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Effects of dietary rapeseed meal on growth performance, digestion and protein metabolism in relation to gene expression of juvenile cobia (*Rachycentron canadum*)

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ABSTRACT

A 60-day feeding trial in seawater floating cages (1.5×1.5×2.5 m) was conducted to investigate the effects of dietary rapeseed meal (RM) levels on feed intake, growth, survival, digestion and protein metabolism in relation to gene expression of juvenile cobia (initial body weight 94.6 g). Five isonitrogenous (crude protein 450 g kg^{-1} of dry matter) and isoenergetic (20 kJ g^{-1}) practical diets were formulated by replacing 0 (the control), 125, 250, 375 and 500 g kg^{-1} fish meal protein with RM protein. Each diet was randomly fed to triplicate groups of fish, and each cage was stocked with 20 fish. Fish were fed twice daily (06:00 and 18:00) to apparent satiation. The survival ranged from 96.7 to 98.3%, and no significant difference was observed among dietary treatments ($P>0.05$). With increasing dietary RM levels, feed intake (FI), specific growth rate (SGR) and feed efficiency (FE) decreased. Fish fed the diet with 250 g kg^{-1} or more protein from RM had significantly lower SGR and FE than the control group ($P<0.05$), but there was no significant difference in FI at this level compared with the control group ($P>0.05$). Apparent digestibility coefficients (ADCs) of dry matter (DM), crude protein and energy significantly decreased with increasing dietary RM levels ($P<0.05$). Fish fed the diet with 250 g kg^{-1} or more protein from RM had significantly lower ADC values of crude protein and energy compared with the control group ($P<0.05$). Whole-body crude protein and crude lipid decreased with increasing dietary RM levels. Fish fed the diet with 500 g kg^{-1} protein from RM had significantly lower whole-body crude protein and crude lipid compared with the control group ($P<0.05$). However, whole-body moisture and ash showed opposite trends with crude protein and crude lipid. Moisture, crude protein and crude lipid contents in cobia muscle showed similar trends with those in whole body. There were no significant differences in plasma ammonia, urea, cholesterol and amino acids among fish fed the experimental diets ($P>0.05$). Fish fed the diet with 500 g kg^{-1} protein from RM had significantly lower aspartate aminotransferase (AST) activity in liver than the control group ($P<0.05$). Hepatic insulin-like growth factor I (IGF-I) gene expression level was significantly decreased in fish fed the diet with 500 g kg^{-1} protein from RM compared with the control group ($P<0.05$). However, IGF-I gene expression level in dorsal muscle was significantly increased in fish fed this diet compared with the control group ($P<0.05$). No significant differences were observed in target of rapamycin (TOR) expression levels in cobia liver and dorsal muscle at different RM levels ($P>0.05$). Results of the present study indicated that protein from RM could substitute 125 g kg^{-1} fish meal protein without influencing the growth, feed utilization and protein metabolism in cobia. The higher substitution levels of RM induced negative influences on feed intake, growth and hepatic IGF-I expression level.

1. Introduction

Rapeseed meal (RM) is one of important protein sources with protein content varying between 320 and 450 g kg^{-1} of dry matter (Burel et al., 2000). Compared with some other commercially available plant proteins, RM has a relatively favorable amino acid profile (Friedman, 1996), and is also the source of minerals, vitamins and other microelements. However, as most plant protein source, RM also contains many anti-nutritional factors (ANFs) such as fiber, oligosaccharides, sinapine, tannins, phytic acid and glucosinolates (GLS) which limit its utilization. Many studies reported that with increasing dietary RM levels, fish growth performance decreased (Cheng et al., 2010; Satoh et al., 1998; Webster et al., 1997).

Protein deposition is the main determinant of live weight (biomass) gain in fish (Dumas et al., 2007). Hence, the lower growth of fish could be attributed to the lower protein deposition. Protein deposition is determined by the balance between the processes of protein synthesis and degradation which are regulated by interactions among hormonal, nutritional, and other influences through cellular signaling pathways (Liu and Barrett, 2002). Recent studies have suggested that the target of rapamycin (TOR) signaling pathway plays

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an important role in protein synthesis and degradation in mammalian animals, and TOR protein was the central in TOR signaling pathway. TOR protein is a conserved Ser/Thr kinase, and can relay a permissive nutritional signal to downstream targets which modulate the initiation and elongation phases of translation (Wullschleger et al., 2006). Some studies on rainbow trout (Lansard et al., 2009, 2010; Seiliez et al., 2008) and Jian carp (Chen et al., 2011; Wu et al., 2011) indicated that nutrition status could regulate the TOR signaling pathway in fish as in mammals. Insulin-like growth factors (IGFs), which regulate the activity of TOR protein via IRS-PI3K-Akt pathway, are important upstream regulators of the TOR signaling pathway (Wullschleger et al., 2006). Among IGFs, IGF-I, which was principally synthesized in the liver, was the major anabolic agent responsible for tissue growth (Thissen et al., 1999). Nutritional status had a profound effect on IGF-expression in fish (Duan et al., 2010), and some studies have demonstrated that food deprivation, high plant protein and lower lysine intake could decrease hepatic IGF-I expression levels of fish (Gómez-Requeni et al., 2004; Hevrøy et al., 2007, 2008; Matthews et al., 1997; Pedroso et al., 2006).

