pump (ÍSAGA EHF)

Calcium-HMB (MaxiNutrition), which is a calcium supplemented HMB, was added directly to the water and also impregnated into the food of fish in the treated tank. The concentration of calcium-HMB in the water was 2,5mg/l. In fact, calcium-HMB solubility was lower than we expected, hence, HMB may not be dissolve in the water. For addition of calcium-HMB into the food, the concentration of HMB in food was 50mg/kg. Calcium-HMB was dissolved in ethanol at 0.1g/ml. Food (Inicio 4mm, BioMar, Denmark) was soaked in this solution and incubated at room temperature until the ethanol had evaporated. The fish were fed with the equivalent of approximately 4.3% of their body weight (i.e. 634,1g of food per tank per day). Control tank fish were fed with normal food without calcium-HMB (Inicio 4mm BioMar, Denmark).

3.2 Experiment-1: Experimental procedure and sample collection

Gill-lamella samples from all the 1330 fishes were collected to measure CATH-2 basal expression.

The control/treatment experiment was performed when fish reached 150g. The experiment lasted three days, hence, fish were exposed to the treatment for 72 hours (**Figure 7**). At 72 hours, fish were sacrificed and gill samples from control and treated fish were collected in order to measure Cathelicidin-2 expression.

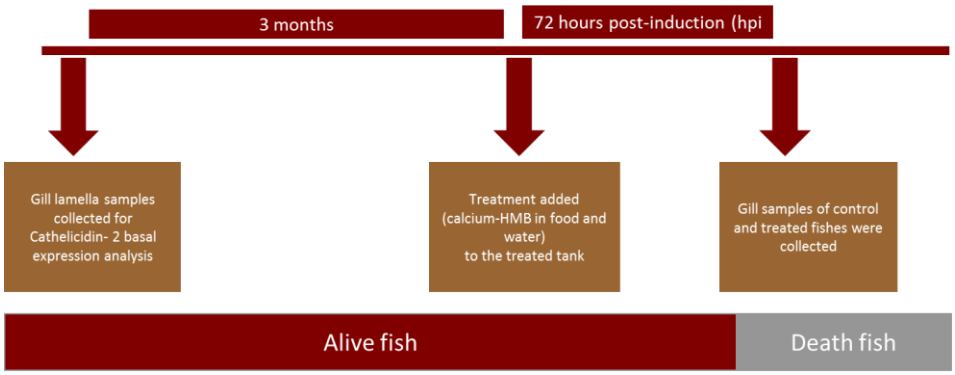

Figure 7. Experimental procedure of Experiment-1. DPI means days post-induction.

3.3 Experiment-2: Feeding and fish maintenance

Juvenile Atlantic salmon were obtained from the Stofnfiskur HF, fresh water farm in Kollafjördur. Experiment 2 was performed with 12 families (6 QTL for IPNV and 6 non-QTL for IPNV). QTL families were under QTL breeding selection for IPNV. QTL families are resistant to the viral disease IPN whereas non-QTL families are not. 12 individuals per family were chosen (6 individuals for control tank and 6 for treatment tank), adding up to a total of 144 individuals (72 in the control tank and 72 in the treatment tank) (**Figure 8)**

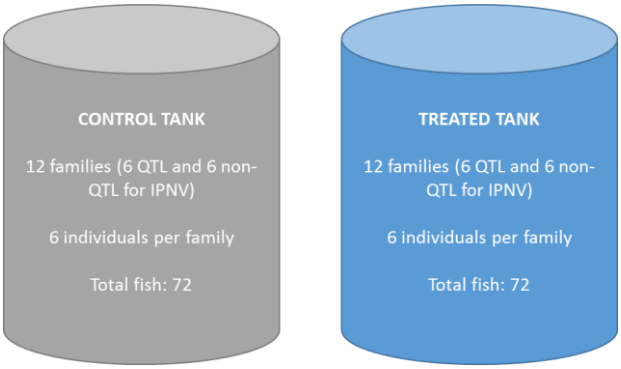

Figure 8. Fish arrangement in two different tanks (control and treated tank).

The average weight of the fish was 37g. Fish were divided into two different tanks (control tank and treated tank). Both control and treatment tank were maintained at 4-5°C and the water was oxygenated by an air pump (ÍSAGA ehf).

In contrast to Experimet-1, HMB treatment (TheProteinWorks), whose composition is specified in **Appendix 8.1**, was this time without supplemented calcium and has higher solubility. Hence, HMB could be dissolved in the water and act properly as an immune-modulator. It was directly added to the water and also impregnated into the food of the treated tank. HMB concentrations and HMB addition were performed following the protocol described for Experiment 1, where HMB was added to the water and food of the treatment tank while no HMB was added to the control tank. Fish in the control tank were fed with normal food (Inicio 2.5mm, BioMar, Denmark).

3.4 Experiment-2: Experimental procedure and sample collection

Treatment was performed for 5 days. At 3 days-post-induction (DPI), water was changed and HMB was added again to avoid bacterial growth and maintain HMB concentration. At 5 DPI, fish were sacrificed and samples from control and treated fish were collected (**Figure 9**).

Four different samples were collected: **Gill samples** and **skin samples** were collected and placed in tubes with 150µl RNAlater to measure CATH-2 expression in gill and skin, and Hepcidin-1 and iNOS expression in skin. **Water samples** were collected on the first day, at 2DPI and at 5DPI in order to measure bacterial growth or water contamination due to the fact that fish were in stagnant water for 5 days. The samples were sent to be analyzed by Sýni, an accredited laboratory located in Reykjavik, Iceland. Finally, **fin samples** were collected and placed in tubes with ethanol to isolate DNA and perform ddRAD-Sequencing in order to obtain possible SNP markers for strong innate-immune system selection.

The rest of the control and treated fish were store at -80°C, for subsequent analysis of different organs such us kidney, heart, skin and spleen as performed in previous experiments.

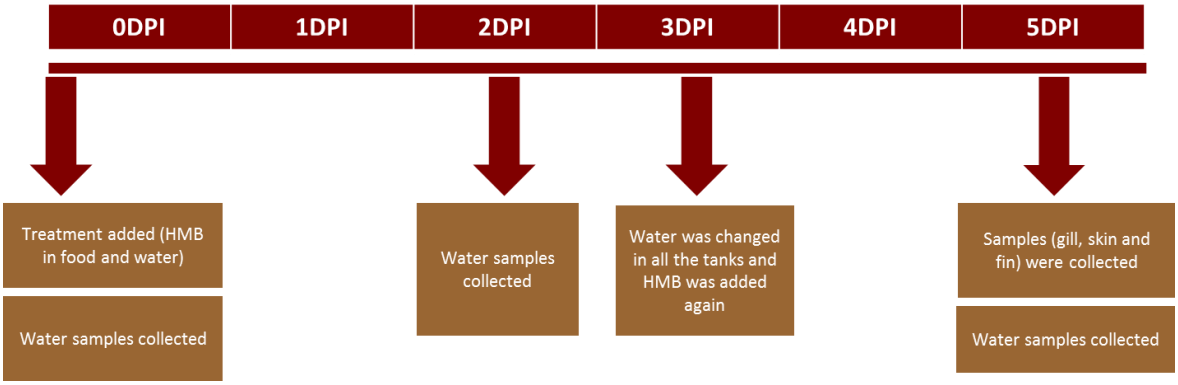

Figure 9. Experimental procedure of Experiment-2. DPI means days post-induction.

3.5 Collection of tissues, RNA extraction and cDNA synthesis

RNAlater was removed from gill samples from Experiment-1 and gill and skin samples from Experiment-2. Samples were then placed into 150µl of Tri Reagent® Solution (Sigma-Aldrich) and homogenized using stainless steel Beads (Berani Uster, CH) in a Minibeadbeater (Bioespec products). Then, RNA was extracted following a standard protocol. RNA quantity and purity were checked using a nanodrop ND1000 (Lab Tech) spectrophotometer. The extracted RNA was incubated with DNase (New England Biolabs) to remove DNA contamination. First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

3.6 qPCR

The total amount of salmon cDNA, was used to perform qPCR using power SYBR Green PCR master mix (Applied Biosystems) with different gene specific primers according to **Table 2**. It is worth noting the cDNA preparations used for basal expression measurements and the control/treatment part of Experiment-1 were not identical. 0.625ng of cDNA were used to perform qPCR of basal expression samples while 5ng of cDNA were used for qPCR of control/treatment-experiment samples. In Experiment 2, the total amount of cDNA used for qPCR was 5ng for all the samples.

Table 2. Primers used for qPCR

Gene	Nucleotide sequence (5' – 3')
csCath-2	Fwd: ATGGGAAACGAATGATGTGC Rev: CGGTCAGTGTTGAGGGTATT
asCath-2	Fwd: TACTGAGCACTCAGAAGATTCGGA Rev: TCTTTACTACCCATCTTAGAGCCC
Hepcidin-1	Fwd: GCTTCTGCTGCAAATTCTGAGG Rev: GTACAAGATTGAGGTTGTGCAG
iNOS	Fwd: AACGAGAGCCAACAGGTGTC Rev: GGTGCAGCATGTCTTTGAGA
EF-1α	Fwd: TGCCCCTCCAGGATGTCTAC Rev: CACGGCCCACAGGTACTG

The reactions consisted of 0.625 or 5ng of cDNA and 9µl of master mix solution in a final volume of 10µl. The qPCR was run in a 7500 Real Time PCR System (Applied Biosystems). qPCR was started with a 2-min hold at 50°C followed by a 10 min hot start at 95°C. Subsequently the amplification was performed with 40 cycles of 15 sec denaturation at 95°C and 1 min annealing/extension at 60°C. For each sample a dissociation step (60°C-95°C) was performed at the end of the amplification phase to identify a single specific melting point for each primer set.

A reference gene test was performed in order to choose the best housekeeping genes between RPS-20, β-actin and EF-1α. Efficiency and response to the different samples were evaluated following some of the ideas put forward by Radonić et al in (2004). Although the three housekeeping genes worked fine, EF-1α was chosen as a reference gene because the Cycle Threshold (Ct) behavior between different samples was more regular than for β-actin and RPS-20.

3.7 Determination of qPCR efficiency

Real Time PCR was performed to determine the efficiency of the different primer sets (csCath-2, asCATH2, Hepcidin-1, iNOS, EF-1α, β-actin). We chose RNA samples from different days post-induction from Experiment-1 and pooled them to make dilution series from 1:2 down to 1:2048.

The Ct values resulting from the RT-qPCR of the dilution series were plotted against the decimal logarithm (log10) of the original RNA input. Based on the following equation, we obtained the efficiency of the different primer pairs:

$$\text{Efficiency} = 10^{1/\text{slope}}$$

3.8 Data and statistical analysis

In Experiment-1 only biological replicates were assessed. In contrast, two technical replicates and biological replicates were assessed in Experiment-2. Data analysis was performed using Ct values. After checking that the data were normally distributed, results were analyzed using the method of Pfaffl (Pfaffl 2001).We used Ct values of the target genes (asCATH2, Hepcidin-1, iNOS) and the Ct values of the reference gene (EF-1α) to calculate ΔCt values for each biological and technical replicates according to the following equation:

$$\Delta Ct = Ct\ (\text{reference gene}) - Ct\ (\text{target gene})$$

The ΔCt mean of the controls (controlW1, controlW2 and controlW3) and the ΔCt of the biological and technical replicates were used to calculate $\Delta\Delta C_t$.

$$\Delta\Delta Ct = \Delta Ct\ (\text{control}) - \Delta Ct\ (\text{treated fish})$$

In addition, relative expression values, referred to as relative quantity (RQ) were calculated from the ΔΔCt values according to the following equation:

$$\text{Relative quantity (RQ)} = 2^{-\Delta\Delta Ct}$$

Furthermore, we log2 transformed the resulting values to obtain Fold Difference (FD) values that can be used for t-tests to assess the statistical significance of the results. The equation is as follows:

$$\text{Fold Differences (FD)} = \text{Log}_2 RQ$$

For basal expression analysis, FD and the standard error (SE) of FD of each individual were used. Individual FD values were separated according to families and a comparison between FD values of the different families was performed by running ANOVA and Nested Linear Model in R version 3.0.1. Also, sciplot was drawn with the FD value means of all the selected families.

Fold Differences and the SE of FD were used to show the effect of calcium- HMB (Experiment-1) or HMB (Experiment-2) on the expression of the genes studied. Firstly, to analyze the data of the calcium-HMB and HMB effect in Cathelicidin-2, Hepcidin-1 and iNOS, FD values of the control and treated individuals were obtained and further split according to the selected families. FD values of the different families were evaluated by performing a two-ways ANOVA (factor 1: level, i.e. control or treated fish; factor 2: family). In addition to the ANOVA, we performed analysis with a Nested Linear Model to integrate all the variables (FD values, level, family and individuals) and obtain more detailed results. Finally, sciplots (bar graph, which use standard error) were drawn in R version 3.0.1 in order to show whether the results for induction of the different genes were statistically significant by comparing gene expression of treated with non-treated (control) fish.

Finally, Genetic parameters (heritability) were analyzed from 457 individuals which were offspring of 63 dams and 34 sires. REML was used to fit mixed linear models using DMU package version 6, release 5.2 (Madsen & Jensen 2013). The model used for the analysis was as follows: $\boldsymbol{y = Xb + Za + e}$, where $\boldsymbol{y}$ is the vector of the FD value of csCATH-2 basal expression of the 62 different families, which is the log10 value of the RQ values; $\boldsymbol{b}$ is a vector of fixed effects, which is in this model equals 1; $\boldsymbol{a}$ is the vector of random additive genetic effects on individual animals; and $\boldsymbol{e}$ is uncorrelated residuals error. FD value was treated as a normal distributed trait.

3.9 Infection assays with cultured cells

3.9.1 Cells, viruses and treatments

CHSE-214 and Atlantic salmon Kidney cells (ASK) were grown as monolayers at 20°C in Eagle‘s minimal supplemented medium (EMEM) with Earle´s salts and non-essential amino acids (GIBCO), Grand Island, NY, 10nM HEPES (pH 7.0) (Sigma, St Louis, MO), sodium bicarbonate (1 mg/ml), gentamicin (50 ug/ml) and 10% of fetal bovine serum (FBS) (GIBCO). Cells were grown on 24 well plastic plates with 12 mm circular glass coverslips or 35 mm diameter plastic dishes. Importantly, cells were grown in a reference laboratory for IPN virus infections (*Centro de Investigación y Gestión de Recursos Naturales, CIGREN),* which is located in the University of Valparaiso, Chile. We used two 24-well plates with CHSE-214 cells for IPNV infection and one 24-well plate with ASK cells for ISAV infection. Organization of plates is shown in **Figures 10** and **11**.

CHSE-214 cells and RTS-11 cells, which are composed of non-adherent monocytes and adherent macrophages were grown as monolayers at 20°C in Leibovits L-15 (GIBCO) supplemented with 15% of FBS (GIBCO) with pH 7,3 in the Aquaculture pathogens reference laboratory for ISA virus and SRS research, which is located at the Pontificia Universidad Católica de Valparaíso (PUCV). Cells were grown on 24 well plastic plates with 12 mm circular glass coverslips or 35 mm diameter plastic dishes. We used two 24-well plates with CHSE-214 cells and RTS-11 cells for SRS infection. Plate organization is showed in **Figure 12**.

The cells were pre-treated with: 20 mM β-Hydroxy-β-methyl butyrate (HMB), 4 mM 4-phenylbutyrate (PBA) (Tocris) and 20 nM 1,25-dihydroxyvitamin D3 (VitD3) (Tocris) 48h pre-infection as shown in **Figure 10, 11** and **12**. Treatments were continued throughout the virus replication period. Concentrations were established in previous experiments performed by Rosana Estévez (unpublished results). Controls were performed by adding EMEM medium without any treatment. Six replicates ere made per treatment for CHSE-214 cells infected by IPNV (**Figure 10**) and 3 replicates per treatment for ASK cells infected by ISA (**Figure 11**).

VitD3	VitD3	VitD3	VitD3	VitD3	VitD3
PBA	PBA	PBA	PBA	PBA	PBA
HMB	HMB	HMB	HMB	HMB	HMB
Control	Control	Control	Control	Control	Control

CHSE-214

18hpi

6replicates
per treatment

VitD3	VitD3	VitD3	VitD3	VitD3	VitD3
PBA	PBA	PBA	PBA	PBA	PBA
HMB	HMB	HMB	HMB	HMB	HMB
Control	Control	Control	Control	Control	Control

CHSE-214

72hpi

6replicates
per treatment

Figure 10. Plates organization for treatment and IPNV infection. Both Plate 1 and 2 were used for IPNV infection but one was kept for 18hpi and the other for 72hpi, as explained bellow.

VitD3	VitD3	VitD3	VitD3	VitD3	VitD3
PBA	PBA	PBA	PBA	PBA	PBA
HMB	HMB	HMB	HMB	HMB	HMB
Control	Control	Control	Control	Control	Control

ASK

3replicates
per treatment

27hpi

72hpi

Figure 11. Plate organization for treatment and ISAV infection. Three replicates per treatment were selected and the plate was divided in two by leaving half of the plate 27hpi and the other half 72hpi,as explained bellow.

