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ORIGINAL RESEARCH

Chitosan-based delivery of fish codon-optimised *Caenorhabditis elegans* FAT-1 and FAT-2 boosts EPA and DHA biosynthesis in *Sparus aurata*

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Abstract Omega-3 long-chain polyunsaturated fatty acids (*n*-3 LC-PUFA) are essential fatty acids required in healthy balanced diets for humans. To induce sustained production of *n*-3 LC-PUFA in gilthead seabream (*Sparus aurata*), chitosan-tripolyphosphate (TPP) nanoparticles encapsulating plasmids expressing fish codon-optimised *Caenorhabditis elegans* FAT-1 and FAT-2 were intraperitoneally administered every 4 weeks (3 doses in total, each of 10 µg plasmid per g of body weight). Growth performance and metabolic effects of chitosan-TPP complexed with pSG5 (empty plasmid), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 were assessed 70 days post-treatment. Tissue distribution analysis showed high expression levels of fish codon-optimised FAT-1 and FAT-2 in the liver (> 200-fold). Expression of *fat-1* and *fat-1* + *fat-2* increased weight gain. Fatty acid methyl esters assay revealed that co-expression of *fat-1* and *fat-2* increased liver production and muscle accumulation of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total *n*-3 LC-PUFA, while decreased the *n*-6/*n*-3 ratio. Co-expression of *fat-1* and *fat-2* downregulated *srebf1* and genes encoding rate-limiting enzymes for de novo lipogenesis in the liver, leading to decreased circulating triglycerides and cholesterol. In contrast, FAT-2 and FAT-1 + FAT-2 upregulated hepatic *hnf4a*, *nr1h3* and key enzymes in glycolysis and the pentose phosphate pathway. Our findings demonstrate for the first time efficient and sustained production of EPA and DHA in animals after long-term treatment with chitosan-TPP-DNA nanoparticles expressing FAT-1 and FAT-2, which enabled the production of functional fish rich in *n*-3 LC-PUFA for human consumption.

Keywords Chitosan · Functional food · Gene therapy · Omega 3 long-chain polyunsaturated fatty acids · *Sparus aurata*

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Introduction

All organisms can synthesise saturated and monounsaturated fatty acids. However, the biosynthetic rate of long-chain polyunsaturated fatty acids (LC-PUFA) in vertebrates is markedly low and cannot cover physiological demands. Linoleic acid (18:2*n*-6,

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LA) and α-linolenic acid (18:3*n*-3, ALA) are precursors for the synthesis of omega-6 (*n*-6) and omega-3 (*n*-3) LC-PUFA series, respectively, and essential fatty acids for vertebrates, which lack Δ12/*n*-6 and Δ15/*n*-3 desaturases required to synthesise LA from oleic acid (18:1*n*-9c, OA) and ALA from LA (Castro et al. 2016; Tocher et al. 2019). LC-PUFA are critical components for growth and development, acting as bioactive components of membrane phospholipids, precursors of signalling molecules and modulators of gene expression. Although the specific physiological roles of *n*-3 LC-PUFA remain unclear, eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA) are thought to exert protective roles preventing atherosclerosis, stroke, obesity, type-2 diabetes, inflammation and autoimmune diseases, among others (Calder 2018; Djuricic and Calder 2021). In contrast, *n*-6 LC-PUFA, particularly arachidonic acid (20:4*n*-6, ARA), are precursors of local hormones promoting acute and chronic inflammation. ARA is a highly abundant fatty acid in the membranes of many cell types. Inflammatory stimuli releases ARA from cell membranes and let cyclooxygenase, lipoxygenase and cytochrome P450 pathways convert ARA to eicosanoids, a family of mediators and regulators of the inflammatory response that include prostaglandins, thromboxanes and leukotrienes. Therefore, many eicosanoids are linked to inflammatory diseases, although some ARA-derived metabolites also seem to be involved in the resolution of inflammation (Djuricic and Calder 2021). The role of ARA on inflammation has been widely studied in mammals. In contrast, knowledge of ARA function is still scarce in fish and cannot be directly inferred from mammalian physiology. Nevertheless, it is well-known the role of eicosanoids, mainly prostaglandins, in the regulation of fish immunity and inflammation (Xu et al. 2022). Apart from plants, fungi and some aquatic microorganisms, few other organisms such as the nematode *Caenorhabditis elegans* and some invertebrates can synthesise de novo *n*-3 and *n*-6 LC-PUFA in significant amounts. Vegetable oils are rich in LA and ALA, and often contain high levels of *n*-6 LC-PUFA, but are devoid of significant amounts of *n*-3 LC-PUFA, particularly EPA and DHA unless obtained from transgenic crops. Therefore, trophic transfer from microalgae and plankton to marine fish and seafood are major sources of LC-PUFA, notably *n*-3 LC-PUFA, in the human diet (Tocher et al. 2019; Osmond and Colombo 2019).

Shortage of *n*-3 LC-PUFA and increased *n*-6/*n*-3 ratio in fish fillets due to substitution of fish oil (rich in *n*-3 LC-PUFA) by vegetable oils (poor in *n*-3 LC-PUFA, but frequently rich in *n*-6 LC-PUFA) in aquafeeds is nowadays a major challenge for the aquaculture sector. To face this problem and increasing demands of functional food with high nutritional value, intense research is being conducted in order to improve the *n*-3 LC-PUFA content in farmed fish, including dietary incorporation of microalgae, genetically modified organisms (GMOs) such as yeast and algae, and plant GMO-derived oils, such as oil from false flax expressing microalgal genes (Betancor et al. 2016; Tocher et al. 2019; Osmond and Colombo 2019; Sales et al. 2021; Carvalho et al. 2022). Production in large-scale fermenters and the supply of balanced amounts of EPA and DHA constrains the use of microalgae biomass in aquafeeds (Tocher et al. 2019). Transgenesis of fish fatty acid desaturases and elongases aiming to increase EPA and DHA levels was assayed in zebrafish (Alimuddin et al. 2007, 2008; Cheng et al. 2015). However, fish desaturases and elongases act on both *n*-3 and *n*-6 fatty acid series and generally do not substantially change the *n*-6/*n*-3 ratio (Pang et al. 2014). Efficient conversion of *n*-6 PUFA into *n*-3 PUFA in transgenic mice expressing *Caenorhabditis elegans n*-3 fatty acid desaturase *fat-1* (FAT-1), an *n*-3 fatty acid desaturase absent in vertebrates (Kang et al. 2004), led to use synthetically humanised and fish codon-optimised *C. elegans* FAT-1 to generate transgenic zebrafish (Pang et al. 2014), common carp (Zhang et al. 2019), channel catfish (Xing et al. 2023), and other vertebrates, including mice, cattle, pigs and sheep (Lai et al. 2006; Ji et al. 2009; Chen et al. 2013; Liu et al. 2016, 2017; Li et al. 2018; Tang et al. 2019; Luo et al. 2020; Sun et al. 2020; You et al. 2021). Transgenesis of *C. elegans fat-1* in zebrafish, common carp, channel catfish, pigs and sheep efficiently increases EPA and DHA, while decreases the *n*-6/*n*-3 ratio (Lai et al. 2006; Pang et al. 2014; Li et al. 2018; Zhang et al. 2019; Luo et al. 2020; Xing et al. 2023). The effect is potentiated in zebrafish and pigs by double transgenesis with codon-optimised *C. elegans* Δ12 fatty acid desaturase *fat-2* (FAT-2), a Δ12 desaturase that converts OA into LA and which is also absent in vertebrates (Pang et al. 2014; Tang et al. 2019).

Given that there are major concerns on environmental risk, sustainability, fish welfare, food safety as well as consumer perception and acceptance of GMOs (Tocher et al. 2019; Osmond and Colombo 2019), in recent years we developed an alternative methodology to GMO generation based on the production of chitosan-tripolyphosphate (TPP)-DNA nanoparticles for transient modification of the expression of target genes in the liver of gilthead seabream (*Sparus aurata*) (González et al. 2016; Gaspar et al. 2018; Silva-Marrero et al. 2019). Chitosan is a cationic polymer of glucosamine and N-acetylglucosamine derived from chitin by deacetylation. Chitosan is increasingly used as carrier for delivering nucleic acids in vivo due to its well-known mucoadhesion, low toxicity, biodegradability and biocompatibility (Wu et al. 2020).

With the aim to promote sustained production of *n*-3 LC-PUFA in *S. aurata*, in the present study chitosan-TPP nanoparticles encapsulating plasmids expressing fish codon-optimised *Caenorhabditis elegans* FAT-1 and FAT-2 were intraperitoneally administered every 4 weeks to *S. aurata* (3 doses in total). Seventy days post-treatment, the effect of chitosan-TPP-DNA nanoparticles was assessed on growth parameters, intermediary metabolism and fatty acid content in the liver and skeletal muscle of *S. aurata*.

Materials and methods

Animals

S. aurata juveniles (7.7 g ± 0.2, mean weight ± SEM) were obtained from Piscicultura Marina Mediterranea (AVRAMAR Group, Burriana, Spain) and maintained at 20 °C in 250-L aquaria supplied with running seawater in the aquatic animals facility of the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB) as described (Silva-Marrero et al. 2017). Fish were fed with commercial diet (Dibaq Microbaq 165, Dibaq, Segovia Spain), containing 52% protein, 18% lipids, 12% carbohydrates, 10% ash, 8% moisture and 21.3 kJ/g gross energy. For the acclimation regime, fish were fed twice daily (9:00 and 17:00) at a ration of 5% body weight (BW). Two weeks before experimental treatments, the ration was adjusted and kept to 3% BW until the end of the experiment. Fish were weighted every 2 weeks to readjust the feed amount. To study the long-term effect of fish codon-optimised *C. elegans fat-1* and *fat-2* expression, 4 groups of fish were intraperitoneally injected up to 3 times (once every 4 weeks) with chitosan-TPP nanoparticles complexed with pSG5 (empty plasmid, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2. Every single administration consisted of 10 µg plasmid per gram BW. Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed by cervical section, blood was collected and the liver, intestine, skeletal muscle and brain were dissected out, frozen in liquid nitrogen and kept at − 80 °C until use. To prevent stress, fish were anesthetised by tricaine methanesulfonate (MS-222; 1:12,500) before handling.

Preparation and characterisation of chitosan-TPP-DNA nanoparticles

Fish codon-optimised FAT-1 and FAT-2 cDNA sequences (GenBank accession nos. ON374024 and ON374025, respectively) were synthesised based on *C. elegans* FAT-1 and FAT-2 using GeneArt Instant Designer (Thermo Fisher Scientific, Waltham, MA, USA) and ligated into pSG5 (Agilent Technologies, Palo Alto, CA, USA). The resulting constructs (pSG5-FAT-1 and pSG5-FAT-2) were verified by cycle sequencing on both sides. Chitosan-TPP nanoparticles encapsulating pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 were prepared by ionic gelation (González et al. 2016). For each experimental condition, 1 mg of plasmid was mixed with 4 mL of 0.84 mg/mL TPP (Sigma-Aldrich, St. Louis, MO, USA). Chitosan-TPP-DNA nanoparticles were formed upon dropwise addition of the TPP-DNA solution into 10 mL of 2 mg/mL low molecular weight chitosan (Sigma-Aldrich, St. Louis, MO, USA)-acetate buffer (pH 4.4) solution. Nanoparticles were sedimented by centrifugation at 36,000 g for 20 min at 15 °C and resuspended in 2 mL of 2% w/v mannitol, which acted as cryoprotector during lyophilisation. Nanoparticles were subjected to a freeze–drying process at − 47 °C. Particle size and *Z* potential were determined by dynamic light scattering and laser Doppler electrophoresis, respectively, using Zetasizer Nano ZS fitted with a 633 nm laser (Malvern Instruments, Malvern, UK). Chitosan-TPP-DNA

nanoparticles were resuspended in 0.9% NaCl before intraperitoneal administration to *S. aurata*.

Body composition

For moisture determination, fish were dried at 85 °C until constant weight was reached (Busacker et al. 1990; Lucas 1996). Moisture was calculated as [wet weight (g) − dry weight (g)]*100/wet weight (g). Dried samples were further used for assaying nitrogen (N), lipid and ash. N content was determined with FlashEA 1112 analyser (Thermo Fisher Scientific, Waltham, MA, USA) and was subsequently used to estimate crude protein by multiplying N content by a factor of 6.25. Crude lipid was extracted with petroleum ether using a Soxhlet extractor. For ash determination, samples were incinerated in a Hobersal 12PR/300 muffle furnace (Hobersal, Caldes de Montbui, Spain) at 550 °C for 12 h (Busacker et al. 1990; Lucas 1996). Crude protein, lipid and ash are expressed as percentage of dry weight.

