Life-stage associated remodeling of lipid metabolism regulation in Atlantic salmon

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Abstract

Atlantic salmon migrates from rivers to sea to feed, grow and develop gonads before returning to spawn in freshwater. The transition to marine habitats is associated with dramatic changes in the environment, including water salinity, exposure to pathogens and shift in dietary lipid availability. Many changes in physiology and metabolism occur across this life-stage transition, but little is known about the molecular nature of these changes. Here, we use a long-term feeding experiment to study transcriptional regulation of lipid metabolism in Atlantic salmon gut and liver in both fresh- and saltwater. We find that lipid metabolism becomes significantly less plastic to differences in dietary lipid composition when salmon transitions to saltwater and experiences increased dietary lipid availability. Expression of genes in liver relating to lipogenesis and lipid transport decreases overall and becomes less responsive to diet, while genes for lipid uptake in gut become more highly expressed. Finally, analyses of evolutionary consequences of the salmonid-specific whole-genome duplication on lipid metabolism reveal several pathways with significantly different ($p < .05$) duplicate retention or duplicate regulatory conservation. We also find a limited number of cases where the whole-genome duplication has resulted in an increased gene dosage. In conclusion, we find variable and pathway-specific effects of the salmonid genome duplication on lipid metabolism genes. A clear life-stage-associated shift in lipid metabolism regulation is evident, and we hypothesize this to be, at least partly, driven by nondietary factors such as the preparatory remodelling of gene regulation and physiology prior to sea migration.

Introduction

Atlantic salmon lives a ‘double life’. It starts its life in rivers, before transforming its physiology and behavior and migrating to sea to grow and accumulate resources for reproduction. This shift in environment requires preparatory remodeling of physiology prior to sea migration (referred to as smoltification), which encompasses a suite of coordinately regulated processes involving hormonal changes and large scale alteration of gene expression. The resulting adaptations to a marine environment include transformation of salt-tolerance, coloration, behavior, growth rate, and metabolism (reviewed in Stefansson et al., 2008).

A key difference between freshwater and sea-habitats is the dietary availability of essential long-chain polyunsaturated fatty acids. Salmon in rivers mostly eat invertebrates that are low in physiologically critical n-3 and n-6, 20 and 22 carbon long-chain polyunsaturated fatty acids (n-3LC-PUFA and n-6LC-PUFA), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3) and docosahexaenoic (22:6n-3), while marine habitat food chains are high in available LC-PUFAs. Possibly as an adaptation to this (Leaver et al., 2008), salmon have evolved a high capacity for endogenous production of LC-PUFAs by elongation and desaturation of essential dietary 18 carbon precursor linoleic and linolenic acids (18:2n-6 and 18:3n-3; Figure 4) and the ability to increase or decrease endogenous production of n-3LC-PUFA from dietary 18:3n-3 (linolenic acid) as a response to the dietary availability (Kennedy et al., 2006; Leaver et al., 2008; Morais et al., 2011; Ruyter et al., 2000; Tocher et al., 2001; Tocher et al., 2002; Zheng et al., 2005). During smoltification and after sea-migration, Atlantic salmon have been shown to undergo transformation of lipid metabolism function, by decreasing lipid syntheses and increasing lipid breakdown (Sheridan, 1989). However, very little is known about the molecular nature of this life-stage associated transformation physiological function.

The evolution of novel traits in salmonids, such as increased plasticity and the ability to migrate to sea, may have been facilitated by their ancestral whole genome duplication (called Ss4R) some 80 million years ago (Allendorf & Thorgaard, 1984; Lorgen et al., 2015; Macqueen & Johnston, 2014; Robertson et al., 2017). Gene duplication can give rise to new adaptive phenotypes in different ways: through evolution of novel functions or gene regulation, subdivision and/or specialization of function among duplicates, or via an adaptive increase in gene dosage. The Atlantic salmon genome contains ~10,000 pairs of Ss4R gene duplicates, of which ~50% have evolved some novel regulation (Lien et al., 2016; Robertson et al., 2017). Indeed, in the context of lipid metabolism, it has recently been shown that a Ss4R duplicate of elovl5, a key enzyme in LC-PUFA syntheses, has gained expression compared to its ancestral regulation with likely implications for the ability to synthesize LC-PUFAs (Carmona-Antoñanzas et al., 2016). This is believed to have facilitated evolution of novel traits, including flexible phenotypes necessary for an anadromous life history (Stefansson et al., 2008).However, no systematic genome wide study has yet been conducted to assess the importance of the Ss4R in evolution of salmon lipid metabolism.

In this study, we integrate comparative genomics with transcriptomic data from a feeding trial carried out across the fresh to saltwater transition to build a functional annotation of lipid metabolism pathway genes in salmon. We use this annotation to elucidate (i) the nature of the transformation of lipid metabolism from freshwater to saltwater life-stages and (ii) the impact of whole genome duplication on evolution of the lipid gene repertoire and metabolic function. Our results indicate a programmed shift in lipid metabolism after transition to seawater, and show that lipid pathways differ with respect to selection pressure on gene duplicates from the salmonid whole genome duplication.

Materials and methods

Orthogroup prediction

Protein sequences were obtained from seven teleost fish species; *Danio rerio* (zebrafish), *Gasterosteus aculeatus* (three-spined stickleback), *Oryzias latipes* (medaka), *Oncorhynchus mykiss* (Rainbow trout), *Oncorhynchus kisutch* (coho salmon), *Salmo salar* (Atlantic salmon), *Thymallus thymallus* (grayling), *Esox lucius* (northern pike), and two mammalian outgroup species; *Homo sapiens* (human), *Mus musculus* (house mouse). Human, mouse, zebrafish, medaka and stickleback protein fasta data were obtained from ENSEMBL (release 83). Atlantic salmon (RefSeq assembly GCF_000233375.1, Annotation Release 100) and northern pike (RefSeq assembly GCF_000721915.2, Annotation Release 101) proteins were obtained from NCBI RefSeq. Rainbow trout proteins were obtained from an assembly and annotation of the genome (Berthelot et al., 2014). Grayling proteins were obtained from an assembly and annotation of the genome (Varadharajan et al., 2017). The coho salmon transcriptome (Kim, Leong, Koop, & Devlin, 2016) was obtained from NCBI (GDQG00000000.1). Where transcriptome data was used, protein sequences were translated using TransDecoder (v2.0.1, http://transdecoder.github.io/). Protein fasta files were filtered to retrieve only the longest protein isoform per gene. Orthofinder (v0.2.8) (Emms et al., 2015) assigned groups of orthologs based on protein sequence similarity. Proteins within an orthogroups were further aligned using MAFFT (v7.130) (Katoh et al., 2002) and maximum likelihood trees were estimated using FastTree (v2.1.8) (Price et al., 2010).

Annotation of salmon lipid metabolism genes

A list of zebrafish proteins obtained from 19 manually selected zebrafish KEGG pathways related to lipid metabolism (Table S1) were used to search for Atlantic salmon orthologs. Orthogroups that contained a selected zebrafish protein were identified. Salmon proteins within those orthogroups were assigned as

orthologs of the closest zebrafish protein based on the orthogroup tree distance. A lipid metabolism gene list was created including salmon orthologs to the selected zebrafish genes. Additional salmon genes related to lipid metabolism not included in KEGG pathways (e.g. regulators or transporters, SREBP, LXR, FABP, etc.) were manually searched for through NCBI and added to the list.

