Growth performance, fatty-acid composition, lipid deposition and hepatic-lipid metabolism-related gene expression in juvenile pond loach *Misgurnus anguillicaudatus* fed diets with different dietary soybean oil levels

Y. Li*, Z. Jia*, X. Liang*, D. Matulic†, M. Hussein*‡ and J. Gao*§

*College of Fisheries, Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education/Key Lab of Freshwater Animal Breeding, Ministry of Agriculture, Huazhong Agricultural University, Wuhan 430070, China, †Department for Fisheries, Beekeeping, Game Management and Special Zoology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia and ‡Faculty of Veterinary Medicine, Benha University, Egypt

A 10 week feeding trial was conducted to evaluate the effects of different dietary soybean oil (SO) levels on growth performance, fatty-acid composition and lipid deposition in viscera, histology and histochemistry of liver, intestine and hepatic-lipid metabolism-related gene expressions in pond loach *Misgurnus anguillicaudatus* juveniles. *Misgurnus anguillicaudatus* (mean ± s.d. mass 0.40 ± 0.01 g) were fed five experimental diets containing SO at different concentrations: 0, 20, 32, 56 and 100% SO and a diet containing 100% fish oil (100% FO). The mass gains and specific growth rates of *M. anguillicaudatus* fed 20% SO and 100% FO diets were significantly higher than those of the other groups ($P < 0.05$). The lipid content of viscera and the amount of cytoplasmic vacuolation in the liver increased with incremental dietary SO level. Meanwhile, increasing dietary SO levels up-regulated the messenger (m)RNA levels of lipogenic genes (such as *Δ6fad*, *scd*, *pparγ*, *fas* and *srebp-1*) and down-regulated the mRNA levels of the lipolytic genes (such as *pparα*, *cpt1*, *atgl* and *hsl*) in the liver. The percentage of 20:4n-6 significantly ($P < 0.05$) increased with increasing dietary SO level, which might be correlated with the up-regulation of the mRNA level of *Δ6fad*. The highest levels of dietary SO, however, had a negative effect on growth performance, lipid deposition of viscera and histology and histochemstry of liver and intestine. The increased lipid accumulation induced by incremental dietary SO level probably occurred through different strategies for lipid metabolism as a result of competition between lipolysis and lipogenesis and between export and import of lipids in this species.

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Key words: lipid accumulation; lipogenic genes; lipolytic genes; *Misgurnus anguillicaudatus*; soybean oil.

INTRODUCTION

Aquculture has widely used diets containing large amounts of marine-based ingredients such as fish oil (FO) for fin-fish species. Fish oil is traditionally the main lipid

§Author to whom correspondence should be addressed. Tel.: +86 027 87282113; email: gaojian@ mail.hzau.edu.cn

source used in aquafeeds, which acts as the predominant source of energy and essential fatty acids (EFA) (Sargent *et al.*, 2002). In the aquaculture industry, one of the challenges that fish nutritionists face is the need to partially or totally replace FO with less expensive, non-traditional animal or plant-oil sources. Therefore, reducing FO inclusion levels and replacing FO with soybean oil (SO), which is cost effective in feedstuffs, could be possible in freshwater fin-fish diets (Li *et al.*, 2015*b*; Li *et al.*, 2016). Plant oil is traditionally used in aquaculture feeds because of its high poly-unsaturated fatty acids (PUFA), especially linoleic acid (LA, 18:2n-6) and linolenic acid (LNA, 18:3n-3), EFAs that maintain normal cell function in freshwater fishes (Izquierdo *et al.*, 2005; Mourente & Bell, 2006). Most freshwater species have the capacity to convert LA and LNA to n-6 and n-3 long-chain (LC)-PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. Thus, LA and LNA are required in the diets for fish growth and survival.

Increasing dietary lipid will, within certain limits, promote protein-sparing and higher growth rates of fishes (Li *et al.*, 2010). High dietary lipid content, however, often causes excessive lipid deposition in liver (or other tissues), which could cause serious health problems and reduce harvest yields in farmed fishes (Luo *et al.*, 2005; Du *et al.*, 2008; Li *et al.*, 2015*b*). Du *et al.* (2008) found that the hepatic-lipid deposition of grass carp *Ctenopharyngodon idella* (Valenciennes 1844) tended to increase with incremental dietary SO level. Similarly, a previous study demonstrated that high levels of dietary SO increased hepatic-lipid content and decreased growth performance in blunt snout bream *Megalobrama amblycephala* Yih 1955 juveniles (Li *et al.*, 2016).

Generally, lipid deposition results from the balance between synthesis of fatty acids and fat catabolism *via* β-oxidation and many key enzymes and transcription factors are involved in these metabolic processes (Lu *et al.*, 2013; Zheng *et al.*, 2014; Chen *et al.*, 2015). These enzymes include lipogenic enzymes (such as fatty-acid synthase, FAS; acetyl-CoA carboxylase, ACC; Δ-6 fatty acyl desaturase, Δ6FAD and acyl-CoA Δ-9 desaturase, SCD) and lipolytic enzymes (such as carnitine palmitoyltransferase 1, CPT1; hormone-sensitive lipase, HSL; and adipose triacylglyceride lipase; ATGL) (Elliott & Elliott, 2009). On the other hand, fatty-acid binding protein (FABP) and several transcription factors such as peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) and sterol-regulator element-binding protein-1 (SREBP-1) play a pivotal role in lipid homeostasis, by regulating the gene transcription of enzymes involved in this pathway (Chen *et al.*, 2015). In addition, lipoprotein lipase (LPL) hydrolyzes triglyceride present in plasma lipoproteins and supplies free fatty acids for storage in adipose tissues, or for oxidation in other tissues and plays a pivotal role in regulating lipid content in fishes (Zheng *et al.*, 2014). At present, although many studies have investigated the effect of dietary SO on lipid deposition in fishes (Mo *et al.*, 2014; Li *et al.*, 2016), the underlying molecular processes involved in the alteration of lipid deposition in the liver and tissues as a response to dietary SO are poorly studied.

The demand for pond loach *Misgurnus anguillicaudatus* (Cantor 1842) has developed vigorously in recent years and it is now one of the most important cultured freshwater species in China. It is a popular traditional Chinese medicine to be used in folk remedies for the treatment of hepatitis, carbuncles, inflammations and cancers (Qin *et al.*, 2002; Gao *et al.*, 2012). Recently, increasing hepatic-lipid deposition, which may reduce the quality of harvested *M. anguillicaudatus*, is limiting the profitability of fish farming. Therefore, this study aimed to investigate the effects of different dietary SO levels on the growth, fatty-acid composition of viscera, histology and the mechanism of

TABLE I. Formulations and proximate composition of the experimental soybean (SO) diets for *Misgurnus anguillicaudatus*

