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Original Research

Transcriptome Analysis of Hepatopancreas Provides Insights into Differential Metabolic Mechanisms of *Eriocheir sinensis* Feeding on Trash Fish and Formulated Feed

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Abstract

Background: *The Chinese mitten crab, Eriocheir sinensis* (*E. sinensis*), is a popular crab species in both domestic and foreign markets. Trash fish are essential for *E. sinensis* breeding, but have caused serious water pollution. The municipal party committee for the main production areas of *E. sinensis* implemented a ban on feeding on trash fish since 2020. **Methods**: In this study, we performed a culture experiment of *E. sinensis* feeding on trash fish and formulated feed, with comparative transcriptome analysis on hepatopancreas of *E. sinensis*. **Results**: The results indicate that formulated feed causes no significant difference in growth, survival rate or content of amino acids in the muscles of adult *E. sinensis*. Formulated feed caused a slight downregulation of pathways involved in amino acid metabolism, development, energy metabolism and homeostasis maintenance. **Conclusions**: On the whole, formulated feed can serve as an undifferentiated substitution for trash fish. This study provides a theoretical foundation for optimizing research on *E. sinensis* feed.

Keywords: *Eriocheir sinensis*; trash fish; formulated feed; hepatopancreas; comparative transcriptome

1. Introduction

The Chinese mitten crab, Eriocheir sinensis (*E. sinensis*), belonging to Phylum Arthropoda, Class Crustacea, Genus Eriocheir, is an important fishery resource in the Yangtze River of China [1,2]. The wild *E. sinensis* population has suffered serious damage due to various factors such as over-fishing and environmental pollution. The Ministry of Agricultural and Rural Affairs issued a notice in 2019 banning commercial fishing of *E. sinensis*. Restoration and protection of wild *E. sinensis* is urgent. However, *E. sinensis*, which has a high nutritional value and unique flavor, is popular in both domestic and foreign markets. Artificial breeding of new and special varieties of *E. sinensis* has become central in the breeding industry [3]. With the continuous expansion of *E. sinensis* culturing, environmental protection has become an increasingly prominent issue. Trash fish are essential for *E. sinensis* breeding, but entail obvious disadvantages: freshness is not guaranteed, the probability of their carrying bacteria is high, there is a food safety risk, nutritional value is unbalanced, and they have unstable qualities. These issues have led to trash fish being considered increasingly unsuitable for *E. sinensis* culturing [3,4]. The main production area for high-quality *E. sinensis* in China is the Suzhou Yangcheng Lake area in the Jiangsu Province. In 2020, the Suzhou Municipal Party Committee placed a ban on feeding on trash fish for *E. sinensis* breeding. The advantages of formulated feed over trash fish include a more stable source, more convenient transportation, and less environmental pollution. In the future, feeding on formulated feed during the entire breeding period is a promising culturing method for *E. sinensis* [5–8]. There has been some research on the effect of trash fish and formulated feed on *E. sinensis*, specifically regarding the effect of formulated feed and trash fish on growth, digestive enzyme activity, reproductive performance, and meat quality [9–13]. There have also been reports about the effect of different ingredients and additional supplements, e.g., oilseed, fructooligosaccharide, vegetable oil, lipid sources, protein-to-energy ratio, substitution of fish meal with soybean meal replacement, linoleic acid, CpG oligodeoxynucleotides on meat composition, flavor, growth performance, immunity, digestive enzyme activity, and composition of intestinal flora [14–23].

The hepatopancreas is important for organ function because it regulates energy storage, metabolism, growth, immunity, and digestive ability [11]. The present research

reported the effect of differential compositions of feed, (such as protein-to-energy ratio; different adding levels, including oilseed; substitution of fish oil with vegetable oil) on the activity of aspartate aminotransferase, alanine aminotransferase, steapsin, and trypsinase, and on the composition and odor of hepatopancreas [14,18,20]. No prior research has systematically reported the differential molecular regulation mechanism of *E. sinensis* feeding on trash fish and formulated feed. Herein, we carried out experiments throughout the entire culture cycle from juvenile *E. sinensis* to adult crab (the longest such experiment compared to relevant reports) [9,11] and performed the first comparative transcriptome analysis on hepatopancreas of *E. sinensis* feeding on the two kinds of feeds to explore the differential molecular regulation mechanism. The results indicate that there were no significant phenotypical differences, including growth, survival rate and content of various amino acids, in the muscle of harvested adult *E. sinensis*. At the mRNA level, formulated feed caused a slight downregulation of regulatory pathways and differentially expressed genes (DEGs) primarily involved in amino acid metabolism, development, energy metabolism and homeostasis maintenance. However, the quantity of the downregulated genes was low, and foldchange values were also small. Overall, feeding on formulated feed will not influence normal growth and development of *E. sinensis*. Formulated feed can serve as an undifferentiated substitution for trash fish. The present study provides theoretical guidance for optimizing research on feed of *E. sinensis* and lays a theoretical foundation for improving the breeding industry of *E. sinensis*, providing high-quality juvenile crab for restoration of wild *E. sinensis* in the Yangtze River of China.

