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Journal:	*Lipids*
Manuscript ID	LIPIDS-16-0270.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
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Keywords:	Fatty acid metabolism < Metabolism, Fish nutrition < Nutrition, n-3 Fatty acids < Nutrition, Desaturases < Specific Lipids, Elongases < Specific Lipids

EPA, DHA, and lipoic acid differentially modulate the n-3 fatty acid biosynthetic pathway in Atlantic salmon hepatocytes

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ABSTRACT

The aim of the present study was to investigate how EPA, DHA, and lipoic acid (LA) influence the different metabolic steps in the n-3 fatty acid (FA) biosynthetic pathway in hepatocytes from Atlantic salmon fed four dietary levels (0 %, 0.5 %, 1.0 % and 2.0 %) of EPA, DHA or a 1:1 mixture of these FA. The hepatocytes were incubated with [1-^{14}C] 18:3n-3 in the presence or absence of LA (0.2 mM). Increased endogenous levels of EPA and/or DHA and LA exposure both led to similar responses in cells with reduced desaturation and elongation of [1-^{14}C] 18:3n-3 to 18:4n-3, 20:4n-3, and EPA, in agreement with reduced expression of the Δ6 desaturase gene involved in the first step of conversion. DHA production, on the other hand, was maintained even in groups with high endogenous levels of DHA, possibly due to a more complex regulation of this last step in the n-3 metabolic pathway. Inhibition of the Δ6 desaturase pathway led to increased direct elongation to 20:3n-3 by both DHA and LA. Possibly the route by 20:3n-3 and then Δ8 desaturation to 20:4n-3, bypassing the first Δ6 desaturase step, can partly explain the maintained or even increased levels of DHA production. LA increased DHA production in the phospholipid fraction of hepatocytes isolated from fish fed 0 % and 0.5 % EPA and/or DHA, indicating that LA has the potential to further increase the production of this health-beneficial FA in fish fed diets with low levels of EPA and/or DHA.

KEYWORDS: desaturases, elongases, fatty acid metabolism, fish nutrition, *in vitro*, n-3 fatty acids

ABBREVIATIONS

ASP	Acid–soluble products
CE	Cholesterol esters
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
FO	Fish oil
LA	Lipoic acid
MDG	Monoacylglycerols and diacylglycerols
NL	Neutral lipids
PL	Phospholipids
PUFA	Polyunsaturated fatty acids
TAG	Triacylglycerol
VLC-PUFA	Very lLong chain polyunsaturated fatty acids
VO	Vegetable oil

1. INTRODUCTION

Norwegian farmed Atlantic salmon (*Salmo salar* L) has faced major changes in their feed composition during the last decades, changing from a purely marine-based diet in the 1990s to diets containing 70 % plant ingredients [1]. As a result, the levels of health-promoting omega-3 ~~very~~ long chain polyunsaturated fatty acids (n-3 ~~V~~LC-PUFA) eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids decreased significantly in salmon organs and tissues [2]. Nevertheless, the lipid composition of an organism is not only affected by ingested lipids, but also by the capacity of organs or tissues to transform these lipids through desaturation and elongation pathways and by the endogenous capacity to synthesize lipids. Vertebrates lack the necessary enzymes to produce n-3 LC-PUFA *de novo*, and thus their production ~~of VLC-PUFA~~ is dependent on biosynthesis from essential preformed C_{18} PUFA obtained from the diet [3].

Endogenous production of ~~V~~LC-PUFA differs notably among species, and is determined to a large extend by the repertoire of fatty acyl elongase (Elovl) and desaturase (Fad) enzymes and their substrate specificities [4]. Salmonids, including Atlantic salmon, are able to elongate and desaturate C_{18} PUFA to C_{20} and C_{22} PUFA [5, 6], and knowledge of the key enzymes involved is available [7-11]. Both bioactive ~~V~~LC-PUFA arachidonic acid (ARA; 20:4n-6) and EPA are synthesized by the same enzymes, requiring a Δ6 desaturation of 18:2n-6 and α-linolenic (ALA; 18:3n-3) precursors, respectively, followed by chain elongation and a further Δ5 desaturation. Alternatively, ~~20:5n-3~~EPA can be produced via elongation of 18:3n-3 to 20:3n-3 followed by Δ8 and Δ5 desaturation [12, 13]. DHA synthesis from EPA requires two further elongations, a Δ6 desaturation and a peroxisomal β-oxidation chain-shortening step [14]. However, a more direct pathway for DHA production from EPA via elongation to 22:5n-3 and Δ4 desaturase has been recently described in several teleost species [15-19] as well as in humans [20]. There are s~~S~~everal ~~are the~~ factors controlling the n-3 fatty acid (FA) biosynthetic pathway. Although the same desaturases and elongases compete for FA substrates of the n-6 and n-3 families, in general with a preference for n-3 [3], the dietary FA ~~dietary~~ composition is known to influence enzyme activity. For instance, hepatocytes from Atlantic salmon fed diets with high levels of n-6 FA presented a higher capacity to increase the products of Δ6-desaturase from 18:3n-3 [21, 22]. In addition, the desaturation and elongation of 18:2n-6 and 18:3n-3 have been shown to be markedly enhanced by EFA deficiency [6].

Lipid-sensing transcription factors such as sterol regulatory element binding protein 1 (SREBP1) play a role in the transcriptional regulation of ~~V~~LC-PUFA biosynthesis in Atlantic salmon [23]. The expression of genes of the LC-PUFA biosynthetic pathway (*elovl* and

fads2) is regulated by SREBP1 in salmon, and the *srebp1* gene is transcriptionally activated by diets containing vegetable oils (VO) [23]. VO-based diets have been consistently reported to increase enzymatic activity of desaturases and elongases to produce EPA and DHA from ~~ALA~~ 18:3n-3 [24, 25]. Two explanations have been proposed: no inhibitory effect from dietary ~~V~~LC-PUFA, or a stimulatory effect from high concentrations of C_{18} substrates [25-27]. Despite the stimulatory effect on the enzymes, FO-based feeds result in higher DHA tissue levels than those obtained with VO-diets [2, 28]. Similar results are observed in mammals, in which 18:3n-3 supplementation increases EPA and DHA levels but to a lower degree than that attained with direct use of the preformed n-3 ~~V~~LC-PUFA [29]. Thus, a better understanding of FA bioconversion capabilities would allow improved dietary FA utilization in farmed fish, thereby providing a significant contribution towards more efficient use of marine resources in fish feeds.

Different strategies to optimize the innate capacities for EPA and DHA production from ALA can be used. For example, ~~T~~the FA composition of the diet may be optimized or a bioactive component to stimulate the pathway may be included. Lipoic acid (LA) is a promising bioactive molecule that plays a role in controlling lipid homeostasis [30]. In addition, LA possesses important antioxidant properties [31]. LA was shown to increase the nutritional value of the South American pacu (*Piaractus mesopotamicus*) by increasing EPA levels in the muscle [32], indicating a role in the regulation of the n-3 pathway.

In the present study we aimed to test the hypothesis that both optimized diet composition and use of bioactive components such as LA play an important role in modulating the capacity of Atlantic salmon hepatocytes to produce EPA and DHA from 18:3n-3.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Radiolabeled FA [1-^{14}C] 18:3n-3 (50 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). α-Lipoic acid (racemic form), essential FA-free bovine serum albumin (BSA), fetal bovine serum (FBS), Leibovitz-15 (L-15), 20,70-dichlorfluorescein, 20,70-dichlorfluorescein, collagenase, phosphate buffer saline (PBS), phenylethylamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), BHT, sodium bicarbonate solution, L-glutamine, Trypan blue, antibiotics, and total protein kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell flasks and cell scrapers were obtained from Nalge Nunc International (Rochester, NY, USA). Metacain MS-222 was purchased from Norsk Medisinaldepot (Norway). Perchloric acid ($HClO_4$), thin-layer

chromatography (TLC) plates, and all solvents and other chemicals for FA analysis were purchased from Merck (Darmstadt, Germany). FA peaks were identified by comparison with the standard mixtures GLC-85 and GLC-463 obtained from Nu-chek Prep (Elysian, MN, USA). Ecoscint A scintillation liquid was purchased from National Diagnostics (Atlanta, GA, USA). PureLink Pro 96 RNA Purification Kit and PureLink DNase were obtained from Invitrogen (Carlsbad, CA, USA), TaqMan Reverse Transcription Reagents kit from Applied Biosystems (Foster City, CA, USA), and LightCycler 480 SYBR Green I Master from Roche Applied Science (Mannheim, Germany).

2.2 Fish and feeding

Atlantic salmon with a mean initial weight of 52.8 g were kept in indoor tanks with seawater from smoltification and grown to approximately 400 g at Nofima Research Station in Sunndalsøra, Norway. Fish were fed for 26 weeks on one of 10 experimental diets. The experimental diets were isoproteic (46.7 %), isolipidic (25.2 %), and isoenergetic (22.2 MJ/kg) and were formulated to cover the nutritional requirements for amino acids and minerals according to the National Research Council [33]. The experimental diets selected in the present study were formulated to test four dietary levels of EPA, DHA, or a 1:1 mixture of EPA and DHA (0 %, 0.5 %, 1.0 %, and 2.0 % in all dietary groups) (Table 1). The content of 18:3n-3, the precursor of LC-PUFA EPA and DHA, was kept at approximately the same level in all diets (4.7 % of total fatty acids and 1.2 % of the diet). A detailed description of the experimental conditions and dietary composition is given by Bou et al. [34]. At the end of the experiment, three fish per dietary treatment were anesthetized in a MS-222 solution (0.2 g/L) for 5 to 10 min prior to isolation of hepatocytes. The average fish weight was 379.7 ± 96.5 g and no major differences in growth between dietary treatments were observed. The experiment was conducted according to the National Guidelines for Animal Care and Welfare published by the Norwegian Ministry of Education and Research (Forsøksdyrforvaltningens tilsyns- og søknadssystem (FOTS) approval 5354).

Hepatocytes isolated from Atlantic salmon fed 10 different experimental diets with different levels of EPA and/or DHA were incubated with radiolabeled ~~α-linolenic acid (~~18:3n-3~~)~~ in the presence and absence of LA. The radiolabeled 18:3n-3 was used to measure changes in FA metabolism, desaturation and β-oxidation. In addition, a parallel experiment was done with hepatocytes incubated in the presence or absence of LA to analyze the transcript levels of some lipid related genes. The details of the different methodological and analytical steps are described below.

2.3 Isolation of hepatocytes

Cells were isolated from three fish per dietary condition and one independent culture per fish was performed. Livers were perfused following a modified two-step collagenase procedure [35, 36] and conducted as previously described [37]. After collagenase perfusion, parenchymal cells were isolated by gently shaking the digested liver in L-15 medium. The suspension of parenchymal cells obtained was filtered through a 100-µm mesh nylon filter, washed three times in L-15 medium, sedimented by centrifugation for 2 min at 50 × *g*, and resuspended in L-15 medium containing 10 % FBS, 0.9 mM sodium bicarbonate, 2 mM L-glutamine, 1 % penicillin-streptomycin solution, and 5 mM HEPES. Cell viability was assessed with 0.4 % Trypan blue. Approximately $4 \cdot 10^5$ hepatocytes/cm^2 were placed on flasks or six-well plates (25 cm^2 and 9.6 cm^2/well, respectively), coated with laminin, and left to attach for 16 h at 13 ºC. Two cell flasks and two wells from each fish were seeded for the experiments. Furthermore, 1 mL of hepatocyte suspension was used to evaluate the effects of the experimental diets on the FA composition of the hepatocytes.