Ingredients (%)	Experimental diets					
	0% SO	20% SO	32% SO	56% SO	100% SO	100% FO
Defatted fishmeal [a]	10·0	10·0	10·0	10·0	10·0	10·0
Soya concentrate	35·0	35·0	35·0	35·0	35·0	35·0
Casein	10·0	10·0	10·0	10·0	10·0	10·0
Activated gluten	10·0	10·0	10·0	10·0	10·0	10·0
CMC [b]	5·0	5·0	5·0	5·0	5·0	5·0
α-Starch	10·0	10·0	10·0	10·0	10·0	10·0
Dextrin	8·0	8·0	8·0	8·0	8·0	8·0
Soybean oil [c]	0	1·6	2·6	4·5	8·0	0
Fish oil [d]	0	0	0	0	0	8·0
Lard oil	8·0	6·4	5·4	3·5	0	0
$Ca(H_2PO_4)$	2·0	2·0	2·0	2·0	2·0	2·0
Minerals mixture [e]	1·0	1·0	1·0	1·0	1·0	1·0
Vitamins mixture [f]	1·0	1·0	1·0	1·0	1·0	1·0
Proximate composition (%)						
Crude protein (dry mass)	50·5	50·8	50·4	50·7	50·3	50·2
Ash (dry mass)	7·4	7·3	7·2	7·0	7·5	7·4
Moisture	11·7	12·0	12·4	11·6	12·2	12·1
Total lipid (dry mass)	8·2	8·1	8·1	8·1	8·2	8·0

FO, Fish oil.

[a]Fishmeal had been skimmed;

[b]carboxy methyl cellulose;

[c]Hebei Meilin Lipid Co., Ltd., Hebei, China;

[d]Riken Vitamin, Tokyo, Japan;

[e]mineral mixture [mg kg^{-1} diet: $MgSO_4$, 3380 mg; Na_2HPO_4, 2153·33 mg; K_2HPO_4, 5913·33 mg; Fe Citrate, 733·33 mg; Ca Lactate, 8060 mg; $Al(OH)_3$, 6·67 mg; $ZnSO_4$, 86·67 mg; $CuSO_4$, 2·67 mg; $MnSO_4$, 20 mg; $Ca(IO_3)_2$, 6·67 mg; $CoSO_4$, 26·67 mg];

[f]vitamin mixture (mg kg^{-1} diet: β-carotene, 32·12 mg; vitamin C, 230 mg; vitamin D_3, 3·24 mg; menadione $NaHSO_3 \cdot 3H_2O$ (K_3), 15·28 mg; DL-α-tochopherol acetate (E), 12·68 mg; thiamine-nitrate (B_1), 19·24 mg; riboflavin (B_2), 64·12 mg; pyridoxine-HCl (B_6), 15·28 mg; cyanocobalamine (B_{12}), 0·04 mg; d-biotin, 1·92 mg; inositol, 1283·04 mg; niacine (nicotic acid), 256·56 mg; Ca panthothenate, 89·34 mg; folic acid, 4·8 mg; choline choloride, 2623·12 mg; ρ-aminobenzoic acid, 127·76 mg].

hepatic-lipid metabolism in *M. anguillicaudatus* juveniles. The results were expected to provide novel information on the potential involvement and mechanisms of lipid deposition modulated by dietary SO in freshwater fish.

MATERIALS AND METHODS

DIET PREPARATION

Ingredients and proximate compositions of the test diets are presented in Table I. Six iso-nitrogenous (50·5%) and iso-lipidic (7·3%) semi-purified experimental diets were formulated to contain 0% (the control diet), 20, 32, 56 and 100 SO and 100% fish oil (100% FO) as

TABLE II. Mean ± S.E. fatty-acid composition (% of total fatty acids) of the experimental soybean (SO) diets for *Misgurnus anguillicaudatus*

Fatty acids	Experimental diets					
	0% SO	20% SO	32% SO	56% SO	100% SO	100% FO
14:00	2·0 ± 0·1[c]	1·8 ± 0·2[c]	1·5 ± 0·0[b]	1·3 ± 0·1[b]	0·2 ± 0·0[a]	6·3 ± 0·2[d]
16:00	29·7 ± 0·1[c]	26·2 ± 0·0[c]	23 ± 0·4[bc]	20·4 ± 0·3[b]	10·3 ± 0·4[a]	15·9 ± 0·3[ab]
17:00	0.2 ± 0·0	0·4 ± 0·1	0·3 ± 0·0	0·2 ± 0·0	0·2 ± 0·0	0·3 ± 0·0
18:00	15·5 ± 0·1[d]	12·2 ± 0·1[c]	10·7 ± 0·4[bc]	8·8 ± 0·4[b]	2·9 ± 0·1[a]	1·8 ± 0·1[a]
Σ SFAs	47·3 ± 0·1[f]	40·6 ± 0·3[e]	35·5 ± 0·5[d]	30·7 ± 0·6[c]	13·6 ± 0·4[a]	24·3 ± 0·4[b]
16:1n-7	1·0 ± 0·0[a]	1·0 ± 0·0[b]	0·6 ± 0·0[a]	0·6 ± 0·0[a]	0·1 ± 0·0[a]	8·3 ± 0·1[b]
17:1n-9	0·2 ± 0·0	0·2 ± 0·0	0·2 ± 0·0	0·1 ± 0·0	0·2 ± 0·0	0·2 ± 0·0
18:1n-7	20·5 ± 0·2[d]	18·7 ± 0·2[c]	17·2 ± 0·4[bc]	16·6 ± 0·2[bc]	12·4 ± 0·5[b]	4·3 ± 0·1[a]
18:1n-9	22·8 ± 0·3[c]	20·7 ± 0·3[b]	18·7 ± 0·3[ab]	18·8 ± 0·5[ab]	15·8 ± 0·3[a]	16·7 ± 0·3[a]
20:1n-7	nd	nd	nd	nd	nd	3·9 ± 0·2
20:1n-9	nd	nd	nd	nd	nd	6·9 ± 0·1
22:1n-11	nd	nd	nd	nd	nd	11·7 ± 0·4
Σ MUFAs	44·4 ± 0·5[c]	40·7 ± 0·4[bc]	36·8 ± 0·6[b]	36·1 ± 0·4[b]	28·6 ± 0·8[a]	52·0 ± 0·4[d]
18:2n-6(LA)	6·6 ± 0·4[b]	15·9 ± 0·1[c]	23·6 ± 0·1[d]	30·4 ± 0·2[e]	55·5 ± 0·8[f]	3·5 ± 0·1[a]
20:4n-6	nd	nd	nd	nd	nd	0·5 ± 0·1
Σ n-6 PUFAs	6·6 ± 0·4[a]	15·9 ± 0·1[b]	23·6 ± 0·1[c]	30·4 ± 0·2[d]	55·5 ± 0·8[e]	4·0 ± 0·2[a]
18:3n-3(LNA)	0·1 ± 0·0[a]	0·8 ± 0·0[b]	1·3 ± 0·1[bc]	2·4 ± 0·1[c]	4·2 ± 0·2[d]	2·5 ± 0·1[c]
20:4n-3	nd	nd	nd	nd	nd	0·5 ± 0·1
20:5n-3(EPA)	nd	nd	nd	nd	nd	7·7 ± 0·2
22:6n-3(DHA)	nd	nd	nd	nd	nd	5·2 ± 0·1
Σ n-3 PUFAs	0·1 ± 0·0[a]	0·8 ± 0·0[b]	1·3 ± 0·1[c]	2·4 ± 0·1[d]	4·2 ± 0·2[e]	15·8 ± 0·2[f]
Σ HUFAs	6·7 ± 0·4[a]	16·7 ± 0·2[b]	24·9 ± 0·1[c]	32·8 ± 0·2[d]	59·7 ± 0·9[e]	19·8 ± 0·3[b]

Means in the same row with different lower case superscript letters are significantly different ($P < 0.05$).

