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Modulation of lipid metabolism, immune parameters, and hepatic *transferrin* expression in juvenile turbot (*Scophthalmus maximus* L.) by increasing dietary linseed oil levels

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A B S T R A C T

There is an increasing trend to replace fish oil (FO) in fish diets with vegetable oils (VO), driven by the short supply of FO derived from wild fish stocks. However, little is known about its influences on fish health and metabolism. In the present study, FO was increasingly replaced by linseed oil (LO) in juvenile turbot's diet, with substitution levels at 0, 33.3%, 66.7% and 100%. The 66.7% FO could be replaced by LO without affecting fish growth and feed efficiency. Turbot in three LO replacing groups (33.3%, 66.7% and 100%LO) showed decreased body protein content compared to 100% FO group, while 33.3% LO group showed the highest body lipid content. In 100% LO group, both fatty acid desaturase and sterol O-acyltransferase2 gene expression significantly increased, while carnitine palmitoyltransferase 1α gene expression significantly decreased ($P < 0.05$) compared to 100% FO group. These all suggest the inhibition of fatty acid β-oxidation and promotion of lipid biosynthesis when FO was completely replaced by LO. Gene expression of sterol-regulatory element binding protein 1, which works as a regulating factor, significantly decreased in low replacing level (33.3% LO group) while remained similar to SREBP1 expression levels of the 100% FO group. Serum metabolites such as TCHO and HDL in 33.3% LO group were significantly higher than the 100% LO group, while serum AST activities significantly increased with increasing LO level ($P < 0.05$). For the immune parameters, both serum lysozyme activity and total antioxidant capacity in 100% LO group were significantly lower than 100% FO group, while superoxide dismutase activity was not significantly affected by fish oil replacement. Compared to 100% FO group, hepatic *transferrin* gene expression increased in 33.3% LO group but decreased in 100% LO group, which might be the reason of slight anaemia in 100% LO group. Further studies are still needed to illustrate the inner mechanism of *transferrin* regulation.

Statement of Relevance: In commercial aquaculture, fish oil replacement has been widely conducted but less is known about its effect on lipid metabolism and immunity. Our research found that fish oil could be replaced by linseed oil at 66.7% in juvenile turbot's diet without affecting fish growth performance and immunity.

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1. Introduction

Over the past several decades, fish oil (FO) has been the principal lipid source for fish diets in intensively farmed fish (Sargent and Tacon, 1999). However, such high reliance on fish oil restrains the sustainability of aquaculture, due to the declined fishery resources and limited fish oil supplies (Naylor et al., 2000; FAO, 2006). Searching for alternative substitutes of FO has been an important research aspect of fish nutritionists, and vegetable oils (VOs) are the most practical substitutes for its low price and wide distribution (Tacon and Metian, 2008). Certain studies have shown that dietary FO can be fully or partially substituted with VOs without affecting growth performance of several fish species such as yellowtail (*Seriola* sp.) (Watanabe, 2002), turbot (*Psetta maxima*) (Regost et al., 2003), European sea bass (*Dicentrarchus labrax*) (Yildiz and Sener, 2004; Montero et al., 2005; Mourente et al., 2005), sharpsnout sea bream (*Diplodus puntazzo*) (Piedecausa et al., 2007), red sea bream (*Pagrus auratus*) (Glencross et al., 2003) and gilthead sea bream (*Sparus aurata*) (Caballero et al., 2003; Izquierdo et al., 2003; Izquierdo et al., 2005). However, the effects of VOs on fish metabolism, physiology and ultimately fish health are not well understood.

Fatty acids regulate the hepatic expression of genes involved in fatty acid desaturation and elongation (Zheng et al., 2004). A few studies showed that FO replacement by VOs in fish diets will increase expression of genes involved in cholesterol biosynthesis such as sterol O-acyltransferase2 (Soat2) and highly unsaturated fatty acid biosynthesis

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such as fatty acid desaturase (FADS) in salmonids (Zheng et al., 2004; Jordal et al., 2005). Fatty acid β-oxidation indicated by carnitine palmitoyltransferase 1α (Cpt1α) and several regulating factors such as peroxisome proliferators-activated receptor α (PPARα) and sterol regulatory element binding protein 1 (SREBP1) were also reported to be affected when fish oil was replaced by LO (Stubhaug et al., 2007). However, effects of fish oil substitution on fish growth and lipid metabolism is sometimes species-specific and may follow different parameters (Montero et al., 2008; Turchini et al., 2009). Further studies on hepatic metabolic gene expression in response to fish oil substitution are required for fish species other than salmonids.

Fish health, especially immune activity, has received more attention in recent years (Trichet, 2010; Kiron, 2012), and fish immune response has been shown to be modulated by various nutrients such as protein (Pérez-Bárcena et al., 2008), lipids (Watkins et al., 2001; Montero et al., 2003), carbohydrates (Kumar et al., 2007), antioxidant vitamins, carotenoids and minerals (Hung et al., 2007). Effects of fatty acids on the fish immune system depend on several biological and methodological factors, including type and concentration of fatty acids, cell types, and species of experimental animals (Calder, 2007). Indeed, dietary n-6/n-3 ratios have been shown to influence immune cell phospholipids (Montero et al., 2003), signal transduction pathways (Brassard et al., 2007), immune cell function (Lin et al., 2007) and finally fish pathogen resistance (Brandsen et al., 2003). However, little is known about the effect of dietary fatty acids on other processes, such as those related with bacterial infection. Transferrins are a group of serum proteins in eukaryotes which act as important modulating elements of nutritional immunity, antimicrobial and antioxidant activity, operating by restricting iron availability (Stafford and Belosevic, 2003; Ong et al., 2006). *Transferrin* genes have been cloned from a variety of fish species, including salmonids (Lee et al., 1998), Atlantic cod (Denovan-Wright et al., 1996), *Cirrhinus mrigala* (Sahoo et al., 2009), roughskin sculpin (Liu et al., 2012), red seabream (Neves et al., 2009) and turbot (Low et al., 2003). Transferrin protein is mainly synthesized in liver and secreted into blood, but high expression has also been found in other tissues including the brain and central nervous system, testes, ovary, spleen, mammary land and kidney (Lambert et al., 2005). However, the effect of dietary lipid sources on fish immune ability including the antibacterial responses has seldom been studied.

