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# Dietary effects of astaxanthin on gonadal development in female broodstock of *Macrobrachium rosenbergii*

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ABSTRACT

This study examines the effects of dietary astaxanthin on ovarian development and reproductive health in female *Macrobrachium rosenbergii*, a key aquaculture species. Five isonitrogenous and isolipidic diets with astaxanthin levels of 0, 27, 62, 140, and 310 mg/kg (AS0, AS27, AS62, AS140, AS310) were tested over 56 days. Weight gain and specific growth rates were significantly higher in AS27, AS62, and AS140 compared to AS0 ($P < 0.05$). Survival rates were significantly higher in AS62, AS140, and AS310 compared to AS0 and AS27 ($P < 0.05$). The AS62 group exhibited the highest gonadosomatic index and estradiol levels, while vitellogenin levels were elevated in AS62 and AS140 ($P < 0.05$). Testosterone was significantly higher in AS27 and AS62 compared to AS310 ($P < 0.05$). Oxidative stress indicators showed lower malondialdehyde levels and higher superoxide dismutase activity in AS62, with total antioxidant capacity peaking in AS140 ($P < 0.05$). Transcriptome analysis revealed upregulation of genes involved in the pentose phosphate pathway, glutathione metabolism, and ubiquinone/terpenoid-quinone biosynthesis in AS140. Specifically, genes such as *g6pd*, *fbp*, *gpx*, and *anpep* were significantly upregulated ($P < 0.05$). Optimal astaxanthin levels (114.30–165.14 mg/kg) enhanced gonadal development, antioxidant capacity, and sex hormone regulation. This study provides insights into the molecular pathways through which astaxanthin supports reproductive health in broodstock *M. rosenbergii*.

## 1. Introduction

The giant freshwater prawn (*Macrobrachium rosenbergii*), belonging to the order Decapoda, family Palaemonidae, and genus Macrobrachium, was regarded as the largest freshwater prawn species worldwide. It's rapid growth rate, broad dietary adaptability, and delicious taste made it highly popular among consumers (Yang et al., 2012). In recent years, the healthy farming industry development of the *M. rosenbergii*

was greatly limited by various factors such as germplasm degradation, rampant diseases, and outdated nutritional technology (Banu and Christianus, 2016). The reproductive performance of broodstock was not only related to germplasm quality but also closely tied to nutritional enhancement before reproduction (Song et al., 2024). Currently, broodstock nutritional enhancement primarily depends on natural feed, which may be affected by pollution, lacks balanced nutrition, and can serve as a vector for disease. In contrast, formulated feed provides consistent quality, is pathogen-free, and enhances broodstock reproductive performance and offspring quality by allowing targeted regulation of specific nutrients (Tavabe et al., 2020).

Nutrients such as proteins, lipids, vitamins, and minerals, in the feed directly influence the gonadal development of broodstock, with both excessive and insufficient nutrition shown to reduce reproductive performance (Bombardelli et al., 2017). Astaxanthin is a naturally occurring red carotenoid pigment, characterized by a long chain of conjugated double bonds in its molecular structure, which provides strong antioxidant properties, and it must be obtained from feed as fish and crustaceans lack the ability to synthesize astaxanthin on their own (Lim et al.,

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2018). It had previously been proven that astaxanthin could enhance the growth, antioxidant capacity,coloration, and immune function of fish and crustaceans(Cheng and Wu, 2019; Long et al., 2017; Zhang et al., 2013). Recent studies showed that astaxanthin enhanced the reproductive performance of aquatic animals (Long et al., 2017; Sawanboonchun et al., 2008). In the diet containing 60.99 ~ 97.43 mg/kg astaxanthin, the gonadal index and levels of related serum hormones (estradiol, follicle-stimulating hormone, testosterone, luteinizing hormone, and vitellogenin) in largemouth bass were increased. Transcriptomic analysis of ovarian tissue revealed significant enrichment of pathways such as "ovarian steroidogenesis" and "steroid hormone biosynthesis," with upregulation of genes such as follicle-stimulating hormone receptor (*fshr*), insulin-like growth factor 1 (*igf1*), and steroidogenic acute regulatory protein (*star*). This indicated that the appropriate addition of astaxanthin promoted the synthesis of related steroid hormones, thereby accelerating ovarian development (Tao et al., 2024). In the study of the promoting effect of astaxanthin on ovarian development and its regulatory mechanism in Nile tilapia (*Oreochromis niloticus*), it was found that a diet containing 150 mg/kg astaxanthin increased the gonadal index, relative fecundity, and oocyte diameter. It affected the MAPK signaling pathway, oocyte meiotic division pathway, and progesterone-mediated oocyte maturation pathway, and promoted the expression of related genes such as *igf1* and *fsh* in these pathways. This indicated that the appropriate addition of astaxanthin in the feed stimulated the upregulation of genes such as *igf1* and *fsh*, which in turn stimulated granulosa cells/theca cells to produce estradiol and progesterone, promoting the proliferation of granulosa cells and oocytes (Qiang et al., 2022). However, research on the effects of dietary astaxanthin supplementation on ovarian development in broodstock is relatively limited, and the mechanisms of action of astaxanthin remain poorly understood. Therefore, this research aimed to investigate the impact of astaxanthin supplementation in feed on the gonadal development of *M. rosenbergii* broodstock, specifically focusing on the underlying mechanisms of action by which astaxanthin influences ovarian growth, antioxidant capacity, and hormone regulation. The findings provide valuable data to support the formulation of specialized broodstock diets.

## 2. Materials and methods

This research was conducted in strict accordance with the Guidance for the care and use of laboratory animals in China. The experimental protocol was approved by Huzhou University's Committee on Ethics of Animal Experiments (20220916).

### 2.1. Preparation of experimental feed

The astaxanthin used in this study (10 % concetration) was provided by Zhejiang NHU Company Ltd., with the remaining 90 % consisting of chemically synthesized lignosulfonate, sugars, and starch.

Five isonitrogenous (47 %) and isolipidic (9.5 %) diets were formulated with actual astaxanthin contents were 0, 27, 62, 140, and 310 mg/kg, designated as AS0, AS27, AS62, AS140, and AS310, respectively (Table 1). The fish meal, soybean meal, and shrimp meal were ground and sieved through a 60-mesh screen, while calcium dihydrogen phosphate and trace elements were sieved through an 80-mesh screen. The ingredients were mixed using a stepwise expansion method, followed by the addition of soybean oil, soybean lecithin, fish oil, and astaxanthin. The mixture was thoroughly blended, and then water was added for further mixing. The blends were extruded using a twin screw extruder (F-26, South China University of Technology, Guangzhou, China) (Luo et al., 2023). The pellets were subsequently dried in an oven at 40 ℃ until the moisture content dropped below 10 %, then stored at −20 ℃ prior to feeding.

**Table 1**
Ingredients and proximate compositions of the experimental diets (g/100, dry basis).

| Ingredients | Diet groups | | | | |
|---|---|---|---|---|---|
| | AS0 | AS27 | AS62 | AS140 | AS310 |
| Fish meal | 40 | 40 | 40 | 40 | 40 |
| Soybean meal | 10 | 10 | 10 | 10 | 10 |
| Shrimp meal | 8 | 8 | 8 | 8 | 8 |
| Wheat gluten meal | 15 | 15 | 15 | 15 | 15 |
| Fish oil | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Soybean oil | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Soy lecithin | 2 | 2 | 2 | 2 | 2 |
| Alpha-starch | 15 | 15 | 15 | 15 | 15 |
| Cholesterol | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin mix[a] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Mineral mix[b] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Choline chloride | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| $Ca(H_2PO_4)_2$ | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Carboxymethyl cellulose | 2 | 2 | 2 | 2 | 2 |
| Microcrystalline cellulose | 1.5 | 1.45 | 1.4 | 1.3 | 1.1 |
| Astaxanthin (10 %) | 0 | 0.05 | 0.1 | 0.2 | 0.4 |
| Total | 100 | 100 | 100 | 100 | 100 |
| Proximate composition | | | | | |
| Crude protein (%) | 47.16 | 47.41 | 46.59 | 46.20 | 47.32 |
| Crude lipid (%) | 9.68 | 9.54 | 9.53 | 9.53 | 9.40 |
| Ash (%) | 12.45 | 12.48 | 12.63 | 11.05 | 11.49 |
| Astaxanthin (mg/kg) | 0 | 27 | 62 | 140 | 310 |

[c]The astaxanthin in the feed provided the following per 100 g: 10 g astaxanthin (10 % concentration), 90 g of other components including chemically synthesized lignosulfonate, sugars, and starch, supplied by Zhejiang NHU Company Ltd

[a] Vitamin premix provided the following per kg of diets: Vitamin A 16000 IU, Vitamin C 150 mg, Vitamin $D_3$ 2000 IU, Vitamin E 360 mg, Vitamin $K_3$ 10 mg, Vitamin $B_1$ 16 mg, Vitamin $B_2$ 45 mg, Vitamin $B_6$ 20 mg, Vitamin $B_{12}$ 0.4 mg, Calcium pantothenate 70 mg, nicotinamide 80 mg, Folic acid 5 mg, Biotin 1 mg, Inositol 320 mg, Zeolite meal 3886.6 mg.

