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Characterization of the genomic responses in early Senegalese sole larvae fed diets with different dietary triacylglycerol and total lipids levels

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ABSTRACT

The aim of this work was to evaluate the genomic responses of premetamorphic sole larvae (9 days post-hatching, dph) fed diets with different lipid and triacylglycerol (TAG) content. For this purpose, two diets with high (rotifers enriched with a fish oil-based emulsion; referred to as HTAG) and low (rotifers enriched with a krill oil-based emulsion; LTAG) levels of total lipids and TAG were evaluated. Lipid class and fatty acid (FA) profiles, histological characterization of intestine, liver and pancreas and expression patterns using RNA-seq were determined. Discriminant analysis results showed that larvae could be clearly differentiated on the basis of their FA profile as a function of the diet supplied until 9 dph although no difference in growth was observed. RNA-seq analysis showed that larvae fed HTAG activated coordinately the transcription of apolipoproteins (*apob*, *apoa4*, *apoc2*, *apoe*, and *apobec2*) and other related transcripts involved in chylomicron formation, likely to facilitate proper lipid absorption and delivery. In contrast, larvae fed LTAG showed higher mRNA levels of several pancreatic enzymes (*try1a*, *try2*, *cela1*, *cela3*, *cela4*, *chym1*, *chym2*, *amy2a* and *pnlip*) and appetite modulators (*agrp1*) and some intra- and extracellular lipases. Moreover, KEGG analysis also showed that several transcripts related to lipid metabolism and glycolysis were differentially expressed with a higher abundance in larvae fed LTAG diet. All these data suggest that early larvae were able to establish compensatory mechanisms for energy homeostasis regulating key molecules for FA and TAG biosynthesis, FA uptake and intracellular management of TAG and FA to warrant optimal growth rates.

1. Introduction

Lipids represent a structurally and functionally diverse class of metabolites that, along with proteins, are the major constituents of cells. They play different important functional roles including the formation and maintenance of cell membranes, energy storage and cellular signalling (Sargent et al., 2002). Among all lipid classes, neutral glycerolipids and phospholipids (PL) play a pivotal role during larval development promoting cell proliferation and providing the energy required for an active anabolic metabolism during exponential growth (Tocher, 2003). Absorption of dietary lipids implies their active hydrolization by pancreatic lipases in the gut lumen. Released fatty acids (FA) are later taken up and re-esterified into triacylglycerols (TAG) and PL in the endoplasmatic reticulum of enterocytes. To be transported in the blood, lipids are assembled into lipoproteins, macromolecular complexes formed by specific carrier proteins, apolipoproteins, with varying amounts of PL, cholesterol esters, and TAG. As a result, four main types of lipoproteins with different density and size can be synthetized including chylomicrons (CM), very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Apolipoproteins are major components of these particles and they can be classified into five major classes, A through E, according to their structure and function, similarly to mammals (Chapman, 1980). Apolipoproteins not only act as lipid transporters but can also target lipoproteins to specific tissues throughout specific binding to lipoprotein receptors and activation of lipolytic enzymes (Gursky, 2005). Mechanisms behind lipid digestion, absorption and transport have not been extensively studied in fish and they are generally assumed to be similar to those described in mammals (Tocher, 2003; Tocher et al., 2008). Nevertheless, a better knowledge of the mechanisms modulating lipid digestion and transport is required to produce high-quality larvae.

Lipids and amino acids are the main energy sources during larval development although TAGs are considered as the most relevant lipid

Abbreviations: CM, chylomicrons; DPH, days post hatch; DEPC, diethyl pyrocarbonate; DET, differentially expressed transcripts; DMA, dimethylacetal; FA, fatty acids; GO, gene ontology; HDL, high-density lipoproteins; HTAG, high triacylglycerol diet; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDL, low-density lipoproteins; LTAG, low triacylglycerol diet; LV, lipid vacuoles; NGS, Next Generation Sequencing; NL, neutral lipids; PI, posterior intestine; PL, phospholipids; rRNA, ribosomal RNA; SD, standard deviation; SNI, supranuclear inclusions; TAG, triacylglycerol; VLDL, very low-density lipoproteins; ZG, zymogen granules.

