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Philosophiae Doctor (PhD) Thesis 2012:06

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Cardiomyopathy syndrome (CMS) in Atlantic salmon, *Salmo salar* L.: functional genomics studies of host-pathogen responses and disease markers

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Ås, December 2011 Gerrit Timmerhaus

Table of Contents

1 Aims of the study

The overall aim of the work presented in this thesis was to increase general knowledge of host-virus responses in Atlantic salmon, and in particular responses to the causative virus of cardiomyopathy syndrome (CMS). The focus lay on characterization of immune responses of infected fish which was examined by gene expression analysis.

The first aim was to establish a microarray platform, which was used in the further experiments of the project. Sequences and annotation of the genes on the microarray were collected in a central database and provided an important resource for this thesis. (**Paper I**)

A CMS challenge trial at the beginning of the study provided most of the biological material for the following experiments. (**Paper II-IV**)

The biological material from the challenge trial was used to examine the immune responses in CMS positive fish over a period of twelve weeks. The regulation of the key immune pathways was examined over the first ten weeks of the challenge in fish with most severe pathology in the heart. The focus was on heart tissue, but responses in other relevant organs were examined as well. (**Paper II**)

Severe pathology associated with CMS was not observed in all of the challenged fish. The aim of **Paper III** was to compare individual responses to infection and disease by examination of transcriptional differences between fish with and without such pathology, in order to identify potential protective and pathological mechanisms.

In order to identify genes that are commonly activated in the early response to different viral diseases of Atlantic salmon, a comparative genomic study of early antiviral genes in fish challenged with CMS, heart and skeletal muscle inflammation (HSMI), infectious salmon anaemia (ISA), infectious pancreatic necrosis (IPN) and the synthetic viral mimic of double-stranded RNA poly I:C was conducted. (**Paper IV**)

2 List of papers

I. Krasnov A, Timmerhaus G, Afanasyev S, Jørgensen SM: **Development and assessment of oligonucleotide microarrays for Atlantic salmon (*Salmo salar* L.).** *Comp Biochem Physiol Part D Genomics Proteomics* 2011, **6:**31-38.

II. Timmerhaus G, Krasnov A, Nilsen P, Alarcon M, Afanasyev S, Rode M, Takle H, Jørgensen SM: **Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon.** *BMC Genomics* 2011, **12:**459.

III. Timmerhaus G, Krasnov A, Takle H, Afanasyev S, Nilsen P, Rode M, Jørgensen SM. **Comparison of Atlantic salmon individuals with different outcomes of cardiomyopathy syndrome (CMS).** *Manuscript submitted to BMC Genomics*

IV. Krasnov A, Timmerhaus G, Schiøtz BL, Torgersen J, Afanasyev S, Iliev D, Jørgensen J, Takle H, Jørgensen SM: **Genomic survey of early responses to viruses in Atlantic salmon, *Salmo salar* L.** *Mol Immunol* 2011, **49:**163-174.

3 Abbreviations

aa	Amino acid
AB	Antibody
AG	Antigen
APC	Antigen presenting cell
bp	Base pairs
cDNA	Complementary DNA
CMC	cell mediated cytotoxicity
CMS	Cardiomyopathy syndrome
DC	Dendritic cell
dsRNA	Double stranded RNA
GLV	*Giardia lamblia* virus
GO	Gene ontology
HR	High responders (fish with high pathology)
HSMI	Heart and skeletal muscle inflammation
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IFN	Interferon
IHN	Infectious haematopoietic necrosis
IMNV	Penaeid shrimp infectious myonecrosis virus
IPN	Infectious pancreatic necrosis
IPNV	Infectious pancreatic necrosis virus
ISA	Infectious salmon anaemia
ISAV	Infectious salmon anaemia virus
LPS	Lipopolysaccharides
LR	Low responders (fish with low pathology)
MA	Microarray
MHC	Major histocompatibility complex
NK	Natural killer (cell)
ORF	Open reading frame
PBL	Peripheral blood leucocytes
PD	Pancreas disease
PMCV	Piscine myocarditis virus
poly I:C	polyinosinic polycytidylic acid

PRR	Pattern recognition receptor
PRV	Piscine reovirus
qPCR	Quantitative real-time RT-PCR
RBC	Red blood cell
RdRp	RNA dependent RNA polymerase
SIQ	Salmon Immunity and Quality (microarray platform)
SPDV	Salmon pancreas disease virus
ssRNA	Single stranded RNA
STARS	Salmon and Trout Annotated Reference Sequences
t	Metric tonne
T_C	Cytotoxic T cell
T_H	T helper cell
TLR	Toll-like receptor
VRG	Virus responsive gene
VHS	Viral hemorrhagic septicemia
VHSV	Viral hemorrhagic septicemia virus
wpi	Weeks post infection

4 Summary

Since the first description of CMS in the mid 1980s, little research has been conducted to approach an understanding of host-pathogen responses in affected Atlantic salmon. The research presented in this thesis aimed to address this topic based on the recent success to reproduce CMS in experimental infection trials.

Transcriptome analysis based on microarrays represents a powerful method for characterization of host responses to complex and unknown traits like infectious diseases. **Paper I** describes the development of an oligonucleotide microarray with 20,000 unique probes to genes of Atlantic salmon. Pilot experiments assessed performance of this platform. In parallel, a bioinformatics system was constructed. The raw data from microarray experiments are processed and stored in a custom-build database. This database combines the possibilities of annotating genes automatically and manually as well as comparing different experiments to each other and was used in **Paper II-IV**.

At the beginning of the studies reported in this thesis, the etiology of CMS was still unknown. We conducted a challenge trial by injecting fish with material from cell culture that was expected to contain the causative virus. The challenged fish developed myocardial changes typical for CMS, which were diagnosed by histopathological examination. The biological material of this trial provided the basis of the **Papers II** and **III** as well as parts of **Paper I** and **IV**. Before **Papers II-IV** were published, the putative CMS causative virus (piscine myocarditis virus, PMCV) was discovered and sequenced, and viral loads could be measured with real-time RT-PCR.

Paper II describes the regulation of immunological pathways and the development of pathology and viral load in CMS challenged fish during 10 weeks of infection. Myocardial pathology associated with CMS was significant from 6 wpi and peaked at 8-9 wpi followed by a recovery. Viral RNA was detected in all organs from 4 wpi suggesting a broad tissue tropism. High correlation between viral load and cardiac histopathology score suggested that cytopathic effect of infection was a major determinant of the myocardial changes. Strong and systemic induction of antiviral and IFN-dependent genes from 2 wpi that leveled off during infection, was followed by a biphasic activation of pathways for B cells and MHC antigen presentation, both peaking at clinical pathology. This was preceded by a distinct cardiac activation of complement at 6 wpi, suggesting a complement-dependent activation of humoral AB-responses. Peak of cardiac pathology and viral load coincided with cardiac-specific upregulation of T cell response genes and splenic induction of complement genes. Preceding the reduction in viral load and pathology, these responses were probably important for viral clearance and recovery.

While **Paper II** focused on the immune response of pooled groups of fish developing strongest pathology and infection, **Paper III** describes a study of individual differences between fish with (so called high responder fish, HR) and without (low responder fish, LR) pathological changes in heart tissues. Global gene expression in HR and LR hearts during infection was compared, in order to characterize differences in the host response and to identify genes with expression patterns that could explain or predict the different outcomes of disease. Virus-responsive genes involved in early antiviral and innate immune responses were upregulated equally in LR and HR at the first stage (2-4 wpi), reflecting the initial increase in virus replication. Repression of heart muscle development was identified by gene ontology enrichment analyses, indicating the early onset of pathology. By six weeks both responder groups had comparable viral load, while increased pathology was observed in HR fish. This was reflected by induced expression of genes implicated in apoptosis and cell death mechanisms, presumably controlling lymphocyte regulation and survival. At the late stage of infection, increased pathology and viral load in HR was accompanied by a broad activation of genes involved in adaptive immunity and particularly T cell responses, probably reflecting the increased infiltration and homing of virus-specific T cells to the infected heart. In contrast, LR fish showed viral clearance and recovery at the late stage, which was associated with activation of genes involved in energy metabolism while adaptive immunity genes were not expressed. These studies (**Paper II** and **III**) provide the first characterization of the temporal and spatial regulation of host-virus responses during CMS, as well as correlates and markers of pathology and protection.

Paper IV reports the identification and characterization of early virus responsive genes (VRGs) that are commonly activated in different viral diseases. CMS-related gene expression at early time points was represented by data published in **Paper II**. The expression data from other studies of viral diseases (heart and skeletal muscle inflammation (HSMI), infectious salmon anaemia (ISA) and infectious pancreatic necrosis (IPN)) and poly I:C treatment were from other experiments stored in the internal database. A total of 117 VRGs were identified, characterized by a rapid induced expression, dependence on the virus level and low tissue specificity. Expression of these genes strongly correlated to expression of IFNα. Some of the highest ranked genes of this study have not been described in the context of virus or immune responses in fish before. According to phylogenetic analyses, a large part of the VRGs has undergone rapid evolution and sequence divergence.

5 General introduction

5.1 Atlantic salmon aquaculture

Aquaculture of Atlantic salmon (*Salmo salar* L.) was established in Norway in the 1970s and has expanded since. The total production of fish in aquaculture increased from 367,000 t in 1997 to 1,006,000 t in 2010 (Figure 1A) [1]. Atlantic salmon has been the most produced species with 928,000 t in 2010 (Figure 1A) and this makes Norway the biggest producer of Atlantic salmon in the world with a share of ca. 50% of the worldwide production [2]. The economic value of the production was 28.1 billion NOK in 2010 (Figure 1Figure 1B). Compared to 6.8 billion NOK in 1997, it increased more than four times within 14 years [1]. The highest increase was observed between the years 2008 to 2010: as a result of raising market prices and increasing production, the market value of Norwegian salmon raised by 85%. While the traditional fishery stagnates during many years, fish farming has been expanding and with decreasing fish resources in the oceans it will probably gain even more importance in the future [3, 4].

While the amount of produced Atlantic salmon raised every year, the mortality rate kept remarkably stable since 1998 [1] (Figure 2A). The rate fluctuated between 11.5% in 2000 and 16.6% in 2003, with an annual average of 14%. Diseases remained the most serious threat for farmed Atlantic salmon. Ca. 90% of the total production loss in 2009 was related to diseases [5]. Besides different bacterial diseases and the ectoparasitic salmon louse (*Lepeophtheirus salmonis*), diseases caused by viruses are the major reason for losses in the production process [6]. The most important virus diseases are infectious pancreatic necrosis (IPN), pancreas disease (PD), infectious salmon anaemia (ISA), heart and skeletal muscle inflammation (HSMI), and cardiomyopathy syndrome (CMS). Official statistics of affected farm sites in Norway was calculated by the Norwegian Veterinary Institute for ISA and PD outbreaks since 1998 [6]. In 2002 IPN was added and since 2005 HSMI. Statistics about CMS was included since 2007. The development of standardized diagnosis methods and the systematical examinations of cultured fish, led to a better overview of the health state of Norwegian fish farms. The total number of affected farm sites was relatively stable between 2007 and 2010 (Figure 2B), which indicates that viral diseases are a persistent problem for the fish industry.

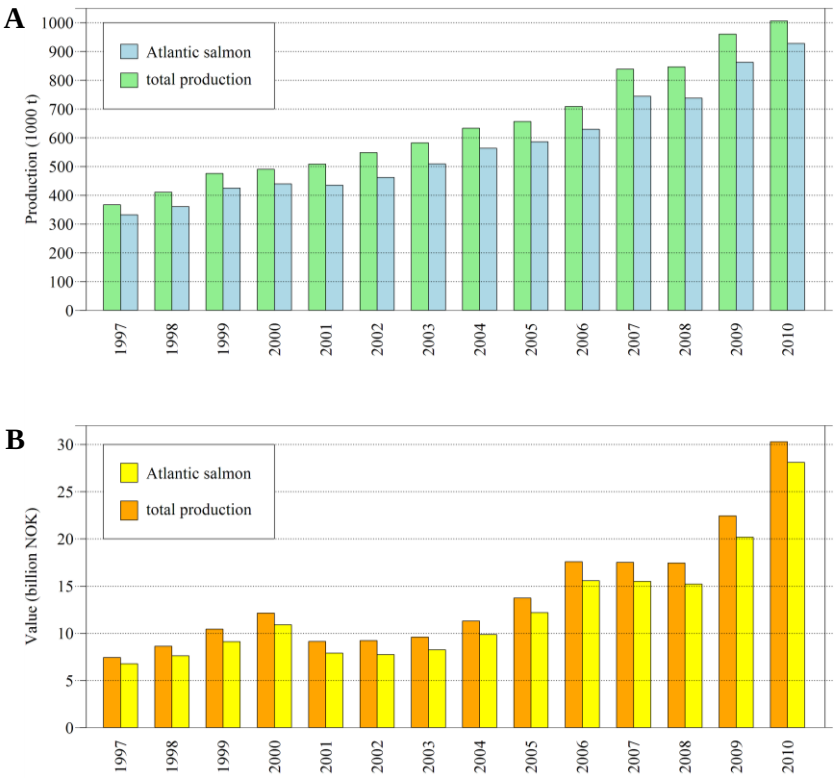

Figure 1: Production of fish in Norwegian aquacultures. The total amounts (slaughter weight) of all fish species and the amounts of Atlantic salmon are shown in figure A. The market value of the production is shown in figure B [1].

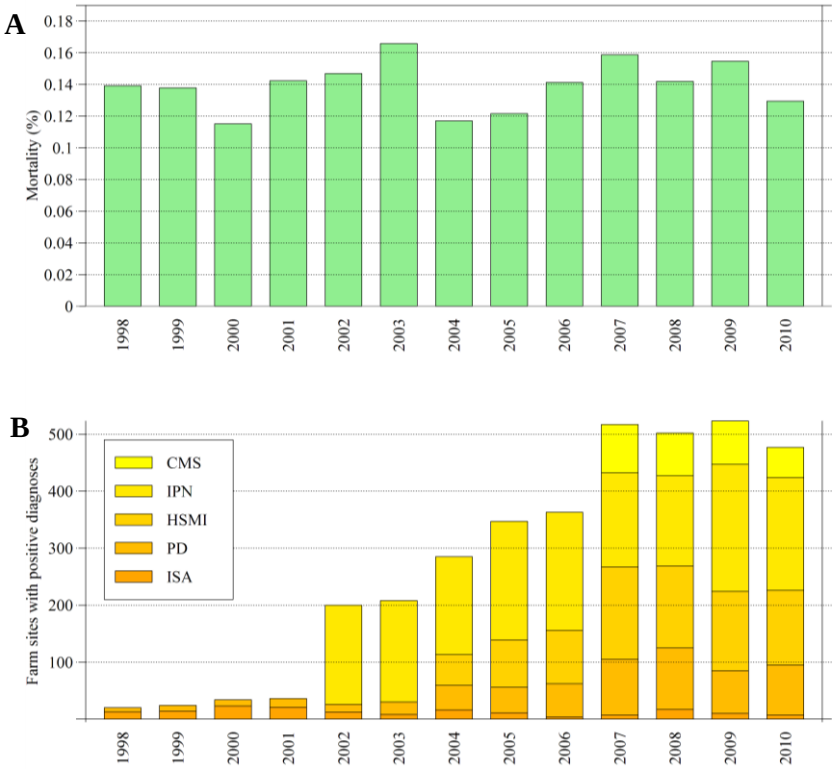

Figure 2: Mortality rate of all Atlantic salmon in Norwegian aquacultures (A) [1] and number of positively diagnosed farm sites for different virus diseases (B) [6].

5.2 Viral diseases

5.2.1 Overview of diseases

Viruses are small infectious particles, consisting of two main components: the genetic material and the outer coat, a hull of proteins. Some types of viruses possess an additional layer of lipids around the protein coat called envelope. The complete viral particles are also called virions. Viruses are classified according to the type of the genetic material they carry, which is either DNA or RNA. The most important pathogenic viruses of Atlantic salmon contain RNA. The viruses causing HSMI, IPN and CMS are double stranded RNA (dsRNA) viruses, while the viruses causing ISA and PD have single stranded RNA (ssRNA) genomes. All viruses need living cells for replication and most viruses infect specific cell types by binding to distinct cell surface proteins. After binding, the virus penetrates the cell membrane and enters the cell. The next step is the uncoating, which releases the viral nucleic acids in the host cell. In case of RNA viruses, the following replication of the virus genome occurs usually in the cytoplasm, but the underlying mechanism differs if the genome of the virus is in sense or anti-sense form. RNA virus genomes include a gene for an RNA dependent RNA polymerase (RdRp), which is needed for replication of the viral genome. Other genes code for coating proteins and, in most cases, for suppressors of defense mechanisms of the host cell. Virus genes are translated by the host cells' ribosomes. Most viruses assemble in complete virions inside the host cell and will finally be released by lysis of the cell or, in case of enveloped viruses, by budding.

The virus causing ISA (ISAV) is an antisense (-)ssRNA virus, thus, its genome must be transcribed to sense RNA before its genes can be translated [7]. For that purpose, the virions include functional RNA polymerases. After uncoating, a single RNA molecule can be transcribed rapidly several times to the sense form, providing many templates for viral proteins. ISAV infects red blood cells (RBCs), which unlike to mammals contain DNA and the replication machinery, and causes severe anemia. The mortality rate in an affected farm can reach 100%. Post mortem examinations of the blood usually show high amounts of lysed RBCs. The virions can survive in sea water and can be transmitted from fish to fish by their secretions. ISA has been a major reason for the fish farming crisis in Chile, therefore the production of Atlantic salmon decreased from 400,000 t in 2005 to 100,000 in 2010 [8].

In contrast to ISAV, the PD virus (salmon pancreas disease virus (SPDV)) is a sense (+)ssRNA virus of the Alphavirus family. At least two different subtypes were identified in Ireland, Scotland and Norway [9] but the pathology is similar. Once released, (+)ssRNA genome can directly be read by the ribosomes of the host cell as an mRNA molecule. The first translated gene codes for an RdRp, which replicates the viral genome subsequently. A SPDV infection in salmon can lead to acinar pancreatic necrosis and fibrosis and a range of myopathies (cardiac, skeletal, oesophageal) [10, 11]. The myopathy of the heart tissue can be similar to other diseases as HSMI and CMS. The mortality rates at

farms with a PD outbreak vary significantly between less than 5% and 40% and many fish recover after several months. However the recovered fish often become thin and dark, which reduces the market value. PD is one of the major commercially important diseases in European fish farms.

The viruses causing HSMI and IPN both contain dsRNA genomes and belong to the Reo- and Birnavirus families respectively. dsRNA virions carry their genomes usually in two or more segments. The IPN virus (IPNV) consists of two segments, which contain genes for different proteins, including the RdRp in gene VP1 and the most important structural and immunogenic protein VP2 [12]. The pathology develops mainly in young fish, within the first 20 weeks in sea water and can cause high losses. Older fish can carry the virus without developing symptoms. In Norway, 40–70% of all fish farming sites in seawater experienced IPN outbreaks during the years 1994– 2004. The outbreaks resulted in an average accumulated site mortality of 10–20%. However, the mortalities vary considerably, from negligible to almost 100% [12]. An IPN infection is characterized by necrosis of pancreatic tissues and thick mucus in the intestines.

Recently the piscine reovirus (PRV) was identified as a possible causative agent for HSMI [13]. Ten segments of the dsRNA genome were sequenced and PRV was assigned to the *Reoviridae* family, equally distant from the orthoreovirus and aquareovirus subfamilies. HSMI appears 5 to 9 months after transfer from fresh water to ocean pens [14] and the pathology is characterized by myocardial necrosis, epi-, endo- and myocarditis, myositis and necrosis of red skeletal muscle. The mortality reaches up to 20% [15]. The pathology of the heart of fish with HSMI is in overall similar to fish with CMS however with more severe inflammation of the epicardium.

5.2.2 Cardiomyopathy syndrome

5.2.2.1 History of CMS

CMS was first diagnosed in Norway in 1985 [16] but the etiology remained unknown. Fifteen years later, approximately 100 fish farms were found to be affected by CMS, of a total of 600 fish farms in Norway [16, 17]. In December 1997 CMS was diagnosed for the first time in Scotland [18]. In this first Scottish outbreak, mortalities of 60% were observed among fish weighting 5-7kg. After the year 2000, first cases have also been found in wild Atlantic salmon and Chinook salmon (*Oncorhynchus tschawytscha)* in Canada [19, 20]. Official statistics of CMS outbreaks in Norway have been available since 2006.

A viral origin of CMS was proposed already after its first discovery. Intranuclear eosinophilic inclusion bodies were observed in unaffected myocardial cells situated adjacent to degenerated cells, which indicated a viral infection [16]. However, viral particles were not found by transmission

electron microscope examinations in two independent studies [17, 18]. Isolation of viral particles from heart, spleen or kidney inoculums in cell culture failed as well [18]. In one study from 1997, viral particles were observed in hearts with CMS and described as nodavirus-like [21]. However, they could not be confirmed as the causative agent. Later studies with immunohistochemical methods with antisera against *Nodaviridae* on cardiac tissue with CMS have not given positive results [18]. First experimental transmissions of CMS to healthy fish was performed before the year 2000 [22, 23], however the results were not published in a scientific form. In addition, CMS was proposed as a late state of HSMI in 2006 [24] and connections to previous outbreaks of IPN at the same fish farm were considered [20]. The latter observation was probably a result of the high prevalence of IPNV in Norwegian fish farms [25]. Besides of a viral origin, autoimmune reactions have been proposed to be the reason for CMS. The observed connection to previous IPN outbreaks was discussed as a late autoimmune reaction subsequently to the IPNV infection. In humans, several viral infections are known to be a possible cause for myocarditis [26, 27]. However, the viral origin of CMS remained under question for a long time.

The first successful experimental transmission of CMS was published in 2009 [28]. In this study, heart homogenate from a field outbreak was used to infect healthy fish. The infected fish developed CMS specific pathologies in the atrium within 6 weeks after the infection and after 12 weeks, first pathologies were found in the spongious layer of the ventricle. The successful transmissibility of CMS supported the hypothetical viral origin.

Finally in the year 2011, a novel virus of the *Totiviridae* family was successfully identified from fish with CMS. This virus was named piscine myocarditis virus (PMCV) and is the most likely causative agent [29].

5.2.2.2 Pathology

The diagnosis of CMS is based on histopathological changes of the Atlantic salmon heart (Figure 3). Early lesions are characterized by a multifocal pattern of the affected tissue of the atrium, clearly separated by healthy tissue (Figure 4C). Multifocal lesions gradually develop into diffuse and extensive lesions (Figure 4D, E). In later stages, affected fish display extensive inflammation and severe myocardial damage, leaving almost no intact cells in the atrium and spongy layer of the ventricle (Figure 4F). Affected myocardial cells show loss of striation, eosinophilia and lysis of contractile fibers. Hypertrophic nuclei and trabecular fibrosis are also observed, and are thought to represent compensatory and regenerative processes [17]. The epicardium of the heart is commonly not affected. Inflammatory infiltrates consist of mononuclear cells, probably lymphocytes and macrophages. The compact layer of the ventricle is usually less affected, and always occurs later than changes in the spongious layer [17].

Clinical signs are very seldom observed and the sick fish may die suddenly due to tamponade or rupture of the atrium or *sinus venosus* (Figure 5). Farmed fish is usually affected between 12 and 18 month after transfer to sea cages. Thus, CMS mostly affects large fish a few months before reaching slaughter weight. In most cases, apparently healthy fish die without any clinical signs [16, 17]. Ultrasound examination and echocardiography of tranquilized fish showed that the atrium is dilated and the cardiac ventricle is compressed in terminal stages of CMS. Pericardial fluid may also be observed by these techniques, as well as the presence of pericardial clots [30]. Other symptoms like skin haemorrhage, raised scales and oedema have also been reported in some cases in connection to CMS [18, 31]. At necropsy, atrial thrombosis, ascetic fluid, fibrinous peritonitis and blood clots on the liver and heart are typical findings [17, 18, 31]. Experimentally infected fish also develop anaemia as evidenced by a significant reduction in hematocrit levels (unpublished data). Other organs may show changes such as congestion of the spleen and gills, possibly due to circulatory failure [16]. However, only the histopathological examination of heart tissue is sufficient for diagnosis of CMS. Many of the other symptoms are also - and usually more significantly - associated with other virus diseases. Especially symptoms of fish with HSMI or PD may be very similar to symptoms of fish with CMS. However, in HSMI and PD, all layers of the ventricle of the heart, including the *epicardium*, show histopathological changes.

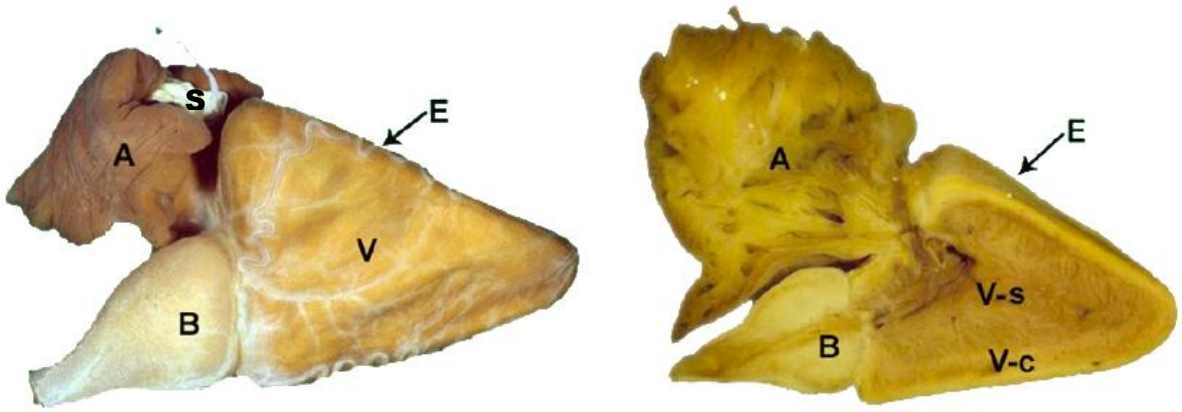

Figure 3: The Atlantic salmon heart. Venous blood flows from the body into the heart through the *sinus venosus* (S) into the atrium (A). From there, it flows into the ventricle (V) and leaves the heart through the *bulbus ateriosous* (B) to the gills. The picture on the right shows a longitudinal cut of the heart. The outer layer of the ventricle, the e*picardium*, is marked with "E", while the compact layer is marked with "V-c". The inner layer of the heart, the spongious layer, is marked with "V-s". Photo: Trygve Poppe

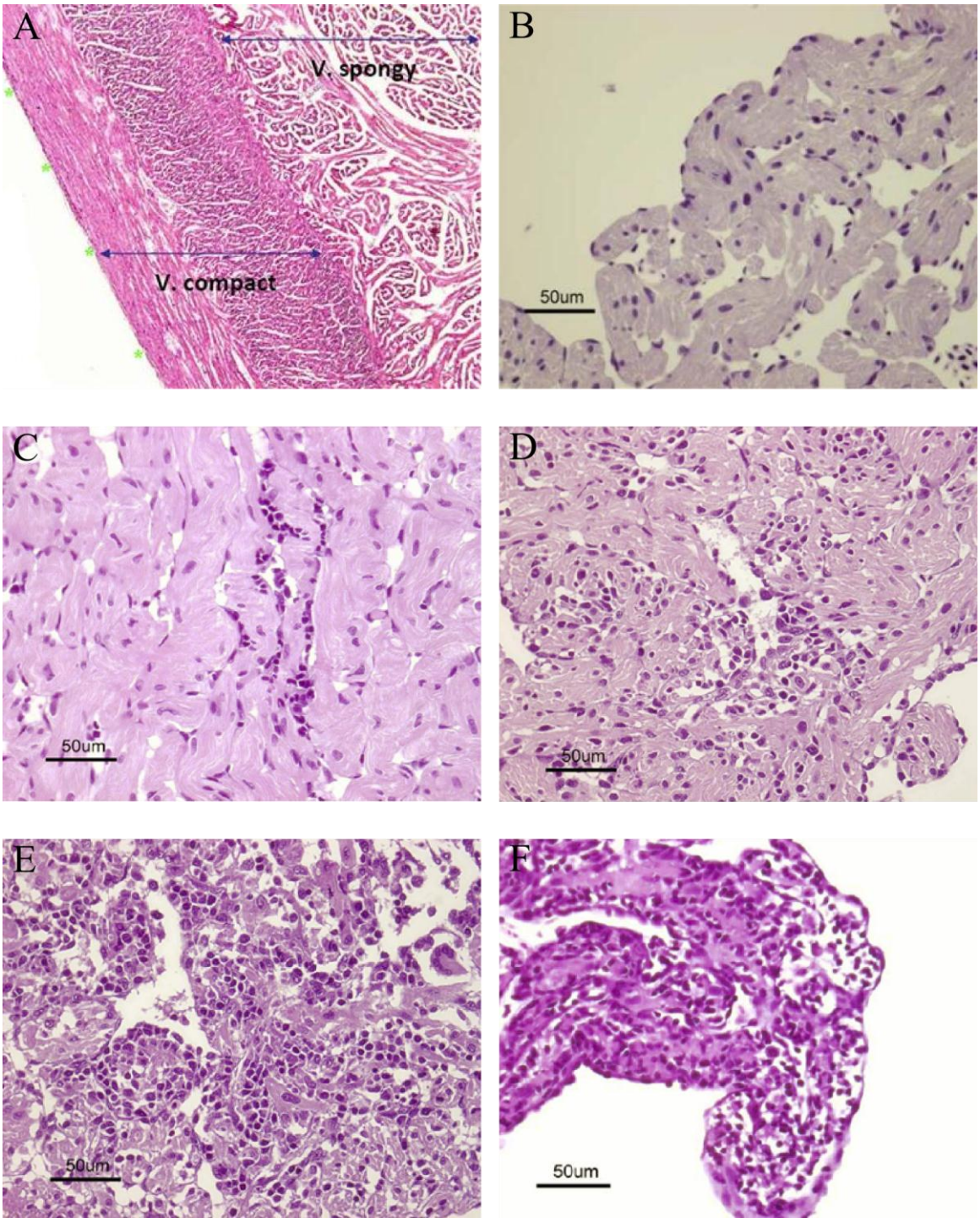

Figure 4: Histological pictures of the Atlantic salmon heart. The three layers of the ventricle are shown in picture A. The spongy and compact layers are labeled in the picture, while the *epicardium* is marked by green stars. Pictures B-F show tissue of the atrium in healthy and pathological stages. The tissue in picture B shows no pathological findings. In picture C, focal lesions with increased number of leukocytes are visible. Several distinct lesions and a moderate increase in number of leukocytes is shown in picture D. Picture E shows inflamed tissue with confluent lesions and moderate to severe increase in number of leukocytes. Massive leukocyte infiltration and severe confluent lesions comprising most of the tissue are shown in picture F. Photos: Trygve Poppe/Marta Alarcon

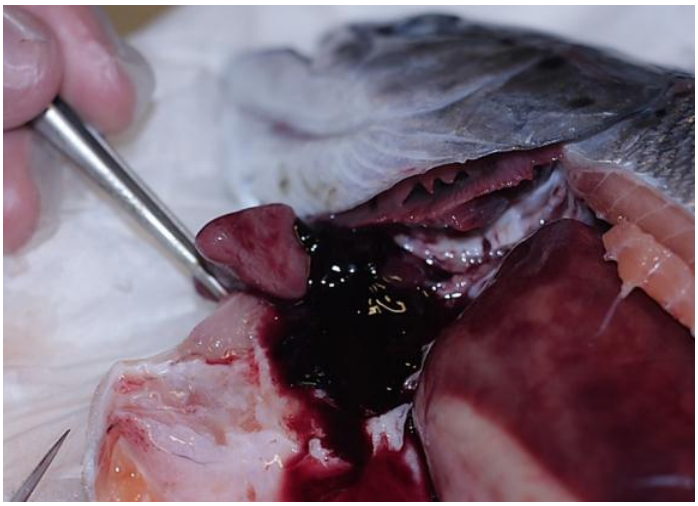

Figure 5: Picture of a *post-mortem* examination of an Atlantic salmon with CMS. The ventricle of the heart is hold up by the forceps and the ruptured atrium and blood clots are visible in the center of the picture. Photo: Sven Martin Jørgensen

5.2.2.3 Epidemiology

Before 2006, no official statistics was available for farm sites with CMS outbreaks [32]. The first field survey of CMS outbreaks in Norway was published in Brun *et al.* [20]. In this study, 25 cases were detected in the first year of the study 1998; elevated numbers were found for the following years 2001 with 103 cases and 2002 with 101 cases. No obvious changes over the four years of the survey in the distribution along the coast line were found; however, higher numbers of cases in autumn and spring were detected, with lowest numbers in the summer months. The reported average accumulated mortality in farms with CMS was with 6.1% against 2.5% in farms without CMS. The highest reported mortality rate was 19.9%. Most of the cases occurred about 400 days post sea transfer, with a range from 253 to 595 days. According to the economic model of the study, CMS caused financial losses of 4–8 million € (or 33-66 million NOK) each year in Norway [20].

CMS occurs along whole Norwegian coastline, but the counties Møre, Sør-, and Nord-Trøndelag are frequently strongest affected. In a prospective study, covering 12 month from September 2000 to 2001, 14,3% of the farms in Sør-Trøndelag were diagnosed with CMS, while only 2.2% of the farms in Troms were affected [33].

According to the yearly fish health report of the Norwegian Veterinary Institute [6], the number of farms diagnosed with CMS in Norway was equal to 85 in 2007. In the following two years, the numbers were relatively stable with 75 and 76 cases. In the most recent report, 53 cases of CMS for the year 2010 were counted [34], indicating a decreasing tendency of outbreaks. Most of the cases in 2010 occurred in mid Norway (Figure 6), similarly distributed as in the previous years.

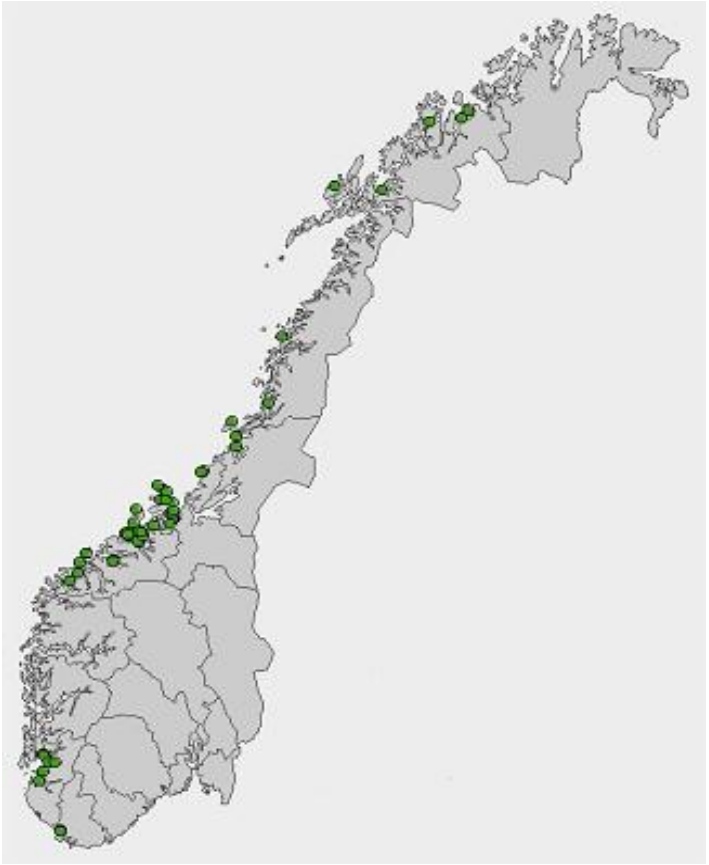

Figure 6: CMS outbreaks in Norway 2010. The green dots represent affected fish farms. Source: Veterinary Institute.

5.2.2.4 The Piscine Myocarditis Virus

Recently a novel virus associated with CMS was identified and named piscine myocarditis virus (PMCV) [29]. The sequenced genome of PMVC included one RNA segment with a length of 6,688 nucleotides. The isolated nucleic acids of PMCV, IPNV and ISAV were digested with different nucleases, and the PMCV genome showed the same digestion pattern as the dsRNA-IPNV, while the ssRNA-ISAV was digested only by single strand specific nucleases. Thus, it was concluded that PMCV was a dsRNA virus [29].

The genome of the PMCV contains three ORFs (Figure 7). ORF 1 encodes the putative coat (CP) protein with a length of 861 amino acids (aa) on reading frame 1. It overlaps with ORF 2, which encodes the RdRp. The overlap region of ORF 1 and 2 contain a "shifty heptamer motif" six bases upstream of the stop codon of ORF 1. A similar motif was also found in the genome of the closely related (see below) putative totivirus penaeid shrimp infectious myonecrosis virus (IMNV) [35]. It causes a -1 frame shift while the translation process and makes the translation of a fusion protein possible [36]. This feature was also proposed for PMCV [29]. ORF 3 encodes a protein of 302 aa length and similarities to other members of the *Totiviridae* family were not found so far. Only weak

homology to a C-X-C motif chemokine 11 was detected; however, the function of the protein remains unknown [29].

The putative RdRp of PMCV is a protein with a length of 726 aa and shows closest homology with the gag-pol fusion protein of the *Giardia lamblia*[1] virus (GLV), a virus of the totivirus family, and to the RdRp of IMNV. Totiviruses are known to infect mostly fungi and protozoa [37]. Phylogenetic analyses with a sequence of 480 aa of the PMCV RdRp, containing the conserved motifs of the putative protein, and 23 other members of the *Totiviridae* family resulted in a cluster containing PMCV, GLV and IMNV [29]; however, this cluster was the most distant to all other totiviruses.

In a challenge trial with PMCV i.p. injected and cohabitating fish, it could be shown that the cohabitants also develop CMS typical symptoms, proposing a horizontal transfer of the virus. Probable transmissibility of the virus was also shown in heart cell culture experiments in the same study [29]. Interestingly, the development of vacuoles in the infected cultured cells was observed while viral replication proceeded without any cytopathic effects. This indicates that the virus is released by other mechanisms than cell lysis. The release of viral particles by budding is frequently observed for enveloped viruses; however, *Totiviridae* are described as non-enveloped. This findings show that more research on PMCV is needed to understand the replication cycle and to confirm the phylogenetic classification.

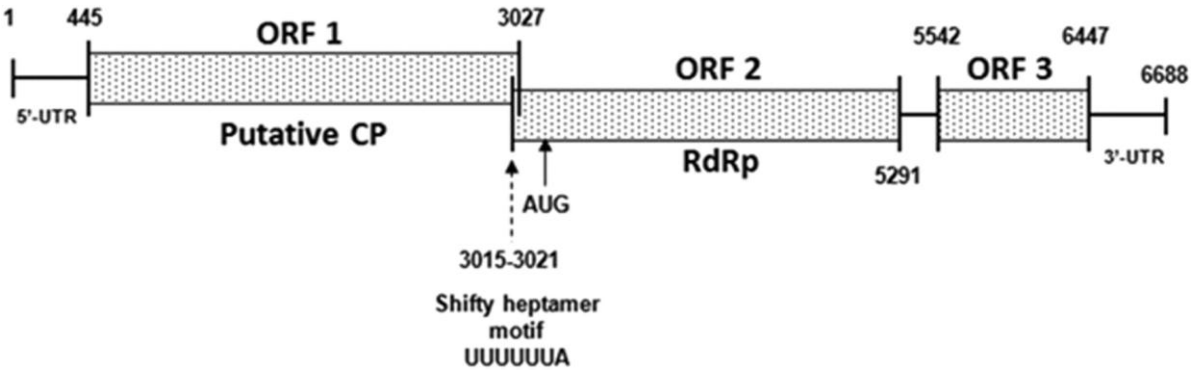

Figure 7: Genome organization of PMCV. The genome has a total length of 6688 bp and contains three ORFs. ORF 1 and 2 are overlapping and are putatively encoding for the coat protein (CP) and the RNA dependent RNA polymerase (RdRp). The function of ORF 3 is unknown. It was proposed that ORF 1 and 2 are translated as a fusion protein. [29]

[1] *Giardia lamblia* is an internal protozoan parasite, living in the guts of mammals, including humans.

5.3 Prevention and control strategies

5.3.1 Challenges

Diseases caused by viruses are responsible for the highest losses in the fish farming industry [6, 8] and new diseases have emerged continuously in Atlantic salmon aquaculture in the last ten years. When a potentially novel disease is diagnosed, its exploration begins with the description of clinical symptoms and pathology. The first description of CMS [16] followed this approach. However, diseases caused by different pathogens may have similar symptoms (e.g. similar cardiac myopathies in fish with PD, HSMI and CMS). Thus, if a potentially novel disease is found, an important question is if it may be a known disease with atypical symptoms. Qualitative and quantitative detection of pathogens with known genomic sequences is performed with qPCR. This method is also used to distinguish between subtypes of the same virus, a practical example is the PD causing SPDV subtypes [9]. However, co-infections or multiple infections can take place in sea farms. Frequently more than one pathogen is found in diseased fish. In one of the first attempts to identify the virus causing CMS, it was found together with PRV, the probable causative virus of HSMI [38]. When an unknown virus is the suspected cause of a novel disease, high throughput sequencing represents an efficient method to obtain genomic sequences from the putative virus, as shown for PMCV and PRV [13, 38]. Using this strategy, total RNA from a putatively infected fish or cell culture is sequenced. All known fish sequences are removed and the remaining (unknown) sequences are tested for homology with known viruses. If such homologies are found, the new sequences can be assembled to a full genome of the virus. Based on the new sequence information, a new qPCR assay for detecting the virus is developed.

Another challenge for diagnostics and disease surveillance is the common observation that fish may carry viral particles in persistent state without developing clinical symptoms. The carrier fish appears healthy, but the disease can break out any time, often triggered by external factors including various stressors. Often fish viruses can be detected in the head kidney of carrier fish in low amounts. For IPNV, it was shown that the virus can replicate and be released from head kidney macrophages without being cytolytic [39, 40].

5.3.2 Disease surveillance

In Norwegian aquaculture diseases must be diagnosed by an authorized laboratory from veterinarians or fish health biologists [6, 41]. This allows for reliable statistics of outbreaks and a national overview over the disease situation. The diagnosis of each disease uses specific methods. Most viral and bacterial diseases are diagnosed with aid of histopathological or molecular methods. Highly standardized screening qPCR assays are available for many diseases. For the main viral diseases

affecting Atlantic salmon, sensitive qPCR assays for the PD causing salmonid alphaviruses [42], IPNV [43, 44] and ISAV [45] have been developed. Before PMCV was sequenced, CMS diagnosis was based on histopathological examinations of the heart and muscle tissue, to be distinguishable from HSMI or PD. With the fully sequenced genome of PMCV, a new standard assay for CMS screening will be developed in the near future.

5.3.3 Vaccines

The use of vaccines for farmed Atlantic salmon was the main reason for the reduction of antibiotics usage in the 1990s and is today one of the most important tools to prevent outbreaks of many bacterial and viral diseases [46-48]. Today, nearly all farmed Atlantic salmon are routinely vaccinated against a number of bacterial and viral diseases (IPN, ISA and PD) [49]. However, negative side effects of oil-adjuvanted vaccines have been shown, including reduced appetite and growth [50, 51]. Furthermore, adhesions between intraperitoneal organs and melanin deposits on internal organs and on the abdominal wall may occur, leading in the most severe cases to reduced slaughter quality [52, 53]. These side effects are associated with prolonged inflammation caused by persistent antigens from the vaccine [54, 55]. Besides, systemic autoimmune reactions, induced by oil-adjuvant vaccines have been observed [56, 57]. The negative effects of vaccines can depend on time of vaccination, vaccine formulation, water temperature and fish condition [50, 55, 58]. However, the benefits of vaccines outweigh the negative effects. Interestingly, it is not entirely clear, if long term protection is mediated by B and T cells as in mammals or if other mechanisms are involved [59].

