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Diet high in α-linolenic acid up-regulates fatty acids biosynthesis-relate gene expression in the hepatopancreas of Yellow River carp *(Cyprinus carpio)*

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Keywords: α-linolenic acid, fatty acid, gene expression, hepatopancreas, Yellow River carp

Abstract

Little information on the effects of a high α-linolenic acid (C18:3n-3) diet on fatty acid composition and lipid-producing gene expression in the hepatopancreas from Yellow River carp (*Cyprinus carpio*). In the study, 60 common carp juveniles were fed for 50 days, a high α-linolenic acid diet supplemented with linseed oil (LO), and a control diet without the addition of linseed oil to evaluate the effect on lipid composition and gene expression of peroxisome proliferator-activated receptors (PPAR-α), delta-6 fatty acid desaturase (delta-6-Fad), stearoyl-CoA-desaturase (SCD), and fatty acid desaturase-5 (Elovl5) in the hepatopancreas. The results showed that increased levels of C20:4n-6, C18:3n-3, C20:5n-3, and C22:5n-3 were observed in LO-treated Yellow River carp hepatopancreas. PPAR-α, delta-6-Fad and Elovl5 gene expression was up-regulated in the Yellow River carp's hepatopancreas fed with a high α-linolenic acid diet ($P < 0.05$), while SCD gene was down-regulated. In conclusion, the addition of α-linolenic acid to the Yellow River carp diet affected gene expression (SCD, PPAR, delta-6-Fad, and Elov15) involved in regulating fatty acid (FA) profile Yellow River carp. It is suggested that delta-6-Fad, Elovl5, and PPAR-α be essential messengers responsible for translating nutritional stimuli into gene expression changes in the hepatopancreas.

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Introduction

Polyunsaturated fatty acids (PUFA) and their metabolites have many physiological functions, including membrane structure, energy supply, gene expression regulation, and cell signal transduction. PUFA is an essential component in advanced eukaryotes, giving cell membrane fluidity, selective permeability, and flexibility (Poulos, 1995). The de novo metabolism in mammalian tissues cannot meet the requirements of PUFA. Animals rely on plants to supply the two main precursors of n-3 and n-6 fatty acids, namely, C18:3n-3 (α-linolenic acid, ALA) and C18:2n-6 (linoleic acid, LA) (Poulos, 1995).

The diet can meet some of the daily needs of highly unsaturated fatty acids (HUFA) in animals. However, most of the HUFA of animal tissues comes from biosynthesis pathways, which involve the transformation of plant-supplied essential fatty acids (EFA) precursors (C18:3n-3 and C18:2n-6) into their respective C20 and C22 products. HUFA, such as eicosapentaenoic acid (C20:5n-3, EPA), arachidonic acid (C20:4n-6, ARA), and docosahexaenoic acid (C22:6n-3, DHA) play an important role in many biological functions (Spector, 1999). HUFA affects many cell and physiological processes in both animals and plants.

The animal organisms that synthesize HUFA from linoleic acid and α-linolenic acid are mainly regulated by the delta-6 desaturases (delta-6-Fad) and elongation enzyme-5 (Elovl5), through the mechanism of nutrition and hormone stimulation (Brenner, 2003). Therefore, fatty acid desaturases enzymes that introduce double bonds into long-chain fatty acids are preserved across boundaries (Nakamura et al., 2004). Palmitic acid (16:0, PA) is the most common saturated fatty acid (SFA) and can be provided in the diet or synthesized endogenously from other fatty acids, carbohydrates, and amino acids. PA represents 20–30% of total fatty acids (FA) in membrane phospholipids (PL) and adipose triacylglycerols (TAG) (Carta et al., 2015).

The liver is one of the significant parts of HUFA metabolism. Many key enzymes are significant regulators of hepatic lipid metabolism. In the lipid metabolic pathways, the regulation of crucial gene expression plays a crucial role in adaptive responses and altering enzyme activity. In the past decade, key transcription factors have been identified to regulate this adaptive response. The critical transcription factors include sterol regulatory element-binding protein (SREBP) (Horton et al., 2002), peroxisome proliferator-activated receptors (PPAR) (Desvergne et al., 2006), carbohydrate response element-binding protein (CHREBP) (Clarke et al., 1993), and stearoyl-CoA-desaturase (SCD).

