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Dietary selenium supplementation alleviates low salinity stress in the Pacific white shrimp *Litopenaeus vannamei*: growth, antioxidative capacity and hepatopancreas transcriptomic responses

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Abstract

se is an essential trace element associated with animal growth and antioxidant and metabolic processes. However, whether se, especially organic se with higher bioavailability, can alleviate the adverse effects of low salinity stress on marine economic crustacean species has not been investigated. Accordingly, juvenile Pacific white shrimp (*Litopenaeus vannamei*) were reared in two culture conditions (low and standard salinity) fed diets supplemented with increasing levels of L-selenomethionine (0·41, 0·84 and 1·14 mg/kg se) for 56 d, resulting in four treatments: 0·41 mg/kg under standard seawater (salinity 31) and 0·41, 0·84 and 1·14 mg/kg se under low salinity (salinity 3). The diet containing 0·84 mg/kg se significantly improved the survival and weight gain of shrimp under low salinity stress and enhanced the antioxidant capacity of the hepatopancreas. The increased numbers of B and R cells may be a passive change in hepatopancreas histology in the 1·14 mg/kg se group. Transcriptomic analysis found that L-selenomethionine was involved in the regulatory pathways of energy metabolism, retinol metabolism and steroid hormones. In conclusion, dietary supplementation with 0·84 mg/kg se (twice the recommended level) effectively alleviated the effects of low salinity stress on *L. vannamei* by regulating antioxidant capacity, hormone regulation and energy metabolism.

Key words: **Antioxidant capacity: *Litopenaeus vannamei*: Low salinity: L-selenomethionine: Health**

The Pacific white shrimp *Litopenaeus vannamei* is a major global marine economic species. *L. vannamei* is a euryhaline species; although the optimal salinity range has not been determined, it is generally believed that a salinity of 15–35 is more conducive to growth and survival[1–4]. The shortage of aquaculture areas and the increase in shrimp epidemics and pollution in coastal areas[5] have led to the rapid development of extensive and intensive farming systems using underground saline waters. Alternative farming conditions bring new challenges to shrimp aquaculture. Various adverse effects of low salinity have been reported, including a lower specific growth rate, feed consumption[6], reduced weight gain[4] and impaired survival[3,7]. Low salinity reduces the diffusion of ions to body tissues or the water environment and results in water absorption in the tissues, causing swelling and damage to the cells[8]. In addition, a change in environmental osmotic stress can cause tissue damage due to the production of free radicals (ROS)[9]. These adverse effects have caused slow growth, low digestion and absorption capacity[6], weak immunity[10] and disruption of gut microbiota homeostasis[11], ultimately increasing the risks of death and disease. Therefore, it is crucial to alleviate the adverse effects of low salinity stress on *L. vannamei*.

Se is a necessary trace mineral element to support growth, oxidation resistance, absorption and utilisation of Ca, glucose, lipids and vitamins in animals. se is an important component of the enzyme glutathione peroxidase (GPx)[12]. GPx catalyses the conversion of hydrogen peroxide and fatty acid hydroperoxides into water and fatty acid alcohol by using reduced glutathione, thereby protecting cell membranes against oxidative damage[13,14]. Therefore, se is an important nutrient that supports the antioxidant system in scavenging excess reactive oxygen species (ROS). se deficiency can decrease GPx activity, increase free radical content, reduce mobility and increase the permeability of biofilms[15], but excessive se is toxic to animals[16–18].

Abbreviations: DEG, differentially expressed gene; MDA, malondialdehyde content; ROS, reactive oxygen species.

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At present, the optimal se level of many aquatic animals has been reported[19–21], and these studies show that adding adequate levels of dietary se can improve growth performance, stress tolerance and immunity. For *L. vannamei*, appropriate dietary se levels (0·45 mg/kg) are beneficial to maintain normal antioxidant capacity[18]. In addition, a previous study found that organic se (L-selenomethionine) is a more suitable se source for *L. vannamei*[22]. However, little is known about the effects of se on shrimp under low salinity stress. The optimal dietary se supplementation level and the mechanism of se tolerance of shrimp under low salinity stress remain unclear.

Therefore, this study investigated the effects of dietary L-selenomethionine on the growth, antioxidant capacity, hepatopancreas health and stress resistance of *L. vannamei*. The results explore the regulatory mechanism of se to alleviate the adverse effects of low salinity stress on *L. vannamei* and provide a reference for dietary manipulation of inland white shrimp at low salinity.

Materials and methods

Diet preparation

The purified experimental basal diet contained 38·67 % crude protein and 4·68 % total lipid. The ingredients and proximate composition of the basal diet are shown in Table 1. Three different levels of L-selenomethionine (CAS: 3211-76-5, Rhawn) were added to the experimental basal diets of 0·4 (control), 0·8 and 1·2 mg/kg se. The analytical se content of feeds was determined by hydride atomic fluorescence spectrometry (GB/T 13883-2008, National Standard of the People's Republic of China) and consisted of average of the two results. The analytical se levels in each experimental diet were 0·41, 0·84, and 1·14 mg/kg, respectively. All dry ingredients were ground, weighed, blended, sifted through 60 mesh screens, mixed with oils and deionised water (300 ml/kg diet) and manufactured into pellets (2 mm diameter) using a press pelleting machine. The pellets were air-dried in a cool place until 10 % moisture was reached and then stored in a −20°C refrigerator.

Rearing and experimental conditions

L. vannamei were obtained from a commercial shrimp farm in Wenchang. First, shrimp were acclimated for 14 d in seawater (salinity 31) in experimental cylindrical tanks (500 l). Then, three-fourth of the shrimp were acclimated to salinity 3 by decreasing two units/d. After reaching a salinity of 3 and feeding for 3 d, shrimp with similar body weights (1·20 ± 0·14 g) were randomly divided into four groups with 3 replicates/group and 30 shrimp/tank. Shrimp were reared in two culture conditions (standard and low salinity) and fed diets supplemented with increasing levels of se (0·41, 0·84 and 1·14 mg/kg se) for 56 d, resulting in four treatments, namely, 0·41 standard salinity (31; positive control), 0·41 low salinity (3, negative control), 0·84 low salinity and 1·14 low salinity. Shrimp were hand-fed with the experimental diets 4 times a day (08:00, 12:00, 16:00 and 20:00) with a total daily ration of 4 % of the body weight. Half of the seawater in the positive control and treatment groups was replaced daily at 13:00. Water quality was maintained at 28 ± 2°C, pH at 8·0 ± 0·3 and nitrite at 0·08 ± 0·02 mg/l. Feces and undigested feed were discharged along with the circulating seawater.

