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Transcriptional regulation of lipid metabolism when salmon fry switches from endogenous to exogenous feeding

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Abstract

The onset of first feeding is a critical period for Atlantic salmon (*Salmo salar*). The fish goes through a dramatic transition, from using the yolk sac as an energy and nutrient resource to rely on exogenous food. However, the digestive system of salmon is not fully developed at start feeding. This is especially true for digestion and absorption of dietary lipids. To optimize lipid composition of the initial feed for better growth and development of salmon, it is

important to have a systemic understanding of lipid metabolism. The present study combines transcriptomics and lipid composition data to obtain an overview of how lipid metabolism pathways change when salmon switch from endogenous to exogenous feed. Transition to exogenous feeding had higher influence on gene expression and fatty acids composition in pyloric caeca relative to liver, suggesting metabolism is more responsive in pyloric caeca when diet switches. The pathways of phospholipid and lipoprotein synthesis were both up-regulated in pyloric caeca, while phospholipid content unchanged. Because phospholipids are a major component of intestinal lipoproteins, it suggests that a higher level of dietary phospholipids is required for optimal lipid transport at first feeding stages. *De-novo* cholesterol synthesis pathway was up-regulated in pyloric caeca after exogenous feeding, while a down-regulation of bile acid synthesis pathway was found in liver. This suggests a higher requirement of cholesterol in salmon fry after switching to exogenous feeding. The present study has provided new insights on the systemic changes of lipid synthesis and transport pathways in salmon fry when switching from endogenous to exogenous feeding.

Keywords: Atlantic salmon, first feeding, transcriptomic, lipid class, fatty acids, lipid metabolism

1. Introduction

The onset of first feeding is among the most critical stages during the life cycle of salmon, in which the fish switches from endogenous to exogenous feeding and enters a phase of exponential growth. Unlike most marine fish species in aquaculture that require live zooplankton as first feed, salmon have a longer embryonic period and is able to utilize formulated pelleted feed when the yolk sac is depleted (Sahlmann, Gu, Kortner, Lein,

Krogdahl, Bakke, 2015). This simplifies the feeding regimes, but it also represents a challenge to design a feed composed to maximize growth and maintain a healthy development of early life stage. For example, there is still a lack of knowledge on the changes of gene expression and lipid compositions when salmon switches from yolk sac to an exogenous diet.

Lipids are major nutrients for salmon, playing an essential role in energy production, cell structure and metabolic regulation (Sargent, Tocher, Bell, 2002). After feed digestion in the intestinal lumen, lipids are absorbed into enterocytes mainly in the pyloric caeca (Denstadli, Vegusdal, Krogdahl, Bakke-McKellep, Berge, Holm, Hillestad, Ruyter, 2004). The absorbed lipids are then re-constructed and embedded into lipoproteins and then excreted into the circulatory system (Sargent, Henderson, Tocher, 1989). Three main classes of lipids are available in the feed; triacylglycerol provides fatty acids for energy production through β-oxidation processes (Sargent, Henderson, Tocher, 1989), phospholipids are instrumental for many structural and biological functions including cell membranes (Tocher, Bendiksen, Campbell, Bell, 2008), and cholesterol is an essential component of cell membranes. Cholesterol degradation pathways also leads to bile acids which are needed during lipid digestion in the intestine (Yun, Mai, Zhang, Xu, 2011). However, salmon fry may often have insufficient ability to synthesize essential lipids *de-novo* at the stage of first-feeding, which can lead to higher requirement of dietary lipids (Jin, Olsen, Østensen, Gillard, Korsvoll, Santi, Gjuvsland, Vik, Torgersen, Sandve, Olsen, 2018; Sahlmann, Gu, Kortner, Lein, Krogdahl, Bakke, 2015; Taylor, Martinez-Rubio, del Pozo, Walton, Tinch, Migaud, Tocher, 2015). For example, salmon fry require dietary supply of long chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), eicosatetraenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), as they are involved in many biological

functions like maintenance of membrane fluidity and production of eicosanoids, and they are key components of neural tissues (Izquierdo, 1996; Sargent, Tocher, Bell, 2002). Furthermore, traditional salmon feed with low levels of phospholipids have been associated with non-optimal growth and development for early stages of salmon fry as their intestine have insufficient ability to *de-novo* synthesize phospholipids, as a result limit production of lipoproteins for lipid transport (Olsen, Dragnes, Myklebust, Ringo, 2003; Poston, 1990; Taylor, Martinez-Rubio, del Pozo, Walton, Tinch, Migaud, Tocher, 2015).

The lipid metabolism is a highly complex network of interactions and feedback mechanisms. To improve our understanding of the biosynthesis, degradation and transport patterns of different lipid classes in the specific life stage, we need to integrate information from system-wide omics approaches with lipid measurements. A challenge for such approaches at the genetic and enzymatic levels is the whole genome duplications ancestral to all salmonids (Lien, Koop, Sandve, Miller, Kent, Nome, Hvidsten, Leong, Minkley, Zimin, Grammes, Grove, Gjuvsland, Walenz, Hermansen, von Schalburg, Rondeau, Di Genova, Samy, Olav Vik, Vigeland, Caler, Grimholt, Jentoft, Vage, de Jong, Moen, Baranski, Palti, Smith, Yorke, Nederbragt, Tooming-Klunderud, Jakobsen, Jiang, Fan, Hu, Liberles, Vidal, Iturra, Jones, Jonassen, Maass, Omholt, Davidson, 2016). Around 60% of genes involved in salmon lipid metabolism are still retained as functional duplicates, referred to as orthologs, in the genome (Gillard, Harvey, Gjuvsland, Jin, Thomassen, Lien, Leaver, Torgersen, Hvidsten, Vik, Sandve, 2018). The orthologs are believed to perform the same chemical reactions and remain under variable regulatory control in different tissues or at different developmental stages (De Santis, Taylor, Martinez-Rubio, Boltana, Tocher, 2015; Gillard, Harvey, Gjuvsland, Jin, Thomassen, Lien, Leaver, Torgersen, Hvidsten, Vik, Sandve, 2018; Lien, Koop, Sandve, Miller, Kent, Nome, Hvidsten, Leong, Minkley, Zimin, Grammes, Grove, Gjuvsland, Walenz, Hermansen,

von Schalburg, Rondeau, Di Genova, Samy, Olav Vik, Vigeland, Caler, Grimholt, Jentoft, Vage, de Jong, Moen, Baranski, Palti, Smith, Yorke, Nederbragt, Tooming-Klunderud, Jakobsen, Jiang, Fan, Hu, Liberles, Vidal, Iturra, Jones, Jonassen, Maass, Omholt, Davidson, 2016).

