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Characterization of lipid metabolism genes and the influence of fatty acid supplementation in the hepatic lipid metabolism of dusky grouper (*Epinephelus marginatus*)

Bruno C. Araújo[a,b,*], Nicholas M. Wade[c], Paulo H. de Mello[a,b], Jandyr de A. Rodrigues-Filho[d], Carlos E.O. Garcia[a,b], Mariana F. de Campos[a,b], Natasha A. Botwright[c], Diogo T. Hashimoto[e], Renata G. Moreira[a,b]

[a] Instituto de Biociências, Departamento de Fisiologia da Universidade de São Paulo, Rua do Matão, trav. 14, n.321, SP 05508-090, Brazil
[b] Centro de Biologia Marinha da Universidade de São Paulo (CEBIMar/USP), Rodovia Manoel Hipólito do Rego, km 131,5, São Sebastião, SP CEP 11600-000, Brazil
[c] CSIRO Agriculture and Food, Queensland Bioscience Precinct, 306 Carmody Rd, St Lucia, QLD 4067, Australia
[d] Fundação Instituto de Pesca do Estado do Rio de Janeiro, Praça Fonseca Ramos, s/n., Centro, Niterói, RJ 24030-020, Brazil
[e] Universidade Estadual Paulista "Júlio de Mesquita Filho", Centro de Aquicultura (CAUNESP), Via de acesso Prof. Paulo Donato Castelane s/n., Jaboticabal, SP CEP 14884-900, Brazil

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ABSTRACT

Dusky grouper is an important commercial fish species in many countries, but some factors such as overfishing has significantly reduced their natural stocks. Aquaculture emerges as a unique way to conserve this species, but very little biological information is available, limiting the production of this endangered species. To understand and generate more knowledge about this species, liver transcriptome sequencing and *de novo* assembly was performed for *E. marginatus* by Next Generation Sequencing (NGS). Sequences obtained were used as a tool to validate the presence of key genes relevant to lipid metabolism, and their expression was quantified by qPCR. Moreover, we investigated the influence of supplementing different dietary fatty acids on hepatic lipid metabolism. The results showed that the different fatty acids added to the diet dramatically changed the gene expression of some key enzymes associated with lipid metabolism as well as hepatic fatty acid profiles. *Elongase 5* gene expression was shown to influence intermediate hepatic fatty acid elongation in all experimental groups. Hepatic triglycerides reflected the diet composition more than hepatic phospholipids, and were characterized mainly by the high percentage of 18:3n3 in animals fed with a linseed oil rich diet. Results for the saturated and monounsaturated fatty acids suggest a self-regulatory potential for retention and oxidation processes in liver, since in general the tissues did not directly reflect these fatty acid diet compositions. These results indicated that genes involved in lipid metabolism pathways might be potential biomarkers to assess lipid requirements in the formulated diet for this species.

1. Introduction

Currently several factors, such as overfishing, high commercial value and reproductive characteristics have resulted in a population decline of dusky grouper (*Epinephelus marginatus*) and the inclusion of this species on the red list of the IUCN (The International Union for Conservation of Nature) as an endangered species (Cornish and Harmelin-Vivien, 2004; http://www.iucnredlist.org/; Rodrigues-Filho et al., 2009). Therefore, captive production can be considered a feasible option to conserve their natural stocks. However, information about the genetics, functional genomics and nutritional requirements of *E. marginatus* are scarce, limiting the production and development of this species.

In recent years, transcriptome studies by Next Generation Sequencing (NGS) have become a powerful molecular tool for researchers. *De novo* transcriptome analysis has been effective across a wide range of fields, such as the identification of molecular markers, discovery of new genes, comparative analysis and gene expression analysis, which have then been applied to many key areas such as conservation, evolution, quantitative genetics, selective breeding, functional genomics, reproductive biology and nutrition (Calduch-Giner et al., 2013; Fox et al., 2014; Leaver et al., 2008; Qian et al.,

* Corresponding author at: Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, SP 05508-090, Brazil.
E-mail address: brunocavar@usp.br (B.C. Araújo).

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2014; Rotlland et al., 2015). This includes fish species of commercial interest such as *Gadus morhua* (Johansen et al., 2011), *Sparus aurata* (Calduch-Giner et al., 2013), *Salmo trutta* (Uren Webster et al., 2013), *Dicentrarchus labrax* (Magnanou et al., 2014) and specifically in nutritional studies in *Lates calcarifer* (Wade et al., 2014), *Senegalese sole* (Richard et al., 2014) and *Salmo salar* (De Santis et al., 2015).

Lipid nutrition is the main bottleneck in marine fish production. Among other processes, knowledge of lipid metabolism in lipogenic and lipolytic tissues such as liver, through the identification of genes related to the fatty acid (FA) synthesis and oxidation process, directly contributes to the development of specific diets in aquaculture, thereby improving the production chain of commercial species. Lipid metabolism in fish is modulated by global metabolic processes, such as energy generation from FA oxidation, production of FA molecules from specific substrates and bioconversion of biologically important FA from their precursors (Glencross, 2009; Nelson and Cox, 2005; Tocher, 2003; Turchini and Francis, 2009). Some studies showed the direct influence of dietary FA on the expression of genes related to FA synthesis and oxidation pathways, and consequently in the hepatic FA profile (Stubhaug et al., 2006, 2007; Torstensen and Stubhaug, 2004). These studies suggest that the mechanisms that regulate FA synthesis, oxidation and deposition have a preference for some FA instead of others, and are modulated by the differential expression of genes like acetyl-CoA carboxylase (ACC), stearoyl CoA desaturase (SCD), fatty acid synthase (FAS), acyl-CoA oxidase (ACOX), elongases (ELOVL), fatty acid desaturase (FADS) and acyl CoA dehydrogenase very long chain (ACADVL). Current research is focused on a species specific understanding of essential FA in marine fish nutrition, mainly 18:3n3 α-linolenic acid (ALA) and 22:6n3 docosahexaenoic acid (DHA) (Glencross, 2009; Taylor et al., 2015; Tocher, 2003; Turchini and Francis, 2009), and the changes in expression of key regulatory genes after dietary fatty acid manipulation (Alhazzaa et al., 2011; Araújo et al., 2016; Jin et al., 2017; Li et al., 2016; Salini et al., 2015b, 2016a; Yan et al., 2017).

