

Parental nutritional programming and a reminder during juvenile stage affect growth, lipid metabolism and utilisation in later developmental stages of a marine teleost, the gilthead sea bream (*Sparus aurata*)

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(Submitted 1 May 2017 – Final revision received 9 August 2017 – Accepted 11 August 2017)

Abstract

Nutrition during periconception and early development can modulate metabolic routes to prepare the offspring for adverse conditions through a process known as nutritional programming. In gilthead sea bream, replacement of fish oil (FO) with linseed oil (LO) in broodstock diets improves growth in the 4-month-old offspring challenged with low-FO and low-fishmeal (FM) diets for 1 month. The present study further investigated the effects of broodstock feeding on the same offspring when they were 16 months old and were challenged for a second time with the low-FM and low-FO diet for 2 months. The results showed that replacement of parental moderate-FO feeding with LO, combined with juvenile feeding at 4 months old with low-FM and low-FO diets, significantly ($P < 0.05$) improved offspring growth and feed utilisation of low-FM/FO diets even when they were 16 months old: that is, when they were on the verge of their first reproductive season. Liver fatty acid composition was significantly affected by broodstock or reminder diets as well as by their interaction. Moreover, the reduction of long-chain PUFA and increase in α -linolenic acid and linoleic acid in broodstock diets lead to a significant down-regulation of hepatic lipoprotein lipase ($P < 0.001$) and elongation of very long-chain fatty acids protein 6 ($P < 0.01$). Besides, fatty acid desaturase 2 values were positively correlated to hepatic levels of 18:4n-3, 18:3n-6, 20:5n-3, 22:6n-3 and 22:5n-6. Thus, this study demonstrated the long-term nutritional programming of gilthead sea bream through broodstock feeding, the effect of feeding a 'reminder' diet during juvenile stages to improve utilisation of low-FM/FO diets and fish growth as well as the regulation of gene expression along the fish's life-cycle.

Key words: Nutritional adaptation of offspring: Long-term effects of parental nutrition: Hepatic gene expression: Fatty acid desaturase: Epigenetics in aquaculture

Aquaculture is the fastest-growing animal production sector accounting, at present, for more than 50% of worldwide fish consumption⁽¹⁾, but one major issue concerning such development is the over-dependence of fish feeds on capture fishery-derived raw materials such as fishmeal (FM) and fish oil (FO)⁽²⁾. The established beneficial roles of FO on human health⁽³⁾ and the use of FO, albeit in small proportions, in other animal production systems, have led to an increase in the demand for this raw material, consequently, raising the prices. Despite great achievements in the reduction of FM in diets of marine fish species^(4–8), complete replacement of FO still remains a major challenge. Moreover, complete substitution of FO negatively affects the immune system and stress and disease resistance⁽⁹⁾ as well as reduces the fillet content of long-chain $n-3$ PUFA ($n-3$ LC-PUFA, includes twenty or more carbon atoms), such as

EPA and DHA, negatively affecting the nutritional value of fish flesh for humans^(10–13).

FO is rich in $n-3$ LC-PUFA, whereas vegetable oil (VO) sources, except in some cases⁽¹⁴⁾, lack the essential fatty acids (EFA) for marine fish such as EPA and DHA but can have significant amounts of α -linolenic acid (ALA), 18:3n-3, and linoleic acid (LA), 18:2n-6, which are biological precursors of EFA. Yet, the bioconversion of 18-carbon PUFA to EPA and DHA depends on the elongation and desaturation capacity of the fish species^(15,16). Generally speaking, whereas freshwater fish possess the ability to convert ALA and LA into LC-PUFA^(17,18), marine fish do not possess the sufficient enzyme activity⁽¹⁹⁾. Nonetheless, LC-PUFA synthesis capacity also appears to differ among marine species^(20–23). The higher LC-PUFA biosynthesis capacity in freshwater fish in comparison with that of marine fish could be

Abbreviations: ALA, α -linolenic acid; f, fish oil-based diet; *elovl6*, elongation of very long-chain fatty acids protein 6; *fads2*, fatty acid desaturase 2; FM, fishmeal; FO, fish oil; HL, 20% fish oil–80% linseed oil; LA, linoleic acid; LL, 40% fish oil–60% linseed oil; LO, linseed oil; lpl, lipoprotein lipase; v, reminder diet; VO, vegetable oil.

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related to differences in the feeding habits and nutrient intake, with marine fish having a continuous access to LC-PUFA-rich sources throughout their lives^(24,25). Besides, these differences among fish species have been related to the diverse evolution of certain genes involved in lipid biosynthesis⁽¹⁶⁾.

Recent evidence suggests that environmental factors experienced by the parents can have long-lasting effects in the offspring or in the later generations^(26,27). Thus, early environmental signs, such as available nutrients during reproduction, can modulate metabolic routes and offspring phenotype^(27–29). This type of metabolic regulation, known as ‘nutritional programming’, has been principally derived from mammalian models, because of their potential effects on development of metabolic disorders in humans in later life⁽³⁰⁾. Therefore, better understanding of the outcomes of parental nutrition and the underlying mechanisms can contribute to the prevention of consequences in the offspring. Besides, nutritional programming may also have potential applications in animal production⁽³¹⁾. In aquaculture, one of the potential beneficial applications of nutritional programming may be the production of individuals better prepared to use some feedstuffs supplying or lacking in specific nutrients, such as VO and plant-protein sources. For instance, specific fat and dietary fatty acid supply during embryonic and offspring development may adjust fish metabolism for better utilisation of 18-carbon fatty acids later in life. Thus, parental nutritional interventions can modulate epigenetic mechanisms that control ‘metabolic decisions’ that are meiotically and mitotically stable through life in humans⁽³²⁾, rodents^(33,34) and cattle⁽³⁵⁾. In fish, nutritional programming studies have mostly focused on early feeding^(36–43), whereas parental nutritional interventions are scarcer^(44–46).

The gilthead sea bream (*Sparus aurata*), is a multi-batch spawner whose oligolecitic eggs largely depend on their continuous intake of nutrients during reproduction⁽⁴⁷⁾. For this reason, egg nutrient content of the gilthead sea bream can be markedly affected by the parental diet even during the spawning season, in turn, affecting early embryonic development⁽⁴⁸⁾. Our previous studies have demonstrated that feeding gilthead sea bream broodstock with high-linseed oil (LO) diets markedly affects fecundity, spawn quality and growth of 45-d-after-hatch larvae and 4-month-old juveniles⁽⁴⁵⁾. Interestingly, when 4-month-old juveniles were challenged with a low-FM and low-FO diet, offspring from parents fed a replacement of 60%-FO with LO showed a faster growth and better feed utilisation than those whose parents had been fed with FO⁽⁴⁵⁾. However, the potential persistence of the effects of broodstock nutritional history on the offspring later in life is still unknown.