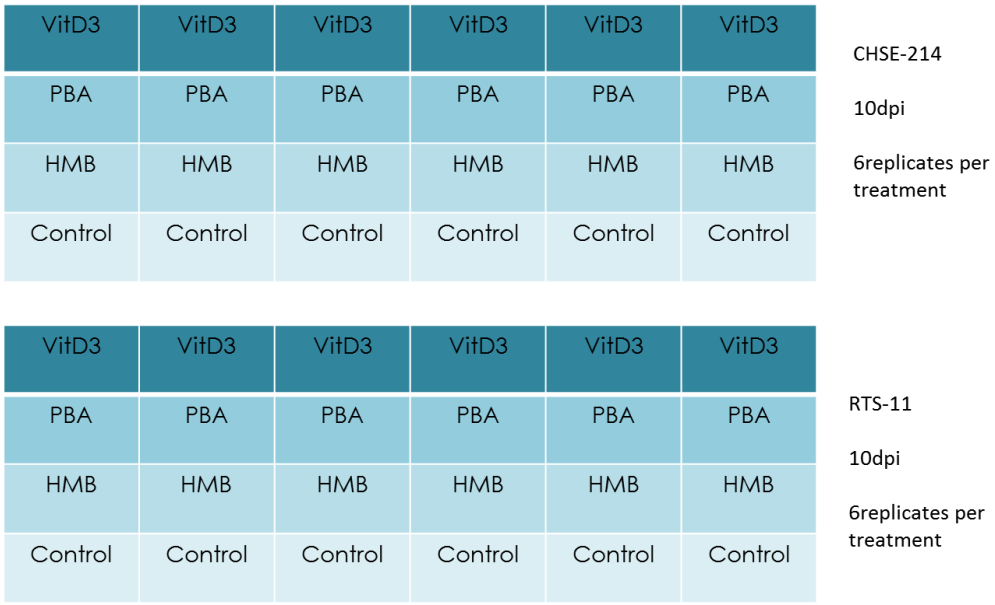

Figure 12. Plate organization for treatment and SRS infection. Six replicates were used per treatment and two different kind of cells were selected (CHSE-214 and RTS-11 cell lines).

3.9.2 Infection and Experimental procedure

The IPNV used in this study was isolated in Chile and it is identical to the VR-299 serotype (Espinoza et al., 1985). IPNV infection was performed following the protocol of Espinoza and Kuznar in (2002). In brief: virions were allowed to attach to CHSE-214 cells at a MOI of 0.1 PFU/cell for 1 hour. After attachment, virus was removed and cells were washed once with 500ul of EMEM medium and further incubated in the presence or absence of the different stimulants for 18 or 72 hours (**Figure 10**). After infection, the coverslips were removed and Indirect Fluorescent Antibody Test (IFAT) was performed on all the cell monolayers of the coverslips following the standardized protocol (Espinoza & Kuznar 2002) in order to quantify infection levels. The remaining CHSE-214 cells that were not on the coverslips were removed, placed in a tube with RNA later and stored at -80°C for qPCR analysis. Experimental procedure is shown in **Figure 13**.

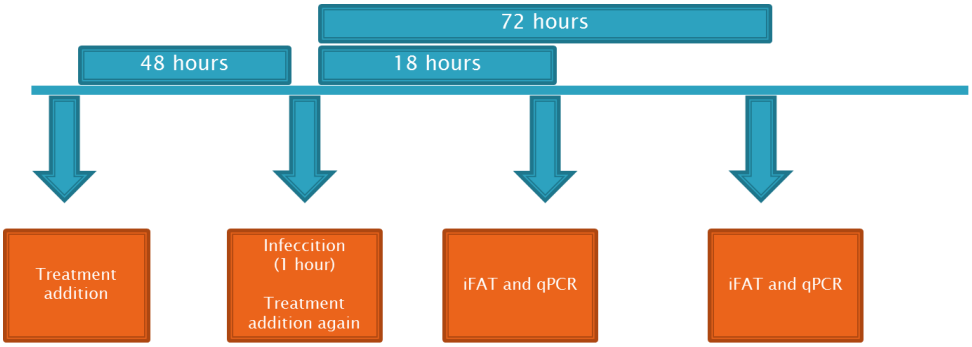

Figure 13. Experimental procedure performed for IPNV infection.

The ISAV used in this study was a Chilean outbreak isolate of the subtype HPR- 7B. ISAV infection was performed according to standard protocol from the CIGREN reference laboratory, in brief: The virions were allowed to attach to the ASK-cells at a MOI of 0.1 PFU/cell for 3 hours. After attachment, virus was removed and cells were washed with 500ul of EMEM medium once and further incubated in the presence or absence of the inducer for 27 or 72 hours (**Figure 11**). After infection, the coverslips were removed and Indirect Fluorescent Antibody Test (IFAT) was performed as described above. The remaining ASK cells that were not on the coverslips were removed, placed in a tube with RNA later and stored at -80°C for qPCR analysis. Experimental procedure is shown in **Figure 14**.

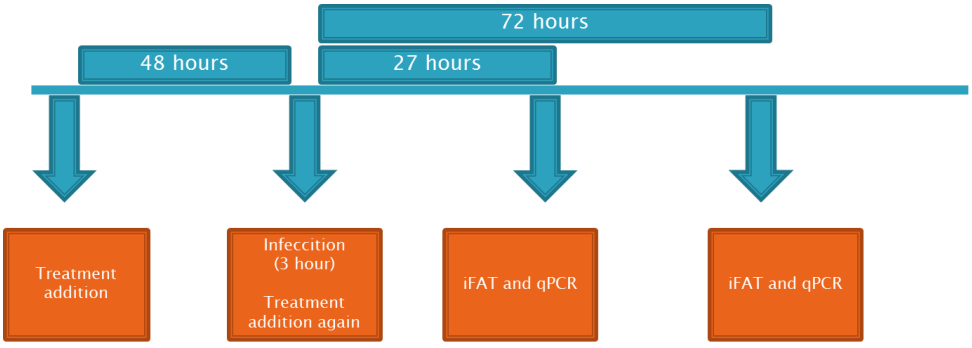

Figure 14. Experimental procedure performed for ISA infection

The stained samples were examined using an Olympus BX60 epifluorescence microscope. The infected cells were counted examining the complete area of the glass (113,1mm^2) with a 10× objective. For IPNV infection analysis, 5 focal fields per well were selected and infected cells were counted. The final cell count was multiplied by 27, 6 focal fields that compose a coverslips. For ISAV infection analysis, 3 focal fields per well were selected and infected cells were counted as well. The obtained values were multiplied by 113,1 which is the total are of the coverslip. Data obtained from both IPNV and ISAV infection was analyzed and statistical tests were performed in R version 3.0.1.

P. salmonis strain from the Molecular Genetic and Immunology laboratory of Pontifical Catholic University of Valparaíso was maintained in blood agar plates (5 g / L peptone, 5 g / L yeast extract, 15 g / L tryptone, 10 g / L glucose, 12 g / L agar, 5% sheep blood), supplemented with 0.1% L-cysteine at 20 ° C (modified Mauel et al., 2008). SRS infection was achieved by transferring a single bacterial colony and growing it in complex medium 1 (MC1): yeast extract 7 g / L, peptone 5 g / L, NaCl 10 g / L, MgSO4 0.4 g / L, CaCl 0.081 g / L, K2HPO4 1 g / L FeSO4 0.08 g / L. The culture grew for 24 hours at 23 ° C at 100rpm and bacterial growth was determined by turbidimetry, reaching an OD 600 between 0.7-0.8. CHSE-214 and RTS-11 cells were infected with 100µl of bacterial culture and incubated at 23ºC for 3 hours. After attachment, bacteria were removed and cells were washed three times with sterile 1X PBS and further incubated in the presence or absence of the different stimulants for additional 10 days. Subsequently, cells from the different wells were removed, RNA was extracted and c-DNA was synthesized as described above. Finally, RT-PCR was performed using EF-1α as a reference gene and ITS as a target using primers, described by Marshall et al, 1998. The experimental procedure is outlined in **Figure 15.**

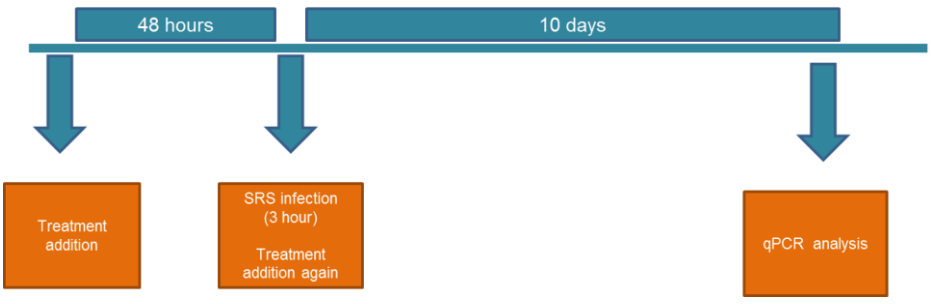

Figure 15. Experimental procedure performed for SRS infection

3.10 ddRAD Sequencing

3.10.1 DNA isolation and double digestion of DNA samples

160 fin samples were collected in Experiment 2 from the parents and offspring of 12 different families (family 143,275,276, 395, 396, 403, 453, 455, 493, 494, 495 and 496) in order to perform DNA isolation. Total genomic DNA (gDNA) was extracted using a standard phenol-chloroform-extraction protocol adapted by Kristen Marie Westfall (**Appendix 8.2**). gDNA concentrations were measured using Nanodrop 1000 and 500ng of gDNA were run on a 2% agarose gel to assess quality.

Total genomic DNA was diluted to 100ng/µL and 1000 ng were double-digested in a 40 µL total reaction volume. *Sau3AI* was added to the reaction with New England Biolabs (NEB) Buffer #3 and incubated for four hours at 37°C, followed by addition of *Ape KI* and a further four hour incubation period at 75°C.

3.10.2 ddRAD library preparation and sequencing

100ng of digested DNA was used for ligation of adapters with NEB T4 DNA ligase. Combinatorial barcoding was employed to attach a unique tag to each individual. Barcodes were the following: TTCTC, TCGTT, AGCCC, GTATT, CTGTA, ACCGT, GCTTA, GGTGT, AGGAT, ATTGA, CATCT, CCTAC, TGCGA, GAGGA, CGCTT, GGAAC, ACAACC, TAGAGC, TATCTC, AGGCAT, TCCACT and TTAGGT. The adapter sequences are listed in Elshire et al. (2011). Adapters were added in a 5:1 molar ratio to sticky ends and the 40µL reaction was incubated at 21°C for 12 hours followed by enzyme inactivation at 65°C, decreasing temperature by 1°C every 45 seconds until 12°C.

Individual ligations were combined into pools of 50 individuals and purified using Sera-Mag magnetic beads following the manufacturer's protocol. Each pool was run on one lane on the Pippin Prep system (Sage Technologies) and size -selected for fragments ranging from 300bp to 400bp using a 2.0% agarose gel without ethidium bromide following the manufacturer's protocol. Eluates from two lanes each were pooled (one pool per 100 individuals) prior to PCR. Pooled and size-selected fragments were amplified in 10 PCR reactions per pool, following the protocol of Elshire et al. (2011) except that the number of PCR cycles was reduced to 10. Primers from (Elshire et al., 2011) are as follows:

a) 59-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

and

b) 59-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

PCR products from each individual pool were pooled and purified using the Sera-Mag magnetic beads according to the manufacturer's protocol. Each pool was eluted in a final volume of 40µL and concentration determined using a SYBR gold quantification procedure (**Appendix 8.3**). Each individual pool was sequenced in two lanes on an Illumina HiSeq2000 at DeCODE Genetics.

3.10.3 ddRAD data analysis

Data obtained from DeCode Genetics were analyzed using Stacks (**Figure 16**), a software platform that was built to identify sequence polymorphisms in RADseq data and to easily incorporate additional data (Catchen et al. 2011). First, raw sequence reads were demultiplexed and cleaned (*process_radtags*) to remove low quality sequences and to separate reads from different samples that were individually barcoded. Data obtained from the demultiplexed process was further aligned to the Atlantic salmon reference genome using Bowtie (Langmead et al. 2009). The aligned reads from each individual were grouped into loci, and polymorphic nucleotide sites were identified by using *pstacks*. Then, loci were grouped together across individuals to generate a catalogue (*cstacks*). *Sstacks* was then used in order to match loci from each individual against the catalogue in order to determine the allelic state at each locus in each individual. Finally, allelic states were converted into

genotypes using *populations* for population genetic statistical analysis. Furthermore, MySQL database, described by Catchen et al. (2011), was used to visualize the data.

Detailed analysis of the data is beyond the scope of this project.

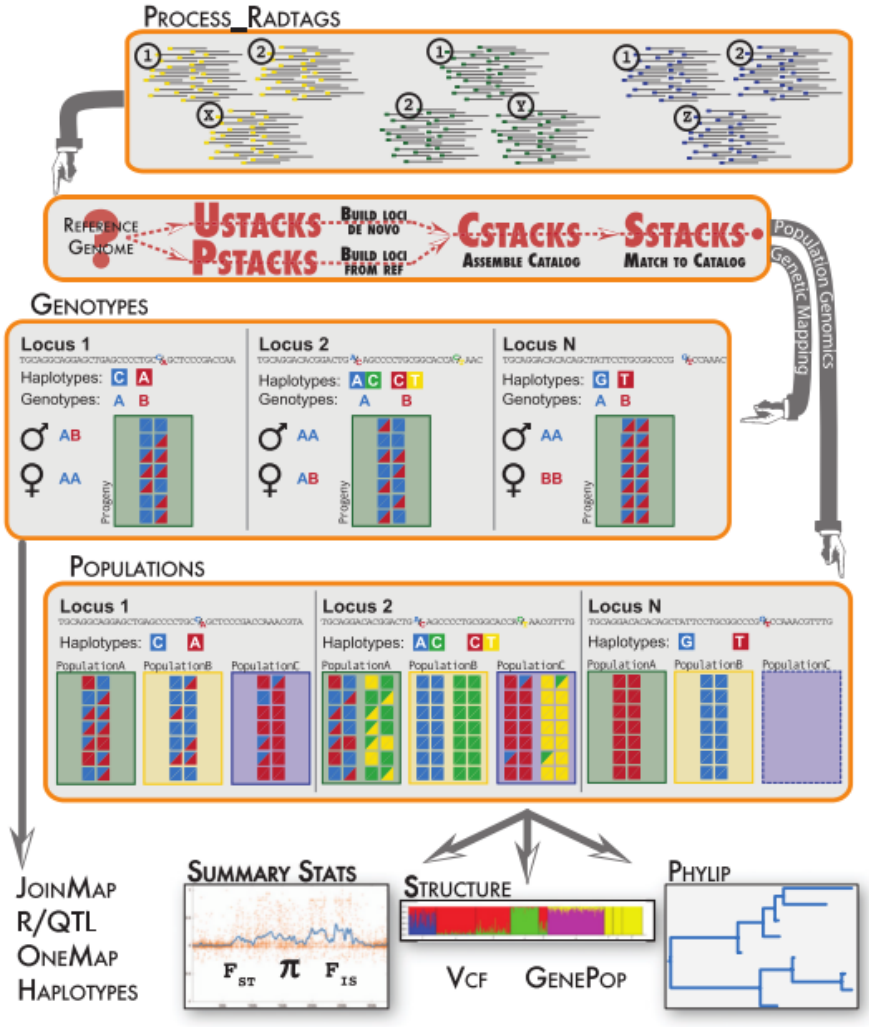

Figure 16. The Stacks pipeline, proceeding in five major stages. Figure obtained from (Catchen et al. 2013). Figure reprinted with permission.

4 Results

4.1 Primer efficiency

Primer efficiencies were evaluated before use in qPCR analysis. The efficiencies of csCATH-2, asCATH-2, Hepcidin-1, iNOS and EF1α were obtained and values were 100.86, 88.77, 94.42, 112.83 and 107.84 percent respectively. See graphs in **Appendix 8.4.** These values are all within an acceptable range of 100%. Melt curve analysis showed a single amplicon generated by qPCR. Therefore, the primers were acceptable for use.

4.2 Experiment-1 results: CATH-2 basal expression analysis and calcium-HMB test as immune-modulator

Cathelicidin-2 (CATH-2) basal expression in selected families and basal expression changes over time were studied in order to find out whether CATH-2 basal expression can be used as a molecular tool to select the most robust families with better innate immunity for breeding stock. In addition, heritability analyses were performed to elucidate genetic influence of CATH-2 basal expression. Finally, the expression of CATH-2 was further analyzed in 14 Atlantic salmon families comparing untreated (control) and calcium-HMB treated fish to evaluate whether calcium-HMB had any effect as an immune-modulator, boosting the innate immune system.

4.2.1 CATH-2 basal expression

Although gill lamella samples of 133 families (10 individuals per family) were collected, only 62 families were selected to be evaluated for CATH-2 basal expression. We used FD values and their standard error (SE) for the different families, which were normally distributed, and compared them to the mean of all the FD values and plotted all these values in a bar graph (**Figure 17**). Results shown in figure 20 show that CATH-2 expression varies between families. Some families (1, 2, 11, 33 and 60) have less expression than the average (FD values < 0), while other families (21, 22, 23, 45 and 66) have higher expression

compared with the average (FD values > 0). We hypothesize that these families have stronger innate immunity. Therefore, these would be the families, which would fulfill the selection requirement for enhanced components of innate immunity (robustness).

In addition, CATH-2 expression values were analyzed by one-way ANOVA and Tukey test, with FD values and Family as variables, in order to calculate the significance of the results. ANOVA analysis determines that there is variability in CATH-2 expression among families and it is highly significant with $p < 0.001$ (**Figure 18**). By doing a more exhaustive analysis, there are some families which stand out from the rest. Families 25, 28, 31, 39 and 54 have higher CATH-2 expression than the FD mean with $p < 0,05$. Families 20, 30, 44, 66, 67 and 85 are also significant with $p < 0,01$. The highest CATH-2-basal-expressions is in families 21, 22, 23, 24 and 45 with $p < 0,001$ (**Figure 19**).

