Carmona-Antoñanzas G, Taylor J, Martinez Rubio L & Tocher DR (2015) Molecular mechanism of dietary phospholipid requirement of Atlantic salmon, Salmo salar, fry, *Biochimica et Biophysica Acta (BBA)- Molecular and Cell Biology of Lipids*, 1851 (11), pp. 1428-1441.

DOI: 10.1016/j.bbalip.2015.08.006

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

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Keywords: Atlantic salmon; dietary requirement; gene expression; phosphoglycerides; phospholipids; qPCR

Running title: Dietary phospholipid requirement in Atlantic salmon fry.

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.

1. INTRODUCTION

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

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

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

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

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

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

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

2. MATERIALS & METHODS

2.1 Fish, diets and sampling protocols

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

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

2.2 Identification of phospholipid biosynthetic genes in Atlantic salmon

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

2.3 Sequence and phylogenetic analysis of PtdSer synthases

2.3.1 Sequence analysis

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

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

2.3.2 Multiple sequence alignment

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

2.3.3 Phylogenetic tree

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

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

2.4 Quantitative RT-PCR

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

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

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

2.4.1 Data analysis and statistical tests

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

3. RESULTS

3.1 Pathways of phospholipid biosynthesis in Atlantic salmon

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

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

3.2 Phylogenetic analysis of PtdSer synthase genes

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

3.3 Gene expression: Phosphoglyceride biosynthesis

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

3.3.1 Cluster 1

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

3.3.2 Clusters 2 and 7

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

3.3.3 Cluster 3 and cluster 8

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

3.3.4 Cluster 4

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

3.3.5 Cluster 5

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

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

3.3.6 Cluster 6

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

3.3.7 Gene expression of transcription factors

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

4. DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

5. CONCLUSIONS

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

ACKNOWLEDGEMENTS

This study, GCA, JFT and LMR were partly funded by the European Commission FP7 Integrated Project No. 288925, Advanced Research Initiatives for Nutrition & Aquaculture (ARRAINA).

FIGURE LEGENDS

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

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

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

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

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

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

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

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

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

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

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871

872

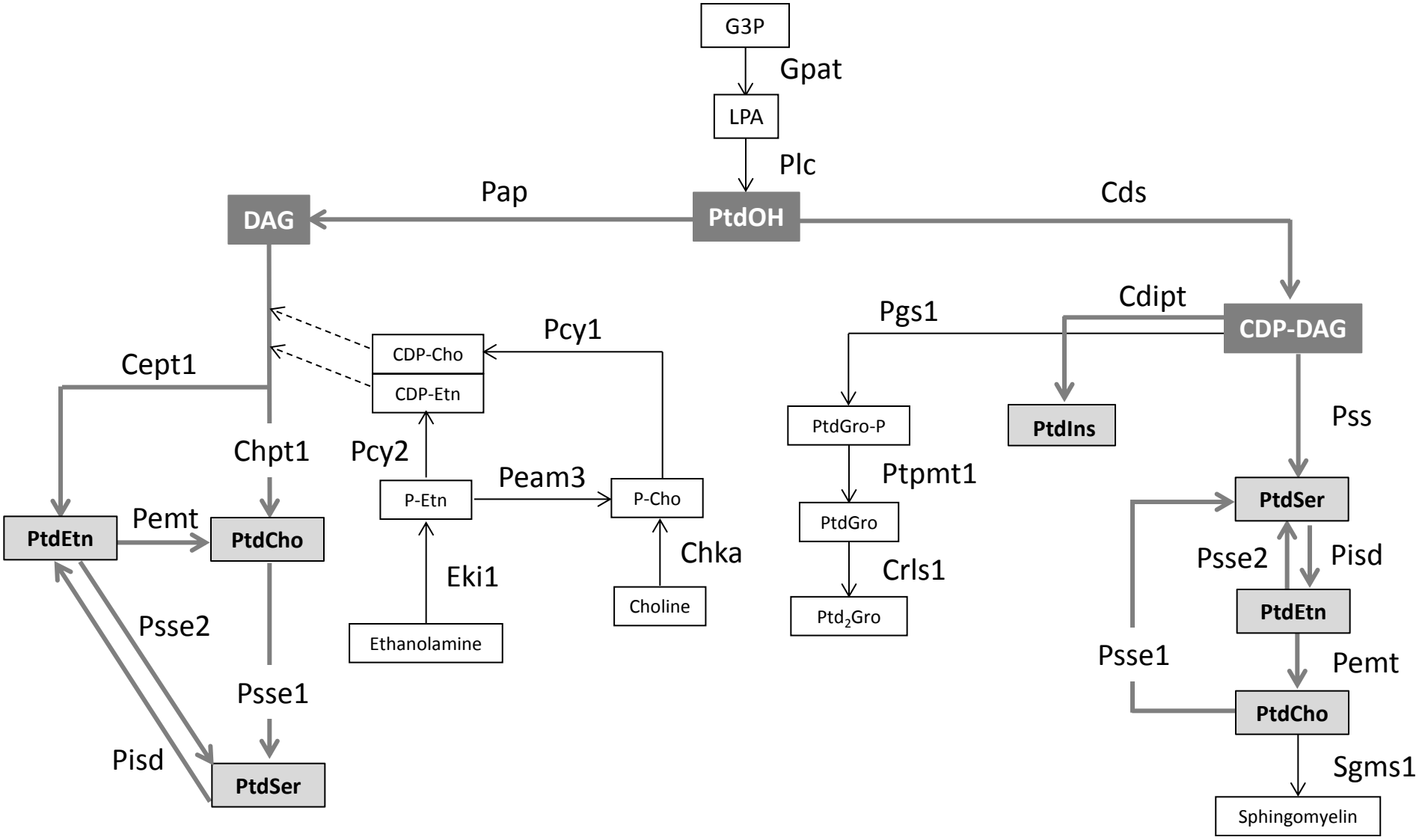
873 **Table 1. Details of primer pairs used for the qPCR analysis.**

