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Docosapentaenoic (22:5 n-6) and docosahexaenoic (22:6 n-3) acids exhibit highly lipogenic properties in rainbow trout preadipocytes

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ABSTRACT

Dietary polyunsaturated fatty acids are essential for fish health. Adipose tissue is the major tissue for fatty acid storage in rainbow trout (*Oncorhynchus mykiss*), and its development and function can be impacted by the fatty acids themselves. In the present study, the effects of seven fatty acids, oleic (OA, 18:1 n-9), α-linolenic (ALA, 18:3 n-3), eicosapentaenoic (EPA, 20:5 n-3), docosahexaenoic (DHA, 22:6 n-3), linoleic (LA, 18:2 n-6), arachidonic (AA, 20:4 n-6), and docosapentaenoic (DPA, 22:5 n-6) acids, on adipogenesis were investigated in primary cultures of rainbow trout preadipocytes. In terms of lipid accumulation, DPA and DHA appeared to be the most lipogenic fatty acids, while all treatments modified the fatty acid composition of the cellular phospholipids and neutral lipids. The fatty acid of interest added to the culture medium was the most abundant in preadipocytes, while the first bioconversion products were detected in lower amounts. In terms of transcriptional effects, DPA increased the expression of the early transcription factor CCAAT/enhancer binding protein δ, while DHA upregulated the expression of genes involved in neutral lipid synthesis, notably lipoprotein lipase, fatty acid transport protein 1 and glycerol-3-phosphate dehydrogenase. Both fatty acids decreased the expression of fatty acid synthase. These results highlight that DPA and DHA exert a significant effect on lipid deposition in rainbow trout preadipocytes, potentially through different pathways, and confirm that fatty acids have major impacts on preadipocyte lipid metabolism and adipogenesis.

1. Introduction

Lipids, and especially fatty acids, fulfill several important functions in fish. Depending on their characteristics, they can (i) provide energy through fatty acid β-oxidation, (ii) represent essential constituents of the phospholipid membranes, (iii) be nuclear receptor ligands able to modulate the expression of genes, and (iv) exhibit signaling properties as precursors of bioactive molecules such as eicosanoids (Tocher, 2003). Lipids represent a significant part of the fish diet. However, their nature has evolved over the last decades in aquaculture. Fish oil has been increasingly replaced by plant-derived oils for environmental and economic reasons (Turchini et al., 2009). Fish oil replacement highly modifies the fatty acid composition of fish diet. Indeed, polyunsaturated fatty acids (PUFA) with eighteen carbons typical of plant-derived oils, notably linoleic (LA, 18:2 n-6) and, to a lesser extent, α-linolenic (ALA, 18:3 n-3) acids, become major fatty acids in fish diet whereas they are present in low proportions in fish oil-based diets. The amount of eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids, two major n-3 long chain PUFA found only in fish oil, will therefore vary depending on the feed formulation (Glencross, 2009). The efficiency of bioconversion of LA and ALA into longer and more unsaturated fatty acids depends on the species. Salmonids can efficiently produce EPA and DHA from ALA (Fig. 1) (Monroig et al., 2011). The amount of PUFA synthesized by the fish itself is, however, limited and the fatty acid composition of the fish and its organs mainly reflects that of the diet, which can have an impact on the cellular pathways and

Abbreviations: AA, arachidonic acid (20:4 n-6); ACSL, long-chain acyl-CoA synthetase; ALA, α-linolenic acid (18:3 n-3); BSA, bovine serum albumin; C/EBP, CCAAT/enhancer binding protein; cPEPCK, cytosolic phosphoenolpyruvate carboxykinase; DHA, docosahexaenoic acid (22:6 n-3); DPA, docosapentaenoic acid (22:5 n-6); EPA, eicosapentaenoic acid (20:5 n-3); FABP, fatty acid binding protein; FASN, fatty acid synthase; FATP, fatty acid transport protein; FBS, foetal bovine serum; GPDH, glycerol-3-phosphate dehydrogenase; LA, linoleic acid (18:2 n-6); LPL, lipoprotein lipase; MUFA, monounsaturated fatty acids; OA, oleic acid (18:1 n-9); ORO, Oil Red O; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids..

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health of the fish (Ferain et al., 2021; Tinant et al., 2023; Tocher, 2015).

Adipose tissue, and primarily perivisceral deposition, is the major site of lipid storage in salmonids and is an essential endocrine organ involved in energy homeostasis (Harwood Jr, 2012; Weil et al., 2013). Lipids are stored in the large lipid droplet of adipocytes, the main cell type in this tissue, in the form of neutral lipids. Two mechanisms enable the expansion of adipose tissue, for example during positive energy balance periods. On the one hand, hyperplasia involves the recruitment of new precursor cells to increase the number of adipocytes and, on the other hand, hypertrophy occurs through the accumulation of lipids in existing adipocytes, leading to cell size increase (Otto and Lane, 2005). The whole pathway is called adipogenesis and it begins with the proliferation of mesenchymal stem cells before their commitment into the adipocyte lineage during the determination phase. These cells, called preadipocytes, are still able to proliferate until the differentiation phase begins and the cells become mature adipocytes (Otto and Lane, 2005). The differentiation is a tightly regulated process. In particular, CCAAT/enhancer binding protein (C/EBP) β and δ are first activated and induce the upregulation of peroxisome proliferator-activated receptor (PPAR) γ and C/EBPα (Bou et al., 2017; Rosen et al., 2000). The transcriptional cascade results in the activation of several adipocyte-related genes, leading to the adipocyte phenotype.

In fish, fatty acids are required to induce differentiation, as demonstrated with primary cultures of preadipocytes (Bouraoui et al., 2008; Vegusdal et al., 2003). These fatty acids mainly come from the diet and differently affect the adipocyte differentiation based on their nature (Salmerón, 2018). It was generally observed that the *in vitro* incubation of fish preadipocytes with C_{18} fatty acids increased the intracellular lipid accumulation in contrast to fatty acids typical of fish oil (i.e., EPA and DHA) (Bou et al., 2020; Riera-Heredia et al., 2020; Todorčević et al., 2008). Fatty acids also modulate the expression of genes involved in adipogenesis (e.g., C/EBPα and δ) as well as in fatty acid transport and metabolism (e.g., lipoprotein lipase (LPL), fatty acid transport protein (FATP) 1, fatty acid synthase (FASN)). However, contrasting results were observed in the literature. As an example, DHA decreased expression of C/EBPα, FASN and LPL in Atlantic salmon (*Salmo salar*) and large yellow croaker (*Pseudosciaena Crocea* R.) (Huang et al., 2010; Wang et al., 2012) whereas no effect was observed in rainbow trout (Riera-Heredia et al., 2020). In addition, little is known about the effects of fatty acids resulting from bioconversion, such as arachidonic (AA, 20:4 n-6) and docosapentaenoic (DPA, 22:5 n-6) acids (Fig. 1).

The changes in the fatty acid profile of fish diets resulting from the replacement of fish oils by plant-derived oils most likely impact the development and function of adipose tissue. The present study was designed to obtain novel insight into the adipogenic and lipogenic effects of fatty acids characteristic of fish oils and plant-derived oils and those originating from the bioconversion pathways (Fig. 1). Seven fatty acids were chosen, one monounsaturated fatty acid (MUFA) (oleic acid (OA, 18:1 n-9)), three n-3 PUFA (ALA, EPA, and DHA), and three n-6 PUFA (LA, AA, and DPA). To the best of our knowledge, this is the first time that the precursors (ALA and LA) and the final products (DHA and DPA) of the n-3 and n-6 bioconversion pathways along with the most represented MUFA in the fish diet (OA) were simultaneously studied for their effects on lipid accumulation and fatty acid metabolism in a fish model system. These effects were investigated in primary cultured rainbow trout preadipocytes. Specifically, lipid accumulation, fatty acid composition of the lipid droplets and membranes, and expression of several adipocyte-related genes were studied.

2. Material & methods

2.1. Experimental design

An *in vitro* model of primary culture of preadipocytes (see Section 2.2) was used in the present study to investigate the effects of seven fatty acids on adipogenesis and preadipocyte lipid metabolism in rainbow trout (*Oncorhynchus mykiss*). At confluence (day 0), preadipocytes were incubated for two days with a hormonal cocktail supplemented in lipid mixture to induce their differentiation (see Section 2.3). Cells were then cultured during 12 to 14 days with one specific fatty acid and a reduced level of lipid mixture. Preadipocytes were treated with either 75, 150, 300 and 600 μM of OA (18:1 n-9), ALA (18:3 n-3), EPA (20:5 n-3), DHA (22:6 n-3), LA (18:2 n-6), AA (20:4 n-6) or DPA (22:5 n-6) to evaluate the dose-dependent lipid accumulation. The upper concentration (600 μM) was chosen based on previous publications investigating the effects of n-3 and n-9 fatty acids in Atlantic salmon preadipocytes (Huang et al., 2010; Todorčević et al., 2008) and serial dilutions by a factor of two were then conducted. Since the highest concentration showed the greatest effect for the majority of the treatments, the fatty acid composition and the expression of several adipocyte- and lipid metabolism-related genes were evaluated at 600 μM. Lipid accumulation and fatty acid composition were measured at the end of the incubation period whereas gene expression was assessed throughout the culture period (i.e., days 0, 2, 4, 8 and 14). At least three independent biological replicates were performed.

