

1 CBP ms.21484 Revised – part B

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3 **Dynamics of PPARs, fatty acid metabolism genes and lipid classes in eggs and**
4 **early larvae of a teleost**

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6 **Cunha I^{a*}, Galante-Oliveira S^{a§}, Rocha E^{a,b}, Planas M^c, Urbatzka R^a, Castro**
7 **LFC^a**

8

9 *^a Interdisciplinary Centre for Marine and Environmental Research (CIIMAR), CIMAR Associate*
10 *Laboratory, University of Porto (U.Porto), Portugal*

11 *^b Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Portugal*

12 *^c Instituto de Investigaciones Marinas (CSIC), Vigo, Spain*

13 *[§] Present address: Department of Biology & Estudos do Ambiente e do Mar (CESAM), University of*
14 *Aveiro, Portugal,*

15

16 *Corresponding author:

17 Isabel Cunha, Rua dos Bragas 289, 4050-123 Porto, Portugal.

18 phone: +351 22 340 18 18, fax: +351 22 339 06 08

19 E-mail address: isabel.cunha@ciimar.up.pt

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21 **Abbreviations:** dpf – days post fertilization; FA, fatty acids; FFA, free fatty acids; HUFA,
22 highly unsaturated fatty acids; LPA, Lysophosphatidic acid; MUFA, monounsaturated fatty
23 acids; PL, phospholipids; PPAR – peroxisome proliferator activated receptor; PUFA,
24 polyunsaturated fatty acids; SE, Sterol esters; SL, sterols; SFA, saturated fatty acids; TG,
25 triglycerides; WE, wax esters.

26

27 **Abstract**

28 Dietary long chain polyunsaturated fatty acids (FA) have been recognized of crucial importance
29 in early development of vertebrates, contributing to the impressive morphological and
30 physiological changes both as building blocks and to energy production. The importance of
31 lipids along development depends on ontogenetic, phylogenetic and environmental parameters.
32 The expression patterns of FA metabolism genes have not been characterized in developing fish
33 embryos nor compared to lipid class profiles. Full lipid metabolism only occurred after

1 hatching, as revealed by alterations in lipid profiles and FA gene expression. Nonetheless,
2 transcriptional changes of some FA genes were already present in embryos at notochord
3 formation. Many genes displayed an expression profile opposed to the decrease of lipids along
4 the development, while others responded solely to starvation. Transcription of most genes
5 involved in FA metabolism had a strong correlation to PPARs mRNA levels ($\alpha 1$, $\alpha 2$, β , γ). The
6 comparison of mRNA expression of the genes with the lipid profiles produced new insights into
7 the FA metabolism and regulation during the development of turbot larvae, providing the basis
8 for future studies including comparative approaches with other vertebrate species.

9

10 **Keywords:** development – embryo - fatty acids – fish larvae - gene transcription - lipid
11 metabolism - peroxisome proliferator activated receptors - *Scophthalmus maximus*

12

13 **1. Introduction**

14

15 Dietary long chain polyunsaturated fatty acids (PUFAs) are very important to the nutritional
16 health, physiology and reproduction of vertebrates (Burr, 1981; Simopoulos, 2000) and of
17 crucial importance in early development, due the occurrence of impressive morphological and
18 physiological changes (Tocher, 2003). Highly unsaturated fatty acids (HUFAs) have long been
19 recognized as essential components in fish larval diets (Sargent et al., 1999), playing both an
20 energetic or structural role, depending on the lipid class molecules they are incorporated in. As
21 components of phospholipids (PL) integrated in cell membranes, docosahexaenoic (22:6n-3) and
22 arachidonic (20:4n-6) acids are particularly required for proper neural development and function
23 (Lauritzen et al., 2001).

24 Peroxisome proliferator activated receptors (PPARs) are involved in many processes related to
25 ontogenesis, such as skeletal formation and differentiation, cell proliferation and epithelial cell
26 growth and differentiation (Michalik et al., 2002; Burdik et al., 2006), lipid metabolism
27 regulation, lipid transport, lipid and glucose oxidation, adipogenesis, lipid homeostasis, (Jump,
28 2002; Varga et al., 2011; Cour Poulsen et al., 2012; Cho et al., 2012) peroxisomal biogenesis
29 (Schrader et al., 2012) and immune functions (Kostadinova et al., 2005). PUFA, oxidized PUFA
30 and eicosanoids are ligands of all PPAR isoforms in mammals and amphibians (Hihi et al.,
31 2002) thereby serving as major transcriptional sensors of fatty acids (FA) (Jump, 2008; Xu et
32 al., 1999; Schupp and Lazar, 2010). Interestingly, the three PPAR subtypes display distinct but
33 overlapping expression and functions (Cour Poulsen et al., 2012). In mammals, PPAR α and
34 PPAR β activate lipid catabolism by regulating expression of target genes encoding enzymes
35 involved in peroxisomal and mitochondrial β -oxidation of FA, the former mainly in liver and

1 the second ubiquitously distributed (Wang et al., 2008; Mandard et al., 2004), while
2 PPAR γ controls lipid accumulation and regulates adipogenesis and osteogenesis (Nedergaard et
3 al., 2005; Ji et al., 2011). It is not very clear whether PPARs have a similar role in mice and
4 humans and to what extent the regulation of PPAR target genes is shared between the two
5 species (Rakhshandehro et al., 2009). Whether the repertoire of PPAR target genes in teleosts is
6 similar to that of mammals is even more uncertain. Compounds that induce proliferation of
7 peroxisomes in rodents, such as fibrates, halogenated hydrocarbons, plasticizers, herbicides
8 and pesticides, may have small or no effect in other species (Lake et al., 1989) due to
9 differences in relative expression of PPAR α (Kliewer et al., 1994; Tugwood et al., 1998).
10 Moreover, affinities of the ligands to the receptors can vary considerably among species and
11 PPAR isotypes, mainly due to differences in the ligand binding domain (Krev et al., 1997). In
12 mammals, PPARs consist of three isotypes, PPAR α , β , and γ while in teleosts an additional
13 PPAR α is present due to the teleost specific whole genome duplication (Robinson-Rechavi et
14 al., 2009).

15 Turbot is an economically important fish species in Spain, France and Portugal. The Food and
16 Agriculture Organization of the United Nations estimated the world aquaculture production in
17 2009 as 69,557 T (FAO, 2011). Nevertheless, continued research and development effort is
18 required in various areas, namely the fry production, with the aim of increasing larval survival
19 rates and reliability of the process. Various malformations (impaired eye migration, anomalies
20 in visual and central nervous system development, skeletal deformities), malpigmentation,
21 decreased growth and poor feeding rates, reduction of stress tolerance and immune system
22 efficiency, and unexpected high mortality rates are some of the problems faced - many of them
23 related to larvae or broodstock nutritional problems related to HUFAs (Estevéz et al., 1995;
24 Izquierdo et al., 1996; Rainuzzo et al., 1997; Naes and Lie 1998; Estevez et al., 1999; Schields
25 et al., 1999; Hamre et al., 2007; Sargent et al., 1999).

26 In turbot eggs, lipids are present in two distinct forms, namely in the yolk and in the oil globule
27 (Silversand et al., 1996). Lipoprotein yolk lipids are primarily polar lipids, especially
28 phosphatidylcholine and phosphatidylethanolamine (Wiegand, 1996). In contrast, the oil globule
29 consists of neutral lipids such as triglycerides (TG), sterol esters (SE) and wax esters (WE)
30 (Wiegand, 1996). It was estimated that 55 to 60% of the lipids in turbot eggs are confined to the
31 oil globule (Silversand et al., 1996). PL appear to constitute 40 to 50% of the total lipids present
32 in turbot eggs (Devauchelle et al., 1988; McEnvoy et al., 1993; Planas et al., 1993). Since no PL
33 are present in the oil globule, it is reasonable to suggest that they are exclusively in the yolk and
34 that yolk lipid of turbot eggs, to a large extent, consist of PL (Silversand et al., 1996).

1 Molecular approaches may be helpful to clarify various aspects of FA metabolism in fish
2 development as well as to identify physiological differences between distinct phylogenetic
3 clades (Castro et al., 2011; Castro et al., 2012; Morais et al., 2012). Clarifying species specific
4 nutritional requirements, effects of nutrients deficiency and starvation, and response to chemical
5 contaminant exposure are important steps to understand the mechanisms, control and regulation
6 of lipid metabolism. In this study, the mRNA transcription was analyzed for the four isoforms of
7 PPAR ($\alpha 1$, $\alpha 2$, β and γ) and for 24 genes involved in various pathways of FA metabolism during
8 the early development of turbot larvae under starving conditions, spanning the period from
9 notochord formation to 9 days post fertilization (dpf). In parallel, the lipid profile was
10 investigated including measurements of total lipids, main lipid classes and FAs fractions. The
11 aim of the study was to compare the lipid profile with the mRNA expression pattern of genes
12 involved in the FA metabolism during the early larval stages of turbot. Here we provide for the
13 first time a comprehensive molecular and analytical snapshot of FA metabolism and regulation
14 during the development of turbot larvae, which should serve as a solid basis for future studies in
15 turbot and other teleost fish, but also for vertebrate classes in comparative approaches.

16

17 **2. Material and methods**

18 2.1. Egg Incubation and Larval Rearing

19 Fertilized eggs of *Scophthalmus maximus* were obtained from a commercial hatchery (Insuiña,
20 Pescanova S.A.) at Mougás – Spain. Larvae were reared from fertilized eggs to 9 dpf in 60 L
21 fibre glass tanks in artificial salt water (35 psu) with gentle and constant aeration at $15 \pm 1^\circ\text{C}$.
22 50% of the artificial salt water was changed daily. Larvae were not feed after mouth opening.
23 Samples were collected at 4 key developmental events: notochord formation (2.5 dpf), hatching
24 (4.5 dpf), mouth opening (7 dpf) and 50% mortality (9 dpf) for yolk sac and oil globule
25 measurements, lipid and FA analysis and molecular biology.

26 2.2. Yolk sac and oil globule measurements

27 Fifteen to twenty eggs/larvae were photographed under a microscope (Olympus IX71®) at each
28 developmental point in order to measure yolk sac and oil globule. Yolk sac volume was
29 calculated from length and width measures assuming a prolate ellipsoid ($4/3\pi a^2b$), where a is
30 the equatorial radius and b is the polar radius). Oil globule volume was calculated from the
31 diameter assuming a spherical form.

32 2.3. Lipid Classes and FA Analysis

33 Lipid extraction was performed following Blight and Dyer (1959). Total lipid content was
34 determined gravimetrically in duplicate samples of 175-400 larvae/eggs. Five lipid classes were
35 quantified on the total lipids fraction: triglycerides (TG), phospholipids (PL), sterol esters + wax

1 esters (SE+WE), sterols (SL), and free fatty acids (FFA). Lipid classes were analysed by thin
2 layer chromatography-densitometry using the Freeman and West (1966) plate staining method.
3 Tripalmitin, cholesterol, cholesterol palmitate and palmitic acid (Sigma) were used as standards
4 for TG, SL, SE+WE and FFA, respectively. PL were quantified following Holland and Gabbot
5 (1971) and Hausen and Grasshoff (1983). SE and WE could not be separated by the solvent
6 mixture and are therefore reported together as a sum. FA were transesterified to methyl esters on
7 total lipid extracts with methanolic hydrogen chloride (Christie, 1982) and, subsequently
8 analysed by gas chromatography, using a programmed-temperature vaporizer injector (PVT,
9 Perkin-Elmer) in the solvent split mode, as described by Herraiz et al. (1987). Henecosanoic
10 acid (21:0) was used as internal standard. Duplicate samples were analysed both for lipid classes
11 and FA analysis.

12 2.4. RNA isolation and cDNA synthesis

13 Three samples of 50 to 100 pooled eggs or 25 to 30 pooled larvae were collected at each
14 sampling point. RNA extraction was performed using an Illustra RNAspin mini isolation kit
15 (GE Healthcare). RNA integrity was verified on 1% agarose gels stained with GelRed
16 (Biotium). RNA concentrations were measured with a Qubit fluorometer platform (Invitrogen)
17 and the Quant-IT RNA BR kit (Invitrogen). The same concentration of total RNA (1 µg) was
18 used per sample. Conversion of total RNA into first strand cDNA was performed using the
19 iScript cDNA synthesis kit (Bio-Rad) following the manufacturer recommendations.

20 2.5. Isolation of gene sequences in turbot

21 Partial sequences of the target genes in turbot were obtained through a degenerate RT-PCR
22 strategy. Degenerate primers (sequences available upon request) were designed based on
23 sequences of the same target genes from other teleost species obtained in databases (Ensemble,
24 NCBI). Block Maker (Henikoff et al., 1995) and Codehope (Rose et al., 1998) software's were
25 used to find conservative sequences on the aligned input sequences and select degenerated
26 primers. Phusion-Taq (Finnzymes, Finland) was used to amplify sequences between each pair of
27 primers. Two microliter of a 1:10 diluted cDNA was used in PCR reactions with the following
28 cycle conditions: 98°C 10sec, 55°C 5 sec, 72°C 30 sec for 40 cycles. PCR products were
29 separated on 1% agarose gels, and single bands were cut and gel-purified using the GFX PCR
30 DNA and Gel Band Purification kit (GE healthcare). Purified PCR products were forwarded for
31 automated sequencing at StabVida (Portugal). Sequences were deposited on GenBank (NCBI)
32 (S1).