Growth parameters

Specific growth rate (SGR), feed conversion ratio (FCR), hepatosomatic index (HSI), protein retention (PR), lipid retention (LR) and protein efficiency ratio (PER) were calculated according to the following equations:

$$SGR = \left(\ln W_f - \ln W_i\right) * 100/T;$$

where W_f and W_i are mean final and initial body fresh weight (g) and T is time (days)

FCR = dry feed intake (g)/wet weight gain (g)

HSI = liver fresh weight (g) ∗ 100/fish body weight (g)

PR = body protein gain (g) ∗ 100/protein intake (g)

LR = body lipid gain (g) ∗ 100/lipid intake (g)

PER = weight gain (g)/feed protein provided (g)

Enzyme activity assays and metabolites

Enzyme activity assays and metabolites were spectrophotometrically determined at 30 °C in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Liver crude extracts were obtained by homogenisation of powdered frozen tissue (1:5, *w/v*) in 50 mM Tris-HCl (pH 7.5), 4 mM, EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 250 mM sucrose, 30 s at 4 °C using PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland). Following centrifugation at 10,000 g for 30 min at 4 °C, the supernatant was collected for enzyme activity assays. Reaction mixtures for 6-phosphofructo-1-kinase (Pfkl), fructose-1,6-bisphosphatase (Fbp1) and total protein were as previously described (Metón et al. 1999b). Enzyme activities were expressed as specific activity (U/g protein). One unit of Pfkl activity was considered the amount of enzyme needed to oxidise 2 µmol of NADH per min. One unit of Fbp1 activity of was defined as the amount of enzyme necessary for transforming 1 µmol of substrate per min. Serum glucose, triglycerides and cholesterol were measured with commercial kits (Linear Chemicals, Montgat, Spain).

Reverse transcription coupled to quantitative real-time PCR (RT-qPCR)

Total RNA from *S. aurata* tissues was isolated using HigherPurity Tissue Total RNA Purification Kit (Canvax, Cordoba, Spain) and reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The mRNA expression levels of genes listed in Table 1 were determined using QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The reaction mixture contained 0.4 µM of each primer (Table 1), 5 µL of SYBR Green (Thermo Fisher Scientific, Foster City, CA, USA), 0.8 µL of diluted cDNA and sterilized milli-Q water to final volume of 10 µL. The amplification cycle was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min. For each gene, standard curves for determining efficiency of the amplification reaction were generated with serial dilutions of control cDNA. Amplification of single products was confirmed by checking dissociation curves after each experiment. Amplicon size was checked by agarose gel electrophoresis. *S. aurata* ribosomal subunit 18S (*18s*), β-actin (*actb*) and elongation factor 1 alpha (*eef1a*) were used as endogenous controls to normalise the mRNA levels for genes of interest in liver samples. For tissue distribution, normalisation

Table 1 Primer sequences used for RT-qPCR in the present study

Gene	Forward sequences (5′–3′)	Reverse sequences (5′–3′)	GenBank Accession
acaca	CCCAACTTCTTCTACCACAG	GAACTGGAACTCTACTACAC	JX073712
acacb	TGACATGAGTCCTGTGCTGG	GCCTCAGTTCGTATGATGGT	JX073714
actb	CTGGCATCACACCTTCTACAACGAG	GCGGGGGTGTTGAAGGTCTC	X89920
cpt1a	GAAGGGCAGATAAAGAGGGGC	GCATCGATCGCTGCATTCAGC	JQ308822
eef1a	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	AF184170
elovl4a	AAGAACAGAGAGCCCTTCCAG	TGCCACCCTGACTTCATTG	MK610320
elovl4b	TCTACACAGGCTGCCCATTC	CGAAGAGGATGATGAAGGTGAC	MK610321
elovl5	GGGATGGCTACTGCTCGACA	CAGGAGAGTGAGGCCCAGAT	AY660879
fads2	CACTATGCTGGAGAGGATGCC	TATTTCGGTCCTGGCTGGGC	AY055749
fasn	GTAGAGGACACGCCCATCGAT	TGCGTATGACCTCTTGGTGTGCT	JQ277708
fat-1	TTCAACCCCATTCCTTTCAGCG	TAGGCGCACACGCAGCAGCA	ON374024
fat-2	AAGAGGACTACAACAACAGAACCGCCA	CGAACAGTCTGCTCCAAGGCCAA	ON374025
fbp1	CAGATGGTGAGCCGTGTGAGAAGGATG	GCCGTACAGAGCGTAACCAGCTGCC	AF427867
gck	TGTGTCAGCTCTCAACTCGACC	AGGATCTGCTCTACCATGTGGAT	AF169368
g6pc1	GCGTATTGGTGGCTGAGGTCG	AAGGAGAGGGTGGTGTGGAAG	AF151718
g6pd	TGATGATCCAACAGTTCCTA	GCTCGTTCCTGACACACTGA	JX073711
hmgcr	ACTGATGGCTGCTCTGGCTG	GGGACTGAGGGATGACGCAC	MN047456
hnf4a	GTGGACAAAGACAAGCGAAATC	GCATTGATGGATGGTAAACTGC	FJ360721
nr1h3	GCATCTGGACGAGGCTGAATAC	ACTTAGTGTGCGAAGGCTCACC	FJ502320
pck1	CAGCGATGGAGGAGTGTGGTGGGA	GCCCATCCCAATTCCCGCTTCTGTGCT CCGGCTGGTCAGTGT	AF427868
pfkfb1	TGCTGATGGTGGGACTGCCG	CTCGGCGTTGTCGGCTCTGAAG	U84724
pfk1	TGCTGGGGACAAAACGAACTCTTCC	AAACCCTCCGACTACAAGCAGAGCT	KF857580
pklr	CAAAGTGGAAAGCCGGCAAGGG	GTCGCCCCTGGCAACCATAAC	KF857579
ppara	GTGAGTCTTGTGAGTGAGGGGTTG	AGTGGGGATGGTGGGCTG	AY590299
srebf1	CAGCAGCCCGAACACCTACA	TTGTGGTCAGCCCTTGGAGTTG	JQ277709
scd1a	TCCCTTCCGCATCTCCTTTG	TTGTGGTGAACCCTGTGGTCTC	JQ277703
18s	TTACGCCCATGTTGTCCTGAG	AGGATTCTGCATGATGGTCACC	AM490061

was performed against *S. aurata 18s* expression. The standard $\Delta\Delta C_T$ method was used to calculate variations in gene expression (Pfaffl 2001).

Fatty acid methyl ester (FAME) analysis

Fatty acid profiles of liver and muscle were analysed by gas chromatography with flame ionisation detection as previously described (Silva-Marrero et al. 2019), using GC-2025 (Shimadzu, Kyoto, Japan) with capillary column BPX70, 30 m × 0.25 mm × 0.25 µm (Trajan Scientific and Medical, Ringwood, Australia). Oven temperature started at 60 °C for 1 min and then it was raised to 260 °C (rate: 6 °C/min). Injector (AOC-20i, Shimadzu, Japan) and detector temperatures were 260 °C and 280 °C, respectively. Sample (1 µL) was injected with helium as carrier gas and split ratio 1:20. Supelco 37 Component FAME Mix (Sigma-Aldrich, St. Louis, MO, USA) was used as reference for identifying fatty acids.

Statistics

To identify significant differences between treatments, the SPSS Version 25 software (IBM, Armonk, NY, USA) was used to submit experimental data to one-way analysis of variance followed by the Duncan post-hoc test (> 2 groups). Statistical significance was considered when $P < 0.05$.

Results

Delivery of chitosan-TPP complexed with pSG5-FAT-1 and pSG5- FAT-2 increases fish codon-optimised FAT-1 and FAT-2 mRNA levels in *S. aurata*

To assess the metabolic effects resulting from expression of *C. elegans fat-1* and *fat-2* in the liver of *S. aurata*, we designed fish codon-optimised *C. elegans* FAT-1 and FAT-2 cDNA sequences for further ligation into pSG5 and prepared chitosan-TPP nanoparticles complexed with empty pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 by ionic gelation. Particle size and *Z* potential of naked chitosan-TPP, expressed as mean ± SEM ($n = 3$), was 214.6 nm ± 20.2 and 37.5 mV ± 0.6, respectively. Incorporation of plasmid DNA to chitosan-TPP did not significantly modify particle size, which was 262.7 nm ± 74.0 (mean ± SEM, $n = 3$), but decreased *Z* potential to 12.0 mV ± 0.8 (mean ± SEM, $n = 3$).

For long-term sustained expression of fish codon-optimised *fat-1* and *fat-2* in the liver of *S. aurata*, each experimental group of fish received every 4 weeks up to 3 intraperitoneal injections of chitosan-TPP complexed with 10 µg/g BW of the corresponding plasmid (pSG5, pSG5-FAT-1, pSG5-FAT-2 or pSG5-FAT-1 + pSG5-FAT-2). The dosing schedule was based on preliminary studies showing that 28 days post-administration of chitosan-TPP-pSG5-FAT-1 and chitosan-TPP-pSG5-FAT-2 (10 µg/g BW of plasmid) to *S. aurata* increased the hepatic mRNA levels of fish codon-optimised *fat-1* and *fat-2* to levels even higher than those found at 72 h post-treatment. Expressed as mean ± SEM ($n = 3$), fold increase over control values at 72 h and 28 days post-treatment were 31.6 ± 4.1 and 74.8 ± 29.0, respectively, for *fat-1* mRNA levels, while for *fat-2* mRNA levels fold increase was 20.3 ± 2.8 and 70.2 ± 7.0, respectively. Seventy days after the beginning of the experiment (14 days following the last injection), the mRNA levels of fish codon-optimised *fat-1* and *fat-2* were determined by RT-qPCR in several tissues of treated fish, including the liver, intestine, skeletal muscle and brain (Fig. 1).

When compared with control fish, chitosan-TPP nanoparticles complexed with pSG5-FAT-1 and pSG5-FAT-2 significantly increased the mRNA levels of *fat-1* and *fat-2*, respectively, in the liver and intestine of *S. aurata*. Specifically, *fat-1* mRNA abundance in the liver of fish administered with pSG5-FAT-1 was 201.8-fold higher than in control fish, while treatment with pSG5-FAT-2 upregulated *fat-2* 297.4-fold. For the intestine, pSG5-FAT-1 and pSG5-FAT-2 upregulated 10.6-fold *fat-1* and 24.7-fold *fat-2*, respectively. Nanoparticle administration did not exert effects on the skeletal muscle and brain.

Effect of fish codon-optimised FAT-1 and FAT-2 expression on whole-body composition, growth performance and serum metabolites in *S. aurata*

Sustained expression of fish codon-optimised FAT-1 + FAT-2 in the liver of *S. aurata* caused a moderate but significant 7.6% decrease of whole-body crude protein values observed in control fish. No effect was observed in moisture, ash and crude lipid body composition (Table 2). Analysis of growth performance parameters showed significantly increased weight gain values in fish expressing *fat-1* (18% of increase) and *fat-1* + *fat-2* (26% of increase) compared to control fish. No significant difference was found between controls and treatment with FAT-2. Similarly, the highest SGR was found in fish treated with FAT-1 + FAT-2, followed by fish treated with FAT-1, controls and fish treated with FAT-2. Fish expressing *fat-2* also presented the lowest PER. HSI significantly decreased in fish treated with FAT-1 and FAT-1 + FAT-2 to 72% of control values. No significant differences were observed in PR and LR.

Serum glucose, triglycerides and cholesterol were also determined in 70-day treated *S. aurata*. Any of the treatments assayed affected blood glucose levels. However, co-expression of *fat-1* + *fat-2* significantly decreased 1.8-fold triglycerides and 1.5-fold cholesterol compared to control levels (Fig. 2).

