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Effects of short-time fasting and feeding frequencies within 24 hours on histology, cholecystokinin and trypsin enzyme activities of digestive organs in black bream, Megalobrama pellegrini (Tchang, 1930), juvenile

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Abstract

To study the regulation and feedback mechanism of cholecystokinin and trypsin in black bream, *Megalobrama pellegrini (Tchang, 1930)* 60 days after hatching under 15 days short-term fasting and different feeding frequencies within 24 hours during the same period, *M. pellegrini* (wet weight 183.75 ± 61.16 mg, total length 20.74 ± 4.08 mm) developed in a recirculating aquaculture system were selected as the subject. In the short-term fasting trial, the body weight, trypsin, and CCK of the feeding control group (FCG) were higher than those of the fasting test group (FTG). Trypsin and CCK in FTG reached the lowest value on the ninth day and the CCK content reached the highest value on the 11th day. A negative feedback regulation of CCK and trypsin had not been found in this trial. The degree of damage to intestinal chorionic epithelial cells was higher than that of hepatopancreas, and detachment of epithelial cells and the striatal border was the main damage. In the 24-hour daily rhythm experiment, juvenile fish were randomly assigned to (A) once feeding, (B) twice feeding, (C) three times feeding, (D) fasting. CCK showed a minimum value at $1:00^{+}$ in group A, while a peak occurred at night in group B\C\D and a maximum value in group C, and a single satiety stimulus can lead to increased hunger. The four treatment groups had an apparent closed-loop regulation while the control point of the fasting group (D) shifted forward to the next day. Different feeding frequencies in a single day had no direct effect on the long-term fluctuation of CCK and trypsin diurnal rhythm. Feeding three times a day was beneficial to the individual growth of juvenile *M. pellegrini*, a better growth results may be produced in the long term. This study aimed to provide a reference for the feeding strategy of juvenile *M. pellegrini* in the RAS.

1. Introduction

In the middle and upper reaches of the Yangtze River in China, black bream *Megalobrama pellegrini (Tchang, 1930)*, belongs to Cypriniformes, Cyprinidae, Culterinae, and Megalobrama. *M. pellegrini* is a unique economic fish in the upper reaches of the Yangtze River (Li et al., 2007b). The meat of *M. pellegrini* is fresh and tender, with high nutritional value, and rich in unsaturated fatty acids. It is a species of high-quality freshwater fish with considerable breeding advantages and wide breeding prospects. With the implementation of the fishing ban in the Yangtze River basin in 2020, how to achieve the factory-intensive production of *M. pellegrini* to meet the growing consumer demand for high-quality aquatic products becomes urgent (Liu, 2004). Recirculating aquaculture system (RAS) is a common aquaculture mode in aquaculture factories. Freshwater larvae and juveniles can obtain a stable growth and development environment in RAS. There are few studies on the digestive physiology of *M. pellegrini* juvenile in the RAS environment, which makes the breeding process lack theoretical reference and hinders the development of high-quality breeding and low cost.

CCK is considered to be one of the most important stimulators of pancreatic enzyme secretion in mammals, along with many hormones in the gastrointestinal tract. CCK has a variety of biological effects, mainly stimulating the secretion and synthesis of pancreatic enzymes, increasing pancreatic bicarbonate secretion and gastric emptying, stimulating gallbladder contraction and relaxation of the sphincter of Oddi, stimulating hepatic bile secretion, regulating small intestinal and colonic motility. It

also acts as a satiety factor to regulate food intake (H et al., 2005). The CCK gene was first found in the gastrointestinal tract of dogs (Ivy and Oldberg, 1927) and has been reported in humans (Y et al., 1985), *Mus musculus* (M et al., 1991), *Oncorhynchus mykiss* (H et al., 2001), *Salmo salar* (Murashita et al., 2008) and *Megalobrama amblycephala* (Ji et al., 2015). Chaudhri (Owais et al., 2006) confirmed that CCK is a typical postprandial satiety signaling factor. Murashita (Murashita et al., 2008) proposed that the expression pattern of CCK in different species was dynamic and changed with environmental factors, including day/night temperature difference, feeding status, diet composition, sampling time, and day/night time. Liddle R.A, et al (A et al., 1985) have shown that the concentration of CCK in human plasma increased from 0.2 pm to 6.0 pm 15 min after a meal. Tartaglia L.A. (Tartaglia et al., 1995) reported that CCK can affect digestion and feeding in aquatic animals. Murashita K (Murashita et al., 2008) pointed out that in Atlantic salmon, the expression of CCK in the brain increased significantly in three hours after a meal. Feng (Ke et al., 2012) found that the expression of CCK in the hypothalamus of grass carp significantly decreased after 72 hours of fasting, which was consistent with the changing trend of CCK in the brain or hypothalamus of fish such as large yellow croaker (Cui, 2013), *Pelteobagrus fulvidraco* (Gong et al., 2013), *Megalobrama amblycephala* (Ji et al., 2015) after fasting. Murashita (Murashita et al., 2008) found that the expression of CCK in the foregut decreased significantly after 72 hours of fasting.

As a typical satiety and antifeedant factor, cholecystokinin (CCK) is closely related to the regulation of trypsin secretion in the hepatopancreas, and the degree of starvation or changes in diet will affect its physiological function to some extent. Long-term studies have shown that it plays an important role in gallbladder contraction, intestinal peristalsis, delayed gastric emptying, and pancreatic enzyme secretion in mammals (N. and L., 1994) and adult fish (S et al., 1997). Trypsin is a protease that acts as a digestive enzyme in vertebrates. It is synthesized in the pancreas as a precursor of the enzyme trypsinogen. As well as acting as a digestive enzyme, it also inhibits and breaks down the precursors of other enzymes such as chymotrypsinogen, carboxypeptidase, and phospholipase, and activates them. After feed ingestion, trypsin is secreted as its inactive precursor, trypsinogen, from the acini of the pancreas into the intestinal lumen and is activated automatically or by enteropeptidase. After nutrients enter the intestine, CCK is released from the enteroendocrine cells of the enterocytes into body fluids and acts on target cells in the pancreas to induce the secretion of digestive enzyme precursors into the intestinal lumen. High intestinal trypsin activity acts as a negative feedback control on CCK release, suggesting a regulatory loop between these two factors in mammals. This regulatory loop has also been reported in sea bass (Tillner et al., 2014) *Gadus morhua*(Tillner et al., 2013), *Atlantic salmon* (S et al., 1997) et al. Although CCK-producing cells are located in distinct regions of the larval gut of several fish species, such as *Gadus morhua* (B et al., 2009), less is known about the regulatory mechanism between CCK and trypsin in developing fish larvae compared to mammals and adult fish. Few reports have focused on understanding the changes between CCK and trypsin, and most have focused on seawater fisheries. The secretion of CCK does not follow any specific rules, but when the fasting period reaches a certain level, the secretion level will continue to decrease after a short-term increase due to functional damage to the digestive system.

Feeding frequency is another important component of aquaculture management that affects fish growth, feed utilization, and management costs. Feeding frequency affects the metabolic state and body composition of the fish. In intensive culture, good feeding frequency can reduce the size difference between individuals in the same batch and effectively improve water quality. The study found that CCK is involved in the endocrine regulation of the individual digestive process and that the fluctuation rhythm in a single day was affected by feeding frequency and was not independent of feeding behavior (Rojas-García et al., 2011). A negative feedback loop between CCK and trypsin was observed in the daily rhythm test of juvenile Senegalese sole (Neda et al., 2021). The effects of fasting and feeding frequency on the digestive system of fish have been comprehensively reported, and our research group has also done a lot of research on fasting and re-feeding of *M. pellegrini*. However, there are relatively few studies on monitoring CCK content or feedback regulation by fasting and changing feeding frequency, and most of them focus on marine fishes. For example, feedback regulation has been monitored in the ontogeny of *Gadus morhua* (Tillner et al., 2013) or *Senegalese sole* juveniles (Neda et al., 2021) al., under different feeding frequencies during diurnal and nocturnal, and short-term daily regulation in *Clupea harengus L* (Rojas-García et al., 2011) by changing the feeding strategy. The effects of fasting and refeeding on the growth and digestive enzyme activities of juvenile *M. pellegrini* have been huge reported, and the effects of refeeding on biochemical and non-specific immune parameters have also been reported by usual research. Qin L, et al (Li et al., 2015) investigated the effects of fasting followed by feeding on the growth, hematology, biochemistry, and non-specific immunity of juvenile *M. pellegrini*. Another report (Li et al., 2013) described the effects of starvation and re-feeding on the growth and digestive enzyme activity of juvenile *M. pellegrini*. At the same time, Qu et al. (Qu et al., 2021) conducted a series of analyses on the body weight and optimal feeding frequency of juvenile *M. pellegrini* in a stream culture mode. The overall growth performance of bream was inferior to that of other fish of the same genus (Li et al., 2007a).