2.4 Incubation of hepatocytes with radiolabeled 18:3n-3 and lipoic acid

Isolated hepatocytes in flasks were washed with L-15 medium without serum supplementation, and then incubated for 48 h with 21 nmol [1-^{14}C] 18:3n-3 (7 µM final concentration) and with or without 0.2 mM LA in a total volume of 3 mL of L-15 medium with 2 % FBS. LA dose and incubation time were selected based on previous studies [38]. Radiolabeled FA substrate (1.8 µCi, 50 mCi/mmol) was added to the medium as a sodium salt bound to FA-free BSA at a molar ratio of FA to BSA of 2.7:1. After incubation, the culture medium was transferred from the culture flasks to vials and centrifuged for 5 min at 50 × *g*. The supernatants (culture media) were immediately frozen at -80 ºC and stored for determination of radiolabeled lipids and oxidation products. Hepatocytes supplemented with 18:3n-3 were washed twice in PBS with 1 % albumin, once with regular PBS, harvested in 2 mL PBS, and stored at -80 ºC until lipid analysis.

Aliquots of 10, 20, 30, 40, and 50 µL of medium containing radioactive 18:3n-3 were transferred before incubation to vials with 8 mL Ecoscint A scintillation liquid to determine total and specific radioactivity (cpm/nmol FA). Samples were counted in a TRI-CARB 1900 TR scintillation counter (Packard Instrument Co., North Chicago, IL, USA).

2.5 Lipid extraction and analysis

Total lipids were extracted from culture media and cells incubated with radiolabeled 18:3n-3 as previously described [39]. The chloroform phase was dried under nitrogen gas and the residual lipid extract was redissolved in 1 mL chloroform. Fifty µL of chloroform were transferred to vials containing 8 mL scintillation liquid and the remaining volume was used for lipid analysis. Free fatty acids (FFA), phospholipid (PL), monoacylglycerols and~~,~~

diacylglycerols (MDG), and triacylglycerol (TAG) were separated by thin-layer chromatography (TLC) using petroleum ether, diethyl ether, and acetic acid (113:20:2 v/v/v) as the mobile phase. Samples were applied on silica gel TLC plates. Lipids were identified by comparison with known standards using a Bioscan AR-2000 Radio-TLC & Imaging Scanner and quantified with the WinScan Application Version 3.12 (Bioscan Inc., Washington, DC, USA). The esterified FA, PL, and NL fractions within the media will be denoted as secreted FA. Spots corresponding to PL and TAG from the cellular lipids were scraped off into glass tubes and trans-methylated for 16 h with 2,2-dimethoxypropane, methanolic HCl, and benzene at room temperature as previously described [40, 41].

Total levels of non-labeled lipids were determined by extraction of PL and NL as described above from hepatocytes isolated from three fish in each dietary group. Immediately after isolation, the cells were washed twice in PBS, centrifuged for 2 min at 1000 x *g*, the supernatant was removed, and the cells were stored at -80 ºC until lipid analysis. TLC plates containing these samples were sprayed with 0.2 % (w/v) 2',7'-dichlorofluorescein in methanol and viewed under UV light to identify lipids by comparison with known standards.

2.6 FA composition analysis

The radioactive FA composition of the PL and NL fractions were determined by reverse-phase HPLC as previously described [42]. The mobile phase contained acetonitrile/H_2O (85:15 v/v, isocratic elution) and was set to a flow rate of 1 mL/min at 30 ºC. A reverse-phase Symmetry 3.5 µm C-18 HPLC column from Waters was used. Radioactive FA levels were measured in an A100 radioactive flow detector (Tri-Carb 1900TR; Packard Instruments). FA were identified by comparing sample and FA standards retention times. Nonradioactive FA standards were detected by absorbance at 215 nm in a UV detector (Waters 2996 PDA Detector).

Unlabeled methyl esters of FA from the PL and NL fractions of hepatocytes were separated in a GC (Hewlett Packard 6890) with a split injector, an SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm, and film thickness 0.25 µm), a flame ionization detector, and HP Chem Station software. Helium was the carrier gas. The injector and detector temperatures were set to 280 ºC. The oven temperature was raised from 50 ºC to 180 ºC at a rate of 10 ºC/min, and then raised to 240 ºC at a rate of 0.7 ºC/min. The relative amount of each FA was expressed as a percentage of the total amount of FA in the analyzed sample and the absolute amount of FA per gram of tissue was calculated using C23:0 methyl ester as internal standard.

2.7 Measurement of $^{14}CO_2$ and acid-soluble products from [1-^{14}C] 18:3n-3

The levels of β-oxidation of 18:3n-3 were measured by counting oxidation products (^{14}C-labeled acid-soluble products (ASP) and $^{14}CO_2$ formed) essentially as previously described [43]. The amount of gaseous [1-^{14}C] CO_2 produced during incubation was determined by transferring 1.5 mL of medium to a glass vial which was then sealed. The glass vial contained a central well with a Whatman filter moistened with 0.3 mL of phenylethylamine/methanol (1:1, v/v). The medium was acidified with 0.3 mL 1 M $HClO_4$, the samples were incubated for 1 h, and then the wells containing the filters were placed into vials for scintillation counting.

The levels of [1-^{14}C] ASP were determined by acidifying 1 mL of the medium with 0.5 mL ice-cold 2 M $HClO_4$ and incubating the sample for 60 min at 4 ºC. The medium was then centrifuged, and an aliquot of the supernatant was collected for scintillation counting.

2.8 Protein content

Protein content of cells was determined using the Total Protein kit (Micro Lowry/Peterson's modification) [44, 45] and absorbance at 540 nm in a Titertek Multiscan 96-well plate reader (Labsystem, Finland).

2.9 Total RNA extraction, cDNA synthesis, and real-time PCR

Isolated hepatocytes in six-well plates were washed with L-15 medium without serum and then incubated for 48 h with or without 0.2 mM LA in 3 mL L-15 medium with 2 % FBS. After incubation, the hepatocytes were washed twice in PBS, harvested in 1 ml Trizol, and stored at -80 ºC until RNA extraction.

Total RNA was isolated using PureLink Pro 96 RNA Purification Kit according to the manufacturer's instructions. RNA was treated with PureLink DNase to remove any contaminating DNA. RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples used in our experiments had A260/280 ratios between 2.02 and 2.14. Total RNA (450 ng) was reverse-transcribed into cDNA in a 20-µL reaction using the TaqMan® Reverse Transcription Reagents kit according to the manufacturer's protocol.

PCR primers (Table ~~1~~2) were designed using the Vector NTI software (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. The efficiency was checked in ten-fold serial dilutions of cDNA for each primer pair. Real-time PCR was performed in a LightCycler 480 (Roche Applied Science, Germany). The PCR master mix consisted of 1 µL forward and reverse primers (final concentrations of 0.5 µM), 4 µL of a 1:10 dilution of cDNA, and 5 µL LightCycler 480 SYBR Green I Master mix. All samples were analyzed in duplicate with a non-template control (NTC) for each gene. The reaction conditions were 95 ºC for 5 min, and

45 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The specificity of PCR amplification was confirmed by melting curve analysis (95 ºC for 5 s, 65 ºC for 1 min, and then 97 ºC). Rpol2, Ef1α, and Etif3 were evaluated for use as reference genes, and it was found that the latter was the most stable. Relative quantification of transcript abundance was calculated using the ΔΔCT method and the formula ΔΔCT = - [(Ct $_{\text{target gene}}$ - Ct $_{\text{Etif3}}$) $_{\text{treatment}}$ - (Ct $_{\text{target gene}}$ - Ct $_{\text{Etif3}}$) $_{\text{control}}$ [46].

2.10 Statistical analysis

Flasks or wells were used as experimental units (n=3). Changes in FA composition of the PL and NL fractions of hepatocytes were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's honest significant difference *post hoc* test to detect differences within dietary groups. All other data were analyzed by a two-way ANOVA using diet and presence or absence of lipoic acid as effects. Spearman's correlation coefficients were calculated to estimate the association of cellular EPA or DHA and 18:3n-3 with different FA products. Differences were considered statistically significant at $P<0.05$. Values are shown as mean ± SEM. All statistical analyses were conducted using the software JMP® version 11.2.1 (SAS Institute Inc., Cary, NC, 1989-2007).

3. RESULTS

3.1 Endogenous FA composition of hepatocytes

To test whether FA content of fish was affected by dietary FA, the endogenous FA composition in salmon hepatocytes was determined. The results show that FA content was significantly affected by dietary FA. The n-6/n-3 ratio gradually increased in the PL fraction of hepatocytes of fish fed diets containing less n-3 ~~V~~LC-PUFA (Table ~~2~~3), and increasing dietary levels of EPA and/or DHA significantly increased ~~FA~~the content of DHA in the PL fraction. In contrast, ~~20:5n-3~~EPA levels in the PL fraction from fish fed the DHA diets were similar to those from fish fed the 0 % diet. ~~On the other hand, significantly higher 22:6n-3 levels were observed in membranes of fish fed diets with increasing levels of EPA and/or DHA, indicating an active conversion from EPA to DHA~~. Nevertheless, the highest levels of ~~22:6n-3~~DHA were detected in fish fed a diet with 2.0 % DHA. The ~~22:6n-3~~DHA content in the membranes of the deficient group (0 % diet) was reduced three-fold when compared to that from the 2.0 % DHA dietary group. In contrast, fish fed the 0 % diet ~~more than~~almost doubled the amount of n-6 FA compared to that of fish fed the 2.0 % diets (2.0% EPA, 2.0% DHA, and 2.0% EPA+DHA diets). This increase in n-6 FA content was mainly due to increased levels of 20:4n-6 and 20:3n-6, followed by 18:2n-6. This was reflected on the n-6

desaturation index, with the highest value corresponding to hepatocytes isolated from fish fed the 0 % diet and gradually decreasing as the dietary n-3 ~~V~~LC-PUFA were increased. The FA composition of the NL fraction was less affected by dietary lipid (Table ~~2~~3), although the amount of PUFA gradually decreased in the NL fraction of hepatocytes as the fish received diets containing less n-3 ~~V~~LC-PUFA. This decrease in PUFA was followed by an increase in MUFA, mainly 18:1 n-9. The amount of PUFA ~~and MUFA~~ was less ~~and more~~ abundant~~,~~ ~~respectively,~~ in the NL than in the PL fraction, whereas the amount of MUFA was more abundant in the NL than in the PL fraction.~~.~~ The levels of the 18:3n-3 precursor remained unaltered regardless of dietary treatment in both fractions. The relative lipid class distribution between total PL and NL fractions, determined using an internal standard GC approach, ~~-~~was not altered by dietary treatment, with the majority being in the PL form and representing ~75 %.

3.2 Effect of endogenous FA composition and LA supplementation on the metabolism of [1-^{14}C] 18:3n-3

To study the dietary and LA effects on the uptake and metabolism of 18:3n-3, Atlantic salmon hepatocytes isolated from fish fed 10 different diets containing different levels of EPA and/or DHA were incubated with [1-^{14}C] 18:3n-3 in the presence or absence of LA for 48 h. Table ~~3~~ 4 shows the total uptake and radioactivity distribution from 18:3n-3 recovered in cellular lipids, water-soluble oxidation (ASP + CO_2) products, and secreted lipids in the culture media. The majority of 18:3n-3 was taken up by hepatocytes and incorporated into cellular lipids (12.4 ± 0.17 nmol, corresponding to 59.2 % of added substrate). The level of incorporation into cellular lipids did not differ between LA-supplemented and control cells. In contrast, the endogenous FA composition had a significant effect on cellular incorporation of radiolabeled FA, with the 0 % dietary group presenting the highest incorporation (13.7 ± 0.26 nmol; average value between control and LA-supplemented cells). The level of radiolabeled secreted lipids did not differ between LA-supplemented and control cells. Nevertheless, the TAG secretion average in control cells was 1.48 ± 0.51 nmol while that from LA-supplemented cells was 1.03 ± 0.50 nmol (data not shown). LA addition promoted the formation of ASP but decreased the production of CO_2 from 18:3n-3. In addition, the amount of ASP was higher in hepatocytes isolated from fish fed diets containing 1.0 % and 2.0 % n-3 ~~V~~LC-PUFA (EPA and/or DHA) than in the other dietary groups.

