



## Original Research Article

# Dietary choline activates the Ampk/Srebp signaling pathway and decreases lipid levels in Pacific white shrimp (*Litopenaeus vannamei*)



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## ABSTRACT

An 8-week feeding trial was conducted in Pacific white shrimp (*Litopenaeus vannamei*) to evaluate the effects of dietary choline supplementation on choline transport and metabolism, hepatopancreas histological structure and fatty acid profile, and regulation of lipid metabolism. Six isonitrogenous and isolipidic diets were formulated to contain different choline levels of 2.91 (basal diet), 3.85, 4.67, 6.55, 10.70 and 18.90 g/kg, respectively. A total of 960 shrimp (initial weight,  $1.38 \pm 0.01$  g) were distributed randomly into twenty-four 250-L cylindrical fiber-glass tanks, with each diet assigned randomly to 4 replicate tanks. The results indicated that dietary choline significantly promoted the deposition of choline, betaine and carnitine ( $P < 0.05$ ). The diameters and areas of R cells, total lipid and triglyceride contents in hepatopancreas, and triglyceride and non-esterified fatty acid contents in hemolymph were negatively correlated with dietary choline level. The contents of functional fatty acids in hepatopancreas, the activity of acetyl-CoA carboxylase (Acc), and the mRNA expression of *fas*, *srebp* and *acc* were highest in shrimp fed the diet containing 4.67 g/kg choline, and significantly higher than those fed the diet containing 2.91 g/kg, the lowest level of choline ( $P < 0.05$ ). The number of R cells, content of very low-density lipoprotein (VLDL), activities of carnitine palmitoyl-transferase (Cpt1), lipoprotein lipase and hepatic lipase, and the mRNA expression levels of *cpt1*, *fabp*, *fatp*, *ldlr*, and *ampk* in hepatopancreas increased significantly as dietary choline increased ( $P < 0.05$ ). In addition, hepatopancreas mRNA expression levels of *ctl1*, *ctl2*, *oct1*, *badh*, *bhmt*, *ck*, *cept*, and *cct* were generally up-regulated as dietary choline level increased ( $P < 0.01$ ). In conclusion, dietary choline promoted the deposition of choline and its metabolites by up-regulating genes related to choline transport and metabolism. Moreover, appropriate dietary choline level promoted the development of hepatopancreas R cells and maintained the normal accumulation of lipids required for development, while high dietary choline not only promoted hepatopancreas lipid export by enhancing VLDL synthesis, but also promoted fatty acid  $\beta$ -oxidation and inhibited de novo fatty acid synthesis by activating the Ampk/Srebp signaling pathway. These findings provided further insight and understanding of the mechanisms by which dietary choline regulated lipid metabolism in *L. vannamei*.

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## 1. Introduction

Choline, a positively charged quaternary amine, was officially recognized as an essential nutrient by the Institute of Medicine in the United States in 1998 (Zeisel and Da-costa, 2009). Choline is found widely in plants and animals, mainly in the form of choline-containing compounds, among which phosphatidylcholine (PC) accounts for more than 90%, while other sources include sphingomyelin, phosphorylcholine, glycerol phosphatidylcholine and

acetylcholine (ACH), with only about 1% existing in the free form (Bremer et al., 1960; Wurtman, 1979). In liver, PC can be synthesized by the phosphatidylethanolamine N-methyltransferase (PEMT) pathway, which also represents the only known route for endogenous choline synthesis (Li et al., 2005). However, a pathway for endogenous synthesis of a nutrient does not guarantee that requirements can be met fully through this source alone (Sherriff et al., 2016), and numerous studies have shown that dietary supplementation of choline is essential for the optimum development of crustaceans (An et al., 2019; Gong et al., 2003; Qi, 2013; Xia, 2014).

Although the mechanisms underlying choline transport have not been completely elucidated, three protein-mediated, and thus saturable, uptake systems displaying Michaelis–Menten kinetics have been reported (Deves and Krupker, 1979; Fisher et al., 1992; Grassl, 1994; Simon et al., 1976). The first mechanism is facilitated diffusion, driven by a choline concentration gradient (Deves and Krupker, 1979), while the second is a high-affinity, Na<sup>+</sup>- and energy-dependent “active transport” system coupled to the biosynthesis of acetylcholine primarily in neuronal tissues (Simon et al., 1976). The third active transport system has a somewhat lower affinity for choline and operates in most cells as a means of choline uptake for phospholipid synthesis (Grassl, 1994; Porter et al., 1992). As alluded to, active choline transport through the cell membrane is mediated and regulated by several transporters including choline transporter-like protein family (CTL), high-affinity choline transporter family (ChT), and organic cation transporter family (OCTs) (Michel et al., 2006). The CTL protein family comprises 5 genes, SLC44A1 to SLC44A5, and is assumed to supply choline for cellular phospholipid synthesis (Michel and Bakovic, 2012), while ChT1, solute carrier family SLC5, mediates Na<sup>+</sup>-dependent choline transport and is expressed mainly in cholinergic neurons and keratinocytes (Haga, 2014). The OCTs, also known as solute carrier family 22 proteins, are generic transporters of heavy metals and organic cations, with only low specificity and rate for transporting choline (Gorboulev et al., 1997; Sweet et al., 2001).

After being transported to tissues and cells, choline can have various biological roles through involvement in oxidation, phosphorylation and acetylation pathways (Li and Vance, 2008). Briefly, the majority of cellular choline is phosphorylated by choline kinase (CK) to form phosphorylcholine (PCho), to which cytidine triphosphate (CTP) can then be added by phosphate cytidylyltransferase 1 to yield cytidine diphosphate choline (CDP-choline), which then reacts with diacylglycerol (DAG) to form PC, an essential component of all membranes (Zeisel and Da-costa, 2009). Studies of PC anabolism in mice led to the estimation that 70% of hepatic synthesis of PC is derived from the CDP-choline pathway requiring dietary choline (Zeisel et al., 2003).

In nerve cells, choline can be acetylated by the enzyme choline acetyltransferase to form ACH, the main mediator of the parasympathetic nervous system (Lockman and Allen, 2002). In liver and kidney, choline can be irreversibly oxidized by choline dehydrogenase and betaine aldehyde dehydrogenase (BADH) to betaine (Dragolovich, 1994; Zeisel, 1990), which is a methyl group donor that acts as a regulator of methionine and S-adenosyl methionine metabolism, DNA and protein methylation (Smith et al., 1994). Thus, transport and metabolism of choline are closely related to cell membrane phospholipid synthesis, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid-cholesterol transport and metabolism (Zeisel and Blusztajn, 1994). Although *ctl* and *oct*-related transcripts have been found in Pacific white shrimp *Litopenaeus vannamei*, their roles in the transport of choline in crustaceans are yet to be determined and verified.

As choline-derived lipids play important roles in maintaining cell structure and lipid transport in and out of cells (NRC, 2011),

modifying the intake of choline nutrients could alter lipid metabolism, especially in the case of choline deficiency (Niculescu et al., 2006). It has been shown in both human and animal studies that choline deficiencies contribute to pathologies such as non-alcoholic fatty liver disease and various neurodegenerative diseases (Riley et al., 1997). In addition, recent studies reported that dietary choline supplementation affected hepatic lipid content and body lipid accumulation in various fish species (Koca et al., 2008; Wu et al., 2016; Zhao et al., 2016). Moreover, Corbin et al. (2014) further explained the effect of choline on fatty liver using omics technologies, which suggested that the influence of choline on fatty liver symptoms was impacted by single nucleotide polymorphisms in specific genes of choline and folic acid metabolism. Although fatty liver problems in farmed aquatic animals can be treated by dietary supplementation of choline, few studies investigated the possible mechanisms whereby dietary choline affected lipid metabolism in crustaceans.

The importance of choline as an essential nutrient has been well established, but our understanding of the interplay between nutrient metabolism and dietary choline requirements is only beginning, especially in crustaceans. Pacific white shrimp, *L. vannamei*, is one of the most widely farmed shrimp species all over the world (Zhao et al., 2018), and studies have shown that dietary choline supplementation improved growth performance, antioxidant capacity, and non-specific immunity (An et al., 2019; Xia, 2014). While some studies have reported choline requirements for *L. vannamei*, the values varied widely (Lu et al., 2022). In general, shrimp fed with practical diets exhibited higher choline requirements than those fed purified or semi-purified diets (An et al., 2019; Gong et al., 2003; Xia, 2014). Previous research in our laboratory showed that practical diets (30% fishmeal) containing 3.3 to 9.5 g/kg choline improved the growth performance of *L. vannamei*, and the optimal choline requirement was suggested to be at least 3.3 g/kg (Xia, 2014). Although progress has been made in understanding the nutritional requirements for choline of *L. vannamei*, there is very limited understanding of the mechanisms of choline transport and metabolism, and the role of dietary choline in the regulation of lipid metabolism. The present study expanded the range of dietary choline levels based on previous results, with the specific aim to elucidate the mechanisms whereby dietary choline could impact the regulation of lipid metabolism of *L. vannamei*, particularly from the perspective of choline transport and metabolism.

## 2. Materials and methods

### 2.1. Animal ethics

All experimental procedures followed the Standard Operation Procedures for the use of Experimental Animals of Ningbo University. The Ethics Scientific Committee approved the study for Experiments on Animals of Ningbo University.

