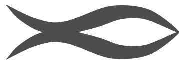

www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 15: 255-264 (2015) DOI: 10.4194/1303-2712-v15_2_07

Cloning and Expression Patterns of MRFs and Effect of Replacing Dietary Fish Oil with Vegetable Oils on MRFs Expression in Grass Carp (*Ctenopharyngodon idellus*)

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Received 03 December 2014
Accepted 07 May 2015

Abstract

In order to investigate characterization and tissue distribution of myogenic regulatory factors (MRFs) in grass carp (*Ctenopharyngodon idellus*), full-length of Myf5, MyoG, Myf6 cDNAs were cloned and expression patterns of MRFs in different tissues were studied. Meanwhile the effect of dietary oil sources (fish oil, FO ; linseed oil, LO; groundnut oil, GO; olive oil, OO) on MRFs gene expression in muscle of grass carp were studied by feeding experiment. The results showed that nucleotide sequence of Myf5, MyoG and Myf6 contained a basic DNA-binding motif and a helix-loop-helix dimerization domain (bHLH) respectively. MRFs were mainly expressed in muscle and a little were expressed in other tissues tested. In red muscle MRFs was mostly expressed in OO group and in white muscle they were mostly expressed in GO group. These results showed that MRFs gene and amino acid in grass carp shared high conservation and it mostly expressed in muscle. Dietary oil sources have a great impact on expression of MRFs genes in muscle of grass carp, probably indicating that OO or GO would be more suitable than FO to be oil sources in diet of grass carp.

Keywords: Myogenic regulatory factors (MRFs); clone; gene expression pattern; fish oil, vegetable oils; grass carp (*Ctenopharyngodon idellus*).

Introduction

Skeletal muscle growth, including both the recruitment of new muscle fibers (hyperplasia) and the growth of existing fibres (hypertrophy) and being called myogenesis, had been reported to be controlled by numerous extracellular signals together with intracellular factors, means myogenic regulatory factors (MRFs). The MRFs includs MyoD (MyoD1, Myf3), myogenin (MyoG, Myf4), Myf5 and Myf6 (MRF4 or herulin) and the sequence identity and role of MRFs had been reported among vertebrates including teleosts (Holterman and Rudnicki 2005). MRFs expression was shown to be in a sequential-manner during myogenesis in mice and zebrafish (Hinits *et al.,* 2009; Roy *et al.,* 2002; Jin *et al.,* 2007). Simultaneously the expression patterns of MRFs had been reported to be different in different manmals (Sabourin and Rudnicki 2000), cat fish (Gregory *et al.,* 2004) and in many other animals, i.e., duck, channel catfish, carp, gilthead seabream, zebrafish and flounder (*Paralichthys olivaceus*) (Liu *et al.,* 2011; Rescan 2001; Kobiyama *et al.,* 1998; Du *et al.,* 2003; Zhang *et al.,* 2010).

Grass carp (*Ctenopharyngodon idellus*) is one of the four major Chinese carps in freshwater aquaculture which had very high annual production from aquaculture. In grass carp, Gong *et al.* (2012) had reported the cloning and tissues expression patterns of MyoG gene, while the other genes encoding MRFs, i.e., MyoD, Myf5 and Myf6, have not been cloned, and the sequences encoding these MRFs have not been reported. Furthermore the information on MRFs expression pattern in adult grass carp tissues is lacking, which is mismatching with the high commercial interest in grass carp.

Lipids or oils are the necessary nutrients in animals and it had been reported that grass carp can live in many kinds of oil sources in their diets without detrimental effect on growth (Cao *et al.,* 1996), while little is known about its effect on MRFs which regulate the growth of muscle and is more subtle to show growth than increase of body weight. Accordingly the present study were conducted and the aim was to investigate the characterization and tissue distribution of MRFs by clone and Q-PCR and to achieve the effect of dietary oil sources on MRFs gene expression in red and white muscle of grass carp by feeding experiments.

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Materials and Methods

Cloning and Sequence Analysis of MRFs Genes in Muscle of Grass Carp

Total RNA of muscle from 6 marketable grass carp (500±16 g), being collected in Chengdu, Sichuan Province, China, were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instruction manual. The cDNA was synthesized by reverse transcription from 1μg of total RNA as described in the manufacturer's protocol (Fermentas Life Science, Hanover, MD, US). The primers for PCR were designed based on the mRNA sequence of Myf5 and MyoG in Cyprinus carpio and on the mRNA sequence of Myf6 in zebrafish, the GenBank number being AB012883, AB012881, NM_001003982 respectively, shown in table 3.

PCR amplification was performed in standard conditions: denaturizing at 95°C for 5 min, then 38 cycles of amplification including 95°C for 45 s, 55.3°C for 1 min, and 72°C for 1.0 min. The amplification was followed by a final extension at 72°C for 10 min.

The PCR fragments were separated by 1% agarose gel electrophoresis, cloned into pMD19-T vector (TaKaRa, Dalian, China) and transformed into *E.coli* DH5α. For each fragment, five clones were selected and sequenced (Shanghai Sangon Biological Engineering Technology Co. Ltd., Shanghai, China). The amino acid sequences, isoelectric points and molecular weights of corresponding proteins were analyzed using ExPASy-Tools (http://www.expasy.org/tools). Protein domains were characterized by lnterproscan (http://www.ebi.ac.uklinterpro/). Signal peptides were identified by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al.,* 2011). Amino acid sequence homology was constructed with the BioEdit software version 5.0.6 (Hall 2001).

