Microarray-based identification of differentially expressed genes in families of turbot (*Scophthalmus maximus*) after infection with viral haemorragic septicaemia virus (VHSV)

Díaz-Rosales P. [1], Romero A. [1], Balseiro P., Dios S., Novoa B., Figueras A[*].

Instituto de Investigaciones Marinas (IIM), CSIC. Eduardo Cabello, 6. 36208 Vigo, Spain.

[1] : These authors contributed equally to this work

Marine Biotechnology (2012) 14:515-529

*Corresponding author: Antonio Figueras

Telephone number: (34) 986214462

Fax number: (34) 986292762

E-mail: antoniofigueras@iim.csic.es

Abstract

Viral hemorrhagic septicemia virus (VHSV) is one of the major threats to the development of the aquaculture industry worldwide. The present study was aimed to identify genes differentially expressed in several turbot (*Scophthalmus maximus*) families showing different mortality rates after VHSV. The expression analysis was conducted through genome-wide expression profiling with an oligo-microarray in the head-kidney. A significant proportion of the variation in the gene expression profiles seemed to be explained by the genetic background, indicating that the mechanisms by which particular species and/or populations can resist a pathogen(s) are complex and multifactorial. Before the experimental infections, fish from resistant families (low mortality rates after VHSV infection) showed high expression of different antimicrobial peptides, suggesting that their pre-immune state may be stronger than fish of susceptible families (high mortality rates after VHSV infection). After infection, fish from both high- and low-mortality families showed an up-modulation of the interferon-induced Mx2 gene, the IL-8 gene and the VHSV-induced protein 5 gene compared with control groups. Low levels of several molecules secreted in the mucus were observed in high-mortality families, but different genes involved in viral entrance into target cells were down-regulated in low-mortality families. Moreover, these families also showed a strong down-modulation of marker genes related to VHSV target organs, including biochemical markers of renal dysfunction and myocardial injury. In general, the expression of different genes involved in the metabolism of sugars, lipids and proteins were decreased in both low- and high-mortality families after infection. The present study serves as an initial screen for genes of interest and provides an extensive overview of the genetic basis underlying the differences between families that are resistant or susceptible to VHSV infection.

Keywords: *Scophthalmus maximus*, VHSV, families, resistance, infection

Introduction

Viral hemorrhagic septicemia virus (VHSV) is one of the most serious pathogens of finfish worldwide in terms of its wide host range, pathogenicity, disease course and mortality rate (Smail 1999). VHSV is thus one of the main threats to the development of the aquaculture industry worldwide. In general, this virus causes severe necrosis in the hematopoietic tissues of affected fish (Yasutake 1975). Although VHSV has historically been considered a disease of farmed rainbow trout (*Oncorhynchus mykiss*) in continental Europe, the host range appears to be broad, and several marine fish species have now been reported to carry VHSV, including turbot (*Scophthalmus maximus*) (Ross et al. 1994; Schlotfeld et al. 1991). In fact, Tafalla et al. (1998) reported that VHSV is able to replicate in turbot blood leukocytes and kidney macrophages.

Although there have been several attempts to develop traditional vaccines against viral diseases based on inactivated or attenuated viruses, the protective effect of these vaccines has been limited or inconsistent (Lorenzen and Olesen 1997; Winton 1997). The most efficient vaccines against VHSV to date have been DNA vaccines using the viral G glycoprotein (Lorenzen et al. 2001, 2002a, 2002b, 2005). However, many consumers do not understand the difference between DNA-vaccinated animals and a genetically modified organism (GMO), and the use of DNA vaccines is still problematic for fish farmers. Therefore, a better understanding of the host immune response is required to develop a more efficient means of VHSV prevention and treatment.

Improving infectious disease resistance by genetic means is an attractive alternative approach because of its prospects for prolonged, sustained protection. There are several strong indications that genetic traits are involved in the resistance of rainbow trout (*O. mykiss*) against VHSV (Jørgensen et al. 2011). Using gynogenesis, it has been possible to obtain highly resistant families of trout after only two generations (Dorson et al. 1995; Slierendrecht et al. 1995). The finding that only a few generations seem to be required to obtain highly resistant or highly susceptible families of offspring may further indicate that only a few genes play a major role. However, the underlying mechanisms of the observed resistance differences are not known, and only a limited number of studies have investigated this topic to date. Attempts to identify the mechanism of resistance against VHSV have focused on allelic variations of the complement component C3 and MHC between resistant and susceptible families of rainbow trout, but these attempts have not been successful (Slierendrecht et al. 1996, 2001). Moreover, a genome-wide analysis of families of trout with different susceptibility to VHSV has recently been published (Jørgensen et al. 2011). However, the basis of this genetically determined VHS resistance has yet to be elucidated. A global understanding of the changes in the expression of host cell genes after viral infection should improve our understanding of the virus/host cell interactions.

Despite the great number of reports in which microarrays have been used to study complex biological mechanisms in fish at the genomic level, few studies have focused on global gene expression in immune cells after VHSV challenge. Only two fish species, the Japanese flounder (*Paralichthys olivaceus*) (Aoki et al. 2011; Byon et al. 2005, 2006) and the zebrafish (*Danio rerio*) (Encinas et al. 2010), have been studied in this manner.

The present study aimed to identify genes that are differentially expressed in low- and high-mortality families of turbot during VHSV infection by genome-wide expression profiling with microarrays. This

characterization could identify genes that constitute relevant VHSV resistance markers and help prioritize candidates of interest for further functional and genetic (e.g. searches for quantitative trait loci) studies. To our knowledge, this is the first report on the global transcriptional response to VHSV infection in turbot.

Materials and methods

Cell culture and virus

The epithelioma papulosum cyprinid (EPC) fish cell line, derived from common carp (*Cyprinus carpio* L.) (Tomasec and Fijan 1971), was obtained from the European collection of cell cultures (ECACC no. 93120820) and used in this work. EPC cells were maintained at 20 ºC in Eagle's minimum essential medium (MEM; Invitrogen, GIBCO) containing 10% foetal bovine serum (FBS; Invitrogen, GIBCO), penicillin (100 IU/ml) (Invitrogen, GIBCO), and streptomycin (100 µg/ml) (Invitrogen, GIBCO), buffered with 7.5% sodium bicarbonate (Invitrogen, GIBCO).

VHSV strain UK 860-94, isolated from turbot in Scotland (*S. maximus*) (Ross et al. 1994), was used for the experimental infections. The virus was replicated in EPCs grown in MEM with antibiotics and 2% FBS at 14 ºC. When the cytopathic effect became extensive, the supernatant was harvested and centrifuged to eliminate cell debris. Clarified supernatants were then used for subsequent experiments. The virus stock was titrated in 96-well plates according to Reed and Müench (1938).

Fish

Six different families of turbot (*S. maximus*) were obtained from a commercial fish farm (Stolt Sea Farm S.A.) in Spain. A total of 120 juvenile fish (3 g mean) per family were stocked in 400 l tanks. Fish were acclimatized to laboratory conditions for 2 weeks prior to the experiments. No clinical signs were ever observed during this period. Fish were maintained at 16.5 (± 2) ºC with aeration and fed daily with commercial dry pellet food (Skretting).

Experimental infections

Fish from each of the six families were divided into four groups containing 50 l of static UV-treated seawater. The fish were lightly anesthetized with 0.15 gl^{-1} of tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA, USA) and infected by intraperitoneal (i.p.) injection with 100 µl of VHSV solution (10^6 $TCID_{50}g^{-1}$) (two groups of n=30). Control groups were injected with the same volume of culture medium (two groups of *n*=30). The mortalities were monitored for 30 days post-infection (p.i.). Surviving fish (considered "resistant") and control fish (considered "naive") from each family were again divided into two groups and re-challenged with the same viral solution or treated with culture medium. Twenty-four hours after the second injection, five fish from each treatment and family were sacrificed, and head kidneys were removed (Fig. 1a). Thus, samples from 24 h p.i. represent the naive infected group (NI); those from 30 days p.i. correspond to the resistant control group (RC), and the

resistant-infected group (RI) is represented by survivor fish re-challenged with the same viral solution. The naive control group (NC) serves as the control for each sampling point (Fig. 1b). Fish care, viral infections and fish sampling were conducted in accordance with the guidelines of the CSIC National Committee on Bioethics.

RNA extraction and reverse transcription

Turbot kidneys were collected in TRIzol reagent (Invitrogen) and stored at -80 °C until use. Total RNA was extracted following the manufacturer´s instructions, and RNA was purified using the RNeasy mini kit (Qiagen) after DNase I treatment to remove genomic DNA (RNase-free DNase set, Qiagen). The purified RNAs were quantified using a Nanodrop ND-1000 (Thermo Scientific). To generate cDNA, RNAs from five fish were pooled (2 µg of RNA per fish). Reverse transcription was performed with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen).

Microarray analysis

An 8 x 15K *S. maximus* Oligo Array v 1.0 was used (AMADID 24698) (Millán et al. 2010; Hagenaars et al. 2011). In brief, this array contains 60-mers from 4,305 genes, each represented at least three times. A total of 16 microarray-8x15K slides were hybridized. RNAs from five fish per group were pooled, and their quality was assessed with a 2100 bioanalyzer (Agilent) before microarray analysis. RNA labelling (2 µg at ~50 µg/ml) and hybridization were conducted with the University of Santiago de Compostela microarray platform using Agilent one-color Gene Expression Analysis, as described previously (Millán et al. 2010), in compliance with Minimum Information about a Microarray Experiment (MIAME) standards (Brazma 2001).

Signal was captured, processed and segmented using an Agilent scanner (G2565B, Agilent Technologies) by the Agilent Feature Extraction Software (v9.5) with protocol GE1-v5_95, extended dynamic range and preprocessing by the Agilent Feature Extraction v9.5.5.1. Fluorescence intensity data and quality annotations were imported into GeneSpring GX version 11.0.2 (Agilent Technologies). All control features (positive, negative, landing lights, *etc.*) were excluded from subsequent analyses. Prior to normalization, all intensity values lower than 1 were set to 1.0; data were normalized by percentile shifts at the 75th percentile and were filtered both by flags and by expression between the 20 and 100th percentile in the raw data.

To assess differential gene expression, the normalized log intensity ratios were analyzed with a three-way ANOVA and Student's *t* tests. The three-way ANOVA was performed in order to explore the effects of family genetic background and the response and resistance to a viral infection on the gene expression profiles ($P<0.05$ and fold-change ratios ≥ 2). Student's *t* tests were performed to identify genes that were differentially expressed between high- and low-mortality families ($P<0.05$ and fold-change ratios ≥ 2) using two or four biological replicates. The data presented in this publication and the MIAME-compliant information have been deposited in the NCBI´s Gene Expression Omnibus and are accesible under the accession number GSE35804.

171

172 Gene expression analysis by real time PCR

173 A group of genes was selected for RT-qPCR expression analysis to validate the results of the
174 microarray analysis. Amplification was performed using specific primers, which were designed using
175 Primer3 software (Rozen and Skaletsky 2000) according to known qPCR restrictions (amplicon size, T_m
176 difference between primers, GC content and self-dimer or cross-dimer formation). The selected genes and
177 primer sequences are shown in Table 1. Primer efficiency was validated with seven serial five-fold
178 dilutions of cDNA and calculated from the slope of the regression line of C_t versus the relative
179 concentration of cDNA (Pfaffl 2001). A melting curve analysis was performed to verify that no primer
180 dimmers were amplified.