It has been reported that the head compartment, representing the neural pool, was quantitatively dominant (80% of the total CCK content), while the digestive tract pool represented 6–10% in aquatic animals (Rojas-García et al., 2011). In order to more accurately explore the regulation mechanism of fasting and feeding frequency on CCK and trypsin in the intestine, and eliminate the influence of head hormones, the dissection steps of special treatment (head, back tissue, and tail peduncle of the individuals were all cut off) were taken for juvenile *M. pellegrini* in sampling. To study the histological effects of short-term fasting and daily rhythm on the digestive system of juvenile *M. pellegrini* and the interaction between intestinal CCK and trypsin feedback regulation, to provide a feeding reference for breeding at *M. pellegrini* in the RAS.

2. Materials and methods

2.1. Larval rearing in RAS

M. pellegrini larvae were obtained from Dongping Aquaculture Co., Ltd, Chongqing, China. The RAS was established in a 28°C constant temperature rearing room. Black bream hatched from domesticated brood fish were randomly placed in the glass tanks of the RAS on the fifth day after hatching (DAH 5) after

treatment with 5000 ppm salt water for half an hour. Each tank contained 32 liters of water with a flow rate of 0.2 $L^{-s^{-1}}$. The RAS used LED tubes and was illuminated at 1200 lx daily from 7:00 to 19:00. Individuals were grown in tanks until DAH59 prior to the experiment and the culture density was gradually reduced to 1.25 ind L^{-1} as the individuals grew. Commercial mash (crude protein ≧ 36, crude fat ≧ 3.5) was fed to the fish three times a day. *M. pellegrini* were fed to apparent satiation three times a day (9:00, 13:00, and 17:00) and the bait remaining in the cylinder was removed after each satiation. Dead fish were cleaned daily and data was recorded.

2.1.1 Short-term fasting trial

Six glass tanks with a water volume of 32 liters in the RAS were selected to prepare for the short-term fasting trial on DAH59. 240 juvenile individuals were randomly divided into the 15-day fasting test group (FTG) and the three times a day feeding control group (FCG), with three replicates for each (1.25 ind L^{-1} in each tank, with an initial body weight of 183.75 ± 61.16 mg, length of 20.74 ± 4.08 mm, mean and SD). The growth data such as WGR and SGR during the test period were measured, and the measurement method refers to He, et al (He et al., 2022). The tanks were emptied after each feeding. In the fasting trial, three individuals were randomly sampled from every three tanks of the two experimental groups. Three individuals were collected from ever group randomly in the morning on 1, 3, 5, 7, 9, 11, 13 and 15 days from DAH60 to DAH75. Each sampling was in triplicate for analyze.

2.1.2 Daily rhythm experiment

Four glass tanks with a water volume of 32 liters of RAS were selected to prepare for the different feeding regimes within 24 hours. 160 normally reared juvenile individuals (DAH62) were randomly divided into four groups (1.25 ind L^{-1} in each tank, with an initial body weight of 238.71 ± 68.84 mg, length of 22.81 ± 2.42 mm, mean and SD). After one-day fasting, four tanks were assigned to each of the following feeding regimes on DAH64: (A) feeding once at 9:00, (B) feeding twice at 9:00 and 17:00, (C) feeding three times at 9:00, 13:00 and 17:00, (D) fasting. The fish were fed to apparent satiation at each feeding time, the proportion of feed was approximately 5.6% of individual body weight. Sampling was divided as 9:00, 13:00, 17:00, 21:00, $1:00^{+1}$, $5:00^{+1}$, and $9:00^{+1}$ into seven points of different feeding regimes for daily rhythm within 24 hours. Three individuals were randomly selected from each sampling point from four groups and analyzed in triplicate.

2.2. Sampling preparation

The tests and procedures of *M. pellegrini* in this study were approved by the Laboratory Animal Centre of Southwest University and obtained the qualification of Laboratory Animal Practitioner (SWU _ LAC-20210794).

Body weight and total length were measured at each sampling. Body weight was calculated by weighing the sub-sample of individuals using a millimeter analytical balance. The total length (mm, from the tip of the maxilla to the end of the notochord) was measured using a caliper. Each sample was taken before

feeding and individuals were anesthetized with tricaine MS-222 (120 mg/L) and transferred to a 2 ml enzyme-free test tube after the head, dorsal muscles, and tail were dissected on ice, frozen with liquid nitrogen, transferred to a -80°C ultra-low temperature refrigerator and stored until analyzed.

Individual samples were analyzed for CCK and trypsin enzyme activity using a combination of highly sensitive methods as reported by Neda Gilannejad (Neda et al., 2021), allowing resolution of both factors at the individual level. Previous studies have shown that a relatively large amount of CCK can be found in the head (mainly in the central nervous system), which may mask changes in CCK in the gastrointestinal tract (Rojas-García et al., 2011). Therefore, all analyses in the present study were performed on dissected juveniles excluding the head, back muscles, and tail.

2.3. Analysis of CCK and trypsin enzyme activity

Each sample was homogenized with a tissue grinder in 0.05M PBS (pH 7.4) at a ratio of 1:9. Samples were centrifuged at 2500 rpm for 20 minutes at 4°C and analyzed immediately. All assays were performed in triplicate.

The measurement of trypsin activity was based on the need to detect total protein content. Total protein content and trypsin enzyme activity were analyzed using assay kits from Nanjing Jiancheng Bioengineering Institute. Before the experiment, the diluted supernatant was diluted tenfold with 0.05 M PBS (pH7.4). Enzyme activity was calculated from the change in absorbance.

The detection method was based on the practice of Neda Gilannejad (Neda et al., 2021). Samples from all groups were analyzed using the Fish Cholecystokinin (CCK) Elisa Assay Kit from Nanjing Jiancheng Bioengineering Institute, which uses the competition method to detect the content of CCK.

2.4 Histology

M. pellegrini in the short-term fasting trial were subjected to routine histology and hematoxylin-eosin (H-E) staining, fixed in 4% paraformaldehyde for 24 hours, and then washed, dehydrated, cleared, and embedded in paraffin. Sagittal sections (5 μm) were cut on a conventional with a microtome (RM2016, Shanghai Leica Instrument Co., Ltd.), placed on gelatin-coated slides, rehydrated, and stained. Tissue sections were observed under a light biomicroscope (B302, Chongqing Aote Optical Instrument Co., Ltd.) and analyzed using a Leica four-camera digital camera and ImageJ.

2.5. Statistical analysis

CCK content and trypsin enzyme activities in the abdominal extracts of *M. pellegrini* were expressed as mean ± SD. Tests for homogeneity of variance and normality were performed. The specific activity of CCK was evaluated by one-way ANOVA. Multiple comparisons of enzymatic activity over time were performed using the Duncan test. Independent samples t-test was used for the analysis of variance between groups. All statistics were analyzed using IBM SPSS Statistics 26 and graphs using Origin 2021. A significance level of $P<0.05$ was used.

3. Results

3.1. Short-term fasting trial

3.1.1 Trypsin enzyme activity and CCK Content

All groups showed an increasing trend in body weight in *M. pellegrini* from DAH60 to DAH75 (Fig 1), and there was a significant difference between groups ($P < 0.05$). The weight gain of the two groups did not show a significant increase during the first three days. The weight gain of the FCG was significantly higher than that of the FTG ($P < 0.01$). There was no significant difference in the weight gain of FTG ($P > 0.05$), and the SGR of FTG was 3.32%. The SGR of FCG reached 6.72%, which was significantly higher than that of FTG.