Tissue expression

Atlantic salmon RNA-seq samples from 15 different tissues (liver, gut, pyloric caeca, heart, kidney, muscle, gill, eye, skin, ovary, nose, testis, brain, head kidney, spleen) were obtained from NCBI SRA (PRJNA72713) (Lien et al., 2016). Fastq files were adapter trimmed before alignment to the Atlantic salmon genome (RefSeq assembly GCF_000233375.1) (Lien et al., 2016) using STAR (v2.5.2a) (Dobin et al., 2013). HTSeq-count (v0.6.1p1) (Anders et al., 2015) counted the sum of uniquely aligned reads in exon regions of each gene in the annotation (RefSeq Annotation Release 100). Gene FPKM values were calculated based on the gene count over the samples effective library size (see TMM method from edgeR (Robinson et al., 2010) user manual) and the mean gene transcript isoform length.

Feed trial

Atlantic salmon fry were obtained from AquaGen Breeding Centre, Kyrksæterøra, Norway and reared in the Norwegian Institute for Water Research (NIVA), Solbergstranda, Norway in four partitioned 1000 liter tanks on vegetable oil (VO) or fish oil (FO) based diets continuously from first feeding (fry weight <0.2 g). Daily feed amount was calculated based on total biomass in each tank and decreased as the fish grew, from 3% at first feeding to 1.2% by the end of the trial. Fish were euthanized periodically throughout the experiment to maintain appropriate levels of dissolved oxygen. VO based feeds contained a combination of linseed oil and palm oil at a ratio of 1.8:1 and FO based feeds contained only North Atlantic fish oil. All feeds were formulated and produced by EWOS innovation (Supplementary File 3). Local groundwater was UV sterilized for use in the freshwater life-stage and water from the Oslofjord

taken from 60 meters below sea surface (~3-3.5% salinity) was UV sterilized for use in the saltwater life-stage. Fish were raised under constant light and water temperature (~12°C) for 26 weeks. Then, 40 pre-smolt salmon (~50g) from each control tank (~240 fish per control tank) were switched to the contrasting diet (VO to FO and vice versa) by physically moving them to the empty partition of the tank receiving the appropriate feed (Figure 8a). Five fish from each of the control tanks (2 VO tanks and 2 FO tanks) were sampled before switching feeds (D0), then fish from both control and feed switch conditions were similarly sampled 1, 2, 5, 9, 16, and 20 days after switching feeds (5 fish x 2 replicate tanks x 4 conditions = 40 fish per time point, figure 8b). Two weeks after freshwater sampling (31 weeks after first feeding), smoltification was triggered by 5 weeks of winter-like conditions with decreased light (12 hours per day) and water temperature (~8°C), immediately followed by 5 weeks of spring-like conditions, returning to normal light (24 hours per day) and water temperature (~12°C). All salmon from the control groups (VO or FO) were then switched to saltwater and allowed to acclimate for 3 weeks. The feed switch was repeated in saltwater by transferring half (~40 fish) of the post-smolt salmon (~200 g) from each control tank to the contrasting feed condition. Again, pre-switch control samples were taken (D0) followed by sampling 1, 2, 6, 9, 16, and 20 days post-diet switch (Figure 8b). For both freshwater and saltwater samplings, feeding was stopped in the mornings of each of the sampling days. All fish were euthanized by a blow to the head and samples of liver and midgut (gut section between pyloric caeca and hindgut) were flash frozen in liquid nitrogen and stored under -80 °C. A subset of the samples taken were used for further RNA-seq analysis (see figure 8c for details).

RNA-sequencing

Total RNA was extracted from selected feed trial samples (see figure 8c for details) using the RNeasy Plus Universal kit (QIAGEN). Quality was determined on a 2100 Bioanalyzer using the RNA 6000 nano kit (Agilent). Concentration was determined using a Nanodrop 8000 spectrophotometer (Thermo Scientific). cDNA libraries were prepared using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina). Library

mean length was determined by running on a 2100 Bioanalyzer using the DNA 1000 kit (Agilent) and library concentration was determined with the Qbit BR kit (Thermo Scientific). Single end sequencing of sample libraries was completed on an Illumina HiSeq 2500 with 100 bp reads.

Differential expression analysis between feed conditions and life-stages

To analyze gene expression differences between feed conditions and life-stages, samples from the feed trial were selected for RNA-seq. Liver and gut tissue RNA were sequenced from fish fed each of the feeds (FO, VO) at day 0 of the diet switch, both before (freshwater) and after (saltwater) smoltification (See figure 8c for the number of RNA-seq replicates and sampling details). Fastq files were processed to produce gene count and FPKM data using the same protocol described under the tissue expression method section. For the feed comparison, changes in gene expression were tested between FO and VO feed conditions for both freshwater and saltwater samples, and liver and gut tissues. For the life-stage comparison, changes in gene expression were tested between freshwater and saltwater stages for both FO and VO feed conditions, and liver and gut tissues. Using RNA-seq gene count data, lowly expressed genes were filtered prior to testing, retaining genes with a minimum of one read count per million (CPM) in two or more samples. Differential expression analysis was carried out using a standard edgeR (Robinson et al., 2010) protocol. Effective library sizes were calculated using the edgeR TMM-normalisation procedure allowing effective comparison of expression data between different sample types (see edgeR user manual). An exact test between expression levels of a pair of conditions gave the log2 fold change, P-value and false discovery rate (FDR) for each gene. Genes with FDR < 0.05 were considered differentially expressed genes (DEGs).

Identification of Ss4R duplicates

To identify putative gene duplicates stemming from the Ss4R, we used the same approach as in Lien et al. (2016). All-vs-all protein blast was run with e-value cutoff of 1e-10 and pident (percentage of identical matches) ≥80 and blast hit coverage of ≥50% of protein length. Only the best protein hits between the 98

defined synteny blocks (see Lien et al., 2016) were considered as putative Ss4R duplicates. Blast result ranking was done using the product of pident times bitscore to avoid spurious 'best blast matches' with low pident (<85) but high bitscore.

Duplicate analysis

Genes from the lipid metabolism gene list were paired together with their putative Ss4R duplicates identified above. The retention of gene duplicates (i.e. whether both genes in a pair were retained, or just one) was compared between all identified duplicates in the salmon genome annotation and the lipid metabolism gene list. Pathway-level retention was explored by comparing the number of genes in each of the 19 selected KEGG pathways (Table S1) in a duplicate pairing to that of the total list of lipid genes, to find pathways with significantly less or more duplicate retention (Fisher's exact test, P-value < 0.05). Regulatory conservation of lipid gene duplicates was explored by correlation of gene expression changes between duplicates over the course of the feed trial described above. RNA-seq data was generated from liver samples of salmon from 38 sampling time points (19 in freshwater and 19 in saltwater). Fastq files were processed to produce gene count and FPKM data using the same protocol described under the tissue expression method section. For each duplicate pair, mean FPKM values were retrieved for each time point and used to calculate a freshwater and saltwater correlation value. Duplicates with Pearson correlation ≥ 0.6 were considered correlated (P-value < 0.003 from 19 sample points). The number of duplicates with correlated expression profiles was counted for each pathway and compared to all lipid genes to find pathways with significantly less or more correlated duplicates (Fisher's exact test, P-value < 0.05). The effect of gene duplication on gene dosage was estimated by calculating a dosage ratio between the FPKM value of a salmon ortholog (sum of gene expression in duplicate pairs) over the FPKM value of the non-duplicated ortholog from northern pike. For salmon, the RNA-seq data from the freshwater and saltwater FO feed trial was used (samples used in differential expression analysis section). For pike, RNA-seq from livers of four individuals were aligned (see tissue expression section for protocol) to their respective

genomes (see genomes in ortholog prediction section). RSEM (v1.2.31) (Li & Dewey, 2011) was used to generate FPKM values for genes so that non-uniquely mapped reads between salmon duplicate genes were not ignored but instead assigned proportionately to each gene to match the proportions of uniquely mapped reads between the genes. Gene dosage levels for duplicate pairs with correlated expression (see above), non-correlated expression and single genes were compared for all lipid metabolism genes and for each pathway.

