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Effects of dietary lipid profile on larval performance and lipid management in Senegalese sole

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ABSTRACT

The aim of this study was to evaluate the short- and long-term effects of diets consisting in rotifers enriched with different oil emulsions (differing mainly in triacylglycerols levels) and provided during the first week of life. Survival, growth, metamorphosis progress, lipid profile and molecular regulatory pathways were evaluated. For comparison purposes, a diet used routinely in the aquaculture industry based on microalgae was also carried out. The present study shows that dietary triacylglycerols (TAG) levels and fatty acid composition modulate larval survival and growth when supplied during larval pelagic stages. Histological observation and gene expression pattern showed that early larvae regulated intestinal lipid transport, showing a coordinate activation of apolipoproteins transcripts. Multivariate analyses identified the major contribution of *apoEa* to discriminate samples by dietary treatments, pointing out the importance of this apolipoprotein as key molecular marker for intestinal lipid mobilization. However, our data indicate that transport capacity is limited in early larvae, increasing in anterior intestine as intestine maturates with larval development, while the increase of fat deposits in the posterior intestine in older larvae supports the limited capacity of this gut section for lipid transport. The accumulation of TAG, phosphocholine (PC) and oleic acid (OA) along pre- and early metamorphosis indicates that these lipids influences positively daily mortality rate, eye-migration progress and growth. Moreover, larval TAG amounts correlate highly with *apoA-IVBa3* mRNA levels, indicating an active role of this apolipoprotein in endogenous lipid mobilization during development. These data provide new evidence that TAG, PC and OA dietary levels are important for larval development and complement the available information about energy management in sole larvae reinforcing the rotifer feeding period as critical to produce high-quality larvae.

Statement of relevance: This paper provides new clues about the mechanisms involved in lipid management in larvae and their consequences during their life-cycle to produce high-quality fry and optimize husbandry procedures.

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1. Introduction

Dietary lipids play a key role during marine fish larval development since they act as the primary source of energy and provide the phospholipids (PL) and essential fatty acids (FA) required to build new cellular structures and organs (Tocher 2003; Tocher et al. 2008). The triacylglycerols (TAG) are the most important lipid class for energy provision in teleosts (Hamre et al. 2013; Mourente and Vázquez 1996; Sheridan 1988; Tocher 2003; Tocher et al. 2008). These glycerolipid species consist of three molecules of FA esterified to the three alcohol groups of glycerol. In fish, saturated and monounsaturated FA are mainly located in the *sn*1 and *sn*3 positions, whereas polyunsaturated fatty acids (PUFAs) are preferentially located in *sn*2. Saturated and monounsaturated FA are readily catabolized in mitochondria whereas β-oxidation of PUFAs occurs in the mitochondria or peroxisomes (Tocher 2003). In addition, each FA follows a different metabolism in fish larvae. So, long chain-PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are mostly retained in body tissues, while oleic acid (OA) is mostly incorporated into TAG to be catabolized for energy supply (Izquierdo, et al., 2001; Morais et al. 2005a; Morais et al. 2006a). Non-catabolized and newly synthesized TAG molecules are stored in depot organs such as liver, muscle or mesenteric fat (Flynn et al. 2009; Sheridan 1988).

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; CHO, cholesterol; CM, chylomicrons; DET, differentially expressed transcripts; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DS, development stage; DT, dietary treatment; DW, dry weight; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; FO, fish oil; HPTLC, high performance thin layer chromatography; LA, linoleic acid; LC, lipid class; MA, microalgae; ML, marine lecithin; MA, microalgae; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PCA, principal components analysis; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PLA, palmitoleic acid; PS, phosphatidylserine; PUFAs, polyunsaturated fatty acids; SA, stearic acid; TAG, triacylglycerol; VLDL, very low density lipoproteins.

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Lipid mobilization, lipolysis or lipid biosynthesis represent key regulatory steps in lipid metabolism. Lipid transport is mediated by macromolecules complex named lipoproteins (Chapman 1980). These lipoproteins contain specific carrier proteins (apolipoproteins) and different amounts of PL, TAG and cholesterol esters. Apolipoproteins represent a complex multigene family that have evolved after gene tandem duplications together the three rounds of whole genome duplications that occurred during teleost evolution. Although this family is not fully characterized in fish, some reports have observed some gene- and paralog-specific responses to dietary lipid contents as consequence of sub- and neofunctionalization processes (Otis et al. 2015; Roman-Padilla et al. 2016a; Roman-Padilla et al. 2016b). On the other hand, lipid metabolism pathways change as larvae develop with early stages exhibiting limited capacity for PL biosynthesis that, in turn, restricts intestinal lipid transport (Tocher et al. 2008). In this sense, Carmona-Antonanzas et al. (2015) demonstrated that some genes involved in intestinal PL biosynthesis were lower expressed in early developmental stages (fry) than in salmon parr highlighting the importance of optimal dietary PL levels to compensate this deficient endogenous biosynthesis in these stages.

