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Dietary methionine level influences growth and lipid metabolism via GCN2 pathway in cobia (*Rachycentron canadum*)

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ABSTRACT

This study investigated the effect of dietary methionine level on growth and lipid metabolism via the general control nonderepressible2 kinase (GCN2) pathway in cobia (*Rachycentron canadum*). Cobia were fed diets with six levels of methionine (0.62%, 0.84%, 1.02%, 1.15%, 1.25% and 1.42% of dry diet) with a constant cystine level (0.42% dry diet). The feeding experiment began in September 2013 and ended in December 2013; during the experiment, cobia were fed ad libitum twice daily (7:00 and 18:00 h) for 10 weeks. Cobia fed the diet with 1.02% methionine showed elevated weight gain (WG) and feed efficiency ratio (FER) compared with those fed the other diets ($P < 0.05$). The content of liver lipid, total triglyceride, and total cholesterol were first enhanced significantly with increasing dietary methionine level from 0.62% to 1.02%, and then decreased markedly with higher levels of dietary methionine level (1.02% to 1.42%). Crude lipid was markedly elevated when the dietary methionine level was 1.02%, and then plateaued with higher dietary methionine level. The expression of genes associated with hepatic lipid synthesis (sterol regulatory element binding protein-1, peroxisome proliferator activated receptor γ, fatty acid synthetase, and stearoyl-CoA desaturase-1) were markedly up-regulated in fish fed the diet containing 1.02% methionine, whereas the transcriptional levels of lipolytic genes (peroxisome proliferator activated receptor α, carnitine acyl transferase-1, and lipase lipoprotein lipase) were elevated in fish fed the methionine-deficient diet (0.62%; $P < 0.05$). The expression of insulin-like growth factor-I (IGF-I) was suppressed by the methionine-deficient diet, whereas the hepatic mRNA expression levels of genes related to amino acid responses (AAR), i.e., GCN2, activating transcription factor 4 (ATF4), CCAAT enhancer binding protein β (C/EBPβ), and asparagine synthetase (ASNS), were significantly up-regulated. In conclusion, the dietary methionine requirement of cobia was estimated to be 1.04% and 1.15% of dry matter (2.23% and 2.45% dietary protein) on the basis of WG and FER, respectively. Results of this study suggested that methionine deficiency could suppress growth, decrease lipid content, and inhibit expression of IGF-I and some genes related to lipid synthesis in cobia; these changes might be regulated by inducing the expression of genes related to the GCN2 pathway (GCN2, ATF4, C/EBPβ, and ASNS).

Statement of relevance

The present study was conducted to investigate the effect of dietary methionine on growth performance, plasma biochemical indexes, lipid content and gene expression involved in lipid metabolism and GCN2 pathway in cobia (*Rachycentron canadum*). Our findings have showed that methionine deficiency could suppress growth, decrease lipid content and inhibit expressions of IGF-I and some lipid synthesis related genes of cobia, which may be regulated by inducing the mRNA expressions of GCN2 pathway related genes (GCN2, ATF4, C/EBPβ and ASNS). The results are reliable and of both theoretical and practical importance.
The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. I have read and have abided by the statement of ethical standards for manuscripts submitted to Aquaculture.

Abbreviations: SREBP-1, sterol regulatory element binding protein-1; PPARγ, peroxisome proliferator activated receptor γ; FAS, fatty acid synthetase; ACC-1, acetyl-coenzyme A carboxylase-1; SCD-1, stearoyl-CoA desaturase-1; G6PD, glucose-6-phosphate dehydrogenase; PPARα, peroxisome proliferator activated receptor α; CPT-1, carnitine acyl transferase-1; HSL, hormone sensitive lipase; LPL, lipase lipoprotein lipase; GCN2, general control non-derepressible kinase 2; ATF4, activating transcription factor 4; C/EBPβ, CCAAT enhancer binding protein β; ASNS, asparagine synthetase; IGF-I, insulin-like growth factor-I; AMPK, adenosine monophosphate activated protein kinase; TOR, target of rapamycin.

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1. Introduction

Cobia, an economically important carnivorous marine species, has been widely reared in south China. Recently, nutritional studies of cobia have been conducted intensively (Chi et al., 2011; Chi et al., 2014; Luo, 2013; Zhou et al., 2006). However, no information is available on the effect of amino acid nutrition on lipid metabolism and amino acid responses of cobia.

Soybean protein is globally known as the largest output of plant protein; it has been widely used for replacement of fish meal with the rapid development of aquatic feed and aquaculture (Gatlin et al., 2007; Hardy, 2010; Naylor et al., 1998; Naylor et al., 2000). However, the essential amino acid (EAA) profile of soybean protein differs from that of fish meal. The limited amount of methionine in soybean protein is the main shortcoming when substitution of fishmeal reaches a certain level. Recent studies have shown that high level of substitution of fish meal with soybean protein caused decreased lipid synthesis and suppressed growth in several cultured fish species (Aoyama et al., 2000; Dias et al., 2005; Robaina et al., 1995; Vilhelmsson et al., 2004; Xu, 2014). The limited amount of methionine in soybean protein might be one of the reasons for the changes in lipid metabolism and inhibition of growth when substitution of fishmeal reaches a certain level (Dias et al., 2005; Xu, 2014).

Previous studies have shown that single EAAs, including methionine, arginine, tryptophan, and branched chain amino acids, could regulate lipid metabolism and glucose utilization in mammals (Y. Cheng et al., 2010; Z. Cheng et al., 2010; Du et al., 2012; Dudek and Semenkovich, 1995; Guo and Cavener, 2007; Jousse et al., 2004). Studies in mammals indicated that the lipid content of liver, plasma, and body as well as the expression of genes associated with de novo lipogenesis and triglyceride synthesis were significantly decreased, but the lipid oxidation ability was enhanced by methionine deficiency (Hirche et al., 2006; Lees et al., 2014; Plaisance et al., 2012). Most studies in fish have investigated the effects of methionine on the growth performance, nutrient utilization, body composition, as well as lipid deposition (Ahmed et al., 2003; Ahmed, 2014; Luo et al., 2005; Rolland et al., 2015; Ruchimat et al., 1997; Zhou et al., 2006). Moderate amount of methionine has been shown to enhance fish growth. However, the effects of methionine on lipid content differ markedly across different fish species. Furthermore, little information is available on the effects of dietary methionine level on the expression of genes related to lipid metabolism (Craig and Moon, 2013; Espe et al., 2010; Lansard et al., 2011). Espe et al. (2010) showed that limited amount of methionine in diets enhanced the ratio of 18:1 to 18:0 fatty acids, fatty acid synthetase (FAS) activity, and triglyceride level in the liver of salmon (*Oncorhynchus kisutch*). However, the expression of FAS was upregulated by the administration of the combination of insulin and methionine in hepatocytes of rainbow trout (*Oncorhynchus mykiss*; Lansard et al., 2011). Craig and Moon (2013) reported that methionine restriction down-regulated the expression of genes related to hepatic lipid synthesis (sterol regulatory element binding protein-1 (SREBP-1) and FAS) and up-regulated the expression of carnitine palmitoyl transferase-1 (CPT-1), a rate-limiting enzyme of fatty acid oxidation, in rainbow trout.

