Diet and life stage associated remodeling of lipid metabolism regulation in the duplicated Atlantic salmon genome

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Abstract

Atlantic salmon migrates from rivers to sea to feed, grow and develop gonads before returning to spawn in freshwater. These habitat shifts require great phenotypic plasticity. To address the unresolved question of how the shift in diet between fresh and saltwater affects the regulation of metabolic function, we fed salmon contrasting diets in each of the two life stages. Combining transcriptomics with comparative genomics, we found that lipid metabolism undergoes a concerted shift between fresh- and saltwater stages. Lipogenesis and lipid transport become less active in liver after transition to saltwater, while genes involved in uptake of dietary lipids in gut greatly increases in lipid-rich seawater environments. We assess how the whole-genome duplication ancestral to salmonids has impacted the evolution of lipid metabolism, and find signatures of pathway-specific selection pressure on gene duplicates and a limited number of cases of increased gene dosage.

Introduction

36 Atlantic salmon lives a 'double life'. It starts its life in rivers, before it transforms its physiology and
37 behavior and migrates to sea to grow and accumulate resources for reproduction. This shift in
38 environment requires preparatory remodeling of physiology prior to sea migration (referred to as
39 smoltification), which encompasses a suite of coordinately regulated processes involving hormonal
40 changes and large scale remodeling of gene expression. The resulting adaptations to a marine
41 environment include shifts in salt-tolerance, coloration, behavior, growth rates, and metabolism.

42

43 Salmon transforms its lipid metabolism function during smoltification (Sheridan, 1989). This is likely
44 related to differences in diet between environments. Salmon in rivers mostly eat invertebrates that are low
45 in essential long-chain polyunsaturated fatty acids (LC-PUFA). Possibly as an adaptation to this (Leaver,
46 Bautista, et al., 2008), salmon has evolved a greater capacity for endogenous production of LC-PUFAs
47 than many other fish species, and several studies have demonstrated that salmon has the ability to increase
48 or decrease endogenous omega-3 synthesis as a response to the dietary availability of LC-PUFAs
49 (Kennedy et al., 2006; Leaver, Villeneuve, et al., 2008; Morais et al., 2011; Ruyter, Røsjø, Måsøval,
50 Einen, & Thomassen, 2000; D.R. Tocher, Bell, MacGlaughlin, McGhee, & Dick, 2001; Douglas R.
51 Tocher et al., 2002; Zheng et al., 2005). In contrast to rivers, marine habitat food chains are high in
52 available LC-PUFAs, and it has therefore been hypothesized that smoltification-associated lipid
53 metabolism changes are linked to preparation to this new dietary situation. However, little is known about
54 the extent and nature of the lipid metabolism remodeling associated with the life stage shift from
55 freshwater to sea.

56

57 The evolution of novel traits in salmonids may have been facilitated by their ancestral whole genome
58 duplication (called Ss4R) some 80 million years ago (Allendorf & Thorgaard, 1984; Lorgen et al., 2015;
59 Macqueen & Johnston, 2014). Gene duplication can give rise to new adaptive phenotypes in several
60 ways: through evolution of novel functions or gene regulation, subdivision of function and specialization,
61 or because of an adaptive increase in gene dosage. The Atlantic salmon genome contains ~10,000 pairs of
62 Ss4R gene duplicates (Lien et al., 2016), of which ~50% have evolved some novel regulation (Lien et al.,
63 2016; Robertson et al., 2017). Indeed, in the context of lipid metabolism, it has recently been shown that a
64 Ss4R duplicate of elovl5, a key enzyme in LC-PUFA syntheses, has gained expression compared to its
65 ancestral regulation with likely implications for the ability to synthesize LC-PUFAs (Carmona-
66 Antoñanzas, Zheng, Tocher, & Leaver, 2016). This is believed to have facilitated evolution of novel
67 traits, including flexible phenotypes necessary for an anadromous life history (Stefansson, Björnsson,

68 Ebbesson, & McCormick, 2008). However, no systematic genome wide study has been conducted to

69 assess the importance of the Ss4R in evolution of salmon lipid metabolism.

70

71 In this study, we integrate comparative genomics with transcriptomic data from feeding trials carried out

72 across the fresh to saltwater transition to build a functional annotation of lipid metabolism pathway genes

73 in salmon. We use this annotation to elucidate (i) the nature of the lipid metabolism transformation from

74 freshwater to saltwater life stages of salmon and (ii) the impact of whole genome duplication on evolution

75 of the lipid gene repertoire and metabolic function. Our results indicate a life-stage programmed shift in

76 lipid metabolism after transition to seawater, and identify differences between lipid pathways with respect

77 to selection pressure on gene duplicates from the salmonid whole genome duplication.

78

Results and discussion

79

Annotation of lipid metabolism genes

80

81 To identify genes involved in lipid metabolism in Atlantic salmon, we initially assembled groups of

82 orthologous genes (orthogroups) from a selection of four salmonid species, Northern pike (their closest

83 unduplicated relative), as well as teleost and mammalian outgroups (Figure 1a). Next, we aligned

84 orthogroup proteins and constructed maximum likelihood gene trees. The majority (82-98%) of proteins

85 from each species were represented in 23,782 ortholog gene trees. The salmonid species had significantly

86 higher number of proteins included in ortholog gene trees compared to non-salmonid fish (Figure S1),

87 reflecting the salmonid specific whole genome duplication. We then used the evolutionary distances in

88 gene trees to infer the most likely salmon sequence orthologs of zebrafish genes selected from 19 KEGG

89 pathways involved in lipid metabolism (Table S1). This resulted in the annotation of 1421 salmon lipid

90 metabolism genes, of which 326 (23%) showed a 2:1 ortholog ratio between salmon and zebrafish (Figure

91 1b). Only 87 (6%) of the zebrafish genes could not be assigned a salmon ortholog.

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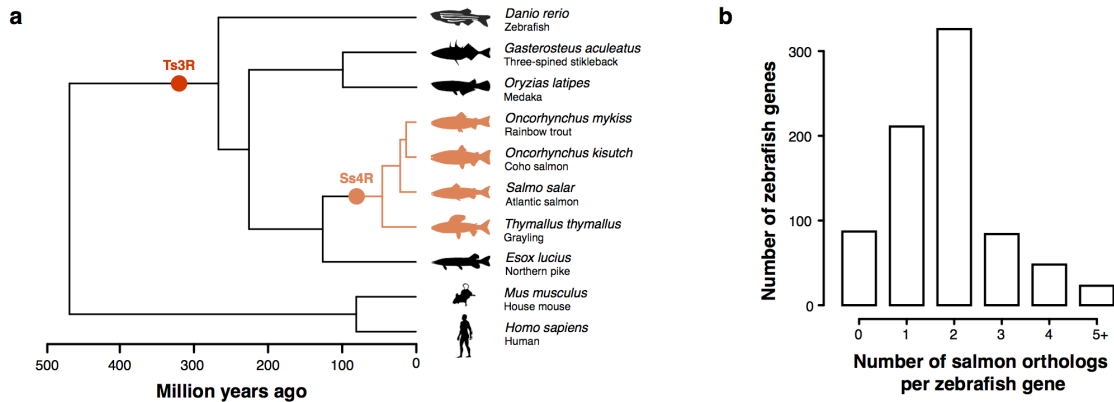
Figure 1: Ortholog annotation (a) Species used to construct ortholog groups and their evolutionary distance. Points in the phylogenetic tree show the time of the teleost specific (Ts3R) and salmonid specific (Ss4R) whole genome duplications. (b) The number of salmon orthologs found (1421 genes in total)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

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 in wild-type Atlantic salmon, and found agreement with known properties of vertebrate lipid metabolism. 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, and heart) (Glatz, Luiken, & Bonen, 2010; Rimoldi, Benedito-Palos, Terova, & Pérez-Sánchez, 2016; Douglas R 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

119 further refinement through manual community curation, we have created an interactive web-server

120 available online: (url).

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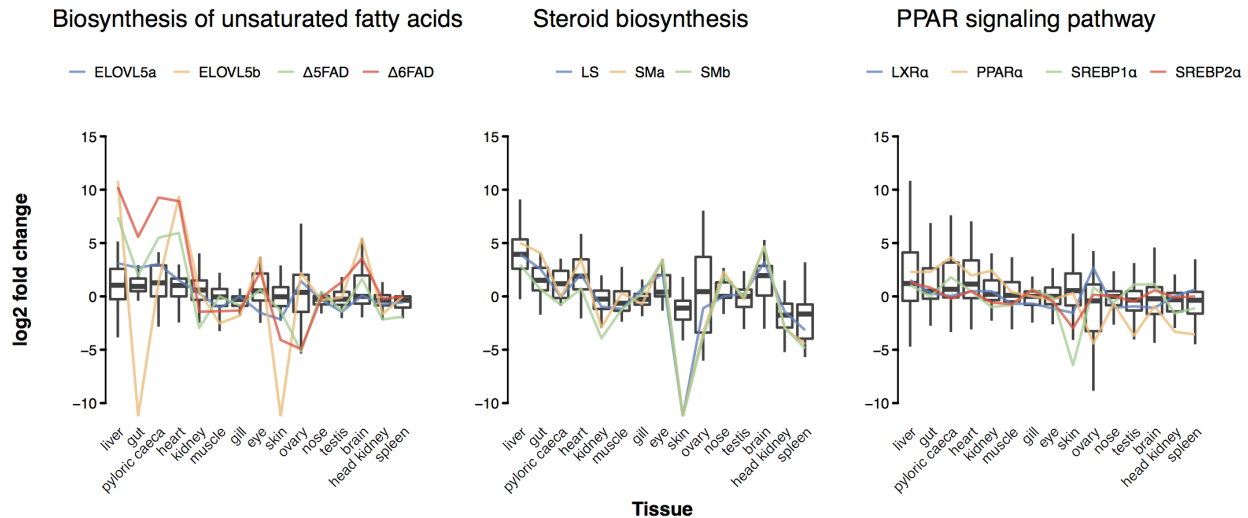
Figure 2: Tissue expression profiles of salmon genes in lipid metabolism pathways

124 Tissue expression profiles of our annotated lipid metabolism genes were consistent with expectations. Gene

125 expression levels are shown as the log2 fold change difference between the FPKM value of each tissue and the

126 median FPKM across all tissues. Expression profiles for selected genes in each pathway are shown (see Figure S2

127 for gene details).

