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Molecular mechanism of dietary phospholipid requirement of Atlantic salmon, *Salmo salar*, fry

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ABBREVIATIONS

Aa, amino acid; bp, base pair; BLA-Pss, bacterial-like animal Pss; CDP, cytidine diphosphate; CA-PPS, CDP-alcohol dependent phospholipid phosphodiester synthase; Cds, CDP-DAG synthetase; Cept, CDP-ethanolamine:diacylglycerol phosphotransferases; Chka, choline kinase; Chpt1, CDP-choline:diacylglycerol phosphotransferases; Cdipt, phosphatidylinositol synthase; CL, cardiolipin (Ptd_2Gro); Crls1, cardiolipin synthase; DAG, diacylglycerol; DHA, docosahexaenoic acid; Eki1, ethanolamine kinase; ER, endoplasmic reticulum; EST, expressed sequence tag; G3P, glycerol-3-phosphate; Gpat, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; Pap, phosphatidic acid phosphatase; Pcy1, phosphocholine cytidylyltransferase; Pcy2, phosphoethanolamine cytidylyltransferase; Peam3, phosphoethanolamine methyltransferase; Pemt, phosphatidylethanolamine methyltransferase; Pgs1, phosphatidylglycerol phosphate synthase; Pisd, phosphatidylserine decarboxylase; PL, phospholipid; Plc, 1-acyl-sn-glycerol-3-phosphate acyltransferase; Pss, phosphatidylserine synthase; Psse, phosphatidylserine synthase via base-exchange; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; Ptd_2Gro, cardiolipin (CL); PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; Ptpmt1, phosphatidylglycerol phosphate phosphatase; SDC, serine decarboxylase; Sgms1, sphingomyelin synthase; TF, transcription factor; TGA, triacylglycerol; TSA, transcriptome shotgun assembly.

ABSTRACT

The phospholipid (PL) requirement in fish is revealed by enhanced performance and stress resistance and reduced occurrence of deformities observed when larvae are provided PL-enriched diets. To elucidate the molecular mechanism underlying PL requirement in Atlantic salmon, *Salmo salar*, were fed a minimal PL diet and tissue samples from major lipid metabolic sites were dissected from fry (2.5 g, 1990 ° day post fertilisation, dpf) and parr (10 g, 2850 °dpf) for gene expression analysis. *In silico* analysis and cloning techniques demonstrated that salmon possess a full set of enzymes for the endogenous production of PL, including a bacterial-like phosphatidylserine (PtdSer) synthase. The gene expression data indicated that major PL biosynthetic genes of phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn) and phosphatidylinositol (PtdIns) including cholinephosphotransferase and phosphatidylcholine methyltransferase, and display lower expression in intestine during the early developmental stage (fry). This is consistent with the hypothesis that the intestine of salmon is immature at the early developmental stage with limited capacity for endogenous PL biosynthesis. The results also indicate that intact PtdCho, PtdEtn and PtdIns are required in the diet. PtdCho and sphimgomyelin constitute the predominant PL in chylomicrons, involved in the transport of dietary lipids from the intestine to the rest of the body. As sphingomyelin can be produced from PtdCho in intestine of fry, our findings suggest that supplementation of dietary PtdCho alone during early developmental stages of Atlantic salmon would be sufficient to promote chylomicron formation. This would support efficient transport of dietary lipids, including PL precursors, from the intestine to the liver where biosynthesis of phosphoglycerides such as PtdEtn, PtdSer, and PtdIns is not compromised in fry as in intestine facilitating efficient utilisation of dietary energy and the endogenous production of membrane PL for the rapidly growing and developing animal.

80 1. INTRODUCTION

81 Dietary lipid is required by all vertebrates, including fish, to satisfy major roles
82 including the provision of metabolic energy and the formation of membranes.
83 Phospholipids (PL) are key structural constituents of cellular membranes and lipoproteins,
84 such as chylomicrons and very high density lipoproteins (VLDL) involved in the transport of
85 dietary lipid from the intestine and liver, respectively, to the rest of the body (Kindel et al.,
86 2010; Mansbach and Siddiqi, 2010; Thiam et al., 2013; Tocher, 2003; Zehmer et al.,
87 2009). The term phospholipid covers all lipids containing phosphorus including
88 sphingolipids (*i.e.* sphingomyelin) and phosphoglycerides, which are characterised by a
89 common backbone of phosphatidic acid (PA) produced by esterification of two activated
90 fatty acids (acyl-CoA) to glycerol-3-phosphate (Lykidis, 2007; Tocher et al., 2008). The
91 major phosphoglycerides of animal tissues phosphatidylcholine (PtdCho),
92 phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer) and phosphatidylinositol
93 (PtdIns) are formed by the esterification of polar head groups choline, ethanolamine,
94 serine or inositol to the phosphate group of PA through a complex sequence of enzymatic
95 reactions (Lykidis, 2007; Tocher et al., 2008).

96 It has long been known that the inclusion of intact phospholipids, specifically
97 glycerophospholipids, in the diet can improve culture performance of many fish species
98 (Coutteau et al., 1997). Thus, dietary lecithin supplementation to diets for larval and
99 juvenile fish indicated that PL significantly enhanced growth performance, survival and
100 stress resistance, and reduced the occurrence of spinal deformities in marine and
101 freshwater species, including Atlantic salmon (*Salmo salar*) (Cahu et al., 2003; Kanazawa,
102 1993; Kanazawa et al., 1983, 1981; Poston, 1990; Rinchard et al., 2007; Takeuchi et al.,
103 1992). Generally, dietary PtdCho enhanced growth and survival whereas PtdIns
104 supplementation has been primarily associated with decreased deformities in fish (Azarm
105 et al., 2013; Geurden et al., 1998a; Kanazawa, 1993). Dietary enrichment with
106 phosphorus, choline or essential fatty acids did not substitute for intact PL in early larval
107 stages showing the requirement was not based on the provision of these essential
108 nutrients (Azarm et al., 2013; Poston, 1990).

109 To date, the precise molecular mechanisms underlying PL requirement in early life
110 stages of fish has not be elucidated although a series of careful studies led to a plausible
111 hypothesis. These studies showed that diets deficient in PL could lead to lipid

112 accumulation in intestinal enterocytes in fish larvae (Fontagné et al., 1998; Liu et al., 2002;
113 Olsen et al., 1999; Salhi et al., 1999). In carp larvae, the intestinal steatosis induced by
114 phospholipid-deficient diets was prevented by supplementing diets with PC and, to a
115 lesser extent, PI (Fontagné et al., 1998). Based on these studies it was suggested that
116 dietary phospholipids were required for the efficient export of dietary lipid from enterocytes
117 (Fontagné et al., 1998; Geurden et al., 1998b; Olsen et al., 1999; Salhi et al., 1999). It was
118 proposed that early life stages of fish have limited ability for endogenous *de novo*
119 biosynthesis of PL backbones and that these were required to be provided by the uptake
120 of PL digestion products (lyso-PL and free fatty acids) to facilitate lipoprotein assembly,
121 specifically the outer PL "coat", and enable efficient export of dietary lipid from enterocytes
122 (Coutteau et al., 1997; Fontagné et al., 1998; Geurden et al., 1995, 1999). This situation is
123 a consequence of larval rearing methods in aquaculture where dietary lipid is primarily
124 supplied as triacylglycerols (TAG), not PL, dietary lipid is primarily supplied as
125 triacylglycerols (TAG), not PL, including to larvae and early developmental stages, and this
126 may not reflect normal diets and actually have insufficient dietary PL to support optimum
127 (Tocher et al., 2008).

128 Although first studied 25 years ago (Poston, 1990,1991) a very recent study has
129 provided considerable new insight to the dietary PL requirement in early development of
130 Atlantic salmon (Taylor et al., 2015). Salmon fry fed a low phospholipid diet from first
131 feeding showed lowest growth and survival, highest level of spinal deformities and
132 displayed intestinal steatosis. Supplementary phospholipid increased growth, improved
133 survival, reduced spinal deformities and prevented steatosis. The data on growth and
134 steatosis indicated that the requirement for dietary phospholipid was restricted to fish of up
135 to 2.5 g. The beneficial effects of dietary phospholipid were associated with PC up to an
136 inclusion level of around 2.5 % of diet (Taylor et al., 2015).

137 As described above, previous studies have suggested that early life stages of fish
138 have limited ability for endogenous *de novo* biosynthesis of PL. However, we further
139 suggest that this impairment cannot be systemic as this would likely be incompatible with
140 life. Our contention is that the limitation in PL biosyntyhesis is restricted to intestinal
141 tissues. Therefore, our overarching hypothesis is that the intestine in early life stages of
142 salmon (early fry) is immature and enterocytes have low capacity for the *de novo*
143 biosynthesis of PL, limiting the assembly of chylomicrons and thus compromising the
144 transport of dietary lipids from intestine to the tissues (Thiam et al., 2013). To understand

145 changes in PL metabolism associated with development, it is of fundamental importance to
146 elucidate the molecular mechanisms of phosphoglyceride biosynthesis in fish. Functional
147 and genomic data has indicated that *de novo* production of PL is greatly conserved in
148 animals and can occur by two main pathways that initially proceed from PA (Holub et al.,
149 1976, 1975; Iijima et al., 1983; Lykidis, 2007; Oxley et al., 2005). The pathways differ
150 depending upon which PL substrate molecules are acivated for assembly. Thus, one
151 pathway utilises a cytidine-activated polar head group and a diacylglycerol molecule
152 (DAG) and the other utilises CDP-activated DAG and a polar head group (Fig. 1). The
153 specific aim of the present study was, firstly, to identify and reconstruct the enzymatic
154 machinery of phosphoglyceride biosynthesis in Atlantic salmon and, secondly, to
155 characterise PL gene expression patterns in major tissues of lipid absorption, biosynthesis
156 and transport, specifically intestine and liver, during critical early developmental stages in
157 order to elucidate the molecular mechanisms of PL requirement of Atlantic salmon.

158

159 ## 2. MATERIALS & METHODS

160 ### *2.1 Fish, diets and sampling protocols*

161 Atlantic salmon eggs were provided by Landcatch Natural Selection (Ormsary,
162 Scotland) and salmon larvae and fry were maintained in the University of Stirling
163 freshwater trial facilities (Howietoun Hatchery and Niall Bromage Freshwater Research
164 Facility, Stirling, UK). All experimental procedures were conducted in compliance with the
165 Animals Scientific Procedures Act 1986 (HomeOffice Code of Practice. HMSO: London
166 January 1997) in accordance with EU regulation (EC Directive 86/609/EEC) and approved
167 by the Animal Ethics and Welfare Committee of the University of Stirling. From first feeding
168 the fish were fed a basal diet designed to satisfy the nutritional requirements of salmonid
169 fish (NRC, 2011), but containing minimum (unsupplemented) levels of PL (Taylor et al.,
170 2015). The diets were formulated and synthesised by BioMar AS (Tech Centre, Brande,
171 Denmark) at appropriate pellet sizes to satisfy gape size of salmon fry (Taylor et al., 2015).
172 Amounts fed were determined on the basis of total body weight according to
173 manufacturer’s protocols and adjusted according to prevailing water temperature. Salmon
174 fry were euthanised by an overdose of MS-222 (PHARMAQ, UK) and samples of liver and
175 intestine (mid-gut), major organs involved in uptake, transport and synthesis of lipids, were
176 dissected at approximately 2.5 g (1990 ° day post fertilisation, dpf) and 10 g (2850 °dpf).

177 Six fish were randomly selected from each time point. Tissue samples were immediately
178 and rapidly disrupted in 1 mL of TriReagent (Sigma, UK) using a BeadBeater homogeniser
179 (BioSpec, Oklahoma, USA) for 30 s, and stored at – 80 °C prior to RNA extraction. The
180 dietary trial and all procedures of Atlantic salmon conformed to European ethical
181 regulations regarding the care and use of farmed animals in research.