33 2.6. Quantitative gene expression

34 Gene expression levels were determined by real-time PCR on an iQ5 real-time thermal cycler
35 (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Two microliter of a 1:10 diluted cDNA

1 was used in PCR reactions with the following cycle conditions: 94°C 30sec, 55°C 30 sec, 72°C
2 30 sec for 40 cycles. Three calibrators + 4 blanks were run in every 96-well plate. Real-time
3 primers were designed using Beacon Designer software version 7.51 (Premier Biosoft) to out-
4 flank intron conserved locations. Primer characteristics and amplicon size are listed at the
5 Supplementary Material (Table S1). Target gene expression was normalized through geometric
6 averaging of multiple internal control genes using GeNorm Software (Vandesompele et al.,
7 2002). From 8 normalizing genes analyzed – *EF1a*, *18S*, *UB2L3*, *RPL8*, *β-actin*, *GAPDH*, *TBP*
8 and *TubB2C* – 5 were chosen according to the GeNorm algorithm: *EF1a*, *18S*, *UB2L3*, *RPL8*
9 and *β-actin*. Gene expression (mean of 2 replicates) of the 27 target genes (Table S1) is
10 presented relatively to the expression at notochord formation.

11 2.7. Statistics

12 One-way ANOVA, followed by Tuckey's post-hoc test, were used to compare data among
13 different developmental stages. When some of the ANOVA assumptions (i.e. normality and
14 homoscedasticity) were not met by data, the Kruskal-Wallis one-way analysis of variance on
15 ranks were used followed by Dunn's test.

16 Principal components analysis (PCA) was employed to study the inter-correlations of the
17 variables (transcription of 27 genes) by clustering them into common factors. The variables
18 within each factor are highly correlated and each factor explains a different part of the total
19 variance in the data. Factors were Varimax rotated and Kaiser normalized. Correlations between
20 each PPARs and the various target genes are presented graphically using CIRCOS software
21 (Krzywinski et al., 2009).

22

23 3. Results

24

25 3.1. Yolk sac and oil globule volume

26 Yolk sac and oil globule dimensions are presented in Table 1. At hatching, the yolk sac was
27 $0.278 \pm 0.020 \mu\text{l}$ and was almost entirely consumed from hatching to mouth opening ($p < 0.01$)
28 (Fig. 1A). At mouth opening, less than one tenth of its original volume was still present ($p <$
29 0.05), surrounding the oil globule (Fig. 1B). By day 9, the yolk sac was completely consumed
30 (Fig. 1C). The oil globule volume remained constant until hatching. From hatching on, it started
31 to be consumed at a linear rate until the end of the experimental period ($p < 0.05$). At mouth
32 opening, half of its original volume was still present, and by 9 dpf, it was reduced to 1.02 ± 0.36
33 nl (Table 1). At this point, the variability of the oil globule volume was quite large since some
34 larvae presented a visible oil globule of near 1/5 of its volume measured at notochord formation
35 (Fig. 1C), while others did not have it at all. Values in between these extremes were rare.

1

2 *3.2. Lipid content, lipid classes and fatty acids*

3 Total lipid content was stable between notochord formation and hatching, and started to decline
4 significantly ($p < 0.05$) at linear rate from hatching until the last stage at 9 dpf (Table 1). All
5 lipid classes remained constant from notochord formation to hatching (Table 2). The most
6 abundant lipid classes in eggs and newly hatched larvae were PL followed by TG, while the
7 least abundant was FFA. From hatching until 9 dpf (50% mortality), TG and SE+WE content
8 decreased significantly ($p < 0.05$). PL, SL and FFA increased from hatching to mouth opening
9 ($p < 0.05$ for PL and SL). PL content decreased significantly ($p < 0.05$) from mouth opening
10 until 9 dpf, while SL and FFA level remained constant during the same period. At the last stage
11 (9 dpf), the lipid fraction was almost entirely composed by PL and SL, while TG and SE+WE
12 were reduced to vestigial levels. On what concerns FA with different degrees of desaturation
13 (Table 3), all three groups (SFA, MUFA and PUFA) also maintained their level stable from
14 notochord formation until hatching. From hatching on, both MUFA and PUFA declined linearly
15 from hatching, with significant differences at mouth opening and 9 dpf ($p < 0.05$). In contrast,
16 SFA levels remained constant until mouth opening but decreased significantly at 50% mortality
17 ($p < 0.05$). N-3 PUFA series was the most abundant at the beginning of development, 8 times
18 more abundant than n-6. Both n-3 and n-6 series remain constant from notochord formation to
19 hatching and then declined linearly up to 50% mortality. At this point, n-6 series content was
20 almost residual while n-3 was still considerable.

21

22 *3.3. Gene expression*

23 The 27 target genes analysed covered a major number of metabolic pathways related to FA and
24 lipids metabolism, including FA hydrolysis and activation, β -oxidation, biosynthesis,
25 desaturation, elongation, hydrolysis of TG and PL, FA esterification to TG and PL and nuclear
26 receptors involved on FA metabolism control (Fig. 2).

27

28 *3.3.1. FA activation and FA hydrolysis*

29 Relative expression levels of two fatty acyl-CoA thioesterases (*Acots*) responsible for FA
30 hydrolysis, and two acyl-CoA synthetase long-chain (*Acls*) responsible for FA activation, are
31 presented in Figure 3. *Acot7* mRNA expression decreased significantly from notochord
32 formation until hatching ($p < 0.05$), and returned to its initial level in later larval stages. *Acot8*
33 mRNA expression was highest at notochord formation, diminished significantly at hatching and
34 remained constant at this expression level until 50% mortality ($p < 0.05$). *Acls12* and *Acls4*

1 mRNA level did not change during development, with the exception of a higher level ($p < 0.05$)
2 in *Acsl2* at 50% mortality (Fig. 3).

3

4 3.3.2. β -Oxidation

5 The mRNA expression rate of Acyl-CoA oxidase 1 (*Acox1*) increased significantly from egg
6 stage to mouth opening, and from there to 50% mortality ($p < 0.05$). Carnitine
7 palmitoyltransferase 1a-related (*Cpt1a-rel*; Boukouvala et al., 2010) remained unchanged during
8 the whole developmental period (Fig. 3).

9

10 3.3.3. FA Biosynthesis and desaturation

11 The mRNA expression FA synthetase (*Fasn*) was constant until mouth opening, but increased
12 twice at 50% mortality ($p < 0.05$; Fig. 4). The transcription of 3 FA desaturases, stearoyl-CoA
13 desaturase members 1a and 1b (*Scd1a*, *Scd1b*) and FA desaturase 2 (*Fads2*) increased
14 significantly between hatching and mouth opening ($p < 0.05$; Fig. 4), while *Fads2* and *Scd1b*
15 further increased at 50% mortality ($p < 0.05$). *Fads2* expression at mouth opening was around
16 12-fold greater than at notochord formation, and 25-fold greater at 50% mortality.

17

18 3.3.4. FA elongation

19 All 6 FA elongases (*Elovl*) increased their expression along the developmental stages (Fig. 5),
20 with significant differences ($p < 0.05$) between hatching and mouth opening (with the exception
21 of *Elovl1a*: significant against notochord formation, $p < 0.05$). *Elovl4b* expression rate increased
22 the most, nearly 25-fold from eggs stage to 50% mortality. *Elovl1*, *Elovl4a*, *Elovl4b* and *Elovl6*
23 expression rate did not change between mouth opening and 50% mortality. However, the
24 expression of *Elovl5* and *Elovl6-like* gene increased significantly between mouth opening and
25 50% mortality ($p < 0.05$), with a 9- fold difference for *Elovl6-like* gene from notochord
26 formation until end of the experiment.

27

28 3.3.5. Hydrolysis of TG and PL

29 The expression rate of the various lipases displayed different patterns with development. While
30 lipoprotein lipase (*Lpl*) expression rate increased during the whole period with significant
31 changes between notochord, mouth opening and 50% mortality ($p < 0.05$), lipase A (*LipA*) and
32 lipase E (*LipE*) remained at the same mRNA transcription level along development (Fig. 6).
33 Hepatic lipase (*LipH*) expression remained stable until mouth opening and then increased
34 abruptly 13-fold afterwards ($p < 0.05$).

35

1 3.3.6. FA esterification to TG and CE

2 Diacylglycerol O-acyltransferase homolog 1 (*Dgat1*) expression remained stable from
3 notochord formation to mouth opening and then increased 1.5-fold at 50% mortality ($p < 0.05$),
4 whereas *Dgat2* decreased from notochord formation to hatching and increased to 2-fold at
5 mouth opening and 50% mortality ($p < 0.05$; Fig. 7).

6 Sterol O-acyltransferase2 (*Soat2*) displayed a decreased mRNA expression as development
7 progressed, from notochord formation to hatching ($p < 0.05$) and remained constant until 50%
8 mortality.

9

10 3.3.7. FA metabolism control

11 The four analyzed *PPAR* isoforms displayed different expression patterns along the
12 development. *PPAR α 1* and *PPAR γ* expression rates increased with significant differences
13 between notochord formation, hatching and mouth opening ($p < 0.05$). In contrast, *PPAR β*
14 remained steady in all larval stages and, *PPAR α 2*, decreased from notochord formation to
15 hatching ($p < 0.05$) and then maintained this low mRNA expression level until 50% mortality
16 (Fig. 8). *PPAR γ* was the gene whose expression increased mostly (90-fold) during the whole
17 experimental period.

18

19 3.4. Principal components analysis

20 The principal components analysis extracted two factors, PC1 and PC2 that explained 76.49%
21 of total data variation (43.46 and 33.03% respectively) (Fig. 9). Based on the examination of the
22 graphical representation of the factor loadings for each variable, genes were grouped according
23 to their relative positions (Fig. 9). At least three groups were initially defined according to their
24 mRNA expression pattern along the larval development. The most determinant variables were
25 *Cpt1a-rel*, *Elovl5*, *Elovl6*, *Fads2*, *Fasn*, *LipH* and *Scd1b* for PC1, and were *Acot8*, *LipA*, *LipE*,
26 *Soat2* and *PPAR α 2* for PC2 (supplementary material 2 - S2). Group 1 included genes whose
27 transcription tended to increase along the developmental stages, either from NT formation or
28 from hatching on. This group includes *PPAR α 1*, *PPAR γ* , and many genes positively correlated
29 to them or inversely correlated to *PPAR α 2* (*Elovl4a*, *Elovl4b*, *Elovl5*, *Elovl6*, *Elovl6l*, *Lpl*,
30 *Scd1a*, *Scd1b*, *Fads2*, *Acox*). Group 2 includes genes whose transcription remained stable along
31 development (*PPAR β* , *Cpt1a-rel*, *Acls2*), and whose transcription increased only at 50%
32 mortality of the starved larvae (*Fasn*, *LipH*, *Dgat1* and *Acls4*) and those whose transcription was
33 quite stable but increased or decreased significantly in some moment during development
34 (*Acot7* and *Dgat2*). Group 3 is composed by those genes whose transcription decreased along

1 development including *PPARα2* and other genes directly correlated to it (*Soat2*, *LipA*, *LipE* and
2 *Acot8*).

3 The correlation factors of the transcription of PPARs with that of the other genes are presented
4 on [supplementary material 3 \(S3\)](#). The correlation of PPAR's transcription to other genes ($p <$
5 0.05) is represented in the diagram of [Figure 10](#). *PPARα1* is the nuclear receptor that was
6 correlated to the highest number of genes, followed by *PPARγ*, *PPARα2* and *PPARβ*. The
7 analysis demonstrated that many genes were correlated or highly correlated to more than one
8 PPAR (Fig. 10, S3). Two genes were even highly correlated to all 4 PPARs (*Elovl4a* and
9 *Elovl4b*). Most commonly, genes are related to 3 PPARs (9 genes) or 2 PPARs (6 genes).
10 *PPARα1* and *PPARγ* correlated together to 17 genes that are mostly involved in FA elongation
11 or desaturation (8 genes).

12

13 **4. Discussion**

14

15 During embryogenesis and until mouth opening, embryos and larvae are theoretically in optimal
16 nutritional conditions. Yolk and oil globule are considered to contain the nutrients and energy
17 required for an adequate development until the stage of mouth opening. Proteins, PL and
18 cholesterol are present in the yolk to be used as building blocks in new cells, tissues and organs,
19 while TG, SE and WE are present both in the oil globule and in yolk to produce energy ([Finn,](#)
20 [1994](#); [Silversand et al., 1996](#)). In our study, the levels of total lipids and lipid classes started to
21 change from hatching to mouth opening (Table 2) and consequently, the transcriptional level in
22 the majority of analysed genes changed significantly at this period (Figs. 3 to 8). These results
23 suggest that the main regulations of lipid metabolism started between hatching and mouth
24 opening at the transcript and lipid level. This outcome is in agreement with previous published
25 studies on lipid content analysis and respirometry in turbot larvae, which pointed out that the
26 start of lipid metabolism occurs by the time of hatching ([Finn, 1994](#); [Finn et al., 1996](#)). It was
27 suggested that fish species with oviparous progeny evolved two types of energetic strategies for
28 their offspring ([Finn, 1994](#)). Type I which includes
29 Atlantic cod and Atlantic halibut, involves only deposition of yolk in the egg, that is resorbed
30 continuously by embryos and larvae, while in type II case, oil globule is only consumed when
31 yolk is exhausted. In this later strategy fall turbot and seabass (*Dicentrarchus labrax*), where
32 free amino acids appeared to be a significant energy substrate during the egg and the early yolk-
33 sac stages while FA from neutral lipids derived from the oil globule seemed to be the main
34 metabolic fuel after hatching ([Finn, 1994](#); [Rønnestad et al., 1998](#)). Also, neutral lipids were

1 dominant for turbot at hatching, while phospholipids predominated prior to first feeding, in
2 accordance to [Rainuzzo et al. \(1993\)](#).