Analysis of MRFs mRNA Level in 7 Tissues of Grass Carp by Quantitative Real-Time ((RT-PCR)

Total RNA was extracted from the heart, liver, muscle, adipose, kidney, intestines and brain of grass carp (n=6 per tissue) as above, respectively. The primers were designed according to Myf5, MyoG , Myf6 and MyoD mRNA sequences of grass carp (Table 3). Real-time analysis was performed by a fluorescence temperature cycler (Bio-Rad, Hercules, CA, USA) using the following procedure: pre-denatured at 95°C for 1 min, followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 54°C /63.5°C for 30s and then extending at 72°C for 30 s. The amplification mixture contained 1 μL of RT reaction mix, 10μL of SYBR® Premix Ex Taq TM (2×) (TaKaRa, Dalian, China), 0.5 μL of 10 μmol/L primers and 8.5 μL ddH_2O. The threshold cycle (CT) was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Effect of Replacing Dietary Fish Oil with Vegetable Oils on Relative Expression of MRFs in Grass Carp

Experimental Diets

Four diets (Table 1) were formulated to contained a proportion of oil (3% total diet) and the oil sources were originated from fish oil (FO) and vegetable oils (linseed oil, LO; groundnut oil ,GO; olive oil, OO) respectively. Lipids from diets were extracted by homogenization in chloroform/methanol (2:1 v/v) (Folch *et al.*, 1957). After extraction of total lipid, an aliquot was saponified and methylated using 12% BF3 in methanol. The fatty acid composition of total lipids was analyzed using methods described previously by Lie and Lambertsen (1991). Briefly, methyl esters were separated using a trace gas chromatograph 2000 (cold on column injection, 60°C for 20s, 25°C /min; 160°C for 28 min, 25°C /min;

Table 1. Primers for cloning and quantitative real-time PCR(qPCR)

Names	Sequences	Annealing temperature(℃)	Utilizations
Myf5-F1	5'- ATGGACGTATTCTCTACATCCC -3'	56	For cloning of Myf5 cDNA
Myf5- R1	5'- TCACAGGACGTGGTAGACTG -3'		
MyoG-F1	5'- GGTGGACTCTTATTCCAGC -3'	57.5	For cloning of Myog cDNA
MyoG -R1	5'-CAGTGGACATAAGCAAATAAATG -3'		
Myf6-F1	5'-ATGATGGACCTGTTTGAGACC -3'	59.5	For cloning of MRF4 cDNA
Myf6-R1	5'-TCACTTCTCTGAGATCTGGCTG -3'		
MyoD-F	5'- TGAGGGAGAGGAGACGACT -3'	54	For qPCR
MyoD-R	5'- GCTCCAGAACAGGGTAGTAGT -3'		
Myf5-F2	5'- GGAGAGCCGCCACTATGA -3'	63.5	For qPCR
Myf5-R2	5'- GCAGTCAACCATGCTTTCAG -3'		
MyoG -F2	5'- AGAGGAGGTTGAAGAAGGTC -3'	59	For qPCR
MyoG-R2	5'- GTTCCTGCTGGTTGAGAGA -3'		
Myf6-F2	5'-GAAAATCTGCTCCAACCGA -3'	60	For qPCR
Myf6-R2	5'-CGCTGCGTAAAATCTCCA -3'		
β-actin-F	5'- ATCCTCCGTCTGGACTTGG -3'	55	Endogenous control
β-actin-R	5'- TCCGTCAGGCAGCTCATAG -3'		

Table 2. Composition and nutrient levels of experimental diets (air-dry basis) %

Raw material	Fish oil (FO)	Linseed oil (LO)	Groundnut oil (GO)	Olive oil (OO)
Ingredients				
Fish meal	3	3	3	3
Soybean Meal	33.92	33.92	33.92	33.92
Rapeseed meal	19	19	19	19
Cottonseed meal	12	12	12	12
Wheat middling and red dog	15.96	15.96	15.96	15.96
Wheat meal	10	10	10	10
Linseed oil		3		
Groundnut oil			3	
Olive oil				3
Fish oil	3			
NaCl	0.3	0.3	0.3	0.3
Calcium dihydrogen phosphate	1.5	1.5	1.5	1.5
Mineral[1)] and vitamin[2)] mix	1.3	1.3	1.3	1.3
Envelope VC	0.02	0.02	0.02	0.02
Total	100	100	100	100
Proximate composition[3)]				
Crude protein	34.54±0.02	34.93±0.51	35.35±0.09	35.06±0.01
Crude fat	6.00±0.21	6.09±0.35	6.08±0.43	6.00±0.32
Moisture	8.11±0.57	8.50±0.64	7.94±0.42	7.91±0.57
Ash	6.34±0.04	6.36±0.12	6.60±0.07	6.49±0.19

1) Minerals (g or mg /kg diet): Fe (iron sulphate) 140mg; Cu(copper sulphate) 2.5mg; Zn (zinc oxide) 65mg, Mn (manganese oxyde) 19mg; Mg (magnesium sulphate) 230mg; Co (cobalt sulphate) 0.1mg; I (potassium iodide) 0.25mg; Se (sodium selenite) 0.2mg.
2)Vitamins (mg or i.u. /kg diet): vitaminA 4000 i.u., vitamin D_3 800 i.u., vitamin E 50 i.u., vitamin B_1 2.5mg, vitamin B_2 9mg, vitamin B_6 10mg, vitamin C 250mg, nicotinic acid 40mg, pantothenic acid 30mg, biotin 100μg, choline 1000mg.
3) Results are means±S.D.(n=2 for proximates)

190°C for 17min, 25°C/min; 220°C for 9min), equipped with a 50-m CP-sil 88 (Chromopack) fused silica capillary column (i.d., 0.32 mm). The fatty acids were identified by retention time using standard mixtures of methyl esters (Nu-Chek-Prep) and quantified using Totalchrom software (version 6.2, Perkin Elmer). The amount of fatty acid per gram tissue was calculated using 19:0 methylester as an internal standard. The fatty acid composition of the diets was shown in Table 2.

Feeding Trial and Sampling Procedures

Eighty juvenile grass carp with an initial mean body mass of 56.9 g were randomly and equally divided into four groups and were reared in indoor tanks (112 cm×34 cm×54 cm) in our experimental facilities (experimental fish farm in Fishery Science Department of Northwest A&F University, Shaanxi, China) under a natural photoperiod (July to August) and fed by hand three times a day for 60 days to visual satiation. The water of 2/3 the tank volume was changed every morning and the water temperature was about 20°C -25°C. The water was continuously aerated 24 h per day and the dissolved oxygen was kept in the level of 5-8 mg/L. Grass carp were counted and weighed every 20 days after 24 h fasting to adjust feed amount. Six grass carp (mean body weight 96.7±20.7 g) were sampled per diet and sacrificed by a blow to the head 18 h after the last meal and the red and white muscle were quickly sampled and frozen with liquid nitrogen and stored at -80°C prior to RNA extraction and cDNA synthesis.