Cobia (*Rachycentron canadum*) is a carnivorous marine fish and can grow from fingerling to 4–6 kg marketable size in 1 year with high feed efficiency (Chou et al., 2001). With the success of artificial propagation and larval production, the culture of cobia has become widely distributed in the southern coastal provinces of China (Zhou et al., 2005). Some studies on replacing FM with dietary plant protein in diets of cobia have been reported (Chou et al., 2004; Lunger et al., 2006, 2007a,b; Romarheim et al., 2008; Salze et al., 2010; Zhou et al., 2005). As far as we know, currently there is no published information on the use of RM in the diets of cobia. Therefore, the present study was to evaluate RM as a partial replacement for fish meal in diets of cobia by examining feed intake, growth, survival, digestion, and protein metabolism in relation to gene expression.

2. Materials and methods

2.1. Experimental diets

Five isonitrogenous (crude protein 450 g kg^{-1} of dry matter) and isoenergetic (20 kJ g^{-1}) experimental diets were formulated replacing 0 (the control), 125, 250, 375 and 500 g kg^{-1} of protein from Peruvian fish meal (FM) with roasted rapeseed meal (RM; *Brassica napus*, China). The ingredients, proximate composition and amino acid profile of ingredients are given in Tables 1 and 2. Crystalline amino acids were supplemented to meet the essential amino acid requirements based on the whole-body amino acid composition of cobia. And monocalcium phosphate was supplemented to meet the phosphorous requirement of cobia (Zhou et al., 2004a). In addition, 1 g kg^{-1} yttrium oxide (Y_2O_3, Fluka Chemicals®) was used as an inert tracer in each diet for determining apparent digestibility of nutrients.

Ingredients were ground into fine powder through a 246-μm mesh. All the ingredients were thoroughly mixed with oil, and water was added to produce stiff dough. The dough was then pelleted with an experimental feed mill (F-26 (II), South China University of Technology, China). Cold-pressed pellets (4.0 mm diameter) were air-dried to about 10% moisture, and were stored at −20 °C prior to use in the feeding trial.

2.2. Feeding trial procedures

Disease-free cobia juveniles (*R. canadum*) were obtained from Jiufu Fish Hatchery in Sanya (Hainan, China). The control diet was fed to all fish during a 1-week conditioning period. At the start of the experiment, fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10,000) (Shanghai Reagent Corporation, China). Fish of homogenous size (94.6 g) were randomly distributed into 15 seawater floating cages (1.5×1.5×2.5 m), and each cage was stocked with 20 fish. Each diet was randomly assigned to three replicate cages. Fish was hand-fed to apparent satiation twice (06:00 and 18:00) daily for 60 days. During the experimental period, rearing water temperature ranged from 29.5 to 32.0 °C, salinity was 24 to 26‰, and dissolved oxygen was approximately 7 mg l^{-1}.

Table 1
Formulation and proximate composition of the experimental diets (g kg^{-1} dry matter).

Ingredients	Diet no. (protein substitution level, g kg^{-1})				
	Diet 1 (0)	Diet 2 (125)	Diet 3 (250)	Diet 4 (375)	Diet 5 (500)
Fish meal (Peruvian)[a]	560	490	420	350	280
Rapeseed meal[a]	0	130	260	390	520
Wheat meal[a]	285	230	175	120	65
Fish oil	6.4	14.8	23.2	31.6	40
Soybean oil	7.2	5.4	3.6	1.8	0
Soybean lecithin oil	20	20	20	20	20
α-cellulose	64.7	48.6	32.5	16.4	0. 3
Mineral premix[b]	20	20	20	20	20
Vitamin premix[b]	20	20	20	20	20
Ethoxyquin	0.5	0.5	0.5	0.5	0.5
Sodium alginate	10	10	10	10	10
Betaine	3	3	3	3	3
DL-methionine	0	0.8	1.6	2.4	3.2
L-lysine (78%)	2.2	3.4	4.6	5.8	7
Monocalcium phosphate	0	2.5	5	7.5	10
Y_2O_3	1	1	1	1	1
Proximate composition (dry matter)[c]					
Crude protein (g kg^{-1})	449	451	453	454	456
Crude lipid (g kg^{-1})	95	94	95	95	96
Gross energy (kJ g^{-1})	19.5	19.5	19.7	19.9	20.0
Digestible phosphorus (g kg^{-1})	9.1	8.9	8.5	8.4	8.2
Ash (g kg^{-1})	125	122	120	118	115
Tannins (g kg^{-1})	0.2	1.7	3.1	4.6	5.8
Phytic acid (g kg^{-1})	2.2	7.8	11.4	15.5	18.7

[a] Guangdong Yuehai Feed Group Co. Ltd., Guangdong, China. Fish meal composition (dry matter basis): crude protein, 706 g kg^{-1}; crude lipid, 119 g kg^{-1}. Rapeseed meal composition (dry matter basis): crude protein, 451 g kg^{-1}; crude lipid, 14 g kg^{-1}; crude fiber, 121 g kg^{-1}; tannins, 12 g kg^{-1}; phytic acid, 31 g kg^{-1}. Wheat meal composition (dry matter basis): crude protein, 152 g kg^{-1}; crude lipid, 10 g kg^{-1}.
[b] According to Ren et al. (2011).
[c] Means of three analyses.

2.3. Analyses and measurement

2.3.1. Sample collection

Before the experiment, five fish from the same population were randomly selected for determination of initial whole-body proximate composition. At the termination of the experiment, the fish were fasted for 24 h before harvest. Total number and body weight of fish in each cage were counted and measured. Four fish per cage were

Table 2
Amino acid composition of the ingredients and 450 g kg^{-1} protein (g kg^{-1} dry weight) from cobia whole body.