3 However, a former transcriptional activity was observed between notochord formation and
4 hatching, where 7 out of 27 genes related to FA metabolism presented significant differences at
5 the transcription level. Those genes are involved in FA metabolism control (*PPAR α 1*, *α 2*), FA
6 hydrolysis (*Acot7*, *Acot8*), hydrolysis of TG and PL (*Lpl*), and FA esterification (*Soat2*, *Dgat2*).
7 Most of the transcriptional changes were characterized by a higher transcript level at notochord
8 formation as compared to hatching (*Acot7*, *Acot8*, *Soat2*, *Dgat2*) and included *PPAR α 2*, which
9 suggested a transcriptional regulation. The altered mRNA expression of genes does not reflect a
10 change in their functionality ([Nikinmaa and Rytkoenen, 2011](#)), and the correlation between
11 mRNA expression and enzyme activity is generally low ([Vogel and Marcotte, 2012](#)). However,
12 mRNA expression in our study can serve as a first indication at which steps the regulation of the
13 lipid metabolism might be altered during the larval development. In this light, it could be
14 speculated that the decreasing transcript level of *Acots* at hatching might lead to an increased
15 FA-CoAs pool, which could be used by many other FA metabolism genes at their onset of
16 expression at mouth opening. *Lpl* was up-regulated between notochord formation and hatching,
17 which seemed to follow the mRNA expression of *PPAR α 1* which could be interpreted as the
18 first signs of hydrolysis of TG/PL. Transcriptional changes at early embryonic stages suggested
19 that important processes of FA metabolism would have started already before hatching. Indeed,
20 a decrease in PL and cholesterol content during turbot embryogenesis was reported by [Finn et](#)
21 [al. \(1996\)](#) in a more detailed study on lipid sources. According to our results, PL hydrolysis
22 might be attributed to *Lpl* activity.

23 PUFA and MUFA decreased significantly between hatching and mouth opening. Interestingly,
24 the genes related to FA elongation (all *Elovs*) and FA desaturation (*Fads2*, *Sdc1a*, *Sdc1b*) (Fig. 4
25 and 5) showed the opposite pattern of unsaturated FA during the same period, being
26 significantly up-regulated at mouth opening. It is feasible that the FA profile contained in the
27 lipid reserves matched the initial demands of the embryos and larvae and, that at a certain
28 developmental time, the larvae started to elongate and desaturate FA, to produce, to some
29 extent, some of those FA no long available in the reserves. Here, the first signs of FA retailoring
30 in turbot larvae were observed at the stage between hatching and mouth opening. In agreement
31 with this, a great increase in desaturase (*Δ 6 desaturase/Fads2*) and elongase (*Elovl5*) expression
32 is also observed in tuna larvae (*Thunnus thymus*) after hatching ([Morais et al., 2011](#)). From
33 notochord formation to mouth opening PUFA are heavily used for the formation of nervous
34 tissue in turbot larva, including eyes which are fully functional at mouth opening ([Al-](#)
35 [Maghazachi and Gibson, 1988](#)). Most *Elovl*s increased their activity 1.5 to 2.5-fold from

1 hatching to mouth opening, while *Elovl4b* increased more than 6-fold. At mouth opening the
2 eyes of turbot larvae get functional to help on foraging behaviour and in accordance, analysis by
3 whole-mount *in situ* hybridisation in zebrafish embryos showed that *Elovl4b* is specifically
4 expressed in photoreceptor cells of retina (Monroig et al., 2010). Furthermore, fads-like gene
5 transcripts in nibe croaker (*Nibe mitsukurii*) larvae fed on oleic acid-enriched Artemia were
6 significantly higher than those in larvae fed on 100% 22:6n-3-enriched Artemia, indicating that
7 the *Fads2* gene was controlled by negative feedback from the quantity of 22:6n3 stored in the
8 larval body (Yamamoto et al., 2010). This finding is also in accordance with our data, where the
9 decrease of PUFA was accompanied by an increase in FA desaturases. In contrast to our results,
10 where *Elovl5* pattern was also inverse to FA decrease, no significant differences were observed
11 in the transcript levels of the *Elovl5* gene in nibe croaker fed on 22:6n-3-enriched Artemia
12 (Yamamoto et al., 2010).

13 From hatching to mouth opening, energetic lipids (TG, SE) were consumed, whereas structural
14 lipids (PL, SL) slightly increased. Surprisingly, most genes involved in hydrolysis of TG, PL
15 and SE remained unchanged during this period (*LipA*, *LipH*, *LipE*). *Lpl* was the only lipase with
16 transcriptional changes during this period and could have played an important role in the
17 catabolism of energetic lipids. Accordingly, *Acox1* expression increased from hatching, and later
18 after mouth opening, in parallel to *Lpl* mRNA expression, which may be indicative of β -
19 oxidation at the protein level and FFA generated by TG hydrolysis (Owen et al., 1979).
20 Therefore, evidence is present at transcriptional level that hydrolysis of TG and PL started
21 between hatching and mouth opening. However, other lipases might be involved in the
22 hydrolysis of SE in the early stages of larval development of turbot, e.g. patatin-like enzymes as
23 in birds (Saarela et al., 2008).

24 Genes involved in the FA esterification to TG, PL and SE (*Dgat1*, *Dgat2*, *Soat2*) showed
25 different expression patterns between hatching and mouth opening. *Soat2* decreased in parallel
26 to the decrease of TG and SE. Free cholesterol is esterified into SE by *Soats* in the endoplasmic
27 reticulum and stored in lipid droplets (Ikonen, 2008). In humans, SOAT2 is limited to the liver
28 and intestine, converting free cholesterol to esters destined for VLDL assembly in the liver or to
29 chylomicron assembly in the intestine (Leon et al., 2005). In turbot larvae, *Soat2* seems to have
30 an initial role before hatching and loses significance afterwards. At hatching, turbot hepatocytes
31 contain few endoplasmic reticulum membranes (Segner et al., 1994) and the intestine is not
32 functional yet, which may justify low *Soat2* transcription levels.

33 During the last stage of development (mouth opening to 50% mortality), larvae were in
34 starvation and consequently all lipid classes diminished significantly. Especially, mRNA
35 expression of *LipH*, *Fasn* and *Dgat1* seemed to be nutritionally regulated, by the decreasing

1 level of substrate (negative feedback) during starving conditions. The transcription of these
2 genes was stable until mouth opening, but increased when extreme starvation conditions were
3 reached. *LipH* hydrolyzes specifically phosphatidic acid to produce 2-acyl lysophosphatidic acid
4 (LPA, a potent bioactive lipid mediator) and a non-specific FA (Aoki et al., 2008; Shinkuma et
5 al., 2010). It has been described that LPA synthesized intracellularly via the pathway of TG or
6 PL biosynthesis can activate PPAR γ (Stapleton et al., 2011). It is plausible that LPA resultant
7 from PL and TG catabolism through *LipH* catalysis might be one of the causes of the strong
8 PPAR γ transcription induction occurring between mouth opening and 50% mortality.

9 The expression of various other genes related to FA synthesis and storage, such as *Elovl5*,
10 *Elovl6l*, *Scd1b* and *Fads2* changed after mouth opening in starving larvae. The expression of
11 these genes had already increased from notochord formation to mouth opening, but starvation
12 further induced their expression levels. In contrast to *LipH*, *Fasn* and *Dgat1*, which seemed to
13 be clearly nutritionally regulated, it is impossible to differentiate if the up-regulation of these
14 genes is ontogenetically programmed during these larval stages or, if they might be induced by
15 low levels of a substrate or product. A recently cloned $\Delta 4Fad$ gene in *Solea senegalensis* was
16 found to be highly responsive to low levels of n-3 long chain PUFA (Morais et al., 2012) and a
17 *Fads2* gene in *N. mitsukurii* was also controlled by negative feedback from the amount of
18 substrate (Yamamoto et al., 2010). However, the possibility of a developmentally fixed pattern
19 of both *Fads* and *Elovl*s to ensure right 22:6n3 levels for neurogenesis independently of dietary
20 supply must not be discharged.

21 Between mouth opening and 50% mortality, significant changes were also observed in *Acox1*
22 mRNA expression (Fig. 3), which might indicated that during starvation β -oxidation of very
23 long and long chain FAs in the peroxisome contributed to the energy production. It is also
24 possible that *Acox1* was to be used not only for FA oxidation but also for FA
25 elongation/shortening, through the Sprecher's shunt (Sprecher, 1992) to produce DHA in the
26 peroxisome, since an intense transcription of *Elovl*s (including *Elovl5* and *Elovl4b*) and *Fads*
27 was detected at the final stage of starvation. Interestingly, the mRNA expression of the different
28 PPARs did not change at the end of the starvation period. This might imply that a transcriptional
29 response of PPARs is not involved in the regulatory response to starvation. The regulatory
30 response might be a direct effect on the target genes driven by the absence of substrates or by
31 other metabolic signals of starvation. In this aspect, the regulation of fasting in teleost fish might
32 be different from mammals. It has been shown in PPAR α null mice that PPAR α is especially
33 important for the adaptive response to fasting by stimulating hepatic FA oxidation and
34 ketogenesis (Kersten et al., 1999; Hashimoto et al., 2000; Leone et al., 1999).

1 In contrast to the starvation period, the different PPARs demonstrated different expression
2 patterns between notochord formation and mouth opening. Transcriptional activity of *PPARα2*
3 was high at notochord formation and decreased 4-fold to a low value from then on. The mRNA
4 expression profile of *PPARα1* was the opposite of *PPARα2*, and increased significantly from
5 notochord to hatching and mouth opening. Fernández *et al.* (Fernandez et al., 2011) observed a
6 strong increase in *PPARα1* transcriptional activity in *Sparus aurata*, from days 2 to 7 after
7 hatching, which confirms our data for *PPARα1*. The different transcription profile observed on
8 the two *PPARα* isoforms is probably indicative of a gene sub-functionalization.

9 In mammals it is well known that high levels of n-3 PUFA suppress transcription of various
10 enzymes of FA metabolism e.g. *Elovl6*, *Fasn*, *Scd1*, through ligation to *PPARα* (Wang et al.
11 2005; Jump et al., 2006). Interestingly, we observed here that the levels of n-3 PUFA decreased
12 linearly from hatching to 50% mortality while the mRNA expression of those enzymes
13 increased significantly, as well as others also involved in desaturation, elongation and β-
14 oxidation of FA (e.g. *Fads2*, *Acox1*, *Acsl4*, *Elovl1* and *Elovl5*). The substrate of these enzymes
15 would possibly be other saturated, monounsaturated and polyunsaturated FA already present,
16 that are transformed in others that perform essential specific functions, taking in to account that
17 vertebrates lack Δ15 desaturase, that some elongases and desaturases have a very limited
18 efficiency in marine fish (Ghioni et al., 1999; Agaba et al., 2005; Tocher et al., 2003), and that
19 fish are not likely to biosynthesize fatty acids *de novo* to any significant extent, if they do so at
20 all (Tocher, 2003), from acetyl-CoA and malonyl-CoA precursors.

21 The principal component analysis revealed that *PPARα1* shared with *PPARγ* a common group
22 of several lipid metabolic genes (Fig. 9). The group consists of 12 genes, which includes most
23 genes related to FA elongation and desaturation. From all the genes studied, *PPARγ* was the one
24 that most changed during the experimental period (~100-fold). This gene is known to be
25 involved in lipid anabolism regulation but is also required for development of various tissues,
26 namely, placental, cardiac, adipose tissue development and skeletogenesis (Fernandez et al.,
27 2011; Barak et al., 1999).

28 *PPARβ* activity was stable during the whole developmental period. Similarly, it was reported
29 that the mRNA level of *PPARβ* was not elevated in the jejunum during the postnatal
30 development of the rat (Mochizuki et al., 2001). In the seabream, *Sparus aurata*, *PPARβ*
31 transcription also seems to be relatively stable during the first 60 days after hatching (Fernandez
32 et al., 2011).

33 Correlation analysis pinpointed that the transcription of various genes may be controlled by
34 more than one *PPAR*, more commonly, by two or three PPARs (Fig 10 and S3). *PPARα1*
35 transcription is highly correlated to *PPARβ* and especially to *PPARγ* and *PPARα2*. *PPARα1* and

1 *PPAR γ* are the nuclear receptors whose transcription correlated to more genes simultaneously.
2 Moreover, all genes correlated to *PPAR α 2* transcription were also inversely correlated to
3 *PPAR α 1* and *PPAR γ* , which might indicate that they co-ordinately modulate the transcription of
4 various genes together, but in opposite directions. To our knowledge, this is the first study in a
5 teleost fish species providing evidence for a regulation of FA metabolism genes by a joint action
6 of different PPARs. In other animal models, it is known that some genes are regulated by more
7 than one PPAR. It has been suggested that *Acox1*, *L-Fabp* and other *PPAR*-dependent genes
8 may be co-ordinately modulated in the small intestine of developing rats, during postnatal
9 development, by the disproportional expression of *PPAR α* over *PPAR β* (Mochizuki et al.,
10 2001). Also, the human LPL promoter is regulated differentially by the binding of *PPAR α* or
11 *PPAR γ* together with the retinoic acid X receptor (RXR) heterodimer (Schoonjans et al., 1996).
12 Also, thiazolidinediones predominantly affected adipocyte LPL production through activation of
13 *PPAR γ* , while fibrates exerted their effects mainly in the liver via activation of *PPAR α*
14 (Schoonjans et al., 1996). Genes that were correlated uniquely to *PPAR γ* (Fig 10 and S3) were
15 related to FA *de novo* synthesis (*Fasn*) and to PL hydrolysis (*LipH*). Genes correlated to
16 *PPAR β* alone are related to FA activation and deactivation (*Acs12* and *Acot7*).

