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Dietary supplementation of selenium yeast enhances the antioxidant capacity and immune response of juvenile *Eriocheir Sinensis* under nitrite stress

Xiaodan Wang[a], Zhenhua Shen[a], Chunling Wang[a], Erchao Li[a,b], Jian G. Qin[c], Liqiao Chen[a,*]

[a] Laboratory of Aquaculture Nutrition and Environmental Health, School of Life Sciences, East China Normal University, Shanghai, 200241, China
[b] Department of Aquaculture, College of Marine Sciences, Hainan University, Haikou, Hainan, 570228, China
[c] School of Biological Sciences, Flinders University, Adelaide, SA, 5001, Australia

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ABSTRACT

This study elucidates the response to nitrite stress and the effect of dietary selenium supplements on the growth, antioxidant activity, immunity and transcriptome of juvenile Chinese mitten crab *Eriocheir sinensis*. In the control group, the crabs were fed the diet without selenium supplementation and there was no nitrite addition to the water. In the test group, the crabs were fed diets with three levels of selenium 0 (N1), 0.5 (N2) and 1.0 (N3) mg/kg in the water containing 2 mg/L NO_2—N as a stress factor for eight weeks. Feed conversion ratio (FCR) was improved by adding dietary selenium. There was no significant difference in specific growth rate and weight gain between N1 and the control groups, or among different selenium levels in the test group. The superoxide dismutase (SOD) activity was significantly lower, but malondialdehyde (MDA) was higher in the N1 group than those in the serum and hepatopancreas of the control group. The activities of SOD, glutathione peroxidase (GPx) and acid phosphatase increased at the medium level of selenium but decreased as the level of dietary selenium increased to 1.0 mg/kg. The serum lysozyme (LZM) activity increased but the MDA content in both serum and hepatopancreas decreased with the increase of selenium levels. The total clean reads of the crabs in the control group, N1 and N3 groups reached 390.7M and were assembled into 106 471 transcripts. Compared with the control group, 1196 gene were significantly expressed (588-up and 608-down) in the N1 group under nitrite stress. Between the N1 and N3 groups, the expression of 1537 genes (751-up and 786-down) were significantly different. KEGG pathway analysis reveals that 11 and 19 pathways were significantly different between N1 and control and between N3 and N1 groups, respectively. Transcriptome results demonstrate that nutrient metabolism is much more active in crabs fed additional selenium under nitrite stress. This study indicates that dietary selenium can improve both antioxidant capacity and immune response and alter the protein and carbohydrate metabolism of *E. sinensis* under nitrite stress.

1. Introduction

Nitrite (NO_2^-) is an important component in the nitrogen cycle in ecosystems since it is an intermediate product of the bacterial nitrification and denitrification processes [1]. It can be accumulated in aquatic systems and becomes toxic to aquatic animals in intensive aquaculture. Decapod crustaceans release ammonia as nitrogenous waste in protein catabolism, which can be converted to nitrite to deteriorate water quality [2,3]. The Chinese mitten crab *Eriocheir sinensis* is an important species in aquaculture and its production reached 796 622 metric tons in 2014, with a value of over 5.5 billion US dollars [4,5]. However, with the increased demand and development of intensive aquaculture, nitrite pollution has become a serious threat to crab health and survival in aquaculture.

The problem of nitrite for freshwater fish and crustaceans is derived from the fact that NO_2^- competes with the Cl^- uptake mechanism in the gills, and part of Cl^- uptake would be replaced by NO_2^- uptake when nitrate is high in the environment [1,6]. Nitrite would be accumulated in the blood plasma, liver, gills, brain and muscle, and its concentration in the blood could be more than 60 times higher than the surrounding medium [6]. In crustacean, nitrite diffuses and oxidises copper in hemocyanin, converting to meta-hemocyanin, which impairs the ability to deliver oxygen to other tissues [7]. This will suppress the immune response, increase susceptibility to pathogens, and induce

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[*] Corresponding author.
E-mail address: lqchen@bio.ecnu.edu.cn (L. Chen).

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apoptosis in hemocytes mediated by reactive oxygen species (ROS) [8–11]. Unlike vertebrates, crustaceans lack an acquired immune system in the true sense, and they must rely on non-specific immune mechanism to deal with environmental stresses [12]. Hemocytes play an important role in the innate immunity of crustaceans, and total hemocyte counts were used as stable immune parameter to evaluate the stress response [13].

Selenium is an effective exogenous antioxidant to help the removal and prevention of oxidative stress [14]. Moreover, selenium is an integral component of glutathione peroxidase (GPx) that plays a crucial role to reduce cellular damage by ROS [15,16]. There are two forms inorganic and organic selenium, and organic selenium has higher bioavailability in improving growth and antioxidant capacity [17,18]. Although the functions of selenium in regulating immunity, disease resistance, stress remission and improvement of nutritional quality in *E. sinensis* have been investigated [19,20], there is little research to reveal the integrated molecular mechanism on the negative effect of nitrite stress and the beneficial effect of selenium in crab.

The ability to cope with environmental stress is largely dependent on the capacity of transcriptomic response and gene expression [21,22]. RNA sequencing (RNA-Seq) has been widely applied to the study of mapping and quantifying transcriptome [23]. This powerful new technology provides a platform to study the genetic and molecular response to a challenging environment for a species [24]. In crustaceans, the hepatopancreas is a vital detoxicating center for xenobiotics, and it is also a crucial metabolic center for eliminating excess ROS in the immune system [25,26]. Therefore, in the current study, the hepatopancreas was used for transcriptome analysis to investigate the transcriptional response in the crab to nitrite stress. In the present study, the growth performance, antioxidant capacity, immune response and transcriptional response were analyzed to evaluate the comprehensive response of *E. sinensis* to nitrite stress and the role of selenium in alleviating the stress impact.

2. Materials and methods

2.1. Experimental diets

Three isonitrogenous practical diets (35.8% crude protein and 7.7% crude lipid) were formulated with three concentrations of selenium (0, 0.5 and 1.0 mg/kg diet, which were named as N1, N2 and N3) in the form of selenium yeast. Fish meal, soybean meal and cottonseed meal were used as the main protein sources. Lysine and methionine were added to adjust the balance of amino acids in the diets (Table 1). Dietary ingredients were ground with 80-μm mesh and all dry ingredients were finely ground and mixed thoroughly before adding oil and water. Diets were wet-extruded into 2.5-mm-diameter pellets using a double helix plodder (F-26, SCUT industrial factory, Guangdong, China), air-dried at room temperature to a moisture content of less than 10%, sieved to various sizes by 6, 10 and 12 mesh sieves and stored at −20 °C until use.

