Aquaculture 469 (2017) 59–66

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Effects of dietary phospholipids on growth performance and expression of key genes involved in phosphatidylcholine metabolism in larval and juvenile large yellow croaker, *Larimichthys crocea*

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ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 1 December 2016
Accepted 3 December 2016
Available online 7 December 2016

Keywords:
Large yellow croaker
Phosphatidylcholine metabolism
Different stages

ABSTRACT

The objective of this study was to investigate the effects of dietary phospholipids (PLs) on survival rate, growth performance and expression of key genes related with phosphatidylcholine (PC) metabolism in larval and juvenile large yellow croaker, *Larimichthys crocea*. Three isonitrogenous and isolipidic diets with graded levels of PLs (2.53%, 6.32% and 12.7%) were randomly allocated to triplicate groups of larvae (initial body weight: 3.86 ± 0.24 mg) eight times daily for 30 days. Five isonitrogenous and isolipidic diets with graded levels of PLs (1.32%, 3.05%, 5.86%, 7.4% and 9.63%) were randomly allocated to triplicate groups of juveniles (initial body weight: 7.36 ± 0.33 g) twice daily to apparent satiation for 60 days. Results showed that survival rate and specific growth rate of larvae fed the diet with 2.53% PLs were significantly lower compared with those of larvae in other groups ($P < 0.05$). However, specific growth rate and survival rate were not significantly different among dietary treatments in juveniles. No significant difference was observed in the transcript levels of key genes involved in PC *de novo* synthesis, including CTP: choline phosphate cytidylyltransferase and phosphatidylethanolamine *N*-methyltransferase, in both the whole larvae body and the livers of juveniles fed diets with different concentrations of PLs. Although no significant difference was found, the mRNA expression levels of 1, 2-diacylglycerol choline phosphotransferase increased with the increasing levels of dietary PLs in the livers of juveniles, with acyl-CoA: diacylglycerol acyltransferase 2 showing the opposite trend. These results confirmed that the beneficial effects of dietary PLs on survival and growth performance of large yellow croaker were restricted to fish larvae. Dietary PLs might reduce lipid deposition in the liver of juvenile large yellow croaker through utilizing more diacylglycerol for PC synthesis, rather than triglyceride synthesis.

Statement of relevance: The present study was conducted to investigate the effects of dietary phospholipids (PLs) on survival rate, growth performance and expression of key genes related to phosphatidylcholine (PC) biosynthesis in larval and juvenile large yellow croaker, (*Larimichthys crocea*). The results are reliable and of both theoretical and practical importance. For "theoretical importance", our study provided the first evidence that dietary PLs might reduce lipid deposition in the liver of juvenile large yellow croaker through utilizing more diacylglycerol for PC synthesis, rather than triglyceride synthesis, which could contribute to better understanding the mechanisms underlying the discrepancy in requirements for phospholipids between larval and juvenile large yellow croaker. For "practical importance", our study confirmed that the beneficial effects of dietary PLs on growth performance of large yellow croaker were restricted to larvae rather than juveniles (at least 7.36 g) and these findings could be applied to reduce the supplementation of PLs in commercial feeds for large yellow croaker, which in turn might reduce the cost of the commercial feeds for large yellow croaker.

The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. I have read and have abided by the statement of ethical standards for manuscripts submitted to Aquaculture.

Abbreviations: PLs, phospholipids; SGR, specific growth rate; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CCT, CTP: choline phosphate cytidylyltransferase; CPT, 1,2-diacylglycerol choline phosphotransferase; DGAT 2, diacylglycerol *O*-acyltransferase 2; $sPLA_2$ IB, secretory phospholipase A_2 group IB; PEMT, phosphatidylethanolamine *N*-methyltransferase.

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http://dx.doi.org/10.1016/j.aquaculture.2016.12.002
0044-8486/

1. Introduction

Phospholipids (PLs) can act as components essential for eukaryotic biological membranes formation (Fagone and Jackowski, 2013), provide energy for embryonic and early larval development (Fraser et al., 1988; Rainuzzo et al., 1992; Tocher et al., 1985) and act as important precursors of a range of highly biologically active mediators of metabolism and physiology including eicosanoids, diacylglycerol (DAG), inositol phosphates and platelet activating factors (Tocher et al., 2008). Dietary PLs exert beneficial effects on growth, survival, stress resistance, lipid absorption and distribution in a variety of fish larvae species (Cahu et al., 1994; Daprà et al., 2011; Geurden et al., 1997; Harada, 1987; Kanazawa, 1997; Poston, 1990; Seoka et al., 2008; Zhao et al., 2013). The growth-promoting effects of PLs appear to depend on the developmental stage and be restricted to larvae and early juveniles, although the requirements for PLs in juvenile and (or) adult fish are largely unstudied. Some researchers suggest that the requirement for PLs during larval stage might be due to the limited capacity to *de novo* synthesize PLs (Tocher et al., 2008; Daprà et al., 2011).

