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

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

Abstract
Dietary long chain polyunsaturated fatty acids (FA) have been recognized of crucial importance in early development of vertebrates, contributing to the impressive morphological and physiological changes both as building blocks and to energy production. The importance of lipids along development depends on ontogenetic, phylogenetic and environmental parameters. The expression patterns of FA metabolism genes have not been characterized in developing fish embryos nor compared to lipid class profiles. Full lipid metabolism only occurred after
hatching, as revealed by alterations in lipid profiles and FA gene expression. Nonetheless, transcriptional changes of some FA genes were already present in embryos at notochord formation. Many genes displayed an expression profile opposed to the decrease of lipids along the development, while others responded solely to starvation. Transcription of most genes involved in FA metabolism had a strong correlation to PPARs mRNA levels (α1, α2, β, γ). The comparison of mRNA expression of the genes with the lipid profiles produced new insights into the FA metabolism and regulation during the development of turbot larvae, providing the basis for future studies including comparative approaches with other vertebrate species.

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

1. Introduction

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

Peroxisome proliferator activated receptors (PPARs) are involved in many processes related to ontogenesis, such as skeletal formation and differentiation, cell proliferation and epithelial cell growth and differentiation (Michalik et al., 2002; Burdik et al., 2006), lipid metabolism regulation, lipid transport, lipid and glucose oxidation, adipogenesis, lipid homeostasis, (Jump, 2002; Varga et al., 2011; Cour Poulsen et al., 2012; Cho et al., 2012) peroxisomal biogenesis (Schrader et al., 2012) and immune functions (Kostadinova et al., 2005). PUFA, oxidized PUFA and eicosanoids are ligands of all PPAR isoforms in mammals and amphibians (Hihi et al., 2002) thereby serving as major transcriptional sensors of fatty acids (FA) (Jump, 2008; Xu et al., 1999; Schupp and Lazar, 2010). Interestingly, the three PPAR subtypes display distinct but overlapping expression and functions (Cour Poulsen et al., 2012). In mammals, PPARα and PPARβ activate lipid catabolism by regulating expression of target genes encoding enzymes involved in peroxisomal and mitochondrial b-oxidation of FA, the former mainly in liver and
the second ubiquitously distributed (Wang et al., 2008; Mandard et al., 2004), while PPARg controls lipid accumulation and regulates adipogenesis and osteogenesis (Nedergaard et al., 2005; Ji et al., 2011). It is not very clear whether PPARs have a similar role in mice and humans and to what extent the regulation of PPAR target genes is shared between the two species (Rakhshandehro et al., 2009). Whether the repertoire of PPAR target genes in teleosts is similar to that of mammals is even more uncertain. Compounds that induce proliferation of peroxisomes in rodents, such as fibrate drugs, halogenated hydrocarbons, plasticizers, herbicides and pesticides, may have small or no effect in other species (Lake et al., 1989) due to differences in relative expression of PPARa (Kliewer et al., 1994; Tugwood et al., 1998).

Moreover, affinities of the ligands to the receptors can vary considerably among species and PPAR isotypes, mainly due to differences in the ligand binding domain (Krev et al., 1997). In mammals, PPARs consist of three isotypes, PPARα, β, and γ while in teleosts an additional PPARα is present due to the teleost specific whole genome duplication (Robinson-Rechavi et al., 2009).

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

In turbot eggs, lipids are present in two distinct forms, namely in the yolk and in the oil globule (Silversand et al., 1996). Lipoprotein yolk lipids are primarily polar lipids, especially phosphatidylcholine and phosphatidylethanolamine (Wiegand, 1996). In contrast, the oil globule consists of neutral lipids such as triglycerides (TG), sterol esters (SE) and wax esters (WE) (Wiegand, 1996). It was estimated that 55 to 60% of the lipids in turbot eggs are confined to the oil globule (Silversand et al., 1996). PL appear to constitute 40 to 50% of the total lipids present in turbot eggs (Devauchelle et al., 1988; McEnvoy et al., 1993; Planas et al., 1993). Since no PL are present in the oil globule, it is reasonable to suggest that they are exclusively in the yolk and that yolk lipid of turbot eggs, to a large extent, consist of PL (Silversand et al., 1996).
Molecular approaches may be helpful to clarify various aspects of FA metabolism in fish development as well as to identify physiological differences between distinct phylogenetic clades (Castro et al., 2011; Castro et al., 2012; Morais et al., 2012). Clarifying species specific nutritional requirements, effects of nutrients deficiency and starvation, and response to chemical contaminant exposure are important steps to understand the mechanisms, control and regulation of lipid metabolism. In this study, the mRNA transcription was analyzed for the four isoforms of PPAR (α1, α2, β and γ) and for 24 genes involved in various pathways of FA metabolism during the early development of turbot larvae under starving conditions, spanning the period from notochord formation to 9 days post fertilization (dpf). In parallel, the lipid profile was investigated including measurements of total lipids, main lipid classes and FAs fractions. The aim of the study was to compare the lipid profile with the mRNA expression pattern of genes involved in the FA metabolism during the early larval stages of turbot. Here we provide for the first time a comprehensive molecular and analytical snapshot of FA metabolism and regulation during the development of turbot larvae, which should serve as a solid basis for future studies in turbot and other teleost fish, but also for vertebrate classes in comparative approaches.