Effect of fish codon-optimised FAT-1 and FAT-2 on the fatty acid profile in the liver and skeletal muscle

The effect of long-term expression of *fat-1* and *fat-2* was analysed on the fatty acid profile of the liver and skeletal muscle of *S. aurata*. Table 3 shows the fatty acid composition in the liver of *S. aurata* long-term treated with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2, and pSG5-FAT-1 + pSG5-FAT-2. Among

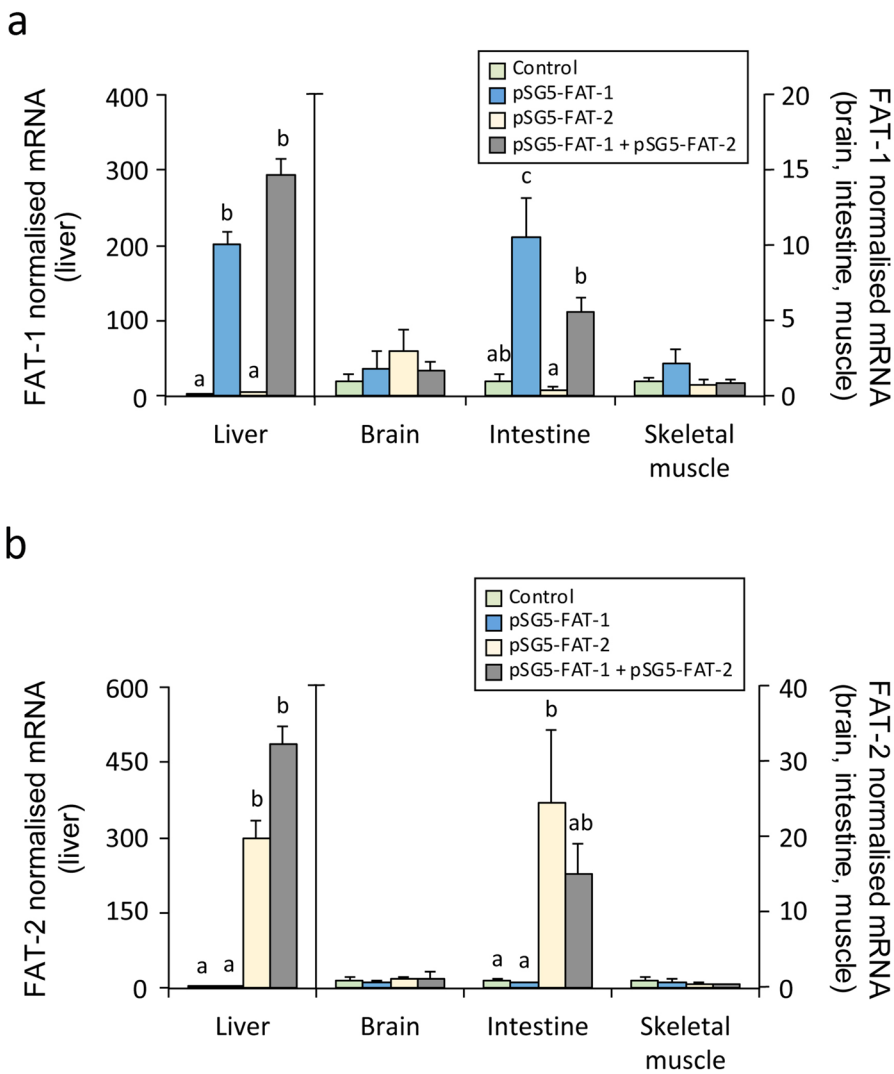

Fig. 1 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the mRNA levels of fish-codon optimised *C. elegans* FAT-1 and FAT-2 in *S. aurata* tissues (brain, skeletal muscle, liver and intestine). Fourteen days after the last injection and 24 h following the last meal, exogenous *fat-1* (**a**) and *fat-2* (**b**) expression was assayed by RT-qPCR, normalised to the *S. aurata 18 s* mRNA levels and represented as mean ± SEM ($n = 4$). For each tissue, homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

30 different fatty acids identified in this study, treatment with FAT-1 and FAT-1 + FAT-2 significantly increased EPA (1.5-fold and 1.6-fold, respectively), DHA (2.4-fold and 2.3-fold, respectively) and total *n*-3 fatty acids (1.7-fold in both cases). The *n*-6/*n*-3 ratio significantly decreased in fish expressing *fat-1* (to 60.3% of control values), *fat-2* (66.9%) and *fat-1* + *fat-2* (63.7%). A moderate 1.2-fold increase of *cis*-10-heptadecenoic acid (17:1*n*-7) was also observed in FAT-1 + FAT-2 treated fish, while expression of *fat-1* decreased palmitoleic acid (16:1*n*-7) to 58.4% of control levels.

The effect of long-term hepatic expression of *fat-1*, *fat-2* and *fat-1* + *fat-2* on the fatty acids profile in the skeletal muscle is shown in Table 4. Twenty-one out of 29 fatty acids identified in the skeletal muscle were significantly affected by co-expression of *fat-1* and *fat-2*. Total saturated fatty acids significantly decreased to 57.1% of control levels, mostly resulting from the low content in myristic acid (14:0; 34.4% of controls), palmitic acid (16:0; 56.3% of controls) and margaric acid (17:0; 50.0% of controls). In addition, treatment with FAT-1 and FAT-2 also decreased margaric acid to 50.0% of control values. In contrast, longer saturated fatty acids (with more than 17 carbons) presented increased values than in controls. Thus, stearic acid (18:0) increased 1.2-fold, while arachidic acid (20:0), behenic acid (22:0), tricosylic acid (23:0) and lignoceric acid (24:0) rised from non-detectable levels in control

Table 2 Growth performance, nutrient retention and body composition of *S. aurata* after intraperitoneal injection of chitosan-TPP complexed with empty vector (pSG5, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2

	Control	FAT-1	FAT-2	FAT-1 + FAT-2
Initial body weight (g)	9.97 ± 0.69	11.42 ± 0.90	9.74 ± 0.44	11.04 ± 0.55
Final body weight (g)	34.64 ± 1.03^{a}	40.51 ± 1.63^{b}	31.46 ± 1.57^{a}	42.15 ± 1.25^{b}
Weight gain (g)	24.67 ± 0.51^{a}	29.09 ± 1.06^{b}	21.73 ± 1.23^{a}	31.11 ± 0.73^{b}
SGR (%)	1.79 ± 0.05^{ab}	1.85 ± 0.08^{ab}	1.59 ± 0.04^{a}	2.01 ± 0.03^{b}
FCR	1.40 ± 0.03^{ab}	1.36 ± 0.04^{ab}	1.53 ± 0.08^{b}	1.29 ± 0.03^{a}
HSI (%)	1.55 ± 0.12^{b}	1.12 ± 0.08^{a}	1.49 ± 0.18^{ab}	1.12 ± 0.15^{a}
PR (%)	24.98 ± 3.24	21.96 ± 0.85	18.90 ± 2.45	21.81 ± 1.56
LR (%)	28.00 ± 7.50	31.47 ± 1.12	25.17 ± 2.03	26.24 ± 2.02
PER	1.40 ± 0.03^{ab}	1.45 ± 0.05^{ab}	1.31 ± 0.07^{a}	1.52 ± 0.04^{b}
Moisture (%)	71.34 ± 1.22	70.68 ± 0.29	72.03 ± 0.98	71.10 ± 1.28
Ash (%)	13.72 ± 1.47	13.93 ± 0.12	13.07 ± 0.73	12.94 ± 0.45
Protein (%)	62.20 ± 3.88^{b}	58.81 ± 1.80^{ab}	57.98 ± 1.04^{ab}	57.50 ± 1.24^{a}
Lipid (%)	27.28 ± 1.44	29.28 ± 0.55	26.09 ± 1.85	25.02 ± 2.3

All fish were fed twice a day at a total daily ration of 3% BW. Every 2 weeks, fish were individually weighted to readjust the feed amount. Data are expressed as mean ± SEM ($n = 3$). Different superscript letters indicate significant differences between groups ($P < 0.05$)

SGR specific growth rate, *FCR* feed conversion ratio, *HSI* hepatosomatic index, *PR* protein retention, *LR* lipid retention, *PER* protein efficiency ratio

fish to low but detectable levels in fish treated with FAT-1 + FAT-2.

Monounsaturated, PUFA and total *n*-3, *n*-6 and *n*-9 fatty acids increased 1.3-fold, 1.3-fold, 2.2-fold, 1.1-fold and 1.5-fold, respectively, in the skeletal muscle of fish expressing *fat-1* + *fat-2*. As a result of greater effect on *n*-3 series than on *n*-6 fatty acids, the *n*-6/*n*-3 ratio significantly decreased to 52.0% of control levels. Considering unsaturated fatty acids with a content greater than 1% for any assayed treatment, expression of *fat-1* + *fat-2* significantly increased EPA (1.7-fold) and DHA (3.0-fold) from the *n*-3 series, LA (18:2*n*-6c; 1.1-fold) and OA (18:1*n*-9c; 1.5-fold), while decreased palmitoleic acid to 61.5% of control levels. For less abundant unsaturated fatty acids (content between 0.1 and 1%), treatment with FAT-1 + FAT-2 also resulted in significant increases of *cis*-10-heptadecenoic acid (1.5-fold), gondoic acid (20:1*n*-9; 3.2-fold), erucic acid (22:1*n*-9; 5.2-fold), eicosadienoic acid (20:2*n*-6; 5.5-fold) and ARA (20:4*n*-6; 1.4-fold).

Effect of fish codon-optimised FAT-1 and FAT-2 on the expression of key genes in de novo lipogenesis and fatty acid oxidation in the liver

The effect of chitosan-TPP-DNA nanoparticles expressing *fat-1* and *fat-2* was also assessed on the hepatic expression of genes involved in de novo lipogenesis and fatty acid oxidation. As shown in Fig. 3, treatment with FAT-1 significantly decreased the mRNA levels of elongation of very long chain fatty acids protein 4a (*elovl4a*; to 43.3% of control values), elongation of very long chain fatty acids protein 4b (*elovl4b*; to 45.4%), elongation of very long chain fatty acids protein 5 (*elovl5*; to 62.3%), sterol regulatory element-binding protein 1 (*srebf1*; to 41.3%) and peroxisome proliferator-activated receptor alpha (*ppara*; to 46.0%), while treatment with FAT-2 decreased *elovl5* (to 43.6%) and *ppara* (to 56.3%) mRNA levels (Fig. 3f–h, k, l). Co-expression of *fat-1* and *fat-2* also significantly down-regulated acetyl-CoA carboxylase 1 (*acaca*; to 31.4% of control values), acetyl-CoA carboxylase 2 (*acacb*; to 65.0%), acyl-CoA 6-desaturase (*fads2*; to 69.4%), *elovl4b* (to 59.1%), *elovl5* (to 41.8%), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*hmgcr*; to 64.2%) and *srebf1* (to 41.9%) (Fig. 3a, b, e, g, h, j, k). No significant differences were found for fatty acid

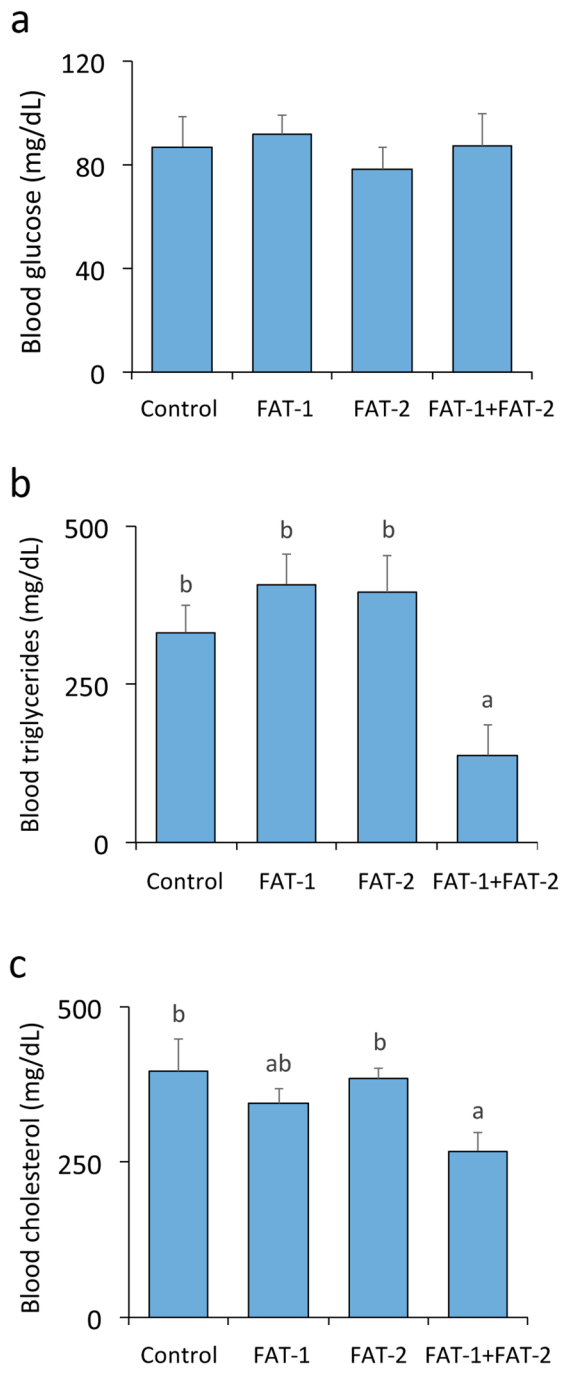

Fig. 2 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on serum glucose (**a**), triglycerides (**b**) and cholesterol (**c**) in *S. aurata*. Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed and the blood was collected. Values are represented as mean ± SEM ($n = 6$–7). Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

synthase (*fasn*), stearoyl-CoA desaturase-1a (*scd1a*) and carnitine O-palmitoyltransferase 1, liver isoform (*cpt1a*) (Fig. 3c, d, i).

Effect of fish codon-optimised FAT-1 and FAT-2 on glycolysis-gluconeogenesis, the pentose phosphate pathway, *hnf4a* and *nr1h3* in the liver

Figure 4a–h shows the effect of long-term expression of fish codon-optimised *fat-1* and *fat-2* on the hepatic expression of rate-limiting enzymes in glycolysis-gluconeogenesis. Due to the pivotal role of the enzymes that control the flux through the fructose-6-phosphate/fructose-1,6-bisphosphate cycle in the regulation of glycolysis-gluconeogenesis, we measured both the mRNA levels and the enzyme activity of Pfkl and Fbp1. Gene expression of *pfkl* and *fbp1* was not significantly affected by the treatments (Fig. 4d, e). However, when considering the Pfkl/Fbp1 activity ratio, fish treated with FAT-1 + FAT-2 exhibited a significant increased glycolytic flux (29.9%) compared to control fish (Fig. 4f). Similarly, treatment with FAT-2 showed a trend to increase the Pfkl/Fbp1 activity ratio (22.7%).

