Transcriptional development of phospholipid and lipoprotein metabolism in different intestinal regions of Atlantic salmon (*Salmo salar*) fry

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Abstract

Background: It has been suggested that the high phospholipid (PL) requirement in Atlantic salmon (*Salmo salar*) fry is due to insufficient intestinal *de-novo* synthesis causing low lipoprotein (LP) production and reduced transport capacity of dietary lipids. However, there has not been performed any in-depth ontological analysis of intestinal PL and LP synthesis with development of salmon. Therefore in this paper we used RNA-seq technology to test the hypothesis that the high PL requirement in salmon fry was associated with undeveloped PL synthesis and LP formation pathways in intestine. There was a special focus on the understanding homologous genes, especially from salmonid-specific fourth vertebrate whole-genome duplication (Ss4R), contribution to salmonid specific features of regulation of PL metabolic pathways. The study was performed in stomach, pyloric caeca and hindgut at 0.16g (1 day before first-feeding), 2.5g and 10g of salmon.

Results: In general, we found an up-regulation of *de-novo* phosphatidylcholine (PtdCho) synthesis, phosphatidylethanolamine (PtdEtn) and LP formation pathways in pyloric caeca of salmon between 0.16g and 10g. Thirteen genes in these pathways were highly (q<0.05) up-regulated in 2.5g salmon compared to 0.16g, while only five more significant (q<0.05) genes were found when the fish grew up to 10g. Different homologous genes were found dominating in stomach, pyloric caeca and hindgut. However, the expression of dominating genes in PL and LP synthesis pathways was much higher in pyloric caeca than stomach and hindgut. Salmon-specific homologous (Ss4R) genes had similar expression during development, while other homologs had more diverged expression.

Conclusions: An increasing capacity for PL synthesis and LP formation was confirmed in pyloric caeca. The up-regulation of the *de-novo* PtdCho pathway confirms that the salmon fry have increasing

64 requirement for dietary PtdCho compared to adult. The similar expressions between Ss4R
65 homologous genes suggest that the functional divergence of these genes was incomplete compared to
66 homologs derived from other whole genome duplication. The results of the present study have
67 provided new information on the molecular mechanisms of phospholipid synthesis and lipoprotein
68 formation in fish.
69
70
71 **Keywords:** Atlantic salmon; biosynthesis; gene expression; homologous genes; intestinal regions;
72 lipoprotein; phospholipids; RNA-seq; whole genome duplication
73

74 **Background**

75 Phospholipids (PL) are the main constituent of all biological cell membranes and separate the
76 intracellular and extracellular aqueous environment. In addition to providing a structural scaffold for
77 cell membranes, PL are also involved in numerous biological functions, such as provision of
78 metabolic energy, cell membrane transport, and regulation of metabolism [1]. PL are also key
79 structural components of lipoproteins (LP), which are involved in transport of dietary lipids from
80 intestinal enterocytes to liver and peripheral tissues [2, 3]. It is well known that dietary inclusions of
81 PL can improve growth and performance in many fish species including Atlantic salmon *Salmo salar*
82 [4], Atlantic cod *Gadus morhua* [5] and rainbow trout *Oncorhynchus mykiss* [6]. Dietary and biliary
83 PL is mainly digested by intestinal phospholipase A_2 in fish resulting in 1-acyl lyso-phospholipids
84 (lyso-PL) and free fatty acids (FFA). Subsequently, both lyso-PL and FFA are absorbed into intestinal

85 enterocytes, re-esterified into PL before being exported to the rest of the body [7]. In addition, PL can
86 be synthesized *de-novo* in enterocytes from glycerol-3-phosphate (G-3-P) in fish [8].
87 A minimum requirement of PL is associated with early developmental stages of fish, but so far no
88 minimum requirement has been demonstrated in adult fish [8]. In line with this, dietary PL has been
89 shown to enhance growth and survival in Atlantic salmon fry up to 2.5 g, but not in larger fish [4, 9].
90 It has been suggested that the higher PL requirement is due to insufficient ability of *de-novo* synthesis
91 in the intestine leading to low LP production and consequently reduced transport capacity of dietary
92 lipids [8]. This was supported by previous histological studies in salmonids, showing lipid
93 accumulation in intestinal enterocytes when fed PL deficient diet [9, 10]. However, these differences
94 were not evident in a previous transcriptomic study where gene expressions in PL biosyntheiss
95 pathways were not changed in 2.5g salmon fed by PL-supplemented diet [11]. No study has
96 demonstrated the gene expression in salmon smaller than 2.5g.
97 The intestinal tract of salmon consists of several regions, with different functions in lipid digestion,
98 absorption and transport. It is generally believed that pyloric caeca (PC), rather than stomach (SM) or
99 hindgut (HG), is the predominant region for lipid absorption and transport in salmon [12, 13].
100 Therefore, PL and LP were assumed to be mostly synthesized in PC region. However, other tissues
101 like SM and HG could also have ability of synthesizing PL due to its structural roles in cell
102 membranes [1]. LP has occasionally been observed in hindgut, suggesting some lipid absorption and
103 transport activities in the region [14]. So far no study has demonstrated the PL metabolic pathways in
104 SM and HG of fish.

