Hindawi
Aquaculture Research
Volume 2023, Article ID 3134932, 16 pages
https://doi.org/10.1155/2023/3134932

Research Article

Screening and Analyzing of Genes and Signaling Pathways Associated with Size Differentiation of Adult Male Prawn *Macrobrachium nipponense*

Gang Jiang,[1] **Yongming Zhu**,[1] **Yucai Xue**,[1] **Ying Lu**,[1,2,3] **Zhiwei Liu**,[1] **and Xuxiong Huang**[1,2,3]

[1]*Centre for Research on Environmental Ecology and Fish Nutrition (CREEFN) of the Ministry of Agriculture and Rural Affairs (Shanghai Ocean University), Shanghai 201306, China*
[2]*Building of China-ASEAN Belt and Road Joint Laboratory on Mariculture Technology and Joint Research on Mariculture Technology, Shanghai 201306, China*
[3]*National Demonstration Center for Experimental Fisheries Science Education (Shanghai Ocean University), Shanghai 201306, China*

Correspondence should be addressed to Xuxiong Huang; xxhuang@shou.edu.cn

Received 26 September 2022; Revised 11 January 2023; Accepted 16 January 2023; Published 4 February 2023

Academic Editor: Liqiao Chen

To better understand the size differentiation mechanism of adult male prawn *Macrobrachium nipponense*, transcriptome sequencing was performed from the second pereiopod, hepatopancreas, and muscle of adult male prawn *M. nipponense* with significant differences in size (large: 1.47 ± 0.12 g; small: 0.28 ± 0.04 g). A total of 46,557 unigenes with an average length of 1,312 bp were obtained. Based on pereiopod of large male (LP) versus pereiopod of small male (SP), muscle of large male (LM) versus muscle of small male (SM), and hepatopancreas of large male (LH) versus hepatopancreas of small male (SH), a total of 805, 473, and 1,037 significantly upregulated DEGs were identified, respectively. The KEGG pathway analysis indicated that pathways such as the PI3K-Akt pathway, the cAMP pathway, the Hippo pathway, and the Ras signaling pathway were enriched in all three comparisons, which may be key size regulators in adult male prawn *M. nipponense*. Furthermore, as potential candidates for size differentiation in adult male prawns, twenty-eight DEGs involved in these four pathways were screened out, and their expressions were verified by quantitative real-time PCR (qPCR). The results of this study preliminarily elucidate the molecular mechanism of male *M. nipponense* in size differentiation and provide a theoretical reference for improving the commercial size of cultured male prawn *M. nipponense*.

1. Introduction

The oriental river prawn, *Macrobrachium nipponense* (Crustacea, Decapoda, Palaemonidae), is widely distributed in freshwater and low-salinity estuarine regions of China, Japan, and Southeast Asia [1, 2]. Due to its delicious taste, high nutritional value, and high local market prices, *M. nipponense* has become one of the most important commercial aquaculture species in China [3]. Like *Macrobrachium rosenbergii*, *M. nipponense* shows a sexually dimorphic growth pattern [4], and male prawns usually grow faster, as well as reach larger sizes at harvest time [5]. Generally, culturing male prawns has higher profitability than culturing females. While in practice, the adult male *M. nipponense* exhibits heterogeneous individual growth (HIG). The adult male individuals in a population polarize into large-size males and small-size males, which are even smaller than the females. A similar phenomenon also has

been reported in *M. rosenbergii* [6]. Therefore, it is necessary to better understand the factors and potential molecular mechanisms behind the size differentiation of male prawns.

Previously, it was reported that some factors such as breeding density [6], social hierarchy, food, and motor activity [7] as well as genetic variation [8] induced heterogeneous growth of male prawns in a population. Selecting high-quality germplasm [9], nutritional enhancement [10], eyestalk ablation [11], and gene regulation [12] have been applied to manage the prawn's size. Although the above-mentioned measurements have been applied in practice, the size-related genes and regulatory mechanisms in the adult male *M. nipponense* are still unclear. A better understanding of the molecular regulation mechanisms on size differentiation in crustaceans will help to culture large-size individuals, thereby increasing productivity and profit [13]. With the rapid development of bioinformatics technology, high-throughputRNA-sequencing has become an important means to reveal the regulatory mechanisms of biological genetics.

In this study, to better understand the molecular regulation mechanisms on size differentiation in adult male prawn *M. nipponense*, a high-throughputRNA-sequencing technique was used to analyze the gene expression differences of the second pereiopod, hepatopancreas, and muscle from adult male *M. nipponense* which have significant differences in size. The identification of pathways and differentially expressed genes in the different sizes of adult male prawns will help build a more complete understanding of the regulatory mechanisms associated with size differentiation and will provide a molecular basis for further research on the growth regulation of male *M. nipponense*.

2. Materials and Methods

2.1. Sample Preparation and RNA Isolation. The broodstock of *M. nipponense* was obtained from Shatian Lake Farm in Qingpu District, Shanghai. Inbred offspring of a monophyletic sibling was selected for the experiment. A berried female was incubated in a 5 L aquarium. After hatching, all the zoea larvae were transferred to and cultured in a 10 L polyethylene tank. After the zoea larvae were completely metamorphosed into postlarvae, the seed was cultured in a recirculating aquarium system (temperature $26 \pm 0.5^{\circ}$C, pH 7.5–8.0, and dissolved oxygen > 5 mg/L, respectively) in the lab with reasonable stock density. The aquatic plant *Ceratophyllum demersum* L. was provided as a shelter for *M. nipponense*. Enough *Artemia* and commercial diet (crude protein, $38.0\% \pm 0.5\%$; crude lipid, $5.0\% \pm 0.5\%$; ash, $16.0\% \pm 0.2\%$; crude fiber, $6.0\% \pm 0.2\%$; and moisture, $12.0\% \pm 0.6\%$) were fed during the culture period which is nearly 180 days.

During the cultural period, the adult male prawns were sorted into large-size and small-size groups. Each male prawn was cocultured with an adult female in a 10 L polyethylene tank for a period to check whether the male was sexually mature. If the cocultured female became a berried female for more than one week, the male was regarded as sexually mature and was sampled for analysis. Prawns were anesthetized on ice for 5 min before being sacrificed by dissection. The muscle, hepatopancreas, and the second pereiopod from the large (BW 1.47 ± 0.12 g) and the small-size adult males (BW 0.28 ± 0.04 g) were, respectively, sampled and directly immersed in liquid nitrogen until used for RNA extraction. Tissues were homogenized with TRIzol reagent (Sangon Biotech, Shanghai, China) to extract total RNA, following the manufacturer's instructions. NanoDrop spectrophotometers (Thermo Fisher, MA, USA) and Qubit 3.0 Fluorometer (Life Technologies, CA, USA) were used to measure the RNA concentration and purity. RNA integrity and quantity were assessed by a Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.2. Library Construction and Sequencing. RNA purification, reverse transcription, library construction, and sequencing were performed at Wuhan BGI Biotechnology Co., Ltd. (Wuhan, China). To ensure the sequence quality, three biological replicates were sequenced for all samples. Two cDNA libraries for small and large size of adult male *M. nipponense* were constructed. The RNA was broken into small random fragments using oligo-dT-attached magnetic beads, and then, cDNA was synthesized. Short fragments were purified with Takara's PCR extraction kit (Takara, Japan). Sequencing adapters were ligated to short fragments and resolved by agarose gel electrophoresis. Suitable fragments were selected for PCR amplification to create the final cDNA library template. Both libraries were sequenced on the BGISEQ-500 platform.

2.3. Functional Annotation Analysis. All the raw data were assembled by the Trinity software. The reads with adaptors, empty reads, and low quality (quality scores < 30) were cleaned [14]. Transcripts longer than 300 bp were used for subsequent analysis. Unigenes were obtained through filtration of isoform and paralog for all the assembled transcripts. Moreover, all unigenes of predicted protein-coding sequences were obtained by searching against different databases, including the National Center for Biotechnology Information (NCBI), nonredundant protein sequence (Nr) and nucleotide (Nt), the SwissProt, the Interpro, the Cluster of Orthologous Groups for Eukaryotic Complete Genomes (KOG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the BLASTx tool (E value cutoff 10^{-5}) [15].

2.4. DEG Analysis of Size-Related Gene Expression. Differences in gene expressions of muscle, hepatopancreas, and second pereiopod in the large- and small-size adult male *M. nipponense* were calculated using DESeq [16]. The Benjamini–Hochberg correction method was used to correct the significance of the p value of the original test hypothesis to obtain the false discovery rate (FDR) [17]. Fold change was used to present the expression ratio between the comparison groups. FDR < 0.001 were considered as the differentially expressed genes (DEGs), and $\log_2^{\text{foldchange}} \geq 1$ was upregulated, and $\log_2^{\text{foldchange}} \leq -1$ was downregulated;

Table 1: Summary of the transcriptome sequencing data (data showed average) of adult male prawn *M. nipponense*.

Group	Total raw reads (*M*)	Total clean reads (*M*)	Q20 (%)	Q30 (%)	GC content (%)
Large-size male prawn	556.16	466.29	96.81	88.39	40.10
Small-size male prawn	507.19	423.91	96.87	88.55	40.27

then, the upregulated DEGs were used for further analysis. Annotation methods were similar to those mentioned above in terms of unigene annotation. Biological repetitive analysis was carried out to evaluate the quality of sequence data.

2.5. Gene Interaction Network Construction. The DEGs filtrated out for the potential size-related genes were from the key signaling pathways in muscle, hepatopancreas, and the second pereiopod of the large- and small-size adult male *M. nipponense*, and it was suggested that these DEGs in the three tissues may participate in the growth and development of male *M. nipponense*. The DEGs list was subjected to analysis by the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://www.string-db.org/).

2.6. qRT-PCR Verification. qRT-PCR was used to measure the relative mRNA expression of the candidate genes in two significantly different sizes (large: 4.75 ± 0.30 g; small: 2.49 ± 0.07 g) of the adult male prawns, *M. nipponense*, which were wild-captured from a river in Lingang city, Shanghai. Independent RNA samples from muscle, hepatopancreas, and second pereiopod of each prawn were extracted using TRIzol reagent (Sangon Biotech, Shanghai, China), and the cDNA was synthesized using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio Inc.). The primers were designed using the Primer 5 software [18], and *ß*-actin was used as an internal control (S1). The qRT-PCR was performed with Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (YEASEN Biotech, Shanghai, China) in the Agilent AriaMx Real-Time PCR System. The PCR reaction systems (20 μL) consisted of 10 μL SYBR Green Master Mix, 0.4 μL of each gene-specific primer (10 nmol), 1 μL of cDNA template, and 8.2 μL of DEPC treated water. qRT-PCR was performed in a total volume of 20 μL, and cycling conditions were 95°C for 2 min, followed by 30 cycles at 95°C for 10 s and 60°C for 20 s. At the end of the PCR cycles, melting curve analyses were performed, and the relative expression levels of the targeted genes were quantified using the $2^{-\Delta\Delta Ct}$ method [19]. The value of $\log_2$-foldchanges was used for the graphing (fold change = $2^{-\Delta\Delta Ct(largesize)}/2^{-\Delta\Delta Ct(smallsize)}$). Three replicates were performed for each tissue.

