Transcriptomic and physiological analyses of hepatopancreas reveal the key metabolic changes in response to dietary copper level in Pacific white shrimp *Litopenaeus vannamei*

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Abstract

All living organisms require copper for growth and development, but the gene expression profiles and molecular mechanisms underpinning dietary copper are poorly investigated. Therefore, the present study aimed to determine the potential metabolic changes in response to dietary copper based on analysis of hepatopancreas transcriptome in *Litopenaeus vannamei*. Three practical diets were formulated to supplement 0 (control diet; C-Cu) and 40 mg kg^{-1} inorganic Cu ($CuSO_4 \cdot 5H_2O$; I-Cu) and copper amino acid chelate (O-Cu), with analyzed Cu being 12.4, 49.8 and 50.0 mg kg^{-1}, respectively. Shrimp fed I-Cu and O-Cu diets had higher percent weight gain and Cu concentration in tissues. Some essential amino acids (lysine, methionine, isoleucine, leucine, valine) and non-essential amino acids (tyrosine, glycine, aspartic acid, proline and serine) in hepatopancreas significantly increased in shrimp fed the copper supplemented diets. Transcriptome analysis indicated a total of 742 and 912 genes were differentially expressed ($q < 0.001$; $\log_2$fold change ≥ 2) in shrimp fed the I-Cu and O-Cu diets, respectively, in comparison to shrimp fed the control diet. Five and eight significantly changed pathways were annotated in the C-Cu vs. I-Cu and C-Cu vs. O-Cu comparisons, with metabolism the leading category for both. Similarly, the proportion of differentially expressed genes revealed that most were enriched in the category of metabolism. Further analysis revealed that dietary copper mainly affected amino acid and glycerophospholipid metabolism. Moreover, two significantly changed pathways (phagosome and IL-17 signaling pathway) related to the immune system were identified in shrimp fed the O-Cu diet. The present study analyzing the hepatopancreas transcriptome identified potential roles of dietary copper on amino acid and glycerophospholipid metabolism and provided new insight that will be valuable in future studies to further elucidate the nutritional molecular basis of copper.

Keywords: Copper, *Litopenaeus vannamei*, Metabolism, Transcriptome, Immune system

1. Introduction

Copper has been recognized as an essential nutrient for animals as a wide variety of biological processes depend on an adequate supply of copper. Virtually, all organisms require copper as a catalytic cofactor in enzymes such as cytochrome *c* oxidase, necessary for respiration; dopamine β-hydroxylase, involved in the production of catecholamine and therefore nerve and metabolic function; superoxide dismutase, promoting the dismutation of potentially damaging oxygen radicals produced in normal metabolic reactions; tyrosinase, required for pigmentation; ceruloplasmin, a potential extracellular free-radical scavenger (Evans, 1973; Puig and Thiele, 2002). In addition, a small fraction of copper is bound to amino acids, confirmed by Neumann and SassKortsak (1967) and Lau and Sarkar (1971), who reported that the affinity between amino acids and copper had important significance in biological copper transport. The homeostasis of copper in the body is strictly controlled due to the potential toxicity of copper to living systems (Kamunde et al., 2002). While numerous studies have investigated the toxicological effect of waterborne copper exposure in animals (Meng et al., 2014; Chen et al., 2016; Wang et al., 2017; Sonnack et al., 2018), there has been little emphasis on the potential effect of dietary copper in shrimp. However, compared with waterborne copper, dietary sources have more significant physiological effects on growth, immunity, reproduction and health of animals (Kamunde et al., 2002).

Organic trace minerals have been viewed as a positive alternative to inorganic minerals due to higher bioavailability and immunity enhancement. Previous studies have demonstrated metal amino acid complexes enhanced disease resistance mainly based on the improvement of immune enzyme activity in channel catfish *Ictalurus punctatus* (Paripatananont and Lovell, 1995), rainbow trout *Onocrhynchus mykiss* (Apines et al., 2003; Apines-Amar et al., 2004), red sea bream *Sparus aurata* (Sarker et al., 2005) and Pacific white shrimp *Litopenaeus vannamei* (Yuan et al., 2020a; Yuan et al., 2018). However, the

specific mechanisms and potential pathways of how organic trace elements enhance immunity have not been clearly identified.

Pacific white shrimp *L. vannamei* are a commercially important farmed marine species due to rapid growth, disease tolerance and adaptability to high density culture (NRC, 2011). The nutrition and feeding of *L. vanname* under semi-intensive or intensive conditions has received a great deal of attention (Pedrazzoli et al., 1998). However, compared with macronutrients such as protein, lipid and carbohydrate, less attention has been paid to micronutrient nutrition, especially trace mineral elements. In comparison to vertebrates, copper has more important physiological functions for crustaceans as it is also involved in the formation of the respiratory pigment hemocyanin and maintains the mineralization of carapace during the molting process (Rao and Anjaneylu, 2008). Therefore, it is important to clarify the nutritional, biological and molecular impacts of dietary copper in shrimp.

With the rise of high-throughput technologies, bioinformatics and associated improvements in computational power, characterizing and analyzing massive amounts of data has become increasingly efficient and easy (Jiménez-Chillarón et al., 2014). Modern transcriptome analysis uses next-generation sequencing to capture and annotate gene sequences as a means of understanding the molecular mechanism of specific physiological processes, giving information on how genes are regulated, and revealing details of an organism's biology (Kanehisa et al., 2007). So far, there are no studies specifically investigating the regulatory mechanisms of dietary copper on nutritional metabolism in aquatic animals. Therefore, screening and identifying differentially expressed genes (DEGs) in the transcriptome, and validating the changed pathways will be helpful to uncover the metabolic regulation and physiological process of dietary copper in *L. vannamei*, and improve our knowledge and understanding of the molecular mechanisms of dietary copper in organisms.

2. Materials and methods

2.1 Experimental diets

Three isonitrogenous (~ 42.5 % crude protein) and isolipidic (~ 8.5 % crude lipid) experimental diets were formulated to contain the same dose of two different forms of copper (inorganic, diet I-Cu; organic, diet O-Cu) in comparison to a control diet with no supplemental copper (diet C-Cu). Thus, 40 mg kg^{-1} $CuSO_4 \cdot 5H_2O$ (Cu content = 25.6 %; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and copper amino acid chelate (Cu content = 10.9 %; Zinpro Corp., USA) were added to the control (unsupplemented) diet, with the analyzed values of Cu being 12.4, 49.8 and 50.0 mg kg^{-1} in C-Cu, I-Cu and O-Cu diets, respectively. The formulations and proximate compositions of the experimental diets are shown in Table S1. The diets were prepared following the protocol described in detail previously (Shi et al., 2020). The experimental diets were sealed in vacuum-packed bags and stored at -20 °C until used in the feeding trial.

2.2 Shrimp rearing and experimental conditions

L. vannamei juveniles (initial weight 0.90 ± 0.00 g) were obtained from a local commercial hatchery (Chia-Tai Ningbo Company, Ningbo, China) and, prior to the start of the feeding trial, were reared in cement pools and fed a commercial diet (40 % protein, 8 % lipid; Yue-Hai Aquafeed Corp., Jiaxing, China) for two weeks to acclimate to the experimental conditions. The feeding trial was conducted at the breeding base of Ningbo Ocean and Fishery Science and Technology Innovation Center (Zhejiang, China). A total of 600 juveniles were randomly allocated to 15 tanks (300-L cylindrical fiber-glass tanks filled with 250-L of seawater) at a stocking density of 40 shrimp per tank, and each experimental diet randomly assigned to five replicates. Shrimp were hand-fed daily at 8:00, 12:00 and 17:00, with a daily

105 ration of 6-8 % of biomass with the morning and evening rations providing 70 % of the total given.
106 Shrimp in each tank were weighed every two weeks with daily ration adjusted accordingly. Dead shrimp
107 were immediately removed, weighed and recorded. Over 70 % of the tank seawater was exchanged daily
108 by siphoning out the waste material and exuviae prior to the morning feed. During the 8-week feeding
109 trial, seawater conditions including temperature (28-32 °C), salinity (25-28 g L^{-1}), pH (7.6-7.8), dissolved
110 oxygen level (not less than 6.0 mg L^{-1}) and ammonia nitrogen (lower than 0.05 mg L^{-1}) were measured
111 by YSI Proplus (YSI, Yellow Springs, Ohio, USA).

112 *2.3 Sample collection*

113 At the termination of the experiment, shrimp were fasted for 24 h and anesthetized with 10 mg L^{-1}
114 eugenol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) before sampling. All shrimp in each
115 tank were individually counted and weighed to determine survival, percent weight gain (PWG) and feed
116 conversion ratio (FCR). Hepatopancreas were collected from three shrimp in each tank (15 per treatment)
117 into 1.5 ml centrifuge tubes, rapidly frozen in liquid nitrogen and stored at -80℃. The 15 hepatopancreas
118 samples were collected per treatment to provide 3 replicates of 5 shrimp for analysis of transcriptome (n
119 = 3). Similarly, hepatopancreas, muscle and carapace from five shrimp in each tank were pooled and
120 used to determine Cu concentration in tissues. Hepatopancreas of another three shrimp from each tank
121 was collected and pooled for analysis of amino acids. Hemolymph samples from five shrimp in each tank
122 were taken from the pericardial cavity using a 1-ml syringe, placed in 1.5-ml microfuge tubes and
123 centrifuged at 850 g for 10 min at 4 °C (Eppendorf centrifuge 5810R, Germany). The supernatant was
124 collected and stored at -80 °C until analysis of hemolymph copper.

125 *2.4 Copper concentration analysis*

126 Copper concentrations in tissues (hepatopancreas, muscle and carapace), experimental diets and seawater

were measured using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer, PE 2100DV, Perkin Elmer, USA) in Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences (Ningbo, China). Briefly, tissues and experimental diets were freeze-dried before acid digestion, where samples were digested in 70 % HNO_3 solution at 80 ℃ for 4 hr. Samples were cooled to room temperature, quantitatively transferred solution to 25 ml volumetric flasks, and filtered through an aqueous phase syringe filter (SCAA-102, ANPEL Laboratory Technologies Inc, China). Finally, samples were transferred into clean 10 ml tubes for on-board testing. Hemolymph copper was determined using the relevant kit (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions. The concentration of Cu in seawater ranged from 2.05 μg L^{-1} to 2.21 μg L^{-1} among three groups fed diets containing different Cu levels.

2.5 Identification and quantification of total amino acids of hepatopancreas

Amino acid profiles of diets (Table S2) and hepatopancreas were determined using a High-speed Amino Acid Analyzer (L-8900, Hitachi High-Technologies Co., Tokyo, Japan) based on the method described previously with a few modifications (Yuan et al., 2020b). Briefly, approximately 50 mg of freeze-dried sample was weighed into a 15 ml glass thread screw neck vial with an 18 mm screw cap containing a translucent blue silicone septa gasket (CNW, Germany). Five ml HCl (6 N) was added, the tube sealed under N_2, and immersed in a sand bath at 110 ℃ for 24 h for digestion. After cooling, the digested samples were washed into a 50 ml volumetric flask using ultrapure water. One ml of this solution was transferred into a 4 ml ampoule bottle (CNW, Germany), dried in a Termovap sample concentrator (MIULAB NDK200-1 N, Hangzhou, China), resuspended in 1 ml HCl (0.02 N) and filtered through a 0.22 μm membrane using a hydrophilic polyether sulfone syringe filter (CNW, Germany) to remove residue and impurity. Finally, samples were transferred into clean 1.5 ml screw vials (HAMAG, Germany)

for on-board testing. The packed column was a Hitachi ion-exchange resin 2622 (4.6 mm × 60 mm, particle size 5 μm) and ninhydrin coloring solution was the reactive reagent for the detection of amino acids. Results were expressed as g/100 g dry matter with all determinations performed in duplicate, with the coefficient of variation within 1 %.

2.6 Transcriptional analysis of hepatopancreas

2.6.1 RNA extraction and qualification

Total RNA was extracted from 60 mg hepatopancreas with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA quality and quantity were determined by spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, USA) and Agilent 2100 bioananlyzer (Thermo Fisher Scientific, MA, USA). The integrity of isolated RNA checked by electrophoresis on a 1.2 % denatured agarose gel and Molecular Imager® Gel Doc™ XR System (Bio-Rad, USA).