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	TCTGGCCAGGACGCATCTCACACT	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				
act*β*	ACATCAAGGAGAAGCTGTGC	GACAACGGAACCTCTCGTTA	141 bp	NM_001123525.1
ef1α	CTGCCCCTCCAGGACGTTTACAA	CACCGGGCATAGCCGATTCC	175 bp	BT072490.1
cfl2	AGCCTATGACCAACCCACTG	TGTTCACAGCTCGTTTACCG	224 bp	BT125570.1
polr2f	CCAATACATGACCAAATATGAAAGG	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.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Peam3
P-Etn
P-Cho
Pgs1
Cdipt
CDP-DAG
PtdGro-P
PtdIns
Pss
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PtdEtn
Pemt
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PtdGro
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Choline
Crls1
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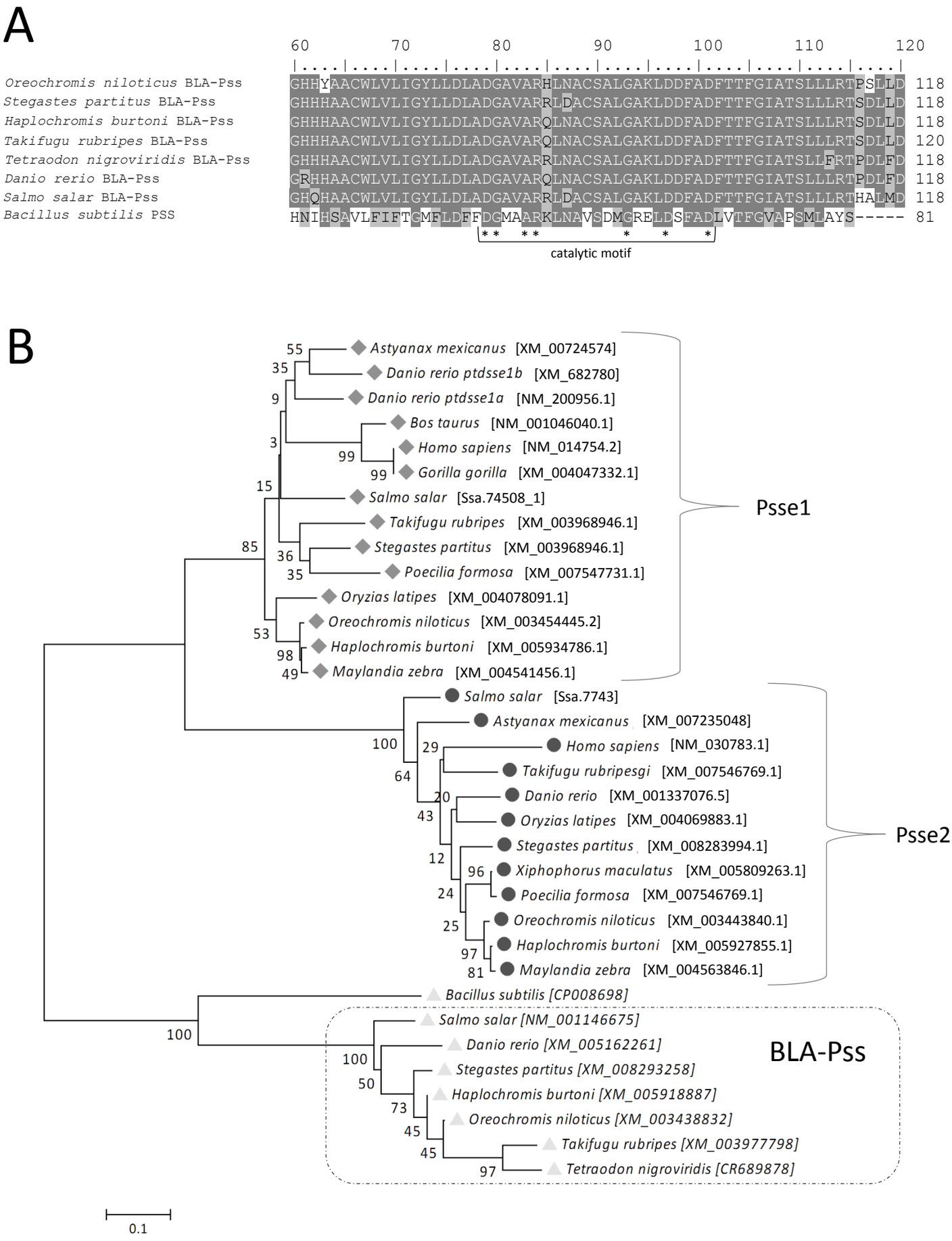
Figure 2.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Bacillus subtilis PSS HNIHSAVLFIFTGMFLDFFDGMAARKLNAVSDMGRELDSFADLVTFGVAPSMLAYS----- 81
catalytic motif
B
Astyanax mexicanus [XM_00724574]
Danio rerio ptdsse1b [XM_682780]
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Bos taurus [NM_001046040.1]
Homo sapiens [NM_014754.2]
Gorilla gorilla [XM_004047332.1]
Salmo salar [Ssa.74508_1]
Takifugu rubripes [XM_003968946.1]
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Figure 3.

