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# 1 Life-stage associated remodeling of lipid metabolism 2 regulation in Atlantic salmon

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23 **Running title:** Adaptive remodeling of lipid metabolism

24

## 25 Abstract

26 Atlantic salmon migrates from rivers to sea to feed, grow and develop gonads before returning to  
27 spawn in freshwater. The transition to marine habitats is associated with dramatic changes in the  
28 environment, including water salinity, exposure to pathogens, and shift in dietary lipid availability.  
29 Many changes in physiology and metabolism occur across this life-stage transition, but little is  
30 known about the molecular nature of these changes.

31 Here we use a long term feeding experiment to study transcriptional regulation of lipid metabolism  
32 in Atlantic salmon gut and liver in both fresh- and saltwater. We find that lipid metabolism  
33 becomes significantly less plastic to differences in dietary lipid composition when salmon  
34 transitions to saltwater and experiences increased dietary lipid availability. Expression of genes in  
35 liver relating to lipogenesis and lipid transport decrease overall and become less responsive to diet,  
36 while genes for lipid uptake in gut become more highly expressed. Finally, analyses of  
37 evolutionary consequences of the salmonid specific whole-genome duplication on lipid  
38 metabolism reveals several pathways with significantly different ( $p < 0.05$ ) duplicate retention or  
39 duplicate regulatory conservation. We also find a limited number of cases where the whole genome  
40 duplication has resulted in an increased gene dosage.

41 In conclusion, we find variable and pathway-specific effects of the salmonid genome duplication  
42 on lipid metabolism genes. A clear life-stage associated shift in lipid metabolism regulation is  
43 evident, and we hypothesize this to be, at least partly, driven by non-dietary factors such as the  
44 preparatory remodeling of gene regulation and physiology prior to sea migration.

45

## 46 Introduction

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

54  
55 A key difference between freshwater and sea-habitats is the dietary availability of essential long-  
56 chain polyunsaturated fatty acids. Salmon in rivers mostly eat invertebrates that are low in  
57 physiologically critical n-3 and n-6, 20 and 22 carbon long-chain polyunsaturated fatty acids (n-  
58 3LC-PUFA and n-6LC-PUFA), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3) and  
59 docosahexaenoic (22:6n-3), while marine habitat food chains are high in available LC-PUFAs.  
60 Possibly as an adaptation to this (Leaver et al., 2008), salmon have evolved a high capacity for  
61 endogenous production of LC-PUFAs by elongation and desaturation of essential dietary 18  
62 carbon precursor linoleic and linolenic acids (18:2n-6 and 18:3n-3; Figure 4) and the ability to  
63 increase or decrease this endogenous production as a response to the dietary availability (Kennedy  
64 et al., 2006; Leaver et al., 2008; Morais et al., 2011; Ruyter et al., 2000; Tocher et al., 2001; Tocher  
65 et al., 2002; Zheng et al., 2005). During smoltification and after sea-migration, Atlantic salmon  
66 have been shown to undergo transformation of lipid metabolism function, by decreasing lipid  
67 syntheses and increasing lipid breakdown (Sheridan, 1989). However, very little is known about  
68 the molecular nature of this life-stage associated transformation physiological function.

69

70 The evolution of novel traits in salmonids, such as increased plasticity and the ability to migrate  
71 to sea, may have been facilitated by their ancestral whole genome duplication (called Ss4R) some  
72 80 million years ago (Allendorf & Thorgaard, 1984; Lorgen et al., 2015; Macqueen & Johnston,  
73 2014; Robertson et al., 2017). Gene duplication can give rise to new adaptive phenotypes in  
74 different ways: through evolution of novel functions or gene regulation, subdivision and/or  
75 specialization of function among duplicates, or via an adaptive increase in gene dosage. The  
76 Atlantic salmon genome contains ~10,000 pairs of Ss4R gene duplicates, of which ~50% have  
77 evolved some novel regulation (Lien et al., 2016; Robertson et al., 2017). Indeed, in the context of  
78 lipid metabolism, it has recently been shown that a Ss4R duplicate of *elovl5*, a key enzyme in LC-  
79 PUFA syntheses, has gained expression compared to its ancestral regulation with likely  
80 implications for the ability to synthesize LC-PUFAs (Carmona-Antoñanzas et al., 2016). This is  
81 believed to have facilitated evolution of novel traits, including flexible phenotypes necessary for  
82 an anadromous life history (Stefansson et al., 2008). However, no systematic genome wide study  
83 has yet been conducted to assess the importance of the Ss4R in evolution of salmon lipid  
84 metabolism.

85

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

92 pathways differ with respect to selection pressure on gene duplicates from the salmonid whole  
93 genome duplication.

94

## 95 Results and discussion

### 96 Annotation of lipid metabolism genes

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

111

112 To validate our ortholog annotation pipeline used to identify lipid metabolism genes, we analyzed  
113 the tissue specificity of these genes using gene expression data from 15 tissues (File S3) of Atlantic  
114 salmon (Lien et al., 2016). Genes in certain fatty acid metabolism related pathways (*fatty acid*  
115 *metabolism*, *PPAR signaling pathway*, *fat digestion and absorption*) had higher overall  
116 expression in tissues known to have high lipid metabolism activity (i.e. pyloric caeca, liver, heart,  
117 and brain) (Glatz et al., 2010; Benedito-Palos & Pérez-Sánchez, 2016; Tocher, 2003) (Figure 2).  
118 Examples include: 1) Liver was the site of highest expression for all genes in the LC-PUFA  
119 biosynthesis pathway (the desaturases  $\Delta 6$ FAD and  $\Delta 5$ FAD, and the elongases elovl5, elovl2 and  
120 elovl4). 2) Bile acids are essential for fat digestion in the gut, but are synthesized in liver. As  
121 expected, the rate limiting step for bile syntheses, cytochrome P450 7A1 (CYP7A1), has the  
122 highest expression in the liver. 3) Cholesterol, an essential component of cell membranes and  
123 precursor to bile acids, is known to be synthesized in all tissues, but primarily in liver, intestine,  
124 and brain (Brown & Sharpe, 2016). This is reflected in our annotation by high expression of the  
125 key cholesterol biosynthesis genes 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR),  
126 isopentenyl-diphosphate  $\Delta$ isomerase (IDI1), squalene epoxidase (SM), and lanosterol synthase  
127 (LS) in these tissues. 4) Several known regulators of lipid metabolism show high expression in  
128 liver, heart, brain and pyloric caeca, as expected, including liver X receptor (LXR), peroxisome  
129 proliferator-activated receptor alpha (PPAR $\alpha$ ), sterol regulatory element binding protein 1  
130 (SREBP1), and sterol regulatory element binding protein 2 (SREBP2). Taken together, the tissue  
131 distribution of lipid metabolism gene expression is in line with knowledge about vertebrate  
132 physiology in general, and support the validity of our annotation of lipid metabolism genes in  
133 salmon. To make all data underlying our annotation easily available, and to facilitate further

134 refinement through manual community curation, we have created an interactive web-server  
135 available online ([goo.gl/8Ap89a](https://goo.gl/8Ap89a)).