As one of the most abundant phospholipid classes in regular PLs sources, phosphatidylcholine (PC) is physiologically important as the principle component of cellular membranes, the precursor of signaling molecules (Robinson et al., 1989; Van-Meer et al., 2008) as well as the key element of lipoproteins (Skipski et al., 1967), bile (Alvaro et al., 1986) and lung surfactant (Pérez-Gil, 2008). There is evidence demonstrating that PC is the main limiting factor driving the requirement for intact PLs (Azarm et al., 2013; Taylor et al., 2015). Like mammals, fish possesses a full set of enzymes for endogenous production of PC and can synthesize PC *de novo* through two pathways: cytidine diphosphate-choline (CDP-choline) pathway and phosphatidylethanolamine (PE) methylation pathway. CTP: choline phosphate cytidylyltransferase α (CCTα) and 1, 2-diacylglycerol choline phosphotransferase (CPT) constitute the two most critical enzymes during the biosynthesis of PC in CDP-choline pathway (Tocher et al., 2008). CCTα catalyzes the formation of CDP-choline and CPT catalyzes the production of PC via the condensation of CDP-choline with DAG (Cole et al., 2012). DAG can be also converted to triglyceride by diacylglycerol O-acyltransferase 2 (DGAT 2), which means CPT may be competing with DGAT 2, ultimately changing the ratio of PC to triglyceride (Oxley et al., 2007). Previous studies have proven the existence of CCT in liver of trout (Holub et al., 1975), brain and liver of goldfish (Ghosh et al., 2006) and intestine and liver of Atlantic salmon (Carmona-Antoñanzas et al., 2015). An alternative pathway for PC biosynthesis is the conversion of phosphatidylethanolamine (PE) to PC by PE-methyltransferase (PEMT) (Vance, 2013). This alternative pathway for PC synthesis occurs only in the liver and contributes to about 30% of total hepatic PC synthesis (CDP-choline pathway provides the remaining 70%) in mammals (Vance, 2013).

Pancreatic enzyme secretory phospholipase A_2 group IB ($sPLA_2$ IB) accounts for the digestion of dietary PLs, resulting into the production of a non-esterified fatty acid and a lysophospholipid (Murakami and Kudo, 2002). Besides being found in Atlantic cod and red sea bream, the existence of $sPLA_2$ IB has been also reported in large yellow croaker (Cai et al., 2015; Sæle et al., 2011; Fujikawa et al., 2012). So far, no studies have evaluated the regulation of key genes expression involved in PC metabolism in response to dietary PLs at transcriptional level, especially at both larval and juvenile stages.

Large yellow croaker (*Larimichthys crocea*) is a carnivorous marine fish species that has been widely cultured in southeast China due to its delicious taste and commercial value (Ai et al., 2008; Xie et al., 2011; Zuo et al., 2012a, 2012b). Although optimal requirement for dietary PLs has been established in large yellow croaker larvae in our previous study (Zhao et al., 2013), n-3 HUFA content failed to keep constant in that study, which might affect the accuracy of the results. Furthermore, little information is available about the effects of dietary PLs on PC metabolism, especially under a comparative study in fish larvae and juveniles. Thus, the present study was conducted to compare the effects of dietary PLs on survival, growth performance, lipid deposition and expression of genes related with PC metabolism in larval and juvenile large yellow croaker, which was expected to contribute to better understanding the discrepancy of requirement for PLs at this two different stages.

2. Materials and methods

2.1. Feed ingredients and diet formulation

For larvae, three isonitrogenous (56% crude protein) and isolipidic (19% crude lipid) diets were formulated by adding graded levels of PLs (0%, 5%, and 12%) and the final dietary PLs concentrations were 2.53%, 6.32% and 12.7%, respectively (Table 1). Fatty acid composition of the three experimental diets is given in Table 2. Low temperature processed white fish meal, krill meal, squid meal and hydrolyzed fish meal (Haiyun bios., Zhejiang, China) were chosen as the primary protein sources. Fish oil, soybean oil and soybean lecithin were used as the lipid sources. Microdiets were manufactured by micro-bonding technology. The particle size of the formulated diets ranged from 150 to 250 μm for fish from 15 to 25 days after hatch (DAH) and 200 to 350 μm for fish thereafter.

For juveniles, five isonitrogenous (44% crude protein) and isolipidic (13.5% crude lipid) diets were formulated by adding graded levels of

Table 1
Formulation and chemical composition of larval experimental diet (% dry matter).

	Dietary phospholipids level (%)		
Ingredient % dry diet	2.53%	6.32%	12.7%
LT-White fish meal[a]	21	21	21
LT-Krill meal[a]	14	14	14
LT-Squid meal[a]	12	12	12
Casein	20	20	20
Hydrolyzed fish meal[a]	4	4	4
LT-Yeast	3	3	3
α-starch	4	4	4
Alginae sodium	1.5	1.5	1.5
Vitamin premix[b]	1.5	1.5	1.5
Mineral premix[c]	1.5	1.5	1.5
Ascorbyl polyphosphate	0.2	0.2	0.2
Attractant mixture[d]	2	2	2
Mld inhibitor	0.05	0.05	0.05
Antioxidant	0.05	0.05	0.05
Choline chloride	0.2	0.2	0.2
Fish oil[e]	3	3	3
Soybean oil[e]	12	7	0
Soybean lecithin[e]	0	5	12
Proximate analysis			
Crude protein	56.35	56.12	56.43
Crude lipid	19.39	18.98	19.44
lecithin	2.53	6.32	12.7

[a] Low temperature white fish meal: crude protein 71.2% dry matter and crude lipid 5.3% dry matter; Low temperature krill meal: crude protein 63.8% dry matter and crude lipid 13% dry matter; Low temperature squid meal: crude protein 59.6% dry matter and crude lipid 2.0% dry matter; Hydrolyzed fish meal: crude protein 77.1% dry matter and crude lipid 1.3% dry matter. All the materials above were bought from Haiyun bios., Zhejiang, China.

