Article

Effect of *Enterocytozoon Hepatopenaei* Infection on Hormonal Regulation in Pacific White Shrimp *Litopenaeus Vannamei*

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Abstract: *Enterocytozoon hepatopenaei* (EHP) is an obligate intracellular parasite classified as a fungal pathogen, which causes hepatopancreatic microsporidiosis (HPM) in shrimp, resulting in large economic losses to the shrimp culture industry. However, the molecular mechanisms of how EHP affects hormonal regulation in shrimp were rarely reported. Thus, in the present study, we investigated the transcriptomic data of the hepatopancreas of EHP-infected and uninfected shrimp to study the hormonal regulation corresponding to EHP infection. The results showed that there were 157 differentially expressed genes (DEGs) at the hormonal regulatory level, of which 32 genes were upregulated and 125 genes were downregulated. Functional enrichment analysis showed that many DEGs were enriched in ecdysis-related pathways, including EGFR tyrosine kinase inhibitor resistance, thyroid hormone signaling pathway, and steroid hormone biosynthesis. Ten genes were randomly selected for qRT-PCR to verify the transcriptome sequencing results, and the results demonstrated that the expression of these genes were consistent with the results of the transcriptome. Our study provides an important dataset that contributes to further understanding of how EHP affects shrimp at the level of hormonal regulation. It provides a basis for further research and control of HPM.

Keywords: Penaeus vannamei; Enterocytozoon hepatopenaei; Transcriptome; Hormone; Molting

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important crustaceans in aquaculture and is widely farmed around the world [1]. It is the most productive of the three major artificially farmed shrimp species in the world [2]. *Enterocytozoon hepatopenaei* (EHP) is a pathogen that infects shrimp hepatopancreatic tissue and causes hepatopancreatic microsporidiosis (HPM). It mainly infects the epithelial cells of the hepatopancreas of shrimp and causes slow growth or even stagnation of shrimp [3]. In 2013, EHP infection was found in shrimp farmed in China, and in the same year, shrimp farming industries in Liaoning, Zhejiang, and Jiangsu were affected by EHP, causing huge losses [4,5]. In recent years, researchers have conducted a series of studies on the epidemiological investigation and detection methods of EHP, but the mechanism of slow growth in shrimp caused by EHP has not been fully investigated.

EHP belongs to Microsporidia, which are an obligate intracellular parasite, marked by their extremely simplified organelles and dependence on the outside world for energy. Dominic Wiredu Boakye et al. also found that EHP cannot produce ATP through glycolysis or oxidative phosphorylation in a comparative analysis of the EHP genome [6,7]. Therefore, early studies mainly speculated that EHP causes slow host growth by utilizing host ATP [8].

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With the development of microbiome, transcriptome, proteome, and metabolomics technologies, the effects of EHP infection on hepatopancreas have been frequently reported recently. Y Duan et al. found that *Bacillus* in the gut flora may be involved in the metabolic homeostasis of *L. vannamei* in response to EHP infection through a combined microbiome and transcriptome analysis [9]. L Wang et al. found that EHP possesses abundant transporter proteins, such as ABC transporter proteins among membrane transporter proteins. These transporter proteins can help microsporidia species steal nutrients and energy from their hosts [10]. Ning M et al. found that there were multiple pathways causing stunting in *L. vannamei* after infection with EHP, such as juvenile hormone esterase-like carboxylesterase 1 (JHEC1) and ecdysteroid regulated-like protein (ERP), which affects *L. vannamei* at the level of hormonal regulation [11]. However, there is still a research gap regarding the transcriptome analysis related to hormonal regulation after EHP infection.

The hepatopancreas is the main target organ of EHP infection, and the life stages of EHP are mainly present in the tubule epithelial cells of the hepatopancreas [12]. In crustaceans, the hepatopancreas is an important immune organ for the synthesis of steroid hormones as well as certain biosynthetic steps [13]. It is also an important organ for the absorption and storage of nutrients and is essential for molting activities [14]. However, the hormonal regulation after EHP infection in shrimp hepatopancreas remains unclear. In this study, we explored the transcriptome of EHP-infected *L. vannamei* by generating de novo assembly using the Illumina platform. Our aim was to explore the differences in gene expression between infected and control groups regarding hormone regulation and to provide relevant transcriptomic data. These results will contribute to a better understanding of the interactions between *L. vannamei* and EHP, providing a wealth of data for further studies and new insights into the interactions between *L. vannamei* and EHP.

2. Materials and Methods

2.1. Pacific white shrimp samples

EHP-infected and uninfected Pacific white shrimp samples were collected from Dafeng district, Yancheng city, Jiangsu Province. The samples were reared separately after collection, with the system at a constant temperature of 24 ± 1 °C and salinity of 12 ± 2 PPT. Samples from the healthy and infected groups were sampled and tested by PCR 5 days before the experiment to confirm the absence of *Vibrio* infection and then used for omics study [15]. Nine shrimp individuals were randomly selected in the infected and control groups, respectively, according to the manufacturer's protocol, and the hepatopancreas was collected and stored in liquid nitrogen until RNA extraction.

2.2. RNA extraction and Quality control

Total RNA was extracted from the samples using TRIzol® Reagent (Invitrogenl, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of the extracted RNA was tested using Nanodrop2000, followed by agarose gel electrophoresis for RNA integrity. Agilent2100 was used to determine the RIN value. Onlyhigh-quality RNAsample (OD_{260}/OD_{280} = 1.8~2.2, $OD_{260}/OD_{230} \geq 2.0$, RIN ≥ 8.0, 28S:18S ≥ 1.0, >1ug) was used to construct sequencing library.