Until recently, mechanisms underlying the modulation of fish lipid metabolism by methionine were poorly understood (Espe et al., 2010; Craig and Moon, 2013; Lansard et al., 2011). In rainbow trout, methionine deficiency inhibited lipogenesis likely due to the activation of adenosine monophosphate activated protein kinase (AMPK) (Craig and Moon, 2013; Hasek et al., 2010). However, Lansard et al. (2011) confirmed that the up-regulation of FAS, ATP citrate lyase (ACLY), and SREBP-1 by supplementation of EAAs and insulin-like growth factor (IGF) were associated with the activation of target of rapamycin (TOR) signaling in hepatic cells of rainbow trout. The general control nonderepressible kinase 2 (GCN2) pathway plays a primary role in the regulation of amino acid metabolism and translation initiation in response to amino acid deprivation by binding to uncharged tRNA (Anthony et al., 2004; Kilberg et al., 2005). Guo and Cavener (2007) found that the GCN2 pathway could reduce the expression of genes related to lipid synthesis in mice fed leucine-deficient diet. Methionine deficiency has been reported to play a similar role in lipid metabolism by activating the GCN2 pathway (Hao et al., 2005; Lees et al., 2014; Plaisance et al., 2012). However, no studies have investigated this aspect in mariculture fish species. Therefore, this study investigated the mechanism of how methionine regulates lipid metabolism via the GCN2 pathway in cobia.

2. Materials and methods

2.1. Experimental design

The protein in the basal diet was provided by fish meal, soybean meal, gelatin, and amino acid mixture with the lowest methionine content. The oil in the basal diet was supplied by fish oil and soy oil. Six isonitrogenous and isolipidic diets containing six levels of methionine (0.62%, 0.84%, 1.02%, 1.15%, 1.25%, and 1.42% dry diet) were prepared by supplementation of crystalline methionine (Tables 1 and 2). Isonitrogenous diets were obtained by adjusting the levels of glycine, one of the nonessential amino acids. The pH of the diets was adjusted to 7.0 by adding 6.0 N NaOH (solution). Ingredients were intensively mixed with fish oil and water, and then pelleted and dehumidified for about 12 h at 45 °C. Dried diets were sieved into two specific pellet sizes (4.0 × 8.0 mm and 6.0 × 8.0 mm) and stored in plastic bags in a refrigerator at −20 °C until use.

2.2. Fish rearing and sampling

The feeding experiment was performed in accordance with the experimental procedures of the Key Laboratory of Mariculture (Ocean University of China), as described by Zuo et al. (2013). Six hundred disease-free juvenile cobia (120 days old) were purchased from a fishery near Zhanjiang, Guangdong, China, and acclimatized for 14 days.

Table 1
Formulation and chemical proximate composition of the experimental diets (dry matter %).

Ingredient	Diet number and methionine supplementation level %					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
	0.00	0.20	0.40	0.60	0.80	1.00
Fish meal	25.00	25.00	25.00	25.00	25.00	25.00
Soybean meal	25.00	25.00	25.00	25.00	25.00	25.00
Wheat flour	19.30	19.30	19.30	19.30	19.30	19.30
Gelatin	6.00	6.00	6.00	6.00	6.00	6.00
Beer yeast	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix[1]	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[1]	2.00	2.00	2.00	2.00	2.00	2.00
Amino acid mixture[2]	4.28	4.28	4.28	4.28	4.28	4.28
Fish oil	4.00	4.00	4.00	4.00	4.00	4.00
Soy oil	4.00	4.00	4.00	4.00	4.00	4.00
Mold inhibitor	0.10	0.10	0.10	0.10	0.10	0.10
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05
Lecithin	2.50	2.50	2.50	2.50	2.50	2.50
Glycine	1.00	0.80	0.60	0.40	0.20	0.00
L-methionine	0.00	0.20	0.40	0.60	0.80	1.00
Microcrystalline cellulose	1.77	1.77	1.77	1.77	1.77	1.77
Total	100.00	100.00	100.00	100.00	100.00	100.00
Proximate analysis (n = 3)						
L-methionine (%)	0.62	0.83	1.02	1.15	1.25	1.42
L-cystine (%)	0.40	0.41	0.38	0.39	0.42	0.41
Crude protein (%)	46.57	46.64	46.45	46.38	46.56	46.41
Crude lipid (%)	12.84	12.79	12.87	12.69	12.77	12.78
Moisture (%)	9.31	9.40	9.42	9.26	9.20	9.33

[1] Mineral mixture and vitamin mixture according to Peng et al. (2014).
[2] Amino acid mixture (g 100 g-1 diet): L-histidine, 0.043; L-isoleucine, 0.318; L-lysine, 1.26; L-Phenylalanine, 0.243; L-threonine, 0.312; L-valine, 0.392; L-tyrosine, 0.479; L-tryptophan 0.35. Amino acids obtained from Huayang Chemical (Hebei, China), the form of L-lysine is L-lysine·HCl.

Table 2
Amino acid (AA) composition of the test ingredient used in the experiment (% dry matter).