In the present study we carried out and compared results of transcriptomic and lipid analyses on pyloric caeca and liver tissues of salmon fry before and after onset of first-feeding. The objectives were to: 1) evaluate the systemic regulation of lipid absorption, biosynthesis, degradation, and transport and 2) identify bottlenecks in lipid metabolism pathways that could reduce utilization of dietary lipids and growth in early stages of salmon. We did this by implementing a metabolic network level approach by computing the combined transcript abundance from salmon orthologs and using this new "sum of transcripts" measurement to overcome the challenges of gene redundancy in the partially tetraploid salmon genome.

2. Material and methods

2.1 Fish, diet and sampling procedure

The study was carried out within the Norwegian animal welfare act guidelines, in accordance with EU regulation (EC Directive 2010/63/EU), approved by the Animal Ethics and Welfare Committee of the Norwegian University of Science and Technology (case number 16/10070). A fast-growing strain of Atlantic salmon was provided by the breeding company AquaGen AS (Trondheim, Norway). The eggs were hatched and cultivated at Ervik hatchery (Frøya, Norway). When the yolk sac was depleted, the fish were fed a normal commercial diet (Skretting AS, Norway) which satisfied the nutritional requirement of salmon. Thirty

individuals (0.2g) were sampled both at yolk sac stage just before initiation of first feeding (unfed salmon) and at fry stage ~20 hours after first feeding (fed salmon). The fish were sacrificed by exposure to 40mg/L Benzocaine (BENZOAK VET, ACD Pharmaceuticals AS, Oslo, Norway). All sampled fed salmon contained feed in their intestine, which was carefully squeezed out by using back of the No.11 scalpel blades (Swann Morton Ltd, Sheffield, UK) before further sampling of tissues. Pyloric caeca and liver of both fed and unfed salmon were immediately dissected and placed in 1mL RNAlater for RNA-seq analysis or immediately put into dry ice for lipid analysis. The dissection process of each fish took 3 minutes in average. Tissues in RNAlater were stored for 24 hours at 4°C for sufficient penetration of the solution and then transferred to -80°C before further analysis.

2.2 Lipid class and fatty acid analysis

Tissues from five individuals were merged to obtain sufficient lipid for analysis. Total lipid was extracted from two replicate pooled samples (2 x 5 fish in each group) based on the method of Folch, Lees, Stanley (1957). Extracted total lipid was then applied onto 10 x 10 cm silica plates (Merck, Darmstadt, Germany) for lipid class separation (Olsen, Henderson, 1989). For lipid class analysis, the plates were sprayed with 3% cupric acetate in 8% phosphoric acid, followed by charring at 160℃ for 20 min (Olsen, Henderson, 1989). Subsequently, the plate was imaged on G:BOX Chemi XX6 (Syngene, Cambridge UK) with Epi LED white lights. The images were quantified using GeneTools image analysis software (Syngene, Cambridge UK). For fatty acids analysis of the major lipid classes, the plates were exposed to iodine vapor and bands were marked with a pencil. Lipid bands of phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn) and triacylglycerol (triacylglycerol) were separately sampled for fatty acid analysis (Li, Olsen, 2017). Fatty acid

methyl esters (FAME) of each lipid class were prepared by acid-catalyzed transesterification at 50˚C for 16 hours (Christie, 1973) before quantified by a Agilent 7890B gas chromatograph with flame ionization detector (Agilent Technologies, Santa Clara, CA). The quality of the fatty acids and lipid class were checked before further analysis, where no sign of oxidized lipids was observed on silica plates or fatty acid chromatogram.

2.3 RNA extraction and sequencing

Tissues from five individuals were pooled to provide enough RNA for sequencing. Four biological replicates (4 x 5fish) were used in each group. The RNA extraction and library preparation was completed in Centre for Integrative Genetics (CIGENE), Ås, Norway. Total RNA was extracted by using RNeasy Plus Universal Kits (QIAGEN, Hilden, Germany), followed by library preparation using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA). RNA concentration and quality were assessed by Nanodrop 8000 (Thermo Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples were sequenced using 100bp single-end high-throughput mRNA sequencing (RNA-seq) on Illumina Hiseq 2500 (Illumina, San Diego, CA, USA) in Norwegian Sequencing Centre (Oslo, Norway).

2.4 Analyses of gene expression and lipid data

The raw sequences are publicly available on ArrayExpress under accession number E-MTAB-7312. The method for handling RNA-sequencing (RNA-seq) data has been described in detail in a previous study (Jin, Olsen, Østensen, Gillard, Korsvoll, Santi, Gjuvsland, Vik, Torgersen, Sandve, Olsen, 2018). Read sequences were quality trimmed

before being aligned to the salmon genome (ICSASG_v2). Raw genes counts were generated using HTSeq-counts (v0.6.1pl) and the NCBI salmon genome annotation (available for download at http://salmobase.org/Downloads/Salmo_salar-annotation.gff3).