This study is part of a program that aims to develop and improve the production potential and conservation status of the dusky grouper through expansion of knowledge of nutritional requirements and the development of specific commercial diets. Therefore, to develop tools to understand the metabolic responses of dusky grouper to dietary FA, we performed a *de novo* transcriptome assembly from liver tissue and investigated the metabolic and molecular responses to dietary FA manipulation through the use of different raw materials. Considering the importance of FA for aquatic organisms, the main objective of this study was to understand the FA mobilization, deposition, synthesis and oxidation pathways in the dusky grouper with a particular focus on long chain n3 FA. These results improve the knowledge of lipid nutrition and metabolism, and contribute to improving the production and conservation of this species.

2. Material and methods

2.1. Experimental design and sample material

Twenty-four adult female dusky grouper (*E. marginatus*) (2.6 ± 0.6 kg and 54 ± 3.6 cm) were sampled by artisanal fishing in São Sebastião city, São Paulo state, Brazil. The fish were kept for 20 weeks (approximately 140 days) in 2000 L tanks under natural temperature (23 ± 1.5 °C) and photoperiod at the Marine Biology Center at the University of São Paulo (CEBIMar/USP). Experimental animals were divided, according to the biomass, in four experimental groups (in duplicated tanks), with a total of eight animals each group. The base diet used in the experiment was composed of sardines without head and viscera, as the wild animals did not accept a commercial diet. Experimental diets were made by the introduction of capsules containing the respective experimental oil (2000 mg total) placed inside the sardines. The first group (S) was fed with a diet composed of only sardines; the second group (LO) was fed with sardines supplemented with linseed oil (rich in 18:3n3); the third group (FO) was fed with sardines supplemented with fish oil (rich in 20:5n3 and 22:6n3); and the last group (CO) was fed with sardines supplemented with coconut oil (rich in 12:0). The feed was supplied every 72 hours, in proportion to 4% of tank biomass. Twenty minutes after feeding, the diet that was not ingested by the experimental animals was removed from the tank, previously dried in filter paper and weighed to calculate the food consumption. After 20 weeks, three fish from each tank were anesthetized with 4 g of benzocaine diluted in 40 ml of ethanol and mixed in 40 L of saltwater, and were posteriorly killed by spinal cord section. Four aliquots of liver (from the same animal) were collected and immediately frozen in liquid nitrogen and subsequently stored at 80 °C for the metabolic and molecular analysis. The experiment was performed in accordance with the Animal Ethics Committee of the Biosciences Institute of the University of São Paulo (Protocol N°. 055/2008).

2.2. Fatty acid analysis

Total lipids from the diets and liver were extracted using a mix of chloroform/methanol/water (2:1:0.5) (modified Folch et al., 1957). Lipid extract from the tissues was fractionated in triacylglycerol (TAG) and phospholipid (PL) using thin layer chromatography (Yang, 1995). TAG and PL extracts from the liver were methylated using 5% HCl methanol prepared by dissolving 10%vol of acetylchloride in methanol, according to Christie (2003). FA analysis was carried out with a Varian gas chromatograph (GC, Model 3900, Walnut Creek, CA, USA), coupled with a flame ionization detector (FID) and a CP-8410 auto sampler. FAMEs analysis was performed using a capillary column (CP Wax 52 CB, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length) and hydrogen was used as the carrier gas at a linear velocity of 22 cm/s. The column was programmed to start in 170 °C for 1 min, followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The auto-injector and FID temperatures were kept at 250 and 260 °C, respectively. FAMEs were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualimix, PUFA fish M, Menhaden Oil, Larodan). Data are presented as percentage of total lipid based on peak areas analyzed.

2.3. RNA extraction and normalization

Total RNA from liver was extracted using RNeasy Lipid Tissue kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. The quantity of extracted RNA was assessed by Nanodrop™ Spectrophotometer (Thermo Fisher Scientific, USA), and the average RIN (RNA integrity number) of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany).

2.4. Library preparation and sequencing

RNA from all 24 fish, with equal concentration (1000 ng/μl), was pooled and used for library construction using the TruSeq RNA Sample Preparation kit (Illumina Inc., USA) according to the manufacturer's specifications. The library quality was validated based on the RIN (> 6.8) using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Transcriptome sequencing was performed with Illumina Miseq® platform (Illumina Inc., USA) using a 300 bp (base pairs) paired-end sequencing strategy. The raw sequence data is available in the NCBI Sequence Read of Archive (SRA) under the accession number SRX1452893.

2.5. Assembly of transcripts, annotation and identification of lipid relevant genes

Quality control was completed on raw data prior to assembly including removing adaptor sequences, removing reads with unknown

nucleotides > 5% and low quality reads with PHRED score < 10, using CLC Genomics Workbench version 7.0.3. (CLC Bio, Aarhus, Denmark). Clean reads were assembled using CLC Genomics Workbench version 7.0.3. (CLC Bio, Aarhus, Denmark) with default local alignment settings (mismatch cost of 2, insertion cost of 3, deletion cost of 3). Short contigs (< 200 bp) and low quality contigs (0.12% of reads containing a PHRED score ≤ 20) were removed. Contigs assemblies were annotated using Blast2GO (Conesa et al., 2005) and the basic alignment search tool (BLAST) algorithm (Altschul et al., 1990). Transcriptome *de novo* assembly, to obtain genes, was carried out using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark), followed by BLASTX alignment against the protein database of non-redundant (nr), zebrafish (*Danio rerio*) and fugu (*Takifugu rubripes*). The best alignment results were selected to determine the sequences to be used in quantitative qPCR (minimum similarity of 85%).

2.6. Quantitative RT-PCR

Reverse transcription was performed on 1 μg of total RNA using Superscript III (Invitrogen, CA, USA) with a master mix containing 25 μM of oligo (dT) 20 and 25 μM of random hexamers, along with 400 pg of control luciferase RNA (L4561, Promega, WI, USA). PCR amplification was performed for each gene-specific primer using a pool of DNAse-treated RNA samples to ensure the absence of contaminating genomic DNA. Primers for each specific gene (*Em ELOVL5*, *Em ACC*, *Em FAS*, *Em SCD*, *Em CPT1a*, *Em ACADVL* and *Em ACOX1*), were designed using PerlPrimer (Marshall, 2004), with the following conditions: temperature: 59–61 °C, distance 155 between primer pair: 100–300 bp and, GC%: 45–55%. The primers for selected genes are detailed in Table 1. For all genes, PCR efficiency was optimized to be between 95% and 105% using the slope of a standard curve over a five-fold serial dilution of a pooled cDNA sample containing all samples analyzed.

