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# Effect of dietary cholesterol and phospholipids on feed intake, growth performance and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.)

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ABSTRACT

Dietary phospholipids play important roles in growth and nutrition metabolism in fish. Previous studies showed that moderate dietary cholesterol supplementation improved the growth performance of juvenile turbot fed plant-based or defatted fishmeal diets. Therefore, we examined the effect of dietary cholesterol and phospholipids on feed intake, growth performance and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.) (initial body weight 5.18 ± 0.01 g) during a 10-week period. Nine isonitrogenous and isolipidic diets were formulated to contain 0.0, 1.0 and 2.0% cholesterol (LC, MC and HC), and each with 0.0, 2.0 and 4.0% phospholipids (LP, MP and HP), respectively. A significant interaction between dietary cholesterol and phospholipids on weight gain rate was shown in the present study ($P = 0.049$). However, no significant interaction between dietary cholesterol and phospholipids was found in feed intake ($P = 0.332$) though the expression of the orexigenic factor ghrelin in the intestine was significantly affected by the interaction ($P = 0.011$). Except for total cholesterol, free cholesterol and cholesterol ester, both high-density lipoprotein cholesterol (HDL-C) ($P = 0.012$) and low-density lipoprotein cholesterol (LDL-C) ($P = 0.002$) in serum were significantly affected by the interaction between dietary cholesterol and phospholipids. Regarding the key genes involved in cholesterol and bile acid synthesis, no interaction between dietary cholesterol and phospholipids was found in the expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*) ($P = 0.380$) and cholesterol 7α-hydroxylase (*cyp7a1*) ($P = 0.587$) in liver. In conclusion, there is a significant interaction between dietary cholesterol and phospholipids on the growth performance and the HDL-C and LDL-C involved in cholesterol transport, while no significant interaction was found on the feed intake in juvenile turbot.

## 1. Introduction

Aquaculture accounts for a growing proportion of the total fishery production, increasing by an annual rate of 6.2% from 2000 to 2012 according to FAO (FAO Fisheries and Aquaculture Department, 2014). However, the production of traditional ingredients (fishmeal and fish oil) was limited (Shepherd and Jackson, 2013) and they were unable to meet the increasing demand for aquafeeds. Plant ingredients have been widely used to replace fishmeal and fish oil for the sustainable development of aquaculture (Tacon et al., 2010). However, their substitutes cause great nutritional changes and a number of studies have been conducted with regard to this issue. Cholesterol is an essential component of cell membranes and a precursor of several bioactive compounds, but it is virtually absent from plant ingredients. Some studies showed that cholesterol in plasma (Deng et al., 2013a; Kortner et al., 2014), bile acid synthesis (Kortner et al., 2014) and disease resistance (Deng et al., 2013b) were significantly affected by dietary cholesterol supplementation. However, the effects on growth performance were mixed depending on the fish species, basal diets and developmental stages (Bjerkeng et al., 1999; Deng et al., 2013a; Norambuena et al., 2013; Sealey et al., 2001; Twibell and Wilson, 2004). In previous studies with turbot in our laboratory, it was observed that a moderate cholesterol supplementation in either plant-based diets (Yun et al., 2011) or defatted fishmeal diets (Zhu et al., 2014) improved the growth performance of juvenile turbot.

On the other hand, the normal growth and development of various larval and juvenile crustaceans and fish require dietary phospholipids (Tocher, 1995; Tocher et al., 2008). It has been proven that phospholipids improve survival and growth performance, and prevent deformities (Coutteau et al., 1997). Similar to the process in mammals,

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dietary cholesterol and phospholipids in mixed micelles diffuse to the intestinal mucosa and are absorbed by enterocytes (Tocher et al., 2008), and then are transported by various lipoproteins in plasma (Babin and Vernier, 1989; Tocher, 1995). In addition to the important role of phospholipids as membrane components, as a source of energy, and as precursors of bioactive compounds including eicosanoids, diacylglycerol (DAG), inositol phosphates and platelet activating factors (PAFs), phospholipids also function as emulsifiers during lipid absorption and transportation (Coutteau et al., 1997; Tocher et al., 2008). Hence a potential interaction between dietary cholesterol and phospholipids may exist in fish. The main type of phospholipids in lecithin from soya is phosphatidylcholine, which mainly drives the requirement of phospholipids in fish (Taylor et al., 2015). In lobsters fed lecithin-free diets, higher cholesterol in the midgut gland and lower cholesterol in the hemolymph were found. This was probably due to the decreased rate of cholesterol out of the midgut gland into the hemolymph (D'Abramo et al., 1985). Correspondingly, higher cholesterol in serum and lipoproteins was found in lobsters fed diets with lecithin supplementation (Baum et al., 1990). In *Penaeus japonicus*, Teshima et al. (1986) reported that the mobilization of dietary triglycerides and cholesterol from the gut and hepatopancreas to the hemolymph was increased by the dietary soybean lecithin (Teshima et al., 1986). In *Litopenaeus vannamei*, the interaction between dietary phospholipids and cholesterol was found to significantly affect growth, as well as the lipid content in hepatopancreas and muscle (Gong et al., 2000). On the contrary, some studies did not find any interaction between dietary phospholipids and cholesterol in shrimp growth (Briggs et al., 1988; Chen and Jenn, 1991). However, very few studies were conducted on the interaction between dietary cholesterol and phospholipids in fish. Therefore, the present study aims to investigate the effect of dietary cholesterol and phospholipids on feed intake, growth performance and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus* L.), which is a species of high economic value and widely reared in both China and Europe.