Essential amino acids	Ingredient (g kg^{-1})			45 g kg^{-1} whole body protein (g kg^{-1})
	Fish meal	Rapeseed meal	Wheat meal	
Arginine	40.8	25.4	6.1	27.2
Histidine	17.4	10.6	3.0	11.0
Isoleucine	34.2	18.6	4.9	19.1
Leucine	54.4	30.1	8.9	34.7
Lysine	54.3	22.8	3.6	35.0
Phenylalanine	29.7	17.2	6.3	18.8
Threonine	30.4	19.2	3.7	21.4
Valine	37.9	32.6	5.5	19.9
Methionine	21.4	7.5	1.9	12.3

randomly collected and stored frozen at −20 °C to determine whole-body proximate composition. Another four fish from each cage were anesthetized with eugenol (1:10,000), and blood samples were collected from the fish heart using 2-ml heparinized syringes to obtain plasma samples after centrifugation (4000 *g* for 10 min) at 4 °C and immediately stored at −80 °C until analysis. The liver, intestinal tract and dorsal muscle were obtained from four fish per cage, and chyme was removed from the gut using distilled water, then immediately stored frozen at −80 °C.

After the sample collection described above, fecal collection and treatment were conducted according to the method described by Ren et al. (2011). The remaining fish were fed with the same diet assignments in order to determine the apparent digestibility coefficients (ADCs) for dry matter, protein, energy and phosphorus. Following a 1-week acclimation period, fish from each replicate were anesthetized with eugenol (1:10,000), and manually stripped of feces 5–7 h after feeding. Feces were collected once a week until sufficient dried feces had been collected for analysis. Pooled feces from each replicate were dried for 12 h at 50 °C and stored at −20 °C.

2.3.2. Analysis of diets, feces, fish body and muscle composition

Analyses of ingredients, diets, fecal samples, fish body and muscle composition were made following the usual procedures (Association of Official Analytical Chemists (AOAC), 1995): samples were dried to a constant weight at 105 °C to determine the dry matter content; crude protein was determined by measuring nitrogen (N×6.25) using the Kjeldahl method (Kjeltec TM 8400, FOSS, Tecator, Sweden); crude lipid was measured by ether extraction using Soxhlet method; ash by combustion at 550 °C for 16 h; energy by an adiabatic bomb calorimeter (PARR1281, USA). Duplicate analyses were conducted for each sample. Essential amino acids, except for methionine, were determined according to the method of Cheng et al. (2010) using Biochrom 30 amino acid analyzer (Biochrom Ltd, UK), and methionine was determined according to the method of Mai et al. (2006) using reverse-phase high-performance liquid chromatography (HPLC, HP1100, USA). Y_2O_3 and phosphorus contents in the diets and feces were determined according to the method of Cheng et al. (2010) using inductively coupled plasma-atomic emission spectrophotometer (ICP-OES, VISTA-MPX, VARIAN, USA) after perchloric acid digestion.

2.3.3. Digestive enzyme assay

Protease activity of intestine was analyzed following the method of Kumar et al. (2006). Tyrosine was used as the standard, and one unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1 μg of tyrosine per 1 min. Alpha-amylase activity (E.C. 3.2.1.1) was determined according to the method described by Cheng et al. (2010) using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). Protein concentration was assayed according to the method of Bradford (1976) using bovine serum albumin (Sigma A-2153) as a standard.

2.3.4. Activities of protein metabolism enzymes

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method described by Cheng et al. (2010) using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China).

2.3.5. Plasma metabolites assay

Plasma cholesterol was determined spectrophotometrically using commercially available kits (Zhejiang Dongou bioengineering Co., Zhejiang, China). Plasma ammonia, urea and amino acids were determined according to the method described by Melo et al. (2006) using Multiskan spectrum (Thermo, USA).

2.3.6. RNA extraction, cDNA synthesis and partial sequence cloning of TOR and IGF-1 gene

Total RNA was extracted from cobia liver using Trizol Reagent (Invitrogen, USA), and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. Three microgram of total RNA was subjected to reverse transcription by PrimerScript® RT Enzyme using Oligo-dT primer (Takara, Japan) in 10 μl volume according to reagent's instructions. The degenerate primer pairs of TOR (forward: 5′-CGC CAA CAA GAT CCT GAA GAA YAT GTG YGA-3′; reverse: 5′-GGC CCT TCA GCA GGA ACA YRA AYT CRT G-3′) and IGF-I (forward: 5′-GGC ATT KRT GTG ATG TCT T-3′; reverse: 5′-TCR CAG CTY TGR AAG CAG CA-3′) were designed using the CODEHOP software. The polymerase chain reaction (PCR) was conducted on an Eppendorf Mastercycler gradient (Eppendorf, Germany) to amplify cDNA fragment of TOR and IGF-I. The PCR conditions as follows, TOR: initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 59 °C for 30 s, primer extension 72 °C for 1 min with a final 5 min extension at 72 °C; IGF-I: initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s, primer extension 72 °C for 30 s with a final 5 min extension at 72 °C. The PCR fragments were subjected to electrophoresis on a 1.2% agarose gel for length difference and cloned into the pEASY-T1 vector (TransGen Biotech, China). After transforming into the competent cells of *Escherichia coli* TOP10, the recombinants were identified through blue–white color selection in ampicillin-containing LB plates and confirmed by PCR. Three positive clones in each PCR fragment were sequenced in both directions and these resulting sequences were verified and subjected to cluster analysis in NCBI.

