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Article

Effects of Dietary Choline on the Endogenous Phospholipids Synthesis in Juvenile Chinese Mitten Crab (*Eriocheir sinensis*)

Yang Xu [1], Mengyu Shi [1], Ping Wu [2], Yuanqin Zhang [3], Samwell Limbu [4], Jinyun Ye [1] and Changle Qi [1,*]

[1] National-Local Joint Engineering Laboratory of Aquatic Animal Genetic Breeding and Nutrition, Zhejiang Provincial Key Laboratory of Aquatic Resources Conservation and Development, School of Life Sciences, Huzhou University, Huzhou, Zhejiang 313000, China

[2] Key Laboratory of Aquatic Animal Nutrition, Jiangsu, College of Basic Medicine and Biological Sciences, Suzhou University, Suzhou, 215123, China

[3] Huzhou Academy of Agricultural Sciences, Huzhou 313000, China

[4] Department of Aquaculture Technology, School of Aquatic Sciences and Fisheries Technology, University of Dar es Salaam, P. O. Box 60091, Dar es Salaam, Tanzania

* Correspondence: 02862@zjhu.edu.cn

Abstract

In order to investigate the effects of choline on the synthesis of endogenous phospholipids in *Eriocheir sinensis*, this experiment was conducted by adding 0%, 0.2% and 0.4% choline to low phospholipid (0% PL) and normal phospholipid (2% PL) diets, respectively, and feeding the crabs with an initial body weight of 0.4 g for 8 weeks. The results showed that diets supplementation with 0.4% choline significantly upregulated the relative mRNA expression of neuropathy target enzyme 1 (*nte1*), phospholipase A2 (*pla2*) and phospholipase B (*plb*) in the low phospholipids condition. In addition, dietary 0.4% choline significantly increased the relative mRNA expression of hepatopancreatic fatty acid binding protein 3 (*fabp3*), fatty acid transporter protein 4 (*fatp4*), carnitine palmitoyltransferase-2 (*cpt-2*), carnitine acetyltransferase (*caat*), carnitine palmitoyltransferase-1a (*cpt-1a*) and carnitine palmitoyltransferase-1b (*cpt-1b*) in juvenile Chinese mitten crab. Dietary 0.2% choline significantly up-regulating the relative expression of fatty acid synthase (*fas*), and fatty acid elongase 6 (*elovl6*) mRNA relative expressions in the 2% PL diets. This study shows that dietary supplementation with 0.4% choline could improve phospholipids synthesis of Chinese mitten crab under low phospholipids condition, and 0.2% Choline could improve the decomposition and remodeling of phospholipids in the normal phospholipids condition.

Keywords: choline; phospholipids synthesis; lipid metabolism; Chinese mitten crab

Key Contribution: Exogenous choline could affect the synthesis of endogenous phospholipids and lipid metabolism in Chinese mitten crabs.

1. Introduction

Phospholipids are a type of complex lipid, which is essential for the growth and development in aquatic animals [1,2]. There are two ways for animals to obtain phospholipids, the endogenous synthesis and exogenous synthesis pathways [3]. Generally, it is believed that the endogenous phospholipid synthesis capacity of aquatic animals is limited, and the phospholipids mainly obtained from the diets [4]. Therefore, a large number of studies have reported the functions of dietary phospholipids in fish and crustaceans, and have demonstrated the significance of exogenous phospholipids for the growth and development of crustaceans [5]. However, the types of dietary

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phospholipids and the unsaturation degree of the fatty acids are quite different from those required for the biological membranes of fish, shrimp and crab. Therefore, it is speculated that they need to go through a process of "decomposition -synthesis-remodeling" before being utilized by aquatic animals [6]. Furthermore, an increasing number of studies have reported that as long as the diets contain sufficient substances for the synthesis of phospholipids, some crustaceans are fully capable of meeting their needs through endogenous synthesis of phospholipids [7]. Therefore, the significance of endogenous synthesis of phospholipids has gradually been concerned.

Phospholipids are mainly composed of polar lipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Among them, phosphatidylcholine is the most predominant component [8]. In mammals, the process of phosphatidylcholine synthesis is relatively well understood. It is mainly synthesized through the Kennedy pathway, which consists of two routes: CDP-choline and CDP-ethanolamine [3]. Choline is the main precursor substance for this process, and this process begins with choline, which is then converted into phosphatidylcholine through a series of enzymatic reactions. This pathway has been extensively studied in mammals, fruit flies, yeast, and nematodes [3,9,10]. The CTP-phosphocholine cytidylyltransferase (CCT) is the rate-limiting enzyme in this pathway [11]. In aquatic animals, although the process of phosphatidylcholine synthesis has not been fully studied, related enzymes and protein families for phosphatidylcholine synthesis have also been discovered in pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*), and Atlantic salmon (*Salmo salar L.*) [12,13]. Therefore, some studies reported that the synthesis pathway of phosphatidylcholine in fish is similar to that in mammals [14].