The RNA-seq analysis was performed in R (v3.4.1). Only genes with a minimum counts level of at least 1 count per million (CPM) in two or more samples were kept for differential expressional analysis (DEA). DEA was performed separately on pyloric caeca and liver by comparing fed and unfed stages of salmon, using R package edgeR. Genes with a false discovery rate (FDR), an adjusted *p* value (*q*) <0.05 were considered to be differentially expressed genes (DEGs) between the two test conditions. Subsequently, a KEGG ontology enrichment analysis (KOEA) was conducted using same package. Significant values ($p<0.05$) were generated based on number of DEGs compared to total genes annotated to each KO term. For visually comparing expression levels between different genes and tissues, normalized counts in the form of transcripts per million (TPM) values were generated. Raw gene counts were first divided by their mRNA length in kilobases to normalize for transcript length, and then divided by the total number of counts from each library to normalize for sequencing depth (Jin, Olsen, Østensen, Gillard, Korsvoll, Santi, Gjuvsland, Vik, Torgersen, Sandve, Olsen, 2018).

The present study had a special focus on the lipid metabolism pathway, where 353 salmon lipid genes were selected from previous ortholog annotations to KEGG database terms (Gillard, Harvey, Gjuvsland, Jin, Thomassen, Lien, Leaver, Torgersen, Hvidsten, Vik, Sandve, 2018). The list of lipid genes with their names and NCBI identifiers is shown in Supplementary File 1. For generating a systemic overview of lipid metabolism pathways, a combined measurement of the expression was generated by summarizing the relative change

of all genes in each chemical reaction of each tissue, and the relative change of each gene (X) was generated by multiplying its relative TPM value (relative TPM of the highest expressed gene in the group) by log2 fold change (Log2FC). The names of the gene group are shown in capital italic letters. The equation to calculate summarized expression is described as follows:

$$Sum\ of\ expression\ (X) = \sum^{n} TPM\frac{X_k}{(X)} * Log2FC(X_k)$$

The analysis of lipid class and fatty acid data was also performed in R (v3.4.1) by using one-way ANOVA with Tukey's multiple comparison test, and differences were considered significant when $p < 0.05$. A pathway diagram of gene expression and lipid composition data was drawn manually using Pathviso (v3.2.4).

3. Results

3.1 Lipid and fatty acid composition of pyloric caeca and liver

The onset of exogenous feeding of an external diet had large influence on the compositions of lipid classes in salmon pyloric caeca and liver (Table 1). In pyloric caeca, the percent of neutral lipids increased significantly (p<0.05) following feeding. This was due to increased monoacylglycerol and free fatty acid contents. Other neutral lipids like triacylglycerol and cholesterol were not changed. The percent of total polar lipids decreased in pyloric caeca following external feeding, this mainly as a result of increased neutral lipids. The composition of polar lipids was unchanged when the fish switched to exogenous feeding.

In contrast, neutral lipid contents decreased in liver after exogenous feeding. This included reductions in triacylglycerol that were reduced by more than 50%. Other neutral lipids like monoacylglycerol and free fatty acids increased, while cholesterol remained unchanged. The composition of total polar lipid was increased after feeding. Regarding the composition of polar lipids, phosphatidylinositol and sphingomyelin increased slightly after external feeding while the lyso-phospholipids decreased. Other polar lipids were unchanged.

First-feeding on exogenous diets changed the composition of fatty acid of salmon pyloric caeca and liver (Table 2), and the fatty acids composition in both tissues changed towards the composition of the diet. The change was generally higher in pyloric caeca than in liver. Regardless of tissue, fatty acid composition of triacylglycerol had clear resemblance with that of the diet, but with some notable differences. Most polyunsaturated fatty acids (18:2n-6, 20:4n-3, 22:5n-3, and DHA) decreased both in pyloric caeca and liver after external feeding, but the amount of EPA was stable. More interestingly, both ARA and 18:4n-3 increased in pyloric caeca after feeding, while no change was observed in liver. Saturated fatty acids (14:0, 16:0, and 18:0) of triacylglycerol all increased in pyloric caeca after feeding, while only 16:0 and 18:0 increased in liver. The major monounsaturated fatty acid 18:1n-9 decreased both in pyloric caeca and liver. The amount of 16:1n-7 increased in pyloric caeca, but decreased in liver after initial feeding.

In general, the percent composition of fatty acids in PtdCho and PtdEtn was less influenced by external diet compared to the fatty acid composition in triacylglycerol. In PtdCho, ARA and 18:4n-3 increased in pyloric caeca but remained stable in liver. The same pattern was found for linoleic acid (18:2n-6), which increased both in pyloric caeca and liver following initial feeding. Furthermore, DHA decreased in PtdCho of liver, but not in pyloric caeca. The

saturated fatty acids (14:0, 16:0, and 18:0) in PtdEtn all increased in liver, but not in pyloric caeca. The percent of DHA in PtdEtn also decreased only in liver.

3.2 Differential gene expression and KEGG ontology enrichment analysis

An average of 15 million reads in total were mapped on to the salmon genome ICSASG_v2. From a total of 81597 annotated genes, 30970 genes passed the minimum level of read counts for differential expression analysis (DEA). In general, 930 differential expressed genes (DEGs, q<0.05) were identified in pyloric caeca when the salmon switched from endogenous to exogenous feeding, while 4050 DEGs were identified in liver.

The KEGG ontology enrichment analysis (KOEA) show that the introduction of feed significantly (p<0.05) regulated similar numbers of KEGG pathways in pyloric caeca and liver of salmon (Figure 1). Higher numbers of up-regulated than of down-regulated pathways were found in pyloric caeca after external feeding, whereas in liver more down-regulated pathways were identified (Supplementary File 2). Furthermore, pyloric caeca contained higher numbers of regulated pathways involved in amino acid and lipid metabolism, whereas liver was more active in carbohydrate, cofactors and vitamins metabolism pathways. Regarding the pathways of lipid metabolism, 6 pathways were significantly (p<0.5) up-regulated in pyloric caeca, while 2 down-regulated pathways were identified in liver. These pathways included fatty acid, sphingolipid and glycerophospholipid metabolisms in pyloric caeca and glycerolipid metabolism and steroid biosynthesis in liver.