Table ~~4~~ 5 shows the relative distribution of esterified radiolabeled lipids derived from [1-^{14}C] 18:3n-3. The majority of radiolabeled FA was found in PL, in which an average of 86 % and 76 % of radiolabeled substrate was incorporated in ~~non-supplemented~~control and LA-supplemented hepatocytes, respectively. Close to 12 % and 21 % of radiolabeled substrate

was used for TAG production in hepatocytes incubated in ~~LA free~~control or LA-supplemented medium, respectively on average. Only a minor part of radiolabeled 18:3n-3 was used for MDG (2 %) and cholesterol esters (CE) (0.4 %) production in all conditions studied; non-esterified free FA were below the detection threshold. In general, a gradual increase in the relative production of TAG and a concomitant decrease in PL was found in hepatocytes isolated from fish fed with increasing levels of EPA and/or DHA. On the other hand, LA consistently decreased the relative production of PL and favored that of all the other ~~neutral lipids~~NL analyzed.

3.3 Effect of endogenous FA composition and LA supplementation on the desaturation and chain-elongation of [1-^{14}C] 18:3n-3

The main products of 18:3n-3 in the PL fraction were ~~20:5n-3~~EPA and ~~22:6n-3~~DHA, followed by 20:4n-3 and 20:3n-3 (Table ~~5~~6). The PL fraction of hepatocytes isolated from fish fed the 0 % diet presented the highest content of 18:3n-3 regardless of being cultivated in the presence or absence of LA. A gradual decrease in 18:3n-3, 18:4n-3, 20:4n-3, ~~20:5n-3~~EPA, and 22:5n-3 content was observed in the PL fraction of hepatocytes isolated from fish fed with increasing levels of EPA and/or DHA, while that of 20:3n-3 gradually increased. LA supplementation in the medium affected 18:3n-3 metabolism (Fig. 1A) in a similar fashion as resulting from increasing the dietary content of n-3 ~~V~~LC-PUFA (Fig. 2A). Thus, LA further reduced the levels of 18:3n-3, 18:4n-3, 20:4n-3, ~~20:5n-3~~EPA, and 22:5n-3 in the PL fraction of hepatocytes, whereas it increased 20:3n-3 levels. Surprisingly, endogenous FA composition had no significant effect on the production of ~~22:6n-3~~DHA. ~~Indeed, the tendency found was contrary to what would have been expected, with a~~A slightly higher production was observed in cells from fish fed with the highest levels of n-3 ~~V~~LC-PUFA. However, a significant interaction between LA and diet was observed in which LA only promoted the presence of ~~22:6n-3~~DHA in the PL fraction of hepatocytes from fish fed 0 % and 0.5 % EPA and/or DHA. Interestingly, the DHA contents in the PL fraction of hepatocytes from fish fed 0.5 % EPA, 0.5 % DHA, and 0.5 % EPA+DHA diets were fairly similar (19.7 ± 0.52 mol%; average value of the aforementioned diets), indicating that endogenous DHA content may be the factor modulating the effects of LA.

The main product from 18:3n-3 found in the NL fraction was 20:3n-3, ~~followed by 22:6n-3~~but smaller amounts of DHA, ~~20:5n-3~~EPA, and 18:4n-3 were also produced (Table ~~6~~7). However, the production of 18:4n-3 and ~~20:5n-3~~EPA was not affected by endogenous FA composition or by LA addition. A gradual increase in the deposition of 18:3n-3, 20:3n-3, and ~~22:6n-3~~DHA contents ~~was observed~~ in the NL fraction of hepatocytes isolated from fish fed with increasing dietary levels of EPA and/or DHA or supplemented with LA was observed. ~~LA~~

addition to the medium consistently promoted the production of these three FA in all dietary groups, further stimulating the effect of dietary n-3 VLC-PUFA. On the other hand, 20:4n-3 and 22:5n-3 levels were below the detection threshold in several experimental groups. LA supplementation and dietary n-3 LC-PUFA had similar effects on the picomoles recovered in 18:3n-3 and its products in the NL fraction (Fig. 1B and 2B).Fig. 1B and 2B show the main effects on the NL fraction of LA and dietary n-3 VLC-PUFA, respectively, and Fig. 3 shows The total DHA production represented as the sum of recovered picomoles in PL and NL showed that LA supplementation increased DHA production in hepatocytes isolated from fish fed ≤ 0.5% EPA and/or DHA (Fig. 3).

To determine the association strength between cellular EPA or DHA and the desaturation and elongation of 18:3n-3 substrate to its different FA products, Spearman's correlation coefficients were calculated (Table 78). DHA cellular content had a stronger association with all FA produced compared to that from EPA cellular content., indicating that endogenous DHA influences the pathway activity to a higher degree than endogenous EPA.

3.4 Effect of endogenous FA composition and LA supplementation on the transcriptional regulation of the n-3 biosynthetic pathway

Because differences in 18:3n-3 desaturation and elongation were observed in hepatocytes isolated from fish fed with different levels of EPA and/or DHA cultivated in LA-free or LA-supplemented medium, we further investigated whether these differences were associated with changes in transcript abundance of genes encoding proteins related to lipid metabolism. Transcript levels of genes coding for *srebp1, aco, elovl2, elovl5b, Δ5fad*, and *Δ6fad_a* are shown in Fig. 4. Transcript levels of all evaluated genes changed as a result of diet composition and, consequently, of endogenous FA composition. mRNA levels of *srebp1* and *aco* decreased in all dietary groups compared to those of the 0 % diet group, whereas *Δ5fad* mRNA levels increased. *Δ6fad_a, elovl2*, and *elovl5b* gene transcripts were modulated in a dose-dependent manner, with decreasing levels as n-3 VLC-PUFA levels increased. LA addition only increased gene transcript levels of the two elongases assessed.

4. DISCUSSION

One of the main aims of the study was to investigate the effects of different dietary levels of EPA and/or DHA on the fish 18:3n-3 metabolism. The endogenous FA composition of hepatocytes was influenced by dietary FA composition. These results are in agreement with several studies in salmonids where different lipid sources were tested [5, 25, 37, 47, 48]. In general, increasing levels of dietary EPA increased cellular levels of EPA, 22:5n-3DPA, and

DHA, whereas increasing DHA dietary levels only increased cellular DHA content. In addition, decreasing dietary n-3 levels increased the levels of ~~the pro-inflammatory FA~~ 20:3n-6 and 20:4n-6 in the PL fraction of hepatocytes, showing a stimulation of the n-6 pathway by 18:2n-6 when EPA and DHA are lacking. It has been extensively reported that feeding fish with a VO-based diet leads to increased activity of the n-6 and n-3 biosynthetic pathways [2, 24, 25, 49, 50]. Two main explanations have been proposed for the stimulation of the pathway: an increase in C_{18} substrate availability, and a lack of C_{20} and C_{22} PUFA ~~that would otherwise lead to a decrease caused by product inhibition~~. However, determining which of these two factors might have a bigger effect is not trivial, because so far the practical diets tested with high levels of n-3 LC-~~H~~PUFA had low levels of C_{18} PUFA and vice versa. In the present study, the dietary levels of 18:3n-3 were kept constant at 1.2 ± 0.02 % in all tested diets. In addition, the levels of 18:2n-6 were kept stable at 5.4 ± 0.08 %, providing a steady 18:2n-6/18:3n-3 ratio of 4.5. These conditions allowed us to rule out a possible effect caused by competition between both substrates for the enzymes and to relate changes in the endogenous n-3 biosynthetic pathway activity directly to dietary and cellular n-3 ~~V~~LC-PUFA content.

Hepatocytes isolated from fish fed with increasing levels of n-3 ~~V~~LC-PUFA showed a significant decrease in the production of radiolabeled 20:4n-3, EPA, and 22:5n-3~~DPA~~ in the PL fraction, and the production of these FA was further reduced in cells supplemented with LA. These results are in agreement with previous studies showing a reduction in the FA biosynthetic pathway linked to ~~V~~LC-PUFA availability [25, 26]. Strikingly, neither diet nor LA supplement had any effect on the levels of radiolabeled esterified DHA in the PL fraction. However, these two factors interacted, and thus LA exposure led to increased elongation and desaturation of 18:3n-3 to DHA in hepatocytes from fish fed diets containing 0 % and 0.5 % EPA and/or DHA. In contrast, LA exposure of cells from fish fed with higher dietary levels of EPA and/or DHA resulted in a reduced cellular capacity to transform 18:3n-3 into DHA. It is noteworthy that the endogenous DHA contents in the PL fractions of fish fed 0.5% EPA, 0.5 % DHA, and 0.5 % EPA+DHA were fairly similar (19.7 ± 0.52 mol%), suggesting that cellular DHA may be modulating the effects of LA. In a recent study, dietary LA supplementation also increased the DHA content in the liver of diet-induced ~~non~~ nonalcoholic fatty liver disease mice [51]. Despite the observed stimulation of DHA synthesis by LA, it had no effect on the transcriptional regulation of desaturase genes. Incubation of salmon hepatocytes with sesamine, another bioactive component, was reported to also increase the conversion of 18:3n-3 to DHA but, paradoxically, decrease *Δ5fad* and *Δ6fad* gene transcripts [52]. On the other hand, the NL fraction of hepatocytes isolated from fish fed with increasing levels of n-3 ~~V~~LC-PUFA showed a significant increase in DHA production that was further increased by

LA addition. However, this LA-induced increase did not compensate for the aforementioned decrease in DHA production in the PL fraction of hepatocytes isolated from fish fed diets containing EPA and/or DHA at dietary levels of 1.0 % or above.