2.7. Statistical Analysis. Data from qPCR verification were analyzed using the SPSS statistical software (version 25.0). Data were tested by one-way ANOVA and Duncan's new multiple range test and showed as mean ± standard error (SE). A significant change was observed with $P < 0.05$.

2.8. Ethical Statement. The present experimental procedures were carried out in strict accordance with the recommendations in the ethical guidelines of EU Directive 2010/63/EU for animal experiments. Permission was obtained from the ethical guidelines of Shanghai Ocean University for the care and use of experimental animals during the experimental programs (SHOU-DW-2019-013).

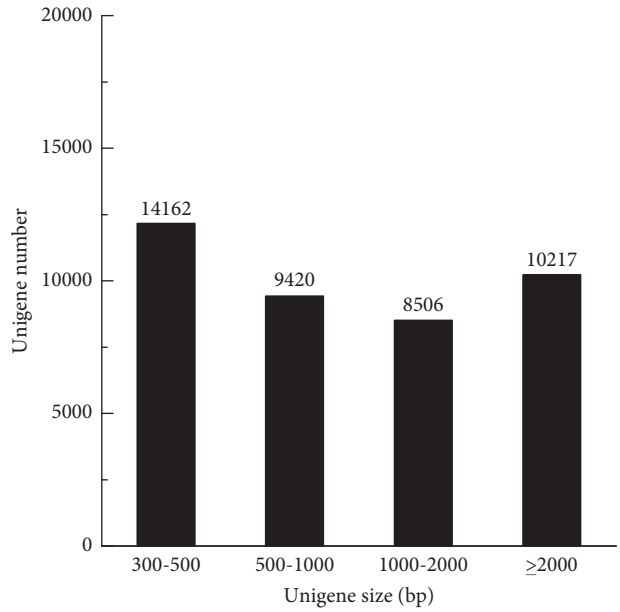


Figure 1: Length distribution of unigenes of transcriptome resulted from de novo assembly of adult male prawn *M. nipponense*.

Table 2: Summary of the annotation statistics of the unigenes of the transcriptome of adult male prawn *M. nipponense*.

	Number of annotated unigenes	(%)
Annotated in NR	22,827	48.82
Annotated in NT	12,640	27.15
Annotated in SwissProt	15,829	34.40
Annotated in KEGG	17,321	37.20
Annotated in KOG	15,485	33.26
Annotated in PFAM	16,257	34.92
Annotated in GO	14,968	32.15
Annotated in all databases	1,975	4.24
Total unigenes	46,557	100

3. Results

3.1. Transcriptome Sequencing. The total number of raw reads achieved for large- and small-size of adult male *M. nipponense* transcriptome was 1063.35 million. After the

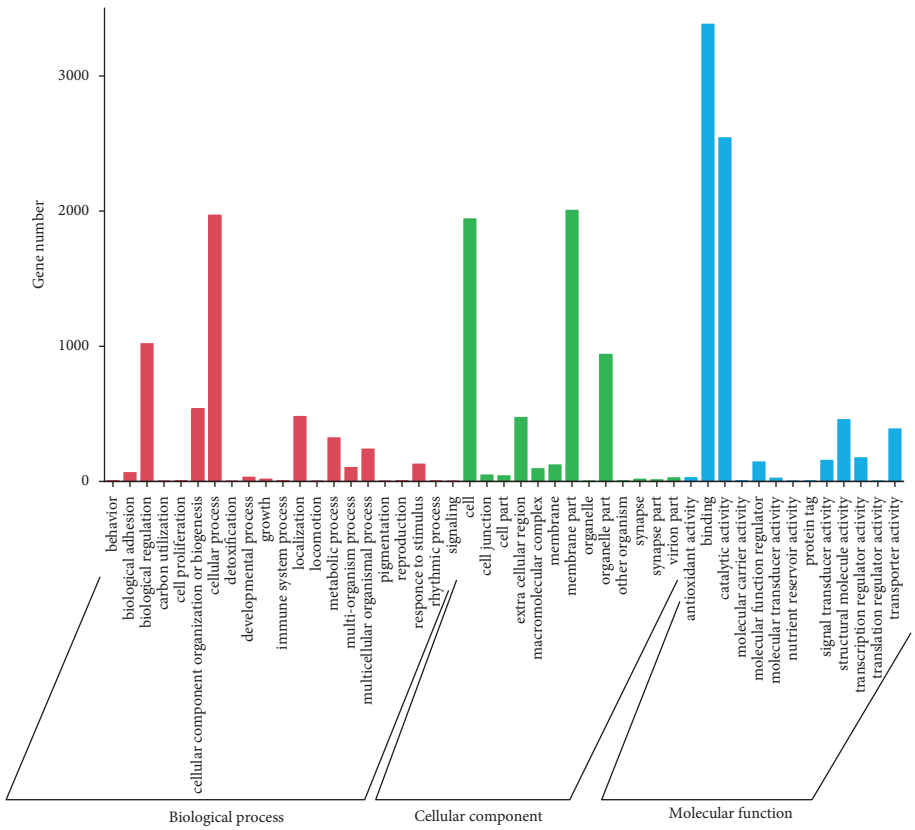


FIGURE 2: GO classification of unigenes. Note: The abscissa is the second level term under the three categories of GO. The ordinate represents the number of genes annotated to the term.

removal of the low-quality reads, approximately 466.29 million clean reads and 429.91 million clean reads were generated from the large- and small-sized prawns, respectively. The quality evaluation analysis showed that the Q20 and Q30 values of each sample exceeded 96.17% and 86.78%, respectively, and the GC contents were both above 40% (Table 1). 46,557 unigenes with an average length of 1,312 bp were finally obtained. The majority of the unigenes were 301–500 bp (30.42%) in length, followed by >2,000 bp (21.95%) and 501–1000 bp (20.23%) (Figure 1). The complete and duplicated BUSCOs of this assembled transcriptome reached 93%, indicating the completeness of this assembled transcriptome.

3.2. Functional Annotation of Assembled Unigenes. All unigenes (46,557) were annotated based on the *M. nipponense* genome [20], and a total of 37,413 (80.36%) unigenes matched known sequences in the *M. nipponense* genome. All the unigenes were then blasted against seven protein databases including NR, NT, SwissProt, KOG, KEGG, GO, and PFAM for annotation analysis (Table 2). The result showed that the NR database provided the highest number of gene annotations (22,727, 48.82%), followed by the KEGG (17,321, 37.20%) and PFAM (16,257, 34.92%) databases. The NT and GO databases yielded fewer unigene annotations than the other five databases.

3.3. GO, KOG, and KEGG Classification of Transcriptome Sequences. DEGs were clustered by the GO, KOG, and KEGG databases to describe their functional attributes. By GO analysis, there were 27.48% categorized into biological processes, of which "cellular processes" and "biological regulation" were the dominant groups; 31.86% were categorized into cellular components, of which "membrane part" and "cells" accounted for the most; and 40.66% were involved in the molecular function category, of which the "binding" and "catalytic activity" were the most abundant groups (Figure 2).

According to sequence homology, 19,954 unigenes were annotated and classified into 25 functional categories in the KOG database, with the "general function prediction only" representing the major group (up to 3,728 unigenes), followed by "signal transduction mechanisms" (2,280 unigenes). The smallest cluster was "cell motility," which only contained 84 unigenes (Figure 3).

KEGG analysis was applied to identify the unigenes related to biological pathways. A total of 46,062 unigenes were annotated into six major pathways including "cellular processes" (12.23%), "environmental information processing" (9.80%), "genetic information processing" (7.90%), "human disease" (31.59%), "metabolism" (15.28%), and "organismal systems" (23.20%) (Figure 4).

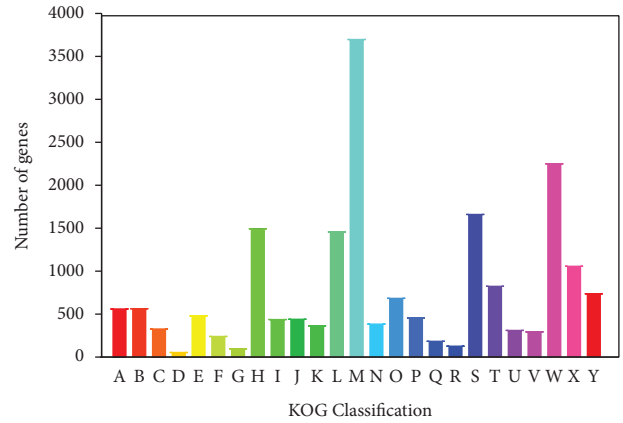
3.4. Analysis of DEGs in Large- and Small-Size Adult Male Prawns from Three Tissues. Compared with those of the small-size male prawns, the DEGs in the three tested tissues of the large-size male prawns were mainly downregulated (Figure 5). A total of 2,550 DEGs were identified between the second pereiopod of a large male (LP) and the second pereiopod of a small male (SP), including 1,745 downregulated genes and 805 upregulated genes. A total of 1,638 DEGs were identified between the muscle of a large male (LM) and the muscle of a small male (SM), including 1165 downregulated genes and 473 upregulated genes. A total of 2,667 DEGs were identified between the hepatopancreas of large males (LH) and hepatopancreas of small males (SH), including 1,630 downregulated genes and 1,037 upregulated genes.

3.5. Identification of Size-Related Pathways and Genes. KEGG analysis revealed that amoebiasis was the main enriched pathway based on LH versus SH, and the PI3K-Akt signaling pathway was the main enriched pathway both based on LM versus SM and LP versus SP. The Hippo signaling pathway, the cAMP signaling pathway, the PI3K-Akt signaling pathway, and the Ras signaling pathway were enriched in all three comparisons (Figure 6). These signaling pathways may play important roles in the size differentiation of male *M. nipponense*. Twenty-eight DEGs were screened out from these pathways, which were significantly upregulated in large-size adult males (Table 3). These DEGs could be considered as potential size-related candidate genes in adult male *M. nipponense*.

