

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 (CuSO₄·5H₂O; 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 ($q < 0.001$; log₂fold 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 *Oncorhynchus 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. vannamei* 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 CuSO₄·5H₂O (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

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

2.3 Sample collection

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

2.4 Copper concentration analysis

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 % HNO₃ solution at 80 °C 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 N₂, and immersed in a sand bath at 110 °C 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 hepatopaneas

2.6.1 RNA extraction and qualification

Total RNA was extracted from 60 mg hepatopaneas 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 bioanalyzer (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

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

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

2.6.4 Identification of differentially expressed genes (DEGs)

DEGs were screened between two groups (C-Cu vs. I-Cu, C-Cu vs. O-Cu) using the DEGseq method (Wang et al., 2010). The significant *p*-value was corrected for False Discovery Rate, and the *q*-value (corrected *p*-value) serve as key indicators to obtain DEGs. Genes were considered as significantly differentially expressed with log₂fold change ≥ 2 and *q*-value ≤ 0.001 (Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003).

2.6.5 Kyoto Encyclopedia of Genes and Genomes enrichment analysis of DEGs

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

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

2.6.6 Transcriptome validation with qRT-PCR

To further validate the veracity and reliability of transcriptomic data, quantitative real-time PCR was preformed, and phosphoserine aminotransferase 1 (*psat1*), serine hydroxymethyltransferase (*shmt*), methionine synthase (*mtr*), tropomyosin-1 (*tpm1*) and phosphate cytidyltransferase 1 (*pcyt1*) were selected for qRT-PCR analysis. The housekeeping gene β -actin was used as an internal normalization control, and the specific primers for the candidate genes used for qPCR were designed by Primer Premier 5.0 (Table S3). The qPCR was carried out in a quantitative thermal cycler system (Light cycler® 96, Roche, Switzerland) with each 20 μ l reaction volume containing 10 μ l of 2 \times ChamQ SYBR Green Master Mix (Vazyme), 0.4 μ l (each) gene-specific forward and reverse primers (10 μ m), 8.4 μ l DEPC water and 0.8 μ l of 1:4 diluted cDNA. The quantitative PCR program was 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s. The amplification efficiency was measured as following: $E=10^{(-1/\text{slope})}-1$, and amplification efficiencies of all genes ranged from 98.4 % to 105.7 %. The relative gene expression values were normalized by β -actin-expressed transcripts, and calculated using the $2^{-\Delta\Delta C_t}$ method as described by Livak and Schmittgen (2001).

2.7 Calculations and statistical analysis

The parameters were calculated as follows:

PWG (%) = $100 \times [\text{final body weight (g)} - \text{initial body weight (g)}] / \text{initial body weight (g)}$;

FCR = feed consumption (g, dry weight) / $[\text{final body weight (g)} - \text{initial body weight (g)}]$;

Survival (%) = $100 \times (\text{final number of shrimp}) / (\text{initial number of shrimp})$

Results are presented as means \pm 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 phenylalanine (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.

3.4.2 Functional annotation and classification of the transcriptome

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

3.4.3 Identification of DEGs

To identify DEGs in response to dietary Cu, comparative transcriptome analysis was performed between 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 and 912 DEGs for the two comparisons, respectively (Fig. 4). Specifically, shrimp fed the diet containing I-Cu showed 271 significantly up-regulated and 471 down-regulated genes, whereas 542 genes were significantly up-regulated and 370 unigenes significantly down-regulated in shrimp fed the diet supplemented with O-Cu, compared to shrimp fed the control (unsupplemented) diet.

3.4.4 KEGG enrichment analysis of DEGs

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

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

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

Glycerophospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are major components of biological membranes (Hermansson et al., 2001). Liver is a major site for the biosynthesis of PC and PE, which are quantitatively the most important phospholipids in animal tissues (Tijburg et al., 1989). The main route for the biosynthesis of PC and PE is from diacylglycerol via a pathway involving phosphate cytidyltransferase (PCYT) followed by choline phosphotransferase (CHPT) or ethanolamine phosphotransferase, respectively. However, another pathway for PC biosynthesis is via methylation of PE catalyzed by the enzyme of phosphatidylethanolamine *N*-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

mechanism of killing (Hodgkinson and Petris, 2012). Overall, results of the present study showed that diets supplemented with copper (I-Cu or O-Cu) promoted the deposition of hepatopancreas Cu and affected nutrient metabolism of *L. vannamei*. Further analysis revealed that dietary copper supplementation affected amino acid (Ser, Gly, Thr, Ile, Leu, Val and Met) and glycerophospholipid (PC and PE) metabolism. In addition, the diet supplemented with O-Cu enhanced the immune system of *L. vannamei* most likely through the pathways of IL-17 signaling and phagosome (Fig. 8).

5. Conclusion

In conclusion, the present study successfully constructed and sequenced the *L. vannamei* hepatopancreas transcriptome and identified the key genes and metabolic pathways responding to dietary copper level. Dietary copper supplementation could promote growth, copper deposition in tissues (hepatopancreas, muscle, carapace) and hemolymph, and the content of amino acids in hepatopancreas. Transcriptome analysis revealed that a total of 742 and 912 DEGs were observed in the comparative treatments (C-Cu vs. I-Cu and C-Cu vs. O-Cu). Diets supplemented with I-Cu or O-Cu affected nutrient metabolism (mainly amino acid and glycerophospholipid metabolism) of *L. vannamei*. Furthermore, O-Cu is a positive alternative to I-Cu, as it may mediate the IL-17 signaling pathway and phagocytosis to improve immune response of *L. vannamei*. The findings contribute to increasing our understanding of the nutritional molecular basis of dietary copper and set the foundation for further in-depth functional studies.

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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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479 **Reference**

- 480 Abdel-Hameid, N.A.H., Zehra, S., Khan, M.A., 2017. Dietary copper requirement of fingerling *Channa*
481 *punctatus* (Bloch) based on growth, feed conversion, blood parameters and whole body copper
482 concentration. Aquacult. Res. 48(6), 2787-2797. <https://doi.org/doi:10.1111/are.13112>
- 483 Abdi, H., 2007. The Bonferonni and Šidák corrections for multiple comparisons. Encycl. Meas. Stat. 1,
484 1-9.
- 485 Aggarwal, S., Gurney, A.L., 2002. IL-17: prototype member of an emerging cytokine family. J. Leukoc.
486 Biol. 71(1), 1-8. <https://doi.org/10.1189/jlb.71.1.1>
- 487 Al-Othman, A.A., Rosenstein, F., Lei, K.Y., 1992. Copper deficiency alters plasma pool size, percent
488 composition and concentration of lipoprotein components in rats. J. Nutr. 122(6), 1199-1204.
489 <http://dx.doi.org/10.1093/jn/122.6.1199>
- 490 Apines, M.J.S., Satoh, S., Kiron, V., Watanabe, T., Aoki, T., 2003. Availability of supplemental amino
491 acid-chelated trace elements in diets containing tricalcium phosphate and phytate to rainbow trout,
492 *Oncorhynchus mykiss*. Aquaculture 225, 431-444. [https://doi.org/10.1016/S0044-8486\(03\)00307-7](https://doi.org/10.1016/S0044-8486(03)00307-7)
- 493 Apines-Amar, M.J.S., Satoh, S., Caipang, C.M.A., Kiron, V., Watanabe, T., Aoki, T., 2004. Amino acid
494 chelates: a better source of Zn, Mn and Cu for rainbow trout *Oncorhynchus mykiss*. Aquaculture
495 240, 345-358. <https://doi.org/10.1016/j.aquaculture.2004.01.032>
- 496 Baker, Z.N., Cobine, P.A., Leary, S.C., 2017. The mitochondrion: a central architect of copper
497 homeostasis. Metallomics 9(11), 1501-1512. <https://doi.org/10.1039/c7mt00221a>
- 498 Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful
499 approach to multiple testing. J. R. Statist. Soc. 57 (1), 289-300. [23](https://doi.org/10.1111/j.2517-</p></div><div data-bbox=)

6161.1995.tb02031.x

Bharadwaj, A.S., Patnaik, S., Browdy, C.L., Lawrence, A.L., 2014. Comparative evaluation of an inorganic and a commercial chelated copper source in Pacific white shrimp *Litopenaeus vannamei* (Boone) fed diets containing phytic acid. *Aquaculture* 422-423, 63-68. <https://doi.org/10.1016/j.aquaculture.2013.11.025>