2. Material and methods

2.1. Egg Incubation and Larval Rearing
Fertilized eggs of Scophthalmus maximus were obtained from a commercial hatchery (Insuiña, Pescanova S.A.) at Mougás – Spain. Larvae were reared from fertilized eggs to 9 dpf in 60 L fibre glass tanks in artificial salt water (35 psu) with gentle and constant aeration at 15 ± 1°C. 50% of the artificial salt water was changed daily. Larvae were not feed after mouth opening. Samples were collected at 4 key developmental events: notochord formation (2.5 dpf), hatching (4.5 dpf), mouth opening (7 dpf) and 50% mortality (9 dpf) for yolk sac and oil globule measurements, lipid and FA analysis and molecular biology.

2.2. Yolk sac and oil globule measurements
Fifteen to twenty eggs/larvae were photographed under a microscope (Olympus IX71®) at each developmental point in order to measure yolk sac and oil globule. Yolk sac volume was calculated from length and width measures assuming a prolate ellipsoid (4/3πa2b), where a is the equatorial radius and b is the polar radius). Oil globule volume was calculated from the diameter assuming a spherical form.

2.3. Lipid Classes and FA Analysis
Lipid extraction was performed following Blight and Dyer (1959). Total lipid content was determined gravimetrically in duplicate samples of 175-400 larvae/eggs. Five lipid classes were quantified on the total lipids fraction: triglycerides (TG), phospholipids (PL), sterol esters + wax
esters (SE+WE), sterols (SL), and free fatty acids (FFA). Lipid classes were analysed by thin layer chromatography-densitometry using the Freeman and West (1966) plate staining method. Tripalmitin, cholesterol, cholesterol palmitate and palmitic acid (Sigma) were used as standards for TG, SL, SE+WE and FFA, respectively. PL were quantified following Holland and Gabbot (1971) and Hausen and Grasshoff (1983). SE and WE could not be separated by the solvent mixture and are therefore reported together as a sum. FA were transesterified to methyl esters on total lipid extracts with methanolic hydrogen chloride (Christie, 1982) and, subsequently analysed by gas chromatography, using a programmed-temperature vaporizer injector (PVT, Perkin-Elmer) in the solvent split mode, as described by Herraiz et al. (1987). Henecosanoic acid (21:0) was used as internal standard. Duplicate samples were analysed both for lipid classes and FA analysis.

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

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

2.6. Quantitative gene expression
Gene expression levels were determined by real-time PCR on an iQ5 real-time thermal cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Two microliter of a 1:10 diluted cDNA
was used in PCR reactions with the following cycle conditions: 94°C 30 sec, 55°C 30 sec, 72°C 30 sec for 40 cycles. Three calibrators + 4 blanks were run in every 96-well plate. Real-time primers were designed using Beacon Designer software version 7.51 (Premier Biosoft) to out-flank intron conserved locations. Primer characteristics and amplicon size are listed at the Supplementary Material (Table S1). Target gene expression was normalized through geometric averaging of multiple internal control genes using GeNorm Software (Vandesompele et al., 2002). From 8 normalizing genes analyzed – EF1a, 18S, UB2L3, RPL8, β-actin, GAPDH, TBP and TubB2C – 5 were chosen according to the GeNorm algorithm: EF1a, 18S, UB2L3, RPL8 and β-actin. Gene expression (mean of 2 replicates) of the 27 target genes (Table S1) is presented relatively to the expression at notochord formation.

2.7. Statistics
One-way ANOVA, followed by Tuckey’s post-hoc test, were used to compare data among different developmental stages. When some of the ANOVA assumptions (i.e. normality and homoscedasticity) were not met by data, the Kruskal-Wallis one-way analysis of variance on ranks were used followed by Dunn’s test.
Principal components analysis (PCA) was employed to study the inter-correlations of the variables (transcription of 27 genes) by clustering them into common factors. The variables within each factor are highly correlated and each factor explains a different part of the total variance in the data. Factors were Varimax rotated and Kaiser normalized. Correlations between each PPARs and the various target genes are presented graphically using CIRCOS software (Krzywinski et al., 2009).