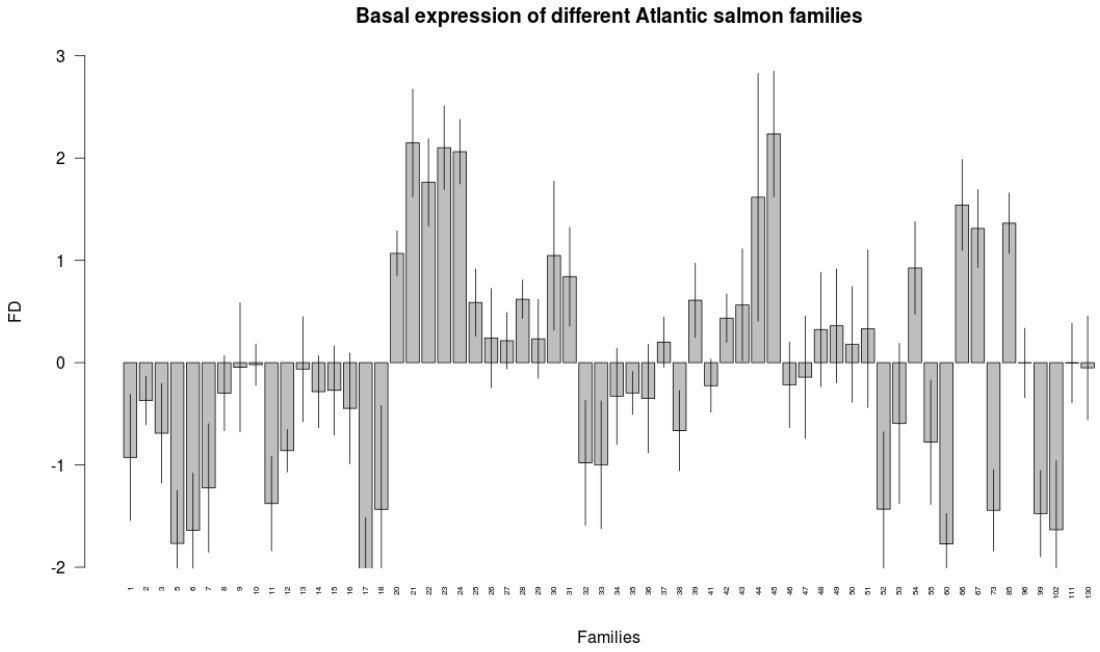

Figure 17. Basal expression of CATH-2 in 62 families. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data was plotted using Fold Differences (FD) and its standard error (SE) to depict the differences of basal expression between the different families. N = 6-8 fish per group. Vertical whiskers are SE.

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Family	61	503.27	8.2504	4.6649	< 2.2e-16	***
Residuals	395	698.60	1.7686			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 18. One-way ANOVA results performed with the FD values of CATH-2 as a target gene and EF-1α as a reference gene. Family was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

lm(formula = data$FD ~ data$Family, data = data)

Residuals:

Min	1Q	Median	3Q	Max
-3.9711	-0.7882	-0.0485	0.7718	4.9261

Coefficients:

Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	-0.92650	0.59474	-1.558	0.120080
data$Family2	0.55705	0.77870	0.715	0.474815
data$Family3	0.23670	0.77870	0.304	0.761317
data$Family5	-0.84052	0.84109	-0.999	0.318256
data$Family6	-0.71147	0.89212	-0.798	0.425635
data$Family7	-0.29831	0.77870	-0.383	0.701863
data$Family8	0.62720	0.74178	0.846	0.398319
data$Family9	0.88075	0.75815	1.162	0.246055
data$Family10	0.90671	0.74178	1.222	0.222305
data$Family11	-0.45107	0.74178	-0.608	0.543476
data$Family12	0.06602	0.74178	0.089	0.929124
data$Family13	0.86344	0.74178	1.164	0.245122
data$Family14	0.64265	0.74178	0.866	0.386813
data$Family15	0.65600	0.75815	0.865	0.387423
data$Family16	0.47939	0.75815	0.632	0.527548
data$Family17	-1.16261	0.77870	-1.493	0.136233
data$Family18	-0.50700	0.77870	-0.651	0.515369
data$Family20	1.99502	0.74178	2.690	0.007459 **
data$Family21	3.07509	0.75815	4.056	6.02e-05 ***
data$Family22	2.69001	0.74178	3.626	0.000325 ***
data$Family23	3.02773	0.75815	3.994	7.76e-05 ***
data$Family24	2.98846	0.74178	4.029	6.73e-05 ***
data$Family25	1.51510	0.74178	2.043	0.041762 *
data$Family26	1.16737	0.75815	1.540	0.124422
data$Family27	1.14125	0.74178	1.539	0.124718
data$Family28	1.54597	0.74178	2.084	0.037789 *
data$Family29	1.15965	0.77870	1.489	0.137232
data$Family30	1.97360	0.75815	2.603	0.009584 **
data$Family31	1.76779	0.75815	2.332	0.020218 *
data$Family32	-0.05344	0.77870	-0.069	0.945317
data$Family33	-0.07199	0.74178	-0.097	0.922737
data$Family34	0.59867	0.80529	0.743	0.457667
data$Family35	0.62996	0.74178	0.849	0.396251
data$Family36	0.57591	0.75815	0.760	0.447935
data$Family37	1.12731	0.74178	1.520	0.129377
data$Family38	0.26198	0.75815	0.346	0.729869
data$Family39	1.53611	0.77870	1.973	0.049232 *
data$Family41	0.70122	0.75815	0.925	0.355576
data$Family42	1.36170	0.75815	1.796	0.073246 .
data$Family43	1.49070	0.77870	1.914	0.056299 .
data$Family44	2.54396	0.89212	2.852	0.004578 **
data$Family45	3.16239	0.75815	4.171	3.73e-05 ***
data$Family46	0.71005	0.89212	0.796	0.426555
data$Family47	0.78419	0.97121	0.807	0.419901
data$Family48	1.25008	0.74178	1.685	0.092729 .
data$Family49	1.28684	0.84109	1.530	0.126828
data$Family50	1.10456	0.84109	1.313	0.189863
data$Family51	1.25823	0.84109	1.496	0.135468
data$Family52	-0.50562	0.80529	-0.628	0.530447
data$Family53	0.33143	0.77870	0.426	0.670616
data$Family54	1.85187	0.80529	2.300	0.021990 *
data$Family55	0.14891	0.75815	0.196	0.844389
data$Family60	-0.84545	0.97121	-0.871	0.384550
data$Family66	2.46766	0.77870	3.169	0.001649 **
data$Family67	2.23859	0.75815	2.953	0.003338 **
data$Family73	-0.51667	0.75815	-0.681	0.495967
data$Family85	2.28964	0.75815	3.020	0.002692 **
data$Family96	0.92368	0.77870	1.186	0.236263
data$Family99	-0.54926	0.77870	-0.705	0.481009
data$Family102	-0.70730	0.75815	-0.933	0.351430
data$Family111	0.92252	0.74178	1.244	0.214362
data$Family130	0.87599	0.75815	1.155	0.248612

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 19. One-way ANOVA detailed results (Tukey test) performed with the FD values of CATH-2 as a target gene and EF-1α as a reference gene. Family was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.2.2 Heritability of traits (CATH-2 basal expression)

The heritability for FD values of 62 different families was estimated to be $h^2 = 0.61$ with a standard error (SE) = 0.061, which is significantly different from zero. Therefore, the result shows high additive genetic variance for FD values, meaning that the expression of CATH-2 is highly influenced by genetic background among these Atlantic salmon families.

4.2.3 Comparison between CATH-2 basal expression and control fish

Both CATH-2 basal (at time -3 months) and control expression values (at time 0) were obtained from some of the fish, from 14 families selected for control/treated experiment. Both sets of values in essence represent basal expression the significant differences being time of sample collection (three months apart) and type of sample (gill lamella or the whole gill). Therefore, CATH-2 basal expression values come from fish with an average of 50g of weight and csCATH-2 control values come from the same fish (three months later) with an average of 150g of weight.

FD values and their standard errors of 11 families were compared in order to see if there were significant changes of CATH-2 basal expression over time. These values, plotted in a bar graph, showed significant differences in basal expression over time for several families (**Figure 20**).

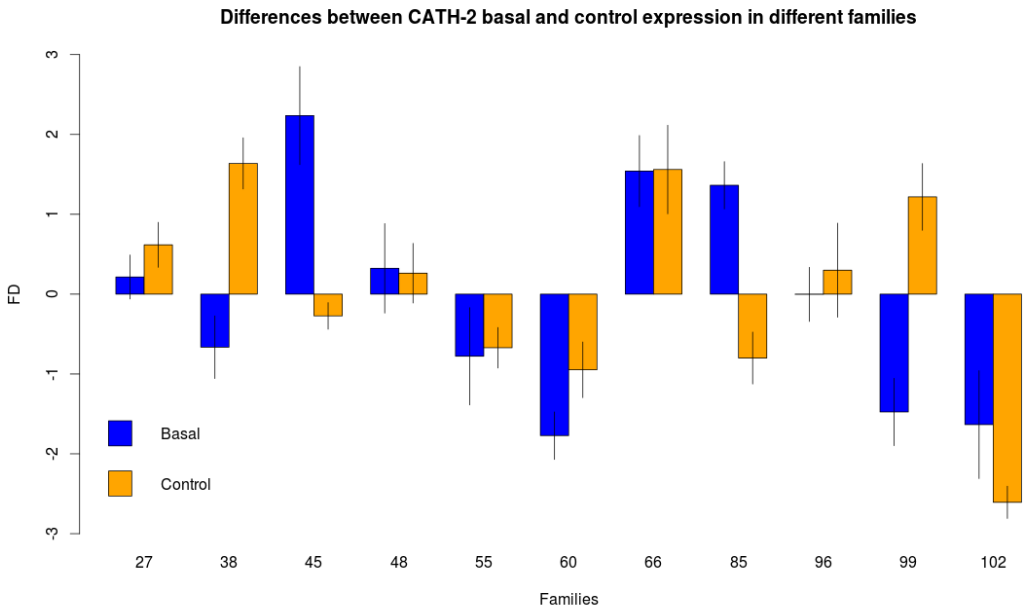

Figure 20. CATH-2 basal and control expression of 11 families in gill tissue (both are CATH-2 basal expression values with 3 months difference in time). Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using Fold Differences (FD) and its standard error to depict the different expression of CATH-2 basal expression over time. n = 4 fish per group. Vertical whiskers are standard error (SE). Different letters denote significant differences (two –way ANOVA, p < 0,001).

Two-way ANOVA (**Figure 21**) showed that there are no general significant differences between basal and control CATH-2 expression considering all the families but there are significant differences (p< 0.001) between family-pattern of behavior. We can see in **Figure 21** that there are some families (Family 38, 60 and 99) that increase CATH-2 expression over time and this increase is significant. However, there are also families (Family 45, 85 and 102) that decrease CATH-2 expression significantly over time.

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Comparison	1	0.619	0.6188	0.4415	0.5078	
data$Family	10	126.923	12.6923	9.0555	9.887e-11	***
data$Comparison:data$Family	10	63.718	6.3718	4.5461	2.215e-05	***
Residuals	109	152.776	1.4016			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 21. Two-way ANOVA results performed with the FD values of CATH-2 gene as a target gene and EF-1α as a reference gene. Comparison (meaning CATH-2 control and basal expression) and Family were the factors (Factor 1 and Factor 2 respectively). Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.2.4 CATH-2 expression in control and treated fish

CATH-2 expression in 14 families was analyzed by comparing the FD values and their standard error (SE) of the control fish with each individual FD value and SE of the control and treated fish. The main objective was to analyze the effect of calcium-HMB in the different families of Atlantic salmon by adding it to water and food.

FD values of control and treated fish were plotted as a bar graph (**Figure 22**), showing that calcium-HMB treatment did not lead to increased CATH-2 expression. We cannot see a clear increase of CATH-2 expression in the different families. The fact that water was white is noteworthy. We believed that calcium-HMB was not dissolve in water due to its low solubility and this may haave affected the role of HMB as an immunostimulant.

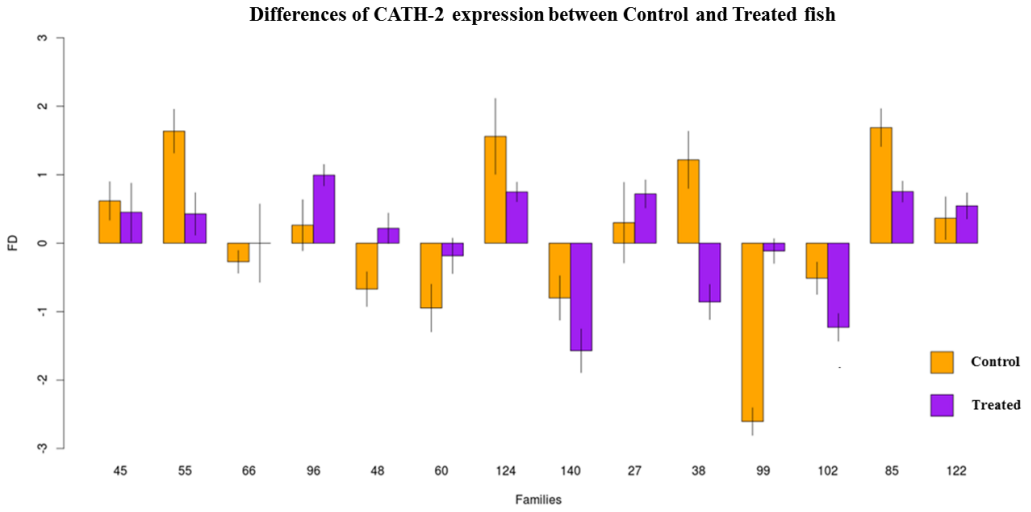

Figure 22. The expression of CATH-2 after 72hpi feeding these 14 families with calcium-HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Shown are Fold Differences (FD) and its standard error to depict the different expression of CATH-2 between control and treated fish. n = 4-6 fish per group. Vertical whiskers are standard error (SE).

CATH-2 expression data was further analyzed by two-way ANOVA, using FD values, family and level (differences between control and treated fish) as variables, in order to see whether the responses to treatment were significantly different between families. Generally, no significant differences were detected between control and treated fish, hence, treatment was not effective. CATH-2 expression was significantly up-regulated in some families e.g. family 99. However, this up-regulation seemed to be meaningless.

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Level	1	0.289	0.2893	0.3207	0.5718	
data$Family	13	127.277	9.7905	10.8517	< 2.2e-16	***
data$Level:data$Family	13	52.645	4.0496	4.4886	9.124e-07	***
Residuals	218	196.682	0.9022			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 23. Two-way ANOVA results performed with the FD values of CATH-2 gene as a target gene and EF-1α as a reference gene. Level (meaning control and treated fish) and Family were the factors (Factor 1 and Factor 2 respectively). Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

To sum up the results, we revealed that CATH-2 basal expression in gills could be used as a molecular marker to further select fish with higher CATH-2 basal expression, fact that has previously been related with stronger innate immune responses. To our surprise, no significant differences were seen between control and treated fish, consequently, calcium-HMB did not induce CATH-2. This could be explained because HMB was, likely, not dissolved in the water due to the low solubility of calcium-HMB, hence, HMB could not boost innate immune responses by increasing CATH-2 gene expression.

4.3 Experiment-2 results: AMPs and iNOS basal expression analysis and HMB test as immune-modulator

Even though we revealed that CATH-2 basal expression could be used as a molecular marker to select families with higher CATH-2 basal expression, a more complete picture was required to draw final conclusion. Therefore, we extended the analysis of basal expression to include Hepcidin-1 and iNOS in Experiment-2. We decided to perform these analyses with gill and skin tissues due to their important role for the innate immunity system.

Due to the fact that calcium-HMB treatment did not work as immunostimulant, HMB was used in this experiment instead.

The Atlantic salmon families selected for Experiment-2 belonged to Stofnfiskur. They were under QTL breeding selection for IPNV as described before. We hypothesized that QTL families should have higher AMP genes expressions compared to non-QTL families. Therefore, QTL and non-QTL families of Atlantic salmon were selected to test this hypothesis.

4.3.1 Results of asCATH-2 expression analysis in gill tissue

CATH-2 basal expression was evaluated in order to reproduce and verify the results of Experiment-1. In addition, the effect of HMB on CATH-2 expression was tested by comparing CATH-2 expression of control and treated fish.

4.3.2.1 asCATH-2 basal expression analysis

asCATH-2 basal expression (control values) of 12 different families was studied in gill tissue in order to see whether there were any differences between families. FD values and their standard errors were plotted as a bar graph (**Figure 24**). Clearly, there were differences between families; for instance, family 275, which is a QTL-family, has a lower basal expression level compared to family 495, which is a non-QTL family. Surprisingly, QTL families have lower asCATH-2 expression than non-QTL families, which disagrees with our hypothesis formulated above.