2.3.7. Real-time quantitative PCR analysis of TOR and IGF-I mRNA expression

Total RNA from liver and dorsal muscle was extracted using Trizol Reagent (Invitrogen, USA), and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA was treated with Recombinant DNase I (RNase-free) (Takara, Japan) to remove possible DNA contaminant according to the manufacturer's instructions. The quantity and quality of the total RNA were assessed using the Nano Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratios of all samples range from 1.86 to 2.00 indicating a satisfactory purity of the RNA samples. Purified RNA was reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan). The real-time PCR primer pairs were designed using Primer Premier 5.00 based on nucleotide sequences of cloning TOR and IGF-I gene of cobia. Real-time PCR assays were carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany) in a final volume of 25 μl containing 2×SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara, Japan), 0.5 μl each of primers (10 μmol l^{-1}), 1 μl of cDNA mix. Gene-specific primers for TOR (forward: 5′-AAG TAC ATG CGC TCT GGA AAC G-3′; reverse: 5′-GGA TGC GGA TGA TAG GCT GG-3′) and IGF-I (forward: 5′-GAG CGC GAT GTG CTG TAT CT-3′; reverse: 5′-GTC CAC AAT GCC GCG TGA CC-3′) were applied to evaluate the mRNA levels of TOR and IGF-I in liver and dorsal muscle. There were no significant differences in transcript levels of β-actin gene (forward: 5′-TGC GTG ACA TCA AGG AGA AGC-3′; reverse: 5′-TAC CGA GGA AGG AAG GCT GG-3′) in liver and dorsal muscle among dietary treatments (Fig. 1), and was used as internal control. The real-time PCR amplification began with 30 s at 95 °C, followed by 35 cycles of 5 s at 95 °C, 25 s at 60.2 °C, and 30 s at 72 °C. No template controls were run for each PCR assay. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analyzed according to the following equation $E = 10^{(-1/Slope)} - 1$. The expression levels of TOR and IGF-I were calculated by $2^{-\Delta\Delta CT}$ method, and the value stood for n-fold difference relative to the calibrator (Livak and Schmittgen, 2001).

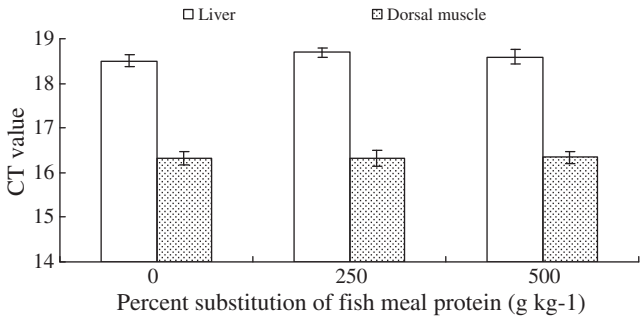

Fig. 1. The transcript levels (C_T value) of β-actin gene in liver and dorsal muscle of cobia fed diets with graded levels of rapeseed meal for 60 days. Values are means ± S.E.M. (n = 3).

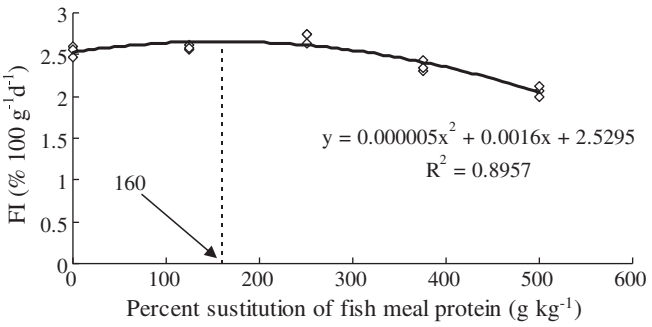

Fig. 2. Regression of dietary substitution of fish meal protein (g kg^{-1}) and FI of cobia.

2.4. Calculations and statistical methods

The following variables were calculated:

$$\text{Survival}(\%) = 100 \times (\text{final amount of fish})/(\text{initial amount of fish})$$

$$\text{Specific growth rate (SGR)} = (\text{Ln}W_t - \text{Ln}W_0) \times 100/t$$

$$\text{Feed intake (FI)} = 100 \times \text{dry feed intake} \times 2/((W_0 + W_t) \times t)$$

$$\text{Feed efficiency (FE)} = (W_t - W_0)/\text{dry feed intake}$$

$$\text{Protein productive value (PPV)} = 100 \times (\text{protein gain}/\text{protein consumption}).$$

Apparent digestibility coefficient (ADC) values of dry matter and nutrients were determined using the following equations:

$$\text{ADC of nutrients or energy (\%)} = (1 - (\text{dietary}Y_2O_3/\text{fecal}Y_2O_3) \times \text{fecal nutrient or energy}/\text{dietary nutrient or energy}) \times 100$$

where W_t and W_0 were final and initial body weights, respectively; t was duration of experimental days.

All data were subjected to analysis of variance using SPSS 17.0 for Windows. Differences among the means were tested by Tukey's multiple range tests. The level of significance chosen was $P<0.05$. Second-degree polynomial regression analysis was performed on SGR and FI against dietary substitution level of FM protein by RM protein.