[b] Mineral premix provided the following per kg of diets: $FeSO_4 \bullet 7\ H_2O$ 124.13 mg, $CuSO_4 \bullet 5\ H_2O$ 9.77 mg, $MnSO_4 \bullet H_2O$ 26.15 mg, $ZnSO_4 \bullet 7\ H_2O$ 154.53 mg, $Na_2SeO_3$ 0.44 mg, $Ca(IO_3)_2$ 2.31 mg, $CoCl_2 \bullet 6\ H_2O$ 1.6 mg, $MgSO_4 \bullet 7\ H_2O$ 1224.49 mg, Zeolite meal 3456.59 mg.

### 2.2. Culture system and feeding regime

The *M. rosenbergii* broodstock was provided by Zhejiang Zhongyi Aquatic Seed Technology Co., LTD., and the feeding trial conducted in their facility in Huzhou City, China. Six hundred healthy *M. rosenbergii*, averaging 10.51 ± 0.03 g, were separated into five dietary treatment groups, each with four replicates of 30 prawn per replicate (The female-to-male ratio was 2:1) and placed in concrete tanks (1.5 m × 1 m × 1 m). A 56-day feeding trial was conducted based on the growth condition of *M. rosenbergii*. The prawns were fed twice daily at 8:00 AM and 5:00 PM, with the feeding amount adjusted to 1.5–2 % of their body weight (adjusted every two weeks). Feeding was done manually. Thirty minutes after feeding, the feeding behavior of the prawns was observed, and any residual feed in the aquaculture water system was collected. Water temperature was kept at 28–29 °C, dissolved oxygen at 5–6 mg/L, pH at 7.8–8.4, and ammonia nitrogen ≤ 0.3 mg/L during the trial.

### 2.3. Sample collection

After 56 days of feeding , female prawns were taken out and placed in an ice bath for 10 minutes to induce anesthesia.Following anesthesia, their body weight and length were measured and recorded. Hemolymph was drawn from the pericardial cavity using a sterile syringe, chilled in an ice box, then centrifuged at 3500 r/min for 10 min, and the serum was stored at −80°C for later analysis.

The gonads and hepatopancreas were dissected and accurately weighed and were immediately placed in liquid nitrogen for temporary storage and later transferred to a −80°C freezer for biochemical and genetic analysis. Furthermore, all female prawns were sampled and their

gonadal developmental stages were assessed. The prawns with the same ovarian development stage were pooled for sample analysis. Based on observation (Table 2), the gonads of *M. rosenbergii* were primarily in stage III; therefore, only stage III samples were selected for all subsequent analyses. The growth performance data calculations are as follows:

Survival rate (SR, %) = 100 × Final number of prawns / Initial number of prawns ;
Weight gain (WG, %) = 100 × (Final weight − Initial weight) / initial weight;
Specific growth rate (SGR, % day-1) = [(ln·Final body weight (g) - ln·Initial body
weight (g)) / Experimental days] × 100;
Specific growth rate (SGR, % $day^{-1}$) = [(ln·Final body weight (g) - ln·Initial body
weight (g)) / Experimental days] × 100;
Gonadosomatic index (GSI, %) = Gonad wet weight / Body wet weight × 100;
Hepatosomatic index (HSI, %) = Hepatopancreas wet weight/ Body wet weight × 100.

### 2.4. Indicators and measuring methods

Astaxanthin content in the feed was measured by Shandong Bayong Biotechnology Co., Ltd. The samples were extracted with a mixed solution of dichloromethane and dimethyl sulfoxide, and cholesterol esterase was added. After the enzymatic hydrolysis, anhydrous sodium sulfate and petroleum ether were added for centrifugation, dried with nitrogen, dissolved in acetone, and passed through a 0.22 μm (Nylon) organic filter. After separation by a C30 reversed-phase liquid chromatography column, it was determined by liquid chromatography.

The drying method was used to measure the moisture content at 105 ℃ (GB/T 6435–2006/ISO 6496:1999). The Dumas nitrogen analyzer was used to calculate the crude protein content (GB/T 6432–94). The Soxhlet extractor was used to determine crude fat content (GB/T 6443–2006/ISO 6492:1999). A 550 °C muffle furnace was used to measure the ash content (GB/T 6438–2007/ISO 5984:2002).

The contents of ovarian vitellogenin (VTG), testosterone (T) and serum estradiol ($E_2$) were analyzed using assay kits produced by Jiangsu Meimian Industrial Co., Ltd. The total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and malondialdehyde (MDA) levels in the hepatopancreas were assessed with kits supplied by Nanjing Jiancheng Bioengineering Institute.

### 2.5. Ovarian transcriptome analysis

After sampling, the analysis of phenotypic data revealed that the AS140 group achieved the highest specific growth rate and gonadosomatic index. Therefore, ovarian samples from the AS0 and AS140 groups were selected for transcriptomic analysis. Ovarian transcriptome analysis of AS0 group and AS140 (4 samples in each group) was performed by Shanghai Meiji Biotechnology Co., Ltd. for RNA extraction, quality control, library construction, and sequencing. RSEM software was utilized to quantify transcript and gene expression levels. After obtaining the Read Counts for genes, differential expression was analyzed using the DESeq2 software, with the default criteria for significantly differentially expressed genes being: FDR < 0.05 and |log2FC= ≥ 1. When a gene met both of these criteria, it was considered a differentially expressed gene (DEG). The Python scipy package was used to statistically analyze the enrichment of genes that were differentially expressed in KEGG and GO pathways. The software Goatools was used to perform GO and KEGG enrichment analysis on the gene set, thereby identifying the main GO functions and KEGG pathways associated with the genes in the set. The Fisher's exact test was employed as the method, and a GO function or KEGG pathway was considered significantly enriched when the $P <$ 0.05.

**Table 2**
The proportion of prawns with gonad development from stage I to stage V.

| Diet Groups | I | II | III | IV | V |
|---|---|---|---|---|---|
| AS0 | 8.9 | 15.6 | 57.8 | 13.3 | 4.4 |
| AS27 | 2.0 | 24.5 | 57.1 | 8.2 | 6.1 |
| AS62 | 3.4 | 34.5 | 55.2 | 3.4 | 3.4 |
| AS140 | 2.1 | 27.1 | 66.7 | 2.1 | 2.1 |
| AS310 | 1.7 | 38.3 | 51.7 | 3.3 | 5.0 |

### 2.6. Quantitative RT-PCR analysis

To validate the RNA sequencing results, five differentially expressed genes were chosen for quantitative RT-PCR analysis. Primers were designed based on the Unigene sequences obtained by sequencing, using Premier 6 software (Table 3), with the β-actin gene serving as an internal reference. The UltraSYBR Mixture kit (Kangwei Century) was used to carry out real-time quantitative PCR. The comparative CT method ($2^{-\triangle\triangle ct}$) was employed to calculate the relative levels of gene expression (Livak and Schmittgen, 2001).

### 2.7. Statistical analysis

All data were analyzed using SPSS 25.0, with results reported as mean ± standard error (SEM). Each variable was evaluated with analysis of variance (ANOVA), and multiple comparisons were made using Duncan's test. Significance was set at $P < 0.05$. Orthogonal polynomial contrasts assessed trends, and if significant effects (linear, quadratic, or cubic) were found, regression analysis identified the best-fitting model.