In regard of other glycolytic-gluconeogenic enzymes, expression of *fat-2* and *fat-1* + *fat-2* significantly upregulated 1.5-fold and 1.8-fold, respectively, the mRNA levels of liver pyruvate kinase (*pklr*) and 1.4-fold those of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (*pfkfb1*) (Fig. 4c). Compared to controls, the expression levels for glucokinase (*gck*), glucose-6-phosphatase catalytic subunit (*g6pc1*) and phosphoenolpyruvate carboxykinase (*pck1*) were not affected (Fig. 4a, b, h).

In addition, expression of *fat-2* also significantly increased the hepatic mRNA levels (2.0-fold) of glucose-6-phosphate dehydrogenase (*g6pd*), the rate-limiting enzyme in the oxidative phase of the pentose phosphate pathway (Fig. 4i), while treatment with FAT-2 and FAT-1 + FAT-2 upregulated hepatocyte nuclear factor 4-alpha (*hnf4a*; 1.5-fold) and oxysterols receptor LXR-alpha (*nr1h3*; 1.6-fold) mRNA levels, respectively (Fig. 4j–k).

Discussion

With the aim to induce sustained production of *n*-3 LC PUFA in *S. aurata*, the ionotropic gelation technique was used to obtain chitosan-TPP nanoparticles

Table 3 Effect of chitosan-TPP complexed with empty vector (pSG5, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the fatty acid profile of *S. aurata* liver

Fatty acid	Control	FAT-1	FAT-2	FAT-1 + FAT-2
14:0	9.38 ± 0.46	7.31 ± 1.45	7.43 ± 0.60	7.40 ± 1.45
15:0	0.10 ± 0.10	0.32 ± 0.12	0.37 ± 0.13	0.32 ± 0.11
16:0	27.49 ± 0.66	27.09 ± 3.37	26.33 ± 1.42	25.29 ± 2.50
17:0	0.21 ± 0.03	0.11 ± 0.04	0.22 ± 0.11	0.11 ± 0.04
18:0	3.73 ± 0.39	3.88 ± 0.30	4.20 ± 0.38	4.03 ± 0.33
20:0	0.02 ± 0.02	0.20 ± 0.13	0.08 ± 0.03	0.08 ± 0.04
21:0	0.06 ± 0.06	0.16 ± 0.06	0.12 ± 0.07	0.11 ± 0.04
22:0	0.00 ± 0.00	0.06 ± 0.04	0.11 ± 0.04	0.05 ± 0.05
23:0	0.02 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
24:0	0.01 ± 0.01	0.03 ± 0.02	0.06 ± 0.02	0.04 ± 0.02
14:1*n*-5	0.06 ± 0.05	0.00 ± 0.00	0.14 ± 0.09	0.13 ± 0.04
15:1*n*-5	0.06 ± 0.02	0.02 ± 0.01	0.05 ± 0.03	0.05 ± 0.02
16:1*n*-7	6.93^{b} ± 0.27	4.05^{a} ± 1.23	5.58^{ab} ± 0.48	5.38^{ab} ± 0.42
17:1*n*-7	0.20 ± 0.02	0.22 ± 0.00	0.26 ± 0.02	0.23 ± 0.01
18:1*n*-9c	21.24 ± 1.24	22.04 ± 1.60	22.45 ± 1.15	21.84 ± 1.89
18:1*n*-9t	0.06 ± 0.03	0.04 ± 0.01	0.08 ± 0.02	0.04 ± 0.01
20:1*n*-9	0.58 ± 0.08	0.83 ± 0.02	0.71 ± 0.13	0.72 ± 0.14
22:1*n*-9	0.17 ± 0.03	0.30 ± 0.08	0.25 ± 0.05	0.31 ± 0.08
24:1*n*-9	0.00 ± 0.00	0.07 ± 0.05	0.07 ± 0.03	0.05 ± 0.03
18:2*n*-6c	23.23 ± 0.52	23.55 ± 1.4	22.42 ± 1.36	24.19 ± 1.29
18:2*n*-6t	0.26 ± 0.22	0.09 ± 0.03	0.09 ± 0.04	0.13 ± 0.05
20:2*n*-6	0.17 ± 0.05	0.25 ± 0.03	0.21 ± 0.05	0.19 ± 0.07
22:2*n*-6	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
18:3*n*-3	1.29 ± 0.10	1.26 ± 0.25	1.76 ± 0.45	1.16 ± 0.20
18:3*n*-6	0.76 ± 0.17	0.65 ± 0.14	0.81 ± 0.06	0.72 ± 0.14
20:3*n*-3	0.00 ± 0.00	0.06 ± 0.06	0.00 ± 0.00	0.07 ± 0.07
20:3*n*-6	0.23 ± 0.04	0.29 ± 0.06	0.21 ± 0.06	0.25 ± 0.02
20:4*n*-6	0.22 ± 0.02	0.29 ± 0.10	0.40 ± 0.14	0.30 ± 0.11
20:5*n*-3	1.79^{a} ± 0.09	2.76^{b} ± 0.29	2.43^{ab} ± 0.26	2.89^{b} ± 0.33
22:6*n*-3	1.70^{a} ± 0.15	4.02^{b} ± 0.65	3.15^{ab} ± 0.74	3.87^{b} ± 0.66
Saturated	41.02 ± 0.58	39.18 ± 4.49	38.92 ± 1.64	37.45 ± 3.54
Monounsaturated	29.3 ± 1.10	27.57 ± 2.60	29.59 ± 1.30	28.75 ± 1.79
PUFA	29.67 ± 0.60	33.25 ± 2.51	31.49 ± 1.39	33.80 ± 1.94
n-3	4.78^{a} ± 0.28	8.11^{b} ± 0.99	7.34^{ab} ± 0.99	8.00^{b} ± 0.93
n-6	24.89 ± 0.47	25.14 ± 1.63	24.15 ± 1.48	25.8 ± 1.54
n-9	22.05 ± 1.18	23.28 ± 1.74	23.56 ± 1.07	22.96 ± 2.06
n-6/*n*-3	5.26^{b} ± 0.28	3.17^{a} ± 0.26	3.52^{a} ± 0.65	3.35^{a} ± 0.42

Data are expressed as percentage of total fatty acids and represented as mean ± SEM ($n = 4$). Different superscript letters indicate significant differences between groups ($P < 0.05$)

complexed with plasmids expressing fish codon-optimised *C. elegans fat-1* and *fat-2*. *C. elegans fat-1* and *fat-2* were chosen to improve *n*-3 LC PUFA biosynthesis in *S. aurata* on the basis of their functionality in transgenic vertebrates, including fish. In fact, methyl-end desaturases from nematodes, including *C. elegans fat-1* and *fat-2*, are considered one of the three main clades in the evolution of animal methyl-end desaturase genes, together with those from two distinct gene lineages of cnidarians and some lophotrochozoans and arthropods (Kabeya et al. 2018). Even though knowledge on pathways of PUFA biosynthesis in non-vertebrate animals is still limited, increasing availability of genomic data and functional characterisation of methyl-end desaturases from marine invertebrates will contribute to a better understanding

Table 4 Effect of chitosan-TPP complexed with empty vector (pSG5, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the fatty acid profile of *S. aurata* skeletal muscle

Fatty acid	Control	FAT-1	FAT-2	FAT-1 + FAT-2
14:0	$8.83^{b\pm}0.37$	$7.86^{b\pm}1.39$	$7.44^{b\pm}1.22$	$3.04^{a\pm}0.10$
16:0	$29.58^{b} \pm 1.31$	$27.00^{b} \pm 3.05$	$26.93^{b} \pm 2.85$	$16.64^{a} \pm 0.34$
17:0	$0.18^{b} \pm 0.01$	$0.09^{a} \pm 0.00$	$0.09^{a} \pm 0.00$	$0.09^{a} \pm 0.00$
18:0	$2.77^{a} \pm 0.04$	$2.78^{a} \pm 0.15$	$2.96^{a} \pm 0.11$	$3.29^{b} \pm 0.08$
20:0	$0.00^{a} \pm 0.00$	$0.11^{a} \pm 0.07$	$0.09^{a} \pm 0.07$	$0.28^{b} \pm 0.03$
22:0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.10^{b} \pm 0.01$
23:0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.09^{b} \pm 0.03$
24:0	$0.00^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.02^{a} \pm 0.02$	$0.08^{b} \pm 0.00$
14:1*n*-5	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.00
15:1*n*-5	0.02 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.05 ± 0.00
16:1*n*-7	$6.63^{b} \pm 0.16$	$6.42^{b} \pm 0.68$	$6.14^{b} \pm 0.56$	$4.08^{a} \pm 0.05$
17:1*n*-7	$0.16^{a} \pm 0.01$	$0.20^{ab} \pm 0.03$	$0.20^{ab} \pm 0.01$	$0.24^{b} \pm 0.00$
18:1*n*-9c	$19.6^{a} \pm 0.53$	$21.98^{a} \pm 2.33$	$21.65^{a} \pm 2.01$	$28.45^{b} \pm 0.30$
18:1*n*-9t	$0.11^{b} \pm 0.04$	$0.06^{ab} \pm 0.03$	$0.11^{b} \pm 0.04$	$0.00^{a} \pm 0.00$
20:1*n*-9	$0.26^{a} \pm 0.16$	$0.48^{ab} \pm 0.12$	$0.54^{ab} \pm 0.10$	$0.84^{b} \pm 0.01$
22:1*n*-9	$0.12^{a} \pm 0.01$	$0.20^{a} \pm 0.09$	$0.21^{a} \pm 0.09$	$0.62^{b} \pm 0.05$
24:1*n*-9	$0.00^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.00^{a} \pm 0.00$	$0.08^{b} \pm 0.01$
18:2*n*-6c	$24.49^{a} \pm 0.62$	$24.57^{a} \pm 0.64$	$25.29^{a} \pm 0.67$	$27.98^{b} \pm 0.26$
18:2*n*-6t	0.40 ± 0.23	0.16 ± 0.16	0.17 ± 0.17	0.00 ± 0.00
20:2*n*-6	$0.06^{a} \pm 0.02$	$0.12^{a} \pm 0.05$	$0.13^{a} \pm 0.04$	$0.33^{b} \pm 0.02$
22:2*n*-6	$0.00^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.02^{a} \pm 0.02$	$0.10^{b} \pm 0.01$
18:3*n*-3	0.86 ± 0.26	0.89 ± 0.17	0.83 ± 0.16	1.11 ± 0.13
18:3*n*-6	0.97 ± 0.13	0.99 ± 0.06	0.90 ± 0.07	1.12 ± 0.04
20:3*n*-3	0.00 ± 0.00	0.04 ± 0.04	0.04 ± 0.04	0.09 ± 0.01
20:3*n*-6	0.13 ± 0.01	0.13 ± 0.03	0.13 ± 0.02	0.20 ± 0.02
20:4*n*-6	$0.23^{a} \pm 0.01$	$0.24^{a} \pm 0.03$	$0.28^{ab} \pm 0.03$	$0.32^{b} \pm 0.01$
20:5*n*-3	$2.36^{a} \pm 0.13$	$2.62^{a} \pm 0.44$	$2.79^{a} \pm 0.45$	$4.07^{b} \pm 0.03$
22:6*n*-3	$2.25^{a} \pm 0.31$	$2.97^{a} \pm 0.99$	$2.98^{a} \pm 0.98$	$6.69^{b} \pm 0.21$
Saturated	$41.36^{b} \pm 1.46$	$37.86^{b} \pm 4.21$	$37.53^{b} \pm 3.86$	$23.62^{a} \pm 0.38$
Monounsaturated	$26.9^{a} \pm 0.80$	$29.39^{a} \pm 1.95$	$28.92^{a} \pm 1.68$	$34.38^{b} \pm 0.34$
PUFA	$31.75^{a} \pm 0.67$	$32.75^{a} \pm 2.26$	$33.55^{a} \pm 2.19$	$42.00^{b} \pm 0.38$
n-3	$5.47^{a} \pm 0.19$	$6.52^{a} \pm 1.59$	$6.64^{a} \pm 1.62$	$11.96^{b} \pm 0.16$
n-6	$26.27^{a} \pm 0.82$	$26.23^{a} \pm 0.74$	$26.91^{a} \pm 0.76$	$30.04^{b} \pm 0.26$
n-9	$20.09^{a} \pm 0.65$	$22.73^{a} \pm 2.53$	$22.51^{a} \pm 2.16$	$29.99^{b} \pm 0.32$
n-6/*n*-3	$4.83^{b} \pm 0.30$	$4.52^{b} \pm 0.69$	$4.59^{b} \pm 0.76$	$2.51^{a} \pm 0.03$

Data are expressed as percentage of total fatty acids and represented as mean ± SEM ($n = 4$). Different superscript letters indicate significant differences between groups ($P < 0.05$)

of *n*-3 LC-PUFA biosynthesis in marine ecosystems (Monroig et al. 2022).