[b] Vitamin premix (IU or g kg^{-1} premix) (provided by Great Seven Bio-Tech., Qingdao, China): retinal palmitate, 3,000,000 IU; cholecalciferol, 1,200,000 IU; DL-α-tocopherol acetate, 40.0 g kg^{-1}; menadione, 8.0 g kg^{-1}; thiamin-HCl, 5.0 g kg^{-1}; riboflavin, 5.0 g kg^{-1}; D-calcium pantothenate, 16.0 g kg^{-1}; pyridoxine-HCl,4.0 g kg^{-1}; *meso*-inositol, 200.0 g kg^{-1}; D-biotin, 8.0 g kg^{-1}; folic acid, 1.5 g kg^{-1}; *para*-aminobenzoic acid, 5.0 g kg^{-1}; niacin, 20.0 g kg^{-1}; cyanocobalamin, 0.01 g kg^{-1}; ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g kg^{-1}.

[c] Mineral premix (g kg^{-1} premix) (provided by Great Seven Bio-Tech., Qingdao, China): $Ca(H_2PO_4)_2 \cdot H_2O$, 675.0; $CoSO_4 \cdot 4H_2O$, 0.15; $CuSO_4 \cdot 5H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 50.0; KCl, 50.0; KI, 0.1; $MgSO_4 \cdot 2H_2O$, 101.7; $MnSO_4 \cdot 4H_2O$, 18.0; NaCl, 80.0; $Na_2SeO_3 \cdot H_2O$, 0.05; $ZnSO_4 \cdot 7H_2O$, 20.0.

[d] Attractant mixture (g kg^{-1} premix) (provided by Great Seven Bio-Tech., Qingdao, China): betaine, 500.0; glycine, 150.0; alanine, 100.0; argine, 100.0; taurine, 100.0; inosine-5′-monophosphoric acid, 50.0.

[e] Provided by Great Seven Bio-Tech., Qingdao, China.

Table 2
Fatty acid composition of larval experimental diet (% total fatty acids).[g]

	Dietary phospholipids level (%)		
	2.53%	6.32%	12.7%
C14:0	2.83	3.09	3.69
C16:0	15.09	17.22	20.47
C18:0	0.13	0.13	0.15
∑SFA[a]	18.05	20.44	24.31
C16:1	4.40	4.84	5.98
C18:1	26.52	25.43	23.21
C20:1	1.96	2.27	2.37
∑MUFA[b]	32.88	32.54	31.55
C18:2n-6	33.85	31.55	27.08
C20:4n-6	0.25	0.23	0.29
∑n-6 PUFA[c]	34.10	31.78	27.37
C18:3n-3	4.62	4.05	2.75
C20:5n-3	3.44	3.77	4.64
C22:6n-3	3.58	3.83	4.79
∑n-3 PUFA[d]	11.65	11.65	12.19
∑n-3/∑n-6	0.34	0.37	0.45
n-3HUFA[e]	7.02	7.60	9.43
DHA/EPA[f]	1.04	1.02	1.03

[a] SFA: saturated fatty acids.
[b] MUFA: mono-unsaturated fatty acids.
[c] n- 6 PUFA: n- 6 poly-unsaturated fatty acids.
[d] n- 3 PUFA: n- 3 poly-unsaturated fatty acids.
[e] n- 3 HUFA: n- 3 highly-unsaturated fatty acids.
[f] DHA/EPA: 22:6n- 3/20:5n- 3.
[g] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C20:0, C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C18:3n-6, C20:3n-6, C18:4n-3, C22:5n-3, are not listed in the table.

PLs (0%, 2%, 4%, 6% and 8%) and the final dietary PLs concentrations were 1.32%, 3.05%, 5.86%, 7.4% and 9.63% (Table 3). White fish meal and soybean meal were chosen as the main protein sources. Fish oil, soybean oil and soybean lecithin were chosen as the primary lipid sources. Fatty acid composition of the five experimental diets is given in Table 4. All formulated diets for larval and juvenile large yellow croaker were packed in separate silver bags to keep light off and stored at −20 °C until use.

2.2. Experimental procedures

Larvae used in this study were obtained and reared at Ningde Fufa Fishery co., Ltd., Fujian, China. All larvae in the hatchery were fed with rotifers, *Brachionus plicatilis* ($0.5–1.5 \times 10^4$ individual L^{-1}) from 3 to 8 DAH, *Artemia nauplii* ($1.0–1.5 \times 10^3$ individual L^{-1}) from 6 to 11 DAH, and live copepods, *Calanus sinicus* and a commercial pellet diet (Otohime A, Nissin Marinetec, Yokohama, Japan) from 10 to 14 DAH, and then the larvae were weaned onto the experimental diets. The experiment was carried out in 9 white plastic tanks (water volume 300 L) at a stocking density of 3000 larvae (mean body weight 3.86 ± 0.12 mg) per tank. They were supplied with seawater that had been filtered through two-grade sand filter. During the rearing period, water temperature ranged from 20 to 24 °C, pH from 7.8–8.2 and salinity from 21 to 25‰. About 150–300% of the water volume was renewed daily and there was an air stone in each tank. Larvae were reared under 14 h light: 10 h dark dial cycle photoperiod. Fluorescent light intensity was 8.5 W m^{-2} maximum during daytime at the water surface. The surface water was skimmed with a polyvinylchloride pipe regularly to remove the suspended waste. Also, accumulation of feeds and feces at the tank bottoms was siphoned twice daily. From 15 to 45 DAH, larvae were manually fed to satiation with the experimental diets eight times (6:00, 7:00, 8:00, 9:00, 13:00, 14:00, 15:00 and 16:00) daily.

