Effect of β-Hydroxy-β-methyl butyrate (HMB) on innate immune response in different Atlantic salmon families

MIRIAM GUADALUPE CONTRERAS MOSTAZO

Faculty of Life and Environmental Sciences
University of Iceland 2013

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20 ECTS thesis submitted in partial fulfillment of a
Baccalaureus Scientiarum degree in Biology

Supervisor
Zophonías O. Jónsson

Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
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Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
University of Iceland
Háskóli Íslands
Askja, Sturlugötu 7
101, Reykjavik
Iceland

Telephone: 525 4000

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Abstract

The aim of this project was to investigate the effect of β-Hydroxy-β-methyl butyrate (HMB) on the expression of antimicrobial peptides (AMP) and inducible nitric oxide synthase (iNOS) in Atlantic salmon (*Salmo salar),* administering it in the diet and water. We studied two different families of *S. salar* and fish were divided into four groups. Fish were sampled over a period of three weeks. The expression of cathelicidin-2 (asCATH2), Hepcidin-1 and iNOS, both important components for the first line of immune defenses, was measured in gill, kidney, spleen and heart tissues. Up-regulation of asCATH2 and iNOS was observed after HMB treatment with increases of expression at different times. In addition, the responses varied throughout days but the variation between families was not significant. Hepcidin-1 expression rather than being enhanced, was in some cases, down-regulated, nevertheless, in one of the two families it was significant enhanced during the last days-post-induction. The study provides us with a powerful molecular tool to evaluate the innate immunity system of different salmon families. Further investigations will be undertaken to confirm whether higher AMP gene expression, or other defense mechanisms of innate immunity, are valuable as a natural protection against common diseases.

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Abbreviations

asCATH2: Cathelicidin-2

AMP: antimicrobial peptides

DPI: days post-induction

HMB:β-Hydroxy-β-methyl butyrate

IFN: Interferon

Ig: Immunoglobulin

iNOS: Inducible nitric oxide synthase

KIC: Ketoisocaproate.

NO: Nitric oxide

PKA: Potential killing activity

RBA: Respiratory burst activity

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Finally, I want to thank my family and friends who supported me and helped me in all my decisions.

1 INTRODUCTION

Immune responses are divided into two types: the innate (non-specific) and the acquired (specific). The innate immunity system in fish is a strong defense mechanism. Innate response, which generally precedes the adaptive response, activates and determines the nature of the adaptive response and co-operates in the maintenance of homeostasis. The essential difference between both systems is the means by which they recognize microorganisms (Fearon Douglas 1996). Although fish have both responses (innate and acquired), the acquired response in fish has a limited antibody repertoire, affinity maturation and memory and a slow lymphocyte proliferation (Magnadóttir 2006). The fish`s acquired immune response is therefore sluggish compared to the instant innate immune response. Moreover the innate immune system is divided into physical barriers, cellular and humoral components.

The aquatic environment is full of pathogens and the skin of teleost fish is unique and histologically diverse and is necessary as a first physical barrier of defense (Fast et al. 2002). The cutaneous mucus layer plays an important role in the defense against pathogens. This mucus besides acting as a natural, physical, biochemical, dynamic and semipermeable barrier (Ángeles Esteban 2012), also has an important immune function, containing biologically active substances like antibodies, lysozymes (bacteriolytic enzymes also found serum and leukocyte-rich tissues such as kidney and gut), complement or C-reactive protein (Fernández & Ruiz 2002). Despite this, the mucus layer can be shed or digested by various pathogens (Ángeles Esteban 2012)

Another important non-specific humoral factor is a large class of glycoproteins known as cytokines. Interferons (IFN) are cytokines named after their ability to “interfere” by inhibiting viral nucleic acid replication. Interferons have been found in the serum of virus infected rainbow trout . IFNs also have the capacity to activate immune cells, such as natural killer cells and macrophages by up-regulating antigen presentation to T lymphocytes, and thereby increasing recognition of infection (Fernández & Ruiz 2002).

1.1 ANTIMICROBIAL PEPTIDES (AMPs)

Antimicrobial peptides (AMPs) are an important component of innate immunity, described as part of the innate immune system which seems to be found in all eukaryotic organisms. AMPs are evolutionarily conserved among different species and present in both the animal and plant kingdoms (Zasloff 2002), although they were initially characterized in insects (Boman 1998). They are usually less than 50 amino acids and cationic.

AMPs are synthesized as inactive pre-proteins and are cleaved to release the active peptide after stimulation (Scocchi et al., 1992). The fundamental secondary structure of the AMPs is the clustering of cationic and hydrophobic amino acids into distinct domains making the AMPs amphipathic. Due to the basic character of the peptides they distinguish between the cell membranes of multicellular animals and microbes. This property comes from their positive charge which recognizes the negative one of the microbial membranes exposed to the outer world. On the other hand animal and plants membranes have the head groups of the phospholipids facing towards the inner leaflet to the cytoplasms, which makes it possible to discriminate between microbial and animal or plant membranes (Zasloff 2002).

The positive charge of cationic AMPs enters into an electrostatic interaction and disrupts the osmotic balance of the microbial membrane (Casadei et al. 2013) . The peptides migrate through the membrane of the microbes to the interior of the cell and disturb intracellular targets. The end result is the destabilization of the membrane which causes cell lysis (Zasloff 2002). Antimicrobial peptides are among most potent and rapidly lethal host defense chemicals that have been described in animals (Noga et al. 2010).

AMPs are divided into four families: defensins, cathelicidins, piscidins and liver expressed antimicrobial peptides (LEAP), including hepcidin and LEAP-2 (Casadei et al. 2013). Since we required an AMP that could be expressed without infection, cathelicidin-2 was one of our genes of choice due to its constitutive expression. Hepcidins were chosen in order to have another expression reference of AMP to compare. Finally, the choice of iNOS was due to their capacity of increase the expression when ectoparasites infect the gills and/or the skin of the fish (Braden et al. 2012).

1.2 Inducible nitric oxide synthase (iNOS)

In past few years nitric oxide (NO) has been found to play a diverse role in infection and immunity. Specifically NO acts as a potent antimicrobial molecule (Bogdan 2001) . NO generation is property of immune-system cells (i.e. dentritic cells, NK cells, mast cells and phagocytic cells). Inducible nitric oxide synthase (iNOS) is produced by stimulated macrophages and functions to synthesize NO.

Besides being fundamental in some infectious disease processes, iNOS also helps to control excessive immune reactions, protects against autoimmunity to some degree and functions as an intra- and intercellular signaling molecule shaping the immune response (Bogdan 2001).

iNOS expression is regulated by cytokines such as IL-1β and TNF-α and is activated by the binding of NF-κB. Also, the expression is regulated by cell-cell contact, immune complexes, microbial and viral products, among other things (Bogdan 2001).

Furthermore, high levels of iNOS have been reported in the gills and skin of fish infected with ectoparasite, for example *Lepeophtherius salmonis* (Braden et al. 2012).

1.3 HEPCIDIN

Hepcidin is a cysteine-rich AMP which has been studied in humans and other species; however, little is known about its role in fish. In humans, hepcidin controls extracellular iron by regulating its intestinal absorption, placental transport and recycling by macrophages. In spite of the controlling role, hepcidins are induced during inflammation and act to trap iron in macrophages, decrease plasma iron concentrations and also contribute to the anemia of inflammation (Ganz & Nemeth 2006). Moreover, high expression found in the liver and spleen can explain important functions in the innate immune response of catfish (Bao et al. 2005). The catfish hepcidin gene was recently described and consists of two introns and three exons that encode a 96 amino acid peptide. Moreover, while hepcidin expression in humans can be found in the liver, hepcidin in

catfish expresses in a wide range of tissues with the notable exception that it is not expressed in the brain (Bao et al. 2005; Ganz & Nemeth 2006).

1.4 CATHELICIDINS

Cathelicidin antimicrobial peptides are multifunctional peptides that are extremely important in the innate immune system of mammals (Scocchi et al. 2009). They range in size from 12 to 80 amino acid residues (Zanetti 2004; Lai & Gallo 2009). In addition, they have been found in vertebrates such as birds, reptiles, amphibia and the primitive vertebrate Atlantic hagfish (Uzzell et al. 2003).