Red and White Muscle MRFs Expression by Quantitative Real-Time (RT-PCR) Analysis

Total RNA and cDNA of red muscle and white muscle in grass carp fed with different oil sources based diets were prepared by the methods described above. Primers of myf5, MyoG, Myf6 and MyoD gene were presented in Table 3 and real-time analysis of these genes was performed by a fluorescence temperature cycler followed the procedure in 2.2.

Statistic Analysis

Data were showed as mean±S.E. Statistical differences were firstly determined by one way ANOVA then by Tukey's post-hoc test. All statistical analyses were performed using SPSS13.0 for Windows Software (SPSS, Chicago, IL, USA). Result were considered significant at $P<0.05$.

Results

Clone and Characterization of Myf5, MyoG and Myf6 Genes in Grass Carp

The clone of grass carp Myf5 gene covered the entire ORF and the nucleotide sequences were displayed in GenBank (GU290227). Myf5 nucleotide sequence in grass carp encoded 240 amino acids polypeptide and contained a basic DNA-binding motif and a helix-loop-helix dimerization domain (bHLH) located at amino acid positions 4-123 (Figure 1). Myf5 protein was predicted to have a molecular weight of 26.23 kDa and its iso-electric point was

Table 3. Fatty acid composition in different oil based diets (% of total fatty acid)

	Fish oil (FO)	Linseed oil (LO)	Groundnut oil (GO)	Olive oil (OO)
Fatty acid composition				
14:0	5.06	0.44	0.46	0.40
16:0	18.78	9.54	13.27	12.22
16:1 n-7	5.52	0.71	0.75	1.09
18:0	3.13	3.40	3.59	3.23
18:1 n-9[1)]	18.14	23.84	33.27	55.62 9)
18:2 n-6	19.09	34.36	38.53	19.87
18:3 n-3	4.27	22.75	2.58	3.06
20:1 n-9	4.18	1.07	1.29	0.94
20:3 n-6	0.57	--8)	--	--
20:4 n-6	--	--	--	--
20:5 n-3	6.87	0.94	2.31	0.79
22:6 n-3	8.48	0.46	0.55	0.48
SFA[2)]	26.96	13.37	17.32	15.85
MUFA[3)]	27.85	25.62	35.31	57.65
PUFA[4)]	39.28	58.52	43.97	24.19
HUFA[5)]	15.35	1.41	2.86	1.26
n-6[6)]	19.66	34.36	38.53	19.87
n-3[7)]	19.62	24.15	5.44	4.32
n-3/n-6	1.00	0.70	0.14	0.22