Life-stage dependent remodelling of lipid metabolism

129 We conducted a feeding trial to study how salmon adjusts its lipid metabolism to different levels of LC-

130 PUFA in freshwater and saltwater. Groups of salmon were fed contrasting diets from hatching until after

131 transition to seawater. One feed was vegetable oil based (VO) and hence low in LC-PUFA, similar to

132 river ecosystem diets, whereas the other was based on fish oil (FO) and high in LC-PUFA as expected in

133 a marine-type diet (see Supplementary table 3 for details on feed composition). VO based diets are also

134 known to contain lower cholesterol levels (Ciftci, Przybylski, & Rudzińska, 2012; Verleyen et al., 2002).

135 In total, 32 and 23 fish were sampled for RNA-seq of liver and gut, respectively, including eight

136 biological replicates from each diet and life-stage (freshwater and saltwater). Fish in the different dietary

137 groups were given FO and VO feed from first feeding (<0.2g) until sampling.

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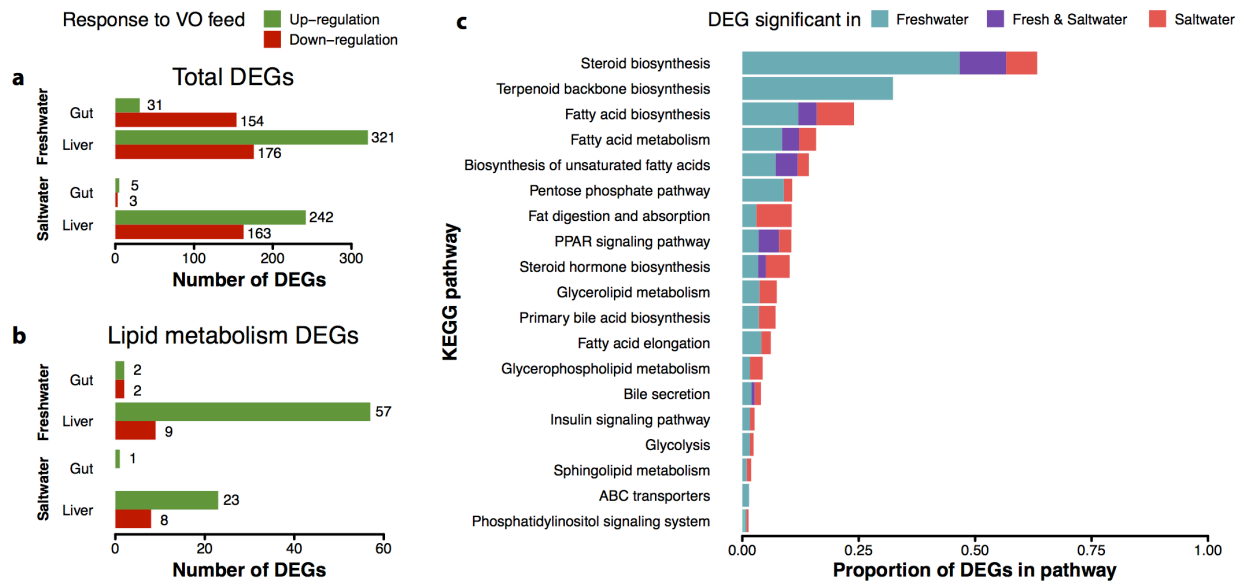
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140 **Figure 3: Gene regulation in response to feed type.** (a) Total number of significant (FDR < 0.05) differentially

141 expressed genes (DEGs) between fish oil (FO) and vegetable oil (VO) fed salmon in the liver and gut tissues of

142 freshwater and saltwater stage Atlantic salmon. (b) As above, but for lipid-associated genes only. (c) Proportions of

143 genes in each KEGG pathway that had significantly different liver expression between the two feed types only in

144 freshwater, only in saltwater, or in both stages.

145

146 In general, global gene expression levels were more affected by dietary composition in liver than in gut

147 (which was largely unresponsive), and the effect was more pronounced in freshwater than in saltwater

148 (Figure 3a). VO diets increased lipid-metabolism related gene expression compared to FO diets, with 57

149 genes (86% of the differentially expressed genes, DEGs) upregulated in freshwater and 23 genes (74% of

150 DEGs) upregulated in saltwater (Figure 3b). The increased activity of liver lipid metabolism under VO

151 diets confirm the well-known ability of salmon to regulate endogenous synthesis of LC-PUFA and

152 cholesterol in fresh water (Kortner, Björkhem, Krasnov, Timmerhaus, & Krogdahl, 2014; Leaver,

153 Villeneuve, et al., 2008; Zheng et al., 2005).

154

155 Freshwater and saltwater fish shared a relatively small number of DEGs for each pathway (Table S4). We

156 found that some pathways had more DEGs in freshwater ('*fatty acid biosynthesis*', '*steroid biosynthesis*',

157 and its precursor '*terpenoid backbone biosynthesis*'), whereas others had more DEGs in saltwater

158 ('*steroid biosynthesis*', '*fatty acid biosynthesis*', and '*fat digestion and absorption*') (Figure 3c). Out of 71

159 lipid metabolism DEGs in the dietary contrast, 78% (51 genes) were freshwater specific, 11% (8 genes)

160 saltwater specific, and 11% (8 genes) shared dietary response (Table S4). For example, only two genes in

161 the FA and LC-PUFA biosynthesis pathways (Δ6FADa and Δ5FAD) shared response to dietary

162 availability in fresh- and saltwater (Figure 4). A similar trend was seen for diet-induced expression

163 changes in the pathways responsible for cholesterol biosynthesis (Figure 5). The few genes that showed

164 diet-effects specific to saltwater included bile salt activated lipase, responsible for the hydrolysis of free

165 fatty acids from TAG obtained from the diet (Douglas R Tocher, 2003). Two of these genes, carboxyl

166 ester lipase, tandem duplicate 2a (CEL2a) and b (CEL2b), are highly upregulated in saltwater in response

167 to VO diet. Taken together, our results present clear evidence of life-stage dependent remodeling of

168 dietary-inducible flexibility in endogenous liver lipid metabolism, and is in line with the idea of a post-

