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n-3 essential fatty acids in Nile tilapia, *Oreochromis niloticus*: Bioconverting LNA to DHA is relatively efficient and the LC-PUFA biosynthetic pathway is substrate limited in juvenile fish

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ABSTRACT

This study aimed to gain a better understanding of the long-chain ($\geq C_{20}$) polyunsaturated fatty acid (LC-PUFA) biosynthetic metabolism, with a specific focus on the metabolic fate of linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) and the relationship between these two substrates in the process of LC-PUFA biosynthesis in juvenile Nile tilapia, *Oreochromis niloticus*. Fish were fed four isoproteic (37.5%) and isolipidic (6.2%) diets formulated with fixed levels of LA (0.60%) and gradual levels of LNA (0.10, 0.63, 1.56 and 2.04% of dry weight, respectively) for 10 weeks. The whole-body fatty acid balance method was used to evaluate fish *in vivo* fatty acid metabolism. The results showed that most of dietary LNA was bioconverted to LC-PUFA when tilapia receiving not enough LNA for optimal growth. In contrast, when fish receiving sufficient dietary LNA, most of the LNA was β-oxidized, with only < 10% LNA being bioconverted. The *in vivo* apparent △-5/-6 desaturase and elongase activities on n-3 and n-6 PUFA, respectively, exhibited a significant linear increase and decrease with the increasing dietary LNA levels, clearly indicating that for all of the enzyme activities, n-3 PUFA is a preferred substrate and the LC-PUFA biosynthetic pathway is substrate limited in Nile tilapia. Dietary higher inclusion of LNA could block or at least slow down n-6 LC-PUFA biosynthesis from LA, indicating that a direct substrate competition for accessing the Δ-6 desaturase and elongase exists between these two substrates. Furthermore, Nile tilapia were relatively efficient in bioconversion from LNA to DHA, and the DHA biosynthesis from EPA may be more direct or faster than the production of EPA from LNA. Despite of the active bioconversion of LNA up to DHA, this metabolic effort was still insufficient to compensate for the significant reduced dietary intake of n-3 LC-PUFA. No significant difference was observed in the gene expression of fatty acyl desaturase (*fads2*) and fatty acid elongase (*elovl5*) among all groups, which suggests that the total *in vivo* apparent enzyme activities are directly and positively affected by substrate availability rather than the extent of the enzyme gene expression.

1. Introduction

It is commonly accepted that fish is the major readily available and edible source of health-promoting omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) for human consumption (Calder, 2014; Nordøy and Dyerberg, 2015; Tur et al., 2012). Considering this realization, the global consumption and demand for fish products is rising (FAO, 2014). However, as a traditional lipid source necessary for the production of n-3 LC-PUFA rich farmed fish, fish oil used by the aquafeed industry is increasingly recognized as an environmentally unsustainable and economically unviable practice (Tocher, 2015; Turchini et al., 2009). In this context, a significant global attention has been focusing on finding possible alternative oils to replace fish oil in the aquafeed formulation; and undoubtedly, vegetable oils are the most sustainable alternatives given their ready availability, low cost and absence of dioxins and pollutants (Olsen, 2011; Nasopoulou and Zaetakis, 2012). However, when fish oil is substituted with vegetable oils, the fish fillet fatty acid composition is normally characterized by significantly decreased levels of n-3 LC-PUFA, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Al-Souti et al., 2012; Chen et al., 2017; Li et al., 2016). Thus, the regulation of *in vivo* n-3 LC-PUFA biosynthesis from dietary C_{18} PUFA precursor, namely linolenic acid (LNA, 18:3n-3), has globally attracted significant research attention.