Monthly intraperitoneal administration of 3 doses of chitosan-TPP-DNA nanoparticles allowed long-standing high expressional levels of the exogenous proteins in the liver, mild expression in the intestine and barely detectable levels in the skeletal muscle and brain. Biodistribution of fish codon-optimised *fat-1* and *fat-2* expression shows that the particle size of chitosan-TPP-DNA complexes used in the present study favoured liver retention in *S. aurata*, which confirms previous reports where we analysed the acute effect of expressing exogenous SREBP1a and silencing of endogenous cytosolic alanine aminotransferase and glutamate dehydrogenase (González et al. 2016; Gaspar et al. 2018; Silva-Marrero et al. 2019). Possibly, discontinuous endothelia of the intestine enables chitosan-TPP-DNA nanoparticle absorption and transportation to the liver through portal circulation (Hagens et al. 2007), while the tight morphology of

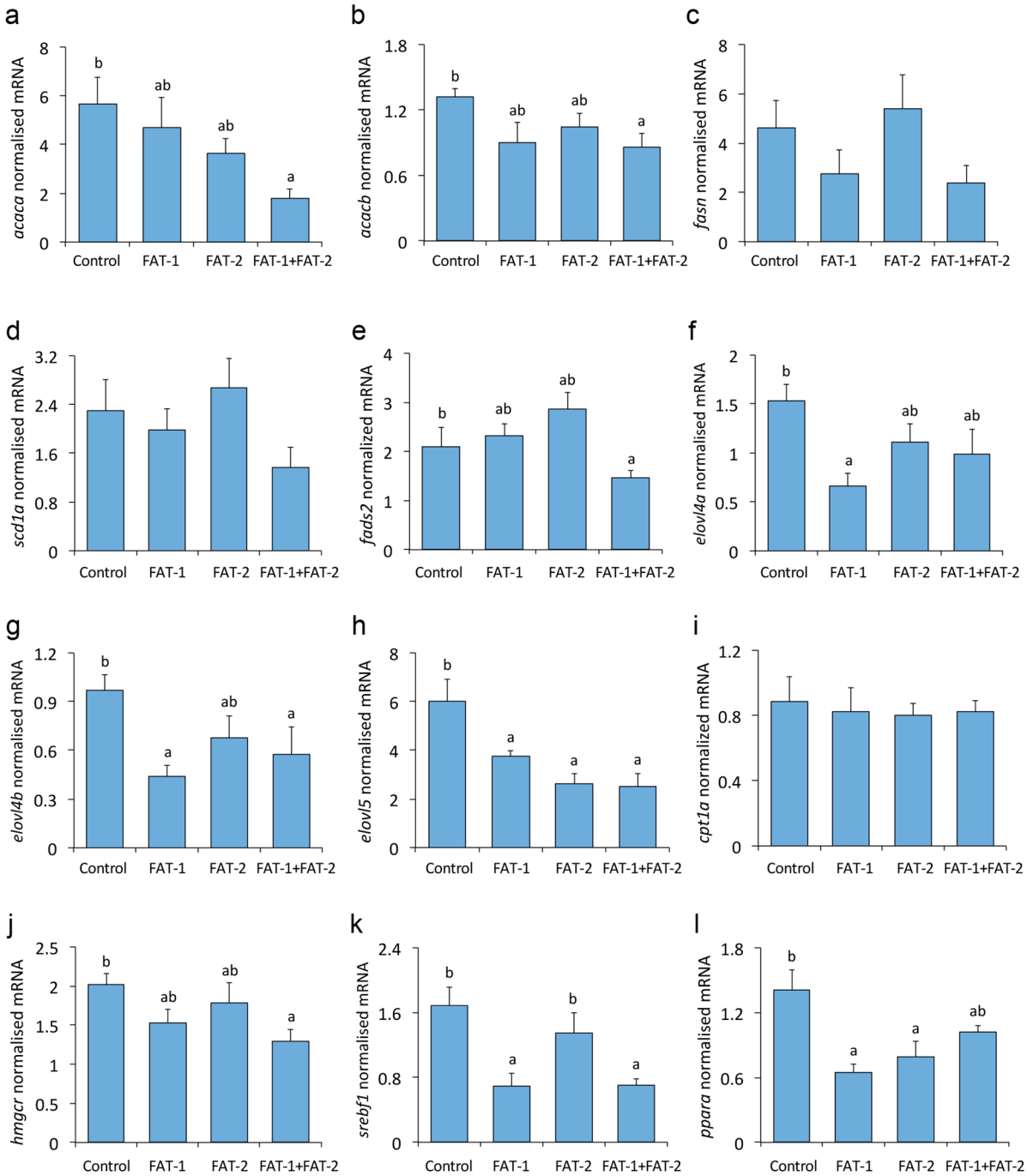

Fig. 3 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the expression of key genes in de novo lipogenesis and fatty acid oxidation in the liver of *S. aurata*. (a-l) Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed and the liver were collected. Data are means ± SEM ($n = 6$). Expression data were normalised by the geometric mean of *S. aurata 18 s*, *actb* and *eef1a* mRNA levels. Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

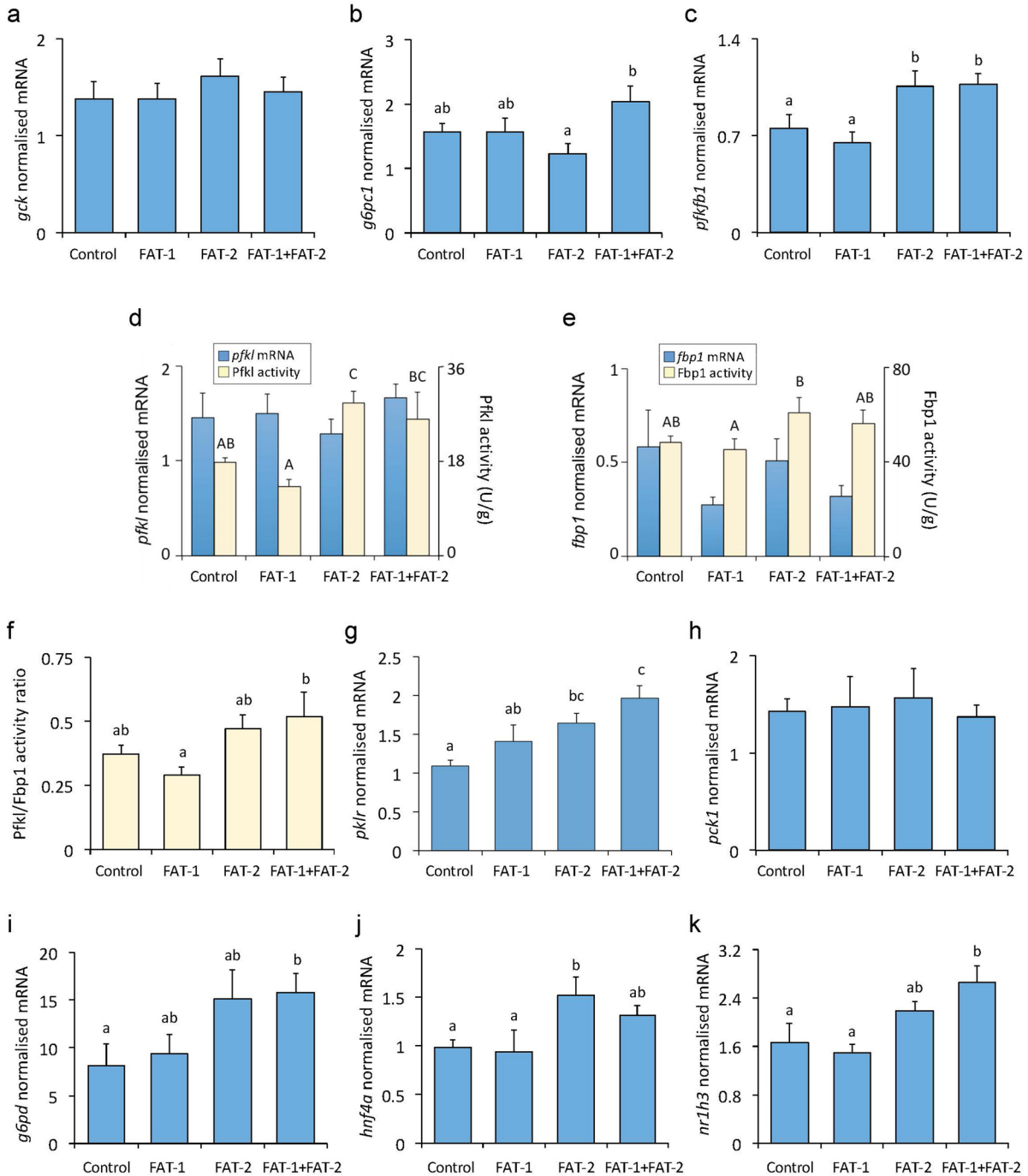

Fig. 4 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the expression of key genes in glycolysis-gluconeogenesis and the pentose phosphate pathway, *hnf4a* and *nr1h3* in the liver of *S. aurata*. (**a**–**k**) Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed and the liver were collected. Hepatic mRNA levels and enzyme activity of Pfkl and Fbp1 are presented as mean ± SEM ($n = 6$). Expression data were normalised by the geometric mean of *S. aurata 18 s*, *actb* and *eef1a* mRNA levels. Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

capillary endothelium in the muscle and brain may limit the transfer of nanoparticles and result in the scarce levels of transcript (Kooij et al. 2005). The feasibility of implementing chitosan-TPP-DNA administration in aquaculture relies on the fact that chitosan is recognised as safe by the U.S. Food and Drug Administration, on one hand, and that a procedure similar to that currently used to administer commercialised DNA vaccines for fish, could be applied to induce endogenous production of *n*-3 LC PUFA, on the other.

Transgenesis of *fat-1*, a gene that facilitates the conversion of *n*-6 to *n*-3 fatty acids, scarcely affected body growth in zebrafish, common carp, mice, pig and lamb (Bhattacharya et al. 2006; Ji et al. 2009; Liu et al. 2016; Zhang et al. 2018, 2019; Sun et al. 2020). However, in the present study long-term hepatic expression of *fat-1* and *fat-1*+*fat-2* significantly increased weight gain but not lipid content in *S. aurata*. A trend to increase SGR was observed in fish co-expressing *fat-1* and *fat*-2, while SGR values in control fish were in agreement with those reported for *Sparus aurata* under similar experimental conditions but non-treated with chitosan nanoparticles (Caballero-Solares et al. 2015; Sáez-Arteaga et al. 2022). Our findings suggest that increased levels of *n*-3 LC-PUFA and decreased *n*-6/*n*-3 fatty acid ratio resulting from expression of fish codon-optimised *fat-1* and *fat-2* may contribute to increased growth performance in *S. aurata*. In support of this hypothesis, substitution of fish oil (rich in *n*-3 LC-PUFA) by vegetable oil enhances the *n*-6/*n*-3 ratio and lowers *n*-3 LC-PUFA and weight gain in *S. aurata* (Houston et al. 2017), as well as in other marine fish such as cobia (Trushenski et al. 2012) and the anadromous Atlantic salmon (Qian et al. 2020). However, dietary fish oil does not significantly affect growth in other fish species such as zebrafish (Meguro and Hasumura 2018), common carp (Ljubojević et al. 2015), red hybrid tilapia (Al-Souti et al. 2012) and rainbow trout (Richard et al. 2006). Different adaptative responses to dietary *n*-3 LC-PUFA and the specific ability for converting *n*-3 and *n*-6 C_{18} PUFA into highly unsaturated long-chain fatty acids may result at least in part from functional diversification of Fads2 activity among teleosts, which in turn may have been influenced by a variety of factors such as phylogeny, trophic level, habitat (marine vs. freshwater) and trophic ecology (Castro et al. 2016; Garrido et al. 2019). Better growth performance of *S. aurata* submitted to sustained expression of fish codon-optimised *fat-1* and *fat-1*+*fat-2* may also result from improved health condition due to increased *n*-3 LC-PUFA and decreased *n*-6/*n*-3 ratio. Consistently, *fat-1* transgenesis prevents liver steatosis and lipid deposition in the abdominal cavity of zebrafish by a mechanism involving hepatic downregulation of lipogenic-related genes and upregulation of steatolysis-related genes (Sun et al. 2020). Moreover, *fat-1* transgenesis prevents glucose intolerance, insulin resistance, non-alcoholic fatty liver disease and allergic airway responses in mice (Bilal et al. 2011; Kim et al. 2012; Romanatto et al. 2014; Boyle et al. 2020), and exerts protective vascular effects on pigs and cattle by reducing inflammatory factors and improving the immune system (Liu et al. 2016, 2017). Accordingly, *S. aurata* treated with FAT-1 and FAT1 + FAT-2 showed decreased HSI levels, which therefore may essentially result from lower lipid deposition in the liver of fish expressing *fat-1*.

