RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2024.043

ScienceAsia 50 (2): 2024: ID 2024043: **1–10**

Transcriptome profiles of the hepatopancreas of the Chinese swamp shrimp (*Neocaridina denticulata*) under different diurnal rhythms

Heng Zhang[a], **Changyue Yu**[b], **Yingdong Li**[b,*], **Qing Liu**[a,*]

[a] Key Laboratory of Hydrobiology in Liaoning Province, College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023 China

[b] Key Laboratory of Livestock Infectious Diseases in Northeast China, Ministry of Education, College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866 China

[*]Corresponding authors, e-mail: liyingdong@syau.edu.cn, liuqing@dlou.edu.cn

Received 3 Jul 2022, Accepted 5 Mar 2024
Available online 3 May 2024

ABSTRACT: Diurnal rhythm affects many physiological functions in crustaceans. To study its effects on physiological functions in *Neocaridina denticulata*, a small shrimp with high ornamental value, we analyzed its hepatopancreatic transcriptome under the daily rhythm using high-throughput sequencing. Hepatopancreas were collected from the shrimps at four time points (06:00, 12:00, 18:00, and 24:00) within a 24-h period. Interestingly, numerous circadian-related unigenes were annotated, including *Per*, *Tim*, *Clk*, *Cyc*, *Cry*, and *5-HT*. We used the whole genes identified at each time point as a group and performed pairwise comparative analysis to screen for differentially expressed genes under the daily rhythm. The largest number of differentially expressed genes (DEGs) (983) was identified between 12:00 and 18:00. The Gene Ontology functional enrichment analysis of DEGs indicated that the sugar and chitin metabolism processes in *N. denticulata* were impacted by its daily rhythm; the Kyoto Encyclopedia of Genes and Genomes enrichment analysis showed that the DEGs were mainly enriched in the 'phototransduction-fly' and 'thermogenesis' pathways. To the best of our knowledge, this is the first comparative transcriptome analysis of the hepatopancreas of *N. denticulata* under daily rhythms; our findings will provide molecular genetic evidence for the study of diurnal rhythms in shrimps. Thus, our study will be useful for future neurophysiology studies using environmental stimuli or overcoming environmental stress.

KEYWORDS: daily rhythm, shrimps, comparative transcriptomics, crustacean, immune system

INTRODUCTION

Like all organisms, members of Crustacea coordinate their physiology and behavior to reflect cyclical patterns of their adaptation to changes in the environment. This coordination of the organism with its environment is achieved through the action of an innate, genetically encoded time-keeping system [1]. Although many biological clocks have been described, the most important and crucial is the circadian system, which is responsible for regulating the physiological and behavioral events that are synchronized with the day-night cycle and recur within a cycle of approximately 24 h [2]. In crustaceans, several physiological changes and activity processes, such as locomotion [3], digestion [4], and immune regulation [5], display a 24-h periodicity and are regulated by the circadian systems. However, only a few studies have described the genes and proteins required to establish circadian signaling systems in members of Crustacea, most of which originate from neurological regions. Research regarding the diurnal regulatory mechanisms in crustaceans is still relatively limited.

Omics-based sequencing has been widely used to study the physiological mechanisms of crustaceans. Homologous cloning and RNA-sequencing (RNA-Seq) methods have been used to sequence and characterize core circadian clocks and clock-related genes [6]. Interestingly, recent studies have revealed the impact of dynamic changes in the physiological functions of the gut bacterial community of the Chinese mitten crab *Eriocheir sinensis*, a crustacean species with high economic value, under a 24-h circadian rhythm [7]. In addition, the hemolymph transcriptomic study of crustaceans further suggests that crustaceans may show differences in immunity and digestibility under a 24-h diurnal variation [8].

Hepatopancreas is an important organ in crustaceans for studying their digestive, metabolic, and immune processes [9]. Previous studies hypothesized that various external stresses could promote the immunological response in shrimps and directly impose on the hepatopancreas; therefore, the transcript-level variation under these stress conditions was investigated [10]. Furthermore, measuring physiological indicators of the hepatopancreas of shrimps during starvation can reveal their physiological, metabolic, and behavioral changes and elucidate the dynamics of their main energy reserves [11]. Notably, liquid chromatography-mass spectrometry (LC-MS)-based metabolomics has been used to investigate the metabolic mechanism of the changes in hepatopan-

creas metabolites during the daily cycle, which suggests that the hepatopancreas of crustaceans is regulated by circadian rhythms affecting its immune and digestive functions [12]. Besides, many genes that function in circadian regulation have been identified in some crustaceans. The first core clock gene identified was *Clk* in the prawn *Macrobrachium rosenbergii* [13]. The recognition of rhythmic transcripts was performed in the lobster *Nephrops norvegicus* [14] and the Antarctic krill *Euphausia superba* [15]. The tissue-specific clock system components of the American lobster *Homarus americanus* have been identified recently, and the sequences of the transcripts/proteins *Clk*, *Cry2*, *Cyc*, *Per*, and *Tim* were validated [16]. However, there is still a lack of mechanistic investigation into the circadian rhythm of shrimps.

N. denticulata, a shrimp species novel to Taiwan, Japan, and Hawaii, is a fully-fledged crustacean model useful for understanding the molecular mechanisms of diel physiology because of its ontogenic characteristics, availability, and ease of culture and genetic manipulation [17]. Life-history traits and ecological characteristics of this ornamental shrimp species have been recently surveyed .Genes associated with growth and reproduction have also been identified in this species [18]. Furthermore, a research on its reproduction and population dynamics was conducted. However, to date we have not obtained any molecular dataset on the circadian rhythm of this shrimp, even though some scientists have started to focus on the interesting subject of crustacean circadian rhythms. Hence, in this study, we aimed to provide a transcriptome profile of the Chinese swamp shrimp *N. denticulata* under a 24-h daily rhythm to elucidate the variation in physiological processes affected by daily rhythms. These results will be useful for future neurophysiology studies using environmental stimuli or overcoming environmental stress.

MATERIALS AND METHODS

Ethics statement

No endangered or protected species were collected in this study. All experiments were conducted following the Guidelines for the Care and Use of Animals for Scientific Purposes established by the Animal Ethics Committee of Shenyang Agricultural University, with minimal animal suffering.

Shrimps

A total of 3,000 healthy *N. denticulata* shrimps (average weight, 1.37 ± 0.28 g) were obtained from a local aquarium market in Shenyang, Liaoning Province, China, in October 2021. The shrimps were transported to the aquaculture laboratory of Shenyang Agricultural University and placed in tanks with a recirculating tap water system for two weeks of acclimatization. The shrimps were kept at natural temperature (14 ± 4 °C) under a 12 h/12 h light/dark cycle. Each day, the temperature in the laboratory peaked at approximately 12:00 (18 °C) and dropped to a minimum (10 °C) at 24:00 (midnight). During the first two weeks of the experiment, the shrimps were fed an artificially tailored diet, with the last feeding time being 48 h prior to the experiment. We randomly sampled 150 shrimps every 6 h during 24 h, i.e., at 06:00, 12:00, 18:00, and 24:00. Immediately after sampling, their hepatopancreas tissues were harvested under natural light at 06:00 and 12:00. Sampling was performed in winter, when it was already dark at approximately 18:00 in the Shenyang, China. Thus, we ensured that the sampling process was performed under darkness at 18:00 and 24:00 and used only infrared light for assistance to prevent the shrimps from seeing the light. Sixty hepatopancreas samples were collected from each group and placed in three separate 2 ml RNase-free tubes, which were subsequently frozen in liquid nitrogen for subsequent RNA sequencing. The whole process is illustrated in Fig. 1.

RNA extraction, cDNA library construction, and deep sequencing

Total RNA extraction from each of the collected hepatopancreas samples was performed using a Trizol Kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The RNA quality of each sample was examined by performing agarose gel electrophoresis using the Agilent 2100 Bioanalyzer (Agilent, Shanghai, China). Transcriptome sequencing was then conducted using the Illumina HiSeq 4000 sequencing platform to generate 150-bp paired-end reads. In each group, we categorized the remaining 20 individuals in pairs and pooled the RNA of each pair to construct sequencing libraries per group: A6 1–2 for the 06:00 group, B12 1–3 for the 12:00 group, C18 1–3 for the 18:00 group, and C24 1–2 for the 24:00 group. The RNA-seq procedures were performed by Personalbio Technology Co., Ltd. (Shanghai, China).

De novo assembly and gene annotation

After eliminating the low-quality sequences and adapter sequences, the clean reads were *de novo* assembled using Trinity to obtain a reference sequence for subsequent analysis. The reads for each sample were separately subjected to transcriptome assembly on the SOAP-*de novo* software. The concordance between the RNA-seq data in the four groups was assessed using the two methods principal component analysis (PCA) and pairwise correlation analysis R-language package (version 4.0.3). Subsequently, Basic Local Alignment Search Tool (BLAST) alignment against NR (non-redundant) (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA), PFAM (protein family) (http://pfam.xfam.org), Swiss-Prot (protein sequences) (http://www.

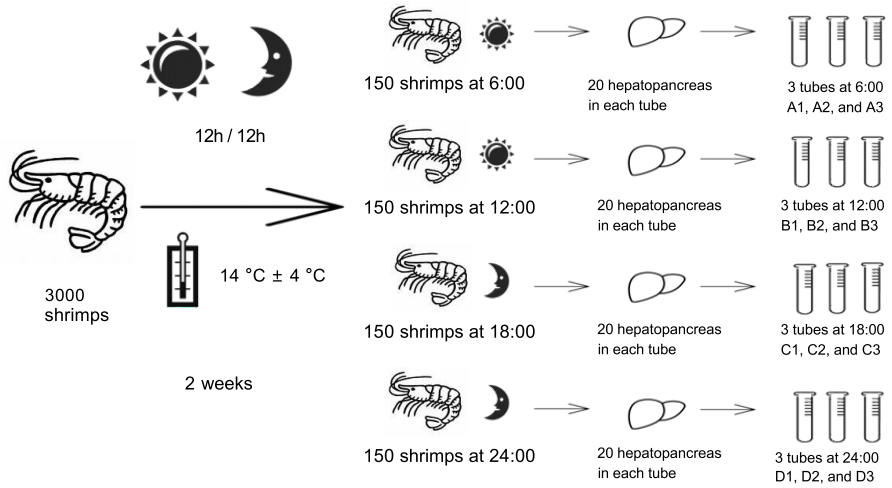

Fig. 1 Flow diagram and summary of this study.

gpmaw.com/html/swiss-prot.html), KEGG (Kyoto Encyclopedia of Genes and Genomes) (https://www.kegg.jp), KO (KEGG orthologue database) (https://www.genome.jp/kegg/ko.html), and GO (Gene Ontology) (http://geneontology.org), and eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) (http://eggnog5.embl.de) was performed.