Results and discussion

Annotation of lipid metabolism genes

To identify genes involved in lipid metabolism in Atlantic salmon, we initially assembled groups of orthologous genes (orthogroups) using protein sequence similarity. We included proteins from four salmonid species sharing the Ss4R genome duplication, in addition to four non-salmonid fish genomes and two model mammalian outgroup species (Figure 1a) to aid in distinguishing Ss4R copies from other gene duplicates. Next, we aligned orthogroup proteins and constructed maximum likelihood gene trees. The majority (82-98%) of proteins from each species were represented in 23,782 ortholog gene trees. The salmonid species had significantly higher number of proteins included in ortholog gene trees compared to non-salmonid fish (Figure S1), reflecting the salmonid specific whole genome duplication. We then used the evolutionary distances in gene trees to infer the most likely salmon sequence orthologs of zebrafish genes selected from 19 KEGG pathways involved in lipid metabolism (File S1). This resulted in the annotation of 1421 (File S2) salmon lipid metabolism genes, of which 326 (23%) showed a 2:1 ortholog ratio between salmon and zebrafish (Figure 1b). Only 87 (6%) of the zebrafish genes could not be assigned a salmon ortholog.

To validate our ortholog annotation pipeline used to identify lipid metabolism genes, we analyzed the tissue specificity of these genes using gene expression data from 15 tissues (File S3) of Atlantic salmon (Lien et al., 2016). Genes in certain fatty acid metabolism related pathways (*'fatty acid metabolism'*, *'PPAR signaling pathway'*, *'fat digestion and absorption'*) had higher overall expression in tissues known to have high lipid metabolism activity (i.e. pyloric caeca, liver, heart, and brain) (Glatz et al., 2010; Benedito-Palos & Pérez-Sánchez, 2016; Tocher, 2003) (Figure 2). Examples include: 1) Liver was the site of highest expression for all genes in the LC-PUFA biosynthesis pathway (the desaturases Δ6FAD and Δ5FAD, and the elongases elovl5, elovl2 and elovl4). 2) Bile acids are essential for fat digestion in the gut, but are synthesized in liver. As expected, the rate limiting step for bile syntheses, cytochrome P450 7A1 (CYP7A1), has the highest expression in the liver. 3) Cholesterol, an essential component of cell membranes and precursor to bile acids, is known to be synthesized in all tissues, but primarily in liver, intestine, and brain (Brown & Sharpe, 2016). This is reflected in our annotation by high expression of the key cholesterol biosynthesis genes 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), isopentenyl-diphosphase Δisomerase (IDI1), squalene epoxidase (SM), and lanosterol synthase (LS) in these tissues. 4) Several known regulators of lipid metabolism show high expression in liver, heart, brain and pyloric caeca, as expected, including liver X receptor (LXR), peroxisome proliferator-activated receptor alpha (PPARα), sterol regulatory element binding protein 1 (SREBP1), and sterol regulatory element binding protein 2 (SREBP2) . Taken together, the tissue distribution of lipid metabolism gene expression is in line with knowledge about vertebrate physiology in general, and support the validity of our annotation of lipid metabolism genes in salmon. To make all data underlying our annotation easily available, and to facilitate further refinement through manual community curation, we have created an interactive web-server available online (goo.gl/8Ap89a).

Life-stage dependent remodeling of lipid metabolism

We conducted a feeding trial to study how salmon adjusts its lipid metabolism to different levels of LC-PUFA in freshwater and saltwater (See figure 8 for experimental details). Groups of salmon were fed contrasting diets from hatching until after transition to seawater. One feed was vegetable oil based (VO) and hence low in LC-PUFA, similar to river ecosystem diets, whereas the other was based on fish oil (FO) and high in LC-PUFA as expected in a marine-type diet (see Table S2 for details on feed composition). VO based diets are also low in cholesterol (Ciftci, et al., 2012; Verleyen et al., 2002). In total, 32 and 23 fish were sampled for RNA-seq of liver and gut, respectively, including up to eight biological replicates from each diet and life-stage (freshwater and saltwater, see figure 8c for details). Fish in the different dietary groups were given FO and VO feed from first feeding (<0.2 g body weight) until sampling.

In general, global gene expression levels were more affected by dietary composition in liver than in gut (which was largely unresponsive), and the effect was more pronounced in freshwater than in saltwater (Figure 3a). VO diets, compared to FO diets, increased lipid-metabolism related gene expression in liver. In freshwater, 66 genes were differentially expressed with 57 (86%) of these upregulated, while in saltwater 31 genes were differentially expressed with 23 (74%) of these upregulated (Figure 3b). The increased activity of liver lipid metabolism under VO diets confirm the well-known ability of salmon to regulate endogenous synthesis of LC-PUFA and cholesterol in response to VO diets (Kortner et al., 2014; Leaver et al., 2008; Zheng et al., 2005).

Fish sampled in freshwater and saltwater shared a relatively small number of differentially expressed genes (DEGs) for each pathway (Table S3). We found that most pathways had more DEGs in freshwater (‘*fatty acid biosynthesis’*, ‘*steroid biosynthesis*’, and its precursor ‘*terpenoid backbone biosynthesis’*), whereas few had more DEGs in saltwater (‘*fat digestion and absorption’* and *‘steroid hormone biosynthesis’*) (Figure 3c). Of 87 lipid metabolism DEGs in the dietary contrast, 56 (64%) were freshwater specific, 21

(24%) saltwater specific, and 10 (11%) shared dietary response. For example, only two genes in the FA and LC-PUFA biosynthesis pathways (D6FADa and D5FAD) shared response to diet in freshwater and saltwater (Figure 4). Similarly, in the pathways responsible for cholesterol biosynthesis, there were more DEGs between diets in FW (21 DEGs in FW, 4 shared and no SW-specific; Figure 5). The few genes that showed diet effects specific to saltwater included bile salt-activated lipase, responsible for the hydrolysis of free fatty acids from TAG obtained from the diet (Tocher, 2003). Two of these genes, carboxyl ester lipase, tandem duplicate 2a (CEL2a) and b (CEL2b), are highly upregulated in saltwater in response to VO diet. Taken together, our results show higher metabolic plasticity in parr stage salmon, suggesting a life-stage-associated remodelling of lipid metabolism in liver. This corroborates the idea of a postsmoltification phenotype adapted to an environment with a surplus of n-3LCPUFA.

To further investigate the life-stage-associated changes in lipid metabolism, we tested for differential expression between salmon in freshwater and saltwater fed diets with identical n-3LC-PUFA profiles (Figure 6). Liver and gut showed contrasting effects of saltwater on lipid gene expression with extensive downregulation in liver and upregulation in gut (Figure 6b). The number of DEGs in each tissue was similar for the environment comparison (Figure 6a), unlike for the diet comparison (Figure 3).