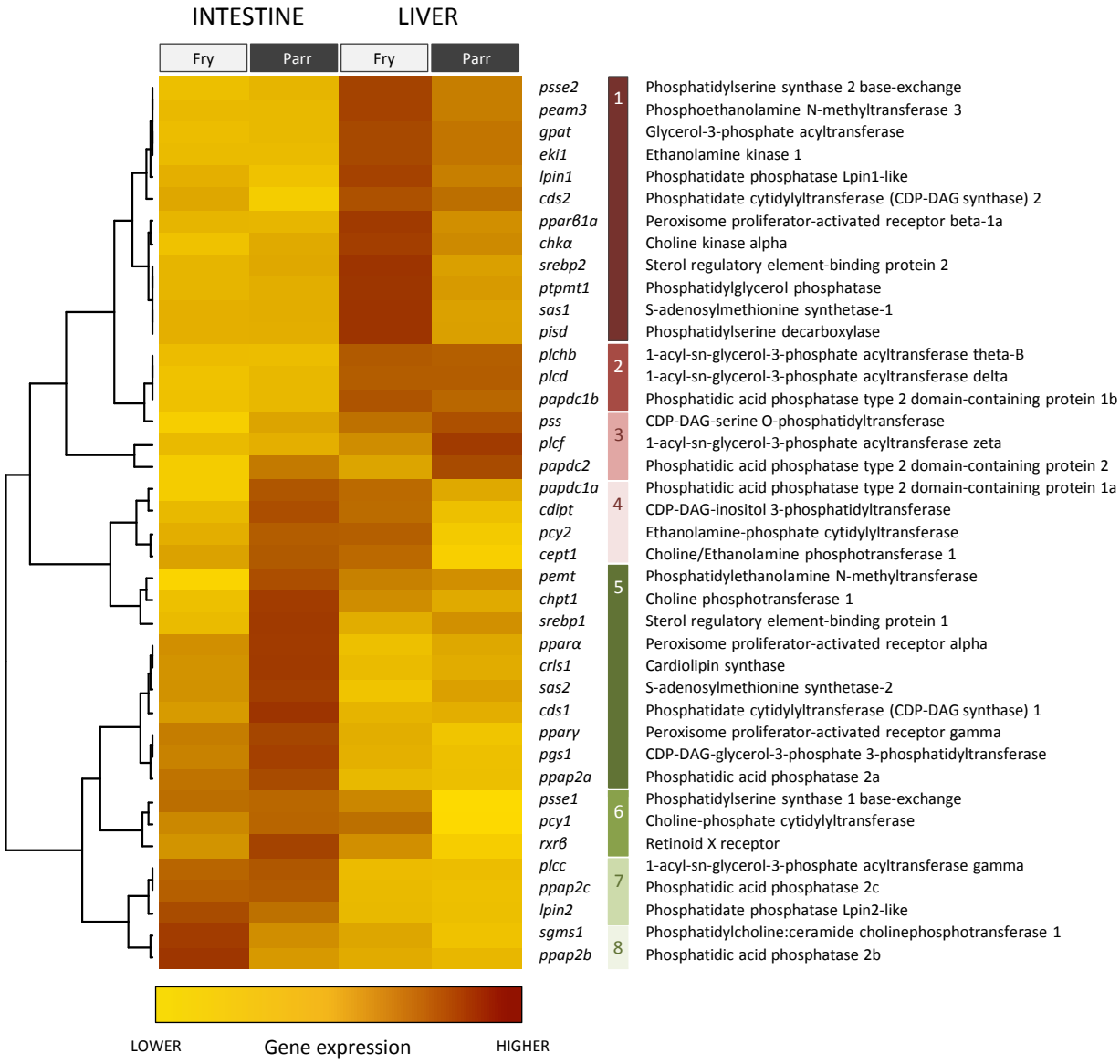

INTESTINE
LIVER
Fry
Parr
Fry
Parr
psse2 Phosphatidylserine synthase 2 base-exchange
peam3 Phosphoethanolamine N-methyltransferase 3
gpat Glycerol-3-phosphate acyltransferase
eki1 Ethanolamine kinase 1
lpin1 Phosphatidate phosphatase Lpin1-like
cds2 Phosphatidate cytidylyltransferase (CDP-DAG synthase) 2
pparβ1α Peroxisome proliferator-activated receptor beta-1a
chkα Choline kinase alpha
srebp2 Sterol regulatory element-binding protein 2
ptpmt1 Phosphatidylglycerol phosphatase
sas1 S-adenosylmethionine synthetase-1
pisd Phosphatidylserine decarboxylase
plchb 1-acyl-sn-glycerol-3-phosphate acyltransferase theta-B
plcd 1-acyl-sn-glycerol-3-phosphate acyltransferase delta
papdc1b Phosphatidic acid phosphatase type 2 domain-containing protein 1b
pss CDP-DAG-serine O-phosphatidyltransferase
plcf 1-acyl-sn-glycerol-3-phosphate acyltransferase zeta
papdc2 Phosphatidic acid phosphatase type 2 domain-containing protein 2
papdc1a Phosphatidic acid phosphatase type 2 domain-containing protein 1a
cdipt CDP-DAG-inositol 3-phosphatidyltransferase
pcy2 Ethanolamine-phosphate cytidylyltransferase
cept1 Choline/Ethanolamine phosphotransferase 1
pemt Phosphatidylethanolamine N-methyltransferase
chpt1 Choline phosphotransferase 1
srebp1 Sterol regulatory element-binding protein 1
pparα Peroxisome proliferator-activated receptor alpha
crls1 Cardiolipin synthase
sas2 S-adenosylmethionine synthetase-2
cds1 Phosphatidate cytidylyltransferase (CDP-DAG synthase) 1
pparγ Peroxisome proliferator-activated receptor gamma
pgs1 CDP-DAG-glycerol-3-phosphate 3-phosphatidyltransferase
ppap2a Phosphatidic acid phosphatase 2a
psse1 Phosphatidylserine synthase 1 base-exchange
pcy1 Choline-phosphate cytidylyltransferase
rxrβ Retinoid X receptor
plcc 1-acyl-sn-glycerol-3-phosphate acyltransferase gamma
ppap2c Phosphatidic acid phosphatase 2c
lpin2 Phosphatidate phosphatase Lpin2-like
sgms1 Phosphatidylcholine:ceramide cholinephosphotransferase 1
ppap2b Phosphatidic acid phosphatase 2b
1
2
3
4
5
6
7
8
LOWER
Gene expression
HIGHER