Differential gene expression and enrichment analysis

The DESeq algorithm in R (R Core Team, 2020) (https://www.R-project.org) was used to analyze differentially expressed genes (DEGs) with ($|\log_2|$ fold change > 1, p-value < 0.05) as a condition for filtering out DEGs with differentially expressed multiples. Subsequently, statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test to determine significant expression level means of the peak-bottom differences. The R-language Pheatmap package (version 4.0.3) was used to cluster the associations of different genes and samples for all groups compared. Distances were calculated using the Euclidean method and clustering was performed using stratified clustering methods. The DEGs were subjected to KEGG and GO pathway enrichment analysis. Enrichment results were checked manually against NR annotations using public databases and literature searches.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA of the samples used in our transcriptomic study was extracted at four time points of the day (06:00, 12:00, 18:00, and 24:00 h), with three groups per time point and five biological replicates per group. Four circadian-regulated genes were selected; their expression was determined using qRT-PCR. The primer sequences for each gene were designed using Primer Premier 5.0 (Table S1). β-actin was used as the internal reference and each sample was tested in triplicate. The cDNA templates obtained at 06:00, 12:00, 18:00, and 24:00 were reverse transcribed using PrimeScript RT reagent (Takara Bio, Shiga, Japan). The SYBR Green RT-PCR assays were performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative changes in gene expression levels were determined using the $2^{-\Delta Ct}$ method. Statistical significance was calculated using the ANOVA test to determine the differences in the expression levels. All statistical analyses were performed using SPSS 20.0 software (Chicago, USA. version 22.0).

RESULTS

Illumina sequencing, read assembly, and functional annotation

Table S2 summarizes the total raw readings and clean reads generated for each sample. After filtering and cleaning the raw readings, 41,293,707 (92.4%) high-quality reads were selected for the assembly (Table S3). The reads for each sample were first separately subjected to transcriptome assembly. Subsequently, all the reads were combined to construct unique transcripts (termed as unigenes) among all the samples (Table S4). The Illumina paired-end reads [Biosample: SAMN27771230] have been uploaded to the National Center for Biotechnology Information.

Using the BLAST tools, 26,506, 15,484, 11,598, 15,949, 22,342, and 16,401 unigenes were annotated to the NR, GO, KEGG, Pfam, eggNOG, and Swiss-Prot databases, respectively, and a total of 8,133 unigenes were annotated as common to all strains (Table S5). For most reads in NR, similar unigenes were matched with those of *Penaeus vannamei* (5,999; 22.63%), *Pe-*

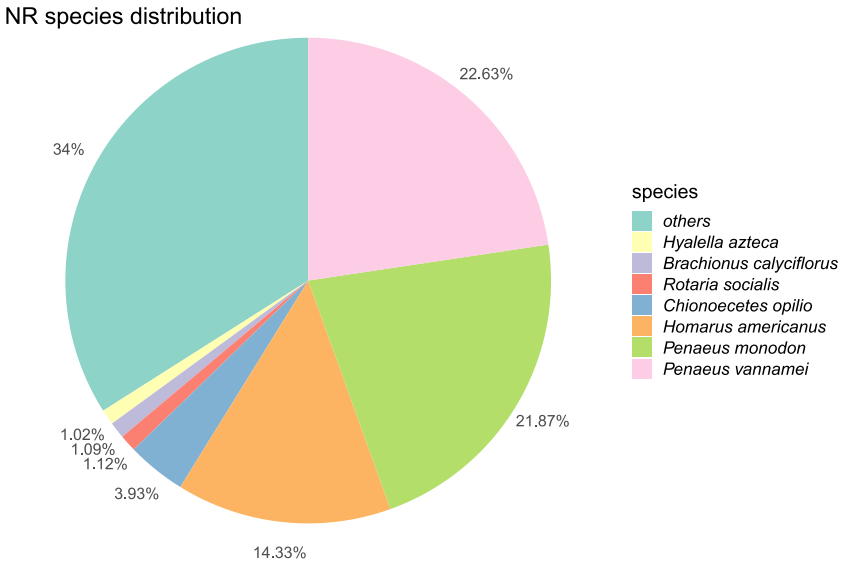

Fig. 2 NR annotation of assembled genes. The percentages marked in the fan diagram imply the similarity in the gene sequences of this species with those of closely related species.

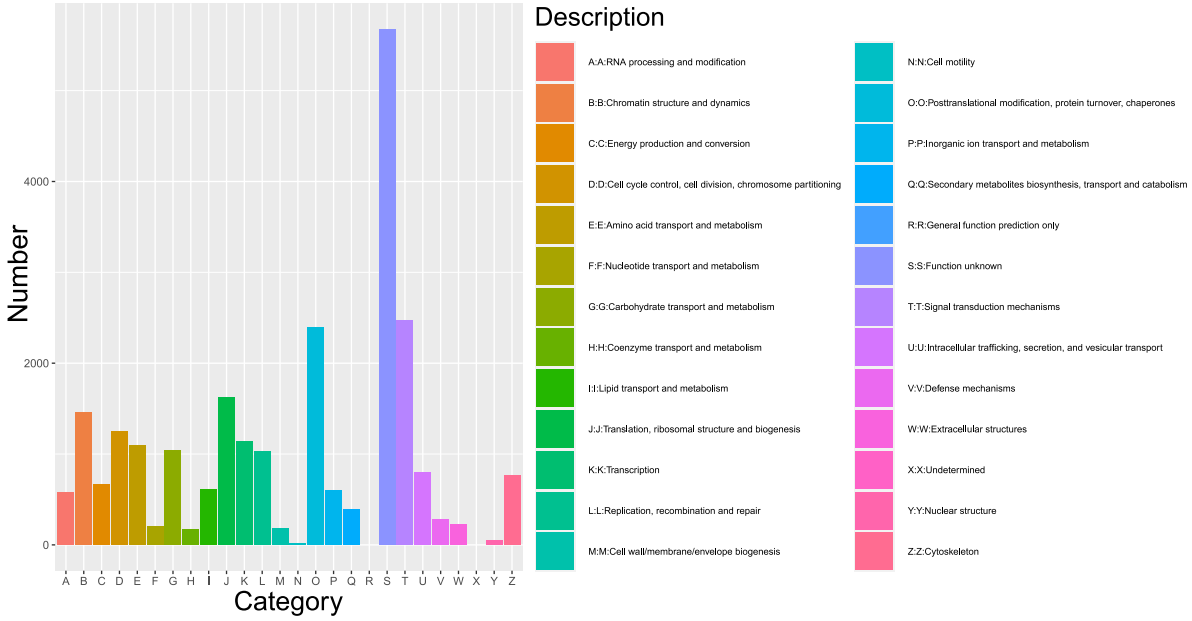

Fig. 3 EggNOG annotation of assembled genes. The genes were annotated by eggNOG alignment, and the eggNOG numbers of the best alignment results were assigned to the corresponding genes. The correspondence between the eggNOG numbers and the eggNOG taxonomy was used to assign each gene to the eggNOG taxonomy.

naeus monodon (5,796; 21.87%), *Homarus americanus* (3,798; 14.33%), *Chionoecetes opilio* (1,042; 3.93%), *Rotaria socialis* (298; 1.12%), *Brachionus calyciflorus* (290; 1.09%), *Hyalella azteca* (271; 1.02%), and other species (9,012; 34%) (Fig. 2). The eggNOG functional analysis classified 19,037 unigenes into 25 groups, with the largest group (2,475; 13%) classified as signal transduction mechanisms, followed by post-translational modification, protein turnover, and chaperones (2,398; 12.60%), and translation, ribosomal structure, and biogenesis (1626; 8.54%) (Fig. 3). GO analysis showed that 14,438; 13,526; and 11,610 unigenes were mapped into the cellular component, biological process, and molecular function categories, respectively (Fig. 4). The statistical results of the KEGG pathway analysis showed that "immune