The activities of the transcription factors must be regulated not only by nutrients and metabolites but also by hormones (Kersten, 2001). PPARs can induce the proliferation of peroxisomes in cells. This process is accompanied by activating the acyl-coenzyme oxidase gene (ACOX1) promoter, which encodes the key enzyme for the peroxidase of long-chain fatty acids oxidation (Bionaz et al., 2013). PPAR-α regulates the biosynthesis of HUFA, which indicates that PPAR-α has multiple effects on the regulation of lipid metabolism (Nakamura et al., 2004).

Yellow River carp, initially an important freshwater fish in North China, is one of the major freshwater fish farmed in Henan Province of China because of its fast growth, disease resistance, golden scales, and fresh meat. In the study, the hypothesis was that the supplement of linolenic acid to Yellow River carp diets would change lipid deposition or gene expression in the hepatopancreas. Changing the activities of SCD, PPAR-α, delta-6-Fad, and Elovl5 activity can increase the production of n-3 fatty acids and reduce the content of saturated fatty acids (SFA), thereby improving fatty acids profiles of the tissue in animals. However, a clearer understanding of SCD, PPAR-α, delta-6-Fad and Elovl5 gene expression in the Yellow River carp hepatopancreas may provide new information about the regulation relationship between genes and the synthesis of fatty acid synthesis to the improvement of Yellow River carp products.

In this study, our specific goal was to determine any associations between SCD, PPAR-α, delta-6-Fad, and Elov15 genes expression and the deposition of n-3 fatty acids in the hepatopancreas of Yellow River carp.

Materials and Methods

Animal and diets

Sixty Yellow River carp juveniles (45~50g) were fed with a circulating water system in six 500L glass tanks (3 replicate tanks for each treatment). The same essential components (35% protein and 6% oil) were fed the two diets, mixed with different oils, fish oil (FO) and linseed oil (LO) were prepared (**Table1**), respectively. During the trial, the carps were fed twice daily at 8:30 and 14:30 (3% of body weight) and weighed every two weeks to adjust the feeding rate. The water temperature was 25±1°C, PH was 6.8±0.2, ammonia nitrogen was less than 0.5 mg / L, dissolved oxygen was more than 5 mg / L. During the trial, there was no mortality in the trial groups. At the end of the experiment, the carps were fed for 50 days, fasted overnight (24 hours), and then slaughtered. Hepatopancreas were removed immediately after slaughter and frozen in liquid nitrogen at-80 °C until fatty acid analysis, and RNA extraction was performed for gene expression analysis.

Table 1 Ingredients and chemical composition of the experimental diets

Ingredients (% of DM)	*FO（fish oil）*	*LO（Linseed oil）*
Casein	32	32
Gelatin	8	8
Dextrin	28	28
Cellulose	19	19
Fish oil	6	6
Linseed oil	0	6
Carboxy methyl cellulose	2	2
Mineral premix	4	4
Vitamin premix	1	1
Proximate composition (%)		
Crude protein	35.76	35.39
Crude lipid	5.86	5.49
Ash content	6.78	6.57
Fatty acid composition (% of total fatty acids)		
C14:0 myristic	8.05	0.08
C16:0 palmitic	30.29	7.05
C16:1 palmitoleic	7.76	0.16
C18:0 stearic	5.49	3.87
C18:1n-9 oleic	18.17	23.74
C18:2n-6 linoleic	2.72	18.38
C18:3n-3 a-linolenic	0.96	45.46