Bono, H., Ogata, H., Goto, S., Kanehisa, M., 1998. Reconstruction of amino acid biosynthesis pathways from the complete genome sequence. *Genome Res.* 8(3), 203-210. <https://doi.org/10.1101/gr.8.3.203>

Bridgers, W.F., 1970. The relationship of the metabolic regulation of serine to phospholipid and one-carbon metabolism. *Int. J. Biochem.* 1(4), 495-505. [https://doi.org/10.1016/0020-711x\(70\)90065-0](https://doi.org/10.1016/0020-711x(70)90065-0)

Cadenas, C., Vosbeck, S., Hein, E. M., Hellwig, B., Langer, A., Hayen, H., et al., 2012. Glycerophospholipid profile in oncogene-induced senescence. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 1821(9), 1256-1268. <https://doi.org/10.1016/j.bbalip.2011.11.008>

Cao, J., Miao, X., Xu, W., Li, J., Zhang, W., Mai, K., 2014. Dietary copper requirements of juvenile large yellow croaker *Larimichthys croceus*. *Aquaculture* 432, 346-350. <https://doi.org/10.1016/j.aquaculture.2014.05.032>

Chen, Q.L., Luo, Z., Huang, C., Pan, Y.X., Wu, K., 2016. De novo characterization of the liver transcriptome of javelin goby *Synechogobius hasta* and analysis of its transcriptomic profile following waterborne copper exposure. *Fish Physiol. Biochem.* 42(3), 979-994. <https://doi.org/10.1007/s10695-015-0190-2>

Cole, L.K., Vance, J.E., Vance, D.E., 2012. Phosphatidylcholine biosynthesis and lipoprotein metabolism. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 1821(5), 754-761.

522 <https://doi.org/10.1016/j.bbalip.2011.09.009>

523 Dallman, P.R., Goodman, J.R., 1970. Enlargement of mitochondrial compartment in iron and copper
 524 deficiency. *Blood* 35(4), 496-505. <https://doi.org/10.1182/blood.V35.4.496.496>

525 Davies, N.T., Lawrence, C.B., Mills, C.F., 1985. Studies on the effects of copper deficiency on rat liver
 526 mitochondria. II. Effects on oxidative phosphorylation. *Biochim. Biophys. Acta, Bioenerg.* 809(3),
 527 362-368. [https://doi.org/10.1016/0005-2728\(85\)90186-0](https://doi.org/10.1016/0005-2728(85)90186-0)

528 Davis, D.A., Lawrence, A.L., Gatlin III, D.M., 1993. Dietary copper requirement of *Penaeus vannamei*.
 529 *Nippon. Suisan. Gakkaishi.* 59, 117-122. <https://doi.org/10.2331/suisan.59.117>

530 Evans, G.W., 1973. Copper homeostasis in the mammalian system. *Physiol. Rev.* 53(3), 535-570.
 531 <https://doi.org/10.1152/physrev.1973.53.3.535>

532 Finkelstein, J.D., Kyle, W.E., Harris, B.J., Martin, J.J., 1982. Methionine metabolism in mammals:
 533 concentration of metabolites in rat tissues. *J. Nutr.* 112(5). <http://dx.doi.org/1011-1018>.
 534 doi:10.1093/jn/112.5.1011

535 Gallagher, C., Reeve, V.E., 1971. Copper deficiency in the rat effect on synthesis of phospholipids. *Aust.*
 536 *J. Exp. Biol. Med. Sci.* 49(1), 21-31. <http://dx.doi.org/10.1038/icb.1971.3>

537 Goodman, J.R., Warshaw, J.B., Dallman, P.R., 1970. Cardiac hypertrophy in rats with iron and copper
 538 deficiency: quantitative contribution of mitochondrial enlargement. *Pediat. Res.* 4(3), 244-256.
 539 <https://doi.org/10.1203/00006450-197005000-00003>

540 Hermansson, M., Hokynar, K., Somerharju, P., 2011. Mechanisms of glycerophospholipid homeostasis
 541 in mammalian cells. *Prog. Lipid Res.* 50(3), 240-257.
 542 <http://dx.doi.org/10.1016/j.plipres.2011.02.004>

543 Humphrey, B.D., Klasing, K.C., 2004. Modulation of nutrient metabolism and homeostasis by the

544 immune system. World's Poult. Sci. J. 60(01), 90-100. <https://doi.org/10.1079/wps20037>

545 Hutson, S., 2001. Structure and function of branched chain aminotransferases. Prog. Nucleic Acid Res.

546 Mol. Biol. 175-206. [http://dx.doi.org/10.1016/s0079-6603\(01\)70017-7](http://dx.doi.org/10.1016/s0079-6603(01)70017-7)

547 Hodgkinson, V., Petris, M. J., 2012. Copper homeostasis at the host-pathogen interface. J. Biol. Chem.

548 287(17), 13549-13555. <http://dx.doi.org/10.1074/jbc.R111.316406>

549 Jaeken, J., Detheux, M., Van Maldergem, L., Frijns, J.P., Alliet, P., Foulon, M., Carchon, H., Schaftingen,

550 E.V., 1996. 3-Phosphoglycerate dehydrogenase deficiency and 3-phosphoserine phosphatase

551 deficiency: Inborn errors of serine biosynthesis. J. Inherited Metab. Dis. 19(2), 223-226.

552 <https://doi.org/10.1007/bf01799435>

553 Jiménez-Chillarón, J.C., Díaz, R., Ramón-Krauel, M., 2014. Chapter 4 - omics tools for the genome-

554 wide analysis of methylation and histone modifications. Compr. Anal. Chem. 63, 81-110.

555 <https://doi.org/10.1016/B978-0-444-62651-6.00004-0>

556 Kamunde, C., Clayton, C., Wood, C.M., 2002. Waterborne vs. dietary copper uptake in rainbow trout and

557 the effects of previous waterborne copper exposure. Am. J. Physiol. Regul., Integr. Comp. Physiol.

558 283(1), R69-R78. <https://doi.org/10.1152/ajpregu.00016.2002>

559 Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S.,

560 Okuda, S., Tokimatsu, T., Yamanishi, Y., 2007. KEGG for linking genomes to life and the

561 environment. Nucleic Acids Res. 36, D480-D484. <https://doi.org/10.1093/nar/gkm882>

562 Kim, S.G., Kang, J.C., 2004. Effect of dietary copper exposure on accumulation, growth and

563 hematological parameters of the juvenile rockfish, *Sebastes schlegeli*. Mar. Environ. Res. 58(1), 65-

564 82. <http://dx.doi.org/10.1016/j.marenvres.2003.12.004>

565 Kohlmeier, M., 2015. Nutrient metabolism: structures, functions, and genes. Academic Press.

566 Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357-
 567 359. <https://doi.org/10.1038/nmeth.1923>

568 Lau, S.J., Sarkar, B., 1971. Ternary coordination complex between human serum albumin, copper (ii),
 569 and l-histidine. J. Biol. Chem. 246(19), 5938-5943.

570 Lawrence, C.B., Farquharson, C., 1988. The effects of reserpine upon the cardiac enlargement of copper
 571 deficiency. Proc. Soc. Exp. Biol. Med. 189(2), 173-182. [https://doi.org/10.3181/00379727-189-](https://doi.org/10.3181/00379727-189-42794)
 572 42794

573 Lee, M.H., Shiau, S.Y., 2002. Dietary copper requirement of juvenile grass shrimp, *Penaeus monodon*,
 574 and effects on non-specific immune responses. Fish Shellfish Immunol. 13(4), 259-270.
 575 <https://doi.org/10.1006/fsim.2001.0401>

576 Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without
 577 a reference genome. BMC Bioinf. 12, 323. <https://doi.org/10.1186/1471-2105-12-323>

578 Lin, Y.H., Shie, Y.Y., Shiau, S.Y., 2008. Dietary copper requirements of juvenile grouper, *Epinephelus*
 579 *malabaricus*. Aquaculture 274(1), 161-165. <https://doi.org/10.1016/j.aquaculture.2007.11.006>

580 Liu, F., Liang, D., Sun, F., Li, H., Lan, X., 1990. Effects of dietary copper on the prawn *Penaeus orientalis*.
 581 Oceanol. Limnol. Sin. 21, 404-410.

