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ORIGINAL ARTICLE

Molecular Mechanism Involved in Carotenoid Metabolism in Post-Smolt Atlantic Salmon: Astaxanthin Metabolism During Flesh Pigmentation and Its Antioxidant Properties

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Abstract

A better understanding of carotenoid dynamics (transport, absorption, metabolism, and deposition) is essential to develop a better strategy to improve astaxanthin (Ax) retention in muscle of Atlantic salmon. To achieve that, a comparison of post-smolt salmon with (+Ax) or without (−Ax) dietary Ax supplementation was established based on a transcriptomic approach targeting pyloric, hepatic, and muscular tissues. Results in post-smolts showed that the pyloric caeca transcriptome is more sensitive to dietary Ax supplementation compared to the other tissues. Key genes sensitive to Ax supplementation could be identified, such as *cd36* in pylorus, *agr2* in liver, or *fbp1* in muscle. The most modulated genes in pylorus were related to absorption but also metabolism of Ax. Additionally, genes linked to upstream regulation of the ferroptosis pathway were significantly modulated in liver, evoking the involvement of Ax as an antioxidant in this process. Finally, the muscle seemed to be less impacted by dietary Ax supplementation, except for genes related to actin remodelling and glucose homeostasis. In conclusion, the transcriptome data generated from this study showed that Ax dynamics in Atlantic salmon is characterized by a high metabolism during absorption at pyloric caeca level. In liver, a link with a potential of ferroptosis process appears likely via cellular lipid peroxidation. Our data provide insights into a better understanding of molecular mechanisms involved in dietary Ax supplementation, as well as its beneficial effects in preventing oxidative stress and related inflammation in muscle.

Keywords Atlantic Salmon · Carotenoids · Astaxanthin · Absorption · Antioxidant

Introduction

Astaxanthin (Ax) is widely used as a feed additive to give a characteristic red flesh pigmentation to farmed Atlantic salmon (Bjerkeng et al. 1992; Torrissen 1989; Schiedt et al. 1985). The degree of flesh pigmentation is defined as one of the most desirable quality criteria for consumers (Alfnes et al. 2006). However, Ax is a rather expensive ingredient with a relatively low muscle retention averaging about 10% of total intake (Torrissen 1989; Storebakken and No 1992; Christiansen et al. 1995). Therefore, a better understanding of carotenoid dynamics is essential to develop a better strategy to improve its retention in the muscle. Beyond flesh pigmentation, Ax has other known functions including its role as provitamin A (Schiedt et al. 1985, 1989), antioxidant (Christiansen et al. 1995), inhibitors of oxidative stress (Kalinowski et al. 2019), and reproduction (Torrissen 1989).

The biochemical and physiological mechanisms involved in absorption, tissue deposition, and metabolism of Ax are not fully elucidated. The absorption process for carotenoids is considered similar to that of lipids. The major steps involve disruption of food matrix and molecular linkages, uptake in lipid droplets, micelle formation, and uptake from micelles into enterocytes and incorporation for transport into chylomicrons (van het Hof et al. 2000). Several dietary and physiological factors affect the Ax absorption in fish, such

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as the food matrix, dietary lipid and cholesterol levels, fatty acid composition, fibre, and fish size (Torrissen 1989; Olsen et al. 2005; Chimsung et al. 2014). It appears that dietary fibres reduce the bioavailability of carotenoids, while cholesterol and α-tocopherol improve their absorption (Chimsung et al. 2014; van het Hof et al. 2000; Bjerkeng et al. 1999; Riedl et al. 1999).

The large difference (approximately 30–50%) between digestibility and their retention (Kyoon No and Storebakken 1991) implies that higher levels of carotenoids are absorbed compared to that deposited in muscle by salmonids (Torrissen 1989; Kyoon No and Storebakken 1991; Storebakken and No 1992). The possible bioconversion of Ax to vitamin A (Schiedt et al. 1985; Al Khalifa and Simpson 1988) suggests that the intestine may be an important site for carotenoid metabolism. Extensive knowledge of carotenoid absorption and metabolism in humans and animal models is generally derived from studies conducted with beta-carotene (Van Vliet 1996; Yeum and Russell 2002; Desmarchelier and Borel 2017). After the enzymatic intestinal digestion of the food matrix and the release of carotenoids, their transition occurs from lipid droplets to mixed micelles. Several dietary and genetic factors influence carotenoid digestion including genes responsible for the expression of digestive enzymes (e.g. gastric lipase, cholesterol esterase, pancreatic lipase) and bile acid formation that aid in carotenoid micellization, intestinal cellular uptake, and lymphatic transport (Van Vliet 1996; Yeum and Russell 2002; Harrison 2012; Desmarchelier and Borel 2017). More recent reports suggest that several proteins including scavenger receptor class B type 1 proteins (SCARB1) that are temporarily present at the apical membrane facilitate the carotenoid uptake (Reboul and Borel 2011), which is also partially responsible for transport of lipids and cholesterol from tissues to lipoproteins and vice versa. The cluster of differentiation 36 (CD36) may facilitate beta-carotene uptake (Borel et al. 2013). Also involved in the carotenoid metabolic pathway, the carotenoid cleavage enzyme BCMO1 showed to be linked to flesh pigmentation (Olson and Hayaishi 1965). The modulation of the expression of the gene encoding this protein may be responsible for the polymorphism of flesh colour, as shown by Madaro et al. (2020) and Helgeland et al. (2014) in Chinook salmon and Atlantic salmon respectively. Other studies in animals (Von Lintig et al. 2005), or more specifically in zebrafish or in mammals (Kumar et al. 2012), have described the metabolism of beta-carotene involving several genes related to their catabolism: retinol dehydrogenases (*rdh*) catalyzing the transformation of retinol to retinal, or the retinaldehyde dehydrogenases (*raldh*) catalyzing the transforamtion of retinal to retinoic acid. The latter is capable of activating the nuclear receptor RAR/RXR gene resulting in regulation of gene expression. In fish intestinal cells, *abcg2* has also been described as responsible for modulating flesh colour by translocating astaxanthin from enterocytes to the intestinal lumen (Zoric 2017).

After absorption from the gastrointestinal tract, the retinol formed in the intestine is transported by the retinol-binding protein (RBP) encoded by the *rbp* gene mainly expressed in the liver, for delivery to the other organs (Lubzens et al. 2003). Non-metabolized carotenoids are transported by lipoproteins in blood to the liver and other tissues for deposition or further metabolism. However, the exact mechanism of carotenoid transport by lipoproteins in blood, tissue uptake, and deposition in various organs is poorly understood. The ability to metabolise and accumulate carotenoids in muscle and skin changes with life stage and physiological status (Storebakken and No 1992). Before the smolt stage, carotenoids are deposited mainly in the skin (Storebakken et al. 1987; Bjerkeng et al. 1992). As the fish grow, the ability to deposit carotenoids in the muscle increases and the concentration in the flesh increases while the concentration in the skin is reduced (Bjerkeng et al. 1992). The flesh is depigmented in connection with sexual maturation when the carotenoids are transferred to the gonads and to the skin (Storebakken et al. 1987).

Information on the direct effect of smolt production on Ax deposition in salmon flesh is scarce. However, Bjerkeng et al. (1992) found an increased rate of deposition of carotenoids in the muscle of rainbow trout (*Oncorhynchus mykiss*) following smoltification and seawater transfer. In Atlantic salmon, metabolic capacity for carotenoid transformation has been identified to be higher in small fish as demonstrated by the decline of the carotenoid metabolite idoxanthin with fish size (Schiedt et al. 1989; Ytrestøyl et al. 2005).

Further knowledge and understanding of the mechanisms involved in the deposition of Ax in the muscle target tissue are needed to devise new strategies to improve Ax retention. Microarray analyses have already been performed in Atlantic salmon brain, gills, kidney, and proximal intestine to evaluate the effect of smoltification or seawater transfer on the host transcriptome and have suggested effects on growth, metabolism, oxygen transport (Seear et al. 2010), and the immune functions of Atlantic salmon (Johansson et al. 2016). Very recently, Ytrestøyl et al. (2021) published a study of transcriptome-based data from Ax-free post smolt Atlantic salmon compared to Ax-fed post-smolt Atlantic salmon. This study showed that the intestinal and muscle transcriptomes were more affected than the liver. Genes associated with immunity, lipid and iron metabolism such as ladderlectins genes of *gimaps* were down-regulated in salmon without Ax. In the liver, the heme-oxygenase gene (*hmox1*) involved in immunity was up-regulated by Ax supplementation. In muscle, genes associated with stress, immunity or motility was found to be also modulated without Ax, such as up-regulations of *cd97* or *cd209*

or the gene encoding the coronin. The present work was designed to study the host transcriptome after smoltification in response to high dietary Ax supplementation in similar tissues. Assessment of the three key tissue levels, pyloric caeca, liver, and muscle was undertaken to investigate the molecular mechanisms involved in the absorption, transport, metabolism, and deposition of Ax, in order to better understand the factors affecting the low retention of Ax in Atlantic salmon flesh.

