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Research Article

Effects of Light and Feeding on Expressions of Clock Genes and Metabolic Genes in the Ridgetail Shrimp, *Exopalaemon carinicauda*

Yongshuai Wang, Yang Bo, Jun Lu, and Yongjian Xu

School of Marine Sciences, Key Lab of Applied Marine Biotechnology of MOE, Ningbo University, Ningbo 315832, China

Correspondence should be addressed to Yongjian Xu; xuyongjian@nbu.edu.cn

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The changes of the circadian clock genes expression, growth, and muscle composition in *Exopalaemon carinicauda* under different photoperiods and feeding time were investigated by real-time fluorescence quantitative PCR and muscle biochemical composition analysis, respectively. Results showed that four clock genes of *Clock*, *Cry1*, *Tim*, and *Per2* showed significant circadian rhythms in both eyestalk and hepatopancreas. Their expression rhythms were highly correlated with the photoperiod in the eyestalk, but in the hepatopancreas, it was significantly correlated with the feeding time. Moreover, the expressions of several metabolic genes were also variable; adipose triglyceride lipase (*ATGL*) and amyloglucosidase (*AGL*) changed significantly with the shift of feeding time, whereas methionine synthase (*MS*) and succinate dehydrogenase (*SDH*) changed significantly with the photoperiod. This reflected the close relationship between the metabolic and circadian clock in *E. carinicauda*; Alterations in photoperiod or feeding time lead to changes in the biological clock systems, which affects the daily expression of metabolic genes at the downstream metabolic pathway and closely correlates their expression rhythms with epi-environmental factors such as feeding. It can be seen from the results that the feeding was also a significant factor that affected the crude protein and crude fat contents in muscle tissue of *E. carinicauda*; their highest values ($18.69\% \pm 0.13\%$, $1.81\% \pm 0.02\%$) were also occurred in the treatment with feeding at 19:00. Differences in muscle composition through a similar mechanism of action, i.e., the biological clock system are affected by the feeding and then regulated changes in downstream metabolic processes. As a result, the optimized conditions for the cultivation of *E. carinicauda* were a short light-phase photoperiod (8L : 16D) with feeding after sunset (19:00).

1. Introduction

Almost all organisms have a precise circadian clock system [1]. This periodic oscillation is synchronized with changes in environmental factors, giving them an adaptive advantage and better survival in nature [2]. The system has great importance for their physiology, behavior, and metabolism [3–5], what is disrupted always produces a variety of physical problems, including metabolic disorders, behavioral impairments, etc. [6, 7]. In the circadian clock system, biological rhythms are generated by regular oscillations of the pacemaker, which bases its activity on the rhythmic expression or repression of clock genes. The clock system typically maintains normal rhythms through a negative feedback regulatory loop that regulates the transcriptional activity of individual clock genes. The expression or repression of clock genes can modulate the oscillatory pattern of the pacemaker [8, 9]. The circadian clock system is divided into central and peripheral clock systems, the former's main pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and the latter is located in various organs all over the body, such as the hepatopancreas, kidney, heart, muscle, etc. [10], and there is a close connection between the two systems.

The change in environment always leads to a change in clock rhythm, and the desynchronization between the two systems is believed to lead to unpreferable conditions such as metabolic disorders [11]. In crustaceans, a variety of endogenous and exogenous factors, such as temperature, light, and food, can all regulate the transcription levels of clock genes [12, 13]. In mammals, fish, and some crustaceans, some studies proved that light and feeding were the main entrainment factors of central and peripheral pacemakers, respectively [14–16]. Light can reset the time of the central pacemaker,

Table 1: Primer pair sequence and annealing temperature (T_m) for real-time qPCR.

Name	Sequence (5′-3′)	T_m (°C)
Clock genes		
Clock	F:CTCGGTGACCCACAGTCAAAR: ACCTTGGTTGTGGTGAGACG	58
Cry1	F:GACTTGCCTCCTTTACCTR: TGTCAAATCCGAGTTCCT	56
Tim	F:CCTAAAGAGGAGGAGTCAGR: CACATTCAGAGCCAGTCG	58
Per2	F:GCGGAAAGCGAGCAAGAAATR: TACTACCACGAGCAGACCCA	58
Metabolic genes		
MS	F:AGCGATAGCGGATAGATTGGR: GGTCAGGTTGGCTTGGATAG	56
ATGL	F:ATGTATGGTTGGCTAATGCGR: TCCCAGAATGCCTCTTCACTT	58
AGL	F:TTTTGGCTTCAGTCTATTCR: ATTTCTTCCAGGTTCTTGT	57
SDH	F:TCTGTGGCTCCTGTTCAAR: GTCCATCCTCCCTCTGTAA	53
β-Actin	F:AACTTTCAACACCCCAGCCAR: TCTCCAGAGTCCAGCACGAT	58

while the feeding signals entrained hepatopancreas clocks rapidly. Most known genes with rhythmic expression can encode enzymes or regulatory proteins involved in food processing and metabolism, such as the 7α-hydroxylase ([17] the rate-limiting enzyme in bile acid synthesis), the serine dehydratase, which is involved in protein and amino acid metabolism, and transcription factors involved in fat metabolism, PPARα, and spot 14 [18], are all under stringent circadian control, proved that there is a close relationship between biological clock system and metabolism system [19]. Therefore, the aim of this study was to forcibly change the expression phase of the hepatic clock by altering the feeding time to synchronize it with the central clock, which may reduce the occurrence of diseases such as metabolic disorders and have important implications for promoting practical production.