## 2. Materials and methods

### 2.1. Feed ingredients and diet formulation

Defatted fish meal was produced by lipid extraction from fishmeal with hot ethanol (Sheen, 2000). The diet was formulated with 65% defatted fishmeal and 21% wheat flour. According to previous studies (Geurden et al., 1997; Zhu et al., 2014), nine isonitrogenous and isolipidic diets were formulated by supplementing of graded levels of cholesterol (0, 1, 2%) and phospholipids (0, 2, 4%). The 0, 1, and 2% cholesterol supplementation in diets were regarded as low cholesterol (LC), moderate cholesterol (MC) and high cholesterol (HC), respectively, and the 0, 2 and 4% phospholipid supplementation in diets were regarded as low phospholipid (LP), moderate phospholipid (MP) and high phospholipid (HP), respectively. Soybean oil was used to balance the lipid content and 2% fish oil was added to meet the essential fatty acid (EFA) requirements of juvenile turbot. 0.11% $D_L$-methionine and 0.04% L-valine (crystalline amino acids) were supplemented to meet the essential amino acid (EAA) requirement of juvenile turbot based on the whole body amino acid profile (Kaushik, 1998). (Table 1).

All ingredients were ground well through 180 μm mesh and then blended thoroughly with the oil containing different levels of cholesterol and phospholipids. Water was added to produce stiff dough which was then 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 use.

### 2.2. Fish, experimental procedure and conditions

Juvenile turbot were obtained from Haiyang fish farm (Haiyang, Shandong, China) and cultivated in Experimental base of Ocean University of China (Qingdao, China). Before the experiment, fish were acclimated for two weeks by feeding twice a day to satiation with commercial diet (Qihao Biotech Co., LTD, Shandong, China). At the beginning of the experiment, fish were fasted for 24 h and then those with similar sizes (initial body weight 5.17–5.19 g) were distributed randomly into the 27 tanks with 30 fish each tank. 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 (~12 h light: ~12 h darkness). During the experimental period of ten weeks, fish were fed with the experimental diet to apparent satiation twice (08:00 and 18:00 h) a day. No feeding behavior towards the pellets was regarded as the satiation of fish. The feed consumption in each tank was recorded. The collection of uneaten feed and leaching loss was estimated according to the previous study (Yun et al., 2012). During the rearing period, temperature, salinity, pH and dissolved oxygen averaged 16.7 ± 0.9 °C, 31.4 ± 0.9‰, 7.7 ± 0.1, 6.4 ± 0.3 mg/L, respectively. Ammonia nitrogen and nitrite were controlled lower than 0.1 mg/L.

### 2.3. Sample collection

At the end of the feeding trial, five fish were randomly sampled from each tank after being fasted for 24 h. Blood was taken from the caudal vein and then centrifuged at 4000*g* and 4 °C for 10 min to obtain serum samples. The serum and the tissues including liver, intestine and brain were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. The fecal collection was conducted after four weeks of feeding trial. About 1 h after feeding, the intact strands of feces were carefully siphoned with a fine mesh net and collected in plastic bottles. The fecal samples from each tank were stored in −20 °C and pooled. At the end of the experiment, the feces were freeze-dried, ground and stored in −20 °C until analysis (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, 2000). 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. Three fish from the same tank were homogenized in a pooled sample for body composition analysis. Three samples per treatment were analyzed, and duplicate analyses were conducted for each sample.

#### 2.4.2. 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 biotechnology and science Inc., Beijing, China.

Lipids were extracted from 500 mg liver and feces with chloroform: methanol (2:1, v/v) (Folch et al., 1957) for total cholesterol analysis. After drying 1 mL lipids with the pure nitrogen stream, the residue was dissolved into 1 mL isopropyl alcohol containing 100 g Triton X-100/L (Reagent Grade). Then the same kit for total cholesterol in serum was used to analyze the total cholesterol in liver and feces.

Plasma from three fish of the same tank was collected in the same tube and regarded as a pooled sample for plasma parameter analysis. The livers from three fish of the same tank were regarded as a pooled

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

| Ingredients % | LP-LC | LP-MC | LP-HC | MP-LC | MP-MC | MP-HC | HP-LC | HP-MC | HP-HC |
|---|---|---|---|---|---|---|---|---|---|
| Defatted fishmeal | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 |
| Wheat flour | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |
| Fish oil | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Soybean oil | 8.57 | 7.57 | 6.57 | 6.57 | 5.57 | 4.57 | 4.57 | 3.57 | 2.57 |
| Soybean lecithin | 0 | 0 | 0 | 2 | 2 | 2 | 4 | 4 | 4 |
| Cholesterol | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 |
| Ca(H2PO4)2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Vitamin premix[a] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Mineral premix[b] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Choline chloride | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 | 0.13 |
| Calcium propionate | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Ethoxyquin | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Met | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 |
| Val | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 |
| *Proximate analysis(dry matter basis)* | | | | | | | | | |
| Crude protein | 48.66 | 48.63 | 48.49 | 48.59 | 48.63 | 48.49 | 49.18 | 49.11 | 49.04 |
| Crude lipid | 10.62 | 10.88 | 10.64 | 10.79 | 11.10 | 10.88 | 11.24 | 10.85 | 10.78 |

[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.

sample for cholesterol content analysis. Feces collected in the same tank were regarded as a pooled sample for cholesterol content analysis. Duplicate analyses were conducted for each sample.