Materials and Methods

Experimental Design, Treatments, and Sampling

Parrs were produced in a flow through system with 1-m square (400 L) tanks. The average water temperature was of 10 ± 0.3 ℃. They were fed the commercial diet devoid of astaxanthin (2 mm Skretting Nutra Olympic, Stavanger, Norway) until reaching 25 g. They were then split into two groups (Table 1):

- One group received a crushed cod diet until 50 g and then sampled (Parr group).
- The other group was smoltified over 12 weeks during which time they continued to be fed the commercial diet. They were then transferred to full strength seawater at 9.4 °C ± 0.5 and fed a diet in triplicate groups that was devoid of astaxanthin from 122 g until 400 g (Betancor et al. 2017). The remaining fish were equally distributed into 6 tanks and fed the experimental diets (triplicate groups) consisting of a cod diet coated with astaxanthin (+ Ax group) or a cod diet without added Ax (− Ax group). The experiment lasted for 4 weeks, and the fish grew from 400 to 550 g. The group fed Ax received pellets that were vacuum coated with CAROPHYLL Pink 10%-CWS (DSM Nutritional Products) to a concentration of 100 mg Ax/kg feed.

At sampling, pyloric caecum, liver, and muscle tissues were collected from 8 individual fish from each tank in each of the three groups. The muscle samples were taken according to the Norwegian quality cut. Samples were preserved into RNAlater. Six samples per treatment were dedicated to microarray and 8 per treatment for validation by qPCR.

This work was conducted in accordance with the laws and regulations controlling experiments and procedures on live animals in Norway.

Table 1 Experimental treatments

Groups	Supplementation	Inclusion level
Parr Pre-smolt salmon	-	-
+ Ax Post-smolt salmon	Astaxanthin	100 mg/kg
− Ax Post-smolt salmon	-	-

Methods for the Determination of the Different Parameters

RNA Extraction

One hundred milligrams of tissue were collected for gene expression measurement of the whole transcriptome.

Total RNA was extracted from tissues (stored at − 20 °C in RNA later) by lysing tissue with FastPrep® 24 (MP Biomedicals, Illkirch, France), using the phenol–chloroform method (TRIzol reagent; Invitrogen, Invitrogen, Cergy Pontoise, France) followed by purification using RNeasy columns by automated method with the Qiacube HT (Qiagen, Courtaboeuf, France).

The concentration of RNA was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Illkirch, France), and the purity was estimated by A260/A280 ratio. RNA integrity was assessed by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). The threshold of the RNA Integrity Number (RIN) was set at 7.5 to validate sufficient quality of the RNAs.

Microarray Analysis

Gene expression was performed by one-color microarray-based analysis using the Salmon (V2) Gene Expression Microarray, 4 × 44 K (Agilent: G2519F-026441) ($n = 6$ per treatment). Two hundred nanograms of total RNA was labelled with Low Input Quick Amp Labelling Kit following manufacturer's instructions. The quality of the Cy-3 labelled cRNA was checked using NanoDrop, and the yield and specific activity were calculated. Hybridization and scanning were done following the protocol described by Agilent using the Agilent G4900DA SureScan Microarray Scanner System. Signal intensities obtained were extracted using Feature Extraction software version 12.1.

Differential Gene Expression Analysis and Pathway Analysis

In addition to the analysis of the raw data with the Partek Genomics suite software version 7, the extracted data were also analysed by the bioinformatics data analysis provider Strombus Genomics.

Examination of differentially expressed genes (DEG) was evaluated, as well as outliers and batch effect assessment, using PCA plots and hierarchical clustering.

The analysis was based on 3 experimental group comparisons:

- Post-smolt fed Ax (+ Ax) compared to post-smolt without Ax (− Ax)
- Post-smolt fed Ax (+ Ax) compared to pre-smolt without Ax (Parr)
- Post-smolt without Ax (− Ax) compared to pre-smolt without Ax (Parr)

The genes with a threshold set at 1.5 of fold change and with a *p*-value of less than 0.05 were considered to determine the number of DEG, the Venn diagrams and the heatmaps comparing all pairs of conditions (+ Ax vs − Ax, + Ax vs Parr, and − Ax vs Parr). The significance of gene expression was assessed by one way ANOVA, and the *p*-values are false discovery rate (FDR) tested using the Benjamini–Hochberg method (Benjamini and Hochberg 1995).

Functional enrichment in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways was assessed based on the list of the significant DEG ($p < 0.05$). As with differential expression analysis, significance values were FDR tested using Benjamini-Hochberg. KEGG pathways were significantly enriched if they had FDR adjusted *p*-values of less than 0.05.

The evaluation of the DEG allowed us, first, to determine the Venn diagrams combining the three different comparisons. The displayed numbers reflect the number of entities with a fold change of at least 1.5 or − 1.5 and an unadjusted *p*-value of 0.05, with or without gene annotation. Only the fully annotated genes will be used for further pathway analysis.

qPCR Confirmation of Selected Genes

The selected target genes (*cd36*, *bcmo1*, *agr2*, *gbp1*, *fbp1*, and *c1ql4)* were analysed by quantitative PCR to validate the microarray results according to their relevance in carotenoid metabolism or due to their strong modulation. The reverse transcription was performed using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Marnes-la-Coquette, France) with 100 ng of RNA ($n = 8$ per treatment), following the manufacturer's instructions. The resultant cDNAs were diluted 5 times to be amplified by real-time PCR. qPCR reactions were performed for all individual samples in triplicate, with 20 µL reaction volume, using SsoAdvanced Universal Sybr Green Master Mix (Bio-Rad, Marnes-la-Coquette, France), with a final concentration of 500 nM of each primer. Sequences of the primers used are given in Supplementary Table 1. The thermal cycling was run on a Light Cycler 96 (Roche Diagnostics, Meylan, France) with the following program: 95 °C 30 s, followed by 40 cycles of denaturation at 95 °C 15 s, and hybridization/elongation at 60 °C 60 s. A melting curve was performed to check the amplicon specificity. Analysis of qPCR data was carried out by averaging the three technical replicates of the Ct values for each of the 8 samples per treatment. Each mean Ct value of the target genes was normalized to the geometric mean Ct value of the housekeeping genes for the same sample. The Delta Ct method was used to determine expression of target genes (Livak and Schmittgen 2001).

Results

Differentially Expressed Genes

To initiate the microarray analysis, the RIN of the samples were determined to ensure further transcriptomic analysis. All RIN had a value of at least 8.3 reflecting high quality of RNAs. The means of the RIN were very similar between the three organs, with 9.3 in pylorus, 9.8 in liver, and 9.9 in muscle.

To determine the number of differentially expressed genes (DEG), three different comparisons were made. The comparison − Ax vs. Parr would reflect mainly the effect of the developmental status of the fish (life stage), while the + Ax vs. − Ax one would reflect the effect of astaxanthin supplementation in post-smolt. The comparison + Ax vs. Parr combined the effect of the life stage and Ax dietary supplementation.

In pylorus (Fig. 1), the highest numbers of DEG were found between post-smolts without Ax and parrs (− Ax vs Parr 941) and between post-smolts with and without Ax (+ Ax vs − Ax 716).

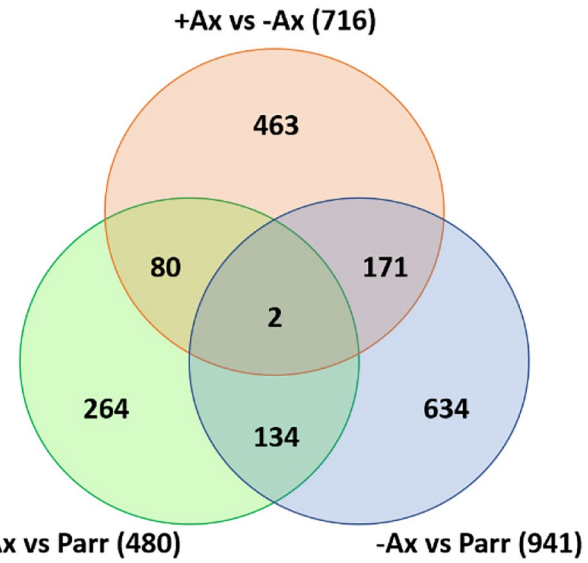

Fig. 1 Venn diagram related to DEGs in pylorus

In liver, the highest numbers of DEG were observed between post-smolts without Ax compared to parrs (− Ax vs Parr 3119), and post-smolts with Ax compared to parrs (+ Ax vs Parr 2361) (Fig. 2).

In muscle, the highest numbers of DEG were clearly observed in the comparison + Ax vs Parr 3413 (Fig. 3).

In liver and muscle, the relative difference in DEG count is higher between − Ax vs Parr and + Ax vs − Ax comparisons, compared to pylorus. Indeed, in the liver we observed 3 times more DEG in − Ax vs Parr 3119 DEG than + Ax vs − Ax 1023 DEG and, in muscle, 2 times more DEG in − Ax vs Parr 1238 DEG than + Ax vs − Ax 654 DEG (Figs. 1, 2 and 3). In pylorus, the difference in DEG counts is less evident between − Ax vs Parr 941 DEG and + Ax vs − Ax 716 DEG comparisons.