Choline is an important substrate for the endogenous synthesis of phosphatidylcholine in aquatic animals, which is associated with a series of physiological functions. On the one hand, choline plays a crucial role on the growth performance of aquatic animals. It has been reported that dietary 6000 mg/kg choline significantly increased the growth of largemouth bass [15]. However, some other studies have shown that dietary choline reduced the growth of pacific white shrimp (*Litopenaeus vannamei*) [16]. The differences in the growth-promoting functions of choline in aquatic animals are not only due to the differences among species, but although the understanding of the function of choline is still not deep enough. On the other hand, choline can reprogram the lipid metabolism of aquatic animals. The deficiency of choline in the fish diet could lead to the lipid metabolism disorders, and resulting in lipid accumulation in the liver of fish [17,18]. Some similar results were reported in the shrimps [19]. These results indicate that choline is closely related to the lipid metabolism of aquatic animals. Besides, choline is the main source of methyl donors in the biological organism, and plays a significant role in neural regulation and the immune system [20,21]. The lack of choline in the diets could affect the immunity and health of aquatic animals [22,23]. The potential mechanism is that choline could enhance the immunity by up-regulating the expressions of genes related with the mTOR signaling pathway [24]. In summary, choline is one of the important nutrients that affect the synthesis of phospholipids, lipid metabolism, growth and health in aquatic animals.

The Chinese mitten crab (*Eriocheir sinensis*) is an important species for aquaculture [25]. Unfortunately, due to the quality issues of the commercial crab diets, the adoption rate of the commercial diets in production has not been high so far. Therefore, clarify the precise lipid nutrition, especially phospholipid nutrition, is particularly crucial for the high-quality diet's formulation. Based on the above discussion, the purpose of this study is to determine whether exogenous choline could affect the synthesis of endogenous phospholipids and lipid metabolism in Chinese mitten crabs. The results may provide theoretical references for precise phospholipids nutrition of crab.

2. Materials and Methods

2.1. Experimental Diets

In order to investigate the effects of choline on the synthesis of endogenous phospholipids, six experimental diets were formulated by adding 0%, 0.2% and 0.4% choline to low phospholipid (0%

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PL) and normal phospholipid (2% PL) diets, respectively. The formulation and proximate nutrient composition of diets were shown in the **Error! Reference source not found.**. In order to prepare the experimental diets, all ingredients were ground into power and sieved through 60 mesh strainers. After when, the ingredients in were precisely weighed according to the formulation and mixed using an electric mixer. Choline is added to the diets by dissolving it in water. Subsequently, 2.5 mm diameter diets were pelleted with a screw-press pelletizer (F-26, South China University of Technology, Guangzhou, China). Finally, all the diets were air dried in room for approximately 48 h to reduce the moisture to less than 10%. After drying, all diets were stored at -20 °C until used.

Table 1. Ingredients and proximate compositions of the experimental diets (%).

Ingredients	Low phospholipids (0% PL)			Normal phospholipids (2% PL)		
	0% CHO	0.2% CHO	0.4% CHO	0% CHO	0.2% CHO	0.4% CHO
Casein	36	36	36	36	36	36
Gelatin	9	9	9	9	9	9
Corn starch	26	26	26	26	26	26
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
Fish oil	4.5	4.5	4.5	3.5	3.5	3.5
Soybean oil	4.5	4.5	4.5	3.5	3.5	3.5
Arginine	1.8	1.8	1.8	1.8	1.8	1.8
Methionine	0.5	0.5	0.5	0.5	0.5	0.5
Lysine	0.5	0.5	0.5	0.5	0.5	0.5
[d] Vitamin premixes	1.5	1.5	1.5	1.5	1.5	1.5
[e] Mineral premixes	1.5	1.5	1.5	1.5	1.5	1.5
Sodium carboxymethyl cellulose	2	2	2	2	2	2
[c] Attractant	3	3	3	3	3	3
[b] Butylated hydroxytoluene	0.1	0.1	0.1	0.1	0.1	0.1
Soybean lecithin	0	0	0	2	2	2
[a] Choline	0	0.2	0.4	0	0.2	0.4
Microcrystalline cellulose	8.6	8.4	8.2	8.6	8.4	8.2
Nutritional analysis (%)						
Crude protein	49.88	49.49	50.87	50.40	51.08	50.62
Crude lipid	10.46	9.45	9.77	10.77	10.51	10.07
Ash	3.06	3.02	2.95	3.18	3.07	3.18
Moisture	7.65	7.38	7.86	8.68	8.91	8.59