## 3. Results

### 3.1. The effect of astaxanthin on the growth and gonadal development of *M. rosenbergii*

No significant differences in HSI and FBW were detected among the five dietary groups (Table 4, $P > 0.05$). The IBW of the AS0 and AS310 groups was significantly higher than that of the AS62, and AS140 groups. As the astaxanthin levels in the feed increased, the GSI initially rose and then decreased, reaching its highest point in the AS62 group, which showed a significant difference from the AS0 and AS27 groups ($P < 0.05$). The SR first increased and then levelled off as the astaxanthin supplementation increased, with the AS0 and AS27 groups being significantly lower than the other groups ($P < 0.05$). The SGR and WG showed a pattern of initially increasing and then decreasing with rising astaxanthin levels, with values in AS27, AS62, and AS140 being significantly higher than those in AS0 ($P < 0.05$).

**Table 3**
Nucleotide sequences of the primers used to assay gene expressions by real-time PCR.

| Gene | Primer sequence(5′-3′) | Gene Bank |
|---|---|---|
| *gpx* | GCAACCAGTTCGGCAAGCAAGA<br>AACATGATGTCGTTCCTGATCTCGTC | QIH05099.1 |
| *fbp* | TGGCAATGGTGTCAATGGCTTCA<br>GGAACGCTGATGAATGTCTGTTGGA | XP_037784493 |
| *g6pd* | GAGGTCCCAGTTAGTCGTCTGAAAG<br>CACAGAAGCCACAGCGTAGGATAT | XP_042214326 |
| *hpd* | CGTTGATGATTCTCAGGTCCACACTAA<br>TGTTCATATCCTCCACGATCTTCACTG | XP_037799122 |
| *anpep* | ACTGTCTCAACAATTCCGCTACTCTC<br>TCCAGCCGTAACTGACTTGATGATTC | XP_042215803 |
| *β-actin* | TCCGTAAGGACCTGTATGCC<br>TCGGGAGGTGCGATGATTTT | AY651918.2 |

**Table 4**
Effects of astaxanthin on growth and gonadal development of *Macrobrachium rosenbergii*.

| Item | Astaxanthin level (mg/kg) | | | | | PSE | P-values | Linear | Quadratic | Cubic |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 27 | 62 | 140 | 310 | | | | | |
| IBW(g) | 11.026$^{a}$ | 10.002$^{bc}$ | 9.628$^{c}$ | 9.516$^{c}$ | 10.748$^{ab}$ | 0.152 | 0.001 | 0.860 | 0.927 | 0.985 |
| FBW (g) | 21.266 | 22.406 | 21.388 | 22.141 | 21.814 | 2.856 | 0.930 | 0.860 | 0.927 | 0.985 |
| SR (%) | 69.250$^{b}$ | 68.250$^{b}$ | 80.000$^{a}$ | 80.000$^{a}$ | 80.000$^{a}$ | 1.809 | 0.030 | 0.033 | 0.021 | 0.053 |
| WG (%) | 92.946$^{b}$ | 124.201$^{a}$ | 122.776$^{a}$ | 132.496$^{a}$ | 103.621$^{ab}$ | 4.683 | 0.035 | 0.897 | 0.014 | 0.026 |
| SGR(%/day) | 1.168$^{b}$ | 1.434$^{a}$ | 1.419$^{a}$ | 1.487$^{a}$ | 1.255$^{ab}$ | 0.038 | 0.029 | 0.846 | 0.014 | 0.022 |
| GSI (%) | 0.203$^{c}$ | 0.249$^{bc}$ | 0.361$^{a}$ | 0.308$^{ab}$ | 0.298$^{ab}$ | 0.015 | 0.005 | 0.159 | 0.020 | 0.006 |
| HSI (%) | 6.817 | 7.849 | 7.590 | 7.257 | 6.977 | 0.168 | 0.276 | 0.458 | 0.470 | 0.248 |

Note: All data were expressed as mean ± SE (n = 4). Mean values within the same row with different lower superscripts showed significant differences ($P < 0.05$). IBW, Initial Body Weigh; FBW, Final Body Weight; GSI, gonadosomatic index; HSI, hepatosomatic index; SGR, specific growth rate.

### 3.2. The effect of astaxanthin on the serum hormones of M. rosenbergii

The $E_2$ content in the serum of the AS62 and AS140 groups was significantly higher than in the AS0, AS27, and AS310 groups (Table 5, $P < 0.05$). The T levels in the serum of the AS27 and AS62 groups were significantly higher than those in the AS310 group ($P < 0.05$). The VTG content in the serum of the AS62 and AS140 groups was significantly higher than that of the AS0 group ($P < 0.05$). The T content in the serum peaked at the 114.30 mg/kg astaxanthin, the $E_2$ content peaked at 164.39 mg/kg, and the VTG content peaked at 165.14 mg/kg (Fig. 1).

### 3.3. The effect of astaxanthin on the antioxidant activity in the hepatopancreas of M. rosenbergii

Liver SOD activity initially increased and then decreased with rising astaxanthin levels in the feed, peaking in the AS62 group, which showed significant differences compared to the AS0, AS27, and AS310 groups (Table 6, $P < 0.05$). Similarly, as the astaxanthin level increased, the hepatopancreas T-AOC capacity first increased and then decreased, peaking in the AS140 group and showing significant differences compared to the AS0 and AS27 groups ($P < 0.05$). The hepatopancreas MDA content initially decreased and then increased, with the lowest content in the AS140 group, significantly different from the AS0 group ($P < 0.05$).

### 3.4. The effect of astaxanthin on the hepatopancreas and gonadal tissues of M. rosenbergii

In the AS0 group, the hepatopancreatic tubules of *M. rosenbergii* were sparsely arranged, with deformed star-shaped lumens, fewer B cells, and an unclear basement membrane (Fig. 2). In the AS27 and AS62 groups, the hepatopancreatic tubules were arranged relatively neatly and tightly, with a clear star-shaped lumen structure and normal cell organization. In the AS140 group, the hepatopancreatic tubules were tightly arranged, the basement membrane between the tubules was intact, and B cells were abundant. In the AS310 group, the hepatopancreatic tubules were sparsely arranged, B cells numbers decreased, and the basement membrane was unclear. No significant changes were observed in R cells among the experimental groups.

The AS140 group exhibited a significant reduction in oogonia in the ovaries of *M. rosenbergii* compared to other groups (Fig. 3). The group showed a higher number of endogenous vitellogenic oocytes with significantly larger average area, arranged more compact structure. Additionally, follicle cells surrounded each oocytes, creating a well-defined follicular cavities. No significant changes were observed in the previtellogenesis oocytes among all groups.

### 3.5. Transcriptome analysis of the effect of astaxanthin on gonadal development

Transcriptome analysis revealed the molecular mechanism by which astaxanthin in the feed regulated the gonadal development of *M. rosenbergii*. The sequencing error rates for the quality control data of the eight samples ranged from 0.0121 % to 0.0123 %, with Q20 values between 98.58 % and 98.68 %, Q30 values from 95.00 % to 95.83 %, and GC content ranging from 40.07 % to 42.91 % (Table 7). These results indicated that the sequencing data from the eight samples met the standards for transcriptome analysis. The statistical results of the DEGs in the gonads of *M. rosenbergii* revealed 615 upregulated genes and 496 downregulated genes between the AS0 and the AS140 group (Fig. 4). The obtained 50,100 unigenes were compared against six databases, including GO, KEGG, eggNOG, NR, Swiss-Prot, and Pfam, resulting in the annotation of 9653, 8862, 11,005, 14,522, 9599, and 11,033 unigenes in each database, respectively (Table 8). Statistical analysis of the comparison between *M. rosenbergii* unigenes and the NR database revealed that the highest proportion of homologous sequences was with American Lobster (*Homarus americanus*), reaching 23.18 %, followed by Red Swamp Crayfish (*Procambarus clarkii*), kuruma prawn (*Penaeus japonicus*), and Pacific white shrimp (*Penaeus vannamei*) with homologous sequence comparison proportions of 17.18 %, 13.13 %, and 11.29 %, respectively. The results indicate that the ovarian unigenes of *M. rosenbergii* exhibit a high degree of homology with the sequences of *American Lobster* (Fig. 5). To elucidate the physiological regulatory mechanism of gonadal development in *M. rosenbergii*, GO functional enrichment analysis of the DEGs was performed (Fig. 6), identifying associations with terms such as "extracellular region," "pigment binding," "glucose-6-phosphate dehydrogenase activity," and "protein peptidyl-prolyl isomerization" ($P < 0.05$). Additionally, KEGG pathway enrichment analysis of the DEGs (Fig. 7) highlighted pathways like the "pentose phosphate pathway," "glutathione metabolism," and "ubiquinone and other terpenoid-quinone biosynthesis" ($P < 0.05$), all of which influenced the gonadal development of *M. rosenbergii*.