Cathelicidins are produced as prepropeptides with a signal sequence (Fig. 1). They are composed of two parts, a conserved cathelin-like N-terminal domain called cathelin propart, and a diverse C-terminal antimicrobial domain called mature antimicrobial peptide. The activating processing enzyme releases the antimicrobial peptide from the conserved cathelicidin region. The responsible protease is usually elastase (Chang et al. 2006)

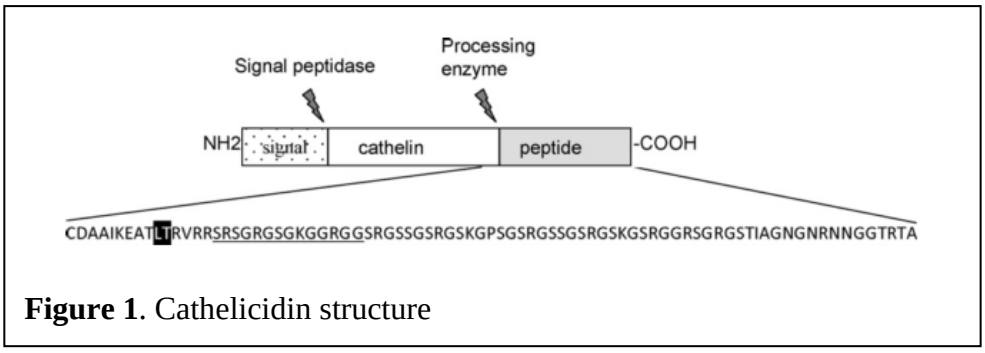

Figure 1. Cathelicidin structure

In 2006 Chang and colleagues found two different cathelicidins, CATH1 and CATH2 in rainbow trout (*Oncorhynchus mykiss)* and in Atlantic salmon (*Salmo salar)* (as) (Chang et al. 2006). Bridle et al (2011) showed that asCATH_2 in contrast to asCATH_1 is constitutively expressed in non-infected fish and in a wide range of organs such as kidney, gill, spleen, intestine, brain, muscle and head. The expression of both is induced upon infection with *Yersinia ruckeri*, a causative agent of yersiniosis and enteric redmouth disease (Tobback et al. 2007).

In addition, the potent antimicrobial activity of both cathelicidins is different depending on the fish pathogen. For example, both cathelicidins have a direct microbicidal activity against *E. coli* and *Vibrio anguillarum* while the microbicidal activity against *Yersinia ruckeri* was little or nonexistent (Bridle et al. 2011)

We can measure the basal levels of its expression in order to further study whether there is any correlation between natural resistance towards different pathogens due to the fact that asCATH-2 is constitutively expressed.

1.5 DISEASE PREVENTION

Innate defense mechanisms can be activated by different substances such as β-glucans, bacterial products, bile acids, vitamin D etc. and the result is the production of antimicrobial molecules.

It is often stated that the effect of immune stimulants on the developing immune system is minimal and also that administering immune stimulants to an animal, that is still growing, is detrimental (Bricknell & Dalmo 2005). In spite of that, there are successful examples of the use of immune stimulants in aquaculture providing resistance to pathogens (e.g. peptidoglycan (Casadei et al. 2013)).

A way to improve defense mechanisms with immune stimulants is the up-regulation of AMP expression. The stimulation of the immune mechanism before the stress appears makes it possible to provide resistance to pathogens during periods of high stress, including reproduction and sea transfer, irrespective of the speed of the AMP response. The idea is to anticipate the immunosuppressive event in order to achieve a stronger immune response (Noga et al. 2010).

β-Hydroxy-β-methyl butyrate (HMB) is a catabolite of the amino acid leucine whose precursor in the cytosol of cells is α-ketoisocaproate (KIC) , the result of leucine transamination. HMB is responsible for many of the regulatory roles of leucine in cell function and is also responsible for an increase in the immune defenses (Siwicki et al. 2000; Siwicki et al. 2006).

The effect of HMB on the respiratory burst activity (RBA), the potential killing activity (PKA) and lymphocyte proliferation have been studied (Siwicki et al. 2000). Also, the effect on the total immunoglobulin (Ig) levels in plasma were analyzed (Siwicki et al. 2003). These studies have demonstrated that HMB significantly increases the response on RBA and PKA activities compared to control-fed fish. In addition, time plays a role in the effects of HMB. For example, some responses, like lysozyme activity, peaked at about the double time of the control-fed fish after 2 weeks of feeding HMB, while PKA had the highest value in the first week (Siwicki et al. 2003).

In the present study we examined the influence of feeding *Salmo salar* with HMB on the expression of Cathelicidin-2, Hepcidin and iNOS key regulators of the innate defenses of *S. salar*.

2 PROJECT AIM

The aim of the project was to investigate the effect of feeding *Salmon salar* with β-Hydroxy-β-methyl butyrate(HMB) and analyze the effect on the expression of three different AMP genes: asCATH_2, Hepcidin and iNOS.

The specific aims were:

- To show if HMB is an immunostimulant.
- To analyze the effect of HMB on the different families of genes.
- To analyze the effect of HMB specifically in two families of *S. salar*.
- To understand the variability within families and between families.

3 MATERIALS AND METHODS

3.1 Fish maintenance and feeding

Juvenile *S. salar* were obtained from Stofnfiskur HF, fresh water farm in Kollafjördur. Two families were chosen for the experiment, one containing a QTL (family 1) that confers resistance to the Infectious Pancreatic Necrosis Virus (IPNV) and the other a non QTL control family (family 2). Four different tanks (35 fish per tank) were set up for the experiment, two control tanks and two tanks with stimulant HMB.

The average weight of the fish was 1.80 g. The fish of first two tanks were maintained at 4-5°C and the water was oxygenated by an EHEIN air pump 400. The water was changed every third day. The fish of the control tanks were maintained at 4-5° C with constant water flow.

HMB was directly added to the water and also impregnated into the food of the first two tanks (family 1 and family 2 tanks), the concentration of HMB in the water was 2mM. HMB was dissolved in ethanol to 0.1g/ml. The food (Inicio 1.5mm, BioMar, Denmark) was soaked in this solution and incubated at room temperature until the ethanol was evaporated. The fish were fed with the equivalent of approximately 4.3% of their body weight (i.e. 2.7g of food per tank and per day). The two control tanks (control families) were not fed HMB.

The experiment lasted three weeks. Three fish were sacrificed per sampling according to Fig. 2. Four samples were collected in the first week (2,3,5,6,10,13,17 and 20 days post-induction (DPI)), and two samples in weeks three and four for treated fish. Control fish were sampled once a week. Only 5DPI, 17DPI and 20DPI were taken to examine hepcidin-1 and iNOS expression in addition to the controls for the three different weeks (controlW1, controlW2 and controlW3).

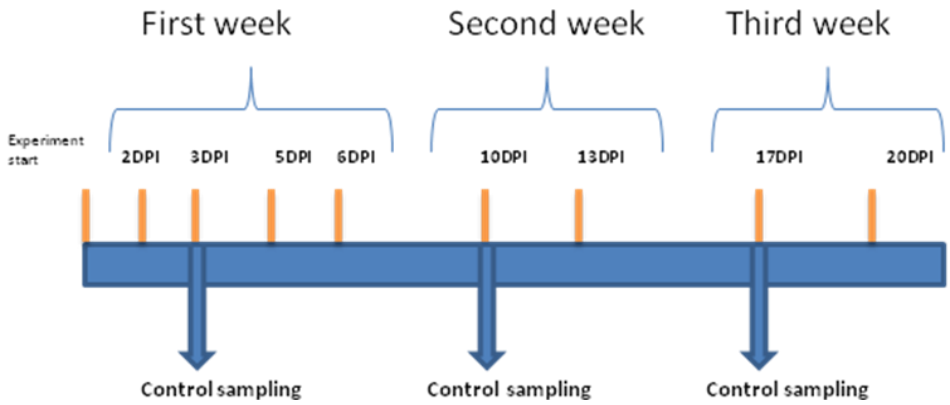

Figure 2. Experimental procedure

3.2 Collection of tissues, RNA extraction and cDNA synthesis

Samples of heart, kidney, spleen and gill were collected and placed into 150µl of Tri Reagent® Solution (Sigma-Aldrich) and homogenized using stainless steel Beads (Berani Uster, CH) in a mixer Minibeadbeater (Bioespec products). Furthermore, RNA quantity and purity were checked using a nanodrop ND1000 (Lab Tech) spectrophotometer. The extracted material was further incubated with DNase (New England Biolabs). First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcriptions Kits (Applied Biosystems), starting from a total of 500ng salmon RNA.

