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Effect of eicosapentaenoic acid on innate immune responses in Atlantic salmon cells infected with infectious salmon anemia virus

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Abstract

Aquaculture is one of the world's fastest-growing sectors in food production but with multiple challenges related to animal handling and infections. The disease caused by infectious salmon anemia virus (ISAV) leads to outbreaks of local epidemics, reducing animal welfare, and causing significant economic losses. The composition of feed has shifted from marine ingredients such as fish oil and fish meal towards a more plant-based diet causing reduced levels of eicosapentaenoic acid (EPA). The aim of this study was to investigate whether low or high levels of EPA affect the expression of genes related to the innate immune response 48 h after infection with ISAV. The study includes seven experimental groups: ± ISAV and various levels of EPA up to 200 µM. Analysis of RNA sequencing data showed that more than 3000 genes were affected by ISAV alone (without additional EPA). In cells with increasing levels of EPA, more than 2500 additional genes were differentially expressed. This indicates that high levels of EPA concentration have an independent effect on gene expression in virus-infected cells, not observed at lower levels of EPA. Analyses of enriched biological processes and molecular functions (GO and KEGG analysis) revealed that EPA had a limited impact on the innate immune system alone, but that many processes were affected by EPA when cells were virus infected. Several biological pathways were affected, including protein synthesis (ribosomal transcripts), peroxisome proliferator activated receptor (PPAR) signaling, and ferroptosis. Cells exposed to both increasing concentrations of EPA and virus displayed gene expression patterns indicating increased formation of oxygen radicals and that cell death via ferroptosis was activated. This gene expression pattern was not observed during infection at low EPA levels or when Atlantic salmon kidney (ASK) cells were exposed to the highest EPA level (200 µM) without virus infection. Cell death via ferroptosis may therefore be a mechanism for controlled cell death and thus reduction of virus replication when there are enough polyunsaturated fatty acids (PUFAs) in the membrane.

Keywords Atlantic salmon, Polyunsaturated fatty acid, Eicosapentaenoic acid, Virus, Infectious salmon anemia virus, Transcriptomics, Viral disease, Ferroptosis

Introduction

Since the first reports of an Atlantic salmon anemia disease and the identifications of the causative agent, infectious salmon anemia virus (ISAV) [1], this virus has created serious health and fish welfare problems on both sides of the Atlantic [2, 3]. Using strict management procedures, the initial wave of outbreaks was reduced [4] and vaccines have been developed [5], but this disease is still a serious problem for the salmon aquaculture industry.

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ISAV belongs to the Orthomyxoviridae negative sense segmented RNA viruses which also includes the influenza genera [6]. Detailed studies of host and tissue tropism [2, 7, 8], uptake [9, 10], replication [11, 12] as well as innate [13–15] and adaptive immune responses during ISAV infections [16–19] have been reported but many questions regarding virus-host interactions remains to be investigated. The recent publication of the first reverse genetics system for ISAV will certainly open new avenues for deeper molecular characterization and vaccine development [20]. Another important area of research into virus-host interactions is the role of dietary and cellular fatty acids on innate and adaptive immune responses during infection. Results from both experimental [21–25] and clinical studies [26–29] suggest that polyunsaturated fatty acids (PUFA) like eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) and their metabolites play important roles in host responses against a range of infections [30]. This focus on the interplay between metabolism and immunity have led to development of a branch of immunology called immunometabolism [31] and many of these insights are relevant for aquaculture as much as the feed is the one of the main elements in the production chain of fish [32]. As in mammals, multiple studies suggest a role for PUFAs in the maintenance of growth and health in Atlantic salmon [33–35] as well as immunity [36–38]. To protect limited marine raw materials (like herring and capelin) for salmon feed production, plant and algae based raw materials with lower PUFA levels are taking over [32]. Although long chain PUFAs like EPA and DHA have been regarded as essential for optimal growth and development in vertebrates [33], the dietary demand for EPA in Atlantic salmon has recently been questioned [39]. Recent findings concerning the intersection of energy metabolism with innate immunity to viral infections like the interaction of *STING* (stimulator of interferon genes) with *FADS2* (fatty acid synthase 2) [40] and the role of lactate in regulation of MAVS (mitochondrial antiviral signaling protein) [41] suggest that dietary lipids play a role in innate immunity. Likewise, expression levels of an enzyme involved in production of endogenous fatty acids (oleoyl-acyl-carrier-protein (ACP) hydrolase (OLAH)) was associated with severity of multiple viral respiratory functions via effects on macrophage lipid droplet dynamics [42]. The regulated cell death pathway named ferroptosis [43] occurring during various forms of viral infections [44] have also been linked to the level of cellular PUFAs. These recent developments incited us to investigate the role of EPA in antiviral immunity in Atlantic salmon kidney (ASK) cells. The cellular levels of EPA may affect antiviral signaling responses in at least three separate ways. Firstly, as ligands for peroxisome proliferator-activated receptors (PPARs) or G-protein coupled receptor 120 (GPR120) [45]. Secondly, as metabolic precursors of immune modulators like resolvins and eicosanoids [46] and lastly, by altering the composition of membrane microdomains called "rafts" where membrane bound signaling proteins like toll-like receptors (TLRs) and MAVS anchor and signal from [47, 48]. Previous studies of PUFA effects on innate immunity in tissues or cells from Atlantic salmon are not conclusive as EPA may confer detrimental [33, 49], neutral, [50, 51] or supportive [52] effects, depending on developmental stage and type of stressor. To gain a more mechanistic view of the interplay between EPA and innate immunity to viral infection in Atlantic salmon, we measured transcriptional responses to ISAV infection at five different cellular EPA levels. One of the main findings not observed with virus or high EPA alone (only in combination) were the enrichment of transcripts related to the ferroptosis and PPAR pathways. This may suggest that the combined stress of high PUFA and viral infection initiates iron dependent lipid peroxide formation and cell death as a host defense mechanism to control viral replication.

Materials and methods

Cell culture

Knut Falk (Norwegian Veterinary Institute) kindly provided the Atlantic salmon kidney (ASK) cell line used in this project. The cells were cultivated at 20 °C and split (1:2) once a week. The cell media consisted of Leibovitz L-15 medium (Lonza BioWhittaker, Verviers, Belgium) supplemented with L-glutamine (4 mM—Lonza BioWhittaker, Verviers, Belgium), fetal bovine serum (10%—Gibco, Life Technologies, Bleiswijk, The Netherlands), 2-mercaptoethanol (40 µM—Gibco, Life Technologies, Bleiswijk, The Netherlands) and gentamicin (50 mg/mL—Lonza BioWhittaker, Walkersville, USA).

The cells were acclimatized one week before the experiment started; the cultivation temperature was reduced to 15 °C, and the content of fetal bovine serum in the media was reduced to 2%. These conditions were also used during the experimental period.

Virus propagation

The ISAV strain used in this experiment was Glesvær 2/90, which has been shown to result in high mortality in Atlantic salmon [53]. The virus was produced and isolated as described by Andresen et al. [54].

