

## Effect of ration size on fillet fatty acid composition, phospholipid allostasis and mRNA expression patterns of lipid regulatory genes in gilthead sea bream (*Sparus aurata*)

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### Abstract

The effect of ration size on muscle fatty acid (FA) composition and mRNA expression levels of key regulatory enzymes of lipid and lipoprotein metabolism have been addressed in juveniles of gilthead sea bream fed a practical diet over the course of an 11-week trial. The experimental setup included three feeding levels: (i) full ration until visual satiety, (ii) 70% of satiation and (iii) 70% of satiation with the last 2 weeks at the maintenance ration. Feed restriction reduced lipid content of whole body by 30% and that of fillet by 50%. In this scenario, the FA composition of fillet TAG was not altered by ration size, whereas that of phospholipids was largely modified with a higher retention of arachidonic acid and DHA. The mRNA transcript levels of lysophosphatidylcholine acyltransferases, phosphatidylethanolamine *N*-methyltransferase and FA desaturase 2 were not regulated by ration size in the present experimental model. In contrast, mRNA levels of stearoyl-CoA desaturases were markedly down-regulated by feed restriction. An opposite trend was found for a muscle-specific lipoprotein lipase, which is exclusive of fish lineage. Several upstream regulatory transcriptions were also assessed, although nutritionally mediated changes in mRNA transcripts were almost reduced to PPAR $\alpha$  and  $\beta$ , which might act in a counter-regulatory way on lipolysis and lipogenic pathways. This gene expression pattern contributes to the construction of a panel of biomarkers to direct marine fish production towards muscle lean phenotypes with increased retentions of long-chain PUFA.

**Key words:** Muscle: Lipid metabolism: Lipoprotein lipase: Stearoyl-CoA desaturase

Global fisheries are in decline and farmed fish constitute an increasing proportion of fish in the human food basket<sup>(1)</sup>. Thus, to assure the continuous growth of aquaculture production, the industry is obliged to find suitable alternatives to fish meal and fish oil in fish feeds. Plant products are the obvious choice, but vegetable oils are devoid of *n*-3 long-chain PUFA (LC-PUFA), and fillet fatty acid (FA) composition of farmed fish points towards a reduction in EPA (20:5*n*-3), docosapentaenoic acid (22:5*n*-3) and DHA (22:6*n*-3) content. Therefore, the sustainable development of aquaculture and the preservation of health benefits of fish consumption represent a complex trade-off, which is sometimes difficult to reconcile. Indeed, high levels of *n*-3 LC-PUFA are important quality factors in human foods, and there is increasing pressure to include EPA and DHA in the finishing diets of salmonids and freshwater fish, though they do not have specific *n*-3

LC-PUFA requirements<sup>(2–4)</sup>. This reinforces the interest for reliable FA descriptors linking dietary and muscle FA composition. In particular, the association between dietary and fillet FA composition is stronger in oily fish than in lean fish, and direct and highly predictable effects are likely to be found in marine fish due to their limited capacity to convert C<sub>18</sub> PUFA of *n*-6 and *n*-3 series to their LC-PUFA<sup>(5)</sup>. According to this, fillet FA composition of gilthead sea bream juveniles is highly predictable for a given class of fish size and adiposity<sup>(6)</sup>, and bivariate regression approaches with FA diet composition and fillet lipid content, as independent variables effectively mirror the year-round fillet FA composition of mature and immature fish<sup>(7)</sup>.

Ration size triggers significant variations in muscle lipid levels of gilthead sea bream<sup>(8–11)</sup>. In salmonids, this outcome has also been broadly described, and concomitant changes in

**Abbreviations:** EL, endothelial lipase; FA, fatty acid; *FADS2*, fatty acid desaturase 2; FAME, fatty acid methyl esters; IATS, Institute of Aquaculture Torre de la Sal; LC-PUFA, long-chain PUFA; *LPCAT1*, lysophosphatidylcholine acyltransferase 1; *LPCAT2*, lysophosphatidylcholine acyltransferase 2; *LPCAT3*, lysophosphatidylcholine acyltransferase 3; *LPL-like*, lipoprotein lipase-like; *LXR $\alpha$* , liver X receptor  $\alpha$ ; PC, phosphatidylcholine; *PMT*, phosphatidylethanolamine *N*-methyltransferase; PL, phospholipids; R<sub>100</sub> group, full ration until visual satiety; R<sub>70</sub> group, 70% of satiation; R<sub>70-20</sub> group, 70% of satiation with the last 2 weeks at 20% of satiation level; *SCD1a*, stearoyl-CoA desaturase 1a; *SCD1b*, stearoyl-CoA desaturase 1b; *SREBP1*, sterol regulatory element-binding protein-1; TL, total lipids.

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the FA composition of muscle total lipids (TL) have been reported<sup>(12,13)</sup>. Most of these changes in FA signatures have been primarily attributed to switches in the relative proportion of phospholipids (PL) and TAG<sup>(14,15)</sup>. However, the FA composition of these two lipid fractions is differentially regulated by nutritional and/or environmental conditions due to their different roles in cellular and lipid metabolism<sup>(5,16)</sup>. In fact, the FA composition of TAG usually bears a close resemblance to dietary lipids, whereas that of PL is highly influenced by environmental factors including temperature and osmolarity<sup>(17,18)</sup>, which may be attributed, at least in part, to changes in feeding levels. In this way, the aim of the present study was to evaluate the specific effects of ration size on fillet FA composition and mRNA gene expression of key regulatory enzymes of lipid and lipoprotein metabolism. For that purpose, lipid classes and FA composition of TL, TAG and PL were analysed in juveniles of gilthead sea bream fed from satiety to maintenance ration. In parallel, to gain knowledge of the regulation of lipid metabolism and the nutritional value of flesh, the expression pattern of fourteen selected genes was assessed by means of candidate gene array focused on tissue FA uptake, FA biosynthesis, PL synthesis and remodelling and upstream regulatory transcription factors. This issue was made after exhaustive searches in our gilthead sea bream transcriptome database, which rendered new sequences annotated as lipoprotein lipase-like (*LPL-like*), lysophosphatidylcholine acyltransferase 1, 2 and 3 (*LPCAT1-3*), phosphatidylethanolamine *N*-methyltransferase (*PEMT*), stearoyl-CoA desaturase 1a (*SCD1a*) and 1b (*SCD1b*) and sterol regulatory element-binding protein-1 (*SREBP1*), and uploaded to GenBank with accession numbers JQ277704–JQ277707, JQ277709, JQ390609 and JQ390612. The study also presented phylogenetic analyses of gene-lineage subfamilies (extracellular TAG lipases and SCD1 enzymes) for which mammalian orthologous genes do not exist yet or remain unclear.

## Experimental methods

### *Animal care and experimental setup*

Juvenile gilthead sea bream (*Sparus aurata* L.) of Atlantic origin (Ferme Marine de Douhet) were acclimatised to laboratory conditions for 25 d before the start of trial (May–July) in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS). Following the acclimatisation period, fish of 17 g initial mean body weight were randomly distributed into 500-litre tanks in triplicate groups of fifty fish each. Fish were fed from May to August (11 weeks) with a commercial diet (D-2 Excel 1P; Skretting) twice per d at three different feeding levels: (i) full ration until visual satiety (R<sub>100</sub> group), (ii) 70% of satiation (R<sub>70</sub> group) and (iii) 70% of satiation with the last 2 weeks at the maintenance ration (20% of the satiation level; R<sub>70-20</sub> group). Maintenance ration was conveniently calculated according to fish size and temperature (23–25°C) at this last stage of the experimental trial. The FA composition of the diet is reported in Table 1.