3.3 Real time PCR

The resulting cDNA was used to perform real time PCR (qPCR) using power Syber Green PCR master mix (Applied Biosystems) with different gene specific primers according to Table 1:

Table.1

Gene	Nucleotide sequence (5'-3')
***as*CATH_2**	Fwd: ATGGGAAACGAATGATGTGC Rev: CGGTCAGTGTTGAGGGTATT
Hepcidin-1	Fwd: GCTTCTGCTGCAAATTCTGAGG Rev: GTCCAAGATTGAGGTTGTGCAG
iNOS	Fwd: AACGAGAGCCAACAGGTGTC Rev: GGTGCAGCATGTCTTTGAGA
EF-1α	Fwd:GCTGTGCGTGACATGAGG Rev: ACTTTGTGACCTTGCCGC

The reactions consisted of 1µl of cDNA and 9µl of master mix solution. The RT-qPCR was run in a 7500 Real Time PCR System (Applied Biosystems). The RT-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 temperature for each primer set.

Elongation factor-1α (EF-1α) was selected as housekeeping gene to use for the data normalization in this experiment

3.4 Efficiency determination

Real Time PCR was performed to determine the efficiency of the different primers (asCATH2, hepcidin-1, iNOS and EF-1α). We choose RNA sample of the different days

post-induction from our experiment and pooled them to make the dilution series from 1:2 up to 1:2048.

The CT-values resulting from the performed 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 genes:

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

3.5 Data and statistical analysis

Data analysis was performed using Cycle Threshold (Ct) values. After checking the data was normally distributed, it was analyzed using the Pfaffl method (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 replicate 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 three biological replicates in the different days post-induction per week 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, also called relative quantity (RQ) were calculated according to the ΔΔCt values according to the following equation:

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

Furthermore, we also calculated fold change (FD) to obtain the statistical significance of the results by doing a t-test. The equation is as follows:

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

Relative quantification (RQ) and the standard deviation of RQ were used to show the effect of HMB in the expression of the genes studied. Firstly, to analyze the data of the HMB effect in asCATH2, we obtained RQ of the different days post-induction compared to the control for each week. We used the RQ mean of the three biological replicates of 2DPI, 3DPI, 5DPI and 6DPI compared to the RQ of the controlW1 ; the RQ mean of the three biological replicates of 10DPI and 13DPI compared to the RQ of the controlW2; and the RQ mean of the three biological replicates of 17DPI and 20DPI compared to the RQ of the controlW3. In the second analysis, we used the RQ mean of the three biological replicates of all the days post-induction compared to the RQ mean of the second and third week controls due to the variability found in the expression of the different week controls. Furthermore, to analyze the data of the HMB effect in hepcidin-1 and iNOS we used the RQ mean of the three biological replicates of 5DPI, 13DPI and 20DPI compared to the RQ mean of all the controls

ΔCt values were also evaluated by ANOVA followed by a Tukey´s post hoc test when appropriate, to analyze control and treated fish, time effects and differences between families in order to define significant expression differences. Firstly, a two-ways ANOVA was performed for the expression data of the different genes (factor 1: level, meaning control or treated fish; factor 2: days post-induction; and factor 3: family). Then, a one-way ANOVA was performed to show the particular effect of the different factors (level, days pots-induction and family) separately. All statistical tests were run in R version 3.0.1.

4 RESULTS

4.1 PRIMER EFFICIENCY

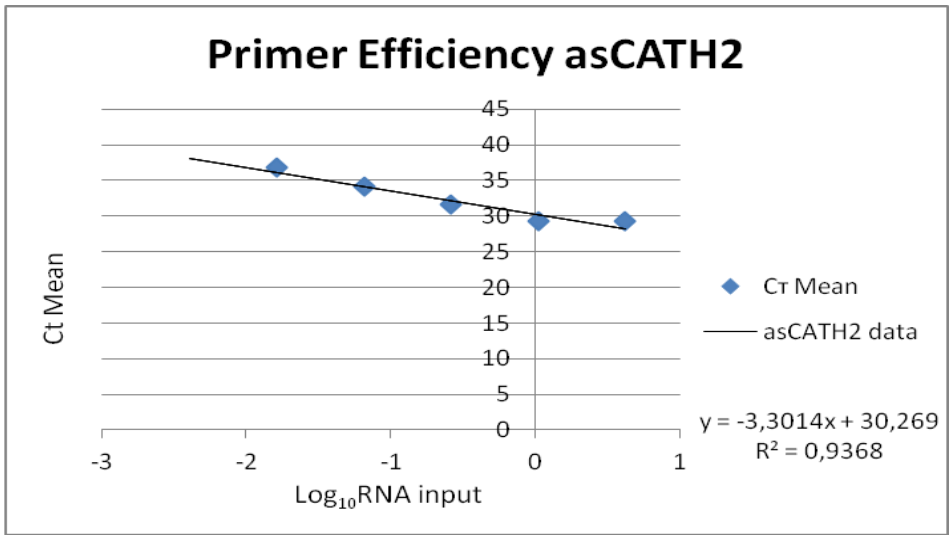

Fig.3 Efficiency of asCATH2 primer, Ct mean values plotted against the logarithm (log10) of the original RNA input, linear regression line and equations to obtain the slope and determine the efficiency.

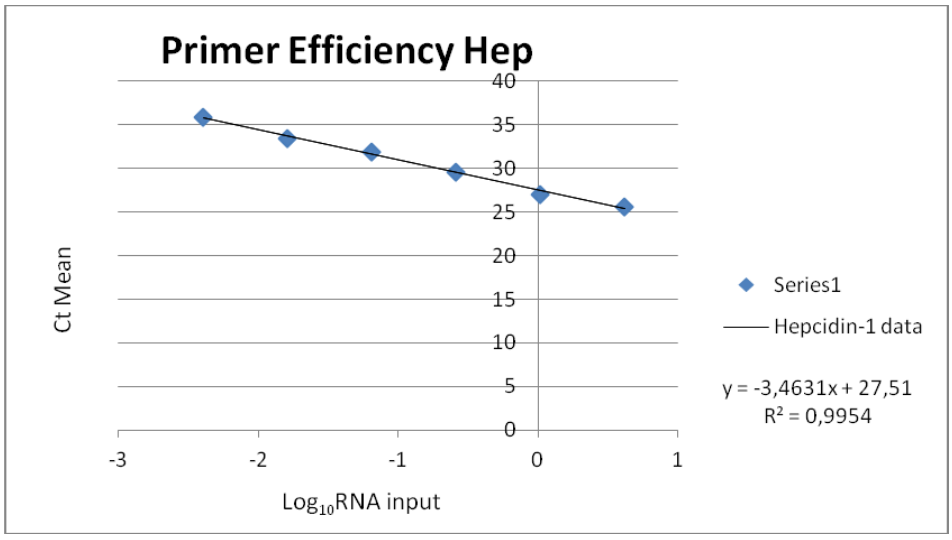

Fig.4 Efficiency of Hepcidin-1primer (target gene), Ct mean values plotted against the logarithm (log10) of the original RNA input, linear regression line and equations to obtain the slope and determine the efficiency.

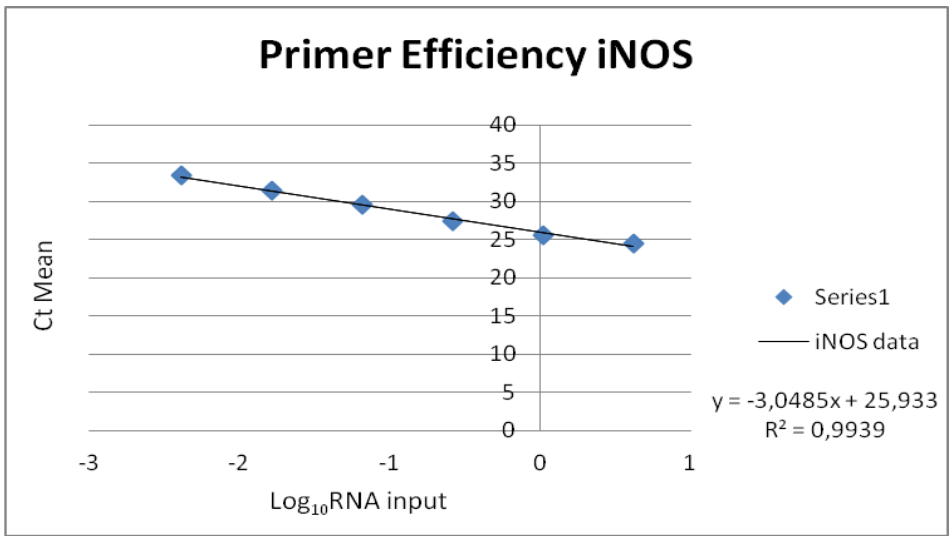

Fig.5 Efficiency of iNOS primer (target gene), Ct mean values plotted against the logarithm (log10) of the original RNA input, linear regression line and equations to obtain the slope and determine the efficiency.