**Table 5**
Effects of astaxanthin on serum hormones of *Macrobrachium rosenbergii*.

| Item | Astaxanthin level (mg/kg) | | | | | PSE | P-values | Linear | Quadratic | Cubic |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 27 | 62 | 140 | 310 | | | | | |
| $E_2$ (ng/L ) | 37.587$^{b}$ | 36.465$^{b}$ | 40.864$^{a}$ | 41.332$^{a}$ | 37.587$^{b}$ | 0.601 | 0.012 | 0.854 | 0.016 | 0.044 |
| T (nmol/L) | 5.682$^{ab}$ | 6.169$^{a}$ | 6.483$^{a}$ | 6.033$^{ab}$ | 5.195$^{b}$ | 0.146 | 0.034 | 0.043 | 0.015 | 0.013 |
| VTG (ug/l) | 420.307$^{b}$ | 436.157$^{ab}$ | 444.041$^{a}$ | 449.707$^{a}$ | 431.042$^{ab}$ | 3.488 | 0.048 | 0.687 | 0.009 | 0.020 |

Note: All data were expressed as mean ± SE (n = 4). Mean values within the same row with different lower superscripts showed significant differences ($P < 0.05$). $E_2$, estradiol; T, testosterone; VTG, vitellogenin.

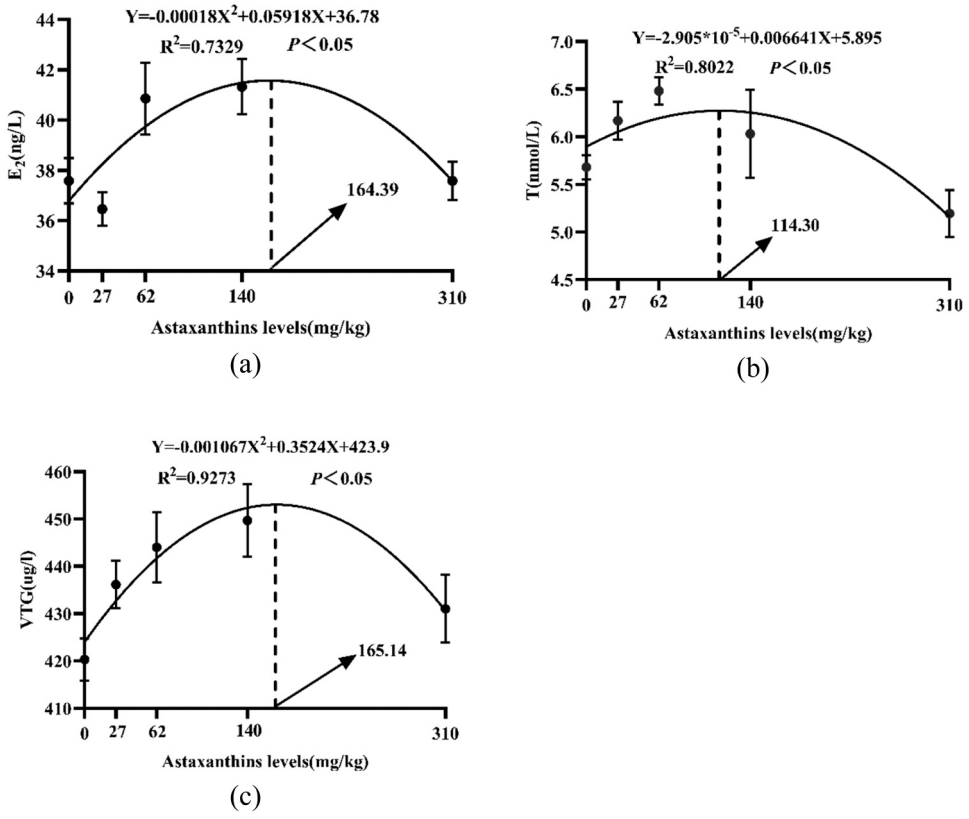


**Fig. 1.** Effects of astaxanthin on serum hormones of *Macrobrachium rosenbergii*. Note: T, testosterone; $E_2$, estradiol; VTG, vitellogenin.

**Table 6**
Effect of astaxanthin on liver antioxidant of *Macrobrachium rosenbergii*.

| Item | Astaxanthin level (mg/kg) | | | | | PSE | *P*-values | Linear | Quadratic | Cubic |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 27 | 62 | 140 | 310 | | | | | |
| MDA (nmol/mgprot) | $14.429^{a}$ | $11.377^{ab}$ | $9.225^{b}$ | $8.865^{b}$ | $9.695^{b}$ | 0.676 | 0.034 | 0.080 | 0.011 | 0.013 |
| T-AOC (U/mgprot) | $0.785^{c}$ | $0.902^{bc}$ | $1.348^{ab}$ | $1.492^{a}$ | $1.271^{abc}$ | 0.091 | 0.042 | 0.088 | 0.009 | 0.027 |
| SOD (U/mgprot) | $128.190^{b}$ | $132.400^{b}$ | $194.406^{a}$ | $161.795^{ab}$ | $120.170^{b}$ | 9.009 | 0.031 | 0.451 | 0.058 | 0.067 |

Note: All data were expressed as mean ± SE (n = 4). Mean values within the same row with different lower superscripts showed significant differences ($P < 0.05$). MDA, malondialdehyde; T-AOC, total antioxidant capacity; SOD, superoxide dismutase.

### 3.6. DEGs were verified by fluorescence quantitative PCR

To verify the accuracy of the transcriptomic data, five DEGs were randomly selected from the significantly enriched KEGG pathways for qPCR validation analysis, with the results shown in Fig. 8. Compared with the AS0 group, Fructose-1,6-Bisphosphatase (*fbp*), Aminopeptidase N (*anpep*), Glutathione Peroxidase (*gpx*), Glucose-6-Phosphate Dehydrogenase (*g6pd*), and Hydroxyphenylpyruvate Dioxygenase (*hpd*) were significantly elevated in the AS140 group ($P < 0.05$). This consistency demonstrated that the transcriptomic sequencing analysis results were accurate and reliable.

## 4. Discussions

The widespread use of astaxanthin as a feed supplement in aquaculture was fully recognized. Earlier research demonstrated that supplementing with a suitable level of astaxanthin could enhance the growth performance of *Penaeus monodon* (Huang et al., 2023), *Litopenaeus vannamei* (Eldessouki et al., 2022), *Procambarus clarkii* (Cheng and Wu, 2019), and *Paralithodes camtschaticus* (Daly et al., 2013). In this study, adding various levels of astaxanth to the diet significantly improved the specific growth rate of *M. rosenbergii*, with the AS140 group exhibiting the best growth performance, aligning with findings in *Litopenaeus vannamei* (Zhang et al., 2013a,b). The growth-enhancing effect of astaxanthin in aquatic animals was attributed to the fact that astaxanthin is a non-vitamin A carotenoid. Carotenoids have been shown to shorten the molting cycle in crustaceans and influence NADPH metabolism in aquatic animals, thereby decreasing energy expenditure and enhancing growth performance (Hertrampf and Piedad-Pascual, 2000; Mao, 2017). The GSI is commonly used as a key metric to assess the gonadal development of aquatic animals (Du et al., 2018). Findings from this study elucidated that the AS62 group had the highest GSI, with significant differences from the AS0 and AS27 groups, which was similar to the results in Nile tilapia (*Oreochromis niloticus*) (Qiang et al., 2022). However, in the AS140 and AS310 groups, the gonadosomatic index did not further increase, possibly because the astaxanthin content in the AS62 group had already met the requirements for gonadal development in *M. rosenbergii*. In studies on the reproductive performance of half-smooth tongue sole (*Cynoglossus semilaevis*) broodstock, the addition of astaxanthin-rich krill meal to the feed did not significantly improve the gonadosomatic index (Xu et al., 2017). In contrast, in studies on discus fish (*Symphysodon aequifasciatus*), the gonadosomatic index continued to increase with higher astaxanthin supplementation (Haque et al., 2023). This difference may be attributed to the varying astaxanthin requirements among different species. During the broodstock nutrition enhancement phase, gonadal development requires a