3.6. Interaction Network of Potential Size-Related Genes. Based on information in the STRING database, the interaction network of potential size-related genes for male *M. nipponense* contained 28 nodes and 53 edges with an average node degree of 3.79. The nodes indicated the DEGs, and the edges indicated the interactions among the DEGs. The core genes were ranked according to the predicted scores. The top 6 high-degree hub nodes included NOTCH1, GSK3B, HSP90AA1, RHOA, YWHAE, and PPKC1. Furthermore, NOTCH1 showed the highest node degree of 10. The modules of the gene interaction network are shown in Figure 7.

3.7. mRNA Expression Profiling of Potential Size-Related Genes. The potential size-related gene expression profiles of LH, SH, LM, SM, LP, and SP cDNA libraries were compared by complete hierarchical linkage cluster analysis (Figure 8). The results showed that the expression profiles of the same gene were tissue-specific. There were 7, 5, and 5 genes upregulated only in hepatopancreas, muscle, and the second pereiopod, respectively. However, 5 genes were upregulated in all three tested tissues at the same time, including PPP2R1, RAC1, IRS1, RHOA, and HSP90AA1. These DEGs may play important roles in the development of *M. nipponense*.

3.8. qRT-PCR Validation. qRT-PCR analysis was used to verify the expressions of size-related genes in muscle, hepatopancreas, and the second pereiopod from the large and small size of wild-captured adult male prawns. Generally, the results exhibited a good consistency with the trend of high-throughput sequencing data (Figure 9), which indicated that the results of the RNA-seq experiments were accurate and reliable.



(A) Amino acid transport and metabolism
(B) Carbohydrate transport and metabolism
(C) Cell cycle control, cell division, chromosome partitioning
(D) Cell motility
(E) Cell wall/membrane/envelope biogenesis
(F) Chromatin structure and dynamics
(G) Coenzyme transport and metabolism
(H) Cytoskeleton
(I) Defense mechanisms
(J) Energy production and conversion
(K) Extracellular structures
(L) Function unknown
(M) General function prediction only
(N) Inorganic ion transport and metabolism
(O) Intracellular trafficking, secretion, and vesicular transport
(P) Lipid transport and metabolism
(Q) Nuclear structure
(R) Nucleotide transport and metabolism
(S) Posttranslational modification, protein turnover, chaperones
(T) RNA processing and modification
(U) Replication, recombination and repair
(V) Secondary metabolites biosynthesis, transport and catabolism
(W) Signal transduction mechanisms
(X) Transcription
(Y) Translation, ribosomal structure and biogenesis

Figure 3: KOG function classification of unigenes. Note: The abscissa is the classification content of KOG, and the ordinate is the number of unigenes.

4. Discussion

It was reported that during the grow-out of the *M. rosenbergii*, the individual males will transform primarily into the large male morphotypes, i.e., the blue-claw (BC) and orange-claw (OC) males, whereas the laggards primarily become small males (SM) [21]. Similar to *M. rosenbergii*, the prawn *M. nipponense* also displays the HIG phenomenon,

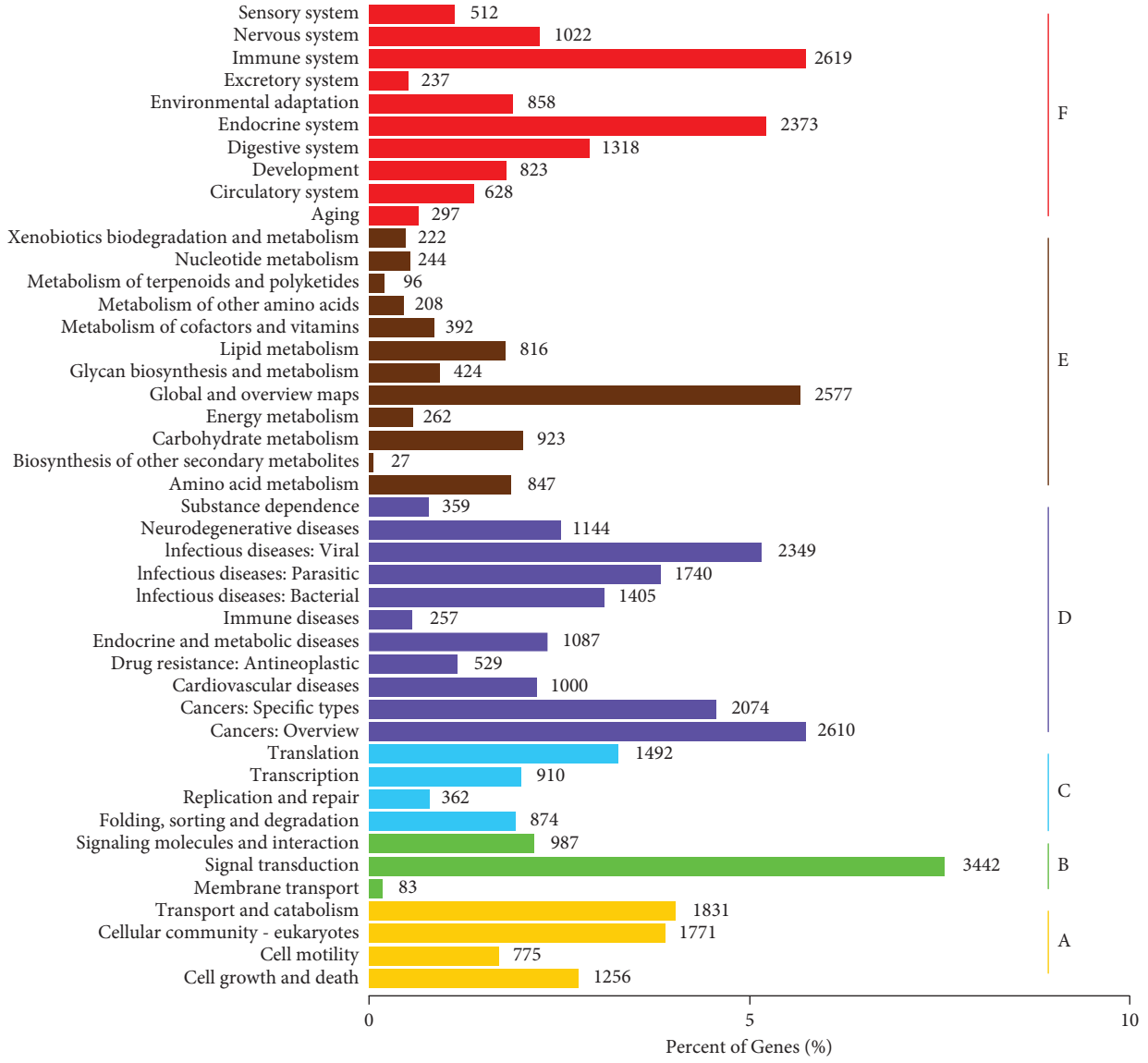


Figure 4: KEGG pathway distribution of unigenes. Note: The ordinate (left) is the name of the KEGG signal pathway, the ordinate (right) is the categories of KEGG, and the abscissa is the number of unigenes annotated to the pathway and the proportion of the number of genes annotated to the total number of genes annotated. (A) Cellular processes, (B) environmental information processing, (C) genetic information processing, (D) human diseases, (E) metabolism, and (F) organismal systems.

and the adult males in a population exhibit an obvious difference in size. The price of commercial prawn *M. nipponense* is closely dependent on individual size. Therefore, the differentiation mechanism of adult male prawn *M. nipponense* warrants investigation.

After sequencing and analysis, the number of unigenes that varied from large- and small-size groups was 46,557 with an average length of 1,312 bp. This is considerably lower than the sum of unigenes (90,491) obtained from the ovary of *M. nipponense* reported by Qiao et al. [22]. This discrepancy was probably due to the difference in tissues of *M. nipponense* used in these two studies.

The number of DEGs showed a clear separation between the large and small size of adult male prawns (Figure 5). Compared with the small-size group, there were much more downregulated DEGs in large-size adult male prawns. This result was consistent with the research in *Pinctada maxima* [23]. Wang et al. suggested that downregulated genes may also promote cell proliferation and biosynthetic processes [24]. By KEGG analysis, the upregulated DEGs in the tested three tissues were mostly predicted to be involved in apoptosis, metabolism, cell proliferation, and cell growth signal pathways (Figure 6), which implied the large differences in metabolism between the large- and small-size adult male prawns. The growth and development of crustaceans are orchestrated by a variety of regulators and intricated processes involving multiple signaling pathways [25]. It was speculated that these DEGs might converge onto some key signaling pathways to regulate the size differentiation of male *M. nipponense*. KEGG analysis of the DEGs showed

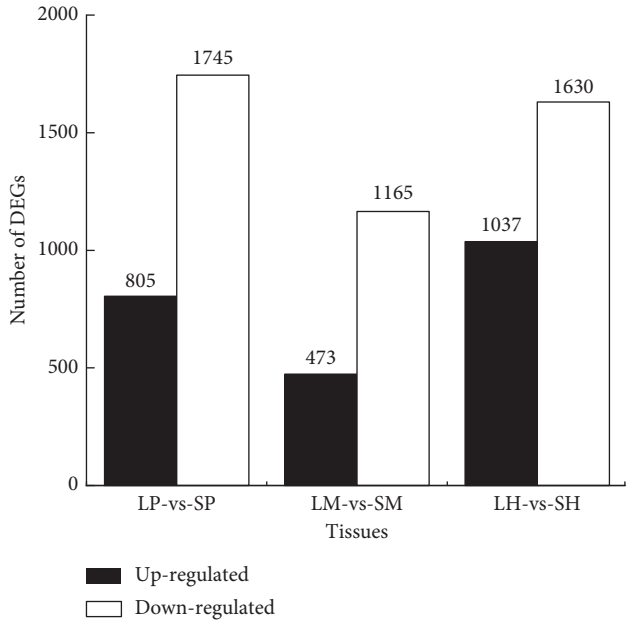


FIGURE 5: The number of differentially expressed genes in the three tested tissues from adult male prawn *M. nipponense* with different sizes. Note: the upregulated and downregulated DEGs are shown in black and white, respectively. The abscissa presents the samples from three tissues; the ordinate shows the number of DEGs. LP: second pereiopod of large-size adult male; SP: second pereiopod of small-size adult male; LM: muscle of large-size adult male; SM: muscle of small-size adult male; LH: hepatopancreas of large-size adult male; SH: hepatopancreas of small-size adult male.

that the PI3K-Akt signaling pathway, the Hippo signaling pathway, the cAMP signaling pathway, and the Ras signaling pathway were enriched in all three tested tissues from different sizes of adult male prawn *M. nipponense* (Figure 6). These predicted biological processes, and pathways were of great significance to explain the relationships between related genes and biochemical metabolic networks. The identification of candidate genes related to size from the annotated genes can provide the theoretical basis for further research and applications on the functional genes of the prawn.