2.2. Experimental animals and management procedure

Juvenile *E. sinensis* were obtained from a local crab company, Shanghai, China. All crabs were acclimated in tanks (300 L) in the Biological Station of East China Normal University for one week. Six hundred healthy crabs (2.19 ± 0.05 g) were randomly assigned to 20 tanks (250 L) with corrugated plastic pipes as shelters to reduce attacking behavior (12 cm long and 25 mm diameter). There was one control and three selenium treatments (N1, N2 and N3) with five replicate tanks, and each tank contained 30 crabs. The crabs in the control group were fed with 0 mg/kg selenium diet without nitrite stress in the ambient water, while the NO_2—N concentration of the other 15 tanks (N1, N2 and N3) were kept at 2 mg/L by adding sodium nitrite. During the experiment, crabs were fed twice daily at 09:00 a.m. and 6:00 p.m., and daily rations were adjusted to be slightly over satiation based on the amount of feed left in the previous day. Two hours after feeding, uneaten diet was removed with a siphon tube. Daily water exchange rate was 1/3 of the tank volume. The incoming fresh water was aerated thoroughly before entering the water recirculation system. The concentration of NO_2—N was measured and adjusted every 8 h. The water quality parameters across all feeding treatments were maintained at 22.0–25.6 °C, 7.2–8.7 pH, dissolved oxygen > 8.5 mg/L and ammonia-N < 0.05 mg/L.

Table 1
Ingredient formulation (g/kg dry basis) and proximate composition (%) of the basal diet.

Ingredients	g/kg	Ingredients	g/kg
Fish meal	250	Cholesterol	5
Soybean meal	190	Lecithin	5
Cottonseed meal	190	Choline chloride	5
Microcystic lysine	4	Vitamin premix[a]	20
Microcystic methionine	6	Mineral premix[b]	30
Corn starch	150	Cellulose	85
Fish oil	20	Sodium carboxymethyl cellulose	20
Soybean oil	20		
Proximate composition (%)			
Crude protein	35.8	Crude lipid	7.77

[a] Vitamin premix: Vitamin premix (per 100 g premix): retinol acetate, 0.043 g; thiamin hydrochloride, 0.15 g; riboflavin, 0.0625 g; Ca pantothenate, 0.3 g; niacin, 0.3 g; pyridoxine hydrochloride, 0.225 g; *para*-aminobenzoic acid, 0.1 g; ascorbic acid, 0.5 g; biotin, 0.005 g; folic acid, 0.025 g; cholecalciferol, 0.0075 g; α-tocopherol acetate, 0.5 g; menadione, 0.05 g; inositol, 1 g. All ingredients are filled with α-cellulose to 100 g.

[b] Mineral premix: Mineral premix (per 100 g premix): KH_2PO_4, 21.5 g; NaH_2PO_4, 10.0 g; $Ca(H_2PO_4)_2$, 26.5 g; $CaCO_3$, 10.5 g; KCl, 2.8 g; $MgSO_4 \cdot 7H2O$, 10.0 g; $AlCl_3 \cdot 6H_2O$, 0.024 g; $ZnSO_4 \cdot 7H2O$, 0.476 g; $MnSO_4 \cdot 6H_2O$, 0.143 g; KI, 0.023 g; $CuCl2 \cdot 2H_2O$, 0.015 g; $CoCl2 \cdot 6H_2O$, 0.14 g; Calcium lactate, 16.50 g; Fe-citrate, 1 g. All ingredients are diluted with α-cellulose to 100 g.

2.3. Sample collection

At the end of the eight-week trial, all crabs in each tank were counted and deprived of feed for 24 h to evacuate the gut content before body-weight determination. Crabs were anesthetized on ice for 10 min, and six crabs were sampled in each tank. The 1-ml syringe with 1:1 pre-cooled anticoagulant solution (0.20 mol/L NaCl, 0.17 mol/L glucose, 50.00 mmol/L sodium citrate, 43.33 mmol/L citric acid, 16.67 mmol/L EDTA·2Na, pH6.5) was used to collect the hemolymph from the base of last walking legs of the crab. Part of hemolymph samples was used to count the number of total hemocytes, and the rest were centrifuged at 2800 × *g* for 20 min at 4 °C to collect the serum. The crabs were dissected to obtain the hepatopancreas, and all hepatopancreases and serum samples were stored at − 80 °C for further biochemical and molecular analyses. The use of animals in this research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

2.4. Growth performance

The indexes for the assessment of growth performance were calculated as follows:

Weight gain rate (%) = 100 × (Wt− W0)/W0, where, W0 is the initial weight and Wt is the final weight;

Crab survival rate (%) = 100 × (final number)/(initial number);

Feed conversion ratio (FCR) = dry feed weight/wet weight gain;

2.5. Total hemocyte counts (THC)

THC were obtained on a hemocytometer. Each hemolymph sample was repeated three times and the mean value was recorded for