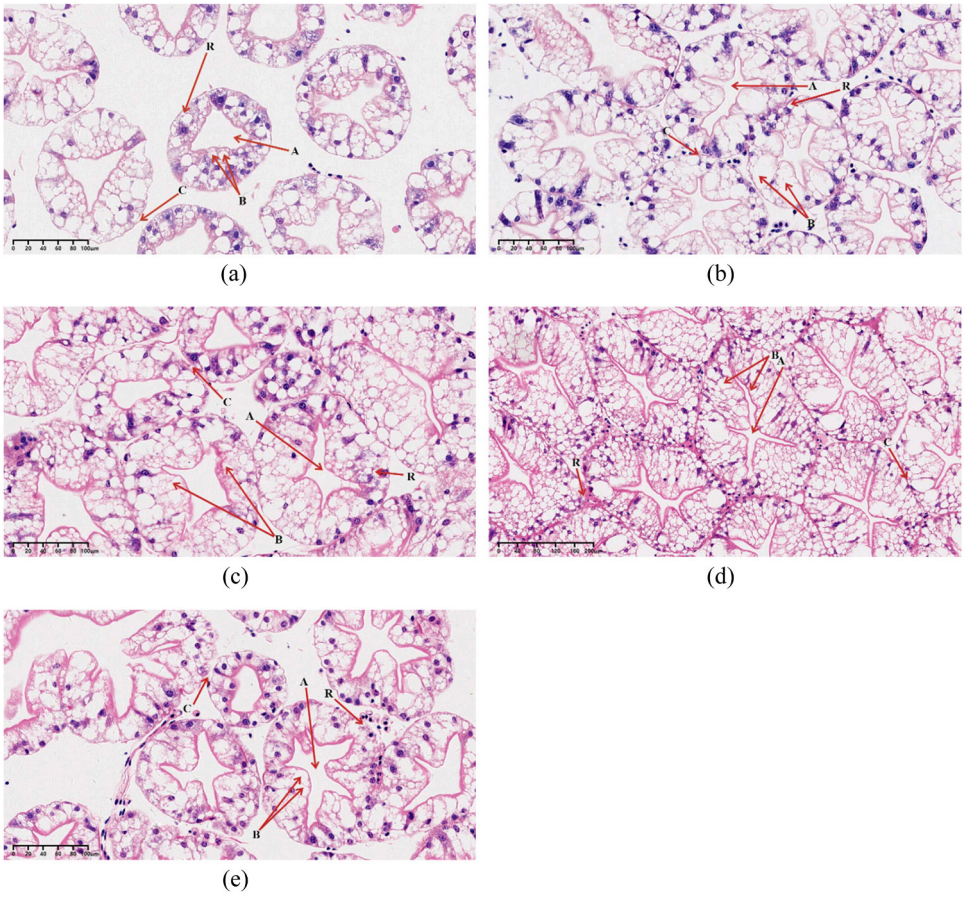


**Fig. 2.** Hepatic histology in *Macrobrachium rosenbergii* fed with diets containing different levels of astaxanthin for 56 days. Representative images of hematoxylin/eosin-stained liver tissue from female shrimp feed with diets containing astaxanthin (magnification ×20, scale bar: 100 μm). (a)–(e) correspond to groups fed with diets containing astaxanthin at 0, 27, 62, 140, and 300 mg/kg, respectively. A: star-shaped lumen; B: B cells (secretory cells); C: basement membrane; R: R cells (storage cells.

large amount of nutrients, with the nutrients in the hepatopancreas are transferred to the ovaries (Wouters et al., 2001). However, no significant correlation was observed between GSI and HSI in this study. A possible reason is that during reproduction, the body prioritizes the supply of nutrients needed for ovarian development, and after reproduction, the hepatopancreas may continue accumulating nutrients in preparation for the next reproductive cycle (Jiang, 2022).

The development of shrimp ovaries was influenced by a complex neuroendocrine regulatory network, and steroid hormones were proven to play a key role in the regulation of reproductive endocrinology (Köhler et al., 2007; Liu et al., 2021; Wierman, 2007). The main steroid hormones included estradiol ($E_2$), testosterone (T), progesterone (P), and cortisol (C). Among these steroid hormones, $E_2$ played a crucial regulatory role in ovarian development and vitellogenesis (Levavi-Sivan et al., 2006; Pankhurst, 2011). Previous studies showed that an appropriate amount of astaxanthin could increase the $E_2$ content in the serum of Tongue Sole (*Cynoglossus semilaevis*) (Xu et al., 2017) and enhance the synthesis and secretion of $E_2$ in cow follicles (Abdel-Ghani et al., 2019). This study demonstrated that $E_2$ and T levels in the serum of *M. rosenbergii* were highest in the 62 mg/kg, with significant differences from the 310 mg/kg. The changes in serum T levels mirrored those of $E_2$, primarily because T is the precursor of $E_2$ in crustaceans, and through the action of aromatase, T can be converted into $E_2$ (Kortner and Arukwe, 2007; Tsai et al., 2000). Estradiol in the serum primarily bound to estrogen receptors in hepatopancreas cells, activating the expression of the vitellogenin gene and promoting the synthesis of vitellogenin, thereby regulating the development and proliferation of oocytes (Örn et al., 2003). Studies on vertebrates such as largemouth bass (*Micropterus salmoides*), Nile tilapia (*Oreochromis niloticus*), and half-smooth tongue sole (*Cynoglossus semilaevis*) revealed that dietary astaxanthin mainly exerted its regulatory effects on gonadal development and maturation through the feedback mechanism of steroid hormones via the hypothalamus-pituitary-gonadal axis (HPG). These findings indicated that there were significant differences in the steroid hormone regulatory mechanisms between vertebrates and crustaceans, which might be related to their physiological structures and the evolutionary adaptation of their endocrine systems(Qiang et al., 2022; Tao et al., 2024; Xu et al., 2017). VTG is the main yolk protein precursor during the oocyte development of crustaceans, and the hepatopancreas is the primary organ responsible for the synthesis of VTG in crustaceans (Dominguez et al., 2012). In female crustaceans, after stimulation by sex hormones (such as $E_2$), cells in the hepatopancreas began to transcribe and translate VTG genes in large quantities, synthesizing VTG. VTG synthesized by hepatopancreatic cells was secreted into the hemolymph, which then transported it to the ovaries, where it accumulated and transformed into yolk proteins, providing essential nutrients for the oocytes (Gen et al., 2001; Mizuta et al., 2013). In this study, the VTG content in the serum of *M. rosenbergii* was highest in the140 mg/kg, with significant differences from the control group. A hypothesis was raised that adding an appropriate amount of astaxanthin promotes VTG synthesis in the hepatopancreas.

The hepatopancreas is an important digestive organ in crustaceans, functioning to secrete digestive enzymes, promote digestion and absorption, and store nutrients (Zhang, 2019). The hepatopancreatic tubule was the basic structural and functional unit, with each tubule being encapsulated and separated by a thin layer of connective tissue. The