2.2. Primary culture of preadipocytes

The experiments were approved by the Animal Care and Use Committee of the Université catholique de Louvain (Permit number:

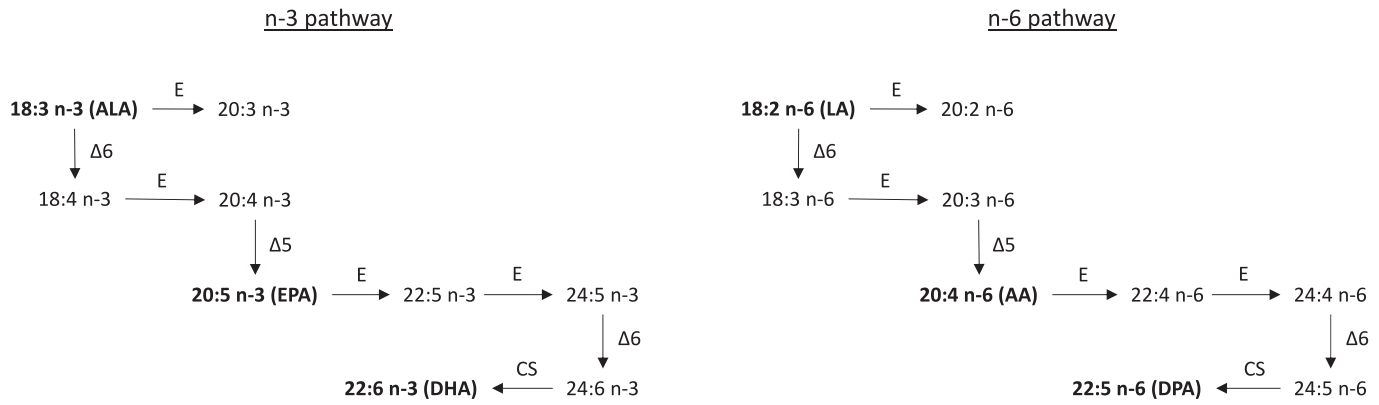

Fig. 1. Bioconversion pathways of n-3 and n-6 fatty acids in fish (adapted from Glencross (2009) and Ferain et al. (2016)). These metabolic pathways primarily occur in hepatocytes and the same enzymes act on both pathways. The vertical arrows represent the desaturation reactions and the horizontal arrows represent the elongation (right arrows)/shortening (left arrows) reactions. The fatty acids of interest in the present study are in bold. Abbreviations: Δ, microsomal fatty acid desaturase enzyme-mediated reactions; AA, arachidonic acid; ALA, α-linolenic acid; CS, peroxisomal fatty acid chain shortening via β-oxidation; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; E, microsomal fatty acid elongase-mediated reactions; EPA, eicosapentaenoic acid; LA, linoleic acid.

153201). Eggs of female rainbow trout were purchased at 'Pisciculture Charles Murgat' (Beaufort, France) and bred at 'Plateforme technologique en biologie aquicole Marcel Huet' (Université catholique de Louvain, Louvain-la-Neuve, Belgium) (Permit number for animal facilities: 1220034). Fish were fed daily with a commercial diet (Skretting, Stavanger, Norway) and maintained in a flow-through system at 12 ± 1 °C under a natural photoperiod. Several fish were sacrificed for each primary culture via a blow to the head followed by the destruction of the brain. Perivisceral adipose tissue was collected and processed as previously described in Tinant et al. (2021) and based on Bouraoui et al. (2008). Briefly, tissue underwent two enzymatic digestions with type II collagenase (400 units/mL) (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), bovine serum albumin (BSA) (1%, w:v) (Sigma-Aldrich, Saint-Louis, MO, USA), HEPES buffer solution (10 mM), antibiotics and antimycotics, for successively 1 h and 30 min at 19 °C. Afterwards, several steps of centrifugation and filtration allowed for the isolation of precursor cells. Remaining erythrocytes were removed with lysis buffer containing 154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM disodium EDTA. Cells were seeded at 20×10^3 cells/cm^2 in 12-well (for Oil Red O (ORO) quantification) or 6-well (for other endpoints) plates (Corning® CellBIND® surface) (VWR, Radnor, PA, USA) in growth medium and incubated at 19 °C. Growth medium was composed of Leibovitz's L-15 medium containing GlutaMAX™ (Life Technologies) supplemented with 10% foetal bovine serum (FBS) (reference 10270–106) (Life Technologies), antibiotics and antimycotics. Medium was renewed after 24 h and then every 2–3 days until confluence (after approximately one week).

2.3. Induction of cell differentiation and fatty acid treatments

At confluence (day 0), hormones (10 μg/mL insulin (Sigma-Aldrich), 10 μM ciglitizone (Sigma-Aldrich) and 0.25 μM dexamethasone (Sigma-Aldrich)) and lipid mixture (4 μL/mL) (reference L5146) (Sigma-Aldrich) were added to growth medium deprived of GlutaMAX™ to induce adipocyte differentiation. The lipid mixture was composed of 4.5 g/L cholesterol, 10 g/L cod liver oil fatty acid methyl esters (FAMEs), 25 g/L polyoxyethylenesorbitan monooleate, and 2 g/L D-α-tocopherol acetate. After a 2-day induction, medium was replaced by growth medium deprived of GlutaMAX™ and enriched with a specific fatty acid (OA, ALA, EPA, DHA, LA, AA or DPA) at 75, 150, 300 or 600 μM and lipid mixture (2 μL/mL). All fatty acids were purchased at Larodan (Solna, Sweden). The fatty acids were supplied as complexes with BSA in the molar proportion of 1:4 (BSA: fatty acid) in phosphate buffered saline (PBS). The BSA used was initially free of fatty acids. Fatty acid-enriched medium was renewed six times throughout the culture (about every two days). Control cells received only the BSA and, as sole sources of fatty acids, lipid mixture and FBS. The potential cytotoxicity of fatty acids was evaluated via the Cytotoxicity Detection Kit from Roche Diagnostics (Basel, Switzerland). The activity of lactate dehydrogenase released by the cells was measured following the manufacturer's instructions and compared to a full toxicity control consisting of control cells lysed by exposure to 1% Triton X-100 (Sigma-Aldrich). No treatment appeared cytotoxic (≤ 10% of toxicity compared to full toxicity control) (data not shown).

2.4. Quantification of total neutral lipids by ORO staining

Culture medium was removed and cells were washed twice with PBS at 19 °C. Cells were fixed with formaldehyde solution in PBS (4%, v:v) for 1 h at room temperature. After removal of formaldehyde, cells were washed and conserved with deionized water at 4 °C until coloration. Stock solution of dye was prepared with ORO (Sigma-Aldrich) dissolved in isopropanol (0.5%, w:v). Working solution was prepared daily by diluting stock solution in deionized water (3:2, v:v) and filtering through 0.22 μm filters. Cells were incubated for 1 h with ORO solution at room temperature and then thoroughly washed and stored with deionized water at 4 °C until ORO quantification. Dye accumulated in cells was extracted with isopropanol and the absorbance of the resulting suspension was measured at 510 nm. Lipid accumulation was proportional to the absorbance and results were expressed in percent of control cells.

2.5. Determination of fatty acid composition and concentrations

Fatty acid profile was determined as previously described in Tinant et al. (2021) with minor modifications. Briefly, after removing medium and washing cells twice with PBS at 19 °C, cells were collected in lysis buffer containing 35 mM sodium dodecyl sulfate, 60 mM Tris buffer and 10 mM sodium EDTA and stored at −20 °C until further analysis. Firstly, total lipids were extracted from 800 μL of cellular suspension with methanol:chloroform:water (2:2:1.8, v:v:v) (Bligh and Dyer, 1959). One millilitre of a solution of 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine (Larodan) and triheptadecanoin (Larodan) was added to each sample as an internal standard. Secondly, extracted lipids were separated into neutral lipid and phospholipid fractions by solid phase extraction (Bond Elut-NH_2, 200 mg, 3 mL) (Agilent Technologies, Santa Clara, CA, USA) (Schneider et al., 2012). Neutral lipids were eluted with chloroform:2-propanol (2:1, v:v) and phospholipids by methanol. Thirdly, fatty acids were methylated for 1 h in basic conditions (0.1 M KOH solution in methanol) and for 15 min in acidic conditions (1.2 M HCl solution in methanol) at 70 °C (Schneider et al., 2012). FAMEs were finally extracted with hexane and an injection standard (methyl-undecanoate) (Larondan) was added in each sample. The FAMEs were separated by gas chromatography (Trace 1310) (Thermo Fisher Scientific, Milan, Italy) equipped with an autosampler TriPlusAS (Thermo Fisher Scientific) and a Rt®-2560 capillary column (biscyanopropyl polysiloxane, 100 m length, 0.25 mm internal diameter, 0.2 μm film thickness) (Restek, Bellefonte, PA, USA) continuously supplied with H_2 as carrier gas at constant pressure (200 kPa). The oven temperature program started at 80 °C. The temperature then increased to 175 °C at a rate of 25 °C/min and stayed at 175 °C for 25 min, before rising to 200 °C (10 °C/min) and staying at this temperature for 20 min. Afterwards temperature increased to 220 °C (10 °C/min) and stayed at 220 °C for 5 min, before increasing again to 235 °C (10 °C/min) and staying at 235 °C for 15 min. Finally, temperature decreased to 80 °C (20 °C/min). Chromatograph was coupled with a Flame Ionisation Detector (Thermo Fisher Scientific) maintained at 255 °C and supplied with air (350 mL/min), H_2 (35 mL/min) and N_2 (40 mL/min) to detect the FAMEs. Chromatograms were processed with ChromQuest software 5.0 (Thermo Fisher Scientific) and each peak was identified and quantified by comparison to an external standard composed of 42 FAMEs (Larodan). Concentrations that were below the limit of detection or quantification were set at 0. Results were expressed in μmol of fatty acids per g of cellular proteins. Proteins were quantified in the same sample as the fatty acid profile via the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Serial dilutions of BSA standard were used for quantification. In addition to cellular samples, the concentration of the fatty acid of interest was measured in the corresponding stock solution. The actual concentrations of the stock solutions differed by up to 11% from the theoretical concentrations, with the exception of the AA solution, whose concentration was 22% lower than the theoretical concentration.