3. Results

3.1. Yolk sac and oil globule volume
Yolk sac and oil globule dimensions are presented in Table 1. At hatching, the yolk sac was 0.278 ± 0.020 µl and was almost entirely consumed from hatching to mouth opening (p < 0.01) (Fig. 1A). At mouth opening, less than one tenth of its original volume was still present (p < 0.05), surrounding the oil globule (Fig. 1B). By day 9, the yolk sac was completely consumed (Fig. 1C). The oil globule volume remained constant until hatching. From hatching on, it started to be consumed at a linear rate until the end of the experimental period (p < 0.05). At mouth opening, half of its original volume was still present, and by 9 dpf, it was reduced to 1.02 ± 0.36 nl (Table 1). At this point, the variability of the oil globule volume was quite large since some larvae presented a visible oil globule of near 1/5 of its volume measured at notochord formation (Fig. 1C), while others did not have it at all. Values in between these extremes were rare.
3.2. Lipid content, lipid classes and fatty acids

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

3.3. Gene expression

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

3.3.1. FA activation and FA hydrolysis

Relative expression levels of two fatty acyl-CoA thioesterases (Acots) responsible for FA hydrolysis, and two acyl-CoA synthetase long-chain (Acsls) responsible for FA activation, are presented in Figure 3. Acot7 mRNA expression decreased significantly from notochord formation until hatching (p < 0.05), and returned to its initial level in later larval stages. Acot8 mRNA expression was highest at notochord formation, diminished significantly at hatching and remained constant at this expression level until 50% mortality (p < 0.05). Acsl2 and Acsl4
mRNA level did not change during development, with the exception of a higher level (p < 0.05) in Acsl2 at 50% mortality (Fig. 3).

### 3.3.2. β-Oxidation

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

### 3.3.3. FA Biosynthesis and desaturation

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

### 3.3.4. FA elongation

All 6 FA elongases (Elov1) increased their expression along the developmental stages (Fig. 5), with significant differences (p < 0.05) between hatching and mouth opening (with the exception of Elov1α: significant against notochord formation, p < 0.05). Elov14b expression rate increased the most, nearly 25-fold from eggs stage to 50% mortality. Elov11, Elov14a, Elov14b and Elov16 expression rate did not change between mouth opening and 50% mortality. However, the expression of Elov5 and Elov6-like gene increased significantly between mouth opening and 50% mortality (p < 0.05), with a 9-fold difference for Elov6-like gene from notochord formation until end of the experiment.

### 3.3.5. Hydrolysis of TG and PL

The expression rate of the various lipases displayed different patterns with development. While lipoprotein lipase (Lpl) expression rate increased during the whole period with significant changes between notochord, mouth opening and 50% mortality (p< 0.05), lipase A (LipA) and lipase E (LipE) remained at the same mRNA transcription level along development (Fig. 6). Hepatic lipase (Liph) expression remained stable until mouth opening and then increased abruptly 13-fold afterwards (p < 0.05).
3.3.6. FA esterification to TG and CE

Diacylglycerol O-acyltransferase homolog 1 (Dgat1) expression remained stable from notochord formation to mouth opening and then increased 1.5-fold at 50% mortality (p < 0.05), whereas Dgat2 decreased from notochord formation to hatching and increased to 2-fold at mouth opening and 50% mortality (p < 0.05; Fig. 7). Sterol O-acyltransferase2 (Soat2) displayed a decreased mRNA expression as development progressed, from notochord formation to hatching (p < 0.05) and remained constant until 50% mortality.

3.3.7. FA metabolism control

The four analyzed PPAR isoforms displayed different expression patterns along the development. PPARα1 and PPARγ expression rates increased with significant differences between notochord formation, hatching and mouth opening (p < 0.05). In contrast, PPARβ remained steady in all larval stages and, PPARα2, decreased from notochord formation to hatching (p < 0.05) and then maintained this low mRNA expression level until 50% mortality (Fig. 8). PPARγ was the gene whose expression increased mostly (90-fold) during the whole experimental period.

