Aquaculture 479 (2017) 774–779

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Expression of long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis genes and utilization of fatty acids during early development in rabbitfish *Siganus canaliculatus*

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ARTICLE INFO

Keywords:
Rabbitfish
LC-PUFA biosynthesis
Early development
Fatty acids
Δ6/Δ5 *fad*
Δ4 *fad*
elovl5
elovl4

ABSTRACT

Rabbitfish *S. canaliculatus* is the first marine fish reported to possess long-chain polyunsaturated fatty acids (LC-PUFA) biosynthetic ability, but the initiation of the LC-PUFA biosynthesis in this species is still unknown. In this study, the gene expression of Δ5/Δ6 fatty acid desaturase (Fad), Δ4 Fad and two fatty acid elongases, four key enzymes for LC-PUFA biosynthesis, were measured during the embryonic and unfed larval development of rabbitfish. Correspondingly, the fatty acid composition of rabbitfish embryos was determined. The results showed that DHA was the primary fatty acid utilized at the zygotic stages. The mRNA expression of the four key genes was detected from 8 hpf (hours post-fertilization) onwards, with a low level of *elovl5* mRNA contrary to the high expression of Δ4 *fad* mRNA. Subsequently, the elevated expression of Δ5/Δ6 *fad* and two elongase genes was observed after hatching, and then a great increment in the expression of all four key genes was detected at 3–4 dph (days post-hatching). These results indicate the production of DHA from EPA via the "Δ4 Fad" pathway as well as the alternative "Sprecher" pathway at the yolk-sac larval stages. Furthermore, the entire LC-PUFA biosynthetic pathway of converting C_{18} PUFA precursors to DHA is activated just after the onset of first-feeding. This study provides information on LC-PUFA biosynthesis during early development in rabbitfish, and offers an approach to determine the nutritional requirements of first-feeding larvae for rabbitfish and other marine fish.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are a cluster of polyunsaturated fatty acids (PUFA) containing carbon chain length no fewer than twenty and double bonds no fewer than two, widely known as arachidonic (ARA; 20:4n-6), eicosapentaenoic (EPA; 20:5n-3), and docosahexaenoic (DHA; 22:6n-3) acids. LC-PUFA play key roles in numerous animal metabolic and physiological processes, including the production of energy, the maintenance of cellular membrane integrity, precursors for eicosanoids and roles as cellular signal and transcription factor ligands (Jump, 2002; Morais et al., 2012). So, they are pivotal for normal function and development of vertebrates.

In fish, the source of LC-PUFA is derived from food supply and endogenous biosynthesis. As with all vertebrates, fish cannot synthesize α-linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) de novo due to the lack of Δ12 and Δ15 desaturases. However, they showed variation in their ability to transform C_{18} PUFA into LC-PUFA (Tocher, 2003). It is widely believed that freshwater fish and salmonid species have a capacity of converting the dietary ALA and LA into LC-PUFA varieties, while marine fish are deficient or weak in such an ability (Tocher, 2003, 2015). The synthesis of EPA or ARA from the C_{18} PUFA precursors is achieved by a first Δ6 desaturation and then an elongation followed by a Δ5 desaturation (Cook and McMaster, 2004), and DHA synthesis from EPA requires two further elongation steps, a second Δ6 desaturation and a peroxisomal β-oxidation process through the so-called "Sprecher" pathway (Sprecher, 2000). Further fatty acyl desaturase (Fad) with Δ4 activity found in *Siganus canaliculatus* (Li et al., 2010), *Solea senegalensis* (Morais et al., 2012), *Chirostoma estor* (Fonseca-Madrigal et al., 2014) and *Channa striata* (Kuah et al., 2015) offers an alternative, simpler pathway to produce DHA from EPA. It is suggested that the relatively richer LC-PUFA marine food chain, the piscivorous feeding behavior and the higher trophic levels in marine fish than in freshwater fish might contribute to the irrelevance and subsequent loss of their LC-PUFA biosynthesis capacity, rendering deficiencies of desaturase or elongase enzyme activities at the molecular level in marine fish (Java-Ram et al., 2011; Li et al., 2010; Morais et al., 2012; Tocher, 2010).