Table 1. The compositions of the experimental basal diets

Term	Content (%)
Ingredient	
Casein*	34·00
Gelatin	7·60
Wheat starch	25·00
Fish oil	3·00
Soyabean oil	3·00
Soyabean lecithin	1·00
Cholesterol	0·50
Choline chloride	2·00
Glycine	0·60
L-Alanine	0·60
L-Glutamic acid	0·60
Lycine	1·20
Vitamin premix†	2·00
Mineral premix‡	2·00
Calcium carbonate pentahydrate	4·00
Butylated hydroxytoluene	0·01
Microcrystalline cellulose	12·89
Nutrient levels	
Crude protein	38·67
Total lipid	4·68
Ash	2·63
Moisture	11·22

* Casein: crude protein ≥92 %, total lipid ≤2 %, Hua Ling Dairy Corporation, Lanzhou, China.

† Vitamin premix (per kg of diet): vitamin A: 4800 mg; L-ascorbyl-2-polyphosphate 35 % Active C: 35·71 g; folic acid: 0·18 g; biotin: 0·05 g; riboflavin 3 g; DL Ca-pantothenate L: 5 g; pyrodoxine HCl B_6:1 g; vitamin B_{12}:0 002 g; thiamine HCl: 0·5 g; Menadione K_3:2 g; DL-α-tocopheryl acetate: 20 mg; inositol: 5 g; nicotinamide: 5 g; vitamin D: 8000 mg.

‡ Mineral premix (per kg of diet): $ZnSO_4 \cdot H_2O$: 20·585 g; $_{Ca(IO3)2}$:0 117 g; $CuSO_4 \cdot 5H_2O$: 0·625 g; $MnSO_4 \cdot H_2O$: 1·225 g; $MgSO_4 \cdot H_2O$: 39·86 g; $CoCl_2$:0 01 g; $FeSO_4 \cdot H_2O$: 11·179 g; $CaHPO_4 \cdot 2H_2O$: 166·442 g.

Sample collection

To avoid postpandrial responses, shrimp were fasted for 24 h before sampling at the end of the 8-week trial[23]. All shrimp were anesthetised on ice. Body and hepatopancreas weights and body lengths were measured. Five shrimp/tank were preserved at −20°C to determine proximate body composition. Three hepatopancreases were dissected and placed in 4 % paraformaldehyde for 24 h. Other hepatopancreases were rapidly immersed in liquid nitrogen and stored at −80°C for antioxidant enzymes and transcriptome analysis. The animal ethics protocol was approved by the East China Normal University Experimental Animal Ethics Committee (No. F20201001).

The following formulas were used to calculate the growth parameters:

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

$$\text{Weight gain}(\%) = (\text{final weight} - \text{initial weight})/\text{initial weight} \times 100$$

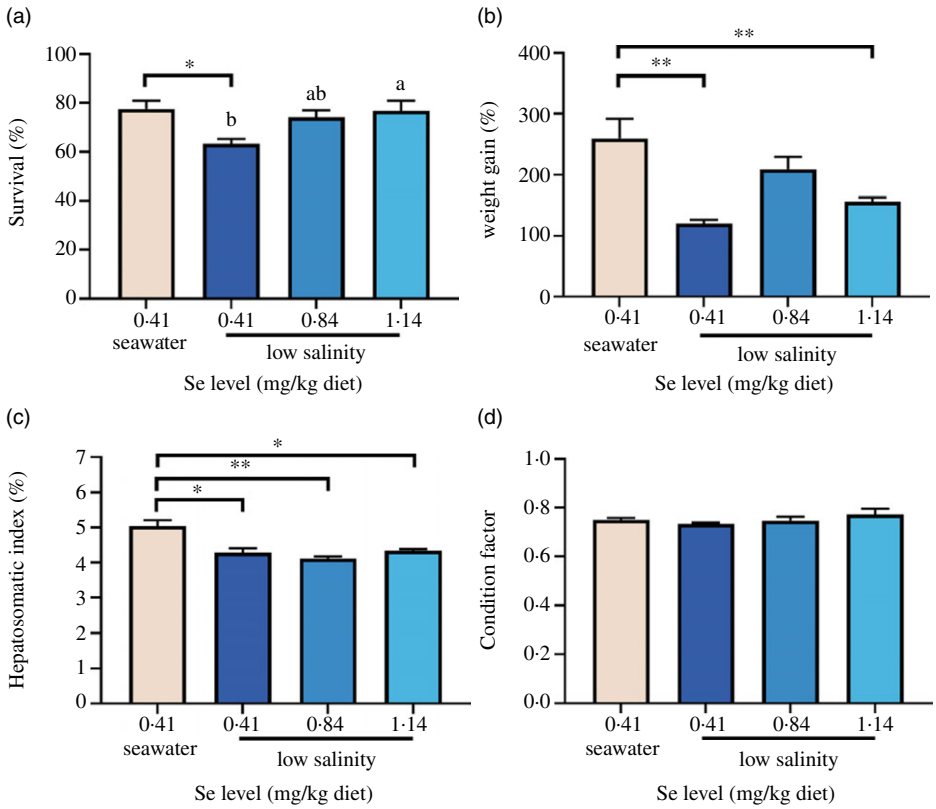

Fig. 1. Effects of different dietary Se levels of L-selenomethionine on (a) survival; (b) weight gain; (c) hepatosomal index; (d) condition factor of *L. vannamei*. Different letters (a, b) indicate significant differences ($P < 0·05$) among the three low salinity groups. Asterisks (*) represent a significant difference of $P < 0·05$ between the low salinity treatments and the seawater control. Double asterisks (**) represent a significant difference of $P < 0·01$ between the low salinity treatments and the seawater control.