FO, Fish oil; LA, linoleic acid; ARA, arachidonic acid; LNA, linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; HUFAs, highly unsaturated fatty acids; nd, not detected.

the dietary lipid source. The experimental diets were formulated and prepared as previously described by Li *et al.* (2016). All experimental diets were stored at −20°C until the time of feeding. No differences were observed in any diets with regard to physical quality. The fatty-acid (FA) compositions of the test diets are given in Table II.

EXPERIMENTAL ANIMALS AND FEEDING TRIAL

The wild-adult *M. anguillicaudatus* were collected from waters near Ezhou city, Hubei province in China. All *M. anguillicaudatus* were subjected to flow cytometry (Beckman Coulter; www.beckmanncoulter.com) for estimating their ploidy levels according to the method described previously by Zhu *et al.* (2012). The breeding method for *M. anguillicaudatus* was conducted according to Gao *et al.* (2014). The experiment was initiated at 30 days post hatching (dph). A total of 360 healthy fish (mean ± s.d. initial body mass 0·4 ± 0·01 g) were divided at the same density (20 fish per tank) into 18 tanks (50 l water volume) with a flow-through system, with triplicate tanks per diet. The freshwater flow to the tanks was 0·1 l min^{-1}, while artificial aeration and natural light–dark regime were applied. During the experimental period, water-quality variables were: temperature 25·0–28·0°C, pH 7·0–7·5 and dissolved oxygen remained at 6·5 mg l^{-1}. Fish were fed the diets to satiation, by hand, four times per day (0800, 1200, 1600 and 2000 hours) for 10 weeks. Diet particle sizes were adjusted according to fish gape, based on visual observation. The amount of feed consumed by the fish in each tank was recorded daily and dead fish were weighed and food consumed (C_F) and feed conversion rate (R_{FC}) were calculated.

SAMPLING

At the end of the 10 week period, 24 h after the last feeding, fish were anaesthetised in an ice slurry. All fish were counted and weighed in batches to determine the survival rate, mass gain (G_M) and specific growth rate (G_{SR}). Then, three fish per tank were randomly selected and the liver and intestine were collected and then fixed in 10% neutral buffered formalin and frozen in liquid nitrogen for histological and histochemical observation, respectively. Another five fish per tank were randomly chosen to dissect the liver and were stored at −80°C for total RNA extraction after having been kept in liquid nitrogen for 8 h. Remaining fish in each replicate tank were dissected to obtain the viscera samples and finally pooled together and kept at −80°C for lipid analysis. The liver and viscera of all fish were weighed to calculate viscera-somatic index (I_{VS}) and hepato-somatic index (I_{HS}).

HISTOLOGICAL AND HISTOCHEMICAL ANALYSES

For histological observation, livers and anterior intestines were fixed in 10% neutral buffered formalin for 24 h and then dehydrated in graded ethanol concentrations and embedded in paraffin wax. Sagittal sections of 5 μm thickness were stained with haematoxylin−eosin (H&E) and then prepared for light microscopy, according to the method of Chen *et al.* (2012). For oil red O staining, frozen livers and anterior intestines were cut on a cryostat microtome. Sections measuring 8 μm in thickness were fixed in cold 10% buffered formalin for 120 min at 4°C and stained with the oil red O solution and then prepared for light microscopy, according to the method of Song *et al.* (2014).

PROXIMATE AND LIPID ANALYSIS

Moisture, crude protein, crude lipid and ash of the experimental diets were determined using standard methods (AOAC, 1995). Total lipids of the diets and viscera were measured following the method of Gao *et al.* (2012). Fatty-acid methyl esters (FAME) were then produced from total lipid aliquots and methylated with boron trifluoride (BF_3) in methanol. The fatty-acid composition of total lipid in the diets and viscera were determined using a gas chromatograph with an OmegawaxTM320 column (Agilents Technologies Inc.; www.agilent.com) according to the method of Gao *et al.* (2012). The temperatures of the injector and detector (FID) were set at 250 and 260°C, respectively. The temperature programme was 200°C (40 min) to 240°C (15 min) at 4°C min^{-1}. High-purity helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The samples (1·0 μl) were manually injected into an injection port and identified FAs were presented as area percentage of total FAs.

MRNA EXPRESSION ANALYSIS

Analyses on transcript levels of the genes included *fas*, *acc*, *Δ6 fad*, *scd*, *cpt1*, *hsl*, *atgl*, *pparα*, *pparγ*, *srebp-1*, *fabp* and *lpl* were conducted by quantitative real-time PCR (qrt-PCR) method. Frozen liver were powdered in a liquid nitrogen-chilled mortar and pestle. Total RNA was extracted from the liver using RNAiso Plus (Takara; www.takara.com) based on the acid guanidinium thiocyanate−phenol−chloroform extraction method. RNA samples were reverse-transcribed to cDNA with oligo-dT primers and a cDNA Synthesis Kit (Takara), following the manufacturer's instructions.

TABLE III. Nucleotide sequences of the primers used to assay gene expression in *Misgurnus anguillicaudatus* by real-time PCR

Target gene	Forward (5′-3′)	Reverse (5′-3′)	Size (bp)	T_M (°C)
fas	AACAGTTGGTTCAGGAGAG	TTCGGCAAGTGAGTTAAGT	160	59
acc	GTATCGTATCATCATCGGTAAC	CCTGCTCGCTTCACATAG	219	59
Δ6 *fad*	AAGTTCAAGGACGCAGAC	TTCAGAGACGACAGACATC	161	59
scd	CCAAGATGATGAGCAAGAAG	CTGATGAAGAAGAGGAGTGA	209	59
cpt1	CCATCTCTTCTGCCTCTAC	GCCACACCATAACCATCA	204	59
hsl	GATTCGCTGGCATCTACC	CGTCTGTTGGAAGTGATATAC	136	59
atgl	TGAACTCATCCAGGCTCTTGT	CACTAATACCACCATCTACATAACG	104	59
pparα	GCTGCCATCATCTGTTGT	GTGTCCTCGGTCTTCTTG	221	59
pparγ	GTGAACCTGGACCTGAAC	CAAGATGATAACGGCAAGAA	261	59
srebp-1	ATACCAGAAGCAGCAGAGT	CCAGAAGAGGACCGATGAT	162	59
fabp	AAGACAACGACCGTCATG	CCTTACAGCAACAACATCAC	144	59
lpl	ACCTGGCTGTAACCTTCA	AACGGCATCATATCTCTGG	294	59

FO, Fish oil; *fas*, fatty acid synthase; *acc*, acetyl-CoA carboxylase; Δ6 *fad*, delta-6 fatty acyl desaturase; *scd*, acyl-CoA delta-9 desaturase; *cpt1*, carnitine palmitoyltransferase 1; *hsl*, hormone-sensitive lipase; *atgl*, adipose triacylglyceride lipase; *ppar*, peroxisome proliferator-activated receptor; *srebp-1*, sterol-regulator element-binding protein-1; *fabp*, fatty acid binding protein; *lpl*, lipoprotein lipase.