105 Many homologous genes in mammals were found belong to gene families controlling the same
106 enzymatic process but have distinct regulation in different tissues and developmental stages [1]. In
107 this respect the Atlantic salmon has another layer of functional genome complexity as it experienced
108 two extra rounds of whole genome duplication (WGD) compared to mammals, at the base of all
109 teleost (Ts3R) and in a common ancestor of all salmonids ~100-80 Mya (Ss4R) [15, 16]. Of the Ts3R
110 and Ss4R gene duplicates, ~20 and 55%, respectively, are still retained as expressed genes in the
111 genome [17]. This dramatic increase in the number of homologous genes in salmon thus necessitates a
112 careful annotation of PL synthesis and LP formation pathway genes and their tissue-specific
113 expression regulation to improve our understanding of salmon PL metabolism.
114 In this paper we annotate and characterize gene regulation involved in PL synthesis and LP formation
115 in different intestinal regions (SM, PC and HG) during early developmental stages of salmon. Our
116 aims are to (I) improve our understanding of the homologous genes, especially from Ss4R,
117 contribution to salmonid specific features of regulation of PL metabolism pathways and to (II)
118 specifically test the hypotheses that PL requirements in early-developmental stages of Atlantic salmon
119 is associated with insufficient PL synthesis and LP formation pathways.
120

121 ## Methods

122 ### Fish, diet and sampling procedure

123 Atlantic salmon eggs were hatched and cultivated at AquaGen Breeding Centre (Kyrksæterøra,
124 Norway). From first-feeding, the fish were fed a normal commercial diet which satisfies the
125 nutritional requirement of salmon, but without any additional PL supplement (Additional file 1). The

126 diet was produced by EWOS AS (Bergen, Norway). Three salmon individuals (n=3) were sampled at
127 sizes of 0.16g (1 day before first feeding), 2.5g (65 days after first feeding) and 10g (100 days after
128 first feeding). The fish were euthanized by 1g/L MS-222 (FINQUEL, Argent chemical labs,
129 Washington, USA) buffered with same amount of sodium bicarbonate before dissection. Samples of
130 SM, PC and HG were dissected and immediately placed in 1mL RNALater. Tissues were stored for
131 24h at 4℃ for sufficient penetration of RNALater before being transferred to -80℃ for long-term
132 storage.
133
134 **RNA extraction, library preparation and transcriptome sequencing**
135 The RNA extraction and library preparation were carried out in Centre for Integrative Genetics
136 (CIGENE, Ås, Norway). Total RNA was extracted from SM, PC and HG using RNeasy Plus
137 Universal Kits (QIAGEN, Hilden, Germany), according to manufacturer's instruction. RNA
138 concentration and purity were assessed by Nanodrop 8000 (Thermo Scientific, Wilmington, USA).
139 RNA integrity was checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA,
140 USA). All samples have RIN value >8, which were sufficient for transcriptome analysis. RNA
141 libraries were prepared by using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA,
142 USA), according to manufacturer's instruction. Samples were sequenced using 100bp single-end
143 high-throughput mRNA sequencing (RNA-seq) on Illumina Hiseq 2500 (Illumina, San Diego, CA,
144 USA) in Norwegian Sequencing Centre (Oslo, Norway).
145
146 **Identification of genes and phylogenetic analysis**

147 The genes involved in PL synthesis and LP formation in Atlantic salmon (*Salmo salar*) were manually
148 annotated by matching salmon proteins to zebrafish (*Danio rerio*) orthologs from the KEGG reference
149 pathway of glycerolphospholipid metabolism and other studies [18-20]. Ortholog group predictions
150 were carried out using Orthofinder (v0.2.8) on proteins from seven fish species: zebrafish (*Danio*
151 *rerio*), stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), Northern Pike (*Esox lucius*),
152 grayling (*Thymallus thymallus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*),
153 and two mammal outgroup species: human (*Homo sapiens*) and mouse (*Mus musculus*). The protein
154 sequences within orthogroups were aligned to each other using MAFTT and maximum likelihood
155 trees were estimated using FastTree. Orthogroup trees were subsequently split into smaller clan trees
156 using an in-house R script (clanfinder.R, available from github). For zebrafish proteins in selected
157 KEGG reference pathways, salmon proteins within the same protein clan tree were annotated using
158 the zebrafish KEGG Orthology terms. The detailed information on Orthogroup prediction and
159 phylogenetic analysis was published elsewhere [19]. All annotated salmon genes was grouped into a
160 PL gene list and used for gene expression analysis.
161
162 **RNA-sequencing data and statistical analysis**
163 Read sequences were quality trimmed, removing any Illumina TruSeq adapter sequence and low
164 quality bases (Phred score < 20) from read ends and length filtered (minimum length 40 bases) using
165 cutadapt (v1.8.1), before being aligned to the salmon genome (ICSASG_v2) using STAR (v2.5.2a).
166 Raw gene counts per sample were generated from read alignments using HTSeq-count (v0.6.1p1) and
167 the NCBI salmon genome annotation (available for download at

http://salmobase.org/Downloads/Salmo_salar-annotation.gff3). The uniquely mapped reads, aligned to exon regions, were counted for each gene in the annotation.

For each tissue type (SM, PC, HG), a differential expression analysis (DEA) was performed comparing 2.5g and 10g samples to the 0.16g samples. Genes were filtered prior to DEA testing by a minimum count level of at least 1 count per million (CPM) in two or more samples, to remove genes with too few counts for testing. From raw counts, DEA was conducted using R package edgeR (v3.8.6) with pairwise exact tests to produce gene fold changes and *p* values. Genes with a false discovery rate (FDR) adjusted *p* value (q) < 0.05 were considered to be differentially expressed between two test conditions. The PL gene list was applied to retrieve DEA results for genes involved in PL and LP synthesis.

For comparing expression levels between different genes and tissues, normalized counts in the form of transcripts per million (TPM) values were generated. Raw gene counts were first divided by their mRNA length in kilobases to normalize for transcript length, then divided by the total number of counts from each library to normalize for sequencing depth.

All RNA-seq data analysis was preformed using R (v3.2.4) and Bioconductor (v3.3). The pathway maps of PL and LP synthesis were produced using PathVisio. The heatmap was drawn using R with package pheatmap. All other figures were produced using SigmaPlot for Windows Version 13.0.