Further, little is known on the physiological or molecular mechanisms involved in the effect of parental diets on the metabolic performance of the offspring. When *n*-3 LC-PUFA are limited and the 18-C atom fatty acids are available in gilthead sea bream diet, the gene for fatty acid desaturase 2 (*fads2*), the key-limiting enzyme for LC-PUFA synthesis, is up-regulated⁽⁴⁹⁾. Long-chain fatty acid synthesis also involves chain-elongation catalysed by elongases (Elovl) with different substrate preferences⁽⁵⁰⁾. Among them, Elovl6 is a key lipogenic enzyme that elongates long-chain SFA and MUFA of 12, 14 and 16 carbon

atoms, which has received much attention because of its importance in metabolic disorders⁽⁵¹⁾. Besides, LC-PUFA may have a direct effect on the expression of other genes related to lipid or carbohydrate metabolism⁽⁵²⁾. Lipoprotein lipase (*lpI*) facilitates the tissue uptake of circulating fatty acids⁽⁵³⁾ from lipoproteins and its expression in the liver can be regulated by *n*-3 LC-PUFA⁽⁵⁴⁾. The provision of energy is accomplished by β -oxidation of free fatty acids transported into the mitochondria in the form of fatty acyl-carnitine esters by carnitine acyltransferases, such as carnitine palmitoyltransferases⁽¹⁵⁾. Replacement of FO with VO changes the fatty acid composition of liver and muscle, affecting the β -oxidation capacity and regulating the expression of *cptI* and *cptIII* genes^(17,55–57). β -Oxidation also takes place in the peroxisome and is modulated by PPAR. A total of three different PPAR isoforms (α , β , γ) have been characterised in gilthead sea bream, *ppara* being the major form expressed in the liver⁽⁵⁸⁾. PPAR are nuclear receptors that regulate differentiation, growth and metabolism and, in mammals, epigenetic mechanisms have been described to regulate these processes involving all the PPAR isoforms⁽⁵⁹⁾. For instance, feeding pregnant rats a protein-restricted diet reduces methylation of the *ppara* promoter in the offspring and the hypomethylation persists into adulthood⁽⁶⁰⁾. Finally, another gene potentially regulated by LC-PUFA is cyclo-oxygenase-2 (*cox2*), a key enzyme in prostanoid biosynthesis⁽⁶¹⁾.

The objective of the present study was to explore the potential persistence of nutritional programming through parental feeding in offspring later in life and to analyse the physiological or molecular mechanisms implied. For this purpose, the offspring of gilthead sea bream broodstock fed diets with different FO/LO levels were followed up for 18 months until the beginning of first gonad development and nutritionally challenged at 4 and 16 months with low FM and FO diets. The effects of both broodstock feeding and nutritional challenge on growth, chemical and fatty acid composition of muscle and liver as well as on the expression of selected genes in the liver were investigated.

Methods

Experimental animals

All fish were obtained from spontaneous spawns of gilthead sea bream broodstock fed three diets with three levels of FO substitution with LO: 100% FO, 40% FO–60% LO (LL) and 20% FO–80% LO (HL)⁽⁴⁵⁾. Offspring from all groups were fed the same commercial diet during larval rearing, weaning and during the growing period until they reached 4 months of age (120 d)⁽⁴⁵⁾. At this stage, triplicate groups of juveniles were nutritionally challenged for 1 month with either a high-FM/FO diet (f) or with a high-VM/LO diet (v) named as ‘reminder diet’ in the present study. Details of the broodstock feeding, juvenile nutritional challenge (reminder) at 4 months and feed formulation have been reported earlier⁽⁴⁵⁾. After this first nutritional challenge (reminder), fish were maintained separately in 1000 litre tanks and fed the same commercial diet until they were 16 months old for use in the present study. A schematic view of this nutritional programming history is shown in Fig. 1.



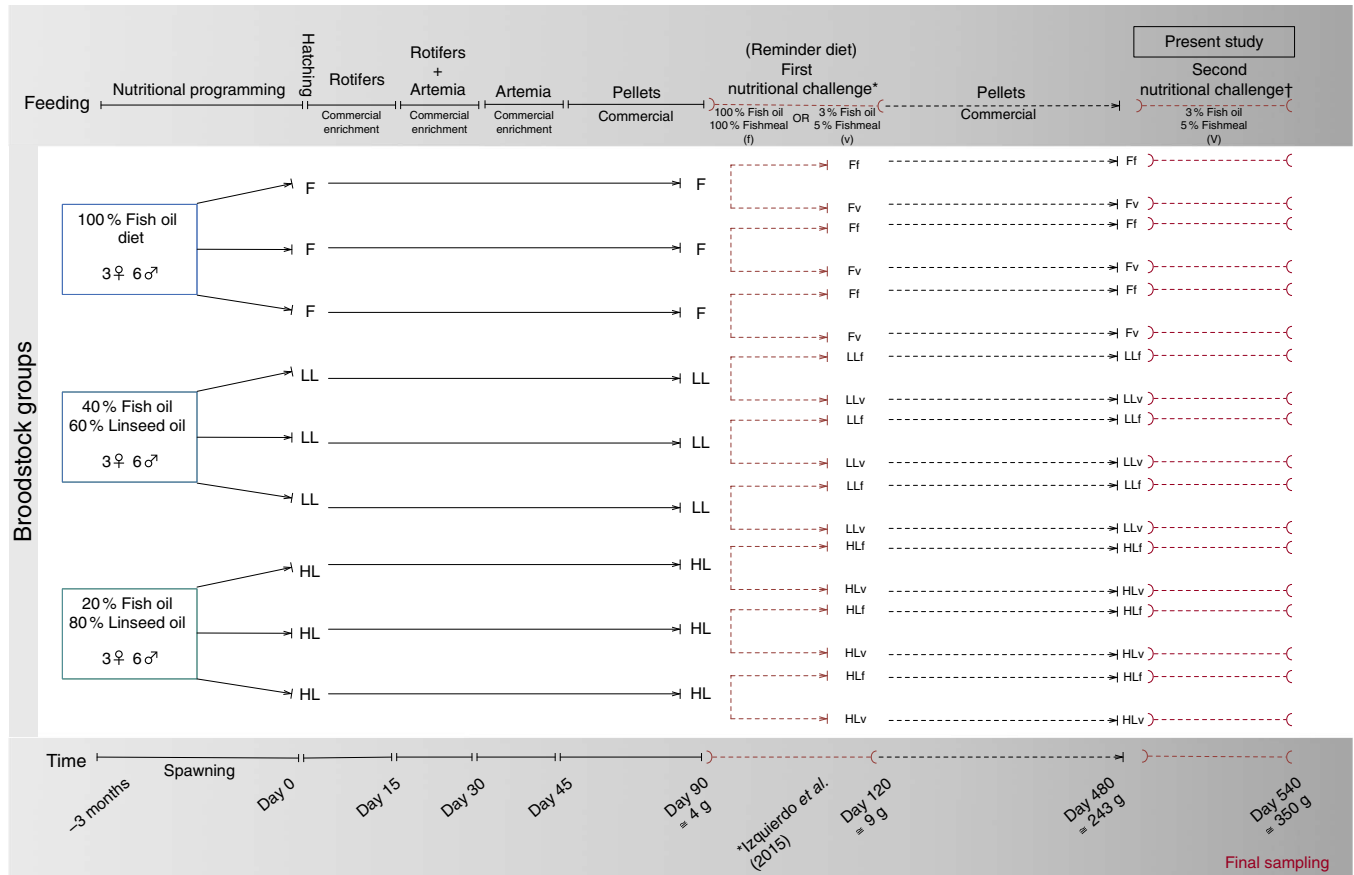


Fig. 1. Schematic view of the nutritional programming history of gilthead sea bream. F, 100% fish oil; LL, 40% fish oil–60% linseed oil; and HL, 20% fish oil–80% linseed oil; f, fish oil-based diet; v, reminder diet. * Previous publication, Izquierdo *et al.* (2015)⁽⁴⁵⁾. † Time of the present study.