Qrt-PCR assays were carried out in a Bio-Rad CFX96 quantitative thermal cycler (www.bio-rad.com) with a 20 μl reaction volume containing 10 μl SYBR green real-time PCR master mix (Bio-Rad), 2 μl of cDNA and 0·2 μm of each primer. Primers sequences are given in Table III. The thermal programme included 1 min at 95°C, 40 cycles at 95°C for 10 s, 59°C for 30 s and a melt-curve step from 65°C gradually increasing 0·5°C s^{-1} to 95°C, with acquisition data every 6 s. All amplicons were initially separated by agarose gel electrophoresis to ensure that they were of correct size. The amplification efficiencies of all genes were approximately equal and ranged from 97·3 to 102·8%. Relative quantification of the target gene transcripts was done using *β-actin* gene and *gapdh* as the reference, which was stably expressed in the present experiment. Normalized gene expression of the control group (0% SO) was set to 1 and the expressions of each target gene for the other groups were expressed relative to the control group. Optimized comparative C_t ($2^{-\Delta\Delta Ct}$) value method (Livak & Schmittgen, 2001) was used here to estimate gene-expression levels. All amplifications were performed in triplicate for each RNA sample.

STATISTICAL ANALYSES

All data were subjected to Levene's test of equality of error variances and one-way ANOVA followed by Tukey's test using SPSS 19·0 (SPSS; www.ibm.com/). Probability values <0·05 were considered statistically significant.

RESULTS

GROWTH PERFORMANCE AND BIOMETRIC PARAMETERS

Growth performance of *M. anguillicaudatus* is shown in Table IV. The final body mass (M), G_M and G_{SR} in fish fed 20% SO diet and 100% FO diet were significantly

TABLE IV. Mean $\pm$ S.E. growth performance of juvenile *Misgurnus anguillicaudatus* (three replicates of 20 fish per tank) fed the experimental soybean (SO) diets over 10 weeks

	Experimental diets					
	0% SO	20% SO	32% SO	56% SO	100% SO	100% FO
Initial body mass (M_0, g)	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Final body mass (M_t, g)	1.41 ± 0.03^a	1.79 ± 0.05^b	1.59 ± 0.05^{ab}	1.50 ± 0.09^{ab}	1.49 ± 0.05^a	1.77 ± 0.06^b
G_M (%)[1]	253.5 ± 9.6^a	348.8 ± 32.9^c	297.7 ± 19.3^b	273.1 ± 19.3^{ab}	274.0 ± 21.6^{ab}	337.8 ± 29.1^c
G_{SR}[2]	0.8 ± 0.0^a	0.9 ± 0.0^c	0.9 ± 0.0^b	0.8 ± 0.0^{ab}	0.8 ± 0.0^{ab}	0.9 ± 0.0^c
R_{FC}[3]	2.0 ± 0.2^c	1.6 ± 0.1^a	1.8 ± 0.1^b	2.0 ± 0.1^c	2.0 ± 0.1^c	1.6 ± 0.1^a
FI (g fish^{-1} 70 days^{-1})[4]	2.1 ± 0.1	2.2 ± 0.0	2.1 ± 0.0	2.2 ± 0.0	2.1 ± 0.0	2.1 ± 0.0
Survival (%)	78.3 ± 7.3	88.3 ± 6	86.7 ± 3.3	78.3 ± 8.8	85 ± 5.8	81.7 ± 7.3

Means in the same row with different lower case superscript letters are significantly different ($P < 0.05$).

FO, Fish oil; [1]G_M, Mass (M) gain $= (M_t - M_0)M_0^{-1}100$, where t is the culture period; [2]G_{SR}, specific growth rate $= [(\ln M_t - \ln M_0)100]t^{-1}$; [3]$R_{FC}$, feed conversion ratio $= [\text{total diet fed (g)}](M_t - M_0)^{-1}$; [4]FI, Feed intake.

higher than those in other groups ($P < 0.05$). Meanwhile, the food conversion ratio (R_{FC}) in fish fed 20% SO and 100% FO diets was significantly lower than that in other groups. Survival of fish fed the test diets, however, did not show any significant differences among all treatments ($P > 0.05$). The I_{HS} and I_{VS} were significantly increased ($P < 0.05$) with incremental dietary SO levels (Fig. 1).

LIPID CONTENT AND FATTY-ACID COMPOSITIONS OF VISCERA

Lipid content and FA composition of viscera is presented in Table V. The visceral lipid content significantly increased ($P < 0.05$) with incremental dietary SO levels, with the highest being in fish fed 100% SO. The visceral lipid content of fish fed 20%
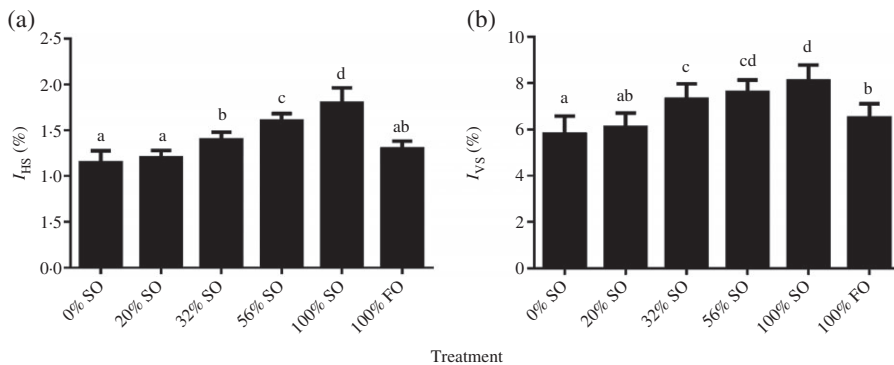

FIG. 1. Means + S.E. ($n = 9$) (a) hepato-somatic index (I_{HS}) and (b) viscera-somatic index (I_{VS}) of juvenile *Misgurnus anguillicaudatus* fed different dietary soybean (SO) levels over 10 weeks. Different lower case letters above columns indicate significant differences among groups ($P < 0.05$). FO, fish oil.