181 Quantitative PCR assays were performed using a 7300 Real Time PCR System (Applied Biosystems).
182 In a final volume of 25 µl, 1 µl of 5-fold diluted cDNA template was mixed with 0.5 µl of each primer (10
183 µM) and 12.5 µl of SYBR green PCR master mix (Applied Biosystems). Amplification was performed
184 under standard cycling conditions of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C
185 for 1 min. Three technical replicates were performed in each sample.

186 Relative gene expression levels were calculated as described by Pfaffl (2001), using elongation factor
187 1-α as housekeeping gene that was expressed constitutively (i.e., not affected by the treatment). Fold-
188 change units were calculated by dividing the normalized expression values in infected tissues by the
189 normalized expression values in the controls.

190

191

192 **Results**

193

194 Mortality

195 The results obtained in the experimental infections showed differences in susceptibility to VHSV
196 between the six turbot families. Meanwhile, families 1 and 4 presented very low mortality rates at 30 d
197 p.i. (3.3% ± 4.6 in both families), with families 2 and 3 showing the highest cumulative mortalities
198 (56.5% ± 23.3 and 46.6% ± 18.8, respectively). Families 5 and 6 had intermediate sensitivity to viral
199 infection, reaching mortality values of 26.6% (± 9.4) and 9.9% (± 4.7), respectively, at the end of the
200 experiment (Fig. 2). Moreover, moribund and dead fish from families 2 and 3 showed external clinical
201 signs of infection, such as haemorrhage at the base of the fins and around the eye orbit and mouth, as well
202 as internal haemorrhage in the liver, in adipose tissue and within the muscle. No external clinical signs of
203 infection were detected in low-mortality families (1 and 4). Thus, we chose to further study the
204 transcriptome profile after VHSV infection in four families: two high-mortality families (families 2 and
205 3) and two low-mortality families (families 1 and 4).

206

207

Overview of transcriptome analysis

Multiple statistical comparisons were performed using a three-way ANOVA in the four selected families in order to explore the effects of family genetic background (factor "Family"), the response to a viral infection at 24 h p.i. (factor "Infection") and previous contact with the virus (factor "Resistance"), as well as the interactions among these factors, on gene expression profile. The three-way ANOVA was performed to compare gene expression in naive-infected (NI or fish treated once with VHSV), resistant controls (RC; i.e., fish treated once with VHSV and sampled 30 days p.i.) and resistant-infected (RI; i.e., survivor fish that were re-challenged with the same viral solution) groups to the expression in the naive controls group (NC) within each family.

Two different comparisons were performed by Student's *t* test analyses considering families with similar mortality rates to be biological replicates. In the first analysis, the values obtained for each treatment group (NC, NI, RC and RI) of the high-mortality families were compared with those obtained in the same treatment group of the low-mortality families. These comparisons identified genes that were differentially expressed between high- and low-mortality families before infection (NC groups), at 24 h p.i. (NI groups), at 30 days p.i. (RC groups) and at 24 h after a re-challenge (RI groups).

Student's *t* tests were also used to compare gene expression in the different treatment groups within the same families. In this context, the NI *vs.* NC and RC *vs.* NC comparisons were used to identify genes that were differentially expressed at 24 h and 30 d after one exposure to the virus, respectively. The genes involved in resistance to the viral infection (RI *vs.* NI) as well as the genes that were stimulated 24 h after a second viral exposure (RI *vs.* RC) were also analyzed.

Three-way ANOVA

The results of the three-way ANOVA analysis determined that the factor "Family" was involved in the greatest number of gene expression differences (Fig. 3). This factor was able to explain the modulation of 45.54% (261 out of 573 genes) of all differentially expressed genes. The combinations "Family-Resistance" and "Family-Infection" explained 9.59% and 8.9% of the changes, respectively. Thus, genetic background alone and in combination with the other factors was involved in the regulation of 70.33% (403 genes) of the differentially expressed genes (Fig. 3). Meanwhile, the factor "Infection" explained the differences in expression in 80 genes (13.96%), and the factor "Resistance" was only involved in 11.86% of the changes (68 genes). Only 36 genes (6.28%) were affected by the combination of these three factors (Fig. 3). Only annotated genes were taken into account in the data analysis because most of these modulated genes were not homologous to any genes in public databases. Moreover, genes with similar and consistent behaviour in both high- and low-mortality families and showing a fold-change ratio ≥ 2 were described.

Although the factor "Family" was associated with the greatest number of differences in the transcriptome, only 19 of these genes were homologous to known sequences (7.27%), and most of them were related to metabolic processes (Supplementary Table 1). Only two genes showed different behaviour between high- and low-mortality families. The UTP-glucose-1-phosphate uridylyltransferase and the

secreted trypsin-like serine protease were always significantly down-modulated in resistant families (1 and 4) (Table 2).

Only 14 of the 80 genes that were regulated by the factor "Infection" (17.5 %) had homology to sequences that have been described in databases (Supplementary table 1). Six of these were significantly down-modulated after infection in low-mortality families (1 and 4). Viral infection significantly reduced the expression of the 60 S ribosomal protein L21, a sex hormone-binding globulin, an aldo/keto reductase, a SPRY domain-containing protein, complement component C9 and an antimicrobial peptide precursor (Table 2). Meanwhile, the interferon-induced protein Mx2 was up-modulated by infection in both low- and high-mortality families (Table 2).

Only ten of the 68 genes (14%) associated with the factor "Resistance" had previously been annotated in databases (Supplementary Table 1). Cathepsin L.1 and the interleukin-8 precursor were up-modulated in both RC and RI groups within the low-mortality families (1 and 4). Moreover, the antitrypsin-α1 gene was highly down-modulated in the same families (Table 2).

Only one gene was linked to the interaction of the "Family" and "Infection" factors. In this analysis, the complement component C8γ was down-regulated in the RC groups within families 1 and 4 (Table 2). The interaction of "Family" and "Resistance" factors did not significantly modify the expression of any gene in low-mortality families compared to high-mortality families (Supplementary Table 1).

As expected, the interaction between the factors "Infection" and "Resistance" induced a large number of changes in the transcriptome. Approximately 60 % of the modulated genes (13 out of 22 genes) showed homology to known sequences, and 11 genes were modulated differently in high- and low-mortality families (Table 2). All of the genes were down-modulated by infection in the different groups within each family (NI, RC and RI groups). The expression levels registered in low-mortality families (1 and 4) were significantly much lower than those obtained in high-mortality ones (Table 2).

Finally, the genes regulated by the interaction of the three factors ("Family", "Infection" and "Resistance") were mostly related to metabolism (Supplementary Table 1). Only 12 out of 36 genes (33.3 %) showed homology to known sequences, and only three genes behaved differently between high- and low-mortality families. The hepcidin gene, a trypsin-like serine protease and a notch homolog protein 2-like gene were down-modulated after infection in low-mortality families only (Table 2). Importantly, the up-modulation of virus-induced protein 5 was observed in all families, as was expected (Table 2).

Student's *t* test analysis

A large amount of information was obtained in all the different comparisons conducted with the Student's *t* tests. The first analysis provided information on genes that were differentially expressed between high- and low-mortality families (Table 3). When not infected (NC groups), the low-mortality families showed higher basal expression of different antimicrobial peptides (2- to 5.9-fold change) than did high-mortality families (Table 3). High-mortality families showed lower expression levels of a sodium/hydrogen exchanger protein gene (2.06 times lower), the complement component C8 gene (2.63 times lower) and the butyrophilin member A1 precursor gene (4.73 times lower) (Table 3). The differences in gene expression between low- and high-mortality families at 24 h after viral infection were analyzed (NI groups). Interestingly, while the low-mortality families exhibited up-modulation of several

genes (selectin P, 5-aminolevulinate synthase and arrestin domain-containing protein 2), the high-mortality families exhibited a strong down-modulation of the lily-type lectin gene (21.99 times lower than low-mortality families), an alcohol dehydrogenase gene (10.77 times lower), and a myeloid-associated differentiation marker gene (6.86 times lower) (Table 3) and a weak down-regulation of other genes (prostasin-like gene, the factor B/C2B gene and the apomucin gene) (Table 3). Among the genes that were differentially expressed 30 d after viral infection (RC groups), low-mortality families showed a significant up-modulation of cathepsin L.1 and ribonuclease T (3.09- and 2.96-fold increase, respectively (Table 3)). High-mortality families still exhibited a down-modulation of the complement component C8 gene (4.94 times lower), the lily-type lectin gene (4.63 times lower), the prostasin-like gene (2.7 times lower) and the apomucin gene (2.74 times lower). Moreover, two genes related to lipid metabolism were down-regulated (7-dehydrocholesterol reductase gene and the HRAS-like suppressor 2 gene) (Table 3). No differences in the gene expression profiles were registered between the surviving fish from high- and low-mortality families 24 h after the second viral infection (RI groups).

The second analysis provided information on genes that were differentially expressed at 24 h and 30 days p.i. (NI *vs.* NC and RC *vs.* NC, respectively), genes related to resistance (RI *vs.* NI) and genes that were stimulated after a second viral exposure (RI *vs.* RC) within either the susceptible or the resistant families (Table 4). Twenty-four hours after infection (NI *vs.* NC), a general down-regulation of gene expression was observed in all families (Table 4). While the changes registered in the high-mortality families ranged between 2.03 to 4.00 (fold-decrease), a much stronger down-modulation was observed in low-mortality families (ranging from 5.03 to 53.09 times lower). Low-mortality families showed the highest down-modulation of antimicrobial peptides (53.09 times lower than controls), several complement components (38.21 times lower) and the perforin gene (32.65 times lower). Moreover, other important genes were highly down-modulated, such as the warm temperature acclimation-related 65-kDa protein (WAP65) (30.37 times lower), the tributyltin binding protein type 1 (29.43 times lower) and the Ca^{2+} dependent complex C1R/C1S (17.60 times lower) (Table 4). High-mortality families showed an up-modulation of factor B/C2B (2.66 times) but a generally low down-modulation of several genes involved in different metabolic pathways (prosaposin, 2-hydroxyacyl-CoA lyase 1, aminotransferases, dehydrogenases and a Na^+/K^+ ATPase) and in the maintenance of the ionic balance (two pore segment channel 1, solute carrier family 25 member 42 and a Na^+/H^+ exchanger isoform 3) (Table 4).

Thirty days after infection (RC *vs.* NC), a significant down-modulation of the tropomyosin and troponin genes (20.43 and 11.65 times lower, respectively) and other genes such as the cytochrome P450 (3.11 times lower) was observed in the low-mortality families. At this time, high-mortality families showed a significant up-modulation of matrix metallopeptidase 13 (2.27 times) and an inhibition of the apolipoprotein E1 (2.27 times lower) (Table 4). Genes related to resistance were identified by comparing the RI *vs.* NI datasets. In this situation, the low-mortality families showed an up-modulation of a myeloid-associated differentiation marker (3.79 times) and a general inhibition of apolipoprotein A1, tributyltin-binding protein type 2, an aldo-keto reductase and the WAP65. High-mortality families showed an up-modulation of two-pore segment channel 1, the probable mitochondrial protein COQ10 and proline dehydrogenase 2. The expression of complement component C9 was also inhibited (2.22 times lower) (Table 4). Differential expression after a second viral infection (RI *vs.* RC) included an up-regulation of

Mx2 and virus-induced protein 5 (3.42 and 3.13 times) in high-mortality families and a down-modulation of coagulation factors (2.77 times lower) in low-mortality families (Table 3).