169 smoltification phenotype adapted to an environment with surplus of essential lipids in the food-chain.
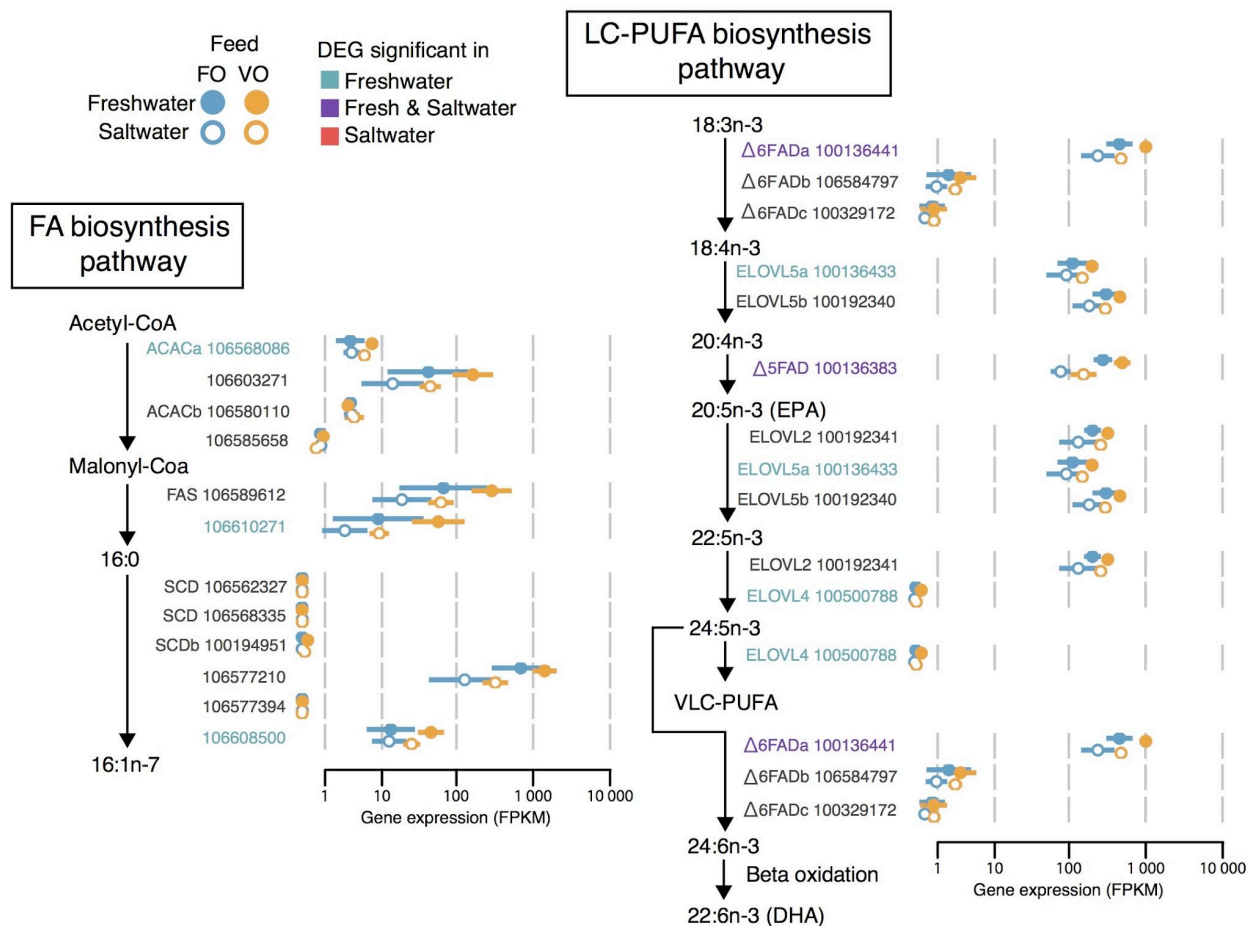
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171 **Figure 4: Diet and life stage effects on FA and LC-PUFA biosynthesis in salmon liver.** Core fatty acid (FA) biosynthesis and

172 biosynthesis of unsaturated fatty acids pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names

173 followed by NCBI gene numbers). Gene expression levels are shown as mean (point) and standard deviation (line) of expression

174 in eight samples (measured in log(FPKM + 1)) from each diet (FO, VO feeds) and life stage (freshwater, saltwater) combination.

175 Genes significantly (FDR<0.05) differentially expressed (DEG) between diets in a life stage are highlighted.

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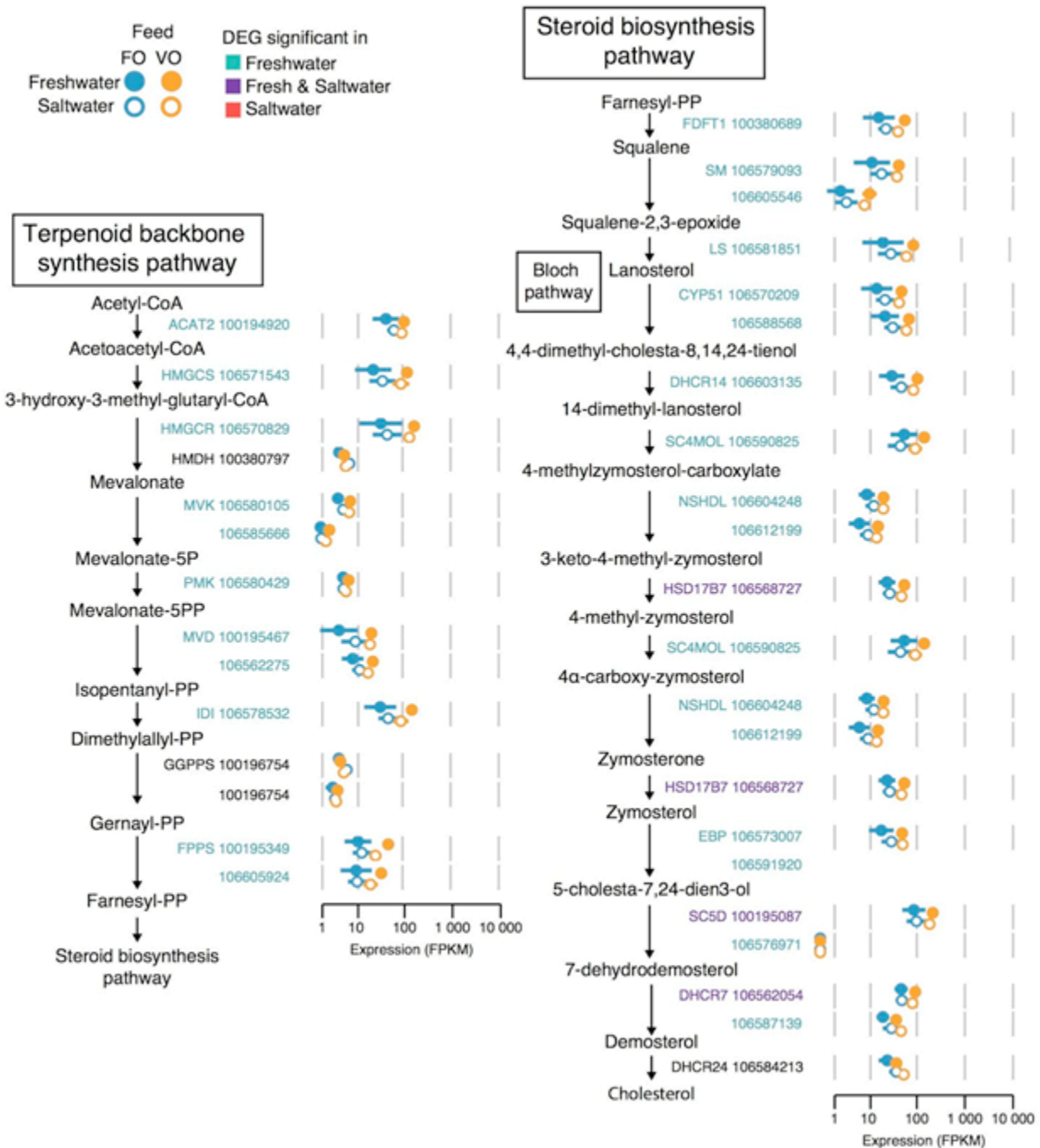
Figure 5: Diet and life stage effects on cholesterol biosynthesis in salmon liver. Terpenoid backbone synthesis and steroid biosynthesis pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names followed by NCBI gene numbers). Gene expression levels are shown as mean (point) 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.

183

184   To further investigate the life-stage associated changes in lipid metabolism we tested for differential
185   expression between freshwater and saltwater in salmon fed the same diets (Figure 6). Liver and gut
186   showed contrasting effects of saltwater on lipid gene expression: Mostly downregulation in liver,
187   upregulation in gut (Figure 6b). The number of DEGs in each tissue were similar for the environment
188   comparison (Figure 6a), unlike for the diet comparison (Figure 3).

189

190   Further examination of key lipid metabolism genes revealed that the system-wide lipid metabolism
191   remodeling represented a concerted shift in the metabolic role of liver and gut. As the salmon entered the
192   marine stage, lipogenic activity in the liver was significantly ramped down, as evident by the markedly
193   lower expression (2.2-3.3 fold) of the master regulator of lipid metabolism SREBP1, a 5-fold decrease in
194   expression of fatty acid synthase, and a 2-3 fold decrease in rate-limiting enzymes in LC-PUFA syntheses
195   (i.e. Δ5FAD, Δ6FADa) (Figure 4). Liver and gut also increased their catabolic activity in saltwater,
196   upregulating the uptake of fatty acids into mitochondria for β-oxidation (uptake carried out by carnitine
197   palmitoyltransferase 1 and 2) (Lehner & Quiroga, 2016). Finally, expression of lipid transport genes
198   shifted from liver to gut with the transition to seawater (apolipoproteins, pathway "Fat digestion and
199   absorption" in Figure 1). Four apolipoproteins (out of 11 annotated) were differentially regulated in liver
200   between different diets; these all decreased their expression 2.4-5 fold in saltwater compared to
201   freshwater. In stark contrast, nine of the diet-regulated apolipoproteins in gut increased their expression in
202   saltwater between 1.8-9.7 fold. These results suggest that salmon's decreased ability to synthesize LC-
203   PUFAs after fresh-to-saltwater transition is compensated by an increased ability to take up lipids in the
204   gut.