Further examination of key lipid metabolism genes revealed that after life-stage transition, the system wide lipid metabolism remodeling represented a concerted shift in the metabolic role of liver and gut. As the salmon entered the marine stage, lipogenic gene expression in the liver was significantly decreased, as evident by the markedly lower expression (2.2-3.3 fold) of the master regulator of lipid metabolism SREBP1, a 5-fold decrease in expression of fatty acid synthase, and a 2-3 fold decrease in rate-limiting enzymes in LC-PUFA synthesis (i.e. Δ5FAD, Δ6FADa) (Figure 4). Liver and gut gene expression also indicated increased catabolic activity in saltwater, with upregulation of the carnitine palmitoyltransferase 1 and 2 genes, responsible for uptake of fatty acids into mitochondria for β-oxidation (Lehner & Quiroga, 2016). Finally, expression of lipid transport genes shifted from liver to gut with the transition to seawater

(apolipoproteins, pathway "Fat digestion and absorption" in Figure 6). Four apolipoproteins (out of 11 annotated) were differentially regulated in liver between different life-stages, with a 2.4-5 fold decrease in saltwater compared to freshwater. In stark contrast, nine of the diet-regulated apolipoproteins in gut increased their expression in saltwater between 1.8-9.7 fold. The results point to an adaptive shift in lipid metabolism, with increased ability to take up lipids in the gut after Atlantic salmon migrates to sea where lipid availability is higher. Remodelling of lipid metabolism across life stages is likely the result of a combination of factors, including the direct regulatory effect of dietary fat itself, effect of salinity and smoltification-induced physiological changes influencing gene regulation. Although the relative importance of these factors is undetermined in our study, the fact that DEGs in the VO versus FO feed contrast were mostly lifestage- specific (Figure 3) supports that factors other than the diet itself contribute significantly to the freshwater and seawater metabolic phenotypes.

Interestingly, diet had a strong influence on the number and direction of gene expression changes between freshwater and saltwater (Figure 6). In gut, about twice as many DEGs (with respect to the fresh- to saltwater transition) were observed in salmon when fed FO diet than VO diet (Figure 6a). In liver, the diet effect was less pronounced, with the FO group containing 46% more DEGs than the VO group (Figure 6a). This diet effect pattern was reflected in the lipid metabolism genes with 89% and 16% more DEGs in the FO group for gut and liver, respectively (Figure 6b). As this diet and life-stage interaction is a genome wide trend, and more pronounced in gut tissue than in liver, this pattern could also be related to differences in osmoregulation and adaptation to saltwater. Two studies have suggested that Atlantic salmon raised on VO based feeds more closely resembling riverine diets adapt to saltwater sooner and better than salmon raised on FO based diets (Bell et al., 1997; Tocher et al., 2000). Conversely, there has been evidence that VO based diets can reduce markers for stress response upon saltwater challenge, resulting in reduced osmoregulatory capacity (Oxley et al., 2010). Regardless of the effect, it is clear that diet can modulate the smoltification process and could explain the discrepancy between diets in number of life-stage related

DEGs. Another possibility is that the different levels of fatty acids in the diets, for example DHA, affect DNA-methylation and thus trigger genome wide divergence in gene regulation (Kulkarni et al., 2011).

Our results clearly demonstrate very different baseline lipid metabolic functions in pre- and post-smolt salmon, as well as life-stage associated changes in the plasticity of lipid metabolism, e.g. the ability to regulate endogenous LC-PUFA synthesis as a response to changes in diet (i.e. fatty acid composition). As opportunistic carnivores, salmon tend to eat whatever the local environment provides. Thus, in freshwater, insects and amphipods provide variable, mostly low amounts of essential LC-PUFA and total fat (Jonsson & Jonsson, 2011; Sushchik et al., 2003), favoring a metabolic function that can efficiently regulate endogenous lipid synthesis based on dietary availability (Carmona-Antonanzas et al., 2014). Conversely, in marine environments, amphipods and smaller fish provide a higher, more stable source of n-3LC-PUFA and total fat (Baeza-Rojano, Hachero-Cruzado, & Guerra-Garcıa, 2014; Jonsson & Jonsson, 2011), promoting a metabolic function that allocates less energy to endogenous synthesis of essential lipids.

Selection on gene duplicates after whole genome duplication

Carmona-Antonanzas et al. (2014, 2016) proposed that the salmonid whole-genome duplication may have adaptively increased the potential for endogenous lipid synthesis. We pursued this hypothesis by searching for distinct signatures of selection pressure on lipid metabolism genes in salmon. Specifically, we compared pathways in terms of their tendency to retain both duplicates of gene pairs, in terms of whether duplicates showed similar regulation (expression patterns across diets and environments), and in terms of total gene dosage (for the one or two genes retained of a pair) in salmon compared to pike, its closest unduplicated sister lineage.

To assess the level of Ss4R duplicate retention, we first defined 10,752 Ss4R duplicate pairs (21,504 genes) in the NCBI refseq annotation using the same approach as Lien et al. (2016). Of the 1,421 annotated lipid metabolism genes, 867 (61%) were retained as duplicated genes after Ss4R (Figure 7a) (in contrast to 47% of the 45,127 salmon genes assigned to ortholog groups). Moreover, our results showed large variation in the proportion of retained duplicates in each lipid metabolism pathway (Figure 7), with the most extreme case being '*fat digestion and absorption*' with 80% retained duplicates and '*steroid hormone biosynthesis*' with only 27% retained Ss4R duplicates.

The regulatory conservation of the duplicates was then estimated by calculating co-expression correlation between Ss4R duplicates from RNA-seq data representing a time course of dynamic changes in gene expression and lipid metabolism function in liver. Fish in the same feeding trial were switched from VO to FO feed and vice versa, in both fresh and saltwater conditions (see Figure 8 for details). In total, 38 sampling time points (20 in freshwater and 18 in saltwater) from the feed switch experiment were used. Pathway-level analyses showed that regulatory conservation was not associated with duplicate retention (Figure 7). For example, the *'biosynthesis of unsaturated fatty acids'* pathway had significantly fewer duplicates retained than expected by chance (P-value < 0.0234), but a significant overrepresentation of duplicate pairs that display highly similar regulation (P-value < 0.0142 and < 0.0361 in freshwater and saltwater, respectively). Interestingly, the 'insulin signaling pathway' also showed higher than expected duplicate co-regulation. This pathway has been shown to be important in regulating uptake and transport of FAs in adipose tissue, liver and muscle of Atlantic salmon (Sánchez-Gurmaches et al., 2011). Other pathways showing signatures of increased duplicate co-regulation were *'terpenoid backbone biosynthesis'*, *'steroid biosynthesis'*, *'fat digestion and absorption'*, and *'fatty acid metabolism'* (Figure 7b-c). Overall, the distinct differences in duplicate retention and conservation of regulatory mechanisms across the lipid metabolism pathways suggest differences in selective pressures shaping duplicate evolution following Ss4R. Moreover, the pathways with highly conserved duplicate co-regulation were also those that were most responsive to dietary differences in fatty acid composition (Figure 3).

Finally, to link duplicate retention and co-regulation to signals of increased gene dosage following Ss4R, we used RNA-seq data from the Northern pike (*Esox lucius*), a species that belongs to the unduplicated sister lineage (see methods for details). For each duplicate pair, we computed the ratio between the sum of Ss4R duplicate expression and its non-duplicated ortholog in pike and compared these ratios to those observed for salmon genes that had not retained two Ss4R duplicates. In total 69 duplicate pairs from 18 different lipid-metabolism related pathways displayed a combined dosage increase relative to single copy genes, of which 26 had highly conserved regulation (i.e. correlated expression) (File S8). We saw no systematic effect of gene dosage when comparing the total gene expression of duplicate pairs with that of single-copy genes; nor did co-regulation of duplicates associate with increased gene dosage (Figure 7d). This pattern was also true for most individual lipid pathways (Figure S4-S5), except for *'biosynthesis of unsaturated fatty acids'*, *'fatty acid metabolism'* and *'fatty acid elongation'*. These three pathways showed a link between co-regulation of duplicated genes and higher total gene dosage (Figure S4-S5, Figure 7d). Underlying this link were three genes with co-regulated dosage effects shared between all three pathways; trifunctional enzyme alpha subunit b (hadhab), elovl6, and the previously identified elovl5 (Carmona-Antonanzas et al., 2014; Carmona-Antoñanzas et al., 2016). Only elovl5 is known to be directly involved in core PUFA biosynthesis. Hadhab is involved in mitochondrial β-oxidation/elongation and elovl6 is involved in elongation of saturated and monounsaturated fatty acids (Bond et al., 2016). Although we do not see a general trend of increased gene dosage effects on lipid metabolism genes after whole genome duplication, it is likely that an increased dosage of elovl5 and the 68 other duplicate pairs has affected the function of lipid metabolism in salmon.

