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Fat deposition pattern and mechanism in response to dietary lipid levels in grass carp, *Ctenopharyngodon idellus*

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Abstract This study aimed to evaluate the fat deposition pattern and lipid metabolic strategies of grass carp in response to dietary lipid levels. Five isonitrogenous diets (260 g kg^{-1} crude protein) containing five dietary lipid levels (0, 20, 40, 60, 80 g kg^{-1}) were fed to quadruplicate groups of 15 fish with initial weight 200 g, for 8 weeks. The best growth performance and feed utilization was observed in fish fed with lipid level at 40 g kg^{-1}. MFI and adipose tissue lipid content increased with increasing dietary lipid level up to 40 g kg^{-1}, and higher lipid level in diet made no sense. Fish adapted to high lipid intake through integrated regulating mechanisms in several related tissues to maintain lipid homeostasis. In the present study, grass carp firstly increased *PPARγ* and *CPT1* expressions in adipose tissue to elevate adipocyte differentiation and lipolysis to adapt to high lipid intake above 40 g kg^{-1}. In liver, fish elevated hepatic lipid uptake but depressed biosynthesis of hepatic FAs, resulted in no difference in HSI and liver lipid content among the groups. Only in muscle, fish showed a significant fat deposition when the lipid intake above 40 g kg^{-1}. The excess lipid, derived from enhanced serum TC and TG contents, was more likely to induce deposition in muscle rather than lipid uptake by adipose tissue in grass carp fed with high dietary lipid, indicating the muscle of grass carp might be the main responding organ to high lipid intake.

Keywords Growth performance · Lipid metabolism · Fat deposition · Gene expression · Protein sparing

Abbreviations

B2M	Beta-2-microglobulin
β-actin	Actin isoform B
CPT1	Carnitine palmitoyltransferase 1
FAS	Fatty acid synthetase
FE	Feed efficiency
FI	Feed intake
HDL	High-density lipoprotein
HMBS	Hydroxymethyl-bilane synthase
HSI	Hepatopancreasomatic index
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MFI	Mesenteric fat index
PE	Protein efficiency

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PPARα	Peroxisome proliferator-activated receptor type α
PPARγ	Peroxisome proliferator-activated receptor type γ
RPL13A	Ribosomal protein L13a
SDHA	Succinate dehydrogenase complex, subunit A
SGR	Specific growth ratio
SREBP1	Sterol regulatory element binding protein 1
TC	Total cholesterol
TG	Total triglyceride
Tubα 1	Tubulin alpha 1
VSI	Viscerosomatic index
WG	Weight gain

Introduction

The management of fat deposition is a significant challenge for both human health and farm animal breeding. In humans, obesity expressed as overexpansion of the adipose tissue is often associated with the development of metabolic disorders, which is becoming increasingly prevalent worldwide (World Health Organization 2006). The management of fat deposition in farm animals, including fish, also has become highly significant, especially in terms of fish fillet quality, since lipid storage in skeletal muscle affects the nutritional value and sensory properties of fish fillet (Wood et al. 2008). Dietary manipulation is currently one of the main approaches used to manage muscle fat content in farm animals (Kolditz et al. 2010).

Animals have developed an accurate and complicated metabolic system to adapt to different nutritional states (Soengas 2014). As in man and birds, endogenous lipids in fish are mainly synthesized in the liver, and then transported to peripheral tissues by the bloodstream (Henderson and Sargent 1981; Hillgartner et al. 1995; Ferré and Foufelle 2010; Weil et al. 2013). The liver plays a central role in metabolic homeostasis and coordinates body metabolism in response to various dietary conditions. Feed composition and feeding level affect biochemical composition of the body tissue, with an obvious change in percentages of fat (Jobling 2001). In mammals, the mechanism of lipid metabolism responding to different dietary fat has been extremely discussed (Lin et al. 2000; Buettner et al. 2007). An increase in dietary lipid generally leads to modification of lipid metabolism in most animals, with inhibition of lipogenic enzymes (Hillgartner et al. 1995; Clarke and Hembree 1990; Gélineau et al. 2001), and stimulation of fatty acid oxidation (Kim et al. 2004).

Increasing dietary energy supply always promotes whole-body fat deposition in most fish (Ackman 1995; Robb et al. 2002). Lipid deposition in fish tissues involves several metabolic processes: lipogenesis, lipid transport by lipoproteins, tissue lipid uptake (mediated by lipoprotein lipase (LPL)) and storage of lipids (Sheridan 1988; Tocher 2003). So far, a number of lipid metabolic genes in some fishes have been cloned, and preliminary functions have also been illustrated (Morash et al. 2009; Leng et al. 2012; He et al. 2015a). Sterol regulatory element binding protein 1 (SREBP1), the main transcription factor that controls the lipogenic pathway, is essential for the transcriptional control of genes encoding enzymes of lipid biosynthesis such as fatty acid synthetase (FAS) and ATP-citrate lyase (ACLY) (Egea et al. 2008; Leng et al. 2012; Dong et al. 2015). Peroxisome proliferator-activated receptor type γ (PPARγ) functions in adipocyte differentiation (Tsai et al. 2008; He et al. 2015a). Carnitine palmitoyltransferase 1 (CPT1) is considered to be the main regulatory enzyme in mitochondrial fatty acid oxidation (Morash et al. 2009), and PPARα regulates the expression of a number of genes essential for lipid and lipoprotein metabolism (Ji et al. 2011). Although a lot of existing literature dealing with lipid metabolism in many teleost species (Wang et al. 2005; Du et al. 2006; Morash et al. 2009; Chatzifotis et al. 2010), little is known about the molecular mechanisms regulating fat deposition in different tissues.