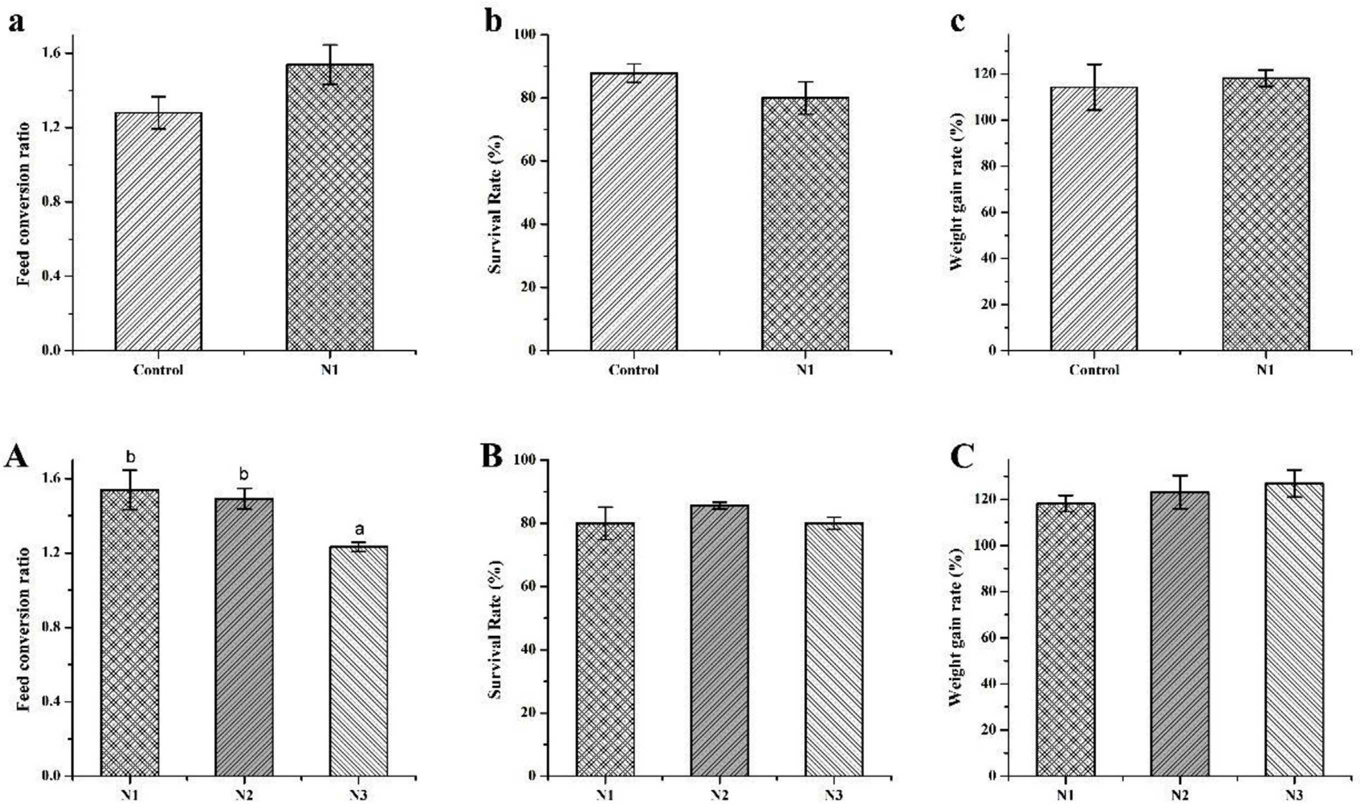

Fig. 1. Effects of nitrite stress and dietary selenium on feed conversion ratio (a, A), survival rate (b, B) and weight gain rate (c, C) of E. sinensis. The results were presented as mean ± SE and different lowercase letters mean significant differences by Duncan's test ($P < 0.05$). Control: control group; N1: 0 mg/kg dietary selenium with nitrite stress; N2: 0.5 mg/kg dietary selenium with nitrite stress; N3: 1.0 mg/kg dietary selenium with nitrite stress.

statistical analysis.

2.6. Biochemical analysis

The superoxide dismutase (SOD), malondialdehyde (MDA) and GPx of serum and hepatopancreases were measured by the iodine starch colorimetric method following the protocols of commercial assay kits. The acid phosphatase (ACP), alkaline phosphatase (ALP) and lysozyme (LZM) activity of serum were also tested by commercial kits by the disodium phenyl phosphate hydrate and hydrolysis method.

2.7. RNA extraction, library conduction and Illumina sequencing

The hepatopancreases of crabs in the control, N1 and N3 groups were chosen for the RNA-seq analysis. Total RNA of the hepatopancreases was extracted using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen), and the extracted RNA was treated with DNase I (Takara, Japan) to remove genomic DNA. The quality and quantity of total RNA were assessed by OD260/OD280 using a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, DE, USA).

The RNA-seq transcriptome library was prepared following the TruSeq™ RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA. The mRNA was isolated according to the poly A selection method using Oligo (dT) beads and then was fragmented using fragmentation buffer.

Single-stranded cDNA was synthesized with random hexamers using RNA as a template. Double-stranded cDNA was synthesized with the effect of dNTPs, DNA polymerase I, RNase H and buffer, and it was purified by AMPure XP beads. A single (A) was added using the Klenow buffer.

Adaptor-modified fragments were selected by AMPure XP beads, and PCR amplification was performed for 15 cycles. After being quantified by Qubit 2.0, the sequencing library was diluted to 1.5 ng/μl. The insert size of the library was tested by Agilent 2100, and was quantified by the Q-PCR method to check the quality of the sequencing library. The RNA-seq sequencing library was sequenced using Illumina HiSeq 4000. The SRA number for data uploaded into NCBI was SRP141254.

2.8. Gene expression analysis and functional enrichment

As there is no reference genome for the Chinese mitten crab, the sequenced reads were spliced using Trinity first (Grabherr et al., 2011), and was taken the hierarchical cluster analysis by Corset (https://code.google.com/p/corsetproject/) (Nadia M Davidson, Alicia Oshlack, 2014). To identify differential expression genes between the two different treatments in two tissues, RSEM (http://deweylab.biostat.wisc,edu/rsem/) was used to quantify gene abundance. The expression level of each transcript was calculated according to the Fragments Per Kilobase of transcript sequence per million base pairs sequenced (FRKM) method. Differential expression analysis was conducted using DESeq2 with p-value ≤ 0.05. Gene Ontology (GO) analysis (http://www.geneontology.org/) was performed to facilitate elucidating the biological implications of unique genes in the significant or representative profiles of the differentially expressed gene. The KEGG (Kyoto Encyclopedia of Genes and Genomes) was performed for functional-enrichment analysis in the metabolic pathways at FDR ≤ 0.05. The KEGG pathway analysis was carried out using KOBAS (http://kobas.cbi.pku.edu.cn/home.do).

2.9. Statistical analysis

All results were tested for normality and homogeneity of variance by Levene's equal variance test. Two hypotheses were tested for this study. T-test was used between the control and N1 groups to test if the nitrite level of 2 mg/L could have a significant impact on the performance of crabs. Then, a one-way ANOVA was used between N1, N2 and N3 groups to test if supplementation of selenium could alleviate the impact of nitrite stress on crabs, using SPSS 20.0 (IBM, Armonk, NY, USA) followed by Duncan's multiple range tests. Data were presented as means ± standard error (SE), and the value of $P < 0.05$ was considered as statistical significance (*) and $P < 0.01$ as greatly significant (**).

3. Results

3.1. Growth performance

The FCR of crabs in the N1 group was significantly higher than the control group, and it decreased with the supplementation of dietary selenium (Fig. 1A, a). The FCR of crabs in the N3 group was significantly lower than that in the N1 and N2 groups. There was neither significant difference in SR and WG between the control and N1 groups, nor among N1, N2 and N3 groups (Fig. 1b, B, c, C). But the WG showed an increase tendency with the increase of selenium supplementation (Fig. 1C). The WG of crabs in the N2 and N3 groups were higher than in the control group, though they were not statistically significant.

3.2. Antioxidant capacity of serum and hepatopancreases

The SOD activity was significantly lower and the MDA were higher in the N1 group than that in the control group in both tissues (Fig. 2A, B, a, b). The hepatopancreases GPx activity was significantly higher in the N1 group than in the control, while no significant difference was observed in the serum (Fig. 2C, c). The SOD activity was first increased and then decreased with the increase of dietary selenium in both serum and hepatopancreases (Fig. 3A, a). The MDA content decreased with the supplementation of selenium in both tissues (Fig. 3B, b). The GPX activity was also first increased and then decreased with the increase of dietary selenium (Fig. 3C, c).

3.3. Immune status

Although the THC was lower in the N1 group than that in the control group, there was no significant difference (Fig. 4a). The LZM, ACP and AKP activities in the N1 group were much lower than those in the control group (Fig. 4b, c, d). Dietary selenium supplementation did not affect the THC, but THC showed an increasing trend with the increase of selenium (Fig. 4A). On the other hand, the LZM activity increased with the increase of dietary selenium (Fig. 4B). The ACP also showed a first increase and then a decrease tendency with the increase of selenium, but the activity in the N3 group was still higher than that in the N1 group (Fig. 4C) No significant difference was observed on the AKP activity among N1, N2 and N3 groups (Fig. 4D).

