



Influence of Dietary Long-Chain Polyunsaturated Fatty Acids and $\omega 6$ to $\omega 3$ Ratios on Head Kidney Lipid Composition and Expression of Fatty Acid and Eicosanoid Metabolism Genes in Atlantic Salmon (*Salmo salar*)

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The interaction of dietary eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA) levels with omega-6 to omega-3 ratios ($\omega 6:\omega 3$), and their impact on head kidney lipid metabolism in farmed fish, are not fully elucidated. We investigated the influence of five plant-based diets (12-week exposure) with varying EPA+DHA levels (0.3, 1.0, or 1.4%) and $\omega 6:\omega 3$ (high $\omega 6$, high $\omega 3$, or balanced) on tissue lipid composition, and transcript expression of genes involved in fatty acid and eicosanoid metabolism in Atlantic salmon head kidney. Tissue fatty acid composition was reflective of the diet with respect to C₁₈ PUFA and MUFA levels (% of total FA), and $\omega 6:\omega 3$ (0.5–1.5). Fish fed 0.3% EPA+DHA with high $\omega 6$ (0.3% EPA+DHA $\uparrow\omega 6$) had the highest increase in proportions (1.7–2.3-fold) and in concentrations (1.4–1.8-fold) of arachidonic acid (ARA). EPA showed the greatest decrease in proportion and in concentration (by $\sim 1/2$) in the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish compared to the other treatments. However, no differences were observed in EPA proportions among salmon fed the high $\omega 3$ (0.3 and 1.0% EPA+DHA) and balanced (1.4% EPA+DHA) diets, and DHA proportions were similar among all treatments. Further, the transcript expression of *elov15a* was lowest in the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish, and correlated positively with 20:3 $\omega 3$, 20:4 $\omega 3$ and EPA:ARA in the head kidney. This indicates that high dietary 18:3 $\omega 3$ promoted the synthesis of $\omega 3$ LC-PUFA. Dietary EPA+DHA levels had a positive impact on *elov15a*, *fadsd5* and *srebp1* expression, and these transcripts positively correlated with tissue Σ MUFA. This supported the hypothesis that LC-PUFA synthesis is positively influenced by tissue MUFA levels in Atlantic salmon. The expression of *ppara* was higher in the 0.3% EPA+DHA $\uparrow\omega 6$ compared to the 0.3% EPA+DHA $\uparrow\omega 3$ fed fish. Finally, significant correlations between head kidney fatty acid composition and the expression of eicosanoid synthesis-related transcripts

(i.e., *5loxa*, *5loxb*, *cox1*, *cox2*, *ptges2*, *ptges3*, and *pgds*) illustrated the constitutive relationships among fatty acids and eicosanoid metabolism in salmon.

Keywords: salmon aquaculture, omega-6/omega-3 ratio, EPA+DHA, lipid metabolism, head kidney transcript expression, nutrigenomics, metabolomics, fatty acids

INTRODUCTION

Long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA, 20:5 ω 3), docosahexaenoic (DHA, 22:6 ω 3) and arachidonic (ARA, 20:4 ω 6) acids play important roles in fish growth, metabolism (Sargent et al., 2002), neural development, health and reproduction (Sargent et al., 1999; Tocher, 2010, 2015). They are also important structural components of cell membranes (Calder, 2013), and act as precursors to eicosanoid metabolites such as prostaglandins, leukotrienes, thromboxanes, docosanoids, maresins and resolvins which regulate inflammatory and immune response processes (Calder, 2007, 2013; Arts and Kohler, 2009; Martinez-Rubio et al., 2013; Holen et al., 2015; Montero et al., 2019). The levels of EPA and DHA in fish feeds showed a large reduction (from ~ 20 to 5% of dietary lipid) over the past ~3 decades (Einen and Thomassen, 1998; Ytrestøyl et al., 2015; Sprague et al., 2016), due to decreasing global availability and rising market price of their fish oil (FO) sources (Turchini et al., 2009; Tacon and Metian, 2015; Froehlich et al., 2018). The reduction in FO and fish meal (FM), and the concomitant rise in incorporation of plant-based ingredients have influenced fish health (e.g., liver steatosis, altered phagocytic activity, and modulation of immune-related transcript expression) (Montero et al., 2003; Ruyter et al., 2006; Caballero-Solares et al., 2017), and their nutritional quality for human consumers (Sprague et al., 2016; De Roos et al., 2017).

Salmonids have a capacity to desaturate and elongate the precursors 18:3 ω 3 and 18:2 ω 6 to LC-PUFA metabolites (Tocher et al., 2000; Hixson et al., 2014; Katan et al., 2019). The enzymes involved in the bioconversion of both ω 3 and ω 6 pathways are the fatty acyl desaturases (FADS2D5, FADS2D6a, FADS2D6b and FADS2D6c; Zheng et al., 2005; Monroig et al., 2010) and fatty acyl elongases (ELOVL5a, ELOVL5b, ELOVL2, ELOVL4a and ELOVL4b; Hastings et al., 2004; Morais et al., 2009; Carmona-Antoñanzas et al., 2011; Zhao et al., 2019). Hepatic LC-PUFA synthesis in Atlantic salmon (*Salmo salar*) is influenced by precursor (18:2 ω 6 and/or 18:3 ω 3) availability, as well as the concentration of LC-PUFA (i.e., EPA, ARA, DHA) (Jordal et al., 2005; Glencross et al., 2015; Katan et al., 2019). This pathway is regulated at the transcriptional level and is mediated by transcription factors [e.g., liver X receptor (LXR), sterol regulatory element binding protein (SREBP) 1 and 2, and peroxisome proliferator-activated receptor (PPAR) α , β and γ (Carmona-Antoñanzas et al., 2014; Hixson et al., 2017; Katan et al., 2019; Emam et al., 2020)]. However, the constitutive regulation of this pathway in salmon head kidney is less well-understood (Betancor et al., 2014), and requires further investigation given the central role of this organ in haematopoietic

and immune processes (Tort et al., 2003; Zapata et al., 2006; Gjøen et al., 2007).

Plant oils used in aquafeeds generally contain high levels of ω 6 FA, and as such, these oils and farmed fish consuming them may provide inadequate ratios of ω 6 to ω 3 (ω 6: ω 3) fatty acids for the consumer (Pickova and Mørkøre, 2007; Weaver et al., 2008; Young, 2009). Further, ω 6: ω 3 plays an important role in fish immune response [through their conversion into pro- and anti-inflammatory eicosanoids (Furne et al., 2013; Holen et al., 2018)] and FA metabolism [e.g., impacting LC-PUFA synthesis and transcript expression of genes involved in this pathway (Torstensen and Tocher, 2010; Vagner and Santigosa, 2011; Katan et al., 2019)]. Previous studies revealed that diets with varying ω 6: ω 3 ratio (i.e., ~0.3–4.0) influenced the transcription of inflammation- and FA metabolism-related genes in Atlantic salmon head kidney (Martinez-Rubio et al., 2013; Holen et al., 2018). However, the interaction of dietary ω 6: ω 3 with EPA+DHA levels, and their nutritional impacts on eicosanoid and fatty acid metabolism in salmon, have not been fully elucidated.

Emam et al. (2020) recently showed that diets with different combinations of ω 6: ω 3 (high ω 6, high ω 3, or balanced) and EPA+DHA levels (0.3, 1.0, or 1.4%) affected muscle lipid composition, and the hepatic transcript expression of fatty acid synthesis-related genes in Atlantic salmon. The current study used the same diets and fish population as in Emam et al. (2020), with the aim to investigate how diets with low FO inclusion levels impact head kidney lipid composition, and the transcript expression of genes involved in fatty acid and eicosanoid metabolism. Furthermore, correlation analyses were used to relate head kidney lipid composition with transcript expression.

MATERIALS AND METHODS

Experimental Diets and Animals

Five experimental diets were manufactured by Cargill Canada (formerly EWOS Canada; Surrey, BC, Canada), top-coated with different oil mixes at the Chute Animal Nutrition Centre (Dalhousie University, Truro, NS, Canada), and stored at -20°C until needed. Experimental diets contained different levels of FO, poultry fat, and vegetable oils (VO; i.e., soy oil, linseed oil, and rapeseed oil) to generate three levels of EPA+DHA (0.3, 1.0, and 1.4%, as formulated) and contrasting ω 6: ω 3 ratios (high ω 6, high ω 3, and balanced). The diets were as follows: 0.3% EPA+DHA with higher ω 6 (0.3% EPA+DHA $\uparrow\omega$ 6), 0.3% EPA+DHA with higher ω 3 (0.3% EPA+DHA $\uparrow\omega$ 3), 1.0% EPA+DHA with higher ω 6 (1% EPA+DHA $\uparrow\omega$ 6), 1.0% EPA+DHA with higher ω 3 (1% EPA+DHA $\uparrow\omega$ 3), and 1.4% EPA+DHA with a more balanced ω 6: ω 3 ratio (1.4% EPA+DHA/balanced). All diets were

formulated to be isonitrogenous and isoenergetic and to meet the nutritional requirements of salmonids (National Research Council (NRC), 2011). Dietary formulations and their lipid compositions were published previously (Emam et al., 2020). However, as they pertain to the current study, they are included as **Supplementary Material** here (**Supplementary Tables 1, 2**).

Atlantic salmon smolts were transported from a regional salmon farm to the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland, Canada; October 2016). After their arrival, fish were graded by size in order to select the most uniform population, PIT (Passive Integrated Transponder; Easy AV, Avid Identification Systems, Norco, CA, USA)-tagged for individual identification, and kept in 3,800-litre tanks until the beginning of the feeding trial. Then, salmon post-smolts [210 ± 44 g mean initial weight \pm standard deviation (SD)] were randomly distributed into twenty 620-litre tanks (40–41 fish tank⁻¹), and subjected to an 8-week acclimation period during which they were given a commercial diet (EWOS Dynamic S, Cargill Inc., MN, USA). After the completion of the acclimation period, fish were switched from the commercial feed and fed with the experimental diets (4 tanks diet⁻¹) for 12 weeks. At all stages, fish were fed to satiation overnight using automatic feeders (AVF6 Vibrating Feeders, Pentair Aquatic Eco-Systems, Inc., Florida, USA). Uneaten pellets were collected every morning, and the daily amount of feed was adjusted based on the number of uneaten pellets. All tanks were supplied with 12°C flow-through filtered seawater at 12 l min⁻¹; the dissolved oxygen level was ~ 10 mg l⁻¹, and the photoperiod was maintained at 24-h light. Mortalities were weighed and recorded throughout the trial. Ethical treatment of fish in this experiment was carried out in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (protocol # 16-75-MR).

Sample Collection

Salmon samples were collected from 10 opportunistically netted fish (each from a different tank) at week 0 (the day before fish were exposed to the experimental diets), and from 5 fish per tank at week 12. Sampling occurred after the fish were starved for 24 h and euthanized with an overdose of MS-222 (400 mg l⁻¹; Syndel Laboratories, Vancouver, BC, Canada). For lipid analyses, head kidney samples were collected in 15 ml glass test tubes that had been rinsed three times with methanol, followed by three rinses with chloroform. Samples were stored on ice during sampling, their wet weights recorded, and they were covered with 2 ml of chloroform (HPLC-grade). Finally, the glass test tubes were filled with nitrogen, the Teflon-lined caps were sealed with Teflon tape, and samples were stored at -20°C . For gene expression analyses, head kidney (50–100 mg sample⁻¹) tissues were collected in 1.5 ml nuclease-free tubes, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extractions were performed.