182 *2.2 Identification of phospholipid biosynthetic genes in Atlantic salmon*

183 The phospholipid biosynthetic pathway in Atlantic salmon was reconstructed *in*
184 *silico* based on previously described eukaryotic genomes (Lykidis, 2007). A wide range of
185 enzymes including phosphatases, kinases, acyltransferases, phosphotransferases,
186 cytidylyltranferasas, methyltransferase and genes involved in methyl group transfers were
187 successfully retrieved from the Atlantic salmon transcriptome shotgun assembly and
188 databases including expressed sequence tags (EST) and transcriptome shotgun assembly
189 (TSA) available from NCBI. Generally, homologous vertebrate sequences from fish
190 species were used as BLAST (megablast) queries under default search parameters and
191 only sequences that exhibited identity > 80 % and revealed > 500 nucleotides of coverage
192 were considered for molecular analysis. The genomes of cichlids damselfish, *Stegastes*
193 *partitus*, and Nile tilapia, *Oreochromis niloticus* were preferentially used as BLAST queries
194 due to their nearly-complete genome and well annotated transcriptome.

195 *2.3 Sequence and phylogenetic analysis of PtdSer synthases*

196 *2.3.1 Sequence analysis*

197 Most eukaryotes synthesise PtdSer by a base-exchange mechanism in which
198 serine substitutes for the choline and ethanolamine groups of PtdCho and PtdEtn,
199 respectively. In vertebrates, two enzymes have been identified: PSSE1 which catalyses
200 the exchange reaction with PtdCho and PSSE2 which utilises PtdEtn (Lykidis, 2007). An
201 alternative pathway for PtdSer synthesis derived from CDP-diacylglycerol and free serine
202 has been described in bacteria e.g. the *Bacillus* PSS enzymes. Interestingly, three
203 homologous genes to the bacterial *pss* genes were identified in the genomes of zebrafish
204 *Danio rerio*, gi¡68402375, (designated BLA-PSS, for Bacillus-Like Animal PSS), which
205 possessed a CDP-alcohol phosphotransferase motif corresponding to the *B. subtilus pss*
206 previously described [Genebank: CP008698] (Williams and McMaster, 1998). BLASTN
207 computational searches of all available PtdSer synthases in the Atlantic salmon

208 transcriptome were performed as previously described. However, less stringent
209 parameters were applied to retrieve a salmon BLA-Pss enzyme,using the aforementioned
210 bacterial protein as query.

211 *2.3.2 Multiple sequence alignment*

212 Generally, it is more informative to compare protein sequences as they can
213 identify homologous sequences from organisms that last shared a common ancestor over
214 1 billion years ago compared to DNA sequences (Pevsner, 2009). Thus, the amino acid
215 (aa) sequence deduced from Atlantic salmon *pss* [Genbank: NM_001146675.1] was
216 aligned with fish orthologs, including *Oreochromis niloticus* [XM_003438832.1],
217 *Haplochromis burtoni* [Genbank: XM_005918887], *Danio rerio* [Genbank:
218 XM_005162261], *Takifugu rubripes* [Genbank: XM_003977798], *Tetraodon nigroviridis*
219 [Genbank: CR689878], *Stegastes partitus* [Genbank: XM_008293258] and bacterial PSS
220 enzymes. Similarly, serine-exchange enzymes from Atlantic salmon Psse1 [Transcriptome
221 assembly: Icl | Ssa.51746_2] and Psse2 [Transcriptome assembly: Icl | Ssa.7743] were
222 aligned with fish homologous species. The ClustalW algorithm (BioEdit 7.1.3, Tom Hall,
223 Ibis Biosciences, Abbott Laboratories) is a progressive alignment method that uses the
224 global alignment approach of Needleman and Wunsch (Needleman and Wunsch, 1970) to
225 create pairwise alignment scores of all sequences applying the BLOSUM62 protein
226 similarity matrix that accounts for the probability of mutation and the biophysical properties
227 of amino acids (Thompson et al., 1994).
228

229 *2.3.3 Phylogenetic tree*

230 A phylogenetic tree was constructed including vertebrate base-exchange PtdSer
231 synthases, PSSE1 and PSSE2, and *Bacillus*-like BLA-Pss from fish species and *B.*
232 *subtilus*. To classify the PtdSer synthases in eukaryotes based on the catalytic activity, the
233 phylogenetic tree was constructed on the basis of the protein sequence of 16 taxa and
234 only the regions corresponding to the catalytic motifs (plus 10 amino acids, aa, up and
235 downstream) were included in the analyses according to Williams and McMaster (1998).
236 The evolutionary history was inferred applying the distance-based Neighbour-Joining (NJ)
237 algorithm (Saitou and Nei, 1987) in MEGA4 (Tamura et al., 2007). For this, the variation
238 among sites was modelled using a JTT substitution matrix (Jones et al., 1992) that
239 integrates observed probabilities of amino acid substitutions obtained from local

240 alignments of large protein databases and uniform evolutionary rates among lineages
241 assumed. A consensus tree was inferred from 1000 bootstrap replicates (Felsenstein,
242 1985).

243 *2.4 Quantitative RT-PCR*

244 Gene expression was determined by quantitative real-time PCR (qPCR). Total RNA
245 was isolated from liver and intestine from Atlantic salmon fry (2.5 g) and parr (10 g) (n = 6)
246 by guanidinium/phenol extraction procedure (TriReagent, Sigma, Poole, UK). RNA
247 integrity and quantity was assessed by electrophoresis and spectrophotometry (Nanodrop
248 1000, Thermo Scientific, Wilmington, USA). Two micrograms of total RNA were reverse
249 transcribed into cDNA using TaqMan Reverse Transcription Reagents (Invitrogen, Paisley,
250 UK) and primed with random hexamers and oligo(dT) in a 3:1 molar ratio. The resulting
251 cDNA was diluted 20-fold with nuclease-free water.

252 For qPCR, oligonucleotide primers spanning exon/exon boundaries for target genes
253 and housekeeping genes (ribosomic proteins *rpl1*, *rpl2* and *rpl3*; polymerase (RNA) II
254 (DNA directed) polypeptide F, *polr2f*; elongation factor 1-alpha, *ef1α*; cofilin2, *cfl2*; beta-
255 actin, act*β* and beta-2-microglobulin, *β2m*) (Table 1) were used at 0.3 μM with 1/200 of the
256 cDNA synthesis reaction (2 μl of a 1:20 dilution) and 5 μl of SYBR-green qPCR mix
257 (Luminaris Color HiGreen qPCR, Thermo Scientific, USA) in a total volume of 10 μL.
258 Reactions were run in a Mastercycler RealPlex^2 (Eppendorf, UK). Amplifications were
259 carried out including systematic negative controls containing no cDNA (NTC, no template
260 control) and omitting reverse transcriptase enzyme (– RT) to check for DNA
261 contamination. UDG pre-treatment at 50 °C for 2 min preceded thermal cycling, which was
262 initiated at 95 °C for 10 min, followed by 40 cycles with a denaturing step at 95 °C for 15 s,
263 annealing at 60 °C for 30 s and extension at 72 °C for 10 s. After the amplification cycle, a
264 melting curve was performed with 0.5 °C increments ranging between 60 °C and 95 °C to
265 ensure the amplification of a single product. In addition, the qPCR product sizes were
266 checked by agarose gel electrophoresis and the identity of random samples was
267 confirmed by sequencing (GATC Biotech, Germany). No primer-dimer formation occurred
268 in the NTC. Gene expression quantification was achieved by including a parallel set of
269 reactions containing serial dilutions from all pooled cDNA experimental samples and
270 assigning each dilution the appropriate value of relative units (RU). As a result, an
271 estimated number of relative copies, corrected for the efficiency of the reaction, was

272 automatically calculated for each sample. The normalised expression values were
273 generated by the ΔCt method (Pfaffl, 2001) and the results expressed as mean normalised
274 ratios (± SE) between the RUs of target genes and a reference gene index calculated from
275 the geometric mean of the three most stable reference genes. Housekeeping gene stability
276 (Supplementary Table 1) was determined applying a correction for efficiency to the raw Ct
277 standard deviation (Pfaffl, 2004) using BestKeeper (Pfaffl et al., 2004).The stability values
278 suggested a different reference index to be calculated for each tissue: *polr2f*, *rpl1* and *rpl2*
279 for intestine, and *polr2f*, *rpl1* and *cofilinβ* for liver.

280 *2.4.1 Data analysis and statistical tests*

281 Gene expression differences between tissues and between time-points
282 (developmental stages) were analysed by pairwise comparisons applying one-way
283 analysis of variance (ANOVA) (PASWS 18.0, SPSS Inc., USA). Similarly, pairwise
284 comparisons were performed within each developmental stage across tissues. Genes that
285 exhibited significant expression differences between and/or within experimental conditions
286 were subject to hierarchical clustering (Pearson's correlation) and presented as a heat
287 map using "gplots" package (Warnes et al., 2014). Gene expression was presented as the
288 relative expression ratio of each gene (relative units). A significance of $P \leq 0.05$ was
289 applied to all statistical tests performed.

290

291 3. RESULTS

292 *3.1 Pathways of phospholipid biosynthesis in Atlantic salmon*

293 Computational analysis in the Atlantic salmon transcriptome shotgun assembly
294 (NCBI) elucidated several gene families participating in phospholipid biosynthesis in
295 Atlantic salmon: lipid phosphatases, cytidylyltransferases, phosphotransferases, kinases,
296 decarboxylase, base-exchange enzymes and methyltransferases. In addition, enzymes
297 involved in methyl group transfers and transcription factors engaged in the regulation of
298 lipid metabolism were considered. Figure 1 outlines the *in silico* reconstruction of the
299 phospholipid biosynthetic pathway in Atlantic salmon.

300 First, phospholipid biosynthesis starts with the successive acylation of glycerol-3-
301 phospate (G3P) and 1-lysophosphatidic acid (LPA) to produce phosphatidic acid (PtdOH).
302 These two steps are catalysed by acyltransferases including glycerol-3-phosphate
303 acyltranferase (Gpat), followed by esterification of fatty acids in position *sn*-2 by 1-acyl-sn-
304 glycerol-3-phosphate acyltransferases (Plcc, Plcd, Plcf and Plchb paralogs). Also, Lpcat2
305 acyltransferase exhibits dual activity over G3P or G3P-Cho. Subsequently, PtdOH can be
306 metabolised by either CDP-diacylglycerol (CDP-DAG) or diacylglycerol (DAG) pathways.
307 The partitioning of PtdOH is regulated by the activity of CDP-DAG synthases (Cds1 and
308 Cds2) and PtdOH phosphatases including lipins and Paps. Once dephosphorylated, DAG
309 can be converted to PtdCho and PtdEtn by the action of CDP-choline:DAG and CDP-
310 ethanolamine:DAG phosphotransferases (Chpt1 and Cept1, respectively) in the presence
311 of CDP-activated choline and ethanolamine. For this, choline and ethanolamine have to be
312 previously phosphorylated by kinases (Etn kinase, Eki1 and Cho kinase, Chka) and
313 activated with CTP via choline-phosphate and ethanolamine-phosphate
314 cytidylyltransferases (Pcy1 and Pcy2, respectively). PtdCho is also produced by the
315 successive methylation of PtdEtn by PtdEtn *N*-methyltranfese (Pemt). Similarly,
316 phosphoethanolamine (P-Etn) can be methylated to phosphocholine (P-Cho) by P-Etn *N*-
317 methyltranfese (Peam3) prior CTP-activation to enter the DAG pathway. In Atlantic
318 salmon, PtdSer can be synthesised either via a base-exchange mechanism from PtdCho
319 and PtdEtn or directly from CDP-DAG. The former route is mediated by serine exchange
320 enzymes (Psse1 and Psse2), present in most eukaryotes, whereas the latter is catalysed
321 by PtdSer synthase *Bacillus-like* animal Pss (BLA-Pss) (Lykidis, 2007). An alternative
322 pathway for the biosynthesis of phosphoglycerides is via the CDP-DAG route. Relevant in
323 the biosynthesis of PtdIns through the action of CDP-DAG-inositol-3-
324 phosphatidyltranferase (Cdipt) and cardiolin via CDP-DAG-glycerol-3-
325 phosphatidyltranferase (Pgs1), phosphatidylglycerol phosphatase (Ptpmt1) and cardiolipin
326 synthase (Crls1), this pathway is secondary in the biosynthesis of PtdCho, PtdEtn or
327 PtdSer in mammals as they lack a bacterial-like PtdSer synthase. From PtdSer, PtdEtn
328 can be synthesised by the action of PtdSer decarboxylase (Pisd). Sphingomyelin, the most
329 common sphingolipid, can be synthesised from PtdCho via PtdCho:ceramide
330 cholinephosphotransferase (Sgms1).