2. Materials and Methods

2.1 Culture Experiment and Sample Collection

Juvenile *E. sinensis* were provided by Suzhou Yangcheng Lake Modern Agriculture Development Co., Ltd., Jiangsu, China. They were raised in a circulating water system (crab apartment) in March 2018, one crab per crab room. The crabs in the control group and experimental group were fed on trash fish and formulated feed, respectively (six males and six females in each group). The analysis of composition of trash fish and formulated feed (including content of moisture, crude protein and ash) was carried out according to the method reported by Helrich [24]. Total lipid was extracted with the method of chloroform-Methanol (V/V = 2:1). The trash fish and formulated feed were analyzed in duplicate for composition. The average values are shown in Table 1. Feeding was stopped one day before the experiment. The initial sizes of *E. sinensis* are shown in Table 2. The crabs were fed twice each day. During the entire culture period, dissolved oxygen was maintained at around 6 mg/L, while ammonia and nitrite concentrations were monitored daily and maintained at below 0.4 mg/L and below 0.2 mg/L, respectively. The adult crabs were harvested in November. We calculated the survival rate, specific growth rate, and feed coefficient of each crab. One female and one male crab were caught from each replicate and we collected three male and three female crabs in the control and experimental groups, respectively. The body size of twelve sampled *E. sinensis* in the control and experimental groups were also measured for RNA-seq analysis. The crabs were anesthetized in MS-222 solution (Kuer Bioengineering, Beijing, China) at a concentration of 30 mg/L for 20 s. After measurement of weight, carapace length, and width, the hepatopancreas was sampled, placed in liquid nitrogen and stored at –80 °C for further experiment, while the muscle was sampled for amino acid analysis.

Table 1. Composition of trash fish and formulated feed for *E. sinensis*.

Ingredients	TF (%)	FF (%)
Moisture	80.06 ± 0.51	8.16 ± 0.16
Crude protein^	61.98 ± 1.41	41.1 ± 0.75
Total lipid^	19.68 ± 1.19	8.09 ± 0.11
Ash^	13.69 ± 0.88	13.16 ± 0.07

Note: "^" indicated percentage of dry body mass.
TF, trash fish; FF, formulated feed.

2.2 Analysis of Amino Acid in Muscle of E. sinensis Fed with Trash Fish and Formulated Feed

External standard method was used to determine the composition and content of amino acid in the muscle of *E. sinensis* [25]. The proteolytic sample (600 mg ± 36 mg) was placed in a customized hydrolyzation tube with 8 mL HCl. The hydrolyzation tube was rotated and then vacuumed. After five minutes, the tube was sealed at the alcohol burner. The sample was hydrolyzed for 22–24 h under (110 ± 1) °C; the hydrolyzation tube was then cut and the solution was transferred into a 25 mL volumetric flask, followed by filtration with double-layer filter paper. One mL filtrate was added into a 25 mL beaker and dried in a vacuum dryer with NaOH (water temperature not exceeding 50 °C). The solution was dissolved in 1 mL HCl (pH 2.2) and transferred to a 1.5 mL centrifuge tube. After centrifugation at 10,000 r/min for 10 min, 0.5 mL supernatant was added in a sample bottle and measured using Agilent HPLC (Agilent Technologies, Palo alto, CA, USA). Each sample, including TFF (female crab fed with trash fish), TFM (male crab fed with trash fish), FFF (female crab fed with formulated feed) and FFM (male crab fed with formulated feed) had three replicates.

2.3 RNA Extraction, Sequencing, Data Filtering, and de novo Assembly

Total RNA was extracted from each collected hepatopancreas of the control and experimental groups (in total, 12) using RNAiso reagent (Takara, Kusatsu-Shiga, Japan)

Table 2. Statistics of *E. sinensis* growth parameters.

No.	Initial size			Harvesting				
	Weight (g)	Carapace length (mm)	Carapac width (mm)	Weight (g)	Carapace length (mm)	Carapac width (mm)	Survival rate (%)	Feed coefficient
C1	9.1 ± 0.51	26.1 ± 0.65	31.1 ± 0.71	200 ± 4.3	67.1 ± 1.98	71.6 ± 2.06	63 ± 1.5	3.9 ± 0.5
C2	8.9 ± 0.49	25.6 ± 0.59	30.8 ± 0.68	197.5 ± 3.6	66.9 ± 1.89	71.1 ± 1.85	60.5 ± 1.2	3.5 ± 0.41
C3	9.3 ± 0.56	26.6 ± 0.69	32.1 ± 0.75	208 ± 4.9	67.6 ± 2.16	71.9 ± 1.96	61.6 ± 1.35	3.8 ± 0.46
E1	8.8 ± 0.46	25.9 ± 0.61	30.9 ± 0.71	173.5 ± 2.8	57.7 ± 1.55	62.1 ± 2.08	58 ± 1.1	2.9 ± 0.32
E2	9 ± 0.52	26.1 ± 0.65	31.2 ± 0.73	184.5 ± 3.5	62.6 ± 1.69	67.2 ± 2.06	56.5 ± 0.86	3.1 ± 0.35
E3	9.3 ± 0.56	26.3 ± 0.68	31.5 ± 0.74	177.5 ± 3.1	58.5 ± 1.6	63.9 ± 1.73	59 ± 1.18	3.6 ± 0.43

Note: C1–C3: the control group that fed on trash fish; E1–E3: the experimental group that fed on formulated feed.

Feed coefficient: ratio of quantity of feed and increment in weight.