Experimental design

ASK cells (passages 40–50) were seeded in 14 wells (35 mm, 6-well plates), with a density of 1.5×10^5 cells per well. The cells were cultivated overnight (15 °C) for adhesion. Thereafter, EPA (Sigma-Aldrich, St. Louis, MO,

USA) (bound to BSA) of different concentrations (0, 25, 50, 100, 200 µM) was added to the wells ($n=2$ for 25, 50 and 100 µM, $n4$ for 0 and 200 µM), and the cells were incubated for 7 days. The wells were washed three times with sterile PBS (QIAGEN, Hilden, Germany), before performing the in vitro infection.

A virus suspension with a multiplicity of infection (MOI) of 1 in serum-free L-15 medium was added to 10 of the wells (0, 25, 50, 100, 200 µM). The extra wells without EPA ($n=2$) and with 200 µM EPA ($n=2$) were used as uninfected controls. The infected wells were incubated for 4 h to allow for virus adsorption, followed by addition of previous culture medium (L-15 supplemented ± EPA).

The cells were incubated for 48 h post-infection (9 days of cultivation in total), then washed three times with PBS, lysed using buffer RLT (QIAGEN, Hilden, Germany) and stored at −20 °C until RNA isolation. This experiment was repeated three times, which resulted in six technical replicates per sample ($n=6$), and 42 samples in total.

Fatty acid analysis

ASK cells were grown in flasks (75 cm^2) with L-15 supplemented medium (2% FBS) and EPA (0, 25, 50, 100, 200 µM) for one week. The total lipids of the cells and cell culture media were extracted as described by Folch et al. [55], by homogenizing the tissue with 2:1 chloroform–methanol (v/v). The chloroform phase was isolated, and nitrogen was used to evaporate the solvent, resulting in the residual lipid extract. Benzene was used to re-dissolve the lipids, and 2,2-dimethoxypropane and methanolic HCl were added for transesterification overnight at room temperature, as described by Mason and Waller and by Hoshi et al. [56, 57]. A gas chromatograph (Hewlett Packard 6890) with helium as a carrier gas, a split injector, a SGE BPX70 capillary column (length: 60 m, internal diameter: 0.25 mm, thickness of film: 0.25 µM), a flame ionization detector (FID) and the HP Chem Station software was used to separate the fatty acid methyl esters and monitor the process. The detector and injector of the chromatograph had a temperature of 300 °C, while the oven temperature was raised from 50 to 170 °C (4 °C / min) and then further raised to 200 °C (0.5 °C /min).

Peaks appeared in the chromatogram as the different compounds eluted from the column and passed through the detector. Individual fatty acid methyl esters were identified by reference to well-characterized standards. The relative amount of each fatty acid was expressed as a percentage of the total amount of fatty acid in the analyzed sample, and the absolute amount of fatty acid per gram of tissue was calculated using C23:0 methyl ester as the internal standard.

Total RNA isolation

Total RNA was extracted for sequencing and qPCR using RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's tissue protocol, with an optional on-column DNase I digestion to remove gDNA (RNase-Free DNase Set, QIAGEN, Hilden, Germany). The RNA samples were eluted in 50 µL RNase free distilled water, and the RNA concentrations were measured using a PicoDrop Pico100 (PicoDrop Technologies, Cambridge, UK).

RNA sequencing

The RNA samples ($n=42$) were sent to the Norwegian Sequencing Centre (NSC). The RNA qualities were checked using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA), confirming sample quality (RIN > 8) and purity (no additional peaks). The cDNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina Inc., San Diego, USA) and sequenced to 150 bp paired end reads with the Illumina HiSeq 4000 sequencer.

Qualitative PCR (qPCR)

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States) was used to make cDNA, using the manufacturer's protocol. The LightCycler 480 and SYBR Green Master Mix (both from Roche Diagnostics, Basel, Switzerland) was used to perform qPCR in 96-well plates. The initial heating lasted for 5 min (95 °C), and the cycling conditions (40 cycles) were 95 °C (10 s), 60 °C (10 s), and 72 °C (10 s), and the melting curves were measured at 95 °C (5 s) and 65 °C (1 min). The qPCR experiment was repeated three times with two technical replicates per sample. The cycle threshold (Ct) values of the samples were obtained to calculate the relative expression levels of the genes (delta-delta Ct method) [58], with *18S* and *ef1a* as reference genes [59]. The primers used are listed in Table 1.

Bioinformatics and statistics

HISAT2 [65] was used to map the FASTQ sequence read files from the RNA sequencing to the Atlantic salmon genome (GCF_000233375.1_ICSASG_v2_genomic.fna). The existing Atlantic salmon annotation file (GCF_000233375.1_ICSASG_v2_genomic.gff) was used to assemble the transcripts with StringTie [66]. Both the genome and the annotation file were downloaded from NCBI (annotation release 100). Following the alignment and assembly of transcripts, the R software package DESeq2 (version 1.40.2) was utilized to quantify the differential expression of genes between the samples and against the controls [67, 68]. The resulting gene expression tables were filtered using a threshold of median > 10, to remove genes that had zero or low

Table 1 List of primers used in qPCR analysis

Genes	Direction	Sequence 5′ → 3′	Accession number	Amplicon	Melting temp [°C]	References
ef1a	F	CACCACCGGCCATCTGATCTACAA	AF321836	77	58.8	[60]
	R	TCAGCAGCCTCCTTCTCGAACTTC			60.6	
18S	F	TGTGCCGCTAGAGGTGAAATT	AJ427629.1	61	54.8	[61]
	R	GCAAATGCTTTCGCTTTCG			52.9	
ifna1	F	CCTGCCATGAAACCTGAGAAGA	AY216594	107	55.4	[59]
	R	TTTCCTGATGAGCTCCCATGC			55.1	
isg15	F	ATGGTGCTGATTACGGAGCC	AY926456	151	54.2	[62]
	R	TCTGTTGGTTGGCAGGGACT			53.9	
mx1/2	F	TGATCGATAAAGTGACTGCATTCA	NM_001123690.1/ NM_001123693.1	80	54.8	[63]
	R	TGAGACGAACTCCGCTTTTTCA			55.9	
ifih1	F	GAGAGCCCGTCCAAAGTGAA	XM_014164134	389	53.9	[54]
	R	TCCTCTGAACTTTCGGCCAC			53.9	
ISAVseg5	F	GAAAGCCCTGCTCTGGC	HQ259675.1	50	51.8	[64]
	R	TCCTCAAGTCTGCTTCGGGA			55.2	
ISAVseg6	F	AGGCCAAAAACGGAAATGGA	HQ259676.1	118	51.6	[64]
	R	CCGTCAGTGCAGTCATTGGTT			54.9	
ISAVseg7	F	GAAATGGACAGAGACGGCGTATCA	HQ259677.1	124	57.9	[64]
	R	GCTCAACTCCAGCTCTCTCATTGT			59.0	