Amino acids[1]	Diet number and methionine supplementation level %					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
	0.00	0.20	0.40	0.60	0.80	1.00
EAAs[2]						
Lysine	3.31	3.48	3.44	3.41	3.49	3.33
Histidine	0.87	0.92	0.91	0.90	0.91	0.89
Arginine	2.51	2.64	2.62	2.65	2.66	2.62
Leucine	3.12	3.38	3.33	3.28	3.39	3.29
Isoleucine	1.66	1.83	1.76	1.79	1.82	1.75
Methionine	**0.62**	**0.83**	**1.02**	**1.15**	**1.25**	**1.42**
Valine	1.93	2.08	2.07	2.10	2.13	2.02
Phenylalanine	1.89	2.03	2.06	1.97	2.05	1.97
Threonine	1.62	1.73	1.73	1.70	1.76	1.70
NEAAs[3]						
Aspartic acid	3.23	3.50	3.42	3.40	3.51	3.36
Cystine	0.40	0.41	0.38	0.39	0.42	0.41
Serine	1.62	1.69	1.70	1.67	1.73	1.68
Gulmatic acid	6.24	6.61	6.59	6.57	6.72	6.53
Glycine	3.78	3.84	3.69	3.65	3.43	3.08
Alanine	2.24	2.40	2.40	2.45	2.48	2.33
Tyrosine	1.49	1.58	1.63	1.57	1.57	1.59

The boldface is the variable quantity of the experiment.
[1] Tryptophan was not analyzed.
[2] EAAs: essential amino acids.
[3] NEAA: non-essential amino acids.

Of these, 360 cobia (150.9 ± 0.8 g) were randomly allocated to 18 sea cages (1.5 m × 1.5 m × 2.5 m) with 20 fish per sea cage. The feeding experiment was initiated in September 2013 and ended in December 2013; the fish were well fed for 10 weeks. The fish were slowly and evenly fed by hand to apparent satiation twice daily at 07:00 and 18:00 h. Each diet was randomly assigned to triplicate cages. During the feeding trial, the photoperiod was natural (light–dark cycle), the rearing water temperature fluctuated from 28 °C to 31 °C, dissolved oxygen was >6.5 mg/L, and salinity was 26‰ to 31‰.

At the termination of the 70-day feeding trial, fish were stopped feeding for 24 h before sampling. Subsequently, all fish from each cage were counted and weighed after they were anesthetized with eugenol (1:10,000). The sampling procedures were performed as described by Zuo et al. (2013). The fish were killed by cervical section, and three fish from each cage were randomly collected and stored at −20 °C for the estimation of the final carcass composition. A batch of four fish was used to obtain blood samples from the caudal vein by using 2 mL heparinized syringes and centrifuged to collect plasma. Four fish per cage were dissected, and their livers were pooled into 1.5 mL tubes, frozen in liquid nitrogen, and then stored in a refrigerator at −80 °C. Livers and muscles from eight fish per cage were filled into 4 tubes (10 mL), frozen in an ice-bath, and then stored at −20 °C.

2.3. Chemical analysis

Crude lipid, crude protein, ash, and moisture contents were analyzed using the established standard methods (AOAC, 1990). The lipid content of the liver and muscle samples was extracted using chloroform/methanol (2:1, *v*/*v*) and evaporated in a heat block (Herzig et al., 2003; Peng et al., 2014). The amino acid composition of the diets (except tryptophan) was analyzed according to Y. Cheng et al. (2010), Z. Cheng et al. (2010) using an L-8900 Automatic Amino Acid Analyzer (Hitachi, Japan). The fatty acid composition of the muscle and liver samples was determined according to the established method (Metcalfe et al., 1966; Ai et al., 2008) and analyzed using gas chromatography (GS; HP6890; USA). Plasma triglyceride (TG), total cholesterol, total protein (TP), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol were analyzed using BS180 Auto Detecter (BS-400; Mindray, China) using the matching commercial reagents and kits.

2.4. Quantitative PCR

The liver was ground in liquid nitrogen, and total RNA was extracted using Trizol Reagent (Takara, Japan). The quality of total RNA was evaluated using a Nano Drop® ND-1000 spectrophotometer and agarose gel electrophoresis (1.2%). Subsequently, Prime Script ™ RT reagent Kit (Takara, Japan) was used to synthesize complementary DNA (cDNA). The cDNA was diluted with RNAase-free water to 80 ng/μL. The reaction system was implemented in a total volume of 25 μL (primer: 1 μL; cDNA: 1 μL; 2× SYBR® premix Ex Taq™ II: 12.5 μL; water: 9.5 μL). The quantitative polymerase chain reaction (PCR) program was performed using a quantitative PCR machine (Mastercycler® ep realplex; Eppendorf, Germany) as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 10 s at 95 °C, 10 s at 59 °C, and 20 s at 72 °C. The specific primer sequences for β-actin, FAS, SERBP-1, peroxisome proliferator activated receptor α (PPARα), peroxisome proliferator activated receptor γ (PPARγ), carnitine acyl transferase-1 (CPT-1), acetyl-coenzyme A carboxylase-1 (ACC1), glucose-6-phosphate dehydrogenase (G6PD), stearoyl-CoA desaturase-1 (SCD-1), hormone sensitive lipase (HSL), lipase lipoprotein lipase (LPL), insulin-like growth factor-I (IGF-I), activating transcription factor (ATF4), GCN2, asparagine synthetase (ASNS), and CCAAT enhancer binding protein β (C/EBPβ) are listed in Table 3. At the end of each PCR, melting curve analysis was performed for confirming the single DNA fragment product. A standard curve was obtained using 5-fold serial dilutions (in triplicate) of cDNA was used to calculate the amplification efficiency by using the following equation: $E = 10(-1 / \text{slope}) - 1$. The amplification efficiencies ranged between 0.92 and 1.05. The data of transcript expression were calculated using comparative CT method ($2^{-\Delta\Delta CT}$ method) as described by Zuo et al. (2013).

2.5. Calculations and statistical analysis

$$\text{Survival rate (SR\%)} = 100 \times F_N/I_N$$
$$\text{Weight gain (WG\%)} = 100 \times (w_t - w_0)/w_0$$
$$\text{Feed intake (FI,\%/day)} = 100 \times F_C(g)/[(w_0 + w_t)]/t$$
$$\text{Feed efficiency ratio (FER)} = (w_t - w_0)(g)/F_C\,(g)$$
$$\text{Hepatosomatic index (HSI,\%)} = 100 \times \text{wet liver weight (g)}/\text{wet body weight (g)}$$

where F_N is the final fish number, I_N is the initial fish number, w_t is the wet final weight, w_0 is the wet initial weight, F_C is the dry feed consumed and t is the experimental period (day).

SPSS 18.0 software was used for all statistical treatments. All treatments were analyzed using one-way analysis of variance (ANOVA) and then by Tukey's multiple-range test. Values with $P < 0.05$ were considered to be significant. All data are shown as the mean ± standard errors. The quadratic regression model was used to estimate the optimum methionine requirement on the basis of weight gain (WG) and feed efficiency ratio (FER).