Real-time polymerase chain amplification reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), 0.2 μM of each primer and an equivalent of 7.5 ng of the reverse-transcribed RNA. The reaction was incubated for 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 40 s at 60 °C. Each reaction was performed in triplicate in an ExicyclerTM96 (Bioneer, Republic of Korea), using the software Exicycler Diagnosis3 (Bioneer, Republic of Korea). Normalization was performed using the ΔCq method (where Cq is the quantification cycle) (De Santis et al., 2011). Gene expression was normalized to a pooled reference sample containing cDNA from all samples and log2-transformed (Livak and Schmittgen, 2001). Elongation factor 1 alpha (EF1α) was used as an endogenous reference, according to previous studies performed with the same species (Garcia et al., 2013). Expression levels of each gene relative to each another were determined by normalizing the cycle threshold values for each gene to EF1α.

2.7. Statistical analyses

Data were presented as mean ± SEM (standard error of the mean). The comparisons between diets, hepatic fatty acid profiles and gene expression of different experimental groups was performed by one-way analysis of variance (ANOVA), followed by the Tukey's HSD test using the software SIGMASTAT for Windows version 3.5 (SigmaStat Software, CA, USA), with a significance level of 5% ($P < 0.05$).

3. Results

3.1. Transcriptome assembly

Sequencing generated 52,022,852 nucleotide paired-end reads, with an average length of 200.9 bp. After trimming, 51,931,408 reads were retained and used to *de novo* assemble the *E. marginatus* liver transcriptome, using the default local alignment settings (mismatch cost of 2, insertion cost of 3 and deletion cost of 3). *De novo* assembly resulted in 92,887 high quality contigs of 646 bp average size, with a total of 14,019 > 999 bp (Table 2).

3.2. Functional annotation and identification of orthologous lipid relevant genes

It was observed that a higher percentage of GO terms of *E. marginatus* sequences matched with zebrafish (23.3%), compared with fugu (14.3%). For both zebrafish and fugu, a higher representation of GO (gene ontology) terms relating to biological processes was observed (50.2% and 45.6%, respectively), followed by molecular function (30.6% and 34.3%, respectively) and cellular components (18.1% and 20.0%, respectively) (Supplementary file 1). The results generated by Blast2GO also showed that the most frequent GO terms related to biological processes, molecular function or cellular component were cellular processes, binding and cell, respectively.

To identify putative *E. marginatus* genes and gene transcripts that act

Table 1
Target genes and primer sequences for quantitative polymerase chain reaction (qPCR) of lipid synthesis and β-oxidation in dusky grouper (*Epinephelus marginatus*).

Gene name	Gene Abbreviation	Genbank accession number	Primer name	Primer sequence
Synthesis				
Elongase 5	*Em ELOVL5*	KY623454	*Em ELOVL5* For	TCACACTCATCTTCCTCTTCTC
			Em ELOVL5 Rev	GGTTTCTCAAATGTCAATCCAC
Acetyl CoA carboxylase	*Em ACC*	KY623451	*Em ACC* For	CATCTTGACTGAACTCACCC
			Em ACC Rev	CATCCTGACAACCTGATTACTG
Fatty acid synthase	*Em FAS*	KY623455	*Em FAS* For	CTCGCAACTTATTGATGGTG
			Em FAS Rev	ATGTAAATAGCCTGAACCCT
Stearoyl CoA desaturase (delta9)	*Em SCD*	KY623452	*Em SCD* For	TCACCAACTATTAGCCACAG
			Em SCD Rev	TATTGCCTGTAGAGAAACCT
β-Oxidation				
Carnitine palmitoyltransferase	*Em CPT1a*	KY623450	*Em CPT1a* For	CAACAATGATCTGCCTTCGT
			Em CPT1a Rev	CACAAATCACAAACATTCAGCC
Acyl CoA dehydrogenase (very long chain)	*Em ACADVL*	KY623449	*Em ACADVL* For	TTGCCATTCTTCAGTTACCA
			Em ACADVL Rev	TTTCACTCTTCAACATCTCCA
Acyl-CoA Oxidase	*Em ACOX1*	KY623453	*Em ACOX1* For	TTGTTTGTAGACCTCCACCA
			Em ACOX1 Rev	ATTGTGTCCTATCTGAATGAAC
Control				
Luciferase	*Luc*	–	Luc qPCR For	GGTGTTGGGCGCGTTATTTA
			Luc qPCR Rev	CGGTAGGCTGCGAAATGC
Elongation factor 1 alpha	*EF1α*	–	*Em EFIα* For	TCGGAGGTATTGGAACTG
			Em EFIα Rev	CCTCAGTGGTCAGGTTGC

Table 2
Summary of Illumina Miseq® sequencing of *E. marginatus* liver tissue, after trimming and *de novo* transcriptome assembly using CLC Genomics Workbench.

	Total
Illumina Miseq	
Read number	52,022,852
Nucleotides (Gb)	10.45
Read average length (bp)	200.9
After trimming	
Read number	51,931,408
Nucleotides (Gb)	9.93
Read average length (bp)	191.2
De novo assembly	
Contigs	92,887
Average length (bp)	646
N50	1029

in lipid synthesis and β-oxidation, transcripts associated with GO terms under lipid metabolic process (GO:006629), phospholipid metabolic process (GO:0006644), lipid modification (GO:0030258), lipid catabolic process (GO:0016042) and neutral lipid metabolic process (GO:0006638) were selected. A total of 1446 lipid-relevant genes were identified against the NCBI generated zebrafish database, among which 858 were related to lipid metabolic processes, 285 were related to phospholipid metabolic processes, 137 were related to lipid modification, 120 were related to lipid catabolic processes and 46 were related to neutral lipid metabolic processes. In contrast, 719 *E. marginatus* transcripts were matched to the NCBI generated fugu database, among which 365 were related to lipid metabolic processes, 151 were related to phospholipid metabolic processes, 101 were related to lipid modification, 67 were related to the lipid catabolic processes and 35 were related to neutral lipid metabolic processes (Fig. 1).

