The Discovery of Circadian Rhythm of Feeding Time on Digestive Enzymes Activity and Their Gene Expression in *Sinonovacula constricta* Within a Light/Dark Cycle

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The circadian rhythm has a great impact on the growth, metabolism and development of animals, but little is known about the circadian rhythm of marine bivalves. Understanding of the feeding rhythm is of great significance to increase the yield of razor clam *Sinonovacula constricta*, an economically important bivalve mollusk. The aim of this experiment was to study the effects of circadian rhythm of feeding time on digestive enzymes activities and their gene expression in *S. constricta* within a light (ZT8-ZT20)/dark (ZT20-ZT8) cycle. The present results showed that circadian rhythm of feeding rate (FR) was highly associated with digestive enzyme activities and relative expression of their genes. The highest values of FR were basically observed in the night from ZT0-ZT2 and ZT6-ZT8, which were significantly higher than those values in the daytime from ZT12-ZT14 and ZT18-ZT20 (*P* < 0.05). The digestive enzymes activities displayed the highest values at ZT2 and ZT8, and the lowest at ZT14 and ZT20. Among them, cellulase and pepsin were found to have significantly different activities (*P* < 0.05), rather than amylase and lipase. Notably, the relative expression of digestive enzyme genes shared the similar pattern with the activities of digestive enzymes. The highest values of relative gene expression of amylase (AMY), lipase (LIP), cellulase (CEL), and pepsin (PEP) were found at ZT2 and ZT8 in the night, while the lowest values were found at ZT14 during the day. It is therefore suggested that the biological clock may regulate the process from feeding to digestion. Furthermore, it might be better to feed at night to reduce cultivating cost and increase economic benefits in the farming industry of *S. constricta*.

**Keywords:** *Sinonovacula constricta*, circadian rhythm, feeding rate, digestive enzyme, gene expression

**INTRODUCTION**

The circadian rhythm is a 24-h cyclical change in environmental factors such as light and temperature caused by the rotation of the earth, which has an important impact on the physiology and behavior of organisms (Fustin et al., 2013). The circadian rhythms inside and outside the organism are closely related to the rhythm changes of the external environment. When the rhythm...
of environmental factors is changed, the nervous system and endocrine system of animals will change accordingly, leading to changes in their behaviors, lifestyles, and physiological conditions (Wu et al., 2013). The feeding rhythm of animals affected by cyclical change factors (e.g., light and tide) is essential for the establishment of scientific feeding mode (Mistlberger, 1994; Sanchezvazquez, 1995; Wang, 2004; Connor and Gracey, 2011). Many activities such as feeding, oxygen consumption and digestion have been used to study rhythmic behavior in European oyster Ostrea edulis, New Zealand cockle Austravenus stutchburyi, clam Saxidomus purpuratus, etc (Morton, 1971; Williams and Pilditch, 1997; Kim et al., 2003). Meanwhile, the absorption of food after feeding is closely related to the process of digestion and metabolism. Digestive enzyme activity is an important indicator of digestion and absorption capacity, which determines the ability to digest and absorb nutrients for food (Bobrowska et al., 2011; Wu et al., 2013). The relationship between the activity of digestive enzymes and the feeding pattern has been studied in cockle Cerastoderma edule (Ibarrola et al., 1998), clam Ruditapes decussatus and Venerupis pullastra (Albentosa and Moyano, 2008), and scallop Patinopecten yessoensis (Li et al., 2010). In addition, some previous studies have found that the activities of digestive enzymes such as pepsin, amylase, cellulase, and lipase are served as indicators that can measure the digestion and absorption of protein and carbohydrates in bivalves (Supannapong et al., 2008; Tizon et al., 2013). For animals, digestion is a complex process that requires signal transduction regulation inside and outside the body. The enzyme-related protein precursor mRNA is regulated to stimulate transcription and synthesis after ingestion, so that the enzyme precursors are released to promote digestion, absorption, and growth (Yúfera et al., 2018). For example, amylase (AMY) gene is proved to be involved in the growth of razor clam Sinonovacula constricta and Pacific oyster Crassostrea gigas, while cellulase (CEL) can improve the digestibility of food and synthesize glucose to provide energy for the body (Meenu et al., 2014; Thongsai klaing et al., 2014; Rong et al., 2015; Liu et al., 2017). Moreover, lipase (LIP) gene expression is correlated with feeding status, whereas pepsin (PEP) has important digestive functions in both vertebrates and invertebrates (Li et al., 2018). The presence of various forms of pepsin precursors such as pepsin A and pepsin C may be related to food or feeding habits (Carginale et al., 2004).