Lipid Extractions

Lipid samples were extracted according to Parrish (1999). Samples were homogenized in a 2:1 mixture of ice-cold chloroform:methanol using a Polytron PCU-2-110

homogenizer (Brinkmann Instruments, Rexdale, ON, Canada). Chloroform-extracted water was added to bring the ratio of chloroform:methanol:water to 8:4:3. Each sample was sonicated for 4 min in an ice bath and centrifuged at 3,000 rpm for 2 min at room temperature. The bottom, organic layer was removed using a double pipetting technique, placing a 2 ml lipid-clean glass Pasteur pipette inside a 1 ml glass pipette, in order to remove the organic layer without disturbing the top, aqueous layer. Chloroform was then added back to the extraction test tube, and the entire procedure was repeated three times. All organic layers were pooled into a separate lipid-clean vial. Finally, samples were concentrated under a flow of nitrogen gas.

Lipid Class Separation

Iatroscan Mark 6 TLC-FID (thin-layer chromatography-flame ionization detector) (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), and a three-step development method were used to determine the lipid class composition (Parrish, 1987). Lipid extracts and standards were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane:diethyl ether:formic acid (98.95:1.0:0.05). The rods were developed for 25 min, removed from the system for 5 min to dry, and replaced for 20 min. The second development was for 40 min in hexane:diethyl ether:formic acid (79:20:1). The final development system had two steps—the first was in 100% acetone for two 15 min time periods, followed by two 10 min periods in chloroform:methanol:chloroform extracted water (5:4:1). Before using each solvent system, the rods were dried in a constant humidity chamber. After each development system, the rods were partially scanned in the Iatroscan, and the data were collected using Peak Simple software (SRI Instruments, version 3.67, Torrance, CA, USA). The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, MO, USA).

Fatty Acid Methyl Ester (FAME) Derivatization

Lipid extracts (50 μl) were transferred into lipid-clean 15 ml glass vials and concentrated under a flow of nitrogen to complete dryness. Then, 1.5 ml of methylene chloride and 3 ml of Hilditch reagent (1.5 sulfuric acid: 98.5 anhydrous methanol) were added to each sample vial, and this was followed by a brief vortexing and 4 min sonication (Fisher Scientific FS30, Pittsburgh, PA, USA). Vials were then filled with nitrogen, capped and heated at 100°C for 1 h. Subsequently, 0.5 ml of saturated sodium bicarbonate solution and 1.5 ml of hexane were added to each vial, followed by a brief vortexing, and then the upper, organic layer was removed into a separate lipid-clean glass vial. Each sample was then dried and refilled with ~ 0.5 ml of hexane. Finally, vials were filled with nitrogen, capped, sealed with Teflon tape, and sonicated for 4 min to resuspend the fatty acids. All solvents were OmniSolv grade (VWR International, Mississauga, Ontario, Canada).

All FAMES were analysed on a HP 6890 GC-FID (gas chromatograph-flame ionization detector) system equipped with a 7683 autosampler (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). Additional details regarding the

GC conditions (e.g., column length, diameter, temperature, carrier gas) are described in Hixson et al. (2017). Fatty acid peaks were identified against known standards (PUFA 1, PUFA 3, BAME and a Supelco 37 component FAME mixture: Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Finally, chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2 (Walnut Creek, CA, USA). FA data were expressed as area percentage of FAME.

RNA Extraction, DNase Treatment, Column Purification and cDNA Synthesis

Head kidney samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), using the TissueLyser II system at 25 Hz for 2.5 min, using 5 mm stainless steel beads (QIAGEN, Mississauga, ON, Canada), and subjected to RNA extraction. This was followed by DNaseI treatment and column purification using the RNase-free DNase Set (QIAGEN) and the RNeasy Mini Clean-up Kit (QIAGEN), respectively. All procedures were conducted according to manufacturer instructions and as described in Xue et al. (2015). RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity and quantity were assessed by Nanodrop UV spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). DNaseI-treated and column-purified RNA samples had A260/280 and A260/230 ratios of 1.8–2.3 (data not shown). All cDNAs were prepared by reverse transcription of 1 µg of DNaseI-treated, column-purified total RNA for each sample, using 1 µl of random primers (250 ng; Invitrogen), 1 µl of dNTPs (0.5 mM final concentration; Invitrogen), 4 µl of 5X first-strand buffer (1× final concentration; Invitrogen), 2 µl of DTT (10 mM final concentration; Invitrogen), and 1 µl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U; Invitrogen) at 37°C for 50 min. The total reaction volume was 20 µl, following manufacturer instructions, and as described in Xue et al. (2015). Finally, all cDNAs were diluted 40 times with nuclease-free water (Invitrogen) prior to real-time quantitative polymerase chain reaction (qPCR).

QPCR Analysis

Expression of 25 genes of interest (GOI) (Table 1) related to FA and eicosanoid metabolism was quantified by qPCR using head kidney cDNA templates from fish fed the five dietary treatments at week 12. For each dietary treatment, eight individuals (two from each tank) were used in the qPCR study. Only head kidney samples from fish that had specific growth rates within 1.5 standard deviations below and above the mean value of each tank were selected for the qPCR study, in order to reduce biological variability. The sequences of all primer pairs used in qPCR analyses, GenBank accession numbers of sequences used for primer design, and other details are presented in Table 1. Notably, primers for the transcripts *fadsd6a*, *elovl2*, and *elovl5b* (GenBank accession numbers AY458652, FJ237532 and FJ237532, respectively) failed quality testing due to low transcript expression levels, and thus were not included in the qPCR study. Each primer pair was quality-tested to verify that a single product was amplified with no primer dimers, and included standard curves and dissociation curves, as described

in Rise et al. (2010) and Booman et al. (2011). In brief, the amplification efficiency (Pfaffl, 2001) of each primer pair was determined using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA. Two pools were generated (i.e., 0.3% EPA+DHA $\uparrow\omega$ 6 and 0.3% EPA+DHA $\uparrow\omega$ 3), and each pool consisted of 8 fish (with each fish contributing an equal quantity to the pool). The reported primer pair amplification efficiencies were an average of the two pools, except if one pool showed poor efficiency or spacing due to low expression levels (i.e., the 0.3% EPA+DHA $\uparrow\omega$ 3 pool was used for *fadsd6b* and *fadsd6c*, while the 0.3% EPA+DHA $\uparrow\omega$ 6 pool was used for *lxrb*).

To select the most suitable normalizer genes, six candidate normalizers were tested based on our previous qPCR studies (*rpl32*, *eef1a-1*, *eef1a-2*, *actb*, *ef3d*, *pabpc1*) (Xue et al., 2015; Caballero-Solares et al., 2017), and salmon literature on reference genes (*actb*, *eef1a-1*, *eef1a-2*) (Olsvik et al., 2005). Their qPCR primers were quality-tested as mentioned above. Half of the fish population involved in the qPCR study was utilized for normalizer testing (i.e., 20 total which consisted of 4 fish per treatment). Cycle threshold (C_T) values were measured using cDNA corresponding to 5 ng of input total RNA. Expression stability was then analysed using the geNorm algorithm (Vandesompele et al., 2002). *Eif3d* and *pabpc1* were shown to be the most stable (i.e., geNorm M-values of 0.23 and 0.22, respectively) among the six candidate genes, and therefore were selected as normalizers.

All qPCR amplifications were performed in a total reaction volume of 13 µl and consisted of 4 µl of cDNA (5 ng input total RNA), 50 nM each of forward and reverse primer, 1× Power SYBR Green PCR Master Mix (Applied Biosystems), and nuclease-free water (Invitrogen). QPCR reactions, including no-template controls, were performed in technical triplicates using the ViiA 7 Real-Time PCR System (384-well format) (Applied Biosystems, Foster City, CA, USA) and the Power SYBR Green I dye chemistry. The Real-Time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles (of 95°C for 15 s and 60°C for 1 min), with the fluorescence signal data collection after each 60°C step. When a C_T value within a triplicate was >0.5 cycle from the other two values, it was considered to be an outlier, discarded and the average C_T of the remaining two values was calculated. The relative quantity (RQ) of each GOI was calculated using a qBase relative quantification framework (Hellemans et al., 2007; Booman et al., 2014) with primer amplification efficiencies incorporated (Table 1). The expression levels of each GOI were normalized to both normalizer genes, and the sample with the lowest normalized expression was used as the calibrator sample (i.e., RQ = 1.0) for each GOI (Rise et al., 2015). Transcript expression data are presented as RQ values relative to the calibrator. QPCR fold-change values were calculated by dividing the mean RQ value of each dietary treatment by that of the 0.3% EPA+DHA $\uparrow\omega$ 6 fish.

Statistical Analyses

Tissue Lipid Composition and qPCR Data

Nested general linear models were used with tank nested in diet (Minitab 17 Statistical Software, State College, PA, USA).

TABLE 1 | qPCR primers.

