Aquaculture 428–429 (2014) 290–296

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Feed intake, growth performance and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.) fed defatted fish meal diets with graded levels of cholesterol

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ARTICLE INFO

Article history:
Received 9 January 2014
Received in revised form 17 March 2014
Accepted 20 March 2014
Available online 28 March 2014

Keywords:
Cholesterol
Juvenile turbot
Feed intake
Growth performance
Ghrelin
Gene expression

ABSTRACT

A 10-week growth trial was conducted to examine the effects of dietary cholesterol on feed intake, growth performance and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.) (initial body weight 5.18 ± 0.01 g). Five isonitrogenous and isolipidic experimental diets were formulated with supplementation of 0.0, 0.5, 1.0, 1.5 and 2.0% cholesterol, and the final dietary cholesterol concentrations were 0.38, 0.76, 1.30, 1.80 and 2.22%, respectively. WGR of fish fed diets with 1.30, 1.80 and 2.22% cholesterol was significantly higher than that of fish fed the diet with 0.38% cholesterol, but no significant differences of WGR were found among fish fed diets with 1.30, 1.80 and 2.22% cholesterol. Also no significant differences were found in feed intake (FI) in all treatments. With dietary cholesterol increasing, a general upward trend of total cholesterol (TC), free cholesterol (FC), cholesterol ester (CE), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in serum was exhibited. However, these kinds of cholesterol in serum were plateaued when fish fed diets with approximately over 1.30 to 1.80% cholesterol. The results of TC in liver and feces showed that, with increasing dietary cholesterol, only TC in feces exhibited an increasing trend. Quantitative PCR (qPCR) was used to assess the effects of dietary cholesterol on rate limiting enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase in cholesterol synthesis and cholesterol 7α-hydroxylase (CYP7A1) in bile acid synthesis. HMG-CoA reductase expression in liver was significantly suppressed with increasing dietary cholesterol, while CYP7A1 expression in liver was significantly enhanced as dietary cholesterol increased. There were no significant differences of ghrelin expression in both gut and brain. It can be concluded that moderate dietary cholesterol was beneficial to growth performance, while feed intake promotion effect was limited in juvenile turbot in the present study. Feedback control might exist in the cholesterol metabolism of turbot to keep cholesterol in homeostasis.

1. Introduction

Cholesterol is an important component of cell membranes (Cheng and Hardy, 2004) and the substrate for many substances, including bile acid, steroid hormones and vitamin D (Hernández et al., 2004; Holme et al., 2006). After being obtained from diets (exogenous) and self-synthesis (endogenous) in which HMG-CoA reductase is the rate limiting enzyme (Maita et al., 2006), cholesterol is transferred in blood by several kinds of lipoproteins in fish. Among them, LDL-C carries cholesterol from liver to peripheral tissues for utilization, while HDL-C carries cholesterol from peripheral tissues to liver (Chen et al., 2003; Deng et al., 2010) for the removal of cholesterol. Then, the cholesterol delivered to liver is degraded to bile acid in which CYP7A1 is the rate limiting enzyme (Matsumoto et al., 2005) and excreted from the body.

In feed industry, fish meal (FM) is a major protein source. However, increasing demand, uncertain availability and high price with the expansion of aquaculture made it necessary to search for alternative protein sources for reducing the level of FM in feed. Though significant progress has been made over the past decade, further studies on the continued replacement of FM with plant protein will be required (Hardy, 2010). As the concentration of cholesterol in plant protein was lower than that in FM (Cheng and Hardy, 2004) and the growth-depressing effect of plant protein diet was generally accompanied by a hypocholesterolemic effect in many fish species (Afuang et al., 2003; Kaushik et al., 2004; Sagstad et al., 2008; Sitjà-Bobadilla et al., 2005; Wang, 2007; Yagi et al., 2006), which indicated that the low concentration of cholesterol in diets could be one of the reasons leading to the

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http://dx.doi.org/10.1016/j.aquaculture.2014.03.027
0044-8486/

poor growth performance of fish when FM was replaced by plant protein.

Many experiments have been conducted to study the effects of dietary cholesterol on feed intake and growth performance of fish. Results showed that when fish were fed diets of high plant protein, the growth performance and feed intake were enhanced by a moderate supplementation of dietary cholesterol, such as juvenile turbot (*Scophthalmus maximus* L.) (Yun et al., 2011), Japanese flounder (*Paralichthys olivaceus*) (Deng et al., 2010) and juvenile channel catfish (*Ictalurus punctatus*) (Twibell and Wilson, 2004), however, when fish were fed diets of FM, the growth performance or feed intake were not enhanced by the supplementation of dietary cholesterol, such as Japanese flounder (*P. olivaceus*) (Deng et al., 2010), Atlantic salmon (*Salmon salar* L.) (Bjerkeng et al., 1999) and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Sealey et al., 2001). Therefore, for further studies on the effects of dietary cholesterol and in terms of the facts that cholesterol is not considered to be an essential nutrient because it can be synthesized by fish (NRC, 1993; Sealey et al., 2001), the present study was conducted on basis of defatted FM and graded levels of cholesterol supplementation.

According to previous studies, hypothalamic region of the brain produces key factors that either stimulate or inhibit feed intake in fish (Volkoff et al., 2005). First reported in rats by Kojima in 1999 (Kojima et al., 1999), ghrelin is an important orexigenic factor involved in the regulation of feed intake by hypothalamus. In fish, having been identified in several species including goldfish (*Carassius auratus*) (Unniappan et al., 2002), tilapia (*Oreochromis niloticus*) (Parhar et al., 2003) and Japanese eel (*Anguilla japonica*) (Kaiya et al., 2003a,b), ghrelin mainly expressed in gut and brain and also played an important role in feed intake stimulation (Unniappan and Peter, 2005). Therefore, in the present study, ghrelin expressions in gut and brain were detected to study the effects of dietary cholesterol on ghrelin and feed intake.

