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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*)

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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.,

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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

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