136

## 137 Life-stage associated remodeling of lipid metabolism

138 We conducted a feeding trial to study how salmon adjusts its lipid metabolism to different levels  
139 of LC-PUFA in freshwater and saltwater (see figure 8 for experimental details). Groups of salmon  
140 were fed contrasting diets from hatching until after transition to seawater. One feed was vegetable  
141 oil based (VO) and hence low in LC-PUFA, similar to river ecosystem diets, whereas the other  
142 was based on fish oil (FO) and high in LC-PUFA as expected in a marine-type diet (see Table S2  
143 and S3 for details on feed composition). VO based diets are also low in cholesterol (Ciftci, et al.,  
144 2012; Verleyen et al., 2002). The proportion of fat in feed also increased between FW and SW  
145 (Table S2), as is standard practice in the aquaculture industry to maintain optimal growth  
146 conditions (Storebakken, 2002). Moreover, total lipid availability is also expected to increase  
147 between natural riverine and marine ecosystem diets. The contrasting levels of EPA/DHA between  
148 FO and VO diets remained constant across life-stages. In total, 32 and 23 fish were sampled for  
149 RNA-Seq of liver and gut, respectively, including up to eight biological replicates from each diet  
150 and life-stage (freshwater and saltwater, see figure 8c for details). Fish in the different dietary  
151 groups were given FO and VO feed from first feeding (<0.2 g body weight) until sampling.

152

153 In general, global gene expression levels were more affected by dietary composition in liver than  
154 in gut (which was largely unresponsive), and the effect was more pronounced in freshwater than  
155 in saltwater (Figure 3a). VO diets, compared to FO diets, increased lipid-metabolism related gene



156 expression in liver. In freshwater, 66 genes were differentially expressed with 57 (86%) of these  
157 upregulated, while in saltwater 31 genes were differentially expressed with 23 (74%) of these  
158 upregulated (Figure 3b). The increased activity of liver lipid metabolism under VO diets confirm  
159 the well-known ability of salmon to regulate endogenous synthesis of LC-PUFA and cholesterol  
160 in response to VO diets (Kortner et al., 2014; Leaver et al., 2008; Zheng et al., 2005).

161  
162 Fish sampled in freshwater and saltwater shared a relatively small number of differentially  
163 expressed genes (DEGs) for each pathway (Table S4). We found that most pathways had more  
164 DEGs in freshwater (*'fatty acid biosynthesis'*, *'steroid biosynthesis'*, and its precursor *'terpenoid*  
165 *backbone biosynthesis'*), whereas few had more DEGs in saltwater (*'fat digestion and absorption'*  
166 and *'steroid hormone biosynthesis'*) (Figure 3c). Out of 87 lipid metabolism DEGs in the dietary  
167 contrast, 56 (64%) were freshwater specific, 21 (24%) saltwater specific, and 10 (11%) shared  
168 dietary response. For example, only two genes in the FA and LC-PUFA biosynthesis pathways  
169 ( $\Delta 6$ FADa and  $\Delta 5$ FAD) shared response to diet in fresh- and saltwater (Figure 4). Similarly, in the  
170 pathways responsible for cholesterol biosynthesis there were more DEGs between diets in FW (21  
171 DEGs in FW, 4 shared, and no SW specific) (Figure 5). The few genes that showed diet-effects  
172 specific to saltwater included bile salt activated lipase, responsible for the hydrolysis of free fatty  
173 acids from TAG obtained from the diet (Tocher, 2003). Two of these genes, carboxyl ester lipase,  
174 tandem duplicate 2a (CEL2a) and b (CEL2b), are highly upregulated in saltwater in response to  
175 VO diet. Taken together, our results show higher metabolic plasticity in parr-stage salmon,  
176 suggesting a life-stage associated remodeling of lipid metabolism in liver. This corroborates the  
177 idea of a post-smoltification phenotype adapted to an environment with a surplus of n-3LC-PUFA.  
178

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

185

186 Further examination of key lipid metabolism genes revealed that after life-stage transition the  
187 system-wide lipid metabolism remodeling represented a concerted shift in the metabolic role of  
188 liver and gut. After the salmon entered the marine stage, lipogenic gene expression in the liver was  
189 significantly decreased, as evident by the markedly lower expression (2.2-3.3 fold) of the master  
190 regulator of lipid metabolism SREBP1, a 5-fold decrease in expression of fatty acid synthase, and  
191 a 2-3 fold decrease in rate-limiting enzymes in LC-PUFA synthesis (i.e.  $\Delta 5$ FAD,  $\Delta 6$ FADa) (Figure  
192 4). Liver and gut gene expression also indicated increased catabolic activity in saltwater, with  
193 upregulation of the carnitine palmitoyltransferase 1 and 2 genes, responsible for uptake of fatty  
194 acids into mitochondria for  $\beta$ -oxidation (Lehner & Quiroga, 2016). Finally, expression of lipid  
195 transport genes shifted from liver to gut with the transition to seawater (apolipoproteins, pathway  
196 "Fat digestion and absorption" in Figure 6). Four apolipoproteins (out of 11 annotated) were  
197 differentially regulated in liver between different life-stages, with a 2.4-5 fold decrease in saltwater  
198 compared to freshwater. In stark contrast, nine of the diet-regulated apolipoproteins in gut  
199 increased their expression in saltwater between 1.8-9.7 fold. The results point to an adaptive shift  
200 in lipid metabolism, with increased ability to take up lipids in the gut after Atlantic salmon migrates  
201 to sea where lipid availability is higher. Remodeling of lipid metabolism across life-stages is likely

202 the result of a combination of factors, including the direct regulatory effect of dietary fat itself,  
203 effect of salinity, and smoltification-induced physiological changes influencing gene regulation.  
204 Although the relative importance of these factors is undetermined in our study, the fact that DEGs  
205 in the VO versus FO feed contrast were mostly life-stage specific (Figure 3), supports that factors  
206 other than the diet itself contribute significantly to the fresh and seawater metabolic phenotypes.

207  
208 Interestingly, diet had a strong influence on the number and direction of gene expression changes between  
209 freshwater and saltwater (Figure 6). In gut, about twice as many DEGs (with respect to the fresh- to  
210 saltwater transition) were observed in salmon when fed FO diet than VO diet (Figure 6a). In liver, the diet  
211 effect was less pronounced, with the FO group containing 46% more DEGs than the VO group (Figure 6a).  
212 This diet effect pattern was reflected in the lipid metabolism genes with 89% and 16% more DEGs in the  
213 FO group for gut and liver, respectively (Figure 6b). As this diet and life-stage interaction is a genome wide  
214 trend, and more pronounced in gut tissue than in liver, this pattern could be related to differences in  
215 osmoregulation and adaptation to saltwater. Two studies have suggested that Atlantic salmon raised on VO  
216 based feeds more closely resembling riverine diets adapt to saltwater sooner and better than salmon raised  
217 on FO based diets (Bell et al., 1997; Tocher et al., 2000). Conversely, there has been evidence that VO  
218 based diets can reduce markers for stress response upon saltwater challenge, resulting in reduced  
219 osmoregulatory capacity (Oxley et al., 2010). Regardless of the effect, it is clear that diet can modulate the  
220 smoltification process and could explain the discrepancy between diets in number of life-stage related  
221 DEGs. Another possibility is that the different levels of fatty acids in the diets, for example DHA, affect  
222 DNA-methylation and thus trigger genome wide divergence in gene regulation (Kulkarni et al., 2011).

223  
224 Our results clearly demonstrate very different baseline lipid metabolic functions in pre- and post-  
225 smolt salmon, as well as life-stage associated changes in the plasticity of lipid metabolism, e.g. the  
226 ability to regulate endogenous LC-PUFA synthesis as a response to changes in diet (i.e. fatty acid

227 composition). As opportunistic carnivores, salmon tend to eat whatever the local environment  
228 provides. Thus, in freshwater, insects and amphipods provide variable, mostly low amounts of  
229 essential LC-PUFA and total fat (Jonsson & Jonsson, 2011; Sushchik et al., 2003), favoring a  
230 metabolic function that can efficiently regulate endogenous lipid synthesis based on dietary  
231 availability (Carmona-Antonanzas et al., 2014). Conversely, in marine environments, amphipods  
232 and smaller fish provide a higher, more stable source of n-3LC-PUFA and total fat (Jonsson &  
233 Jonsson, 2011; Baeza-Rojano, et. al., 2014), promoting a metabolic function that allocates less  
234 energy to endogenous synthesis of essential lipids.