The trial was conducted under the natural photoperiod and temperature conditions at IATS latitude (40°5N; 0°10E). Water

**Table 1.** Diet fatty acid (FA) composition (% total FA methyl esters)

FA (%)	Diet
14:0	5.01
15:0	0.11
16:0	17.57
16:1n-7	4.81
16:2	0.77
16:3	0.17
16:4	1.17
17:0	0.86
18:0	4.14
18:1n-9	15.55
18:1n-7	2.90
18:2n-6	21.4
18:3n-6	0.19
18:3n-3	2.38
18:4n-3	0.89
20:0	0.26
20:1n-7	0.23
20:1n-9	1.17
20:2n-6	0.13
20:3n-6	0.14
20:3n-3	0.05
20:4n-6	0.61
20:4n-3	0.34
20:5n-3	7.53
22:0	0.23
22:1n-9	0.29
22:1n-11	1.0
22:5n-3	0.9
22:6n-3	4.5

flow was 20 litres/min and oxygen content of water effluents was always higher than 85% saturation. At the end of the trial period and following overnight fasting, nine randomly selected fish per dietary treatment were anaesthetised with 3-aminobenzoic acid ethyl ester (MS-222, 100 µg/ml). The entire fillet from fish left side (denuded from skin) was taken, vacuum packed and stored at –80°C until lipid analyses. A representative portion of the skeletal muscle (dorsal white muscle) from the right side of the fish was taken under RNase-free conditions, frozen with liquid N<sub>2</sub> and stored at –80°C until gene expression analyses. Nine additional fish per dietary treatment were used for whole-body composition analyses.

All procedures were carried out according to the national (IATS-CSIC Review Board) and present EU legislation on the handling of experimental animals.

### *Lipid analyses*

Fillets and specimens for whole-body analyses were ground and small aliquots were dried to estimate the moisture content. The moisture content was determined by drying in an oven at 105°C for 24 h. The remaining samples were freeze-dried and the TL content was determined gravimetrically using the Soxhlet 4001046 Auto extraction apparatus (Selecta) with 50 ml diethyl ether at 120°C, according to the manufacturer's instructions.

Fillet lipids for analyses of FA and lipid classes were extracted by the method of Folch *et al.*<sup>(19)</sup>, with

chloroform–methanol (2:1) containing 0.01% butylated hydroxytoluene as antioxidant. The analysis of lipid classes was performed by high-performance TLC using a one-dimensional double development, as described by Olsen & Henderson<sup>(20)</sup>. Briefly, TL were separated on TLC plates (10 × 10 cm, silica gel (200 μm); Merck) using methyl acetate–isopropanol–chloroform–methanol–0.25% aqueous potassium chloride (25:25:25:10:9, by vol.) and hexane–diethyl ether–acetic acid (85:15:1.5, by vol.) as developing solvent mixtures for PL and neutral lipids, respectively. The separated lipid fractions were charred for 10 min at 160°C after spraying the plate with 3% (w/v) cupric acetate in 8% (v/v) phosphoric acid<sup>(21)</sup> and were quantified by calibrated scanning densitometry (Bio-Rad GS670 Imaging densitometer; Bio-Rad). The quantity of each lipid class was expressed on wet matter basis.

The FA from TL were fractionated by TLC (Silica gel G 60, 20 × 20 cm glass plates; Merck), using hexane–diethyl ether–acetic acid (85:15:1.5, by vol.) as a solvent system. The PL bands were scraped and extracted with chloroform–methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. The TAG were scraped and extracted with hexane–diethyl ether (1:1, v/v). After the addition of non-adeanoic FA (Sigma) as internal standard, fractions of TL, TAG and PL were subjected to acid-catalysed transmethylation for 16 h at 50°C using toluene and 1% (v/v) H<sub>2</sub>SO<sub>4</sub> in methanol<sup>(22)</sup>. The FA methyl esters

(FAME) were extracted with hexane–diethyl ether (1:1, v/v), and those derived from TL were purified by TLC using hexane–diethyl ether–acetic acid (85:15:1.5, by vol.) as a solvent system. The FAME were then analysed with a gas chromatograph (GC 8000 Series; Fisons Instruments) equipped with a fused-silica 30 m × 0.25 mm open tubular column (film thickness: 0.25 μm; Tracer, TR-WAX; Teknokroma) and a cold on-column injection system. Helium was used as a carrier gas and temperature programming was from 50 to 180°C at 40°C/min and then to 220°C at 3°C/min. Peaks were recorded in a personal computer using a software package (Azur, Datalys). Individual FAME were identified by reference to well-characterised fish oil standards in the analysed sample.

Marinol (sardine oil) from the Fishing Industry Research Institute (Rosebank) and FAME 37 from Supelco were used as fish oil standards. Butylated hydroxytoluene and internal standard (19:0) were obtained from Sigma-Aldrich. All lipid solvents were of HPLC grade and were obtained from Merck.

### Gene expression analysis

For the two extreme groups (R<sub>100</sub> and R<sub>70-20</sub>), total RNA from skeletal muscle was extracted using the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems) with a DNase step. The RNA yield was 30–50 μg, with absorbance measures

**Table 2.** Forward and reverse primers for quantitative real-time PCR

Gene	Symbol	Accession number	Primer sequence (5'–3')	
			Forward	Reverse
Fatty acid desaturase 2*	<i>FADS2</i>	AY055749	GCA GGC GGA GAG CGA CGG TCT GTT CC	AGC AGG ATG TGA CCC AGG TGG AGG CAG AAG
Lipoprotein lipase†	<i>LPL</i>	AY495672	CGT TGC CAA GTT TGT GAC CTG	AGG GTG TTC TGG TTG TCT GC
Lipoprotein lipase-like†	<i>LPL-like</i>	JQ390609	CAG AGA TGG AGC CGT CAC TCA C	TCT GTC ACC AGC AGG AAC GAA TG
Liver X receptor α‡	<i>LXRα</i>	FJ502320	GCA CTT CGC CTC CAG GAC AAG	CAG TCT TCA CAC AGC CAC ATC AGG
Lysophosphatidylcholine acyltransferase 1§	<i>LPCAT1</i>	JQ390612	CGT GAT AGC CTT ATC TGT CGT ATG C	CCG TCC TCC TCT GCC TCA A
Lysophosphatidylcholine acyltransferase 2§	<i>LPCAT2</i>	JQ277705	GAC TGC CGC CTG ATG ATC TC	ATG GTG ATG CGT CCT CCT TTA C
Lysophosphatidylcholine acyltransferase 3§	<i>LPCAT3</i>	JQ277706	TGA CGG CGG GCA GGA ACC ATC	CCC ACC AGG AAG CCT CCA TAG AAG TAG
Peroxisome proliferator-activated receptor α‡	<i>PPARα</i>	AY590299	TCT CTT CAG CCC ACC ATC CC	ATC CCA GCG TGT CGT CTC C
Peroxisome proliferator-activated receptor β‡	<i>PPARβ</i>	AY590301	AGG CGA GGG AGA GTG AGG ATG AGG AG	CTG TTC TGA AAG CGA GGG TGA CGA TGT TTG
Peroxisome proliferator-activated receptor γ‡	<i>PPARγ</i>	AY590304	CGC CGT GGA CCT GTC AGA GC	GGA ATG GAT GGA GGA GGA GGA GAT GG
Phosphatidylethanolamine N-methyltransferase§	<i>PEMT</i>	JQ277707	TTG GTG CCA GTC CTG TTG GTC TC	TGA TAG ATC AGT CCA GTG AAT GGT CCT TC
Stearoyl-CoA desaturase 1a*	<i>SCD1a</i>	JQ277703	CGG AGG CGG AGG CGT TGG AGA AGA AG	AGG GAG ACG GCG TAC AGG GCA CCT ATA TG
Stearoyl-CoA desaturase 1b*	<i>SCD1b</i>	JQ277704	GCT CAA TCT CAC CAC CGC CTT CAT AG	GCT GCC GTC GCC CGT TCT CTG
Sterol regulatory element-binding protein-1‡	<i>SREBP1</i>	JQ277709	AGG GCT GAC CAC AAC GTC TCC TCT CC	GCT GTA CGT GGG ATG TGA TGG TTT GGG
β-Actin	<i>ACTB</i>	X89920	TCC TGC GGA ATC CAT GAG A	GAC GTC GCA CTT CAT GAT GCT

\* Fatty acid desaturases.

