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Effects of light cycle on circadian feeding activity and digestive physiology in *Haliotis discus hannai*

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ABSTRACT

The abalone *Haliotis discus hannai* is a typical nocturnal animal. It generally feeds and moves at night but evades light by hiding in the shade during the day. However, the effects of different light cycles on the feeding physiology and potential molecular mechanisms responsible for such effects in *H. discus hannai* have not yet been reported. We examined the feeding patterns of abalones under different light (L)/dark (D) cycles (0 L:24D, 12 L:12D, 24 L:0D) using an infrared camera and analyzed the relationships between circadian digestive enzyme activities, gene expression levels of neuropeptide Y (*NPY*) and its receptor (*NPYR*), which can promote appetite, and feeding rhythms. Feeding in abalones, including searching for food and, food capture and intake, was examined by observation of videos. The percentage of abalones feeding under different light/dark cycles had two peaks, at 20:00–22:00 and 02:00–04:00. The ingestion rate was significantly higher in abalones maintained under 12 L:12D compared with 24 L:0D conditions. The percentage of feeding individuals and food intake were significantly lower in the "daytime" compared with at "night" irrespective of the light/dark cycle, suggesting the existence of an independent internal timing mechanism and the possible involvement of circadian clock genes in the regulation of periodic feeding activity in abalones. In abalones maintained under 12 L:12D and 24 L:0D cycles, protease, cellulase, and α-amylase activities started to increase from 18:00, 2 h before the first peak of food intake. Expression levels of *NPY* peaked at 20:00 and expression levels of *NPYR* increased again at 04:00, suggesting that *NPY* and *NPYR* might be induced before and after the two peak periods of food intake in abalones, respectively. These findings demonstrate the feeding rhythm and ingestion rate of abalones under different light/dark cycles, as well as the relationships among digestive enzyme activities, gene expression levels of *NPY* and *NPYR*, and feeding activity, which in turn provides a useful reference for the development of optimal feeding strategies and improved food utilization in abalone aquaculture production.

1. Introduction

The abalone *Haliotis discus hannai* is one of the most economically important marine shellfish species in China, with an aquaculture production of 163,300 tons, accounting for >90% of the total global yield in 2018 (FAO, 2019). The abalone aquaculture industry has expanded rapidly in recent years, but has encountered problems, including increased mortality associated with higher temperatures and lower oxygen levels in the scenario of global climate change (Chen et al., 2016; Shen et al., 2019). Intensified efforts to understand the basic physiological ecology of abalones will help to guide an effective response to the issues of the abalone aquaculture industry.

In their natural environment, abalone prefer to inhabit rocky reefs and cracks including various kinds of seaweed and with unobstructed water flow, and are unlikely to gather in large numbers in areas exposed to direct sunlight and wind. As a nocturnal animal, abalones usually feed and move at night, and avoid light and hide in the shade during the day (Wang and Wang, 2008). Considering these nocturnal habits, abalones are often fed at night under artificial indoor breeding conditions, and the light environment can be controlled by placing a black shading net over the aquaculture tanks (Wu and Zhang, 2016). It is unclear if abalone feeding activity continues to show an obvious circadian rhythm under artificial breeding conditions, with controllable light conditions, absence of predators, and an adequate food supply, but this information is crucial for developing a suitable feeding strategy.

Understanding the feeding rhythm is essential for establishing a

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scientific feeding practice, involving feeding time, amount, and frequency, and further impacts on food efficiency and the aquaculture water environment (Ebrahimi et al., 2017; Fortes-Silva et al., 2010; Santos et al., 2016). Periodic changes in certain environmental factors, such as light and temperature, may impact on the feeding rhythm, and thus, ultimately on the food intake of the animals (Kitagawa et al., 2015; Mattos et al., 2016). Light is a critical ecological factor influencing feeding behavior in aquatic organisms. Light levels show exceptionally high variability over short time periods, associated with the day/night rhythm (Ceinos et al., 2019; Wei et al., 2019). The survival and ingestion rates of *Haliotis corrugata* larvae kept in the dark were significantly higher than those under constant lighting (Gorrostieta-Hurtado et al., 2009). Furthermore, the food intake by *H. discus hannai* kept under a 0 Light:24 Dark (0 L:24D) cycle was significantly higher compared with those kept under 4 L:20D, 12 L:12D, and 24 L:0D, but their food conversion efficiency was significantly lower than that in animals kept at 4 L:20D (Gao et al., 2018). Garcia-Esquivel et al. (2007) reported that *H. fulgens* showed the highest specific growth rate and food intake under 0 L:24D. Most previous studies of feeding activity in abalones have thus focused on food intake amount and food conversion efficiency based on long-term breeding experiments. However, data on how and when abalones feed, and the circadian rhythm and ingestion rate, are lacking.

Animal growth and food utilization are closely associated with the digestive function of the intestinal tract. Digestive enzyme activity reflects the digestion and absorption capacities of the intestinal tract, which in turn determine the ability of an organism to digest and absorb nutrients from its food (da Silva Reis et al., 2019; Guerra-Santos et al., 2017; López-Olmeda et al., 2012). Under red light, protease and cellulase activities in *H. discus hannai* kept at 8 L:16D were significantly higher than at 0 L:24D, 12 L:12D, and 16 L:8D (Gao et al., 2017). In addition to digestive enzymes, other factors may have a critical impact on food intake and growth of aquatic animals. For example, neuropeptide Y (NPY) not only participates in the feeding and reproductive behavior of animals, but also acts on pro-opiomelanocortin neuronal synapses through its nerve fibers, leading to hyperpolarization of the cell membrane, preventing the transmission of anorexia signals (Ghamari-Langroudi et al., 2005). NPY can activate signaling pathways and induce a variety of physiological functions by binding to its receptor. NPY receptor type 1 (NPYR-1) is a key receptor impacting feeding behavior in vertebrates, and small changes in NPYR-1 brain levels inhibited thermogenesis and lipolysis, leading to a significant increase in food intake in mice (Gicquiaux et al., 2002; Lundell et al., 1995). Molluscan NPF has been reported in *Deroceras reticulatum* (Ahn et al., 2017), *Pinctata fucata*, and *Crassostrea gigas* (Stewart et al., 2014). Additionally, NPF/NPY have been reported to play roles in feeding, metabolism, reproduction, and stress responses in both invertebrates and mammals (Nässel and Wegener, 2011), and they are thought to participate in regulating energy flow in the mollusc *Lymnaea stagnalis* (De Jong-Brink et al., 2001). However, the relationship between NPY and food intake behavior in abalones has not yet been reported, and there is thus an urgent need to gain further insights into the feeding rhythms of abalones under different light/dark cycles in relation to changes in expression levels of NPY and NPYR.