The lists of the most modulated genes between the different groups and in the different tissues are presented with their fold change and *p*-value in Supplementary Tables 2, 3, 4, 5, 6, 7, 8, 9, 10. Some gene expression modulations are detailed below:

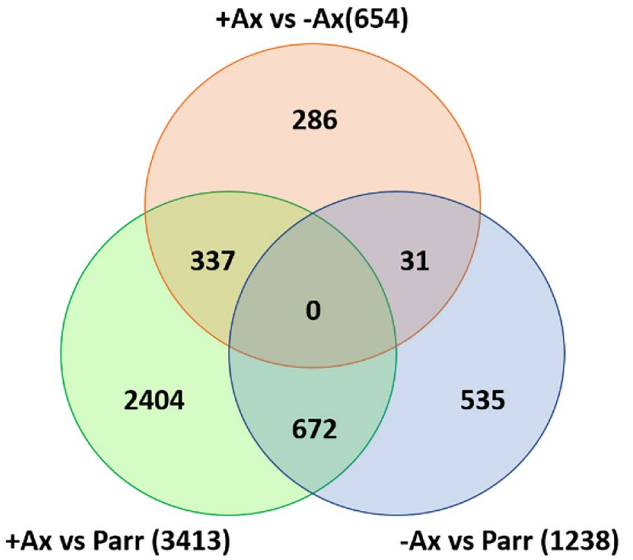

Fig. 3 Venn diagram related to DEGs in muscle

Pyloric Tissue

Regarding the genes differentially expressed between the + Ax and − Ax groups, we observed that the highest increase in mRNA expression was found for the gene encoding the CD36 protein ($p = 0.001$; FC = 3.81) involved in the transport of carotenoids in enterocytes.

However, we also observed that neither *bcmo1* nor the scavenger receptor gene *scarb1* and were modulated in the Ax-fed group. Interestingly, the gene encoding for the LDL receptor (LDLR) was significantly up-regulated ($p = 0.03$; FC = 3.32).

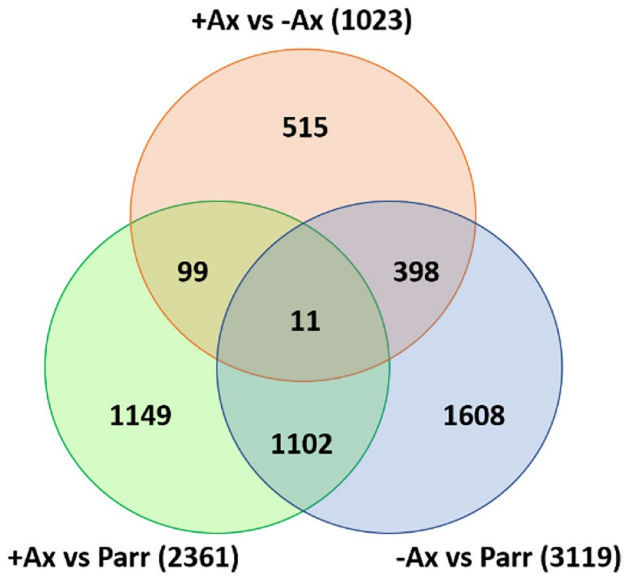

Fig. 2 Venn diagram related to DEGs in liver

Other up-regulated genes related to carotenoid metabolism were observed following Ax feeding in post-smolts, such as the genes encoding the retinol dehydrogenases RDH3 ($p = 0.05$; FC = 1.67) and RDH8 ($p = 0.06$; FC = 2.78) and also the retinal dehydrogenase ALDH1A2 ($p = 0.025$; FC = 2.22). Similarly, *abcg2*, encoding the protein responsible for the efflux of carotenoid back to the lumen, was up-regulated but not significantly ($p = 0.076$; FC = 2.18).

Among the most modulated genes, the *hmox1* gene encoding the heme-oxygenase (HO-1) ($p = 0.001$, FC = −2.17), the *gbp1* gene encoding the guanylate-binding protein (GBP) ($p = 0.03$, FC = −1.96), or the MHC class II transactivator gene (CIITA, $p = 0.03$; FC = −2.08) were down-regulated. The gene encoding the transcription factor basic transcription element binding (BTEB) was also observed as one of the most down-regulated genes in the + Ax group compared to the − Ax group ($p = 0.004$; FC = −1.96).

Hepatic Tissue

When examining differentially expressed genes, two genes encoding motor proteins, myosin ($p = 0.01$; FC = 2.06) and dynein ($p = 0.02$; FC = 2.03), were up-regulated in liver of + Ax compared to − Ax. We also observed that the gene encoding plasma retinol binding-protein 2 (*rbp2*) was significantly down-regulated in + Ax vs − Ax ($p = 0.03$; FC = −2.29).

Our data showed that *agr2* is the most up-regulated gene in liver of the + Ax group compared to − Ax ($p = 0.03$;

FC = 3.58) and, among the most up-regulated genes, we also observed the nuclear receptor coactivator 4 gene (*ncoa4*), which was significantly up-regulated in fish supplemented with Ax ($p = 0.015$; FC = 1.87).

Among the most down-regulated genes between + Ax and − Ax, our data showed also that the heme-oxygenase gene (*hmox1*) was significantly down-regulated ($p = 0.047$; FC = −3.23), and we observed a down-regulation of the *sat1* gene ($p = 0.01$, FC = −2.70) encoding the diamine acetyltransferase 1 as observed in the pylorus.

Muscular Tissue

With regard to the DEGs, we can observe that only a few genes were down-regulated in + Ax group compared to − Ax group. Most of the DEGs were therefore up-regulated. The gene with the highest response in the muscle of post-smolts fed Ax compared to controls seems to be associated with the fructose bisphosphatase 1 ($p = 0.04$; FC = 3.57). The gene encoding the actin-binding protein, coronin 2A, was also among the most up-regulated genes in the muscle of Ax-fed salmon ($p = 0.01$; FC = 1.94). Similarly, genes encoding tumour necrosis factor receptor (TNFR) and transcription factor D-Jun were significantly up-regulated in post-smolts fed + Ax ($p = 0.04$; FC = 2.44) and ($p = 0.03$; FC = 2.15).

Induction Effect of Ax at Post-Smolt Stage

Regarding the smoltification effect, in the pylorus and liver, both up- and down-regulations are in the same range of fold changes, from − 33 to + 36, whether the fish are fed Ax or not in comparison to parrs. Among the top modulated genes, a strong regulation down to a FC of −1429 was observed in muscle.

In the pylorus, *bcmo1* expression in + Ax compared to Parr was down-regulated ($p = 0.05$; FC = −1.82). When comparing − Ax to Parr, a strong increase of the expression of chemokines genes *scya113* and *scya109* ($p = 0.005$; FC = 8.10 and $p = 0.002$; FC = 10.04, respectively) was noticed. Ax supplementation attenuates the up-regulations of these chemokine-like genes when comparing + Ax vs Parr ($p = 0.03$; FC = 3.02, and $p = 0.07$; FC = 3.44).

In muscle, the gene encoding the collagenase 3 (*mmp13*) was the most up-regulated gene in + Ax compared to Parr, and when examining this gene in liver, it was also highly up-regulated in + Ax vs Parr ($p = 0.009$; FC = 9.42) but less in − Ax vs Parr ($p = 0.091$; FC = 4.25). Similarly, when examining the data in muscle and liver, the gene encoding the CD9 antigen (*cd9)* was strongly up-regulated in both smolt groups compared to parrs.

Finally in muscle, when focusing on a cell stress-related gene, the one encoding the DNA-damage inducible transcript 4-like showed a 24-fold ($p = 0.04$) change in up-regulation in the − Ax group while in the + Ax group, the fold-change decreased to 7.5 ($p = 0.08$).

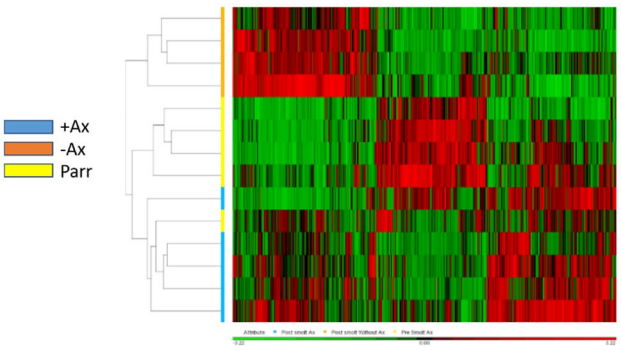

Fig. 4 Heatmap of gene expression pattern in pylorus

Quantitative PCR performed on the six target genes confirmed the differential gene expression observed in microarray. On the 12 selected significant modulations measured by microarray, the qPCR analysis confirmed 9 of them and 1 measurement was close to being significant ($p = 0.06$ for *bcmo1* in pylorus (− Ax vs Parr) (Supplementary Table 11).

Gene Expression Pattern Clustering (Heatmaps and Volcano Plots)

In pylorus, the first clustering is occurring between − Ax and samples from Parr and + Ax (Fig. 4). The second separation of the dendrogram shows a very strong clustering between samples from + Ax and the Parr.