2.6.2 Library preparation and sequencing

After extraction, mRNA was purified from total RNA using oligo (dT)-attached magnetic beads and fragmented with fragmentation buffer. First-strand cDNA was generated using random N6 primed reverse transcription. Buffer, dNTPs, RNase H and DNA polymerase I were added to synthesize second-strand cDNA. The cDNA fragments obtained were amplified by PCR, and products purified by Ampure XB Beads (Beckman Coulter; Beverly, MA, USA), then dissolved in EB solution. The library quality was validated on an Agilent Technologies 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). Sequencing of the cDNA library transcriptome was performed on an Illumina HiSeq4000 sequencer according to the manufacturer's specifications (Illumina). Library construction and RNA sequencing were performed by BGI-Shenzhen Company (Beijing Genomics Institute, Shenzhen, China).

2.6.3 Reads mapping to the reference genome and functional annotation

171 The sequencing data was filtered with Trimmomatic software (version 0.36) by removing low quality
172 reads (bases with quality less than 10 accounted for around 20 % of the total bases), and reads with
173 adapter and unknown base reads (unknown bases were just over 5 %). Clean reads obtained were stored
174 in FASTQ format and used for quantitative analysis, and the Q20 (percentage of phred quality score >
175 20) and Q30 (percentage of phred quality score > 30) of clean data were calculated (Table 1).

176 The reference genome of *L. vannamei* (GCF_003789085.1_ASM378908v1) was downloaded from
177 the National Center for Biotechnology Information (NCBI). High-quality clean reads were aligned to the
178 reference coding gene set using short reads alignment tool of Bowtie 2 (version 2.2.5) (Langmead and
179 Salzberg, 2012), then expression levels of genes were calculated and normalized into fragment per
180 kilobase of transcript per million base pairs sequenced (FPKM) by RSEM software (Li and Dewey, 2011;
181 Langmead and Salzberg, 2012). Gene functions were annotated based on the following databases: NCBI
182 non-redundant (Nr) protein sequences, Gene Ontology (GO) classification, Kyoto Encyclopedia of Gene
183 and Genomes (KEGG) metabolic pathway analysis and Animal Transcription Factor (TF) database.

184 *2.6.4 Identification of differentially expressed genes (DEGs)*

185 DEGs were screened between two groups (C-Cu vs. I-Cu, C-Cu vs. O-Cu) using the DEGseq method
186 (Wang et al., 2010). The significant *p*-value was corrected for False Discovery Rate, and the *q*-value
187 (corrected *p*-value) serve as key indicators to obtain DEGs. Genes were considered as significantly
188 differentially expressed with $\log_2$fold change ≥ 2 and *q*-value ≤ 0.001 (Benjamini and Hochberg, 1995;
189 Storey and Tibshirani, 2003).

190 *2.6.5 Kyoto Encyclopedia of Genes and Genomes enrichment analysis of DEGs*

191 A functional-enrichment analysis was performed to excavate significantly enriched metabolic pathways
192 or signal transduction pathways. Pathway enrichment analysis between two groups (C-Cu vs. I-Cu, C-

193 Cu vs. O-Cu) were based on KEGG enrichment analysis performed by Phyper using the hypergeometric
194 test. A *q*-value ≤ 0.05 with a rigorous threshold by Bonferroni was defined as the significance level in
195 the corresponding pathway of DEGs (Abdi, 2007).

196 *2.6.6 Transcriptome validation with qRT-PCR*

197 To further validate the veracity and reliability of transcriptomic data, quantitative real-time PCR was
198 preformed, and phosphoserine aminotransferase 1 (*psat1*), serine hydroxymethyltransferase (*shmt*),
199 methionine synthase (*mtr*), tropomyosin-1 (*tpm1*) and phosphate cytidylyltransferase 1 (*pcyt*1) were
200 selected for qRT-PCR analysis. The housekeeping gene *β*-actin was used as an internal normalization
201 control, and the specific primers for the candidate genes used for qPCR were designed by Primer Premier
202 5.0 (Table S3). The qPCR was carried out in a quantitative thermal cycler system (Light cycler® 96,
203 Roche, Switzerland) with each 20 μl reaction volume containing 10 μl of 2 × ChamQ SYBR Green
204 Master Mix (Vazyme), 0.4 μl (each) gene-specific forward and reverse primers (10 μm), 8.4 μl DEPC
205 water and 0.8 μl of 1:4 diluted cDNA. The quantitative PCR program was 95 °C for 2 min, followed by
206 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s. The amplification efficiency was measured
207 as following: $E=10^{(-1/slope)}-1$, and amplification efficiencies of all genes ranged from 98.4 % to 105.7 %.
208 The relative gene expression values were normalized by *β-actin*-expressed transcripts, and calculated
209 using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

210 *2.7 Calculations and statistical analysis*

211 The parameters were calculated as follows:

212 PWG (%) = 100 × [final body weight (g) - initial body weight (g)] / initial body weight (g);

213 FCR = feed consumption (g, dry weight) / [final body weight (g) - initial body weight (g)];

214 Survival (%) = 100 × (final number of shrimp) / (initial number of shrimp)

Results are presented as means ± S.E.M. of five replicates (n = 5). All data were checked for normality and homogeneity of variances, and were normalized when appropriate. Data were analyzed using one-way analysis of variance ANOVA to investigate differences among treatments followed by Duncan's multiple range test at a significance level of $P < 0.05$ (IBM SPSS Statistics 20). Hierarchical cluster analysis and heat map visualization were performed using the online program ImageGP (http://www.ehbio.com/ImageGP/index.php/).

3. Results

3.1 Growth performance and feed utilization

Growth performance and feed utilization of *L. vannamei* fed the experimental diets are shown in Fig. 1. Shrimp fed the control diet had significantly lower percent weight gain (PWG) and higher feed conversion ratio (FCR) than those fed the diets supplemented with Cu (I-Cu or O-Cu), and there were no significant differences in PWG and FCR between shrimp fed the I-Cu and O-Cu diets. No statistical differences were found in survival among all treatments.

3.2 Cu concentrations in tissues and hemolymph

Copper concentrations in tissues and hemolymph are shown in Fig. 2. Shrimp fed the diet supplemented with I-Cu had the highest concentrations of copper in hepatopancreas and muscle, and the lowest concentrations were recorded in shrimp fed the control diet (C-Cu). Copper concentrations in carapace and hemolymph were significantly higher in shrimp fed the diets supplemented with Cu (I-Cu or O-Cu) than those fed the control diet.

3.3 Identification and quantification of total amino acids of hepatopancreas

The amino acid composition of hepatopancreas of *L. vannamei* fed different dietary copper levels are

presented in Fig. 3 and Table S4. A total of 17 amino acids were detected in hepatopancreas including 9 essential amino acids (EAA) and 8 non-essential amino acids (NEAA). The predominant EAA in *L. vannamei* hepatopancreas were leucine (Leu), lysine (Lys), valine (Val) and threonine (Thr), and the predominant NEAA were glutamic acid (Glu), aspartic acid (Asp), proline (Pro) and serine (Ser). In addition, the contents of amino acids in hepatopancreas were significantly affected by dietary copper level. Shrimp fed the control diet had significantly lower contents of Lys, methionine (Met), isoleucine (Ile), Leu, Val, tyrosine (Tyr), glycine (Gly), Asp, Pro, Ser and total amino acid (TAA) than those fed the I-Cu and O-Cu diets. Furthermore, shrimp fed the diet supplemented with O-Cu had significantly higher contents of Lys, Ile, Leu, Val, Tyr, Gly and Asp than those fed the other diets. No statistical differences were found in the contents of phenyalanine (Phe), Thr, arginine (Arg), histidine (His), cystine (Cys), Glu and alanine (Ala).

3.4 Transcriptional analysis of L. vannamei hepatopancreas

3.4.1 Sequencing and mapping

An overview of the sequencing and mapping data is summarized in Table 1. Nine cDNA libraries were established for hepatopancreas including three C-Cu libraries (C-Cu-1, C-Cu-2, C-Cu-3), three I-Cu libraries (I-Cu-1, I-Cu-2, I-Cu-3) and three O-Cu libraries (O-Cu-1, O-Cu-2, O-Cu-3). All raw reads were deposited to the Short Read Archive (SRA) of the NCBI (PRJNA658481). In total, 47.3 to 50.8 million raw reads were obtained for the nine libraries. After filtering, the number of clean reads ranged from 43.0 to 45.9 million, and 80.9 % - 83.8 % were matched in comparison with the reference genome. The clean bases of each sample reached 6.43 Gb, with the percentages of Q20 and Q30 higher than 96.7 % and 80.2 %, respectively. The sequence lengths of unigenes, shown in Fig. S1, indicated most transcripts were longer than 3000 nt.

259 *3.4.2 Functional annotation and classification of the transcriptome*

260 According to GO terms, a total of 30164 unigenes were classified into three major functional categories,
261 including cellular components (42.1 %), biological processes (30.4 %) and molecular function (27.5 %)
262 (Fig. S2A). To characterize biological pathways in the transcriptome, KEGG analysis was performed, by
263 which 27301 unigenes were grouped into six categories including human diseases (29.2 %), organismal
264 systems (21.2 %). metabolism (19.6 %), cellular processes (11.5 %), environmental information
265 processing (9.9 %) and genetic information processing (8.6 %) (Fig. S2B).

266 *3.4.3 Identification of DEGs*

267 To identify DEGs in response to dietary Cu, comparative transcriptome analysis was performed between
268 two groups (C-Cu vs. I-Cu, C-Cu vs. O-Cu) with $\log_2$fold change ≥ 2 and q-value ≤ 0.001, revealing 742
269 and 912 DEGs for the two comparisons, respectively (Fig. 4). Specifically, shrimp fed the diet containing
270 I-Cu showed 271 significantly up-regulated and 471 down-regulated genes, whereas 542 genes were
271 significantly up-regulated and 370 unigenes significantly down-regulated in shrimp fed the diet
272 supplemented with O-Cu, compared to shrimp fed the control (unsupplemented) diet.

273 *3.4.4 KEGG enrichment analysis of DEGs*

274 Hierarchical cluster analysis was performed with heat map visualization, showing the three replicate
275 samples in each group clustered together, with the O-Cu group clustering further from the C-Cu group
276 with the I-Cu group intermediate (Fig. 5A). The analysis of DEGs is shown in Fig. 5B, indicating that
277 most DEGs were enriched in the metabolism category. Further subdividing the metabolic pathways, the
278 proportion of DEGs involved in amino acid, lipid, carbohydrate, cofactor and vitamin metabolism of *L.*
279 *vannamei* fed I-Cu and O-Cu diets were 18.1 %, 12.4 %, 12.4 %, 9.3 % and 16.1 %, 12.9 %, 11.7 %,
280 9.8 %, respectively.

DEGs were mapped to the KEGG database to discriminate the significantly changed pathways. Together, five and eight significantly changed pathways were annotated in the comparison groups (C-Cu vs. I-Cu and C-Cu vs. O-Cu), respectively (q-value < 0.05) (Table 2 and Fig. S3). According to their functional annotations, the five significantly changed pathways between the C-Cu and I-Cu groups mainly related to metabolism, including amino acid metabolism, biotin metabolism and folate biosynthesis. The DEGs between the C-Cu and O-Cu groups were mapped into eight significantly enriched pathways relating to metabolism (biotin metabolism, drug metabolism-other enzymes, tyrosine metabolism, glycine, serine and threonine metabolism), cellular processes (phagosome) and organismal systems (IL-17 signaling pathway, melanogenesis, fat digestion and absorption). Combining the results in Fig. 5B and Table 2, showed that diets supplemented with copper mainly affect metabolism of *L. vannamei*, especially amino acid and lipid metabolism.