2.3. Library preparation and Illumina sequencing

After extracting total RNA, mRNA can be isolated from total RNA by using magnetic beads with oligo (dT) for A-T style base pairing with polyA. MRNA is then broken under suitable conditions by adding fragmentation buffer. Under the action of reverse transcriptase, one-strand cDNA is synthesized in reverse using mRNA as the template, followed by two-strand synthesis to form a double-stranded structure using random primers. After that, End Repair Mix is added to patch, the ends to be flat, and an A base is added at the 3' end for ligating the Y-shaped connector. After ligating the adapter, the products are

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purified and fragment sorted, and then the sorted products are used for PCR amplification and purification to obtain the final library. Finally, mapped data (reads) were obtained that could be used for subsequent transcript assembly, expression level calculation, etc.

2.4. *De novo assembly and gene annotation*

Low quality reads (Qphred < 20) and reads containing N (module base) were removed from the raw reads using fastp (https://github.com/OpenGene/fastp). Clean data was obtained and analyzed by HiSat2 (http://ccb.jhu.edu/software /hisat2/index.shtml) for comparison with the reference genome to obtain mapped data (reads) for subsequent transcript assembly, expression calculation, etc [16]. The assembly of transcripts was then performed using StringTie (https://ccb.jhu.edu/software/stringtie/) [17], while the results were evaluated for quality.

Gene functions were annotated in view of the following databases: Nr (non-redundant protein database, NCBI) [18], Pfam (Protein family) [19], KOG/COG (Clusters of Orthologous groups of proteins) [20], Swiss-Prot (A manually annotated and reviewed protein sequence database) [21], KO (KEGG Ortholog database) [22]and GO (Gene Ontology) [23], six databases The transcripts were annotated.

2.5. *Identification of differentially expressed genes*

To calculate the expression levels of transcripts in different groups, RSEM (http://deweylab.github.io/RSEM/) software was used , and the expression level of genes was determined according to the Fragments Per Kilobase of exon model per million mapped fragments (FPKM) method.

The software used for variance expression was DESeq2 (http://bioconductor.org/packages/stats/bioc/DESeq2/) and the analysis method was based on a negative binomial distribution model [24]. The criteria for significantly differentially expressed genes were: FDR < 0.05 (DESeq2/edgeR/ Limma) / FDR < 0.001 (DEGseq) / Prob > 0.8 (NOIseq) & $|\log2(FC)| \geq 1$, and a gene was considered differentially expressed genes (DEGs) when it met both conditions.

2.6. *Quantitative RT-PCR analysis*

To further validate our transcriptome data, RNA samples from transcriptome sequencing were analyzed by using quantitative real-time RT- PCR (qRT-PCR). Ten specific genes were selected, including SERCA, Action-C-A3a, 3β-HSD, HSP90, P450-9e2, P450-6k-1, BIP, ATP1B, UGT-2C1 & 17β-HSD, which are involved in immunity, molting, etc. 18s RNA was used as a reference gene for qRT-PCR analysis. Primers were designed using Primer Premier 5 software (Table 1). the qRT-PCR reaction system (20 μl) consisted of 2 × Power SYBR Green PCR Master mix, 1 μl of upper and lower primers, and 1 μl of cDNA template. PCR amplification conditions were: 5 min at 95 °C, followed by 10 s at 95 °C and 20 s at 57 °C. All reactions were performed in parallel three times.

Sequences of primers used in this study.

Primer name	Sequence (5′-3′)
SERCA-F	GGAGGACGCACATACCTATTC
SERCA-R	GGCAGTTCATTCGGACCATAC
Action-C-A3a-F	CATCCACGAGACCACCTACAAC
Action-C-A3a-R	GAGCGAGGGCAGTGATTTCC
3β-HSD-F	TGACGGAGTGCGGAACTTG
3β-HSD-R	ATCTTGACGGCGAGAGTGC
HSP90-F	GAAGTTGGAGAGAGGCTGTTGG
HSP90-F	GGAATGCGTCTGCGAGGTTAC
P450-9e2-F	TGGCACTGACCTGACGAT
P450-9e2-R	TCATGCTGGCCCTGTTCT

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P450-6k-1-F	CCTTGCCATGAGGTTTGC
P450-6k-1-R	CCAGGCTCCAGCACGATT
BIP-F	GTTGTCACTGTCCCTGCCTAC
BIP-R	CTTCTCGCCGTCCTTCTTGTC
ATP1B-F	CACCCAAGCAGACTCCAGAATG
ATP1B-R	AGGCAATGAAGCAGAGGATAGC
UGT-2C1-F	GAGTCCTTCTACCACACCAC
UGT-2C1-R	TTGAGGCTGTTGACGATGAG
17β-HSD-F	GGAGGAGGCAGTGGCATAG
17β-HSD-R	ACTCCACGGTTTCTTCAGC

2.7.Statistical Analysis

Statistical analysis was performed using SPSS 18.0 software. Data were expressed as mean ± standard deviation (SD). Significant differences between samples were analyzed by one-way analysis of variance (ANOVA) and Duncan's test with a significance level of 0.05.