No significant difference in trypsin enzyme activity was found in FCG, the final level of trypsin activity in the control group was higher than the initial value (Fig 4). A higher level was found in FCG compared to FTG. The trypsin activity of FTG showed a decreasing trend on the first day of fasting and reached the lowest value of 279.43 U/mgprot on the ninth day of fasting (DAH72), which was significantly different from other time points (P 0.05). After a slight increase, it fell back to a level lower than the initial trypsin activity on the fifteenth day of fasting. The trypsin enzyme activity showed a significant difference between FTG and FCG (P 0.05). The values of each point in FCG were greater than those in FTG (Fig 2).

The trypsin enzyme activity test showed significantly higher levels in FCG on days 11 and 13, with peaks higher than adjacent points (Fig 2). The enzyme activity levels of all sampling points in FCG were higher than the baseline value. FTG showed two downward trends, reaching a low of 279.43 U/mgprot on the ninth day of fasting. The enzyme activity level of FTG was lower than the baseline value on the fifteenth day of fasting (Fig 2). The enzyme activity level of FCG was always higher than that of FTG, and a very significant difference was found between the two groups (Fig 4).

The CCK content in FCG showed two upward fluctuations and the second peak occurred at DAH70, reaching the highest value of 18583.85 ng/L (Fig 3). A continuous upward trend was observed in FTG after the lowest value of 77.41 ng/L on day 9. The large fluctuation of CCK was not detected in FTG during the first 14 days, but the highest value of 3777.26 ng/L appeared on the 15th day, which was still significantly lower than that in FCG. The CCK content in FCG remained significantly higher than that in FTG when compared with the mean values (Fig 4). The final values of both groups were significantly higher than the initial values.

3.1.2 Histology

Intact intestinal chorion and a single layer of enterocytes were observed on the first day of fasting (Fig 5. a), The initial appearance of the intestinal wall was healthy, and goblet cells were located in the enterocytes of the small intestine, the intercellular distribution, muscularis mucosa and basement membrane were tightly arranged, and the connective tissue of the lamina propria was dense. On the ninth

day of fasting (Fig 5. b), there was evacuation between the monolayer of enterocytes of *M. pellegrini*, the number of goblet cells was significantly reduced compared to the first day of fasting and the muscular mucosa was loosely arranged within the chorion. On the 13th day of fasting, partial detachment of the striated border was noted (Fig 5. c), the degree of villus shrinkage became larger and the villus height decreased. Enterocytes were necrotic and sloughed off, the lamina propria of the intestinal mucosa was edematous, and the lamina propria showed a looser shape than on the 9th day of fasting (Fig 5. b). On the 15th day of fasting, complete goblet cells are difficult to observe (Fig 5.d), although there was no significant change in the area of a single goblet cell (Table 1). The intestinal chorion had partially fallen off, the monolayer of columnar epithelial cells was vacuolated and degenerated, and the loss of the striated border was more obvious than on the 13th day of fasting (Fig 5. c). The connective tissue of the lamina propria was loose, and the mucous membrane and the basement membrane were damaged to some extent. The mucosal muscle was loose and deformed, and the gap between the mucosal muscle and the lamina propria became larger on day 15.

Table 1. Effects of short-term fasting on intestinal epithelial cells

	villus height (μm)	villus width (μm)	goblet cells area (μm)	muscle layer thickness (μm)
1	932.47 ± 190.76 ^{a}	264.56 ± 21.28 ^{a}	672.45 ± 161.81 ^{ab}	167.42 ± 65.87 ^{a}
3	762.42 ± 68.75 ^{b}	256.93 ± 38.17 ^{ab}	993.00 ± 398.69 ^{a}	72.39 ± 18.06 ^{b}
9	663.48 ± 126.43 ^{b}	259.12 ± 50.46 ^{ab}	298.17 ± 108.76 ^{b}	52.16 ± 14.45 ^{b}
13	514.05 ± 49.97 ^{c}	221.91 ± 39.68 ^{b}	950.20 ± 529.88 ^{a}	75.30 ± 21.63 ^{b}
15	448.41 ± 87.25 ^{c}	141.26 ± 20.42 ^{c}	552.35 ± 336.93 ^{b}	137.44 ± 22.78 ^{a}

The pancreas of *M. pellegrini* was not evenly distributed in the liver, but independently and diffusely distributed, called hepatopancreas. On the first day of fasting, the hepatocytes were filled with large lipid vacuoles, the nuclei were pushed aside and the cells were compact (Fig 6. a). The liver cells showed obvious cord-like connections, and the dense pancreas was embedded in them by day three (Fig 6. b). By day 9, the volume of the liver cells had significantly decreased, intracellular fat had decreased, intercellular spaces had become loose, cells had become irregularly arranged, cell borders had become blurred, and the alveolar cells of the pancreas had shrunk (Fig 6. c). The degree of vacuolar degeneration of the liver cells increased, the pancreatic cells gradually shrank and became loose, and the nucleoli of the pancreatic cells were deformed and displaced on day 11 (Fig 6.d). After 13 days of fasting, it could be seen that the vacuoles of the liver cells were degenerated, the cell borders were blurred (Fig 6. e), the nucleoli were severely displaced and the zymogen particles were reduced (Fig 6. f). The hepatocytes had almost no cytoplasm on the last day of fasting, the intercellular space of liver cells increased and the vacuoles were almost damaged and ruptured (Fig 6. h). Pancreatic acinar cells gradually shrunk (Fig 6. g).

3.2. Daily rhythm experiment

No significant difference in body length and weight was found in the initial values of the four groups in the daily rhythm experiment, and each group was not statistically significant throughout the process.

The CCK content of the first group (Fig 7. A) peaked at 17:00, which was the only peak at this time among the four groups, then showed a downward trend until an upward trend began after the lowest level appeared at $1:00^{+1}$, the CCK content at $1:00^{+1}$ and $5:00^{+1}$ was significantly different from other nodes. The trypsin activity of group A tended to increase after the first feeding and to decrease after 17:00, but there was no significant difference between the trypsin activities in this group.

The CCK content of the second group showed an increasing and then decreasing trend and peaked at 21:00 (Fig 7. B), the value of this point being significantly different from that of the other three groups. A significant decreasing trend was found after 21:00, which was significantly different from the trypsin activity at $5:00^{+1}$. A difference was found between 21:00 and $9:00^{+1}$ which was different from the other three groups.

Two peaks of CCK at 13:00 and $1:00^{+1}$ were found in group C (Fig 7. C), and the CCK content at $1:00^{+1}$ was significantly increased. Trypsin activity peaked at 13:00 and showed a continuous downward trend until $1:00^{+1}$. The trypsin activity in group C showed the highest level in the first 12 hours than the others.

The CCK content of the fasting group (Fig 7. D) reached a maximum at $1:00^{+1}$ and continued to fall to a minimum at $9:00^{+1}$. The 17: 00 value of this group was significantly lower than that of the third group. The CCK content at $9:00^{+1}$ was 236.01 ng/L, significantly lower than that of the second and third groups. Two peaks of trypsin activity were detected at 17:00 and $1:00^{+1}$ in the fasting group. The peak at $1:00^{+1}$ was significantly higher than that before and after, and there was a significantly low level at $5:00^{+1}$. The change in CCK content and baseline at $5:00^{+1}$ was less than that at $9:00^{+1}$.

4. Discussion

The aim of this study was to investigate the feedback-regulatory effects of short-term fasting and different feeding frequencies in a single day on the secretion of CCK and trypsin in juvenile *M. pellegrini*. The initial body weight of the juveniles was 183.75 ± 61.16 mg. This was not strictly a growth test, but the FCG still showed a better growth trend. This may indicate that the experimental individuals were still in the early stages of ontogeny.

4.1 Short-term fasting trial

Fasting directly damages the structure of the epithelium of the digestive tract and the hepatopancreas. As the duration of fasting increases, damage to the digestive system correlates approximately positively with the degree of hunger. Although there are many reports on the fasting experiment in Cyprinidae, there

are relatively few studies on the fasting experiment at the early stage of individual development. In order to obtain more reasonable experimental data, this study selected *M. pellegrini*, which has entered the juvenile stage, as the test object. The SGR results showed that the SGR of the fasting group was only half that of the control group, indicating that at the juvenile stage of *M. pellegrini*, the individual was in the rapid development stage of nutrient accumulation. Under the premise of feeding the same commodity feed, hunger has become a direct obstacle to individual development and the most important negative factor. For this reason, the combination of ELISA detection of enzyme activity with histological observation of the intestinal tract and hepatopancreas has become essential for a more intuitive analysis of the correlation between changes in tissue structure and fluctuations in CCK and trypsin in the fasting experiment.