3.3. Fatty acid profile

3.3.1. Diets and hepatic fatty acid profile

No significant difference in food consumption was observed between the experimental groups. Total lipid of livers from S, LO, FO and CO animals were 59.6 ± 18.3, 45.6 ± 17.3, 38.6 ± 10.7 and 52.3 ± 17.3 mg/g respectively, and no significant differences were observed between the groups. The sardine (total lipid 27.5 ± 7.2 mg/g) FA profile contained 14.40% and 7.86% of 22:6n3 DHA and 20:5n3 eicosapentaenoic acid (EPA), respectively, while the FO (total lipid 91.8 ± 16.2 mg/g) diet contained 15.96% of DHA and 13.02% of EPA. The LO (total lipid 89.3 ± 21.2 mg/g) diet contained 9.87% of 18:3n3, far in excess of the other experimental diets that showed a percentage between 1 and 1.7% (Table 3) (the CO diet total lipid was 99.7 ± 18.6 mg/g).

3.3.2. FA profile of hepatic phospholipids

Hepatic phospholipids (PL) from CO fed fish contained a significantly higher proportion of 14:0 and 16:0 compared with the others groups. Conversely, CO fed fish contained a significantly lower proportion of 18:0 compared with animals LO fed fish. Taken together, these changes did not result in a significant change in total saturated FA (SFA) between experimental groups. Significant alterations were observed in total monounsaturated FA (MUFA), with a higher percentage of these fatty acids in animals fed CO. This difference is mainly attributed to the higher percentage of 16:1 compared to LO and FO groups, and 18:1n9 compared with the other groups. For polyunsaturated FA (PUFA), a significantly higher percentage of DHA was present in the liver phospholipid fraction of fish fed the FO diet when compared to fish fed the CO diet. Lastly, the LO fed fish showed a higher percentage of 18:2n6 in the liver phospholipid fraction when compared to animals from other experimental groups (Table 4).

3.3.3. FA profile of hepatic triglycerides

Hepatic triglycerides (TG) showed significant changes in total SFA, total MUFA and total PUFA. The proportion of 14:0 was significantly higher in the CO fed fish compared to animals from the other experimental diet groups. Animals fed the CO diet showed a significantly higher proportion of 16:0 in the liver when compared to animals fed the LO diet. CO fed fish contained a significantly lower proportion of hepatic 18:0 compared to the FO fed fish. In contrast with phospholipids, significant differences in total SFA content were detected in the TG fraction, with a significantly lower deposition of these SFAs in the liver of animals fed the LO diet compared to the other groups, and a significantly higher proportion of SFA in the liver of animals fed the CO diet, than animals from the S and LO diet groups. In MUFA, a significantly lower proportion of 16:1 was observed in the liver of animals from the LO diet group compared to the animals from the CO and S diet groups. The other MUFA that showed significant changes between the groups was 18:1n7 which was present in the liver of LO fed animals at a significantly lower proportion compared to the S and FO fed fish. These changes resulted in a higher proportion of total MUFA in animals fed CO compared to those fed LO. Regarding PUFA n3, the proportion of 18:3n3 ALA in the liver of LO fed fish was 12.58% of total FAs which was significantly higher than for the other groups that recorded below 1%, In the fish fed the LO diet, there was a significantly lower percentage of 22:5n3 compared with fish fed the S or FO diets. The sum of n3 PUFA, mainly influenced by the changes observed in 18:3n3 percentage, was significantly higher in fish fed the LO diet than those fed the CO diet.

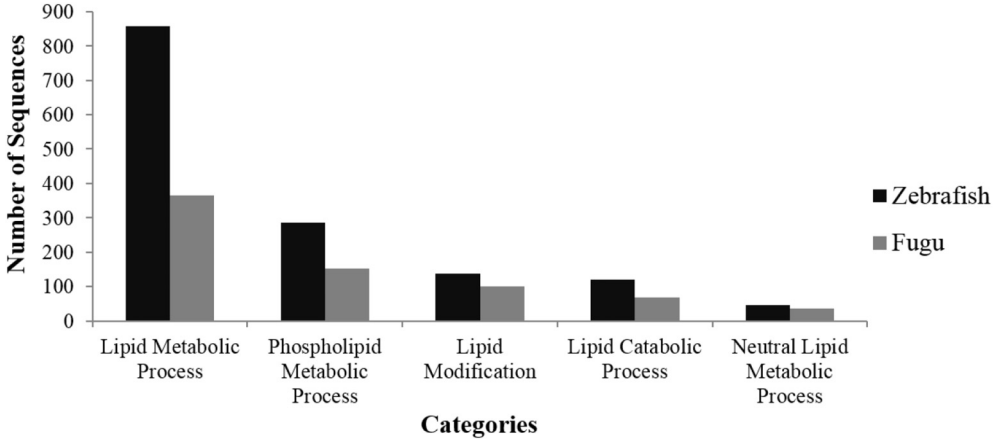

Fig. 1. Distribution of lipid relevant categories from the transcriptome of *Epinephelus marginatus* liver against zebrafish (*Danio rerio*) and fugu (*Takifugu rubripes*) protein databases.

Table 3
Fatty acid profile (%) of the base and experimental diets.