Turbot (*S. maximus* L.) is a carnivorous fish species that is widely cultured in Europe and East Asia because of its appreciated flesh and rapid growth. However, with the high requirement of dietary protein (Cho et al., 2005; Lee et al., 2003) and high demand of FM in practical diets (Bonaldo et al., 2011) for turbot, it is necessary to replace FM with appropriate plant protein and improve the effects of high plant protein diets, on which cholesterol might play an important role. Therefore, it is important to study the effects of dietary cholesterol on feed intake and growth performance in turbot.

2. Materials and methods

2.1. Feed ingredients and diets formulation

Cholesterol (>99%) was obtained from Qingdao Fulin Biochemical Co., Ltd. Defatted fish meal was obtained by extracting lipid from fish meal with ethanol (Sheen, 2000). All diets contained 65% defatted fish meal and 21% wheat flour. 2% fish oil was added to meet the essential fatty acids (EFA) requirements of juvenile turbot. 0.11% DL-methionine and 0.04% L-valine (crystalline amino acids) were supplemented to meet the essential amino acids (EAA) requirement of juvenile turbot based on the whole body amino acid profile (Kaushik, 1998). Five isonitrogenous and isolipidic diets were formulated with supplementation of graded levels of cholesterol (0.00, 0.50, 1.00, 1.50, 2.00%), and the final dietary cholesterol concentration is 0.38, 0.76, 1.30, 1.80 and 2.22%, respectively. They were named as C-0.38, C-0.76, C-1.30, C-1.80 and C-2.22, respectively (Table 1).

All ingredients were ground into fine powder through a 180 μm mesh. Ingredients of each diets were blended thoroughly first by hand and then mechanically. Cholesterol was dissolved into oil and then mixed with all ingredients. At last, water was added into the mixture to produce stiff dough. Then the dough was pelleted by experimental feed mill (F-26 (II), South China University of Technology, China) and dried for about 12 h in a ventilated oven at 45 °C, and stored in freezer at −20 °C until used.

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

Ingredients	C-0.38	C-0.76	C-1.30	C-1.80	C-2.22
Defatted fish meal	65	65	65	65	65
Wheat flour	21	21	21	21	21
Fish oil	2	2	2	2	2
Soybean oil	6.57	6.07	5.57	5.07	4.57
Soybean lecithin	2	2	2	2	2
Cholesterol	0	0.5	1	1.5	2
$Ca(H_2PO_4)_2$	1	1	1	1	1
Vitamin premix[a]	1	1	1	1	1
Mineral premix[b]	1	1	1	1	1
Choline chloride	0.13	0.13	0.13	0.13	0.13
Calcium propionate	0.1	0.1	0.1	0.1	0.1
Ethoxyquin	0.05	0.05	0.05	0.05	0.05
Met	0.11	0.11	0.11	0.11	0.11
Val	0.04	0.04	0.04	0.04	0.04
Analyzed nutrients compositions (dry matter basis)					
Crude protein	48.59	48.99	48.63	49.09	48.49
Crude lipid	11.53	10.91	11.10	10.95	10.88
Cholesterol	0.38	0.76	1.30	1.80	2.22

[a] Vitamin premix supplied the diet with (mg kg^{-1} diet) the following compounds: retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopherol acetate, 240; vitamin K_3, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin B_{12} (1%), 10; L-ascorbyl-2-monophosphate-Na (35%), 2000; calcium Pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; choline chloride (50%), 2500; cellulose, 2473.

[b] Mineral premix consisted of (mg kg^{-1} diet) the following ingredients: $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $CuSO_4 \cdot 5H_2O$, 10; $MnSO_4 \cdot H_2O$, 45; KI, 60; $CoCl_2 \cdot 6H_2O$ (1%), 50; Na_2SeO_3 (1%), 20; $MgSO_4 \cdot 7H_2O$, 1200; calcium propionate, 1000; zeolite, 2485.

2.2. Fish, experimental procedure and conditions

Juvenile turbot were bought form Haiyang fish farm (Haiyang, Shandong, China) and reared in Experimental base of Ocean University of China (Qingdao, China). A 2-week acclimatization was conducted prior to the experiment. During this period fish were fed twice a day to satiation with commercial diet produced by Qihao Biotech Co., Ltd. (Shandong, China). The experiment was conducted in the indoor seawater recirculating system with fiberglass circular tanks (400-L and flat bottom), which were provided with continuous aeration and maintained under natural photoperiod.

Fish were fasted for 24 h at the beginning of the experiment. Fish with similar sizes (initial body weight 5.18 ± 0.01 g) were distributed randomly into the 15 tanks, and each tank had 30 fish. Each tank was randomly ordered, and fish were hand-fed with the experimental diet to apparent satiation twice (08:00 and 18:00 h) a day for 10 weeks. No feeding behavior of fish towards the pellets was regarded as the sign of satiation. The consumption of feed in each tank was recorded. The uneaten feed was collected 1 h later and then weighed after dried to constant weight at 70 °C. Leaching loss in the uneaten diet was estimated by leaving five samples of each diet in tanks without fish for 1 h, recovering, drying and reweighing. During the experimental period, water temperature ranged from 15.0 to 18 °C, salinity from 30 to 33‰, pH from 7.5 to 8.0, ammonia nitrogen was lower than 0.1 mg/L, nitrite was lower than 0.1 mg/L, dissolved oxygen was higher than 6.0 mg/L.

2.3. Sample collection

Before the experiment, ten fish were randomly selected from the population for the determination of initial whole body proximate composition. At the termination of the experiment, fish were fasted for 24 h and anesthetized with eugenol (1:10,000) (purity 99%, Shanghai Reagent, China) before sampling. Total number and weight of fish in each tank were recorded. Five fish were randomly sampled in each tank and kept in −20 °C for whole body composition analysis. Blood

samples were taken from the caudal vein using heparinized syringes. Serum samples were obtained after centrifugation (4000 *g* for 10 min) at 4 °C and immediately frozen in liquid nitrogen, then stored at −80 °C until analysis. Liver samples were also frozen in liquid nitrogen and then stored at −80 °C for the analysis of cholesterol content, HMG-CoA reductase and CYP7A1 gene expression. Similarly, gut and brain samples were stored for the analysis of ghrelin gene expression. The feces collection method was the same as described by Deng et al. (2010).