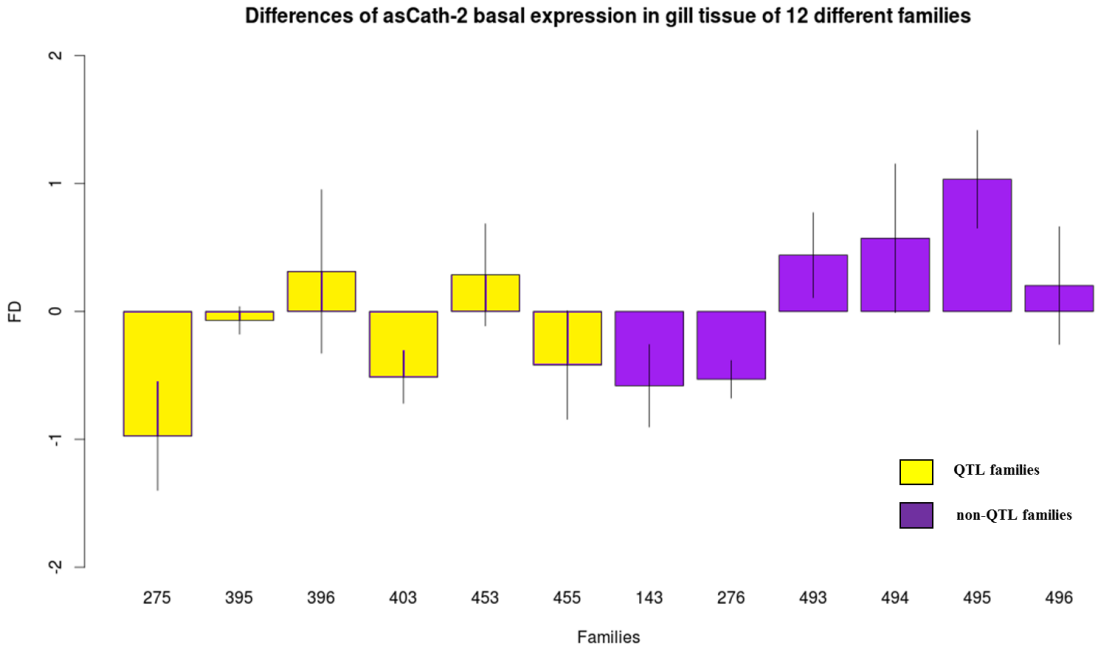

Figure 24. asCATH-2 basal expresision of 12 families in gill samples, where families 275, 395, 396, 403, 453 and 455 are QTL families for IPNV and families 143, 276, 493, 494, 495 and 496 are non-QTL families for IPNV. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data was plotted using Fold Differences (FD) and their standard error (SE) to depict the differences of basal expression between the different families. N = 5-6 fish per group. Vertical whiskers are SE.

One-way ANOVA was performed with the FD values using family as a variable. Results (**Figure 25**) showed that asCATH-2 basal expression differences between families are statistically significant with $p < 0.05$. However, FD values were not as meaningful as we expected.

Analysis of Variance Table

Response: dat$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
dat$Family	11	21.960	1.99635	2.0236	0.04333	*
Residuals	55	54.258	0.98651			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

lm(formula = dat$FD ~ dat$Family, data = dat)

Residuals:

Min	1Q	Median	3Q	Max
-1.88607	-0.57489	-0.03234	0.49401	2.66871

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	-0.9748	0.4055	-2.404	0.019612	*
dat$Family[T.275]	0.9052	0.6014	1.505	0.138010	
dat$Family[T.276]	1.2880	0.5734	2.246	0.028735	*
dat$Family[T.395]	0.4610	0.5734	0.804	0.424878	
dat$Family[T.396]	1.2613	0.6014	2.097	0.040589	*
dat$Family[T.403]	0.5567	0.5734	0.971	0.335897	
dat$Family[T.453]	0.3941	0.6014	0.655	0.515000	
dat$Family[T.455]	0.4446	0.6411	0.694	0.490902	
dat$Family[T.493]	1.4155	0.5734	2.468	0.016708	*
dat$Family[T.494]	1.5475	0.5734	2.699	0.009230	**
dat$Family[T.495]	2.0086	0.5734	3.503	0.000923	***
dat$Family[T.496]	1.1776	0.5734	2.054	0.044788	*

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 25. One-way ANOVA detailed results performed with the FD values of asCATH-2 gene as a target gene and EF-1α as a reference gene. Family was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.1.2 asCATH-2 expression in gill tissue of control and treated fish

Analysis of asCATH-2 expression of control and treated fish (fed with HMB) in gill samples indicated that differences between the two were in all cases statistically significant, meaning that HMB treatment induced asCATH-2 expression in the gills (**Figure 26**).

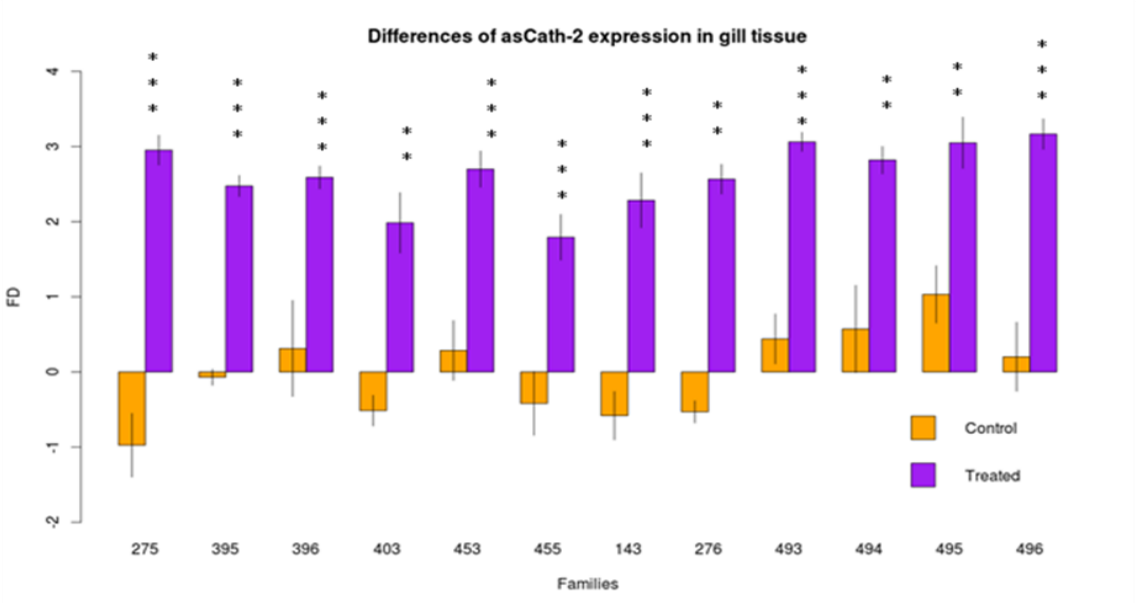

Figure 26. The expression of asCATH_2 in gill samples after 5 days-prt-induction (DPI) feeding these 12 families with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using Fold Differences (FD) and its standard error (SE) to depict the different expression of asCATH-2 between control and treated fish. Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 6 fish per group. Vertical whiskers are SE.

Two-way ANOVA (**Figure 27**) shows significantly higher expression in treated fish ($p < 0.001$). However, as we can see in **Figure 26,** not all the differences between control and treated fish are highly significant in all the families. For instance, differences in family 275, 395, 396 and 493 are highly significant with $p < 0.001$ while differences in families 494,495 and 496 are significant with $p < 0.01$. Moreover, all the treated fish of the different families increase asCATH-2 expression in a similar way when they are treated with HMB, even though asCATH-2 basal expression is significantly different between families (seen above in **Figure 24**).

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Level	1	233.856	233.856	349.3043	< 2.2e-16	***
data$Family	11	25.246	2.295	3.4282	0.0003909	***
data$Level:data$Family	11	8.643	0.786	1.1736	0.3132412	
Residuals	113	75.652	0.669			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 27. Two-way ANOVA results performed with the FD values of asCATH-2 gene as a target gene and EF-1α as a reference gene. Level (meaning control and treated fish) and Family were the factors. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.2 Results of AMP and iNOS gene expression analysis in skin tissue

In order to get a more complete picture of AMPs and iNOS basal expression and expression increase due to HMB treatment, skin tissue was selected due to its important role in innate immune responses.

4.3.2 .1 asCATH-2 basal expression

A new analysis of basal expression was performed with skin samples from the same 12 families. As we can see in **Figure 28** (FD values and standard errors plotted in a bar graph), the asCATH-2 expression pattern varies in the different families.

One-way ANOVA of FD values shows that these asCATH-2 basal expression differences between families are significant ($p < 0.05$) (**Figure 29**). However, these differences are not significant in all the families, only Family 455 and 494 are significantly different from the others with a pvalue of 0.05. Again, there is no clear difference in mean asCATH-2 basal expression between QTL and non-QTL families, leading us to conclude that basal expression of asCATH-2 is not a good indicator of disease tolerance (at least to IPNV virus).

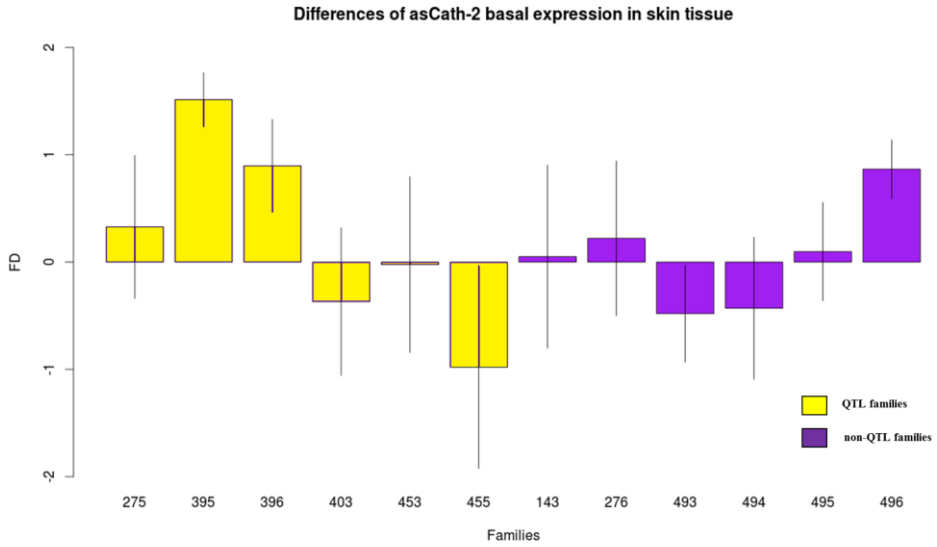

Figure 28. asCATH-2 basal expresision of 12 families in skin samples, where families 275, 395, 396, 403, 453 and 455 are QTL families for IPNV and families 143, 276, 493, 494, 495 and 496 are non-QTL families for IPNV. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data was plotted using Fold Differences (FD) and its standard error (se) to depict the differences of basal expression between the different families. N = 5-6 fish per group. Vertical whiskers are standard error (SE).

Analysis of Variance Table

Response: dat$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
dat$Family	11	97.731	8.8846	2.5813	0.01117 *
Residuals	50	172.096	3.4419		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Detailed analysis

lm(formula = dat$FD ~ dat$Family, data = dat)

Residuals:

Min	1Q	Median	3Q	Max
-4.3156	-0.8756	-0.0145	1.1921	3.2515

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.3948	0.9276	1.504	0.1390
dat$Family[T.275]	-0.6975	1.2445	-0.560	0.5777
dat$Family[T.276]	-2.1905	1.1976	-1.829	0.0733 .
dat$Family[T.395]	-0.8302	1.1976	-0.693	0.4914
dat$Family[T.396]	0.6238	1.2445	0.501	0.6184
dat$Family[T.403]	-2.1869	1.1976	-1.826	0.0738 .
dat$Family[T.453]	0.7243	1.3119	0.552	0.5833
dat$Family[T.455]	-3.1027	1.2445	-2.493	0.0160 *
dat$Family[T.493]	-2.0875	1.1976	-1.743	0.0875 .
dat$Family[T.494]	-3.4541	1.3119	-2.633	0.0112 *
dat$Family[T.495]	-2.2027	1.2445	-1.770	0.0828 .
dat$Family[T.496]	-0.8151	1.1976	-0.681	0.4992

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 29. One-way ANOVA results performed with the FD values of asCATH-2 gene as a target gene and EF-1α as a reference gene. Family was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.2 .2 Comparison between gill and skin asCATH-2 basal expression values

Although no generally significant differences between asCATH-2 basal expression of gill and skin samples were found in two-way ANOVA results (**Figure 31**), FD values and their standard errors were plotted in a bar graph (**Figure 30**) to confirm that there are some families, whose difference between asCATH-2 basal expression of gill and skin, is statistically significant. This comparison interesting in terms of selecting the appropriate tissue to further analyze and use asCATH-2 basal expression as a selection marker, if it can indeed be done. Further experiments involving challenges with pathogens are need in order to reach a conclusion but the amount of fluctuation in basal expression between individuals and between the two tissues makes asCATH-2 basal expression unlikely to be a useful selection marker.

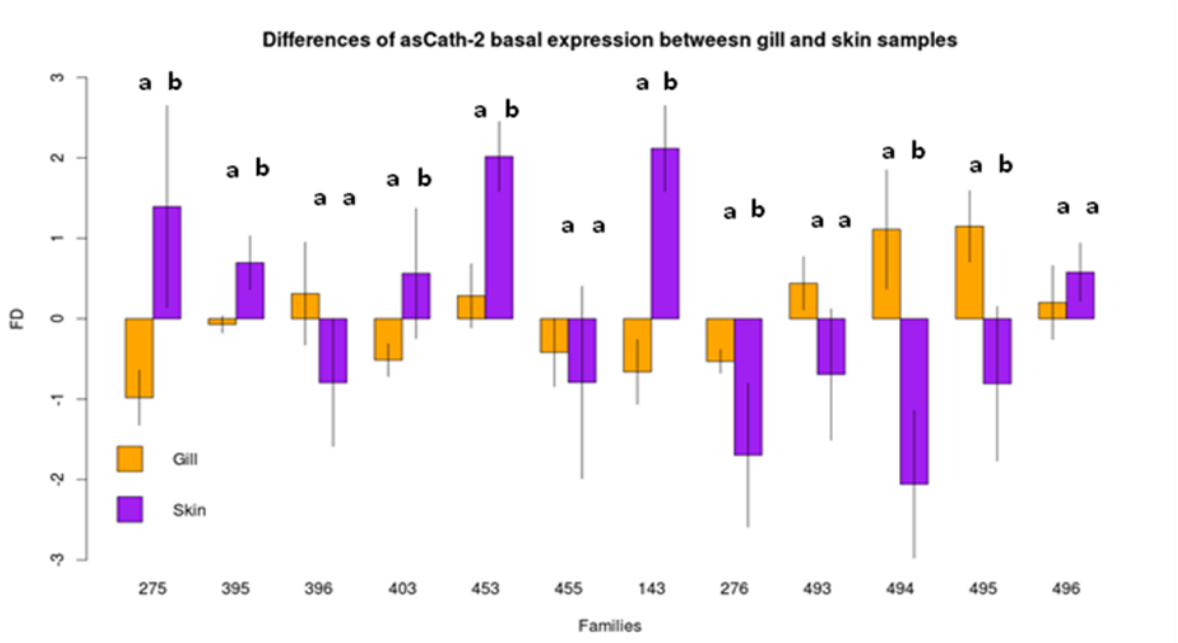

Figure 30. asCATH-2 basal expression of 12 families in gill and skin tissue. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using Fold Differences (FD) and its standard error (SE) to depict differences of gill and skin tissue asCATH-2 basal expression. n = 5-6 fish per group. Vertical whiskers are SE. Different letters denote significant differences (*two–way ANOVA, $p < 0,05$*).

Analysis of Variance Table

Response: set$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
set$Comparison	1	0.008	0.0081	0.0036	0.951997	
set$Family	11	37.839	3.4399	1.5445	0.127959	
set$Comparison:set$Family	11	80.013	7.2739	3.2659	0.000774	***
Residuals	98	218.271	2.2273			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 31. Two-way ANOVA results performed with the FD values of asCATH-2 gene as a target gene and EF-1α as a reference gene. Comparison (meaning asCATH-2 basal expression of gill and skin samples, being gill=1 and skin=2) and Family were the factors (Factor 1 and Factor 2 respectively). Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.2 .3 asCATH-2 expression in skin tissue of control and treated fish

Two-way ANOVA analysis (**Figure 33**) corroborated that there was not a general up-regulation of asCATH-2 expression in skin when fish were treated with HMB. However, by plotting FD values and standard errors in a bar graph (**Figure 32**), we realized that there were significant differences between control and treated fish in some families; for instance family 395, 396, 455, 276 and 494.

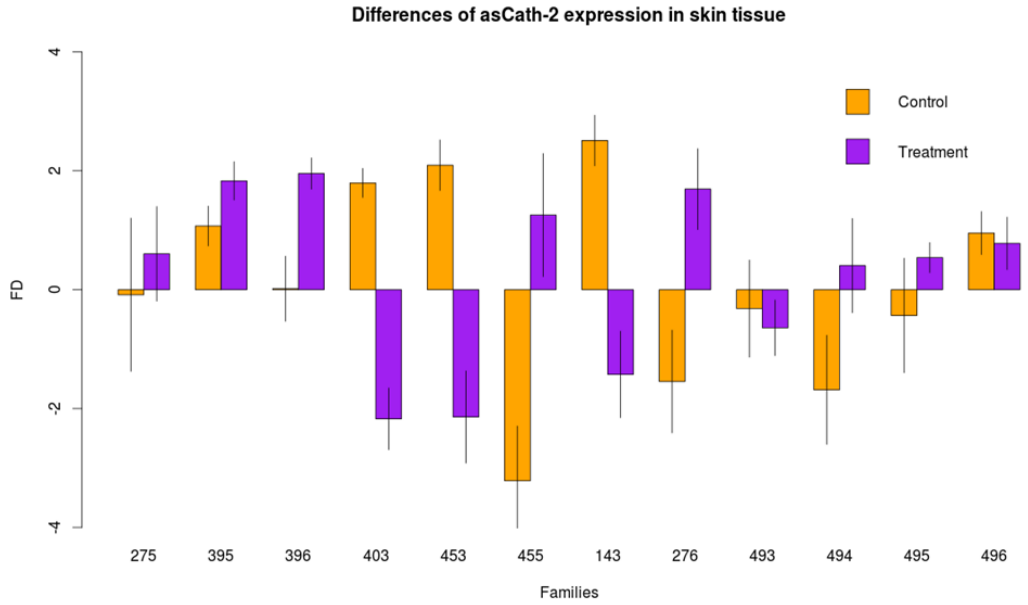

Figure 32. The expression of asCATH_2 in skin samples after 5 DPI feeding these 12 families with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using Fold Differences (FD) and its standard error (SE) to depict the different expression of asCATH-2 between control and treated fish. n = 6 fish per group. Vertical whiskers are SE.

