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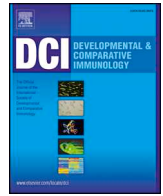
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Kinetics of transcriptional response against poly (I:C) and infectious salmon anemia virus (ISAV) in Atlantic salmon kidney (ASK) cell line

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ABSTRACT

Vaccine adjuvants induce host innate immune responses improving long-lasting adaptive immunity against vaccine antigens. *In vitro* models can be used to compare these responses between adjuvants and the infection targeted by the vaccine. We utilized transcriptomic profiling of an Atlantic salmon cell line to compare innate immune responses against ISAV and an experimental viral vaccine adjuvant: poly (I:C). Induction of interferon and interferon induced genes were observed after both treatments, but often with different amplitude and kinetics. Using KEGG ortholog database and available software from Bioconductor we could specify a complete bioinformatic pipeline for analysis of transcriptomic data from Atlantic salmon, a feature not previously available. We have identified important differences in the transcriptional profile of Atlantic salmon cells exposed to viral infection and a viral vaccine adjuvant candidate, poly (I:C). This report increases our knowledge of viral host-pathogen interaction in salmon and to which extent these can be mimicked by adjuvant compounds.

1. Introduction

Aquaculture, including fish farming, is the world's fastest growing sector in food production of animal origin and will soon provide more seafood than the global fish capture (FAO, 2018). In 2016, total production of fish and other aquatic animals, reached 170 million tonnes and, from that total, 80 million tonnes came from aquaculture (FAO, 2018). This expansion of fish farming was only possible due to extensive vaccine development, which has resulted in effective prevention against bacterial diseases and decline in the use of antibiotics by the North-Atlantic salmon industry (Adams, 2019; Sommerset et al., 2005). While there are many effective vaccines against bacterial diseases on the market, only a few effective vaccines against viruses are commercially available, making viral infections responsible for great losses for aquaculture (Kibenge et al., 2012; Rodger, 2016). A new disease affecting farmed Atlantic salmon causing anemia and, in many cases, high mortalities was first detected in Norway in 1984. Few years later, it was established that the disease could be transferred by injection of liver homogenates from diseased fish and that non-injected fish could also be infected by cohabitation with injected fish. These findings suggested an infectious etiology and the disease was called infectious salmon anemia (ISA) (Thorud and Djupvik, 1988). It took more than ten years for the virus to be isolated and characterized (Dannevig et al., 1995; Mjaaland et al., 1997) and it is still, 35 years later, causing loss to Atlantic salmon

aquaculture (Rimstad and Markussen, 2019). ISAV belongs to the *Orthomyxoviridae* family, same family as influenza virus, and causes a highly contagious disease in Atlantic salmon (*Salmo salar*) farms. ISAV causes a range of symptoms like severe anemia, lethargy and hemorrhage, and cumulative mortality in ISAV outbreaks varies from 10 to 90% (Aamelfot et al., 2014; Krossoy et al., 1999; OIE, 2019), whereas up to 95% mortality have been observed in experimental settings (Aamelfot et al., 2014; Kibenge et al., 2006; Krossoy et al., 1999). Although there are some commercially available vaccines against ISAV, and data from manufacturers show protection, documentation of efficacy under farming conditions, in scientific peer reviewed articles, are scarce or nonexistent (Dhar, 2011; Gomez-Casado et al., 2011; Mikalsen et al., 2005).

Immunization with live attenuated pathogens often induces strong protection, but their usage can be limited by safety issues. Inactivated or subunit vaccines are not always very easy and cheap to produce, and their capacity to induce protective responses is frequently limited (Bøgwald and Dalmo, 2019; Evensen, 2016; Sommerset et al., 2005). The use of nucleic acid-based vaccines may expand in the future, but it remains modest, with very few DNA vaccines authorized in Europe (Collins et al., 2019). Efficient and cheap vaccines is still lacking against several fish diseases including ISA, hence there is a considerable interest to understand the mechanisms of action of adjuvants and to develop new ones. Adjuvants are substances that increase the response

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induced by immunization. Mineral and plant oil-based formulations are commonly used in fish commercial vaccines. These oil emulsions influence vaccine outcome in time, place and concentration, improving vaccines immune-availability (Schijns and Lavelle, 2011; Tafalla et al., 2013). Host innate antiviral immune responses are crucial during viral infections and are responsible for controlling virus propagation. The interferon (IFN) system is activated after recognition of virus nucleic acids or glycoproteins through pattern recognition receptors (PRRs), resulting in secretion of type I IFN followed by induction of interferon stimulated genes (ISGs) with antiviral activity (Langevin et al., 2013; Robertsen, 2018; Schoggins and Rice, 2011). Double-stranded RNA (dsRNA) is a molecular pattern associated with viral infections and is recognized by several PRRs including several RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). Poly (I:C) (Polyriboinosinic acid:polyribocytidylic acid) is a synthetic analogue of dsRNA recognized by mammalian TLR3, MDA5 (melanoma differentiation-associated gene 5), RIG-I, LGP2 (laboratory of genetics and pathology 2) (Alexopoulou et al., 2001; Kato et al., 2008). These receptors were also identified in a variety of fish species and, together with TLR22 (only found in fish), have been shown to be upregulated after stimulation with poly (I:C) (Chang et al., 2011; Matsuo et al., 2008; Shen et al., 2016; Vidal et al., 2015). Poly (I:C) has been widely used to stimulate and study antiviral immunity and interferon responses (Matsumoto and Seya, 2008; Poynter et al., 2015; Svingerud et al., 2012). There are also many studies and clinical trials using poly (I:C) as adjuvant for virus infection and cancer vaccine/treatment in humans showing good results (Cheng et al., 2018; Maeda et al., 2019; Smith et al., 2018). Poly (I:C) has been used as both mimic of viral infection as well as adjuvant for new fish experimental vaccine formulations. Promising results showed that many genes related to viral immunity are upregulated when poly (I:C) is administered to fish or fish cell lines, and the use of different platforms of delivery, including nanoparticles, can both diminish the minimal dose required as well as result in more efficient vaccines (Arnemo et al., 2017; Hafner et al., 2013; Kavaliauskis et al., 2016; Zhu et al., 2019). The adjuvant activity of type I IFN in vaccination against ISAV has been shown by others, where fish injected with plasmid expressing IFN together with plasmid expressing ISAV HE (hemagglutinin-esterase) resulted in enhanced protection of salmon against ISAV, lowering mortality, increasing specific IgM antibodies and resulting in increased duration of the Mx/ISG15 response (Chang et al., 2015; Robertsen et al., 2016). To develop better vaccines against viral diseases in fish it is therefore important to understand the differences between the antiviral innate responses induced by infection and those elicited by vaccine adjuvants like poly (I:C). Uptake of double stranded poly (I:C) from the cell culture medium will engage endosomal PRRs like TLR3, RIG-I and MDA-5 (DeWitte-Orr et al., 2010; Hwang et al., 2012). The recent discovery of endosomal dsRNA transporters pumping polynucleotides into the cytosol (Nguyen et al., 2017) may explain why cytosolic sensors like RIG-I and MDA-5 are important sensors of extracellularly added interferon inducing polynucleotides like poly (I:C) (Gitlin et al., 2006). Likewise, although ssRNA viruses like ISAV and influenza orthomyxovirus use nuclear replication, sequencing of nucleic acids bound to immunoprecipitated cytosolic PRRs like RIG-I reveals binding of all viral genomic segments to this sensor (Baum et al., 2010). In addition, each segment 3' and 5' termini contain partial inverted complementary sequences forming panhandle or corkscrew dsRNA structures capable of binding to RIG-I (Killip et al., 2015). These observations may explain why responses to poly (I:C) and to the virus largely overlap. However, viral infections deeply affect cell metabolism and can be detected by sensor binding viral proteins (Eisenreich et al., 2019). Thus, they can modify the expression of many genes that are not directly up or downregulated by nucleic acid sensing. While both ISAV and poly (I:C) should trigger type I IFN pathway, they likely lead to distinct transcriptional responses.

We, therefore, compared transcriptome responses to ISAV, and important viral pathogens of Atlantic salmon, and poly (I:C), a compound

used to mimic the induction of type I IFN response. These differences observed between these responses will provide new insights into pathways triggered in different immunization contexts. We report here a detailed temporal study of ISAV and poly (I:C) response (by qPCR) in Atlantic salmon kidney (ASK) cell line up to 96 h after stimulation. In addition, two time points: 12 and 48 h, were subjected to full transcriptome analysis of regulated genes by RNA sequencing, providing a comprehensive picture of important differences between responses to RNA virus and its mimic poly (I:C).

2. Materials and methods

2.1. Cell culture

Atlantic salmon kidney cells (ASK) (Devold et al., 2000) were kindly provided by Knut Falk (Norwegian Veterinary Institute). Cells were routinely split once a week (1:2) and cultivated at 20 °C in Leibovitz L-15 medium supplemented with 4 mM L-glutamine (both reagents from Lonza Biowhittaker, Verviers, Belgium), 10% fetal bovine serum, 40 µM 2-mercaptoethanol (both from Gibco, Life Technologies, Bleiswijk, The Netherlands) and gentamicin (50 µg/ml - Lonza Biowhittaker, Walkersville, USA). Cells were placed at 15 °C one week before the experiment started, for acclimatization, and for all the duration of the experimental period.

2.2. Virus propagation

For this experiment we used ISAV strain Glesvær 2/90, as it has been shown to result in high mortality in Atlantic salmon (Mjaaland et al., 2005). For virus production, ASK cells (passage 52) were inoculated with virus that was allowed to adsorb for 4 h at 15 °C in serum free medium, before cell culture media with 10% serum was added. Supernatants from the cell flasks were collected after 14 days, cleared by centrifugation at 4000 × g for 20 min, aliquoted and frozen at -80 °C. An aliquot, from a single freeze-thaw cycle, was used for titration on ASK cells using tissue culture infectious dose method (TCID₅₀) (Reed and Muench, 1938).

2.3. Time series analysis

ASK cells were incubated at 15 °C for a week before the experiment started. Cells were then counted, seeded in 6-well plates (1.5 × 10⁵ cells per well, passages 40–50) and left overnight to adhere at 15 °C. *In vitro* infection was performed by washing the wells three times with sterile PBS, adding a multiplicity of infection (MOI) 10 virus suspension in serum-free L-15 medium and, after 4 h (to allow adsorption), culture medium (L-15 supplemented) was added and plates were incubated until sampling. For stimulation with poly (I:C) (P1530 Sigma, St. Louis, MO), 30 µg/ml was added in the wells and plates were incubated until sampling time. Time points used in this study: 0, 3, 6, 12, 24, 48, 72 and 96 h. At each time point, wells were washed three times with PBS, cells lysed with RT buffer (QIAGEN, Hilden, Germany) and samples were stored at -20 °C, for a few days, until analysis. Control cells, time point zero, were cells not infected with virus and not stimulated with poly (I:C). The two groups were named PolyIC and ISAV for cells stimulated by poly (I:C) and for cells infected by ISAV, respectively. The experiment was repeated three times and each time point had 2–4 technical replicates (cell culture wells).