Procedures and analyses

For fatty acid analysis, total fat was extracted from hepatopancreas with chloroform/methanol (2:1, V / V) homogenate containing 0.01% butylated hydroxytoluene as an antioxidant. The Lipid extract was washed by the Folch method (Folch et al, 1957) and was methylated in 1% sulfuric acid in methanol at 70 °C for 3 hours to prepare fatty acid methyl ester (FAMEs). FAMEs was extracted with Heptane and analyzed with a gas chromatograph (GC-2010; Shimadzu, Japan) equipped with an automatic sampler and a hydrogen flame ionization detector. The GC is equipped with 30 m × 0.25 mm × 0.25 μ m capillary column (vf-23ms, Varian, USA). The carrier gas is nitrogen, and the combustion

supporting gas is air and hydrogen. The temperature of the injector and detector is 250 ° C. The column temperature was maintained at 120 °C for 3 minutes at first and then increased to 190 °C at the rate of 10 °C / Min. The rate was then increased to the final temperature of 220 °C at a rate of 2 °C / Min and maintained for 15 minutes. Individual fatty acids were identified by comparison with commercial standards (Sigma, USA) and quantified using the CLASS-GC10 GC workstation (Shimadzu, Japan).

Hepatopancreas gene expression

A tissue homogenizer was used to homogenize approximately 50 mg of quick-frozen hepatopancreas samples from each animal. Total RNA was extracted using Trizol Reagen kit (Takara, Japan), and 2 μg of total RNA was reverse transcribed into cDNA using PrimeScript RT-PCR kit (Takara, Japan). Gene expression was measured by real-time quantitative PCR (RT-qPCR) (SYBR Green II) on the Thermal Cycler Dice Real-Time System (TaKaRa Code. TP800) using β-actin as a housekeeping gene. Design primers for gene expression analysis were shown in **Table 2**. Each sample was run in triplicate and PCR reactions without template addition were used as negative controls. β-actin expression was used to normalize the mRNA expression level of genes in each sample and was calculated using the comparative threshold cycle (Ct) method (Pfaffl, 2001).
Data analysis.

Table 2 Names and sequences of the primers used in this study

primer	*sequence (5′→3′)*
delta-6Fad	F : ATCGGACACCTGAAGGGAGCG
delta-6Fad	R : CATGTTGAGCATGTTGACATCCG
Elovl5	F : GTACCAATGGGAGGTTCGGCAC
Elovl5	R : GAGTTGAAGGTTTGGATGAAATGCATG
Scd	F : GTCTGTGCTGCTGATGTGCT
Scd	R : GCACTCGTGTTGAACTCCAC
PPAR-a	F : GGGAAAGAGCAGCACGAG
PPAR-a	R : GCGTGCTTTGGCTTTGTT
β-actin	F : CGCCCCAGACATCAGGGTG
β-actin	R : CACAGATCATGTTTGAGACCTTCAACAC

Statistics

All statistical analyses were conducted using SPSS V17.0 (SPSS Inc., Chicago, IL, USA). The data were reported as mean ± standard error (M ± SD). T-test was used to compare the significance of the mean value. $p>0.05$was considered significant.

Results

Dietary fatty acid composition

About 30% of C16: 0 in the FO diet group was the main component of saturated fatty acids (SFA), and 18.17% of C18: 1n-9 was the main component of total monounsaturated fatty acids, 2.72% of C18:2n-6, and 0.96% of C18:3n-3 which were the main contributor of PUFA (**Table 1**). In the LO diet group, the percentage of C18:3n-3 increased to 45.46%.

Hepatopancreas fatty acid composition

The fatty acid composition of the hepatopancreas in carp fed the FO and LO diets were shown in **Table 3**. The major fatty acids detected in the hepatopancreas were C18: 2n-6, C20: 4n-6, C18: 3n-3, and C22: 5n-3 with significant differences between the treatment groups. There was no significant effect on the percentages of C20: 5n-3, C22: 6n-3, and C22: 5n-3 ($p>0.05$). However, the levels of C20: 5n-3, C22: 6n-3, and C22: 5n-3 in the LO group were higher than those in the FO group, but the differences were not statistically

significant (p>0.05). There was no significant difference in total saturated fatty acids (SFA) between the treatment groups (p>0.05). There were significant differences in total monounsaturated fatty acids (MUFA), total n-3, and total n-6 polyunsaturated fatty acids (PUFA) between the treatment groups (p>0.05). Increasing LO content in the diet will produce higher percentages of C18: 3n-3 and n-3 PUFA accompanied by higher proportions of C20: 4n-6 and C18: 2n-6.