The same letter in one column indicates no significant difference ($p > 0.05$).

following the manufacturer's instructions. The quality of extracted RNA was checked using RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo alto, CA, USA), RNA concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and contaminant genomic DNA was removed with Recombinant DNaseI (Takara, Kusatsu-Shiga, Japan). The mRNA was isolated using magnetic beads and then broken into fragments and reverse transcribed into cDNA with added adapters. The obtained twelve cDNA libraries were constructed and sequenced on the Illumina HiSeq^{TM} 2500 platform (Illumina, San Diego, CA, USA). Paired-end data were generated. The obtained raw data were submitted to NCBI (NCBI, Bethesda, MD, USA) with the accession number of SRP256042. The raw data were tested for quality control using FASTQC (Babraham Institute, Cambridge, Cambridgeshire, UK). Some low-quality vectors (including adapters and/or primers), contaminated reads, low-quality bases at the 3' end, empty reads, and ambiguous 'N' nucleotides were removed, and the cutoff value for length control was set as 35 bp. NGS QC TOOLKIT v2.3.3 (Roche, Pleasanton, CA, USA) [26] was used to filter the data. Transcriptome assembly was performed using the Trinity software (Broad Institute, Cambridge, Cambridgeshire, UK) [27]. BUSCO analysis was made for assessment of transcriptome completeness [28].

2.4 Function Annotation

Unigenes were aligned according to the following priority: non-redundant protein (Nr), non-redundant nucleotide (Nt), Swiss-Prot (http://www.uniprot.org/downloads), clusters of orthologous groups for eukaryotic complete genomes (KOG, ftp://ftp.ncbi.nih.gov/pub/COG/KOG/kyva), Gene Ontology (GO, http://www.geneontology.org/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway.html) using BlastX with an E-value $<10^{-5}$ [29,30]. GO annotation was performed using Blast2GO software (Biobam, Valencia, New Mexico, Spain) [31].

2.5 Gene Quantification and Differential Expression Analysis

The obtained unigenes were put in a constructed library, and the abundance of expression of each unigene in each sample was measured using Bowtie2 software (version no. 2.2.9) (http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml) (Ben Langmead, Maryland, College Park, USA) [32] and eXpress software (version no. 1.5.1) (http://www.rna-seqblog.com/express-a-tool-for-quantification-of-rna-seq-data/) (California University, Berkeley, CA, USA) [33]. Gene expression levels were evaluated as fragments per kilobase of transcript per million mapped reads (FPKM) [34].

Differential expression quantification was calculated using the DESeq software package (version no. 1.18.0) [35] (http://bioconductor.org/packages/release/bioc/html/DESeq.html). The parameters for DESeq were $|\log_2\text{FoldChange}| > 1$. The fold change for the hepatopancreas was calculated as the ratio between the expression level of genes in the hepatopancreas sample of the experimental group and that of the control group sample, where $|\log_2\text{FoldChange}| > 1$ was used as the cutoff threshold to identify DEGs. All DEGs are listed in **Supplementary File 1**. GO terms and KEGG pathway enrichment analyses were performed on DEGs ($p < 0.05$). GO terms were classified into three categories: BP, CC, and MF. GO terms and KEGG terms were sequenced according to $-\log_{10} p$, and the top 30 GO terms (10 terms per category) and top 10 KEGG pathways were filtered according to the $-\log_{10}$ (p-value) and then sequenced according to DEGs numbers.

2.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Validation

To validate the accuracy of differential expression results from transcriptomic sequencing data analysis, the expression levels of ten DEGs were measured by qRT-PCR. Ten DEGs were randomly selected and analyzed by the Thermal Cycler Real Time System (TaKaRa, Kusatsu-Shiga, Japan). The primers were designed with Primer Premier 5.0 software (Premier Biosoft, California, USA).

Table 3. Body size parameters of sampled *E. sinensis* for RNA-seq.

Sample No.	Weight (g)	Carapace length (mm)	Carapace width (mm)
CF1	199 ± 4.2	67 ± 1.98	72.3 ± 2.1
CF2	197.5 ± 3.7	66.3 ± 1.86	71.6 ± 1.89
CF3	202.5 ± 4.5	66.1 ± 2.05	72.1 ± 1.99
CM1	205 ± 4.2	69.5 ± 2.09	75.1 ± 2.6
CM2	201 ± 3.7	66.9 ± 1.9	72.1 ± 1.96
CM3	206 ± 4.5	67.2 ± 2.15	72.6 ± 2.1
EF1	175 ± 2.9	58.1 ± 1.59	63.3 ± 2.16
EF2	179.5 ± 3.1	62.6 ± 1.63	67.9 ± 2.1
EF3	181 ± 3.3	59.1 ± 1.9	64.6 ± 1.79
EM1	180 ± 2.9	58.9 ± 1.66	64.1 ± 2.25
EM2	186 ± 3.1	63.5 ± 1.75	69 ± 2.29
EM3	185 ± 3.3	59.8 ± 2.09	65.1 ± 1.86

Note: "C" refers to control group feeding on trash fish; "F" refers to female; "M" refers to male; "E" refers to experimental group feeding on formulated feed.

The primer sequences are listed in **Supplementary File 2** (**Supplementary Table 1**). β-actin was used as internal reference gene. The amplification procedures for the detected DEGs were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s, and 72 °C for 50 s. Every sample was detected in triplicate, and the $2^{-\Delta\Delta CT}$ method was used to calculate the gene expression level [36].