2.4. Total RNA isolation and sequencing

Total RNA was extracted using Rneasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's tissue protocol. A step for removal of genomic DNA was included by adding Dnase I (Rnase-Free Dnase Set, QIAGEN, Hilden, Germany). RNA was eluted in 50 µl Rnase-free distilled water. RNA concentration was measured using PicoDrop

Pico 100 (PicoDrop Technologies, Cambridge, UK). For sequencing, two time points (12 h and 48 h) were chosen for virus (called V12h and V48h) and for poly (I:C) (P12h and P48h) plus a common control (Control). Four experimental replicates (each containing RNA from two cell culture wells) by group, 20 samples in total, were sent to Norwegian Sequencing Centre (NSC), where RNA quality were verified with Agilent 210e0 Bioanalyser (Agilent, USA), and library preparation was performed using TruSeq™ Stranded mRNA Library Prep Kit (Illumina Inc., San Diego, USA). Libraries were then sequenced on Illumina HiSeq 4000 sequencer resulting in 150-bp paired-end reads.

2.5. Quantitative PCR (qPCR)

RNA was reverse transcribed to cDNA using high-capacity RNA-to-cDNA kit (Applied Biosystems Inc., United States), following manufacturers protocol. qPCR was performed in 96-well plates on LightCycler 480 using SYBR Green Master Mix (both from Roche Diagnostics, Basel, Switzerland) using RNase-free water and no-RT as negative controls. Cycling conditions were: 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Melting curve was measured by 95 °C for 5 s and 65 °C for 1 min to evaluate the specificity of the primers (single peak in melt curve slope change). All qPCR experiments were performed using 2–3 experimental replicates (cell culture wells). Cycle threshold (Ct) values were obtained and used to calculate correlation with RNA-seq. For calculation of relative expression levels, delta-delta Ct method was used assuming 100% primer efficiency (Pfaffl, 2001). *18s* and *ef1a* were used as reference genes (as they have shown consistent performance in these types of experiments with Ct values ranging from 8 to 10 for *18s* and from 17 to 19 from *ef1a*) (Jorgensen et al., 2006a). Primers used are listed in Table 1.

2.6. Bioinformatics and statistics

Fastq files containing reads from the RNA-seq were mapped to the Atlantic salmon genome (GCF_000233375.1_ICASAG_v2_genomic.fna) using the HISAT-Stringtie pipeline (Pertea et al., 2016). Transcripts

were assembled on the existing Atlantic salmon annotation file (GCF_000233375.1_ICASAG_v2_genomic.gff). Both files were downloaded from NCBI (Annotation release 100). After mapping and assembly of full and partial transcripts, the R package Deseq2 (version 1.22.1) was used to quantify differential expression between groups, at each time point, against the control (Love et al., 2014, 2015). Gene expression tables from Deseq2 were cleaned using median > 10 as a cut off, to get rid of genes with zero or low counts. Using Benjamin-Hochberg (BH) correction to calculate adjusted *p*-value (padj), genes with adjusted *p*-value (padj) below 0.01 were regarded as differentially expressed genes (DEGs). For gene regulation, we filtered out genes with less than twofold change ($0.5 < FC < 2$) in expression (upregulated genes had \log_2 fold change (Log_2FC) > 1 and downregulated genes with $\text{Log}_2\text{FC} < -1$). All scripts for transcriptome analysis are available in Supplementary file 1, exploratory plots and sample analysis in Supplementary file 2. R package clusterProfiler (Yu et al., 2012) was used for both gene ontology (GO) and KEGG pathway analysis with 0.01 as *p*value and *q*value cutoffs, BH adjusted. Pathview R package was used to draw KEGG pathway maps. The same Fastq files were also mapped against the ISA Glesvær 2/90 virus genome (Accession HQ259671, HQ259672, HQ259673, HQ259674, HQ259675, HQ259676, HQ259677, HQ259678) (Merour et al., 2011). An ISAV annotation file was generated from the NCBI sequence files.

3. Results

3.1. Time series analysis of innate immunity transcripts

To examine the kinetics of anti-viral responses induced by ISAV and poly (I:C) in ASK cells, a time course study was performed. The expressions of genes known to be dysregulated during virus infection: *ifna*, *mx1*, *isg15* and *tnfa1* (Kileng et al., 2007; Purcell et al., 2004; Xu et al., 2012), were analyzed at eight time points (0, 3, 6, 12, 24, 48, 72 and 96 h) by qPCR (Fig. 1). Stimulation with poly (I:C) resulted in a rapid but transient upregulation of *ifna1* and *tnfa1*. After 3 h, the expression levels of these genes were significantly higher than in the

Table 1
Primers for quantitative polymerase chain reaction (qPCR) analysis.

Genes	Direction	Sequence 5'→3'	GenBank accession number	Amplicon length	Reference
<i>ef1a</i>	F	CACCACCGGCCATCTGATCTACAA	AF321836	77 bp	Jorgensen et al. (2007a)
	R	TCAGCAGCCTCCTTCTCGAACTTC			
<i>18S</i>	F	TGTGCGCTAGAGGTGAAATT	AJ427629.1	61 bp	Jorgensen et al. (2007a)
	R	GCAAATGCTTTTCGCTTTTCG			
<i>ifna1</i>	F	CCTGCCATGAAACCTGAGAAGA	AY216594	107 bp	Jorgensen et al. (2006b)
	R	TTTCCTGATGAGCTCCCATGC			
<i>tnfa1</i>	F	GCGGAGCGTACCACCTCCTC	DQ787157.1	51 bp	*
	R	GGAGTCCGAATAGCGCCAA			
<i>isg15</i>	F	ATGGTGCTGATTACGGAGCC	AY926456	151 bp	Schiotz et al. (2009)
	R	TCTGTTGGTTGGCAGGACT			
<i>mx1</i>	F	TGATCGATAAAGTGACTGCATTCA	SSU66477	80 bp	Jorgensen et al. (2006b)
	R	TGAGACGAACTCCGCTTTTTC			
<i>ccl19</i>	F	ATCGCAGAATGGAAAGGCCA	XM_014128861	76 bp	*
	R	TCAGGGATCACCCACAGTCT			
<i>cxcl11</i>	F	AGAGGCTCCATTTGCCAAGA	XM_014143455	159 bp	*
	R	GGCTGTCTTCAGGCAGTTTT			
<i>ifih1</i>	F	GAGAGCCGTCCAAAGTGAA	XM_014164134	389 bp	*
	R	TCCTCTGAACTTTTCGGCCAC			
<i>ifit5</i>	F	GCCGTAGATCTGAAGCCCTC	XM_014179291	200 bp	*
	R	GCCCTGCCCTCATCTTTCTT			
<i>irf4</i>	F	TAACTGAACGGACTGTAAGCCT	XM_014135096	79 bp	*
	R	AACGCAITTTCTTGACCGGC			
<i>rsad2</i>	F	CTGCCAGATGATGTTTGTATG	XM_014186947	106 bp	*
	R	AAAGCTGTGATGCAGGGCTC			

* Primers designed for this study. *ef1a* - elongation factor 1 alpha; *18s* - 18S ribosomal RNA; *ifna1* - interferon alpha1; *tnfa1* - tumor necrosis factor alpha-1; *isg15* - interferon-stimulated gene 15; *mx1* - interferon-induced GTP-binding protein Mx1; *ccl19* - cc motif chemokine ligand 19; *cxcl11* - c-x-c motif chemokine ligand 11; *ifih1* - interferon induced with helicase C domain 1; *ifit5* - interferon-induced protein with tetratricopeptide repeats 5; *irf4* - interferon regulatory factor 4 and *rsad2* - radical S-adenosyl methionine domain-containing protein 2.

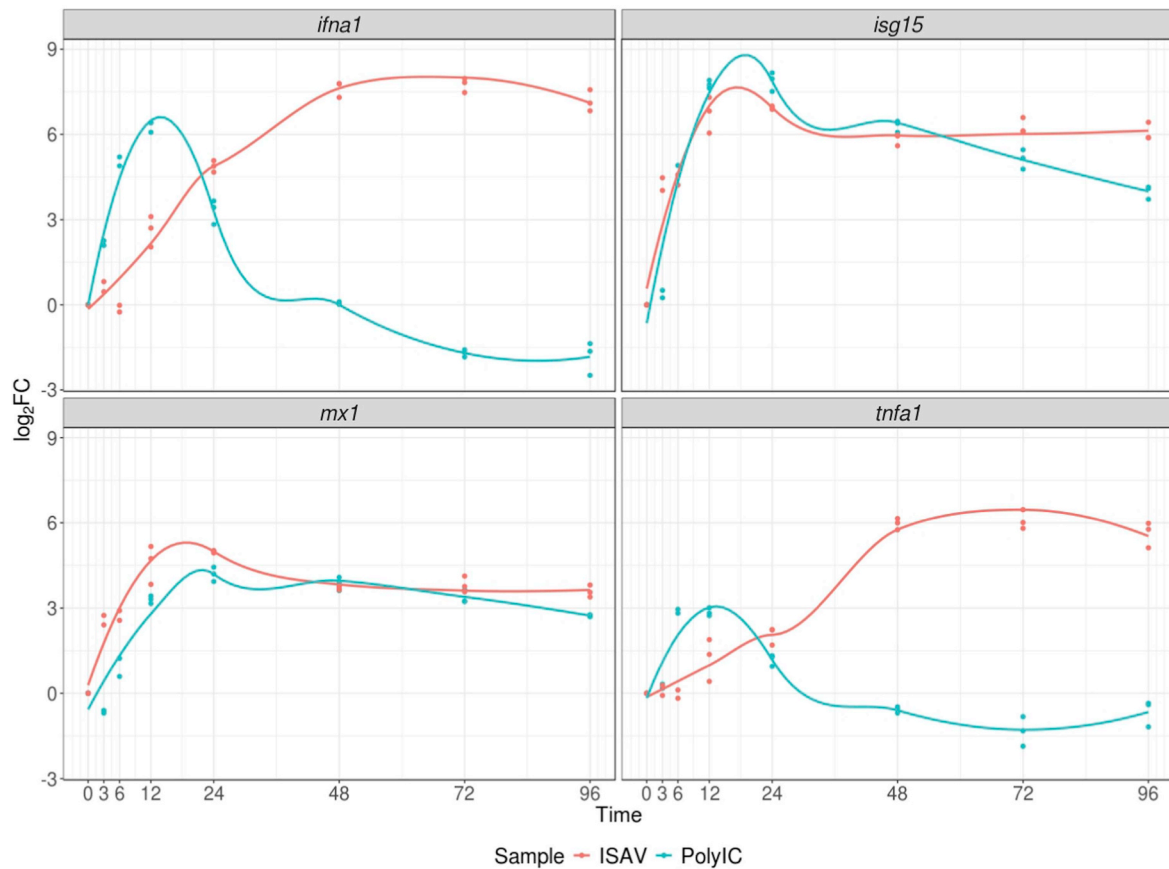


Fig. 1. Time series analysis of innate immunity transcripts. Four genes were analyzed in cells stimulated with poly (I:C) (PolyIC group) or infected with ISAV (ISAV group). Time points were 0, 3, 6, 12, 24, 48, 72 and 96 h after stimulation. Genes were analyzed by qPCR using *ef1a* and *18s* as reference genes. Each group with 2–3 replicates (cell culture wells).