Validation of array results by real-time PCR

Real-time PCR analysis was performed to confirm the microarray data (Fig. 4). The expression levels of five selected genes (an antimicrobial peptide, complement component C8 γ-chain, hepcidin, Mx2 and the WAP65) were measured and normalized to the expression of EF-1α, which was found to be expressed constitutively in microarray analyses. The microarray data were confirmed by real-time PCR for all genes, with the obtained expression patterns showing the same directions of response in both methodologies (Figure 4).

Discussion

In order to search for molecular markers linked to resistance in different families of turbot and to explore systemic gene response after VHSV infection, we performed a transcriptional analysis in low- and high-mortality families after infection. Although the effects of VHSV infection on the modulation of several immune functions and some immune-related genes have been described in different fish species including turbot (Abollo et al. 2005; Encinas et al. 2010; Jørgensen et al. 2011; Novoa et al. 2010; Tafalla et al. 2001, 2005; Tafalla and Novoa 2001), the effect of the progression of VHS disease on the global gene expression profiles of resistant and susceptible fish families remains unknown. Only one recent publication has explored the importance of several key innate immune-related genes in the increased survival observed in different families of infected rainbow trout (*O. mykiss*) (Jørgensen et al. 2011).

As reviewed by Douglas (2006), oligonucleotide microarrays have been developed for several fish species, such as zebrafish, salmonids including the rainbow trout and the Atlantic salmon, catfish, seabream and flatfish including turbot, sole and the Japanese flounder (Aoki et al. 2011; Byon et al. 2005, 2006; Cerdá et al. 2010; Douglas et al. 2007; Kurobe et al. 2005; Millán et al. 2010; Osuna-Jiménez et al. 2009; Park et al. 2009). There are few well-annotated and curated flatfish EST and protein sequences available in public databases; this is one reason why a significant percentage of the genes identified in our analyses show no homology to known sequences deposited in databases. This lack of information presents a major challenge for transcriptomic analysis. To understand the importance of the differentially expressed genes obtained through our microarray analysis of natural resistance to a VHSV infection, an overview of all the changes in gene expression determined by different statistical approaches were correlated with different stages of the viral disease.

In this work, infection with a turbot VHSV isolate revealed a differential susceptibility to the disease between the six families of turbot. The high levels of cumulative mortality registered in four out of six families were similar to those described previously (Brudeseth et al. 2005; Castric and de Kinkelin 1984; King et al. 2001; Snow and Smail 1999). Moreover, diseased turbot displayed external clinical signs of VHSV infection and internal lesions (Castric and de Kinkelin 1984; King et al. 2001; Ross et al. 1994;

Smail 1999). We classified families 1 and 4 as "low-mortality" and families 2 and 3 as "high-mortality". Moreover, the observed modulation of different gene expression was consistent with the development of the disease. The up-modulation of the interferon-induced Mx2 gene, the IL-8 gene and the VHSV-induced protein 5 gene in the infected groups (NI and RI groups) in both high- and low-mortality families suggested a response similar to that observed in other fish species. Jørgensen et al. (2011) reported a similar up-modulation of these genes in infected trout. The importance of the Mx proteins in resistance against DNA and RNA viruses has been clearly described (Haller et al. 2007). Moreover, the up-modulation of Mx2, IL-8 and VHSV-induced protein 5 after viral infection was already been reported in different fish species (Abollo et al. 2005; Fernández-Trujillo et al. 2008; O'Farrell et al. 2002; Park et al. 2009; Tafalla et al. 2005; Workenhe et al. 2009). Interestingly, low-mortality families also showed a significant down-regulation of cytochrome P450 expression at 30 days after infection (RC group), suggesting that VHSV could be present inside the fish at this time; previous studies have reported that cytochrome P450 is permanently downregulated in humans during viral and bacterial infections (Armstrong and Renton 1994; Bleau et al. 2001; Morgan 1997).

Our three-way ANOVA analysis showed that the factor "Family" (i.e. genetic background) explained the greatest number of variations in the gene expression profiles, highlighting the importance of the genetic background in the response to viral infection and in the degree of resistance or susceptibility of the different families of turbot. This large-scale screen of changes in mRNA expression revealed different patterns in high- and low-mortality families before and during viral infection. Similar observations in infected rainbow trout (*O. mykiss*) were reported by Jørgensen et al. (2011).

Before the experimental infection, fish from low-mortality families showed a high expression of different antimicrobial peptides, suggesting a pre-immune state higher than that of high-mortality families, which could lead to a faster and stronger immune response after the first viral infection. The opposite situation was observed in high-mortality families, where low levels of complement component C8 and other cellular receptors (butyrophilin A1 precursor gene) could be associated with a higher susceptibility to viral infection, as has been previously described in humans (Botto et al. 2009; Cooper and Nemerow 1989; Jasin 1977; Pickering et al. 2008).

The basements of the fins and the gills are the main sites of VHSV entry in bath infections (Smail 1989). Mucosal secretion protects the fish against bacterial and viral aggression through different specific and non-specific immune substances on the fins, skin and other body surfaces (Palaksha et al. 2008; Salinas et al. 2011; Shephard 1994; St Louis-Cormier et al. 1984). Several mucins, prostasin proteins and lectins play an important role in limiting infectious disease (Linden et al. 2008). Interestingly, low levels of several components of the mucus, such as apomucins, a prostasin-like protein and a lily-type lectin, were registered in high-mortality families of turbot. Moreover, the keratin protein that is a major component of the fins (Saraswat and Ram 1970) and protects the fish by providing a barrier against viruses (Friedman 2006) was also down-regulated in high-mortality families.

Interestingly, a down-regulation of different genes involved in the entrance of the virus into target cells was observed in low-mortality families after infection. High levels of a secreted trypsin-like serine protease have recently been shown to improve the entrance of a pseudorabies virus within the target cells (Glorieux et al. 2011). Some members of the lipocalin family, such as apolipoproteins and the scavenger

receptor cysteine-rich-domain-containing protein, are also essential for virus infectivity (Hill et al. 2007; Hishiki et al. 2010; Owen et al. 2009). Park et al. (2009) also reported an increase in apolipoprotein A1 after a nodavirus infection in trout. In this context, the low expression levels of all these genes in low-mortality families could be an advantage, preventing the spread of the viral infection within the fish.

The high- and low-mortality families also showed different gene expression profiles after VHSV infection. The low-mortality families showed a strong down-regulation of antimicrobial peptides (including the hepcidin gene), perforins (up to 53.09 and 32.65 times lower, respectively) and several components of the complement pathway. Although antimicrobial peptides have been described to possess antiviral activity in fish (Chia et al. 2010; Falco et al. 2009; Pasupuleti et al. 2011; Rajanbou and Chen 2011; Wang et al. 2010; Zhou et al. 2011), it is possible that low-mortality families down-modulated these genes in favor of the expression of other genes that are important to maintain the minimal physiological functions that ensure fish survival.

The anterior kidney is the most important target organ of rhabdoviral infection (Smail 1999), and infection induces extensive haemorrhages, apoptosis and necrosis in the haematopoietic tissue as well as in the renal tubules and glomeruli (Amend and Smith 1975). Normal kidney function is lost during infection, and the physiological dysfunction of the renal elements disrupts electrolyte balance and plasma pH. Moreover, the destruction of haematopoietic tissue also induces changes in the composition of cellular components of the blood (Amend and Smith 1974; Watson et al. 1956). The ultimate cause of death is usually acute kidney failure and loss of osmoregulation. The expression of several proteins has been used to assess renal function and monitor the course of progression of renal diseases in humans. Among them, levels of retinol-binding protein (Ayatse 1991), microglobulin (Miyata et al. 1998; Narisawa et al. 1998; Pless-Mulloli et al. 1998), the tributyltin-binding proteins (Post et al. 2010) and the apolipoproteins (Boes et al. 2006) have been shown to increase during renal disease. In our experimental infections, the low-mortality families (in contrast to the high-mortality ones) showed a strong down-modulation of these three genes at all sampling points (24 h p.i., 30 d p.i. and 24 h after a second viral exposure), suggesting the maintenance of kidney structure and function. The changes in ion channel gene expression did not show a specific expression pattern, likely because the ion transporters along the nephron are very dynamic and respond quickly to fluctuations in electrolytes (McDonough et al. 2003). Moreover, the high levels of expression of the myeloid-associated differentiation marker gene in low-mortality families at 30 d p.i. also suggest the normal haematopoietic function of this tissue because VHSV is able to replicate in kidney macrophages (Tafalla et al. 1998).

A small number of immune-related genes were obtained in our analysis, most probably because the only available oligo-array employed in our analysis was constructed using spleens from *Aeromonas salmonicida*-challenged fish (Millán et al. 2010). Fibrinogens are acute phase proteins (APPs) that are involved in blood coagulation, immune response and inflammation (Baumann and Gauldie 1994). Previous studies that evaluated fibrinogen levels following viral infections (Byon et al. 2005; Park et al. 2009) and after vaccination (Keiser et al. 2009) suggested that fibrinogen expression levels are high during systemic inflammatory response (Xie et al. 2009). Therefore, fibrinogen expression depends on the time after the viral infection, as well as the virus itself. Our oligo-array indicated that several genes involved in coagulation (fibrinogen α and γ chains and the coagulin factor II) are down-regulated in the

low-mortality families, suggesting an alternative role for these components. This extreme down-regulation is possible given that neither external clinical signs nor haemorrhagic lesions were detected in the low-mortality families. Moreover, the low level of fibrinogen could reduce the likelihood of opportunistic bacterial infection because the fibrinogen molecule can act as a receptor for the attachment of bacteria to virus-infected cells (Sanford et al. 1982). It is important to remember that antimicrobial peptides, complement components and other related antibacterial genes were inhibited in low-mortality families. The WAP65 was highly down-regulated in the low-mortality families (up to 275.46 times lower in family 4). WAP65 plays an important role in the acclimation to warm temperature and in immune response after bacterial and viral infections (Park et al. 2009; Sarropoulou et al. 2010; Sha et al. 2008). A recent study in ayu (*Plecoglossus altivelis*) revealed that WAP65 is involved in the activation of the complement system through component C3 (Shi et al. 2011). A general down-regulation of the complement components was registered in both resistant and susceptible families after VHSV infection. Therefore, our results could suggest a regulation of the complement system by WAP65 in low-mortality families after infection. As described by Shi et al. (2011), WAP65 might be involved in fighting microbiological infections through the activation of the complement system.