3. Results

3.1. Transcriptome sequencing and Quality Assessment

The cDNA libraries of hepatopancreas of *Litopenaeus vannamei* with and without EHP infection were sequenced on Illumina sequencing platform. A total of 6 cDNA libraries were constructed (Table 2), and 58.71 Gb of Clean Data were obtained, of which each sample had more than 7.92 Gb of Clean Data, with sequence alignment ratios ranging from 88.19% to 90.19% with the reference genome . The analysis detected a total of 20,356 expressed genes, including 17,922 known genes and 2,434 novel genes; a total of 33,314 expressed transcripts, including 23,571 known transcripts and 9,743 novel transcripts. The above results indicate that the sequence data are of good quality and can be used for further analysis.

Summary of the sequencing data.

Sample	Raw reads	Clean reads	Error rate(%)	Q30(%)	GC content(%)
E4_1	83107974	82056776	0.0241	95.13	46.38
E5_1	81716484	80593678	0.0242	94.98	45.59
E6_1	74812324	73946060	0.0242	95.04	47.73
H1_1	54122398	53619998	0.0237	95.57	53.36
H2_1	54113264	53590994	0.0238	95.44	53.16
H3_1	53803330	53284416	0.0238	95.41	54.31

3.2. Differentially expressed genes (DEGs) identification

A total of 157 differentially expressed genes associated with hormone regulation were identified in the hepatopancreas by analysis of DEGs with |fold change| > 2 and P-adjust < 0.05 by DEGseq2 software, of which 32 and 125 genes were significantly up- and down-regulated, respectively (Figure 1).

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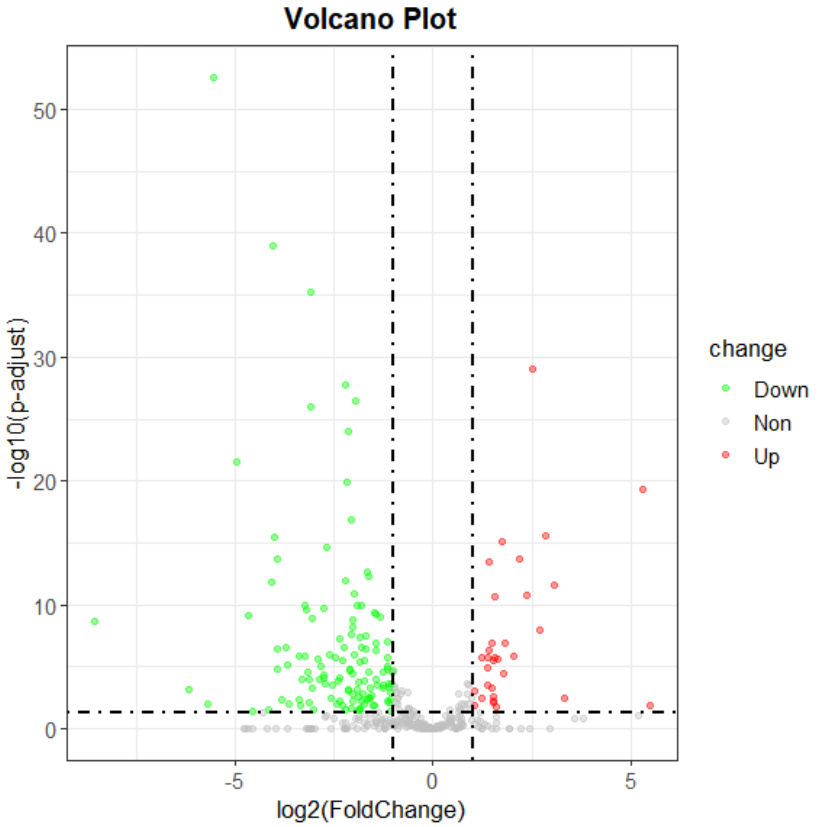

Figure 1. Volcano map of differentially expressed genes of hepatopancreatic hormones in the healthy and EHP-infected groups of *Litopenaeus vannamei*.

3.3. COG category annotation of DEGs

As shown in (Figure 2), among the hormone-regulated genes in the hepatopancreas, a total of 553 genes were mapped to 19 different COG categories. Except for the gene categories with unknown functions, the most mapped COG groups were the "Cytoskeleton" group (95 unigenes), followed by the "Transcription" group (43 unigenes), and the "Signal transduction mechanisms" group (39 unigenes). "Signal transduction mechanisms" group (39 unigenes). The least mapped COG group was the "Coenzyme transport and metabolism" group (1 unigenes).