control and continued to increase until reaching a maximum at 12 h. At this time point, *ifna1* and *tnfa1* were upregulated 64-fold and 8-fold, respectively. At 48 h both genes were back to pre-stimulated levels. Completely different profiles were observed for the same genes when cells were infected with ISAV. The expression levels of both genes initially increased slowly, reaching their maximum levels at 48 h and remained highly upregulated throughout the time course (96 h). For *isg15* and *mx1*, ISAV infected cells showed higher expression levels than poly (I:C) stimulated cells already at 3 h. At 12 h both groups exhibited similar high expression levels, which continued throughout the experiment.

3.2. RNA-seq analysis of expression kinetics after viral and poly (I:C) stimulation

The quality of the RNA-seq data was analyzed via multiple exploratory plots before analysis of DEGs. This analysis confirmed good sample replication and data distribution. Both sample and feature (genes) were clustered in PCA plots/heatmaps, confirming consistency in the data (Supplementary file 2). Based on the results from the time series analysis, RNA-seq was used to analyze the full ASK cells transcriptome at 12 and 48 h after infection with ISAV or stimulation with poly (I:C). The number of sequenced reads from each sample varied from 18 to 28 million achieving an average alignment rate of 76% (range 50–83%) of the reads mapped to Atlantic salmon genome (Table 2). Most of the samples showed alignment around 80% to the Atlantic salmon genome, except from the 48 h samples from ISAV infected cells, which exhibited lower percentage of alignment (around 50%). This discrepancy was resolved when the same samples were mapped to the ISAV genome. In the 48 h samples, about 25–30% of the

Table 2

Total sequenced reads and alignment rate of mapping.

Sample	Total reads	Alignment rate Atlantic salmon	Alignment rate ISAV	Time	Group
Control 1	28,382,485	83.16%	0.0%	0	Control
Control 2	21,197,107	81.12%	0.0%	0	Control
Control 3	22,191,845	84.37%	0.0%	0	Control
Control 4	2,5563,727	83.24%	0.0%	0	Control
P12h1	22,622,810	82.69%	0.0%	12	PolyIC
P12h2	18,694,282	83.61%	0.0%	12	PolyIC
P12h3	24,184,310	84.05%	0.0%	12	PolyIC
P12h4	20,331,953	83.54%	0.0%	12	PolyIC
P48h1	22,416,777	81.94%	0.0%	48	PolyIC
P48h2	22,902,401	81.94%	0.0%	48	PolyIC
P48h3	28,722,626	81.34%	0.0%	48	PolyIC
P48h4	24,926,479	82.16%	0.0%	48	PolyIC
V12h1	25,380,095	82.89%	1.44%	12	ISAV
V12h2	23,478,048	81.43%	1.91%	12	ISAV
V12h3	21,565,623	80.67%	1.83%	12	ISAV
V12h4	18,474,473	79.79%	1.68%	12	ISAV
V48h1	25,174,167	50.45%	33.77%	48	ISAV
V48h2	24,260,934	55.31%	29.93%	48	ISAV
V48h3	25,244,357	54.99%	22.83%	48	ISAV
V48h4	24,947,205	58.06%	27.11%	48	ISAV

reads mapped to the ISAV genome, filling the gap for a comparable overall alignment rate in all samples to 80–85%.

3.3. Identification of differentially expressed genes (DEGs)

Deseq2 package was used to estimate changes in expression levels of the genes (DEGs) in each group (for each time point/treatment) against

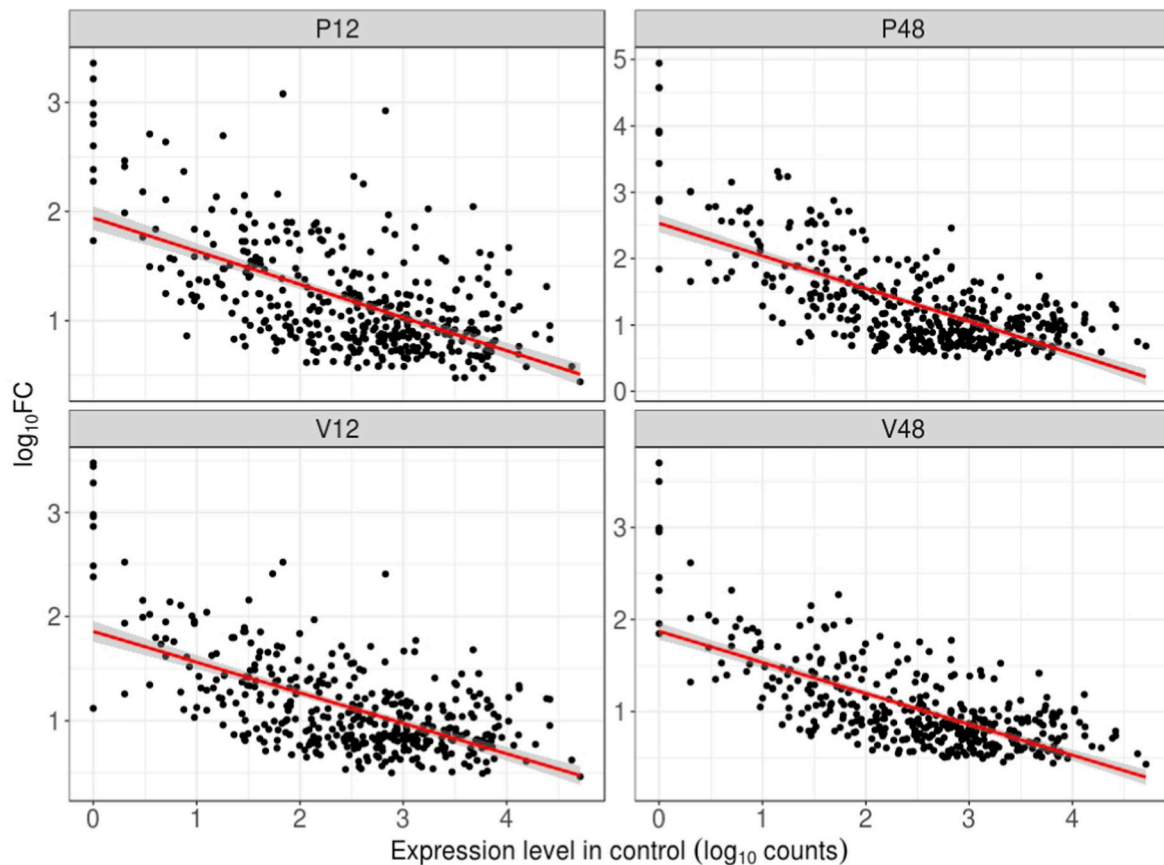


Fig. 2. Correlation between expression level and degree of upregulation. Relationship between expression level in control cells and degree of upregulation (average fold change) in ASK cells infected with ISAV or stimulated with poly (I:C) for 12 h and 48 h. Data points are from the set of genes with significant change in all four groups (404 genes).

the control. Before analyzing the time and treatment dependent effects we analyzed the aggregated samples (both groups and both time points together against control) to reveal the dominant trend of transcriptional changes induced by these treatments (stimulated cells vs control). This test identified 495 significantly perturbed genes (many previously described as virus or interferon induced genes), all upregulated with a mean 18-fold change (Supplementary Table 1). These treatments therefore predominantly induce gene upregulation more strongly than transcriptional inhibition. We next tested the effect of time and treatment against control. Filtering out low counts genes (median < 10) and non-significant genes (adjusted p value > 0.01) resulted in a table containing 3111 genes that were significantly modulated, when compared to control, in at least one of the groups (Supplementary Table 2). There was a general trend in all samples that genes with low expression in control showed higher fold change induced by the infection or poly (I:C) stimulation (Fig. 4).

Among the DEGs, we identified 404 genes that appeared in all four groups (Fig. 3), comprising genes encoding proteins related to immune response like chemokines (*ccl19*, *cxcl11*), multiple TRIM proteins, interferon stimulated genes (*ifi44*, *irf1*, *irf7*, *isg15*, *rsad2* and *mx*) as well as PRRs such as *tlr-8* and *lgp2* were upregulated in both time points (12 h and 48 h), for both groups (PolyIC and ISAV). The kinetics of some of the genes regulated by poly (I:C) and/or virus infection are shown in Fig. 4.

Poly (I:C) stimulation generally resulted in quicker and stronger response, with more genes being up or downregulated both at 12 h and 48 h than for infection with ISAV (Fig. 5). For the PolyIC group, we identified 1164 upregulated and 387 downregulated DEGs at 12 h (P12). At 48 h, poly (I:C) treated cells still showed strong dysregulation, with many genes upregulated (1,216) and an increased number of

downregulated genes (807 DEGs). This was also observed for the ISAV group where upregulated DEGs were 755 at 12 h and 819 at 48 h, whereas the number of downregulated genes jumped from only 11, at 12 h, to 211 at 48 h. A large majority of genes regulated by ISAV were also regulated by Poly (I:C), indicating that most of genes modulated by the viral infection were type I IFN dependent.