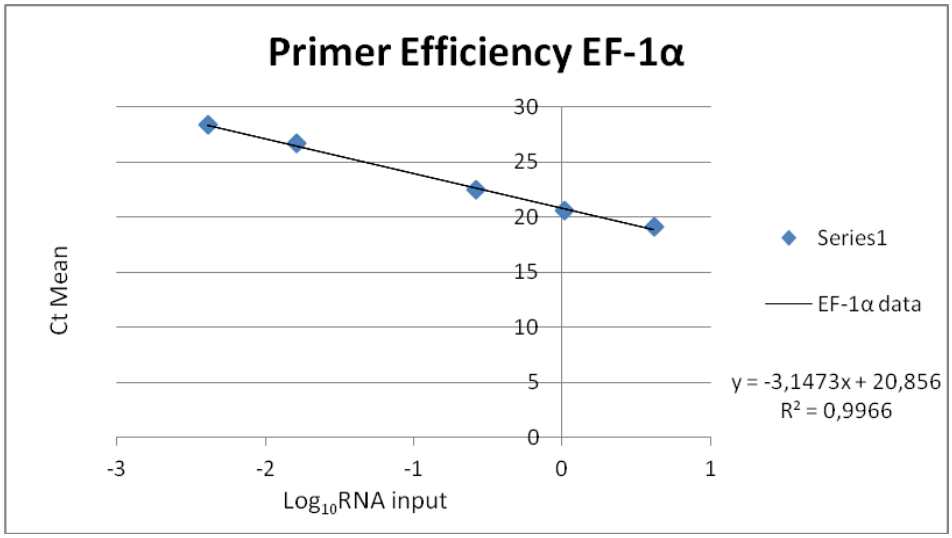

Fig.6 Efficiency of EF-1α primer (reference gene), Ct mean values plotted against the logarithm (log10) of the original RNA input, linear regression line and equations to obtain the slope and determine the efficiency.

The efficiencies of asCATH2, hepcidin-1, iNOS and EF-1α were obtained and the values were 100,86% , 94.42% , 112,83% and 107.84% respectively.

4.2 EXPERIMENT RESULTS

The expression of three different genes (asCATH2, Hepcidin-1 and iNOS) was studied in two different fish families comparing untreated (control) and HMB treated fishes. Moreover, the behavior of the different genes in the different families was compared in order to establish the pattern of expression.

4.2.1 EFFECT OF HMB ON asCATH_2 EXPRESSION

Relative quantifitation (RQ) of the treated fish compared to the controls per week and the standard deviation of RQ were used in order to explain the effect of HMB in *S. salar* during the feeding days. In addition to this, one-way and two-way ANOVAs and plost hoc tests were used to calculate the significance of the results.

Family 1 showed a gradual increase in expression of asCATH2 in the first week, day 5 being the highest, with a relative quantity (RQ) of 50, using the RQ mean of the three biological replicate fish. In all the post-induction days the up-regulation is statistically significant with $p<0.05$ in 2DPI, 3DPI and 6DPI; and with $p<0.01$ in 5DPI. These results were corroborated with t-tests using the fold change (FD) mean of the controls and the FD mean of the three biological replicates per day post-induction. The second week has undoubtedly the most up-regulated expression of asCATH2 with a mean value of 470 times of the control W2 (second week control) at 10DPI, and of 1110 times the controlW2 value at 13DPI. Even though these results clearly show a large increase in asCATH2 expression, we should consider that this effect could be in part influenced by the decreased expression of asCATH2 in control W2 (see Fig.7). The up-regulation in the third week is striking, with values of 121 times at 17DPI and 208 times at 20DPI compared to the control W3 (third week control). Both these values are statistically significant ($p<0.05$).

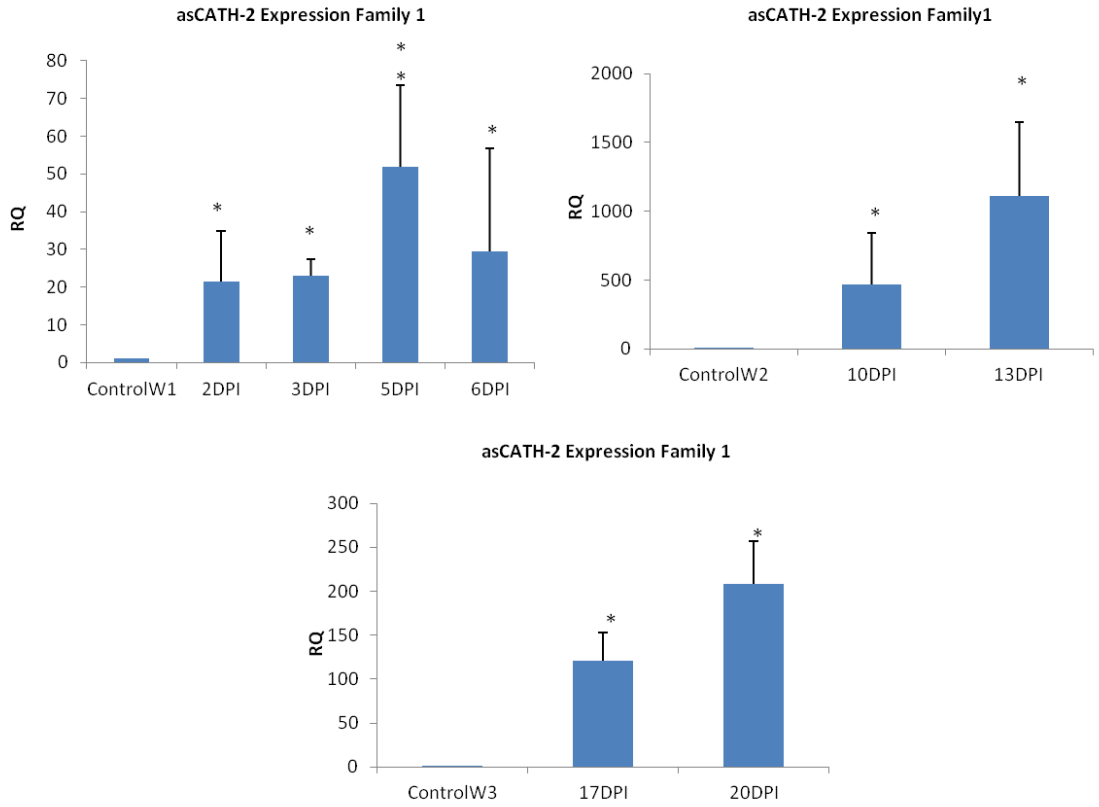

Fig.7 The expression of asCATH_2 during the three weeks feeding the family1 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of asCATH_2 in the graph . Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

Results for family 2, were similar to those for family 1, with a powerful expression of asCATH2 evident. RQ mean values were 171 times higher than the control at 10DPI and 419 at 13DPI, relative to the RQ mean value of controlW2. We obtained the highest value of the first week at 5DPI. All the results are statistically significant with p values of <0.01 for 3, 6, 10 and 17 DPI and p<0.001 for 2, 5, 13 and 20 DPI