Thus, the aim of this study was to evaluate the effect of FO substitution with linseed oil (LO) on metabolic gene expression and some immune parameters, including hepatic *transferrin* expression in juvenile turbots.

2. Methods and materials

2.1. Experimental diets

Fishmeal was defatted (<2%) with alcohol to minimize the effects of fish oil that was contained in fishmeal, and then used as main protein source along with soybean meal. FO and LO served as the main lipid sources of the diets. As shown in Table 1, FO was increasingly replaced by LO at 0% (Diet 1), 33.3% (Diet 2), 66.7% (Diet 3) and 100% (Diet 4) replacing levels. All four experimental diets were iso-protein (appropriately 50%) and iso-lipid (12.5%) and met the nutritional requirements of turbot (Lee et al., 2003; Regost et al., 2001). A composite attractant (betaine: dimethyl-β-propiothetin: threonine: glycine: inosine-5′-diphosphate trisodium salt = 3:4:1:1:1) was used to improve the diets' palatability (Wang et al., 2015). All the ingredients were mixed step by step, and then lipids were distributed to the diets homogeneously. After water was added to the compound, diets were manufactured using a twin-screw extruder in the form of 3-mm diameter pellets and then stored in −20 °C freezer. Ingredient and proximate composition of the diets are reported in Table 1 and fatty acid compositions of experimental diets are shown in Table 2.

Table 1
Ingredient and nutrient composition of the experimental diets.

	Diet1	Diet2	Diet3	Diet4
Defatted fishmeal[a]	46	46	46	46
Soybean meal	20	20	20	20
Wheat flour	13.75	13.75	13.75	13.75
Beer yeast	2	2	2	2
Fish oil	9	6	3	0
Linseed oil	0	3	6	9
Soy lecithin	2.5	2.5	2.5	2.5
Vitamin premix[b]	2	2	2	2
Mineral premix[c]	2	2	2	2
Monocalcium phosphate	1	1	1	1
Attractants[d]	0.8	0.8	0.8	0.8
Binder	0.5	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2
Yttrium trioxide	0.1	0.1	0.1	0.1
Calcium propionate	0.1	0.1	0.1	0.1
Ethoxy quinoline	0.05	0.05	0.05	0.05
Proximate composition				
Crude protein	50.16	50.19	50.17	50.18
Crude lipid	12.57	12.59	12.53	12.55

[a] Fish oil was defatted with ethanol and <2% lipid content was left in defatted fish meal.
[b] Supplied the following(mgkg^{-1} diet):retinyl acetate, 32; cholecalciferol, 5; all-rac-a-tocopheryl acetate, 240;menadione sodium bisulphite, 10; ascorbic acid, 120; cyanocobalamin, 10; biotin, 60; choline dihydrogen citrate, 7 g; folie acid, 20; inositol, 800; niacin, 200; D-Ca-pantothenate, 60; pyridoxine HCl, 20; riboflavin, 45; thiamin HCl, 25,Microcrystalline cellulose, 16,473.
[c] Supplied the following(mgkg^{-1} diet): MgSO4-7H2O, 1200; CuSO4-7H2O,10; FeSO4-7H2O, 80; ZnSO4-H2O, 50;MnSO4-H2O, 45; CoCl2, 5; Na2SeO3, 20;Calcium Iodate , 60; Zeolite powder, 8485.
[d] Supplied the following(% diet): betaine, 0.4; DMPT ,0.2; threonine,0.2; Glycine, 0.1; Inosine-5′-diphosphate trisodium salt, 0.1.

2.2. Experimental procedure

Juvenile turbot were purchased from a fish-rearing farm (Laizhou, Shandong, China) and experiments were conducted at the National Oceanographic Center (Qingdao, China). All fish were acclimated to experimental conditions feeding Diet 1 for two weeks before experiments. At the beginning of the experiment, thirty turbot (5.00 g ± 0.10) were randomly allotted to each tank (300*L*) with 3 tanks per treatment. All tanks were connected to a circulating seawater system with flow rate set at 0.5 l/min, oxygen concentration at over 85% saturation. Daylight

Table 2
Fatty acid composition (% total fatty acids) of the experimental diets.[a]

Fatty acid	Diet1	Diet2	Diet 3	Diet 4
C14:0	6.05	3.99	2.40	0.42
C16:0	24.39	21.30	15.08	9.89
C18:0	4.26	4.20	6.55	4.31
C20:0	3.06	2.12	0.34	0.22
ΣSFA[b]	37.76	31.61	24.37	14.84
C16:1	6.66	5.03	2.71	1.10
C18:1	18.35	18.76	17.60	22.02
ΣMUFA[c]	25.01	23.79	20.31	23.12
C18:2n-6	16.99	17.58	17.45	20.51
C20:4n-6	0.55	0.32	0.17	0.05
Σn-6PUFA[d]	17.54	17.90	17.62	20.56
C18:3n-3	3.27	13.98	24.59	34.51
C20:5n-3	5.24	3.51	2.20	0.77
C22:6n-3	6.18	4.21	2.89	1.25
Σn-3PUFA[e]	14.69	21.70	29.67	36.53
ΣSFA/ΣPUFA	1.17	0.80	0.52	0.26
EPA/DHA	0.85	0.83	0.76	0.61

[a] Values are mean ± S.E. of 3 replicate tanks. Values with the different superscript are statistically different ($p < 0.05$).
[b] SFAs: saturated fatty acids.
[c] MUFAs: mono-unsaturated fatty acids.
[d] n − 6 PUFAs: n − 6 poly-unsaturated fatty acids.
[e] n − 3 PUFAs: n − 3 poly-unsaturated fatty acids.

followed natural changes over trial (July 9–September14). Water temperature was maintained at 18 ± 1 °C.