TABLE V. Mean ± S.E. ($n = 20$) lipid content and fatty acid composition in viscera of juvenile *Misgurnus anguillicaudatus* fed the experimental soybean (SO) diets over 10 weeks

	Experimental diets					
	0% SO	20% SO	32% SO	56% SO	100% SO	100% FO
Lipid content (% of wet mass)	7·5 ± 0·7[a]	8·2 ± 0·3[ab]	10·6 ± 0·4[c]	13·4 ± 0·3[d]	15·4 ± 0·7[e]	8·9 ± 0·4[b]
Fatty acid (% of total fatty acids)						
14:0	1·9 ± 0·1[bc]	1·6 ± 0·1[ab]	1·7 ± 0·3[ab]	1·4 ± 0·1[ab]	1·2 ± 0·1[a]	2·4 ± 0·2[c]
16:0	22·3 ± 0·9[c]	20·0 ± 0·5[c]	19·0 ± 1·7[bc]	17·8 ± 1·2[b]	15·1 ± 0·6[a]	17·8 ± 0·6[b]
17:0	0·8 ± 0·1	0·6 ± 0·1	0·7 ± 0·2	0·5 ± 0·0	0·6 ± 0·1	0·7 ± 0·0
18:0	3·6 ± 0·2[c]	3·5 ± 0·3[c]	3·3 ± 0·5[bc]	2·8 ± 0·1[b]	2·5 ± 0·2[ab]	2·2 ± 0·1[a]
Σ SFAs	28·6 ± 0·9[d]	25·7 ± 0·7[c]	24·7 ± 1·7[c]	22·5 ± 1·2[b]	19·4 ± 0·6[a]	23·2 ± 0·7[b]
16:1n-7	8·0 ± 0·2[d]	7·0 ± 0·6[c]	6·4 ± 1·3[b]	6·0 ± 0·6[b]	4·7 ± 0·2[a]	6·7 ± 0·4[bc]
17:1n-9	0·8 ± 0·1	0·6 ± 0·0	0·8 ± 0·0	0·7 ± 0·4	0·4 ± 0·1	0·5 ± 0·2
18:1n-7	3·4 ± 0·1	3·3 ± 0·2	3·2 ± 0·2	3·5 ± 0·1	3·0 ± 0·4	3·7 ± 0·3
18:1n-9	36·4 ± 1·5[e]	34·0 ± 0·6[d]	32·5 ± 2·5[c]	28·8 ± 0·4[b]	26·5 ± 0·8[a]	24·0 ± 0·8[a]
20:1n-7	0·8 ± 0·2[b]	0·7 ± 0·1[b]	0·5 ± 0·1[ab]	0·4 ± 0·1[a]	0·4 ± 0·0[a]	1·5 ± 0·1[c]
20:1n-9	1·2 ± 0·3[a]	0·9 ± 0·0[a]	1·2 ± 0·2[a]	1·2 ± 0·1[a]	1·1 ± 0·2[a]	3·3 ± 0·2[b]
Σ MUFAs	50·6 ± 1·0[e]	46·5 ± 1·0[d]	44·6 ± 1·7[c]	40·5 ± 1·5[b]	36·1 ± 1·3[a]	42·5 ± 0·3[bc]
18:2n-6(LA)	14·7 ± 0·9[a]	20·4 ± 1·3[b]	22·7 ± 2·2[c]	28·3 ± 1·6[d]	33·6 ± 1·1[e]	22·3 ± 1·6[c]
20:2n-6	0·2 ± 0·2[a]	0·6 ± 0·1[b]	0·9 ± 0·1[c]	1·3 ± 0·1[d]	1·8 ± 0·1[e]	0·7 ± 0·1[b]
20:4n-6(ARA)	0·8 ± 0·1[a]	1·4 ± 0·1[b]	1·8 ± 0·0[c]	3·0 ± 0·0[d]	4·2 ± 0·1[e]	0·6 ± 0·1[a]
Σ n-6 PUFAs	15·6 ± 0·8[a]	22·3 ± 1·3[b]	25·4 ± 2·3[c]	32·6 ± 1·6[d]	39·6 ± 1·0[e]	24·3 ± 1·5[c]
18:3n-3(LNA)	0·3 ± 0·0[a]	0·3 ± 0·1[a]	0·2 ± 0·0[a]	0·2 ± 0·0[a]	0·3 ± 0·0[a]	0·7 ± 0·1[b]
18:4n-3	0·5 ± 0·0	0·6 ± 0·1	0·6 ± 0·0	0·6 ± 0·0	0·6 ± 0·1	0·5 ± 0·0
20:4n-3	0·5 ± 0·1[a]	0·5 ± 0·1[a]	0·6 ± 0·1[a]	0·6 ± 0·1[a]	0·6 ± 0·1[a]	2·7 ± 0·3[b]
20:5n-3(EPA)	0·8 ± 0·2	0·7 ± 0·1	1·0 ± 0·1	0·8 ± 0·1	0·8 ± 0·1	0·9 ± 0·0
22:5n-3	0·4 ± 0·1	0·3 ± 0·1	0·2 ± 0·0	0·3 ± 0·1	0·3 ± 0·0	0·3 ± 0·1
22:6n-3(DHA)	1·3 ± 0·1[a]	1·4 ± 0·1[a]	1·3 ± 0·1[a]	1·2 ± 0·3[a]	1·1 ± 0·1[a]	3·5 ± 0·7[b]
Σ n-3 PUFAs	3·8 ± 0·4[a]	3·9 ± 0·4[a]	3·9 ± 0·2[a]	3·7 ± 0·5[a]	3·6 ± 0·3[a]	8·5 ± 1·1[b]

Means in the same row with different lower case superscrip letterts are significantly different ($P < 0.05$).

FO, Fish oil; LA, linoleic acid; ARA, arachidonic acid; LNA, linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

SO diet, however, was not significantly different from that of fish fed 100% FO diet ($P > 0.05$). Dietary SO supplementation significantly increased in the percentage of LA, 20:2n-6, arachidonic acid (ARA) and total n-6 PUFAs in viscera and significantly decreased the percentage of 16:0, 18:1n-9, saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA). In addition, viscera of fish fed 100% FO diet presented significantly higher ($P < 0.05$) levels of LNA, DHA and total n-3 PUFAs, compared to all SO groups.

HISTOLOGY AND HISTOCHEMISTRY OF THE LIVER AND INTESTINE

Liver and intestine of juvenile *M. anguillicaudatus* stained with H&E staining are shown in Figs 2 and 3. The liver structure from 0, 20% SO and 100% FO groups showed normal histology and polygonal cells possessed round nuclei with prominent nucleoli (Fig. 2). Cytoplasmic vacuolation, however, was presented in the 32, 56 and 100% SO groups and the amount of cytoplasmic vacuolation was increased with the dietary SO
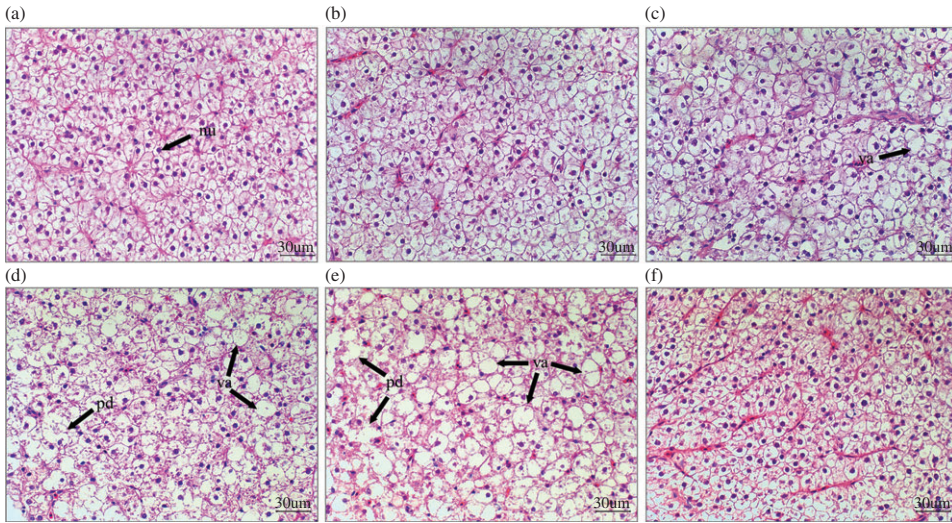
FIG. 2. H&E staining of the liver tissue sections of juvenile *Misgurnus anguillicaudatus* fed diets with different levels of dietary soybean (SO) over 10 weeks: (a) 0% SO; (b) 20% SO; (c) 32% SO; (d) 56% SO; (e) 100% SO; (f) 100% fish oil (FO). Nu, nucleus; pd, parenchyma disorganization; va, vacuole.