235

## 236 Selection on gene duplicates after whole genome duplication

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

244

245 To assess the level of Ss4R duplicate retention, we first defined 10,752 Ss4R duplicate pairs  
246 (21,504 genes) in the NCBI RefSeq annotation using the same approach as Lien et al. (2016). Of  
247 the 1,421 annotated lipid metabolism genes, 867 (61%) were retained as duplicated genes after  
248 Ss4R (Figure 7a) (in contrast to 47% of the 45,127 salmon genes assigned to ortholog groups).

249 Moreover, our results showed large variation in the proportion of retained duplicates in each lipid  
250 metabolism pathway (Figure 7), with the most extreme case being '*fat digestion and absorption*'  
251 with 80% retained duplicates and '*steroid hormone biosynthesis*' with only 27% retained Ss4R  
252 duplicates.

253

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

272 duplicate co-regulation were also those that were most responsive to dietary differences in fatty  
273 acid composition (Figure 3).

274

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

295 likely that an increased dosage of *elov15* and the 68 other duplicate pairs has affected the function  
296 of lipid metabolism in salmon.

## 297 Conclusion

298 Atlantic salmon needs great plasticity of physiology and behavior to adapt for migration between  
299 freshwater and sea. By analyzing transcriptomic changes through the transition from fresh- to  
300 saltwater and the associated increase in dietary lipids, we identified an overall remodeling of lipid  
301 metabolism, with liver reflecting higher lipid metabolic plasticity and higher capacity of  
302 endogenous synthesis of LC-PUFAs in freshwater, while gut lipid uptake genes become more  
303 active in saltwater. These results indicate adaptive optimization of the Atlantic salmon lipid  
304 metabolism to account for life-stage specific dietary availability. Moreover, we found signatures  
305 of pathway-specific selection pressure on gene duplicates, including a gene dosage increase in  
306 three genes involved in fatty acid metabolism. This illustrates possible adaptive consequences of  
307 the salmonid whole-genome duplication for the evolution of lipid metabolism. Future studies  
308 should attempt to decipher how the life-stage related metabolic reprogramming is controlled (for  
309 example through epigenetic mechanisms). Understanding this will have important implications for  
310 understanding evolution of genome regulatory processes in anadromous salmonids and potentially  
311 have economically important implications for Atlantic salmon aquaculture.

## 312 Materials and methods

### 313 Orthogroup prediction

314 Protein sequences were obtained from seven teleost fish species; *Danio rerio* (zebrafish),  
315 *Gasterosteus aculeatus* (three-spined stickleback), *Oryzias latipes* (medaka), *Oncorhynchus*  
316 *mykiss* (Rainbow trout), *Oncorhynchus kisutch* (coho salmon), *Salmo salar* (Atlantic salmon),  
317 *Thymallus thymallus* (grayling), *Esox lucius* (northern pike), and two mammalian outgroup  
318 species; *Homo sapiens* (human), *Mus musculus* (house mouse). Human, mouse, zebrafish, medaka  
319 and stickleback protein fasta data were obtained from ENSEMBL (release 83). Atlantic salmon  
320 (RefSeq assembly GCF\_000233375.1, Annotation Release 100) and northern pike (RefSeq  
321 assembly GCF\_000721915.2, Annotation Release 101) proteins were obtained from NCBI  
322 RefSeq. Rainbow trout proteins were obtained from an assembly and annotation of the genome  
323 (Berthelot et al., 2014). Grayling proteins were obtained from an assembly and annotation of the  
324 genome (Varadharajan et al., 2017). The coho salmon transcriptome (Kim, Leong, Koop, &  
325 Devlin, 2016) was obtained from NCBI (GDQG00000000.1). Where transcriptome data was used,  
326 protein sequences were translated using TransDecoder (v2.0.1, <http://transdecoder.github.io/>).  
327 Protein fasta files were filtered to retrieve only the longest protein isoform per gene. Orthofinder  
328 (v0.2.8) (Emms et al., 2015) assigned groups of orthologs based on protein sequence similarity.  
329 Proteins within an orthogroups were further aligned using MAFFT (v7.130) (Katoh et al., 2002)  
330 and maximum likelihood trees were estimated using FastTree (v2.1.8) (Price et al., 2010).



## 331 Annotation of salmon lipid metabolism genes

332 A list of zebrafish proteins obtained from 19 manually selected zebrafish KEGG pathways related  
333 to lipid metabolism (Table S1) were used to search for Atlantic salmon orthologs. Orthogroups  
334 that contained a selected zebrafish protein were identified. Salmon proteins within those  
335 orthogroups were assigned as orthologs of the closest zebrafish protein based on the orthogroup  
336 tree distance. A lipid metabolism gene list was created including salmon orthologs to the selected  
337 zebrafish genes. Additional salmon genes related to lipid metabolism not included in KEGG  
338 pathways (e.g. regulators or transporters, SREBP, LXR, FABP, etc.) were manually searched for  
339 through NCBI and added to the list.

## 340 Tissue expression

341 Atlantic salmon RNA-Seq samples from 15 different tissues (liver, gut, pyloric caeca, heart,  
342 kidney, muscle, gill, eye, skin, ovary, nose, testis, brain, head kidney, spleen) were obtained from  
343 NCBI SRA (PRJNA72713) (Lien et al., 2016). Fastq files were adapter trimmed before alignment  
344 to the Atlantic salmon genome (RefSeq assembly GCF\_000233375.1) (Lien et al., 2016) using  
345 STAR (v2.5.2a) (Dobin et al., 2013). HTSeq-count (v0.6.1p1) (Anders et al., 2015) counted the  
346 sum of uniquely aligned reads in exon regions of each gene in the annotation (RefSeq Annotation  
347 Release 100). Gene FPKM values were calculated based on the gene count over the samples  
348 effective library size (see TMM method from edgeR (Robinson et al., 2010) user manual) and the  
349 mean gene transcript isoform length.

## 350 Feed trial

351 Atlantic salmon fry were obtained from AquaGen Breeding Centre, Kyrksæterøra, Norway and  
352 reared in the Norwegian Institute for Water Research (NIVA), Solbergstranda, Norway in four  
353 partitioned 1000 liter tanks on vegetable oil (VO) or fish oil (FO) based diets continuously from  
354 first feeding (fry weight <0.2 g). Daily feed amount was calculated based on total biomass in each  
355 tank and decreased as the fish grew, from 3% at first feeding to 1.2% by the end of the trial. Fish  
356 were euthanized periodically throughout the experiment to maintain appropriate levels of dissolved  
357 oxygen. VO based feeds contained a combination of linseed oil and palm oil at a ratio of 1.8:1 and  
358 FO based feeds contained only North Atlantic fish oil. Percent protein in feed decreased with fish  
359 size from 56% at first feeding to 41% at the end of the trial. This corresponded with an increase in  
360 percent lipid from 16% at first feeding to 31% at the end of the trial. At the time of sampling, the  
361 proportion of lipid in the feed was 22% in freshwater and 31% in saltwater (Table S2). Increasing  
362 lipid proportion in feed with fish size is standard practice in the aquaculture industry as this  
363 maintains optimal growing conditions by decreasing the digestible protein to digestible energy  
364 ratio (Storebakken, 2002). All feeds were formulated and produced by EWOS innovation  
365 (Supplementary File 3). Local groundwater was UV sterilized for use in the freshwater life-stage  
366 and water from the Oslofjord taken from 60 meters below sea surface (~3-3.5% salinity) was UV  
367 sterilized for use in the saltwater life-stage. Fish were raised under constant light and water  
368 temperature (~12°C) for 26 weeks. Then, 40 pre-smolt salmon (~50g) from each control tank  
369 (~240 fish per control tank) were switched to the contrasting diet (VO to FO and vice versa) by  
370 physically moving them to the empty partition of the tank receiving the appropriate feed (Figure  
371 8a). Five fish from each of the control tanks (2 VO tanks and 2 FO tanks) were sampled before  
372 switching feeds (D0), then fish from both control and feed switch conditions were similarly