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Tilapia, one of the most widely and successfully cultured freshwater fish worldwide, is known to be capable of transforming parts of dietary LNA, which can be found rich in some vegetable oils, into n-3 LC-PUFA (Olsen et al., 1990; Tocher et al., 2002a). However, the final contents of n-3 LC-PUFA in fillets of tilapia fed fish oil deprived diets were still inevitably significantly lower than that of fish fed with fish oil-based diets (Al-Souti et al., 2012; Bahurmiz and Ng, 2007; Teoh et al., 2011; Teoh and Ng, 2016). Therefore, it is important to gain a better understanding of the mechanism of n-3 LC-PUFA biosynthesis in tilapia for maximizing the n-3 LC-PUFA content of fish fillets when formulating feed without fish oil.

In vivo, the biosynthetic pathway of LC-PUFA generally involves a series of enzymatic steps catalyzed by two fatty acyl desaturases (Δ-6 desaturase and Δ-5 desaturase), two fatty acid elongases (Elovl5 and Elovl2) and a peroxisomal β-oxidation for fatty acid chain shortening (Sargent et al., 2002; Sprecher, 2000). In this pathway, two essential fatty acids (EFA), linoleic acid (LA, 18:2n-6) and LNA, can be simultaneously bioconverted into n-6 LC-PUFA (such as arachidonic acid, ARA, 20:4n-6) and n-3 LC-PUFA (such as EPA and DHA), respectively. Δ-6 desaturase is known as a rate-limiting enzyme because of its involvement in the first step of biosynthesizing LC-PUFA by desaturating the precursors, LNA and LA (Hastings et al., 2001). Many studies reported that the efficiency and affinity of Δ-6 desaturase is dependent on the availability of the enzymatic substrates, as well as the competition between these substrates (LNA and LA) for accessing the Δ-6 desaturase (Francis et al., 2009; Thanuthong et al., 2011; Turchini et al., 2006). The substrate competition for Δ-6 desaturase also extends to the 24 carbon fatty acids (24:5n-3 and 24:4n-6) at the second use of the enzyme in the biosynthesis of DHA and 22:5n-6, respectively (Portolesi et al., 2007). It is well known that Δ-6 desaturase has a higher affinity towards LNA than LA in many fish, such as Murray cod (Senadheera et al., 2011), Nile tilapia (Karapanagiotidis et al., 2007; Ng et al., 2013) and Rainbow trout (Thanuthong et al., 2011). Dietary inclusion of a high LNA/LA ratio leaded to a greater Δ-6 desaturase activity (Bell et al., 1997, 2001; Li et al., 2008), and excessive supply of dietary LNA could block or at least slow down Δ-6 desaturase activity on LA (Karapanagiotidis et al., 2007; Thanuthong et al., 2011; Zheng et al., 2005; Tocher et al., 2002a). Conversely, an excess of dietary LA may also inhibit the desaturation of LNA in salmons (Ruyter et al., 2000; Thanuthong et al., 2011; Tocher et al., 2001). Furthermore, Emery et al. (2013) suggested that the activity of Δ-6 desaturase on n-3 and n-6 substrates is independent and there is none or minor competition between substrates for Δ-6 desaturase in rainbow trout. Evidence on substrate competition for Δ-6 desaturase in teleosts appears to be somewhat inconsistent (Emery et al., 2013; Vagner and Santigosa, 2011), and further investigation is still needed to gain a better understanding of the regulation of LC-PUFA biosynthesis.