CCK is secreted by intestinal epithelial cells. In this study, fasting for 15 days was more severe on intestinal tissue damage, especially in the post-intestinal mucosa. damage to the lamina propria and muscle layer may lead to intestinal peristalsis and the efficiency of digestion and absorption of food. Damage to the hepatopancreas leads to the regulation of body fat and glycogen supply, and glycogen storage in the hepatopancreas is completely replaced by gluconeogenesis. However, experiments have shown that short-term fasting causes more damage the mucosal cells in the intestine than to the hepatopancreas.

It has been reported that a short fasting period has no effect on the nutritional status of juvenile *M. pellegrini*, but prolonged fasting may cause the starvation effect to continue to act on the fish, thereby affecting the nutritional status of juvenile *M. pellegrini* (Li et al., 2015). The reason why the monolayer columnar epithelial cells in the intestinal chorion had a relatively fast metabolism and were more susceptible to changes in food intake, such as shedding and damage, is that fasting for 15 days did not make the *M. pellegrini* individuals truly hungry. As the hepatopancreas was a relatively stable and reproducible digestive organ, and the juvenile *M. pellegrini* was in a state of highly developed organs, 15 days of fasting may not be enough to cause serious pathological changes in the hepatopancreas, resulting in irreversible effects on digestion and metabolism.

Although the trypsin activity in the fasting group showed a trend of decreasing first and then increasing, in general, the trypsin activity in the fasting group was lower than that in the normal feeding group. The experimental data showed that the level of CCK reached the lowest level on the ninth day of the fasting group, which was consistent with the trend reported in *Trachinotus blochii* (Ye et al., 2019) about the increase in fasting, the overall degeneration of the fish digestive tract and its accessory glands can be caused, but the lowest value appeared with a slight lag. However, in our experiment, the content of CCK showed a downward trend within one week of fasting, but there was no significant difference, and it did not reach a very low value until the ninth day. Differ from *Atlantic herring* (Rojas-García et al., 2011), *yellow catfish* (Gong et al., 2013), *Megalobrama amblycephala* (Ji et al., 2015), and *Salmo salar* (Murashita et al., 2008), which were observed that the expression of CCK decreased significantly after 72 hours of fasting. Mature individuals were usually used by those studies that the enterocytes were more

sensitive to the secretion response of CCK, which may be the reason why our experiment was different from other results.

According to the HE staining results, the intestinal tissue structure was damaged on day 9. The CCK content gradually decreased during the fasting period and dropped to a significantly low level on day 9, which was consistent with the trypsin enzyme activity and histological results. Due to the lack of dietary nutrient intake, the fat not metabolized in the early stage was deposited in the hepatopancreas, causing the nucleus to shift and vacuoles to appear. The CCK content and trypsin enzyme activity of FCG showed an upward trend on day 11, and the CCK content was significantly higher than the previous point, which may be related to the growing weight of FCG showed a strong upward trend on day 11 (DAH70). The CCK content of FTG showed a highly significant increase on day 11, and the trypsin enzyme activity was also higher than the initial value of the experiment on day 11, suggesting that the juveniles had reached the mature stage and the degree of fasting was not enough to affect their normal development process. Trypsin activity was also significantly higher on day 11 at the beginning of the experiment. Meng Q et al. (Meng and Tang, 1986) pointed out that the development of olfactory bulbs, intestines, gills, and other organs of *M. pellegrini* during the same period became more and more mature. The development of the fish was not only gradual and continuous but also intermittent and sudden. This change in the growth period may explain why the CCK content and the trypsin activity level recovered simultaneously after reaching the lowest value on the ninth day. Li W et al. (Li et al., 2005) reported that *M. pellegrini* entered the adult stage on the 68th day after hatching, which may also explain the phenomenon of our experiment that the individuals were in the adult stage, but this node was slightly later than the mentioned node.

In this experiment, juvenile *M. pellegrini* was small in size, and the short-term fasting time was not long enough for CCK and trypsin to have obvious negative feedback. Subsequent experiments will extend the fasting time under the same experimental conditions to explore the functional damage caused by the damage of the digestive structure and add CCKR monitoring, in order to start to explore the problems that have not been understood in this experiment.

4.2 Daily rhythm experiment

The feedback regulation of CCK involved in exogenous feeding has been confirmed by Polakof and Khoa, et al (Kurokawa and Suzuki, 2002; Polakof et al., 2011). To explore the potential regulatory feedback between intestinal trypsin and CCK in the short-term stable state of juvenile *M. pellegrini*, such as a gastrointestinal regulatory loop during *M. pellegrini* development, and whether the regulatory loop is affected by feeding frequency. The cholecystokinin-releasing peptide secreted by the small intestinal mucosa is very sensitive to trypsin, and trypsin can inactivate the CCK-releasing peptide. Therefore, after the CCK-releasing peptide has caused CCK release and pancreatic enzyme secretion to increase, trypsin will inactivate it again, thereby feedback-regulating the further secretion of CCK and pancreatic enzymes. The physiological significance of feedback regulation of pancreatic enzyme secretion is to prevent excessive secretion of pancreatic enzymes.

According to this report, the endogenous rhythm throughout the day was independent of dietary treatment or short-term fasting, which is consistent with the results in sea bass (Galaviz et al., 2011). The negative feedback loop between CCK and trypsin has also been demonstrated in the development and experiment of *Senegalese sole* juveniles (Neda et al., 2021), *Gadus morhua* larvae (Tillner et al., 2013), and *Clupea harengus L* (Rojas-García et al., 2011). The fluctuation of trypsin was not much different from that of CCK content, which may be because the hepatopancreas was in a rapid developmental stage, whereas CCK as a satiety factor responds quite quickly to hunger, resulting in negative feedback between the two without obvious synchronization, which was different from the cosine curve of *Senegalese sole* juveniles reported by Neda Gilannejad et al (Rojas-García et al., 2011).

The CCK of group A fed once in the morning showed an extremely low value at $1:00^{+1}$, which was close to the extremely low value of group D at $9:00^{+1}$, indicating that the single feeding may have favored the stimulation of larval hunger. The CCK content of the different feeding strategy groups all showed a downward trend during the night. The C group showed an extremely high peak which was more than twice as high as the B group. This peak may be due to the fact that the feeding frequency met their satiation needs, so there was no extreme hunger at night. What is difficult to explain is that the completely fasting group (D) and the twice fed group (B) both had a peak at night with no statistical difference, but the fed group (B) was slightly higher than (D) and the peak occurred earlier than (D). Regular feeding in the early stages causes the secretion of the digestive glands in the individual digestive system to have a biological rhythm, so that even in a state of complete fasting, CCK will still show a changing trend similar to that after ingestion in the short term, which becomes the explanation for this phenomenon. The fasting period in group D was longer than in the others, but there was no significant difference between the CCK content at $9:00^{+1}$ and the initial value. Although the CCK level in group D was presumably due to the longer fasting time, which led to the forward shift of the decline time node, it showed a closed-loop adjustment level between the baseline and $5:00^{+1}$, then continued to show a downward trend, but no significant decrease. Therefore, the closed-loop feedback regulation of this test group could be considered to end at $5:00^{+1}$, which was the sixth sampling point. This may be since under relatively long time fasting conditions, the secretion level of CCK was greatly reduced, resulting in a decrease in the sensitivity of CCK secretion compared to the other three groups, which led to the forward shift of the time point of closed-loop regulation in the D group.

This was similar to the observation of Carlos R. et al. (Rojas-García et al., 2011) that CCK is involved in the regulation of the digestive process of herring larvae, but CCK did not seem to have a circadian rhythm independent of feeding and no rhythm of CCK secretion was found without food stimulation. It is worth noting that synchronization was found between the peak of CCK levels and the peak of trypsin activity in each group.