1) It is mainly composed of C18:1 n-9, including a little of C18:1 n-7 ; 2)SFA means saturated fatty acids and it includes C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0 ; 3) MUFA means monounsaturated fatty acids and totals include C16:1 n-9, C18:1 n-9, C20:1 n-9 ; 4) PUFA means polyunsaturated fatty acids and totals include C18:2 n-6, C18:3 n-3, C18:3 n-6, C20:4 n-6, C20:5 n-3, C22:6 n-3 ; 5) HUFA totals include highly unsaturated fatty acids, C20:4 n-6,C20:5 n-3, C22:6 n-3 ; 6) n-6 totals include C18:2 n-6, C20:3 n-6, C20:4 n-6 ; 7) n-3 totals include C18:3 n-3, C20:5 n-3, C22:6 n-3 ; 8) means it was not detected ;9) showed the highest amount of fatty acid in each dietary group.

```
Cte   ---MD--VFS TSQIFYDSAC ASSPEA---L DFGPG----- -----GELAG SEEDEHVRAP 42
Cyp   ---MD--VFS TSQIFYDSTC ASSPEA---L EFGPG----- -----GELAG SEEDEHVRVP 42
Sch   ---MD--VFS TSQIFYDSTC SSSPEA---L EFGPG----- -----GELDG SEEDEHIRAP 42
Dan   ---MD--VFS TSQIFYDSTC ASSPED---L EFGAS----- -----GELTG SEEDEHVRAP 42
Onc   ---MD--VFS QSQVFYDSAC ASSPED---L DFGP------ -----RELDG SEEDEHVRVP 41
Sal   ---MD--VFS QSQIFYDSAC ASSPED---L DFGP------ -----GELDG SEEDEHVRVP 41
Hom   MDVMDGCQFS PSEYFYDGSC IPSPEGEFGD EFVPR-VAAF GAHK-AELQG SDEDEHVRAP 58
Mus   MDMTDGCQFS PSEYFYEGSC IPSPEDEFGD QFEPR-VAAF GAHK-AELQG SDDEEHVRAP 58
Gal   MEVMDSCQFS PSELFYDSSC LSSPEGEFPE DFEPRELPPF GAPAPTEPAC PEEEEHVRAP 60

Cte   GAPHQPGHCL QWACKACKRK ASTVDRRRAA TMRERRRLKK VNHAFEALRR CTSANPSQRL 102
Cyp   GAPHQPGHCL QWACKACKRK ASTVDRRRAA TMRERRRLKK VNHAFEALRR CTSANPSQRL 102
Sch   GAPHQPGHCL QWACKACKRK ASTVDRRRAA TMWERRRLKK VNHAFEALRR CTSANPSQRL 102
Dan   GAPHQPGHCL QWACKACKRK ASTVDRRRAA TMRERRRLKK VNHAFEALRR CTSANPSQRL 102
Onc   GTPHQAGHCL QWACKACKRK SSTVDRRRAA TMRERRRLKK VNHGFEALRR CTSANPSQRL 101
Sal   GTPHQAGHCL QWACKACKRK SSTVDRRRAA TMRERRRLRK VNHGFEALRR CTSANHSQRL 101
Hom   TGHHQAGHCL MWACKACKRK STTMDRRKAA TMRERRRLKK VNQAFETLKR CTTTNPNQRL 118
Mus   TGHHQAGHCL MWACKACKRK STTMDRRKAA TMRERRRLKK VNQAFETLKR CTTTNPNQRL 118
Gal   SGHHQAGHCL MWACKACKRK STTMDRRKAA TMRERRRLKK VNQAFETLKR CTTANPNQRL 120
            Basic domain

Cte   PKVEILRNAI QYIESLQELL REQVENYYSL PMESSSEPAS PSSSCSESMV DCNSP-VWPQ 161
Cyp   PKVEILRNAI QYIESLQELL REQVENYYSL PMESSSEPAS PSSSCSESMV DCNSP-VWPQ 161
Sch   PKVEILRNAI QYIESLQELL REQVENYYSL PMESSSEPAS PSSSCSESMI DCNSP-VWPQ 161
Dan   PKVEILRNAI QYIESLQELL REQVENYYSL PMESSSEPAS PSSSCSESMV DCNSP-VWPQ 161
Onc   PKVEILRNAI QYIESLQELL HEHVENYYGL PGESSSEPGS PSSSCSDSMV DCNSPVVWPQ 161
Sal   PKVEILRNAI QYIESLQELL HEHVENYYGL PGESSSEPGS PSSSRSDSMV DCNIPVVWPQ 161
Hom   PKVEILRNAI RYIESLQELL REQVENYYSL PGQSCSEPTS PTSNCSDGMP ECNSP-VWSR 177
Mus   PKVEILRNAI RYIESLQELL REQVENYYSL PGQSCSEPTS PTSNCSDGMP ECNSP-VWSR 177
Gal   PKVEILRNAI RYIESLQELL REQVENYYHL PGQSCSEPTS PSSSCSDVMA DSRSP-VWPA 179
           Helix-loop-Helix domain

Cte   MNPNFGNNYN FDAQSASAVD RTPGVSSLQC LSSIVDRLSS VDTGVAMGMR NMVALSP-TG 220
Cyp   MNPNFGNNYN FEAQNASAVD RTPGASSLQC LSSIVDRLSS VDTGVAMGMR NMVALSP-TG 220
Sch   MNPNFGNNYN FEAQNASAVD RIPGASSLQC LSSIVDRLSS VDTGVAMGMR NMVTLSP-TG 220
Dan   MNQNYGNNYN FDVQNASTME RTPGVSSLQC LSSIVDRLSS VDP---AGMR NMVVLSP-TG 217
Onc   MNTSYGNNYS Y-TKNVSSGE RGAGASSLAC LSSIVDRLSS VDASAPAGLR DMLTFSP-SS 219
Sal   MNTSYGNNYS Y-TKNVSSGE RGAGASSLAR LSNIVDRLSS VDASAPAGLR DMLTFSP-SS 219
Hom   KSSTFDSIYC PDVSNVYATD KN-SLSSLDC LSNIVDRITS SEQP-GLPLQ DLASLSPVAS 235
Mus   KNSSFDSIYC PDVSNACAAD KS-SVSSLDC LSSIVDRITS TEPS-ELALQ DTASLSPATS 235
Gal   RGSSFEAGYC REMPHGYATE QSGALSSLDC LSSIVDRLSP AEEP-GLPLR HAGSLSPGAS 238

Cyp   SDSQCSSADS PSNRPVYHVL 240
Sch   SDSQCSSPDS PSNRPVYHVL 240
Dan   SDSQSSSPDS PNNRPVYHVL 237
Onc   TDSQPCTPES PGTRPVYHVL 239
Sal   TDSQPCTTES PGTRPVYHVL 239
Hom   TDSQPATPGA SSSRLIYHVL 255
Mus   ANSQPATPGP SSSRLIYHVL 255
Gal   IDSGPGTPGS PPPRRTYQAL 258
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Figure 1. Comparison of the amino acid sequences and domains of the Myf5 protein among vertebrates. The sequence of highly conserved basic helix-loop-helix domain is underlined. The putative amino acid sequence of grass carp Myf5 (GenBank accession no. ADB56965) is compared to the amino acid sequences of Cyp (*Cyprinus carpio*, BAA33566); Sch (*Schizothorax prenanti*,AFL56776); Dan (*Danio rerio*,AF270789), Onc (*Oncorhynchus mykiss*, NP_001118001); Sal (*Salmo salar*,NP_001117116); Hom (*Homo sapiens*, NP_005584); Mus (*Mus musculus* ,CAA39643); Gal (*Gallus gallus*, NP_001025534). The alignment was generated using vector pMD19-T 7.0 software.

common carp, mud carp, zebra fish, etc, and it shared relatively lower identity with other vertebrate species, such as human, mouse and chicken (Figure 1).

Grass carp MyoG gene covered the entire ORF and its nucleotide sequences were displayed in GenBank (JQ793897). The cDNA of MyoG in grass carp contained a 762 bp ORF. The deduced amino acid sequence revealed a 253 amino acid polypeptide with a predicted molecular weight of 28.03 kDa and an isoelectric point (pI) of 6.09. The bHLH domain located at amino acid positions of 1-158. No signal peptide was found through Signal P 4.0 analyse on line. The MyoG amino acid in grass carp exhibited higher degrees of sequence identities with other fishes (73%~95%) and lower degrees of sequence identities with mammals and poultry (53%~57%). The amino acids homology in basic domains was significantly different among different species while HLH domain was not significantly different, showing that amino acids in HLH domain was highly conserved (Figure 2).

Myf6 gene in grass carp covered the entire ORF and the nucleotide sequences were displayed in GenBank (JQ793896). A 720 bp nucleotide sequence of Myf6 in grass carp was isolated by RT-PCR and it contained an ORF encoding a predicted protein of 239 amino acids with a bHLH domain located at amino acids 2-148. The molecular weight and the theoretical pI deduced were 26.93 KDa and 5.93 respectively. No signal peptide was found in the predicted protein. Alignment of Myf6 amino acid sequences homolog revealed that Myf6 in grass carp shared higher identity with other fish and lower identity with other vertebrates, such as mammals or poultry (Figure 3).

```
Cte   MEPFETNPYF FADQRFYEGG DNFFQTRLTG GFDQAGYQDR S-SMMGLCG- -DGRL-LSNG  56
Cyp   MELFETNPYF LADQRFYEGG DNFFQSRLTG GFDQTGYQDR S-SMMGLCG- -DGRL-LSNG  56
Dan   MELFETNPYF FNDQRFYEGA DNFFQSRING GFEQAGYQDR N-SMMGLCG- -DGRM-LTTT  56
Ict   MELFETNPYF FPEQRFYESG ENFFPSRLTG GFDQGGYQDR S-SMVGLCG- -DGRL-LSSN  56
Ame   MELFETNPYF FPEQRFYESG ENFFPSRLTG GFDQGGYQDR S-SMVGLCA- -DGRL-LSSN  56
Onc   MELFETNPYF FPDQRFYEGG DNFYQSRLPG GYDQGGYQER GGSMMGLCGG LSGGVGVGLG  60
Sal   MELFETNPYF FPDQRFYEGG DNFYQSRLPG GYDQGGYQER GGSMMGLCGG LSGRVGVGLG  60
Hom   MELYETSPYF YQEPRFYD-G ENYLPVHLQG -FEPPGY-ER T--ELTLSP- -------EAP  47
Mus   MELYETSPYF YQEPHFYD-G ENYLPVHLQG -FEPPGY-ER T--ELSLSP- -------EAR  47
Gal   MELFETNPYF FPEQRFYD-G ENFLGSRLQG -YEAAAFPER P--EVTLCP- -------ESR  48