3.3 Systemic change of pathways involved in lipid metabolism

The change from endogenous to exogenous feeding resulted in a significant (q<0.05) regulation of many genes related to lipid metabolism in pyloric caeca and liver of salmon (Supplementary File 3). In general, more DEGs related to lipid metabolism were found in liver than in pyloric caeca, in agreement with the total DEG differences between the two tissues. However, by using the sum of expression of duplicates involved in the same chemical reaction, we found higher changes of lipid metabolism pathways in pyloric caeca than in liver (Figure 2). This is in agreement with KOEA result where pyloric caeca was found to be more active in lipid metabolism. By combining results of lipid class and gene expression, we could estimate the systemic changes of lipid metabolism in pyloric caeca and liver of salmon after the onset of external feeding.

In pyloric caeca, the pathway of beta-oxidation was slightly up-regulated in salmon fry after onset of first feeding, though DEGs were only identified in the last step of the pathway. This could relate to the increased percent of fatty acids in pyloric caeca of salmon after feeding on external diet. The composition of PtdCho, PtdEtn and PtdSer was unchanged in pyloric caeca after external feeding, whereas the pathways for *de-novo* and lyso- synthesis of the phospholipids were up-regulated. The pathways for *de-novo* synthesis of cholesterol was up-regulated in pyloric caeca after onset of first feeding, and 5 DEGs were identified in the pathway. The subsequent pathways, which consume cholesterol for synthesizing cholesterol ester or bile acid, were also up-regulated after external feeding. However, the percentage of cholesterol in the pyloric caeca was not changed. An increase in monoacylglycerol was found in pyloric caeca of salmon after external feeding, but the pathway for synthesizing triacylglycerol from monoacylglycerol was strongly down-regulated. The expression of *MTP*, *APOA1*, *APOA4*, *APOB-I* and *FABP10* involved in intestinal lipoprotein formation was all up-regulated in pyloric caeca. Apart from *FABP10*, other genes encoding fatty acid binding

proteins such as *FABP1*, *FABP2*, *FABP3*, *FABP6* and *FABP7*, were all down-regulated after feeding. Genes encoding transcriptome factors, *PPARB* and *SREBP2*, were up-regulated after external feeding, while *PPARG*, *RXRA* and *SREBP1* were down-regulated.

The pathway of beta oxidation was also up-regulated in liver. This was in line with the increased content of free fatty acids after onset of first feeding. The *LPCAT* was also up-regulated in liver as in pyloric caeca, though the contents of PtdCho, PtdEtn, PtdSer were unchanged and the content of lyso-phospholipids was decreased. Neither the content of cholesterol nor the pathway for *de-novo* synthesis of cholesterol was changed in liver. However, a strong down-regulation of *CYP7A1* was found in fed salmon compared to unfed, suggesting a down-regulated pathway for synthesizing bile acid from cholesterol. The *ABCB11* involved in export of bile acid was slightly up-regulated in liver. Following the decreased percentage of triacylglycerol in liver after external feeding, the *MTP* involved in transporting triacylglycerol was up-regulated. The *APOA1* was also up-regulated after external feeding, suggesting an increased number of apolipoprotein A1 produced in liver. The genes involved in fatty acid transport proteins like *FATP2*, *FATP4* and *FATP6* were all down-regulated in liver after onset of first feeding. For genes encoding transcriptome factors in liver, *PPARG* and *SREBP1* were down-regulated after external feeding, while *PPARA*, *RXRA* and *RXRG* were up-regulated.

4. Discussion

The main objective of the present study was to explore the changes of lipid metabolism in salmon fry caused by the change from endogenous feeding to exogenous feeding on an external diet. By integrating transcriptomic, fatty acid and lipid class data, we identified many

changes in the metabolism of fatty acids, phospholipids, triacylglycerol and cholesterol in pyloric caeca and liver of salmon fry after onset of exogenous feeding. The gene expression changes were mostly correlated to the difference in lipid and fatty acid composition of salmon before and after exogenous feeding. However, several uncorrelated relationships were also found between gene expression and lipid composition, suggesting a certain extent of active control on lipid metabolism in pyloric caeca and liver of salmon fry. Furthermore, the levels of gene expression and fatty acid changes were both higher in pyloric caeca than in liver, suggesting pyloric caeca to be more active in lipid metabolism when the fish change from endogenous to exogenous feeding.

The present study was the first to report lipid class and fatty acid analysis for pyloric caeca and liver separated from salmon fry body tissues at the first-feeding stage (Gillard, Harvey, Gjuvsland, Jin, Thomassen, Lien, Leaver, Torgersen, Hvidsten, Vik, Sandve, 2018). Compared to triacylglycerol, phospholipids are mostly structural lipids and are often less influenced by the fatty acid composition of the diet (Tocher, Bendiksen, Campbell, Bell, 2008). Therefore, it was surprising to see the rapid incorporation of dietary fatty acids into the phospholipids, especially in the phospholipids of liver, 20 hours after start of feeding. The level of incorporation was, however, very low. It is generally believed that LC-PUFA like DHA and EPA are selectively retained in the phospholipids (Stubhaug, Lie, Torstensen, 2007). This is in agreement with the present finding of less changes in DHA and EPA contents in phospholipids than in triacylglycerol, after onset of first feeding. Moreover, the content of DHA in phospholipids was stable in pyloric caeca, while it slightly decreased in liver after onset of feeding. This was probably a result of an increasing amount of newly synthesized PtdCho and PtdEtn in pyloric caeca, which selectively esterify LC-PUFA from the total fatty acids pool (Tocher, Bendiksen, Campbell, Bell, 2008). All salmon tissues, including pyloric

caeca and liver, are capable of elongating and desaturating linoleic acid 18:2n-6 or linolenic acid 18:3n-3 to the respective C20 and C22 fatty acids (Sargent, Tocher, Bell, 2002; Tocher, 2015). However, the pathway of elongation and desaturation showed little change after external feeding. This is probably because the capacity of synthesizing LC-PUFA through elongation and desaturation is very low at first feeding stage.