Body fatty acid composition is affected by multiple factors, including de novo fatty acid synthesis, physiological requirements and dietary fatty acid profile. Single-gene expression of either *fat-1* or *fat-2* enhanced fatty acid desaturation and, consequently, *n*-3 LC-PUFA synthesis in transgenic mice (Pai et al. 2014), pig (Tang et al. 2019), zebrafish (Pang et al. 2014), and common carp (Zhang et al. 2019). Similarly, *S. aurata* long-term treated with chitosan-TPP-DNA nanoparticles expressing either *fat-1* or *fat-2* showed a general trend to increase liver and muscle EPA, DHA, and total *n*-3 fatty acids and PUFA, while decreased the *n*-6/*n*-3 ratio and saturated fatty acids, conceivably by conversion into unsaturated fatty acids. Most of these effects were potentiated by hepatic co-expression of *fat-1* and *fat-2*. Combined activities of FAT-1 and FAT-2 decreased saturated fatty acids such as 14:0, 16:0 and 17:0, while increased unsaturated fatty acids, particularly *n*-3, and to a lesser extend *n*-9 and *n*-6. Thus, co-expression of *fat-1* and *fat-2* promoted a synergistic effect that favoured liver production of *n*-3 LC-PUFA and its accumulation in the muscle, particularly EPA and DHA. Given that *fat-1* and *fat-2* mRNA levels were scarcely detected in the skeletal muscle of *S. aurata*, changes in the muscle fatty acid profile between treatments may ascribe to hepatic fat exportation forming part of very low density lipoproteins (VLDL).

In contrast to most animals where *fat-1* transgenesis generally results in a significant decrease of the *n*-6 fatty acid series (Kang et al. 2004; Lai et al. 2006; Liu et al. 2016, 2017; Li et al. 2018; You et al. 2021), the effect of *fat-1* transgenesis in fishes seems species-dependent. Similarly as in *S. aurata*, transgenesis of *fat-1* did not affect total *n*-6 fatty acids in the muscle of channel catfish (Xing et al. 2023), while a slight decrease was observed in zebrafish muscle (Pang et al. 2014). In contrast, *fat-1* transgenesis largely decreased total *n*-6 PUFAs in common carp muscle (Zhang et al. 2019). The effect of *fat-2* transgenesis in the fatty acid profile also depended on the fish species. Thus, *fat-2* transgenesis in channel catfish decreased palmitic acid (16:0) and, as in *S. aurata*, led to a not significant trend to increase total *n*-6 and *n*-3 series in the muscle (Xing et al. 2023). However, *fat-2* increased total *n*-6 PUFAs while did not affect the *n*-3 series in zebrafish muscle (Pang et al. 2014). Divergent effects of *C. elegans fat-1* and *fat-2* among fish species may be attributed to the interaction between exogenous enzymes and the diversity of endogenous desaturases and elongases that are distributed within different teleost taxonomic groups, and which in turn are responsible for different specific abilities for converting *n*-3 and *n*-6 C_{18} PUFA into highly unsaturated LC-PUFAs in teleosts (Monroig et al. 2022).

Among biochemical parameters improved by *fat-1* transgenesis, reduced circulating levels for triglycerides and cholesterol were reported in mice (Romanatto et al. 2014), pigs (Liu et al. 2016), and cattle (Liu et al. 2017), while decreased hepatic triglycerides and cholesterol ester were found in zebrafish (Sun et al. 2020). Similarly, hepatic co-expression of *fat-1* and *fat-2* reduced serum triglycerides and cholesterol in *S. aurata*. In this regard, the present study showed that long-term co-expression of *fat-1* and *fat-2* promoted a general decrease of the expression of key enzymes for de novo lipogenesis in the liver of *S. aurata*. In addition, decreased serum triglycerides may also be attributed in part to the increase of *n*-3 LC-PUFA in the liver, where *n*-3 fatty acids are generally thought to reduce the production of VLDL and induce fatty acid β-oxidation (Shearer et al. 2012).

Treatment with FAT-1 + FAT-2 significantly downregulated *hmgcr*, which encodes the rate-limiting enzyme in cholesterol synthesis, and key genes in fatty acid synthesis, such as *acaca* and *acacb*, which catalyse conversion of acetyl-CoA into malonyl-CoA in the cytosol and mitochondrion, respectively, fatty acid elongases (*elovl4b* and *elovl5*) and *fads2* desaturase. Although no significant, the expression of *scd1a*, which catalyses the insertion of a *cis* double bond at the Δ9 position into saturated C_{16} and C_{18} fatty acyl-CoA (Wang et al. 2005), also showed a trend to decrease in fish treated with nanoparticles expressing *fat-1* + *fat-2*. Furthermore, the expression of the three fatty acid elongases herein analysed (*elovl4a*, *elovl4b* and *elovl5*) strongly decreased by sustained expression of *fat-1*. In transgenic animals, the effect of FAT-1 seems to depend on a variety of factors including the species, environmental conditions and dietary lipid content. Similarly as in *S. aurata*, a high-fat diet (13.4% of crude lipid *versus* 18.0% used in the present study) downregulated the hepatic expression of *acaca*, *fasn* and *scd1* in *fat-1* transgenic zebrafish. However, a low-fat diet (3.1% of crude lipid) caused the opposite effects, upregulating the expression of the three genes (Sun et al. 2020). In line with our findings, *fat-1* transgenesis in mice decreased the levels of phosphorylated ACACA and FASN (Romanatto et al. 2014). However, *fat-1* transgenic common carp showed upregulation of *fads2*, *elovl5* and *elovl2* in the liver, and transgenic pigs co-expressing *fat-1* and *fat-2* also presented increased expression levels of *elovl5* and *elovl2* in the muscle, skin and fat (Zhang et al. 2019; Tang et al. 2019).

Downregulation of *acacb* in *S. aurata* treated with FAT-1 + FAT-2 suggests a limited synthesis rate of mitochondrial malonyl-CoA. Any of the treatments affected the mRNA abundance of *cpt1a*, which is essential for the mitochondrial uptake of long-chain fatty acids and their subsequent β-oxidation in the mitochondrion. However, given that malonyl-CoA is a potent allosteric inhibitor of CPT1A (Saggerson 2008), our data suggest that in addition to decrease de novo lipogenesis, sustained co-expression of *fat-1* and *fat-2* may increase fatty acid oxidation in the liver of *S. aurata* fed medium- or high-fat diets. Similar results were reported for zebrafish fed a high-fat diet, where *fat-1* transgenesis stimulated lipolysis-related genes and mitochondrial energy metabolism-related genes while downregulated the hepatic expression of genes related with lipogenesis and lipid deposition (Sun et al. 2020). Accordingly, upregulation of hepatic fatty acid oxidation-related genes by *fat-1* transgenesis was also reported in common carp

(Zhang et al. 2019), mice (Romanatto et al. 2014; Boyle et al. 2020) and goat cells (Fan et al. 2016), as well as in *fat-1* and *fat-2* double transgenic zebrafish (Pang et al. 2014).

In mammals, alternate promoters in the *srebf1* gene generate SREBP1a and SREBP1c, which constitute transcription factors with a major role in de novo lipogenesis activation. SREBP1c primarily transactivates genes required for fatty acid and triglyceride synthesis while SREBP1a is a potent activator of all SREBP-responsive genes, including genes associated with cholesterol synthesis. Consistent with the role of *srebf1* in the transcription of lipogenic genes both in fish and mammals (Carmona-Antoñanzas et al. 2014; Silva-Marrero et al. 2019), downregulation of *srebf1* in the liver of *S. aurata* submitted to long-term expression of fish codon-optimised *fat-1* and *fat-1*+*fat-2* led to a trend to decrease the expression of genes involved in cholesterol synthesis (*hmgcr*) and fatty acid synthesis (*acaca*, *acacb* and *fasn*), desaturation (*scd1a* and *fads2*) and elongation (*elovl4a*, *elovl4b* and *elovl5*). In agreement with our findings, transgenic zebrafish expressing *fat-1* (when feeding a high-fat diet), *fat-2* and *fat-1*+*fat-2* and double transgenic pigs for *fat-1* and *fat-2* also showed downregulated expression levels of *srebf1* (Pang et al. 2014; Tang et al. 2019; Sun et al. 2020). Since DHA suppresses *srebf1* expression and enhances its protein degradation (Jump 2008), increased levels of DHA seem the main responsible for decreased expression of *srebf1* and de novo lipogenic genes in the liver of *S. aurata* expressing *fat-1* and *fat-1*+*fat-2*. Consistently, substitution of fish oil, rich in DHA, by vegetable oil leads to upregulation of *srebf1* and fatty acid synthesis-related genes in *S. aurata* (Ofori-Mensah et al. 2020).

In the present study, FAT-1 and FAT-2 downregulated *ppara* in the liver of *S. aurata*. PPARA is a nuclear receptor activated by a wide range of ligands including fatty acids and fatty acid metabolites, such as eicosanoids. In the mammalian liver, PPARA controls the expression of genes involved in fatty acid uptake, intracellular transport, acyl-CoA formation and fatty acid mitochondrial and peroxisomal oxidation, ketogenesis and lipoprotein metabolism (Bougarne et al. 2018). Supplementation of fish oil to rodents enhances *ppara* expression in the liver (Hein et al. 2010; Kamisako et al. 2012), possibly as a result of increased availability of *n*-3 LC-PUFA, particularly EPA. However, the effect of dietary fish oil on *ppara* expression in fish depends on the species. Similarly as in *S. aurata* expressing *fat-1* and *fat-2*, fish oil was shown to decrease the hepatic mRNA levels of *ppara* in *S. aurata* and juvenile turbot (Peng et al. 2014; Ofori-Mensah et al. 2020), while the opposite effect was reported in large yellow croaker and lean, but not fat, Atlantic salmon (Morais et al. 2011; Du et al. 2017). As pointed out by Peng et al. (2014), fatty acid-derived factors other than EPA-mediated activation may contribute to species-specific regulation of *ppara* expression in fishes.

In spite of limited knowledge of the effect of *C. elegans* FAT-1 and FAT-2 on glucose metabolism, *fat*-1 transgenesis was reported to improve glucose homeostasis by lowering hepatic gluconeogenesis and decreasing blood glucose and insulin in mice (Romanatto et al. 2014). Similarly, reduced plasma glucose was found in *fat*-1 transgenic cattle (Liu et al. 2017). In the present study, we found that long-term expression of fish codon-optimised *C. elegans* FAT-2 and FAT-1+FAT-2 stimulated glycolysis and the expression levels of *hnf4a* and *nr1h3* in the liver of *S. aurata*. By controlling the flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle, *pfkl* and *fbp1* exert critical roles in hepatic glycolysis-gluconeogenesis. Although the mRNA levels of *pfkl* and *fbp1* were not significantly affected by any of the treatments, expression of *fat-2* and *fat-1*+*fat-2* promoted higher levels of Pfkl/Fbp1 activity ratio, possibly as a result of *pfkfb1* upregulation. The bifunctional enzyme *pfkfb1* catalyses the synthesis and degradation of fructose-2,6-bisphosphate, which is a major regulator of glycolysis–gluconeogenesis through allosteric activation of Pfkl and inhibition of Fbp1 (Okar et al. 2004). We previously showed that refeeding and high carbohydrate diets upregulate *pfkfb1* and the kinase activity of the bifunctional enzyme in the liver of *S. aurata*, leading to a concomitant increase in fructose-2,6-bisphosphate levels (Metón et al. 1999a, 2000). As in mammals, fructose-2,6-bisphosphate is an allosteric activator of *S. aurata* PFKL (Mediavilla et al. 2008). Therefore, our results suggest that *pfkfb1* upregulation in the liver of fish expressing *fat-2* and *fat-1*+*fat-2* may be a key step favouring the glycolytic flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle, which in turn will increase the hepatic content

of fructose-1,6-bisphosphate, an allosteric activator of Pklr.

The nuclear receptor HNF4A is a master regulator of liver metabolism through transcriptional regulation of target genes involved in glucose metabolism, lipid metabolism and hepatocyte differentiation (Meng et al. 2016). In mammals, HNF4A transactivates both glycolytic and gluconeogenic genes. Thus, HNF4A-binding to the gene promoter is required for insulin-stimulated upregulation of *gck* and *pklr* in the fed state, while a synergistic action of HNF4A and FOXO1 enhances the transcription of *g6pc1* and *pck1* during fasting (Hirota et al. 2008; Ganjam et al. 2009). Furthermore, HNF4A was previously shown to induce the expression of *nr1h3* (Theofilatos et al. 2016), which encodes LXR-alpha, a nuclear receptor stimulated by insulin that is also involved in glucose and lipid metabolism (Zhao et al. 2012). Indeed, LXR-alpha was shown to upregulate *pklr* mRNA levels in mice (Cha and Repa 2007), and behave as a key regulator of *pfkfb* expression in humans by binding and transactivating the gene promoter of the bifunctional enzyme (Zhao et al. 2012). Therefore, increased *hnf4a* and *nr1h3* mRNA abundance in *S. aurata* expressing *fat-2* and *fat-1*+*fat-2* may enhance hepatic upregulation of *pfkfb1* and *pklr* expression, and thus increase the glycolytic flux in the liver. Consistent with HNF4A-dependent enhancement of glycolysis in *S. aurata*, *hnf4a* expression was previously shown to increase in *S. aurata* under glycolytic conditions *versus* gluconeogenic conditions such as fasting and treatment with streptozotocin (Salgado et al. 2012). Increased levels of *n*-3 LC-PUFA may be a key factor leading to *hnf4a* and *nr1h3* upregulation in the *S. aurata* liver. In agreement, *fat-1* transgenic mice presented increased hepatic mRNA levels of *hnf4a* and to a lesser extend *nr1h3* (Kim et al. 2012). Similarly, dietary supplementation with dried marine algae, rich in *n*-3 LC-PUFA (particularly DHA), induced *hnf4a* expression in the pig liver (Meadus et al. 2011). Furthermore, fish oil upregulated *nr1h3* in *S. aurata* adipocytes (Cruz-Garcia et al. 2011), and in the liver of juvenile turbot, Nile tilapia and mice (Kamisako et al. 2012; Peng et al. 2014; Ayisi et al. 2018).