Results

Annotation of PL pathway orthologs in salmon

In order to get comprehensive knowledge on the expression of genes involved in PL and LP synthesis in salmon, we created a PL gene list of all salmon genes in the pathways based on their zebrafish paralogs identified in previous studies. A total of 62 zebrafish genes involved in PL *de-novo* synthesis, lyso-PL synthesis, PL turnover and LP synthesis pathways were used to identify PL metabolism genes in salmon. In total, 125 corresponding salmon genes were identified and named based on their phylogenetic relationship to human and zebrafish homologs. Due to the Ss4R WGD, 67% of the PL genes contain a salmon-specific duplicate in the genome. Homologous genes controlling same enzymatic reaction were grouped into a family for comparison of gene expression. A summary of identification and nomenclature of PL genes is shown in Additional file 2.

Tissue specific regulation of PL metabolism in the gut

The raw sequencing data are publicly available on European Nucleotide Archive under accession number PRJEB21981. An average total of 22 million reads were collected from each library, out of which ~85% were mapped to the salmon genome ICSASG_v2 (Additional file 3). From a total of 81574 genes currently annotated, 31411 genes passed a minimum level of read counts for use in DEA. DEA was carried out on SM, PC and HG separately to assess the extent of developmentally associated changes in PL metabolism by comparing 2.5g and 10g salmon to 0.16g. The different intestinal regions differed greatly in the numbers of differentially expressed genes (DEGs, q<0.05), with 10% DEGs in SM, and around 30% DEGs in PC and HG (Additional file 4).

Relative expression of genes involved in PL synthesis and LP formation show categorization into three distinct tissue related clusters associated to developmental differences (Fig. 1). The genes in

209 cluster 2 are characterized by having highest expression in PC, while the remaining genes were either
210 highest expressed in SM (Cluster 1) or HG (Cluster 3). Only a few genes describe a reduced
211 expression following development of fish, whereas most genes show onset of expression, especially in
212 cluster 2. The DEGs in SM, PC and HG of 2.5g and 10g compared to 0.16g salmon were also
213 annotated in Figure 1. Similar to the genome-wide changes in expression, PC and HG were much
214 more responsive (40% DEGs) compared to SM (less than 20% DEGs). Most DEGs were shared
215 between PC and HG in Cluster 2, while DEGs in other clusters were much fewer. Moreover, the
216 shared DEGs showed a much larger change in PC than in HG during development, resulting in
217 increasing difference of expression between PC and HG as salmon growth.
218
219 **Regulatory divergence of homologous genes among developmental stages and intestinal sections**
220 **among Ss4R duplicates**
221 The relative expression (TPM value) of all genes in the PL and LP synthesis pathways are
222 summarized in Additional file 5. The salmon-specific (Ss4R) homologous genes showed mostly a
223 similar divergence among developmental stages and intestinal sections. Moreover, the highest
224 expression among the homologous genes was mostly found in PC rather than in SM or HG, which has
225 supported that PC is the most important intestinal section for PL synthesis and LP formation in
226 salmon.
227 *Pcyt1* family is a representative example to demonstrate the regulatory complexity of homologous
228 genes among intestinal sections and developmental stages (Figure 2). The *pcyt1* family has 2 members
229 in mammals (*pcyt1a* and *pcyt1b*), 4 members in zebrafish (*pcyt1aa*, *pcyt1ab*, *pcyt1ba* and *pcyt1bb*)

and 7 members in salmon (*pcyt1aa*, *pcyt1ab_1*, *pcyt1ab_2*, *pcyt1ba_1*, *pcyt1ba_2*, *pcyt1bb_1* and *pcyt1bb_2*). Among all homologous genes, *pcyt1bb_1* had much higher expression levels in PC than in SM and HG. It was also the highest expressed homolog in all tissues. The expression level of *pcyt1bb_1* in PC and HG both more than doubled as the fish grew from 0.16g to 10g. The Ss4R homologous genes, *pcyt1bb_1* and *pcyt1bb_2*, showed similar divergence between tissues and developmental stages. The expressions of *pcyt1ab_1* and *pcyt1ba_1* genes were both higher in SM than PC and HG, while *pcyt1aa*, *pcyt1ab_2* and *pcyt1ba_2* were similarly expressed between the three tissues.

Regulation of PL synthesis and LP formation pathways in PC

It clear from our expression analyses (Figure 1) that PC is the most active tissue, measured as expression level, with regards to PL and LP metabolic pathways. Therefore, PC was selected for a detailed study of the differences in expression regulation of homologous genes between 0.16g, 2.5g and 10g salmon (Figure 3). The homologous genes in eighteen families of key genes in PtdCho, PtdEtn and LP synthesis pathways were thus selected for in depth analyses.

In most gene families, one or two genes had much higher expression than their homologs, with all being up-regulated during development (Figure 3). In the eleven families with two highly expressed genes, they are mostly salmon-specific duplicates from Ss4R WGD. In *de-novo* pathways, highly expressed genes were mostly significantly (q<0.05) up-regulated from 0.16g to 10g (Figure 3 A). The highly expressed genes involved in the PtdCho synthesis pathway (*chk*, *pcyt1* and *chpt* families) showed a much more pronounced increase compared to genes in other *de-novo* pathways. By

comparing TPM of the highly expressed genes, *lpin* and *plpp* had slightly higher expression compared to *cds* family. Genes in *ept* family had much lower expression than other families in the pathway. In lyso-PL synthesis pathways, the expression of *lpcat3a* was largely increased (q<0.05) during development, whereas other *lpcat* genes remained stable (q>0.05, Figure 3 B). *Lpgat1a* and *lpiat1* in other families in lyso-PL pathways were also up-regulated (q<0.05) during development (Additional file 6). All other genes in lyso-PL synthesis pathways were expressed much lower than *lpcat3a*. Genes in LP formation pathway had much higher expression than in PL synthesis pathways (Figure 3 C). All highly expressed genes in LP formation pathway were largely up-regulated (q<0.05) between 0.16g and 10g salmon.