In the present study, 16-month-old offspring sea breams of homogeneous weight were selected and distributed into 18 500-litre light-grey fibreglass cylinder tanks (2.8 kg/m³). Each tank contained thirty fish with mean initial body weight of 243.2 (sd 12.7) g. Tanks were supplied with filtered seawater (37 parts per million (ppm) salinity), which entered from the tank surface and drained from the bottom at a rate of 250 litre/h to maintain a high water quality, which was tested daily and no deterioration was observed. O₂ level, water temperature and pH were monitored in real-time using Miranda aquaculture water quality monitoring system (Innovaqua). Water was continuously aerated (125 ml/min), attaining an average of 6.8 (sd 0.8) ppm dissolved O₂ during the experimental period. The average water temperature and pH for the duration of the trial were 24.6 ± 0.6°C and 7.89, respectively. Natural photoperiod was maintained during the whole experimental period (10-h light).

Experimental diet

The experimental diet was formulated and produced by Biomar to be low in FO (3%) and FM (5%). Thus, the diet was high in oleic acid (18:1*n*-9), LA (18:2*n*-6) and ALA (18:3*n*-3) (Table 1). Juveniles from each group were fed daily until apparent satiation for 60 d, three times a day at 09.00, 13.00 and 17.00 hours. The feed was supplied in small portions

(<5–6 pellets at a time) to ensure that all feed was eaten. After each feeding, uneaten feed was collected, kept in aluminium oven trays, dried overnight at 105°C and weighted to calculate feed intake.

Biochemical analyses

Moisture, protein⁽⁶³⁾ and crude lipid⁽⁶⁴⁾ contents of the tissue samples and diets were analysed. Fatty acid methyl esters were obtained by trans-methylation of crude lipids as previously described⁽⁶⁵⁾. Fatty acid methyl esters were separated using GLC (GC-14A; Shimadzu) following the conditions described previously⁽⁶⁶⁾ and identified by comparison with previously characterised standards and GLC-MS (Polaris QTRACE™ Ultra; Thermo Fisher Scientific).

Molecular studies

Liver samples from three fish per each tank (nine per group) were collected at the beginning (480-d-old fish) and at the end of the feeding challenge (540-d-old fish). Samples were collected on ice from fish kept unfed for 24 h, each tissue sample from one individual was assigned to a corresponding 1.5-ml Eppendorf tube and was snap frozen in liquid N₂ immediately after sampling. The samples were then stored at –80°C until RNA extraction and analyses. RNA was extracted

Table 1. Main ingredients*, energy, protein and % total fatty acids contents of diet for the nutritional challenge of gilthead sea bream juveniles obtained from broodstock fed diets 100% fish oil (FO), 40% FO–60% linseed oil (LO) and 20% FO–80% LO during spawning

Main ingredients (%)	%	Proximate composition	(% DM)
Fishmeal SA† 68 super prime	5.00	Crude lipids	21.7
Fishmeal alternative protein sources‡	54.50	Crude protein	45.1
Rapeseed meal cake	11.30	Moisture	9.0
Wheat	6.89	Ash	5.4
Fish oil SA†	3.00		
Vegetable oil mix§	13.00	Gross energy (MJ/kg, as is)	22.5
% Total fatty acids		% Total fatty acids	
14:0	6.6	18:3n-3	11.8
14:1n-5	0.1	18:4n-3	0.4
15:0	0.1	18:4n-1	0.0
16:0iso	0.0	20:0	0.4
16:0	12.3	20:1n-9	0.0
16:1n-7	2.1	20:1n-7	1.0
16:1n-5	0.1	20:1n-5	0.1
16:2n-4	0.2	20:2n-9	0.0
17:0	0.3	20:2n-6	0.1
16:3n-4	0.1	20:3n-9	0.0
16:3n-3	0.0	20:3n-6	0.0
16:3n-1	0.0	20:4n-6	0.2
16:4n-3	0.4	20:3n-3	0.0
18:0	3.2	20:4n-3	0.1
18:1n-9	32.3	20:5n-3	2.5
18:1n-7	2.3	22:1n-11	0.1
18:1n-5	0.0	22:1n-9	0.3
18:2n-9	0.0	22:4n-6	0.0
18:2n-6	20.3	22:5n-6	0.1
18:2n-4	0.1	22:5n-3	0.3
18:3n-6	0.1	22:6n-3	1.7
18:3n-4	0.0		

* Please see Torrecillas *et al.*⁽⁶²⁾ for the complete list of feed ingredients.

† South American, Superprime (Feed Service).

‡ Blood meal spray (Daka), soya protein concentrates 60% (Svane Shipping), maize gluten 60 (Cargill), wheat gluten (Cargill).

§ Linseed (2.6%) (Ch. Daudruy), rapeseed (5.2%) (Emmelev) and palm oils (5.2%) (Cargill).

using Qiagen RNeasy Mini Kit (Qiagen). Before real-time PCR analysis two different potential housekeeping genes, β -actin (β -act) and ribosomal protein L27 (*rpl27*), were tested. Data from duplicate samples (n 18) using the two candidate housekeeping genes were compared using an online program (<http://leonxie.esy.es/Reffinder/?type=reference>) (Reffinder)⁽⁶⁷⁾ and β -act was selected as the most suitable housekeeping gene for the present study (β -act, threshold cycle (C_t) values: min = 18.9, max = 20.6, mean = 19.64, SD = 0.5; *rpl27*: min = 17.0 max = 21.2, mean = 19.1, SD = 1.01).

Real-time quantitative PCR were performed in an iQ5 Multi-color Real-Time PCR detection system (Bio-Rad) using β -act as the housekeeping gene in a final volume of 15 μ l/reaction well and with 100 ng of total RNA reverse-transcribed to complementary DNA (cDNA). Samples, housekeeping gene, cDNA template and reaction blanks were analysed in duplicates (Table 2). Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:100 and 1:1000). A single ninety-six-well PCR plate was used to analyse each gene, primer efficiency and blank samples (Multiplate; Bio-Rad). Melting-curve

analysis was performed and amplification of a single product was confirmed after each run. Fold expression of each gene was determined by delta-delta C_T method ($2^{-\Delta\Delta C_T}$)⁽⁶⁶⁾. PCR efficiencies were similar and no efficiency correction was required^(71,72) (Table 2). Fold expression was related to that of offspring obtained from FO diet-fed broodstock and fed commercial diets throughout their life (Ff group).