To our knowledge, the effect of *fat-1* and *fat-2* transgenesis on the pentose phosphate pathway was not previously addressed. In the present study, long-term expression of fish codon-optimised *fat-2* and *fat-1*+*fat-2* promoted higher expression levels of *g6pd*, which encodes the rate-limiting enzyme for the production of NADPH in the oxidative phase of the pentose phosphate pathway. Previous reports indicated that dietary carbohydrates are a key factor that enhances G6pd activity in the liver of *S. aurata* (Metón et al. 1999b). Nevertheless, our findings support that fatty acid composition, particularly the *n*-3/*n*-6 ratio, also seems to regulate the hepatic expression of *g6pd*. In agreement with *g6pd* upregulation by *n*-3 LC-PUFA, fish oil stimulated G6PD activity in the rat liver (Yilmaz et al. 2004), and dietary supplementation with *n*-3 PUFA increased *g6pd* mRNA levels in the pig muscle (Vitali et al. 2018). Furthermore, *n*-6 PUFA, particularly LA, decreased *g6pd* mRNA levels in rat hepatocytes (Kohan et al. 2011). Species-specific regulation of *g6pd* expression by fatty acid composition may occur in other fishes. In this regard, total replacement of fish oil by vegetable oil did not affect G6pd activity but increased the mRNA levels in the liver of Nile tilapia (Ayisi et al. 2018), while enhanced G6pd activity in the liver of Atlantic salmon (Menoyo et al. 2005). Bearing in mind a general trend to downregulate de novo hepatic lipogenesis in *S. aurata* co-expressing *fat-1* and *fat-2*, NADPH resulting from *g6pd* upregulation by *n*-3 LC-PUFA may reinforce cellular protection from oxidative stress.

Considered together, the metabolic effects of periodical administration of chitosan-TPP-DNA nanoparticles expressing fish codon-optimised *C. elegans fat-1* and *fat-2* included hepatic downregulation of de novo lipogenesis-related genes, leading to reduced circulating levels of triglycerides and cholesterol, stimulation of fatty acid oxidation and upregulation of glucose oxidation via glycolysis and pentose phosphate pathway without affecting glycemia. Although co-expression of *C. elegans fat-1* and *fat-2* in *S. aurata* reduced blood triglycerides, the action of *fat-1* and *fat-2* may allow protein sparing in the liver of a carnivorous teleost, such as *S. aurata*, by a mechanism involving increased glucose and fatty acid oxidation to obtain energy in fish fed medium- or high-fat diets.

Conclusion

The present study shows that long-term treatment with chitosan-TPP nanoparticles complexed with plasmids expressing fish codon-optimised *C. elegans fat-1* and *fat-2* allowed efficient expression of exogenous FAT-1 and FAT-2 desaturases in the liver of *S. aurata*, which in turn elevated the *n*-3 LC-PUFA content, particularly EPA and DHA, and decreased the *n*-6/*n*-3 ratio both in the liver and the skeletal muscle. Co-expression of fish codon-optimised *fat-1* and *fat-2* promoted the highest weight gain, *n*-3 LC-PUFA accumulation in the muscle, and had metabolic effects that included downregulation of lipid biosynthesis and stimulation of fatty acid and glucose oxidation in the liver. Expression of fish codon-optimised *fat-1* and *fat-1*+*fat-2* downregulated the hepatic expression of *srebf1* and as a consequence, the mRNA levels of key genes in de novo lipogenesis, while *fat-2* and *fat-1*+*fat-2* upregulated *hnf4a*, *nr1h3* and glucose oxidation through glycolysis and the pentose phosphate pathway. Our findings support that chitosan-TPP-DNA nanoparticles co-expressing fish codon-optimised *fat-1* and *fat-2* can alleviate the effect of fish oil replacement with vegetable oil currently occurring in aquafeeds and enable production of functional fish rich in EPA and DHA for human consumption. Future studies will help to determine dosage and the optimal developmental stage to administer *fat-1* and *fat-2* chitosan-TPP-DNA nanoparticles in cultured fish.

Availability of data

Data are available from the corresponding author upon reasonable request.

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Author contribution Conceptualization: IM; methodology: AF, MPA, IM; formal analysis and investigation: YW, AR, MPA, IM; writing—original draft preparation: YW, IM; writing—review and editing: YW, AR, AF, MPA, IM; funding acquisition: IM; supervision: IM.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Experimental procedures involving fish were performed in accordance with the guidelines of the University of Barcelona's Animal Welfare Committee (proceeding #10811, Generalitat de Catalunya), in compliance with local legislation and EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

References

Alimuddin YG, Kiron V et al (2007) Expression of masu salmon Δ5-desaturase-like gene elevated EPA and DHA biosynthesis in zebrafish. Mar Biotechnol 9:92–100. https://doi.org/10.1007/s10126-006-6003-y

Alimuddin KV, Satoh S et al (2008) Cloning and over-expression of a masu salmon (*Oncorhynchus masou*) fatty acid elongase-like gene in zebrafish. Aquaculture 282:13–18. https://doi.org/10.1016/j.aquaculture.2008.06.033

Al-Souti A, Al-Sabahi J, Soussi B, Goddard S (2012) The effects of fish oil-enriched diets on growth, feed conversion and fatty acid content of red hybrid tilapia, *Oreochromis* sp. Food Chem 133:723–727. https://doi.org/10.1016/j.foodchem.2012.01.080

Ayisi CL, Zhao JL, Hua XM, Apraku A (2018) Replacing fish oil with palm oil: effects on mRNA expression of fatty acid transport genes and signalling factors related to lipid metabolism in Nile tilapia (*Oreochromis niloticus*). Aquac Nutr 24:1822–1833. https://doi.org/10.1111/anu.12821

Betancor MB, Sprague M, Montero D et al (2016) Replacement of marine fish oil with de novo omega-3 oils from

transgenic *Camelina sativa* in feeds for gilthead sea bream (*Sparus aurata* L.). Lipids 51:1171–1191. https://doi.org/10.1007/s11745-016-4191-4

Bhattacharya A, Chandrasekar B, Rahman MM et al (2006) Inhibition of inflammatory response in transgenic fat-1 mice on a calorie-restricted diet. Biochem Biophys Res Commun 349:925–930. https://doi.org/10.1016/j.bbrc.2006.08.093

Bilal S, Haworth O, Wu L et al (2011) Fat-1 transgenic mice with elevated omega-3 fatty acids are protected from allergic airway responses. Biochim Biophys Acta Mol Basis Dis 1812:1164–1169. https://doi.org/10.1016/j.bbadis.2011.05.002

Bougarne N, Weyers B, Desmet SJ et al (2018) Molecular actions of PPARα in lipid metabolism and inflammation. Endocr Rev 39:760–802. https://doi.org/10.1210/er.2018-00064

Boyle KE, Magill-Collins MJ, Newsom SA et al (2020) Maternal fat-1 transgene protects offspring from excess weight gain, oxidative stress, and reduced fatty acid oxidation in response to high-fat diet. Nutrients 12:767. https://doi.org/10.3390/nu12030767

Busacker GP, Adelman IR, Goolish EM (1990) Growth. In: Schreck CB, Moyle PB (eds) Methods for fish biology. American Fisheries Society, Bethesda, pp 363–387

Caballero-Solares A, Viegas I, Salgado MC et al (2015) Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 444:79–87. https://doi.org/10.1016/j.aquaculture.2015.03.025

Calder PC (2018) Very long-chain n-3 fatty acids and human health: fact, fiction and the future. Proc Nutr Soc 77:52–72. https://doi.org/10.1017/S0029665117003950

Carmona-Antoñanzas G, Tocher DR, Martinez-Rubio L, Leaver MJ (2014) Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. Gene 534:1–9. https://doi.org/10.1016/j.gene.2013.10.040

Carvalho M, Marotta B, Xu H et al (2022) Complete replacement of fish oil by three microalgal products rich in n-3 long-chain polyunsaturated fatty acids in early weaning microdiets for gilthead sea bream (*Sparus aurata*). Aquaculture 558:738354. https://doi.org/10.1016/j.aquaculture.2022.738354

Castro LFC, Tocher DR, Monroig O (2016) Long-chain polyunsaturated fatty acid biosynthesis in chordates: insights into the evolution of Fads and Elovl gene repertoire. Prog Lipid Res 62:25–40. https://doi.org/10.1016/j.plipres.2016.01.001

Cha JY, Repa JJ (2007) The liver X receptor (LXR) and hepatic lipogenesis. the carbohydrate-response element-binding protein is a target gene of LXR. J Biol Chem 282:743–751. https://doi.org/10.1074/jbc.m605023200

Chen Y, Mei M, Zhang P et al (2013) The generation of transgenic mice with fat1 and fad2 genes that have their own polyunsaturated fatty acid biosynthetic pathway. Cell Physiol Biochem 32:523–532. https://doi.org/10.1159/000354456

Cheng C-L, Huang S-J, Wu C-L et al (2015) Transgenic expression of omega-3 PUFA synthesis genes improves zebrafish survival during *Vibrio vulnificus* infection. J Biomed Sci 22:103. https://doi.org/10.1186/s12929-015-0208-1

Cruz-Garcia L, Sánchez-Gurmaches J, Bouraoui L et al (2011) Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata* L.). Comp Biochem Physiol Part A Mol Integr Physiol 158:391–399. https://doi.org/10.1016/j.cbpa.2010.11.024

Djuricic I, Calder PC (2021) Beneficial outcomes of omega-6 and omega-3 polyunsaturated fatty acids on human health: an update for 2021. Nutrients 13:2421. https://doi.org/10.3390/nu13072421

Du J, Xu H, Li S et al (2017) Effects of dietary chenodeoxycholic acid on growth performance, body composition and related gene expression in large yellow croaker (*Larimichthys crocea*) fed diets with high replacement of fish oil with soybean oil. Aquaculture 479:584–590. https://doi.org/10.1016/j.aquaculture.2017.06.023

Fan Y, Ren C, Wang Z et al (2016) Transgenesis of humanized fat1 promotes n−3 polyunsaturated fatty acid synthesis and expression of genes involved in lipid metabolism in goat cells. Gene 576:249–255. https://doi.org/10.1016/j.gene.2015.10.013

Ganjam GK, Dimova EY, Unterman TG, Kietzmann T (2009) FoxO1 and HNF-4 are involved in regulation of hepatic glucokinase gene expression by resveratrol. J Biol Chem 284:30783–30797. https://doi.org/10.1074/jbc.m109.045260

Garrido D, Kabeya N, Betancor MB et al (2019) (2019) Functional diversification of teleost Fads2 fatty acyl desaturases occurs independently of the trophic level. Sci Rep 91(9):1–10. https://doi.org/10.1038/s41598-019-47709-0

Gaspar C, Silva-Marrero JI, Fàbregas A et al (2018) Administration of chitosan-tripolyphosphate-DNA nanoparticles to knockdown glutamate dehydrogenase expression impairs transdeamination and gluconeogenesis in the liver. J Biotechnol 286:5–13. https://doi.org/10.1016/j.jbiotec.2018.09.002

González JD, Silva-Marrero JI, Metón I et al (2016) Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism. Mar Biotechnol 18:85–97. https://doi.org/10.1007/s10126-015-9670-8

Hagens WI, Oomen AG, de Jong WH et al (2007) What do we (need to) know about the kinetic properties of nanoparticles in the body? Regul Toxicol Pharmacol 49:217–229. https://doi.org/10.1016/j.yrtph.2007.07.006

Hein GJ, Bernasconi AM, Montanaro MA et al (2010) Nuclear receptors and hepatic lipidogenic enzyme response to a dyslipidemic sucrose-rich diet and its reversal by fish oil n-3 polyunsaturated fatty acids. Am J Physiol Endocrinol Metab 298:E429–E439. https://doi.org/10.1152/ajpendo.00513.2009

Hirota K, Sakamaki JI, Ishida J et al (2008) A combination of HNF-4 and Foxo1 is required for reciprocal transcriptional regulation of glucokinase and glucose-6-phosphatase genes in response to fasting and feeding. J Biol Chem 283:32432–32441. https://doi.org/10.1074/jbc.m806179200

Houston SJS, Karalazos V, Tinsley J et al (2017) The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. Br J Nutr 118:1010–1022. https://doi.org/10.1017/s0007114517002975

Ji S, Hardy RW, Wood PA (2009) Transgenic expression of n-3 fatty acid desaturase (fat-1) in C57/BL6 mice: effects on glucose homeostasis and body weight. J Cell Biochem 107:809–817. https://doi.org/10.1002/jcb.22179