Table 2. Proximate whole body composition of *L. vannamei* fed different diets

	Dietary se level (mg se/kg)							
	0·41 (salinity 31)		0·41 (salinity 3)		0·84 (salinity 3)		1·14 (salinity 3)	
Term	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Crude protein (%)	13·37	1·22	13·32	1·27	13·01	0·82	11·01	2·34
Total lipid (%)	0·88	0·11	0·93	0·16	0·80	0·21	0·55	0·19
Ash (%)	3·50	0·10[a]	3·32	0·17[a,b]	2·98	0·23[b]	3·18	0·03[a,b]
Moisture (%)	81·03	1·21	80·90	1·77	82·74	0·72	82·48	1·09

Three whole shrimp bodies from each of the three tanks were tested. The results represent the means ± SE.
[a,b]Indicates a significant difference between se levels ($P < 0·05$).

$$\text{Hepatosomatic index (\%)} = \text{hepatopancreas weight/final weight} \times 100$$

$$\text{Condition factor (\%)} = \text{final weight/length}^3 \times 100,$$
where weight is in g and length is in cm.

Proximate composition

Analytical methods were used for moisture, crude lipid, crude protein and ash[24]. Four shrimp in each group and four fully ground and mixed diets were dried at 105°C to a constant weight. Crude protein content was determined by a Dumas nitrogen analyzer (Elementar Rapid N Excess). Crude lipid content was measured by the Soxhlet method (Buchi 36680) after extraction with ether. The ash content was determined by burning the sample in a muffle furnace for 10 h at 550°C.

Enzyme assays and biochemical analysis

Six hepatopancreas samples in each group (two samples per tank) were homogenised in normal saline (0·86 %) and

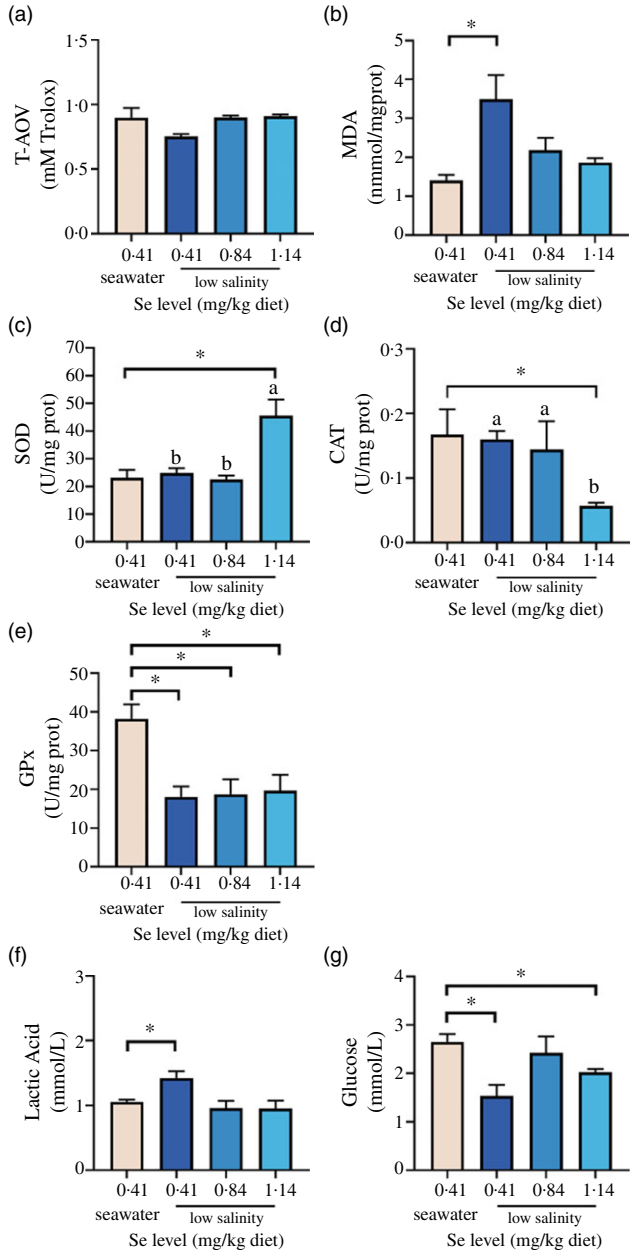

Fig. 2. Effects of different dietary Se levels of L-selenomethionine on the hepatopancreas antioxidative ability of *L. vannamei*. (a) T-AOC; (b) SOD activity; (c) CAT activity; (d) GPx activity; (e) MDA content; (f) serum lactic acid content; (G) serum glucose content. The different letters (a, b) indicate significant differences ($P < 0{\cdot}05$) among groups. Asterisks (*) represent a significant difference of $P < 0{\cdot}05$ between the low salinity treatments and the seawater control. T-AOC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde content.

centrifuged at 4°C at 3000 rpm for 10 min. The supernatant concentration of the homogenate was determined by the weight to volume ratio. The activities of superoxide dismutase and catalase, GPx activity, total antioxidant capacity, malondialdehyde content (MDA) and total protein concentration and the contents of lactic acid and glucose in serum were detected using a commercial kit (Nanjing Jiancheng Co., Ltd.). The results were recorded on a microplate reader (Epoch, BioTek). The specific experimental method was described in a previous study[18].

Hepatopancreas histology

The hepatopancreases from three shrimp per treatment were dehydrated with different gradients of alcohol (80 %, 90 %, 100 %), cleared with xylene and embedded in paraffin to make solid wax blocks. These solid wax blocks were cut into 5-μm thick continuous section blocks in each section by a rotary microtome (Leica RM2125). The segmented tissues were stained with hematoxylin–eosin and examined under a microscope (Nikon, Eclipse 200).

Total RNA isolation and transcriptome analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) from the hepatopancreases (three mixed samples) of nine shrimp randomly selected from each group. Sequencing was performed according to a previous procedure[18]. The cDNA library was sequenced with an Illumina HiSeq™ 4000 instrument. The sequencing data were stored in the NCBI SRA. The registration number is SRP310771.