Cte   VGLEDKPSPS SSLGLSMSPH QEQQHCPGQC LPWACKVCKR KSVTMDRRKA ATLREKRRLK  116
Cyp   VGLEDKPSPS SSLGLSLSPH QEQQHCPGQC LPWACKVCKR KSVTMDRRKA ATLREKRRLK  116
Dan   VGLEDKPSPS SSLGLSMSPH QEQQHCPGQC LPWACKVCKR KSVTMDRRKA ATLREKRRLK  116
Ict   VGLEDKPSPS STLSLSLSPN QEQEHCPGQC LPWACKVCKR KSVSMDRRRA ATLREKRRLK  116
Ame   VGLEDKPSPS STLSLSLSPN QEQEHCPGQC LPWACKVCKR KSVSMDRRRA ATLREKRRLK  116
Onc   GGMEDKATPS G-----LSPH PEP-HCPGQC LPWACKLCKR KTVTMDRRKA ATMREKRRLK  115
Sal   GGMEDKATPS G-----LSPH PEP-HCPGQC LPWACKLCKR KTVTMDRRKA ATMREKRRLK  115
Hom   GPLEDKG--- ---------L GTPEHCPGQC LPWACKVCKR KSVSVDRRRA ATLREKRRLK  95
Mus   GPLEEKG--- ---------L GTPEHCPGQC LPWACKVCKR KSVSVDRRRA ATLREKRRLK  95
Gal   GALEEKD--- ---------S TLPEHCPGQC LPWACKICKR KTVSIDRRRA ATLREKRRLK  96
                    Basic domain

Cte   KVNEAFEALK RSTLMNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEHE QG--NLHYRA  174
Cyp   KVNEAFEALK RSTLMNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEHE QG--NLHYRS  174
Dan   KVNEAFEALK RSTLMNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEHE QG--NLHYRA  174
Ict   KVNEAFEALK RSTLTNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEHE QT--GLHYRS  174
Ame   KVNEAFEALK RSTLTNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEHE QT--GLHYRS  174
Onc   KVNEAFEALK RSTLMNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEND QGTQGLQYRT  175
Sal   KVNEAFEALK RSTLMNPNQR LPKVEILRSA IQYIERLQAL VSSLNQQEND QGTQGLHYRT  175
Hom   KVNEAFEALK RSTLLNPNQR LPKVEILRSA IQYIERLQAL LSSLNQEERD -----LRYRG  150
Mus   KVNEAFEALK RSTLLNPNQR LPKVEILRSA IQYIERLQAL LSSLNQEERD -----LRYRG  150
Gal   KVNEAFEALK RSTLLNPNQR LPKVEILRSA IQYIERLQSL LSSLNQQERE QR--ELRYR-  153
              Helix-loop-Helix domain

Cte   AAPQ---GVS SSSEQGSGST CSSSPEWSSA SEHCAPVYSS THEDLLNDDS SEQTNLRSLT  231
Cyp   TAPQ---AVS SSSDQGSGST CCSSPEWSSA SEQCAPAYSS THEDLLNDDS SEQTNLRSLT  231
Dan   TAAAPHTGVS SSSDQGSGST CCSSPEWSSA SDHCVPAYSS AHEDLLNDDS SEQSNLRSLT  234
Ict   SAAQ---RVS SSNEQGSGST CCSSPEWSTA SDHCTTAYGS THEDLLNEDS SEQANLRSLT  231
Ame   SAAQ---RVS SSNDQGSGST CCSSPEWSTA SDHCTTAYGS THEDLLNEDS SEQANLRSLT  231
Onc   GPAQP--RVS SSSEQGSGST CCSSPEWSNT SDHCAQSYS- -NEDLLSADS PEQTNLRSLT  230
Sal   GPAQP--RVS SSSEQGSGST CCSSPEWSNT SDHCTQSYS- -NEDLLSADS PEQTNLRSLT  230
Hom   GGGPQ---PG VPSECSSHSA SCS-PEWGSA LEFSANPGD- ---HLLTADP TDAHNLHSLT  202
Mus   GGGPQ---PM VPSECNSHSA SCS-PEWGNA LEFGPNPGD- ---HLLAADP TDAHNLHSLT  202
Gal   PAAPQ---PA APSECGSGSS SCS-PEWSTQ LEFGTNPAD- ---HLLSDDQ AEDRNLHSLS  205

Cte   SIVDSITGTE VTPVPY--TV DISK 253
Cyp   SIVDSITGTE VTPVPY--SV DISK 253
Dan   SIVDSITGTE ATPVAY--SV DISK 256
Ict   SIVDSITGTE GAPVAY--SV DITK 253
Ame   SIVDSITGTD GAPVAY--SV DITK 253
Onc   SIVDSITAAE GAPLAYPVPV DIPK 254
Sal   SIVDSITAAE GAPVAYPVPV DIPK 254
Hom   SIVDSIT-VE DVSVAFP-DE TMPN 224
Mus   SIVDSIT-VE DMSVAFP-DE TMPN 224
Gal   SIVESIA-VE DVAVTFP-EE RVQN 227
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Figure 2. Comparison of the amino acid sequences and domains of the MyoG protein among vertebrates. The putative amino acid sequence of grass carp MyoG (GenBank accession no. AFL56778) is compared to the amino acid sequences of Cyp (*Cyprinus carpio*, BAA33564); Dan (*Danio rerio*, NP_571081); Ict (*Ictalurus furcatus* ,AAS48404); Ame (*Ameiurus catus*,AAS67040), Onc (*Oncorhynchus mykiss*, NP_001118199); Sal (*Salmo salar*, NP_001117072); Hom (*Homo sapiens*, AAP35897); Mus (*Mus musculus*,NP_112466); Gal (*Gallus gallus*, NP_989515).