Statistical analysis

Data on growth and biochemical composition were statistically analysed using two-way ANOVA, using broodstock diet and reminder diet as fixed factors in IBM SPSS version 23.0.0.2 for Mac (IBM SPSS Inc.). Data were split into groups based on each fixed factor (broodstock and reminder diet) and compared with one-way ANOVA. Scheffe's *post hoc* multiple comparisons for broodstock and reminder diet, separately, assessed differences between groups. Before the analysis of data, equality of variances was tested using Levene's test and distribution of data using Shapiro–Wilk tests. All data except *cox2* gene expression showed normal distribution and equality of variances.

Gene expression data (except for *cox2*) were analysed by means of two-way ANOVA using broodstock and reminder diet as fixed factors. Expression data were next analysed using Welch's ANOVA and subsequently compared with the Games–Howell test for identification of differences between groups. The fixed factor for Welch's ANOVA was experimental groups in gene expression data analysis. Pearson's correlation test was performed using R (R Foundation for Statistical Computing). Gene expression figures were created using lattice package (version 0.20–33)⁽⁷³⁾ downloaded from the comprehensive R Archive Network library. The sample size for all analysed data was nine and data were expressed as means and standard deviations.

Results

Growth performance

The low-FM/FO diet was well accepted and there were no significant differences in feed intake among fish groups (mean, 3.95 (SD 0.39) kg, $P > 0.05$). At the beginning of the trial, there were no significant differences in fish body weight (mean, 243.2 (SD 12.7) g) among experimental groups ($P > 0.05$) (Table 3). However, after 60 d of feeding the low-FM/FO diet, LLv fish (obtained from broodstock fed low LO and fed at 4 months with the v, high in VM and VO) showed the highest body weight, being significantly ($P < 0.05$) higher than that of fish LLf, from the same broodstock but fed the diet f at 4 months (high in FM and FO) (Table 3). Besides, the growth of LLv fish was also significantly higher than that of fish from Fv or HLv that had been fed the same v, but came from broodstock fed FO or high LO. Thus, the two-way ANOVA analysis of final body weight showed a significant effect of the broodstock diet ($P < 0.05$) and the interaction between broodstock and reminder diet ($P < 0.01$). The specific growth rate of LLv fish was significantly higher than that of LLf, denoting the significant effect of the v, as well as higher than Fv and HLv ($P < 0.05$). Thus, the two-way

Table 2. Primers, RT-PCR reaction efficiencies, and GeneBank accession numbers and reference articles for sequences of target and housekeeping genes

Genes	Primer sequence 5'-3' (F) and 5'-3' (R)	Efficiency (%)	GenBank access no.	References
<i>lpl</i>	CGT TGC CAA GTT TGT GAC CTG AGG GTG TTC TGG TTG TCT GC	98.0	AY495672	(68)
<i>ppara</i>	TCT CTT CAG CCC ACC ATC CC ATC CCA GCG TGT CGT CTC C	102.0	AY590299	(68)
<i>elovl6</i>	GTG CTG CTC TAC TCC TGG TA ACG GCA TGG ACC AAG TAG T		JX975702	(68)
<i>fads2</i>	CGA GAG CCA CAG CAG CAG GGA CGG CCT GCG CCT GAG CAG TT	109.2	AY055749	(40)
<i>cox2</i>	GAG TAC TGG AAG CCG AGC AC GAT ATC ACT GCC GCC TGA GT	107.0	AM296029	(69)
<i>cpt1b</i>	CCA CCA GCC AGA CTC CAC AG CAC CAC CAG CAC CCA CAT ATT TAG	98.0	DQ866821	(70)
<i>β-Act</i>	TCT GTC TGG ATC GGA GGC TC AAG CAT TTG CGG TGG ACG	100.7*	X89920	–

lpl, lipoprotein lipase; *elovl6*, elongation of very long-chain fatty acids protein 6; *fads2*, fatty acid desaturase 2; *cox2*, cyclo-oxygenase-2; *cpt1*, carnitine palmitoyltransferase 1; *β-act*, *β*-actin.

* The average efficiency of housekeeping gene from six RT-PCR runs.

Table 3. Growth performance parameters after 2 months' feeding of very low-fishmeal (5%) and very low-fish oil (FO) (3%) diet in 16-month-old gilthead sea bream (*Sparus aurata*) originated from broodstock fed linseed oil (LO) as a replacement for FO – 0% (100% FO (F)), 60% (40% FO–60% LO (LL)), 80% (20% FO–80% LO (HL)) – and fed either a fishmeal- and FO-based diet (f) or a very low-fishmeal (5%) and very low-FO (3%) 'reminder' diet (v) for 1 month at 4 months of age (Mean values and standard deviations; *n* 3)

Groups	Initial body weight (g)		Final body weight (g)		SGR (%/d)*		FCR†	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ff	243.0	1.9	337.4	3.3	0.7	0.06	1.5 ^A	0.1
LLf	244.1	0.9	336.3 ^B	2.6	0.6 ^B	0.03	1.7 ^B	0.1
HLf	242.5	2.0	339.6	7.2	0.7	0.05	1.6	0.1
Fv	243.6	0.9	319.5 ^b	5.5	0.5 ^b	0.07	1.8 ^{B,b}	0.1
LLv	246.4	0.4	351.3 ^{A,a}	1.2	0.7 ^{A,a}	0.03	1.4 ^{A,a}	0.2
HLv	242.3	1.3	321.3 ^b	4.0	0.6 ^b	0.07	1.9 ^b	0.2
Two-way ANOVA								
Broodstock diet (<i>B</i>)	ND		<i>P</i> < 0.05		ND		ND	
Reminder diet (<i>R</i>)	ND		ND		<i>P</i> < 0.05		ND	
<i>B</i> × <i>R</i>	ND		<i>P</i> < 0.01		<i>P</i> < 0.05		<i>P</i> < 0.01	

SGR, specific growth rate; FCR, feed conversion ratio; ND, no difference.

^{A,B} Mean values with unlike superscript letters were significantly different between fish fed f or v diets during the first nutritional challenge (reminder) coming from the same parental feeding. ^{a,b} Mean values with unlike superscript letters were significantly different between fish coming from different parental feeding and fed the same diet during the first nutritional challenge (reminder) (*P* < 0.05).

* SGR (%/d) = (Ln (final weight (g)) – Ln (initial weight (g)))/(number of days) × 100.

† FCR = (total weight of consumed feed (g))/(weight gain (g)).

ANOVA showed the significant effect of the reminder diet at 4 months as well as the interaction between broodstock and reminder diet (*P* < 0.05). Regarding feed conversion, the best values were also obtained for fish in the LLv, Fv or HLv group. For fish coming from broodstock fed FO, the feed conversion ratio (FCR) was better when fish had been fed FO at 4 months (Ff) than when fed diet v (Fv). The two-way ANOVA showed a strong interaction between broodstock and reminder diets (*P* < 0.01).