The number of segments per million segments in 1000 bases was used to calculate the expression of single genes to determine the differentially expressed genes (DEG) for the three groups: 0·41 (low salinity) group (shrimp in the normal seawater were used as the salinity control for the shrimp fed the control diet under low salinity), 0·84 group (shrimp fed the control diet under low salinity were used as the control for those fed the diet with 0·84 mg/kg se) and 1·14 group (shrimp fed the control diet under low salinity were used as the control for those fed the diet with 1·14 mg/kg se). The R statistical software package edgeR (empirical analysis of digital gene expression in R, http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) was used for RSEM analysis (http://deweylab.biostat.wisc.edu/RSEM). An error rate <0·05 was used as the threshold *P* value to judge the significant difference in gene expression level. Gene set enrichment analysis (www.broadinstitute.org/gsea) was used to identify differential pathways between the control diet in the normal seawater and low salinity seawater, 0·41 and the diet with 0·84 mg/kg se under low salinity, and 0·41 and 1·14 mg/kg se under low salinity.

A comprehensive value > 0·2 was obtained, and the top 50 DEG related to metabolism were screened. Cytoscape version 3.7.1[25] and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database[26] were used to construct a protein–protein interaction network to obtain known and predicted protein interactions. The confidence score cut-off applied for interactions was 0·9 (highest confidence). Single nodes, doublets and triplets detached from the main network cluster were removed.

Statistical analysis

All data were calculated as the mean ± standard error (SE). Data were checked for normality and homogeneity of variances. Two hypotheses were tested: (1) Student's *t* test was used to compare the positive control (standard salinity seawater) group with negative control (low salinity) group and other two low salinity groups (i.e., 0·41 standard salinity *v.* 0·41 low salinity; 0·41 standard salinity *v.* 0·84 low salinity; and 0·41 standard salinity *v.* 1·14

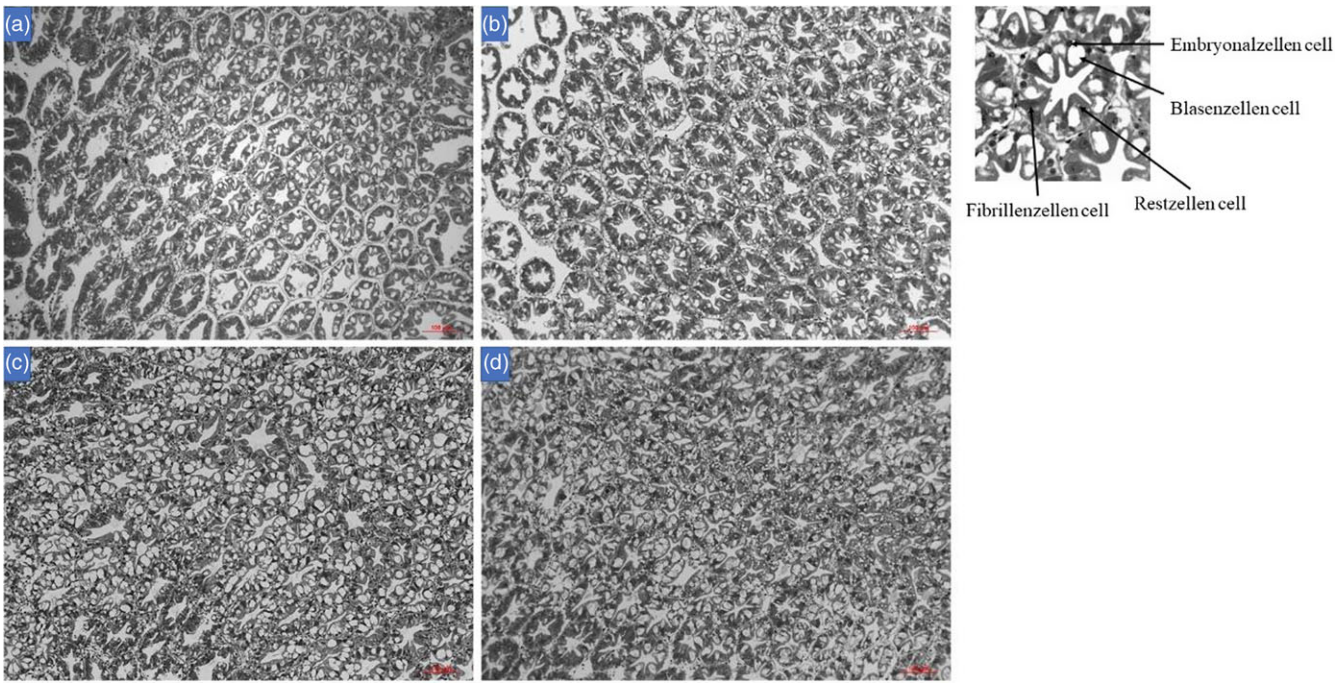

Fig. 3. Effects of different dietary Se levels of L-selenomethionine on hepatopancreas histology. (a) Seawater group; (b) diet with 0·41 mg/kg se under low salinity; (c) diet with 0·84 mg/kg se under low salinity; (d) diet with 1·14 mg/kg se diet under low salinity. The illustration at the upper right shows four types of cells visible in a cross-section of the hepatopancreas tubule.

low salinity); and (2) one-way ANOVA was used in shrimp fed diets with 0·41, 0·84 and 1·14 mg/kg se under low salinity. SPSS statistics 22.0 software (IBM Inc.) was used to conduct statistical analyses. A multiple comparison test (Duncan) was performed to compare the significant differences among the low salinity treatments. P values <0·05 were considered statistically significant.

Results

Growth performance and whole-body proximate composition

Individual comparisons between the positive control and the rest of the dietary treatments demonstrated a reduced survival of shrimp fed the negative control diet ($P < 0·05$), while no significant differences were found in the 0·84 and 1·14 mg/kg se groups (Fig. 1(a)). Compared with the seawater group, low salinity stress significantly decreased the weight gain of shrimp fed the 0·41 and 1·14 mg/kg se diets ($P < 0·01$), while there was no significant change in the 0·84 mg/kg se group. Shrimp fed the 0·84 mg/kg se diet exhibited a significant decreased in hepatosomatic index ($P < 0·01$), while the 0·41 and 1·14 mg/kg se groups showed a significant decrease in hepatosomatic index compared with the seawater group ($P < 0·05$, Fig. 1(b) and (c)). No significant change was found in the condition factor among all treatment groups ($P > 0·05$; Fig. 1(d)). The ash content of shrimp fed 0·84 mg/kg se under low salinity stress was significantly lower than that in the salinity control, and the contents of crude protein, total lipids and moisture were not significantly different among all groups ($P > 0·05$, Table 2).