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class for energy provision (Hamre et al., 2013; Mourente and Vázquez, 1996; Tocher et al., 2008). However, dietary TAG levels must be provided to larvae within an optimum range since several authors have reported detrimental effects on larval growth and development when they are provided in excess (Gawlicka et al., 2002; Izquierdo et al., 2000; Olsen et al., 2000; Pousão-Ferreira et al., 1999). Larvae fed high levels of TAG exhibit a high fat accumulation in the enterocytes that interfere with nutrient traffic throughout the gut and, consequently, an inadequate absorption of protein and other essential nutrients can occur (Gisbert et al., 2008; Hamre et al., 2013; Izquierdo et al., 2000; Morais et al., 2007). Moreover, a reduced capacity of Senegalese sole (*Solea senegalensis*) larvae to digest and absorb FA esterified to TAG compared to PL has been suggested to be probably associated with a deficient emulsification of TAG and a lower activity of neutral lipases versus phospholipases (Morais et al., 2005a).

Senegalese sole is an important species in aquaculture with high growth rates in the pelagic stage and negligible mortalities until complete metamorphosis (approximately three weeks after hatching) (Parra and Yúfera, 2001; Yufera et al., 1999). This high performance during early development has been associated to an early maturation of the digestive capacity as well as to an adequate management of energy reserves to accomplish successfully the metamorphic process. Studies on pancreatic enzymes showed that the activation of the expression and activity of zymogens occurs during development, including pancreatic proteases, amylases and lipases (Conceição et al., 2007; Gamboa-Delgado et al., 2011; Manchado et al., 2008) making feasible the early assimilation of lipids and proteins at similar rates (Yufera et al., 1999). Moreover, some studies have evaluated lipid requirements and the role of essential FA, PL and neutral lipids (NL) in sole larvae, providing new relevant information on the effects of these lipids on the digestive capacity, lipid absorption, FA composition and their effects on sole larvae development (Boglino et al., 2012; Morais et al., 2006; Morais et al., 2005a; Morais et al., 2005b; Mourente and Vázquez, 1996; Navarro-Guillen et al., 2014; Parra et al., 1999; Vazquez et al., 1994; Villalta et al., 2005a; Villalta et al., 2005b). Nevertheless, little is known about the overall physiological and genomic responses associated to dietary lipid components. To address this question, Next Generation Sequencing (NGS) technologies offer important advantages to evaluate wide transcriptomic responses in non-model species in a cost-effective manner (Cerda and Manchado, 2013).

The aim of this work was to evaluate the physiological, metabolic and molecular responses induced by two diets differing in their TAG and total lipid contents in young sole larvae at 9 days post-hatching (dph). Lipid classes, FA profiles and wide-gene expression patterns using RNA-seq were determined. Histological characterization of the intestine, liver and pancreas and RT-qPCR were also carried out to evaluate the results.

2. Material and methods

2.1. Larval rearing and experimental diets

Fertilized eggs were obtained from naturally spawning Senegalese sole broodstock (IFAPA Centro El Toruño). Eggs were collected early in the morning (9:00 a.m.) and transferred to a 1000 ml measuring cylinder to separate buoyant (viable) from non-buoyant (non-viable) eggs. The number of eggs in each fraction was estimated using volumetric methods (1100 eggs ml^{-1}). Eggs were incubated at a density of 2000 embryos L^{-1} in 300 L cylinder-conical tanks with gentle aeration and complete water exchange every two hours. Newly hatched larvae (one day post-hatch (dph)) were then transferred to six 300 L tanks (three replicates per treatment) at an initial density of 30 larvae L^{-1}. Lights were kept off until the onset of external feeding at 3 dph. After that, a 18 L:6D photoperiod with a light intensity of 200 lx was established. Temperature and salinity were 19.0 °C and 37.6, respectively. Water was filtered through sand filters and 10 μm and 3 μm nominal retention cartridges to maintain the water quality. Water was kept stagnant until 7 dph followed by a daily water exchange of 40%. Tanks were provided with a central draining pipe with a 250 μm mesh and gentle aeration.