The process of lipid digestion and absorption in salmon is believed to be similar to that of mammals (Tocher, Bendiksen, Campbell, Bell, 2008). Dietary triacylglycerol is broken down by lipases in the intestinal lumen and absorbed into intestinal enterocytes in the form of free fatty acids and monoacylglycerol (Li, Olsen, 2017). The enterocytes contain monoacylglycerol- and diacylglycerol-acyltransferase (encoded by *MOGAT* and *DGAT* genes), which can re-synthesize absorbed monoacylglycerol to triacylglycerol (Li, Olsen, 2017).

However, there is likely an upper-limit for the enterocytes to store triacylglycerol. Overloaded triacylglycerol in enterocytes seemed to suppress *MOGAT* and *DGAT* genes and cause accumulation of monoacylglycerol, as observed in the present study. This is controlled by peroxisome proliferator-activated receptor gamma (PPARγ) encoded by the *PPARG* genes which protect against excess triacylglycerol accumulation by suppressing expression of *MOGAT* and *DGAT* (Greenstein, Majumdar, Yang, Subbaiah, Kineman, Cordoba-Chacon, 2017). The accumulation of triacylglycerol will form lipid droplets in enterocytes and is suggested to be caused by insufficient synthesis of the lipoprotein for exporting triacylglycerol into circulatory system (Olsen, Dragnes, Myklebust, Ringo, 2003; Tocher, Bendiksen, Campbell, Bell, 2008). This was supported by the present study, as we observed up-regulated pathways for synthesizing PtdCho, PtdEtn and lipoprotein in pyloric caeca after

onset of first feeding. However, the percent of PtdCho and PtdEtn, the major component of lipoprotein, was stable in pyloric caeca. This suggests a dietary requirement of phospholipid for salmon fry, as the capacity of *de-novo* synthesizing phospholipid in enterocytes is not sufficient (Abdulkadir, Tsuchiya, 2008; Jin, Olsen, Østensen, Gillard, Korsvoll, Santi, Gjuvsland, Vik, Torgersen, Sandve, Olsen, 2018; Poston, 1990; Taylor, Martinez-Rubio, del Pozo, Walton, Tinch, Migaud, Tocher, 2015).

After entering the plasma membrane, free fatty acids bound to fatty acid binding proteins (FABPs) are transported to cell compartments. Many FABPs are identified which have different activities among tissues and difference preference among fatty acids and other lipids like monoacylglycerol, lyso-phospholipids and bile acids (Vance, Vance, 2016). Many genes encoding FABPs was changed in pyloric caeca and liver of salmon fry after onset of first feeding. The *FABP10* gene is annotated as a paralog of the *FABP1* gene (encoding liver fatty acids binding protein, L-FABP) and is only found in vertebrates (Sharma, Liu, Thisse, Thisse, Denovan-Wright, Wright, 2006). Our study also found a high expression of *FABP10* gene in both pyloric caeca and liver. More interestingly, the *FABP10* gene was strongly up-regulated in pyloric caeca after onset of first feeding, while all other *fabp* genes were down-regulated. This is consistent with that the L-FABP, encoded by *FABP10*, can also bind monoacylglycerol and lyso-phospholipids, which are the major lipids the fish absorb from diets (Lagakos, Guan, Ho, Sawicki, Corsico, Kodukula, Murota, Stark, Storch, 2013). Furthermore, L-FABP also plays a crucial role in initiating the budding of newly formed pre-lipoprotein from endoplasmic reticulum membranes of intestinal enterocytes, which then are transported to the Golgi apparatus for further maturation (Neeli, Siddiqi, Siddiqi, Mahan, Lagakos, Binas, Gheyi, Storch, Mansbach, 2007). This is also consistent with the up-regulation of genes involved in lipoprotein synthesis in pyloric caeca.

Like all vertebrates, the cholesterol homeostasis in salmon is often achieved by balancing dietary intake, *de-novo* synthesis in the body and excretion as bile acids. After external feeding, salmon seemed to have higher requirement of cholesterol, as the *de-novo* synthesis pathway was up-regulated in pyloric caeca and the cholesterol 7 alpha-hydroxylase (*CYPT7A1*) gene for bile acid synthesis was down-regulated in liver. These were likely regulated by Farnesoid X receptor (FXR) and retinoid X receptor alpha (RXR) which participate in cholesterol homeostasis (Ananthanarayanan, Balasubramanian, Makishima, Mangelsdorf, Suchy, 2001) and both genes were up-regulated in liver of salmon after external feeding. However, the cholesterol *de-novo* synthesis pathway was unchanged in liver, which might due to the lower activities of lipid metabolism genes in liver.

The present study used "summed expression of homologous genes" instead of traditional "fold change of single gene" to describe the changes of gene expression in lipid metabolism pathways when salmon switches from endogenous to exogenous feeding. The "sum of expression" provided a general change of gene expression of each reaction in lipid metabolism pathways, which helps to achieve a systemic overview of lipid metabolism changes in pyloric caeca or liver of salmon when switching from endogenous to exogenous feeding. On the other hand, we are aware that the different expression and regulation of homologous genes are likely due to differences in function, subcellular localization, and developmental stages of tissues (Gillard, Harvey, Gjuvsland, Jin, Thomassen, Lien, Leaver, Torgersen, Hvidsten, Vik, Sandve, 2018; Jin, Olsen, Østensen, Gillard, Korsvoll, Santi, Gjuvsland, Vik, Torgersen, Sandve, Olsen, 2018). The significantly regulated single genes (DEGs) were also taken into consideration in the description of the general changes of lipid metabolism pathway after exogenous feeding.

This study is the first to compare gene expression and lipid composition before and after the onset of exogenous feeding in salmon. A rapid change was found in the lipid metabolism of pyloric caeca and liver, as reflected by both gene expression and lipid composition. The PtdCho and PtdEtn and lipoprotein synthesis pathways were up-regulated in pyloric caeca after external feeding while the percent of PtdCho and PtdEtn was unchanged. A up-regulation of *de-novo* cholesterol synthesis pathway was found after external feeding, while the bile acid synthesis pathway was down-regulated. Based on these results, we suggest that salmon fry might have higher dietary requirement of phospholipid and cholesterol. Future studies could focus on manipulation of the composition of the salmon diet based on physiological requirement. We also suggest future feeding trials on dietary requirement of phospholipids and cholesterol in salmon fry, as the fish might have insufficient capacities of phospholipid and cholesterol synthesis after external feeding.