The juveniles were obtained and reared at a commercial farm in Xiangshan bay, Ningbo, China. Juveniles were fed a commercial diet for two weeks to acclimate to the experimental conditions and feeds. Juvenile large yellow croaker with similar size (7.36 ± 0.33 g) were distributed into 15 sea cages (1 m × 1 m × 1.5 m) and each cage was

Table 3
Formulation and chemical composition of juvenile experimental diet (% dry matter).

	Dietary phospholipids level (%)				
Ingredient % dry diet	1.32%	3.05%	5.86%	7.4%	9.63%
Fish meal[a]	43	43	43	43	43
Soybean meal[a]	15	15	15	15	15
Wheat meal[a]	24.35	24.35	24.35	24.35	24.35
Yeast meal	3	3	3	3	3
Attractant[b]	0.3	0.3	0.3	0.3	0.3
Mold inhibitor[c]	0.1	0.1	0.1	0.1	0.1
Antioxidant	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.2	0.2	0.2	0.2	0.2
Mineral premix[d]	2	2	2	2	2
Vitamin premix[e]	2	2	2	2	2
Fish oil[f]	2	2	2	2	2
Soybean oil[f]	8	6	4	2	0
Soybean lecithin[f]	0	2	4	6	8
Proximate analysis					
Crude protein	44.15	43.79	44.93	44.43	45.14
Crude lipids	13.71	13.56	13.65	13.18	13.06
Leithin	1.32	3.05	5.86	7.4	9.63

[a] Fish meal: crude protein 69.92% dry matter and crude lipid 6.9% dry matter; soybean meal: crude protein 54.7% dry matter and crude lipid 1.7% dry matter; wheat meal: crude protein 17.1% dry matter and crude lipid 1.1% dry matter. All the materials were provided by Great Seven Bio-Tech., Qingdao, China.
[b] Attractant (provided by Great Seven Bio-Tech., Qingdao, China): glycine and betaine.
[c] Mold inhibitor (provided by Great Seven Bio-Tech., Qingdao, China): contained 50% calcium propionic acid and 50% fumaric acid.
[d] Mineral premix (mg or g kg^{-1}diet) (provided by Great Seven Bio-Tech., Qingdao, China): $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H_2O$, 80 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $MnSO_4 \cdot H_2O$, 45 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; $Na_2SeO_3 \cdot H_2O$, 20 mg; calcium iodate, 60 mg; zeolite, 18.485 g.
[e] Vitamin premix (mg or g kg^{-1}diet) (provided by Great Seven Bio-Tech., Qingdao, China): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B12, 10 mg; vitamin B6, 20 mg; folic acid, 20 mg; vitamin B1, 25 mg; vitamin A, 32 mg; vitamin B2, 45 mg; pantothenic acid, 60 mg; biotin, 1.2 mg; niacin acid, 200 mg; α-tocopherol, 120 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g kg^{-1}.
[f] Provided by Great Seven Bio-Tech., Qingdao, China.

Table 4
Fatty acid composition of juvenile experimental diet (% total fatty acids).[g]

	Dietary phospholipids level (%)				
Fatty acid	1.32%	3.05%	5.86%	7.4%	9.63%
C14:0	1.92	2.04	2.14	2.32	2.62
C16:0	14.32	15.32	16.66	18.40	20.26
C18:0	0.09	0.10	0.10	0.11	0.12
∑SFA[a]	16.33	17.45	18.91	20.83	22.99
C16:1	3.80	4.09	4.42	4.83	5.40
C18:1	27.25	24.30	25.67	22.09	20.39
C20:1	0.09	0.10	0.10	0.11	0.12
∑MUFA[b]	32.44	30.05	31.41	28.26	27.17
C18:2n-6	36.03	34.73	33.29	31.48	29.52
C20:4n-6	0.41	0.44	0.48	0.50	0.56
∑n-6 PUFA[c]	36.44	35.17	33.77	31.98	30.08
C18:3n-3	4.73	4.44	4.10	3.65	2.89
C20:5n-3	2.09	2.21	2.39	2.52	2.83
C22:6n-3	4.28	4.64	5.05	5.44	6.04
∑n-3 PUFA[d]	11.10	11.28	11.54	11.60	11.75
∑n-3/∑n-6	0.30	0.32	0.34	0.36	0.39
n-3HUFA[e]	6.37	6.85	7.44	7.95	8.87
DHA/EPA[f]	2.04	2.10	2.11	2.16	2.14

[a] SFA: saturated fatty acids.
[b] MUFA: mono-unsaturated fatty acids.
[c] n -6 PUFA: n- 6 poly-unsaturated fatty acids.
[d] n -3 PUFA: n- 3 poly-unsaturated fatty acids.
[e] n- 3 HUFA: n- 3 highly-unsaturated fatty acids.
[f] DHA/EPA: 22:6n- 3/20:5n- 3.
[g] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C20:0, C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C18:3n-6, C20:3n-6, C18:4n-3, C22:5n-3, are not listed in the table.

Table 5
qPCR primers used in this study.