2.6. RNA extraction and cDNA synthesis

RNA samples were treated as previously described in Tinant et al. (2021). Briefly, culture medium was removed and cells were washed with PBS at 19 °C before being collected in TRIzol® Reagent (Life Technologies) and stored at −80 °C until further analysis. RNA was extracted with chloroform. The upper phase containing RNA was collected and 70% ethanol was added to the samples. Following steps of RNA extraction were performed with Aurum™ Total RNA Mini Kit from Bio-Rad Laboratories (Hercules, CA, USA). Samples were loaded onto

RNA binding columns and washed with low and high stringency wash solutions. Potential remaining genomic DNA was removed via DNase treatment following the manufacturer's recommendations. RNA was eluted using diethyl pyrocarbonate (Sigma-Aldrich)-treated water. The 260/280 and 260/230 ratios were assessed to verify the purity of RNA samples. The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) was used to reverse transcribe 1 μg of total RNA into cDNA. The following temperature program was used: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. cDNA samples were stored at −20 °C.

2.7. RT-PCR analysis

The expression of genes of interest was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR). Analysis was performed with a StepOnePlus™ Real-Time PCR System (Life Technologies) in 96-well plates and samples were run in technical duplicates. Features of primers are recorded in Table A.1. A mix of cDNA, forward and reverse primers and SYBR® Select Master Mix (Life Technologies) was prepared in a final volume of 20 μL per well. The following temperature program was applied: 2 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at a gene-specific annealing-extension temperature (Table A.1) followed by a melting curve analysis (15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C). Each primer pair was experimentally validated before RT-PCR analysis as in Tinant et al. (2021). Amplification results were processed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The reference gene was rRNA 18S that showed high stability across treatments. Control cells at day 0 (i.e., before any treatment) were used as reference condition.

2.8. Statistical analyses

Statistical analyses were performed with the R version 3.5.1 and JMP® Pro 16.2.0 software. Normality and homoscedasticity of the residuals were checked before analysis. Data transformation (log, square root or inverse) was performed if needed. In case of heteroscedasticity, a correction was added to the linear mixed model estimated for each parameter via the *lme* function from the *nlme* package in R (Pinheiro et al., 2018). The relation between the samples coming from the same primary culture was included into the estimated model. The inter-group differences were tested with the *contrast* function in R (Kuhn et al., 2016) or Student's *t*-tests in JMP®. The multiplicity of tests was taken into account through a Bonferroni-Holm correction applied via the *p.adjust* function. Results were considered significant when the corrected *p*-value was below 0.05.

3. Results & discussion

3.1. Hormones and fatty acids are required for trout adipocyte differentiation and lipid accumulation

At confluence (day 0), a hormonal cocktail containing insulin, ciglitizone and dexamethasone, and supplemented with the lipid mixture was added to the culture medium to induce differentiation. The mRNA expression of several genes was modulated by this cocktail. After two days, the expression of the transcription factor C/EBPδ involved in early adipogenesis (Rosen et al., 2000) was highly increased (x7.1, Fig. 2), as previously observed in Tinant et al. (2021). The expression of this gene was also upregulated in Atlantic salmon preadipocytes in the late stage of differentiation (Huang et al., 2010). The expression of FATP1 and fatty acid binding protein 11a (FABP11a) that support the uptake and intracellular transport of fatty acids, respectively, was significantly increased at day 2 compared to day 0 (x3.4–4.6, Fig. 2 and Fig. 3). An upregulation of the FATP1 expression was also observed in Atlantic salmon preadipocytes, while the expression of FABP11 increased later in adipogenesis (Huang et al., 2010). Moreover, at day 2, the cocktail significantly increased the expression of glycerol-3-phosphate dehydrogenase (GPDH) (x2.1, Fig. 2), responsible for glycerol-3-phosphate production. This effect highlights the need for glycerol-3-phosphate, used as the carbon backbone in triglyceride synthesis (Fig. 3). On the contrary, the expression of FASN, the key enzyme in the fatty acid *de novo* synthesis, and long-chain acyl-CoA synthetase 1 (ACSL1), which activates fatty acids into acyl-CoA, decreased at day 2 (x0.2–0.7, Fig. 2). In the same *in vitro* model, the expression of FASN was also reduced after 24 h in the presence of a lipid mixture, suggesting a downregulation of the expression of this enzyme when fatty acids are available in the culture medium (Lutfi et al., 2017). Both ACSL1 and FATP1 display an acyl-CoA synthetase activity (Mashek et al., 2007). The decreased ACSL1 expression at day 2 may be offset by the simultaneous upregulation of the FATP1expression.

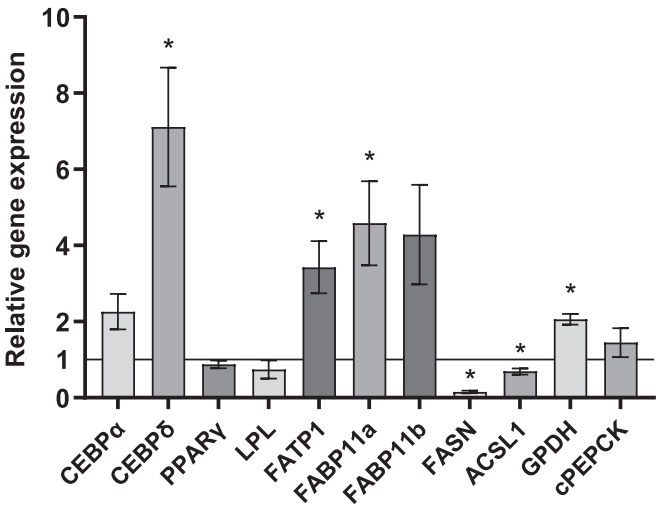

Fig. 2. Relative expression of genes involved in transcriptional cascade of the adipogenesis (C/EBPα and δ, PPARγ), fatty acid metabolism (LPL, FATP1, FABP11a and b, FASN, ACSL1) and glyceroneogenesis (GPDH, cPEPCK) in preadipocytes after 2-day incubation with hormonal cocktail and lipid mixture. Stars indicate significant effect of the hormonal cocktail and lipid mixture after 2 days ($p < 0.05$). The $2^{-\Delta\Delta Ct}$ method was applied for relative gene expression analysis (Livak and Schmittgen, 2001), using 18S as reference gene and cells at day 0 as reference condition. The solid line refers to the relative gene expression of the reference condition. Data are presented as mean ± standard error of the mean ($n = 4$). Abbreviations: ACSL1, long-chain acyl-CoA synthetase 1; C/EBPα and δ, CCAAT/enhancer binding protein α and δ; cPEPCK, cytosolic phosphoenolpyruvate carboxykinase; FABP11, fatty acid binding protein 11; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; GPDH, glycerol-3-phosphate dehydrogenase; LPL, lipoprotein lipase; PPARγ, peroxisome proliferator-activated receptor γ.

At the end of the experiment, neutral lipid levels were significantly higher in cells incubated with individual fatty acids (600 μM) compared to control cells (Table 1). Similar results were obtained after a 7-day incubation with ALA, EPA, DHA or LA at 100 μM in rainbow trout preadipocytes (Riera-Heredia et al., 2020). Overall, these results confirm that exogenous fatty acids are required to differentiate rainbow trout primary preadipocytes into adipocytes capable of accumulating fatty acids in the form of neutral lipids, as previously observed (Bouraoui et al., 2008; Tinant et al., 2021).