373 sampled 1, 2, 5, 9, 16, and 20 days after switching feeds (5 fish x 2 replicate tanks x 4 conditions  
374 = 40 fish per time point, figure 8b). Two weeks after freshwater sampling (31 weeks after first  
375 feeding), smoltification was triggered by 5 weeks of winter-like conditions with decreased light  
376 (12 hours per day) and water temperature ( $\sim 8^{\circ}\text{C}$ ), immediately followed by 5 weeks of spring-like  
377 conditions, returning to normal light (24 hours per day) and water temperature ( $\sim 12^{\circ}\text{C}$ ). All salmon  
378 from the control groups (VO or FO) were then switched to saltwater and allowed to acclimate for  
379 3 weeks. The feed switch was repeated in saltwater by transferring half ( $\sim 40$  fish) of the post-smolt  
380 salmon ( $\sim 200$  g) from each control tank to the contrasting feed condition. Again, pre-switch  
381 control samples were taken (D0) followed by sampling 1, 2, 6, 9, 16, and 20 days post-diet switch  
382 (Figure 8b). For both freshwater and saltwater samplings, feeding was stopped in the mornings of  
383 each of the sampling days. All fish were euthanized by a blow to the head and samples of liver and  
384 midgut (gut section between pyloric caeca and hindgut) were flash frozen in liquid nitrogen and  
385 stored under  $-80^{\circ}\text{C}$ . A subset of the samples taken were used for further RNA-Seq analysis (see  
386 figure 8c for details).

## 387 RNA-sequencing

388 Total RNA was extracted from selected feed trial samples (see figure 8c for details) using the  
389 RNeasy Plus Universal kit (QIAGEN). Quality was determined on a 2100 Bioanalyzer using the  
390 RNA 6000 nano kit (Agilent). Concentration was determined using a Nanodrop 8000  
391 spectrophotometer (Thermo Scientific). cDNA libraries were prepared using the TruSeq Stranded  
392 mRNA HT Sample Prep Kit (Illumina). Library mean length was determined by running on a 2100  
393 Bioanalyzer using the DNA 1000 kit (Agilent) and library concentration was determined with the

394 Qbit BR kit (Thermo Scientific). Single end sequencing of sample libraries was completed on an  
395 Illumina HiSeq 2500 with 100 bp reads.

## 396 Differential expression analysis between feed conditions and life-stages

397 To analyze gene expression differences between feed conditions and life-stages, samples from the  
398 feed trial were selected for RNA-Seq. Liver and gut tissue RNA were sequenced from fish fed  
399 each of the feeds (FO, VO) at day 0 of the diet switch, both before (freshwater) and after (saltwater)  
400 smoltification (See figure 8c for the number of RNA-Seq replicates and sampling details). Fastq  
401 files were processed to produce gene count and FPKM data using the same protocol described  
402 under the tissue expression method section. For the feed comparison, changes in gene expression  
403 were tested between FO and VO feed conditions for both freshwater and saltwater samples, and  
404 liver and gut tissues. For the life-stage comparison, changes in gene expression were tested  
405 between freshwater and saltwater stages for both FO and VO feed conditions, and liver and gut  
406 tissues. Using RNA-Seq gene count data, lowly expressed genes were filtered prior to testing,  
407 retaining genes with a minimum of one read count per million (CPM) in two or more samples.  
408 Differential expression analysis was carried out using a standard edgeR (Robinson et al., 2010)  
409 protocol. Effective library sizes were calculated using the edgeR TMM-normalisation procedure  
410 allowing effective comparison of expression data between different sample types (see edgeR user  
411 manual). An exact test between expression levels of a pair of conditions gave the log<sub>2</sub> fold change,  
412 P-value and false discovery rate (FDR) for each gene. Genes with FDR < 0.05 were considered  
413 differentially expressed genes (DEGs).

## 414 Identification of Ss4R duplicates

415 To identify putative gene duplicates stemming from the Ss4R, we used the same approach as in  
416 Lien et al. (2016). All-vs-all protein blast was run with e-value cutoff of  $1e-10$  and pident  
417 (percentage of identical matches)  $\geq 80$  and blast hit coverage of  $\geq 50\%$  of protein length. Only the  
418 best protein hits between the 98 defined synteny blocks (see Lien et al., 2016) were considered as  
419 putative Ss4R duplicates. Blast result ranking was done using the product of pident times bitscore  
420 to avoid spurious ‘best blast matches’ with low pident ( $< 85$ ) but high bitscore.

## 421 Duplicate analysis

422 Genes from the lipid metabolism gene list were paired together with their putative Ss4R duplicates  
423 identified above. The retention of gene duplicates (i.e. whether both genes in a pair were retained,  
424 or just one) was compared between all identified duplicates in the salmon genome annotation and  
425 the lipid metabolism gene list. Pathway-level retention was explored by comparing the number of  
426 genes in each of the 19 selected KEGG pathways (Table S1) in a duplicate pairing to that of the  
427 total list of lipid genes, to find pathways with significantly less or more duplicate retention  
428 (Fisher’s exact test, P-value  $< 0.05$ ). Regulatory conservation of lipid gene duplicates was explored  
429 by correlation of gene expression changes between duplicates over the course of the feed trial  
430 described above. RNA-Seq data was generated from liver samples of salmon from 38 sampling  
431 time points (19 in freshwater and 19 in saltwater). Fastq files were processed to produce gene count  
432 and FPKM data using the same protocol described under the tissue expression method section. For  
433 each duplicate pair, mean FPKM values were retrieved for each time point and used to calculate a  
434 freshwater and saltwater correlation value. Duplicates with Pearson correlation  $\geq 0.6$  were

435 considered correlated (P-value < 0.003 from 19 sample points). The number of duplicates with  
436 correlated expression profiles was counted for each pathway and compared to all lipid genes to  
437 find pathways with significantly less or more correlated duplicates (Fisher's exact test, P-value <  
438 0.05). The effect of gene duplication on gene dosage was estimated by calculating a dosage ratio  
439 between the FPKM value of a salmon ortholog (sum of gene expression in duplicate pairs) over  
440 the FPKM value of the non-duplicated ortholog from northern pike. For salmon, the RNA-Seq  
441 data from the freshwater and saltwater FO feed trial was used (samples used in differential  
442 expression analysis section). For pike, RNA-Seq from livers of four individuals were aligned (see  
443 tissue expression section for protocol) to their respective genomes (see genomes in ortholog  
444 prediction section). RSEM (v1.2.31) (Li & Dewey, 2011) was used to generate FPKM values for  
445 genes so that non-uniquely mapped reads between salmon duplicate genes were not ignored but  
446 instead assigned proportionately to each gene to match the proportions of uniquely mapped reads  
447 between the genes. Gene dosage levels for duplicate pairs with correlated expression (see above),  
448 non-correlated expression and single genes were compared for all lipid metabolism genes and for  
449 each pathway.