Target genes	Forward (5′–3′)	Reverse (5′–3′)	GenBank number or publication
CCTα	CGCCAGAGTTTCTCGCAAGACATCG	CGTGGACAAGGTGAAGAGGAAGGTGC	KF006239
CPT	GCATCTCCACGATTTAGCCTTC	CTGACACAGTAACGCACCAAG	KJ563920
DGAT 2	TTCGGTGCTTTCTGCAACTTCG	AAGGATGGGGAAGCGGAAGT	Yan et al. (2015)
PEMT	GTGGGCACTCTACTGGTTGTC	TCGGGTTCTCCATGATGTTGAAG	KJ563918
$sPLA_2$ IB	TCTGGACAGGTGCTGTCAGG	AAGATGGGCCAGCACTCAGG	Cai et al. (2015)
β-actin	CTACGAGGGTTATGCCCTGCC	TGAAGGAGTAACCGCGCTCTGT	Yan et al. (2015)

stocked with 60 fish. Each diet was randomly allocated to triplicate cages of fish. Fish were hand-fed twice daily (05:00 and 17:00) to apparent satiation. The feeding trial lasted for 60 days. During the experimental period, the water temperature, salinity and dissolved oxygen were measured daily. The water temperature ranged from 25 to 30 °C and salinity from 32‰ to 36‰. The dissolved oxygen was approximately 7 mg L^{-1}.

2.3. Sampling and dissection

At the end of the experiment, survival was determined by counting the individuals remaining in each tank or cage for both larvae and juveniles. All larvae were deprived of food for 24 h before sampling to empty their guts. Fifty individuals were randomly sampled from each tank to measure wet body weight (BP 210S, Sartorius, Germany). Fifty individuals per tank were sampled and immediately frozen in liquid nitrogen and stored at −80 °C for the whole body lipid content analysis. Another fifty individuals per tank were sampled and immediately frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

Large yellow croaker juveniles were fasted for 24 h and anaesthetized in eugenol (1:10,000) (Shanghai Reagent, China) for wet weight determination (JA61001, Shanghai precision &scientific instrument Co., Ltd., China). The livers and muscle were dissected from five fish from each cage and immediately frozen in liquid nitrogen and then stored at −80 °C for lipid content analysis. The livers and muscle of three fish per cage were sampled and immediately frozen in liquid nitrogen and then stored at −80 °C for gene expression analysis.

2.4. Biochemical analysis

The crude lipid contents of the whole larvae body and the lipid levels of liver and muscle of juveniles were measured through ether extraction using the Soxhlet method (Soxhlet Extraction System B-811, BUCHI, Switzerland). The fatty acid profiles of the experimental diets were analyzed using the procedures described by Metcalfe et al. (1966) with some modification (Ai et al., 2008; Zuo et al., 2012a, 2012b). About 100 mg freeze-dried samples were added into a 20 mL volumetric screwed tube with cover. Then 3 mL potassium hydroxide methanol (1 N) was added and heated on 72 °C water bath for 20 min. After that, 3 mL HCL–methanol (2 N) was added and the mixture was heated on 72 °C water bath for another 20 min. Finally, 1 mL hexane was added into the mixture above, shaken vigorously for 1 min, and then allowed to separate into two layers. Fatty acid methyl esters were separated, and quantified by a HP6890 gas chromatograph (Agilents Technologies Inc., Santa Clara, California, USA) with a fused silica capillary column (007-CW, Hewlett Packard, Palo Alto, CA, USA) and a flame ionization detector. The column temperature was programmed to rise from 150 °C up to 200 °C at a rate of 15 °C/min, from 200 °C to 250 °C at a rate of 2 °C/min. Injector and detector temperature was 250 °C, respectively.

PL content was measured by determining phosphorus content (phosphoru × 25) using molybdenum blue method (Li et al., 2005; Zhao et al., 2013). Briefly, about 100 mg freeze-dried samples was digested with nitric acid and perchloric acid and added with molybdenum blue reagent. The phosphorus content can be calculated by assaying the absorbance at 830 nm with the UV-2401PC spectrophotometer (Shimadzu Corporation, Japan).

2.5. RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction

Total RNA was extracted using Trizol Reagent (Invitrogen, USA) and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA was treated with RNase-Free DNase (Takara, Japan) to remove DNA contaminant and reversely transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan) following the instructions. First strand cDNA was diluted by 4 times using sterilized double-distilled water. Real-time quantitative polymerase chain reaction was carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). The operational approach referred to Zuo et al. (2012a, 2012b). The primers sequences for CCT, CPT and PEMT were designed and synthesized based on the published sequences from GenBank and the primers for β-actin, DGAT 2 and $sPLA_2$ IB were directly synthesized based on the corresponding sequences in published papers (Cai et al., 2015; Yan et al., 2015) (Table 5). At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. The fluorescence data acquired during the extension phase were normalized to β-actin via $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relative mRNA expression of target genes in fish fed no PL-supplemented diet was selected as the calibrator.

2.6. Statistical analysis

Statistical analysis was performed by using SPSS 17.0 for Windows (SPSS Incorporation, USA). Following testing for homogeneity of variance, all the data were subjected to a one-way ANOVA and differences between the means were tested by Duncan's multiple-range test. The level of significance was set at $P < 0.05$ and the results were presented as mean values with their standard errors.

3. Results

3.1. Survival and growth performance

In the present study, larvae fed diets with 6.32% and 12.7% PLs showed higher survival rate (SR) and specific survival rate (SGR) than

Table 6
Effects of dietary phospholipids on survival, SGR and lipid composition of larval large yellow croaker (mean ± S.E.M., n = 3).*

	Dietary phospholipids level (%)		
	2.53%	6.32%	12.7%
Initial weight (mg)	3.86 ± 0.12	3.86 ± 0.12	3.86 ± 0.12
Final weight (mg)	23.75^a ± 1.12	62.61^b ± 2.33	70.17^b ± 4.43
Specific growth rate (%/day)	6.06^a ± 0.16	9.29^b ± 0.12	9.67^b ± 0.21
Survival (%)	12.8^a ± 1.31	23.3^b ± 1.29	22^b ± 2.11
Whole body lipid content (%)	9.61^a ± 0.52	13.22^b ± 0.68	14.72^b ± 0.97

* Data with the different superscript letters in the same row are significantly different ($P < 0.05$).