### 2.2. Experimental diet preparation

The experimental diets were designed based on the nutritional requirements of *L. vannamei* with formulations and proximate compositions shown in Table 1. Choline chloride (analytical reagent grade, 99%) was used as a choline source and supplemented to the basal diet at 0 (basal diet), 1.5, 3.0, 6.0, 12.0, and 24.0 g/kg, and the corresponding choline levels in the diets were 2.91, 3.85, 4.67, 6.55, 10.70 and 18.90 g/kg, respectively. The diets were prepared following the detailed procedures described by Lu et al. (2020). Dietary choline concentration was determined by the China

**Table 1**  
Formulation and proximate composition of experimental diets (dry matter, g/kg).

| Item  | Dietary choline level, g/kg |        |        |        |        |        |
|---|-----------------------------|--------|--------|--------|--------|--------|
|   | 2.91                        | 3.85   | 4.67   | 6.55   | 10.70  | 18.90  |
| <b>Ingredients</b>  |                             |        |        |        |        |        |
| Fish meal <sup>1</sup>  | 300.0                       | 300.0  | 300.0  | 300.0  | 300.0  | 300.0  |
| Krill meal <sup>1</sup>                                       | 60.0                        | 60.0   | 60.0   | 60.0   | 60.0   | 60.0   |
| Soybean meal <sup>1</sup>                                     | 270.0                       | 270.0  | 270.0  | 270.0  | 270.0  | 270.0  |
| Wheat flour <sup>1</sup>                                      | 269.9                       | 269.9  | 269.9  | 269.9  | 269.9  | 269.9  |
| Fish oil <sup>1</sup>   | 30.0                        | 30.0   | 30.0   | 30.0   | 30.0   | 30.0   |
| Cholesterol <sup>1</sup>                                      | 5.0                         | 5.0    | 5.0    | 5.0    | 5.0    | 5.0    |
| Soybean lecithin <sup>1</sup>                                 | 10.0                        | 10.0   | 10.0   | 10.0   | 10.0   | 10.0   |
| Cellulose <sup>1</sup>  | 24.0                        | 22.5   | 21.0   | 18.0   | 12.0   | 0.0    |
| Myo-inositol <sup>2</sup>                                     | 1.1                         | 1.1    | 1.1    | 1.1    | 1.1    | 1.1    |
| Vitamin premix <sup>3</sup>                                   | 5.0                         | 5.0    | 5.0    | 5.0    | 5.0    | 5.0    |
| Mineral premix <sup>4</sup>                                   | 10                          | 10     | 10     | 10     | 10     | 10     |
| Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> <sup>5</sup> | 15.0                        | 15.0   | 15.0   | 15.0   | 15.0   | 15.0   |
| Choline chloride <sup>5</sup>                                 | 0                           | 1.5    | 3.0    | 6.0    | 12.0   | 24.0   |
| Sum   | 1000.0                      | 1000.0 | 1000.0 | 1000.0 | 1000.0 | 1000.0 |
| <b>Analyzed proximate composition</b>                         |                             |        |        |        |        |        |
| Dry matter  | 938.0                       | 951.1  | 927.7  | 942.1  | 941.9  | 919.5  |
| Protein   | 409.9                       | 410.5  | 403.1  | 415.5  | 416.4  | 407.8  |
| Lipid   | 101.7                       | 109.4  | 109.0  | 100.3  | 105.5  | 100.4  |
| Ash   | 100.6                       | 101.8  | 101.8  | 102.2  | 100.3  | 102.2  |
| Analyzed choline  | 2.91                        | 3.85   | 4.67   | 6.55   | 10.70  | 18.90  |

<sup>1</sup> The ingredients were bought from Ningbo Tech-Bank Feed Co., Ltd., Ningbo, China.

<sup>2</sup> Myo-inositol was bought from Chengyi Aquatic Science and Technology Co., Ltd., Guangzhou, China.

<sup>3</sup> Per kilogram of vitamin premix (g/kg): nicotinamide (99%), 20; biotin (2%), 6.5; DL- $\alpha$ -tocopherol acetate (50%), 40; vitamin C phosphate (35%), 114; cyanocobalamin (1%), 4; calcium-D-pantothenate (99%), 21; thiamine nitrate (99%), 12; folic acid (97%), 1.1; riboflavin (80%), 40; menadione (43%), 7; retinyl acetate (500,000 IU/g), 4.5; cholecalciferol (500,000 IU/g), 0.03; pyridoxine hydrochloride (99%), 20.

<sup>4</sup> Per kilogram mineral mixture (g/kg): FeC<sub>6</sub>H<sub>5</sub>O<sub>7</sub>, 4.57; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 9.43; MnSO<sub>4</sub>·H<sub>2</sub>O (99%), 4.14; CuSO<sub>4</sub>·5H<sub>2</sub>O (99%), 6.61; MgSO<sub>4</sub>·7H<sub>2</sub>O (99%), 238.97; KH<sub>2</sub>PO<sub>4</sub>, 233.2; NaH<sub>2</sub>PO<sub>4</sub>, 137.03; C<sub>6</sub>H<sub>10</sub>CaO<sub>6</sub>·5H<sub>2</sub>O (98%), 34.09; CoCl<sub>2</sub>·6H<sub>2</sub>O (99%), 1.36.

<sup>5</sup> The ingredients were bought from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

National Analytical Center (Guangzhou, China) according to standard GB/T 14924.11-2001.

### 2.3. Shrimp culture and condition monitoring

Juvenile Pacific white shrimp were obtained from Hainan Lutai Marine Biological Technology Co., Ltd. (China) and fed a commercial diet (40% protein, 8% lipid; Yue-Hai Aquafeed Corp, Jiaxiang, China) for 7 d to acclimatize to the experimental conditions. After that, 960 juvenile *L. vannamei* (initial weight, 1.38 ± 0.01 g) were randomly assigned to twenty-four 250-L cylindrical fiber-glass tanks, with each diet randomly assigned to 4 replicate tanks. Shrimp were fed 3 times a day (06:00, 12:00, and 18:00) for 56 d at a daily rate of 7% to 10% of body weight that was adjusted fortnightly according to the weight of shrimp in each tank. Feces (by siphoning) and dead shrimp were removed, and approximately 60% to 70% of seawater in each tank was exchanged daily. During the feeding trial period, seawater temperature, salinity, pH, ammonia nitrogen, and dissolved oxygen were maintained at 26 to 31 °C, 20 to 22 g/L, 7.5 to 7.8, and 0 to 0.05 mg/L and not less than 6.0 mg/L, respectively.

### 2.4. Sample collection

At the end of the feeding trial, hemolymph samples (1 mL hemolymph per shrimp) from 9 shrimp per tank were collected immediately using the method described by Yuan et al. (2019). The hemolymph was kept at 4 °C for 24 h before serum samples were obtained by centrifugation (956 × g, 10 min, 4 °C) and stored

at –80 °C until analysis of antioxidant and non-specific immunity enzyme activity. Hepatopancreas samples from 9 shrimp per tank were collected into sterile microcentrifuge tubes, frozen immediately in liquid nitrogen, and then stored at –80 °C for analysis of gene expression and biochemical parameters. Muscle samples were dissected from the same 9 shrimp per tank and stored at –80 °C prior to analysis of muscle carnitine.

### 2.5. Proximate composition analysis

Diet and hepatopancreas proximate compositions were determined following the methods of the Association of Official Analytical Chemists (AOAC, 2006). Briefly, the moisture content of diets was determined by drying a given weight of samples to a constant weight at 105 °C. The crude protein content of diets was determined by the Dumas combustion method using a protein analyzer (FP-528, Leco, USA). Crude lipid contents of diets and total lipid contents in shrimp hepatopancreas were determined by the Soxhlet extraction method. Crude ash of diets was determined by combustion in a muffle furnace (550 °C, 8 h).

### 2.6. Biochemical parameter assays

Triglyceride (TG), cholesterol (CHO), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), non-esterified fatty acid (NEFA) and ACH contents, and lipoprotein lipase (Lpl) and hepatic lipase (HL) activities were measured by commercial assay kits (Nanjing Jiancheng Co, Nanjing, China). Choline, betaine, PC, carnitine and very low-density lipoprotein (VLDL) contents, fatty acid synthase (Fas), acetyl-CoA carboxylase (Acc), and carnitine palmitoyl-transferase (Cpt1) activities were estimated spectrophotometrically using commercial shrimp ELISA kits (Cusabio, Wuhan, China).

### 2.7. Hepatopancreas histological determinations

Hepatopancreas tissue was fixed in 4% paraformaldehyde solution for 72 h, dehydrated using a gradient of ethanol concentration, embedded in paraffin, sliced at 4 μm, dried in a constant temperature oven at 37 °C, and stained with hematoxylin and eosin (H&E). The hepatopancreas tissue sections were examined in an Olympus microscope (DP72) and analyzed using ImageJ to measure the diameter, area, and number of hepatopancreas R cells. For each of these indicators, 10 measurements were acquired ( $n = 40$ ) per tissue sample.

### 2.8. Fatty acid determinations

In brief, total lipids were first extracted from freeze-dried hepatopancreas samples with chloroform:methanol (2:1, vol:vol) solution, followed by preparation of fatty acid methyl esters (FAME) by the addition of methanolic sulfuric acid (Wang et al., 2021). Finally, FAME were separated and analyzed on a gas chromatograph mass spectrometer (Agilent 7890B-5977A, Agilent Technologies, USA), as described in detail by Wang et al. (2021).