2.7 Statistical Analysis

Statistical analyses were performed using SPSS 21.1 software (SPSS, Chicago, IL, USA), and the results are shown as Mean ± SE. Statistical analysis was performed using One-Way ANOVA and Student's *t*-test. The minimum significance level was set at 0.05.

3. Results

3.1 Statistics on the Growth Parameters and Body Size of E. sinensis Feeding on Trash Fish and Formulated Feed

The growth parameter and survival rate of all harvested *E. sinensis*, as well as the feed coefficient when harvesting, are shown in Table 2. The body size of sampled *E. sinensis* for RNA-seq analysis is shown in Table 3. As shown in Table 2, there were no significant differences in growth performance, survival rate, or feed coefficient of *E. sinensis* between feeding on trash fish and formulated feed, indicating that formulated feed will not cause a significant phenotypical difference in the development of *E. sinensis*.

3.2 Analysis of Amino Acids in Muscle of E. sinensis Fed with Trash Fish and Formulated Feed

The composition and content of amino *acids* in the muscle of *E. sinensis* fed with two kinds of feeds are shown in Table 4. The results indicate that there was no significant difference in composition and content of amino acid in the muscle of *E. sinensis* between the control and the experimental group. Likewise, there was no significant difference in essential amino acids (EAA), including threonine, valine, methionine, phenylalanine, isoleucine, leucine, and lysine. There was also no significant difference in content of flavor amino acids (FAA), including aspartic acid, glutamic acid, glycine, alanine, tyrosine and phenylalanine. As for flavor amino acids, the content of glutamic acid and aspartic acid were relatively high. As for EAA, the content of lysine, threonine and valine were relatively high.

3.3 Sequencing and Assembly of Hepatopancreas Transcriptome of E. sinensis

As shown in Table 5, a total of 87,668,204,100 clean data were generated. Phred quality score was used as an index for the base calling accuracy and calculated using the FastQC software version 0.10.1 (Babraham Institute, Cambridge, UK). In this study, a Q30 value larger than 93% indicated that base calling accuracy for each replicate had reached 99.9% and met the requirement for further analysis. After assembly, we obtained 41,656 unigenes. Among these, 24,415 unigenes were longer than 500 bp, max length was longer than 15,582 bp, the average length was 933.38 bp, and N50 was 1304 bp. BUSCO analysis result was shown in Fig. 1, Top 10 species distribution of unigenes against the Nr database in NCBI was shown in Fig. 2.

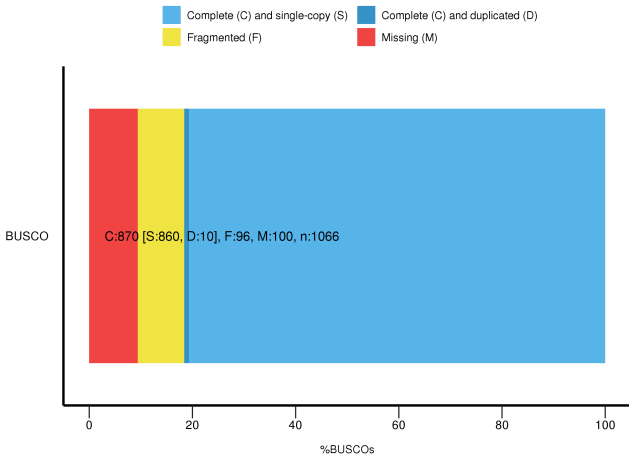

Fig. 1. BUSCO assessment results.

3.4 Differential Expression Analysis of DEGs

3.4.1 Enrichment Analysis of the Top 30 GO

As shown in Fig. 3, most of the top 30 GO terms were downregulated. Among them, 22 terms were completely downregulated, another four terms contained both up- and down-regulated DEGs, and there were seven up-regulated DEGs in total. The top 10 biological process (BP) terms were mainly involved in amino acid modification and biosynthesis, muscle development, cytoskeleton, and regulation of cellular homeostasis. The top 10 cellular component (CC) terms were involved in biosynthesis; processing

Table 4. The content of amino acids in the muscles of harvested *E. sinensis* fed with TF and FF (wet weight, g/100 g).

