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Integrate Analysis of Eyestalk Proteome and Metabolome in Precocious and Formal Juvenile Female *Eriocheir sinensis*

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Abstract: The Chinese mitten crab (*Eriocheir sinensis*) is an economically important crustacean. With the development of the *E. sisnensis* industry, precocity has become a significant challenge in juvenile crab culturing. In this study, the eyestalks of female *E. sinensis* from precocious (PE) and normal juvenile (NE) groups were used for proteome and metabolome analyses. In total, 731 up-regulated and 657 down-regulated differentially expressed proteins (DEPs) were identified in the PE and NE groups. In addition, 110 differentially expressed metabolites (DMs) were up-regulated and 256 were down-regulated in the PE group. An integrated analysis showed 5667 significant correlations between the metabolites and proteins and 109 common pathways in the proteome and metabolome. The proteins were mostly associated with the mechanistic target of rapamycin (mTOR) pathway, longevity regulation, autophagy, and the pyrimidine and purine metabolism pathways. The metabolites were primarily enriched in amino acid and lipid metabolisms. These results demonstrated the differences in the PE and NE groups at two omics levels and will be useful for the *E. sinensis* industry.

Keywords: protein; metabolite; precocity; one year old; Chinese mitten crab

Key Contribution: An integrated analysis showed 5667 significant correlations between the metabolites and proteins and 109 common pathways in the proteome and metabolome, indicating their importance in the formation of precocity.

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1. Introduction

Eriocheir sinensis is vital freshwater crustacean in China that has been consumed for a long time due to its delicious taste and nutritional value [1]. As such, its production has increased from 232,400 tons in 2000 to 888,629 tons in 2023 [2]. Although production has significantly increased, like disease, precocity still exists in the crab culture. Precocious *E. sinensis* is a sexually mature crab in the first year and grows slowly in the second year [3]. Normal female and male *E. sinensis* can grow for 24 and 22 months, respectively [4]. Because the growing period of precocious *E. sinensis* is shorter than its normal form, its body weight is less than 50 g and has less economic value [4,5]. In a previous study, the prevalence rates of precocious *E. sinensis* in wild and aquaculture environments were 5~10% and 18.2~98%, respectively [6]. The mortality rate of precocious *E. sinensis* reached 60~90% before June in the second year [7].

The development of precocious *E. sinensis* is associated with intrinsic and extrinsic factors. Genes and endocrine glands have been reported as intrinsic factors [8–11]. The 18S and 28S rRNA of the testes showed higher methylation and lower hydroxymethylation rates in precocious *E. sinensis* [8]. Xu et al. [10] reported an SNP in a gene encoding a molt-inhibiting hormone associated with precocious *E. sinensis*. Combined transcriptome and metabolome analyses showed that phenylalanine metabolism and neuroactive ligand–receptor interactions are the key pathways in precocious *E. sinensis* formation [12].

The aquaculture environment and nutrition are the main extrinsic factors for precocious *E. sinensis* [4,13–16]. The precocity rate of *E. sinensis* under natural light was significantly higher than that under low or medium light conditions [13]. In addition, the percentage of precocious *E. sinensis* increased significantly when the stocking density increased from 0 to 60 individuals per m^2 [14]. The precocity rate was also shown to be negatively correlated with the growth rate, according to a protein-to-energy ratio experiment [15]. A higher precocity rate was observed when juvenile *E. sinensis* were not fed phospholipid and unsaturated fatty acid supplementation [16].

The eyestalk is a multifunctional organ in crustaceans that produces neurohormones and regulates gonadal development, growth, and molting [17]. According to a transcriptome analysis, five neuropeptide genes have been identified in the eyestalks of precocious and normal juvenile *E. sinensis* [11]. A previously study showed that genes associated with neuropeptide hormones, lipid transport, and lipid metabolic processes were up-regulated in the eyestalk of precocious *E. sinensis*, indicating that they are part of the neuroendocrine system [18]. As Jia reported, the eyestalk is essential for the ovarian development of *Exopalaemon carinicauda*, and seven pathways related to oogenesis and oocyte rapid growth are enriched in the ovaries after eyestalk ablation [19]. When *E. sinensis* was challenged with different photoperiod regimes, 1809 differentially expressed genes (DEGs) and genes related to crustacean hyperglycemic and juvenile hormones were enriched in the eyestalk [20].