In the n-3 FA pathway, 18:3n-3 can either be desaturated via Δ6 desaturase to 18:4n-3 or elongated via Elovl5 to 20:3n-3. Desaturation was favored by low dietary levels of n-3 ~~V~~LC-PUFA in the PL fraction of hepatocytes, whereas the addition of LA had no effect in the production of 18:4n-3. In contrast, elongation of 18:3n-3 to 20:3n-3 was promoted in both lipid fractions by increasing dietary levels of n-3 ~~V~~LC-PUFA and by LA addition. This is in agreement with previous studies showing that hepatocytes isolated from Atlantic salmon fed FO-based diet rich in n-3 ~~V~~LC-PUFA [48], supplemented with DHA in the culture medium [5], or supplemented with LA [38], significantly enhanced the production of 20:3n-3. Furthermore, the cellular DHA content in control cells was strongly correlated (0.883; $P<0.0001$) to the amount of 20:3n-3. This FA was considered to be a dead-end product of the pathway. However, it was recently shown that not only mammalian [13], but also teleostei, Δ6 Fads possess Δ8 desaturase activity [12], and thus 20:3n-3 can be desaturated to 20:4n-3, which can then be reincorporated into the pathway for further Δ5 desaturation. Even though the activity of the Δ8 pathway in freshwater/diadromous species has been reported to be low compared to that from other marine teleosts [12], it might provide an alternative route for the synthesis of ~~20:5n-3~~EPA from 18:3n-3 that does not involve a Δ6 desaturation. As suggested by the correlation coefficients in our study, cellular DHA content seems to have a major effect modulating the activity of the pathway by regulating the conversion of 18:3n-3 to either 20:3n-3 or 18:4n-3. The decrease in content of the desaturation product 18:4n-3 with increasing dietary n-3 ~~V~~LC-PUFA is also consistent with the decrease in *Δ6fad_a* transcript abundance. However, the n-3 ~~V~~LC-PUFA dietary stimulation of 20:3n-3 production was not accompanied by an up-regulation of *elovl5b* transcripts in the present study. LA supplementation, on the contrary, increased both *elovl5b* transcript abundance and 20:3n-3 production. A recent study suggested that land-locked salmon, which remain in freshwater their whole life and thus are naturally surrounded by lower levels of n-3 PUFA, might have a higher Δ8 activity compared to their farmed counterparts [53]. In the present study, radioactivity recovered in 20:4n-3 was significantly reduced in the PL fraction of cells by increasing levels of n-3 ~~V~~LC-PUFA, and the addition of LA reduced it further. Since radioactivity in 20:4n-3 could be the result of either Δ6 or Δ8 activity, it is difficult to draw conclusions. Knowledge of Δ8 activity regulation is still limited, and thus the Δ8 desaturase activity of salmon *Δ6fad_a* or how diet interacts with this alternative pathway remain to be explored, emphasizing the need for further research. In control cells, despite the decrease in 20:4n-3, ~~20:5n-3~~EPA, and *Δ6fad_a* mRNA levels with increasing dietary n-3 ~~V~~LC-PUFA, an

increase in radiolabeled DHA was observed. Therefore, it is possible that inhibition of Δ6 activity is compensated by Δ8 or Δ4 activity to provide DHA. A functional *Δ4fads* was first identified in several teleost species [15-18], and recently it has also been characterized in human cancer cells [20]. However, if this desaturation step is of importance in non-cancer human cells is currently unknown. In addition, whether Atlantic salmon possess this ability, and if so, what is the capacity of this direct pathway remain to be explored. Dietary PUFA play a role as ligands of key transcription factors, including SREBP1 [23]. The transcript levels of this transcription factor gene were decreased by dietary n-3 ~~V~~LC-PUFA. These results are in agreement with the regulation described in mammals, in which SREBP1 is activated by low levels of cholesterol and is inhibited by high levels of PUFA [54].

After entering the cell, radiolabeled FA substrate can be used for energy purposes by going through β-oxidation and extensive carbon recycling, or can be esterified into cellular lipids. In the present study, radiolabeled 18:3n-3 or its FA products were preferentially incorporated into PL with increasing percentages found in hepatocytes isolated from fish fed with decreasing levels of n-3 ~~V~~LC-PUFA, and that consequently contained significantly lower amounts of these FA. In contrast, little radioactivity was recovered in TAG, with increasing percentages observed in hepatocytes isolated from fish fed with increasing levels of n-3 ~~V~~LC-PUFA. These results are in agreement with several previous studies in salmonid hepatocytes and muscle cells showing that PUFA are predominately incorporated into PL [47, 55, 56]. In contrast, another study showed that radioactivity was mostly recovered in salmon hepatocytes in the form of TAG [48]. However, in this last study, hepatocytes in suspension were incubated with radiolabeled FA for only 2 h, which may explain the discrepancy in the results.

In this study, we also demonstrated clear effects of LA supplementation on FA metabolism in Atlantic salmon hepatocytes. Even though the amount of radiolabeled cellular lipids was not influenced by LA supplementation, LA significantly reduced the esterification of 18:3n-3 and its products into PL and increased the content of these compounds in storage depots. In addition, this increased incorporation into cellular TAG was paralleled by a decrease in TAG secretion to the media. In mammals, even though the exact mechanisms are still unclear, strong evidence supports the effects of LA on TAG metabolism [57]. Reduced levels of esterified radiolabelled TAG in the media indicate that LA reduce the secretion of TAG-rich VLDL from hepatocytes to blood, in agreement with ~~Despite of some conflicting results, t~~the majority of the studies in mammals ~~show that LA reduce blood TAG~~ [57-59]~~., which is in agreement with the effect of LA in salmon hepatocytes observed in the present study.~~

Formation of ASP oxidation products was generally enhanced in hepatocytes with the highest endogenous level of DHA, whereas no dietary modulation of CO_2 production was observed. LA supplementation, on the other hand, significantly increased ASP and decreased CO_2 production. LA has been shown to decrease lipid accumulation in non-adipose tissues by stimulating hepatic β-oxidation in mice [60] and in rat skeletal muscle [61]. On the other hand, LA inhibited the oxidation of FFA in primary rat hepatocytes and increased pyruvate oxidation [62]. Because in our experiments LA significantly increased the production of ASP, we speculate that LA may increase DHA production by stimulating peroxisomal β-oxidation [56]. However, the gene transcript abundance of acyl-CoA oxidase (*aco*), the rate-limiting enzyme of peroxisomal β-oxidation, decreased by n-3 ~~V~~LC-PUFA, whereas addition of LA did not have any effect on its regulation. Lack of regulation of this enzyme by FA at both protein and transcript levels has been reported in rainbow trout hepatocytes [47] and Atlantic salmon hepatocytes [52], despite showing an increased production of β-oxidation products.

This study ~~strongly~~ indicates that LA plays a role influencing n-3 FA metabolism in Atlantic salmon hepatocytes by enhancing the production of DHA, but this production is restricted by high cellular DHA content. In addition, increasing dietary levels of EPA and/or DHA reduced salmon's innate production of 18:4n-3, 20:4n-3, EPA, and ~~DPA~~22:5n-3, but DHA production was maintained, even showing a slight increase with high dietary EPA and/or DHA. To determine the exact mechanisms by which LA and dietary n-3 ~~V~~LC-PUFA increase the levels of health-beneficial ~~V~~LC-PUFA, further research on the Δ6, Δ8, and Δ4 activities is required.

ACKNOWLEDGMENTS

The authors would like to thank Målfrid Tofteberg Bjerke and Inger Øien Kristiansen for their skilful technical assistance. This work was carried out with support from the Norwegian Research Council (grant number NFR 224913). There are no conflicts of interest to report.

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713

714

715 **FIGURE LEGENDS**

716 **Fig.1** Main changes produced by lipoic acid supplementation in esterification of [1-^{14}C] 18:3n-
717 3 and its products into phospholipids (A) and neutral lipids (B). Data are shown as mean ±
718 SEM (n=3).

719 **Fig. 2** Main changes produced by increasing dietary levels of EPA and/or DHA in
720 esterification of [1-^{14}C] 18:3n-3 and its products into phospholipids (A) and neutral lipids (B).
721 Data are shown as mean ± SEM (n=6).

722 **Fig. 3** Total radiolabeled docosahexaenoic acid (DHA; 22:6n-3) esterified into cellular lipids
723 (PL+NL) in hepatocytes incubated with [1-^{14}C] 18:3n-3 in the presence or absence of lipoic
724 acid. Cells were isolated from fish fed diets containing different levels of EPA and/or DHA for
725 26 weeks before the experiment. Data are shown as mean ± SEM (n=3).

726 **Fig. 4** Relative changes in mRNA transcript abundance of genes involved in the n-3 fatty
727 acid biosynthetic pathway. Atlantic salmon hepatocytes cultivated in the presence or
728 absence of lipoic acid were isolated from fish fed 10 experimental diets containing different
729 levels of EPA and/or DHA for 26 weeks. Samples (n=3) were analyzed using real-time qPCR
730 and data are presented as -ΔΔCt ± SEM. Cells isolated from fish fed the 0 % diet were used
731 as control and values were set to zero. Results are compared by two-way analysis of
732 variance (diet and lipoic acid as factors; P<0.05).

TABLES

Table 1. Fatty acid composition (mol%) in the experimental diets.

	0%	0.5% EPA	1.0% EPA	2.0% EPA	0.5% DHA	1.0% DHA	2.0% DHA	0.5% EPA+DHA	1.0% EPA+DHA	2.0% EPA+DHA
16:0	17.7	17.2	16.8	15.9	17.3	16.9	16.0	17.2	16.8	16.0
18:0	4.2	4.1	4.0	3.9	4.2	4.2	4.2	4.2	4.1	4.1
SFA[1]	23.0	22.4	21.9	20.7	22.3	22.3	21.3	22.5	22.1	21.1
18:1n-9	44.5	43.4	41.7	38.3	43.5	42.0	39.4	43.1	41.8	39.0
MUFA[2]	47.3	45.8	44.2	41.0	46.3	44.9	42.4	45.8	44.7	41.6
18:2n-6	23.7	23.0	22.5	21.3	23.2	22.8	21.5	23.1	22.5	21.5
18:3n-3	5.3	5.1	4.9	4.9	5.1	4.9	4.6	5.1	5.0	4.6
C18 PUFA[3]	29.0	28.3	27.7	26.5	28.5	27.8	26.3	28.4	27.6	26.3
20:5n-3	0.0	2.2	4.3	8.4	0.4	0.7	1.4	1.4	2.5	5.1
22:6n-3	0.1	0.6	1.1	1.9	1.8	3.6	7.4	1.2	2.3	4.7
LC-PUFA[4]	0.7	3.5	6.3	11.8	2.9	5.0	10.1	3.4	5.6	11.0

[1]Includes 14:0, 20:0, 22:0, 24:0.
[2]Includes 20:1n-9, 20:1n-11, 22:1n-11.
[3]Includes 18:3n-6.
[4]Includes 20:2n-6, 20:3n-6, 20:4n-6, 22:5n-3.

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Table ~~1~~2. Atlantic salmon primer sequences used for real-time PCR.

Gene	Accession no.	Direction	Primer sequence 5´→ 3´
ef1α	AF321836	Forward	CACCACCGGCCATCTGATCTACAA
		Reverse	TCAGCAGCCTCCTTCTGAACTTC
etif3	DW542195	Forward	CAGGATGTTGTTGCTGGATGGG
		Reverse	ACCCAACTGGGCAGGTCAAGA
rpol2	CA049789	Forward	TAACGCCTGCCTCTTCACGTTGA
		Reverse	ATGAGGGACCTTGTAGCCAGCAA
aco	DQ364432	Forward	CCTTCATTGTACCTCTCCGCA
		Reverse	CATTTCAACCTCATCAAAGCCAA
Δ5fad	AF478472	Forward	GCTTGAGCCCGATGGAGG
		Reverse	CAAGATGGAATGCGGAAAATG
Δ6fad_a	AY458652	Forward	TCCCCAGACGTTTGTGTCAGATGC
		Reverse	GCTTTGGATCCCCCATTAGTTCCTG
elovl2	TC91192	Forward	CGGGTACAAAATGTGCTGGT
		Reverse	TCTGTTTGCCGATAGCCATT
elovl5b	NM_001136552	Forward	GCAACCTTGACCCAAACAGG
		Reverse	CCTTGTCTCTACGCAAGGGA
srebp1	NM_001195818	Forward	AGCTGCACGGCTTCCAGCAG
		Reverse	TCCTCCGTCTTGGCTCCGGG

Elongation factor 1 alpha (*ef1α*) eukaryotic translation initiation factor 3 (*etif3*), RNA polymerase II polypeptide (*rpol2*), acyl-CoA oxidase (*aco*), Δ5 desaturase (*Δ5fad*), Δ6 desaturase isoform a (*Δ6fad_a*), elongase 2 (*elovl2*), elongase 5b (*elovl5b*), sterol regulatory element binding protein 1 (*srebp1*).