Biochemical composition

At the end of the study, protein, lipid and ash contents of liver or muscle were similar (*P* > 0.05) (Table 4). However, liver fatty acid composition was significantly affected by broodstock or reminder diets as well as by their interaction (Table 5). For instance, LO increase in broodstock diet significantly reduced liver contents on 16:4*n*-3, a product of EPA *β*-oxidation, and increased 16:3*n*-1 or

18:0, whereas the interaction of broodstock and reminder diets affected the ratios 18:0:16:0 and 18:1:16:1, indicators of *elovl6* activity, and the related ratio 16:1:16:0 (Table 5). The *fads2* products 20:3*n*-6 and 20:4*n*-3 were significantly reduced by the reminder diet (*P* = 0.018) and its interaction with the broodstock diet (*P* = 0.015), respectively, whereas 20:4*n*-6, 20:5*n*-3 and 22:6*n*-3 tended to be higher in offspring fed the reminder diet, but were not significantly different (*P* = 0.17) (Table 5). Muscle fatty acid composition did not differ significantly among the different experimental groups (*P* > 0.05) (Table 6).

Gene expression

Reduction of LC-PUFA and increase in ALA and LA in broodstock diets lead to a significant (*P* < 0.001) down-regulation of hepatic *lpl* (Fig. 2), which was significantly (*P* < 0.01) emphasised by feeding the 4-month-old juveniles the v diet, based on

Table 4. Biochemical composition of liver and muscle tissue after 2 months' feeding with a very low-fishmeal (5%) and very low-fish oil (FO) (3%) diet in 16-month-old gilthead sea bream (*Sparus aurata*) originated from broodstock fed linseed oil (LO) as a replacement for FO – 0% (100% FO (F)), 60% (40% FO–60% LO (LL)), 80% (20% FO–80% LO (HL)) – and fed either a fishmeal- and FO-based diet (f) or a very low-fishmeal (5%) and very low-FO (3%) 'reminder' diet (v) for 1 month at 4 months of age (Mean values and standard deviations; $n=3$)

Groups	Moisture (%)		Protein (%)		Lipids (%)		Ash (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Liver*								
Ff	64.2	2.0	11.1	0.3	14.9	3.0	2.5	0.5
LLf	66.6	5.9	10.1	2.4	12.9	5.9	2.7	1.2
HLf	66.5	0.1	12.1	0.4	11.7	1.8	2.9	0.3
Fv	66.6	4.2	11.2	1.3	11.0	2.2	2.9	0.4
LLv	68.4	2.8	12.2	1.4	10.9	4.9	2.7	0.8
HLv	67.1	4.1	10.7	1.3	12.7	4.4	2.4	0.1
Muscle*								
Ff	72.3	0.1	21.6	0.1	5.1	0.5	1.4	0.1
LLf	72.7	0.5	20.6	0.9	5.2	0.7	1.4	0.1
HLf	72.5	0.8	21.3	0.2	5.4	1.1	1.4	0.1
Fv	73.0	0.1	21.3	0.3	4.7	0.2	1.5	0.1
LLv	72.5	1.0	21.3	0.4	5.2	0.5	1.5	0.1
HLv	72.9	0.1	21.4	0.2	4.9	0.5	1.4	0.1

* No significant differences were found for broodstock diet ($P>0.05$), reminder diet ($P>0.05$) and interaction of these two factors ($P>0.05$) using the two-way ANOVA analysis.

plant ingredients and with low LC-PUFA and high ALA and LA contents. Thus, the lowest relative expression of *lpl* was found in HLv and HLf fish. Similarly, the origin of the fish based on different broodstock diets significantly ($P<0.01$) down-regulated hepatic *elovl6* (Fig. 2), with the lowest relative expression of *elovl6* found in LLv and HLf fish. Besides, *elovl6* expression was significantly correlated to liver contents of 18:1n-3 (18:1n-3) ($r=0.89$) and 18:2n-6 (18:2n-6) ($r=0.89$), ratios of product: substrate of *elovl6* activity. There was no significant effect of either the broodstock or the reminder diet on hepatic expression of *fads2* (Fig. 2), but their values were positively correlated to hepatic levels of 18:4n-3 (18:4n-3) ($r=0.86$) and 18:3n-6 (18:3n-6) ($r=0.8$), products of the *fads2* activity, as well as to the end desaturation products 20:5n-3 (20:5n-3) ($r=0.98$), 22:6n-3 (22:6n-3) ($r=0.95$) and 22:5n-6 (22:5n-6) ($r=0.95$). Besides, *fads2* expression values were negatively correlated ($r=-0.52$) to *elovl6*. Regarding fatty acid catabolism biomarkers, reduction of LC-PUFA and increase in ALA and LA acids in broodstock diets lead to a significant ($P<0.001$) down-regulation of hepatic *cpt1b* (Fig. 2), which was significantly ($P<0.05$) emphasised by the reminder diet. Moreover, the relative expression of *cpt1b* was highly correlated to 18:1n-9 (18:1n-9) ($r=0.82$) and negatively correlated to 20:5n-3 (20:5n-3) ($r=-0.62$). No significant differences were found in the relative expression of *ppara* or *cox2*, which were negatively correlated ($r=-0.57$ and $r=-0.73$, respectively) to *cpt1b* expression.

The overall response showed similar trends for *lpl*, *cpt1b* and *elovl6* expressions, whose values showed a high correlation in their relative gene expression between *lpl* and *elovl6* ($r=0.52$), *cpt1b* and *elovl6* ($r=0.72$) and *lpl* and *cpt1b* ($r=0.74$).

Discussion

In animal production, nutritional programming can be useful to improve offspring adaptation to farm conditions^(74,75). As the limited availability of FM and FO is the main constraint in fish

production, modulation of offspring phenotype through parental feeding for an improved utilisation of low-FM and low-FO diets can have important advantages⁽³¹⁾. Previous studies in gilthead sea bream have demonstrated that it is possible to improve low-FM and low-FO feed utilisation in the offspring coming from broodstock fed with increased substitution of FO with LO⁽⁴⁵⁾. This adaptation included the regulation of expression of genes for key metabolic enzymes in the liver such as *fads2*⁽⁴⁵⁾ or glucocorticoid receptor (*gr*) (S Turkmen *et al.*, unpublished results). However, the persistence of these phenotypic or metabolic changes later in life had not been studied yet. The present study shows that replacement of parental feeding with moderate-FO with LO combined with juvenile feeding with low-FM and low-FO diets improves offspring growth and feed utilisation of low-FM/FO diets even when they are 16 months old: that is, when they are on the verge of their first reproductive season. Thus, among fish fed the low-FM/FO diet during the juvenile stages, those obtained from parents fed moderate LO levels showed the highest growth, denoting the persistent effect of parental nutrition. However, higher LO levels (80% replacement of FO) in broodstock diets did not improve the growth of 16-month-old offspring, in agreement with previous studies⁽⁴⁵⁾. Thus, feeding broodstock with this high-LO diet markedly reduced spawning quality, larval survival and larval and juvenile growth⁽⁴⁵⁾, as a consequence of the deleterious effects of very low *n-3* HUFA levels in broodstock diets⁽⁴⁷⁾. The present study demonstrated the persistence of these negative effects of early EFA deficiencies during offspring life. On the contrary, 60% FO substitution with LO in sea bream broodstock diets did not negatively affect spawning quality or larval growth and produced 4-month-old juveniles with a better ability to utilise low-FM/FO diets⁽⁴⁵⁾, in agreement with the present study.