Grass carp (*Ctenopharyngodon idella*) is a typical herbivorous finfish without stomach. In the present study, the effects of dietary lipid level on growth parameters, tissue fat deposition and gene expression of several key actors involved in fatty acids uptake by tissue (*LPL*), lipogenesis (*SREBP1, FAS* and *PPARγ*), and fatty acid oxidation (*CPT1* and *PPARα*) were evaluated in liver and adipose tissue. In fish, the liver and adipose tissue are both principal sites of lipid synthesis and storage, involved in energy balance and lipid homeostasis (Sheridan and Kao 1998; Nanton

et al. 2007). The objective of the present study was to evaluate the fat deposition pattern and lipid metabolic strategies of grass carp in response to dietary lipid levels.

Materials and methods

Experimental diets

Composition and chemical analysis of the five experimental diets are shown in Table 1. Using fish meal and casein as protein sources, corn starch and α-starch as the carbohydrate sources, fish oil and soybean oil as the lipid sources, five isonitrogenous diets with approximately 260 g kg^{-1} crude protein were prepared. Five artificial diets were formulated to contain five crude lipid levels (0, 20, 40, 60 and 80 g kg^{-1}), named as diet L0, L2, L4, L6 and L8. The ingredients were from mainland of China and purchased from Shentianyu and Fulong Dietary Company (Wuhan, China). Ingredients were ground into fine powder through 250 μm mesh using the 9FQ20-16F type feed mill (Tieshi grinding machinery Ltd., Hebei, China). The diets were pelleted (4 mm diameter) by the SLH-200B type ring mode pellet feed pressing machine (Kailing Ltd., Wuxi, China) within 30 min after the ingredient was mixed. Then the pellets were air-dried

Table 1 Compositions of diets added with different levels of lipid

Experimental diets	L0	L2	L4	L6	L8
Ingredients (g kg^{-1})					
Fish meal	155.2	155.2	155.2	155.2	155.2
Casein[a]	260.2	260.2	260.2	260.2	260.2
Fish oil	0	10	20	30	40
Soybean oil	0	10	20	30	40
DL-Met (99 %)	1.6	1.6	1.6	1.6	1.6
α-Starch[b]	40	40	40	40	40
Corn starch	386.5	386.5	386.5	386.5	386.5
Cellulose	100	80	60	40	20
Ca $(H_2PO_4)_2$	20	20	20	20	20
Mineral mix[c]	20	20	20	20	20
Vitamin mix[d]	10	10	10	10	10
Choline chloride	6	6	6	6	6
Ethoxyquin	0.5	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000	1000
Proximate composition					
Dry matter (DM) (%)	88.95	89.44	89.79	89.97	90.16
Crude protein (% DM)	26.01	26.18	26.01	25.93	25.84
Crude lipid (% DM)	0.53	2.62	4.35	6.89	8.83
Carbohydrate (% DM)	42.29	40.39	38.80	37.12	35.89
Ash (% DM)	11.93	11.79	11.58	11.48	11.46
Gross energy (kJ g^{-1})	14.41	14.67	15.59	16.01	16.44

[a] Crude protein and lipid content of casein was 84.4 and 0.6 %, respectively

[b] Crude protein and lipid content of α-starch was 0.3 and 0.2 %, respectively

[c] Vitamin premix (per kg of diet): vitamin A, 2000 IU; vitamin B1 (thiamin), 5 mg; vitamin B2 (riboflavin), 5 mg; vitamin B6, 5 mg; vitamin B12, 0.025 mg; vitamin D3, 1200 IU; vitamin E 21 mg; vitamin K3 2.5 mg; folic acid, 1.3 mg; biotin, 0.05 mg; pantothenic acid calcium, 20 mg; inositol, 60 mg; ascorbic acid (35 %), 110 mg; niacinamide, 25 mg

[d] Mineral premix (per kg of diet): $MnSO_4$, 10 mg; $MgSO_4$, 10 mg; KCl, 95 mg; NaCl, 165 mg; $ZnSO_4$, 20 mg; KI, 1 mg; $CuSO_4$, 12.5 mg; $FeSO_4$, 105 mg; Na_2SeO_3, 0.1 mg; Co, 1.5 mg

and divided into small bags, and stored in a freezer at −20 °C until used.