#### 2.4.3. RNA extraction, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted from liver, gut and brain samples using Trizol Reagent (Invitrogen, USA) and was quantified by Nano Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) (absorbance at 260 nm). The integrity of the samples was assessed using 1.2% denaturing agarose gel electrophoresis. 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.

The primers sequences of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*), cholesterol 7α-hydroxylase (*cyp7a1*) and *ghrelin* for real-time quantitative PCR and the operational approach referred to the previous study (Zhu et al., 2014) (Table 2). Real-time quantitative 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. Melting curve analysis was performed to confirm the single and specific PCR product at the end of the PCR reaction. The fluorescence data were normalized to the beta-actin gene (AY008305.1) by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and the relative mRNA expression of target genes in fish fed LC-LP diet was selected as the calibrator.

### 2.5. Calculations and statistical methods

Growth parameters were calculated as follows:

Survival rate (%) = 100 × final fish number/initial fish number.

Weight gain rate (%) = 100 × (final body weight − initial body weight) /initial body weight.

Feed intake (%/d) = 100 × feed consumed (g) /[(initial body weight + final body weight)/2)]/days.

Feed conversion ratio = dry feed consumed (g)/wet weight gain (g).

All data were analyzed by two-way analysis of variance (ANOVA), and means were subsequently separated by Tukey's test. Differences were regarded as significance when $P < 0.05$. All data are presented as mean values with S.E.M. Software SPSS 16.0 (SPSS Inc.) was used for all statistical evaluations.

## 3. Results

### 3.1. Survival rate, growth performance and feed intake

Survival rate (SR) was higher than 95% in all treatments, independent of both the levels of dietary cholesterol and phospholipids. The final body weight (FBW) was not significantly affected by the interaction between cholesterol and phospholipids ($P = 0.060$) but was significantly promoted with increasing dietary cholesterol ($P = 0.001$). Fish fed diet with moderate and high cholesterol showed significantly higher FBW than fish fed low cholesterol regardless of the level of phospholipids. The interactive effect between cholesterol and phospholipids on WGR (weight gain rate) was found ($P = 0.049$). Feed intake (FI) was significantly affected by increasing dietary cholesterol, showing a significantly higher FI in fish fed moderate and high cholesterol diet than fish fed low cholesterol diet ($P < 0.001$). However, dietary phospholipids and its interaction with cholesterol exerted no effect on the FI ($P = 0.332$). The feed conversion ratio (FCR) was

**Table 2**
qPCR primers in the present study.

| Target genes | Forward (5′–3′) | Reverse (5′–3′) | Publication or Genbank number |
|---|---|---|---|
| *hmgcr* | CCACGAGCAATGTTGTCCC | TTAGGCATCGCTGGTCTTTT | Yun et al. (2012) |
| *cyp7a1* | TCAAATAGCCAGCGGCAAAC | CCATGACAGCTTCGACCCTC | Zhu et al. (2014) |
| *ghrelin* | TTTCCTCAGCCCTTCACA | TGCTGTCTCCGTGTTTCC | Miao (2013) |
| *β-actin* | TAGGTGATGAAGCCCAGAGC | CTGGGTCATCTTCTCCCTGTT | AY008305.1 |

**Table 3**
Effect of the interaction between dietary phospholipids and cholesterol on feed intake, growth and survival of juvenile turbot (*Scophthalmus maximus* L.) (n = 3).[1]

| Phospholipids | Cholesterol | IBW[2] (g) | FBW[3] (g) | WGR[4] (%) | FI[5] (%/day) | FCR[6] | SR[7] (%) |
|---|---|---|---|---|---|---|---|
| Individual treatment means | | | | | | | |
| LP | LC | 5.17 | 32.57 | 529.69$^{ab}$ | 1.59 | 0.77 | 98.89 |
| LP | MC | 5.19 | 32.66 | 529.62$^{ab}$ | 1.67 | 0.81 | 98.89 |
| LP | HC | 5.18 | 33.96 | 555.22$^{a}$ | 1.70 | 0.81 | 98.89 |
| MP | LC | 5.17 | 27.99 | 440.95$^{b}$ | 1.56 | 0.80 | 97.87 |
| MP | MC | 5.17 | 34.31 | 563.54$^{a}$ | 1.74 | 0.83 | 95.56 |
| MP | HC | 5.16 | 36.06 | 598.63$^{a}$ | 1.70 | 0.80 | 96.67 |
| HP | LC | 5.19 | 32.12 | 518.66$^{ab}$ | 1.54 | 0.75 | 98.89 |
| HP | MC | 5.20 | 34.70 | 567.56$^{a}$ | 1.67 | 0.79 | 98.89 |
| HP | HC | 5.18 | 35.40 | 582.97$^{a}$ | 1.69 | 0.80 | 100.00 |
| Pooled S.E.M | | 0.00 | 0.55 | 10.52 | 0.01 | 0.01 | 0.48 |
| Means of main effect | | | | | | | |
| LP | | 5.18 | 33.06 | 538.18 | 1.65 | 0.80 | 96.67 |
| MP | | 5.17 | 32.79 | 534.38 | 1.67 | 0.81 | 98.89 |
| HP | | 5.19 | 34.07 | 556.40 | 1.63 | 0.78 | 99.26 |
| | LC | 5.19 | 30.89$^{b}$ | 496.43$^{b}$ | 1.56$^{b}$ | 0.77$^{b}$ | 97.78 |
| | MC | 5.18 | 33.89$^{a}$ | 553.57$^{a}$ | 1.69$^{a}$ | 0.81$^{a}$ | 98.52 |
| | HC | 5.18 | 35.14$^{a}$ | 578.94$^{a}$ | 1.70$^{a}$ | 0.80$^{a}$ | 98.52 |
| ANOVA: *P*-Values | | | | | | | |
| Phospholipids | | 0.142 | 0.374 | 0.439 | 0.184 | 0.063 | 0.095 |
| Cholesterol | | 0.614 | 0.001 | 0.001 | < 0.001 | 0.007 | 0.781 |
| Phospholipids*Cholesterol | | 0.900 | 0.060 | 0.049 | 0.332 | 0.403 | 0.906 |