Figure 4. Cluster 1

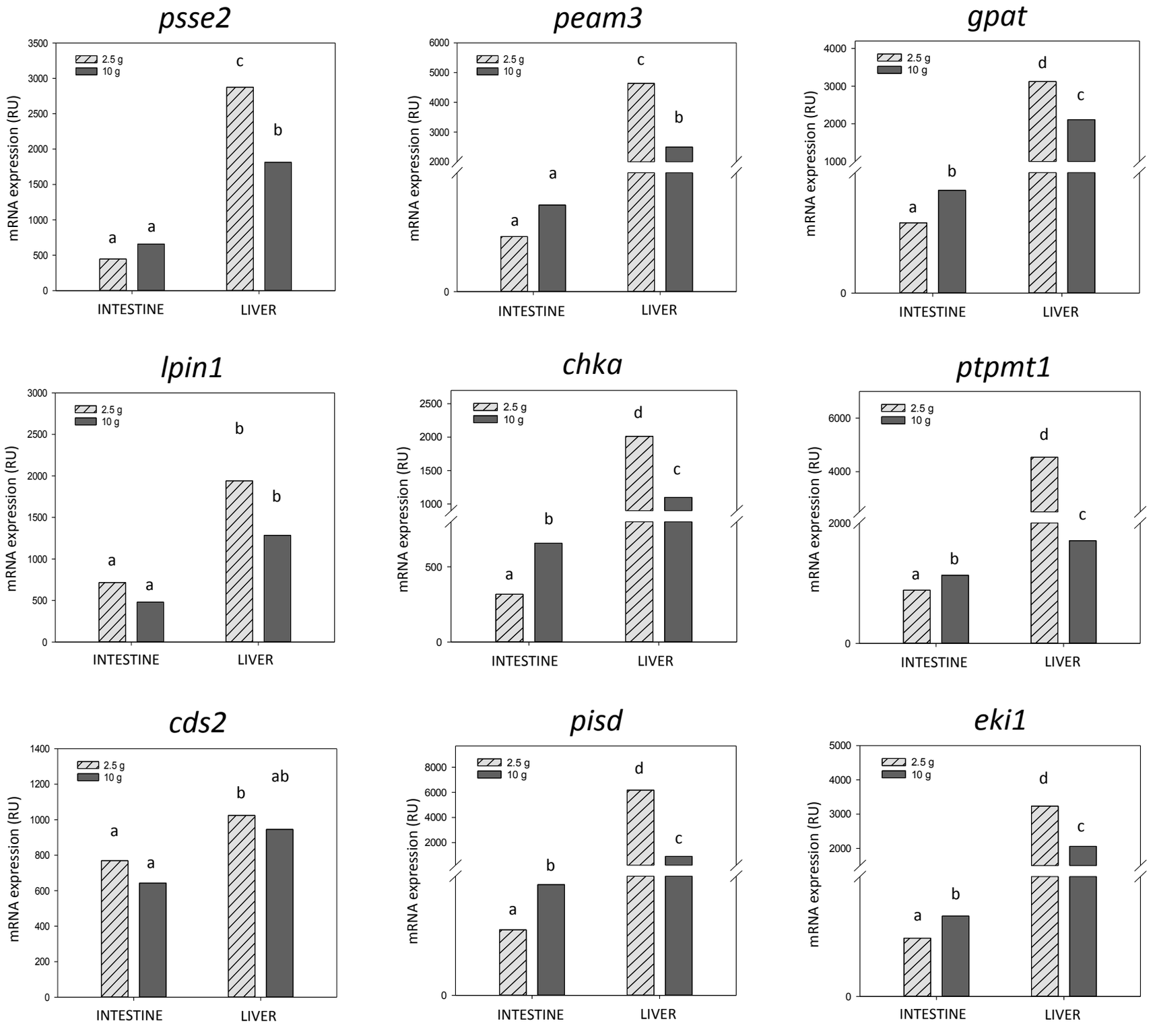


Figure 5. Cluster 2,7