The above-mentioned growth improvement in 16-month-old fish obtained from broodstock fed moderate LO levels and the reminder low-FM/FO diet at 4 months of age was also



Table 6. % Total fatty acids of muscle after 2 months' feeding of a very low-fishmeal (5%) and very low-fish oil (FO) (3%) diet in 16-month-old gilthead sea bream (*Sparus aurata*) originated from broodstock fed linseed oil (LO) as a replacement for fish oil – 0% (100% FO (F)), 60% (40% FO–60% LO (LL)), 80% (20% FO–80% LO (HL)) – and fed either a fishmeal- and FO-based diet (f) or a very low-fishmeal (5%) and very low-FO (3%) 'reminder' diet (v) for 1 month at 4 months of age (Mean values and standard deviations; n 3)

FA (%)*	Groups											
	Ff		LLf		HLf		Fv		LLv		HLv	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	4.83	0.15	4.90	0.15	5.17	0.15	4.99	0.33	4.98	0.03	4.97	0.35
14:1n-7	0.08	0.00	0.08	0.00	0.08	0.00	0.08	0.00	0.07	0.00	0.08	0.00
14:1n-5	0.08	0.00	0.09	0.03	0.08	0.02	0.09	0.01	0.08	0.00	0.08	0.01
15:0	0.21	0.01	0.23	0.01	0.21	0.01	0.22	0.02	0.22	0.00	0.22	0.02
15:1n-5	0.02	0.00	0.03	0.00	0.03	0.00	0.02	0.01	0.03	0.00	0.03	0.00
16:0iso	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.01
16:0	14.69	0.42	15.03	0.41	14.52	0.21	15.03	0.12	15.18	0.31	14.88	0.48
16:1n-7	4.89	0.31	5.12	0.18	4.51	0.14	4.92	0.64	4.89	0.36	4.87	0.72
16:1n-5	0.08	0.00	0.09	0.00	0.08	0.00	0.08	0.01	0.08	0.01	0.08	0.01
16:2n-6	0.05	0.04	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
16:2n-4	0.36	0.07	0.33	0.02	0.29	0.01	0.31	0.05	0.31	0.01	0.31	0.04
17:0	0.28	0.03	0.30	0.01	0.27	0.00	0.29	0.04	0.28	0.01	0.29	0.03
16:3n-4	0.14	0.00	0.14	0.00	0.14	0.01	0.14	0.01	0.14	0.00	0.14	0.01
16:3n-3	0.05	0.00	0.05	0.01	0.04	0.00	0.05	0.02	0.04	0.01	0.05	0.01
16:3n-1	0.05	0.02	0.07	0.01	0.07	0.01	0.09	0.01	0.07	0.00	0.08	0.02
16:4n-3	0.35	0.11	0.34	0.03	0.31	0.01	0.33	0.06	0.34	0.01	0.30	0.09
16:4n-1	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.01
18:0	3.50	0.04	3.45	0.11	3.55	0.11	3.55	0.07	3.53	0.00	3.61	0.10
18:1n-9	27.27	0.21	27.23	0.92	28.47	0.99	27.23	1.37	27.77	0.28	27.66	1.00
18:1n-7	2.85	0.06	2.90	0.01	2.74	0.04	2.84	0.16	2.82	0.08	2.84	0.14
18:1n-5	0.39	0.45	0.13	0.01	0.11	0.01	0.13	0.02	0.12	0.01	0.13	0.02
18:2n-9	0.22	0.02	0.25	0.06	0.22	0.01	0.24	0.02	0.24	0.02	0.22	0.02
18:2n-6	11.52	0.40	11.46	0.55	12.77	0.49	11.62	1.36	11.74	0.62	11.79	1.59
18:2n-4	0.13	0.01	0.14	0.00	0.13	0.01	0.13	0.01	0.13	0.01	0.14	0.01
18:3n-6	0.35	0.05	0.36	0.05	0.33	0.01	0.35	0.03	0.34	0.02	0.32	0.00
18:3n-4	0.15	0.01	0.14	0.00	0.13	0.00	0.15	0.01	0.13	0.01	0.14	0.01
18:3n-3	5.11	1.11	4.53	0.51	5.48	0.47	4.57	1.16	4.76	0.40	4.75	1.18
18:3n-1	0.18	0.30	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
18:4n-3	0.69	0.04	0.73	0.03	0.64	0.03	0.69	0.07	0.70	0.05	0.67	0.06
18:4n-1	0.22	0.13	0.14	0.00	0.14	0.01	0.13	0.01	0.14	0.01	0.15	0.01
20:0	0.24	0.01	0.24	0.01	0.24	0.01	0.24	0.00	0.24	0.01	0.24	0.00
20:1n-9	0.28	0.02	0.29	0.03	0.25	0.03	0.31	0.09	0.28	0.03	0.28	0.07
20:1n-7	2.36	0.13	2.44	0.24	2.19	0.10	2.43	0.38	2.37	0.11	2.35	0.34
20:1n-5	0.16	0.01	0.17	0.01	0.16	0.01	0.17	0.02	0.16	0.01	0.16	0.02
20:2n-9	0.31	0.01	0.31	0.02	0.31	0.03	0.30	0.02	0.30	0.01	0.28	0.01
20:2n-6	0.60	0.29	0.42	0.02	0.45	0.00	0.42	0.02	0.42	0.00	0.43	0.02
20:3n-9	0.03	0.01	0.03	0.01	0.05	0.01	0.04	0.02	0.04	0.03	0.06	0.03
20:3n-6	0.23	0.00	0.22	0.01	0.24	0.02	0.22	0.01	0.21	0.01	0.21	0.03
20:4n-6	0.46	0.04	0.46	0.04	0.42	0.06	0.46	0.06	0.44	0.00	0.46	0.05
20:3n-3	0.33	0.08	0.28	0.00	0.32	0.00	0.28	0.04	0.28	0.01	0.30	0.04
20:4n-3	0.58	0.00	0.58	0.01	0.54	0.02	0.55	0.05	0.55	0.03	0.57	0.05
20:5n-3	4.36	0.21	4.42	0.19	3.88	0.27	4.15	0.62	4.22	0.17	4.26	0.61
22:1n-11	1.42	0.16	1.53	0.24	1.21	0.12	1.52	0.40	1.45	0.15	1.41	0.36
22:1n-9	0.56	0.02	0.57	0.06	0.53	0.01	0.59	0.07	0.56	0.01	0.55	0.05
22:4n-6	0.10	0.01	0.10	0.01	0.10	0.01	0.11	0.01	0.10	0.00	0.11	0.01
22:5n-6	0.12	0.01	0.13	0.00	0.11	0.01	0.13	0.01	0.12	0.01	0.13	0.00
22:5n-3	2.07	0.15	2.10	0.20	1.88	0.15	2.08	0.27	1.99	0.10	2.09	0.26
22:6n-3	6.99	0.73	7.41	0.66	6.53	0.89	7.64	1.13	7.03	0.12	7.27	0.81

* No significant differences were found for broodstock diet ($P > 0.05$), reminder diet ($P > 0.05$) and interaction of these two factors ($P > 0.05$) using the two-way ANOVA analysis.