The proposed regulation of PL synthesis and LP formation pathways is summarized in Figure 4. By summarizing the change of the highly expressed genes in PC, we found up-regulations of *de-novo* PtdCho and PtdEtn synthesis pathways and down-regulations of *de-novo* PtdSer and PtdGro synthesis pathways between 0.16g and 10g salmon (Figure 4 A). Other *de-novo* synthesis pathways were not changed during development. The Lyso-cardiolipin synthesis pathway was not changed, while other lyso-PL synthesis pathways were all up-regulated. The phospholipid turnover pathways were not changed during development. The LP formation pathway was up-regulated between 0.16g and 10g (Figure 4 B).

Discussions

The objective of the present study was to explore the transcriptional changes of PL synthesis and LP formation pathways in different intestinal regions of salmon during early developmental stages. By

integrating RNA-seq data with a manually curated automated sequence ortholog prediction, we identified many DEGs in SM, PC and HG during development of salmon. Most of those changes occurred between start feeding at 0.16g and 2.5g, and continued up to 10g. This shows that the maturation of lipid metabolic pathways progressed for a substantial period following the completion of yolk sac reabsorption. By comparing the highly expressed genes in each family, we found a continuous increased capacity of PtdCho, PtdEtn and LP synthesis in PC from 0.16g to 10g. This implies an increased capacity for PL and LP synthesis after onset of start feeding.

Our results are in line with previous qPCR-based single gene studies in salmon where several major PL biosynthetic genes were up-regulated in PtdCho and PtdEtn synthesis between 2.5g and 10g [18]. However, we did not observe a clear levelling-off of expression at 10g, suggesting that the completion of intestinal maturation might take longer time to accomplish. This supports the hypotheses of a higher dietary PL requirement in salmon fry compared to larger fish previously put forward [10].

The present study was the first to investigate expression differences between homologous genes in PL and LP synthesis pathways in salmon. Several homologous genes were identified controlling the same enzymatic step in PL and LP synthesis. In most cases, we found that one or two genes had much higher expression levels than their homologs for a given tissue, suggesting these genes to be key regulators in the pathways. However, the expression levels of the homologous genes appear to vary with both intestinal regions and developmental stages. This resulted in different genes dominating in SM, PC and HG. The diverged expressions are likely due to different functions of homologous genes in the tissues, subcellular location and developmental stages [1]. More interestingly, the salmon-specific homologous genes derived from Ss4R WGD expressed similarly in different tissues

293 and developmental stages, while other homologous genes seemed to have more diverged function.
294 This may suggest that the functional divergence of Ss4R homologous genes was incomplete compared
295 to homologs from other WGDs. This supports the recent study in which 55% of the Ss4R homologous
296 genes were found to have similar expression levels among 15 tissues in salmon [17].
297 The present study was the first to utilize RNAseq to investigate roles of different intestinal regions in
298 PL metabolism. PC is the predominant region for lipid absorption and transport in salmon [12, 13].
299 This is consistent with higher expression of genes involved in PtdCho, PtdEtn and LP synthesis
300 pathways in PC rather than SM and HG. As PtdCho is the predominant lipid class forming the
301 membrane fraction of LP, the higher expression of PtdCho synthesis pathway in PC verifies a high
302 rate of LP production [3, 8]. This has been confirmed by histological observation of large lipid
303 droplets accumulating in midgut enterocytes of PL-deficient fish, while little droplets were found in
304 fish fed dietary PtdCho [9, 10]. The expression of genes involved in PL synthesis in SM and HG were
305 most likely related to other biological function such as cell maintenance and metabolism. Almost no
306 expression of LP formation genes were found in SM, agrees with the general observation that SM is
307 not involved in lipid digestion, uptake and transport. However, despite being expressed at low levels,
308 many genes of the LP formation pathways were found in HG, suggesting some capacity to absorb and
309 transport lipid in this intestinal region [14].
310 Several highly expressed genes in PL and LP synthesis pathways of salmon do not conform to the
311 known function and regulation of these pathways in mammals. For example, it is believed that choline
312 kinase (CK) α enzyme is critical for PtdCho maintenance in most tissues in mammals, whereas CKß
313 enzyme is only essential for muscle tissue [21, 22]. In salmon, the expression of the *chkb* gene,

314 encoding for CKß, was significantly elevated in PC after start feeding, whereas *chka* genes were
315 unchanged. Similarly, the expressions of *pcyt1a* genes were relatively low in PC during early
316 development, while *pcyt1bb_1* notably increased in PC after start feeding. Therefore, we assume that
317 the production of PtdCho for LP synthesis is probably through a compensatory pathway controlled by
318 *chkb* and *pcty1b* genes and activated in PC after switching to external feeding. On the other hand,
319 there may be another pathway controlled by *chka* and *pcyt1a* genes, which produce PtdCho to
320 maintain cell growth and survival. This suggestion agrees with previous studies pointing to the
321 subcellular location of the enzymes [23, 24]. The CTP: phosphocholine cytidylyltransferase (CCT) α,
322 which is the product of the *pcyt1a* gene, is predominantly located in the nucleus. On the other hand,
323 *pcyt1b*-encoded CCTß is localized in the endoplasmic reticulum (ER) and the cytosol, which could be
324 utilized in synthesizing PtdCho for LP formation. However, as the level of gene expression does not
325 always directly reflect relative importance of two similar enzymes in a pathway, the posttranslational
326 modification like phosphorylation of CCT could also be critical in regulating the activity of enzymes
327 without affecting the mRNA level [1].
328
329 **Conclusions**
330 The present study has provided new information on the molecular mechanisms of PL synthesis and
331 LP formation in salmon fry. By comparing the expression levels of homologous genes, we identified
332 several genes which had highly expression among their homologs in PtdCho, PtdEtn and LP synthetic
333 pathways in PC of salmon. Those highly expressed genes were all up-regulated during development,
334 confirming the increasing capacity for PL synthesis and LP formation. Taken the lowered activity of