[1] Treatment means represent the average values of three tanks per treatment. Tukey's test was conducted for individual means only if there was a significant interaction. Means followed by the same letter are not significantly different ($P > 0.05$).
[2] IBW: Initial body weight.
[3] FBW: Final body weight.
[4] WGR: Weight gain weight.
[5] FI: Feed intake.
[6] FCR: Feed conversion ratio.
[7] SR: Survival rate.

significantly lower in fish fed low cholesterol diet than those fed moderate and high cholesterol diet ($P = 0.007$). Neither the phospholipids ($P = 0.063$) nor the interaction ($P = 0.403$) between cholesterol and phospholipids significantly affected FCR (Table 3).

### 3.2. Body composition

Other than body crude lipid, the dietary cholesterol, phospholipids, and their interaction exerted no significant effect on the body crude protein and ash ($P > 0.05$). The body crude lipid was significantly affected by the dietary phospholipids. Fish fed diet with low phospholipids showed significantly higher crude lipid than fish fed diet with high phospholipids ($P = 0.002$) (Table 4).

### 3.3. Cholesterol in serum, liver and feces

Total cholesterol (TC) in serum was significantly enhanced with the increasing level of dietary cholesterol ($P < 0.001$). By contrast, high dietary phospholipids significantly decreased the TC in serum ($P = 0.037$). However, there was no interactive effect between dietary cholesterol and phospholipids on TC ($P = 0.111$). Regarding the two important lipoproteins responsible for cholesterol transport in plasma, the concentration of high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) showed a similar pattern affected by dietary cholesterol, phospholipids and their interaction between each other. Both HDL-C and LDL-C in plasma were significantly increased with the increasing dietary cholesterol ($P < 0.05$) but decreased with the increasing dietary phospholipids ($P < 0.05$). Further, they were significantly affected by the interaction between dietary cholesterol and phospholipids as well ($P < 0.05$). The HDL-C/LDL-C ratio was significantly affected by the dietary cholesterol ($P < 0.001$) rather than the phospholipids ($P = 0.118$). The fish fed diets with high and moderate cholesterol showed a significantly lower HDL-C/LDL-C than those fed low cholesterol diet ($P < 0.001$) and no interactive effect was observed on this ratio ($P = 0.384$). Free cholesterol (FC) was found to be significantly affected by the dietary cholesterol ($P < 0.001$) instead of phospholipids ($P = 0.105$). The moderate and high dietary cholesterol resulted in a significantly higher FC in the serum of fish ($P < 0.001$). The interaction between dietary cholesterol and phospholipids exerted no effect on FC in serum ($P = 0.584$). Cholesterol ester (CE) in serum was significantly enhanced with increasing dietary cholesterol ($P < 0.001$) but significantly decreased in fish fed high dietary phospholipids ($P = 0.007$). However, there was no interactive effect on the CE in serum ($P = 0.170$). TC in liver was significantly affected by both dietary cholesterol and phospholipids ($P < 0.05$) however, there was no interaction ($P = 0.153$). Fish fed diet with high and moderate cholesterol showed a significantly higher TC in liver than those fed low cholesterol diet ($P < 0.001$). Further, the TC in liver was significantly higher in fish fed high phospholipids than those fed low phospholipids ($P = 0.034$). TC in feces was only affected by dietary cholesterol, increasing significantly with the higher dietary cholesterol ($P < 0.001$). No significant interaction between dietary cholesterol and phospholipids was observed on the TC in feces ($P = 0.179$). (Table 5).

### 3.4. Expression of hmgcr, cyp7a1 and ghrelin

The expression of *hmgcr* in liver was significantly inhibited in fish fed moderate and high cholesterol diet ($P < 0.001$) regardless of the phospholipids level. The dietary phospholipids ($P = 0.357$) and its interaction with cholesterol ($P = 0.380$) did not affect the expression of *hmgcr* in liver (Fig. 1). Contrary to *hmgcr*, the expression of *cyp7a1* in liver was significantly promoted by dietary cholesterol ($P < 0.001$). The dietary phospholipids ($P = 0.958$) and its interaction with cholesterol ($P = 0.587$) did not affect the expression of *cyp7a1* in liver, either (Fig. 2). Dietary phospholipids significantly affected the expression of