2.3. Sampling procedure

At the end of the rearing experiment, turbot were fasted for 24 h and anesthetized with MS222 (1:10,000) (Shanghai Reagent, China). Then all fish per tank were weighted together to calculate the growth parameters, within which 10 fish were further sampled for other analysis. Four fish per tank with 3 tanks per treatment were sacrificed for tissue samples such as liver, muscle and intestine. Blood samples from 3 fish per tank were transferred into heparin sodium pre-coated tubes and used for hematological analysis right away. Blood samples from another 3 fish were transferred into non-coated tubes, centrifuged at 3000 ×*g* for 10 min at 4 °C·All tissue samples including tissue and serum were stored in liquid nitrogen and transferred into −80 freezer until analysis. Another 3 fish per tank were collected and stored in −20 °C freezer for whole body composition analysis.

2.4. Growth parameters

As mentioned above, all fish were weighted together after anesthetization to calculate the average weight. Weights of feeds, which have been fed to turbot in each tank, were also used to calculate FI and FCR.

Specific growth rate (SGR) = (ln (final body weight)-ln (initial body weight))/rearing days × 100%;

Feed conversion ratio (FCR) = dry matter intake/wet weight gain;

Feed intake (FI) = 100 × dry matter intake × 2/((initial weight + final weight) × rearing days).

2.5. Biochemical analysis

Chemical composition of whole body and tissues were measured following standard methods (AOAC, 2012). Dry matter was analyzed by drying the samples to constant weight at 105 °C. Crude protein was determined by using the Kjeldahl method (Kjeltec TM 8400, FOSS, Sweden) and estimated by multiplying nitrogen by 6.25. Crude lipid was measured after diethyl ether extraction using Soxhlet method (Buchi 36680, Switzerland). Ash was determined after combustion in a muffle furnace at 550 °C for 16 h.

2.6. Blood analysis

2.6.1. Hematological index

Hematological index such as red blood cell number (RBC), was determined by a blood cell counter (Mindray BC-3000 Plus, Shenzhen, China).

2.6.2. Serum metabolites assay

Serum biochemical parameters including total cholesterol (TCHO), high-density lipo-protein (HDL), along with activities of serum alkaline phosphatase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed by Automatic biochemical analyser (Sysmex-800,Sysmex Corporation, Kobe, Japan) using the Diagnostic Reagent Kits (Sysmex Wuxi, Wuxi, China).

2.7. Functional immune assay

2.7.1. Lysozyme (LMZ) activity

LMZ activity in serum was measured according to the method of Ellis (Ellis, 1990). Briefly, a sample of 0.05 ml serum was added to a 1.4 ml suspension of Micrococcus lysodeikticus (Sigma) (0.2 mg ml/1) in a 0.1 M sodium phosphate buffer (pH 6.8). The reaction was carried at 25 °C and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer. Each unit is defined as the amount of sample causing a decrease in absorbance of 0.001 per minute.

2.7.2. Superoxide dismutase (SOD) activity

Serum SOD activity was measured spectrophotochemically by the ferricytochrome C method of Zuo et al. (2012) using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome C and 0.024 IU ml/1 xanthine oxidase. The reaction was triggered by the addition of the xanthine oxidase. Results are expressed in units of SOD per milliliter serum and each unit is defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate measured at 550 nm.

2.7.3. Total antioxidant capacity (TAC)

TAC was assayed by 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) method as described by Luo et al. (2012). ABTS was oxidized by reactive oxygen species (ROS) to green $ABTS^{+}$ while this process might be inhibited by antioxidant. Trolox solution acted as the standard to assay TAC,with all results expressed as equal to mM trolox.

2.8. Quantitative RT-PCR for lipid metabolism and transferrin expression

Total RNA sample was extracted from hepatocytes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and oligo dT primers (Promega, Charbonnie `res, France) according to the manufacturers' instructions. Primers for genes involved in lipid metabolism and transferrin are listed in Table 3. Elongation factor 1α (EF1α) was employed as a non-regulated reference gene and no changes in EF1α gene expression were observed in our investigations (data not shown). Target gene expression levels were determined by quantitative RT-PCR. The RT-PCR method was according to Wang et al. (2014). To calculate the expression of hepatic genes, the comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used.

2.9. Statistics analysis

All data were analyzed by one-way ANOVA for the effects of fish oil replacement by linseed oil. Homogeneity of variance test was conducted to ensure that variance is homogeneous. Tukey's test was utilized to

Table 3
Primers used in this study.

Genes	Sense primer	Reversed antisense primer	References
Fads2	TGTCCTACTATCTTCGCTTCTTC	TAACCAGTCCTTGTGCTTCTC	Cunha et al. (2013)
Soat2	GCTCGTGATGTTCGTCTAC	TGAATGGAGGACAAGATTAACC	Cunha et al. (2013)
Cpt1a	ATGGGAAGAGTGGACTGAATG	GCTGGAAGGCATCTGTGG	Cunha et al. (2013)
PPARα	GCGTCCCTTCAGTGATAT	CTCCACAGCAGATGATAG	Cunha et al. (2013)
SREBP1	GCCATTGACTACATCCGTTAC	CATCAGCCTGTCCATCTACTTC	Cunha et al. (2013)
Transferrin	CTGTCGCTGTGGCGAAGAAGG	CGATGGGCGCTGTGCTGTTAT	Low et al. (2003)
EF-1a	TATTAACATCGTGGTCATTGG	CAGGCGTACTTGAAGGAG	Dang and Sun (2011)

Table 4
Effect on growth performance and feed intake on turbot fed gradient dietary linseed oil. (Values in the same row with the different superscript were statistically different ($p < 0.05$)).

	Diet1	Diet 2	Diet 3	Diet 4	F-value	P-value
Initial body weight(g)	5.11 ± 0.01	5.10 ± 0.01	5.11 ± 0.01	5.11 ± 0.01		
Final body weight(g)	28.18 ± 1.32b	28.93 ± 0.44 b	26.81 ± 1.90 b	20.67 ± 0.90a	26.47	0.00
SGR(%/day)[a]	2.71 ± 0.07 b	2.75 ± 0.03 b	2.63 ± 0.11 b	2.22 ± 0.07 a	29.32	0.00
FI(%/day)[b]	1.05 ± 0.01	1.04 ± 0.01	0.99 ± 0.03	1.05 ± 0.02	4.07	0.06
FCR[c]	0.95 ± 0.01a	0.94 ± 0.01a	0.922 ± 0.02a	1.08 ± 0.04b	15.08	0.00

[a] SGR: Specific growth rate.
[b] FI: Feed intake.
[c] FCR: Feed convertion ratio.