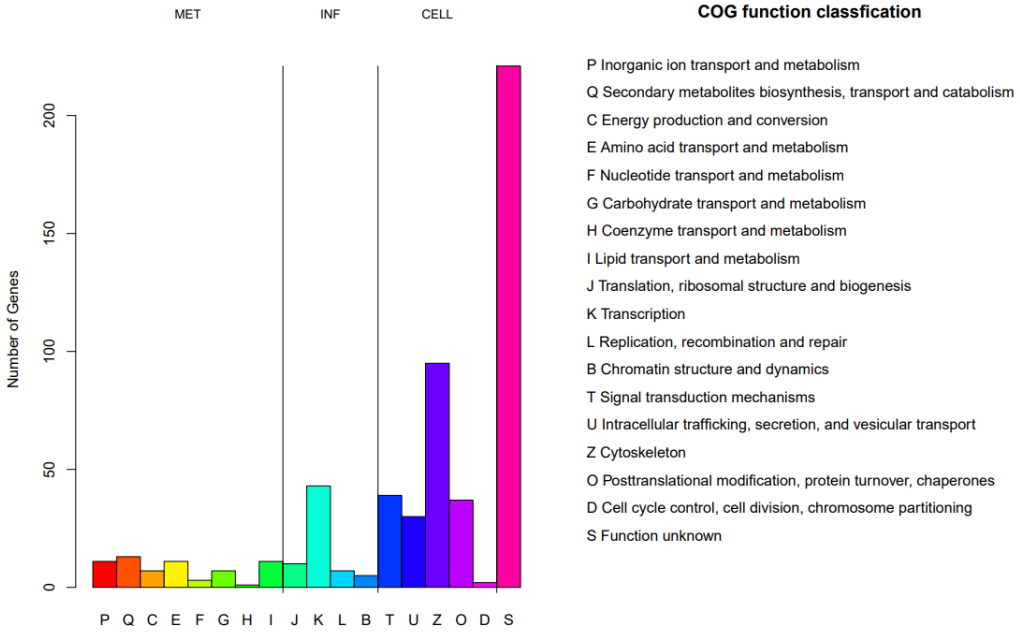

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Figure 2. COG classification of hormone regulation-related single genes in the hepatopancreas of EHP-infected and uninfected *Litopenaeus vannamei* x-axis indicates COG categories, y-axis indicates the number of unigenes. (Note: The figure shows MET for Metabolism, INF for Information Storage And Processing, CELL for Cellular Processes And Signaling).

3.4. GO term annotation for DEGs

As shown in (Figure 3), a total of 604 single genes were mapped to 42 gene ontology (GO) terms, including three major categories: molecular function, cellular components, and biological processes. Among them, molecular function is the most annotated, divided into 11 subclasses, involving binding (401), catalytic activity (212), molecular function regulator (97), molecular transducer activity (80), etc. Cellular components include 14 subclasses, divided into cell parts (246), membrane part (197), organelles (176), extracellular regions (83), etc. And among the 17 subclasses of biological processes, the main ones are cellular process (193) and biological regulation (184).

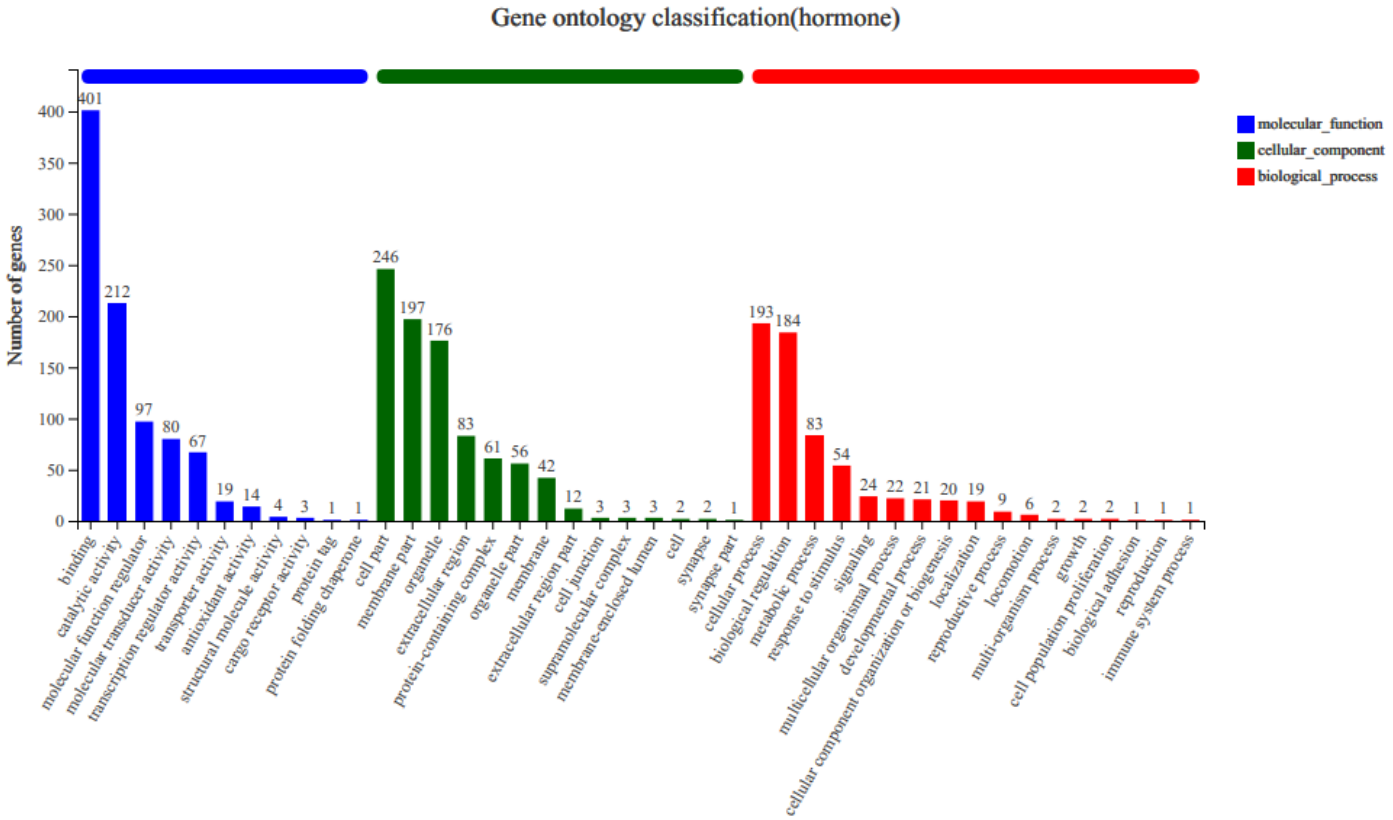

Figure 3. GO functional annotation analysis of differentially expressed genes related to hepatopancreas hormone regulation in the *Litopenaeus vannamei*.