Table 7
Effects of dietary phospholipids on survival, SGR and lipid content of juvenile large yellow croaker (mean ± S.E.M., n = 3)*.

	Dietary phospholipids level (%)				
	1.32%	3.05%	5.86%	7.4%	9.63%
Initial weight (g)	7.36 ± 0.33	7.36 ± 0.33	7.36 ± 0.33	7.36 ± 0.33	7.36 ± 0.33
Final weight (g)	36.23 ± 2.30	35.78 ± 1.07	31.88 ± 2.92	35.95 ± 3.59	37.12 ± 1.72
Specific growth rate (%/day)	2.65 ± 0.10	2.63 ± 0.05	2.43 ± 0.15	2.63 ± 0.17	2.69 ± 0.08
Survival (%)	95.56 ± 1.47	91.67 ± 5.36	84.44 ± 2.94	89.44 ± 3.38	94.44 ± 2.42
Liver lipid content (%)	21.35^{a} ± 0.47	21.27^{a} ± 0.25	18.60^{b} ± 0.67	18.34^{bc} ± 0.51	16.14^{c} ± 0.25
Muscle lipid content (%)	8.82 ± 0.51	8.73 ± 0.45	7.90 ± 0.49	7.81 ± 0.14	7.14 ± 0.13

* Data with the different superscript letters in the same row are significantly different ($P < 0.05$).

larvae fed the diet with 2.53% PLs ($P < 0.05$, Table 6). SR and SGR were comparable between larvae fed diets with 6.32% and 12.7% PLs ($P > 0.05$, Table 6). However, for juvenile large yellow croaker, SR and SGR were not significantly different among dietary treatments ($P > 0.05$, Table 7).

3.2. Lipid content

In the present study, larvae fed diets with 6.32% and 12.7% PLs showed higher whole body lipid content than larvae fed the diet with 2.53% PLs ($P < 0.05$, Table 6). The whole body lipid content was comparable between larvae fed diets with 6.32% and 12.7% PLs ($P > 0.05$, Table 6). In juvenile stage, no significant difference was found in muscle lipid content among dietary treatments ($P > 0.05$, Table 7). Liver lipid levels significantly decreased with the increasing levels of dietary PLs ($P < 0.05$, Table 7), with larvae fed the diet with 9.63% PLs showing the lowest liver lipid content.

3.3. Expression of genes related with phosphatidylcholine biosynthesis and DGAT2

In larvae, dietary PLs did not significantly affect the mRNA expression of CCTα, as well as the transcript levels of CPT, DGAT 2 and PEMT ($P > 0.05$, Figs. 1A and 2A). In the liver of juvenile large yellow croaker, the transcript levels of PC biosynthesis-related genes (CCTα, CPT and PEMT) and DGAT 2 were also not significantly affected by dietary PLs ($P > 0.05$, Figs. 1B and 2B).