**Table 4**
Effect of the interaction between dietary phospholipids and cholesterol on the composition of the whole body of juvenile turbot (*Scophthalmus maximus* L.) (n = 3).[1]

| Phospholipids | Cholesterol | Moisture (%) | Crude protein (%) | Crude lipid (%) | Ash (%) |
|---|---|---|---|---|---|
| Individual treatment means | | | | | |
| LP | LC | 77.83 | 15.77 | 3.11 | 3.78 |
| LP | MC | 77.77 | 15.74 | 3.17 | 3.75 |
| LP | HC | 77.92 | 15.77 | 3.11 | 3.72 |
| MP | LC | 78.41 | 15.17 | 2.38 | 3.90 |
| MP | MC | 78.00 | 15.52 | 2.79 | 3.70 |
| MP | HC | 77.67 | 15.81 | 3.20 | 3.60 |
| HP | LC | 78.30 | 15.51 | 2.40 | 3.83 |
| HP | MC | 76.93 | 16.32 | 2.79 | 3.98 |
| HP | HC | 77.96 | 15.60 | 2.52 | 3.80 |
| Pooled S.E.M | | 0.14 | 0.11 | 0.08 | 0.03 |
| Means of main effect | | | | | |
| LP | | 77.84 | 15.76 | $3.13^{a}$ | 3.75 |
| MP | | 78.03 | 15.50 | $2.79^{b}$ | 3.73 |
| HP | | 77.73 | 15.81 | $2.57^{b}$ | 3.87 |
| | LC | 78.18 | 15.48 | 2.63 | 3.84 |
| | MC | 77.57 | 15.86 | 2.91 | 3.81 |
| | HC | 77.85 | 15.72 | 2.94 | 3.71 |
| ANOVA: *P*-Values | | | | | |
| Phospholipids | | 0.708 | 0.492 | 0.002 | 0.182 |
| Cholesterol | | 0.251 | 0.403 | 0.062 | 0.233 |
| Phospholipids*Cholesterol | | 0.436 | 0.513 | 0.107 | 0.392 |

[1] Treatment means represent the average values of three tanks per treatment. Means followed by the same letter are not significantly different ($P > 0.05$).

ghrelin in brain ($P < 0.001$). A significantly higher expression of ghrelin in brain was found in fish fed moderate phospholipids than those fed either low or high phospholipids. However, dietary cholesterol ($P = 0.787$) and its interaction with phospholipids ($P = 0.476$) showed no significant effect on ghrelin expression in the brain. (Fig. 3) Unlike in the brain, ghrelin expression in the intestine was significantly affected by the interactive effect between dietary cholesterol and phospholipids ($P = 0.011$) (Fig. 4).

## 4. Discussion

Consistent with the previous study (Zhu et al., 2014), the growth performance of fish fed the moderate and high cholesterol diets (1% and 2% cholesterol) was increased significantly regardless of dietary

**Table 5**
Effect of the interaction between dietary phospholipids and cholesterol on serum, liver, feces cholesterol of juvenile turbot (*Scophthalmus maximus* L.) (n = 3).[1]

| Phospholipids | Cholesterol | Serum | | | | | | Liver | Feces |
|---|---|---|---|---|---|---|---|---|---|
| | | TC[2] | HDL-C[3] | LDL-C[4] | HDL-C/LDL-C | FC[5] | CE[6] | TC | TC |
| Individual treatment means | | | | | | | | | |
| LP | LC | 1.16 | $0.35^{e}$ | $0.23^{de}$ | 1.59 | 0.65 | 0.50 | 0.82 | 0.32 |
| LP | MC | 2.57 | $0.78^{cd}$ | $1.02^{a}$ | 0.77 | 1.39 | 1.17 | 1.46 | 0.57 |
| LP | HC | 3.34 | $0.90^{c}$ | $1.00^{ab}$ | 0.91 | 1.33 | 2.01 | 1.37 | 1.09 |
| MP | LC | 1.21 | $0.35^{e}$ | $0.20^{e}$ | 1.78 | 0.61 | 0.60 | 1.00 | 0.32 |
| MP | MC | 2.34 | $0.57^{de}$ | $0.73^{bc}$ | 0.77 | 1.05 | 1.29 | 1.66 | 1.02 |
| MP | HC | 3.54 | $0.82^{c}$ | $1.00^{ab}$ | 0.82 | 1.18 | 2.36 | 1.60 | 1.27 |
| HP | LC | 1.28 | $0.47^{e}$ | $0.22^{de}$ | 2.22 | 0.73 | 0.56 | 0.99 | 0.41 |
| HP | MC | 2.00 | $0.52^{e}$ | $0.50^{cd}$ | 1.10 | 1.22 | 0.78 | 1.62 | 0.67 |
| HP | HC | 2.77 | $0.78^{cd}$ | $0.97^{ab}$ | 0.81 | 1.25 | 1.52 | 2.34 | 1.18 |
| Pooled S.E.M | | 0.17 | 0.04 | 0.07 | 0.11 | 0.06 | 0.13 | 0.10 | 0.08 |
| Means of main effect | | | | | | | | | |
| LP | | $2.36^{a}$ | $0.68^{a}$ | $0.75^{a}$ | 1.09 | 1.13 | $1.23^{a}$ | $1.21^{b}$ | 0.66 |
| MP | | $2.36^{a}$ | $0.58^{b}$ | $0.64^{b}$ | 1.12 | 0.95 | $1.42^{a}$ | $1.42^{ab}$ | 0.87 |
| HP | | $2.02^{b}$ | $0.59^{b}$ | $0.56^{b}$ | 1.38 | 1.07 | $0.95^{b}$ | $1.65^{a}$ | 0.75 |
| | LC | $1.21^{c}$ | $0.39^{c}$ | $0.22^{c}$ | $1.87^{a}$ | $0.66^{b}$ | $0.55^{c}$ | $0.93^{b}$ | $0.35^{c}$ |
| | MC | $2.30^{b}$ | $0.62^{b}$ | $0.75^{b}$ | $0.88^{b}$ | $1.22^{a}$ | $1.08^{b}$ | $1.58^{a}$ | $0.75^{b}$ |
| | HC | $3.22^{a}$ | $0.83^{a}$ | $0.99^{a}$ | $0.85^{b}$ | $1.26^{a}$ | $1.96^{a}$ | $1.77^{a}$ | $1.18^{a}$ |
| ANOVA: *P*-Values | | | | | | | | | |
| Phospholipids | | 0.037 | 0.030 | 0.003 | 0.118 | 0.105 | 0.007 | 0.034 | 0.053 |
| Cholesterol | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| Phospholipids*Cholesterol | | 0.111 | 0.012 | 0.002 | 0.384 | 0.584 | 0.170 | 0.153 | 0.179 |