3.5. GO term enrichment analysis and KEGG pathway enrichment analysis

The GO enrichment analysis of the genes related to hormone regulation in the hepatopancreas of Litopenaeus vannamei was performed in three aspects: molecular function, cellular composition, and biological process, respectively, using R language. At the molecular function level, most of the hormone genes were enriched in molecular_function binding and ion binding functions (Figure 4a). At the cellular component level, most of the hormone genes were enriched in cellular_component, cellular anatomical entity, and intracellular organelle functions (Figure 4b). In the case of biological processes, most of them are enriched in biological regulation, regulation of biological process, and regulation of cellular process functions (Figure 4c).

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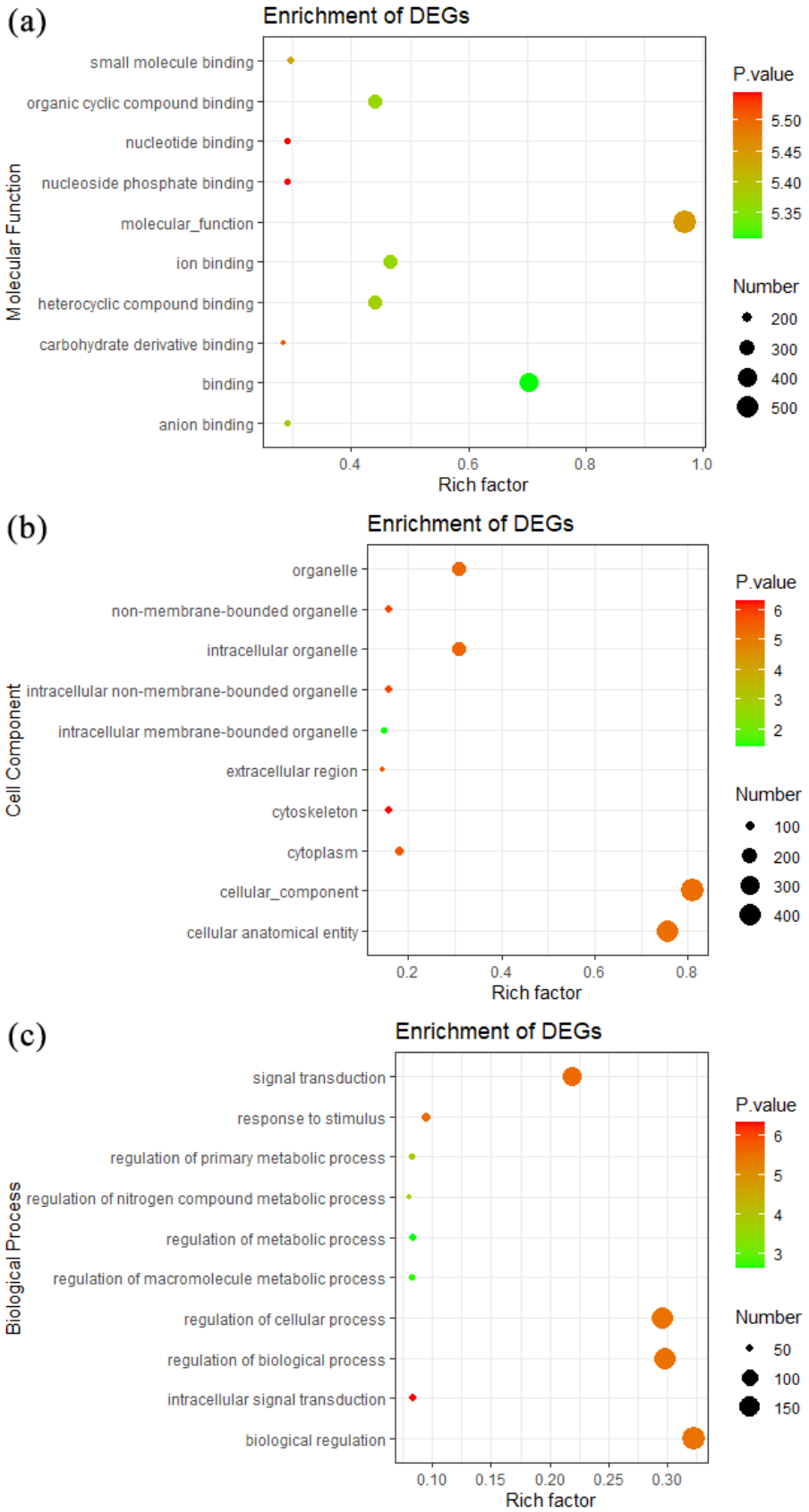

Figure 4. GO enrichment analysis of differentially expressed genes associated with hormone regulation. (a). Top 10 significantly enriched GO molecular function in DEGs. (b). Top 10 significantly enriched GO cellular components in DEGs. (c). Top 10 significantly enriched GO biological processes in DEGs. X-axis indicates the enrichment factor of the corresponding pathway, and Y-axis indicates the KEGG pathway name. Different colors of the dots indicate q values, and the number of DEGs in each pathway is indicated by the size of the dot.