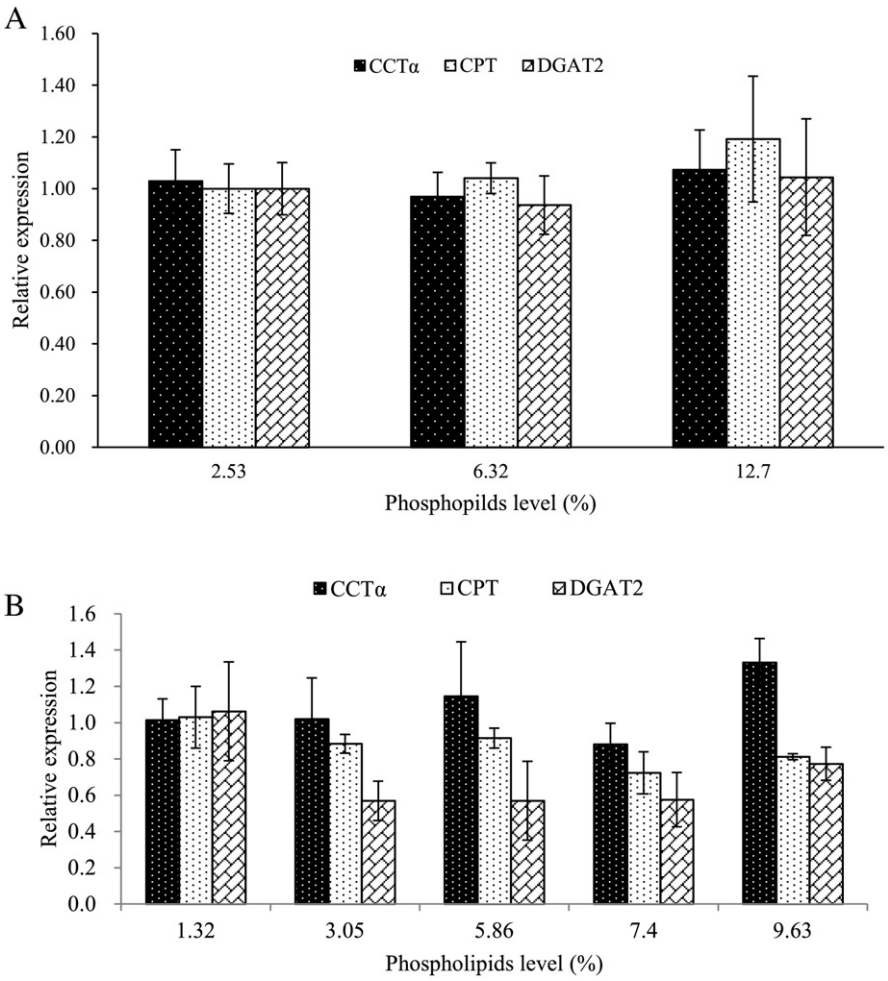

Fig. 1. Relative mRNA expression of CCTα, CPT and DGAT 2 in larval large yellow croaker (A) and liver of juvenile large yellow croaker (B). Values are presented as means ± S.E.M. (n = 3). Different letters above the bars represent significant difference between experimental groups ($P < 0.05$). CCTα: CTP: choline phosphate cytidylyltransferase; CPT: 1, 2-diacylglycerol choline phosphotransferase; DGAT2: diacylglycerol *O*-acyltransferase 2.

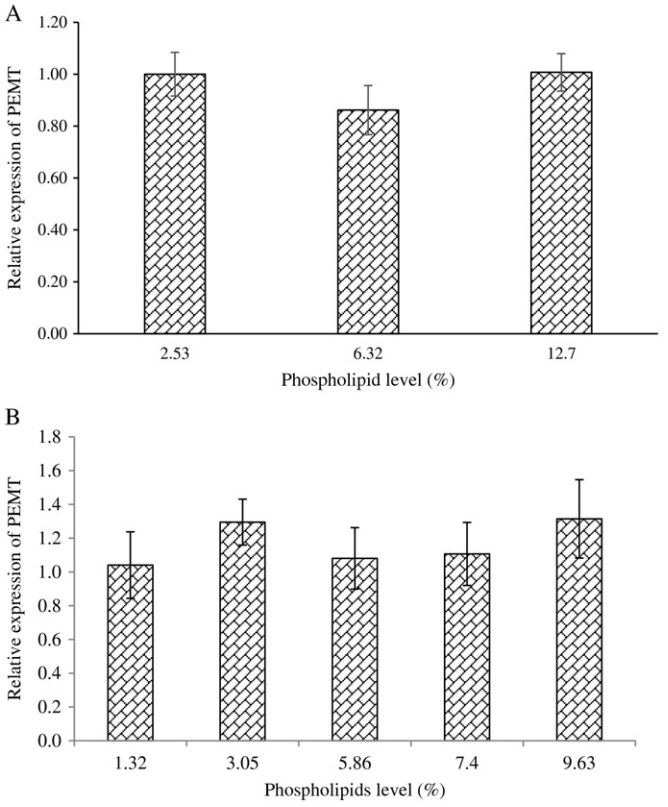

Fig. 2. Relative mRNA expression of PEMT in larval large yellow croaker (A) and liver of juvenile large yellow croaker (B). Values are presented as means ± S.E.M. (n = 3). Different letters above the bars represent significant difference between experimental groups ($P < 0.05$). PEMT: phosphatidylethanolamine *N*-methyltransferase.

3.4. Expression of genes related with phosphatidylcholine digestion in larvae

The transcript levels of $sPLA_2$ IB of larvae fed diets with 6.32% and 12.7% were significantly lower than that of larvae fed the diet with 2.53% PLs ($P < 0.05$, Fig. 3). The transcript levels of $sPLA_2$ IB were comparable between larvae in 6.32% and 12.7% PLs groups.

4. Discussion

In the present study, SR and SGR of larvae fed diets with 6.32% and 12.7% PLs were significantly higher than those of larvae fed the diet

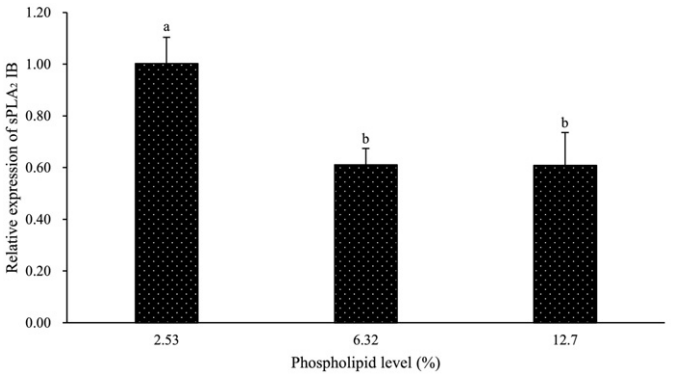

Fig. 3. Relative mRNA expression of $sPLA_2$ IB larval large yellow croaker. Values are presented as means ± S.E.M. (n = 3). Different letters above the bars represent significant difference between experimental groups ($P < 0.05$). $sPLA_2$ IB: secretory phospholipase A2 group IB.

with 2.53% PLs, which confirmed a requirement for PLs in large yellow croaker larvae (Zhao et al., 2013). However, SR and SGR increased with the increasing levels of dietary PLs from 2.6% to 5.72% and thereafter declined in the previous study (Zhao et al., 2013). Fish oil was used to keep the experimental diets isolipidic in that study, which led to decreasing levels of n-3 HUFA from 2.51% to 1.17% dry diet with increment of dietary PLs (Zhao et al., 2013). Relatively lower content of n-3 HUFA in the highest level of PLs group might inhibit the growth performance of larvae. On the contrary, the present study used soybean oil to keep n-3 HUFA content of the experimental diets constant and SR and SGR were not compromised by the highest level of dietary PLs. These results emphasized the importance to maintain constant n-3 HUFA content of the experimental diets in order to better understand the role of intact PLs. In addition, it should be noticed that even though some fatty acids levels (C16:0, C18:1, C18:2n-6 and C18:3n-3) seemed different between diets supplemented with 6.32% and 12.7% PLs, no significant differences in survival and growth performance were observed in these two treatments, indicating that these fatty acids might play less important role in large yellow croaker larvae comparing with n-3 HUFA.