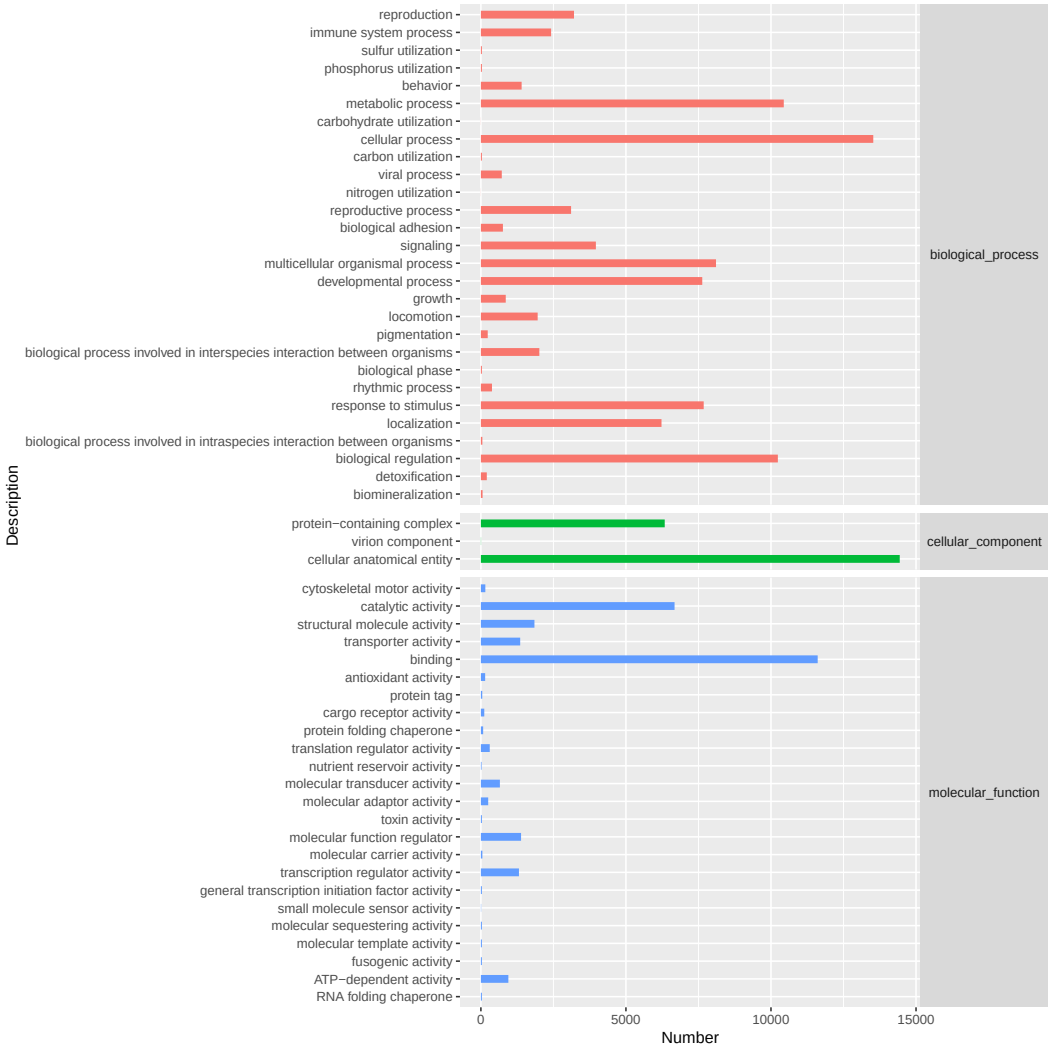

Fig. 4 GO annotation of assembled genes. The GO annotation results correspond to GO term, and the number of genes annotated to the second level of classification is counted.

Table 1 Unigenes related to the daily rhythm.

Unigene ID	Length	Protein
TRINITY_DN772_c0_g1	4646	clock-interacting pacemaker [*Chionoecetes opilio*]
TRINITY_DN11229_c0_g1	1932	clock [*Macrobrachium rosenbergii*]
TRINITY_DN1354_c0_g2	4670	protein timeless homolog [*Penaeus monodon*]
TRINITY_DN4336_c0_g1	6894	timeless [*Macrobrachium nipponense*]
TRINITY_DN9857_c0_g1	2074	timeless-interacting protein-like [*Penaeus monodon*]
TRINITY_DN8067_c0_g2	5355	period [*Macrobrachium nipponense*]
TRINITY_DN45956_c0_g1	302	period circadian protein-like [*Penaeus monodon*]
TRINITY_DN45956_c0_g3	444	period circadian protein-like [*Penaeus monodon*]
TRINITY_DN11080_c0_g1	4177	cryptochrome precursor [*Penaeus vannamei*]
TRINITY_DN1733_c3_g1	3238	cryptochrome-1-like [*Penaeus vannamei*]
TRINITY_DN16207_c0_g1	2661	5-hydroxytryptamine receptor 2C-like [*Penaeus monodon*]
TRINITY_DN2372_c0_g1	4664	5-hydroxytryptamine receptor 1 [*Drosophila melanogaster*]
TRINITY_DN47063_c0_g2	2626	5-hydroxytryptamine receptor 1 [*Drosophila melanogaster*]
TRINITY_DN48453_c0_g1	875	5-hydroxytryptamine receptor 2A [*Bos taurus*]
TRINITY_DN49700_c0_g1	396	circadian locomoter output cycles protein kaput-like [*Penaeus monodon*]
TRINITY_DN22297_c0_g1	525	circadian locomoter output cycles protein kaput-like [*Penaeus monodon*]

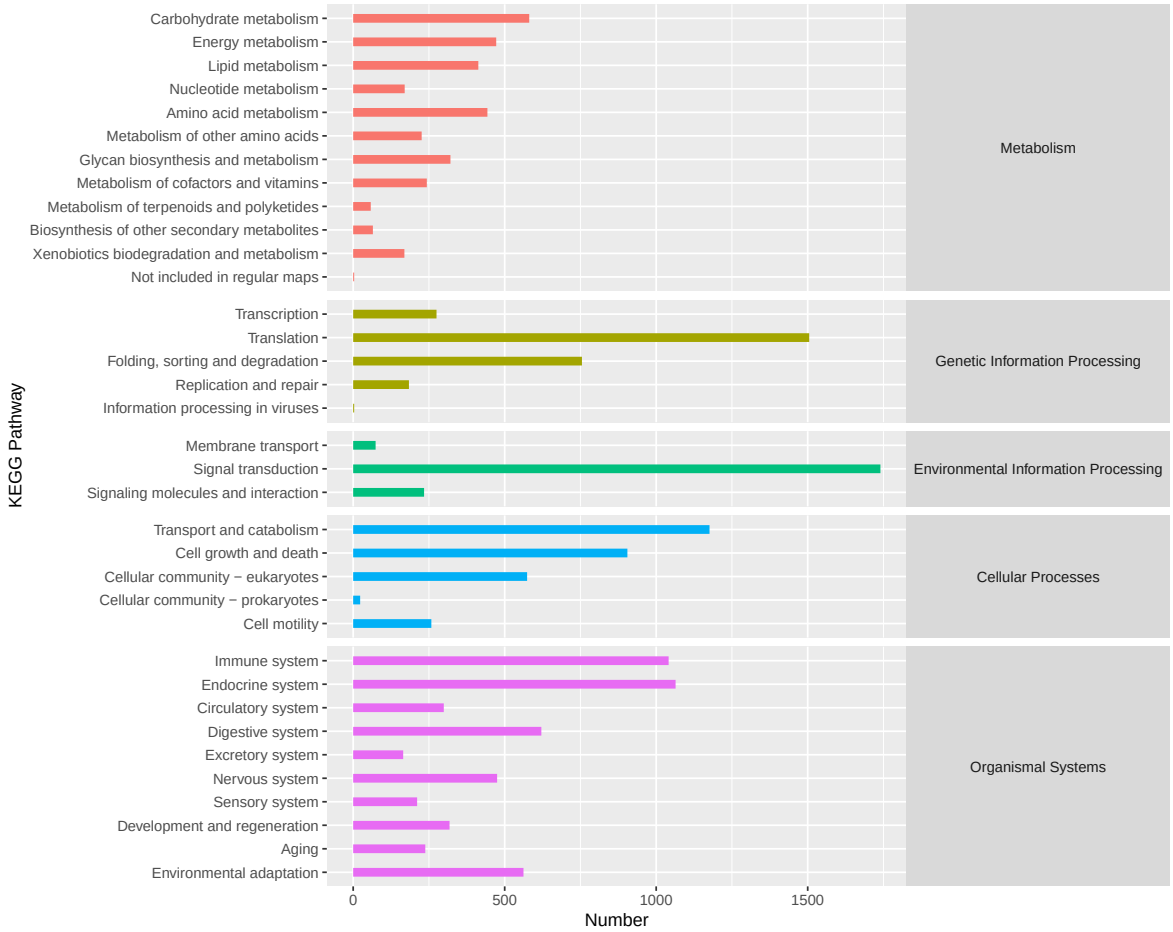

Fig. 5 KEGG annotation of assembled genes.

system" (1,041; 20.85%), "endocrine system" (1,064; 21.31%), and "digestive system" (621; 12.43%) were the top three enriched pathways in "organismal systems". In "metabolism", "carbohydrate metabolism" (581; 18.38%), "energy metabolism" (472; 14.93%), and "amino acid metabolism" (443; 14.01%) were the top three enriched pathways (Fig. 5). Notably, in the present study, numerous unigenes involved in circadian regulation were identified. *Per*, *Tim*, *Clk*, and *Cyc*, which are typical circadian-regulation genes, were annotated in the hepatopancreas transcriptome of *N. denticulata sinensis* shrimps. Moreover, circadian-related cryptochrome and 5-hydroxytryptamine (5-HT) were also determined in the present annotation (Table 1).

Differential gene expression and functional enrichment analysis

To identify the DEGs, we used all the genes identified at each time point as a group and ran a two-by-two group comparative analysis. The heatmap clustering of all differential genes is shown in Fig. 6. The largest number of DEGs (983) was identified between 12:00 and 18:00; the expression of 588 and 350 genes were upregulated and downregulated, respectively (Fig. S1).

A total of 449 DEGs were identified in the 06:00 vs. 12:00 group; of these, the expression of 190 and 259 were upregulated and downregulated, respectively. Based on GO annotation of the DEGs, the GO term for the most significant enrichment was associated with molting and sugar metabolic processes such as chitin binding (Fig. S2). These related differential genes were annotated to most of the significant terms under the categories of biological processes, cellular components, and molecular functions. We performed a KEGG enrichment analysis of the DEGs. The significantly-changed pathway included 'phototransduction-fly', which is involved in biological circadian rhythms (Fig. S3).

Between 06:00 and 18:00 h, 677 DEGs were identified, 384 of them were upregulated and the rest were downregulated. GO enrichment analysis showed that the downregulated genes were primarily enriched in biosynthetic process terms (Fig. S4). In particular, the

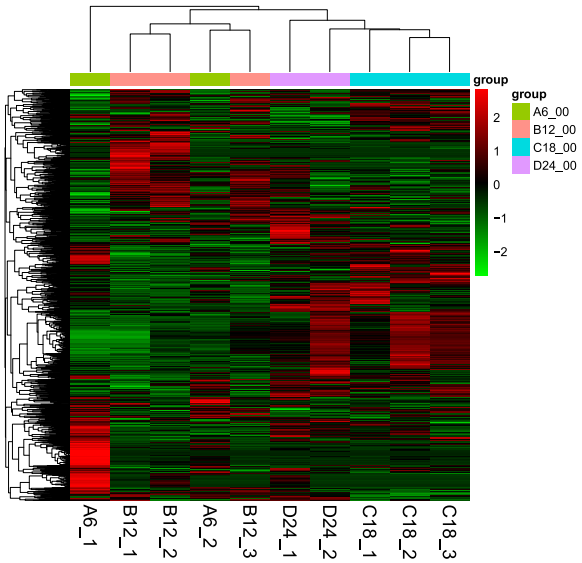

Fig. 6 Heatmap clustering of all differentially expression genes (DEGs). The genes are represented horizontally, one sample per column, with red indicating high-expression genes and green indicating low-expression genes.