3.2. Fatty acids do not equally impact intracellular lipid accumulation

Seven specific fatty acids were studied in the present research, one n-9 MUFA (OA), three n-3 PUFA (ALA, EPA and DHA) and three n-6 PUFA (LA, AA and DPA). The number of carbon atoms and double bonds increase from ALA to DHA, and from LA to DPA. Preadipocytes were incubated with increasing fatty acid concentrations (75, 150, 300 and 600 μM). A dose-dependent accumulation of intracellular lipids was observed by ORO staining with the three n-3 PUFA as well as with LA

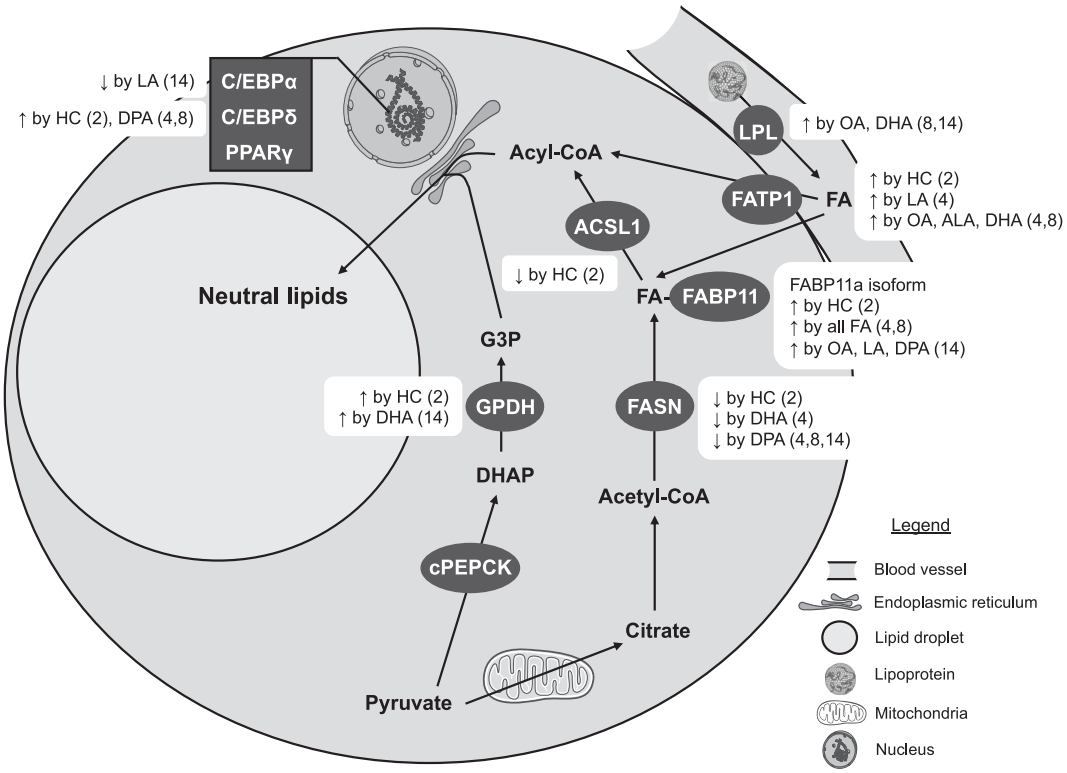

Fig. 3. Proteins encoded by the genes of interest involved in adipocyte development and lipid metabolism. The effects on gene expression of incubating pre-adipocytes with (i) the hormonal cocktail supplemented with lipid mixture during 2 days, and then with (ii) one specific fatty acid (OA, ALA, DHA, LA, or DPA) at 600 μM (the sampling day (i.e., day 4, 8 or 14) is specified in brackets in the figure) are indicated for each gene (increase "↑" or decrease "↓") compared to control cells. Detailed results are presented in Figs. 2 and 5. The figure is adapted from Tinant et al. (2021) and has been made with illustrations from Servier Medical Art. Abbreviations: ACSL1, long-chain acyl-CoA synthetase 1; ALA, α-linolenic acid; C/EBPα and δ, CCAAT/enhancer binding protein α and δ; cPEPCK, cytosolic phosphoenolpyruvate carboxykinase; DHA, docosahexaenoic acid; DHAP, dihydroxyacetone phosphate; DPA, docosapentaenoic acid; FA, fatty acid; FABP11, fatty acid binding protein 11; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; G3P, glycerol-3-phosphate; GPDH, glycerol-3-phosphate dehydrogenase; HC, hormonal cocktail; LA, linoleic acid; LPL, lipoprotein lipase; OA, oleic acid; PPARγ, peroxisome proliferator-activated receptor γ.

and DPA (Fig. 4), highlighting that the impact on lipid storage function in differentiating preadipocytes is proportional to the amount of fatty acids supplied. DPA and DHA appeared to be the most lipogenic fatty acids with the greatest effect reached at 600 μM.

Comparable results to those observed with ORO staining were obtained when analyzing the fatty acid content of the neutral lipid fraction, which mainly comprises triglycerides present in lipid droplets (Table 1). The impact of fatty acids (at 600 μM) on the intracellular neutral lipid accumulation decreased as follows: DPA > DHA > LA, OA > EPA, ALA ≥ AA > control cells (Table 1). DPA and, to a lesser extent, DHA strongly increased lipid accumulation, upwards of +490% and + 370% compared to the control, respectively (Table 1). To the best of our knowledge, this is the first time that the effect of DPA was investigated in fish preadipocytes. Similar to our results, RTL-W1 liver cells enriched with DPA accumulated two to three times more neutral lipids than cells incubated with other PUFA (i.e., AA, ALA, EPA and DHA). The differences were, however, not significant (Ferain et al., 2016). Previous studies highlighted differential impacts of the other fatty acids on intracellular lipid accumulation in fish adipocytes. The incubation of salmon preadipocytes with OA (100 μM, 72 h in Bou et al. (2020) and 600 μM, 21 days in Todorčević et al. (2008)) led to a higher amount of cellular lipids than with EPA, which is in line with our results. Nevertheless, Todorčević et al. (2008) showed that the lipid content was lower with DHA as compared to OA and EPA, as opposed to our observations. Other contradictory results were obtained in rainbow trout pre-adipocytes incubated with fatty acids at 100 μM, where ALA induced the highest accumulation of neutral lipids compared to EPA and LA, while no significant difference was observed compared to DHA (Riera-Heredia et al., 2020). Differences in fatty acid exposure protocols (48 h without prior hormonal exposure in Riera-Heredia et al. (2020) vs. 12–14 days plus two initial days with hormonal cocktail in the present study) could explain variations between the two studies. Indeed, it has been shown that fatty acid metabolism can change during adipocyte differentiation, from fatty acid β-oxidation to fatty acid deposition, and can be fatty acid-dependent (Todorčević et al., 2008). Notably, EPA and DHA are mostly oxidized in preadipocytes and stored in adipocytes (Todorčević et al., 2008). In the present study, the proportion of each specific fatty acid that is oxidized is unknown and should be part of a future research with labelled fatty acids. AA appeared to be the least lipogenic fatty acid. Similarly, preadipocytes of grass carp (*Ctenopharyngodon idellus*) incubated *in vitro* with AA (300 μM) contained less intracellular lipids after 8 days of culture than preadipocytes incubated with OA (Tian et al., 2016). It should be noted that the lower lipid content detected in the present study in the AA-enriched cells may be due to the lower effective concentration in the culture medium, as described in the Material & Methods Section 2.5.

The impact of the treatment on the total fatty acid content of the phospholipid fraction, which corresponds to the plasma membrane bilayer and the lipid droplet monolayer, differed from that on the total fatty acid content of the neutral lipid fraction. Notably, cells exposed to fatty acids did not contain a higher amount of phospholipids in their membranes than control cells, except for those enriched with AA (Table 2). The total fatty acid content of the phospholipid fraction was also significantly higher in cells enriched in AA than in OA, ALA, and DHA (Table 2). Likewise, EPA-enriched cells showed a significantly increased content of the total fatty acids compared to DHA-enriched

Table 1
Concentration of fatty acids in neutral lipid fraction of preadipocytes (in µmol of fatty acids per g of cellular proteins) after incubation with one specific fatty acid (OA, ALA, EPA, DHA, LA, AA, or DPA) at 600 µM during 12–14 days, or with no specific fatty acid (Control).