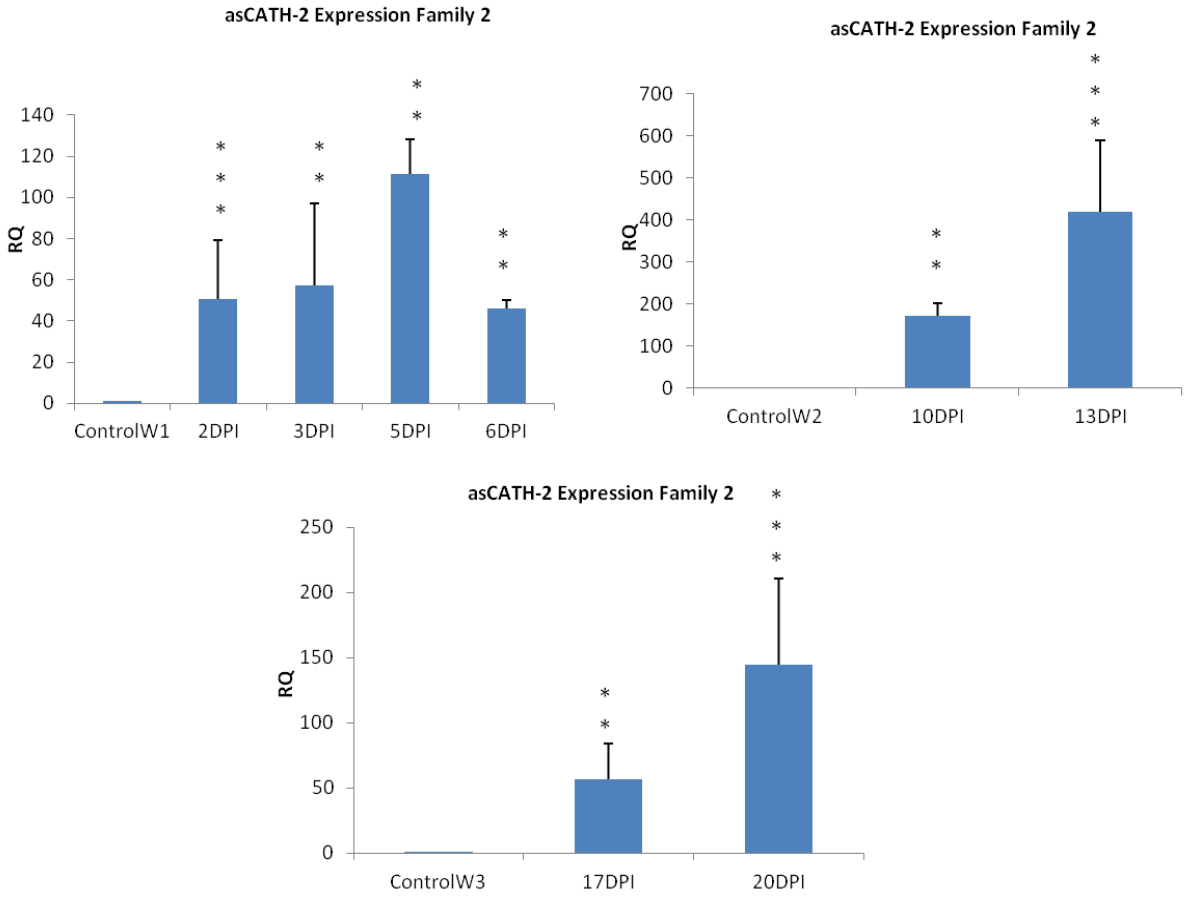

Fig. 8 The expression of asCATH_2 during the three weeks feeding the family2 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of asCATH_2 in the graph . Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

asCATH_2 expression data was further analyzed by ANOVA to determine the variability between families. The differences between controls and treated fish and the differences among days post-induction were highly significant with a $p<0.001$ (Fig.9). Also, the differences between families were significant ($p<0.001$) (Fig. 9). Nevertheless, when we performed the one-way ANOVA with family variable as a factor, the differences were non-significant (Fig.10). Despite this, the results of the two-way and one-way ANOVA with Level and Day as a factor were statistically significant ($p<0.001$) (Fig. 10).

```
Analysis of Variance Table

Response: data$dCT
                                Df Sum Sq Mean Sq  F value    Pr(>F)
data$Level                       1 506.97  506.97 645.4044 < 2.2e-16 ***
data$Day                         7  60.67    8.67  11.0330 3.468e-07 ***
data$Family                      1   4.86    4.86   6.1922  0.017891 *
data$Level:data$Day              1   3.02    3.02   3.8409  0.058255 .
data$Level:data$Family           1   8.04    8.04  10.2293  0.002987 **
data$Day:data$Family             7  18.05    2.58   3.2831  0.008955 **
data$Level:data$Day:data$Family  1   0.01    0.01   0.0180  0.893950
Residuals                       34  26.71    0.79
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Fig.9 Two-way ANOVA results performed with the ΔCt values of asCATH2 gene as a target gene and EF-1α as a reference gene. Level, Day and Family were the factors (Factor 1, Factor 2 and Factor 3 respectively.

```
Analysis of Variance Table

Response: data$dCT
           Df Sum Sq Mean Sq F value    Pr(>F)
data$Level  1 489.64  489.64  137.45 < 2.2e-16 ***
Residuals  58 206.61    3.56
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Analysis of Variance Table

Response: data$dCT
          Df Sum Sq Mean Sq F value    Pr(>F)
data$Day   7 297.32  42.474  5.5364 8.478e-05 ***
Residuals 52 398.93   7.672
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Analysis of Variance Table