Whether the feeding situation is better or not may be reflected in the secretion of trypsin. The trypsin in group C showed a downward trend at night, which was consistent with the situation after several feedings by Robert Tillner (Tillner et al., 2014). At the same time, the C group had higher trypsin levels

during the day than the other three groups. It can be assumed that this feeding frequency can better activate the digestive process and is a superior feeding schedule. In the test group fed three times a day, the trypsin level during the day was at the highest level, so it could be inferred that feeding three times a day was more conducive to this growth stage of *M. pellegrini* Growth and development, which was consistent with studies on *Arapaima gigas* (Pedrosa et al., 2019), *Coreius guichenoti* (Zhao et al., 2022), *Micropterus salmoides* (Sun et al., 2023), *Oncorhynchus mykiss*(Mozhdeh et al., 2022), *Ctenopharyngodon idellus* (Wu et al., 2021) and others.

Overall, in the four daily rhythm experiments with different feeding frequencies, there was no significant difference between the initial value and the final value of CCK and trypsin after 24 h, which means that the short-term change of feeding strategy did not affect the regulation mechanism of trypsin and CCK in juvenile fish. It is worth mentioning that, except for the fact that the final CCK value of group B tended to increase compared to the initial value and the final CCK value of group D tended to decrease compared to the initial value, there was no extremely significant difference between the initial and final data of CCK and trypsin in the same group, which provided a reliable basis for the subsequent discussion of the closed-loop regulation of the two parameters in the daily rhythm. Although the negative feedback regulation between CCK and trypsin may not show very sensitive negative feedback regulation in the short-term fasting test due to the change in developmental stage, the feedback regulation of CCK in the gut of *M. pellegrini* was very sensitive in the daily rhythm test, and the signal feedback for satiety was fast, which is consistent with the findings that the high expression of CCK in golden pompano (Ye et al., 2019) was associated with appetite control. The biorhythm levels of CCK and trypsin would change over the course of long-term individual development, although the fluctuations would only exist in different periods of a single day. At the same time, we speculated that after a certain period of fasting, due to functional damage to the digestive system, the secretion levels of CCK and trypsin would be reduced and the feedback regulation of the two would be affected accordingly. An appropriate feeding strategy could maintain normal levels of trypsin and thus normal digestive function. Based on the results of this experiment, it was recommended that *M. pellegrini* juveniles be fed three times a day during this period to maintain normal trypsin secretion levels.

The results of this study provided a more complete understanding of the feedback regulation mechanism of CCK and trypsin in the short-term fasting and one-day rhythm of *M. pellegrini* and provided a basis for optimizing feeding and feeding programs.

5. Conclusion

In the short-term fasting trial, there was no obvious negative feedback phenomenon between CCK and trypsin, and the damage to the digestive organs was relatively mild. It can be inferred that the 15-day fasting was not enough to cause *M. pellegrini* to produce real hunger. The daily rhythm experiment in the same period showed that CCK and trypsin had a negative feedback regulatory loop in the circadian rhythm of *M. pellegrini*, and feeding three times a day may produce better growth effects and a single feeding in the morning may induce hunger earlier than fasting throughout the day. Based on the two

trials, it was speculated that during the rapid development of juvenile *M. pellegrini*, the fluctuation of CCK and trypsin activity in each day had no obvious relationship with the rhythm during the highly growth level for a long time.

Declarations

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Author contribution statement

Conceptualization: Q Li, H Tang, W Li. Sampling: W Li, X Lin, K Zhang, W Zhang. Data analysis: W Li, X Lin. Preparation of figures and tables: W Li, X Lin. Conducting the research, data interpretation, writing: W Li, Q Li, H Tang. Supervision: Q Li, H Tang.

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Declaration of Competing Interest.

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

Data Availability Statement

The data of the present study are available from the authors upon reasonable request.

Conflicts of Interest Statement

The authors declare no conflict of interest.

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Figures

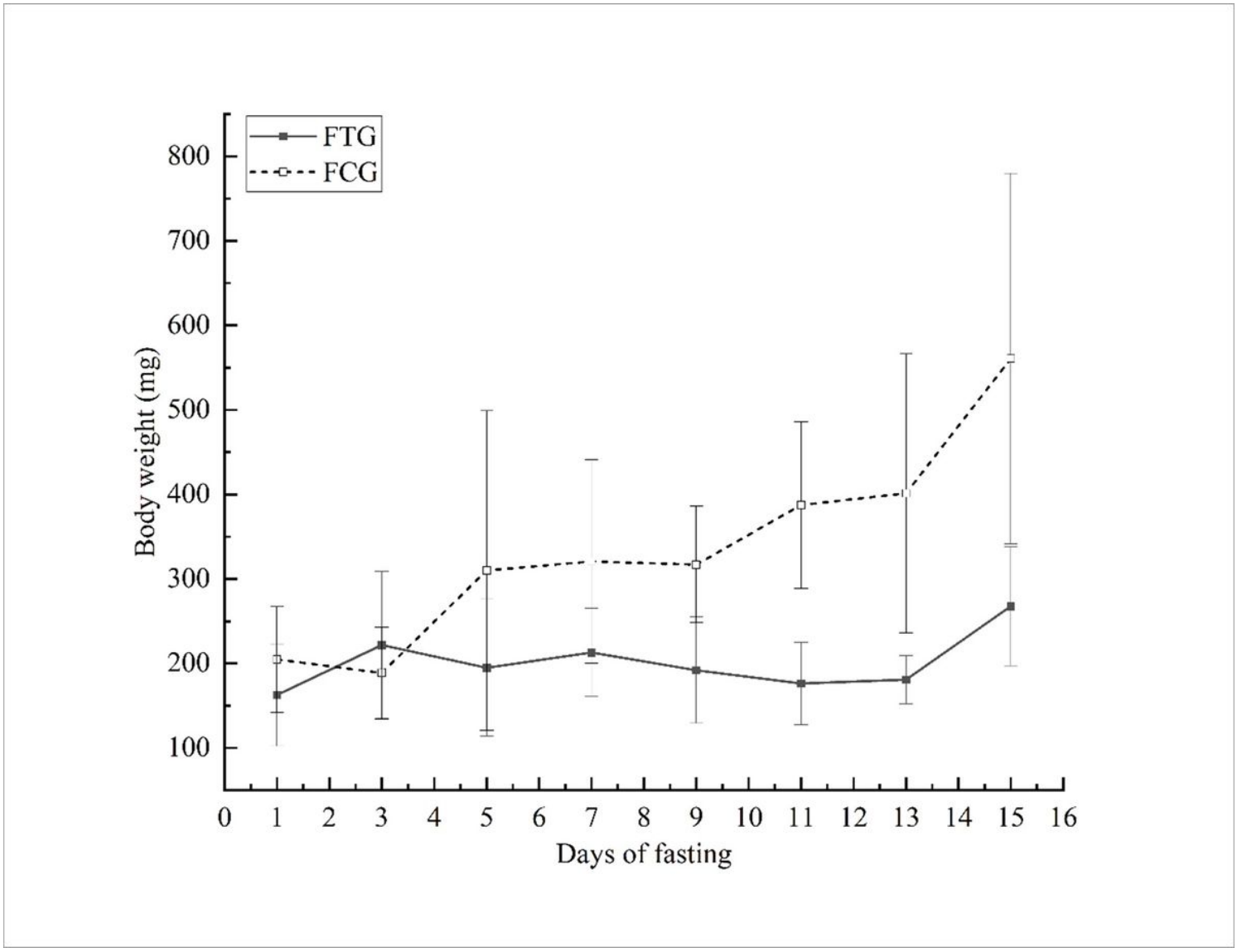

Figure 1

Body weight (mg) of FTG and FCG within 15 days of the fasting trial. Feeding in FCG was performed as usual at 9:00, 13:00, and 17:00 three times a day. The dotted line represented FCG, and the solid line represented FTG. Data were presented as mean ± standard deviation (n = 3 individuals, all groups n = 3

tanks). and $P < 0.05$ is used. The growth rate and specific growth rate during the experiment were as follows ("-T" denotes FTG, "-C" denotes FCG):

WGR(%) = (FBW-IBW) /W_0

SGR(%) =[(lnFBW - lnIBW) / t]×100%

WGR-T=64.56%

SGR-T=3.32%

WGR-C=173.8%

SGR-C=6.72%

Including FBW is the final weight of the juvenile. IBW is the initial weight of the juvenile and 't' is the time.

