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

I. Cunha [a,*], S. Galante-Oliveira [a,1], E. Rocha [a,b], M. Planas [c], R. Urbatzka [a], L.F.C. Castro [a]

[a] Interdisciplinary Centre for Marine and Environmental Research (CIIMAR), CIMAR Associate Laboratory, University of Porto (U. Porto), Portugal
[b] Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U. Porto), Portugal
[c] Instituto de Investigaciones Marinas (CSIC), Vigo, Spain

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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.

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; Burdick et al., 2006), lipid metabolism regulation, lipid transport, lipid and glucose oxidation, adipogenesis, lipid homeostasis, (Jump, 2002; Varga et al., 2011; Cho et al., 2012; Cour Poulsen 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 isosoforms in mammals and amphibians (Hihi et al., 2002) thereby serving as major transcriptional sensors of fatty acids (FA) (Xu et al., 1999; Jump, 2008; 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 β-oxidation of FA, the former mainly in liver and the second ubiquitously distributed (Wang et al., 2003; Mandard et al., 2004), while *PPARγ* 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,

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.

* Corresponding author at: Rua dos Bragas 289, 4050-123 Porto, Portugal. Tel.: +351 22 340 18 18; fax: +351 22 339 06 08.
E-mail address: isabel.cunha@ciimar.up.pt (I. Cunha).
[1] Present address: Department of Biology and CESAM, University of Aveiro, Portugal.

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plasticizers, herbicides and pesticides, may have small or no effect in other species (Lake et al., 1989) due to differences in relative expression of PPARα (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 (Krey 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., 2001).

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 (Estévez and Kanazawa, 1995; Izquierdo, 1996; Rainuzzo et al., 1997; Næss and Lie, 1998; Estévez et al., 1999; Sargent et al., 1999; Shields et al., 1999; Hamre et al., 2007).

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; McEvoy 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, 2012; Morais et al., 2012). Clarifying species specific nutritional requirements, effects of nutrient 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 FA 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 fiber-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 fed 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 ($4/3\pi a2b$), 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 lipid fraction: triglycerides (TG), phospholipids (PL), sterol esters + wax esters (SE + WE), sterols (SL), and free fatty acids (FFA). Lipid classes were analyzed 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 Gabbott (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 analyzed 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 analyzed 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 was used to find conservative sequences in the aligned input sequences and select degenerated primers. Phusion-Taq (Finnzymes, Finland) was used to amplify sequences between each pair of primers. Two microliters of a 1:10 diluted cDNA was used in PCR reactions with the following cycle conditions: 98 °C—10 s, 55 °C—5 s, and 72 °C—30 s 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) (Table S1).