Table 5
Crude composition of turbot fed gradient dietary linseed oil. (Values in the same row with the different superscript were statistically different ($p < 0.05$)).

	Diet1	Diet 2	Diet 3	Diet 4	F-value	P-value
Moisture[a]	77.67 ± 0.13	77.15 ± 0.10	77.22 ± 0.07	77.22 ± 0.21	2.97	0.10
Protein[b]	70.57 ± 0.37b	66.88 ± 0.08a	67.01 ± 0.60a	66.89 ± 0.26a	23.18	0.00
Lipid[b]	12.36 ± 0.73ab	13.93 ± 0.86b	10.59 ± 0.41a	10.34 ± 0.75a	5.64	0.02
Ash[b]	17.25 ± 0.28	17.02 ± 0.19	17.47 ± 0.33	18.16 ± 0.52	1.96	0.20

[a] Percentage of wet weight.
[b] Percentage of dry weight.

compare individual means. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Growth performance

All diets in four experimental groups were well accepted by turbot and no significant differences in FI were found among different diets (Table 4). FO substitution by LO did not affect fish growth (SGR and FBW) when the substituting level was not higher than 66.7%, however, turbot fed diet with 100% LO showed decreased growth compared to those fed 100% FO. In agreement with these results, the FCR of turbot in the 100% LO group was significantly higher than those in 100% FO group, while turbot in 33.3% LO and 66.7% LO groups showed a similar FCR like 100% FO (Table 4).

3.2. Whole body composition

Turbot body composition was also significantly influenced by FO replacement. As shown in Table 5, fish body protein contents significantly decreased when FO was replaced by LO. Moreover, turbot in 33.3% LO group showed the highest lipid content and was significantly higher than 66.7% LO and 100% LO group. Moisture and ash contents of whole body were not affected by fish oil replacement.

3.3. Blood analysis

As shown in Table 6, red blood cell numbers in 100% LO groups were significantly lower than 33.3% LO and 100% FO group.

Serum TCHO concentration was significantly higher in 33.3% LO group than 100% LO group. HDLC in serum of 66.7% LO and 100% LO group were significantly lower than that in 33.3% LO group. In all, 33.3% LO group showed the highest serum TCHO and HDLC levels. Amino acid metabolism enzymes such as serum AST activities showed a trend to increase with increasing replacing level, and AST activities in 66.7% and 100% LO replacing level were significantly higher than 100% FO group. ALT activities were not significantly affected by linseed oil replacement.

3.4. Lipid metabolism gene expression

FADS which plays a key role in high unsaturated fatty acid biosynthesis, showed a trend to increase with increasing dietary LO level and 66.7% and 100% LO group showed higher expression levels than 100% FO group (Fig. 1). Soat2 which controls FA incorporation into cholesteryl esters, i.e., cholesterol synthesis, decreased in 33.3% LO group but increased in 100% LO group ($P < 0.05$), compared to 100% FO group. Cpt1α which controls fatty acid β-oxidation was significantly lower in the 100% LO group, whereas expression levels in the 33.3% and 66.7% groups were similar to the 100% FO group. For the regulating factors, SREBP1 showed a significant decrease at 33.3% replacing level while remained similar to SREBP1 expression levels of the 100% FO group at 100% replacing level. PPARα did not show any significant changes after fish oil replacement.

3.5. Functional immune assay and transferrin gene expression

LMZ activity in serum was significantly decreased in the 66.7% LO and the 100% LO group, compared to the 100% FO group (Table 7). Serum total SOD activity was not significantly affected by different

Table 6
Red blood cell number and serum metabolites in turbot fed gradient dietary linseed oil. (Values in the same row with the different superscript were statistically different ($p < 0.05$)).

	Diet1	Diet 2	Diet 3	Diet 4	F-value	P-value
Hematological index						
RBCs(10^{12}/l)	144.89 ± 2.02b	142.89 ± 2.17b	134.78 ± 1.73ab	112.63 ± 2.01a	22.65	0.00
Serum metabolites						
TCHO (mmol/l)	1.70 ± 0.21ab	1.99 ± 0.14b	1.69 ± 0.13ab	1.14 ± 0.09a	5.67	0.02
HDLC (mmol/l)	1.33 ± 0.02ab	1.44 ± 0.01b	1.31 ± 0.01a	1.31 ± 0.04a	5.91	0.02
AST (U/l)	13.00 ± 1.53a	21.00 ± 3.51ab	28.00 ± 3.61bc	34.67 ± 2.60c	10.05	0.00
ALT (U/l)	7.33 ± 0.88	5.33 ± 0.33	6.00 ± 0.57	5.00 ± 1.15	1.67	0.25