5.3.4 Selective breeding

Since the first collection of Atlantic salmon eggs in different Norwegian rivers in the early 1970s [60], the brood stock of salmon for fish farming was continually selected for different characteristics. This improved growth and survival rates, robustness and quality of the farmed fish. This period of time is very short in comparison to other domestic animals [61]. For fish in aquaculture, disease resistance is of particular importance [62]. Classical breeding programs based on survival rates of fish in field trials or controlled challenge tests have improved resistance to several diseases, e.g. IPN [63, 64]. In addition, molecular methods became available in the recent years. The marker assisted selection is based on the search for quantitative trait loci (QTL) in fish families with high and low survival rates in challenge test. In this context, most research has been conducted for QTLs related to IPN [65, 66] and ISA resistance [67, 68]. Knowledge about QTLs associated with disease resistance allows a selection

of the most promising brood stock fish for egg production and fixation of trait in subsequent generations.

5.3.5 Clinical nutrition

In addition to preventive measures that reduce losses due to diseases in aquacultures (e.g. vaccination, reduction of stress), different feeding strategies and diet formulations are being used. Today, health diets are offered from all feed producers for general disease purposes or targeting specific diseases, as for example lice, winter ulcer and PD. During PD outbreaks, highest mortality rates have been observed, when fish resume feeding after a period of inappetance [69, 70]. Thus, many farmers withhold feed when PD is suspected at a farm. However, this strategy may cause unnecessary losses in production [70]. In addition, supplementing vitamin E and C in the diet, may aid tissue reparation and recovery [71, 72]. No clinical nutrition is described for IPN infected fish yet; however, low vitamin E levels have been found in fish in acute infection phase [73]. Thus, a diet with elevated vitamin E levels may be beneficial for IPNV infected fish. Direct beneficial effects of the nutrition additive L-cysteine ethyl ester have been shown for Atlantic salmon, suffering amoebic gill disease [74].

Not much research has been conducted with clinical nutrition of salmon; however, modern analytic methods may enhance the research in this field. It has been shown in microarray experiments that nutrients influence gene expression mainly by activation or suppression of transcription factors [75]. Thus, studying the effects of nutrients on the transcriptome in fish may provide new insights in this field.

5.4 Fish immunology

5.4.1 The innate immune system

The innate immune system represents the evolutionary older defense strategy. It can be found in all classes of plants, fungi and animals and is independent of previous contact to invading pathogens. It reacts very quickly because many of the components are expressed in the host continuously and the induction of distinct inflammatory signaling molecules happens rapidly after infection. The innate response is also relatively temperature independent, which is important for ectothermic vertebrates like fish. In contrast, the adaptive immune response of fish is more temperature dependent and usually needs 4-6 weeks to develop first anti-pathogenic effects at optimum temperatures. The innate immune

system of fish can detect and react against a broad spectrum of pathogens and is of higher importance than in mammals, which depend largely on the adaptive immune system.

The innate defense against viruses employs basically two mechanisms. One is the antiviral response of nonspecific cytotoxic cells to virus infected cells and the other is the production of antiviral proteins (e.g. interferons, lectins etc.). The complement system can play an important role in response to some viruses and especially in neutralization of viruses in later stages.

Virions or viral components are detected by pattern recognition receptors (PRRs), which can be found as transmembrane receptors on the cell-surface, the intracellular membrane system and not membrane bound in the cytosol. Toll-like receptors (TLRs) are an important group of PRRs, which can sense viral components. Several TLRs that detect different virus specific molecules have been identified. Some detect viral nucleic acids and are localized on vesicles such as endosomes (TLR 3, 7 and 9) [76]. TLR 2 and 4 are expressed on the cell surface and recognize viral envelope components and TLR 3, 7 and 8 recognize viral RNA. Unmethylated DNA with CpG motifs, which is specific for DNA viruses, can be detected by TLR 9. TLR 3 and TLR 7 detect dsRNA or ssRNA respectively [77]. Activation of many TLRs leads to activation of key regulators of the immune response including IRF7 and NF-κβ. These proteins activate the transcription of type I IFNs and proinflammatory cytokines [78]. Conserved functions of TLRs in mammals and fish have been shown for several members of this family. Stimulation of TLR 9 of Japanese flounder with CpG DNA caused activation of a TNF promoter [79] and expression of TLR 3 in rainbow trout [80] and zebrafish [81] were up-regulated after stimulation with poly I:C and virus challenge. TLR 22 in fish was proposed as a functional substitute of human TLR 3 for sensing viral dsRNA [82]. However, functions of many fish TLRs are waiting for exploration.

Two important cytosolic receptors for viral RNA are retinoic acid inducible protein-I (Rig-I) [83] and melanoma differentiation associated gene 5 (Mda-5) [84]. Both are RNA helicases of the Rig-I like helicases (RLH) protein family [85]. Rig-I and Mda-5 possess, besides of the RNA helicase domain, a caspase recruitment domain, which is important for downstream signaling [86]. Rig-I but not Mda-5 is able to sense the 5′ phosphate of viral RNAs (mRNAs of the host are capped and do not possess 5'phosphate ends). Mda-5 recognizes viral ssRNA [87]. After activation, the caspase recognition domain of Rig-I and Mda-5 interacts with other proteins to activate the interferon system [88], Rig-I and Mda-5 like EST sequences have been found in different fish species, including salmonids [89]

The type I interferon system is an important part of innate defense against viral infections in vertebrates [90]. It consists of cytokines which are rapidly produced by many cell types after a viral infection. One of the strongest inducers of expression of interferons (IFNs) is dsRNA, as it is present in cells with replicating viruses, including viruses with ssRNA genomes [91]. Experimental induction of IFNs is often conducted by treatment with the synthetic dsRNA polyinosinic polycytidylic acid

(poly I:C) and allows the study of the interferon response without the pathological effects of a virus infection. IFN expression rises very quickly after infection. This was shown for example in a study with Atlantic cod, where transcripts of IFN regulatory factors were induced greater than 10-fold after 6 hours of injection with poly I:C [92]. IFNs activate transcription factors, which induce the expression of antiviral proteins. One of these transcription factors is Signal Transducer and Activator of Transcription 1 (STAT1). The Atlantic salmon STAT1 homolog was examined in several studies. For instance, early induction and peak expression after injection of ISAV and IPNV occurred after 6 and 4 days respectively [93]. Some of the antiviral proteins, activated by STAT1 inhibit the translation of viral mRNA, including the well described Mx proteins [94]. Mx proteins have been found in different fish species, including rainbow trout [95, 96] and Atlantic salmon [97]. Measuring the expression of Mx proteins, using qPCR, is an established method to detect IFN response in fish. [97]. Within two days after a viral infection, a rapid induction of IFNs has been shown in rainbow trout, experimentally infected with viral hemorrhagic septicemia virus (VHSV) [98]. And in an experiment with isolated Atlantic salmon macrophages stimulated with poly I:C, peak interferon production occurred within 24 h and peak Mx protein production after 48 h [99]. In another study, salmon were injected with poly I:C and the production of Mx protein in various tissues within 2 days and for at least 14 days was detected [97]. The early induction indicated possible correlation between the levels of interferon and the resistance against the viral pathogen; however, in most cases the IFN levels were correlated to the viral load and not to resistance. The highest IFN levels are observed in most infected fish [98]. The IFN system is activated with PRRs but many viruses have developed counter measures against the innate immune response. It was shown that a constitutively expressed Mx gene in Atlantic salmon cell culture was capable of inhibiting the replication of IPNV [100] and another article reported that IPNV encoded proteins inhibited an Mx gene of Atlantic halibut, which was previously stimulated by dsRNA injection [101]. Two proteins of IPNV (VP4 and VP5) were identified to be potential antagonists of the Mx promoter activators [102]. These results indicate that inhibition of Mx expression is essential for IPNV replication and is of great interest for the development of an IPNV treatment for fish in aquaculture. A beneficial effect of Mx expression has also been reported for ISAV infections, by reducing cytopathic effects [103]. However, another study with ISAV infected Atlantic salmon resulted in significant induction of type I IFN genes but a restriction of virus replication was not observed [104]. An IFN-stimulated gene, which is potentially involved in reducing the spread of ISAV, is ISG-15. It was up-regulated after infection and the gene product was capable to bind to the ISAV nucleoprotein, which indicates an antiviral role [105].

The antiviral response of nonspecific cytotoxic cells is well described in higher vertebrates. Natural killer (NK) cells limit the spread of virions by lysing infected cells (cell mediated cytotoxicity (CMC)) in early stages of the infection. Very little work has been done on NK-cells in fish. However, a significant elevation of the CMC of kidney leucocytes in both Atlantic salmon and rainbow trout cell

cultures has been shown, after the cell lines were infected with IPNV [106, 107]. In another study, autologous lymphoid cell lines of channel catfish, infected with channel catfish virus, were killed by peripheral blood leukocytes, while the same uninfected cell lines were not [108]. These studies are not sufficient to conclude that the same NK-cell mechanisms exist in fish as in mammals; however, they suggest the existence of non-specific CMC in fish.

Antiviral effects of the complement system in fish have not been studied thoroughly yet. However, in humans the classical complement pathway can be activated directly by viruses and virus infected cells [109, 110]. Furthermore, the complement of mammals plays an important role in the neutralization of viruses and killing of virus-infected cells [111]. One study with rainbow trout has shown that the neutralization of rhabdoviruses (VHSV and infectious hematopoietic necrosis virus) by antibodies is dependent on the presence of complement [112]. However, this may be a result of the enveloped nature of rhabdoviruses and depends on the membranolytic activity of complement lysing the envelope of the virus particle. The complement system is an important bridge between the innate and the adaptive immune response.

The innate immune system plays an important role in bacterial infections as well. A broad spectrum of anti-microbial substances and complement components are always present in the blood of vertebrates. As reaction to distinct molecules on the surface of the bacterial membrane, increased production of acute phase proteins, release of cytokines, non-classical complement activation, and phagocytosis takes place. Such surface molecules are detected by PRRs, which are present on various cells. However, many pathogenic bacteria have developed mechanisms to avoid recognition and immune responses.

The most important serum defense factor against bacteria is the complement system. While its effect against viruses is still not completely clear, the anti-bacterial effects are better understood. The alternative complement pathway can be directly activated by lipopolysaccharides (LPS), which is an important constituent of the cell wall of Gram-negative bacteria. Fully activated, this pathway causes lysis of bacteria by integrating pores in the bacterial cell membranes. Some of bacterial pathogens in fish are resistant to this mechanism [113]. However, complement component C3a remains on the bacterial surface and reacts as an opsonin for phagocytic cells. The released C5a components are chemotaxins that attract macrophages and neutrophils. The activity of the alternative pathway in fish serum is higher compared to mammals [114] suggesting this pathway is an important defense mechanisms of fish against bacteria.

Some pathogenic bacteria of fish (e.g. bacteria of the genus *Vibrio*) use adhesins to attach themselves to the cells of the host, mostly by binding to carbohydrates [115]. After attachment, they are internalized in the host cell by endocytosis, can start grow and cause the disease [116]. A possible countermeasure of the host against adhesins is mediated by lectins. This is a group of proteins with

different specificities for binding carbohydrates [115] and have been found in salmon serum [117-119] and mucus of ayu (*Plecoglossus altivelis)* [120]. The binding to the carbohydrates on the surface of bacteria may block the attachment and subsequent invasion of the host. Lectins can have other immune roles in fish that are waiting for exploration.

5.4.2 The adaptive immune system

The adaptive immune system relies on vastly diverse receptors and can memorize previously detected components of pathogens. Fish possess a similar adaptive immune system as mammal, consisting of B and T lymphocytes and antigen presenting cells (APCs), like macrophages and dendritic cells (DCs). Macrophages seem to be the most important APCs in fish, but DCs have also been described for rainbow trout [121]. Phagocytosed antigens are presented by APCs to lymphocytes over the MHC-II pathway, while intrinsic antigens are presented by the MHC-I pathway by most cells of the organism. B cells are responsible for the humoral response while T cells mediate the cellular response. In mammals, lymphocytes are generated in the red bone marrow, but only T lymphocytes migrate to the thymus for maturation. Lymphocytes are maintained and immune responses are initiated in lymphatic organs (e.g. lymph nodes, spleen) [122]. In contrast, teleosts do not have red bone marrow or lymph nodes. Head kidney, spleen and thymus are considered as the major lymphatic organs of teleosts [123]. The head kidney exhibits functional similarities with red bone marrow of mammals and is considered as the primary B cell organ. The head kidney has important functions in collecting and presentation of antigens from the circulation and is the major site of antibody production ([124], review).

Fish lack the possibility of isotypic antibody (AB) switching, which reduces the variability of the antibody response [125]. Furthermore, the immunoglobulin (Ig) repertoire of teleost differs from mammals in different aspects. ([126], review). Mammals express 5 different Ig classes and several sub-classes, while only three classes were found in fish. The tetrameric IgM is the first identified and most prevalent Ig in fish serum [127]. It is also the most universal Ig in vertebrates [128]. Two populations of IgD^+ B cells (IgM^+/IgD^+ and IgM^-/IgD^+) have been identified in channel catfish; however, the function of IgD is still not fully understood [129]. IgA is the most important Ig in reacting against parasites in mammals; however, it was not found in fish. The recently discovered Ig class IgT is unique in fish and is involved in immune reactions in the gut and mucus [130]. This class is also known as IgZ in zebrafish [131] and is likely the functional equivalent to the mammal IgA.

Fish also have no lymph nodes or germinal centers, a restricted immunological memory and the secondary response is limited or absent [132]. However, some interesting features of the teleost immune system were found, which are missing in the mammalian system. For example, Ig-like molecules have been found in fish, which may increase the diversity of B-cell recognition capacity

[133, 134]. In addition, phagocytic activity of B lymphocytes has been shown for some fish, which indicates diverse roles of B cells [135].

T cells are supposed to be the most important driving force of the adaptive immune response against viruses. The key mechanism to prevent further spread of virus particles is CMC, executed by $CD8^+$ T cells, also called cytotoxic T cells (T_C). Nearly all cells of an organisms express MHC-I molecules on the cell surface [136]. These are membrane bound complexes, presenting protein fragments of degraded proteins to T cells. In case of virus infected cells, also fragments of viral peptides are presented. $CD8^+$ T_C are negatively selected during their development for self-recognition, resulting in a population of T cells, which do not (except for autoimmune reactions) react on host specific peptides. Though, if non-self peptides are detected by specific T_C, CMC is induced, to kill the infected cell, preventing the further spread of the virus. The recognition is a complex process, which involves in addition to the MHC-I molecules on the host cell different receptors on the T_C cell, including the specific T cell receptor (TCR) in complex with the CD3-receptor and CD8. The process is not very well explored in fish yet; however, sequence homologies have been found for genes of the MHC-I complex between mammals and fish (reviewed in [137]), for TCR in rainbow trout [138, 139] and Atlantic salmon [140] and for CD8 in rainbow trout [141]. Three genes of Atlantic salmon CD3 have also been characterized [142]. In addition, high expression levels for TCR genes have been detected in the thymus of Atlantic cod [143] and for CD8 in rainbow trout [141], indicating that T cell selection and development of mammals and fish occur in the same organ. In a study, using immunohistochemistry with ISAV infected Atlantic salmon tissues, an early mobilization (day 17 post challenge) of cellular immunity was shown by elevated concentrations of $CD8^+$ cells in the head kidney [144].

In mammals, most of the T lymphocytes express either CD8 or CD4 on their cell surface. While $CD8^+$ T cells are mainly responsible for CMC, $CD4^+$ T cells, also known as T helper cells (T_H), can stimulate T_C cells and B cells. T_H cells are highly important to activate the proliferation of T_C cells and antibody class switching of B cells in mammals. They are activated by APCs and B cells that present processed antigens via the MHC-II pathway. For recognition, the T_H cell receptors TCR/CD3 and CD4 are needed. The existence of T_H cells in fish is not entirely confirmed yet. However, their existence is likely since a CD4 homolog was found in fugu [145] and a CD4-like gene in rainbow trout [146]. In Atlantic salmon, different genes encoding two CD4 molecules were identified (CD4-1 and CD4-2) and were strongest expressed in thymus and spleen [147].

The signaling system between cells of the adaptive immune system is highly complex and based on small signaling peptides, called cytokines. Some important cytokines of mammals, involved in activation of leukocytes are interleukin (IL)-2, IL-6 and interferons. T_H cells release IL-2 after activation by APCs, which stimulates CD8+ T_C cells [148]. IL-6 can be released by various cells,

including monocytes, fibroblasts, macrophages and lymphocytes and is important for induction of fever and inflammation [149]. IL-2 and IL-6 are also inducers of differentiation of T cells to T_C cells [150]. In addition to earlier described roles of IFNs in repression of viral replication, IFNs also induce the expression of MHC-I [151, 152] and prevent apoptosis in T_H cells [153]. Thus, IFNs are also involved in stimulation of the adaptive immune system. Apart from IFNs, other fish cytokines were described. IL-2-like genes were found in rainbow trout and fugu [154, 155] and an IL-6 homolog of tiger puffer was cloned [156].

5.5 Functional genomics

DNA microarrays have been developed since the 1990s. In early studies, the expression of relatively small amounts of genes could be measured (e.g. 45 Arabidopsis genes in 1995 [157]). In the following years, new technologies have been established and genome-wide expression analyses became possible. Today, different commercial and custom microarray platforms are available, representing transcriptomes of various organisms. The number of publications, including microarray data was increasing rapidly between the years 1999 to 2006 (Figure 8). In the following years until today, microarrays became an established method and relative numbers of published articles has remained stable.

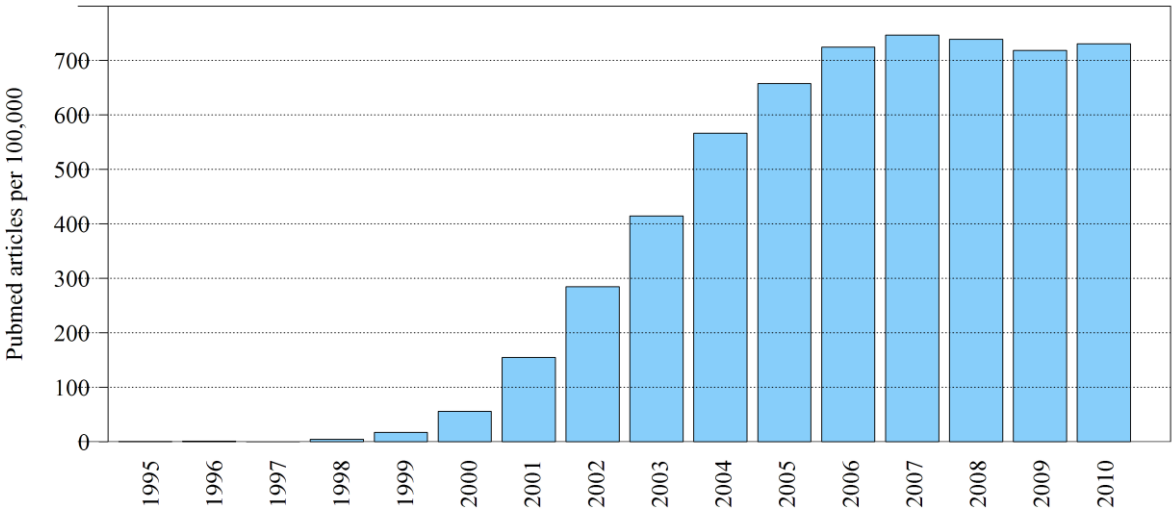

Figure 8: Number of articles in PubMed database including the keyword "microarray" per year and per 100.000 published articles. Data was collected from PubMed [158].

5.5.1 DNA microarrays: technology and methods

DNA microarrays (MA) are based on DNA sequences anchored to a solid surface, such as glass, nylon or plastic. The sequences of the DNA strands are complementary to the corresponding mRNAs and the positions of the DNA probes on the MA are known. For early MA platforms, probes were produced by PCR and afterwards spotted on the array (called "cDNA MA"). The probes were long and thus resulted in a relatively high risk of unspecific binding. Modern MAs commonly use synthetic oligonucleotides of a defined length between 20 and 60 nucleotides (called "oligonucleotide MA"). For design of a new MA, the first step is the selection of mRNA sequences. Next, the probe sequences are calculated by computer programs, which is similar to qPCR primer design. Finally, the probes are synthesized directly on the MA surface.

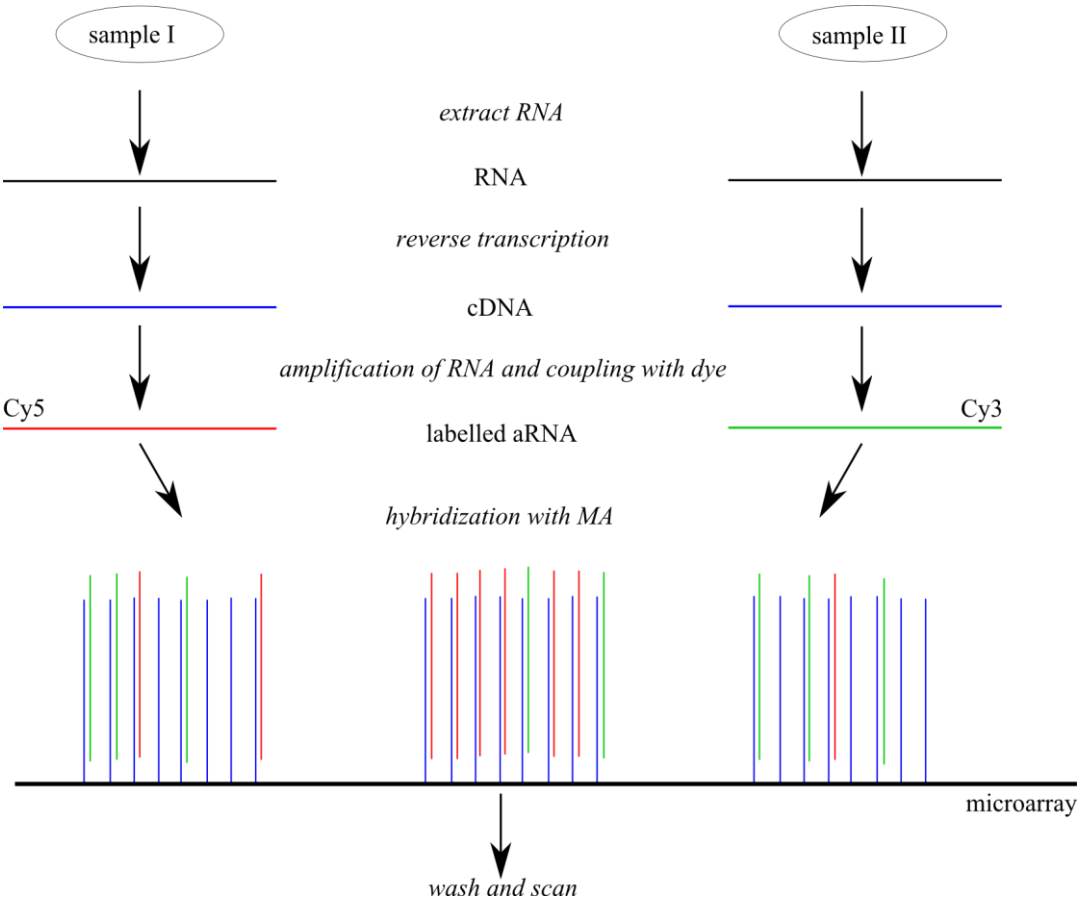

Figure 9: Experimental workflow of a microarray experiment.

For measuring the gene expressions, total RNA is extracted and labeled with a fluorophore (Figure 9). Analyses with oligonucleotide MA include also linear amplification of mRNA. In case of one-color

experiments, all samples are labeled with the same fluorophore, commonly Cyanine 3 (Cy3) and hybridized with the MAs. In two-color experiments the test and control samples are labeled with Cy3 or Cy5 and each MA is hybridized with a mixture of equal amounts of Cy3 and one Cy5 labeled RNA. During hybridization the labeled RNAs bind to their complementary DNA probes. After the reaction the MAs are washed to remove unspecific binding und scanned with a high resolution scanner. Cy3 and Cy5 have distinct emission wavelength and can be detected separately. For data analysis, the emission intensities for each of the spots on the array are calculated. In case off two-color experiments, the ratio between the two dyes corresponds to the relative expression of the respective gene between the two samples. In one-color experiments, the emission values of different MAs are used to calculate the relative gene expression.

The experimental design is critical for a successful MA experiment. One-color experiments are more flexible because the samples to be used for estimation of relative gene expression can be chosen after the analyses. For two-color hybridization, the control must be chosen in advance. Both approaches have advantages and drawbacks and as a rule, produce similar outcomes [159]. Two basic types of two-color experiments are direct and reference design (Figure 10). For direct design, two Cy3/Cy5-labeled RNA samples, which should be compared directly to each other (e.g. infected vs. not infected fish), are hybridized on the same MA. The expression ratio can be calculated directly for each of the MAs. In reference designs, the same RNA sample, usually a pool of many different samples (the reference RNA), is used on all MAs of the experiment. For calculation of the final expression ratio between two MAs, the difference to the reference RNA is compared. The reference design is more robust than the direct design because the samples to compare can be chosen after the experiment, similar to one-color experiment.

Figure 10: Examples for a direct and a reference design with two-color microarrays. Samples "S" are infected samples, while "C" represents control samples. "C P" is a pool of controls samples and "C R" a common reference. Each gradually colored green-red bar represents one MA experiment.

5.5.2 Microarray studies in teleosts

5.5.2.1 Overview (species, technology)

A number of microarrays have been developed for different fish species in the last years. The first custom designed MAs were cDNA MAs that included from several hundred to several thousand genes. Often the selection of genes was adapted to the aims of studies. After custom oligonucleotide MAs became available, the number of genes per MA increased to several tens of thousands genes and production of genome-wide platforms is feasible at present.

Transcriptome studies with fish have been conducted in different research areas and MAs for a broad variety of model and commercially important fish species have been developed, including salmonids, zebrafish, medaka, carp, sea bream and sea bass, catfish and flatfish ([160], review). One example for a custom designed oligonucleotide MA platform with 44k probes, based on a broad variety of Atlantic salmon and rainbow trout sequences, is described in **Paper I** of this thesis [161]. The Genomic Research on All Salmons Project (GRASP) is another example for the development of MAs for salmonids [162, 163]. Different custom designed MA platforms for flatfish have been reviewed in Cerda *et al.* [164]. Both cDNA and oligonucleotide MAs are described, which are partly specific for distinct tissues and aspects. Furthermore, the features of ten different MA, each specific for a different fish species have been reviewed in Miller and Maclean 2008 [165].

5.5.2.2 Research areas and results

MAs are used in different areas of fish research. The best described model organism among teleosts is zebrafish. This organism is of special interest of developmental studies [166] and, more recently, for studies of the immune response as well [167]. However, fish are the most diverse and species-rich class of vertebrates, which makes species specific studies of the immune response necessary, if applications as the development of vaccines or identifying disease markers is the intention. A general finding in fish transcriptomics is a high variation of gene expression between individuals. For example, a study with wild killifish (*Fundluus heteroclitus)* revealed significant differences between individuals in transcription of metabolic genes [168] and another study, comparing transcriptomics in the liver of European flounder from the Baltic and the North sea, revealed differences in the transcription of distinct immune genes, even though the genetic differences between the populations were very low [169].

Salmonids are of high commercial interest, therefore transcriptome studies often aim at reduction of losses in fish farms and optimization of the production process. Different genome wide [161, 163, 170, 171] and specialized MA platforms [172, 173] were developed. Studying the reaction of fish to distinct nutrition components on the transcriptional level is one of the possible approaches to reduce

the need of natural resources for fish feed and optimization of nutrition of farmed fish [174, 175]. Other studies addressed the reproduction processes [173, 176], responses to stress [177] and toxic substances [172, 178, 179]. Host-pathogen response studies are of particular interest and importance for fish aquaculture. Studies performed in this field are reviewed in the following section.

5.5.2.3 Functional genomics studies of host-pathogen responses

The interaction between pathogens and the host is very complex. Functional genomics provides powerful tools to study these interactions. The main benefit of MAs is the large number of genes, which can be analysed in a single experiment. The results are highly dependent on the annotation quality of the differentially expressed genes and must be interpreted carefully. A part of genes are specific for distinct types of cells, while others may be transcribed in diverse cells. Therefore relative abundance of transcripts may show changes in the cellular composition of tissues, which is of special interest for studies of the immune system.

A number of studies used microarrays to examine host immune responses by measuring the transcription rate of thousands of genes simultaneously. Most of these studies with teleosts were focused on diseases affecting salmonids, Japanese flounder and zebrafish. One of these studies examined the immune response of Atlantic salmon against ISAV by comparing individuals with early and late mortality after infection [180]. Similar as in Kileng *et al.* [104], fish with highest virus replication rates showed also the highest activation of innate immune response, but this did not provide protection. Subsequent protection and clearing of ISAV was most likely dependent an adaptive immunity processes [180]. Another study with ISAV infection was conducted with Atlantic salmon macrophage-like cell lines. Macrophages are important targets of ISAV *in vivo* and reacted with rapid induction of pro-inflammatory and antiviral immune genes. Furthermore, increased oxidative stress was proposed to be an important determinant for the cytopathic outcome of an ISAV infection [181]. Macrophage/dendritic cell lines were also used in another infection study with high and low virulence ISAV strains. Comparison of the immune reaction to both strains resulted in similar strong antiviral immune reactions. However, several differences in the reactions suggested virus virulence mechanisms to avoid the immune system [182]. Other studies examined the immune response of rainbow trout against IHNV. Complementary DNA microarrays were used to investigate and compare the host response in the head kidney after 24 and 72 hours against IHNV, attenuated IHNV and LPS. The attenuated IHNV triggered an immune response, which was described as ‘mixed’ between the anti-viral IHNV and the anti-bacterial LPS response [183]. In another study, the gene expression in different tissues of rainbow trout after injection with an IHNV DNA vaccine was examined, resulting in a systemic up-regulation of the type I IFN system and an unspecific immune response [184]. Microarray based gene expression analysis was also conducted for the host response

of Japanese flounder against VHSV. Protective effects of DNA vaccines, based on VHSV glycoprotein containing plasmid DNA vectors, were reported in two articles. Gene expression analyses showed both humoral and cellular immune response activation and fish that were highly resistant against the virus [185, 186].

Host immune responses against bacterial pathogens have also been analyzed by transcriptomic tools. In two studies, cDNA microarrays were used to study innate immune reactions of Atlantic salmon in different tissues against *Aeromonas salmonicida*. Novel genes, involved in anti-bacterial immune reactions were identified and differences in the gene expression between the examined tissues were found [187, 188]. Biomarkers for *Piscirickettsia salmonis* infected Atlantic salmon were reported in another article. A panel of genes related to the immune system was differentially expressed in macrophages and head kidney and some of them can be specific for the response against this intracellular pathogen [189]. Another genus of intracellular bacteria, causing enteric septicaemia in catfish and Japanese flounder, is *Edwardsiella* (*Enterobactericea* family). The immune response against *Edwardsiella ictaluri* was addressed in two studies. A 26k oligonucleotide microarray with 19k channel catfish and 7k blue catfish probes was used to examine the immune response in the liver. In the first study, genes of the iron homeostasis and different immune pathways were highly induced during the acute phase of the disease in channel catfish. Among the induced immune genes were the complement components, including membrane attack complex proteins, pattern recognition receptors and chemokines [190]. In the following study with *Edwardsiella ictaluri* infected blue catfish, a similar immune response was reported and in addition MHC class I pathways showed early activation [191]. In another study, the differences in immune reaction between vaccinated and unvaccinated Japanese flounder after infection with *Edwardsiella tarda* were examined by measuring the gene expression in PBLs using cDNA microarrays. Different immune pathways were induced. However, the reported differences between the vaccinated and unvaccinated fish were limited and did not allow for a clear conclusion about the protection and efficacy of the vaccine.

Besides of virus and bacteria diseases, immune responses against other pathogens have been addressed in microarray experiments. Saprolegniasis, caused by fungal *saprolegniaceae* species, occurs in farmed Atlantic salmon. Transcriptomic studies of diseased fish showed up-regulation of unspecific immune pathways. Induction of genes with unknown function was found as well and some of these are potentially specific for response to this fungal disease [192]. In a transcriptomic study of Atlantic salmon gills affected by amoebic gill disease, the tumor suppressor p53 was found to be down-regulated and a possible role of p53 in disease pathogenesis was discussed [193]. Immune responses against the whirling disease causing parasite *Myxobolus cerebralis* in rainbow trout skin was examined by cDNA microarrays and revealed up-regulation of different immune genes, including genes of ubiquitin-like protein 1 and IFN regulation factor [194]. A study of the immune responses of Japanese flounder against the parasite *Neoheterobothrium hirame* revealed a broad range of genes,

involved in specific and non-specific immune pathways and potential infection markers [195]. The crustacean ectoparasite salmon louse causes wounds in the skin of Atlantic salmon and subsequent inflammation. The transcriptomic responses have been examined in the infected skin, liver, spleen and head kidney using cDNA microarrays and qPCR analysis. A fast induction of adaptive immune reactions was reported in the damaged skin and was described as T_{H2}–pathway specific. In addition, systemic inflammation reactions and elevated stress, witnessed by high transcription rates of metalloproteinases has been reported [196].

Zebrafish is an established model organism for developmental processes and has moved more into focus as a promising model for immunology in the last years. Due to a short generation time, a spectrum of established gene knockdown methods, small body size and a similar immune system to mammals, the zebrafish model allows both the study of a fully developed immune system and solely of the innate response. The adaptive immune system of zebrafish is not active before 2-4 weeks after fertilization and the organism remains transparent, which makes the early larval stage a good model for innate defense reactions [197, 198]. This feature has been used in two zebrafish-*salmonella* disease studies. One study used oligonucleotide microarrays to identify genes of the innate immune response, which were induced after experimental *salmonella* infection of zebrafish larvae. Further knockdown experiments of the flagellin receptor TLR5 and the adaptor MyD88 revealed that different genes are activated downstream of the knocked down genes, including the matrix metalloproteinase *mmp9* and *il-8* [199]. Another study focused on the role of the key modulator of development and immunity TNF receptor-associated factor 6 (Traf6), which is well described for mammals [200]. The zebrafish homolog of *traf6* was knocked down before an experimental *salmonella* infection of zebrafish larvae. To identify genes of the innate immunity, which were affected by the knockdown, microarray and deep sequencing experiments were performed. Analyses resulted in finding of a wide spectrum of genes including well known anti-microbial and inflammatory genes. This study confirmed that Traf6 was an important immune regulator in fish [201]. Another study investigated the immune reaction of zebrafish against mycobacterium strains at different live stages to compare the limited innate response of larvae to the fully functional immune response of adult fish. Overall, the immune responses at the different live stages were highly similar [202]. In addition, further genome wide immune profiles of adult zebrafish against mycobacterium [167] and *Streptococcus suis* [203] have been published.

The high number of genome wide studies addressing host-pathogen interaction in fish by using microarrays highlights importance of this method for understanding immune processes as defence mechanisms against all kinds of pathogens. Sequencing of genomes of commercially important species including Atlantic salmon and further improvement of the annotations will strengthen research and create new possibilities.

6 Results and general discussion

6.1 The SIQ oligonucleotide microarray

6.1.1 Microarray development and description

The development of a new microarray platform, based on 60mer oligonucleotide DNA probes, was important for the examination of the immune response of Atlantic salmon (**Paper I**). Basis for selection of sequences was Salmon and Trout Annotated Reference Sequences (STARS) system, which includes a relational database and programs for sequence annotation and management of microarray data. The sequences in the database were mainly mRNA sequences from UniGene [204]. Additional sequences were collected from GenBank [205], The Gene Indices [206] and own ESTs. STARS contained sequences from different fish species; however, the microarrays (MAs) were designed with Atlantic salmon sequences with addition of rainbow trout sequences. The MAs were called Salmon Immunity and Quality (SIQ), which referred to the first fields of application. All MA versions were fabricated by Agilent technologies (Santa Clara, USA) with four MAs per glass slide and 44,000 spots per MA. The first SIQ 1.0 MA contained 10,316 genes with four replicates each and was successfully used in pilot experiments. The following versions aimed at full coverage of the known protein encoding sequences of Atlantic salmon and the number of replicates was reduced to two. For experiments, described in **Paper II-IV,** SIQ MAs of the versions 2.0, 3.0 and 4.0 were used. The resources for SIQ2.0 and SIQ3.0 are described in **Paper I**. For version 4.0, only a small amount of new sequences was added in comparison to version 3.0.

The pilot study with the newly developed SIQ1.0 platform (**Paper I**) aimed at quality control of the new MAs, detection of genes, specifically transcribed in leukocytes and identification of virus responsive genes. In 104 MA experiments, 60% of the salmon and 38% of the trout specific spots were hybridized with labeled RNA in average. These results showed that salmon sequences gave a significantly higher success rate; however, many of the trout sequences gave reliable signals as well and can fill some of the gaps in the salmon transcriptome. Besides of the performance test, a list of leucocyte specific genes was identified by hybridizing microarrays with RNA, extracted from mixture of tissues and RNA from peripheral blood leucocytes (PBL). This first study also identified a number of virus responsive genes (VRG). Samples were taken from salmon with different diseases, including CMS and HSMI that had unknown etiology by the time of this study. A comprehensive search for VRG based on a large number of experiments and analyses is presented in **Paper IV**.

6.1.2 Confirmation of microarray results by qPCR

Important findings need to be confirmed with at least one independent method and therefore qPCR verification is commonly included in MA studies. In **Paper II** and **III**, the expression of six genes was analyzed with both qPCR and MA. These results were combined in Figure 11. The values are highly correlated to each other and confirm the reliability of the microarray results. However, MA tended to under estimate high expression ratios and therefore the slope of the regression function was lower than 1 (0.82). This is due to a limited dynamic range of MA since spots with probes tend to saturate at relatively high abundance of labeled transcripts.

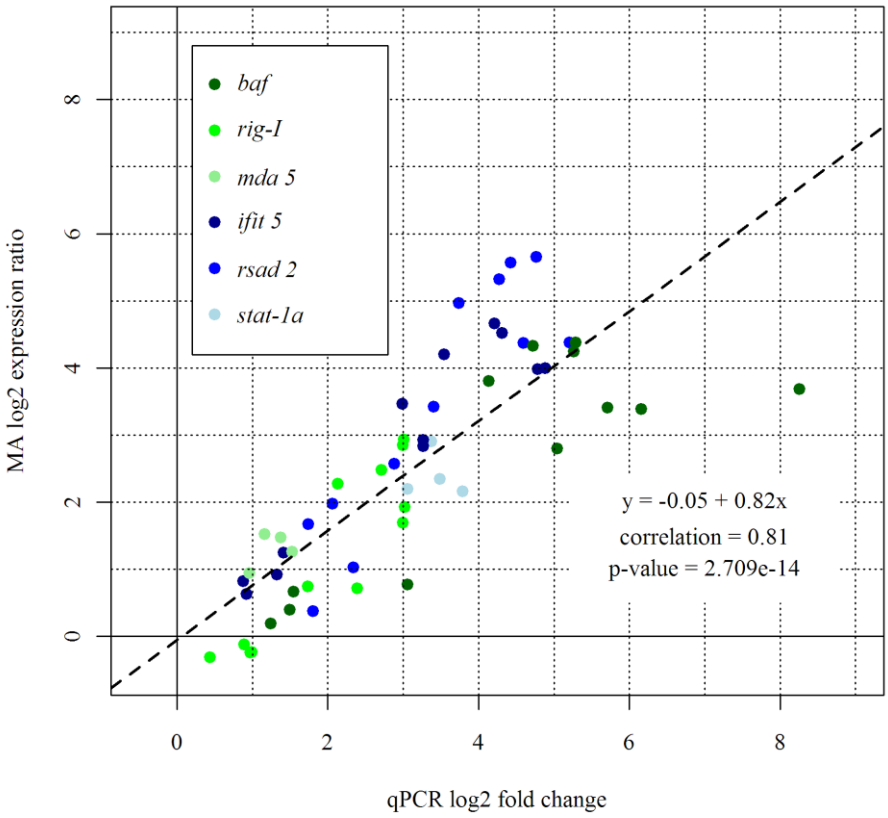

Figure 11: MA confirmation by qPCR. Values of qPCR (log_2 fold change) are plotted against their respective values from microarray (log_2 expression ratio) experiments. Expression of six genes was tested in four (*stat-1a* and *mda5*) or twelve (the other four genes) different samples. Values are combined from MA-qPCR confirmations in *Paper II* and *III*. Formula of regression function of all values, correlation coefficient and p-value are shown in the lower right corner.

6.1.3 Microarray experimental design

Two different experimental designs were used. The design in **Paper II** was direct and relied on pools of RNA, while individual fish were tested in a reference design for **Paper III**. In the direct design,

control pools were composed from eight to ten control fish and were hybridized together with smaller pools of infected fish. Though individual differences in the controls were not examined, this design appeared appropriate for the aim of the study. Large gene groups representing different immune pathways were identified among differentially expressed genes and this provided an overview of the host immune response of Atlantic salmon against PMCV. A reference design was needed for the aim of **Paper III**. In this study, individual differences were in focus with respect to the development of CMS. Relatively high individual variation of gene expression of fish was known [168, 169] and was confirmed in this study. Using the reference design control individuals were examined independently from the infected fish. This increased statistical power and excluded a number of genes which would be erratically qualified as differentially expressed in the direct design. Three control fish (one from each of the time points 4, 6 and 10 wpi) showed abnormal expression profiles and they were removed from the data set. In general, a reference design with individual fish is preferable. However, MA experiments are still expensive and reference design demands a larger amount of MAs for the controls.

6.2 Host-virus responses to PMCV

The experimental infection of Atlantic salmon with PMCV in a challenge trial under controlled conditions provided the biological material for studies described in **Paper II** and **III** as well as parts of **Paper I** and **IV**. The successful induction of pathology typical for CMS was confirmed by histopathological examination of heart tissue. In addition, strong correlation between the virus load (measured by qPCR) and severity of heart pathology (histology score) was found (**Paper II** and **III**). While clinical outbreaks of CMS from field typical give mortalities of 5-20% [20], no fish died in the present challenge trial. This suggests that other stressors, fish in a different life stage or a higher number of viral particles are needed to cause mortality.

The temporal and spatial regulation of immune responses in PMCV infected fish was examined in **Paper II** over a period of ten weeks. In this study, fish developing the strongest CMS pathology were analyzed. Six gene sets representing different immune pathways and showing significant temporal and spatial regulation were identified, including the antiviral and IFN response, complement response, B cell response, MHC AG presentation, T cell response and apoptosis. All challenged fish mounted a similar antiviral status (confirmed by gene expression and viral load) in earlier stages of the disease but developed different outcomes in terms of cardiac pathology. Therefore, in **Paper III**, we examined individual differences between fish with high and low pathologies at different stages of CMS. These groups were termed high and low responders (HR and LR). HR fish developed severe cardiac pathology (histopathology score 2 and 3) from 6wpi and onwards, while LR fish did not develop any pathology (score 0 and 1).