Proteomics and metabolomics have become common methods for analyzing differentially expressed proteins (DEPs) and differentially expressed metabolites (DMs). According to Liu et al. [21], 150 DEPs were identified in the spermatozoa of precocious *E. sinensis* and were significantly enriched in substance and energy metabolism. Some DEPs related to nutritional metabolism and ovarian development have also been identified at different ovarian stages in *E. sinensis* [22]. In addition, neuroactive-ligand receptor interactions and phenylalanine metabolism are two essential pathways in the ovaries between precocious and normal sexually mature *E. sinensis* [12]. To date, a combined analysis of the eyestalk proteome and metabolome for precocious *E. sinensis* has not been reported. In this study, the proteome and metabolome of the eyestalk in precocious and normal juvenile *E. sinensis* were sequenced, and a combined analysis of the two omics approaches was performed.

2. Materials and Methods

2.1. Ethics Approval

One-year-old precocious and normal juvenile female *E. sinensis* were approved by the Experimental Animal Welfare and Ethics committee of the Anhui Academy of Agricultural Sciences (Hefei, China) for use in this experiment.

2.2. Experimental Design

The experiment *E. sinensis* were ten months old and cultured in an earth pond at the density of 120,000 megalpa larva per acre. All the megalpa larva of the experiment *E. sinensis* were from the same breeding farm in Anhui Province. A total of 40 crabs in the PE and NE groups were collected from the Fisheries Institute of Anhui Academy of Agricultural Sciences (Hefei, China). The mean body weight in the PE and NE groups was 21.6 ± 2.3 g and 11.2 ± 1.6 g, respectively. All crabs were anesthetized on ice before sampling. The eyestalks were used for proteome and metabolome analyses. Four biological replicates with ten organisms mixed per replicate were performed for the two groups. All the samples were placed in liquid nitrogen and stored at −80 °C.

2.3. Proteome Analysis

The data independent acquisition (DIA) proteome method [23] was used for eyestalk proteome analysis. DIA data were acquired in the diaPASEF mode. Raw DIA data were processed and analyzed using Spectronaut 18 (Biognosys AG, Zurich, Switzerland) with default settings, and the retention time prediction type was set to dynamic iRT. The cutoff level of the q value for precursors and proteins was 1%. A proteome principal component

analysis (PCA) was performed to determine the differences between the PE and NE groups. DEPs were selected according to fold change ≥1.5 and p value < 0.05.

2.4. Nontargeted Metabolomic Analysis

Tissues (100 mg) were ground with liquid nitrogen, and the homogenate was resuspended in pre-chilled 80% methanol and 0.1% formic acid. The samples were incubated on ice for 5 min and then were centrifuged at 15,000 rpm, 4 °C for 5 min. A portion of the supernatant was diluted to a final concentration containing 53% methanol. The samples were transferred to a fresh Eppendorff tube and centrifuged at 15,000 rpm, 4 °C for 10 min. Finally, the supernatant was subjected to liquid chromatography–mass spectrometry (LC-MS/MS). UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, Bremen, Germany) coupled with an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher, Germany). The raw data files generated by UHPLC-MS/MS were processed using Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. Statistical analyses were performed using the statistical software R (R version R-3.4.3) and Python (Python 2.7.6 version).

2.5. Integrative Analysis of Proteome and Metabolome

Correlation analyses between the proteins and metabolites were performed using R (R version R-3.4.3) for the PE and NE groups. Significant KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were identified based on an integrated proteome and metabolome analysis using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/, accessed on 10 May 2024).

3. Results and Discussion

3.1. Proteome Annotation

The data-independent acquisition analysis of eyestalks from one-year-old *E. sinensis* females in the PE and NE groups was performed. The data were filtered with standards of a 1.0% false discovery rate for the precursor and protein thresholds at the peptide and protein levels for qualitative analysis. After merging the filtered data from the PE and NE groups, 6254 proteins were identified (Supplementary Table S1). Raw proteomic data (PXD055120) were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/, accessed on 8 May 2024).

3.2. Identification of DEPs

PCA indicated a variation of 43.02% at the proteome level. Inter- and intra- group variations at the proteome level were demonstrated by PC1 and PC2, respectively. The PCA results for the DEPs between the PE and NE groups indicated that the samples in each group clustered together, and the two groups were clearly separated (Figure 1).