ef1a—elongation factor 1 alpha, *18S*—18S ribosomal RNA, *ifna1* – interferon alpha-1, *isg15*—interferon-stimulated gene 15, *mx1/2*—interferon-induced GTP-binding protein Mx1/2, *ifih1*—interferon induced with helicase C domain 1, *ISAVseg5* – infectious salmon anemia virus gene segment 5, *ISAVseg6* – infectious salmon anemia virus gene segment 6, *ISAVseg7* – infectious salmon anemia virus gene segment 7

counts. The adjusted p-value (padj) was calculated using the Benjamini-Hochberg (BH) procedure [69], and the genes classified as differentially expressed genes (DEGs) had a p-value (padj) below 0.01. A gene was considered upregulated if the log2 fold change (Log2FC) > 1, and downregulated if Log2FC < −1. The sample analysis and the exploratory plots are shown in Supplementary file 1. The gene ontology and KEGG pathway analysis was performed using the R package clusterProfiler [70], with 0.01 as a cutoff for the p-values and q-values (BH adjusted). The KEGG pathway maps were visualized using the R package Pathview [71].

Results

Verification of infection

To verify that cells were infected and responsive before submitting the samples for sequencing, we analyzed a few well known viral and interferon induced salmon transcripts by qPCR [54]. Figure 1 shows that all four transcripts were robustly upregulated by ISAV at 48 h p.i. suggesting that the cells were indeed infected. We also confirmed infection by qPCR of three ISAV genomic RNA segments, and the cells were positive for all (not shown). Figure S8 shows cellular morphology at various time points after infection with ISAV. The cytopathic effect begins to appear at day 3, so RNA samples were isolated at day 2 to avoid the presence of too many dead cells in the samples.

Fatty acid analysis

The EPA content (%) of cells and cell culture media is presented in Supplementary file 1 (Fig. S1). The cellular content of EPA increased from 2% when no EPA was added, to 21% of total fatty acids when EPA was supplemented at 200 μM.

RNA sequencing

An exploratory analysis of the RNA-seq raw data is presented in Supplementary file 1 (Fig. S2-S7). Briefly, the samples were sequenced to a depth of about 15–20 million reads (150 nt, double reads) with a mapping frequency to the Atlantic salmon genome of about 80%. Counts were log normally distributed and tests of replicate correlation showed good agreement between technical replicates. PCA and clustering analysis suggested that ISAV infection was the main driver of variability, but EPA also displayed an effect. One of the samples had corrupted sequencing files and were removed from further analyses (EPA, 50 μM, replicate 6).

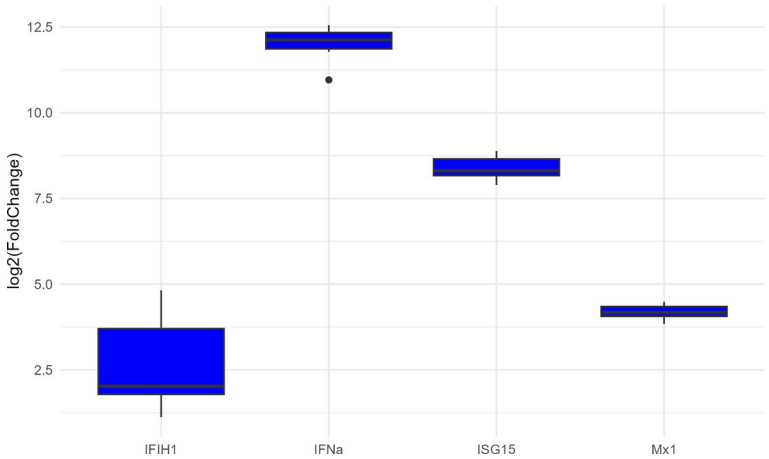

Fig. 1 Relative expression of interferon induced with helicase C domain 1 (IFIH1), interferon alpha-1 (IFNa), interferon-stimulated gene 15 (ISG15) and interferon-induced GTP-binding protein Mx1 (Mx1) in infected control vs. non-infected control (0 µM EPA). Expression level is calculated as relative expression to two housekeeping genes (ef1a—elongation factor 1 alpha and 18S—18S ribosomal RNA) using the delta-delta Ct method. Data are displayed as median (horizontal line), 25 and 75% percentiles (box) and 5 and 95% percentiles (whiskers) log2 fold change. All four transcripts were significantly different from control (Wilcoxon rank sum test, $p < 0.01$, $n = 6$)

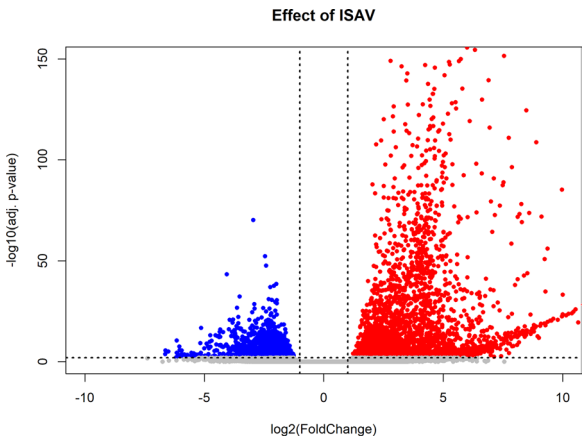

Fig. 2 Volcano plot of DEGs in ASK cells 48 h after infection with ISAV. Red dots are upregulated (2090 transcripts), blue dots are downregulated genes (1331 transcripts). Grey dots are not significantly changed (adjusted *p*-value > 0.01)

Transcriptome effects of ISAV infection and EPA alone

As previously shown by Andresen [54], infection of ISAV in these cells had strong transcriptional effects with more than 3000 genes dysregulated at 48 h p.i. (Fig. 2).

This virus elicits expression of transcripts that were enriched in antiviral and immune system responses related to biological processes and pathways (Fig. 3). All significantly affected genes are listed in Supplementary 2.

As a control, the effects of only 200 µM EPA for 9 days without virus was also analyzed. Only 268 transcripts were moderately differentially expressed in this group, suggesting that EPA treatment alone did not stress the cells (not shown).

Transcriptome effects of increasing levels of EPA during ISAV infection

When cells exposed to increasing levels of EPA (0–200 µM) for 7 days were infected with ISAV, a total of 2921 transcripts were affected by the levels of this fatty acid (adjusted p-value < 0.01) (Supplementary 2). Compared to the effects of virus alone, the changes in expression levels were modest but many transcripts displayed a clear dose response to EPA levels (Figs. 4, 5). Transcripts that showed a positive correlation to EPA levels included antimicrobial peptides (cathelicidin), *MMP9*, and *ARF4* among others. Transcripts with a negative correlation to cellular EPA levels included GTP binding signaling proteins, thrombospondin, and thioredoxin interacting protein.

