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Effects of different dietary lipid sources on fatty acid composition and gene expression in common carp

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Abstract: The effects of fatty acid composition in artificial feed on the change in the fatty acid composition of carp muscles and the relationship between *Δ6-Fad* and *Elovl5* genes participating in the regulation of fatty acid synthesis were studied. Juveniles were fed three semi-purified diets (D1–D3) for 6 weeks with different lipid sources: D1, fish oil with high highly unsaturated fatty acids (HUFA); D2, corn oil with high linoleic acid (18:2n-6, LA), D3, linseed oil with high α-linolenic acid (18:3n-3, LNA); then, samples were taken to explore the molecular mechanism and the factors which affect the synthesis of carp HUFA. The content of LA and arachidonic acid (20:4n-6, AA) in common carp fed Diet 2 was higher than in carp receiving D3 ($P < 0.05$), but the contents of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) were lower than in carp fed D1 and D2 ($P < 0.05$). The liver transcript abundance of *Δ6-Fad* and *Elovl5* in fish fed D2 and D3 at the end of 6 weeks was generally higher than the abundance in the initial stage and in the fish fed D1 ($P < 0.05$). The results suggest that the common carp can biosynthesise HUFA, and the type and content of fatty acids in feed affected not only the composition and content of fatty acids in common carp muscles, but also the *Δ6-Fad* and *Elovl5* gene expression involved in the biosynthesis of HUFA. Feeding high levels of n-3 HUFA diet can increase the body content of EPA and DHA in common carp. The results of this research may provide a theoretical basis for choosing an appropriate source of lipid for common carp feeds.

Keywords: common carp; feed fatty acids; Δ6-desaturase; *Elovl5* elongase; gene expression

Fish are an important source of highly unsaturated fatty acids (HUFA) in human food. HUFA are beneficial to cardiovascular health, immune function, neurodevelopment and anti-inflammation (Eilander et al. 2007; Kim et al., 2019; Zhao et al. 2019). Common carp (*Cyprinus carpio*) is one of the most important freshwater fishes cultured for human food and an important source of HUFA. Artificial feeds are primary nutritional sources, therefore it is important to study the relationship between the fatty acid composition of carp and the fatty acids in diet. It is generally accepted that the freshwater fish can convert C18 to HUFA. For example, 18:3n-3 (α-linolenic acid, LNA) and 18:2n-6 (linoleic acid, LA) for $C_{20/22}$ HUFA, including 20:4n-6 (arachidonic acid, AA), 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) (Cook et al. 1996). However, this conversion ability differs in different fish species, primarily because of their own fatty acid desaturase and fatty acid chain elongation enzymes (Tocher et al. 2003). The fatty acid desaturase enzymes can introduce double bonds into fatty acids, whereas the fatty acid chain elongation enzyme can extend the fatty acid chain (Jakobsson et al. 2006; Monroig and Kabeya 2018). Among them, Δ6-fatty acid desaturase enzyme (*Δ6-Fad*) and elongation enzyme-5 (*Elovl5*) are highly unsaturated fatty acid rate-limiting enzymes, and different species have different ability to transform

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to HUFA. Polyunsaturated fatty acids (PUFA) are an essential part of the vertebrate diet, but the amount of HUFA requirement varies from fish to fish. In aquaculture, the composition of fatty acids in fish mainly depends on feed and their own synthesising capacity (Tocher 2010). Carp are fast-growing benthic omnivores, however, in the body composition of fatty acids, in carp raised in fishponds and fed formulated diets the DHA and DHA/EPA ratios are generally lower than those in marine and some freshwater fish (Liu 1991; Luo 2001; Ackman 2002; Guler et al. 2008; Donmez 2009; Prchal et al. 2018; Shaliutina-Kolesova et al. 2019). Although some freshwater fish have been reported to synthesise HUFA, the mechanism of HUFA synthesis in carp has been scarcely documented.

We studied the effects of fatty acid composition in artificial diets on the changes in the composition of fatty acids in carp muscle. We also explored the relationship between the *Δ6-Fad* and *Elovl5* genes involved in regulating the fatty acid synthesis and the composition of fatty acids in feed to provide a theoretical basis for choosing an appropriate source of lipids for the common carp feeds.