Antioxidant capacity and related biochemical components

The results of antioxidant parameters and biochemical components are shown in Fig. 2. The total antioxidant capacity in shrimp fed the negative control diet was numerically lower than that in shrimp fed the positive control diet, but the difference was insignificant. No significant difference was found in the total antioxidant capacity ($P > 0·05$; Fig. 2(a)). The MDA content in shrimp fed the negative control diet was significantly higher than that in shrimp fed the positive control diet ($P < 0·05$), and the MDA content gradually decreased with increasing se supplementation to a similar level (Fig. 2(b)). Low salinity did not affect the superoxide dismutase and catalase activities of shrimp fed the positive control diet, while superoxide dismutase activity was significantly improved, and catalase activity was inhibited by the diet with 1·14 mg/kg se compared with other groups ($P < 0·05$; Fig. 2(c) and (d)). Low salinity significantly inhibited GPx activity ($P < 0·05$), and the increase in se levels numerically enhanced GPx activity, but the difference was insignificant (Fig. 2(e)). Compared to the positive control, low salinity significantly increased the lactic acid content in the serum ($P < 0·05$), and the diets with 0·84 or 1·14 mg/kg se corrected this effect ($P < 0·05$; Fig. 2(f)). Low salinity also significantly decreased the glucose content in the serum ($P < 0·05$), and the diet with 0·84 mg/kg se significantly increased the glucose content under low salinity stress ($P < 0·05$; Fig. 2(g)).

Hepatopancreas histology

The hepatopancreas histology of *L. vannamei* is shown in Fig. 3. Salinity significantly affected the four kinds of cells in hepatopancreas tubules: embryonalzellen cells (E cells), restzellen cells

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(R cells), fibrillenzellen cells (F cells) and blasenzellen cells (B cells). The hepatopancreas tubules of shrimp in seawater were arranged neatly, and the B cells were full in shape, evenly distributed and abundant (Fig. 3(a)), while the B-cell radius decreased in shrimp fed the negative control diet under low salinity (Fig. 3(b)). In the shrimp-fed diets with 0·84 and 1·14 mg/kg se in low salinity, the hepatopancreas tubules were arranged more closely. A larger size of B cells was found in shrimp fed the diet with 0·84 mg/kg se (Fig. 3(c)), while tighter spaces between the hepatopancreas tubules were observed in shrimp fed 1·14 mg/kg se (Fig. 3(d)).

Hepatopancreas transcriptome analysis

The RNA sequence analysis of the hepatopancreas produced 536,394,450 raw reads and 80,995,561,950 raw bases. A total of 528,036,952 clean reads and 77,445,110,324 clean bases were produced. Clean reads were compared to the genome of *L. vannamei*, and the ratio was 88·29 %–93·60 %. The Venn diagram showed common and unique DEG among the three groups (Fig. 4(a)). In total, 555 differentially expressed genes were identified in the three low-salinity feeding groups (Fig. 4(b)). The negative control contained seventy-one upregulated and ninety-eight downregulated genes (Fig. 4(c)). The diet with 0·84 mg/kg se group contained 153 upregulated and 162 downregulated genes (Fig. 4(c)). The diet with 1·14 mg/kg se group contained eighty upregulated and ninety-one downregulated genes (Fig. 4(c)). Gene set enrichment analysis was used to identify genes at the overall level and provide clues for significantly related DEG and biological processes (Table 3). Compared with the positive control, the DEG in the shrimp fed the negative control diet at low salinity were significantly enriched in metabolic pathways, such as glutathione metabolism, fructose and mannose metabolism, retinol metabolism and oxidative phosphorylation. Compared with the shrimp fed 0·84 mg/kg se at low salinity, the DEG in the negative control diet at low salinity were significantly enriched in the metabolic pathways of retinol metabolism, steroid hormone biosynthesis and multiple carbohydrate metabolism-related pathways (e.g., pentose, glucuronic acid conversion, biosynthesis of other O-glycans and biosynthesis of glutenin-type O-glycans). The differentially expressed genes in shrimp fed 1·14 mg/kg se at low salinity were significantly enriched in fructose and mannose metabolism, oxidative phosphorylation, glutathione metabolism, steroid hormone biosynthesis and multiple carbohydrate metabolism (e.g., biosynthesis of other O-glycans and biosynthesis of mucin-type O-glycans). The heatmap shows the changes in all DEG in each group and clusters the genes with the same expression pattern into three types (Fig. 4(d)).

Protein–protein interaction analysis of DEG

We further analysed the interactions between the DEG. Protein products from fifty metabolism-related DEG produced a total of forty-seven links (Fig. 5). The names of nodes are the KEGG Orthologs names of the genes, and the full descriptions and corresponding KEGG pathways are shown in Table 4. The DEG ndufv1 and ost3, followed by atp6s14, had the highest scores for betweenness centrality, indicating that they play an important connecting role in the corresponding protein clusters. Among them, ndufv1 has the highest degree of connection with other proteins. Its connected protein clusters are associated with oxidative phosphorylation. The ost3 and atp6s14 protein clusters were associated with protein processing pathways in the endoplasmic and mTOR signalling pathways, respectively.

Discussion

Growth performance

Excessive deviation of environmental osmotic pressure from the isotonic point can adversely affect the growth and survival of organisms. *L. vannamei* has an extraordinary tolerance to low salinity, but the negative effects of low salinity on the growth of *L. vannamei* should be considered. For example, acute salinity changes can directly lead to an increase in the mortality of *L. vannamei*[7]. In the 50 d culture experiments at high, medium and low salinity (32, 17 and 3), the weight gain in *L. vannamei* at salinity 17 was significantly higher than that at salinity 3, and the lowest survival occurred at salinity 3[27]. In this study, the survival, weight gain and hepatosomatic index of shrimp fed different levels of L-selenomethionine under low salinity stress were significantly lower than those in the salinity control, except for the 0·84 mg/kg se group. With the increase in dietary se, the survival of shrimp under low salinity stress gradually increased. The weight gain of shrimp fed 0·84 mg/kg se was equivalent to that of the positive control, indicating that increasing the dietary se level can promote the growth of shrimp in a low salinity environment. This is the same change as the se requirement of fish under stress[28]. Based on weight gain, 0·84 mg/kg se is the optimal se requirement for *L. vannamei* at salinity 3.