3.4. Transcriptome sequencing and de novo assembly

A total of 138.2M, 128.1M and 124.4M clean reads were obtained from the control, N1 and N3 respectively after the removal of low-quality reads. The mean GC (%) of these three groups was 52.20%, 52.17% and 51.15%, respectively. In total, 106 471 transcripts were obtained and analyzed by *de novo* assembly. The summary of RNA-Seq results is shown in Table 2 and the accession number of *de novo* was SRP141254.

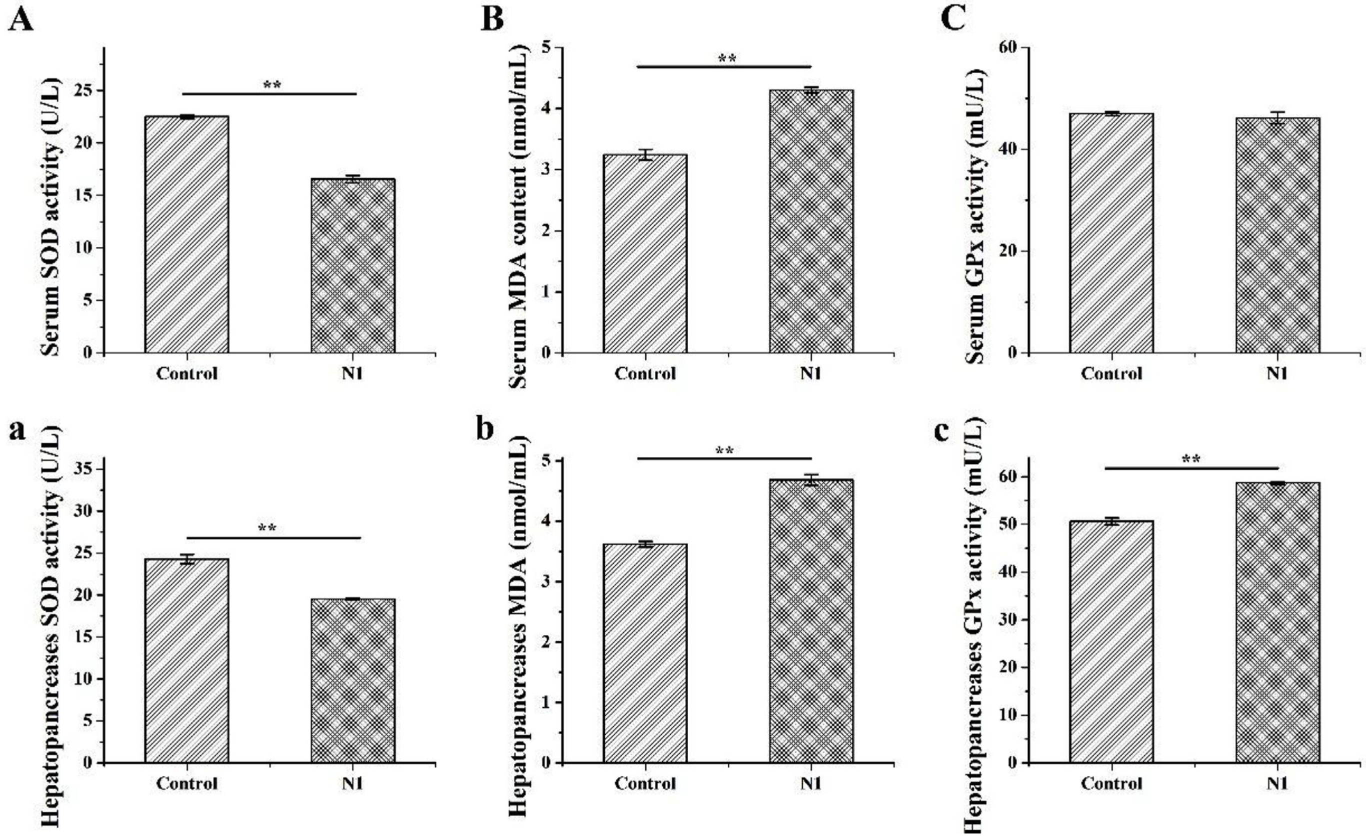

Fig. 2. Effects of nitrite stress on the activities of superoxide dismutase (SOD) (A, a), malondialdehyde (B, b) and glutathione peroxidase (C, c) in the serum and hepatopancreases of E. sinensis. Capital letter represents the enzyme activity in serum and lowercase number represents the activity in hepatopancreases. The results were presented as mean ± SE (n = 9) and different lowercase letters above each column mean significant differences by Duncan's test ($P < 0.05$). Control: control group; N1: 0 mg/kg dietary selenium with nitrite stress.