FA	S	LO	FO	CO	P value
10:0	N.D.	N.D.	N.D.	1,38 ± 0,12	–
12:0	N.D.	N.D.	N.D.	19,42 ± 1,08	–
14:0	10,07 ± 1,03[a]	5,75 ± 0,52[b]	8,21 ± 0,75[c]	15,99 ± 1,16[d]	< 0.001
16:0	32,56 ± 1,21[a]	29,98 ± 1,32[b]	24,39 ± 0,68[c]	25,77 ± 1,39[c]	< 0.001
18:0	5,97 ± 0,46	6,75 ± 0,85	4,78 ± 0,88	5,83 ± 0,79	0,084
20:0	0,42 ± 0,07[a]	0,59 ± 0,09[b]	0,37 ± 0,07[a]	0,45 ± 0,09[ab]	0,031
Σ SAT	**59,03 ± 0,85[a]**	**43,07 ± 1,17[b]**	**37,74 ± 0,77[c]**	**68,83 ± 0,96[d]**	**< 0.001**
16:1	5,9 ± 0,87	5,26 ± 0,75	7,74 ± 0,78	4,04 ± 0,52	0,070
18:1n9	6,38 ± 0,24[a]	12,53 ± 0,74[b]	7,72 ± 0,76[a]	8,16 ± 0,64[a]	< 0.001
18:1n7	2,58 ± 0,15	2,79 ± 0,54	2,82 ± 0,09	1,96 ± 0,87	0,501
20:1n9	0,57 ± 0,09	0,67 ± 0,10	0,80 ± 0,23	0,57 ± 0,14	0,217
Σ MUFA	**15,44 ± 0,75[a]**	**21,25 ± 1,30[b]**	**19,08 ± 1,15[ab]**	**14,73 ± 0,71[a]**	**0,007**
18:3n3 (ALA)	1,67 ± 0,25[a]	9,87 ± 0,99[b]	1,71 ± 0,15[a]	1,04 ± 0,15[a]	< 0.001
20:5n3 (EPA)	7,86 ± 0,46[a]	4,70 ± 0,30[b]	13,02 ± 1,40[c]	3,07 ± 0,73[d]	< 0.001
22:5n3	0,81 ± 0,09	0,60 ± 0,13	1,31 ± 0,54	0,40 ± 0,20	0,749
22:6n3 (DHA)	14,40 ± 1,00[a]	8,62 ± 0,65[b]	15,96 ± 1,44[a]	5,27 ± 0,97[c]	< 0.001
Σ PUFA n3	**25,32 ± 0,87[a]**	**24,28 ± 1,33[a]**	**32,79 ± 0,33[b]**	**10,09 ± 0,33[c]**	**< 0.001**
18:2n6	1,97 ± 0,37[a]	5,34 ± 0,59[b]	1,96 ± 0,47[a]	2,14 ± 0,20[a]	< 0.001
18:3n6	0,21 ± 0,11[a]	0,22 ± 0,04[b]	0,17 ± 0,02[b]	0,09 ± 0,03[b]	0,002
20:4n6 (ARA)	1,37 ± 0,18[ab]	0,95 ± 0,21[ab]	1,34 ± 0,19[a]	0,61 ± 0,11[b]	0,033
22:4n6	0,86 ± 0,13	0,60 ± 0,14	0,73 ± 0,08	0,37 ± 0,07	0,073
Σ PUFA n6	**4,66 ± 0,54[ac]**	**7,36 ± 0,69[b]**	**4,40 ± 0,57[c]**	**3,40 ± 0,17[a]**	**< 0.001**
Σ PUFA	**30,04 ± 0,50[a]**	**31,65 ± 1,43[b]**	**37,20 ± 0,50[c]**	**13,49 ± 0,49[d]**	**< 0.001**
Others	**5,98 ± 0,29[a]**	**4,38 ± 0,86[b]**	**6,62 ± 0,12[a]**	**3,44 ± 0,33[c]**	**0,002**
Σ n3/Σ n6	**5,44 ± 1,22[a]**	**3,30 ± 0,43[b]**	**7,45 ± 0,89[a]**	**2,96 ± 0,10[b]**	**0,002**

S (sardine), LO (linseed oil), FO (fish oil) and CO (coconut oil), ΣSFA. ΣMUFA, ΣPUFA, ΣPUFA n3, ΣPUFA n6, are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n3, polyunsaturated n6 respectively. [a,b] Different letters represents values significantly different. N.D., fatty acids not detected.

Table 4
Fatty acid profile (%) of hepatic phospholipids (PL).

FA	S	LO	FO	CO	P value
14:0	0.92 ± 0.33[a]	0.87 ± 0.50[a]	0.78 ± 0.11[a]	2.93 ± 1.44[b]	≤0.001
16:0	17.45 ± 2.26[a]	15.64 ± 4.70[a]	17.09 ± 3.35[a]	21.24 ± 3.55[b]	0.042
18:0	15.40 ± 1.41[ab]	18.20 ± 3.40[a]	16.53 ± 3.16[ab]	11.30 ± 4.80[b]	0.022
20:0	0.28 ± 0.08	0.25 ± 0.06	0.28 ± 0.12	0.20 ± 0.10	0.485
Σ SFA	**34.05 ± 1.49**	**34.96 ± 3.80**	**34.68 ± 1.49**	**35.67 ± 1.06**	**0.834**
16:1	3.20 ± 0.50[ab]	2.28 ± 1.39[a]	2.42 ± 0.32[a]	6.40 ± 2.8[b]	0.044
18:1n9	4.02 ± 0.75[a]	3.79 ± 0.67[a]	3.59 ± 0.50[a]	7.59 ± 3.46[b]	0.004
18:1n7	1.96 ± 0.54	1.61 ± 0.57	1.95 ± 0.38	2.43 ± 0.61	0.116
20:1n9	0.35 ± 0.13	0.33 ± 0.22	0.39 ± 0.08	0.51 ± 0.18	0.368
Σ MUFA	**9.53 ± 1.65[a]**	**8.01 ± 2.70[a]**	**8.35 ± 1.60[a]**	**16.93 ± 8.70[b]**	**0.015**
18:3n3 (ALA)	N.D.	2.12 ± 1.78	N.D.	0.85 ± 0.29	0.270
20:5n3 (EPA)	6.71 ± 1.46	7.65 ± 2.30	6.96 ± 2.52	7.36 ± 2.11	0.883
22:4n3	1.67 ± 0.73	1.49 ± 0.44	1.17 ± 0.53	1.18 ± 0.24	0.320
22:5n3	1.19 ± 0.30	0.97 ± 0.40	1.18 ± 0.49	1.75 ± 0.81	0.128
22:6n3 (DHA)	29.68 ± 1.43[ab]	27.26 ± 3.62[ab]	32.50 ± 5.22[a]	23.27 ± 6.20[b]	0.025
Σ PUFA n3	**39.25 ± 1.87**	**39.50 ± 5.30**	**41.81 ± 1.87**	**34.07 ± 6.66**	**0.129**
18:2n6	0.98 ± 0.32[a]	3.40 ± 1.07[b]	0.80 ± 0.21[a]	1.42 ± 0.28[a]	≤0.001
18:3n6	1.37 ± 0.62	1.14 ± 0.48	1.01 ± 0.38	0.65 ± 0.29	0.136
20:4n6 (ARA)	10.76 ± 2.40	8.89 ± 0.82	8.74 ± 1.89	7.12 ± 3.05	0.089
22:4n6	1.04 ± 0.17	1.20 ± 0.53	1.15 ± 0.57	1.51 ± 0.34	0.405
Σ PUFA n6	**14.6 ± 2.32**	**14.63 ± 1.16**	**11.69 ± 2.32**	**10.71 ± 3.26**	**0.064**
Σ PUFA	**54.61 ± 2.29**	**54.93 ± 6.5**	**54.63 ± 2.29**	**45.41 ± 9.15**	**0.055**
Others	**3,01 ± 0.41**	**2.88 ± 0.39**	**3.54 ± 0.41**	**3.06 ± 0.32**	**0.374**
Σ n3/Σ n6	**2.88 ± 0.89**	**2.70 ± 0.34**	**3.70 ± 0.89**	**3.31 ± 1.38**	**0.100**

S (sardine), LO (linseed oil), FO (fish oil) and CO (coconut oil). ΣSFA, ΣMUFA, ΣPUFA, ΣPUFA n3, ΣPUFA n6 are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n3 and polyunsaturated n6 respectively. [a,b] Different letters represents values significantly different. N.D., fatty acids not detected.