Amino acids	TFF[a]	TFM[a]	FFF[a]	FFM[a]
Aspartic acid	7.11 ± 0.21	6.86 ± 0.29	7.08 ± 0.3	7.29 ± 0.45
Glutamic acid	11.01 ± 0.49	10.99 ± 0.39	10.85 ± 0.53	11.05 ± 0.61
Glycine	5.99 ± 0.26	5.69 ± 0.31	5.71 ± 0.41	5.85 ± 0.36
Alanine	4.56 ± 0.31	4.49 ± 0.25	4.35 ± 0.36	4.41 ± 0.43
Tyrosine	2.21 ± 0.18	2.16 ± 0.22	2.01 ± 0.29	2.19 ± 0.21
Phenylalanine	2.79 ± 0.11	2.86 ± 0.16	2.68 ± 0.21	2.75 ± 0.2
∑FAA	33.67 ± 3.31	33.05 ± 3.28	32.63 ± 3.19	33.54 ± 3.26
Threonine	3.39 ± 0.18	3.28 ± 0.24	3.21 ± 0.11	3.18 ± 0.26
Valine	3.36 ± 0.06	3.29 ± 0.19	3.21 ± 0.08	3.3 ± 0.16
Methionine	1.78 ± 0.09	1.71 ± 0.19	1.69 ± 0.11	1.68 ± 0.15
Phenylalanine	2.79 ± 0.11	2.86 ± 0.16	2.68 ± 0.21	2.75 ± 0.2
Isoleucine	2.98 ± 0.16	2.88 ± 0.21	2.81 ± 0.19	2.89 ± 0.28
Leucine	5.06 ± 0.21	5.1 ± 0.3	5.01 ± 0.28	5.03 ± 0.19
Lysine	5.46 ± 0.25	5.39 ± 0.26	5.41 ± 0.19	5.33 ± 0.22
∑EAA	24.82 ± 1.35	24.51 ± 1.26	24.2 ± 1.23	24.16 ± 1.25
Cysteine	0.29 ± 0.06	0.26 ± 0.1	0.23 ± 0.13	0.21 ± 0.09
Histidine	1.33 ± 0.09	1.39 ± 0.16	1.32 ± 0.12	1.36 ± 0.08
Arginine	7.56 ± 0.32	7.49 ± 0.3	7.52 ± 0.43	7.55 ± 0.35
Serine	3.19 ± 0.11	3.23 ± 0.1	3.21 ± 0.15	3.22 ± 0.09
Proline	3.58 ± 0.29	3.66 ± 0.22	3.69 ± 0.16	3.73 ± 0.26
∑TAA	74.44 ± 2.16	73.49 ± 2.33	72.62 ± 2.35	73.77 ± 2.25

Note: The letter "a" at top of each column indicates non-significant difference ($p > 0.05$).

TFF, female crab fed with trash fish; TFM, male crab fed with trash fish; FFF, female crab fed with formulated feed; FFM, male crab fed with formulated feed. ∑FAA, total content of flavor amino acids; ∑EAA, total content of essential amino acids; ∑AA, total content of all amino acids.

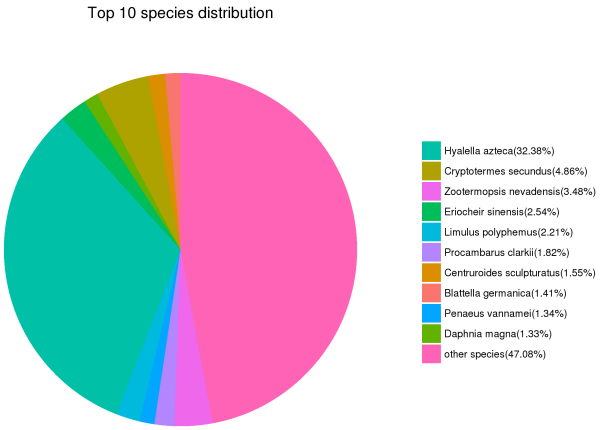

Fig. 2. Top 10 species distribution of unigenes against the Nr database in NCBI.

and modification of protein, including endoplasmic reticulum (ER); ER lumen; and substance transportation, such as lipid droplet. Among the top 10 molecular function (MF) terms, nine were downregulated and only two were upregulated DEGs. Seven terms directly involved in amino acid metabolism were downregulated, including essential amino acids, such as valine, isoleucine, leucine, and flavor amino acids, such as glutamic acid.

3.4.2 Enrichment Analysis of the Top 10 KEGG Pathways

As shown in Fig. 4, the top 10 KEGG pathways mainly concerned metabolism. Eight pathways were downregulated. Among of the total 33 DEGs, two were upregulated. Akin to the top 30 GO terms, the top 10 pathways were primarily involved in amino acid metabolism, including essential amino acids such as methionine (Met), valine (Val), leucine (Leu), isoleucine (Ile), threonine (Thr), and non-essential amino acids such as proline (Pro), cysteine (Cys), glycine (Gly), serine (Ser), and arginine (Arg). In addition, the top 10 KEGG pathways were also involved in glycan and carbon metabolism, which were downregulated.

3.4.3 Key DEGs in the Top 30 GO and Top 10 KEGG Pathways

Comprehensive analysis of the function of DEGs involved in the top 30 GO terms and top 10 KEGG pathways showed that they were mainly involved in amino acid metabolism, development, energy metabolism, and homeostasis maintenance (Table 6).

3.5 qRT-PCR Validation of Gene Expression

Ten DEGs were randomly selected and validated using qRT-PCR (Fig. 5). Relative expression levels of ten DEGs

Table 5. Summary of sequencing of hepatopancreas transcriptome of *E. sinensis*.