MATERIAL AND METHODS

Experimental feed, animals and design. A formulated semi-purified feed is described in Table 1 was administered. Casein was used as a protein source; fish oil, corn oil, and flaxseed oil were used as sources of essential fatty acids. Isonitrogenous feeds included three different kinds of fatty acids. Diet 1 contained 2.70% LA, 0.96% LNA, 0.56% AA, 4.70% EPA and 6.24% DHA. Diet 2 contained 52.30% and 0.61% LA. Diet 3 contained 18.38% and 45.46% LNA (Table 2). Feed was prepared once a week, and amounts of 2–3% of the fish body weight were stored in a plastic bag in a refrigerator at −18 °C. Fish were fed twice daily at 08:00 and 14:00 h by mixing with water and forming a small cake. Uneaten feed and faeces were siphoned before each feeding.

The experimental fish weighed 45–50 g and were collected from a local common carp breeding farm in Mengjin county in Luoyang City, Henan province, China, and all carps coming from the same pond shared the same genetic background. The fish were randomly allocated into three treatment groups, with 3 replications of 20 fishes each, and

Table 1. Ingredients and composition of the experimental diets (D1, D2 and D3) for common carp[1]

Ingredients (g/100 g diet)	Experimental diet		
	D1	D 2	D3
Casein	32	32	32
Gelatin	8	8	8
Dextrin	28	28	28
Cellulose	19	19	19
Fish oil	6	0	0
Corn oil	0	6	0
Linseed oil	0	0	6
Carboxymethyl cellulose	2	2	2
Mineral premix[2]	4	4	4
Vitamin premix[3]	1	1	1
Proximate composition (%)			
Crude protein	35.76	35.44	35.39
Crude lipid	5.86	5.67	5.49
Ash content	6.78	6.89	6.57

[1]all the ingredients and chemicals used were purchased from Sangon and East China Pharmaceuticals Company, Shanghai, China; [2]mineral premix consisted of (g/kg of premix): aluminum chloride, 0.45; cobalt chloride, 0.2; copper sulphate, 2.0; ferrous sulphate, 19.5; potassium iodide, 0.5; potassium chromium sulphate, 0.3; magnesium sulphate, 300.0; manganese sulphate, 7.5; sodium selenite, 0.02; zinc sulphate, 37; cellulose, 632.5; [3]vitamin premix consisted of (mg/kg of premix): thiamin hydrochloride, 10; riboflavin, 20; calcium pantothenate, 40; nicotinic acid, 50; pyridoxine hydrochloride, 10; folic acid, 5; inositol, 400; choline chloride, 2 000; menadione, 10; cholecalciferol, 1 500 IU; biotin, 1; vitamin B12, 0.02; vitamin A, 3 000 IU; vitamin E, 50 IU; vitamin C, 200

180 test fishes were stocked in nine glass tanks of 100 × 80 × 60 cm in size. The growth experiment was conducted indoors with the temperature controlled at 20 ± 2 °C. The recirculating culture system maintained water with pH 6.5 ± 0.2, ammonia nitrogen at less than 0.5 mg/L, and dissolved oxygen at more than 5 mg/L; the light cycle was controlled at 12 h light/12 h darkness. The fish were acclimated for 2 weeks before the 6-week feeding experiment.

Sample preparation. Six experimental fishes were randomly selected at the beginning of the experiment and their livers and muscles were removed. All livers were mixed together, immediately frozen in liquid nitrogen and stored in a refrigerator at −80 °C as the initial samples for

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Table 2. Fatty acid composition (percentage of total fatty acids) of the experimental diets