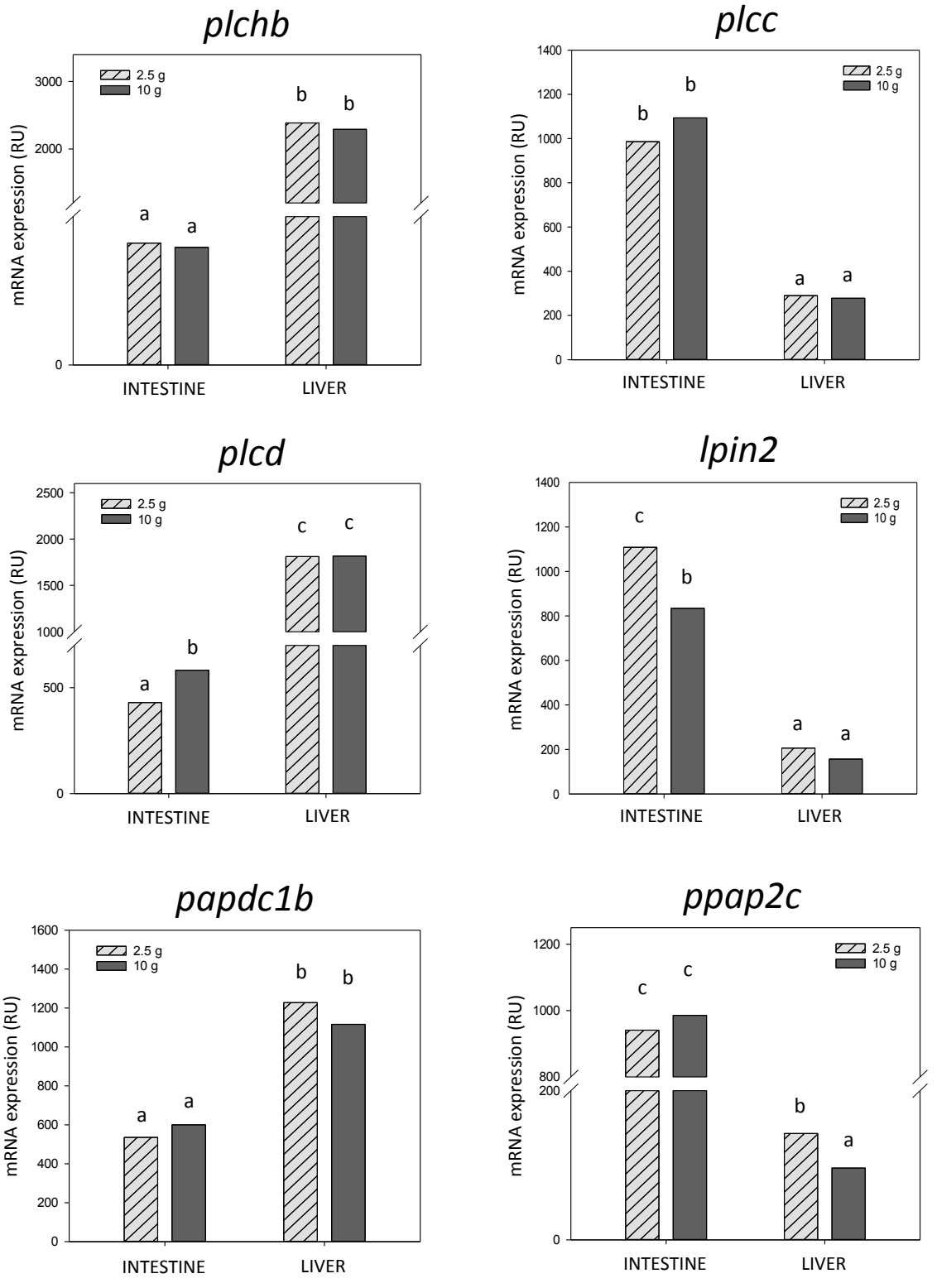


Figure 6. Cluster 3,4, 8