Fish and experimental conditions

Grass carp were obtained from the Fish Center of Xiantao, Hubei, China. Prior to the experiment, healthy fish with similar body weight were selected and distributed randomly into 20 pond cages (2 m × 2 m × 2 m) for acclimation. The fish were fed to apparent satiation with a commercial diet (32.0 % protein; 9.0 % fat; 6.9 % moisture; 7.6 % ash) twice a day at 08:00 and 16:00 (Beijing time) during the acclimation period. During the acclimation period, the oxygen saturation value was 84.51 ± 2.17 %, the temperature was 22.5 ± 0.3 °C. After the 2-week acclimation, fish were then starved for 24 h to measure the body length and weight at the beginning of the experiment. The stocking density was 25 fish (200.69 ± 6.28 g) per cage, and each diet was fed to four randomly assigned cages. The fish were fed by hand twice daily at 08:00 and 16:00 for 8 weeks. Fish were hand-fed slowly little by little to prevent waste of dietary pellets. When the experimental feed was supplied, the fish would swim to the water surface to ingest the feed. As long as fish were fed to satiation, they would never come up to water surface again. Hence, their apparent satiation could be judged by feeding behavior observation. During the experimental period, the oxygen saturation value was 85.84 ± 3.23 %, the temperature was 23.6 ± 0.4 °C, the ammonia content was about 0.27 ± 0.02 mg L^{-1} and pH ranged from 7.31 ± 0.23.

Sample collection and chemical analyses

At the end of the 8-week feeding trial, approximately 24 h after the last feeding, all the fish were anaesthetized with 200 mg L^{-1} MS-222 (Argent Chemical Laboratories, Redmond, WA, USA) and then weighed and counted. In each cage, six fish were randomly captured and stored in a freezer at −20 °C until used for whole-body chemical analysis. About 2 g of viscera, livers, muscle (dorsal fillet without skin), and adipose tissue from another six fish were dissected and separated for tissue lipid contents detection; other four fish for molecular experiments were randomly captured and killed by the spinal cord dissected for measure and dissection, and then stored at −80 °C until used. About 0.3 g fish liver and mesenteric adipose tissue samples for gene expression assay were immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation and subsequent analysis. The fish were anesthetized with 100 mg L^{-1} MS-222 and blood samples collected from the caudal vein of other three fish removed at the sampling time from each cage by using heparinized syringe. Blood was centrifuged (Heraeus Multifuge X1R, Thermo Electron LED GmbH, Osterode, Germany) at 3500 g for 10 min, and then serum was separated and stored at −80 °C until used.

Dietary composition was determined by standard methods (A.O.A.C. 1995). Moisture was determined by oven drying at 105 °C for 6 h. Ash was measured using a muffle furnace (SX2-4-10, Zhengda Electric Technology Co., Ltd., Longkou, China) at 550 °C for 12 h. Crude protein (N × 6.25) was determined following the Kjeldahl method after an acid digestion using a Kjeltec system (K9860 Kjeltec Analyzer, Hanon Instruments, China). Crude lipid was evaluated by the ether-extraction method using Soxtec System HT (Soxtec System HT6, Tecator, Sweden). Energy content of the diets was measured by bomb calorimetry using a Parr 6200 calorimeter equipped with a Parr 1108 Oxygen Bomb and a Parr 6510 water handling system (Parr Instrument Company, Moline, IL, USA).

Serum total cholesterol (TC), total triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) contents were determined using an automatic biochemical analyser [Abbott Aeroset Analyzer (Abbott Laboratories, Abbott Park, IL, USA)] in the Zhongnan Hospital of Wuhan University (Wuhan, Hubei, China).

Gene expression assay

Total RNA of the liver was extracted by SV Total RNA Isolation System kit (Promega, USA) following the manual. The quantity and quality of 2ul RNA obtained were checked by spectrophotometric analysis with the Eppendorf Biophotometer Plus (Eppendorf AG, Hamburg, Germany); ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) was used to assess purity of RNA and A_{260}/A_{280} ratios above 2.0 for RNA were controlled. Then 1 μg of the RNA was reverse transcribed to cDNA using SuperScript™ II RT reverse transcriptase (Takara, Japan).

Real-time PCR was applied to evaluate the expression level of gene expression assay using gene-specific primers as shown in Table 2. A set of six housekeeping genes (actin isoform B, *β-actin*; ribosomal protein L13a, *RPL13A*; tubulin alpha 1, *Tubα 1*; beta-2-microglobulin, *B2 M*; hydroxymethyl-bilane synthase, *HMBS*; succinate dehydrogenase complex, subunit A, *SDHA*) were selected from the transcriptome assemblies (Vandesompele et al. 2002) in order to test their transcription stability for the treatment series and tissue panel. GeNorm software was then used to compute the expression stability values (M) for each gene where a lower M value corresponds to more stable gene expression. Real-time PCR assays were carried out in a quantitative thermal cycler (MyiQ^{TM} 2 Two-Color Real-Time PCR Detection System, BIO-RAD, USA) with a 20 µL reaction volume containing 10 µL of 2 × SYBR$^{®}$ Premix Ex Taq^{TM} (TaKaRa BIO, Tokyo, Japan), 1 µL of template (50 ng/µL), and 0.4 µL of each forward and reverse primer (10 µM). The PCR parameters were 95 °C for 1 min followed by 40 cycles at 95 °C for 10 s, 57 °C for 30 s and a melt curve step (from 95 °C, gradually reducing 0.5 °C s^{-1} to 57 °C, with acquisition data every 6 s). The amplification efficiencies of control and target genes were ranged from 98.7 to 103.5 %.