### 2.9. Total RNA extraction, reverse transcription and real-time quantitative PCR

The detailed procedures used for total RNA extraction, reverse transcription, and real-time quantitative PCR were performed as described previously (Yuan et al., 2019). Briefly, total RNA was extracted and reverse transcribed by TRIzol Reagent and HiScript RT SuperMix Reagent kit (Vazyme, China) following the manufacturer's protocol. Real-time quantitative PCR was performed on a

quantitative thermal cycler (Lightcycler 96, Roche, Switzerland). Specific primers used in the experiment were designed using Primer Premier 5.0 and synthesized commercially (Tsingke Biotech Co., Ltd., Hangzhou, China) (Table S1). Standard curves were generated using 6 different dilutions of the cDNA samples, and amplification efficiency was determined using the equation  $E = 10^{(-1/\text{Slope})} - 1$ . Amplification efficiencies of all genes were approximately equal and ranged from 90% to 110%. As  $\beta$ -actin was stably expressed in all tissues of *L. vannamei*, it was used as a reference gene to normalize cDNA loading. The obtained data were normalized to  $\beta$ -actin and quantified by the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

### 2.10. Statistical analyses

Results are presented as means  $\pm$  standard error (mean  $\pm$  SEM). Before one-way analysis of variance (ANOVA), the acquired data was checked by Levene's test to ensure normality of distribution and homogeneity of variance. In addition, to determine if the effects were linear and/or quadratic, regression analysis using orthogonal polynomial contrasts was performed. Mean differences among treatments were compared using Tukey's range test with a significance level of  $P < 0.05$  (IBM SPSS Statistics 19).

## 3. Results

### 3.1. Effects of dietary choline level on growth performance and feed utilization

Dietary choline level significantly affected the growth performance of *L. vannamei* (Table S2). There were significant quadratic relationships between dietary choline level and final body weight (FBW), percent weight gain (PWG), specific growth rate (SGR) and feed efficiency (FE), where the highest values for FBW, PWG, SGR, and FE were observed in shrimp fed the diet containing 4.67 g/kg choline. There were no statistical differences in the survival of *L. vannamei* among all diets ( $P > 0.05$ ). Two slope broken-line regression analyses of PWG against dietary choline level indicated that the optimal dietary level of *L. vannamei* was calculated to be 4.17 g/kg choline (Fig. S1).

### 3.2. Effects of dietary choline level on contents of choline and choline metabolites

Dietary choline level significantly affected choline (Fig. 1A), betaine (Fig. 1B) and PC (Fig. 1C) contents in the hepatopancreas of *L. vannamei*. There was a significant linear and quadratic relationship between dietary choline level and choline content in the hepatopancreas (Table S3), where choline content increased continuously as dietary choline level increased from 2.91 to 6.55 g/kg and remained stable with further increased dietary choline level. Shrimp fed the diets containing 4.67, 6.55, 10.70 and 18.90 g/kg choline had significantly higher choline content than shrimp fed the diet containing the lowest (2.91 g/kg) choline level ( $P < 0.01$ ). There was a significant linear and quadratic relationship between dietary choline level and hepatopancreas betaine content ( $P < 0.01$ ), but no significant linear, or quadratic relationship between dietary choline level and PC ( $P > 0.05$ ) (Table S3). The contents of betaine and PC in the hepatopancreas increased initially and then decreased as dietary choline level increased. Highest betaine and PC contents in hepatopancreas were observed in shrimp fed diets with 10.70 and 4.67 g/kg choline, respectively, and were significantly higher than those fed the diet with 2.91 g/kg choline ( $P < 0.05$ ). Hepatopancreas carnitine content showed a similar pattern to choline and thus increased as dietary choline

increased from 2.91 to 6.55 g/kg and then remained stable with further increased dietary choline (Fig. 1E), whereas, in contrast, the content of carnitine in muscle continued to increase as dietary choline level increased ( $P < 0.05$ ) (Fig. 1F). Hemolymph ACH content (Fig. 1D) was not affected by dietary choline level ( $P > 0.05$ ).

### 3.3. Effects of dietary choline level on mRNA expression of choline transport and metabolism-related genes in hepatopancreas

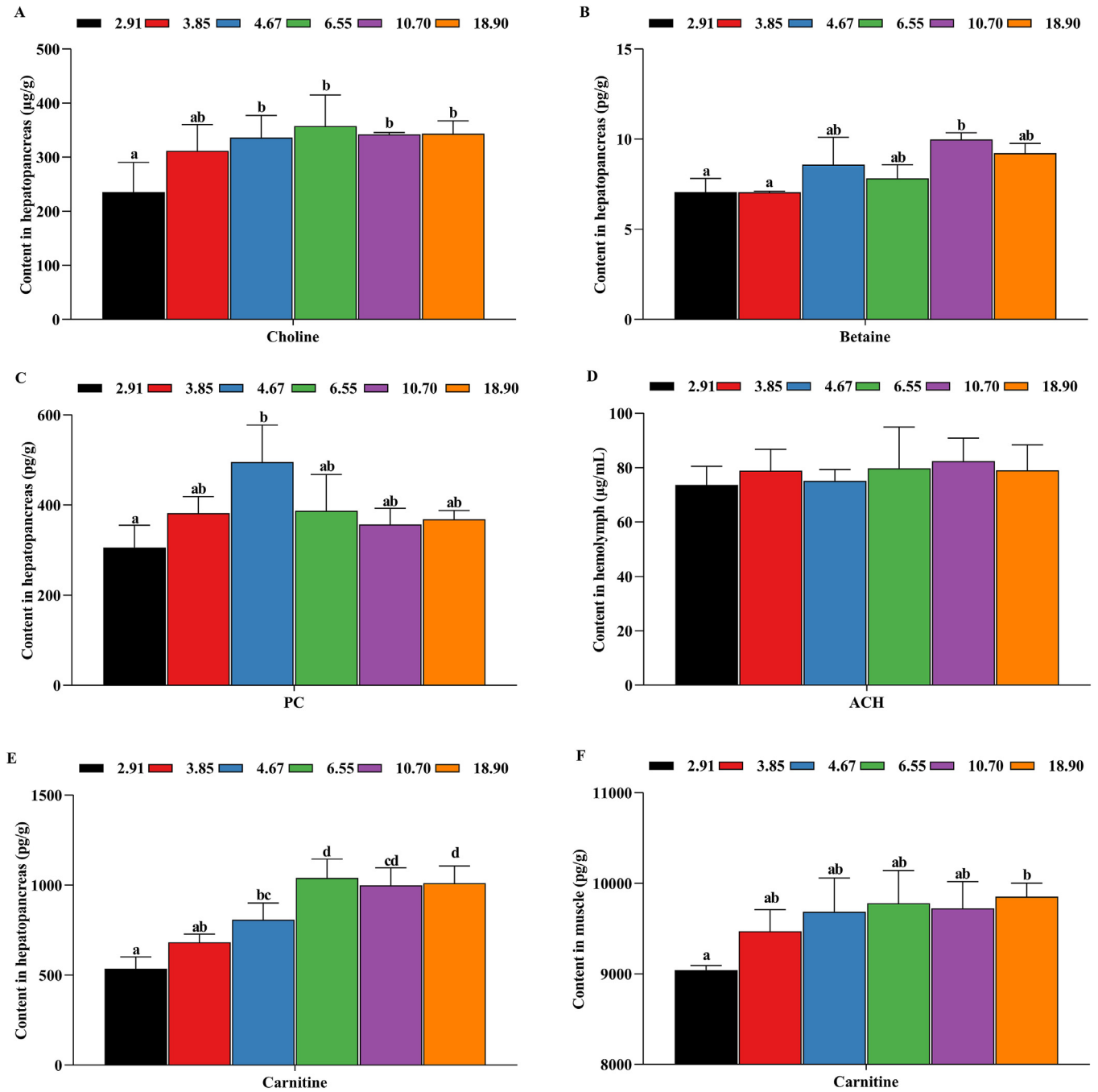
Hepatopancreas mRNA expression levels of genes related to choline transport and phosphorylation pathway increased linearly with increasing dietary choline level (Fig. 2; Table S3). Shrimp fed diets containing 10.7 and 18.9 g/kg choline had significantly higher mRNA expression levels of CTL-like protein 1 (*ctl1*) than shrimp fed diets containing fed 2.91 and 3.85 g/kg choline ( $P < 0.01$ ). The mRNA expression levels of CTL-like protein 2 (*ctl2*) in shrimp fed the 6.55, 10.70, and 18.90 g/kg choline diets were significantly higher than in shrimp fed diets containing the lower levels of choline (2.91, 3.85 and 4.67 g/kg choline) ( $P < 0.001$ ). Compared with the control group fed the diet containing the lowest level of choline (2.91 g/kg), the mRNA expression levels of organic cation transporter 1-like (*oct1*), *ck*, choline/ethanolamine phosphotransferase (*cept*) and CTP:phosphocholine cytidyltransferase (*cct*) were significantly up-regulated in shrimp fed the 4.67, 6.55, 10.70 and 18.90 g/kg choline diets ( $P < 0.01$ ). There was a significant quadratic relationship between dietary choline level and the expression levels of oxidative pathway-related genes. The highest expression level of *badh* was observed in shrimp fed the diet containing 4.67 g/kg choline, while the highest mRNA expression level of betaine-homocysteine S-methyltransferase (*bhmt*) was observed in shrimp fed the 6.55 g/kg choline diet, which was significantly higher than the levels in shrimp fed the control diet with the lowest choline ( $P < 0.05$ ).