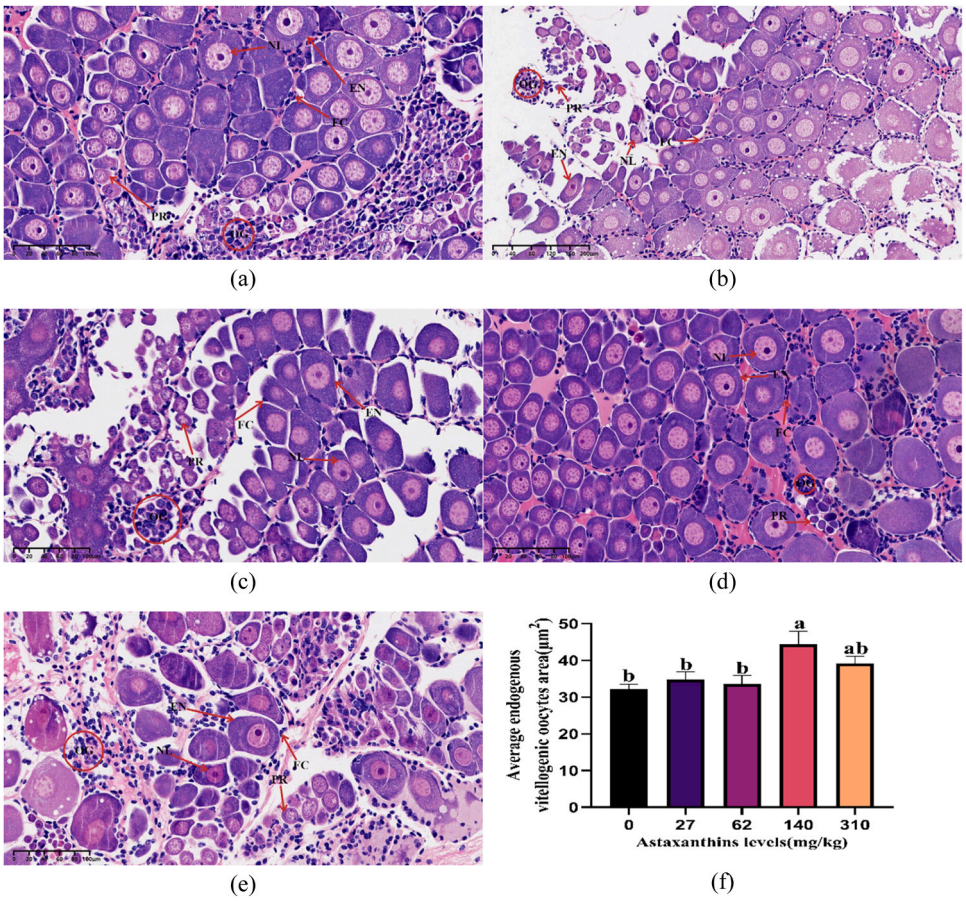


**Fig. 3.** Gonad histology in *Macrobrachium rosenbergii* fed with diets containing different levels of astaxanthin for 56 days. Representative images of hematoxylin/eosin-stained liver tissue from female shrimp feed with diets containing astaxanthin (magnification ×20, scale bar: 100 μm). (a)–(e) correspond to groups fed with diets containing astaxanthin at 0, 27, 62, 140, and 300 mg/kg, respectively. OG: oogonia; PR: previtellogenic oocytes; EN: endogenous vitellogenic oocytes; FC: follicular cell; NL: nucleus.

**Table 7**
Quality assessment of the sequencing data of ovary samples in different treatments.

| Diet Groups | Raw reads | Raw bases | Clean reads | Clean bases | Error rate (%) | Q20 (%) | Q30 (%) | GC content (%) |
|---|---|---|---|---|---|---|---|---|
| AS0–1 | 55085510 | 8317912010 | 54773970 | 8205614593 | 0.0121 | 98.67 | 95.8 | 42.91 |
| AS0–2 | 55712340 | 8412563340 | 55388582 | 8302099355 | 0.0122 | 98.65 | 95.75 | 40.73 |
| AS0–3 | 54640836 | 8250766236 | 54285584 | 8130976953 | 0.0123 | 98.59 | 95.57 | 42.12 |
| AS0–4 | 53212890 | 8035146390 | 52886678 | 7948623954 | 0.0123 | 98.56 | 95.5 | 40.07 |
| AS140–1 | 66947436 | 10109062836 | 66537752 | 9951354261 | 0.0122 | 98.62 | 95.64 | 42.29 |
| AS140–2 | 64177724 | 9690836324 | 63802690 | 9535742904 | 0.0121 | 98.68 | 95.83 | 40.92 |
| AS140–3 | 54731248 | 8264418448 | 54396334 | 8162604727 | 0.0123 | 98.58 | 95.51 | 41.57 |
| AS140–4 | 56673528 | 8557702728 | 56303882 | 8437447996 | 0.0122 | 98.61 | 95.64 | 42.06 |

hepatopancreatic tubule mainly consisted of secretory cells (B cells), storage cells (R cells), fibrocytes (F cells), and embryonic cells (E cells), with B cells and R cells being the most abundant (Romano et al., 2015). The function of R cells was to absorb and store nutrients and participate in lipoprotein metabolism, while B cells were responsible for nutrient absorption and served as the main site for digestive enzyme synthesis (Khalil et al., 2014). The findings from this study demonstrated that compared to other groups, the AS140 group had more compactly arranged hepatopancreatic tubules, a well-defined stellate lumen structure, an intact basement membrane between the hepatopancreatic tubules, and an abundant number of B cells. These findings support the hypothesis that addition of an appropriate amount of astaxanthin was beneficial to the absorption and storage of nutrients in the hepatopancreas of *M. rosenbergii*, improving its growth and ovarian development. The ovarian development of *M. rosenbergii* was closely related to the maturity of oocytes, with the oocytes in the ovaries gradually developing from the initial oogonia and undergoing stages of previtellogenesis, endogenous vitellogenesis, and exogenous vitellogenesis, eventually forming mature oocytes (Guimarães, 2020; Martins et al., 2007). Previous studies demonstrated that the addition of an appropriate amount of astaxanthin could accelerate the development of Oreochromis oocytes to stage V and reduce atretic follicles (Qiang et al., 2022). In this study, the number of oogonia in the ovaries of the AS140 group was significantly reduced, while the number of oocytes in the endogenous vitellogenic oocytes phase was higher, and the average area increased significantly, with a more compact arrangement. This suggested that the appropriate addition of astaxanthin in the feed could accelerate the growth and development of oocytes, thus promoting ovarian maturation.

The health of the hepatopancreas relied on an effective antioxidant

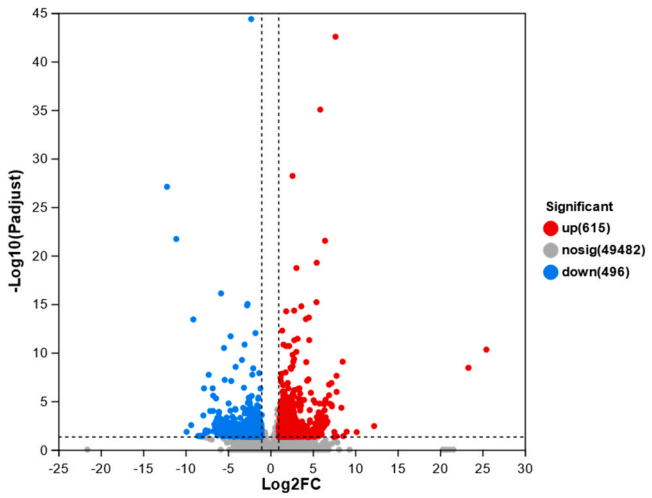


**Fig. 4.** The number of differentially expressed genes in Macrobrachium rosenbergii gonad tissue between the AS0 group and the AS140 group.

**Table 8**
Statistics of unigene annotations based on each database.

| Database category | Total unigene | Annotation unigene | Annotation percent ( % ) |
|---|---|---|---|
| GO | 50100 | 9653 | 0.1927 |
| KEGG | 50100 | 8862 | 0.1769 |
| eggNOG | 50100 | 11005 | 0.2197 |
| NR | 50100 | 14522 | 0.2899 |
| Swiss-Prot | 50100 | 9599 | 0.1916 |
| Pfam | 50100 | 11003 | 0.2196 |