[a] Shanghai Bioengineering Co. Ltd., Shanghai, China. [b] Antioxidant: 2,6-di-tert-butyl-p-cresol. Shanghai Bioengineering Co., Ltd., Shanghai, China. [c] Attractants: glycine, alanine, glutamic acid, betaine. [d] Vitamin premix (per 100 g premix): Ca pantothenate, 0.3 g; p-aminobenzoic acid, 0.1 g; cholecalciferol, 0.0075 g; riboflavin, 0.0625 g; menaquinone, 0.05 g; ascorbic acid, 0.5 g; biotin, 0.005 g; vitamin A acetate, 0.043 g; folic acid 0.025 g; pyridoxine nicotinate, 0.225 g; thiamine hydrochloride, 0.15 g; hydrochloric acid, 0.3 g; α-tocopheryl acetate, 0.5 g; The remainder is supplemented with α-cellulose up to 100 g. [e] Mineral premix (per 100 g): KI, 0.023 g; CuCl2·2H2O, 0.015 g; Ca(H2PO4)2, 26.5 g; MnSO4·6H2O, 0.143 g; AlCl3·6H2O, 0.024 g; KH2PO4, 21.5 g; NaH2PO4, 10.0 g; CoCl2·6H2O, 0.14 g; KCl, 2.8 g; ZnSO4·7H2O, 0.476 g; Calcium lactate, 16.50 g; CaCo3, 10.5 g; MgSO4·7H2O, 10.0 g; Fe-citrate, 1 g; The remainder was supplemented with α-tocopheryl supplement to 100 g.

2.2. Feeding Trial and Sampling

The feeding experiment was carried out in the Aquaculture Center of Huzhou University (Huzhou, Zhejiang). Crabs were obtained from a local farm. The experimental water was aerated fully before use. Prior to the feeding trial, all of the experimental crabs were stocked in some 300 L tanks (100 × 80 × 60 cm) to acclimatize the experimental environment. After then, crabs (0.4 ± 0.03 g,

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mean ± S.D.) with intact appendages were randomly distributed into six dietary treatments. Each treatment was set 4 paralleled tanks, and each tank containing 25 crabs. Four bundles of corrugated plastic pipes and arched tiles were placed in each tank as shelters to reduce attacking behavior. Diets with a daily ration of 4% body weight were hand-fed to crabs twice (8:00 and 18:00). Feces were removed in the morning (09:00), and the water of 30% tank volume was exchanged daily. During the experimental period, the water temperature varied from 18 °C to 24 °C. The pH value varied between 7.0 and 8.0. Dissolved oxygen was above 7 mg/L. The ammonia was below 0.2 mg/L. The feeding duration was 8 weeks.

At the end, all the crabs from each tank were anesthetized with crushed pieces of ice, and then counted and group-weighed by tank. Following, 10 crabs from each tank were randomly selected for sampling. The hepatopancreas samples were collected and put into liquid nitrogen immediately and then stored at -80 °C for the analyses of gene expression.

2.3. Determination of Growth Performance

Weight gain (WG, %) = (final crab weight – initial crab weight) × 100 / initial crab weight;

Specific growth rate (SGR, %) = (Ln (final crab weight) - Ln (initial crab weight)) × 100 / days;

Hepatopancreas somatic index (HSI, %) = hepatopancreas weight / final whole-body weight × 100.

2.4. Proximate Nutrient Composition of Experimental Diets

The proximate nutrient compositions of experimental diets were determined by the standard procedures using proximate composition analysis [26]. Each diet was randomly collected four duplicate samples for proximate nutrient composition analysis (n = 4). All the samples were spread out evenly in a glass petri dish and dried until they reached a constant weight (at 105 °C). The lipid of each diet was extracted using the chloroform-methanol method. The crude lipid was quantified using a KjeltecTM 8200 (Foss, Hoganas, Sweden). Ash was measured using a muffle furnace (PCD-E3000 Serials, Peaks, Japan) at 550 °C for 6 h.

2.5. Gene Expression

Total RNA was extracted from the hepatopancreas using the RNAiso Plus (CAT # 9109, Takara, Japan) according to the manufacturer's protocol. The total RNA concentration and quality were estimated using the Nano Drop 2000 spectrophotometer (Thermo, USA). If the ratio of A260/A280 was between 1.8 to 2.0, the sample was used for reverse transcribed using a PrimeScript™ RT master mix reagent kit (Tiangen, Beijing, China). The specific primers for the genes of E. sinensis were designed based on the NCBI data base (https://www.ncbi.nlm.nih.gov/assembly/GCA_024679095.1/) using NCBI Primer BLAST (**Table 1**). The RT-PCR amplification reactions were performed using an CFX96 Real-Time PCR system (Bio-rad, Richmond, CA). Samples were run in quintuplicate and normalized with the control gene β-actin and glyceraldehyde-phosphate dehydrogenase (GAPDH). The gene expression levels were calculated by geometric averaging of multiple internal control genes [27].

Table 1. Primer sequences of the experimental genes.