† Key enzymes on tissue fatty acid uptake.

‡ Transcription factors.

§ Enzymes involved in phospholipid synthesis and remodelling.

**Table 3.** Characteristics of assembled sequences according to basic local alignment search tool (BLAST) searches

Contig(s)	F*	Size (nt)	Annotation†	Best match‡	E§	CDS
C2_29860	42	2518	LPL-like	BAB20996	0.0	28–1515
C2_14455	24	1862	LPCAT1	XP_003443759	0.0	369– > 1862
C2_8782	55	2091	LPCAT2	XP_003437708	$3 \times 10^{-157}$	< 1–914
C2_13645	23	1441	LPCAT3	XP_003450693	0.0	< 1–1296
C2_36050	6	1571	PEMT	ACI68830	$9 \times 10^{-118}$	129–830
C2_81189	3	367	SREBP1	CAG03157	$9 \times 10^{-12}$	< 1– > 367
C2_3937	99	1382	SCD1a	CBN81527	0.0	156–1163
C2_5624	120	1665	SCD1b	CBM40644	0.0	148–1155

nt, Nucleotides; CDS, coding domain sequence.

\* Number of sequences.

† Gene identity determined through BLAST searches.

‡ Best BLAST-X protein sequence match.

§ Expectation value.

( $A_{260/280}$ ) of 1.9–2.1. Reverse transcription of 500-ng total RNA was performed with random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was performed with an iCycler IQ Real-time Detection System (Bio-Rad), as described elsewhere<sup>(23)</sup>. Briefly, diluted reverse transcription reactions were conveniently used for PCR reactions in 25  $\mu$ l volume, in combination with a SYBR Green Master Mix (Bio-Rad). Specific primers for target genes and  $\beta$ -actin (housekeeping gene) were used at a final concentration of 0.9  $\mu$ M (Table 2). The  $\Delta\Delta C_t$  method<sup>(24)</sup> was used for the normalisation procedure, and expression values were referred to *PEMT* as reference gene. The set of primers for *LPL*, liver X receptor  $\alpha$  (*LXR $\alpha$* ) and *PPAR $\alpha$* , *PPAR $\beta$*  and *PPAR $\gamma$*  were the same as previously used<sup>(25,26)</sup>. New sets of primers were designed for FA desaturase 2 (*FADS2*) and additional sequences were generated following the update (November 2011) of the IATS complementary DNA gilthead sea bream database (<http://www.siginae.org/iats>). These new sequences were submitted to GenBank as partial (*LPCAT1*, *LPCAT2*, *LPCAT3*, *SREBP1*) or complete (*LPL-like*, *PEMT*, *SCD1a*, *SCD1b*) coding sequences (Table 3). Of note, the best hit for *LPL-like* is a red sea bream sequence (AB054063) annotated as *LPL2* by Oku *et al.*<sup>(27)</sup>. *SCD1a* (JQ277703) and *SCD1b* (JQ277704) sequences were annotated according to the revised nomenclature for the stearoyl-CoA desaturase family<sup>(28)</sup>.

### Phylogenetic analysis

Phylogenetic analysis was carried out on the basis of amino acid differences (p-distance) with the unweighted pair group method with arithmetic mean algorithm (pairwise deletion) in MEGA software (version 5.0; [www.megasoftware.net](http://www.megasoftware.net)). A total of thirty-seven sequences from fourteen species were included in the phylogenetic analyses of SCD1 enzymes and extracellular TAG lipases. Reliability of phylogenetic trees was assessed by bootstrapping using 1000 bootstrap replications.

### Statistical analysis

Data on growth performance and lipid composition analyses were analysed by one-way ANOVA followed by the Student–Newman–Keuls test ( $P < 0.05$ ). Data on gene expression were

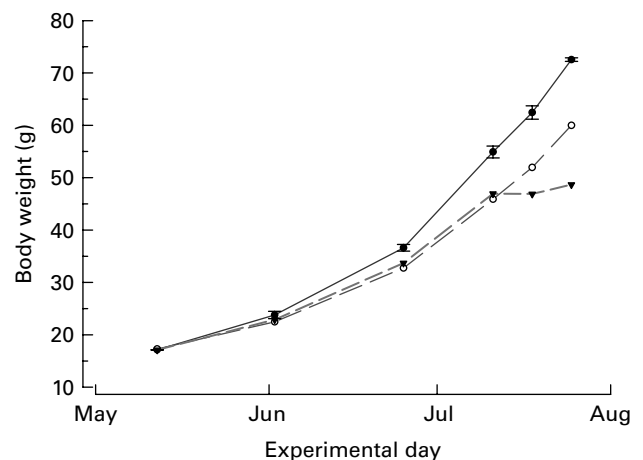
analysed by Student's *t* test. All analyses were performed using the SPSS 17.0 program (SPSS, Inc.).

## Results

### Growth performance and lipid analyses

Data on body weight are shown in Fig. 1. Fish of the R<sub>100</sub> group grew from 17 (SD 0.12) to 72 (SD 0.34) g, with a feed efficiency (wet weight gain/dry feed intake) of 0.89 (SD 0.07). The 30% feed restriction (R<sub>70</sub> group) had a lesser final body weight (60 (SD 0.02) g) with a slight, but statistically significant ( $P < 0.05$ ), improvement in feed efficiency (0.97 (SD 0.02)). In the R<sub>70-20</sub> group, the feed intake was adjusted in the last 2 weeks to maintenance ration, and the final body weight (48.6 (SD 0.22) g) was accordingly lower. For more details on growth and bioenergetics, see Bermejo-Nogales *et al.*<sup>(29)</sup>.

As shown in Table 4, whole-body lipid content in wet matter basis was significantly lower in R<sub>70-20</sub> group (8.8%) in comparison to the other two experimental groups (12.3–11.7%). In parallel, the water content increased significantly and progressively with the reduction of ration size from 65.3% in the R<sub>100</sub> group to 69.8% in the R<sub>70-20</sub>. Similar trends were found for moisture and lipid content in fillet



**Fig. 1.** Body weight over the course of the growth trial of fish fed a practical diet with different feeding levels. ●—, Full ration until visual satiety (R<sub>100</sub>); -○-, 70% of saturation (R<sub>70</sub>); -▼-, 70% of saturation with the last 2 weeks at 20% of saturation level (R<sub>70-20</sub>).