defense mechanism; when the activity of antioxidant enzymes was maintained at a high level, the hepatopancreas could withstand damage caused by reactive oxygen species (ROS) and maintain its normal function (Ma et al., 2024; Shen et al., 2010; Zhang et al., 2013a,b). SOD effectively prevented oxidative stress damage to cells by eliminating superoxide free radicals (Yang et al., 2010). MDA is the final product of lipid peroxidation, is commonly used as an indicator of cellular oxidative damage (Kong et al., 2019). T-AOC reflected the overall ability of the antioxidant system to combat ROS and other free radicals; the higher the total antioxidant capacity, the stronger the body's defense against oxidative stress, thereby more effectively protecting cells and tissues from oxidative damage (Duan et al., 2015). Therefore, the levels of MDA, T-AOC, and SOD activity were used to assess the body's antioxidant capacity. Astaxanthin, a carotenoid containing multiple conjugated double bonds and phenolic hydroxyl groups, effectively neutralized free radicals, thus exhibiting powerful antioxidant properties (Ambati et al., 2014; Cheng et al., 2003). Li et al. (2022) found that the addition of 100 mg/kg astaxanthin to the feed improved the SOD and T-AOC capacity in the hepatopancreas of *Penaeus vannamei*, while the MDA level significantly decreased. Jiang (2020) reported that adding 7.5–11.25 mg/kg astaxanthin to the feed could enhance the T-AOC capacity in the hepatopancreas of juvenile *Eriocheir sinensis*, with a significant reduction in MDA levels. The results of this study indicated that adding 62–140 mg/kg of astaxanthin to the feed significantly increased the activity of superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) in the hepatopancreas of *M. rosenbergii*, while significantly decreasing the content of malondialdehyde (MDA). This finding was consistent with the conclusions of previous studies, suggesting that an appropriate amount of astaxanthin could effectively enhance the antioxidant capacity of the organism and reduce oxidative damage. However, when the astaxanthin supplementation in the feed reached 310 mg/kg, the antioxidant capacity of *M. rosenbergii* declined. This phenomenon may be related to the "pro-oxidant effect" of high concentrations of astaxanthin. During the process of scavenging free radicals, astaxanthin might generate unstable intermediates, such as astaxanthin radicals, which not only fail to effectively scavenge free radicals but also induce oxidative stress, leading to a reduction in antioxidant capacity (Hussein et al., 2006). This mechanism was further validated in a study on *Marsupenaeus japonicus*: when the feed contained a high dose of astaxanthin, the MDA content in the shrimp significantly increased, while the SOD activity significantly decreased (Chen et al., 2023). Similarly, in studies on *Penaeus vannamei*, high doses of astaxanthin were also observed to increase MDA levels, further confirming that high concentrations of astaxanthin might cause oxidative damage (Xie et al., 2018). These findings collectively suggest that the antioxidant effect of astaxanthin is dose-dependent: appropriate supplementation can effectively enhance antioxidant capacity, while excessive supplementation may induce a pro-oxidant effect, leading to a decrease in antioxidant capacity.

The mechanism of ovarian development has been widely studied through transcriptome sequencing technology in species such as Red Swamp Crayfish (*Procambarus clarkii*) (Kang et al., 2019) and rainbow trout (*Oncorhynchus mykiss*) (Nynca et al., 2023). In this study, KEGG functional enrichment analysis of the transcriptome showed that many differentially expressed genes were concentrated in pathways such as

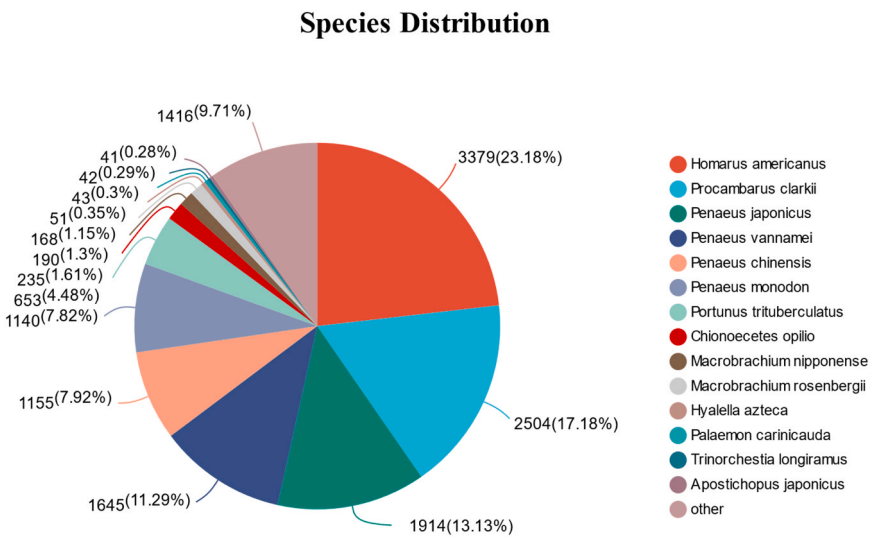


**Fig. 5.** Statistical Comparison of Species Category Map.

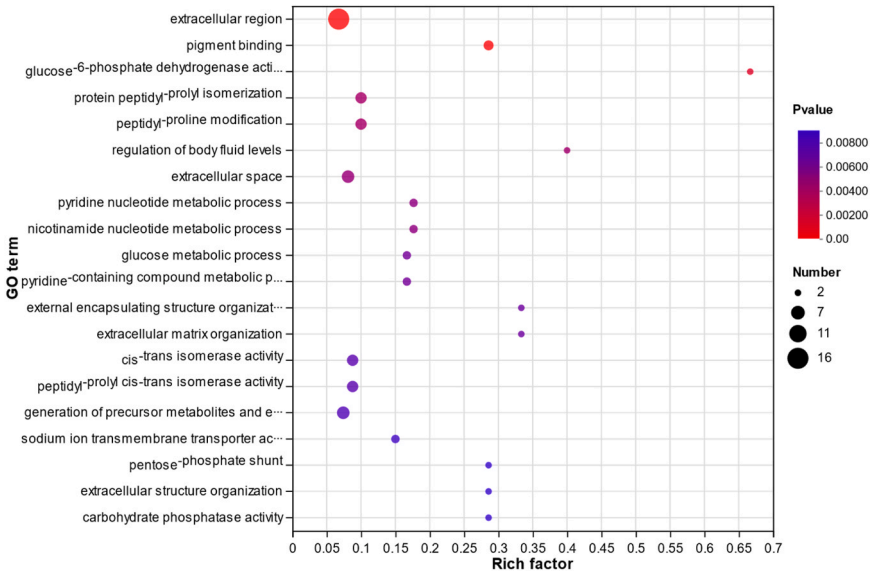


**Fig. 6.** GO classification of differentially expressed genes in the gonad tissues between the AS0 group and the AS140 group.

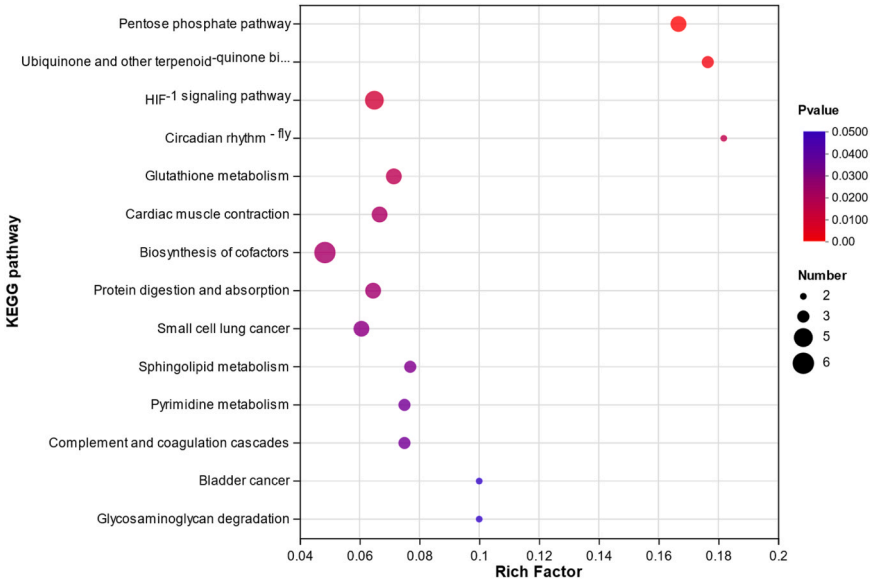


**Fig. 7.** KEGG enrichment subclasses and signaling pathways of differentially expressed genes in the gonad tissues between the AS0 group and the AS140 group.