PL genes in early life of salmon and the link to PL requirement, it seems likely that the highest requirement is during early first feeding, then reducing as the fish grows. The expression levels of the homologous genes in PL synthesis and LP formation pathways appeared to vary with both intestinal regions and developmental stages. This resulted in different genes dominating in SM, PC and HG. The salmon-specific homologous genes derived from Ss4R WGD expressed similarly among different tissues and developmental stages, while homologous genes from other WGD seemed to have more diverged function. More studies on both gene and protein levels are required to confirm these relationships during early stages of salmon development. Considering the present results on identification of key regulating genes in PL synthesis and lipid transport, we suggest a future study on the dietary requirement of PL at first-feeding stage, which focuses on the changes of key regulating genes involved in PL and LP synthesis pathways in PC of salmon.

List of abbreviations

agpat, 1-acylglycerol-3-phosphate acyltransferases**; cd36,** cluster of differentiation 36**; CDP,** cytidine diphosphate**; cdipt,** cdp-diacylglycerol-inositol 3-phosphatidyltransferase**; CDP-DAG,** CDP-diacylglycerol**; CDP-Cho,** CDP-choline**; CDP-Etn,** CDP-ethanolamine**; cds,** CDP-DAG synthase**; cept,** CDP-choline/ethanolamine: diacylglycerol phosphotransferase**; CH,** cholesterol**; chk,** choline kinase**; Cho-P,** phosphocholine**; chpt,** CDP-choline: diacylglycerol phosphotransferase**; crls,** cardiolipin synthase**; etnk,** ethanolamine kinase**; ER,** endoplasmic reticulum**; ept,** CDP-ethanolamine**:** diacylglycerol phosphotransferase**; Etn-P,** phosphoethanolamine**; fabpl,** fatty acid binding protein, liver**; DAG,** diacylglycerol**; G-3-P,** glycerol-3-phosphate**; gpat,** glycerol-3-phosphate acyltransferase**;**

lclat, lysocardiolipin acyltransferase**; lpcat,** lysophosphatidylcholine acyltransferase**; mboat,** membrane bound o-acyltransferase**; mtp,** microsomal triglyceride transfer protein**; pcyt1,** choline-phosphate cytidylyltransferase**; pcyt2,** ethanolamine-phosphate cytidylyltransferase**; pemt,** phosphatidylethanolamine methyltransferase**; pgp,** phosphatidylglycero phosphatase**; pgs,** CDP-diacylglycerol: glycerol-3-phosphate phosphatidyltransferase**; pmt,** phosphoethanolamine n-methyltransferase**; plpp,** phosphatidate phosphatase**; pss,** CDP-diacylglycerol:serine phosphatidyltransferase**; ptdss,** phosphatidylserine synthase**; pisd,** phosphatidylserine decarboxylase**; PtdCho,** phosphatidylcholine**; PtdEtn,** phosphatidylethanolamine**; PtdGro,** phosphatidylglycerol**; PtdIns,** phosphatidylinositol**; PtdOH,** phosphatidic acid**; PtdSer,** phosphatidylserine**; sar1,** secretion associated, RAS related GTPase 1**; TAG,** triacylglycerol**; taz,** tafazzin.

Declarations

Ethics approval and consent to participate

The study was carried out within the Norwegian animal welfare act guidelines, in accordance with EU regulation (EC Directive 86/609/EEC) and approved by the Norwegian Animal Research Authority (NARA).

Consent for publication

Not applicable

Availability of data and materials

377 The raw gene counts data of the current study is available for download at

378 http://salmobase.org/Downloads/Salmo_salar-annotation.gff3

379 The orthogroup prediction and phylogenetic analysis of salmon genes are included in this published

380 article and its supplementary information files:

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382 TR, Vik JO et al: Diet And Life Stage Associated Remodeling Of Lipid Metabolism Regulation In

383 The Duplicated Atlantic Salmon Genome. bioRxiv 2017. https://doi.org/10.1101/140442

384

385 **Competing interests**

386 The authors declare that they have no competing interests.

387

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391

392 **Authors' contributions**

393 YJ, RO, NS and YO comprehended the idea and designed the experiments. YJ sampled fish, prepared

394 RNA-seq and analyzed RNA-seq data, and was a major contributor in writing the manuscript. MØ

395 assisted in fish sampling. SK assisted in fish rearing and maintenance. GG and SS assisted in

396 RNA-seq data analysis. Annotation of salmon genes including orthogroup prediction and

397 phylogenetic analysis were done by SS, AG, JV and JT. SS, RO and YO provided directions and

398 critiques. All authors read and approved the final manuscript.