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744 Table ~~2~~3. Fatty acid composition (mol%) in the PL and NL fraction of hepatocytes from Atlantic salmon fed the experimental diets for 26 weeks
745 (means ± sem; n = 3)

PL fraction	0%	0.5% EPA	1% EPA	2% EPA	0.5% DHA	1% DHA	2% DHA	0.5% EPA+DHA	1% EPA+DHA	2% EPA+DHA	ANOVA
16:0	18.4 ± 0.29^{b}	19.2 ± 0.28^{ab}	19.4 ± 0.07^{ab}	19.8 ± 0.53^{ab}	19.6 ± 0.21^{ab}	20.1 ± 0.51^{ab}	20.5 ± 0.41^{a}	20.0 ± 0.21^{ab}	20.2 ± 0.35^{a}	19.5 ± 0.18^{ab}	0.015
18:0	8.3 ± 0.23	7.6 ± 0.28	7.8 ± 0.32	7.2 ± 0.46	7.1 ± 0.21	7.1 ± 0.14	6.8 ± 0.54	7.4 ± 0.38	7.1 ± 0.44	6.9 ± 0.12	0.150
SFA[1]	29.8 ± 1.70	27.4 ± 0.53	27.7 ± 0.42	27.7 ± 0.92	27.3 ± 0.08	27.6 ± 0.64	27.7 ± 0.15	27.9 ± 0.26	27.9 ± 0.22	27.5 ± 0.85	0.541
18:1n-9	19.1 ± 1.28^{a}	18.9 ± 0.57^{ab}	17.0 ± 0.19^{abc}	14.8 ± 0.15^{c}	18.3 ± 0.87^{ab}	16.8 ± 1.04^{abc}	14.7 ± 0.46^{c}	19.4 ± 0.55^{a}	16.4 ± 0.25^{abc}	15.7 ± 0.33^{bc}	0.0002
MUFA[2]	24.2 ± 2.36^{a}	21.4 ± 0.22^{ab}	19.0 ± 0.51^{b}	17.0 ± 0.26^{b}	20.4 ± 0.87^{ab}	18.5 ± 0.83^{b}	17.0 ± 0.71^{b}	21.4 ± 0.70^{ab}	17.8 ± 0.27^{b}	17.7 ± 0.73^{b}	0.0004
18:2n-6	9.9 ± 0.57^{ab}	9.7 ± 0.75^{ab}	8.7 ± 0.47^{ab}	7.2 ± 0.53^{b}	10.8 ± 0.59^{a}	10.1 ± 0.15^{ab}	8.8 ± 0.11^{ab}	10.6 ± 1.28^{a}	10.0 ± 0.38^{ab}	8.1 ± 0.27^{ab}	0.007
18:3n-3	0.7 ± 0.04	0.7 ± 0.07	0.7 ± 0.04	0.8 ± 0.12	0.7 ± 0.07	0.7 ± 0.03	0.9 ± 0.05	0.8 ± 0.16	0.8 ± 0.06	0.9 ± 0.06	0.537
20:3n-3	0.04 ± 0.04^{b}	0.12 ± 0.02^{b}	0.14 ± 0.00^{ab}	0.20 ± 0.01^{ab}	0.14 ± 0.00^{ab}	0.15 ± 0.02^{ab}	0.40 ± 0.16^{a}	0.14 ± 0.03^{ab}	0.18 ± 0.01^{ab}	0.23 ± 0.04^{ab}	0.02
20:3 n-6	5.9 ± 0.71^{a}	5.5 ± 0.10^{ab}	4.0 ± 0.12^{bc}	2.0 ± 0.21^{de}	5.5 ± 0.27^{ab}	3.6 ± 0.24^{c}	1.8 ± 0.20^{e}	5.2 ± 0.29^{ab}	3.4 ± 0.24^{cd}	1.5 ± 0.09^{e}	<0.0001
20:4n-6	11.9 ± 1.03^{a}	8.7 ± 0.84^{ab}	7.2 ± 0.12^{bcd}	4.6 ± 0.10^{d}	8.9 ± 0.75^{ab}	7.8 ± 0.90^{bcd}	5.6 ± 0.50^{bcd}	8.6 ± 1.13^{abc}	6.8 ± 0.22^{bcd}	5.2 ± 0.28^{cd}	<0.0001
20:5n-3	1.7 ± 0.07^{e}	4.1 ± 0.09^{cd}	5.7 ± 0.38^{bc}	9.4 ± 0.30^{a}	1.8 ± 0.21^{e}	2.4 ± 0.39^{e}	2.2 ± 0.23^{e}	2.8 ± 0.12^{de}	4.6 ± 0.41^{bc}	6.1 ± 0.59^{b}	<0.0001
22:5n-3	2.6 ± 0.68^{ab}	1.9 ± 0.18^{abc}	2.3 ± 0.07^{ab}	2.7 ± 0.12^{a}	0.9 ± 0.13^{c}	0.8 ± 0.09^{c}	0.7 ± 0.09^{c}	1.5 ± 0.09^{bc}	1.4 ± 0.03^{bc}	1.5 ± 0.11^{abc}	<0.0001
22:6n-3	11.2 ± 0.84^{g}	18.5 ± 0.45^{f}	22.8 ± 0.48^{de}	26.6 ± 0.41^{bc}	21.3 ± 0.83^{ef}	26.4 ± 1.21^{bcd}	33.3 ± 0.46^{a}	19.2 ± 0.53^{ef}	25.5 ± 0.39^{cd}	29.2 ± 1.07^{b}	<0.0001
PUFA[3]	46.1 ± 2.18^{b}	51.2 ± 0.65^{a}	53.3 ± 0.64^{a}	55.3 ± 0.89^{a}	52.3 ± 0.83^{a}	53.9 ± 0.49^{a}	55.4 ± 0.56^{a}	50.7 ± 0.93^{b}	54.3 ± 0.34^{a}	54.7 ± 0.54^{a}	<0.0001
n-6 PUFA	24.0 ± 1.40^{a}	20.5 ± 0.16^{bcd}	17.8 ± 0.41^{def}	13.9 ± 0.51^{g}	22.0 ± 0.41^{ab}	19.9 ± 0.55^{bcd}	16.4 ± 0.47^{efg}	21.3 ± 0.37^{abc}	18.6 ± 0.26^{cde}	15.6 ± 0.09^{fg}	<0.0001
n-6/n-3	1.5 ± 0.08^{a}	0.8 ± 0.01^{bc}	0.6 ± 0.01^{def}	0.4 ± 0.01^{g}	0.9 ± 0.03^{b}	0.7 ± 0.03^{cd}	0.4 ± 0.01^{efg}	0.9 ± 0.02^{b}	0.6 ± 0.02^{de}	0.4 ± 0.01^{fg}	<0.0001
n-6 DI[4]	0.64 ± 0.01^{a}	0.59 ± 0.03^{ab}	0.57 ± 0.01^{abcd}	0.48 ± 0.03^{cd}	0.57 ± 0.02^{abc}	0.53 ± 0.02^{abcd}	0.46 ± 0.01^{d}	0.57 ± 0.05^{abcd}	0.50 ± 0.02^{bcd}	0.46 ± 0.01^{d}	0.0001
NL fraction											
16:0	17.9 ± 4.75	13.5 ± 1.05	15.3 ± 1.01	13.0 ± 0.50	14.7 ± 1.17	14.5 ± 1.44	16.2 ± 0.79	14.4 ± 0.46	19.3 ± 0.70	17.3 ± 0.65	0.267
18:0	11.1 ± 2.67	9.9 ± 0.32	10.0 ± 0.25	9.1 ± 0.77	8.1 ± 0.29	9.1 ± 0.93	9.9 ± 1.48	10.0 ± 0.38	8.4 ± 1.01	8.6 ± 0.10	0.715
SFA[1]	31.5 ± 8.03	24.4 ± 0.81	26.0 ± 1.02	22.7 ± 1.33	23.8 ± 1.01	24.5 ± 1.91	26.7 ± 1.55	26.3 ± 1.39	28.3 ± 0.17	26.7 ± 0.85	0.604
18:1n-9	41.8 ± 5.43	35.2 ± 5.58	33.0 ± 1.85	30.5 ± 6.96	35.0 ± 1.86	33.6 ± 3.17	26.3 ± 0.92	35.8 ± 3.74	27.1 ± 3.70	21.2 ± 1.29	0.068
MUFA[2]	46.6 ± 6.63	39.9 ± 6.26	37.6 ± 2.39	35.3 ± 8.33	39.3 ± 2.38	37.0 ± 3.00	29.6 ± 0.80	40.9 ± 5.11	30.0 ± 4.10	24.1 ± 1.41	0.101
18:2n-6	10.1 ± 0.58	10.1 ± 0.47	11.0 ± 0.83	10.2 ± 0.42	11.7 ± 0.38	12.8 ± 0.50	12.4 ± 0.56	11.1 ± 1.44	12.3 ± 0.94	10.2 ± 0.96	0.122
18:3n-3	0.7 ± 0.39	0.9 ± 0.06	1.2 ± 0.09	1.3 ± 0.09	1.0 ± 0.07	1.3 ± 0.17	1.6 ± 0.22	1.0 ± 0.14	1.5 ± 0.23	1.3 ± 0.16	0.097
20:3n-3	nd	nd	0.10 ± 0.05	0.27 ± 0.01	0.09 ± 0.05	0.07 ± 0.07	0.10 ± 0.10	0.14 ± 0.07	0.06 ± 0.06	0.22 ± 0.11	
20:3n-6	1.9 ± 0.93^{b}	3.4 ± 0.53^{a}	2.5 ± 0.17^{ab}	1.5 ± 0.10^{b}	3.6 ± 0.32^{a}	2.4 ± 0.23^{ab}	1.5 ± 0.17^{b}	3.2 ± 0.56^{a}	2.5 ± 0.35^{ab}	1.5 ± 0.08^{b}	0.010
20:4n-6	2.7 ± 0.20	5.9 ± 1.09	4.4 ± 0.77	4.4 ± 1.45	5.8 ± 0.49	4.9 ± 0.98	4.6 ± 0.21	4.8 ± 1.18	4.6 ± 1.26	5.3 ± 0.24	0.487
20:5n-3	0.6 ± 0.33^{c}	2.3 ± 0.63^{bc}	3.7 ± 0.37^{ab}	5.7 ± 1.15^{a}	1.2 ± 0.30^{bc}	1.6 ± 0.21^{bc}	1.8 ± 0.28^{bc}	1.4 ± 0.34^{bc}	3.7 ± 0.64^{ab}	5.2 ± 0.58^{a}	<0.0001
22:5n-3	0.1 ± 0.11^{c}	1.1 ± 0.21^{abc}	1.4 ± 0.09^{ab}	2.0 ± 0.34^{a}	0.6 ± 0.11^{bc}	0.4 ± 0.18^{bc}	0.6 ± 0.09^{bc}	0.7 ± 0.20^{bc}	0.8 ± 0.40^{bc}	1.4 ± 0.09^{ab}	<0.0001
22:6n-3	4.2 ± 1.49^{c}	9.8 ± 2.93^{abc}	10.1 ± 1.54^{abc}	14.4 ± 4.24^{abc}	10.3 ± 0.60^{abc}	12.5 ± 2.79^{abc}	18.9 ± 1.65^{ab}	8.0 ± 2.59^{bc}	14.1 ± 3.37^{abc}	22.0 ± 2.88^{a}	0.005

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PUFA[3]	21.9 ± 2.03[b]	35.7 ± 5.59[ab]	36.4 ± 3.34[ab]	42.0 ± 7.06[ab]	36.9 ± 1.37[ab]	38.5 ± 3.17[ab]	43.7 ± 1.20[a]	32.8 ± 6.50[ab]	41.7 ± 4.26[ab]	49.2 ± 1.95[a]	0.017
n-6 PUFA	14.4 ± 1.41	18.3 ± 1.28	17.6 ± 1.40	17.2 ± 1.32	20.1 ± 0.29	20.4 ± 0.54	19.3 ± 0.39	18.4 ± 2.67	19.1 ± 0.28	17.9 ± 0.54	0.12
n-6/n-3	2.9 ± 0.82[a]	1.4 ± 0.24[ab]	1.1 ± 0.04[b]	0.8 ± 0.19[b]	1.5 ± 0.09[ab]	1.3 ± 0.16[ab]	0.8 ± 0.05[b]	1.9 ± 0.45[ab]	1.0 ± 0.17[b]	0.6 ± 0.06[b]	0.004
n-6 DI[4]	0.30 ± 0.04	0.47 ± 0.04	0.38 ± 0.02	0.36 ± 0.07	0.44 ± 0.03	0.36 ± 0.04	0.33 ± 0.02	0.41 ± 0.03	0.36 ± 0.07	0.40 ± 0.03	0.25

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$) by ANOVA followed by Tukey's honestly significant difference *post hoc* test. nd, not detectable levels.