The early development periods of fish involve the embryonic stages (fertilization to hatching), the yolk-sac larval stages (hatching to first-

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http://dx.doi.org/10.1016/j.aquaculture.2017.07.028
Received 17 February 2017; Received in revised form 17 July 2017; Accepted 19 July 2017
Available online 19 July 2017
0044-8486/

feeding), the pelagic larval stages and the post-larval stages (Morais et al., 2012). LC-PUFA are necessary for the embryonic and larval development of fish, in which LC-PUFA are utilized primarily for cell division and organogenesis as well as energy. For example, large amounts of LC-PUFA, particularly DHA, are specifically accumulated and are required for building phospholipids – the fundamental components of cell membranes, especially that of neural and visual systems during embryogenesis and larval development (Mourente et al., 1999; Sargent et al., 2002; Tocher, 2010), as fish larvae are visual hunters and their head/eyes constitute the major fraction of body mass for effective prey capture (Morais et al., 2012). Thus, the provision of adequate LC-PUFA is generally required through live prey enrichments to improve the survival and to promote growth and development of the planktonic larvae for marine fish. The developmental stage from fertilization to the start of exogenous feeding is a critical period characterized by extremely fast growth and extensive organogenesis, which is entirely dependent on endogenous nutrient. However, little is known about the endogenous LC-PUFA biosynthesis systems and their roles during embryonic and yolk-sac larval development of fish, except for few studies on zebrafish *Danio rerio* (Monroig et al., 2009, 2010; Tan et al., 2010), cobia *Rachycentron canadum* (Monroig et al., 2011), Atlantic bluefin tuna *Thunnus thynnus* (Morais et al., 2011) and Senegalese sole *Solea senegalensis* (Morais et al., 2012). Moreover, investigation on the production and utilization of endogenous LC-PUFA during embryogenesis and yolk-sac larval development may shed light on the nutritional requirements of first-feeding larvae and further provide insights into the development of live prey enrichments (Sargent et al., 2002; Tocher, 2003).

Siganus canaliculatus, commonly known as the rabbitfish, belongs to the family Siganidae. It is a marine reef fish distributed throughout the Indo-Pacific, including the coastal areas around Hong Kong and Taiwan (Grandcourt et al., 2007). *S. canaliculatus* larvae normally feed on zooplankton and turn to consuming algae and seagrasses when they reach juvenile stage. It is the first marine fish reported to possess the LC-PUFA biosynthetic ability with all the key genes (Δ5/Δ6 *fad*, Δ4 *fad*, *elovl5* and *elovl4*) encoding the enzymes required for LC-PUFA biosynthesis (Li et al., 2008, 2010; Monroig et al., 2012). However, there is no information on when and how the endogenous LC-PUFA biosynthesis system is activated and the following roles in LC-PUFA supply during the embryonic and larval development in rabbitfish. The present study aims to elucidate the activation patterns of the LC-PUFA biosynthetic pathway and the utilization of endogenous fatty acids by investigating the mRNA expression of the fatty acid desaturase and fatty acid elongase genes involved in LC-PUFA biosynthesis, and the fatty acid composition during embryonic and yolk-sac larval stages in *S. canaliculatus*. The results will provide information on LC-PUFA biosynthesis during early development in rabbitfish, and offer an approach to determine the nutritional requirements of first-feeding larvae for rabbitfish and other marine fish.

2. Materials and methods

2.1. Experimental animals and sampling

One hundred individuals of *S. canaliculatus* (body mass interval of 400–600 g) were collected from the coastal areas of Rao Ping, Chaozhou city of Guangdong province in southern China during March 2014. The broodstock were kept in captivity in a floating cage in the local offshore cage farm and fed with frozen small fish and macroalgae two times a day. On 11th May 2014, 57 mature individuals (500 ± 20 g) were selected and were injected with hormone (50 IU/kg HCG and 2 μg/kg LHRH-A3 per individual) from the base of pectoral fin. All female and male broodstock were then kept together in a close-meshed net in the floating cage for natural insemination. Spawning was observed on May 12th and the following day; only the batch of fertilized eggs spawned on May 13th were collected and transferred into an 800 L plastic aquarium for further incubation and larval rearing. The incubation temperature was maintained at 26–27 °C, while the rearing temperature ranged from 27 °C to 30 °C. The salinity was kept constant at 28 ppt and dissolved oxygen ≥ 5 mg/L was maintained by gentle aeration. Approximately 20% volume of the water was exchanged daily in the morning throughout the larval rearing period.