We observed that most of induced genes were upregulated less than 10-fold, but many genes were induced to higher expression levels (from 10 to 100 times). Only a few genes showed fold changes higher than 100 and 500 times, but many of these genes were not expressed in control cells and the fold change value of the treatment groups can therefore be disproportionately affected (Deseq2 adds a pseudo-count of 1 to non-expressed genes before calculating fold change). However, some genes related to immune response with significant expression in control cells, like, *c-x-c motif chemokine 10-like*, *c-x-c motif chemokine 11*, *c-c motif chemokine 20-like*, *receptor-transporting protein 3-like* and *isg15-like* showed actually strong expression exhibiting fold changes of more than 100 times to basal level expression of control group (Fig. 6 & Supplementary Table 2).

DEGs comprised many genes belonging to the TRIM family. Many members of this large multigene family are involved in antiviral defense in mammals. In fish, several large TRIM subsets have been described, with members implicated in the type I IFN response (Boudinot et al., 2011; Sardiello et al., 2008). In Atlantic salmon, the power of our analysis was decreased by the incomplete genome annotation, with many protein models likely truncated. Such issues are typical of large gene families with many genes of similar sequence, which makes the assembly challenging. However, combining blast search with phylogenetic analyses based on current salmon protein models, well supported clusters also comprising zebrafish/human *TRIM39/btr*, *TRIM25*,

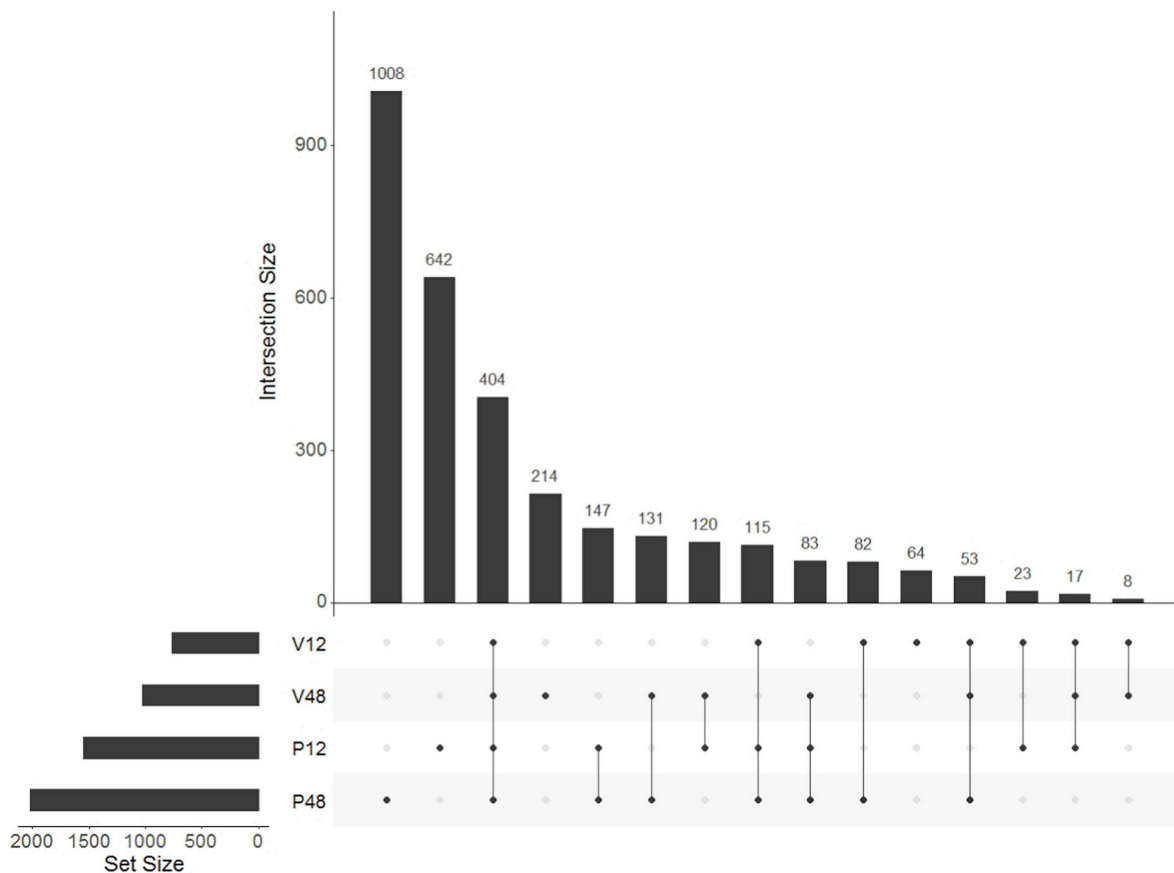


Fig. 3. UpSet plot. Plot showing overlapping of genes identified for each group. The bars show the overlap between the indicated motifs below: V12/V48 (cells infected with ISAV for 12 and 48 h, respectively) and P12/P48 (cells stimulated with poly (I:C) for 12 and 48 h, respectively).

TRIM47 and *fintrim* (*fintrim* 14, 16, 67) in distance trees could be identified (Supplementary Fig. 1). Most salmon trim genes differentially expressed belong to *fintrim*, and cluster with known zebrafish and rainbow trout members of this subset. Another group corresponding to *btr/trim39* was identified, with two genes not significantly modulated in P12 but highly upregulated in P48. Those two *btr/trim39* were also upregulated in both virus groups (V12, V48) but with a lower relative expression than in P48. Finally, the well-known ISG *trim25* is represented in our dataset by two genes upregulated in all four groups with similar level of expression.

3.4. Classifying interferon-stimulated genes among DEGs

In an attempt to identify ISGs in our list of DEGs, we disregarded the different time points and considered only the different treatments (stimulation with poly (I:C) versus infection with ISAV). A Venn diagram was drawn from this analysis revealing three clusters of genes: 1797 genes exclusively regulated by poly (I:C), 286 genes solely up/down-regulated by ISAV (ISAV group) and 1028 genes that were common for the two groups (Fig. 7a). These three groups were then compared with lists of known ISGs from human (Schoggins et al., 2011) and zebrafish (Levraud et al., 2019). As a result, we identified 82 genes in the “Poly IC only” group, 13 genes in the “ISAV only” group and 261 genes shared by both groups for which orthologs are present in ISG lists reported from human and zebrafish (Fig. 7b and Supplementary Table 3). Within this list of salmon ISGs, 89 genes had human orthologs associated to antiviral activity (Schoggins, 2019; Schoggins and Rice, 2011), many of them being duplicated. Fig. 8 displays the kinetic profiles for a selection of these ISGs with known antiviral activity in human, illustrating the diversity of induction modes after poly (I:C) versus viral stimulation.

3.5. Enrichment analysis showed that poly (I:C) and ISAV DEGs are generally associated with same biological processes and pathways

To identify the pathways in which DEG could be implicated, a gene ontology (GO) analysis was performed using *enrichGO* function in the R package *clusterProfiler*. The *compareCluster* function was applied to compare enriched functional categories of gene clusters between downregulated and upregulated genes. After running GO enrichment analysis, using the whole list of significant genes from *DeSeq2* (3111 DEGs), 185 unique GO terms for the different GO categories were identified: Biological Process (BP), Molecular Function (MF) and Cellular Components (CC). GO terms with a *q*value lower than 0.01 were considered significant; 156 GO terms belonging to BP, 34 to MF and 5 as part of CC category. One hundred and fourteen BP enriched terms were significant in the cluster of upregulated DEGs with most terms related to immune response. Fig. 9 shows ten GO terms with highest *q*value of each group (P12, P48, V12, V48), but because some groups may have lower (yet significant) *q*value they may not appear in the dotplot (for a complete table with Biological Process terms see Supplementary Table 4). P12 and V48 groups displayed more upregulated genes (Counts) in most of the immune-related terms. Upregulated terms involved with regulation of macrophage/leukocyte/cell activation were over-represented in the P48 and V12 groups. Most of the terms in the BP downregulated category were related to metabolic processes and signal transduction. Although signal transduction terms were enriched in the P12 group, metabolic processes terms were enriched in downregulated DEGs in the P12, P48 and V48 groups. V12 group does not appear in any downregulated gene cluster analysis because of the low number of DEGs in this group (11 downregulated DEGs). GO analysis for MF category resulted in 34 unique enriched