205

206   Interestingly, diet had a strong influence on the number and direction of gene expression changes between
207   freshwater and saltwater (Figure 6). In gut, the transcriptional changes between fresh- and seawater was
208   twice as high when fed FO diet than VO diet (Figure 6a). In liver, the diet effect was less pronounced,
209   with the FO group containing 46% more DEGs than the VO group (Figure 6a). Identical patterns were
210   seen for lipid metabolism genes with 89% and 16% more DEGs in FO group for gut and liver,
211   respectively (Figure 6b). As this diet and life-stage interaction is a genome wide trend, and more
212   pronounced in external barrier gut tissue than in liver, this pattern could be related to differences in
213   osmoregulation and adaptation to saltwater. The higher levels of essential omega-6 FA linoleic acid (18:2
214   n-6, LA) in VO diets could result in increased levels of arachidonic acid (20:4 n-6, ARA). ARA is a
215   precursor to eicosanoids with a multitude of biological functions including osmoregulation (Mustafa &
216   Srivastava, 1989; Oxley et al., 2010). Another possibility is that the different levels of fatty acids in the

217   diets, for example DHA, affect DNA-methylation and thus trigger genome wide divergence in gene

218   regulation (Kulkarni et al., 2011).
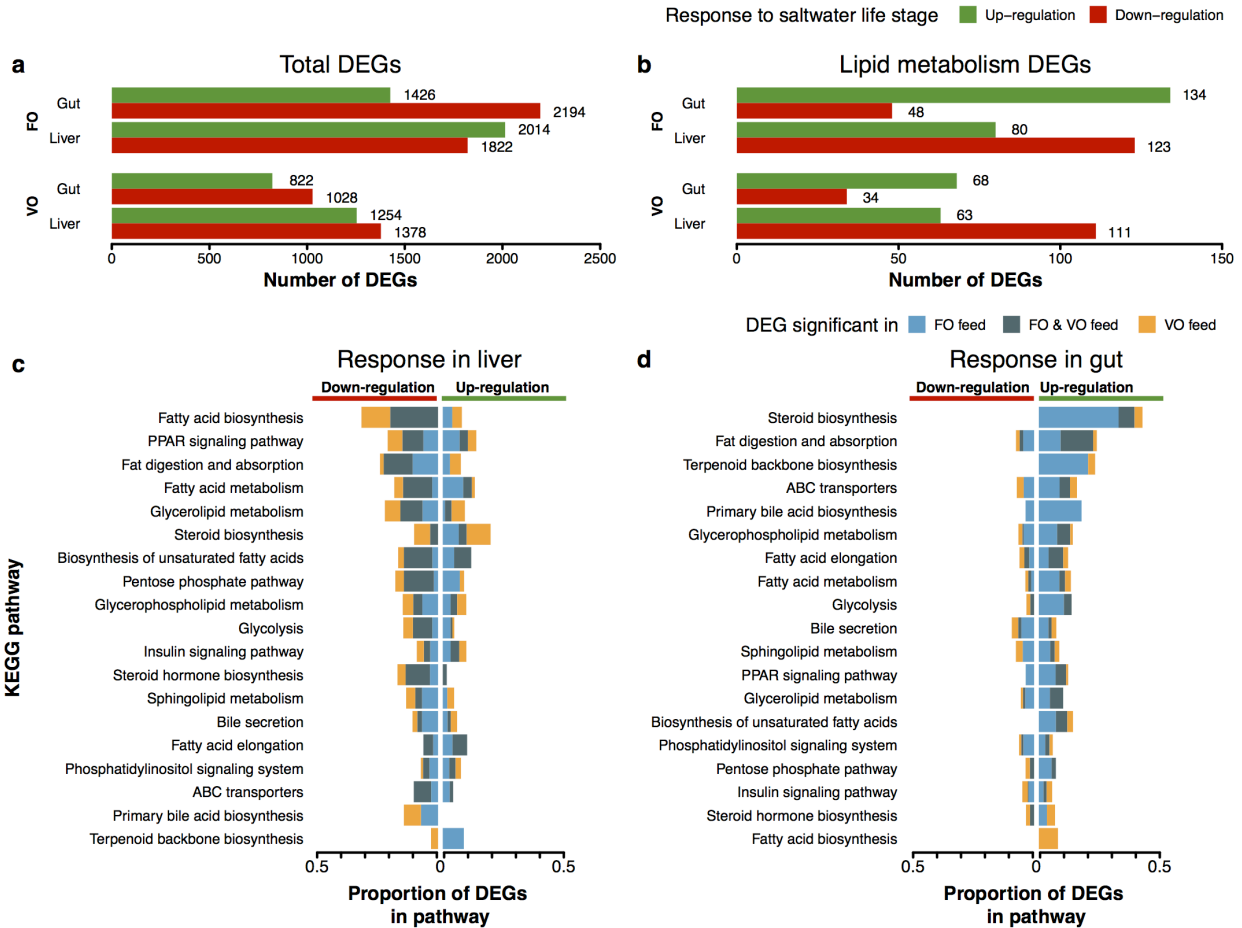
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220   **Figure 6: Gene regulation in response to life stage.** (a) Total number of significant (FDR < 0.05) differentially

221   expressed genes (DEGs) between freshwater and saltwater life stages in the liver and gut tissues of Atlantic salmon

222   fed fish oil (FO) or vegetable oil (VO) diets. (b) As above, but for lipid metabolism DEGs. (c) Proportion of genes

223   in each KEGG pathway that are DEGs in liver and (d) gut, coloured by DEG significance in only FO, only VO, or

224   both diets, and separated into up- or down-regulation in saltwater samples.

225

226   Our results clearly demonstrate very different baseline lipid metabolic functions in pre- and post-smolt

227   salmon, as well as life-stage associated changes in the plasticity of lipid metabolism, e.g. the ability to

228   regulate endogenous LC-PUFA synthesis as a response to changes in diet (i.e. lipid content). As

229   opportunistic carnivores, salmon tend to eat whatever the local environment provides. Thus, in

230   freshwater, insects and amphipods provide variable, mostly low amounts of essential LC-PUFA (Jonsson

231   & Jonsson, 2011; Sushchik, Gladyshev, Moskvichova, Makhutova, & Kalachova, 2003), favoring a

232   metabolic function that can efficiently regulate endogenous lipid synthesis based on dietary availability

233   (Carmona-Antonanzas, Tocher, Martinez-Rubio, & Leaver, 2014). Conversely, marine environments

234   provide a stable source of essential dietary lipids, promoting a metabolic function that allocate less energy

235   to endogenous synthesis of essential lipids.

Selection on gene duplicates after whole genome duplication

237   Carmona-Antonanzas et al (2014, 2016) proposed that the salmonid whole-genome duplication may have

238   adaptively increased the potential for endogenous lipid synthesis. We pursued this hypothesis by

239   searching for distinct signatures of selection pressure on lipid metabolism genes in salmon. Specifically,

240   we compared pathways in terms of their tendency to retain both duplicates of gene pairs, in terms of

241   whether duplicates showed similar regulation (expression patterns across tissues, diets and environments),

242   and in terms of total gene dosage (for the one or two genes retained of a pair) in salmon compared to pike,

243   its closest unduplicated relative.

244

245   To assess the level of Ss4R duplicate retention, we first defined 10,752 Ss4R duplicate pairs (21,504

246   genes) in the NCBI refseq annotation using the same approach as Lien et al. (2016). Of the 1,421

247   annotated lipid metabolism genes, 867 (61%) were retained as duplicated genes after Ss4R (Figure 7a) (in

248   contrast to 47% of the 45,127 salmon genes assigned to ortholog groups). Moreover, our results showed

249   large variation in the proportion of retained duplicates in each lipid metabolism pathway (Figure 7), with

250   the most extreme case being '*fat digestion and absorption*' with 80% retained duplicates and '*steroid*

251   *hormone biosynthesis*' with only 27% retained Ss4R duplicates.

252

253   The regulatory conservation of the duplicates was then estimated from RNA-seq data representing a time

254   course of dynamic changes in gene expression and lipid metabolism function in liver. Fish in the same

255   feeding trial as described above were switched from VO to FO feed and vice versa, in both fresh and

256   saltwater conditions (see methods and supplementary data for details). In total, 38 sampling time points

257   (19 in freshwater and 19 in saltwater) from the switch experiment were used to calculate co-expression

258   correlation for Ss4R duplicate pairs. Pathway-level analyses showed that conservation at the regulatory

259   level was not associated with duplicate retention (Figure 7). For example, the '*biosynthesis of unsaturated*

260   *fatty acids*' pathway had significantly fewer duplicates retained than expected by chance (P-value <

261   0.0234), but a significant overrepresentation of duplicate pairs that display highly similar regulation (P-

262   value < 0.0142 and < 0.0361 in freshwater and saltwater, respectively). Other pathways showing

263   signatures of increased duplicate co-regulation were '*insulin signalling pathway*', '*terpenoid backbone*

264   *biosynthesis*', '*steroid biosynthesis*', '*fat digestion and absorption*', and '*fatty acid metabolism*' (Figure

265   7b-c). Over all, the distinct differences in duplicate retention and conservation of regulatory mechanisms

266 across the lipid metabolism pathways suggest differences in selective pressures shaping duplicate

267 evolution following Ss4R. Moreover, the pathways with highly conserved duplicate co-regulation was

268 also those that were most responsive to dietary differences in fatty acids? (Figure 3).