Fatty acids	Diet 1	Diet 2	Diet 3
14:0	8.05	0.12	0.08
16:0	30.29	13.19	7.05
16:1	7.76	0.22	0.16
18:0	5.49	1.76	3.87
18:1	18.17	30.19	23.74
20:0	0.86	0.35	0.23
20:1	2.84	0.38	0.21
22:1	3.18	nd	nd
18:2n-6	2.72	52.30	18.38
18:3n-6	nd	0.85	0.07
20:4n-6	0.56	nd	nd
18:3n-3	0.96	0.61	45.46
20:5n-3	4.70	nd	nd
22:5n-3	0.53	nd	nd
22:6n-3	6.24	nd	nd
Total SFA	44.69	15.42	11.23
Total MUFA	31.95	30.79	24.11
Total n-3	12.43	0.61	45.46
Total n-6	3.28	53.15	18.45
n-3/n-6	3.78	0.01	2.46

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; nd = not detected

mRNA determination of *Δ6-Fad* and *Elovl5* genes. On days 3 and 14 and at the end of the experiment, three fishes were taken randomly from each tank, and their livers and muscles were dissected; these tissues were mixed into one sample and immediately frozen in liquid nitrogen and stored in a refrigerator at –80 °C for the determination of fatty acids and for the quantitative analysis of genes.

RNA extraction and real-time quantitative PCR (RT-qPCR). Total RNA was extracted using a TRIzol Reagent Kit (TaKaRa, Japan), and 2 μg of total RNA was reversely transcribed into cDNA using a PrimeScript RT-PCR Kit (TaKaRa). Expression of the genes was measured by real-time quantitative PCR (RT-qPCR) (SYBR Green II) on a Thermal Cycler Dice TP800 sequence detection system (TaKaRa), using *β-actin* as a housekeeping gene. Primers for gene expression analysis (Table 3) were designed based on the full-length of Δ6-desaturase cDNA. Each sample was run in triplicate, and PCR reaction without the addition of the template was used as negative controls. The relative mRNA expression levels of Δ6-desaturase in each sample were normalised with *β-actin* expression and calculated by the comparative threshold cycle (Ct) method.

Table 3. Primers for determining mRNA content of *Δ6-Fad* and *Elovl5*

Primers	Sequence (5' → 3')
Δ6-Fad F	ATCGGACACCTGAAGGGAGCG
Δ6-Fad R	GAGTTGAAGGTTTGGATGAAATGCATG
Elovl5 F	GTCCTGACCATGTTCCAGACATCTTG
Elovl5 R	CATGAAGCTCCTCTACTGCGCTG
β-actin F	CGCCCCAGACATCAGGGTG
β-actin R	CACAGATCATGTTTGAGACCTTCAACAC

Fatty acid analysis. Total lipid was extracted from muscle tissue by homogenisation in chloroform/methanol (2 : 1, v/v), which contained 0.01% butylated hydroxytoluene as an antioxidant. The lipid extracts were washed according to the Folch procedure (Folch et al. 1957), and methylated in 1% sulphuric acid in methanol at 70 °C for 3 h to prepare fatty acid methyl esters (FAMEs). FAMEs were extracted in heptanes and analysed using a gas chromatograph GC-2010 (Shimadzu, Japan) equipped with an autosampler and a hydrogen flame ionization detector. The GC was fitted with a 30 m × 0.25 mm × 0.25 μm capillary column VF-23ms (Varian, USA). The carrier gas was N_2, and the combustion-supporting gases were air and H_2. The injector and detector temperatures were both 250 °C. The column temperature was initially held at 120 °C for 3 min, followed by an increase at a rate of 10 °C/min to 190 °C. The temperature was then increased at a rate of 2 °C/min to final 220 °C and maintained for 15 min. Individual fatty acids were identified by comparison against commercial standards (Sigma, USA) and quantified using the CLASS-GC10 GC workstation (Shimadzu).

Statistical analysis. The results were analysed by one-way ANOVA in SPSS Version 17.0, and Tukey's multiple comparisons were conducted when the differences were significant ($P < 0.05$).