Based on $p \leq 0.05$ and fold change ≥1.5 or ≤0.67, 1388 DEPs were identified in the PE and NE groups. Among all DEPs, 731 were up-regulated and 657 were down-regulated (Supplementary Table S2). Some were related to molting, including eclosion and molt-inhibiting hormones. In addition, many DEPs were associated with the growth hormone-regulated TBC protein, insulin-like growth factor-binding protein complex acid labile subunit, multiple epidermal growth factor-like domain protein 6, ras-related and estrogen-regulated growth inhibitor-like isoform X1, growth hormone-inducible transmembrane protein, growth_differentiation factor 8-like isoform X1, crustacean hyperglycemic hormone, diuretic hormone, and pigment-dispersing hormone 1 peptide-like isoform X1.

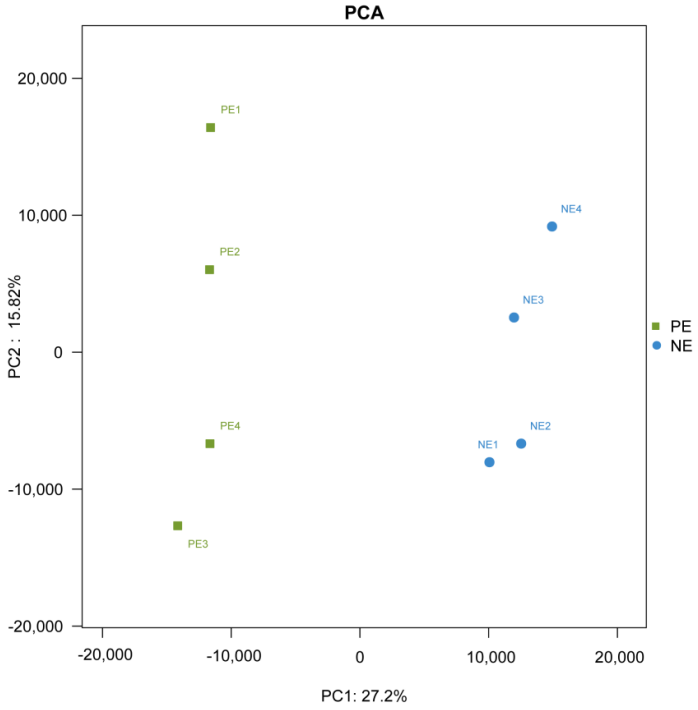

Figure 1. Principle component analysis of proteome abundance in the precocious and normal juvenile groups. Green plot (PE) indicates the precocious Chinese mitten crab; blue plot (NE) indicates the normal juvenile Chinese mitten crab.

DEPs in the eyestalks of samples from the PE group were significantly enriched ($p < 0.05$) in 852 GO terms. The most enriched biological process items were the "cellular process", "metabolic process", and "multicellular organismal process". The most enriched cellular components were "intracellular", the "protein containing complex", and "cellular anatomical entity". The dominant molecular function included "binding", "catalytic activity", "transporter activity", and "structural molecule activity" (Figure 2).

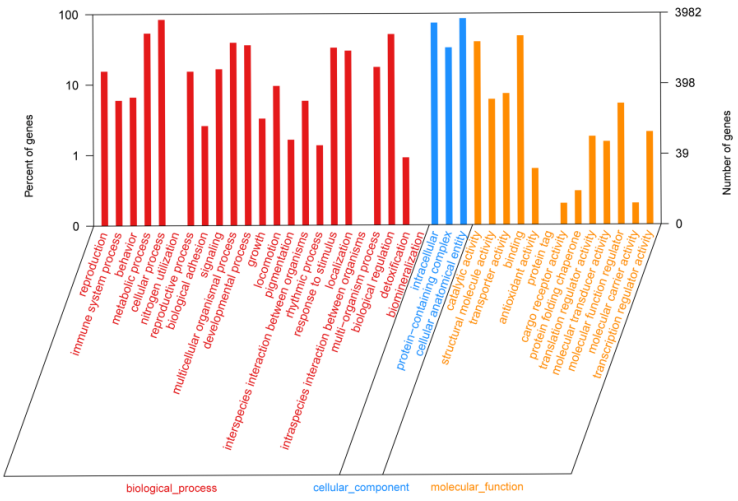

Figure 2. Gene ontology terms for DEPs between the precocious group and the normal juvenile group.