Response: data$dCT
            Df Sum Sq Mean Sq F value Pr(>F)
data$Family  1  11.35  11.350  0.9611  0.331
Residuals   58 684.90  11.809
```

Fig.10 One-way ANOVA performed with the ΔCt values of asCATH2 gene as a target gene and EF-1α as a reference gene. Level, Day and Family were used as factors for each test.

4.2.1.1 ACCURACY OF asCATH2 CONTROLS

The expression of the *as*CATH2 gene in the controls varied with respect to the different weeks in such a way that the relative quantity (RQ) of the controls was higher in the first week and decreased along the following two weeks (Fig. 11). The control variability in the two different families is also distinct; hence, there is a bigger difference between the first week control and the rest of the controls in family 1 than in family 2. In spite of this fact, the difference is not statistically significant.

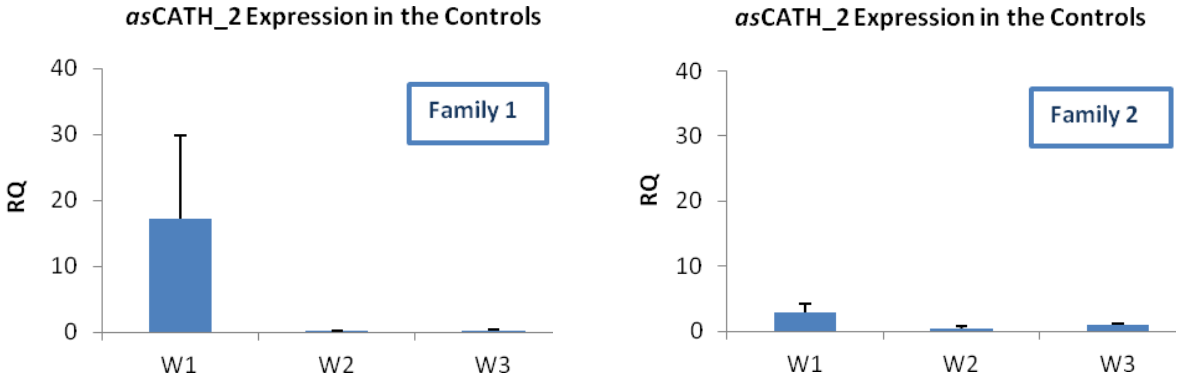

Fig.11 Control expression of the two different families in ech of the three weeks of the experiment. Real time PCR was performed and data were normalized relative to the expression of elongation factor (EF-1α). Data were analyzed using the Pfaffl method.

We removed the first week controls due to the high expression of asCATH2 gene of the first week controls compared to the remaining controls, particularely in family 1, in order to avoid the distortion of the final conclusions. A new analysis of asCATH2 data was performed using the RQ mean of the different week controls compared with the RQ values of the post-induction days. In family 1, the expression of asCATH2 increased markedly during the first six days post-induction with high RQ mean values of 5200 at 5DPI or 2900 at 6DPI. From the 10DPI, asCATH2 expression drecreased to RQ mean values of 240 or 405 at 10DPI and 20DPI respectively, although the increase of expression was still significant with $p<0.01$ (Fig.12). In family 2, asCATH2 expression also increased during the first days post-induction up to RQ mean values of 288 at 3DPI and 562 at 5DPI. The increase of expression is maintained at high levels, for example, 366 RQ mean value at 13DPI and 231 at 20DPI (Fig.13).

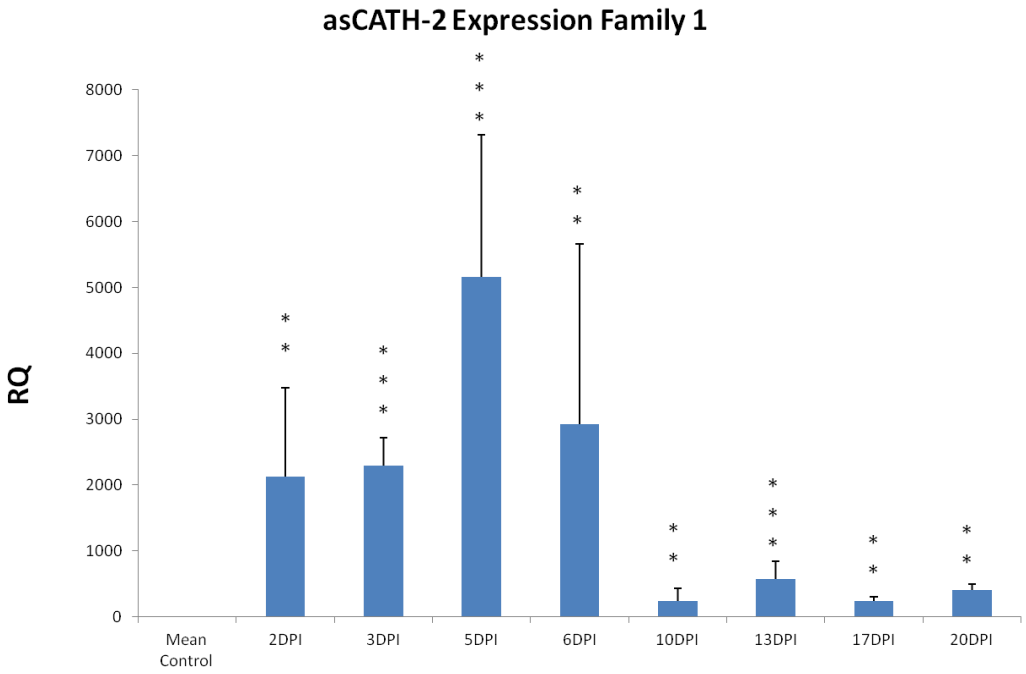

Fig. 12 The expression of asCATH2 along the different post-induction days feeding the family1 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of asCATH_2 in the graph . Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

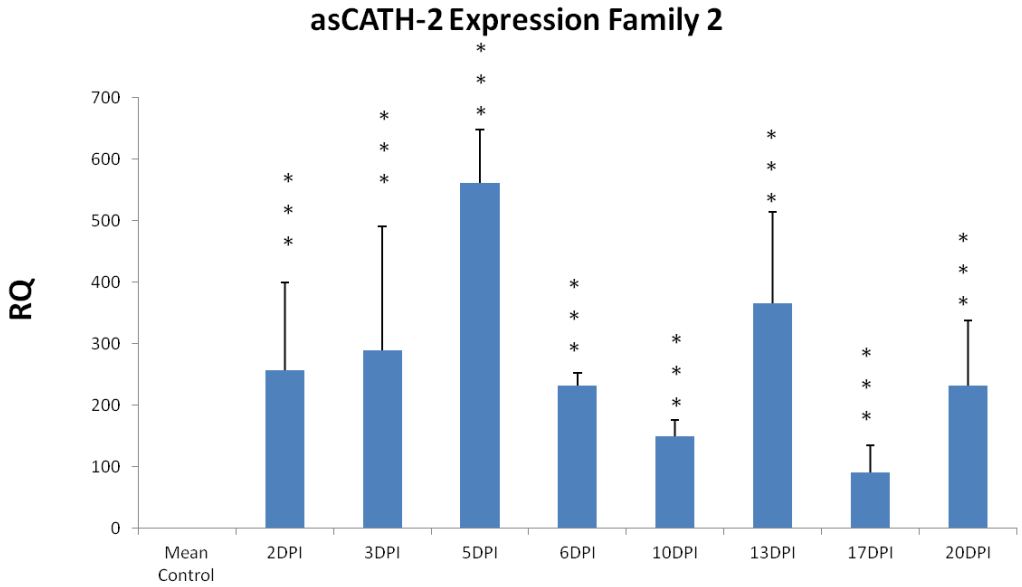

Fig. 13 The expression of asCATH2 along the different post-induction days feeding the family2 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of asCATH_2 in the graph . Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

4.2.2 EFFECT OF HMB ON HEPCIDIN-1 EXPRESSION

Analysis of hepcidin-1 expression in control fish and in fish feeding with HMB indicated that the difference between both was, in almost all cases, not statistically significant. In family 1, hepcidin-1 expression decreases with time post-induction. At 13DPI and 20DPI , the expression was below the control level, meaning that HMB does not have an effect on the APM hepcidin-1 (Fig. 14). The results from family 2, nevertheless, were different from the results from family 1 in such a way that the increase of hepcidin-1 expression at 20DPI was statistically significant with $p<0.05$ (Fig. 15).

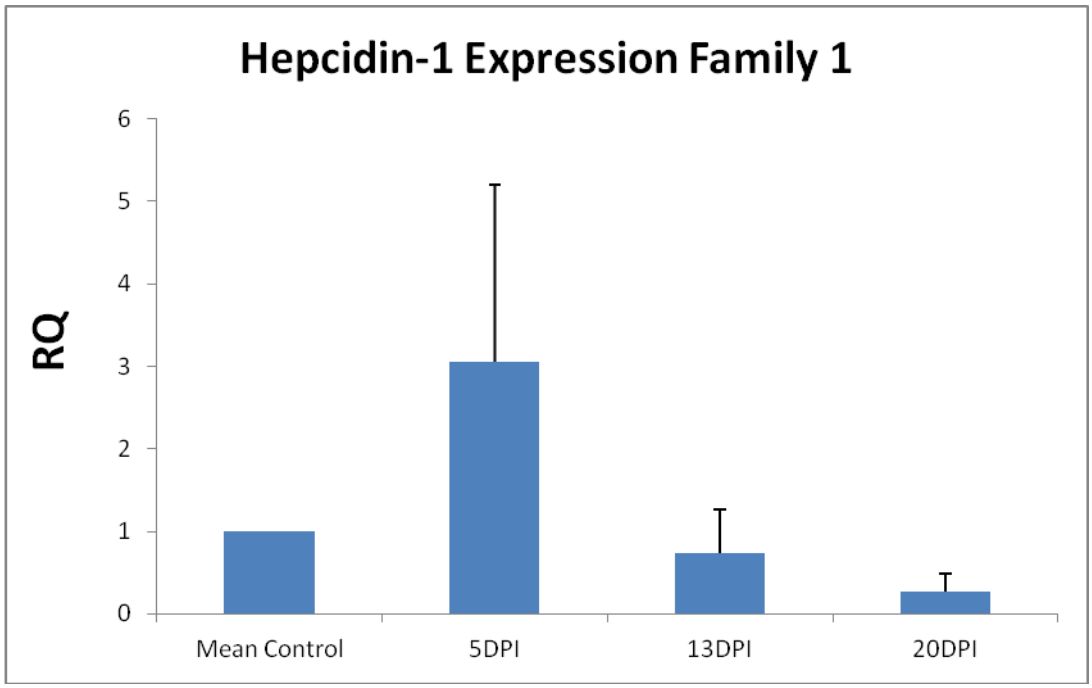

Fig.14 The expression of hepcidin-1 during the different days post-induction feeding the family1 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of Hepcidin-1 in the graph . Asterisks indicate significant differences between control and treated fish, with *=$p<0.05$, **=$p<0.01$ and ***$p<0.001$ respectively. n = 3 fish per group.

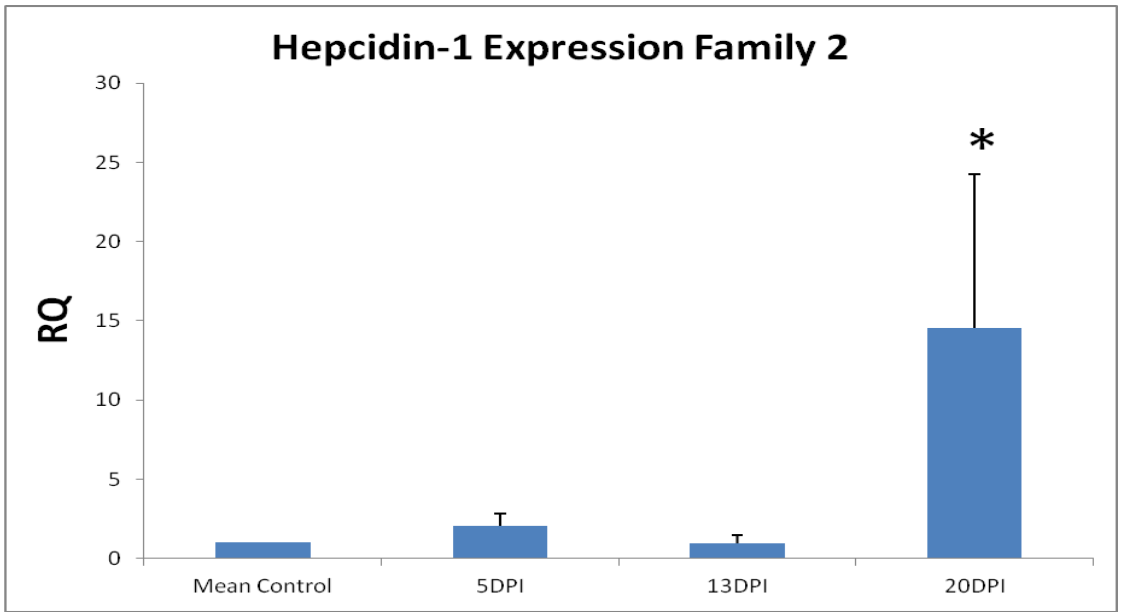

Fig.15 The expression of hepcidin-1 during the different days post-induction feeding the family2 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor 1α (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of Hepcidin-1 in the graph . Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

Results from two-way ANOVA and the three one-way ANOVAs indicate that Hepcidin-1 was not significantly up-regulated (Figs.16 and 17). In Fig. 16, variable Day was significantly different between the families, hence, the pattern behavior of the different post-induction days changed depending on the families.