It has previously reported that tilapia can be a net producer of n-3 LC-PUFA despite being fed LC-PUFA deprived diets (Chen et al., 2013; Teoh et al., 2011); and genetically improved farmed Nile tilapia has a greater ability for fatty acid neogenesis, elongation and Δ-5/6 desaturation than red hybrid tilapia (*Oreochromis sp.*) (Teoh et al., 2011). The gene of fatty acyl desaturases (*fads2*) in Nile tilapia has been cloned and demonstrated to exhibit bifunctional enzymatic activities of Δ-6 and Δ-5 (Tanomman et al., 2013). Additionally, the Δ-4 desaturase was recently isolated and functionally determined in Nile tilapia by Oboh et al. (2017), suggesting the existence of an alternative route — Δ-4 pathway for DHA biosynthesis from EPA in Nile tilapia. Teoh and Ng (2016) suggested that in Nile tilapia, the enzyme activities of desaturase and elongase acting on n-3 PUFA were always higher than those on n-6 PUFA. It is also well known that the bioconversion pathway is inhibited by high dietary LC-PUFA but stimulated by high concentrations of dietary LNA and LA (Tocher, 2015). However, such an increased endogenous biosynthesis from fatty acid precursors was insufficient to compensate for n-3 LC-PUFA deficiencies in fish fed fish oil deprived diets (Karapanagiotidis et al., 2007; Al-Souti et al., 2012; Ng et al., 2013). Consequently, it is envisaged that a solution for the crisis around fish oil shortages can be achieved through a better understanding of fish LC-PUFA biosynthetic metabolism. Therefore, the objective of the present study was to evaluate the effects of graded amounts of dietary LNA with a constant amount of LA in LC-PUFA deprived diets on the fatty acid metabolism, and with emphasis on the fate of LNA and LA metabolism and the relationship between the two substrates, LNA *versus* LA in the process of LC-PUFA biosynthesis in juvenile Nile tilapia, *O. niloticus*.

2. Materials and methods

2.1. Animals, diets and experimental design

Nile tilapia (*O. niloticus*) fingerlings were obtained from a local hatchery and kept in the feeding facility of South China Agricultural University. After two weeks of acclimation with a basal lipid-free diet, 40 fish were randomly selected and anesthetized with MS–222, snap frozen by liquid nitrogen and then stored at −80 °C for later chemical analysis. Fish with initial body weight of 2.10 ± 0.01 g were then randomly distributed into 16 recirculating tanks (30 fish per tank), assigned in quadruplicate to each of the semi-purified experimental diets containing graded levels of LNA (0.10, 0.63, 1.56 and 2.04% of dry weight). All isoproteic (appr. 37.5% crude protein) and isolipidic (appr. 6.2% total lipid) diets contained similar levels of 18:2n-6 (0.60%) which was essential for normal growth of Nile tilapia (Takeuchi et al., 1983). The ingredients and fatty acid composition of the experimental diets were shown in Table 1. Feeding rate was 10% of body weight in the first 2 weeks, 8% in the following 4 weeks, and then gradually reduced to 5% in the next 4 weeks. Fish were fed three times daily at 8:30, 12:00 and 17:00 for 10 weeks and daily food consumption was recorded. After 6 weeks of the feeding trial, fecal samples (intact strands) collected from each tank were pooled, freeze-dried, finely ground and stored at −20 °C until analyzed. At the end of the feeding trial, fish were fasted for 24 h, anesthetized and individually weighed, and then four fish from each tank were randomly selected and snap frozen in liquid nitrogen and stored at −80 °C for subsequent chemical analysis. Liver tissues were excised from another four fish of each tank and immersed in liquid nitrogen and subsequently stored at −80 °C for RNA extraction.

2.2. Chemical analysis

Total fatty acid composition of experimental diets, whole fish and fecal samples from each tank were analyzed by gas chromatography–mass spectrometry (GC–MS) after chloroform/methanol extraction, saponification and methylation with boron trifluoride as previously described in detail (Chen et al., 2013). Fatty acids were identified by retention indices and by comparing of their mass spectra with the NIST 05 spectral database and quantified relative to the internal standard (19:0). The apparent digestibility of individual fatty acids was assessed using the inert marker yttrium oxide and calculated from the standard formulas as previously described by Codabaccus et al. (2010). Total yttrium contents were determined in the diet and fecal samples after nitric acid digestion using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin Elimer Optia 3000DV; Perkin Elimer, Wellesley, MA, USA).

2.3. Whole-body fatty acid balance calculations

The whole-body fatty acid balance method was used to assess the apparent *in vivo* fatty acid metabolism (elongation, desaturation and β-oxidation) in Nile tilapia fed diets with graded levels of LNA as previously described in detail by Turchini et al. (2007) and Turchini and Francis (2009). Briefly, after calculating the individual fatty acid (FA) intake (= g of feed intake × mg of FA per g of feed), excretion (= mg of