For n6 FAs, the 18:2n6 proportion was significantly higher in fish fed the LO diet, and contained 7.12% of total hepatic TG, while the other groups showed approximately 1.5% of these FAs. The difference observed in 18:2n6 had the most effect on total n6 PUFA, and resulted in a significantly higher proportion in the liver TG fraction of fish fed the LO diet compared to the other diet groups. These changes in n3 and n6 PUFA resulted in a significantly higher proportion of total PUFA within the hepatic TG fraction in fish fed the LO diet compared to the other experimental groups (Table 5).

3.4. Quantitative qPCR

Many significant changes were observed in the expression of genes related to FA synthesis and oxidation in *E. marginatus* liver in response to the different diets (Fig. 2, Table 6). Expression of *Em ACC* was significantly up-regulated in fish fed the CO diet compared with fish fed the FO diet ($P < 0.05$). Likewise, the expression of *Em SCD* was significantly up-regulated in fish fed the S group compared with fish fed the CO diet ($P < 0.05$). There were no significant changes in the expression of *Em FAS* and *Em ELOVL5* between groups ($P > 0.05$).

Table 5
Fatty acid profile (%) of hepatic triglycerides (TG).

FA	S	LO	FO	CO	P value
14:0	3.71 ± 1.12[a]	2.74 ± 0.43[a]	4.13 ± 0.92[a]	7.87 ± 2.52[b]	0.001
16:0	23.03 ± 2.36[ab]	18.72 ± 2.25[a]	25.98 ± 5.28[ab]	26.64 ± 1.07[b]	0.048
18:0	4.35 ± 1.19[ab]	3.93 ± 0.68[ab]	4.97 ± 1.37[a]	2.74 ± 0.29[b]	0.030
20:0	0.14 ± 0.04	0.11 ± 0.2	0.20 ± 0.07	0.11 ± 0.03	0.340
Σ SFA	**31.42 ± 2.1[a]**	**25.60 ± 2.4[b]**	**35.51 ± 5.6[ac]**	**37.54 ± 2.8[c]**	**0.048**
16:1	11.05 ± 3.55[a]	6.42 ± 1.72[b]	9.84 ± 1.94[abc]	14.15 ± 3.11[ca]	0.012
18:1n9	12.42 ± 2.37	12.16 ± 0.84	9.71 ± 1.87	12.73 ± 1.47	0.066
18:1n7	3.56 ± 0.40[a]	2.81 ± 0.38[b]	3.51 ± 0.47[a]	3.19 ± 0.35[ab]	0.049
20:1n9	0.80 ± 0.18	0.59 ± 0.10	0.73 ± 0.18	0.68 ± 0.07	0.949
Σ MUFA	**28.07 ± 5.0[ab]**	**22.20 ± 2.7[a]**	**24.06 ± 3.9[ab]**	**31.04 ± 4.3[b]**	**0.038**
18:3n3 (ALA)	0.70 ± 0.23[a]	12.58 ± 3.12[b]	0.61 ± 0.12[a]	0.70 ± 0.22[a]	≤0.001
20:5n3 (EPA)	6.76 ± 1.47	5.92 ± 1.18	6.87 ± 3.35	4.92 ± 0.53	0.382
22:4n3	1.3 ± 0.23	1.06 ± 0.08	1.12 ± 0.26	0.96 ± 0.07	0.067
22:5n3	3.40 ± 0.57[a]	2.15 ± 0.14[b]	3.22 ± 0.87[a]	2.73 ± 0.60[ab]	0.006
22:6n3 (DHA)	19.08 ± 3.98	16.48 ± 2.39	18.47 ± 4.73	13.37 ± 1.49	0.142
Σ PUFA n3	**31.53 ± 5.5[ab]**	**38.60 ± 4.7[a]**	**30.96 ± 7.5[ab]**	**23.17 ± 2.0[b]**	**0.012**
18:2n6	1.44 ± 0.33[a]	7.12 ± 1.62[b]	1.58 ± 0.25[a]	1.70 ± 0.29[a]	≤0.001
18:3n6	0.19 ± 0.03	0.22 ± 0.11	0.27 ± 0.11	0.11 ± 0.03	0.063
20:4n6 (ARA)	2.33 ± 0.34	1.58 ± 0.29	2.30 ± 0.86	2.00 ± 0.51	0.054
22:4n6	2.03 ± 0.74	1.71 ± 0.76	1.96 ± 1.07	1.42 ± 0.43	0.615
Σ PUFA n6	**6.20 ± 0.9[a]**	**11.00 ± 1.90[b]**	**6.40 ± 0.9[a]**	**5.50 ± 0.7[a]**	**≤0.001**
Σ PUFA	**38.40 ± 6.00[a]**	**50.10 ± 4.40[b]**	**37.96 ± 8.00[a]**	**29.40 ± 1.60[a]**	**0.046**
Others	**3,73 ± 0.60**	**3,71 ± 0.30**	**4,53 ± 0.10**	**3,97 ± 0.30**	**0.468**
Σ n3/Σ n6	**5.08 ± 1.4**	**3.60 ± 1.00**	**4.70 ± 1.00**	**4.10 ± 0.08**	**0.052**

S (sardine), LO (linseed oil), FO (fish oil) and CO (coconut oil). ΣSFA, ΣMUFA, ΣPUFA, ΣPUFA n3, ΣPUFA n6, are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n3 and polyunsaturated n6 respectively. [a,b] Different letters represents values significantly different.

For the β-oxidation genes, significant changes were observed in the gene expression of *Em ACADVL*, with significant up-regulation in fish fed the S diet compared to the other groups ($P < 0.05$). Additionally, the expression of *Em ACADVL* was significantly down-regulated in fish fed the CO diet, compared with fish fed the LO or FO diets ($P < 0.05$). *Em ACOX1* expression was significantly down-regulated in fish fed the S diet compared to the other experimental groups ($P < 0.05$) (Fig. 2, Table 6).