Previous studies have confirmed that the Ras signaling pathway plays a vital role in the metabolism, growth, and differentiation of somatic cells [26, 27]. As a major component of the cell membrane, the Ras family function may be realized by mediating crosstalk with other pathways and finally regulating cell proliferation, differentiation, migration, and apoptosis [27, 28]. During the development of crustaceans, they undergo a series of moltings, with every molting, the body gets longer and heavier [29]. Jeong et al. also demonstrated that Ras-related proteins were involved in the development and molting-mediated in animals [30]. In this study, eight DEGs were enriched in this signaling pathway including Ral guanine nucleotide dissociation stimulator-like 1 (RGL1), Ras homolog gene family member A (RHOA), Ras-related C3 botulinum toxin substrate 1 (RAC1), Ras association domain-containing protein 1 (RASSF1), Ras-specific guanine nucleotide-releasing factor 1 (RASGERF1), angiopoietin 1 (ANGPT1), and calmodulin (CALM). Among these DEGs, five DEGs belonging to the Ras homolog gene family implied that the Ras family played positive regulatory roles in the development and growth of adult male prawn *M. nipponense*. Furthermore, CALM is a well-known Ca^{2+} binding protein, which can mediate various cell-signaling pathways by interacting with its targets, and this binding may be a key element in the modulation of Ras signaling [31, 32]. Several studies have proved that the CALM gene could interact with other proteins and affect the growth, migration, and malignancy of cells [31, 33]. This may explain why the CALM gene was screened out from the Ras signaling pathway, and it showed a significantly high expression in the large-size*M. nipponense*. Therefore, the gene CALM may play an important role in promoting the development of male *M. nipponense*.

The formation of proteins mainly involves two signaling pathways, one is the Ras pathway and the other is downstream of PI3 kinase (PI3K). The PI3K-Akt pathway is widely presented in animal cells, which can regulate various biological processes such as apoptosis and metabolism [34–36]. As the upstream signal, Ras signals to the PI3K-Akt signaling pathway cooperatively regulate the size of the organism [37]. The PI3K-Akt pathway may also provide feedback to activate the Ras signaling cascade. In this study, nine DEGs involve the PI3K-Akt pathway, such as phosphoenolpyruvate carboxy kinase (PCK), reelin (RELN), FMS-like tyrosine kinase 1 (FLT1), serine/threonine-protein phosphatase 2A regulatory subunit A (PPP2R1), fatty acid synthase (FASN), molecular chaperone HtpG (HSP90AA1), von Willebrand factor (VWF), cyclic AMP-dependent transcription factor ATF-2 (CREB), and insulin receptor substrate 1 (IRS1). PPP2R1 showed high expression levels in large-size male prawns. Protein serine/threonine phosphatase 2A (PP2A) has been reported to activate some growth factors [26, 38]; therefore, the high expression of PPP2R1 may exhibit a positive in the growth and development of male *M. nipponense*. PCK regulates glycolytic flux and involves in the synthesis of glucose. PCK can also affect the metabolism rate of hepatic glucose [39]. Compared with the small-size male prawns, the mRNA expression levels of PCK significantly upregulated in hepatopancreas in large-size prawns, suggesting an enhanced rate of glucose metabolism in large-size prawns. The hepatopancreas is an important organ, which can affect the digestion and absorption of nutrients and directly or indirectly correlate with growth promotion [40, 41]. The upregulated PCK gene in hepatopancreas indicated that the large-size adult male prawns had a more effective ingestion capability. Heat shock proteins (HSPs) are a highly conserved multigene superfamily that can be found in almost all organisms [42]. Various HSP forms are also involved in many vital biological processes, such as metabolism, growth, differentiation, and cell apoptosis [43, 44]. In crustaceans, studies on HSPs mainly focus on innate immune responses and environmental stress [45–47]. As an evidence for HSP90AA1 involved in the size development of male *M. nipponense,* HSP90AA1 mRNA expression levels were a significantly higher expression in the large-size male prawns. Spees et al. reported that the

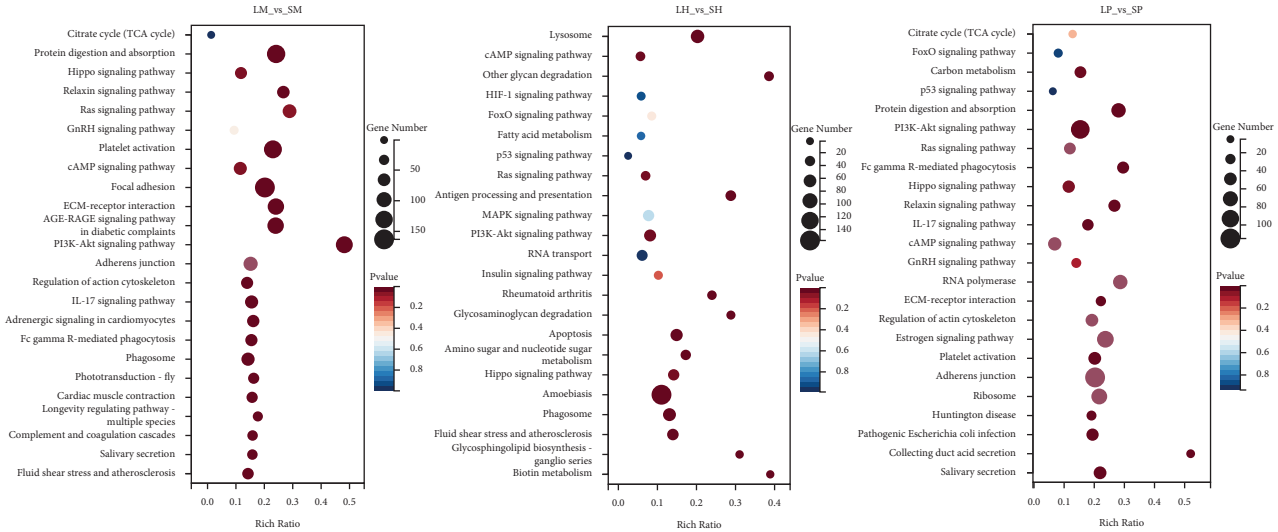


FIGURE 6: DEGs in KEGG pathway enrichment from the second pereiopod, muscle, and hepatopancreas between large- and small-size adult male prawns. Note: The ordinate (left) is the name of the KEGG signal pathway, the ordinate (right) is the number of unigenes annotated to the pathway and the (*P*) value, and the abscissa is the rich ratio. LP: second pereiopod of large-size adult male; SP: second pereiopod of small-size adult male; LM: muscle of large-size adult male; SM: muscle of small-size adult male; LH: hepatopancreas of large-size adult male; SH: hepatopancreas of small-size adult male.

HSP90 mRNA level was significantly induced in the premoult relative to the intermoult phase in American lobster [48], which indicated that HSP90 may also be involved in the growth and development of crustaceans. The insulin family includes insulin-related peptide receptors and epidermal growth factor receptors, which control body size and organ growth in metazoans [49–51]. The insulin family may directly act on the PI3K signaling pathway through the stimulation of growth factors, thereby promoting the body's energy metabolism [52]. IRS1 is a major downstream signaling protein for insulin, which can convey the growth signals to the PI3K-Akt pathway [53]. In this study, compared to the small-size prawns, the large-size individuals displayed higher expressions on the IRS1 gene in all tested tissues. Li et al. demonstrated that the expression level of insulin-like peptide (ILP) was significantly higher in large-size prawns than in small-size prawns [29]. Hyun [54] also reported that insulin signaling nonautonomously regulated muscle growth by modulating muscle-specific insulin receptors (InR). Thus, the function of IRS1 may be similar to the insulin family, and IRS1 plays an important role in regulating the growth and development of adult male *M. nipponense*.

The cAMP signaling pathway could be mediated by the Ras family positively and negatively [55]. Han confirmed that the cAMP pathway was involved in regulating protein synthesis in *Callinectes sapidus*Y-organs [56]. The Y-organs (YO) can produce ecdysteroids, which are essential for crustacean growth and postembryonic development including molting and regeneration [57]. Inhibiting the protein synthesis in Y-organs will decrease ecdysone production and finally hinder the development and growth of crustaceans. The effect of cAMP is a general suppression of overall protein synthesis [56]. Therefore, compared with other signaling pathways, there were fewer DEGs screened out from the large-size male prawns in this pathway. Azmitia reported that serotonin (5-hydroxytryptamine receptor 1, HTR1E) can activate Akt pathways, and it plays an important role in cell survival and proliferation [58]. It could be speculated that the gene HTR1E upregulated in hepatopancreas may play the signaling transduction medium, which transmits the growth signals to AKT and affects the growth and development of the male *M. nipponense*.

Gene transcription between the extracellular and intracellular is an important process of cell number regulation [59]. The Ras signaling pathway plays a vital role in this process, which can transduce extracellular signals into nuclear events and regulate many important cellular processes [37]. As one of the important metabolic pathways in the nucleus, the Hippo pathway has been delineated and proved to be the key to controlling the organ size in *Drosophila* by regulating both cell proliferation and apoptosis [60], but few studies in *M. nipponense*. This result showed the Hippo signaling pathway related to the size differentiation of adult male prawn *M. nipponense*. Meng et al. revealed that the core of the Hippo pathway is mainly composed of some protein kinases, which activate the Hippo–YAP pathway and regulate the organ size [61, 62]. In this pathway, eight DEGs were screened out including Notch 1 (NOTCH 1), lipoma-preferred partner (LPP), atypical protein kinase C iota type (PRKCI), casein kinase 1 delta protein kinases (CSNK1D), protocadherin Fat 4 (FAT4), 14-3-3 protein epsilon (YWHAE), glycogen synthase kinase 3 beta (GSK3B), and protein scribble (SCRIB). As the function of YWHAE, YWHAE may act as a bridge between proteins and regulate different biologic processes, including apoptosis, mitogenic signal transduction, and

Table 3: Selected size-related genes in the *M. nipponense* transcriptome.