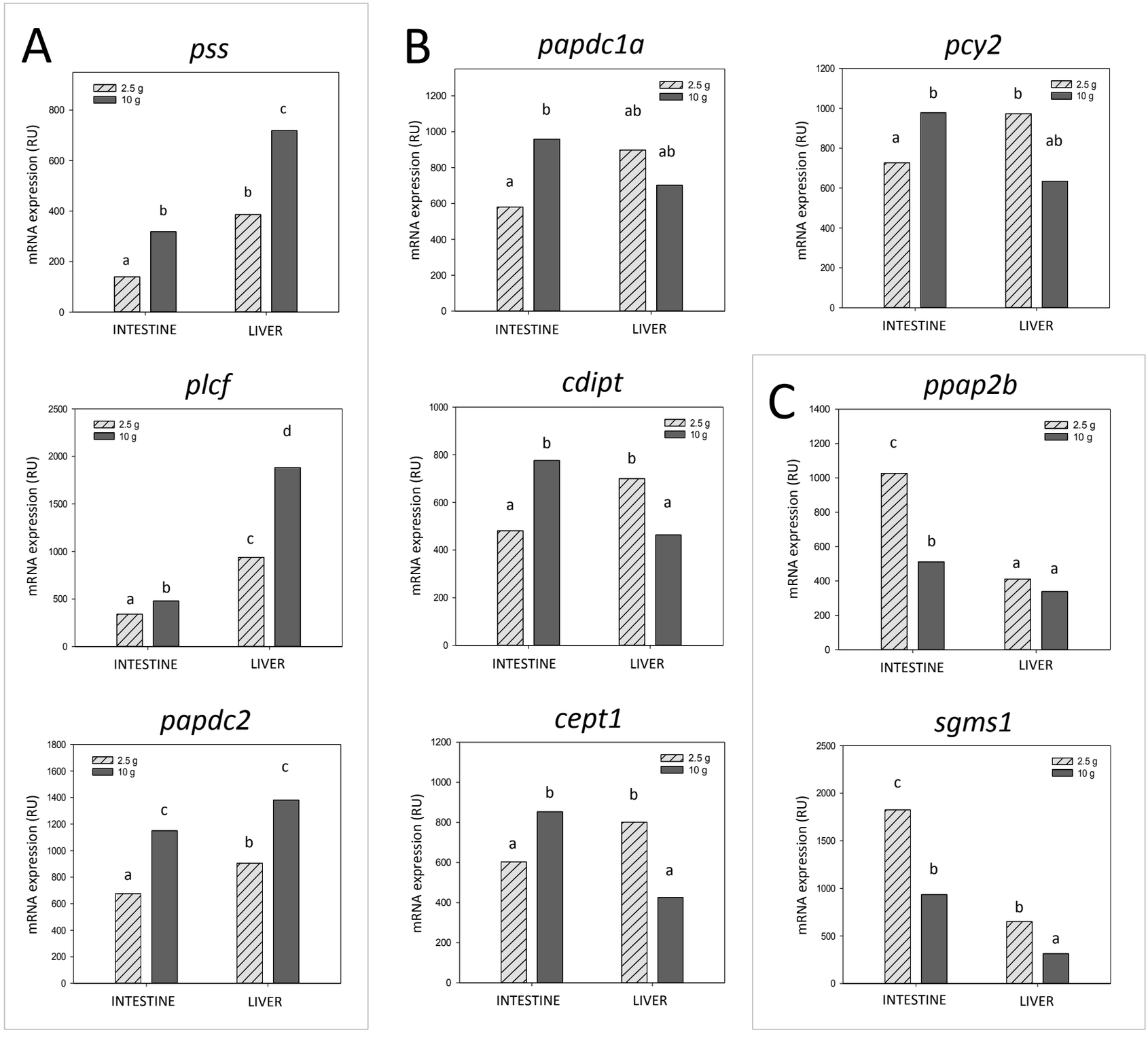


Figure 7. Cluster 5, 6