17 In accordance to our results, three developmental periods in the early turbot larvae were
18 characterized as follows (Figure 11):

- 19 a) Embryos – from notochord formation to hatching – the oil globule was not consumed,
20 analytical lipid parameters were constant, but the first transcriptional alterations in lipid
21 metabolism genes were observed. First transcriptional signs for hydrolysis of TG and PL (*Lpl*)
22 were detected together with a high expression of *PPAR α 2*.
- 23 b) Eleutheroembryos – larvae feeding on endogenous reserves – lipids started to be
24 metabolized, which could be observed by the decrease of the lipid classes levels related to
25 energy storage (TG, SE+WE, MUFA and PUFA) as well as the reduction of oil globule and
26 yolk sac volumes. Moreover, structural lipids (i.e PL, SL) increased significantly during this
27 stage. Many genes related to lipid anabolism and catabolism were up-regulated, and no gene
28 was down-regulated during this stage. First transcriptional signs for FA β -oxidation (*Acox1*), FA
29 desaturation (*Fads2*, *Scd1a*, *Scd1b*) and FA elongation (all *Elovl*s) were observed.
- 30 c) Larvae – mouth opened but not fed – oil globule was consumed until exhaustion. The level of
31 energy storage lipids (SE+WE and TG) decreased until exhaustion. PL decrease, and only SL
32 levels remain steady. *LipH*, *Dgat1* and *Fasn*, whose transcriptional activity did not change
33 during the whole development, responded strongly to starvation at this stage.

34

35 **5. Conclusions**

1
2 In conclusion, full lipid metabolism only occurs after hatching, as revealed by alterations in lipid
3 profiles and FA gene expression; Transcriptional changes of some FA genes were already
4 present in embryos at notochord formation; Many of the studied genes displayed an expression
5 profile, which is opposed to the decrease of lipids along the development due to the
6 consumption of yolk reserves and oil globule, suggesting a negative feedback of substrates on
7 FA gene expression; Other genes (e.g. *LipH*, *Fasn* and *Dgat1*) were not altered during
8 development, but responded strongly to starvation and; Transcription of most genes involved in
9 FA metabolism had a strong correlation to PPARs mRNA levels ($\alpha1$, $\alpha2$, β , γ) and evidence is
10 suggested for an orchestrated control by two or three PPARs isoforms.

11
12 **Acknowledgements.** This work was developed under the research project
13 PTDC/MAR/68885/2006, funded by the Portuguese Foundation for Science and Technology
14 (FCT) and by the “Programa Operacional Ciência e Inovação 2010” (POCI 2010), co-financed
15 by the FEDER European Community fund. We would like thank Insuiña – Pescanova S.A., for
16 kindly supply the embryos used on the experiments described on this paper.

17

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44

45 **Figure captions**

46

1 **Fig. 1** – Photographs of turbot larva yolk-sac (YS) and oil globule (OG) along development. A –
2 at hatching (4.5 dpf); B – at mouth opening (7 dpf); C – at 50% mortality (9 dpf). H – heart; IL –
3 intestinal loop; OT – otolith; PI – posterior intestine; S - stomach.

4
5 **Fig. 2** – Schematic representation of the analyzed gene families, positioned at their respective
6 cellular components and catalyzing a biochemical process on lipid metabolism.

7
8 **Fig. 3** – Transcription level of genes involved in FA hydrolysis (*Acot7*, *Acot8*), FA activation
9 (*Acs12*, *Acs14*) and β -oxidation (*Acox1* and *Cpt1a-rel*), represented as mean values (\pm standard
10 error) along turbot larvae development, from notochord formation (NT) to 50% mortality
11 (50%M). H – hatching; MO – mouth opening. One-way ANOVA F-test statistic and test
12 probability (p) are presented. Different letters (a, b, c) mean significant differences on
13 expression levels between developmental stages after Tukey test ($p < 0.05$).

14
15 **Fig. 4** - Transcription level of genes involved in FA biosynthesis (*Fasn*) and desaturation
16 (*Fads2*, *Scd1a* and *Scd1b*) represented as mean values (\pm standard error) along turbot larvae
17 development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO –
18 mouth opening. One-way ANOVA F-test statistic and test probability (p) are presented.
19 Different letters (a, b, c) mean significant differences on expression levels between
20 developmental stages after Tukey test ($p < 0.05$).

21
22 **Fig. 5** - Transcription level for genes involved in FA elongation (*Elovl1*, *Elovl4a*, *Elovl4b*,
23 *Elovl5*, *Elovl6* and *Elov6l*) represented as mean values (\pm standard error) along turbot larvae
24 development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO –
25 mouth opening. One-way ANOVA F-test statistic or one-way Kruskal-Wallis H-statistic and
26 tests probability (p) are presented. Different letters (a, b, c) mean significant differences on
27 expression levels between developmental stages after Tukey or Dunn's test ($p < 0.05$).

28
29 **Fig. 6** - Transcription level of genes involved in hydrolysis of TG and PL (*LipA*, *LipE*, *LipH* and
30 *Lpl*) represented as mean values (\pm standard error) along turbot larvae development, from
31 notochord formation (NT) to 50% mortality (50%M). H – hatching; MO – mouth opening. One-
32 way ANOVA F-test statistic or one-way Kruskal-Wallis H-statistic and tests probability (p) are
33 presented. Different letters (a, b, c) mean significant differences on expression levels between
34 developmental stages after Tukey or Dunn's test ($p < 0.05$).

35

1 **Fig. 7** - Transcription level of genes involved in FA esterification to TG and CE (*Dgat1*, *Dgat2*
2 and *Soat2*) represented as mean values (\pm standard error) along turbot larvae development, from
3 notochord formation (NT) to 50% mortality (50%M). H – hatching; MO – mouth opening. One-
4 way ANOVA F-test statistic and test probability (p) are presented. Different letters (a, b, c, d)
5 mean significant differences on expression levels between developmental stages after Tukey test
6 ($p < 0.05$).

7

8 **Fig. 8** - Transcription level of genes involved in FA metabolism control (*PPAR α 1*, *PPAR α 2*,
9 *PPAR β* and *PPAR γ*) represented as mean values (\pm standard error) along turbot larvae
10 development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO –
11 mouth opening. One-way ANOVA F-test statistic or one-way Kruskal-Wallis H-statistic and
12 tests probability (p) are presented. Different letters (a, b, c, d) mean significant differences on
13 expression levels between developmental stages after Tukey or Dunn's test ($p < 0.05$).

14

15 **Fig. 9** – Principal Component (PC) plot representing variables in the rotated plan after Principal
16 Component Analysis. Rotation method was Varimax with Kaiser Normalization. Near each PC
17 it is presented the percentage of data variation explained by it. G1, G2 and G3 correspond to the
18 3 variables' groups defined.

19

20 **Fig. 10** – Circos diagram showing correlations between each PPAR and the various target genes.
21 The degree of correlation is set by the ribbon color in 4 levels corresponding to 4 quartiles, from
22 the lowest to the highest correlated: Q1 - quartile ribbons have color grey, Q2 - quartile ribbons
23 have color yellow, Q3 - quartile ribbons have color orange, Q4 - quartile ribbons have color red.
24 Only significant correlations (level < 0.05) are presented. Segments on the extreme of the
25 ribbons represent the color in the opposite side. *Cpt1a-rel* and *Dgat2* are not presented since
26 they are not significantly correlated to any *PPAR*.

27

28 **Fig. 11** – Schematic description of the morphological, biochemical and molecular events along
29 larval development from notochord formation in the egg to 50% mortality in starving (non feed)
30 larvae at 3 days after mouth opening.

31

1 **Table 1**

2 Yolk sac volume, oil globule volume and total lipid content along early development of turbot
 3 (dpf – days post fertilization). Values correspond to the mean \pm standard deviation of 15-20
 4 larvae for yolk sac and oil globule volume and, of 2 pooled samples (n = 175-400 eggs or
 5 larvae) for total lipids and total fatty acids. Different superscript letter in the same row indicate
 6 significant differences (p < 0.05).

7

Days Post Fertilization (dpf)	2.5	4.5	7	9
Developmental event	notochord formation	hatching	mouth opening	50% mortality
Yolk sac (μ l)	-	0.278 \pm 0.020 ^a	0.022 \pm 0.002 ^b	-
Oil Globule (nl)	4.77 \pm 0.90 ^a	4.85 \pm 0.11 ^a	2.22 \pm 0.05 ^b	1.02 \pm 0.36 ^c
Total Lipids (μ g/egg or larva)	9.63 \pm 0.28 ^a	9.98 \pm 0.37 ^a	6.90 \pm 0.23 ^b	3.92 \pm 0.03 ^c
Total Fatty Acids (μ g/egg or larva)	5.30 \pm 0.22 ^a	5.25 \pm 0.04 ^a	3.76 \pm 0.19 ^{ab}	1.62 \pm 0.15 ^b

12

1 **Table 2**

2 Triglycerides, phospholipids, sterols, Sterols esters + Wax esters and free fatty acids content
 3 ($\mu\text{g}/\text{larvae}$ or egg) along early development of turbot (dpf – days post fertilization). Values
 4 correspond to the mean \pm standard deviation of 2 pooled samples ($n = 175\text{-}400$ eggs or larvae)
 5 for lipid classes analysis. Different superscript letter in the same row indicate significant
 6 differences ($p < 0.05$).

7

Days Post Fertilization (dpf)	2.5	4.5	7	9
Developmental event	notochord formation	hatching	mouth opening	50% mortality _g
Triglycerides (TG)	1.20 \pm 0.05 ^a	1.23 \pm 0.04 ^a	0.62 \pm 0.09 ^b	-
Phospholipids (PL)	1.65 \pm 0.02 ^a	1.75 \pm 0.07 ^{ab}	2.08 \pm 0.11 ^b	1.47 \pm 0.18 ¹⁰
Sterols (SL)	0.40 \pm 0.01 ^a	0.35 \pm 0.01 ^a	0.54 \pm 0.05 ^b	0.51 \pm 0.02 ^b
Sterols Esters + Waxes Esters (SE+W)	1.11 \pm 0.01 ^a	1.15 \pm 0.09 ^a	0.40 \pm 0.05 ^b	0.07 \pm 0.01 ^c
Free Fatty Acids (FFA)	-	-	0.03 \pm 0.02	0.01 \pm 0.02

1 **Table 3**

2 Saturated, monounsaturated, polyunsaturated, n-3 series and n-6 series fatty acids content
 3 ($\mu\text{g}/\text{larvae}$ or egg) along early development of turbot (dpf – days post fertilization). Values
 4 correspond to the mean \pm standard deviation of 2 pooled samples ($n = 175\text{-}400$ eggs or larvae)
 5 for fatty acids analysis. Different superscript letter in the same row indicate significant
 6 differences ($p < 0.05$).
 7

Days Post Fertilization (dpf)	2.5	4.5	7	9
Developmental event	notochord formation	hatching	mouth opening	50% mortality
Saturated Fatty Acids	1.80 ± 0.07^a	1.83 ± 0.03^a	1.47 ± 0.09^a	0.75 ± 0.09^b
Monounsaturated Fatty Acids	1.55 ± 0.06^a	1.49 ± 0.02^a	0.90 ± 0.07^b	0.28 ± 0.03^c
Polyunsaturated Fatty Acids	1.98 ± 0.09^a	2.11 ± 0.01^a	1.43 ± 0.05^b	0.59 ± 0.07^c
n-3 series Fatty Acids	1.76 ± 0.01^a	1.85 ± 0.03^a	1.29 ± 0.07^b	0.51 ± 0.02^c
n-6 series Fatty Acids	0.22 ± 0.00^a	0.25 ± 0.01^a	0.15 ± 0.02^b	0.07 ± 0.01^c