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The top 20 KEGG pathways enriched are shown in (Figure 5). Among the hormone regulation-related genes, thyroid hormone signaling pathway (207) was the most enriched pathway. Meanwhile, Proteoglycans in cancer (135), Rap1 signaling pathway (132), Oxytocin signaling pathway (123), Platelet activation (123), Shigellosis (123) and other signaling pathways were also enriched.

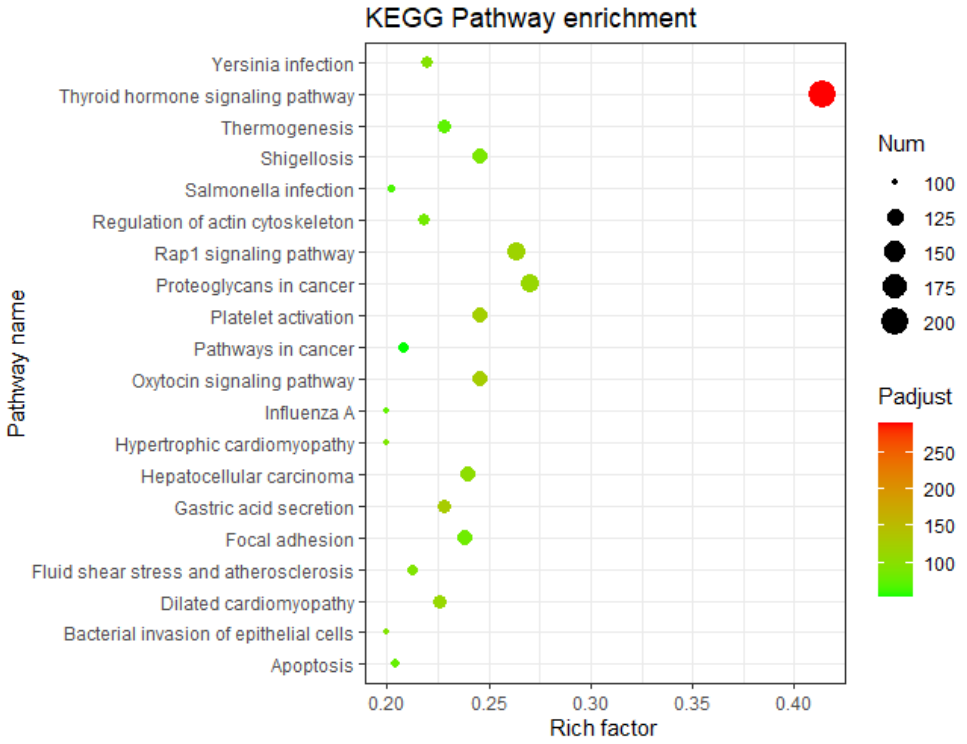

Figure 5. KEGG enrichment analysis of hormone-regulation-related differentially expressed genes. X-axis indicates the enrichment factor of the corresponding pathway, and Y-axis indicates the KEGG pathway name. Different colors of the dots indicate q values, and the number of DEGs in each pathway is indicated by the size of the dot.

KEGG pathway analysis was performed on the significantly up- and down-regulated and hormone-regulated related genes, respectively, to identify the relevant signaling pathways. As shown in (Figure 6), among the up-regulated DEGs, the pathways related to immune and information processing functions were more affected, and the genes were mainly concentrated in two pathways, EGFR tyrosine kinase inhibitor resistance (5), and thyroid hormone signaling pathway (4). Among the downregulated DEGs, pathways related to environmental information processing and metabolic functions were more affected, with genes mainly concentrated in Steroid hormone biosynthesis (13), Pentose and glucuronate interconversion (10), EGFR tyrosine kinase inhibitor resistance (9), and other pathways. EGFR tyrosine kinase inhibitor resistance pathway was significantly affected in both upregulated and downregulated DEGs.

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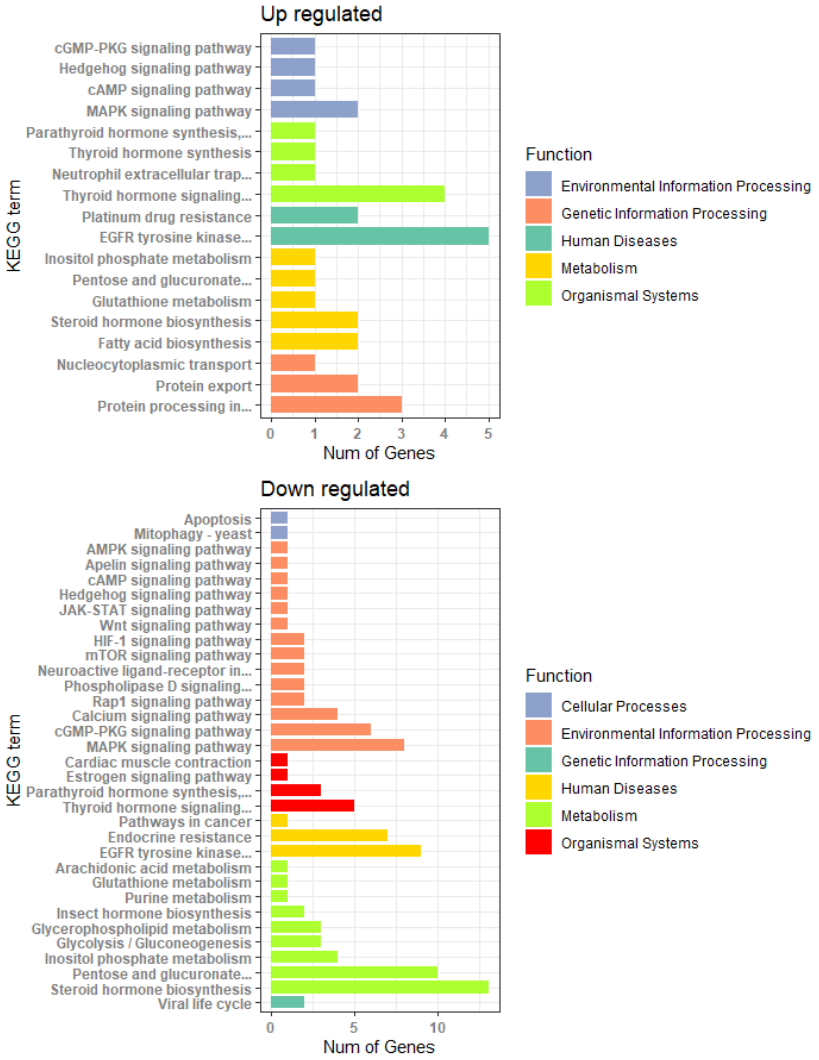