### 3.4. Effects of dietary choline level on lipid class contents and physicochemical indices

The contents of lipid (Fig. 3A) and TG (Fig. 3B) in hepatopancreas decreased significantly and showed linear and quadratic relationships (Table S3). Shrimp fed the diet with the highest content of choline (18.9 g/kg) had the lowest lipid and TG contents in hepatopancreas, significantly lower than in shrimp fed the 2.91, 3.85, 4.67, and 6.55 g/kg choline diets ( $P < 0.001$ ). The VLDL contents of hepatopancreas and hemolymph increased significantly with increasing dietary choline level and showed a linear relationship (Fig. 3B and C; Table S3), while TG and NEFA contents in hemolymph decreased significantly with linear relationships (Fig. 3C). There were no significant differences in the contents of lipid in muscle, CHO in hepatopancreas and hemolymph, and HDL-C and LDL-C in hemolymph among the dietary treatments ( $P > 0.05$ ).

### 3.5. Hepatopancreas histological structure

Hepatopancreas tubule structures including embryonic cells (Ec), fibrous cells (Fc), and blasenzellen cells (Bc), hepatopancreas tubule lumen structure (L), and basal membrane (Bm) were observed and examined in shrimp fed the diets with different choline levels (Fig. 4). However, a notable reduction in the number of vacuoles (V) was found in shrimp fed the higher levels of choline supplementation compared to shrimp fed the control diet with lowest choline (2.91 g/kg) (Fig. 4A–F). The quantitative analysis of hepatopancreatic R cell characteristics showed that the number of R cells increased as the dietary choline level increasing from 2.91 to 4.67 g/kg, and remained stable with the further increased level of dietary choline (Fig. 4G). A positive linear trend was found between dietary



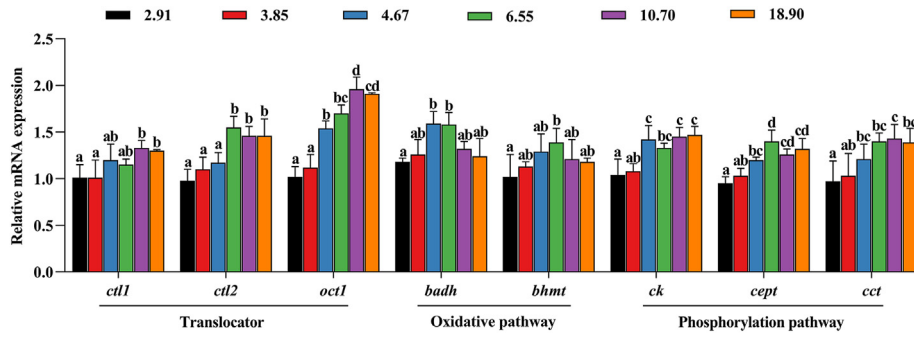
**Fig. 1.** Effects of dietary choline (g/kg diet) on contents of (A) choline, (B) betaine and (C) PC in hepatopancreas, (D) ACH in hemolymph, and carnitine in (E) hepatopancreas and (F) muscle of *Litopenaeus vannamei* fed the experimental diets. PC = phosphatidylcholine; ACH = acetyl choline. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>a-d</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).

choline level and the number of R cells. Shrimp fed the 4.67 g/kg choline diet had significantly higher R cell numbers than shrimp fed the 2.91 g/kg choline diet ( $P < 0.05$ ). The diameters and areas of R cells in the hepatopancreas were negatively correlated with dietary choline level. Shrimp fed the diet with 18.90 g/kg choline had R cells with lowest diameter and area ( $P < 0.01$ , Fig. 4H and I).

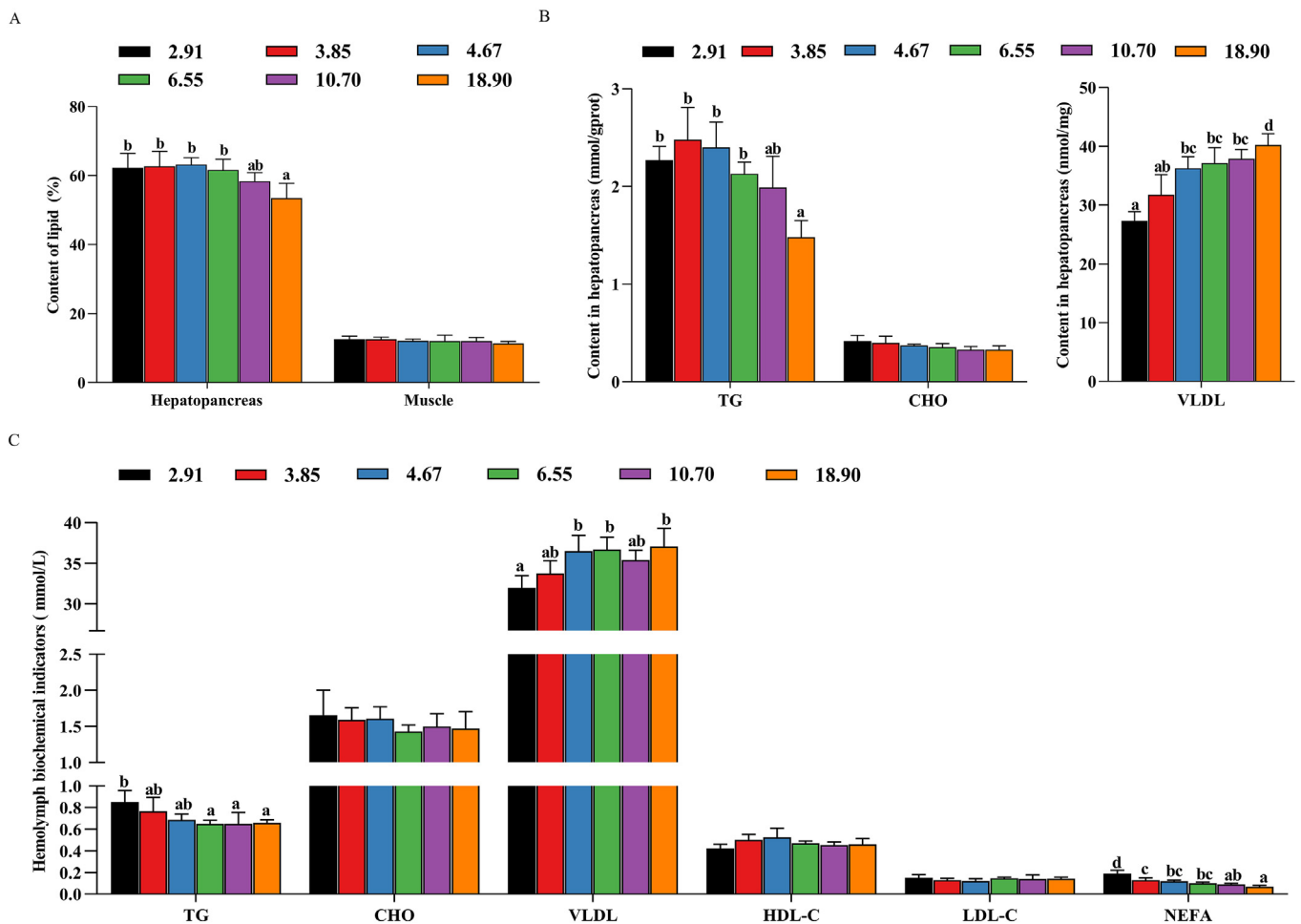
### 3.6. Effects of dietary choline level on fatty acid profile of hepatopancreas

The effects of dietary choline level on the profile of selected fatty acids of the hepatopancreas of *L. vannamei* are shown in Fig. 5, and