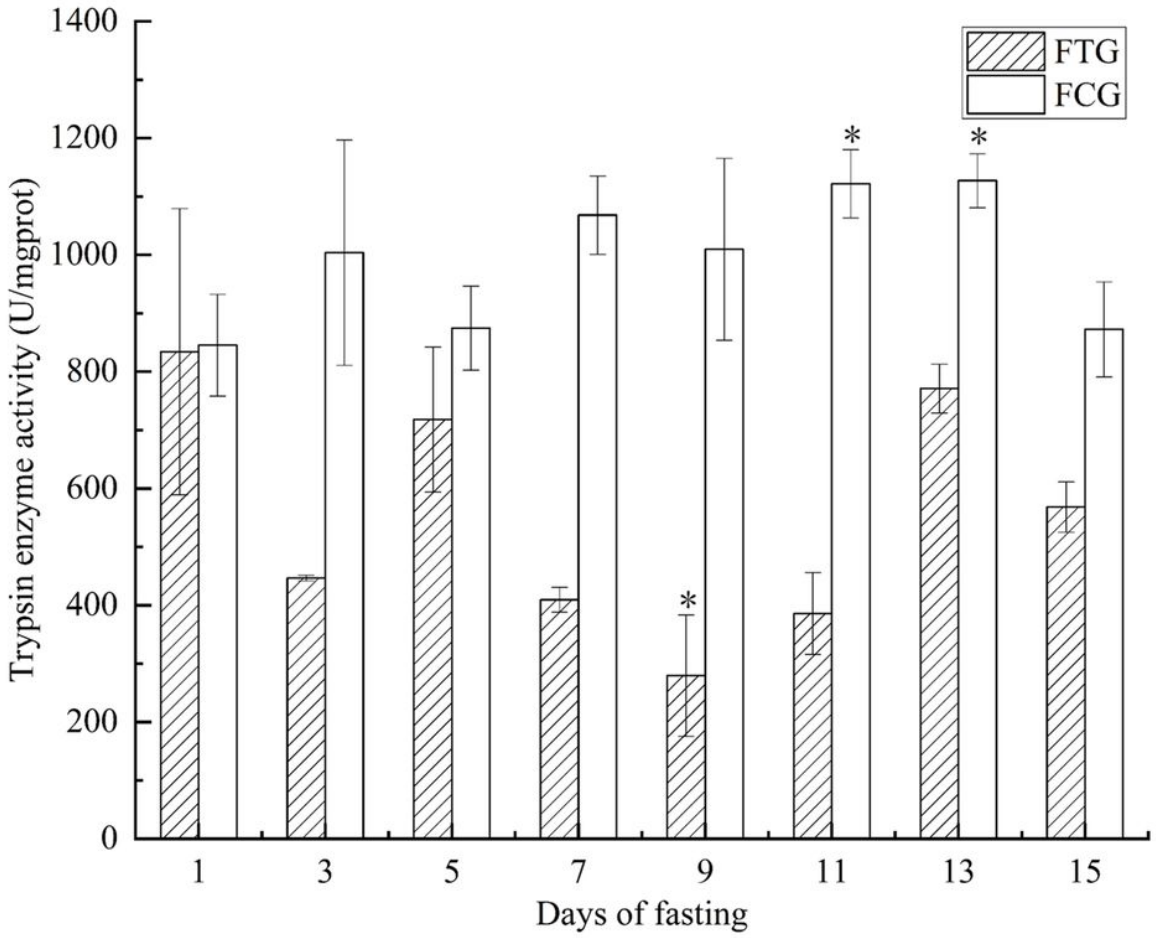

Figure 2

The trypsin activity (U/mgprot) of FTG and FCG of the fasting study. The grey stripe indicates the experimental group and the white stripe indicates the control group (feeding at 9:00, 13:00, and 17:00, three times a day as usual). Data are presented as mean ± standard deviation (n = 3 individuals, all feeding treatments n = 3 tanks) and P < 0.05 is used, "*" represents the significant difference within a group.

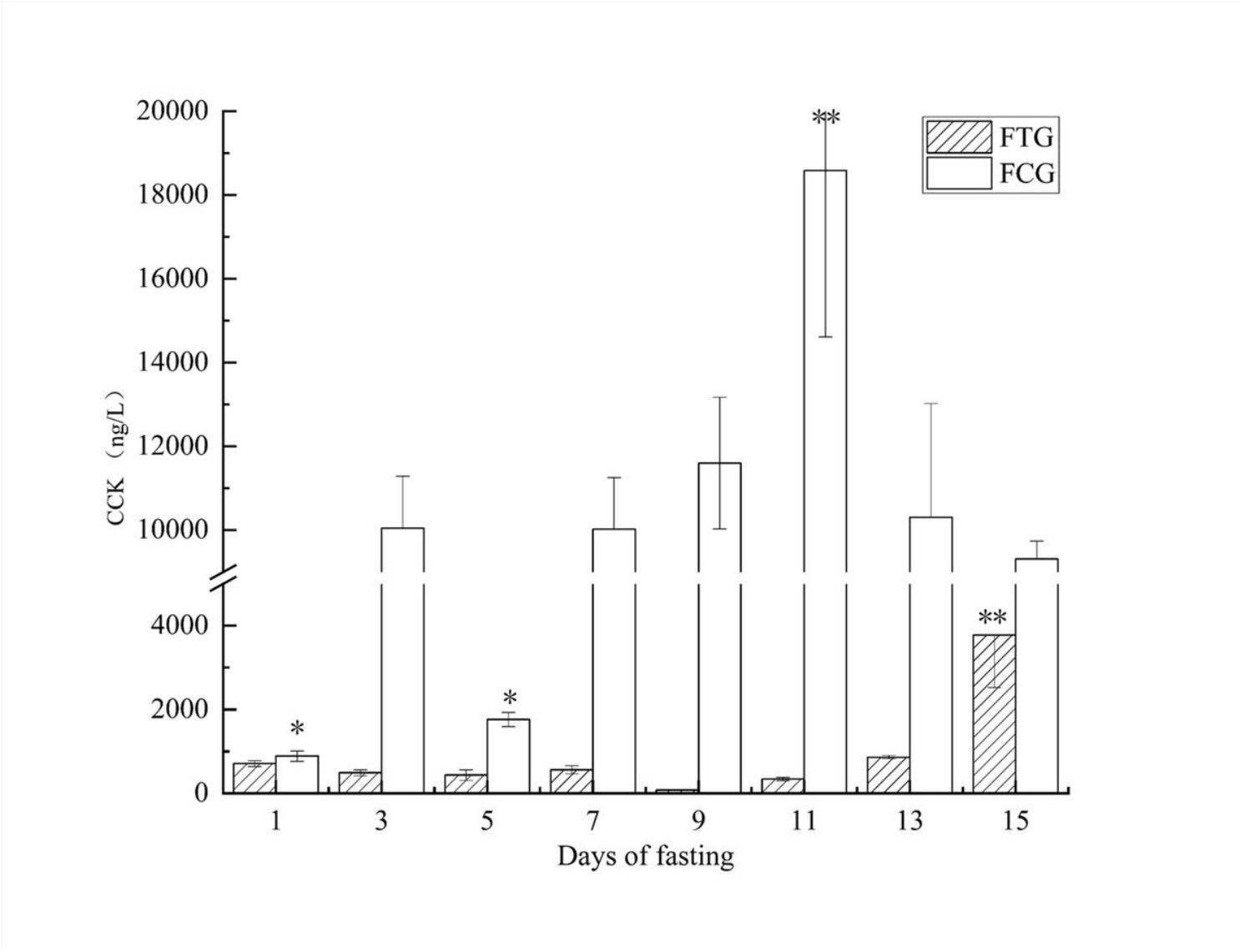

Figure 3

CCK content in FTG and FCG of the fasting trial. The grey strip indicated FTG and the white strip indicated FCG (feeding at 9:00, 13:00, and 17:00, three times a day as usual). Y-axis coordinates were processed with breakpoints between 5000 and 9000 scales. Data are presented as mean ± standard (n = 3 individuals, all feeding treatments n = 3 tanks), and $P < 0.05$ is used, "*" represents the significant difference within a group, ' ** ' indicates an extremely significant difference.