2.4. Biochemical analysis

2.4.1. Ingredients, diets and body composition assays

Dry matter, crude protein, crude lipid, and ash were analyzed for ingredients, experimental diets and fish samples (AOAC, 1995). Dry matter was analyzed by drying the samples to constant weight at 105 °C. Crude protein was determined using the Kjeldahl method and estimated by multiplying nitrogen by 6.25. Crude lipid was quantified by ether extraction using Soxhlet method. Ash was examined by combustion in a muffle furnace at 550 °C for 16 h. Duplicate analyses were conducted for each sample.

2.4.2. Cholesterol assays

2.4.2.1. Serum cholesterol assays. The concentration of total cholesterol (TC), free cholesterol (FC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in serum were determined by colorimetric enzymatic methods using commercial kits. TC kit was supplied by Dong'ou Biological Engineering Co., Ltd., Zhejiang, China. FC kit was supplied by Applygen Technologies Inc., Beijing, China. Then the amounts of cholesterol esters were calculated by subtracting the FC value from the TC value. The HDL-C and LDL-C kits were supplied by Biosino Bio-technology and Science Inc., Beijing, China.

2.4.2.2. Dietary, hepatic and fecal cholesterol assays. After the extraction of lipids from 500 mg liver, diets and feces with chloroform: methanol (2:1, v/v) (Folch et al., 1957), 10 mL lipid solution was obtained. Then, 1 mL of the lipid solution was sampled to dry under a pure nitrogen stream. The obtained residue was mixed with 1 mL isopropyl alcohol containing 100 g Triton X-100/L (Reagent Grade). Afterwards, TC content in liver, diets and feces was determined using the same kit as for serum. Duplicate analyses were conducted for each sample.

2.4.3. Real-time quantitative PCR

2.4.3.1. RNA extraction and cDNA synthesis. Total RNA was extracted from liver, gut and brain samples using Trizol Reagent (Invitrogen, USA), and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. 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 ranged from 1.90 to 2.07, indicating a satisfactory purity of the RNA samples. Purified RNA was subjected to reverse transcription to cDNA by TransScript® One-Step gDNA Removal and cDNA synthesis SuperMix (TransGen Biotech, China) according to the reagent's instructions.

2.4.3.2. Real-time quantitative PCR analysis of HMG-CoA reductase expression. The real-time qPCR primer of HMG-CoA reductase gene was according to Yun et al. (2012). Real-time PCR assays were carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, German) in a final volume of 25 μL containing 12.5 μL 2 × TransStart™ Top Green qPCR SuperMix (TransGen Biotech, China), 1 μL each of primers (10 μmol/L), 1 μL of cDNA mix. HMG-CoA reductase gene-specific primers F (5′-CCACGAGCAATGTTGTCCC-3′), R (5′-TTAGGCATCGCTGGTCTTTT-3′) were applied to evaluate the mRNA levels of HMG-CoA reductase in liver. Reference Beta-actin gene (AY008305.1) (F: 5′-TAGGTGATGAAGCCCAGAGC-3′, R: 5′-CTGGGTCA TCTTCTCCCTGTT-3′) was used as internal control. The real-time qPCR amplification began with 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 56 °C, and 20 s at 72 °C. No template controls were run for each PCR assay. A four-fold serial dilution was used to assess PCR efficiencies for each assay, quantifying 5 concentrations in triplicate. The primer amplification efficiency was analyzed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$, the value was 1.0019 for HMG-CoA reductase and 0.9948 for Beta-actin in liver. The absolute ΔC_T value (HMG-CoA reductase C_T − Beta-actin C_T) of the slope is 0.0498 for liver, which indicated that the $\Delta\Delta C_T$ calculation for the relative quantification of HMG-CoA reductase could be used. The expression levels of HMG-CoA reductase was calculated by $2^{-\Delta\Delta CT}$ method, and the value stood for n-fold difference relative to the calibrator (Livak and Schmittgen, 2001).

2.4.3.3. Partial sequence cloning and real-time quantitative PCR analysis of CYP7A1 expression. The degenerate primer pair of CYP7A1 F (5′-ATYCCHTACCTBGGCTGTGC-3′) and R (5′-GCACTCTTGAABABATGRA TGGG-3′) was designed based on CYP7A1 nucleotide sequence of human *Homo sapiens* (NM_000780.3), chicken *Gallus gallus* (NM_001001753.1), India medaka *Oryzias melastigma* (JX454629.1), rainbow trout *Oncorhynchus mykiss* (AB675933.1) and zebrafish *Danio rerio* (NM_201173.1). The polymerase chain reaction (PCR) to obtain the fragment of CYP7A1 was conducted on an Eppendorf Mastercycler gradient (Eppendorf, German) to amplify CYP7A1 cDNA fragment. The PCR conditions were as follows: initial denaturation step at 94 °C for 30 s, primer annealing at 55 °C for 30 s, primer extension 72 °C for 40 s with a final 10 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. 3 positive clones in each PCR fragment were sequenced in both directions and these resulting sequences were verified and subjected to cluster analysis in NCBI. At last, a sequence containing 576 nucleotides was obtained.

The real-time qPCR primer was designed using Primer Premier 5.00 based on nucleotide sequences of cloning CYP7A1 gene of turbot. CYP7A1 gene-specific primers F (5′-TCAAATAGCCAGCGGCAAAC-3′), R (5′-CCATGACAGCTTCGACCCTC-3′) were applied to evaluate the mRNA levels of CYP7A1 in liver. The reference gene was also Beta-actin (AY008305) (F: 5′-TAGGTGATGAAGCCCAGAGC-3′; R: 5′-CTGGGTCATC TTCTCCCTGTT-3′). The real-time qPCR amplification began with 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 56 °C, and 20 s at 72 °C. The primer amplification efficiency was 0.8358 for CYP7A1 and 0.9035 for Beta-actin in liver. The absolute ΔC_T value (CYP7A1 C_T − Beta-actin C_T) of the slope is −0.0473 for liver which indicated that the $\Delta\Delta C_T$ calculation for the relative quantification of CYP7A1 could be used as well. The other analyzing processes were the same as mentioned above.