3. Results

3.1. Survival rate, growth performance, and methionine requirement

Survival rate (93.3%–96.7%) was independent of dietary methionine level ($P > 0.05$). WG increased markedly as dietary methionine increased up to 1.02% ($P < 0.05$) and decreased gradually thereafter ($P < 0.05$). FER showed a similar tendency as WG (Table 4).

Quadratic regression analysis was used to evaluate the methionine requirement of cobia based on WG and FER, and the following equations were obtained: $Y = -98.948x^2 + 206.45\times + 10.202$, $R^2 = 0.826$ and $Y = -0.3958x^2 + 0.9005\times + 0.3461$, $R^2 = 0.943$, respectively. The dietary methionine requirement of cobia was estimated to be 1.04% of diet (Fig. 1) and 1.14% of diet (Fig. 2), 2.23% and 2.45% of dietary protein, respectively.

Table 3
Nucleotide sequence of primers for real-time quantitative PCR amplification.

Gene	Nucleotide sequence (5′-3′)	Amplicon size (bp)	GenBank reference or publication
FAS	F: ACGGTTACGCCAACTCATC R: TGCTTCGCTCTTCACCACC	225	FJ842648.1
SERBP-1	F: GGCTACTGTGACTGGCTGTGA R: TGTCGCTGTGGCTCAAACTCA	203	KT075048
PPARα	F: CCTGTGTGGAGACCTGATGGAG R: AGTGGAAGCCTGAAGCCTTGT	199	EF680883.1
PPARγ	F: CCGCTCGTATCCTCCATCTCTT R: ATGCCGCCACCGTATCATCC	172	EF680884.1
CPT-1	F: TGCTGTTGCCACGGGAGATT R: CGCTGCTCGGTGTCATCAAG	180	KT075040
ACC1	F: CAGGTCCAGGCAGAAGTTCCA R: AGCCAGCTTCACAGCACACT	190	KT075038
G6PD	F: TCAGGGACGAGGAGGCTACTT R: ACCCAACACCACATCAGACAGT	147	KT075042
SCD-1	F: GACAGACGCAGACCCTCACAA R: AAGCACATTAGCAGCACAGACG	143	KT075047
HSL	F: GCAGCCAACCACAGAGCATC R: AGCAAGCAGGGAGACGAAGG	166	KT075045
LPL	F: TGAGCACGCAGATGACCAGAG R: AGTCCCTTGATCCCTTCCAGTG	170	KT075046
IGF-I	F: GAGCGCGATGTGCTGTATCT R: GTCCACAATGCCGCGTGACC	211	(Luo, 2013)
ATF4	F: GCCACATCTCTTACCACCGACA R: GCCAGCAACTGACTCAATTCCT	169	KT075039
GCN2	F: TGAGGATACGGTGATGCCAACA R: AAGGAGGCTTGAGGAAGGAGTG	186	KT075044
ASNS	F: TGGTTGCTGCTACACTGGTGAA R: GAGGCTCGGATGGTGGTGATAT	188	KT075043
C/EBPβ	F: ATCAGCAGCAGCAGCAGCA R: CTCAGTGTAGCCCAGCGAGTAG	156	DQ350620.1
β-actin	F: TGCGTGACATCAAGGAGAAGC R: TACCGAGGAAGGAAGGCTGG	180	(Luo, 2013)

FAS: fatty acid synthetase; SREBP-1: sterol regulatory element binding protein-1; PPARγ: peroxisome proliferator activated receptor γ; CPT-1: carnitine acyl transferase-I; ACC-1: acetyl-coenzyme A carboxylase-1; SCD-1: stearoyl-CoAdesaturase-1; G6PD: Glucose-6-phosphate dehydrogenase; PPARα: peroxisome proliferator activated receptor α; HSL: hormone sensitive; LPL: lipase lipoprotein lipase; GCN2: genes: general control nonderepressible2; ATF4: activating transcription factor; C/EBPβ: CCAAT enhancer binding protein β; ASNS: asparagine synthetase; IGF-I: insulin like growth factor-I.

3.2. Body composition

Crude protein and lipid content of whole body increased markedly as dietary methionine increased from 0.62% to 1.02% ($P < 0.05$), and then plateaued when dietary methionine level was above 1.02% ($P > 0.05$). Moisture content showed an opposite trend from that of protein and lipid contents. The hepatic lipid content was elevated as dietary methionine increased from 0.62% to 1.02%, and thereafter decreased when dietary methionine increased from 1.02% to 1.42%. The lipid content of muscle was not significantly different among the dietary treatments ($P > 0.05$; Table 5).

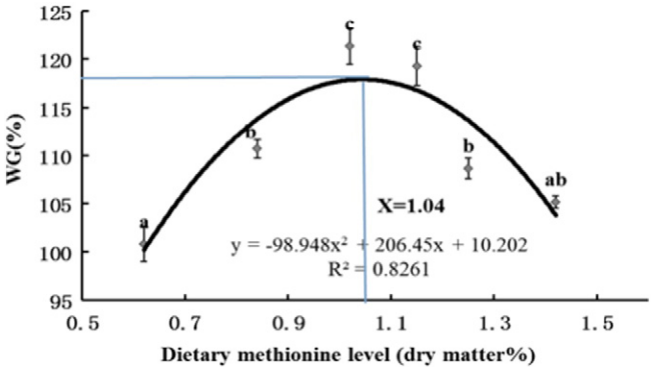

Fig. 1. Relationship between dietary methionine level and weight gain rate of cobia (*Rachycentron canadum*) fed experimental diets for 10 weeks based on quadratic regression analysis, and indicated that the optimum dietary methionine level is 1.04%.

3.3. Liver fatty acid profile

The hepatic content of 18:1 and monounsaturated fatty acid in 1.02% methionine group was the highest among all treatments ($P < 0.05$). The content of n-6 polyunsaturated fatty acids in the liver was higher in fish fed the diet with moderate level of methionine (1.02%) than in the methionine-deficient group (0.62%; $P < 0.05$; Table 6).

3.4. Plasma biochemical indexes

TP and glucose contents in the plasma of fish fed the diet with 1.14% methionine were significantly higher than those in the control (0.62%) and the excessive (1.42%) methionine groups ($P < 0.05$). TG and HDL-C in the plasma were positively correlated to dietary methionine level (from 0.62% to 1.02%; $P < 0.05$) and decreased with further increase of dietary methionine level (Table 7).