[1]Includes 14:0 and 20:0

[2]Includes 16:1 n-7 and 20:1 n-9

[3]Includes 18:3 n-6 and 20:2 n-6

[4]n-6 DI = (20:3 n-6 + 20:4 n-6) / (18:2 n-6 + 20:3 n-6 + 20:4 n-6)

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Table ~~3~~4. Fate of radioactivity from [1-^{14}C] 18:3n-3 incubated in Atlantic salmon hepatocytes in the presence or absence of lipoic acid isolated from fish fed 10 experimental diets containing different levels of EPA and/or DHA for 26 weeks. Values are means ± sem (n = 3).

	Cellular lipids (nmol)		Secreted lipids in media (nmol)		CO_2 in medium (nmol)		ASPF in medium (nmol)		Recovery (%)	
	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA
0 %	13.9 ± 0.3	13.5 ± 0.4	2.40 ± 0.12	2.56 ± 0.12	0.021 ± 0.003	0.014 ± 0.002	0.56 ± 0.02	0.56 ± 0.04	80.5 ± 1.0	79.4 ± 2.8
0.5% EPA	12.6 ± 0.3	12.2 ± 0.3	2.60 ± 0.12	2.78 ± 0.08	0.013 ± 0.001	0.008 ± 0.001	0.49 ± 0.03	0.54 ± 0.07	74.8 ± 1.4	73.9 ± 2.0
1.0% EPA	12.1 ± 0.5	12.0 ± 0.4	3.36 ± 0.06	3.40 ± 0.17	0.017 ± 0.002	0.010 ± 0.001	0.56 ± 0.06	0.93 ± 0.24	76.4 ± 2.3	78.0 ± 1.7
2.0% EPA	13.4 ± 1.0	13.7 ± 0.7	2.47 ± 0.27	2.90 ± 0.59	0.016 ± 0.003	0.015 ± 0.003	0.52 ± 0.05	0.63 ± 0.08	78.0 ± 3.5	81.9 ± 0.4
0.5% DHA	12.1 ± 0.9	12.6 ± 0.4	2.87 ± 0.57	2.91 ± 0.70	0.019 ± 0.001	0.013 ± 0.001	0.57 ± 0.05	0.73 ± 0.12	74.1 ± 2.1	77.3 ± 2.2
1.0% DHA	11.2 ± 0.5	10.9 ± 0.5	4.12 ± 0.48	4.06 ± 0.15	0.018 ± 0.001	0.012 ± 0.001	0.62 ± 0.03	0.78 ± 0.06	76.2 ± 1.2	75.1 ± 3.2
2.0% DHA	12.4 ± 1.1	12.0 ± 0.8	3.31 ± 0.42	3.82 ± 0.19	0.021 ± 0.003	0.016 ± 0.003	0.63 ± 0.04	0.87 ± 0.07	78.0 ± 5.5	79.4 ± 4.4
0.5% EPA+DHA	13.1 ± 1.3	13.4 ± 0.2	2.19 ± 0.30	2.14 ± 0.30	0.038 ± 0.023	0.011 ± 0.000	0.51 ± 0.03	0.56 ± 0.09	75.6 ± 4.8	76.7 ± 2.7
1.0% EPA+DHA	11.4 ± 1.1	11.4 ± 0.7	3.52 ± 0.45	3.55 ± 0.53	0.019 ± 0.002	0.015 ± 0.001	0.61 ± 0.03	0.74 ± 0.06	74.1 ± 3.8	74.6 ± 1.0
2.0% EPA+DHA	12.6 ± 0.8	12.0 ± 1.0	3.43 ± 0.55	3.74 ± 0.57	0.017 ± 0.001	0.020 ± 0.004	0.58 ± 0.01	0.80 ± 0.02	79.2 ± 1.7	79.0 ± 2.4
ANOVA P_{Diet}	0.013		0.0002		0.58		0.02		0.32	
ANOVA P_{LA}	0.69		0.36		0.01		< 0.0001		0.52	
ANOVA $P_{Diet \times LA}$	0.99		0.99		0.47		0.47		0.99	

~~Values are means ± sem (n = 3).~~ ASPF, acid- soluble products~~fraction~~. CONT, control cells; LA, cells supplemented with lipoic acid.

Equal amount of cells was seeded for each experimental condition, corresponding to 5.6 ± 0.25 mg protein per flask (mean ± SEM).

Table 45. Percentage distribution between phospholipids (PL), mono- and diacylglycerol (MDG), triglycerides (TAG), and cholesterol esters (CE) produced from [1-^{14}C] 18:3n-3 in Atlantic salmon hepatocytes in the presence or absence of lipoic acid from fish fed different levels of EPA and/or DHA for 26 weeks prior to the experiment (means ± sem; n = 3)

	PL (%)		MDG (%)		TAG (%)		CE (%)	
	CONRTROL	LA	CONRTROL	LA	CONRTROL	LA	CONRTROL	LA
0 %	90.9 ± 0.9	86.8 ± 0.7	1.3 ± 0.6	1.5 ± 0.4	7.5 ± 0.9	11.5 ± 0.5	0.3 ± 0.0	0.1 ± 0.1
0.5% EPA	89.2 ± 1.8	80.2 ± 1.9	0.5 ± 0.3	1.9 ± 0.2	10.2 ± 1.9	17.7 ± 1.9	nd	0.2 ± 0.2
1.0% EPA	87.7 ± 0.5	75.4 ± 2.4	2.0 ± 0.3	3.0 ± 0.8	10.2 ± 0.3	21.2 ± 1.6	0.1 ± 0.1	0.4 ± 0.1
2.0% EPA	74.8 ± 4.1	63.7 ± 1.5	1.4 ± 0.3	3.2 ± 1.1	23.5 ± 3.7	32.2 ± 1.0	0.4 ± 0.1	0.9 ± 0.2
0.5% DHA	89.8 ± 2.4	81.3 ± 1.5	1.3 ± 0.1	2.3 ± 0.1	8.8 ± 2.3	15.8 ± 1.4	0.1 ± 0.1	0.6 ± 0.0
1.0% DHA	88.6 ± 2.5	75.3 ± 0.6	2.7 ± 0.7	3.5 ± 0.6	8.4 ± 1.8	20.6 ± 1.1	0.2 ± 0.1	0.6 ± 0.3
2.0% DHA	84.3 ± 0.9	74.7 ± 2.1	1.8 ± 0.2	2.9 ± 0.3	13.6 ± 0.6	21.7 ± 1.8	0.4 ± 0.2	0.8 ± 0.1
0.5% EPA+DHA	84.0 ± 6.3	77.1 ± 6.7	1.6 ± 0.9	2.0 ± 1.2	14.2 ± 5.3	20.3 ± 5.6	0.1 ± 0.1	0.6 ± 0.0
1.0% EPA+DHA	87.5 ± 2.0	77.0 ± 2.8	2.4 ± 1.0	2.7 ± 0.8	10.0 ± 2.6	19.6 ± 2.1	0.1 ± 0.0	0.8 ± 0.2
2.0% EPA+DHA	80.3 ± 2.8	69.9 ± 1.9	2.7 ± 1.4	3.6 ± 1.2	16.7 ± 2.8	25.6 ± 3.4	0.3 ± 0.1	0.9 ± 0.3
ANOVA P_{Diet}	< 0.0001		0.14		< 0.0001			
ANOVA P_{LA}	< 0.0001		0.009		< 0.0001			
ANOVA $P_{Diet\ x\ LA}$	0.91		0.99		0.91			

Total radioactivity recovered in the different lipid classes was set to 100% for each dietary group (PL+ MDG + TAG + CE = 100%). The different *P*-values are significance levels from two-way ANOVA. nd = non detectable amounts; CONT = control cells; LA = cells supplemented with lipoic acid.

Table ~~5~~6. Percentage of substrate added recovered in PUFA from the phospholipid fraction of hepatocytes incubated with [1-^{14}C] 18:3n-3 in lipoic acid free or supplemented media (means ± sem; n = 3). The fish had been fed diets containing different levels of EPA and/or DHA for 26 weeks prior to the experiment.

	18:3n-3		18:4n-3		20:3n-3		20:4n-3		20:5n-3		22:5n-3		22:6n-3	
	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA
0%	36.4 ± 0.9	30.1 ± 0.7	1.0 ± 0.13	1.4 ± 0.12	0.5 ± 0.03	2.6 ± 0.4	5.4 ± 0.33	4.4 ± 0.29	10.2 ± 0.5	6.8 ± 0.4	1.0 ± 0.08	1.1 ± 0.20	5.3 ± 0.5	8.2 ± 1.1
0.5% EPA	28.6 ± 1.2	21.9 ± 2.0	0.8 ± 0.09	0.9 ± 0.23	0.6 ± 0.28	3.7 ± 0.71	3.5 ± 0.62	2.5 ± 0.32	12.7 ± 0.5	7.4 ± 0.7	0.9 ± 0.11	0.8 ± 0.18	6.0 ± 0.7	8.4 ± 0.6
1.0% EPA	26.6 ± 0.9	22.1 ± 0.1	0.6 ± 0.19	0.5 ± 0.17	1.8 ± 0.47	4.7 ± 0.59	2.3 ± 0.83	1.5 ± 0.55	10.8 ± 2.0	6.4 ± 1.7	0.6 ± 0.11	0.4 ± 0.13	7.3 ± 0.1	7.0 ± 0.6
2.0% EPA	23.8 ± 1.4	19.6 ± 1.4	0.4 ± 0.10	0.4 ± 0.06	3.8 ± 1.11	8.3 ± 1.42	1.7 ± 0.30	1.0 ± 0.14	8.0 ± 1.3	3.9 ± 0.6	0.5 ± 0.06	0.2 ± 0.04	8.0 ± 0.1	6.4 ± 1.0
0.5% DHA	27.6 ± 1.1	24.3 ± 0.8	0.7 ± 0.27	0.7 ± 0.27	1.2 ± 0.33	4.5 ± 0.71	3.1 ± 0.96	2.0 ± 0.50	10.4 ± 2.4	6.4 ± 1.3	1.0 ± 0.27	0.7 ± 0.18	7.0 ± 0.4	8.3 ± 1.4
1.0% DHA	26.2 ± 1.0	22.0 ± 1.0	0.5 ± 0.03	0.4 ± 0.09	2.1 ± 0.24	2.6 ± 1.08	1.4 ± 0.12	1.0 ± 0.09	9.4 ± 1.4	7.3 ± 1.6	0.3 ± 0.05	0.1 ± 0.09	6.9 ± 0.7	5.3 ± 0.6
2.0% DHA	26.6 ± 3.0	22.2 ± 1.8	0.4 ± 0.05	0.3 ± 0.04	5.0 ± 0.95	8.5 ± 1.84	1.3 ± 0.30	0.7 ± 0.06	5.7 ± 0.9	3.3 ± 0.5	0.2 ± 0.19	0.1 ± 0.07	8.7 ± 0.5	5.7 ± 0.4
0.5% E+D	27.3 ± 1.5	22.2 ± 2.9	1.1 ± 0.42	1.3 ± 0.31	0.7 ± 0.33	3.7 ± 1.42	4.1 ± 0.62	3.1 ± 0.40	11.0 ± 0.4	6.6 ± 0.5	1.1 ± 0.20	0.7 ± 0.15	6.0 ± 1.2	10.0 ± 1.9
1.0% E+D	27.1 ± 0.0	21.5 ± 1.3	0.5 ± 0.00	0.4 ± 0.09	2.3 ± 0.00	5.9 ± 1.62	2.0 ± 0.00	1.1 ± 0.04	8.4 ± 0.0	4.6 ± 0.9	0.7 ± 0.00	0.4 ± 0.11	7.9 ± 0.0	6.9 ± 1.0
2.0% E+D	25.1 ± 2.7	20.6 ± 2.6	0.4 ± 0.06	0.3 ± 0.04	4.7 ± 0.38	8.5 ± 0.35	1.5 ± 0.26	0.8 ± 0.13	6.9 ± 1.1	3.5 ± 0.7	0.3 ± 0.10	0.1 ± 0.02	8.8 ± 1.3	5.6 ± 0.7
ANOVA P_{Diet}	< 0.0001		< 0.0001		< 0.0001		< 0.0001		0.0003		< 0.0001		0.75	
ANOVA P_{LA}	< 0.0001		0.81		< 0.0001		0.0002		< 0.0001		0.01		0.97	
ANOVA $P_{Diet \times LA}$	0.99		0.95		0.69		0.99		0.96		0.85		0.0016	