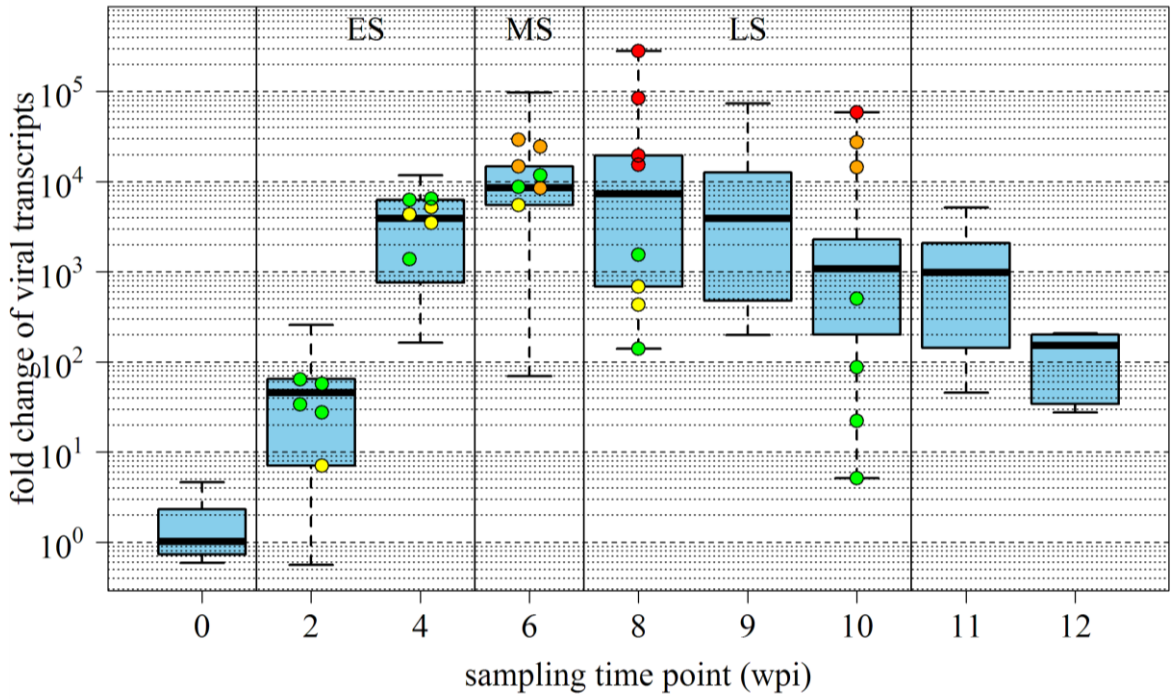

Figure 12: Boxplot of virus load in infected fish heart tissue, relative to median of non-infected 0 wpi results. Numbers of measured fish differed between 6 and 14 (values from *Paper II* and *III*). Values of individual fish of *Paper III* are plotted as dots with colors according to histopathological state of the heart (green and yellow = low pathology, orange and red = high pathology). The disease stages, described in *Paper III*, are indicated at the top (ES = early stage, MS = mid stage, LS = late stage).

6.2.1 General and antiviral responses at early stage (2-4wpi)

At the time when **Paper I** was published, PMCV was not identified and the etiology of CMS was still unknown. However, the pilot study with the novel SIQ MAs revealed a typical induction pattern of antiviral immune response at the earliest time point, which supported the hypothesis of a viral origin of CMS.

After identification and sequencing of PMCV [29], it was possible to measure the amount of virus in the infected fish. The concentration of viral transcripts in the heart was measured for the challenged fish by qPCR (**Paper II-III** and Figure 12). The virus load increased from 0 to 2 wpi by an average of 33 fold in heart tissue and reached >5000 fold at 4 wpi (compared to 0 wpi). However, although a standardized dose of inoculum was used, we did not know exactly how many viral transcripts were injected in each fish at the beginning of the challenge, because sufficient cultivation and titration of the virus is not yet possible. Thus, the virus load per fish may have been very low after injection, which is a possible explanation of the relatively slow replication of virus. Nonetheless, the expression of six genes involved in the early cellular response to viral RNA was equally induced in 20 individuals, suggesting that all fish were mounting a similar antiviral response to PMCV (**Paper II**). In addition,

all individuals examined in **Paper III** showed a similar virus replication within the first 4 wpi and no significant differences in histopathology.

Paper II and **III** provided additional data for the early immune response against PMCV. A group of 85 genes, involved in antiviral and IFN response (VRGs), is described in **Paper II**, which was strongest induced at the early time points (Figure 13). Many of these were also among genes induced in individual fish in the early stage described in **Paper III**, including the PRR genes *rigI*, *mda5* and *tlr 3* as well as genes of the IFN response *stat-1a* and *mx*. Expression of these genes was induced throughout infection, but the magnitude of expression gradually decreased towards the end. In fish with strong pathology, VRGs were upregulated until the end of the trial (**Paper II-III**). However, VRGs were not expressed in fish without pathology and lower viral load at 8-10 wpi (LR fish) (**Paper III**). Most members of this gene group were also upregulated early in response to other viral diseases (**Paper IV**). These results show that VRGs are strongest induced short after infection and are correlated to the virus load in later stages.

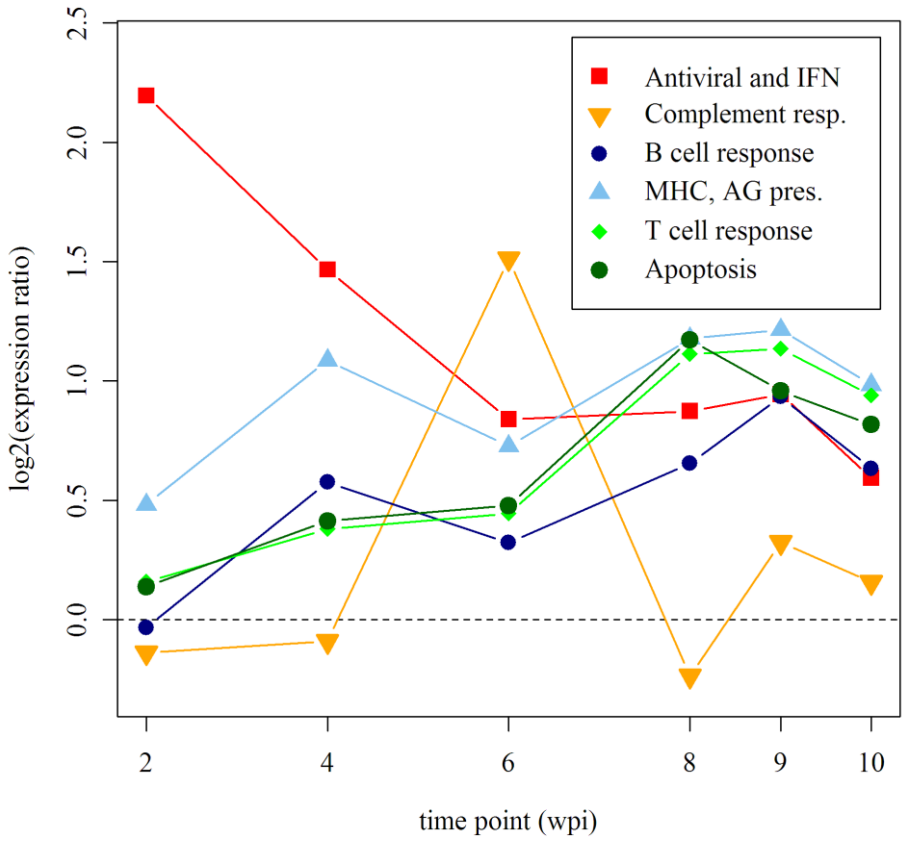

Figure 13: Simplified figure of the immune response in Atlantic salmon, infected with PMCV (*Paper II*). The medians of the expression ratios of the six gene sets are plotted against the sampling time points.

The presence of viral particles activates AG processing and presentation pathways via MHC in humans [151, 152] and the same was observed in Atlantic salmon during ISAV infection [207]. A group of genes, involved in MHC AG presentation, was upregulated at 2 wpi and even stronger at 4 wpi during the PMCV infection challenge (**Paper II,** Figure 13). Gene ontology (GO) enrichment analysis confirmed significant induction of AG presentation at all stages of the disease, except for late stage LR (**Paper III**). This indicates that the same relationship of innate antiviral responses and MHC-dependent presentation of AG exists in fish as well and that the activation of adaptive immune response begins already at early stages of the disease and is correlated to the virus load.

The first (weaker) peak of B cell response genes was detected at 4 wpi and was correlated with the expression pattern of MHC AG presentation (**Paper II**). This finding may reflect an early influx of B cells to the heart tissue. B cells are mainly responsible for AG presentation and production of specific ABs, however since B cells of fish have phagocytic activity [135], the presence of B cells in the heart may also be connected to this purpose.

The GO enrichment of repressed genes in the early stage of the disease showed repression of genes involved in cellular developmental processes, cytoskeleton organization and cardiac muscle development, possibly reflecting the infection-related stress and initial cellular events of pathological changes (**Paper III**).

6.2.2 Early pathology at mid stage (6wpi)

Viral load in heart increased further from 4 to 6 wpi; however, this increase was lower than between the earlier time points (**Paper II**).

Twenty-two genes related to the complement response were significantly induced specifically in heart at 6 wpi (Figure 13). Genes encoding different components in the complement system were identified. The time point of induction supported a role for complement as a bridge between the innate and adaptive immune system, since it occurred after the first induction of B cell- and MHC antigen presentation genes and prior to the development of cardiac pathology and peak of T and B cell responses. The stronger second peak of induction of B cell response genes subsequent to the activation of complement genes might also indicate that a potential humoral response based on antibody-dependent cellular cytotoxicity and virus neutralization was complement-dependent.

At 6 wpi, the first cases of severe pathological changes occurred in HR fish, while viral loads were equal between LR and HR (**Paper III**). Thus, this was the time point prior to the different development of infection and pathology in HR and LR, and differentially expressed genes at this stage might represent early predictors of pathology or recovery/clearance. Nine genes were identified, which

were significantly higher induced in fish with severe pathology (HR) than in LR. Most of them were related to immune functions (**Paper III**) and have been implicated in the control of lymphocyte regulation and survival. Strong inflammatory response and T cell activity was found in the late stage of the disease in HR fish (**Paper II-III**). This outcome may be already set in the mid stage.

Genes with stronger induction in LR than in HR might represent possible early predictors of viral clearance and recovery (**Paper III**). However, only seven genes were identified, with no clear functional relation to disease and immunity.

6.2.3 Peak pathology (8-9 wpi)

The second and stronger peak of B cell and AG presentation components was evident at 8-9 wpi, which was probably a consequence of the higher influx of leukocytes and the high virus load in the heart tissue (**Paper II**). The strongest induction of T cell and apoptotic pathways components coincided with peak pathology and B cell markers. Genes encoding CD8 and CD4 were induced, which indicated the activation of both T_C and T_H cells. The coregulation of apoptotic pathways is possibly a reflection of controlled cell death of T lymphocytes and/or host target cells. T_C cell mediated CMC is an important process to inhibit the spread of viral particles, however controlled cell death of T cells is also a fundamental process of the immune system to maintain homeostasis [208]. Several genes of Rho GTPases were coregualted with T cells markers. These proteins are important regulators of T cells and are involved in TCR signaling, T cell migration and apoptosis [209]. This finding supports the importance of these proteins in the immune regulation of fish.

The majority of genes induced in late stage (8-10 wpi) HR but not in late stage LR, was also related to adaptive immune response pathways (**Paper III**). In particular, T cell related genes were over-represented and likely reflect the increased infiltration and homing of virus-specific T cells to the infected tissue, analogous to the results of **Paper II**. In addition, GO enrichment analysis of functional groups in HR showed that immune system process/immune response and more specifically, activation of lymphocytes and leukocytes were positively correlated to viral load in the late stage. Consequently, while having an important role in the clearance of virus in infected heart tissue, virus-specific immune cells such as T and B cells may at the same time lead to increased tissue damage and burden for the host. Whether the strong pathology accompanied by induced expression of T cell response genes in HR fish is reflecting immunopathology could not be concluded based on the present data. The balance between immunopathology and immunity in humans is an important field of research ([208], review) and should be subject to further research in Atlantic salmon.

6.2.4 Recovery and virus clearance

After 10 wpi, viral load decreased in fish with most severe CMS pathology, and the six immune pathways were less strongly induced. This indicated that virus replication was under control of the hosts' immune system and virus clearance was proceeding (**Paper II**). In LR fish, decreasing virus levels were observed from 8 wpi and onwards (**Paper III**). This coincided with a complete lack of upregulation of adaptive immune response genes, which were found to be significantly induced and correlated to the elevated pathology in HR at late stages. However, some immune related genes were still induced also in LR, such as *granzyme A* (*gzmA*). GzmA is a serine protease and important inducer of antiviral and apoptotic pathways in infected cells, produced by cytotoxic T cells and NK cells [210]. Even though the expression was on a lower level in LR compared to HR, it indicated cytotoxic activity also in LR fish. Genes that were induced in LR and not in HR were mostly involved in energy metabolism and muscle regeneration and can be considered as markers for recovery and protection. Cell respiration and metabolic processes were also correlated to the viral load in LR (GO enrichment analysis). Thus, LR fish seem to cope with the infection by immune responses in the preceding stages and/or by a different composition or regulation of the late response, and managed to activate cardiac energy metabolism for recovery and regeneration of infected tissue in the late stage. A summary of host-virus responses during CMS based on the results of Paper II and III is shown in Figure 14.

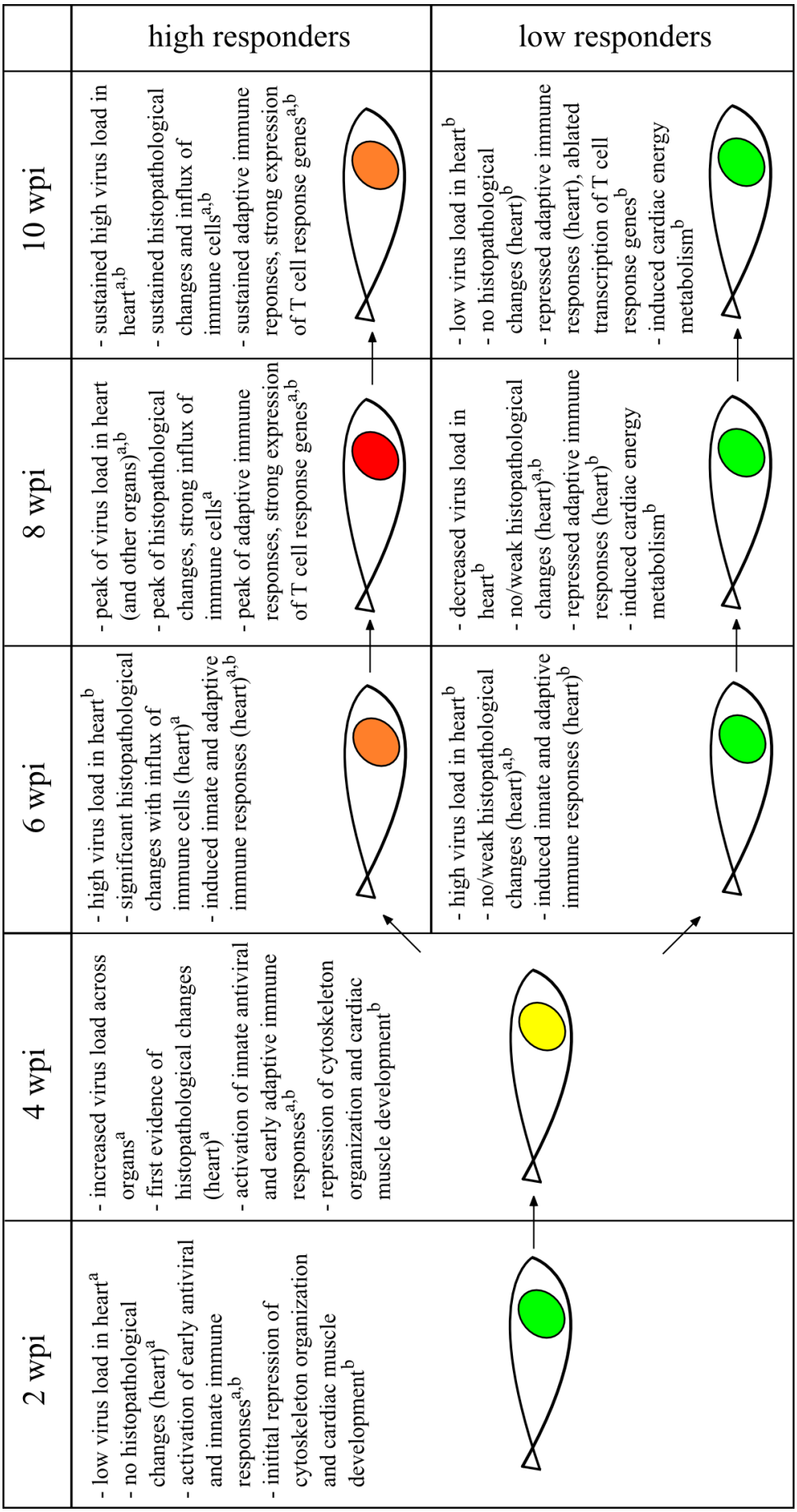

Figure 14: Summary of host-virus responses in high and low responder fish based on findings from *Paper II* and *Paper III*, as indicated with superscripted letters a and b, respectively. Colored circles in fish illustrate histopathology (atrium score level), analogous to Figure 13.

6.2.5 Infection and host responses in different organs and blood

The immune responses in different tissues in addition to the heart (liver, head kidney, spleen) and blood (RBCs and PBLs) were examined in **Paper II**. Time points 4 and 8 wpi were chosen for representing pre-clinical state and peak pathology. The group of early antiviral genes was stronger induced in the earlier time point for all tissues (Figure 15), indicating a systemic infection with PMCV. A similar pattern, but less significant, was found for genes of the MHC AG presentation pathway in kidney, spleen and PBLs. The head kidney of fish is the main organ for collecting antigens and maturation of leucocytes and is, as the spleen, a primary lymphatic organ ([124], review). The presented results likely reflect these mechanisms. However, the heart was the only organ with increasing induction of MHC AG pathway genes between 4 and 8 wpi.

Interestingly, complement components expression was induced at peak pathology in the spleen. In humans, complement component C3 has an important role of regulating the maturation of B cells in the spleen [211]. Thus, splenic complement may be involved in signaling and activation events of AG presentation and possibly of production of virus-specific antibodies by B cells.

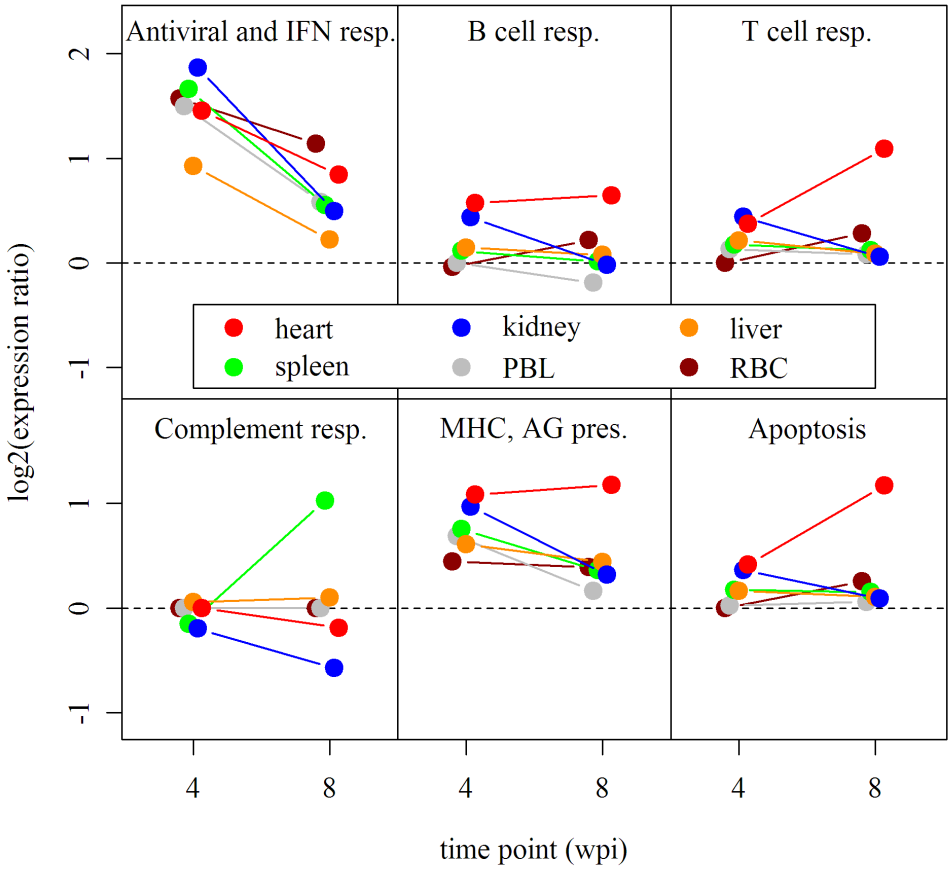

Figure 15: Simplified figure of the immune response in Atlantic salmon against PMCV in different organs and blood (*Paper II*). The medians of the expression ratios of the six gene sets at 4 and 8 wpi are shown. The dots are slightly distributed over the x-axis for better visualization.

Induced expression of genes of B cell, MHC AG presentation, T cell and apoptosis pathways were found at 4 wpi in the kidney, while it was significantly decreased at 8 wpi. Among these, the T cell specific gene group shows the strongest opposite trend to the regulation in heart. This may indicate that lymphocytes mature to effector cells in the kidney and migrate to the heart subsequently, where the elimination of virus infected cells occurs.

The highest levels of virus load were found in kidney, spleen and heart (**Paper II**). Heart is the only organ with an increasing amount of virus transcripts between the two time points. This finding supports that the heart is the main site of propagation for PMCV [29]. High levels of viral transcripts in head kidney and spleen are common for viral fish diseases. This is probably connected to the important role of both organs in attracting APCs and priming of lymphocytes [212]. The relative high transcription rate of genes of MHC AG presentation pathways supports this assumption.

6.3 Gene markers of early viral infection in Atlantic salmon

A comparative study of the expression of VRGs in response to different virus diseases (CMS, ISAV, IPN and HSMI) and poly I:C is described in **Paper IV**. The tissue samples were taken from different organs of infected Atlantic salmon and salmon cell cultures. The response to CMS was represented by the data from analyses partly published in **Paper II**. Many of the genes which were part of the antiviral and IFN responsive gene group in fish with CMS (**Paper II)** were also upregulated in other viral diseases, indicating a common role in the early response to viruses (**Paper IV**). In total, 117 VRGs that were strongly induced in all diseases and poly I:C treated cells were identified (**Paper IV**). Approximately a third of these genes were specialized antiviral genes, or genes related to the immune system. The remaining genes were not directly linked to the immune system or had unknown functions. VRGs showed rapid activation, as evidenced from cell cultures showing strong induction one day after exposure to poly I:C and ISAV. In fish with CMS and HSMI, VRGs were upregulated several weeks before symptoms of disease could be seen and sustained until the end of challenge (10 wpi). This was also supported at the protein level for four VRGs (immunofluorescence staining, cardiac tissue), showing induced cardiac expression from early virus replication to peak pathology stage during CMS. VRGs were activated by diverse viruses and the presence of exogenous RNA appeared to be the main if not the only requirement for activation. The level of VRG induction was related to the virus load and no activation was seen at low infection levels in fish without pathological symptoms (asymptomatic carrier fish). Furthermore, the expression of VRGs in several organs confirmed low tissue specificity. Expression of VRGs was highly correlated to gene expression of IFNa, which led to the assumption that IFNs and VRGs are co-regulated and stimulated downstream by activated PRRs. However, a further positive feedback of VRG activation by IFNs is also likely.

In common, these features suggested a potential for VRGs as diagnostic markers for identification of a positive infection status of fish. At present, diagnostics of viral diseases is based on clinical symptoms, histopathology and detection of virus proteins or genes, which may have complications and limitations being prone to both false positive and false negative findings. Fish infected with protracted or non-pathogenic virus strains do not develop disease while assays may give positive results. CMS, HSMI and other emerging diseases are of particular challenge due to a significant time lag between the first occurrence and clinical symptoms. While the presented data were not sufficient for practical recommendations regarding diagnostic implementation of VRGs, this will be interesting to follow up in the future.

A large proportion of VRGs were not directly linked to the immune system or had unknown functions. Immune genes in fish have traditionally been identified by search for putative homologs to well-characterized mammalian genes, however recent transcriptome studies by us (**Paper IV**) and others have identified many unexpected and putative novel immune genes in fish. While presumption on the phylogenetic conservation of functions is still accepted as a guideline in fish biology, the immune system is probably one of the biological structures being most highly evolved and adapted to vertebrate classes and species. Examples of homologous genes that might have changed functions in the course of vertebrate evolution were found among the salmon VRGs, by sequence comparison of the distribution of homologous genes in different species. Results suggested a rapid evolution of VRGs in comparison with the entire proteome, and VRGs emerged both before and after separation of teleosts and tetrapods. However, the transcriptome of Atlantic salmon is still incomplete and the release of the fully sequenced genome in the near future will improve this approach.

7 Concluding remarks

Two major aims have been achieved in the work, as presented in this thesis. The first was the development of a microarray platform for Atlantic salmon research. This platform has been proven to produce reliable results and has been used successfully in different experiments.

The second aim was to increase the knowledge of host-pathogen responses of Atlantic salmon during CMS and infection with PMCV. This goal was achieved by examination of the temporal and spatial regulation of different immune pathways from transcriptome studies of experimentally infected fish. In one series of experiments, the immune response in highly affected fish was analyzed and in another, individual differences between fish with and without pathological changes were examined. These results provide new insights into host-virus responses during CMS, leading to a better understanding of the pathogenesis and correlates of pathology and protection.

Furthermore, early virus responsive genes (VRGs) that are commonly activated in different viral diseases of Atlantic salmon were identified and characterized. This provided an overview of the cellular and molecular events activated upon viral infections, resulted in many novel genes and markers of antiviral immunity as well as insight into the evolution of genes in this important functional class.

Collectively, these findings provide knowledge and tools that will improve our understanding of Atlantic salmon immunity against viruses in general and PMCV infection in particular.

8 Future perspectives

The microarray platform described in this thesis is highly adaptable to new sequence information and annotation. This allows a continuous development and improvement of the platform and if necessary also an adaptation to special needs of future experiments. Especially the release of the full genome sequence of Atlantic salmon in the near future should allow a further reduction of redundant probes and a more complete coverage of all genes. With a growing number of experiments with different diseases, improved cross-comparisons will be possible. This may result in new insights into the immune responses of Atlantic salmon.

The knowledge about PMCV is largely incomplete. The surprising family membership needs further confirmation and the virus propagation and life cycle remains unclear. These fields are of interest for future research.

The gene sets which were used in this work can be used as a basis for further experiments with other diseases to examine and compare specific immune responses.

Additional challenge trials with PMCV and Atlantic salmon may be conducted with larger fish and with cohabitation, to allow more natural conditions for a CMS outbreak. In this context, prevention strategies for CMS related mortality could be tested. Feeding strategies and reduction of stress should be considered.

Disease specific gene induction has not been identified yet. However, this can be an important field for future research. Genetic markers for distinct diseases would be precious for diagnosis of disease affected fish farms. Many viruses can be found ubiquitously and the detection of them is often not sufficient for diagnosis. Disease specific marker genes would improve the diagnostic remarkably. With a growing database of disease specific transcriptional regulation and increasing knowledge of host-virus responses, discovery of such markers are more likely.

We examined differences in gene expression between fish with and without strong pathological reactions against PMCV. We could identify T cell activity as the most remarkable difference in both groups of fish, however the reason for protection/recovery and immunity in fish without pathology remains unclear and is an interesting field of research in the future. Furthermore, possible regulators between immunity and immunopathology are of high interest.

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Paper I

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Development and assessment of oligonucleotide microarrays for Atlantic salmon (*Salmo salar* L.)☆

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ABSTRACT

The cDNA microarrays have played a major role in functional genomics of fish and contributed substantially to different areas of aquaculture research. However at present these platforms are gradually substituted with oligonucleotide microarrays (ONM), which represent the most cost-efficient, flexible, powerful and accurate tool for multiple gene expression profiling, especially in species with rich genomic resources. This paper describes the development and assessment of ONM platforms for Atlantic salmon. The process started with the establishment of a bioinformatic system, selection of a low redundancy set of nucleotide sequences providing coverage of transcriptomes of several fish species, their identification by protein products and annotations. Pilot experiments were performed to address issues that are essential for development of ONM: gene composition, quality assessment, hybridization success of homologous and heterologous probes, optimum numbers of spot replicates and processing, management and mining of gene expression data. Performance of microarrays was evaluated in two experiments with Atlantic salmon. Comparison of peripheral blood leukocytes with a mixture of other tissues was conducted for characterization of the leukocyte transcriptome. Analyses of salmon infected with different viral diseases identified virus-responsive genes that can be used as markers for diagnostics of infected status of fish. Data mining with functional annotations confirmed the relevance of these findings.

1. Introduction

Microarray analyses measure the expression of large numbers of genes in parallel. This methodology, which combines hypotheses-driven and hypotheses-free research strategies, is used for inference of molecular mechanisms, classification of samples, diagnostics and search for novel biomarkers. Due to the use of standard platforms, laboratory protocols and procedures for processing of primary data, the results of microarrays analyses are well suited for database management and meta-analysis across multiple experiments, whilst data mining is based on powerful statistical procedures with support from functional and structural annotations of genes. Salmonid cDNA microarrays were constructed shortly after large-scale sequencing of salmon and trout cDNA libraries from several research institutes. Genome-wide (von Schalburg et al., 2005; Taggart et al., 2008) and specialized (Koskinen et al., 2004; Cavileer et al., 2009) platforms have been applied for a wide range of tasks in diverse research areas including fish nutrition (Leaver et al., 2008; Kolditz et al., 2010), reproduction (Bonnet et al., 2007; Cavileer et al., 2009), stress physiology (Krasnov et al., 2005) and toxicology (Koskinen et al., 2004; Krasnov et al., 2007; Finne et al., 2007), development (Vuori et al., 2006) and differentiation of primary cell cultures (Todorcević et al., 2010). Studies of fish diseases and immunity have addressed responses to bacteria (Rise et al., 2004; Vanya Ewart et al., 2008), viruses (Jørgensen et al., 2008; Workenhe et al., 2009), fungi (Roberge et al., 2007) and parasites (Skugor et al., 2008), vaccines (Purcell et al., 2006; Skugor et al., 2009), cytokines (Martin et al., 2007) and inflammatory stimulators (MacKenzie et al., 2006; Djordjevic et al., 2009). However despite impressive achievements, cDNA platforms suffer from limitations and disadvantages. At present most research group working with salmonids and other aquaculture species do not have full access to clones required for fabrication of cDNA microarrays. Maintenance and PCR amplification of large clone sets is expensive and labour consuming while risk of errors is high. Probably the most important drawback of cDNA microarrays is their limited ability to discriminate paralogs since long probes cross-hybridize with highly similar transcripts from members of multi-gene families (Skugor et al., 2009). In salmonids this problem is aggravated with a large number of expressed duplicated genes. These complications can be resolved with

Abbreviations: BLAST, basic local alignment search tool; EST, expressed sequence tag; ONM, oligonucleotide microarray; PBL, peripheral blood leukocytes; SI, signal intensity; VRG, virus-responsive genes.

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oligonucleotide microarrays (ONM), which in addition provide greater accuracy and reproducibility of analyses. Until recently, use of ONM was hampered with high prices but at present they are rapidly substituting the cDNA platforms. First salmonid (rainbow trout) microarray contained 1672 elements representing more than 1400 genes (Tilton et al., 2005).

Construction of ONM platforms begins with establishment of mRNA sequence sets aiming at comprehensive coverage of transcriptomes at low redundancy. Next stage is identification of genes from search across protein databases and annotation by functions, pathways and structural features. For successful development and use of ONM it is necessary to define the gene composition and optimum number of spot replicates and to choose criteria for quality assessment. We worked on these issues taking advantage of experience obtained with cDNA chips. Computer programs were developed since the existing bioinformatic resources are not adapted for a number of tasks that are essential for functional genomics research in farmed fish species. Construction of relational databases is required for the management of diverse information including gene sequences, features and annotations results of microarray analyses, and complex iterative searches for large groups of genes, custom annotations and comparative genomics. The latter is especially important for aquaculture that operates with a large number of species. We are working with two most commercially important salmonids, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Both species are represented with large numbers of mRNA sequences in public databases. Despite substantial overlap, many genes have been identified in either salmon or trout and the use of heterologous probes can provide better coverage of transcriptomes. However unlike cDNA microarrays, which work well with closely related species, oligonucleotide probes are more sensitive to sequence divergence. Here we describe the construction and assessment of salmon microarrays based on the Agilent Technology system. Pilot experiments addressed key technical issues including gene composition and selection of genes, quality and variance of measurements, performance of heterologous probes and ways for updating the ONM platform. In addition, these studies produced results that are of significant interest for aquaculture research. Limited availability of peripheral blood leukocyte (PBL) markers is a well recognised problem of fish immunology. We compared the transcriptomes of PBL and other tissues to search for genes with preferential expression in leukocytes. Microarray analyses of salmon with several viral diseases identified transcripts with responses to viruses which may be used as diagnostic markers of infected status.

2. Material and methods

2.1. Selection and annotation of mRNA sequences, design of oligonucleotide microarrays for Atlantic salmon

To accomplish sequence processing and annotation in designing of OMN platforms and data analyses, computer programs were developed that included a relational database (MySQL) and applications written in Delphi programming language. Initially this system was constructed for salmonids and named STARS (Salmon and Trout Annotated Reference Sequences). Later two fish species were added: zebrafish (*Danio rerio*) and Atlantic cod (*Gadus morhua*). STARS consists of three modules; STARS-SA (Sequence Analyses) which is a stand-alone BLAST client with parser, STARS-MA (MicroArray) for primary processing, database storage and management of microarray results and experiments (meta-analysis), and STARS-GI (Gene Index) for work with genes, annotations and statistical analyses of microarray gene expression data. Beta version of STARS is publically available on request. The main sets of sequences from the four fish species were retrieved from UniGene (http://www.ncbi.nlm.nih.gov/unigene), the division of GenBank, which stores mRNA clusters, each represented with the longest sequence (Wheeler et al., 2003). The mRNA sequences from other sources — GenBank, The Gene Indices (Quackenbush et al., 2000; http://compbio.dfci.harvard.edu/tgi/tgipage.html) and own ESTs (described below) — were compared with sequences from UniGene, and those not found in UniGene (blastn, $e<-80$) were clustered with blastclustn and included in STARS. For identification and annotation, the nucleotide sequences were searched with blastx ($e<-20$) across four sets of proteins; human proteins from Uniprot (http://www.uniprot.org/) and RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/), zebrafish proteins from RefSeq and own proteins (salmonid and Atlantic cod) from GenBank. Results of blastx analyses were used for annotations. Fish genes were linked to Gene Ontology — GO (Ashburner et al., 2000) and InterPro, the database of multi-gene families (Apweiler et al., 2001) via RefSeq and Uniprot proteins using respectively Gene Ontology Annotation — GOA (Camon et al., 2003; http://www.ebi.ac.uk/GOA/) and the Sequence Retrieval System – SRS (Etzold et al., 1996; http://srs.ebi.ac.uk). Human Uniprot proteins were also used for annotations by pathways defined in Kyoto Encyclopaedia of Genes — KEGG (Ogata et al., 1999; http://www.genome.jp/kegg/). To date, three salmon ONM versions have been designed based on the 4×44 k format from Agilent Technologies (Santa Clara, CA, USA). These arrays included either genes selected by functional annotations or all protein-identified genes with addition of unidentified sequences derived from a leukocyte EST library (made from viral and bacterial infected leukocyte populations) that we recently constructed and sequenced (5000 clones, unpublished data). Rainbow trout sequences that did not match to Atlantic salmon sequences (blastn, $e<-80$) and corresponded to proteins that were not represented in the salmon genes were included in these ONMs. The sense and anti-sense orientation of sequences was determined from blastx analyses. The 60-mer probes were designed with aid of the Agilent eArray program (https://earray.chem.agilent.com/earray/). Currently used platform (Design ID 027139) is publically available on this web site.

2.2. Origin and preparation of samples

Tissue samples for the study on virus-responsive genes were from controlled and approved (http://www.fdu.no) challenge trials performed at VESO research station (Vikan, Norway), using Atlantic salmon smolt (average weight 50–100 g) infected with infectious salmon anaemia virus (cohabitation, ISAV Glesvaer2/90 isolate), salmonid alpha virus/PD-virus (cohabitation, SAV3 isolate) and cardiomyopathy syndrome (intraperitoneal injection of CMS agent, kindly provided by Pharmaq). Samples of fish infected with heart and skeletal muscle inflammation (HSMI) were from a previous study (Kongtorp and Taksdal, 2009) kindly provided by Dr. Kongtorp (National veterinary institute, Oslo, Norway). From the respective challenge trials above, samples from parallel tanks with uninfected control groups were collected and used as references for microarray hybridizations. All samples were collected from anaesthetized fish according to RNAse-free procedures and preserved in RNAlater (Applied Biosystems/Ambion, Austin, TX, USA) until preparation. Blood samples for the leukocyte–transcriptome study were from the same ISAV, CMS-infected and control fish as described above. PBL fractions were freshly isolated from blood as described (Fischer and Koellner, 2007) and RNA immediately extracted. To search for leukocyte-specific transcripts, PBL were compared with a normalized mixture of RNA from tissues (heart, liver, spleen, skeletal muscle, skin and intestine). Heart and PBL of virus-infected salmon were hybridized to samples of corresponding tissues from uninfected control fish.

2.3. RNA extraction and microarray analyses

Tissue samples (~10 mg) were placed in 2 ml tubes with screw caps (Precellys24, Bertin Technologies, Orléans, France) containing 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA). Two steel beads (diameter 2 mm) were added to each tube and samples were homogenized in Precellys®24 homogenizer for 2×25 s at 5000 rpm with a break of 5 s between rounds. RNA was extracted from the homogenized tissues

Table 1
Summary of genomic resources represented in the STARS database.

Species	Number of all sequences	Number of UniGene sequences	Number of identified sequences	Number of proteins
Atlantic salmon	75,953	33,647	25,921	11,308
Rainbow trout	57,972	26,681	20,523	10,950
Atlantic cod	19,367	15,382	4849	3464
Zebrafish	51,141	51,141	28,095	14,960

Table 2
Features of salmonid oligonucleotide microarrays presented in the article.

ONM version	SIQ1.0	SIQ2.0	SIQ3.0
Total probe number	10,316	21,013	21,323
Atlantic salmon	7575	17,608	18,132
Rainbow trout	2741	3405	3191
Spot replicates	4	2	2
Analyzed samples[a]	4	100	40

[a] By date of manuscript submission.

using PureLink RNA mini kits (Invitrogen) according to the manufacturer's protocol. Concentration of total RNA was measured with NanoDrop 1000 Spectrometer (Thermo Scientific, Waltham, MA, USA) and quality was assessed using Agilent 2100 Bioanalyzer with RNA Nano kits (Agilent Technologies). Samples with RNA integrity number (RIN) of 8 or higher were accepted for microarray analyses. Unless specified otherwise, all reagents and equipment used for microarray analyses were from Agilent. RNA amplification and labelling were performed using Quick Amp Labelling Kits, Two-Colour and RNA Spike-In Kits, Two-Colour following the manufacturer's protocol for 4×44k microarrays; each reaction used 500 ng of total RNA. Gene Expression Hybridization Kit was used for fragmentation of labelled RNA. Hybridizations to microarrays were performed in hybridization oven (Agilent Technologies) at 65 °C and rotation speed of 10 rpm. After hybridization, arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA). GenePix software was used for spot-grid alignment, feature extraction and quantification. Assessment of spot quality was done with aid of GenePix flags and by ratio (R) between the difference of signal and background intensities ($SI-SB$) and sum of their standard deviations (SD_I+SD_B). After filtration of low quality spots, Lowess normalization of $\log_2$-expression ratios (ER) was performed. Analyses presented in this paper include 3 microarrays (leukocyte–transcriptome study) and 9 microarrays (virus–responsive genes study).

3. Results

3.1. Design and performance of Atlantic salmon oligonucleotide microarrays

Designing of ONM platforms for Atlantic salmon was based mainly on publically available mRNA sequence resources. We added ESTs from our leukocyte library (2.7% of all Atlantic salmon sequences) to improve presentation of leukocyte-specific transcripts. By using UniGene supplemented with sequences from GenBank and Gene Indices we aimed at a comprehensive coverage of the sequence identified fish genes. The STARS database is updated after each new release of UniGene, new sequences are processed and annotated using standard procedures (see Section 2.1). The genomic resources used for designing ONM are summarized in Table 1. The ratio of protein-identified sequences was highest in zebrafish (0.55), intermediate in two salmonid species (0.34 in Atlantic salmon and 0.35 in rainbow trout) and lowest in Atlantic cod (0.25). Total number of reference proteins identified in both salmonids (14,974) was similar to that in zebrafish, the teleost species with the greatest amount of mRNA sequences. The overlap of sets of proteins represented in Atlantic salmon and rainbow trout was equal to 65%. Therefore, use of heterologous probes in ONM substantially improved coverage of the transcriptomes in both species.

To date, three microarray platforms have been designed and used for Atlantic salmon (Table 2). First microarray (SIQ1.0 — Salmon Immunity and Quality) included selected genes from the functional classes and pathways of importance for these areas and individual genes suggested by research partners. We also included 2043 EST sequences from our leukocyte library, 1252 of which were unidentified. SIQ1.0 consisted of 10,316 probes, each in 4 replicates. Next versions, SIQ2.0 and SIQ3.0 aimed at a complete coverage of protein-identified genes. SIQ3.0 was designed taking into account results produced with previous microarrays and new sequences that appeared in UniGene after last update in September 2009.

Results produced in pilot analyses were used to assess the performance of OMN and to establish procedures for data processing. In our work with cDNA microarrays we compared several methods for evaluation of quality and eventually selected the $(SI-SB)/(SD_I+SD_B)$ ratio (R) and GenePix flags. With respect to ONM, both procedures produced curves of similar shapes and the results were nearly identical at R equal to 0.15 (Fig. 1). Both methods can be used, however R-filtration

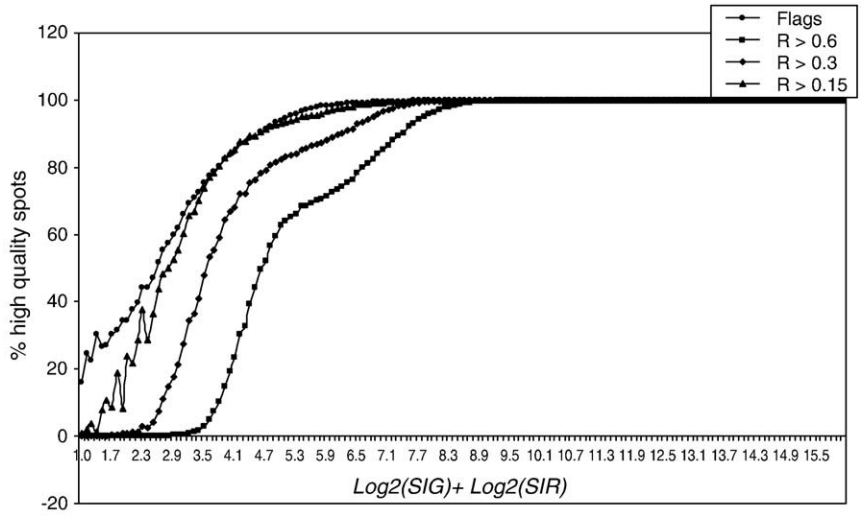

Fig. 1. Comparison of procedures for filtration of spots. Quality was evaluated with GenePix flags and with R-ratio. Panel presents relationship between the percentage of high quality spots and the sum of $\log^2$-SI in green (SIG) and red (SIR) channels.