3.4. Principal components analysis

The principal components analysis extracted two factors, PC1 and PC2 that explained 76.49% of total data variation (43.46 and 33.03% respectively) (Fig. 9). Based on the examination of the graphical representation of the factor loadings for each variable, genes were grouped according to their relative positions (Fig. 9). At least three groups were initially defined according to their mRNA expression pattern along the larval development. The most determinant variables were Cpt1a-rel, Elovl5, Elov6l, Fads2, Fasn, LipH and Scd1b for PC1, and were Acot8, LipA, LipE, Soat2 and PPARa2 for PC2 (supplementary material 2 - S2). Group 1 included genes whose transcription tended to increase along the developmental stages, either from NT formation or from hatching on. This group includes PPARα1, PPARγ, and many genes positively correlated to them or inversely correlated to PPARα2 (Elov4a, Elov4b, Elov5, Elov6l, Elov6l, Lpl, Scd1a, Scd1b, Fads2, Acox). Group 2 includes genes whose transcription remained stable along development (PPARβ, Cpt1a-rel, Acls2), and whose transcription increased only at 50% mortality of the starved larvae (Fasn, LipH, Dgat1 and Acsl4) and those whose transcription was quite stable but increased or decreased significantly in some moment during development (Acot7 and Dgat2). Group 3 is composed by those genes whose transcription decreased along
development including $PPAR\alpha_2$ and other genes directly correlated to it ($Soat2$, $LipA$, $LipE$ and $Acot8$).

The correlation factors of the transcription of PPARs with that of the other genes are presented on supplementary material 3 (S3). The correlation of PPAR’s transcription to other genes ($p < 0.05$) is represented in the diagram of Figure 10. $PPAR\alpha_1$ is the nuclear receptor that was correlated to the highest number of genes, followed by $PPAR\gamma$, $PPAR\alpha_2$ and $PPAR\beta$. The analysis demonstrated that many genes were correlated or highly correlated to more than one PPAR (Fig. 10, S3). Two genes were even highly correlated to all 4 PPARs ($Elovl4a$ and $Elovl4b$). Most commonly, genes are related to 3 PPARs (9 genes) or 2 PPARs (6 genes).

$PPAR\alpha_1$ and $PPAR\gamma$ correlated together to 17 genes that are mostly involved in FA elongation or desaturation (8 genes).

4. Discussion

During embryogenesis and until mouth opening, embryos and larvae are theoretically in optimal nutritional conditions. Yolk and oil globule are considered to contain the nutrients and energy required for an adequate development until the stage of mouth opening. Proteins, PL and cholesterol are present in the yolk to be used as building blocks in new cells, tissues and organs, while TG, SE and WE are present both in the oil globule and in yolk to produce energy (Finn, 1994; Silversand et al., 1996). In our study, the levels of total lipids and lipid classes started to change from hatching to mouth opening (Table 2) and consequently, the transcriptional level in the majority of analysed genes changed significantly at this period (Figs. 3 to 8). These results suggest that the main regulations of lipid metabolism started between hatching and mouth opening at the transcript and lipid level. This outcome is in agreement with previous published studies on lipid content analysis and respirometry in turbot larvae, which pointed out that the start of lipid metabolism occurs by the time of hatching (Finn, 1994; Finn et al., 1996). It was suggested that fish species with oviparous progeny evolved two types of energetic strategies for their offspring (Finn, 1994). Type I which includes Atlantic cod and Atlantic halibut, involves only deposition of yolk in the egg, that is resorbed continuously by embryos and larvae, while in type II case, oil globule is only consumed when yolk is exhausted. In this later strategy fall turbot and seabass ($Dicentrarchus labrax$), where free amino acids appeared to be a significant energy substrate during the egg and the early yolk-sac stages while FA from neutral lipids derived from the oil globule seemed to be the main metabolic fuel after hatching (Finn, 1994; Rønnestad et al., 1998). Also, neutral lipids were
dominant for turbot at hatching, while phospholipids predominated prior to first feeding, in accordance to Rainuzzo et al. (1993).

However, a former transcriptional activity was observed between notochord formation and hatching, where 7 out of 27 genes related to FA metabolism presented significant differences at the transcription level. Those genes are involved in FA metabolism control (PPARα1, α2), FA hydrolysis (Acot7, Acot8), hydrolysis of TG and PL (Lpl), and FA esterification (Soat2, Dgat2). Most of the transcriptional changes were characterized by a higher transcript level at notochord formation as compared to hatching (Acot7, Acot8, Soat2, Dgat2) and included PPARα2, which suggested a transcriptional regulation. The altered mRNA expression of genes does not reflect a change in their functionality (Nikinmaa and Rytkoenen, 2011), and the correlation between mRNA expression and enzyme activity is generally low (Vogel and Marcotte, 2012). However, mRNA expression in our study can serve as a first indication at which steps the regulation of the lipid metabolism might be altered during the larval development. In this light, it could be speculated that the decreasing transcript level of Acots at hatching might lead to an increased FA-CoAs pool, which could be used by many other FA metabolism genes at their onset of expression at mouth opening. Lpl was up-regulated between notochord formation and hatching, which seemed to follow the mRNA expression of PPARα1 which could be interpreted as the first signs of hydrolysis of TG/PL. Transcriptional changes at early embryonic stages suggested that important processes of FA metabolism would have started already before hatching. Indeed, a decrease in PL and cholesterol content during turbot embryogenesis was reported by Finn et al. (1996) in a more detailed study on lipid sources. According to our results, PL hydrolysis might be attributed to Lpl activity.