Figure 6. KEGG pathway analysis of significantly upregulated versus downregulated DEGs. X-axis indicates the number of genes contained in the pathway and Y-axis indicates the KEGG pathway name.

From the above analysis, the differences of hormone regulation-related genes were mainly in the two functions of metabolism and Environmental Information Processing. Therefore, the expression quantification software RSEM was used to cluster the DEGs of the six samples with the expression index FPKM and the quantification index TPM. Figure 7 shows the hierarchical clustering of the gene expressions of the six samples under the two functions. It can be clearly seen that the six samples were mainly divided into two groups, one group included H1, H2, and H3, and the other group included E1, E2, and E3, indicating that the healthy group was significantly different from the EHP-infected group in the two major functional categories.

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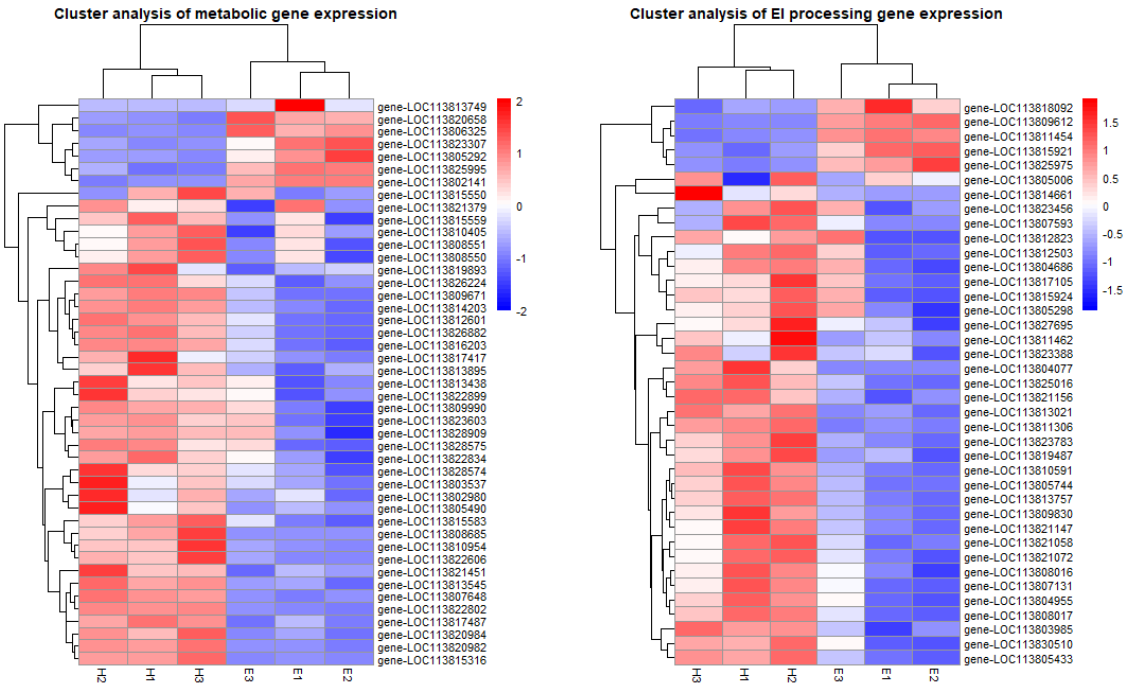

Figure 7. Heat map of clustering of DEGs in two functions: Metabolism and Environmental Information Processing. x-axis H represents uninfected samples, E represents samples infected with EHP, and y-axis represents hormone-regulated related genes in this function.

3.6. Validation of transcriptome data by qRT-PCR

To further evaluate the sequencing results, we screened the top 10 most significantly different genes among the hormone regulation-related genes by qRT-PCR analysis. As shown in (Figure 8), although there were some differences in the expression levels of ATP1B and HSP90, the qRT-PCR results showed the same expression trends as the high-throughput sequencing data. These results reflected the reliability of the RNA-seq results and confirm the accuracy of the expression results of DEGs in high-throughput sequencing analysis.