the "Pentose phosphate pathway," "Glutathione metabolism," and "Ubiquinone and other terpenoid-quinone biosynthesis", which were found to affect the development of gonads in *M. rosenbergii*. The pentose phosphate pathway was shown to provide multiple functions, including supplying reducing power, supporting nucleotide synthesis, and regulating metabolism (González-Ruiz, 2023.; Rufino-Palomares et al., 2013; Sagar et al., 2019; Yu, 2021). *g6pd*, the rate-limiting enzyme of the pentose phosphate pathway (PPP), was demonstrated to generate NADPH to support antioxidant defense and promote lipid synthesis (Wamelink et al., 2008). Previous study on tilapia (*Oreochromis mossambicus*) reported that the upregulation of the *g6pd* gene was found to produce more NADPH, which provided the energy and reducing power necessary for the synthesis of fats and steroids during growth and development (Dawood et al., 2019). In studies on juvenile *M. rosenbergii*, it was also found that the increase in *g6pd* (glucose-6-phosphate dehydrogenase) enzyme activity in the hepatopancreas promotes fatty acid synthesis and energy metabolism (Sagar et al., 2019). Moreover, the hepatopancreas of *M. rosenbergii* serves as a nutrient synthesis center, where synthesized nutrients can be transported to the gonads through the circulatory system, providing essential nutrients for gonadal development. During the ovarian development of *M. rosenbergii*, a large amount of fats and steroids were required to support the growth and development of oocytes. The upregulation of the *g6pd* gene was shown to promote these metabolic processes by increasing the supply of NADPH, thus providing the necessary energy and material basis for gonadal development. *fbp*, a key enzyme in carbohydrate metabolism, was found to have its gene upregulated, which activated the gluconeogenesis pathway, particularly by catalyzing the hydrolysis of fructose-1, 6-bisphosphate to fructose-6-phosphate (Cota-Ruiz et al., 2015). When the sugar supply was insufficient, gluconeogenesis was shown to serve as the primary pathway for synthesizing glucose (Zhang, 2022). Therefore, it was hypothesized that the upregulation of the *fbp* gene in this experiment led to glucose production via the gluconeogenesis pathway, providing energy for the oocytes of *M. rosenbergii* and supporting their growth, development, and normal functions. Astaxanthin activates the pentose phosphate pathway through its antioxidant effects, thereby

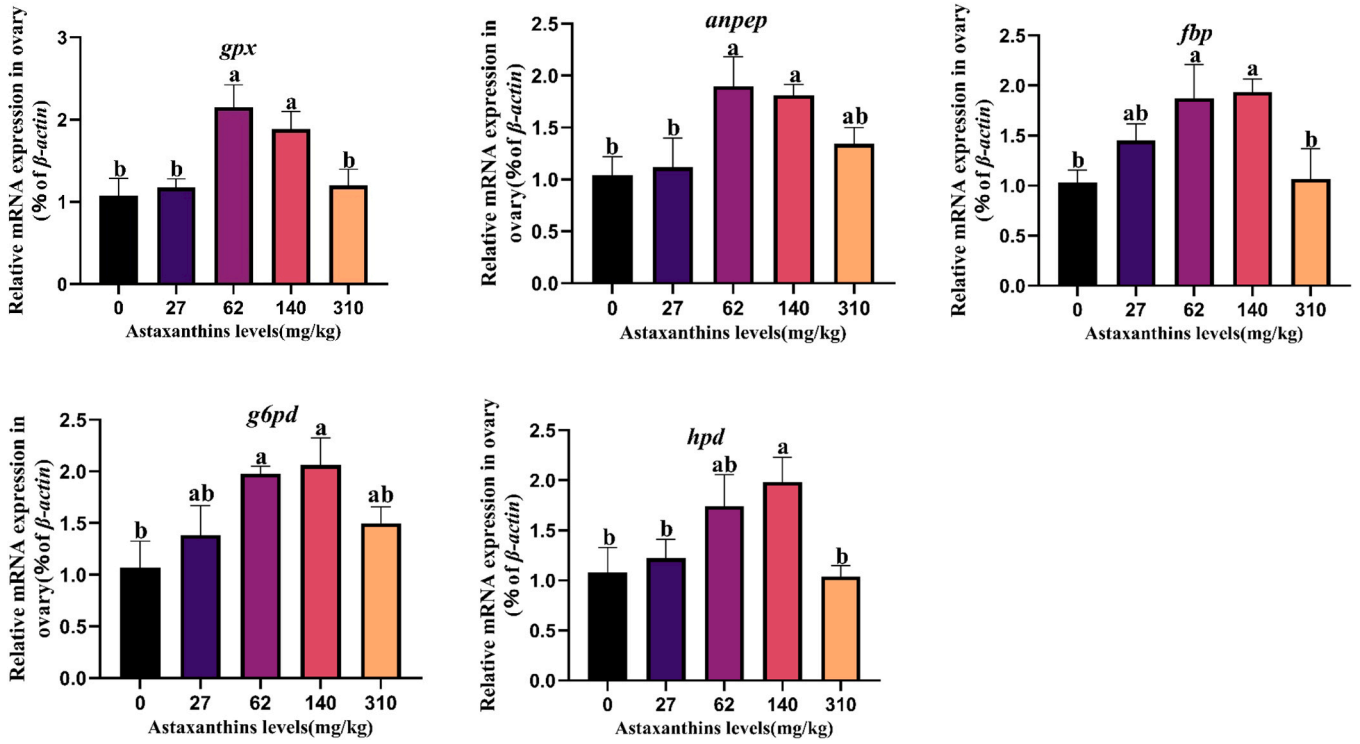


**Fig. 8.** Transcript levels of genes related to Pentose phosphate pathway and Ubiquinone and other terpenoid-quinone biosynthesis in *Macrobrachium rosenbergii* fed with diets containing astaxanthin at different levels for 56 days. All data were expressed as mean ± SE (n = 4). Mean values within the same row with different lower superscripts showed significant differences ($P < 0.05$). Note: Glutathione Peroxidase, gpx; Aminopeptidase N, anpep; Fructose-1,6-Bisphosphatase, fbp; Glucose-6-Phosphate Dehydrogenase, g6pd; Hydroxyphenylpyruvate Dioxygenase, hpd.

promoting the expression of the *g6pd* and *fbp* genes. The upregulation of these genes may provide sufficient energy and antioxidant support for gonadal development, consequently enhancing the gonadal maturation of *M. rosenbergii*. The glutathione metabolism pathway was primarily involved in redox balance, antioxidant protection, and cell function regulation (Liu, 2022). Research indicated that GSH was an important antioxidant that participated in cellular redox reactions and played a key role in multiple physiological processes (Liu et al., 2021). In studies on sows, the addition of glutathione to the culture medium was shown to effectively reduce oocyte lysis and increase the formation rate of blastocysts, thereby improving the quality and developmental competence of oocytes and blastocysts (Li et al., 2014). Similarly, in studies on cows, glutathione in the culture medium was found to significantly increase the proportion of oocytes that developed into morulae and blastocysts, accelerating embryonic development (Ali et al., 2003). These studies demonstrated that glutathione played a positive role in enhancing oocyte quality and embryo development. Findings from this study elucidated that the A140 group showed upregulation of genes such as *anpep* and *gpx* in the glutathione metabolism pathway. *anpep*, as an exopeptidase, was found to support cell repair and division by providing amino acids, thereby promoting cell growth (Frías-Quintana et al., 2021; Mazurais et al., 2015). Meanwhile, *gpx* was shown to reduce cellular inflammation and oxidative stress by eliminating ROS, helping to maintain cellular immune balance (El-Dahhar et al., 2024). Given these functions, it is hypothesized that the astaxanthin supplementation in the AS140 group enhanced the antioxidant capacity of oocytes, reducing the levels of ROS in the body, thus promoting follicle formation and oocyte maturation and development. Overrall, the "pentose phosphate pathway" and "glutathione metabolism" pathways were activated in *M. rosenbergii* in response to astaxanthin, indicating that astaxanthin boosts oocyte antioxidant capacity via multiple mechanisms, significantly reducing oxidative stress damage to ovarian tissues.

## 5. Conclusions

Astaxanthin supplementation in the feed was shown to enhance gonadal development, serum hormone levels, and antioxidant capacity of *M. rosenbergii*. Additionally, the relevant genes in the "Pentose phosphate pathway" and "Glutathione metabolism" pathways were upregulated. Based on VTG, T, and $E_2$ indicators, the optimal astaxanthin content for broodstock *M. rosenbergii* feed is recommended to be between 114.30 mg/kg to 165.14 mg/kg. In conclusion, this study offers new insights into the mechanisms through which astaxanthin influences gonadal development.

## CRediT authorship contribution statement

**Tao Mingwei:** Writing – original draft. **Wei Jie:** Data curation. **de Cruz Clement:** Writing – review & editing, Conceptualization. **Wang Junyi:** Methodology. **Du Houkuan:** Methodology. **Zhou Hangxian:** Data curation. **Xu Qiyou:** Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data Availability

Data will be made available on request.

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