In the current study, we observed and defined the feeding activity of abalones and quantified the food-intake-behavior characteristics in relation to changes in circadian rhythm using infrared camera technology. We also examined digestive enzyme activities and *NPY* and *NPYR* gene expression levels under different light/dark cycles, to gain insights into the potential molecular mechanisms by which these cycles impact the feeding rhythm in abalones. These results are expected to provide insights into abalone feeding habits, and a basis for the development of optimal feeding strategies, reduced water contamination, and improved efficiency in abalone aquaculture.

2. Materials and methods

2.1. Source and acclimation of experimental abalones

Abalones (shell length 8.63 ± 0.96 cm, body weight 78.41 ± 2.79 g) were purchased from Fuda Abalone Aquafarm (Jinjiang, Fujian). All experimental abalones were sourced from the same batch after artificial hatching and placed in culture tanks under natural light/dark cycles to acclimate for 10 days prior to experiments. The seawater was changed once a day and continuous aeration was provided. The water temperature was maintained at 18 °C, salinity: 30 ± 1, pH 7.9, and concentration of dissolved oxygen >6 mg/L. The culture water was sourced from the sea and was used after sedimentation and sand filtration. The abalones were fed with fresh *Gracilaria lemaneiformis* every day at 18:00 at 5% of abalone wet weight to ensure satiation.

2.2. Experimental design and procedures

2.2.1. Light conditions

Three different light cycles were used: 24 Light:0 Dark (24 L:0D), 12 L:12D, and 0 L:24D. For 12 L:12D, lighting apparatuses were switched on and off at 06:00 am and 18:00, respectively, using a time controller. The experimental spaces were enclosed by a blackout cloth throughout, and during light conditions, light was provided by light-emitting diodes (LEDs) (Shenzhen Fluence Technology PLC, white-light lamp, $\lambda_{400\sim780nm}$) to give a light intensity at the bottom of the aquarium of 35.70 ± 6.24 lx. "Day" was defined as the period from 06:00–18:00 and "night" as 18:00–06:00, to simulate light/dark changes in the natural environment.

2.2.2. Observation of feeding activity

Feeding at the bottom of the aquarium (45 cm × 35 cm × 35 cm) was observed for 10 abalones using an infrared camera with a red LED (wavelength 630 nm) light source. Two-thirds of the volume of fresh seawater were exchanged daily, and abalone were cultured in stagnant water with continuous aeration during the experiment. Feeding activity was divided into different stages, and the *bulbus oris* swallowing frequency was determined at night for each food intake for 100 cycles of independent continuous observations.

2.2.3. Food-intake rhythm

Ten abalones were placed in each of 12 aquaria (80 cm × 40 cm × 35 cm), half the seawater was replaced at 08:00 every morning, and excess *G. lemaneiformis* were provided at 5% of abalone wet weight. After acclimation for 3 days, the feeding activities of the abalones were observed under the three different light/dark cycles, each lasting 4 consecutive days. Four aquaria were used for each light cycle treatment. Two infrared cameras were installed above each aquarium, with the field of vision covering the entire bottom surface, and feeding activity was observed under fully dark conditions. The video data were stored on the network video recorder for subsequent analysis. Feeding activity of the abalones was judged according to the video data, based on the location of the abalones in the aquarium and the reductions in amounts of food. The number of abalones ingesting food was recorded once every 30 min, and four consecutive feeding values at 30 min intervals were used to generate a single mean value for each 2 h period. The food intake rhythm was analyzed according to the feeding proportion (percentage of individuals ingesting food relative to total number of individuals) within each period.

2.2.4. Ingestion rate

Six abalones were placed in each of 12 aquaria (45 cm × 35 cm × 35 cm) after 3 days of acclimation, and fed with *G. lemaneiformis* at 8:00 each day for 4 consecutive days. Prior to each feeding, residual food was collected into a plastic cup by siphoning. Residual food was collected at 18:00 and the next morning at 06:00 to determine the ingestion rate of

the abalones during the day and at night, respectively. Four tanks were subjected to each light/dark cycle. The collected residual food was washed with freshwater to remove salt on the surface, transferred to a 50 mL beaker, and dried in an oven at 60 °C to constant weight. The food intake rates at day and night were calculated using the method proposed by Maxwell et al. (2009):

$$\text{Day/night IR} = ((\text{Wo–Wu})/\text{Wsc})/\text{t}$$

where IR is ingestion rate; Wo is the dry weight of offered food (mg); Wu is the dry weight of residual food (mg); Wsc is the wet weight of the abalones in each aquarium (g); and t is the time (h).

The daily ingestion rate of abalones under each light/dark cycle was calculated by the following equation:

$$\text{IR} = \left(\text{IR}_{\text{day}} + \text{IR}_{\text{night}}\right)/2$$

2.2.5. Digestive enzyme activity

A total of 144 abalones from the same batch were placed in 12 aquaria (45 cm × 35 cm × 35 cm), with four replicates for each light/dark cycle ($n = 12$ per aquarium). After acclimation to each light/dark cycle for 7 days, intestinal tract tissues were taken from four randomly selected abalone from each light/dark cycle every 2 h for 24 h. The intestinal tract contents were rinsed out with 0.84% iced saline, and the intestinal tracts of four abalones were transferred to a 2 mL centrifuge tube, placed immediately in liquid nitrogen, and stored at −80 °C for future measurement. To eradicate the effects of hunger and satiation on digestive enzyme activity, abalones were fed with excess *G. lemaneiformis* at 5% wet weight at 08:00 every day.