582 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
 583 quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.
 584 <https://doi.org/10.1006/meth.2001.1262>

585 Luo, J., Zhu, T., Wang, X., Cheng, X., Yuan, Y., Jin, M., Betancor, M.B., Tocher, D.R., Zhou, Q.C., 2020.
 586 Toxicological mechanism of excessive copper supplementation: Effects on coloration, copper
 587 bioaccumulation and oxidation resistance in mud crab *Scylla paramamosain*. J. Hazard. Mater. 395,

588 122600. <https://doi.org/10.1016/j.jhazmat.2020.122600>

589 Matthews, R.G., Drummond, J.T., 1990. Providing one-carbon units for biological methylations:
590 mechanistic studies on serine hydroxymethyltransferase, methylenetetrahydrofolate reductase, and
591 methyltetrahydrofolate-homocysteine methyltransferase. *Chem. Rev.* 90(7), 1275-1290.
592 <https://doi.org/doi:10.1021/cr00105a010>

593 McBride, H.M., Neuspiel, M., Wasiak, S., 2006. Mitochondria: more than just a powerhouse. *Curr. Biol.*
594 16(14), R551-R560. <https://doi.org/10.1016/j.cub.2006.06.054>

595 Medeiros, D.M., Bagby, D., Ovecká, G., McCormick, R., 1991. Myofibrillar, mitochondrial and valvular
596 morphological alterations in cardiac hypertrophy among copper-deficient rats. *J. Nutr.* 121(6), 815-
597 824. <https://doi.org/10.1093/jn/121.6.815>

598 Meng, X., Tian, X., Liu, M., Nie, G., Jiang, K., Wang, B., Wang, L., 2014. The transcriptomic response
599 to copper exposure by the gill tissue of Japanese scallops (*Mizuhopecten yessoensis*) using deep-
600 sequencing technology. *Fish Shellfish Immunol.* 38(2), 287-293.
601 <https://doi.org/10.1016/j.fsi.2014.03.009>

602 National Research Council (NRC) 2011. Nutrient requirements of Fish and Shrimp. Washington DC: The
603 National Academies Press. 176-179.

604 Neumann, P.Z., SassKortsak, A., 1967. The state of copper in human serum: evidence for an amino acid-
605 bound fraction. *J. Clin. Invest.* 46(4), 646-658. <https://doi.org/10.1172/JCI105566>

606 Outteryck, O., de Seze, J., Stojkovic, T., Cuisset, J.M., Dobbelaere, D., Delalande, S., Lacour, A., Cabaret,
607 M., Lepoutre, A.C., Deramecourt, V., Zephir, H., Fowler, B., Vermersch, P., 2012. Methionine
608 synthase deficiency: A rare cause of adult-onset leukoencephalopathy. *Neurology* 79(4), 386-388.
609 <http://dx.doi.org/10.1212/wnl.0b013e318260451b>

610 Paripatananont, T., Lovell, R.T., 1995. Responses of channel catfish fed organic and inorganic sources
 611 of zinc to *Edwardsiella ictalurid* challenge. J. Aquat. Anim. Health 7(2), 147-154.
 612 [https://doi.org/10.1577/1548-8667\(1995\)007<0147:ROCCFO>2.3.CO;2](https://doi.org/10.1577/1548-8667(1995)007<0147:ROCCFO>2.3.CO;2)

613 Pearce, J., Jackson, N., Stevenson, M.H., 1983. The effects of dietary intake and of dietary concentration
 614 of copper sulphate on the laying domestic fowl: Effects on some aspects of lipid, carbohydrate and
 615 amino acid metabolism. Br. Poult. Sc. 24(3), 337-348. <https://doi.org/10.1080/00071668308416748>

616 Pedrazzoli, A., Molina, C., Montoya, N., Townsend, S., León-Hing, A., Parades, Y., Calderón, J., 1998.
 617 Recent advances on nutrition research of *Penaeus vannamei* in ecuador. Rev. Fish. Sci. 6(1-2), 143-
 618 151. <https://doi.org/10.1080/10641269891314258>

619 Peña, M.M.O., Lee, J., Thiele, D.J., 1999. A delicate balance: homeostatic control of copper uptake and
 620 distribution. J. Nutr. 129(7), 1251-1260. <https://doi.org/10.1093/jn/129.7.1251>

621 Puig, S., Thiele, D.J., 2002. Molecular mechanisms of copper uptake and distribution. Curr. Opin. Chem.
 622 Biol. 6(2), 171-180. [https://doi.org/10.1016/s1367-5931\(02\)00298-3](https://doi.org/10.1016/s1367-5931(02)00298-3)

623 Rao, M.S., Anjaneylu, N., 2008. Effect of copper sulfate on molt and reproduction in shrimp *Litopenaeus*
 624 *vannamei*. Int. J. Biol. Chem. 2, 35-41. <https://doi.org/10.3923/ijbc.2008.35.41>

625 Ruiz, L.M., Jensen, E.L., Rossel, Y., Puas, G.I., Gonzalez-Ibanez, A.M., Bustos, R.I., Ferrick, D.A.,
 626 Elorza, A.A., 2016. Non-cytotoxic copper overload boosts mitochondrial energy metabolism to
 627 modulate cell proliferation and differentiation in the human erythroleukemic cell line K562.
 628 Mitochondrion 29, 18-30. <https://doi.org/10.1016/j.mito.2016.04.005>

629 Sarker, S.A., Satoh, S., Kiron, V., 2005. Supplementation of citric acid and amino-acid chelated trace
 630 elements to develop environment-friendly feed for red sea bream, *Pagrus major*. Aquaculture 248,
 631 3-11. <https://doi.org/10.1016/j.aquaculture..04.012>

632 Shao, X., Liu, W., Xu, W., Lu, K., Xia, W., Jiang, Y., 2010. Effects of dietary copper sources and levels
 633 on performance, copper status, plasma antioxidant activities and relative copper bioavailability in
 634 *Carassius auratus gibelio*. Aquaculture 308(1-2), 60-65.
 635 <https://doi.org/10.1016/j.aquaculture.2010.07.021>
 636 Shi, B., Jin, M., Jiao, L., Betancor, M.B., Tocher, D.R., Zhou, Q., 2020. Effects of dietary Zn level on
 637 growth performance, lipolysis and expression of genes involved in the Ca^{2+} /CaMKK β /AMPK
 638 pathway in juvenile Pacific white shrimp. Br. J. Nutr. 1-31.
 639 <https://doi.org/10.1017/s0007114520001725>
 640 Sonnack, L., Klawonn, T., Kriehuber, R., Hollert, H., Schäfers, C., Fenske, M., 2018. Comparative
 641 analysis of the transcriptome responses of zebrafish embryos after exposure to low concentrations
 642 of cadmium, cobalt and copper. Comp. Biochem. Physiol, Part D: Genomics Proteomics 25, 99-108.
 643 <https://doi.org/10.1016/j.cbd.2017.12.001>
 644 Storey, J.D., Tibshirani, R., 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci.
 645 100(16), 9440. <https://doi.org/10.1073/pnas.1530509100>
 646 Sun, S., Qin, J., Yu, N., Ge, X., Jiang, H., Chen, L., 2013. Effect of dietary copper on the growth
 647 performance, non-specific immunity and resistance to *Aeromonas hydrophila* of juvenile Chinese
 648 mitten crab, *Eriocheir sinensis*. Fish Shellfish Immunol. 34(5), 1195-1201.
 649 <https://doi.org/10.1016/j.fsi.2013.01.021>
 650 Tan, X.Y., Luo, Z., Liu, X., Xie, C.X., 2011. Dietary copper requirement of juvenile yellow catfish
 651 *Pelteobagrus fulvidraco*. Aquacult. Nutr. 17(2), 170-176. <https://doi.org/10.1111/j.1365-2095>
 652 Tang, Q.Q., Feng, L., Jiang, W.D., Liu, Y., Jiang, J., Li, S.H., Kuang, S.Y., Tang, L., Zhou, X.Q., 2013.
 653 Effects of dietary copper on growth, digestive, and brush border enzyme activities and antioxidant

654 defense of hepatopancreas and intestine for young grass carp (*Ctenopharyngodon idella*). Biol.
655 Trace. Elem. Res. 155(3), 370-380. <https://doi.org/10.1007/s12011-013-9785-6>

656 Tijburg, L.B.M., Geelen, M.J.H., van Golde, L.M.G., 1989. Regulation of the biosynthesis of
657 triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. Biochim. Biophys.
658 Acta, Lipids Lipid Metab. 1004(1), 1-19. [http://dx.doi.org/10.1016/0005-2760\(89\)90206-3](http://dx.doi.org/10.1016/0005-2760(89)90206-3)