[1] Treatment means represent the average values of three tanks per treatment. Tukey's test was conducted for individual means only if there was a significant interaction. Means followed by the same letter are not significantly different ($P > 0.05$).

[2] TC: Total cholesterol.

[3] HDL-C: High-density lipoprotein cholesterol.

[4] LDL-C: Low-density lipoprotein cholesterol.

[5] FC: Free cholesterol.

[6] CE: Cholesterol ester.

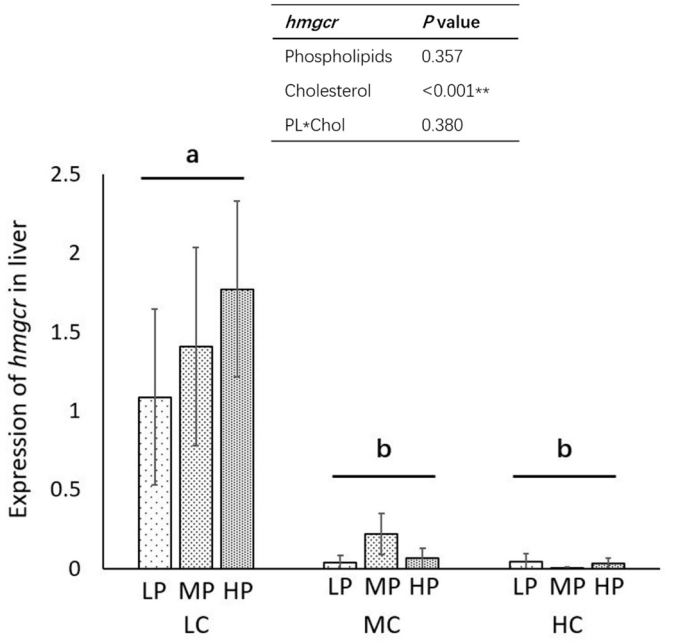


**Fig. 1.** Effects of dietary cholesterol and phospholipids on relative mRNA expression of *hmgcr* in the liver of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n 3), with their standard errors represented by vertical bars.[a, b] Mean values with unlike letters were significantly different ($P < 0.05$; Tukey's test). Values labeled with "**" are statistically significant with $P < 0.01$. PL, phospholipids; Chol, cholesterol.

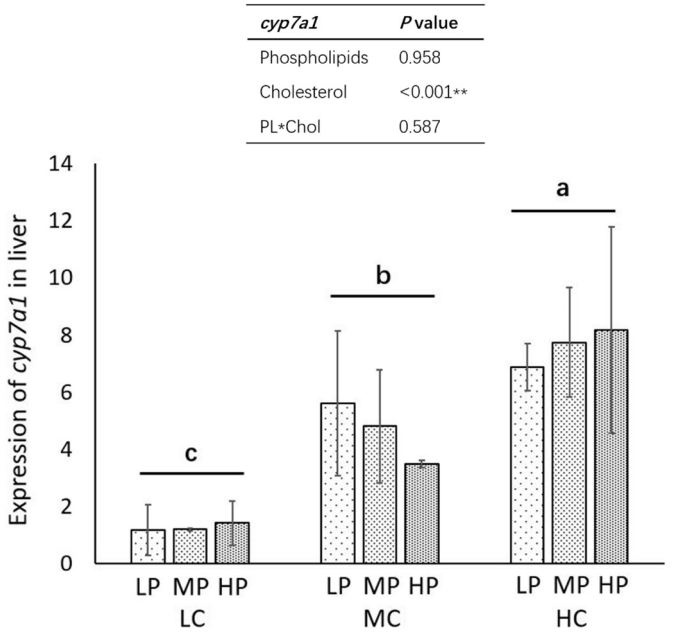


**Fig. 2.** Effects of dietary cholesterol and phospholipids on relative mRNA expression of *cyp7a1* in the liver of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n 3), with their standard errors represented by vertical bars.[a, b, c] Mean values with unlike letters were significantly different ($P < 0.05$; Tukey's test). Values labeled with "**" are statistically significant with $P < 0.01$. PL, phospholipids; Chol, cholesterol.

phospholipids. This growth promoting effect of dietary cholesterol can be attributed to the increased feed intake, which was higher in fish fed moderate and high cholesterol as well. On the other hand, dietary phospholipids exerted no effect on the final body weight and feed intake of juvenile turbot, which is different from the growth promoting effect of dietary phospholipids in larval fish (Coutteau et al., 1997; Feng et al., 2017). The higher ability to synthesize phospholipids in juvenile fish may be the main reason for such a difference between juvenile and larval fish. The interaction between dietary cholesterol and phospholipids in terms of weight gain rate was significant, although the *P* value was on the edge of significance. This is an important finding and further studies are required to elucidate the specific mechanisms underlying the interaction with regard to growth performance.