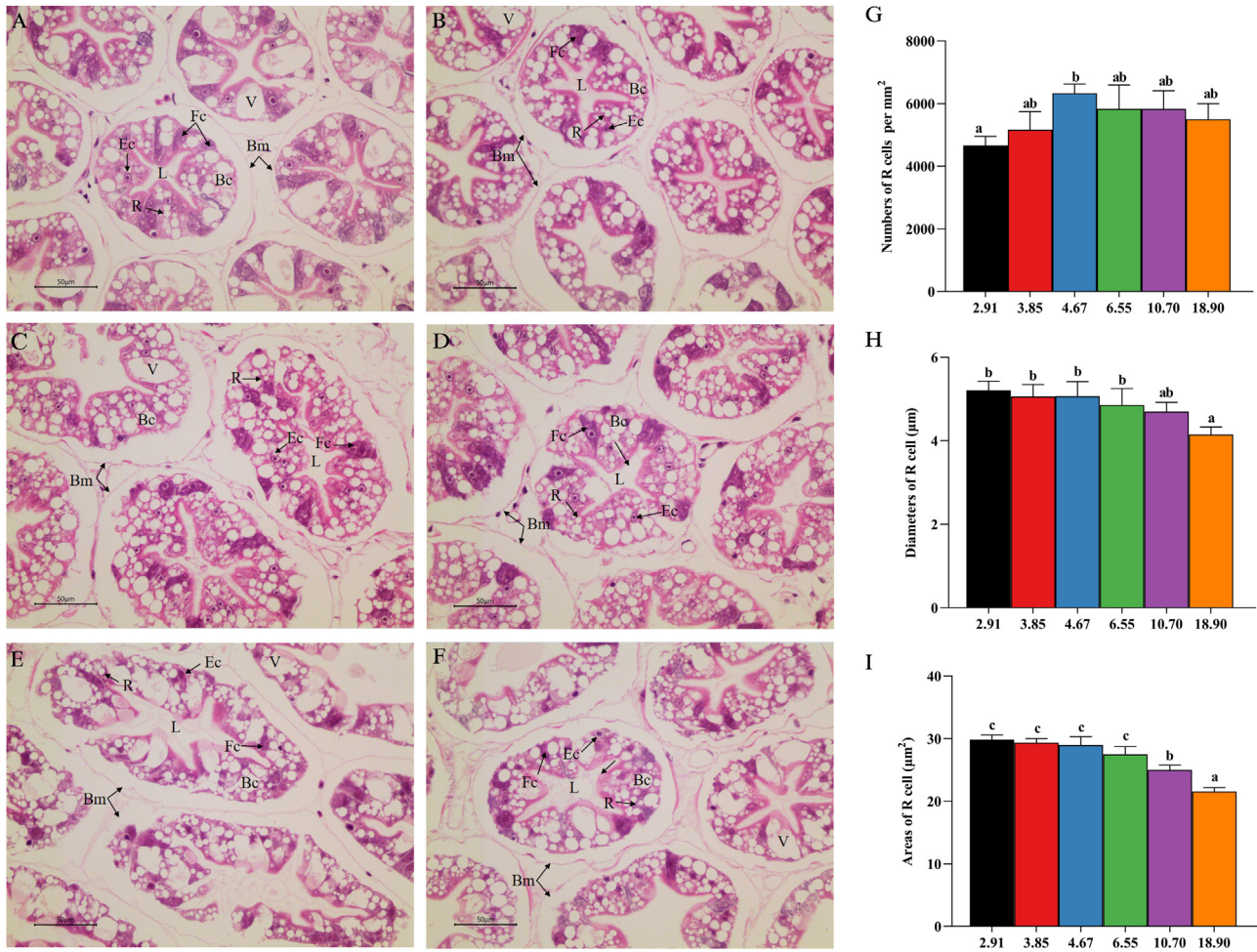
full fatty acid compositions are presented in Table S4. Contents of n-3 PUFA, n-6 PUFA, long-chain polyunsaturated fatty acids (LC-PUFA), EPA (20:5n-3), and DHA (22:6n-3) in hepatopancreas showed significant linear and quadratic relationships with dietary choline level. The contents of n-3 PUFA, n-6 PUFA, LC-PUFA, EPA, and DHA in hepatopancreas increased as dietary choline level increased from 2.91 to 4.67 g/kg and then decreased with further increased dietary choline (Fig. 5A and B). The content of SFA, monounsaturated fatty acids (MUFA), ARA (20:4n-6), and the n-3/n-6 PUFA and DHA/EPA ratios were not affected significantly by dietary choline level in *L. vannamei*. Hierarchical cluster analysis and heat maps showed that there was a significant difference in



**Fig. 2.** Effect of dietary choline (g/kg diet) on mRNA expression levels of genes related to choline transport and metabolism in the hepatopancreas of *Litopenaeus vannamei*. *ctl1* = CTL-like protein 1; *ctl2* = CTL-like protein 2; *oct1* = organic cation transporter 1-like; *badh* = betaine aldehyde dehydrogenase; *bhmt* = betaine-homocysteine S-methyltransferase; *ck* = choline kinase; *cept* = choline/ethanolamine phosphotransferase; *cct* = CTP:phosphocholine cytidyltransferase. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>a-d</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).



**Fig. 3.** Effects of dietary choline (g/kg diet) on (A) total lipid contents in hepatopancreas and muscle, (B) hepatopancreas lipid class contents, and (C) hemolymph biochemical parameters of *Litopenaeus vannamei*. TG = triglyceride; CHO = cholesterol; VLDL = very low-density lipoprotein; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; NEFA = non-esterified fatty acid. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>a-d</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).



**Fig. 4.** Effects of dietary choline at (A) 2.91, (B) 3.85, (C) 4.67, (D) 6.55, (E) 10.70 and (F) 18.90 g/kg diet, respectively, on hepatopancreas histological structure of *Litopenaeus vannamei* (400× magnification). Scale bar, 50 µm. Data on R cells are provided in panels (G) numbers, (H) diameters, and (I) areas of R cell ( $n = 40$ ). Bc = blasenzellen cell; Bm = basal membrane; Ec = embryonic cell; Fc = fibrous cell; L = lumen structure; R = restzellen cell; V = vacuole. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>a-c</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).

hepatopancreas fatty acid composition between shrimp fed the diet containing 4.67 g/kg choline and shrimp fed the 18.90 g/kg choline diet (Fig. 5C).

### 3.7. Effects of dietary choline level on hepatopancreas lipid metabolic enzyme activities

The activities of Fas, Acc, Cpt1, Lpl, and Hl in hepatopancreas were all affected significantly by dietary choline level (Fig. 6). Hepatopancreas Fas and Acc showed significant quadratic relationships, while Cpt1, Lpl, and Hl showed significant linear relationships with dietary choline level (Table S3). The activities of Fas and Acc increased as dietary choline level increased from 2.91 to 4.67 g/kg and decreased with further increased dietary choline level. The activities of Cpt1, Hl, and Lpl increased consistently as dietary choline level increased. Activities of Cpt1, Hl, and Lpl in shrimp fed the diets containing 10.70 and 18.90 g/kg choline were significantly higher than in shrimp fed the 2.91 g/kg choline diet ( $P < 0.01$ ).

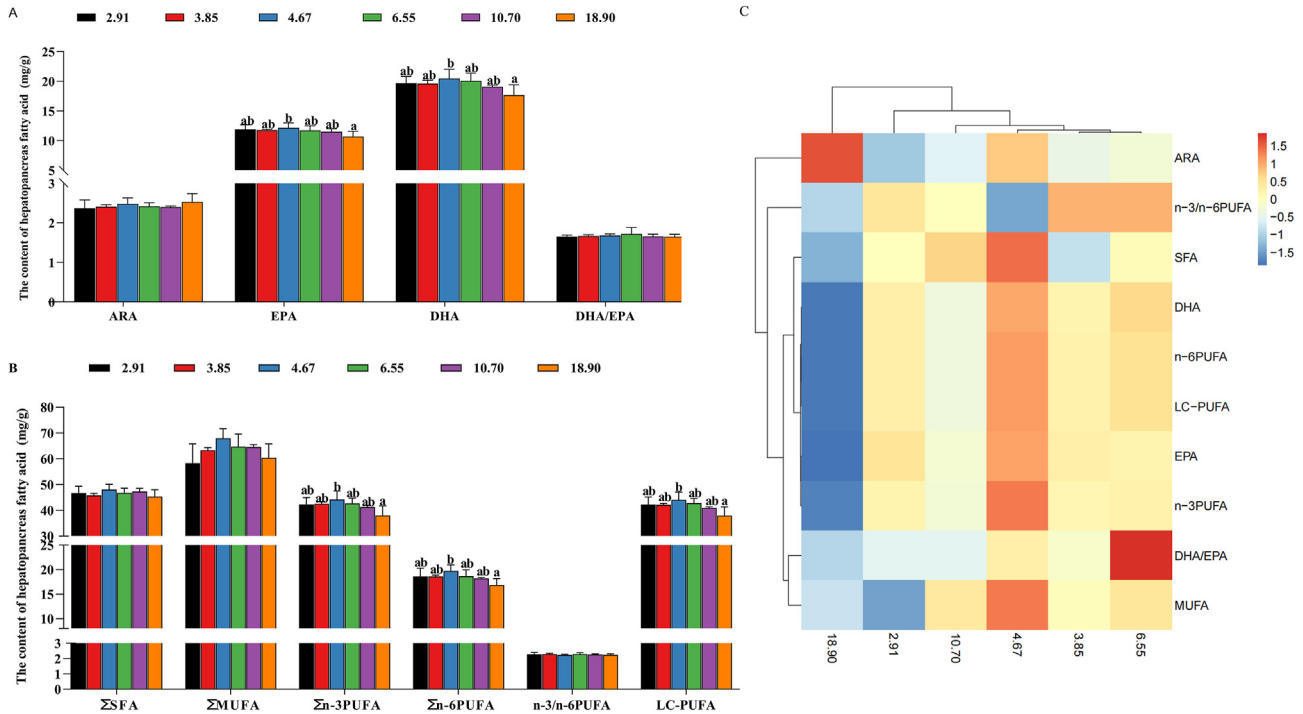
### 3.8. Hepatopancreas lipid metabolic related gene expression

The expression levels of genes related to lipid anabolism (Fig. 7A), lipid catabolism (Fig. 7B), transport of fatty acid and lipids

(Fig. 7C), and regulatory factors (Fig. 7D) were all significantly affected by dietary choline level. The mRNA expression levels of *fas*, *acc*, *cpt1*, low-density lipoprotein receptor (*ldlr*), sterol regulatory element-binding protein (*srebp*), fatty acid binding protein (*fabp*), fatty acid transport protein (*fatp*), 5'-AMP-activated protein kinase subunit alpha (*ampkα*), 5'-AMP-activated protein kinase subunit beta (*ampkβ*), and 5'-AMP-activated protein kinase subunit gamma (*ampkγ*) showed significant linear and quadratic relationships (Table S3). The mRNA expression levels of *srebp*, *fas* and *acc* were up-regulated as dietary choline levels increased from 2.91 to 4.67 g/kg and down-regulated with further increased dietary choline ( $P < 0.01$ ). The mRNA expression levels of *cpt1*, *fabp*, *fatp*, *ldlr*, *ampkα*, *ampkβ*, and *ampkγ* were significantly up-regulated as dietary choline level increased ( $P < 0.01$ ), with the highest expression levels of *cpt1*, *fabp*, and *fatp* observed in shrimp fed the diet containing 18.90 g/kg choline.