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609

## 610 Data Accessibility:

- 611 - Supplementary files have been deposited to [datadryad.org](http://datadryad.org) under the accession:  
612 [doi:10.5061/dryad.j4h65](https://doi.org/10.5061/dryad.j4h65)
- 613 - All gene expression results can be accessed through the interactive shiny web server:  
614 <https://goo.gl/8Ap89a>
- 615 - Lipid metabolism gene annotation can be accessed from <https://goo.gl/VVUVWr>
- 616 - Raw RNA-Seq data has been deposited into European Nucleotide Archive (ENA) under  
617 the project accession number PRJEB24480

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622

## 623 Legends Figures

624

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

629

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

631 Tissue expression profiles of our annotated lipid metabolism genes were consistent with expectations. Gene expression  
632 levels are shown as the log<sub>2</sub> fold change difference between the FPKM value of each tissue and the median FPKM  
633 across all tissues. Expression profiles for selected genes in each pathway are shown (see Figure S2 and S3 for all  
634 pathways and gene details).

635

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

641

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

647

648 **Figure 5: Diet and life-stage effects on cholesterol biosynthesis in salmon liver.** Terpenoid backbone synthesis and steroid  
649 biosynthesis pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names followed by NCBI gene

650 numbers). Gene expression levels are shown as mean (point) and standard deviation (line) of expression in eight samples (measured  
651 in  $\log(\text{FPKM} + 1)$ ) from each diet (FO, VO feeds) and life-stage (freshwater, saltwater) combination. Genes significantly  
652 ( $\text{FDR} < 0.05$ ) differentially expressed (DEG) between diets in a life-stage are highlighted.

653

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

659

660 **Figure 7: Gene duplication in lipid metabolism pathways.** For the total list of lipid metabolism genes in Atlantic salmon, and  
661 sets of genes belonging to different KEGG pathways: (a) Number and percentage of genes with a duplicate homolog from the Ss4R  
662 duplication. (b) Number and percentage of duplicate genes with correlated liver expression response to feed in freshwater and (c)  
663 saltwater (Correlation  $\geq 0.6$ , P-value  $< 3.306\text{e-}3$ , using 19 time points from feed trial for each water condition). Fisher's exact test  
664 was used to detect pathways with significant enrichment compared to all gene (P-value  $< 0.05$ ) (d)  $\text{Log}_2$  gene dosage ratios  
665 (salmon:pike) in liver from fish in freshwater, where the ratio is computed between expression in the salmon duplicates (FPKM,  
666 sum of the two duplicates) and the expression of the corresponding pike ortholog. Ratios were computed for all lipid metabolism  
667 genes and genes in the pathway '*biosynthesis of unsaturated fatty acids*'. For comparison, ratios were also computed for genes  
668 without retained duplicates, i.e. with a 1:1 orthology between salmon and pike. Duplicates were grouped into correlated (corr.) or  
669 non-correlated (non-corr.) based on saltwater correlation result in (c). Dosage ratios (points) greater than the 95% quantile of single  
670 gene dosages are marked in red.

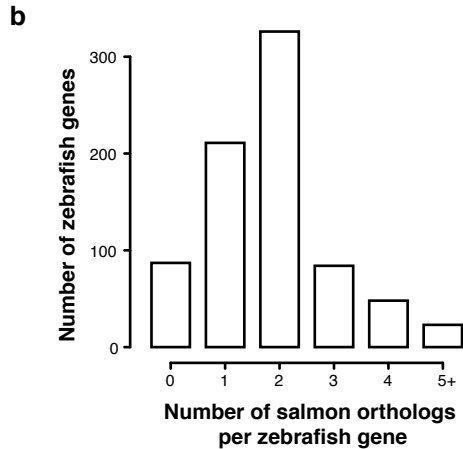
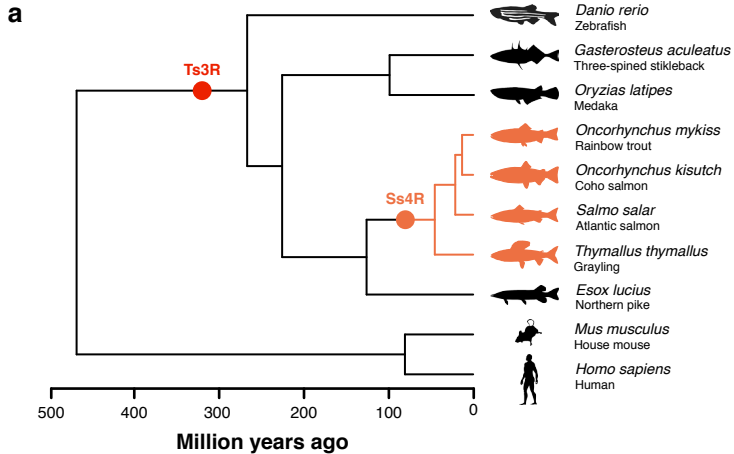
671

672 **Figure 8: Overview of feed trial experiment.**

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

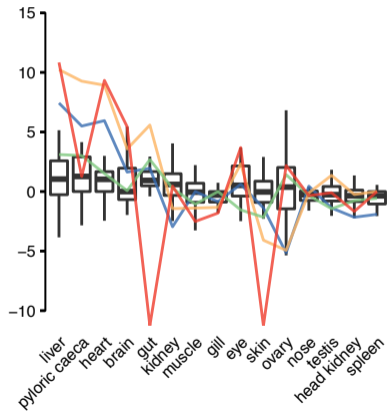
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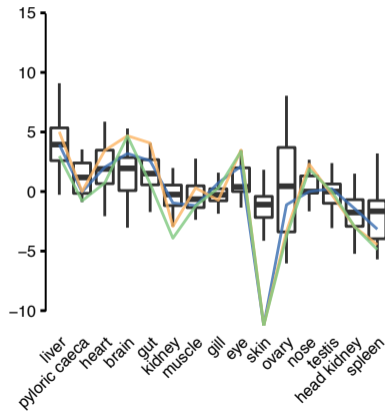
## Biosynthesis of unsaturated fatty acids

—  $\Delta 5$ FAD —  $\Delta 6$ FAD — ELOVL5a — ELOVL5b



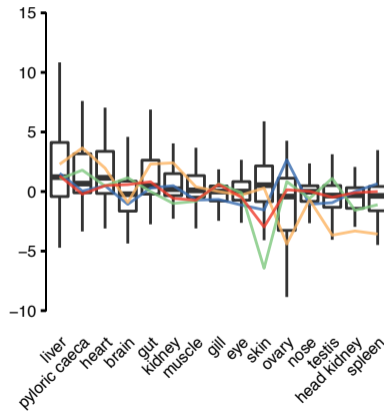
## Steroid biosynthesis

— LS — SMa — SMb



## PPAR signaling pathway

— LXR $\alpha$  — PPAR $\alpha$  — SREBP1 $\alpha$  — SREBP2 $\alpha$



Tissue

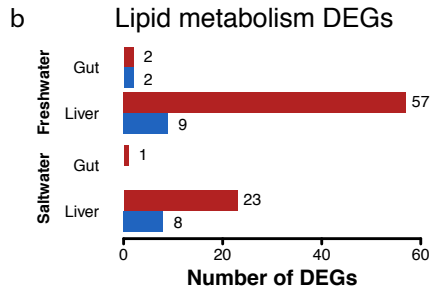
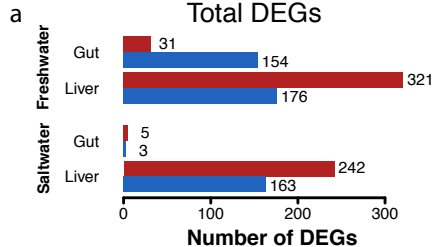
Response to VO feed