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Level	1	2.519	2.5188	1.0088	0.31747	
data$Family	11	61.755	5.6141	2.2485	0.01684	*
data$Level:data$Family	11	229.627	20.8752	8.3607	2.018e-10	***
Residuals	106	264.663	2.4968			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 33. Two-way ANOVA results performed with the FD values of asCATH-2 gene as a target gene and EF-1α as a reference gene. Level (meaning control and treated fish) and Family were the factors. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.2 .4 Hepcidin-1 basal expression in skin tissue

Significant differences of Hepcidin-1 basal expression in 9 different families with $p < 0.001$ were verified by one-way ANOVA analysis (**Figure 34**). Results were plotted in a bar graph (**Figure 35**) in order to have a clearer picture of which families were significantly different from the others.

In **Figure 35,** QTL families have higher Hep-1 basal expression compared with non-QTL families. One-way ANOVA test (**Figure 36**) and a bar graph (**Figure 37**) showed that there is a statistically difference in Hepcidin-1 basal expression between QTL and non-QTL families with $p < 0.001$. Nevertheless, family 143 showed the highest basal expression. Therefore, even though significant differences between Hepcidin-1 basal expressions were found between QTL and non-QTL families, these differences were not as meaningful as we expected them to be.

Analysis of Variance Table

Response: dat$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
dat$Family	8	172.883	21.610	8.7208	1.837e-06 ***
Residuals	35	86.731	2.478		

Figure 34. One-way ANOVA results performed with the FD values of Hep-1 gene as a target gene and EF-1α as a reference gene. Family was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

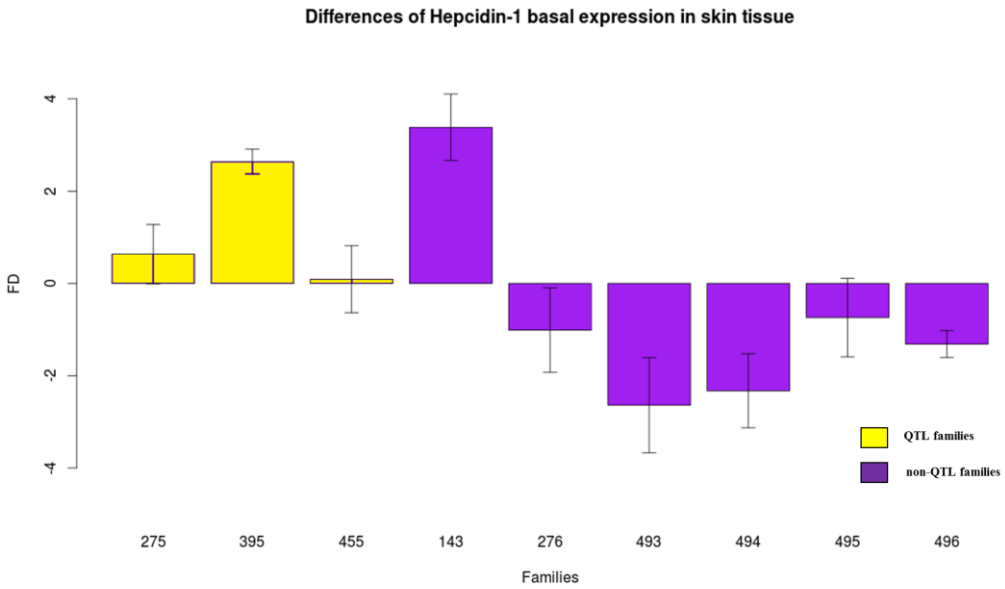

Figure 35. Hep-1 basal expresision of 9 families in skin samples, where families 275, 395 and 455 are QTL families for IPNV and families 143, 276, 493, 494, 495 and 496 are non-QTL families for IPNV. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data was plotted using Fold Differences (FD) and its standard error (SE) to depict the differences of basal expression between the different families. N = 5-6 fish per group. Vertical whiskers are SE.

Analysis of Variance Table

Response: da$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
da$QTL	1	79.399	79.399	18.504	9.898e-05	***
Residuals	42	180.215	4.291			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 36. One-way ANOVA results performed with the FD values of Hep-1 gene as a target gene and EF-1α as a reference gene. QTL (meaning the difference between QTL and non-QTL families) was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

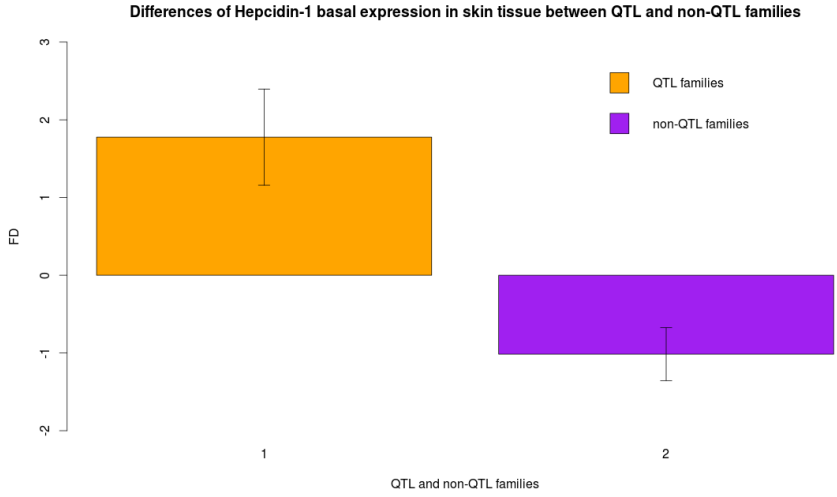

Figure 37. Differences of Hep-1 basal expression in skin tissue between QTL and non-QTL families considering 9 families (275, 395 and 455 as QTL families for IPNV and families 143, 276, 493, 494, 495 and 496 as non-QTL families). Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data was plotted using the mean Fold Differences (FD) values and its standard error (SE). n.1 = 3 families and n.2= 6 families. Vertical whiskers are SE.

4.3.2 .5 Hepcidin-1 expression in skin tissue of control and treated fish

None of the families analyzed for Hep-1 expression showed up-regulation when treated with HMB, as there were no significant differences between control and treated fish in any of the families, shown in the two-way ANOVA test (**Figure 38**) and bar graph (**Figure 39**) results.

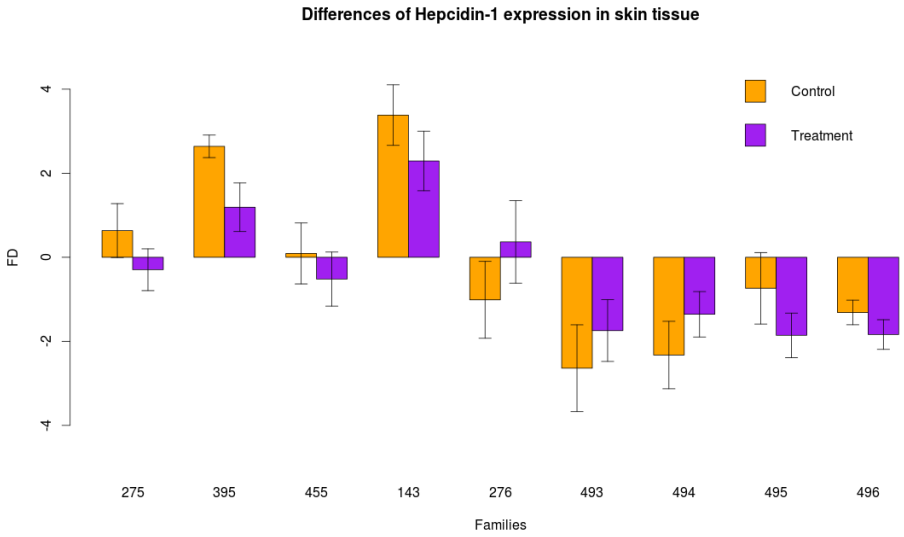

Figure 38. The expression of Hepcidin-1 in skin samples after 5 DPI feeding these 9 families with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using Fold Differences (FD) and its standard error (SE) to depict the different expression of Hepcidin-1 between control and treated fish. Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 6 fish per group. Vertical whiskers are SE.

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Level	1	2.432	2.4322	1.0026	0.3199	
data$Family	8	252.371	31.5464	13.0043	1.274e-11	***
data$Level:data$Family	8	22.886	2.8608	1.1793	0.3227	
Residuals	76	184.364	2.4258			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Figure 39. Two-way ANOVA results performed with the FD values of the Hepcidin-1 gene as a target gene and EF-1α as a reference gene. Level (meaning control and treated fish) and Family were the factors. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.2 .6 iNOS basal expression in skin tissue

Results shown in the bar graph in **Figure 40** and one-way ANOVA (**Figure 41**) showed that there were no significant differences in iNOS basal expression between the three families that were studied.

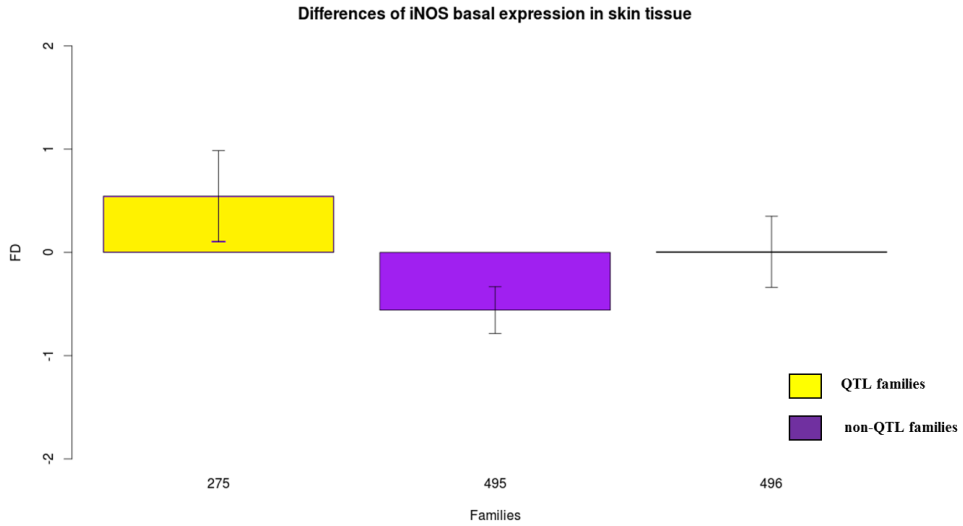

Figure 40. iNOS basal expresision of 3 families in skin samples, where families 275 is QTL families for IPNV and families 495 and 496 are non-QTL families for IPNV. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data was plotted using Fold Differences (FD) and its standard error (SE) to depict the differences of basal expression between the different families. n = 5-6 fish per group. Vertical whiskers are SE.

Analysis of Variance Table

Response: dat$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
dat$Family	1	1.8463	1.84633	3.0883	0.1024
Residuals	13	7.7719	0.59784		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 41. One-way ANOVA results performed with the FD values of iNOS gene as a target gene and EF-1α as a reference gene. Family was the only factor. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.3.2 .7 iNOS expression in skin tissue of control and treated fish

Differences in iNOS expression between control and treated fish were statistically significant with

$p < 0.001$. Therefore, HMB treatment did increase iNOS expression. In addition, as shown in **Figure 42**, iNOS expression in family 275 (QTL family) was not up-regulated due to HMB treatment. In contrast, iNOS expression in family 495 and 496 (non-QTL families) was up-regulated significantly.

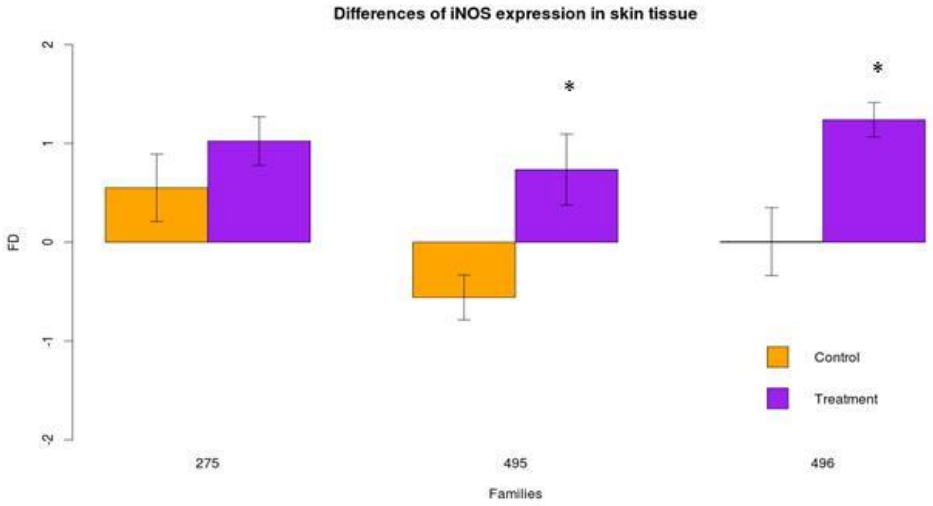

Figure 42. The expression of iNOS in skin samples after 5DPI feeding these 3 families with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using Fold Differences (FD) and its standard error (SE) to depict the different expression of iNOS between control and treated fish. Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 6 fish per group. Vertical whiskers are SE.

Analysis of Variance Table

Response: data$FD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Level	1	8.7003	8.7003	17.7413	0.0002243	***
data$Family	2	2.6442	1.3221	2.6960	0.0843599	.
data$Level:data$Family	2	1.2084	0.6042	1.2321	0.3065024	
Residuals	29	14.2215	0.4904			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 43. Two-way ANOVA results performed with the FD values of iNOS gene as a target gene and EF-1α as a reference gene. Level (meaning control and treated fish) and Family were the factors. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

Taken together, CATH-2 basal expression in gill was significantly different between families as we expected due to the results of Experiment-1. In addition, QTL families were shown to have lower CATH-2 basal expression compared with non-QTL families, which is not the expected outcome of our hypothesis that higher basal expression of CATH-2 is what protects against IPNV in the QTL families. AMPs and iNOS basal expression results in skin showed that CATH-2 and Hepcidin-1 basal expression were significantly different between families and, interestingly, for Hepcidin-1 basal expression, QTL families appear to have higher Hepcidin-1 basal expression compared to non-QTL families. Contrarily, iNOS basal expression was not generally different between Atlantic salmon families.

Our results show that feeding Atlantic salmon with HMB increases CATH-2 expression in gills of all families tested. Surprisingly, HMB neither up-regulated CATH-2 nor Hepcidin-1 expression in skin. In contrast, results from iNOS expression analysis showed up-regulation due to HMB treatment, with a stronger up-regulation in non-QTL families.

4.3.3 Water sample results

Fish were maintained in stagnant water for 5 days, although water was exchanged at 3DPI in order to maintain HMB concentrations and avoid possible bacterial growth. To assess water quality, samples were collected at the 1st, 2nd and 5th DPI.

Results sent by Sýni (**Appendix 8.5**), an accredited laboratory located in Reykjavik, showed that there was no increase of bacterial growth, in control and treated tank at 1st and 2nd DPI. In addition, even though water of control and treated tanks was changed at 3 DPI, there was an increase of bacterial growth (measured by total plate count at 22°C) at 5 DPI. The estimated number was approximately 5 times higher in the treated tank than control tank. These differences are unlikely to be significant but we cannot rule out the possibility of an effect on the fish. Moreover, coliform bacteria were found in the control tank at 1 DPI, and treated tank at 2 DPI and 5 DPI; and *E. coli* in control tank at 1 DPI and treated tank at 5 DPI but concentrations were so low that they are not of concern.

HMB treatment is the only difference between both tanks. Therefore, the fact that higher bacterial growth in treated tank could be due to HMB treatment should be considered. However, this is likely to be a result of an exposure to other external factors (e.g. handling, water change, etc).

4.4 Infection assays with cultured cells results

To further elucidate if the increased expression of these genes due to the treatment had a defense role against bacterial and viral infections in general or if the effect was specific to the disease, infection assays were performed with three different pathogens (the viruses IPNV and ISAV; and the bacterium *P. salmonis*) using different cell lines (CHSE-214, ASK and RTS-11). Cells were differently treated before and after infection with three treatments (HMB, PBA and vitaminD3) (shown in **Figure 10, 11** and **12**).

4.4.1 IPNV infection results

Application of all the different treatments (HMB, PBA and vitaminD3) before and after IPNV infection showed a significant reduction of infected cells at 18 hpi, reduction by HMB treatment was the most significant with a reduction of 53% in the number of infected cells compared to the control. PBA and vitaminD3 reduced infected cell numbers slightly but much less than HMB treatment; approximately by 17 and 20%, respectively, compared to control (**Figure 44**). Results from 72 hpi showed that almost all the cells were infected irrespective of treatment. This is due to a second cycle of replication of the virus which was not inhibited by any of the treatments.