331 *3.2 Phylogenetic analysis of PtdSer synthase genes*

332 Transcriptomic resources revealed partial sequences of homologous base-
333 exchange PtdSer synthases in Atlantic salmon, Psse1 [Ssa.74508_1] and Psse2
334 [Ssa.7743] and a 1,472 bp full-length cDNA sequence BLA-Pss [NM_001146675]. The
335 salmon bacterial-like Pss open reading frame (ORF) encodes a putative protein of 241 aa
336 that shares 83 % to 89 % identity to other teleost BLA-Pss and a lower identity value with
337 the *Bacillus* sp. catalytic site (56 %). The multiple alignment elucidated the presence of
338 seven aa residues (Fig. 2A) that have been established to be highly conserved across
339 eukaryote and prokaryote phospho- and phosphatidyltransferase genes (Williams and
340 McMaster, 1998). Phylogenetic analysis of the catalytic motifs showed that teleost and
341 *Bacillus* sp Pss enzymes cluster together according to accepted taxonomy as displayed in
342 the phylogenetic tree (Fig. 2B) with putative Pss enzymes forming a separate clade and
343 thus in agreement with Lykidis (2007). Similarly, vertebrate Psse1 and Psse2 homologs
344 constituted separate phylogenetic clusters, although more closely related to each other
345 than to the BLA-Pss branch. The aa identity between salmon Psse1 and Psse2 catalytic
346 site was 53 %, whereas salmon Psse1 exhibited aa identities ranging between 90 % to 95
347 % to vertebrate species and Psse2 between 90 % and 97 %. The lowest identity levels
348 were identified for some teleost orthologs, including *Oryzias latipes* and *Poecilia formosa*
349 and not necessarily mammalian members of the Psse family suggesting a high level of
350 sequence conservation across vertebrates.

351 *3.3 Gene expression: Phosphoglyceride biosynthesis*

352 To study the biosynthesis of phosphoglycerides we focussed on the expression
353 values for key biosynthetic genes in intestine and liver, major tissues involved in the
354 absorption, synthesis and transport of lipids (Tocher, 2003; Tocher et al., 2008).
355 Hierarchical clustering of PL biosynthetic genes and TFs based on their standardised
356 expression revealed eight clear patterns of gene expression (Fig. 3). Only *lxrα* and *lpcat2*
357 were not included in the heat map as no significant differences in expression of these
358 genes were observed between experimental conditions. Two major clusters contained the
359 majority of genes studied: 1) genes that displayed lower expression in intestine (greater
360 expression in liver) during the fry stage (Fig. 3, “cluster 1”), and 2) genes with an increased
361 mean expression in the intestine of parr compared to any other conditions (Fig. 3, “cluster
362 5”).

363 3.3.1 Cluster 1

364 One of the two larger gene-expression clusters was characterised by genes with
365 constitutively lower expression levels in the intestine compared to the liver (Fig. 4). A clear
366 subdivision could be made within this group when differences in expression between
367 developmental stages are present in intestine (higher in parr), as in phosphoglyceride
368 kinases, *chka* and *eki1*, PtdSer decarboxylase (*pisd*) involved in PtdEtn synthesis,
369 phosphatidylglycerol phosphatase, *ptpmt1*, and the step-limiting acyltransferase *gpat*. In
370 addition, the expression pattern of the aforementioned genes suggested that as the gene
371 expression increased in the intestine of parr it decreased in the liver.

372 3.3.2 Clusters 2 and 7

373 Certain genes, including acyltransferases *plcd*, *plchb* and *papdc1b* PtdOH phosphatase
374 were mainly transcribed in the liver, whereas *plcc*, *lpin2* and *ppap2c* exhibited greater
375 expression in the intestine compared to the liver (Fig. 5). The characteristic of the
376 aforementioned genes is that the tissue expression in liver and intestine, respectively, did
377 not show any developmental changes.

378 3.3.3 Cluster 3 and cluster 8

379 On the other hand, the transcript expression detected for PtdSer synthase BLA-Pss, Plcf
380 and Papdc2 enzymes increased in direct correlation with physiological development from
381 fry to parr stage (Fig. 6A). Whereas, the expression of *ppap2b* and *sgms1*, involved in
382 sphingomyelin synthesis, showed the opposite trend with parr displaying lower expression
383 than fry (Fig. 6B).

384 3.3.4 Cluster 4

385 These genes exhibited similar expression patterns to the constituents of cluster 1, which
386 revealed inverse expression patterns between liver and intestine. In this case, the
387 expression values in the intestine increased and conversely decreased in the liver as the
388 salmon developed including genes *papdc1a*, *cdipt*, *pcy2* an *cept1* (Fig. 6C).

389 3.3.5 Cluster 5

390 Cluster 5 in Figure 3 grouped a set of genes in which greater differences were observed
391 between fry and parr intestine, whereas no variation was observed in liver (Fig. 7A). Pemt,
392 involved in PtdEtn methylation to PtdCho, choline phosphotransferase (Chpt1) critical for
393 PtdCho synthesis from DAG, DCP-DGA synthase 1 (Cds1), Sas2 methyl donor,

394 phosphatase *ppap2a* and cardiolipin biosynthetic genes as *crls1* and *pgs1* were
395 significantly more expressed in parr intestine.

396 3.3.6 Cluster 6

397 Pcy1, P-choline cytidylyltransferase and PtdSer synthase via base-exchange, Psse1,
398 displayed a particular expression pattern with similarly high transcription levels in intestine
399 and fry liver, but lower in the liver of parr (Fig. 7B).

400 3.3.7 Gene expression of transcription factors

401 Transcription factors: sterol regulatory element-binding proteins, Srebp1 and Srebp2, liver
402 X receptor (Lxrα), peroxisome proliferator-activated receptors (Pparα, Pparβ1a and Pparγ)
403 and retinoid X receptor (Rxrβ) have been previously characterised in Atlantic salmon
404 (Carmona-Antoñanzas et al., 2013a; Cruz-Garcia et al., 2009; Leaver et al., 2007;
405 Minghetti et al., 2011). Srebp2 and Pparβ1were primarily expressed in the liver, whereas
406 Srebp, Pparα and Pparγ exhibited greater expression in intestine and significantly higher
407 in parr than in fry similar to the expression pattern described above for cluster 5 (Fig. 8).
408 Rxr, on the other hand, did not show a clear pattern of expression and only a mild increase
409 was noticed from fry to parr.

410

411 4. DISCUSSION

412 In aquaculture, the lipid component in feeds are supplied by oils rich in TAG as a
413 major source of energy and essential fatty acids for growth (Polakof et al., 2012).
414 However, the necessity to provide dietary intact PL to support normal/optimal growth and
415 development of larvae and early developing stages of many marine and freshwater fish
416 species has long been known and this has been attributed to limited ability to
417 endogenously produce PL in young fish (Coutteau et al., 1997; Tocher et al., 2008).
418 Despite this being the case, the biochemical mechanisms of PL biosynthesis in fish and
419 the impairment in early developmental stages had not been elucidated. The present study
420 aimed to address this lack and to identify gene candidates for the deficient steps in PL
421 biosynthesis in early development of fish, specifically Atlantic salmon. In addition, the
422 study tested the hypothesis that the limitation was due to a deficiency in PL biosynthesis
423 specifically in intestinal tissue, rather than a systemic lack in all tissues, and was simply a

424 reflection of an immature gut in early developmental stages of fish. Thus the expression of
425 around 40 genes involved in PL biosynthesis and the regulation of lipid metabolism was
426 determined in intestine and liver of salmon fry (1990 °dpf) and parr (2850 °dpf). The
427 expression patterns of phosphoglyceride biosynthetic genes provided clear evidence of
428 crucial genes of PtdCho (i.e. choline phosphotransferase and phosphatidylethanolamine
429 methyltransferase), PtdEtn (i.e. ethanolamine phosphate cytidylyltransferase,
430 ethanolamine phosphotransferase), PtdIns (i.e. inositol phosphatidyltransferase), PtdSer
431 (i.e. phosphatidylserine synthase) and PtdGro (i.e. glycerol-3-phosphate
432 phosphatidyltransferase) being marginally transcribed in the intestine of salmon fry, which
433 could limit endogenous production of PL from dietary precursors.

434 PL biosynthetic mechanisms are strongly conserved across lineages, although
435 certain phylogenetic differences have been reported previously between distant clades.
436 Only plants and algae, for instance, express a serine decarboxylase (SDC) that catalyses
437 the conversion of serine to ethanolamine, and most higher vertebrates lack
438 methyltrasferases to catalyse the successive methylation of phosphoethanolamine into
439 phosphocholine, which subsequently enters the CDP-choline pathway (Lykidis, 2007).
440 Similarly, bacteria possess the ability to produce PtdSer via two different processes: a
441 base-exchange mechanism similar to that identified in higher vertebrates catalysed by
442 Psse1 and Psse2 acting on PtdCho and PtdEtn, respectively, or from CDP-DAG mediated
443 by serine phosphatidyltransferase (PSS), which belongs to a large family of phospholipid
444 phosphodiester synthases (PPS). Phylogenetic analysis demonstrated that the putative
445 bacterial-like animal Pss in Atlantic salmon clustered separately from the serine-exchange
446 phosphatidyltransferases and was more closely related to the *Bacillus* sp. PSS enzymatic
447 motif. Genomic studies performed by Lykidis (2007) discovered the presence of bacterial
448 Pss proteins in *Danio*, *Fugu* and *Tetraodon* that contained a complete PPS active site,
449 DG-X_2-AR-X_8-G-X_3-D-X_3-D (Williams and McMaster, 1998) in contrast to other PSS
450 proteins identified in mammals. Accordingly, knockout mammalian cells lacking both PSSE
451 forms are PtdSer auxotrophs (Saito et al., 1998). This additional mechanism might offer
452 teleosts an alternative route to PtdSer formation, albeit yet to be confirmed functionally.
453 The expression of *pss* in Atlantic salmon suggested the intestine of early salmon fry might
454 not be capable of producing PtdSer from CDP-DAG until further developed, thus requiring
455 Psse enzymes to compensate. Interestingly, Psse1 exhibited considerably high expression

456 in the intestine at both time points, whereas low expression of Psse2 in the intestine
457 suggested it might be more involved in the synthesis of PtdSer in the liver along with Pss.

458 In vertebrates, acyltransferases and phosphatases comprise large gene families
459 whose members exhibit distinct tissue expression patterns (Takeuchi and Reue, 2009), a
460 common outcome observed among duplicated genes (Carmona-Antoñanzas et al.,
461 2013b). Gpat, considered the rate-liming enzyme in the acylation of glycerol-3-phosphate
462 in mammals (Coleman and Lee, 2004), is poorly expressed during early developmental
463 stages in the intestine especially during the fry phase possibly limiting acylation of PL.
464 Among the LPA acyltransferases, *plcc* is generally expressed in the intestine, whereas
465 orthologs *plcd*, *plcf* and *plchb* were mainly expressed in liver during this period
466 development up to 10 g. A similar pattern of functional complementation through tissue
467 partition of paralogous genes was observed among phosphatases involved in the
468 synthesis of DAG suggesting that such enzymatic steps are not compromised at any
469 developmental stage. Interestingly, two phosphatidate cytidylyltransferases, *cds1* and
470 *cds2*, were identified in Atlantic salmon. *Cds1*, exhibited a clear expression pattern
471 suggesting an inability to synthesise PL efficiently during the fry stage; however,
472 compensated by the predicted constant activity of Cds2 to produce CDP-DAG from PtdOH
473 throughout tissue and developmental stage.

474 In eukaryotes, the formation of cardiolipin (Ptd_2Gro, CL) proceeds from CDP-DAG
475 and intermediates PtdGroP and PtdGro (Lykidis, 2007). The expression of
476 phosphatidylglycerophosphate synthase (Pgs1), the rate-liming reaction of CL
477 biosynthesis forming PtdGroP from CDP-DAG and G3P, and cardiolipin synthase (Crls1)
478 (Chang, 1998) exhibited ~ 2-fold increase in the parr intestine compared to the fry intestine
479 or liver. The expression data suggested immaturity of the intestine in Atlantic salmon fry
480 and was consistent with the predominance of intestine over liver in the production of CL,
481 the mitochondrial-specific PL (Horvath and Daum, 2013). However, failure to synthesise
482 and remodel CL in the inner mitochondrial membrane has been shown to be tissue
483 specific resulting in skeletal and cardiac myopathies and growth disturbance in mammals
484 (Kelley et al., 1991; Schlame, 2013), abnormal cardiac development in zebrafish and
485 irregular morphology of germ cells and *C. elegans* (Khuchua et al., 2006; Sakamoto et al.,
486 2012).