Gene name (symbol)	Nucleotide sequence (5'–3') ^a	Amplification efficiency (%)	Amplicon size (bp)	GenBank Accession number
Elongation of very long chain fatty acids 4 b (<i>elovl4b</i>) ^b	F:TAGCAGAGTTGGGGATCAGC R:CGAGATTAGGTGCGTGTACTG	99.4	101	XM_014144655
Elongation of very long chain fatty acids 5 a (<i>elovl5a</i>) ^c	F:CAGTGTGGTGGGACAAAG R:TTCCCTCATGGACAAGCA	90.2	115	AY170327
Delta-5 fatty acyl desaturase (<i>fadsd5</i>) ^c	F:GTCTGGTTGTCCGTTCTGTT R:GAGGCGATCAGCTTGAGAAA	89.0	135	AF478472
Delta-6 fatty acyl desaturase b (<i>fadsd6b</i>) ^c	F:TGACCATGTGGAGAGTGAGG R:CCTCAAGCAAGGCCTCTAGT	90.1	95	NM001172281
Delta-6 fatty acyl desaturase c (<i>fadsd6c</i>)	F:CCAGTTGGAATCAGTACTGC R:GTGTGTCTCCAGGACGAAG	102.7	162	NM001171780
Sterol regulatory element-binding protein 1 (<i>sreb1</i>) ^c	F:TCAACAAGTCGGCAATTCTG R:GACATCTTCAGGGCCATGTT	103.1	100	HM561860
Sterol regulatory element-binding protein 2 (<i>sreb2</i>) ^c	F:GAGTGCTGAGGAAAGCCATC R:TCTCCACATCGTCAGACAGC	103.1	129	HM561861
Liver X receptor a (<i>lxra</i>) ^c	F:GGGCAAGATGGACAGATCAT R:CCTCACCAGGACCAACATCT	102.6	126	FJ470290
Liver X receptor b (<i>lxrb</i>) ^d	F:CTCGCCTGTGTTCTGTTTT R:GAAACGCAAGACCTTCTGCT	92.6	103	GE770391
Peroxisome proliferator-activated receptor alpha a (<i>pparaa</i>) ^b	F:CCCTGGTGGCTAAGATGGT R:AGACTTGGCGAACTCGGTTA	103.1	132	XM_014124067
Peroxisome proliferator-activated receptor beta 1 (<i>pparb1</i>)	F:CAGCTGATCAACGGTACGAC R:TGCTCTTGGCAAACTCAGTG	93.4	112	NM001123635
Peroxisome proliferator-activated receptor beta 2 a (<i>pparb2a</i>) ^c	F:CCGTTTGTGATCCATGATGT R:GTGCACTGACAGCGGTAAAA	103.7	128	NM001123559
Fatty acid synthase a (<i>fasa</i>) ^c	F:GGAGGGCACAATGGAGTAAA R:TGAGACAGTGAATCGGATGG	97.7	136	DW563978
Fatty acid synthase b (<i>fasb</i>) ^c	F:TGCCATACAAGTGATGTCCTG R:AGTGGGCACCAACATGAAC	99.1	105	EG872804
Acyl-coenzyme A oxidase 1 (<i>acox1</i>) ^b	F:GTGCACCTACGAGGGAGAGA R:TAGGACACGATGCCACTCAG	99.9	111	DW555884
Carnitine palmitoyltransferase 1 a (<i>cpt1a</i>) ^b	F:CGGTGGCAGATGATGGATATG R:GAGTGCTTGCTGGAGATGTG	104.6	82	XM_014176428
Cytosolic phospholipase A2 (<i>cplA2</i>)	F:GACGTGGCAGATTGAGACAA R:GAACGAGAGAGATGGCAGGT	92.3	147	NM_001141333
Cyclooxygenase-1 (<i>cox1</i>) ^c	F:CTCATGAGGGTGGTCTCAC R:AGGCACAGGGGTAGGATAC	104.1	135	BT045745
Cyclooxygenase-2 (<i>cox2</i>) ^e	F:ACCTTTGTGCGAAACGCTAT R:GAGTAGGCCTCCAGCTCTT	97.0	113	AY848944
Arachidonate 5-lipoxygenase a (<i>5loxa</i>) ^c	F:CTGCTCACCATGCTGCTGTC R:GTGTGGGAGGAGGCTTCC	97.0	93	NM001139832
Arachidonate 5-lipoxygenase b (<i>5loxb</i>) ^c	F:ACTGTGTGGGTTTCCCAAG R:GACAGCAGCGTGATGTGCAG	102.6	98	DW555519
Prostaglandin-D synthase (<i>pgds</i> , alias <i>lipocalin-type pgds</i>) ^c	F:GGTGCTCAACAAGCTCTACA R:GCAGGAAAGCGATGTTGTCA	90.3	114	BT048787
Prostaglandin E synthase 2-like (<i>ptges2</i>)	F:TTCTGCGCTGTTACCCAGAG R:GTACATCGTCTGACCTTCAG	99.8	112	XM_014171682/014160437
Prostaglandin E synthase 3 (<i>ptges3</i>)	F:TGGCCTAGGCTAACGAAAGA R:TTGCCTAGTTCCTCGTCTGAG	97.7	101	BT056895
Leukotriene A4 hydrolase (<i>lkha4</i> , alias <i>lta4h</i>)	F:AAGGTCTCCAAGTAACAGC R:AATGGCAGTGATCTCCAA	94.8	97	NM_001140120

(Continued)

TABLE 1 | Continued

Gene name (symbol)	Nucleotide sequence (5'–3') ^a	Amplification efficiency (%)	Amplicon size (bp)	GenBank Accession number
Eukaryotic translation initiation factor 3 subunit D (<i>eif3d</i>)^e	F:CTCCTCCTCCTCGTCCTCTT R:GACCCCAACAAGCAAGTGAT	94.2	105	GE777139
Polyadenylate-binding protein cytoplasmic 1 (<i>pabpc1</i>)^e	F:TGACCGTCTCGGGTTTGTAG R:CCAAGGTGGATGAAGCTGTT	93.6	108	EG908498

^aF is forward and R is reverse primer.

^bPrimers that were previously published in Emam et al. (2020).

^cPrimers that were previously published in Katan et al. (2019).

^dPrimers that were previously published in Hixson et al. (2017).

^ePrimers that were previously published in Caballero-Solares et al. (2017). Normalizer genes are in bold font.

This was followed by Tukey *post-hoc* tests ($p < 0.05$) to identify significant differences among treatments at week 12 (Minitab 17 Statistical Software). However, when a significant tank effect was identified ($p < 0.05$), a one-way ANOVA followed by Tukey *post-hoc* tests was performed (Minitab 17 Statistical Software, State College, PA, USA). To identify significant differences in head kidney lipid composition between week 0 (i.e., initial) and week 12, a one-way ANOVA followed by Tukey *post-hoc* tests was used. To show the effects of dietary EPA+DHA (i.e., 0.3 and 1%) and $\omega 6:\omega 3$ (i.e., high $\omega 6$ and high $\omega 3$) factors on the transcript expression of each GOI, a two-way ANOVA was performed (Minitab 17 Statistical Software). The 1.4% EPA+DHA/balanced treatment was not included in this analysis since it was not formulated following the 2×2 factorial design of the other four treatments (i.e., 0.3 or 1.0% EPA+DHA vs. high $\omega 6$ or high $\omega 3$).

For the qPCR analysis, each dietary treatment was tested for outliers using Grubb's test ($p < 0.05$). In total, 7 RQ values were identified as statistical outliers in the entire dataset (i.e., out of 1,000 values comprising all samples and all GOIs), and excluded from the study. All GOIs had a sample size of 7–8 per dietary treatment. Finally, residuals were tested to verify normality, independence and homogeneity of variance. Normality was examined using the Anderson-Darling test. If the test failed ($p < 0.05$), a one-way ANOVA on ranks was performed, and followed by a Kruskal-Wallis test (SigmaPlot, Systat Software, Inc., Version 13, San Jose, CA, USA). In all cases, differences were considered statistically significant when $p < 0.05$.

Multivariate and Pearson Correlation Analyses

Principal coordinate analysis (PCoA) was used to describe head kidney lipid and FA composition in the five dietary treatments (PRIMER, Plymouth Routines in Multivariate Ecological Research; PRIMER-E Ltd., version 6.1.15, Ivybridge, UK). A similarity matrix was used, and the first two PCO axes (i.e., PCO1, PCO2) were plotted. SIMPER (Similarity of Percentages Analysis) was performed to quantify differences among treatments in lipid and FA data, while PERMANOVA (Permutational Multivariate Analysis of Variance) was used to perform pairwise tests between treatments (9,999 permutations). In all cases, the non-parametric Bray-Curtis similarity was used.

Pearson correlation analysis was performed to identify relationships between transcript expression (i.e., qPCR RQ data) and tissue lipid composition (i.e., % FA and lipid classes) in the

head kidney, using individual fish. All GOIs and lipid classes [i.e., triacylglycerols (TAG), sterols (ST) and phospholipids (PL)] were used in the correlation analysis. However, transcripts and lipid classes with no significant correlations were removed. Individual saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) that accounted for >5% of the total FA, and all $\omega 6$ and $\omega 3$ FA were included in the analysis. IBM SPSS (IBM SPSS Statistics, Version 25, Armonk, New York, USA) was used for the correlation analyses. In order to separately group transcripts and lipid composition (% fatty acids and lipid classes), hierarchical clustering was used with group average in PRIMER (Version 6.1.15, Ivybridge, UK).

RESULTS

Head Kidney Lipid Composition

The predominant lipid class in the head kidney was TAG (46.6–52.0%), followed by PL (22.8–28.6%), and ST (18.4–23.7%) (Table 2). No significant differences were observed among dietary treatments in any lipid class or in total lipids (mg g^{-1}) at week 12 ($p = 0.09$ – 0.72). ST showed a decrease from week 0 (initial) to week 12 in all treatments ($p < 0.0001$, Table 2).

Individual SFA showed differences among treatments (i.e., 14:0 and 18:0; $p < 0.0001$ and 0.001, respectively) at week 12. The 1.4% EPA+DHA/balanced treatment had higher 14:0 than the 0.3% EPA+DHA $\uparrow \omega 6$, 0.3% EPA+DHA $\uparrow \omega 3$ and 1.0% EPA+DHA $\uparrow \omega 6$ fed fish. Lower 18:0 levels were observed in the 1.4% EPA+DHA/balanced compared to the high $\omega 6$ (i.e., with 0.3% and 1.0% EPA+DHA) treatments. However, 16:0 and total SFA (Σ SFA) were similar among dietary treatments ($p = 0.60$ and 0.45, respectively) at week 12 (Table 2).

Salmon fed the 1.4% EPA+DHA/balanced diet had the highest level of total MUFA (Σ MUFA), while the 1% EPA+DHA $\uparrow \omega 3$ fed fish showed higher Σ MUFA than the 0.3% EPA+DHA $\uparrow \omega 6$ fish at week 12 ($p < 0.0001$). These differences were also observed in some individual MUFA (i.e., 18:1 $\omega 7$, 20:1 $\omega 11$, 20:1 $\omega 9$, 22:1 $\omega 11$; $p < 0.0001$). The 0.3% EPA+DHA $\uparrow \omega 3$ fish had higher 18:1 $\omega 9$ compared to fish fed the high $\omega 6$ diets (i.e., 0.3 and 1% EPA+DHA), and this FA was higher in the 1.4% EPA+DHA/balanced than the other treatments (not including 0.3% EPA+DHA $\uparrow \omega 3$; $p < 0.0001$). All treatments showed a significant decrease in Σ MUFA, 18:1 $\omega 9$ and other MUFA (i.e.,

TABLE 2 | Lipid and fatty acid composition (%) of Atlantic salmon head kidney^a before (Initial) and after 12 weeks of feeding diets with different ratios of $\omega 6:\omega 3$ and levels of EPA+DHA.