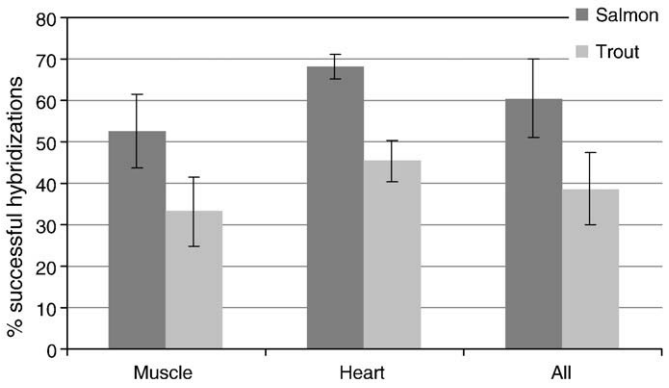

Fig. 2. Success of hybridizations to Atlantic salmon and rainbow trout probes evaluated as percentage of spots that passes quality control in skeletal muscle (36 samples), heart (42 samples) and all results (104 samples). Data are mean ± SD.

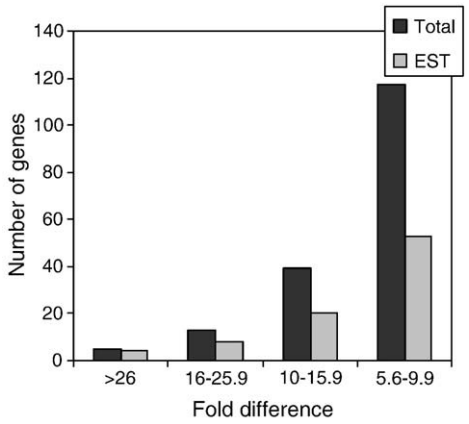

Fig. 4. The numbers of genes with different expression ratios between PBL and other tissues.

appears more stringent at weak signals. The major fraction of disqualified spots was located in the area of low *SI*. Therefore, if labelling is uniform the probability of accurate detection and measurement of transcripts depends mainly on their abundance. Success of hybridizations was evaluated for each probe by percentage of samples that passed quality control and as expected the results differed between tissues (Fig. 2). However the number of probes that were filtered in all analyzed samples was small. Overall, homologous (Atlantic salmon) probes showed consistently greater success of hybridization though many rainbow trout probes produced high *SI* and reliable results. Analysis of variation in technical replicates is important for evaluation of accuracy and reproducibility of results (Fig. 3). The variation of expression ratio was relatively high at low *SI*, then decreased substantially and stabilized at *SI* above the threshold indicated with arrow.

3.2. Comparative transcriptome analysis of leukocytes and tissues

The SIQ1.0 array was used to search for candidate markers of leukocytes by comparison of PBL against pooled normalized mixtures of tissues (see Section 2.2). Three hybridizations were performed and 174 probes showed >5.6-fold ($\log_2 - ER > 2.5$) greater expression in all analyzed samples. In total, 6215 probes passed quality check in all samples and 1235 probes of these (19.9%) corresponded to ESTs from our leukocyte library. These ESTs comprised 85 of 174 selected transcripts (49.9%) and enrichment of probes from this set was greatest among transcripts with highest expression differences between PBL and other tissues (Fig. 4); 33 ESTs with PBL-specific expression have not been identified by protein products. The complete list of genes with preferential expression in PBL can be found in the supplementary data and the 20 genes with greatest expression differences (>15-fold) are presented in Table 3. This list includes 14 ESTs, 5 of which are unknown. Highest expression differences were observed in a C-X-C chemokine receptor and leukocyte cell-derived chemotaxin 2, and in several genes involved in leukocyte recruitment and migration. Integrins are essential for leukocyte interactions with other cells and extracellular matrix (ECM). Fermitin homolog or kindlin-3 belongs to a family of proteins that mediate integrin activation (Larjava et al., 2008). Matrix metalloproteinases MMP9 and MMP13 destroy ECM thus facilitating infiltration of leukocytes in solid tissues, and hyaluronidase may have a related function. CD40, a member of the TNF-receptor superfamily, is a costimulatory protein required for antigen presentation. JunB can be involved in a multitude of immune processes as a part of AP1 transcription factor. The list also includes three genes encoding effector proteins. Neutrophil cytosolic factor and myeloperoxidase are required for production of pathogens destructing free radicals while complement factor D is a component of the alternative pathway. High expression level of fructose-1,6-bisphosphatase may indicate the importance of gluconeogenesis in Atlantic salmon leukocytes. The search for GO classes, InterPro families and KEGG pathways enriched among the PBL-specific transcripts (Table 4) confirmed high relevance for this group of

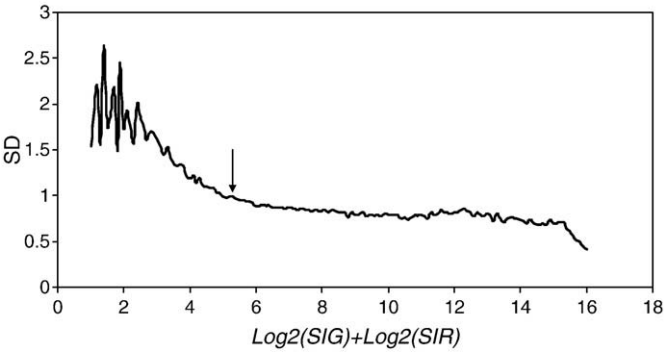

Fig. 3. Standard deviation of $\log_2$-expression ratio (SD, *y*-axis) versus signal intensity (*x*-axis). Results of all hybridizations were analyzed; spots were filtered with GenePix flags. Lower threshold value for acceptable signal intensity is indicated with an arrow.

Table 3
Genes with highest expression differences (>15-fold change) between PBL and other tissues.

GenBank accession	Gene name	Fold change
NM_001165293.1	C-X-C chemokine receptor type 4[a]	69.75
BT049525.1	Leukocyte cell-derived chemotaxin 2 (LECT2)[a]	60.76
GO058305	Matrix metalloproteinase-9 (MMP9)[a]	27.64
BT045762.1	Fermitin family homolog 3[a]	22.29
BT059155.1	Unknown[a]	22.02
DY697071	Integrin beta-1 precursor	21.72
BT046016.1	Collagenase 3 precursor (MMP13)[a]	19.32
BT046046.1	Neutrophil cytosolic factor 1[a]	18.52
BT072012.1	Myeloperoxidase precursor[a]	18.13
EG836561	Apoptosis inducing protein D	17.63
EG922433.1	Complement factor D precursor	17.53
DY720173.1	Unknown[a]	16.98
EG891184.1	CD40	16.88
BT044843.1	Transcription factor jun-B[a]	16.04
EG906557.1	Unknown[a]	15.87
DY706460	Family with sequence similarity 5C	15.68
BT072459.1	Fructose-1,6-bisphosphatase 1[a]	15.68
DW543769.1	Unknown	15.29
BT045573.1	Hyaluronidase-2[a]	15.18
DY694368.1	Unknown[a]	15.07

[a] ESTs from our leukocyte library.

Table 4
Enrichment of functional classes (GO), multi-gene families (Interpro) and pathways (KEGG) in the leukocyte transcriptome.

	No. genes	*p* Value[a]
Gene ontology		
C-X-C chemokine receptor activity	4	0
G-protein coupled receptor signaling	7	0.012
Integrin complex	5	0.00015
Regulation of apoptosis	4	0.006
Interpro		
Toll-Interleukin receptor	3	0
Peptidoglycan binding-like	4	0
Matrixin and adamalysin	4	0
KEGG		
Acute myeloid leukemia	6	0.001
Arachidonic acid metabolism	4	0.03
B cell receptor signaling pathway	5	0.04
Cytokine–cytokine receptor interaction	8	0
ECM–receptor interaction	6	0.03
Hematopoietic cell lineage	8	0
Jak-STAT signaling pathway	11	0
Leukocyte transendothelial migration	9	0.002
Neuroactive ligand–receptor interaction	4	0.03

[a] Yates' corrected chi square test.

genes. In addition to the functional groups mentioned above, there were genes involved in hematopoiesis and maturation of B cells, Jak-STAT pathway and arachidonic acid metabolism that includes production of lipid inflammatory regulators.

3.3. Identification of virus-responsive genes

Results of microarray analyses in the hearts of salmon with PD, CMS and HSMI and PBL from fish infected with ISAV were used for identification of virus-responsive genes (VRG). We found 95 transcripts with >2-fold induction in at least 6 of 8 analyzed samples (Supplementary data). Most of these genes have known roles in immune responses or their association with anti-viral responses can be predicted from stimulation with IFN and virus (VHSV). The list of the 20 most up-regulated transcripts is shown in Table 5. Barrier-to-autointegration factor (BAF) facilitates integration of retroviruses in host genomes (Segura-Totten and Wilson, 2004). The DHX58 helicase is a putative homolog to mammalian LGP2, which binds viral dsRNA and regulates virus-sensing with the cytoplasmic receptor RIG-1 (Satoh et al., 2010). The IFN-induced GTPase Mx is one of the best studied anti-viral genes in salmonid fish, which is widely used for detection and monitoring of responses to pathogens (Robertsen, 2006). Another gene belongs to the family of large inducible GTPases. Radical S-adenosyl methionine domain-containing protein, also known as viperin, inhibits release of viruses from plasma membranes of infected cells (Wang et al., 2007). This gene showed dramatic up-regulation in ISAV-infected TO cells as well as several genes with unknown functions: sacsin and IFN-induced IFIT-5 and Gig2 (Workenhe et al., 2009). Marked responses to viruses were shown by closely related but structurally different proteins from several multi-gene families. Two transcripts encoding 52 kDa Ro proteins belong to a large TRIM family that includes genes with anti-viral activities in fish (van der Aa et al., 2009) and higher vertebrates (Nisole et al., 2005). Several TRIM-encoding transcripts were induced by ISAV in salmon TO cells (Workenhe et al., 2009). Ubiquitin-like proteins are involved in a multitude of defensive responses in virus-infected cells. We analyzed enrichment of GO and KEGG terms among VRG, but in contrast to the leukocyte transcriptome this search did not reveal any significant functional groups or pathways.

Table 5
Virus-responsive genes.

GenBank accession	Gene	Fold increase		
		Mean	Min	Max
CB500614.1	52 kDa Ro protein-1	20.86	6.46	51.07
DY704952	52 kDa Ro protein-2	15.12	4.31	27.83
BT049316.1	Barrier-to-autointegration factor (BAF)	18.02	13.99	21.53
BT044881.1	IFN-induced GTP-binding protein Mx	11.87	9.67	28.81
BT046021	IFN-induced protein with tetratricopeptide repeats 5 (IFIT-5)	18.54	11.05	25.38
BT044026.1	IFN-inducible protein Gig2-like-1	46.06	12.59	103.57
EG815123.1	IFN-inducible protein Gig2-like-2	9.24	5.64	15.96
111553317	KIAA1593 protein (similar to bloodthirsty)	7.29	3.63	21.26
209154815	ATP-dependent RNA helicase DHX58	10.55	6.55	23.64
209733083	Radical S-adenosyl methionine domain-containing 2	32.89	12.86	74.70
209734507	Receptor-transporting protein 3-1	17.23	11.52	29.13
DW538275.1	Receptor-transporting protein 3-2	68.19	46.62	119.20
117833400	Sacsin	18.12	10.13	36.73
117509696	Similar to very large inducible GTPase 1	18.18	13.75	28.34
209735329	Ubiquitin-like protein 1-1	17.32	13.48	20.84
209737699	Ubiquitin-like protein 1-2	16.10	12.47	21.24
117504401	Ubiquitin-like protein	27.31	22.25	32.50
BT072288.1	VHSV-inducible protein	13.38	9.46	18.95
117459355	Zinc finger, NFX1-type containing 1	7.83	4.73	17.94
DY694368.1	Unknown	16.34	13.08	20.17

4. Discussion

4.1. Development of salmon ONM

The key prerequisite for successful development of ONM is the selection of sequences providing an appropriate coverage of expressed genes at minimal redundancy. Unlike species with completely sequenced genomes, most salmonids genes are currently represented in public databases with only mRNA sequences. We selected UniGene as the core set since GenBank provides sequence assembly of high quality and this database most likely will be maintained and updated in future. Use of a common source by different research groups working in functional genomics of salmonids would standardize the identifiers of genes thus promoting exchange of gene expression data and meta-analysis. At present it is impossible to evaluate precisely the redundancy of the sequence sets that we have formed for Atlantic salmon and rainbow trout. The ratio of numbers of identified nucleotide sequences to the numbers of proteins in STARS, which is close to two in both salmonid species, provides an overvalued estimate. Part of sequences that match the same reference proteins in the blastx analyses corresponds to duplicated genes or members of multi-gene families. Use of contigs instead of unique sequences from UniGene would probably reduce the number of probes corresponding to the same genes. UniGene does not produce contigs since forced merge of sequences from different clones is error prone especially when dealing with members of multi-gene families with high sequence conservation, recently duplicated genes and splice variants. In our view moderate redundancy in ONM platforms is acceptable and moreover, independent probes corresponding to same loci are valuable for assessment of quality and reproducibility of measurements.

Judging by the numbers of identified proteins, coverage of transcriptomes in Atlantic salmon and rainbow trout is at present close to that in zebrafish. Actual numbers of protein coding sequences in STARS is greater since many genes most likely have not been identified with blastx. The number of reference salmonid protein sequences in public databases is still small. Part of salmonid genes either lack orthologs in other taxae or their identification is hampered with high sequence divergence. However it is noteworthy that the number of zebrafish proteins represented in UniGene is almost half of the number of reference proteins in RefSeq, a large part of which were predicted from genomic sequences. Some of these proteins can be unexpressed. However it is likely that new genes will be identified

after sequencing of libraries from specialized cell types and developmental stages. Complete coverage of Atlantic salmon and rainbow trout genes will become available after the genome is fully sequenced and annotated. Then it will be possible to design unique probes to all transcribed loci and to analyze the whole transcriptome excluding genes whose expression levels are below detection thresholds.

Identification and annotation of nucleotide sequences are important tasks that can be implemented in different ways. It is customary to perform blastx searches across the complete set of proteins deposited in GenBank or Uniprot using Blast2GO (Conesa et al., 2005) or similar programs. We did not use this approach since a large part of proteins with best matches are predicted sequences from genome projects that lack sensible names and reliable annotations. Furthermore, the composition of protein databases changes rapidly, especially with the advent of new genome sequencing projects. Searches performed in different times produce different results. This impedes standardization of gene names and comparison of results produced with different ONM platforms. We chose reference human proteins as the least redundant and best annotated set, which most likely will not be subject to major changes in the future. Naming and annotation of zebrafish proteins are still far from completion. However since genome of this species has been sequenced one may assume that most part of proteins have been identified at the sequence level. We used RefSeq since this database provides low redundancy sets of proteins with concise and informative names. The Uniprot proteins were included in analyses since they are linked to KEGG, while GO and InterPro annotations via RefSeq and Uniprot produced slightly different results.

When designing ONM one may choose between genome-wide and specialized platforms, using all or protein-identified sequences. In our previous work with cDNA microarrays we preferred to select genes by their functional roles. The limited number of genes (1800 in the last version, SFA2.0) made it possible to print each clone in six spot replicates and to our experience this was important for the technical accuracy of gene expression analyses. With respect to research tasks pursued in this paper, full coverage appeared a more relevant approach than selection of genes by annotations. The probability of finding markers among leukocyte ESTs was noticeably higher in comparison with genes selected by the functional annotations, though genes from all immune-related GO classes and KEGG pathways were included in the microarrays. The shortage of annotations in public sources is obvious, particularly for immune-related genes as exemplified with the salmonid VRGs described in this study. Many of these genes contained terms “IFN-” and “VHSV-inducible” in their names and mining of databases and publications found their association with viral infections. However none of these VRG were annotated as virus-responsive in GO and the most part is not included in KEGG pathways. Due to large numbers of probes in genome-wide ONM the number of spot replicates was decreased to two. However given a small variation of measurements at signal intensity levels above the threshold, this probably did not affect accuracy of results for a large part of genes. Microarray formats provided by Agilent (15 k, 44 k and 244 k) are well compatible with genome-wide platforms. At present we are using a 21 k platform as the main version however modifications will continue. Until now preference has been given to the protein-identified and annotated genes since this facilitates interpretation of results. However screening of unknown sequences is important for gene discovery and identification of novel diagnostic markers. Despite relatively small number of unknown sequences in our microarrays they were represented between both leukocyte markers and VRGs. Multiple spot replicates are recommended for genes expressed at low levels since the probability of error increases substantially at low SI. With the accumulation of data it will be possible to define genes that are not expressed at detectable levels in particular tissues and cell types and these can be excluded from specialized platforms. In addition to genome-wide and tissue-specific microarrays, application-targeted ONMs with small numbers of genes can be considered for standard research and diagnostic-related research tasks. In addition to Atlantic salmon ONM we have designed platforms for rainbow trout and Atlantic cod. Standard procedures are being used for processing and annotation of nucleotide sequences and new species can be easily incorporated in our bioinformatic system.

Substitution of cDNA platforms with ONM opens opportunities for solution of most urgent problems of functional genomics of fish. We propose an approach based on integration of the instruments (microarray) and bioinformatic tools, which in our view can provide conceptual advances in this research area. Already at present salmonid ONM cover a substantial fraction of transcriptome while simple and explicit procedures for updates of Gene Index and composition of microarrays ensure sustainable development. Standardization of gene names and annotations facilitates an exchange of data between research groups and promote meta analyses thus increasing the value of each separate study. Cross-species sequence comparison and identification of homologous genes open the way to comparative genomics adding a new dimension to functional genomics of fish. Biological interpretation of gene expression profiles is the most important part of transcriptomic research. We have achieved more detailed annotations of fish sequences in comparison with those produced with other tools. Given limitations of public databases, our bioinformatic system is adapted for custom annotations based on literature mining and analyses of the gene expression profiles.

4.2. Results of pilot studies

Studies reported in this paper were conducted mainly to work on the technical issues and to validate the performance of the ONM platforms developed for Atlantic salmon. However pilot analyses produced results that can be valuable for fish immunology and functional genomics. Availability of markers specific for subpopulations of lymphoid and myeloid cells is a prerequisite for basic research on the salmonid immune system and practical monitoring and diagnostics of fish health and disease. Although antibodies against leukocyte subpopulations have been published for several fish species (see Fischer and Koellner, 2007), there is a limitation of such tools for fish and in particular for salmonid species. Existing tools are also based on search for homologs to mammalian proteins which may have obvious limitations given the high probability of species-specific adaption of immune molecules and functions in phylogenetically remote groups. Thus many salmonid immune genes and proteins apparently do not have homologs in higher vertebrates. Functional genomics and microarrays provides a promising strategy for identification of novel immune genes and markers since high numbers of candidate transcripts with both known and unknown functions (as judged from homology search) can be effectively screened and compared across samples. Pilot microarray analyses in this study identified a panel of transcripts with higher expression levels in PBL, and a simple search for enriched GO classes and KEGG pathways confirmed high relevance of the results. The transcripts identified as candidate markers are involved in the full range leukocyte activities. Identification of cell-specific genes is essential for correct interpretation of microarray results. Microarrays may find differences in the abundance of transcripts, which can be caused by regulation of expression and/or changes in the cellular composition. Earlier we observed dramatic changes of LECT2, MMP9 and MMP13 under various conditions including handling stress (Krasnov et al., 2005), treatment with lipopolysaccharide (MacKenzie et al., 2006), exposure to toxicity (Krasnov et al., 2007) and infestation with parasites (Skugor et al., 2008). Given the high expression of these transcripts in PBL, one may conclude that changes were caused largely by infiltration of leukocytes or activation of resident monocytes/macrophages. To continue the search for leukocyte markers it will be necessary to perform the analyses of PBL from healthy salmon and from fish with different diseases using ONM with probes to both

identified and unidentified transcripts. Identification of tissue- and cell-specific genes is a common task in different areas of fish biology including reproduction and development. Our results suggest that combination of library sequencing and microarray analyses can be recommended as an efficient approach. Inclusion of ESTs in ONM improves the probability of finding candidate markers.

Search for VRG is important given the challenges related to proper diagnostics of infected status of fish in aquaculture. At present this task is accomplished with the finding of pathogens. New viral diseases appear in salmonid aquaculture continuously while the identification of pathogens and development of diagnostic assays take time. Tests based on host responses to viruses would be valuable for the monitoring of fish health and disease status. Analyses with ONM identified a group of genes with similar up-regulation in salmon with different viral diseases. Importantly, these genes increased expression in salmon with CMS and HSMI, novel diseases with presumably viral aetiology. We selected transcripts that responded to different infections and a large part of these showed similar induction in salmon cells infected with ISAV (Workenhe et al., 2009). Close resemblance of gene lists produced in studies that used different experimental material and microarrays suggested that a finite set of actors is involved in generalized innate protection against viruses. A large part of VRGs have unknown roles, which are awaiting further investigation.

5. Conclusions

The main goal of the work reported in this paper was to promote the development and application of oligonucleotide microarrays to aquaculture research. We have developed a bioinformatic pipeline (STARS), which assists the designing and updates of microarray platforms for the key farmed fish species, processing of primary results and data mining. Results of pilot studies confirmed a high power and accuracy of gene expression profiling with a salmon ONM (SIQ) and produced sound results. Use of this system by different groups would facilitate exchange of data and meta-analysis thus increasing the value of each separate study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbd.2010.04.006.

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Paper II

Paper II

BMC Genomics

RESEARCH ARTICLE Open Access

Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon

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Abstract

Background: Cardiomyopathy syndrome (CMS) is a disease associated with severe myocarditis primarily in adult farmed Atlantic salmon (*Salmo salar* L.), caused by a double-stranded RNA virus named piscine myocarditis virus (PMCV) with structural similarities to the *Totiviridae* family. Here we present the first characterisation of host immune responses to CMS assessed by microarray transcriptome profiling.

Results: Unvaccinated farmed Atlantic salmon post-smolts were infected by intraperitoneal injection of PMCV and developed cardiac pathology consistent with CMS. From analysis of heart samples at several time points and different tissues at early and clinical stages by oligonucleotide microarrays (SIQ2.0 chip), six gene sets representing a broad range of immune responses were identified, showing significant temporal and spatial regulation. Histopathological examination of cardiac tissue showed myocardial lesions from 6 weeks post infection (wpi) that peaked at 8-9 wpi and was followed by a recovery. Viral RNA was detected in all organs from 4 wpi suggesting a broad tissue tropism. High correlation between viral load and cardiac histopathology score suggested that cytopathic effect of infection was a major determinant of the myocardial changes. Strong and systemic induction of antiviral and IFN-dependent genes from 2 wpi that levelled off during infection, was followed by a biphasic activation of pathways for B cells and MHC antigen presentation, both peaking at clinical pathology. This was preceded by a distinct cardiac activation of complement at 6 wpi, suggesting a complement-dependent activation of humoral Ab-responses. Peak of cardiac pathology and viral load coincided with cardiac-specific upregulation of T cell response genes and splenic induction of complement genes. Preceding the reduction in viral load and pathology, these responses were probably important for viral clearance and recovery.

Conclusions: By comparative analysis of gene expression, histology and viral load, the temporal and spatial regulation of immune responses were characterised and novel immune genes identified, ultimately leading to a more complete understanding of host-virus responses and pathology and protection in Atlantic salmon during CMS.

Background

Cardiomyopathy syndrome (CMS) is a severe cardiac disease affecting Atlantic salmon (*Salmo salar* L.). Since its first diagnosis in Norway 1985 [1], it has also been diagnosed in sea farms in Scotland, the Faroe island, Denmark and Canada [2]. CMS primarily affects farmed fish from 12 to 18 months after transfer to sea water [3,4], but cases of CMS in wild salmon have also been observed [5].

The diagnosis of CMS is based on cardiac histopathology, characterised by severe inflammation and necrosis of the spongy myocardium of the atrium and ventricle [6]. Inflammatory infiltrates consist of mononuclear cells, probably lymphocytes and macrophages. The compact layer of the ventricle is usually less affected, and always occurs later than changes in the spongious layer [6,7]. Farmed salmon suffering from CMS often lack clinical signs and may die suddenly due to rupture of

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the atrium or sinus venosus resulting in cardiac tamponade [1,6]. Other symptoms like skin haemorrhages, raised scales and oedema have also been reported [3,5]. At necropsy, ascitic fluid, fibrinous perihepatitis and blood clots on the liver and heart are typical findings [3,5,6]. The first study indicating a transmissible nature of the disease, showed typical cardiac lesions in salmon post-smolts six weeks post injection of cardiac and kidney homogenate from CMS-diseased fish [7].

Recently a novel virus associated with CMS was cultured and identified [8]. The proposed virus named piscine myocarditis virus (PMCV) is a double-stranded RNA virus with structural similarities suggesting assignment to the *Totiviridae* family. In this study, viral RNA could be detected by quantitative real-time RT-PCR (qPCR) from 2 weeks post challenge, peaking at 6-8 weeks post challenge, coinciding with the increase of histopathological lesions in the heart. Virus particles were also detected by *in situ* hybridization in degenerate and necrotic cardiac myocytes from field outbreaks of CMS.

In the present study, the same PMCV inoculum was used to experimentally reproduce CMS and to characterise the host immune response in infected salmon post-smolts. To gain an understanding of the immune response and host-virus interaction, a genome-wide approach based on oligonucleotide microarrays was used [9]. Six gene sets representing different arms of the immune response were identified, and temporal and spatial regulation was evaluated in combination with histology and relative quantification of viral RNA. The findings provide a comprehensive understanding of the immune response against PMCV in Atlantic salmon, and pathological and protective correlates thereof.

Results

Experimental CMS infection

No mortality or clinical signs associated with CMS was observed. Potential contamination by other pathogens was excluded by qPCR for known viruses and bacteria from relevant organs and numbers of samples. Histopathological examination of heart was scored 0-3 according to severity of CMS lesions, as summarised in Figure 1. Results were used for evaluation of the infection challenge and for design of gene expression analyses. In control groups, one fish had moderate to severe cardiac lesions at 10 wpi, and was graded score 2 in the spongy layer of the ventricle and score 3 in the atrium. For all the other control fish, only score 0 and 1 were observed. No statistical difference between replicate control groups was found.

Groups receiving PMCV inoculum developed cardiac lesions consistent with CMS from 6 wpi and onwards. At 6 wpi, 63% of the infected fish had moderate lesions (score 2) in the atrium (percentages refer to observations, excluding missing values). Lesions were first found in the atrium and subsequently in the spongy layer of the ventricle. The peak of histopathological lesions was observed at 8 wpi, with moderate atrial lesions (score 2) in 36%, and severe lesions (score 3) in 32% of the fish. In the subsequent time points, fewer fish had cardiac lesions, and at 9, 10 and 11 wpi, respectively 7.4%, 4.3% and 3.8% of the fish were scored 3. At 12 wpi, only mild focal lesions (scores 0 and 1) were described in the atrium and spongy ventricle. In general, lesions were first found in the atrium and were more severe than in the spongious layer. Differences between group 3 and 4 were significant for atrial lesions at 10 wpi and epicardial lesions at 11 wpi. Lesions in atrium of control groups 1 and 2 versus infected groups 3 and 4 were statistically different for all time points except 12 wpi, with highest significance between 4 and 11 wpi ($p < 0.01$). A similar difference was found in spongious lesions with highest significance between 6 and 11 wpi. Lesions in epicardium differed significantly between infected and controls at 4, 6 and 9 wpi.

Viral load

PMCV levels were analysed by qPCR to document viral replication in heart during infection and in the different tissues at early infection (4 wpi) and peak pathology (8 wpi) stages (Figure 2). The same six individuals per time point as used for gene expression analyses were tested. Since 0 wpi and the two latest time points (11 and 12 wpi) were not included in microarray analysis, six randomly chosen samples from group 3 and 4 were tested respectively. At 2 wpi, 5 out of 6 fish were positive for viral RNA in heart (median of relative copy number = 20.5 fold, Figure 2a). Levels increased strongly until 4 wpi and then gradually until 6 wpi (median of relative copy number = 11, 583 fold), concurrent with the onset of histopathological changes. Levels reached a plateau phase between 6 and 10 wpi with no significant changes in viral RNA. From 10 to 11 wpi, levels were significantly reduced, indicating a clearance of virus. One week later (12 wpi), both viral load and individual variance were reduced. For most time points, individual variation in viral RNA was observed, analogous to the variation observed for histopathology score. Correlation between histopathology scores and viral C_T levels in heart was highly significant (correlation coefficient: 0.75, $p = 5.5 \times 10^{-11}$) (Figure 3).

Comparison of viral loads between tissues showed highest and equal viral loads in heart, spleen and kidney (Figure 2b). Significantly lower and equal levels of viral RNA were found in blood cells (PBL and RBC) and liver. Except for heart ($p = 0.030$), viral loads were not significantly different between 4 and 8 wpi in any of the tissues investigated.

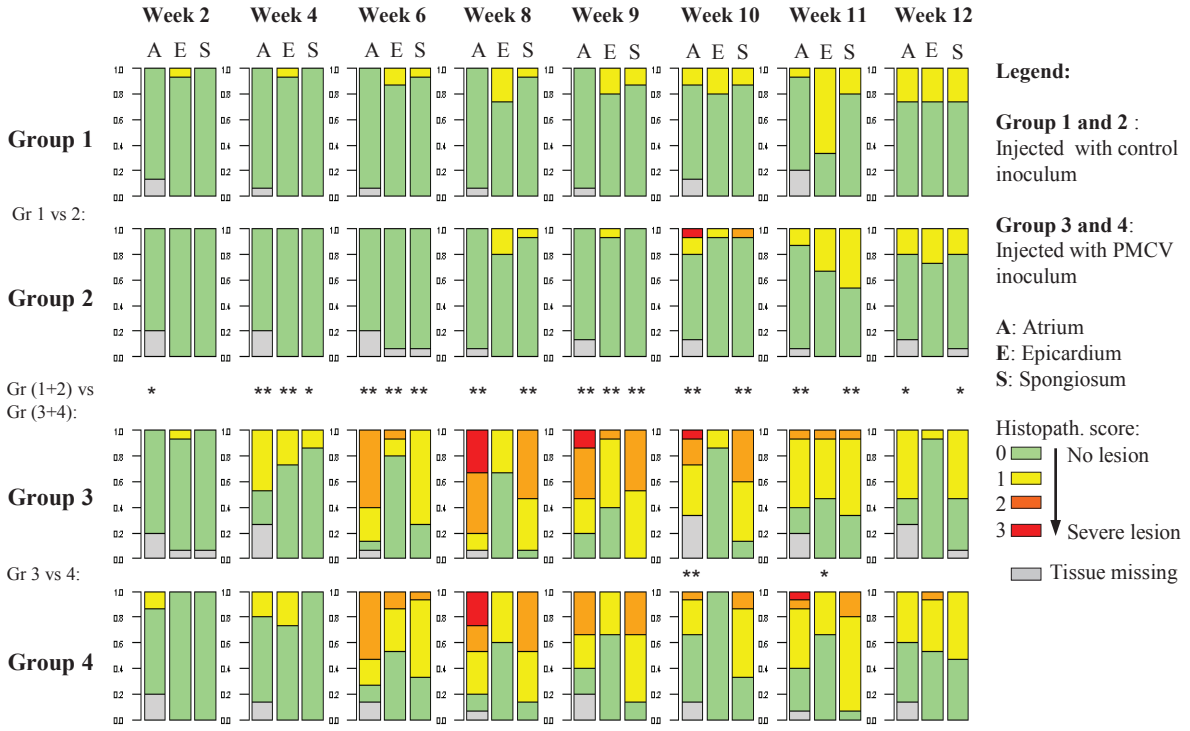

Figure 1 Cardiac histopathology results of PMCV infected and control groups. The proportions of histopathology scores in atrium (A), epicard (E) and spongious (S) were plotted as percentage bars. The bars represent the results of the 15 sampled fish per group and time point. The histopathology scores are shown as colours from green (score 0) to red (score 3), grey indicates missing tissue. Asterisks in the middle row represents the level of significance between groups (*/** = $p < 0.05/0.01$), according to two-sided *t*-tests. The row in the middle represents the difference between the two infected groups and the two control groups.

Identification of gene sets representing immune pathways

The main purpose of the gene expression study was to identify gene sets representing different immune pathways and characterise their regulation over the time course of CMS in the infected organs. Fish were challenged by injection to ensure simultaneous infection and virus dose. Since histomorphological changes were investigated in cardiac tissue, RNA from infected versus control heart samples from six time points (2, 4, 6, 8, 9 and 10 wpi) were used for microarray analysis. In order to examine responses in fish with similar disease status and infection level, individuals with highest histology scores and viral loads were selected from the time points when pathological changes were significant (6-10 wpi). After microarray experiments, 5712 differentially expressed genes with a mean $\log_2$-ER > |0.65| in at least one time point were selected. Genes implicated in different immune pathways were defined in the resulting list using the STARS software package [9], which contains custom annotation of genes on the microarray based on GO classes, KEGG pathways, mining of literature and public databases and experimental evidence (transcription profiles/meta-analysis). Further, immune genes were arranged in seven sets taking into account both functions and the expression profiles. Six gene sets (Additional file 1) showed differential expression between at least two subsequent time points (one-way ANOVA with Newman-Keuls test, Additional file 2), while one gene set (inflammatory components) was excluded since no significant temporal changes were found. The $\log_2$-ER for all genes per gene set and time point were combined from microarray results of the two sample pools (2, 6, 9, 10 wpi) and four sample pools (4 and 8 wpi). The resulting expression profiles of the six gene sets are shown as box plots in Figure 4. Gene composition and temporal regulation for each gene set is presented in the following section.

Composition and temporal regulation of immune pathways

1: Early antiviral and interferon response

This gene set included 85 genes associated with nonspecific innate immunity related to the early antiviral and interferon (IFN) responses. This also included predicted pattern recognition receptors (e.g. toll-like receptors and RIG helicases) and associated genes, and early induced virus-responsive genes known from other salmonid viral

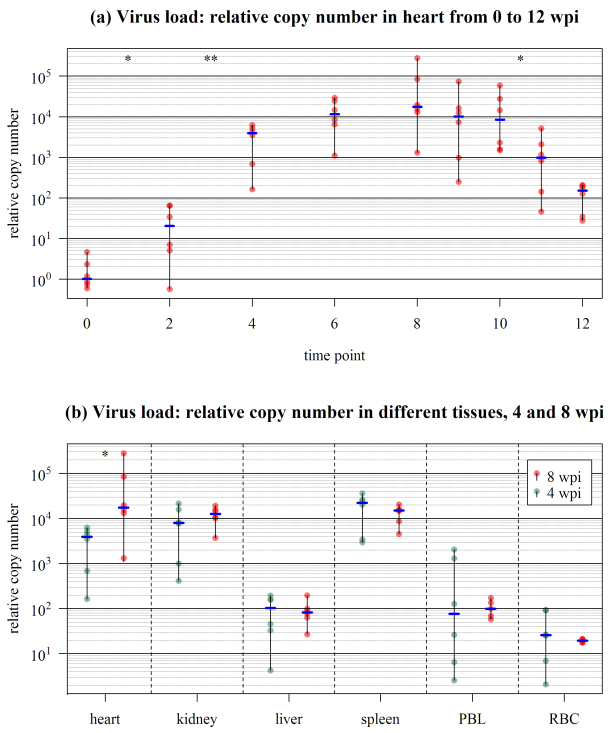

Figure 2 Assessment of viral load by qPCR. (a) Time course study in heart tissue: PMCV levels are shown as relative copy numbers for six individuals per time point. Copy numbers are relative to the median of the 0 wpi values which was set to 1. The black lines show the variance and blue bars represent the median. (b) PMCV levels in different tissues at 4 and 8 wpi. Relative copy numbers are shown as green dots (4 wpi) or red dots (8 wpi). Numbers are relative to median of 0 wpi values set to 1 (a). Levels of significant differences (*t*-test) were calculated for log-transformed values.

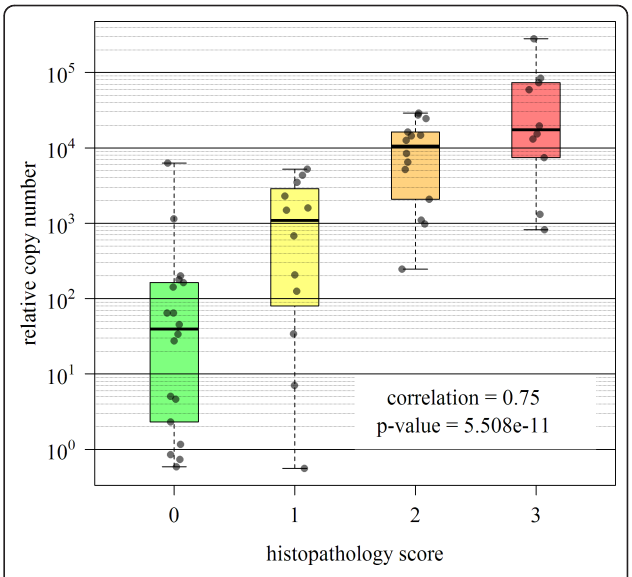

Figure 3 Correlation between cardiac histopathology and viral loads. Histopathology scores were correlated against relative copy numbers of PMCV in infected fish (n = 6) from all time points. Data points are plotted randomly across the box area in x-direction for better visualization. Correlation coefficient and p-value were calculated for log-transformed copy numbers.

disease profiles in our microarray database (e.g. inflammasome/pyrin-like genes such as VHSV-induced and TRIM/RING finger genes). The expression profile showed strongest upregulation at the early stages which levelled off during infection (Figure 4a). A median $\log_2$-ER +2.1 at 2 wpi decreased to +0.7 at 6 wpi. This level remained unchanged until 9 wpi followed by a significant decrease to +0.5 at 10 wpi. A heat map showing the expression of ten genes is given in Figure 5. These were selected either by random or based on their functional importance as evidenced from other studies in fish or higher vertebrates. Early upregulation of the cytoplasmic RNA helicases *retinoic acid inducible gene I* (*rigI*) and *melanoma differentiation-associated gene 5* (*mda5*) involved in sensing and degradation of viral RNA, as well as a gene similar to the membrane-bound *toll-like receptor 3*, implied activation of virus recognition receptors and antiviral signalling. Several genes known to be activated in response to IFN signalling were upregulated, such as *signal transducer and activator of transcription 1a* (*stat1a*), *myxovirus resistance gene Mx*, *interferon-inducible protein Gig2-like* and *radical s-adenosyl methionine domain-containing protein 2* (*rsad2*) also known as *viperin*. A similar expression profile was also observed for a suit of genes known to be induced by IFN but with unknown roles in fish immunity, such as *interferon-induced protein with tetratricopeptide repeats 5* (*ifit5*) and *very large inducible GTPase 1* (*vlig1*). A transcript encoding the 52 kDa Ro protein was one of several TRIM/RING finger genes highly induced at 2 and 4 wpi, supporting the role of this multi-gene family in early virus recognition and host defence [10].

2: Complement response

Twenty-two genes associated with the complement system were not differentially regulated at 2 wpi followed by a gradual upregulation from 4 wpi which peaked at 6 wpi (median $\log_2$-ER +1.5), concurrent with the onset of cardiac pathology. In subsequent time points expression levelled off, with a weak but significant induction at 9 wpi coinciding with pathology peak (Figure 4b). The upregulation at 6 wpi was significantly stronger compared to earlier and later time points. The heat map of representative genes (Figure 5) shows activation of genes with different roles in the complement system: Antigen: antibody-complex binding by C1q; activating enzymes C2b and C1r/s; membrane-binding proteins and peptide-inflammatory mediators C3a/4a/5a, C3, C3-4 and C5 pre-protein; and membrane-attack protein by C8b.

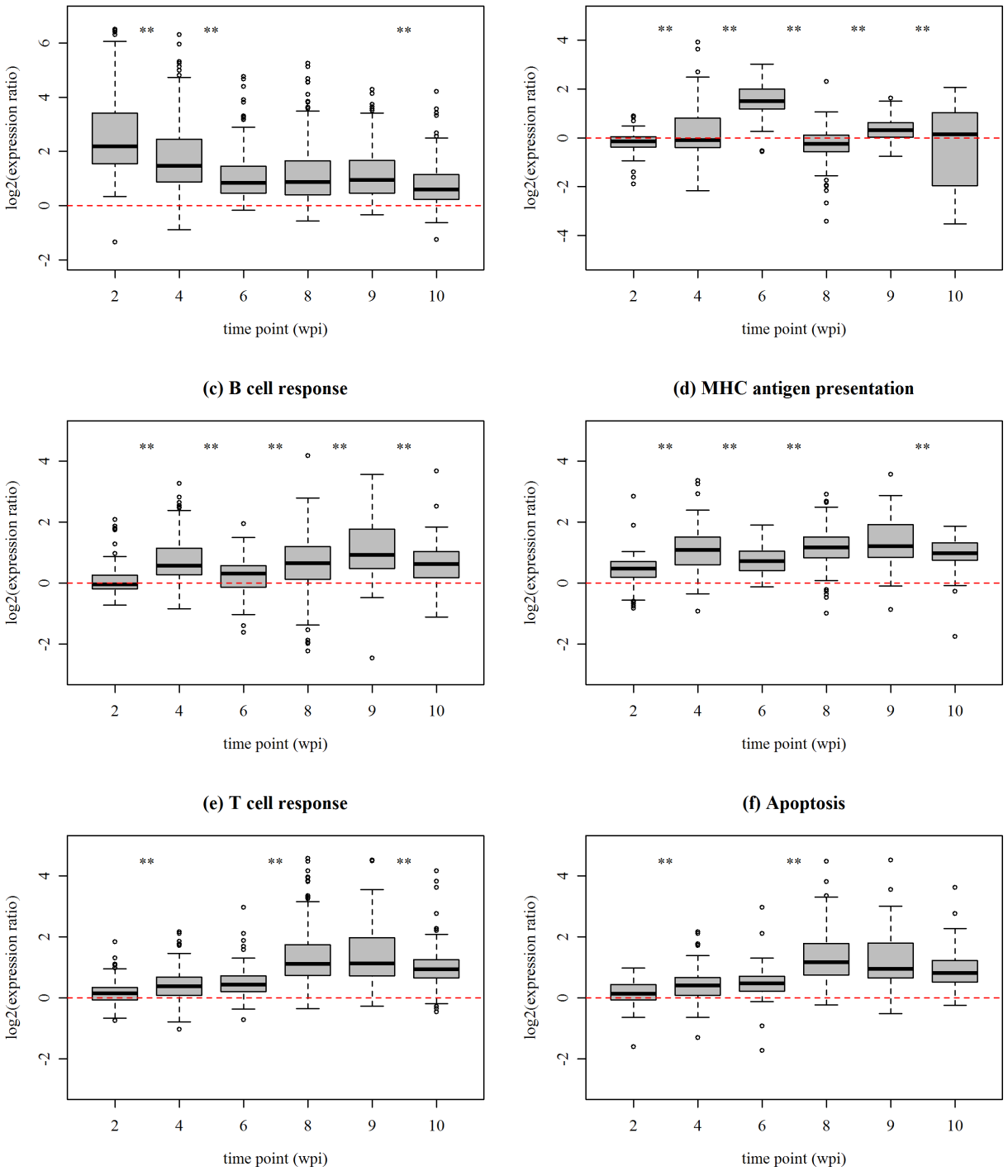

Figure 4 Temporal regulation of immune responses in PMCV infected fish. Box plots show log_2-ER (expression ratios) for all genes included in the six gene sets (a-f) resulting from microarray analysis of infected versus control fish from the time course study in cardiac tissue. Boxes represent 50% of the values, while black bars mark the median log_2-ER. Whiskers indicate the maximum length of 1.5 times the box length. Values beyond whiskers are plotted as circles. Significance levels of *t*-tests between time points are coded with asterisks: * = p-values between 0.01 and 0.05, ** = p-values < 0.01.