487 Phosphatidylinositol (PtdIns), produced directly from CDP-DAG, also precedes
488 synthesis of important intracellular regulators (i.e. phosphoinositides) (Tocher et al., 2008).
489 In fish, PtdIns can be assimilated from yolk sac lipids directly into embryonic or larval
490 tissue lipids without modification (Sargent et al., 2002); however, dietary supplementation
491 with PtdIns resulted in optimal survival and minimal skeletal deformities in common carp
492 (Geurden et al., 1997, 1998a) and larval ayu (Kanazawa et al., 1985) suggesting that
493 dietary requirement of PtdIns during early developmental stages might be dependent on
494 provision of PtdIns from progenitors. In Atlantic salmon, expression of CDP-DAG-inositol
495 3-phosphatidyltransferase (Cdipt) suggested a reduced ability for the salmon fry to
496 synthesise PtdIns in the intestine compared to parr., The gene expression pattern in the
497 liver suggests it could compensate for the deficiencies in intestinal PL metabolism
498 provided intermediate metabolites for the production of PL, including LPA, PtdOH and
499 choline or inositol, which cannot be synthesised by animals *de novo*, are transported from
500 the intestine to the liver. However, results indicated that in the absence of dietary PL,
501 salmon fry accumulated lipid in the enterocytes in the form of droplets suggesting a failure
502 to efficiently transport dietary lipid away from the intestine.

503 Dietary lipids diffuse into the enterocytes where lyso-PL are re-esterified (Hazel et
504 al., 1987; Tocher, 2003) and transported to the endoplasmic reticulum (ER) (Thiam et al.,
505 2013). In the ER membrane, dietary lipids including neutral lipids, such as TAG and sterol
506 esters, are packaged into lipoproteins for distribution to the rest of the body as
507 chylomicrons (Chapman, 1980; Noriaki et al., 1990; Sire et al., 1981). Characterised by
508 similar PL composition, the formation of chylomicrons and lipid droplets is determined by
509 biophysical and structural properties (Guijas et al., 2014). In mammals, chylomicron
510 creation is controlled by the PL to neutral lipid ratio, which in turn is determined by the
511 dietary intake and biosynthetic capacity of the species, essential to maintain the round
512 shape and obtain the greater size and density characteristic of chylomicrons (Guijas et al.,
513 2014; Kindel et al., 2010; Thiam et al., 2013). PtdCho, which constitutes over 80 % of the
514 total PL in lipoproteins (Daum and Vance, 1997; Wood et al., 1964), is required to
515 establish a neutral curvature and stabilise the molecule that protects the hydrophobic core
516 from lipolysis (Guijas et al., 2014) and possibly constitutes the limiting factor in the
517 formation of chylomicrons.

518 Whereas PtdIns is believed to prevent malformations during development, PtdCho
519 is required for growth and development in early life stages of fish (Poston, 1990; Tocher et

520 al., 2008). The two pathways of PtdCho synthesis are: (i) phosphorylation of choline and
521 transfer to a DAG acceptor (CDP-choline pathway), and (ii) sequential methylation of
522 PtdEtn. Choline kinase, *chka*, and especially choline phosphotransferase, *chpt1*, mRNA
523 showed significantly upregulated expression in intestine of parr. Accordingly, mammalian
524 CHPT1 is highly expressed in intestine suggesting an important role for these tissues in
525 the biosynthesis of PtdCho (Henneberry et al., 2000). The existence of methyltransferases
526 provides an alternative route to PtdCho formation from ethanolamine precursors.
527 Phosphoethanolamine methyltransferase (Peam3), specific to teleosts and frogs (Lykidis,
528 2007), is involved in the indirect production of phosphocholine by methylation of
529 phosphoethanolamine predominantly in the liver, whereas phosphatidylethanolamine
530 methyltransferase enables the efficient conversion of PtdEtn to PtdCho in parr intestine
531 with a clear reduction in fry. Similarly, the biosynthesis of PtdEtn through the CDP-
532 ethanolamine pathway proved a clear reduction in the activity of the three major steps
533 during the larval stage in the intestine. This pattern was mirrored in the expression of
534 PtdSer decarboxylase (Pisd) that results in PtdEtn as the final product. The requirement of
535 PtdEtn is regarded secondary during early developmental stages in fish preceded by
536 PtdCho and PtdIns (Tocher et al., 2008).

537 Thus, the lower expression in the fry intestine of several key genes involved in the
538 biosynthesis of PtdCho, PtdIns, PtdEtn and PtdSer, suggested there could be reduced
539 capacity of intestine in fry to efficiently synthesise phosphoglycerides, thus compromising
540 chylomicron formation and resulting in the steatosis observed in salmon when fed a
541 minimal PL diet from hatching (Taylor et al., 2014). Accordingly, dietary PL
542 supplementation increased chylomicrons in rainbow trout, *Oncorhynchus mykiss* (Azarm et
543 al., 2013) supporting the importance of PL in enabling dietary lipids to be transported from
544 the intestine to the liver, adipose, cardiac and skeletal muscle tissue (Chapman, 1980;
545 Noriaki et al., 1990; Sire et al., 1981). Transcription factors Lxr, Ppar and Srebp play
546 important roles in the regulation of lipid metabolism in fish (Carmona-Antoñanzas et al.,
547 2013a; Cruz-Garcia et al., 2012; Leaver et al., 2005; Minghetti et al., 2011). The tissue
548 expression of Ppars in Atlantic salmon indicated that *pparα* and *pparγ* expression is
549 greater in the intestine than in the liver opposite to *pparβ1a*. Although promoter studies
550 were not performed, the tissue expression patterns suggested that Pparα, Pparγ, and
551 specially Srebp1 (with > 2-fold higher expression in the parr intestine compared to fry
552 intestine) might be involved in the transcriptional regulation of genes associated with

553 development-related phosphoglyceride requirements. Accordingly, previous studies
554 indicated that SREBP1a targeted SRE sites of genes from the PL biosynthetic pathway in
555 mammals (Berger and Roberts, 2004; Kast et al., 2001) although only partial dominance
556 was attributed to the interaction with SREBP1a. Methyl donor enzymes provide methyl
557 (CH_3) groups to methyltransferases such as Peam3 and Pemt involved in the biosynthesis
558 of phosphocholine and PtdCho, respectively. This gene expression study indicated that,
559 whereas *sas1* is highly expressed in the liver, intestinal *sas2* might limit methylation rate
560 as it showed lower expression in intestine of fry compared to parr.

561

562 ## 5. CONCLUSIONS

563 In conclusion, Atlantic salmon possess a complete set of enzymes for the endogenous
564 biosynthesis of PL, including a fish-specific phosphocholine methyltransferase and a
565 bacterial-like PtdSer synthase that offers alternative routes for the biosynthesis of PtdCho
566 and PtdSer, respectively. The gene expression data indicated that synthesis of
567 phosphoglycerides was potentially compromised in salmon fry at 2.5 g (1990 °dpf)
568 compared to parr of 10 g (2850 °dpf) given that most biosynthetic genes showed lower
569 expression during the earlier developmental stage, whereas sphingomyelin production, the
570 second key PL in chylomicron formation (Wood et al., 1964), was not. PtdCho is the major
571 PL in lipoproteins (Guijas et al., 2014; Wood et al., 1964) with over 80 % of total PL, and
572 so is likely to be the limiting phosphoglyceride in the formation of chylomicrons and, thus,
573 in the transport of dietary lipids to the rest of the body. Interestingly, most key
574 phosphoglyceride biosynthetic genes exhibited an inverse pattern of expression between
575 intestine in liver, excluding critical PtdCho enzymes cholinephosphotransferase and
576 phosphatidylcholine methyltransferase. Thus, our findings suggest that supplementation of
577 dietary PtdCho during early life stages of Atlantic salmon might be sufficient to promote
578 chylomicron formation over lipid droplet formation, preventing steatosis, and promoting
579 lipid (and energy) transport from the intestine. Hence, transport of dietary lipids, including
580 PL precursors, to the liver where biosynthesis of phosphoglycerides like PtdEtn, PtdSer,
581 and PtdIns is greater would be sufficient to facilitate endogenous production of PL during
582 early stages. Future studies on the dietary requirement of PtdCho and PtdIns during larval
583 stages in fish should focus on physiological responses to PL supplementation and
584 mechanisms of transcriptional regulation.

585

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590

591

592 FIGURE LEGENDS

593 **Figure 1. Atlantic salmon pathways for phospholipid biosynthesis.** Acyltransferases
594 control the successive acylation of glycerol-3-phospate (G3P) and 1-lysophosphatidic acid
595 (LPA) to produce phosphatidic acid (PtdOH). Cds and Pap regulate the distribution of
596 PtdOH between CDP-diacylglycerol (CDP-DAG) and diacylglycerol (DAG).
597 Phosphatidyltransferases utilise CDP-DAG or phospholipids and phosphotransferases
598 utilise DAG as substrates to synthesise phospholipids. Gpat, glycerol-3-phosphate
599 acyltransferase; Plc, 1-acyl-sn-glycerol-3-phosphate acyltransferase; Cds, CDP-DAG
600 synthetase; Pap, phosphatidate phosphatase; Pcy1, phosphocholine cytidylyltransferase;
601 Chka, choline kinase; Chpt1, CDP-choline:diacylglycerol phosphotransferase; Pcy2,
602 phosphoethanolamine cytidylyltransferase; Eki1, ethanolamine kinase; Cept1, CDP-
603 ethanolamine:diacylglycerol phosphotransferase; Pemt, phosphatidylethanolamine
604 methyltransferase; Peam3, phosphoethanolamine methyltransferase; Cdipt,
605 phosphatidylinositol synthase; Ptpmt1, phosphatidylglycerol phosphate phosphatase;
606 Pgs1, phosphatidylglycerol phosphate synthase; Crls1, cardiolipin synthase; Pss,
607 phosphatidylserine synthase; Psse, phosphatidylserine synthase via base-exchange; Pisd,
608 phosphatidylserine decarboxylase; Sgms1, sphingomyelin synthase.

609 **Figure 2. Phylogenetic analysis of phosphatidylserine (PtdSer) synthases in Atlantic**
610 **salmon.** A) Multiple alignment of deduced amino acid sequences of the *Bacillus* Pss with
611 teleost BLA-Pss orthologs was performed using ClustalW (BLOSUM62 substitution matrix)
612 from BioEdit, and identity/similarity was calculated based on a 60 % identity threshold.
613 Identical residues are shaded *dark grey*, and altered residues are shaded in *light grey* if
614 they exhibit the same chemical qualities, or *white* if they do not. Outlined is the catalytic

615 motif and an asterisk indicates each of the 7 amino acid residues conserved across
616 phospho- and phosphatidyltransferases (Williams and McMaster, 1998); –, represents a
617 gap in the sequence. B) Phylogenetic tree revealing the relative position of PtdSer
618 synthases: Psse via base-exchange and *Bacillus*-like animal Pss (BLA-Pss) according to
619 proteins from other vertebrate orthologs. The tree was constructed on the amino acid
620 sequences extracted from the Atlantic salmon shotgun transcriptome (NCBI) using the
621 Neighbour Joining method (Saitou and Nei, 1987). The evolutionary distances were
622 computed using the JTT matrix-based method (Jones et al., 1992). All positions containing
623 gaps and missing data were eliminated from the dataset (complete deletion option). The
624 numbers on the branches represent the frequencies (%) with which the presented tree
625 topology was obtained after bootstrapping (1,000 iterations). Phylogenetic analysis were
626 conducted in Mega4 (Tamura et al., 2007).