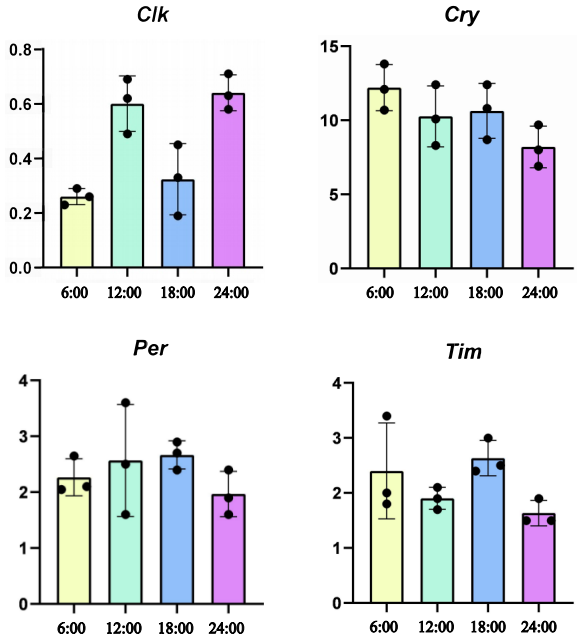

Fig. 7 qRT-PCR verification of four circadian-regulated genes. The groups marked "*" represent a significant difference at $p < 0.05$. Horizontal axis represents time of the day and vertical axis represents expression level.

KEGG enrichment analysis revealed that most DEGs were involved in the metabolic process, like nitrogen metabolism, oxidative phosphorylation, and arginine biosynthesis; downregulated genes were dominant in these pathways. It is worth noting that the thermogenesis pathway (which reflects the external environment, especially temperature variation), was one of the most significantly enriched pathways (Fig. S5).

The smallest number of DEGs (282) was identified in the comparison between 06:00 and 24:00; 223 and 59 genes were upregulated and downregulated, respectively. GO terms with the highest enrichment level were related to sugar metabolism and chitin metabolism process (Fig. S6). According to the KEGG enrichment analysis, half of the top 10 most enriched pathways were involved in metabolism (Fig. S7).

qRT-PCR verification

To verify the accuracy of the transcriptomic data results and to probe the real-time expression of circadian-regulated genes under circadian rhythms, the expression levels of four circadian-regulated genes (*Per*, *Tim*, *Clk*, and *Cry*) were assessed using qRT-PCR. Only the expression of *Clk* fluctuated significantly in response to circadian rhythms. Additionally, the expression trend of each gene was consistent with the results of the transcriptomic data (Fig. 7).

DISCUSSION

The objective of the present study was to perform the transcriptome analysis of the hepatopancreas of *N. denticulata* sinensis shrimps under the daily rhythm. The annotation of unigenes at four different time points during the day was analyzed. The circadian clock enables organisms to anticipate some external environmental changes, schedule their life activities at appropriate time points, and coordinate internal processes with the environment through the regularity of the daily cycle [2]. The molecular mechanism of daily rhythms in animals is best documented in the fruit fly *Drosophila melanogaster* and has been reported to involve the interaction of four transcription factors, *Per*, *Tim*, *Clk*, and *Cyc* [19, 20].

A genetic screen for mutants with altered circadian rhythms led to the discovery of the first clock gene *Per*, which plays a central role in the transcriptional feedback loop [21]. Core clock genes encode proteins that form two diverse heterodimers: *Per* and *Tim* form the *Per-Tim* heterodimer and *Clk* and *Cyc* form the *Clk-Cyc* heterodimer [22]. The *Per* feedback loop is intertwined with the *Clk* feedback loop. The *Per-Tim* heterodimer acts as an auto-regulatory negative feedback loop, leading to a reduction in transcriptional activation of *Clk* and *Cyc* [23]. This negative feedback loop is tuned by the action of *Cry*. *Cry* genes were identified in the present annotation. However, while studying the circadian mechanisms in crustaceans, only a small number of the identified key genes/proteins are required for their circadian regulatory system; these have only been reported in members of Astacidea [1, 24]. The presence of *Per* genes in the retina and

lamina has been previously reported in *Procambarus clarkii* [25]. Similarly, it was demonstrated that in crayfish, *Per*- and *Tim-Clk* are located in the cytoplasm and nucleus of specific neurons in the retina, optic lobe, and brain [26]. In crustaceans, the genes involved in the circadian signal system have mostly been detected in neural tissues. However, recent studies on circadian rhythm-associated genes in non-neural tissues suggest that the regulatory mechanisms of daily rhythms are not only present in the nervous system. Yu et al [8] used transcriptome sequencing methods to study the hemolymph transcriptome of *E. sinensis* collected at different time points over a 24-h period, and several core clock genes (*Per*, *Clk*, *Tim*, and *Cyc*) that interacted in a putative molecular clock feedback loop were identified. This was the first time that putative crustacean clock genes had been identified in the hemolymph. Notably, the circadian-related genes identified in the hemolymph did not show significant differential expression levels, which is consistent with the results of the current study and similar to the results obtained in a previous transcriptome sequencing analysis of the eyestalk of *E. sinensis* [27]. Since the current study is the first exploration of transcript levels in shrimps under daily rhythms, the lack of data regarding clock genes in this species resulted in a somewhat long interval between the sampling time points. The sampling time points should be at shorter intervals when further trends in clock gene expression abundance are explored or the 24-h cyclic expression profile is characterized. It is likely that the differential expression of clock genes in crustaceans is tissue-specific and exists only in the nervous system. The circadian mechanism in shrimps remains unclear, but in general, our results demonstrate the expression of genes of the circadian signal system in a non-neural tissue, the hepatopancreas. Prospective investigations could focus on detecting the expression levels of circadian regulation-associated genes in different tissues, and the expression analysis of circadian regulation-associated genes in non-neural and neural tissues can be performed on a diel basis, along with the examination of the temporal variability.

In the 06:00 vs. 12:00 h and 06:00 vs. 24:00 comparison, the enrichment of DEGs showed that the GO terms with the highest enrichment were related to sugar metabolism and the chitin metabolism process. This suggests that circadian rhythms affect the energy metabolism pathway of shrimps, especially, sugar metabolism and chitin synthesis. In general, the metabolism of crustaceans is not different from the main pathways of higher animal phyla; in particular, the metabolic processes are broadly similar to those of vertebrates, and the similarities outweigh the differences [28]. Sugar metabolism is vital for preserving the energy balance in crustaceans, especially the pentose phosphate pathway for NADPH production and ribose synthesis for the overall energy metabolism process [29]. Crustaceans lack effective glucose homeostasis mechanisms, which tolerate large changes in blood glucose [11]. In general, significant changes in glucose metabolism levels may be due to external environmental changes and a resulting energy crisis. The process of sugar metabolism varies according to the daily rhythm, which was also observed in the hepatopancreas, which is the main tissue for glycogen storage in shrimps [29]. It was reported that change in sugar metabolism is a straightforward and reliable indicator of biological stress in shrimps and can be used as a marker in disease diagnosis [30]. The growth and development of crustaceans is mediated by molting, which is associated with the glycogen content in the hepatopancreas. The content of lipids, glycogen, and several inorganic elements in the hepatopancreas fluctuates with their function and the stage of the molting cycle [31]. For *Litopenaeus vannamei*, sugar metabolic processes have implications for growth, body composition, and ammonia tolerance [32]. Thus, the significant enrichment of DEGs in the terms related to sugar metabolism may suggest that their differential glycol metabolism under daily rhythms is designed for energy compensation during growth and development.

In crustaceans, like all arthropods, growth is accompanied by a molting cycle, and the main physiological activity during molting is the metabolism of chitin, including its synthesis and degradation [31]. Notably, the chitin metabolism process is enriched with numerous DEGs that were identified during the daily rhythm. Chitin synthesis in crustaceans was demonstrated to be correlated with sugar metabolism [33], further indicating that the circadian cycle regulates the molting process in crustaceans and influences their growth and development to a large extent by regulating sugar and chitin metabolism. Several studies have made it clear that the molting process is influenced by circadian cycles [34]. However, whether this effect is continuous or would occur at a specific stage of growth and development warrants further discussion. Methods to influence growth and development through the regulation of circadian rhythms or to benefit the breeding process also presents a future direction for research.

The results of the KEGG enrichment analysis showed that DEGs under circadian rhythms were mainly enriched in the pathways 'phototransduction-fly' and 'thermogenesis', which indicates that the daily rhythm of the shrimps was mainly influenced by external environmental cues, especially light and temperature. Circadian rhythms in crustaceans are driven by endogenous clocks accompanying changes in various environmental factors, such as light [35] and temperature [36]. Light is the strongest environmental factor for resetting the daily rhythm in crustaceans;

hence, many experiments that investigate the mechanism of circadian rhythms involve changing the length of the photoperiod [37, 38]. Temperature is another key factor affecting circadian rhythms in crustaceans. Temperature can affect the circadian rhythm of the red swamp crayfish, *Procambarus clarkii*, by modulating its immune response [39]. Crustaceans regulate their heart rate and locomotor activity according to the diurnal changes in external temperature [5]. This affects the thermal effect of the organism, which may be why thermogenesis was one of the most enriched pathways. However, more mechanistic studies are needed to reveal how these external environmental factors are involved in the circadian regulation of crustaceans, especially, shrimps.

Moreover, in the present study, circadian experiments were performed at 6-h intervals during a day. However, ideally, to observe the diurnal rhythm accurately, more than four timepoints (i.e., a higher resolution than 6-h intervals) should be considered. Thus, further explorations of the oscillations of clock system genes in various crustaceans are needed. However, in the present study, we have provided preliminary details regarding the transcriptome profile variations in a new model shrimp under circadian rhythms.