Primer name	Sequences (5′-3′)	Product length
β-actin F	TGGGTATGGAATCCGTTGGC	101 bp
β-actin R	AGACAGAACGTTGTTGGCGA	
gapdh F	CACCGTGCATGCTGTTACTG	108 bp
gapdh R	ACCAGTGGAGGATGGGATGA	
fabp3 F	CCACCGAGGTCAAGTTCAAGC	195 bp
fabp3 R	TCACACCATCACACTCCGACAC	
fatp4 F	GACGGCAGACACGGAAAGAGA	101 bp

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fatp4 R	CAGGTGGAGGCAAGCAAACTC	
fatp6 F	TGATGGGAAGGCAGGAATGG	119 bp
fatp6 R	TGCGGATGAAGCGAGGTACA	
elovl6 F	TGAGAAGCGGCAATGGATGAAG	164 bp
elovl6 R	TGGAGAAGAGGGCCAGGAAGAC	
Δ9 fad F	TGGCACAACTACCACCACGTCT	160 bp
Δ9 fad R	TCCTCTTCTCGATCATCTCCGG	
cpt-1a F	CATCTGGACACCCACCTCCA	183 bp
cpt-1a R	ATCTCCTCACCCGGCACTCT	
cpt-1b F	GGCATTCTCCTTTGCCATCAC	138 bp
cpt-1b R	ACACCACACCGCACATTGTTC	
cpt-2 F	AGCAGGCAGTGGCTCAGTTTA	169 bp
cpt-2 R	AAGGCAAGGAAGGGGTTGTAG	
caat F	CATCAAGAGCCAGGAGCCCA	172 bp
caat R	CTTCAACAGCAGCCCGCAAA	
cet F	CAAGCTTCTCGACTCTGGCA	140 bp
cet R	TGGCCTTCATGTAAGCAGCA	
cct F	CCCTGGACACTAGAGGACGA	128 bp
cct R	ACGAACATTCCACGAGCCTT	
lpcat F	TATGGCTGATGCTTTGGGGG	145 bp
lpcat R	TGTCTGGCAGGGTAGGTTCT	
pla$_2$ F	GCCTCTGGACGACTGTGAAA	192 bp
pla$_2$ R	TTTGGGGTATTTGGGTCCCG	
plb F	CTGCACCCCTCCTTACAGTG	103 bp
plb R	GTGAGACCTGTGACCCAGTG	
nte1 F	TGTACTTTTCCGCCGCTTCT	156 bp
nte1 R	GGCCGGATGTATTCGCAGTA	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. fabp3: Fatty acid binding protein 3. fatp4: Fatty acid transporter protein 4. fatp6: Fatty acid transporter protein 6. elovl6: Fatty acid elongase 6. Δ9 fad: Fatty acid desaturase 9. cpt-1a: Carnitine palmitoyltransferase-1a. cpt-1b: Carnitine palmitoyltransferase-1b. cpt-2: Carnitine palmitoyltransferase-2. caat: Carnitine acetyltransferase. cet: CTP-phosphoethanolamine. Cytidylyltransferase. cct: CTP-phosphocholine cytidylyltransferase. lpcat: Lysophosphatidylcholine acyltransferase. pla$_2$: Phospholipase A2. plb: Phospholipase B. nte1: Neuropathy target enzyme 1.

2.6. Statistical Analysis

Statistical analysis was performed using the SPSS 25.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA). Data were analyzed by two-way analysis of variance (ANOVA) to determine if there was any interaction between phospholipids levels and choline levels. At the same phospholipids level, one-way analysis of variance (ANOVA) was used to analyze the significant differences among crabs fed the diets with different choline levels. When the means of each treatment were significantly different, Duncan's multiple range test was used to compare means among these treatments. At the choline level, independent-samples T test was used to determine significant differences between crabs fed with the different choline levels. Significance was set at $P < 0.05$. The data were represented as the mean ± standard error of mean (S.E.).

3. Results

3.1. Effects of Dietary Choline on the Growth Performance of Juvenile Chinese Mitten Crab

In the low phospholipids (0% PL) groups, although dietary choline level did not significantly affect the weight gain (WG) and specific growth rate (SGR), but the WG and SGR showed an upward trend with the increasing dietary choline supplementation ($P > 0.05$). However, in the normal

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phospholipids (2% PL) groups, dietary choline decreased the WG and SGR, but there were on significant differences among each choline diets ($P > 0.05$). In the low phospholipids (0% PL) groups, dietary choline levels did not significantly affect the hepatopancreas somatic index (HSI) of crabs ($P > 0.05$). However, in the normal phospholipids (2% PL) groups, dietary choline decreased HSI, but there were on significant differences among each choline diets ($P > 0.05$).