399

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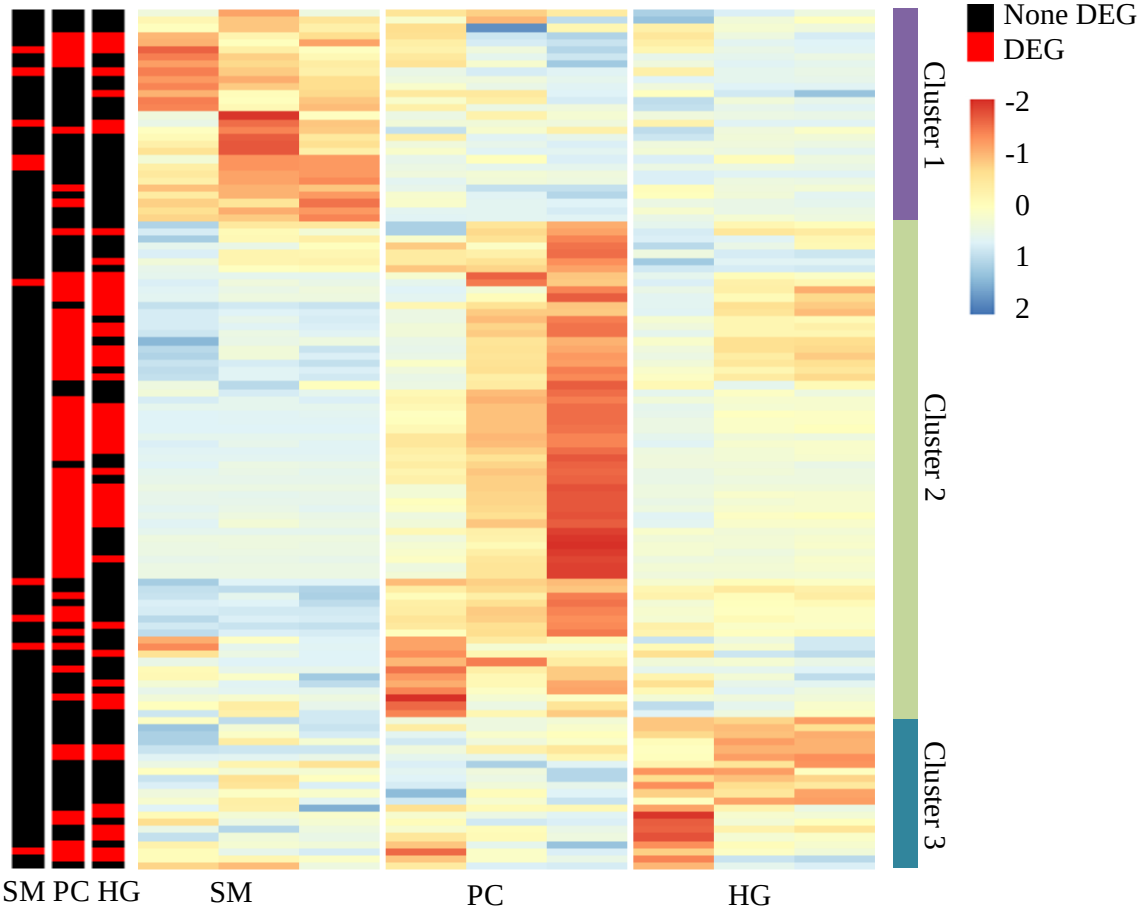
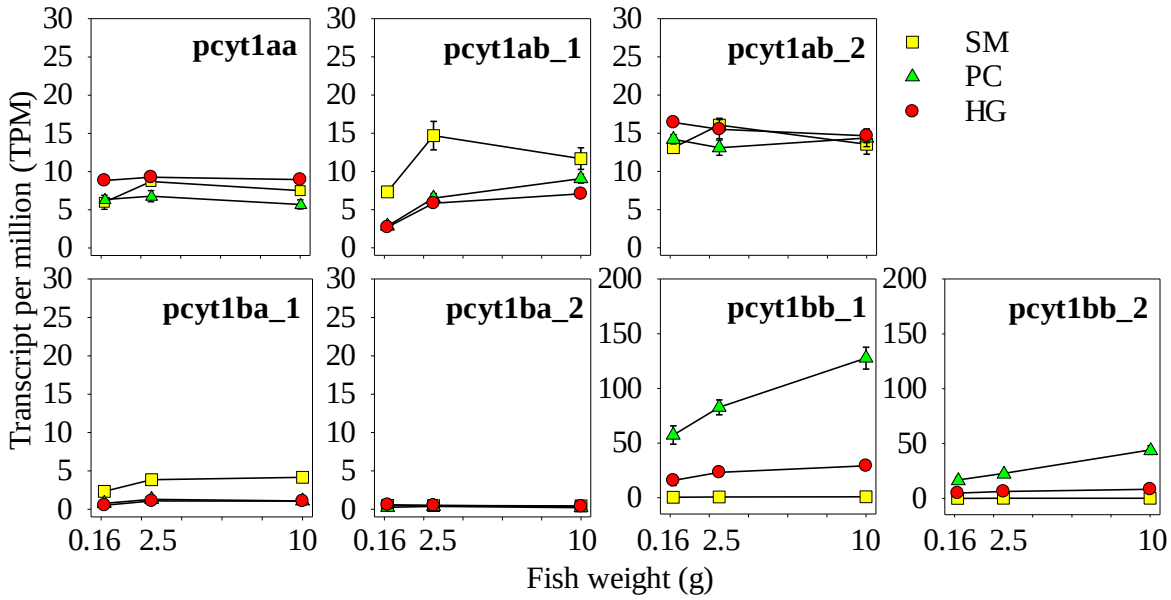


Figure 1 Expression of genes in phospholipid and lipoprotein synthsis pathways between different intestinal regions of salmon. For each tissue, the three columns represent 0.16g, 2.5g and 10g samples from left to right. The color intensity is relative to the standard deviation from mean of TPM over developmental stages and tissues (raw-scaled). Differential expressed genes (DEG, q<0.05) between 0.16g, 2.5g and 10g samples were annotated in three columns, which represent stomach (SM), pyloric caeca (PC) and hindgut (HG) respectively from left to right.



487

488 **Figure 2 Comparison of *pcyt1* genes in TPM over developmental stages and intestinal regions.**

489 The gene expressions were compared in transcript per million (TPM) over three developmental stages

490 (0.16g, 2.5g and 10g) in stomach (SM), pyloric caeca (PC) and hindgut (HG) of salmon. Numbers

491 after underline indicates Ss4R gene duplicates specific in salmonids.