accompanied by an enhanced utilisation of the low-FM/FO diet, as denoted by the better FCR, which could be related to the modulation of lipid and carbohydrate metabolism. There were no large differences in the proximate composition and fatty acid profiles of sea bream liver and muscle, reflecting the profound effect of the diet, regardless of the nutritional history of the different fish groups. Nevertheless, both broodstock diet and reminder diet had a significant effect on some major fatty acids in the liver such as 18:0, a terminal product of lipogenesis, and in the ratios 18:0:16:0 and, particularly, 18:1:16:1. Both 16:0 and 16:1 are substrates for *elovl6*, a key rate-limiting enzyme in the long-chain fatty acid elongation cycle and, therefore, the ratios

18:0:16:0 and 18:1:16:1, are indicators of the activity of this enzyme. These results are in agreement with the hepatic *elovl6* expression, which was down-regulated by the increase in LO in the broodstock diets and was correlated inversely to the 16:0 contents in the liver and directly to the 18:0:16:0 and 18:1:16:1, denoting a significant post-transcriptional effect. Besides, LO increase in broodstock diets also increased the hepatic 18:0:18:1 ratios in the 16-month-old offspring.

These results are in agreement with the 16:0 reduction and 18:0:18:1 increase in mice models with *Elavl6* disruption, which showed protection against a high-SFA diet-induced insulin resistance that lead to hepatosteatosis similar to that of

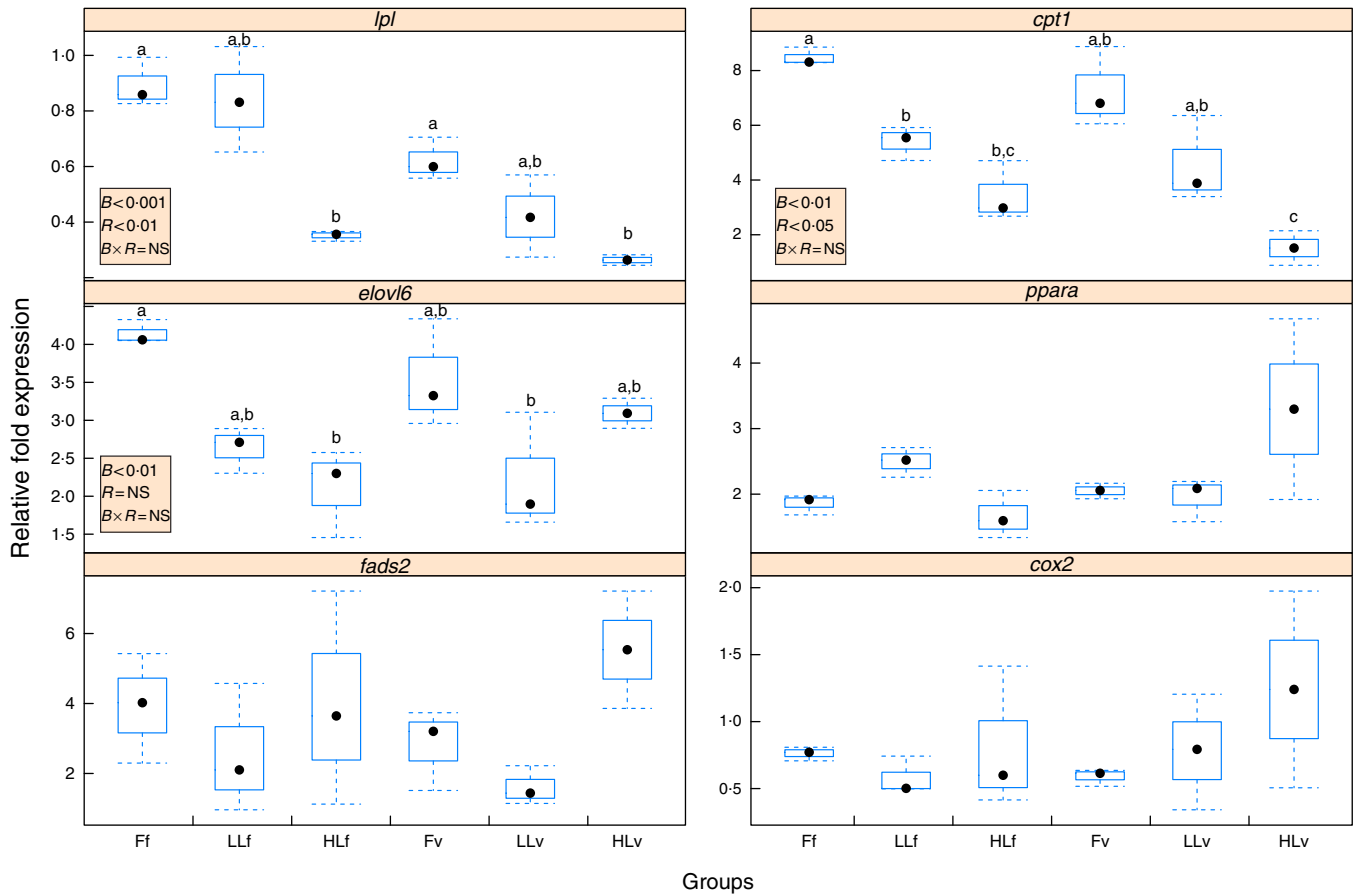


Fig. 2. Box and whisker plots of relative fold expression (groups v. control sample) of six different genes after challenging 16-month-old gilthead sea bream individuals with high-vegetable oil and high-meal feeds for 2 months. *lpl*, lipoprotein lipase; *elov16*, elongation of very long-chain fatty acids protein 6; *fads2*, fatty acid desaturase 2; *cox2*, cyclo-oxygenase-2; *cpt1*, carnitine palmitoyltransferase I; ---, maximum and minimum fold expression; □, upper and lower quartiles; ●, median; □, P values of two-way ANOVA; B, broodstock diet; R, reminder diet; B × R, interaction of these two parameters, ns, $P > 0.05$; F, 100% fish oil; LL, 40% fish oil–60% linseed oil; and HL, 20% fish oil–80% linseed oil; f, fish oil-based diet; v, reminder diet. $n = 3$ for all the groups and genes. ^{a,b,c} Mean values with unlike letters were significantly different between each group, no indications mean no significant difference ($P > 0.05$).