Pathway	Gene ID	DEGs	Gene annotation	Tissue
PI3K-Akt signaling pathway	CL1078.Contig4_All	IRS1	Insulin receptor substrate 1	Hepatopancreas, muscle, second pereiopod
	CL2842.Contig6_All	PCK	Phosphoenolpyruvate carboxy kinase	Hepatopancreas
	Unigene11655_All	RELN	Reelin	Muscle
	CL5151.Contig4_All	PPP2R1	Serine/threonine-protein phosphatase 2A regulatory subunit A	Hepatopancreas, muscle, second pereiopod
	Unigene3487_All	FLT1	FMS-like tyrosine kinase 1	Muscle
	CL4779.Contig8_All	FASN	Fatty acid synthase	Muscle and hepatopancreas
	Unigene42925_All	HSP90AA1	Molecular chaperone HtpG	Hepatopancreas, muscle, second pereiopod
	Unigene3838_All	VWF	von Willebrand factor	Second pereiopod
	Unigene19231_All	CREB	Cyclic AMP-dependent transcription factor ATF-2	Second pereiopod
Ras signaling pathway	CL261.Contig2_All	RGL1	Ral guanine nucleotide dissociation stimulator-like 1	Hepatopancreas
	CL1988.Contig2_All	RHOA	Ras homolog gene family, member A	Hepatopancreas, muscle, second pereiopod
	Unigene5590_All	RAC1	Ras-related C3 botulinum toxin substrate 1	Hepatopancreas, muscle, second pereiopod
	Unigene30897_All	RASGRF1	Ras-specific guanine nucleotide-releasing factor 1	Hepatopancreas
	CL1961.Contig3_All	ANGPT1	Angiopoietin 1	Muscle
	CL915.Contig3_All	RAPGEF2	Rap guanine nucleotide exchange factor 2	Muscle and second pereiopod
	CL124.Contig1_All	RASSF1	Ras association domain-containing protein 1	Muscle and hepatopancreas
	Unigene3276_All	CALM	Calmodulin	Muscle and second pereiopod
cAMP signaling pathway	CL2609.Contig2_All	HTR1E	5-Hydroxytryptamine receptor 1	Hepatopancreas and second pereiopod
	Unigene35825_All	CNGB1	Cyclic nucleotide-gated channel beta 1	Muscle
	CL4768.Contig1_All	PFKFB2	6-Phosphofructo-2-kinase/fructose-2,6 biphosphatase 2	Muscle
Hippo signaling pathway	Unigene45249_All	NOTCH 1	Notch 1	Second pereiopod
	Unigene11861_All	LPP	Lipoma-preferred partner	Hepatopancreas
	CL2310.Contig4_All	PRKCI	Atypical protein kinase C iota type	Hepatopancreas
	CL1152.Contig3_All	CSNK1D	Casein kinase 1, delta	Hepatopancreas
	CL1894.Contig3_All	FAT4	Protocadherin Fat 4	Hepatopancreas
	Unigene43168_All	YWHAE	14-3-3 Protein epsilon	Muscle and second pereiopod
	CL149.Contig1_All	GSK3B	Glycogen synthase kinase-3 beta	Second pereiopod
	CL271.Contig10_All	SCRIB	Protein scribble	Second pereiopod

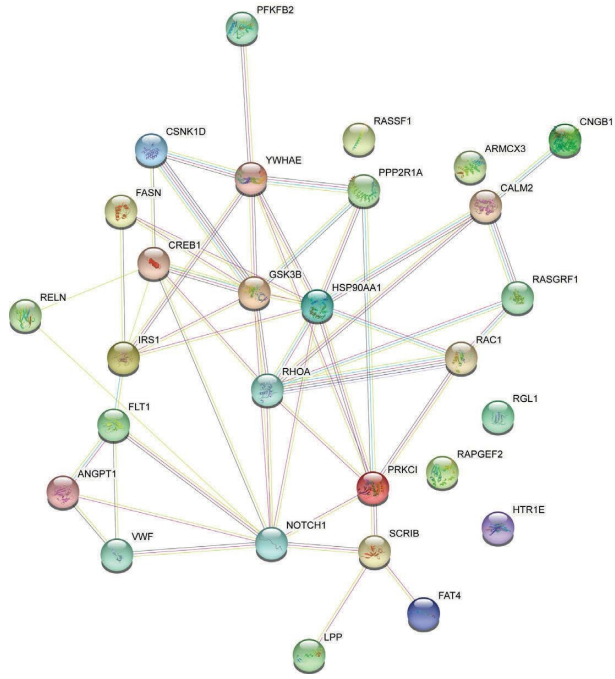


Figure 7: Protein network visualization of the potential size-related genes on the STRING website. Note: the circularity represent the differentially expressed genes (DEGs) in modules, and the lines show the interaction between the DEGs; RAC1: Ras-related C3 botulinum toxin substrate 1; RHOA: Ras homolog gene family, member A; PCK: phosphoenolpyruvate carboxy kinase; PPP2R1: serine/threonine-protein phosphatase 2A regulatory subunit A; IRS1: insulin receptor substrate 1; CSNK1D: casein kinase 1, delta; PFKFB2: 6-phosphofructo-2-kinase/fructose-2,6 biphosphatase 2; RASSF1: Ras association domain-containing protein 1; RAPGEF2: Rap guanine nucleotide exchange factor 2; RELN: reelin; FLT1: FMS-like tyrosine kinase 1; FASN: fatty acid synthase; HSP90AA1: molecular chaperone HtpG; VWF: von Willebrand factor; CREB: cyclic AMP-dependent transcription factor ATF-2; RGL1: Ral guanine nucleotide dissociation stimulator-like 1; RASGRF1: Ras-specific guanine nucleotide-releasing factor 1; ANGPT1: angiopoietin 1; CALM: calmodulin; HTR1E: 5-hydroxytryptamine receptor 1; CNGB1: cyclic nucleotide gated channel beta 1; NOTCH 1: notch 1; LPP: lipoma-preferred partner; PRKCI: atypical protein kinase C iota type; FAT4: protocadherin Fat 4; YWHAE: 14-3-3 protein epsilon; GSK3B: glycogen synthase kinase 3 beta; SCRIB: protein scribble.

cell cycle [63]. Therefore, in the current study, the gene YWHAE is upregulated both in muscle and the second pereiopod of the large-size male *M. nipponense*. Moreover, CSNK1D is encoded by YCK1 and YCK2, and any form of YCK proteins performed essential functions for promoting growth and division [64]; while, in this study, like the PCK gene, the gene CSNK1D just significantly upregulated in the hepatopancreas of large-size adult males (Figure 9), this may be the reason for the male *M. nipponense* developed into the large-size. Therefore, the Hippo signaling pathway may play the important role in the growth and development of adult male *M. nipponense*.

Based on the analysis of the key pathways and candidate genes, the possible overview of the mechanism of size differentiation in adult male *M. nipponense* was constructed (Figure 10). The growth factors could directly act on IRS1 through FLT4; then, the growth signals are transmitted to the PI3K-AKT signaling pathway, which performs different physiological and metabolic processes. Upregulated PCK and PPP2R1 may enhance the body's energy metabolism, and the gene CREB upregulation may inhibit cell apoptosis, both promoting growth and development, and finally induce large-size male prawns. While, the cAMP signaling pathway may play a less important role than other pathways in the size differentiation of male *M. nipponense* and is more of a signal transmission medium. After receiving the signals from CNGB1, the upregulated Ras signal pathway (higher expressions of RASSF1, RHOA, and RAC1) will activate the epidermal growth factors directly or indirectly to promote cell proliferation and inhibit cell apoptosis, which may induce large-size male prawn. Furthermore, in this pathway, the functions of the genes CALM, RASGERF, and RAPGEF2 mainly work as a bridge to transfer the signals. On the other side, the Ras signal pathway may regulate the Hippo signaling pathway through YWHAE. After being stimulated by growth factors, the Hippo signaling pathway performs serious physiological responses and finally can upregulate CSNK1D expression, which will promote cell proliferation to induce large-size prawns.

In conclusion, pathways and candidate genes that are possibly involved in the size differentiation were screened out by transcriptome sequencing of the second pereiopod, hepatopancreas, and muscle in different sizes of adult male prawn *M. nipponense*. Twenty-eight DEGs from the Hippo signaling pathway, the cAMP signaling pathway, the PI3K-Akt signaling pathway, and the Ras signaling pathway were identified. qRT-PCR analysis indicated that these DEGs were significantly highly expressed in the large-size adult male *M. nipponense*. The possible molecular regulation