4. Discussion

The results obtained in this study revealed changes mainly in TG, similar to the results observed in *Salmo salar* (Bell et al., 2001, 2003; Torstensen et al., 2004). Usually in fish there is a FA substrate preference during β-oxidation, with SFA being the primary oxidization targets, followed by MUFA and PUFA (Kiessling and Kiessling, 1993; Tocher, 2003), which explains the higher percentage of MUFA in TG of animals from the CO diet group. Similarly, the data suggests that *E. marginatus* possess apparently high oxidation of SFA in hepatic TG and PL, since the percentage of this FA was lower in liver tissue than in the diets. This corroborates the hypothesis that when offered diet containing SFA excess, such as CO diet, these FAs can be directly oxidized to generate metabolic energy (Bell et al., 2003; Sargent et al., 2002). These results suggest that *E. marginatus* has a self-regulatory potential to store SFA and MUFA in the liver which may be used in β-oxidation processes. The same profile was observed in a congeneric species, *Epinephelus malabaricus* (Wu et al., 2002).

Results for *E. marginatus* 18:3n3 were not similar to those observed in *Salmo salar* (Torstensen et al., 2004), since past work found a higher oxidation of this FA when in dietary excess. Apparently, *E. marginatus* incorporated 18:3n3 from LO diet directly into liver PL, suggesting the retention of this FA. Similar results were observed in *Sparus aurata* and *Dicentrarchus labrax* (Izquierdo et al., 2003). According to Regost et al. (2003) the percentage of n3 PUFA in liver PL of *Psetta maxima* fed with FO rich diet were stored in most tissues, compared with animals fed with low n3 PUFA diets, providing evidence for the importance of these FA to marine fishes. The higher DHA percentage in hepatic PL in fish from FO diet can be justified by the high incorporation of this FA in the diet, since this FA is important in many biological functions, and in fish is commonly spared for biological membranes (Araújo et al., 2016; Salini et al., 2016b). 22:5n3 deposition in liver from all experimental groups can be related mainly to the elongation process from EPA (20:5n3), modulated by ELOVL5 gene expression, since this FA was not present in the experimental diets. Generally, elongation processes are necessary when dietary supply of the long chain FA (LCPUFA) levels are inadequate. The elongation of PUFA precursors, mainly 18 and 20 carbon FA, is modulated by expression of the *ELOVL5* gene. Studies carried out with seabream, seabass (Izquierdo et al., 2003) and barramundi (Salini et al., 2015a), showed high expression of *ELOVL5*, favoring the synthesis of intermediate FAs such as 18:4n3, 18:3n6, 22:5n3 and 22:4n6. In *Sparus aurata*, *Dicentrarchus labrax* (Izquierdo et al., 2003), and *Epinephelus malabaricus* (Wu and Chen, 2012) a specific deposition of 22:5n3 was observed in muscle and liver. This result was observed even when this fatty acid was not present in the diet, which the authors attributed to the elongation of 22:5n3 from EPA, mediated by *ELOVL5* expression. The high percentage of DHA in the FO diet did not influence the TG profile, since no significant difference was observed between the experimental groups. However as seen previously, the excess of this FA present in the FO diet was deposited in PLs, probably due to their importance in the composition of biological membranes.

Fish diets poor in PUFA may exert agonistic and antagonistic effects on different lipid metabolism pathways, and these effects can change according to the species, tissue, age and diet composition (Tocher, 2003). Some studies performed with fish and mammals showed that *ACC* gene expression can be directly modulated by diet composition (Iritani, 1993; Kim, 1997; Seiliez et al., 2013). ACC is an enzyme directly involved in FA synthesis, which provides malonyl Co-A as a substrate for FA biosynthesis. Our results showed that *Em ACC* was up-regulated in fish fed with CO, and inversely down-regulated in animals fed with FO. Similar results were found in rats and in Atlantic salmon (Betancor et al., 2016; Ducasse-Cabanot et al., 2007; Iritani, 1993; Iritani et al., 1998). Stearoyl-CoA desaturase gene (*SCD*) is responsible for the synthesis of the enzyme that convert SFA as 16:0 and 18:0 in MUFA as 16:1n7 and 18:1n9 (Nelson and Cox, 2005; Sargent et al., 2002; Tocher, 2003; Torstensen and Stubhaug, 2004). The SCD