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Cte   -MMDLFETNT YFFNDLRYL- EGDHG---TL DMPGVSPLYE GNDSPLSPGQ DPVPSETGCE  55
Cyp   -MMDLFETNT YFFNDLRYL- EADHG---TL DMPGVSPLYE GNDSPLSPGQ DPVPSETGCE  55
Dan   -MMDLFETNA YFFNDLRYL- EGDHG---TL DMPGVSPLYE GNDSPLSPGQ DPVPSETGCE  55
Tra   -MMDLFETNP YLFNDLRYLE EGDHGPLQHL DMSGVSPLYN GNDSPLSPGQ DNVPSETGGE  59
Tak   -MMDLFETNT YLFNDLRYLE EGDHGPLQHL DMSGVSPLYN GNDSPLSPGQ DNVPSETGGE  59
Sal   -MMDLFETHT YFFNDLRYL- EGDHGPLQHL DMAGVSPLYH GNDSPLSPGG D--PSETGCD  56
Hom   MMMDLFETGS YFF----YL- DGENVTLQPL EVAEGSPLYP GSDGTLSPCQ DQMPPEAGSD  55
Mus   MMMDLFETGS YFF----YL- DGENVTLQPL EVAEGSPLYP GSDGTLSPCQ DQMPQEAGSD  55
Gal   MMMDLFETGS YFF----YL- DGENGALQQL EMAEGSPLYP GSDGTLSPCQ DQLPPEAGSD  55

Cte   SSGEEHVLAP PGLQP-HCEG QCLMWACKIC KRKSAPTDRR KAATLRERRR LKKINEAFDA  114
Cyp   SSGEEHVLAP PGLQP-HCDG QCLMWACKIC KRKSAPTDRR KAATLRERRR LKKINEAFDA  114
Dan   SSGEEHVLAP PGLQA-HCEG QCLMWACKIC KRKSAPTDRR KAATLRERRR LKKINEAFDA  114
Tra   SSGEEHVLAP PGLRA-HCDG QCLMWACKVC KRKSAPTDRR KAATLRERRR LKKINEAFEV  118
Tak   SSGDEHVLAP PGLRS-HCEG QCLMWACKIC KRKSAPTDRR KAATLRERRR LKKINEAFDA  118
Sal   SSGEEHVLAP PGLQP-HCEG QCLIWACKVC KRKSAPTDRR KAATLRERRR LKRINEAFDA  115
Hom   SSGEEHVLAP PGLQPPHCPG QCLIWACKTC KRKSAPTDRR KAATLRERRR LKKINEAFEA  115
Mus   SSGEEHVLAP PGLQPPHCPG QCLIWACKTC KRKSAPTDRR KAATLRERRR LKKINEAFEA  115
Gal   SSGEEHVLAP PGLQPPHCPG QCLIWACKTC KRKSAPTDRR KAATLRERRR LKKINEAFEA  115
            Basic domain

Cte   LKKKTVPNPN QRLPKVEILR SAINYIEKLQ DLLHTLDEQE QNSDSDPYTY NVKENH-VAP  173
Cyp   LKKKTVPNPN QRLPKVEILR SAINYIEKLQ DLLHTLDEQE HNSESEPYTY NVKENH-VVP  173
Dan   LKKKTVPNPN QRLPKVEILR SAINYIEKLQ DLLHSLDEQE QSNDTDPYTY NLKENH-VTP  173
Tra   LKRKTVANPN QRLPKVEILR SAISYIEGLQ DLLQTLDEQE KPQNGS--TH KYKEHS-VAG  175
Tak   LKRKTVANPN QRLPKVEILR SAISYIERLQ DLLQTLDEQE RSQSGASDTR NDKEQNRPSG  178
Sal   LKKKTVPNPN QRLPKVEILR SAINYIEQLQ DLLHTLDEQE NPPQNG---Y NVKEHH-ASN  171
Hom   LKRRTVANPN QRLPKVEILR SAISYIERLQ DLLHRLDQQE KMQELGVDPF SYRSKQENLE  175
Mus   LKRRTVANPN QRLPKVEILR SAISYIERLQ DLLHRLDQQE KMQELGVDPY SYKPKQEILE  175
Gal   LKRRTVANPN QRLPKVEILR SAISYIERLQ DLLHRLDQQD KMQEVAADPF SFSPKQGNVP  175
          Helix-loop-Helix domain

Cte   NEYHWKKTCQ SWQGNPDHSN SQMAGHREGA -AVESSASSS LRRLSSIVDS ISTEETKARC  232
Cyp   IEYHWKKTCQ NWQGIPDHSN SQMAGHREEA -ALESSSSSS LRHLSSIVDS ISTEETKARC  232
Dan   SEYHWKKTCQ SWQENPDHSS SQMAGHREGA -VLESSESSS LRRLSSIVDS ISTEEPKARC  232
Tra   HEYHWKKSSE TWPTSADHST AAMIHQREG- -ASESSGASS LLRLSSIVDS ITNDD-KIHF  232
Tak   GDYRWKKASN TWPTSADHS- -AIINQRDG- -NCESSATSS LLCLSSIVSS IS-DD-KTNL  232
Sal   KEYHWKKNCQ NWQTSADHSN APMTNQREG- -FTESSASTS LLRLSSIVDS ISSEE-KPTC  228
Hom   GADFLRTCSS QWPSVSDHSR GLVITAKEGG ASIDSSASSS LRCLSSIVDS ISSEERKLPC  235
Mus   GADFLRTCSP QWPSVSDHSR GLVITAKEGG ANVDASASSS LQRLSSIVDS ISSEERKLPS  235
Gal   GSDFLSTCGS DWHSASDHSR ALGGSPKAGG SMVESSASSS LRCLSSIVDS ISSDEPKLPG  235

Cte   PSQISEK  239
Cyp   PSQISEK  239
Dan   PSQISEK  239
Tra   SEGVSED  239
Tak   RQGVQED  239
Sal   NEEVSEK  235
Hom   VEEVVEK  242
Mus   VEEVVEK  242
Gal   AEEAVEK  242
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Figure 3. Comparison of the amino acid sequences and domains of the Myf6 protein among vertebrates. The putative amino acid sequence of grass carp Myf6 (GenBank accession no. AFL56777) is compared to the amino acid sequences of Cyp (*Cyprinus carpio*, ADC38865); Dan (*Danio rerio*, NP_001003982); Tra (*Trachidermus fasciatus*, AFP28938); Tak (*Takifugu rubripes*, NP_001027943); Sal (*Salmo salar*, NP_001117079); Hom (*Homo sapiens*, CAG46563); Mus (*Mus musculus*, NP_032683); Gal (*Gallus gallus*, NP_001025917).