```
Analysis of Variance Table

Response: data$dCT
                      Df Sum Sq Mean Sq F value   Pr(>F)
data$Level             1  0.387  0.3870  0.3023 0.589215
data$Day               4 28.101  7.0252  5.4876 0.004549 **
data$Family            1  2.920  2.9204  2.2813 0.148301
data$Level:data$Family 1  3.093  3.0932  2.4162 0.137491
data$Day:data$Family   4 46.619 11.6547  9.1039 0.000333 ***
Residuals             18 23.043  1.2802
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Fig.16 Two-way ANOVA performed with the ΔCt values of hepcidin-1 gene as a target gene and EF-1α as a reference gene. Level, Day and Family were the factors (Factor 1, Factor 2 and Factor 3 respectively)

```
Analysis of Variance Table

Response: data$dCT
            Df  Sum Sq Mean Sq F value Pr(>F)
data$Level   1   0.387  0.3870  0.1044  0.749
Residuals   28 103.776  3.7063
```

```
Analysis of Variance Table

Response: data$dCT
           Df Sum Sq Mean Sq F value Pr(>F)
data$Day    5 28.488  5.6975  1.8069 0.1496
Residuals 24 75.676  3.1532
```

```
Analysis of Variance Table

Response: data$dCT
             Df Sum Sq Mean Sq F value Pr(>F)
data$Family   1   2.92  2.9204  0.8077 0.3765
Residuals    28 101.24  3.6158
```

Fig.17 One-way ANOVA performed with the ΔCt values of hepcidin-1 gene as a target gene and EF-1α as a reference gene. Level, Day and Family were used as a factor in each test.

4.2.3 EFFECT OF HMB ON iNOS EXPRESSION

As with asCATH2 expression, HMB had a clear effect on iNOS expression. The behavior of the two families was similar although there were some peculiarities throughout the weeks. Family1 showed us a gradual increase (Fig. 18). After 5DPI we can see RQ mean values as much as 50 times higher than the mean RQ of the controls. The highest value was observed in the third week with a RQ mean value of 2553 times higher than controls. The RQ mean value of the second week was also high: 2062. The expression of iNOS at 17 and 20 DPI was enhanced significantly ($p<0.001$) while the increase of expression at 5DPI was statistically significant with a $p<0.0.1$. iNOS expression in family 2 also increased gradually with days post-induction. The highest RQ mean value of family 2 was found at 20DPI (1269) (Fig. 19). All the changes in iNOS expression in family 2 were significant ($p<0.05$ at 5DPI and $p<0.001$ at 13 and 20DPI). Furthermore, family 1 fish had a higher and more rapid increase in RQ values compared to family 2.

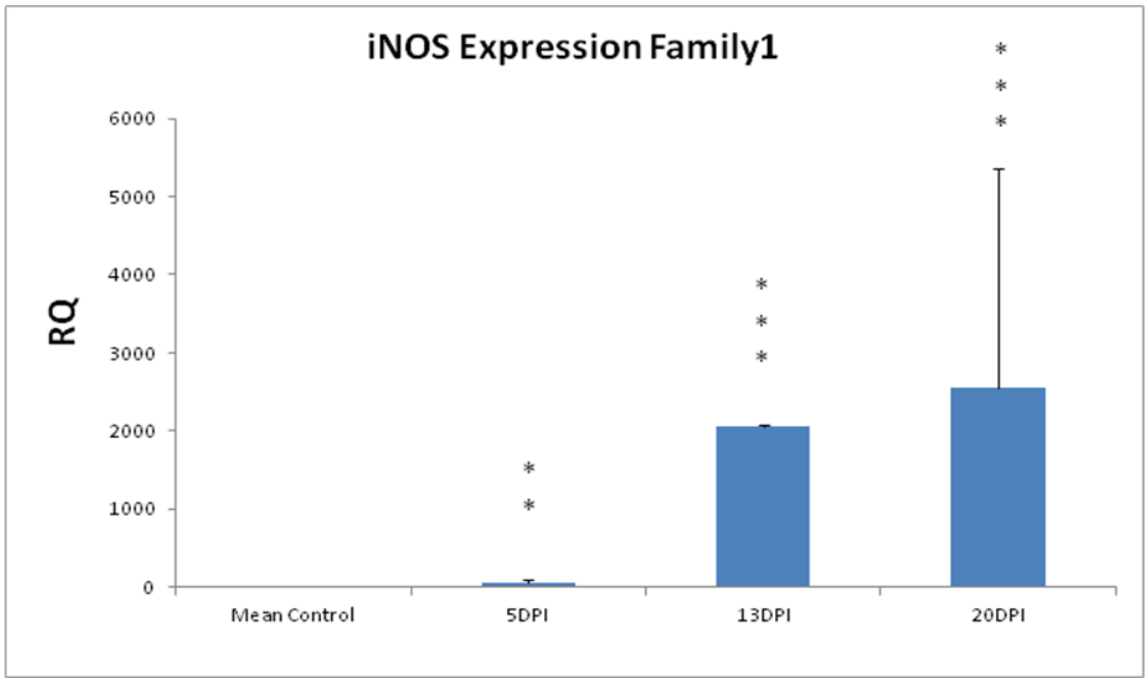

Fig.18 The expression of iNOS during the different days post-induction feeding the family1 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of iNOS in the graph . Asterisks indicate significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

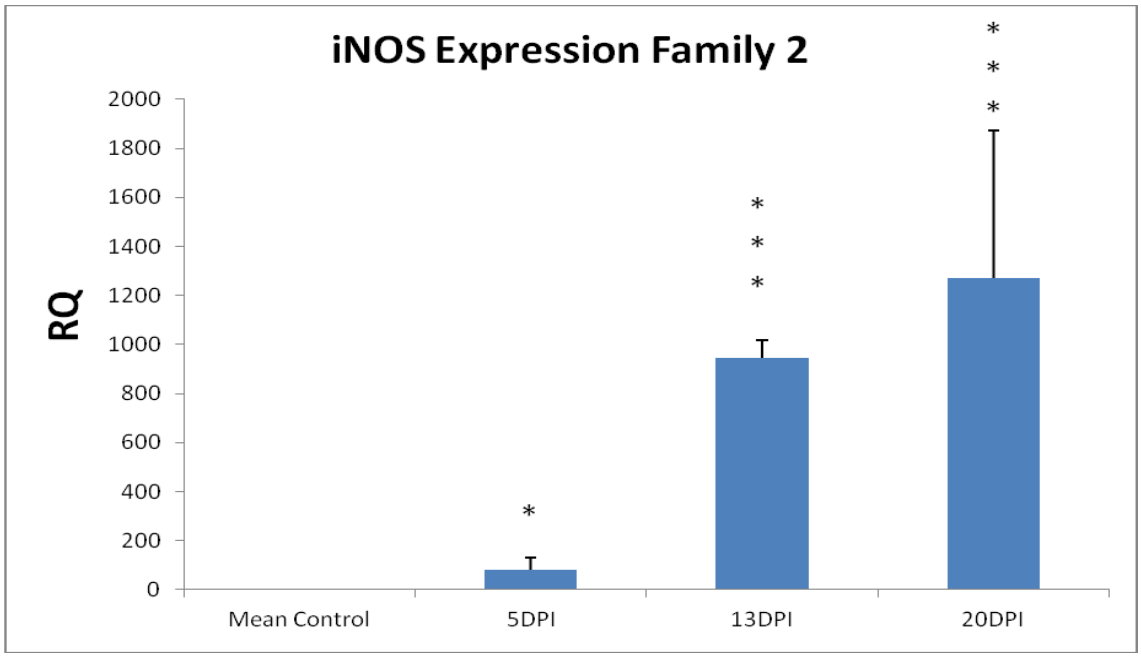

Fig.19 The expression of iNOS during the three weeks feeding the family2 fish with HMB. Real time PCR was performed and data were normalized relative to the expression of elongation factor (EF-1α). Data were analyzed using the Student`s Test-T using fold change(FD) to obtain the significance and relative quantifitation (RQ) to depict the different expression of iNOS in the graph . Asterisks indicates significant differences between control and treated fish, with *=p<0.05, **=p<0.01 and ***p<0.001 respectively. n = 3 fish per group.