**Table 4.** Fillet lipid class composition (% wet weight), moisture (%) and lipid content (% fillet wet weight) of whole body and fillets of fish fed at three different feeding levels (Mean values and standard deviations,  $n$  9)

	R <sub>100</sub>		R <sub>70</sub>		R <sub>70-20</sub>	
	Mean	SD	Mean	SD	Mean	SD
Whole-body moisture	65.34 <sup>a</sup>	0.17	66.53 <sup>b</sup>	0.31	69.80 <sup>c</sup>	0.42
Whole-body fat	12.31 <sup>a</sup>	0.38	11.68 <sup>a</sup>	0.39	8.79 <sup>b</sup>	0.28
Fillet moisture	72.44 <sup>a</sup>	0.36	74.75 <sup>b</sup>	0.44	75.51 <sup>b</sup>	0.53
Fillet fat	6.50 <sup>a</sup>	0.63	4.49 <sup>b</sup>	0.33	3.36 <sup>c</sup>	0.24
Fillet lipid class						
CE	0.25	0.13	0.22	0.13	0.18	0.15
TAG	4.00 <sup>a</sup>	1.20	2.16 <sup>b</sup>	0.76	2.37 <sup>b</sup>	0.89
NEFA	0.11 <sup>a</sup>	0.05	0.07 <sup>a,b</sup>	0.06	0.02 <sup>b</sup>	0.01
COL	0.53 <sup>a</sup>	0.09	0.37 <sup>b</sup>	0.11	0.40 <sup>b</sup>	0.09
DG	0.15 <sup>a</sup>	0.05	0.08 <sup>b</sup>	0.05	0.07 <sup>b</sup>	0.03
MG	0.06	0.01	0.04	0.01	0.04	0.02
PE	0.31 <sup>a</sup>	0.05	0.25 <sup>b</sup>	0.06	0.24 <sup>b</sup>	0.04
PA/CL	0.08	0.03	0.07	0.02	0.06	0.01
PI	0.08	0.02	0.07	0.02	0.07	0.01
PS	0.04	0.01	0.04	0.01	0.04	0.01
PC	0.48	0.07	0.41	0.10	0.41	0.09
SM	0.05	0.04	0.03	0.02	0.03	0.02
Total NL	5.10 <sup>a</sup>	0.47	2.94 <sup>b</sup>	0.34	3.08 <sup>b</sup>	0.38
Total PL	1.04	0.06	0.87	0.07	0.85	0.06

R<sub>100</sub>, full ration until visual satiety; R<sub>70</sub>, 70% of satiation; R<sub>70-20</sub>, 70% of satiation with the last 2 weeks at 20% of satiation level; CE, cholesteryl esters; COL, cholesterol; DG, diacylglycerols; MG, monoacylglycerols; PE, phosphatidyl ethanolamine; PA/CL, phosphatidic acid/cardioliipin; PI, phosphatidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl choline; SM, sphingomyelin; NL, neutral lipids; PL, polar lipids.

<sup>a,b,c</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ).

samples, but in this case, the reduction of lipid content was higher, changing progressively and significantly from 6.5% in the R<sub>100</sub> group to 3.3% in the R<sub>70-20</sub>. The reduction of ration size also affected the lipid class composition, mainly due to changes in neutral lipids, with a 5.1% reduction in the R<sub>100</sub> group and 2.9–3.1% in the other two experimental groups. A slight but significant reduction of phosphatidylethanolamine was found with the intermediate and severe reduction of ration size, but significant changes were not found for PL, considered globally as the sum of phosphatidylethanolamine, phosphatidylcholine (PC) and other minor polar lipid fractions.

The changes in ration size modified the fillet FA composition of TL (Table 5), with a statistically significant reduction in %FAME of 14:0, 16:1 $n$ -7, 18:1 $n$ -9, 18:2 $n$ -6, 18:3 $n$ -6 and 18:3 $n$ -3. This trend was the opposite for DHA, which achieved a maximum of 8.4% in the R<sub>70-20</sub> group and lower levels (7.2–7.3%) in R<sub>70</sub> and R<sub>100</sub> groups.

Experimental data evidenced minor changes in the FA composition of TAG (Table 6). Of note, only four FA (16:4, 20:1 $n$ -9, 22:1 $n$ -9, 22:6 $n$ -3) varied significantly and progressively with the reduction of ration size. This contrasted with the observations made in PL, where a high number of FA, including 14:0, 17:0, monoenes (16:1 $n$ -7, 18:1 $n$ -9, 18:1 $n$ -7, 20:1 $n$ -9) and most PUFA of  $n$ -3 (18:3 $n$ -3, 18:4 $n$ -3, 20:4 $n$ -3, 20:5 $n$ -3) and  $n$ -6 series (18:2 $n$ -6, 18:3 $n$ -6, 20:2 $n$ -6, 20:3 $n$ -6) decreased significantly in R<sub>70-20</sub> fish. This trend was opposite for 20:4 $n$ -6 (arachidonic acid) and DHA, which varied between two extreme groups from 2.4 to 2.2% and from 23.7 to 19.2%, respectively.

### Phylogenetic and gene expression analyses

Phylogenetic analysis of the endogenous extracellular TAG lipase family was performed with representative sequences of reptiles, mammals and fish, and the resulting tree revealed three major nodes of LPL, hepatic lipase and endothelial lipase (EL), according to the present hierarchy of vertebrates (Fig. 2(a)). A fourth node, closely related to LPL but exclusive of fish lineage, was identified. This additional node, named LPL-like, is presently represented by LPL sequences from zebrafish, tuna and sparid fish (red sea bream and gilthead sea bream).

As shown in Fig. 2(b), phylogenetic analysis of SCD1 sequences from modern teleost fish identified two major clades of SCD1a and SCD1b isoforms, according to the present hierarchy of teleosts. The analysis was carried out with sequences derived from model and non-model fish species, including those derived from gilthead sea bream. As expected, representative mammalian SCD1 sequences are fitted as outgroup when they are included in the analysis.

The muscle gene expression profile of the two extreme groups is shown in Fig. 3. Irrespective of experimental condition, the highest mRNA levels for the analysed lipid enzymes were encountered for *SCD1b*, *LPL-like* and *LPCAT3*, whereas lower levels were found for *FADS2* and then for *PEMT* and *SCD1a* (Fig. 3(a)). As concerns transcriptional regulatory factors (Fig. 3(b)), the highest mRNA expression was found for *SREBP1* and the lowest for *LXR $\alpha$* , with intermediate values for PPAR isoforms. When the gene expression pattern was compared between R<sub>100</sub> and R<sub>70-20</sub> groups, no differences were found at the level of *PEMT*, *LPCAT1*, *LPCAT2*, *LPCAT3*,

**Table 5.** Fatty acid (FA) composition (% total FA methyl esters) of total lipids from filets of fish fed different feeding levels: full ration until visual satiety (R<sub>100</sub> group), 70% of satiation (R<sub>70</sub>) and 70% of satiation with the last 2 weeks at 20% of satiation level (R<sub>70-20</sub>)

(Mean values and standard deviations, *n* 9)