Antioxidant capacity

Diets containing 0·84 and 1·14 mg/kg se displayed strong antioxidant capacity in this study. se plays a vital role in the antioxidant defense system of animals[29]. It is the active center of the antioxidant enzyme GPx. Lipid peroxidation caused by ROS produces MDA, which normally acts as a marker of oxidative stress. Generally, salinity stress can cause an increase in ROS, resulting in MDA production. The antioxidant enzyme system developed by animals during evolution can reduce the generation of oxidative stress by scavenging ROS[12,30]. However, there was no significant difference in GPx activity under low salinity, which was speculated to be because 0·41 mg/kg se in the diet had reached the activity threshold of GPx and could not continue to increase its activity. Similar results were found in rainbow trout[28]. This result also indicates that GPx activity can be used as a marker of se when animals are deficient in se, and when the maximum requirement of se is exceeded, GPx activity cannot reflect tissue se levels. The increased ROS can also cause tissue damage. However, the same histological changes can occur in hepatopancreas tubules[9]. se has a limited effect on hepatopancreas histology under low salinity stress. The increased numbers of B and R cells may be a passive change in tissue histology to provide more active energy for metabolism in response to low salinity stress. Metabolic changes may be the cause of low weight gain at low salinities.

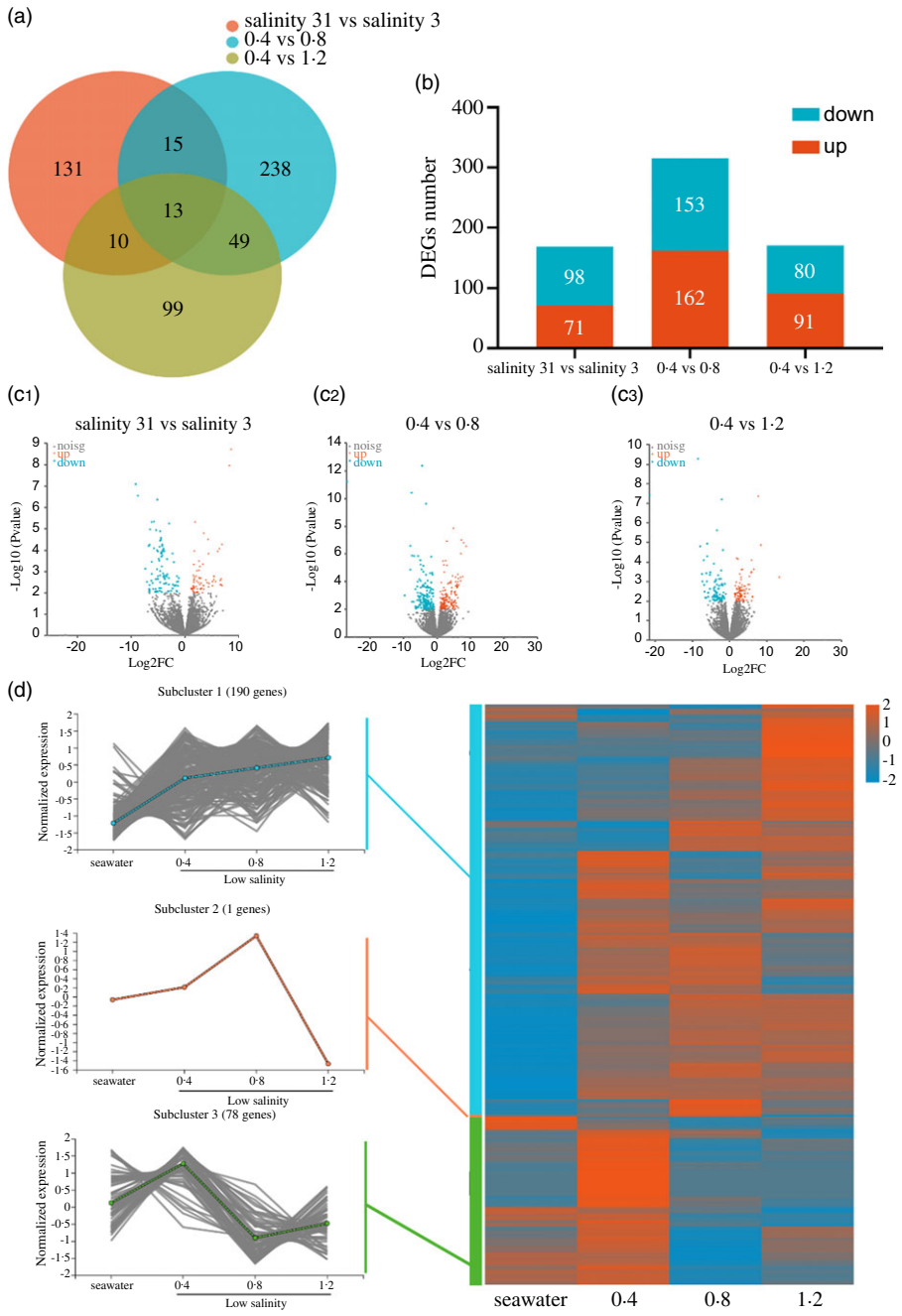

Fig. 4. Transcriptome analysis of the effect of different dietary Se levels on the hepatopancreas in *L. vannamei*. (a) Venn diagram; (b) Volcano diagram; (c) Up- and downregulated differentially expressed genes in each group; (d) Heatmap of all differentially expressed genes in the three groups. Genes were clustered into three expression patterns. GSEA, Gene set enrichment analysis.