## 4. Discussion

The results of previous studies in this species showed that dietary choline level significantly affected the growth performance of *L. vannamei*, and shrimp fed 4.67 g/kg choline diet had significantly higher PWG, SGR and FE than shrimp fed the control diet with lowest choline (2.91 g/kg) and the diet with highest choline

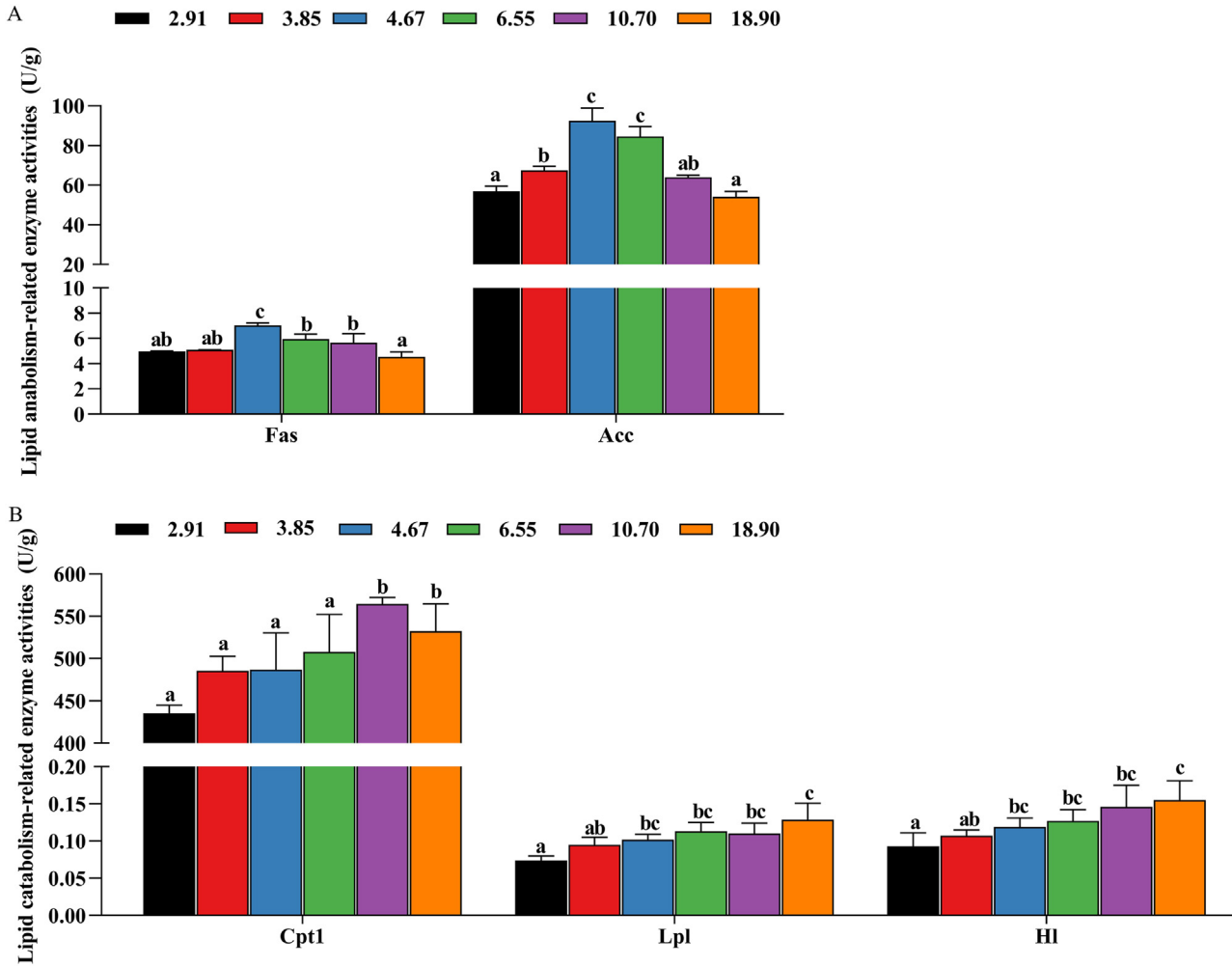


**Fig. 5.** Effects of dietary choline (g/kg diet) on fatty acid profile of hepatopancreas of *Litopenaeus vannamei*. (A) Fatty acid profile of hepatopancreas, (B) hierarchical cluster analysis (HCA), and (C) heat map visualization of hepatopancreas fatty acid composition. ARA = arachidonic acid; EPA = 20:5n-3; DHA = 22:6n-3; DHA/EPA = ratio of 22:6n-3 to 20:5n-3; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acids; n-3/n-6 PUFA = ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids; LC-PUFA = long-chain polyunsaturated fatty acids. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>ab</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).

(18.90 g/kg), which suggested that both deficiency and excess of dietary choline had negative effects on growth of *L. vannamei* (Lu et al., 2022). The impact of dietary choline level on growth is likely to be closely related to the metabolism of choline in *L. vannamei*. It is well known that after dietary choline is absorbed in the intestine, free choline is transported to cells and, depending upon the tissue or organ, produces corresponding metabolites, including PC, ACH and betaine, which have a range of functions (Hollenbeck, 2012). Given that the hepatopancreas is the first organ to receive nutrients after absorption and is a site of major metabolic activity in shrimp (Caceci et al., 1988), the contents of choline and its metabolites in the hepatopancreas of *L. vannamei* were determined. In the present study, the lowest level (2.91 g/kg) of dietary choline reduced the content of choline, betaine, and PC in the hepatopancreas, consistent with a recent study by Yuan et al. (2021), which demonstrated that dietary choline deficiency reduced the levels of choline, betaine and PC in the intestine and gill of grass carp (*Ctenopharyngodon idella*). It is worth noting that, although dietary choline supplementation can increase choline and choline metabolite contents in tissues, these were not always positively correlated with dietary choline levels. Hepatopancreas choline contents increased as dietary choline level increased from 2.91 to 4.67 g/kg and then plateaued as dietary choline level increased from 4.67 to 18.9 g/kg, suggesting that choline deposition in the hepatopancreas of *L. vannamei* reached saturation after meeting the maximum growth demand. Similarly, numerous studies have reported that choline concentration in the liver of some fish, such as juvenile cobia (*Rachycentron canadum*), parrot fish (*Oplegnathus fasciatus*) and blunt snout bream (*Megalobrama amblycephala*), increased linearly with increasing dietary choline level up to a plateau after which the amount of available choline exceeded the level required to satisfy growth potential (Jiang et al.,

2013; Khosravi et al., 2015; Mai et al., 2009). In addition, it was found that hepatopancreas PC and betaine contents increased initially and then decreased as dietary choline level increased, with the break points being 4.67 and 10.70 g/kg choline, respectively. These results suggested that the dietary choline level that maintains optimal levels of choline metabolites in the hepatopancreas varies depending upon the metabolite and its biological functions. Interestingly, serum ACH content was not influenced by dietary choline level, which may be related to the hemolymph homeostatic balance of *L. vannamei*. In contrast, carnitine, a vital part of the mitochondrial membrane fatty acid transport system, was affected by dietary choline level. Research has shown that a single intraperitoneal injection of choline chloride (100 pmol) into rats fed a choline-deficient diet restored near-normal concentrations of hepatic carnitine within 90 min, while intraperitoneal injections of betaine were ineffective within 90 min, suggesting that this rapid recovery effect was unique to choline and was essentially a redistribution of pre-existing carnitine independent of direct biosynthesis (Carter and Frenkel, 1978). Daily et al. (1998) found that supplementing choline (3 g/kg) over and above normal dietary levels (1.85 g/kg) enhanced carnitine accretion in guinea pig tissues, which suggested that the changes in hepatopancreas carnitine concentration in the present study could result from either increased de novo synthesis of carnitine in hepatopancreas or altered transport into and/or out of the tissue. In the present study, hepatopancreas carnitine content increased as dietary choline level increased up to 6.55 g/kg and then remained stable with further increased dietary choline, while the content of carnitine in muscle continued to increase as dietary choline level increased further. Thus, the data support the hypothesis that dietary choline alters carnitine metabolism and/or tissue distribution in shrimp. In general, carnitine is synthesized predominantly by the liver, which is