In the liver, the heatmap confirms the observations of DEG in Venn diagrams (Fig. 5) with a less clear separation between post-smolts groups: the first clustering is grouping one part of the post-smolt salmon without Ax from the rest of the samples, and then a final separation of samples from parrs.

In muscle, the clustering due to Ax supplementation is less pronounced (Fig. 6), with a first very clear clustering of samples from parrs from the other two groups. The final

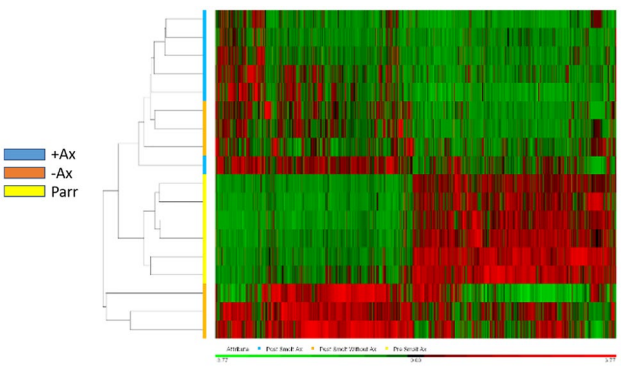

Fig. 5 Heatmap of gene expression pattern in liver

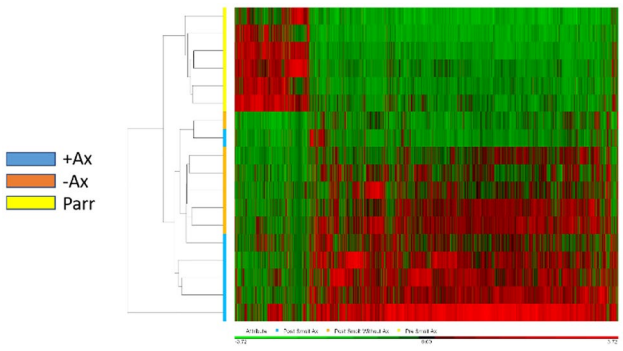

Fig. 6 Heatmap of gene expression pattern in muscle

separation of the samples due to Ax supplementation is visible by the clustering of samples from + Ax to – Ax.

The Volcano plots confirm our previous observations (Supplementary Figs. 1, 2, 3, 4, 5, 6, 7, 8 and 9) of the number of DEG and the transcriptome heatmap of the different tissues, as we see more effects in the pylorus than in the liver and the muscle for + Ax vs –Ax compared to Parr vs + Ax or – Ax.

Pathway Analysis

Comparing – Ax against those + Ax resulted in 6 significantly enriched pathways in pylorus, 1 pathway in the liver, and none in muscle, involving between 2 and 4 DEGs per pathway (Tables 2 and 3). Ferroptosis pathway was interestingly enriched in both pylorus and liver.

Comparison of post-smolts without astaxanthin (– Ax) with parrs showed a total of 8 significantly enriched pathways in pylorus, 3 in the liver, and 13 in muscle, involving between 2 and 32 DEGs per pathway (Tables 4, 5 and 6).

Comparison of post-smolts fed astaxanthin (+ Ax) with parrs revealed no significantly enriched pathway in pylorus while 6 in the liver and 11 in muscle were found, involving between 7 and 35 DEGs per pathway (Tables 7 and 8).

Table 2 Enriched pathways in pylorus of post-smolt salmon fed diets with and without astaxanthin (+ Ax vs – Ax)

Description	*Count*	*p. adjust*	*Gene ID*
DNA replication	4	2.2e – 03	prim2, dpod1, pola2, rfc2
Homologous recombination	3	2.2e – 02	dpod1, brip1, atm
Nucleotide excision repair	3	2.6e – 02	dpod1, ercc2, rfc2
One carbon pool by folate	2	4.8e – 02	gart, mthfd1l
Mismatch repair	2	4.8e – 02	dpod1, rfc2
Ferroptosis	3	4.8e – 02	LOC106590030, LOC106608673, LOC106563588

Table 3 Enriched pathways in liver of post-smolt salmon fed diets with and without astaxanthin (+ Ax vs – Ax)

Description	*Count*	*p. adjust*	*Gene ID*
Ferroptosis	3	4.8e – 02	LOC106590030, LOC106563588, ncoa4

Discussion

Differentially Expressed Genes and Transcriptome Clustering

In pylorus, the number of DEG suggests that, in addition to the impact of smoltification (here intended as the transition between parr and post-smolt life stages and not the smoltification process itself), there is also a major Ax-induced effect on gene expression patterns in addition to the impact of life stage. Indeed, the difference in DEG between – Ax vs Parr and + Ax vs – Ax comparisons is less evident than in the other organs. However, life stage impacted liver gene expression more than Ax although Ax-induced effect is still significant in liver response. In muscle, the high number of DEG between post-smolts fed Ax and parrs reflects the highest effect of the life stage or the combined effects of life stage and dietary Ax supplementation.

In the pylorus, the differences in DEG between (– Ax vs Parr) and (+ Ax vs – Ax) were less evident, which suggests that the effect of Ax supplementation in this organ was higher than in the other tissues. It appears that smoltification affected the gene response predominantly in the liver and muscle. The transcriptome heatmap results confirm that the strongest effect of Ax supplementation was in pylorus compared to the other organs. It clearly shows that the effect of Ax supplementation is as important as the effect of smoltification. In the liver, the heatmap also shows similar effects of smoltification and Ax supplementation. In muscle, the heatmap confirms that the main DEG pattern occurred before and after smoltification. Looking only at the effect of Ax supplementation in post-smolt salmon, Ytrestøyl et al. (2021) observed the highest number of DEG muscle. This difference could potentially be due to the level of Ax in feed which is lower in Ytrestøyl et al. (2021) than in the present study.

Effect of Astaxanthin on Pylorus of Post-Smolt Salmon (+ Ax vs – Ax)

From the results obtained from the gene expression response detailed below, a proposed mechanism of absorption and metabolization is summarized in Fig. 7.

There were several up-regulated genes in pylorus of + Ax supplemented fish compared to – Ax, that are involved in lipid/carotenoid metabolism and absorption. CD36, whose

Table 4 Enriched pathways in pylorus of post-smolt salmon without astaxanthin compared to parrs without astaxanthin (− Ax vs Parr)

Description	*Count*	*p. adjust*	*Gene ID*
Fatty acid degradation	4	9.9e − 03	acat2, LOC106602847, LOC106585052, cpt2
Taurine and hypotaurine metabolism	3	9.9e − 03	gadl1, LOC106605019, ggt1
Fatty acid metabolism	4	4.0e − 02	acat2, fadsd6, LOC106585052, cpt2
beta-Alanine metabolism	3	4.0e − 02	gadl1, LOC106602847, upb1
Phagosome	6	4.0e − 02	tap2a1, LOC106604436, LOC106565699, tap1, lamp2, LOC106569947
Pantothenate and CoA biosynthesis	2	4.6e − 02	gadl1, upb1
Valine, leucine, and isoleucine degradation	3	4.6e − 02	acat2, LOC106602847, LOC106585052
Arginine and proline metabolism	3	4.9e − 02	LOC106602847, gamt, LOC106563588

mRNA expression was most increased, is a key regulator of lipid uptake and is involved in the intestinal transport of beta-carotene (Borel et al. 2013). Our observation could therefore suggest that dietary Ax would lead to an up-regulation of this gene, improving its absorption into the intestinal cell.

After absorption of Ax from the digestive tract, carotenoids are transported by lipoproteins to the liver and other tissues (Harrison 2012). The LDL receptor gene was also interestingly up-regulated in + Ax fish, which suggests an involvement in the intestinal carotenoid/astaxanthin uptake and transport. The LDL receptors are involved in carotenoid uptake in the liver (Dallinga-Thie et al. 2016; Thomas and Harrison 2016) but such mechanism has not been described in intestinal tissue. In human intestinal epithelial cells, LDL receptors are present mainly on the basolateral membrane and to a lower extent in microvilli (Fong et al. 1995). We could hypothesize that Ax is associated to LDL synthesis in enterocytes and transported through basolateral LDL receptors.

Madaro et al. (2020) showed that the red phenotype of Chinook salmon compared to white phenotype had a higher expression of *scarb1*, compared to the white phenotype probably reflecting higher bioavailability of Ax. Neither *bcmo1* nor *scarb1* showed modulation in our data but are known to be regulated by the transcription factor intestine-specific homeobox (ISX). When activated by high quantities of intracellular all-trans retinoic acid, it will repress *bcmo1* and *scarb1* expression. The negative feedback reduces carotenoid uptake leading to decreased intracellular retinoic acid concentration (Widjaja-Adhi et al. 2015). In addition, Madaro et al. (2020) showed also that, in red chinook salmon as opposed to white, *bcmo1* was up-regulated and could be linked to the production of vitamin A and then to all-trans retinoic acid (Fig. 7) without influencing the pigmentation. This was also suggested by Helgeland et al. (2019). From these observations, it appears pertinent to suggest that gene expressions of *bcmo1* and *scarb1* are regulated by a negative feedback. This supports the hypothesis of utilization of carotenoid to generate all-trans retinoic acid.