In general, the expressions of different genes involved in the metabolism of sugars, lipids and proteins were decreased in both high- and low-mortality families. Low-mortality families showed a strong down-modulation of several genes related to sugar metabolism, while the high-mortality families showed a less dramatic down-regulation of genes related to lipid metabolism. Although the relationship between glucose and lipid metabolism and viral infection is not clear (Anty et al. 2007; Bernard et al. 1988), Encinas et al. (2010) reported a general up-regulation of glycolytic enzymes in susceptible zebrafish after VHSV infection. The different result observed here could be the consequence of a combined response between the change in metabolic rates induced by viral infection and the "natural predisposition of the resistant families to reduce the metabolic rates to inhibit the spread of the disease. The importance of metabolic regulation in the VHSV susceptibility of different families of turbot cannot be well explained, but these observations highlight a dramatic difference in metabolic requirements between the two family groups (high and low mortality).

In summary, the present work confirms that the mechanisms by which particular species and/or populations can resist a pathogen(s) are complex and multifactorial. Taking into account all of the data described in this work, the final consequence of the infection (life or death) will be determined by multiple interactions between genes involved in many non-immune processes to ensure the preservation of essential physiological functions. When the vital functions are ensured, the activity of immune-related genes will be able to address infections in low-mortality families, as was described by Jørgensen et al. (2011). In this sense, families with a genetic background that improves the maintenance of kidney, heart, and liver function and stimulates genes related to inhibiting viral entrance will have more opportunities to overcome VHSV infection. The present study is an initial screen for genes of interest and provides an extensive description of the genetic basis underlying the differences between turbot families that are resistant or susceptible to VHSV infection.

Acknowledgments

We wish to thank the Spanish Ministerio de Ciencia e Innovación for funding through project CSD2007-00002 "Aquagenomics" of the Consolider-Ingenio 2010 program. We also wish to thank Ana Riaza from Stolt Sea Farm for providing the turbot families and Paulino Martínez from Universidad de Santiago de Compostela for his support. Dr. P. Díaz-Rosales wishes to thank the Spanish National Research Council (CSIC, Spain) for a JAE-Doc contract.

References

Abollo E, Ordás C, Dios S, Figueras A, Novoa B (2005). Molecular characterisation of a turbot Mx cDNA. Fish Shellfish Immunol 19:185-190

Amend DF, Smith L (1974). Pathophysiology of Infectious Hematopoietic Necrosis Virus Disease in Rainbow Trout (*Salmo gairdneri*): Early changes in blood and aspects of the immune response after injection of IHN virus. J Fish Res Board Can 31:1371-1378

Amend DF, Smith L (1975). Pathophysiology of infectious hematopoietic necrosis virus disease in rainbow trout: hematological and blood chemical changes in moribund fish. Infect Immun 11:171-179

Anty R, Gelsi E, Giudicelli J, Mariné-Barjoan E, Gual P, Benzaken S, Saint-Paul MC, Sadoul JL, Huet PM, Tran A (2007) Glucose intolerance and hypoadiponectinemia are already present in lean patients with chronic hepatitis C infected with genotype non-3 viruses. Eur J Gastroenterol Hepatol 19:671-677

Aoki T, Hirono I, Kondo H, Hikima J, Jung TS (2011). Microarray technology is an effective tool for identifying genes related to the aquacultural improvement of Japanese flounder, *Paralichthys olivaceus*. Comp Biochem Physiol Part D Genomics Proteomics. 6: 39-43

Armstrong SG, Renton KW (1994). Factors involved in the down-regulation of cytochrome P450 during *Listeria monocytogenes* infection. Int J Immunopharmacol. 16:747-754

Ayatse JO (1991) Human retinol-binding protein: its relationship to renal function in renal diseases. West Afr J Med 10:226-231

Baumann H, Gauldie J (1994) The acute phase response. Immunology Today 15: 74-80

Bernard A, Zwingelstein G, Meister R, Wild TF (1988). Hyperinsulinemia induced by canine distemper virus infection of mice and its correlation with the appearance of obesity. Comp Biochem Physiol B 91:691-696

Bleau AM, Fradette C, El-Kadi AO, Côté MC, du Souich P (2001) Cytochrome P450 down-regulation by serum from. Drug Metab Dispos 29:1007-1012

Boes E, Fliser D, Ritz E, König P, Lhotta K, Mann JF, Müller GA, Neyer U, Riegel W, Riegler P, Kronenberg F (2006). Apolipoprotein A-IV predicts progression of chronic kidney disease: the mild to moderate kidney disease study. J Am Soc Nephrol 17:528-536

Botto M, Kirschfink M, Macor P, Pickering MC, Wurzner R, Tedesco F (2009). Complement in human diseases: Lessons from complement deficiencies. Mol Immunol 46:2774-2783

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M (2001) Minimum information about a microarray experiment (MIAM) – toward standards for microarray data. Nat Genet 29:365-371

Brudeseth BE, Raynard RS, King, JA, Evensen O (2005) Sequential pathology after experimental infection with marine Viral Hemorrhagic Septicemia Virus isolates of low and high virulence in turbot (*Scophthalmus maximus* L.) Vet Pathol 42:9–18

Byon JY, Ohira R, Hirono I, Aoki T (2005) Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. Fish Shellfish Immunol 18:135-147

Byon JY, Ohira T, Hirono I, Aoki T (2006) Comparative immune responses in Japanese flounder *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using microarray analysis. Vaccine 24:921-930

Castric J, de Kinkelin P (1984) Experimental study of the susceptibility of two marine fish species, sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) to viral haemorrhagic septicaemia. Aqua 41:203-212

Cerdà J, Douglas S, Reith M. (2010) Genomic resources for flatfish research and their applications. J Fish Biol 77:1045-1070

Chia, TJ, Wu, YC, Chen, JY, Chi, SC (2010). Antimicrobial peptides (AMP) with antiviral activity against fish nodavirus. Fish and Shellfish Immunology 28:434-439

Cooper NR, Nemerow GR (1989). Complement and infectious agents: a tale of disguise and deception. Complement Inflamm 6:249-258.

Dorson M, Quillet E, Hollebecq MG (1995) Selection of rainbow trout resistant to viral haemorrhagic septicaemia virus and transmission of resistance by gynogenesis. Vet Res 26:361-368

Douglas, SE. (2006). Microarray studies of gene expression in fish. OMICS. 2006 Winter 10:474-489

Douglas SE, Knickle LC, Kimball J, Reith ME.(2007) Comprehensive EST analysis of Atlantic halibut (*Hippoglossus hippoglossus*), a commercially relevant aquaculture species. BMC Genomics 8:144

Encinas P, Rodríguez-Milla MA, Novoa B, Estepa A, Figueras A, Coll J (2010) Zebrafish fin immune responses during high mortality infections with viral haemorrhagic septicemia rhabdovirus. A proteomic and transcriptomic approach. BMC Genomics 11:518

Falco A, Ortega-Villaizan M, Chico V, Brocal I, Perez L, Coll JM, Estepa A (2009) Antimicrobial peptides as model molecules for the development of novel antiviral agents in aquaculture. Mini Rev Med Chem 9:1159-1164

Fernandez-Trujillo A, Ferro P, Garcia-Rosado E, Infante C, Alonso MC, Bejar J, Borrego JJ, Manchado M (2008) Poly I:C induces Mx transcription and promotes an antiviral state against sole aquabirnavirus in the flatfish Senegalese sole (Solea *senegalensis* Kaup). Fish Shellfish Immunol 24:279-285

562 Friedman HM (2006) Keratin, a dual role in herpes simplex virus pathogenesis. J of Clin Virol
563 35:103–105
564 Glorieux S, Favoreel HW, Steukers L, Vandekerckhove AP, Nauwynck HJ (2011). A trypsin-like
565 serine protease is involved in pseudorabies virus invasion through the basement membrane barrier of
566 porcine nasal respiratory mucosa. Vet Res 42:58
567 Hagenaars A, Meyer IJ, Herzke D, Pardo BG, Martinez P, Pabon M, De Coen W, Knapen D (2011).
568 The search for alternative aqueous film forming foams (AFFF) with a low environmental impact:
569 physiological and transcriptomic effects of two Forafac(®) fluorosurfactants in turbot. Aquat Toxicol
570 194:168-176
571 Haller O, Stertz S, Kochs G (2007). The Mx GTPase family of interferon-induced antiviral proteins.
572 Microbes Infect 9:1636-1643
573 Hill JM, Bhattacharjee PS, Neumann DM (2007) Apolipoprotein E alleles can contribute to the
574 pathogenesis of numerous clinical conditions including HSV-1 corneal disease. Exp Eye Res 84:801-811
575 Hishiki T, Shimizu Y, Tobita R, Sugiyama K, Ogawa K, Funami K, Ohsaki Y, Fujimoto T, Takaku H,
576 Wakita T, Baumert TF, Miyanari Y, Shimotohno K (2010) Infectivity of hepatitis C virus is influenced by
577 association with apolipoprotein E isoforms. J Virol 84:12048-12057
578 Jasin HE (1977). Absence of the eighth component of complement in association with systemic lupus
579 erythematosus-like disease. J Clin Invest 60:709-715
580 Jørgensen HB, Sørensen P, Cooper GA, Lorenzen E, Lorenzen N, Hansen MH, Koop BF, Henryon M
581 (2011). General and family-specific gene expression responses to viral hemorrhagic septicaemia virus
582 infection in rainbow trout (*Oncorhynchus mykiss*). Mol Immunol 48:1046-1058
583 Keiser PB, Miller LB, Biggs-Cicatelli S, Zollinger WD (2009) Plasma fibrinogen levels after
584 vaccination with a native outer membrane vesicle vaccine for *Neisseria meningitidis*. Vaccine 27:6809-
585 6813
586 King JA, Snow M, Skall HF, Raynard RS (2001) Experimental susceptibility of Atlantic salmon
587 *Salmo salar* and turbot *Scophthalmus maximus* to European freshwater and marine isolates of viral
588 haemorrhagic septicaemia virus. Dis Aquat Org 47:25–31
589 Kurobe T, Yasuike M, Kimura T, Hirono I, Aoki T (2005). Expression profiling of immune-related
590 genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. Dev Comp
591 Immunol 29:515-523
592 Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA (2008). Mucins in the mucosal barrier
593 to infection. Mucosal Immunol 1:183-197
594 Lorenzen N, Olensen NJ (1997) Immunization with viral antigens: viral haemorrhagic septicaemia.
595 Dev Biol Stand 90:201-209
596 Lorenzen N, Lorenzen E, Einer-Jensen K (2001) Immunity to viral haemorrhagic septicaemia (VHS)
597 following DNA vaccination of rainbow trout at an early life-stage. Fish Shellfish Immunol 11:585-591
598 Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE (2002a) DNA vaccines as a tool for analysing
599 the protective immune response against rhabdoviruses in rainbow trout. Fish Shellfish Immunol 12:439-
600 453

Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE (2002b) Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Dev Comp Immunol 26:173-179

Lorenzen E, Lorenzen N, Einer-Jensen K, Brudeseth B, Evensen Ø (2005) Time course study of in situ expression of antigens following DNA-vaccination against VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) fry. Fish Shellfish Immunol 19:27-41

McDonough AA, Leong PKK, Yang LE (2003). Mechanisms of Pressure Natriuresis. Ann N Y Acad Sci 986:669–677