627 **Figure 3. Hierarchical clustering based on gene expression patterns of**
628 **phosphoglyceride biosynthetic genes.** Columns represent the normalised expression
629 values for each of the experimental conditions (Tissue, intestine or liver; developmental
630 stage; fry, ~2.5 g or parr, ~10 g fish) and rows represent single genes in Atlantic salmon.
631 The cluster dendogram was constructed based on the distances between two genes
632 across the conditions using Pearson's correlation method in R (package "gplots", Warnes
633 et al., 2014). Colour bars to the right indicate the location of gene clusters with significant
634 developmental meaning.

635 **Figure 4. Expression of phosphoglyceride biosynthetic genes in major lipid**
636 **metabolic tissues of Atlantic salmon fry depicted in heatmap as Cluster 1.** Gene
637 expression was expressed as relative units (RU) calculated from the mean normalised
638 ratios (n = 6, ±SE) between the estimated copy numbers of target genes and the
639 estimated copy numbers of the reference genes. Columns represent the normalised
640 expression values for each of the experimental conditions (tissue, intestine or liver;
641 developmental stage; fry, ~2.5 g or parr, ~10 g). Bars bearing different letters are
642 significantly different (One-way ANOVA; $P < 0.05$).

643 **Figure 5. Expression of phosphoglyceride biosynthetic genes in major lipid**
644 **metabolic tissues of Atlantic salmon fry depicted in heatmap as Clusters 2 and 7.**
645 Gene expression was expressed as relative units (RU) calculated from the mean
646 normalised ratios (n = 6, ±SE) between the estimated copy numbers of target genes and

647 the estimated copy numbers of the reference genes. Columns represent the normalised
648 expression values for each of the experimental conditions (tissue, intestine or liver;
649 developmental stage; fry, ~2.5 g or parr, ~10 g). Bars bearing different letters are
650 significantly different (One-way ANOVA; $P < 0.05$).

651 **Figure 6. Expression of phosphoglyceride biosynthetic genes in major lipid**
652 **metabolic tissues of Atlantic salmon fry depicted in heatmap as Clusters 3, 4 and 8.**
653 Panels A. and B. include gene clusters "4" and "5" from Figure 3. Gene expression was
654 expressed as relative units (RU) calculated from the mean normalised ratios ($n = 6$, ±SE)
655 between the estimated copy numbers of target genes and the estimated copy numbers of
656 the reference genes. Columns represent the normalised expression values for each of the
657 experimental conditions (tissue, intestine or liver; developmental stage; fry, ~2.5 g or parr,
658 ~10 g). Bars bearing different letters are significantly different (One-way ANOVA; $P <$
659 0.05).

660 **Figure 7. Expression of phosphoglyceride biosynthetic genes in major lipid**
661 **metabolic tissues of Atlantic salmon fry depicted in heatmap as Cluster 5 and 6.**
662 Panels A. and B. include gene clusters "4" and "5" from Figure 3. Gene expression was
663 expressed as relative units (RU) calculated from the mean normalised ratios ($n = 6$, ±SE)
664 between the estimated copy numbers of target genes and the estimated copy numbers of
665 the reference genes. Columns represent the normalised expression values for each of the
666 experimental conditions (tissue, intestine or liver; developmental stage; fry, ~2.5 g or parr,
667 ~10 g). Bars bearing different letters are significantly different (One-way ANOVA; $P <$
668 0.05).

669 **Figure 8. Expression of transcription factors in major lipid metabolic tissues of**
670 **Atlantic salmon fry.** Gene expression was expressed as relative units (RU) calculated
671 from the mean normalised ratios ($n=6$, ±SE) between the estimated copy numbers of
672 target genes and the estimated copy numbers of the reference genes. Columns represent
673 the normalised expression values for each of the experimental conditions (tissue, intestine
674 or liver; developmental stage; fry, ~2.5g or parr, ~10g). Bars bearing different letters are
675 significantly different (One-way ANOVA; $P < 0.05$).

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871

872

873 **Table 1. Details of primer pairs used for the qPCR analysis.**

874

Transcript	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon size	Accession no.
PL biosynthetic genes				
cdipt	GGAACGAGCTCTTCTTCTGC	AGAACGCTGATGGCAGACTT	125 bp	GE776220
cept1	GCAGGCCAGACGAACCAATA	TGAACATGCCAGCGAAACAGC	161 bp	NM_001139869
cds1	ACCTGCTCGCTCAGTTCCAG	CTGGACAGCGAAGGGAAATG	131 bp	GBRB01044576.1
cds2	CCTGTTCCAGCTGCAGGACT	GGCAAAGAAGCCTCCGAAAGG	149 bp	DW572582
chkα	GACACGGGTGAGCTGAGCAT	TGTGGGACTCCCTGGTGAAG	169 bp	DY706802
chpt1	GTACGCCTTCCCCATCTTGG	TGAGGCCGATGTGCATACCT	149 bp	GBRB01045139.1
crls1	GCTCCAGTCCTGGGAGTCCT	CAGCCAATGGGTCAAGTGCG	158 bp	NM_001173887
eki1	GCCTTCGACATAGGCAACCA	AGCCCCACACTGTGCTTGTA	134 bp	NM_001141805
lpin1	GTCAACGAGCGAGGAACCAT	TTGGGGTGGAACAGTTGCTTG	152 bp	DW543824
lpin2	CTCCCTCAGCTCGGGAAAGA	CCGGTCGTTCACCCAGTACA	153 bp	DY703144
ppap2a	GGTCTTTGGAGAGTGCCTTTC	ATGTCAGTGAGCGATTGGCTC	141 bp	BT044703, BT045534
ppap2b	AGTGTCATGGGCCTGAGAGC	CTGGACTGCAGGTAGAACACC	112 bp	EG847528
ppap2c	GCTGGCTGCAGTCACCATCT	CTGCCCCGAACAGGAAGGTA	152 bp	NM_001140716
papdc1a	TCCAGCCAGAGGAGATGTGG	TCAACGTCACAGCCAGAGAGG	177 bp	NM_001146657
papdc1b	TGCGTGGCTTACTGCCAGAG	GAGAGGTGGGGACATGGTCA	156 bp	NM_001139847
papdc2	GAACCCCTCCACCCACTCTC	GCCGCATACAATCCTCTTCGG	150 bp	CB516677
pcy1	GTGGGCGTGTGTAGTGACGA	TGTCGTCATGGGCAACAAAGTC	184 bp	BT045986, BT045054
pcy2	CCAGTTTGCCTCAGGACAGG	AGTGCAGTCCCACAATGACG	149 bp	NM_001173569
peam3	CTGGGCCTGGACCTGTCA	AACGAGGCCTCTGGGAACTC	125 bp	NM_001173879
pemt	GAAGGACCTTTCACCGAGGA	TGGTGTGTGGCCTGGTAGTC	141 bp	NM_001141302
pgs1	CAACACATCGCCGAATCAGA	CCTGCATCAGTCAGCAAACG	149 bp	NM_001173895
pisd	CGGAAACTCAAACCGGCTATC	GTGGTCCCAGGAAGGTCTCC	154 bp	NM_001173606
pss	CTGGATGCATGCTCTGCACT	CGGACAATCACAGACAGGACA	146 bp	NM_001146675
ptdsse1	TGGATGTTTGGGGCCATAGC	ACCGCACCATCCCATACAGA	151 bp	GBRB01071318.1
ptdsse2	CATATACGACCCGGGGAACA	TGCTCCAGACTGTACTCCAGGAA	174 bp	GBRB01018987.1
ptpmt1	GCACTCAGTCACCGGGAAAA	GGTCGGACAGAGGCCAACAT	149 bp	BT048109
sgms1	CAGTGGCCACACAGTCATGC	AGCCACCACCACGTCTATGG	163 bp	DW546744
lpcat2	TTCCTCAAGCTGCCCATCAG	AGCTGTGCGGAGGACCTCTT	150 bp	NM_001173913
plcc	TGCACTGCAGGAGCACTACG	GCTGACAAACACCCCACAGG	151 bp	NM_001140138, BT059719
plcd	GGGAACACGCTTCACAGAGG	CCCCAGGGTTCCATACCTGT	153 bp	NM_001141264
plcf	GCAGTAACCGGGACCTGGAC	AGGAAGCCGTAGCGGATCAC	229 bp	BT045376
plchb	CCAGAGGGAGGGTGGCTCTA	GCGCTGAGTCACCTGGTCAT	154 bp	NM_001141489.2
gpam	GGAGGACCAGGAGGAGCTGA	CGGGGGATAGGCTCACTTTG	155 bp	DY720751.1
sas1	ATGACATCGGAGCCGGAGAT	CACTCCCCATTGGCAGACAG	136 bp	BT058661.1
sas2	ACCCAGTGGACGCTTTGTCA	CAGCAGAGCGGTCCACCTTA	149 bp	BT059080.1
Transcription factors				
srebp1	GCCATGCGCAGGTTGTTTCTTCA	TCTGGCCAGGACGCATCTCACAC T	151 bp	NM_001195818.1
srebp2	CACCTACCCTTACCCCTGCTGACA	GATGGTGGTGCCCCCGCTGAG	147 bp	NM_001195819.1
pparα	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	111 bp	DQ294237
pparβ1a	GAGACGGTCAGGGAGCTCAC	CCAGCAACCCGTCCTTGTT	151 bp	NM_001123635.1
pparγ	TGCTGCAGGCTGAGTTTATG	CAGGGGAAAGTGTCTGTGGT	107 bp	NM_001123546.1
lxrα	GCCGCCGCTATCTGAAATCTG	CAATCCGGCAACCAATCTGTAGG	209 bp	NM_001145421.1
rxrβ	GTGGAGTGGGCCAAGAGGAT	AGCTCATTGGCCAGGAGGAC	152 bp	BT043993.1
Housekeeping genes				
actβ	ACATCAAGGAGAAGCTGTGC	GACAACGGAACCTCTCGTTA	141 bp	NM_001123525.1
ef1α	CTGCCCCTCCAGGACGTTTACAA	CACCGGGCATAGCCGATTCC	175 bp	BT072490.1
cfl2	AGCCTATGACCAACCCACTG	TGTTCACAGCTCGTTTACCG	224 bp	BT125570.1
polr2f	CCAATACATGACCAAATATGAAAG G	ATGATGATGGGGATCTTCCTGC	156 bp	BT057259.1
β2m	TCCCAGACGCCAAGCAG	TGTAGGTCTTCAGATTCTTCAGG	138 bp	BT046451.2
rpl1	ACTATGGCTGTCGAGAAGGTGCT	TGTACTCGAACAGTCGTGGGTCA	120 bp	NM_001140826.1
rpl2	TAACGCCTGCCTCTTCACGTTGA	ATGAGGGACCTTGTAGCCAGCAA	110 bp	BT049591.1
rpl3	GGCAAGAAGCAGCTGGAGAA	TTACGCAGACCACGATGGGT	326 bp	NM_001001590

GenBank [http://www.ncbi.nlm.nih.gov/].

Figure 1.