Previous studies in fish showed that dietary phospholipid deficiency resulted in lipid accumulation in the intestinal enterocytes and suggested that efficient lipid exportation from the intestine required phospholipids (Olsen et al., 1999; Salhi et al., 1999; Stéphanie et al., 1998). In the present study, the higher body lipid in fish fed the low phospholipid diet may be due to lipid accumulation in the intestine or liver, suggesting that phospholipids play an important role in suppressing lipid accumulation in juvenile turbot. However, further studies using histological methods are proposed to verify this point.

An interesting finding is that the interaction between cholesterol and phospholipids affected levels of high-density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C) cholesterol in serum rather than total cholesterol, free cholesterol and cholesterol ester in serum. Like mammals, after being digested by several lipases in the intestine, the exogenous lipids including cholesterol and phospholipids are absorbed by enterocytes in fish. Then cholesterol, phospholipids and triglycerides are transported from the intestine to the liver by chylomicrons. VLDL formed in the liver then transports the lipids to the extrahepatic tissues via the circulating system during which triglycerides in the VLDL are hydrolyzed, resulting in the LDL whose main function is to provide cholesterol for peripheral tissues (Babin and Vernier, 1989; Tocher, 1995). On the other hand, lecithin cholesterol acyltransferase (LCAT) catalyzes the esterification of cholesterol in initial HDL by transferring fatty acid from phospholipids to free cholesterol, resulting in the maturation of HDL (Eisenberg, 1984). After that, mature HDL transports the cholesterol ester from peripheral tissues to the liver and steroidogenic organs (Stein and Stein, 1999). Therefore, phospholipids and cholesterol are not only required for HDL and LDL but also interact with each other during their transport, which may result in this interaction between dietary phospholipids and cholesterol on HDL-C and LDL-C.

Total cholesterol, free cholesterol and cholesterol ester in serum were all positively influenced by the dietary cholesterol, which is in accordance with previous studies on turbot (Yun et al., 2011; Zhu et al., 2014), suggesting a positive correlation between dietary cholesterol and cholesterol in serum. This differs from studies in mammals showing that dietary cholesterol exerted a very small effect on the level of cholesterol in plasma (Hegsted, 1986; Kern Jr, 1991). The reason for this may be due to the lipoproteinemia associated with low protein levels in the plasma of fish compared to mammals, resulting in the more susceptibility to dietary cholesterol (Babin and Vernier, 1989). However, further studies should be implemented to elucidate this difference between fish and mammals.

Furthermore, total cholesterol and cholesterol ester were decreased with increasing dietary phospholipids. However, free cholesterol was not affected by dietary phospholipids, indicating this lowering effect on total cholesterol in serum by dietary phospholipids was mainly attributed to a decrease in cholesterol ester. Similarly, the reduction of cholesterol in plasma by soybean phospholipids was also found in rats (Iwata et al., 1992) and humans (Childs et al., 1981).

A much lower HDL-C/LDL-C in plasma appeared in fish fed moderate and high cholesterol, indicating that cholesterol tends to move towards the peripheral tissues. Together with the finding that more cholesterol was found in feces when fish were fed increasing dietary cholesterol, these results suggested that both cholesterol elimination and transport to peripheral tissues were promoted when fish were fed a high cholesterol diet. Furthermore, as liver is the major organ involved in cholesterol metabolism and the synthesis of bile acids in the liver accounts for the largest proportion in cholesterol catabolism (Russell,

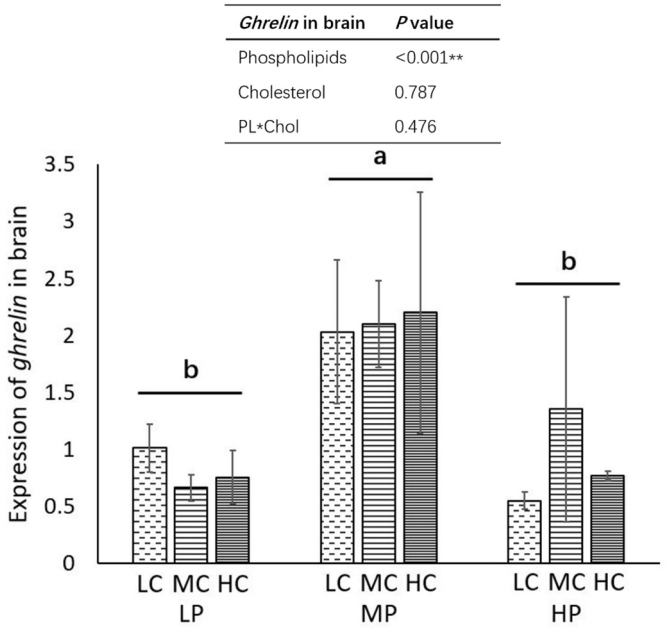


**Fig. 3.** Effects of dietary cholesterol and phospholipids on relative mRNA expression of *ghrelin* in the brain of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n 3), with their standard errors represented by vertical bars.[a, b] Mean values with unlike letters were significantly different ($P < 0.05$; Tukey's test). Values labeled with "**" are statistically significant with $P < 0.01$. PL, phospholipids; Chol, cholesterol.