After fertilization, the 8-cell stage was attained in 30 min, the morula stage in 85 min, the blastula stage in 2 h, and the gastrula stage in 4.5 h in rabbitfish (Hara et al., 1986). In the present study, the sample was first obtained at about 8 hpf (hours post-fertilization) and subsequently collected at every about 3 h interval until 21 hpf during the zygotic stages, i.e. 8, 11, 14, 18 and 21 hpf. After the eggs hatched at about 22 hpf, the yolk-sac larvae were sampled daily. For example, 0 dph (days post-hatching) larvae were 0-days or several hours old; 1 dph larvae were one-day-old, etc. At 36–48 h after hatching (2 dph), the yolk-sac larvae would start first-feeding (Bagarinao, 1986). The survival of the unfed larvae was observed to decline greatly at 3 dph, and only a few viable larvae (≤ 100 individuals) were successfully obtained at 4 dph. Thus, four samples of the unfed larvae were obtained at different larval stages (0 dph, 1 dph, 3 dph and 4 dph). All samples were flash-frozen in liquid nitrogen and stored at − 80 °C until further analysis. The five embryo samples (8, 11, 14, 18 and 21 hpf) were used for fatty acid analysis, and the total nine samples of embryos and yolk-sac larvae were used for further gene expression analysis. Moreover, the unfertilized eggs from three females were mixed and used for fatty acid analysis as an initial reference.

2.2. Fatty acid composition of rabbitfish embryos

To monitor the changes in fatty acid composition during embryonic development, approximately 1 g of embryos from each stage (8, 11, 14, 18 and 21 hpf) was collected for fatty acid analysis, and the unfertilized eggs were used as a control group. Fatty acid methyl esters (FAME) were prepared by boron trifluoride methanol complexness (Sigma-Aldrich, USA) catalyzed transesterification of total lipids as described previously (Li et al., 2005, 2008). After purification, FAME were separated and quantified using gas chromatography (GC-17A; Shimadzu, Kyoto, Japan) equipped with an auto-sampler and a hydrogen flame ionization detector. The detailed GC parameters were as described previously (Li et al., 2005, 2008). Individual fatty acids were identified by comparison with known commercial standards (Sigma-Aldrich, USA) and quantified using CLASS-GC10 GC workstation (Shimadzu, Kyoto, Japan).

2.3. Temporal expression of Fad and Elovl genes involved in rabbitfish LC-PUFA biosynthesis

The expression of four genes, namely Δ6/Δ5 *fad*, Δ4 *fad*, *elovl5* and *elovl4*, encoding key enzymes required for LC-PUFA biosynthesis during the embryonic and yolk-sac larval development of rabbitfish was studied by real time quantitative PCR (qPCR). Total RNA was extracted from embryos and larvae from 8 hpf to 4 dph using Trizol reagent (Invitrogen, USA) according to the manufacturer's specifications. One microgram of total RNA was reverse transcribed into cDNA using the FastQuant® RT kit (Tiangen, China) with a genomic DNA elimination reaction. Specific primers (Table 1) were designed based on the open reading frame (ORF) cDNA sequences of Δ6/Δ5 *fad* (GenBank accession no. EF424276), Δ4 *fad* (GenBank accession no. GU594278), *elovl5* (GenBank accession no. GU597350) and *elovl4* (GenBank accession no. JF320823) of rabbitfish (Monroig et al., 2012; Xie et al., 2015). The housekeeping gene *β-actin* of rabbitfish was used as a reference gene (GenBank accession no. EU107278.1) (Xie et al., 2015). The qPCR was performed on a Lightcycler 480 system (Roche, Switzerland) in a total reaction volume of 20 μL containing 10 μL of SYBR Green Supermix (Roche, Switzerland), 1 μL of each primer (10 μM), 6 μL ddH_2O and 2 μL of cDNA (10 ng μL^{-1}). The qPCR program consisted of an initial