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Valid bases (%)	Q30 (%)	GC (%)
CF1	49,122,402	7,368,360,300	49,072,716	7,360,907,400	93.11	93.44	49.50
CF2	49,598,290	7,439,743,500	49,022,956	7,353,443,400	92.68	93.88	49.85
CF3	49,432,092	7,414,813,800	49,133,104	7,369,965,600	93.91	93.93	50.27
CM1	49,679,350	7,451,902,500	49,289,810	7,393,471,500	92.95	93.55	49.82
CM2	49,576,946	7,436,541,900	49,282,474	7,392,371,100	93.27	93.73	49.04
CM3	49,746,760	7,462,014,000	49,544,122	7,431,618,300	93.36	93.89	49.18
EF1	49,627,882	7,444,182,300	48,383,012	7,257,451,800	93.59	94.15	50.55
EF2	49,749,968	7,462,495,200	48,494,264	7,274,139,600	93.43	94.02	50.08
EF3	49,792,528	7,468,879,200	48,519,364	7,277,904,600	93.45	94.17	51.09
EM1	49,360,850	7,404,127,500	48,103,300	7,215,495,000	93.80	93.93	50.95
EM2	48,933,986	7,340,097,900	47,682,148	7,152,322,200	93.77	93.84	50.39
EM3	49,309,502	7,396,425,300	47,927,424	7,189,113,600	92.89	93.55	48.53

Note: CF1–3: three female crabs of replicates feeding on trash fish; CM1–3: three male crabs of replicates feeding on trash fish; EF1–3: three female crabs of replicates feeding on formulated feeds; EM1–3: three male crabs of replicates feeding on formulated feeds.
Valid bases: valid base ratio.

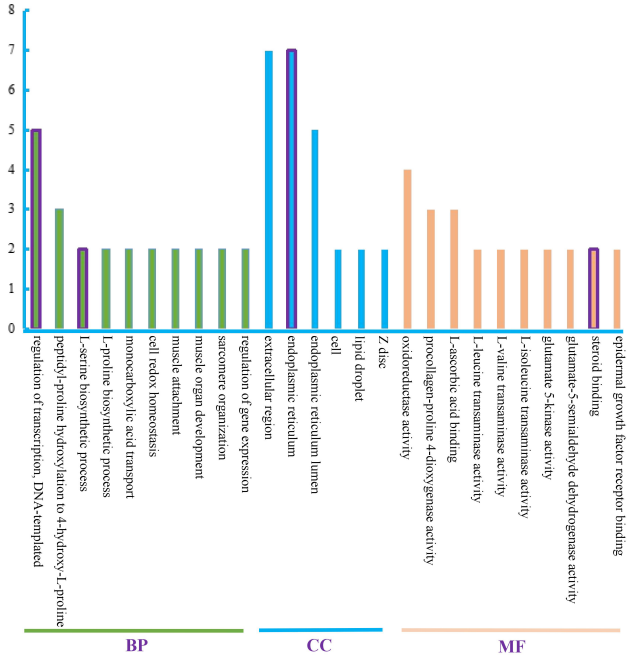

Fig. 3. Top 30 GO terms. The top 30 GO terms were classified into three categories; BP, CC, and MF. Each category is shown in a different color. The terms containing both upregulated and downregulated DEGs are shown in the purple box. The remaining terms were completely downregulated.

measured by qRT-PCR were mostly consistent with those determined by Illumina sequencing. The results of the correlation analysis were as follows: $y = 1.0441x + 0.0259$ ($R^2 = 0.956$). These results indicate that the RNA-seq data were reliable.

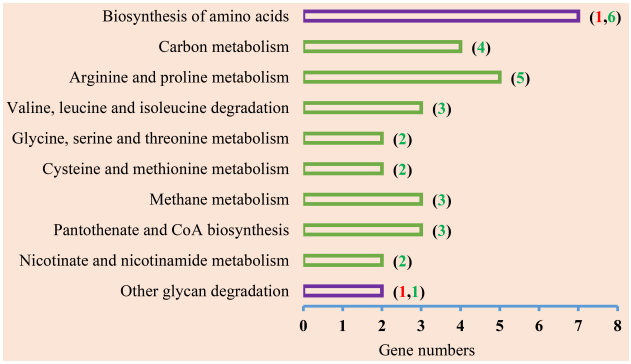

Fig. 4. Top 10 KEGG pathways. The KEGG pathways containing both upregulated and downregulated DEGs are shown in the purple box. Downregulated terms are shown in the green box. The numbers in brackets are gene numbers. Red shows an upregulated gene number, green shows a downregulated gene number.

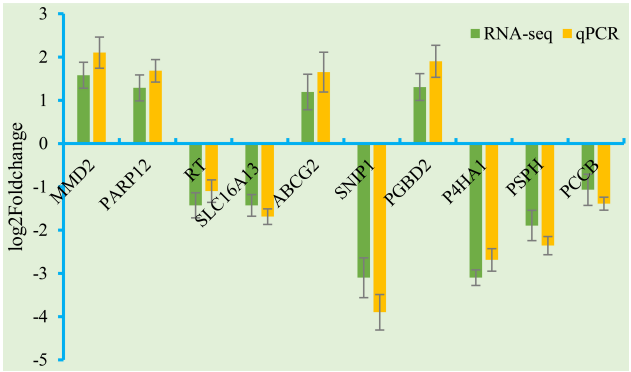

Fig. 5. Validation of DEGs by qRT-PCR. The horizontal axis shows gene names. The vertical axis shows relative expression.

Table 6. Key DEGs in hepatopancreas transcriptome of *E. sinensis*.