Author Contributions

Yang Jin, Yngvar Olsen, Rolf Erik Olsen, Simen Rød Sandve and Olav Vadstein designed and performed the research. Yang Jin, Gareth Benjamin Gillard and Thomas Nelson Harvey performed the transcriptomic analysis. Yang Jin and Keshuai Li performed the lipid and fatty acid analysis. Jon Olav Vik and Simen Rød Sandve guided the transcriptomic analysis and revised the manuscript. Yngvar Olsen and Rolf Erik Olsen guided the lipid analysis and revised the manuscript. Mari-Ann Østensen and Nina Santi provided input on the experimental design, carried out the experiment and sampling and reviewed the manuscript. All authors participated in the revision of this paper by providing comments and editing.

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Table 1 Lipid class composition of pyloric caeca and liver in unfed yolk sac alevin and fed fry. Numbers indicate percentage contents of total lipid.

	Pyloric caeca		Liver	
	alevin	fry	alevin	fry
Percent of total lipids				
Total neutral lipids	38.7±1.1	44.5±1.8*	40.8±0.7	24.4±3.6*
Total polar lipids	61.3±1.1	55.5±2.3*	59.2±0.7	69.9±3.1*
Triacylglycerol	20±1.3	19.3±0.5	26.5±0.2	11.5±1.1*
Free fatty acids	2.4±0.1	4.8±0*	1.6±0.1	2.4±0.1*
Cholesterol	13.3±0.2	12.2±0.8	9.5±0	10.9±0
Monoacylglycerol	1.6±0.1	5.1±0.3*	1.3±0.2	3.8±1.2*
Other neutral lipids	1.3±0.1	2±0.3	1.4±0.1	1±0.4
Percent of total polar lipids				
Lyso-phospholipids	3.1±0.2	5.4±1.4	5.6±0.5	3.6±0.2*
PtdCho	34.2±1.3	33.1±3.9	40.3±0.1	39.6±1.1
PtdEtn	19.6±0.4	19.2±0.8	20.6±0.6	21.2±0.3
Phosphatidic acid	6.5±1.2	8.2±0.1	9±1	8±0
Phosphatidylglycerol	9.7±0.7	8.2±0.5	8.3±0.5	10.4±0.6
Phosphatidylinositol	10.5±1.3	9.6±0	6.4±0.1	7.6±0.3*
Phosphatidylserine	6±0	7.3±1.9	1.8±0.4	1±0
Sphingomyelin	10.4±0.4	9±0.9	8±0	9.6±0*

* Significantly (p<0.05) different lipid class composition in fed salmon fry compared to unfed alevin in Pyloric caeca and Liver tissues.

PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine

Table 2 FA composition in phosphatidylcholine (PtdCho) phosphatidylethanomine (PtdEtn) and triacyclglycerols (triacylglycerol) of pyloric caeca and liver of unfed alevins and fed salmon fry. Total FA composition in the diet is also shown.

	PtdCho				PtdEtn				triacylglycerol				
	Pyloric caeca		Liver		Pyloric caeca		Liver		Pyloric caeca		Liver		
	alevin	fry	alevin	fry	alevin	fry	alevin	fry	alevin	fry	alevin	fry	Diet
14:0	1.2±0.1	0.8±0.1	1.2±0.4	1.0±0.4	0.3±0.0	0.3±0.0	0.2±0.0	0.7±0.1*	1.9±0.3	3.4±0.2*	1.8±0.1	1.9±0.3	6.2±0.1
16:0	30.7±0.9	28.2±0.4	28.0±0.4*	29.4±0.4*	14.7±0	15.2±0.3	11.2±0	13.2±0.3*	11.8±0.3	16.2±0.2*	10.2±0.1	13.3±0*	20.0±0.7
18:0	5.3±0	5.1±0.1	3.7±0	4.8±0.8	8.8±0.2	9.3±0.1	5.2±0	7.3±0.4*	6.3±0.2	7.5±0.5	5.9±0.1	9.8±0.2*	3.7±0.2
16:1n-7	1.2±0.1	1.3±0.1	2.0±0	1.7±0.2	0.7±0	0.7±0	0.8±0	0.7±0*	5.2±0	8.1±0.4*	4.8±0	3.8±0.2*	6.3±0
18:1n-9	10.8±0.1	10.7±0.5	11.6±0	12.5±0.6	10.9±0.2	10.7±0.1	12.1±0	12.1±0.1	27.0±0.5	22.9±1.1*	27.0±0.5	25.0±1.3*	11.8±0.2
18:1n-7	1.6±0	2.0±0*	1.6±0	1.7±0.1	4.6±0.1	5.2±0.2	4.1±0	4.2±0.4	4.4±0.1	5.8±0.8	4.2±0.1	6.5±1	3.4±0.1
20:1n-9	0.3±0	0.4±0	0.2±0	0.2±0	1.0±0.1	1.0±0.2	0.5±0	0.5±0	1.0±0	1.3±0.2	0.9±0	0.7±0*	1.1±0
22:1n			0.1±0	0.2±0		0.2±0							