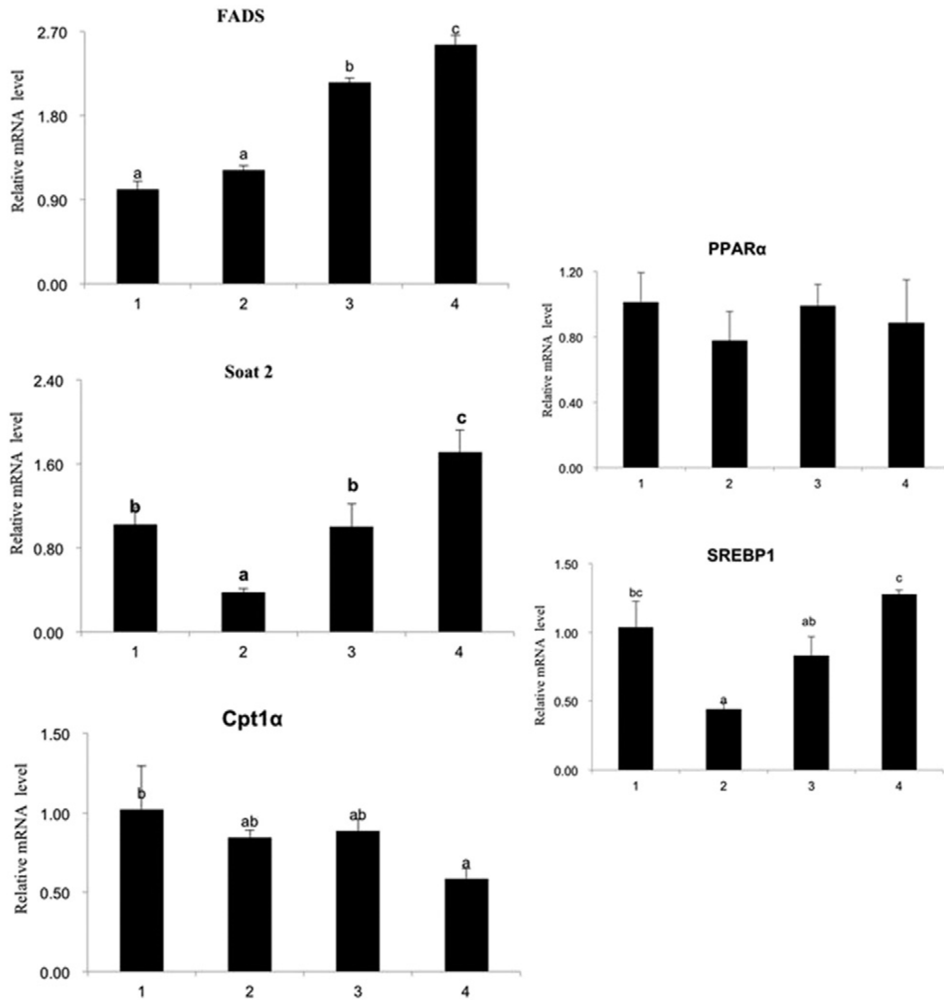

Fig. 1. Hepatic metabolic gene expression in turbot fed increasing dietary linseed oil. Values are mean ± S.E. ($n = 3$). Values with the different superscript are statistically different ($p < 0.05$).

Table 7
Serum function immune activity of turbot fed gradient dietary linseed oil. (Values in the same row with the different superscript were statistically different ($p < 0.05$).

	Diet1	Diet 2	Diet 3	Diet 4	F-value	P-value
LMZ (units/ml)	175.42 ± 1.90b	178.65 ± 1.03b	153.61 ± 2.79a	136.56 ± 0.84a	117.92	0.00
SOD (units/ml)	76.78 ± 1.03	77.24 ± 1.14	75.88 ± 0.53	73.92 ± 0.57	1.38	0.32
T-AOC (mM)	12.61 ± 0.40b	13.37 ± 0.24b	12.82 ± 0.19b	10.77 ± 0.14a	18.50	0.00

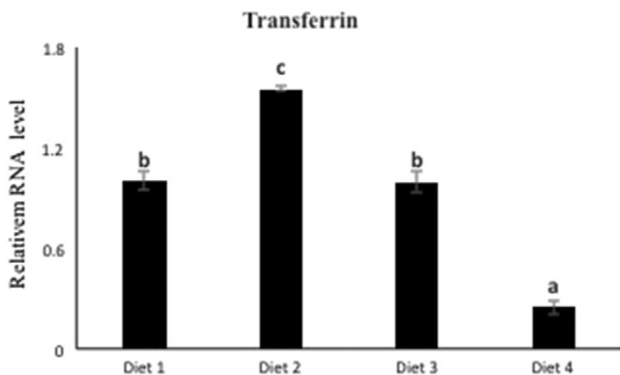

Fig. 2. *Transferrin* gene expression in turbot fed increasing dietary linseed oil. Values are mean ± S.E. ($n = 3$). Values with the different superscript are statistically different ($p < 0.05$).

dietary lipid sources. In addition, T-AOC activity was significantly lower in the 100% LO group than the 100% FO group (Table 7).

As shown in Fig. 2, *Transferrin* gene expression was significantly affected by fish oil replacement. Compared to 100% FO group, *Transferrin* gene expression showed significant increase in 33.3% LO while decreased in 100% LO.

4. Discussion

In the present study, we demonstrated that up to 66.7% dietary fish oil could be substituted by linseed oil without affecting the growth performance and feed efficiency of turbot, while complete fish oil substitution reduced fish growth and feed utilization; which is in agreement with previous studies in gilthead sea bream (Izquierdo et al., 2003; Izquierdo et al., 2005), sea bass (Yildiz and Sener, 2004), and salmonids(Bell et al., 2001). Conversely, Regost et al. (2003) reported that complete fish oil replacement did not affect the growth of turbot.

The differences reported in these two studies may be due to differences in fish size, lipid content of diets assayed and/or the source of fish meal included in the experimental diets (Montero et al., 2008). Studies have shown that fish of different size have different requirements for nutrition and juvenile fish might need more essential fatty acid than larger fish (NRC, 2011). In our experiment, the initial turbot body weight was only 5.11 g, much smaller than earlier studies with 579 g turbot (Regost et al., 2003). In addition, fish meal were defatted before usage with <1% lipid remaining in the fishmeal prior to inclusion in the diets. As a consequence, the 100% LO group cannot get essential fatty acids such as EPA and DHA from fishmeal, which resulted in growth-inhibition. It has been shown that fatty acid composition of fillet (Zuo et al., 2012) and even intestine (Montero et al., 2010) can reflect dietary fatty acid composition. In our study, lipid composition was also significantly affected by diet and the 33.3% LO group showed a significant higher lipid content than the 100% LO group. The 33.3% LO group showed highest lipid content, showing the good fatty acid balance to lipid deposition by partial linseed oil inclusion, which may suggest dietary inclusion of partial vegetable oil is even good for fish lipid deposition (Nasopoulou and Zabetakis, 2012).