According to KEGG pathway enrichment analyses, DEPs were significantly enriched in "DNA replication"; the "cell cycle"; "mismatch repair"; "base excision repair"; the "cytosolic DNA-sensing pathway"; "butanoate metabolism"; "RNA polymerase"; "pyrimidine metabolism"; "amino sugar and nucleotide metabolism"; and "glycine, serine, and threonine metabolism" (Figure 3).

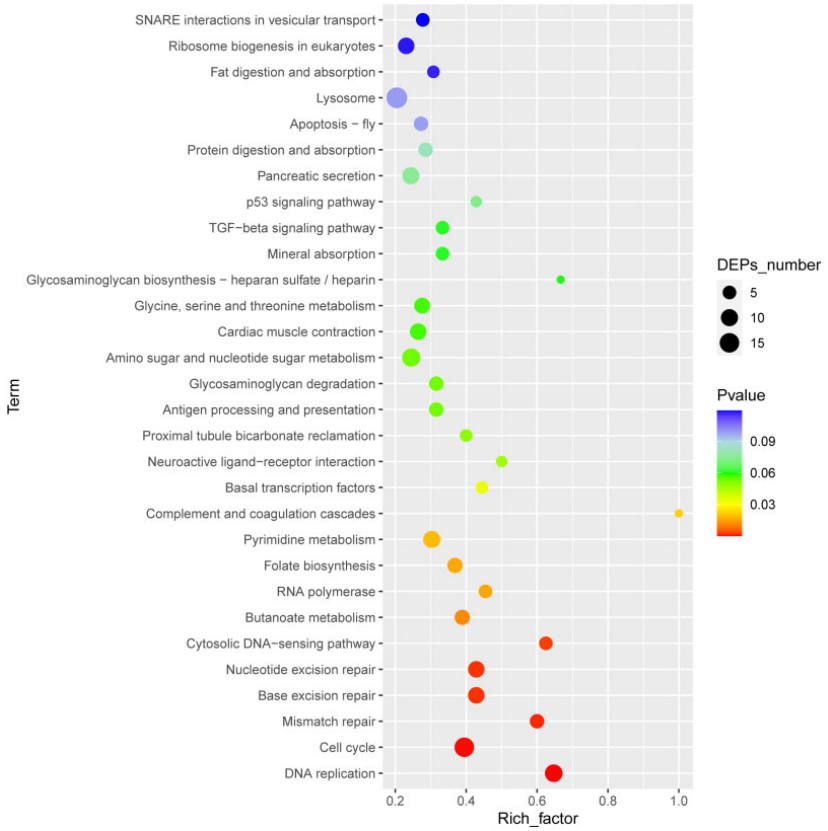

Figure 3. Top 30 KEGG pathways of DEPs.

3.3. Metabolite Analysis

A total of 1317 unique metabolites were identified using untargeted metabolomics LC-MS (Supplementary Table S3). An orthogonal partial least squares discriminant analysis (OPLS-DA) mode was employed to filter the DMs. In the OPLS-DA score plot, samples from the PE and NE groups can be easily distinguished (Figure 4).

According to OPLS-DA analyses, the 396 metabolites (110 up-regulated and 286 down-regulated, Supplementary Table S4) showed significant variation (OPLS-DA VIP $\geq$ 1, $p \leq 0.05$ and (FC $\geq$ 1.2 or FC $\leq$ 1/1.2)) in the two groups (Figure 5). The most up-regulated metabolites were lipids (Fucoxanthin, 11-Deoxy prostaglandin, 15-Keto prostaglandin, LDGTS 16:0, Stearic acid, Dibutyl sebacate, Tetradecanedioic acid, and 13-HPODE) and amino acids (N-acetyl-L-ornithine, Pantethine, D-pantethine, biocytin, and N-Acetyl-L-leucine). The most down-regulated metabolites were amino acids (Calcium D-Panthotenate, N-lactoyl-phenylalanine, argininosuccinic acid, S-(Methyl) glutathione, S-lactoyglutathione, bialaphos, N-Acetyl-DL-phenylalanine, and gamma-glutamyltyrosine) (Supplementary Table S4). More importantly, several of these DMs significantly correlated with steroid hormone levels, including 7-ketolithocholic acid, progesterone, dehydroepiandrosterone, methandrostenolone, 17alpha-hydroxyprogesterone, and dihydrotestosterone levels. These results indicated that the direct association of the DMs changed in the precocious crab and can provide an essential reference for further exploration of DMs in the precocious crab. The raw sequencing data were deposited in the China National GeneBank Sequence Archive (CNSA) of the China National GeneBank DataBase (CNGBdb) with the accession number CNP0005949.