Conclusion

Atlantic salmon needs great plasticity of physiology and behaviour to adapt for migration between freshwater and sea. By analysing transcriptomic changes through the transition from fresh- to saltwater and

the associated increase in dietary lipids, we identified an overall remodelling of lipid metabolism, with liver reflecting higher lipid metabolic plasticity and higher capacity of endogenous synthesis of LC-PUFAs in freshwater, while gut lipid uptake genes become more active in saltwater. These results indicate adaptive optimization of the Atlantic salmon lipid metabolism to account for life-stage-specific dietary availability. Moreover, we found signatures of pathway-specific selection pressure on gene duplicates, including a gene dosage increase in three genes involved in fatty acid metabolism. This illustrates possible adaptive consequences of the salmonid whole-genome duplication for the evolution of lipid metabolism. Future studies should attempt to decipher how the life-stage-related metabolic reprogramming is controlled (e.g., through epigenetic mechanisms). Understanding this will have important implications for understanding evolution of genome regulatory processes in anadromous salmonids and potentially have economically important implications for Atlantic salmon aquaculture

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Author Contributions

S.R.S., J.O.V., A.G., and J.S.T. conceived of the study and designed the feeding trial experiment. S.R.S., J.O.V., and A.G. carried out orthogroup prediction and lipid metabolism annotation. T.N.H., Y.J., J.S.T., and J.O.V. carried out the feeding trial. T.N.H. and Y.J. prepared the samples for RNA sequencing. M.T., S.L., and M.L. provided input on the experimental design and helped interpreting the results from the transcriptomic and gene duplicate analysis. G.G. and T.R.H. performed the bioinformatic analyses. T.N.H., G.G., S.R.S., and T.R.H. wrote the manuscript. All authors reviewed the final manuscript draft.

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Data Accessibility:

- Supplementary files have been deposited to datadryad.org under the accession: doi:10.5061/dryad.j4h65
- All gene expression results can be accessed through the interactive shiny web server: https://goo.gl/8Ap89a
- Lipid metabolism gene annotation can be accessed from https://goo.gl/VVUVWr
- Raw RNA-Seq data have been deposited into European Nucleotide Archive (ENA) under the project Accession no. PRJEB24480

Figures

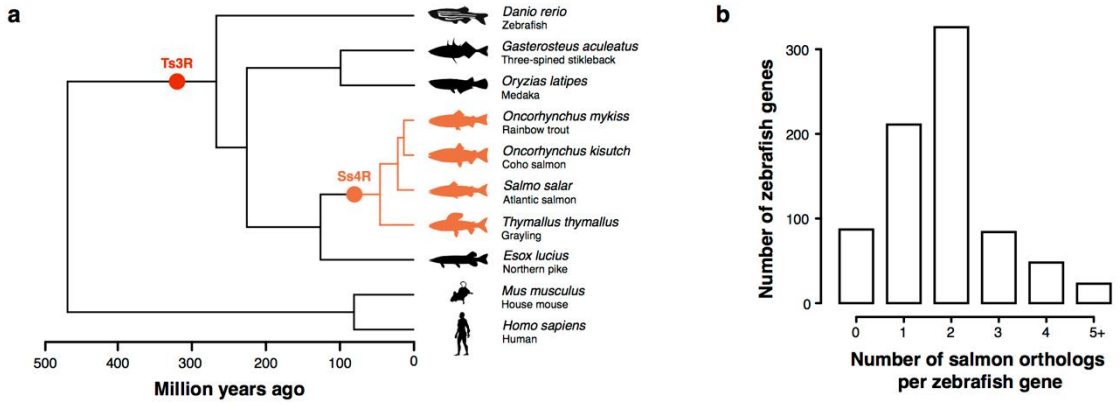


Figure 1: Ortholog annotation (a) Species used to construct ortholog groups and their evolutionary distance. Points in the phylogenetic tree show the time of the teleost specific (Ts3R) and salmonid specific (Ss4R) whole genome duplications. (b) The number of salmon orthologs found (1421 genes in total) per zebrafish gene in 19 selected KEGG pathways involved in lipid metabolism.

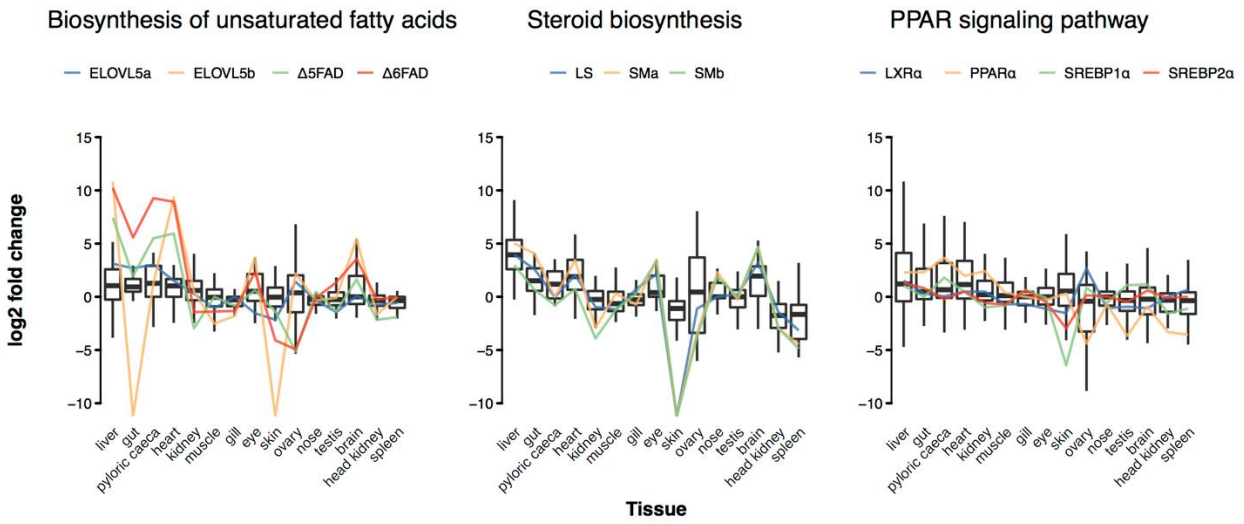


Figure 2: Tissue expression profiles of salmon genes in lipid metabolism pathways

Tissue expression profiles of our annotated lipid metabolism genes were consistent with expectations. Gene expression levels are shown as the log2 fold change difference between the FPKM value of each tissue and the median FPKM across all tissues. Expression profiles for selected genes in each pathway are shown (see Figure S2 and S3 for all pathways and gene details).