3.4.5 Identification DEGs in amnio acid and lipid metabolism

Hierarchical cluster analysis and heat map visualization of DEGs involved in amino acid and lipid metabolism are shown in Fig. 6, showing that diets supplemented with copper (I-Cu and O-Cu) mainly affected glycine, serine and threonine metabolism and glycerophospholipid metabolism. Genes involved in glycine, serine and threonine metabolism (*3-pgdh*, *shmt*, *psph*, *itae*, *psat1*) and glycerophospholipid metabolism (*pemt*, *chpt1*, *pcyt1*, *psd1*) were higher in shrimp fed the copper supplemented diets than those fed the control diet. The network diagram of pathways showed that seven genes including 3-phosphoglycerate dehydrogenase (*3-pgdh*), phosphoserine aminotransferase 1 (*psat1*), phosphoserine phosphatase (*psph*), glycine hydroxymethyltransferase (*shmt*), threonine aldolase (*itae*), branched-chain amino acid aminotransferase (*bcat*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*mtr*) were at the critical nodes of amino acid biosynthesis and mainly involved in the synthesis of serine,

glycine, threonine, valine, leucine, isoleucine and methionine. In addition, phosphate cytidylyltransferase 1 (*pcyt1*), cholinephosphotransferase 1 (*chpt1*), phosphatidylethanolamine N-methyltransferase (*pemt*) and phosphatidylserine decarboxylase 1 (*psd1*) were at the critical nodes involved in the synthesis of phosphatidylcholine and phosphatidylethanolamine (Fig. 7).

3.4.6 Verification of transcriptome data by qRT-PCR

To further validate the transcriptomic data, five genes including *psat1*, *shmt*, *mtr*, *pcyt1* and tropomyosin-1 (*tpm1*) were randomly selected for qRT-PCR analysis. The gene expression patterns revealed by qRT-PCR analysis were similar to the RNA-Seq results (Fig. S5), confirmed by the fact that mRNA expression levels of *psat1*, *shmt*, *mtr*, *pcyt1* were significantly up-regulated and *tpm1* was significantly down-regulated in shrimp fed the I-Cu and O-Cu diets, supporting the reliability of the RNA-Seq data.

4. Discussion

4.1 Dietary copper promotes growth and copper deposition in tissues

Copper is essential for survival and growth for all organisms, and appropriate dietary copper intake improving growth performance and feed utilization has been reported in numerous species (NRC, 2011). In the present study, shrimp fed the I-Cu and O-Cu diets had higher PWG and lower FCR compared to those fed the control unsupplemented diet, suggesting copper insufficiency in the control diet retarded growth and reduced feed utilization in *L. vannamei*. Similar results were also reported in crustaceans including juvenile grass shrimp *Penaeus monodon* (Lee and Shiau, 2002), *L. vannamei* (Bharadwaj et al., 2014), juvenile Chinese mitten crab *Eriocheir sinensis* (Sun et al., 2013) and various fish species (Shao et al., 2010; Tang et al., 2013; Tan et al., 2011; Wang et al., 2016; Abdel-Hameid et al., 2017; Lin et al., 2008; Cao et al., 2014). In practical diets, the optimal copper requirement for prawn *Penaeus orientalis*

was 53 mg kg^{-1} diet (Liu et al., 1990), while in purified diets, Cu requirements for maximum growth and normal non-specific immune responses of *P. monodon* were around 15 - 21 and 10 - 30 mg kg^{-1}, respectively (Lee and Shiau, 2002). Sun et al. (2013) reported *E. sinensis* fed a semi-purified diet containing 40.34 mg kg^{-1} Cu had the highest weight gain among all treatments. Dietary copper requirement was estimated to be 34 mg kg^{-1} for *L. vannamei* maximum weight gain when fed a semi-purified diet, and 128 mg kg^{-1} Cu had no adverse effects on shrimp growth or survival (Davis and Lawrence, 1993). Furthermore, Zhou et al. (2017) reported that a high level of Cu (257 mg kg^{-1}) did not appear to be detrimental to *L. vannamei*. All organisms need energy to grow and maintain normal metabolism, primarily met by mitochondria that continuously adjust energy output according to cellular energy demands and ongoing metabolic processes (Ruiz et al., 2016). Previous studies have shown mitochondria to be a focus of damage such as vacuolation and hypertrophy in dietary copper deficiency, (Goodman et al., 1970; Dallman and Goodman, 1970; Davies et al., 1985; Lawrence and Farquharson, 1988; Medeiros et al., 1991). Aerobic metabolism depends on cellular copper homeostasis and distribution because this element is a critical component of cytochrome *c* oxidase involved in ATP production (Peña et al., 1999).

In the present study, copper concentration in tissues (hepatopancreas, muscle and carapace) and hemolymph were correlated with the dietary copper levels, particularly in hepatopancreas. The highest Cu concentration was found in hepatopancreas, followed by carapace and muscle, indicating hepatopancreas is the main site for copper deposition. This is consistent with previous studies in *L. vannamei* (Bharadwaj et al., 2014), mud crab *Scylla paramamosain* (Luo et al., 2020), tilapia *Oreochromis mossambicus* (Tsai et al., 2013), juvenile rockfish *Sebastes schlegeli* (Kim and Kang, 2004) and crucian carp *Carassius auratus gibelio* (Shao et al., 2010). The absorption of copper occurs primarily

in small intestine after digestion of dietary copper in stomach, with most of the ingested copper being rapidly deposited in liver (Wapnir, 1998). Therefore, hepatopancreas is the appropriate tissue for studying the impacts of dietary copper in *L. vannamei*.

4.2 Transcriptome analysis revealed that Cu affected metabolism and immune system of L. vannamei

Few studies have elucidated the molecular mechanisms and gene expression profiles underpinning dietary copper intake in animals. However, it was reported that dietary copper deficiency down-regulated genes involved in mitochondrial and peroxisomal fatty acid beta-oxidation and up-regulated genes related to plasma cholesterol transport in rat intestine transcriptome (Tosco et al., 2010). In the present study, results indicated that 271 and 471 genes were up- and down-relgulated in shrimp fed the I-Cu diet, and those DEGs were mainly enriched in metabolism pathways. Similarly, shrimp fed the diet supplemented with O-Cu showed 542 and 370 up- and down-relgulated genes, and these DEGs were significantly annotated in eight pathways including biotin metabolism, drug metabolism, tyrosine metabolism, glycine, serine and threonine metabolism, phagosome, IL-17 signaling pathway, melanogenesis, lipid digestion and absorption, four of which were related to metabolism. The same results were also found in the proportions of DEGs, showing that most DEGs were enriched in the metabolism category. Therefore, transcriptome analysis indicated that supplementation of copper (I-Cu or O-Cu) in the diet mainly affected nutrient metabolism of *L. vannamei*.

The nutrients in food are required for normal physiological functions and growth, for example, amino acids and their derivatives serve virtually all categories of signaling, metabolism, and functional support (Kohlmeier, 2015). The interplay among nutrients, metabolites and gene expression is involved in the coordination of animal growth (Yuan et al., 2013). Many compounds can be used as precursors for amino acid synthesis in organisms, such as the 3-phosphoglycerate, produced by the glycolysis pathway,

which is a precursor of serine, glycine, threonine, while pyruvate, another substance produced by glycolysis, is the carbon skeleton for valine, leucine and isoleucine (Bono et al., 1998). Serine itself is the first amino acid produced in the serine family, with glycine and threonine produced by the modification of serine (Bridgers, 1970). The biosynthesis of serine starts with the oxidation of 3-phosphoglycerate to 3-phosphohydroxylpyruvate by 3-phosphoglycerate dehydrogenase (3-PGDH) (Jaeken et al., 1996). This ketone is reductively aminated by phosphoserine aminotransferase 1 (PSAT1) to yield phosphoserine, which is hydrolyzed to serine by phosphoserine phosphatase (PSPH) (Jaeken et al., 1996). The hydroxymethyl group of serine is replaced with methyl by serine hydromethyltransferase (SHMT) to form glycine, afterwards glycine and acetaldehyde are catalyzed by threonine aldolase (ITAE) to produce threonine (Matthews, 1990). Additionally, branched-chain amino acid aminotransferase (BCAT) is an enzyme that catalyzes the synthesis of branched-chain amino acids such as leucine, isoleucine and valine (Hutson, 2001). Methionine is an EAA and plays a critical role in protein synthesis in many species and can be converted to homocysteine, high levels of which can cause hyperhomocysteinemia in humans (Finkelstein et al., 1982; Outteryck et al., 2012). There are several ways in organisms to degrade homocysteine, one of which is the regeneration of methionine catalyzed by 5-methyl tetrahydrofolate-homocysteine methyltransferase (MTR) (Zhang et al., 2012). In the present study, shrimp fed the I-Cu and O-Cu diets displayed up-regulation of *3-pgdh*, *psat1*, *psph*, *shmt*, *itae*, *bcat* and *mtr*, indicating dietary copper might promote the biosynthesis of serine, glycine, threonine, valine, leucine, isoleucine and methionine in hepatopancreas. This result was largely consistent with the amino acid profiles of hepatopancreas, with shrimp fed the diets supplemented with copper showing increased contents of EAA (lysine, methionine, isoleucine, leucine, valine) and NEAA (tyrosine, glycine, aspartic acid, proline and serine). The effects of dietary copper on amino acid metabolism has not been

391 widely reported, but a few studies have shown that copper supplementation affected hepatic amino acid
392 metabolism enzymes (Evans, 1973; Pearce et al., 1983). Copper deficiency inhibited amino acid
393 biosynthesis mainly due to ATP depletion resulting from impairment of mitochondrial respiration, which
394 reduced synthesis of endogenously encoded amino acids (Weisenberg et al., 1980). Mitochondria have
395 long been recognized for their role in energy production and contain known cupro-enzymes cytochrome
396 *c* oxidase (COX) (McBride et al., 2006). The catalysis and assembly of the COX requires the insertion
397 of multiple metal co-factors that include two copper sites (CuA and CuB) (Baker et al., 2017). COX is
398 the terminal enzyme of the electron transport chain, and it accepts electrons from cytochrome *c* to
399 generate the bulk of energy (Baker et al., 2017). Therefore, a possible mechanism for dietary copper
400 induced promotion of amino acid synthesis is that copper can maintain the integrity of the mitochondrial
401 respiratory chain, thereby providing energy for amino acid biosynthesis. Studies that further clarify the
402 relationship between copper and amino acid and energy metabolism, and characterize how they are
403 regulated, will be of great significance to advancing our understanding of copper's biological functions
404 and nutritional value.

405 Glycerophospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are
406 major components of biological membranes (Hermansson et al., 2001). Liver is a major site for the
407 biosynthesis of PC and PE, which are quantitatively the most important phospholipids in animal tissues
408 (Tijburg et al., 1989). The main route for the biosynthesis of PC and PE is from diacylglycerol via a
409 pathway involving phosphate cytidylyltransferase (PCYT) followed by choline phosphotransferase
410 (CHPT) or ethanolamine phosphotransferase, respectively. However, another pathway for PC
411 biosynthesis is via methylation of PE catalyzed by the enzyme of phosphatidylethanolamine *N*-
412 methyltransferase (PEMT). Furthermore, PE can be produced from phosphatidylserine via the action of

phosphatidylserine decarboxylase (PSD) (Wellner et al., 2013; Vance and Tasseva, 2013; Cole et al., 2012). A growing body of evidences have suggested that copper deficiency depresses phospholipid synthesis in rat liver (Gallagher and Reeve, 1971; Al-Othman, 1992). The decrease in the molar ratio of phospholipid to cholesterol is generally associated with reduced membrane fluidity and leads to the loss of mitochondrial functions (Cadenas et al., 2012). The present study has revealed that dietary copper may promote the biosynthesis of PC and PE in hepatopancreas, confirmed by the increased mRNA expression levels of *pemt*, *pcyt1*, *chpt1* and *psd1* in shrimp fed the I-Cu and O-Cu diets. Appropriate glycerophospholipid contents, compositions and proportions are required to support the integrity of the mitochondrial membrane and maintain ATP production, thereby providing the energy required for amino acid biosynthesis and growth.