The ridgetail shrimp (*Exopalaemon carinicauda*), widely distributed in coastal waters of China, is one of the Chinese native shrimps with high economic value and has diurnal and nocturnal habits. As an emerging pond culture species, its aquaculture sustainability is severely limited by the feeding technology, most of which still extends and relies on the farming experience of other shrimp species, and its circadian clock-related research is even more weak. Based on the above background, this study investigated the expression rhythms of circadian clock genes and some metabolic genes in the eyestalk and hepatopancreas of *E. carinicauda* with quantitative polymerase chain reaction (qPCR), evaluated the synchrony changes of two circadian clock systems under different feeding pattern combinations, and conducted the muscle composition, aiming to fill the gap in research on biological clock and find a feeding time and photoperiod suitable for synchronization of the biological clock system to refine the feeding strategy of *E. carinicauda*.

2. Materials and Methods

2.1. Shrimp Sources and Sampling. The ridgetail shrimp (body length 3.91 ± 0.28 cm, body weight 0.74 ± 0.09 g) was provided from a farming earth pond in Sanmen, Zhejiang Province, China, and cultured temporarily under laboratory conditions for 1 week. During this period, fed once a day, and maintained aeration and water circulation to ensure the water quality. Healthy and vigorous individuals were selected for the follow-up experiment.

2.2. Experimental Design and Breeding Management. The experiment included two tests. The purpose of the first test was to find the appropriate condition of photoperiod, which included two treatments (G1 and G2). In G1, the photoperiod was 12L : 12D with a light phase from 6:00 to 18:00. The photoperiod of G2 was 8L : 16D with a light phase from 9:00 to 17:00. Both treatments had the same feeding time at 19 o′clock. The other test was to find the appropriate condition of feeding time, which included six treatments (F5, F10, F14, F19, F22, and F2) corresponding to the six feeding time (5, 10, 14, 19, 22, and 2 o′clock, respectively) under 8L : 16D. Each treatment had three replicates.

More than 1,200 shrimps (average weight: 0.74 ± 0.09 g) were average and randomly assigned to 24 square glass aquaria (length × width × height: 55 × 35 × 50 cm, average number: 50, condition: pH 7.0–9.0, dissolved oxygen > 4 mg/L, nitrite nitrogen < 0.15 mg/L, and ammonia nitrogen < 0.5 mg/L) and then put them into six light incubators (temperature of 25 ± 1°C, salinity of 23 ± 1, light intensity of 1,000 lux). Exchanged the saltwater about 50% every day, fed once a day, and maintained aeration (Tianbang brand white shrimp 1# feed, feed particle size : diameter 1 mm × length 1 cm, tank side feeding).