The overlap of transcripts between DEGs induced by ISAV alone and DEGs affected by the level of EPA was limited (Fig. 6). Only 120 of 3421 transcripts changed by viral infection were modulated by the cellular levels of EPA, suggesting that alternative signaling pathways were activated with increasing levels of the fatty acid.

When analyzing the levels of viral transcripts (ISAV segments 5, 6, and 7) at 48 h p.i. we did not observe a significant effect of EPA levels on viral replication (Fig. 7).

KEGG pathway enrichment analysis with gene sets that were affected by EPA revealed that processes related to protein synthesis, amino acid, RNA metabolism, PPAR pathway, fatty metabolism, and ferroptosis were enriched with transcripts stimulated by EPA (Fig. 8). Cell cycle,

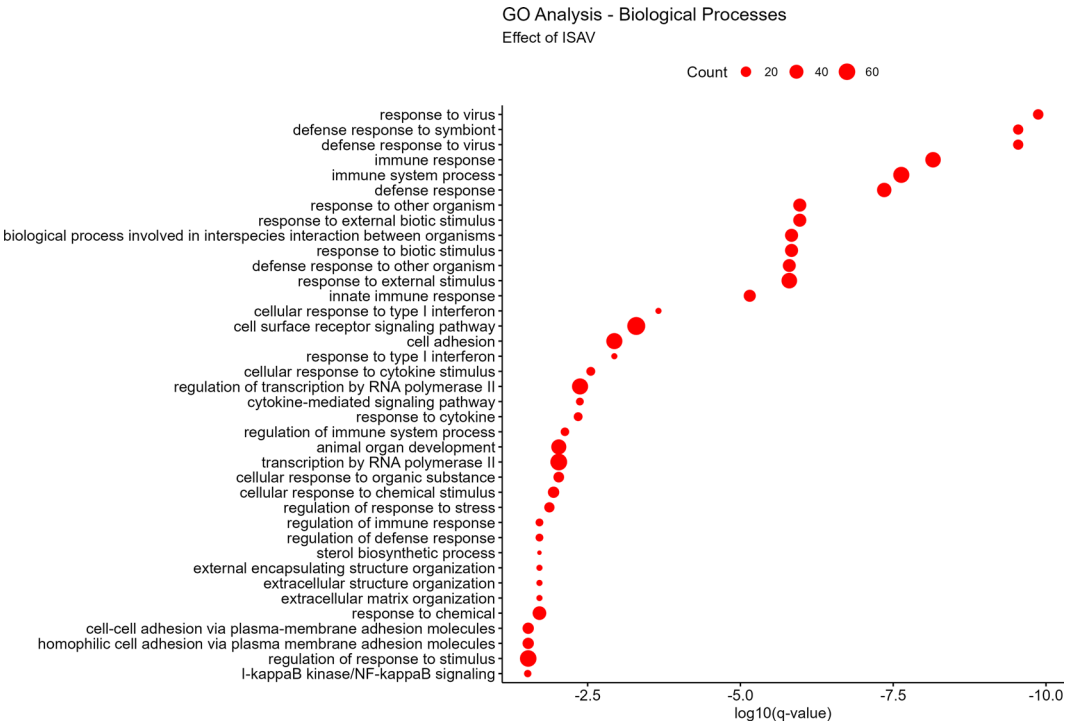

Fig. 3 GO enrichment analysis of DEGs in ISAV infected ASK cells

p53 pathway, and TGF-beta signaling pathways were enriched with transcripts inhibited by EPA.

Taking a closer look at the affected transcripts in the PPAR pathway revealed that all transcripts connected to this pathway were upregulated by EPA. Target genes for *PPAR-alpha, -delta, and -gamma* were affected by EPA in ISAV infected cells (Fig. 9).

Enrichment of transcripts in the ferroptosis pathway was another interesting feature of transcriptional changes observed with increasing EPA levels not seen in control cells infected with ISAV. Ferroptosis is a regulated iron dependent cell death pathway characterized by reduced antioxidant capacity, accumulation of lipid peroxides, and reactive oxygen species [43]. High levels of ferritin combined with reduced levels of the system Xc^- subunit *SLC7A11* (transporter for glutathione precursor cysteine) and glutathione peroxidase 4 (*GPX4*) was observed in EPA treated cells infected with ISAV, which may trigger activation of this pathway (Fig. 10).

Discussion

Recent developments in the understanding of the interplay between dietary fatty acids and the immune responses at the cellular and organismal levels [40, 72, 73] requires further investigations of these relationships in farmed aquatic animals where feed is one of the main material factors [32, 74]. Previous reports suggest that salmon feeds with higher levels of EPA may confer a protective effect against viral [75, 76] and bacterial infections [77]. However, the minimal dietary requirements for EPA in salmon feed are not firmly established and may be dependent on developmental stage and other environmental factors [78–80]. Some reports conclude that EPA is not essential for normal health and growth of Atlantic salmon [39]. Nevertheless, that study was a short-term study (14 weeks) and used small fish of approximately 53 g at the beginning and concluded when fish reached approximately 200 g at the end of the feeding trial. Further, prior to the experiment a commercial diet was used. Commercial diets at this life stage are typically rich in n-3 VLC-PUFAs and this may have provided enough n-3 PUFAs to be sustained by the fish during the short feeding trial.

In this report we have studied the transcriptomic responses to ISAV in salmon cells under various cellular levels of EPA (from 2 to 21% of total fatty acids). We first confirmed the infection model by qPCR of established interferon regulated transcripts and viral genomic segments and found that the cells were robustly infected. RNA-seq analysis also confirmed