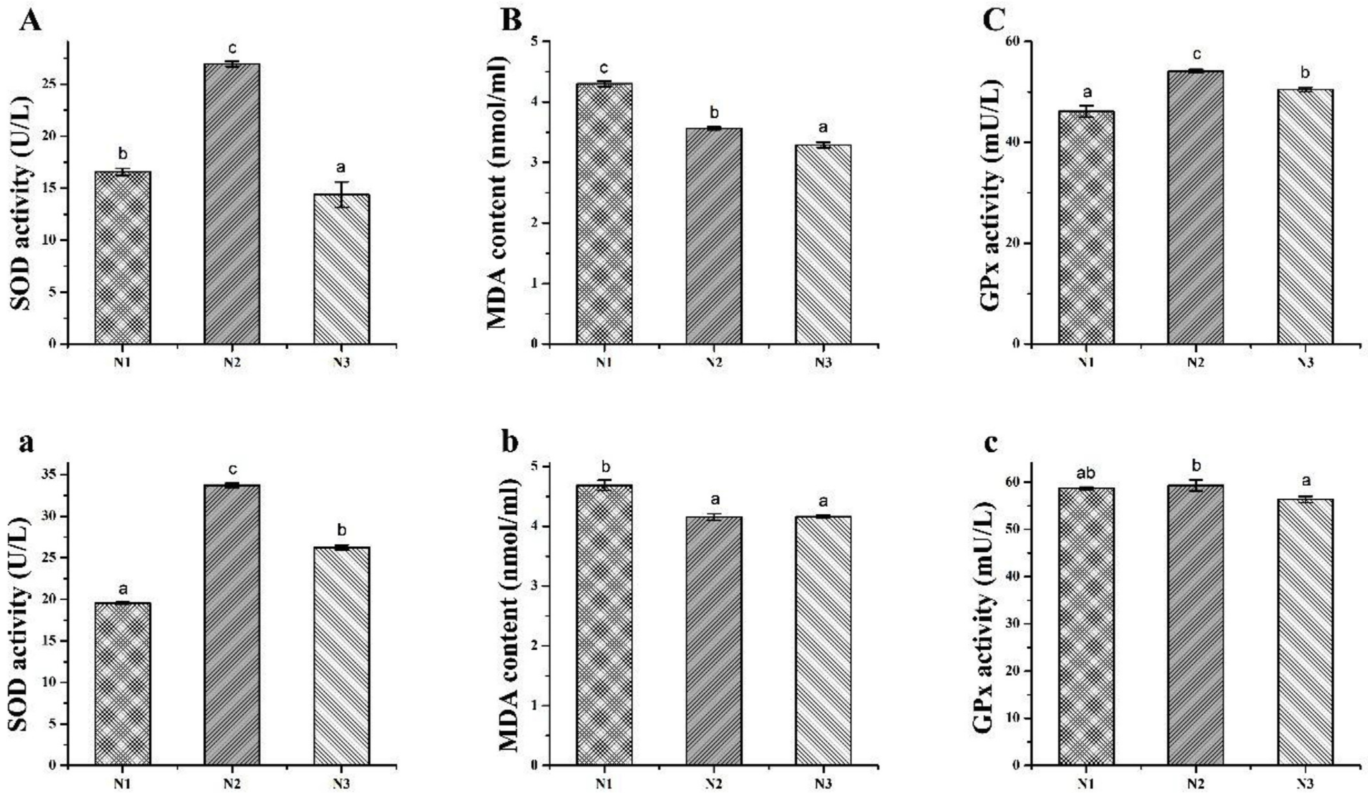

Fig. 3. Effects of dietary selenium on the activities of Superoxide dismutase (SOD) (A, a), malondialdehyde (B, b) and glutathione peroxidase (C, c) in the serum and hepatopancreases of E. sinensis. Capital letter represents the enzyme activity in serum and lowercase number represents the activity in hepatopancreases. The results were presented as mean ± SE (n = 9) and different lowercase letters above each column mean significant differences by Duncan's test (P < 0.05). N1: 0 mg/kg dietary selenium with nitrite stress; N2: 0.5 mg/kg dietary selenium with nitrite stress; N3: 1.0 mg/kg dietary selenium with nitrite stress.

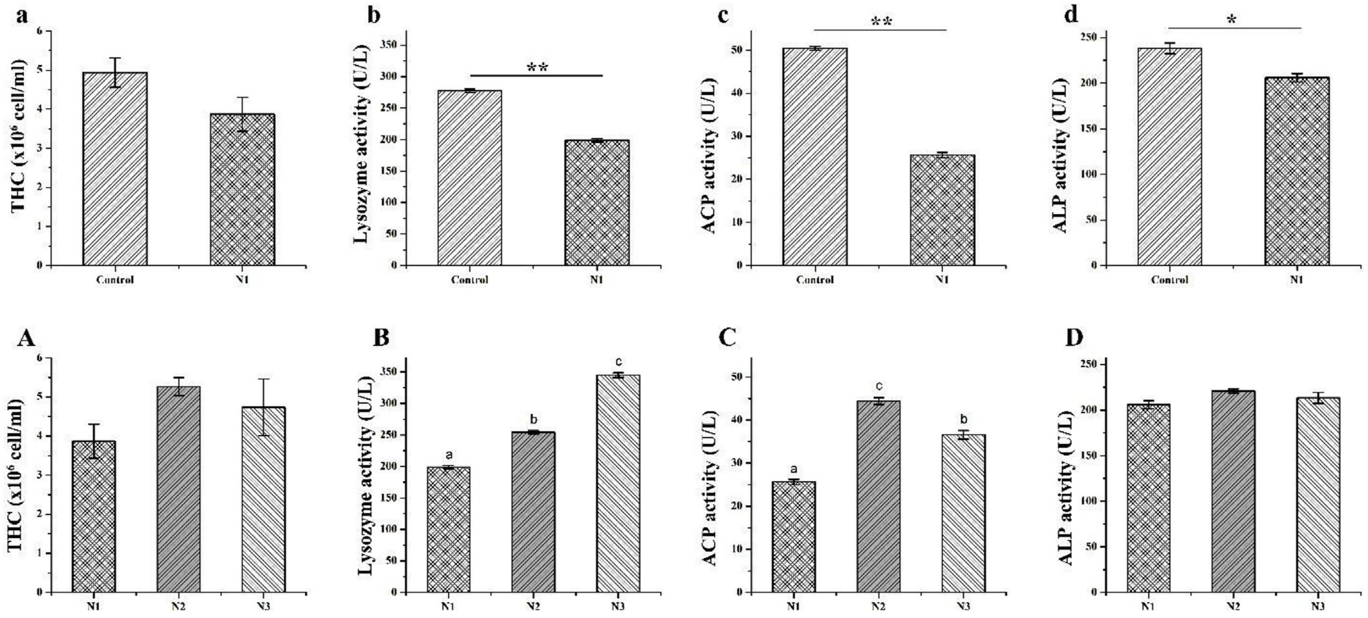

Fig. 4. Effects of nitrite stress and dietary selenium on the total hemocyte counts (a, A), lysozyme (b, B), the acid phosphatase (ACP) (c, C) and the alkaline phosphatase (ALP) (d, D) in the serum of E. sinensis. The results were presented as mean ± SE (n = 9) and different lowercase letters mean significant differences by Duncan's test (P < 0.05). Control: control group; N1: 0 mg/kg dietary selenium with nitrite stress; N2: 0.5 mg/kg dietary selenium with nitrite stress; N3: 1.0 mg/kg dietary selenium with nitrite stress.

3.5. Analysis of gene expression

The mean mapping ratio of the control, N1 and N3 was 69.38%, 70.02% and 66.92% respectively. The expression of 1196 genes (588-up and 608-down) were significantly different in the hepatopancreases of the crab between N1 and the control groups (N1 vs Control) ($P < 0.05$, Fig. 5A, a). The expression of 1537 genes (751-up and 786-down) were significantly different between N1 and N3 groups (Fig. 5B,

Table 2
Basic information of the transcriptome analysis.

	Min length (bp)	Median length (bp)	Max length (bp)	Mean length (bp)	N50 (bp)	N90 (bp)	Total nucleotides (bp)	Total numbers
Transcripts	201	364	21 201	714	1241	273	118 114 867	165 452
Genes	201	562	21 201	974	1584	404	101 271 435	103 960