Table 3 Fatty acid composition of hepatopancreas from Yellow River carp fed experimental diets (% of total fatty acids).

Fatty acids	*Dietary treatments*	
	FO	*LO*
14:0	0.69±0.03[a]	0.47±0.06[b]
16:0	20.02±0.86	19.92±0.84
16:1	3.22±0.01[a]	2.35±0.09[b]
18:0	6.03±0.12	6.59±0.18
18:1	24.32±0.81	23.91±1.76
20:0	0.19±0.01	0.14±0.01
20:1	1.54±0.02	1.39±0.07
22:1	0.30±0.05	0.18±0.02
18:2n-6	16.94±0.77 [b]	18.16±0.79 [a]
18:3n-6	0.28±0.01[a]	0.19±0.02[b]
20:4n-6	3.36±0.00 [b]	4.58±0.21[a]
18:3n-3	0.96±0.01[b]	3.25±0.14[a]
20:5n-3	2.02±0.10	2.03±0.22
22:5n-3	1.12±0.02	1.28±0.18
22:6n-3	11.02±0.84	11.30±0.82
Total SFA	26.92±0.93	27.12±0.89
Total MUFA	29.38±0.90 [a]	27.83±0.70 [b]
Total n-3	15.12±0.93[b]	16.86±0.28[a]
Total n-6	20.58±0.77 [b]	22.93±0.60 [a]

Note: Lowercase letters indicate significant difference at level (p<0.05) .

Hepatopancreas gene expression

Real-time PCR measurements of gene expression showed that delta-6-fad, Elovl5, and PPAR-αgenes were more expressed in the hepatopancreas of LO-fed Yellow River carp. The expression of Delta-6-Fad in the hepatopancreas of Yellow River carp Fed with LO was 3.37 times as high as that of the FO group. In the hepatopancreas of Yellow River carp fed with LO, the expression of Elovl5 was 3.34 times as high as the FO group. The expression of PPAR-α in the hepatopancreas of common carp fed with LO was 1.84 times as high as that of the FO group. The variation of the SCD gene in the LO Group was 0.46 times lower than that in the FO group (**Figure 1**).

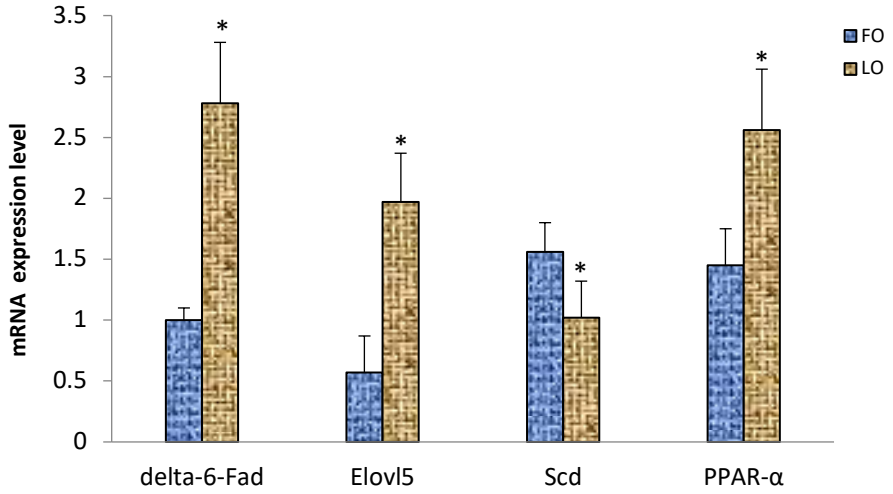

Figure 1 Comparisons of delta-6-Fad, Elovl5, SCD, and PPAR-α gene expression in the hepatopancreas of Yellow River carp. Values indicated by the * show significant difference compared with the FO group (p<0.05).