Category	Gene name	Gene definition	log_2FoldChange	*p*-value
Amino acid metabolism	*BCAT*	Branched-chain-amino-acid aminotransferase	–1.17	0.02
	PHGDH	D-3-phosphoglycerate dehydrogenase	–1.60	0
	ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	–1.00	0.01
	ALH-13	Probable delta-1-pyrroline-5-carboxylate synthase	–1.13	0.01
	P4HA1	Prolyl 4-hydroxylase subunit alpha-1	–3.10	0.03
	P4HA2	Prolyl 4-hydroxylase subunit alpha-2	–1.91	0.05
	PCCB	Propionyl-CoA carboxylase beta chain, mitochondrial	–1.07	0.03
	IFI30	Gamma-interferon-inducible lysosomal thiol reductase	–1.99	0.05
Development regulation	*NINAB*	Carotenoid isomerooxygenase	–4.89	0.01
	SMT1	Probable cycloartenol-C-24-methyltransferase 1	–1.45	0
	TMEM8B	Transmembrane protein 8B	–1.78	0.04
	ZNF219	Zinc finger protein 219	–4.82	0.01
	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	–1.63	0.05
	MARF1	Meiosis regulator and mRNA stability factor 1	–2.64	0.03
	AB	Protein abrupt	–1.13	0.01
	MLP84B	Muscle LIM protein Mlp84B	–1.43	0.05
	RT	Protein O-mannosyltransferase 1	–2.29	0.01
Energy metabolism and homeostasis maintenance	*NAMPT*	Nicotinamide phosphoribosyltransferase	–1.82	0.04
	NAS-4	Zinc metalloproteinase nas-4	–1.16	0.04
	SLC16A13	Monocarboxylate transporter 13	–1.43	0
	ALD	Fructose-bisphosphate aldolase	–1.25	0.04
	CG12206	Glutaredoxin domain-containing cysteine-rich protein CG12206	–2.47	0.02
	TIMP3	Metalloproteinase inhibitor 3	–1.07	0.03

4. Discussion

In this study, differentially enriched KEGG pathways and DEGs in hepatopancreas of *E. sinensis* were downregulated and can be classified into amino acid metabolism, development, energy metabolism and homeostasis maintenance.

4.1 Amino Acid Metabolism in Hepatopancreas after Feeding on Formulated Feed

Pathways and DEGs related to amino acid metabolism were found to be mainly involved in EAA metabolism, including Met, Val, Leu, Ile and Thr. In addition, they were also relevant to some non-essential amino acids such as Pro, Cys, Gly, Ser, and Arg, and relevant regulatory DEGs (*BCAT1*, *PHGDH*, *ALDH18A1*, *ALH-13*, *P4HA1*, *P4HA2*, *PCCB*, *IFI30*, etc.).

BCAT1, as a key enzyme in the catabolism of branched amino acids, can reversely catalyze the generation of ketoacid and glutamic acid from branched amino acids and α-ketoglutaric acid. It plays a regulatory role in the regulation of the cell cycle and catalyzes the catabolism of essential amino acids such as Val, Leu and Ile [37]. *PHGDH* functions in the catalytic reaction of L-serine biosynthesis [38]. *ALDH18A1* plays an important role in the biosynthesis of Arg and Pro [39]. *ALH-13* is essential for proline biosynthesis [40]. *P4HA1* and *P4HA2* play a key regulatory role in the formation of 4-hydroxyproline in collagen [41,42]. *PCCB* plays a regulatory role in the metabolism of Val, Ile, and Thr [43]. *IFI30*, as an important reductase, plays a positive regulatory role in the unfolding of targeted proteins to be degraded in lysosome [44].

Varieties and contents of amino acids are always important indicators for evaluating the nutrition and taste of meat. Amino acids are non-volatile active substances and participate in the formation of flavor [45–47]. As shown in Table 3, formulated feed caused no significant difference in composition and content of various amino acids in the muscle of *E. sinensis* fed with two kinds of feeds. Exploring the molecular regulation mechanism of these two feeding modes, our results indicate that formulated feed caused a slight downregulation of amino acid metabolism on mRNA level, but the quantity of the downregulated genes was low, and foldchange values were small (Table 5). This could explain why there were no significant phenotypical differences between *E. sinensis* feeding on trash fish and formulated feed. However, there is still space for improvement of *E. sinensis* feed, considering that the DEGs were primarily involved in the biosynthesis of amino acids such as glutamic acid, Ser, Arg and Pro. Future research on molecular regulation mechanism and expanded corresponding amino acids can improve the quality of *E. sinensis* formulated feed.

4.2 Development Regulation and Formulated Feed

According to our results, development-relevant regulatory pathways and DEGs were involved in muscle development, including *MLP84B* and *RT*, and universal reg-

ulatory genes relevant to development, such as *NINAB*, *TMEM8B*, *ZNF219*, *EFEMP1*, *MARF1*, and *AB*. *MLP84B*, a microtubule-associated protein, plays a regulatory role in cell differentiation during late stage of myogenesis [48]. *RT*, a protein O-mannosyltransferase, plays a regulatory role in the generation and maintenance of muscle development [49]. *NINAB* plays an essential role in maintenance of proper photoreceptor development and biosynthesis of vitamin A [50]. *TREM8B* plays an important role in cell growth, adhesion, and proliferation [51]. *ZNF219*, a transcriptional regulator, functions in the differentiation of chondrocyte [52]. *EFEMP1* plays a negative regulatory role in chondrocyte differentiation [53]. *MARF1*, as an indispensable regulator of oogenesis, plays an important regulatory role in germline integrity [54]. *AB* plays a regulatory role in embryonic muscle attachments [55].