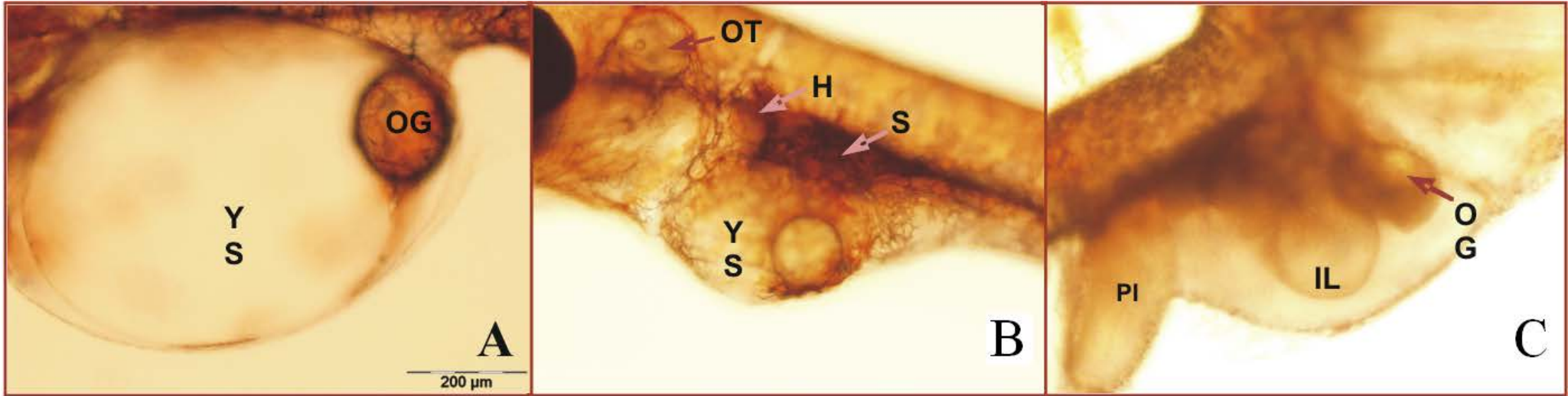


Fig. 1

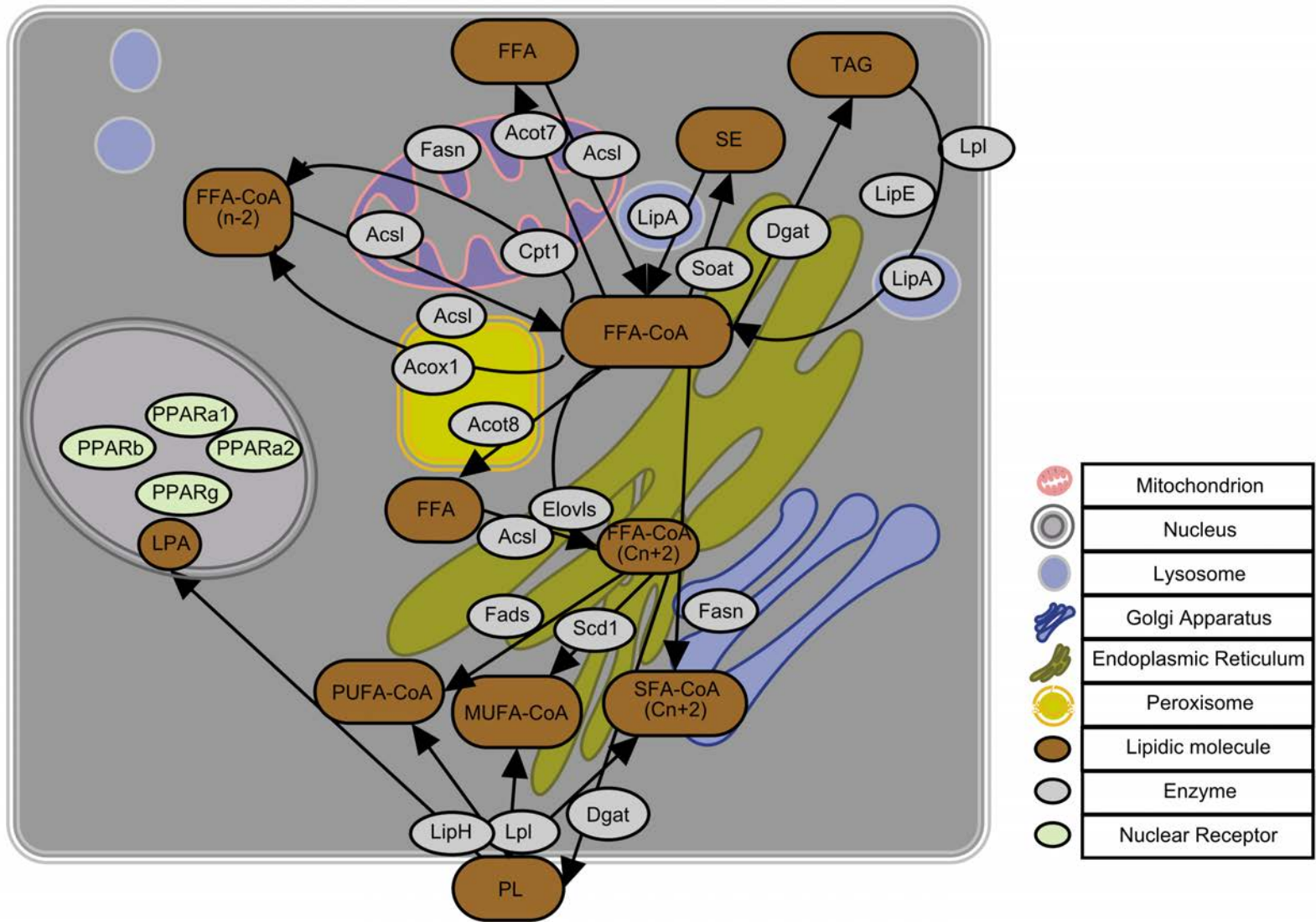
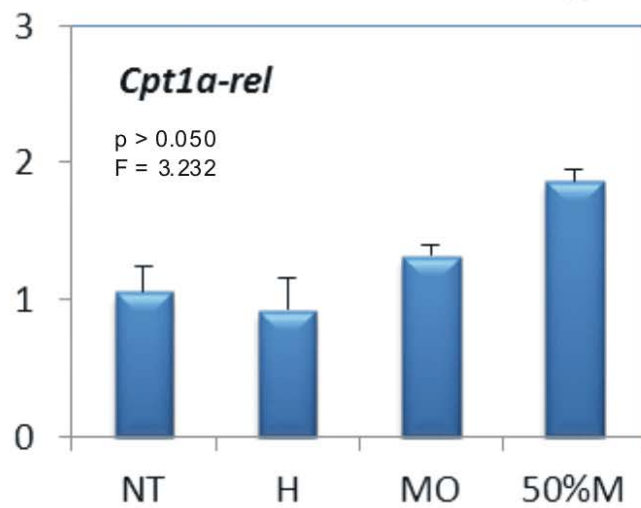
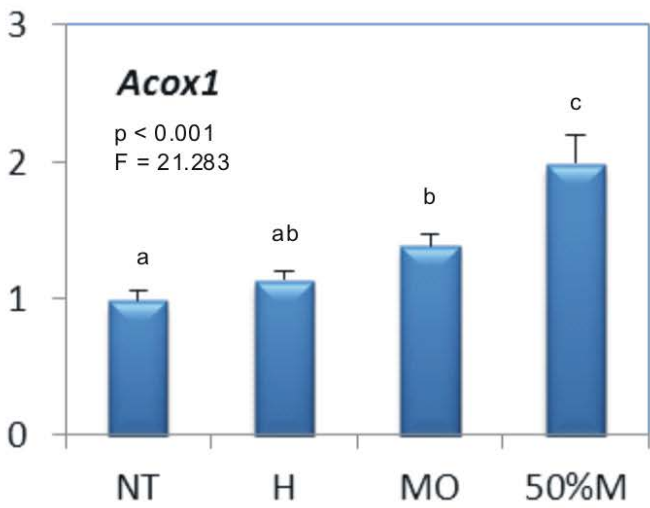
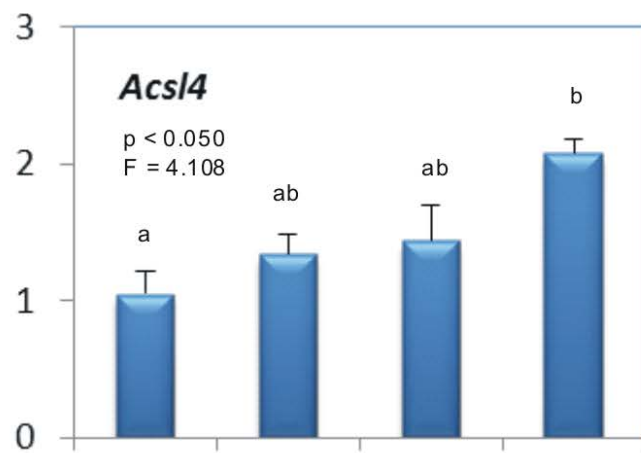
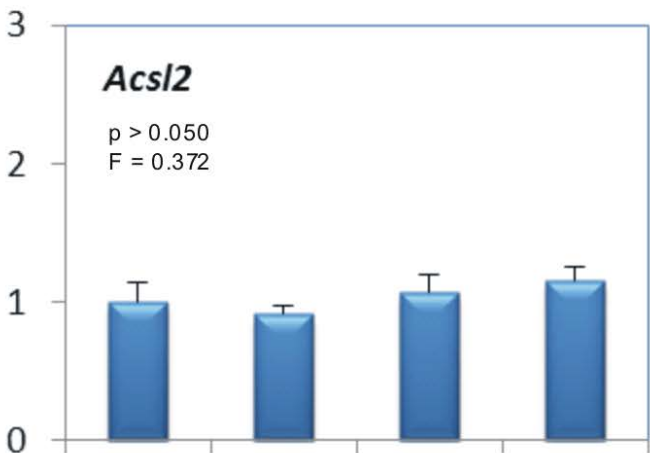
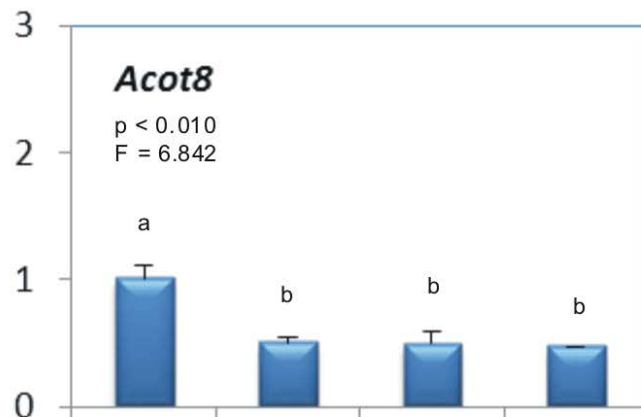
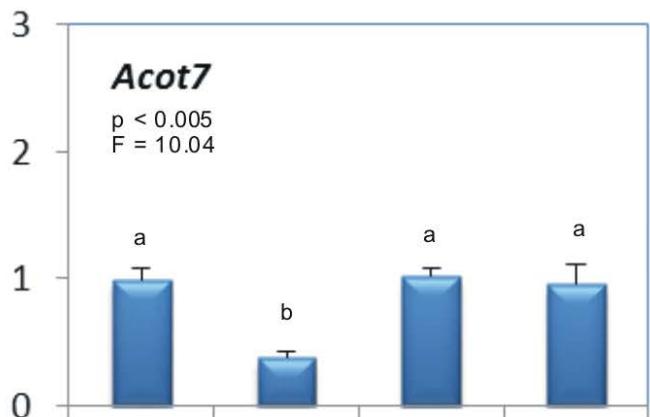