Gene expression levels were quantified relative to the expression of housekeeping genes using the optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method (Livak and Schmittgen 2001). All amplifications were performed in triplicate for each RNA sample. Data from three replicate RT-PCR samples were analyzed using CFX Manager TM software (Version 1.0). The ΔCt (differences in the Ct value between target gene and housekeeping genes) for each sample was subtracted from that of the calibrator, which was called ΔΔCt, gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ and the value represented an n-fold difference were normalized to the defined control L0 group. Modifications of gene expression are represented with respect to the same calibrator (plasmid vector), which is assumed to have the value of 1 A.U. (arbitrary unit).

Statistical analysis

All data were presented as mean ± S.E.M (standard error of the mean). We performed a nested ANOVA, with diet as the main factor and the factor cage nested into the factor diet. Nested design was possible using SPSS 17.0 software. However, the factor cage proved to be non-significant, so we change to one factor (diet) ANOVA.

For growth rates, the mean weight of fish in each cage was used as the basis to calculate the growth rates (n = 4), as indicated above. To establish differences between diets, we performed a simple ANOVA, with

Table 2 Primer sequences for the quantitative real-time PCR

Gene name	Primer	Sequence 5′–3′	Tm (°C)	Product size (bp)	*E*-values (%)	Genbank accession no.
SREBP1	SREBP1-F	CCTCTGGGTCATCGTTTCT	56	80	103.5	GU339498.1
	SREBP1-R	CCTGCCTGGCTGTAGATG				
FAS	FAS-F	GATGGGTCTACAGCCTGATGG	58	208	98.9	HM802556.1
	FAS-R	GACACCCTGTGGACATTGAGC				
PPARγ	PPARγ-F	GATGGTTGGCATGTCACAC	54.5	206	101.7	GQ220296.1
	PPARγ-R	TTCCTGACAGTATGGCTC				
PPARα	PPARα-F	TCAGCGGGAAAGAGGAG	56	148	102.6	FJ231987.1
	PPARα-R	ACGAAAGGCGGAGTGC				
CPT1	CPT1-F	GCCACTGTAAAGGAGAACC	55	272	99.5	JF728839.1
	CPT1-R	GGATGCCTCATAAGTCAAG				
LPL	LPL-F	TACAGCGGCGTTCACACTTG	57	169	98.7	FJ436077.1
	LPL-R	CTACATGAGCACCAAGACTG				
β-actin	β-act-F	CGTGACATCAAGGAGAAG	57	299	100.3	M25013.1
	β-act-R	GAGTTGAAGGTGGTCTCAT				

diet as a single factor. Differences between diets were deduced from a Fisher post hoc test, with significance established at $P = 0.05$. The same design was used to analyze the other data.

The normality of data was assessed by using SPSS software with the Shapiro–Wilk test. All data were subjected to one-way analysis of variance (one-way ANOVA) using SPSS 17.0 software. Differences between the means were tested by Duncan's multiple range test after homogeneity of variances was checked. Statistical significance was determined at the 5 % level.

Results

Effects of dietary lipid levels on growth performance and feed utilization

After 8-week feeding trial, weight gain (WG), specific growth ratio (SGR), feed intake (FI), feed efficiency (FE) and protein efficiency (PE) of grass carp fed with different lipid levels are presented in Table 3. WG and SGR were highest in grass carp fed diets with L4 diet, followed by L6 and lowest in fish fed with the lipid-free diet. The highest FI was observed in L2 and L4 groups. FE and PE were highest in grass carp fed diets with L4 and L6 diets.

Slaughter indices, including viscerosomatic index (VSI), hepatopancreasomatic index (HSI) and mesenteric fat index (MFI), are presented in Table 4. Significant higher VSI was observed in L6 group than in L0 group. The MFI was increased with the increasing dietary lipid level from 0 to 4 %. No significant difference was seen in HSI among all the groups.

Effects of dietary lipid levels on whole-body composition and tissue lipid contents

Whole-body and muscle lipid contents were increased with the increasing dietary lipid level (Table 5). No

Table 3 Growth performance and feed utilization of grass carp fed with different dietary lipids

Item	L0	L2	L4	L6	L8
IW	203.67 ± 3.48	202.42 ± 3.18	197.04 ± 7.02	199.89 ± 2.19	200.56 ± 1.57
FW	380.92 ± 9.86^{c}	427.03 ± 12.13^{b}	472.48 ± 15.02^{a}	442..85 ± 6.88^{ab}	422.45 ± 7.28^{b}
WG (%)	86.98 ± 1.82^{d}	110.63 ± 2.84^{c}	139.90 ± 1.12^{a}	121.85 ± 4.23^{b}	110.70 ± 2.82^{c}
SGR (%)	0.98 ± 0.02^{d}	1.16 ± 0.02^{c}	1.37 ± 0.01^{a}	1.24 ± 0.03^{b}	1.16 ± 0.02^{c}
FI (g fish^{-1})	421.55 ± 6.01^{b}	464.02 ± 7.53^{a}	464.00 ± 11.52^{a}	416.48 ± 1.96^{b}	419.05 ± 11.98^{b}
FE	0.42 ± 0.02^{c}	0.48 ± 0.01^{b}	0.59 ± 0.02^{a}	0.58 ± 0.02^{a}	0.53 ± 0.01^{b}
PE	1.62 ± 0.08^{d}	1.84 ± 0.05^{c}	2.28 ± 0.06^{a}	2.25 ± 0.07^{a}	2.05 ± 0.03^{b}