Interactions between nutrition and immunity are particularly important for normal animal growth and productivity (Humphrey and Klasing, 2004). The interleukin 17 (IL-17) family is a subset of cytokines consisting of IL-17A-F and involves many immune signaling molecules, playing an important role in protecting the host against extracellular pathogens (Aggarwal and Gurney, 2002). Moreover, phagocytosis is one of the main mechanisms of innate immune defense and is the first process responding to infection in invertebrates, mainly through the process of ingestion of large particles by cells thereby removing pathogens and cell debris (Wu et al., 2008). A study reported previously that organic copper improved innate immunity more than inorganic copper, confirmed by the increased activity of phenol oxidase in *L. vannamei* hepatopancreas (Yuan et al., 2018). The present study further clarified the potential mechanism whereby dietary O-Cu enhanced the immune system might be mediated by the pathways of IL-17 signaling and phagosome (Table 2 and Fig. S4). A previous study reported that phagosome accumulates copper during bacterial infection, which may constitute an important

435 mechanism of killing (Hodgkinson and Petris, 2012). Overall, results of the present study showed that
436 diets supplemented with copper (I-Cu or O-Cu) promoted the deposition of hepatopancreas Cu and
437 affected nutrient metabolism of *L. vannamei*. Further analysis revealed that dietary copper
438 supplementation affected amino acid (Ser, Gly, Thr, Ile, Leu, Val and Met) and glycerophospholipid (PC
439 and PE) metabolism. In addition, the diet supplemented with O-Cu enhanced the immune system of *L.*
440 *vannamei* most likely through the pathways of IL-17 signaling and phagosome (Fig. 8).
441
442 **5. Conclusion**
443 In conclusion, the present study successfully constructed and sequenced the *L. vannamei*
444 hepatopancreas transcriptome and identified the key genes and metabolic pathways responding to dietary
445 copper level. Dietary copper supplementation could promote growth, copper deposition in tissues
446 (hepatopancreas, muscle, carapace) and hemolymph, and the content of amino acids in hepatopancreas.
447 Transcriptome analysis revealed that a total of 742 and 912 DEGs were observed in the comparative
448 treatments (C-Cu vs. I-Cu and C-Cu vs. O-Cu). Diets supplemented with I-Cu or O-Cu affected nutrient
449 metabolism (mainly amino acid and glycerophospholipid metabolism) of *L. vannamei*. Furthermore, O-
450 Cu is a positive alternative to I-Cu, as it may mediate the IL-17 signaling pathway and phagocytosis to
451 improve immune response of *L. vannamei*. The findings contribute to increasing our understanding of
452 the nutritional molecular basis of dietary copper and set the foundation for further in-depth functional
453 studies.
454
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Conflicts of interest

The authors declared that there were no conflicts of interest.

Ethics approval

The study was performed in strict accordance with the Standard Operation Procedures (SOPs) of the Guide for Use of Experimental Animals of Ningbo University. The experimental protocol and procedures were approved by the Institutional Animal Care and Use Committee of Ningbo University.

Authors' contributions

B.S. formulated the research question, designed the study, carried out the study, analyzed the data and wrote the article. M.J. designed the study, assisted in the correction and developed the questions. Y.Y. and D.Y.S. assisted in the correction. M.B.B. developed the questions, and revised the manuscript. D.R.T. assisted in developing the research questions and revising the manuscript. L.F.J. developed the questions and revised the manuscript. Q.Z. formulated the research question, designed the study, and revised the manuscript. All the authors read and approved the final version of the manuscript.

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Supplementary Materials:

Table S1 Formulations and proximate compositions of the experimental diets

Table S2 Amino acid compositions (g/100g dry matter) of the experimental diets

Table S3 Primers used for quantitative real-time PCR

Table S4 Amino acid compositions (g/100g, dry matter) of hepatopancreas of *L. vannamei* fed the experimental diets

Fig. S1 Overview the distributions of *L. vannamei* transcriptome sequence length. The x-axis shows the transcript length, and y-axis shows the corresponding number of transcripts.

Fig. S2 Gene ontology (GO) annotations (**A**) and Kyoto encyclopedia of genes and genomes (KEGG) classification (**B**) of unigenes in hepatopancreas transcriptome of *L. vannamei*. The number of genes in each GO and KEGG subcategory are shown in x-axis, and y-axis shows the corresponding GO function

723 and KEGG pathway.

724 **Fig. S3** KEGG pathway enrichment analysis of the top 20 pathways in C-Cu vs. I-Cu (**A**) and C-Cu vs.
725 O-Cu (**B**). The x-axis indicates enrichment ratio, and the y-axis represents the top 20 pathways. The size
726 of the bubble represents the number of DEGs in the corresponding pathway, and the color of the bubble
727 represents the *q*-value of the corresponding pathway, with $q \leq 0.05$ considered as significant enrichment.

728 **Fig. S4** The significantly changed KEGG pathways of IL-17 signaling (**A**) and phagosome (**B**), with
729 genes marked in orange indicating up-regulation.

730 **Fig. S5** Validation of differentially expressed genes by qRT-PCR in hepatopancreas transcriptome of *L.*
731 *vannamei* (C-Cu vs. I-Cu (**A**) and C-Cu vs. O-Cu (**B**)). The red bars indicate RNA-seq, and blue bars
732 indicate gene expression levels normalized against reference genes *β*-actin. Error bars indicate standard
733 deviations of averages from three replicates. *psat1*, phosphoserine aminotransferase 1; *shmt*, serine
734 hydromethyltransferase; *mtr*, methionine synthase; *tpm1*, tropomyosin-1; *pcyt1*, choline-phosphate
735 cytidylyltransferase.

Table 1

Summary of the transcriptome sequencing and mapping for *Litopenaeus vannamei*

Groups	Sample	Raw Reads (10^6)	Clean Reads (10^6)	Clean Bases (Gb)	Q20 (%)	Q30 (%)	Clean Reads Ratio (%)	Total Mapping (%)
C-Cu	C-Cu-1	47.33	43.16	6.47	96.89	88.83	91.20	83.84
	C-Cu-2	50.83	45.87	6.88	96.85	88.68	90.25	81.21
	C-Cu-3	47.33	42.88	6.43	96.97	89.11	90.60	80.89
I-Cu	I-Cu-1	47.33	43.23	6.48	97.19	89.68	91.34	82.06
	I-Cu-2	49.08	45.02	6.75	97.04	80.21	91.73	81.73
	I-Cu-3	47.33	43.00	6.45	96.84	89.00	90.85	82.61
O-Cu	O-Cu-1	49.08	44.42	6.66	96.78	88.57	90.50	81.22
	O-Cu-2	47.33	43.10	6.46	96.87	88.72	91.06	81.40
	O-Cu-3	47.33	43.05	6.46	96.72	88.59	90.97	82.41

742 **Table 2**

743 The significantly enriched pathways (*q*-value < 0.05) in C-Cu vs. I-Cu and C-Cu vs. O-Cu

Pathway ID	Pathway name	KEGG level 1 classify	KEGG level 2 classify	*Q*-value [1]	Rich ratio [2]
C-Cu vs. I-Cu					
Ko00260	Glycine, serine and threonine metabolism	Metabolism	Amino acid metabolism	0.00004	0.13830
Ko04975	Fat digestion and absorption	Organismal systems	Digestive system	0.00318	0.13043
Ko00780	Biotin metabolism	Metabolism	Metabolism of cofactors and vitamins	0.01193	0.20833
Ko00790	Folate biosynthesis	Metabolism	Metabolism of cofactors and vitamins	0.01193	0.11940
Ko01230	Biosynthesis of amino acids	Metabolism	Amino acid metabolism	0.02800	0.08264
C-Cu vs. O-Cu					
Ko00780	Biotin metabolism	Metabolism	Metabolism of cofactors and vitamins	0.00000	0.37500
Ko00983	Drug metabolism-other enzymes	Metabolism	Xenobiotics biodegradation and metabolism	0.00185	0.08837
Ko00350	Tyrosine metabolism	Metabolism	Amino acid metabolism	0.00329	0.13889
Ko00260	Glycine, serine and threonine metabolism	Metabolism	Amino acid metabolism	0.02314	0.10638
Ko04145	Phagosome	Cellular processes	Transport and catabolism	0.02456	0.06583
Ko04657	IL-17 signaling pathway	Organismal systems	Immune system	0.02496	0.05587
Ko04916	Melanogenesis	Organismal systems	Endocrine system	0.02928	0.08824
Ko04975	Fat digestion and absorption	Organismal systems	Digestive system	0.02947	0.11594

744 [1] only enriched KEGG pathways with *q*-value < 0.05 according to Bonferroni is displayed. [2] Rich ratio = term candidate gene number/term gene number.

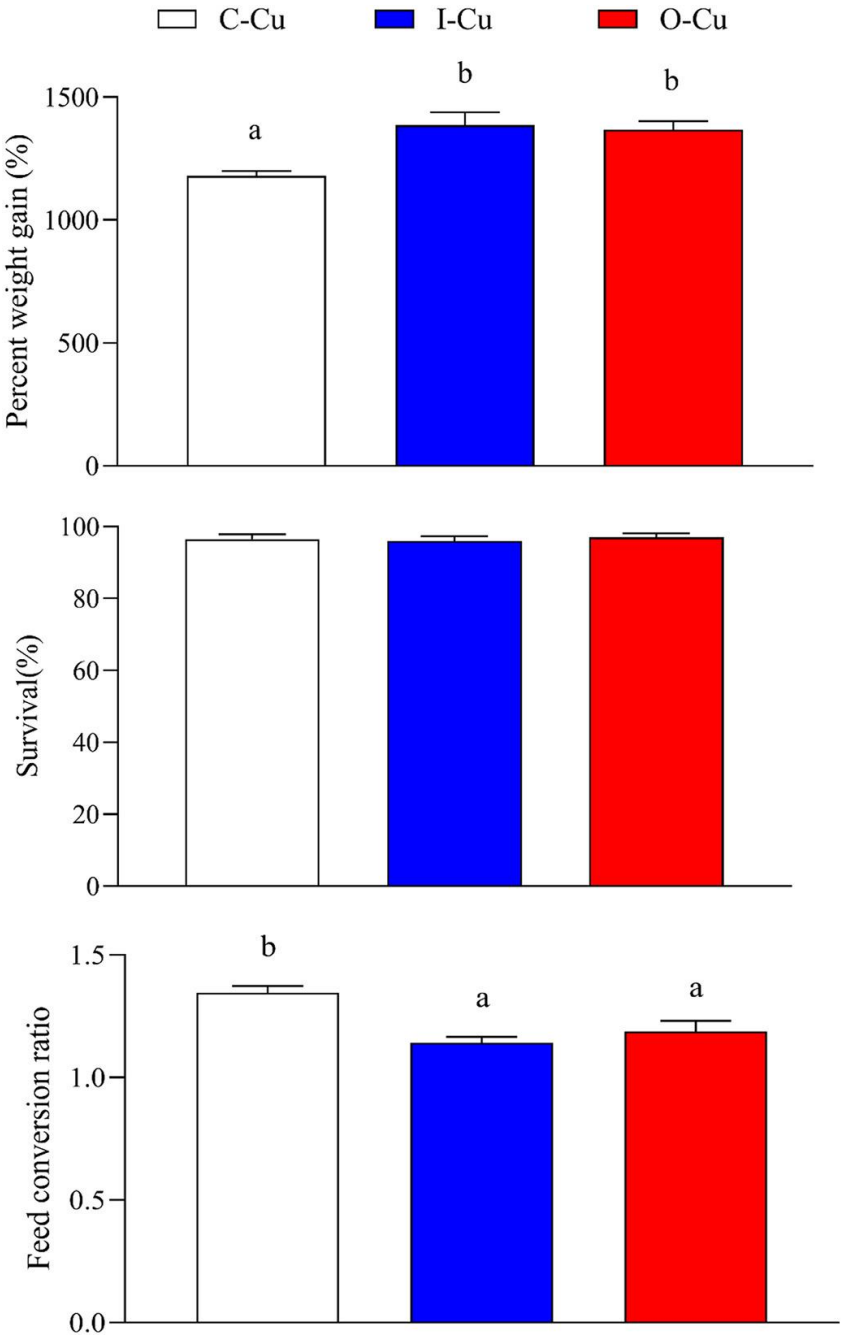
C-Cu
I-Cu
O-Cu
Percent weight gain (%)
1500
1000
500
0
a
b
b
Survival(%)
100
80
60
40
20
0
Feed conversion ratio
1.5
1.0
0.5
0.0
b
a
a