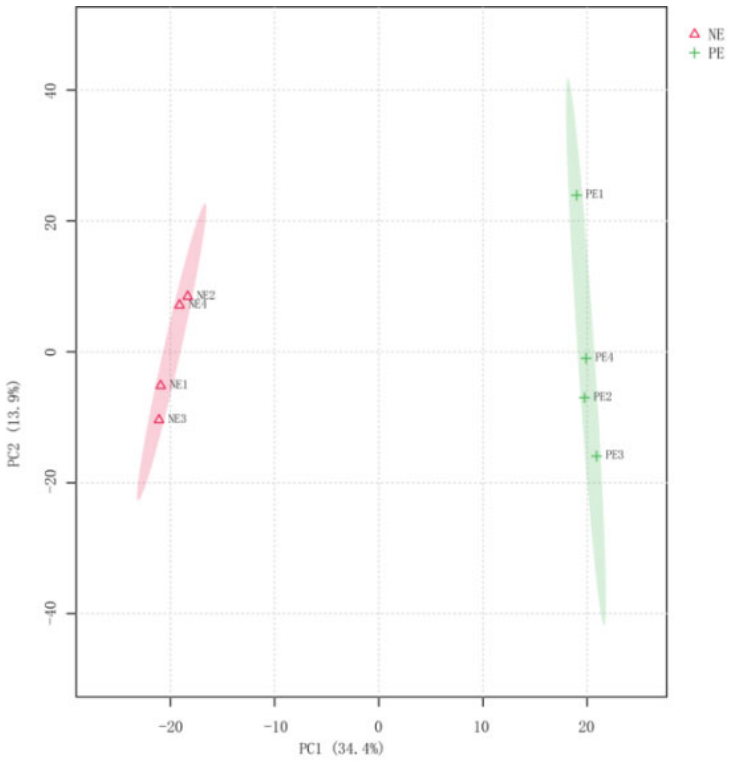

Figure 4. Orthogonal partial least squares (OPLS-DA) score plots for precocious (blue) and normal juvenile (red) groups.

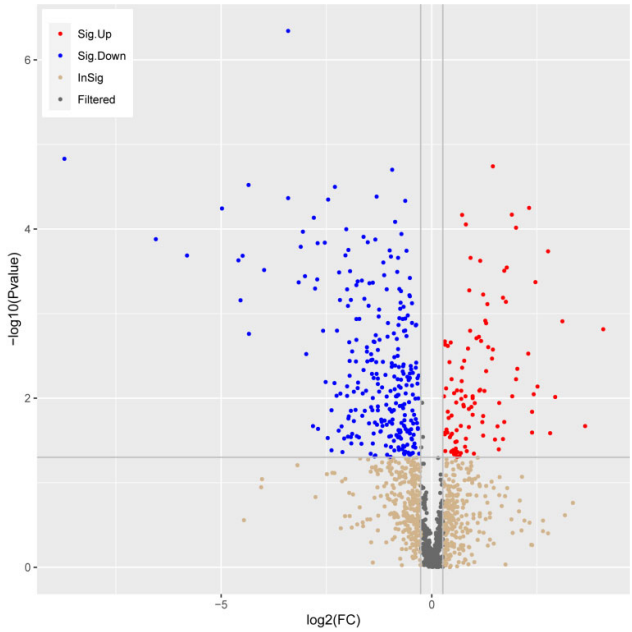

Figure 5. Volcano plots of DMs for the precocious group and normal juvenile group. The *x*-axis and *y*-axis represent fold change and −log10(*p*-value), respectively. In the volcano plot, red dots, blue dots, tawny dots, and grey dots represent significant up-regulated, significant down-regulated, non-significant different metabolites, and fitered metabolites, respectively.

The KEGG pathway enrichment analysis showed that different metabolites were enriched in "carbon metabolism", "glyoxylate and dicarboxylate metabolism", "fatty acid biosynthesis", "glutathione metabolism", the "pentose phosphate pathway", "porphyrin

metabolism", the "biosynthesis of alkaloids derived from histidine and purine", "fat digestion and absorption", and "fatty acid elongation" (Figure 6).