Gene Expression Patterns of MRFs in Different Tissues of Grass Carp

The gene expression of MyoD, Myf5, MyoG and Myf6 in grass carp was found in heart, liver, muscle, adipose, kidney, intestines and brain and exclusively they were mostly expressed in muscle (Figure 4).

Growth Performance of Grass Carp Fed with Four Oil Based Diets

The FO, LO, GO and OO diets were most rich in (HUFA, 20:5n-3+22:6n-3), 18:3n-3, 18:2n-6 and 18:1n-9 respectively (Table 2). The final mean weight among these dietary groups were 92.4±25.7 g, 96.7±28.3 g, 98.9±18.8 g and 96.5±25.2 g in FO, LO, GO and OO respectively and there were no significant difference among them (P>0.05) (data not shown).

MRFs Relative Expression in Muscle of Grass Carp Fed with Four Different Oil Based Diets

Figure 5 showed that relative expression of Myf5, MyoG, Myf6 and MyoD in red muscle of grass carp was mainly expressed in OO group and relative expression of MRFs in white muscle were significantly higher expressed in GO group (P<0.05). MRFs expression in FO group was relatively low both in white muscle and in red muscle.

Discussion

Characterization of Myf5, MyoG and Myf6 in grass carp muscle showed that all of these genes in

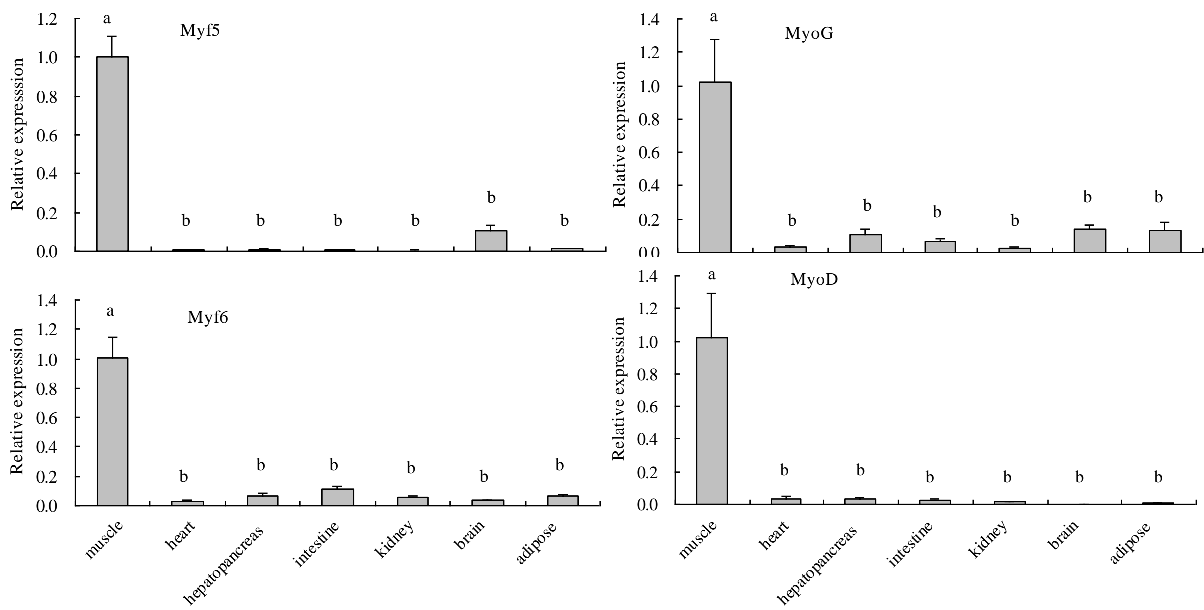

Figure 4. Expression of Myf5, MyoG, Myf6 and MyoD in 9 kinds of tissues of grass carp by quantitative real-time RT-PCR analysis.