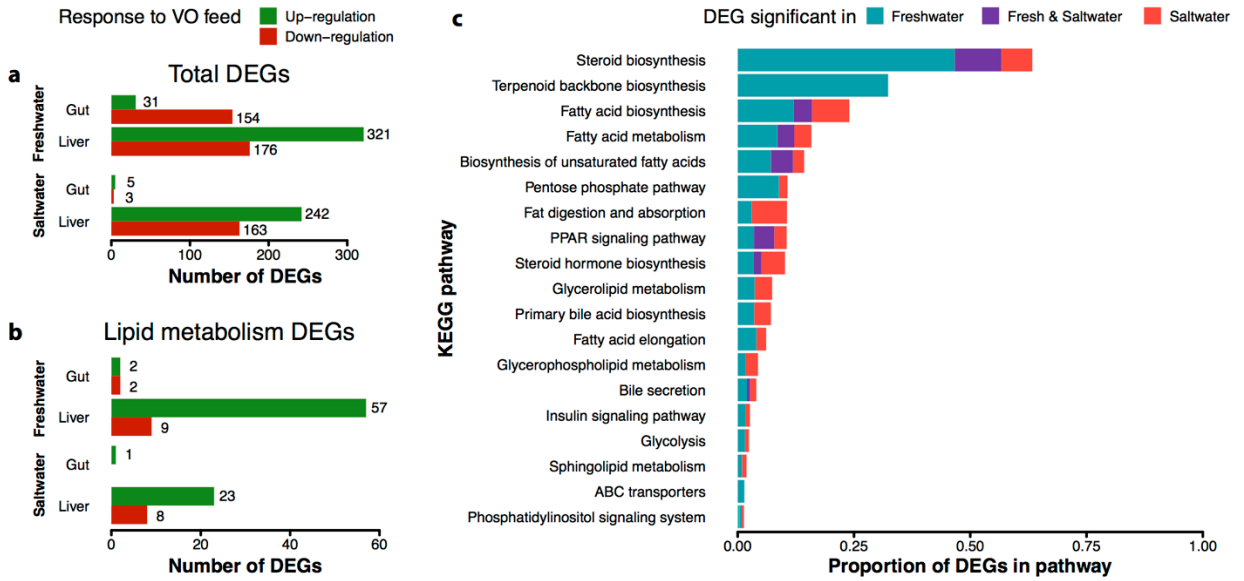


Figure 3: Gene regulation in response to feed type. (a) Total number of significant (FDR < 0.05) differentially expressed genes (DEGs) between fish oil (FO) and vegetable oil (VO) fed salmon in the liver and gut tissues of freshwater and saltwater stage Atlantic salmon (see Files S4 (liver) and S5 (gut) for underlying data). (b) As above, but for lipid-associated genes only. (c) Proportions of genes in each KEGG pathway that had significantly different liver expression between the two feed types only in freshwater, only in saltwater, or in both stages.

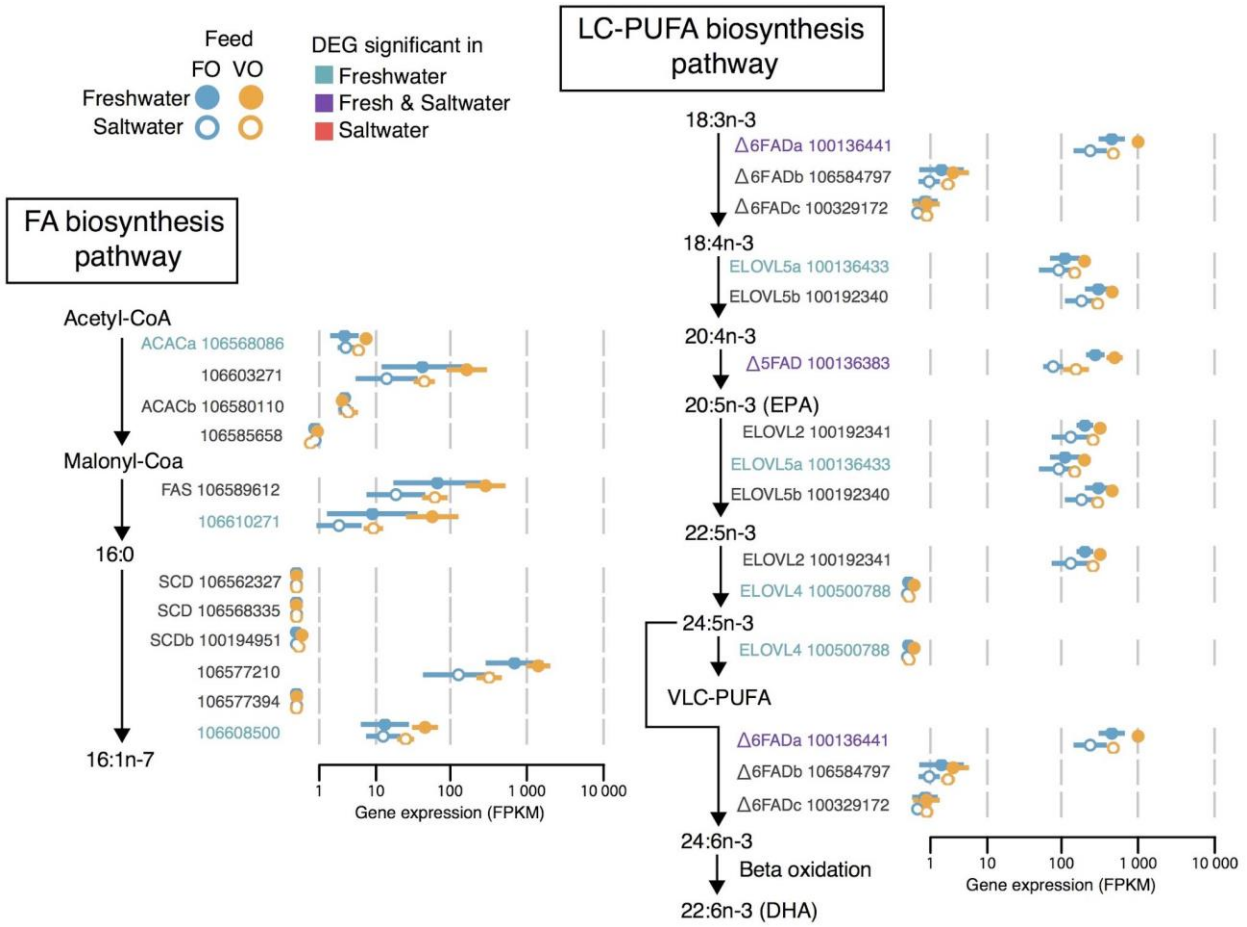


Figure 4: Diet and life-stage effects on FA and LC-PUFA biosynthesis in salmon liver. Core fatty acid (FA) biosynthesis and biosynthesis of unsaturated fatty acids pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names followed by NCBI gene numbers). Gene expression levels are shown as mean (point) and standard deviation (line) of expression in eight samples (measured in log(FPKM + 1)) from each diet (FO, VO feeds) and life-stage (freshwater, saltwater) combination. Genes significantly (FDR<0.05) differentially expressed (DEG) between diets in a life-stage are highlighted.