- Up-regulation
- Down-regulation

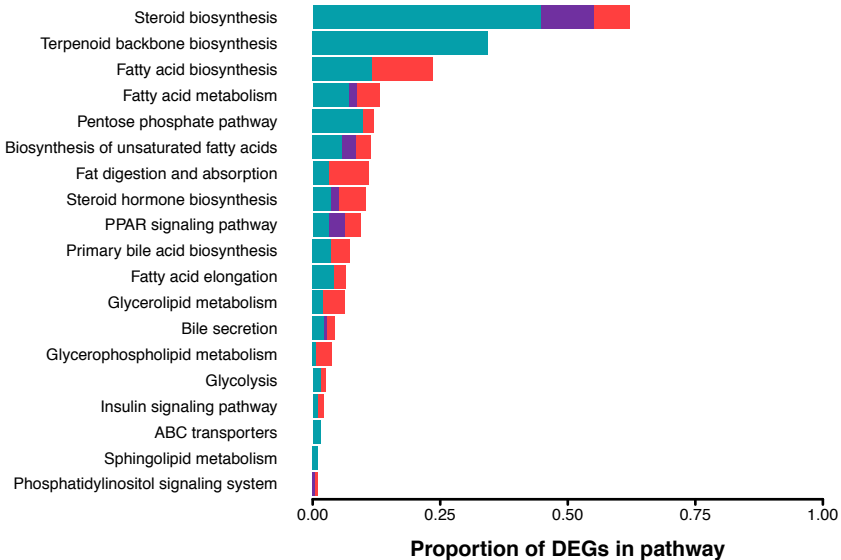
C

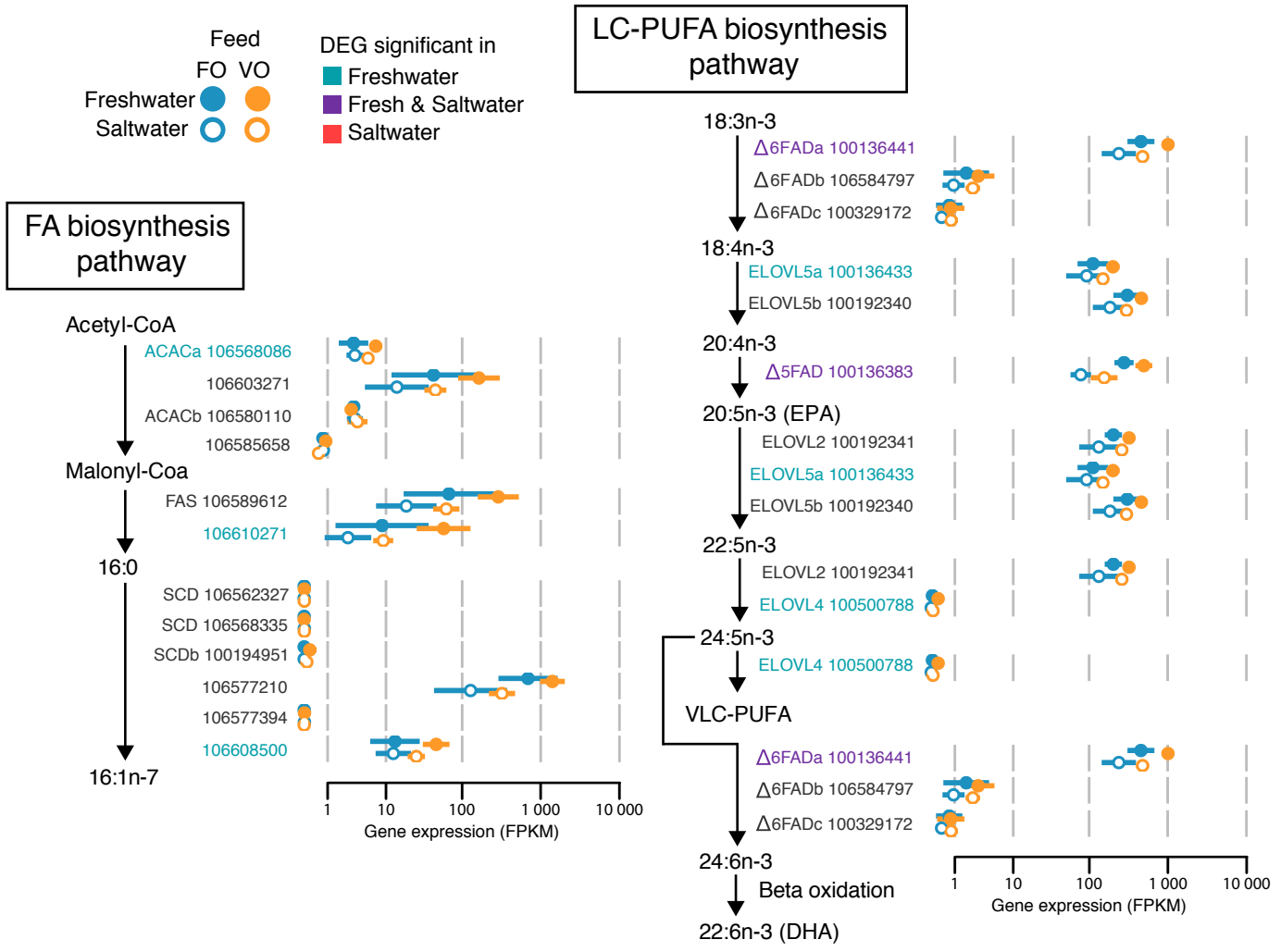
DEG significant in

- Freshwater
- Fresh & Saltwater
- Saltwater



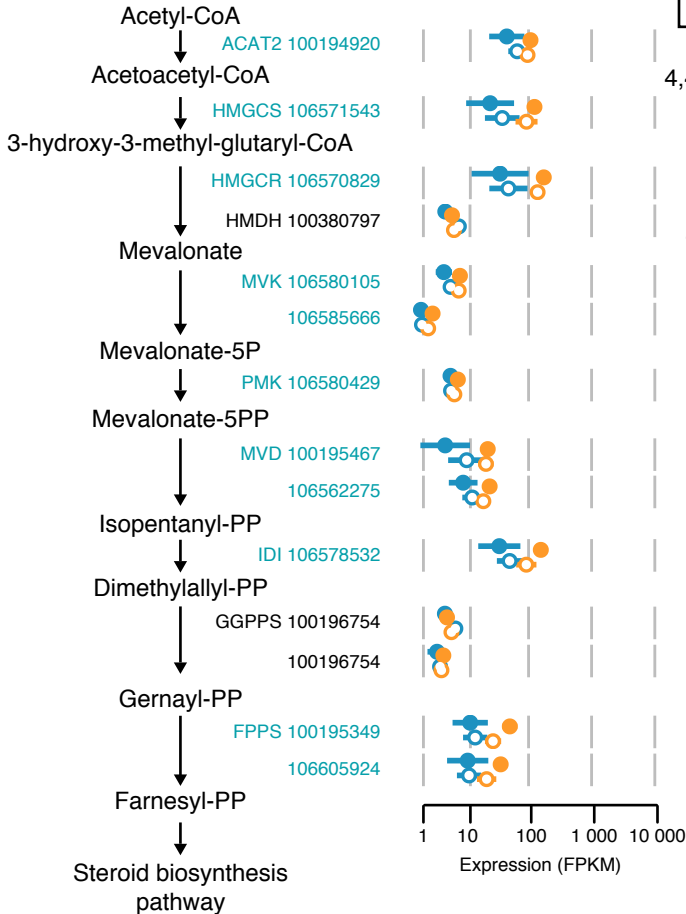
**KEGG pathway**



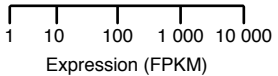