PUFA and MUFA decreased significantly between hatching and mouth opening. Interestingly, the genes related to FA elongation (all Elovs) and FA desaturation (Fads2, Sdc1a, Sdc1b) (Fig. 4 and 5) showed the opposite pattern of unsaturated FA during the same period, being significantly up-regulated at mouth opening. It is feasible that the FA profile contained in the lipid reserves matched the initial demands of the embryos and larvae and, that at a certain developmental time, the larvae started to elongate and desaturate FA, to produce, to some extent, some of those FA no long available in the reserves. Here, the first signs of FA retailoring in turbot larvae were observed at the stage between hatching and mouth opening. In agreement with this, a great increase in desaturase (Δ6 desaturase/Fads2) and elongase (Elov5) expression is also observed in tuna larvae (Thunnus thymus) after hatching (Morais et al., 2011). From notochord formation to mouth opening PUFA are heavily used for the formation of nervous tissue in turbot larva, including eyes which are fully functional at mouth opening (Al-Maghazachi and Gibson, 1988). Most Elovs increased their activity 1.5 to 2.5-fold from
hatching to mouth opening, while Elovl4b increased more than 6-fold. At mouth opening the
eyes of turbot larvae get functional to help on foraging behaviour and in accordance, analysis by
whole-mount in situ hybridisation in zebrafish embryos showed that Elovl4b is specifically
expressed in photoreceptor cells of retina (Monroig et al., 2010). Furthermore, fads-like gene
transcripts in nibe croaker (Nibea mitsukurii) larvae fed on oleic acid-enriched Artemia were
significantly higher than those in larvae fed on 100% 22:6n-3-enriched Artemia, indicating that
the Fads2 gene was controlled by negative feedback from the quantity of 22:6n3 stored in the
larval body (Yamamoto et al., 2010). This finding is also in accordance with our data, where the
decrease of PUFA was accompanied by an increase in FA desaturases. In contrast to our results,
where Elovl5 pattern was also inverse to FA decrease, no significant differences were observed
in the transcript levels of the Elovl5 gene in nibe croaker fed on 22:6n-3-enriched Artemia
(Yamamoto et al., 2010).

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

Genes involved in the FA esterification to TG, PL and SE (Dgat1, Dgat2, Soat2) showed
different expression patterns between hatching and mouth opening. Soat2 decreased in parallel
to the decrease of TG and SE. Free cholesterol is esterified into SE by Soats in the endoplasmic
reticulum and stored in lipid droplets (Ikonen, 2008). In humans, SOAT2 is limited to the liver
and intestine, converting free cholesterol to esters destined for VLDL assembly in the liver or to
chylomicron assembly in the intestine (Leon et al., 2005). In turbot larvae, Soat2 seems to have
an initial role before hatching and loses significance afterwards. At hatching, turbot hepatocytes
contain few endoplasmic reticulum membranes (Segner et al., 1994) and the intestine is not
functional yet, which may justify low Soat2 transcription levels.
During the last stage of development (mouth opening to 50% mortality), larvae were in
starvation and consequently all lipid classes diminished significantly. Especially, mRNA
expression of LipH, Fasn and Dgat1 seemed to be nutritionally regulated, by the decreasing
level of substrate (negative feedback) during starving conditions. The transcription of these
genes was stable until mouth opening, but increased when extreme starvation conditions were
reached. LipH hydrolyzes specifically phosphatidic acid to produce 2-acyl lysophosphatidic acid
(LPA, a potent bioactive lipid mediator) and a non-specific FA (Aoki et al., 2008; Shinkuma et
al., 2010). It has been described that LPA synthesized intracellularly via the pathway of TG or
PL biosynthesis can activate PPARγ (Stapleton et al., 2011). It is plausible that LPA resultant
from PL and TG catabolism through LipH catalysis might be one of the causes of the strong
PPARγ transcription induction occurring between mouth opening and 50% mortality.
The expression of various other genes related to FA synthesis and storage, such as Elovl5,
Elovl6l, Scd1b and Fads2 changed after mouth opening in starving larvae. The expression of
these genes had already increased from notochord formation to mouth opening, but starvation
further induced their expression levels. In contrast to LipH, Fasn and Dgat1, which seemed to
be clearly nutritionally regulated, it is impossible to differentiate if the up-regulation of these
genes is ontogenetically programmed during these larval stages or, if they might be induced by
low levels of a substrate or product. A recently cloned Δ4Fad gene in Solea senegalensis was
found to be highly responsive to low levels of n-3 long chain PUFA (Morais et al., 2012) and a
Fads2 gene in N. mitsukurii was also controlled by negative feedback from the amount of
substrate (Yamamoto et al., 2010). However, the possibility of a developmentally fixed pattern
of both Fads and Elovl5 to ensure right 22:6n3 levels for neurogenesis independently of dietary
supply must not be discharged.
Between mouth opening and 50% mortality, significant changes were also observed in Acox1
mRNA expression (Fig. 3), which might indicated that during starvation β-oxidation of very
long and long chain FAs in the peroxisome contributed to the energy production. It is also
possible that Acox1 was to be used not only for FA oxidation but also for FA
elongation/shortening, through the Sprecher’s shunt (Sprecher, 1992) to produce DHA in the
peroxisome, since an intense transcription of Elovl5s (including Elovl5 and Elovl4b) and Fads
was detected at the final stage of starvation. Interestingly, the mRNA expression of the different
PPARs did not change at the end of the starvation period. This might imply that a transcriptional
response of PPARs is not involved in the regulatory response to starvation. The regulatory
response might be a direct effect on the target genes driven by the absence of substrates or by
other metabolic signals of starvation. In this aspect, the regulation of fasting in teleost fish might
be different from mammals. It has been shown in PPARα null mice that PPARα is especially
important for the adaptive response to fasting by stimulating hepatic FA oxidation and
ketogenesis (Kersten et al., 1999; Hashimoto et al., 2000; Leone et al., 1999).
In contrast to the starvation period, the different PPARs demonstrated different expression patterns between notochord formation and mouth opening. Transcriptional activity of PPARα2 was high at notochord formation and decreased 4-fold to a low value from then on. The mRNA expression profile of PPARα1 was the opposite of PPARα2, and increased significantly from notochord to hatching and mouth opening. Fernández et al. (Fernandez et al., 2011) observed a strong increase in PPARα1 transcriptional activity in Sparus aurata, from days 2 to 7 after hatching, which confirms our data for PPARα1. The different transcription profile observed on the two PPARα isoforms is probably indicative of a gene sub-functionalization.