The Gene set enrichment analysis results showed that "glutathione metabolism" was significantly downregulated in *L. vannamei* fed the control diet after 8 weeks of low salinity stress, while it was significantly upregulated in shrimp fed the diet with 1·14 mg/kg se. Glutathione is a functional substance in animals that can maintain normal immune function, antioxidant capacity and detoxification. Previous studies have found that appropriate se supplementation contributes to the synthesis of glutathione[18], which is consistent with the results of this study, suggesting that se supplementation can contribute to glutathione synthesis. se supplementation is beneficial to support the antioxidant system. In addition, "retinol metabolism"-related genes in shrimp fed the positive control diet were significantly upregulated, while those fed the diet with 0·84 mg/kg se were significantly downregulated. Known as vitamin A, retinol is an essential lipid-soluble antioxidant in animals. The upregulation of "retinol metabolism" under salinity stress indicates that retinol is involved in alleviating low salinity stress as a supplement to

Table 3. Metabolism-related gene set enrichment analysis

Pathways	NES	*P* value	Regulate
0·41 mg/kg (salinity 31) *v.* 0·41 mg/kg (salinity 3)			
Glutathione metabolism	1·47	0·00	Down
Fructose and mannose metabolism	−1·36	0·01	Up
Retinol metabolism	−1·32	0·01	Up
Oxidative phosphorylation	−1·27	0·02	Up
0·41 mg/kg (salinity 3) *v.* 0·84 mg/kg (salinity 3)			
Retinol metabolism	1·20	0·00	Down
Steroid hormone biosynthesis	1·22	0·00	Down
Pentose and glucuronate interconversions	1·24	0·00	Down
Ascorbate and aldarate metabolism	1·49	0·00	Down
Fructose and mannose metabolism	1·73	0·00	Down
Oxidative phosphorylation	1·24	0·00	Down
Glycine, serine and threonine metabolism	1·42	0·00	Down
Metabolism of xenobiotics by cytochrome P450	1·26	0·00	Down
Butanoate metabolism	1·58	0·02	Down
Other types of O-glycan biosynthesis	−1·33	0·02	Up
Mucin type O-glycan biosynthesis	−1·35	0·04	Up
Glycosaminoglycan biosynthesis - heparan sulfate/heparin	−1·38	0·04	Up
0·41 mg/kg (salinity 3) *v.* 1·14 mg/kg (salinity 3)			
Fructose and mannose metabolism	1·58	0·00	Down
Oxidative phosphorylation	1·26	0·00	Down
Other types of O-glycan biosynthesis	−1·42	0·00	Up
Mucin-type O-glycan biosynthesis	−1·46	0·01	Up
Pentose and glucuronate interconversions	1·43	0·01	Down
N-Glycan biosynthesis	−1·33	0·02	Up
Glutathione metabolism	−1·29	0·03	Up
Steroid hormone biosynthesis	1·25	0·05	Down

NES, normalised enrichment score. The enrichment score calculated for each gene subset was standardised according to the size of the gene set to obtain the standardised enrichment score.
The |NES| is the analysis result, shows that the higher the value, the better the reliability.

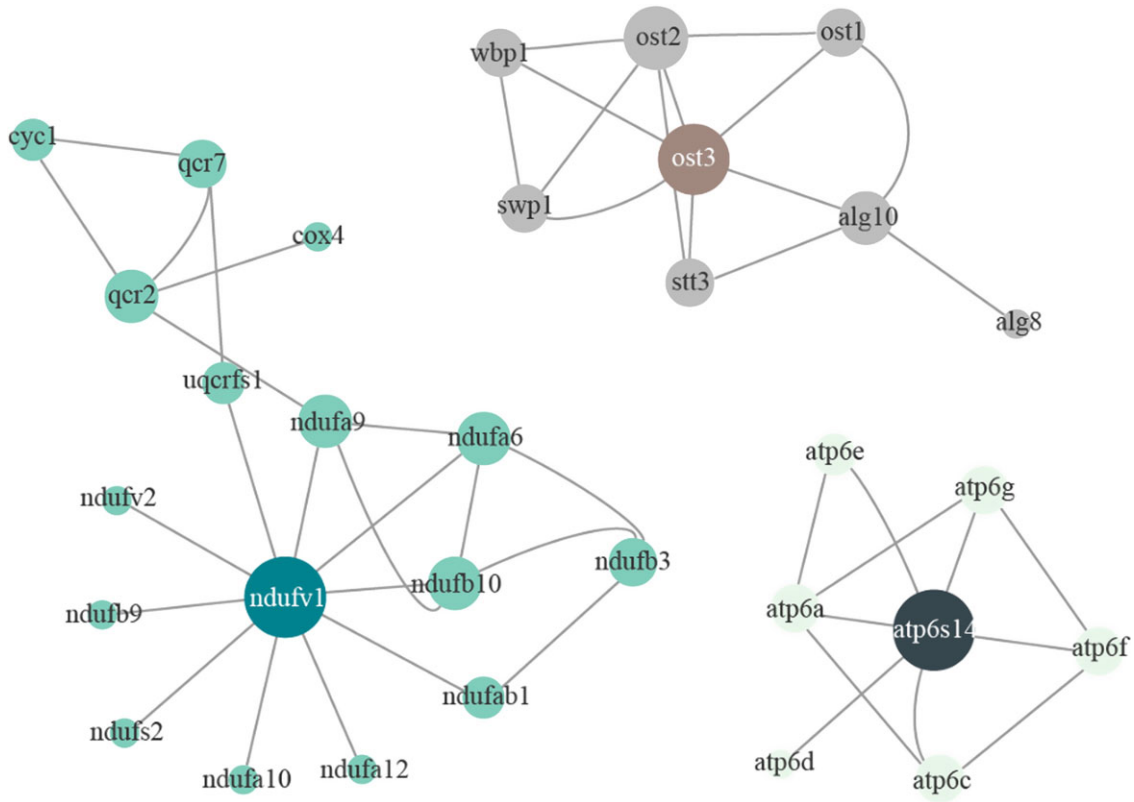

Fig. 5. Three main protein–protein interaction network clusters of differentially expressed genes in three comparisons. Nodes represent genes, and edges represent the interaction between the two proteins that express genes. The more connections there are, the larger the node area. The colour of the most connected gene in each cluster was darkened, indicating that this gene may be the core gene in the cluster. The detailed notes of each node are shown in Table 4.