3.5. Expression of genes related to hepatic lipid metabolism in cobia

Expression of genes involved in hepatic lipid synthesis (SREBP-1, PPAR γ, FAS, and SCD-1) was markedly higher in fish fed the diet with 1.02% methionine (Fig. 3a), whereas the transcriptional levels of lipolytic genes (PPARα, CPT-1, and LPL) were elevated in fish fed the methionine-deficient diet (0.62%; $P < 0.05$; Fig. 3b).

3.6. Expression of genes related to the GCN2 pathway and IGF-I in cobia

The transcriptional levels of genes involved in the GCN2 pathway (GCN2, ATF4, C/EBPβ, and ASNS) in the liver were the highest in fish fed the diet containing 0.62% methionine, which were significantly higher than those of fish fed diets containing 1.02% and 1.42% methionine

Table 4
Effects of dietary methionine levels on growth and feed utilization of cobia (*Rachycentron canadum*) for 10 weeks.

Index	Dietary methionine level (dry weight %)					
	0.62	0.84	1.02	1.15	1.25	1.42
SR[1]	95.00 ± 2.88	93.33 ± 1.67	96.67 ± 1.67	95.00 ± 1.67	96.67 ± 3.33	96.67 ± 3.33
WG[2]	100.84 ± 1.83[a]	110.72 ± 0.94[b]	121.38 ± 1.90[c]	119.28 ± 2.07[c]	108.68 ± 1.10[b]	105.18 ± 0.65[ab]
FER[3]	0.75 ± 0.01[a]	0.81 ± 0.02[b]	0.86 ± 0.02[c]	0.87 ± 0.02[c]	0.84 ± 0.01[bc]	0.83 ± 0.01[bc]
FI[4]	1.81 ± 0.11	1.77 ± 0.23	1.73 ± 0.30	1.76 ± 0.22	1.80 ± 0.13	1.80 ± 0.10
HSI[5]	1.29 ± 0.03	1.30 ± 0.08	1.28 ± 0.15	1.46 ± 0.20	1.64 ± 0.11	1.12 ± 0.04

[a,b,c]Data in the same row sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
[1] SR: survival rate.
[2] WG: weight gain rate.
[3] FER: feed efficiency ratio.
[4] FI: feed intake.
[5] HSI: hepatosomatic index.

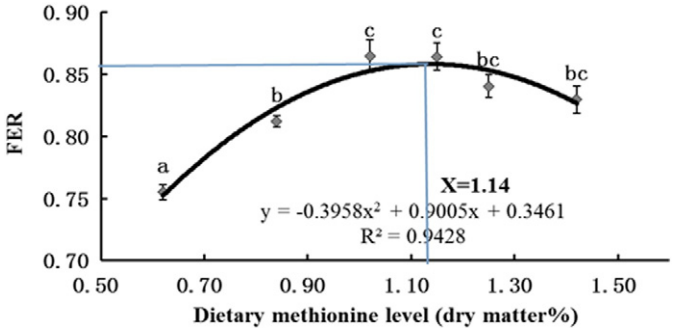

Fig. 2. Relationship between dietary methionine level and feed efficiency rate of cobia (*Rachycentron canadum*) fed experimental diets for 10 weeks based on quadratic regression analysis, and indicated that the optimum dietary methionine level is 1.14%.

($P < 0.05$). No significant difference was observed between the 1.02% and 1.42% methionine groups (Fig. 4a). The expression of IGF-I was up-regulated in the liver as dietary methionine increased up to 1.02% ($P < 0.05$), and no significant difference was found between 1.02% and 1.42% methionine groups (Fig. 4b).

4. Discussion

The growth and feed utilization of fish were increased by supplementation with moderate content of methionine (1.02%), but reduced when dietary methionine level was equal to or above 1.15%; this was similar to the findings obtained in smaller cobia (initial weight of 11.61 ± 0.16; Zhou et al., 2006). Based on WG and FER, the dietary methionine requirement of cobia was 1.04% and 1.14% of dry diet (2.23% and 2.45% dietary protein), respectively. These estimated values are lower than 1.19% (dry diet) for smaller cobia (Zhou et al., 2006), but within the range (1.90%–5.32% of dietary protein) of other cultured fish species (Alam et al., 2001; Borlongan and Coloso, 1993; Liao et al., 2014; Tang et al., 2009; Twibell et al., 2000; Zhou et al., 2006; Zhou et al., 2011). Relatively lower methionine content in diets inhibited the expression of IGF-I, similar to that reported in salmon (Hevrøy et al., 2007) and rainbow trout (Rolland et al., 2015), which might be the reason for the growth suppression of fish. The reduction of weight gain in fish fed diets with higher levels of methionine might be due to toxic effects, nutritional stress and increasing energy expenditure for the degradation of extra methionine (Baker, 2006; Zhou et al., 2006).

However, the limited amount of EAAs in diets might exert suppressive effects on the growth of fish by inhibiting protein synthesis. Fish growth is mainly attributed to the accumulation of protein via synthesis and accretion (Rønnestad et al., 1999). The GCN2 signaling pathway plays a regulatory role in translation initiation, the limiting step of protein synthesis (Anthony et al., 2004; Plaisance et al., 2012; Lees et al., 2014). An important response to amino acid starvation in both mammals and fish is the inhibition of protein synthesis by phosphorylation of the α-subunit of translation initiation factor 2, which is an important component of the GCN2 pathway (Anthony et al., 2004; Xu, 2014). Another possible mechanism for the regulation of growth by GCN2 is that ATF4 is upregulated after the activation of GCN2; it then binds to tuberous sclerosis complex 2, an important upstream factor of TOR, which could inhibit TOR (the main regulator of growth and protein synthesis; Jewell and Guan, 2013). In this study, the protein content and TP were enhanced with increasing dietary methionine level, which indicated suppression of protein synthesis by methionine deficiency. A reverse expression pattern of genes related to the GCN2 pathway was found in the liver, suggesting the activation of this pathway by methionine deficiency. These results suggested that the poor growth of fish fed the methionine-deficient diet (0.62%) might be due to low protein synthesis owing to the activation of the GCN2 pathway. Moderate methionine (1.02%) could benefit the growth performance of fish by suppressing the GCN2 pathway, which is characterized by lower transcriptional levels of GCN2, ATF4, C/EBP β, and ASNS. Lower expression levels of genes related to the GCN2 pathway could be responsible for the relatively better fish growth. Hence, the GCN2 pathway plays a dominating regulatory role in growth and protein synthesis that shape innate and adaptive amino acid responses (Anthony et al., 2004; Kilberg et al., 2005). The findings of this study indicated that dietary methionine deficiency could activate the GCN2 pathway, which eventually suppressed the growth performance of cobia.