The lipid levels were significantly lower in the livers of juveniles fed diets with higher levels of dietary PLs (5.86–9.63% dry diet). Previous studies in mammals have demonstrated that dietary PLs could inhibit hepatic triglyceride synthesis and promote hepatic triglyceride oxidization to reduce lipid content in the liver of rats (Buang et al., 2005; Rossmeisl et al., 2014). Alternatively, as the most abundant PL class on the surface of lipoprotein, PC can promote lipid transport from liver to perihepatic tissues by enhancing the formation of very low density lipoprotein (Yao and Vance, 1988). Thus, dietary PLs might also reduce lipid content of the liver in juvenile large yellow croaker by optimizing lipid metabolism. In the present study, larvae fed diets with 6.32% and 12.7% PLs showed higher whole body lipid content than larvae fed the diet with 2.53% PLs, which was in accordance with the findings of Gao et al. (2014) and Zhao et al. (2013). Previous studies demonstrated that PLs could facilitate lipid digestion and absorption in the intestine of the fish larvae via improved emulsification (Hung et al., 1997; Koven et al., 1993). However, the exact mechanisms need to be further investigated.

$sPLA_2$ IB is responsible for the digestion of dietary PLs and can hydrolyze the fatty acid ester bond at the sn-2 position of PL and produce a non-esterified fatty acid and a lysophospholipid (Cai et al., 2015). In the present study, the transcript levels of $_sPLA_2$ IB of larvae fed diets with 6.32% and 12.7% PLs were significantly lower than that of larvae fed the diet with 2.53% PLs. This was inconsistent with some studies. Buchet et al. (2000) found that the increasing levels of PLs could induce a large increase in $sPLA_2$ IB activity in red drum *Sciaenops ocellatus* larvae. In addition, the mRNA expression of $sPLA_2$ IB was positively correlated with PLs content in the diets of seabass larvae (Cahu et al., 2003). One possible reason for this may be because large yellow croaker larvae in the 2.53% PLs group tended to maximize the hydrolysis of low content of dietary PLs for utilization through enhancing the expression of $sPLA_2$ IB via negative feedback mechanism. Different fish species and diet formulation might be other reasons.

CCTα catalyzes the rate-limiting step in the CDP-choline pathway. Nevertheless, the last step catalyzed by CPT can become rate limiting if the supply of diacylglycerol is restricted (Gibellini and Smith, 2010). In addition, PC produced from PE methylation pathway can compensate for PC production in the liver. The limited capacity of fish larvae to *de novo* synthesize PC is the main limiting factor driving the requirement for dietary intact PLs. Thus, in order to provide new insights into the mechanisms related to the requirement for PLs in larvae, we detected the transcript levels of genes encoding key enzymes involved in endogenous synthesis of PC in large yellow croaker fed diets with graded levels of PLs, especially at both larval and juvenile stage. There were apparently no significant effects of dietary PLs concentrations on the expression of key genes associated with PC synthesis (CCTα, CPT and PEMT) for larval large yellow croaker. In a recent study, intestinal

transcriptome in Atlantic salmon fed diets with different levels of PLs was conducted and the results also showed that dietary treatments did not significantly affect the transcript levels of any genes related with phospholipids metabolism in the intestine of 2.5 g fry (De Santis et al., 2015). Furthermore, Daprà et al. (2011) reported that the expression of CPT was not significantly affected by dietary PLs in rainbow trout larvae. One explanation with the results obtained with the expression of CCTα, CPT and PEMT was that the expression of these genes was not only regulated at transcriptional level. It has been reported that the activity of CCT can be regulated efficiently by translocation on and off membranes (Sugimoto et al., 2008). Further studies are needed to determine protein expression and enzyme activities of CCT, CPT and PEMT. Although no significant difference was also observed in the expression of PC synthesis-related genes in the liver of juvenile large yellow croaker, the transcript levels of CPT in the liver increased with the increasing levels of dietary PLs, accompanied by the decreasing mRNA expression levels of DGAT 2, which is involved in triglyceride synthesis. CPT may compete with DGAT 2 for diacylglycerol in the fish enterocytes (Oxley et al., 2007), which implied that hepatocytes of large yellow croaker fed diets with higher levels of PLs might utilize more diacylglycerol for the synthesis of PC rather than triglyceride, ultimately reducing lipid accumulation in the liver.

In conclusion, the beneficial effects of dietary PLs on survival and growth performance of large yellow croaker were restricted on larval stage. Dietary PLs could not affect the PC synthesis of both larvae and juveniles at transcriptional level. Dietary PLs might reduce lipid deposition of liver through utilizing more diacylglycerol for PC synthesis, rather than triglyceride synthesis.

Conflict of interest

There are no competing interests.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grant no.: 31172425 and 31372541) and the National Basic Research Program of China (973 Program, grant no. 2014CB138600).

We thank K.K. Men, J.J. Cao, S.J. Xing, S.Q. Lin, L. Cheng and staff in Ningde Fufa Fishery co., Ltd. for their help during the experiment.

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