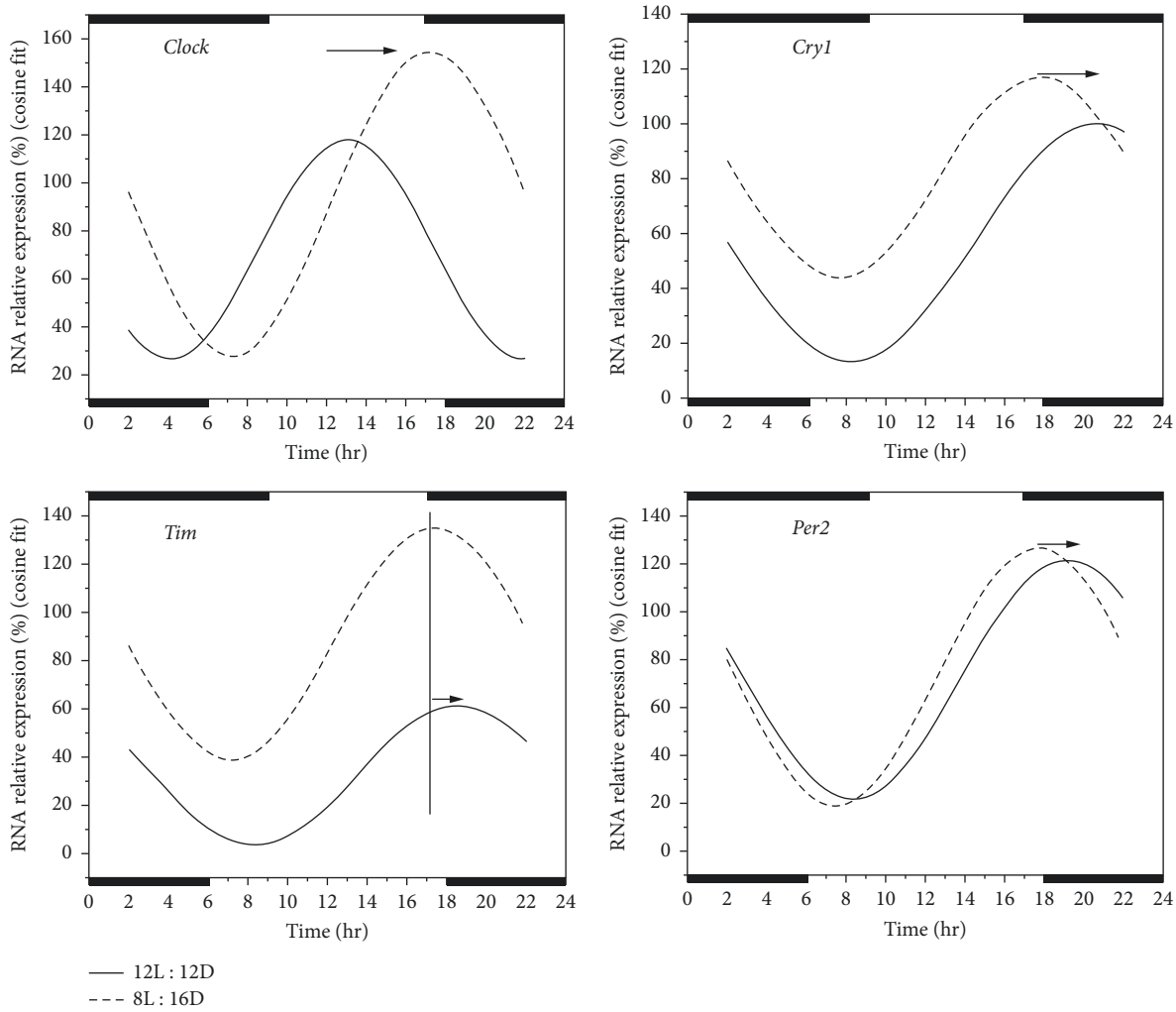

Figure 1: The cosine fit curve of four clock gene expressions in the eyestalk under two photoperiods of 12L : 12D and 8L : 16D. Based on the expression curve fitted by Acro software, the existence of cosine wave indicates that there is a significant rhythm. Black thick line indicates dark night.

Both tests lasted 30 days. Stopped feeding 1 day before sampling, and the sampling starting point in each treatment was specified at 6:00, then sampled every 4 hr (the samples were marked as ZT6, ZT10, ZT14, ZT18, ZT22, and ZT2, respectively). Six shrimps (mixed two individuals per sample × three repetition samples) were sampled each time and killed with the excessive anesthetic. Their eyestalk and hepatopancreas tissues were collected and stored in liquid nitrogen for 5 min and then transferred to a −80°C refrigerator for subsequent analysis. Another five shrimps were randomly selected from each aquarium to measure their body length and body weight before and after the test. Then, the crude protein was determined by the Kjeldahl method (Foss, Denmark). Crude fat was determined by Soxhlet extraction, and ash was determined by combustion in the MAF furnace method [20]. The water content was determined by the normal pressure drying method.

2.3. RNA Extraction and cDNA Synthesis. Utilizing the Trizol technique for RNA extraction. The samples were taken out from the refrigerator, thawed with the ice bath, added RNAiso Plus solution (9108Q, Takara), and transferred the supernatant after centrifugation to a new centrifuge tube and added chloroform; then, centrifuged again to take out the upper layer of colorless solution and placed it in another new tube. Added an equal volume of isopropanol and mixed evenly for precipitating RNA. After centrifugation, the white precipitate of RNA would be attained. After being washed twice with ethanol, the RNA precipitate was incorporated into the DEPC water finally. A spectrophotometer was used to measure its absorbance values at 260 and 280 nm wavelengths, and the purity and integrity of the total RNA of the sample were checked by 1% agarose electrophoresis. After meeting the quality requirements, its cDNA was synthesized with an appropriate amount of total RNA, gDNA remover, random primers, and RNase-free water, according to the instructions of the TRANS reverse transcription kit (TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (one-step gDNA removal)). The obtained cDNA was diluted eight times for performing a qPCR.