-9	0.2±0	0.1±0*			0.4±0		0.1±0	0.3±0.	0.1±0	0.3±0*	0.1±0	0.4±0.	0.5±0
					.3			1*				1*	
22:1n		0.0±0	0.0±0	0.0±0		0.0±0		0.0±0					
-11	0.0±0				0.0±0		0.0±0		0.1±0	0.5±0.	0.1±0	0.1±0.	1.3±0
										1*		1	
18:2n			2.2±0			4.5±0							
-6	1.5±0	1.8±0.		2.5±0.	4.4±0		6.9±0	7.0±0.	7.1±0	6.5±0*	7.6±0	6.7±0.	7.6±0
		1*		1*	.1		.1	2				1*	
18:3n			0.0±0							0.1±0			
-6	0.1±0	0.4±0.		0.4±0.	0.4±0	0.3±0.	0.4±0	0.6±0.	0.3±0		0.1±0	0.6±0.	0.3±0
		3		4	.5	4	.1	3	.1			1*	
20:2n		0.3±0	0.2±0	0.2±0		0.7±0		0.7±0				0.7±0	
-6	0.2±0				0.7±0		0.8±0		0.7±0	0.6±0*	0.7±0		0.2±0
					.1								
20:3n		0.4±0	0.3±0			0.5±0		0.4±0					
-6	0.3±0			0.3±0.	0.4±0		0.4±0		0.4±0	0.3±0*	0.5±0	0.5±0.	0.0±0
				1								2	
20:4n			1.9±0	1.9±0		2.4±0		2.1±0				1.4±0	
-6	1.6±0	1.9±0*			2.2±0		2.2±0		1.4±0	1.6±0*	1.4±0		0.9±0
					.1								
18:3n		0.5±0	1.1±0			0.8±0							
-3	0.4±0			1.1±0.	0.8±0		2.2±0	2.0±0.	2.5±0	1.8±0.	2.7±0	1.9±0.	1.2±0
				1	.1		.1	1	.2	4		3	
18:4n			0.1±0	0.1±0									
-3	0.0±0	0.2±0*			0.0±0	0.1±0*	0.0±0	0.1±0*	0.5±0	1.2±0.	0.3±0	0.3±0.	1.9±0
										2*	.1	1	
20:3n		0.1±0	0.1±0	0.1±0		0.2±0		0.3±0					

-3	0.1±0				0.2±0		0.3±0		0.3±0	0.2±0*	0.4±0	0.2±0*	0.0±0
20:4n			0.7±0	0.6±0		0.6±0							
-3	0.6±0	0.6±0.			0.6±0		0.9±0	0.7±0*	2.1±0	1.2±0.	2.3±0	1.5±0.	0.5±0
		1							.1	1*	.1	2*	
20:5n			10.4±0										13.0±
-3	7.8±0	8.6±0.		9.8±0.	4.7±0	4.5±0.	5.7±0	5.2±0.	7.3±0	6.1±0.	8.5±0	7.9±1.	0.1
	.2	3		4	.1	1	.1	2	.3	6	.3	1	
22:5n								3.4±0					
-3	3.1±0	3.0±0.	3.7±0.	3.3±0.	2.9±0	2.5±0.	3.6±0		5.8±0	3.6±0.	6±0.1	4.6±0.	1.3±0
	.1	1	1	1		1*			.2	2*		3*	
22:6n	30.6±	30.6±0		26.7±1	40.5±	39.6±0	42.2±	37.9±1	13.1±	10.0±0	14.1±	12.2±0	
-3	0.9	.4	29.1±0	.3	0.1	.2	0.1	*	0.3	.1*	0.2	.2*	9.2±0
			.1										.1
24:1n						0.1±0		0.1±0					
-9	2.0±0	2.4±0.	1.6±0.	1.3±1.	0.1±0		0.1±0		0.2±0	0.3±0*	0.1±0	0.2±0*	0.4±0
	.2	4	1	6									

* Significantly (p<0.05) different FA composition in fed salmon compared to unfed.