	Initial	0.3% EPA+DHA $\uparrow\omega 6$	0.3% EPA+DHA $\uparrow\omega 3$	1% EPA+DHA $\uparrow\omega 6$	1% EPA+DHA $\uparrow\omega 3$	1.4% EPA+DHA Balanced
Lipid classes composition (% of total lipid)						
TAG (%) ^b	38.9 ± 18.7	46.6 ± 12.5	48.5 ± 12.8	47.8 ± 9.8	47.0 ± 15.9	52.0 ± 14.7
ST (%) ^c	36.7 ± 15.2	<u>23.7 ± 6.9</u>	<u>20.5 ± 7.2</u>	<u>23.5 ± 6.0</u>	<u>21.8 ± 6.8</u>	<u>18.4 ± 6.8</u>
PL (%) ^d	23.5 ± 11.4	27.2 ± 14.1	28.6 ± 11.2	22.8 ± 9.1	28.2 ± 12.3	25.4 ± 11.0
TL (mg g ⁻¹) ^e	32.1 ± 12.0	26.9 ± 7.2	33.8 ± 9.0	31.9 ± 8.3	35.1 ± 14.7	36.2 ± 13.4
Fatty acid composition (% of total FA)						
14:0	2.4 ± 0.5	<u>0.9 ± 0.4^a</u>	<u>1.1 ± 0.3^a</u>	<u>1.4 ± 0.3^{ab}</u>	<u>1.7 ± 0.8^{bc}</u>	2.1 ± 0.9 ^c
16:0	15.4 ± 2.0	14.9 ± 1.2	14.1 ± 0.8	15.6 ± 3.2	14.9 ± 3.7	14.8 ± 2.7
16:1 $\omega 7$	5.6 ± 1.0	<u>2.1 ± 0.8^a</u>	<u>2.9 ± 0.5^{bc}</u>	<u>2.4 ± 0.8^{ab}</u>	<u>3.1 ± 1.0^{bc}</u>	<u>3.4 ± 0.9^c</u>
18:0	5.0 ± 0.7	5.6 ± 1.0 ^a	5.2 ± 0.4 ^{ab}	5.7 ± 1.9 ^a	4.9 ± 0.5 ^{ab}	4.4 ± 0.3 ^b
18:1 $\omega 9$	31.5 ± 4.7	<u>20.7 ± 3.1^a</u>	<u>24.8 ± 2.3^{bc}</u>	<u>20.0 ± 2.9^a</u>	<u>22.6 ± 2.5^{ab}</u>	<u>25.7 ± 2.5^c</u>
18:1 $\omega 7$	3.4 ± 0.4	<u>2.2 ± 0.2^a</u>	<u>2.3 ± 0.1^{ab}</u>	<u>2.4 ± 0.3^{ab}</u>	<u>2.4 ± 0.2^b</u>	3.0 ± 0.1 ^c
18:2 $\omega 6$ (LNA)	10.1 ± 1.1	<u>19.3 ± 3.1^a</u>	11.6 ± 1.2 ^c	<u>16.3 ± 3.6^b</u>	10.5 ± 1.4 ^c	9.7 ± 1.0 ^c
18:3 $\omega 6$	0.3 ± 0.1	<u>0.9 ± 0.2^a</u>	<u>0.5 ± 0.1^b</u>	<u>0.5 ± 0.1^b</u>	0.3 ± 0.1 ^c	0.3 ± 0.1 ^c
18:3 $\omega 3$ (ALA)	1.1 ± 0.3	1.9 ± 0.4 ^a	<u>6.5 ± 1.2^b</u>	1.7 ± 0.5 ^a	<u>6.3 ± 1.5^b</u>	1.7 ± 0.3 ^a
18:4 $\omega 3$	0.6 ± 0.2	0.7 ± 0.2 ^a	<u>1.8 ± 0.4^c</u>	0.7 ± 0.2 ^a	<u>1.5 ± 0.3^b</u>	<u>0.9 ± 0.2^a</u>
20:1 $\omega 11$	0.5 ± 0.1	<u>0.2 ± 0.1^a</u>	0.2 ± 0.3 ^a	<u>1.0 ± 0.3^b</u>	<u>0.9 ± 0.3^b</u>	<u>1.5 ± 0.4^c</u>
20:1 $\omega 9$	2.9 ± 0.5	<u>1.2 ± 0.4^a</u>	<u>1.4 ± 0.2^{ab}</u>	<u>1.6 ± 0.3^b</u>	<u>1.6 ± 0.5^b</u>	<u>2.1 ± 0.4^c</u>
20:2 $\omega 6$	0.6 ± 0.1	<u>1.4 ± 0.3^a</u>	0.7 ± 0.1 ^b	<u>1.2 ± 0.4^a</u>	0.6 ± 0.2 ^b	0.8 ± 0.1 ^b
20:3 $\omega 6$ (DGLA)	0.4 ± 0.1	<u>2.6 ± 0.7^a</u>	<u>1.0 ± 0.2^c</u>	<u>1.5 ± 0.5^b</u>	0.7 ± 0.2 ^d	0.7 ± 0.1 ^{cd}
20:4 $\omega 6$ (ARA)	1.2 ± 0.8	<u>4.3 ± 1.7^a</u>	<u>2.5 ± 0.6^b</u>	<u>2.4 ± 0.8^b</u>	2.0 ± 0.7 ^b	1.9 ± 0.6 ^b
20:3 $\omega 3$	0.1 ± 0.04	0.1 ± 0.03 ^a	<u>0.4 ± 0.1^b</u>	0.2 ± 0.03 ^a	<u>0.4 ± 0.1^b</u>	<u>0.2 ± 0.02^a</u>
20:4 $\omega 3$	0.4 ± 0.2	0.4 ± 0.1 ^a	<u>1.0 ± 0.2^c</u>	0.6 ± 0.1 ^{ab}	<u>0.9 ± 0.3^c</u>	<u>0.7 ± 0.2^b</u>
20:5 $\omega 3$ (EPA)	2.9 ± 1.7	2.6 ± 0.6 ^a	3.7 ± 0.7 ^b	3.8 ± 1.2 ^b	<u>4.4 ± 1.3^b</u>	<u>4.3 ± 1.1^b</u>
22:1 $\omega 11$	2.4 ± 0.4	<u>0.7 ± 0.3^a</u>	<u>0.8 ± 0.3^a</u>	<u>1.4 ± 0.3^b</u>	<u>1.4 ± 0.5^b</u>	<u>1.8 ± 0.5^c</u>
22:4 $\omega 6$	0.1 ± 0.05	<u>0.2 ± 0.1^a</u>	0.1 ± 0.01 ^b	0.2 ± 0.1 ^{ab}	0.1 ± 0.1 ^b	0.2 ± 0.2 ^{ab}
22:5 $\omega 6$ ($\omega 6$ DPA)	0.1 ± 0.1	<u>0.4 ± 0.1^a</u>	0.2 ± 0.05 ^b	0.2 ± 0.04 ^b	0.2 ± 0.05 ^b	0.2 ± 0.04 ^b
22:5 $\omega 3$ ($\omega 3$ DPA)	1.0 ± 0.5	0.8 ± 0.3 ^a	1.0 ± 0.1 ^{abc}	1.0 ± 0.4 ^{ab}	1.1 ± 0.4 ^{bc}	1.3 ± 0.4 ^c
22:6 $\omega 3$ (DHA)	6.8 ± 4.3	<u>12.4 ± 2.9</u>	<u>12.8 ± 3.0</u>	<u>13.5 ± 3.4</u>	<u>13.6 ± 3.8</u>	<u>14.0 ± 3.8</u>
Σ SFA ^f	23.8 ± 2.9	22.3 ± 2.2	21.2 ± 1.0	23.8 ± 5.6	22.4 ± 4.9	22.2 ± 3.7
Σ MUFA ^g	48.5 ± 6.9	<u>28.3 ± 4.7^a</u>	<u>33.8 ± 3.0^{ab}</u>	<u>30.7 ± 4.5^{ab}</u>	<u>33.7 ± 3.6^b</u>	<u>39.3 ± 3.7^c</u>
Σ PUFA ^h	27.3 ± 8.8	<u>49.0 ± 4.5^a</u>	<u>44.6 ± 2.4^{ab}</u>	<u>45.1 ± 8.3^{ab}</u>	<u>43.6 ± 5.0^b</u>	<u>38.1 ± 4.3^c</u>
$\Sigma \omega 3$	13.1 ± 7.2	<u>19.1 ± 3.0^a</u>	<u>27.4 ± 2.9^c</u>	<u>21.7 ± 5.0^{ab}</u>	<u>28.5 ± 4.8^c</u>	<u>23.4 ± 4.4^b</u>
$\Sigma \omega 6$	12.9 ± 1.9	<u>28.9 ± 3.4^a</u>	<u>16.4 ± 0.8^c</u>	<u>22.1 ± 4.6^b</u>	14.3 ± 1.2 ^{cd}	13.6 ± 0.5 ^d
$\omega 6:\omega 3$	1.2 ± 0.7	<u>1.5 ± 0.3^a</u>	<u>0.6 ± 0.1^c</u>	<u>1.0 ± 0.2^b</u>	<u>0.5 ± 0.1^c</u>	<u>0.6 ± 0.1^c</u>
EPA+DHA	9.7 ± 6.0	<u>15.0 ± 3.3</u>	<u>16.5 ± 3.4</u>	<u>17.3 ± 4.4</u>	<u>18.0 ± 4.8</u>	<u>18.3 ± 4.6</u>
DHA:EPA	2.4 ± 0.2	<u>4.9 ± 1.1^a</u>	<u>3.4 ± 0.6^b</u>	<u>3.7 ± 0.7^b</u>	3.2 ± 0.7 ^b	<u>3.3 ± 0.5^b</u>
EPA:ARA	2.4 ± 0.6	<u>0.7 ± 0.3^a</u>	<u>1.6 ± 0.3^b</u>	<u>1.6 ± 0.3^b</u>	2.2 ± 0.4 ^c	2.3 ± 0.5 ^c

^aMean ($n = 10-20$) ± standard deviation (SD). Different superscripts in the same row indicate significant differences among treatments at week 12. Underlining indicates values that are significantly different to week 0 (Initial) ($p < 0.05$).

^bTriacylglycerol.

^cSterols.

^dPhospholipids.

^eTotal lipids.

^fTotal saturated fatty acids.

^gTotal monounsaturated fatty acids.

^hTotal polyunsaturated fatty acids.

16:1 $\omega 7$, 18:1 $\omega 7$, 20:1 $\omega 9$, 22:1 $\omega 11$) when comparing week 12 to week 0 ($p < 0.0001$).

The levels of LC-PUFA precursors 18:2 $\omega 6$ and 18:3 $\omega 3$ reflected experimental diet levels (i.e., highest 18:2 $\omega 6$ in the

high $\omega 6$ fed salmon; highest 18:3 $\omega 3$ in the high $\omega 3$ fed salmon; $p < 0.0001$; **Table 2**). Further, compared with week 0, 18:2 $\omega 6$ and 18:3 $\omega 3$ increased after 12 weeks of feeding high $\omega 6$ (i.e., 0.3% EPA+DHA $\uparrow\omega 6$ and 1% EPA+DHA $\uparrow\omega 6$) and high $\omega 3$ (0.3%

EPA+DHA $\uparrow\omega 3$ and 1% EPA+DHA $\uparrow\omega 3$) diets, respectively ($p < 0.0001$; **Table 2**). The intermediate $\omega 6$ (18:3 $\omega 6$, 20:2 $\omega 6$ and 20:3 $\omega 6$) and $\omega 3$ PUFA (18:4 $\omega 3$, 20:3 $\omega 3$ and 20:4 $\omega 3$), and the $\omega 6:\omega 3$ ratios were different among treatments at week 12 ($p < 0.0001$) and responded to dietary changes in $\omega 6$ and $\omega 3$ PUFA. The proportion of 20:4 $\omega 6$ was highest in fish fed 0.3% EPA+DHA $\uparrow\omega 6$ diet ($p < 0.0001$), while no significant differences were observed among the remaining four treatments at week 12. In contrast, the 0.3% EPA+DHA $\uparrow\omega 6$ fish showed the lowest 20:5 $\omega 3$ ($p < 0.0001$), and similar levels of this $\omega 3$ LC-PUFA were observed among the remaining four treatments ($p = 0.18$) at week 12. Further, after 12 weeks of feeding, no differences were shown among dietary treatments in 22:6 $\omega 3$ ($p = 0.53$). Compared with week 0, 20:4 $\omega 6$ showed an increase after 12 weeks of feeding high $\omega 6$ (i.e., 0.3 and 1% EPA+DHA) and the 0.3% EPA+DHA $\uparrow\omega 3$ diet, while 20:5 $\omega 3$ increased in the 1% EPA+DHA $\uparrow\omega 3$ and 1.4% EPA+DHA/balanced fed fish. All 5 treatments increased in 22:6 $\omega 3$ at week 12 compared to week 0 ($p < 0.0001$). Fish fed the 0.3% EPA+DHA $\uparrow\omega 6$ diet had the highest $\omega 6:\omega 3$, and the high $\omega 6$ fish (i.e., 0.3 and 1% EPA+DHA) had higher $\omega 6:\omega 3$ compared to high $\omega 3$ (i.e., 0.3 and 1% EPA+DHA) and 1.4% EPA+DHA/balanced fed fish at week 12 ($p < 0.0001$). The DHA:EPA ratio was higher in the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish compared with the other four treatments at week 12 ($p < 0.0001$; **Table 2**). Finally, EPA:ARA was highest in the 1% EPA+DHA $\uparrow\omega 3$ and the 1.4% EPA+DHA/balanced treatments, and lowest in the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish ($p < 0.0001$).