659 Tosco, A., Fontanella, B., Danise, R., Cicatiello, L., Grober, O.M.V., Ravo, M., Weisz, A., Marzullo, L.,
660 2010. Molecular bases of copper and iron deficiency-associated dyslipidemia: a microarray analysis
661 of the rat intestinal transcriptome. Genes Nutr. 5(1), 1-8. [https://doi.org/10.1007/s12263-009-0153-](https://doi.org/10.1007/s12263-009-0153-2)
662 2

663 Tsai, J.W., Ju, Y.R., Huang, Y.H., Deng, Y.S., Chen, W.Y., Wu, C.C., Liao, C.M., 2013. Toxicokinetics of
664 tilapia following high exposure to waterborne and dietary copper and implications for coping
665 mechanisms. Environ. Sci. Pollut. Res. 20(6), 3771-3780. [https://doi.org/10.1007/s11356-012-](https://doi.org/10.1007/s11356-012-1304-3)
666 1304-3

667 Vance, J.E., Tasseva, G., 2013. Formation and function of phosphatidylserine and
668 phosphatidylethanolamine in mammalian cells. Biochim. Biophys. Acta, Mol. Cell Biol. Lipids
669 1831(3), 543-554. <http://dx.doi.org/10.1016/j.bbalip.2012.08.016>

670 Wang, H., Li, E., Zhu, H., Du, Z., Qin, J., Chen, L., 2016. Dietary copper requirement of juvenile Russian
671 sturgeon *Acipenser gueldenstaedtii*. Aquaculture 454, 118-124.
672 <https://doi.org/10.1016/j.aquaculture.2015.12.018>

673 Wang, L., Feng, Z., Wang, X., Wang, X., Zhang, X., 2010. DEGseq: an R package for identifying
674 differentially expressed genes from RNA-seq data. Bioinformatics 26 (1), 136-138.
675 <https://doi.org/10.1093/bioinformatics/btp612>

676 Wang, T., Long, X., Chen, X., Liu, Y., Liu, Z., Han, S., Yan, S., 2017. Integrated transcriptome, proteome
 677 and physiology analysis of *Epinephelus coioides* after exposure to copper nanoparticles or copper
 678 sulfate. *Nanotoxicology* 11(2), 236-246. <https://doi.org/10.1080/17435390.2017.1290291>
 679 Wapnir, R.A., 1998. Copper absorption and bioavailability. *Am. J. Clin. Nutr.* 65(5), 1054S-1060S.
 680 <https://doi.org/10.1093/ajcn/67.5.1054S>
 681 Weisenberg, E., Halbreich, A., Mager, J., 1980. Biochemical lesions in copper-deficient rats caused by
 682 secondary iron deficiency. Derangement of protein synthesis and impairment of energy metabolism.
 683 *Biochem. J.* 188(3), 633-641. <https://doi.org/10.1042/bj1880633>
 684 Wellner, N., Diep, T.A., Janfelt, C., Hansen, H.S., 2013. N-acylation of phosphatidylethanolamine and
 685 its biological functions in mammals. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 1831(3), 652-
 686 662. <http://dx.doi.org/10.1016/j.bbalip.2012.08.019>
 687 Wu, W., Zong, R., Xu, J., Zhang, X., 2008. Antiviral phagocytosis is regulated by a novel rab-dependent
 688 complex in shrimp *penaeus japonicus*. *J. Proteome. Res.* 7(1), 424-431.
 689 <https://doi.org/10.1021/pr700639t>
 690 Yuan, H.X., Xiong, Y., Guan, K.L., 2013. Nutrient sensing, metabolism, and cell growth control. *Mol.*
 691 *Cell* 49(3), 379-387. <https://doi.org/10.1016/j.molcel.2013.01.019>
 692 Yuan, Y., Jin, M., Xiong, J., Zhou, Q., 2018. Effects of dietary dosage forms of copper supplementation
 693 on growth, antioxidant capacity, innate immunity enzyme activities and gene expressions for
 694 juvenile *Litopenaeus vannamei*. *Fish Shellfish Immunol.* <https://doi.org/10.1016/j.fsi.2018.10.075>
 695 Yuan, Y., Luo, J., Zhu, T., Jin, M., Jiao, L., Sun, P., Ward, T.L., Ji, F., Xu, G.Z., Zhou, Q.C., 2020a.
 696 Alteration of growth performance, meat quality, antioxidant and immune capacity of juvenile
 697 *Litopenaeus vannamei* in response to different dietary dosage forms of zinc: Comparative

advantages of zinc amino acid complex. Aquaculture 735120.
<https://doi.org/10.1016/j.aquaculture.2020.735120>

Yuan, Y., Wang, X., Jin, M., Jiao, L., Sun, P., Betancor, M.B., Tocher, D.R., Zhou, Q., 2020b. Modification of nutritional values and flavor qualities of muscle of swimming crab (*Portunus trituberculatus*): Application of a dietary lipid nutrition strategy. Food Chem. 125607.
<https://doi.org/10.1016/j.foodchem.2019.125607>

Zhang, Z., Tian, C., Zhou, S., Wang, W., Guo, Y., Xia, J., Liu, Z.M., Wang, B., Wang, X.W., Golding, B.T., Griff, B.T., Du, Y.S., Liu, J.Y., 2012. Mechanism-based design, synthesis and biological studies of N5-substituted tetrahydrofolate analogs as inhibitors of cobalamin-dependent methionine synthase and potential anticancer agents. Eur. J. Med. Chem. 58, 228-236.
<http://dx.doi.org/10.1016/j.ejmech.2012.09.027>

Zhou, Y., Rhodes, M.A., Liu, J., Davis, D.A., 2017. Effects of various dietary levels of copper hydroxychloride on growth performance and tissue response in Pacific white shrimp *Litopenaeus vannamei* fed practical diets. Aquacult. Nutr. 23(1), 171-180. <https://doi.org/10.1111/anu.12378>

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

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.

Fig. S4 The significantly changed KEGG 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.

736 **Table 1**

737 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

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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
<i>C-Cu vs. I-Cu</i>					
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
<i>C-Cu vs. O-Cu</i>					
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.