Figure 1

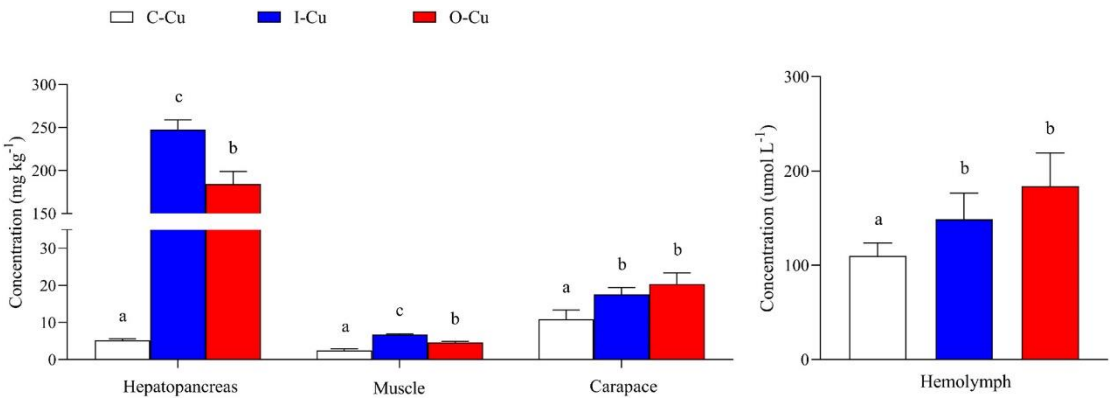
C-Cu
I-Cu
O-Cu
Concentration (mg kg-1)
300
250
200
150
30
20
10
0
a
c
b
a
c
b
a
b
b
Hepatopancreas
Muscle
Carapace
Concentration (umol L-1)
300
200
100
0
a
b
b
Hemolymph

Figure 2

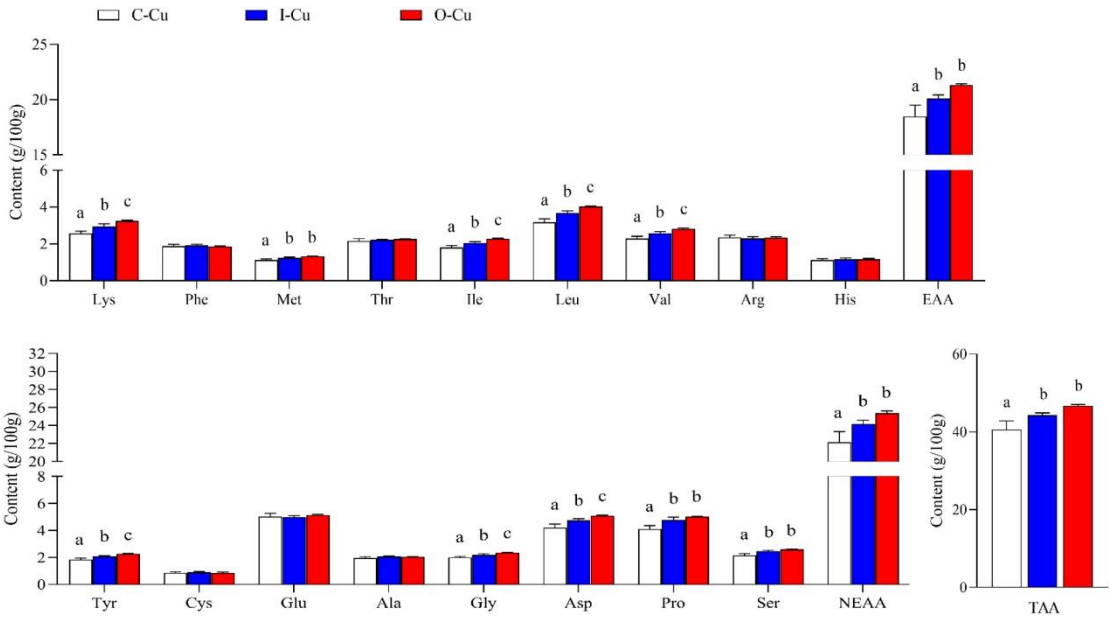
C-Cu
I-Cu
O-Cu
Content (g/100g)
Lys
Phe
Met
Thr
Ile
Leu
Val
Arg
His
EAA
Tyr
Cys
Glu
Ala
Gly
Asp
Pro
Ser
NEAA
TAA

Figure 3

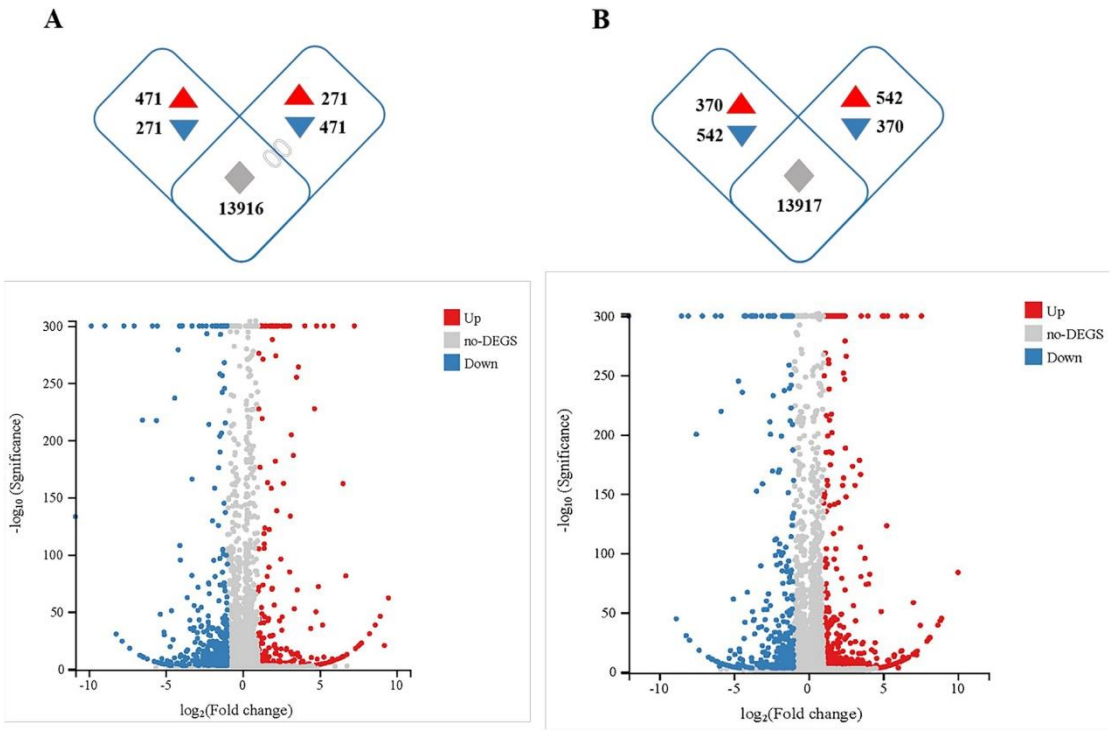
A
471
271
271
471
13916
B
370
542
542
370
13917
Up
no-DEGS
Down
-log10 (Significance)
log2(Fold change)

Figure 4

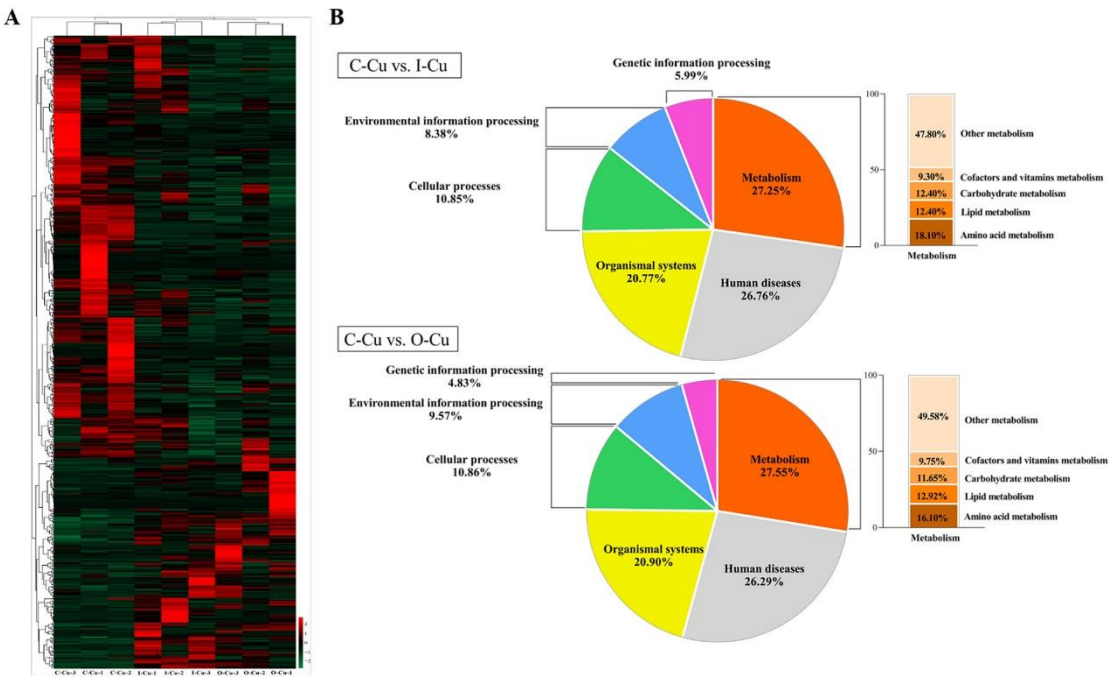
A
B
C-Cu vs. I-Cu
Genetic information processing
5.99%
Environmental information processing
8.38%
Cellular processes
10.85%
Metabolism
27.25%
Organismal systems
20.77%
Human diseases
26.76%
47.80% Other metabolism
9.30% Cofactors and vitamins metabolism
12.40% Carbohydrate metabolism
12.40% Lipid metabolism
18.10% Amino acid metabolism
Metabolism
C-Cu vs. O-Cu
Genetic information processing
4.83%
Environmental information processing
9.57%
Cellular processes
10.86%
Metabolism
27.55%
Organismal systems
20.90%
Human diseases
26.29%
49.58% Other metabolism
9.75% Cofactors and vitamins metabolism
11.65% Carbohydrate metabolism
12.92% Lipid metabolism
16.10% Amino acid metabolism
Metabolism