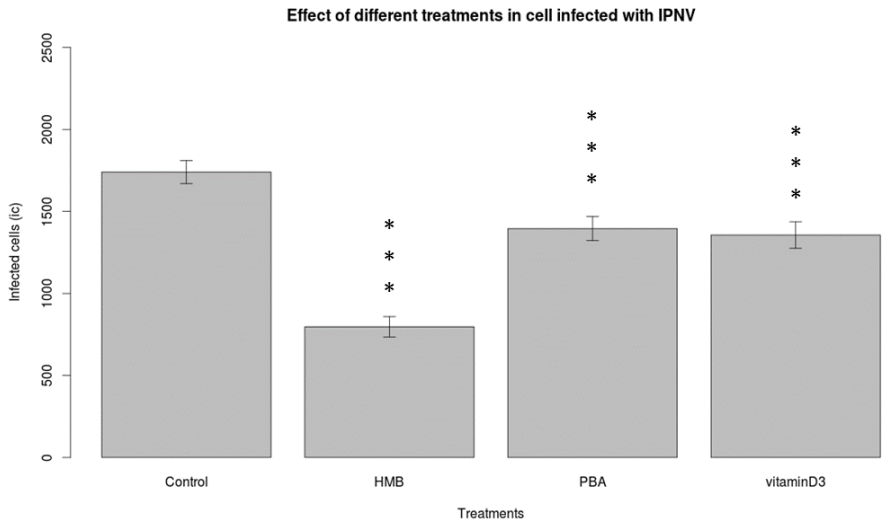

Figure 44. Infected cell number of the different treatments and the control in IPNV infection of CHSE-214 cells at 18 hpi are showed in a bargraph using the mean of infected cells and its standard error. Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 6 wells per group. Vertical whiskers are standard error (SE).

ANOVA analysis demonstrated that reduction of infected cells in all the treatments was highly significant with $p < 0.001$ compared with the control at 18hpi. In addition, ANOVA analysis corroborated that HMB treatment is more effective at preventing infection than PBA or vitaminD3 (shown in **Figure 45**).

Analysis of Variance Table

Response: data$IC

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Treatment	3	13557924	4519308	38.3240	2.548e-16	***
data$Well	5	1969680	393936	3.3406	0.008000	**
data$Treatment:data$Well	15	4495958	299731	2.5417	0.003238	**
Residuals	95	11202747	117924			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

lm(formula = IC ~ Well + Treatment:Well, data = data)

Residuals:

Min	1Q	Median	3Q	Max
-800.40	-204.24	-16.56	198.72	783.84

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	1893.36	153.57	12.329	< 2e-16	***
Well[T.2]	82.80	217.19	0.381	0.703876	
Well[T.3]	55.20	217.19	0.254	0.799921	
Well[T.4]	-237.36	230.36	-1.030	0.305444	
Well[T.5]	-298.08	217.19	-1.372	0.173149	
Well[T.6]	-535.44	217.19	-2.465	0.015482	*
Well1:Treatment[T.HMB]	-1335.84	217.19	-6.151	1.82e-08	***
Well2:Treatment[T.HMB]	-1153.68	217.19	-5.312	7.13e-07	***
Well3:Treatment[T.HMB]	-1280.64	217.19	-5.897	5.66e-08	***
Well4:Treatment[T.HMB]	-618.24	230.36	-2.684	0.008588	**
Well5:Treatment[T.HMB]	-673.44	217.19	-3.101	0.002541	**
Well6:Treatment[T.HMB]	-585.12	217.19	-2.694	0.008345	**
Well1:Treatment[T.PBA]	-1054.32	217.19	-4.854	4.73e-06	***
Well2:Treatment[T.PBA]	-402.96	217.19	-1.855	0.066644	.
Well3:Treatment[T.PBA]	-568.56	217.19	-2.618	0.010297	*
Well4:Treatment[T.PBA]	-38.64	230.36	-0.168	0.867146	
Well5:Treatment[T.PBA]	138.00	217.19	0.635	0.526694	
Well6:Treatment[T.PBA]	-126.96	217.19	-0.585	0.560223	
Well1:Treatment[T.vitaminD3]	-794.88	217.19	-3.660	0.000414	***
Well2:Treatment[T.vitaminD3]	-336.72	217.19	-1.550	0.124375	
Well3:Treatment[T.vitaminD3]	-557.52	217.19	-2.567	0.011819	*
Well4:Treatment[T.vitaminD3]	-325.68	230.36	-1.414	0.160693	
Well5:Treatment[T.vitaminD3]	-546.48	217.19	-2.516	0.013540	*
Well6:Treatment[T.vitaminD3]	270.48	217.19	1.245	0.216052	

Figure 45. On the left, two-way ANOVA results of IPNV infection performed with the infected cell number of the different groups (Control, HMB, PBA and vitaminD3) at 18 hpi. Treatment and Well (replicates) were the factors (Factor 1 and Factor 2 respectively). Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively. On the right, nested linear model perfomed with infected cells numer and using the formula: *Y(ic values)= Well + Treatment:Welll*. Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.4.2 ISAV infection results

Treated cells infected with ISAV did not show the same extent of inhibition of infection as in IPNV infection. Fewer infected cells were counted in wells with HMB treatment at 27 hpi compared to the control (26% fewer infected cells), as shown in **Figure 46** and **Figure 47**, and this difference between Control and HMB treatment was statistically significant with $p < 0.05$. PBA and vitaminD3 did not reduce infection by ISAV in ASK cells. Interestingly, vitaminD3 was significantly different ($p < 0.05$) from the control but increasing instead of decreasing ISAV infection at 27hpi (**Figure 47**). Results from 72hpi showed that almost all the cells were infected, most likely due to viral replication in the wells. These results are supported by two-way ANOVA analysis (**Figure 47**).

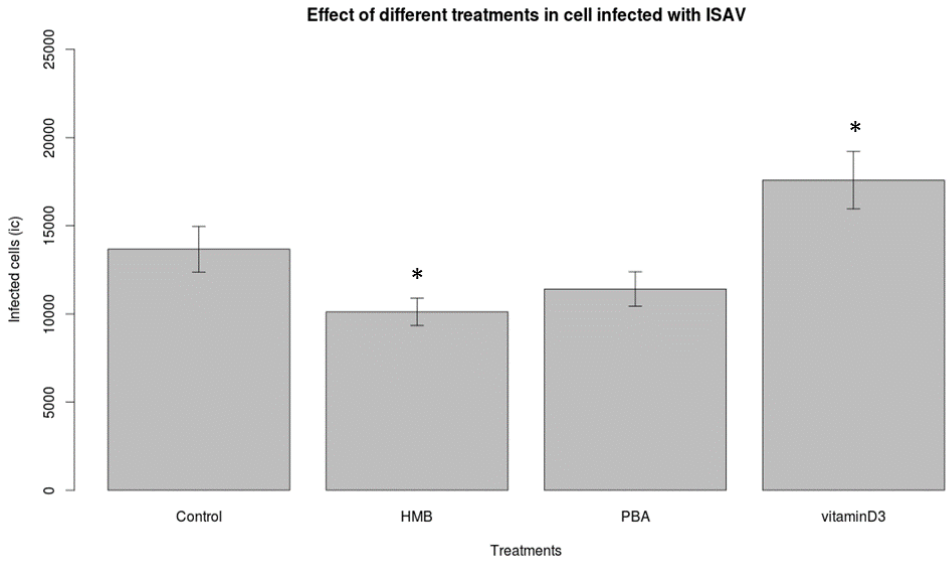

Figure 46. Infected cell number of the different treatments and the control in ISAV infection of ASK cells at 27 hpi are showed in a bargraph using the mean of infected cells and its standard error. Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 wells per group. Vertical whiskers are standard error (SE).

Analysis of Variance Table

Response: data$IC

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$Treatment	3	289138710	96379570	7.3790	0.00114	**
data$Well	2	42559818	21279909	1.6292	0.21703	
data$Treatment:data$Well	6	67067122	11177854	0.8558	0.54066	
Residuals	24	313471195	13061300			

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Detailed analysis of Variance

lm(formula = IC ~ Treatment, data = data)

Residuals:

Min	1Q	Median	3Q	Max
-8872	-2155	-44	2416	7025

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	13672	1212	11.280	1.1e-12	***
TreatmentHMB	-3556	1714	-2.075	0.0461	*
TreatmentPBA	-2262	1714	-1.320	0.1963	
TreatmentvitaminD3	3908	1714	2.280	0.0294	*

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure 47. Two-way ANOVA results of ISAV infection performed with the infected cell number of the different groups (Control, HMB, PBA and vitaminD3) at 27hpi. Treatment and Well (replicates) were the factors (Factor 1 and Factor 2 respectively). Significance levels are *=p<0.05, **=p<0.01 and ***p<0.001 respectively.

4.4.3 SRS infection results

Unfortunately the culture of *P. salmonis* caused a severe infection of CHSE-214 and RTS-11 cells at the dilution that it was used and, at the moment of analysis, our internal housekeeping gene control was severely degraded preventing us from obtaining any meaningful results or drawing any conclusions. It would be worthwhile to repeat the experiment with lower titers of bacteria.

In summary, even though all three treatments (HMB, PBA and vitaminD3) decreased the number of infected cells due to IPNV infection in the CHSE-214 cell line, HMB was the most powerful decreasing the number of infected cells almost by half. For the ISAV infection assay, results were different. Only HMB treatment decreased the number of infected ASK cells and the decrease was not as marked as in the IPNV infection assay. To our surprise, vitaminD3 increased the number of infected ASK cells rather than decrease it.

Unfortunately, SRS results could not be obtained due to the severe infection caused by *P. salmonis.*

4.5 ddRAD Sequencing results

Raw sequence reads, obtained from DeCode, were demultiplexed and cleaned by using *process_radtags* in order to remove low quality sequences and to separate reads from different samples that were individually barcoded.

Results of both lanes (**Figures 48** and **49**) showed that 2-6 million reads were obtained for the majority of samples, which would be considered a suitable number of reads for further analysis. However, some of the samples yielded less than 500.000 thousands reads while others yielded very high numbers up to 24 million of reads. Samples with number of reads far away (higher or lower) from the average should be removed, at least for the initial analysis, as samples with low read numbers will not yield useful data whereas excessively high number of reads will slow down analysis significantly.

The aim with this part of the project was to utilize the available material to establish the ddRADseq method. The raw data look very promising and experimentally things went well. Detailed analysis of the data, searching for SNPs that are informative about various traits, including disease tolerance is the next step. This is however beyond the scope of this M.S. thesis.

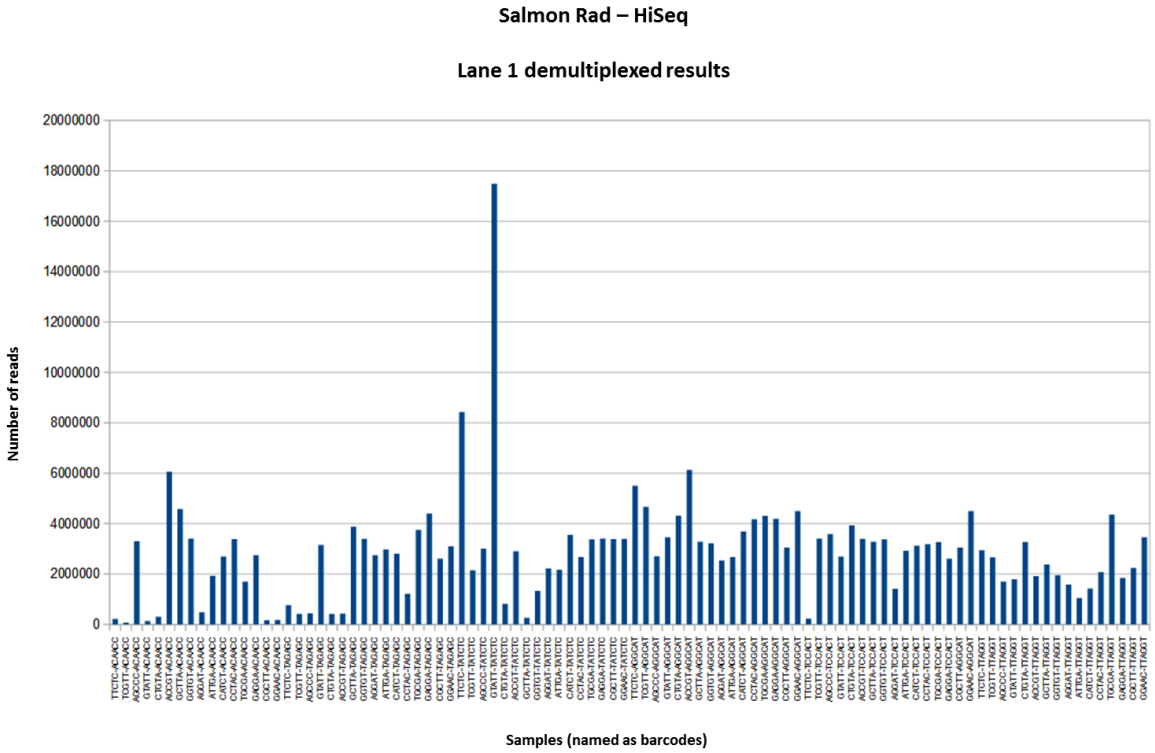

Figure 48. Demultiplexed results (Lane 1) performed with ddRAD-Sequencing data of Atlantic salmon samples from different families and their offspring using *Stacks* software.

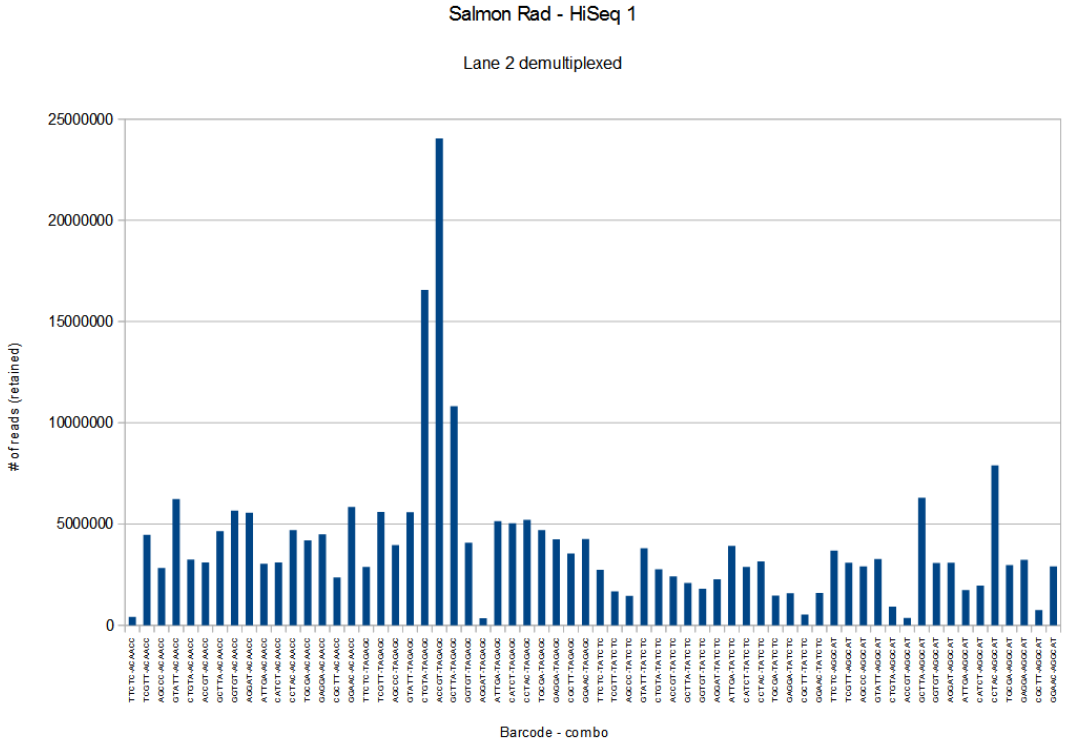

Figure 49. Demultiplexed results (Lane 2) performed with ddRAD-Sequencing data of Atlantic salmon samples from different families and their offspring using *Stacks* software.

5 Discussion

Disease outbreaks constitute one of the most relevant problems for aquaculture. General management (e.g. biosecurity, genetics, water quality, nutrition, etc.) is the first step in order to avoid pathogen infections but it may not always be effective. Therefore, when opportunistic pathogens present in the environment infect the fish, the innate immune system is the first chance to stop pathogen invasion, preventing disease outbreaks. However, when the innate immunity is not strong enough to prevent the infection, antibiotics and vaccines must be used. Fish rely on innate defenses for initial protection against pathogen invasion because their acquired immune system has a poor immunological memory and a short-lived secondary response (Du Pasquier 2001). However, the innate immunity mechanisms have many components, AMPs are known to be extremely important as innate defenses in both vertebrates and invertebrates (Zasloff 2002), being some of the most potent and rapidly lethal host defense chemicals that have been described in animals (Noga et al. 2011).

Our main objective was to find out if the differential expression of AMP genes, which are known to play a crucial role in the defense system in vertebrates, could be used as a molecular tool to select the most robust families for breeding purposes. CATH-2 expression has an important role on the innate immune system (Scocchi et al. 2009). For instance, Ramanathan et al (2002) showed that mammalian cathelicidins possess a broad antimicrobial activities capacitating defense against a range of Gram negative and Gram positive bacteria, fungi, parasites and viruses . Hepcidin-1 participates in many immune mechanisms (inflammation, trapping iron in macrophages and decreasing plasma iron concentrations) (Ganz & Nemeth 2006). Moreover, iNOS is a relevant component in some infectious disease processes and controls excessive immune reaction (Bogdan 2001). Therefore, CATH-2, Hepcidin-1 and iNOS were our selected genes.