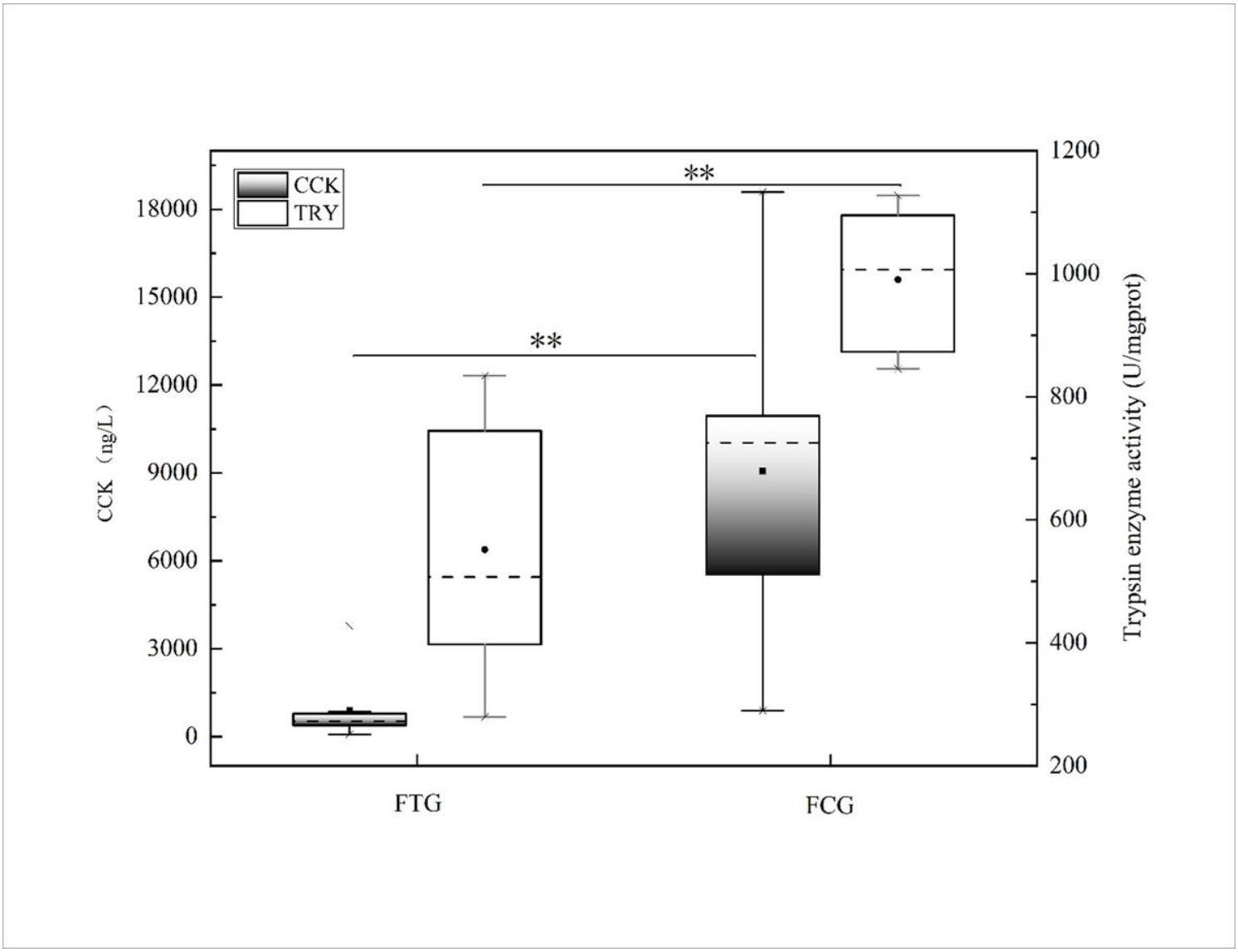

Figure 4

CCK content and trypsin activity of FTG and FCG are statistically analyzed between groups. Dark grey represents CCK content, white box line represents TRY. Data are presented as mean ± standard and $P < 0.05$ is used, ' ** ' indicates an extremely significant difference.

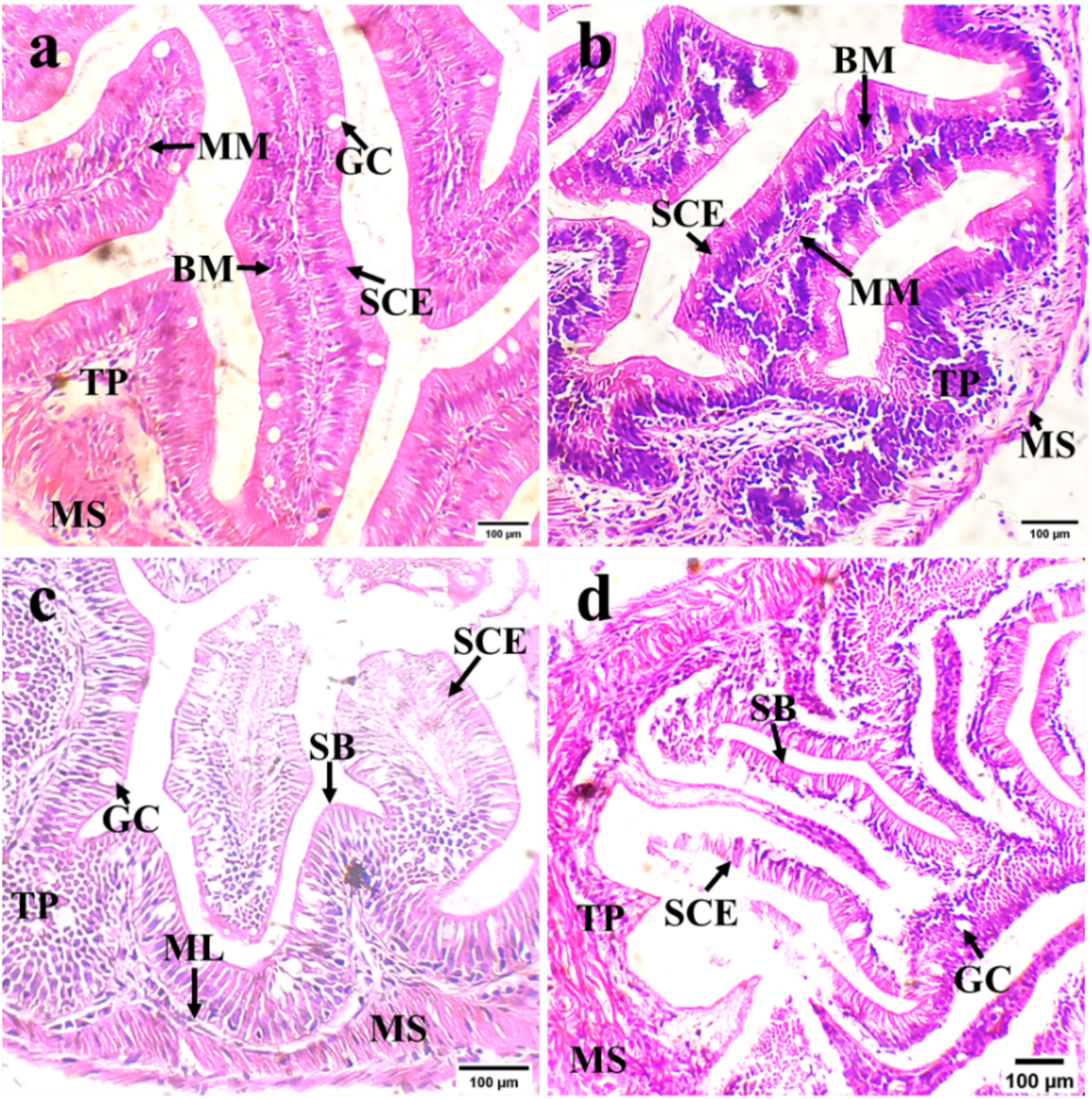

Figure 5

The first day of the fasting trial intestinal (middle and anterior intestinal tract) corresponds to figure a, figure b is the ninth day of fasting intestinal slices, figure c is the 13th day of fasting intestinal slices, and the 15th day of fasting intestinal slices was shown in figure d. HE staining, microscope eyepiece (× 40) observation.