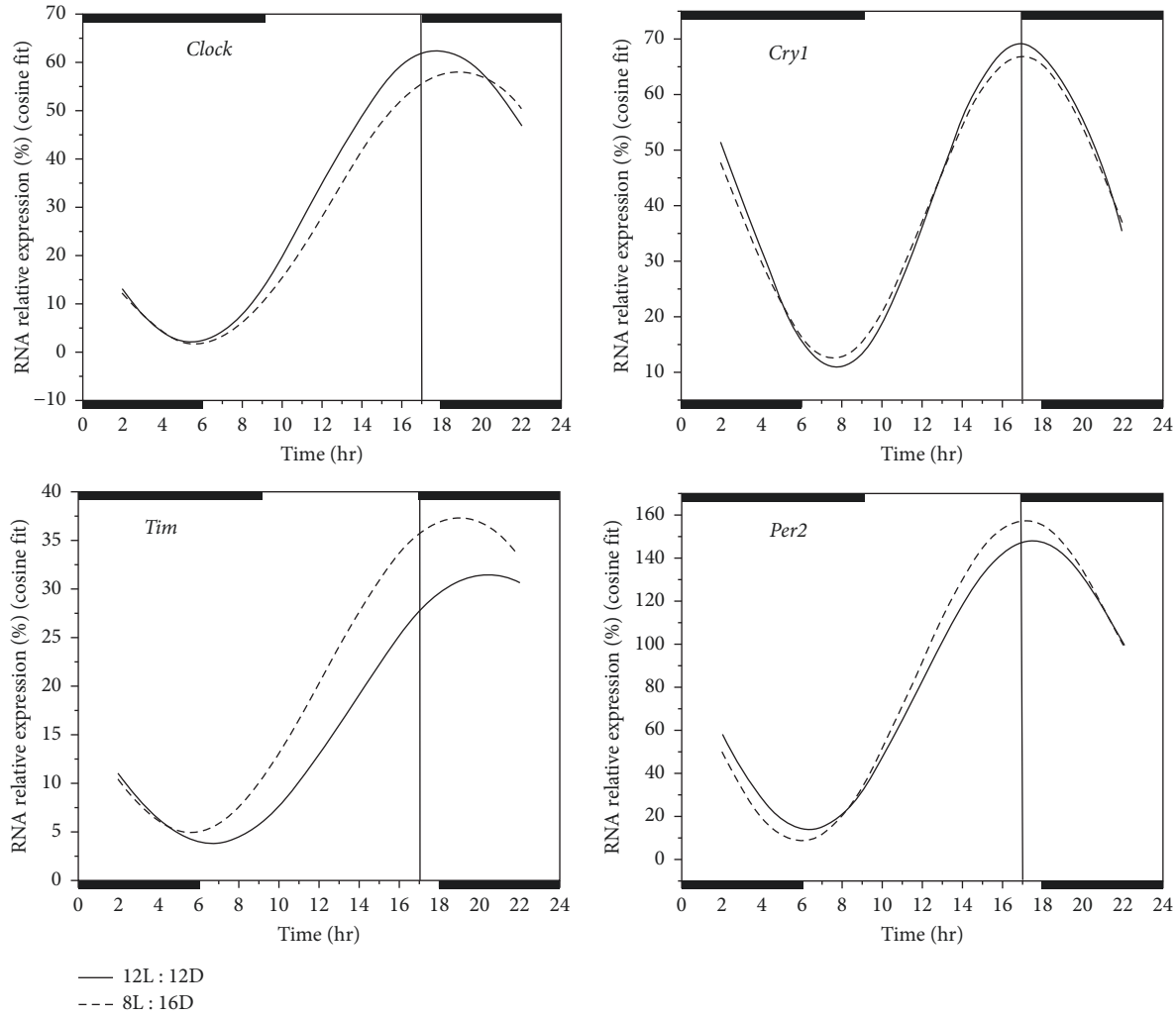

FIGURE 2: The cosine fit curve of clock gene expressions in the hepatopancreas under two photoperiods of 12L : 12D and 8L : 16D. Based on the expression curve fitted by Acro software, the existence of cosine wave indicates that there is a significant rhythm. Black thick line indicates dark night.

2.4. Real-Time qPCR. Four clock genes (*Clock*, *Cry1*, *Tim*, *Per2*) and metabolic genes (*MS*, *ATGL*, *AGL*, *SDH*) sequences had been screened and obtained in this experiment. Transcriptome sequencing was used to obtain all of the gene sequences included in this study, which are now available in the NCBI database with the accession number PRJNA887190.

The NCBI's blast sequence alignment service was used to determine all gene identities, and the Primer Premier 5 software was used to design their specific primers (Table 1). Diluted part of cDNA into a concentration gradient to evaluate the amplification efficiency of primers for each gene to determine whether or not the amplification efficiency was >85%. Real-time qPCR was performed on the Eppendorf Mastercycler Realplex, using a 2x SYBR Green qPCR Master Mix kit (US Everbright RT mix with DNase). The reaction system of qPCR was: 2x SYBR Green Master Mix 10 μl, forward and reverse primers 1 μl each, cDNA 1.5 μl, and RNase-free water 6.5 μl. The qPCR reaction conditions were 2 min at 95°C, 40 cycles: 10 s at 95°C, 20 s at TM, and 25 s at 72°C. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of the target gene.

2.5. Data Analysis. All treatments used the same control to facilitate comparative analysis of expression levels of each gene among treatments. The Acro circadian rhythm analysis program was used to analyze raw data to determine whether the expression of the target gene meets the cosine function and to fit the time point of the peak expression (acrophase) of the target gene through the cosine wave. Unidirectional analysis of variance was used to determine the statistical difference between different sampling times for each gene, and Tukey's test was used to determine the significance of the differences between sample time points and the average values of different sample sets. Growth performance and muscle composition data were statistically processed using SPSS version 26.0 software, and one-way analysis of variance (ANOVA) was performed. Duncan's test method was used to compare the significance of the difference between treatments,