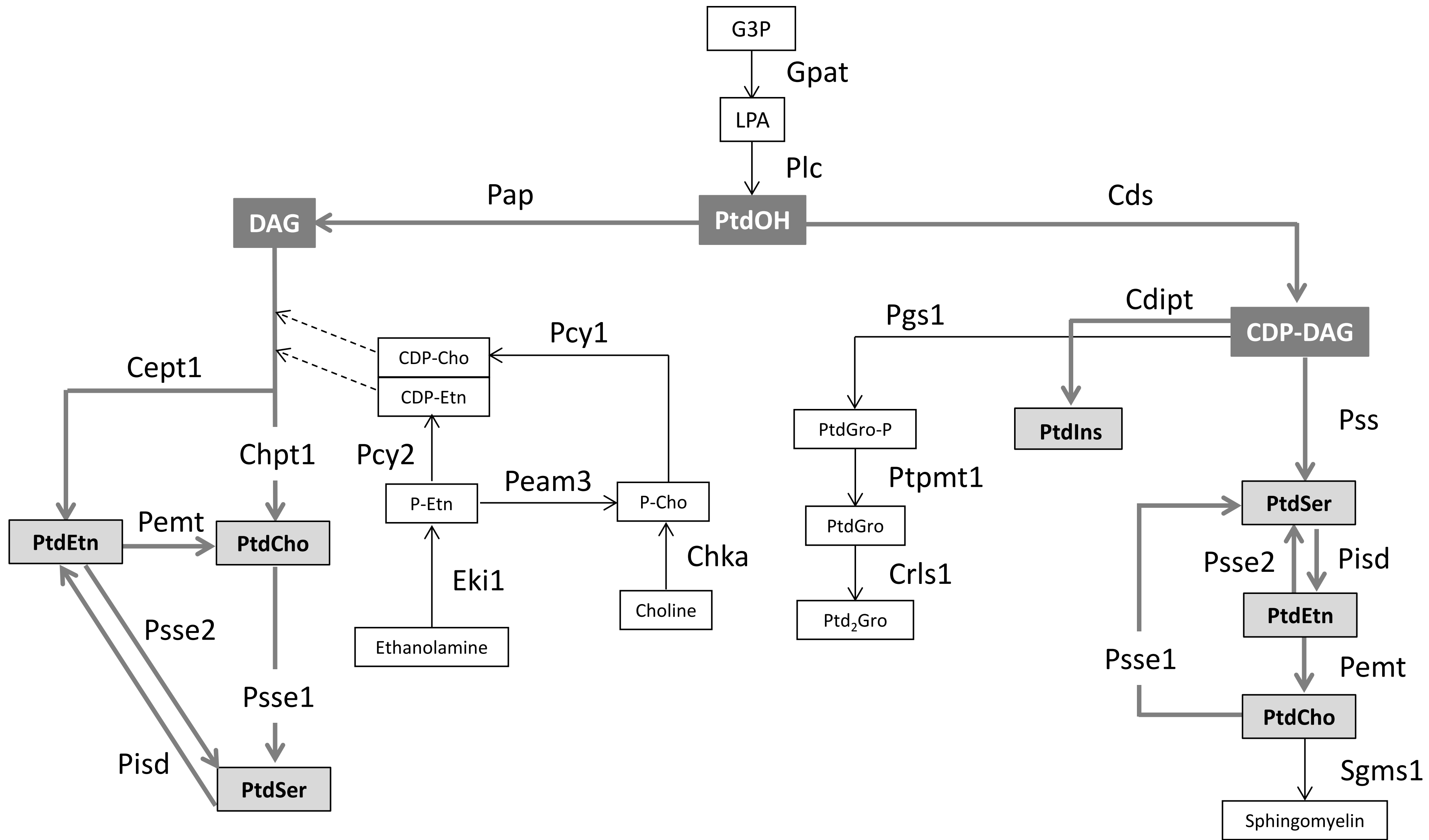
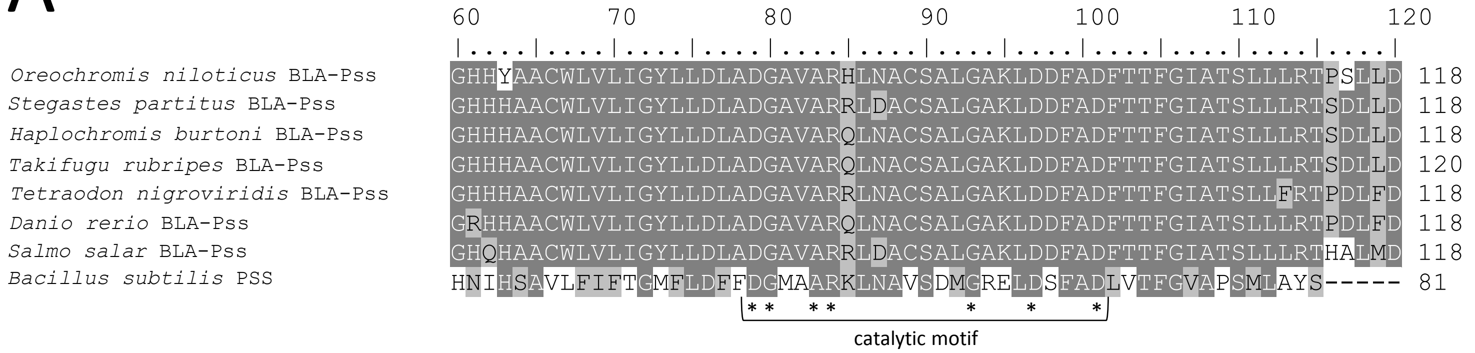


Figure 2.



B

55 *Astyanax mexicanus* [XM_00724574]
35 *Danio rerio ptdsse1b* [XM_682780]
9 *Danio rerio ptdsse1a* [NM_200956.1]
Bos taurus [NM_001046040.1]
3
99 *Homo sapiens* [NM_014754.2]
99 *Gorilla gorilla* [XM_004047332.1]
15 *Salmo salar* [Ssa.74508_1]
Takifugu rubripes [XM_003968946.1]
85
36 *Stegastes partitus* [XM_003968946.1]
35 *Poecilia formosa* [XM_007547731.1]
Oryzias latipes [XM_004078091.1]
53 *Oreochromis niloticus* [XM_003454445.2]
98 *Haplochromis burtoni* [XM_005934786.1]
49 *Maylandia zebra* [XM_004541456.1]

Psse1

Salmo salar [Ssa.7743]
Astyanax mexicanus [XM_007235048]
100
29 *Homo sapiens* [NM_030783.1]
64 *Takifugu rubripesgi* [XM_007546769.1]
20 *Danio rerio* [XM_001337076.5]
43 *Oryzias latipes* [XM_004069883.1]
Stegastes partitus [XM_008283994.1]
12
96 *Xiphophorus maculatus* [XM_005809263.1]
24 *Poecilia formosa* [XM_007546769.1]
25 *Oreochromis niloticus* [XM_003443840.1]
97 *Haplochromis burtoni* [XM_005927855.1]
81 *Maylandia zebra* [XM_004563846.1]

Psse2

Bacillus subtilis [CP008698]
100
Salmo salar [NM_001146675]
100 *Danio rerio [XM_005162261]*
50 *Stegastes partitus [XM_008293258]*
73 *Haplochromis burtoni [XM_005918887]*
45 *Oreochromis niloticus [XM_003438832]*
45 *Takifugu rubripes [XM_003977798]*
97 *Tetraodon nigroviridis [CR689878]*

BLA-Pss

0.1

Figure 3.

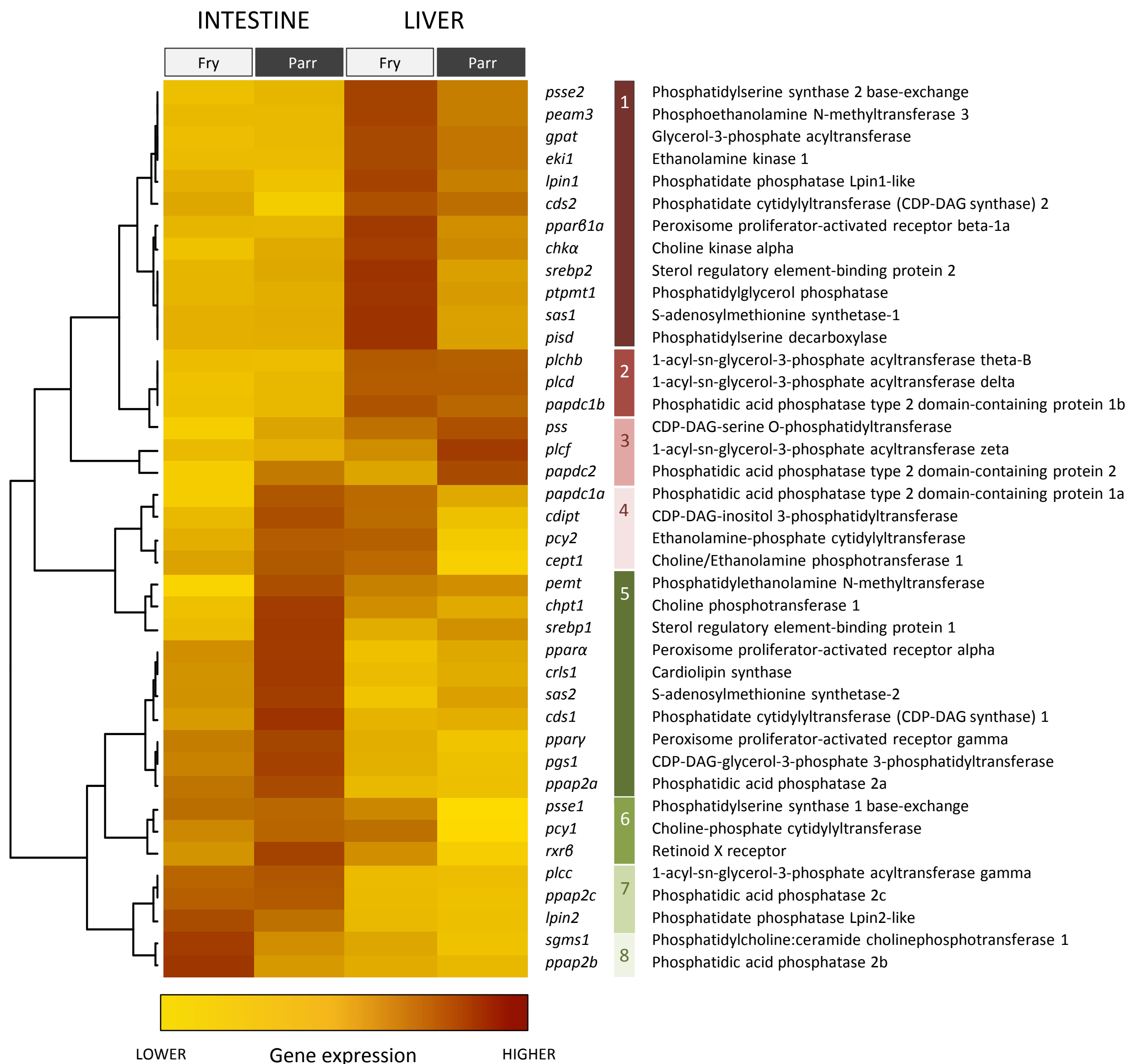
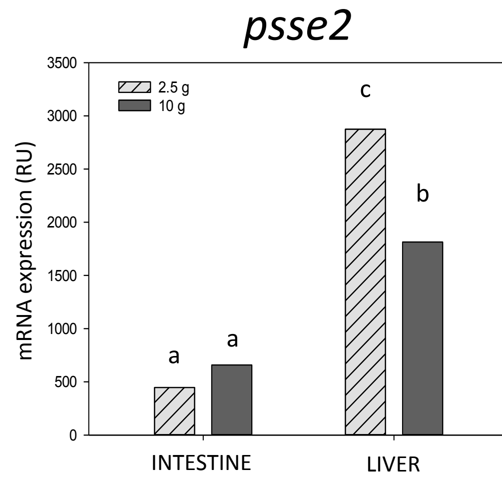
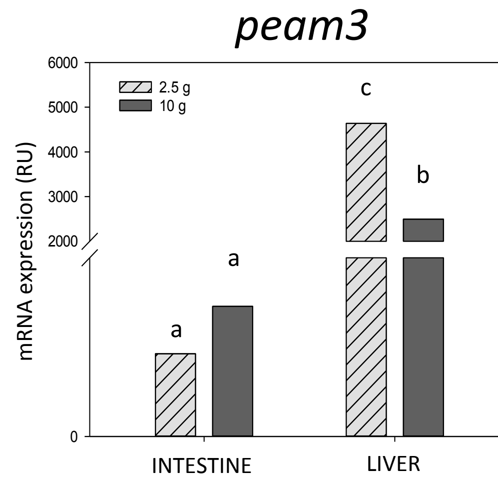
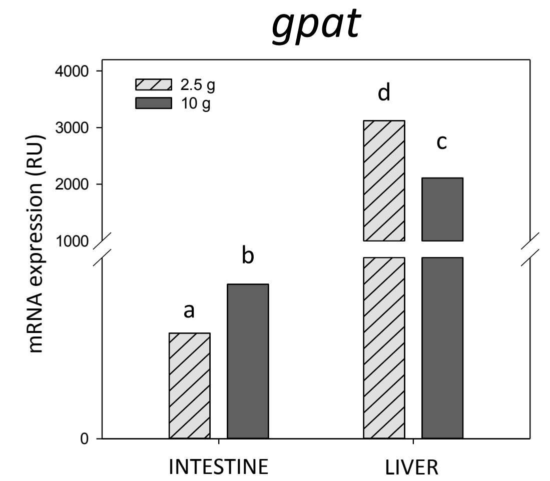
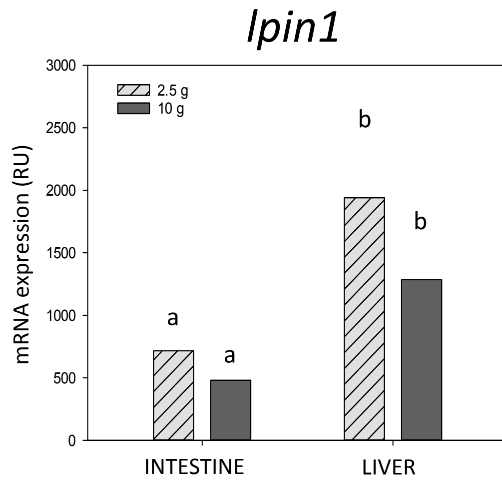