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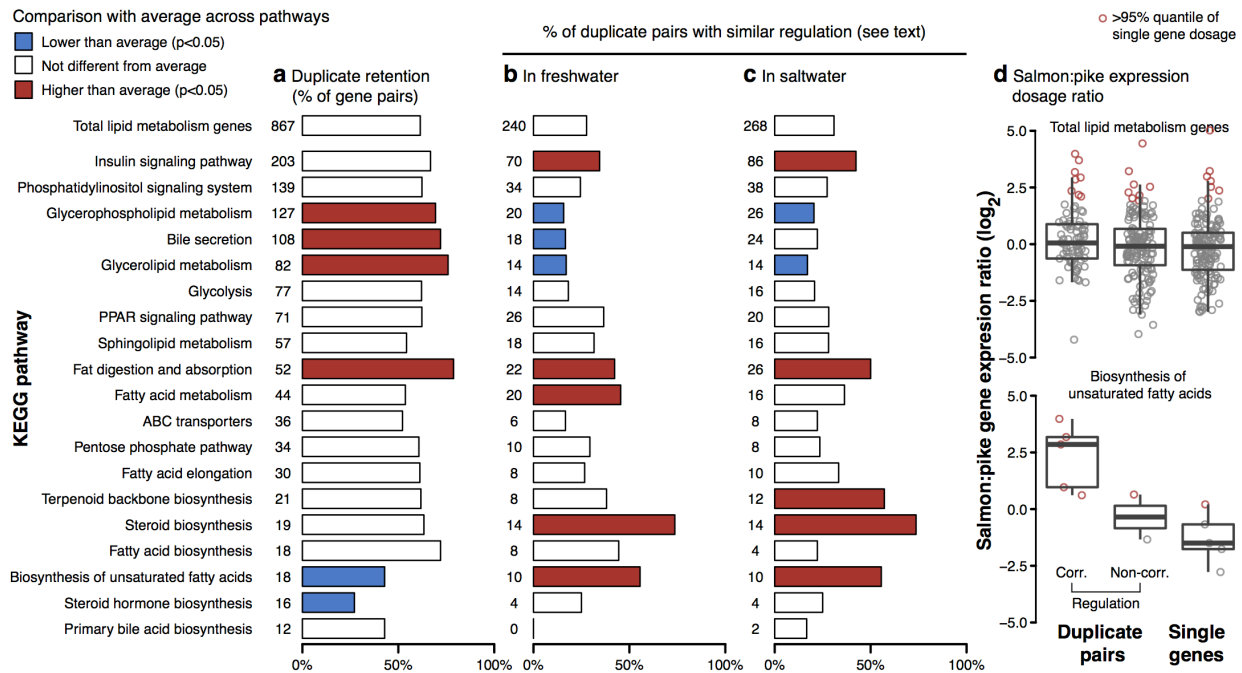
Figure 7 - Gene duplication in lipid metabolism pathways. For the total list of lipid metabolism genes in Atlantic salmon, and sets of genes belonging to different KEGG pathways: (a) Number and percentage of genes with a duplicate homolog from the Ss4R duplication. (b) Number and percentage of duplicate 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.

282

283 Finally, to link duplicate retention and co-regulation to signals of increased gene dosage following Ss4R,

284 we used RNA-seq data from the Northern pike (*Esox lucius*), a species that belongs to the unduplicated

285 sister lineage (see methods for details). For each duplicate pair we computed the ratio between the sum of

286 Ss4R duplicate expression and its non-duplicated ortholog in pike and compared these ratios to those

287 observed for salmon genes that had not retained two Ss4R duplicates. In total 69 duplicate pairs from 18

288 different lipid-metabolism related pathways displayed a combined dosage increase relative to single copy

289   genes, of which 26 had highly conserved regulation (i.e. correlated expression) (Supplementary file 10).

290   Compared to single copy genes, the analyses of expression sum of duplicate pairs did not reveal a

291   systematic increase in gene dosage, nor did co-regulation of duplicates associate with increased gene

292   dosage (Figure 7d). This pattern was also true for most individual lipid pathways (Figure S4-S5), except

293   for *'biosynthesis of unsaturated fatty acids'*, *'fatty acid metabolism'* and *'fatty acid elongation'*. These

294   three pathways showed a link between co-regulation of duplicated genes and higher total gene dosage

295   (Figure S4-S5, Figure 7d). Underlying this links were three genes with co-regulated dosage effects shared

296   between all three pathways; trifunctional enzyme alpha subunit b (hadhab), elovl6, and the previously

297   identified elovl5 (Carmona-Antonanzas et al., 2014; Carmona-Antoñanzas et al., 2016). Only elovl5 is

298   known to be directly involved in core PUFA biosynthesis. hadhab is involved in mitochondrial β-

299   oxidation/elongation and elovl6 is involved in elongation of saturated and monounsaturated fatty acids

300   (Bond, Miyazaki, O'Neill, Ding, & Ntambi, 2016). Although we do not see a general trend of increased

301   gene dosage of lipid metabolism genes after whole genome duplication, it is likely that an increased

302   dosage of *elovl5* and the 68 other duplicate pairs has affected the function of lipid metabolism in salmon.

303

Conclusion

305   Seagoing salmon needs great plasticity of physiology and behaviour to adapt for migration between

306   freshwater and sea. By analyzing transcriptomic changes through the transition from fresh- to saltwater,

307   we identified an overall remodeling of lipid metabolism, with liver being more active in freshwater to gut

308   being more active in saltwater. This baseline remodelling was modulated by diet: Our experiment also

309   included life-long dietary contrasts showing that lipid metabolism was significantly more responsive to

310   dietary differences in lipid composition in fresh water than in saltwater. These results indicate adaptive

311   optimization of the Atlantic salmon lipid metabolism to account for life-stage specific dietary availability.

312   Moreover, we found signatures of pathway-specific selection pressure on gene duplicates, including a

313   gene dosage increase in three genes involved in fatty acid metabolism. This illustrates possible adaptive

314   consequences of the salmonid whole-genome duplication for the evolution of lipid metabolism. Future

315   studies should attempt to decipher how this life-stage related metabolic reprogramming is controlled, e.g.

316   to what extent it reflects a "hard coded" physiological transformation before sea migration (for example

317   through epigenetic remodeling).

318

Materials and methods

Orthogroup prediction

Protein sequences were obtained from seven teleost fish species; *Danio rerio* (zebrafish), *Gasterosteus aculeatu* (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.*, awaiting publication of data). 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, Misawa, Kuma, & Miyata, 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 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, such as genes 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

346

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

Feed trial

356

357 Atlantic salmon fry were obtained from AquaGen Breeding Centre, Kyrksæterøra, Norway and reared in
358 the Norwegian Institute for Water Research (NIVA), Solbergstranda, Norway on vegetable oil (VO) or
359 fish oil (FO) based diets from first feeding (fry weight <0.2g). VO based feeds contained a combination
360 of linseed oil and palm oil at a ratio of 1.8:1 and FO based feeds contained only North Atlantic fish oil.
361 All feeds were formulated and produced by EWOS innovation. Smoltification was triggered by 5 weeks
362 of winter-like conditions with 12 hours of light per day followed by spring-like conditions with 24 hours
363 of light per day. Salmon were then immediately switched to saltwater and allowed to acclimate for 3
364 weeks before first sampling. Pre-smolt salmon in freshwater (~50g) and post-smolt salmon in saltwater
365 (~200g) were either switched to the contrasting feed condition (VO to FO and vice versa) or maintained
366 on the same feed as a control. Fish samples were taken on days 0, 1, 2, 6, 9, 16, and 20 post each diet
367 switching, with five fish from each of two replicate tanks per treatment (5*2). All fish were euthanized by
368 a blow to the head and samples of liver and midgut (gut section between pyloric caeca and hindgut) were
369 flash frozen in liquid nitrogen and stored under -80℃ for further analysis.

RNA sequencing

370

371 Total RNA was extracted from selected feed trial samples using the RNeasy Plus Universal kit
372 (QIAGEN). Quality was determined on a 2100 Bioanalyzer using the RNA 6000 nano kit (Agilent).
373 Concentration was determined using a Nanodrop 8000 spectrophotometer (Thermo Scientific). cDNA
374 libraries were prepared using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina). Library mean
375 length was determined by running on a 2100 Bioanalyzer using the DNA 1000 kit (Agilent) and library

376 concentration was determined with the Qbit BR kit (Thermo Scientific). Single end sequencing of sample

377 libraries was completed on an Illumina HiSeq 2500 with 100bp reads.

378 Differential expression analysis of feed and life stages

379 To analyse gene expression differences between feed types and life stages, samples from the feed trial

380 were selected for RNA-seq. The liver and gut tissue of a number of replicate fish were sequenced for each

381 of the feeds (FO, VO) at day 0 of the diet switch, both before (freshwater) and after (saltwater)

382 smoltification. Fastq files were processed to produce gene count and FPKM data using the same protocol

383 described under the tissue expression method section. For feed comparison, changes in gene expression

384 were tested between FO and VO feeds for both freshwater and saltwater samples, and liver and gut

385 tissues. For life stage (water condition) comparison, changes in gene expression were tested between

386 freshwater and saltwater stages for both FO and VO feed samples, and liver and gut tissues. Using RNA-

387 seq gene count data, lowly expressed genes were filtered prior to testing, retaining genes with a minimum

388 of one read count per million (CPM) in two or more samples. Differential expression analysis was carried

389 out using a standard edgeR (Robinson et al., 2010) protocol. Effective library sizes were calculated using

390 the edgeR TMM-normalisation procedure allowing effective comparison of expression data between

391 different sample types (see edgeR user manual). An exact test between expression levels of a pair of

392 conditions gave the log2 fold change, P-value and false discovery rate (FDR) for each gene. Genes with

393 an FDR < 0.05 were considered a differentially expression gene (DEG).