Figure 5 Temporal regulation of representative genes. Heat map showing the temporal expression of ten selected genes from each immune pathway (gene set) from Figure 4, as referred to in the Results section. Data are mean $\log_2$-ER of replicate pools of heart tissues from the six time points (2 to 10 wpi). Graded levels from gray to red indicate upregulation, and graded levels from gray to blue indicate downregulation. The scale of the colors is shown at the bottom of the figure.

3: B cell response

This gene set included 37 genes involved in differentiation and regulation of B cells and antigen recognition by immunoglobulins. The expression profile was characterised by upregulation at two time points; during early infection 4 wpi and at peak pathology 9 wpi (Figure 4c). The later peak was stronger with median log_2-ER +0.93 compared to +0.57 at 4 wpi. The two peaks were separated by the complement activation at 6 wpi. Immunoglobulin-related genes, represented with 21 distinct transcripts, comprised a large part of this group (Additional file 1). Genes related to antigen receptor signalling included *hematopoietic lineage cell-specific (Lyn substrate 1) protein (hs1)* and *kelch-like protein 6 (klhl6)* (Figure 5). Similar function was predicted for several genes with Src homology-3/2 (Sh3/2) domains and activities, such as *src kinase-associated phosphoprotein 2* (*skap2*) and *dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide* (*dapp1*). The *tyrosine-protein kinase lyn* also plays a regulatory role in B cell receptor response after antigen binding. The *CD9 antigen*, which is expressed in many B cell subsets and in plasma cells in mammals [11,12], was strongest induced at the early peak. The opposite was found for *CD97 antigen precursor*, suggesting that it may have a role in activated B and T cells.

4: MHC antigen presentation

This gene set included 34 genes involved in processing and presentation of viral antigens via MHC class I and II. The expression profile was similar to that of B cell response, but with less difference in average induction levels between the two peaks at 4 and 9 wpi, respectively log_2-ER +1.09 and +1.21 (Figure 4d). Besides, these genes were significantly upregulated already at the earliest time point (2 wpi). The gene set was dominated by genes related to the MHC class I pathway, such as antigen processing by proteasome components PSMBs/TAPs, and antigen presentation by the *MHC class I heavy chain* and light chain *beta-2-microglobulin* (Figure 5). Examples of MHC class II related genes were a salmon homologue to the *HLA class II histocompatibility antigen gamma chain* and *cathepsin s precursor*, a lysosomal cysteine peptidase involved in degradation of peptides for antigenic presentation on MHC class II molecules [13].

5: T cell response

The fifth gene set included 69 genes with known or presumed roles in the regulation and effector functions of T lymphocytes. The expression profile showed a slight but significant upregulation from 2 to 4 wpi which increased by additional +1 median log_2-ER at 8 wpi and reached maximum of +1.4 log_2-ER induction at 9 wpi (Figure 4e). This peak coincided with highest levels of the MHC antigen presentation and B cell response genes, and the time points when viral load and cardiac pathology were peaking. From 9 to 10 wpi gene expression dropped significantly. All classes of effector T cells seemed to be activated from 8 wpi onwards; cytotoxic (CTL) cells by induction of *interferon gamma, granzyme* and *CD8 beta* and T helper cells by induction of *CD4 T cell surface glycoprotein* (Figure 5). Upregulation of other genes with common regulatory roles in T cell activation included CD3 antigens, T cell receptor genes, *CD28 T-cell specific surface glycoprotein* and the *proto-oncogene tyrosine-protein kinase lck*.

6: Apoptosis

A group of 25 genes functionally linked to apoptotic pathways showed a coregulated expression pattern with the T cell response gene set, and was assumed to be involved in controlling cell death of T lymphocytes and/or host target cells, as their maximum induction coincided with the histopathology peak (Figure 4f). This gene set included several genes from the family of TNF receptors and caspases, with central roles in the execution phase of apoptosis (Figure 5). Interestingly, the majority of genes was linked to the family of Rho GTPases, with recently established roles in controlling T cell regulation and apoptosis, e.g. *rho-related GTP-binding protein RhoF* and *G precursors*, *CDC42 small effector 2*, *rho GTPase-activating protein 15*, *regulator of G-protein signalling 1*, and several genes related to the Ras superfamily (Additional file 1). Other important regulators of programmed cell death in immunity which were activated included the *tnf decoy receptor (tnfrsf6b)* and the *programmed cell death 1 ligand 1 precursor CD274*.

Tissue regulation of immune pathways

Next, we analysed the tissue-specific features of immune transcriptome responses during CMS. Two RNA sample pools (n = 3 each pool, same individuals as analysed in the time course study) from the same organs as tested for viral load were analysed by microarrays from two time points; before the onset of cardiac pathology at 4 wpi and at peak of cardiac pathology/viral load at 8 wpi. The six gene sets outlined in the time course study were examined (Additional file 1), and their expression profiles are shown as box plots in Figure 6. Early antiviral and IFN-dependent genes were induced in all tissues, with significantly higher median log_2-ER at 4 wpi compared to 8 wpi (Figure 6a). Levels at 4 wpi were similar in kidney, heart, spleen and blood, being lower in the liver. MHC antigen presentation also responded to infection in all examined tissues and, except for heart, levels were generally stronger at 4 versus 8 wpi (Figure 6d). The remaining functional groups showed restricted expression changes. The complement response was upregulated in spleen at the peak of pathology 8 wpi (Figure 6b). Genes associated with B cells were upregulated in heart and at both time points (Figure 6c). They

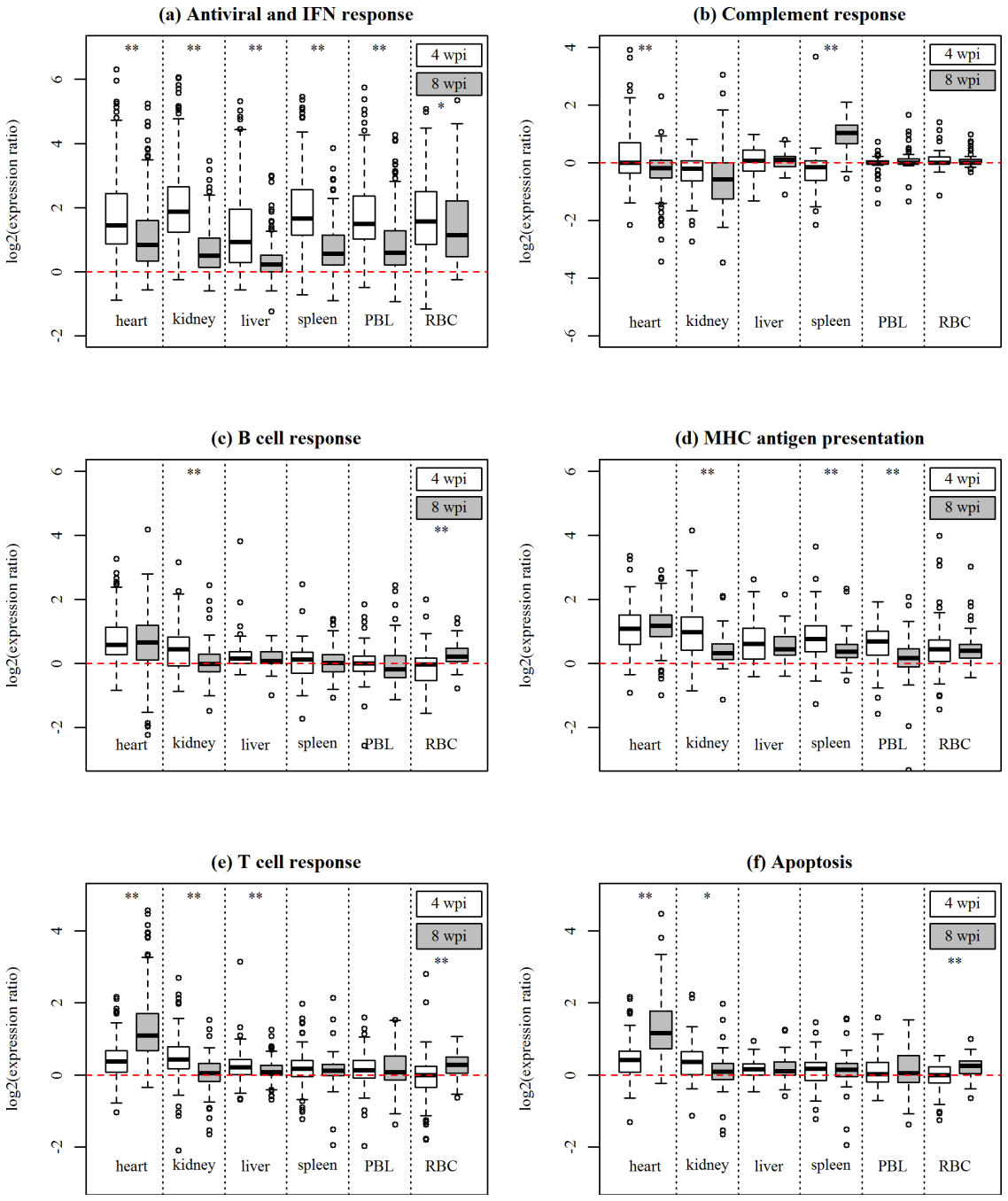

Figure 6 Regulation of immune responses in different tissues. Box plots show log_2-ER (expression ratios) for all genes included in the six gene sets (a-f) resulting from microarray analysis of infected versus control fish from the study in different tissues and from two time points; early infection stage (4 wpi) and peak cardiac pathology/viral load (8 wpi). Details on the box plots are described in the legend of Figure 4.

also showed a weak but significant induction in kidney at 4 wpi and in RBC at 8 wpi. The T cell and apoptosis gene sets showed similar expression profiles, with induction in heart which was strongest at peak pathology 8 wpi when compared to 4 wpi (Figure 6e-f). In addition, a significant though relatively weak increase was found in RBC between 4 and 8 wpi. Similar to the B cell response, kidney showed a transient induction at 4 wpi.

Real-time qPCR analyses

To verify the microarray results, six differentially expressed genes were analysed by qPCR in the four sample

pools from 4 wpi. The results produced with two independent methods were in close concordance (Figure 7). The coefficient of linear regression was close to unity (0.97) while correlation and linear dependency were highly significant (Pearson r: 0.85, $p = 3.8 \times 10^{-6}$). The qPCR analyses also assessed the individual variation and relationship between viral load and gene expression changes at 4 wpi. Six genes encoding putative antiviral and IFN-dependent genes from gene set 1 were selected due to high induction levels at this early time point. Relative expression of *rig-I, mda5, stat1a, ifit5, rsad2* and *baf* was determined in 20 individuals from CMS infected groups 3 and 4 versus the same control pool as used for the microarray experiments (n = 10) (Figure 8). These genes were strongly induced in all fish with median fold changes from +3 (*mda5*) to +52.5 (*baf*). At this time point, no significant histopathological changes were observed, and equal numbers of individuals had histopathology scores of 0 or 1. As expected, none of the analysed genes showed significantly different expression between fish with histopathological scores 0 and 1 (both corresponded to a normal state of heart). Viral load in heart varied between C_T 19-25 in these individuals, and gene expression levels and virus C_T values were strongly correlated for all six genes (Table 1). This implied that genes were activated as a result of increased viral replication and suggested that they might represent markers of early infection status.

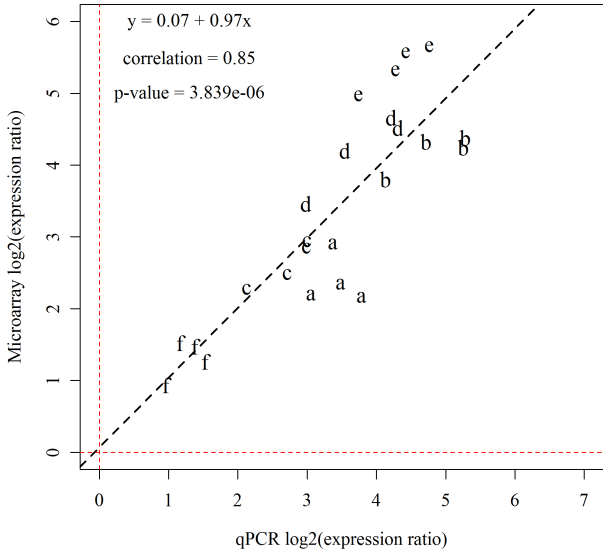

Figure 7 Confirmation of microarray results by qPCR. Expression levels of six genes were compared, as listed in Additional file 4. Log_2-ER from microarrays was plotted on the y-axis, while log_2-transformed fold changes from real-time qPCR were plotted on the x-axis. The four replicate pools of heart tissue from 4 wpi against control pools were measured for each gene. The dashed black line represents the regression function of the measured values. The function is shown in the top left corner of the plot. In addition, the Pearson correlation and the corresponding p-value are shown.

Discussion

This study addressed the temporal and spatial development of immune responses assessed by transcriptome changes during experimental piscine myocarditis virus infection. The regulation of immune pathways was compared to the disease status evaluated by histopathology and viral load, aiming at a comprehensive understanding of the host-virus interactions. These results provide a framework for in-depth functional studies on immunity and for evaluation of preventive strategies such as vaccination and nutritional intervention during CMS in Atlantic salmon.

Challenge trial and infection

Since the discovery of CMS, its diagnosis has been based on clinical findings and cardiac histopathology [4]. A virus with structural similarities to the *Totiviridae* family named PMCV was recently identified as the presumed causative agent of CMS [8]. Thus, pathogenesis and disease progression can now be more thoroughly evaluated by combining virus-specific qPCR with histology. It should be noted that due to difficulties with PMCV cultivation *in vitro*, virus titration has not been successful yet (M. Rode, personal communication). Consequently, the relative expression of viral RNA in this study could not be related to actual numbers of viral particles. Cardiac histopathology showed moderate to severe lesions consistent with CMS (score 2 or 3) exclusively in infected groups, with only one exception in control groups at 10 wpi. Furthermore, replicate groups were very similar to each other with respect to histopathology score. Significant differences between group replicates were only observed between infected groups at two time points (atrium at 10 wpi and epicardium at 11 wpi). The differences between infected and control groups were mainly associated with lesions in the atrium and spongy layer of the ventricle, which were highly significant from 4 to 11 wpi and 6 to 11 wpi, respectively. As expected, no lesions were observed in the compact layer of the ventricle, and lesions in epicardium were less prominent. These results are coherent with the pathology as described from clinical outbreaks and previous challenge trials with CMS [4,7,8].

After the peak in histology score at 8-9 wpi, lesions declined gradually suggesting the onset of a recovery phase. This was supported from qPCR analysis of viral load which followed the same pattern: increased replication until 6 wpi followed by a plateau phase until 10 wpi, and finally decreasing levels to the end point of the challenge trial. Thus, the strong correlation between histopathology and viral load which peaked concurrently

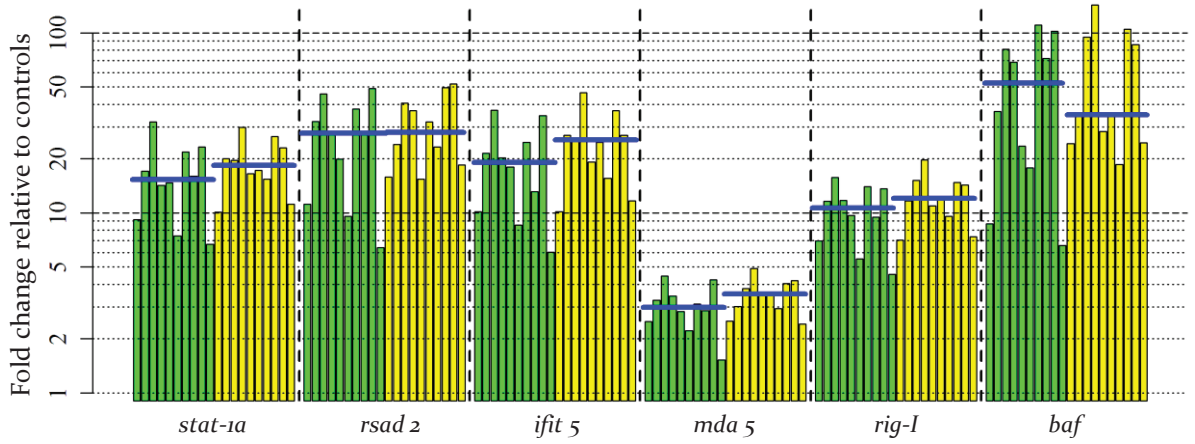

Figure 8 Assessment of individual expression of antiviral genes. Gene expression in 20 infected fish (from groups 3 + 4 at 4 wpi) is shown as fold change relative to uninfected controls based on real-time qPCR. The y-axis is plotted logarithmic. Bars are colored according to the histopathology score of atrium for respective fish; green = score 0, yellow = score 1. The median fold change for each histopathology score per gene is indicated with a blue line.

with the activation of T cell pathway genes, suggest that the observed cardiac lesions resulted from virus cytopathic effects and necrosis of infected myocytes, triggering an inflammatory response followed by activation of T-cell mediated immunity. Examination of viral loads across different tissues showed equally high levels of viral RNA in kidney and spleen as compared to heart, while liver and blood cells had lower levels. However, increased replication from 4 to 8 wpi was only observed in heart, supporting that this organ was the main site of virus propagation [8]. However, heart may not be the primary replication site, since viral RNA was detected in all tissues and blood cells early after infection. High infection levels in kidney and spleen are typical for viral diseases in salmon, and are probably related to their roles in attracting primary infected/antigen presenting cells and priming lymphocytes for specific immunity. From a challenge trial with the recently described piscine reovirus (PRV) [14], higher viral loads were found in spleen and kidney as compared to lower but equal levels between heart and liver [15]. While belonging to different families, both PRV and PMCV cause necrosis and inflammation in heart muscle. The lower levels of PRV in heart may reflect a more persistently infecting nature compared to PMCV [8,15].

Table 1 Correlation between expression levels of antiviral genes and viral load.

Gene	Correlation coefficient	p-value
stat-1a	0.863419	0.001287
rsad 2	0.887746	0.000605
ifit 5	0.854039	0.001659
rig-I	0.909621	0.000261
baf	0.903799	0.000333
mda 5	0.836384	0.002561

mRNA levels (fold change relative to controls, real-time qPCR) of six genes (from early antiviral and IFN gene set) from 6 individual fish were tested for correlation (Pearson) against viral loads (non-normalized C_T values, real-time qPCR) at 4 wpi.

While clinical CMS outbreaks typically give 5-20% mortality [4], no fish died in the present challenge trial. This suggested that during natural CMS outbreaks, either larger fish (or different life stages), higher numbers of infectious viral particles or possibly additional stressors must be present to give mortality. Coherence between distinct stress factors and viral infection resulting in higher mortality has been shown for other diseases in farmed Atlantic salmon [16,17]. An interesting observation was the high proportion of fish with no or moderate cardiac lesions at time points when viral loads and histopathological scores were significant. Thus, fish obviously exhibited different outcomes of infection. Comparison of these groups is currently under investigation. In the present study, fish with strongest pathology and viral infection were selected at each time point, in order to characterise immune responses at the transcriptome level in fish at similar stages of the disease process and with representative CMS pathology. Fish was challenged by injection to ensure simultaneous infection of all fish and since cohabitation had shown to give slower development of disease and weaker overall pathology ([8] and unpublished results). The individual qPCR analysis of six antiviral genes in 20 fish 4 wpi showed similar levels of upregulation, supporting that all fish had mounted equal antiviral responses following infection and were in similar disease state. This was further supported by a significant increase in viral loads of fish between week 2 and 4.

Temporal development of immune responses

Early antiviral and IFN responses were activated at every time point and across tissues during infection. However, the overall expression profile showed declined mRNA levels with time in spite of increased virus replication. This contrasted the strong correlation between expression levels of six selected genes and viral load observed at 4 wpi, implying that activation of these genes with increased production of viral RNA was predominant at the early stage and possibly related to autocrine effects, such as pathogen recognition and induction of signalling pathways. The subsequent reduction in transcriptional activity might be due to increased paracrine effects of proteins in the induced innate responses, including both effector functions for clearance of virus and recruitment of immune cells for development of humoral and adaptive immunity. The IFN type I responses to different viral diseases have been reported in salmonids, with particular focus on IFN alpha and Mx protein [18-21]. We identified a suite of putative IFN-dependent genes with stronger upregulation which have been unknown or scarcely investigated in salmon. Most of these genes have shown responses to other viral diseases in salmon [9]. Strongest induction at 2 wpi was found for *ifit5* and *rsad2*, also known as *viperin*. Both genes are known to be induced by IFN and involved in defence against viruses [22,23]. Little is known about *gig1*- and *gig2-like* genes in fish, but they were induced by viral infection in grass carp cells [24]. Members of this gene family were also strongly induced in rainbow trout 24 h post infection with the parasite causing whirling disease [25]. Four different genes from the tripartite motif (TRIM) family C-IV were also significantly induced over several time points. One of them, TRIM25, has been implicated in the RIG-I pathway by regulating the capability of RIG-I to activate type I IFN [26,27]. Several genes belonging to families of IFN-inducible GTPases were also early induced, including two transcript similar to very large inducible GTPase 1 (VLIG1) and eight transcripts similar to GTPase IMAP family member 7. The role of these novel GTPases in vertebrate infection is gaining interest [28,29].

Proteins of the complement system bind and opsonize viral particles, marking them for phagocytosis by APCs. Binding to antigen-antibody complexes makes the complement system a bridge between the innate and the adaptive immune system. This is in line with results of the present study, where upregulation of complement genes at 6 wpi took place shortly after the first activation of B cell- and MHC antigen presentation genes and the onset of cardiac histopathology. In subsequent time points, activation of the adaptive immune response was most prominent. This distinct sequence of immune events was evidence for a coordinated regulation of responses, and the 'bridging' role of the complement system between the early innate response and the fully activated adaptive response. Coincidence with the first occurrence of moderate cardiac lesions (histopathology score 2), suggests complement genes as candidates disease markers for the early clinical stage of CMS.

The immediate activation of antigen presentation as has been observed during early virus infection in salmon [19], was supported by the upregulation of proteasome and MHC class I pathway genes that coincided with the early IFN/antiviral response at 2 wpi. This was analogous to the typical development of an adaptive immune response: while IFNs are strongest activated and elicit antiviral effects very early after infection, they also have an activating effect on antigen processing and presentation [30]. Activation of antigen presentation is also the first step in the cellular immune response mediated by B and T lymphocytes. The first peak of B cell activity was detected at 4 wpi following the typical pattern of a humoral immune response in teleost fish, usually expected between 4 and 6 weeks after infection [31]. However, the co-regulated B cell- and MHC antigen presentation genes showed a biphasic expression, with a second and even stronger activation at 8 and 9 wpi when the clinical signs were also peaking. This observation is probably explained by the higher influx of leukocytes and level of inflammatory reactions in heart tissue as supported by histology. Interestingly, stronger second peak of induction occurred after the activation of complement genes at 6 wpi. This may indicate that a potential humoral response based on antibody-dependent cellular cytotoxicity and virus neutralization is complement-dependent. Future development of tools for assessment of virus-specific antibody titers may confirm this. Most of the representative genes (Figure 5) followed the typical regulatory pattern for B cell and antigen presentation components. For example, the strongest induction of CD9 was found at 2 and 4 wpi, before the strongest T cell activation was detected. It has been shown that CD9 is induced downstream of the antigen receptor during T-independent humoral B cell response [32]. However, the majority of genes showed highest upregulation in heart at peak pathology and viral load, 8 and 9 weeks after infection, indicating their role in B cell responses and presentation of viral antigens to effector T cells. One example was CD97, a surface protein of both B and T lymphocytes which is expressed at low levels in inactive cells but rapidly induced after activation [33]. Thus, it can be used as a marker of general activation of lymphocytes. In this study it was a representative marker gene for the overall expression profile of B/T lymphocyte-related responses.

The co-regulatory pattern of T cell- and apoptosis-related genes correlating with histopathology score was

a prominent feature of the immune response in CMS hearts. Control of cell death by apoptosis is a fundamental process for regulation of the T cell response and for maintaining homeostasis in the immune system after it has expanded to combat infections [34]. Importantly, dysfunctional control of T cell function and apoptosis is associated with immunopathology [35]. Thus, the apoptosis-related profile coinciding with the T cell profile in this study may represent novel genes involved in regulation of effector function and controlled cell death of T cells in salmon. Of particular interest were several genes encoding TNF-related proteins and programmed cell death ligand 1 (aka B7-H1/CD274). The dominance of genes encoding Rho GTPases was interesting, since they have been implicated in the regulation of TCR signaling, T cell cytoskeletal reorganization, T cell migration and T cell apoptosis [36]. It seemed to be a borderline from 6 to 8 wpi when expression of T cell/apoptosis pathways was significantly induced, coinciding with the first occurrence of histopathology scores 3 and virus C_T values below 20. According to this pattern, the first severe inflammation and cytopathic effects caused by the virus (histopathology score 2) at 6 wpi was probably the priming event for a strong influx of lymphocytes to the infected heart tissue. Cardiac elevation of mRNA levels for CD8, granzyme and IFN gamma at 8-10 wpi indicated activity of cytotoxic $CD8^+$ T cells. Genes encoding CD4 were also induced, but at lower levels. One week after elevation of T cell activity (9 wpi, median of relative fold change of PMCV = 10, 021) viral load and histopathology score were decreasing (10 wpi, median PMCV fold change = 8, 404), and the first significant decrease was evident at 11 wpi (median PMCV fold change = 983). This indicated that the cellular effector response mediated by T cells, and in particular $CD8^+$ T cells, was contributing to a successful clearance of the virus infection. Among other interesting genes in this group, TNF decoy receptor showed the highest correlation versus histopathology score and viral load. However, the function of this receptor in salmon immunity is not known.

Tissue regulation of immune responses

The systemic induction of early antiviral and IFN-dependent genes was expected, given the observed replication of PMCV in all tissues and the fact that most of these genes are presumably activated in the presence of viral RNA. The stronger induction at early infection 4 wpi compared to peak viral load 8 wpi was common for all tissues and blood, and has already been discussed. The functional relation between IFNs and MHC antigen presentation pathways was supported by their similar expression profiles across tissues and time points. The only exception was the cardiac expression of the latter, which was equally induced between time points (4 and 8 wpi). Proteasome and MHC components are commonly induced by IFNs during viral infection [19,37]. In addition to heart tissue, where pathology developed, these genes were equally induced in spleen and kidney at 4 wpi supporting the importance of these tissues for lymphocyte maturation and priming of the immune response [38]. The observation that this induction was not in sync with viral load may further suggest that these responses were time-dependent, e.g. related to the stage of disease rather than viral load and pathology. Little is known about the expression of complement components in Atlantic salmon during viral infections. In common carp and channel catfish, the highest expression of complement was found in the liver [39,40]. In humans, liver is also the main source of complement component C3, but production in macrophages and endothelial cells has also been shown [41]. During CMS, complement genes were only activated in extrahepatic tissue and more specifically in cardiac tissue, where virus infection was most prominent. Interestingly, complement genes were induced in the spleen during clinical phase, suggesting that splenocytes (e.g. macrophages) represent an important source of complement and can play a role in this response in salmonid virus infection. This induction of complement was also independent of viral load, which was equal between 4 and 8 wpi. In humans, the complement component C3 has an important role in regulating the maturation of B cells in the spleen [42]. Thus, the induction of splenic complement might reflect signalling events between activation of antigen presenting cells such as B cells and possibly production of virus-specific antibodies. However, more research is needed to understand this process. Tissue regulation of adaptive immune responses as represented by expression of B cell, T cell and apoptosis gene sets shared some common features. Most notable was the opposite regulation of these responses in heart and kidney between the early and clinical stage, which was characterised by an induced expression from 4 to 8 wpi in heart, and reduced expression from 4 to 8 wpi in kidney. Interestingly, twelve genes (among them CD8, CD37, granzyme and TNF decoy receptor) showed no regulation in heart but induced expression in kidney at 4 wpi. On the contrary, at 8 wpi induction was restricted to heart while no expression changes were found in kidney. This could be evidence for an early clonal expansion and maturation of effector T cells in kidney which then migrated to the heart for elimination of virus-infected cells four weeks later. The adaptive immune responses in kidney was activated at the early stage of infection despite equally high levels of viral

RNA at both 4 and 8 wpi, further suggesting a specific role for kidney in the early priming and maturation of cellular immunity.

Conclusions

We used oligonucleotide microarrays to assess transcriptome changes in Atlantic salmon experimentally infected with PMCV, inducing cardiac pathology consistent with CMS and transient viraemia. From comparative analysis of gene expression, histology and viral load, the temporal and spatial regulation of immune responses were characterised and novel immune genes identified, ultimately leading to a more complete understanding of host-pathogen responses and pathology and protection in Atlantic salmon during CMS.

Methods

Experimental infection and sampling

The infection trial was performed at VESO Vikan (Veterinary Science Opportunities, Namsos, Norway), a GLP-certified research station for infectious challenge experiments on aquatic organisms. The trial was approved by The National Animal Research Authority http://www.fdu.no according to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (EST 123). The experimental design with selection of sampling times and PMCV inoculum was based on results from two previous pilot trials [7] (and unpublished results). From both of these experiments, histopathological lesions associated with CMS were significant from week 6 until week 10 post challenge (injection). Therefore, in the present study we sampled weekly from 8 until 12 weeks post challenge, aiming to cover the period with CMS pathology. Biweekly sampling after infection (2, 4, 6 wpi) was done in order to cover the early phase before pathology. Unvaccinated Atlantic salmon (*Salmo salar* L., standard strain from Aqua Gen AS) were smoltified (seawater-adapted) according to standard procedures and kept at 12°C under standardised conditions (light, feeding, water flow, salinity and fish density). Fish were acclimatised in respective tanks for at least one week before challenge.

The trial was conducted in four separated tanks; one infected and one control group in duplicates. Each tank contained 120 fish with an average weight of 50 g at the beginning of the experiment. Injection of PMCV was performed after sedation (benzocaine, 30-40 mg L^{-1}). Infected groups received an intraperitoneal (i.p.) injection dose (0.2 ml) of a supernatant from a GF-1 cell line (derived from the fin tissue of orange-spotted grouper, *Epinephelus coioides* [43]) infected with PMCV as described [8]. In short, heart tissue from freshly dead Atlantic salmon was collected from a clinical field outbreak of CMS (diagnosed by histopathological examination, score > 3). Tissue was homogenised, centrifuged to remove cellular debris (4000 g at 4°C for 20 min) and filtrated (0.22 μm filter) before inoculation onto GF-1 cells. Cells were grown in plug seal cap culture vessels at 15°C in L-15 supplemented with 1% L-glutamine (2 mM), 0.1% gentamicin sulphate (50 μg ml^{-1}) (all from Sigma Aldrich, St. Louis, USA) and 10% fetal bovine serum (Invitrogen, CA, USA). Cytopathic effect (CPE) was evident by accumulation of cytoplasmic vacuoles from 6 until 21 days post inoculation, when supernatant and cell lysate were harvested. CPE was reproduced when passaged onto fresh cells. Inoculation of cells with heart tissue homogenate prepared from healthy Atlantic salmon (confirmed by histopathology, score 0) did not give CPE. Tanks with control groups were injected i.p. with the same dose of conditioned medium from uninfected cell culture prepared as described above. Both PMCV and control inoculums were tested negative for salmonid alphavirus subtype 3, infectious pancreatic necrosis virus, infectious salmon anaemia virus, piscine reovirus and grouper nervous necrosis virus by qPCR.

An overview of samplings and analyses is given in Additional file 3. Tissues and blood were sampled at eight time points: 2, 4, 6, 8, 9, 10, 11 and 12 wpi, in order to cover early infection phase (biweekly sampling) and clinical phase with improved coverage (weekly sampling). In addition, samples were taken from fish before the experiment started (0 wpi). From each time point, 15 fish from each of the four tanks were sedated (as described above) and euthanized by decapitation. Standardised samples from heart, mid-kidney, liver and spleen were snap-frozen in liquid nitrogen for RNA, and fixed in formalin (10% neutral phosphate-buffered) for histology. Blood was sampled from the caudal vein in heparinized vacutainers and kept on ice. Peripheral blood leukocytes (PBL) were separated from red blood cells (RBC) as described [44] and stored at -80°C until RNA was extracted.

Histopathology

Formalin-fixed heart samples were prepared by paraffin wax embedding and standard histological techniques [45]. Sections were stained with haematoxylin and eosin. From each fish, a longitudinal section of the whole heart was evaluated. All cardiac compartments were examined and classified histologically using a visual analogue scale. Atrium, epicardium, compact and spongy layers of the ventricle were graded from 0 to 3 according to the severity of the lesions [7]. Score 0 and 1 was considered normal, with no histopathological findings (score 0), or a single or few focal lesions (score 1). Score 2 represented several distinct lesions and increased mononuclear infiltration. Score 3 represented multifocal to

confluent lesions in > 50% of tissue and moderate to severe leukocyte infiltration. Sections were coded and evaluation was randomised and blinded.

RNA extraction

Tissue samples for microarray hybridization and qPCR were stored at -80°C prior to RNA extraction. Standardised tissue sections of 10 mg from each organ and 5-10 × 10^6 blood cells (PBL and RBC) were prepared under sterile/RNase-free conditions. Tissue sections from heart consisted of an equal mix of ventricle and atrium with all compartments included. Frozen sections were transferred directly to 1 ml chilled TRIzol (Invitrogen) in 2 ml tubes with screw caps (Precellys®24, Bertin Technologies, Orléans, France). Two steel beads (2 mm diameter) were added to each tube and tissue was homogenized in a Precellys®24 homogenizer for two times 25 sec at 5000 rounds per minute with a pause of 5 sec between rounds. Blood samples were homogenized in 1 ml chilled TRIzol by repetitive pipetting up and down. RNA was extracted from the homogenized tissues using PureLink RNA Mini kits according to the protocol for TRIzol-homogenised samples (Invitrogen). The concentration of extracted total RNA was measured with a NanoDrop 1000 Spectrometer (Thermo Scientific, Waltham, MA, USA). The integrity of total RNA was determined using an Agilent 2100 Bioanalyzer with RNA Nano kits (Agilent Technologies, CA, USA). Samples with RNA integrity number (RIN) of 8 or higher were accepted.

Design of microarray experiments

An overview of microarray analyses is given in Additional file 3. The salmonid oligonucleotide microarray (SIQ2.0, NCBI GEO platform GPL10679) was used, consisting of 21 K features printed in duplicates on 4 × 44 K chips from Agilent Technologies [9]. Two-color design was used, where pooled infected fish labelled with fluorescent Cy5 dye and non-infected pooled control fish from the same time point labelled with Cy3 dye were competitively hybridised on the array. Microarray hybridizations were divided in two experimental lines. The first was a time course study in heart tissue from 2, 4, 6, 8, 9 and 10 wpi. These time points were selected based on the results from histopathological examination (Figure 1), and covered the early infection phase and peak of cardiac pathology. From each time point, biological replicates of test (infected) samples consisted of two RNA pools with each pool consisting of three individual fish. For time points 4 and 8 wpi, representing respectively the early and clinical phase of infection, two new pools were added, each consisting of two fish (different than those used in the first two pools). The individual fish were selected for maximum heart histopathology score at the time points when this was significant (from 6 wpi onwards, see Figure 1). Reference samples were pooled RNA (equimolar amounts of total RNA) from eight to ten fish from groups 1 and 2 (non-infected controls) per each time point. The second experimental line focused on tissue responses in mid-kidney, liver, spleen, PBL and RBC (in addition to heart as described above) at the early and clinical phase of infection, respectively 4 and 8 wpi. Similar to the time course study, biological replicates were two pools of RNA, each consisting of three individual fish, per each tissue and time point. The two pools were RNA from the same six individuals as used for the time course study. Reference sample pools were prepared in the same manner, with RNA from the same non-infected control individuals as used for the time course study. Recording of microarray experiment metadata was in compliance with the Minimum Information About a Microarray Experiment (MIAME) guidelines [46].

Microarray hybridization and data processing

Unless specified otherwise, all reagents and equipment used for microarray analyses were from Agilent Technologies according to manufacturer's protocol. Labelling and amplification of RNA was performed on 500 ng total RNA using Quick Amp Labeling Kits, Two-Color and RNA Spike-In Kits, Two-Color. For fragmentation of labelled RNA, the Gene Expression Hybridization Kit was used. Hybridizations were performed for 17 hours in an Agilent hybridization oven set to 65°C with a rotation speed of 10 rounds per minute. Arrays were washed for one minute with Gene Expression Wash Buffer I at room temperature, and one minute with Gene Expression Wash Buffer II at 37°C. Slides were scanned immediately after washing using a GenePix Personal 4100 A scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 μm resolution and with manually adjusted laser power to ensure an overall intensity ratio close to unity between Cy3 and Cy5 channels and with minimal saturation of features. The GenePix Pro software (version 6.1) was used for spot-grid alignment, feature extraction of fluorescence intensity values and assessment of spot quality. After filtration of low quality spots, data were exported into the STARS platform [9] for data transformation, normalization and quality filtering. The values in spot replicates were averaged, Lowess normalization of $\log_2$-expression ratios (ER) was performed, and differentially expressed genes (DEG) were selected based on mean $\log_2$-ER > |0.65| in at least one time point and tissue, spot signal quality threshold, number of positive spots and one-sample *t*-test ($p < 0.05$, H_0: $\log_2$-ER = 0). Corrections for false discovery rate were not employed as previous microarray studies in Atlantic salmon have demonstrated them to be overly

conservative [47,48]. The final list of DEG used for further analysis included 5712 genes. Data was submitted to GEO (submission number GSE28843).

Gene sets and annotations

For this work, functional subgroups or gene sets were compiled from the list of 5712 differentially expressed genes (Additional file 1). These were created by the use of the STARS software package customized for mining of microarray gene expression data [9]. STARS contain custom annotations of genes on the microarray based on GO classes, KEGG pathways, mining of literature and public databases and experimental evidence (transcription profiles/meta-analyses).

Quantitative real-time RT-PCR

The following section relates to the analysis of host gene expression. Experiments were conducted according to the MIQE guidelines [49]. Synthesis of cDNA was performed on 0.2 μg DNAse-treated total RNA (Turbo DNA-freeTM, Ambion, Austin, TX, USA) using the TaqMan® Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in 25 μl reactions with random hexamer priming according to manufacturer's protocol. Complementary DNA was stored undiluted at -80°C in aliquots to avoid repeated freeze-thawing. To avoid risk for presence of residual DNA contamination, control reactions without RT was tested on respective tissues and qPCR primers were possibly designed to span introns. Oligonucleotide primers were designed with the program eprimer3 from the EMBOSS program package (version 5.0.0, http://emboss.sourceforge.net/). Amplicon size was set to 80-160 and melting temperature to 59-61°C. Primers were purchased from Invitrogen (Additional file 4). *In silico* analysis of gene targets was performed using a customised program for BLAST and sequence alignments [9]. PCR amplicon size and specificity were confirmed by gel electrophoresis and melting curve analysis (Tm calling; LightCycler 480, Roche Diagnostics, Mannheim, Germany). QPCR was conducted using 2× SYBR® Green Master Mix (Roche Diagnostics) in an optimised 12 μl reaction volume, using 5 μl of 1:10 diluted cDNA, and primer concentrations of 0.42 μM. PCR reactions were prepared manually and run in duplicates in 96-well optical plates on a Light Cycler 480 (Roche Diagnostics) with the following conditions: 95°C for 5 min (pre-incubation), 95°C for 5 sec, 60°C for 15 sec, 72°C for 15 sec (amplification, 45 cycles) and continuous increase from 65°C to 97°C with standard ramp rate (melting curve). Cycle threshold (C_T) values were calculated using the second derivative method. For evaluation of the results, the mean of duplicates was used. Duplicate measurements that differed more than 0.5 C_T values were removed and reanalysed. Relative expression ratios of test samples versus the average of the controls were calculated according to [50]. Elongation factor 1α (GenBank ID: BT072490.1) was used as reference gene [51], and was found to be stably transcribed in control and test samples according to the BestKeeper software [52]. The efficiency of the PCR reactions was estimated for all primer pairs by six times 1:5 dilution series of a cDNA mix of all used samples. The efficiency values were estimated by using the LightCycler® 480 Software (version 1.5.0.39). All measured efficiencies were between 1.905 and 1.999.

Viral load

Relative quantification of PMCV was employed by qPCR on RNA isolated as described above from selected samples (heart; weeks 0, 2, 4, 6, 8, 9, 10, 11, 12, n = 6, kidney/liver/spleen/PBL/RBC; weeks 4 and 8, n = 6). Each sample's RNA concentration was normalized to 62.5 ng per 20 μl cDNA synthesis reaction, which was part of the SuperScript® III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen). In order to reduce secondary structures, RNA was heat denatured at 95°C - 5 min and then cooled down to 4°C prior to addition of RT enzyme and master mix. cDNA synthesis reaction conditions and temperature cycling were kept in line with manufacturer's guidelines. qPCR was performed on each sample in triplicate reactions containing 12.5 μl 2× Platinum® SYBR® Green qPCR SuperMix-UDG, ROX reference dye was added to the master mix to give a final reaction concentration of 50 nM, 1.25 μl 6 μM ORF2-3F (5'-GGAAGCAGAAGTGGTGGAGCGT-3') and 1.25 μl 6 μM ORF2-3R (5'-CCGGTTTTGCG CCCTTCGTC-3'). Ten μl 1:10 dilution of cDNA was added per reaction. The reaction conditions were UDG-incubation at 50°C for 2 min, activation of the hot-start polymerase at 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec, primer annealing for 15 sec and extension for 45 sec at 60°C. Melting curve analysis was performed to confirm formation of expected amplicon. The viral loads were expressed as a relative copy number with non-infected controls (0 wpi) set to 1, calculated by the formula $2^{\wedge}(C_{T(0wpi\ median)} - C_{T(sample)})$.

Statistical analyses

Histopathology scores and pair-wise comparison of gene sets were tested for significant differences by an independent two-sample t test using the function *t*-test() in the R software STATS-package (version 2.10.1, http://www.cran.r-project.org/). In addition, one-way ANOVA followed by Newman-Keuls test was used to assess differences between time points and tissues for each gene set. Correlations and respective p-values were calculated by the cor.test() function in R. For all tests, significance

levels of the resulting p-values with $p < 0.05$ and $p > 0.01$ are marked with single asterisk (*), and $p < 0.01$ are marked with double asterisk (**) in all figures. The function of the regression line and the respective p-value for the confirmation of the microarray experiments by qPCR were calculated by the lm()-function ("linear model") in R.

Additional material

Additional file 1: Complete list of immune gene sets. Gene composition and respective log_2-ER for the six gene sets representing immune pathways regulated over time (in heart) and in different tissues, as referred to in the Results section.

Additional file 2: Results from ANOVA on gene sets. Results of one-way ANOVA with Newman-Keuls test for the log_2-ER values of the six gene sets from time points and tissues.

Additional file 3: Experimental outline. Overview of sampling strategy and number of biological replicates for the different analyses.

Additional file 4: Real-time qPCR primers used in the study. The first column refers to the letters used in Figure 7 for plotting of expression values per each gene.

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Authors' contributions
GT and SMJ drafted the manuscript. GT, SMJ and AK conducted gene expression analysis. MR, HT and SMJ designed the challenge test. MR and PN prepared PMCV inoculum and analysed viral load. MA performed histopathological analysis. SA developed the software for processing of microarray data and performed parts of the statistical analyses. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Paper III

Paper III

Comparison of Atlantic salmon individuals with different outcomes of cardiomyopathy syndrome (CMS)

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Abstract

Background

Cardiomyopathy syndrome (CMS) is a severe disease of Atlantic salmon (*Salmo salar* L.) associated with significant economic losses in the aquaculture industry. CMS is diagnosed with a severe inflammation and degradation of myocardial tissue caused by a double-stranded RNA virus named piscine myocarditis virus (PMCV), with structural similarities to the *Totiviridae* family. In the present study we characterized individual host responses and genomic determinants of different disease outcomes.