492

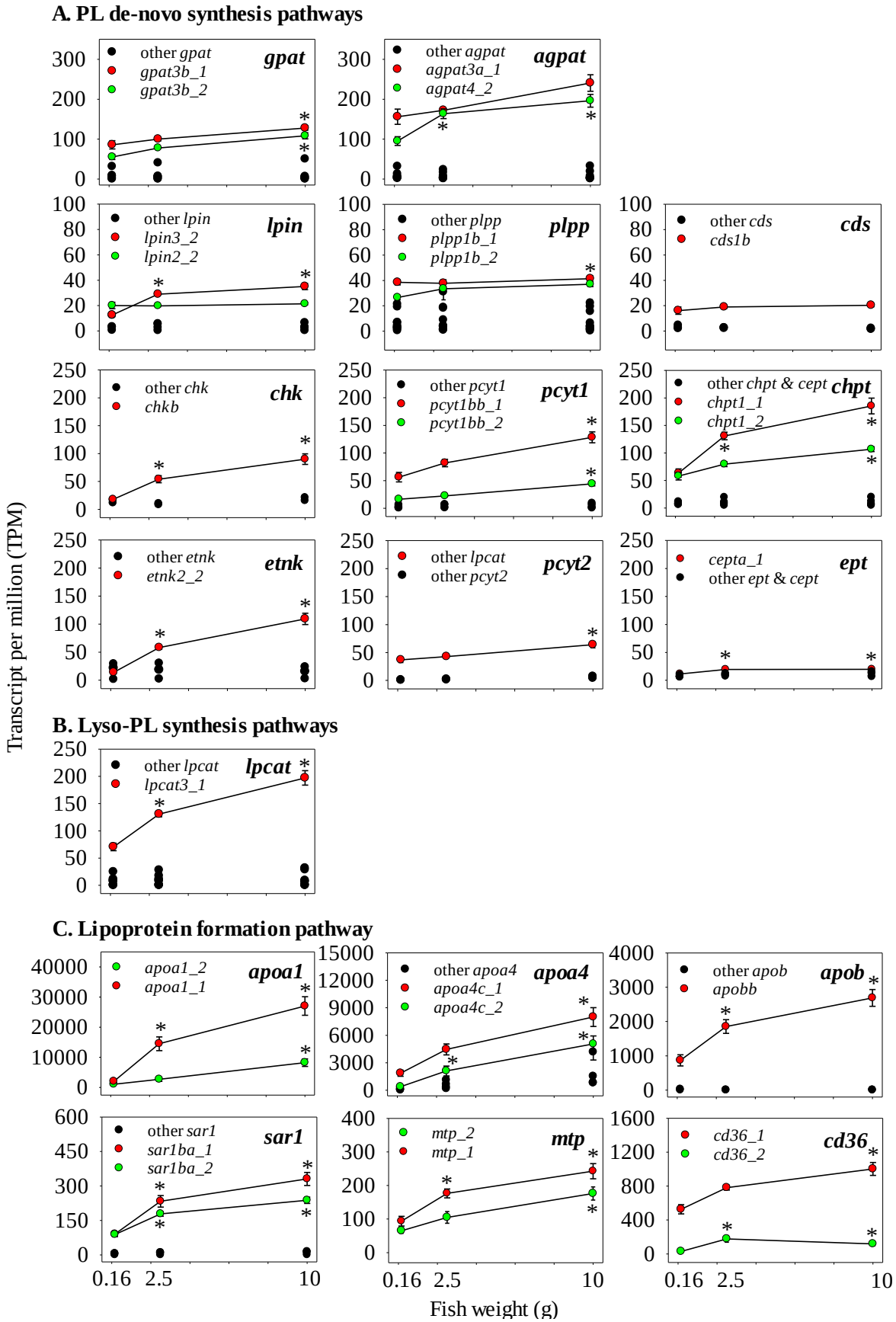


Figure 3 Expressions of key genes in phospholipid and lipoprotein synthesis pathways in pyloric

caeca at early stage of salmon. Eighteen families of homologous genes in phospholipid (PL) *de-novo* synthesis, lyso-phospholipid (lyso-PL) synthesis and lipoprotein formation pathways are shown for comparing their expression between 0.16g, 2.5g and 10g fish. Genes with high TPM are marked in red and green, while other genes were all marked in black.