394 Identification of Ss4R duplicates

395 To identify putative gene duplicates stemming from the Ss4R we used the same approach as in Lien et al.

396 (2016). All-vs-all protein blast was run with e-value cutoff of 1e-10 and pident (percentage of identical

397 matches) ≥80 and blast hit coverage of ≥50% of protein length. Only the best protein hits between the 98

398 defined synteny blocks (see Lien et al., 2016) were considered as putative Ss4R duplicates. Blast result

399 ranking was done using the product of pident times bitscore to avoid spurious 'best blast matches' with

400 low pident (<85) but high bitscore.

401 Duplicate analysis

402 Gene from the lipid metabolism gene list were paired together with their putative Ss4R duplicate

403 identified above. The retention of gene duplicates (number of genes in duplicate pairings) was compared

404 between all identified duplicates in the salmon genome annotation and the lipid metabolism gene list.

405 Pathway-level retention was explored by comparing the number of genes in each of the 19 selected

406 KEGG pathways (Table S1) in a duplicate pairing to that of the total list of lipid genes to find pathways

407 with significantly less or more duplicate retention (Fisher's exact test, P-value < 0.05). Regulatory

408 conservation of lipid gene duplicates was explored by correlation of gene expression changes between

409 duplicates over the course of the feed trial described above. RNA-seq data was generated from liver

410 samples of salmon from 38 sampling time points (19 in freshwater and 19 in saltwater). Fastq files were

411 processed to produce gene count and FPKM data using the same protocol described under the tissue

412 expression method section. For each duplicate pair, mean FPKM values were retrieved for each time

413 point and used to calculate a freshwater and saltwater correlation value. Duplicates with Pearson

414 correlation ≥ 0.6 were considered correlated (P-value < 0.003 from 19 sample points). The number of

415 duplicates with correlated expression profiles was counted for each pathway and compared to all lipid

416 genes to find pathways with significantly less or more correlated duplicates (Fisher's exact test, P-value <

417 0.05). The effect of gene duplication on gene dosage was estimated by calculating a dosage ratio between

418 the FPKM value of a salmon duplicate (sum of gene expression in duplicate pairs) over the FPKM value

419 of the non-duplicated ortholog from northern pike. For salmon, the RNA-seq data from the freshwater and

420 saltwater FO fed trial was used (samples used in differential expression analysis section). For pike, RNA-

421 seq from livers of four individuals were aligned (see tissue expression section for protocol) to their

422 respective genomes (see genomes in ortholog prediction section). RSEM (v1.2.31) (Li & Dewey, 2011)

423 was used to generate FPKM values for genes so that non-uniquely mapped reads between duplicate genes

424 were not ignored but instead assigned to each gene to match the proportions of uniquely mapped reads

425 between the genes. Gene dosage levels for duplicate pairs with correlated expression (see above), non-

426 correlated expression and single genes was compared for all lipid metabolism genes and for each

427 pathway.

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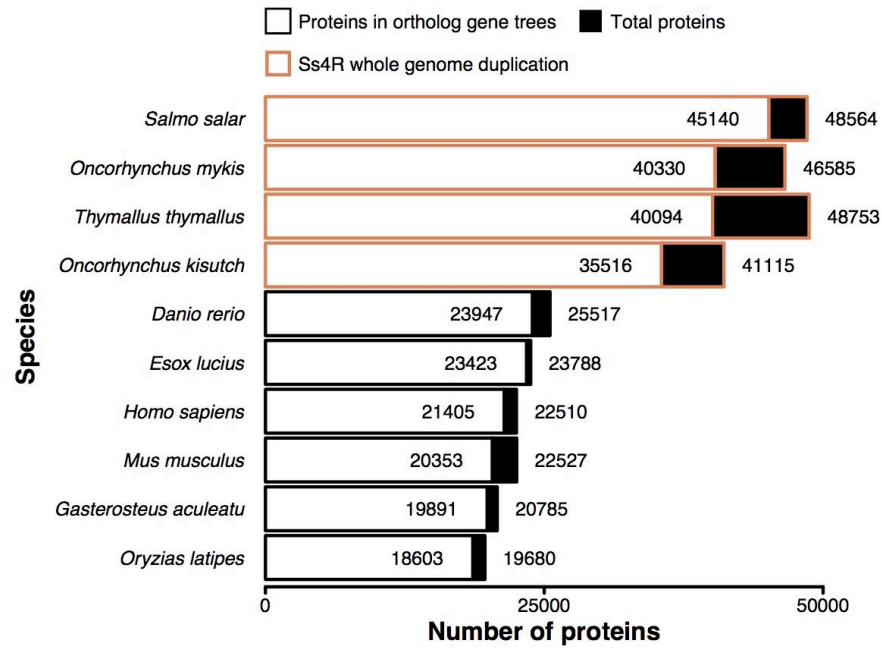535   13–24. http://doi.org/10.1016/j.bbalip.2005.01.006

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543 # Supplemental material

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545   Figure S1: Number of proteins in ortholog gene trees for each species
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| KEGG ID	Pathway name
dre00010	Glycolysis
dre00561	Glycerolipid metabolism
dre04070	Phosphatidylinositol signaling system
dre04910	Insulin signaling pathway
dre00600	Sphingolipid metabolism
dre01212	Fatty acid metabolism
dre03320	PPAR signaling pathway
dre00564	Glycerophospholipid metabolism
ko04976	Bile secretion
dre02010	ABC transporters
dre00900	Terpenoid backbone biosynthesis
ko04975	Fat digestion and absorption
dre00100	Steroid biosynthesis
dre00061	Fatty acid biosynthesis
dre00140	Steroid hormone biosynthesis
dre00062	Fatty acid elongation
dre00120	Primary bile acid biosynthesis
dre01040	Biosynthesis of unsaturated fatty acids
dre00030	Pentose phosphate pathway