Table 3
Growth performance of cobia fed diets with graded levels of rapeseed meal.[1]

Diet no. (substitution level, g kg^{-1})	FBW[2] (g)	SGR[3] (% d^{-1})	FI[4] (% 100 $g^{-1}d^{-1}$)	FE[5]	PPV[6] (%)	Survival (%)
Diet 1 (0)	320.4^{d}	2.03^{d}	2.57^{c}	0.71^{d}	28.52^{d}	98.3
Diet 2 (125)	314.0^{d}	2.00^{d}	2.59^{c}	0.69^{d}	28.24^{d}	98.3
Diet 3 (250)	282.5^{c}	1.82^{c}	2.64^{c}	0.61^{c}	24.64^{c}	98.3
Diet 4 (375)	208.7^{b}	1.32^{b}	2.36^{b}	0.53^{b}	20.51^{b}	96.7
Diet 5 (500)	169.2^{a}	0.97^{a}	2.06^{a}	0.46^{a}	16.60^{a}	96.7
ANOVA[7]						
Pooled S.E.M.[8]	16.1	0.11	0.06	0.03	1.25	0.67
P value	<0.001	<0.001	<0.001	<0.001	<0.001	0.871

[1] Values are means of three replicates. Means in each column with same superscripts have no significant differences ($P>0.05$).
[2] FBW: final body weight.
[3] SGR: specific growth rate.
[4] FI: Feed intake.
[5] FE: feed efficiency.
[6] PPV: Protein productive value.
[7] ANOVA: One-way analysis of variance.
[8] S.E.M.: standard error of mean.

3. Results

3.1. Survival and growth performance

The survival ranged from 96.7 to 98.3%, and no significant difference was observed among dietary treatments ($P>0.05$) (Table 3). FI of fish was significantly affected by the replacement level of FM protein with RM protein. There was no significant difference in FI among fish fed diets with 0, 125 and 250 g kg^{-1} protein from RM, but when substitution level was more than 250 g kg^{-1}, the FI was significantly lower compared with the control group (Diet 1) ($P<0.05$). A second-degree regression analysis indicated that when substitution level was 160 g kg^{-1}, FI had the maximum value (Fig. 2). With increasing dietary RM levels, SGR showed a second-degree polynomial regression model (Fig. 3). And fish fed the control diet showed the best SGR, however, fish fed the diet with 125 g kg^{-1} protein from RM (Diet 2) had comparable growth with the control group ($P>0.05$). When RM replaced more than 125 g kg^{-1} protein from FM, SGR was significantly lower compared with the control group ($P<0.05$). The FE and PPV showed similar trends as SGR (Table 3). Fish fed the diet with more than 125 g kg^{-1} protein from RM had significantly lower FE and PPV compared with the control group ($P<0.05$).

3.2. The ADC of dry matter and nutrients

The ADC of dry matter decreased with increasing dietary RM levels (Table 4). The ADC of dry matter in fish fed diets containing more than 250 g kg^{-1} protein from RM was significantly lower than that in the control group ($P<0.05$). Moreover, when the substitution level was more than 125 g kg^{-1}, the ADC values of crude protein and energy were significantly lower compared with the control group ($P<0.05$). No significant difference in ADC of phosphorus (68.9–72.4%) were observed ($P>0.05$) among dietary treatments ($P>0.05$).

3.3. Whole body and muscle composition

Fish whole-body composition analysis showed that with increasing dietary RM levels, whole-body moisture and ash contents increased, and whole-body crude protein and crude lipid contents decreased. Compared with fish fed the control group, fish fed the diet with 500 g kg^{-1} protein from RM had significantly higher whole-body moisture and ash contents, and significantly lower crude protein and crude lipid contents ($P<0.05$) (Table 5).

Muscle composition analysis, except for ash, showed similar trends with those in whole body. Compared with fish fed the control

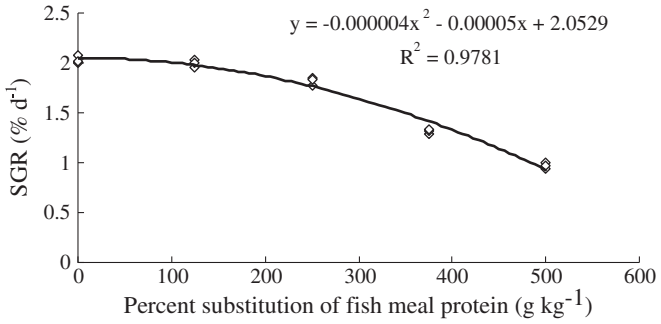

Fig. 3. Regression of dietary substitution of fish meal protein (g kg^{-1}) and SGR of cobia.

group, fish fed the diet with 375 g kg^{-1} or more protein from RM had significantly higher whole-body moisture content, and significantly lower crude protein and crude lipid contents ($P<0.05$) (Table 5). No significant difference was observed in muscle ash among dietary treatments ($P>0.05$).

3.4. Plasma metabolites

No significant differences were observed in plasma ammonia (0.266–0.285 μmol ml^{-1}), urea (1.37–1.66 μmol ml^{-1}), cholesterol (1.82–2.03 μmol ml^{-1}) and amino acids (12.50–15.15 μmol ml^{-1}) among dietary treatments ($P>0.05$) (Table 6).

Table 4
Apparent digestibility coefficients (ADC) of dry matter and nutrients by cobia fed diets with graded levels of rapeseed meal.[1]

Diet no. (substitution level, g kg^{-1})	ADC of dry matter (%)	ADC of crude protein (%)	ADC of energy (%)	ADC of phosphorus (%)
Diet 1 (0)	61.8^{b}	85.7^{c}	77.8^{c}	68.9
Diet 2 (125)	59.2^{b}	84.3^{bc}	77.7^{c}	70.4
Diet 3 (250)	57.4^{b}	80.7^{ab}	74.3^{b}	72.3
Diet 4 (375)	50.6^{a}	79.6^{a}	73.5^{b}	71.9
Diet 5 (500)	48.0^{a}	78.0^{a}	70.9^{a}	72.4
ANOVA[2]				
Pooled S.E.M.[3]	1.468	0.847	0.731	0.576
P value	<0.001	0.001	<0.001	0.353

[1] Values are means of three replicates. Means in each column with same superscripts have no significant differences ($P>0.05$).
[2] ANOVA: One-way analysis of variance.
[3] S.E.M.: standard error of mean.