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

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

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

Correlation analysis pinpointed that the transcription of various genes may be controlled by more than one PPAR, more commonly, by two or three PPARs (Fig 10 and S3). PPARα1 transcription is highly correlated to PPARβ and especially to PPARγ and PPARα2.
PPARγ are the nuclear receptors whose transcription correlated to more genes simultaneously. Moreover, all genes correlated to PPARα2 transcription were also inversely correlated to PPARα1 and PPARγ, which might indicate that they co-ordinately modulate the transcription of various genes together, but in opposite directions. To our knowledge, this is the first study in a teleost fish species providing evidence for a regulation of FA metabolism genes by a joint action of different PPARs. In other animal models, it is known that some genes are regulated by more than one PPAR. It has been suggested that Acox1, L-Fabp and other PPAR-dependent genes may be co-ordinately modulated in the small intestine of developing rats, during postnatal development, by the disproportional expression of PPARα over PPARβ (Mochizuki et al., 2001). Also, the human LPL promoter is regulated differentially by the binding of PPARα or PPARγ together with the retinoic acid X receptor (RXR) heterodimer (Schoonjans et al., 1996). Also, thiazolidinediones predominantly affected adipocyte LPL production through activation of PPARγ, while fibrates exerted their effects mainly in the liver via activation of PPARα (Schoonjans et al., 1996). Genes that were correlated uniquely to PPARγ (Fig 10 and S3) were related to FA de novo synthesis (Fasn) and to PL hydrolysis (LipH). Genes correlated to PPARβ alone are related to FA activation and deactivation (Acsl2 and Acot7).

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

a) Embryos – from notochord formation to hatching – the oil globule was not consumed, analytical lipid parameters were constant, but the first transcriptional alterations in lipid metabolism genes were observed. First transcriptional signs for hydrolysis of TG and PL (Lpl) were detected together with a high expression of PPARα2.

b) Eleutheroembryos – larvae feeding on endogenous reserves – lipids started to be metabolized, which could be observed by the decrease of the lipid classes levels related to energy storage (TG, SE+WE, MUFA and PUFA) as well as the reduction of oil globule and yolk sac volumes. Moreover, structural lipids (i.e PL, SL) increased significantly during this stage. Many genes related to lipid anabolism and catabolism were up-regulated, and no gene was down-regulated during this stage. First transcriptional signs for FA β-oxidation (Acox1), FA desaturation (Fads2, Scd1a, Scd1b) and FA elongation (all Elovl) were observed.

c) Larvae – mouth opened but not fed – oil globule was consumed until exhaustion. The level of energy storage lipids (SE+WE and TG) decreased until exhaustion. PL decrease, and only SL levels remain steady. LipH, Dgat1 and Fasn, whose transcriptional activity did not change during the whole development, responded strongly to starvation at this stage.