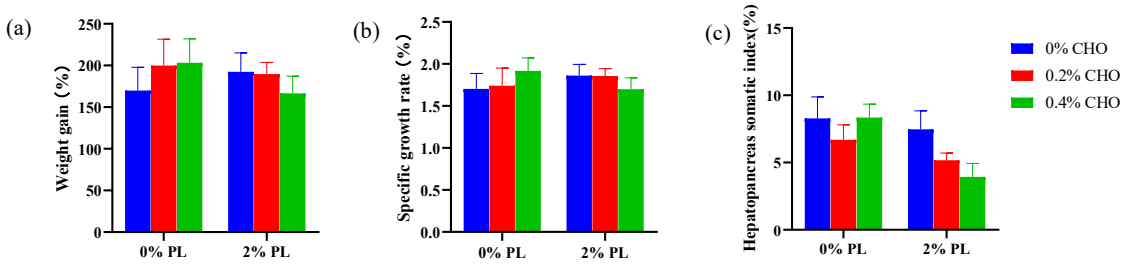

Figure 1. Effects of dietary choline on the growth performance of juvenile Chinese mitten crab.

3.2. Effects of Dietary Choline on the Relative mRNA Expression of Genes Related to Phospholipid Synthesis in Juvenile Chinese Mitten Crab

Dietary choline levels and phospholipids levels significantly affected the relative mRNA expressions of CTP-phosphoethanolamine cytidylyltransferase (*cet*) and lysophosphatidylcholine acyltransferase (*lpcat*) (significant main effects) and there is a significant interaction between them ($P < 0.05$). The further analysis showed that in the 0% choline and 0.2% choline groups, the relative mRNA expressions of *cet* and *lpcat* of crabs fed the 2% PL diets were significantly up-regulated compared with the crabs fed the 0% PL diets ($P < 0.05$). The dietary choline level did not have significant main effect on the relative mRNA expression of CTP-phosphocholine cytidylyltransferase (*cct*) ($P > 0.05$). While, the dietary phospholipids level had a significant main effect on the relative mRNA expression of (*cct*) ($P < 0.05$). In the normal phospholipids (2% PL) groups, the relative expressions of *cet* and *lpcat* of crabs fed the 0.2% CHO diets were significantly up-regulated compared with the crabs fed the 0% CHO and 0.4% CHO diets ($P < 0.05$).

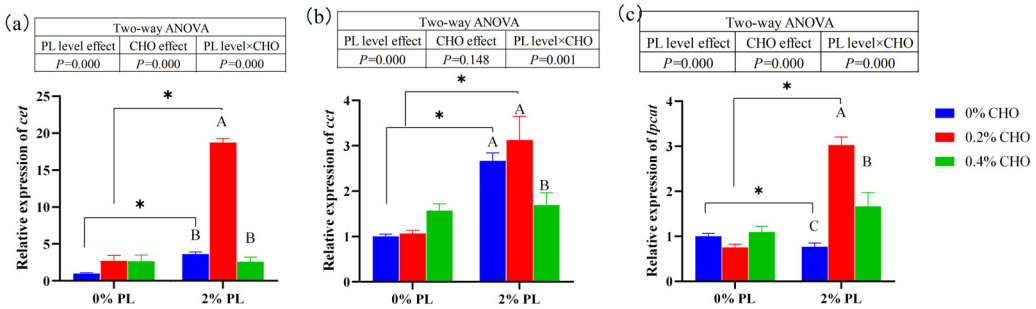

Figure 2. Effects of dietary choline on the relative mRNA expression of genes related to phospholipid synthesis in juvenile Chinese mitten crab. Note: *cet*: CTP-phosphoethanolamine cytidylyltransferase; *cct:* CTP-phosphocholine cytidylyltransferase; *lpcat*: lysophosphatidylcholine acyltransferase. Different letters at the top of the bar graph indicate significant differences ($P < 0.05$), the connected lines are significant differences, $P < 0.05$ (marked *), the same below. Number of biological replicates (n = 6).

3.3. Effects of Dietary Choline on the Relative mRNA Expression of Genes Related to Phospholipid Catabolism in Juvenile Chinese Mitten Crab

As shown in the **Figure 3**, dietary choline levels and phospholipids levels had significant main effects on the relative mRNA expression of neuropathy target enzyme 1 (*nte1*), phospholipase A2

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(*pla2*) and phospholipase B (*plb*), and there is a significant interaction between them ($P < 0.05$). The further analysis showed that in the 0% choline and 0.2% choline groups, the relative mRNA expressions of *nte1* and *pla2* of crabs fed the 2% PL diets were significantly up-regulated compared with the crabs fed the 0% PL diets ($P < 0.05$). Moreover, in the 0% PL diets, the relative mRNA expressions of nte1 and *pla2* of crabs fed the 0.4% CHO diets were significantly up-regulated compared with the crabs fed the 0% CHO and 0.2% CHO diets ($P < 0.05$). While, in the 2% PL diets, the highest mRNA expressions of *nte1* and *pla2* were observed in the crabs fed the 0.2% CHO ($P < 0.05$). The relative mRNA expressions of *plb* of crabs fed the 2% PL diets were significantly up-regulated compared with the crabs fed the 0% PL diets under the 0.2% CHO and 0.4% CHO diets ($P < 0.05$). In the 0% PL diets, crabs fed the 0.2% CHO diets showed the lowest mRNA expressions of *plb*, *nte1* and *pla2* ($P < 0.05$).