Table 6
Plasma metabolites of cobia fed diets with graded levels of rapeseed meal.[1]

Diet no. (substitution level, g kg^{-1})	Ammonia μmol ml^{-1}	Urea μmol ml^{-1}	Cholesterol μmol ml^{-1}	Amino acids μmol ml^{-1}
Diet 1 (0)	0.266	1.66	1.92	15.15
Diet 2 (125)	0.276	1.51	2.03	13.26
Diet 3 (250)	0.285	1.37	1.92	12.50
Diet 4 (375)	0.283	1.43	2.03	13.26
Diet 5 (500)	0.284	1.40	1.82	12.50
ANOVA[2]				
Pooled S.E.M.[3]	0.003	0.045	0.043	0.489
P value	0.323	0.257	0.561	0.463

[1] Values are means of one composite sample from four fish in each of three replicate groups.
[2] ANOVA: One-way analysis of variance.
[3] S.E.M.: standard error of mean.

3.5. Activities of digestive enzymes and protein metabolism enzymes

There were no significant differences in activities of protease (1.60–2.01 U/mg protein) and alpha-amylase (0.62–0.69 U/mg protein) in intestine among dietary treatments ($P>0.05$) (Table 7).

The activity of AST in liver significantly decreased with increasing dietary RM levels ($P<0.05$). Fish fed the diet with 500 g kg^{-1} protein from RM had significantly lower hepatic AST activity compared with the control group ($P<0.05$). The activity of ALT in liver showed a decreased trend with increasing dietary RM levels, however, no significant difference was observed among dietary treatments ($P=0.093$).

3.6. Cloning of partial cDNA sequence of TOR and IGF-I gene

The PCR product amplified by the degenerate primers for TOR was 596 bp, and its nucleotide sequences was significantly homologous to tilapia *Oreochromis niloticus* (GenBank accession number: XM_003449131) (identities 93%), zebra fish *Danio rerio* (GenBank accession number: AB290031) (identities 83%) and common carp *Cyprinus carpio* (GenBank accession number: FJ899680) (identities 83%).

The PCR product for IGF-I was 256 bp, and its nucleotide sequences was significantly homologous to red striped snapper *Lutjanus erythropterus* (GenBank accession number: JN383435) (identities 98%), yellowtail *Seriola quinqueradiata* (GenBank accession number: AB439208) (identities 97%), Atlantic halibut *Hippoglossus hippoglossus* (GenBank accession number: EU682475) (identities 97%) and Asian bass *Lates calcarifer* (GenBank accession number: EU136126) (identities 97%).

Table 5
Whole-body and muscle composition of cobia fed diets with graded levels of rapeseed meal.[1]

Diet no. (substitution level, g kg^{-1})	Whole-body				Muscle			
	Moisture (%)	Crude protein (% ww[2])	Crude lipid (% ww[2])	Ash (% ww[2])	Moisture (%)	Crude protein (% ww[2])	Crude lipid (% ww[2])	Ash (% ww[2])
Diet 1 (0)	74.2^{a}	17.5^{bc}	4.9^{b}	3.4^{a}	74.9^{a}	21.1^{b}	3.3^{c}	1.8
Diet 2 (125)	73.7^{a}	17.8^{c}	5.3^{b}	3.5^{ab}	75.5^{a}	20.9^{b}	2.6^{bc}	1.8
Diet 3 (250)	74.6^{ab}	17.6^{bc}	4.5^{ab}	3.5^{ab}	75.6^{a}	21.1^{b}	2.5^{bc}	1.8
Diet 4 (375)	75.9^{ab}	17.1^{ab}	3.7^{ab}	3.7^{b}	76.9^{b}	20.2^{a}	1.9^{ab}	1.8
Diet 5 (500)	76.9^{b}	16.6^{a}	2.8^{a}	4.1^{c}	77.8^{b}	20.4^{a}	1.0^{a}	1.8
ANOVA[3]								
Pooled S.E.M.[4]	0.378	0.131	0.284	0.065	0.182	0.107	0.223	0.009
P value	0.010	0.001	0.013	<0.001	<0.001	<0.001	0.001	0.435

[1] Values are means of one composite sample from four fish in each of three replicate groups. Means in each column with same superscripts have no significant differences ($P>0.05$).
[2] ww: wet weight.
[3] ANOVA: One-way analysis of variance.
[4] S.E.M.: standard error of mean.

Table 7
The activities of digestive enzymes in intestine and protein metabolism enzymes in liver of cobia fed diets with graded levels of rapeseed meal.[1]

Enzyme (U/mg protein)	Diet no. (protein substitution level, g kg^{-1})					ANOVA[2]	
	Diet 1 (0)	Diet 2 (125)	Diet 3 (250)	Diet 4 (375)	Diet 5 (500)	Pooled S.E.M.[3]	*P* value
Protease	1.99	2.01	1.88	1.78	1.60	0.079	0.637
Alpha-amylase	0.62	0.69	0.63	0.68	0.64	0.025	0.906
ALT	27.85	34.13	20.78	23.97	19.38	1.977	0.093
AST	37.87^{b}	36.41^{b}	30.50^{ab}	26.62^{ab}	23.86^{a}	1.737	0.012

[1] Values are means of one composite sample from four fish in each of three replicate groups. Means in each line with same superscripts have no significant differences ($P>0.05$).
[2] ANOVA: One-way analysis of variance.
[3] S.E.M.: standard error of mean.