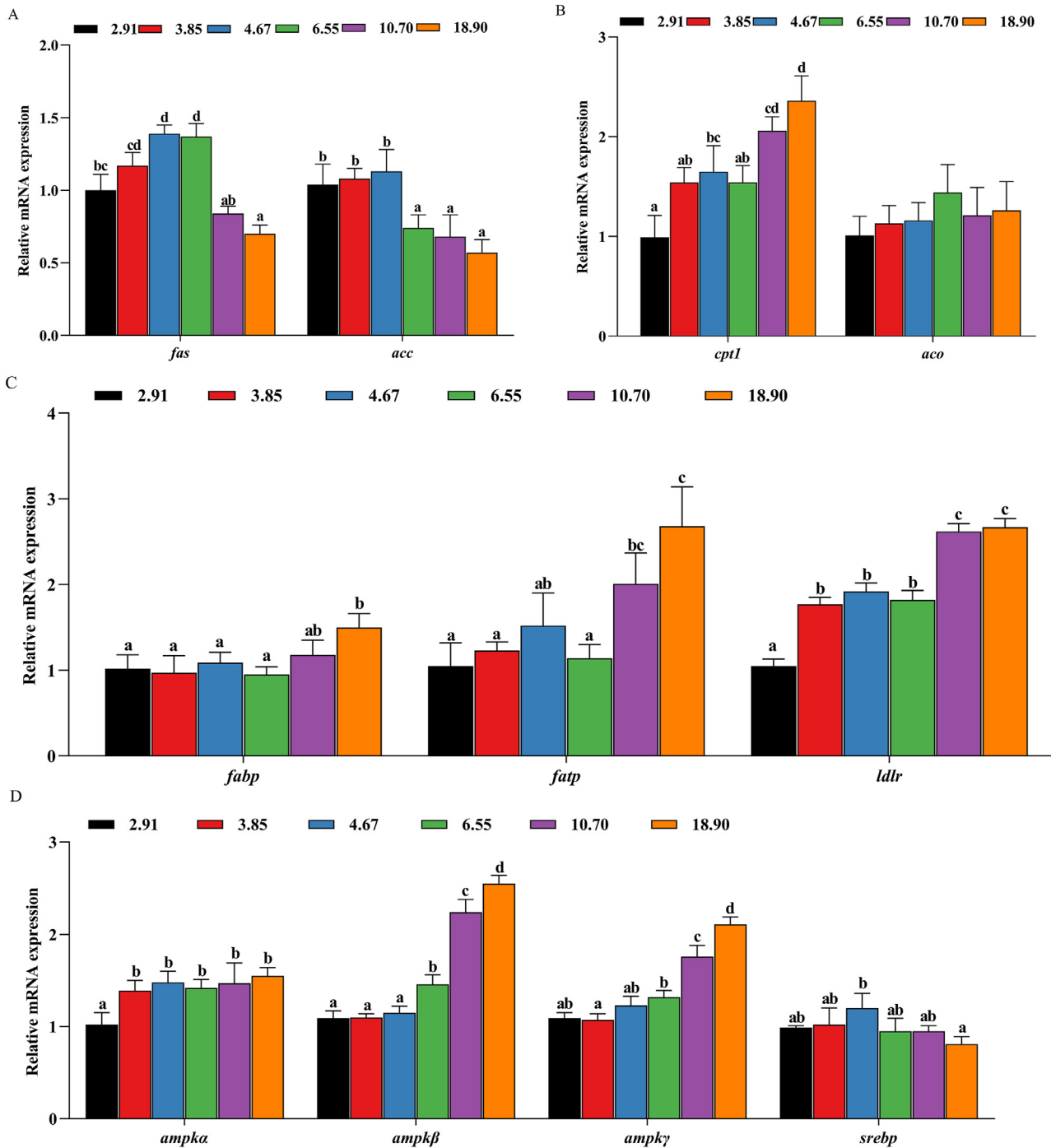
**Fig. 6.** Effects of dietary choline (g/kg diet) on hepatopancreas activities of lipid metabolic related enzymes of *Litopenaeus vannamei*. Fas = fatty acid synthase; Acc = acetyl-CoA carboxylase; Cpt1 = carnitine palmitoyl-transferase; Lpl = lipoprotein lipase; HI = hepatic lipase. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>a-c</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).

the primary supplier of carnitine for plasma and tissues (Carter and Frenkel, 1978). Therefore, when dietary choline level exceeded 6.55 g/kg, the hepatopancreas carnitine content remained stable, while the muscle carnitine content continued to increase, suggesting that muscle tissue may act as a reservoir of carnitine. However, further studies are necessary to study the mechanism of dietary choline's effect on carnitine metabolism and distribution in *L. vannamei*.

Choline is a hydrophilic cation at physiological pH and cannot diffuse across cellular membranes to any great extent so as a result, membrane transport plays an essential role in choline metabolism in cells (Lockman and Allen, 2002). Biosynthesis of PC is one of the important pathways of choline metabolism, which occurs on the cytoplasmic side of the endoplasmic reticulum membrane (ER) through a cascade of three enzymatic steps: choline phosphorylation by CK, conversion of PCho to CDP-choline by CCT and, finally, addition of CDP-choline to DAG to form PC catalyzed by CEPT (Vance and Vance, 2004). The CCT enzyme exists in the nucleus as an inactive soluble form, but can move to the cytoplasm and become membrane-bound and activated to catalyze the synthesis of CDP-choline from PCho and cytidine triphosphate, the rate-limiting step in normal cells (Borkenhagen and Kennedy, 1957; Cornell and Northwood, 2000). However, CK is mainly located in the cell cytoplasm of tissues and catalyzes the production of PCho

by phosphorylation of free choline (Wittenberg and Kornberg, 1953) and can, under some circumstances, be rate-limiting and have a regulatory role in the biosynthesis of PC (Aoyama et al., 2004; Hosaka et al., 1992). Therefore, CK and choline transporters can, in some cases, play important regulatory roles (Kent, 1997). In the present study, changes in the expression levels of choline transport-related genes (*ctl1*, *ctl2*, and *oct1*) and PC synthesis-related genes (*ck*, *cct*, and *cept*) in response to dietary choline were similar to the trend of choline deposition in the hepatopancreas. Therefore, it is reasonable to assume that dietary choline supplementation promoted choline deposition and PC biosynthesis in the hepatopancreas by increasing the expression levels of choline transport-related genes and PC synthesis-related genes. Although the individual contribution of *ctl1*, *ctl2* and *oct1* to choline supply into cells, it is likely that, between them, *ctl1*, *ctl2* and *oct1* play important roles in maintaining homeostasis PC biosynthesis.

Choline is also essential for the synthesis of betaine, which is produced by the oxidation of choline in a two-step reaction in which choline is first converted to betaine aldehyde by mitochondrial choline oxidase, and then betaine aldehyde is further oxidized to betaine by BADH in the cytoplasm or mitochondria (Ueland et al., 2005). Betaine participates in the methionine cycle as a methyl donor in the conversion of homocysteine to methionine via the action of BHMT (Nocianitri et al., 2002). In the present study, the



**Fig. 7.** Effects of dietary choline (g/kg diet) on mRNA expression levels of hepatopancreas lipid metabolic related genes of *Litopenaeus vannamei*, related to (A) lipid anabolism, (B) lipid catabolism, (C) transport of fatty acid and lipids, and (D) regulatory factors. *fas* = fatty acid synthase; *acc* = acetyl-CoA carboxylase; *cpt1* = carnitine palmitoyl-transferase; *aco* = acyl-CoA oxidase; *fabp* = fatty acid binding protein; *fatp* = fatty acid transport protein; *ldlr* = low-density lipoprotein receptor; *ampkα* = 5'-AMP-activated protein kinase subunit alpha; *ampkβ* = 5'-AMP-activated protein kinase subunit beta; *ampkγ* = 5'-AMP-activated protein kinase subunit gamma; *srebp* = sterol regulatory element-binding protein. Mean values are based on 4 replicates, and standard errors are represented by vertical bars. <sup>a-d</sup> Bars with different superscript letters differ significantly ( $P < 0.05$ ).

expression levels of oxidative pathway-related genes *badh* and *bhmt* were up-regulated initially and then down-regulated as dietary choline level increased, with the break points being 4.67 and 6.55 g/kg choline, respectively. It is unclear why high levels of dietary choline lead to reduced expression of genes associated with oxidative pathways. However, homeostasis of choline in the hepatopancreas is achieved by balancing choline, PC, betaine anabolism, and catabolism and so it could be speculated that high

(excessive) dietary choline may shift the balance toward phospholipid synthesis relative to the oxidation pathway.

In some studies in fish, dietary choline prevented and reduced hepatic lipid deposition (Jin et al., 2019; Liu et al., 2021; Luo et al., 2016). However, there are few reports on the role of choline in regulating lipid metabolism in crustaceans although, in the present study, high dietary choline (18.90 g/kg) decreased the contents of lipid and TG in hepatopancreas. Similar results were reported in the

Chinese mitten crab (*Eriocheir Sinensis*), where whole-body lipid content and hepatopancreas TG and total CHO contents decreased as dietary choline increased (Qi, 2013). It is well known that PC synthesis is important for the production of VLDL particles responsible for transporting TG and CHO from the liver to peripheral organs (Smallwood et al., 2016). In the present study, the contents of VLDL in hepatopancreas and hemolymph were positively correlated with dietary choline level. Thus, the decrease of lipid and TG in shrimp fed high choline may be related to the fact that PC promoted VLDL synthesis in the hepatopancreas. Moreover, the decreased contents of TG and NEFA in hemolymph were also consistent with the view that high dietary choline prevents excess lipid deposition. While CHO is also transported by LDL and HDL in the hemolymph in crustaceans (Tian et al., 2020), dietary choline had no major impact on CHO, LDL-C, and HDL-C contents in hemolymph in the present study.