2.4.3.4. Real-time quantitative PCR analysis of ghrelin expression. The real-time qPCR primer of ghrelin gene was according to Miao (2013) Ghrelin gene-specific primers F (5′-TTTCCTCAGCCCTTCACA-3′), R (5′-TGCTGT CTCCGTGTTTCC-3′) were applied to evaluate the mRNA levels of ghrelin in gut and brain. Reference Beta-actin gene (AY008305.1) (F: 5′-TAGG TGATGAAGCCCAGAGC-3′, R: 5′-CTGGGTCATCTTCTCCCTGTT-3′) was used as internal control. The real-time qPCR amplification began with 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 54 °C, and 20 s at 72 °C. The primer amplification efficiency was 0.9400 for ghrelin and 0.9740 for Beta-actin in gut, and 0.8760 for ghrelin and 0.9763 for Beta-actin in brain. The absolute ΔC_T value (ghrelin C_T − Beta-actin C_T) of the slope is −0.0889 for gut and −0.0706 for brain, which indicated that the $\Delta\Delta C_T$ calculation for the relative quantification of ghrelin could be used as well. The other analyzing processes were also the same as mentioned above.

2.5. Calculations and statistical methods

Growth parameters were calculated as follows:

$$\text{Survival rate}(\%) = 100 \times \text{final fish number}/\text{initial fish number}.$$

$$\text{Weight gain rate}(\%) = 100 \times (\text{FBW} - \text{IBW})/\text{IBW}.$$

$$\text{Feed intake}(\%/\text{day}) = 100 \times \text{feed consumed}(\text{g})/[(\text{IBW} + \text{FBW})/2)]/\text{days}.$$

$$\text{Feed efficiency ratio} = \text{wet weight gain}(\text{g})/\text{dry feed consumed}(\text{g}).$$

Software SPSS 16.0 (SPSS Inc.) was used for all statistical evaluations. All data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. Differences were regarded as significance when $P < 0.05$. All data are presented as mean values with standard error of means.

3. Results

3.1. Survival rate and growth performance

The survival rate ranged from 95.6 to 100% and no significant differences were observed among dietary treatments. With dietary cholesterol increasing from 0.38 to 1.30%, feed intake (FI) increased from 1.56 to 1.74%/day. Then a slight fluctuation (from 1.74 to 1.66 then to 1.70%/day) appeared when dietary cholesterol increased from 1.30% to 2.22%. However, no significant differences were observed among dietary treatments ($P > 0.05$). As dietary cholesterol increased, an upward trend was observed in final body weight (FBW). When the concentration of cholesterol in diet was equal to or more than 1.30%, the final body weight (FBW) (34.31 to 36.05 g) was significantly higher than the fish fed the diet with 0.38% cholesterol (27.99 g) ($P < 0.05$), and no significant differences were observed among fish fed diets with equal to or higher than 1.30% cholesterol ($P > 0.05$). An upward trend of weight gain rate (WGR) (from 440,95 to 598.63%) with increasing dietary cholesterol was also showed in the present study, moreover, WGR of fish fed diets with over 1.30% cholesterol (563.54 to 598.63%) was significantly higher than that of fish fed the diet with 0.38% cholesterol (440.95%) ($P < 0.05$). However, no significant differences of WGR were found among fish fed diets with 0.76, 1.30, 1.80 and 2.22% cholesterol. The feed efficiency ratio (FER) (ranging from 1.21 to 1.29) showed no significant differences among dietary treatments ($P > 0.05$) (Table 2).

3.2. Body composition

Fish body composition analysis showed that the carcass lipid significantly increased from 2.38 to 3.20% as the dietary cholesterol increased from 0.38 to 2.22% ($P < 0.05$). By contrast, carcass moisture (from 78.41 to 77.67%) and ash (from 3.90 to 3.60%) had decreasing trend though no significant differences were exhibited in carcass moisture and ash ($P > 0.05$). Similarly, carcass protein had no significant differences in all dietary treatments as well ($P > 0.05$) (Table 3).

3.3. Plasma, liver and feces cholesterol

Total cholesterol (TC), free cholesterol (FC), cholesterol ester (CE), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in serum were all in a general upward trend with increasing dietary cholesterol and were plateaued in fish fed diets with cholesterol levels around 1.30 to 1.80%. Fish fed diets with equal to or more than 1.30% cholesterol had significantly higher TC in serum (2.34 to 3.54 mmol/L) than those fed the diets with cholesterol below 1.30% (1.15 and 1.21 mmol/L) ($P < 0.05$). HDL-C in serum of fish fed the 1.80 and 2.22% cholesterol diets (0.75 and 0.82 mmol/L) were significantly higher than that of fish fed the 0.38 and 0.76% cholesterol diets (0.35 and 0.47 mmol/L) ($P < 0.05$). Similarly, when dietary cholesterol was equal to or more than 1.30%, LDL-C in the serum (0.73 to 1.00 mmol/L) of fish was significantly higher than that (0.20 to 0.24 mmol/L) of fish fed the diets with cholesterol below 1.30% ($P < 0.05$). However, no significant differences were observed in serum LDL-C among the dietary treatments with cholesterol more than 1.30% ($P > 0.05$). FC and CE in serum significantly increased from 0.61 to 1.18 mmol/L and 0.43 to 2.36 mmol/L respectively with the increase of dietary cholesterol ($P < 0.05$). Likewise, TC in feces exhibited an increasing trend from 0.32 to 1.27 mmol/L as dietary cholesterol increased from 0.38 to 2.22%, Furthermore, TC in feces of fish fed diets with 1.30% cholesterol and above were significantly higher than that of fish fed diets with 0.38 and 0.76% cholesterol ($P < 0.05$). No significantly increasing trend was exhibited in liver TC. However, TC in liver of fish fed the diet with 1.80% cholesterol (2.01 mmol/L) was significantly higher than that of fish fed the diet with 0.76% cholesterol (0.95 mmol/L) ($P < 0.05$) (Table 4).