Millán A, Gómez-Tato A, Fernández C, Pardo BG, Álvarez-Dios JA, Calaza M, Bouza C, Vázquez M, Cabaleiro S, Martínez P (2010). Design and performance of a turbot (*Scophthalmus maximus*) oligo-microarray based on ESTs from immune tissues. Mar Biotechnol 12:452-465

Miyata T, Jadoul M, Kurokawa K, Van Ypersele C (1998) Microglobulin in Renal Disease. J Am Soc Nephrol 9:1723-1735

Morgan ET (1997) Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev 29:1129–1188

Narisawa T, Murakami A, Aiba M, Takaba T (1998). Evaluation of highly damaged renal function following extracorporeal circulation. Usefulness of alpha 1-microglobulin index. Jpn J Thorac Cardiovasc Surg 46:1097-1104

Novoa B, Mackenzie S, Figueras A (2010). Inflammation and innate immune response against viral infections in marine fish. Curr Pharm Des 16: 4175-4184

O'Farrell C, Vaghefi N, Cantonnet M, Buteau B, Boudinot P, Benmansour A (2002). Survey of transcript expression in rainbow trout leukocytes reveals a major contribution of interferon-responsive genes in the early response to a rhabdovirus infection. J Virol 76:8040-8049

Osuna-Jiménez I, Williams TD, Prieto-Alamo MJ, Abril N, Chipman JK, Pueyo C. (2009) Immune- and stress-related transcriptomic responses of *Solea senegalensis* stimulated with lipopolysaccharide and copper sulphate using heterologous cDNA microarrays. Fish Shellfish Immunol 26:699-706

Owen DM, Huang H, Ye J, Gale M Jr (2009) Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. Virology 394:99-108

Palaksha KJ, Shin GW, Kim YR, Jung TS (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol 24:479-488

Park KC, Osborne JA, Montes A, Dios S, Nerland AH, Novoa B, Figueras A, Brown LL, Johnson SC. (2009) Immunological responses of turbot (*Psetta maxima*) to nodavirus infection or polyriboinosinic polyribocytidylic acid (pIC) stimulation, using expressed sequence tags (ESTs) analysis and cDNA microarrays. Fish Shellfish Immunol 26:91-108

Pasupuleti M, Schmidtchen A, Malmsten M (2011). Antimicrobial peptides: key components of the innate immune system. Crit Rev Biotechnol. Doi: 10.3109/07388551.2011.594423

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002-2007

639 Pickering MC, Macor P, Fish J, Durigutto P, Bossi F, Petry F, Botto M, Tedesco F. (2008)
640 Complement C1q and C8beta deficiency in an individual with recurrent bacterial meningitis and adult-
641 onset systemic lupus erythematosus-like illness. Rheumatology (Oxford) 47:1588-1589
642 Pless-Mulloli T, Boettcher M, Steiner M, Berger J.(1998) alpha-1-Microglobulin: epidemiological
643 indicator for tubular dysfunction induced by cadmium? Occup Environ Med 55:440-445
644 Post FA, Wyatt CM, Mocroft A (2010) Biomarkers of impaired renal function. Curr Opin HIV AIDS
645 5:524-30
646 Rajanbabu V, Chen JY (2011) Antiviral function of tilapia hepcidin 1-5 and its modulation of
647 immune-related gene expressions against infectious pancreatic necrosis virus (IPNV) in Chinook salmon
648 embryo (CHSE)-214 cells. Fish Shellfish Immunol 30:39-44
649 Reed LJ, Müench H (1938) A simple method of estimating 50% endpoints. Am J Hyg 27:493-497
650 Ross K, McCarthy U, Huntly PJ, Wood BP, Stuart D, Rough EI, Smail DA, Bruno DW (1994) An
651 outbreak of viral haemorrhagic septicaemia (VHS) in turbot (*Scophthalmus maximus*) in Scotland. Bull
652 Eur Assoc Fish Pathol 14:213
653 Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers.
654 In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: Methods in molecular biology.
655 Humana Press, Totowa, New Jersey
656 Salinas I, Zhang YA, Sunyer JO (2011). Mucosal immunoglobulins and B cells of teleost fish. Dev
657 Comp Immunol. 2011 Nov 26. [Epub ahead of print]
658 Sanford BA, Davison VE, Ramsay MA (1982). Fibrinogen-mediated adherence of group A
659 Streptococcus to influenza A virus-infected cell cultures. Infect Immun. 38:513-520
660 Saraswat RC, Ram N (1970) Amino acid composition of some skeletal elements of the carp *Cirrhina*
661 *Mrigala* (Ham). Indian J Fish 9:22-25
662 Sarropoulou E, Fernandes JM, Mitter K, Magoulas A, Mulero V, Sepulcre MP, Figueras A, Novoa B,
663 Kotoulas G (2010) Evolution of a multifunctional gene: The warm temperature acclimation protein
664 Wap65 in the European seabass *Dicentrarchus labrax*. Mol Phylogenet Evol 55:640-649
665 Schlotfeldt HJ, Ahne W, Jørgensen PEV, Glende W (1991) Occurrence of viral haemorrhagic
666 septicaemia in turbot *Scophthalmus maximus* – a natural outbreak. Bull Eur Ass Fish Pathol 11:105-107
667 Sha Z, Xu P, Takano T, Liu H, Terhune J, Liu Z (2008). The warm temperature acclimation protein
668 Wap65 as an immune response gene: its duplicates are differentially regulated by temperature and
669 bacterial infections. Mol Immunol 45:1458-69
670 Shephard KL (1994) Functions for fish mucus. Rev Fish Biol Fish 4:401-429
671 Shi YH, Chen J, Li CH, Yang HY, Lu XJ (2011) The establishment of a library screening method
672 based on yeast two-hybrid system and its use to determine the potential interactions of liver proteins in
673 ayu, *Plecoglossus altivelis*. Fish Shellfish Immunol 30:1184-1187
674 Slierendrecht WJ, Lorenzen N, Glamann J, Koch C, Rombout JHWM (1995) Immunocytochemical
675 analysis of a monoclonal antibody specific for rainbow trout (*Oncorhynchus mykiss*) granulocytes and
676 thrombocytes. Vet Immunol Immunopathol 46:349-360

677 Slierendrecht WJ, Olesen NJ, Lorenzen N, Jorgensen PEV,Gottschau A, Koch C (1996) Genetic
678 alloforms of rainbow trout (*Oncorhynchus mykiss*) complement component C3 and resistance to viral
679 haemorrhagic septicaemia under experimental conditions. Fish Shellfish Immunol 6:235-237
680 Slierendrecht WJ, Olesen NJ, Juul-Madsen HR (2001) Rainbow trout offspring with different
681 resistance to viral haemorrhagic septicaemia. Fish Shellfish Immunol 11:155-167
682 Smail DA (1999) Fish diseases and disorders. In: Woo PTK, Bruno DW (eds) Viral, bacterial and
683 fungal infections. CAB International Publishing, New York
684 Snow M, Smail DA (1999). Experimental susceptibility of turbot *Scophthalmus maximus* to viral
685 haemorrhagic septicaemia virus isolated from cultivated turbot. Dis Aquat Organ 38:163-168
686 St Louis-Cormier EA, Osterland CK, Anderson PD (1984). Evidence for a cutaneous secretory
687 immune system in rainbow trout (*Salmo gairdneri*). Dev Comp Immunol. 8:71-80
688 Tafalla C, Novoa B (2001) Respiratory burst of turbot (*Scophthalmus maximus*) macrophages in
689 response to experimental infection with viral haemorrhagic septicaemia virus (VHSV). Fish Shellfish
690 Immunol 11:727-734
691 Tafalla C, Figueras A, Novoa B (1998) In vitro interaction of viral haemorrhagic septicaemia virus
692 and leukocytes from trout (*Oncorhynchus mykiss*) and turbot (*Scophthalmus maximus*). Vet Immunol
693 Immunopathol 62:359-366
694 Tafalla C, Figueras A, Novoa B (2001) Viral hemorrhagic septicemia virus alters turbot *Scophthalmus*
695 *maximus* macrophage nitric oxide production. Dis Aquat Organ. 47:101-107
696 Tafalla C, Coll J, Secombes CJ (2005) Expression of genes related to the early immune response in
697 rainbow trout (*Oncorhynchus mykiss*) after viral haemorrhagic septicemia virus (VHSV) infection. Dev
698 Comp Immunol 29:615-626
699 Tomasec J, Fijan N (1971) Virusne bolestiriba (viral diseases of fish). Final report on research under a
700 part of project 6n/1966, Zagreb
701 Wang YD, Kung CW, Chen JY (2010) Antiviral activity by fish antimicrobial peptides of epinecidin-
702 1 and hepcidin 1–5 against nervous necrosis virus in medaka. Peptides 31:1026–1033
703 Watson ME, Guenther RW, Royce RD (1956) Hematology of healthy and virus-diseased Sockeye
704 salmon, *Onchorhynchus nerka*. Zoologica 41: 27-38.
705 Winton JR (1997) Immunization with viral antigens: infectious haemotopoietic necrosis. Dev Biol
706 Stand 90:211-220
707 Workenhe ST, Hori TS, Rise ML, Kibenge MJT, Kibenge FSB (2009) Infectious salmon anaemia
708 virus (ISAV) isolates induce distinct gene expression responses in the Atlantic salmon (*Salmo salar*)
709 macrophage/dendritic-like cell line TO, assessed using genomic techniques. Mol Immunol 46:2955-2974
710 Xie FJ, Zhang ZP, Lin P, Wang SH, Zou ZH, Wang YL (2009) Identification of immune responsible
711 fibrinogen beta chain in the liver of large yellow croaker using a modified annealing control primer
712 system. Fish Shellfish Immunol 27:202-209.
713 Yasutake WT (1975) Fish virus diseases: clinical, histopathological and comparative aspects. In:
714 Ribelin WE, Migaki G (eds) The pathology of fishes. University of Wisconsin Press, Madison

Zhou JG, Wei JG, Xu D, Cui HC, Yan Y, Ou-Yang ZL, Huang XH, Huang YH, Qin QW (2011). Molecular cloning and characterization of two novel hepcidins from orange-spotted grouper, *Epinephelus coioides*. Fish Shellfish Immunol 30:559-568

Figures and Tables

Fig. 1 Design of the experimental infections for microarray analysis (**A**) and to analyze the fish immune response to the viral infection process over time (**B**).