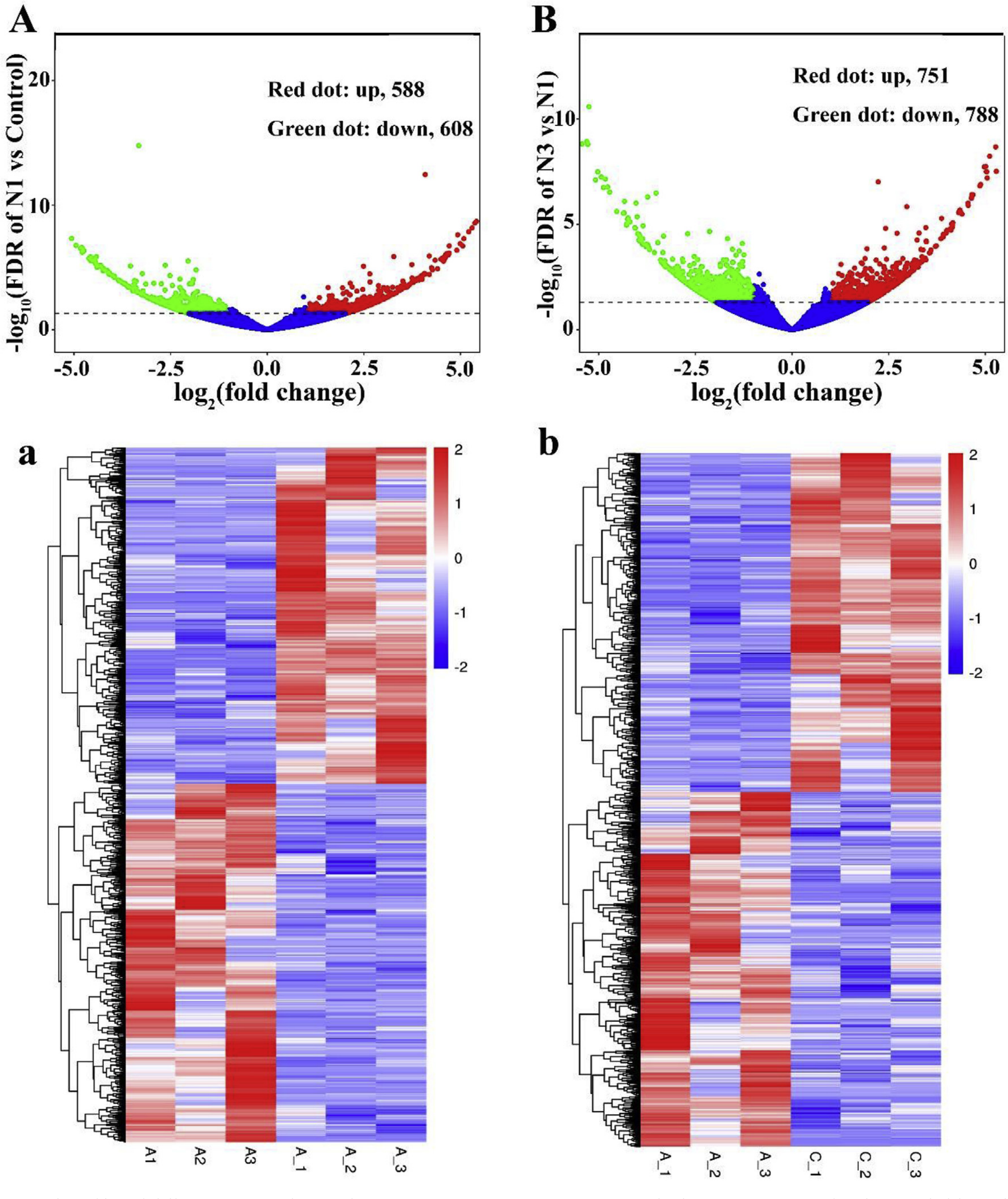

Fig. 5. Transcriptional profiles of different expressed genes between two pair-wise comparisons in the hepatopancreases of crabs revealed by Volcano plots and heatmaps. A and a: N1 vs control group; B and b: N3 vs N1 group. For volcano plots, the X-axis represents log2 (Fold change), and the Y-axis is -log10(P-value). The differently expressed genes are shown as green and red (log2Fold change > 1, p-value < 0.05). The lower panels a and b are heatmaps between two pair-wise comparison based on FPKM units. The color key represents FPKM normalized log2 transformed counts. Each column presents a replicate of each group, and each row represents a gene. A represents the control group; C_ is the N3 group; A_ is the N1 group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

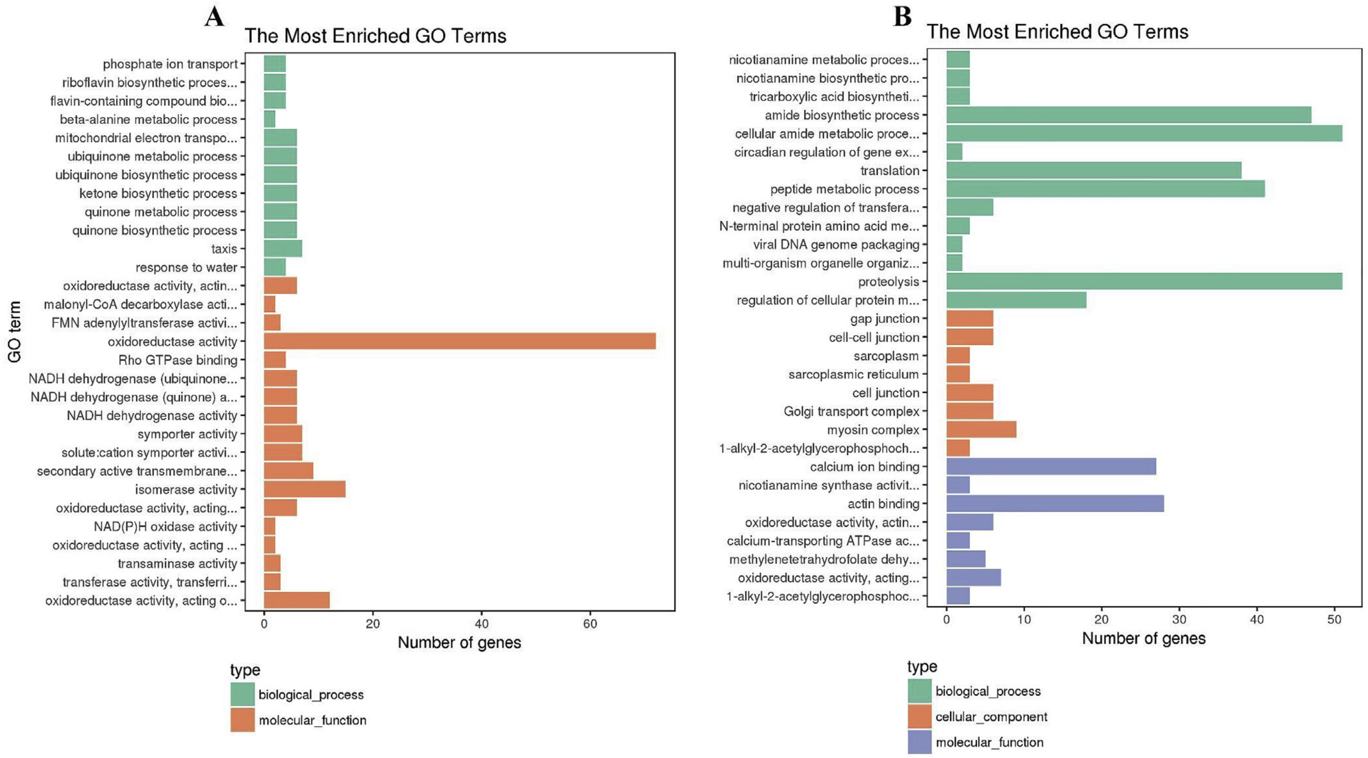

Fig. 6. Gene Ontology (GO) terms for the transcriptomic sequences of E. sinensis under nitrite stress. A: N1 vs Control group; B: N3 vs N1.

b).