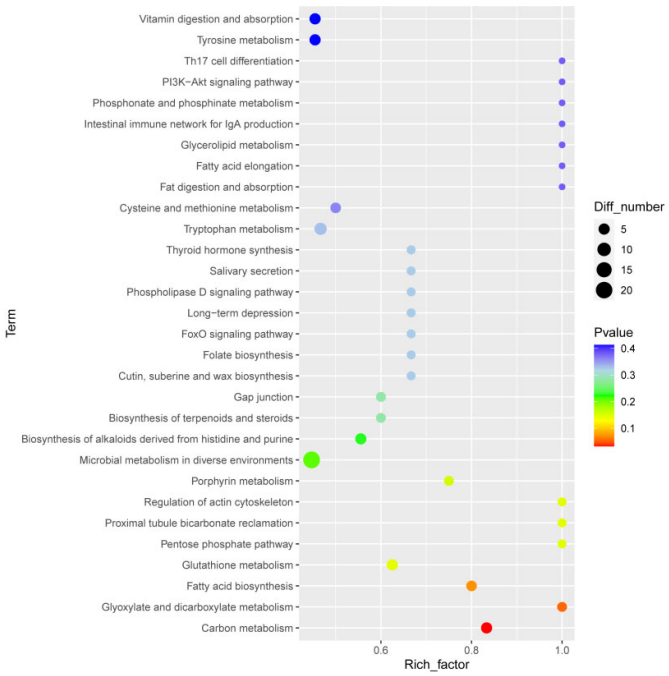

Figure 6. KEGG enrichment analysis of different metabolites in the precocious group and normal juvenile group.

3.4. Multi-Omics Analyses

Integrated proteomic and untargeted metabolomic analyses were performed to identify the relationships between the proteins and metabolites. A total of 5667 significant correlations between metabolites and proteins were identified with $p < 0.05$ and $|\text{Pearson's correlation coefficient}| \geq 0.9$ (Supplementary Table S5). A total of 109 common pathways were identified in the integrated proteome and metabolome analyses (Supplementary Table S6). Functional enrichment analyses showed that the proteins were mainly involved in the mTOR, longevity regulation, animal autophagy, pyrimidine metabolism, purine metabolism, amino sugar and nucleotide sugar metabolism, carbon metabolism, cofactor biosynthesis, and pancreatic secretion pathways. Metabolites such as 5-hydroxyindole-3-acetic acid, 3-methylindole, N-acetylserotonin, xanthurenic acid, acetylcholine, LPC 20:2, LPC 18:1, LPC 17:1, and LPA 20:5 were mostly enriched in amino acid and lipid metabolism.

Both the mTOR and longevity-regulating pathways are related to longevity. The mTOR pathway belongs to the class of evolutionarily conserved threonine and serine kinases that can maintain cell homeostasis and metabolism [24,25]. mTOR is crucial for cell growth and survival, immunity, autophagy, and metabolism [24]. Inhibition of the mTOR pathway extends lifespan and confers protection against a growing list of age-related pathologies [26]. Four DEPs and one DM were in the mTOR signaling pathway between the precocious and normal juvenile groups. Among the four DEPs, the ragulator complex protein LAMTOR5-like and the protein Wnt-4-like were up-regulated, while the GATOR complex protein NPRL3-like isoform X1 and the eukaryotic translation initiation factor 4E type 2-like isoform X1 were down-regulated. There were four DEPs and one DM in the longevity-regulating pathway. Among the four DEPs, RB1-inducible coiled-coil protein 1 and adenylate cyclase type 1-like isoform X4 were up-regulated, while adenylate cyclase type 3-like isoform X1 and eukaryotic translation initiation factor 4E type 2-like isoform X1 were down-regulated in the precocious crabs. The lifespan of precocious crabs is shorter than that of normal juvenile crabs and may be affected by the up-regulated DMs.

5′-Adenylic acid was the only DM that was up-regulated in the mTOR and longevity-regulating pathways in the precocious crab.

Thermogenesis is critical for survival and optimal body temperature [27]. Guanosine-3′,5′-cyclic monophosphate (cGMP) was reported to be essential for thermoregulation [28]. cGMP was the only metabolite that was down-regulated in precocious crabs. In a previous study, cod exposure regulated brown adipose tissue thermogenesis via the cGMP signaling pathway [29]. In the present study, 20 DEPs were identified in the thermogenic pathway. Among the 20 DEPs, cytochrome c oxidase assembly factor 6, cytochrome b-c1 complex subunit 6, protein arginine methyltransferase NDUFAF7, transcription activator BRG1-like isoform X1, cytochrome c oxidase assembly protein COX11, and adenylate cyclase type 3-like isoform X1 were considerably down-regulated.