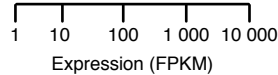
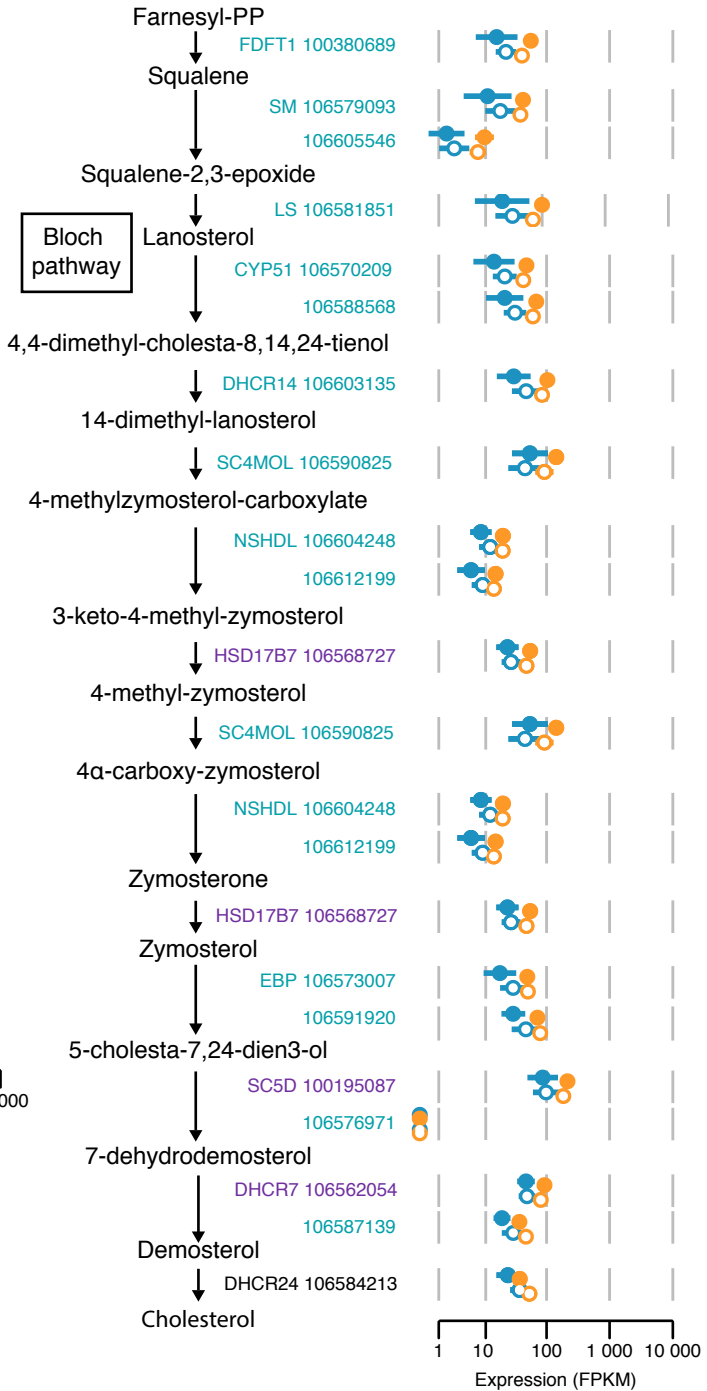




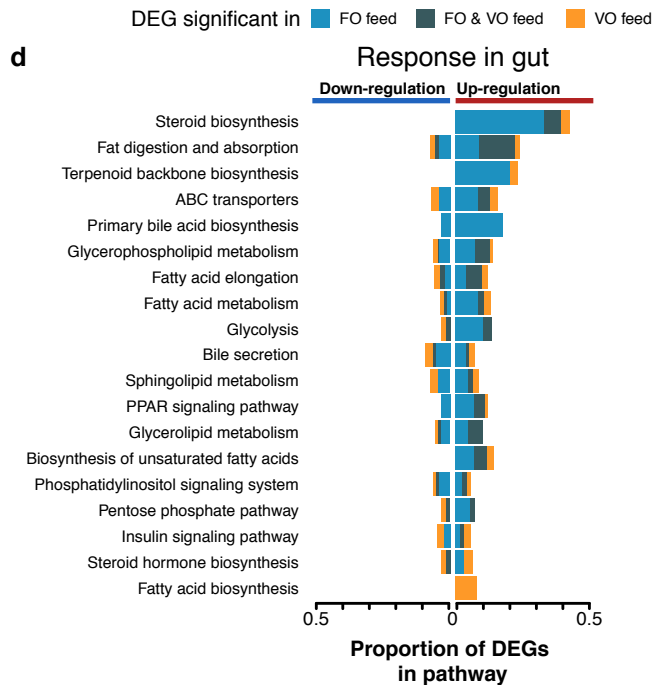
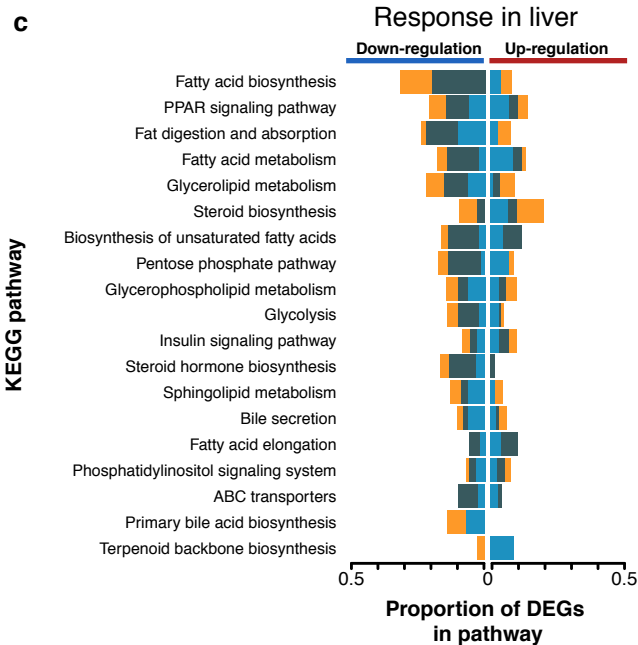
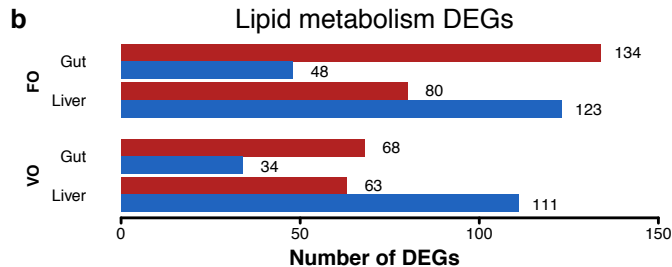
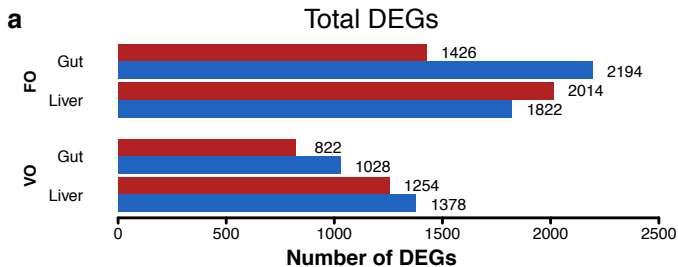
### Terpenoid backbone synthesis pathway