PCoA showed a clear separation between the high $\omega 6$ and high $\omega 3$ treatments (i.e., 0.3% EPA+DHA $\uparrow\omega 6$ and 1% EPA+DHA $\uparrow\omega 6$ on top, and 0.3% EPA+DHA $\uparrow\omega 3$ and 1% EPA+DHA $\uparrow\omega 3$ on the bottom; **Figure 1**). The 1.4% EPA+DHA/balanced fed fish clustered with the high $\omega 3$ treatments. PCO1 and PCO2 accounted for 49.8 and 30.3% of the variability, respectively. PERMANOVA pairwise tests showed that the 0.3% EPA+DHA $\uparrow\omega 3$ were not different from 1% EPA+DHA $\uparrow\omega 3$ fed fish [$p(\text{perm}) = 0.12$]. However, all other treatments were significantly different from each other [$p(\text{perm}) = 0.0001\text{--}0.003$] (**Supplementary Table 3**). PCoA vectors showed that fish fed high $\omega 6$ diets (particularly 0.3% EPA+DHA $\uparrow\omega 6$) were associated with 18:2 $\omega 6$, 20:2 $\omega 6$, 20:3 $\omega 6$, 20:4 $\omega 6$, and $\Sigma\omega 6$, while the high $\omega 3$ and 1.4% EPA+DHA/balanced diet fed fish were associated with 18:3 $\omega 3$, 20:3 $\omega 3$, 20:4 $\omega 3$, 20:5 $\omega 3$, $\Sigma\omega 3$, EPA:ARA, the MUFA 18:1 $\omega 7$, 16:1 $\omega 7$, 18:1 $\omega 9$ and 20:1 $\omega 9$, as well as ΣMUFA (**Figure 1**). The lipid classes ST and PL clustered with the high $\omega 6$ and high $\omega 3$ (including 1.4% EPA+DHA/balanced) treatments, respectively.

SIMPER analysis indicated that the dissimilarity between 0.3% EPA+DHA $\uparrow\omega 6$ and 0.3% EPA+DHA $\uparrow\omega 3$, and between 0.3% EPA+DHA $\uparrow\omega 6$ and 1% EPA+DHA $\uparrow\omega 6$ were mainly caused by $\Sigma\omega 6$, $\Sigma\omega 3$, and the lipid classes TAG, PL and ST (**Supplementary Table 4**). The main FA contributors to the dissimilarities between the remaining high $\omega 6$ and high $\omega 3$ comparisons were $\Sigma\omega 6$ and 18:2 $\omega 6$. Finally, the FA dissimilarity between 1.4% EPA+DHA/balanced and the high $\omega 6$ treatments was mainly driven by $\Sigma\omega 6$, ΣPUFA and ΣMUFA , while ΣPUFA contributed to the FA dissimilarity

between 1.4% EPA+DHA/balanced and the high $\omega 3$ treatments (**Supplementary Table 4**). This analysis also showed that the highest dissimilarity in lipid composition profile was between 0.3% EPA+DHA $\uparrow\omega 6$ and 1.4% EPA+DHA/balanced fed fish (average dissimilarity = 18.7%), while the lowest dissimilarity was between the two high $\omega 3$ (i.e., 0.3% EPA+DHA $\uparrow\omega 3$ and 1% EPA+DHA $\uparrow\omega 3$; average dissimilarity = 10.0%) and the two high $\omega 6$ (i.e., 0.3% EPA+DHA $\uparrow\omega 6$ and 1% EPA+DHA $\uparrow\omega 6$; average dissimilarity = 11.0%) treatments.

QPCR Analysis of Transcripts Involved in Fatty Acid Metabolism and Eicosanoid Synthesis

Dietary Impacts on Transcript Expression

One-way ANOVA indicated that salmon fed 0.3% EPA+DHA $\uparrow\omega 3$, 1.4% EPA+DHA/balanced, and the 1% EPA+DHA diets had higher expression levels (1.31–1.40-fold; $p = 0.01$) of the transcript *elovl5a* compared with the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish (**Figure 2B** and **Supplementary Table 5**). The head kidney transcript expression of *fadsd5* was higher in the 1% EPA+DHA $\uparrow\omega 3$ compared with the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish (1.62-fold; $p = 0.04$; **Figure 2C** and **Supplementary Table 5**), while *srebp1* mRNA levels were higher in the 1% EPA+DHA $\uparrow\omega 3$ and 1.4% EPA+DHA/balanced treatments compared with the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish (1.48- and 1.47-fold, respectively; $p = 0.02$; **Figure 2D** and **Supplementary Table 5**). The transcript *ppara* showed lower expression in the 0.3% EPA+DHA $\uparrow\omega 3$ compared with the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish (0.37-fold; $p = 0.02$; **Figure 2G** and **Supplementary Table 5**). The prostaglandin synthesis-related transcript *ptges3* showed an increasing trend (although not significant) in the 0.3% EPA+DHA $\uparrow\omega 3$ compared with the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish (1.30-fold; $p = 0.06$; **Supplementary Table 5**). Finally, the transcript expression of *elovl4b*, *srebp2* and *lxrb* (**Figures 2A,E,F**, respectively, and **Supplementary Table 5**) was not significantly impacted by diet.

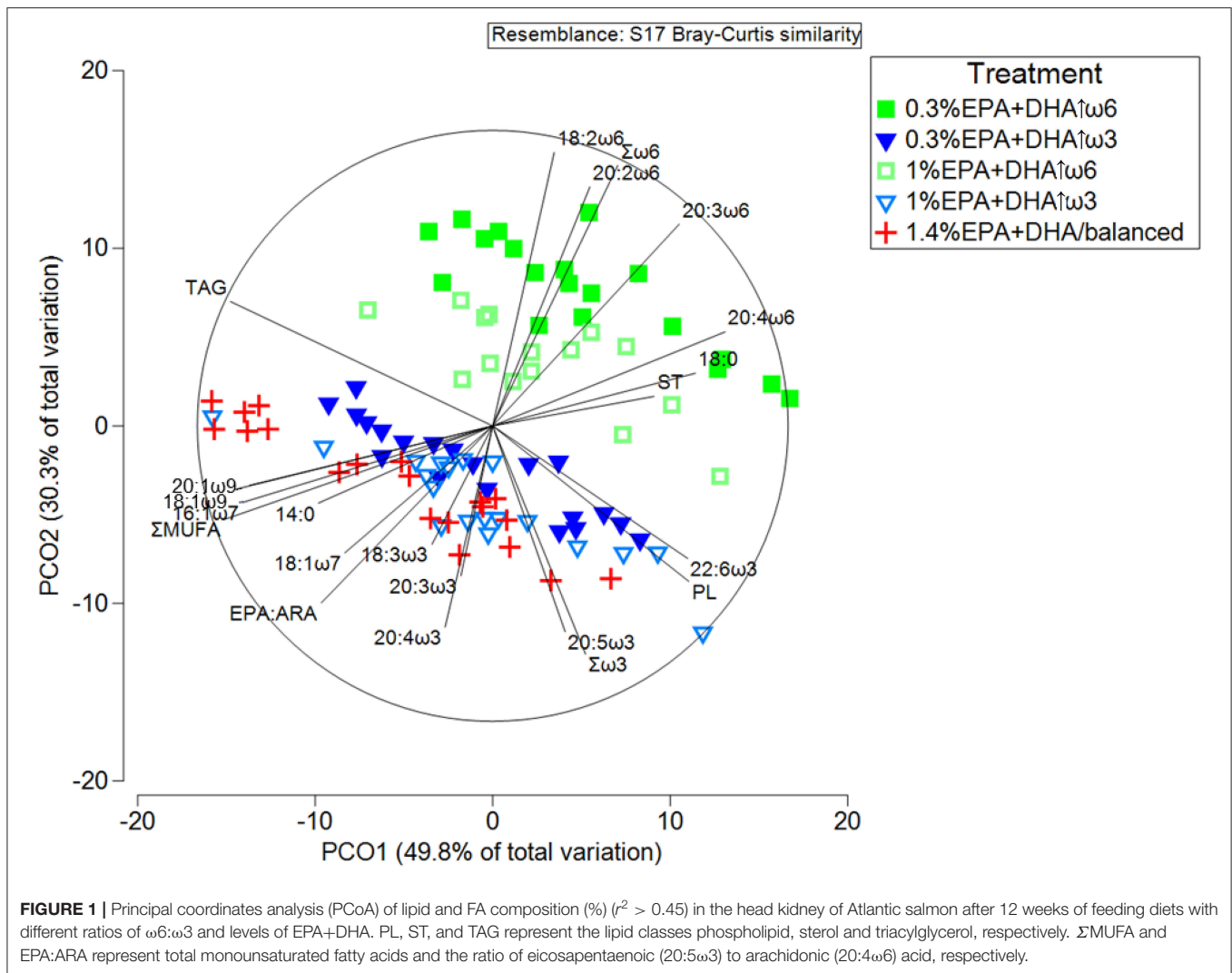
Effects of EPA+DHA and $\omega 6:\omega 3$ Factors on Transcript Expression

Two-way ANOVA analysis showed a significant interaction between the dietary factor EPA+DHA and $\omega 6:\omega 3$, impacting the transcript expression of *elovl4b* (**Figure 2I** and **Supplementary Table 6**). The transcript expression of *elovl5a* was significantly affected by both EPA+DHA and $\omega 6:\omega 3$, while *fadsd5*, *srebp1*, *srebp2* and *lxrb* were significantly affected by dietary EPA+DHA levels. Finally, the transcript expression of *ppara* and *cox1* was significantly affected by the factor $\omega 6:\omega 3$ (**Figure 2I** and **Supplementary Table 6**).

Hierarchical Clustering and Pearson Correlation Analyses

Hierarchical Clustering of Head Kidney Transcript Expression and Lipid Composition

Hierarchical clustering of the qPCR analysed transcripts identified three separate clusters (**Figure 3**). Cluster I consisted of the transcript *pparb2a*, and the eicosanoid metabolism-related



transcripts *ptges3*, *ptges2*, *5loxa*, *5loxb*, and *cox1*. Cluster II included *pparaa* and *lxra*, while cluster III included the lipid metabolism-related transcripts *srebp2*, *elovl5a*, *srebp1*, *acox1*, *fadsd5*, and the eicosanoid synthesis-related *cox2* and *pgds*. Cluster analysis of head kidney lipid composition also showed three clusters. Cluster I was composed of the $\omega 3$ FA (i.e., 20:5 $\omega 3$, 22:6 $\omega 3$, 20:4 $\omega 3$, 18:4 $\omega 3$, 18:3 $\omega 3$, 20:3 $\omega 3$, and 22:5 $\omega 3$), $\Sigma \omega 3$, 18:1 $\omega 9$, Σ MUFA, EPA:ARA, and the lipid class TAG. Cluster II included 18:0, 16:0, Σ SFA and 22:4 $\omega 6$. Cluster III showed the $\omega 6$ FA (i.e., 18:3 $\omega 6$, 20:2 $\omega 6$, 18:2 $\omega 6$, 22:5 $\omega 6$, 20:3 $\omega 6$, 20:4 $\omega 6$), $\Sigma \omega 6$, $\omega 6:\omega 3$, as well as Σ PUFA and the lipid class ST (Figure 3).