CONCLUSION

In the present study, we analyzed the hepatopancreas transcriptome profile of the Chinese swamp shrimp *N. denticulata* under a 24-h daily rhythm. This study is the first large-scale, comprehensive description of the gene expression of the hepatopancreas of the shrimps under a daily rhythm. The experimental design involved sampling animals from four time points within 24 h, which facilitated the identification of genes that are likely candidates most influenced by the daily rhythms of *N. denticulata*. Interestingly, numerous circadian-related unigenes were annotated, including *Per*, *Tim*, *Clk*, *Cyc*, *Cry*, and *5-HT*. The enrichment of DEGs showed that the GO terms with the highest enrichment level were related to sugar and chitin metabolism. The KEGG enrichment analysis showed that DEGs were mainly enriched in the pathways 'phototransduction-fly' and 'thermogenesis'. To the best of our knowledge, this is the first report on the transcriptome analysis of shrimps under a daily rhythm, providing molecular genetic evidence in the study of circadian rhythms in shrimps and other crustaceans.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.043 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA832034.

Acknowledgements: This work was supported by the Liaoning Province "The Open Competition Mechanism to Select the Best Candidates" Project (No. 2021JH1/10400040) and the earmarked fund for Modern Agro-Industry Technology Research System (Grant No. CARS-48). We thank Bio JuXing Aquatic Organism Co., Ltd., for providing the shrimps.

REFERENCES

1. Christie AE, Yu A, Pascual MG (2017) Circadian signaling in the Northern krill *Meganyctiphanes norvegica*: *In silico* prediction of the protein components of a putative clock system using a publicly accessible transcriptome. *Mar Genom* **37**, 97–113.
2. Yoshii T, Hermann-Luibl C, Helfrich-Frster C (2015) Circadian light-input pathways in Drosophila. *Commun Integr Biol* **9**, e1102805.
3. Ziegler TA, Cohen JH, Forward RB (2010) Proximate control of diel vertical migration in phyllosoma larvae of the caribbean spiny lobster *Panulirus argus*. *Biol Bull-US* **219**, 207–219.
4. Farnés OC, Forrellat-Barrios A, Guerrero-Galvan SR, Vega-Villasante F (2007) A review of digestive enzyme activity in penaeid shrimps. *Crustaceana* **80**, 257–275.
5. She Q, Han ZB, Liang SD, Xu WB, Li X, Zhao YY, Wei H, Dong J, et al (2019) Impacts of circadian rhythm and melatonin on the specific activities of immune and antioxidant enzymes of the Chinese mitten crab (*Eriocheir sinensis*). *Fish Shellfish Immun* **89**, 345–353.
6. Christie AE (2020) Identification of the molecular components of a putative *Jasus edwardsii* (Crustacea; Decapoda; Achelata) circadian signaling system. *Invertebr Neurosci* **20**, 1–13.
7. Yu CY, Li LS, Jin JX, Zhang BL, Wei H, Zhao YY, Li X, Li YD (2021) Comparative analysis of gut bacterial community composition during a single day cycle in Chinese mitten crab (*Eriocheir sinensis*). *Aquacult Rep* **21**, 100907.
8. Yu CY, Zhang BL, Zhang ZY, Wang SM, Wei TY, Li LS, Zhao YY, Wei H, et al (2022) Comparative transcriptome analysis reveals the impact of the daily rhythm on the hemolymph of the Chinese mitten crab (*Eriocheir sinensis*). *Chronobiol Int* **1**, 1–13.
9. Wang L, Yan B, Liu N, Li YQ, Wang Q (2008) Effects of cadmium on glutathione synthesis in hepatopancreas of freshwater crab, *Sinopotamon Yangtsekiense*. *Chemosphere* **74**, 51–56.
10. Xu D, Wu J, Sun L, Qin X, Fan X, Zheng X (2021) Combined stress of acute cold exposure and waterless duration at low temperature induces mortality of shrimp *Litopenaeus vannamei* through injuring antioxidative and immunological response in hepatopancreas tissue. *J Therm Biol* **100**, 103080.
11. Sanchez-Paz A, Garcia-Carreno F, Hernandez-Lopez J, Muhlia-Almazan A, Yepiz-Plascencia G (2007) Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). *J Exp Mar Biol Ecol* **340**, 184–193.
12. Yu CY, Zhang BL, Hu N, Li LS, Wang SM, Zhang ZY (2022) Comparative metabolomic analysis of Chinese mitten crab (*Eriocheir sinensis*) during the daily cycle. *Aquac Res* **53**, 2947–2958.
13. Yang J-S, Dai Z-M, Yang F, Yang W-J (2006) Molecular cloning of Clock cDNA from the prawn. *Brain Res* **1067**, 13–24.
14. Sbragaglia V, Lamanna F, Mat A, Rotllant G, Joly S, Ketmaier V, de La Iglesia HO, Aguzzi J (2015) Identification, characterization, and diel pattern of expression of

canonical clock genes in *Nephrops norvegicus* (Crustacea: Decapoda) eyestalk. *PLoS One* **10**, e0141893.
15. Hunt BJ, Özkaya Ö, Davies NJ, Gaten E, Seear P, Kyriacou CP, Tarling G, Rosato E (2017) The *Euphausia superba* transcriptome database, Superba SE: An online, open resource for researchers. *Ecol Evol* **7**, 6060–6077.
16. Christie AE, Yu A, Pascual MG, Roncalli V, Cieslak MC, Warner AN, Lameyer TJ, Stanhope ME, et al (2018) Circadian signaling in *Homarus americanus*: region-specific *de novo* assembled transcriptomes show that both the brain and eyestalk ganglia possess the molecular components of a putative clock system. *Mar Genom* **40**, 25–44.
17. Nathan K, Yung S, Xin S, Zhe Q, Wang W, Chan T, Stephen T, Sebastian S, et al (2014) Genomic sequence and experimental tractability of a new decapod shrimp model, *Neocaridina denticulata*. *Mar Drugs* **12**, 1419–1437.
18. Sin YW, Kenny NJ, Qu Z, Chan KW, Chan KW, Cheong SP, Leung RW, Chan TF, et al (2015) Identification of putative ecdysteroid and juvenile hormone pathway genes in the shrimp *Neocaridina denticulata*. *Gen Comp Endocr* **214**, 167–176.
19. Allada R, Emery P, Takahashi JS, Rosbash M (2001) Stopping time: The genetics of fly and mouse circadian clocks. *Annu Rev Neurosci* **24**, 1091–1119.
20. Rothenfluh A, Young MW, Saez L (2000) A timeless-independent function for period proteins in the Drosophila clock. *Neuron* **26**, 505–514.
21. Young MW (2001) Time zones: a comparative genetics of circadian clocks. *Nat Rev Genet* **2**, 702–715.
22. Shafer OT, Rosbash M, Truman JW (2002) Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of *Drosophila melanogaster*. *J Neurosci* **22**, 5946–5954.
23. Lowrey PL, Takahashi JS (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genom Hum G* **5**, 407–441.
24. Staff PO (2018) Correction: identification, characterization, and diel pattern of expression of canonical clock genes in *Nephrops norvegicus* (crustacea: decapoda) eyestalk. *PLoS One* **13**, e0200981.
25. Aréchiga H, Rodríguez-Sosa L (1998) Circadian clock function in isolated eyestalk tissue of crayfish. *Proc R Soc London Ser B* **265**, 1819–1823.
26. Escamilla-Chimal EG, Velazquez-Amado RM, Fiordelisio T, Fanjul-Moles ML (2010) Putative pacemakers of crayfish show clock proteins interlocked with circadian oscillations. *J Exp Biol* **213**, 3723–3733.
27. Li YD, Han ZB, She QX, Zhao YY, Wei H, Dong J, Xu WB, Li X, et al (2019) Comparative transcriptome analysis provides insights into the molecular basis of circadian cycle regulation in *Eriocheir sinensis*. *Gene* **694**, 42–49.
28. Mantel LH (2012) *Internal Anatomy and Physiological Regulation*, Elsevier, New York.
29. Chiaki N, Shinji N, Hiromichi N (2010) Effects of crustacean hyperglycemic hormone (CHH) on the transcript expression of carbohydrate metabolism-related enzyme genes in the kuruma prawn, *Marsupenaeus japonicus*. *Front Endocrinol* **172**, 293–304.
30. Mathew S, Nair A, Anandan R, Nair NV, Devadasan K (2007) Biochemical studies on changes associated with enzymes of glucose metabolism in white spot syndrome virus (WSSV) infected with *Penaeus monodon* (Fabricius). *Afr J Biotechnol* **6**, 1944–1948.
31. Tian Z, Kang X, Mu S (2012) The molt stages and the hepatopancreas contents of lipids, glycogen and selected inorganic elements during the molt cycle of the Chinese mitten crab *Eriocheir sinensis*. *Fish Sci* **78**, 67–74.
32. Wang XD, Li EC, Xu C, Qin JG, Wang SF, Chen XF, Cai Y, Chen K, et al (2014) Growth, body composition, ammonia tolerance and hepatopancreas histology of white shrimp *Litopenaeus vannamei* fed diets containing different carbohydrate sources at low salinity. *Aquac Res* **47**, 1932–1943.
33. Stevenson JR, Hettick BP (2010) Metabolism of chitin precursors by crayfish tissues during chitin synthesis. *J Exp Zool Part A* **214**, 37–48.
34. Techa S, Chung JS (2015) Ecdysteroids regulate the levels of molt-inhibiting hormone (MIH) expression in the blue crab, *Callinectes sapidus*. *PLoS One* **10**, e0117278.
35. Maciel FE, Geihs MA, Vargas MA, Cruz BP, Ramos BP, Vakkuri O, Meyer-Rochow VB, Nery L, et al (2008) Daily variation of melatonin content in the optic lobes of the crab *Neohelice granulata*. *Comp Biochem Phys A* **149**, 162–166.
36. Aréchiga H, Fernéndez-Quiróz F, de Miguel FF, Rodríguez-Sosa L (1993) The circadian system of crustaceans. *Chronobiol Int* **10**, 1–19.
37. Fuentes-Pardo B, Ramos-Carvajal J (1983) The phase response curve of electroretinographic circadian rhythm of crayfish. *Comp Biochem Phys A* **74**, 711–714.
38. Darnell ZM, Daniel R (2012) Ecological physiology of the circadian pigmentation rhythm in the fiddler crab Uca panacea. *J Exp Mar Biol Ecol* **391**, 112–117.
39. Dong CH, Bai SH, Du LQ (2015) Temperature regulates circadian rhythms of immune responses in red swamp crayfish *Procambarus clarkii*. *Fish Shellfish Immun* **45**, 641–647.