Results

From time course studies of experimentally infected Atlantic salmon post-smolts, fish exhibited different outcomes of infection and disease. High responder (HR) fish were characterized with sustained and increased viral load and pathology in heart tissue. Low responder (LR) fish showed declining viral load from 6-10 weeks post infection (wpi) and absence of pathology. Global gene expression (SIQ2.0 oligonucleotide microarray) in HR and LR hearts during infection was compared, in order to characterize differences in the host response and to identify genes with expression patterns that could explain or predict the different outcomes of disease. Virus-responsive genes involved in early antiviral and innate immune responses were upregulated equally in LR and HR at the first stage (2-4 wpi), reflecting the initial increase in virus replication. Repression of heart muscle development was identified by gene ontology enrichment analyses, indicating the early onset of pathology. By six weeks both responder groups had comparable viral load, while increased pathology was observed in HR fish. This was reflected by induced expression of genes implicated in apoptosis and cell death mechanisms, presumably controlling lymphocyte regulation and survival. At the late stage of infection, increased pathology and viral load in HR was accompanied by a broad activation of genes involved in adaptive immunity and particularly T cell responses, probably reflecting the increased infiltration and homing of virus-specific T cells to the infected heart. In contrast, LR fish showed viral clearance and recovery at the late stage, which was associated with activation of genes involved in energy metabolism while adaptive immunity genes were not expressed.

Conclusions

High responder fish showed high viral load and severe cardiac pathology in the late clinical stage of CMS which was associated with a strong and sustained expression of genes involved in cellular adaptive immune responses. Low responder fish managed to significantly reduce viral load and recover without development of severe cardiac pathology, presumably by immune responses at the earlier stages combined with activation of genes involved in cardiac energy metabolism at the late stage.

Background

Cardiomyopathy syndrome (CMS) is a severe cardiac disease affecting farmed Atlantic salmon (*Salmo salar* L.) primarily in the second year in seawater close to harvest [1, 2]. Since the first diagnosis in Norway in 1985 [3], it has later been diagnosed in Scotland, the Faroe Island, Denmark and Canada [1, 4-6]. Pathology associated with CMS has also been observed in wild Atlantic salmon [7]. CMS is diagnosed based on cardiac histopathology, characterised by a severe inflammation and necrosis of the spongy myocardium of the atrium and ventricle [4]. Inflammatory infiltrates consist of mononuclear cells, probably lymphocytes and macrophages. The compact layer of the ventricle is usually less affected, and always occurs later than changes in the spongious layer [4, 8]. Farmed salmon suffering from CMS often lack clinical signs and may die suddenly due to rupture of the atrium or sinus venosus resulting in cardiac tamponade [3, 4]. A remarkable feature of CMS is the slow development of pathology, which is observed both in the field and under experimental conditions [8-10].

Recently, the causative agent of CMS was identified as double-stranded RNA virus with the proposed name piscine myocarditis virus (PMCV) [9]. The same virus sequence was also identified from high-throughput sequencing of fish with CMS [11]. PMCV has a genome size of 6,688 bp with three open reading frames, the second encoding an RNA-dependent RNA polymerase showing sequence similarities with *Giardia lamblia* virus and infectious myonecrosis virus of penaeid shrimp, suggesting assignment to the *Totiviridae* family [9]. Following experimental challenge with cell culture-grown virus, histopathological changes were observed in heart tissue from 6 weeks post-infection (wpi) with peak severity at 9-10 wpi [9, 10]. Analysis of viral load by quantitative real-time RT-PCR (qPCR) showed

replication of virus in several organs from 4 wpi, suggesting a broad tissue tropism. Peak of viral load occurred in heart, spleen and kidney and coincided with the peak of cardiac pathology [10]. Viral load in the hearts from experimental and clinical field cases correlated well the severity of histopathological changes, suggesting that cytopathic effects of infection was a major determinant of the myocardial changes [9, 10]. From transcriptome analysis of immune responses in fish, developing the strongest pathology and infection, the temporal and spatial regulation of the different arms of immunity during CMS was characterised [10]. It was shown that the peak of cardiac pathology and viral load coincided with a cardiac-specific induction of T cell response genes and splenic induction of complement genes. Activation of these responses was preceding a reduction in viral load and pathology, suggesting that they were important for viral clearance and recovery.

From the same challenge experiment, a significant proportion of the infected fish did not developed cardiac pathology, providing an opportunity for a comparative study of individuals with different outcomes of disease and characterisation of the underlying molecular mechanisms associated with protection versus pathology. Here, we show that fish developing sustained or increased infection and severe pathology (so called high responders, HR) have a different character and regulation of cellular adaptive immunity, when compared to fish without pathology and earlier viral clearance and recovery (so called low responders, LR). In particular, induced transcription of T cell response genes in high responders was associated with elevated pathology and viral load, possibly reflecting an increased infiltration and homing of virus-specific T cells to the affected heart tissue. Lower abundance of these transcripts in heart tissue of low responders combined with activation of energy metabolism was associated with a positive outcome. These results provide a comprehensive understanding of individual responses to CMS and immunological determinants of virus clearance and pathology.

Results

PMCV infection and disease responders

From a challenge trial where fish were infected with intraperitoneal (ip) injection of identical doses of PMCV [10], we observed different outcomes of cardiac pathology between individuals. It was shown that all fish mounted a similar antiviral status (gene expression,

microarrays/qPCR) and cardiac viral load (PMCV RNA, qPCR) until 6 weeks post infection (wpi). At this time point, cardiac pathology associated with CMS (histopathology score 2) was first significant and found positive in 17/27 fish. In the present study, we quantified viral load in an extended number of individuals and found that in the subsequent course of infection (8-10 wpi), fish could be divided in two groups according to differences in the development of disease (histopathology) and infection (viral load) (Figure 1). One group, termed high responders (HR), developed sustained or increased PMCV levels in heart together with elevated pathology that further decreased until 12 wpi [10]. In the other group, termed low responders (LR), viral load declined from 6-10 wpi to levels similar as for fish at 2 wpi, and no pathology was detected. According to these observations, we compared global gene expression in hearts of HR and LR during infection, in order to characterize potential differences in the host response and to identify gene expression patterns that could explain or predict the different disease outcomes.

Overall host responses in high and low responders

As a basis for gene expression analysis, the entire time course of disease was divided into three stages according to the characteristics of infection and pathology for HR and LR (Figure 1): the early stage (2-4 wpi) with equal viral load and histopathology score, the mid stage (6 wpi) with equal viral load but different histopathology score, and the late stage (8-10 wpi) where both viral load and histopathology were different between HR and LR. For initial characterization of host responses in HR and LR, enrichment analysis of gene ontology (GO) classes and KEGG pathways was performed among differentially expressed genes (DEG) at each stage (Figure 2). Common responses at the early stage included activation of innate immune responses; immune signaling pathways (Toll-like receptors, Jak-STATs, chemokines), antigen presentation (MHC class I complex) and processing (proteasome, protein modification- and ubiquitin-dependent processes), complement and phagocytosis. Repressed responses included cellular developmental processes, cytoskeleton organization and cardiac muscle development, possibly reflecting the infection-related stress and initial cellular events of pathological changes. Activation of immune responses was maintained through the mid stage for both HR and LR. At the late stage, major differences in responses were evident between HR and LR. While the number of upregulated immune response categories increased significantly in HR, these were completely ablated or leveled off in LR. Instead, LR fish showed significant induction of different catabolic and metabolic processes, possibly reflecting responses associated with recovery.

Gene markers and predictors of responses and disease outcome

Further, we identified genes associated with the early response to infection and genes with expression differences between HR and LR in mid and late stages. Differentially expressed genes with contrasting expression between HR and LR (*t*-test, $p < 0.05$) and expression profiles that correlated to the early, mid and late stages of infection were selected (Pearson's correlation coefficient > 0.6, Additional file 2). Heat maps of selected genes are shown in Figures 3-5.

i) Markers of early antiviral response

We identified 258 genes that showed significant upregulation in the early stage and gradually decreased expression at the mid and late stages (Additional file 2). These genes were induced with an average $\log_2$-ER of +1.7 at the early stage; expression ratios of 20 representative genes are shown in Figure 3. The majority of these genes were previously identified as virus-responsive [12], and they are part of the early antiviral and interferon (IFN) response to CMS [10]. Other genes in this group encode proteins important for protein degradation and antigen presentation via MHC.

ii) Early pathology and outcome predictors

Since viral clearance occurred in LR at the late stage, genes that were differentially induced or repressed in LR versus HR at the preceding mid stage might represent prognostic markers of disease outcome. Differentially expressed genes at this stage were of particular interest, since PMCV levels in heart correlated with histopathology score [10] and this was the only stage where HR and LR had different histopathology score levels but equal viral loads. Thus, such genes may represent early predictors of pathology or recovery/ clearance. We identified nine genes that were induced at mid stage in HR but not in LR (predictors of early pathology, Figure 4A), and seven genes that were induced in LR but not in HR (early predictors of recovery/clearance, Figure 4B). Genes induced in HR were mainly immune-related by function. *DDX5* (aka *p68*) encodes a RNA helicase regulating many aspects of transcription and shown to interact with HCV replication [13]. The other genes were implicated in apoptosis and thus might be a part of cell death mechanisms controlling lymphocyte regulation and survival. *TNF decoy receptor* (also known as TR6 or *decoy receptor 3*), is a member of the TNF receptor superfamily and an important mediator of T cell immunity and biomarker for inflammatory diseases (reviewed in [14]). Similarly, *GTPase IMAP7 family*

member 7 (or IAN7, GIMAP7) is part of a newly discovered family of cell survival regulators expressed in lymphocytes [15]. *Lymphocyte G0/G1 switch protein 2* (G0S2) was identified as a novel proapoptotic factor induced by TNF-α through NF-κB and shown to antagonize Bcl-2 [16]. Interestingly, G0S2 expression was particularly high in heart and peripheral blood cells, the latter was also observed during measles virus infection in humans [17]. Yet another HR-induced pro-apoptotic gene was *cell death activator CIDE-3* which was also specifically expressed in heart and intestine [18]. Its implication in virus infection has also been reported [19]. Genes that were induced in LR had no clear functional relation to the disease, except for *CD209 antigen-like protein A* and *lectin precursor*, presumably involved in immune-cell signaling (Figure 4B).

iii) Markers of pathology and outcome

To identify genes whose expression patterns were predictive of the outcome of infection, we searched for genes that were induced during declining viral load/pathology at late stage in LR but not in HR (predictors of recovery/clearance), and genes that were upregulated during increased viral load/pathology in HR but not in LR (predictors of pathology). We identified 116 genes that were induced in HR while 33 genes were induced in LR (Additional file 2). Nearly all genes with higher expression in HR were involved in adaptive immune responses (Figure 5A), and were included in our previously identified CMS profiles for T/B cell responses, MHC class I antigen presentation and apoptosis [10]. Genes related to T cell responses were overrepresented, which likely reflected increased infiltration and homing of virus-specific T cells to the infected tissue. Upregulation of genes encoding components of the T-cell receptor (*CD3gammadelta-A, CD3 zeta chain precursor* and *CD28*) substantiated this assumption. In addition, an activator of naïve T cells, *SH2 domain-containing protein 1A* was induced. Genes with putative roles in the regulation of effector function and controlled cell death of T cells included several TNF-related genes and *programmed cell death ligand 1* (aka CD274/B7-H1). Upregulation of genes encoding Rho GTPases was also an interesting finding, since they have been implicated in the regulation of T cell-receptor signaling and cytoskeletal reorganization, cell migration and apoptosis in T cells [20]. To substantiate these results, expression of four genes were evaluated by qPCR in an extended number of fish per group (n=9 HR, n=10 LR, 8 wpi). Genes implicated in T cell responses were tested; *TNF decoy receptor* as marker of early pathology, *CD274* and *TNF-11b* as markers of late pathology, and *granzyme-A* (*GzmA*) as a marker of cytotoxic T cells and the T cell response to CMS [10]. As shown in Figure 6, all genes had significantly higher relative expression ratios

in HR versus LR (*t*-test on $\log_2$-transformed values, $p < 0.01$). In contrast to the other genes, *GzmA* was also upregulated in LR, however significantly lower than in HR. Taken together, induced transcription of these genes suggested an increased population and activity of T cells in infected hearts of HR compared to LR.

Genes that were induced during declining viral replication in LR and not in HR encoded for enzymes of metabolic pathways or cell respiration, and can be considered to represent markers for recovery or protection (Figure 5B). Some of these enzymes are involved in the intermediate pathways of metabolism, e.g. aldolase a, malate dehydrogenase and different isomerases. Components of the electron chain included *ATP synthetase* and *electron transfer flavoprotein*. Three genes are potentially involved in heart muscle regeneration. *Hydroxysteroid dehydrogenase-like protein 2* was induced during myocardial injury following injection of bone marrow mononuclear cells in rats [21]. *Calsequestrin-like protein* is important for the Ca^{2+} regulation in muscle cells and a dramatic decrease of protein concentration was observed in a proteomics study of human dystrophic muscle fibers [22]. β-parvin (aka Affixin) is a integrin-linked protein that is involved in the linkage between integrin and the cytoskeleton and was supposed to be involved in membrane repair mechanisms in human [23].

To further substantiate results, we performed GO analysis of differentially expressed genes in HR and LR to identify functional groups that were positively or negatively correlated to the viral load at the late stage (Figure 7). As expected, positively correlated functional groups in HR included immune system process/immune response and more specifically, activation of lymphocytes and leukocytes. Negatively correlated groups included a large number of categories related to cardiac and skeletal muscle development (Figure 7 and Additional file 1). In LR fish, no immune-related GO categories showed correlation to viral load. In contrast, metabolic processes, generation of precursor metabolites/energy and oxidation reduction were positively correlated categories, while muscle cell development and skeletal myofibril assembly were negatively correlated.

Microarray confirmation by qPCR

To confirm microarray data, $\log_2$-transformed expression levels of four early antiviral genes by qPCR were compared with the respective expression ratios from microarrays (Figure 8). Primers for qPCR (Additional file 3) and oligonucleotide probes on the microarray were

based on the same cDNA sequences. Data from 8 infected fish showed high correlation between the two methods.

Discussion

In this study, we compared the host response in PMCV infected salmon with different outcomes of disease in order to characterize gene expression patterns and responses that could be associated with cardiac pathology versus recovery. Assessment of viral load and histopathology from bi-weekly samples of heart over a period of ten weeks suggested that fish exhibited different development of disease from six weeks post-infection. The so called high responders (HR) group developed severe pathology and increased infection level while the low responder (LR) group showed reduced viral load and retained a normal heart with absence or limited pathology. This could be interpreted as different resistance to the disease since all fish were simultaneously infected using a standardized i.p.-injection with identical dose of virus. Previous analyses of fish from the same challenge trial also showed that all fish (n = 20) mounted a similar level of early antiviral response [10]. The early stage (2-4 wpi), was characterized by a rapid virus replication with no significant changes in myocardial tissue (histology score 0-1). However, results of the GO enrichment analysis showed repression of many functional groups related to cardiac and general muscle development, suggesting an onset of pathology at the molecular/cellular level. This could also be expected, given the strong antiviral response (on transcriptomic level) in the cells at this stage, possibly leading to compromised cell growth. The challenge trial was conducted with young fish with a rapidly growing heart and the virus replication might retard this process. Both GO analysis and gene expression profiling found that the early antiviral response involved genes of the interferon pathway and antigen presentation pathways via MHC, as was observed in our recent study [10]. Most of these genes were identified as virus-responsive with high correlation to expression of IFNa [12]. These are typical early responses to viruses and reflect the innate immune response followed by initiation of the adaptive immune response [24, 25]. Thus, our results show that PMCV-infected cells successfully induced the transcription of many antiviral and IFN-dependent genes, but that this response had little or no direct effect on virus replication and outcome, since their magnitude of expression was negatively correlated with development of infection/disease and their expression remained induced within the whole

challenge trial, except for the late stage in LR which had lowest viral loads. The same was observed in a study with viral haemorrhagic septicaemia virus infected rainbow trout, where genes of the interferon system were correlated to the viral load in affected tissue, however strong expression did not reflect a better protection against virus spread [26]. Nonetheless, the innate antiviral responses are of great importance for mounting of downstream immune pathways.

The mid stage of the disease was characterized by similar viral loads but different histopathology score in LR and HR and gene enrichment analysis of functional classes and pathways showed a similar activation of immune responses in both groups. For the analysis of predictor genes, our main hypotheses were that genes with stronger expression in HR than in LR represent predictors of early pathology, and genes with stronger expression in LR than in HR might represent predictors of protection/recovery. The predicted functional role of the majority of genes with significantly higher expression in HR (Figure 4) were interesting in view of the development of pathology in the subsequent late stage, which in our previous study was characterized by an increased inflammation dominated by influx of T cells [10]. In mammals, these genes are involved in cell death mechanisms and have been implicated in the control of lymphocyte regulation and survival. Induced transcription of these genes at mid stage of CMS in HR was likely a result of the initial rise in lymphocyte infiltration to the infected tissue as reflected from histopathology (this was the first stage with score 2, representing pathology above normal background levels, score 0/1). Since predictor genes were identified based on expression differences between HR versus LR, potential genes with equal expression in HR/LR at this stage were not taken into account. In this respect it is necessary to mention that many genes related to the T cell response in CMS [10] were upregulated already at the mid stage (see Figure 5 and Additional file 2), however differences between HR and LR appeared later. Thus, activation of lymphocytic and inflammatory responses occurred in both responder groups, but different abilities to control or regulate these responses might explain different outcomes in the subsequent stage. The importance of these responses in immunity versus immunopathology is intensively studied in human infections [27], and should deserve more attention in relation to fish viral infections.

The recent studies on CMS showed a high correlation between histopathology score and viral load implying that the cytopathic effect of infection was a major determinant of the myocardial changes [9, 10]. Results of this study further show that the level of pathology and

infection is correlated to the activation of lymphocytes and leukocytes (GO enrichment analysis) and particularly expression of genes associated with the T cell response at the late stage in HR. This was also expected, assuming that this response reflects the migration of virus-specific lymphocytes to the infected tissue. Infiltration of leukocytes/lymphocytes did not occur in LR fish according to histopathology results. This was accompanied by a completely absent expression of a large number of genes mainly related to T and B cell responses in the late stage, which was the most significant difference between HR and LR fish identified in this study. The lack of transcription of many genes involved in the regulation of T cell effector function and cell death/survival was of particular interest. Control of these processes is fundamental for regulation of the T cell response and for maintaining homeostasis in the immune system after it has expanded to combat infections [27, 28]. As already discussed, many of these genes were also expressed at the mid stage of HR but not in LR. Elevated gene expression levels of *granzyme A* (*gzmA*) in LR fish showed that cytotoxic cells were also present in the heart of these fish. GzmA is a serine protease and important inducer of antiviral and apoptotic pathways in infected cells, produced by cytotoxic T cells and NK cells [29]. This suggests that adaptive cellular immune responses occurred in LR fish as well, however on a lower level compared to HR fish. Hence, both different shaping and a lower magnitude of immune responses could explain the different outcome of these groups. In contrast to HR, genes involved in energy metabolism and other catabolic/metabolic processes were induced in LR. In addition, the GO enrichment analysis showed that the same processes were correlated to the decline in viral load in LR. Thus, in contrast to HR, these fish seemed to cope with the infection by immune responses in the preceding stages and/or by a different composition or regulation of the late response, and managed to activate cardiac energy metabolism for recovery and regeneration of infected tissue in the late stage.

In conclusion, we have compared the host response and pathogenesis in PMCV infected salmon with different outcome of disease, and described gene expression patterns and predictors and associated functional pathways underlying these differences. The main findings are summarized in Figure 9, suggesting that very different composition and regulation of adaptive cellular immunity in the late stage of infection was the most prominent feature associated with pathology versus recovery. In our previous CMS study focusing on the most severely diseased fish, we also concluded that the same responses were possibly associated with an observed viral clearance and reduced pathology later on (10-12 weeks post infection) [10]. Consequently, while having an important role in the clearance of virus in infected heart

tissue, virus-specific immune cells such as T and B cells may at the same time lead to increased tissue damage and burden for the host. Whether the stronger immune responses observed in HR was reflecting an immunopathology could not be concluded based upon the present data. However, the importance of understanding the balance between immunity and immunopathology is highly acknowledged in human disease [27], and should be subject to further study in viral diseases of Atlantic salmon. The basis for the positive outcome of LR fish is also not known. A recent field study with family group indicated a genetic basis for survival against CMS (N. Santi, personal communication). Studies of genetic background in combination with host responses and disease development in controlled infection trials will be followed up in future research.

Methods

Experimental infection and sampling

The biological material for this work was taken from a challenge trial previously described in [10]. In this trial, 120 fish were kept in four separated tanks; one infected and one control group in duplicates. The fish had an average weight of 50 gram at start. Each fish of the infected group received an intraperitoneal (i.p.) injection dose (0.2 ml) of an inoculate originating from a passaged cell culture developing cytopathogenic effects after infection with filtered heart homogenate derived from several adult Atlantic salmon with clinical CMS. Control groups received the same dose of conditioned cell culture medium. Tissue samples were collected 2, 4, 6, 8 and 10 wpi. At each sampling date, 15 fish from each of the four tanks were sedated (as described above) and euthanized by decapitation. For this study standardised samples from heart were snap-frozen in liquid nitrogen for RNA and fixed in formalin (10 % neutral phosphate-buffered) for histology.

Histopathology

Scoring of heart lesions by histopathological examination (Figure 1) was taken from a previous study as described [10]. In brief, score 0 and 1 was considered normal, with no histopathological findings (score 0), or a single or few focal lesions (score 1). Score 2 represented several distinct lesions and increased mononuclear infiltration. Score 3 represented multifocal to confluent lesions in > 50 % of tissue and moderate to severe leukocyte infiltration.

RNA extraction

Sampled hearts for microarray hybridization were stored at -80 °C prior to RNA extraction. A piece of circa 10 mg was cut from each frozen sample and transferred directly to 1 ml chilled TRIzol (Invitrogen, Carlsbad, CA, USA) in 2 ml tubes with screw caps (Precellys®24, Bertin Technologies, Orléans, France). Two steel beads (diameter: 2 mm) were added to each tube and the tissue was homogenized in a Precellys®24 homogenizer for two times 25 sec at 5000 rounds per minute with a break of 5 sec between the rounds. RNA was extracted from the homogenized tissues using PureLink RNA Mini kits according to the protocol for TRIzol-homogenised samples (Invitrogen). The concentration of extracted total RNA was measured with a NanoDrop 1000 Spectrometer (Thermo Scientific, Waltham, MA, USA). The integrity of total RNA was estimated, using an Agilent 2100 Bioanalyzer with RNA Nano kits (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA integrity number (RIN) of 8 or higher were accepted.

Quantitative real-time RT-PCR

Experiments were conducted according to the MIQE guidelines [30]. Synthesis of cDNA was performed on 0.2 μg DNAse-treated total RNA (Turbo DNA-free^{TM}, Ambion, Austin, TX, USA) using the TaqMan® Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in 25 μl reactions with random hexamer priming according to manufacturer's protocol. Complementary DNA was stored undiluted at -80 °C in aliquots to avoid repeated freeze-thawing. To avoid risk for presence of residual DNA contamination, control reactions without RT was tested and qPCR primers were possibly designed to span introns. Oligonucleotide primers for genes of Atlantic salmon were designed with the program eprimer3 from the EMBOSS program package (version 5.0.0, http://emboss.sourceforge.net/). Amplicon size was set to 80-200 and melting temperature to 59-61 °C. Primers were purchased from Invitrogen (Additional file 3). *In silico* analysis of gene targets was performed using a customised program for BLAST and sequence alignments [31]. PCR amplicon size and specificity were confirmed by gel electrophoresis and melting curve analysis (Tm calling; LightCycler® 480, Roche Diagnostics, Mannheim, Germany). QPCR was conducted in duplicate reactions as described [10]. Cycle threshold (C_T) values were calculated using the second derivative method. Duplicate measurements that differed more than 0.5 C_T values were removed and reanalysed. For relative quantification, the mean of duplicates was used. Relative expression ratios of test samples versus the average of the controls were calculated

according to the Pfaffl method [32]. Elongation factor 1α (GenBank ID: BT072490.1) was used as reference gene [33], and was found to be stably transcribed in control and test samples according to the BestKeeper software [34]. The efficiency of the PCR reactions was estimated for all primer pairs by six times 1:5 dilution series of a cDNA mix of all used samples. The efficiency values were estimated by using the LightCycler® 480 Software (version 1.5.0.39).

Viral load

Relative quantification of PMCV was assessed by qPCR as described [10]. In brief, total RNA (62.5 ng per sample, isolated as described above) from infected fish (heart; weeks 0, 2, 4, 6, 8, 10, n = 5-8 per time point) were subject to cDNA synthesis (SuperScript® III Platinum® Two-Step qRT-PCR Kit with SYBR® Green, Invitrogen) and qPCR (2X Platinum® SYBR® Green qPCR SuperMix-UDG, Invitrogen) in triplicate reactions. Melting curve analysis was performed to confirm expected amplicon. Viral load was expressed as the relative copy number with non-infected controls (0 wpi) set to 1 and calculated by the formula $2^{\wedge}(C_{T(0wpi\ median)} - C_{T(sample)})$.

Design of microarray experiments

An overview of microarray hybridizations is given in Additional file 4. The salmonid oligonucleotide microarray (SIQ2.0, NCBI GEO platform GPL10679) was used [12], consisting of 21K features printed in duplicates on 4 x 44K chips from Agilent Technologies. Two-colour hybridizations in a reference design were used, where test samples labelled with Cy5 dye and reference samples labelled with Cy3 dye were competitively hybridized per array. As reference samples, pools of equimolar amounts of RNA from heart tissue from 6-8 control fish per time point were used (only fish with histopathology score 0 were included). The examined time points were 2, 4, 6, 8 and 10 wpi. For each time point, 5-8 individuals from both test (high and low responders) and control groups were hybridized against the reference sample, giving a total number of 65 arrays with 33 infected and 32 control individuals.

Microarray hybridization and data analysis

Unless specified otherwise, all reagents and equipment used for microarray analyses were purchased from Agilent Technologies and used according to manufacturer's protocol. In brief, RNA labelling and amplification was performed with Low Input Quick Amp Labeling Kits, Two-Color and RNA Spike-In Kits, Two-Color for 4 x 44K microarrays, using 200 ng of

total RNA per reaction. For fragmentation of the labelled RNA, Gene Expression Hybridization Kits were used. Labelled RNA was hybridized for 17 hours (hybridization oven, Agilent) at 65 °C and rotation speed of 10 rounds per minute. Arrays were washed for one minute with Gene Expression Wash Buffer I at room temperature, and one minute with Gene Expression Wash Buffer II at 37°C. Slides were scanned immediately after washing using a GenePix Personal 4100A scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 µm. The laser power was manually adjusted to ensure an overall intensity ratio close to unity between Cy3 and Cy5 channels and with minimal saturation of features. The GenePix Pro software (version 6.1) was used for spot-grid alignment, feature extraction of fluorescence intensity values and assessment of spot quality.

Subsequent data processing and analyses were performed using the STARS platform [31]. Values of replicate spots passing quality control were averaged and Lowess normalization of $\log_2$-expression ratios (ER) was performed. Initial quality filtering was based on mean spot intensity and number of informative spots, resulting in 11,913 passed features. Outliers among control fish identified by cluster analysis (uncentered correlation, complete linkage using "Cluster 3.0" [35]) were removed. Significant differences between gene expression of infected and control fish were calculated by *t*-tests for each time point and group (HR and LR). The median values of respective control fish were subtracted from the individual values of the infected fish, and median $\log_2$-ER for each gene were calculated per group and time point. The final list of differentially expressed genes (DEG) was selected by filtering for previously mentioned *t*-tests ($p < 0.05$, at least one time point or group) and $\log_2$-ER > |0.7| (in at least one time point or group). Corrections for false discovery rate were not employed as previous microarray studies in Atlantic salmon have demonstrated them to be overly conservative [36, 37]. Data was submitted to GEO (accession number to be confirmed).

For identification of marker/predictor genes in HR and LR, correlation analysis was performed to search for (i) genes associated with the early antiviral response (strong induction at the early stage, moderate induction at the mid stage and late stage HR and no induction at late stage LR), (ii) genes with expression changes in mid and late stage HR and LR (iii) genes that were induced at only one stage and (iv) genes whose expression profiles correlated with virus loads. Equal thresholds were established for all correlations (Pearson's $r > 0.6$).

Enrichment of GO classes and KEGG pathways among DEG were assessed with STARS ($p < 0.05$, Yates' corrected chi square); terms represented with less than five genes were not taken

into consideration. In addition, BLAST2GO [38] with an E-value cut-off for the BLAST searches of 10^{-20} was used for annotations and BiNGO plugin (Version 2.44, [39]) for Cytoscape (http://www.cytoscape.org/, version 2.8.0) for GO enrichment analyses. Only GO terms with the category "biological process" were considered and threshold of significance of the corrected p-value was < 0.05 (Benjamin & Hochberg false discovery rate correction). Multiple hits of identical probes were reduced to the GO term with highest occurrence (for complete lists, see Additional file 1). Part of plots and statistical calculations were made with the R software package (version 2.13.0, www.cran.r-project.org/).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GT, SMJ and AK drafted the manuscript and conducted gene expression analysis. HT and SMJ planned the experimental design. MR and PN analysed viral load. SA developed the software for processing of microarray data and performed parts of the statistical analyses. All authors read and approved the final manuscript.

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Figures

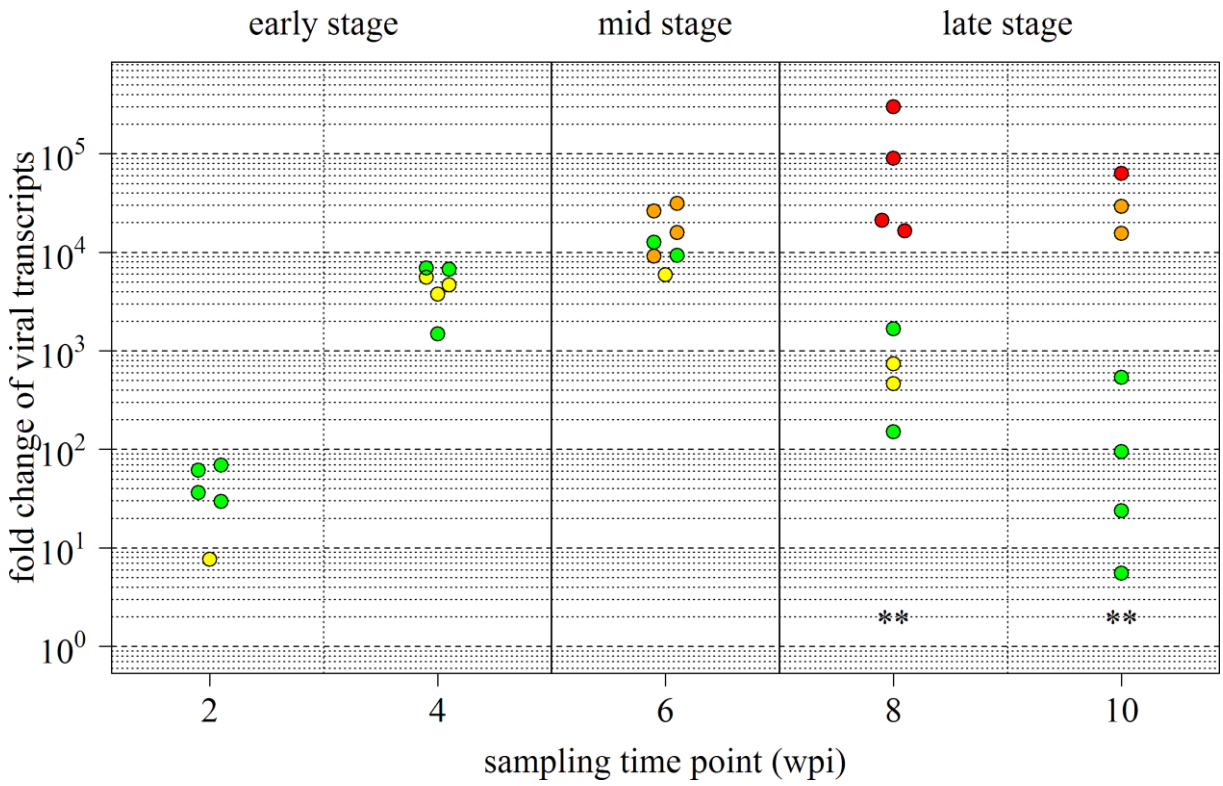

Figure 1. Virus load and pathology of disease responders

PMCV RNA level and pathology score in heart tissue of challenged fish over the time course of infection. The three stages of disease (early = 2-4 wpi, mid = 6 wpi and late = 8-10 wpi) are indicated on top of the figure, as explained in Results. Relative quantification of PMCV by qPCR is given as fold change of viral transcripts relative to the median levels of 0 wpi samples set to 1. Individual values are shown as dots and coloured according to the level of CMS pathology (histopathology score level based on atrium; green = score 0, yellow = 1, orange = 2, red = 3). Significant differences in viral load between high responder fish (HR, orange and red dots) and low responder fish (LR, green and yellow dots) from mid and late stages are marked with asterisks (*t*-test on log-transformed values, $p < 0.01$). Overlapping dots were plotted beside each other.

early stage: 2-4 wpi			mid stage: 6 wpi			late stage: 8-10 wpi			
#	Source	GO name	#	Source	GO name	#	Source	GO name	
38	B2GO	Immune system process	23	B2GO	Immune system process	46	B2GO	Immune system process	High responders
23	B2GO	Immune response	14	ST:GO	Ubiquitin-dependent prot catab process	24	B2GO	Immune response	
18	B2GO	Antigen processing and presentation	13	ST:GO	Endopeptidase activity	21	ST:KEGG	Chemokine signaling pathway	
18	ST:GO	Immune response	13	ST:GO	Proteasome core complex	17	ST:KEGG	Lysosome	
17	ST:GO	Ubiquitin-dependent prot catab process	13	ST:KEGG	Proteasome	16	B2GO	Defense response	
17	ST:KEGG	Proteasome	11	ST:KEGG	Chemokine signaling pathway	16	ST:KEGG	Proteasome	
15	ST:GO	Endopeptidase activity	11	ST:KEGG	Lysosome	15	B2GO	Response to biotic stimulus	
15	ST:GO	Proteasome core complex	10	ST:GO	Immune response	15	ST:KEGG	Fc gamma R-mediated phagocytosis	
14	ST:KEGG	Chemokine signaling pathway	9	ST:KEGG	Fc gamma R-mediated phagocytosis	15	ST:KEGG	Regulation of actin cytoskeleton	
13	ST:KEGG	Antigen processing and presentation	9	ST:KEGG	Jak-STAT signaling pathway	14	B2GO	Antigen processing and presentation	
13	ST:KEGG	Fc gamma R-mediated phagocytosis	9	ST:KEGG	Toll-like receptor signaling pathway				
13	ST:KEGG	Phagosome	8	B2GO	Antigen processing and presentation				
12	ST:GO	Antigen processing and presentation	7	ST:GO	Protein modification process				
12	ST:KEGG	Toll-like receptor signaling pathway	6	ST:GO	Antigen processing and presentation				
11	ST:KEGG	Lysosome	6	ST:GO	MHC class I protein complex	#	Source	GO name	
9	ST:GO	MHC class I protein complex	6	ST:GO	Signal transducer activity	7	B2GO	Cofactor biosynthetic process	
9	ST:GO	Protein modification process	5	ST:GO	Cytoskeleton	7	ST:KEGG	Porphyrin metabolism	
8	ST:KEGG	Jak-STAT signaling pathway	5	ST:GO	Ubiquitin cycle	7	ST:KEGG	ECM-receptor interaction	
6	ST:GO	Ubiquitin cycle	5	ST:KEGG	Antigen processing and presentation	6	B2GO	Oxygen transport	
6	ST:KEGG	Complement and coagulation cascades				6	B2GO	Pigment biosynthetic process	
						5	B2GO	Heme biosynthetic process	
#	Source	GO name	#	Source	GO name	#	Source	GO name	Low responders
35	B2GO	Cell. comp. organization at cell.level	33	B2GO	Immune system process	23	B2GO	Catabolic process	
31	B2GO	Cellular developmental process	17	B2GO	Immune response	15	B2GO	Gen. of prec. metabolites and energy	
30	B2GO	Organelle organization	14	ST:GO	Ubiquitin-dependent prot catab process	15	B2GO	Oxidation reduction	
27	B2GO	Anatomical structure morphogenesis	14	ST:KEGG	Chemokine signaling pathway	14	B2GO	Carboxylic acid metabolic process	
24	B2GO	Cellular component assembly	13	ST:GO	Endopeptidase activity	9	B2GO	Immune response	
22	B2GO	Cell. comp. assembly at cellular level	13	ST:GO	Proteasome core complex	8	B2GO	Carbohydrate catabolic process	
21	B2GO	Cell development	12	B2GO	Antigen processing and presentation	8	B2GO	Coenzyme metabolic process	
21	B2GO	Actin filament-based process	12	ST:GO	Immune response	7	B2GO	Glycolysis	
21	B2GO	Cytoskeleton organization	12	ST:KEGG	Lysosome	7	B2GO	Energy deriv. by ox. of organic comp.	
19	B2GO	Regulation of signal transduction	11	ST:KEGG	Phagosome	6	B2GO	Antigen processing and presentation	
19	B2GO	Actin cytoskeleton organization	10	ST:GO	Protein modification process	6	B2GO	Dicarboxylic acid metabolic process	
19	B2GO	Cellular component morphogenesis	10	ST:KEGG	Antigen processing and presentation	6	B2GO	Coenzyme catabolic process	
17	B2GO	Anat. struc. form. involved in morphog.	10	ST:KEGG	Jak-STAT signaling pathway				
17	B2GO	Muscle structure development	8	ST:GO	Antigen processing and presentation				
16	B2GO	Reg. of small GTPase med. signal trans.	8	ST:KEGG	Fc gamma R-mediated phagocytosis				
16	B2GO	Proteolysis	8	ST:KEGG	Natural killer cell mediated cytotoxicity				
16	B2GO	Muscle cell development	7	ST:GO	MHC class I protein complex				
16	B2GO	Cardiovascular system development	7	ST:GO	Ubiquitin cycle	#	Source	GO name	
15	B2GO	Reg. of Ras protein signal transduction	7	ST:GO	Signal transducer activity	16	B2GO	Ribonucleoside triphos. catab. process	
15	B2GO	Striated muscle cell development				9	B2GO	GTP catabolic process	

Figure 2. Enrichment analysis of gene ontology classes and pathways

Gene enrichment analysis of regulated gene ontology (GO) classes and KEGG pathways in all fish at the early disease stage, and in high and low responder groups at mid and late stages. GO terms highlighted in red indicate upregulated genes and blue indicate downregulated genes. The different bioinformatics sources used for analyses are indicated (column "source"); ST:GO/KEGG: STARS platform, B2GO: BLAST2GO/Cytoscape. Column "#" shows the number of regulated genes behind the respective GO term (primary data in Additional file 1).

#	E	M:HR	M:LR	L:HR	L:LR	cat	Refseq ID	Name
1	**5.3**	**3.9**	**4.3**	**2.5**	**0.8**	V	XP_001344381.1	Interferon-inducible protein Gig2-like [Salmo salar]
2	**5.2**	**3.9**	**3.6**	**2.6**	**0.7**	V	XP_693604.2	Receptor-transporting protein 3 [Salmo salar]
3	**5.0**	**3.9**	**3.5**	**2.7**	**1.2**	V	NP_001020727.1	Radical S-adenosyl methionine domain-containing protein 2 [Salmo salar]
4	**4.8**	**3.9**	**3.4**	**2.9**	**0.8**	V	XP_695770.2	Interferon-induced protein with tetratricopeptide repeats 5 [Salmo salar]
5	**4.0**	**2.3**	**2.4**	**2.0**	**0.9**	V	ACX35594.1	VHSV-inducible protein [Salmo salar]
6	**3.7**	**3.1**	**3.5**	**3.3**	0.5	V	XP_001332607.1	Barrier-to-autointegration factor [Salmo salar]
7	**2.9**	**1.7**	**1.6**	**1.6**	0.3	V	XP_695523.2	Zinc finger, NFX1-type containing 1 [Homo sapiens]
8	**2.6**	**1.6**	**1.7**	**1.5**	0.3	V	XP_682942.2	Interferon-induced protein 44 [Salmo salar]
9	**2.6**	**2.4**	**2.6**	**2.0**	**0.4**	V	NP_001019587.1	PR domain zinc finger protein 9 [Salmo salar]
10	**2.2**	**1.4**	**1.3**	**1.3**	0.3	V	NP_001006049.1	Damage-regulated autophagy modulator [Salmo salar]
11	**1.8**	**0.9**	**0.9**	**0.8**	0.0	V	NP_001001817.1	Galectin-9 [Salmo salar]
12	**1.7**	**0.9**	**0.9**	**0.8**	0.0	V	XP_694124.2	Interferon induced with helicase C domain 1 [Homo sapiens]
13	**1.6**	**0.9**	**0.8**	**0.9**	-0.1	V	NP_001002748.1	Tetraspanin-3 [Salmo salar]
14	**1.6**	**0.7**	**0.8**	**0.8**	0.2	V	XP_001342625.1	Peroxisomal prolif-act rec A-interact comp 285 kDa protein [Salmo salar]
15	**1.5**	**0.9**	**0.8**	**0.8**	0.1	V	NP_571555.1	Signal transducer and activator of transcription 1-alpha/beta [Salmo salar]
16	**1.1**	**0.4**	**0.5**	**0.5**	0.1	V	XP_001342317.1	52 kDa Ro protein [Salmo salar]
17	**4.5**	**4.6**	**4.1**	**3.6**	**1.4**	P	NP_001071272.1	Ubiquitin-like protein precursor [Salmo salar]
18	**3.9**	**1.9**	**2.4**	**2.1**	0.2	P	XP_001343099.1	Hect domain and RLD 6 [Homo sapiens]
19	**1.6**	**1.4**	**1.8**	**1.4**	**0.7**	P	NP_571779.1	MHC class I antigen [Oncorhynchus mykiss]
20	**1.0**	**0.7**	**0.7**	0.5	0.0	P	XP_697222.2	Deltex-3-like [Salmo salar]
L	5.3	2.6	0.0	-2.6	-5.3			

Figure 3. Gene markers of early antiviral response

Heat map of 20 selected genes with expression profile showing upregulation at the early stage and gradually decreased expression during mid and late stages of infection (complete data in Additional file 2). Left columns show the median $\log_2$ expression ratios of genes in the different stages and responder groups ("E" = early, "M:HR" = mid stage HR, "M:LR" = mid stage LR, "L:HR" = late stage HR, "L:LR" = late stage LR). Graded levels from white to red and blue indicate respectively upregulation and downregulation, according to the color scale (row "L"). Significant differences in $\log_2$–ER between control and infected samples (*t*-test, $p < 0.05$) are shown in bold. Column "cat" indicates the category of gene annotation according to [10]: V- early antiviral and IFN response, P- MHC antigen presentation.