3.6. GO and KEGG analysis

Based on GO analysis by GOseq, the functions of the differently expressed genes could be classified into three main categories: biological process, molecular function and cellular component. Thirty most enriched GO terms of different comparative groups (N1 vs control and N3 vs N1) are shown in Fig. 5. In the N1 vs control group, the most enriched GO terms were mainly about biological process and molecular function, and the oxidoreductase activity and isomerase activity were the well-represented terms among molecular function (Fig. 6A). In the N3 vs N1 group, amide biosynthetic process, cellular amide metabolic process, translation, peptide metabolic process and proteolysis were significantly enriched in the biological process, and calcium ion binding and actin binding were the most significant terms in molecular functions (Fig. 6B).

KEGG pathways were analyzed to obtain significantly changed pathways involving the differently expressed genes. Eleven and 19 pathways were significantly changed in the N1 vs control and N3 vs N1 groups, respectively. According to the reported functions of these pathways, most of them participated in the antioxidant and immune functions (Tables 3 and 4). Pathways related with the oxidoreductase activity counted for the vast majority.

Table 4
Anti-oxidation and immune-related pathways between N1 and N3 groups.

Pathway term	Associated genes	P-value
Histidine metabolism	E1.2.1.5|E1.2.1.3|ALDH9A1|	0.0017
Arginine and proline metabolism	DAO, aao|ALDH9A1|E1.2.1.3|rocD, OAT| PRODH|	0.0082
Protein digestion and absorption	DPP4|SLC9A3, NHE3|COL1AS|PRSS|	0.028
beta-Alanine metabolism	E1.2.1.5|E1.2.1.3|ALDH9A1|	0.034
Pyruvate metabolism	E1.2.1.3|ALDH9A1| MDH2|	0.038
Glycolysis/Gluconeogenesis	E1.2.1.3|ALDH9A1| E1.2.1.5|GAPDH, gapA|	0.042
Tyrosine metabolism	MIF|DBH| E1.2.1.5|	0.044
Phenylalanine metabolism	MIF|E1.2.1.5|	0.05

Table 3
Anti-oxidation and immune-related pathways between the N1 group and the control.

Pathway term	Associated genes	P-value
Lysosome	GAA|AGA, aspG|LGMN|CTNS|AP3B|SMPD1, ASM| SUMF1, FGE|ARSB|AP1B1|HGSNAT| SLC11A, NRAMP|	4E-04
Folate biosynthesis	QDPR|queD, ptpS, PTS|MOCS2, moaE|QDPR|GCH1, folE|	0.004
Tryptophan metabolism	E1.2.1.3|CCBL| AADAT, KAT2|AFMID|	0.009
Biosynthesis of unsaturated fatty acids	E1.3.3.6, ACOX1, ACOX3|HSD17B12, KAR, IFA38|	0.015
Focal adhesion	LAMC1|ROCK2|RAC1|PPP1C|COL1AS| FLT1,VEGFR1|LAMA3_5|ACTB_G1| MYLK|MYL12|PARV|PAK1|	0.021
Glycerolipid metabolism	E1.2.1.3|dgkA,DGK|LPIN|GLYCTK|E2.7.1.29, DAK1, DAK2|	0.023
Glycine, serine and threonine metabolism	BHMT|E2.3.1.37, ALAS| GNMT|GLYCTK|	0.034
Circadian rhythm	PER|PRKAG|CRY|	0.036
Carbohydrate digestion and absorption	SLC37A4|MGAM|	0.048

4. Discussion

4.1. Growth, antioxidant capacity and immune response

Environmental stress could increase FCR and affect the feeding efficiency of aquatic animals [27,28]. The negative effect of stress may be mediated by the disruption of metabolic regulation, extra energy expenditure and reduction of nutrient absorption [27]. Nitrite is a significant stress factor causing an increased FCR in this study, but dietary selenium supplementation could effectively alleviate this adverse effect. It seems that an appropriate level of dietary selenium could increase the protein content in epithelial cells on the intestine, and high intracellular protein may improve nutrient absorption [29,30]. The medium level of dietary selenium increased the weight gain in the study, though the increase was not significant compared with no selenium supplementation in the presence of nitrite. Selenomethionine is contained in selenium yeast and can be converted to and stored as selenoprotein via methionine metabolism. The active selenoprotein could interact with iodine to prevent abnormal hormone metabolism and promote growth [31,32].

MDA is a natural product of lipid peroxidation and is commonly used to monitor endogenous oxidative damage [33]. Therefore, the increase of MDA is usually associated with various environmental stress and pathological states of animals [34]. The MDA contents in both serum and hepatopancreas of a carb significantly increased under nitrite stress in this study, indicating that environmental nitrite induces lipid peroxidation in the crab. Environmental stress can trigger the over production of reactive oxygen species (ROS) and result in a severe damage to cells [35]. Unlike vertebrates, invertebrates only have non-specific innate immunity such as the prophenoloxidase-activating defense system, the action of endogenous antimicrobial peptides and phagocytosis, and serine protease clotting processes, but do not have any immune defense system [36,37]. The antioxidant defense system in invertebrates is thus more important than in vertebrates [38]. In crustaceans, antioxidant defense, especially the specialized antioxidant enzymes SOD and GPx, plays a crucial role to eliminate ROS [15,20]. Under nitrite stress, both SOD and GPx activities decreased, but the supplementation of 0.5 mg selenium/kg of diet significantly increased the activities of these two enzymes in the serum and hepatopancreas, suggesting the important role that selenium plays in the antioxidant defense system [16].

With the addition of selenium in the diet, the content of MDA in the serum decreased to a level similar to the control group. It seems that 1.0 mg/kg selenium could decrease the activities of SOD and GPx. Although aquatic animals can maintain a proper growth rate and a strong antioxidant status with selenium addition in the diet, the absence of selenium in the diet would result in slow growth and poor immunity [39–41].

Haemocytes of crustaceans are a crucial part of the cellular immune system for melanization, cytotoxicity, cell recognition and phagocytosis [42,43]. Environmental stress from sulfide, copper, salinity, nitrite or ammonia could lower the THC in crustaceans [8,44–47]. In the present study, the nitrite stress decreased the THC level in the crab. The decrease of THC may impair immune capability or decrease disease or stress resistance [8]. The increase of dietary selenium shows a sign of THC elevation to reduce nitrite stress on *E. sinensis*. The response of THC selenium addition is consistent with the changes of SOD and GPx activities and MDA contents. Due to the lack of an adaptive immune system, LZM together with other molecular effectors is a key component for defending against pathogens and oxidative stress in a crustacean [48]. The ACP and AKP are typical hydrolases involved in the extermination of toxin invasion and pollutant detoxification, and they also play a positive role in the immune system of crustaceans as part of lysosomal enzyme [49–51]. In the present study, a significant response of the activities of LZM, SOD and GPx to nitrite stress was observed, further suggesting that nitrite stress can impair the immune capacity. The observations in the present study are consistent with the previous reports that 0.5–2.5 mM ammonia stress decreased the LZM activity in *Litopenaeus vannamei* and the activity of LZM in *Charybdis japonica* exposed to Na_2S was lower than that in the control [52,53].