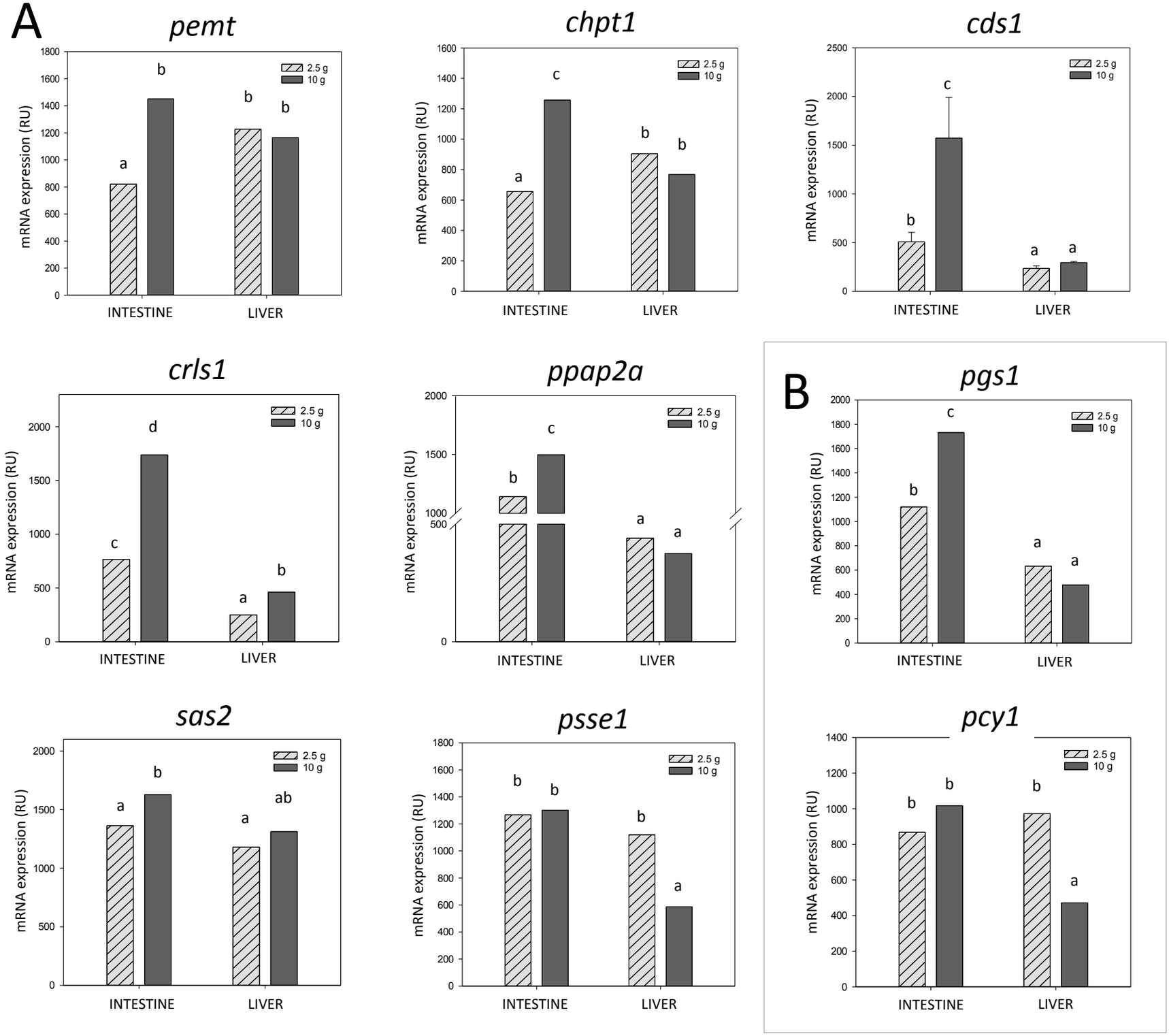
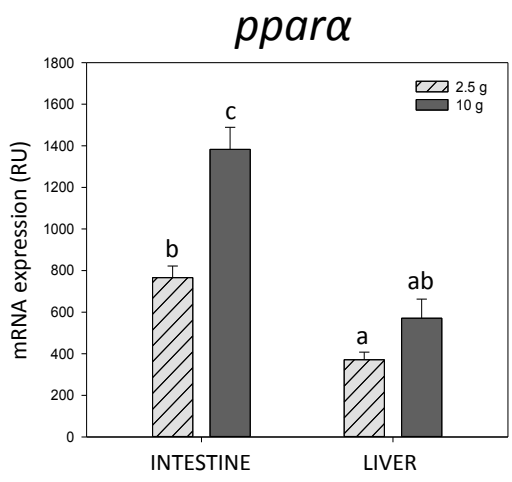