Table 4. The annotation of DEG with the highest connectivity in the protein interaction network

KEGG pathway	Node name	KO id	NR description
Oxidative phosphorylation (map00190)	*cyc1*	K00413	cytochrome c1, heme protein, mitochondrial-like
	qcr7	K00417	cytochrome b-c1 complex subunit 7-like
	qcr2	K00415	cytochrome b-c1 complex subunit 2, mitochondrial-like
	cox4	K02263	cytochrome c oxidase subunit 4 isoform 1, mitochondrial-like
	uqcrfs1	K00411	cytochrome b-c1 complex subunit Rieske, mitochondrial-like
	ndufa9	K03953	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial-like
	nudfa6	K03950	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6-like
	ndufv2	K03943	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial-like
	ndub3	K03959	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3-like
	ndufb10	K03966	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10-like
	ndufv1	K03942	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial-like
	ndufb9	K03965	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9-like
	ndufs2	K03935	NADH-ubiquinone oxidoreductase 49 kDa subunit-like
	ndufa10	K03954	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial-like
	ndufa12	K11352	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12-like
	ndufab1	K03955	acyl carrier protein, mitochondrial-like, transcript variant X1
Protein processing in endoplasmic reticulum (map04141)	*wbp1*	K12670	dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit-like
	ost2	K12668	dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit DAD1-like
	ost1	K12666	dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1-like
	swp1	K12667	dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 2-like, transcript variant X1
	ost3	K12669	tumor suppressor candidate 3-like
	alg10	K03850	putative Dol-P-Glc:Gl
	stt3	K07151	dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit STT3A-like
	alg8	K03849	probable dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1,3-glucosyltransferase, transcript variant X1
mTOR signaling pathway (map04150)	*atp6e*	K02150	V-type proton ATPase subunit E-like
	atp6 g	K02152	V-type proton ATPase subunit G-like
	atp6a	K02145	V-type proton ATPase catalytic subunit A-like
	atp6s14	K02151	V-type proton ATPase subunit F-like
	atp6f	K03661	V-type proton ATPase 21 kDa proteolipid subunit-like
	atp6d	K02146	V-type proton ATPase subunit d 1-like
	atp6c	K02148	V-type proton ATPase subunit C-like

DEG, differentially expressed gene.

antioxidant capacity. In addition, previous studies have shown that se supplementation can improve immune capacity by regulating "retinol metabolism" and "glutathione metabolism" in chickens, which is consistent with these results[31]. Therefore, se can reduce oxidative stress by regulating antioxidant-related pathways.

Energy regulation

Under chronic low salinity stress (salinity 3), *L. vannamei* juveniles require additional energy to resist osmotic pressure from nutrient metabolism[32]. After low salinity stress for 8 weeks, fructose and mannose metabolism and oxidative phosphorylation-related genes were significantly upregulated in shrimp fed the negative control diet compared with the positive control. However, this effect was corrected by higher se supplementation with shrimp-fed diets with 0·84 or 1·14 mg/kg se displaying significantly downregulated fructose and mannose metabolism and oxidative phosphorylation-related genes. Fructose and mannose metabolism is one of the crucial processes of carbohydrate metabolism in animals and can provide energy for the body. Therefore, carbohydrate supplementation can directly meet the higher energy requirements of aquatic animals under stress conditions, especially under low salinity[32]. On the other hand, se deficiency can disrupt glucose homeostasis in the body, resulting in a disruption of energy metabolism[33,34]. 'Oxidative phosphorylation' is the coupling reaction of ADP and inorganic phosphoric acid to synthesise ATP through the energy released during oxidation. Oxidative phosphorylation activity can be regulated by tissue metabolism to maintain energy metabolic homeostasis. Changes in the concentration of oxidative phosphorylation maximally enhance energy metabolism[35]. Additionally, as a crustacean, the oxidative phosphorylation pathway-related DEG of the Chinese mitten crab *Eriocheir sinensis* were significantly enriched, which is the same as this study's result[36].

In the protein–protein interaction analysis, *ndufv1* in all DEG related to oxidative phosphorylation played an important connecting role. As a product of the *ndufv1* gene, the NDUFV1 protein is the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase. The main function of NADH dehydrogenase is to generate ROS. As a product of the ndufv1 gene, the protein NDUFV1 is the core subunit of NADH dehydrogenase in the mitochondrial membrane respiratory chain. Downregulated expression of ndufv1 may lead to loss of energy supply or oxidative stress[37,38]. Cytochrome c oxidase submit 4 (*cox4*) was also found in DEG. Cytochrome c is an essential component of the electron transport chain (*ETC*) in

mitochondria and is involved in energy metabolism[39]. When zebrafish were exposed to microcystin-LR, the expression of *ndufv1* and *cox2* was significantly inhibited, the body changed to anaerobic respiration, and mitochondria were damaged[40], while se-methylselenocysteine containing Se can interfere with the protein expression related to energy metabolism disorder, amino acid metabolism abnormality, synaptic dysfunction and oxidative stress caused by disease in mice[41]. These results indicated that se could alleviate energy metabolism disorders under stress.

Overall, the above results combined with body weight gain, glucose content, lactate content in serum and downregulated oxidative phosphorylation-related genes suggest that se can alleviate low salinity stress by regulating energy metabolism.

Hormone regulation

se supplementation also downregulated the 'steroid hormone biosynthesis' pathway in *L. vannamei* under low salinity stress. Steroid hormones play an essential role in regulating normal metabolism, immune function and osmotic pressure[42–44]. Low salinity was found in a previous study to cause changes in steroid metabolism[32]. The metabolic mechanism of tea Se polysaccharide in rats was closely related to the metabolic mechanism regulating steroid biosynthesis[45], and steroid hormones were involved in regulating osmotic pressure in whales[46]. This suggests that se may alleviate low salinity stress by regulating the metabolism of steroid hormones. However, the mechanism by which se participates in hormone regulation in *L. vannamei* needs further investigation.

Conclusion

In conclusion, se could effectively alleviate the physiological effects of low salinity stress on *L. vannamei* by regulating antioxidant capacity (e.g., retinol metabolism, glutathione metabolism and antioxidant enzyme activity), hormone regulation via the control of steroid hormone synthesis and energy metabolism (e.g., carbohydrate metabolism and oxidative phosphorylation). Furthermore, our findings suggest an adequate supplementation of se at 0·84 mg/kg (L-selenomethionine) to maintain weight gain and enhance stress resistance under low salinity stress.

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Q. Y., F. H. and E. L. conceived the experiment; Q. Y. formulated the experimental diets; Q. Y., F. H. and E. L. conducted the experimental trials and laboratory analysis; F. H., A. R., J. Q., L. C. and E. L. conducted polish work of this manuscript. All authors contributed to and approved the manuscript.

There are no conflicts of interest.

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