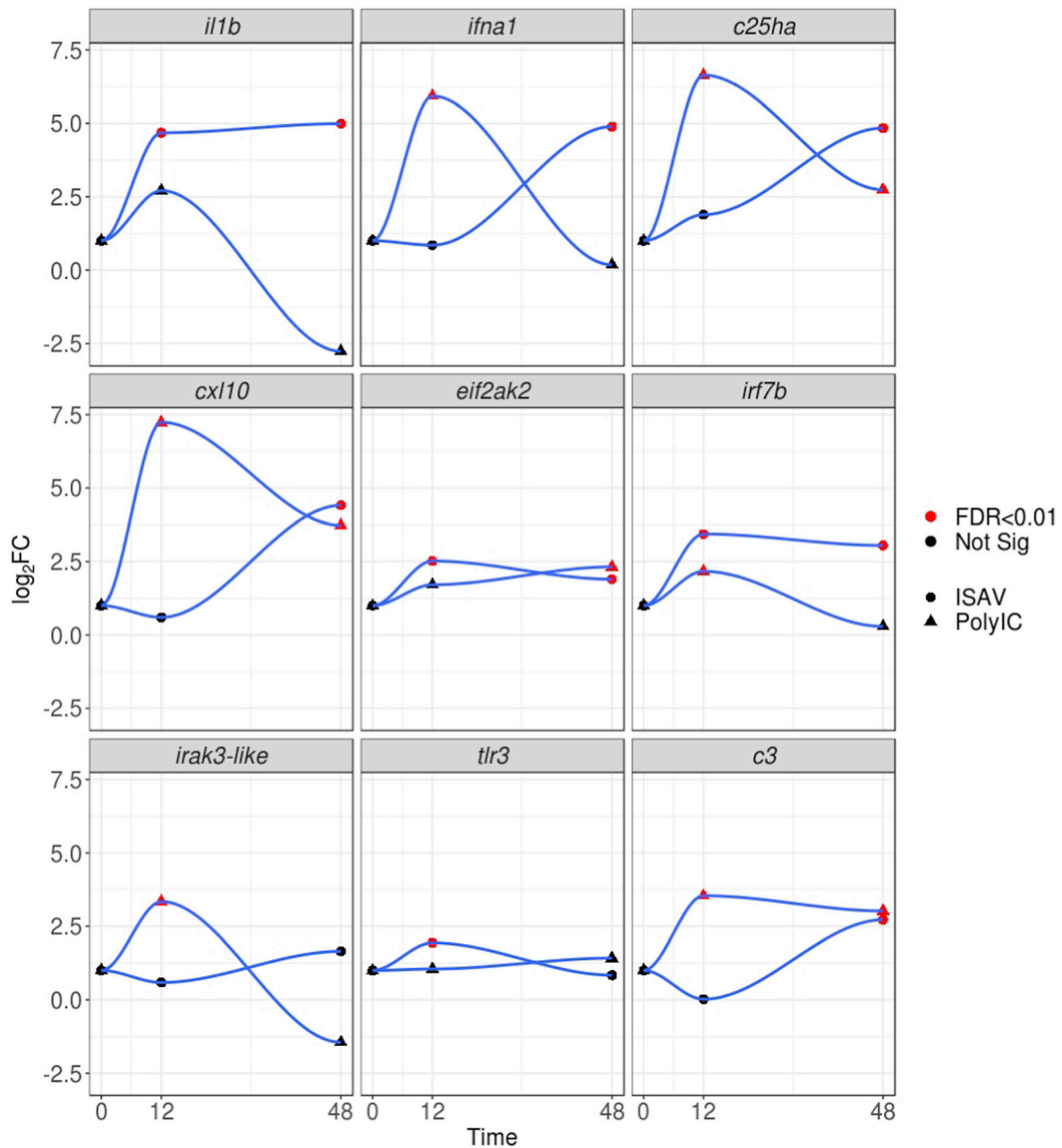


Fig. 4. Kinetics of selected DEGs dysregulated by the treatments. Cells ($n = 4$) were stimulated with poly (I:C) (PolyIC group) or infected with ISAV (ISAV group). Time points were 12 and 48 h after stimulation, plus a control non-stimulated group. DEGs are significant genes with median counts > 10 and $\text{padj} < 0.01$.

terms (Fig. 10), where terms involved with cytokine signaling, like “cytokine activity” and “chemokine activity” were among the upregulated terms.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed to identify to which pathways the significant DEGs belonged. The function `enrichKEGG` was used to perform pathway analysis. A q value cutoff of 0.01 was used to detect significant pathways, resulting in 30 pathways containing 2744 unique DEGs (Fig. 11). The same parameters as used for `enrichGO` were used for running `enrichKEGG` analysis, and a full table with all enriched pathways is provided (Supplementary Table 5). Important pathways related to innate immune response were enriched in the cluster of upregulated genes, as Toll-like, NOD-like and RIG-I-like receptor signaling pathways. Downregulated DEGs from P12 group were part of pathways involved in cell cycle, cellular senescence and some signaling pathways like “Wnt signaling pathway” and “TGF-beta signaling pathway”. P48

and V48 groups shared many of the pathways where their downregulated genes were clustered, as “Fatty acid metabolism”, “PPAR signaling pathway” and “Biosynthesis of unsaturated fatty acids”.

3.6. Validation of RNA-seq analysis by qPCR showed good correlation

To validate our RNA-seq analysis pipeline, the same RNA samples were subjected to qPCR analysis using a panel of primer pairs, showed on Table 1, covering low to high levels of expression in stimulated ASK cells. Fig. 12 shows a good agreement ($R^2 = 0.82$) between these two methods, suggesting satisfactory technical reproducibility of the analyses.

4. Discussion

RNA sequencing technology is improving the fundamental

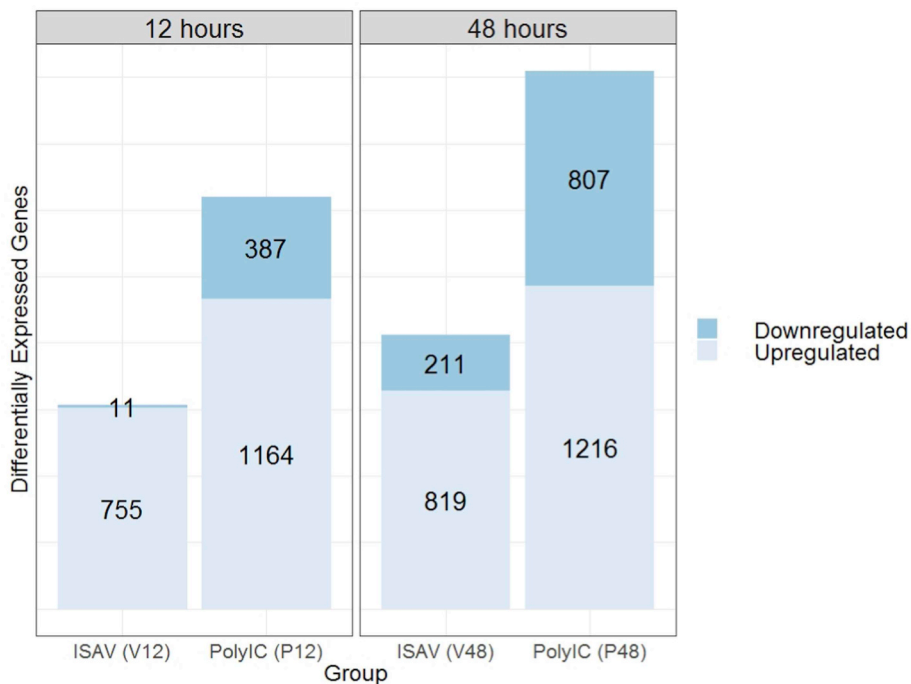


Fig. 5. Differentially expressed genes (DEGs). DEGs at 12 and 48 h in ASK cells stimulated with poly (I:C) (PolyIC group) or infected with ISAV (ISAV group). DEGs are significant genes with median counts > 10 and padj < 0.01. All groups with n = 4.

understanding of host immune response against pathogens as well as expanding our knowledge on how adjuvants work, which is obviously highly interesting for development of new vaccines that are both safe and result in higher protection. High-throughput sequencing has become the method of choice for transcriptome analysis, especially when using species with no reference genes sequences available. Transcriptome analysis in salmonids has specific issues, including incomplete annotation, imperfect genome assembly and high number of paralogs due to salmonid-specific whole-genome duplication around 90

mya (i.e. “4R”) (Allendorf, 1978; Macqueen and Johnston, 2014), making salmonids transcriptome analysis challenging.

In an effort to identify immune components involved in response to ISAV or synthetic dsRNA, we compared ASK cells response to these two stimuli at different time points using RNA sequencing. First a kinetic experiment was conducted with ASK cells stimulated for a range of sampling times (up to 96 h) and gene expression was measured by qPCR analysis for four genes involved in the immune response against virus: *ifna1*, *tnfa1*, *isg15* and *mx1*. With this analysis, we observed that some

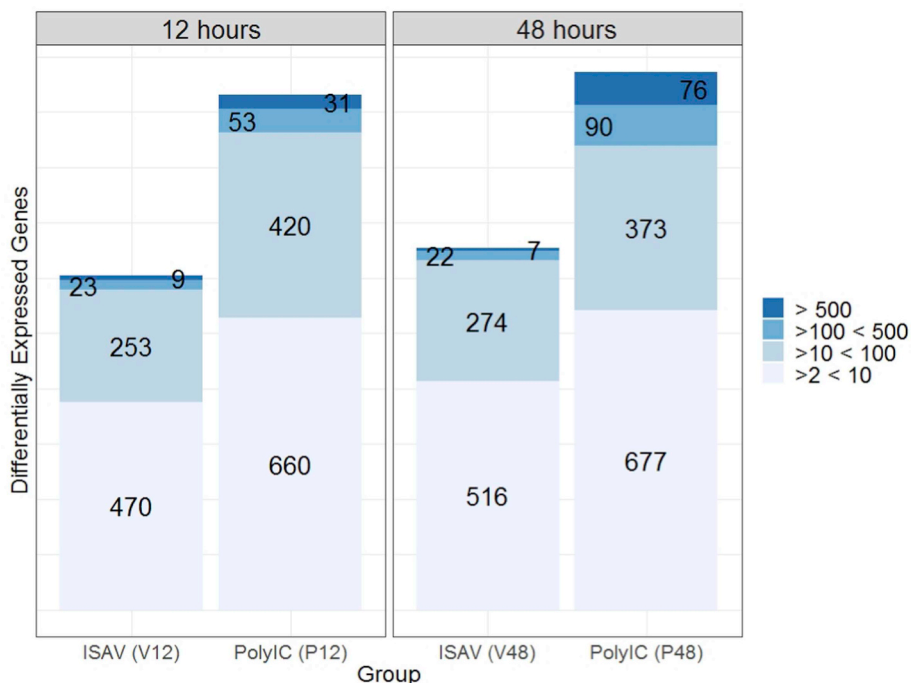


Fig. 6. Level of expression of upregulated genes. DEGs at 12 and 48 h in ASK cells stimulated with poly (I:C) (PolyIC group) or infected with ISAV (ISAV group). Relative expression (fold change) when compared with control cells. DEGs are significant genes with median counts > 10 and padj < 0.01. All groups with n = 4.

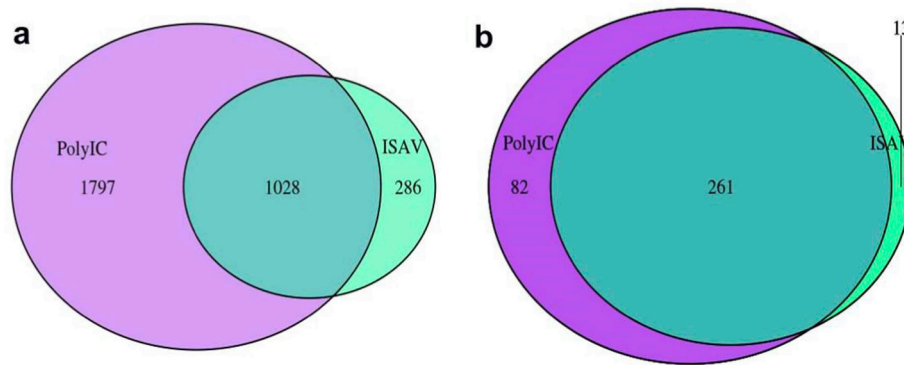


Fig. 7. Venn diagram. Venn diagram showing (a) DEGs from each group (PolyIC and ISAV) without taking time into consideration; (b) shows ISG identified in each of these groups. Poly IC (cells stimulated with poly (I:C)) and ISAV (cells infected with ISAV).

genes (*ifna1* and *tnfa1*) displayed different profiles being regulated early in PolyIC group and late in ISAV group. For the two other tested genes, *isg15* and *mx1*, both groups showed similar kinetics of regulation, but ISAV infection increased the levels of *isg15* and *mx1* transcripts as early as 3 h after stimulation, whereas the PolyIC treated cells showed a delayed response. Other studies have shown the same trend of expression in different Atlantic salmon head kidney cell lines (TO and SHK-1), where early ISAV infection resulted in induction of *mx1* and *isg15* genes through IFN-independent pathways, as their upregulation occurred before *ifna1* levels increased. In the same studies, poly (I:C) effects on the expression of these genes occurred after IFN upregulation (Jensen and Robertsen, 2002; Kileng et al., 2007).