Minor amounts of radioactivity (0.48 ± 0.04%; mean ± sem) were recovered in two non-identified peaks. CONT, control cells; LA, cells supplemented with lipoic acid.

Table ~~6~~7. Percentage of substrate added recovered in PUFA from the neutral lipid fraction of hepatocytes incubated with [1-^{14}C] 18:3n-3 in lipoic acid free or supplemented media (means ± sem; n = 3). The fish had been fed diets containing different levels of EPA and/or DHA for 26 weeks prior to the experiment.

	18:3n-3		18:4n-3		20:3n-3		20:4n-3		20:5n-3		22:5n-3		22:6n-3	
	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA	CONT	LA
0%	3.6 ± 0.5	4.5 ± 0.5	0.5 ± 0.03	0.3 ± 0.07	0.1 ± 0.06	1.6 ± 0.1	0.14 ± 0.14	0.26 ± 0.02	0.58 ± 0.11	0.53 ± 0.20	nd	nd	0.77 ± 0.18	1.10 ± 0.11
0.5% EPA	3.1 ± 0.8	5.6 ± 0.6	0.7 ± 0.32	0.8 ± 0.16	0.5 ± 0.24	2.8 ± 0.50	0.11 ± 0.11	0.14 ± 0.09	0.86 ± 0.21	0.49 ± 0.04	0.10 ± 0.06	0.02 ± 0.02	0.82 ± 0.05	1.46 ± 0.14
1.0% EPA	3.5 ± 0.2	6.6 ± 0.8	0.4 ± 0.08	0.5 ± 0.08	1.0 ± 0.10	3.8 ± 0.32	0.06 ± 0.06	0.04 ± 0.04	0.70 ± 0.14	0.69 ± 0.10	nd	nd	1.15 ± 0.15	2.25 ± 0.22
2.0% EPA	8.6 ± 2.0	11.4 ± 0.6	0.5 ± 0.05	0.6 ± 0.03	3.5 ± 1.20	7.8 ± 1.23	0.10 ± 0.05	0.21 ± 0.16	0.83 ± 0.06	0.57 ± 0.13	0.03 ± 0.03	nd	2.08 ± 0.41	2.76 ± 0.13
0.5% DHA	3.3 ± 0.9	5.5 ± 0.6	0.4 ± 0.12	0.4 ± 0.09	0.4 ± 0.22	2.6 ± 0.44	0.06 ± 0.06	0.14 ± 0.10	0.43 ± 0.17	0.51 ± 0.17	nd	nd	0.86 ± 0.20	1.73 ± 0.19
1.0% DHA	3.5 ± 0.6	6.2 ± 0.7	0.3 ± 0.13	0.5 ± 0.10	0.7 ± 0.45	2.6 ± 0.28	0.04 ± 0.04	0.04 ± 0.04	0.61 ± 0.15	1.16 ± 0.23	nd	nd	1.14 ± 0.44	2.40 ± 0.17
2.0% DHA	4.8 ± 0.2	6.4 ± 0.3	0.3 ± 0.06	0.3 ± 0.07	2.2 ± 0.13	5.1 ± 0.39	nd	0.03 ± 0.03	0.31 ± 0.03	0.34 ± 0.18	nd	nd	1.55 ± 0.18	1.95 ± 0.44
0.5% E+D	5.0 ± 2.2	7.5 ± 2.0	1.4 ± 0.93	1.0 ± 0.67	1.0 ± 0.31	2.9 ± 0.24	0.48 ± 0.48	0.30 ± 0.30	1.05 ± 0.58	0.50 ± 0.27	nd	nd	1.13 ± 0.28	1.61 ± 0.34
1.0% E+D	3.5 ± 0.0	5.9 ± 0.8	0.3 ± 0.00	0.5 ± 0.11	1.2 ± 0.00	3.9 ± 0.77	nd	nd	0.56 ± 0.00	0.36 ± 0.18	nd	nd	1.15 ± 0.00	1.64 ± 0.34
2.0% E+D	6.3 ± 0.7	8.1 ± 0.1	0.4 ± 0.08	0.3 ± 0.09	2.4 ± 0.35	5.8 ± 0.22	0.08 ± 0.08	0.17 ± 0.10	0.75 ± 0.08	0.40 ± 0.11	0.04 ± 0.04	0.07 ± 0.07	1.66 ± 0.17	2.28 ± 0.06
ANOVA P_{Diet}	< 0.0001		0.08		< 0.0001				0.20				< 0.0001	
ANOVA P_{LA}	< 0.0001		0.91		< 0.0001				0.20				< 0.0001	
ANOVA $P_{Diet\ x\ LA}$	0.99		0.99		0.34				0.31				0.65	

Minor amounts of radioactivity (0.12 ± 0.02%; mean ± sem) were recovered in two non-identified peaks. nd = non detectable amounts; CONT = control cells; LA = cells supplemented with lipoic acid.

774 Table ~~7~~8. Spearman's correlation coefficients between cellular EPA or cellular DHA and 18:3n-3 and its FA products in control hepatocytes and
775 hepatocytes supplemented with lipoic acid.

	Cellular EPA		Cellular DHA	
	CONT	LA	CONT	LA
18:3n-3	-0.325 (0.091)	-0.443 (0.014)	-0.519 (0.005)	-0.392 (0.032)
18:4n-3	-0.257 (0.187)	-0.310 (0.096)	-0.663 (0.0001)	-0.754 (< 0.0001)
20:3n-3	0.393 (0.039)	0.454 (0.012)	0.883 (< 0.0001)	0.582 (0.0007)
20:4n-3	-0.284 (0.144)	-0.322 (0.082)	-0.763 (< 0.0001)	-0.820 (< 0.0001)
20:5n-3	-0.085 (0.668)	-0.291 (0.119)	-0.6842 (< 0.0001)	-0.589 (0.0006)
22:5n-3	-0.275 (0.157)	-0.306 (0.100)	-0.775 (< 0.0001)	-0.854 (<0.0001)
22:6n-3	0.390 (0.040)	-0.201 (0.287)	0.767 (< 0.0001)	

776 CONT, control cells; LA, cells supplemented with lipoic acid.

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Fig.1

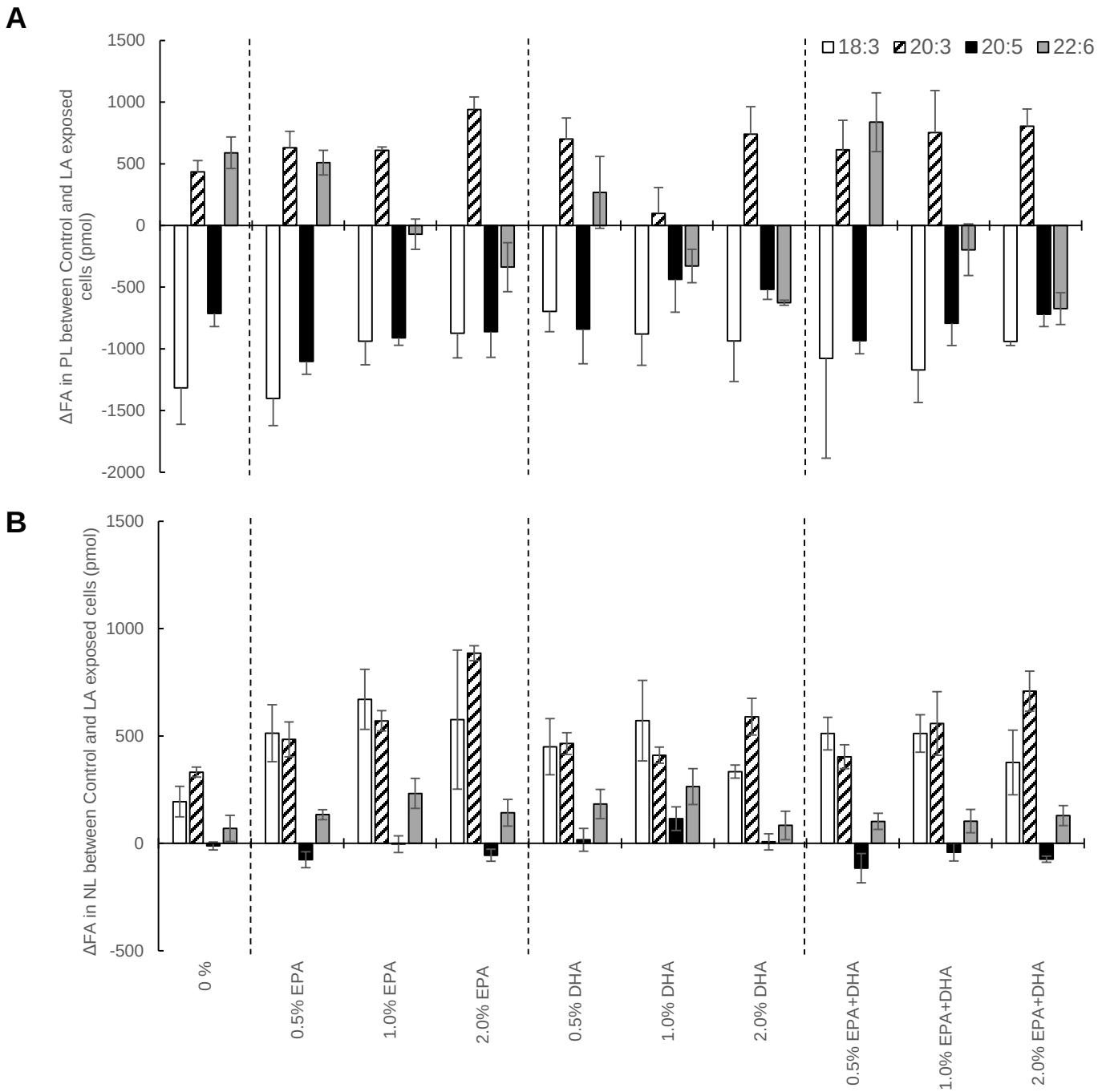

A
B
18:3
20:3
20:5
22:6
ΔFA in PL between Control and LA exposed cells (pmol)
ΔFA in NL between Control and LA exposed cells (pmol)
1500
1000
500
0
-500
-1000
-1500
-2000
0 %
0.5% EPA
1.0% EPA
2.0% EPA
0.5% DHA
1.0% DHA
2.0% DHA
0.5% EPA+DHA
1.0% EPA+DHA
2.0% EPA+DHA