level increasing (Fig. 2). In addition, parenchyma disorganization was also presented in the 56% SO and 100% SO groups (Fig. 2). For intestinal histology, dietary SO supplement tended to increase the amount of goblet cells (Fig. 3). Also 32, 56 and 100% SO treatments presented that the absorptive vacuoles of epithelial cells were irregularly arranged on the edge of simple mucosal folding (Fig. 3). Liver and intestine of juvenile *M. anguillicaudatus* stained with oil red O are respectively shown in Figs 4 and 5. Compared with the control group, oil red O staining indicated that the lipid accumulation in the liver and intestine were significantly increased with the dietary SO supplementation, especially, in the 32, 56 and 100% SO groups.

MRNA EXPRESSION LEVELS OF GENES INVOLVED IN LIPID LIPOLYSIS

The effect of the experimental diets on the mRNA levels of lipolytic genes in the liver of juvenile *M. anguillicaudatus* is shown in Fig. 6. Dietary SO supplementation significantly down-regulated the mRNA levels of *cpt1*, *pparα*, *hsl* and *atgl* in the liver. Compared with 0% SO diet, the 100% FO diet significantly up-regulated the mRNA levels of *cpt1* and *pparα*.

MRNA EXPRESSION LEVELS OF GENES INVOLVED IN LIPID TRANSPORT AND LIPOGENESIS

The effect of the experimental diets on the mRNA levels of *fabp*, *lpl* and lipogenic genes in the liver of juvenile *M. anguillicaudatus* is shown in Fig. 7. Dietary SO supplementation significantly increased the mRNA levels of *lpl*, *Δ6fad, scd, pparγ, fas* and *srebp-1* and reduced the mRNA levels of *fabp* and *acc* in the liver.
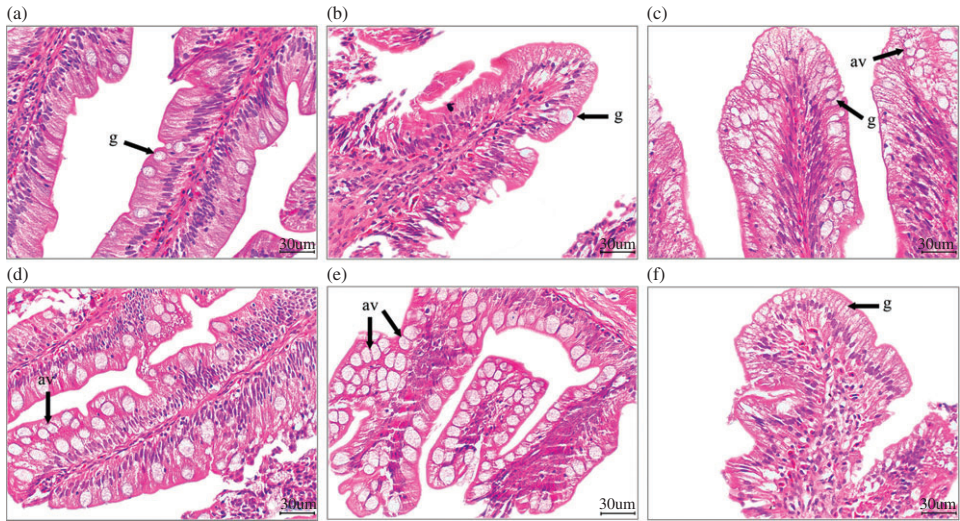
F IG . 3. H&E staining of the intestine tissue sections of juvenile *Misgurnus anguillicaudatus* fed diets with dif-
ferent levels of dietary soybean (SO) over 10 weeks: (a) 0% SO; (b) 20% SO; (c) 32% SO; (d) 56% SO; (e)
100% SO; (f) 100% fish oil (FO). Av, Absorptive vacuole; g, goblet cell.

DISCUSSION

In the present study, the fact that growth of fish fed 20% SO diet was significantly
higher than other SO groups and similar to the 100% FO group, indicated that this level
of dietary SO inclusion maintained normal growth for *M. anguillicaudatus* juveniles.
These results, which could be related to appropriate dietary levels of LA and LNA,
were in agreement with previous studies in *M. amblycephala* (Li *et al.*, 2016). Thus,
dietary LA and LNA concentrations of 1·3 and 0·06% of the total diet, respectively,
might meet the EFA requirements and improve growth in *M. anguillicaudatus* juve-
niles. This is similar to the requirement of dietary LA and LNA concentrations for most
freshwater fish species, which are approximately 1·0 − 1·5 and 0·05 − 1·0% respectively
(Radunzneto *et al.*, 1996; Glencross, 2009; Wang *et al.*, 2014).

This study suggested that the dietary requirement of n-3 PUFA for *M. anguillicau-
datus* was very low, which agreed with the previous study of Radunaneto *et al.*, (1996)
who found that dietary n-3 PUFA requirement for carp *Cyprinus carpio* L. 1758 was
only 0·05%. Conversely, in this study, increasing the levels of SO above 20% affected
the growth negatively. The lower growth performances of fish fed 56 and 100% SO diet
indicated that *M. anguillicaudatus* was unable to utilize higher levels of dietary SO. The
lower mass gain could be explained, at least in part, by the decreased feed utilization,
although it might also have resulted from a poor utilization of excess dietary SO.