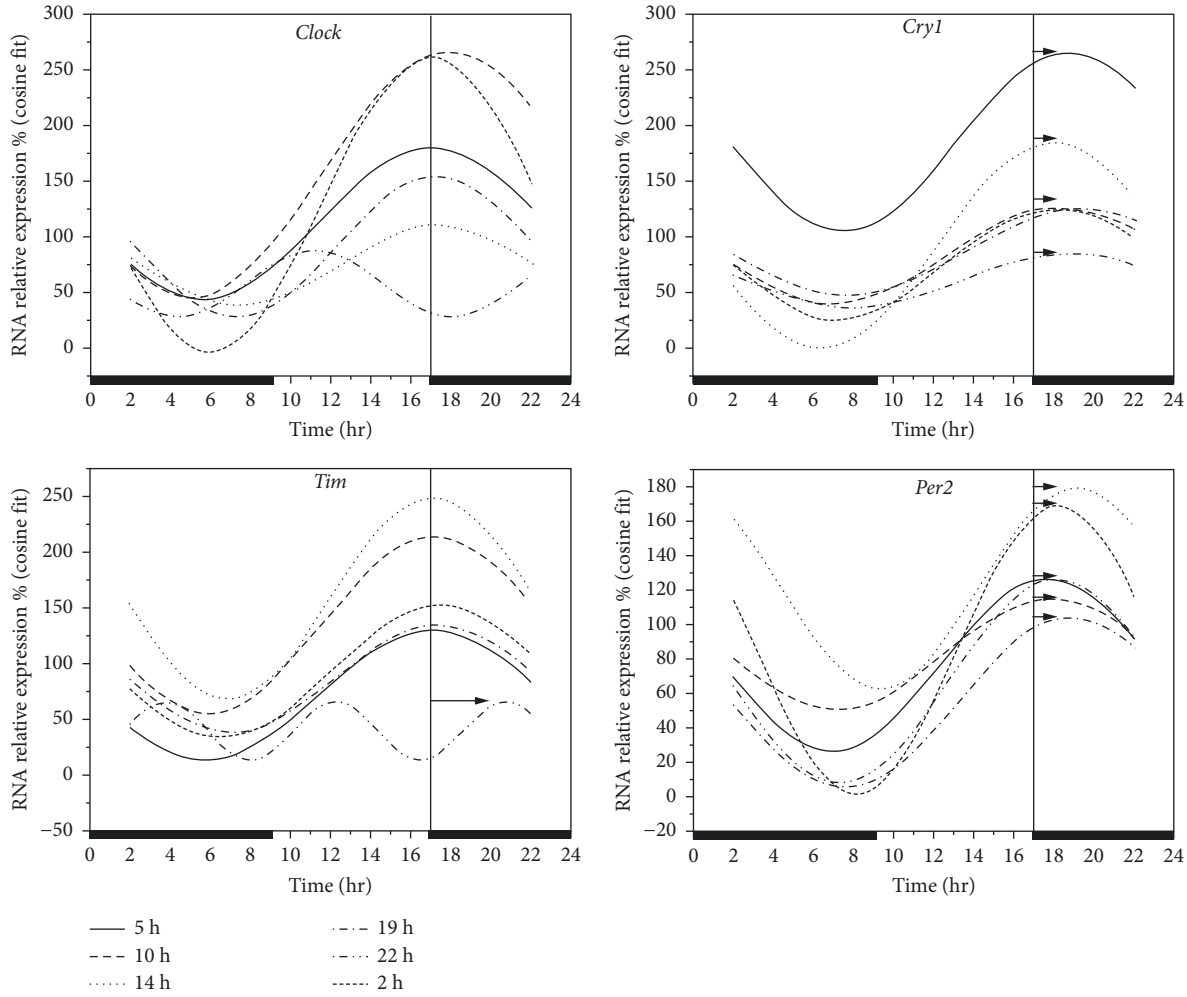

Figure 3: The cosine fit curve of clock gene expressions in the eyestalk at different feeding time. Based on the expression curve fitted by Acro software, the existence of cosine wave indicates that there is a significant rhythm. Black thick line indicates dark night.

with a significant level of $p < 0.05$. The experimental data were expressed as "mean ± standard deviation."

3. Results

3.1. Expressions of Clock Genes in Both Eyestalk and Hepatopancreas under Different Photoperiods. All four genes had a complete rhythm cycle within 24 hr, but their expression levels and acrophase were significantly different between the two photoperiods in the eyestalk (Figure 1). Besides *Per2*, the expression levels of the other three genes were significantly higher under 8L : 16D than 12L : 12D (Figure 1). Furthermore, all genes showed their acrophases, which appeared around ZT18 under 8L : 16D, but their acrophases changed under the photoperiod of 12L : 12D. Among them, *Clock* appeared in the middle of daytime (ZT13), while the other three genes showed their acrophases around ZT20 (Figure 1).

Similar results appeared in the hepatopancreas of *E. carinicauda*. The expression trends of all genes were almost identical between 8L : 16D and 12L : 12D; the acrophases of *Clock* and *Tim* appeared around ZT18 and *Cry1* and *Per2* were around ZT17. Moreover, the expression level of *Tim* significantly decreased under 12L : 12D than 8L : 16D ($p < 0.05$), but the differences in the other three genes were not significant in G1 and G2 (Figure 2). As shown in Figures 1 and 2, it can be seen that the factor of light affected the central clock system more than the peripheral clock system.

3.2. Expressions of Clock Genes in the Eyestalk and Hepatopancreas under Different Feeding Times. In the eyestalk of *E. carinicauda*, all four genes also had a complete rhythm cycle within 24 hr in each treatment of feeding time, except *Clock* and *Tim* in F22 treatment, which had more than one cycle (1.7 and 2.6 cycles within 24 hr, respectively). Their acrophases in all treatments were similar; *Clock* and *Tim* appeared their acrophases around ZT17 and *Cry1* and *Per2* were around ZT18 (Figure 3). However, there was a significant change in expression level; *Clock* had a highest value in the treatments of feeding time at 10:00 and 2:00 (F10 and F2), which was nearly three times higher than its lowest value appeared in F14 treatment. *Tim*, *Cry1*, and *Per2* had their highest values appeared in F14, F5, and F14, respectively (Figure 3). The gene expression level was obviously influenced by the factor of feeding in the eyestalk.