Fish oil replacement significantly affected lipid metabolism, especially the high unsaturated fatty acid biosynthesis (Zheng et al., 2004). In our study, one of the main steps for high unsaturated fatty acid biosynthesis, fatty acids desaturation indicated by FADS gene expression, significantly increased in the 66.7% LO and the100% LO group, similar to results from Atlantic salmon (Leaver et al., 2008); as increased HUFA biosynthetic gene expression can at least in part account for and make up for decreased dietary HUFA level (Tocher et al., 2002). In addition, cholesterol biosynthesis indicted by Soat2 gene expression, firstly decreased and then increased with increasing fish oil replacing level, which is similar to earlier studies in salmonids (Leaver et al., 2008). Furthermore, fatty acid β-oxidation was decreased at complete fish oil replacing level by LO, which is different from results by Stubhaug et al. (2007) on salmonids. The difference might be the result of different sampling time after final feeding, as earlier studies in sea bream reported different mitochondrial and peroxisomal oxidation activity at 6 or 24 h after feeding (Diez et al., 2007). For the regulating factors, SREBP1 in 33.3% LO group was significantly lower than 100% FO group, while 100% LO group and 100% FO group showed similar SREBP1 expression. The result of SREBP1 paralleled with Soat2, which controls FA incorporation into cholesteryl esters, proved that SREBP1 may play a role in controlling FA incorporation into cholesteryl esters (Horton et al., 2002). Another regulating factor, PPARα did not show any significant change after linseed oil replacement. On the other hand, fish oil substitution also significantly changed the serum metabolites, such as serum TCHO and HDLC. TCHO has been shown to play important roles in fish growth (Yun et al., 2011) and HDLC also plays a central role in transferring the cholesterol from the extra hepatic tissues in the liver for metabolism, as high serum HDLC concentration was always correlated with good growth performance (Reyna-Villasmil et al., 2007). In this study, the levels of TCHO and HDLC were highest in 33.3% LO group, suggesting that dietary inclusion of partial LO is even good for fish metabolism.

Complete fish oil substitution has been shown to induce some negative effects on the fish immune system (Fracalossi and Lovell, 1994), despite the inner mechanisms involved being poorly understood (Trichet, 2010). For example, dietary fatty acids affect lipid composition of inflammatory and immune cells (Calder, 2007). Lysozyme is an important defense molecule of fish innate immune system, which is important in mediating protection against microbial invasion (Saurabh and Sahoo, 2008). SOD, CAT and other enzymes play roles in cleaving reactive oxygen and are proposed as biomarkers of oxidative stress in a variety of marine and freshwater organisms (Radovanović et al., 2010). T-AOC reflects the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidants (Ghiselli et al., 2000). In this study, serum LMZ activity was not significantly affected in 33.3% LO group, but significantly decreased in 66.7% LO and 100% LO group, compared to 100% FO group. Serum total SOD activity was not significantly changed. In addition, T-AOC activity showed no significant changes in both partial LO replacing level while significantly decreased in 100% LO replacing level. In vivo antioxidant activity was not influenced at partial replacing level, while decreased during complete fish oil replacement.

Transferrins are a super-family of iron-binding proteins widely distributed in vertebrates and play a basic role in the transport of iron and other metals (Gomme et al., 2005). Through the transport of many irons, transferrin participates in various metabolic processes, including immune regulation, antimicrobial and antioxidant activity (Stafford and Belosevic, 2003). In our studies, *transferrin* gene expression was significantly lower in complete fish oil replacement, suggesting that complete fish oil replacement significantly affected immune-related factors. Studies on rats showed that slight anemia was observed under conditions of sub-chronic treatment with hypolipidemic drugs/peroxisome proliferators, which may be due to the decreased iron availability by decreased *Transferrin* suppression (Hertz et al., 1996). Our study also showed a decreased blood cell number in complete fish oil replacing group which might be due to its decreased *transferrin* gene expression compared to 100% FO group. Further studies are needed to illustrate the inner mechanism between transferrin and anemia along with nutritional immunity.

Overall, under the present experimental condition, 66.7% fish oil could be replaced by linseed oil in juvenile turbot's diet without affecting fish growth, lipid metabolism and immune-related factors. Further studies are still needed to illustrate the roles of transferrin in fish immunity.

Conflict of interest

The authors declare no conflict of interest.

Authorship

Gen He and Kangsen Mai designed research; Qingchao Wang conducted research, analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

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References

AOAC, 2012. Official Methods for Analysis. 19th ed. Association of Official Analytical Chemists, Washington, USA.

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J., Sargent, J.R., 2001. Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid composition and hepatocyte fatty acid metabolism. J. Nutr. 131, 1535–1543.

Brandsen, M.P., Carter, C.G., Nichols, P.D., 2003. Replacement of fish oil with sunflower oil in feeds for Atlantic salmon (*Salmo salar* L.): effect on growth performance, tissue fatty acid composition and disease resistance. Comp. Biochem. Physiol. B 135, 611–625.

Brassard, P., Larbi, A., Grenier, A., Frisch, F., Fortin, C., Carpentier, A.C., Fülöp, T., 2007. Modulation of T-cell signaling by non-esterified fatty acids. Prostag Leukotr Ess 77, 337–343.

Caballero, M.J., Izquierdo, M.S., Kjorskvik, E., Montero, D., Socorro, J., AJ, F.´´n., et al., 2003. Morphological aspect of intestinal cells from gilthead sea bream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225, 325–340.

Calder, P.C., 2007. Immunomodulation by omega-3 fatty acids. Prostag. Leukotr. Ess. 77, 327–335.

Cunha, I., Galante-Oliveira, S., Rocha, E., Planas, M., Urbatzka, R., Castro, L.F., 2013. Dynamics of PPARs, fatty acid metabolism genes and lipid classes in eggs and early larvae of a teleost. Comp. Biochem. Physiol. B 164 (4), 247–258.