Correlations Between Lipid Composition and Fatty Acid Metabolism Transcripts

The head kidney transcript expression of *pparb2a* was correlated positively with 20:4 $\omega 3$ ($p = 0.031$; Figure 3), while *pparaa* showed negative correlations with several $\omega 3$ FA (i.e., 18:3 $\omega 3$, 18:4 $\omega 3$, 20:3 $\omega 3$, 20:4 $\omega 3$) and $\Sigma \omega 3$ ($p = 0.002$ – 0.044), and positive correlations with several $\omega 6$ FA (i.e., 18:2 $\omega 6$, 20:2 $\omega 6$,

20:3 $\omega 6$, 20:4 $\omega 6$, 22:5 $\omega 6$), $\Sigma \omega 6$, and the ratio of $\omega 6:\omega 3$ ($p = 0.009$ – 0.038). The five transcripts *lxra*, *srebp2*, *elovl5a*, *srebp1*, and *fadsd5* showed positive correlations with EPA:ARA ($p = 0.001$ – 0.032 ; Figure 3). The mRNA levels of *elovl5a* also showed positive correlations with 20:3 $\omega 3$, 20:4 $\omega 3$ and Σ MUFA ($p = 0.013$ – 0.044), while *srebp1* correlated positively with 22:5 $\omega 3$ and Σ MUFA and Σ SFA ($p = 0.006$ – 0.047). The transcript *fadsd5* correlated positively with Σ MUFA, Σ SFA, and the FA 16:0 and 18:0 ($p = 0.001$ – 0.032). The same five transcripts (i.e., *lxra*, *srebp2*, *elovl5a*, *srebp1*, and *fadsd5*) and *acox1* correlated negatively with $\omega 6$ FA; *lxra* with 20:2 $\omega 6$, 20:3 $\omega 6$ and $\Sigma \omega 6$ ($p = 0.021$ – 0.042), and *srebp2* with 18:3 $\omega 6$, 20:3 $\omega 6$, 20:4 $\omega 6$, 22:5 $\omega 6$, as well as $\Sigma \omega 6$ and Σ PUFA ($p = 0.0001$ – 0.041). *Elovl5a*, *srebp1*, and *fadsd5* correlated negatively with 18:2 $\omega 6$, 18:3 $\omega 6$, 20:2 $\omega 6$, 20:3 $\omega 6$, 20:4 $\omega 6$, 22:5 $\omega 6$, $\Sigma \omega 6$, $\omega 6:\omega 3$, and Σ PUFA ($p = 0.0001$ – 0.028). The transcript *acox1* showed negative correlations with 18:3 $\omega 6$ and 22:5 $\omega 6$ ($p = 0.011$ and 0.016 , respectively; Figure 3).

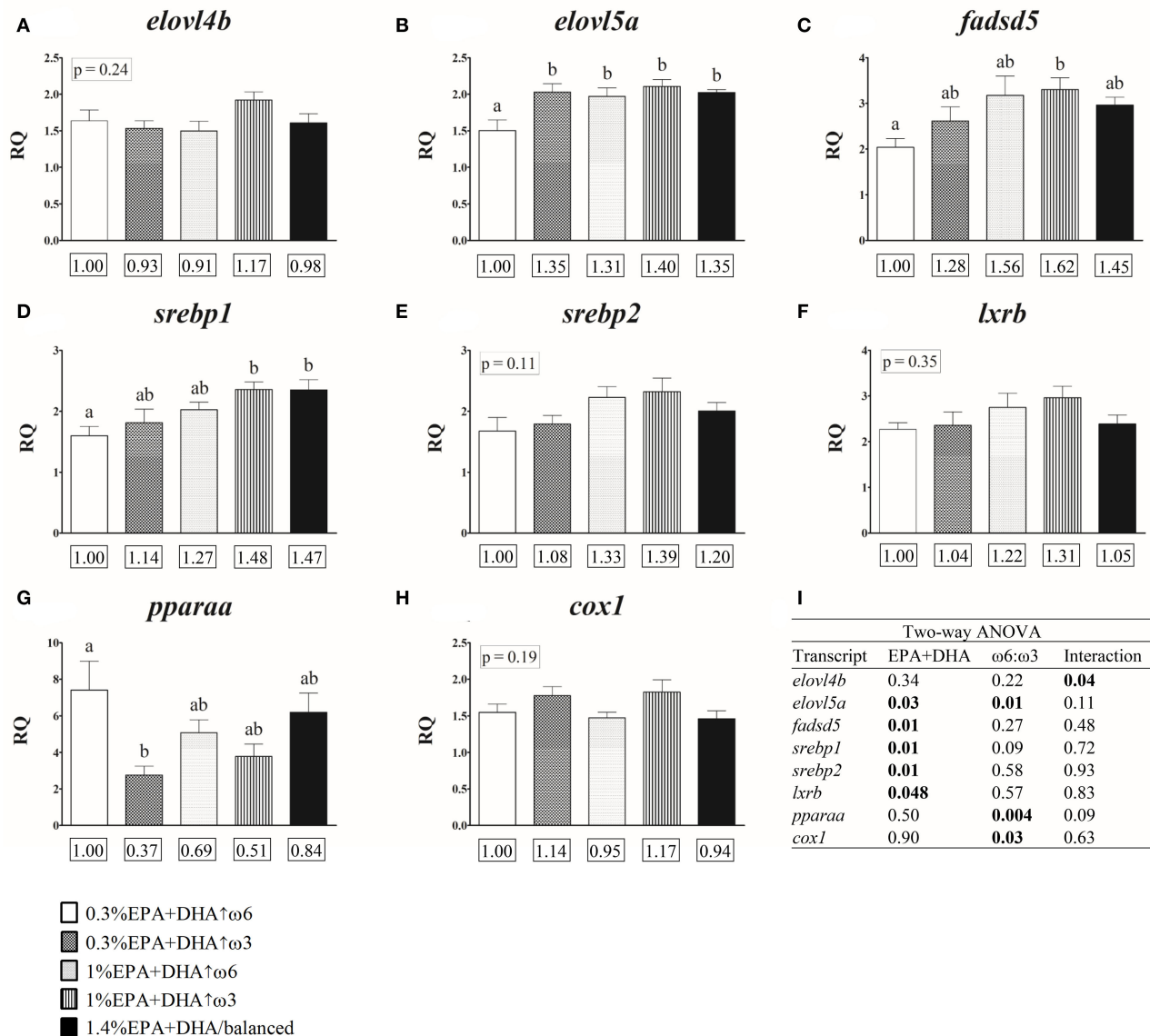
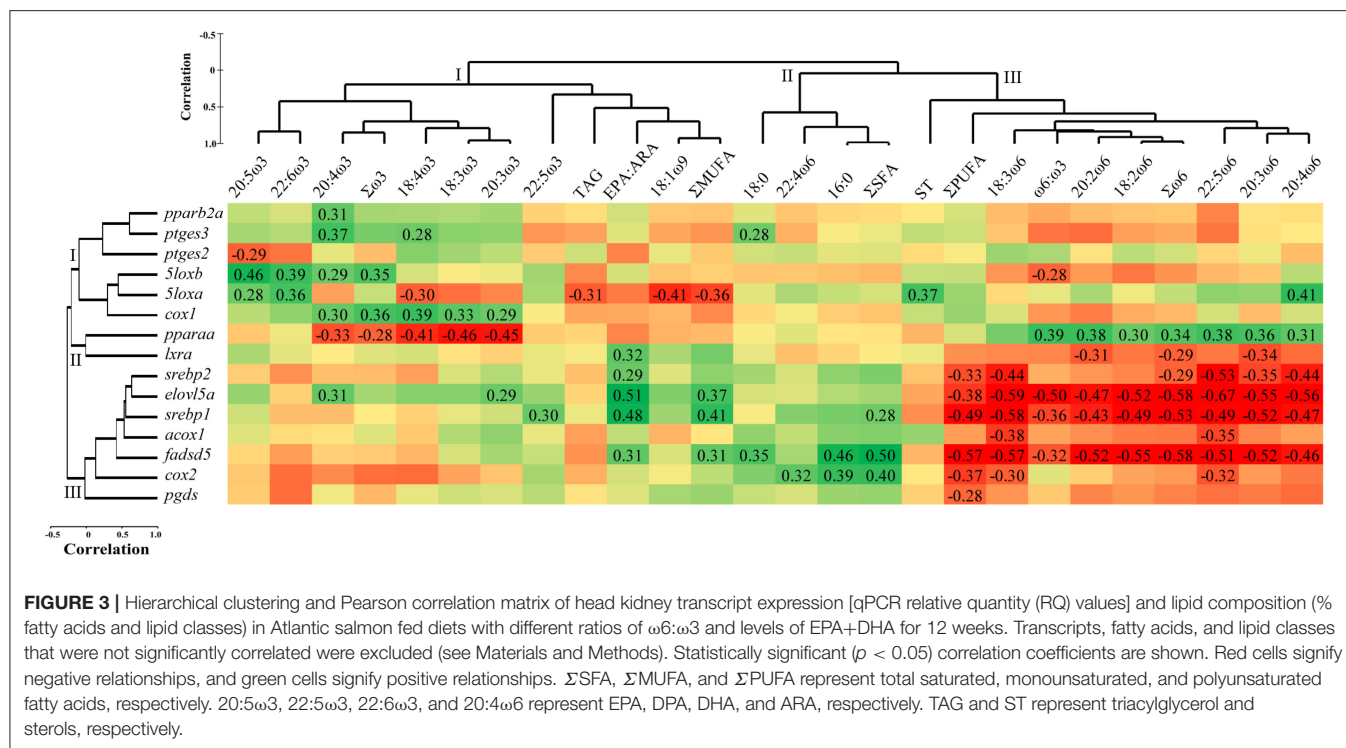


FIGURE 2 | Head kidney qPCR transcripts related to fatty acid metabolism (A–G) and eicosanoid synthesis (H) in salmon fed diets with different ratios of $\omega 6:\omega 3$ and levels of EPA+DHA for 12 weeks. Transcripts identified as statistically significant in the two-way ANOVA analysis are presented (A–I). Transcript expression data of these and other genes of interest (GOIs) are presented in **Supplementary Table 5**. Transcript expression values presented as mean relative quantity (RQ) \pm SE ($n = 7-8$). Different letters above error bars indicate significant differences among treatments. When differences were not statistically significant (one-way ANOVA and Tukey *post-hoc* tests; $p > 0.05$), p -values are shown above error bars. Values below each GOI represent fold-changes relative to the 0.3% EPA+DHA $\uparrow \omega 6$ treatment (see Materials and Methods). (I) Two-way ANOVA results. Significant differences ($p < 0.05$) are shown in bold font. Two-way ANOVA analysis of the entire set of GOIs is presented in **Supplementary Table 6**.