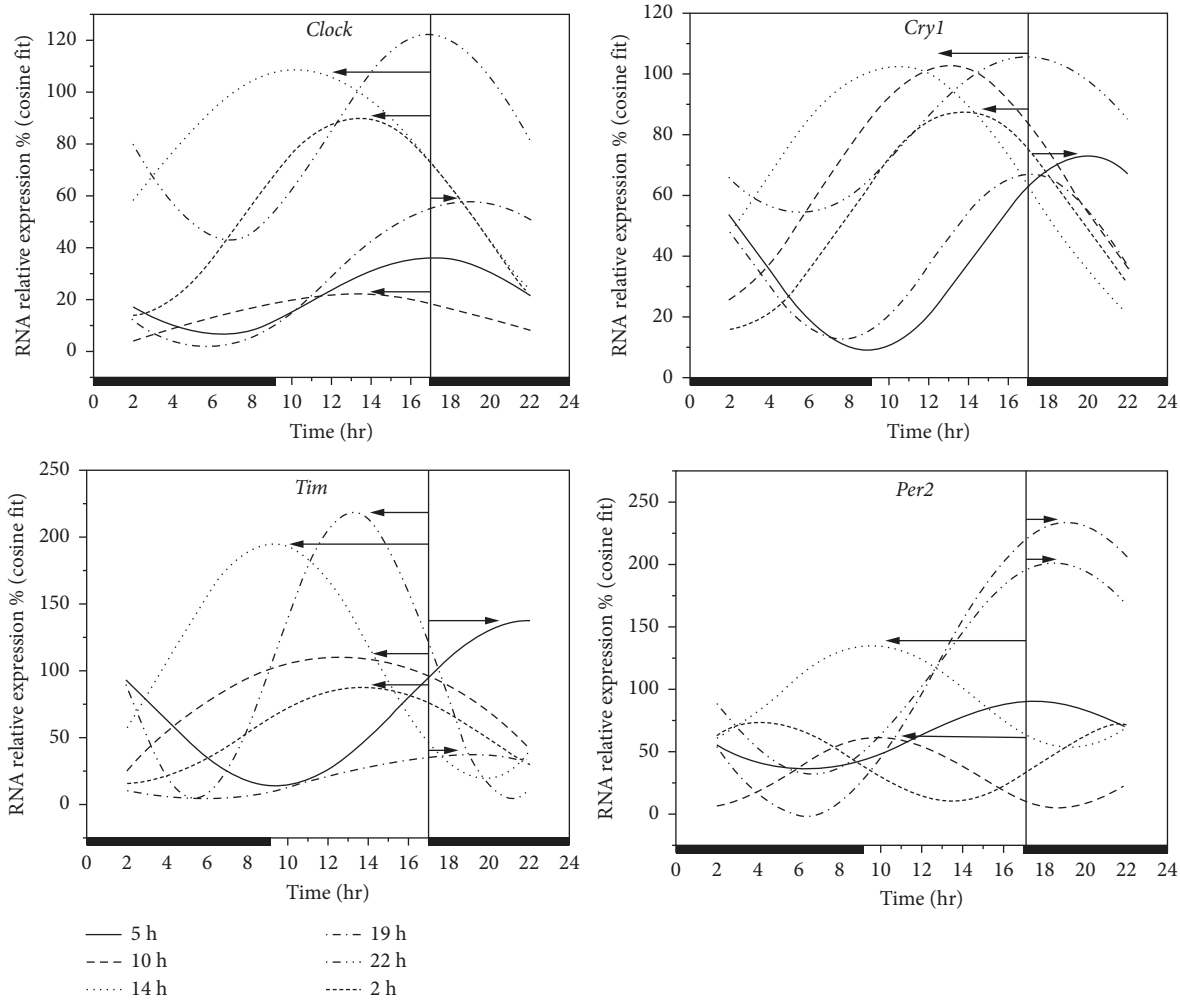

FIGURE 4: The cosine fit curve of clock gene expressions in the hepatopancreas at different feeding time. Based on the expression curve fitted by Acro software, the existence of cosine wave indicates that there is a significant rhythm. Black thick line indicates dark night.

In the hepatopancreas, the expressions of more genes were affected by the factor of feeding (Figure 4). First, the rhythm cycle of genes' expression in several treatments was not a whole cycle within 24 hr, like two genes (*Clock* and *Tim*) in the eyestalk. Their cycles were prolonged in F10 treatment, exceeding 24 hr. All four genes' cycles were slightly shortened (about 22 hr/cycle) in five of all treatments and significantly shortened (14–16 hr/cycle) in the other seven treatments. Second, there was a change of the acrophase. Compared to the eyestalk, the expression peak of each gene varied significantly in the hepatopancreas. *Clock* kept its peak at ZT17 only in P5 and P19 treatments, and no treatment with *Tim's* peak appeared at ZT17. *Cry1* in P19 and P22 peaked at ZT18, and *Per2* also peaked at ZT18 in P5, P19, and P22. Third, there were significant differences in the expression levels of each gene among six treatments with different feeding time. However, the highest expression of all genes occurred in P22 treatment, except for *Per2* in P19.

3.3. Expressions of Metabolic Genes in the Hepatopancreas of E. carinicauda. The expressions of metabolic genes in the hepatopancreas of *E. carinicauda* were also influenced by light and feeding (Figure 5).

MS showed completely opposite trends between the two photoperiods, as well as *SDH*, indicating that both genes were clearly regulated by light (Figure 5). Merely, the rhythm cycle of MS expression did not change (24 hr/cycle) in all treatments, and their acrophases appeared around ZT17-ZT19 under short light-phase photoperiod. Their maximum expression levels were relatively close among different treatments, with the highest value appearing in F10 treatment. *SDH*, on the other hand, showed a shortened rhythm cycle (18–20 hr/cycle) in most treatments, with their acrophases mostly occurring around ZT14 and the highest expression level appearing in F22 treatment. The expressions of *ATGL* and *AGL* were not affected by the photoperiod (no difference between G1 and G2, except for the maximum expression level), but their expressions were confused in all treatments of feeding time. Among them, the rhythm cycles of *ATGL* and *AGL* had slightly shortened (20–22 hr/cycle) in F5, F22, and F10 treatments, but *AGL* was extended to 26 hr/cycle in F22. Their acrophases also appeared variable in each treatment, and so did their highest experession levels (Figure 5).