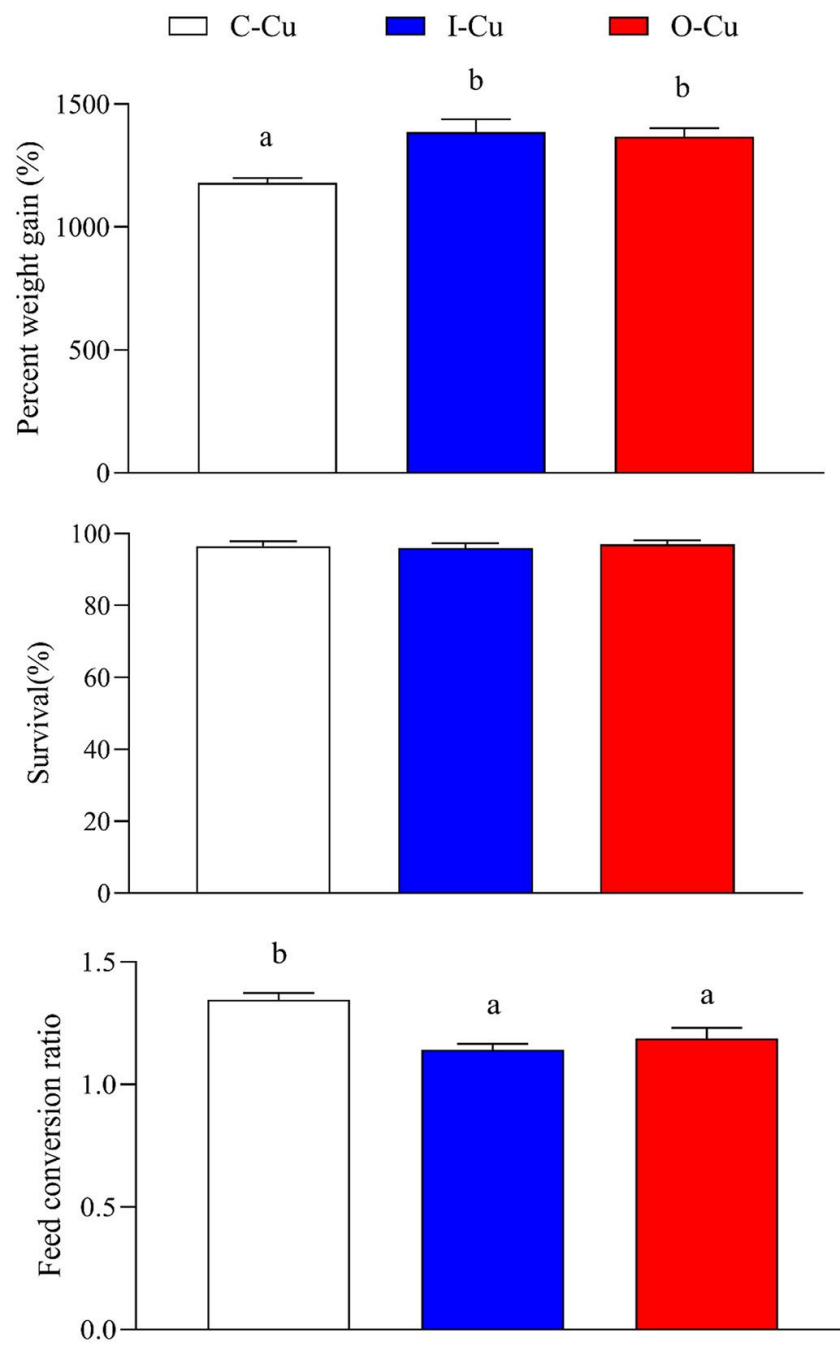


Figure 1

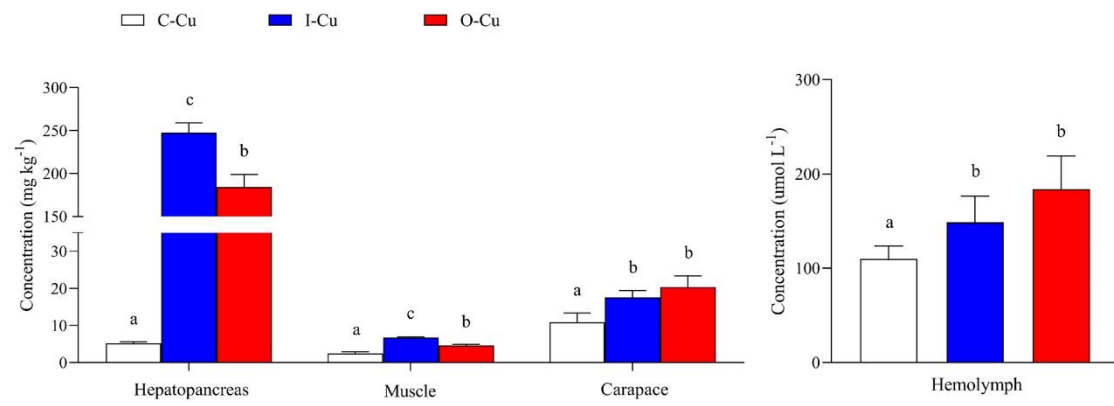


Figure 2

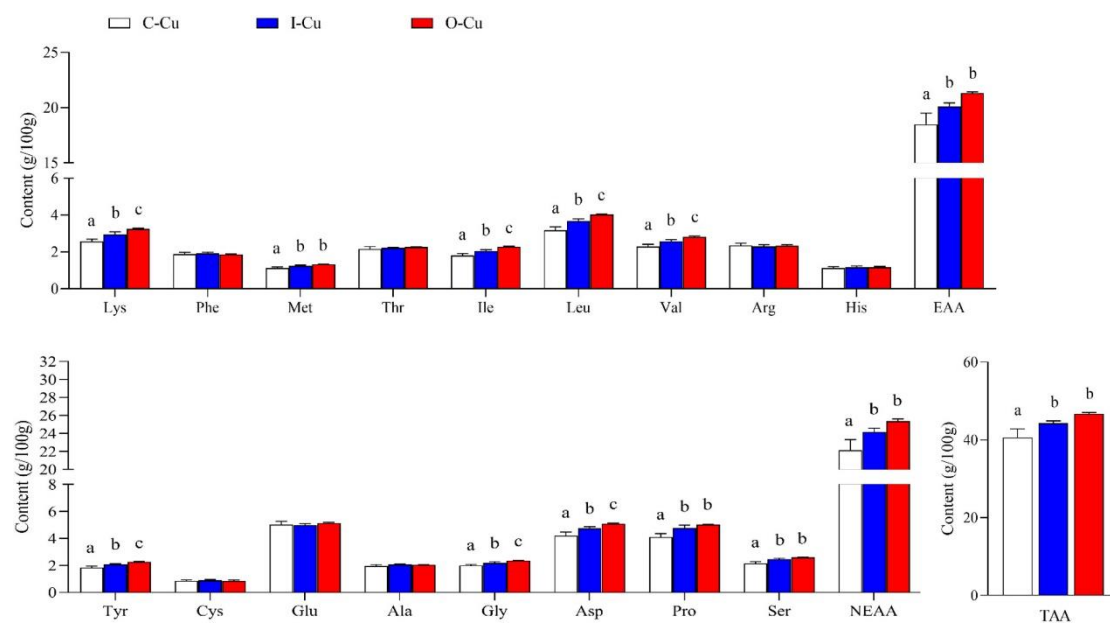


Figure 3

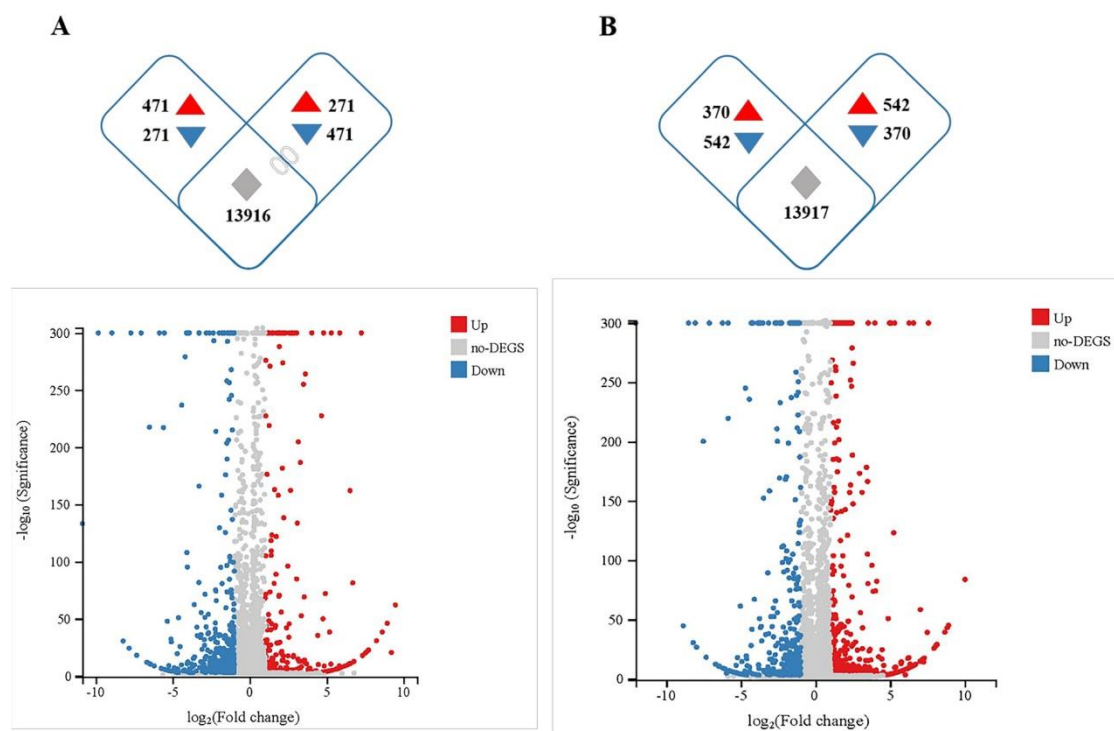


Figure 4

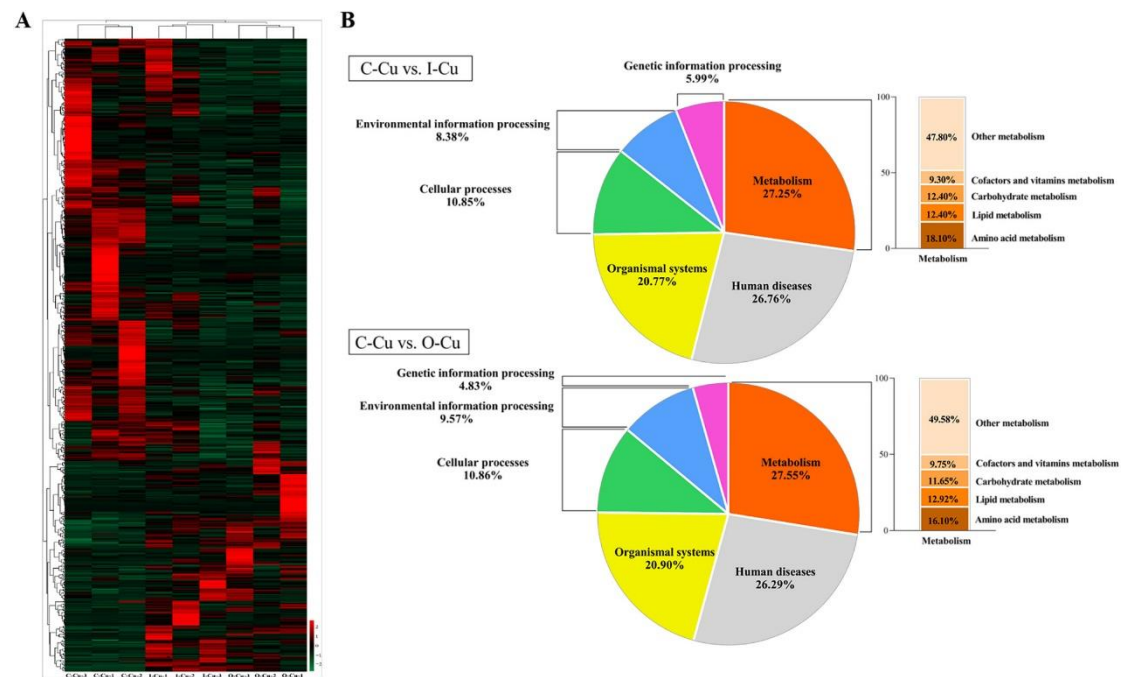


Figure 5

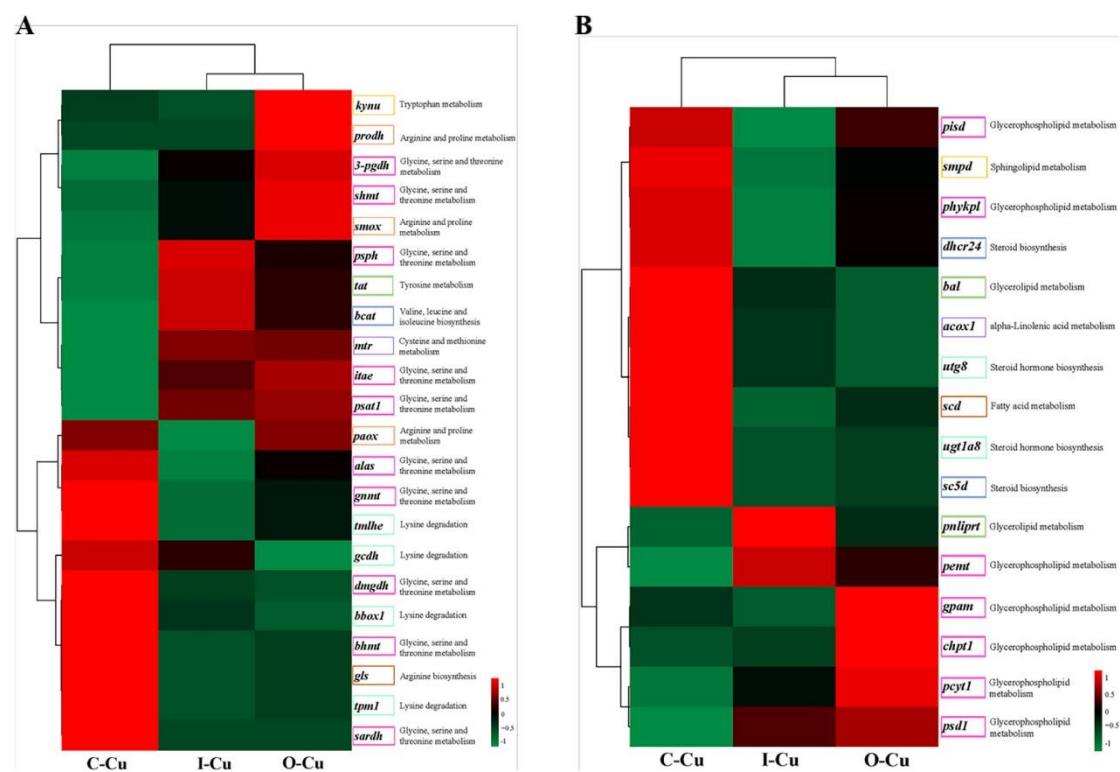


Figure 6

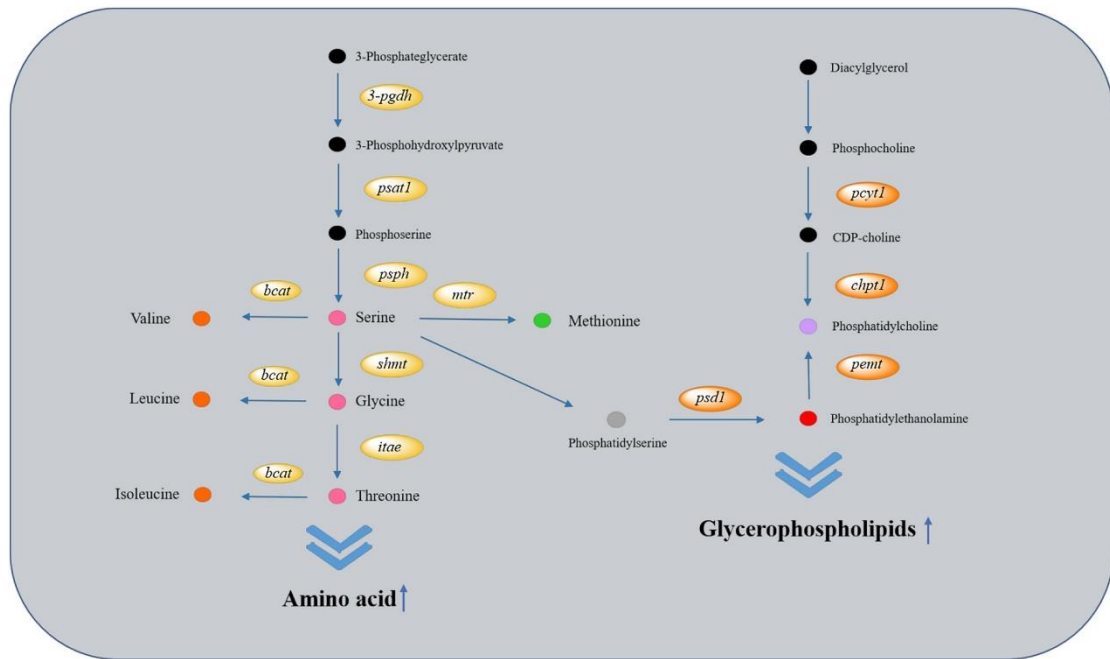


Figure 7

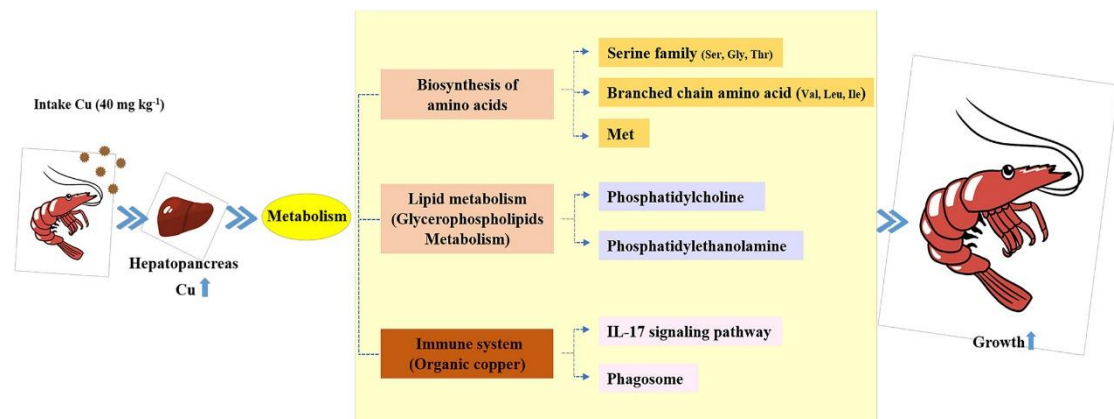


Figure 8

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 ₂ PO 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 ·5H ₂ O 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; K₂SO₄, 2.25; MgSO₄·7H₂O, 3.62; FeSO₄·7H₂O, 0.25; CaCO₃, 0.16; MnSO₄·H₂O, 0.12; ZnSO₄·7H₂O, 0.27; KIO₃ (1%), 0.02; Na₂SeO₃ (1%), 0.07; CoSO₄·7H₂O, 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- α -tocopherol, 100; cyanocobalamin, 0.1; biotin, 6.0; folic acid, 10; retinyl acetate, 5000 IU; cholecalciferol, 2000 IU