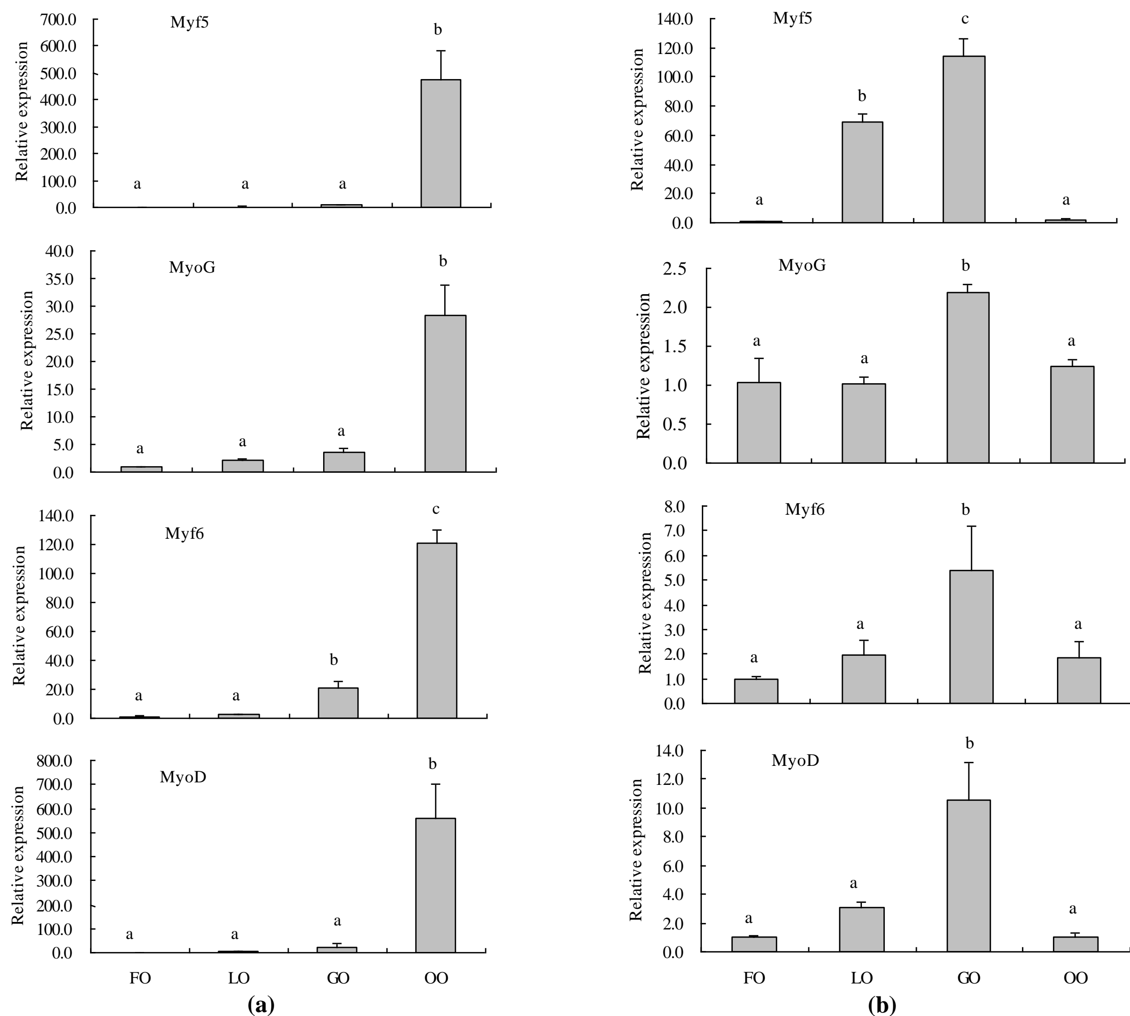

Figure 5. Effect of dietary oil sources on MRFs expression in grass carp. MRFs expression was evaluated in red muscle **(a)** and inwhite muscle **(b)** by quantitative real-time RT-PCR (n=6). A: Myf5, B: MyoG, C: Myf6, D: MyoD. Values not sharing a common letter are significantly different (P<0.05).

indicating that these genes were similar and conserved. Genomic structure, regulatory mechanism and biological function of myf5 had been reported to be well conserved between fish and mammals (Chen *et al.,* 2008).

By multiple sequence alignments the present deduced amino acid sequence of Myf5, MyoG and Myf6 in grass carp muscle compared with other fishes exhibited higher degree of similarity than that degree of similarity compared with other animals or mammals. As previous report showed that molecular structures of myf6 and myf5 proteins are conserved in vertebrates, which deduced the present high degree of similarity in MRFs amino acid sequences in fish.

MyoD, Myf5, MyoG and Myf6 are mainly expressed in muscle of grass carp (Figure 4) in the present study, suggesting that they shares functional homology to MRFs in other species. The present result also showed that MRFs expression had been slightly detected in other tissues of grass carp, i.e., heart, hepatopancreas, intestine, kidney. Although previous reports that MRFs were only expressed in skeletal muscle (Sabourin and Rudnicki 2000; Rescan 2001; Gregory *et al.,* 2004; Kobiyama *et al.,* 1998; Du *et al.,* 2003), it was in accordance with the following findings that MRFs expression had been detected in 14-day duck embryo myocardium (Liu *et al.,* 2011) and in other non skeletal muscel of flounder (*Paralichthys olivaceus*), i.e., heart muscle, spleen, kidney, gill, eye (Zhang *et al.,* 2010). MRFs expression levels in these non skeletal tissues was about 1%-5% of the expression in muscle (Zhang *et al.,* 2010). In line with the present result, Johansen et al., (2005) also found that in rainbow trout Myf5 gene was mainly expressed in red and white muscle and it was very lowly expressed in other tissues, including eye, tongue, liver, heart, gill, kidney and brain etc. Previous results showed that although the expression of Myf5 gene in flounder (*Paralichthys olivaceus*) had not been detected in heart, liver, spleen and kidney by RT-PCR, it had been detected both in skeletal muscle and intestine (Tan *et al.,* 2006). Our previous results (Gong *et al.,* 2012) showed that MyoG gene expression in grass carp could be detected by RT-PCR in red muscle, white muscle, fat, hepatopancreas, kidney, brain, intestines ect.

Lipids rather than protein and carbohydrates have been used as a major non-protein energy source. Previous researches showed that different oil sources or the addition of 1% linoleic acid to the diets had effected the growth of grass carp (Liu *et al.,* 1995; Cao *et al.,* 1996). The present results showed that final mean weight and mean body length was not affected by different oil based diets, while MRFs gene were mostly expressed in red muscle of OO group and in white muscle of GO group (Figure 5 a, b) and MRFs in FO group was relatively low expressed in either white or red muscle.

Previous researches showed that poly-unsaturated fatty acid (PUFA), especially HUFA are agonists for peroxisome proliferator–activated receptors (PPARs) and for sterol-regulatory element binding protein (SREBPs) to regulate fatty acid metabolism (Clarke 2001). PPARγ is reported to be expressed in myoblast cell line and it can suppress muscle specific transcription factors, Myf5, MyoD, myogenin, and MRF4 (myf6) (Hu *et al.,* 1995). SREBPs over-expression in muscle had been reported to leads to atrophy of both differentiated myotubes *in vitro*, and tibialis muscle in vivo, and the expression of MRFs was drastically reduced in these conditions (Lecomte *et al.,* 2010), where SREBP-1 is reported to regulate muscle protein synthesis through the down regulation of the expression of MYOD1, MYOG in human primary myotubes (Dessalle *et al.,* 2012). Therefore it was speculated that the present FO diet, being rich in HUFA, suppressed MRFs gene expression in muscle of grass carp by stimulating the function of PPARγ or SREBPs. More works are necessary to declare the regulation of HUFA on MRFs gene expression.

Conclusion

MRFs gene and amino acid in grass carp shared high conservation with other species and it mostly expressed in muscle. Dietary oil sources have a great impact on the expression of MRFs genes in muscle of grass carp, probably indicating that oil sources of OO and GO was more suitable than FO to be dietary oil sources in diet of grass carp.

Acknowledgements

This work was supported by National Natural Sciences Foundation of China (No.31201990), Applied Basic Research Program of Sichuan Province (No.2014JY0088), Animal Science Discipline Program of Southwest University for Nationalities (2014XWD-S0905) and the Fundamental Research Funds for the Central Universities (Northwest A&F University, QN2011104).

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