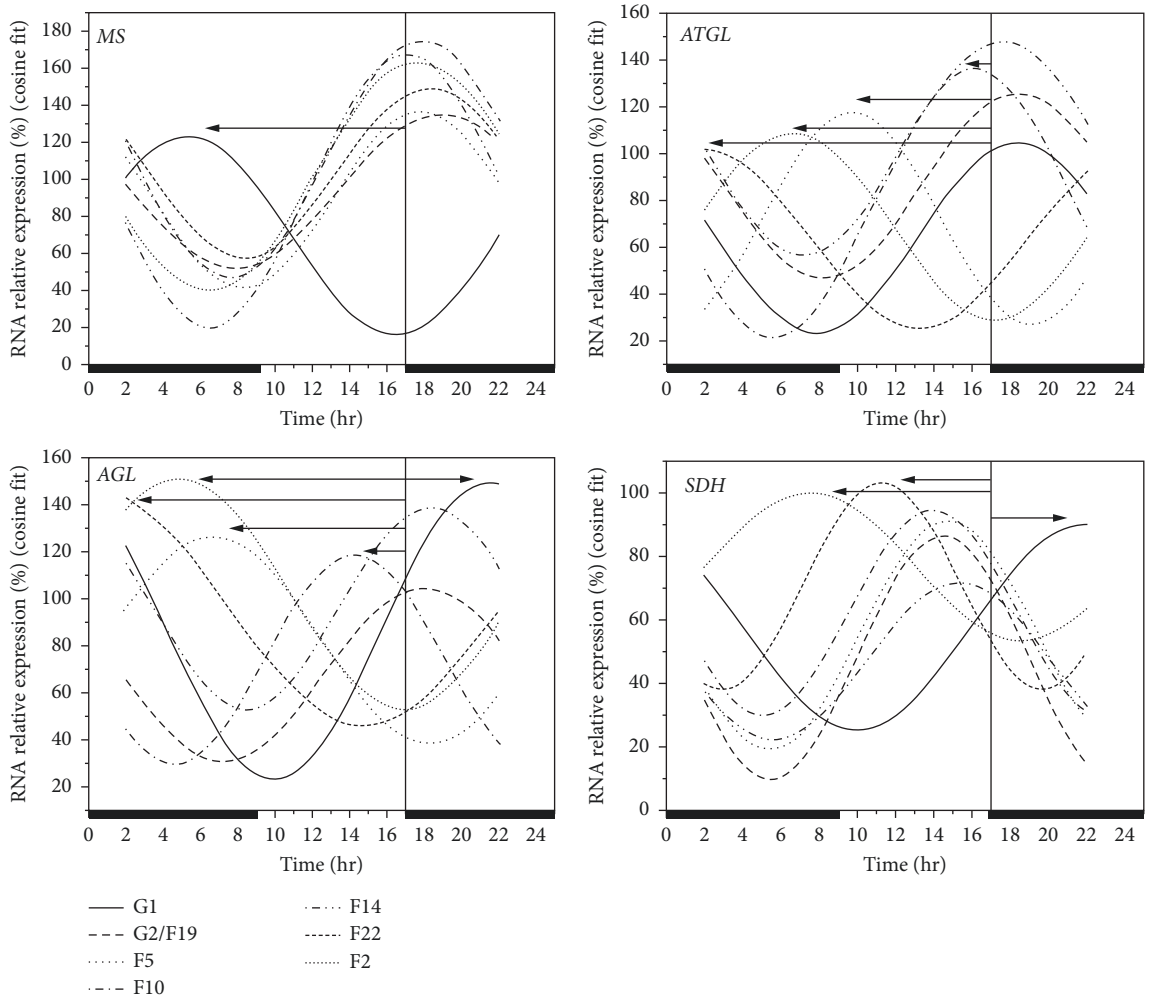

Figure 5: The cosine fit curve of metabolic gene expressions in the hepatopancreas in different treatments. Based on the expression curve fitted by Acro software, the existence of cosine wave indicates that there is a significant rhythm. Black thick line indicates dark night.

Table 2: Comparisons of the muscle composition of *E. carinicauda* in all treatments.

Treatment	Water (%)	Crude protein (%)	Crude fat (%)	Ash (%)
G1	77.28 ± 0.13	18.09 ± 0.18^{b}	1.73 ± 0.08^{b}	1.81 ± 0.09
F2	77.65 ± 0.07	17.82 ± 0.15^{bc}	1.73 ± 0.03^{b}	1.75 ± 0.08
F5	77.81 ± 0.16	18.19 ± 0.24^{b}	1.74 ± 0.03^{b}	1.83 ± 0.07
F10	77.54 ± 0.09	17.51 ± 0.16^{c}	1.72 ± 0.05^{b}	1.78 ± 0.18
F14	77.32 ± 0.09	17.63 ± 0.21^{c}	1.72 ± 0.05^{b}	1.82 ± 0.04
F19 (G2)	77.32 ± 0.15	18.51 ± 0.13^{a}	1.83 ± 0.03^{a}	1.78 ± 0.11
F22	77.31 ± 0.08	18.69 ± 0.13^{a}	1.83 ± 0.05^{a}	1.75 ± 0.11

Note: Different letters showed significant differences among treatments in each column ($p < 0.05$).

3.4. Effects of Light and Feeding on the Muscle Composition of E. carinicauda. The expressions of metabolic genes in the hepatopancreas of *E. carinicauda* were also influenced by light and feeding (Figure 5).