wild-type mice⁽⁵¹⁾. This protection was related to the restoration of hepatic insulin receptor substrate-2, suppression of hepatic protein kinase-C ϵ and restoration of Akt phosphorylation⁽⁵¹⁾, overall, indicating a better utilisation of dietary carbohydrates under conditions of high-fat diet-induced insulin resistance. Indeed, insulin stimulates acetyl-CoA carboxylase that produces malonyl-CoA, which inhibits CPTI activity and affects utilisation of fatty acids and glucose as substrates⁽⁷⁶⁾. Thus, genes related to fatty acid oxidation, such as *Cpt1*, are down-regulated in mice with *Elovl6* disruption⁽⁵¹⁾, whereas up-regulation of *Cpt1* expression caused by intra-uterine growth restriction increases the risk of type 2 diabetes in adulthood⁽⁵⁹⁾. In agreement, in the present study, down-regulation of *elov16* was correlated to down-regulation of *cpt1*, a rate-limiting enzyme for fatty acid oxidation in mitochondria⁽⁷⁷⁾. Moreover, increased LO in broodstock diet and increased plant-protein and lipid sources in the 4-month-old reminder diet induced the down-regulation of *cpt1* in the gilthead sea bream offspring, evidencing a long-term nutritional programming effect. A down-regulation of *cpt1b* gene expression was also found in the liver of juvenile rainbow trout by vitamin supplementation at first feeding showing that nutritional interventions during developmental plasticity (larval

period) may provoke longer-term effects later in life⁽⁷⁸⁾. *Cpt1* expression in the liver of fish is down-regulated by the reduction of dietary PUFA, particularly, LC-PUFA^(57,79). Accordingly, *cpt1* expression is also down-regulated in Atlantic salmon when dietary FO is substituted with VO^(80,81). *In vitro* studies in rainbow trout hepatocytes showed that *ppara* and *cpt1* are up-regulated by MUFA and down-regulated by EPA, among other fatty acids^(80,81). In the present study *cpt1b* expression was highly correlated to 18:1n-9 and negatively correlated to 20:5n-3 in the liver. Besides, LO increase in broodstock diets significantly reduced the liver contents of 16:4n-3, an intermediate product of β -oxidation of EPA. In gilthead sea bream offspring, *cpt1* expression in the liver is negatively correlated to *ppara*, suggesting that nutritional programming by LO reduces β -oxidation in the mitochondria but not in the peroxisomes. In mammals, parental feeding with a high-lipid diet lead to hypomethylation of four specific CpG dinucleotides in *PPARA* and the modification of the mRNA transcript in juvenile offspring⁽⁶⁰⁾. In gilthead sea bream fed a low-FM/FO diet, hepatic *ppara* is reportedly down-regulated, in association with retarded growth⁽⁸²⁾. In the present study, *ppara* in the offspring of HLv fed a low-FM/FO diet was not down-regulated and

growth was even increased instead of being reduced upon parental feeding with LO.

In previous studies, parental feeding of gilthead sea bream with increased substitution of FO with LO significantly up-regulated *fads2* in 1-month-old offspring (S. Turkmen *et al.*, unpublished results). Similarly, in Senegalese sole, parental nutritional history affects growth performance and the expression of $\Delta 4fad$ and *elov15* in the 2-month-old progeny⁽⁴⁴⁾. However, in the present study, *fads2* of the 16-month-old fish did not show significant differences, which could be related to the strong influence of the very low-FM/FO diets fed to the 16-month-old sea bream. Indeed, *fads2* relative expression was high in all fish and up to 5.5 times higher than the values in gilthead sea bream juveniles fed commercial diets containing high levels of FM and FO (data not shown). This is in agreement with the up-regulation of *fads2* expression in fish fed reduced *n-3* LC-PUFA diets rich in linolenic acid or LA⁽⁸³⁾. Dietary changes did not affect *fads2* gene expression in Atlantic cod (*Gadus morhua*) either⁽⁸⁴⁾, reportedly, related to the low dietary FO levels causing an up-regulation of this gene⁽⁴⁹⁾ or to a post-transcriptional regulation⁽⁴⁹⁾ as observed in other marine fish species⁽⁸⁵⁾. Despite a slightly higher *fads2* expression in offspring of broodstock fed LO and the remainder diet, individual differences among fish belonging to the same treatment lead to large variations with no significant differences among groups. Nevertheless, *fads2* expression in the liver was correlated with hepatic contents in 18:4*n-3*, 18:3*n-6*, 20:5*n-3*, 22:6*n-3* and 22:5*n-6*, intermediate and end products of desaturation activity by this enzyme⁽²⁵⁾.

The down-regulation of *lpl* expression in the liver of offspring from broodstock fed feeds with high LO levels, especially in those fish that received a low-FO/FM diet during juvenile stages, was correlated with reduced liver lipid contents, in agreement with the reduced lipid deposition associated with the down-regulation of *lpl* expression in the liver of the gilthead sea bream in previous studies⁽⁸⁶⁾. LPL is a determinant of lipid deposition or catabolism fate⁽⁵⁸⁾. Thus, nutritional programming through regulation of different genes within the pathway of lipid metabolism including *lpl*, *elov16* and *cpt1* may prepare the offspring for a better utilisation of low-FM and low-FO diets, improving VO and VM utilisation and reducing the risk for hepatosteatosis described in gilthead sea bream fed these type of diets⁽⁸⁷⁾. As occurs in mammals, nutritional signals through parental feeding may improve offspring fitness at later stages, triggering a 'predictive adaptive response'⁽⁸⁸⁾. Thus, in gilthead sea bream, offspring of broodstock fed moderate LO levels and fed the low-FM/FO diet during juvenile stages showed improved final body weight and feed utilisation.

To our knowledge, this is the first study that demonstrates the profound effects of *n-3* LC-PUFA profiles in parental diets on long-term effects in fish offspring even later in life: that is, in those on the verge of their first sexual maturation. In mammals, *n-3* LC-PUFA supplementation in maternal diets reduces premature births⁽⁸⁹⁾ and enhances immune health^(90,91), growth, development and pancreatic tissue morphometry in the offspring⁽⁹²⁾. Besides, the nutritional programming effect of LC-PUFA on parental diet and the epigenetic regulation of gene expression has been also demonstrated in mammals⁽⁹³⁾,

implying different epigenetic and physiological mechanisms including cell differentiation, neuro-hormonal regulation, etc.⁽⁹⁴⁾, which have not yet been demonstrated in fish. The present study has also pointed out that nutritional programming through parental feeding interacts with the feeding history during juveniles stages, as feeding a low-FM/FO diet for only one month when fish were 4-months-old affected gene expression and fish performance later when fish were on the verge of reproduction, in agreement with studies in other vertebrates⁽⁹⁵⁾. In summary, partial replacement of FO with LO in parental diets during gilthead sea bream reproduction induced long-term persistent effects on transcription of selected genes in the offspring, which regulate energy metabolism in the liver for a better utilisation of diets high in VO and VM. Moreover, these long-term effects on gene transcription are further enhanced by feeding the offspring juveniles with diets high in VO and VM, which improved growth and feed utilisation. Studies are underway to better understand the potential epigenetic, metabolic and molecular mechanisms involved in the metabolic conditioning of offspring through parental nutrition in gilthead sea bream.

Acknowledgements

The authors wish to thank Dr Sadasivam Kaushik for his suggestions on the final version of the manuscript. Acknowledgement is also owed to an anonymous referee who gave very valuable suggestions.

This work has been (partly) funded under the EU seventh Framework Programme by the Advanced Research Initiatives for Nutrition & Aquaculture (ARRAINA), project no. 288925.

S. T. conducted all experiments, analysed and evaluated all biological, biochemical and molecular analyses. Molecular biology samples were analysed by S. T. with the supervision of M. J. Z. All trials were designed by S. T., D. M. and C. M. H.-C., and M. I. supervised the entire work. L. R. was involved in the formulation and the preparation of the diets. The paper was written by S. T. and M. I.

The authors declare that there are no conflicts of interest. The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

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