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556   Table S1: KEGG pathways related to lipid metabolism selected for gene annotation
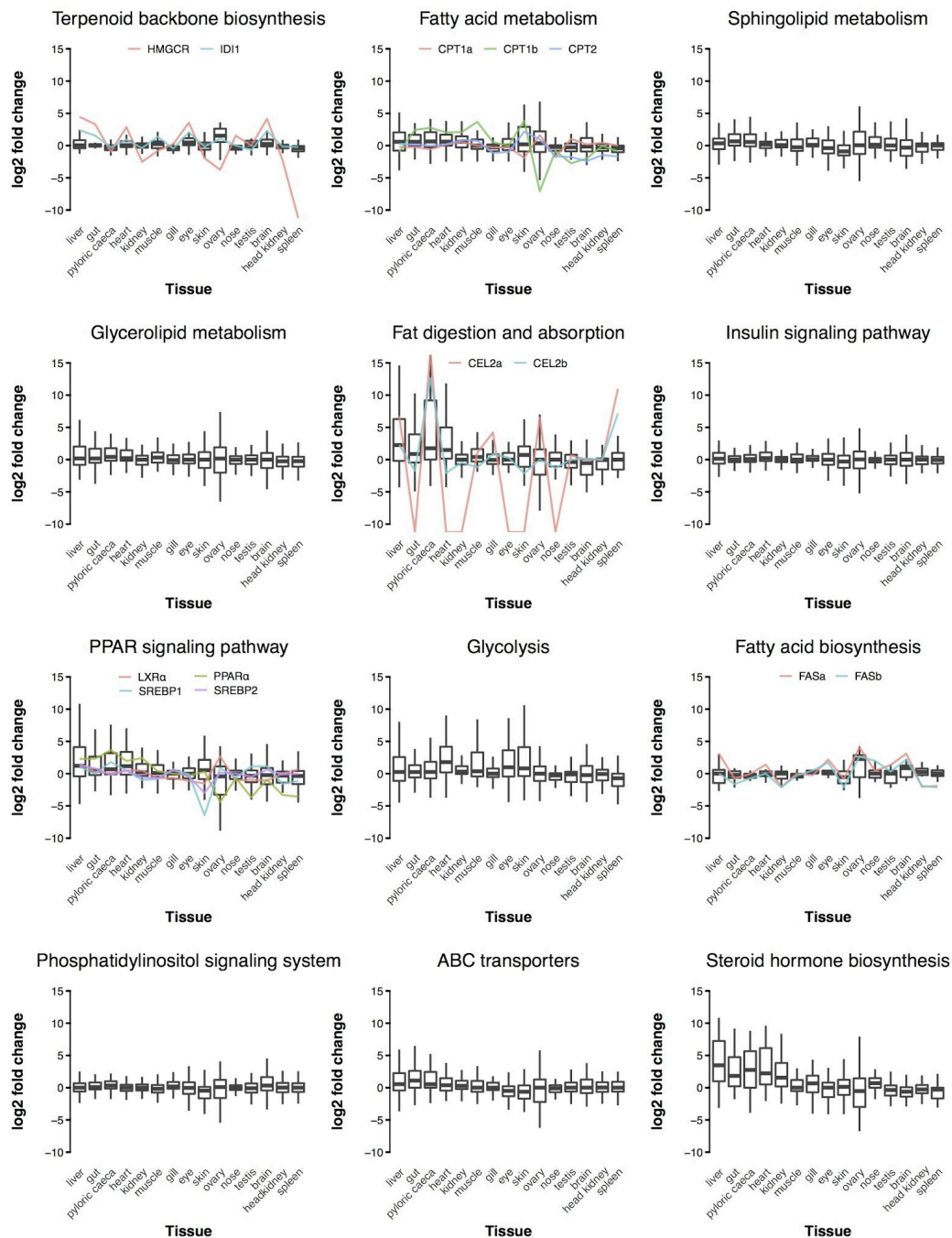
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Figure S2: Tissue expression profiles of salmon genes in lipid metabolism pathways.
Tissue expression profiles of salmon genes in KEGG pathways for *Salmo salar* tissues; pyloric caeca, liver, heart, gut, skin, kidney, muscle, eye, ovary, brain, nose, gill, testis, spleen, and head kidney. Gene expression levels are shown as the log2 fold change difference between each tissue FPKM to the medium FPKM across all tissues. Expression of select genes of interest are overlaid. These are 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), isopentenyl-diphosphase Δisomerase (IDI1), carnitine palmitoyltransferase 1 (CPT1a and CPT1b) and 2 (CPT2), carboxyl ester lipase, tandem duplicate 2 (CEL2a and CEL2b), liver x receptor alpha (LXRαa), peroxisome proliferator-activated receptor alpha (PPARαa), sterol regulatory element binding protein 1 (SREBP1) and 2 (SREBP2), and fatty acid synthase (FASa and FASb).
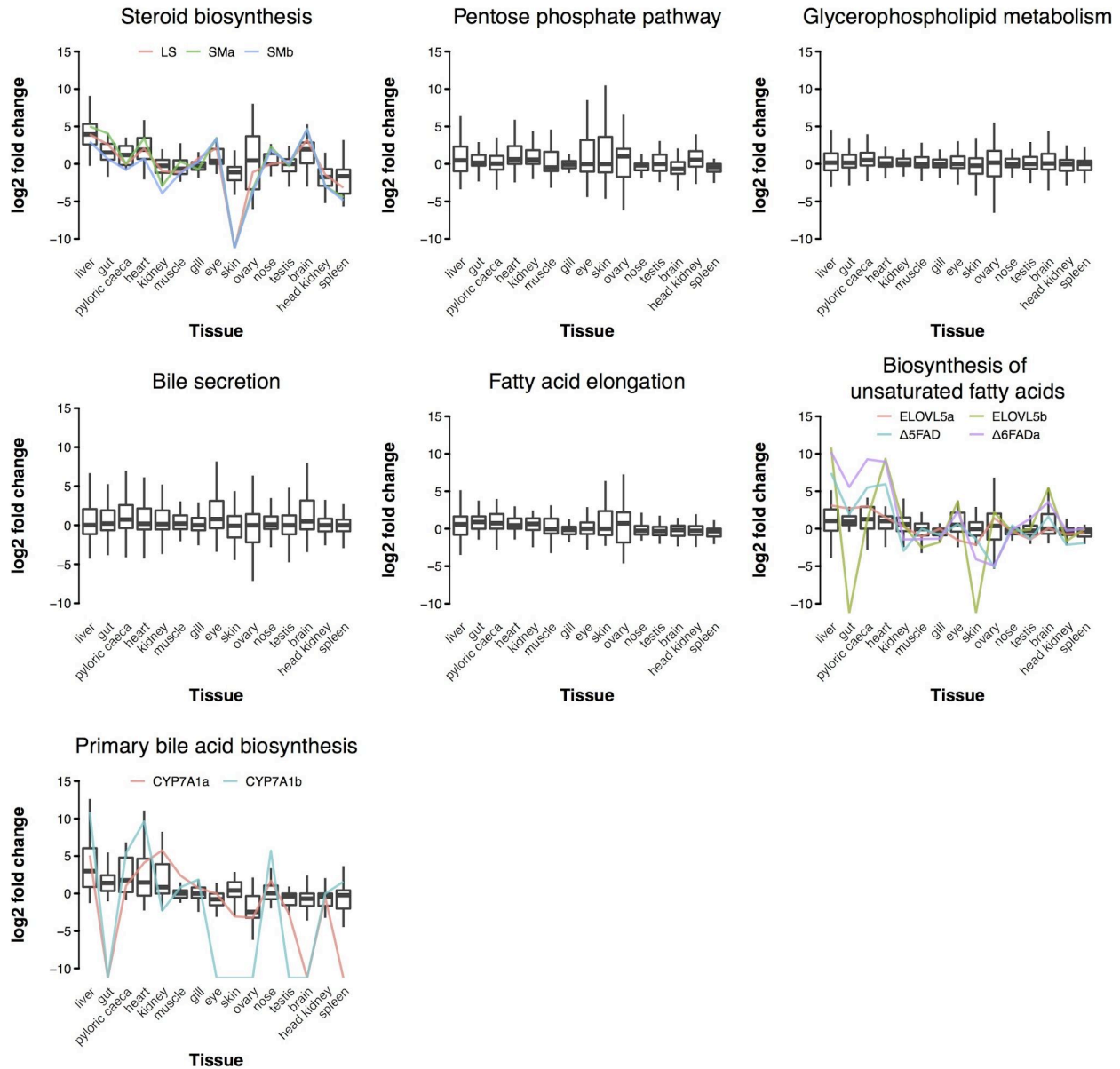
568
569 **Figure S3: Tissue expression profiles of salmon genes in lipid metabolism pathways.** Tissue expression profiles of salmon
570 genes in KEGG pathways for *Salmo salar* tissues; pyloric caeca, liver, heart, gut, skin, kidney, muscle, eye, ovary, brain, nose,
571 gill, testis, spleen, and head kidney. Gene expression levels are shown as the log2 fold change difference between each tissue
572 FPKM to the medium FPKM across all tissues. Expression of select genes of interest are overlaid. These are lanosterol synthase
573 (LS), squalene epoxidase (SMa and SMb), fatty acid elongase 5 (ELOVL5a and ELOVL5b), delta 5 fatty acid desaturase
574 (Δ5FAD), delta 6 fatty acid desaturase (Δ6FAD), and cytochrome P450 7A1 (CYP7A1a and CYP7A1b).

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Fatty Acid	Freshwater		Saltwater	
	MA	VO	MA	VO
14:0	6.4	1.5	6.5	1.6
15:0	0.5	0.1	0.6	0.1
16:0	15.4	18.5	15.3	19.5
18:0	2.8	3.8	3.8	4.8
20:0	0.1	0.2	0.1	0.1
Total Saturates	25.2	24.1	26.3	26.1
16:1n7	4.1	0.9	3.8	0.8
18:1n9	16.9	23.5	14.9	23.5
20:1n9	8	1.4	8.2	1.4
22:1n11	12	2.2	12.4	2.1
Total Monounsaturates	41	28	39.3	27.8
18:2n6	4.6	12.7	4.2	12.5
18:3n6	0.1	0.1	0.1	0.1
20:2n6	2.7	0.5	3	0.5
20:4n6	0.3	0.1	0.3	0.1
Total n-6 PUFA	7.7	13.4	7.6	13.2
18:3n3	2.11	28.8	2.5	26.8
20:3n3	0.2	0.1	0.3	0.1
20:5n3	5.8	1.3	6.2	1.4
22:5n3	0.9	0.2	0.8	0.2
22:6n3	10.7	2.3	11.7	2.7
Total n-3 PUFA	19.71	32.7	21.5	31.2
EPA+DHA	16.5	3.6	17.9	4.1

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583   Table S3: FA composition of feeds. All values are expressed as mass percentage of lipid fraction.