	Control	OA	ALA	EPA	DHA	LA	AA	DPA
14:0	0.9 ± 0.1^{a,b}	1.0 ± 0.2^{a,b}	0.7 ± 0.1^{a}	1.0 ± 0.2^{a,b}	1.7 ± 0.3^{c}	1.0 ± 0.2^{b}	1.0 ± 0.1^{a,b}	2.1 ± 0.3^{d}
16:0	6.3 ± 1.2^{a,b}	8.1 ± 1.6^{a,b,c}	6.6 ± 1.4^{a,b}	7.0 ± 1.0^{a,b}	7.6 ± 1.4^{b,c}	7.8 ± 1.5^{b,c}	7.1 ± 1.6^{a}	7.7 ± 1.1^{c}
18:0	7.0 ± 0.8^{a,b}	8.0 ± 1.1^{a,b}	7.6 ± 1.2^{a,b}	6.5 ± 1.2^{a}	7.7 ± 0.9^{a,b}	8.8 ± 1.1^{b}	6.4 ± 1.4^{a}	7.9 ± 0.9^{a,b}
Total SFA	**14.2 ± 2.0^{a,b}**	**17.1 ± 2.8^{a,b,c}**	**14.8 ± 2.6^{a,b}**	**14.5 ± 2.2^{a}**	**17.0 ± 2.3^{b,c}**	**17.6 ± 2.7^{b,c}**	**14.6 ± 3.1^{a}**	**17.7 ± 1.7^{c}**
16:1 n-7	0.6 ± 0.0^{d}	0.7 ± 0.1^{d}	0.0 ± 0.0^{a,b,c}	0.8 ± 0.1^{c,d}	1.5 ± 0.3^{a}	0.0 ± 0.0^{a,b,c}	0.8 ± 0.1^{d}	2.3 ± 0.4^{b}
18:1 n-9	3.5 ± 0.1^{a}	45.7 ± 9.0^{c}	3.1 ± 0.2^{a}	4.0 ± 0.4^{a}	6.3 ± 1.0^{b}	4.2 ± 0.3^{a}	3.4 ± 0.3^{a}	7.2 ± 0.8^{b}
20:1 n-9	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.1
22:1 n-9	0.8 ± 0.3	1.1 ± 0.4	0.8 ± 0.3	1.0 ± 0.4	0.9 ± 0.3	0.9 ± 0.4	0.9 ± 0.3	0.6 ± 0.2
Total MUFA	**4.9 ± 0.5^{a}**	**48.1 ± 9.3^{d}**	**3.9 ± 0.5^{a}**	**5.9 ± 0.8^{a}**	**8.8 ± 1.7^{b}**	**5.1 ± 0.5^{a}**	**5.2 ± 0.6^{a}**	**10.6 ± 1.3^{c}**
18:3 n-3	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	21.3 ± 5.2^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
20:3 n-3	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.4 ± 0.1^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
18:4 n-3	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.7 ± 0.1^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.4 ± 0.1^{a,b}
20:5 n-3	0.0 ± 0.0^{a,b}	0.9 ± 0.2^{b}	0.0 ± 0.0^{a,b}	25.1 ± 4.3^{c}	2.2 ± 0.5^{a}	0.0 ± 0.0^{a,b}	0.0 ± 0.0^{a,b}	0.8 ± 0.1^{b}
22:5 n-3	0.3 ± 0.0^{a}	0.9 ± 0.1^{c}	0.4 ± 0.1^{a}	1.9 ± 0.2^{d}	1.0 ± 0.2^{c}	0.6 ± 0.1^{b}	0.4 ± 0.0^{a,b}	1.0 ± 0.1^{c,d}
22:6 n-3	0.5 ± 0.0^{a}	0.9 ± 0.1^{e}	0.5 ± 0.1^{a}	0.6 ± 0.1^{a}	66.4 ± 16.5^{c}	0.5 ± 0.1^{a}	0.3 ± 0.0^{b}	1.4 ± 0.2^{d}
Total n-3 PUFA	**0.8 ± 0.1^{c}**	**2.7 ± 0.4^{f}**	**23.2 ± 5.5^{a}**	**27.6 ± 4.6^{a}**	**69.6 ± 17.3^{d}**	**1.1 ± 0.2^{c}**	**0.7 ± 0.0^{b}**	**3.6 ± 0.6^{e}**
18:2 n-6	0.6 ± 0.0^{a,b}	0.7 ± 0.1^{a,b,c}	0.6 ± 0.0^{a,b}	0.7 ± 0.1^{a,b,c}	1.0 ± 0.1^{b,c}	43.4 ± 11.9^{d}	0.5 ± 0.1^{a}	1.1 ± 0.1^{c}
20:2 n-6	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.7 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
18:3 n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.4	0.0 ± 0.0	0.8 ± 0.2
20:4 n-6	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	16.2 ± 0.7^{b}	3.6 ± 0.5^{c}
22:4 n-6	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.9 ± 0.1^{b}	0.6 ± 0.1^{a,b}
22:5 n-6	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	82.9 ± 13.0^{b}
Total n-6 PUFA	**0.6 ± 0.0^{a}**	**0.7 ± 0.1^{a}**	**0.6 ± 0.0^{a}**	**0.7 ± 0.1^{a}**	**1.0 ± 0.1^{a}**	**45.7 ± 12.5^{d}**	**18.7 ± 0.7^{b}**	**89.1 ± 13.8^{c}**
Total PUFA	**1.4 ± 0.1^{b}**	**3.4 ± 0.5^{f}**	**23.9 ± 5.5^{a}**	**28.3 ± 4.7^{a}**	**70.6 ± 17.4^{c}**	**46.8 ± 12.7^{e}**	**19.4 ± 0.7^{a}**	**92.7 ± 14.3^{d}**
n-3/n-6 ratio	**1.5 ± 0.2^{c}**	**3.7 ± 0.6^{e}**	**35.6 ± 7.5^{a}**	**40.4 ± 2.5^{a}**	**68.0 ± 9.4^{d}**	**0.0 ± 0.0^{b}**	**0.0 ± 0.0^{b}**	**0.0 ± 0.0^{b}**
Total FA	**20.5 ± 2.4^{c}**	**68.6 ± 12.5^{f}**	**42.6 ± 7.6^{a}**	**48.7 ± 7.6^{a,b}**	**96.4 ± 21.0^{d}**	**69.5 ± 15.6^{f}**	**39.1 ± 4.0^{b}**	**121.0 ± 16.8^{e}**

Values with no common letters indicate significant effect of the fatty acids treatment ($p < 0.05$). Data are presented as mean ± standard error of the mean (n = at least 3). Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LA, linoleic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

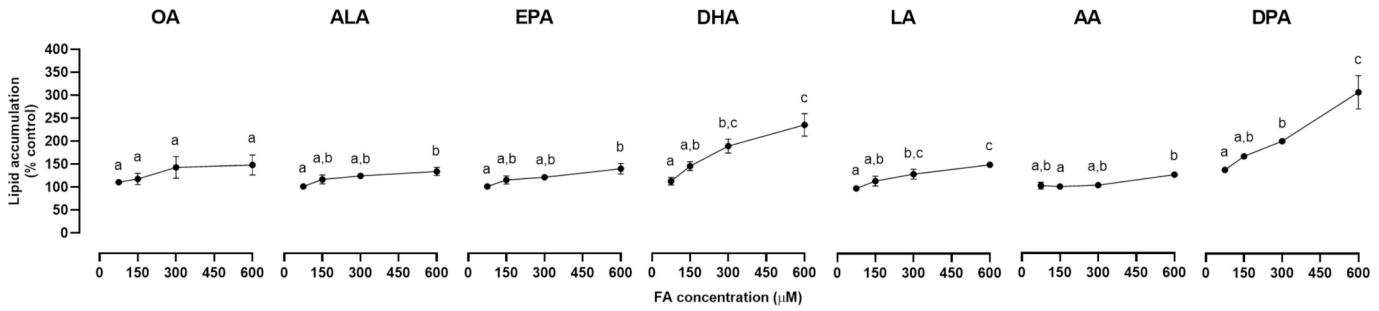

Fig. 4. Lipid accumulation (in % of control cells) quantified by ORO staining in preadipocytes after incubation with one specific fatty acid (OA, ALA, EPA, DHA, LA, AA, or DPA) at increasing concentrations (75, 150, 300, 600 µM) during 12–14 days. For each fatty acid, values with no common letters indicate significant effect of the concentration ($p < 0.05$). Data are presented as mean ± standard error of the mean ($n = 3$ or 4, except for the condition "DPA – 75 µM" where $n = 2$). Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; OA, oleic acid.

cells (Table 2). Although significant, the effect of AA represents only +12% compared with the control condition, which is low in view of the increases of the total fatty acid content in the neutral lipid fraction (+91% to +490%). These results are in line with experiments on RTL-W1 liver cells, in which total phospholipid content remains stable across several PUFA treatments (Ferain et al., 2016). It suggests that the retention of exogenous fatty acids into the membranes mainly occurs via the replacement of existing fatty acids or during the phospholipid turnover, rather than from phospholipid accumulation.

3.3. *Fatty acid treatments increase the content of the fatty acid of interest and its bioconversion products in neutral lipids and phospholipids*

Preadipocytes incubated with each fatty acid (i.e., OA, ALA, EPA, DHA, LA, AA and DPA) exhibited a specific enrichment of the corresponding fatty acid in the neutral lipid and phospholipid fractions compared with the other conditions (Tables 1 and 2). The increase of the total neutral lipid content essentially resulted from the incorporation of this fatty acid within the lipid droplets. Similar *in vitro* results were observed in Atlantic salmon preadipocytes, with a significant incorporation of OA, EPA and DHA (600 µM, 14 days) in neutral lipids and phospholipids (Todorčević et al., 2008). Specific retention of the major dietary fatty acids (ALA, EPA, DHA and LA) was also observed *in vivo* in rainbow trout adipose tissue (Tinant et al., 2023). Several bioconversion products were detected in significant amounts in the neutral lipids and phospholipids of the enriched cells. In particular, the elongation and desaturation products of ALA (i.e., 20:3 n-3 and 18:4 n-3), EPA (i.e., 22:5 n-3 and 24:5 n-3), LA (i.e., 20:2 n-6 and 18:3 n-6), and AA (i.e., 22:4 n-6) were quantified in the corresponding enriched cells (Tables 1 and 2). These results highlight that preadipocytes are capable of carrying out the first steps of n-3 and n-6 fatty acid bioconversion (Fig. 1), although the proportion of the bioconversion products remains minimal compared to the fatty acid initially used for enrichment in *in vitro* preadipocytes. Comparable *in vitro* results were reported for phospholipids

Table 2
Concentration of fatty acids in phospholipid fraction of preadipocytes (in μmol of fatty acids per g of cellular proteins) after incubation with one specific fatty acid (OA, ALA, EPA, DHA, LA, AA, or DPA) at 600 μM during 12–14 days, or with no specific fatty acid (Control).