Flatfish larvae demand a high energy supply during pelagic stages due to their fast growth rates and the extra-energy demands for physical tissue remodelling during metamorphosis (Cañavate et al. 2006; Geffen et al. 2007; Parra and Yúfera 2001; Yufera et al. 1999). To satisfactory fulfill all morphological transformations, the pelagic larvae accumulate lipid reserves that are later used as fuel for eye migration and organ translocation in metamorphosis (Yufera et al. 1999). Several previous studies in sole have dealt with the role of dietary PL and FA to modulate larval growth, metamorphosis and organogenesis (Boglino et al. 2012; Bonacic et al. 2016b; Mourente and Vázquez 1996; Navarro-Guillen et al. 2014; Villalta et al. 2005a; Villalta et al. 2005b). Nevertheless, the mechanisms that early pelagic and metamorphic stages use to manage energy supply are not-fully understood. A previous work in pre-metamorphic Senegalese sole larvae showed that pelagic larvae were able to induce the expression of a set of apolipoproteins to deal with low dietary TAG levels in order to increase lipid reserves (Hachero-Cruzado et al. 2014). However, in this study the evaluation period was restricted to the first week of feeding, and lipid management along the metamorphic stages was not appraised. Bonacic et al. (2016b) also found an up-regulation of genes involved in lipid transport by dietary lipids components in pelagic larvae confirming the relevance of lipid transport mechanisms to properly satisfy larval demands. The aims of this study was to evaluate the short- and long-term effects of diets with different TAG contents provided during the first nine days of life and their consequences on survival, growth, metamorphosis progress, lipid profile and molecular regulatory pathways.

For comparison purposes, a diet used routinely in the aquaculture industry based on microalgae was also carried out. The results obtained provide new clues about the mechanisms involved in lipid management in larvae and their consequences during their life-cycle to produce high-quality fry and optimize husbandry procedures.

2. Material & methods

2.1. Experimental diets and live preys enrichment procedures

Three experimental diets consisting in rotifers enriched with microalgae *Tisochrysis lutea* (T-iso) and two marine emulsions based on fish oil (FO) or marine lecithin (ML) were tested. The marine emulsions were formulated using Vitamin C (1.81 g/100 g; L-Ascorbic acid 6-palmitate 95%, Sigma Aldrich, Steinheim, Germany), Vitamin E (0.36 g/100 g; Vitamin E acetate 97%, Alfa Aesar, Karlsruhe, Germany) and Tween 80 (7.25 g/100 g; Panreac Quimica, Barcelona, Spain) as constant ingredients. Marine Lecithin LC60 (ML, Phosphotech, Saint-Herblain, France) was added at 90.58 g/100 g and Fish Oil (FO, Eurocoyal, Sant Cugat del Valles, Spain) plus DHA (NuaDHA-1000®, Biological Innovations, Vizcaya, Spain) at 63.41 g/100 g FO and 27.17 g/100 g DHA, respectively. All data were related to dry weight (DW) of the emulsion. These dietary components were mixed with distilled water (32% w/w) using an Ultra-turrax® (IKA® T10 basic) and an ultrasonic equipment (Branson ultrasonics, Model 250/450 Sonifer®) to homogenize. Rotifers enrichments were conducted for 3 h adding 0.47 g emulsion wet weight per 10^6 rotifers and a density of 2×10^5 rotifers L^{-1}; or 10^7 cell *T. lutea* per ml of rotifers culture. *Artemia* were enriched for 24 h adding 2×10^6 cell ml^{-1} *T. lutea* and a density of 200 nauplii ml^{-1}. All enrichments were carried out at 20 °C and 35 g L^{-1}.

Rotifers and *Artemia* samples for lipid analysis ($n = 6$) were collected in different days of larval rearing procedures. Samples were roughly washed with clean seawater and ammonium formate solution (1% w/w), frozen in liquid nitrogen and kept at −80 °C until analysis.

2.2. Larval rearing and feeding protocol

The study has been carried out in accordance with EC Directive 86/609/EEC for animal experiments and Spanish regulations on animal welfare. All procedures were approved by the Animal Ethics Committee of IFAPA. 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. At 2 days post-hatch (dph), larvae were transferred to nine 300 L tanks (three replicates per treatment) at an initial density of 28 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 for larvae fed rotifers enriched with marine oil emulsions and 500 lx for larvae fed rotifers enriched with *T-iso* was established. These differences in light intensity were established to prevent light stress in sole larvae since the later group was added the microalgae *Nannochloropsis gaditana B3 strain* (1000 cell ml^{-1}) in order to follow a standard culture procedure in the industry (Fernández-Díaz et al. 2001) and avoid any change in lipid composition for rotifers enriched with marine oil emulsions. Temperature and salinity were 19 °C and 35 g L^{-1}, respectively. Water was kept stagnant until 7 dph followed by a daily exchange of 40% from 7 to 9 dph and increasing progressively up to 150% as larvae grew as reported in Fernández-Díaz et al., (2001). The oxygen level was kept constant (6.8 ppm).

Larvae were fed the experimental diets (rotifers) from 3 (mouth opening) to 11 dph (Fig. 1). Rotifers were added twice a day between 11:00 and 12:00 a.m., and 5:00 and 6:00 p.m. Final concentrations of rotifers in tanks were increased from 2.5 to 25 preys ml^{-1}. From 9 dph, larvae were fed twice a day (10:00 and 15:00) with *Artemia* metanauplii ranging between 0.5 metanauplii ml^{-1} at 9 dph to 3 metanauplii ml^{-1} at 28 dph. To maintain constant live prey concentrations within each experimental tank, three water samples of each tank were sampled before supplying new preys. Estimated average number of preys in each tank was used to adjust total prey concentrations in the tank. The daily mortality rate for fish larvae was calculated using the methodology reported in Buckley et al. (1984).

Larvae were sampled at 9, 12, 16 and 22 dph for lipid analysis; at 9, 12 and 22 dph for histological analyses; and 6, 9, 12, 16, 22 and 29 dph for molecular analyses. Samplings were carried out at 11:00 a.m., before supplying new food (when needed). For lipid determination, two pools of larvae (15–400 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

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