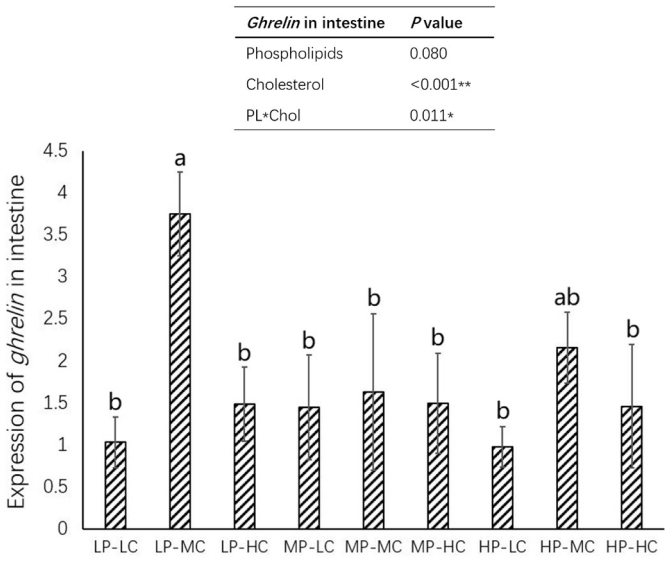


**Fig. 4.** Effects of dietary cholesterol and phospholipids on relative mRNA expression of *ghrelin* in the intestine of juvenile turbot (*Scophthalmus maximus* L.). Values are means (n 3), with their standard errors represented by vertical bars.[a, b] Mean values with unlike letters were significantly different ($P < 0.05$; Tukey's test). Values labeled with "*" are statistically significant with $P < 0.05$. Values labeled with "**" are statistically significant with $P < 0.01$. PL, phospholipids; Chol, cholesterol.

1992), cholesterol accumulation in the liver of fish fed a high cholesterol diet may also suggest a tendency towards cholesterol elimination. Additionally, the liver's cholesterol content was promoted by dietary phospholipids, which may be due to the promoting role of lysophosphatidylcholine on cholesterol efflux (Hara et al., 1997; Stein et al., 1979).

Regarding the gene expression involved in cholesterol metabolism in the liver, HMGCr is the key enzyme responsible for cholesterol synthesis (Bucher et al., 1960) and CYP7A1 is the key enzyme involved in bile acid synthesis, which is an important pathway for cholesterol elimination (Diane F. Jelinek et al., 1990). The increasing dietary cholesterol significantly inhibited the expression of *hmgcr* and promoted the expression of *cyp7a1* regardless of dietary phospholipids. These results showed that dietary phospholipids did not affect the synthesis of cholesterol and bile acid in fish at the level of gene expression. The inhibition of dietary cholesterol on the expression of *hmgcr* is not beyond expectation because consistent results have also been found in mammalian studies (Linn, 1967; Shapiro and Rodwell, 1969; Siperstein and Fagan, 1966). Similarly, the promoting effect of cholesterol on the expression of *cyp7a1* was also well-confirmed in mammalian studies (D. F. Jelinek et al., 1990; Li et al., 1990). Taken together, these changes caused by dietary cholesterol may work in a concerted way and contribute to cholesterol homeostasis. However, only rare studies were reported with regard to the effect of dietary phospholipids on *hmgcr* and *cyp7a1*. Alhough dietary phospholipids did not affect the expression of these two genes significantly in the present study, it would be important to conduct research in this respect since cholesterol metabolism is closely related to phospholipids.

Ghrelin is an orexigenic factor which can facilitate the weight gain and energy accumulation (Korbonits et al., 2004). In fish, the main physiological functions of ghrelin are involved in the regulation of pituitary hormone secretion and food intake (Riley et al., 2002; Unniappan and Peter, 2004). Ghrelin was primarily expressed in the gut and weakly in the brain of fish (Unniappan and Peter, 2005). In the present study, the tissue-specific effect of dietary cholesterol and phospholipids was found in the expression of ghrelin. We found that the interaction between dietary cholesterol and phospholipids affected the ghrelin expression in the intestine. However, ghrelin expression in the brain was only affected by dietary phospholipids, showing a higher expression in fish fed moderate phospholipids. Interestingly, previous studies in gilthead sea bream (Babaei et al., 2017), grass carp (Feng et al., 2013) and blunt snout bream (Ji et al., 2015) also showed that the expression of ghrelin between the intestine and brain was differently affected by starvation. However, the reason for this tissue-specific expression on ghrelin is still unclear. Moreover, ghrelin played an important role in the lipid metabolism, which was found to promote lipid storage and suppress lipid oxidation in mammals (Theander-carrillo et al., 2006) and fish (Matsuda et al., 2011; Riley et al., 2008). In the future, further studies are required to elucidate the effect of dietary cholesterol and phospholipids on ghrelin and other related orexigenic and anorexigenic factors.

In conclusion, there was an interaction between dietary cholesterol and phospholipids on growth performance in juvenile turbot in terms of weight gain rate. No interaction between dietary cholesterol and phospholipids was found in terms of feed intake and ghrelin expression in the brain. However, it significantly affected the ghrelin expression in the intestine of juvenile turbot. The results of HDL-C and LDL-C in serum suggested that cholesterol transport was significantly affected by the interaction between dietary cholesterol and phospholipids. Future studies on larvae or earlier stages of juvenile turbot would be helpful to elucidate the interaction between dietary cholesterol and phospholipids on growth performance and feed intake in fish.

## Conflict of interest

None.

## Authors contributions

Qinghui Ai and Kangsen Mai designed the study. Tengfei Zhu performed the experiments, analyzed the data and wrote the manuscript. Qinghui Ai, Kangsen Mai, Wei Xu and Tengfei Zhu contributed to manuscript corrections.

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