	Control	OA	ALA	EPA	DHA	LA	AA	DPA
14:0	14.2 ± 0.4^{b,c}	4.3 ± 0.1^{d}	5.0 ± 0.3^{a}	12.0 ± 0.3^{c}	15.4 ± 0.6^{b}	5.0 ± 0.2^{a,d}	13.6 ± 0.3^{b,c}	14.0 ± 0.9$^{b,\ c}$
16:0	60.8 ± 0.7^{b}	33.4 ± 0.5^{c}	39.9 ± 1.6^{a}	67.9 ± 0.9^{b}	63.6 ± 1.3^{b}	41.7 ± 1.2^{a}	67.8 ± 2.1^{b}	63.2 ± 2.1^{b}
18:0	27.4 ± 1.2^{c}	19.2 ± 2.4^{a}	20.2 ± 1.5^{a,b}	27.5 ± 2.4^{b,c}	20.5 ± 1.6^{a,b}	20.3 ± 2.5^{a}	21.9 ± 2.8^{a,b}	17.6 ± 0.3^{a}
Total SFA	**102.3 ± 0.8^{c}**	**56.8 ± 2.8^{b}**	**65.1 ± 2.6^{a,b}**	**107.3 ± 2.5^{c}**	**99.6 ± 1.1^{c}**	**66.9 ± 3.0^{a}**	**103.3 ± 4.9^{c}**	**94.9 ± 3.1^{c}**
16:1 n-7	9.5 ± 0.4^{c}	2.4 ± 0.2^{f}	4.1 ± 0.4^{a}	10.5 ± 0.3^{b,d}	9.7 ± 0.5^{c,d}	3.0 ± 0.3^{e}	11.7 ± 0.3^{b}	9.8 ± 0.4^{c,d}
18:1 n-7	10.4 ± 0.2^{c}	3.1 ± 0.3^{e}	3.8 ± 0.3^{a}	9.1 ± 0.2^{c}	6.7 ± 0.3^{b}	2.7 ± 0.2^{e}	6.7 ± 0.1^{b}	4.8 ± 0.2^{d}
18:1 n-9	75.0 ± 2.5^{c}	182.6 ± 9.7^{g}	35.3 ± 2.7^{a}	61.7 ± 0.5^{e}	57.1 ± 2.5^{b}	17.5 ± 1.4^{f}	49.9 ± 1.3^{b}	42.7 ± 2.4^{d}
20:1 n-9	1.5 ± 0.1^{a}	2.6 ± 0.2^{d}	1.6 ± 0.1^{a,b}	3.1 ± 0.2^{c}	1.9 ± 0.1^{b}	1.8 ± 0.1^{b}	3.0 ± 0.2^{c,d}	1.5 ± 0.1^{a}
Total MUFA	**96.3 ± 3.1^{c}**	**190.7 ± 10.2^{f}**	**44.9 ± 3.4^{a}**	**84.4 ± 0.7^{c}**	**75.3 ± 3.3^{b}**	**25.0 ± 1.9^{e}**	**71.2 ± 1.8^{b}**	**58.9 ± 3.1^{d}**
18:3 n-3	0.7 ± 0.0^{b,d}	0.0 ± 0.0^{e}	127.9 ± 5.7^{a}	0.8 ± 0.0^{d}	0.4 ± 0.0^{c}	0.0 ± 0.0^{e}	0.5 ± 0.0^{b,c}	0.5 ± 0.1^{c}
20:3 n-3	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	8.9 ± 0.8^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
18:4 n-3	0.8 ± 0.0^{b}	0.0 ± 0.0^{b}	5.5 ± 0.5^{a}	0.6 ± 0.0^{b}	0.4 ± 0.0^{b}	0.0 ± 0.0^{b}	0.7 ± 0.0^{b}	0.6 ± 0.0^{b}
20:4 n-3	2.4 ± 0.1^{d}	1.0 ± 0.1^{f}	1.6 ± 0.1^{a}	0.9 ± 0.0^{c,f}	0.6 ± 0.0^{b,e}	0.4 ± 0.0^{g}	0.7 ± 0.0^{b,c}	0.6 ± 0.1^{e}
20:5 n-3	27.3 ± 1.6^{c}	15.9 ± 0.9^{h}	13.7 ± 1.0^{a}	92.8 ± 2.3^{f}	11.3 ± 0.4^{d}	7.0 ± 0.4^{g}	2.0 ± 0.2^{b}	1.5 ± 0.1^{e}
22:5 n-3	17.0 ± 1.0^{c}	8.8 ± 0.8^{a}	7.8 ± 0.7^{a}	25.6 ± 1.2^{e}	2.2 ± 0.2^{d}	5.6 ± 0.5^{b}	4.9 ± 0.2^{b}	1.9 ± 0.2^{d}
24:5 n-3	0.2 ± 0.0^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.3 ± 0.0^{c}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
24:6 n-3	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.4 ± 0.0^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
22:6 n-3	29.2 ± 1.7^{c}	15.1 ± 1.2^{a}	17.0 ± 1.4^{a}	9.2 ± 0.3^{f}	95.3 ± 3.7^{d}	10.5 ± 0.8^{f}	5.2 ± 0.2^{b}	4.6 ± 0.3^{e}
Total n-3 PUFA	**77.6 ± 4.2^{c}**	**40.8 ± 3.0^{h}**	**182.5 ± 9.5^{a}**	**130.2 ± 3.9^{f}**	**110.6 ± 4.0^{d}**	**23.4 ± 1.7^{g}**	**14.1 ± 0.5^{b}**	**9.6 ± 0.6^{e}**
18:2 n-6	4.0 ± 0.2^{a,b}	1.4 ± 0.2^{f}	4.8 ± 0.6^{a}	2.5 ± 0.0^{c,d}	2.5 ± 0.1^{c,d}	173.2 ± 5.3^{e}	3.1 ± 0.2^{b,c}	2.2 ± 0.1^{d}
20:2 n-6	0.6 ± 0.0^{b}	0.2 ± 0.0^{e}	0.4 ± 0.0^{a}	0.6 ± 0.0^{b,c}	0.5 ± 0.0^{c}	11.7 ± 0.8^{d}	0.6 ± 0.0^{b,c}	0.4 ± 0.0^{a}
18:3 n-6	0.5 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	3.7 ± 0.2^{b}	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}
20:3 n-6	3.6 ± 0.2^{c}	1.4 ± 0.1^{a}	1.4 ± 0.1^{a}	1.1 ± 0.1^{f}	1.0 ± 0.1^{d}	1.5 ± 0.1^{a}	2.3 ± 0.1^{b}	0.8 ± 0.0^{e}
20:4 n-6	17.3 ± 1.2^{c}	7.4 ± 0.7^{e}	4.5 ± 0.4^{a}	5.4 ± 0.2^{d}	4.3 ± 0.2^{a}	4.2 ± 0.3^{a}	120.0 ± 5.5^{b}	18.5 ± 1.8^{c}
22:4 n-6	1.0 ± 0.0^{c}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.6 ± 0.0^{e}	0.3 ± 0.0^{d}	0.3 ± 0.0^{f}	24.2 ± 2.8^{b}	1.1 ± 0.1^{c}
22:5 n-6	0.7 ± 0.0^{c}	0.0 ± 0.0^{b}	0.5 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	125.0 ± 3.2^{d}
Total n-6 PUFA	**27.7 ± 1.5^{c}**	**10.9 ± 1.0^{a}**	**11.9 ± 1.1^{a}**	**10.2 ± 0.4^{a}**	**8.6 ± 0.4^{d}**	**194.7 ± 6.2^{e}**	**150.7 ± 8.4^{b}**	**148.5 ± 4.5^{b}**
Total PUFA	**105.3 ± 4.7^{c}**	**51.7 ± 4.0^{g}**	**194.5 ± 10.6^{a}**	**140.4 ± 4.2^{e}**	**119.3 ± 4.3^{d}**	**218.2 ± 7.4^{f}**	**164.8 ± 8.9^{b}**	**158.1 ± 5.1^{b,e}**
n-3/n-6 ratio	**2.8 ± 0.2^{c}**	**3.8 ± 0.1^{g}**	**15.6 ± 0.8^{a}**	**12.8 ± 0.1^{d}**	**12.8 ± 0.2^{d}**	**0.1 ± 0.0^{f}**	**0.1 ± 0.0^{b}**	**0.1 ± 0.0^{e}**
Total FA	**303.9 ± 7.6^{a,b}**	**299.2 ± 11.4^{a,b}**	**304.5 ± 15.0^{a,b}**	**332.2 ± 3.5^{b,c}**	**294.1 ± 7.5^{a}**	**310.1 ± 8.6^{a,b,c}**	**339.3 ± 13.1^{c}**	**311.9 ± 11.2^{a,b,c}**

Values with no common letters indicate significant effect of the fatty acids treatment ($p < 0.05$). Data are presented as mean ± standard error of the mean (n = at least 3). Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LA, linoleic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

in fish cell lines such as RTL-W1 liver cells incubated with n-3 (ALA, EPA, DHA) and n-6 (LA, AA, DPA) PUFA (50 μM, 7 days) (Ferain et al., 2016) and fathead minnow epithelial cells incubated with ALA, EPA, DHA, LA and AA (up to 20 μM, 24 h) (Gregory et al., 2011). Bioconversion products were also detected *in vivo* in the neutral lipids and phospholipids of rainbow trout adipose tissue (Tinant et al., 2023). They were, however, not limited to the first desaturated and elongated products. Instead, ALA and EPA were converted to DHA while LA was converted to DPA n-6 (Tinant et al., 2023). This higher level of final metabolites observed *in vivo* could be due to a greater bioconversion efficiency of the tissue *in vivo* or to a redistribution from the liver, which is the primary organ for fatty acid bioconversion (Sargent et al., 2002).