RESULTS

Effect of dietary fatty acids on fatty acid composition. The composition of fatty acids in diet significantly affected the composition of fatty acids in carp muscle (Table 4). The content of LA in muscle of the Diet 2 group (containing higher

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Table 4. Fatty acid composition (percentage of total fatty acids) of muscle from common carp fed experimental diets for 6 weeks (mean ± SEM)

Fatty acid	Initial	Diet 1	Diet 2	Diet 3
14:0	0.55 ± 0.02^{b}	0.69 ± 0.03^{a}	0.50 ± 0.05^{b}	0.47 ± 0.06^{b}
16:0	20.61 ± 0.24	20.02 ± 0.86	19.20 ± 1.02	19.92 ± 0.84
16:1	2.41 ± 0.10^{b}	3.22 ± 0.01^{a}	2.98 ± 0.54^{ab}	2.35 ± 0.09^{b}
18:0	6.53 ± 0.16^{a}	6.03 ± 0.12^{ab}	5.70 ± 0.28^{b}	6.59 ± 0.18^{a}
18:1	23.75 ± 0.56	24.32 ± 0.81	25.02 ± 2.13	23.91 ± 1.76
20:0	0.21 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.14 ± 0.01
20:1	1.82 ± 0.07	1.54 ± 0.02	1.53 ± 0.13	1.39 ± 0.07
22:1	0.3 ± 0.01	0.30 ± 0.05	0.24 ± 0.04	0.18 ± 0.02
6:2n-6	18.68 ± 0.29^{b}	16.94 ± 0.77^{c}	21.35 ± 0.99^{a}	17.16 ± 0.79^{c}
6:3n-6	0.32 ± 0.03^{a}	0.28 ± 0.01^{a}	0.32 ± 0.02^{a}	0.19 ± 0.02^{b}
20:4n-6	3.96 ± 0.15^{b}	4.36 ± 0.00^{ab}	4.66 ± 0.03^{a}	3.58 ± 0.21^{b}
6:3n-3	1.45 ± 0.15^{b}	0.96 ± 0.01^{c}	0.98 ± 0.06^{c}	3.25 ± 0.14^{a}
8:5n-3	1.85 ± 0.21^{a}	2.02 ± 0.10^{a}	1.08 ± 0.19^{b}	2.03 ± 0.22^{a}
22:5n-3	1.09 ± 0.02^{a}	1.12 ± 0.02^{a}	0.75 ± 0.06^{b}	1.28 ± 0.18^{a}
22:6n-3	9.96 ± 0.87^{a}	11.02 ± 0.84^{a}	6.65 ± 1.06^{b}	10.70 ± 0.82^{a}
Total SFA	27.9 ± 0.76	26.92 ± 0.93	25.60 ± 1.25	27.12 ± 0.89
Total MUFA	28.28 ± 0.45	29.38 ± 0.90	29.77 ± 2.44	27.83 ± 1.70
Total n-3	14.35 ± 0.23^{b}	15.12 ± 0.93^{b}	9.46 ± 1.26^{c}	17.26 ± 0.28^{a}
Total n-6	22.96 ± 0.56^{b}	21.58 ± 0.77^{b}	26.26 ± 1.89^{a}	20.94 ± 0.60^{b}

in the same row, values with no letter or the same superscripts mean no significant difference ($P > 0.05$), while those with different superscripts mean a significant difference ($P < 0.05$)

LA) at the end of 6-week feeding was significantly higher than that in the groups of Diet 1, Diet 3 and at the beginning of the trial ($P < 0.05$). After 6 weeks of feeding, the content of LNA in muscle of the Diet 3 group was significantly higher than that in the groups of Diet 1, Diet 2, and at the beginning of the trial ($P < 0.05$). The contents of EPA and DHA in muscle of the diet group with high HUFA content were significantly higher than those of the Diet 2 group after 6 weeks ($P < 0.05$). The results showed that carp can efficiently convert LA to AA, LNA to EPA, then to docosapentaenoic acid (DPA) and DHA. The content of HUFA (AA, EPA and DHA) in the muscle of common carp is closely related to the content of LA and LNA in feed.