Dang, W., Sun, L., 2011. Determination of internal controls for quantitative real time RT-PCR analysis of the effect of Edwardsiella tarda infection on gene expression in turbot (*Scophthalmus maximus*). Fish Shellfish Immunol 30, 720–728.
Denovan-Wright, E.M., Ramsey, N.B., McCormick, C.J., Lazier, C.B., Wright, J.M., 1996. Nucleotide sequence of transferrin cDNAs and tissue-specific expression of the transferrin gene in Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. B 113, 269–273.
Diez, A., Menoyo, D., Pérez-Benavente, S., Calduch-Giner, J.A., de Celis S, V.-R., Obach, A., Favre-Krey, L., Boukouvala, E., MJ, L., DR, T., Pérez-Sanchez, J., Krey, G., JM, B., 2007. Conjugated linoleic acid affects lipid composition, metabolism, and gene expression in gilthead sea bream (*Sparus aurata* L). J. Nutr. 137 (6), 1363–1369.
Ellis, A.E., 1990. Lysozyme assays. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Robertsen, B.S., Van Muiswinkel, W.B. (Eds.), Techniques in Fish Immunology. SOS Publications, Fair Haven, NJ, pp. 101–103.
F.A.O. Food and Agricultural Organization, 2006. State of World Aquaculture. ftp://ftp.fao.org/docrep/fao/009/a0874e/a0874e00.pdf.
Fracalossi, D.M., Lovell, R.T., 1994. Dietary lipid sources influence responses of channel catfish (*Ictalurus punctatus*) to challenge with the pathogen Edwarsiella ictaluri. Aquaculture 119, 287–298.
Ghiselli, A., Serafini, M., Natella, F., Scaccini, C., 2000. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Radic. Biol. Med. 29 (11), 1106–1114.
Glencross, B.D., Hawkins, W.E., Curnow, J.G., 2003. Evaluation of canola oils as alternative lipid resources in diets for juvenile red seabream, *Pagrus auratus*. Aquac. Nutr. 9, 305–315.
Gomme, P.T., McCann, K.B., Bertolini, J., 2005. Transferrin: structure, function and potential therapeutic actions. Drug Discov. Today 10 (4), 267–273.
Hertz, R., Seckbach, M., Zakin, M.M., Bar-Tana, J., 1996. Transcriptional suppression of the transferrin gene by hypolipidemic peroxisome proliferators. J. Biol. Chem. 271 (1), 218–224.
Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. 109 (9), 1125–1131.
Hung, S.-W., Tu, C.-Y., Wang, W.S., 2007. In vivo effects of adding singular or combined anti-oxidative vitamins and/or minerals to diets on the immune system of tilapia (*Oreochromis hybrids*) peripheral blood monocytes-derived, anterior kidney-derived, and spleen-derivedmacrophages. Vet. Immunol. Immunopathol. 115, 87–99.
Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L., Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquac. Nutr. 9, 397–407.
Izquierdo, M.S., Montero, D., Robaina, L., Caballero, M.J., Roselund, G., Gine's, R., 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture 250, 431–444.
Jordal, A.E., Torstensen, B.E., Tsoi, S., Tocher, D.R., Lall, S.P., Douglas, S.E., 2005. Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). J. Nutr. 135, 2355–2361.
Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. Anim. Feed Sci. Technol. 173 (1–2), 111–133.
Kumar, V., Sahu, N.P., Pal, A.K., Kumar, S., 2007. Immunomodulation of *Labeo rohita* juveniles due to a dietary gelatinized and non-gelatinized starch. Fish Shellfish Immun. 23, 341–353.
Lambert, L.A., Perri, H., Halbrooks, P.J., Mason, A.B., 2005. Evolution of the transferrin family: conservation of residues associated with iron and anion binding. Comp. Biochem. Physiol. B 142, 129–141.
Leaver, M.J., Villeneuve, L.A.N., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R., Taggart, J.B., 2008. Functional genomics reveals increased cholesterol and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). BMC Genomics 9, 299.
Lee, J.Y., Tada, T., Hirono, I., Aoki, T., 1998. Molecular cloning and evolution of transferrin cDNAs in salmonids. Mol. Mar. Biol. Biotechnol. 7, 287–293.
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.). Aquac. Nutr. 9, 283–286.
Lin, M.T., Hsu, C.S., Yeh, S.L., Yeh, C.L., Chang, K.J., Lee, P.H., Chen, W.J., 2007. Effects of o-3 fatty acids on leukocyte Th1/Th2 cytokine and integrin expression in rats with gut-derived sepsis. Nutrition 23, 179–186.
Liu, Y., Yu, S., Chai, Y., Zhu, Q., 2012. Transferrin gene expression in response to LPS challenge and heavy metal exposure in roughskin sculpin (*Trachidermus fasciatus*). Fish Shellfish Immunol. 32 (1), 223–229.
Low, C., Wadsworth, S., Burrells, C., Secombes, C.J., 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture 221, 23–40.
Luo, J.G., Li, L., Kong, L.Y., 2012. Preparative separation of phenylpropenoid glycerides from the bulbs of *Lilium lancifolium* by high-speed counter-current chromatography and evaluation of their antioxidant activities. Food Chem. 131 (3), 1056–1062.
Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M.J., Izquierdo, M.S., 2003. Vegetable lipid sources for gilthead sea bream (*Sparus aurata*) diets: effects on fish health. Aquaculture 225, 353–370.
Montero, D., Robaina, L., Caballero, M.J., Gine's, R., MS, I., 2005. Growth, feed utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: a time-course study on the effect of re-feeding period with a 100% fish oil diet. Aquaculture 248, 121–134.
Montero, D., Grasso, V., Izquierdo, M.S., Ganga, R., Real, F., Tort, L., Caballero, M.J., Acosta, F., 2008. Total substitution of fish oil by vegetable oils in gilthead sea bream (*Sparus aurata*) diets: effects on hepatic Mx expression and some immune parameters. Fish Shellfish Immun 24, 147–155.
Montero, D., Mathlouthi, F., Tort, L., Afonso, J.M., Torrecillas, S., Fernández-Vaquero, A., Negrin, D., Izquierdo, M.S., 2010. Replacement of dietary fish oil by vegetable oils affects humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream *Sparus aurata*. Fish Shellfish Immunol. 29 (6), 1073–1081.