As expected, OA-enriched cells exhibited the highest level of total MUFA in phospholipids and neutral lipids due to the high accumulation of the supplemented fatty acid. Cells incubated with DPA, followed by DHA, the two most lipogenic fatty acids in the present study, had the second highest levels of total MUFA in their neutral lipids, due to the high amount of OA and 16:1 n-7. An increased conversion of 16:0 and 18:0 into their monounsaturated counterparts could have contributed to this difference. Similarly, only preadipocytes incubated with DPA showed a significantly higher accumulation of saturated fatty acids (SFA) in neutral lipids, mainly due to a specific retention of 14:0 and 16:0, compared to the control cells. Cells incubated with n-3 PUFA (i.e., ALA, EPA or DHA) showed the highest levels of total n-3 PUFA and the lowest levels of total n-6 PUFA in their neutral lipids and phospholipids (Tables 1 and 2). The opposite situation was observed for cells enriched with n-6 PUFA (i.e., LA, AA or DPA). These results led to a high n-3/n-6 ratio for the n-3 PUFA-enriched cells and a ratio close to zero for the n-6 PUFA-enriched cells. OA-enriched cells and control cells presented an intermediate n-3/n-6 ratio.

AA was detected in the neutral lipids of cells incubated with DPA, whereas it was absent in the other conditions, with the exception of cells incubated with AA (Table 1). Although not yet reported in fish, this could result from the retroconversion of DPA to AA, as observed in *Daphnia magna* (Strandberg et al., 2014) and in rat liver (Tam et al., 2008), or from a specific synthesis or uptake from the medium by the DPA-enriched cells. In the membranes, AA was quantified in all conditions. This could suggest that DPA-enriched cells have taken up AA from the medium and incorporated it not only in membranes, like all other cells, but also in the lipid droplets due to the high lipogenic effect of DPA. Several fatty acids (e.g., all SFA, OA, 22:5 n-3, DHA and LA) were quantified in all experimental conditions, including control (Tables 1 and 2), as a result of their presence in the lipid mixture and/or FBS (Table A.2). They could also come from *de novo* synthesis (SFA and OA) and/or bioconversion (22:5 n-3 and DHA). On the contrary, 24:5 n-3 and 24:6 n-3 were detected only in phospholipids, suggesting that very long chain PUFA were preferentially incorporated into membranes rather than lipid droplets.

3.4. Fatty acids modulate adipocyte differentiation and lipid metabolism at transcriptional level

The expression of several genes involved in adipocyte differentiation and neutral lipid anabolism was assessed in preadipocytes incubated with OA, ALA, DHA, LA or DPA, and in control cells. These fatty acids were chosen because they showed the highest effects on lipid accumulation (DPA, DHA, LA and OA) and are the precursors (ALA and LA) or the final products (DHA and DPA) of the bioconversion pathways (Fig. 1). Gene expression was measured at days 4, 8 and 14 from the initiation of adipocyte differentiation (i.e., day 0), corresponding to a contact time with the fatty acid of interest of 2, 6 and 12 days, respectively.

The expression of three transcription factors, C/EBPα, C/EBPδ and PPARγ, having key roles in adipocyte differentiation in vertebrates

including rainbow trout (Bou et al., 2017), was assessed. C/EBPα expression was not influenced by the fatty acids at days 4 and 8. The situation was similar at day 14, except for LA treatment which significantly reduced C/EBPα expression compared with the control condition (x0.6, Fig. 5). These results are in line with the absence of difference of C/EBPα expression after a 6-h exposure of rainbow trout preadipocytes to ALA, DHA or LA (Riera-Heredia et al., 2020). On the contrary, our results are opposed to those of Huang et al. (2010), who reported a reduced expression of C/EBPα after 14 days of exposure of Atlantic salmon preadipocytes to OA and DHA. At days 4 and 8, C/EBPδ expression was significantly upregulated by DPA compared with the control condition (x1.4) whereas no difference was observed at day 14 (Fig. 5). This result supports the initiation of adipogenesis in DPA-enriched cells and may explain the enhanced lipid accumulation in this condition. The other fatty acids did not affect the expression of C/EBPδ (Fig. 5), which corresponds in part to what was observed for salmon preadipocytes, where OA had no effect on the expression of this gene after 14 days whereas DHA downregulated its expression (Huang et al., 2010). Contrary to the expression of C/EBPα, which remained stable throughout the experiment, the expression of C/EBPδ significantly decreased over time in preadipocytes enriched in ALA, DHA and DPA as well as in control cells (Fig. 5). In the present study, none of the fatty acid treatments had an impact on the expression of PPARγ (Fig. 5). Similar results were obtained in rainbow trout preadipocytes and in gilthead sea bream bone-derived mesenchymal stem cells exposed during 6 h to ALA, DHA or LA (Riera-Heredia et al., 2019; Riera-Heredia et al., 2020).

The fatty acids found within neutral lipids can originate from the circulation (*in vivo*) or the culture medium (*in vitro*). LPL is an enzyme capable of hydrolyzing triglycerides from circulating lipoproteins and is considered as an early marker of adipocyte differentiation whose expression is maintained in differentiated cells (Bouraoui et al., 2012; Oku et al., 2006). None of the fatty acids had an impact on LPL expression at day 4 whereas the treatments with OA and DHA significantly upregulated LPL expression compared with the control condition at days 8 (x2.0 and x2.3, respectively) and 14 (x3.2 and x2.9, respectively) (Fig. 5). As with our day 4 results, no effect was observed in trout preadipocytes and in gilthead sea bream bone-derived mesenchymal stem cells incubated during 6 h with ALA, DHA or LA (Riera-Heredia et al., 2019; Riera-Heredia et al., 2020). In contrast to our findings, LPL expression was downregulated by DHA (up to 200 μM, 6 days) in differentiating preadipocytes of large yellow croaker (Wang et al., 2012). The expression of LPL increased over time in DHA-enriched cells (Fig. 5), supporting the commitment of these cells into the adipocyte lineage. The expression of genes involved in fatty acid uptake and transport was greatly impacted by the treatments. FATP1 expression was highly increased at days 4 and 8 by all fatty acids compared with the control condition (x1.5 for DPA, x1.8 for DHA and LA, x2.2 for ALA, and x2.3 for OA), except by LA at day 8 (only a trend towards an increase) and by DPA at both timepoints, which is surprising given that DPA-enriched cells accumulated large amounts of lipids. The metabolic demand for fatty acids (i.e., the synthesis of acyl-CoA and triglycerides) increased by the DPA-induced adipogenesis could be the driving force of the fatty acid uptake rather than the availability of the substrate (DPA) and the membrane transporters (FATP1) (Mashek and Coleman, 2006). In parallel, OA-enriched cells exhibited a higher FATP1 expression than DPA-enriched cells at days 4 and 8. The effects disappeared at day 14 (Fig. 5). Similar results were obtained for LA and ALA in trout preadipocytes after 6 h of incubation (Riera-Heredia et al., 2020) and in RTL-W1 liver cells after 7 days (Ferain et al., 2018a; Ferain et al., 2018b). Contrary to the present study, DHA decreased FATP1 expression after 14 days in salmon preadipocytes whereas OA had no effect (Huang et al., 2010). At days 4 and 8, all cells enriched with fatty acids showed a significantly higher FABP11a expression than the control cells (x2.3 for ALA, x2.4 for LA, x2.5 for OA, x2.7 for DHA, and x4.0 for DPA). FABP11a expression was also significantly increased by DPA compared with ALA and LA at day 4 and ALA at day 8. At day 14, OA (x3.6), LA (x3.3) and DPA (x2.2) induced a significant increase in FABP11a expression compared with control cells, while LA significantly raised the expression of this gene compared with ALA (Fig. 5). On the contrary, no effect of fatty acids was observed on the expression of the FABP11b isoform (Fig. 5). Different results were obtained in salmon preadipocytes in which DHA increased and OA kept FABP11 expression stable at day 14 (Huang et al., 2010). A reduced expression of FABP11a was observed over time in ALA- and DPA-enriched cells while this effect occurred in all cells for FABP11b (Fig. 5). The high expression of both isoforms in the early stage of differentiation should have increased the availability of FABP11a/b in the cytoplasm, leading to negative feedback in the late stage. Overall, these results indicate that genes involved in fatty acid uptake and transport can be rapidly and highly activated in response to fatty acid supply.

Endogenous fatty acids synthetized by FASN can also be part of the triglycerides stored in lipid droplets. In the present study, cells incubated with DHA (at day 4, x0.5) and DPA (at days 4, 8 and 14, x0.4) exhibited a significantly reduced expression of FASN compared with control cells. Moreover, FASN expression was decreased by LA and DPA compared to ALA at days 8 and 14 (Fig. 5). At day 14, DHA also downregulated FASN expression compared to ALA while the expression was significantly reduced in DPA- compared to OA-enriched cells (Fig. 5). Furthermore, FASN expression decreased over time in the DPA-enriched cells (Fig. 5). FASN expression was also downregulated by DHA (up to 200 μM, 6 days) in differentiating large yellow croaker preadipocytes (Wang et al., 2012). Negative feedback from exogenous fatty acids could lead to inhibition of *de novo* synthesis (Riera-Heredia et al., 2019; Salmerón et al., 2016). These results combined with the fact that the most abundant fatty acid in differentiating preadipocytes is the one intentionally added to the culture medium confirm that fatty acids stored in growing lipid droplets are preferentially retained from culture medium and do not originate from the *de novo* synthesis.