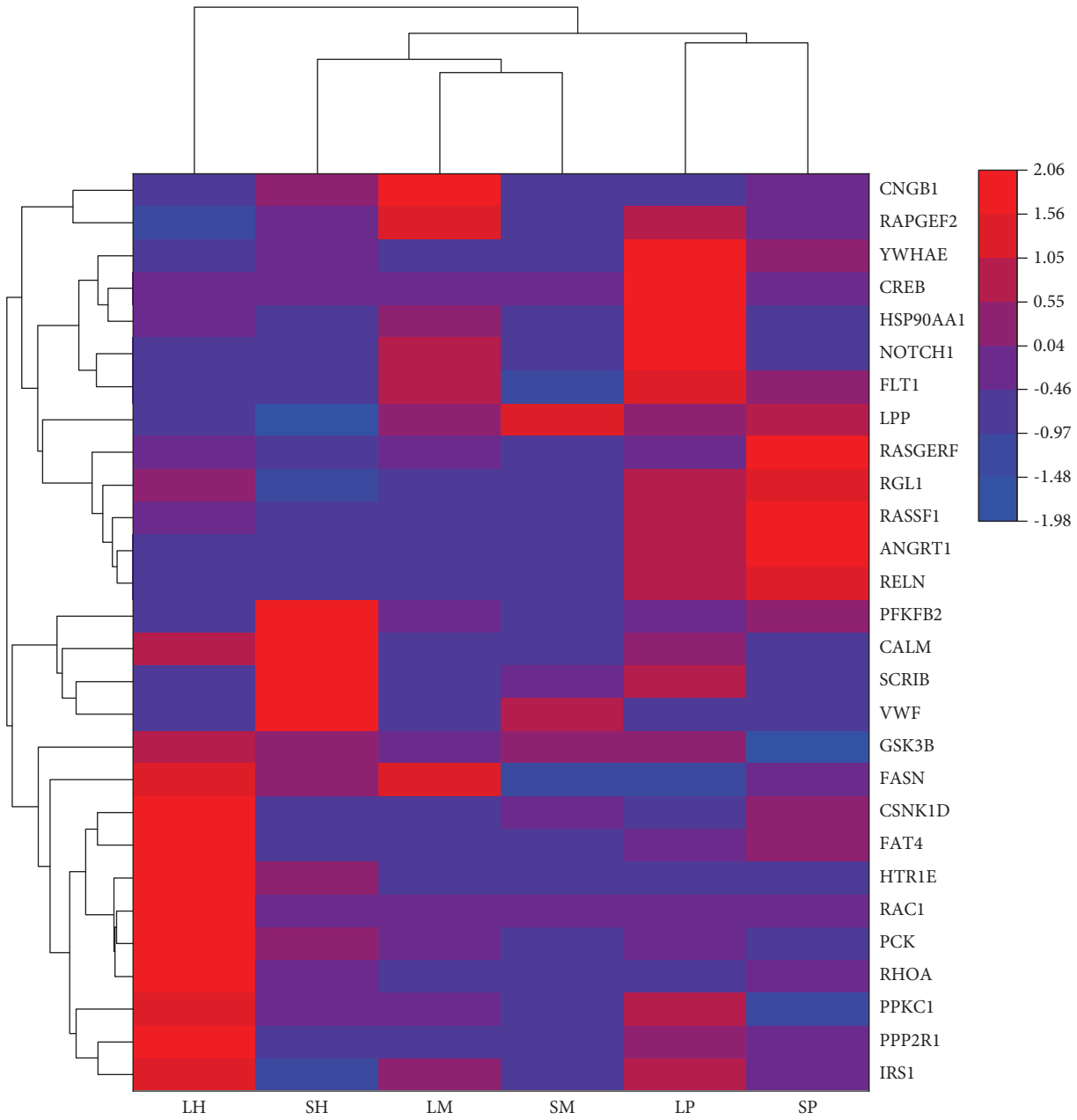


FIGURE 8: Hierarchical clustering analysis for potential size-related genes in the large-size male prawn compared with the small-size adult male prawn. Note: the color indicates the $\log_2$-fold change from high (red) to low (green), as indicated by the color scale. The ordinate (right) is the name of the potential size-related genes, the abscissa is the name of the tested tissues. LP: second pereiopod of large-size adult male; SP: second pereiopod of small-size adult male; LM: muscle of large-size adult male; SM: muscle of small-size adult male; LH: hepatopancreas of large-size adult male; SH: hepatopancreas of small-size adult male; RAC1: Ras-related C3 botulinum toxin substrate 1; RHOA: Ras homolog gene family, member A; PCK: phosphoenolpyruvate carboxy kinase; PPP2R1: serine/threonine-protein phosphatase 2A regulatory subunit A; IRS1: insulin receptor substrate 1; CSNK1D: casein kinase 1, delta; PFKFB2: 6-phosphofructo-2-kinase/fructose-2,6 biphosphatase 2; RASSF1: Ras association domain-containing protein 1; RAPGEF2: Rap guanine nucleotide exchange factor 2; RELN: reelin; FLT1: FMS-like tyrosine kinase 1; FASN: fatty acid synthase; HSP90AA1: molecular chaperone HtpG; VWF: von Willebrand factor; CREB: cyclic AMP-dependent transcription factor ATF-2; RGL1: Ral guanine nucleotide dissociation stimulator-like 1; RASGRF1: Ras-specific guanine nucleotide-releasing factor 1; ANGPT1: angiopoietin 1; CALM: calmodulin; HTR1E: 5-hydroxytryptamine receptor 1; CNGB1: cyclic nucleotide gated channel beta 1; NOTCH 1: notch 1; LPP: lipoma-preferred partner; PRKCI: atypical protein kinase C iota type; FAT4: protocadherin Fat 4; YWHAE: 14-3-3 protein epsilon; GSK3B: glycogen synthase kinase 3 beta; SCRIB: protein scribble.

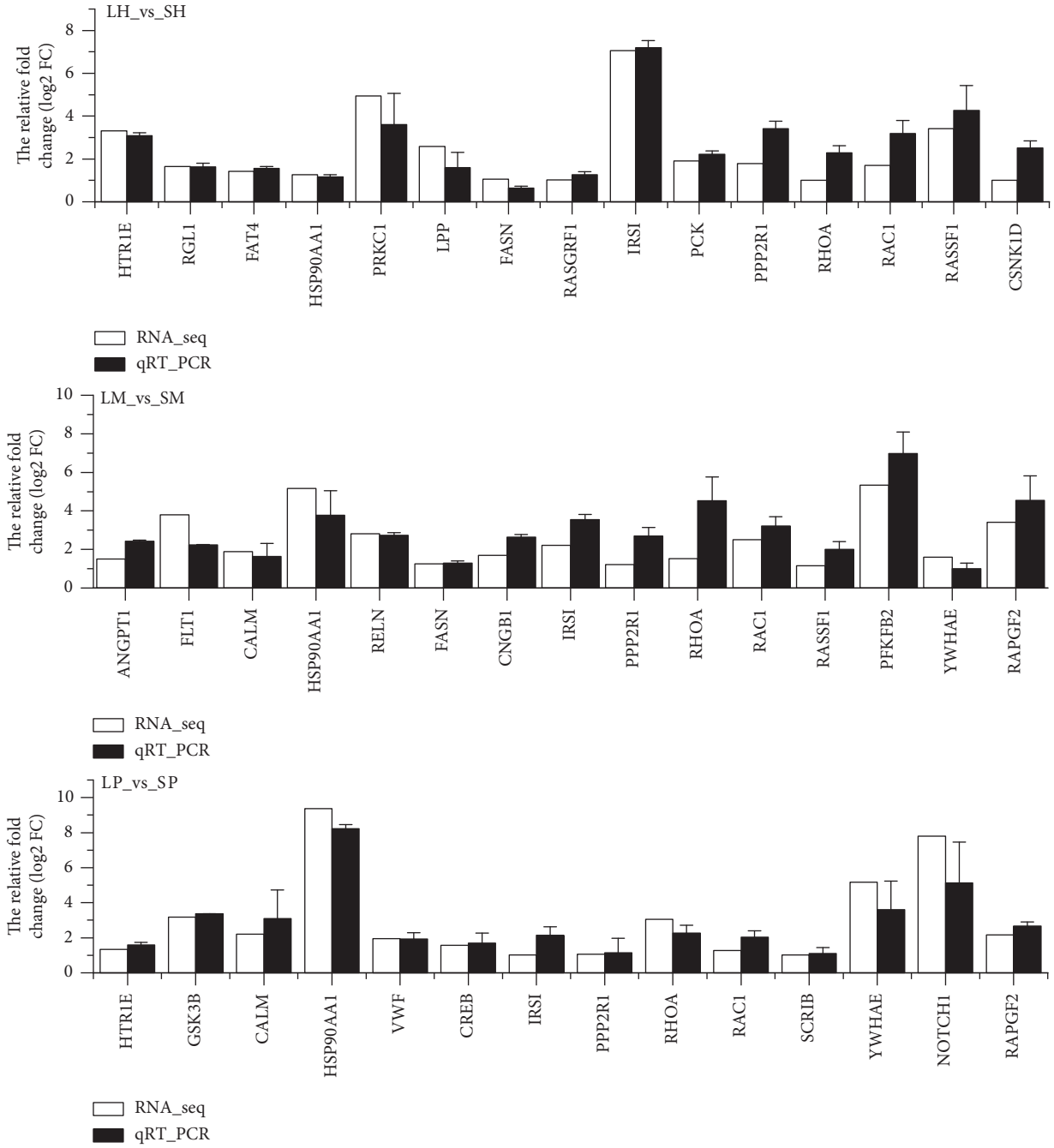


Figure 9: Results comparison on the qRT-PCR and RNA-seq for size-related genes. Notes: the qRT-PCR and RNA-seq results are shown in black and white, respectively. The abscissa presents the name of DEGs; the ordinate shows the expression levels. LP: second pereiopod of large-size adult male; SP: second pereiopod of small-size adult male; LM: muscle of large-size adult male; SM: muscle of small-size adult male; LH: hepatopancreas of large-size adult male; SH: hepatopancreas of small-size adult male; RAC1: Ras-related C3 botulinum toxin substrate 1; RHOA: Ras homolog gene family, member A; PCK: phosphoenolpyruvate carboxy kinase; PPP2R1: serine/threonine-protein phosphatase 2A regulatory subunit A; IRS1: insulin receptor substrate 1; CSNK1D: casein kinase 1, delta; PFKFB2: 6-phosphofructo-2-kinase/fructose-2,6 biphosphatase 2; RASSF1: Ras association domain-containing protein 1; RAPGEF2: Rap guanine nucleotide exchange factor 2; RELN: reelin; FLT1: FMS-like tyrosine kinase 1; FASN: fatty acid synthase; HSP90AA1: molecular chaperone HtpG; VWF: von Willebrand factor; CREB: cyclic AMP-dependent transcription factor ATF-2; RGL1: Ral guanine nucleotide dissociation stimulator-like 1; RASGRF1: Ras-specific guanine nucleotide-releasing factor 1; ANGPT1: angiopoietin 1; CALM: calmodulin; HTR1E: 5-hydroxytryptamine receptor 1; CNGB1: cyclic nucleotide gated channel beta 1; NOTCH 1: notch 1; LPP: lipoma-preferred partner; PRKCI: atypical protein kinase C iota type; FAT4: protocadherin Fat 4; YWHAE: 14-3-3 protein epsilon; GSK3B: glycogen synthase kinase 3 beta; SCRIB: protein scribble.