S. constricta is an economically and ecologically important benthic marine bivalve, which naturally distributes along the western Pacific coasts of China, Japan, and South Korea (Morton, 2010). It has been widely cultivated in the intertidal zone and estuary waters with more than 400 years of cultivating history in the Zhejiang and Fujian provinces, China (Shen et al., 2013). Due to relatively short production cycle and high productive efficiency, it has been become an important marine aquaculture species in China with 852,925 tons of production in 2018 (FAO, 2020). Food intake is one of the important conditions for increasing the yield of industrial aquaculture (Rønnestad et al., 2013). Good ingestion and efficient digestion of nutrients are necessary to meet the high demand of matter and energy, which are able to support the high-growth rate (Navarro-Guillén et al., 2018). In order to determine the optimal feeding time and increase the growth rate of S. constricta, we continuously measured feeding rate, digestive enzyme activity, and relative expression of digestive enzyme genes at different time points in the day and night. The present results will be beneficial for reducing farming cost and increasing farming benefits.

**MATERIALS AND METHODS**

**Experimental Animals**

S. constricta were obtained from Ningbo Ocean and Fishery Science and Technology Innovation Base (Ningbo, Zhejiang province). One-year-old adults (n = 60) with an average shell length of (5.9 ± 0.3) cm were collected to culture for a week in seawater within light and dark (L/D) cycles. The lights in the experimental environment were turned on, and shoot them directly into the tank from ZT (Zeitgeber Time) 8-ZT20 to simulate the daytime, and from ZT20-ZT8 to cover the tank with a black cloth to simulate the night. All clams were not fed before the start of the experiment. During the formal experiment, the clams were fed with the live microalgae of Chaetoceros muilleri with the concentration of (2.5 ± 0.2) × 10⁵ cell/L. The water temperature and salinity were maintained at (25.0 ± 2) °C and (20 ± 1) ppt, respectively. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang Wanli University, China.

**Feeding Rate Experiment**

The clams in similar size were randomly distributed into 12 tanks, having five individuals in each tank. The time setting was to divide 24 h into four time periods, including ZT0-ZT2, ZT6-ZT8, ZT12-ZT14, and ZT18-ZT20. During each time point, directly into the tank from ZT (Zeitgeber Time) 8-ZT20 to simulate the daytime, and from ZT20-ZT8 to cover the tank with a black cloth to simulate the night. All clams were not fed before the start of the experiment. During the formal experiment, the clams were fed with the live microalgae of Chaetoceros muilleri with the concentration of (2.5 ± 0.2) × 10⁵ cell/L. The water temperature and salinity were maintained at (25.0 ± 2) °C and (20 ± 1) ppt, respectively. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang Wanli University, China.

Before and after each time point, water samples of the experimental groups and control groups were sampled to count the concentration of algae on a blood cell counter. The feeding rate (FR) was calculated according to the following formula:

\[
FR = \frac{V(C_{td}-C_{id})}{NT} \times (Riisgård, 1991).
\]

Where \( C_{id} \) and \( C_{td} \): the algae concentration in the control group and experimental group at the end of the experiment (cells/L); \( T \): the experimental time (h); \( V \): the volume of the experimental water (L); \( N \): the number of experimental clam; \( S_d \): the control bait variation coefficient.