Jump DB (2008) N-3 polyunsaturated fatty acid regulation of hepatic gene transcription. Curr Opin Lipidol 19:242–247. https://doi.org/10.1097/mol.0b013e3282ffaf6a

Kabeya N, Fonseca MM, Ferrier DEK et al (2018) Genes for de novo biosynthesis of omega-3 polyunsaturated fatty acids are widespread in animals. Sci Adv. https://doi.org/10.1126/sciadv.aar6849

Kamisako T, Tanaka Y, Ikeda T et al (2012) Dietary fish oil regulates gene expression of cholesterol and bile acid transporters in mice. Hepatol Res 42:321–326. https://doi.org/10.1111/j.1872-034x.2011.00924.x

Kang JX, Wang J, Wu L, Kang ZB (2004) Fat-1 mice convert n-6 to n-3 fatty acids. Nature 427:504–504. https://doi.org/10.1038/427504a

Kim EH, Bae JS, Hahm KB, Cha JY (2012) Endogenously synthesized n-3 polyunsaturated fatty acids in fat-1 mice ameliorate high-fat diet-induced non-alcoholic fatty liver disease. Biochem Pharmacol 84:1359–1365. https://doi.org/10.1016/j.bcp.2012.08.029

Kohan AB, Qing Y, Cyphert HA et al (2011) Chylomicron remnants and nonesterified fatty acids differ in their ability to inhibit genes involved in lipogenesis in rats. J Nutr 141:171–176. https://doi.org/10.3945/jn.110.129106

Kooij G, van Horssen J, de Vries E (2005) Tight junctions of the blood-brain barrier. In: de Vries E, Prat A (eds) The blood-brain barrier and its microenvironment: basic physiology to neurological disease. CRC Press, Boca Raton, pp 69–92

Lai L, Kang JX, Li R et al (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24:435–436. https://doi.org/10.1038/nbt1198

Li M, Ouyang H, Yuan H et al (2018) Site-specific fat-1 knock-in enables significant decrease of n-6PUFAs/n-3PUFAs ratio in pigs. G3 8:1747–1754. https://doi.org/10.1534/g3.118.200114

Liu X, Pang D, Yuan T et al (2016) N-3 polyunsaturated fatty acids attenuates triglyceride and inflammatory factors level in hfat-1 transgenic pigs. Lipids Health Dis 15:89. https://doi.org/10.1186/s12944-016-0259-7

Liu X-F, Wei Z-Y, Bai C-L et al (2017) Insights into the function of n-3 PUFAs in fat-1 transgenic cattle. J Lipid Res 58:1524–1535. https://doi.org/10.1194/jlr.M072983

Ljubojević D, Radosavljević V, Puvača N et al (2015) Interactive effects of dietary protein level and oil source on proximate composition and fatty acid composition in common carp (*Cyprinus carpio* L.). J Food Compos Anal 37:44–50. https://doi.org/10.1016/j.jfca.2014.09.005

Lucas A (1996) Bioenergetics of organisms: methods. In: Priede IG (ed) Bioenergetics of aquatic animals. Taylor & Francis, London, pp 65–81

Luo R, Zheng Z, Yang C et al (2020) Comparative transcriptome analysis provides insights into the polyunsaturated fatty acid synthesis regulation of fat-1 transgenic sheep. Int J Mol Sci 21:1121. https://doi.org/10.3390/ijms21031121

Meadus WJ, Duff P, Rolland D et al (2011) Feeding docosahexaenoic acid to pigs reduces blood triglycerides and induces gene expression for fat oxidation. Can J Anim Sci 91:601–612. https://doi.org/10.4141/cjas2011-055

Mediavilla D, Metón I, Baanante IV (2008) Purification and kinetic characterization of 6-phosphofructo-1-kinase from the liver of gilthead sea bream (*Sparus aurata*). J Biochem 144:235–244. https://doi.org/10.1093/jb/mvn066

Meguro S, Hasumura T (2018) Fish oil suppresses body fat accumulation in zebrafish. Zebrafish 15:27–32. https://doi.org/10.1089/zeb.2017.1475

Meng J, Feng M, Dong W et al (2016) Identification of HNF-4α as a key transcription factor to promote ChREBP expression in response to glucose. Sci Rep 6:23944. https://doi.org/10.1038/srep23944

Menoyo D, López-Bote CJ, Obach A, Bautista JM (2005) Effect of dietary fish oil substitution with linseed oil on the performance, tissue fatty acid profile, metabolism, and oxidative stability of Atlantic salmon. J Anim Sci 83:2853–2862. https://doi.org/10.2527/2005.83122853x

Metón I, Caseras A, Mediavilla D et al (1999a) Molecular cloning of a cDNA encoding 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from liver of *Sparus aurata*: nutritional regulation of enzyme expression. Biochim Biophys Acta 1444:153–165. https://doi.org/10.1016/S0167-4781(98)00270-X

Metón I, Mediavilla D, Caseras A et al (1999b) Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). Br J Nutr 82:223–232. https://doi.org/10.1017/s0007114599001403

Metón I, Caseras A, Fernández F, Baanante IV (2000) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, *Sparus aurata*. Biochim Biophys Acta Gene Struct Expr 1491:220–228. https://doi.org/10.1016/s0167-4781(00)00040-3

Monroig S-C, Kabeya N et al (2022) Desaturases and elongases involved in long-chain polyunsaturated fatty acid biosynthesis in aquatic animals: from genes to functions. Prog Lipid Res 86:101157. https://doi.org/10.1016/j.plipres.2022.101157

Morais S, Pratoomyot J, Taggart JB et al (2011) Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. BMC Genomics 12:1–17. https://doi.org/10.1186/1471-2164-12-255

Ofori-Mensah S, Yıldız M, Arslan M, Eldem V (2020) Fish oil replacement with different vegetable oils in gilthead seabream, *Sparus aurata* diets: effects on fatty acid metabolism based on whole-body fatty acid balance method and genes expression. Aquaculture 529:735609. https://doi.org/10.1016/j.aquaculture.2020.735609

Okar DA, Wu C, Lange AJ (2004) Regulation of the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Adv Enzyme Regul 44:123–154. https://doi.org/10.1016/j.advenzreg.2003.11.006

Osmond ATY, Colombo SM (2019) The future of genetic engineering to provide essential dietary nutrients and improve growth performance in aquaculture: advantages and challenges. J World Aquac Soc 50:490–509. https://doi.org/10.1111/jwas.12595

Pai VJ, Wang B, Li X et al (2014) Transgenic mice convert carbohydrates to essential fatty acids. PLoS ONE 9:e97637. https://doi.org/10.1371/journal.pone.0097637

Pang S-C, Wang H-P, Li K-Y et al (2014) Double transgenesis of humanized fat1 and fat2 genes promotes omega-3 polyunsaturated fatty acids synthesis in a zebrafish model. Mar Biotechnol 16:580–593. https://doi.org/10.1007/s10126-014-9577-9

Peng M, Xu W, Mai K et al (2014) Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. Aquaculture 433:442–449. https://doi.org/10.1016/j.aquaculture.2014.07.005

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. https://doi.org/10.1093/nar/29.9.e45

Qian C, Hart B, Colombo SM (2020) Re-evaluating the dietary requirement of EPA and DHA for Atlantic salmon in freshwater. Aquaculture 518:734870. https://doi.org/10.1016/j.aquaculture.2019.734870

Richard N, Kaushik S, Larroquet L et al (2006) Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). Br J Nutr 96:299–309. https://doi.org/10.1079/bjn20061821

Romanatto T, Fiamoncini J, Wang B et al (2014) Elevated tissue omega-3 fatty acid status prevents age-related glucose intolerance in fat-1 transgenic mice. Biochim Biophys Acta 1842:186–191. https://doi.org/10.1016/j.bbadis.2013.10.017

Sáez-Arteaga A, Wu Y, Silva-Marrero JI et al (2022) Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (*Sparus aurata*). Aquaculture 555:738221. https://doi.org/10.1016/j.aquaculture.2022.738221

Saggerson D (2008) Malonyl-CoA, a key signaling molecule in mammalian cells. Annu Rev Nutr 28:253–272. https://doi.org/10.1146/annurev.nutr.28.061807.155434

Sales R, Galafat A, Vizcaíno AJ et al (2021) Effects of dietary use of two lipid extracts from the microalga *Nannochloropsis gaditana* (Lubián, 1982) alone and in combination on growth and muscle composition in juvenile gilthead seabream, *Sparus aurata*. Algal Res 53:102162. https://doi.org/10.1016/j.algal.2020.102162

Salgado MC, Metón I, Anemaet IG et al (2012) Hepatocyte nuclear factor 4alpha transactivates the mitochondrial alanine aminotransferase gene in the kidney of *Sparus aurata*. Mar Biotechnol 14:46–62. https://doi.org/10.1007/s10126-011-9386-3

Shearer GC, Savinova OV, Harris WS (2012) Fish oil—How does it reduce plasma triglycerides? Biochim Biophys Acta 1821:843–851. https://doi.org/10.1016/j.bbalip.2011.10.011

Silva-Marrero JI, Sáez A, Caballero-Solares A et al (2017) A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). BMC Genomics 18:768. https://doi.org/10.1186/s12864-017-4148-x

Silva-Marrero JI, Villasante J, Rashidpour A et al (2019) The administration of chitosan-tripolyphosphate-DNA nanoparticles to express exogenous SREBP1a enhances conversion of dietary carbohydrates into lipids in the liver of *Sparus aurata*. Biomolecules 9:297. https://doi.org/10.3390/biom9080297

Sun S, Castro F, Monroig Ó et al (2020) fat-1 transgenic zebrafish are protected from abnormal lipid deposition induced by high-vegetable oil feeding. Appl Microbiol Biotechnol 104:7355–7365. https://doi.org/10.1007/s00253-020-10774-x

Tang F, Yang X, Liu D et al (2019) Co-expression of fat1 and fat2 in transgenic pigs promotes synthesis of polyunsaturated fatty acids. Transgenic Res 28:369–379. https://doi.org/10.1007/s11248-019-00127-4

Theofilatos D, Anestis A, Hashimoto K, Kardassis D (2016) Transcriptional regulation of the human liver X receptor α gene by hepatocyte nuclear factor 4α. Biochem Biophys Res Commun 469:573–579. https://doi.org/10.1016/j.bbrc.2015.12.031

Tocher DR, Betancor MB, Sprague M et al (2019) Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: bridging the gap between supply and demand. Nutrients 11:89. https://doi.org/10.3390/nu11010089

Trushenski J, Schwarz M, Bergman A et al (2012) DHA is essential, EPA appears largely expendable, in meeting the n-3 long-chain polyunsaturated fatty acid requirements of juvenile cobia *Rachycentron canadum*. Aquaculture 326–329:81–89. https://doi.org/10.1016/j.aquaculture.2011.11.033

Vitali M, Dimauro C, Sirri R et al (2018) Effect of dietary polyunsaturated fatty acid and antioxidant supplementation on the transcriptional level of genes involved in lipid and energy metabolism in swine. PLoS ONE 13:e0204869. https://doi.org/10.1371/journal.pone.0204869

Wang J, Yu L, Schmidt RE et al (2005) Characterization of HSCD5, a novel human stearoyl-CoA desaturase unique to primates. Biochem Biophys Res Commun 332:735–742. https://doi.org/10.1016/j.bbrc.2005.05.013

Wu Y, Rashidpour A, Almajano MP, Metón I (2020) Chitosan-based drug delivery system: applications in fish biotechnology. Polymers (basel) 12:1177. https://doi.org/10.3390/polym12051177

Xing D, Shang M, Li S et al (2023) CRISPR/Cas9-mediated precision integration of fat-1 and fat-2 from *Caenorhabditis elegans* at long repeated sequence in channel catfish (*Ictalurus punctatus*) and the impact on n-3 fatty acid level. Aquaculture 567:739229. https://doi.org/10.1016/j.aquaculture.2023.739229

Xu H, Meng X, Wei Y et al (2022) Arachidonic acid matters. Rev Aquac 14:1912–1944. https://doi.org/10.1111/raq.12679

Yilmaz HR, Songur A, Özyurt B et al (2004) The effects of n-3 polyunsaturated fatty acids by gavage on some metabolic enzymes of rat liver. Prostaglandins Leukot Essent Fat Acids 71:131–135. https://doi.org/10.1016/j.plefa.2004.03.002

You W, Li M, Qi Y et al (2021) CRISPR/Cas9-mediated specific integration of *Fat-1* and IGF-1 at the *pRosa26* locus. Genes 12:1027. https://doi.org/10.3390/genes12071027

Zhang J, Cui ML, Nie YW et al (2018) CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus. FEBS J 285:2828–2839. https://doi.org/10.1111/febs.14520

Zhang X, Pang S, Liu C et al (2019) A novel dietary source of EPA and DHA: metabolic engineering of an important freshwater species-common carp by fat1-transgenesis. Mar Biotechnol 21:171–185. https://doi.org/10.1007/s10126-018-9868-7

Zhao LF, Iwasaki Y, Nishiyama M et al (2012) Liver X receptor α is involved in the transcriptional regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene. Diabetes 61:1062–1071. https://doi.org/10.2337/db11-1255