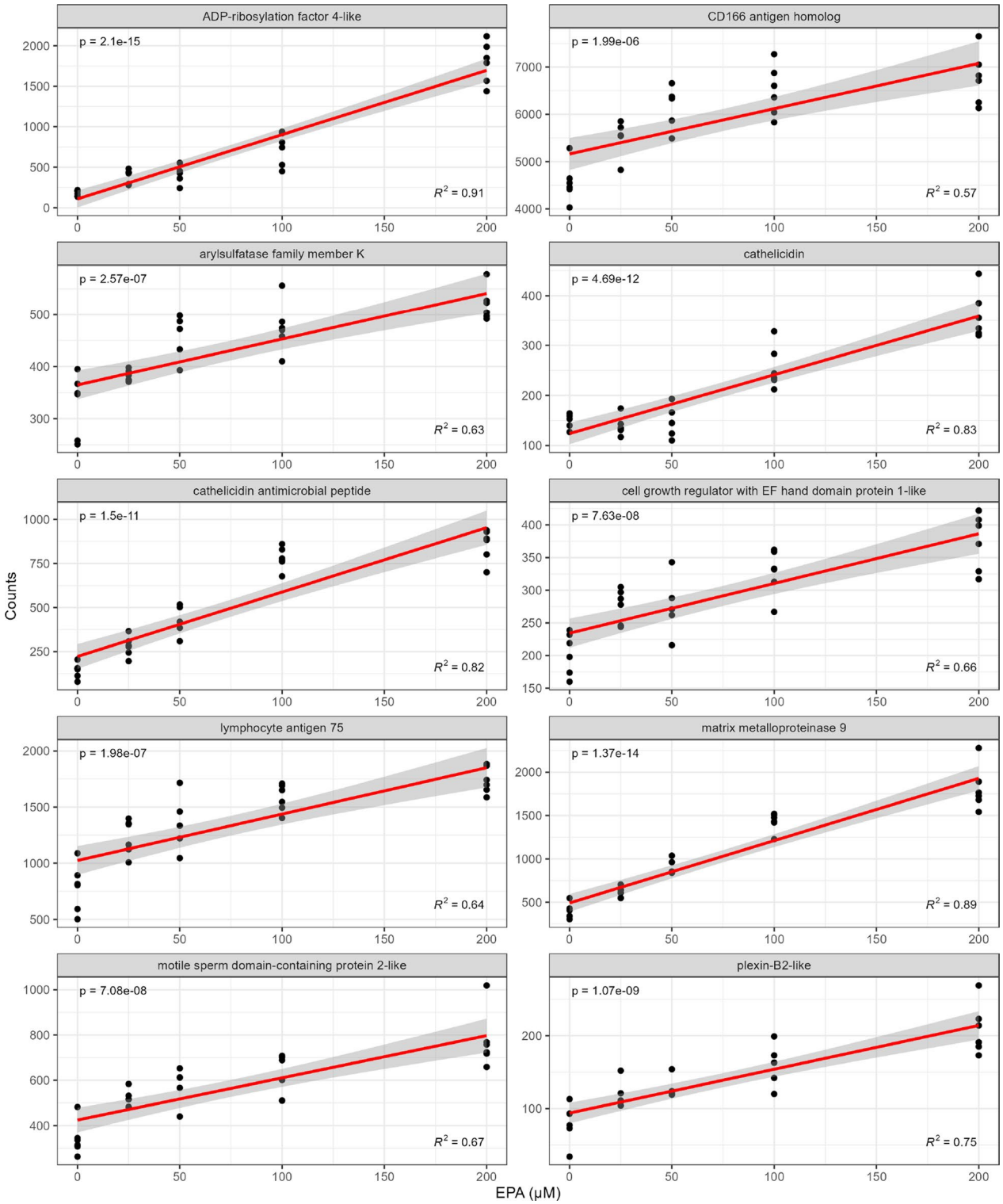

Fig. 4 Plot of raw counts and best fit line for the transcripts most positively correlated to EPA concentration in ISAV infected ASK cells

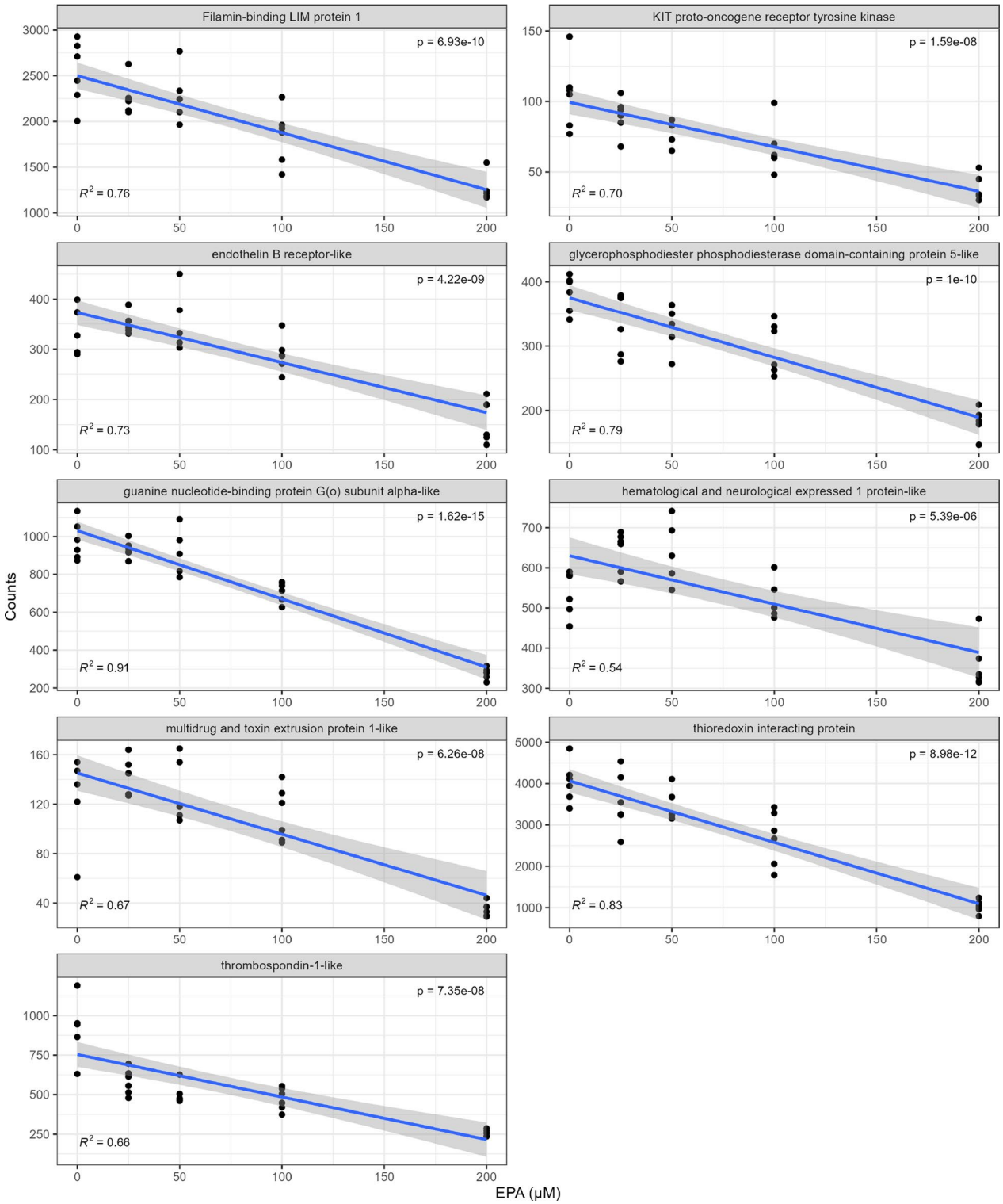

Fig. 5 Plot of raw counts and best fit line for the transcripts most negatively correlated to EPA concentration in ISAV infected ASK cells