Figure 8. TF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10 g
b
c
a
ab
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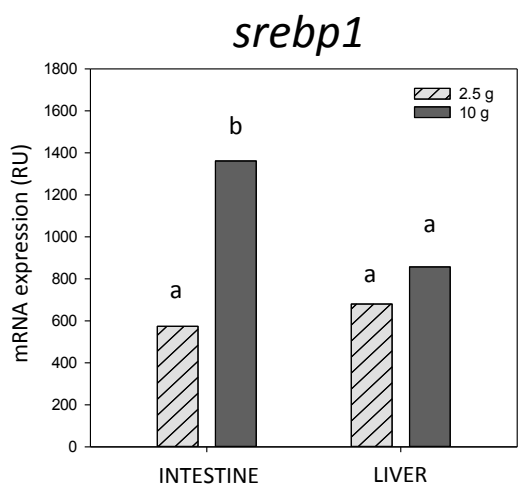
LIVER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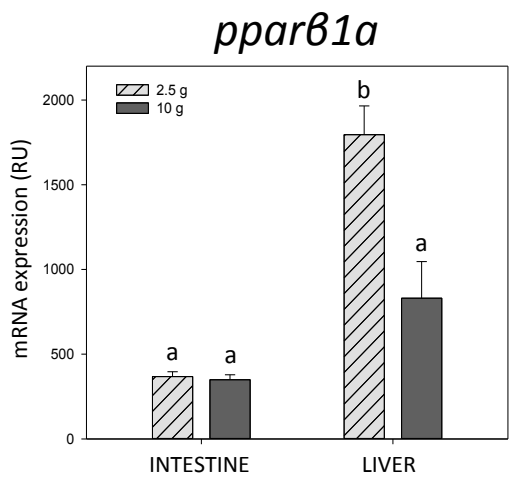
LIVER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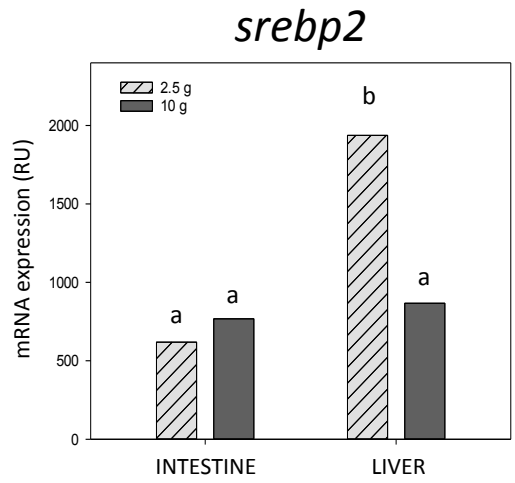
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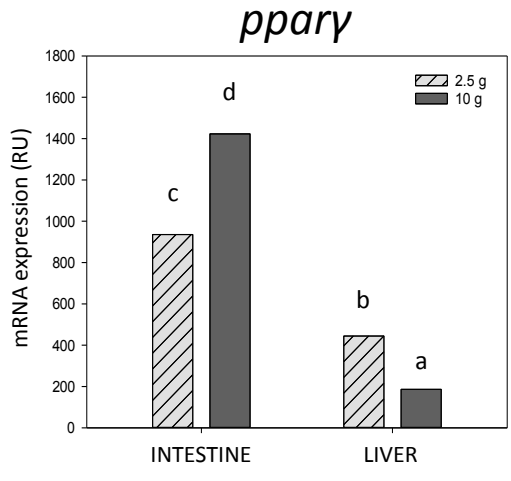
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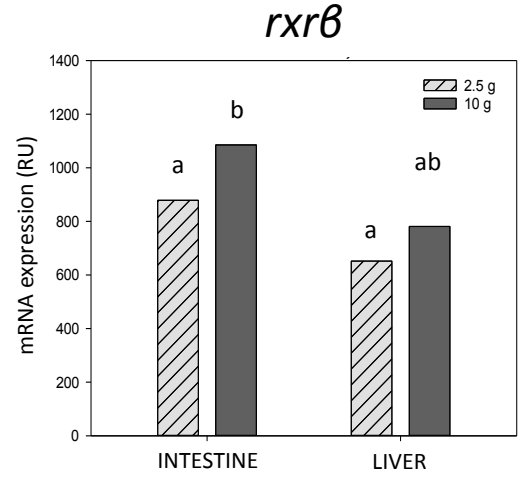
LIVER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mRNA expression (RU)
2.5 g
10 g
a
b
a
ab
INTESTINE
LIVER

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

Data of candidate reference genes (n=12)

		Intestine				Liver			
Genes	*Efficiency*	GeoMean *[Ct]*	Ct Range *[Min, Max]*	SD *[± Ct]*	SD *[± corrected Ct]*	GeoMean *[Ct]*	Ct Range *[Min, Max]*	SD *[± Ct]*	SD *[± 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

880

881