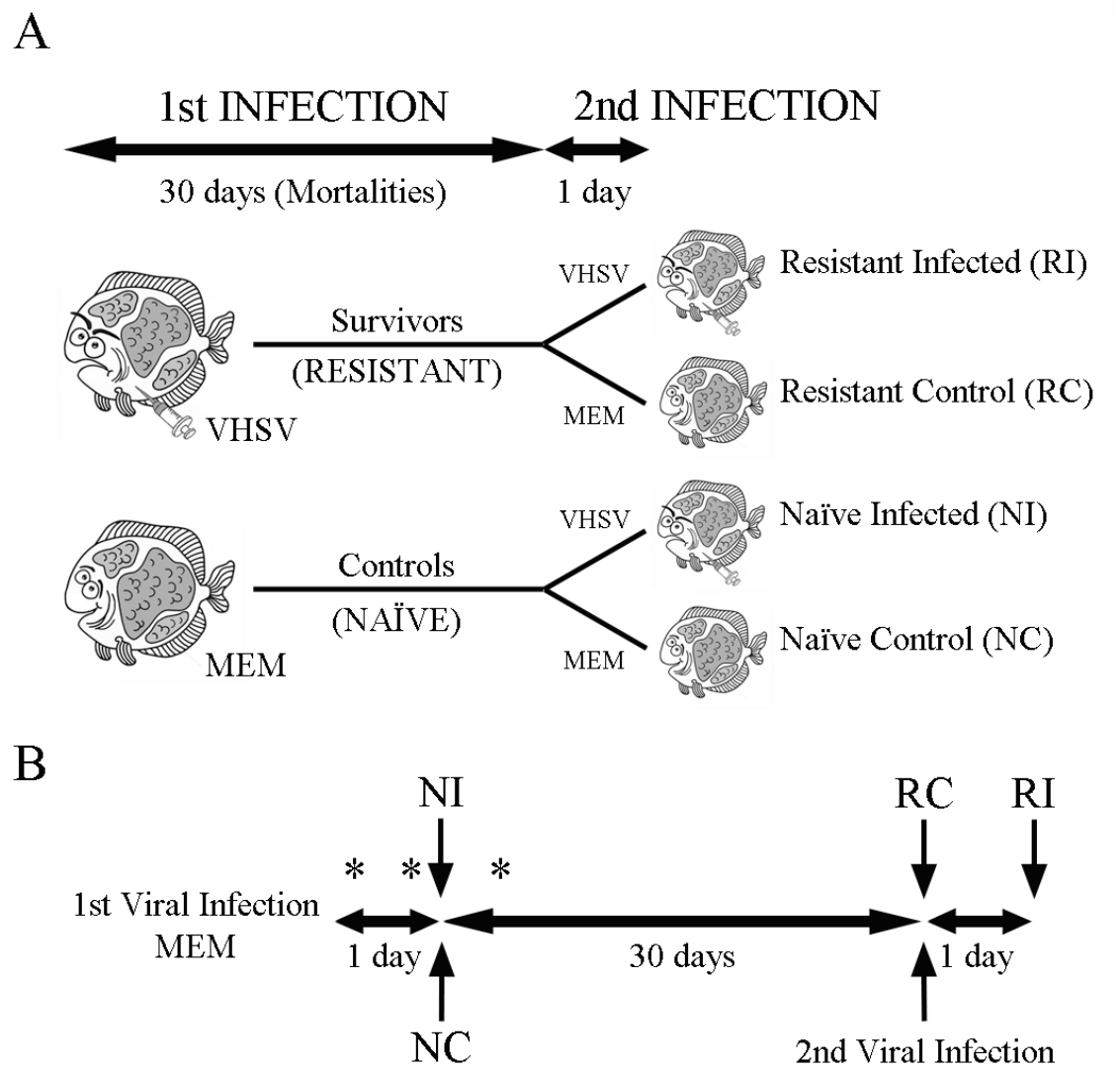

Fig. 2 Cumulative mortalities obtained in the six turbot families at 30 d p.i. with VHSV.

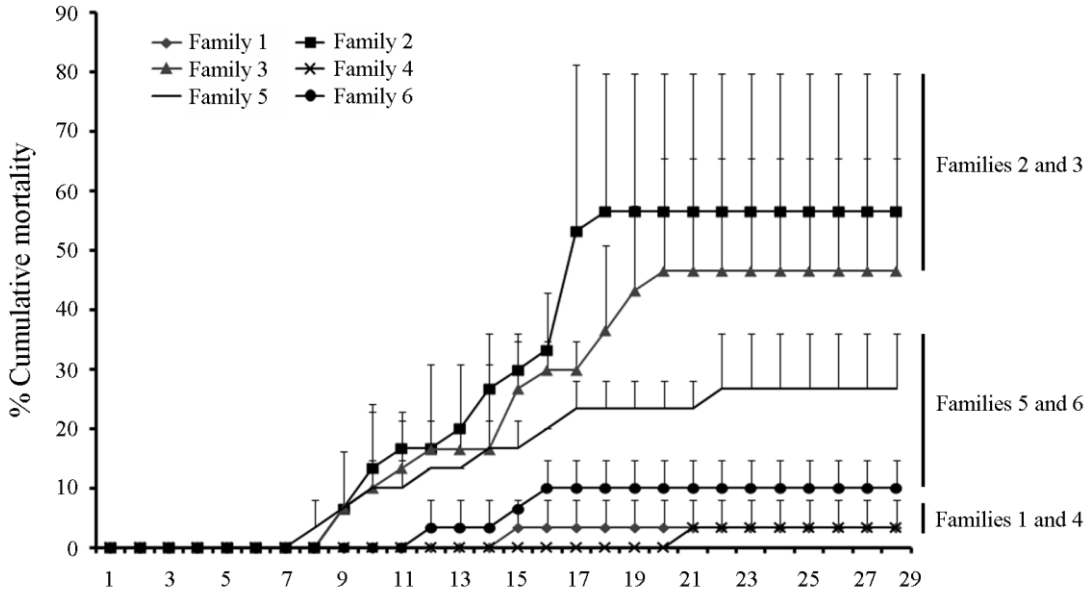

Fig. 3 Venn diagram based on three-way ANOVA analysis, showing the effects of family genetic background (factor "Family"), response at 24 h (factor "Infection") and response at 30 d (factor "Resistance") after VHSV infection on the changes observed in the gene expression profiles.

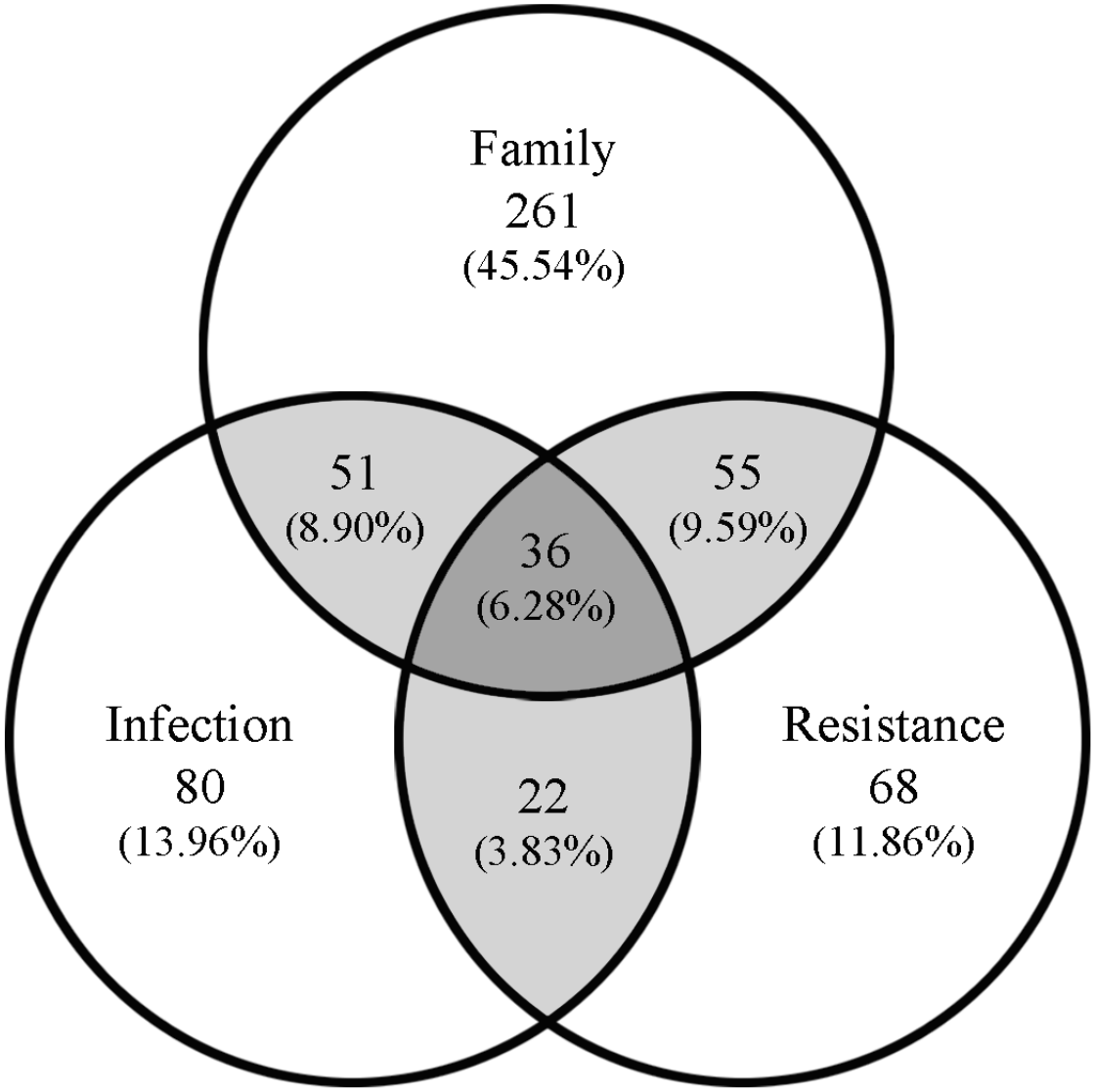

Fig. 4 Validation of microarray results by RT-qPCR. Families 1 and 2 were selected as the resistant and susceptible families, respectively. The expression levels of the selected genes (an antimicrobial peptide, the complement component C8 γ-chain, hepcidin, Mx2 and the 65-kDa warm temperature acclimation protein) were normalized to the housekeeping gene EF-1α.