Fig.2

Relative mRNA expression



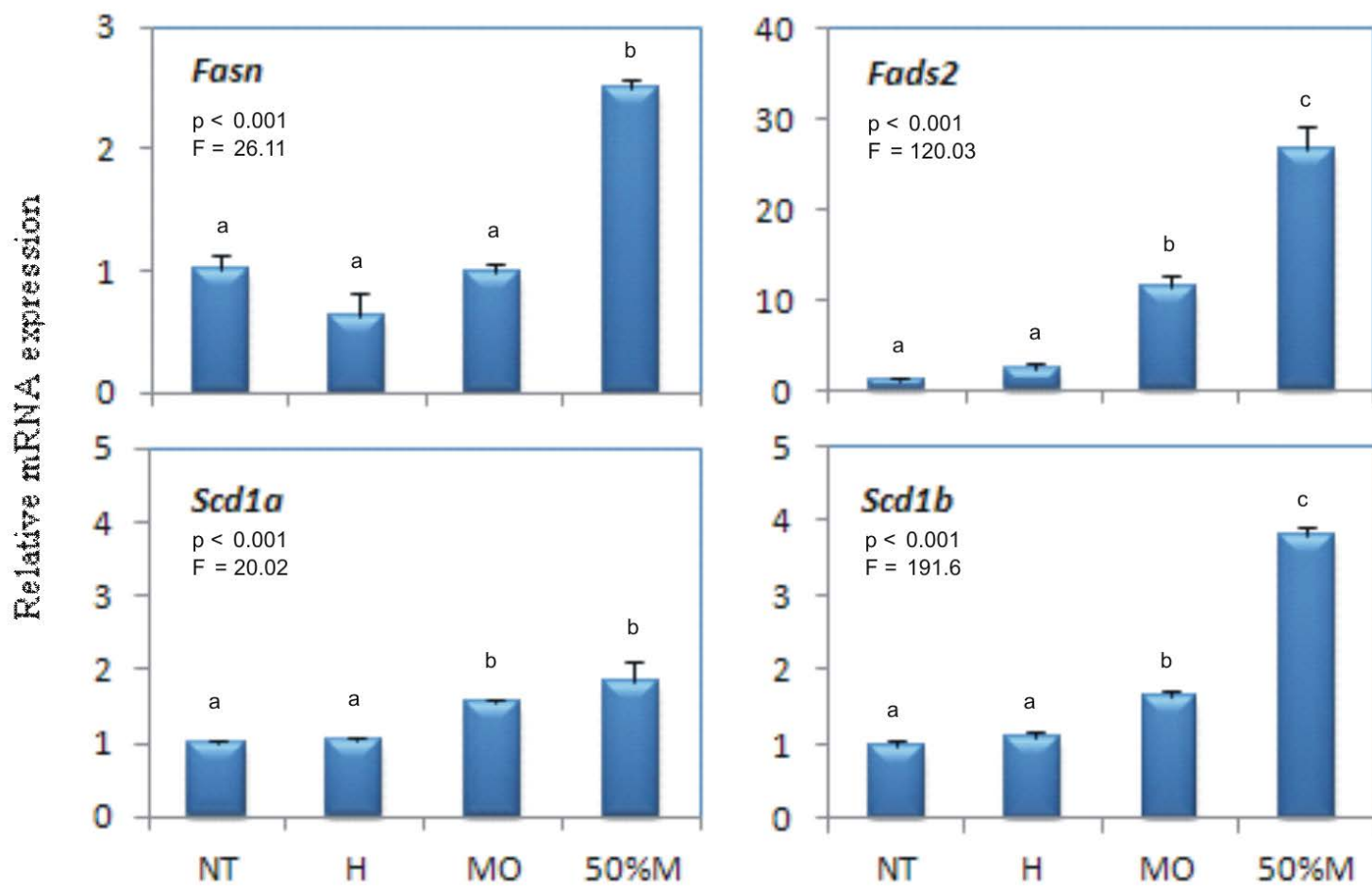


Fig.4

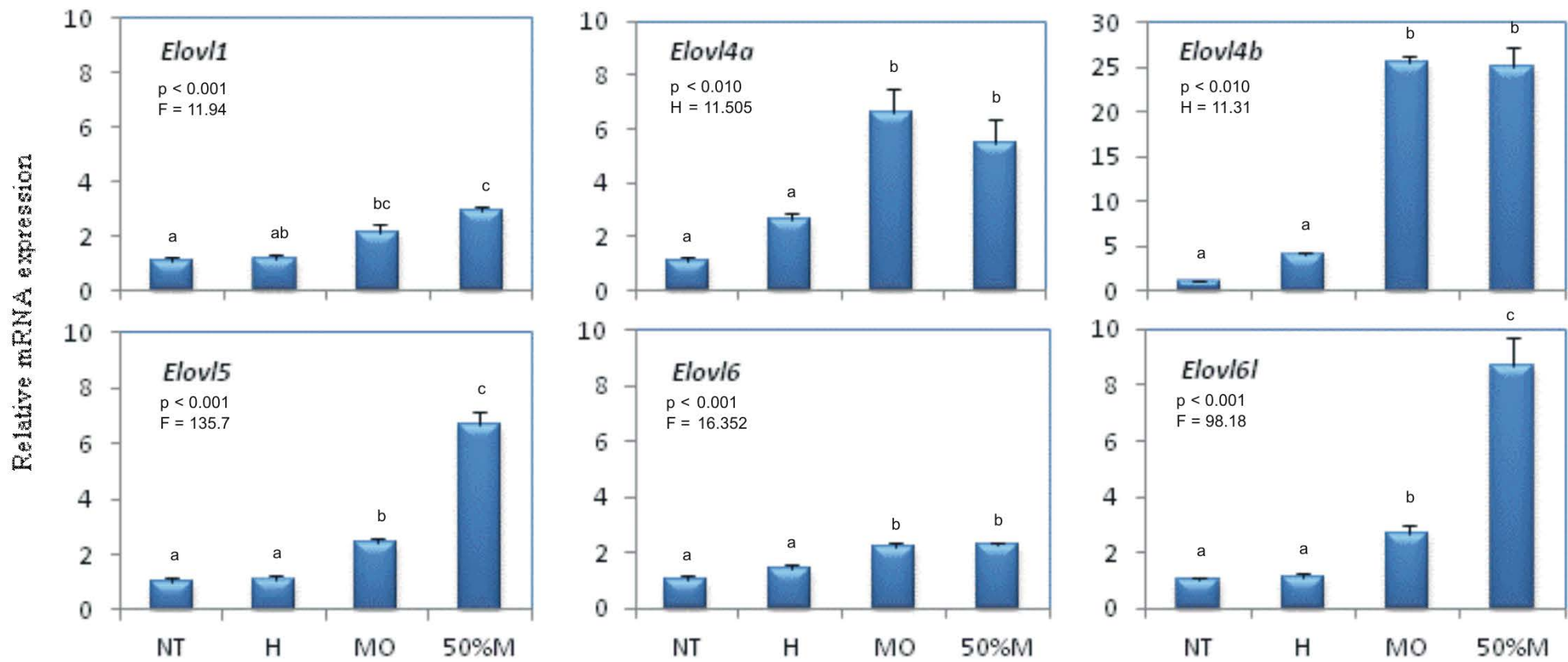


Fig.5

Relative mRNA expression

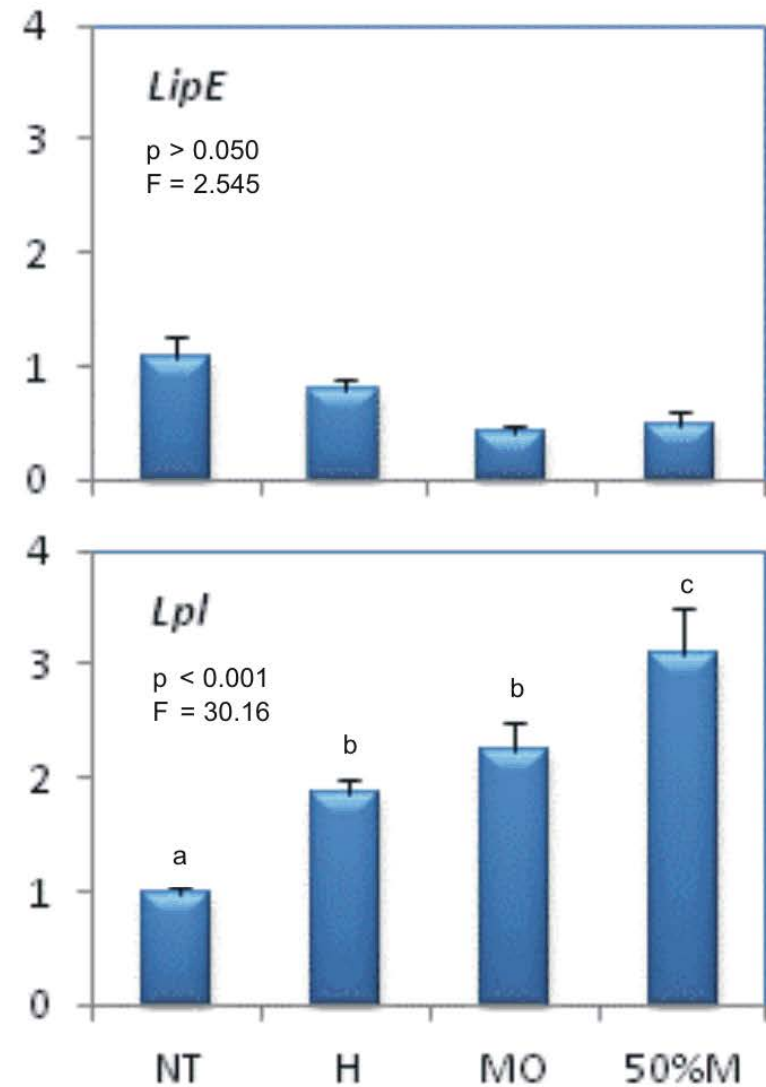
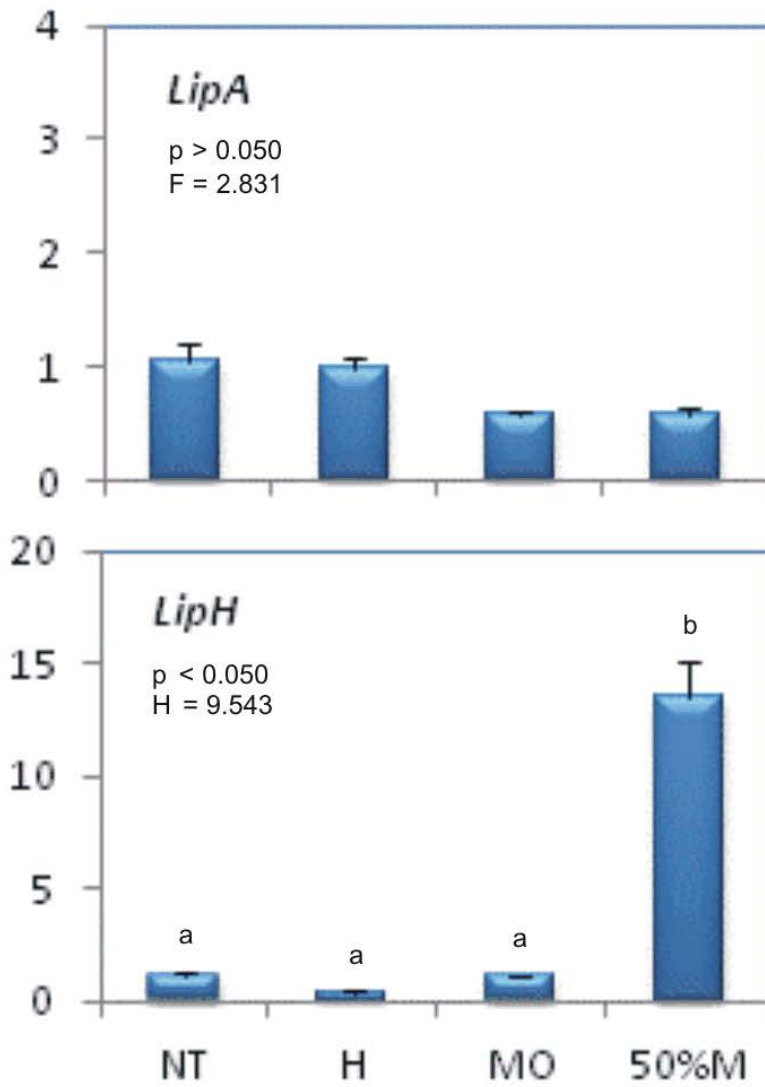