The level of SO in the diet had considerable influence on the lipid deposition in
M. anguillicaudatus. The increased lipid contents in liver and intestine of *M. anguil-
licaudatus* might be due to a high dose of dietary LA in SO diet. Few studies have
been conducted to explore the effects of supplementation of high dose of dietary LA
on lipid deposition in fishes, whereas, a number of studies on mammals have demon-
strated detrimental effects on lipid depositon of high doses of dietary LA (Pérez-Matute
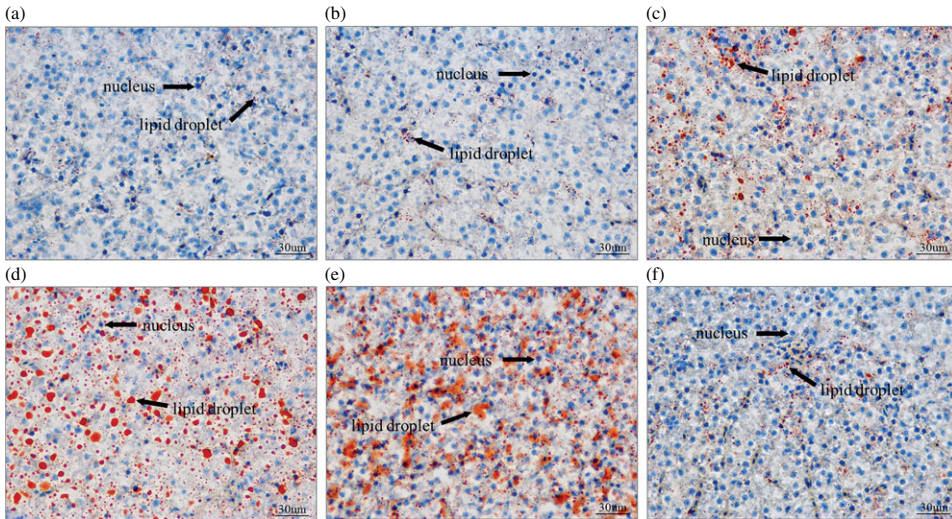*et al.*, 2007; Muhlhausler & Ailhaud, 2013). Alvheim *et al.* (2012) reported a positive

Fig. 4. Oil red O staining of the liver tissue sections of juvenile *Misgurnus anguillicaudatus* fed diets with different levels of dietary soybean (SO) over 10 weeks: (a) 0% SO; (b) 20% SO; (c) 32% SO; (d) 56% SO; (e) 100% SO; (f) 100% fish oil (FO).

correlation between lipogenesis and dietary LA level in rats and Gao *et al.* (2004) indicated that the insulin resistance occurred at high LA accumulation in 3 T3-L1 adipocytes.

Liver plays a central role in lipid metabolism and it is a sensitive organ reflecting dietary lipid change in fishes. *cpt1* is the rate-limiting enzyme for *β*-oxidation of fatty acids (Eaton & Bartlett, 2002), involved in fatty-acid oxidation (Sampath & Ntambi, 2005; Zheng *et al.*, 2014). *hsl* and *atgl* both have the capacity to initiate triacylglycerol (TG) degradation (Reid *et al.*, 2008). In this study, the suppressed hepatic *cpt1*, *hsl* and *atgl* expressions might reflect a decrease in fatty-acid oxidation, TG degradation and eventually reduction of the hepatic-lipid consumption in the higher SO groups, which correlated well with the increased lipid content in their livers. The finding that expression levels of *cpt1*, *hsl* and *atgl* in the 100% FO group were significantly higher than in the 100% SO group may be due to the higher percentage of dietary n-3 LC-PUFA, which could promote transcription of fatty-acid oxidation related genes (Jump, 2004) and mitochondrial proliferation (Vamecq *et al.*, 1993) in the 100% FO diet.

Hepatic-lipid deposition was also connected with the hepatic lipogenesis and uptake of mobilized fatty acids from the adipose tissue (Vyas *et al.*, 2012). LPL is considered as the key regulatory enzyme in the lipid uptake; it hydrolyzes triglyceride present in plasma lipoproteins and supplies free fatty acids for storage in adipose tissue, or for oxidation in other tissues and plays a pivotal role in regulating lipid content in fish (Tan *et al.*, 2009; Zheng *et al.*, 2013). The fact that increasing dietary SO levels significantly up-regulated the mRNA levels of lipogenesis genes (*fas* and *lpl*) is similar to turbot *Scophthalmus maximus* (L. 1758) (Mo *et al.*, 2014) and Atlantic salmon *Salmo salar* L. 1758 (Morais *et al.*, 2011). This indicated that the high percentage of SO in the diet increased the expressions of *lpl*, which could enhance removal of triglyceride rich lipoproteins by the liver tissue (Lu *et al.*, 2013). The increase in liver *fas* and *lpl*
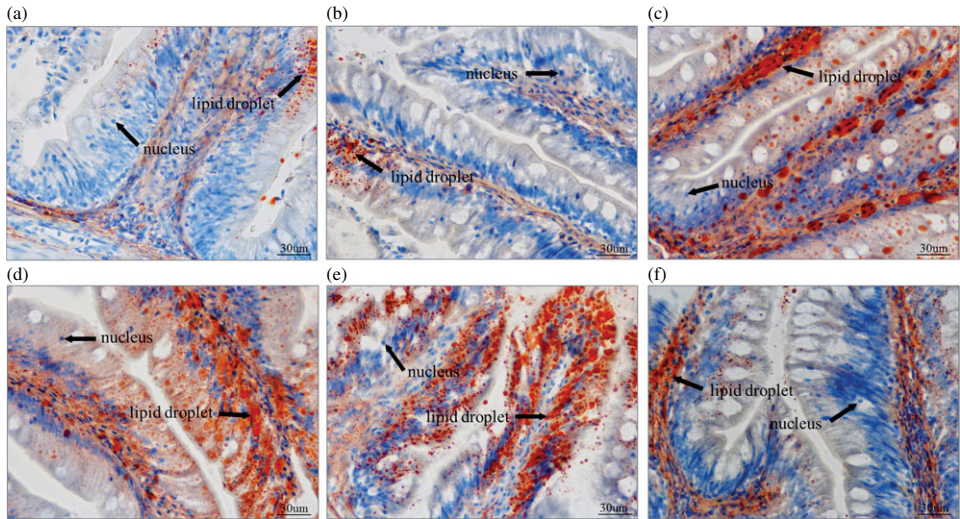
FIG. 5. Oil red O staining of the intestine tissue sections juvenile *Misgurnus anguillicaudatus* fed diets with different levels of dietary soybean (SO) over 10 weeks: (a) 0% SO; (b) 20% SO; (c) 32% SO; (d) 56% SO; (e) 100% SO; (f) 100% fish oil (FO).

activity might contribute to an explanation of the increased liver fat deposition of *M. anguillicaudatus* fed diet with increasing graded levels of substitution of FO with SO.

pparα and *pparγ* are two key transcription factors that are involved in lipid metabolism. *pparα* plays an important role in the catabolism of fatty acids by up-regulating the expression of several key enzymes involved in fatty-acid oxidation (Lu *et al.*, 2013; Li *et al.*, 2015a), while *pparγ* plays pivotal role in the regulation of lipogenesis and promotes lipid storage (Zheng *et al.*, 2013). The finding that dietary SO supplementation down-regulated expression of *pparα* gene but up-regulated *pparγ* mRNA levels in the liver, was consistent with the increased lipid content in the liver. This was in agreement with the result obtained working on *M. amblycephala* by Lu *et al.* (2013), who reported that the *pparα* mRNA expression was suppressed by a high-fat diet. Furthermore, the expressions of *pparα* and *pparγ* in the liver of *M. anguillicaudatus* were closely correlated with those of *cpt1*, *hsl*, *atgl*, *fas* and *lpl*. It further step indicated the importance of *pparα* and *pparγ* in the regulation of lipid metabolism (Tabarin *et al.*, 2005; Zheng *et al.*, 2013). *srebp-1*, *fabps* and *acc* are critical regulators of lipid metabolism, by regulating the enzyme and substrate for *de novo* lipogenesis (Osborne, 2000; Shimano, 2000; Obici *et al.*, 2003). The fact that dietary SO supplementation up-regulated the mRNA level of *srebp-1* may be correlated with the high concentration of HUFAs in the SO diets, especially in the 100% SO diet (Howell *et al.*, 2009). The mRNA levels of *acc* and *fabp*, however, were down-regulated by the increasing level of dietary SO, which were negatively correlated with lipid deposition in the liver. This might correspond to a form of liver protection, limiting excess lipid deposition in the liver.