Partial samples of intestinal tract were removed from storage in liquid nitrogen, and protease, cellulase, and α-amylase activities were determined using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), as follows (Gao et al., 2018): 0.2–0.4 g of tissue and 1.8 mL 0.86% normal saline were ground in a mortar, followed by centrifugation at 2500, 8000, and 2500 *g*/min at 4 °C for 15 min, to separate the supernatant. One unit of pepsin activity was defined as tissue protein per mg decomposed into 1 μg amino acid at 37 °C per minute, cellulase activity as tissue per g catalyzed to 1 μg glucose per minute, and α-amylase activity as protein per mg in tissues reacted with the substrate (3,5-dinitrosalicylic acid, 0.2 mol/L phosphate buffer, 6 mol/L NaOH, 0.3% NaCl) at 37 °C for 30 min to hydrolyze 10 mg amylon. The protein content in the homogenate was determined using Coomassie blue staining, as described by Bradford (1976), with bovine serum albumin as the protein marker.

2.2.6. Appetite-related genes

The remaining intestinal tract tissue samples of four abalone from each light/dark cycle every 2 h were removed from liquid nitrogen, ground, and then mixed quickly with 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA (Gao et al., 2018). The quality and concentration of RNA were assessed using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). The indexes of biotic integrity of all the extracted RNA samples were ≥ 9.4, with RNA ratios (28S/18S) ≥1.8. Total RNA was extracted by removing residual DNA from the samples using RQI RNase-Free DNase (TaKaRa, Shiga, Japan), and the RNA (800 ng) was then reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and used for real-time quantitative polymerase chain reaction (PCR) using a SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus) (TaKaRa) and TaKaRa Thermal Cycler Dice™ Real Time System TP800 instrument. The total volume of the reaction system in the PCR tube was 20 μL, including SYBR® Premix Ex Taq™ II 10 μL, forward primer (10 μM) 0.5 μL, reverse primer (10 μM) 0.5 μL, DNA template 1 μL, and dH_2O 8 μL.

The primer sequences used for real-time fluorescent quantitative PCR assays of *NPY* and *NPYR* were designed using primer3 (v0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Table 1). The samples were mixed evenly in the PCR tube and then placed into a PCR plate (Roche Diagnostics, Indianapolis, IN, USA), and PCR amplification was performed after transient centrifugation. The reaction conditions were initial denaturation at 94 °C for 30 s, followed by cycling at 94 °C for 5 s and 60 °C for 30 s, for 40 cycles in total. Solubility curve analysis was carried out at the end of the experiment. All PCR analyses were repeated three times for each cDNA sample, and mRNA levels of the target genes were calibrated using the real-time PCR Ct ($2^{-\Delta\Delta Ct}$) relative quantitative method, with the β-actin gene as the quantitative standard.

Table 1
Gene specific primers used for qRT-PCR.

Gene	Sequence (5′-3′)	Efficiency (%)	Size (bp)	Reference
NPY	F: CGACCATTCCAGGCTCTTAG R: CACTTTGTCCCCGTTGTAGG	100.17	161	MF066910
NPYR	F: GTCGCATCTTGGTATGGCA R: GTTGGTGCGGTTCTTGTAGCCG	102.93	189	MW386999
β-Actin	F: CCACTTGGTCCATTTCG R: GGACTGGATTTCCGGCCA	98.75	147	MW387000

NPY: neuropeptide Y, NPYR: neuropeptide Y receptor.
F: Forward primer; R: Reverse primer.

2.3. Data analysis

All data were analyzed statistically using SPSS 18.0. Prior to data analysis, the normal distribution and homogeneity of variance of the data were validated by Kolmogorov–Smirnov and Levene's tests. Differences in the percentages of abalones ingesting food, digestive enzyme activity, and expression levels of appetite-related genes at different sampling points and under different light cycles were examined by two-way ANOVA and Tukey's multiple comparison. The ingestion rates of abalones under different light/dark cycles were compared by one-way ANOVA and Tukey's multiple comparison. Logarithmic transformation was used to satisfy the homogeneity test of variances and standard normal distribution for the differences in percentages of abalones ingesting food, and the ingestion rates between day and night were compared using independent sample *t*-tests. All data were presented as mean ± standard deviation, and $P < 0.05$ was considered to indicate a significant difference. Data obtained from this analysis were plotted using SigmaPlot (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Feeding activity of H. discus hannai

Fig. 1 shows sample images from videos of feeding abalone. Based on the analysis of video records, the feeding process of abalones was divided into three stages. Stage I was the search stage, in which the epipodial tentacle was extended radially, and food items were searched for and located by frequent up and down swings of the cephalic tentacle. Stage II was the capture stage, in which the epipodium was raised and extended forward to cover the food item. Stage III was ingestion, during which, after contacting food, the proboscis contracted and closed and placed the food in the mouth, exposing the yellow *bulbus oris*. Food in the mouth was then cut by the jaw plate and entered the esophagus. Residual food and particles were then collected and placed on the anterior mouth by foot movement, and the captured foods were continuously collected and placed on the anterior proboscis by epipodium swings. These steps were repeated in cycles. According to continuous observations of feeding activity in 10 abalones, including 100 separate swallowing processes, the mean swallowing frequency was calculated as 0.74 ± 0.05 times/s.

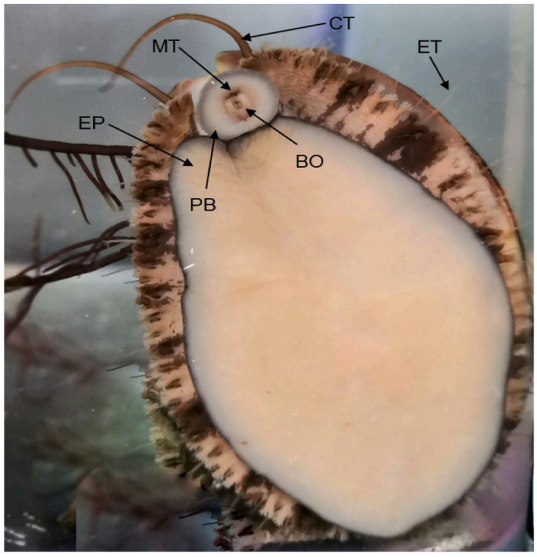

Fig. 1. Sample image obtained from videos of feeding organs of *H. discus hannai*. MT: mouth; EP: epipodium; PB: proboscis; BO: bulbus oris; ET: epipodial tentacle; CT: cephalic tentacle.