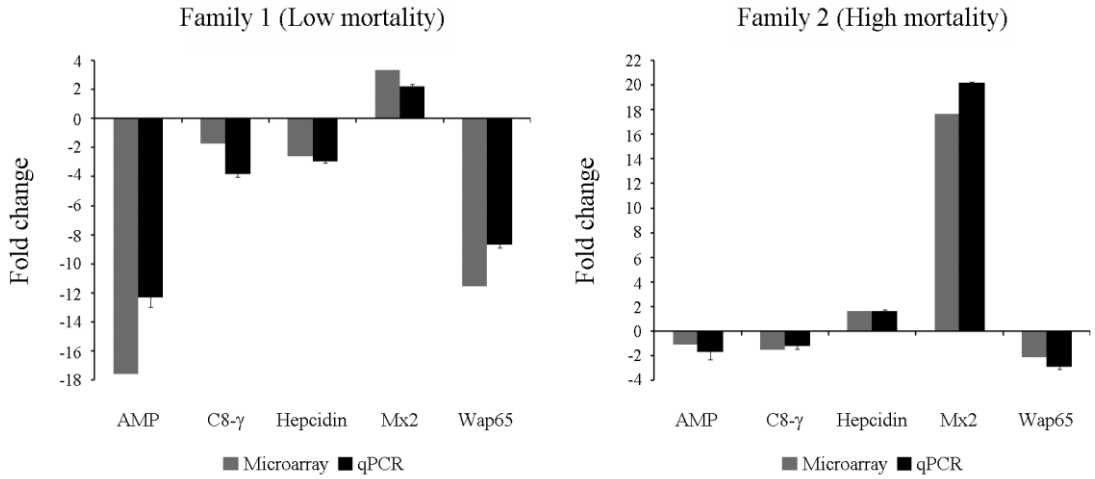

Table 1 Primers used for RT-qPCR

Gene	Primer	Sequence (5'-3')
Antimicrobial peptide	AMP-F	ATG AAG ACT CTC ACC GTT GC
	AMP-R	TTC TGT CTG TTA CTC GGC ATC
Complement component C8-γ	C8g-F	CAC AAA CAG GGC AAG ACA AC
	C8g-R	GTA GGT GGG GAA AGG GAA GA
Hepcidin	Hep-F	CGA GTC ACA TCA GGC AGA AG
	Hep-R	TGC GGG TTA TCC TCA GAA CT
Warm temperature acclimation protein 65 kDa	Wap65-F	GTT AGA CGC CAT CAC CAC TG
	Wap65-R	CGC ATG TAG ACT GGA CCT GA
Mx2	Mx2-F	CTG CCA GAT GCT TCA GGA TA
	Mx2-R	TGC AAC CAA TGT CCA AGT TC
Elongation factor 1-α	EF1a-F	GGAGGCCAGCTCAAAGATGG
	EF1a-R	ACAGTTCCAATACCGCCGATTT

Table 2 Selection of differentially expressed genes determined by three-way ANOVA analysis using $P<0.05$ and a fold-change ratio equal or greater than two. Only genes with homology to any known sequence in public databases and showing similar and consistent behavior between low- and high-mortality families (LM and HM, respectively) are presented. Fold-change ratios lower than two are not represented

	FAMILY 1 (LM)			FAMILY 2 (HM)			FAMILY 3 (HM)			FAMILY 4 (LM)		
Factor "FAMILY"	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**
UTP-glucose-1-phosphate uridylyltransferase	**-3,58**	**-2,54**	**-6,49**			**-3,29**		**-3,60**		**-9,96**	**-4,58**	**-16,77**
Secreted trypsin-like serine protease		**-2,57**	**-3,16**							**-6,30**	**-2,26**	**-12,65**
Factor "INFECTION"												
60S ribosomal protein L21	**-4,33**	**-6,56**	**-8,25**			**-3,29**				**-19,95**	**-4,20**	**-32,73**
Sex hormone-binding globulin	**-6,04**	**-6,53**	**-8,27**			**-2,25**	**2,41**			**-51,83**	**-7,93**	**-69,56**
Aldo/keto reductases, related to diketogulonate reductase		**-2,49**	**-4,02**							**-6,06**	**-3,63**	**-6,63**
SPRY domain containing protein		**-2,35**	**-2,23**							**-22,41**	**-4,78**	**-33,48**
Complement component C9	**-6,06**	**-8,88**	**-8,65**			**-2,07**				**-35,51**		**-33,70**
Antimicrobial peptide precursor	**-17,58**	**-24,10**	**-22,48**		**-4,91**	**-13,85**				**-160,35**		**-248,15**
Mx type 2	**3,34**	**2,32**	**5,03**	**17,66**		**3,24**			**2,78**			**2,07**
Factor "RESISTANCE"												
Cathepsin L.1		**4,12**	**4,17**	**2,81**							**2,50**	
Interleukin-8 precursor		**3,69**	**28,59**						**2,18**		**2,13**	**2,26**
Alpha-1-antitrypsin	**-8,72**	**-19,05**	**-14,52**	**-2,26**	**-3,23**	**-6,87**	**3,01**		**-2,03**	**-43,41**	**-6,99**	**-103,18**
Factors "FAMILY and INFECTION"												
Complement component C8 gamma		**-2,26**								**-4,69**	**-2,13**	**-4,83**
Factors "INFECTION and RESISTANCE"												
Apolipoprotein a	**-3,16**	**-2,09**	**-4,89**							**-9,63**	**-4,60**	**-16,57**
Alpha-1-microglobulin	**-5,03**	**-6,95**	**-5,46**			**-2,38**				**-33,32**	**-6,92**	**-82,90**
Retinol-binding like protein	**-3,94**	**-2,92**	**-8,66**			**-2,06**	**2,28**			**-10,54**	**-5,86**	**-26,63**
Scavenger receptor cysteine rich domain containing protein	**-2,47**	**-2,05**	**-3,05**							**-4,58**	**-3,28**	**-7,15**
Tributyltin binding protein type 1	**-11,27**	**-21,28**	**-19,47**		**-5,85**	**-15,54**	**2,12**		**-2,02**	**-76,85**	**-3,89**	**-156,95**
Fibrinogen alpha chain	**-12,41**	**-22,25**	**-19,94**	**-2,16**	**-3,49**	**-6,29**	**2,66**			**-93,22**	**-4,42**	**-115,41**
Fibrinogen gamma chain		**-24,87**	**-30,58**		**-2,88**	**-5,40**	**2,71**			**-100,87**	**-5,39**	**-178,39**
Serpin peptidase inhibitor, clade A, member 10	**-6,69**	**-8,14**	**-9,89**			**-2,07**	**2,29**			**-54,42**	**-6,09**	**-76,16**
Serine/cysteine proteinase inhibitor	**-10,08**	**-17,63**	**-14,28**	**-2,70**	**-3,25**	**-7,03**	**2,57**	**-2,19**	**-2,36**	**-60,17**	**-10,39**	**-151,30**
Warm temperature acclimation protein 65-2	**-11,53**	**-30,63**	**-20,86**	**-2,09**	**-5,22**	**-23,95**	**2,22**		**-2,01**	**-179,53**	**-4,71**	**-275,46**
MSSP multi-domain protein	**-10,68**	**-44,85**	**-16,42**	**-2,25**	**-5,22**	**-11,14**		**-2,85**	**-3,69**	**-198,20**	**-9,78**	**-567,94**
Factors "FAMILY and INFECTION and RESISTANCE"												
Hepcidin precursor	**-2,61**		**-4,26**							**-9,27**	**-2,21**	**-17,27**
Secreted trypsin-like serine protease			**-3,34**							**-2,85**	**-3,01**	**-6,51**
Neurogenic locus notch homolog protein 2-like	**-3,63**	**-5,11**	**-6,11**		**-2,09**	**-2,56**				**-41,47**	**-3,14**	**-44,63**
Virus-induced protein 5		**2,03**	**9,60**	**4,92**		**2,49**			**3,24**			**2,68**

Table 3 Differences in the gene expression profiles between high- and low-mortality families of turbot to VHSV as determined by Student's *t* test analysis ($P<0.05$). Genes represented had a fold-change ratio ≥2

	Modulated genes in high-mortality families		**Modulated genes in low-mortality families**	
	Gene name	**Fold change**	**Gene name**	**Fold change**
Naive control: basal gene expression before infection	Sodium/hydrogen exchanger isoform 3	**-2.06**	Antimicrobial peptide NK-lysin precursor	**+2.01**
	Bone morphogenetic protein 4	**-2.19**	Antimicrobial peptide precursor	**+5.95**
	Protein tyrosine phosphatase receptor type Q	**-2.56**		
	Complement component C8 gamma	**-2.63**		
	Butyrophilin subfamily 1 member A1 precursor	**-4.73**		
	Gene name	**Fold change**	**Gene name**	**Fold change**
Naïve-infected: early gene expression after 24 h p.i.	Phosducin-like protein 3	**-2.00**	TT ORF1 domain containing protein	**+2.03**
	S100-A1	**-2.07**	Selectin P	**+2.17**
	Prostasin-like	**-2.08**	Two pore segment channel 1	**+2.20**
	Factor B/C2B	**-2.22**	5-Aminolevulinate synthase	**+2.21**
	Apomucin	**-2.71**	Betaine aldehyde dehydrogenase	**+2.40**
	Myeloid-associated differentiation marker	**-6.86**	Arrestin domain-containing protein 2	**+2.61**
	Alcohol dehydrogenase class VI	**-10.77**		
	Lily-type lectin	**-21.99**		
	Gene name	**Fold change**	**Gene name**	**Fold change**
Resistant control: late gene expression after 30 days p.i.	7-Dehydrocholesterol reductase	**-2.36**	Golgi-associated plant pathogenesis-related protein 1	**+2.11**
	15-Hydroxyprostaglandin dehydrogenase	**-2.41**	Exonuc X-T domain containing protein	**+2.96**
	Cytochrome b ascorbate-dependent protein 3	**-2.43**	Cathepsin L1	**+3.09**
	Prostasin-like	**-2.70**		
	Apomucin	**-2.74**		
	DUF566 domain containing protein	**-4.52**		
	Lily-type lectin	**-4.63**		
	HRAS-like suppressor 2	**-4.69**		
	Cationic trypsin-3 precursor	**-4.86**		
	Complement component C8 gamma	**-4.94**		
	Keratin 15	**-5.56**		

Table 4 Differences in the gene expression profiles at 24 h p.i., 30 d p.i. and 24 h after a second infection with VHSV in high- and low-mortality families of turbot, identified through a Student's *t* test analysis ($P<0.05$). Genes represented had a fold-change ratio ≥2

	Modulated genes in high-mortality families		**Modulated genes in low-mortality families**	
	Gene name	**Fold change**	**Gene name**	**Fold change**
NI *vs.* NC	Factor B/C2B	**+2.66**	Fatty acid binding protein H6-isoform	**-2.50**
	O-Fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	**-2.03**	Fructose-bisphosphate aldolase A	**-5.03**
	Prosaposin	**-2.00**	Apolipoprotein A1	**-7.29**
	2-hydroxyacyl-CoA lyase 1	**-2.02**	Ca2+ dependent complex C1R/C1S subunit	**-17.60**
	TT ORF1 domain containing protein	**-2.02**	Tributyltin binding protein type 1	**-29.43**
	Heat shock protein 90	**-2.08**	Warm temperature acclimation-related 65 kDa protein	**-30.37**
	RING finger protein 10	**-2.18**	Perforin	**-32.65**
	Ca2+-binding actin-bundling protein fimbrin/plastin	**-2.19**	Complement regulatory plasma protein	**-38.21**
	Alkaline phosphatase	**-2.21**	Antimicrobial peptide precursor	**-53.09**
	Similar to protein tyrosine phosphatase, receptor Q	**-2.24**		
	Two pore segment channel 1	**-2.24**		
	Histidyl-tRNA synthetase	**-2.24**		
	SEC6-like 1	**-2.37**		
	5-Aminolevulinate synthase	**-2.40**		
	4-Aminobutyrate aminotransferase	**-2.42**		
	Carnitine octanoyltransferase	**-2.47**		
	DUF2457 multi-domain protein	**-2.64**		
	Permeases of the major facilitator superfamily	**-2.67**		
	Similar to hepatitis B virus x associated protein	**-2.71**		
	Butyrophilin subfamily 1 member A1 precursor	**-2.71**		
	Sodium potassium ATPase alpha subunit	**-2.82**		
	Solute carrier family 25 member 42	**-2.95**		
	Proline dehydrogenase (oxidase) 2	**-3.30**		
	Betaine aldehyde dehydrogenase	**-3.74**		
	Sodium/hydrogen exchanger isoform 3	**-4.00**		
	Gene name	**Fold change**	**Gene name**	**Fold change**
RC *vs.* NC	Matrix metallopeptidase 13	**+2.27**	Cytochrome P450	**-3.11**
	Apolipoprotein E1	**-2.27**	Fructose-bisphosphate aldolase A	**-3.95**
			Skeletal muscle atypical fast troponin T isoform 1	**-11.65**
			Tropomyosin 3	**-20.43**
	Gene name	**Fold change**	**Gene name**	**Fold change**
RI *vs.* NI	WD repeat domain 23	**+2.10**	Myeloid-associated differentiation marker	**+3.79**
	Two pore segment channel 1	**+2.24**	Aldo-keto reductase family 1, member A1a	**-2.06**
	Probable mitochondrial protein COQ10	**+2.73**	Chloride channel CLC-5	**-2.07**
	Proline dehydrogenase (oxidase) 2	**+2.94**	Apolipoprotein A1	**-2.22**
	Complement component C9	**-2.22**	Tributyltin binding protein type 2	**-2.25**
			Apolipoprotein A1	**-2.36**
			MID1 interacting G12-like protein	**-2.98**
			Warm temperature acclimation-related 65 kDa protein	**-3.25**