In our first experiment (Experiment-1) we revealed, by analyzing CATH-2 basal expression in gill tissue of 62 families of Atlantic salmon, that there are significant ($p<0,001$) CATH-2 basal expressions differences between families. In addition, CATH-2 basal expression in gills was measured after 3 months in order to see whether changes would happen over time and fish development. Although no general differences were found between both CATH-2 basal expression measurements, some families did present significant differences and,

interestingly, some families increased CATH-2 basal expression whereas others decreased it when we compared then with the average. In our second experiment (Experiment-2), CATH-2 basal expression was measured in gill and skin tissue of 12 families. Even though some families presented significantly different basal expression levels when gill and skin tissue were compared, both basal expression levels were statistically different between families, showing that these findings were not just randomly obtained. Further studies could include different time points of CATH-2 basal expression measurements, so that the most suitable time for CATH-2 basal expression analysis during fish development could be established. For a more complete picture, heritability for CATH-2 basal expression of 62 families was estimated and the result shows a heritability of 0.61 with a SE of 0.0061. Therefore, the basal expression of CATH-2 is clearly and highly affected by genetics among the 62 Atlantic salmon families.

According to the study made on basal expression levels of the remaining two genes, Hepcidin-1 basal expressions in skin was also statistically different between families, hence, Hepcidin-1 basal expression could be used for fish robustness selection. Unfortunately, we could not formulate the same hypothesis for iNOS basal expression because the results were not significantly different between families.

Aquaculture companies are currently using quantitative trait loci (QTL) because of their potential to facilitate the manipulation of important traits in fish breeding and to control traits of economic significance to their production (e.g., disease resistance, fecundity, growth, etc.) (Ozaki et al. 2001). Moen et al (2009), for instance, described a major QTL for resistance against the viral disease IPN in Atlantic salmon, providing a tool for direct selection of fish possessing resistance against this virus. Our selected Atlantic salmon families, which belong to the fish breeding company Stofnfiskur, are also under an IPNV-QTL breeding selection. Therefore, a comparison was performed to further study the differences of Hepcidin-1 and iNOS basal expression in skin tissue between QTL and non-QTL. Hepcidin-1 basal expression was significantly higher in QTL families, indicating a possible future use of Hepcidin-1 basal expression as tool for fish robustness selection. In contrast, iNOS basal expression was higher in non-QTL families. Those results may be due to the low number of families selected. Further experiments should include a larger number of families in order to obtain more statistical power.

Previous studies have shown that HMB could work as a possible immunostimulant. For example, the addition of HMB as food additive for rainbow trout and carp resulted in an increase of RBA, PKA and lymphocyte proliferation (Siwicki et al. 2003). Also, in our previous studies we concluded that by adding HMB in the diet and water of two families of Atlantic salmon, the expression of CATH-2, Hepcidin-1 and iNOS was increased at different time points, implying that HMB could work as an immune-modulator. Two different formulations of HMB are commercially available (calcium-HMB and HMB). We performed experiments using both formulations and chose to sample gill and skin tissue. We increased the number of families to a total of 14 (calcium-HMB, Experiment 1) and 12 (HMB, Experiment 2) to increase statistical power and assess whether genetic background influenced the response. With calcium-HMB, differences between controls and treated fish were not significant. This results, plus the fact that the water was white, indicated that the calcium component of the formulation could had not been dissolved in the water, which may not allow HMB to induce CATH-2 expression. In contrast, Experiment-2 revealed completely different results in gill tissue. It is noteworthy that fish belonging to the 12 Atlantic salmon families used for this study appeared to increase CATH-2 expression approximately up to the same values (two to three Fold Differences), reacting similarly to HMB treatment, even though their initial basal expression levels were significantly different. Therefore, we hypothesized that when fish have low CATH-2 basal expression values, upon HMB treatment, their basal expression will increase to obtain similar level of expression as in families with higher CATH-2 basal expression values. These findings might provide a relatively simple, inexpensive and environmentally friendly means to boost innate immunity in anticipation of stressful events such as transportation, tank exchanges, vaccination or possible infections.

The mucosa-associated lymphoid tissues (physical barrier) in teleost fish include the gut, skin and gills; they are exposed to the external environment and form the initial barrier to pathogen invasion (C. McL.Press and Evensen, 1999; Dalmo et al., 1997). In our study, skin was selected because fish skin mucus acts as a defense barrier, and due to the fact that antimicrobial properties against infectious pathogens (bacteria and viruses) have been demonstrated in different fish species (Ángeles Esteban 2012; Ellis 2001; Su 2011; Wei et al. 2010). Contrary to our expectations, CATH-2 expression analysis in skin showed that CATH-2 is not generally up-regulated under HMB treatment. However, we realized that

some families (Family 395, 396 and 455) did increase CATH-2 expression significantly and, in contrast, two of the families (family 403 and 453) down-regulated CATH-2 expression, which could explain the outcome of this ANOVA analysis.

One interesting observation made in this study was that Hepcidin-1 and iNOS results concurred with results obtained in our previous studies made in a mixture of tissues (heart, kidney, spleen and gill), where Hepcidin-1 was late up-regulated at 20 DPI and iNOS showed up-regulation at 5, 13 and 20 DPI. In this study, HMB treatment did not seem to increase Hepcidin-1 expression in skin tissue may be due to the fact that we collected the samples at 5 DPI, being too early to see an increase of expression. iNOS expression in skin, contrarily, was increased significantly in two of the three selected families. Interestingly, both families that showed increased expression were non-QTL families for IPNV resistance and their basal expression levels were lower than in the QTL family, which coincides with the CATH-2 expression results discussed above.

To our surprise, water samples analysis showed that there was an increase of bacterial growth at 5 DPI, approximately 5 times higher in the treated tank than in the control tank. *E. coli* and other coliform bacteria were also found at specific time points but concentrations were not high enough to be of concern. The somewhat higher total bacterial count at 5 DPI in the treated tank is a fact that we must acknowledge. Bacterial growth in the water is likely to induce AMP expression and although the numbers are relatively low and the difference between control and treated tanks is less than an order of magnitude, we cannot completely exclude the possibility that this may influence the results. Nevertheless, we concluded that HMB treatment had an effect on CATH-2 and iNOS expression, inducing their expression. The reason for the somewhat higher bacterial count in the HMB supplemented tank is unclear. Most likely this is simply a chance occurrence, and in any case the difference is small. Total plate count were done by Sýni at 22ºC whereas the tanks were held at ~5°C. The assay therefore counts the number of mesophilic bacteria which may not be an accurate measurement of the number of relevant species. One intriguing possibility is that HMB may influence the bacterial community favoring mesophiles. Such speculation is, however, premature bases on one data point.

It is known that the use of HDAC inhibitors (butyrate, HMB, PBA, and others) on cells can have an effect on hyperacetylation of histones, leading to changes in chromatin structure,

gene expression, and cellular function. They also enhance nuclear receptor-mediated expression of the CAMP genes (Steinmann et al. 2009). VitaminD3 has been found to boost the antimicrobial activity of human monocytes/macrophages against *Mycobacterium tuberculosis* (Rook et al. 1986). Liu et al. (2007) demonstrated that cathelicidin is required for the vitaminD3-triggered antimicrobial activity against this intracellular bacterium. Furthermore, Steinmann et al (2009) showed that PBA acts synergistically with vitaminD3 and hypothesized that it is a promising drug candidate for treatment of microbial infections. Hence, PBA may be useful as an AMP-inducer, strengthening the innate antimicrobial response. Therefore, in the light of these previous studies and the putative HMB effect on AMPs and iNOS expression up-regulation, we were interested to elucidate whether the increased expression of these genes due to HMB treatment as well as the two other previously described treatments (PBA and vitaminD3), had a protective effect against bacterial and viral infections (IPNV, ISAV and SRS).

Our results showed that HMB, PBA and vitaminD3 treatments decrease significantly the number of infected cells at 18 hpi in an IPNV infection. HMB treatment seemed to be the most powerful treatment with a reduction of 53 percent in the number of infected cells compared with the control. PBA and vitaminD3 treatments worked similarly by reducing the number of infected cells by 17 and 20 percent respectively. On the other hand, results for ISAV infection were not as clear as IPNV infection results. Only HMB had clear protective effect against ISAV infection by reducing significantly the number of infected cells at 27 hpi. PBA and vitaminD3 did not reduce infected cells in ISAV infection, hence, they are not candidates for treatment against pathogen infections. It is important to highlight that vitaminD3 increased the number of infected cells rather than decrease it, which was unexpected as vitaminD3, as explained above, was previously found to strengthen the immune system by boosting antimicrobial activity (Rook et al. 1986). Moreover, a reduction of infected cells was not visible at 72hpi in either viral infections, where all the cells were infected most likely due to a second step of viral replication. No conclusions could be drawn about *P. salmonis* infection because our internal housekeeping gene control was severely degraded due to the violent infection. In addition, we collected all the infected cells from both IPNV and ISAV infections to perform further qPCR analysis and study if treatments added before and after infection had any effect in AMP gene expressions. Unfortunately, analysis are still underway.

In order to study in greater detail whether there is an inhibition of infection caused by the induced expression of these AMPs, on these viral agents (IPNV and ISAV), additional infection trials should be performed using different multiplicity of infections (MOI). Many viruses cause immuno-depression and toxicity by simply over-expressing viral proteins e.g. the classical example of hexons in Adenovirus. It should be considered that we need to infect at very high MOIs and very high fish densities in order to perform reproducible infections. However, these conditions seldom enclutered in natural and normal conditions by free-living marine animals or even in aquaculture. Unfortunately challenge experiments using these pathogens cannot be conducted in Iceland due to the fact that Iceland has the status of a "country free of viral diseases". Therefore, all work with exotic agents (pathogens and non-pathogens) is not allowed and will have to be performed again in Chile or elsewhere, where allowed.

Finally, fin samples from parents of all the 12 families (Experiment-2) and their offspring (a total of 160 samples) were collected to provide a foundation to further search for SNPs, which can be used to select the most robust fish genotypes for salmon farming, rather than performing time consuming and more expensive qPCR expression analysis. Two to six million reads were obtained for the majority of samples in both lanes, (**Figures 49** and **50**) and this number of reads should be enough to perform further analysis.

6 Conclusions

We have revealed that the Atlantic salmon families selected had different CATH-2 basal expression levels and we believe that this could be used as a tool for robustness selection due to it had been suggested in previous studies. Importantly, it was highly affected by genetic relationships among the specific 62 families studied. Moreover, Hepcidin-1 basal expression could also be used as a phenotype marker for better innate immunity selection but further analyses need to be performed in order to draw unequivocal conclusions. In contrast, iNOS basal expression did not seem to work as a selection marker.

An important objective of this study was to test whether the two different formulations of HMB (calcium-HMB and HMB) work as immune-modulators by increasing the expression of AMP genes and iNOS. We showed that HMB treatment in food and water did act as an immune-modulator, stimulating CATH-2 and iNOS expressions in Atlantic salmon families at 5 DPI. On the other hand, HMB did not stimulate Hepcidin-1 expression. A plausible reason is that Hepcidin-1 expression seems to need longer incubation treatment with HMB to increase its expression. Calcium-HMB did not show any enhancing effect on the expression of the selected studied genes, likely due to its lack of solubility. Therefore, it should not be used as immunostimulant.

In addition, we show that HMB, PBA and vitaminD3 treatments significantly decrease the number of IPNV infected CHSE-214 cells when added to the tissue culture medium prior and throughout the infection procedure. Therefore, they may have an up-regulation effect on the innate immune responses. The results from ISA virus infection showed that HMB treatment decreased the number of infected cells, compared with controls; but, contrarily, PBA and vitaminD3 did not reveal any significant decrease of infected cells.

Future aims should include new infection assays with cultured cells test in order to complete the unfinished analysis and to perform new infection trials using different multiplicity of infections (MOI). In addition, detailed analysis of RAD-Sequencing data will be the most important aim of our next project.

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8 Appendix

8.1 HMB treatment (The ProteinWorks): Certificate of Analysis

Certificate of Analysis

Batch Number	20120505
Date of Manufacture	May 2012
Re-Test Date	2 Years from Date of Manufacture
CAS No	135236-72-5
Molecular Formula	C10H18CaO6. H2O
Compound Ingredient	None

Test	Specification	Results
Appearance	Crystalline Powder	Conforms
Colour	White to Off/White	Conform
Aroma	Characteristic	Conforms
Flavour	Characteristic	Conforms
Calcium	Min 16%	15.07%
HMB	Min 84%	84.18%
Total Content	Min 99%	99.25%
Loss on Drying	Max 8%	679%
Particle Size	100% Through 80 Mesh	Conforms
Bulk Density	Min 0.5g/ml	0.52g/ml
Heavy Metals	Max 10ppm	7ppm
Lead (Pb)	Max 3ppm	Conforms
Cadmium (Cd)	Max 1ppm	Conforms
Mercury (Hg)	Max 0.1ppm	Conforms
Arsenic (As)	Max 1ppm	Conforms
Total Plate Count	Max 1,000cfu/g	<1,000cfu/g
Yeast & Mould	Max 100cfu/g	<100cfu/g
E. coli	Negative	Negative
Salmonella	Negative	Negative
Staphylococcus	Negative	Negative
GMO Status	Non-GMO	Non-GMO
Irradiation Status	Non Irradiated	Non Irradiated

This material conforms to the USP/CP standard.

We confirm that the information above is sourced from the original manufacturers/suppliers Specification and Batch Certificate of Analysis.

8.2 Phenol Chloroform Extraction protocol

Warning: Phenol is extremely dangerous. Always work in fume hood and use 2 pairs nitrile gloves (no latex). There is a centrifuge to use inside the fume hood. If you get phenol on your gloves, change the outside pair as soon as possible. If you spill on your skin, do not use water to rinse it off. Apply PEG300 to skin and wipe off with paper towel.

Tissue and cell lysis

1. Ethanol preserved tissue, rinse in water for several minutes and squeeze all liquid out in paper.
2. Cut about 3-4 mm square tissue into small pieces (~100 mg).
3. Add 500uL STE buffer
4. Add 50uL 10% SDS
5. Add 20uL Proteinase K (10 mg/mL)
6. Incubate at 55 degrees until tissue is digested (3-4 hours). Mix gently by inversion every 45 – 60 minutes (can incubate overnight at 37 degrees).

Extraction - use wide bore tips if necessary, cut off the ends of P1000 tips with a blade.

1. Add equal volume phenol:chloroform:isoamyl alcohol (25:24:1) mixture
2. Gently mix by inversion, 3 minutes
3. Centrifuge 10,000g for 3 minutes
4. Remove aqueous layer (carefully, no phenol or cell debris) and add to new tube
5. Repeat steps 1-4
6. Add equal volume chloroform:isoamyl alcohol (24:1)
7. Gently mix by inversion, 2 minutes
8. Centrifuge 10,000g for 5 minutes
9. Remove aqueous layer (very carefully this time) and add to new tube
10. Optional: repeat steps 6-9

Precipitation

1. Add 1/10th volume 3M sodium acetate
2. Add 1mL cold (from freezer) 96% ethanol
3. Put in -20 freezer minimum 2 hours, preferably overnight (increases DNA yield)
4. Centrifuge at maximum speed at 4 degrees for 30 minutes
5. Pipette off liquid being careful not to disturb pellet
6. Add 500uL of cold (from freezer) 70% ethanol
7. Centrifuge at maximum speed at 4 degrees for 5 minutes
8. Pipette off liquid, do not disturb pellet and take away as much liquid as possible

9. Let pellet air dry until completely dry, at least one hour.
10. Add 100-200uL 1X T.E (10mM Tris, 0.1mM EDTA) depending on size of pellet
11. Let pellet dissolve overnight in fridge (or at 37 degrees for 20-30 minutes if you are in a hurry).
12. Optional: Add 10uL RNase-A (10mg/mL), incubate 37 degrees for 1 hour.
13. Check concentration and purity on nanodrop and quality on agarose gel.

8.3 DNA Quantification using SYBR gold

It is necessary to quantify your PCR product to dilute for the Illumina sequencing kit protocol. You can achieve this with an expensive Bioanalyser or by using the inexpensive SYBRgreen/gold method. Since you need to generate a standard curve for *each time* you prepare the SYBR dilutions, wait until you have a sufficient number of samples to run.

You will need:

- Sterile amber or foil covered 1.5 mL polypropylene tubes. You will still need up to 10 tubes for the standard curve.
- SYBR Green or SYBR Gold (at known concentration).
- Sterile 1X TE (10mM Tris, 1mM EDTA).
- Control dsDNA of known concentration (i.e. lambda DNA).
- A clear polyethylene plate for the Genios, minimum well capacity 200 µL.
- Fluorescence microplate reader (i.e. Tecan Genios)

1. Turn on Genios in equipment room (room 352, next to qPCR machine), the laser needs to warm up for at least 20 minutes. Make your SYBR dilutions (2-step dilution).

Calculate how much 1: 4.5 dye solution you will need (at 100uL per sample and 100uL per standard). Use this amount to back calculate how much dye to transfer from the concentrated stock solution of 10,000X. For example, if I need 10mL of 1:4.5 dye solution, I would need to add 222uL of 1:100 dye solution to 9.777 mL 1X TE. To make 222 µL of the 1:100 dye solution, I need to add 2.5 µL of concentrated SYBR (at 10,000X) to 247.5 µL 1X TE. Remember the lowest amount you can reliably pipette is 1uL so the smallest amount of 1:100 dye solution you can make is 100 µL (by adding 1uL of concentrated SYBR to 99 µL 1X TE). Use the rule that each 1mL of 1:4.5 dye solution is 22.2 µL of 1:100 dye solution + 977.7 µL 1X TE.