Table 2
Effects of dietary cholesterol on growth, feed intake and survival of juvenile turbot (*Scophthalmus maximus* L.) (means ± SE, n = 3).[a]

	C-0.38	C-0.76	C-1.30	C-1.80	C-2.22	Pooled[b] S.E.M.	ANOVA[c] F value	ANOVA P value
IBW[d]	5.17 ± 0.01	5.19 ± 0.01	5.17 ± 0.01	5.17 ± 0.01	5.16 ± 0.01	0.01	0.716	0.600
FBW[e]	27.99[a] ± 0.70	31.19[ab] ± 0.64	34.31[b] ± 0.91	35.31[b] ± 1.78	36.05[b] ± 1.23	0.91	8.700	0.003
WGR[f]	440.95[a] ± 12.5	501.15[ab] ± 12.65	563.54[b] ± 17.36	582.46[b] ± 33.49	598.63[b] ± 22.11	17.54	9.582	0.002
FI[g]	1.56 ± 0.02	1.59 ± 0.02	1.74 ± 0.03	1.66 ± 0.08	1.70 ± 0.02	0.02	3.550	0.052
FER[h]	1.26 ± 0.02	1.28 ± 0.01	1.21 ± 0.03	1.29 ± 0.04	1.26 ± 0.02	0.01	1.264	0.346
SR[i]	97.8 ± 1.11	100 ± 0.00	95.6 ± 2.94	98.9 ± 1.11	96.7 ± 1.92	0.77	1.042	0.433

[a] Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).
[b] S.E.M.: standard errors of means.
[c] ANOVA: one-way analysis of variance.
[d] IBW (g): initial body weight.
[e] FBW(g): final body weight.
[f] WGR (%): weight gain rate = 100 × (FBW − IBW) / IBW.
[g] FI (%/day): feed intake = 100 × feed consumed / [(IBW + FBW) / 2] / days.
[h] FER: feed efficiency ratio = wet weight gain (g) / dry feed consumed (g).
[i] SR (%): survival rate = 100 × final fish number / initial fish number.

Table 3
Effects of dietary cholesterol on composition of the whole body of juvenile turbot (*Scophthalmus maximus* L.) (means ± SE, n = 3).[a]

	C-0.38	C-0.76	C-1.30	C-1.80	C-2.22	Pooled[b] S.E.M.	ANOVA[c] F value	ANOVA P value
Moisture	78.41 ± 0.03	78.20 ± 0.12	78.00 ± 0.16	78.15 ± 0.10	77.67 ± 0.39	0.10	1.886	0.190
Crude protein	15.17 ± 0.28	15.82 ± 0.06	15.52 ± 0.22	15.60 ± 0.32	15.81 ± 0.47	0.13	0.769	0.569
Crude lipid	2.38^{a} ± 0.11	2.63^{ab} ± 0.07	2.79^{ab} ± 0.04	2.80^{ab} ± 0.07	3.20^{b} ± 0.28	0.09	4.483	0.025
Ash	3.90 ± 0.14	3.94 ± 0.10	3.70 ± 0.02	3.65 ± 0.08	3.60 ± 0.07	0.05	2.692	0.093

[a] Mean values in the same row with different superscript letters are significantly different (P < 0.05).
[b] S.E.M.: standard errors of means.
[c] ANOVA: one-way analysis of variance.

3.4. Expression of HMG-CoA reductase and CYP7A1 gene in liver, and ghrelin gene in gut and brain

The transcriptional levels of HMG-CoA reductase in the liver decreased significantly with increasing dietary cholesterol. Hepatic HMG-CoA reductase mRNA expression level was significantly decreased by approximately 0.50-fold, 0.20-fold, 0.04-fold and 0.01-fold in the 0.76, 1.30, 1.80 and 2.22% cholesterol treatment groups, respectively (P < 0.05) (Fig. 1). By contrast, the transcriptional levels of CYP7A1 in the liver increased significantly with increasing dietary cholesterol and CYP7A1 mRNA expression level was significantly increased by approximately 1.77-fold, 2.74-fold, 5.18-fold and 5.40-fold in the 0.76, 1.30, 1.80 and 2.22% cholesterol treatment groups, respectively (P < 0.05) (Fig. 2). Ghrelin mRNA levels in the gut of fish fed the 1.80% cholesterol diet and in the brain of fish fed the 0.76% cholesterol diet were higher than other treatment groups, however, there were no significant differences in the transcription of ghrelin in the gut and brain among dietary treatments (P > 0.05) (Figs. 3 and 4).

4. Discussion

Compared with the previous study based on high plant protein diet in which both growth performance and feed intake were significantly improved with increasing dietary cholesterol (Yun et al., 2011), only growth performance in the present study was significantly improved with increasing dietary cholesterol and no significant differences in feed intake were observed though an increasing trend was exhibited. Therefore, a deduction can be made that cholesterol could play a role in phagostimulant in diets on basis of plant protein, however, in diets on basis of FM, the feed intake promotion effects of cholesterol could not be the main factor. Further studies were still needed to explain how dietary cholesterol improve growth performance. Given the growth performance and health condition of juvenile turbot, the suggested level of dietary cholesterol in the present experiment is 1.30% (1% dietary cholesterol supplementation), which was also suggested in juvenile turbot (*S. maximus* L.) (Yun et al., 2011), juvenile channel catfish (*I. punctatus*) (Twibell and Wilson, 2004), and Japanese flounder (*P. olivaceus*) (Chen, 2006).

The process of cholesterol metabolism could be divided into three parts roughly, which include the synthesis, transport and removal of cholesterol. In the present study, several important indexes in the three parts were detected for elucidating the variation in cholesterol metabolism.

HMG-CoA reductase is the rate limiting enzyme in cholesterol synthesis (Maita et al., 2006). In the present study, the expression of HMG-CoA reductase in liver significantly decreased with increasing dietary cholesterol. This was consistent with the study of Maita et al. (2006), who found the expression of HMG-CoA reductase in yellowtail fed plant-based diet was higher than that fed FM diet. This indicated that the endogenous synthesis of cholesterol could be suppressed by the increasing exogenous cholesterol in diet and a negative feedback control to keep the balance of cholesterol might exist in fish.