Effects of light and feeding on the muscle composition of *E. carinicauda* are shown in Table 2. The shrimp had an obviously higher content of crude protein and crude fat in its muscle in 8L : 16D than in 12L : 12D ($p < 0.05$), while the contents of water and ash were not different ($p > 0.05$). The factor of feeding also affects the muscle composition of *E. carinicauda*; the contents of crude protein and crude fat in the muscle had a higher value in both F19 and F22 treatments than in other treatments ($p < 0.05$), but the other composition was similar ($p > 0.05$). Interestingly, the protein content in shrimp muscle was lowest in the treatments that fed in the daytime (F10 and F14), slightly increased in the

treatments that fed before sunrise (F5), and the highest in the treatments that fed after sunset (F19). It showed a significant correlation with the feeding time and also with the light (light intensity).

4. Discussion

4.1. Effects of Light and Feeding on Expressions of Clock and Metabolic Genes in E. carinicauda. *E. carinicauda* has more vigor at night than in the daytime. Although its natural behavior of "diurnal and nocturnal" has been widely recognized, it is still the first time to clarify this natural habit through the circadian clock system. This study described the expressions of clock genes in the eyestalk and hepatopancreas of *E. carinicauda* and confirmed the existence of circadian rhythms in *E. carinicauda* at the molecular level.

In this study, all clock genes had good rhythmicities in the eyestalk of *E. carinicauda* and showed their obvious rhythm difference under different photoperiods, but their expressions did not change significantly with different feeding time. The same results were found in *Penaeus vannamei* [21] and *Lysmata amboinensis* [22], etc., indicated that the expressions of clock genes were driven by light in the eyestalk of shrimp. Similar results were also found in fish, such as Nile tilapia (*Oreochromis niloticus*) [23], marine teleost (*Sparus aurata*) [24], and marine medaka (*Oryzias melastigma*) [25]; the controlled, time-restricted, and planned feeding could change the daily rhythm of clock gene in the hepatopancreas, but did not change the rhythms in the brain, so that the brain central clock system of photoperiod entrainment was decoupled from the hepatopancreas peripheral clock system of food entrainment [26]. *E. carinicauda* had a similar clock response mechanism in the stalk and could be regulated by light but not by feeding. Feeding was a major factor in determining the phase of the peripheral circadian clock [27]. Continuous daytime-restricted feeding could completely change the circadian expression phases of clock genes in peripheral tissues of nocturnal rodents, while the central clock was not affected [28]. Similarly, the expressions of clock genes were significantly altered in the hepatopancreas of Nile tilapia, when changed the feeding time restrictively [23]. Clock genes were similarly strongly entrained by food signals in the hepatopancreas of Gilthead sea bream [15]. However, there were no reports about the effect of feeding on the circadian clock system in crustaceans, and this study was the first evaluation of clock gene expressions in the hepatopancreas of *E. carinicauda*.

Meanwhile, to evaluate whether the metabolic function was regulated by circadian rhythms in *E. carinicauda*, this study also analyzed the expressions of several metabolic genes. Both *ATGL* and *AGL* were changed significantly by feeding, while *MS* and *SDH* were changed significantly by light, indicated the close relationship between the metabolic system and the circadian clock system in *E. carinicauda*. Paredes et al. [15] reported that the circadian rhythms of key genes involved in lipid metabolism in the hepatopancreas of *Sparus aurata* were highly synchronous with light but independent of feeding. Other reports also said that the enzyme activities and endocrine system, which were related to lipid metabolism, could be changed by time-restricted feeding [29]. In crustaceans, the conclusion that carbohydrate metabolism was regulated by circadian rhythms had been previously confirmed in *Procambarus clarkii* [30] and *Antarctic krill* [31]. This study preliminarily confirmed that protein metabolism was regulated by the circadian rhythms and clock system in *E. carinicauda*.

4.2. Effects of Light and Feeding on the Muscle Composition of E. carinicauda. Protein was the most important nutritional component in animal growth, and weight gain was greatly affected by the content of protein [32]. In this study, the protein metabolism enzyme gene (MS) showed a strong circadian rhythm and a strong correlation with light. This suggested that the 8L : 16D treatment resulted in more active protein synthesis and accumulation, which, in turn, suggested faster shrimp growth. Further research is necessary to fully understand how a well-synchronized biological clock system controls metabolic efficiency.

The interaction between biological clock and energy metabolism indicated that feeding had a serious impact on the metabolism of *E. carinicauda* in this study. Several reports said that time-restricted feeding can inhibit obesity and metabolic diseases [33, 34] due to fine-tuning of the biological clock [34]. Much evidence supported the view that feeding affects metabolic status, which was consistent with the objective and results of this study. Although the mechanism underlying the effects of feeding on the metabolic rhythms was still unclear, some evidence remained very enlightening, such as the biological clock system could regulate the expression of the leptin gene in an organism, thereby controlling appetite [35].

5. Conclusion

In summary, light and feeding can regulate the peripheral clock system of *E. carinicauda* and changed its synchronization with the central clock system. The synchronization of the two clock systems had an important impact on the growth and muscle composition of *E. carinicauda*. The growth synchronization results tentatively confirmed the clock synchronization theory that the higher the synchronization between the central clock system and the peripheral clock system, the better the metabolic efficiency of the organism, the better the growth performance, and the better the muscle composition. It provided an important guiding significance for the aquaculture industry. As a result, the optimized conditions for the cultivation of *E. carinicauda* were also obtained: a short light-phase photoperiod (8L : 16D) with feeding after sunset (19:00).

Data Availability

The data are available from the corresponding author on reasonable request.

Ethical Approval

In this study, all animal experiments strictly followed protocols approved by the Animal Welfare Committee of Ningbo University and the study was carried out in strict accordance with the guidelines and regulations established by this committee.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

The authors contributed to all laboratory trials and data analyzing stages.

Acknowledgments

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