Correlations Between Lipid Composition and Eicosanoid Metabolism Transcripts

The transcript expression of *ptges3* was correlated positively with 18:4 $\omega 3$, 20:4 $\omega 3$ and 18:0 ($p = 0.012-0.049$; **Figure 3**), while *ptges2* showed a negative correlation with 20:5 $\omega 3$ ($p = 0.041$). The transcript expressions of *5loxa* and *5loxb* showed positive correlations with 20:5 $\omega 3$ and 22:6 $\omega 3$ ($p = 0.002-0.045$). *5loxb* also correlated positively with 20:4 $\omega 3$ and $\Sigma \omega 3$ ($p = 0.042$ and

0.017, respectively), and negatively with $\omega 6:\omega 3$ ($p = 0.044$), while *5loxa* correlated positively with ST and 20:4 $\omega 6$ ($p = 0.012$ and 0.006, respectively), and negatively with 18:4 $\omega 3$, TAG, 18:1 $\omega 9$ and Σ MUFA ($p = 0.006-0.038$; **Figure 3**). The mRNA levels of *cox1* showed positive correlations with the $\omega 3$ FA: 18:3 $\omega 3$, 18:4 $\omega 3$, 20:3 $\omega 3$, 20:4 $\omega 3$ and $\Sigma \omega 3$ ($p = 0.009-0.043$). The transcript *cox2* correlated positively with 16:0, Σ SFA and 22:4 $\omega 6$ ($p = 0.007-0.029$), and negatively with 18:3 $\omega 6$, 22:5 $\omega 6$ and



Σ PUFA ($p = 0.013$ – 0.037). Finally, *pgds* correlated negatively with Σ PUFA ($p = 0.045$; **Figure 3**).

DISCUSSION

Head Kidney Lipid Composition

The head kidney was mainly composed of TAG (46.6–52.0%), and this was followed by the lipid classes PL (22.8–28.6%), and ST (18.4–23.7%) (**Table 2**). Our lipid class (i.e., TAG and ST) proportions are in the range of what was found in earlier studies on Atlantic salmon head kidney (Martinez-Rubio et al., 2013; Foroutani et al., 2020). Similar to our findings, Foroutani et al. (2020) reported that TAG was the predominant lipid class in Atlantic salmon head kidney. Head kidney is a principal component of the immune system and the major site of haematopoiesis in fish (Tort et al., 2003; Zapata et al., 2006). Therefore, high levels of TAG may allow fish to fulfill the energy required to fuel cell differentiation processes and other immune functions. Interestingly, Thompson et al. (1995) reported that TAG proportions in Atlantic salmon head kidney leucocytes, macrophages, T- and B-cells varied between ~14 and 20%, and indicated that TAG may serve as an energy store and/or a source of free fatty acids that are used in cellular functions in salmon. Our study also showed that lipid class composition [when expressed as % or as concentration (mg g^{-1} wet weight)] was not significantly affected by diet (**Table 2**, **Supplementary Table 7**). This result is in agreement with Betancor et al. (2014) who reported that dietary variation in LC-PUFA did not impact head kidney lipid classes in Atlantic salmon. However, the current study revealed that PL proportions were correlated positively

with 20:5 $\omega 3$ and negatively with 18:2 $\omega 6$, 20:2 $\omega 6$, and $\omega 6:\omega 3$ in the head kidney (**Supplementary Table 8**). Further, PCoA indicated associations between PL and the $\omega 3$ PUFA 22:6 $\omega 3$, 20:5 $\omega 3$, and $\Sigma\omega 3$ (**Figure 1**). These data suggest a preferential incorporation into the membrane of $\omega 3$ over $\omega 6$ PUFA. This can potentially increase the availability of anti-inflammatory eicosanoids in the head kidney (Martinez-Rubio et al., 2013). However, this can only be postulated, as fatty acid composition of the PL portion of the head kidney was not determined in our study.

The fatty acid composition of the head kidney reflected that of the experimental diets. For example, LC-PUFA precursor composition (i.e., 18:2 $\omega 6$ and 18:3 $\omega 3$) and $\omega 6:\omega 3$ ratio of the head kidney reflected the dietary composition (i.e., highest 18:2 $\omega 6$ and $\omega 6:\omega 3$ in the high $\omega 6$ fed fish; highest 18:3 $\omega 3$ in the high $\omega 3$ fed fish). Previous studies also indicated that head kidney lipid composition of Atlantic salmon (Gjøen et al., 2004; Foroutani et al., 2020), gilthead seabream (*Sparus aurata*) (Montero et al., 2003) and Nile tilapia (*Oreochromis niloticus*) (Chen et al., 2016) reflected dietary variation in 18:2 $\omega 6$ and 18:3 $\omega 3$. Gjøen et al. (2004) demonstrated that dietary replacement of FO with soy oil (i.e., 50%) modified head kidney $\omega 6:\omega 3$ (from 0.3 to 1.1), which reflected the dietary $\omega 6:\omega 3$. These ratios are comparable to the $\omega 6:\omega 3$ detected in our study (i.e., 0.5–1.5). At week 12, the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish had 1.7–2.3-fold more 20:4 $\omega 6$ in terms of proportions and 1.4–1.8-fold in terms of concentrations, when compared with the other treatments. However, 20:5 $\omega 3$ proportions (0.6–0.7-fold) and concentrations (0.4–0.6-fold) were lowest in this group, compared to the other treatments. Additionally, both

proportions and concentrations of 20:5 ω 3 were similar between the 0.3% EPA+DHA $\uparrow\omega$ 3 and the 1% EPA+DHA $\uparrow\omega$ 3 fed fish. A similar effect on head kidney 20:4 ω 6 proportions was shown in a previous study where soy oil fed Atlantic salmon had 3-fold higher 20:4 ω 6 than FO fed fish (Gjøen et al., 2004). Furthermore, the differences in 20:4 ω 6 and 20:5 ω 3 proportions are in line with our previous study with Atlantic salmon muscle (Emam et al., 2020). Taken together, these data highlight the importance of dietary LC-PUFA precursors (i.e., 18:2 ω 6 and 18:3 ω 3) when salmon are fed with lower inclusion of EPA and DHA (0.3%), and their impact on head kidney LC-PUFA. Although LC-PUFA synthesis was not quantified in our study, the fact that intermediate ω 6 and ω 3 PUFAs were also significantly different among dietary treatments, and their head kidney proportions were higher than those of the diet, suggest that the LC-PUFA synthesis pathway was activated. Interestingly, 22:6 ω 3 proportions were not affected by diet at week 12, and 22:6 ω 3 concentrations (within the high ω 3 and high ω 6 treatments) did not vary with increasing dietary EPA+DHA levels. This suggests that in addition to elongation and desaturation, selective retention had also occurred. Indeed, earlier studies indicated higher 22:6 ω 3 retention in Atlantic salmon with decreasing dietary EPA+DHA (Glencross et al., 2014; Bou et al., 2017). However, the fact that 1% EPA+DHA/high $\uparrow\omega$ 3 and 1.4% EPA+DHA/balanced treatments showed higher concentration of 22:6 ω 3 than the 0.3% EPA+DHA/high $\uparrow\omega$ 6 fed fish suggests that both dietary EPA+DHA and ω 6: ω 3 influenced head kidney DHA content.

Salmon fed the 1.4% EPA+DHA/balanced diet had the highest level of Σ MUFA, and the 1% EPA+DHA $\uparrow\omega$ 3 fed fish showed higher Σ MUFA than the 0.3% EPA+DHA $\uparrow\omega$ 6 fed fish. These differences in Σ MUFA, and the observed changes in individual MUFA (i.e., 16:1 ω 7, 18:1 ω 7, 18:1 ω 9 and 20:1 ω 9) were also a reflection of the diets. Notably, the 1.4% EPA+DHA/balanced diet contained rapeseed oil which is rich in 18:1 ω 9 (Orsavova et al., 2015), and this may have influenced the observed changes in tissue MUFA. Emam et al. (2020) reported a similar response in salmon muscle, and showed that hepatic MUFA synthesis-related transcript *scdb* was positively correlated with muscle 20:1 ω 7 and 20:1 ω 9, among other MUFA. This suggests that head kidney MUFA differences between treatments in our study were also related to changes in MUFA synthesis. However, further analyses are required in order to test this hypothesis (e.g., transcript expression of MUFA synthesis-related genes in the head kidney).

Transcript Expression of Lipid Metabolism-Related Genes

Transcripts Involved in LC-PUFA Synthesis

Transcript expression levels of key genes encoding enzymes involved in LC-PUFA synthesis were modulated by diet in our study. Salmon fed with 0.3% EPA+DHA $\uparrow\omega$ 6 had the lowest expression of *elovl5a*, while the remaining four treatments had similar levels of this transcript (Figure 2B and Supplementary Table 5). The transcript expression of other genes encoding elongases (*elovl4b*) and desaturases

(*fadsd5*, *fadsd6b* and *fadsd6c*) was also similar among 0.3% EPA+DHA $\uparrow\omega$ 3, 1% EPA+DHA (both high ω 3 and high ω 6) and 1.4% EPA+DHA/balanced fed fish (Supplementary Table 5). Further, *elovl5a* was correlated positively with ω 3 (20:3 ω 3 and 20:4 ω 3), while both *elovl5a* and *fadsd5* were correlated negatively with ω 6 PUFA (18:2 ω 6, 18:3 ω 6, 20:2 ω 6, 20:3 ω 6, 20:4 ω 6, 22:5 ω 6) in the head kidney. These transcript expression changes along with the fatty acid data suggest that 20:5 ω 3 synthesis was lower in fish fed lower dietary levels of 18:3 ω 3 and EPA+DHA (i.e., 0.3%), and further support the notion that higher dietary 18:3 ω 3 promoted the synthesis of ω 3 LC-PUFA in the head kidney. Indeed, LC-PUFA synthesis in salmonids is influenced by the availability of tissue LC-PUFA (i.e., EPA, DHA, ARA) and the precursors 18:3 ω 3 and 18:2 ω 6 (Jordal et al., 2005; Jump et al., 2005; Glencross et al., 2015; Katan et al., 2019). *Fadsd5* expression was higher with 1% EPA+DHA $\uparrow\omega$ 3 compared with 0.3% EPA+DHA $\uparrow\omega$ 6 fed fish, while *srebp1* showed higher expression levels in the 1% EPA+DHA $\uparrow\omega$ 3 and 1.4% EPA+DHA/balanced treatments compared with the 0.3% EPA+DHA $\uparrow\omega$ 6 fed fish. These transcripts, as well as *srebp2* and *lxrb*, were significantly positively impacted by dietary EPA+DHA levels (Figures 2C,D,I). Our results are in contrast with previous studies reporting up-regulation of hepatic *fadsd5* in Atlantic salmon fed plant-based (containing lower EPA and DHA) compared to FO diets (Zheng et al., 2004; Morais et al., 2011; Xue et al., 2015). However, our data is in agreement with Betancor et al. (2014) who reported up-regulation of head kidney *srebp1* and a \sim 1.5-fold increase in *fadsd5* in Atlantic salmon fed higher dietary levels of EPA+DHA (2% compared to 1% of diet). FO diets used in the former studies contained higher proportions of EPA+DHA (\sim 11–24% of total FA) and higher inclusions of FO (\sim 14–30% of diet) compared to our study (EPA+DHA proportions and FO inclusions were 2.8–8.8% and 0.1–6.8%, respectively). These as well as organ (i.e., liver vs. head kidney) differences may explain the discrepancies between studies. Further, dietary ratios of ALA:LNA were shown to influence LC-PUFA accumulation in salmonids and African catfish muscle tissues (Colombo et al., 2018; Sourabié et al., 2019), and this may have played a role in the regulation of LC-PUFA synthesis in our study. Finally, the positive impact of dietary EPA+DHA levels in our study could be related to MUFA levels, as the transcript expression of several lipid metabolism genes (*elovl5a*, *fadsd5*, and *srebp1*) showed a positive correlation with head kidney Σ MUFA (Figure 3). Interestingly, Emam et al. (2020) showed that hepatic *elovl5a* and *srebp2* were positively correlated with the levels of individual MUFA in salmon muscle. Indeed, an earlier study with Atlantic salmon revealed that LC-PUFA synthesis is stimulated by dietary MUFA levels, and suggested that the sparing effect of SFA and MUFA on ω 3 LC-PUFA could be the underlying mechanism (Emery et al., 2016).