TC, HDL-C, LDL-C, FC and CE content in the serum of juvenile turbot all increased with the increasing dietary cholesterol, which were similar to the previous study by Yun et al. (2011) and Chen (2006). These results indicated that the cholesterol concentrations in serum of fish were positively correlated with dietary cholesterol and more cholesterol in diet might contribute to the increase of serum cholesterol, which is a causative factor for atherosclerosis (Mathur et al., 1961). HDL-C/LDL-C ratio can be used as an index to show the direction of cholesterol transport and the risk of atherosclerosis (Goldstein and Brown, 1984; Potter et al., 1993). In the present study, this ratio was in a decreasing trend with increasing dietary cholesterol as a whole. The ratio in 0.76% cholesterol dietary treatment was significantly higher than that in 1.30, 1.80

Table 4
Effects of dietary cholesterol on serum, liver, feces cholesterol of juvenile turbot (*Scophthalmus maximus* L.) (means ± SE, n = 3).[a]

	C-0.38	C-0.76	C-1.30	C-1.80	C-2.22	Pooled[b] S.E.M.	ANOVA[c] F value	ANOVA P value
Serum								
TC[d]	1.21^{a} ± 0.10	1.15^{a} ± 0.06	2.34^{b} ± 0.16	3.03^{bc} ± 0.44	3.54^{c} ± 0.18	0.27	21.742	0.000
HDL-C[e]	0.35^{a} ± 0.03	0.47^{a} ± 0.03	0.57^{ab} ± 0.07	0.75^{bc} ± 0.06	0.82^{c} ± 0.01	0.05	17.293	0.000
LDL-C[f]	0.20^{a} ± 0.03	0.24^{a} ± 0.06	0.73^{b} ± 0.06	0.90^{b} ± 0.12	1.00^{b} ± 0.03	0.09	30.818	0.000
HDL-C/LDL-C	1.78^{ab} ± 0.08	2.24^{b} ± 0.52	0.77^{a} ± 0.03	0.84^{a} ± 0.05	0.82^{a} ± 0.02	0.18	8.148	0.003
FC[g]	0.61^{a} ± 0.03	0.72^{ab} ± 0.10	1.05^{bc} ± 0.05	1.18^{c} ± 0.14	1.18^{c} ± 0.11	0.07	8.221	0.003
CE[h]	0.60^{a} ± 0.10	0.43^{a} ± 0.06	1.29^{ab} ± 0.21	1.85^{bc} ± 0.30	2.36^{c} ± 0.25	0.21	15.986	0.000
Liver								
TC	1.00^{ab} ± 0.10	0.95^{a} ± 0.07	1.66^{ab} ± 0.09	2.01^{b} ± 0.42	1.60^{ab} ± 0.20	0.14	4.371	0.027
Feces								
TC	0.32^{a} ± 0.02	0.51^{a} ± 0.07	1.02^{b} ± 0.03	1.08^{b} ± 0.12	1.27^{b} ± 0.20	0.10	14.010	0.000

[a] Mean values in the same row with different superscript letters are significantly different (P < 0.05).
[b] S.E.M.: standard errors of means.
[c] ANOVA: one-way analysis of variance.
[d] TC: total cholesterol.
[e] HDL-C: high-density lipoprotein cholesterol.
[f] LDL-C: low-density lipoprotein cholesterol.
[g] FC: free cholesterol.
[h] CE: cholesterol ester.

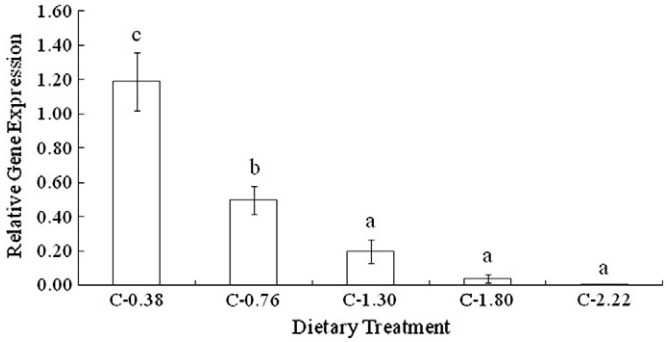

Fig. 1. Effects of dietary cholesterol on relative mRNA expression of HMG-CoA reductase in the liver of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n = 3), with their standard errors represented by vertical bars. [a,b,c]Mean values for the same gene with unlike letters were significantly different ($P < 0.05$; Tukey's test).

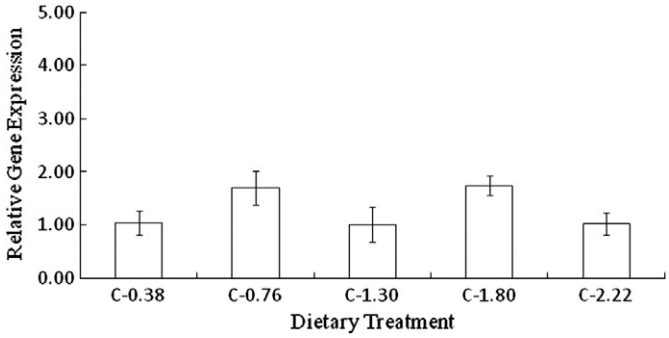

Fig. 3. Effects of dietary cholesterol on relative mRNA expression of ghrelin in the gut of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n = 3), with their standard errors represented by vertical bars.

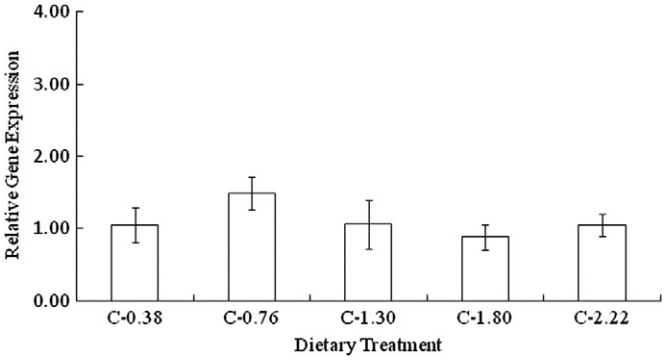

Fig. 4. Effects of dietary cholesterol on relative mRNA expression of ghrelin in the brain of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n = 3), with their standard errors represented by vertical bars.

and 2.22% cholesterol dietary treatments. This indicated that the direction of cholesterol transport tended to accumulate in the peripheral tissue when the dietary cholesterol was equal to or more than 1.30%. Therefore, a high concentration of cholesterol in diet could be detrimental to fish health and the dietary cholesterol should be kept in an appropriate range for fish to take advantage of the cholesterol without health hazard.