3.2. Food-intake rhythm

The percentage of feeding abalones varied significantly under different light/dark cycles and at different sampling points over the course of 24 h ($P < 0.05$) (Table 2). The percentage of abalones feeding in each group reached a maximum at 20:00–22:00, and the percentage of abalones ingesting food was significantly higher under 12 L:12D conditions than under any other light/dark cycle ($P < 0.05$) (Fig. 2A). A second food-intake peak occurred at 02:00–04:00, when the percentage of abalones ingesting food was significantly higher than at 00:00–02:00 and 04:00–06:00 ($P < 0.05$). The percentage of abalones ingesting food at "night" was significantly higher than that in the "daytime", according to *t*-tests ($P < 0.05$, Fig. 2B).

3.3. Ingestion rate

The light/dark cycle had a significant impact on the ingestion rate of abalones ($P < 0.05$) (Fig. 3A). The ingestion rate at 0 L:24D was significantly higher than those at 24 L:0D and 12 L:12D ($P < 0.05$). Comparison of circadian patterns suggested that the ingestion rate of abalones was significantly higher at "night" than in the "daytime" under all light/dark cycles ($P < 0.05$, Fig. 3B).

3.4. Digestive enzyme activities

Protease activity in abalones differed significantly under different light/dark cycles and at different sampling points ($P < 0.05$) (Table 3). Protease activity peaked at 18:00 and reached a minimum at 10:00 in abalones at 24 L:0D, peaked at 20:00 and again at 02:00 in abalones at 12 L:12D, and peaked at 18:00 and 02:00 in abalones at 0 L:24D (Fig. 4A). Protease activity was significantly higher at both 12 L:12D peaks compared with those at 24 L:0D and 0 L:24D ($P < 0.05$).

Cellulase activity also differed according to the light/dark cycle and sampling point (Table 3). Cellulase activities in abalones at 24 L:0D and 12 L:12D reached two peaks at 20:00 and 02:00, with minimum activity at 14:00 (Fig. 4B). In contrast, cellulase activity at 0 L:24D peaked at 16:00 and also showed an increase at 02:00, but the latter level showed no significant difference compared with that at 00:00 ($P > 0.05$).

α-Amylase activity was significantly higher at 20:00 compared with any other time point ($P < 0.05$), followed by a decline, in abalone's maintained under 24 L:0D (Fig. 4C). Activity peaked at 20:00 and again at 02:00 in abalones under 12 L:12D conditions, and was significantly higher at both 12 L:12D peaks compared with at 12 L:0D and 0 L:24D ($P < 0.05$). α-Amylase activity peaked at 18:00 and was significantly higher than at other times ($P < 0.05$), apart from 16:00 and 20:00, in abalones at 0 L:24D ($P < 0.05$).

3.5. Expression of appetite-related genes

Light/dark cycle and sampling point had significant impacts on the expression levels of *NPY* ($P < 0.05$) (Table 4). Gene expression levels of *NPY* reached maximum values at 20:00 in abalones raised at 12 L:12D and 24 L:0D, and expression levels were significantly lower under 24 L:0D compared with 12 L:12D and 0 L:24D. *NPY* expression levels peaked at 18:00 in abalones at 0 L:24D, and were significantly higher at 18:00–04:00 than at any point from 06:00–16:00 (Fig. 5A).

NPYR gene expression levels also varied significantly under different light/dark cycles and at different sampling points (Table 4). Expression levels peaked at 22:00 and were lowest at 18:00 in the 24 L:0D group. The peak at 22:00 in the 12 L:12D group and the expression levels at 18:00–22:00 were significantly higher than in the 24 L:0D and 0 L:24D groups (Fig. 5B). Expression levels of *NPYR* in the 0 L:24D group at 20:00 were significantly higher than at any other sampling point ($P < 0.05$). Expression levels in the 0 L:24D group were significantly higher than those in the 24 L:0D group at all points, except 22:00 ($P < 0.05$).

4. Discussion

Animal behavior represents an immediate response to external stimuli and vital signs (Sakamoto et al., 2011). As a typical nocturnal animal, abalone movement behaviors show an obvious circadian rhythm (Gao et al., 2020). In nature, feeding behavior of aquatic animals involves six steps, including selection of a feeding area, searching for food, accessing possible food, catching the food item, and ingestion and swallowing (Zhang, 2013). Feeding activity of two holothurian species, *Stichopus tremulus* (Gunnerus) and *Mesothuria intestinalis* (Ascanius), was shown to depend on the extent and reach of the tentacles, but feeding rates were affected by morphological differences in the tentacles (Hudson et al., 2005). In the current study, we divided the abalone feeding process into three stages and analyzed the swallowing frequency based on video recordings, to provide reference information regarding their feeding method, ingestion rate, and feeding rhythm. Although there have been no reports of the role of abalone olfactory organs, most aquatic animals rely on chemical signals from small-molecule metabolic products to guide their food intake, with herbivorous and omnivorous organisms mainly sensitive to sugar molecules and amino acids (Derby and Sorensen, 2008; Grasso and Basil, 2002). The current video observations showed that abalone stretched their cephalic tentacle out to detect food, and every feeding activity began with frequent swings of the cephalic tentacle. Once the cephalic tentacle touched the food item, the abalone's epipodium lifted up to capture the food. These observations suggest that the cephalic tentacle plays a crucial role in perceiving and locating food items.

A previous study revealed that the cumulative movement distance and time in *H. discus hannai* peaked at 00:00–03:00 in animals maintained at 12 L:12D, while the lowest movement velocity occurred at 21:00–00:00, with an obvious circadian rhythm (Gao et al., 2020). In the

Table 2
Results of the two-way ANOVA conducted to test the effect of different light cycles and times on feeding proportion.