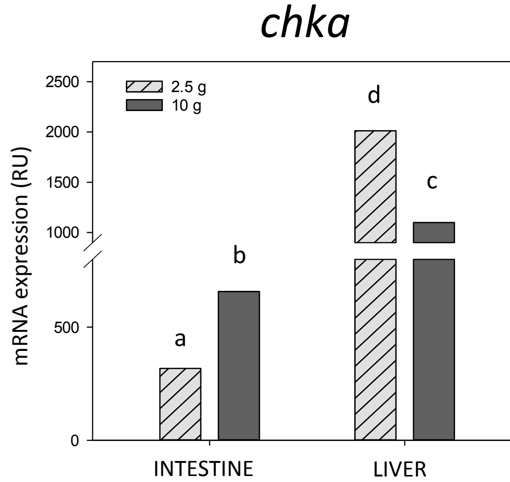
Figure 4. Cluster 1

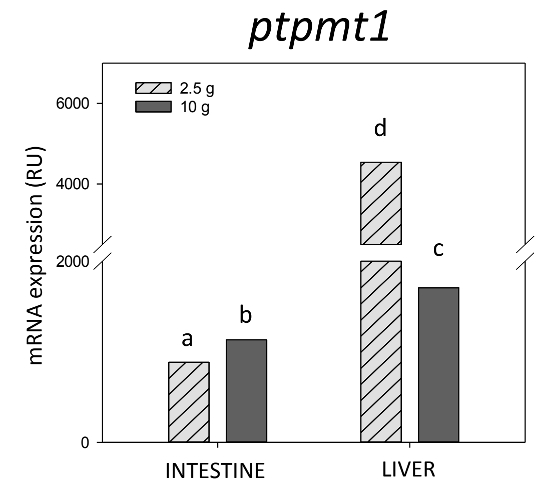


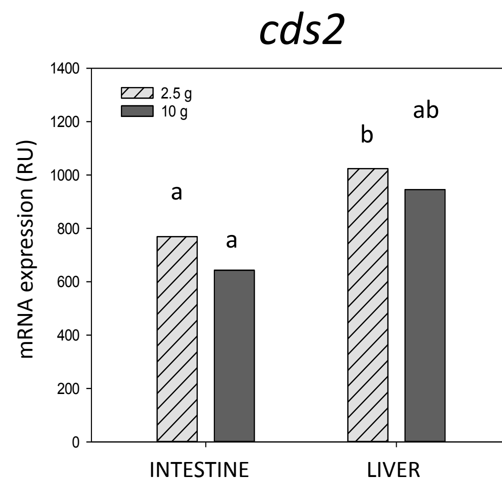


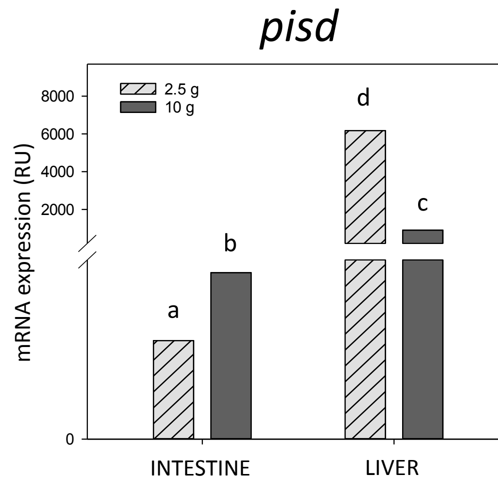












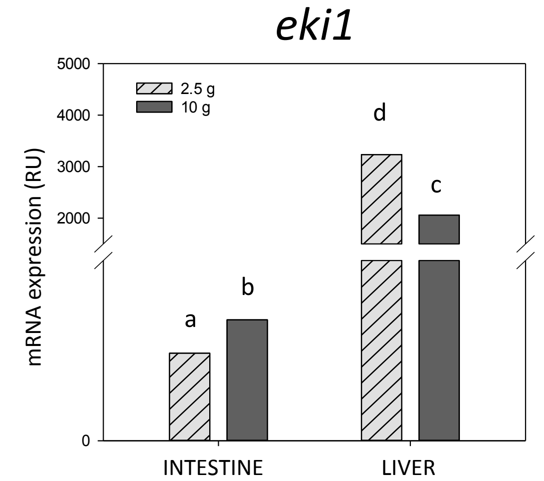
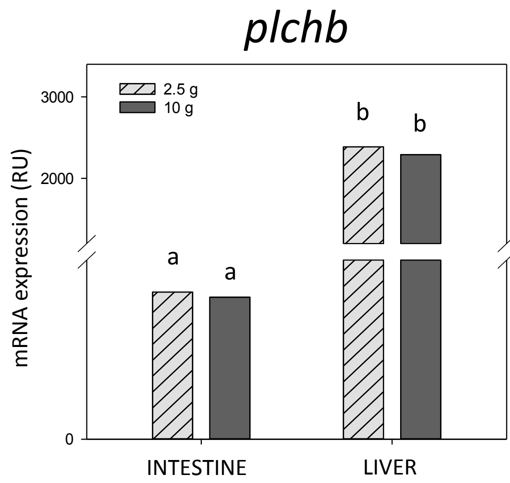
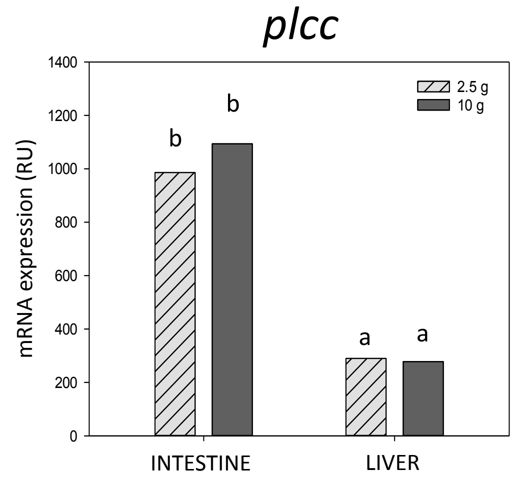
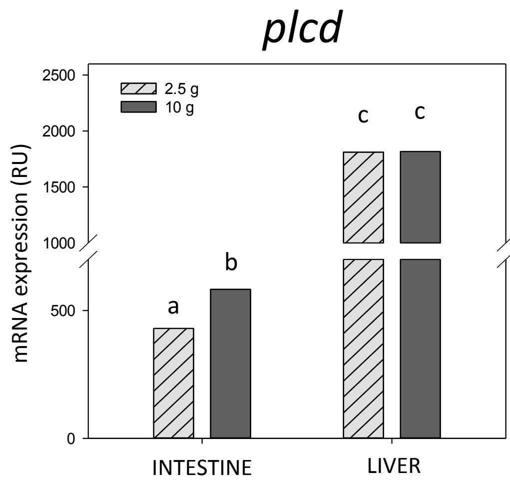
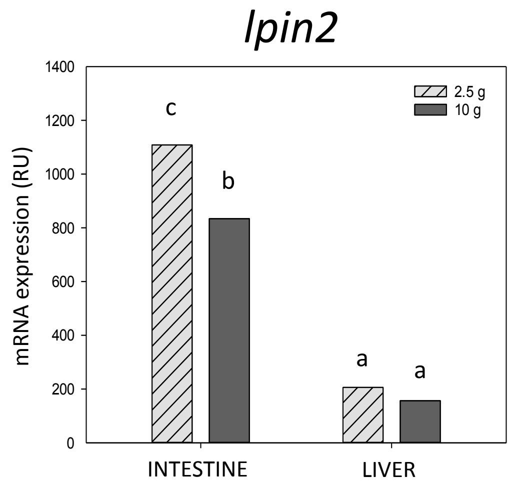