As a final result, the one-way and the two-ways ANOVAs showed us a great difference in iNOS expression between control and treated fish in both families (Figs. 20 and 21). Moreover, iNOS expression changed significantly depending on the post-induction day ($p<0.001$); however, no significant differences were detected between families (Fig. 21).

```
Analysis of Variance Table

Response: data$dCT
                      Df Sum Sq Mean Sq  F value    Pr(>F)
data$Level             1 580.28  580.28 391.9966 1.646e-15 ***
data$Day               4  99.53   24.88  16.8084 1.900e-06 ***
data$Family            1   3.06    3.06   2.0681    0.1645
data$Level:data$Family 1   0.99    0.99   0.6713    0.4214
data$Day:data$Family   4  10.40    2.60   1.7558    0.1738
Residuals             22  32.57    1.48
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Fig.20 Two-way ANOVA performed with the ΔCt values of iNOS gene as a target gene and EF-1α as a reference gene. Level, Day and Family were the factors (Factor 1, Factor 2 and Factor 3 respectively)

```
Analysis of Variance Table

Response: data$dCT
           Df Sum Sq Mean Sq F value   Pr(>F)
data$Level  1 580.28  580.28  126.71 1.16e-12 ***
Residuals  32 146.55    4.58
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Analysis of Variance Table

Response: data$dCT
          Df Sum Sq Mean Sq F value   Pr(>F)
data$Day   5 679.80 135.961  80.966 9.17e-16 ***
Residuals 28  47.02   1.679
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Analysis of Variance Table

Response: data$dCT
            Df Sum Sq Mean Sq F value Pr(>F)
data$Family  1   3.06  3.0615  0.1354 0.7154
Residuals   32 723.76 22.6175
```

Fig.21 One-way ANOVA performed with the ΔCt values of iNOS gene as a target gene and EF-1α as a reference gene. Level, Day and Family were used as a factor for each test.

5 DISCUSSION

The environment experienced by fish is a key determinant of their health, and companies are losing substantial amounts of money due to the diverse range of fish diseases affecting cultured stocks. This study was instigated in order to find an alternative to vaccines and antibiotics, but also to increase the innate immunity system in a more simple and economical way. Many studies have shown improvement of some mechanisms which provide resistance to pathogens during some stages such as high stress, reproduction, sea transfer and in general changes in conditions (Bricknell & Dalmo 2005). Despite this, the use of immunostimulants is a controversial topic.

The aim of this project was to examine the effect of HMB on the innate immunity in *S. salar*, or more specifically, on antimicrobial peptides (AMP) and iNOS. Several previous investigations have found that HMB acts as an immunostimulant which has an effect on innate immunity. In the present study we evaluated the innate-immune response measuring the expression of asCATH2, Hepcidin-1 and iNOS real time PCR (qPCR) in kidney, heart, spleen and gill tissues. AMPs are known to play a crucial role in the defense system in vertebrates (Broekman et al. 2011). Our results clearly show that supplementing HMB in food and water has a strong enhancing effect on the expression of these genes and this effect changes depending on the gene families.

In previous studies we analyzed the HMB effect on different *S. salar* families. Four individuals from 100 families, were fed on HMB mixed in the food-pellets but not in the water. We found large variability of the expression of asCATH2 in the different families that could be attributed to genetic and/or development factors. We also observed that the general increase of the gene expressions was lower in the pooled families than the separated individual families. Therefore the present study was focused mainly on two families, one containing a QTL that confers resistance to the Infectious Pancreatic Necrosis Virus (IPNV) and the other a non QTL control family.

One of the most interesting findings was that HMB does not only act as an immunostimulant in respiratory burst activity (RBA), potential killing activity (PKA),

lymphocyte proliferation and on the total immunoglobulin (A. K. Siwicki et al. 2000) but also acts as an immunostimulant by inducing expression of asCATH2 and iNOS.

asCATH2 expression has an important role on the innate immune-system (Scocchi et al. 2009) and our results indicate that asCATH2 expression was up-regulated by HMB, with a highly significant difference between control and treated fishes. The differences observed in asCATH2 expression in controls between the first week of the experiment and weeks two and three were surprising (see Fig. 11); but could possibly be explained by the maturation of the immune system during the ontogeny (Ángeles Esteban 2012). The data of this study was re-analyzed, ignoring the first week and reanalyzing the data of asCATH2 expression using only the last two weeks, due to the fact that we considered the first week controls as outlier for both families. We believe that something could have happened in the farm where the controls were that caused induction. A high and rapid increase in asCATH2 expression was seen as early as 2-days post- induction, and reached a maximum value at 5DPI in both families. In family 1, the rate of increase decreased throughout the experiment period from the 6DPI whilst in family 2 there was a little decrease from the 10DPI, with asCATH2 expression remaining high. The significance of the asCATH2 expression differences between families differed depending on the type of ANOVA test. Using a two-way ANOVA, the differences between both families were statistically significant ($p<0.05$), whilst differences were non-significat using one-way ANOVA . That is these patterns have different behaviors in the families and this may have imparted some influence on the two-way ANOVA. Moreover, the genetic input in asCATH2 expression turned out not to be relevant for these two chosen families, as we do not see significant differences between family 1 and family 2 comparing treated fish with control fish.

In this study we also examined Hepcidin-1 expression. We decided to reduce the number of samples to one per week (5,13 and 20 DPI) and compare them with the mean of the controls. Hepcidin-1 is naturally induced during inflammation, trapping iron in macrophages, decreasing plasma iron concentrations and also contributing to the anemia of inflammation (Ganz & Nemeth 2006). Comparing the expression results of Hepcidin-1 and asCATH2, we did not find a significant change after the HMB treatment in family 1. This non-enhanced Hepcidin-1 expression due to HMB treatment shows us that different AMPs

have different triggering mechanisms. By contrast, in family 2, the increase of hepcidin-1 expression at 20DPI was statistically significant ($p<0.05$) with a RQ mean value of 14.6 compared with the RQ mean value of the controls, meaning that the enhancement of hepcidin-1 expression was lower and slower than that of asCATH2. The differences between families were however, not significant.

The effect of HMB was also observed in iNOS expression. iNOS is produced by stimulated macrophages and functions to synthesize (NO). It is also fundamental in some infectious disease processes and in the control of excessive immune reaction (Bogdan 2001). Our results showed that HMB in the diet and in the water increased iNOS expression gradually up to maximum RQ mean values of 2500 in family 1 and 1270 in family 2. However family 1 reached higher RQ mean values than family 2, but the differences between families were not statistically significant.

In conclusion, the results of this study strongly suggests that supplemental HMB in food and water acts as an immune-modulator and directly stimulates innate immunity, specifically asCATH2 and iNOS expression, in *S. salar*. Furthermore, these immune-modulators were activated at different times, with asCATH2 activated in the first days post.induction. Hepcidin-1, in family 2, was expressed later, meaning that the activating mechanisms of hepcidin-1 expression needed even more time than iNOS mechanisms.

5.1 FUTURE STUDY AIMS

We have now presented the study of two different families of *S. salar* (yearclass 2012-2, Stofnfiskur) in which we have measured the temporal expression of genes involved in the innate immune response. We studied the constitutive expression of CATH2 in these two families in order to explain inter-family differences in the basal levels of expression. We have performed challenge experiments with two different viruses, infectious Pancreatic Necrosis virus (IPNV) and Pancreas Disease virus (PD) on 30 individuals from each 133 Families from yearclass 2012-2, a total of 3990 fish per challenge. We have found 50% of these families are naturally resistant to IPNV and 40% natural resistant to PD.

We have PITTagged 10 individuals from these 133 families and kept in a separate tank. We will take one gill lamella from each tagged fish and measure the basal expression of the innate immunity gene under study in order to see if there is a correlation between the

naturally virus-resistant families and the expression of CATH2. Later this year we will perform a third challenge experiment on 30 individuals from these 133 families, with the intracellular bacterial pathogen *Piscirickettsia salmonis* that causes salmonid rickettsial septicemia (SRS). In this challenge we will induce the expression of the studied innate-immune genes with HMB in the 10 PITTagged fish one week prior to the challenge and we will study whether induction CATH2, Hepcidin and iNOS expression levels have any effect on SRS.

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