Retinol dehydrogenases (RDH) are involved in the metabolism of β-carotene and vitamin A. In mammals, after β-carotene cleavage by *bcmo1* into all trans-retinoic acid and retinol, the latter is further processed into retinal by RDH (Eroglu and Harrison 2013). As *rdh3* and *rdh8* were up-regulated in + Ax, it is not unlikely that similar processing would occur with Ax absorbed in the pyloric cells. The up-regulation of the genes encoding these retinol dehydrogenases and also the retinal dehydrogenase 2 (*aldh1a2)* in + Ax vs − Ax emphasizes our hypothesis of a significant metabolism of Ax in the pylorus enterocytes, as the latter catalyses the oxidation of retinal to retinoic acid (Moise et al. 2007), then decreasing the availability of Ax for deposition.

Table 5 Enriched pathways in liver of post-smolt salmon without astaxanthin compared to parrs without astaxanthin (− Ax vs Parr)

Description	*Count*	*p. adjust*	*Gene ID*
Protein processing in endoplasmic reticulum	24	4.8e − 07	pkz, LOC106567674, dnaja2, LOC106580431, LOC106598366, LOC106607563, LOC106569608, LOC106583199, LOC106579628, canx, s61a1, LOC106582927, LOC106573818, dnajc3, pdia1, LOC106590419, LOC106584651, LOC106584323, pdia3, LOC106571177, LOC106565722, LOC106567637, LOC100380749, hs90a
Protein export	5	2.0e − 02	LOC106571451, LOC106598366, LOC106583199, s61a1, LOC100380712
Cysteine and methionine metabolism	7	4.5e − 02	LOC106587404, LOC100194641, ahcy, ldhb, LOC106593044, LOC106576140, LOC106580346

Table 6 Enriched pathways in the muscle of post-smolt salmon without astaxanthin compared to parrs without astaxanthin (− Ax vs Parr)

Description	*Count*	*p. adjust*	*Gene ID*
Oxidative phosphorylation	32	3.5e − 06	LOC106602876, LOC106604294, LOC106562006, LOC106567829, LOC106583886, LOC106573122, vato, LOC106612155, ndua4, LOC106561701, LOC106573873, LOC106578938, ndub6, LOC106600492, LOC106576117, LOC106606878, ndufa3, ndua6, LOC106602938, LOC106573095, LOC106568019, atp5e, LOC106569582, ndufs2, LOC106607492, LOC106562286, nduc2, LOC106613744, LOC106610772, LOC106607688, LOC106580140, LOC106565035
Carbon metabolism	30	2.6e − 04	aldh6a1, LOC106564824, LOC100380727, aco1, LOC106599099, LOC106565361, LOC106583035, prps1, rpe, pgp, fbp2, mmsa, pgd, LOC106561216, LOC106573122, LOC100194645, LOC106573440, pgk, tpi1b, LOC100194624, LOC106600492, LOC106581960, LOC106569582, LOC106562156, LOC106563586, LOC106603767, enoa, rgn, LOC106611334, LOC106588431
RNA transport	32	7.1e − 04	4ebp, LOC106604514, rae1, eif3j, LOC100380746, LOC100195680, LOC106578266, LOC106561546, eif3j, pop5, LOC100380723, eif2b5, LOC106585806, LOC106577905, 4ebp, LOC106579473, LOC106607164, LOC106603332, if4e, thoc7, snupn, LOC106574529, ncbp2, if4ea, LOC106577251, ran1, nup54, nbp1a, LOC106582627, LOC106565058, LOC106578327, 4ebp
Ribosome biogenesis in eukaryotes	18	2.0e − 03	utp15, pwp2, LOC106582083, nol5, nog2, rexo2, pop5, LOC100380723, dkc1, nop56, if6, LOC100196059, LOC106585140, LOC106588218, LOC106607124, LOC106580531, LOC106608971, ran1
Glycine, serine, and threonine metabolism	11	1.0e − 02	pmge, cgl, grhpr, LOC106561216, LOC100194645, aldh7a1, LOC106573440, LOC100194731, LOC106583798, gamt, bhmt
Spliceosome	25	1.3e − 02	LOC106596195, u2af2, ppih, LOC106607257, LOC106565348, ppie, bcas2, LOC106570281, LOC106607064, rsmb, snrpa1, dhx8, LOC106588664, hnrpm, sfrs6, ncbp2, LOC106562047, lsm3, sf3a1, sf3b2, ctbl1, nbp1a, LOC106603659, ro32, LOC106585463
Necroptosis	28	1.3e − 02	LOC100136558, stat2, hs90a, LOC106605228, glna, ub7i3, LOC106584177, stat4, LOC106561969, LOC106599099, parp1, LOC100136565, LOC106587129, pkz, parp3, LOC106577590, frih, tnr1a, irf9, LOC100136564, LOC106587134, pygb, dnm1l, LOC106612162, hmgb1, LOC106577200, LOC106608595, LOC106611749
Glyoxylate and dicarboxylate metabolism	10	1.3e − 02	LOC106564824, glna, LOC106584177, hoga1, aco1, pgp, grhpr, LOC106561216, LOC106573440, LOC106562156
Beta-alanine metabolism	9	2.7e − 02	aldh6a1, gadl1, mmsa, aldh3a2, aldh7a1, upb1, LOC100194731, LOC106603767, LOC106611334
Drug metabolism—other enzymes	12	2.7e − 02	kith, LOC106584056, hprt1, gsta3, pyr5, itpa, gstt2b, LOC106569706, ud2a2, upb1, LOC106569196, ndka
Lysosome	22	2.7e − 02	cd68, LOC106605328, LOC106562440, cats, ap3m1, lamp2, LOC106589952, ctsa, slc17a5, ap1m1, gba, LOC106583886, LOC106604436, LOC106565426, LOC106609887, LOC106607342, LOC106567183, lamp1, vato, LOC106602594, LOC106612102, ctsw
Glycolysis/Gluconeogenesis	14	3.7e − 02	pck2, LOC106565361, pmge, fbp2, aldh3a2, LOC100194645, aldh7a1, pgk, tpi1b, LOC100194624, LOC106563586, enoa, ldhb, LOC106588431
Fatty acid degradation	9	4.4e − 02	acsl4, aldh3a2, aldh7a1, LOC106604299, LOC106603767, cpt2, LOC106585052, LOC106611334, acsl1

The up-regulated gene *abcg2* in + Ax vs − Ax fish is known to play a role in the efflux of lipophilic compounds and exogenous substrates (Ando et al. 2007; Mizuno et al. 2007). In Atlantic salmon, Zoric (2017) suggested this ATP-binding cassette, identified as a QTL, could translocate Ax from the enterocytes back into the lumen, potentially explaining the limitation of pigment deposition in muscle. It would be possible, that this up-regulation observed would lead to a decrease of deposition of Ax in the muscle, with this high level of supplementation in the diet. However, in our study, this hypothesis of efflux of Ax into the lumen and the low efficacy of deposition in muscle by a loss of Ax in pylorus cannot be confirmed. It is therefore possible that a lower dietary dose of carotenoids would reduce the loss of Ax by decreasing the export from the enterocyte back to the intestinal flow through ABCG2, or potentially would reduce the metabolization via BCMO1.

Table 7 Enriched pathways in liver of post-smolt salmon fed diets with astaxanthin and parrs not fed astaxanthin (+ Ax vs Parr)

Description	*Count*	*p. adjust*	*Gene ID*
Protein export	7	1.9e − 03	LOC106590555, LOC106587724, LOC106571451, LOC106583199, s61a1, LOC100380712, sc61a
Protein processing in endoplasmic reticulum	22	1.9e − 03	LOC106590555, svip, LOC106567674, LOC106580431, LOC106607563, LOC106569608, LOC106583199, LOC106579628, canx, s61a1, LOC106573818, dnajc3, pdia1, LOC106590419, dnja1, LOC106584323, pdia3, LOC106571177, LOC106565722, LOC106567637, sc61a, LOC100380749
Phagosome	20	1.9e − 03	LOC106590555, stx7, stx12, cats, LOC106604294, LOC106569947, LOC106590025, LOC106604765, LOC106604436, LOC106569608, LOC106583199, spd2a, canx, s61a1, LOC106563067, LOC106572477, LOC106573818, LOC106584323, ncf1, sc61a
Pyruvate metabolism	9	1.9e − 03	LOC106563498, LOC100194641, acyp2, pck2, aldh7a1, LOC106561021, ldhb, LOC106593044, LOC106593429
Glycine, serine, and threonine metabolism	7	2.8e − 02	cgl, gamt, aldh7a1, LOC106561021, LOC106573440, LOC106583798, alas2
Citrate cycle (TCA cycle)	7	4.0e − 02	LOC106563498, LOC100194641, pck2, LOC106561021, LOC106593044, LOC106593429, LOC106570380

We also observed that feeding post-smolts high dietary Ax led to a downregulation of pro- and antioxidant mechanisms. For example, the heme-oxygenase HO-1 (encoded by the *hmox1* gene) is known as a major heat shock/stress response protein and its activity could increase during cellular stress (Elbirt and Bonkovsky 1999). The down-regulation of the related gene *hmox1* by Ax seems to reflect an enhanced beneficial antioxidant capacity at the intestinal level.