Item	Source	MS	*df*	*F*	*P*
Feeding proportion	Light cycle	263.492	2	16,644.551	<0.001
	Time	51.781	11	3270.652	<0.001
	Light cycle × Time	40.733	22	2573.058	<0.001

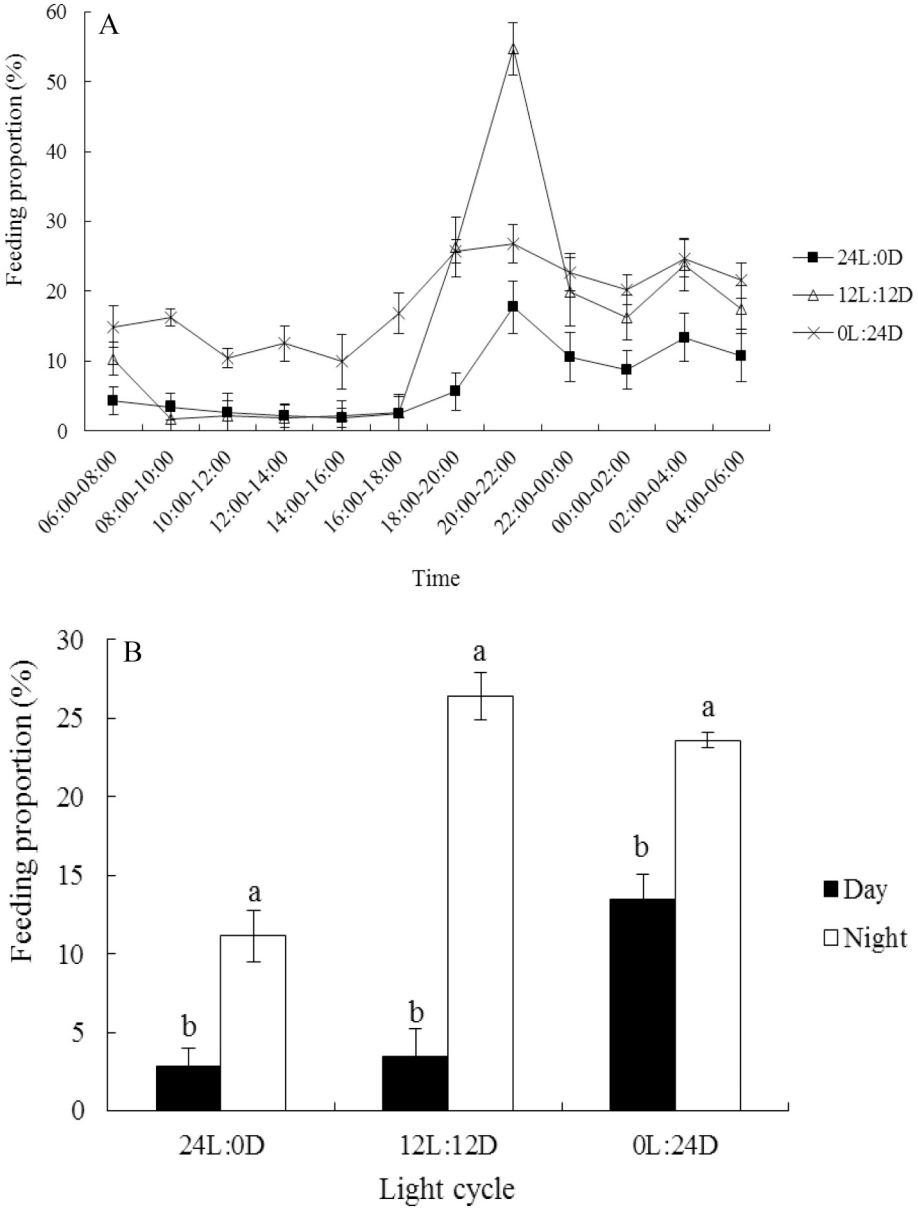

Fig. 2. Feeding proportions at different light cycles. (A) Diurnal changes in the proportion of feeding *H. discus hannai* at 24 L:0D, 12 L:12D, and 0 L:24D based on two-way analysis of variance and Tukey's test. (B) Mean day and night feeding proportions of *H. discus hannai* at different light cycles based on independent samples *t*-test. Different letters within each treatment represent significant differences ($n = 4$, $P < 0.05$).

current study, the percentages of abalones ingesting food in each light cycle condition group peaked at 20:00–22:00 and at 02:00–04:00, respectively, with the latter peak coinciding with the peak in distance moved from 00:00–03:00, suggesting that the animals moved to search for food at this time. The percentages of abalones ingesting food were significantly higher at "night" than in the "daytime", irrespective of the light/dark cycle, further demonstrating their nocturnal characteristics in terms of sensitivity to environmental light. A behavioral study of *Apostichopus japonicus* (Dong et al., 2010) found that the feeding peak occurred at 21:00–00:00, regardless of light intensity. *A. japonicas* also showed nocturnal behavior characteristics under 24 L:0D and 0 L:24D, but its food intake and movement time were shortened or prolonged compared with natural light conditions, and there was no obvious circadian movement rhythm (Dong et al., 2011). The present study showed that the ingestion rate was significantly higher in abalones kept at 0 L:24D compared with 24 L:0D and 12 L:12D, and was significantly lower in the "daytime" compared with at "night" under each light cycle, suggesting that light strongly reduced feeding activity in abalones.

The percentage of feeding animals and ingestion rate were also higher at "night" than in the "daytime" in abalones maintained at 24 L:0D and 0 L:24D, which suggested that, in addition to the influence of light, abalones may also have an internal auto-timing mechanism resulting from long-term evolutionary adaptation, to synchronize the animal's behavior rhythm with periodic environmental changes. Previous evidence showed that some animals can avoid their predators' feeding peaks by regulating their circadian rhythm (Miyajima-Taga et al., 2016; Tertschnig, 1989). Many sea urchins hide in cracks in rock reefs during the day and emerge at night to feed (Livore and Connell, 2012; Tegner, 1989). Mercier et al. (1999) suggested that small individuals of *Holothuria scabra* only fed at night, as a self-protection measure to avoid predation. However, the percentages of abalones feeding in the "daytime" were similar at 24 L:0D and 12 L:12D, suggesting the existence of an internal physiological regulatory mechanism, and possible effects of hunger and other factors. When exposed to light