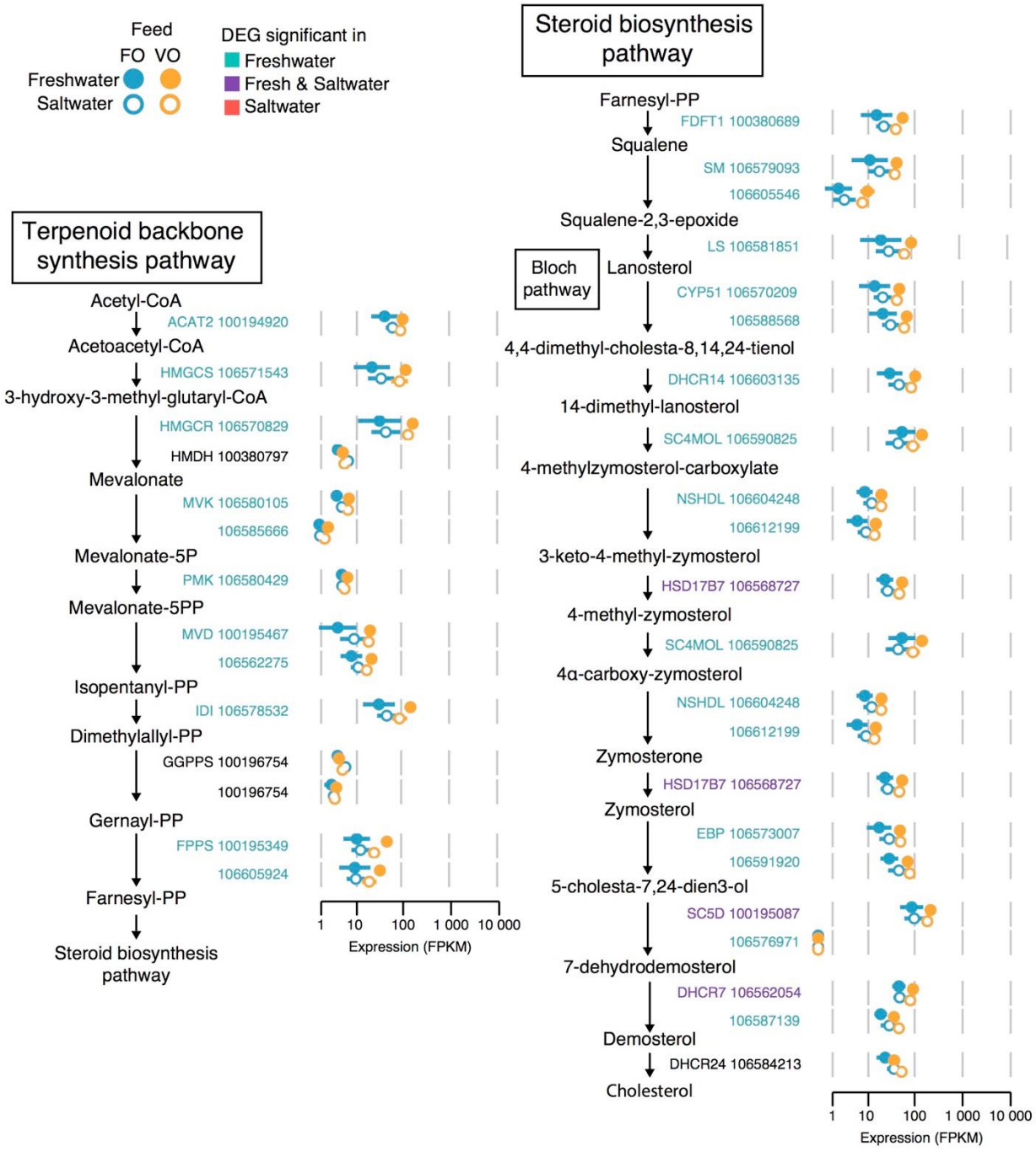


Figure 5: Diet and life-stage effects on cholesterol biosynthesis in salmon liver. Terpenoid backbone synthesis and steroid biosynthesis pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names followed by NCBI gene numbers). Gene expression levels are shown as mean (point) and standard deviation (line) of expression in eight samples (measured in log(FPKM + 1)) from each diet (FO, VO feeds) and life-stage (freshwater, saltwater) combination. Genes significantly (FDR<0.05) differentially expressed (DEG) between diets in a life-stage are highlighted.

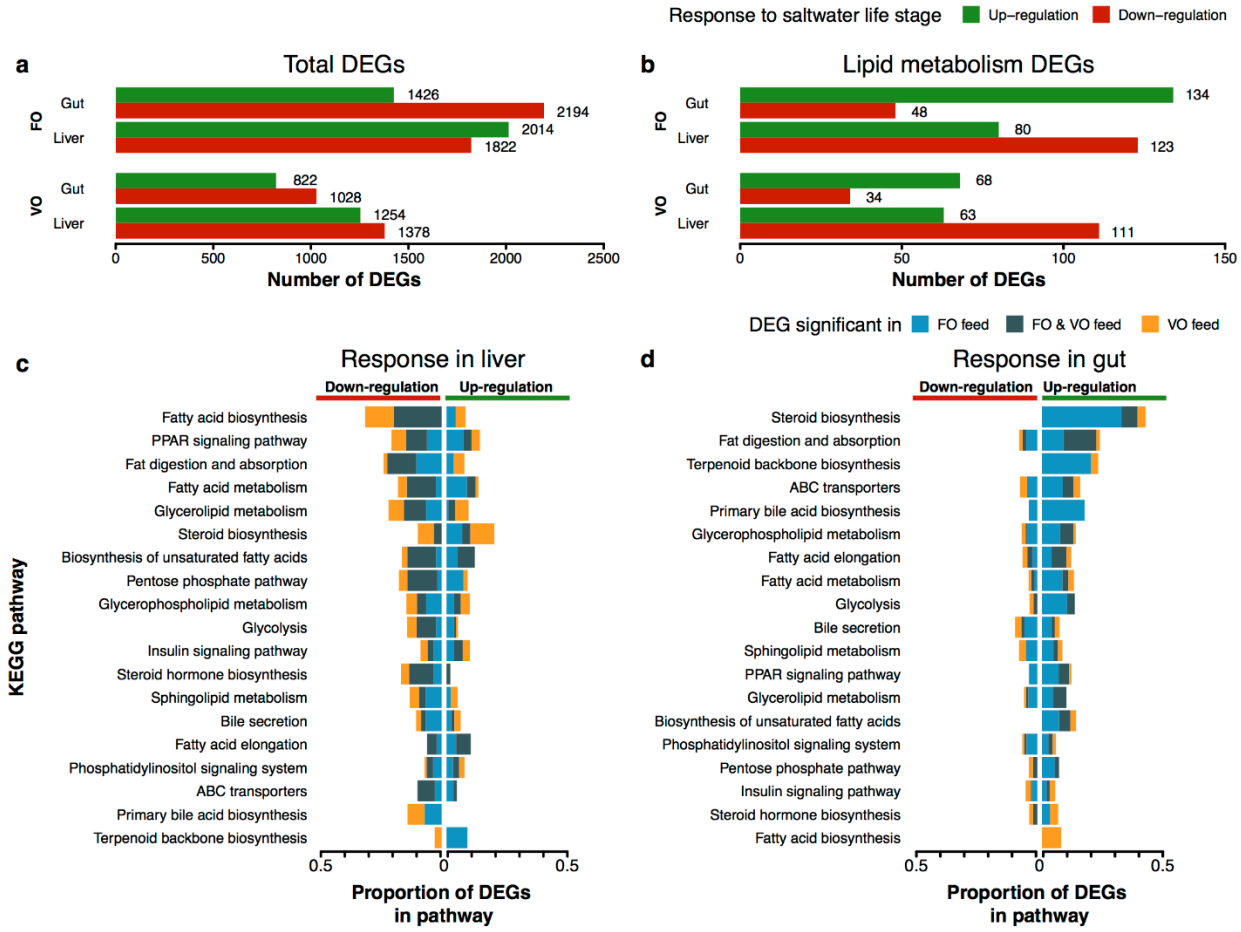


Figure 6: Gene regulation in response to life-stage. (a) Total number of significant (FDR < 0.05) differentially expressed genes (DEGs) between freshwater and saltwater life-stages in the liver and gut tissues of Atlantic salmon fed fish oil (FO) or vegetable oil (VO) diets (see Files S6 and S7 for underlying data). (b) As above, but for lipid metabolism DEGs. (c) Proportion of genes in each KEGG pathway that are DEGs in liver and (d) gut, colored by DEG significance in only FO, only VO, or both diets, and separated into up- or down-regulation in saltwater samples.