Note: DO NOT split 1:4.5 dye solution across 1.5 mL tubes because pipetting variation could give different amounts of dye in each tube. If you need to make more than 1.5 mL, use a 15 mL tube.

2. Prepare a series of 2X serial dilutions of control dsDNA ranging from 2 ng/ml to 2,000 ng/ml in amber tubes.

*In assay describes the final concentration of dsDNA that is present.

dsDNA (ng/mL)	dsDNA (µL)	1X TE	Total Volume (µL)	*in assay dsDNA (ng/mL)
2000.0			178.3	1000.0
1000.0	78.3	78.3	156.6	500.0
500.0	56.6	56.6	113.2	250.0
50.0	13.2	118.8	132.0	25.0
10.0	32.0	128.0	160.0	5.0
4.0	60.0	90.0	150.0	2.0
2.0	50.0	50.0	100.0	1.0

Set up the serial dilutions by adding the volume of dsDNA specified in column 2 from the previous tube, i.e. for the 50.0ng/mL dilution take 13.2 µL from the 500ng/mL dilution and for the 10.0ng/mL dilution take 32 µL from the 50ng/mL dilution, and so on.

**You might be interested in adding one or two more concentrations based on what you are expecting for the sample concentration. For this RADseq protocol, expect between 50 to 70 nM.

4. Record the dsDNA concentrations on each tube. You will have 100 µL of standard dsDNA in each tube.

5. Prepare your samples by adding 1 µL of sample (purified PCR product) to 99 µL of 1X TE in labeled amber tubes, your sample is now a 1:100 dilution. (You might want to prepare some other dilutions if you do not know what concentration to expect, i.e. 1:200 dilutions, but only do this for one or two samples and quantify first before proceeding with all samples). Once you figure out the appropriate dilution for your particular species/protocol, you should not need to alter it.

6. Prepare a reference standard by adding 100 µL of 1X TE into a labeled amber tube.

7. Add 100 µL of 1:4.5 dye solution to all tubes (standards, samples and reference). Your samples are now at 200:1 dilution and the standards are at the concentration in the 5th column. Vortex briefly and incubate at room temperature for 5 minutes.

8. Transfer 200 µL to wells in the plate in preparation for reading on the Genios, recording well ID for standards, samples and reference. Cover plate in aluminum foil.

9. Reading on the Genios. Login password: GENios1.

a. Open the Magellan software.

b. Click on Start Measurement in Wizard.

c. Choose DNAsybrgreen.mth as the method.

*Sometimes the correct filter has been removed from the machine, it is in the top drawer to the right. The correct filter has 485nm wavelength on it, it goes into the filter slot in the machine. Plate and filter slot control is through the screen. The program will not run if the correct filter is not in the machine.

d. Click on start and the machine will read the plate, this takes only 1-2 minutes.

e. Write the output values on a separate sheet of paper as to date I have not figured out a way to export the data in readable format.

Molarity Calculation

Open the excel spreadsheet "DNA_quant.xlsx".

Constructing the standard curve (STD sheet):

1. Enter the standard dsDNA concentration and fluorescence emittance values in the appropriate columns. A curve should appear in the chart on the same sheet. Note: if you have added more standards, remake the curve with all the data.

2. Check the R^2 value is > 0.98. If the R^2 value is < 0.98, read the plate again and enter the values in another table. If the R^2 value is still < 0.98, repeat the whole procedure (including sample preparation). You are fitting a linear regression line to the graph and then using the equation of this line to calculate the concentrations of your samples.

3. Check the accuracy of your standard curve by calculating the concentration of the standards based on the formula of the graph.

The formula of the graph will take the form of $f(x) = mx + b$ where m is the slope of the line and b is the y-intercept. Eq. 1 is the calculation for concentration from emittance and standard curve equation.

Eq. 1 **Concentration (ng/mL) = (emittance – b) / m**

You should have calculated similar concentrations as the standards, do not worry if they are slightly different as long as they are similar.

Calculating Sample Molarity (SAMPLE sheet):

1. Enter the sample names and fluorescence emittance values in the appropriate columns.

2. Calculate the concentration (ng/mL) using Eq. 1 and factoring in the dilution. Now we need to calculate the molarity of the library.

3. Enter the average fragment size (known from the Pippin Prep).

Notes on sample molarity and Illumina sequencing:

There is a calculation error on the quantification spreadsheet. Do not change this! Proceed as normal and report the concentration as seen in the appropriate (nM) column as the true concentration to go through the Illumina MiSeq preparation protocol.

If using Illumina MiSeq, go through the preparation protocol as normal but add 2.5X more volume than required to the flow cell.

8.4 Figures of Primer Efficiency analysis

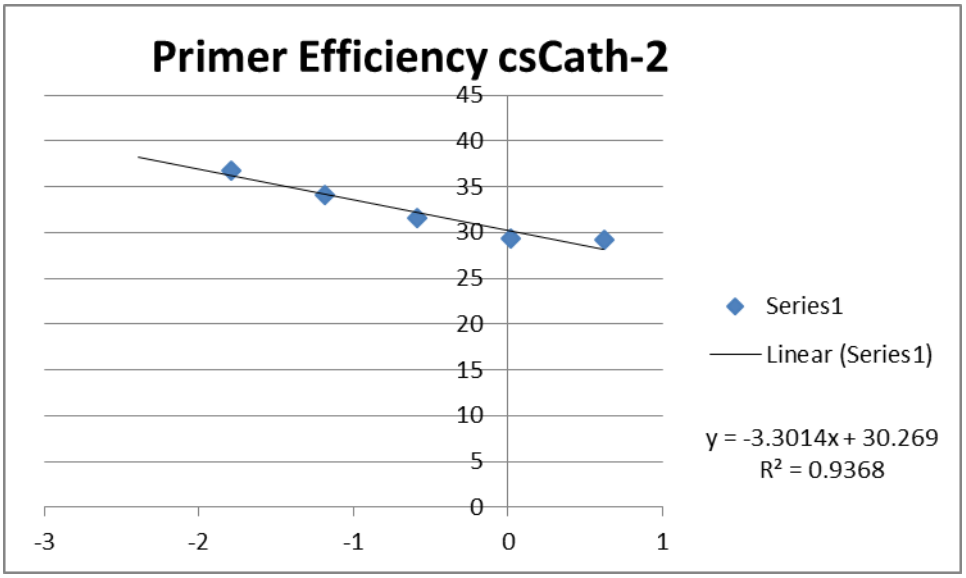

Figure 50. Efficiency of csCath-2 primer (target gene). Ct mean values plotted againts the logarithm (log10) of the original RNA input, linear regresssion line and equations to obtain the slope and determine the effiency

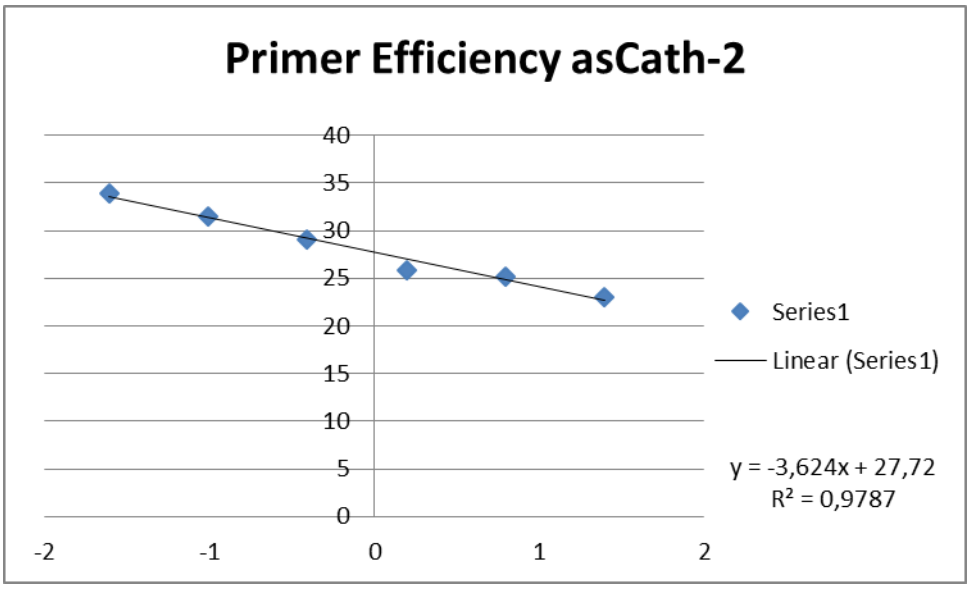

Figure 51. Efficiency of asCath-2 primer (target gene). Ct mean values plotted againts the logarithm (log10) of the original RNA input, linear regresssion line and equations to obtain the slope and determine the effiency

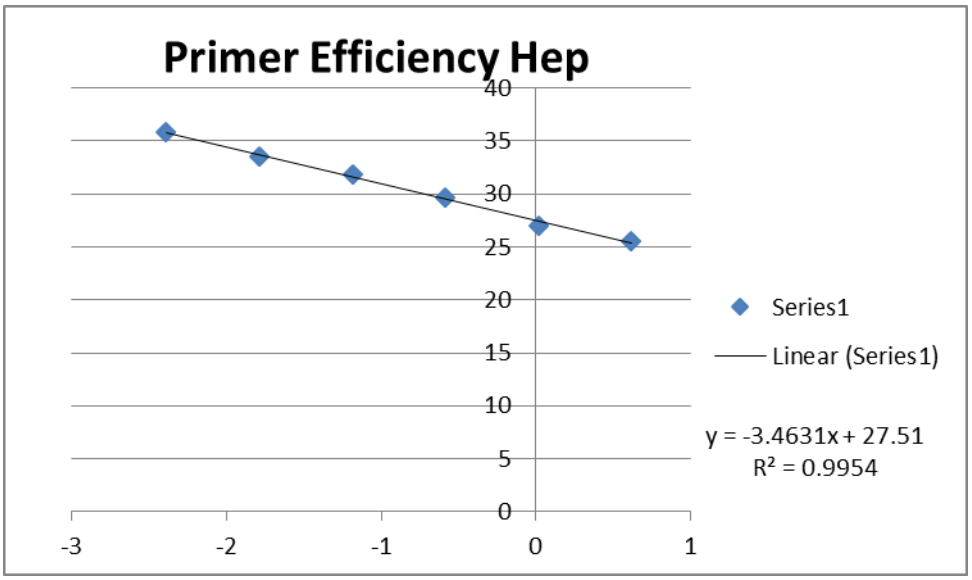

Figure 52. Efficiency of hepcidin primer (target gene). Ct mean values plotted againts the logarithm (log10) of the original RNA input, linear regresssion line and equations to obtain the slope and determine the effiency

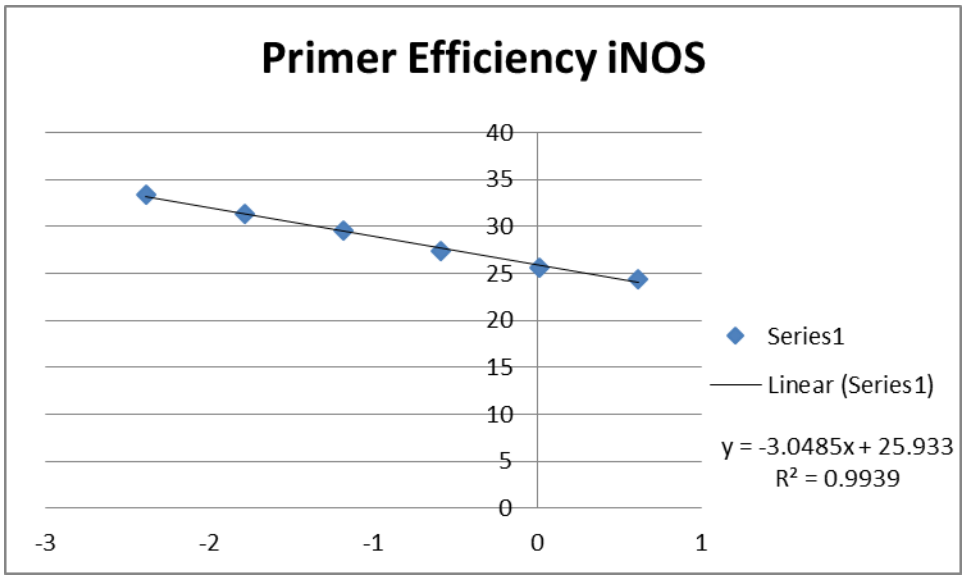

Figure 53. Efficiency of iNOS primer (target gene). Ct mean values plotted againts the logarithm (log10) of the original RNA input, linear regresssion line and equations to obtain the slope and determine the effiency

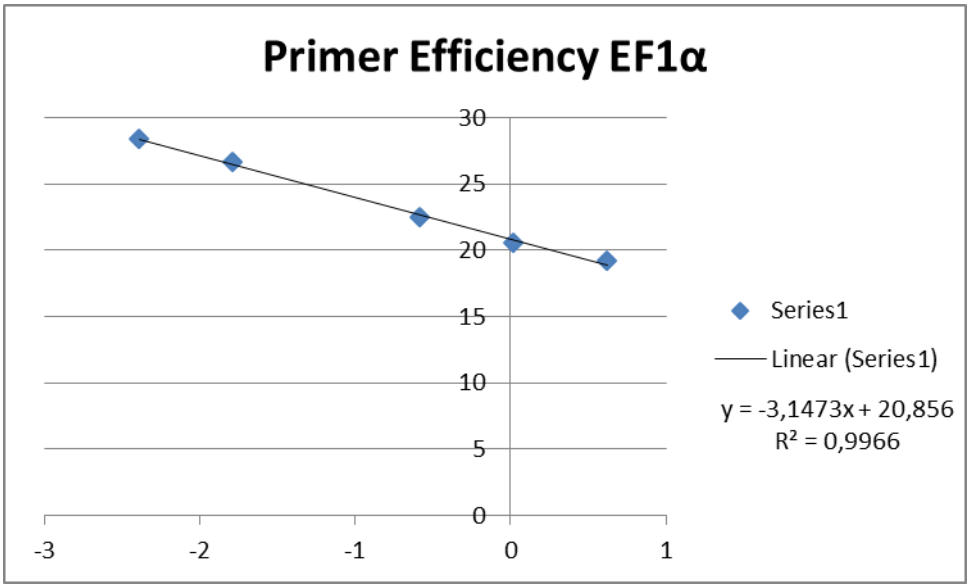

Figure 54. Efficiency of EF1α primer (reference gene). Ct mean values plotted againts the logarithm (log10) of the original RNA input, linear regresssion line and equations to obtain the slope and determine the effiency

8.5 Water samples analysis by Sýni

SWEDAC ACKREDITERING 1701 ISO/IEC 17025

REPORT
issued by an accredited laboratory

SÝNI

Stofnfiskur hf
B.t Eduardo Rodriguez H
Staðarberg 2 – 4
221 Hafnarfirði

TEST RESULTS

Sample no.: 6314-6317-14

Sample type:	Water.	**Received:**	20.06.2014
Sender:	Stofnfiskur hf	**Examined:**	20.06.2014
Sampled by:	Stofnfiskur hf	**Client:**	Stofnfiskur hf

Lab sample no.	Sample ident.	TPC 22°C /ml ISO 6222:1999	Coli-forms* /100 ml MPN	*E.coli** /100 ml MPN
6314	Watersample – Control tank 18.06.2014	52.000	2	2
6315	Watersample – Treated tank 18.06.2014	> 56.000	< 1	< 1
6316	Watersample – Control tank 20.06.2014	17.000	< 1	< 1
6317	Watersample – Treated tank 20.06.2014	22.000	4	1

Comments: Temperature in samples at arrival : 10,4°C
In compliance with guidelines in regulation no. 536/2001 about/regarding drinking water, the water is unfit for consumption in sample: 6314-6317-14

Reykjavík, June 26th 2014

Árný Árnadóttir
Process engineer

Results only relate to the test sample. Information on detection limits is found on www.syni.is *Tests methods marked with an asterisk are not accredited. A written permission by Rannsóknarþjónustan Sýni ehf is required for partial copying. Uncertainty with 95% confidence interval (k=2).

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REPORT
issued by an accredited laboratory

1701
ISO/IEC 17025

SÝNI

Stofnfiskur hf
B.t Eduardo Rodriguez H
Staðarberg 2 – 4
221 Hafnarfirði

TEST RESULTS

Sample no.: 6397-6398-14

Sample type:	Water.	**Received:**	23.06.2014
Sender:	Stofnfiskur hf	**Examined:**	24.06.2014
Sampled by:	Stofnfiskur hf	**Client:**	Stofnfiskur hf

Lab sample no.	Sample ident.	TPC 22°C /ml ISO 6222:1999	Coli-forms* /100 ml MPN	*E.coli** /100 ml MPN
6397	Watersample – Treated tank 23.06.2014	> 3.200.000	6	< 1
6398	Watersample – Control tank 23.06.2014	600.000	< 1	< 1

Comments: Temperature in samples at arrival : 15°C
In compliance with guidelines in regulation no. 536/2001 about/regarding drinking water, the water is unfit for consumption in sample: 6397-6398-14.

Reykjavík, June 30th 2014

Árný Árnadóttir

Árný Árnadóttir
Process engineer

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