Values are means ± SEM of four replicates, and values within the same row with different letters in superscript are significantly different ($P < 0.05$)

IW (g), initial weight; FW (g), final weight; FI (g fish^{-1}), feed intake; Weight gain (WG, %) = 100 × (final weight − initial weight)/initial weight; Specific growth ratio (SGR, %) = 100 × (lnFW − lnIW)/time (days); Feed efficiency (FE) = wet weight gain (g)/feed intake (g); Protein efficiency (PE) = (final weight − initial weight)/protein intake

Table 4 Slaughter variables of juvenile grass carp fed experimental with different dietary lipids

Item	L0	L2	L4	L6	L8
VSI	7.48 ± 0.20^{b}	8.04 ± 0.22^{ab}	8.16 ± 0.19^{ab}	8.27 ± 0.20^{a}	7.79 ± 0.27^{ab}
HSI	2.08 ± 0.16	2.50 ± 0.09	2.15 ± 0.12	2.21 ± 0.14	2.01 ± 0.10
MFI	0.93 ± 0.06^{c}	1.58 ± 0.06^{b}	2.00 ± 0.14^{a}	2.01 ± 0.03^{a}	1.92 ± 0.13^{a}

Values are means ± SEM of four replicates and values within the same row with different letters in superscript are significantly different ($P < 0.05$)

Viscerosomatic index (VSI) = 100 × viscerosomatic weight (g)/body weight (g); Hepatopancreasomatic index (HSI) = 100 × liver weight (g)/body weight (g); Mesenteric fat index (MFI) = 100 × mesenteric fat weight (g)/body weight (g)

Table 5 Body composition of grass carp fed experimental diets with different dietary lipids (% wet weight)

Item	Initial	Experimental diets				
		L0	L2	L4	L6	L8
Whole body						
Moisture	74.13 ± 0.89	74.28 ± 0.49	72.51 ± 0.42	72.95 ± 0.60	72.98 ± 0.61	72.71 ± 0.47
Protein	15.64 ± 0.20	16.12 ± 0.12^{b}	16.13 ± 0.25^{b}	16.25 ± 0.23^{ab}	16.71 ± 0.10^{a}	16.64 ± 0.05^{ab}
Lipid	4.34 ± 0.23	5.09 ± 0.33^{e}	6.69 ± 0.10^{d}	7.31 ± 0.30^{c}	7.94 ± 0.05^{b}	8.63 ± 0.13^{a}
Ash	4.27 ± 0.14	4.40 ± 0.08	4.43 ± 0.15	4.23 ± 0.25	3.96 ± 0.06	4.01 ± 0.09
Back muscle						
Lipid	–	4.93 ± 0.13^{d}	5.47 ± 0.14 ^{cd}	6.07 ± 0.21^{c}	7.97 ± 0.05^{b}	10.19 ± 0.69^{a}
Liver						
Lipid	–	26.23 ± 1.44	24.32 ± 1.47	27.03 ± 1.86	31.12 ± 2.00	28.36 ± 3.15
Viscera						
Lipid	–	40.68 ± 1.29^{c}	57.42 ± 2.89^{b}	71.90 ± 1.70^{a}	65.35 ± 2.64^{ab}	65.76 ± 3.85^{ab}
Adipose tissue						
Lipid	–	85.59 ± 2.37^{b}	86.47 ± 1.23^{b}	89.50 ± 2.09^{ab}	92.23 ± 1.12^{a}	92.15 ± 1.16^{a}

Values are means ± SEM of four replicates and values within the same row with different letters in superscript are significantly different ($P < 0.05$)

Table 6 Serum lipid fractions of grass carp fed experimental diets with different dietary lipids

Item	Diets				
	L0	L2	L4	L6	L8
TC (mmol/L)	5.83 ± 0.06^{b}	5.89 ± 0.17^{ab}	6.47 ± 0.15^{ab}	6.56 ± 0.26^{ab}	6.86 ± 0.30^{a}
TG (mmol/L)	1.67 ± 0.05^{c}	2.01 ± 0.05^{b}	2.07 ± 0.04^{b}	2.58 ± 0.04^{a}	2.83 ± 0.06^{a}
HDL (mmol/L)	1.26 ± 0.02^{b}	1.44 ± 0.03^{ab}	1.43 ± 0.00^{ab}	1.34 ± 0.01^{ab}	1.52 ± 0.07^{a}
LDL (mmol/L)	1.21 ± 0.01^{b}	1.14 ± 0.03^{b}	1.55 ± 0.06^{a}	1.53 ± 0.05^{a}	1.53 ± 0.11^{a}

Values are mean ± S.E.M of four replicates and values within the same row with different letters are significantly different ($P < 0.05$)

TC total cholesterol, *TG* total triglyceride, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