With the purpose of gaining in-depth knowledge of the gene expression response and identifying genes being regulated by ISAV and/or poly (I:C), next-generation sequencing based transcriptome analysis was conducted with two time points, 12 and 48 h, for both groups, plus a common control. We identified a cluster of common host-response genes for both poly (I:C) stimulation and ISAV infection, among these were genes encoding chemokines (*cxcl1*, *cxcl10* and *ccl19*) PRR receptors (*tlr8*, *lgp2* and *nirc5*), many TRIM proteins (belonging to *trim25*, *trim39*, and *fintrim* subsets) and many typical ISGs like *mx*, *isg15*, *isg 56* and *rsad2/viperin*. Many of those genes, like *lgp2*, *nirc5* and ISGs, were also shown to be regulated by ISAV infection and extracellular poly (I:C) by others (Chang et al., 2011; Valenzuela-Miranda et al., 2015).

A strong initial IFN response in PolyIC group was observed, in agreement with previous reports by Zou and collaborators (Zou et al., 2014) where IFN upregulation was recorded as early as 2 h after stimulation. As a result of the magnitude of this initial response, negative cellular feedback loops control the expression of those immune genes to avoid undesired effects and to maintain internal immune homeostasis. Our results show that many genes were downregulated, when comparing to the basal level expression on the control group, at 48 h in both groups, but with more genes being downregulated at this time point in the PolyIC group, including *ifna*, what could indicate a way to prevent overstimulation. Cells also control innate immunity and inflammatory responses via induction of genes that inhibit immune responses. In a review article, Rebl and Goldammer (2018) discuss multiple genes controlling immune homeostasis in mammals, which have also been identified in teleost, many of them with multiple copies. Genes involved in controlling cytokine signaling were identified in all four groups, some being common to all four as *socs1* and *socs3* and others being exclusive to one group, like *irak3* in P12 group. These observations suggest that as poly (I:C) induces an early and strong stimulation of immune genes, cells countermeasure overexpression by upregulating genes involved in control of inflammation, probably to avoid cell damage. Studies with Atlantic salmon TO cells also reported highly upregulation of *socs1* and *socs3* after virus infection and type I IFN

treatment, this upregulation being attributed to regulation of negative feedback of IFN signaling in TO cells (Xu et al., 2015). Downregulation of immune genes, or upregulation of genes involved in modulation of cytokine signaling, may also be caused by the virus in an attempt to weaken the host defense. Viruses' ability to subvert hosts immune system has been shown by others both in mammals as well as in fish cell lines (García-Rosado et al., 2008; Jørgensen et al. (2007b); Mansfield et al., 2010; Pauli et al., 2008).

The innate immune system is the first line of defense against pathogens. It is highly conserved and constituted by lysozymes, complement, lectins, interferon, and pattern recognition receptors (PRR) among others (Medzhitov and Janeway, 2000; Rebl and Goldammer, 2018; Whyte, 2007). Poly (I:C) and viral dsRNA are recognized by TLR3, both in mammals and in *Salmonidae* (Alexopoulou et al., 2001; Vidal et al., 2015), by fish-specific TLR22 (Matsuo et al., 2008) and by RIG-I-like receptors (MDA5, RIG-I and LGP2/dhx58) in the cytoplasm, (Xu et al., 2016; Yoneyama and Fujita, 2008). The result of dsRNA recognition by these receptors trigger signaling pathways that lead to production of type I IFN induction, proinflammatory cytokines and induction of ISGs (Armeno et al., 2014; Gantier and Williams, 2007). Interestingly, genes encoding Mda5 and RIG-I receptors, *ifih1* and *ddx58*, respectively, were found upregulated by poly (I:C) stimulation at both time points, but in virus infected cells these genes were only upregulated at 12 h after infection. Many viruses encode proteins that directly interfere with RIG-I or MDA5 receptors to inhibit interferon production (Childs et al., 2009; Ling et al., 2009; Manokaran et al., 2015; Masatani et al., 2010), and NS1 protein from influenza virus has been shown to suppress RIG-I signaling by inhibiting TRIM25-mediated RIG-I CARD ubiquitination (Gack et al., 2009).

We also compared our list of salmon DEGs with lists of ISGs from studies in human and zebrafish (Levraud et al., 2019; Schoggins et al., 2011). As expected, most of salmon DEGs with ISG orthologs in human/zebrafish were upregulated by both treatments and well-known ISGs like *mx*, *isg15*, *rsad2*, multiple *trim* and *ifit* genes as well as RLRs (*ddx58*, *ifih1* and *dhx58*) were present in this list. Besides those genes that commonly appear in ISGs studies in fish, we found genes that are not regarded as ISGs, like *vegfc-like* (vascular endothelial growth factor C-like), or ISGs not frequently mentioned like *xaf1* (x-linked inhibitor of apoptosis protein (XIAP)-associated factor 1). XAF1 is a natural antagonist of apoptosis inhibitors, enhancing interferon-induced apoptosis (Jeong et al., 2018), and it has been shown to be upregulated by salmonid alphavirus in Atlantic salmon TO cells (Xu et al., 2015). VEGF is the principal proangiogenic factor responsible for enhancing formation of new blood vessels. It has been shown that virus like herpes simplex virus and dengue virus are capable of using host genes for angiogenesis both in tumor progression and disease pathogenesis (Alkharsah, 2018). Some viruses, like: Orf virus (infect sheep, goats and humans), white

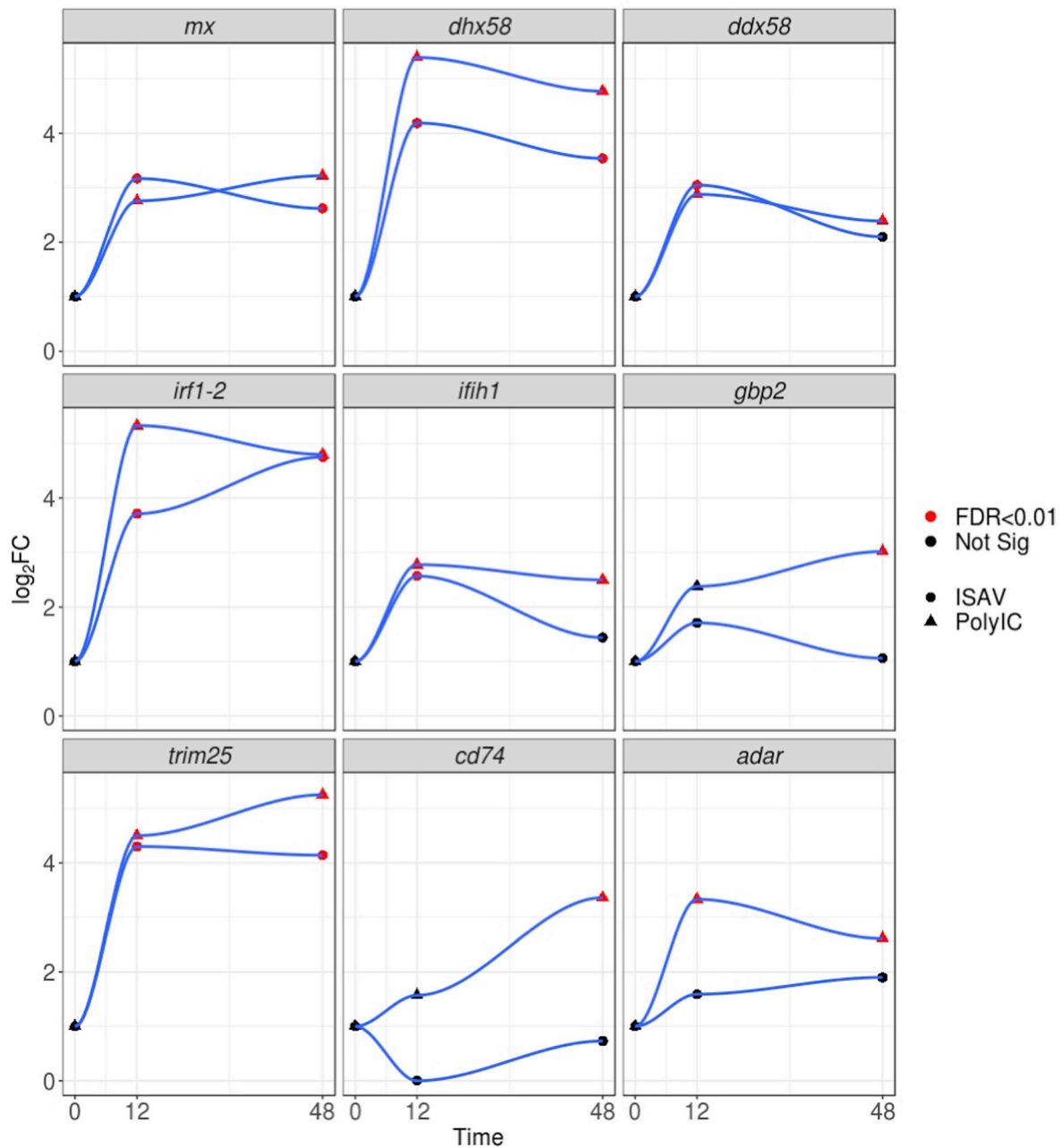


Fig. 8. Expression kinetics of selected interferon stimulated genes. Plots showing the kinetics of ISGs with known antiviral activity. PolyIC group – ASK cells were stimulated with poly (I:C) for 12 and 48 h; ISAV group – ASK cells infected with ISAV for 12 and 48 h. Significant genes with median counts > 10 and padj < 0.01. All groups with n = 4.

spot syndrome virus (infect crustacean) and infectious spleen and kidney necrosis virus (infect fish), have their own *VEGF-like* genes which can induce vascular proliferation and permeability, over-expression of host VEGF and regulation of virus propagation (Li et al., 2017; Mercer et al., 2002; Wang et al., 2008). For the ISGs with antiviral activity in human cells and zebrafish most of the genes were also upregulated in both groups and *ifit1* (*interferon-induced protein with tetratricopeptide repeats 1-like*) and *irf1-2* (*interferon regulatory factor 1 isoform 2*) among the most upregulated. *IFIT1*, also known as *ISG56*, recognize viral RNA and inhibit its translation (Diamond, 2014), while *IRF1* plays a broad function in a variety of biology processes and it participates in interferon signaling (Shi et al., 2011). Although most of the antiviral ISGs found in our results are the classic genes used to study

antiviral response in mammals and fish, like *trim25*, *mx*, *rig-I*, *irf7*, some genes like *jag 1* (*jagged canonical Notch ligand 1*), *nampt* (*nicotinamide phosphoribosyltransferase*), or even *map3k14* (*mitogen-activated protein kinase kinase kinase 14*) have not yet been fully characterized as antiviral ISGs in fish. Thus, our data provide a useful set of new genes that may be involved in antiviral response. What role, if any, these genes play in fish antiviral immunity is a subject for further research.