Pyrimidine is a structural component of nucleic acids, nucleotides, folate, pterin, and vitamins and an essential molecule in cells [30]. Pyrimidine metabolism participates in synthesizing DNA and RNA and forming UDP-sugar for the glycosylation of lipids and proteins [31]. A combined transcriptome and metabolome analysis showed that DEGs and DMs were enriched in the pyrimidine metabolism pathway in the precocious puberty group [32]. Moreover, pyrimidine metabolism is one of the main enriched pathways in the serum of female precocious and normal sexually mature *E. sinensis*, and in the gonads of male precocious and normal sexually mature *E. sinensis* [12]. There were 10 DEPs and four DMs in the pyrimidine metabolism pathway between precocious and normal juvenile crabs. Among the 10 DEPs, thymidine phosphorylase, ribonucleoside-diphosphate reductase subunit M2 B-like isoform X1, pseudouridine-5-phosphate glycosidase-like isoform X1, thymidylate kinase-like isoform X1, CAD protein-like isoform X1, and the ribonucleoside-diphosphate reductase large subunit were markedly down-regulated. Among the four DMs, deoxyuridylic acid (dUMP), methylmalonate, and L-dihydroorotic acid were extensively down-regulated in precocious crabs.

Purine is essential to signaling molecules (cGMP and cAMP), coenzymes, nucleic acids, and energy transfer molecules. The purine metabolic pathways can be divided into *de novo* biosynthetic, catabolic, and salvage pathways [33]. The purine metabolic pathway can affect precocity, and the total number of compounds involved in purine metabolism reached 68 in the high- and low-precocity groups [34]. Purine metabolism is a common pathway in the hepatopancreas of precocious males and normal sexually mature *E. sinensis* [12]. There were nine DEPs and six DMs in the purine metabolism pathway in precocious and normal juvenile crabs. Among the nine DEPs, AMP deaminase 2-like isoform X2, cGMP-dependent 3_5-cyclic phosphodiesterase-like isoform X1, probable 3_5-cyclic phosphodiesterase pde-5 isoform X1, adenylate cyclase type 1-like isoform X4, and snake venom 5-nucleotidase-like were up-regulated. cGMP is a ubiquitous secondary messenger that plays a negative role in controlling ovarian steroidogenesis [35]. Among the six DMs, cGMP, xanthine, deoxyguanosine, xanthosine, and guanine were down-regulated in precocious crabs.

4. Conclusions and Prospects

In conclusion, a combined analysis of the proteome and metabolome of the eyestalk was performed to provide novel insights into the mechanism of action of precocious *E. sinensis*. The combined analysis showed 5667 significant correlations between the metabolites and proteins, and 109 common pathways in the proteome and metabolome. These proteins are involved in the mTOR, longevity regulation, autophagy, pyrimidine metabolism, and purine metabolism pathways. These metabolites were mostly enriched in amino acid and lipid metabolism. This study investigated the proteome and metabolome of female precocious *E. sinensis* and will be useful for clarifying precocious *E. sinensis* mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9110468/s1, Table S1: Proteins identified in the precocious and normal juvenile groups; Table S2: DEPs in the precocious and normal juvenile groups; Table S3: unique metabolites identified in precocious and normal juvenile groups; Table S4: Different expressed metabolites in the precocious and normal juvenile groups; Table S5: A total of 5667 significant

correlation between metabolites and proteins; Table S6: A total of 109 common pathways in proteome and metabolome integrated analysis.

Author Contributions: T.P.: methodology, data curation, and writing—original draft preparation. M.Y.: methodology and writing—review and editing. H.J. and T.L.: writing—review and editing. J.L.: project administration, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All crab experiments were conducted under the national regulations on laboratory animals of China and approved by the Experimental Animal Welfare and Ethical of Anhui Academy of Agricultural Sciences guidelines of use of animals for research (approval code: AAAS2022-20).

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data generated or used during this study appear in the submitted article.

Conflicts of Interest: The authors declare that there are no conflicts of interest regarding the publication of the work described in this manuscript.

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