Effects of dietary fatty acids on gene expression of *Δ6-Fad* and *Elovl5* in common carp. After 3, 14 and 42 days of feeding, the expression of *Δ6-Fad* and *Elovl5* genes in the liver of the test fish showed significant differences between the treatment groups, as well as between different stages of the trial (Figure 1). Compared to the beginning of the experiment, the expression of *Δ6-Fad* gene in the group of Diet 2 increased, and the expression of *Δ6-Fad* gene on days 14 and 42 of the experiment was higher than that at the beginning of the trial ($P < 0.05$). However, the level of *Δ6-Fad* gene expression in the group of Diet 3 was significantly

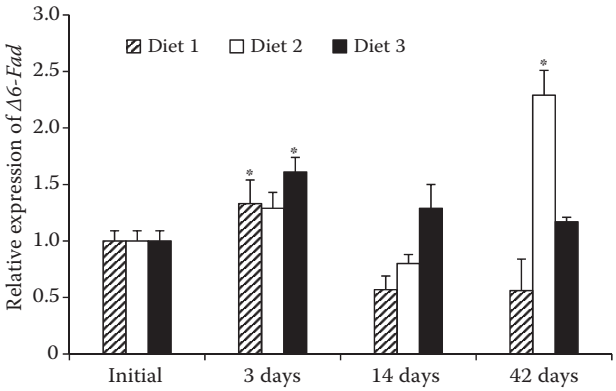

Figure 1. Expression of *Δ6-Fad* relative to *β-actin* in the liver of common carp at the start of the experiment (Initial) and after feeding Diets 1–3 for 3, 14 and 42 days

values are mean ± SEM ($n = 6$); *significant differences ($P < 0.05$) between the column's dietary treatment and the Initial

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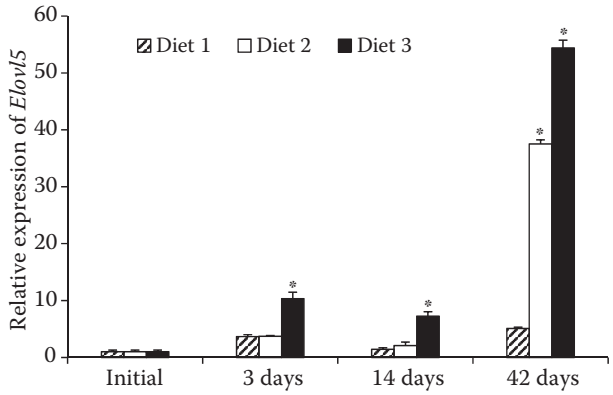

Figure 2. Expression of *Elovl5* relative to *β-actin* in the liver of common carp at the start of the experiment (Initial) and after feeding Diets 1–3 for 3, 14 and 42 days

values are mean ± SEM (n = 6); *significant differences (P < 0.05) between the column's dietary treatment and the Initial

higher than that at the beginning of the experiment (P < 0.05) only on day 3 of the experiment. Later in the experiment, the expression of *Δ6-Fad* gene in the Diet 2 group was significantly higher than in the other groups (P < 0.05).

The expression of *Elovl5* gene in the treatment groups of Diet 2 and Diet 3 showed an obvious upward trend compared to that at the beginning of the experiment, and the expression of *Elovl5* gene in the treatment groups of Diet 2 and Diet 3 increased significantly on day 42 compared to the beginning of the experiment (P < 0.05) (Figure 2).

DISCUSSION

It is generally believed that herbivorous or omnivorous freshwater fish have the capability to convert LA and LNA into AA, EPA and DHA (Sargent et al. 2002), but different fish species differ in their ability to extend their fatty acid desaturase and fatty acid chains (Tocher et al. 2006). However, marine fish are mostly carnivorous and are considered to have a lower capacity to synthesise HUFA, so HUFA must be added through the feed (Mourente et al. 1994). However, as for the freshwater benthic carp, it is a fish species whose diet is omnivorous including partially meat in the artificial culture environment, some food comes from benthic organisms, while HUFA may directly come from aquatic organisms in marine fish. However, there are few reports about the characteristics of HUFA synthesis and the mechanism of *Δ6-Fad* and *Elovl5* genes involved in HUFA synthesis. In this experiment, the contents of EPA, DPA and DHA in the muscle of the Diet 3 group were higher than those of the Diet 2 group, indicating that carp can meet their requirements for essential fatty acids (EFA) by using LA and LNA in diet. In common carp, AA, EPA and DHA can be obtained in two ways: firstly, AA, EPA and DHA are directly incorporated into feed; secondly, AA, EPA and DHA are synthesised by LA and LNA, respectively, under the action of key enzymes. In this experiment, due to different composition of LA and LNA in diet, AA, EPA and DHA levels in the body of the test fish were different at the end of 6-week feeding. As a freshwater fish species, carp has the ability to synthesise HUFA; different fat sources in diet have obvious effects on the body fatty acid composition. Therefore, it can be concluded that the composition of fatty acids in diet significantly affects the composition of fatty acids in the muscle of carp. The *Δ6-Fad* and *Elovl5* genes, the key enzymes involved in HUFA synthesis, were expressed in carp liver. The levels of Diet 2 and Diet 3 were significantly higher than those of Diet 1, and were also higher than those at the beginning of the experiment. The results showed that the *Δ6-Fad* and *Elovl5* genes were involved in the regulation of HUFA synthesis in common carp due to the difference of fatty acids in diets.