	Gene name	Fold change	Gene name	Fold change
RI *vs.* RC	Mx type 2	**+3.42**	Pancreatic carboxypeptidase A1	**-2.03**
	Virus-induced protein 5	**+3.13**	Coagulin factor II	**-2.77**
	Exonuc X-T domain containing protein	**+2.73**		

Supplementary table 1 Differentially expressed genes determined by 3-way ANOVA analysis using P<0.05 and a fold-change ratio equal or greater than two. Only genes with homology to any known sequence in public databases are presented between low- and high-mortality families (LM and HM, respectively). Fold-change ratios lower than two are not represented. The selected genes represented in table 1 are highlighted.

Factor "FAMILY"	FAMILY 1 (LM)			FAMILY 2 (HM)			FAMILY 3 (HM)			FAMILY 4 (LM)		
	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC
UTP-glucose-1-phosphate uridylyltransferase	**-3,58**	**-2,54**	**-6,49**			**-3,29**		**-3,60**		**-9,96**	**-4,58**	**-16,77**
Secreted trypsin-like serine protease		**-2,57**	**-3,16**							**-6,30**	**-2,26**	**-12,65**
Catechol-O-methyltransferase domain-containing protein 1			**2,19**									
JAK3 tyrosine kinase			**2,62**									
C1q domain containing protein												**2,02**
Cationic trypsin-3 precursor							**-2,19**		**-2,03**		**-3,78**	
MAPEG domain containing protein											**2,03**	
Apomucin									**-2,03**			
CDC42 small effector 1			**2,48**									
Cytochrome c oxidase polypeptide precursor			**-2,08**									
Novel protein similar to H.sapiens platelet glycoprotein Ib beta chain									**2,07**			
A type IV collagen										**2,07**		
Polyadenylate-binding protein 1A											**-2,08**	**-2,35**
Transforming, acidic coiled-coil containing protein 3			**2,18**									
Growth factor independent 1									**2,31**	**-2,38**		
Probable oxidoreductase			**-2,80**							**2,25**		
HRAS-like suppressor 2				**-2,07**							**-3,41**	
ADP-ribosylation factor-like protein 8B			**2,60**									
Urocanase domain containing 1			**-2,63**									

Factor "INFECTION"	FAMILY 1 (LM)			FAMILY 2 (HM)			FAMILY 3 (HM)			FAMILY 4 (LM)		
	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC
60S ribosomal protein L21	**-4,33**	**-6,56**	**-8,25**			**-3,29**				**-19,95**	**-4,20**	**-32,73**
Sex hormone-binding globulin	**-6,04**	**-6,53**	**-8,27**			**-2,25**	**2,41**			**-51,83**	**-7,93**	**-69,56**
Aldo/keto reductases, related to diketogulonate reductase		**-2,49**	**-4,02**							**-6,06**	**-3,63**	**-6,63**
SPRY domain containing protein		**-2,35**	**-2,23**							**-22,41**	**-4,78**	**-33,48**
Complement component C9	**-6,06**	**-8,88**	**-8,65**			**-2,07**				**-35,51**		**-33,70**

Antimicrobial peptide precursor	**-17,58**	**-24,10**	**-22,48**		**-4,91**	**-13,85**				**-160,35**		**-248,15**
Mx type 2	**3,34**	**2,32**	**5,03**	**17,66**		**3,24**			**2,78**			**2,07**
Cytochrome P450	**-2,03**		**-2,84**						**-2,04**			
Ca2+-dependent lipid-binding protein, contains C2 domain				**-3,22**				**-2,24**				**-2,07**
Enolase 3-1				**-2,44**				**-2,18**			**-2,30**	**-2,45**
Galactoside-binding soluble lectin 9			**4,85**	**2,15**								
Chymotrypsinogen 1										**-74,41**	**-8,71**	**-111,92**
Ifi-6-16 domain containing protein				**2,32**								
Serine-pyruvate aminotransferase/archaeal aspartate aminotransferase			**-2,19**									

Factor "RESISTANCE"	**FAMILY 1 (LM)**			**FAMILY 2 (HM)**			**FAMILY 3 (HM)**			**FAMILY 4 (LM)**		
	NI/NC	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**
Cathepsin L.1		**4,12**	**4,17**	**2,81**							**2,50**	
Interleukin-8 precursor		**3,69**	**28,59**						**2,18**		**2,13**	**2,26**
Alpha-1-antitrypsin	**-8,72**	**-19,05**	**-14,52**	**-2,26**	**-3,23**	**-6,87**	**3,01**		**-2,03**	**-43,41**	**-6,99**	**-103,18**
Complement component C3-1					**-3,56**				**-2,31**	**-6,10**	**-3,18**	**-12,97**
Prosaposin											**2,62**	**2,57**
BRCA1-associated RING domain protein 1			**2,17**									
V-set domain containing protein		**-2,18**									**2,65**	**3,43**
Hypothetical LOC796684		**-2,54**	**-2,45**									
hATC domain containing protein			**-2,28**									
N-acetylgalactosamine kinase		**2,29**	**4,72**									

Factors "FAMILY and INFECTION"	**FAMILY 1 (LM)**			**FAMILY 2 (HM)**			**FAMILY 3 (HM)**			**FAMILY 4 (LM)**		
	NI/NC	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**	**NI/NC**	**RC/NC**	**RI/NC**
Complement component C8 gamma		**-2,26**								**-4,69**	**-2,13**	**-4,83**
Glyco-transf 64 multi-domain protein				**-2,44**								
Novel protein				**-2,55**		**-2,35**			**2,33**			
CREB binding protein				**-2,82**								
Exo-endo phos domain containing protein				**-2,00**						**-8,27**	**-3,49**	**-10,29**
LSU rRNA				**-2,25**		**-2,15**						
Myosin heavy chain				**-2,37**		**-2,25**						

Factors "FAMILY and RESISTANCE"	FAMILY 1 (LM)			FAMILY 2 (HM)			FAMILY 3 (HM)			FAMILY 4 (LM)		
	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC
Transglutaminase 2, C polypeptide	**12,03**			**16,38**		**-2,34**	**4,72**	**2,62**			**2,61**	**-4,54**
Actin-related protein 2/3 complex subunit 3			**2,39**									

Factors "INFECTION and RESISTANCE"	FAMILY 1 (LM)			FAMILY 2 (HM)			FAMILY 3 (HM)			FAMILY 4 (LM)		
	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC
Apolipoprotein a	**-3,16**	**-2,09**	**-4,89**							**-9,63**	**-4,60**	**-16,57**
Alpha-1-microglobulin	**-5,03**	**-6,95**	**-5,46**			**-2,38**				**-33,32**	**-6,92**	**-82,90**
Retinol-binding like protein	**-3,94**	**-2,92**	**-8,66**			**-2,06**	**2,28**			**-10,54**	**-5,86**	**-26,63**
Scavenger receptor cysteine rich domain containing protein, group B -like	**-2,47**	**-2,05**	**-3,05**							**-4,58**	**-3,28**	**-7,15**
Tributyltin binding protein type 1	**-11,27**	**-21,28**	**-19,47**		**-5,85**	**-15,54**	**2,12**		**-2,02**	**-76,85**	**-3,89**	**-156,95**
Fibrinogen alpha chain	**-12,41**	**-22,25**	**-19,94**	**-2,16**	**-3,49**	**-6,29**	**2,66**			**-93,22**	**-4,42**	**-115,41**
Fibrinogen, gamma polypeptide		**-24,87**	**-30,58**		**-2,88**	**-5,40**	**2,71**			**-100,87**	**-5,39**	**-178,39**
Serpin peptidase inhibitor, clade A (antitrypsin), member 10	**-6,69**	**-8,14**	**-9,89**			**-2,07**	**2,29**			**-54,42**	**-6,09**	**-76,16**
Serine/cysteine proteinase inhibitor	**-10,08**	**-17,63**	**-14,28**	**-2,70**	**-3,25**	**-7,03**	**2,57**	**-2,19**	**-2,36**	**-60,17**	**-10,39**	**-151,30**
Warm temperature acclimation protein 65-2	**-11,53**	**-30,63**	**-20,86**	**-2,09**	**-5,22**	**-23,95**	**2,22**		**-2,01**	**-179,53**	**-4,71**	**-275,46**
MSSP multi-domain protein	**-10,68**	**-44,85**	**-16,42**	**-2,25**	**-5,22**	**-11,14**		**-2,85**	**-3,69**	**-198,20**	**-9,78**	**-567,94**
Galectin-9			**5,60**	**2,30**								
Placental growth factor-like	**-2,40**		**-3,44**	**-2,11**	**-2,02**	**-4,16**					**-2,25**	

Factors "FAMILY and INFECTION and RESISTANCE"	FAMILY 1 (LM)			FAMILY 2 (HM)			FAMILY 3 (HM)			FAMILY 4 (LM)		
	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC	NI/NC	RC/NC	RI/NC

Hepcidin precursor	**-2,61**		**-4,26**							**-9,27**	**-2,21**	**-17,27**
Secreted trypsin-like serine protease			**-3,34**							**-2,85**	**-3,01**	**-6,51**
Neurogenic locus notch homolog protein 2-like	**-3,63**	**-5,11**	**-6,11**		**-2,09**	**-2,56**				**-41,47**	**-3,14**	**-44,63**
Virus-induced protein 5		**2,03**	**9,60**	**4,92**		**2,49**			**3,24**			**2,68**
Fructose-bisphosphate aldolase			**-2,36**									
Glyceraldehyde 3-phosphate dehydrogenase			**-2,56**									
V-set domain containing protein												**2,01**
Cytochrome P450			**-2,56**		,						**-2,18**	
LSU rRNA			**-2,66**									**-2,27**
Ribonucleoside-diphosphate reductase subunit M2											**-2,13**	
RNA-binding proteins (RRM domain)												**-2,16**
Tryptophan 2,3-dioxygenase						**-2,07**			**-2,15**		**-2,33**	**-4,95**