The guanylate-binding protein (GBP) encoded by the *gbp1* gene is part of a family of GTPases that is induced by interferon (IFN)-gamma. GTPases induced by IFN-gamma (interferon-inducible GTPase) are key to the protective immunity against microbial and viral pathogens. These GTPases are classified into three groups: the small 47-KD immunity-related GTPases (IRGs), the Mx proteins (MX1, MX2), and the large 65- to 67-kd GTPases. GBPs fall into the last class (Tripal et al. 2007). The Ax supplementation could therefore reflect a positive effect as the lower expression of the *gbp1* might decrease inflammation through reduced IFNg production, which seems to be also correlated with the lower expression of the transcription factor IRF1 closely related to *ifng* expression. These genes encode proteins that have already been described as important key molecules in immune response (Zhang et al. 2017). Also closely related to IFNs, MHC class II genes are up-regulated by IFNg by activation of the class II transactivator factor (CIITA) (Jørgensen et al. 2007). The down-regulation of the corresponding gene in this trial could therefore suggest the role of Ax in modulating the inflammatory and anti-viral responses. While nowadays, knowledge on this topic in salmon is scarce; the effect of Ax on the inflammatory response related to human diseases has been the subject of attention of many studies. Amar et al. (2012) and Chang and Xiong (2020) have shown that Ax increased the protection of rainbow trout challenged with infectious hematopoietic necrosis virus. The authors hypothesized that Ax may influence the resistance of rainbow trout to viral pathogen via inhibition of virally induced and ROS-mediated oxidative stress. Ytrestøyl et al. (2021) also showed some inflammation effects in salmon without Ax. However, the modulated genes observed were not the same as in our study, as they described, up-regulations of *ladderlectin* and *gimaps* genes, or genes responsive to virus, such as *trim39-like* genes in salmon fed Ax.

The basic transcription element binding (BTEB) is a transcription factor which is involved in the control of a diverse number of genes, such as the basic transcription element gene (*bte*) in the promoter of the carcinogen-metabolizing cytochrome P4501A1 gene (*cyp1a1*), by repressing its expression (Imataka et al. 1992; Kaczynski et al. 2002). Carotenoids are known to impact the induction of cytochrome P450 (Gradelet et al. 1997; Ohno et al. 2011, 2012). Ax may activate the expression of the cytochrome P450A1 gene, by reducing the expression of its related repressor *bteb,* observed in this study. However, Page and Davies (2002) have demonstrated that dietary Ax does not induce xenobiotic-metabolizing enzyme systems in rainbow trout and suggested species specificity in the ability of such substrates to induce the enzyme systems when compared to results in mammalian studies (Ohno et al. 2011, 2012).

Ax Effect in Liver of Post-Smolt Salmon + Ax vs – Ax

In human and animals, lipids droplets (LDs) are transported in hepatic cells by myosin and dynein proteins along actin filaments or microtubules (Kilwein and Welte

Table 8 Enriched pathways in the muscle of post-smolt salmon fed diets with astaxanthin and parrs, not fed astaxanthin (+ Ax vs Parr)

Description	*Count*	*p. adjust*	*Gene ID*
RNA transport	35	3.0e − 05	4ebp, LOC106604514, rae1, eif3j, LOC100380746, LOC100195680, LOC106578266, LOC106561546, pop5, LOC100380723, eif2b5, LOC106585806, LOC106577905, 4ebp, LOC106579473, LOC106607164, LOC106603332, LOC106580370, nup93, LOC106610815, thoc7, snupn, LOC106607644, LOC106574529, ncbp2, LOC106588091, if4ea, LOC106612879, LOC106577251, LOC100380709, ran1, LOC106582627, LOC106565058, LOC106578327, LOC100136389
Spliceosome	28	1.0e − 03	LOC106596195, u2af2, ppih, LOC106607257, LOC106565348, hnrpg, bcas2, LOC106570281, LOC106612977, LOC106607064, rsmb, snrpa1, dhx8, hnrpm, LOC106566253, sfrs6, ncbp2, LOC106588091, LOC106562047, lsm7, lsm3, LOC106589849, srsf9, lsm5, sf3a1, LOC106580853, prp19, LOC106603659
Necroptosis	30	2.6e − 03	LOC100136558, stat2, hs90a, LOC106605228, glna, ub7i3, LOC106584177, stat4, LOC106561969, LOC106599099, parp1, LOC100136565, LOC106587129, pkz, LOC106577590, frih, tnr1a, irf9, LOC106578020, LOC106584536, LOC106602874, LOC100136564, sqstm, pygb, dnm1l, parp2, LOC106612162, spata2, pygma, LOC106611749
Ribosome biogenesis in eukaryotes	17	3.3e − 03	utp15, pwp2, LOC106582083, nol5, nog2, rexo2, pop5, LOC100380723, nop56, rcl1, if6, LOC100196059, LOC106585140, LOC106588218, LOC106608971, ran1, LOC100136389
Apoptosis	30	4.9e − 03	LOC106572477, LOC106613963, LOC106561969, LOC106607598, LOC106605328, LOC106584280, LOC106562440, cats, casp3, parp1, tba1a, LOC106587129, LOC106601178, LOC106603345, mcl-1, tnr1a, atf4, LOC106578020, LOC106565426, rask, LOC106568974, birc5, parp2, LOC100380862, LOC106612162, LOC106561173, tuba8l2, akt2, LOC106573930, LOC106585195
Glycolysis/gluconeogenesis	16	4.9e − 03	LOC100380728, LOC106565361, pmge, LOC106560437, fbp2, aldh3a2, LOC106589759, LOC100194645, LOC106611602, acss1, pgk, tpi1b, LOC100194624, enoa, ldhb, LOC106588431
Lysosome	23	8.2e − 03	cd68, LOC106605328, LOC106562440, cats, ap3m1, lamp2, LOC106589952, ctsa, gns, ap1m1, gba, LOC106583886, LOC100380629, LOC106604436, LOC106565426, LOC106609887, LOC106567183, ap1s3, mprd, gm2a, LOC106579190, vato, LOC106561173
Phagosome	28	8.2e − 03	ncf1, LOC106572477, LOC106569947, LOC106605328, cats, tap1, tba1a, lamp2, LOC106604294, LOC106563067, LOC106567829, LOC106604765, LOC106583886, itb2, LOC106604436, LOC106567937, canx, tbb5, LOC106603053, vatb2, rab5a, stx7, mprd, LOC106579190, rac1, vato, atp6v1f, tuba8l2
Carbon metabolism	23	1.6e − 02	LOC100380728, LOC106599099, LOC106565361, prps1, rpe, pgp, fbp2, mmsa, LOC106574967, LOC106589759, pgd, LOC106561216, LOC100194645, LOC106611602, acss1, LOC106573440, pgk, tpi1b, LOC100194624, enoa, rgn, LOC106611334, LOC106588431
Proteasome	12	2.4e − 02	psmb8b, psma7, psmb9-b, LOC106571922, LOC106580837, LOC106588399, psme1, LOC106612618, LOC106579706, LOC106601269, LOC106569175, LOC100194647
RNA degradation	15	3.7e − 02	LOC106565361, exos2, exosc10, LOC106588450, LOC100136430, btg2, LOC106604950, LOC106580576, dcps, LOC106566956, exos4, lsm7, lsm3, lsm5, enoa

2019), which were found to be up-regulated in + Ax vs − Ax. Granneman et al. (2017) have proposed that lipid droplets are homologous to carotenoid droplets and are distributed by perilipin motor proteins in fish skin. No information exists regarding the presence of these lipid droplets in other fish tissues.

The plasma retinol-binding protein is involved in the transport of retinol from the liver to the plasma (Alapatt et al. 2013; Johansson et al. 2001) and is regulated by the retinol concentration in the liver. The down-regulation of *rbp2* observed in + Ax vs − As could potentially reflect the high level of retinol from the earlier metabolization of Ax arriving at the liver.

Interestingly, several significant top modulated genes were closely related to the p53 protein. As a transcription factor, p53 can be activated by several signals, such as nutrient DNA damage, oxidative stress, nutrient deprivation, or activated oncogenes (Chen 2016). The genes regulated by the activation of p53 are involved in cell cycle arrest, cellular senescence, antioxidant response, or apoptosis (Chen 2016). P53 has also been described as a key regulator of ferroptosis, a lipid peroxidation-induced cell death (Kang et al. 2019).