Appendix A. Supplementary data

Table S1 Primers for qRT-PCR.

gene ID	Primers
β-actin-F	CGAGTGGCCCCCGAGGAGTC
β-actin-R	TCGCAGGATGGCATGAGGGAGAG
Clk-F	AAGCACTTGCTCTTCCAGGT
Clk-R	TGGCGGTGAGCAAAAATCAC
Cry-F	GCGAAGTGCATCATTAGCCG
Cry-R	ACACCACTCACTTGTTCCTCT
Per-F	AAGGCCCTATAGGAGTGCGT
Per-R	AGCAGTCCGGCTCTAATGTA
Tim-F	AGAAGATCCCCCTCTGGTCC
Tim-R	AGACGCATTGTCCGACTTGT

Table S2 The raw data of transcriptomic sequences.

Sample	Reads No.	Bases (bp)	Q30 (bp)	N (%)	Q20 (%)	Q30 (%)
A6_1	45663650	6849547500	6439331997	0.012303	97.88	94.01
A6_2	45083090	6762463500	6329017400	0.015188	97.7	93.59
B12_1	44919242	6737886300	6294700238	0.012383	97.64	93.42
B12_2	44241954	6636293100	6235295298	0.013106	97.85	93.95
B12_3	44026784	6604017600	6218076797	0.012352	97.93	94.15
C18_1	44027164	6604074600	6223812790	0.015068	97.95	94.24
C18_2	44617976	6692696400	6293680657	0.012712	97.89	94.03
C18_3	45140914	6771137100	6374907567	0.012856	97.95	94.14
D24_1	43524386	6528657900	6136320654	0.012437	97.85	93.99
D24_2	45558404	6833760600	6388333489	0.014868	97.62	93.48

Table S3 The statistical data of clean reads.

Sample	Clean reads No.	Clean data (bp)	Clean reads %	Clean data %
A6_1	42,467,670	6,370,150,500	93.00	93.00
A6_2	41,828,970	6,274,345,500	92.78	92.78
B12_1	41,556,384	6,233,457,600	92.51	92.51
B12_2	40,970,092	6,145,513,800	92.60	92.60
B12_3	40,549,944	6,082,491,600	92.10	92.10
C18_1	40,493,198	6,073,979,700	91.97	91.97
C18_2	41,066,498	6,159,974,700	92.04	92.04
C18_3	41,662,030	6,249,304,500	92.29	92.29
D24_1	40,268,272	6,040,240,800	92.51	92.51
D24_2	42,074,012	6,311,101,800	92.35	92.35

Table S4 The summary information of transcriptomic sequences.

	Transcript	Unigene
Total length (bp)	266,748,128	82,691,490
Sequence number	184,887	75,762
Max length (bp)	35,642	35,642
Mean length (bp)	1,443	1,091
N50 (bp)	2,761	2,054
N50 sequence No.	27,632	10,376
N90 (bp)	508	407
N90 sequence No.	115,791	51,970
GC %	38	38

Table S5 The annotation statistics results.

Database	Number	Percentage (%)
NR	26506	34.99
GO	15484	20.44
KEGG	11598	15.31
Pfam	15949	21.05
eggNOG	22342	29.49
Swissprot	16401	21.65
In all database	8133	10.73

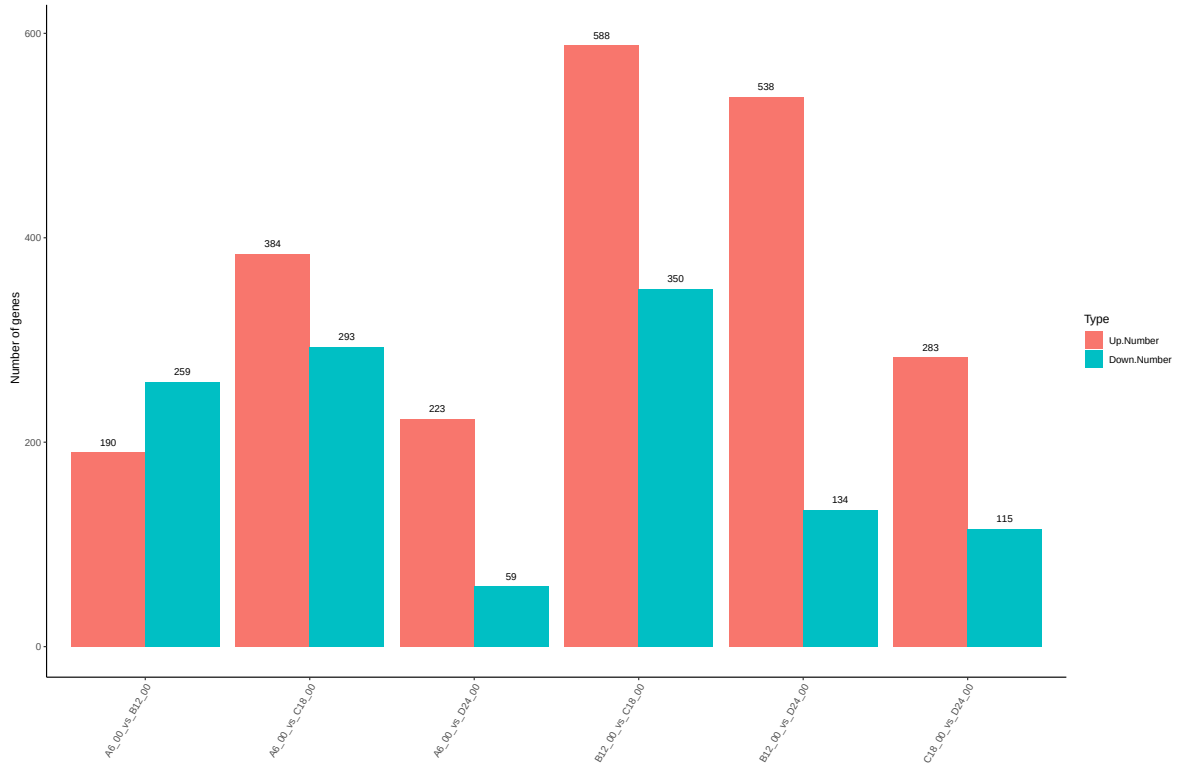

Fig. S1 Number of differentially expression genes (DEGs). The horizontal axis represents the pairwise comparison of different time groups, and the vertical axis represents the number of DEGs identified in each comparison group.

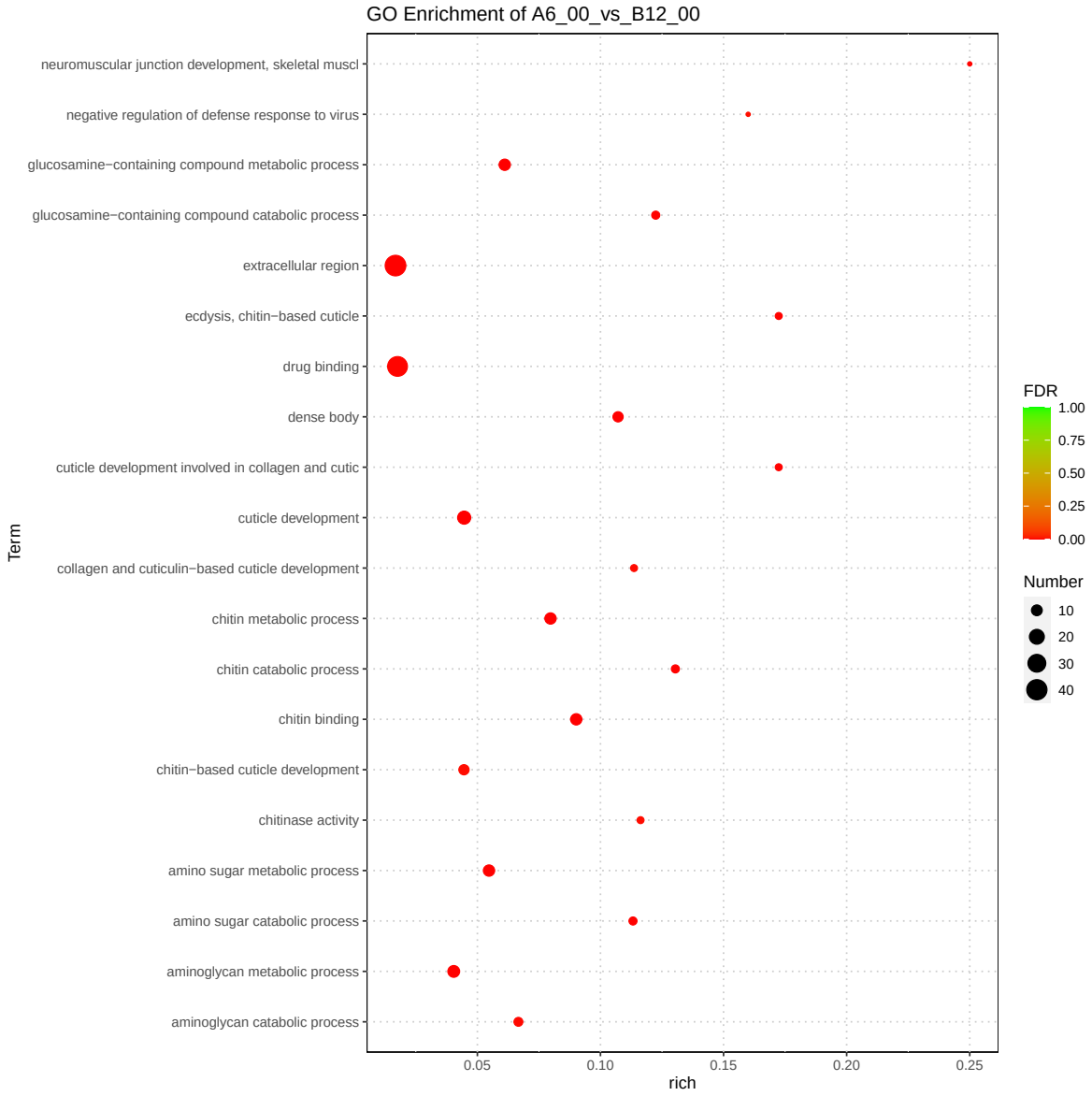

Fig. S2 GO enrichment analysis between 06:00 and 12:00.