FA (%)	R <sub>100</sub>		R <sub>70</sub>		R <sub>70-20</sub>	
	Mean	SD	Mean	SD	Mean	SD
14:0	3.30 <sup>a,b</sup>	0.09	3.50 <sup>a</sup>	0.26	3.16 <sup>b</sup>	0.30
15:0	0.12	0.02	0.12	0.02	0.11	0.02
16:0	16.65	1.00	17.55	1.05	15.23	1.54
16:1 <i>n</i> -7	5.59 <sup>b</sup>	0.09	5.37 <sup>a,b</sup>	0.32	5.21 <sup>a</sup>	0.32
16:2	0.71 <sup>a</sup>	0.02	0.59 <sup>b</sup>	0.05	0.66 <sup>a,b</sup>	0.07
16:3	0.79 <sup>a</sup>	0.03	0.72 <sup>b</sup>	0.04	0.73 <sup>b</sup>	0.05
16:4	0.51 <sup>a</sup>	0.07	0.38 <sup>b</sup>	0.10	0.45 <sup>a</sup>	0.06
17:0	0.36 <sup>a</sup>	0.01	0.40 <sup>b</sup>	0.02	0.35 <sup>a</sup>	0.03
18:0	4.40	0.23	4.79	0.38	4.36	0.49
18:1 <i>n</i> -9	18.27 <sup>a</sup>	0.68	17.41 <sup>b</sup>	1.04	16.83 <sup>b</sup>	0.89
18:1 <i>n</i> -7	2.89	0.07	2.83	0.16	2.74	0.19
18:2 <i>n</i> -6	18.75 <sup>a</sup>	0.20	17.48 <sup>b</sup>	0.92	16.98 <sup>b</sup>	1.16
18:3 <i>n</i> -6	0.24 <sup>a</sup>	0.01	0.21 <sup>b</sup>	0.01	0.21 <sup>b</sup>	0.02
18:3 <i>n</i> -3	2.05 <sup>a</sup>	0.02	1.78 <sup>b</sup>	0.10	1.80 <sup>b</sup>	0.13
18:4 <i>n</i> -3	0.91 <sup>a</sup>	0.15	0.74 <sup>b</sup>	0.07	0.84 <sup>a,b</sup>	0.07
20:1 <i>n</i> -7	0.16	0.03	0.15	0.02	0.14	0.02
20:1 <i>n</i> -9	1.34	0.02	1.34	0.09	1.35	0.13
20:2 <i>n</i> -6	0.50	0.10	0.48	0.06	0.49	0.04
20:3 <i>n</i> -6	0.37 <sup>a</sup>	0.06	0.27 <sup>b</sup>	0.11	0.30 <sup>a,b</sup>	0.04
20:3 <i>n</i> -3	0.09 <sup>a</sup>	0.01	0.07 <sup>b</sup>	0.02	0.08 <sup>a,b</sup>	0.01
20:4 <i>n</i> -6	0.68	0.05	0.66	0.17	0.75	0.09
20:4 <i>n</i> -3	0.52	0.02	0.42	0.14	0.49	0.03
20:5 <i>n</i> -3	6.48	0.34	5.96	0.37	6.34	0.49
22:1 <i>n</i> -9	0.28	0.01	0.26	0.05	0.28	0.03
22:1 <i>n</i> -11	0.84	0.03	0.87	0.11	0.89	0.08
22:5 <i>n</i> -3	2.40	0.15	2.05	0.13	2.33	0.20
22:6 <i>n</i> -3	7.33 <sup>a</sup>	0.56	7.25 <sup>a</sup>	0.83	8.45 <sup>b</sup>	1.07

<sup>a,b</sup> Mean values within experimental groups with unlike superscript letters were significantly different (*P* < 0.05).

*LPL*, *FADS2*, *FAS*, *PPARγ*, *LXRα* and *SREBP1*. In contrast, the expression of *LPL-like*, *PPARα* and *PPARβ* was significantly up-regulated in the R<sub>70-20</sub> group, whereas that of *SCD1a* and *SCD1b* was significantly down-regulated. When data for a given gene in R<sub>70-20</sub> fish were referred to R<sub>100</sub> fish, the fold-changes for the up-regulated genes were 1.93, 2.02 and 1.92 for *LPL-like*, *PPARα* and *PPARβ*, respectively. In the case of down-regulated genes, the fold-changes were 0.17 for *SCD1a* and 0.62 for *SCD1b*.

## Discussion

Muscle lipid content has a considerable interest for the farm animal industry, as it strongly affects meat quality<sup>(30)</sup>. Hence, dietary manipulation and genetic selection are presently the main tools used to manage muscle fat content in farmed fish<sup>(31–33)</sup>. Different flesh lipid deposition rates also modify the nutritional value of the end product and, thereby, the present study was focused on the effect of ration size on the fillet FA profiles of gilthead sea bream. From the present results, it is conclusive that the reduction of ration size entailed the decrease of lipid content of whole body and fillet, which was accompanied by a decrease of water content, whereas that of protein and ash remained unchanged (data not shown). The decrease of TL was closely associated to the

reduction of neutral lipid content, whereas the amount of polar lipids remained unchanged. Regarding FA profiles, our previous gilthead sea bream study highlights the fact that the fillet FA composition of PL remains mostly unaltered when the theoretical requirements of essential FA are met through diet<sup>(34)</sup>. Moreover, the robustness of PL is apparently greater in tissues with higher dietary requirements in essential FA<sup>(35)</sup>. This contrasts with the plasticity of TAG, which closely resembles the FA composition of the diet in a wide range of cultured fish, including gilthead sea bream<sup>(35)</sup>, European sea bass<sup>(36)</sup>, Atlantic cod<sup>(37)</sup>, turbot<sup>(38)</sup>, Atlantic salmon<sup>(39)</sup>, sunshine bass<sup>(40)</sup>, pike perch<sup>(41)</sup> and arctic charr<sup>(42)</sup>. However, ration size and dietary lipid sources affect the FA composition of neutral and polar lipids differentially, at least when evaluated in juvenile gilthead sea bream fed at maintenance ration for 2 weeks in the summer growing period. Indeed, the fillet FA profile of TAG remained mostly unaltered with the reduction in ration size and muscle lipid depots, whereas polar lipids exhibited significant changes that mostly affected arachidonic acid and DHA in the case of LC-PUFA. Earlier studies in rainbow trout<sup>(14)</sup> and chinook salmon<sup>(15)</sup> do not support this finding, and the authors concluded that the effect of ration on muscle FA composition is primarily due to changes in the relative levels of PL and TAG. However, in the present study, changes in the PL profile contribute directly and/or indirectly to the lean phenotype of R<sub>70-20</sub>. It is not easy to explain the differences in lipid metabolism of these apparent fish species, but it should be stressed that the overall literature strongly supports the allostatic regulation of PL in response to different stimuli<sup>(17)</sup>.

Several studies in cultured fish with varying feeding levels<sup>(43,44)</sup> and diet composition<sup>(3,45–48)</sup> denote that both arachidonic acid and DHA can be selectively retained to meet the requirements for tissue membrane FA and function. It has also been observed that lean strains of Atlantic salmon accumulate *n*-3 LC-PUFA more rapidly than fat fish<sup>(33)</sup>. For instance, intervention studies in human subjects and rodents highlight the fact that hypoenergetic diets facilitate the unsaturation of skeletal muscle PL in association with improved insulin sensitivity<sup>(49–52)</sup>. This agrees with the observation that the Pima Indians, a population with a high prevalence of diabetes and obesity, display a low concentration of *n*-3 LC-PUFA in muscle PL<sup>(53)</sup>. Conversely, insulin sensitivity in obese subjects can be facilitated by the incorporation of DHA in the muscle membrane to a level that may exceed the level found in a lean healthy background population<sup>(54,55)</sup>. This functional association has not been demonstrated in fish yet, although the increased incorporation of DHA in the muscle PL of R<sub>70-20</sub> fish can be viewed as part of the complex regulatory mechanisms that orchestrate the change towards energy storage rather than energy mobilisation when nutrient insulin secretagogues become available above a critical threshold level. This type of regulation also operates on a daily basis, and earlier studies in the European sea bass evidenced that the stimulation of feeding behaviour in fish fed at mid-day is accompanied by the phase advancement of circadian insulin rhythms<sup>(56)</sup>.