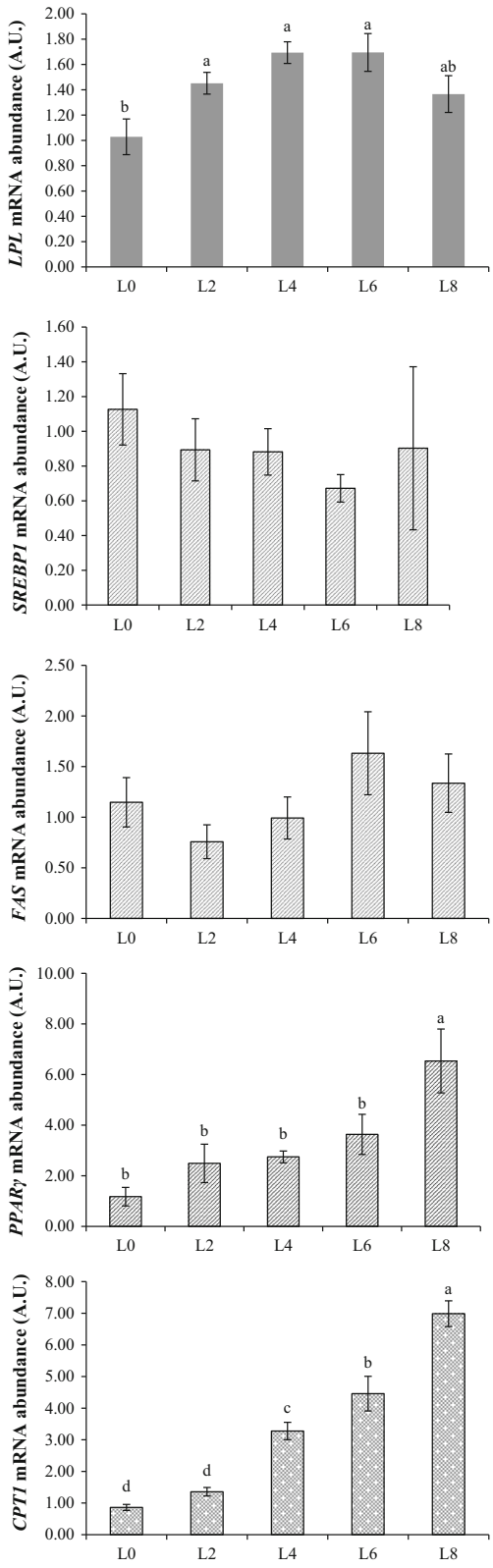
significant difference was seen in moisture and ash contents among all the groups. Fish fed with L6 diet had higher whole-body protein content than fed with L0 and L2 diets. There was no significant difference of liver lipid content among the treatments. The viscera and adipose tissue lipid contents were increased with the increasing dietary lipid level up to 40 g kg^{-1}.

Effects of dietary lipid levels on serum indices

Serum TC, TG, HDL and LDL concentrations are reported in Table 6. Higher TC and HCL concentrations were observed in L8 group than in L0 group. Fish fed with higher dietary lipid level had higher TG and LDL.

Effects of dietary lipid levels on lipid metabolism-related gene expressions

By using geNorm software, gene expressions are normalized to the geometric mean of the best combination of two genes (*β-actin* and *RPL13A*). The relative expressions of genes involved in lipid metabolism in the adipose tissue of grass carp fed with different dietary fat levels were analyzed by real-time PCR (Fig. 1). In the adipose tissue, the expression of *LPL* was significantly higher in L2, L4 and L6 group than that in L0 group. Fish fed with L8 diet had higher *PPARγ* mRNA abundance in adipose tissue than that fed with the other diets. The gene expressions of *CPT1* in adipose tissue increased with the



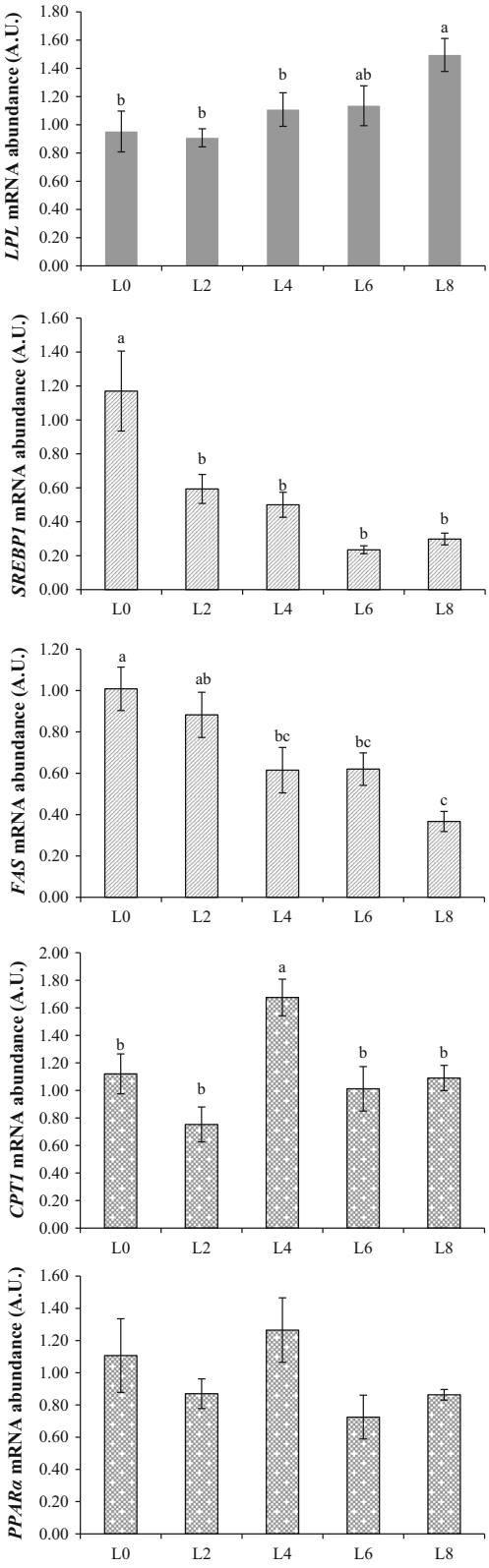
◀**Fig. 1** The relative expressions of genes involved in lipid metabolism in adipose tissue of grass carp fed with different dietary lipid levels. Gene expressions are normalized to the geometric mean of the best combination of two genes (*β-actin* and *RPL13A*). Values are mean ± S.E.M. of four replicates, and values within the same *row* with different *letters* are significantly different ($P < 0.05$)