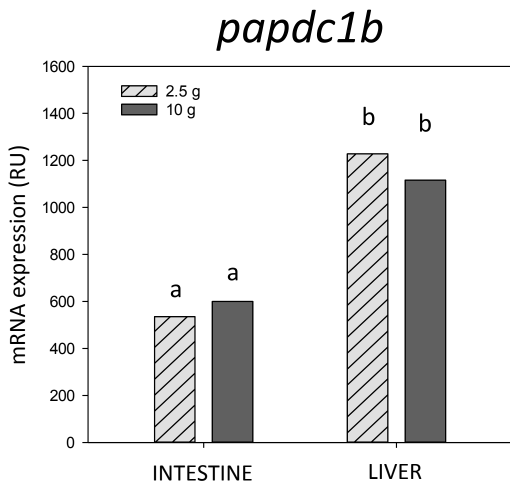
Figure 5. Cluster 2,7











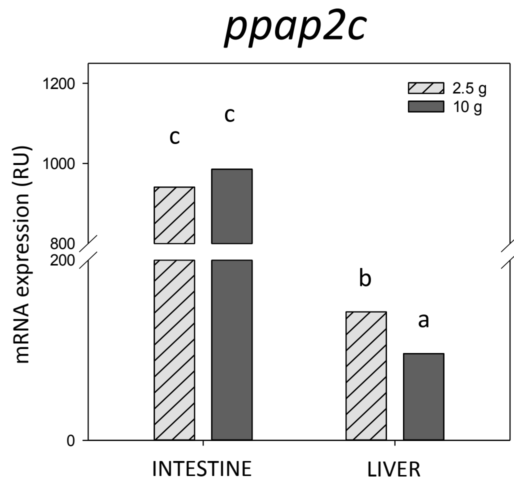


Figure 6. Cluster 3,4, 8

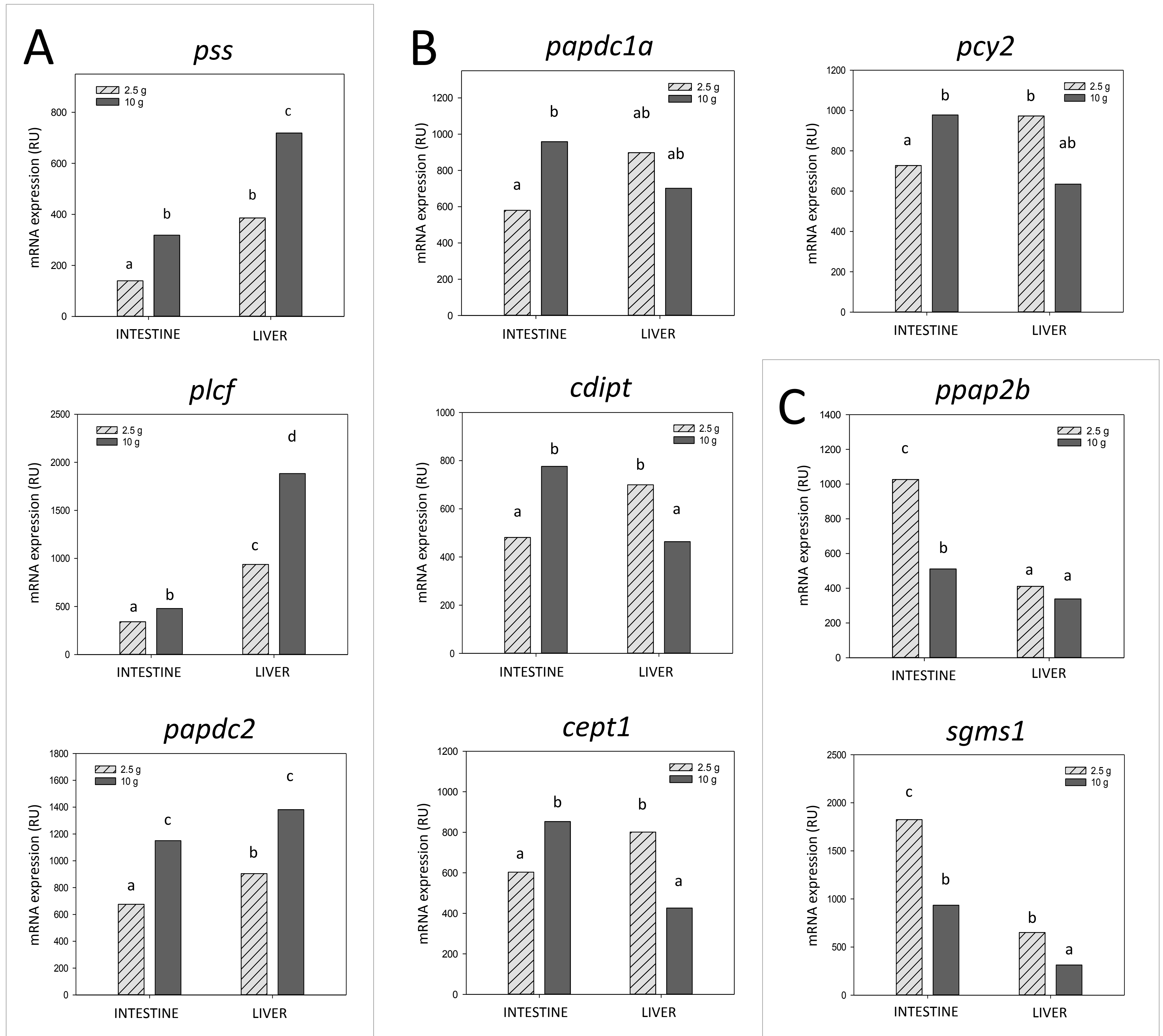


Figure 7. Cluster 5, 6

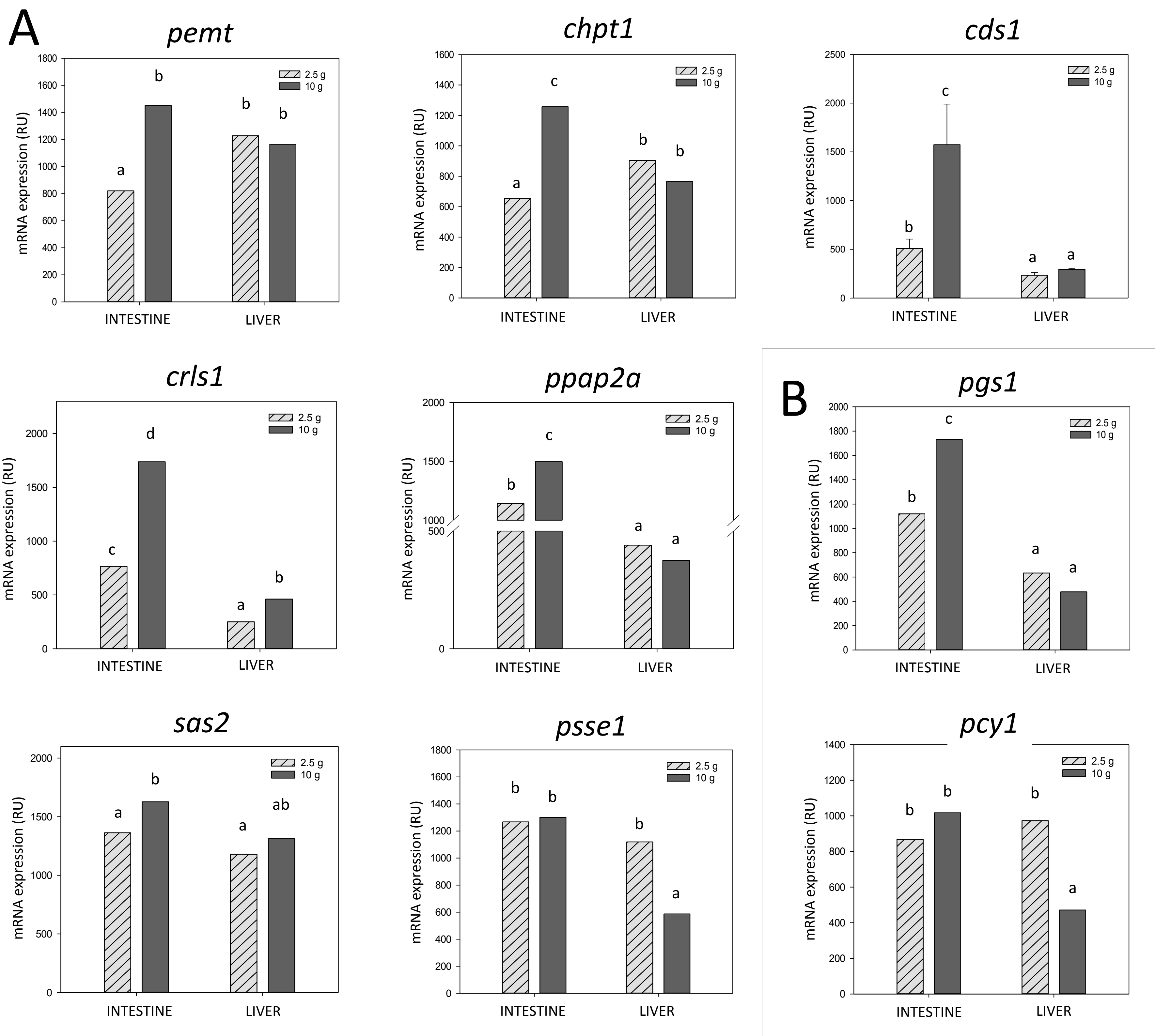
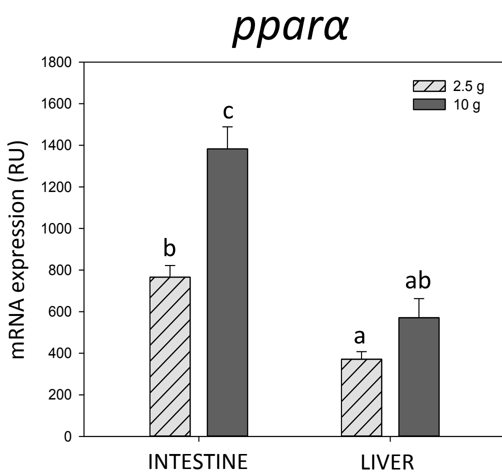
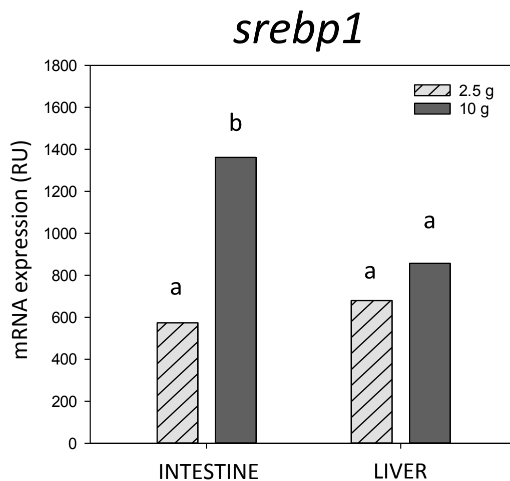
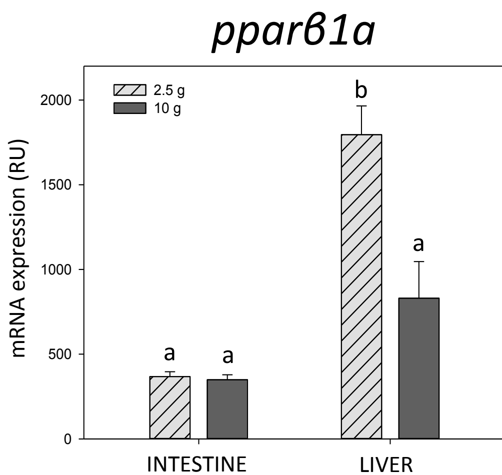
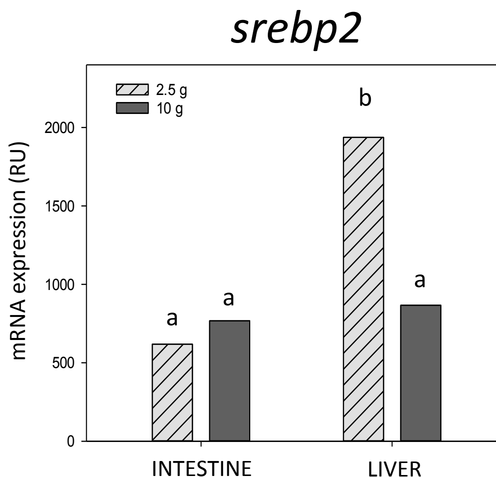


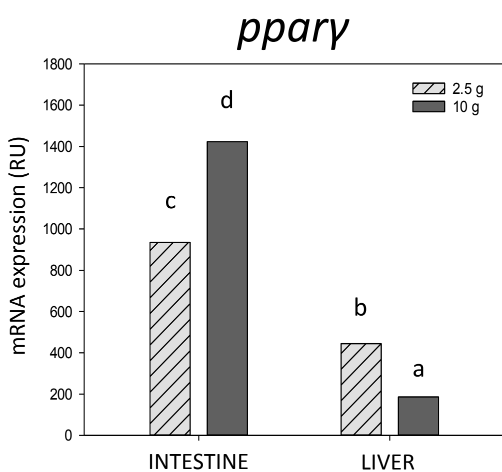
Figure 8. TF

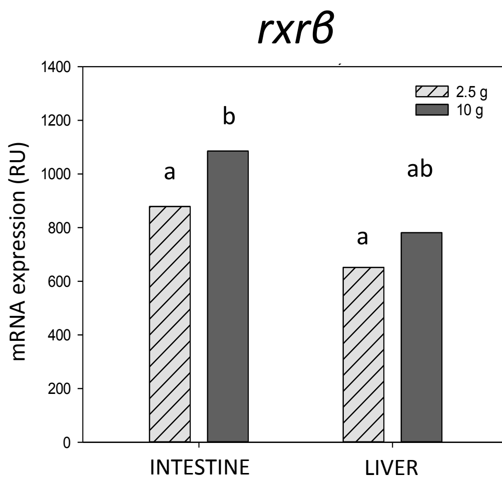












875 **Supplementary Table 1. Details of reference genes used for qPCR.** Expression stability was assessed according to BestKeeper
876 (Pfaffl *et al.*, 2004) calculated on corrected Ct values. ¥, genes used to normalise expression in liver; *, genes used to normalise
877 expression in intestine.

Data of candidate reference genes (n=12)

		Intestine				Liver			
		GeoMean	Ct Range	SD	SD	GeoMean	Ct Range	SD	SD
Genes	*Efficiency*	*[Ct]*	*[Min, Max]*	*[± Ct]*	*[± corrected Ct]*	*[Ct]*	*[Min, Max]*	*[± Ct]*	*[± corrected Ct]*
β actin	1.94	22.28	[21.6, 23.2]	0.467	1.241	25.78	[25.1, 28.3]	0.636	0.115
cofilinβ[¥]	2.00	21.43	[20.8, 22.1]	0.346	0.840	25.56	[24.8, 26.7]	0.431	0.059
ef1a	2.00	20.36	[20.1, 20.8]	0.203	0.953	21.67	[20.8, 22.8]	0.634	1.177
polr2f[*¥]	1.82	29.10	[28.6, 29.5]	0.223	0.038	30.69	[29.6, 26.5]	0.612	0.040
b2m	1.78	22.19	[21.4, 22.7]	0.403	7.176	25.18	[24.5, 26.5]	0.434	1.127
rpl1[*¥]	1.98	27.20	[26.5, 27.8]	0.258	0.018	28.22	[27.3, 29.7]	0.697	0.020
*rpl2**	2.00	32.05	[31.1, 33.0]	0.409	0.075	33.94	[32.1, 26.4]	1.285	0.066
rpl3	2.00	22.13	[21.8, 22.8]	0.267	0.402	23.61	[22.6, 24.9]	0.747	0.405

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