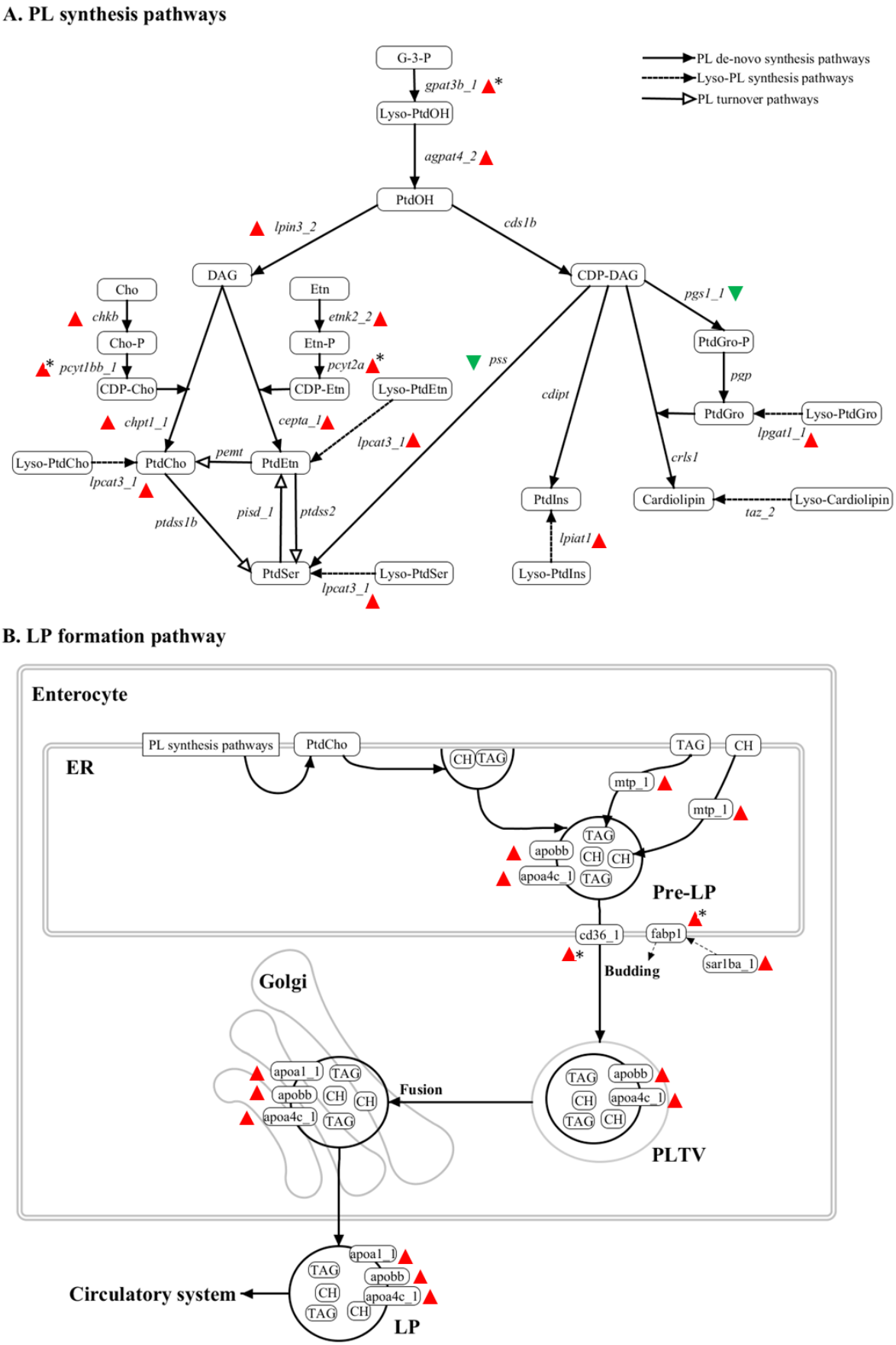


Figure 4 Comparison of phospholipid (PL) synthesis and lipoprotein (LP) formation pathways

between 0.16g, 2.5g and 10g salmon. Colored triangles indicate the significantly (q<0.05) up (red) or down (green) regulation of the highest expressed genes found in each enzymatic step of the pathways. Asterix indicates genes only significantly (q<0.05) changed between 0.16g and 10g. A**)** PL *de-novo* synthesis, lyso-PL synthesis and PL turnover pathways in fish. Glycerol-3-phosphate (G-3-P) is first acylated by acyltransferases to phosphatidic acid (PtdOH), which can be transferred into diacylglycerol (DAG) or CDP-diacylglycerol (CDP-DAG) by phosphatidate phosphatase (plpp and lpin) or CDP-DAG synthetase (cds). DAG is utilized with CDP-choline (CDP-Cho) and CDP-ethanolamine (CDP-Etn) for synthesizing of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). CDP-DAG is utilized for synthesizing of phosphatidylserine (PtdSer), phosphatidylglycerol (PtdGro), phosphatidylinositol (PtdIns) and Cardiolipin. **B)** LP formation pathway in enterocyte of fish. PtdCho is synthesized on the membrane of endoplasmic reticulum (ER) through de-novo synthesis, turnover or lyso-PL pathway before used for pre-lipoprotein (Pre-LP) formation. Pre-LP is a nascent lipoprotein assembled by PtdCho, triacylglycerol (TAG), cholesterol (CH), apolipoprotein B (apob) and apolipoprotein AIV (apoa4). Pre-LP is then targeted to the Golgi apparatus via pre-lipoprotein transport vesicle (PLTV) generated by ER. The maturation of Pre-LP happens in Golgi, where apolipoprotein AI (apoa1) is added before secreting into circulatory system.

Additional files

Additional file 1: Table 1 Composition and nutritional value of the diet used in current experiment. (DOCX 15.5 kb)

523 **Additional file 2: Table 1** List of Atlantic salmon (Ssa) genes involved in phospholipid (PL) *de-novo*
524 synthesis, lyso-PL synthesis and lipoprotein (LP) formation pathways. Nomenclature of salmon genes
525 was based on their human (Hsa) and zebrafish (Dre) paralogs. Numbers after underline in Ssa names
526 indicate salmon-specific gene duplicates. NCBI gene ID of salmon and zebrafish is also listed in table.
527 Reference listed the origin of zebrafish genes used for identification of salmon genes. (DOCX 31.5
528 kb)

529 **Additional file 3: Table 1** Summary of mapping statistics of all 45 samples used for RNA sequencing
530 (3 replicates * 3 tissue in 0.16g fish, 6 replicates * 3 tissue in 2.5g and 10g fish). (DOCX 16 kb)

531 **Additional file 4: Table 1** Number of significantly ($q<0.05$) differentially expressed genes (DEG)
532 between 2.5g, 10g and 0.16g salmon. (DOCX 16.1 kb)

533 **Additional file 5: Table 1** Transcript per million (TPM) of all gene duplicates in phospholipid and
534 lipoprotein synthesis pathways in stomach, pyloric caeca and Hindgut of 0.16g, 2.5g and 10g salmon.
535 (DOCX 32.8 kb)

536 **Additional file 6: Table 1** Log2 fold change (LogFC) and adjusted *p* value (*q*) of all gene duplicates
537 in phospholipid and lipoprotein synthesis pathways in stomach, pyloric caeca and hindgut of 2.5g and
538 10g salmon both compared to 0.16g. (DOCX 35 kb)