³ CuSO₄·5H₂O (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.
<i>β-actin</i>	F: CGAGGTATCCTCACCCCTGAA R: GTCATCTTCTCGCGGTTAGC	176	58.22 58.80	AF300705
<i>psat1</i>	F: CAAACAAAGTGTGAGACGCA R: AATGAAGACTGGAGATGGCA	184	57.18 56.52	XM_027352141
<i>shmt</i>	F: TTGGTCAGACAGTTGAAGCG R: TGATGATTGCGTAGACCTCG	208	58.42 57.51	XM_027371590
<i>mtr</i>	F: TATTCAACCTCGCATCCC R: CCGTCTAACAACCTTCG	252	54.00 53.13	XM_027364132
<i>tpm1</i>	F: GCCAACACAATCCTTAGCAA R: GACGCAAGGGACAGATGGTT	224	56.20 59.60	XM_027362037
<i>pcyt1</i>	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

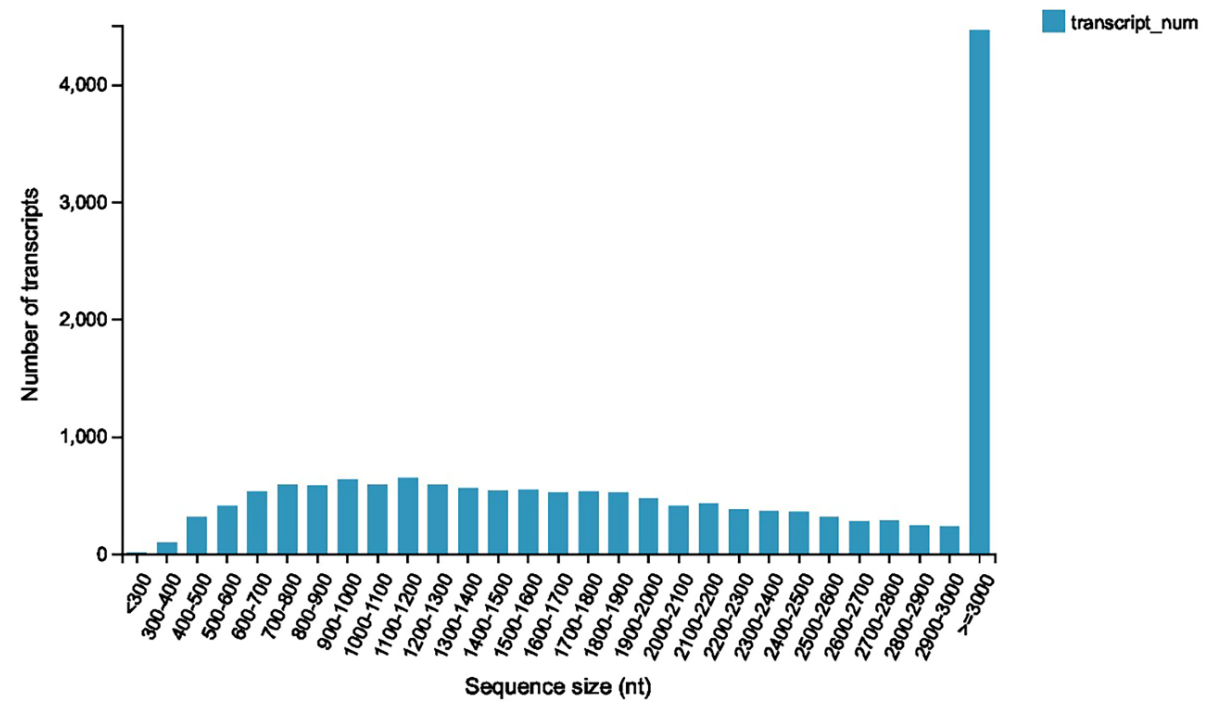


Fig. S1 Overview the distributions of *L. vannamei* transcriptome sequence length. The x-axis shows the transcript length, and y-axis shows the corresponding number of transcripts.

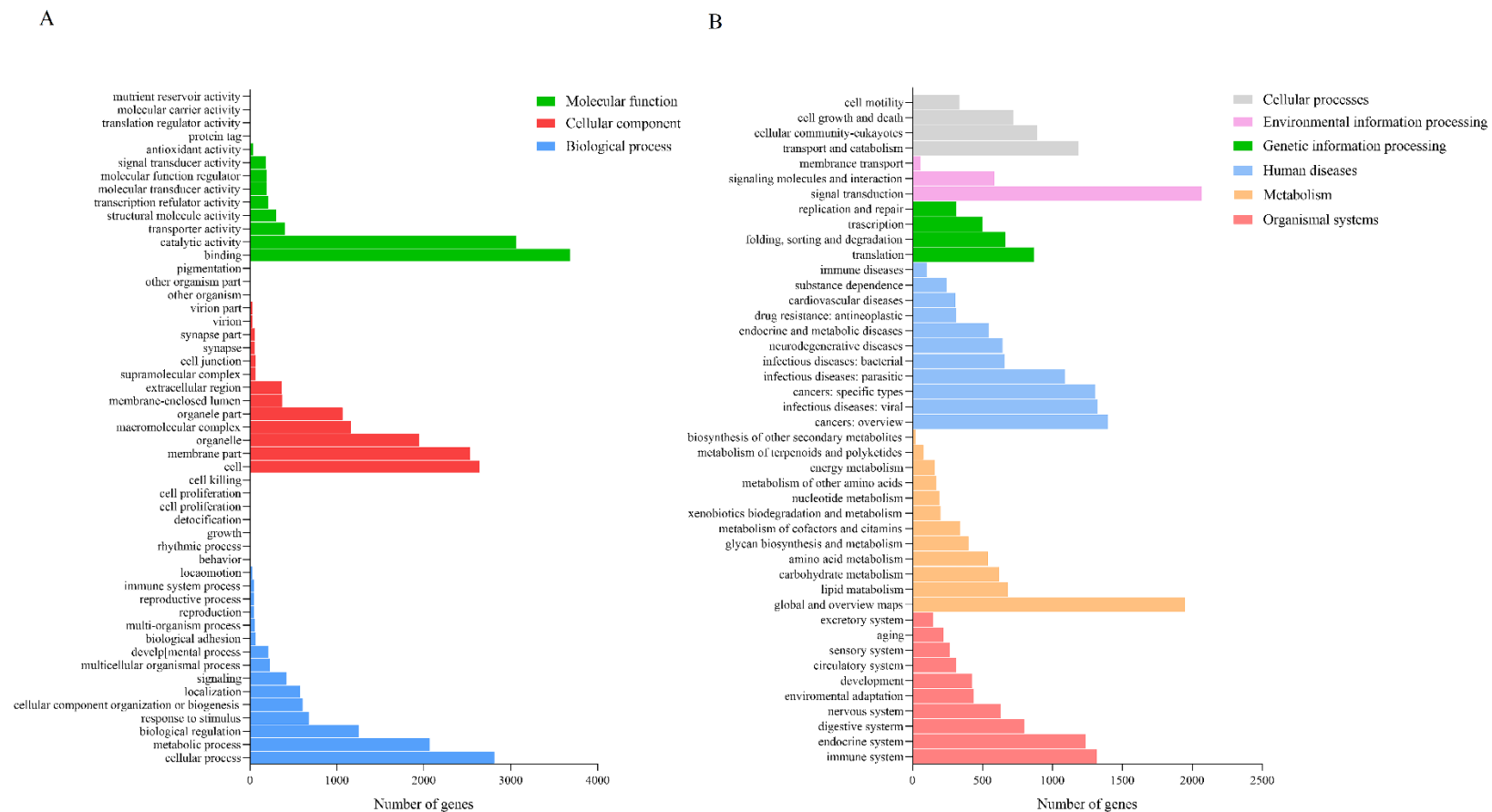
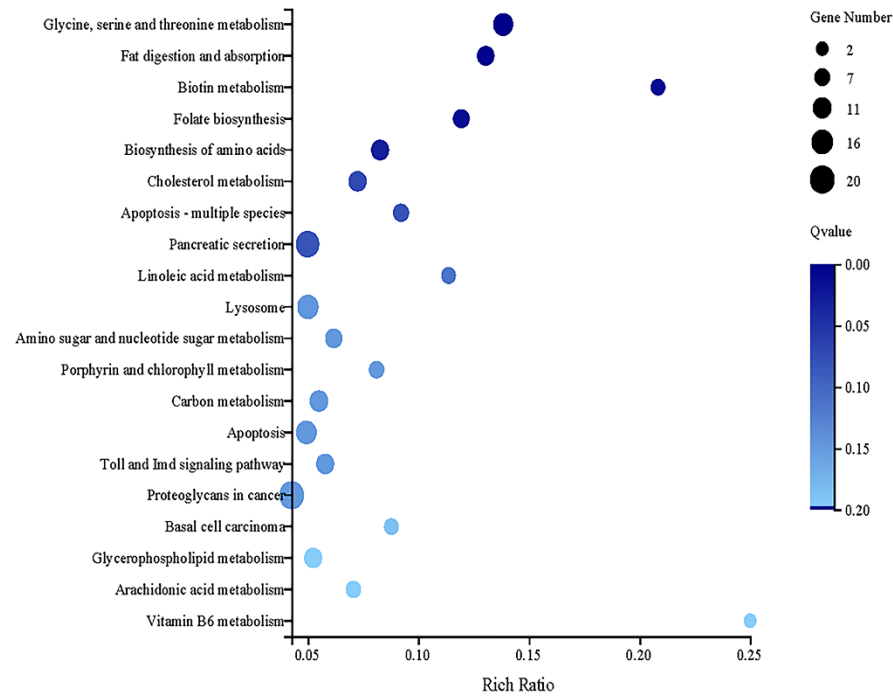