3.7. Expression of TOR and IGF-I gene in liver and dorsal muscle

With increasing dietary RM levels, IGF-I expression in liver decreased. Fish fed the diet with 500 g kg^{-1} protein from RM had significantly lower IGF-I expression in liver compared with fish fed the control group ($P<0.05$). However, IGF-I expression in dorsal muscle showed an opposite trend. Fish fed the diet with 500 g kg^{-1} protein from RM had significantly higher IGF-I expression in dorsal muscle compared with the control group ($P<0.05$) (Fig. 4).

No significant differences were observed in liver TOR expression (0.79–1.01) and dorsal muscle TOR expression (0.92–1.03) among dietary treatments ($P>0.05$). However, a decreased trend was observed in hepatic TOR expression among dietary treatments.

4. Discussion

The present study showed that there was a second-degree polynomial relationship between the growth of cobia and dietary RM levels ($Y=-0.000004X^2-0.00005X+2.0529$, $R^2=0.9781$). There was no

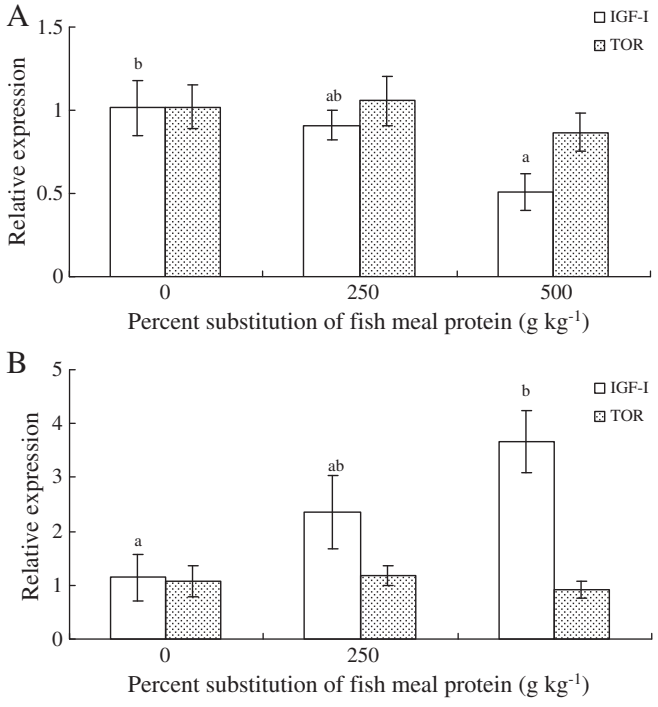

Fig. 4. Relative mRNA expression of IGF-I and TOR in the liver (A) and dorsal muscle (B) of cobia fed diets with graded levels of rapeseed meal for 60 days. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.E.M. (n = 3). Bars of the same gene bearing with same letters are not significantly different by Tukey's test ($P>0.05$).

significant difference in SGR among fish fed diets with 0 and 125 g kg^{-1} protein from RM, but when the protein substitution level was more than 125 g kg^{-1} (the content of RM in diets was more than 130 g kg^{-1}), SGR was significantly lower than the control group. It indicated that protein from RM could substitute 125 g kg^{-1} fish meal protein in commercial diets without influencing the growth of cobia. Similar results have also been obtained in rainbow trout (Hilton and Slinger, 1986), hybrid tilapia (Zhou and Yue, 2010) and Japanese seabass (Cheng et al., 2010). However, there was no significant difference in growth of rainbow trout when fed the diet with 30% dietary RM compared with fish fed the diet with the whole fish meal (Shafaeipour et al., 2008).

Hilton and Slinger (1986) suggested that suppression of feed intake could be the main reason for reduced growth performance of rainbow trout as dietary RM level increased. In the present study, a second-degree regression analysis indicated that when substitution level was 160 g kg^{-1}, FI had the maximum value. And there was no significant difference in FI among fish fed diets with 0, 125 and 250 g kg^{-1} protein from RM, but when substitution level was more than 250 g kg^{-1}, the FI was significantly lower compared with the control group. This result indicated that to some extent feed palatability was affected when more than 250 g kg^{-1} protein replaced FM protein. This result was different from that in Japanese seabass (Cheng et al., 2010), which showed that FI increased with increasing dietary canola meal levels, but similar to the findings in Chinook salmon (Satoh et al., 1998) and rainbow trout (Shafaeipour et al., 2008). Hence, it could be concluded that suppression of feed intake due to decreased palatability partly influenced the growth performance of cobia in the present study.