Total bile acid (TBA) synthesized from cholesterol in liver is an important way for the removal of body cholesterol, in which CYP7A1 is the rate limiting enzyme (Matsumoto et al., 2005). The expression of CYP7A1 in liver was detected in the present study, and the result showed that the expression of CYP7A1 in liver was in a significantly upward trend as the dietary cholesterol increased, which is in accordance with the previous study by Yun et al. (2011) (Yun et al., 2011). These results indicated that the ability of TBA synthesis was strengthened with the increasing cholesterol uptake by fish. A negative feedback mechanism to keep cholesterol in homeostasis was also shown in this process. TC in feces was detected in terms of the cholesterol elimination way. The results showed that the cholesterol excreted in feces increased with the increasing intake of cholesterol, which could be considered as a way for excessive cholesterol elimination.

To elucidate the effects of dietary cholesterol on feed intake, an important orexigenic peptide ghrelin was taken as an assessment in the present study. In fish, ghrelin is known to be involved in the control of feed intake and has been identified in several species as mentioned above. In the present study, the expression of ghrelin in both gut and brain was detected. The results showed that the expression of ghrelin were low and there were no significant differences among the five treatments in gut and brain. This was similar with the result in channel catfish (Peterson et al., 2012), in which gut ghrelin mRNA was also not significantly affected by feeding. Ghrelin is an orexigenic peptide in goldfish (Parhar et al., 2003; Unniappan et al., 2004), so the different results of ghrelin in the present study might be interpreted as the limited effects of dietary cholesterol on ghrelin or the species-specific actions of ghrelin in appetite regulation.

It can be concluded from the present study that 1) though fish are able to synthesize cholesterol, the cholesterol synthesized endogenously may not be enough for the optimal growth of turbot. 2) A moderate dietary cholesterol supplementation is beneficial to the growth performance of turbot 3) Feedback control to keep cholesterol in homeostasis exists in turbot, and cholesterol in serum is positively related to dietary cholesterol. 4) In juvenile turbot, the growth performance promotion by dietary cholesterol is not through the effects on ghrelin in gut and brain.

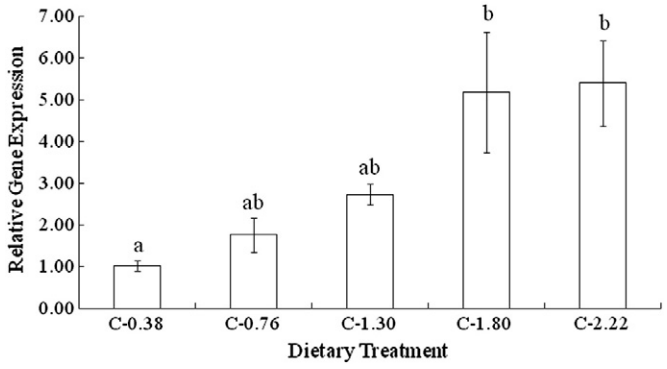

Fig. 2. Effects of dietary cholesterol on relative mRNA expression of CYP7A1 in the liver of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n = 3), with their standard errors represented by vertical bars. [a,b]Mean values for the same gene with unlike letters were significantly different ($P < 0.05$; Tukey's test).

Acknowledgements

This study was financially supported by the China Agriculture Research System (CARS-50-G08), Agricultural Scientific and Technological Achievements into Capital (2010GB23600673) and National Natural Science Foundation of China grants (31072222 and 30901108).

We thank S.H. Feng and K.K. Men for their help in diet production. Thanks are also due to K.K. Zhang, S.Q. Lin, X. Wang, J.J. Cao, K. Lu and L. Wang for their assistance in sampling.

References

Afuang, W., Siddhuraju, P., Becker, K., 2003. Comparative nutritional evaluation of raw, methanol extracted residues and methanol extracts of moringa (*Moringa oleifera* Lam.) leaves on growth performance and feed utilization in Nile tilapia (*Oreochromis niloticus* L.). Aquac. Res. 34, 1147–1159.

Association of Official Analytical Chemists (AOAC), 1995. Official Methods of Analysis of Official Analytical Chemists International, 16th ed. Association of Official Analytical Chemists, Arlington, VA.

Bjerkeng, B., Storebakken, T., Wathne, E., 1999. Cholesterol and short-chain fatty acids in diets for Atlantic salmon *Salmo salar* (L.): effects on growth, organ indices, macronutrient digestibility, and fatty acid composition. Aquacult. Nutr. 181–192.

Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Fontanillas, R., Badiani, A., Gatta, P.P., 2011. Increasing dietary plant proteins affects growth performance and ammonia excretion but not digestibility and gut histology in turbot (*Psetta maxima*) juveniles. Aquaculture 318, 101–108.

Chen, J.H., 2006. Effect of Supplemental Cholesterol in Diets Containing Soybean Meal on Feed Intake and Growth of Japanese Flounder (*Paralichthys olivaceus*). Doctor thesis Ocean University of China, Qingdao, P.R. China pp. 127–139.

Chen, J.Y., Chen, J.C., Wu, J.L., 2003. Molecular cloning and functional analysis of zebrafish high-density lipoprotein-binding protein. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136, 117–130.

Cheng, Z.J., Hardy, R.W., 2004. Protein and lipid sources affect cholesterol concentrations of juvenile Pacific white shrimp, *Litopenaeus vannamei* (Boone). J. Anim. Sci. 82, 1136–1145.

Cho, S.H., Lee, S.M., Lee, J.H., 2005. Effect of dietary protein and lipid levels on growth and body composition of juvenile turbot (*Scophthalmus maximus* L) reared under optimum salinity and temperature conditions. Aquacult. Nutr. 11, 235–240.