Discussion

It is generally accepted that primarily herbivorous or omnivorous fish have the ability to desaturate C18 fatty acids from LA to ARA, and AlA to EPA and DHA (Sargent et al., 2002). Some freshwater fish possess enzymes that elongate and desaturate fatty acids of the n-3 and n-6 series, particularly linolenic acid, to obtain sufficient levels of PUFA for membrane function and fluidity (Ilknur and Nilsun, 2012**).** In contrast, it is widely believed that most carnivorous marine species are less able to convert C18 PUFA organisms into HUFA and therefore need pre-formed HUFA in their diets (Agaba et al., 2004). The contents of n-6 and n-3 fatty acids in the muscle of common carp in ponds fed with natural food were higher, but the contents of n-6 and n-3 fatty acids in common carp fed formula feed were lower (Steffens and Wirth 2007). Buchtová et al (2010) showed that the fatty acid profiles of different common carp genetic populations may be different, so strain, hybridization, and reproduction should be considered when carp are fed with different fatty acids. In this study, the α-linolenic acid levels in the LO diet group were higher than α-linolenic acid levels in FO diet group. Therefore, it can be assumed that the carp feed that contains more α-linolenic acid will absorb more intact α-linolenic acid. The desaturation and elongation of C18:2n-6 (linoleic acid) and C18:3n-3 (α-linolenic acid) plays an important part in fatty acids products biosynthesized ARA, EPA, and DHA in the hepatopancreas.

The fatty acid desaturase and elongase gene expression regulated by nutrition has been reported in fish. The degree to which fish can desaturate and elongase C18 PUFA to HUFA varies from species to species (Tocher et al., 2006). Nutrients have been reported to increase the level of desaturase transcription that occurs in freshwater fish and mammals (Bell et al., 2001). Buzzi et al (1997) demonstrated that when rainbow trout were fed a diet containing olive oil, their liver delta-6-Fad activity increased compared to a diet containing fish oil. The abundance of delta-6-Fad mRNA in the liver of mice fed essential fatty acid diet was twice as high as that of LA mice fed corn oil. However, some studies have shown that HUFA also inhibits delta-6-Fad in freshwater fish and mammals (Ulmann et al., 1992). It has been reported that the Elovl5 gene in fish preferentially uses C20 and C18 PUFA as substrates rather than C22 PUFA (Zheng et al., 2009). In Atlantic salmon, replacing fish oil with linseed oil increases the expression of elovl5 in the liver (Zheng et al., 2004).

As a key transcription factor for different genes involved in the homeostasis of lipids, PPAR-α is known to regulate genes expressed in a specific manner by responsibility-sharing

overlapping cells and tissues, PPAR-α is known to be involved in fatty acid metabolism (Schoonjans et al., 1996). The synthesis of triglyceride (TG) and fatty acid both occur assembly in the liver, fatty acids are synthesized by fatty acid synthesis (Strable et al., 1996). According to the expression of the tested genes, there are more β-oxidation related genes (PPAR-α and SCD) in carp liver. The fatty acid desaturase is regulated by PPAR genes in mammals (Alegret et al., 1995). The n-3 and n-6 PUFA have been proved to be effective inhibitors or inducers of liver gene expression (Grønn et al., 1992), to enhance lipid oxidation by inducing PPAR-α (Zúñiga et al., 2011). The dietary C18:2n-6 (linoleic acid) activated the expression of PPARγ, which subsequently binds to DNA and accommodates gene expression of anti-inflammatory cytokines, thus leading to the inhibition of colonic inflammation (Yang et al., 2021). These findings indicate that the proportion of n-3 and n-6 PUFA in the diet alters the liver genes expression that encodes enzymes associated with fatty acid transformation.

The study showed that α-linolenic acid-rich in Yellow River carp diet results in changing of hepatopancreas fatty acids composition, up-regulation of delta-6-Fad, Elovl5, and PPAR-α, and downregulation of SCD gene. These findings may increase the understanding of the mechanism of α-linolenic acid controlling the expression of specific genes in aquatic animals and provide insights for the development of new nutrition strategies to better manage Yellow River carp in the process of fatty acids regulation.

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