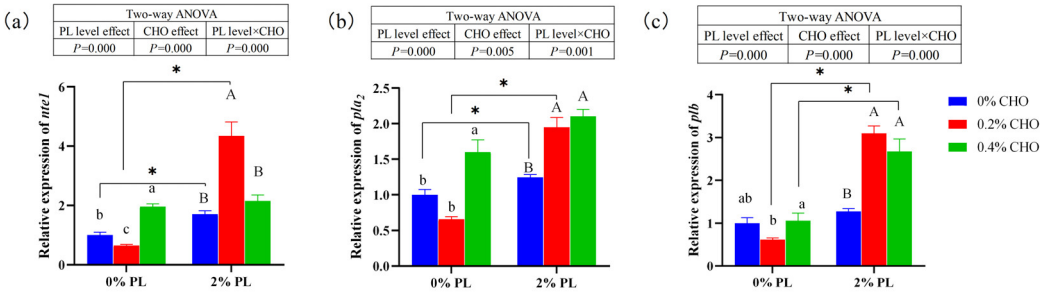

Figure 3. Effects of dietary choline on the relative mRNA expression of genes related to phospholipid catabolism in juvenile Chinese mitten crab.Note: *nte1*: neuropathy target enzyme 1; *pla2*: phospholipase A2; *plb*: phospholipase B.

3.4. Effects of Dietary Choline on the Relative mRNA Expression Genes Related to Fatty Acid Absorption, Decomposition and Synthesis in Juvenile Chinese Mitten Crab

As shown in the **Figure 4** dietary choline levels and phospholipids levels had significant main effects on the relative mRNA expression of fatty acid binding protein 3 (*fabp3*), fatty acid transporter protein 4 (*fatp4*), fatty acid transporter protein 6 (*fatp6*), carnitine acetyltransferase (*caat*), carnitine palmitoyltransferase-1b (*cpt-1b*), carnitine palmitoyltransferase-2 (*cpt2*), fatty acid synthase (*fas*) and fatty acid elongase 6 (*elovel6*), and there is a significant interaction between them ($P < 0.05$). Dietary choline levels had significant main effect on the relative mRNA expression of carnitine palmitoyltransferase-1a (*cpt-1a*) ($P < 0.05$). In the low phospholipids (0% PL) groups, the relative mRNA expression of *fabp3* was significantly up-regulated with the increasing dietary choline supplementation ($P < 0.05$). However, in the normal phospholipids (2% PL) groups, the relative mRNA expression of *fabp3* was significantly down-regulated with the increasing dietary choline supplementation ($P < 0.05$). The relative mRNA expressions of *fatp4*, *caat*, *cpt-1a*, *cpt-1b* and *cpt2* were significantly down-regulated in the crabs fed the 0.2% CHO diet in the low phospholipids (0% PL) groups ($P < 0.05$). On the contrary, the relative mRNA expressions of *fas* and *elovel6* were significantly up-regulated in the 0.2% CHO diet in the low phospholipids (0% PL) groups ($P < 0.05$). Moreover, the relative mRNA expressions of *fatp4*, *fatp6*, *caat* and *cpt2* were significantly up-regulated in the crabs fed the 0.2% CHO diet in the normal phospholipids (2% PL) groups ($P < 0.05$).