Reported studies on the effect of trash fish and formulated feed on *E. sinensis* growth indicate that, overall, feeding on formulated feed during the entire culture-cycle will not influence normal growth and development of *E. sinensis* [4,10]. While our conclusions are in line with those of the previous studies, it is possible that the slight differences of regulatory DEGs can only be reflected on an mRNA level and cannot significantly influence the growth and development of *E. sinensis*.

4.3 Energy Metabolism and Homeostasis Maintenance after Feeding on Formulated Feed

In our study, pathways related to energy metabolism and homeostasis maintenance were nicotinate and nicotinamide (NAD) metabolism, monocarboxylic acid transport, and cell redox homeostasis. The DEGs involved were *NAMPT*, *NAS-4*, *SLC16A13*, and *TIMP3*, etc. NAD forms coenzymes 1 and 2 and participates in numerous biochemical reactions. It is essential for oxidation and glycan catabolism as well as regulation of energy metabolism [56]. NAMPT, a rate-limiting component in NAD biosynthesis pathway, plays a regulatory role in the expression of clock genes [57]. Monocarboxylates such as lactate, pyruvate, and ketone body, are important energy substances that play a key regulatory role in the digestion and absorption of nutrients [58]. SLC16A13 plays an important role in catalyzing transportation of monocarboxylates across the plasma membrane to take part in energy metabolism [59]. NAS-4 functions in the regulation of digestion [60]. TIMP3, an important regulatory enzyme in cellular homeostasis maintenance, participates in tissue-specific acute response [61].

Enzyme preparation, a kind of bioactive additive, is composed of single or multiple enzymes. It can supplement exogenous digestive enzyme, activate secretion of endogenous digestive enzyme and increase feed utilization rate. It can also eliminate the anti-nutrient factor in feed, improve digestive function and enhance the immune resistance of aquatic animals. Enzyme preparation has been widely used in the fish feed industry [62,63]. To date, there have been no reports relevant to application of enzyme preparation on *E. sinensis*. β-glucanase is a widely used enzyme preparation that can catalyze the production of oligosaccharides and glucose to enhance the glucose metabolism of the organism [64]. Metalloproteinase is a protease with good resistance to high temperature and pH and that has strong protein decomposition ability. Therefore, it has been well applied in aquatic animal feed [65,66]. Wu *et al*. [67] carried out research on tilapia feed combined with metalloproteinase. Their results showed that metalloproteinase can significantly improve apparent digestibility, increase the activity of serum antioxidant dismutase, and strengthen the anti-stress ability of fish. Research on the common carp (*Cyprinus carpio*) carried out by Monier *et al*. [68] demonstrated that exogenous enzyme can increase antioxidant capacity of fish and improve the fish intestinal health. According to the present study, adding β-glucanase and metalloproteinase to *E. sinensis* feed should be considered as a means of improving the digestive and anti-stress properties of *E. sinensis* feed.

5. Conclusions

Herein, we performed a culture experiment of *E. sinensis* feeding on trash fish and formulated feed, and comparative transcriptome analysis on hepatopancreas of *E. sinensis*. At a phenotypical level, our results indicate that formulated feed caused no significant differences on growth performance, survival rate or content of various amino acids in the muscle of harvested *E. sinensis*. At the mRNA level, the results showed that formulated feed cause a slight downregulation of regulatory pathways and DEGs that are mainly involved in amino acid metabolism, development, energy metabolism and homeostasis maintenance. However, the quantity of the downregulated genes was low, and fold-change values were also small. In sum, feeding on formulated feed will not influence normal growth and development of *E. sinensis*. Formulated feed can serve as an undifferentiated substitution for trash fish. The present study can be used as a theoretical basis for optimization of *E. sinensis* feed. Future research on molecular regulation mechanism and optimal amino acid levels as well as enzyme preparation such as β-glucanase and metalloprotease on *E. sinensis* can be initiated and enhanced. Doing so should strengthen the theoretical foundation for the development and improvement of *E. sinensis* feed. The present study should, in turn, help promote the development of the *E. sinensis* breeding industry and restoration of wild *E. sinensis*.

Abbreviations

E. sinensis, *Eriocheir sinensis*; DEGs, differentially expressed genes; Nr, Non-redundant protein; Nt, Non-redundant nucleotides; KOG, Clusters of orthologous groups for eukaryotic complete genomes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and

Genomes; FPKM, Fragments per kilobase of transcript per million mapped reads; qRT-PCR, Quantitative real-time polymerase chain reaction; EAA, essential amino acids; FAA, flavor amino acids; BP, biological process; CC, cellular component; ER, endoplasmic reticulum; MF, molecular function; Met, methionine; Val, valine; Leu, leucine; Ile, isoleucine; Thr, threonine; Pro, proline; Cys, cysteine; Gly, glycine; Ser, serine; Arg, arginine; NAD, nicotinate and nicotinamide.

Author Contributions

YT and GX designed the research study. MW, ML and SS performed the research. PX, JG and XM provided advice on the research. MW, ML, JL, FY, HL, CS, NW provided help on the sampling and parameters measurement. MW, ML analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Animal Care and Use Committee of the Freshwater Fisheries Research Center at the Chinese Academy of Fishery Sciences. All the experiments conformed to the Guidelines for the Care and Use of Laboratory Animals set by the Animal Care and Use Committee of the Freshwater Fisheries Research Center (2003WXEP61, Jan 6th of 2003), and the study was carried out under a field permit (No. 20182AC1328).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2708226.

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