Figure 5

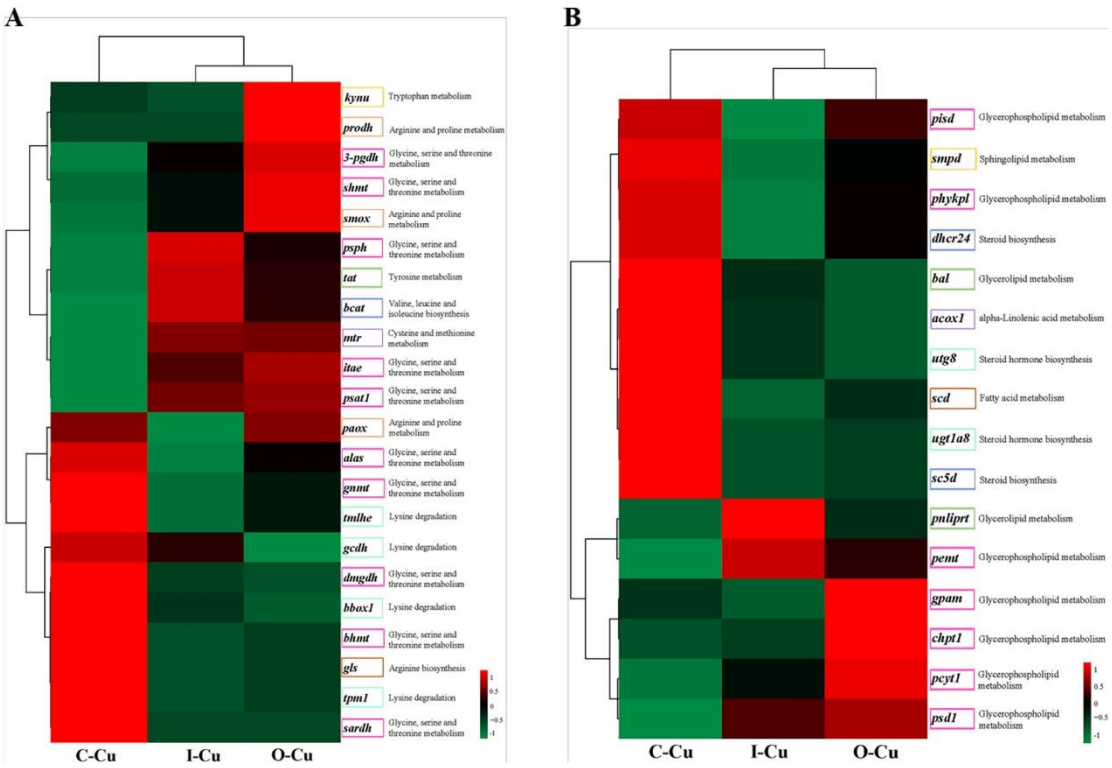
A
kynu
Tryptophan metabolism
prodh
Arginine and proline metabolism
3-pgdh
Glycine, serine and threonine metabolism
shmt
Glycine, serine and threonine metabolism
smox
Arginine and proline metabolism
psph
Glycine, serine and threonine metabolism
tat
Tyrosine metabolism
bcat
Valine, leucine and isoleucine biosynthesis
mtr
Cysteine and methionine metabolism
itae
Glycine, serine and threonine metabolism
psat1
Glycine, serine and threonine metabolism
paox
Arginine and proline metabolism
alas
Glycine, serine and threonine metabolism
gnmt
Glycine, serine and threonine metabolism
tmlhe
Lysine degradation
gcdh
Lysine degradation
dmgdh
Glycine, serine and threonine metabolism
bbox1
Lysine degradation
bhmt
Glycine, serine and threonine metabolism
gls
Arginine biosynthesis
tpm1
Lysine degradation
sardh
Glycine, serine and threonine metabolism
1
0.5
0
−0.5
-1
C-Cu
I-Cu
O-Cu
B
pisd
Glycerophospholipid metabolism
smpd
Sphingolipid metabolism
phykpl
Glycerophospholipid metabolism
dhcr24
Steroid biosynthesis
bal
Glycerolipid metabolism
acox1
alpha-Linolenic acid metabolism
utg8
Steroid hormone biosynthesis
scd
Fatty acid metabolism
ugt1a8
Steroid hormone biosynthesis
sc5d
Steroid biosynthesis
pnliprt
Glycerolipid metabolism
pemt
Glycerophospholipid metabolism
gpam
Glycerophospholipid metabolism
chpt1
Glycerophospholipid metabolism
pcyt1
Glycerophospholipid metabolism
psd1
Glycerophospholipid metabolism
1
0.5
0
−0.5
-1
C-Cu
I-Cu
O-Cu

Figure 6

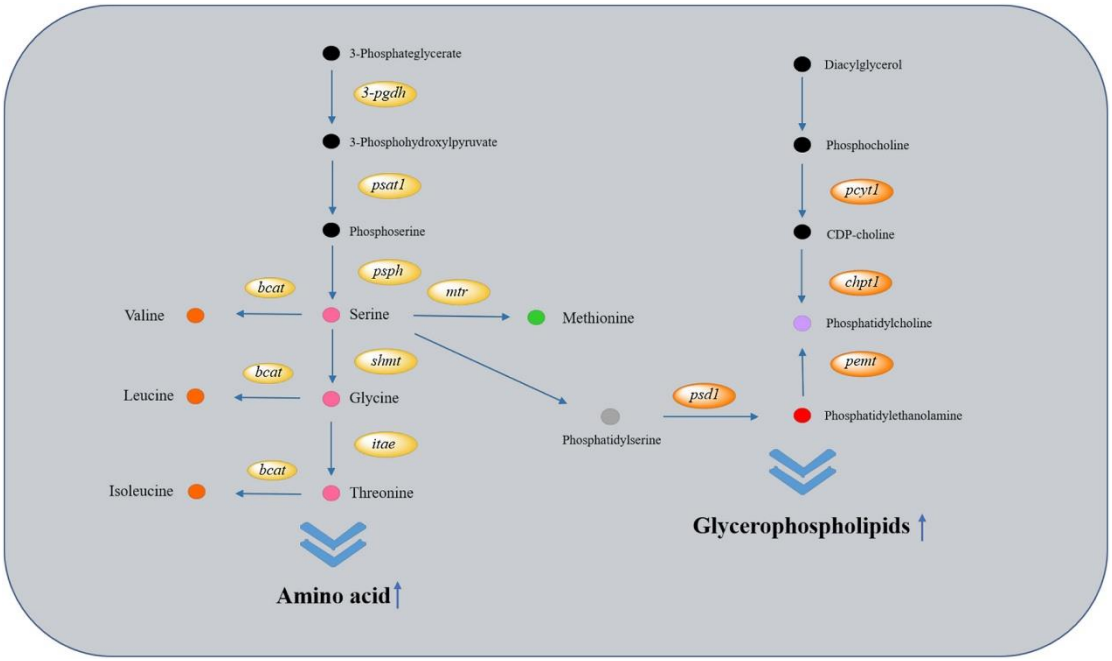
3-Phosphateglycerate
3-pgdh
3-Phosphohydroxylpyruvate
psat1
Phosphoserine
psph
bcat
mtr
Valine
Serine
Methionine
shmt
bcat
Leucine
Glycine
itae
bcat
Isoleucine
Threonine
Amino acid
Phosphatidylserine
psd1
Diacylglycerol
Phosphocholine
pcyt1
CDP-choline
chpt1
Phosphatidylcholine
pemt
Phosphatidylethanolamine
Glycerophospholipids

Figure 7

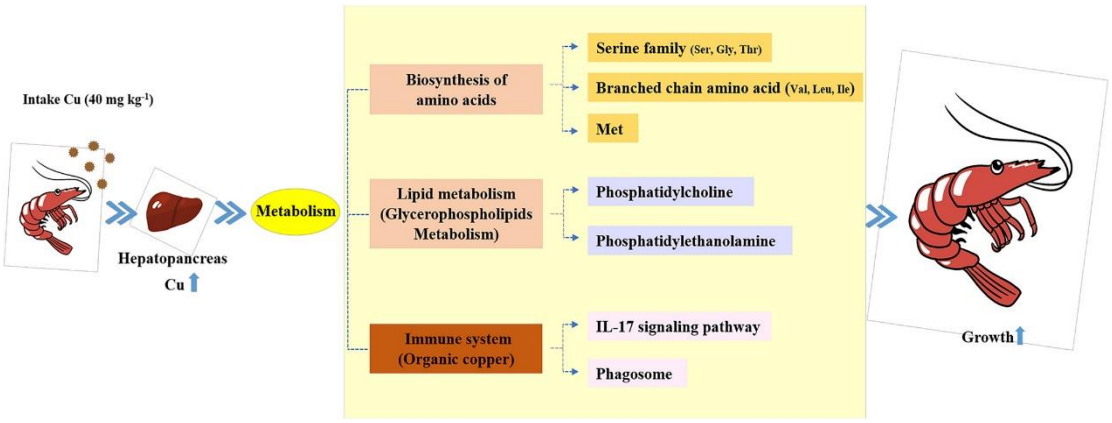
Intake Cu (40 mg kg-1)
Hepatopancreas
Cu
Metabolism
Biosynthesis of amino acids
Serine family (Ser, Gly, Thr)
Branched chain amino acid (Val, Leu, Ile)
Met
Lipid metabolism (Glycerophospholipids Metabolism)
Phosphatidylcholine
Phosphatidylethanolamine
Immune system (Organic copper)
IL-17 signaling pathway
Phagosome
Growth

Figure 8

Transcriptomic and physiological analyses of hepatopancreas reveal the key metabolic changes in response to dietary copper level in Pacific white shrimp *Litopenaeus vannamei*

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Table S1

Formulations and proximate compositions of the experimental diets

Ingredients (g kg^{-1})	C-Cu	I-Cu	O-Cu
Fish meal	200.00	200.00	200.00
Soybean protein concentrate	60.00	60.00	60.00
Soybean meal	230.00	230.00	230.00
Poultry meal	60.00	60.00	60.00
Krill meal	30.00	30.00	30.00
Peanut meal	50.00	50.00	50.00
Wheat flour	286.75	286.75	286.75
Fish oil	15.00	15.00	15.00
Soybean oil	15.00	15.00	15.00
Soy lecithin	20.00	20.00	20.00
Mineral premix [1]	10.00	10.00	10.00
Vitamin premix [2]	5.00	5.00	5.00
Ca $(H_2PO_4)_2$	15.00	15.00	15.00
Choline chloride	3.00	3.00	3.00
Astaxanthin	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$ [3]		0.16	
Copper amino acid chelate [4]			0.37
Proximate compositions (dry matter %)			
Crude protein	42.56	42.77	42.56
Crude lipid	8.65	8.50	8.68
Dry matter	91.39	90.87	90.35
Ash	10.98	11.40	10.65
Analyzed copper (mg kg^{-1})	12.40	49.80	50.00

[1] Mineral premix (g kg^{-1} diet): NaCl, 0.74; K_2SO_4, 2.25; $MgSO_4 \cdot 7H_2O$, 3.62; $FeSO4 \cdot 7H_2O$, 0.25; $CaCO_3$, 0.16; $MnSO_4 \cdot H_2O$, 0.12; $ZnSO_4 \cdot 7H_2O$, 0.27; KIO_3 (1%), 0.02; Na_2SeO_3 (1%), 0.07; $CoSO_4 \cdot 7H_2O$, 0.02; zeolite, 2.44. The mineral premix does not supply Cu

[2] Vitamin premix (mg kg^{-1} diet): D-Ca pantothenate, 120; inositol, 2000; menadione, 60; nicotinic acid, 100; pyridoxine hydrochloride, 100; riboflavin, 50; thiamin nitrate, 60; all-rac-a-tocopherol, 100; cyanocobalamin, 0.1; biotin, 6.0; folic acid, 10; retinyl acetate, 5000 IU; cholecalciferol, 2000 IU

[3] $CuSO_4 \cdot 5H_2O$ (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), Cu content = 25.6 %

[4] Copper amino acid chelate (Zinpro Corp., USA), Cu content = 10.9 %

Table S2

Amino acid compositions (g/100g, dry matter) of the experimental diets

Amino acids	C-Cu	I-Cu	O-Cu
Arg	2.83	2.86	2.84
His	0.86	0.88	0.87
Ile	1.46	1.44	1.44
Leu	2.76	2.72	2.75
Lys	2.27	2.23	2.25
Met	0.83	0.84	0.82
Thr	1.44	1.43	1.46
Phe	1.86	1.88	1.89
Val	1.67	1.66	1.64
Total essential amino acids	15.98	15.94	15.96
Ala	1.95	1.94	1.93
Asp	3.65	3.66	3.64
Cys	0.75	0.77	0.73
Glu	7.02	7.00	7.05
Gly	2.01	2.03	2.07
Pro	2.04	2.03	2.01
Ser	1.71	1.72	1.77
Tyr	1.53	1.54	1.52
Total non-essential amino acids	20.66	20.69	20.72
Total amino acids	36.64	36.63	36.68