**Table 6.** Effect of feeding ration on the fillet fatty acid (FA) profile of TAG and phospholipids (% of total FA methyl esters; mean values and standard deviations of individual fish, *n* 9)

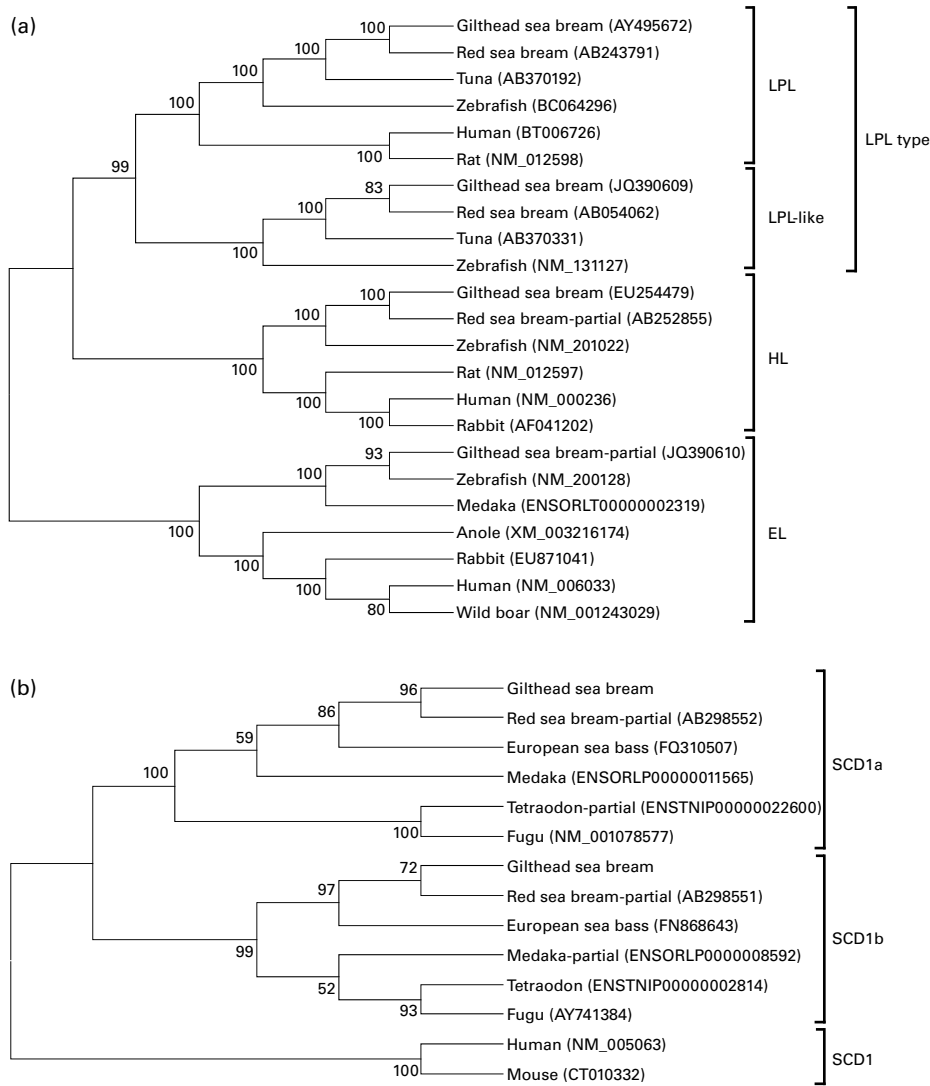
FA	TAG (%)						Phospholipids (%)					
	R <sub>100</sub>		R <sub>70</sub>		R <sub>70-20</sub>		R <sub>100</sub>		R <sub>70</sub>		R <sub>70-20</sub>	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	3.60	0.11	3.63	0.19	3.69	0.10	0.94 <sup>a</sup>	0.12	0.83 <sup>a,b</sup>	0.16	0.75 <sup>b</sup>	0.09
16:0	14.99	0.49	15.05	0.46	14.72	0.30	17.31	0.43	17.53	0.50	17.33	0.55
16:1 <i>n</i> -7	6.16	0.15	6.16	0.09	6.25	0.11	1.55 <sup>a</sup>	0.12	1.50 <sup>a</sup>	0.14	1.22 <sup>b</sup>	0.10
16:2	0.80	0.03	0.79	0.02	0.80	0.03	0.52	0.19	0.39	0.12	0.40	0.10
17:0	0.32	0.02	0.33	0.02	0.33	0.01	0.39 <sup>a</sup>	0.03	0.37 <sup>a,b</sup>	0.03	0.34 <sup>b</sup>	0.03
16:3	0.76	0.29	0.85	0.12	0.76	0.31	0.15	0.07	0.19	0.14	0.13	0.05
16:4	0.76 <sup>a</sup>	0.07	0.74 <sup>a,b</sup>	0.04	0.70 <sup>b</sup>	0.03	0.25	0.03	0.25	0.03	0.27	0.05
18:0	3.23	0.35	3.25	0.12	3.25	0.11	7.74	0.40	7.55	0.25	7.70	0.34
18:1 <i>n</i> -9	19.00	0.97	18.84	0.48	19.10	0.36	11.09 <sup>a</sup>	0.33	10.63 <sup>a,b</sup>	0.82	10.18 <sup>b</sup>	0.40
18:1 <i>n</i> -7	2.73	0.18	2.76	0.07	2.83	0.07	2.90 <sup>a</sup>	0.15	2.68 <sup>a,b</sup>	0.21	2.63 <sup>b</sup>	0.04
18:2 <i>n</i> -6	19.82	0.80	19.14	0.79	19.04	0.42	12.56 <sup>a</sup>	0.50	12.09 <sup>a</sup>	0.51	11.19 <sup>b</sup>	0.56
18:3 <i>n</i> -6	0.26	0.01	0.26	0.01	0.26	0.01	0.18	0.01	0.17	0.01	0.17	0.01
18:3 <i>n</i> -3	2.29	0.20	2.17	0.10	2.14	0.05	0.82 <sup>a</sup>	0.04	0.76 <sup>b</sup>	0.06	0.62 <sup>c</sup>	0.05
18:4 <i>n</i> -3	1.00	0.08	1.01	0.05	1.00	0.04	0.07 <sup>a</sup>	0.11	0.14 <sup>b</sup>	0.02	0.07 <sup>a</sup>	0.06
20:0	0.20	0.04	0.21	0.02	0.22	0.02	0.36 <sup>a</sup>	0.21	0.22 <sup>a,b</sup>	0.06	0.18 <sup>b</sup>	0.05
20:1 <i>n</i> -7	0.19	0.02	0.19	0.02	0.20	0.02	–	–	–	–	–	–
20:1 <i>n</i> -9	1.36 <sup>a</sup>	0.19	1.47 <sup>a,b</sup>	0.14	1.56 <sup>b</sup>	0.10	0.67 <sup>a</sup>	0.11	0.57 <sup>a,b</sup>	0.05	0.55 <sup>b</sup>	0.18
20:2 <i>n</i> -6	0.31	0.03	0.26	0.03	0.27	0.03	0.40 <sup>a</sup>	0.08	0.28 <sup>b</sup>	0.05	0.29 <sup>b</sup>	0.08
20:3 <i>n</i> -6	0.33	0.06	0.29	0.06	0.30	0.03	0.56 <sup>a</sup>	0.10	0.42 <sup>b</sup>	0.11	0.41 <sup>b</sup>	0.07
20:3 <i>n</i> -3	0.10	0.01	0.09	0.01	0.10	0.01	–	–	–	–	–	–
20:4 <i>n</i> -6	0.53	0.03	0.53	0.02	0.54	0.01	2.18 <sup>a</sup>	0.06	2.32 <sup>a,b</sup>	0.21	2.45 <sup>b</sup>	0.10
20:4 <i>n</i> -3	0.55	0.05	0.53	0.04	0.55	0.03	0.35 <sup>a</sup>	0.03	0.32 <sup>a,b</sup>	0.04	0.30 <sup>b</sup>	0.03
20:5 <i>n</i> -3	6.33	0.49	6.36	0.35	6.15	0.21	11.18 <sup>a</sup>	0.14	11.17 <sup>a,b</sup>	0.72	10.49 <sup>b</sup>	0.57
22:0	0.11	0.03	0.12	0.03	0.13	0.01	–	–	–	–	–	–
22:1 <i>n</i> -9	0.30 <sup>a</sup>	0.06	0.32 <sup>a,b</sup>	0.04	0.34 <sup>b</sup>	0.03	–	–	–	–	–	–
22:1 <i>n</i> -11	0.85	0.21	0.97	0.16	1.03	0.12	–	–	–	–	–	–
22:5 <i>n</i> -3	2.53	0.26	2.41	0.21	2.52	0.18	3.10	0.17	2.95	0.15	3.02	0.19
22:6 <i>n</i> -3	6.48 <sup>a</sup>	0.19	6.73 <sup>a,b</sup>	0.34	6.95 <sup>b</sup>	0.28	19.17 <sup>a</sup>	0.45	21.34 <sup>b</sup>	1.46	23.75 <sup>c</sup>	1.39