Fig.2

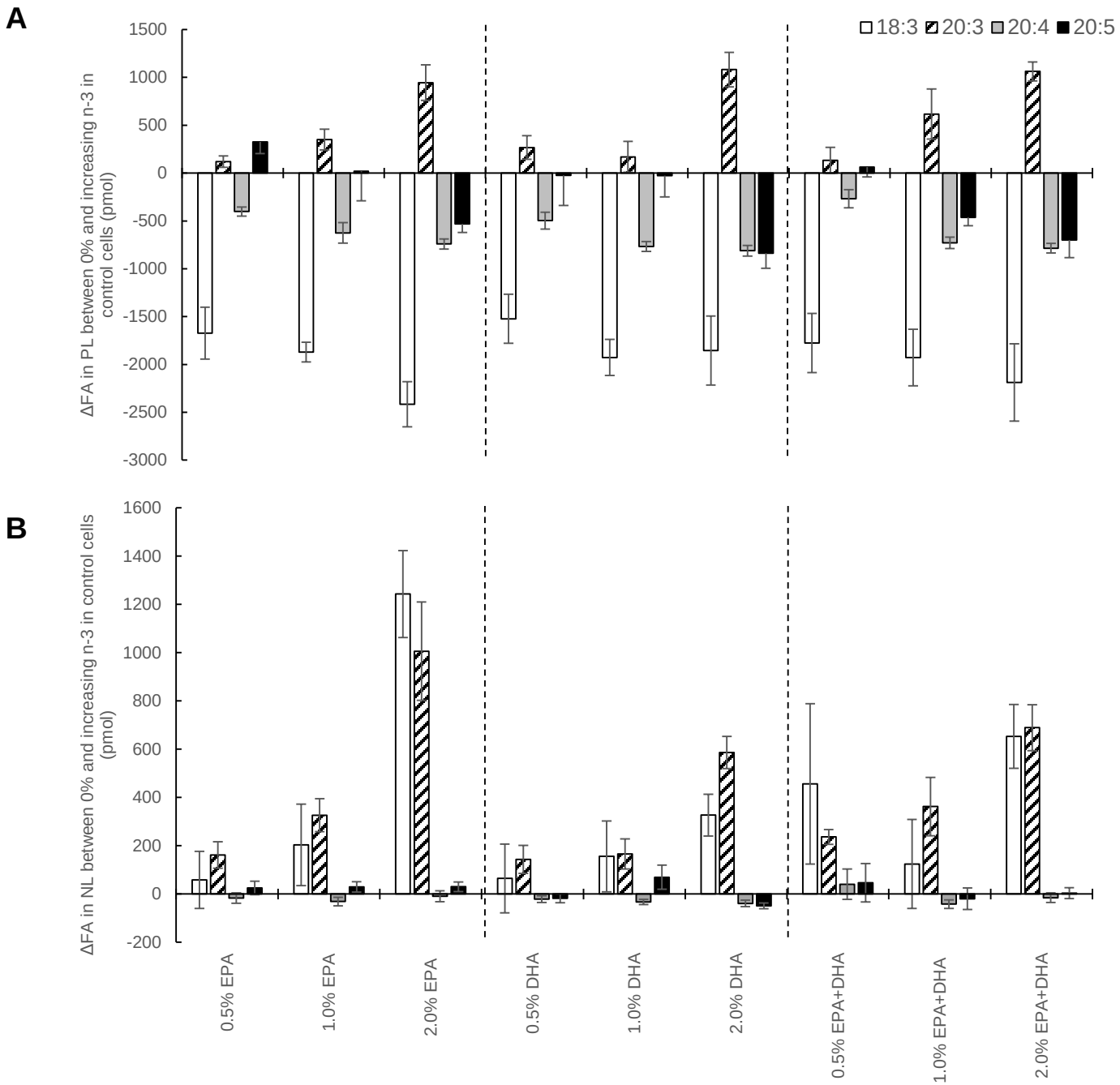

A
18:3
20:3
20:4
20:5
ΔFA in PL between 0% and increasing n-3 in control cells (pmol)
1500
1000
500
0
-500
-1000
-1500
-2000
-2500
-3000
B
ΔFA in NL between 0% and increasing n-3 in control cells (pmol)
1600
1400
1200
1000
800
600
400
200
0
-200
0.5% EPA
1.0% EPA
2.0% EPA
0.5% DHA
1.0% DHA
2.0% DHA
0.5% EPA+DHA
1.0% EPA+DHA
2.0% EPA+DHA

Fig.3

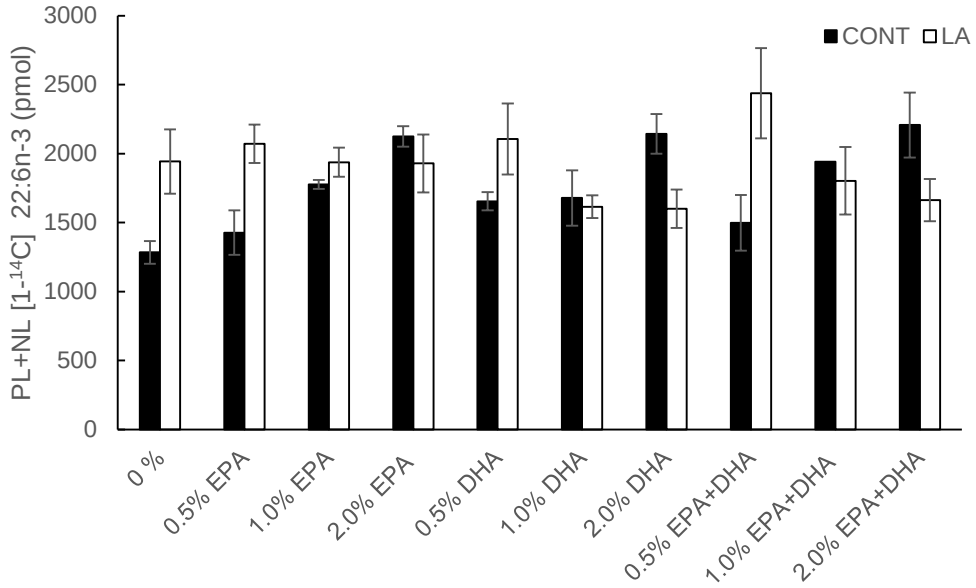
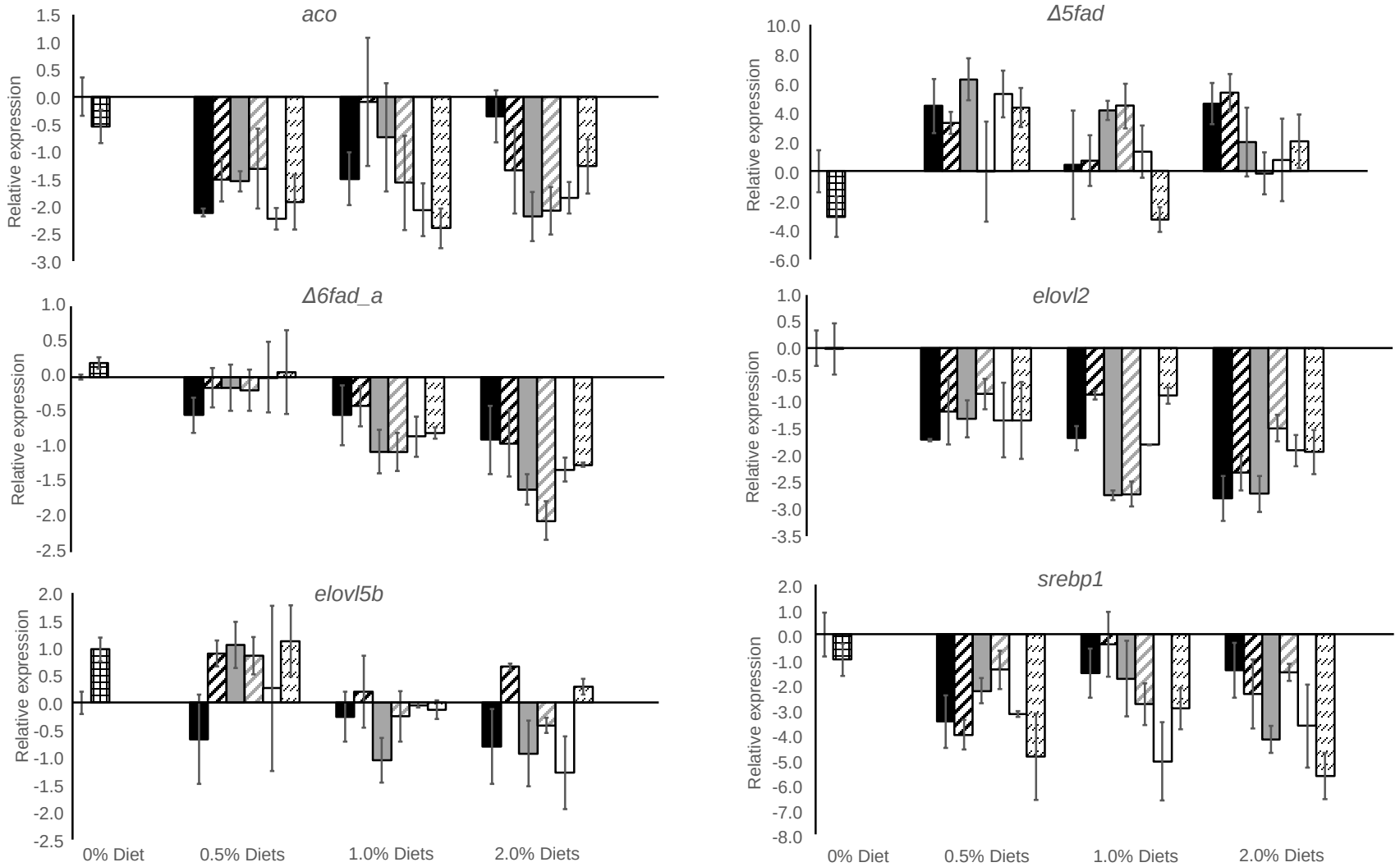

PL+NL [1-14C] 22:6n-3 (pmol)
3000
2500
2000
1500
1000
500
0
CONT
LA
0 %
0.5% EPA
1.0% EPA
2.0% EPA
0.5% DHA
1.0% DHA
2.0% DHA
0.5% EPA+DHA
1.0% EPA+DHA
2.0% EPA+DHA

Fig.4



	P_{Diet}	P_{LA}	$P_{Diet \times LA}$
aco	0.03	0.85	0.62
Δ5fad	0.02	0.11	0.59
Δ6fad_a	< 0.0001	0.72	0.98
elovl2	< 0.0001	0.005	0.47
elovl5b	0.03	0.0006	0.72
srebp1	0.004	0.97	0.21