#	E	M:HR	M:LR	L:HR	L:LR	cat	Refseq ID	Name (A)
1	-0.9	**2.6**	0.1	-1.4	0.0	V	XP_001345161.1	GTPase, IMAP family member 7 [Oncorhynchus mykiss]
2	-0.1	**0.9**	**0.2**	-0.2	-0.7	V	ACN11269.1	Probable ATP-dependent RNA helicase DDX5 [Salmo salar]
3	0.1	**1.0**	**0.4**	-0.2	-0.8	V	ACN11269.1	Probable ATP-dependent RNA helicase DDX5 [Salmo salar]
4	0.2	**2.1**	**0.8**	**2.0**	0.0	T	AAK91758.1	TNF decoy receptor [Oncorhynchus mykiss]
5	0.3	**1.9**	0.3	-0.6	0.1	O	NP_571332.2	Hemoglobin subunit alpha [Salmo salar]
6	1.5	**2.5**	1.1	-0.8	0.3	O	XP_001337352.1	Hemoglobin subunit alpha-4 [Salmo salar]
7	0.2	**1.2**	0.4	-0.4	-0.2	O	NP_001070181.1	Hypothetical protein LOC84293 [Homo sapiens]
8	0.2	**0.8**	0.1	0.1	0.1	O	NP_001071068.1	Phosphatase 1L [Salmo salar]
9	0.2	**0.9**	0.2	-0.2	0.2	M	XP_701038.2	Calponin-1 [Salmo salar]
10	0.0	**1.6**	-0.4	0.1	0.1	L	ACI67778.1	Lymphocyte G0/G1 switch protein 2 [Salmo salar]
11	0.7	**1.7**	-0.9	0.1	-0.3	L	ACI69363.1	Lymphocyte G0/G1 switch protein 2 [Salmo salar]
12	-0.1	**1.3**	0.4	-0.5	-0.6	A	NP_001038512.1	Cell death activator CIDE-3 [Salmo salar]
L	2.6	1.3	0.0	-1.3	-2.6			

#	E	M:HR	M:LR	L:HR	L:LR	cat	Refseq ID	Name (B)
1	**0.8**	0.2	**1.0**	0.4	0.0	V	XP_001336802.1	Fish virus induced TRIM protein [Oncorhynchus mykiss]
2	**0.9**	0.3	**1.0**	**0.4**	-0.1	T	XP_001334880.1	CD209 antigen-like protein A [Salmo salar]
3	0.3	0.4	**1.0**	0.2	0.2	O	NP_955940.1	Integral membrane protein 2B [Salmo salar]
4	0.6	-0.1	**0.8**	-0.1	0.4	O	NP_001070239.1	Seraf-like protein [Oncorhynchus mykiss]
5	-0.4	-0.1	**0.8**	-0.3	-0.4	O	XP_683907.1	Sphingomyelin phosphodiesterase 1, acid lyso. isof. 1 pre. [Homo sapiens]
6	**0.8**	-0.2	**1.3**	0.5	0.0	I	XP_700580.1	Lectin precursor [Salmo salar]
7	0.1	0.1	**1.4**	-0.3	-0.2	E	NP_998887.1	Phosphate carrier protein, mitochondrial precursor [Salmo salar]
8	**-0.2**	0.1	**1.5**	-0.1	-0.3	E	NP_998887.1	Phosphate carrier protein, mitochondrial precursor [Salmo salar]
9	0.0	0.0	**1.0**	0.1	**0.5**	E	NP_998887.1	Phosphate carrier protein, mitochondrial precursor [Salmo salar]
L	1.5	0.8	0.0	-0.8	-1.5			

Figure 4. Early pathology and outcome predictor genes

Heat map of all genes with expression profile showing upregulation in HR (A) and LR (B) fish at the mid stage of infection. Further explanations are given in Figure 3. Column "cat" indicates the category of gene annotation according to [10]: V- early antiviral and IFN response, T- T cell response, M- muscle cytoskeleton development, L- lymphocyte regulation, A- apoptosis, I- implicated in immune response, E- mitochondrial electron chain/energy metabolism, O- other/unknown. Genes may have identical name and Refseq ID match, but represent different cDNA sequences on the array (see Additional file 2).

#	E	M:HR	M:LR	L:HR	L:LR	cat	Refseq ID	Name (A)
1	**0.4**	**1.0**	**0.8**	**1.9**	**0.3**	T	XP_685814.2	Programmed cell death 1 ligand 1 precursor [Salmo salar] CD274
2	**0.4**	0.5	0.6	**1.9**	0.3	T	NP_001117093.1	CD3gammadelta-A [Salmo salar]
3	**0.5**	0.3	**0.5**	**1.8**	**0.5**	T	NP_001108163.1	SH2 domain-containing protein 1A [Salmo salar]
4	0.5	0.3	0.3	**1.6**	-0.1	T	ACI69113.1	CD28 T-cell-specific surface glycoprotein
5	0.4	0.1	0.2	**1.5**	0.4	T	ACI67060.1	CD3 T-cell surface glycoprotein zeta chain precursor
6	**0.5**	**0.5**	**0.6**	**1.4**	0.4	T	NP_957198.1	Fermitin family homolog 3 [Salmo salar]
7	**0.5**	0.3	**0.4**	**1.3**	0.1	T	NP_571395.1	Plastin-2 [Salmo salar]
8	**0.6**	0.4	**0.4**	**1.3**	0.3	T	XP_001338671.1	Caldesmon 1 isoform 4 [Homo sapiens]
9	**0.5**	0.2	0.4	**1.2**	0.2	T	NP_001004510.1	Signal transducer and activator of transcription 4 [Salmo salar]
10	0.3	0.1	0.3	**0.9**	0.2	T	NP_849172.2	NOD3 protein [Homo sapiens]
11	**0.4**	**0.5**	**0.6**	**1.2**	0.0	P	NP_001019580.1	Cathepsin S precursor [Salmo salar]
12	**0.7**	**0.5**	**0.6**	**1.1**	**0.1**	P	XP_684112.2	Tapasin-related protein [Salmo salar]
13	0.8	0.5	**0.6**	**1.0**	0.1	P	NP_571870.2	Proteasome subunit alpha type-6 [Salmo salar]
14	0.3	**0.8**	**0.8**	**1.2**	0.0	B	XP_690443.1	SH3 protein expressed in lymphocytes [Salmo salar]
15	**0.4**	0.3	**0.2**	**0.7**	0.2	B	NP_956922.1	Src kinase-associated phosphoprotein 2 [Salmo salar]
16	**0.8**	**1.3**	**0.6**	**3.0**	0.2	A	XP_001334210.1	Tumor necrosis factor receptor superfam member 11B prec [Salmo salar]
17	**0.6**	**0.6**	**0.6**	**1.3**	0.2	A	NP_001070775.1	Regulator of G-protein signaling 18 [Salmo salar]
18	**0.4**	0.4	0.4	**1.1**	0.4	A	XP_001338932.1	Ras GTPase-activating protein nGAP [Salmo salar]
19	**0.4**	**0.5**	**0.6**	**1.0**	0.2	A	NP_001018478.1	Rho-related GTP-binding protein RhoF precursor [Salmo salar]
20	**0.6**	**0.5**	**0.4**	**0.9**	0.2	A	NP_956668.1	Tumor necrosis factor, alpha-induced protein 8-like protein 2 [Salmo salar]
L	3.0	1.5	0.0	-1.5	-3.0			

#	E	M:HR	M:LR	L:HR	L:LR	cat	Refseq ID	Name (B)
1	0.1	**-0.3**	0.2	0.1	**0.8**	O	NP_001003620.1	Calsequestrin-like [Salmo salar]
2	**-0.2**	-0.5	-0.3	0.0	**0.8**	O	NP_998069.1	Chromatin modifying protein 2B [Homo sapiens]
3	0.5	0.3	0.1	0.0	**0.9**	O	NP_001038439.1	Placental protein 11 precursor [Homo sapiens]
4	-0.1	-0.4	-0.2	-0.2	**0.8**	O	XP_001343674.1	Remodeling and spacing factor 1 [Homo sapiens]
5	0.2	-0.5	-0.3	-0.6	**1.0**	O	NP_570995.1	Retinol-binding protein 2 [Oncorhynchus mykiss]
6	0.8	-0.2	-1.4	0.1	**1.5**	O	NP_001008628.1	Serine/threonine-protein kinase PINK1, mitochondrial prec. [Salmo salar]
7	0.3	0.2	-0.1	0.1	**0.8**	O	ACI67000.1	Transmembrane emp24 domain-containing protein 7 prec. [Salmo salar]
8	0.1	0.5	-0.1	0.1	**0.8**	M	NP_956020.1	Parvin, beta isoform a [Homo sapiens]
9	0.0	-0.3	-0.2	0.3	**0.8**	M	NP_001030338.1	Supervillin [Danio rerio]
10	**0.3**	-0.3	0.0	0.2	**0.8**	E	NP_919358.2	Aldolase a, fructose-bisphosphate 1 [Salmo salar]
11	0.0	0.0	0.0	0.0	**1.0**	E	NP_001070823.1	ATP synthase H+ transp. mito. F1 complex alpha subunit 1 [Salmo salar]
12	0.1	-0.6	-0.9	-0.3	**0.8**	E	XP_689468.2	Electron transfer flavoprotein subunit alpha, mito. precursor [Salmo salar]
13	0.0	-0.1	-0.1	-0.1	**0.8**	E	NP_955893.1	Hydroxysteroid dehydrogenase-like protein 2 [Salmo salar]
14	0.2	0.3	0.2	-0.2	**1.4**	E	ACI33582.1	Malate dehydrogenase, cytoplasmic [Salmo salar]
15	0.1	0.0	-0.1	0.4	**1.0**	E	NP_001002645.1	Peroxisomal 3,2-trans-enoyl-CoA isomerase [Salmo salar]
16	0.0	0.1	0.0	0.0	**0.7**	E	NP_957318.1	Phosphoglycerate mutase 2-2 (muscle) [Salmo salar]
17	0.4	0.2	0.0	0.3	**0.7**	E	NP_001002577.2	Succinate-CoA ligase GDP-forming alpha subunit [Oncorhynchus mykiss]
18	0.0	0.2	0.0	0.1	**1.5**	E	NP_956313.1	Trifunctional enzyme subunit beta, mitochondrial precursor [Salmo salar]
19	0.1	0.3	0.1	-0.2	**0.9**	E	ACM09835.1	Triosephosphate isomerase [Salmo salar]
20	**-0.2**	**-0.5**	-0.1	0.1	**0.9**	I	XP_001340380.1	Tumor protein p53 inducible nuclear protein 2-like [Salmo salar]
L	1.5	0.8	0.0	-0.8	-1.5			

Figure 5. Gene markers of pathology and outcome

Heat map of selected genes with expression profile showing strongest upregulation in HR (A) or LR (B) fish at the late stage of infection. Further explanations are given in Figure 3. Column "cat" indicates the category of gene annotation according to [10]: T- T cell response, P- MHC antigen presentation, B- B cell response, A- apoptosis, M- muscle cytoskeleton development, E- mitochondrial electron chain/energy metabolism, I- implicated in immune response, O- other/unknown.

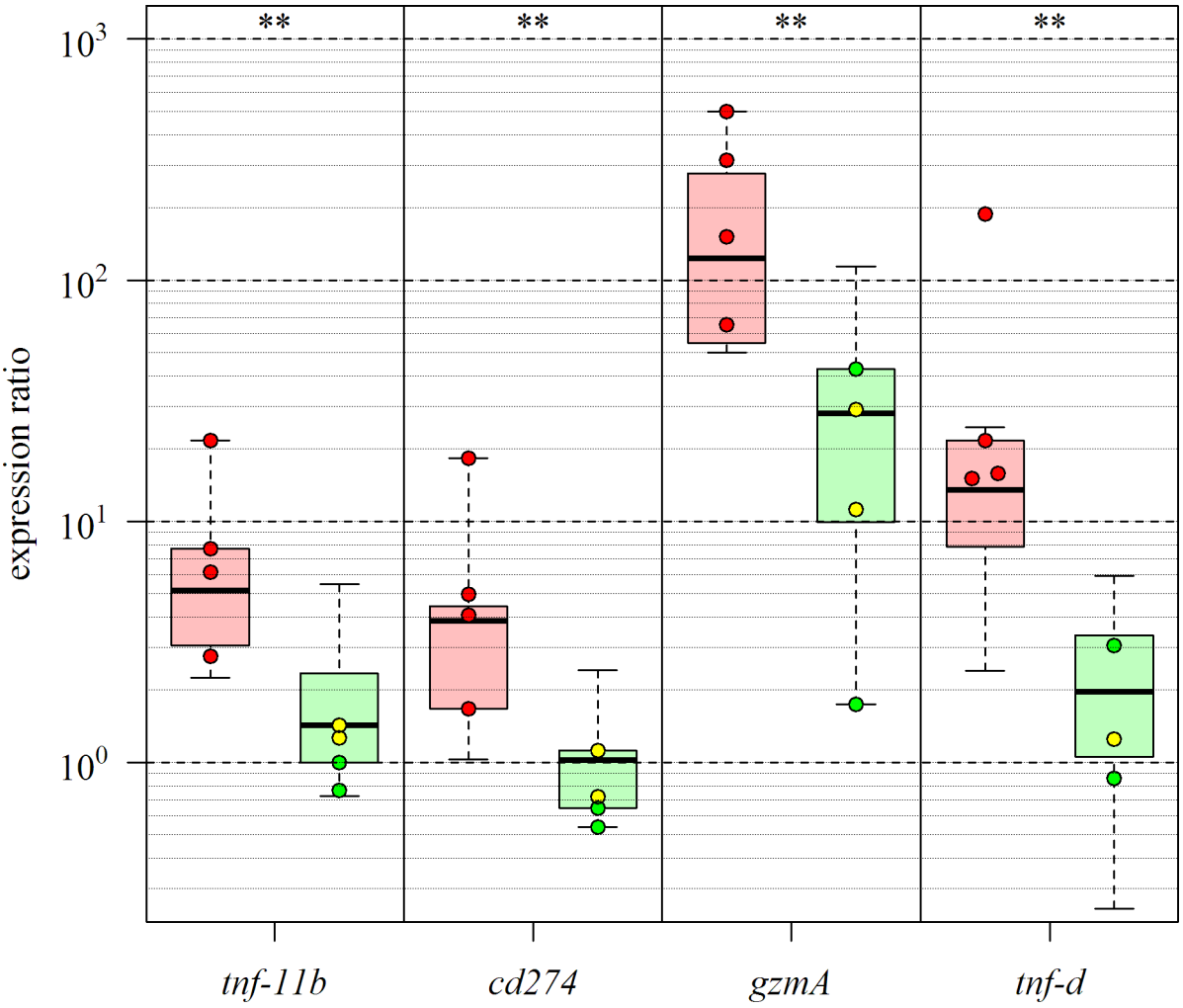

Figure 6. Gene expression of selected markers of late pathology

Real-time qPCR expression of four genes identified as pathology markers from microarrays in an extended number of individuals eight weeks post-infection. Gene abbreviations are; *tnf-11b*: *tumor necrosis factor receptor superfamily member 11b*, *cd274*: *programmed cell death ligand 1*, *gzmA*: *granzyme A*, *tnf-d*: *tumor necrosis factor decoy receptor*. The gene expression ratios are shown as boxplots based on nine HR fish (red) and ten LR fish (green) against the average of control fish. Values of the fish used in the microarray experiments are highlighted with dots and colored according to their histopathology score level (see Figure 1). Boxes represent 50 % of the values, while black bars mark the median log-ER. Whiskers indicate the maximum length of 1.5 times the box length. Significance levels between all HR and LR fish are indicated on top of the plot (*t*-test on log-transformed values, ** = $p < 0.01$).

	positively correlated GOs, late stage			negatively correlated GOs, late stage		
High responders	#	GO number	GO name	#	GO number	GO name
	41	GO:0002376	immune system process	26	GO:0055001	muscle cell development
	23	GO:0006955	immune response	25	GO:0035023	reg. of Rho protein signal transd.
	21	GO:0001775	cell activation	25	GO:0055002	striated muscle cell development
	17	GO:0045321	leukocyte activation	24	GO:0031032	actomyosin structure organization
	14	GO:0046649	lymphocyte activation	24	GO:0010927	cellular comp. Ass. inv. in morph.
	4	GO:0033632	reg. of cell-cell adhesion by integrin	23	GO:0030239	myofibril assembly
				22	GO:0014866	skeletal myofibril assembly
				12	GO:0033058	directional locomotion
				12	GO:0043056	forward locomotion
				12	GO:0007512	adult heart development
Low responders	#	GO number	GO name	#	GO number	GO name
	60	GO:0044281	small molecule metabolic process	11	GO:0055002	striated muscle cell develop.
	27	GO:0055114	oxidation reduction	6	GO:0014866	skeletal myofibril assembly
	21	GO:0006091	gen. of prec. metabolites + energy			
	19	GO:0044271	cellular nitr. Comp. biosyn. process			
	13	GO:0015980	energy deriv. by ox. of organic comp.			
	11	GO:0045333	cellular respiration			
	8	GO:0022900	electron transport chain			
	6	GO:0009060	aerobic respiration			
	6	GO:0043648	dicarboxylic acid metabolic process			
	5	GO:0006103	2-oxoglutarate metabolic process			

Figure 7. Gene ontology classes correlated to viral load at late stage

Gene ontology classes correlated to viral load in responder groups at the late stage of infection. Significantly enriched GO classes (FDR correction, $p < 0.05$) with positive or negative correlation to viral load (Pearson's $r > 0.6$) in HR and LR groups at 8-10 wpi are shown. Column "#" shows the number of genes behind the respective GO term/class, and only the ten GOs with lowest p-values are shown (for completed data, see Additional file 1).

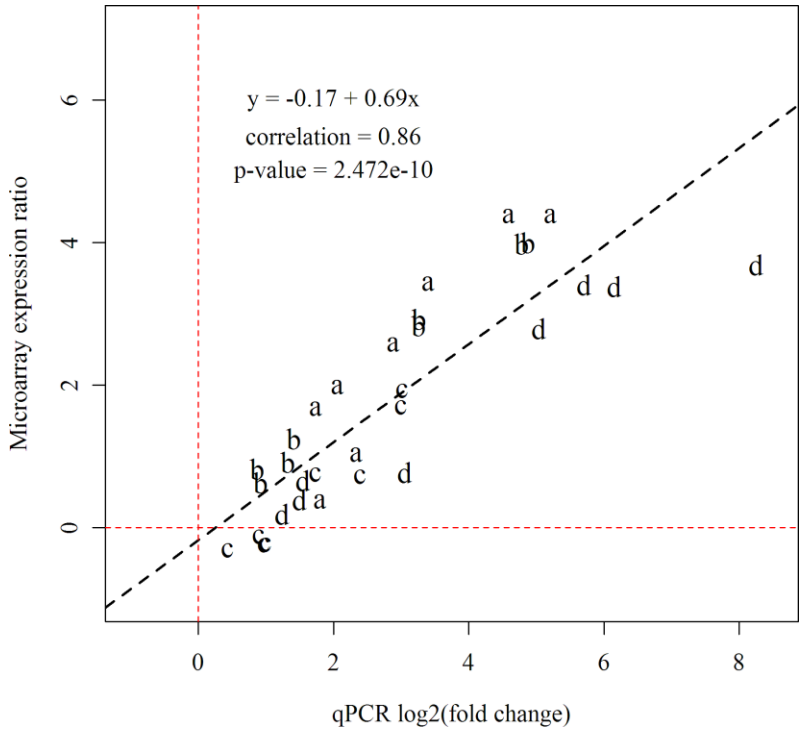

Figure 8. Confirmation of microarray results by qPCR

Comparison of gene expression results obtained with qPCR and microarrays. Log_2-ER of four genes were compared (n = 8, plotted as letters; a = *radical s-adenosyl methionine domain-containing protein 2* (*rsad2*/*viperin*), b = *interferon-induced protein with tetratricopeptide repeats 5* (*ifit 5*), c = *retinoic acid inducible gene I* (*rigI*), d = *barrier to autointegration factor* (*baf*). The dashed black line represents the regression function of the measured values. The regression model, correlation coefficient and p-value are plotted in the graph.

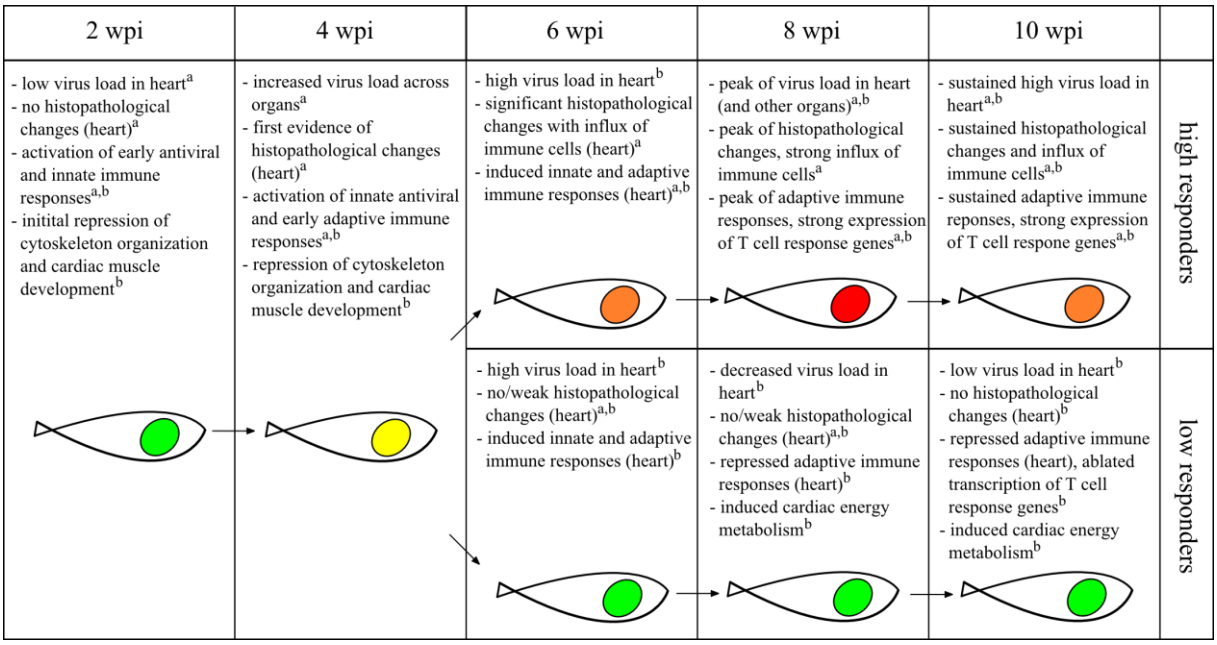

Figure 9. Summary of pathogenesis in responder groups

Summary of host-virus responses in high and low responder fish based on findings from a previous CMS study [10] and the present work, as indicated with superscript letters a and b, respectively. Coloured circles in fish illustrate histopathology (atrium score level), analogous to Figure 1.

Description of Additional files:

Additional file 1. Primary data for GO enrichment analysis

Complete data used for Figures 2 and 7 as referred to in the Figure legends / Results section.

Additional file 2. Primary data for gene markers and predictors

Complete data used for the heat maps in Figures 3-5. Data are median log_2-ER for differentially expressed genes (DEG, *t*-test vs controls, $p < 0.05$) showing correlated expression (Pearson $r > 0.6$, correlation matrix in last sheet) to the different stages of infection in high and low responders (mid and late stages). Grades levels of red and green indicate respectively up- and downregulation.

Additional file 3. Real-time qPCR primers used in the study

Additional file 4. Experimental outline

Overview of experimental groups and number of biological replicates used for the different analyses.

Paper IV

Paper IV

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Genomic survey of early responses to viruses in Atlantic salmon, *Salmo salar* L.

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ABSTRACT

Viral diseases are one of the main problems and risk factors in aquaculture. At present diseases are diagnosed by detection of pathogens and clinical symptoms. Identification of genes involved in early responses to viruses is important for better knowledge of antiviral defence and development of diagnostic tools. The aim of this study was to search for gene markers common for viral infections in Atlantic salmon based on microarray analyses of a wide range of samples. Gene expression profiles from fish and cell cultures infected with different viruses and treated with the synthetic double-stranded RNA poly(I:C) were compared in order to identify virus responsive genes (VRG). The list of VRG defined in this study contained 117 genes with known or unidentified functions. Several genes, including the most highly ranked one (receptor transporting protein), had not been previously reported to be involved in antiviral defence. VRG were characterized by a rapid induction and low tissue specificity, and their expression levels were related to the viral load. Immunofluorescence analyses of proteins encoded by VRG in cardiac tissue of salmon with the viral disease cardiomyopathy syndrome (CMS) revealed a common expression pattern. In head kidney leukocytes VRG showed comparable or equal responses to CpG and poly(I:C), which mimic respectively bacterial DNA and viral RNA. Most VRG showed highly correlated expression with interferon-a (IFNa). Sequence comparison of salmon VRG with those from other species gave an understanding of the evolution of these genes, which showed a remarkably rapid sequence divergence in comparison with the entire proteome. VRG emerged both before and after separation of teleosts and tetrapods, and among genes found exclusively in fish species there were members of several multigene families: tripartite motif proteins, gig1- and gig2-like proteins. Several VRG, including genes with unknown functions and orthologs to mammalian RNA helicase RIG-I and chemokine C-X-C type 10, were present in cyprinid and salmonid fish but not in the phylogenetically advanced orders, suggesting that they have been lost in the evolution of *Teleostei*. Apparently, a number of genes involved in antiviral responses in salmon have acquired different functional roles in higher vertebrates.

1. Introduction

Viral diseases are one of the main problems and risk factor in Atlantic salmon aquaculture. Most of the Atlantic salmon diseases with relatively long history are caused by highly pathogenic RNA viruses such as infectious salmon anaemia (ISA), infectious pancreas necrosis (IPN) and pancreas disease (PD). Other diseases of viral origin have recently gained attention such as the cardiomyopathy syndrome (CMS) and heart and skeletal muscle inflammation (HSMI), that were first recognized in respectively 1985 (Amin and Trasti, 1988) and 1999 (Kongtorp et al., 2004). Both diseases were recently associated with RNA viruses (Haugland et al., 2011; Palacios et al., 2010), although a causative relationship between infection and development of HSMI awaits final

Abbreviations: BLAST, basic local alignment search tool; CMS, cardiomyopathy syndrome; ER, expression ratio; HSMI, heart and skeletal muscle inflammation; IF, immunofluorescence; IPN, infectious pancreas necrosis; IFN, interferon; ISA, infectious salmon anaemia; PRR, pathogen recognition receptor; TRIM, tripartite motif protein; VRG, virus-responsive genes.

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confirmation. Both diseases typically progress slowly, cause low or no mortality and may often have asymptomatic characteristics. Given the high prevalence of unexplained mortalities of salmon during the seawater phase one may presume existence of viral diseases that are still unknown.

The need for better understanding of antiviral defence and tools for diagnostics of the infection status of fish has driven research on host genes that are activated by viruses. Similar to higher vertebrate species, teleost fish possess antiviral responses conferred by genes that are rapidly induced after infection with RNA viruses and treatment with compounds that mimic viral RNA such as poly(I:C) (reviewed in Stein et al., 2007; Verrier et al., in press). Binding of foreign RNA to cytoplasmic (RNA helicases RIG-I, MDA5) or endosomal (Toll-like receptors TLR3, TLR7/8) pathogen recognition receptors (PRRs) triggers signal transduction pathways that activate antiviral genes including type I interferons (IFNs). IFNs augment innate immune responses and establish an antiviral status. A number of VRG were identified in salmonid fish based on molecular cloning of putative homologs of mammalian genes with known roles including IFNs, Mx and ISG-15 (Robertsen, 2006, 2008; Røkenes et al., 2007; Sun et al., 2009). Viral infections also activate host genes regulating diverse processes including cell cycle, apoptosis and signaling pathways (Ghazal et al., 2000). Sequencing of subtracted libraries (O'Farrell et al., 2002) and gene expression analyses with cDNA microarrays (Jorgensen et al., 2008; Workenhe et al., 2009) revealed a suite of salmonid genes induced with viral infections, and many of these were not previously described in fish. Collectively, specialized antiviral and other genes that are activated in response to viruses can be termed virus-responsive genes (VRG). The development of oligonucleotide microarrays (ONM) containing probes to all protein encoding (blastx identified) transcripts of Atlantic salmon stored in public databases (Krasnov et al., 2011) has further expanded the search for VRG. In this paper we report the identification of VRG based on a large number of gene expression analyses in salmon tissues and cells exposed to important viral pathogens and poly(I:C). Expression of these genes was examined with ONM, qPCR and immunofluorescence. Responses of VRG to compounds that mimic bacterial and viral infections were analyzed and their expression profiles were compared with that of IFNa. The application of transcriptomic tools made it possible to identify a large fraction of genes that are involved in early response to viruses. This provided an overview of the cellular and molecular events activated upon viral infections while the wealth of sequence information from fish and higher vertebrates shed light on the evolution of this important functional class.

2. Materials and methods

2.1. Experiments and microarray analyses

Development of Atlantic salmon ONMs and bioinformatic system (STARS) was described in detail elsewhere (Krasnov et al., 2011). In brief, probes were designed to all unique transcripts available from public databases (Unigene clusters, TIGR contigs, GenBank mRNA sequences with complete reading frames) that were identified with blastx search across proteins from Unigene and Refseq (human and zebrafish, *Danio rerio*) and GenBank (salmonid species). Probes to 916 unidentified transcripts from our own cDNA library derived from leukocytes of infected salmon were added. First ONM was designed in January 2009 and the composition was updated periodically to add new sequences and to remove probes that did not produce high quality signals. However these changes were relatively small and a major fraction of probes was present on all platforms, which made possible cross experiment comparison. All microarrays included 21 k unique probes printed in duplicate on 4×44 k chips from Agilent Technologies. Since the salmon genome has not been completely sequenced yet, probes were designed to transcripts, many of which included incomplete reading frames. Though unique transcripts were used for the microarray design it was impossible to completely avoid redundancy, and the exact number of represented genes is unknown.

Protocols for microarray hybridizations were according to Krasnov et al. (2011) and are briefly described in the following. Unless specified otherwise, all microarray analyses used standard reagents, equipment and protocols for dual dye hybridizations from Agilent Technologies (CA, USA), using a design where infected/test samples labelled with fluorescent Cy5 dye and non-infected/control samples labelled with Cy3 dye were competitively hybridised to arrays. Initial sampling and RNA extraction of tissues/cells were based on standardized protocols under sterile/RNase-free conditions, and quality was assessed with Agilent 2100 BioAnalyzer. Scanning of hybridised microarrays was performed with GenePix Personal 4100A and GenePix Pro6.0 (Molecular Devices, Sunnyvale, CA, USA) was used for grid alignment, feature extraction, quality assessment and quantification. Subsequent processing of results and data mining was performed with STARS. After removal of low quality spots flagged with GenePix, $\log_2$-expression ratios (ERs) were calculated and lowess normalization performed. All probes were annotated in STARS by functions (GO) and pathways (KEGG) and linked to the main molecular web databases. STARS is also adapted for custom annotations by expression profiles, results of databases and literature mining and other sources.

To search for VRG, gene expression data from 108 samples were used that were arranged in five groups by treatments; the list and description of samples are in Supplementary Table 1. Controls for all samples were noninfected fish (for cohabitants), sham-injected fish (for i.p.-injected fish) or mock-treated cells (for *in vitro* experiments). The most important viruses causing diseases of Atlantic salmon (ISA, IPN, CMS and HSMI) and poly(I:C) were included. Experimental infections of salmon with HSMI and CMS were described in detail in Kongtorp and Taksdal (2009) and Timmerhaus et al. (submitted manuscript). Liver samples from of 0 year salmon in the early phase following infection with IPNV was kindly provided by Aqua Gen AS (Norway). A standard IPN cohabitation challenge test was performed at Veso Vikan (Norway) and samples were collected from cohabitant fish during 2–10 days post-infection (dpi) covering the immediate host–virus interaction before the onset of mortality. Four of 28 analyzed individuals showed marked activation of VRG. Established Atlantic salmon cell-line ASK was infected with ISAV as described previously (Schiøtz et al., 2008) and treated with 50 μg/ml poly(I:C) (Invivogen); samples were collected and analyzed in duplicate after 1 and 5 days. Results of an independent experiment with ASK and poly(I:C) (50 μg/ml, 3 days, 4 replicates), which used single dye hybridization and Agilent scanner (GEO Omnibus GSE25328) were included in analyses. Atlantic salmon head kidney leukocytes were isolated and cultivated as in Iliev et al. (2010). Cells were either left non-stimulated (control) or treated with 20 μg/ml of poly(I:C) (Invivogen) or 2 μM phosphorothioate CpG-B (Thermo Scientific). Microarray analyses were performed in duplicate in control and CpG (5 days) and poly(I:C) treated cultures (one and five days); all hybridizations used one day control culture as a common reference. The criterion for selection of VRG (mean $\log_2$-ER > 1 in at least three of five groups) was adjusted to achieve an appropriate balance between sensitivity (number of genes) and specificity (preferred responses to viruses).

2.2. Quantitative real-time RT-PCR (qPCR)

Analyses were carried out in salmon with ISA (heart) and IPN (red blood cells, RBC). Primers were designed using Vector NTI

(Invitrogen) and eprimer3 from the EMBOSS program package (version 5.0.0, http://emboss.sourceforge.net/). Amplicon size was set to 80–160 and melting temperature to 59–61 °C (Supplementary Table 2). Primers were fabricated by Invitrogen. Analyses were conducted according to the MIQE guidelines (Bustin et al., 2009). The cDNA synthesis was performed on 0.2 μg DNAse-treated total RNA (Turbo DNA-freeTM, Ambion, Austin, TX, USA) using TaqMan® Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in 25 μl reactions with random hexamer priming. QPCR was conducted using 2× SYBR® Green Master Mix (Roche Diagnostics) in a 12 μl reaction volume, using 5 μl 1:10 diluted cDNA, and primer concentrations of 0.42 μM. PCRs were run in duplicates in 96-well optical plates on a Light Cycler 480 (Roche Diagnostics) under the following conditions: 95 °C for 5 min (pre-incubation), 95 °C for 5 s, 60 °C for 15 s, 72 °C for 15 s (45 cycles) and continuous increase from 65 °C to 97 °C with standard ramp rate (melting curve). Cycle threshold (C_T) values were calculated using the second derivative method. For evaluation of the results, the mean of duplicates was used. Duplicate measurements that differed more than 0.5 C_T values were removed and reanalysed. Relative expression ratios of test samples versus the average of the controls were calculated according to the Pfaffl method (Pfaffl et al., 2002). Elongation factor 1α (GenBank ID: BT072490.1) used as a reference gene showed stable expression in control and test samples according to the Best Keeper software (Pfaffl et al., 2002). The efficiency of the PCRs was estimated for all primer pairs by six times 1:5 dilution series of a cDNA mix of all used samples; the Light Cycler® 480 Software (version 1.5.0.39) was used.

2.3. Immunofluorescence microscopy

Polyclonal antibodies to selected VRG were produced by GL Biochem (Shanghai, China). From a total of eight VRG tested, purified antibodies were obtained for four proteins: galectin-9 (GAL), radical S-adenosyl methionine domain-containing protein 2 (RSAD), gig2-like protein (GIG) and ubiquitin-like protein (ULP). One to three different peptides per target protein were synthesized, conjugated with KLH (keyhole limpet hemocyanin) and injected in two rabbits for a total of 10 weeks with eight immunizations. Antisera obtained at seventh bleeding (day 56) were tested with ELISA, peptides that produced best results are in Supplementary Table 3. Qualified antisera were antigen-affinity purified and reactivity against the peptide and protein (tissue lysate from salmon) was confirmed by SDS-PAGE/Western blotting (Supplementary Fig. 1) and ELISA (test titer > 1:32,000). All antibodies recognized bands corresponding to the expected size of the target proteins.

Tissue expression during viral infection was tested on hearts from Atlantic salmon post-smolts experimentally challenged by i.p.-injection (Timmerhaus et al., submitted manuscript) with CMS ($n = 3$) and compared with healthy non-infected controls ($n = 3$). CMS hearts from two time-points (4 and 8 weeks post-infection) were analyzed, diagnosed with respectively mild and severe histopathology, as scored by microscopic examination of HE-stained paraffin sections (Timmerhaus et al., submitted manuscript). Samples were formalin-fixed heart tissue (10% neutral phosphate-buffered) prepared by standard paraffin wax embedding. Paraffin was removed by Histo-Clear (National Diagnostics, Georgia, USA), followed by rehydration in decreasing ethanol concentration. Heat-induced epitope retrieval was achieved by heating the slides in 10 mM Tris–HCl pH 10 for 20 min in a microwave oven. The slides were cooled to room temperature and permeabilized for 20 min in PBS with 1% Triton 100×. After blocking for 2 h (5% dry milk in PBS) with 0.1% Tween 20 (PBST) the sections were incubated over night at 4 °C with primary antibodies in PBST with 2% dry milk and 1% DMSO. After extensive PBST washing, Alexa-conjugated antibodies (Invitrogen) were applied for 2 h at room temperature followed with cell membrane and nuclear staining with Wheat Germ Agglutinin (WGA) and DAPI, respectively (Invitrogen). The slides were cover slipped using ProLong Gold antifade and microscoped on a Zeiss Axio Observer Z1 equipped with an AxioCam MR5 camera and the Apotome system (Carl Zeiss Microimaging GmbH). High magnification image stacks were deconvolved before creation of extended focus images. Representative images from one fish per group are shown in Section 3.

2.4. Sequence analyses

The Atlantic salmon VRGs were compared with the complete sets of proteins from genomic projects to examine their occurrence in fish species and in higher vertebrates and to evaluate sequence similarities. Proteins of *Ciona intestinalis*, zebrafish, medaka (*Oryzias latipes*), puffer fish (*Takifugu rubripes*), three-spined stickleback (*Gasterosteus aculeatus*), the African clawed frog (*Xenopus tropicalis*), chicken (*Gallus gallus*), human and mouse were downloaded from Ensembl ftp (http://www.ensembl.org/info/data/ftp/index.html). Chicken was omitted since many VRG did not find any match most likely due to incomplete coverage of proteins. Proteins identified in the Atlantic cod (*Gadus morhua*) genome project were retrieved from www.codgenome.no. Sequences with no match to Ensembl proteins or with patchy distribution were searched across GenBank at NCBI. To compare divergence of VRG with the trend for whole genome, a 12.3 k set of Atlantic salmon mRNA sequences with complete reading frames were retrieved from GenBank. Sequence comparisons were performed with blastx at cutoff $e < -10$; STARS was used to run analyses and to parse the output text files. Sequence alignments were performed with clustalx. Phylip was used for phylogenetic analyses that included bootstrap and construction of maximum likelihood trees.

3. Results

3.1. Identification and composition of VRG

Gene expression profiles from different microarray experiments were compared to search for genes that were up-regulated in salmon with different viral diseases (IPN, CMS and HSMI) and responded to ISAV and poly(I:C) in cell cultures. With the applied criteria (mean 2-fold increase in at least three of five treatments), 130 candidate genes were selected. Blastx search across Atlantic salmon mRNAs with complete reading frames from GenBank (this data set is rapidly growing) slightly reduced redundancy of the initial set leaving 117 probes. Complete gene expression data are in Supplementary Table 4 and 25 genes with highest expression changes in all samples are presented in Table 1. It is noteworthy to mention that the list of VRG includes duplicated genes or highly similar paralogs (two nicotinamide phosphoribosyl transferases, three PPARα-interacting proteins, two poly polymerase 12, two RNA helicases RIG-I, three very large inducible GTPases1, two IRF7 and two IFN-induced protein 44) and members of multi-gene families (15 tripartite motif proteins (TRIMs), six RING finger proteins and seven gig2-like proteins). VRG showed differential expression in all analyzed tissues and cells of Atlantic salmon heart, liver, head kidney, spleen, peripheral blood leukocytes (PBL) and red blood cells (RBC), primary (head kidney leukocytes) and passaged (ASK) cell cultures. Early (1 day) activation was observed in poly(I:C) treated and ISAV infected cells. In salmon with CMS and HSMI, high expression levels persisted within the whole period of disease. Induction of VRG with viral infections was confirmed with qPCR and ranking of genes by the magnitude of changes was similar to microarray results (Fig. 1). The thresholds of viral infections that

Table 1
Atlantic salmon VRG with highest expression changes based on all samples included in the study. Genes are ranked by mean expression ratio (log_2-ER, test vs control) in the descending order.

GenBank	Gene	Mean	CMS[a]	ISAV	Poly(I:C)	IPN	HSMI
EG825775	Receptor-transporting protein	4.33	4.48	4.33	6.40	2.88	3.58
EG836160	Ubiquitin-like protein-1	3.86	4.10	4.45	4.47	2.83	3.45
209156101	IFN-induced protein with tetratricopeptide repeats 5-2	3.85	4.01	3.80	5.17	3.00	3.28
BT072288	VHSV-inducible protein-3	3.26	3.68	3.54	3.50	2.70	2.88
EG906096	Sacsin	3.02	4.02	2.27	4.32	2.84	1.67
GO053979	VHSV-inducible protein-4	2.89	3.40	2.42	3.40	2.77	2.44
EG841846	Similar to KIAA1593	2.83	3.13	2.98	3.10	2.98	1.97
EG841455	Very large inducible GTPase 1-1	2.76	3.19	1.21	3.92	3.01	2.48
209154815	ATP-dependent RNA helicase DHX58	2.68	2.77	2.70	3.26	3.09	1.60
DW569595	Gig1-1	2.64	2.58	2.98	2.95	3.05	1.66
S31967511	IFN-induced protein 44-2	2.59	2.82	2.98	3.27		1.31
BT044026	Gig2-1	2.57	3.83	1.11	2.45	2.67	2.78
EG791574	Zinc finger, NFX1-type containing 1	2.54	2.61	2.07	3.67	2.98	1.39
FN178459	Retinoic acid-inducible gene-I	2.53	2.57	2.35	3.53	2.38	1.80
EG831164	PR domain zinc finger protein 9	2.52	2.31	3.56	2.91	1.87	1.96
BT049316	Barrier-to-autointegration factor	2.49	3.42	1.52	2.14	2.44	2.95
BT049703	XIAP-associated factor 1	2.40	2.07	2.57	3.12	2.78	1.47
GO057318	CD9-2	2.31	2.02	3.34	2.68	2.52	0.98
DW556796	IFN-induced protein 44-1	2.31	2.37	2.37	2.81	2.27	1.71
EG871799	Fish virus induced TRIM-1	2.30	1.81	2.60	3.41	2.51	1.17
DY728694	Radical S-adenosyl methionine domain-containing 2	2.27	2.88	1.84	2.92	2.85	0.86
S31981688	PPARA-interacting complex 285 kDa protein	2.25	2.56	1.87	3.32		1.26
BT044022	Gig2-2	2.24	3.20	0.25	2.29	2.67	2.78
BT072557	VHSV-inducible protein-1	2.24	2.60	2.07	2.61	2.32	1.58
209730585	SRK2 tyrosine kinase	2.22	1.82	2.61	3.20	2.46	1.02

[a] Table presents results of microarray analyses in heart (CMS, HSMI), liver (IPN), ASK cells (ISAV, poly(I:C)). Complete list of samples is in Supplementary Table 1.

activate VRG may vary for different pathogens and dsRNA. In the liver of IPNV challenged salmon, we observed up-regulation of VRG at high viral loads: C_T = 13–15 (Fig. 2). Similar results were observed from comparison of high and low virulent isolates of IPNV (Skjesol et al., 2011). VRG showed correlation with virus levels in the heart of salmon experimentally challenged with CMS (Timmerhaus et al., submitted manuscript).

One VRG from our leukocyte cDNA library (DY740074) was not identified with blastx, and judging by multiple stop codons close to the 5′-end it probably did not contain any reading frame. The rest were divided in six groups taking into account functions and known or unknown roles in antiviral defence (see Supplementary Table 4). Classification was performed *ad hoc* exclusively to assist presentation of VRG in this study and can be easily modified in light of new data and findings.