R<sub>100</sub>, full ration until visual satiety; R<sub>70</sub>, 70% of satiation; R<sub>70-20</sub>, 70% of satiation with the last 2 weeks at 20% of satiation level.  
<sup>a,b,c</sup> Mean values for each lipid class with unlike superscript letters were significantly different (*P* < 0.05).

It is well known that PL are first synthesised in the *de novo* Kennedy pathway<sup>(57)</sup>, but their FA composition at the sn-2 position is altered in the remodelling pathway (Land's cycle)<sup>(58)</sup> through the concerted action of acyltransferases and phospholipase A<sub>2</sub> enzymes. Both biosynthetic pathways have also been described in teleost fish<sup>(59,60)</sup>. The discovery of a number of acyltransferases has recently occurred<sup>(61)</sup>, but it appears that both mammals and fish contain a number of LysoPL acyltransferases that exhibit distinct acyl-CoA and lysoPL acceptor specificities<sup>(62)</sup>. Herein, we focused on LPCAT, given that PC is the principal component of animal cellular membranes<sup>(63)</sup>, and interestingly, the three LPCAT reported in human subjects<sup>(64)</sup> are represented by the corresponding orthologues (LPCAT1–3) in our gilthead sea bream transcriptome database. Besides, all of them were expressed at detectable levels in the skeletal muscle of gilthead sea bream (*LPCAT1* < *LPCAT2* < *LPCAT3*), but none was transcriptionally regulated by ration size in the present experimental model, which limits their role as master regulators of PL FA composition. In this respect, it must also be noted that LPCAT are highly selective for PUFA, but do not discriminate well between *n*-6 PUFA and *n*-3 PUFA, which would explain, at least in part, why the membrane FA composition of rat skeletal muscle is highly responsive to the balance between *n*-6 and *n*-3 PUFA in the diet<sup>(65)</sup>.

Some other important regulatory steps of PL synthesis and remodelling are the transmethylation reactions catalysed by the PEMT enzyme to convert phosphatidylethanolamine to PC. Of note, cystidine diphosphate choline is the major source of PC under normal physiological conditions but, interestingly, the expression of PEMT is markedly up-regulated in the liver tissue of human subjects and rodents when dietary choline or PC are deficient<sup>(66–68)</sup>. In consequence, as phosphatidylethanolamine unsaturation is generally higher than that of PC<sup>(60,69)</sup>, a different contribution of cystidine diphosphate choline and PEMT pathways on PC biosynthesis may alter the final composition of plasma membrane PL. Nevertheless, as reported above for LPCAT, the PEMT enzyme was not transcriptionally regulated by severe energy restriction in the present experimental model, which suggests that the observed changes in PL FA composition were more directly related to switches in FA metabolism than PL biosynthesis and remodelling. In that sense, other authors have suggested that PL biosynthesis pathways are limited or inefficient for several fish species (reviewed by Tocher *et al.*<sup>(60)</sup>).

Obviously, one important issue in FA metabolism is the capability for LC-PUFA biosynthesis of different fish species. This has been widely studied in recent years, and a general statement in marine fish, including gilthead sea bream<sup>(70)</sup>, is that the expression of *FADS2* ( $\Delta$ -6 desaturase) is induced at



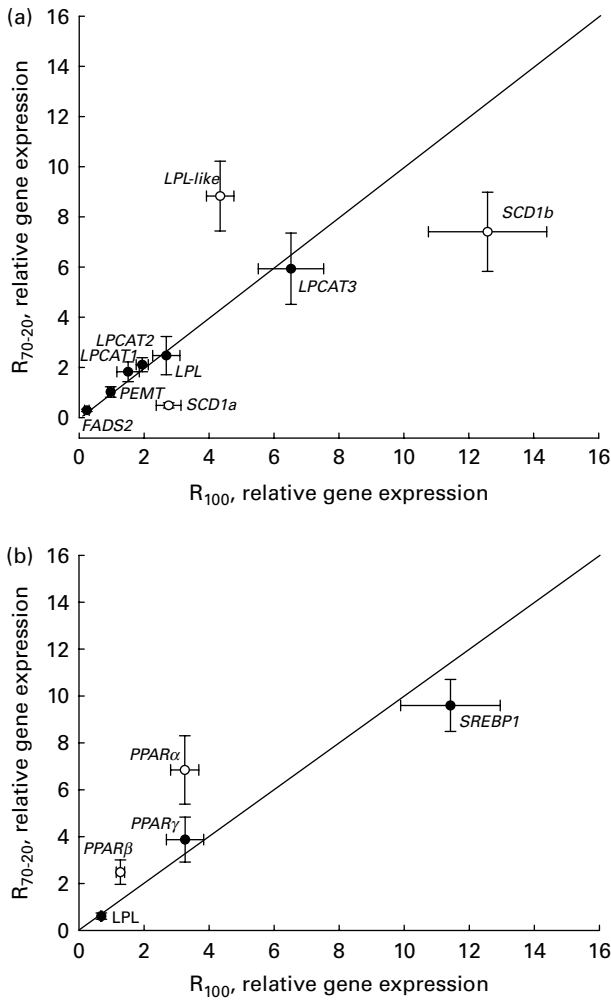
**Fig. 2.** Phylogenetic analyses of (a) endogenous extracellular TAG lipase family and (b) stearoyl-CoA desaturase 1 family. Bootstrap values are shown at each node. LPL, lipoprotein lipase; LPL-like, lipoprotein lipase-like; HL, hepatic lipase; EL, endothelial lipase; SCD1a, stearoyl-CoA desaturase 1a; SCD1b, stearoyl-CoA desaturase b.

very low levels. This, together with the blockage of  $\Delta$ -5 desaturase and  $C_{18}$ - $C_{20}$  elongase steps, greatly limits the flux through LC-PUFA biosynthetic pathways in marine fish<sup>(71,72)</sup>. In the present study, this point of view is reinforced by the reduced mRNA expression of *FADS2* in comparison to other analysed transcripts. Additionally, *FADS2* was little responsive to changes in ration size; therefore, the enhanced incorporation of LC-PUFA (arachidonic acid and DHA) in muscle PL of  $R_{70-20}$  fish cannot be explained by the enhanced biosynthesis of LC-PUFA.