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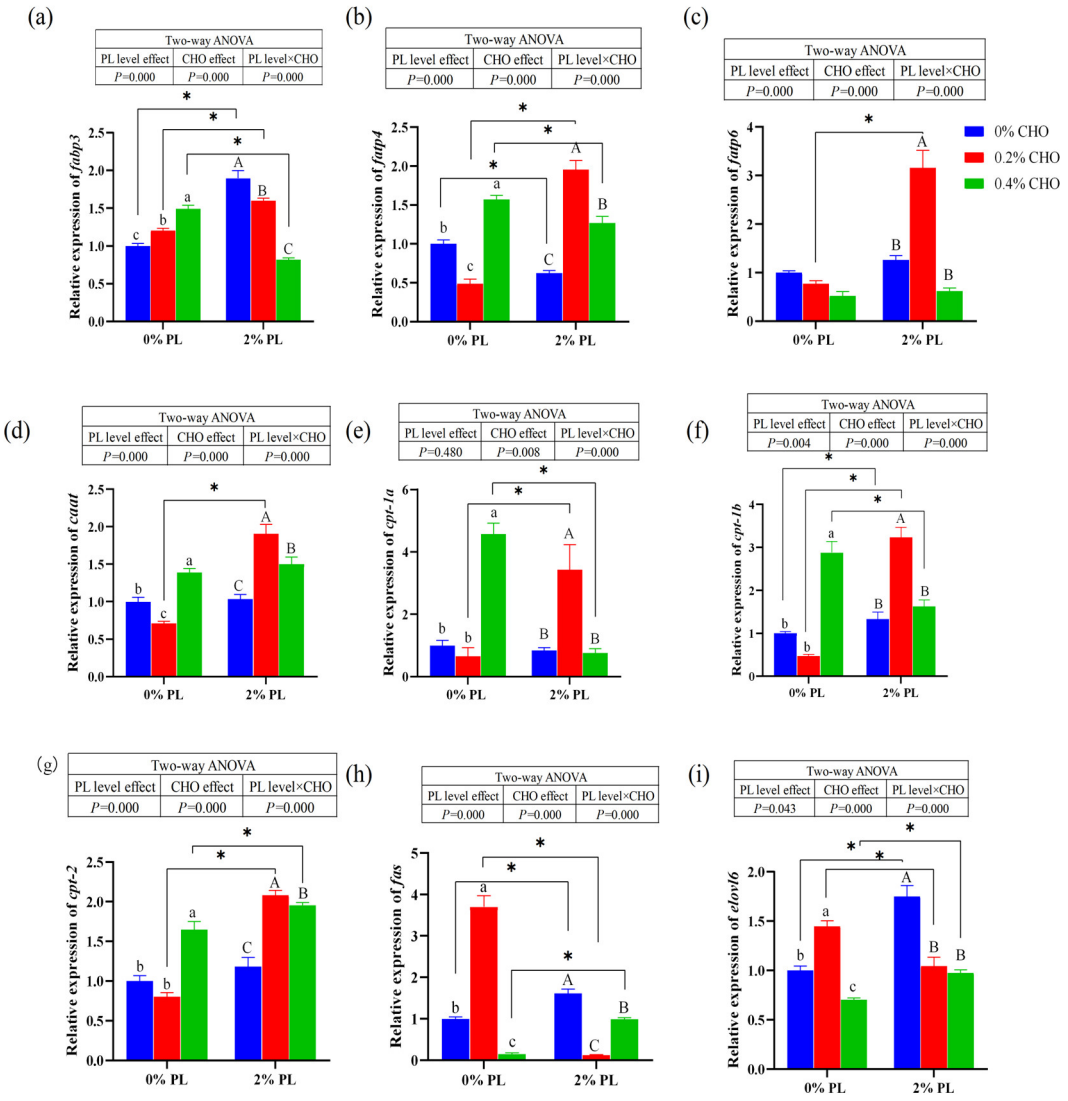

Figure 4. Effects of dietary choline on the relative mRNA expression genes related to fatty acid absorption, decomposition and synthesis in juvenile Chinese mitten crab. *fabp3*: Fatty acid binding protein 3. *fatp4:* Fatty acid transporter protein 4. *fatp6:* Fatty acid transporter protein 6. *caat:* Carnitine acetyltransferase. *cpt-1a:* Carnitine palmitoyltransferase-1a. *cpt-1b:* Carnitine palmitoyltransferase-1b. *cpt-2:* Carnitine palmitoyltransferase-2. *fas:* Fatty acid synthase. *elovl6:* Fatty acid elongase 6.

4. Discussion

Some previous studies had reported that a certain dietary choline could improve the growth of aquatic animals, such as largemouth bass [15] Asian swamp eel [28] and Bluntnose black bream [29]. In the present study, dietary choline did not significantly affect the growth of Chinese mitten crab in the both low phospholipids (0% PL) and normal phospholipids (2% PL) groups. Our result was inconsistent with these studies, which might be caused by the species-species differences. However, choline tends to promote growth in the low phospholipids condition. Therefore, there may also be significant differences in growth performance after an extending feeding period. Some other studies have reported that dietary choline could reduce the growth shrimp. In the present study, dietary choline slightly reduced growth of crabs in the normal phospholipids groups, which is consistent with the result on the pacific white shrimp (*L. vannamei*) [16]. In summary, supplementation of choline in the diet could to some extent promote the growth (no significant differences) of crabs under low phospholipid condition, achieving results similar to those of the normal phospholipid group.

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Moreover, it is not recommended to supplement choline in the diets under the condition of abundant phospholipids.