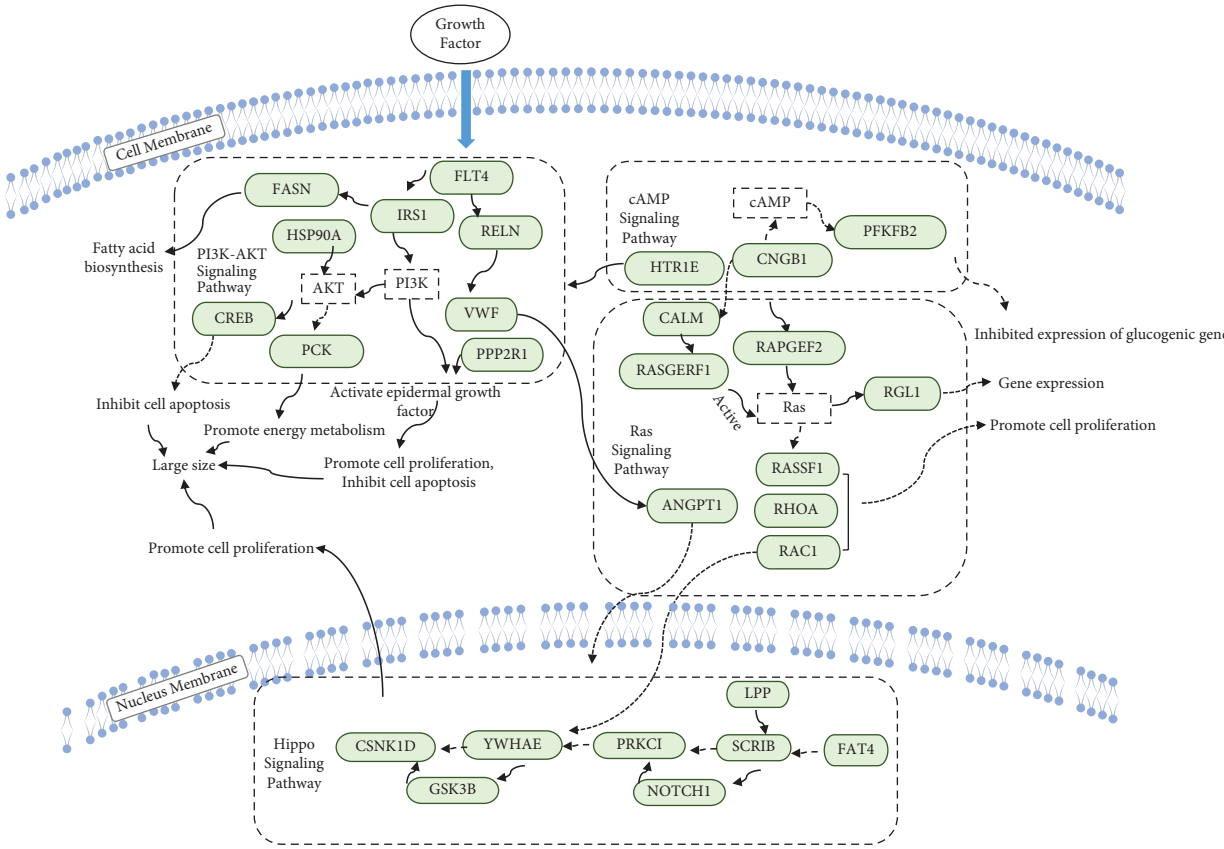


FIGURE 10: Hypothesized scheme of the regulatory process to large size phenotypic of adult male *M. nipponense*. Note: solid lines mean confirmed effects and dotted lines mean possible effects. The blue oval represents the screened candidate genes, and the white dotted box indicates the signal transduction genes in the signal pathway but not screened out; RAC1: Ras-related C3 botulinum toxin substrate 1; RHOA: Ras homolog gene family, member A; PCK: phosphoenolpyruvate carboxy kinase; PPP2R1: serine/threonine-protein phosphatase 2A regulatory subunit A; IRS1: insulin receptor substrate 1; CSNK1D: casein kinase 1, delta; PFKFB2: 6-phosphofructo-2-kinase/fructose-2,6 biphosphatase 2; RASSF1: Ras association domain-containing protein 1; RAPGEF2: rap guanine nucleotide exchange factor 2; RELN: reelin; FLT1: FMS-like tyrosine kinase 1; FASN: fatty acid synthase; HSP90AA1: molecular chaperone HtpG; VWF: von Willebrand factor; CREB: cyclic AMP-dependent transcription factor ATF-2; RGL1: Ral guanine nucleotide dissociation stimulator-like 1; RASGRF1: Ras-specific guanine nucleotide-releasing factor 1; ANGPT1: angiopoietin 1; CALM: calmodulin; HTR1E: 5-hydroxytryptamine receptor 1; CNGB1: cyclic nucleotide gated channel beta 1; NOTCH 1: notch 1; LPP: lipoma-preferred partner; PRKCI: atypical protein kinase C iota type; FAT4: protocadherin Fat 4; YWHAE: 14-3-3 protein epsilon; GSK3B: glycogen synthase kinase 3 beta; SCRIB: protein scribble.

mechanism on size differentiation in male prawns *M. nipponense* was put forward.

Data Availability

All relevant data are available at the NCBI database (accession PRJNA789588).

Disclosure

A preprint has previously been published [65].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Gang Jiang performed the data analysis and drafted the manuscript. Yongming Zhu conducted the feeding experiment and sample collection. Yucai Xue checked the English writing, Ying Lu provided technical assistance on data analysis and refining suggestions for the manuscript, Zhiwei Liu provided technical assistance on qRT-PCR, and Xuxiong Huang designed the research, provided financial support, and refined the manuscript. All authors read, reviewed, edited, and approved the final manuscript.

Acknowledgments

This study was financially supported by the project of Shanghai Municipal Agricultural Commission (2019 No. 2-5) and Establishment of China-ASEAN Belt and Road Joint Laboratory on Mariculture Technology and Joint Research on Mariculture Technology.

Supplementary Materials

Curated versions of the genomes were assembled, annotated, and analyzed during the current study based on the

M. nipponense genome (https://gigadb.org/dataset/100843). The reads of *M. nipponense* transcriptome were submitted to NCBI with the accession number of PRJNA789588. Additional file 1: Table S1: Primers were used for qPCR verification in this study. (*Supplementary Materials*)

References

[1] Y. X. Cai and P. K. L. Ng, "The freshwater palaemonid prawns (Crustacea: Decapoda: caridea) of Myanmar," *Hydrobiologia*, vol. 487, pp. 59–83, 2002.

[2] H. Fu, S. Jiang, and Y. Xiong, "Current status and prospects of farming the giant river prawn (Macrobrachium rosenbergii) and the oriental river prawn (Macrobrachium nipponense) in China," *Aquaculture Research*, vol. 43, pp. 993–998, 2012.

[3] F. Zhang, L. Chen, J. Qin et al., "CDNA cloning and expression analysis of gustavus gene in the oriental river prawn Macrobrachium nipponense," *PLoS One*, vol. 6, Article ID e17170, 2011.

[4] K. Y. Ma, Z. Q. Liu, J. Y. Lin, J. L. Li, and G. F. Qiu, "Molecular characterization of a novel ovary-specific gene fem-1 homolog from the oriental river prawn," *Macrobrachium nipponense. Gene*, vol. 575, pp. 244–252, 2016.

[5] K. Ma, J. Feng, J. Lin, and J. Li, "The complete mitochondrial genome of Macrobrachium nipponense," *Gene*, vol. 487, pp. 160–165, 2011.

[6] R. Banu, S. S. Siraj, A. Christianus, N. F. Ikhsan, and A. H. Rajaee, "Comparison of all morphotype males and various types stocking density of Macrobrachium rosenbergii (De Man) on growth and survival rate," *Aquaculture Reports*, vol. 3, pp. 184–188, 2016b.

[7] I. Karplus, "Social control of growth in Macrobrachium rosenbergii (De Man): a review and prospects for future research," *Aquaculture Research*, vol. 36, pp. 238–254, 2005.

[8] M. R. Banu, A. Christianus, N. F. M. Ikhsan, and A. H. Rajaee, "Effect of cold shock and hormone on growth and male morphotypes of freshwater prawn, Macrobrachium rosenbergii (de Man)," *Aquaculture Research*, vol. 47, pp. 3740–3746, 2016a.

[9] S. B. Jin, N. Wang, H. Qiao, H. T. Fu, and Y. Wu, "Molecular cloning and expression of a full length cDNA encoding crustacean hyperglycemic hormone (CHH) in oriental river pawn (*Macrobrachium nipponense*)," *Journal of Fishery Sciences of China*, vol. 37, pp. 82–92, 2013a.

[10] W. Zhao, Z. Wang, Y. Yu et al., "Growth and antioxidant status of oriental river prawn *Macrobrachium nipponense* fed with diets containing vitamin E," *Chinese Journal of Oceanology and Limnology*, vol. 34, pp. 477–483, 2016.

[11] W. Shen, G. Ren, Y. Zhu, and X. Zhang, "Characterization of MSTN/GDF11 gene from shrimp *Macrobrachium nipponense* and its expression profiles during molt cycle and after eyestalk ablation," *Genes & Genomics*, vol. 37, pp. 441–449, 2015.

[12] H. Qiao, Y. Xiong, W. Zhang et al., "Characterization, expression, and function analysis of gonad-inhibiting hormone in Oriental River prawn, *Macrobrachium nipponense* and its induced expression by temperature," *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, vol. 185, pp. 1–8, 2015.

[13] B. B. Patnaik, J. M. Chung, H. J. Hwang et al., "Transcriptome analysis of air-breathing land slug, Incilaria fruhstorferi reveals functional insights into growth, immunity, and reproduction," *BMC Genomics*, vol. 20, p. 154, 2019.

[14] M. G. Grabherr, B. J. Haas, M. Yassour et al., "Full-length transcriptome assembly from RNA-Seq data without a reference genome," *Nature Biotechnology*, vol. 29, pp. 644–U130, 2011.

[15] S. Jin, H. Fu, Z. Qiao et al., "Transcriptome analysis of androgenic gland for discovery of novel genes from the oriental river prawn, *Macrobrachium nipponense*, using illumina hiseq 2000," *PLoS One*, vol. 8, 2013b.

[16] S. Anders and W. Huber, "Differential expression analysis for sequence count data," *Genome Biology*, vol. 11, 2010.

[17] K. S. Kwong, B. Holland, and S. H. Cheung, "A modified Benjamini-Hochberg multiple comparisons procedure for controlling the false discovery rate," *Journal of Statistical Planning and Inference*, vol. 104, pp. 351–362, 2002.

[18] A. Untergasser, I. Cutcutache, T. Koressaar et al., "Primer3-new capabilities and interfaces," *Nucleic Acids Research*, vol. 40, 2012.

[19] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method," *Methods*, vol. 25, pp. 402–408, 2001.

[20] S. Jin, Y. Fu, Y. Hu et al., "Identification of candidate genes from androgenic gland in *Macrobrachium nipponense* regulated by eyestalk ablation," *Scientific Reports*, vol. 11, Article ID 19855, 2021a.

[21] K. Ilan, H. Gideon, W. Giora, and H. Wohlfarth, "The effect of size-grading juvenile *Macrobrachium rosenbergii* prior to stocking on their population structure and production in polyculture: II. Dividing the population into three fractions," *Aquaculture*, vol. 62, pp. 90341–90348, 1987.

[22] H. Qiao, H. Fu, Y. Xiong et al., "Molecular insights into reproduction regulation of female Oriental River prawns *Macrobrachium nipponense* through comparative transcriptomic analysis," *Scientific Reports*, vol. 7, Article ID 12161, 2017.

[23] R. Hao, Z. Wang, C. Yang et al., "Metabolomic responses of juvenile pearl oyster *Pinctada maxima* to different growth performances," *Aquaculture*, vol. 491, pp. 258–265, 2018.

[24] S. Wang, P. Song, and M. Zou, "AMP-activated protein kinase, stress responses and cardiovascular diseases," *Clinical Science*, vol. 122, pp. 555–573, 2012.

[25] S. Sahni, K. C. Park, Z. Kovacevic, and D. R. Richardson, "Two mechanisms involving the autophagic and proteasomal pathways process the metastasis suppressor protein, N-myc downstream regulated gene 1," *Biochimica et Biophysica Acta, Molecular Basis of Disease*, vol. 1865, pp. 1361–1378, 2019.