The SCD enzymes with  $\Delta$ -9 desaturase activity are ubiquitously found in all living organisms and their mRNA expression, in particular that corresponding to SCD1 enzymes, was highly regulated by ration size in the present experimental model. Major products of SCD activity are palmitoleic acid and oleic acid, but the evolutionary history of these rate-limiting enzymes in the biosynthesis of monoenes is a complex process of duplication and loss events. Thus, as

recently reviewed by Castro *et al.*<sup>(28)</sup>, a different number of paralogous genes (*SCD1*–*SCD5*) have been reported in tetrapods and fish. In particular, SCD enzymes have evolved through the evolution of teleost lineage as duplicated genes of SCD1-type (*SCD1a*, *SCD1b*), as was further corroborated by the phylogenetic analyses of SCD sequences, including those from gilthead sea bream. From the present results, it is also conclusive that the two SCD isoforms of gilthead sea bream were significantly expressed in the skeletal muscle, although clearly the most abundant mRNA was that codifying for the SCD1b isoform. A different tissue expression level has also been reported in the adipose tissue for the two SCD1 enzymes of red sea bream<sup>(73)</sup>, but regardless of this, the two gilthead sea bream SCD1 isoforms were down-regulated by ration size in the skeletal muscle, which was not surprising given that SCD enzymes are strong markers of lipogenesis in human subjects and rodents and low SCD expression and





**Fig. 3.** Relative mRNA expression of (a) lipid enzymes and (b) transcription regulatory factors in the two extreme groups (full ration until visual satiety (R<sub>100</sub>) and 70% of satiation with the last 2 weeks at 20% of satiation level (R<sub>70-20</sub>)) are plotted against each other in the scatter plot. Genes encoding lipoprotein lipase (*LPL-like*), *PPARα* and *PPARβ*, are significantly up-regulated ( $P < 0.05$ ) in R<sub>70-20</sub> fish. Genes encoding stearoyl-CoA desaturase 1a (*SCD1a*) and stearoyl-CoA desaturase 1b (*SCD1b*) are significantly down-regulated ( $P < 0.05$ ) in R<sub>70-20</sub> fish. *LPCAT1*, lysophosphatidylcholine acyltransferase 1; *LPCAT2*, lysophosphatidylcholine acyltransferase 2; *LPCAT3*, lysophosphatidylcholine acyltransferase 3; *PEMT*, phosphatidylethanolamine *N*-methyltransferase; *FADS2*, fatty acid desaturase 2; *SREBP1*, sterol regulatory element-binding protein-1; *LPLα*, lipoprotein lipase α.

activity have been associated to a reduced risk of obesity and insulin resistance<sup>(74–78)</sup>.

A third means of regulation of tissue FA composition is the endogenous extracellular TAG lipases, which act as key-limiting enzymes on tissue FA uptake. The overall literature strongly supports a distinct regulation of LPL, hepatic lipase and EL, with distinct substrate specificities to accommodate the full spectrum of circulating lipoproteins<sup>(79)</sup>. Hence, LPL displays the highest TAG activity and EL the lowest, whilst at the same time becoming more active as phospholipase<sup>(80)</sup>. The expression of lipases is also tissue specific and EL is synthesised by endothelial cells and by other cell types such as macrophages<sup>(81)</sup>, which agrees with the observation that the

gilthead sea bream EL sequence (JQ390610) derived from *de novo* assembly of next-generation sequencing data contains mostly nucleotide reads from head kidney, the equivalent of bone marrow in mammals. Conversely, the expression of hepatic lipase is restricted to liver (A Saera-Vila and J Pérez-Sánchez, unpublished results), whereas LPL is more ubiquitous, and together with the hormone-sensitive lipase drives the reallocation of body fat depots in fish either with lean phenotypes<sup>(26)</sup> or with nutritionally mediated changes in lipid storage capacity<sup>(25,82)</sup>. In mammals, it is also well known that fasting down-regulates the LPL activity of adipose tissue<sup>(83,84)</sup>, with no changes or increases in heart and skeletal muscle<sup>(85,86)</sup>. Likewise, fasting decreases LPL activity and mRNA expression in the adipose tissue of gilthead sea bream, which is reversed by either insulin or arginine (potent insulin secretagogue) treatments<sup>(87)</sup>. In the same study, a role of insulin in the regulation of LPL has not been proved in other tissues, which is not surprising given that results from this and previous studies<sup>(25,88)</sup> highlight a constitutive or low expression of LPL in the white skeletal muscle. In contrast, *LPL-like* is the predominant LPL isoform in the skeletal muscle of gilthead sea bream and its mRNA expression was markedly up-regulated by the reduction of ration size. Experimental evidence also indicates that LPL transcripts are differentially regulated in the liver tissue of Atlantic salmon in response to diet<sup>(89)</sup>. A different tissue distribution has also been reported for LPL and *LPL-like* in red sea bream<sup>(27)</sup>, which suggests that, at least in sparid fish, *LPL-like* is preferentially expressed in muscle tissues in a well-regulated manner. This offers the possibility of a complex tissue regulation that might contribute to modifying the muscle PL FA composition if LPL and *LPL-like* enzymes actually have distinct TAG and PL activities.

TNF-α is a mediator of insulin resistance, and important studies with TNF-α-deficient mice evidenced the improvement of insulin sensitivity in hyperphagic models of rodent obesity<sup>(90)</sup>. In mammals, most of these TNF-α-mediated effects are produced by stimulating lipolysis and inhibiting LPL activity at transcriptional and post-transcriptional levels<sup>(91)</sup>. A lipolytic effect of recombinant human TNF-α has been demonstrated in freshly isolated adipocytes of rainbow trout<sup>(92)</sup> and gilthead sea bream<sup>(88)</sup> and, interestingly, sequence analysis of the 5'-flanking region of gilthead sea bream LPL reveals a strong conservation of some regulatory elements (GC box, CCAAT/NF-Y, OCT-1) with a synergistic effect on the promoter activity of avian and mammalian *LPL* genes. Conserved regulatory elements for LXR, *PPARα* and *SREBP1* have also been reported in the promoter region of SCD enzymes<sup>(93)</sup>, although, unfortunately, SCD promoters remain little studied in fish and in gilthead sea bream, in particular. This makes the interpretation of results arising from upstream regulatory transcriptors more difficult, although it must be noted that *PPARα*, and also *PPARβ*, were responsive to changes in ration size in the present experimental model. In the case of *PPARα*, its increased mRNA expression with the reduction of ration size is not surprising given that this PPAR isoform has been traditionally associated with lipolytic and SCD-deficient phenotypes<sup>(74,78,94)</sup>. More difficult to explain is the response of

PPAR $\beta$ , but given that this more ubiquitous PPAR isoform has recently been associated to lipogenic rather than lipolytic roles<sup>(95,96)</sup>, the reduced but also stimulated expression of PPAR $\beta$  in R<sub>70-20</sub> fish can be viewed as a counter-regulatory response to maintain the muscle lipolytic and lipogenic pathways finely regulated. Indirect evidence for this is provided by the earlier *in vitro* studies, in which the lipolytic TNF- $\alpha$  cytokine exerts dual effects in primary cultures of gilthead sea bream adipocytes that differ among and between lean and fat fish<sup>(26)</sup>. However, at least in one set of fat fish, the lipolytic effects of TNF- $\alpha$  were apparently mediated by the down-regulated expression of PPAR $\beta$ .

In summary, a number of new gilthead sea bream sequences (*EL*, *LPL-like*, *LPCAT*, *PEMT*, *SCD1*, *SREBP1*) with important roles in lipid metabolism have been introduced in public database repositories. Additionally, new insights into muscle FA composition and mRNA expression patterns have been addressed in an experimental model of ration size. Interestingly, the results highlight a distinct regulation of FA composition in neutral and polar lipids, which reinforces the concept of the allostatic regulation of PL. From a mechanistic point of view, it is complex to define the vast array of enzymes and upstream regulatory transcription factors that contribute to modify the membrane FA composition, although PPAR, *LPL-like* and *SCD1* are emerging as reliable target genes to direct marine fish production and selection towards lean muscle fish phenotypes with increased retentions of LC-PUFA.

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