Once inside the cell, fatty acids should be activated to acyl-CoA (Fig. 3). ACSL1 expression remained unchanged across fatty acid treatments (Fig. 5). In DPA-enriched cells, ACSL1 expression decreased at day 14 compared with day 4 (Fig. 5). FATP1, whose expression was upregulated by the hormonal cocktail and lipid mixture, and by several fatty acid treatments, is also capable of activating fatty acids and forming acyl-CoA (Mashek et al., 2007), which may partly explain the lack of effect on ACSL1 expression. Activated fatty acids are then bound to glycerol-3-phosphate synthetised by GPDH from dihydroxyacetone phosphate, itself produced by the cytosolic phosphoenolpyruvate carboxykinase (cPEPCK) (Fig. 3). Increased GPDH expression and activity is known to be an indicator of adipocyte differentiation (Bou et al., 2017; Bouraoui et al., 2008). GPDH expression was only affected by fatty acids at day 14. DHA significantly increased GPDH expression compared with control (x1.8) and LA conditions while DPA raised the expression compared to LA (Fig. 5). These results are consistent with the high lipid content quantified in the DHA- and DPA-enriched cells. Moreover, GPDH expression was downregulated in LA-enriched cells between day 4 and day 14 (Fig. 5). On the other hand, a significantly reduced expression of cPEPCK was observed in DPA-enriched cells compared to ALA-enriched ones at day 8 only (Fig. 5). All fatty acid-enriched cells also showed a reduction of cPEPCK expression over time (Fig. 5). Nonetheless, these results do not exclude a contrasting effect at post-transcriptional level, as observed in rainbow trout preadipocytes in which the steady level of PEPCK mRNA was associated with an increase in the level of the corresponding protein (Bou et al., 2017).

4. Conclusion

The present study confirms that fatty acids are capable of modulating adipocyte differentiation and metabolism in rainbow trout. The specific fatty acids added to the culture medium were well incorporated into cell membranes and lipid droplets. Some bioconversion products were also

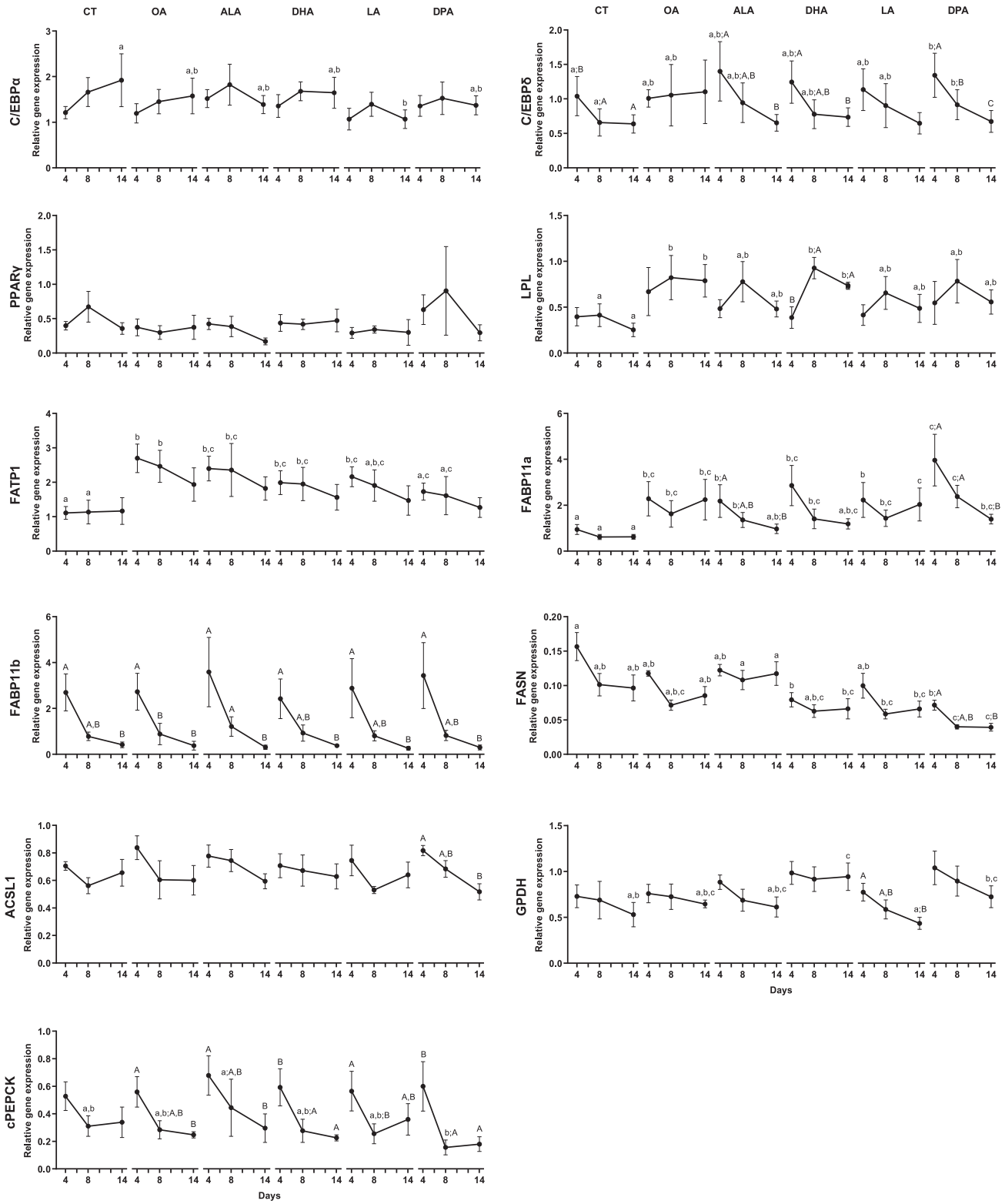

Fig. 5. Relative expression of genes involved in transcriptional cascade of the adipogenesis (C/EBPα and δ, PPARγ), fatty acid metabolism (LPL, FATP1, FABP11a and b, FASN, ACSL1) and glyceroneogenesis (GPDH, cPEPCK) in preadipocytes incubated with one specific fatty acid (OA, ALA, DHA, LA, or DPA) at 600 μM, or with no specific fatty acid (CT) at days 4, 8 and 14. For each gene and each day, values with no common lowercase letters indicate significant effect of fatty acids treatment ($p < 0.05$). For each gene and each fatty acid treatment, values with no common capital letters indicate significant effect of the day ($p < 0.05$). The $2^{-\Delta\Delta Ct}$ method was applied for relative gene expression analysis (Livak and Schmittgen, 2001), using 18S as reference gene and control cells at day 0 as reference condition. Data are presented as mean ± standard error of the mean (n = 4). Abbreviations: ACSL1, long-chain acyl-CoA synthetase 1; ALA, α-linolenic acid; C/EBPα and δ, CCAAT/enhancer binding protein α and δ; cPEPCK, cytosolic phosphoenolpyruvate carboxykinase; CT, control; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; FABP11, fatty acid binding protein 11; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; GPDH, glycerol-3-phosphate dehydrogenase; LA, linoleic acid; LPL, lipoprotein lipase; OA, oleic acid; PPARγ, peroxisome proliferator-activated receptor γ.

detected, though in a much lesser extent than the original fatty acid. Each fatty acid distinctively affected adipocyte differentiation, both in terms of lipid accumulation and gene transcription. DPA and DHA appeared to be the most lipogenic fatty acids, whereas AA showed the slightest effect. As summarized in Fig. 3, DPA upregulated the expression of the early transcription factor C/EBPδ and thus seems to promote adipogenesis through its transcriptional cascade. On the other hand, the lipogenic effect of DPA was not linked to an increase in FATP1 transcription. Regarding DHA, it increased the expression of genes involved in neutral lipids synthesis, notably LPL, FATP1 and GPDH. Both fatty acids positively impacted the expression of FABP11a. These results add to our knowledge of the effects of fatty acids and their bioconversion products on adipogenesis and lipid deposition. This should be of great interest for aquaculture industry in the context of fish oil replacement by plant-derived oils. Indeed, according to our results, this practice can be expected to increase the amount of fatty acids typical of plant-derived oils in adipocytes located in muscle (i.e., in fillets), thus impacting nutritional value for human consumption (lower levels of n-3 long-chain PUFA). LA, itself or after bioconversion into DPA, could also increase both perivisceral and muscle fat deposition affecting fish health and production yields. Further experiments are still required to refine the mechanism of action of DPA and DHA in rainbow trout preadipocytes. As an example, fatty acid oxidation markers should be included in future studies as fatty acids with a high degree of unsaturation are prone to oxidation and adipogenesis could be affected by oxidative stress (De Villiers et al., 2017).

CRediT authorship contribution statement

Gilles Tinant: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ineke Neefs:** Investigation. **Alice De Groote:** Writing – review & editing, Investigation, Data curation. **Melissa M. Page:** Writing – review & editing. **Jean-François Rees:** Writing – review & editing, Visualization, Supervision, Funding acquisition. **Yvan Larondelle:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization. **Cathy Debier:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2025.111087.

Data availability

Data will be made available on request.

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