The accumulation of lipids in fish is primarily associated with lipid synthesis and degradation (Peng et al., 2014). First, synthesis of lipid depends on the expression levels of lipogenic enzymes, which is regulated primarily by SREBP-1 (Brown and Goldstein, 1997; Shimano, 2000) and secondarily by PPAR γ (Herzig et al., 2003) in the liver. In this study, the expression of genes related to hepatic de novo lipogenesis (FAS, SCD-1, and ACC1) and the main regulatory factors of lipid synthesis (SREBP-1 and PPAR γ) were down-regulated both in the low (0.62%) and high (1.42%) methionine groups, indicating the suppression of lipid synthesis. The content of 18:1 fatty acids showed a similar changing pattern with the expression of SCD-1, which further supported the fact that methionine could affect lipid synthesis. These results are in agreement with the observations in mammals (Y. Cheng et al., 2010; Z. Cheng et al., 2010; Du et al., 2012; Guo and Cavener, 2007; Jousse et al., 2004; Plaisance et al., 2012) and fish (Craig and Moon, 2013) suggesting that deficiency of EAAs (methionine, leucine, isoleucine, and valine) could depress the synthesis of lipids. In this study, the lipid content in the liver and plasma was positively related to the expression of these enzymes. Thus, the lower lipid content was likely attributed to the lower expression of lipid synthesis enzymes. Second, β-oxidation is the dominating pathway of fatty acids catabolism in fish and mammals. The basic mechanism and regulation of this pathway appears to be highly conserved in mammals and fish (Nanton et al., 2003). PPARα, an important mediator of the hepatic response to starvation and a nuclear receptor, is responsible for stimulating lipid oxidation and

Table 5
Proximate composition (wet weight %) in whole body and lipid content of the liver and muscle in cobia (*Rachycentron canadum*) fed diets with graded levels of methionine for 10 weeks.

Index	Dietary methionine level (dry weight %)					
	0.62	0.84	1.02	1.15	1.25	1.42
Proximate composition of whole body (wet weight %)						
Moisture	76.42 ± 0.19^{c}	76.64 ± 0.33^{c}	72.76 ± 0.53^{a}	74.24 ± 0.41^{ab}	73.30 ± 0.21^{ab}	74.97 ± 0.52^{bc}
Crude protein	12.93 ± 0.19^{a}	13.25 ± 0.34^{a}	15.43 ± 0.47^{b}	15.36 ± 1.00^{b}	15.14 ± 0.73^{b}	14.91 ± 0.40^{b}
Crude lipid	3.05 ± 0.08^{a}	5.16 ± 0.10^{b}	7.09 ± 0.17^{c}	6.52 ± 0.30^{c}	7.25 ± 0.21^{c}	6.55 ± 0.20^{c}
Ash	4.00 ± 0.16^{ab}	4.07 ± 0.39^{b}	3.58 ± 0.25^{ab}	2.98 ± 0.05^{a}	3.46 ± 0.05^{ab}	3.19 ± 0.14^{ab}
Lipid content (wet weight %)						
Liver lipid	8.50 ± 0.38^{ab}	8.24 ± 0.65^{a}	12.14 ± 0.48^{c}	11.17 ± 0.42^{bc}	8.38 ± 0.91^{a}	8.53 ± 0.52^{ab}
Muscle lipid	3.02 ± 0.13	2.35 ± 0.20	3.84 ± 0.35	3.44 ± 0.56	2.57 ± 0.29	2.76 ± 0.36

^{a,b,c}Data in the same row sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).

Table 6
Effects of dietary methionine on the fatty acid profile (total fatty acids %) of liver in cobia (*Rachycentron canadum*).[1]

Fatty acid	Dietary methionine level (dry weight %)					
	0.62	0.84	1.02	1.14	1.25	1.42
C16:0	19.41 ± 0.21	19.03 ± 1.42	20.34 ± 1.91	18.44 ± 0.52	20.65 ± 2.24	18.19 ± 0.84
C18:0	11.08 ± 0.89	10.95 ± 0.73	10.28 ± 0.78	10.19 ± 1.22	10.19 ± 1.25	10.97 ± 0.76
C20:0	3.37 ± 0.18	2.82 ± 0.17	2.89 ± 0.10	2.91 ± 0.24	2.67 ± 0.27	2.81 ± 0.17
∑SFA[2]	33.87 ± 0.80	32.81 ± 2.28	33.51 ± 2.58	31.54 ± 1.67	32.80 ± 3.71	31.96 ± 1.48
C14:1	3.62 ± 0.21	3.48 ± 0.09	3.43 ± 0.14	3.59 ± 0.06	3.60 ± 0.15	3.16 ± 0.20
C16:1	15.74 ± 0.85	13.0 ± 20.72	14.61 ± 0.85	14.04 ± 0.95	14.55 ± 0.64	13.41 ± 0.49
C18:1	30.19 ± 0.39^{a}	31.57 ± 0.54^{ab}	33.39 ± 0.18^{b}	31.86 ± 0.10^{ab}	31.04 ± 0.42^{a}	30.31 ± 0.68^{a}
∑MUFA[3]	49.56 ± 0.66^{ab}	48.06 ± 0.43^{ab}	51.42 ± 0.91^{a}	49.49 ± 1.03^{ab}	49.19 ± 0.51^{ab}	46.88 ± 1.26^{a}
C18:2n-6	13.19 ± 0.39	14.56 ± 0.55	15.72 ± 0.66	14.87 ± 0.10	15.71 ± 0.49	15.31 ± 0.49
C20:4n-6	0.3 ± 0.07	0.84 ± 0.16	0.93 ± 0.24	0.67 ± 0.05	0.98 ± 0.25	0.85 ± 0.09
∑n-6PUFA[4]	13.49 ± 0.46^{a}	15.41 ± 0.39^{ab}	16.65 ± 0.89^{b}	15.54 ± 0.07^{ab}	16.69 ± 0.74^{b}	16.16 ± 0.56^{ab}
C18:3n-3	1.98 ± 0.34	1.89 ± 0.17	1.85 ± 0.17	1.96 ± 0.10	2.00 ± 0.11	1.98 ± 0.14
C18:4n-3	0.73 ± 0.18	0.82 ± 0.16	0.75 ± 0.20	0.75 ± 0.20	0.77 ± 0.10	1.02 ± 0.20
C20:5n-3(EPA)	1.53 ± 0.19	1.56 ± 0.27	1.47 ± 0.08	1.77 ± 0.17	1.83 ± 0.21	1.68 ± 0.20
C22:6n-3(DHA)	5.55 ± 1.24	5.80 ± 1.22	6.35 ± 0.67	4.56 ± 0.16	5.45 ± 1.67	5.13 ± 1.38
∑n-3PUFA[5]	9.80 ± 0.75	10.08 ± 0.89	10.43 ± 0.52	9.05 ± 0.03	10.05 ± 0.67	9.82 ± 0.99