Induction kinetics by ISAV versus poly (I:C) in ASK cells was often contrasted, and follow a large number of different patterns for genes with typical, well known ISG orthologs in human and/or zebrafish. This is likely due to a combination of mechanisms regulating the expression of ISG: besides response to type I IFN, direct induction by the virus and specific (possibly cell type dependent) regulatory mechanisms also

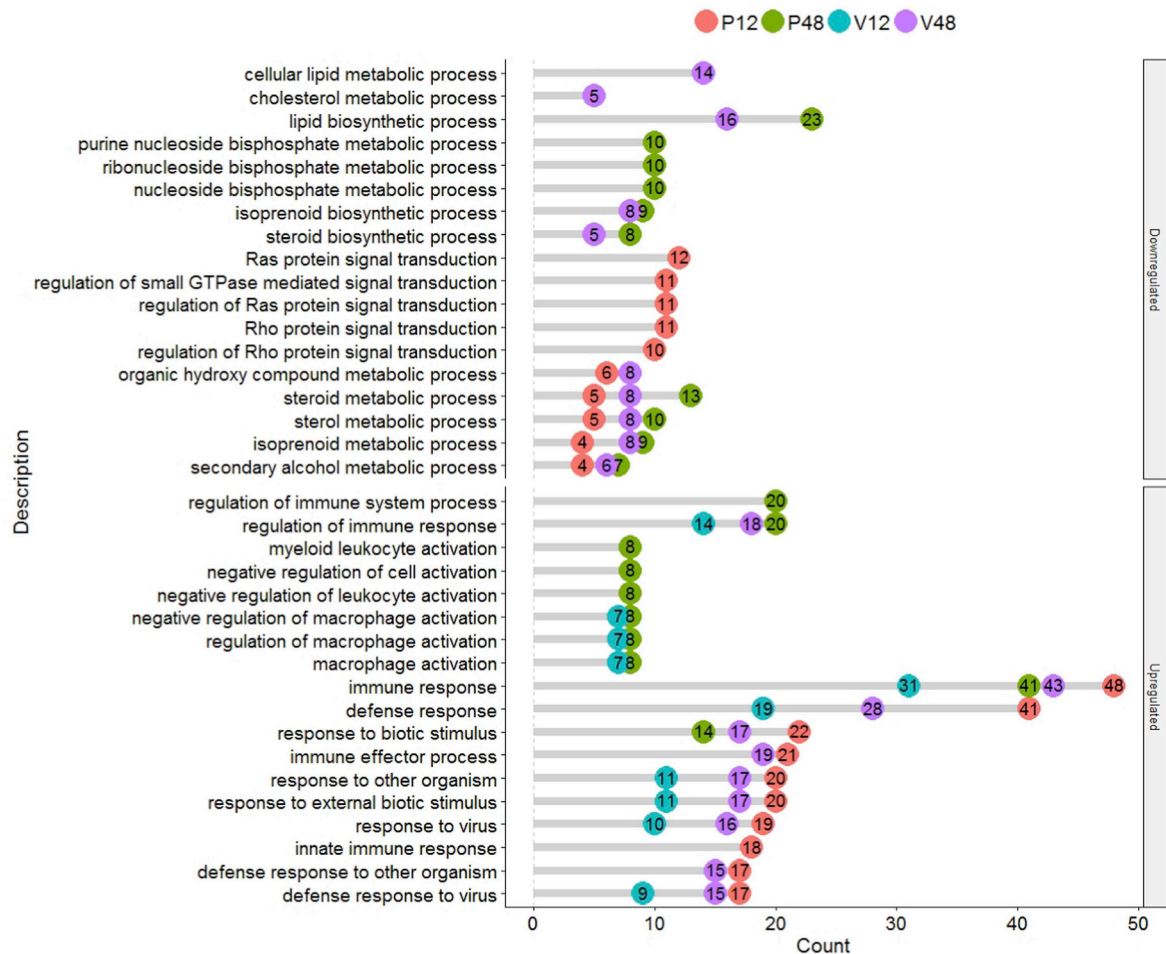


Fig. 9. Gene ontology - Biological process. Gene ontology enrichment analysis of the differentially expressed genes from ASK infected with ISAV (V12, V48) or stimulated with poly (I:C) (P12, P48) for 12 or 48 h. Top 10 terms for each group are shown. GO annotation based on *Salmo salar* OrgDb object. Dots are colored by group and numbers correspond to Count (number of DEGs in each term). Upregulated and downregulated genes are genes with $\text{Log}_2\text{FC} > 1$ or < -1 , respectively.

exert a control. With future high-quality annotation and assembly of the salmon genome, we believe that our data will provide an interesting basis to dissect the relative importance of these mechanisms.

TRIM (tripartite motif containing) proteins are a large family of proteins involved in numerous cellular processes as apoptosis, cell cycle regulation, transcriptional regulation and innate immune response (McNab et al., 2011). They share the capability to act as E3 ligases involved in the ubiquitylation cascade interfering with viral infection by directly targeting viral proteins for degradation (Meroni and Diez-Roux, 2005; Patil and Li, 2019) and they are also upregulated by the immunologically important Type I and Type II interferons (McNab et al., 2011). Versteeg and collaborators (Versteeg et al., 2013), cloned and analyzed all 75 known human TRIMs demonstrating that many, roughly half, of all human TRIMs are important regulators of the innate immune response and that they can act at different steps during the induction of IFN and proinflammatory cytokines. Among our DEGs we identified many TRIM proteins, with a total of 57 entries, and most of them present in multiple copies. It is not surprising that most of the TRIM proteins found among our DEGs are upregulated in both in poly (I:C) and ISAV infected cells, as members of TRIM family have been shown to exhibit antiviral activities (Uchil et al., 2008). It has been demonstrated that TRIM25 is essential not only for RIG-I ubiquitination but also for RIG-I-mediated interferon- β production and antiviral activity in response to RNA virus infection (Gack et al., 2007). Another study showed that TRIM39 play an important regulator on fish immune

response against viruses and cell cycle in *Epinephelus coioides*, overexpression of this TRIM protein was able to inhibit viral gene transcription *in vitro* (Wang et al., 2016). A large family of TRIM proteins are found only in teleost and named fintrims (as fish new TRIMs). They were first identified in rainbow trout as virus induced genes, suggesting antiviral activity (van der Aa et al., 2009). Since this first study many TRIM proteins have been found in different fish species and they contribute to the establishment of antiviral immunity also in fish (Langevin et al., 2017, 2019; Luo et al., 2017). A study comparing IFN versus viral stimulation in zebrafish showed many fintrim genes among the interferon-stimulated genes (Levrud et al., 2019).

When up and downregulated DEGs were further clustered, with significant enrichment analysis (GO and KEGG), based on functions and signaling pathways, we observed that downregulated genes from P12, P48 and V48 fit in with terms and pathways related to metabolism, especially fatty acid metabolism. Fatty acid synthesis and fatty acid oxidation play important roles in the immune system, while the first is involved in inflammatory responses, the latter is preferentially used by non-inflammatory and tolerogenic immune cells (O'Neill et al., 2016). It has been shown that viruses are capable to modulate host's lipid metabolism to increase its replication. Hepatitis C virus (HCV) is known to alter cell lipid metabolism to its own benefit and therapeutics that downregulate fatty acid synthesis can inhibit replication of some viruses (Gaunt et al., 2013; Poynter and DeWitte-Orr, 2016). Studies in mammals have shown that type I IFN response can downregulate lipid