Table S3

Primers used for quantitative real-time PCR

Gene	Primers (5′-3′)	Size (bp)	TM (°C)	Accession no.
β-actin	F: CGAGGTATCCTCACCCTGAA R: GTCATCTTCTCGCGGTTAGC	176	58.22 58.80	AF300705
psat1	F: CAAACAAAGTGTGAGACGCA R: AATGAAGACTGGAGATGGCA	184	57.18 56.52	XM_027352141
shmt	F: TTGGTCAGACAGTTGAAGCG R: TGATGATTGCGTAGACCTCG	208	58.42 57.51	XM_027371590
mtr	F: TATTCAACCTCGCATCCC R: CCGTCTAACAAACCTTCG	252	54.00 53.13	XM_027364132
tpm1	F: GCCAACACAATCCTTAGCAA R: GACGCAAGGGACAGATGGTT	224	56.20 59.60	XM_027362037
pcyt1	F: GATTCCCTAACCCACAGTCG R: TGGCACAACCTCGTCTACAT	99	57.69 59.03	XM_027351723

F, forward primer; R, reverse primer. *psat1*, phosphoserine aminotransferase 1; *shmt*, serine hydromethyltransferase; *mtr*, methionine synthase; *tpm1*, tropomyosin-1; *pcyt1*, choline-phosphate cytidylyltransferase

Table S4

Amino acid compositions (g/100g, dry matter) of hepatopancreas of *L. vannamei* fed the experimental diets

Amino acids	C-Cu	I-Cu	O-Cu
Lys	2.57±0.08[a]	2.94±0.09[b]	3.24±0.03[c]
Phe	1.88±0.06	1.91±0.04	1.87±0.01
Met	1.13±0.03[a]	1.21±0.02[b]	1.33±0.01[b]
Thr	2.17±0.08	2.23±0.01	2.27±0.01
Ile	1.81±0.06[a]	2.05±0.04[b]	2.28±0.02[c]
Leu	3.17±0.11[a]	3.68±0.07[b]	4.03±0.02[c]
Val	2.29±0.07[a]	2.59±0.05[b]	2.82±0.03[c]
His	1.13±0.04	1.17±0.04	1.17±0.03
Arg	2.35±0.07	2.30±0.05	2.32±0.04
Total essential amino acids	18.50±0.58[a]	20.10±0.19[b]	21.32±0.07[b]
Tyr	1.84±0.06[a]	2.08±0.03[b]	2.26±0.02[c]
Cys	0.86±0.04	0.89±0.03	0.86±0.03
Glu	5.02±0.14	4.98±0.06	5.14±0.03
Ala	2.00±0.06	2.07±0.02	2.05±0.01
Gly	1.99±0.04[a]	2.18±0.05[b]	2.36±0.02[c]
Asp	4.21±0.15[a]	4.73±0.08[b]	5.09±0.02[c]
Pro	4.10±0.15[a]	4.76±0.13[b]	5.02±0.02[b]
Ser	2.14±0.08[a]	2.47±0.04[b]	2.60±0.01[b]
Total non-essential amino acids	22.12±0.69[a]	24.17±0.24[b]	25.37±0.15[b]
Total amino acids	40.62±1.28[a]	44.27±0.36[b]	46.69±0.20[b]

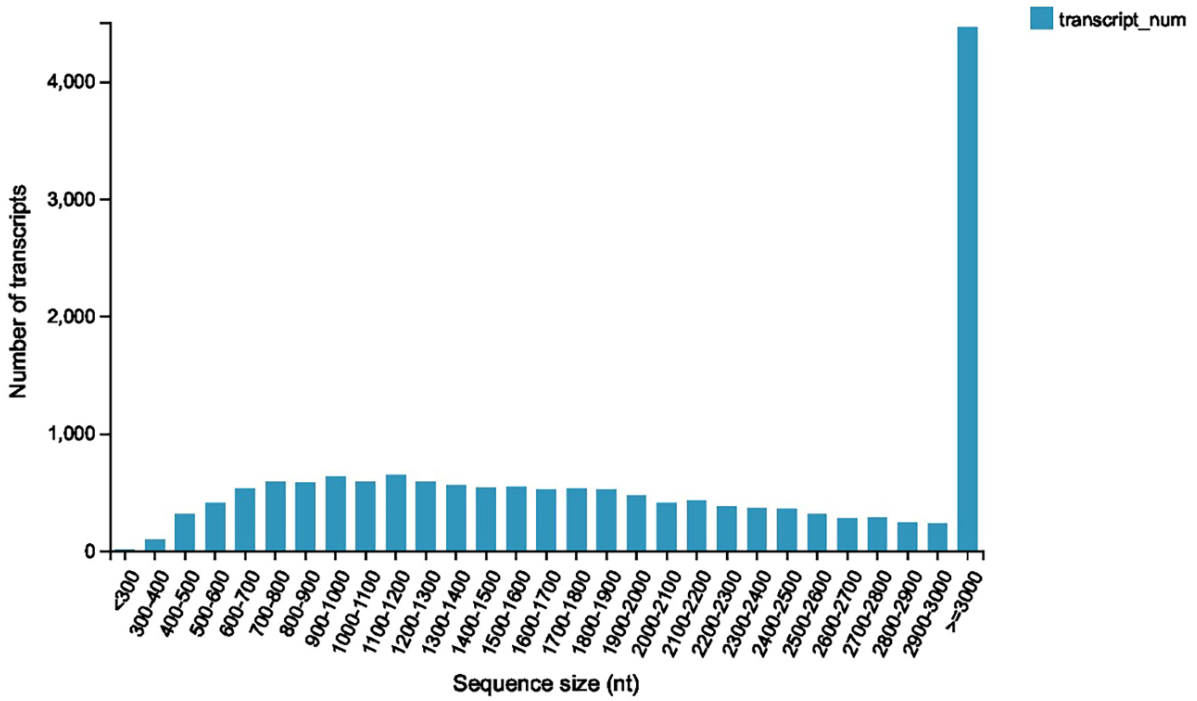


Fig. S1 Overview the distributions of *L. vannamei* transcriptome sequence length. The x-axis shows the transcript length, and y-axis shows the corresponding number of transcripts.

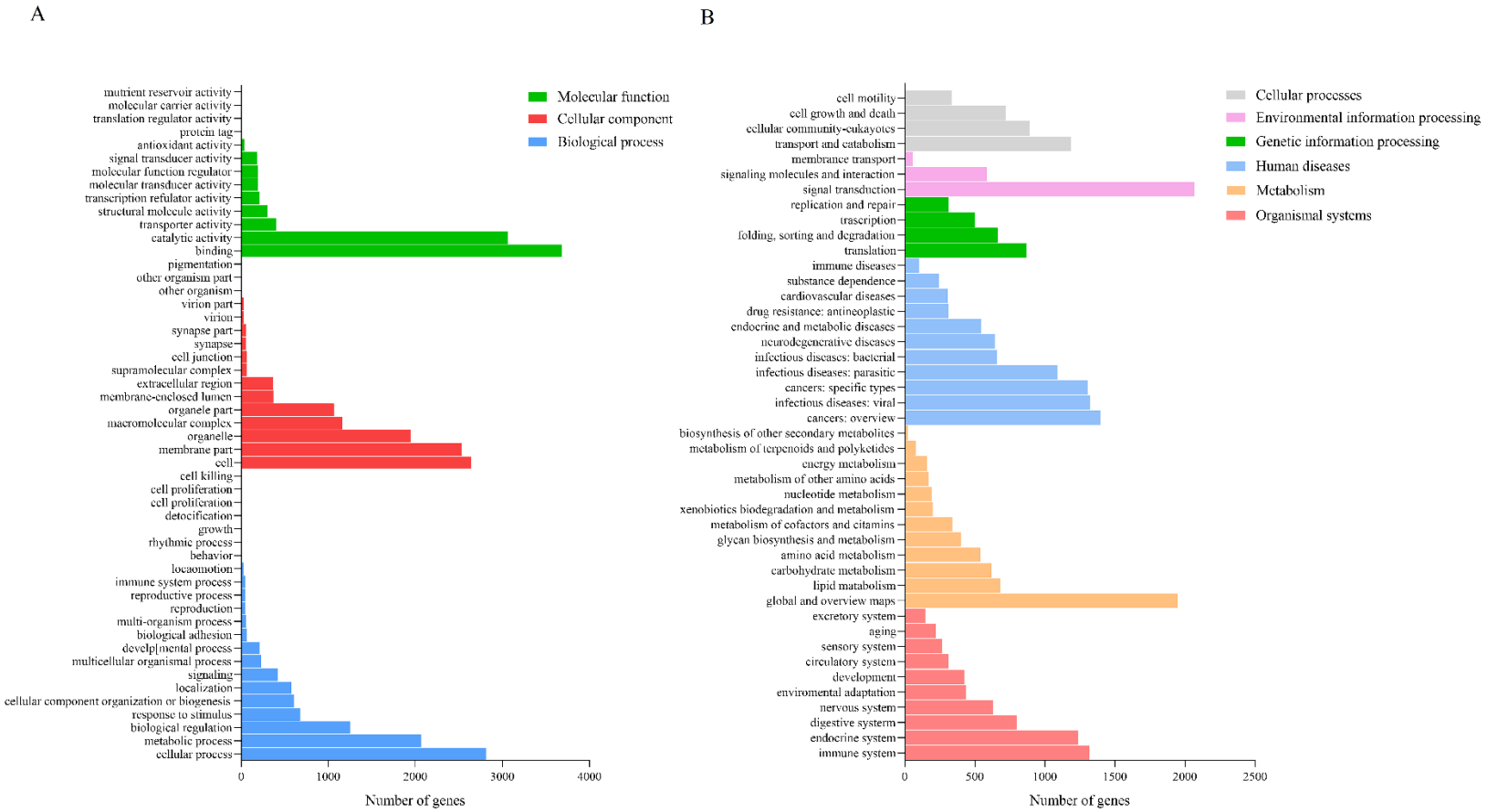


Fig. S2 Gene ontology (GO) annotations (**A**) and Kyoto encyclopedia of genes and genomes (KEGG) classification (**B**) of unigenes in hepatopancreas transcriptome of *L. vannamei*. The number of genes in each GO and KEGG subcategory are shown in x-axis, and y-axis shows the corresponding GO function and KEGG pathway.