### Steroid biosynthesis pathway



Response to saltwater life stage ■ Up-regulation ■ Down-regulation

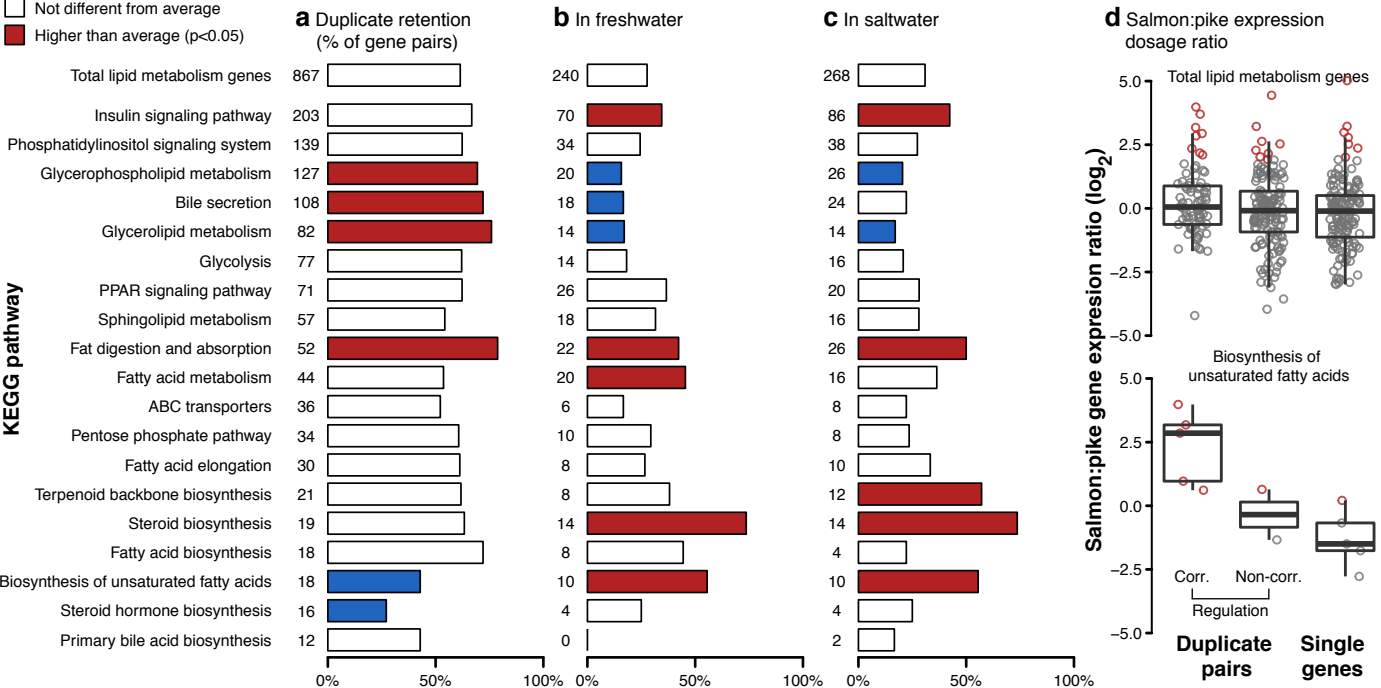


Comparison with average across pathways

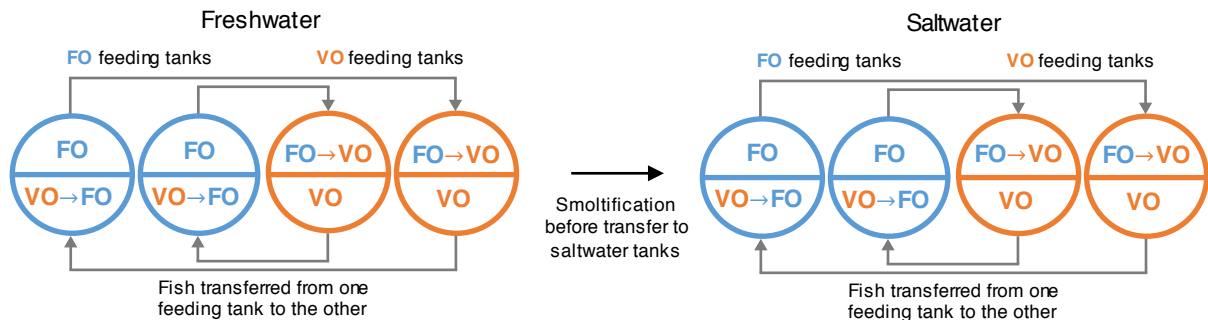
- Lower than average ( $p < 0.05$ )
- Not different from average
- Higher than average ( $p < 0.05$ )

% of duplicate pairs with similar regulation (see text)

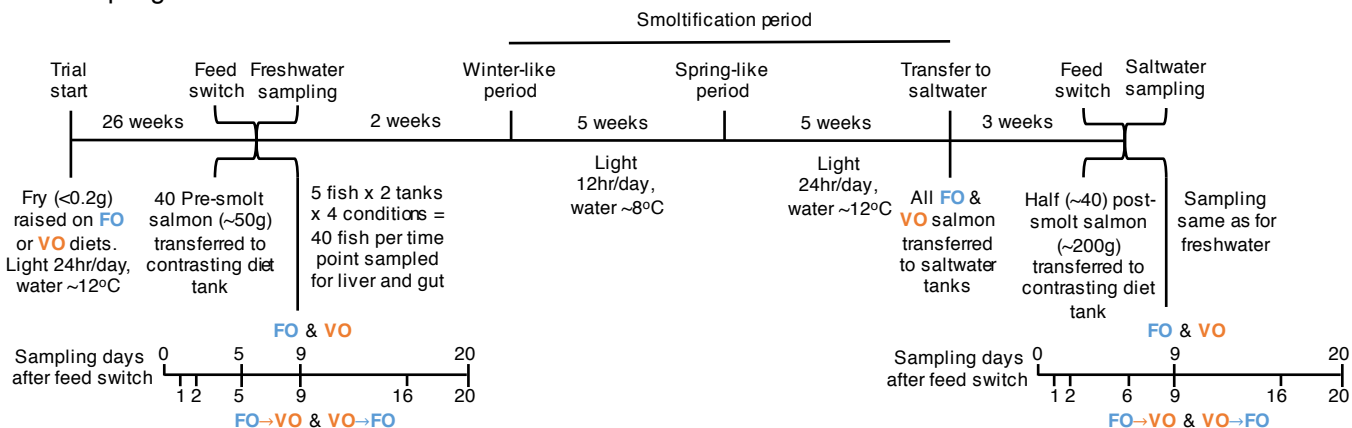
○ >95% quantile of single gene dosage



## a Feeding tank setup



## b Sampling timeline



## c Samples sequenced for total RNA

Feed	Tissue	Freshwater sampling days							Saltwater sampling days						
		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