In recent years, some studies have reported on the genes regulating fatty acid desaturase and fatty chain elongation enzymes by dietary fatty acids, such as the replacement of fish oil with vegetable oil in the diets of freshwater fish, which can enhance the expression of fatty acid desaturase (Zheng et al. 2005a, b). The results have shown that the expression of *Δ6-Fad* was significantly higher in rainbow trout fed olive oil than when they received fish oil (Buzzi et al. 1996). However, some studies suggest that HUFA inhibit the expression of *Δ6-Fad* in freshwater fish (Ulmann et al. 1992). The results of the present study showed that LA and LNA in diet promoted the expression of the *Δ6-Fad* fatty acid desaturase enzyme, while HUFA inhibited its expression. In the early stage of the experiment, the ability of LNA to promote the high expression of *Δ6-Fad* was stronger than that of LA, but in the later stage of the experiment, LA was stronger than LNA (Li et al. 2008). However, in this study, too much LNA was found to inhibit the expression of *Δ6-Fad* in the experiment, and the same results were found in the studies of sea

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bream (Izquierdo et al. 2008); it was suggested that excessive LNA inhibited not only the expression of *Δ6-Fad*, but also the desaturation of LA (Bell et al. 1993). Some studies on Atlantic salmon suggest that diets containing a high proportion LA/LNA from fish oil produce more LA desaturated products than those containing LNA. However, excessive LA in vegetable oil can also inhibit the desaturation of LNA (Ruyter et al. 2000).

Currently, *Elovl5* has been reported in some fish which can extend C18 and C20 PUFA (Alimuddin et al. 2008; Zheng et al. 2009). The study of Atlantic salmon showed that replacing fish oil with linseed oil significantly increased the expression of the *Elovl5* gene in Atlantic salmon (Zheng et al. 2004). In our study, the group on Diet 2 contained higher LA, and LA could synthesise only AA, but not EPA and DHA, and dietary EPA and DHA were also very low. EPA and DHA are essential fatty acids for carp, so *Δ6-Fad*, a key fatty acid synthesis enzyme, always shows a high expression level. The Diet 2 group and the Diet 3 group produced a lot of intermediate products in the early stage of feeding due to the action of fatty acid desaturase, which required higher fatty acid elongase to extend the fatty chain in the later stage. However, there were differences between Diet 2 and Diet 3 because of the differences in synthetic substrates, and the total amount of the Diet 3 group was obviously higher than that of the Diet 2 group, including AA, EPA and DHA. These results may be related to the preference of *Elovl 5* to n-3 substrate (Gregory et al. 2010).

Based on the results of this study, we can conclude that the carp can biosynthesise HUFA, the content and type of fatty acids in the diet can affect the composition and content of fatty acids in carp muscle, as well as the level of *Δ6-Fad* and *Elovl5* gene expression involved in the biosynthesis of HUFA. Feeding diets which contain high levels of n-3 HUFA is beneficial to increase EPA and DHA in carp. This study also suggested that fish oil in carp feed can be replaced by vegetable oil in carp culture. This study provided a theoretical basis for selecting suitable fat sources for carp feed.

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