^{a,b}Data in the same row sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
[1] Some fatty acids, of which the contents are minor, in trace amounts or not detected (such as 22:0, 24:0, 20:1n-9, 22:2n-11, 20:2n-6, 18:3n-6 and 22:5n-3).
[2] SFAs: saturated fatty acids.
[3] MUFAs: mono-unsaturated fatty acids.
[4] n-6PUFAs: n-6 poly-unsaturated fatty acids.
[5] n-3PUFAs: n-3 poly-unsaturated fatty acids.

ketogenesis in the liver (Kersten et al., 1999). The activation of PPARα could induce β-oxidation by up-regulating key enzymes such as CPT-1 (Gulick et al., 1994). In this study, PPARα was markedly up-regulated in the control group than in the other treatments; a similar expression pattern of CPT-1 was found in the liver, which was consistent with the findings of a study conducted in rainbow trout (Craig and Moon, 2013). Moreover, previous studies have shown that methionine deficiency could promote mitochondrial β-oxidation in various tissues of mice (Lees et al., 2014; Plaisance et al., 2012). Taken together, these findings indicated that the reduction of lipid content caused by methionine deficiency was attributed to the combination of improvement of fatty acid oxidation ability and inhibition of lipid synthetic ability. However, the hepatic lipid content was decreased in fish fed the diet with excess methionine (1.42%), which was partly caused by the depression of lipogenic genes at the transcriptional level.

LPL has been considered to be a mediator of fatty acid uptake by tissues; it could interact with triacylglycerol in plasma lipoproteins and release fatty acids (Auwerx et al., 1992; Nilsson-Ehle et al., 1980; Robinson, 1970). In this study, similar expression pattern of PPARα and reverse pattern of TG concentration were found, suggesting that LPL mRNA levels might be regulated by PPARα. Similar results of LPL expression and activity were reported in mammals fed leucine-deficient (Y. Cheng et al., 2010; Z. Cheng et al., 2010) and methionine-deficient (Kawasaki et al., 1998) diets. The expression and activity of hepatic LPL of fish were affected by several nutritional factors (fasting, dietary lipid content, and amino acid profile; Black and Skinner, 1986; Liang et al., 2002; Sabugal et al., 1996; Sheridan and Mommsen, 1991). However, no study has investigated the relationship between methionine and LPL in fish; further studies are warranted to elucidate the underlying mechanism.

As discussed above, the fatty acid oxidation ability was increased and lipid synthesis ability was decreased, as well as the GCN2 pathway was activated, in fish fed the methionine-deficient diet. High level substitution of fish meal with soybean protein caused the depression of growth and inhibition of lipid synthesis; these effects likely were associated with the activation of the GCN2 pathway (Xu, 2014). Payne et al. (2010) reported that C/EBPβ (an important component of the GCN2 pathway) could bind to the SREBP-1 promoter and play a direct key role in the regulation of SREBP-1. C/EBPβ also plays a dual role as a stimulator of cell differentiation (Darlington et al., 1998). The

Table 7
Effects of dietary methionine on the plasma biochemical index in cobia (*Rachycentron canadum*).[1]

Index	Dietary methionine level (dry weight %)					
	0.62	0.84	1.02	1.15	1.25	1.42
TP[1] (mmol/L)	23.90 ± 0.59^{a}	22.70 ± 0.46^{a}	28.30 ± 1.07^{b}	32.17 ± 1.12^{c}	26.80 ± 0.75^{b}	26.73 ± 0.83^{b}
TC[2] (mmol/L)	1.17 ± 0.03	1.19 ± 0.11	1.19 ± 0.02	1.14 ± 0.08	1.16 ± 0.03	1.25 ± 0.11
TG[3] (mmol/L)	0.49 ± 0.02^{a}	0.68 ± 0.03^{b}	0.73 ± 0.03^{b}	0.48 ± 0.03^{a}	0.45 ± 0.03^{a}	0.44 ± 0.02^{a}
GLU[4] (mmol/L)	1.25 ± 0.13^{a}	1.32 ± 0.07^{a}	1.80 ± 0.04^{ab}	2.34 ± 0.20^{b}	1.86 ± 0.30^{ab}	1.97 ± 0.09^{ab}
HDL-C[5] (mmol/L)	0.23 ± 0.01^{ab}	0.20 ± 0.02^{a}	0.41 ± 0.03^{b}	0.32 ± 0.05^{ab}	0.32 ± 0.05^{ab}	0.31 ± 0.01^{ab}
LDL-C[6] (mmol/L)	0.022 ± 0.002	0.025 ± 0.001	0.025 ± 0.002	0.023 ± 0.002	0.025 ± 0.001	0.021 ± 0.001
AST[7] (U/L)	3.03 ± 0.48	3.07 ± 0.07	3.13 ± 0.20	3.00 ± 0.35	3.17 ± 0.36	2.87 ± 0.18
ALT[8] (U/L)	12.67 ± 1.20	13.1 ± 0.32	13.33 ± 0.88	12.00 ± 1.00	13.15 ± 0.62	12.33 ± 0.88

^{a,b}Data in the same row sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
[1] TP: The contents of total protein.
[2] TC: total cholesterol.
[3] TG: total triacylglycerol.
[4] GLU: glucose.
[5] HDL-C: high density lipoprotein cholesterol.
[6] LDL-C: low density lipoprotein cholesterol.
[7] AST: aspartate aminotransferase.
[8] ALT: alanine aminotransferase.