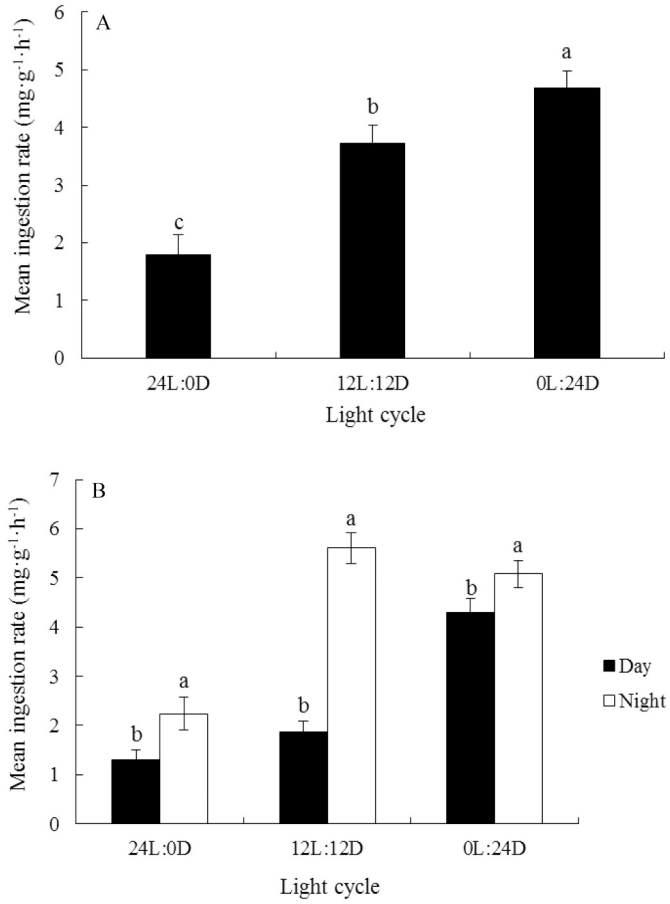

Fig. 3. Mean ingestion rates (IRs) at different light cycles. (A) Mean IR of *H. discus hannai* at different light cycles (24 L:0D, 12 L:12D, and 0 L:24D) based on one-way analysis of variance and Tukey's test. (B) Mean day and night IRs of *H. discus hannai* at different light cycles based on independent samples *t*-test. Different letters within each treatment represent significant differences ($n = 4$, $P < 0.05$).

Table 3
Results of the two-way ANOVA conducted to test the effect of different light cycles and times on selected digestive enzyme activities.

Item	Source	MS	*df*	*F*	*P*
Protease activity	Light cycle	0.031	2	28.706	<0.001
	Time	0.402	11	368.183	<0.001
	Light cycle × Time	0.105	22	95.991	<0.001
Cellulase activity	Light cycle	7.445	2	8126.164	<0.001
	Time	2.171	11	2369.984	<0.001
	Light cycle × Time	0.688	22	751.046	<0.001
α-Amylase activity	Light cycle	17.347	2	11,641.887	<0.001
	Time	6.835	11	4587.262	<0.001
	Light cycle × Time	2.237	22	1501.014	<0.001

for 24 h, some abalones fed during the "daytime". However, the light/dark cycle is the main external factor influencing the feeding activity of abalones under artificial breeding conditions, given the absence of predation pressure, and efforts to regulate the light/dark cycle may therefore promote feeding. These results may thus guide the choice of feeding time and frequency in abalones in aquaculture.

Digestive enzyme activities reflect the digestive physiology of aquatic animals. Changes in environmental factors may increase energy consumption compared with normal metabolism, requiring enhanced digestive enzyme activity to digest the food and ensure an adequate energy supply (Kolkovski, 2001; Vera et al., 2007). In the current study, the light/dark cycle had a significant impact on protease, cellulase, and α-amylase activities. During the 20:00–04:00 period, activities of all three enzymes were significantly lower in abalones kept at 24 L:0D compared with 12 L:12D, suggesting that changes in the light/dark cycle affected digestive physiology. There is generally a relationship between food intake and digestive enzyme activity, with undigested food stimulating the digestive system to secrete digestive enzymes, which in turn promote feeding behaviors (Bolasina et al., 2006). The present results showed that the ingestion rate and the percentage of abalones feeding were both lower at 24 L:0D than at 12 L:12D and 0 L:24D, and changes in digestive enzyme activity were thus closely associated with these behaviors. We aimed to judge the occurrence of feeding activity in abalones according to changes in digestive enzyme activity. Digestive enzyme activity generally began to increase 2 h before the peak food intake, suggesting that the early secretion of digestive enzymes prepared the animal for the subsequent food intake by optimizing digestion and metabolism to allow the organism to complete feeding in as short a time as possible, to minimize the risk of predation (Aranda et al., 2001). Studies of feeding physiology in *Sparus aurata* (Montoya et al., 2010) and *Dicentrarchus labrax* (del Pozo et al., 2012) found similar patterns of digestive enzyme activity, which in turn enhanced the ability to digest and absorb nutrients.

Even in abalones kept at 0 L:24D and 24 L:0D, representing a dramatically unnatural light/dark cycle, digestive enzyme activity tended to be higher at "night" and lower in the "daytime", suggesting an alternative regulatory mechanism influencing changes in digestive enzyme activity, in addition to the direct influence of the light/dark cycle. Circadian clock genes can regulate digestion and absorption and the metabolism and physiological functions of digestive organs, as well as other behaviors associated with metabolism, leading to rhythmic 24-h oscillations, and breakdown of this physiological rhythm would lead to metabolic disorders (Wu et al., 2008; Wu et al., 2009). Maouyo et al. (1993) found that peristalsis of the small intestine was rhythmic and associated with the rhythmicity of pancreatic juice secretion in rats. Furthermore, mRNA expression levels of the intestinal epithelial cell transporter, oligopeptide transporter 1, were also rhythmic, and amino acid and peptide absorption were subject to regulation by the circadian rhythm (Saito et al., 2008). Circadian rhythms have been demonstrated in organisms from bacteria to mammals, and the metabolism of the digestive tract microbiome and its host were both shown to be regulated by circadian rhythms, involving a linked and interactive rhythm mechanism (Thaiss et al., 2014). It can be thus inferred that the regulatory role of the circadian clock is crucial for maintaining stable changes in digestive enzyme activity in abalones, even if the light/dark cycle changes drastically.