The transcript expression of head kidney *ppara* was higher in the 0.3% EPA+DHA $\uparrow\omega$ 6 compared with the 0.3% EPA+DHA $\uparrow\omega$ 3 fed fish (Figure 2G), and correlation analysis indicated that this transcript was positively correlated with ω 6 PUFA (18:2 ω 6, 20:2 ω 6, 20:3 ω 6, 20:4 ω 6, 22:5 ω 6), $\Sigma\omega$ 6 and ω 6: ω 3, and negatively with ω 3 PUFA (18:3 ω 3, 18:4 ω 3, 20:3 ω 3, 20:4 ω 3) and $\Sigma\omega$ 3 (Figure 3). *Ppara* is a transcription factor

that regulates numerous genes involved in lipid-related processes such as FA oxidation, as well as bile acid and triacylglycerol metabolism (Kersten, 2014). Further, several studies in fish demonstrated involvement of *Ppara* in LC-PUFA synthesis (Dong et al., 2017; Zhu et al., 2019). Thus, our data suggest that *pparaa* may have played a regulatory role in $\omega 6$ PUFA synthesis, particularly in the 0.3% EPA+DHA $\uparrow\omega 6$ fed fish. Emam et al. (2020) showed that hepatic *pparaa* expression was higher in the 0.3% EPA+DHA $\uparrow\omega 3$ than the 0.3% EPA+DHA $\uparrow\omega 6$ and 1% EPA+DHA treatments (i.e., high $\omega 3$ and high $\omega 6$), and also a positive correlation between liver *pparaa* and muscle $\omega 3$ PUFA. These contrasting results suggest that *pparaa* response in Atlantic salmon is tissue dependent. Finally, the fact that the transcript expression of *pparaa* was significantly impacted by dietary $\omega 6:\omega 3$ (Figure 2I), and the correlation with head kidney $\omega 6:\omega 3$ (Figure 3), suggest that this transcript may have been involved with inflammatory processes in our study. This is in line with previous studies with mammals (Jones et al., 2002; Becker et al., 2008; Varga et al., 2011) and Atlantic salmon (Martinez-Rubio et al., 2013) which showed that *Ppara* and *ppara* play a role in the resolution of inflammation. More studies are required in order to investigate the dietary-induced impacts of *ppara* on inflammatory processes in Atlantic salmon head kidney.

Transcripts Involved in Eicosanoid Metabolism

Our qPCR results (Supplementary Table 5) are in line with previous studies which indicated no differences in head kidney constitutive transcript expression of several genes related to eicosanoid synthesis (i.e., *5lox*, *cox2*) in fish exposed to varying EPA levels [i.e., 0–20 μ M (Furne et al., 2013), 1.1 and 19 mg g⁻¹ lipid (Salini et al., 2016)] or $\omega 6:\omega 3$ ratios (0.7–4.1) (Holen et al., 2018). However, Holen et al. (2018) reported that Atlantic salmon fed with soybean oil as a FO replacement had higher expression of *pgds* and *ptes* in head kidney leucocytes compared to fish fed palm and rapeseed oils. Differences in dietary and tissue EPA:ARA, EPA+DHA, the tissues/cells examined (head kidney vs. isolated leucocytes), and variation between *in vivo* and *in vitro* models could have contributed to these discrepancies between studies. Further, our study revealed that head kidney transcript expression of *cox1* was negatively impacted by dietary $\omega 6:\omega 3$ levels (Figures 2H,I), and positively correlated with head kidney $\omega 3$ fatty acids (i.e., 18:3 $\omega 3$, 18:4 $\omega 3$, 20:3 $\omega 3$, 20:4 $\omega 3$, and $\Sigma\omega 3$; Figure 3). The enzyme COX1 is constitutively expressed in several tissues (e.g., liver, kidney, spleen, gill, muscle, gut) and has maintenance and homeostatic functions (Tapiero et al., 2002; Olsen et al., 2012). Our data suggest that *cox1* expression could result in production of anti-inflammatory prostaglandins in Atlantic salmon head kidney at the constitutive level. Further, differences among treatments in head kidney EPA:ARA (Table 2) may have altered the production of eicosanoids via *cox1* transcription. Indeed, changes in cell membrane EPA:ARA could alter eicosanoid production and the inflammatory response in vertebrates (Calder, 2003; Arts and Kohler, 2009; Salini et al., 2016). However, since eicosanoid levels were not measured in our study, these hypotheses could not be tested. Further, the implications of FA-transcript correlations on eicosanoid production could not be assessed.

In addition to *cox1*, transcript expression of *5loxa*, *5loxb*, and *ptes3* was also positively correlated with head kidney $\omega 3$ LC-PUFA. However, *5loxa* showed positive correlations with both $\omega 3$ (i.e., 20:5 $\omega 3$, 22:6 $\omega 3$) and $\omega 6$ (20:4 $\omega 6$) LC-PUFA. The latter result is in line with Katan et al. (2019) who reported that hepatic *5loxa* showed a positive relationship ($p = 0.059$) with liver EPA+ARA in Atlantic salmon fed different mixes of plant oils as a FO replacement. However, this is in contrast with Caballero-Solares et al. (2020) who showed that hepatic *5loxa* correlated negatively with liver EPA and DHA in salmon fed terrestrial plant ingredients as FO and FM alternatives. Discrepancies between studies suggest that the interaction of *5loxa* transcription with tissue LC-PUFA in salmon depends on tissue (i.e., head kidney vs. liver) and/or dietary inputs (e.g., protein and lipid sources). Our study also showed positive correlations between $\omega 3$ PUFA (i.e., 18:4 $\omega 3$, 20:4 $\omega 3$) and *ptes3* expression, and negative correlations between EPA and *ptes2*. In mammals, both PTGES2 and PTGES3 convert PGH₂ into PGE₂ (Neuman et al., 2017; Xu et al., 2019). Wei et al. (2016) showed that muscle *ptes3* expression was induced in pigs fed with linseed-enriched diets for 60 days. However, previous studies reported no impacts of dietary fatty acids on the transcript expression of *ptes2* and *ptes3* in bovine oocytes (Ponter et al., 2012) and Eurasian perch (*Perca fluviatilis*) liver, brain and intestine (Geay et al., 2015). Clearly, more research is required to elucidate the influence of dietary and tissue PUFA on the transcript expression of *ptes2* and *ptes3* in fish.

Cox2 showed a positive correlation with 16:0, Σ SFA and 22:4 $\omega 6$, and negative correlations with Σ PUFA, 18:3 $\omega 6$ and 22:5 $\omega 6$ in the current study (Figure 3). This finding supports previous human macrophage studies which indicated that SFA induced *cox2* expression (Lee et al., 2001; Rocha et al., 2016). Astiz et al. (2012) showed that addition of 22:4 $\omega 6$ and 22:5 $\omega 6$ did not impact the production of COX2 in rat testis cells. However, a previous study on mouse brain indicated that reduction in the levels of ARA metabolites 22:4 $\omega 6$ and 22:5 $\omega 6$ reflected a shift of ARA from LC-PUFA biosynthesis prostaglandin or leukotriene synthesis (McNamara et al., 2009). Thus, the negative correlation between 22:5 $\omega 6$ and *cox2* could be a result of higher availability of ARA for COX-2 mediated prostaglandin synthesis. Finally, similarly to *cox2*, *pgds* correlated negatively with Σ PUFA. This latter finding is in line with Katan et al. (2019) who showed that hepatic *pgds* was negatively correlated with liver EPA+ARA+DHA in Atlantic salmon. However, Holen et al. (2015) reported that the combination of EPA+ARA+DHA did not modulate *cox2* transcript expression in Atlantic salmon head kidney cells. In summary, further examination is needed in order to elucidate the influence of ARA metabolites (i.e., 22:4 $\omega 6$ and 22:5 $\omega 6$) on *cox2* expression, and the interactive impacts of PUFA on the transcript expression of *pgds* and *cox2* in Atlantic salmon head kidney.

CONCLUSION

This study investigated head kidney lipid composition and transcript expression of genes involved in fatty acid and

eicosanoid metabolism in Atlantic salmon fed varying dietary levels of EPA+DHA (0.3, 1.0, and 1.4%) and ω 6: ω 3 ratios (high ω 6, high ω 3, and balanced) for 12 weeks. TAG was the predominant lipid class in all treatments, regardless of diet. This suggests that this lipid class could play an important role in immune and/differentiation processes in Atlantic salmon head kidney. Head kidney fatty acid composition was reflective of the diet with respect to C_{18} PUFA and MUFA levels (% of total fatty acids), and responded to ω 6: ω 3 variation. Proportions of 20:5 ω 3 were similar among 0.3% EPA+DHA \uparrow ω 3, 1% EPA+DHA (both high ω 3 and high ω 6 treatments) and 1.4% EPA+DHA/balanced fed fish, although dietary 20:5 ω 3 varied by 2.5- to 3-fold. Tissue fatty acid composition changes agreed with positive correlations between head kidney 20:3 ω 3 and 20:4 ω 3 and *elovl5a* transcript levels. This suggested that high dietary 18:3 ω 3 promoted the synthesis of ω 3 LC-PUFA in salmon fed lower dietary EPA+DHA levels (0.3%). Further, head kidney transcript expression of several genes involved in FA metabolism (e.g., *elovl5a*, *fadsd5* and *srebp1*) was positively impacted by dietary EPA+DHA levels, and showed a positive correlation with head kidney Σ MUFA. This supported the hypothesis that LC-PUFA synthesis is stimulated by increasing MUFA levels in Atlantic salmon.

Several eicosanoid synthesis-related transcripts were significantly correlated with head kidney fatty acid composition. The head kidney transcript expression of *5lox*b correlated positively with ω 3 LC-PUFA and Σ ω 3, and correlated negatively with ω 6: ω 3. *Cox1* and *ptges3* transcript expression correlated positively with ω 3 PUFA, while *cox1* was negatively impacted by dietary ω 6: ω 3 levels. We hypothesize that these transcript expression changes may have increased the production of anti-inflammatory eicosanoids in salmon head kidney. Finally, our study revealed significant positive and negative correlations of *cox2* expression with SFA (i.e., 16:0, Σ SFA) and 22:5 ω 6, respectively. Given the important role of the head kidney in salmon immune responses and the interaction with dietary LC-PUFA, future studies should elucidate how ω 6: ω 3 and EPA+DHA levels affect LC-PUFA synthesis (e.g., stable isotope and FA mass balance methods) and eicosanoid production (e.g., LC-MS/MS) in this organ.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of Memorial University of Newfoundland.

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AUTHOR CONTRIBUTIONS

MR, CP, and RT contributed to conception and design of the study. TK was involved in all aspects of the study, including experimental design, data and statistical analyses, and writing of the first manuscript draft. TK, XX, and AC-S took part in sampling. XX assisted with analyses related to the qPCR study. AC-S assisted with the Pearson correlation matrix. All authors contributed to original manuscript editing, revisions, and they read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2020.602587/full#supplementary-material>

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Conflict of Interest: RT was employed by the company Cargill, but did not have any additional role in the study design, data collection, analysis and interpretation, writing of the manuscript, or in the decision to submit the manuscript for publication.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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