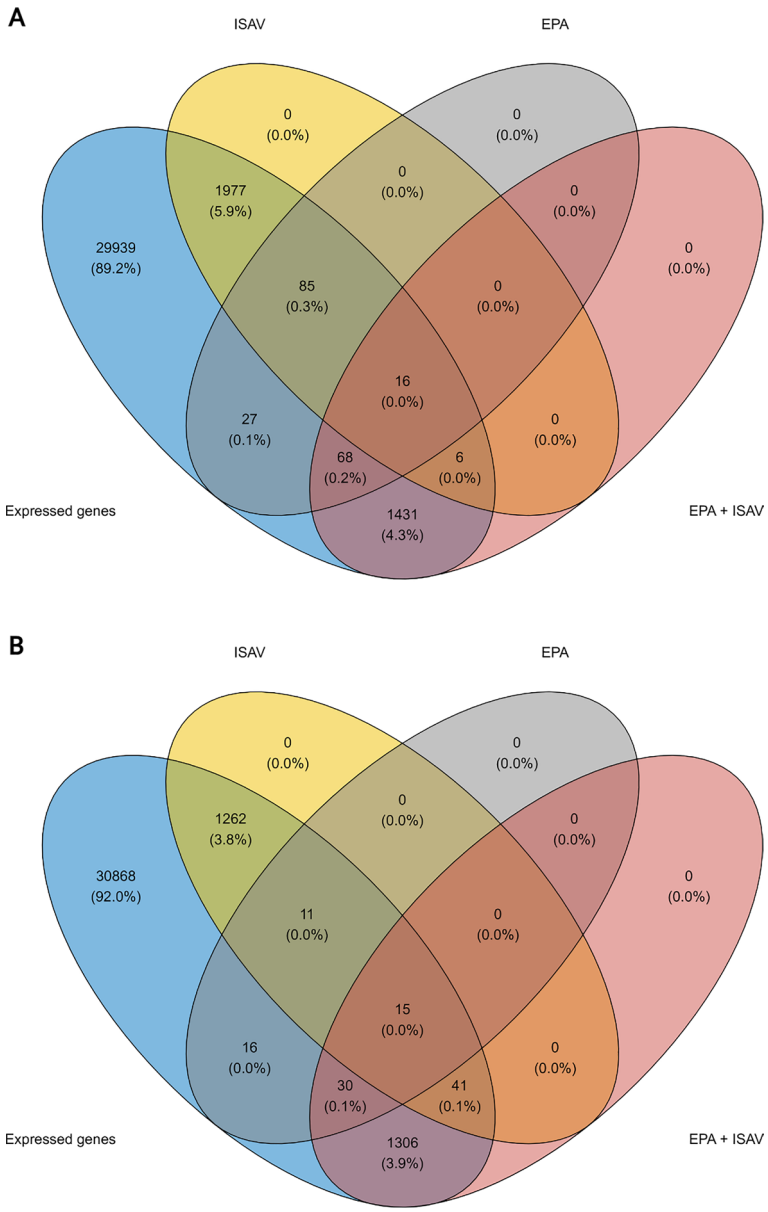

Fig. 6 Venn diagrams showing overlap of significant DEGs in the various experimental groups. **A**: Upregulated genes. **B**: Downregulated genes

the transcriptomic changes resulting from this infection in cells cultured in standard medium [54]. The sampling time point (48 h p.i.) was based on previous kinetic studies [54], capturing the innate transcriptional response in these cells. However, this sample was probably too early to capture effects of EPA on viral replication, as this normally takes about 72 h to develop quantifiable levels [81]. Results from pathway enrichment analysis were similar but not identical to a comparable study using the interferon inducer poly I:C instead of virus as the immunological stimuli [82]. The effects of poly I:C in combination with elevated levels of EPA were more restricted in the sense that multiple pathways like ECM-receptor interaction, autophagy, apelin, and VEGF-signaling were suppressed. Incubation with the highest concentration of EPA alone without any additional inflammatory stimuli had limited effects on the transcriptional profile of the cells and did

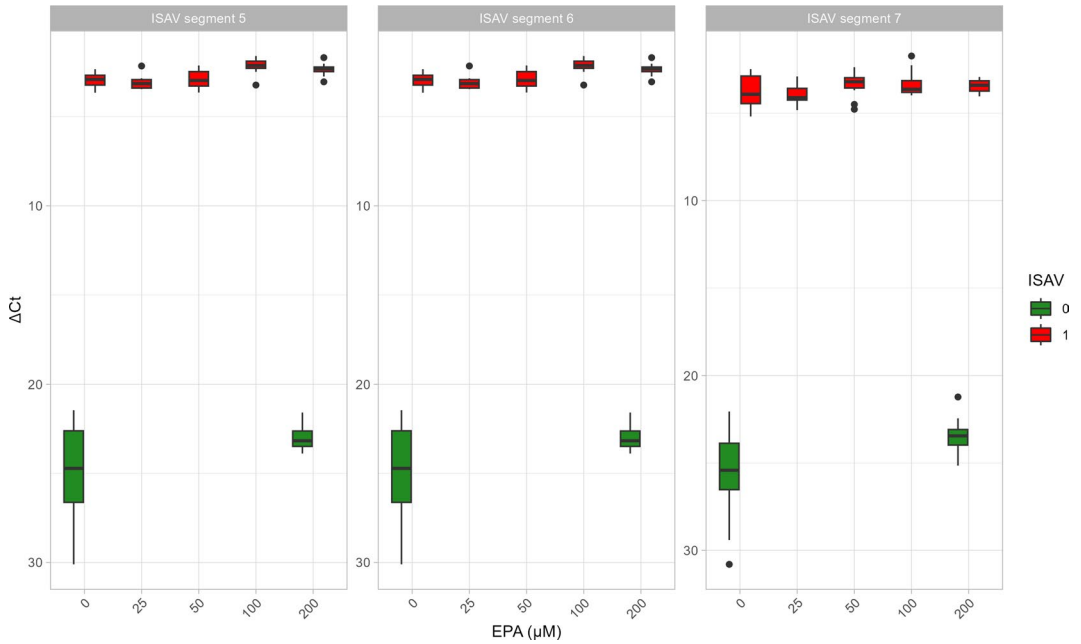

Fig. 7 qPCR analysis of three ISAV gene segments in infected ASK cell cultures (red bars) or noninfected cells (green bars) at various EPA levels at 48 h after infection ($n=6$)