Histology of the hepatopancreas is a key means of assessing the metabolic condition of crustaceans (Rosas et al., 1995). As in other decapod crustaceans, the hepatopancreatic R cell of *L. vannamei* is a primary location for lipid deposition and storage (mainly in the form of lipid droplets), and so R cells are an important tool in understanding metabolic conditions (Caceci et al., 1988). In the present study, the number of hepatopancreas R cells was positively correlated with hepatopancreas PC content, and increased dietary choline did not reduce the number of R cells in hepatopancreas, with fewer R cells observed in the control group fed the lowest level of choline. However, the size of the R cells was reduced by increased dietary choline as evidenced by lower diameters and areas, and the number of vacuoles in the hepatopancreas decreased. Therefore, while choline initially promoted the development of hepatopancreas in *L. vannamei*, the reduction of hepatopancreas lipid level in shrimp fed the high choline diets can be attributed to the size of lipid droplets in hepatopancreas R cells.

Hepatopancreas is the main organ involved in lipid metabolism in crustaceans, and appropriate lipid content is essential for the hepatopancreas to maintain the membrane structure and function of other tissues. As important components of membrane lipids, fatty acids play particularly vital roles in physiological and metabolic regulation of membrane lipid homeostasis (Kültz, 2012). Moreover, PC is a key component of VLDL, which plays a crucial part in the transport of fatty acids from liver tissue to other tissues through circulation (Vance and Vance, 1985; Yao and Vance, 1988). In addition, specific molecular species of PC may serve as endogenous ligands for and activate peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) to suppress de novo fatty acid synthesis and promote fatty acid catabolism (Chakravarthy et al., 2009). In the present study, while the PC content of hepatopancreas was positively correlated with dietary choline, the hepatopancreas contents of n-3 and n-6 PUFA, total LC-PUFA, EPA and DHA initially increased as dietary choline level increased from 2.91 to 4.67 g/kg, then decreased with further increased dietary choline with the minimum level found in *L. vannamei* fed the highest level of choline. These results indicated that high dietary choline levels may enhance the transport of lipids in the form of phospholipids from the hepatopancreas, improve the utilization of fatty acids, and prevent abnormal lipid metabolism in the hepatopancreas. While the distribution of fatty acids in animal tissues generally reflects the composition of fatty acids in the diet (Xu et al., 2020), it is important to highlight that the lipid content and composition of the diets were not changed in the present study, and the only difference between diets was choline content. Therefore, while the increased content of PUFA in hepatopancreas when dietary choline level increased from 2.91 to 4.67 g/kg may partly reflect the increased TG content, it is reasonable to suggest that the alterations in hepatopancreas PUFA content in shrimp fed the different diets may also be

the result of choline regulation of lipid and fatty acid metabolism. For instance, a recent study by Liu et al. (2021) established that moderate dietary choline levels promoted neutral lipid (TG and NEFA) deposition in the liver of yellowtail kingfish (*Seriola lalandi*). In addition, lipid metabolism in the hepatopancreas includes the de novo synthesis of SFA and MUFA and oxidation of all fatty acids, which can alter the contents and relative proportions of fatty acids. This is particularly important in the case of LC-PUFA, especially EPA, DHA, and ARA which are essential nutrients with crucial functions impacting the physiological processes of aquatic animals (Lu et al., 2020; Luo et al., 2021). In this study, the highest levels of DHA, EPA, and LC-PUFA were found in shrimp fed the diet supplying 4.67 g/kg choline, which may reflect an appropriate dietary level of choline.

To further investigate the regulatory effect of dietary choline on hepatopancreas lipid metabolism, the activities of some enzymes and the expression of some key genes related to lipid metabolism were determined. Studies have shown that Fas and Acc are vital regulatory enzymes in the regulation of fatty acid biosynthesis, playing key roles in lipid homeostasis in fish and aquatic animals (Xu et al., 2020). In the present study, as dietary choline increased, the changes in Fas and Acc activities were generally correlated with changes in fatty acid contents, which suggested that choline may promote fatty acid synthesis by up-regulating Fas and Acc activities. In addition to anabolic enzymes, catabolic enzymes may also be affected by dietary choline. Mitochondrial CPT1 is an enzyme responsible for forming acylcarnitines by catalyzing the transfer of the acyl group of a long-chain fatty acyl-CoA from CoA to L-carnitine, which plays a key role in the transport of fatty acids through the inner membrane of mitochondria (Mc-Garry and Brown, 1997). The lipases LPL and HL hydrolyze TG in lipoproteins and provide free fatty acids (FFA) to tissues (Koerner et al., 2019). In the present study, Cpt1, Lpl, and Hl activities increased continually as dietary choline level increased, which might explain the reduced total lipid and TG in the hepatopancreas of shrimp fed a high-choline diet. Therefore, the reduced hepatopancreas lipid content in *L. vannamei* fed the high-choline diet may largely reflect the increased activities of lipolysis-related enzymes and enhanced fatty acid  $\beta$ -oxidation. In general, in addition to the activities of lipid metabolism enzymes, transcriptional levels of genes are also used as indicators to monitor lipid metabolism of animals (Li et al., 2021). Therefore, the mRNA expression of genes related to lipid anabolism (*fas*, *acc*), lipid catabolism (*cpt1*, *aco*), fatty acid and lipid transport (*fabp*, *fatp*, *ldlr*), and regulatory factors (*ampk*, *srebp*) were measured in this study. The mRNA expression levels of *fas*, *acc* and *cpt1* generally showed similar patterns to the corresponding enzyme activities. The mRNA expression levels of *fabp*, *fatp*, and *ldlr* increased continually as dietary choline increased, with highest expression levels observed in shrimp fed the diet with highest choline, 18.90 g/kg. These data suggest that high dietary choline promoted fatty acid and lipid transport as FABP and FATP are key enzymes in the transport of long-chain fatty acids (LCFA) (Dreyer et al., 1993), while LDLR is a cell-surface receptor that binds to apolipoproteins to mediate the endocytosis of CHO-rich LDL particles and clear them from circulation (Brown and Goldstein, 1976). It is well known that the lipoprotein VLDL is synthesized by the liver and transports lipids in the blood circulation, where lipases (HL and LPL) aid in the hydrolysis and uptake of their component lipids into tissues while remnant lipoproteins (LDL-C) are taken up by LDLR (Koerner et al., 2019). It is worth noting that the contents of VLDL in hepatopancreas and hemolymph in the present study were positively correlated with dietary choline level, while the LDL-C content in hemolymph did not change significantly. Therefore, it is reasonable to speculate that the stable level of LDL-C in the hemolymph may be related to the high expression of *ldlr*, that is, LDLR is responsible for removing LDLC obtained by VLDL hydrolysis in the hemolymph and

maintaining the homeostatic balance of the hemolymph. Thus, high dietary choline might regulate hepatopancreas lipid metabolism by down-regulating mRNA expression of genes related to lipid anabolism and up-regulating mRNA expression of genes related to fatty acid  $\beta$ -oxidation and lipid transport. In addition, *ampk*, as a “sensor” and “regulator” of energy in hepatocytes, is known to regulate lipid metabolism by influencing the transcription levels of lipid metabolism-related transcription factors, such as *srebp* and *cpt1* (Hardie et al., 1998) while, in turn, *srebp* controls the expression of key enzymes in de novo fatty acid production, including *acc* and *fas* (Shi et al., 2021). Therefore, an important finding in the present study was that the expression levels of *ampk $\alpha$* , *ampk $\beta$* , and *ampk $\gamma$*  increased continually with the choline level increased, whereas the mRNA expression levels of *srebp* showed a similar pattern to the mRNA expression levels of *fas* and *acc*. This suggested that high dietary choline may activate the Ampk/Srebp signaling pathway, which may therefore be an important mechanism in the action of choline on lipid metabolism.

## 5. Conclusion

In conclusion, dietary choline promoted the deposition of choline and its metabolites in the hepatopancreas in *L. vannamei* and up-regulated the mRNA expression levels of genes related to choline transport and metabolism. An appropriate dietary choline level had positive effects on both lipid synthesis and catabolism and maintained lipid accumulation at the level required for normal shrimp development by promoting the development of hepatopancreatic R cells. Moreover, high dietary choline was more conducive to lipid mobilization, reducing and preventing the deposition of excess lipid in the hepatopancreas. High dietary choline not only promoted hepatopancreas lipid export by enhancing VLDL synthesis via the phosphorylation pathway, but also activated the Ampk/Srebp signaling pathway promoting fatty acid  $\beta$ -oxidation and inhibiting de novo fatty acid synthesis. These findings provided further insight and understanding of the regulation of lipid metabolism by dietary choline in *L. vannamei*.

## Author contributions

**Jingjing Lu:** Formal analysis, Investigation, Writing-original draft, Writing-review and editing. **Qicun Zhou:** Conceptualization, Methodology, Supervision, Writing-review & editing, Project administration, Funding acquisition. **Xinyue Tao, Jiaxiang Luo, Tingting Zhu:** Formal analysis. **Lefei Jiao, Peng Sun:** Resources. **Douglas R. Tocher:** Writing-review & editing. **Min Jin:** Conceptualization, Methodology, Validation and Supervision, Project administration.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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## Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2023.05.014>.

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