**Analysis of Digestive Enzyme Activities**

A total of 240 clams with similar sizes were randomly distributed into three tanks (group A, group B, and group C). Samples were obtained at 6 h intervals (ZT2, ZT8, ZT14, and ZT20) after...
feeding, having continuous sampling for 3 days. Four individuals were randomly selected from each group, and the visceral mass tissue was dissected and immediately frozen in liquid nitrogen, and stored at −80°C.

The enzyme extracts were prepared to measure the enzyme activities of α-amylase, lipase, cellulase, and pepsin. Each sample was weighed at 100 mg, which was mixed with 0.9 mL of normal saline for mechanically homogenization using an automatic sample rapid grinding machine. The samples were centrifuged at 3,500 rpm for 10 min at 4°C. All samples were kept in ice in order to avoid enzymes denaturation or damage. Enzyme extracts were kept at −20°C until analysis within 24 h.

All enzymatic activity analysis was conducted by using the commercial kits from Nanjing Jiancheng Bioengineering Institute. α-amylase activity as protein per mg of visceral mass reacted with the substrate at 37°C for 30 min to hydrolyze 10 mg amylon and recorded at OD 560 nm. The unit of lipase activity was per g tissue protein reacted with methyl resorufin substrate at 37°C and recorded at OD 580nm. The cellulase activity as tissue per g catalyzed to 1 µg glucose per minute and recorded at OD 550nm. One unit of pepsin activity was defined as tissue protein per mg decomposed into 1 µg amino acid at 37°C per minute and recorded at OD 660nm. The protein concentration extracted from the tissue uses the BSA kit. The enzyme activity of α-amylase, lipase, cellulase, and pepsin were all measured according to the standard protocol.

RNA Extraction and Real-Time Quantitative Expression Analysis of Digestive Enzyme Genes

The total RNA was extracted from the visceral mass by using the Trizol reagent following the manufacturer's instructions. The RNA quality was tested by electrophoresis, and the concentration was measured with a nucleic acid detector NanoVue Plus. Total RNA was reverse transcribed to cDNA by RT-PCR kits.

The whole predicted coding sequence (CDS) sequences of AMP, LPS, CEL, and PEP were obtained from the genome of S. constricta (WSYO00000000.1). The primer sequences were designed following the CDS sequences and listed in Table 1. The mRNA expression levels per each gene were assessed by qRT-PCR using ChamQ SYBR qPCR Master Mix. The 20 µL reaction volume for amplification contained 10 µL of SYBR qPCR Master Mix, 1 µL of each primer (10 µM), and 8 µL of cDNA sample (10 ng/µL). Initial denaturation was conducted at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. 18S rRNA gene was selected as the housekeeping gene, and the expression levels of AMP, LPS, CEL, and PEP gene were normalized to that of 18S rRNA by using the 2−ΔΔCT method.

![Figure 1](https://www.roche.com/)

**Statistical Analysis**

Data were presented as the means ± standard deviation, and one-way ANOVA analysis were used to compare the difference in food intake ratio, digestive enzyme activity and digestive enzyme gene expression at different time points in the day and night. GraphPad prism 8 software was used to statistically analysis. P < 0.05 was considered statistically as the significant difference, and P < 0.01 as the extremely significant difference.

### RESULTS

**Analysis of Diurnal Difference of Feeding Rate**

Under the condition of 12 h light/12 h dark (L/D) cycles, the food intake rates at ZT0-ZT2 and ZT6-ZT8 during the night were higher than those at ZT12-ZT14 and ZT18-ZT20 during the day, indicating an obvious circadian rhythm of feeding (Figure 1). For the first day at ZT0-ZT2 and ZT18-ZT20, the food intake rates were 2.15 × 10^8 (cells/h) and 1.225 × 10^8 (cells/h). For the second day at ZT6-ZT8 and ZT12-ZT14, the food intake rates were 2.03 × 10^8 (cells/h) and 0.85 × 10^8 (cells/h). For the third day at ZT6-ZT8 and ZT18-ZT20, the food intake rates were 2.33 × 10^8 (cells/h) and 1.38 × 10^8 (cells/h). In total, the highest and lowest