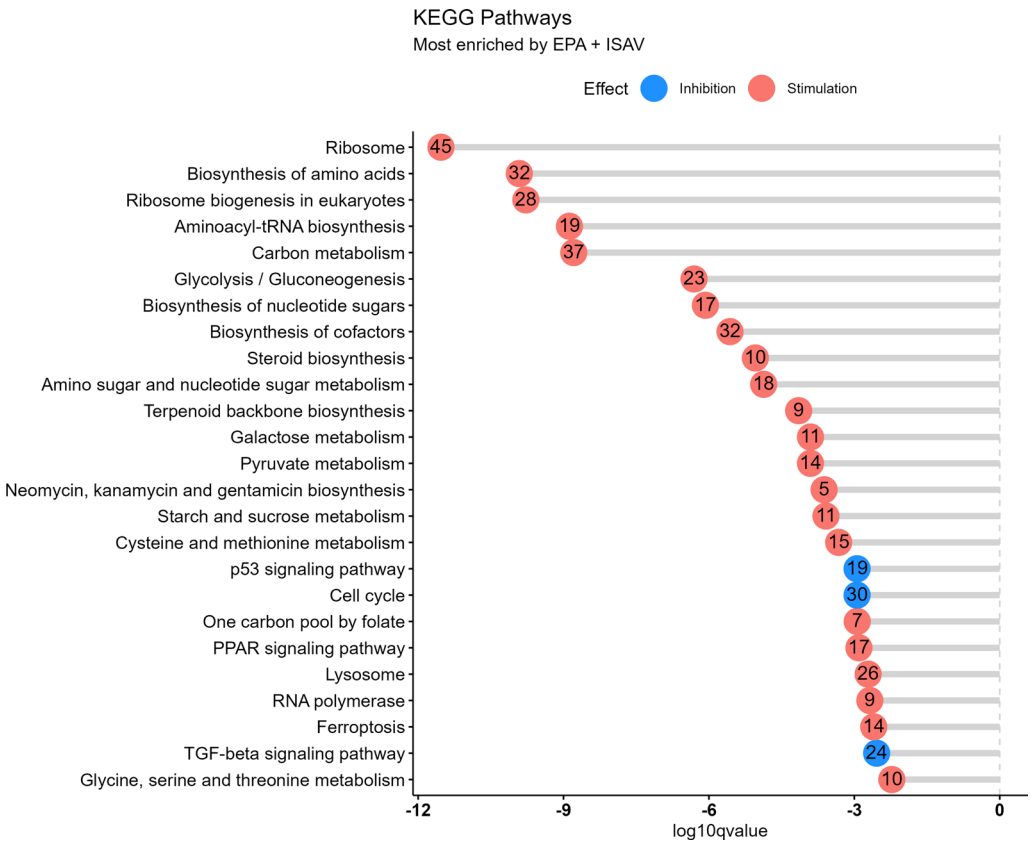

Fig. 8 KEGG pathway enrichment analysis (biological processes) of gene sets significantly affected by EPA levels in ISAV infected ASK cells. Count represents number of transcripts in gene set. Color represents direction of EPA effect (blue = inhibition, red = stimulation)

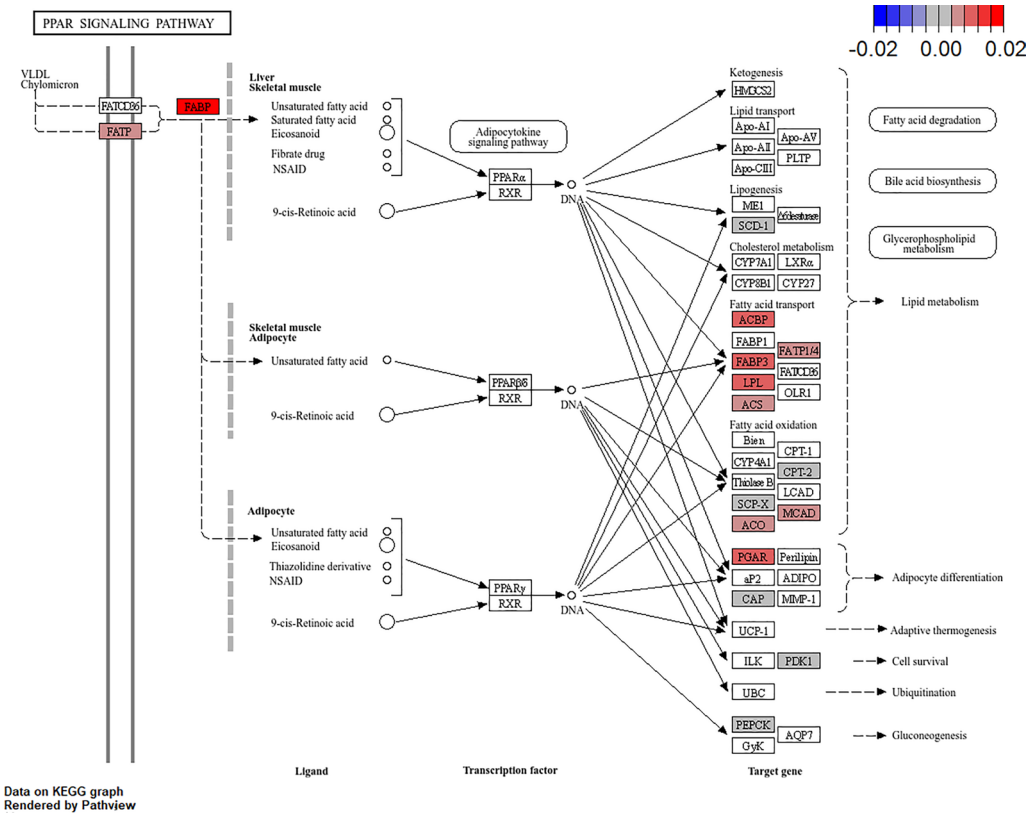

Fig. 9 Effect of EPA on transcripts in the PPAR pathway during ISAV infection in ASK cells

not reveal significantly enriched GO or KEGG terms. This suggests that the highest concentration (200 µM) was well tolerated by the cells. However, combined with ISAV infection as the inflammatory signal, robust transcriptional changes involving multiple metabolic pathways were observed at higher levels of EPA. In addition to more general pathways like ribosome, carbohydrate, amino acid, and fatty acid metabolism, pathways enriched by EPA were ferroptosis, PPAR signaling, and lysosomal pathways. Transcripts downregulated by EPA were involved in cell cycle p53- and TGF-beta signaling (Fig. 7). These observations are in line with results obtained using poly I:C as inflammatory stimuli [82]. Although originally described as regulators of lipid metabolism, PPARs are now also recognized for their role in controlling inflammation induced by lipopolysaccharides [83] or via inhibition of interferon production [84]. In our experimental in vitro model, higher levels of EPA may therefore attenuate inflammatory responses to viral infection via activation of PPAR pathways, similar to effects observed with other viruses [85]. In dietary studies of Atlantic salmon combined with outbreaks of viral disease, a protective effect of higher PUFA levels was observed [86].