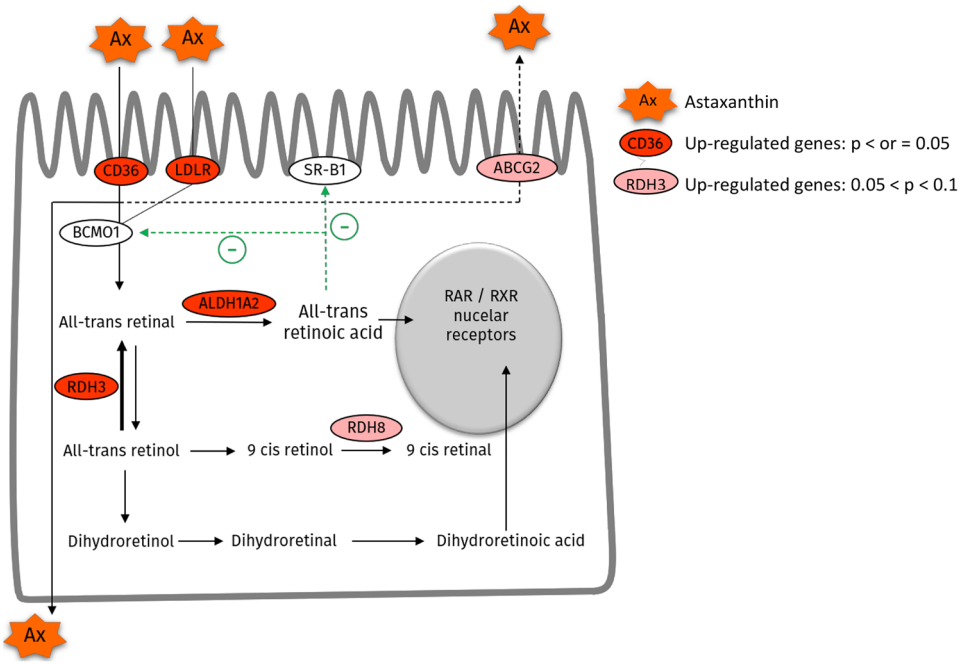

Fig. 7 Proposed summary of modulated genes involved in the carotenoid metabolism in pyloric tissue of Atlantic salmon fed Ax. The genes involved in the transport of Ax, such as *cd36* and potentially *ldlr* at the apical level increased their expression when Ax is present in the intestine. Once inside the cell, either Ax cross the cell reaching the basolateral membrane or its metabolization is initiated firstly by bcmo1 to generate all-trans retinal. Two genes were then up-regulated reflecting either the metabolization into all-trans retinoic acid by the ALDH1A2 to bind to the RAR/RXR transcription factor, or the metabolization into all-trans retinol. Two other genes, *rdh8* and *aldh1a2*, show higher expression to catalyze the transformation of 9 cis retinol to 9 cis retinal or dihydroretinal to dihydroretinoic acid, respectively. This mechanism is regulated by a negative feedback with no modulation of *bcmo1* and *sr-b1*. Another regulation could be possible with higher expression of *abcg2* regulating the efflux back to the intestinal lumen

Upstream of this process, AGR2, encoded by the strongly down-regulated *agr2* gene in + Ax, has been described to inhibit p53 activation, indicating a regulation of the initiation of the further process driven by p53 (Pohler et al. 2004) as described below, and thus reflecting the positive effect of Ax supplementation on this downstream process in the liver, especially on ferroptosis.

Rockfield et al. (2018) suggested that the inactivation of P53 triggers the transcription of *ncoa4*. NCOA4 activates the peroxisome proliferator-activated receptors (PPARs) and co-activates steroid receptors (Kollara and Brown 2012; Tan et al. 2014). Direct links clearly show that carotenoids, and especially Ax, modulate the expression of PPARs, thus regulating the lipid metabolism (Cui et al. 2007; Jia et al. 2012). Our data therefore confirm that the activation of PPARs by Ax would be generated by the up-regulation of *ncoa4* probably from inhibition of p53 through up-regulation of *agr2*. The inhibition of p53 and the subsequent potential activation of NCOA4 was shown to also induce the release of iron from ferritin. This release of iron was described as responsible for the induction of ferroptosis (Latunde-Dada 2017; Santana-Codina and Mancias 2018). The down-regulation of *hmox1*, encoding the heme-oxygenase, strengthens the positive effect of Ax supplementation through the mitigation of the ferroptotic process. Indeed, former evidences suggest that heme-oxygenase, as a cellular stress response protein, would also mediate redox regulation in ferroptosis, via release of iron from heme, thus generating ROS and lipid peroxidation (Kwon et al. 2015; Chang et al. 2018; Chiang et al. 2019). It is well known that supplementation of Ax reduced lipid peroxidation (Karppi et al. 2007; Ni et al. 2015). Interestingly, Ytrestøyl et al. (2021) also observed a modulation of *hmox1* in liver, which in this case, revealed an up-regulation in salmon fed Ax. Nevertheless, based on our results, Ax would therefore impact redox balance also through heme-oxygenase-related pathway (Niu et al. 2018; Wang and Zhuang 2019). Until now, no available data or literature have described possible mechanisms of Ax supplementation on the decrease of lipid peroxidation-induced ferroptosis.

In parallel of the first pathway initiated by p53, a second pathway was impacted by the down-regulation of *sat1*. Previous studies showed that SAT1, activated by p53, could induce lipid peroxidation through the activity of lipid oxygenases (ALOX15) in association with reactive oxygen species (ROS), leading finally to the ferroptotic process (Murphy 2016; Ou et al. 2016), strengthening thus the positive effect of Ax on the reduction of lipid peroxidation.

Altogether, our observations suggest that one of the antioxidant effects of Ax supplementation would be ferroptosis-associated. The effect of Ax on the reduction of lipid peroxidation via the two pathways is described in Fig. 8.

Ax Effect in Muscle of Post-Smolt Salmon + Ax vs – Ax

As the major metabolic tissue of the body, muscle plays an important role in the whole-body metabolism and homeostasis and is therefore responsible, for a large part, of the insulin-induced glucose uptake (Díaz et al. 2009). In muscle, the *fbp1* gene was up-regulated in + Ax vs – Ax and encodes the fructose-1,6-bisphosphatase involved in glycogen synthesis from lactate and in the regulation of glycolysis via futile cycle while it regulates gluconeogenesis in the liver (Marcus and Harrsch 1990; Rakus et al. 2005). The coronin 2A protein is involved in the actin subunit flux leading to an increase of actin network plasticity and filament growth. Actin has been identified as the major Ax-binding protein in Atlantic salmon muscle (Matthews et al. 2006). The dynamics of the actin network are controlled by the action of coronin by spatially targeting Arp2/3 complex and cofilin (Gandhi and Goode 2008). In human muscle cells, Ax enhances insulin sensitivity through IRS-1 activation, that subsequently activates the PI3K, Akt, and the Rho-family GTPase Rac1 (Sztretye et al. 2019). This mechanism has already been described in rat, as being involved in the remodelling of actin network via PAK1 by regulating the Arp2/3 complex and cofilin, and thus having an impact on the translocation of the glucose transporter GLUT4 (Tunduguru et al. 2014). Additionally, Díaz et al. (2009) demonstrated that insulin and IGF-I stimulated the expression of GLUT4 in myoblasts and myotubes of rainbow trout cells, in vitro. Based on these findings in other species and on the up-regulation of the *coro2a* in our study, we could hypothesize that Ax recruits coronin for its transport in salmon muscle cells via actin network polymerization. Ax would induce regulation of the glucose metabolism reflected by increased the expression of *fbp1* as observed in the present study. The Fig. 9 describes these findings and was adapted and completed from the review of Sztretye et al. (2019). The up-regulation of *fbp1* may be potentially due to intracellular glucose uptake leading to either activation of the futile cycle to regulate the glycolysis or activation of gluconeogenesis due to a decrease of glucose in the blood (Newsholme and Crabtree 1970; Jung and Sikora 1984; Rakus et al. 2005; Garrett and Grisham 2010). We can notice that the study of Ytrestøyl et al. (2021) also showed a modulation of the motility-related coronin gene but was found to be down-regulated in salmon fed Ax.

Pooley et al. (2013) studied the recombinant interleukin 1 beta (IL-1β)–induced inflammatory response of primary muscle cell cultures in Atlantic salmon and demonstrates the modulation of the expression of immune-related genes including the down-regulation of TNF alpha gene and the activation of the gene encoding Jun transcription factor. Little is known about Jun-D transcription factor in salmon and even less about its modulation by oxidative stress. In humans, the gene encoding D-Jun transcription factor seems to be down-regulated by oxidative stress and TNFR has been associated with oxidative stress (Fischer and Maier 2015). In our study, the up-regulation of the genes encoding the tumour necrosis factor receptor (TNFR) and the transcription factor D-Jun in post-smolts fed + Ax would however need further studies to determine the modulatory effect of Ax on the response to oxidative stress and related inflammation at the muscle level.