**TABLE 1** Primer sequences used for qRT-PCR analysis.

| Primers | Sequence (5′-3′) | Length (bp) |
|---------|-----------------|-------------|
| AMY-F   | ACCATCGTCACCGTTTC | 201         |
| AMY-R   | CAACAAGGTCGGTCGTC | 223         |
| LPS-F   | AGAGTCGGCAAGTGGTGTTG | 201         |
| LPS-R   | ATGCTGGGGCAGCTGCTG | 139         |
| CEL-F   | TGAGAGTGGTGGAAAGGAA | 207         |
| CEL-R   | TGTTGTCGGGAAGTGGTGCC | 139         |
| PEP-F   | ACCCGTCTCCACCATT | 180         |
| PEP-R   | GCCTGTGATTGGAGCAGAT | 180         |
| 18S-F   | TCGGTTTCATGGCCTGGTGT | 180         |
| 18S-R   | CAGTTGCGATCGTATGGTCA | 180         |

*References:*

1. https://www.roche.com/
2. http://www.njjcbio.com/
3. https://www.thermofisher.cn/
4. https://www.sangon.com/
5. https://www.takarabiomed.com.cn/
6. http://vazyme.bioon.com.cn/
TABLE 2 | Results of one-way ANOVA analysis of feeding rate under the diurnal cycle.

| Feeding rate | 1 days | 2 days | 3 days |
|--------------|--------|--------|--------|
|              | MS     | df     | F      | P      |
| Between ZT   | 0.639  | 3      | 13.50  | 0.0017** |
| Within ZT    | 0.0473 | 8      | 0.221  | 0.083  |
|              | 1.093  | 3      | 49.47  | <0.0001** |
|              | 0.561  | 3      | 6.795  | 0.0137* |
|              | 0.083  | 8      |        |        |

Asterisks indicate significant differences: *P < 0.05 and **P < 0.01.

FIGURE 2 | Analysis of four digestive enzyme activities in 12 h light/12 h dark (L/D) cycles during 3 days. (A) Lipase activity diagram. (B) α-amylase activity diagram. (C) Cellulase activity diagram. (D) Pepsin activity diagram. Values are represented as means ± SD (n = 4). Group A, B, C are three parallel tanks with the same cultivating conditions, and the red circle represents Group A, the blue square represents Group B and black triangle represents Group C. The gray grid represents the dark time point.

Ingestion points were significantly different in the experimental period by one-way ANOVA analysis (P < 0.05), with 1.69–2.39 times between them (Table 2).

The Circadian Rhythm of Four Digestive Enzyme Activities

The results of α-amylase, lipase, cellulase, and pepsin activities (expressed as U mg⁻¹) were shown in Figure 2 and Table 3. These four enzymes have a diurnal variation trend in group A, B, C. The activities of lipase reached the highest value at ZT20 and ZT2, followed by low levels of activity between ZT8 and ZT14. Among all these four enzymes, the activities of lipase were significantly lower than those of other enzymes (P < 0.05). However, only part of the results detected significant differences between the highest and lowest points of lipase activities (Table 3 and Figure 2A). For α-amylase, the maximum values of enzyme activities were generally detected at ZT20 and ZT2, while the minimum values were found at ZT8 and ZT14. Nevertheless, α-amylase activities during the whole day and night cycle were statistically different between time periods, but it was only partially present (Table 3 and Figure 2B). As indicated, cellulase and pepsin had the highest activities, displaying the same changing trend (P < 0.05, Table 3 and Figures 2C,D).

Basically, the enzyme activities were found to be higher in the dark (ZT20-ZT8) than those in the light (ZT8-ZT20).