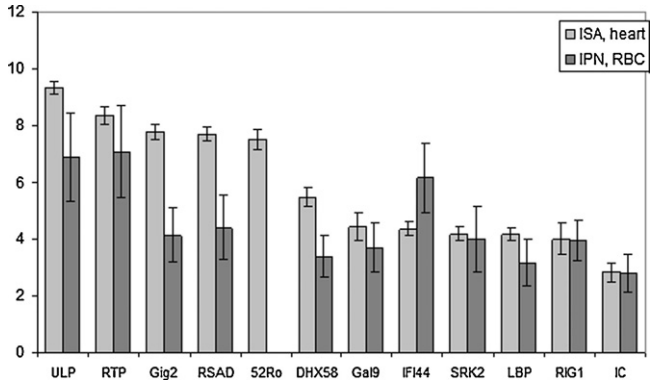

Fig. 1. Quantitative real-time PCR analyses of virus responsive genes (VRG). Bars show change in expression of selected VRG in tissues of Atlantic salmon with the viral diseases ISA (heart) and IPN (red blood cells, RBC) relative to uninfected controls. Gene abbreviations are: ULP, ubiquitin-like protein; RTP, receptor transporting protein; RSAD, radical S-adenosyl methionine domain-containing protein; DHX58, ATP-dependent RNA helicase; GAL9, galectin-9; IFI44, IFN-induced protein 4; SRK2, tyrosine-protein kinase; LBP, galectin-3 binding protein; RIG-I, RNA helicase; IC, IFN induced with helicase C domain 1. Data are $-\Delta\Delta C_T \pm$ SE normalised against elongation factor 1 alpha, all differences from control are significant (*t* test, $p > 0.05$, $n = 8$).

(i) *Specialized antiviral genes* include 28 genes for proteins involved in responses at different levels. RNA helicases RIG-I and DHX58 play a key part in intracellular detection of foreign RNA (Yoneyama et al., 2005), which activates signal transducers including IRF3 and IRF7. The latter stimulate production of IFNa and antiviral effectors, such as very large inducible GTPases – VLG (MacMicking, 2004), proapoptotic XIAP associated factor 1 – XAF1 (Chawla-Sarkar et al., 2003) and ubiquitin-related proteins that take part in modification and degradation of viral components (ubiquitin-like and hect domain proteins, ubiquitin activating enzyme and ligase, KIAA1593). Radical S-adenosyl methionine domain-containing protein 2 (RSAD or viperin) is thought to impair virus budding by disrupting lipid rafts at the plasma membrane (Chin and Cresswell, 2001). IFNa augments antiviral responses via Jak/Stat pathway that includes STAT1, 3 and 6, while suppressor of cytokine signaling SOCS1 inhibits Jak/Stat signaling (Sakamoto et al., 2000).

(ii) Fifteen VRG have *immune functions*, which are not restricted to antiviral defence. MHC class I antigen, transporter associated protein TAP, beta-type proteasome subunits PSMB8 and 9 are involved in antigen processing and presentation. SRK2-like tyrosine kinase belongs to a large family of protein kinases involved in multitude of regulatory processes, its role in salmon needs further exploration. Two genes are similar to CD9, a transmembrane protein that plays an important part in activation, adhesion and aggregation of platelets (Jennings et al., 1994). Another gene denoted as platelet basic protein precursor has been found exclusively in salmon. These genes that are probably associated with thrombocytes showed highest expression changes in this group. Microsialin regulates macrophages functions being involved in phagocytic

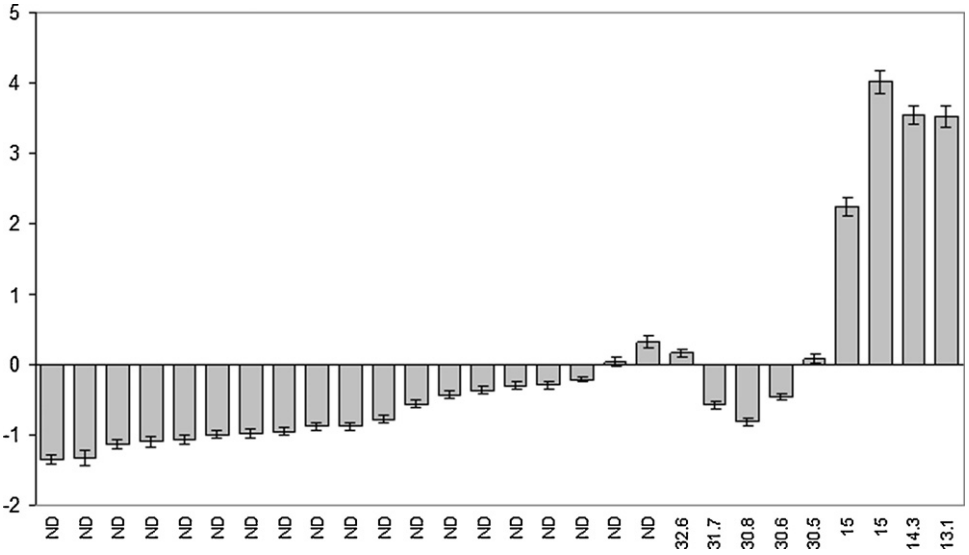

Fig. 2. VRG activated in Atlantic salmon with high viral load. Panel shows expression of VRG in the liver of juvenile salmon challenged with IPNV. Data are mean $\log_2$ ER ± SE (117 genes); microarray hybridizations used a pool of all samples as a reference. Bars correspond to individuals ranged by viral load as determined with qPCR; C_T values are indicated below each bar (ND, not detected).

activities, intracellular lysosomal metabolism and extracellular cell–pathogen interactions (da Silva et al., 1996). It binds to lectins or selectins and directs homing of macrophages. Other genes are related to chemokines and cytokines. The primary role of nicotinamide phosphoribosyltransferase is biosynthesis of NAD. However this enzyme also known as visfatin or pre-B-cell colony-enhancing factor 1 has cytokine activity (Samal et al., 1994). The group also includes IL-10 and IL-20 receptors, TNF receptor-associated protein and several chemokines with unknown functions.

(iii) Ten genes with largely *non-immune* functions are known to be involved in virus responses. Deoxycytidine kinase (DCK) phosphorylates deoxyribonucleosides; this enzyme is required for antiviral action of nucleoside analogs used as chemotherapeutics (Sabini et al., 2003) and a gene resembling LPS-inducible thymidylate kinase probably has similar functions. P2Y purinoreceptors mediate nucleotide signaling. Interestingly, P2Y blocks IFNα production by human plasmacytoid dendritic cells (Shin et al., 2008). Opioid growth factor receptor is involved in a multitude of regulatory processes. This gene is induced with IFNγ in murine and zebrafish macrophages (López-Muñoz et al., 2011). Cholesterol 25-hydroxylase, an enzyme of steroid biosynthesis is activated with type I IFNs in mammalian macrophages (Park and Scott, 2010). Eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) interacts with viral mRNA (Piron et al., 1998). BANF1, a protein with essential roles in organization and maintenance of chromosomes, interacts with retroviruses during their integration into host genomes (Segura-Totten and Wilson, 2004).

(iv) Ten genes have relatively well explored functions but their association with antiviral defence has to our knowledge not been reported. This group includes proteins involved in signal transduction (ras-related C3 botulinum toxin substrate), regulation of gene expression (PPARα-interacting complex 285 or regulator of nonsense transcripts), maturation of mRNA (poly polymerase 12) and lipid metabolism (apolipoprotein L3).

(v) In contrast to previous group, 42 VRG have *unknown functions*, but their virus responses have been documented. This can be seen from their names since many genes are designated as IFN-, fish virus- of VHSV-induced. This group includes 14 genes from the TRIM family characterized by presence of a RING finger domain and zinc finger B-box followed with a coiled coil regions, six genes possess RING finger and nine genes encode proteins with similarity to gig1 and gig2 that were recently identified in cyprinid fish (Zhang and Gui, 2004). The responses of salmon sacsin, galectin 9, galectin-binding protein to viral infections are well documented (Jorgensen et al., 2008; Workenhe et al., 2009) though their roles in antiviral defence remain unclear.

(vi) Finally 13 genes with *unidentified functions* to our knowledge, have *not* been described as *virus responsive*. One of these, receptor-transporting protein 3 (RTP3), showed the highest magnitude of expression changes among all VRGs (Table 1).

3.2. Cell and tissue localization

Polyclonal antibodies were made to peptides retrieved from the salmon sequences of four VRG and evaluated with SDS-PAGE followed by Western blotting (Supplementary Fig. 1). Antibodies for galectin 9 (GAL), radical S-adenosyl methionine domain-containing protein 2 (RSAD), IFN-inducible protein gig2 (GIG) and ubiquitin-like protein (ULP) were used for immunofluorescence analysis of protein expression in hearts from CMS challenged salmon sampled at 4 and 8 weeks after challenge. Week 4 and 8 represents early viraemia and peak pathology stages, respectively (Timmerhaus et al. submitted manuscript). A modest activity was detectable for all proteins investigated in the control specimens, as exemplified by GAL shown in Fig. 3B. Fluorescence activity increased in hearts 4 weeks after infection and by week 8, a considerable increase in fluorescence activity was evident (Fig. 3D and F). Microscopy at a higher magnification showed GAL antibody binding in all cardiac compartments. In the compactum layer, some cardiomyocytes showed strong staining and infiltrating leukocytes had abundant GAL protein expression (Fig. 4A). A similar pattern of GAL activity was also present in the spongiosa and atrium, with cells of the latter being strongly labelled (Fig. 4B and C). Investigation of leukocytes inside vasculature revealed different sub-cellular localization. The majority of these cells displayed fluorescence in a thin ring in the cell membrane, whereas some cells had GAL activity in different compartments including the nuclei (Fig. 4D). ULP showed staining of cardiomyocytes, mainly in the cytosol (Fig. 4E). RSAD showed

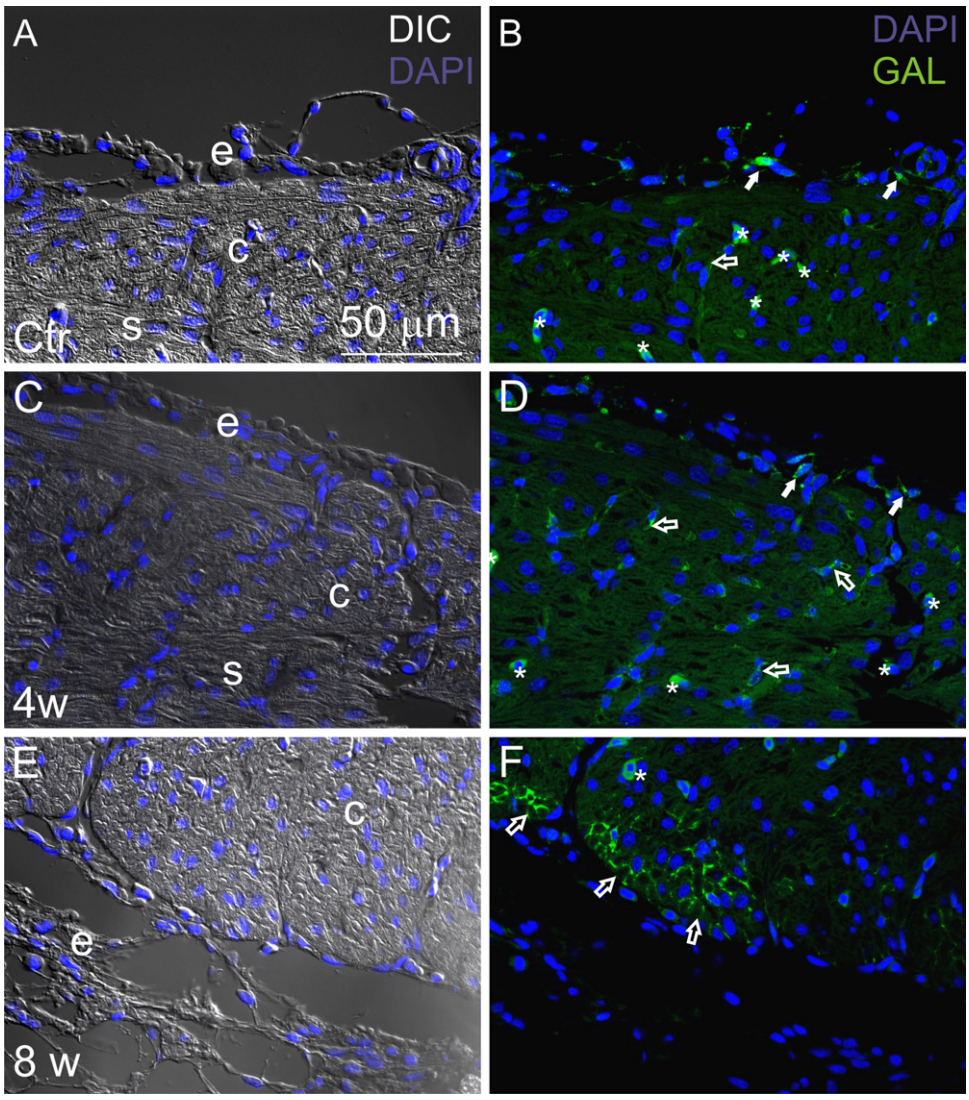

Fig. 3. Immunofluorescence analysis of galectin 9 (GAL) expression in the epicardium and compactum layer of hearts from healthy and infected Atlantic salmon with CMS pathology. (A) Differential Interference Contrast (DIC) image combined with DAPI fluorescent nuclei, uninfected control heart. (B) GAL immunofluorescence and DAPI overlay. Fluorescence in the myocytes (open arrow) is absent or low, stronger signals are visible in leukocytes (arrow) residing in the epicardium. Erythrocytes (asterisk) show high autofluorescence. (C) DIC image with nuclear DAPI staining 4 weeks after CMS challenge. No pathological signs or infiltrating leukocytes are visible. (D) Increased GAL activity is visible in part of the cardiomyocytes of the compactum (open arrow) and in a few epicardial cells (arrow). (E) DIC image with DAPI stained nuclei 8 weeks after challenge show altered myocyte organization but no leukocyte infiltration. (F) Strong fluorescence is visible in a number of myocytes (open arrow).

cytosolic and nuclear localization (Fig. 4F), while GIG was localized in the nuclei with some weak cytosolic staining (Fig. 4G).

3.3. Regulation of VRG expression

For in-depth analysis on intracellular expression of VRG during antiviral responses, primary cultures of head kidney leukocytes treated with poly(I:C) and CpG oligodeoxynucleotides were analyzed with microarrays. These compounds bind to different PRRs in warm blood animals; poly(I:C) interacts with TLR3 and RIG-I (Schröder and Bowie, 2005) and unmethylated CpG binds to TLR9 (Wagner, 2002). A large fraction of VRG was regulated in response to the treatments, and a mean of 2-fold up-regulation was observed for 97 genes. The 20 strongest induced genes are shown in Fig. 5 and results for all VRG are in Supplementary Table 5. These genes were also among the most highly induced genes in virus-infected tissues and cells. The microarray analysis of cell cultures was performed in duplicates and the data were insufficient for accurate comparison of the stimulators by the magnitude of responses, however there were obvious similarities in their effects on VRG expression. Interestingly, a weak induction (1.75-fold) of IFNa was observed only in poly(I:C)-treated cells after one day while IFNγ was induced by all treatments (data not shown). To further examine the relationship between VRG and IFNs, we selected 104 microarray samples of salmon with viral diseases and divided them in two groups, of which 52 samples were considered as IFNa-positive (>1.5-fold up-regulation) while the rest were denoted as IFNa-negative. The most highly ranked VRG (by magnitude of expression) showed strong up-regulation in absence of IFNa induction (Table 2), although expression changes were consistently greater in the IFNa-positive samples ($p < 10^{-5}$, t-test). In overall, VRG showed high correlation with IFNa (Pearson $r = 0.89 \pm 0.01$) while only two genes (SOCS1 and deoxycytidine kinase) tended to co-express with IFNγ ($r = 0.79$ and 0.71). To expand search for co-regulated genes, we calculated correlation of the IFNa expression profile to those of all genes present on the microarrays in the samples of salmon with viral diseases. Genes with high correlation ($r > |0.7|$, 414 and 140 genes with respectively positive and negative

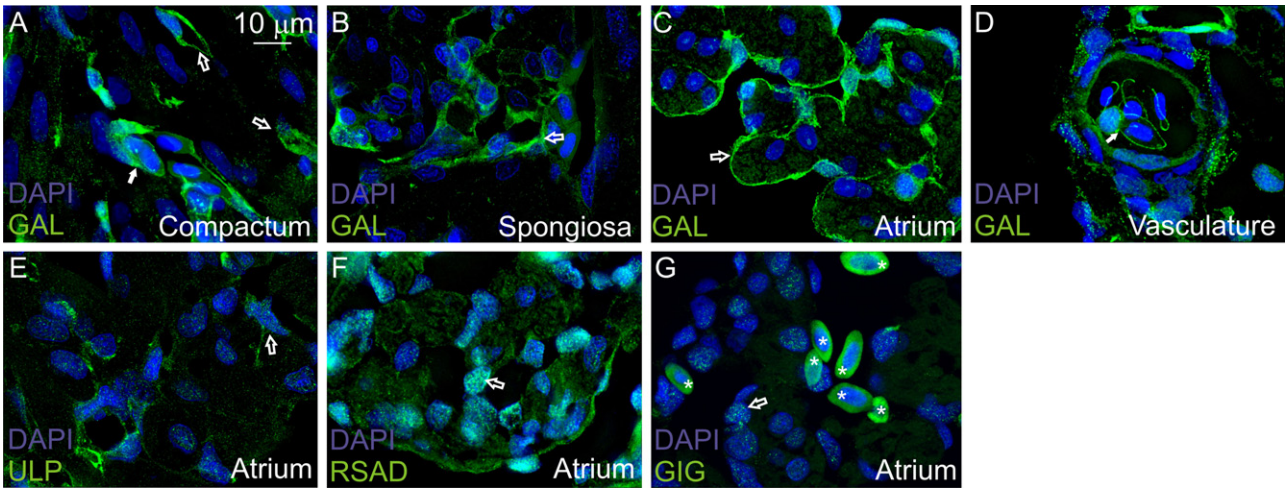

Fig. 4. Immunofluorescence analysis of expression of selected VRG proteins in different cardiac compartments from a representative specimen diagnosed with severe CMS pathology at 8 weeks post-infection. A–D: Galectin 9 (GAL) expression shows membrane bound, cytoplasmic and nuclear fluorescence detected in all cells, with stronger staining in some areas. (A) In the compactum layer, strong fluorescence is visible in both myocytes (open arrow) and leukocytes (arrow). (B) In the spongiosa, GAL is detected in myocytes. (C) Atrial myocytes show high GAL levels and notably in the cell membrane (open arrow). (D) In vasculature, GAL is detected in epithelial cells and leukocytes inside the vessel. Most of these cells show GAL localized to the cell membrane as a ring (arrow). Another leukocyte population reveals fluorescence in all sub cellular compartments. (E) In atrial myocytes Ubiquitin-like protein (ULP) is located mainly to the cytoplasm, with some nuclear signals as well (arrow). (F) Radical S-adenosyl methionine domain-containing protein 2 (RSAD) is detected predominately in the nuclei of atrial myocytes (arrow) and in addition weaker cytoplasmic and membrane signals were seen. (G) IFN-inducible protein gig2 (GIG) shows nuclear localization in the myocytes of atrium. Erythrocytes show strong autofluorescence (asterisk).

correlation) are presented in Supplementary Table 6. Among genes not included in the VRG list, highest correlation ($r > 0.95$) was observed for CD265, E3 ubiquitin-protein ligase LINCR, NF-kappa-B inhibitor alpha, adipophilin, heat shock 70 kDa protein and transcription factor jun-B. Genes with strong negative correlation ($r < -0.85$) have unknown roles (40 kDa nucleolar protein, HemK methyltransferase 1) or unclear association with IFNa (aldehyde dehydrogenase 16A1, serine/threonine-protein phosphatase 2A).

3.4. Structural comparison and evolution of VRG

The blastx search across Ensembl and GenBank protein sequences was used to examine the presence of VRG in different fish species and vertebrate taxa and to evaluate the rates and modes of their evolution; sequence comparison with blastx were performed at low stringency (cutoff $e < -10$), complete results are in Supplementary Table 7. By distribution among species, VRG can be divided in four groups (Fig. 6).

Gene	C, D5	PIC, D1	PIC, D5	CpG, D5
52 kDa Ro protein	-1.6	3.8	1.1	2.2
Barrier-to-autointegration factor	-0.7	3.2	2.3	2.2
C-C motif chemokine 19	-0.7	2.8	1.2	2.9
C-X-C motif chemokine 10	0.1	5.0	1.5	3.2
Deoxycytidine kinase	-0.2	3.7	1.0	2.2
IFN-induced with tetratricopeptide repeats 5	-0.9	5.6	2.1	2.8
Gig2-1	-0.7	4.4	3.3	3.2
Gig2-7	-0.9	4.3	2.1	2.8
Gig2-4	-0.6	4.2	2.1	2.7
Patched domain containing 1	-0.6	2.8	1.0	1.4
PPAR A A-interacting 285 kDa protein	-1.2	4.9	1.6	2.8
ATP-dependent RNA helicase DHX58	-0.8	3.7	1.0	2.4
Radical S-adenosyl methionine domain-containing 2	0.2	6.5	2.4	4.4
Receptor-transporting protein 3	0.0	7.4	4.6	4.3
Sacsin	-0.7	4.5	2.3	3.1
Similar to very large inducible GTPase 1	-0.6	5.3	1.6	3.0
Suppressor of cytokine signaling 1	-0.4	4.6	0.4	2.5
Ubiquitin-like protein precursor	0.2	5.2	2.8	2.9
Vacuolar protein sorting-associated protein	-0.2	2.3	3.3	2.5
XIAP-associated factor 1	-1.0	3.4	1.0	2.0

Fig. 5. Responses of VRG to stimulation in primary cultures of Atlantic salmon head kidney leukocytes. Cells were cultivated for one (D1) or five (D5) days under common conditions (control, C) and with addition of poly(I:C) (PIC) or CpG. Microarray analyses were conducted in duplicates, the data are $\log_2$-ER highlighted with a colour scale.

Table 2
Expression of VRG in virus-infected samples that showed upregulation (IFNa+) or no regulation (IFNa−) of IFNa. Data are mean $\log_2$-ER ± SE (microarrays).

GenBank	Gene	IFNa+	IFNa−
AY216595	Interferon alpha 2	1.26 ± 0.10	0.22 ± 0.03
EG836160	Ubiquitin-like protein-1	4.30 ± 0.08	3.11 ± 0.16
EG825775	Receptor-transporting protein 3	4.93 ± 0.18	3.05 ± 0.25
AF076620	Radical S-adenosyl methionine domain-containing containing protein 2	4.38 ± 0.15	2.68 ± 0.18
BT044026	Gig2-7	4.13 ± 0.08	2.56 ± 0.15
BT072288	VHSV-inducible protein-3	3.94 ± 0.14	2.54 ± 0.19
EG906096	Sacsin	3.94 ± 0.15	2.22 ± 0.19
209156101	IFN-induced protein with tetratricopeptide repeats 5-2	3.89 ± 0.17	2.53 ± 0.18
BT049116	Ubiquitin-like protein-2	3.74 ± 0.11	1.99 ± 0.18
EG841455	Very large inducible GTPase 1-1	3.73 ± 0.13	2.37 ± 0.17
BT049316	Barrier-to-autointegration factor	3.72 ± 0.09	2.64 ± 0.14
BT072201	Gig2-4	3.64 ± 0.09	2.07 ± 0.14
BT044022	Gig2-2	3.51 ± 0.16	2.27 ± 0.16
EG841846	Similar to he Hect domain and RLD3	3.50 ± 0.13	2.03 ± 0.17
DY704952	52 kDa Ro protein-1	3.50 ± 0.17	1.83 ± 0.17
GO053979	VHSV-inducible protein-4	3.38 ± 0.14	2.05 ± 0.15
DW537860	Hect domain and RLD 6	2.94 ± 0.11	1.68 ± 0.16
BT072557	VHSV-induced protein-1	2.93 ± 0.08	1.56 ± 0.14
209154815	Probable ATP-dependent RNA helicase DHX58	2.92 ± 0.13	1.50 ± 0.14
CA045875	Vacuolar protein sorting-associated protein 52-like	2.87 ± 0.09	1.50 ± 0.14
DW569595	Gig1-1	2.84 ± 0.13	1.78 ± 0.13
FN178459	Retinoic acid-inducible gene-I	2.83 ± 0.09	1.66 ± 0.14
EG791574	Zinc finger, NFX1-type containing 1	2.82 ± 0.13	1.44 ± 0.12
209735717	Signal transducer and activator of transcription 1	2.78 ± 0.10	1.84 ± 0.13
DW556796	IFN-induced protein 44-1	2.53 ± 0.09	1.65 ± 0.12
EG831164	PR domain zinc finger protein 9	2.25 ± 0.09	1.73 ± 0.10

A: Matches to the major fraction of Atlantic salmon VRG (89 genes – 72%) were found in all species included in the analyses and 43 genes showed similarity to proteins of a primitive *Chordata*, *Ciona intestinalis*. To evaluate the rates of VRG evolution, zebrafish and human were used as a reference. The median identities between Atlantic salmon VRG and the most similar proteins of these species as determined with blastx in local alignments were equal to respectively 54.1% and 42.6%. Similar comparison of 12.3 k Atlantic salmon transcripts with complete reading frames retrieved from GenBank produced markedly higher median values: 76.9% (zebrafish) and 68.4% (human).

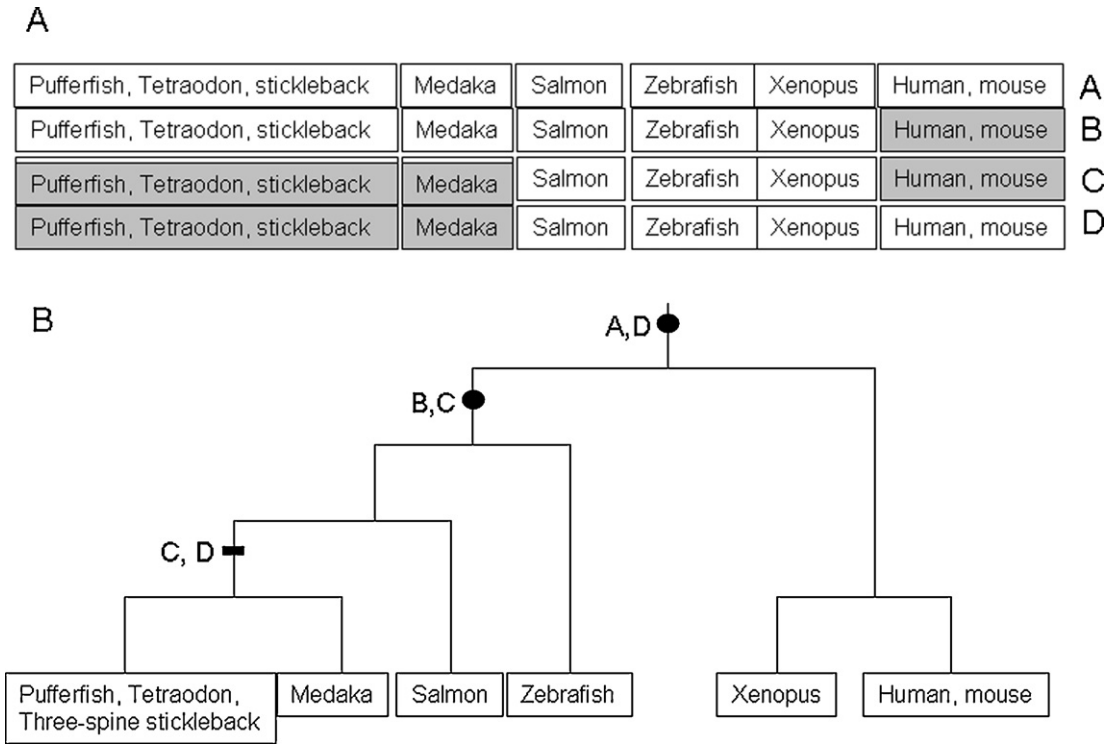

Fig. 6. Structural comparison and evolution of VRG A: division of VRG in four groups (A–D) by distribution in species with sequenced genomes. (A) The major fraction of salmon VRG with matches to all species. (B) VRG found exclusively in fish or in fish or amphibians. (C) VRG found in salmon and zebrafish but not in the advanced teleost groups and higher vertebrates. (D) VRG with homologs in cyprinid fish and higher vertebrates but not in fish species from other orders. Presence and absence of putative homologs is indicated with respectively white and grey background. The species are positioned corresponding to the phylogenetic distance from Atlantic salmon. B: phylogenetic relationship between species. Putative gain (●) and loss (–) of genes are indicated.

B: Genes found exclusively in fish or in fish and amphibians. Blastx search across either ENSEMBL (L3 lipoprotein) or GenBank (ULP, RTP and platelet basic protein similar to C-X-C chemokine) detected related proteins in several fish species from different orders but not in higher vertebrates. A large part of genes in this group belongs to three multi-gene families. TRIM are present in diverse phylogenetic groups while two subtypes of this large multi-gene family appear to be specific for fish (Du Pasquier, 2009; van der Aa et al., 2009). Two families include genes related to gig1 and gig2 that were recently discovered in crucian carp (*Carassius auratus*) CAB cells (Zhang and Gui, 2004); these genes seem to be absent in warm blood animals while presence of one gig2-like protein in *Xenopus* suggests that the latter can be conserved in lower vertebrates. No members of these families were found in pufferfish, while in medaka we detected two gig2 but not gig1. In other fish species the numbers of gig1 and gig2 genes were equal to respectively one and one in three-spine stickleback, one and four in *Tetraodon* and seven and eight in zebrafish. With respect to Atlantic salmon, five gig1 and seven gig2 were represented on the microarrays. All gig2 probes showed responses to viruses. Two gig1 that were not activated by infections share a N-terminal domain, which is absent from the virus responsive genes. Both gig families are characterized with exclusively high divergence (similarity of salmon proteins is within 23–45%) suggesting multiple duplication and rapid accumulation of sequence changes.

C: Genes found in salmon and zebrafish but not in advanced teleost groups and higher vertebrates; these are e.g. vacuolar sorting protein and three different genes that share a name of VHSV-induced protein.

D: Several salmon VRG seem to have homologs in cyprinid fish and higher vertebrates but not in fish species from other orders. A notable example is a putative RIG-I/DDX58, a cytoplasmic helicase with an essential role in virus sensing. Absence of RIG-I in all (Sarkar et al., 2008) or part (Zou et al., 2009) of fish lineages was hypothesized based on search in genomes of three fish species: *Danio*, *Tetraodon* and *Takifugu*. A short protein with greater similarity to RIG-I was found only in *Danio* genome. However functional orthologs to mammalian RIG-I were subsequently identified in cyprinid and salmonid fish (Biacchesi et al., 2009; Yang et al., 2011). We screened the Ensmebl data sets and Atlantic cod sequences and selected proteins that matched to Rig-I/DDX58, MDA5 and LGP2/DHX58. These helicases are structurally related but LGP2/DHX58 lacks caspase recruitment domain (CARD) and works mainly as a negative regulator of virus recognition (Yoneyama et al., 2005). Other blastx-identified fish helicases with remote similarity to salmon RIG-I belonged to different lineages (endoribonuclease Dicer and Fanconi anemia group M protein). The proteins were arranged in three clades with a strong bootstrap support. Putative RIG-I homologs were found in mammals, bird (duck – *Anas platyrhynchos*), salmon and cyprinid fish but not in fish from other orders, while LGP2/DHX58 and MDA5-similar proteins were found in all analyzed species. Several more VRG showed similar distribution including C-X-C chemokine 10 and IFIT-5.

4. Discussion

In this study we compared expression profiles from a large number of samples in order to identify VRG in Atlantic salmon, which allowed us to select a subset of viral infection markers. We also examined regulation of VRG expression, composition and establishment of this functional group in vertebrate evolution. Implementation of these tasks required a large number of analyses that measured expression of the same gene sets in multiple samples producing standardized results that are well accessible for data management and mining. Genome-wide ONM supported with bioinformatic tools provided an efficient and reliable approach to these objectives. The study confirmed the usefulness of *in vitro* models. We searched for VRG characterized with low tissue specificity and cell cultures appeared well suited for a task of this kind. The same panel of genes was up-regulated in virus infected fish and cells treated with poly(I:C) and ISAV. To this end, we selected 117 genes, and close overlap with a gene set found in the microarray study of Atlantic salmon cells infected with ISAV (Workenhe et al., 2009) suggests that a significant part of this functional group has been defined. Importantly, the list included all VRG that were identified from initial studies based on our previous cDNA microarray platform SFA1.0 and 2.0 (Schiøtz et al., 2008; Jorgensen et al., 2008). The list of VRG is obviously incomplete and one may ask if many new important actors can be found in future. The limitations in the search for VRG could be related to both the gene composition of the microarray and the available data set. Microarrays used in our studies include probes to unique transcripts identified with blastx with addition of unidentified sequences from our salmon leukocyte cDNA library. Given that many libraries from virus infected Atlantic salmon have been sequenced, one can expect a good coverage of VRG by mRNA stored in the public databases. However it is possible that part of salmon genes involved in antiviral responses encode unknown proteins and these were not represented on the ONM. Several VRG reported in this study matched only to salmonid proteins stored in GenBank. Given that the number of reference salmonid proteins is still relatively small, it is likely that part of VRG specific for this taxonomic group may remain unidentified and their number is hard to predict. Furthermore, the composition of gene lists compiled by the expression profiles obviously depends on the cut-off parameters and the data sets. Therefore we can expect increase of the VRG list and modification of its content. The complete sequence of the Atlantic salmon genome with predicted transcripts will be released in near future and new ONM will be designed. We shall continue to work with viral diseases including those that are poorly presented (ISA) or absent (PD) in our database. The genes selected in this study may respond to different infections and other stimuli. Slight down-regulation of VRG was observed in skin of salmon infected with ectoparasite salmon louse (Tadiso et al., 2011) and after treatment with cortisol (unpublished results). One may anticipate induction of VRG with bacterial infection. However at present our gene expression database does not contain data from salmon with bacterial diseases and therefore we are unable to assess specificity of responses. Nonetheless, it is already possible to make preliminary conclusions on the composition of VRG, regulation and evolution of this functional group.

Activation of VRG *per se* is not always sufficient for protection of salmon against acute infections with the highly pathogenic viruses. We observed highest expression levels in salmon with low resistance to ISAV (Jorgensen et al., 2008) and in fish infected with high virulence isolate of IPNV (Skjesol et al., 2011). Up-regulation of VRG does not predict the outcome of disease but can be used for detection of infection status. At present, diagnostics of viral diseases is based on clinical symptoms, histopathology and detection of viruses. While being most reliable and consistent, this strategy may have complications and limitations being prone to both false positive and false negative findings. Fish infected with protracted or non-pathogenic virus strains do not develop disease while assays may give positive results. CMS, HSMI and other emerging diseases are of particular challenge due to a significant time lag between the first occurrence and clinical symptoms. Obviously, diagnostics based on host markers that respond commonly to different viruses and are able to identify a positive infection status would be a valuable supplementation to the traditional methods. Such molecular markers need to meet a number of criteria: strong

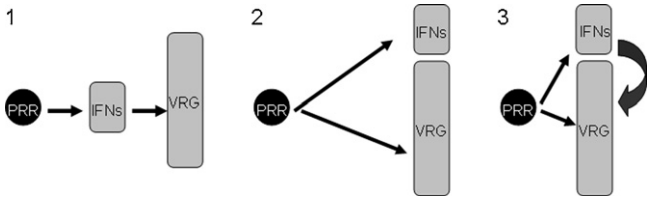

Fig. 7. Possible relationship between regulation of IFNs and VRG following activation through viral pattern recognition receptors (PRR); explanations are in text (Section 4).

and rapid responses to diverse viruses, correlation with the virus levels and severity of disease combined with a low tissue specificity, allowing for detection from different sampling materials. The presented data are not sufficient for practical recommendations. However the highly ranked VRG apparently meet the requirements for diagnostic markers of viral infections. Their responses are rapid as evidenced from cell cultures showing strong induction one day after exposure to poly(I:C) and ISAV. In fish with CMS and HSMI, VRG were upregulated several weeks before symptoms of disease could be seen and sustained until the end of challenge (10 weeks). This was also supported at the protein level for four VRG, showing induced cardiac expression from early viraemia to peak pathology stage. VRGs are activated by diverse viruses and presence of exogenous RNA appears the main if not the only requirement. The levels of VRG induction are related to the virus titers and no activation is seen at low infection levels that do not damage fish. Expression of VRG is stimulated with different synthetic agents including CpG and poly(I:C) that mimic bacterial and viral infections. Similar responses to these compounds suggested an overlap of signaling pathways activated with different PRRs in Atlantic salmon. However this was observed *in vitro* at high concentrations of the stimulating compounds that are probably never achieved *in vivo*. To use VRG for diagnostics it will be necessary to determine the thresholds that distinguish viral infections from other stimuli. In our studies VRG have shown co-ordinated expression profiles hence different genes can be equally used as diagnostic markers. Immunofluorescence analyses of four VRG proteins in heart of salmon with CMS did not reveal any differences in their distribution within the infected tissue. The microscopic observations revealed high expression levels of VRG in a moderate number of cells, which may reflect the low-virulent nature of the piscine myocarditis virus. Assuming that induction of VRG is associated with high levels of virus, it appears that a restricted number of cells may carry high loads of the pathogen. Staining was most prominent in cardiomyocytes, probably since these are the main infected heart cells during CMS (Haugland et al., 2011). However VRG expression was also observed in other cell types. Different sub-cellular localization was observed for different cell types which may reflect distinct functional roles during infection. For example, the membrane staining of GAL in infiltrating leukocytes may reflect its role in signal transduction or cell-cell adhesion, while cytosolic expression of ULP in myocytes may reflect a role in the proteasome. In general, the low tissue specificity observed from microarray and qPCR analyses is an advantage for diagnostic purposes, since different tissues and cell types can be used including red blood cells that can be collected without sacrificing fish.

High correlation of VRG and IFNa is in line with the important role of IFNs in orchestration of antiviral responses. In a simplistic way, three main types of relationship between regulation of VRG and IFNs can be considered (Fig. 7). (1) *IFN-dependent*: binding of viral components to PRRs induces IFNs, which in turn activate downstream VRG, principally through the Jak-Stat pathway. The ability of recombinant salmon IFNa to increase expression of antiviral effectors was confirmed (Ooi et al., 2008). (2) *IFN-independent*: IFNs and VRG are activated through the same or different pathways. Their coregulation is observed in most cases however VRG do not necessarily require IFN. As an example, in ISAV infected TO cells Mx and ISG15 were activated earlier than IFNa (Kileng et al., 2007). However conclusion on IFN-independence is difficult to confirm taking into account the complexity of the Atlantic salmon IFN system, which includes at least eleven genes with different expression profiles (Sun et al., 2009). Salmon cells deficient in IFNa receptor would be required to settle these questions, but this receptor has so far not been identified in salmonid fish. (3) *Positive feedback*: IFNs are coregulated with VRG and further increase their expression. Similar responses to primary and secondary stimuli are typical for cytokine networks. For example, bacterial lipopolysaccharide (LPS) and LPS-induced TNFα cause similar gene expression changes (Magder et al., 2006). Markedly higher expression of VRG in IFNa + samples (Table 2) is consistent with this type of regulation. It is noteworthy to mention the relatively limited number of VRG-positive cells that were detected with immunofluorescence (Figs. 3 and 4). If the paracrine mode of IFNa signaling were preponderant, one would expect more homogenous distribution of positively stained cells in the infected tissue. The observed pattern can be better explained by coregulation of VRG and IFNs in the same cells and further amplification of the antiviral responses through a positive feedback. Another explanation can be transient up-regulation of IFNa, which is commonly observed only within several hours after treatment of cells with virus or poly(I:C). However in contrast to *in vitro* studies, microarray analyses of infected salmon revealed sustained up-regulation of IFNa.

Sequence comparison of salmon genes with Ensembl proteins from different species suggested a relatively rapid evolution of VRG taken as a functional group. Some of the VRG, including highly specialized antiviral proteins, such as RSAD or viperin, showed high sequence conservation across all vertebrate species. However, global comparison with blastx suggested that the similarity of VRG with proteins from other species was markedly lower in comparison with all known salmon genes with complete reading frames. This could be expected since immune genes represent a highly dynamic fraction of genomes which evolves rapidly due to the continuous competition between hosts and pathogens. Comparison of salmon VRG with Ensembl proteins suggested that this functional group was established mainly at the early stage of vertebrate evolution (many VRG find match with *Ciona intestinalis* proteins), however part of genes could be lost in the advanced teleost orders (Fig. 5B). The available sets of fish proteins are incomplete and absence of blastx match is obviously insufficient for definite conclusions. However, the presence of genes in salmonids and cyprinids and absence in five *Acanthopterygii* species with sequenced genomes suggests loss as the most plausible scenario. From search across proteins in the Atlantic cod genome VRG that were also present in four *Acanthopterygii* species were found. Therefore loss of VRG probably took place after separation of *Euteleostei* and *Neoteleostei*. Both phylogenetically young and old genes could be subject to extinction. An interesting example is RIG-I, which shows the importance of sequence data obtained through both genomic projects and targeted cloning. Patchy distribution of RIG-I in sequenced genomes led authors to conclude that this gene had evolved in mammals (Sarkar et al., 2008), which was obviously premature. The finding of RIG-I-like genes in sea urchins (Zou et al., 2009) suggested that they have appeared in pre-vertebrate evolution, though surprisingly, no full-length RIG-I was found in fish genomes. Fully functional RIG-I genes were found in fish species whose genomes have not been sequenced. Currently, many Atlantic salmon VRG are presented with incomplete sequences, which impede structural and phylogenetic analyses. Completion

of the Atlantic salmon genome project will be of great importance for understanding the evolution of this and other functional groups.

Until recently, immune genes in fish have mainly been identified by search for putative homologs to the mammalian genes with well known roles. Presumption on the phylogenetic conservation of functions is still accepted as a guideline in fish biology. Studies based on functional genomics have produced many unexpected results that may question the universal credibility of this paradigm, especially with respect to the immune system. Homologous genes might change functions in course of vertebrate evolution and examples can be found among Atlantic salmon VRG. Sacsin has one of the highest ranks in our studies and its responses to viral pathogens in salmon were reported by other researchers (Workenhe et al., 2009). Human sacsin is described as a protein with important roles in the central neural system and search in PubMed returns publications that explored it exclusively in this context; to our knowledge responses to viruses have not been documented. Sacsin regulates Hsp70 (Parfitt et al., 2009) and interestingly, expression of both genes is tightly correlated with IFNa ($r = 0.92$ and 0.95, see Supplementary Table 6) suggesting that in salmon they can be a part of the IFN pathway. Another VRG with high correlation to IFNa ($r = 0.96$) is similar to cholesterol 25-hydroxylase known principally as an enzyme of steroid and bile acid metabolism. Acquisition of new functions might go in parallel with diversification of multigene families. A well known example is TRIM proteins, which recently received substantial attention in fish biology (Du Pasquier, 2009; van der Aa et al., 2009). This large family is present in all vertebrates, however at least one subtype of TRIM with strong antiviral responses is specific for fish which generally have markedly higher numbers of these genes. Establishment of a novel function was in parallel with rapid increase and diversification of this multigene family. TRIM genes comprise a large part of salmon VRG described in this and other (Workenhe et al., 2009) studies. However shortage of sequences with complete reading frames impedes structural analyses, which needs to be postponed until release of the complete Atlantic salmon genome sequence. Another problem is the absence of a commonly accepted TRIM nomenclature, which impedes comparison of results from different research groups. The finding of multiple gig1- and gig2-like genes showed that families involved in antiviral responses have appeared and undergone extensive diversification during fish evolution, and discoveries of new actors can be expected.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.08.007.

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