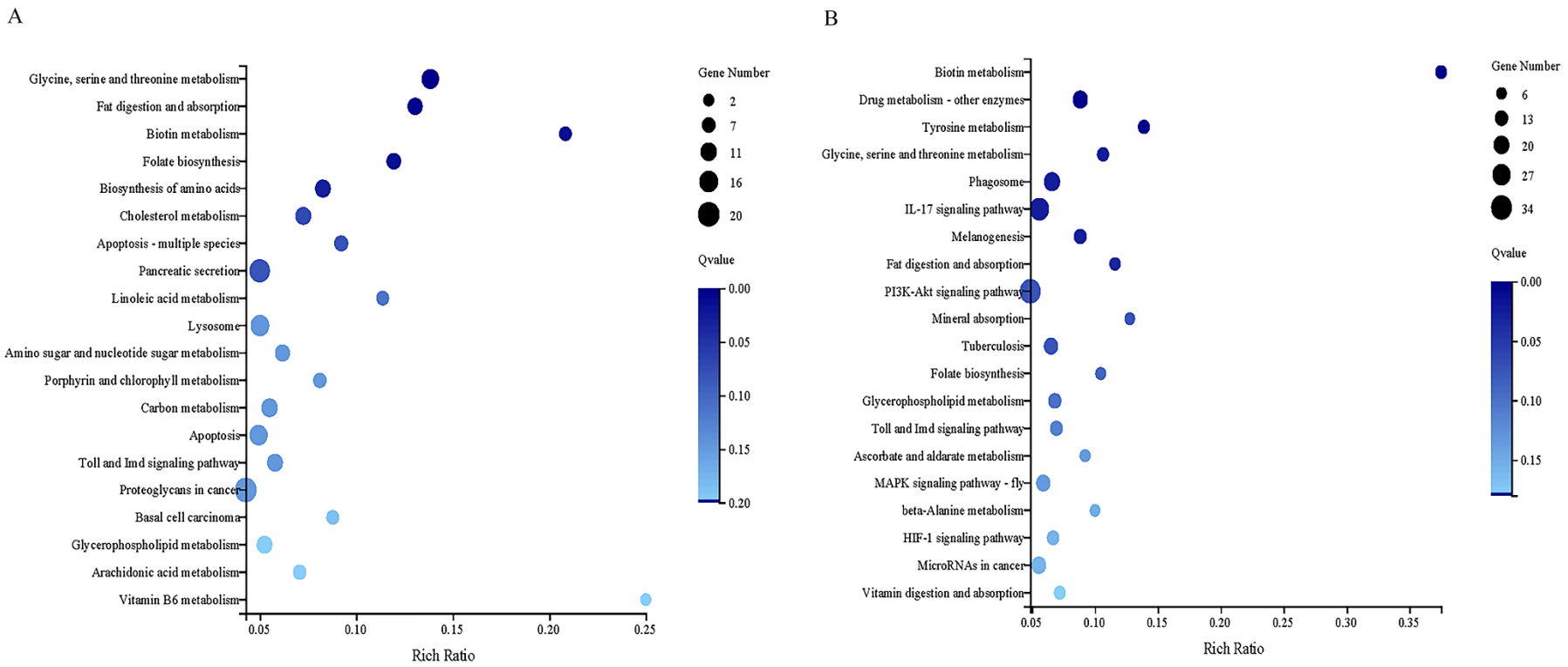
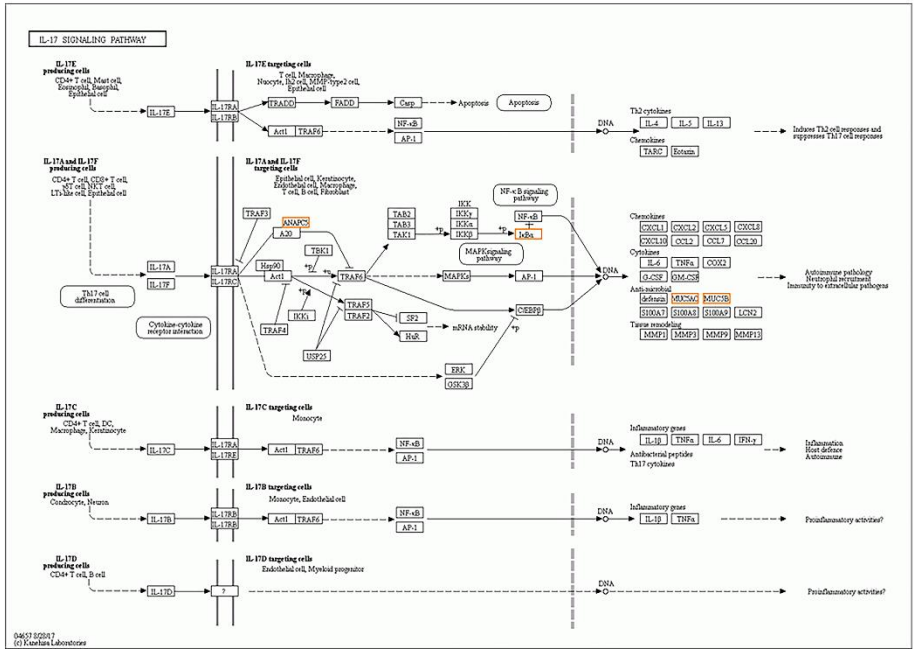


Fig. S3 KEGG pathway enrichment analysis of the top 20 pathways in C-Cu vs. I-Cu (**A**) and C-Cu vs. O-Cu (**B**). The x-axis indicates enrichment ratio, and the y-axis represents the top 20 pathways. The size of the bubble represents the number of DEGs in the corresponding pathway, and the color of the bubble represents the *q*-value of the corresponding pathway, with $q \leq 0.05$ considered as significant enrichment.

A



B

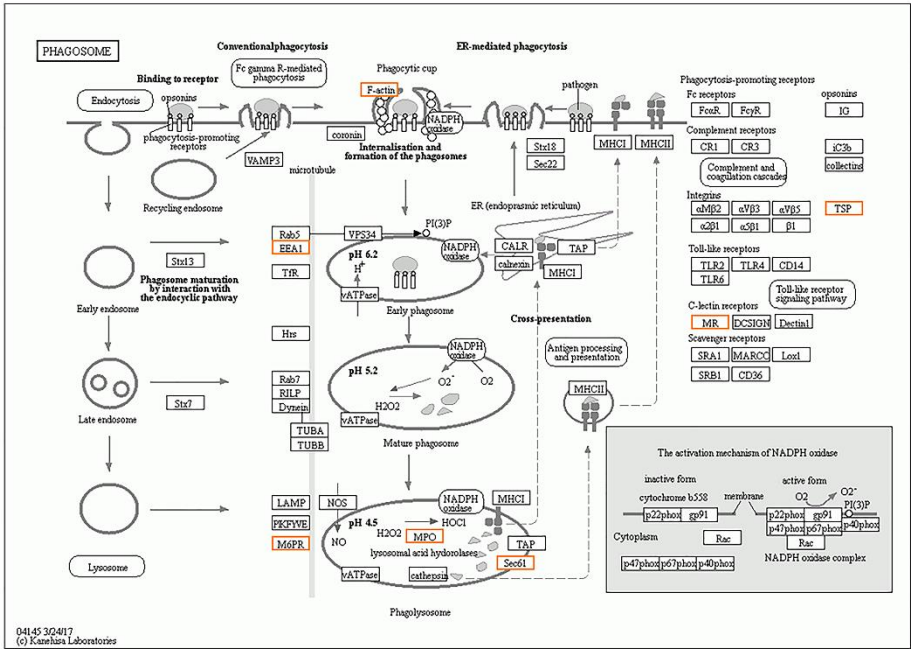


Fig. S4 Significantly changed pathways of IL-17 signaling (**A**) and phagosome (**B**), with genes marked in orange indicating up-regulation.

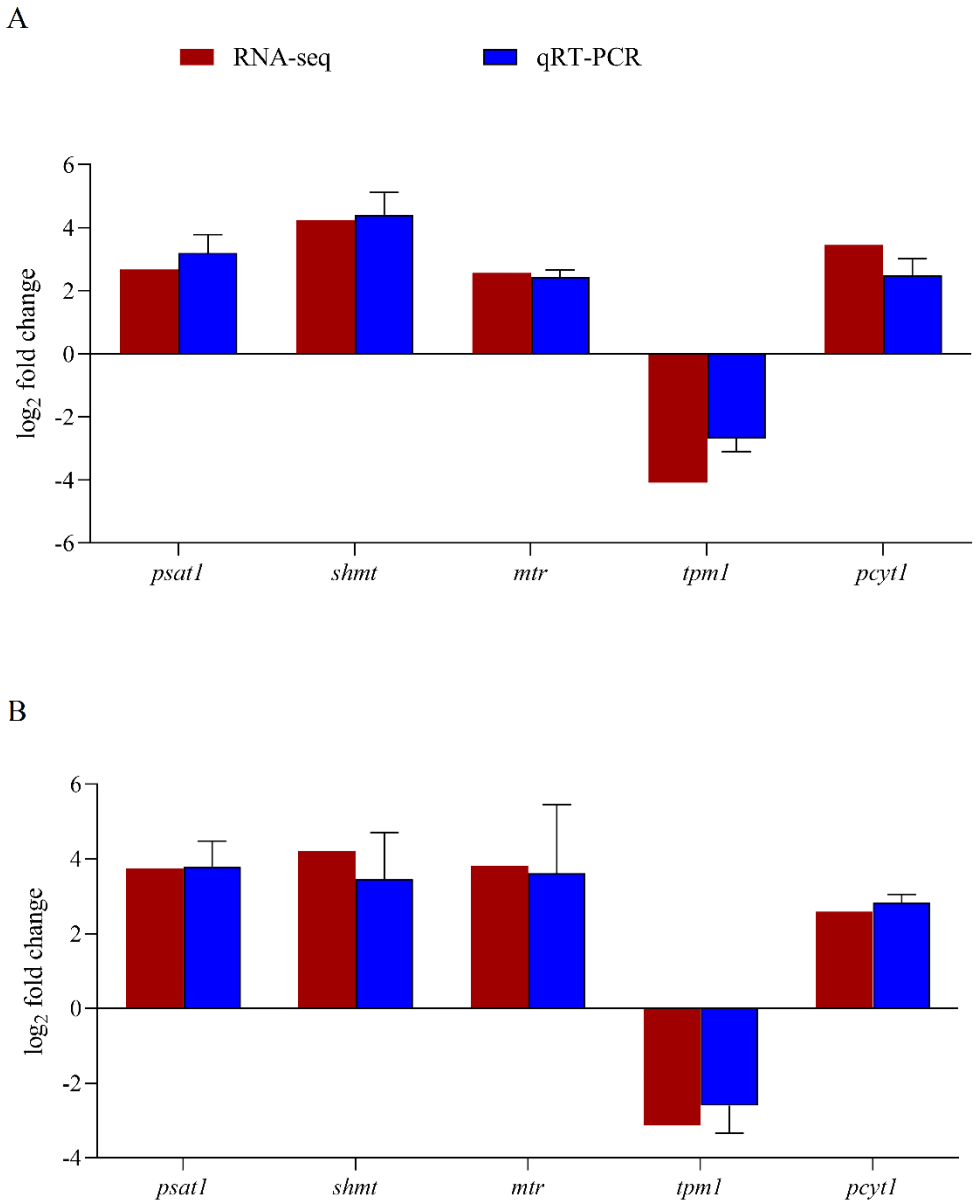


Fig. S5 Validation of differentially expressed genes by qRT-PCR in hepatopancreas transcriptome of *L. vannamei* (C-Cu vs. I-Cu (**A**) and C-Cu vs. O-Cu (**B**)). The red bars indicate RNA-seq, and blue bars indicate gene expression levels normalized against reference genes *β*-actin. Error bars indicate standard deviations of averages from three replicates. *psat1*, phosphoserine aminotransferase 1; *shmt*, serine hydromethyltransferase; *mtr*, methionine synthase; *tpm1*, tropomyosin-1; *pcyt1*, choline-phosphate cytidylyltransferase.

Abbreviations for Figure 6: *kyun*, kynureninase; *prodh*, proline dehydrogenase 1; *3-pgdh*, 3-phosphoglycerate dehydrogenase; *shmt*, serine hydromethyltransferase; *smox*, spermine oxidase; *psph*, phosphoserine phosphatase; *tat*, tyrosine aminotransferase; *bcat*, branched-chain amino acid aminotransferase; *mtr*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *itae*, threonine aldolase; *psat1*, phosphoserine aminotransferase 1; *paox*, peroxisomal N(1)-acetyl-spermine/spermidine oxidase; *alas*, 5-aminolevulinate synthase; *gnmt*, glycine N-methyltransferase; *tmlhe*, trimethyllysine dioxygenase; *gcdh*, glutaryl-CoA dehydrogenase; *dmgdh*, dimethylglycine dehydrogenase; *bbox1*, gamma-butyrobetaine dioxygenase 1; *bhmt*, betaine-homocysteine S-methyltransferase; *gls*, glutaminase liver isoform; *tpm1*, tropomyosin-1; *sardh*, sarcosine dehydrogenase; *pisd*, phosphatidylserine decarboxylase proenzyme; *smpd*, sphingomyelin phosphodiesterase; *phykpl*, 5-phosphohydroxy-L-lysine phospho-lyase; *dhcr24*, delta(24)-sterol reductase; *bal*, bile salt-activated lipase; *acox1*, peroxisomal acyl-coenzyme A oxidase 1; *utg8*, 2-hydroxyacylsphingosine 1-beta-galactosyltransferase; *scd*, stearoyl-CoA desaturase 5; *utg1a8*, UDP-glucuronosyltransferase 1-8-like; *sc5d*, delta(7)-sterol 5(6)-desaturase; *pnliprt*, lipase-related protein 2; *pemt*, phosphatidylethanolamine N-methyltransferase; *gpam*, glycerol-3-phosphate acyltransferase 3; *chpt1*, cholinephosphotransferase 1; *pcyt1*, choline-phosphate cytidylyltransferase 1; *psd1*, phosphatidylserine decarboxylase 1