Relative Expression of Four Digestive Enzyme Genes

The results of relative expression of digestive enzyme genes were shown in Figure 3. The relative expression of AMY, LPS, CEL, and PEP had a regular trend from high to low levels from ZT2 to ZT20 of 3 days, with no significant change in diurnal fluctuations. The relative expression of LPS had the highest expression level at ZT2 and ZT8, the lowest expression at ZT14 (Figure 3A). Meanwhile, AMY was detected to be the highest at ZT2 and the lowest at ZT14 (Figure 3B). Similarly, the gene expression of CEL was found to be the highest at ZT2 and the lowest at ZT14 (Figure 3C). Consistently, PEP showed the highest expression level at ZT2 and the lowest expression at ZT14 and ZT20 (Figure 3D).

DISCUSSION

For most organisms, circadian rhythm is affected by endogenous factors and external environmental factors...
TABLE 3 | Results of one-way ANOVA analysis of digestive enzyme activity under the diurnal cycle.

| Digestive enzyme | Group | 1 days | 2 days | 3 days |
|------------------|-------|--------|--------|--------|
|                  |       | MS     | F      | P      | MS     | F      | P      | MS     | F      | P      |
| α-amylase        | A     | 0.0362/0.0081 | 4.448  | 0.0406* | 0.0296/0.0052 | 5.63  | 0.0226* | 0.0101/0.0145 | 0.7008 | 0.5776 |
|                  | B     | 0.0147/0.0040 | 3.72  | 0.061   | 0.0096/0.0138 | 6.786 | 0.0137* | 0.0036/0.0053 | 7.252  | 0.0114* |
|                  | C     | 0.00074/0.0013 | 5.671  | 0.022*  | 0.0053/0.0093 | 3.543 | 0.0677 | 0.0017/0.0002 | 0.5499 | 0.6622 |
| Lipase           | A     | 12.32/1.308     | 9.417  | 0.050** | 16.66/5.527     | 3.015 | 0.0942 | 5.648/1.961    | 2.88   | 0.1029 |
|                  | B     | 4.54/2.417      | 1.878  | 0.216   | 5.692/5.07      | 1.123 | 0.396  | 51.1/5.248     | 9.738  | 0.0048** |
|                  | C     | 2.339/6.736     | 0.347  | 0.792   | 3.217/0.5558    | 9.042 | 0.006* | 18.51/1.838    | 10.07  | 0.0043** |
| Cellulase        | A     | 31.74/1.791     | 17.72  | 0.0007** | 8.832/3.394     | 2.602 | 0.1243 | 38.26/0.9671   | 39.56  | 0.0001** |
|                  | B     | 5.283/2.831     | 1.866  | 0.2137  | 14.45/3.256     | 4.438 | 0.0406* | 29.01/2.822    | 10.28  | 0.004**  |
|                  | C     | 48.92/5.099     | 19.49  | 0.0005* | 4.717/2.27      | 2.078 | 0.1816 | 9.341/0.928    | 1.07   | 0.0043** |
| Pepsin           | A     | 1.589/0.4292    | 3.701  | 0.0616  | 5.721/0.2232    | 25.63 | 0.0002** | 1.953/0.2985   | 6.545  | 0.0151*  |
|                  | B     | 0.3937/0.0769   | 5.122  | 0.0288* | 0.8881/0.1158   | 7.673 | 0.0097** | 1.181/0.2887   | 4.091  | 0.0493*  |
|                  | C     | 0.6492/0.0234   | 27.74  | 0.0001** | 0.4604/0.1137   | 4.049 | 0.0505 | 0.286/0.02271  | 12.59  | 0.0021** |

Asterisks indicate significant differences: *P < 0.05 and **P < 0.01.

bZT/wZT stands for between ZT and within ZT. All the df values of bZT were three. All the df values of wZT were eight.

FIGURE 3 | Relative gene expression of LPS (A), AMY (B), CEL (C), and PEP (D) in 12 h light/12 h dark (L/D) cycles during 3 days. Values are represented as means ± SD (n = 4). The gray grid represents the dark time point.