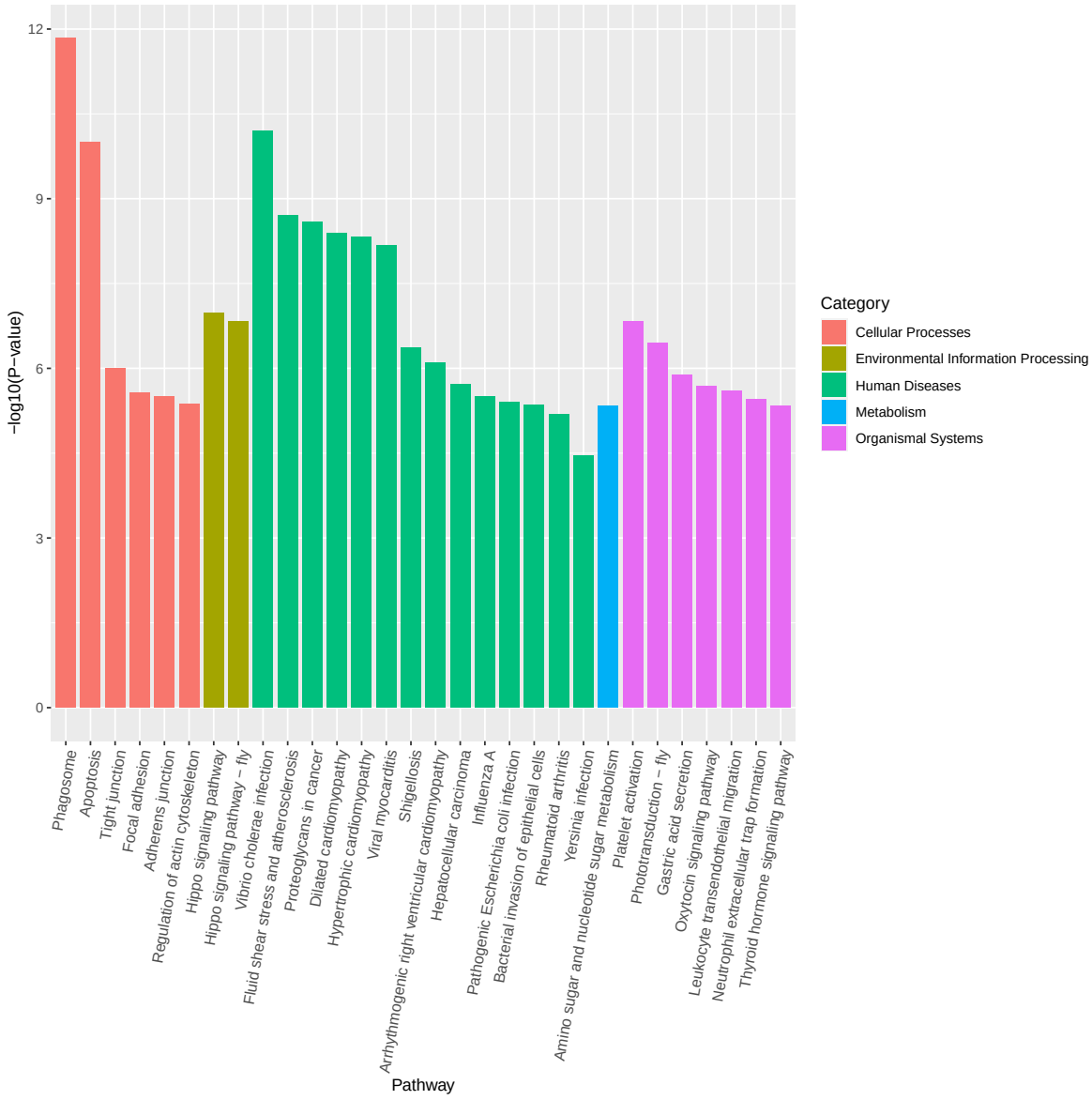

Fig. S3 KEGG enrichment analysis between 06:00 and 12:00.

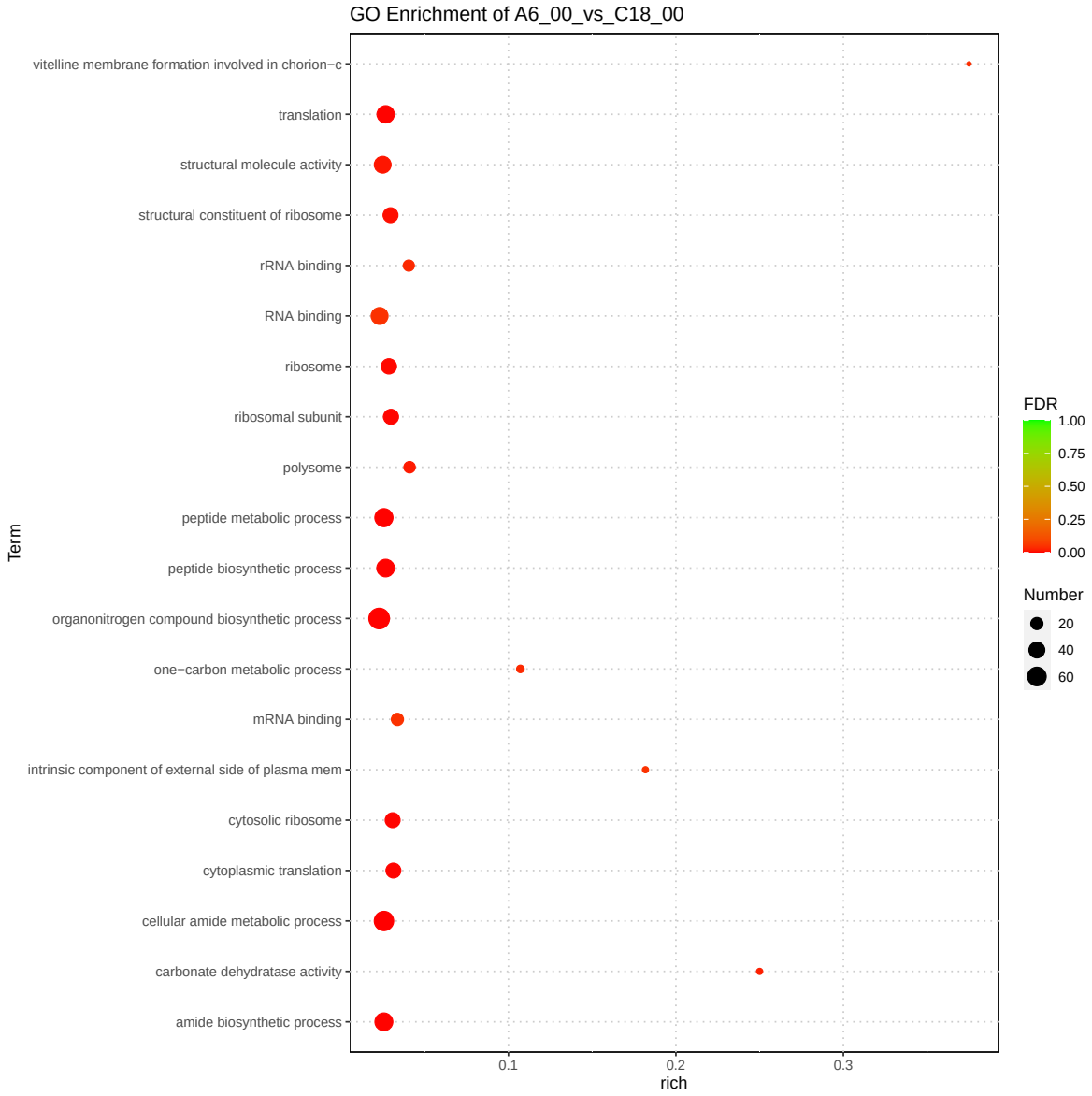

Fig. S4 GO enrichment analysis between 06:00 and 18:00.