increasing dietary lipid. No significant difference in *SREBP1* and *FAS* expressions was observed among the treatments.

The hepatic *LPL* expression was significantly higher in L8 group than that in L0, L2 and L4 group (Fig. 2). Fish fed with L0 diet had higher hepatic *SREBP1* mRNA abundance than that fed with other diets. The gene expression of *FAS* in liver was significantly lower in L8 group than that in L0 and L2 group. The L4 group had the highest hepatic *CPT1* expression, and other groups showed no significant differences. A similar tendency in hepatic *PPARα* expression was found without significant difference.

Discussion

To have a better understanding of fat deposition pattern and lipid metabolic strategies in fish, five isonitrogenous diets containing five dietary lipid levels (0, 20, 40, 60, 80 g kg^{-1}) were fed to quadruplicate groups. The best growth performance and feed utilization was observed in fish fed 40 g kg^{-1}, while fish growth and feed utilization significantly declined with higher dietary lipid level. Previous studies attributed the fish growth-inhibition effects of high dietary lipid to the excessive energy that can cause reduced feed consumption-limited ability to digest and absorb high amounts of lipid and, therefore, growth retardation (Jobling and Wandsvik 1983; Ellis and Reigh 1991; Kaushik and Medale 1994; Paspatis and Boujard 1996; Wang et al. 2005). The increased digestible energy content with lipid supplementation in fish diets has been shown to have a protein sparing effect in many fishes, therefore reducing nitrogen losses to the environment (Cho and Kaushik 1990). Contrary to many other species like salmonids or seabass where a protein sparing has been well demonstrated (Lee and Putnam 1973; Beamish and Medland 1986; Cho and Kaushik 1990; Arzel et al.



◀**Fig. 2** The relative expressions of genes involved in lipid metabolism in liver of grass carp fed with different dietary lipid levels. Gene expressions are normalized to the geometric mean of the best combination of two genes (*β-actin* and *RPL13A*). Values are mean ± S.E.M. of four replicates, and values within the same *row* with different *letters* are significantly different ($P < 0.05$)

1994; Xu et al. 2011), the increase in dietary lipid level from 40 to 80 g kg^{-1} does not appear to improve protein utilization in grass carp.

Cellulose in diet is unlikely to be the cause affecting performance of grass carp. The presence of cellulose activity in grass carp suggested the necessity of providing cellulose in the diet (Lesel et al. 1986; Das and Tripathi 1991; Li et al. 2009; Li et al. (2016b). Stanley (1974) reported that grass carp were highly efficient in converting assimilated food into fish biomass giving it a rapid growth rate despite wasteful digestion. Actually, cellulose was used at levels up to 165 g kg^{-1} for grass carp without affecting growth rates (Gao et al. 2010). In other studies, cellulose was also used at levels up to 400 g kg^{-1} for *Ictalurus punctatus* and *Tilapia zillii* without affecting growth rates (Garling and Wilson 1977; El-Sayed and Garling 1988).

Generally, an increase in dietary lipid levels is correlated with an increase in whole-body lipid content, and excessive dietary lipids result in excessive fat deposition in viscera cavity, liver, and muscle of fishes (Peres and Oliva-Teles 1999; Regost et al. 2001; Pei et al. 2004; Wang et al. 2005; Martins et al. 2007; Song et al. 2009; Ghanawi et al. 2011). In the current study, the viscera cavity, adipose tissue, muscle and whole-body lipid contents increased when dietary lipid levels increased from 0 to 4 %. Only in muscle, fish showed a significant fat deposition when the lipid intake above 40 g kg^{-1}. Similar results have also been reported in other fish species (Luo et al. 2005; Wang et al. 2005; Ding et al. 2010; Xu et al. 2011). Some researchers reported that the major site for fat deposition in some fishes is the liver (Ackman 1995; Li et al. 2016a), but in the present study, no statistical differences were noted in HSI and liver lipid contents among grass carp fed with different dietary lipid levels. The results in this study were similar to earlier studies in other fishes including [Atlantic halibut *Hippoglossus hippoglossus* (Martins et al.

2007), sea bream *Diplodus sargus* (Sá et al. 2006), and meagre *Argyrosomus regius* (Chatzifotis et al. 2010)]. These results suggested that liver does not contribute significantly to fat deposition in grass carp.