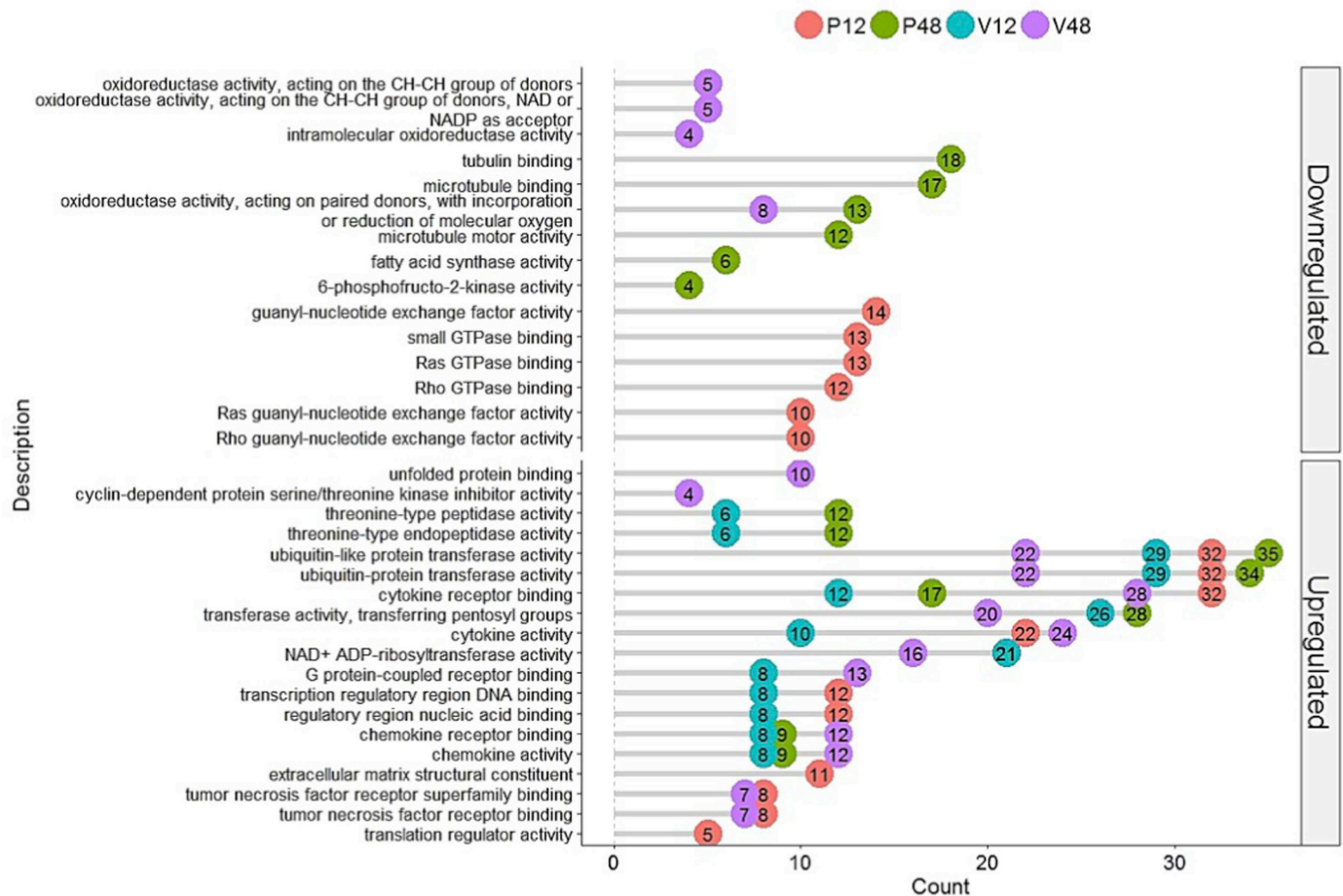


Fig. 10. Gene ontology - Molecular function. Gene ontology enrichment analysis of the differentially expressed genes from ASK infected with ISAV (V12, V48) or stimulated with poly (I:C) (P12, P48) for 12 or 48 h. Top 10 terms for each group are shown. GO annotation based on *Salmo salar* OrgDb object. Dots are colored by group and numbers correspond to Count (number of DEGs in each term). Upregulated and downregulated genes are genes with $\text{Log}_2\text{FC} > 1$ or < -1 , respectively.

metabolism and promote antiviral response (Blanc et al., 2011; York et al., 2015). However, in some cases, virus seems to promote down-regulation of lipid metabolism to stimulate replication. One study compared two strains of influenza virus and the results showed that only the NS1 protein of the highly virulent strain induced down-regulation of lipid metabolism. This may suggest that repression of lipid metabolism contribute to virulence of some viruses as transcription of certain lipid-based proinflammatory mediators were blocked (Billharz et al., 2009). In our data, among the few genes involved in lipid biosynthesis that were upregulated, *prostaglandin G/H synthase 2-like* (*ptgs2-like*) and *cholesterol 25-hydroxylase like protein* (*c25ha-like*) were identified. Cyclooxygenase-2, encoded by *ptgs2* gene is responsible for the formation of prostaglandins that are engaged in inflammatory responses, whereas C25ha protein has been shown to inhibit virus replication and to affect virus entry (Zhang et al., 2019). Alteration on fatty acid metabolism caused by ISAV and poly (I:C) can be a cellular mechanism to limit virus replication, as a robust IFN response and induction of certain ISGs can modulate cellular metabolism and exert antiviral activity by targeting different metabolic pathways (Raniga and Liang, 2018). More studies focusing on the correlation of metabolism and virus infection are necessary to better understand how these metabolic changes can affect immune responses against virus and stimulation by adjuvants.

Our kinetics analysis shows that both poly (I:C) and ISAV treatment induce many genes to react in a similar way and affecting mostly the same pathways. However, there are some important differences, as expression of some ISGs and PRRs, which demonstrate that poly (I:C) mimics certain but not all aspects of viral infection. Elucidation of these differences, especially when regarded to choosing time points to test adjuvants/antigens, will be highly valuable to development of new vaccines adjuvants. This study confirms that ASK is a responsive cell line that can be used not only for virus cultivation and titration but also as an *in vitro* model for studies of innate immune response in Atlantic salmon. It is invaluable to obtain a global gene expression profile of poly (I:C) stimulated Atlantic salmon cells to gain a systematic view of its application as adjuvant in vaccine formulations.

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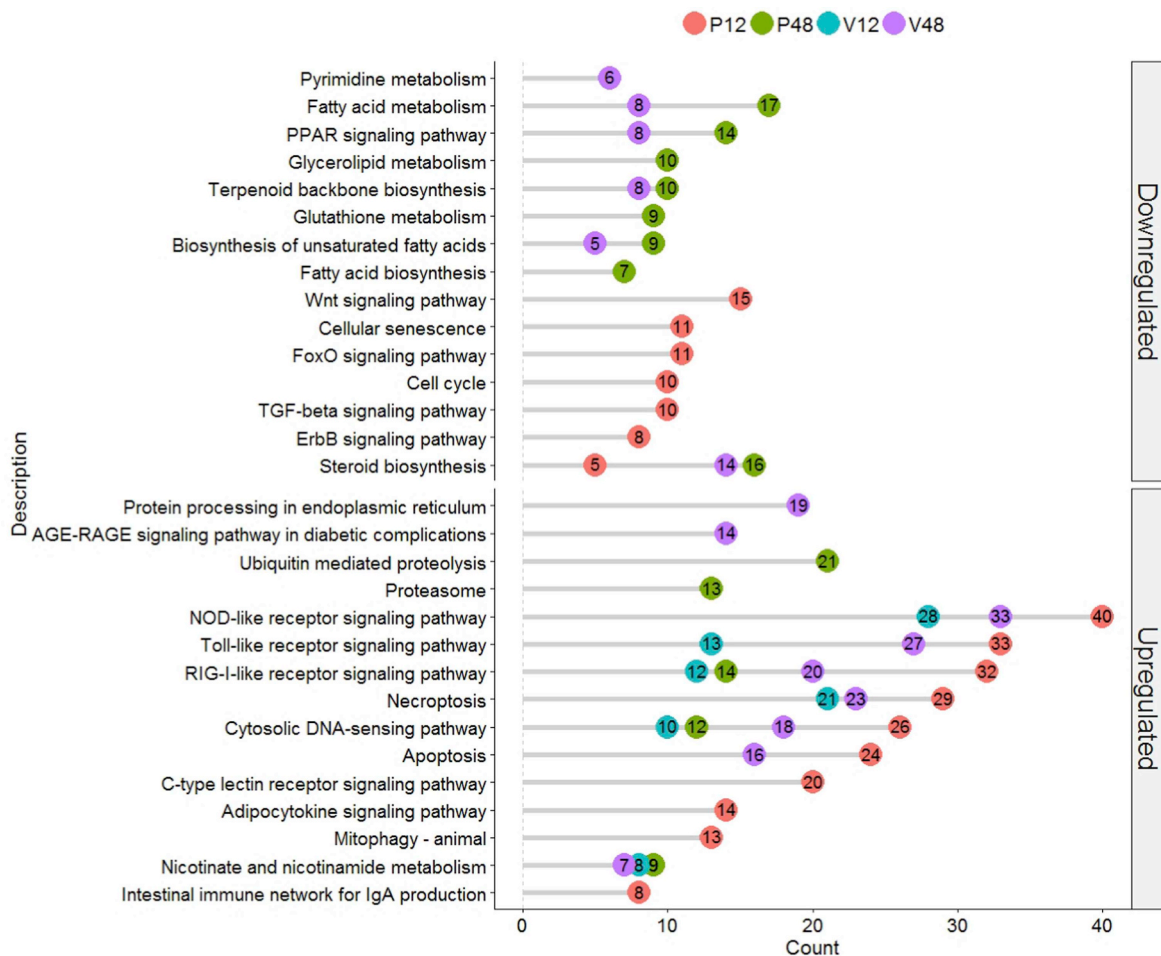


Fig. 11. KEGG analysis of the differentially expressed genes. KEGG pathways of DEGs from ASK infected with ISAV (V12, V48) or stimulated with poly (I:C) (P12, P48) for 12 or 48 h. KEGG pathways built based on *Salmo salar* database found at KEGG website. Dots are colored by group and numbers correspond to Count (number of DEGs in each term). Upregulated and downregulated genes are genes with $\text{Log}_2\text{FC} > 1$ or < -1 , respectively.

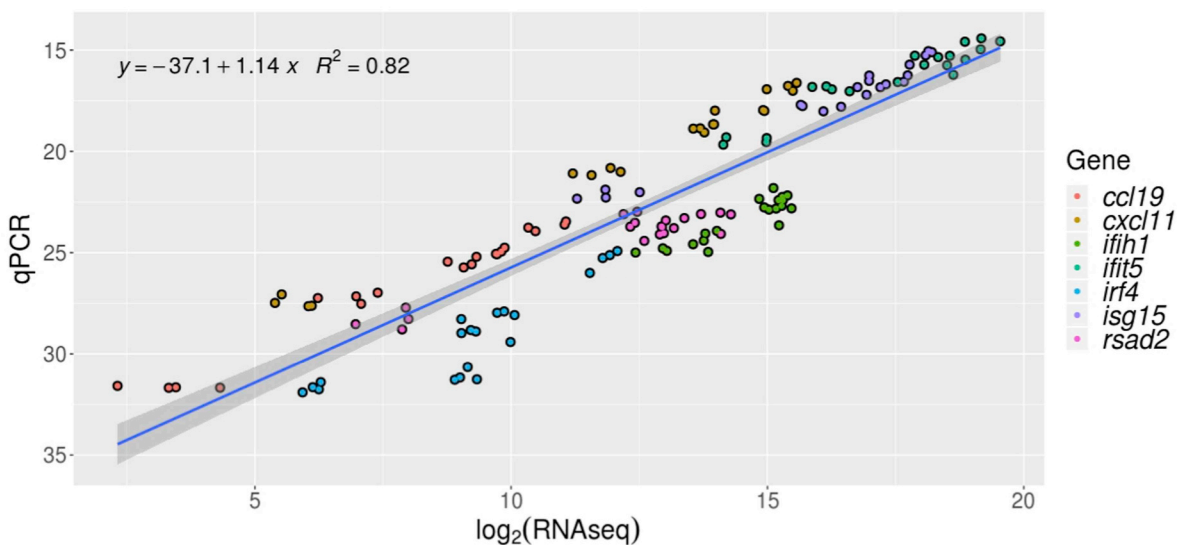


Fig. 12. Validation of RNA-seq analysis by qPCR. Regression analysis of log_2 transformed RNA-seq counts and qPCR Ct values for seven immune genes showed good agreement between the two types of analysis.

Author contribution

AA – study design, performing experiments, acquisition and analysis of data, writing software for statistical analysis and figures creation, writing manuscript.

PB – data analysis, phylogenetic analysis, writing manuscript.

TG - study conception and design, statistical analysis and interpretation of data, writing manuscript.

All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.dci.2020.103716>.

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