5. Conclusions
In conclusion, full lipid metabolism only occurs after hatching, as revealed by alterations in lipid profiles and FA gene expression; Transcriptional changes of some FA genes were already present in embryos at notochord formation; Many of the studied genes displayed an expression profile, which is opposed to the decrease of lipids along the development due to the consumption of yolk reserves and oil globule, suggesting a negative feedback of substrates on FA gene expression; Other genes (e.g. LipH, Fasn and Dgat1) were not altered during development, but responded strongly to starvation and; Transcription of most genes involved in FA metabolism had a strong correlation to PPARs mRNA levels ($\alpha_1$, $\alpha_2$, $\beta$, $\gamma$) and evidence is suggested for an orchestrated control by two or three PPARs isoforms.

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

References


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

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

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

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

Fig. 5 - Transcription level for genes involved in FA elongation (Elovl1, Elovl4a, Elovl4b, Elovl5, Elovl6 and Elovl6l) represented as mean values (± standard error) along turbot larvae development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO – mouth opening. One-way ANOVA F-test statistic or one-way Kruskal-Wallis H-statistic and tests probability (p) are presented. Different letters (a, b, c) mean significant differences on expression levels between developmental stages after Tukey or Dunn’s test (p < 0.05).

Fig. 6 - Transcription level of genes involved in hydrolysis of TG and PL (LipA, LipE, LipH and Lpl) represented as mean values (± standard error) along turbot larvae development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO – mouth opening. One-way ANOVA F-test statistic or one-way Kruskal-Wallis H-statistic and tests probability (p) are presented. Different letters (a, b, c) mean significant differences on expression levels between developmental stages after Tukey or Dunn’s test (p < 0.05).
Fig. 7 - Transcription level of genes involved in FA esterification to TG and CE (Dgat1, Dgat2 and Soat2) represented as mean values (± standard error) along turbot larvae development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO – mouth opening. One-way ANOVA F-test statistic and test probability (p) are presented. Different letters (a, b, c, d) mean significant differences on expression levels between developmental stages after Tukey test (p < 0.05).

Fig. 8 - Transcription level of genes involved in FA metabolism control (PPARα1, PPARα2, PPARβ and PPARγ) represented as mean values (± standard error) along turbot larvae development, from notochord formation (NT) to 50% mortality (50%M). H – hatching; MO – mouth opening. One-way ANOVA F-test statistic or one-way Kruskal-Wallis H-statistic and tests probability (p) are presented. Different letters (a, b, c, d) mean significant differences on expression levels between developmental stages after Tukey or Dunn’s test (p < 0.05).

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

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

Fig. 11 – Schematic description of the morphological, biochemical and molecular events along larval development from notochord formation in the egg to 50% mortality in starving (non feed) larvae at 3 days after mouth opening.
Table 1

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

<table>
<thead>
<tr>
<th>Days Post Fertilization (dpf)</th>
<th>2.5</th>
<th>4.5</th>
<th>7</th>
<th>9</th>
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<tr>
<td>Developmental event</td>
<td>notochord formation</td>
<td>hatching</td>
<td>mouth opening</td>
<td>50% mortality</td>
</tr>
<tr>
<td>Yolk sac (µl)</td>
<td>-</td>
<td>0.278 ± 0.020(^a)</td>
<td>0.022 ± 0.002(^b)</td>
<td>-</td>
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<tr>
<td>Oil Globule (nl)</td>
<td>4.77 ± 0.90(^a)</td>
<td>4.85 ± 0.11(^a)</td>
<td>2.22 ± 0.05(^b)</td>
<td>1.02 ± 0.30(^d)</td>
</tr>
<tr>
<td>Total Lipids (µg/egg or larva)</td>
<td>9.63 ± 0.28(^a)</td>
<td>9.98 ± 0.37(^a)</td>
<td>6.90 ± 0.23(^b)</td>
<td>3.92 ± 0.03(^c)</td>
</tr>
<tr>
<td>Total Fatty Acids (µg/egg or larva)</td>
<td>5.30 ± 0.22(^a)</td>
<td>5.25 ± 0.04(^a)</td>
<td>3.76 ± 0.19(^ab)</td>
<td>1.62 ± 0.15(^b)</td>
</tr>
</tbody>
</table>
Triglycerides, phospholipids, sterols, Sterols esters + Wax esters and free fatty acids content (µg/larvae or egg) along early development of turbot (dpf – days post fertilization). Values correspond to the mean ± standard deviation of 2 pooled samples (n = 175-400 eggs or larvae) for lipid classes analysis. Different superscript letter in the same row indicate significant differences (p < 0.05).