Cheng et al. (2010) demonstrated that decreased nutrient digestibility, especially the ADC of crude protein, could be the main reason for reduced growth performance of Japanese seabass as dietary canola meal level increased. In the present study, there were no significant differences in ADC values of crude protein and energy among fish fed diets containing 0 and 125 g kg^{-1}RM. But when substitution level was more than 125 g kg^{-1}, ADC values of crude protein and energy were significantly lower than those fed the control diet, and linear regression analysis showed that dietary RM level was negatively correlated with the ADC of protein ($Y=-0.1608X+85.651$, $r=0.8055$, $P<0.05$). This result agreed well with the study of Zhou et al. (2004b) which showed that ADC values of dry matter, crude protein and energy for RM were significantly lower than those for FM in cobia. Some studies reported that tannins and phytic acid in RM could reduce the digestibility of nutrients (Francis et al., 2001; McCurdy and March, 1992). In this study, with increasing dietary RM levels, dietary tannins and phytic acid increased from 0.2 to 5.8 g kg^{-1} and 2.2 to 18.7 g kg^{-1} respectively, meanwhile the ADCs of dry matter, crude protein and energy all significantly decreased. Some other ANFs, such as fiber, oligosaccharides and GLS, also could affect the digestibility of nutrients (Francis et al., 2001). Hence, it could be concluded that lower nutrient digestibility due to presence of various ANFs is an important limiting factor when RM was used as the protein source for cobia.

In the present study, the activities of protein metabolism enzymes AST and ALT in liver were reduced with increasing dietary RM levels, and fish fed the diet with 500 g kg^{-1} protein from RM had significantly lower hepatic AST activity compared with the control group ($P<0.05$), which indicated the utilization of dietary protein decreased and the liver was damaged to a certain extent. This result was similar to the findings of Cheng et al. (2010). Aminotransferases, such as ALT and AST, catabolize amino acids and transfer amino groups to alpha-keto acids (reversible catalysis). However, when the available essential amino acids are deficient, the keto acids may be reduced, thereby reducing the activities of ALT and AST (Cheng et al., 2010).

In the present study, hepatic IGF-I expression decreased as RM increased, which paralleled well with the results of growth performance. Fish fed the diet containing 250 g kg^{-1} RM showed lower

hepatic IGF-I expression than the control group, but no significant difference was observed. When substitution level was 500 g kg^{-1}, IGF-I expression in liver was significantly lower than the control group. Hence, it could be concluded that to some extent the activity of protein synthesis decreased with increasing dietary RM levels, which resulted in lower growth. This was confirmed by some previous studies on gilthead sea bream (Gómez-Requeni et al., 2004) and Atlantic salmon (Hevrøy et al., 2008), which showed that fish fed the diet with high plant protein had significantly lower hepatic IGF-I expression compared with the whole fish meal diet. Some studies showed that feed restriction decreased hepatic IGF-I mRNA levels in eel (Duan and Hirano, 1992), coho salmon (Duan and Plisetskaya, 1993), barramundi (Matthews et al., 1997) and grouper (Pedroso et al., 2006) compared with the control, and refeeding of the starved fish led to a rise in hepatic IGF-I mRNA (Duan and Plisetskaya, 1993; Pedroso et al., 2006). In the present study, with increasing dietary RM levels, FI of cobia significantly decreased. Hence, feed restriction could be one of factors for decreased hepatic IGF-I expression for cobia. When FM in diet was replaced by RM, lysine in diets decreased. Hevrøy et al. (2007) demonstrated that lower lysine intake by Atlantic salmon resulted in a significant down-regulation of IGF-I levels in hepatic tissue compared to salmon on higher lysine intake. In the present study, crystalline amino acids were supplemented to meet the essential amino acid requirements based on the whole-body amino acid composition of cobia, but supplemented amino acids did not improve the growth performance of this fish, which indicated that supplemented crystalline amino acids could not be efficiently utilized by this fish, which was in agreement with the study on Japanese seabass (Cheng et al., 2010). Hence, imbalance of essential amino acids could be another factor for decreased hepatic IGF-I expression.

PPV of fish decreased with increasing dietary RM levels. There was no significant difference in PPV among fish fed diets with 0 and 125 g kg^{-1} protein from RM, but when the protein substitution level was more than 125 g kg^{-1}, PPV was significantly lower than the control group. EAA imbalance could be one reason for declining PPV. In the present study, although crystalline amino acids were supplemented to meet the essential amino acid requirements, EAA imbalance might be still exist due to absorption of crystalline amino acids and protein amino acids was not simultaneously. Lower FI could be another reason for declining PPV because of higher proportion of the feed intake used for maintenance. PPV was determined by the balance between the processes of protein synthesis and degradation. With increasing dietary RM levels, plasma ammonia increased, which indicated that to some extent protein degradation increased. In addition, some ANFs in RM, such as fiber, oligosaccharides and GLS, could affect the digestibility of crude protein (Francis et al., 2001), which also decreased the PPV of fish. Whole-body crude protein decreased with increasing dietary RM levels, and fish fed the diet with 500 g kg^{-1} protein from RM had significantly lower crude protein contents. Similar results have also been obtained in channel catfish (Webster et al., 1997) and hybrid tilapia (Zhou and Yue, 2010). However, some previous studies reported that no significant differences in whole-body crude protein were observed as dietary RM level increased (Cheng et al., 2010; Satoh et al., 1998; Webster et al., 2000). These differences could be due to different species, age, RM levels, processing technology and so on. In the present study, whole-body crude lipid decreased with increasing dietary RM levels which paralleled well with the result of the FI and energy digestibility. It could be concluded that decreased whole-body crude lipid was probably due to lower energy intake caused by lower FI and energy digestibility.

In conclusion, there are obvious limitations to the use of RM as an ingredient in diets of marine fish such as the cobia. Suppression of feed intake, low nutrient digestibility caused by the presence of various ANFs and decreased protein synthesis activity related with lower hepatic IGF-I gene expression could be the main reasons for reduced growth performance. From this study, protein from RM could substitute 125 g kg^{-1} fish meal protein without influencing the growth, feed utilization and protein metabolism in cobia.

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