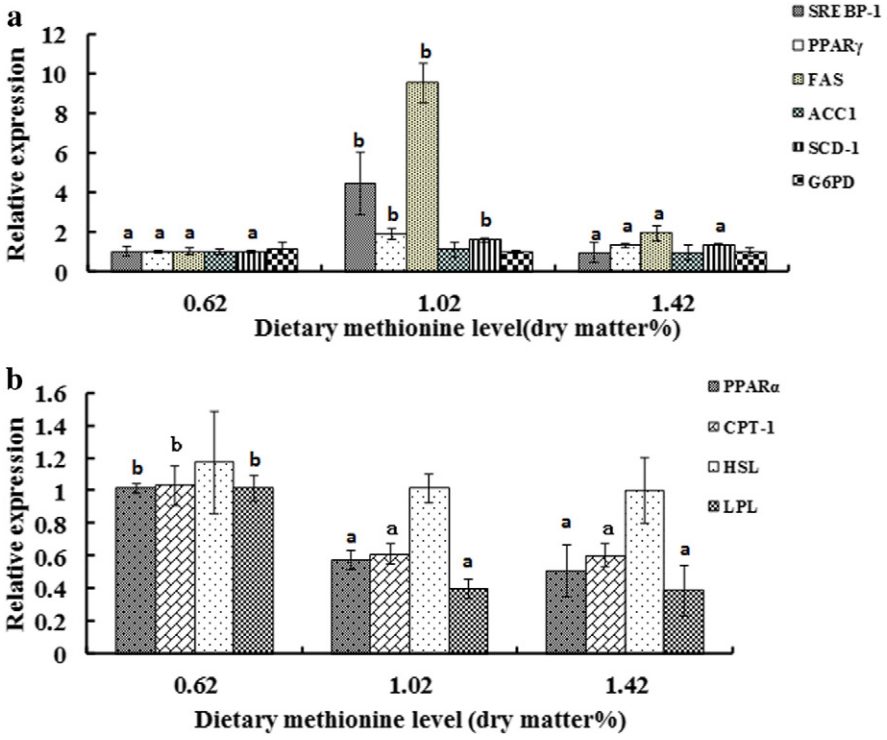

Fig. 3. Effect of cobia (*Rachycentron canadum*) fed diets containing deficient (0.62%), optimum (1.02%) and excess (1.42%) levels of methionine on a) relative expression of hepatic lipogenesis related genes: sterol regulatory element binding protein-1 (SREBP-1), peroxisome proliferator activated receptor γ (PPARγ), fatty acid synthetase(FAS), acetyl-coenzyme A carboxylase-1 (ACC-1), stearoyl-CoA desaturase-1 (SCD-1) and Glucose-6-phosphate Dehydrogenase (G6PD); b) fatty acid oxidation-related genes: peroxisome proliferator activated receptor α (PPARα), carnitine acyl transferase-1 (CPT-1), hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) in liver for 10 weeks. Values (means ± S.E.M.) in bars that have the same letter are not significantly different ($P < 0.05$; Tukey's test) among treatments.

activation of the GCN2 pathway inhibited lipid synthesis and increased the β-oxidation ability by increasing the expression of fibroblast growth factor 21, a critical hormone for the induction of hepatic fat oxidation during starvation and a target gene for ATF4 (Ana et al., 2012; De Sousa-Coelho et al., 2013). Moreover, the results of some previous studies indicated that the inhibition of lipid synthesis by EAA (methionine, leucine, isoleucine, and valine) deficiency was via the activation of the GCN2 pathway (Du et al., 2012; Guo and Cavener, 2007; Plaisance et al., 2012). Further, the activation of the GCN2 pathway could inhibit the TOR signaling pathway, which eventually suppressed lipid synthesis by inhibiting SREBP-1 (Bakan and Laplante, 2012; Jewell and Guan, 2013). These findings suggested that the GCN2 pathway might be involved in the mediation of lipid metabolism and might explain our observation that the decrease of lipid content in fish fed the methionine-deficient diet might be controlled by the activation of the GCN2 pathway. However, further investigations are needed to confirm this hypothesis in fish.

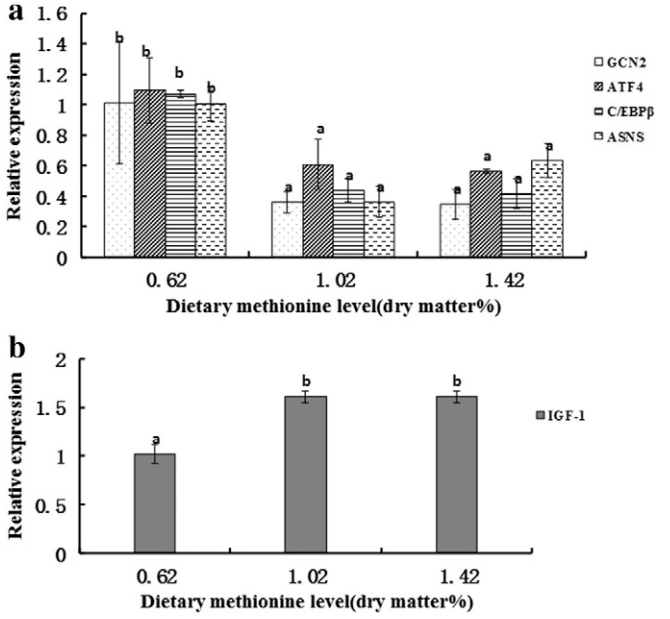

Fig. 4. Effect of cobia (*Rachycentron canadum*) fed diets containing deficient (0.62%), optimum(1.02%) and excess (1.42%) levels of methionine on relative expression a) hepatic Amino acid Response genes: general control nonderepressible2 (GCN2), activating transcription factor (ATF4), CCAAT enhancer binding protein β (C/EBPβ) and asparagine synthetase (ASNS); b) hepatic GH-IGF-I axis gene: insulin like growth factor-1 (IGF-I). Values (means ± S.E.M.) in bars that have the same letter are not significantly different ($P < 0.05$; Tukey's test) among treatments.

In conclusion, the dietary methionine requirement of cobia was determined to be 1.04% and 1.14% of dry matter (2.23% and 2.45% dietary protein) on the basis of WG and FER, respectively. Results of this study suggested that methionine deficiency could suppress growth, decrease lipid content, and inhibit the expression of IGF-I and some genes related to lipid synthesis in cobia; these changes might be regulated by inducing the expression of genes related to the GCN2 pathway (GCN2, ATF4, C/EBPβ, and ASNS).

Conflicts of interest

There are no conflicts of interest to report.

Acknowledgments

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