Two different treatments consisting of rotifers enriched with two marine oil emulsions were tested. Emulsions were formulated with 2 g 100 g^{-1} wet weight Tween 80 (Panreac Quimica S.A., Castellar de Vallès, Spain), 0.1 g 100 g^{-1} wet weight vitamin E (Vitamin E acetate 97%, Alfa Aesar, Karlsruhe, Germany) and 0.5 g 100 g^{-1} wet weight vitamin C (L-Ascorbic acid 6-palmitate 95%, Sigma Aldrich, Steinheim, Germany) as constant ingredients and two different oil bases: krill oil (AkerBiomarine, Oslo, Norway) and fish oil (Sopropêche, Wimille, France) at 25 g 100 g^{-1} emulsion wet weight. In basis to total lipid and TAG contents, fish oil-based diets were named as high TAG diet (HTAG) and krill oil-based diet as low TAG diet (LTAG). Enrichments were conducted at a density of 2×10^5 rotifers L^{-1}, at 20 °C for 3 h, adding 0.31 g emulsion wet weight per liter of rotifers culture. Larvae were fed twice a day, between 11:00 and 12:00 am and 5:00 and 6:00 pm. Final concentration of rotifers in the tank was 1×10^4 preys L^{-1}. To maintain constant rotifer concentration within each experimental tank, three water samples (10 mL) from each tank were sampled before supplying new food. Estimated average number of prey in each sample was used to adjust total prey concentrations in the tank.

Rotifers samples for lipid analysis ($n = 4$) were collected throughout the experimental period. Samples were washed with clean seawater and ammonium formiate solution (1% w/w), frozen in liquid nitrogen and kept at −80 °C until analysis. Larvae were sampled at 3, 6, and 9 dph for lipid analysis whereas for histological and molecular analyses only at the end of the experiment. Samplings were carried out at 11:00 a.m., before supplying new food (when needed). For RNA isolation, three pools of larvae (one per tank including 50 larvae) were randomly collected using a 350-μm-mesh net, washed with DEPC water, frozen in liquid nitrogen and stored at −80 °C until RNA isolation and further analysis. For lipid determination, three pools of larvae (300 larvae in each larval pool) from each tank were randomly collected, washed with clean seawater and ammonium formate solution (1% w/w), frozen in liquid nitrogen and kept at −80 °C until analysis. For histological analysis, 30 larvae of each tank were randomly collected, euthanized with an overdose of tricaine methane sulphonate (MS-222) and fixed in buffered formaldehyde at 4 °C overnight and preserved in ethanol 70% until histological processing.

2.2. Total lipids, lipid classes and fatty acids analyses

Total lipids (TL) were extracted with chloroform:methanol (2:1 v/v) containing 0.01% of butylated hydroxytoluene (BHT) as antioxidant (Christie, 2003). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically. Lipid classes were separated by one dimensional double development high performance thin layer chromatography (HPTLC) using methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9 by vol.), as the polar solvent system and hexane/diethyl ether/glacial acetic acid (80:20:2 by vol.), as the neutral solvent system. Lipid classes were visualized by charring at 160 °C for 20 min after dipping in cupric acetate in 3% phosphoric acid (Olsen and Henderson, 1989). Final quantification was made by densitometry in a CAMAG scanner at a wavelength of 325 nm, and by comparison with external standard (Sigma-Aldrich) (Morillo-Velarde et al., 2013). TL extracts were subjected to acid-catalyzed transmethylation for 16 h at 50 °C, using 1 ml of toluene and 2 ml of 1% sulphuric acid (v/v) in methanol. The resultant FA methyl esters (FAME) and dimethylacetals (DMA) formed from ether lipids were purified by thin layer chromatography (TLC),