As an endogenous appetitive factor, NPY plays a crucial role in feeding activity and regulates the appetite via a complex regulatory network (Dumont et al., 1992; Malmström, 2001). During the 18:00–04:00 period, gene expression levels of *NPY* and *NPYR* were significantly lower in abalones maintained under 24 L:0D compared with 12 L:12D and 0 L:24D. Expression levels of *NPY* at 12 L:12D and 24 L:0D peaked at 20:00, coincident with the highest percentage of feeding abalones, indicating that higher expression levels of *NPY* were accompanied by enhanced feeding activity. Jing et al. (2007) found that neuropeptide F (NPF) was mainly distributed in some parts of *Aplysia californica* brain ganglion, and a neuron, B18, located in the brain ganglion, initiated and regulated the feeding process. When *A. californica* was satiated, the B18 motor neuron mediated the transition from feeding-related to non-feeding movements via the release of NPF. Peterson et al. (2012) found that expression levels of *NPY* in brain tissue in *Ictalurus punctatus* increased 0–4 h before food intake and were positively correlated with hunger time. Narnaware and Peter (2001) proved that expression levels of *NPY* were significantly increased in hypothalamic tissue from goldfish after 72 h of fasting, and decreased to

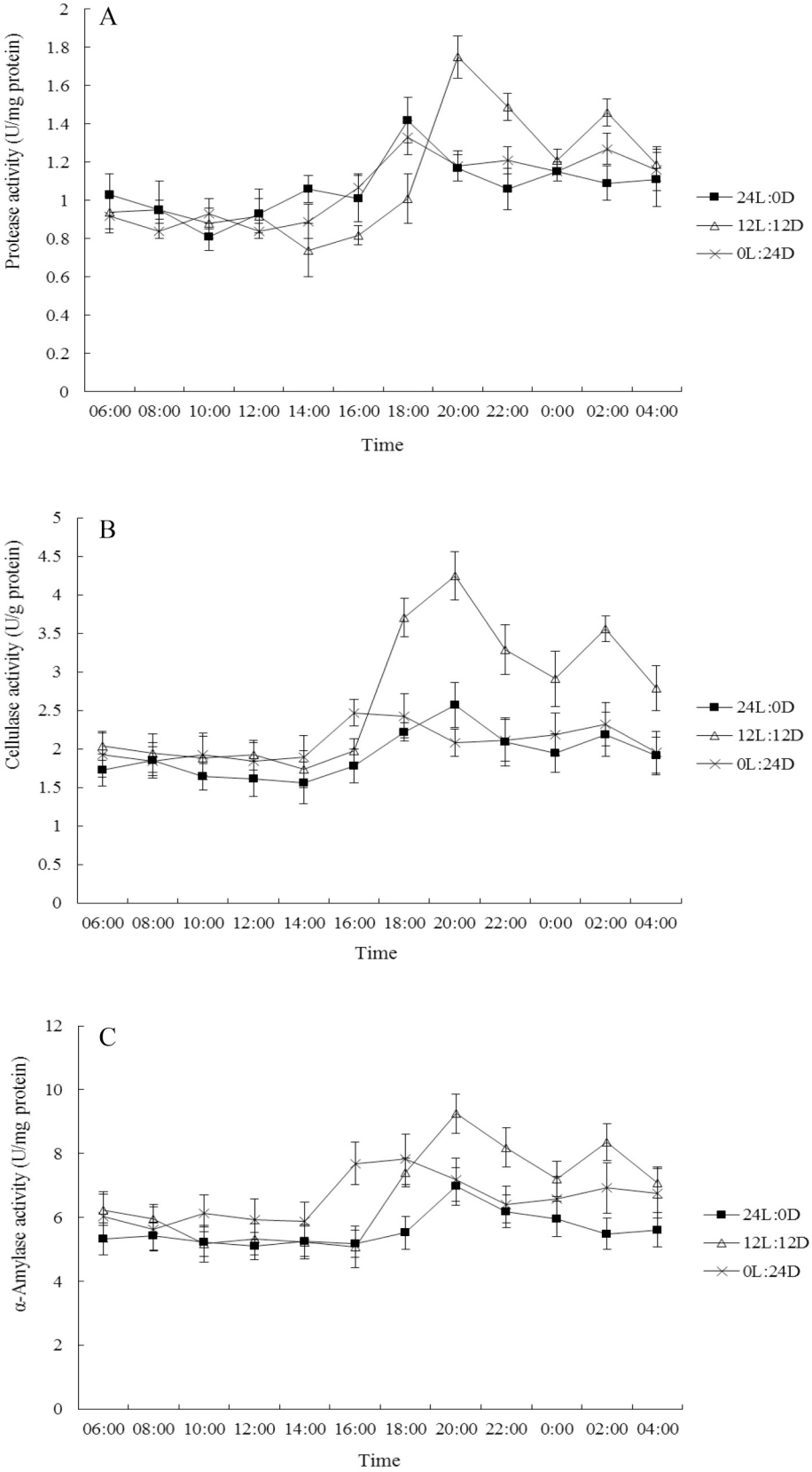

Fig. 4. Digestive enzyme activity levels over time. Diurnal pattern of protease (A), cellulase (B), and α-amylase (C) activities at different light cycles (24 L:0D, 12 L:12D, and 0 L:24D). Data presented as mean ± standard deviation (n = 4).

Table 4
Results of the two-way ANOVA conducted to test the effect of different light cycles and times on the relative expression of *NPY* and *NPYR* in intestinal tract.

Item	Source	MS	*df*	*F*	*P*
NPY	Light cycle	4.876	2	3723.485	<0.001
	Time	1.087	11	830.205	<0.001
	Light cycle × Time	0.167	22	127.175	<0.001
NPYR	Light cycle	3.397	2	2376.101	<0.001
	Time	0.872	11	609.968	<0.001
	Light cycle × Time	0.181	22	125.819	<0.001

normal levels 1 h after subsequent feeding. A similar phenomenon was found in the current study, with expression levels of *NPY* in abalones at 12 L:12D and 24 L:0D gradually decreasing after 20:00, while the percentage of feeding abalones also fell drastically at 22:00–02:00. However, we found no similar trend in expression levels of *NPYR*, which peaked at 22:00 in abalones at 12 L:12D and 24 L:0D, then decreased, but increased again at 04:00. The percentage of feeding abalones also tended to increase at 02:00–04:00, suggesting that *NPYR* had a significant influence on appetite regulation in abalones in this period. In vertebrates, NPYR-1 is a key receptor influencing feeding behavior, while NPYR-2 is also involved in the regulation of feeding activity, and activation of NPYR-2 may lead to the formation of adipose tissue, accumulation of adipocytes, and weight gain (Cerdá-Reverter and Larhammar, 2000; Lavebratt et al., 2006). We therefore inferred that NPY was mainly involved in regulating the first feeding peak in abalones, while NPYR played a dominant role in regulating the second peak. We further found that expression levels of *NPY* and *NPYR* tended to be higher at "night" and lower in the "daytime", irrespective of the light/dark cycle, indicating that the biological rhythm was an internal mechanism adapting the organism to cyclical diurnal changes. Circadian clock genes may be indirectly involved in regulating periodic feeding activity in abalone by controlling the expression levels of *NPY* and *NPYR*. Further studies are needed to gain an insight into the internal relationship between the rhythmic expression of circadian clock genes and feeding activity rhythms in abalone, to provide data to support the development of optimal feeding strategies and to improve productivity by manipulating the light environment.