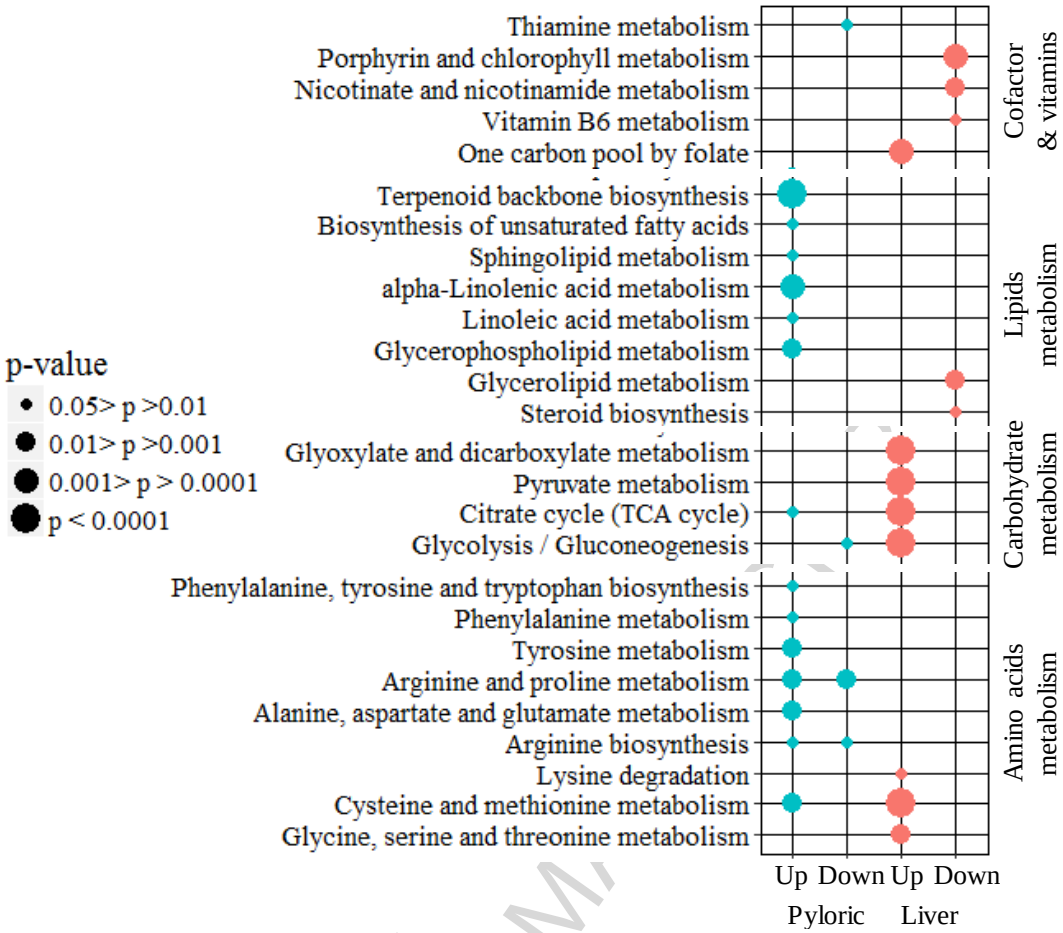

Figure 1 Bubble graph plotting of KEGG ontology enrichment analysis (KOEA) results on pyloric caeca and liver of salmon. Various KEGG pathways involved in lipid, carbohydrate, amino acids and vitamins metabolism were significantly (p<0.05) regulated in salmon fry after initial feeding compared to unfed salmon. Statistical test was applied using hypergeometric test based on number of differential expressed genes versus total genes annotated to each KEGG ontology term.

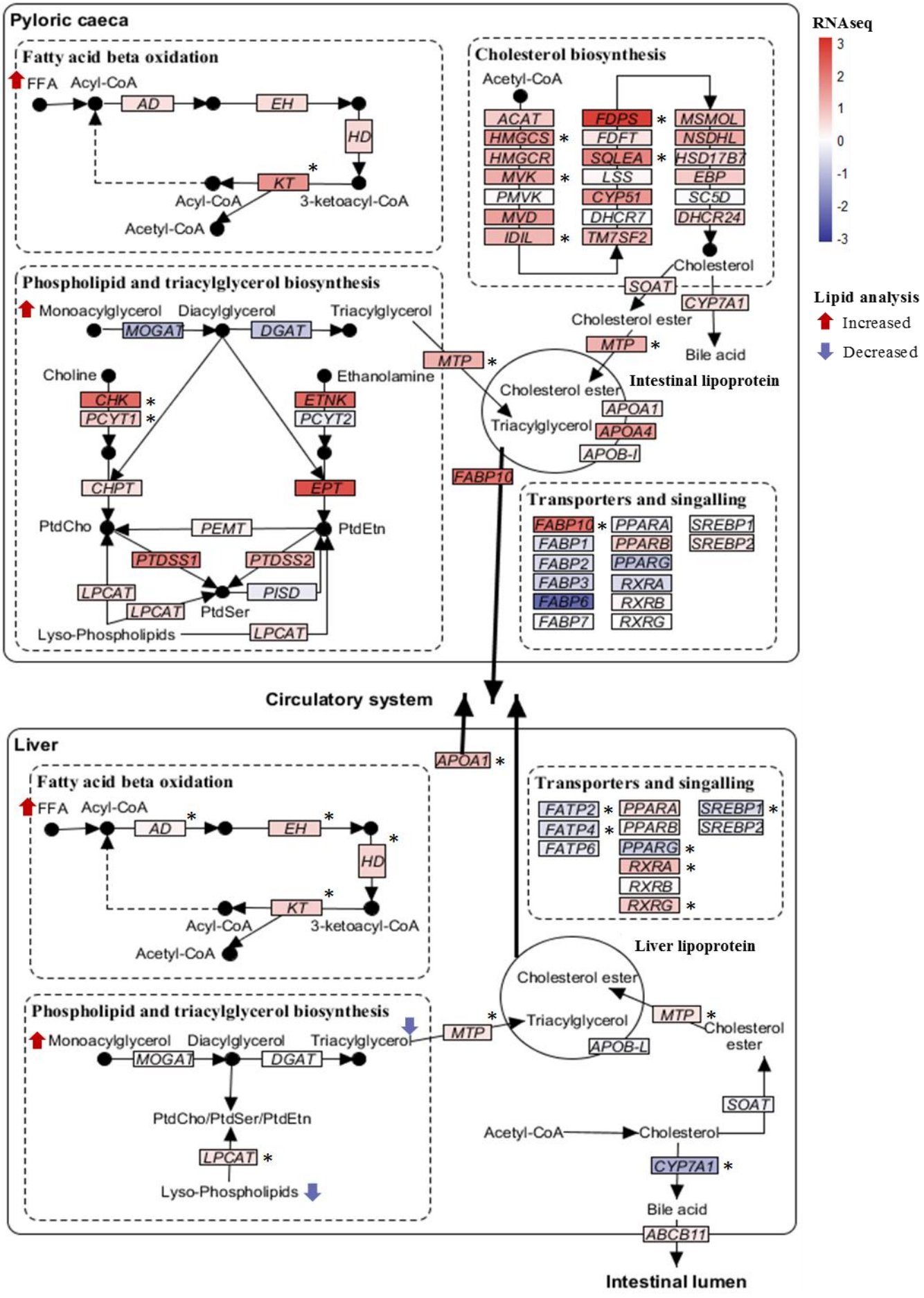

Figure 2 Changes of gene expression and lipid composition in pyloric caeca and liver of salmon fry after onset of external diet compared to unfed salmon. The expressional change of genes in each enzymatic reaction is shown in summarized expressional score (Log2 fold changes * relative expressional levels of the highest expressed homolog) of all homologous gene involved in the same reaction. Reactions that contain at least one differentially expression gene (q<0.05) between fed and unfed salmon are annotated with an asterisk. The increased or decreased lipid classes after exogenous feeding compared to unfed salmon were also annotated into the figure. FFA, free fatty acids; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

Highlights

- The present study aimed to understand the regulation of lipid metabolism pathways when the fish switched from endogenous to exogeneous feeding.
- Increased phospholipid and cholesterol synthesis pathways were found in salmon after exogeneous feeding, suggesting a high requirement of the two lipids for salmon fry.
- The manuscript has combined fatty acids, lipid class and gene expression data together to study the systemic regulation of lipid metabolism in very small salmon.
- In the study we developed a "sum of transcripts" measurement, which combined expression of homologous genes to overcome the challenges of gene redundancy in the partially tetraploid salmon genome.