Mourente, G., Good, J.E., Bell, J.G., 2005. Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass (*Dicentrarchus labrax* L.) effects on flesh fatty acid composition, plasma prostaglandins E2; and F2a, immune function and effectiveness of a fish oil finishing diet. Aquac. Nutr. 11, 25–40.
Nasopoulou, C., Zabetakis, I., 2012. Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. LWT Food Sci. Technol. 47, 217–224.
Naylor, R.L., Goldberg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Libchenko, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017–1024.
Neves, J.V., Wilson, J.M., Rodrigues, P.N., 2009. Transferrin and ferritin response to bacterial infection: the role of the liver and brain in fish. Dev. Comp. Immunol. 33 (7), 848–857.
NRC (National Research Council), 2011. Nutrient Requirements of Fish and Shrimp. National Academy Press, Washington, D.C., USA.
Ong, S.T., Ho, J.Z., Ho, B., Ding, J.L., 2006. Iron-withholding strategy in innate immunity. Immunobiology 211, 295–314.
Pérez-Bárcena, J., Regueiro, V., Marsé, P., Raurich, J.M., Rodríguez, A., Ibáñez, J., de Lorenzo Mateos, A.G., Bengoechea, J.A., 2008. Glutamine as a modulator of the immune system of critical care patients: e¡ect onToll-like receptor expression. A preliminary study. Nutrition 24, 522–527.
Piedecausa, M.A., MJ, M.´n., Garcı'a Garcı'a, B., MD, H.´n., 2007. Effects of total replacement of fish oil by vegetable oils in the diets of sharpsnout seabream (*Diplodus puntazzo*). Aquaculture 263, 211–219.
Radovanović, T.B., Borković-Mitić, S.S., Perendija, B.R., Despotović, S.G., Pavlović, S.Z., Cakić, P.D., Saičić, Z.S., 2010. Superoxide dismutase and catalase activities in the liver and muscle of barbel (*Barbus barbus*) and its intestinal parasite (*Pomphoryinchus laevis*) from the Danube river. Serbia. Arch Biol Sci 62, 97–105.
Regost, C., Arzela, J., Cardinalb, M., Robina, J., Larochec, M., Kaushikd, S.J., 2001. Dietary lipid level, hepatic lipogenesis and flesh quality in turbot (*Psetta maxima*). Aquaculture 193, 291–309.
Regost, C., Arzel, J., Robin, J., Rosenlund, G., Kaushik, S.J., 2003. Total replacement of fish oil by soybean oil with return to fish oil in turbot (*Psetta maxima*) I. Growth performance, flesh fatty acid profile, and lipid metabolism. Aquaculture 217, 465–482.
Reyna-Villasmil, N., Bermúdez-Pirela, V., Mengual-Moreno, E., Arias, N., Cano-Ponce, C., Leal-Gonzalez, E., Souki, A., Inglett, G.E., Israili, Z.H., Hernández-Hernández, R., Valasco, M., Arraiz, N., 2007. Oat-derived β-glucan significantly improves HDLC and diminishes LDLC and non-HDL cholesterol in overweight individuals with mild hypercholesterolemia. Am. J. Ther. 14 (2), 203–212.
Sahoo, P.K., Mohanty, B.R., Kumari, J., Barat, A., Sarangi, N., 2009. Cloning, nucleotide sequence and phylogenetic analyses, and tissue-specific expression of the transferrin gene in *Cirrhinus mrigala* infected with *Aeromonas hydrophila*. Comp. Immunol. Microb. 32, 527–537.
Sargent, J.R., Tacon, A.G.J., 1999. Development of farmed fish: a nutritionally necessary alternative to meat. Proc. Nutr. Soc. 58 (2), 377–383.
Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquac. Res. 39, 223–239.
Stafford, J.L., Belosevic, M., 2003. Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Dev. Comp. Immunol. 27, 539–554.
Stubhaug, I., Lie, Ø., Torstensen, B.E., 2007. Fatty acid productive value and β-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period. Aquac. Nutr. 13, 145–155.
Tacon, A.G.J., Metian, M., 2008. Global overviewof the fish meal and fish oil in industrially compounded aquafeeds: trend and future prospects. Aquaculture 285, 146–158.
Tocher, D.R., Fonseca-Madrigal, J., Bell, J.G., Dick, J.R., Henderson, R.J., Sargent, J.R., 2002. Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem. 26, 157–170.
Trichet, V.V., 2010. Nutrition and immunity: an update. Aquac. Res. 41, 356–372.
Turchini, G.M., Torstensen, B.E., Ng, W.K., 2009. Fish oil replacement in finfish nutrition. Rev. Aquac. 1, 10–57.
Wang, Q., He, G., Wang, X., Mai, K., Xu, W., Zhou, H., 2014. Dietary sulfur amino acid modulations of taurine biosynthesis in juvenile turbot (*Psetta maxima*). Aquaculture 422-423, 141–145.
Wang, Q., He, G., Mai, K., Xu, W., Zhou, H., 2015. Fishmeal replacement by mixed plant proteins and maggot meal on growth performance, target of rapamycin signalling and metabolism in juvenile turbot (*Scophthalmus maximus* L.). Aquac. Nutr. http://dx.doi.org/10.1111/anu.12296.
Watanabe, T., 2002. Strategies for further development of aquatic feeds. Fish. Sci. 68, 242–252.
Watkins, S.M., German, J.B., Shoenfeld, Y., Gerschwin, M.E., 2001. Lipids and immunity. In: Shoenfeld, Y., Wick, G., Harats, D. (Eds.), Atherosclerosis and Autoimmunity. The Research Center of Autoimmune Diseases, Chaim Sheba Medical Center/University of Innsbruck, Institute for General and Experimental Pathology, Israel/Austria, pp. 87–111.
Yildiz, M., Sener, E., 2004. The effect of dietary oils of vegetable origin on the performance, body composition and fatty acid profiles of sea bass (*Dicentrarchus labrax* L., 1758) juveniles. Turk. J. Vet. Anim. Sci. 28, 553–556.

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 (1), 105–110.

Zheng, X., Tocher, D.R., Dickson, C.A., Bell, J.G., Teale, A.J., 2004. Effects of diets containing vegetable oil on expression of genes involved in highly unsaturated fatty acid biosynthesis in liver of Atlantic salmon (*Salmo salar*). Aquaculture 236, 467–483.

Zuo, R., Ai, Q., Mai, K., Xu, W., Wang, J., Xu, H., Liufu, Z., Zhang, Y., 2012. Effects of dietary n-3 highly unsaturated fatty acids on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (Larmichthys crocea) following natural infestation of parasites (*Cryptocaryon irritans*). Fish Shellfish Immun 32 (2), 249–258.