<table>
<thead>
<tr>
<th>Days Post Fertilization (dpf)</th>
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<th>4.5</th>
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</thead>
<tbody>
<tr>
<td>Developmental event</td>
<td>notochord formation</td>
<td>hatching</td>
<td>mouth opening</td>
<td>50% mortality</td>
</tr>
<tr>
<td>Triglycerides (TG)</td>
<td>1.20 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.23 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Phospholipids (PL)</td>
<td>1.65 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.08 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.47 ± 0.18&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sterols (SL)</td>
<td>0.40 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.51 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Sterols Esters + Waxes Esters (SE+W)</td>
<td>1.11 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.15 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Free Fatty Acids (FFA)</td>
<td>-</td>
<td>-</td>
<td>0.03 ± 0.02</td>
<td>0.01 ± 0.02</td>
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</tbody>
</table>

<sup>a</sup> <sup>b</sup> <sup>c</sup>
Table 3

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

<table>
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<tr>
<th>Days Post Fertilization (dpf)</th>
<th>2.5</th>
<th>4.5</th>
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<tbody>
<tr>
<td>Developmental event</td>
<td>notochord formation</td>
<td>hatching</td>
<td>mouth opening</td>
<td>50% mortality</td>
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<td>Saturated Fatty Acids</td>
<td>1.80 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.47 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Monounsaturated Fatty Acids</td>
<td>1.55 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.49 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Polyunsaturated Fatty Acids</td>
<td>1.98 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.11 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.43 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.59 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>n-3 series Fatty Acids</td>
<td>1.76 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.85 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.29 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.51 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>n-6 series Fatty Acids</td>
<td>0.22 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 4

**Fasn**

- p < 0.001
- F = 26.11

**Fads2**

- p < 0.001
- F = 120.03

**Scd1a**

- p < 0.001
- F = 20.02

**Scd1b**

- p < 0.001
- F = 191.6
Fig. 5
Relative mRNA expression

LipA
p > 0.050
F = 2.831

LipE
p > 0.050
F = 2.545

LipH
p < 0.050
H = 9.543

Lpl
p < 0.001
F = 30.16

NT  H  MO  50%M

Fig. 6
**Fig. 7**

- **Dgat1**
  - $p < 0.001$
  - $F = 12.664$
  - Bars labeled with 'a' and 'b'

- **Dgat2**
  - $p < 0.050$
  - $F = 10.81$
  - Bars labeled with 'a', 'b', and 'c'

- **Soat2**
  - $p < 0.001$
  - $F = 47.221$
  - Bars labeled with 'a' and 'b'
Fig. 8

- **Pparα1**: p < 0.001, F = 1781.6
- **Pparα2**: p < 0.010, H = 11.743
- **Pparβ**: p > 0.050, F = 3.224
- **Pparγ**: p < 0.050, H = 10.981

Relative mRNA expression

- NT, H, MO, 50% M
**EMBRYOS**

- Oil globule volume constant;
- Lipid content does not change;
- TG, PL, SL and SE+WE levels do not change;
- First transcriptional changes for genes involved in hydrolysis of TG and PL, and FA esterification
- Upregulation of *PPARα1, Lpl* and *Soat1*;
- Downregulation of *PPARα2, Acots, Dgat2* and *Soat2*.

**ELEUTHEROEMBRYOS**

- Oil globule consumed at linear rate;
- Yolk sac almost completely absorbed;
- Decrease of total lipid content;
- Reduction of TG, SE+WE, MUFA and PUFA;
- Increase of phospholipids and sterols level;
- First transcriptional changes for FA β-oxidation, elongation and desaturation genes;
- Upregulation of *PPARα1, PPARγ, all elongases and desaturases, Acox1, Lpl, Dgat2, Soat2, Acsl4 and Acot8*;
- No genes are downregulated.

**LARVAE**

- Oil globule consumed at linear rate until near exhaustion;
- Decrease of total lipid content
- Decrease of all lipid classes except sterols;
- *LipH, Dgat1* and *Fasn* respond strongly to starvation.
- Upregulation of *Fads2, Scd1b, Elovl5, Elovl6l, Lpl, Acox1* and *Acsl4*;

---

**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 (R²) of the primers’ efficiency and NCBI accession number.

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<tr>
<th>Gene symbol</th>
<th>Sense primer</th>
<th>Reversed antisense primer</th>
<th>Amplicon Size</th>
<th>E (%)</th>
<th>R²</th>
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<tr>
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<td>Elovl1a</td>
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<td>88.0</td>
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<td>0.991</td>
<td>KC189930</td>
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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.

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<th>CP2</th>
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<td>Acsl4</td>
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<td>Acsl2</td>
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**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.

<table>
<thead>
<tr>
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<th>PPARα1</th>
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<th>PPARγ</th>
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