[26] Z. Wang, J. Cui, J. Song et al., "Comparative transcriptome analysis reveals growth-related genes in juvenile Chinese sea cucumber, Russian sea cucumber, and their hybrids," *Marine Biotechnology*, vol. 20, pp. 193–205, 2018.

[27] L. Zheng, Z. Zhao, L. Rong, L. Xue, and Y. Song, "RASSF6-TRIM16 axis promotes cell proliferation, migration and invasion in esophageal squamous cell carcinoma," *Journal of Genetics and Genomics*, vol. 46, pp. 477–488, 2019.

[28] D. T. Leicht, V. Balan, A. Kaplun et al., "Raf kinases: function, regulation and role in human cancer," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1773, pp. 1196–1212, 2007.

[29] F. Li, S. Zhang, C. Fu, T. Li, and X. Cui, "Molecular and functional analysis of the insulin-like peptides gene in the oriental river prawn *Macrobrachium nipponense*," *General and Comparative Endocrinology*, vol. 280, pp. 209–214, 2019.

[30] W. Jeong, H. Bae, W. Lim, F. W. Bazer, and G. Song, "RAS-related protein 1: an estrogen-responsive gene involved in development and molting-mediated regeneration of the

female reproductive tract in chickens," *Animal*, vol. 12, no. 8, pp. 1–8, 2017.

[31] P. Villalonga, C. López-Alcalá, M. Bosch, A. Chiloeches, and N. Agell, "Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates its downstream signaling," *Molecular and Cellular Biology*, vol. 21, p. 7345, 2001.

[32] S. Kang, H. N. Kwon, S. Kang, and S. Park, "Interaction between IDH1 WT and calmodulin and its implications for glioblastoma cell growth and migration," *Biochemical and Biophysical Research Communications*, vol. 524, pp. 224–230, 2020.

[33] M. T. Wang, M. Holderfield, J. Galeas, R. Delrosario, and F. Mccormick, "K-ras promotes tumorigenicity through suppression of non-canonical wnt signaling," *Cell*, vol. 163, pp. 1237–1251, 2015.

[34] L. Chen and J. S. Khillan, "A novel signaling by vitamin A/retinol promotes self renewal of mouse embryonic stem cells by activating PI3K/Akt signaling pathway via insulin-like growth factor-1 receptor," *Stem Cells*, vol. 28, pp. 57–63, 2010.

[35] B. Shalmon, M. Drendel, M. Wolf, A. Hirshberg, and Y. Cohen, "Mutational analysis of PI3K/AKT and RAS/RAF pathway activation in malignant salivary gland tumours with a new mutation of PIK3CA," *International Journal of Oral and Maxillofacial Surgery*, vol. 45, pp. 721–725, 2016.

[36] H.-Z. Long, Y. Cheng, Z.-W. Zhou, H.-Y. Luo, D.-D. Wen, and L.-C. Gao, "PI3K/AKT signal pathway: a target of natural products in the prevention and treatment of Alzheimer's disease and Parkinson's disease," *Frontiers in Pharmacology*, vol. 12, Article ID 648636, 2021.

[37] V. Kumar, M. X. Zhang, M. W. Swank, J. Kunz, and G. Y. Wu, "Regulation of dendritic morphogenesis by ras–PI3K–Akt–mTOR and ras–MAPK signaling pathways," *Journal of Neuroscience the Official Journal of the Society for Neuroscience*, vol. 25, Article ID 11288, 2005.

[38] D. G. Adams, R. L. Coffee, H. Zhang, S. Pelech, S. Strack, and B. E. Wadzinski, "Positive regulation of raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes," *Journal of Biological Chemistry*, vol. 280, pp. 42644–42654, 2005.

[39] S. C. Burgess, T. T. He, Z. Yan et al., "Cytosolic phosphoenolpyruvate carboxykinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver," *Cell Metabolism*, vol. 5, pp. 313–320, 2007.

[40] Y. M. Chai, S. S. Yu, X. F. Zhao, Z. Qian, and J. X. Wang, "Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* response to white spot syndrome virus," *Fish & Shellfish Immunology*, vol. 29, pp. 480–486, 2010.

[41] R. Gomez, M. Bedolla-Cazares, V. Zorrilla, E. Toledo-Cuevas, and M. Canosa, "Effects of increasing the dietary lipid levels on the growth performance, body composition and digestive enzyme activities of the teleost pejerrey (*Odontesthes bonariensis*)," *Aquaculture*, vol. 416, pp. 15–22, 2013.

[42] K., C. Kregel, "Invited Review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance," *Journal of Applied Physiology*, vol. 192, pp. 2177–2186, 2002.

[43] J. C. Ranford, A. Coates, and B. Henderson, "Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones," *Expert Reviews in Molecular Medicine*, vol. 2, pp. 1–17, 2000.

[44] E. S. Christians, Z. Qi, J. P. Renard, and I. J. Benjamin, "Heat shock proteins in mammalian development," *Seminars in Cell & Developmental Biology*, vol. 14, pp. 283–290, 2003.

[45] W. Rungrassamee, R. Leelatanawit, P. Jiravanichpaisal, S. Klinbunga, and N. Karoonuthaisiri, "Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to Vibrio harveyi in *Penaeus monodon*," *Developmental & Comparative Immunology*, vol. 34, pp. 1082–1089, 2010.

[46] J. Zhou, W. N. Wang, W. Y. He et al., "Expression of HSP60 and HSP70 in white shrimp, *Litopenaeus vannamei* in response to bacterial challenge," *Journal of Invertebrate Pathology*, vol. 103, pp. 170–178, 2010.

[47] W. J. Huang, J. H. Leu, M. T. Tsau, J. C. Chen, and L. L. Chen, "Differential expression of LvHSP60 in shrimp in response to environmental stress," *Fish & Shellfish Immunology*, vol. 30, no. 2, pp. 576–582, 2011.

[48] J. L. Spees, S. A. Chang, D. L. Mykles, M. J. Snyder, and E. S. Chang, "Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster," *Cell Stress & Chaperones*, vol. 8, pp. 258–264, 2003.

[49] P. J. Schlueter, G. Peng, M. Westerfield, and C. Duan, "Insulin-like growth factor signaling regulates zebrafish embryonic growth and development by promoting cell survival and cell cycle progression," *Cell Death & Differentiation*, vol. 14, pp. 1095–1105, 2007.

[50] M. Perillo and M. I. Arnone, "Characterization of insulin-like peptides (ILPs) in the sea urchin *Strongylocentrotus purpuratus*: insights on the evolution of the insulin family," *General and Comparative Endocrinology*, vol. 205, pp. 68–79, 2014.

[51] T. E. Ventura, A. Battaglene, C. Stephen, C. Hollander, and C. Lian, "Discovery of a novel insulin-like peptide and insulin binding proteins in the Eastern rock lobster Sagmariasus verreauxi," *General and Comparative Endocrinology*, vol. 215, pp. 76–87, 2015.

[52] S. M. Cheng, T. J. Ho, A. L. Yang et al., "Exercise training enhances cardiac IGFI-R/PI3K/Akt and Bcl-2 family associated pro-survival pathways in streptozotocin-induced diabetic rats," *International Journal of Cardiology*, vol. 167, pp. 478–485, 2013.

[53] A. Gregory, O. Cesarone, E. Prakash, C. Chang-Po, and E. Wickstrom, "Insulin receptor substrate 1 knockdown in human MCF7 ER+ breast cancer cells by nuclease-resistant IRS1 siRNA conjugated to a disulfide-bridgedd-peptide analogue of insulin-like growth factor 1," *Bioconjugate Chemistry*, vol. 18, pp. 1831–1840, 2007.

[54] S. Hyun, "Body size regulation and insulin-like growth factor signaling," *Cellular and Molecular Life Sciences*, vol. 70, pp. 2351–2365, 2013.

[55] B. N. Kholodenko, J. F. Hancock, and W. Kolch, "Signaling ballet in space and time," *Nature Reviews Molecular Cell Biology*, vol. 11, pp. 414–426, 2010.

[56] D. W. Han, N. Patel, and R. D. Watson, "Regulation of protein synthesis in Y-organs of the blue crab (*Callinectes sapidus*): involvement of cyclic AMP," *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, vol. 305, pp. 328–334, 2006.

[57] H. Jung, R. E. Lyons, D. A. Hurwood, and P. B. Mather, "Genes and growth performance in crustacean species: a review of relevant genomic studies in crustaceans and other taxa," *Reviews in Aquaculture*, vol. 5, pp. 77–110, 2013.

[58] E. C. Azmitia, "Modern views on an ancient chemical: Serotonin effects on cell proliferation, maturation, and apoptosis," *Brain Research Bulletin*, vol. 56, pp. 413–424, 2001.

[59] B. Zhao, L. Li, Q. Lei, and K. L. Guan, "The Hippo–YAP pathway in organ size control and tumorigenesis: an updated version," *Genes & Development*, vol. 24, pp. 862–874, 2010.

[60] B. Zhao, Q. Y. Lei, and K. L. Guan, "The Hippo-YAP pathway: new connections between regulation of organ size and cancer," *Current Opinion in Cell Biology*, vol. 20, pp. 638–646, 2008.

[61] Z. Meng, T. Moroishi, and K. L. Guan, "Mechanisms of Hippo pathway regulation," *Genes & Development A Journal Devoted to the Molecular Analysis of Gene Expression in Eukaryotes Prokaryotes & Viruses*, vol. 30, pp. 1–17, 2016.

[62] S. Ma, Z. Meng, R. Chen, K. L. Guan, and R. D. Kornberg, "The Hippo pathway:hlogy and pathophysiology," *Annual Review of Biochemistry, R. D. Kornberg, Ed.*,vol. 88, pp. 577–604, 2019.

[63] S. Rajagopalan, R. S. Sade, F. M. Townsley, and A. R. Fersht, "Mechanistic differences in the transcriptional activation of p53 by 14-3-3 isoforms," *Nucleic Acids Research*, vol. 38, pp. 893–906, 2009.

[64] L. C. Robinson, M. M. Menold, S. Garrett, and M. R. Culbertson, "Casein kinase I-like protein kinases encoded by YCK1 and YCK2 are required for yeast morphogenesis," *Molecular and Cellular Biology*, vol. 13, pp. 2870–2881, 1993.

[65] G. Jiang, Y. M. Zhu, Y. C. Xue, Z. W. Liu, Y. Lu, and X. X. Huang, "Screening and Analyzing of Genes and Signaling Pathways Associated with Size Differentiation of Adult Male Prawn *Macrobrachium Nipponense*," *Research Square*, 2022.