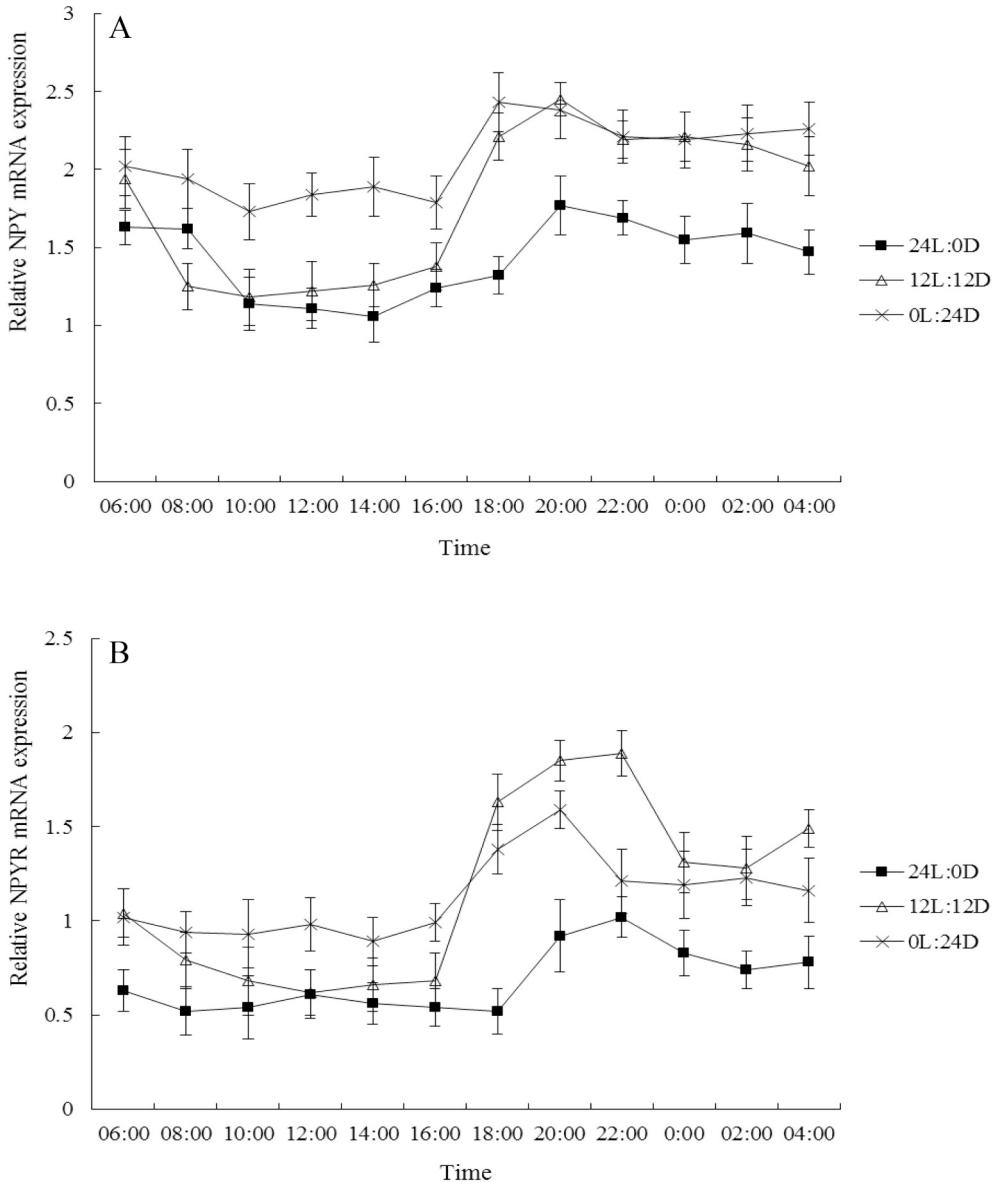

Fig. 5. *NPY* (A) and *NPYR* (B) gene expression in the intestinal tract of *H. discus hannai* under three different light cycles (24 L:0D, 12 L:12D, and 0 L:24D). Each data point represents mean ± standard deviation of four samples normalized to β-actin.

In summary feeding in abalones consists of searching for food followed by food capture and intake, with an average swallowing frequency of 0.74 ± 0.05 times/s. Abalones had two feeding peaks under each light/dark cycle, at 20:00–22:00 and 02:00–04:00, and both the percentage of feeding individuals and the ingestion rate were significantly higher at "night" than in the "daytime", indicating the existence of an internal physiological mechanism. In addition, circadian clock genes may mediate the regulation of feeding in abalones, to maintain synchronization between the body rhythm and cyclic changes in environmental factors. Protease, cellulase, and α-amylase activities peaked at 20:00 and 02:00 in abalones maintained under 12 L:12D conditions, while activities tended to be higher at "night" and lower in the "daytime" in animals at 0 L:24D and 24 L:0D. Digestive enzyme activity also generally increased 2 h before the feeding peak, which could enhance the ability to digest and absorb nutrients. Expression levels of *NPY* reached a maximum at 20:00 and then gradually decreased, in accord with the first feeding peak, while expression levels of *NPYR* increased again at 04:00, suggesting that *NPY* and *NPYR* regulated the two feeding peaks, respectively. In this study, we continuously observed and monitored the feeding rhythm and digestive physiology of abalones under different light/dark cycles to further understand abalone behavioral ecology and to provide guidance for the development of optimal feeding strategies and to improve food utilization in aquaculture practices.

Author statement

XLG and CHK conceived the project. WWY, XL and GWP participated in the design of the study and discussed the results. Measurement of growth and RNA extraction were conducted by GWP. XLG carried out data analyses and wrote the manuscript. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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