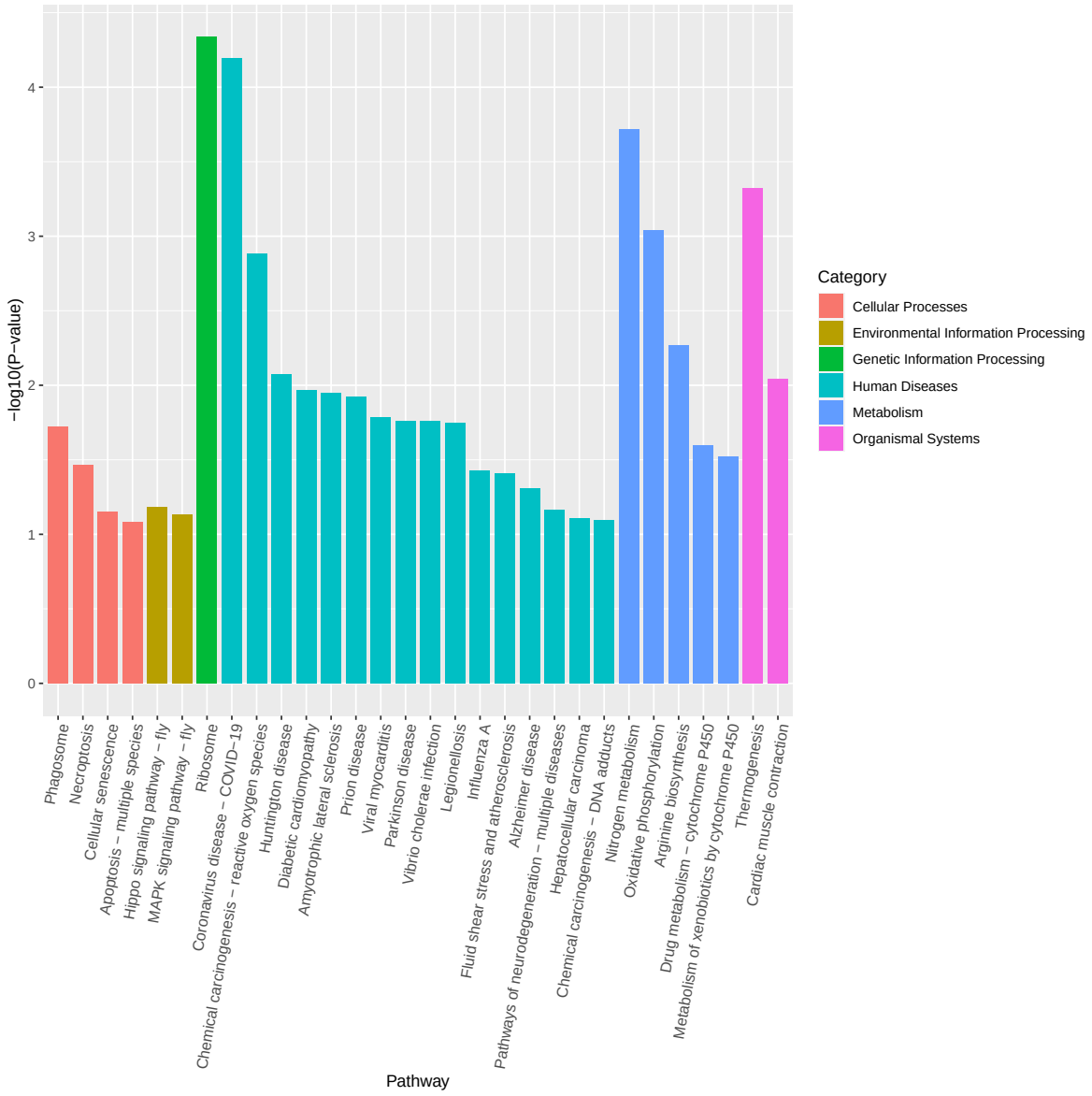

Fig. S5 KEGG enrichment analysis between 06:00 and 18:00.

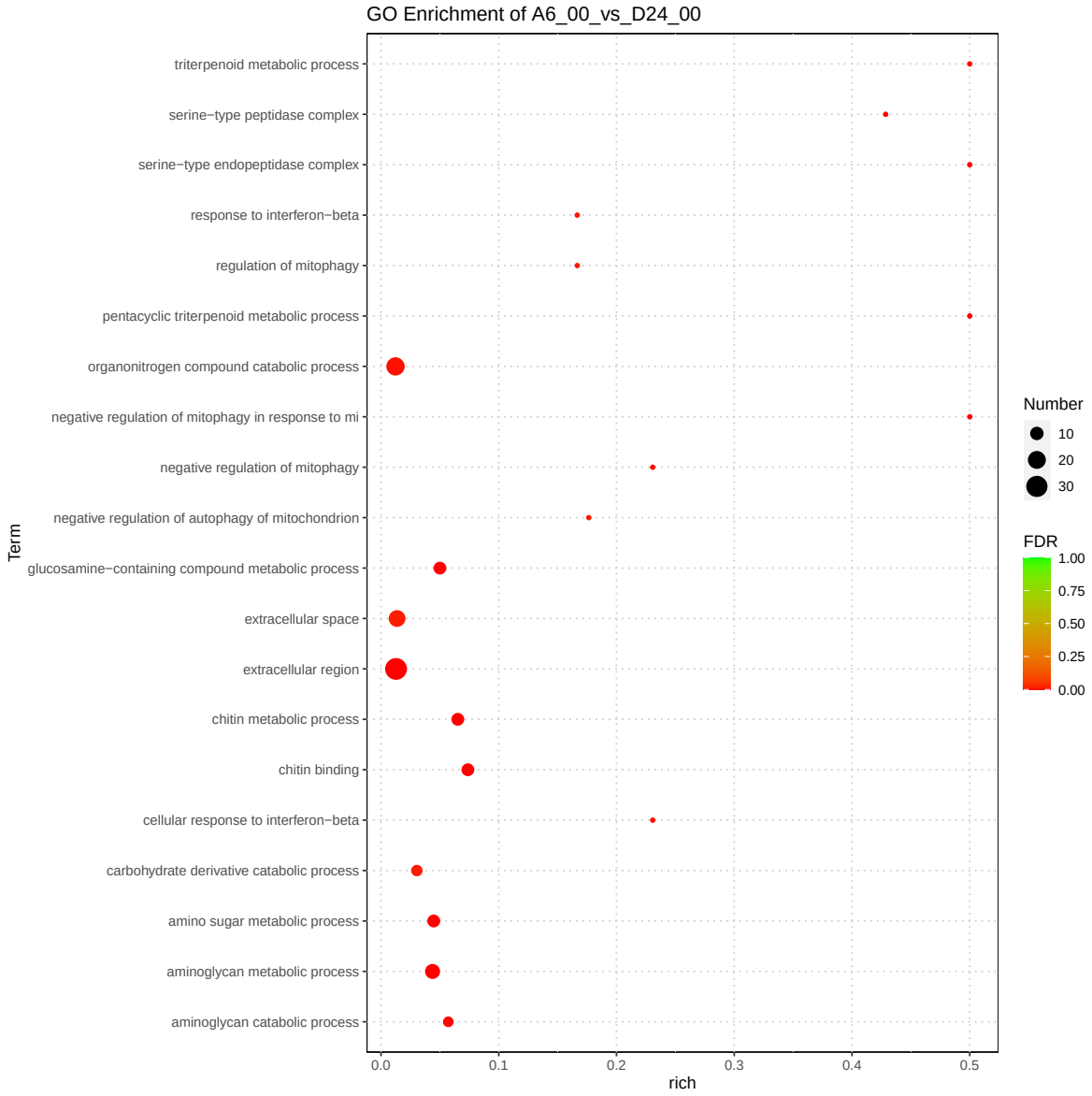

Fig. S6 GO enrichment analysis between 06:00 and 24:00.

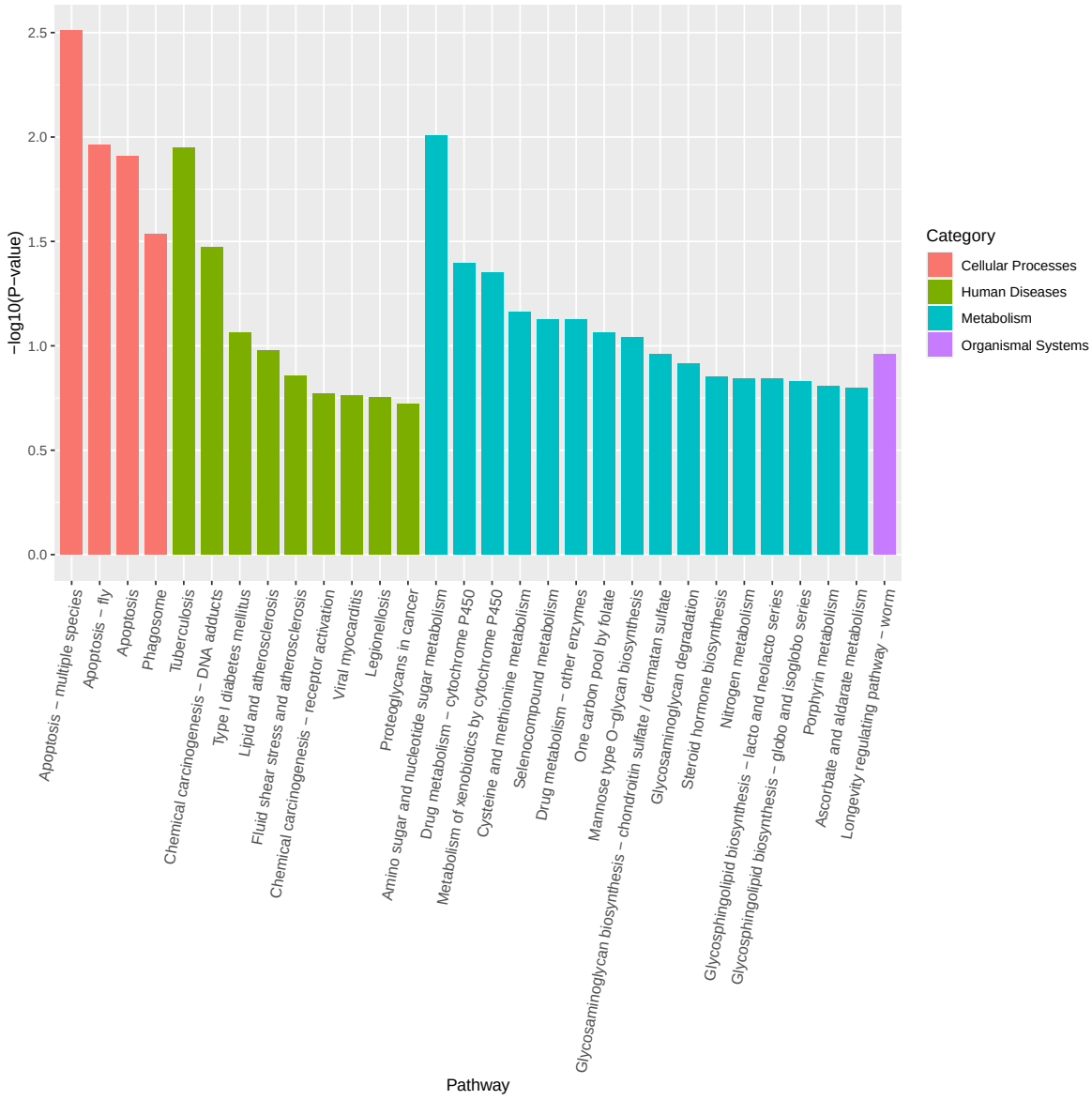

Fig. S7 KEGG enrichment analysis between 06:00 and 24:00.