Nitrite stress also significantly decreased the ACP and AKP activities, but dietary selenium increased the ACP activity. The LZM activity of the crab rose with the increase of dietary selenium, indicating that the immune defense is also enhanced by selenium. Both ACP and AKP showed a pattern of first increase and then decrease with the increase of LZM activity. When the hemocytes, especially granulocyte, underwent "degranulation" during phagocytosis, ACP and AKP would be released from the lysosome into hemolymph [54,55]. Hence, the increased enzyme activities of ACP and AKP in the N2 group may be related to the increase of LZM activity for degranulation. When the dietary selenium reached 1.0 mg/kg, the antioxidant damage was efficiently reduced to the acceptable range, and the degranulation was no longer regulated by LZM. This may be the reason why the activity of ACP and AKP showed a decreasing tendency when dietary selenium increased from 0.5 to 1.0 mg/kg.

4.2. Transcriptional response

Nine pathways related to anti-oxidation and immunity were significantly altered under nitrite stress. Eight pathways related to anti-oxidation and immunity were significantly altered when the crab was supplied with 1.0 mg/kg dietary selenium under nitrite stress and all these pathways are related to metabolism. Moreover, almost all the genes with different expression involved in these eight pathways were up-regulated, indicating that the nutrient metabolism is much more active in the crab fed additional selenium. This may reveal a new insight into the function of selenium in stress resistance.

Under nitrite stress, nine genes involved in peroxisome biogenesis and peroxisomal proteins were significantly up-regulated in the N1 group vs the control. Peroxisomes have the ability to proliferate, multiply or be degraded in response to environmental or nutritional stimuli, because it is an organelle that participates in multiple cellular functions, especially for ROS elimination [56,57]. Except for catalase, antioxidant enzyme SOD was also localized in the peroxisome, an organelle found in all eukaryotic cells, in invertebrates [58]. The environmental nitrite increased the activity of peroxisomes in the current study, suggesting that the enhanced ability of SOD for scavenging ROS. This ubiquitous organelle participates in not only cell rescue from ROS damage but also generation ROS. The β-oxidation of fatty acids contributes to the generation of H_2O_2 in peroxisomes [56]. The peroxisomal acyl Co-A oxidase (ACOX) gene was significantly up-regulated in the N1 group compared with the control, suggesting the enhancement of the β-oxidation and production of H_2O_2. It was found that H_2O_2 could accumulate in the peroxisomes as a result of the imbalance in the peroxide-generating versus peroxide-removing enzymes in Indian catfish [59]. The expression of malonyl-CoA decarboxylase that was related with other-oxidation in the peroxisomes were also up-regulated in the current study. These results showed that nitrite stress simultaneously enhanced ROS production and ROS scavenging in peroxisomes.

Five genes were significantly up-regulated constituting a related line for L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH4) synthesis to produce more BH4. The BH4 has an antioxidant function in the whole host of biological processes, e.g., as a regulator of nitric oxide synthase or as a direct radical scavenger [60]. Besides, BH4 was also reported to directly protect against cell injury induced by H_2O_2 and mitochondrial superoxide [61,62]. The nitrite stress may promote the production of BH4 to improve antioxidant capacity. The amino acid L-tryptophan could enhance growth and stress resistance of aquatic animals [63,64]. It may also exert the stress-mitigating function in *E. sinensis* under nitrite stress. Two other amino acid metabolism pathways, beta-alanine metabolism and glycine, serine and threonine metabolism were also identified in the *E. sinensis* under nitrite stress. Glycine and threonine are important in stress resistance in *L. vannamei* [21,65,66], suggesting that these two amino acids are a prerequisite for stress tolerance but need further investigation for their functionality. Glycerolipid metabolism, which is involved in intracellular signaling processes and membrane formation [67], was also up-regulated under nitrite stress. Similarly, carbohydrate digestion and absorption were also upregulated to satisfy the extra energy demand to cope with stress.

The lysosome is the main catabolic organelle involved in the response to nutrient availability, stress resistance, membrane repair, cell death, development and cell differentiation [68]. This organelle serves as biomarkers for the defense system and health status of crustaceans, and is sensitive to ROS induced by immunological challenge and other sources of stress [69–71]. Genes related to lysosomal acid hydrolases, lysosomal membrane proteins and transport of synthesized lysosomal enzymes were all up-regulated, indicating that the activity of lysosomes might be enhanced by nitrite stress. However, previous biochemical results in the current study showed that the activities of all related enzymes, LZM, ACP and AKP were decreased by the stress.

Almost all the pathways identified in the N3 group and the control were related to metabolism, indicating that the metabolic rate was enhanced by dietary selenium in the crabs. Selenium is a structural component of GPx that plays a crucial role in ROS elimination [72]. GPx can use glutathione (GSH) as a substrate to decrease H_2O_2 in intracellular spaces and to reduce lipid peroxides in cell membranes [72,73]. Selenium can also reduce the immunosuppressive action of oxidative stress [74] and affect nutrient metabolism in animals. In another study, dietary selenium can increase total triglyceride, total cholesterol and nonesterified fatty acids in the liver and adipose tissues and the high-selenium diet can also alter protein metabolism and elevate total protein concentrations in both liver and muscle of pigs [75]. Selenium can affect glycemic control through insulin signaling, glycolytic pathway and pyruvate metabolism in humans [76]. In aquatic animals, dietary selenium can effectively improve flesh quality of rainbow trout (*Oncorhynchus mykiss*) through inhibition of protein degradation [77]. In a previous report, the total contents of protein and amino acids in the muscle of Chinese mitten crab fed selenium-biofortified corn was much higher than in the non-selenium group [19]. In the present study, protein and carbohydrate-related pathways were significantly up-regulated in the crabs fed 1.0 mg/kg dietary selenium under nitrite stress. However, the mechanisms of how the dietary selenium affects metabolism is not conclusive based on the evidence in the current study and further research is needed to investigate the function of selenium to improve antioxidant response and immune capability.

5. Conclusion

Nitrite stress increased FCR and suppressed growth, antioxidation and immunity. The dose of 1.0 mg/kg dietary selenium yeast significantly improved the feed utilization of *E. sinensis* under nitrite stress. The antioxidant balance in both serum and hepatopancreas, and ACP activity in serum showed the higher significant values in the crabs fed 0.5 mg/kg dietary selenium, while the serum LZM activity of crab was better in the 1 mg/kg selenium group. Furtherly, the transcriptional analysis that nine metabolism pathways related to anti-oxidation and immunity were significantly altered under nitrite stress. Eight metabolism pathways related to anti-oxidation and immunity were identified in the crab fed dietary selenium compared to the non-selenium group. Selenium supplementation improved antioxidant capacity and immune response, and also affected metabolism to help crabs better adapt to nitrite stress.

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