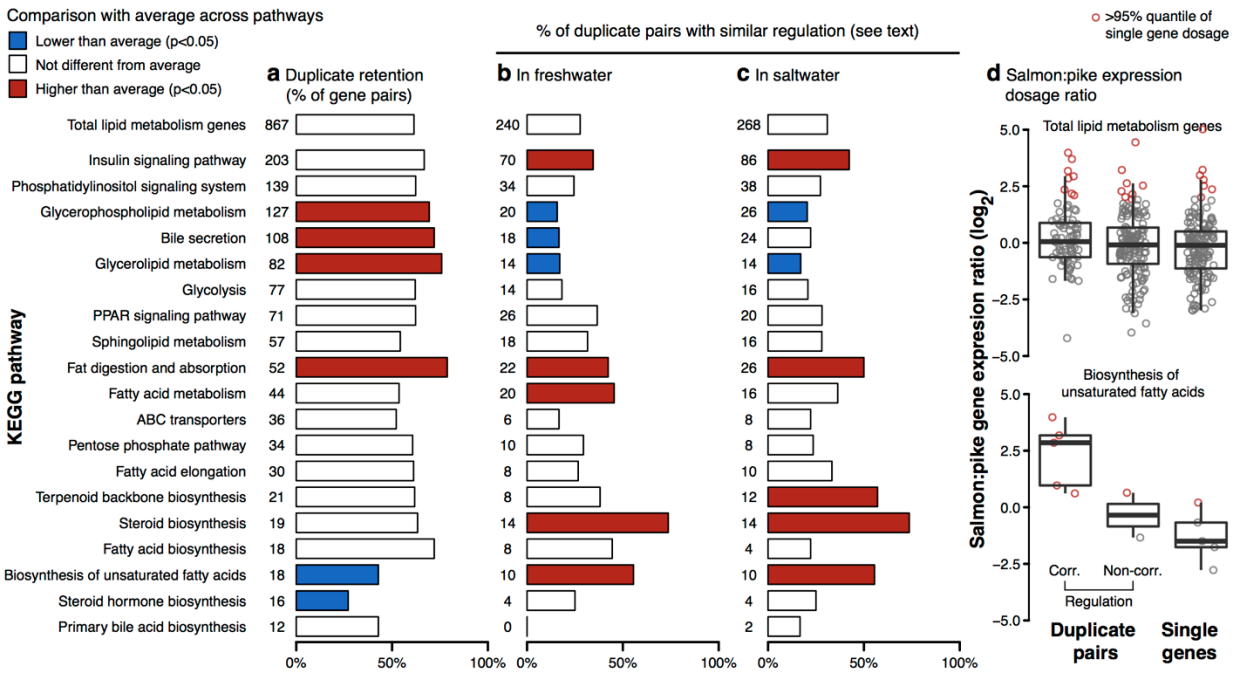
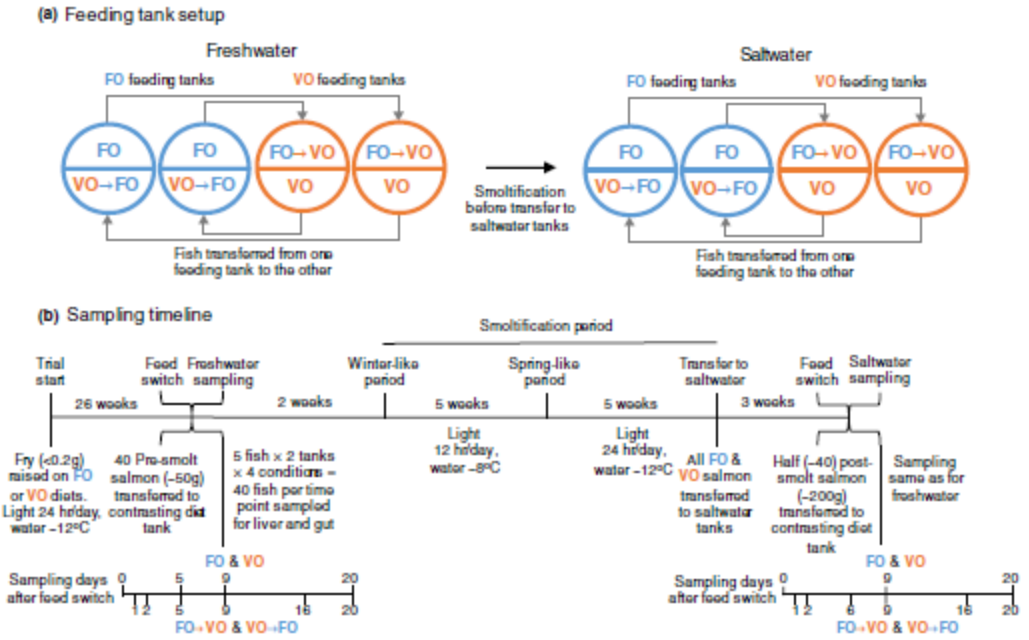


Figure 7: Gene duplication in lipid metabolism pathways. For the total list of lipid metabolism genes in Atlantic salmon, and sets of genes belonging to different KEGG pathways: (a) Number and percentage of genes with a duplicate homolog from the Ss4R duplication. (b) Number and percentage of duplicate genes with correlated liver expression response to feed in freshwater and (c) saltwater (Correlation >= 0.6, P-value < 3.306e-3, using 19 time points from feed trial for each water condition). Fisher's exact test was used to detect pathways with significant enrichment compared to all gene (P-value < 0.05) (d) Log2 gene dosage ratios (salmon:pike) in liver from fish in freshwater, where the ratio is computed between expression in the salmon duplicates (FPKM, sum of the two duplicates) and the expression of the corresponding pike ortholog. Ratios were computed for all lipid metabolism genes and genes in the pathway *'biosynthesis of unsaturated fatty acids'*. For comparison, ratios were also computed for genes without retained duplicates, i.e. with a 1:1 orthology between salmon and pike. Duplicates were grouped into correlated (corr.) or non-correlated (non-corr.) based on saltwater correlation result in (c). Dosage ratios (points) greater than the 95% quantile of single gene dosages are marked in red.



		Freshwater sampling days							Saltwater sampling days						
Feed	Tissue	0	1	2	5	9	16	20	0	1	2	6	9	16	20
FO	Liver	8			8	8		8	8				8		8
	Gut	4				4		4	8				8		8
VO	Liver	8			8	8		8	8				8		8
	Gut	4				4		4	7				8		8
VO→FO	Liver		4	4	4	4	4	4		4	4	4	4	4	4
	Gut		4	4	4	4	4	4		4	4	4	4	4	4
FO→VO	Liver		4	4	4	4	4	4		4	4	4	4	4	4
	Gut		4	4	4	4	4	4		4	4	4	4	4	4

Figure 8: Overview of feed trial experiment.

(a) Atlantic salmon fry were reared in 4 feeding tanks containing freshwater; 2 continuously fed fish oil (FO) and 2 vegetable oil (VO). A feed switch involved the transfer of fish from one tank to an empty partition of another tank fed the opposite diet. After smoltification fish from FO and VO tanks were transferred to 4 new feeding tanks containing saltwater and the feed switch was repeated. (b) Timeline of feed trial showing fish sampling and smoltification periods. Fish were sampled before (D0) and up to 20 days after the fresh- or saltwater feed switch. (c) Total RNA was sequenced from select fish tissue samples. The number of RNA-seq replicates are shown for each, tissue, condition and time point.