Choline is a precursor for the synthesis of phospholipids and the growth-promoting effect of choline may be related to the synthesis of phospholipids. Therefore, our study investigated the effects of choline on the key genes involved in phospholipids synthesis. Our results showed that choline up-regulated the relative mRNA expressions of CTP-phosphoethanolamine cytidylyltransferase (*cet*) and CTP-phosphocholine cytidylyltransferase (*cct*). CCT and CET are the key enzymes for the synthesis of PC and PE, and play a limiting role in the pathway. They can catalyze the formation of cytidine diphosphate choline and cytidine diphosphate ethanolamine from phosphocholine and phosphoethanolamine, and finally produce PC and PE [3]. Therefore, choline may be able to promote the synthesis of phospholipids under low phospholipid condition. Besides, our result showed that dietary supplementation with phospholipids significantly up-regulated the relative mRNA expressions of lysophosphatidylcholine acyltransferase *(lpcat*). IPCAT is a key enzyme for phospholipid remodeling and can participate in the acylation reaction of lysophosphatidylcholine [30]. This result indicated that dietary phospholipids may need to be restructured before they can be utilized due to the types of dietary phospholipids and the unsaturation degree of the fatty acids are quite different from those required for the biological membranes. To verify the above hypothesis, our study investigated the effect of choline on the phospholipid's catabolism. Phospholipase B (*plb*) can hydrolyze lysophospholipids to produce glycerophosphocholine [31]. Moreover, Phospholipase A2 (*pla2*) can specifically hydrolyze the second ester bond in glycerophospholipid molecules, generating products such as lysophospholipid 1 and fatty acids [32]. Neurosin target enzyme 1 (*nte1*) can regulate the degradation of choline phospholipids to maintain the choline cycle and lipid balance, which catalyzes the synthesis of phosphatidylcholine through the CDP-choline pathway [33]. These enzymes are key targets involving in the remodeling and synthesis processes of phospholipids. In the present study, 2% phospholipids significantly up-regulated the relative mRNA expressions of *plb*, *pla2* and *nte1*, which validates the assumptions we made above. Moreover, our results showed that dietary choline phospholipids catabolism showed a significant interaction, indicating that dietary choline could affect the decomposition and remodeling of phospholipids. In summary, choline could promote phospholipid synthesis under low phospholipid condition, and it can facilitate the catabolism and remodeling of phospholipids under high phospholipid condition.

During the process of phospholipid synthesis, there is lipid synthesis and catabolism. Therefore, our study subsequently explored the effects of choline on the lipid metabolism. Some previous studies have reported that fatty acid binding proteins (FABPs) and fatty acid transport proteins (FATPs) are the key factors for the transmembrane transport of fatty acids **[34–36]** In the present study, dietary choline significantly up-regulated the relative mRNA expressions of *fabp3* and *fatp4*, which indicated that choline activated the transport of fatty acids. In addition, dietary 0.4% CHO significantly up-regulated the relative mRNA expression of *cpt-2*, *cpt-1a* and *cpt-1b*. CPT-2, CPT-1a and CPT-1b play crucial roles in the process of fatty acid metabolism, in which CPT-1a and CPT-1b involving the entry of fatty acids into mitochondria [37,38], and CPT-2 can catalyze the conversion of acylcarnitine to acylcoenzyme A, allowing fatty acids to enter the mitochondrial matrix for β-oxidation [39,40]. Thus, we speculated that dietary supplementation with 0.4% choline promoted the fatty acid catabolism in the Chinese mitten crab, and it might be to generate energy for phospholipid synthesis.

Phospholipids synthesis not only requires energy but also specific fatty acids. Therefore, we hypothesize that there is also associated fatty acid synthesis metabolism during the process of phospholipid synthesis. Fatty acid synthase (*fas*) is a key enzyme for the de novo synthesis of fatty acid chain and the fatty acid elongase (*elove6*) is a key rate-limiting enzyme that determines the length of the fatty acid chain [5,41,42]. Our study showed that 0.2% CHO significantly enhanced the expression of *fas* and *elovl6* under low phospholipids condition. Thus, we speculated that choline promoted the de novo synthesis of fatty acids, possibly to provide the necessary substrates for phospholipid synthesis.

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5. Conclusions

This study indicated that dietary supplementation with 0.4% choline could up-regulated the genes related to phospholipids synthesis (*cet* and *cct*), fatty acid catabolism (*cpt-2*, *cpt-1a* and *cpt-1b*) and fatty acid synthesis (*fas* and *elove6*) under low phospholipids condition. While, dietary 0.2% Choline could improve the decomposition and remodeling of phospholipids by up-regulated the mRNA expressions of *plb*, *pla2* and *nte1*.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PL	Phospholipids
WG	Weight gain
SGR	Specific growth rate
HSI	Hepatopancreas somatic index
cet	CTP-phosphoethanolamine cytidylyltransferase
cct	CTP-phosphocholine cytidylyltransferase
lpcat	Lysophosphatidylcholine acyltransferase
nte1	Neuropathy target enzyme 1
pla2	Phospholipase A2
plb	Phospholipase B
fabp3	Fatty acid binding protein 3
fatp4	Fatty acid transporter protein 4
fatp6	Fatty acid transporter protein 6
cpt-2	Carnitine palmitoyltransferase-2
caat	Carnitine acetyltransferase
cpt-1a	Carnitine palmitoyltransferase-1a
cpt-1b	Carnitine palmitoyltransferase-1b
fas	Fatty acid synthase
elovel6	Fatty acid elongase 6
Δ9 fad	Fatty acid desaturase 9

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