(Mata-Sotres et al., 2016). Under natural light conditions, the behavior of most animals is divided into two types: diurnal and nocturnal. Understanding and clarifying the circadian rhythm can provide a scientific basis for revealing accurately the laws and inner mechanisms of many life phenomena. Although the feeding rhythm is of great significance for establishing a scientific feeding mode to farmed animals, there are few studies on the feeding rhythm and their mechanisms in aquatic animals. In fish, as reported, the Gilthead sea bream Sparus aurata showed an feeding rhythm during the day under a light/dark condition (Mata-Sotres et al., 2015). In contrast, the Japanese prawn Penaeus japonicus feeds at night under the same conditions (Reymond and Lagardère, 1990). In mollusks, many species showed similar feeding and metabolic rhythm. For example, Manila clam Ruditapes philippinarum exhibited a 24 h circadian rhythm of feeding rates, having a higher feeding rate at night than that in the day (Jiang et al., 2009). A study on circadian feeding activity and digestive physiology in the abalone Haliotis discus hannai found that the percentages of ingesting food were significantly higher at night than in the daytime, demonstrating their nocturnal characteristics (Gao et al., 2021). An obvious circadian rhythm in water filtration rate was also found in the scallop Chlamys farreri, which had a significantly higher water filtration rate at night than that in daytime (Du et al., 2012). In S. constricta, the obvious diurnal changes in food intake rates were proved by the energy budget study (Li et al., 2006). In the present study, we found that the feeding rate of razor clam in dark was significantly higher than that in light, speculating
under the light/dark cycle found that digestive enzymes on the feeding and digestion physiology of abalone H. discus of next food intake (José Antonio et al., 2016). A recent research the regulation of translation process and may be the anticipation shown that the increase of enzyme genes expression at night was digestive physiology of aquatic animals. Existing research has correlated to their enzyme activities, which directly reflect the intestine, which the ability to hydrolyze lipids is weak.

The expression of digestive enzyme genes is strongly correlated to their enzyme activities, which directly reflect the digestive physiology of aquatic animals. Existing research has shown that the increase of enzyme genes expression at night was the regulation of translation process and may be the anticipation of next food intake (José Antonio et al., 2016). A recent research on the feeding and digestion physiology of abalone H. discus hannai under the light/dark cycle found that digestive enzymes and feeding related genes were significantly higher at night than during the day with rhythmic 24 h oscillations (Gao et al., 2021). In this research, the mRNA levels of four key digestive enzyme genes (AMY, LPS, CEL, and PEP) by qRT-PCR during the light/dark cycles were basically consistent with the production of their enzymes. Furthermore, the genes expression showed a periodic pattern of high at night and low during the day for consecutive 3 days, indicating that circadian rhythm may be the internal mechanism that was regulated by circadian clock genes (Paredes et al., 2014; Qin et al., 2020). Then the circadian clock genes may regulate the expression of AMY, LPS, CEL, and PEP, thereby participating indirectly in the feeding and digestion activities of S. constricta. Although the similar trends of gene expression and activities of key digestive enzymes, and feeding rates in this experiment were discovered, deep understanding of their corresponding relationships still need a lot of in-depth and meticulous research.

**CONCLUSION**

The feeding rate, digestive enzyme activities and relative expression of their genes all had a circadian rhythm in S. constricta, which can be basically determined that ZT0-ZT2 will reach the peak at night and ZT12-ZT14 will reach the lowest value during the day from feeding to digestion. Therefore, it can be speculated that razor clams have higher nighttime activities than during the day, suggesting that the feeding time in the industrial farming can be arranged at night to reduce cultivating costs and increase farming benefits.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**ETHICS STATEMENT**

The adult hard clams Sinonovacula constricta at the age of 1 year were collected from the genetic breeding research center of Zhejiang Wanli University, China. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Wanli University, China.

**AUTHOR CONTRIBUTIONS**

HY and YD conceived and designed the project. TZ collected the samples and contributed reagents. YL performed the experiments and data analysis, and wrote and revised the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.744212/full#supplementary-material

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