Ferroptosis is a mechanism of controlled cell death characterized by increased cellular Fe^{2+} concentration, iron-dependent oxidation of unsaturated membrane fatty acids, and mitochondrial contraction. This cell death can be experimentally induced by inhibition of cysteine uptake (precursor for the antioxidant glutathione) via the amino acid transporter system X_c^-, inactivation/reduction of glutathione peroxidase 4 (*GPX4*), depletion of coenzyme Q10, or lipid peroxidation due to PUFA overload [87] (Fig. 10). The observed downregulation of system X_c^- combined with high levels of ferritin (and hence stored iron) in ISAV infected ASK cells may explain the triggering of ferroptosis observed in our study. How ferroptosis contributes to physiological homeostasis is not

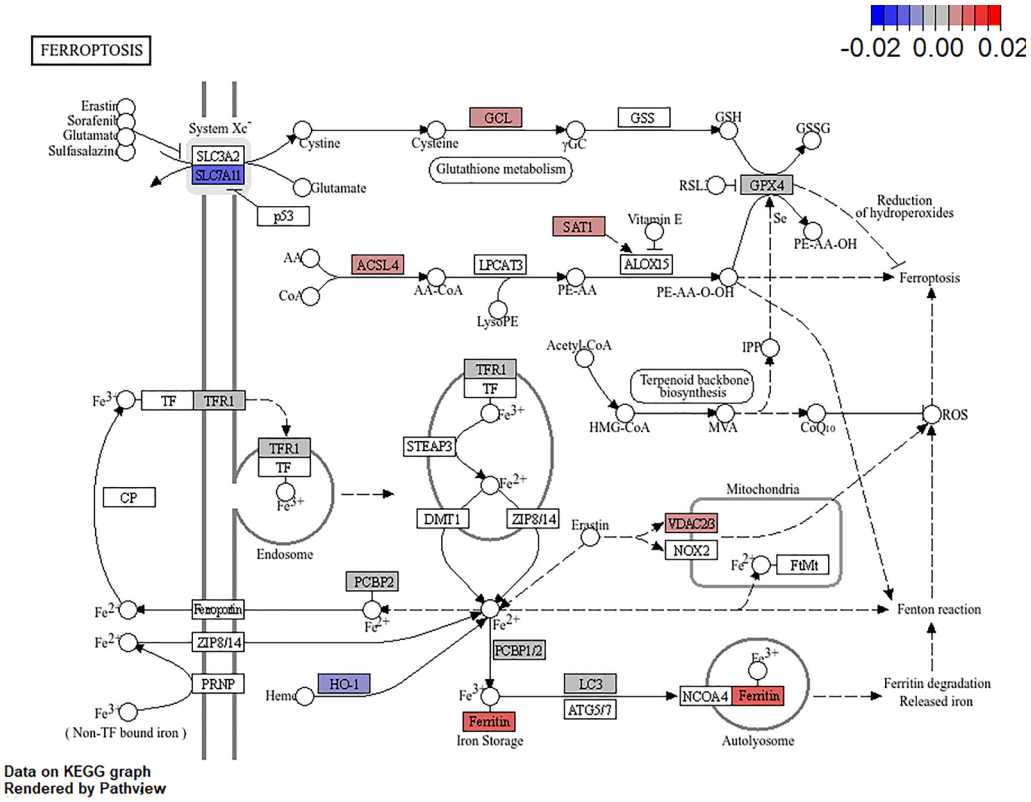

Fig. 10 Effect of EPA on transcripts in the ferroptosis pathway during ISAV infection in ASK cells

completely understood but it may play a role in tumor suppression [88], immunity [89], and development [90]. Recent studies suggest that ferroptosis may limit viral replication and pathogenesis [91] and be a part of the host innate immune response limiting viral spread [92]. The role of ferroptosis during ISAV infection under high levels of PUFAs like EPA observed here needs confirmation by biochemical assays of iron and peroxidation products in addition to analyzing the effects of ferroptosis inhibitors and activators on viral replication. In reviewing the experience with PUFA supplements on murine models of infection the jury is still out on the main effects. Several reports have documented reduced survival of bacteria infected animals on dietary fish oils [93, 94], most probably due to their immunosuppressive effects. However, other studies suggested increased resistance to bacterial infection by PUFA supplementation duet to enhanced cytokine production [95]. Murine models of viral infection also display contrasting effects of PUFAs on disease outcome [96, 97]. This dichotomy demonstrates the fine-tuned response by the immune system tailored to the invading pathogen and that there is no "one size fits all" explanation for the immunomodulatory roles of PUFAs [98]. Given the important role and high levels of PUFAs in ISAVs most important host, farmed Atlantic salmon, further studies are therefore needed to optimize the levels of these feed ingredients to support disease resistance to this virus and other pathogens.

Conclusion

The interplay between lipid metabolism and immunity is receiving increased attention and constitutes a major part of immunometabolism. In this study, we have shown that various cellular levels of EPA in Atlantic salmon cells affect the regulation of multiple transcripts involved in innate immune responses to viral infection. At high levels of EPA, viral infection may precipitate regulated cell death pathways like ferroptosis due to increased oxidative stress. This supports previous studies using other viruses [91] and encourages further investigations on the interplay between metabolism and immunity in this species.

Abbreviations

ACP	Acyl carrier protein
ASK	Atlantic salmon kidney
BH	Benjamini-Hochberg
Ct	Cycle threshold
DEG	Differentially expressed genes
DHA	Docosahexaenoic acid
ECM	Extracellular matrix
EPA	Eicosapentaenoic acid
FADS2	Fatty acid desaturase 2

GO	Gene ontology
GPR120	G-protein coupled receptor 120
GPX4	Glutathione peroxidase 4
IFIH1	Interferon induced with helicase C domain 1
IFNa	Interferon alpha-1
ISAV	Infectious salmon anemia virus
ISG15	Interferon-stimulated gene 15
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAVS	Mitochondrial antiviral-signaling protein
MOI	Multiplicity of infection
Mx1	Interferon-induced GTP-binding protein Mx1
OLAH	Oleoyl-ACP hydrolase
PCA	Principal component analysis
Poly I:C	Polyinosinic-polycytidylic acid
PPAR	Peroxisome proliferator activated receptor
p.i.	Post-infection
STING	Stimulator of interferon genes
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
VLC-PUFA	Very-long-chain polyunsaturated fatty acids

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-024-02619-0.

Supplementary file 1: This file contains results from the fatty acid analysis, the exploratory plots of the RNA-seq data and a picture of virus infected cells.

Supplementary file 2: Tables with significantdifferentially expressed genesin the various experimental groups.

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Author contributions
Conceptualization, B.R., T.K.Ø., M.B. and T.G.; methodology, B.R., T.K.Ø., M.B. and T.G..; software, I.H., S.A. and T.G..; validation, I.H., S.A. and T.G., formal analysis, I.H., S.A., B.R., T.K.Ø., M.B. and T.G., investigation, I.H., S.A., B.R., T.K.Ø., M.B. and T.G.; resources, B.R., T.K.Ø., M.B. and T.G.; data curation I.H., S.A. and T.G.; writing—original draft preparation, I.H. and T.G..; writing—review and editing, I.H., S.A., B.R., T.K.Ø., M.B. and T.G.; visualization, I.H., S.A. and T.G.; supervision, B.R., T.K.Ø., M.B. and T.G.; project administration, B.R., T.K.Ø., M.B. and T.G..; funding acquisition, B.R., T.K.Ø., M.B. and T.G.. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
Raw data from this project is available from the SRA archive (Bioproject ID: PRJNA1113821).

Declarations

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Not applicable.

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Not applicable.

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The authors declare no competing interests.

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