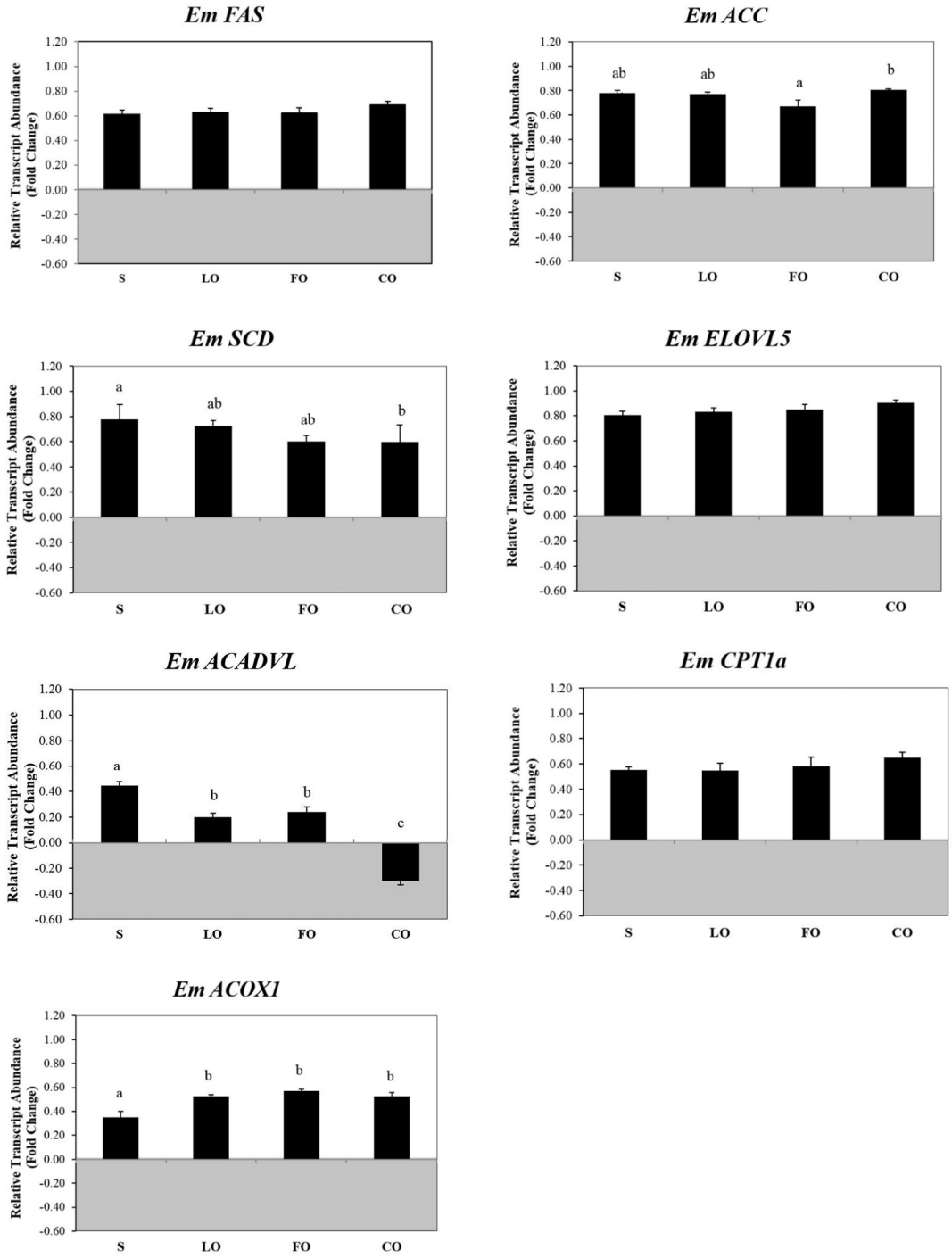

Fig. 2. Relative gene expression levels of fatty acid synthesis and β-oxidation genes. *Em FAS* (*fatty acid synthase*); *Em ACC* (*acetyl-CoA carboxylase*); *Em SCD* (*Stearoyl CoA Desaturase*); *Em ELOVL5* (*elongase 5*); *Em CPT1a* (*carnitine palmitoyltransferase* 1a); *Em ACADVL* (*acyl CoA dehydrogenase very long chain*); *Em ACOX1* (*acyl-CoA oxidase 1*). Transcript levels of each gene were calculated relative to each other using raw cycle threshold values for each gene, normalized against Ef1α (elongation factor 1 alpha). Values shown are the log2-fold change relative to the average *Ct* value for all genes. Superscripts denote significant ($P < 0.05$) differences between different diets.

expression in different groups showed a contradictory profile, since this gene was down-regulated in CO animals compared to the S animals, even with the high percentage of SFA in this diet. Similarly, ELOVL5 expression in *E. marginatus* was contradictory, since FO animals that received a lower amount of SFA in the diet presented the proportional expression compared to the animals from the CO group that presented approximately 69% of this FA class in the diet. In general, no statistical differences in the expression of *Em ELOVL5* were observed between experimental groups, corroborating results observed in *Sparus aurata* and *Dicentrarchus labrax* (Izquierdo et al., 2003) fed with PUFA poor diets. However, there was no evidence to indicate direct synthesis of DHA from 22:5n3, a process that is expected in the majority of marine fishes (Chen et al., 2017; Izquierdo et al., 2003; Salini et al., 2015a; Zheng et al., 2010).

The lipid profile of the diet also directly affects β-oxidation (Frøyland et al., 1998; Madsen et al., 1998; Willumsen et al., 1996). Some fish species show increased peroxisomal oxidation, while others favour mitochondrial oxidation (Stubhaug et al., 2007). Similarly, to the result observed in this study, *CPT1* gene expression in *Salmo salar* was not altered by the PUFA rich diets. The significant down-regulation

Table 6
Overview of the diet influence in gene expression.

Genes/diets	S	LO	FO	CO
Em FAS	N/D	N/D	N/D	N/D
Em ACC	N/D	N/D	↓	↑
Em SCD	↑	N/D	N/D	↓
Em ELOVL5	N/D	N/D	N/D	N/D
Em ACADVL	↑	↑↓[a]	↑↓[a]	↓[a]
Em CPT1a	N/D	N/D	N/D	N/D
Em ACOX1	↓	↑	↑	↑

Em FAS (*fatty acid synthase*); *Em ACC* (*acetyl-CoA carboxylase*); *Em SCD* (*Stearoyl CoA Desaturase*); *Em ELOVL5* (*elongase 5*); *Em CPT1a* (*carnitine palmitoyltransferase* 1a); *Em ACADVL* (*acyl CoA dehydrogenase very long chain*); *Em ACOX1* (*acyl-CoA oxidase 1*). N/D = No significant differences observed between the groups; arrows up = up-regulated expression; arrows down = down-regulated expression.

[a] LO and FO down-regulated compared to S and up-regulated compared to CO.

of *Em ACADVL* in fish fed CO compared to fish fed S suggested that mitochondrial oxidation was significantly modified by diet. In fish with sedentary habits, like *E. marginatus*, liver is the tissue that contributes most to β-oxidation, and ACADVL can be considered a key enzyme in mitochondrial oxidation of LCPUFA. Therefore, the higher SFA percentage, and consequently the lower PUFA percentage in CO diet may be a potential cause of the reduced expression of this gene. Peroxisomal oxidation, evaluated in this study by *Em ACOX1* gene expression, was significantly altered by diet composition. Nutritional studies with *Salmo salar* showed that MUFA, mainly 18:1n9, is the main FA oxidized by the peroxisome in liver, and their concentrations directly influenced the expression of genes related to this pathway (Bell et al., 2003; Stubhaug et al., 2006; Torstensen et al., 2004). Even with *Em ACOX1* up-regulated in LO, FO and CO, gene expression results apparently cannot be related with FA diet composition, since significantly changes in 18:1n9 percentage in CO and FO were not observed, compared to the S diet.

5. Conclusions

NGS and transcriptome assembly proved an excellent discovery tool for finding lipid-relevant genes in *E. marginatus*, since these genes are highly conserved between teleost species. Diets with different FA profiles significantly changed hepatic FA profile and the expression of genes related to synthesis and oxidation in *E. marginatus*. Supplementation of DHA in *E. marginatus* diets is important, mainly because DHA cannot be synthesized directly from elongation and desaturation from their precursors, as shown by the hepatic accumulation of 22:5n3, an intermediate FA between EPA and DHA. The results of this study generated important information about lipid metabolism in the dusky grouper, and provide a basis for future dietary formulation studies to improve their production cycle.

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