Fig.6

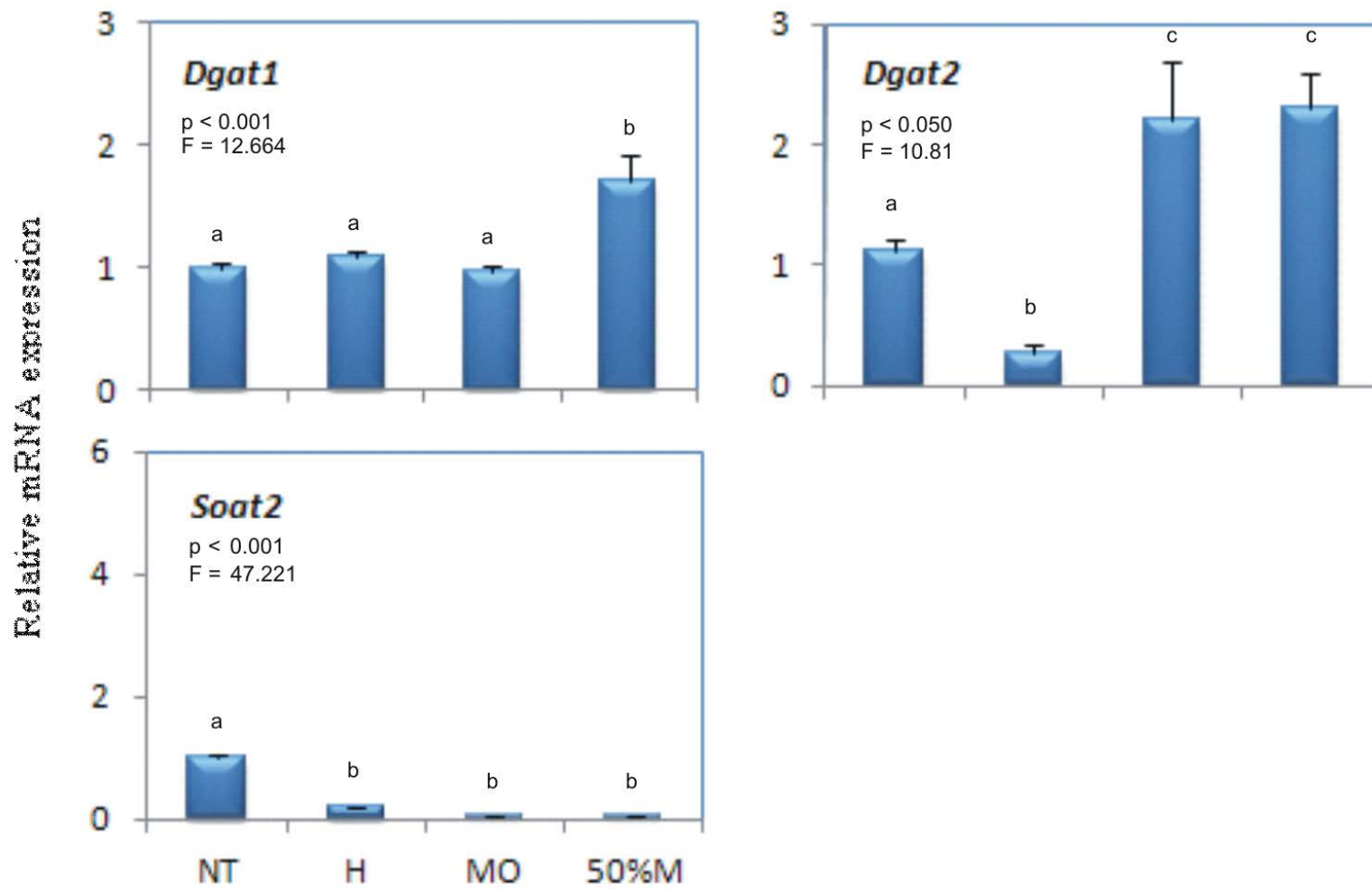


Fig.7

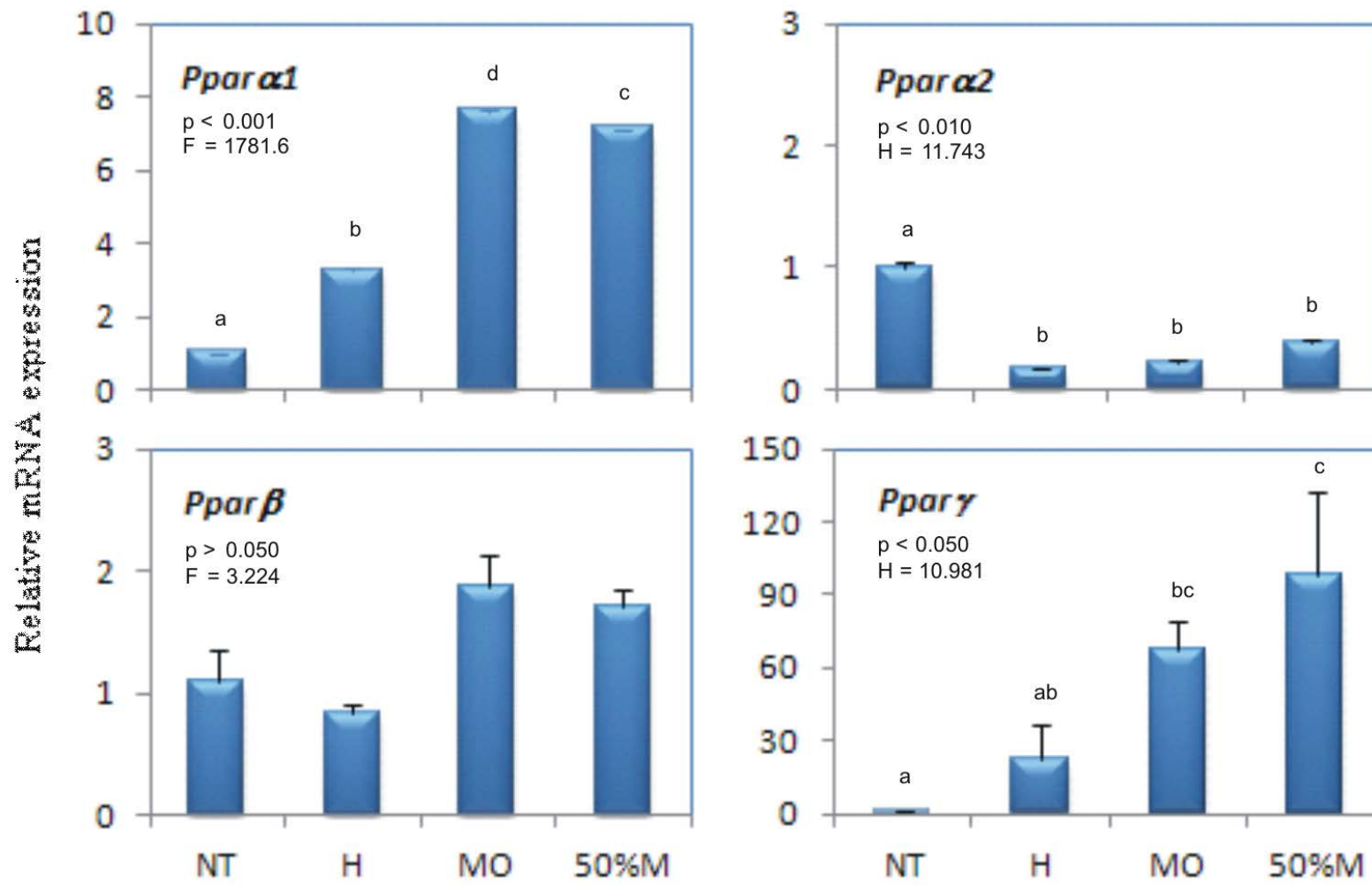


Fig.8

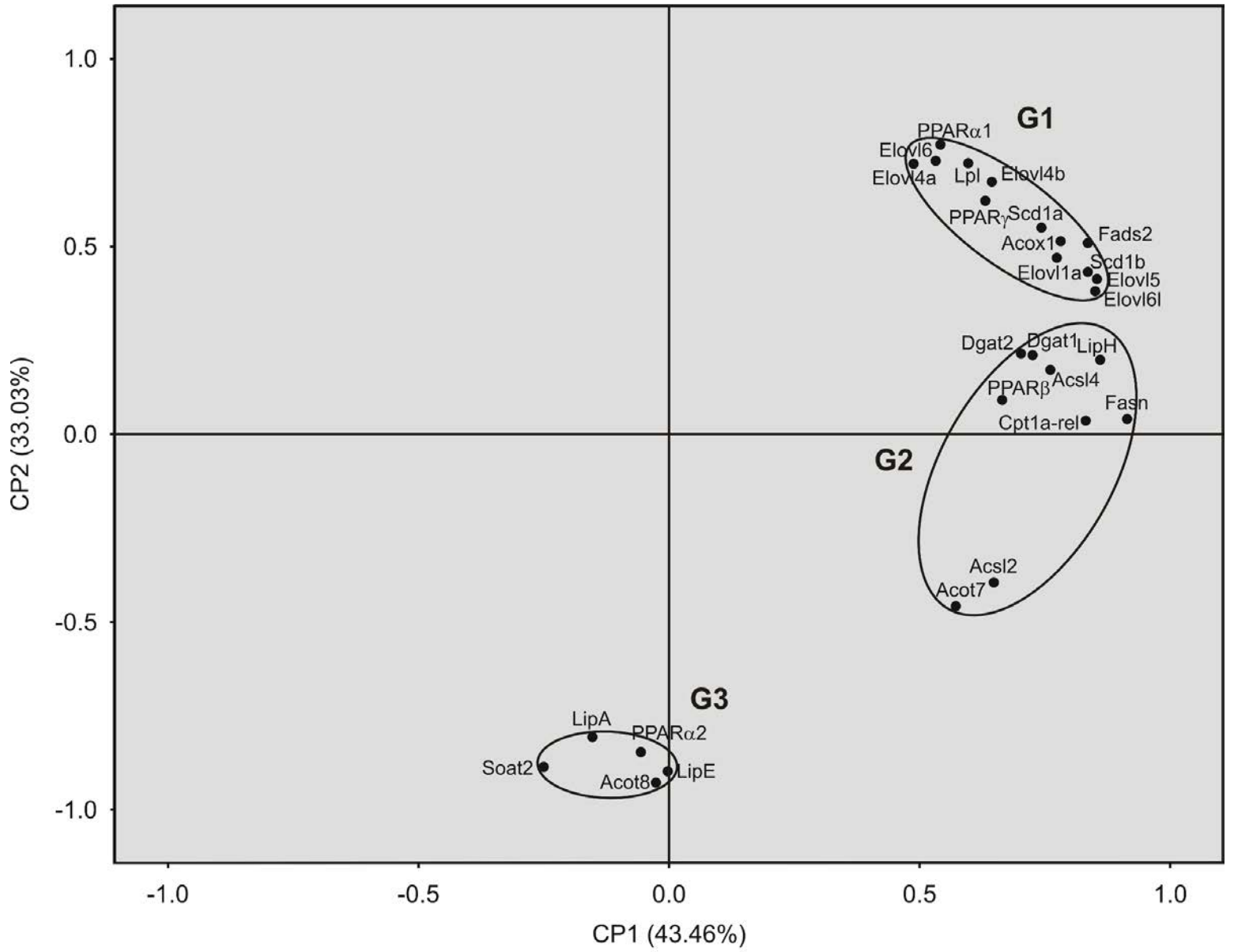


Fig. 9

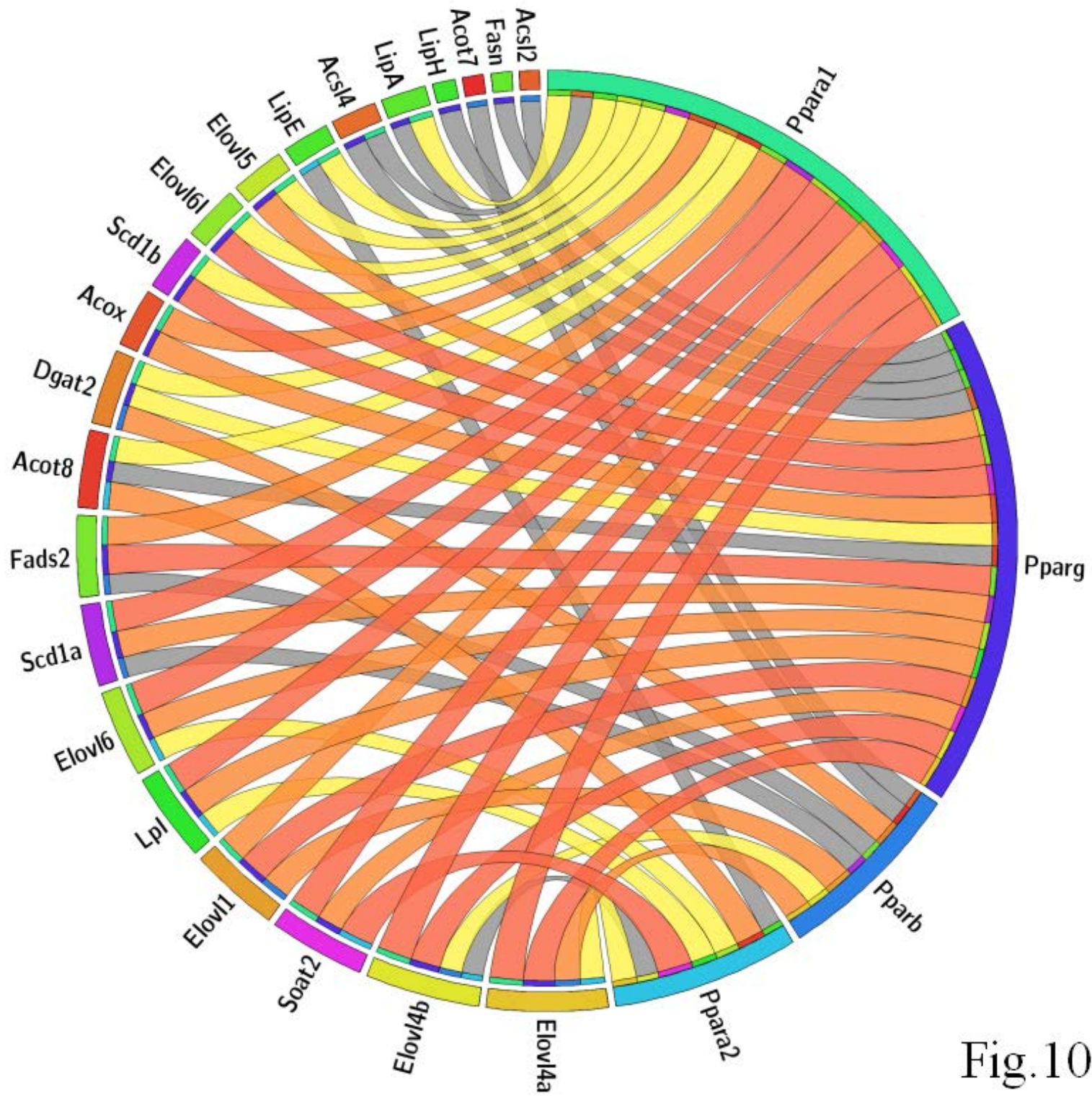


Fig.10

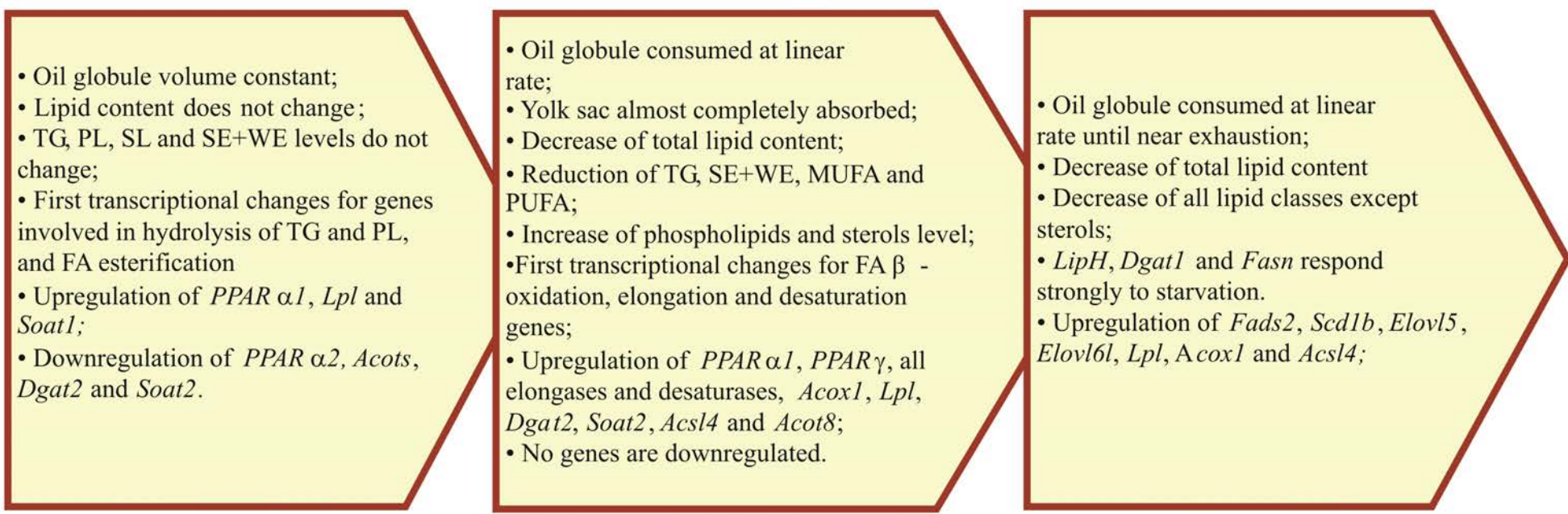
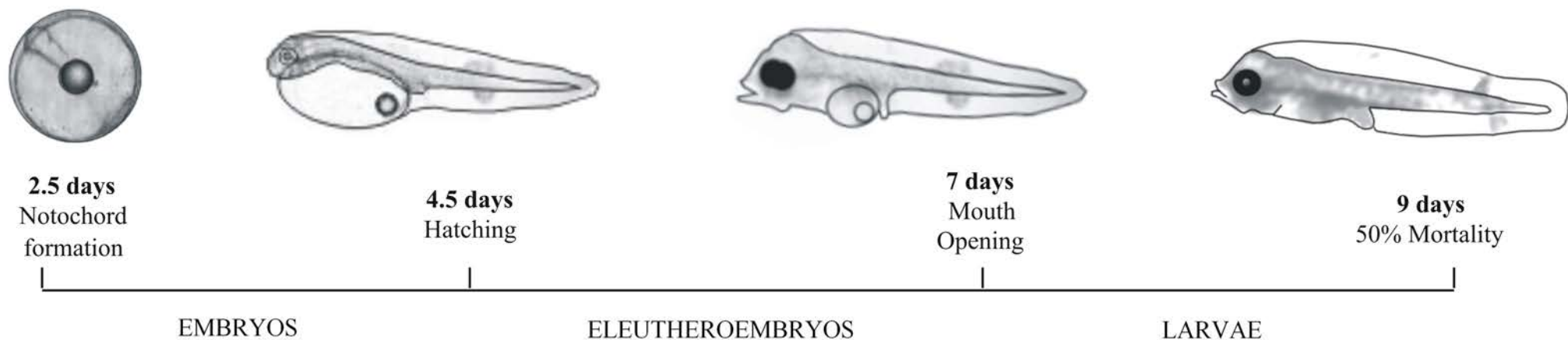


Fig. 11

Supplementary material 1. Details on the primer pairs used for real-time PCR amplification of the target and 5 reference genes, including nucleotide sequence of the sense and antisense (reversed) primers, amplicon size, amplification efficiency (E), coefficient of determination (R^2) of the primers' efficiency and NCBI accession number.

Gene symbol	Sense primer	Reversed antisense primer	Amplicon Size	E (%)	R^2	Accession Number
<u>Hydrolysis of FA-CoA to FA + CoA</u>						
<i>Acot7</i>	CTGGTTCGGGTGGAGAAG	GCTGCCTTGTGGTCATC	167	108.7	0.995	KC189922
<i>Acot8</i>	GCCCACTTCCTCGCATCTC	GCACTCGTACAGCATCCATTC	81	108.8	0.995	KC461231
<u>FA activation to FA-CoA</u>						
<i>Acs14</i>	AGTTCGGCAGTGGATTGG	CAGCGTGGCATAGAATGTC	143	104.2	0.988	KC189924
<i>Acs12</i>	CGGGCTGCTGGCTAAAGG	CTGAGATGATCCACTCTGGTCTG	85	102.1	0.984	KC189923
<u>FA β-Oxidation</u>						
<i>Cpt1a-rel</i>	ATGGGAAGAGTGGACTGAATG	GCTGGAAGGCATCTGTGG	96	105.7	0.996	KC189926
<i>Acox1</i>	CGACCAGGAGAGCCAGAG	AGCCATAGCCAGCAGAGG	78	107.2	0.980	KC189925
<u>FA biosynthesis up to 16:0</u>						
<i>Fasn</i>	AGTGGTAGTGCTGCTGAC	CTATGTTGCCTCCTGGTAG	164	89.3	0.993	KC189927
<u>FA desaturation</u>						
<i>Scd1a</i>	CGTCCGAGGCTTCTTCTTC	CAGACAGCAGGTCGTTGAG	104	102.9	0.989	KC189928
<i>Scd1b</i>	TGGAGATGTTGGACCTGAAAG	TTGAGCACCAGAGCGTATC	178	94.1	0.990	KC189929
<i>Fads2</i>	TGTCCTACTATCTTCGCTTCTTC	TAACCAGTCTTGCTGCTTCTC	170	116.7	0.973	AY546094
<u>FA incorporation into triglycerides</u>						
<i>Elovl1a</i>	GGAAGCCTGATAGTCTACAAC	AATAATCTTTGAGAACCAGAACAG	183	88.0	0.991	KC189930
<i>Elovl4a</i>	TGTTCACGCTCTGGTGGATC	GCCGTAATACAGATACATCAGGAC	107	87.8	0.968	KC189931
<i>Elovl4b</i>	CTGGTGGATTGGCATCAAG	CGTGGAACTGGATCATCTG	179	105.8	0.963	KC189932
<i>Elovl5</i>	AACTGAACACTTACATAGACTC	GATTGTAGACCACCAGGAG	189	106.5	0.978	AF465520
<i>Elovl6</i>	CATCACCGTGTGCTGTAC	GCCCGTAATGCGTAGTAAGAG	120	113.3	0.994	KC189933
<i>Elovl6l</i>	GTACCACTGGATGCACGAG	GCGAACAGCACCAGGTAG	93	91.8	0.982	KC461232
<u>FA incorporation into triglycerides</u>						
<i>Dgat1</i>	ATACTCGTGCCATCTGTGCTC	AGTCGTCTCATCAGGAACCTTAC	177	101.9	0.993	KC189938
<i>Dgat2</i>	TGCTGTGGTCATCGTTATC	CTTGTAGCGTCGTTCTC	163	105.3	0.981	KC189939
<u>FA incorporation into cholesteryl esters</u>						
<i>Soat2</i>	GCTCGTGATGTTGCTCTAC	TGAATGGAGGACAAGATTAACC	129	84.4	0.993	KC189940
<u>Hydrolysis of triglycerides and phospholipids</u>						
<i>LipA</i>	ACGATAGCATTCATAGCATTCTC	GGCAGGACGGACATCTTG	128	95.1	0.991	KC189934
<i>LipE</i>	CATAACAGGCTTGAACAG	GCTCGTAGGAGATTAGAC	179	98.6	0.993	KC189935
<i>LipH</i>	GGCAGGTCCGATGTTTAC	GCTCCTCTCAGTCCGAATG	111	76.9	0.998	KC189936
<i>Lpl</i>	CGCTCTATCCACCTGTTC	GGACCTTGTTGATGTTGTAG	154	109.1	0.987	KC189937
<u>Nuclear receptors involved in FA metabolism</u>						
<i>PPARa1</i>	GCGTCCCTTCAGTGATAT	CTCCACAGCAGATGATAG	137	97.5	0.967	JX975469
<i>PPARa2</i>	AAGTTCCAGTTCGCCACAC	GGTCTCCGACGAGATTATG	90	108.7	0.976	JX975470