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pathway_name	total	FW.sig	SW.sig	FW.SW.sig	FW.p	SW.p	FW.SW.p
Phosphatidylinositol signaling system	223	2	2	1	0.0090	0.0090	0.0045
ABC transporters	69	1	NA	NA	0.0145	NA	NA
Sphingolipid metabolism	105	1	1	NA	0.0095	0.0095	NA
Glycolysis	124	2	1	NA	0.0161	0.0081	NA
Insulin signaling pathway	304	5	3	NA	0.0164	0.0099	NA
Bile secretion	150	4	3	1	0.0267	0.0200	0.0067
Glycerophospholipid metabolism	183	3	5	NA	0.0164	0.0273	NA
Fatty acid elongation	49	2	1	NA	0.0408	0.0204	NA
Primary bile acid biosynthesis	28	1	1	NA	0.0357	0.0357	NA
Glycerolipid metabolism	108	4	4	NA	0.0370	0.0370	NA
Steroid hormone biosynthesis	59	3	4	1	0.0508	0.0678	0.0169
PPAR signaling pathway	114	9	8	5	0.0789	0.0702	0.0439
Fat digestion and absorption	66	2	5	NA	0.0303	0.0758	NA
Pentose phosphate pathway	56	5	1	NA	0.0893	0.0179	NA
Biosynthesis of unsaturated fatty acids	42	5	3	2	0.1190	0.0714	0.0476
Fatty acid metabolism	82	10	6	3	0.1220	0.0732	0.0366
Fatty acid biosynthesis	25	4	3	1	0.1600	0.1200	0.0400
Terpenoid backbone biosynthesis	34	11	NA	NA	0.3235	NA	NA
Steroid biosynthesis	30	17	5	3	0.5667	0.1667	0.1000

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591   Table S4: Table of total and DEG gene counts for each lipid metabolism pathway

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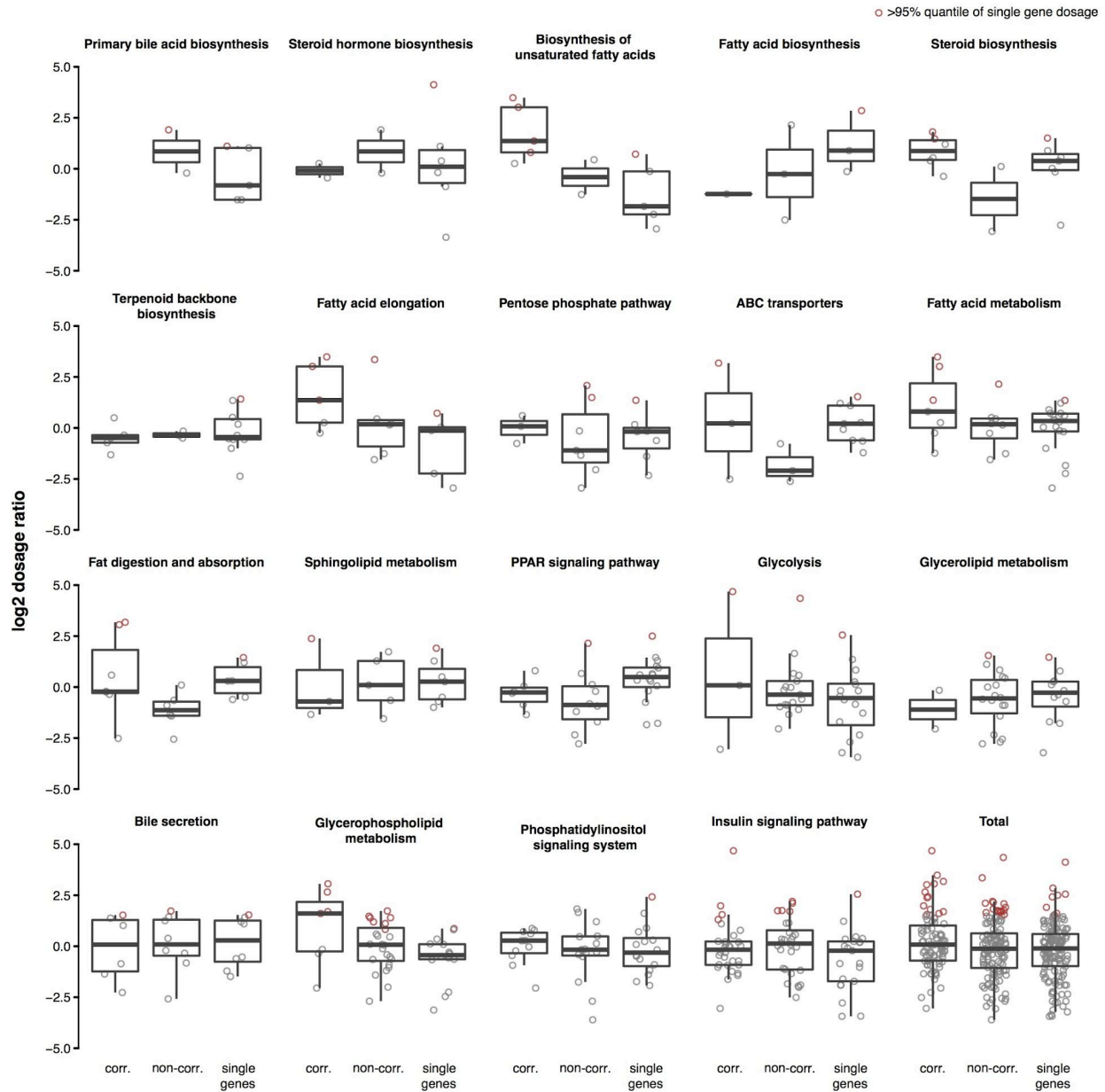
Figure S4: Gene dosage ratios and duplicate correlation for saltwater conditions
595
596 Log2 of gene dosage ratios of saltwater salmon ortholog expression in liver (FPKM, summed for duplicates) to expression of a
597 pike ortholog, for total lipid metabolism genes and genes 19 KEGG pathways. Duplicates were grouped into correlated (corr.) or
598 non-correlated (non-corr.) based on saltwater correlation results. Dosage ratios (points) greater than the 95% quantile of single
599 gene dosages in the plot are marked in red.

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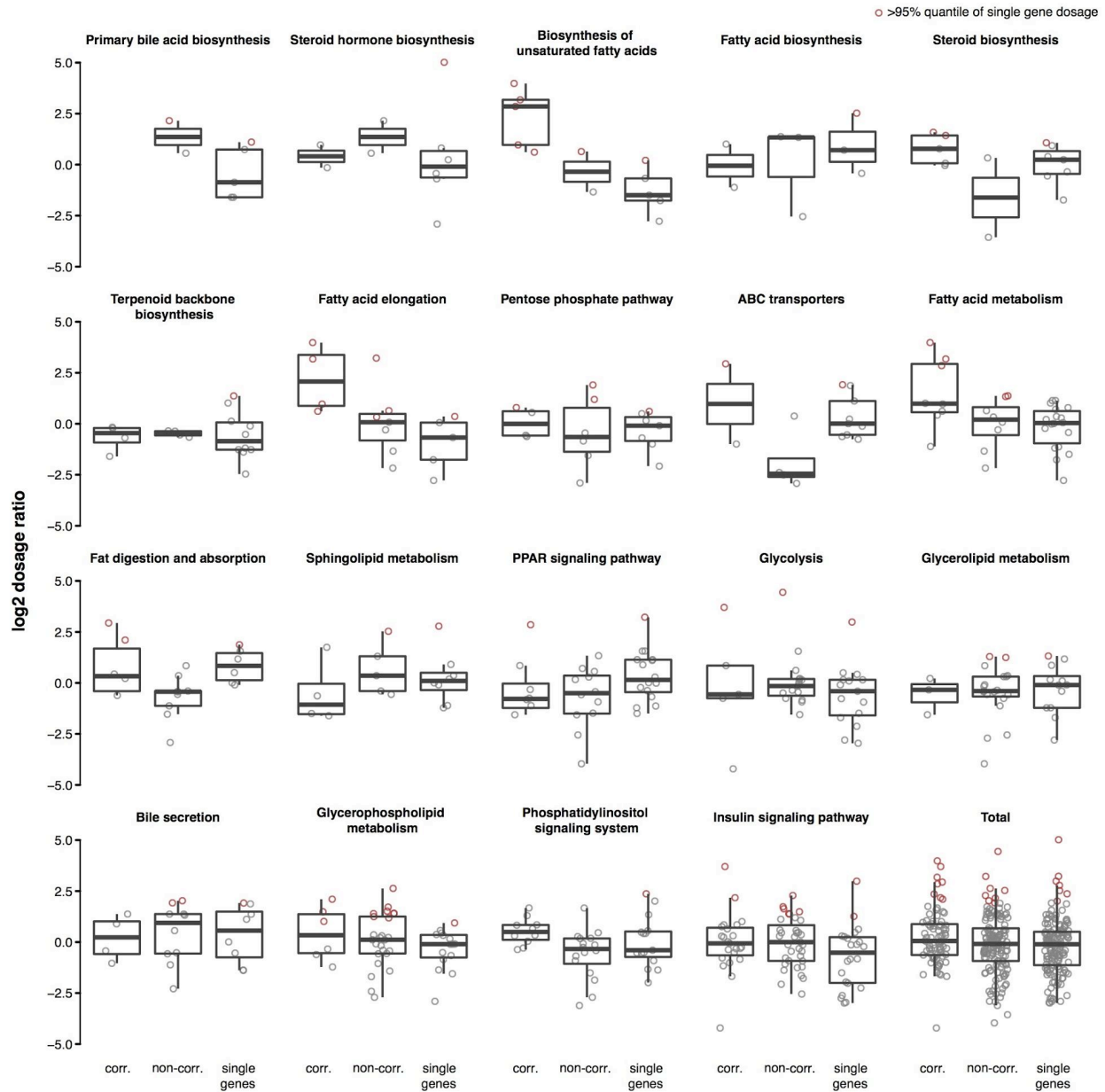
Figure S5: Gene dosage ratios and duplicate correlation for freshwater conditions
Log2 of gene dosage ratios of freshwater salmon ortholog expression in liver (FPKM, summed for duplicates) to expression of a pike ortholog, for total lipid metabolism genes and genes 19 KEGG pathways. Duplicates were grouped into correlated (corr.) or non-correlated (non-corr.) based on freshwater correlation results. Dosage ratios (points) greater than the 95% quantile of single gene dosages in the plot are marked in red.