Under physiological conditions, the desaturase gene family members are evolutionarily conserved and they play an important role in the regulation of fatty-acid metabolism (Guo *et al.*, 2013). In the present study, the increasing graded levels of dietary SO
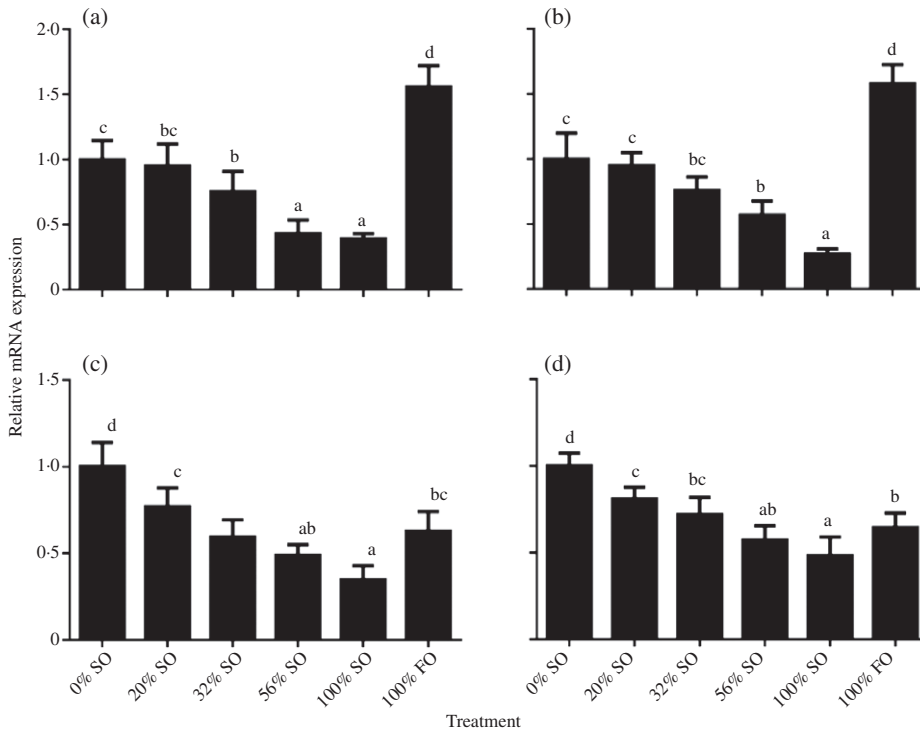
FIG. 6. Mean + S.E. (*n* = 3) relative mRNA expression of genes involved in lipolysis, in liver of juvenile *Misgurnus anguillicaudatus* fed experimental soybean diets over 10 weeks: (a) *pparα*, (b) *cpt1*, (c) *atgl*, (d) *hsl*. Different lower case letters indicate significant differences among groups (*P* < 0·05).

up-regulated the mRNA levels of Δ*6 fad*, which was well correlated with the increase in ARA content of the viscera. This might be due to the higher content of LA, the substrate for desaturation of the n-6 PUFA series in the SO diets. *scd* plays an important role in fatty-acid metabolism by catalysing the conversion of saturated fatty acids to n-9 monounsaturated fatty acids (Roongta *et al.*, 2011; Zhang *et al.*, 2014). Additionally, increased SCD activity has been connected with increased fatty-acid synthesis and decreased fatty-acid oxidation (Sampath *et al.*, 2007; Bjermo & Riserus, 2010). In the present study, a similar trend was also found in hepatic *scd* expression, which was in agreement with the results reported in *M. amblycephala* (Li *et al.*, 2016). It indicated that a high percentage of dietary SO supplementation up-regulated *scd* expression, leading to lipid accumulation in adipose tissues, which was consistent with the results of hepatic-lipid content in this study. The increase in liver *scd* may be related to the high dose of LA in SO fed fish, because a high dose of dietary LA could reduce triacylglycerol breakdown to increase triacylglycerol assembly and to provoke insulin resistance (Harant-Farrugia *et al.*, 2014).

In summary, the present study indicated that supplementation of 20% SO (8·1% dietary lipid) in diet could improve growth performance of *M. anguillicaudatus* juveniles. The higher percentage of SO supplementation, however, was found to reduce growth and to increase lipid content in viscera. The increased lipid content could be attributed to the up-regulation of the mRNA levels of lipogenic genes (such as Δ*6fad,*
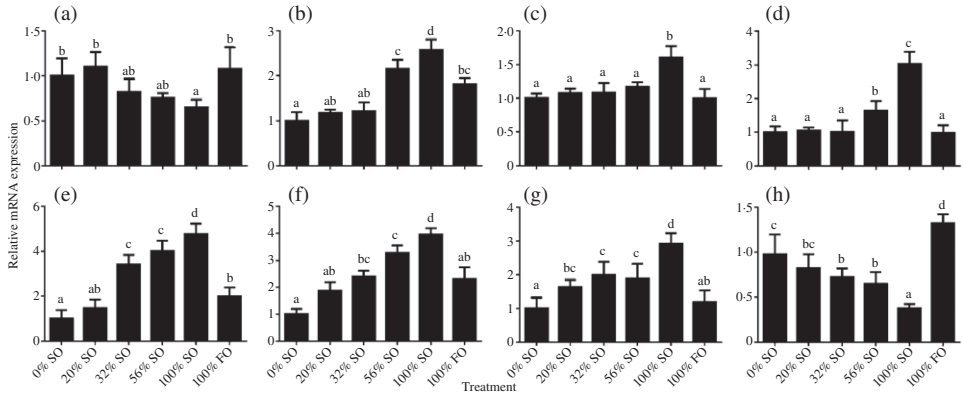
FIG. 7. Mean + S.E. ($n = 3$) relative mRNA expression of genes involved in lipid transport, (a) *fabp* and (b) *lpl* and lipogenesis (c) *6fad*, (d), (e) *pparγ*, (f) *fas*, (g) *srebp-1* and (h) *acc* in liver of juvenile *Misgurnus anguillicaudatus* fed experimental soybean diets over 10 weeks. Different lower case letters indicate significant differences among groups ($P < 0.05$).

scd, pparγ fas and *srebp-1*) and the down-regulation of the mRNA levels of the lipolytic genes (such as *pparα, cpt1, atgl* and *hsl*). Thus, incremental dietary SO level influenced lipid deposition of viscera including liver and intestine probably through different lipid metabolic strategies occurring as a result of competition between lipolysis and lipogenesis and between export and import of lipids in *M. anguillicaudatus*.

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