Deng, J., Mai, K., Ai, Q., Zhang, W., Wang, X., Tan, B., Ma, H., 2010. Interactive effects of dietary cholesterol and protein sources on growth performance and cholesterol metabolism of Japanese flounder (*Paralichthys olivaceus*). Aquacult. Nutr. 16, 419–429.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Goldstein, J.L., Brown, M.S., 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J. Lipid Res. 25, 1450–1461.

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquac. Res. 41, 770–776.

Hernández, P.V., Olvera-Novoa, M.A., Rouse, D.B., 2004. Effect of dietary cholesterol on growth and survival of juvenile redclaw crayfish, *Cherax quadricarinatus* under laboratory conditions. Aquaculture 236, 405–411.

Holme, M.H., Zeng, C., Southgate, P.C., 2006. The effects of supplemental dietary cholesterol on growth, development and survival of mud crab, *Scylla serrata*, megalopa fed semi-purified diets. Aquaculture 261, 1328–1334.

Kaiya, H., Kojima, M., Hosoda, H., Moriyama, S., Takahashi, A., Kawauchi, H., Kangawa, K., 2003a. Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. Endocrinology 144, 5215–5226.

Kaiya, H., Kojima, M., Hosoda, H., Riley, L.G., Hirano, T., Grau, E.G., Kangawa, K., 2003b. Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. J. Endocrinol. 176, 415–423.

Kaushik, S., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat. Living Resour. 11, 355–358.

Kaushik, S.J., Coves, D., Dutto, G., Blanc, D., 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture 230, 391–404.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656–660.

Lee, J.K., Cho, S.H., Park, S.U., Kim, K.D., Lee, S.M., 2003. Dietary protein requirement for young turbot (*Scophthalmus maximus* L.). Aquacult. Nutr. 9, 283–286.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Maita, M., Maekawa, J., Satoh, K., Futami, K., Satoh, S., 2006. Disease resistance and hypocholesterolemia in yellowtail *Seriola quinqueradiata* fed a non-fishmeal diet. Fish. Sci. 72, 513–519.

Mathur, K.S., Wahi, P.N., Gahlaut, D.S., Sharma, R.D., Srivastava, S.K., 1961. Prevalence of coronary heart disease in general population at Agra. Indian J. Med. Res. 49, 605.

Matsumoto, J., Erami, K., Ogawa, H., Kishida, T., Ebihara, K., 2005. The protease-resistant fraction of smoked, dried bonito lowers serum cholesterol in ovariectomized rats fed cholesterol-free diets. J. Food Sci. 70, S467–S474.

Miao, H.J., 2013. Effects of Dietary Carbohydrate on Lipid Metabolism and Ghrelin mRNA Relative Expression Level in Turbot. Master thesis Ocean University of China, Qingdao, P.R. China p. 39.

National Research Council, 1993. Nutrient Requirement of Fish. National Academy of Press, Washington, D.C.(114).

Parhar, I.S., Sato, H., Sakuma, Y., 2003. Ghrelin gene in cichlid fish is modulated by sex and development. Biochem. Biophys. Res. Commun. 305, 169–175.

Peterson, B.C., Waldbieser, G.C., Riley Jr., L.G., Upton, K.R., Kobayashi, Y., Small, B.C., 2012. Pre- and postprandial changes in orexigenic and anorexigenic factors in channel catfish (*Ictalurus punctatus*). Gen. Comp. Endocrinol. 176, 231–239.

Potter, S.M., Jimenez-Flores, R., Pollack, J., Lone, T.A., Berber-Jiménez, M.D., 1993. Protein–saponin interaction and its influence on blood lipids. J. Agric. Food Chem. 41, 1287–1291.

Sagstad, A., Sanden, M., Krogdahl, Å., Bakke-Mckellep, A.M., Frøystad, M., Hemre, G.I., 2008. Organs development, gene expression and health of Atlantic salmon (*Salmo salar* L.) fed genetically modified soybeans compared to the near-isogenic non-modified parental line. Aquacult. Nutr. 14, 556–572.

Sealey, W.M., Craig, S.R., Gatlin, D.M., 2001. Dietary cholesterol and lecithin have limited effects on growth and body composition of hybrid striped bass (*Morone chrysops* × *M. saxatilis*). Aquacult. Nutr. 7, 25–31.

Sheen, S.S., 2000. Dietary cholesterol requirement of juvenile mud crab *Scylla serrata*. Aquaculture 189, 277–285.

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249, 387–400.

Twibell, R.G., Wilson, R.P., 2004. Preliminary evidence that cholesterol improves growth and feed intake of soybean meal-based diets in aquaria studies with juvenile channel catfish, *Ictalurus punctatus*. Aquaculture 236, 539–546.

Unniappan, S., Peter, R.E., 2005. Structure, distribution and physiological functions of ghrelin in fish. Comp. Biochem. Physiol. A 140, 396–408.

Unniappan, S., Lin, X., Cervini, L., Rivier, J., Kaiya, H., Kangawa, K., Peter, R.E., 2002. Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology 143, 4143–4146.

Unniappan, S., Canosa, L.F., Peter, R.E., 2004. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology 79, 100–108.

Volkoff, H., Canosa, L.F., Unniappan, S., Cerda-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142, 3–19.

Wang, C., 2007. Effects of Dietary Replacement of Fishmeal by Soybean Meal and Feeding Strategy on Gibel Carp. Master's thesis Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.

Yagi, H., Matsuoka, S., Yamamoto, K., Jinnai, M., Hosokawa, H., 2006. Inclusion of soy protein concentrate in a diet for spotted halibut *Verasper variegatus*. Suisan Zoshoku 54, 61–68.

Yun, B., Mai, K., Zhang, W., Xu, W., 2011. Effects of dietary cholesterol on growth performance, feed intake and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.) fed high plant protein diets. Aquaculture 319, 105–110.

Yun, B., Ai, Q., Mai, K., Xu, W., Qi, G., Luo, Y., 2012. Synergistic effects of dietary cholesterol and taurine on growth performance and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.) fed high plant protein diets. Aquaculture 324, 85–91.