Fig. S2 Gene ontology (GO) annotations (A) and Kyoto encyclopedia of genes and genomes (KEGG) classification (B) of unigenes in hepatopancreas transcriptome of *L. vannamei*. The number of genes in each GO and KEGG subcategory are shown in x-axis, and y-axis shows the corresponding GO function and KEGG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

A



B

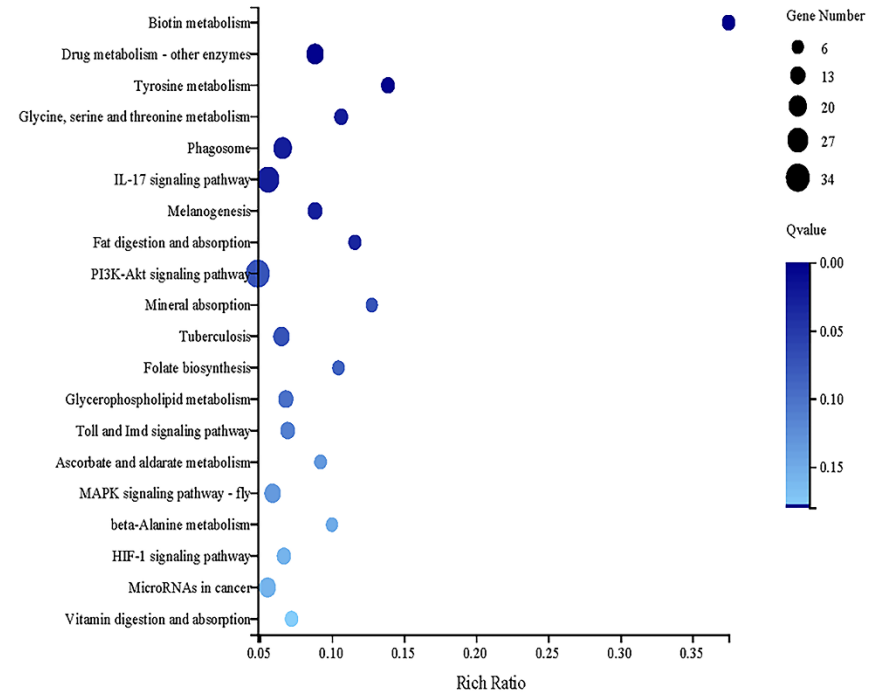


Fig. S3 KEGG pathway enrichment analysis of the top 20 pathways in C-Cu vs. I-Cu (A) and C-Cu vs. O-Cu (B). The x-axis indicates enrichment ratio, and the y-axis represents the top 20 pathways. The size of the bubble represents the number of DEGs in the corresponding pathway, and the color of the bubble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

[illegible][illegible]

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

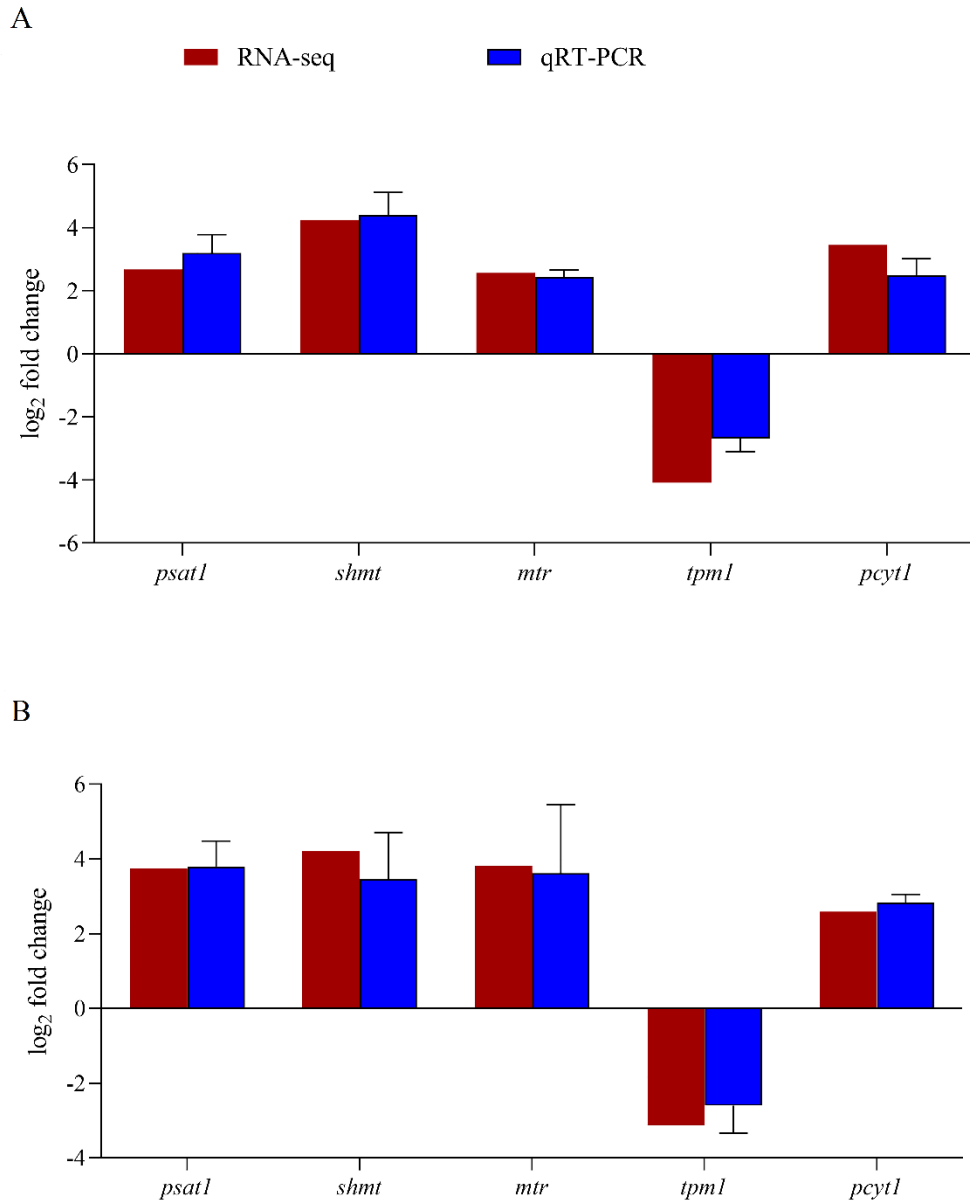


Fig. S5 Validation of differentially expressed genes by qRT-PCR in hepatopancreas transcriptome of *L. vannamei* (C-Cu vs. I-Cu (**A**) and C-Cu vs. O-Cu (**B**)). The red bars indicate RNA-seq, and blue bars indicate gene expression levels normalized against reference genes β -actin. Error bars indicate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

Abbreviations for Figure 6: *kyn*, 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; *psid*, 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 cytidyltransferase 1; *psd1*, phosphatidylserine decarboxylase 1