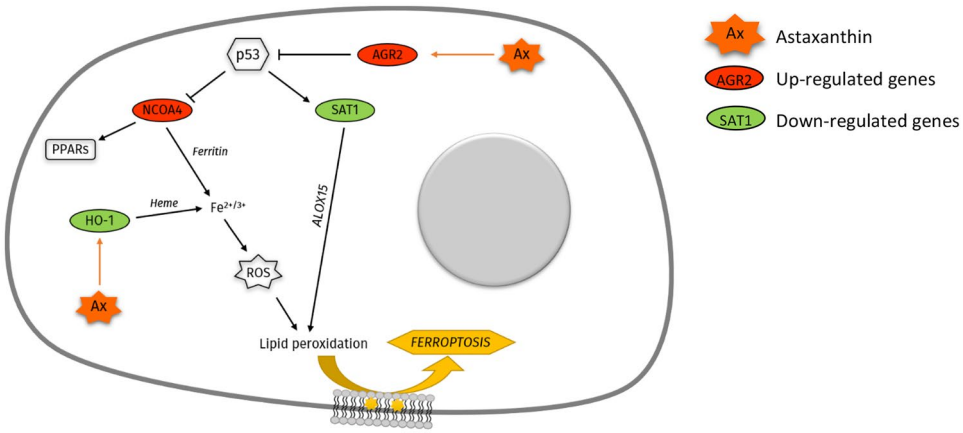

Fig. 8 Proposed summary of modulated genes involved in activation of ferroptosis in liver tissue of Atlantic salmon fed Ax. The Ax in the liver could trigger the up-regulation of *agr2*, regulating thus all the downstream processes driven by p53 leading to ferroptosis as followed: one axis contributes to the up-regulation of *ncoa4* activating PPARs on one side. On the other side, free iron is released from ferritin, balanced by down-regulation of *hmox* (HO-1), limiting the generation of ROS and thus lipid peroxidation. The other modulated axis is characterized by a down-regulation of *sat1*, limiting via ALOX15 the lipid peroxidation-induced ferroptosis

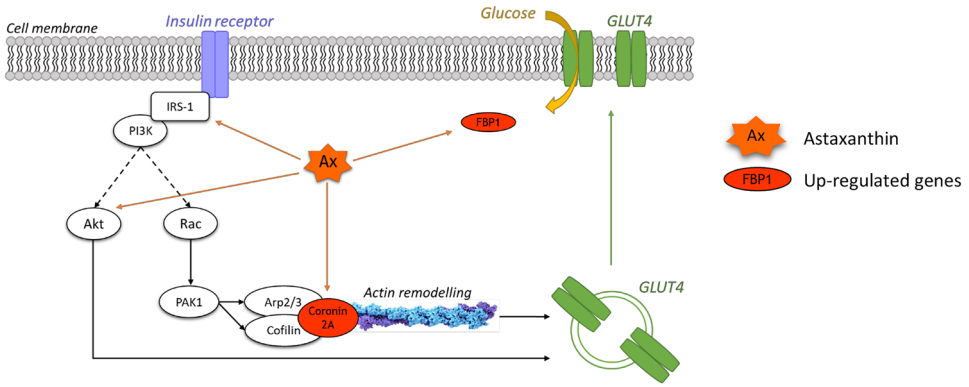

Fig. 9 Proposed involvement of Ax in regulation of glucose uptake in muscular cells (adapted from Sztretye et al. 2019). Ax is known to activate IRS-1/AKT pathway triggering the translocation of GLUT4 in the muscular cell membrane allowing glucose uptake. Here, Ax also induced up-regulation of *fbp1* responsible of glucose homeostasis via gluconeogenesis or glycolysis. Additionally, *coro2a* was up-regulated and participates to the actin remodelling needed for GLUT4 translocation initiated by IRS1/PI3K/AKT pathway

Induction Effect of Ax at Post-Smolt Stage: Relative Impact of Smoltification Only. (+ Ax vs Parr and – Ax vs Parr)

In pylorus, the down-regulation of *bcmo1* in + Ax compared to parr confirms the negative feedback of all-trans RA generated during catabolism of Ax. However, it would be possible that the symmetrical cleavage of Ax into two retinal molecules is of more importance for parrs than post-smolts.

In addition, we observed that smoltification increased *scya113* and *scya109* expression. These CC chemokines have been identified in channel catfish (*Ictalurus punctatus*) and could not be related to any mammalian chemokines. SCYA109 was found only in gills, and SCYA113 was one the chemokines up-regulated in response to a pathogen (*Edwardsiella ictaluri*) and detected in the spleen and head kidney, (Bird and Tafalla 2015). From our study, we could suggest that Ax supplementation mitigates the up-regulation of these chemokine-like genes. However, there is no confirmation of the existence of these chemokines in salmonids.

In the liver, most of the DEG are the expression of the shift from parr to post-smolt life stage. For example, the genes encoding arginase or fucose-binding protein (FBP)-like lectin 4. However, *mmp13*, encoding the collagenase 3, was more up-regulated in + Ax than in – Ax when compared to parr. MMP13 is involved in hepatocyte proliferation and repair (Endo et al. 2011). Collagenase helps to reduce collagen in fibrous tissue leading to an improvement of liver function (Lieber 2004). Such a hepatoprotective effect of Ax related to the reduction of collagen accumulation has been observed in animal models for liver fibrosis (Bae et al. 2018). This up-regulation of *mmp13* would lead to a positive impact of Ax supplementation suggesting that Ax could provide a hepatoprotective effect to the animals in the post-smolt stage.

In muscle, both + Ax vs Parr and – Ax vs Parr comparisons showed again that the major effects observed related to the sea-water life stage only, such as changes in the expression of IFN-stimulated gene factor 15 or α-actinin. Another example is the up-regulation of *cd9*. Dahle and Jørgensen (2019) suggested that this tetraspanin protein (CD9) is involved in the anti-viral response in salmonids. We can speculate that this may be connected to the major challenge that the immune system of post-smolt fish may experience in saltwater (Johansson et al. 2016). Interestingly, the positive impact of Ax through its antioxidant and DNA protection effects is reflected by the stronger up-regulation of the DNA-damage inducible transcript 4-like in + Ax than in – Ax compared to parr.

Pathway Analysis

The pathway analysis was only possible on fully annotated genes of the *Salmo salar* genome with defined gene symbols. Therefore, these results show, only partially, the effects on the gene pathways. This is due to the incomplete identification and annotation of the other modulated genes. However, the number of pathways impacted, as well as the number of DEG in the respective pathways, confirmed the higher impact of Ax supplementation on the host transcriptome at the level of intestinal transport/absorption (pylorus) than at the site of deposition (muscle). Life stage–related differences on the host transcriptome patterns were only observed at muscular level.

The effects of dietary Ax supplementation in post-smolt salmon groups at pylorus level were related to DNA replication/recombination/repair and nucleotide excision repair. This reflects the well-known antioxidant effect of Ax on DNA damage induced by potential oxidative stress (Santocono et al. 2007). The one-carbon (1C) metabolism

pathway, mediated by the folate cofactor was also impacted, supporting multiple physiological processes including biosynthesis (purines and thymidine), amino acid homeostasis (glycine, serine, and methionine), and redox defence. Smoltification effect (− Ax vs Parr) was essentially related to fatty acid metabolism at pylorus level.

In the liver, pathways related to protein processing in endoplasmic reticulum and protein export were affected by smoltification (− Ax vs Parr) and that confirms the observation made earlier from DEG and heatmaps. In muscle, smoltification effect was observed on pathways linked to carbon metabolism, RNA, or ribosome, reflecting potential higher transcription and translation rate after smoltification.

Conclusion

To better understand the molecular mechanisms of absorption, transport, metabolism, and deposition of Ax in Atlantic salmon, Ax-induced was on transcriptome profile of muscle, pylorus, and liver tissues were assessed by comparing post-smolt fed diets supplemented with and without Ax. In addition, we attempted to find evidence of specific changes in Ax metabolism driven by Atlantic salmon life stages by the comparison with salmon parr, not fed Ax. Results demonstrate that pylorus is the major site of the host response to dietary Ax supplementation and metabolism followed by liver and muscle. The post-smolt transformation during the feeding period showed a response with major effects observed at muscular level.

Data suggest that certain molecular mechanisms are triggered at the pyloric caeca level to absorb, transport, and metabolize Ax, which involves several transporters and enzymes that could be linked to cholesterol metabolism. Indeed, enzymes involved in the catabolism of Ax in pylorus are likely retinol dehydrogenase.

Several already known antioxidant effects of Ax on DNA damage were also observed at gene level. Moreover, a potential link appears likely with the ferroptosis process via cellular lipid peroxidation. This mechanism seems to play an important role also in the liver of salmon fed Ax, as we observed a strong modulation of key genes involved in ferroptosis-related lipid peroxidation. This recently described cellular process has not yet been studied in relation to potential Ax effects. Our data clearly demonstrate a beneficial effect of Ax in reducing ferroptosis mechanism. On the basis of these results, it appears that a transport mechanism of Ax to reach muscle likely exists, with the involvement of coronin protein in the intracellular transport of Ax to its deposition in the muscle where it could trigger glucose uptake and homeostasis.

Our data provide insights into a better understanding of molecular mechanisms involved in Ax supplementation. Indeed, key sensitive genes to Ax supplementation could be identified, such as *cd36* in pylorus, *agr2* in the liver, or *fbp1* in muscle. Several hypotheses have been proposed on the basis of this exploratory analysis leading to a better understanding of the molecular mechanisms of Ax transport, absorption, and deposition as well as its beneficial effects in preventing oxidative stress and related inflammation in muscle.

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Availability of Data and Material Microarray data will be deposited in the GEO database.

Declarations

Ethics Approval This study was approved by the appropriate ethics committee to ensure the welfare of animals.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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