MS: muscular, SCE: simple columnar epithelium (enterocytes), ML: mucous layer, BM: basement membrane, TP: tunica propria, SB: striated border, MM: muscularis mucosae, GC: goblet cell

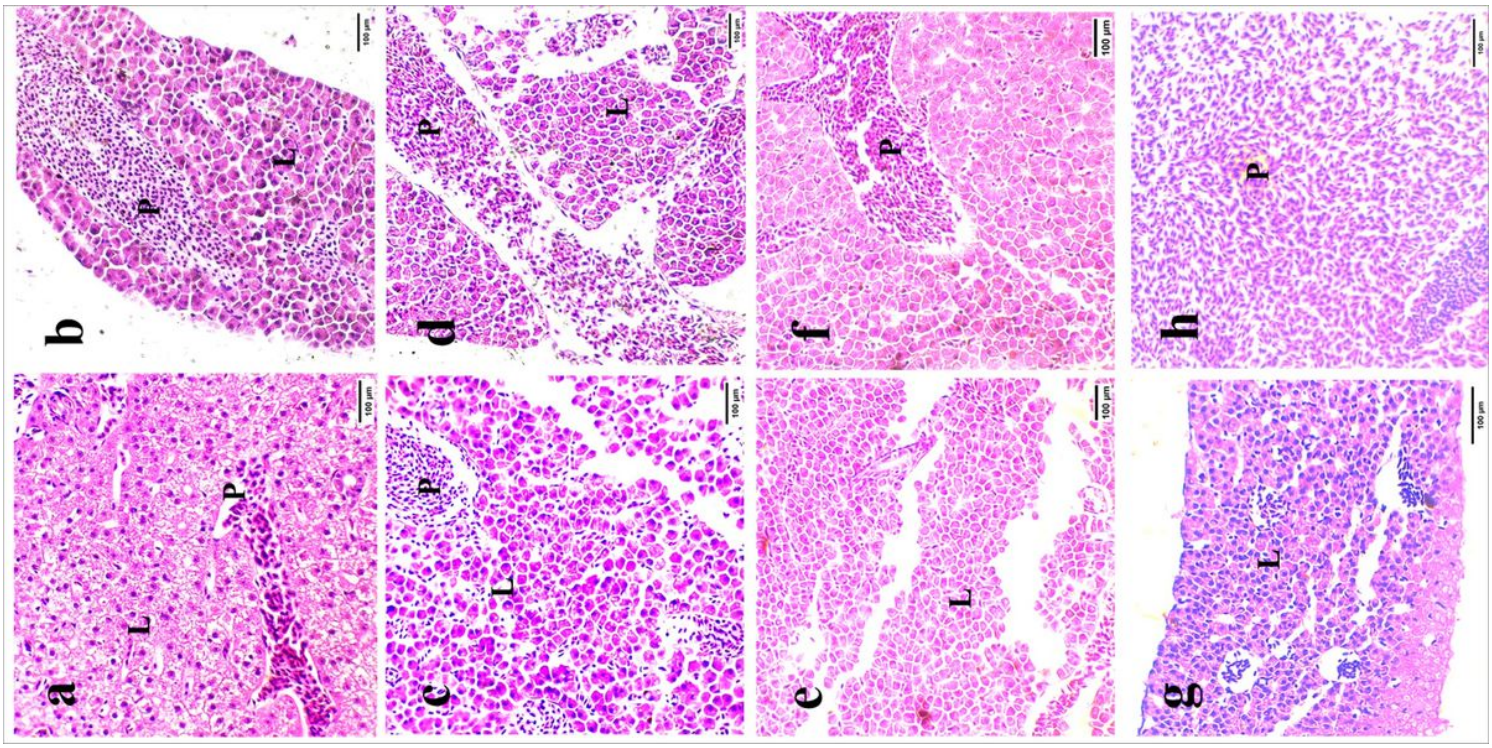

Figure 6

Hepatopancreas sections on days 1 and 3 of fasting are shown in a and b. Hepatopancreas sections on days 9 and 11 are shown in c and d. Hepatopancreas sections on day 13 of fasting are shown in e and f. Hepatopancreas sections on day 15 of fasting are shown in g and h. HE staining, microscope observation (× 40).

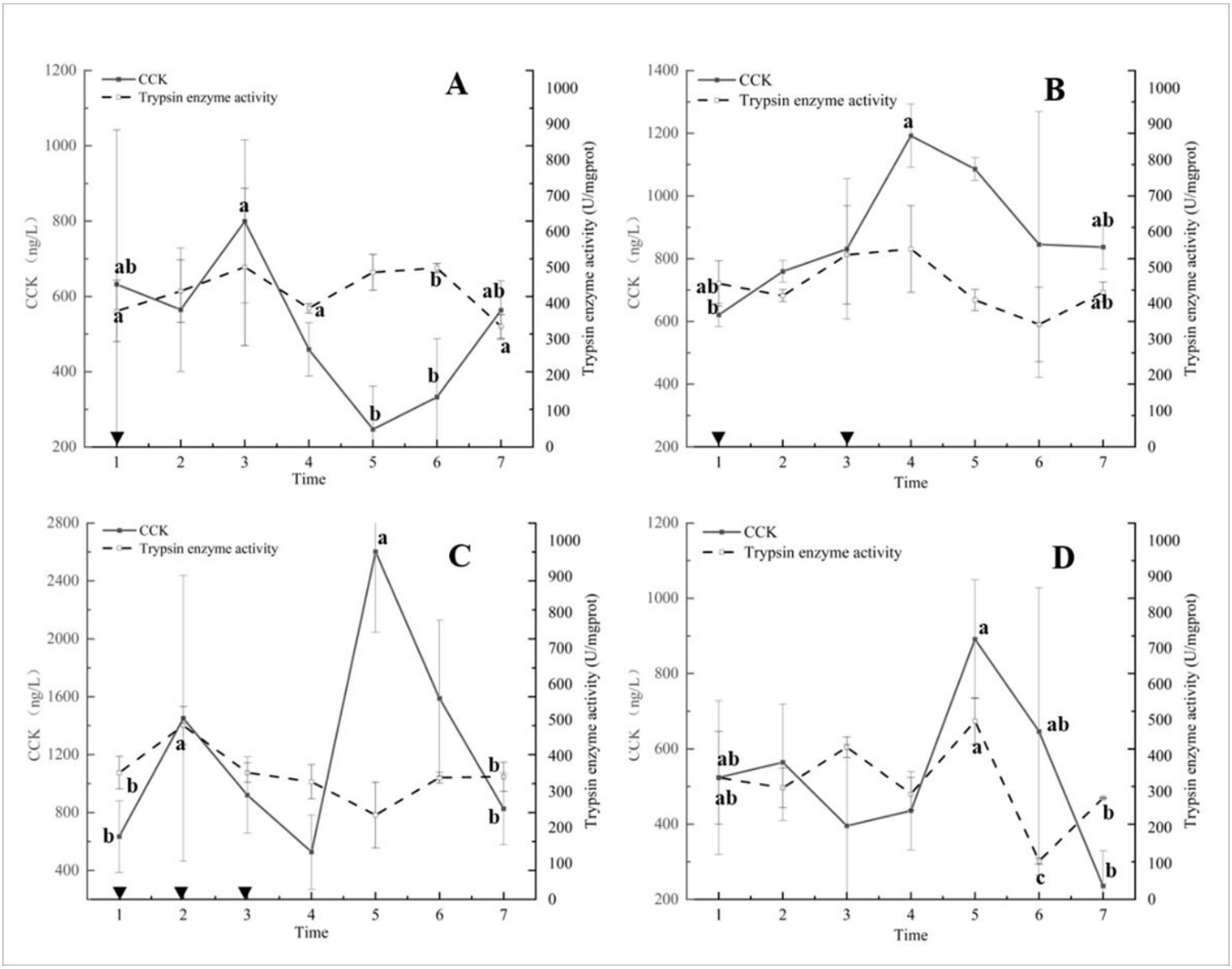

Figure 7

The line graph of changes in CCK and trypsin within 24 hours. In the four daily rhythm experimental groups. "▼" indicates the feeding point, A has one feeding point, B has two feeding points, C is the normal feeding control group and has three feeding points, and D is the fasting trial group, of which 1, 2, 3, 4, 5, 6 and 7 correspond to 9:00, 13:00, 17:00, 21:00, 1:00 $^{+1}$, 5:00 $^{+1}$ and 9:00 $^{+1}$, respectively. There are three sets of Y-axes in each graph and data are presented as mean ± standard. Different superscripts indicate a significant difference in multiple comparisons ($P < 0.05$).