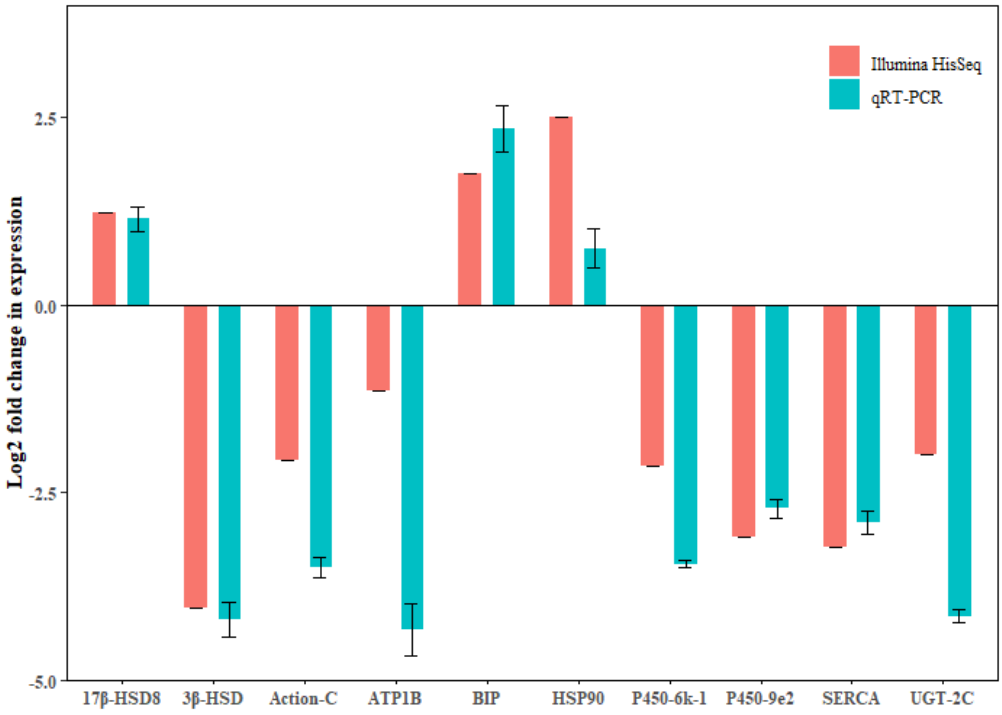

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Figure 8. Comparison of ten genes differential expressions determined by Illumina HisSeq sequencing and qRT-PCR. Relative transcript levels determined by qRT-PCR, using 18s RNA as the internal control, are shown by the blue bars. Data shown are means in triplicate ± SD.

4. Discussion

In this study, 4897 DEGs (2421 up-regulated, 2476 down-regulated) which were most enriched in energy metabolism, amino acid metabolism, carbohydrate metabolism pathways, this was consistent with the study reported by Ning M. Moreover, we identified 157 genes related to hormones from the DEGs since the genes relating to hormones were key factors for growth and molting for shrimp [25], then we found that 32 and 125 genes were significantly up- and down-regulated respectively.

Sarcoreticulum calcium ATPase isoform(SERCA) is a calcium ATPase, playing an important role in the regulating of calcium levels in the cytoplasm, which is crucial for molting of crustaceans [26,27]. In the present study, the SERCA expression was significantly downregulated, meaning the calcium regulation in the hepatopancreas was inhabited, since the hepatopancreas was the main organ for calcium storage and was regulating the calcium level [28]. Thus, we speculated that SERCA was downregulated due to EHP infection and influenced the calcium regulation, eventually resulting to hamper the molting and growth of shrimp.

Hydroxysteroid dehydrogenases including 3 beta-hydroxysteroid dehydrogenase(3β-HSD) and 17-beta-dehydrogenase(17β-HSD) relating to the molting. The high level of 3β-HSD expression relates to ecdysteroid biosynthetic pathways which facilitates the molting of *Carcinus maenas* [29]. However, 3β-HSD in EHP-infected group was significantly lower than that of the healthy group, suggesting that the molting might be hampered since they lack enough ecdysteroid. Moreover, 17β-HSD was related to the process of carapace hardening since it significantly upregulated after the molting of *Scylla paramamosain* [30]. In this study, 17β-HSD in EHP-infected group was significantly higher than that of the healthy group, suggesting the carapace was remaining in a relative high hardness, then the shrimp might be difficult to molt.

Among the upregulated genes, EGFR tyrosine kinase inhibitor resistance and thyroid hormone signaling (THs) pathways were the most enriched. EGFR stimulates ecdysone production in insects and plays an important role in larval growth, molting and metamorphosis [31]. THs has a significant impact on the metamorphosis of larvae molting.

As for the downregulated genes, Steroid hormone biosynthesis、Pentose and glucuronate interconversions, and EGFR tyrosine kinase inhibitor resistance were the most enriched. Ecdysteroid were proved to play important roles in molting periods for crustaceans [32,33]. Notably, EGFR tyrosine kinase inhibitor resistance was also enriched among downregulated genes. We can make a preliminary inference that the regulation of ecdysteroids were in a mess due to EHP infection, although the relationship was still unclear.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Conceptualization, H.S., Y.Q. and X.W.; methodology, Y.D., S.Z.,.; software, Y.Q., B.W., W.S.,; validation, H.C., G.J., and H.L.; formal analysis, L.Z.,; investigation, J.C.; resources, X.F.; data curation, H.S.; writing—original draft preparation, Y.D., L.Z,; writing—review and editing, H.S., Y.Q.,; supervision, H.S.; project administration, H.S.; funding acquisition, H.S.. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data reported in this paper have been deposited in the OMIX, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix: accession no. OMIX002325).

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Conflicts of Interest: The authors declare no conflict of interest.

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