Teleost fish adapt to dietary lipid intake through modulation of the expression of lipid metabolic genes (Alves Martins et al. 2012). To explore the molecular mechanisms involved in dietary lipid regulation and fat deposition, the expressions of several key genes in liver and adipose tissue were analyzed. In the present study, the expressions of *CPT1* in adipose tissue and *LPL* in liver were significantly elevated with increasing dietary lipid level. *CPT1* participates in the transport of long chain fatty acids helping in the process of β-oxidation (Morash et al. 2009) and *LPL* mediates tissue lipid uptake (Sheridan 1988; Tocher 2003) in fish. The enhanced mRNA abundance of *CPT1* in adipose tissue and *LPL* in liver in increasing dietary lipid suggested potential sources of hepatic fatty acids are non-esterified fatty acids coming from the hydrolysis of TGs stored in the adipose tissue and dietary fatty acids arising from uptake (Ferré and Foufelle 2010). Meanwhile, the expression of hepatic *SREBP1* and *FAS* were significantly decreased in L8 group, suggesting lipogenesis in fish liver was decreased with a high lipid intake. The previous studies also pointed out that *SREBP1 and FAS* gene expressions were reduced with increasing lipid intake in other fish liver (Leng et al. 2012; He et al. 2015b). A possible reason was that elevated intake of exogenous lipid reduced the endogenous synthesis to maintain the dynamic balance between direct absorption of exogenous feed and endogenous synthesis (Leng et al. 2012). Moreover, the reduction of dietary carbohydrate level might also be the reason for the decrease expression of hepatic *SREBP1*, as *SREBP1* mediated the conversion of an excess of carbohydrates into fatty acids (Egea et al. 2008; Ferré and Foufelle 2010). Once in the liver, fatty acids are esterified into TGs, which can be stored in lipid droplets into the hepatocytes or secreted as TGs-enriched lipoproteins (TC, TG and LDL) into the bloodstream (seen in higher serum TC, TG and LDL contents in increasing dietary lipid).

In mammals, when energy intake is greater than energy expenditure, adipose tissue swells through increasing the numbers and/or enlarging the size of adipocytes (Rosen and Spiegelman 2006). PPARγ functions in mammalian adipocyte differentiation (Rosen et al. 1999; Wu et al. 1999). A number of studies have indicated that high lipid intake leads to increased expression of hepatic *PPARγ* and a number of PPARγ-targeted genes involved in adipocyte differentiation and lipid storage (Brun and Spiegelman 1997; He et al. 2015a). The highest expression of mRNA of hepatic *PPARγ* was observed in grass carp fed with high lipid diet, suggesting the increased adipocyte differentiation in adipose tissue. Previous study also reported that the high fat feeding not only cause the differentiation of adipocytes, but also induces adipogenesis through elevating FA synthesis *de novo* (Ilich et al. 2014). In the present study, no significant difference in *SREBP1* and *FAS* expressions were observed in grass carp adipose tissue among treatments, indicating lipogenesis was not activated by the current levels of dietary lipid. Therefore, in the case of grass carp, the increase in adipocyte numbers might be the main metabolic solution to deal with high lipid intake.

TG in mammalian serum is lipolysed by *LPL* to generate fatty acids that are mainly taken-up by muscle and adipose tissue for oxidation and esterification into TGs (Den Boer et al. 2004). Fish fed with higher dietary lipid level had higher serum TC, TG, HDL and LDL contents in this study indicated a more active lipid transport. Similar results were also observed by Regost et al. (2001) and Du et al. (2006). However, the incremental serum TC and TG contents could not stimulate *LPL* expression in adipose tissue of grass carp fed with dietary lipid above 40 g kg^{-1}. Therefore, an interesting question remains as where the lipid has gone. The answer might be obtained from the muscle, which was the only fat storage tissue that has a significant fat deposition when the lipid intake above 40 g kg^{-1}. These results indicated that the muscle is likely to be the main responding organ to deal with high lipid intake in grass carp. However, the mechanism of muscle fat deposition needs to be further explored.

In conclusion, an appropriate amount of dietary lipid (40 g kg^{-1} in this study) enhances growth performance and food intake in grass carp weight 200 g. No protein sparing effect was observed in high dietary level (above 40 g kg^{-1}) group. MFI and adipose tissue lipid content increased with increasing dietary lipid level up to 40 g kg^{-1}, and higher lipid level in diet made no sense. Fish adapted to high lipid intake through integrated regulating mechanisms in

several related tissues to maintain lipid homeostasis. In the present study, grass carp firstly increased *PPARγ* and *CPT1* expressions in adipose tissue to elevate adipocyte differentiation and lipolysis to adapt to high lipid intake above 40 g kg^{-1}. In liver, fish elevated hepatic lipid uptake but depressed biosynthesis of hepatic FAs, resulted in no difference in HSI and liver lipid content among the groups. Only in muscle, fish showed a significant fat deposition when the lipid intake above 40 g kg^{-1}. The excess lipid, derived from enhanced serum TC and TG contents, was more likely to induce deposition in muscle rather than lipid uptake by adipose tissue in grass carp fed with high dietary lipid, indicating the muscle of grass carp might be the main responding organ to high lipid intake. Therefore, too high dietary lipid should be avoided as excess lipid might deposit into muscle to affect the fillet taste, although not hamper the lipid metabolism in liver and adipose tissue.

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