<i>PPARb</i>	ACAAGTCAACACAGCCTAC	CGCACCTGGAAGTAACTG	166	98.7	0.990	KC189941
<i>PPARg</i>	TAATGGAAGGAGAGCAGTTC	CTGTGGAAGAAGCGTAGC	193	130.9	0.970	KC189932
<u>Reference Genes</u>						
<i>b-Actin</i>	TGGCATCATACCTTCTACAATG	TACGACCAGAGGCATACAG	187	99.4	0.999	EU686692
<i>EF-1a</i>	TATTAACATCGTGGTCATTGG	CAGCGTACTTGAAGGAG	153	90.3	0.991	AF467776
<i>RPL8</i>	CTCCGCCACATTGACTTC	GCCTTCTGCCACAGTAG	197	101.2	0.986	DQ848874
<i>UB2L3</i>	GGTCTGCCTGCCTATCATC	TGTATTCTTCTGCCAGGTCTG	137	102.5	0.985	KC355244
<i>18S</i>	CCAACACGGGAAACCTCAC	ATCGCTCCACCAACTAAGAAC	111	105.8	0.990	EF126038

Supplementary material 2. Rotated Matrix with variables' factor loadings. Extraction method was Principal Component Analysis. Rotation method was Varimax with Kaiser Normalization. Rotation converged in 3 iterations. Red italic marked loadings are greater than 0.8.

	CP1	CP2
<i>Acot7</i>	0.572	-0.457
<i>Acot8</i>	-0.026	<i>-0.927</i>
<i>Acox1</i>	0.782	0.515
<i>Acsl4</i>	0.761	0.172
<i>Acsl2</i>	0.648	-0.394
<i>Cpt1a-rel</i>	<i>0.831</i>	0.037
<i>Dgat1</i>	0.702	0.216
<i>Dgat2</i>	0.725	0.212
<i>Elovl1</i>	0.773	0.471
<i>Elovl4a</i>	0.488	0.721
<i>Elovl4b</i>	0.644	0.673
<i>Elovl5</i>	<i>0.854</i>	0.414
<i>Elovl6</i>	0.532	0.729
<i>Elovl6l</i>	<i>0.850</i>	0.381
<i>Fads2</i>	<i>0.836</i>	0.510
<i>Fasn</i>	<i>0.914</i>	0.042
<i>LipA</i>	-0.153	<i>-0.806</i>
<i>LipE</i>	0.003	<i>-0.898</i>
<i>LipH</i>	<i>0.860</i>	0.199
<i>Lpl</i>	0.597	0.723
<i>Scd1a</i>	0.743	0.551
<i>Scd1b</i>	<i>0.836</i>	0.433
<i>Soat2</i>	-0.251	<i>-0.885</i>
<i>PPARα1</i>	0.541	0.773
<i>PPARα2</i>	-0.056	<i>-0.846</i>
<i>PPARβ</i>	0.665	0.092
<i>PPARγ</i>	0.631	0.623

Supplementary material 3. Correlation coefficient of the relationship between the transcription rate of each PPAR and the various target genes analyzed (N = 28). Red bold marked correlation factors are significant at 0.01 level and blue italic marked ones are significant at 0.05 level.

	<i>PPARα1</i>	<i>PPARα2</i>	<i>PPARβ</i>	<i>PPARγ</i>
<i>Acot 7</i>	0.082	0.468	<i>0.564</i>	0.042
<i>Acot8</i>	-0.706	0.787	0.100	<i>-0.565</i>
<i>Acox1</i>	0.767	-0.432	0.452	0.784
<i>Acsl4</i>	<i>0.601</i>	-0.432	0.449	<i>0.609</i>
<i>Acsl2</i>	0.204	0.065	<i>0.540</i>	0.244
<i>Cpt1a-rel</i>	0.493	-0.180	0.455	0.498
<i>Dgat1</i>	0.392	-0.180	0.124	0.425
<i>Dgat2</i>	0.664	-0.132	0.712	<i>0.622</i>
<i>Elovl1</i>	0.790	-0.399	0.768	0.833
<i>Elovl4a</i>	0.941	-0.683	0.721	0.860
<i>Elovl4b</i>	0.974	<i>-0.609</i>	<i>0.626</i>	0.825
<i>Elovl5</i>	0.688	-0.337	0.425	0.746
<i>Elovl6</i>	0.911	<i>-0.654</i>	0.396	0.742
<i>Elovl6l</i>	<i>0.658</i>	-0.318	0.497	0.816
<i>Fads2</i>	0.797	-0.436	<i>0.556</i>	0.815
<i>Fasn</i>	0.416	-0.004	0.384	<i>0.550</i>
<i>LipA</i>	-0.667	0.477	-0.227	<i>-0.535</i>
<i>LipE</i>	<i>-0.659</i>	<i>0.571</i>	-0.035	<i>-0.505</i>
<i>LipH</i>	0.474	-0.152	0.347	<i>0.601</i>
<i>Lpl</i>	0.857	-0.700	0.441	0.813
<i>Scd1a</i>	0.862	-0.444	<i>0.571</i>	0.741
<i>Scd1b</i>	0.685	-0.341	0.440	0.818
<i>Soat2</i>	-0.867	0.955	-0.272	-0.730
<i>PPARα1</i>		-0.749	<i>0.588</i>	0.847
<i>PPARα2</i>			-0.180	<i>-0.583</i>
<i>PPARβ</i>				<i>0.586</i>