



Correlation between digestive system development and feedback regulation of cholecystokinin and trypsin in *Megalobrama pellegrini* larvae and juveniles

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ABSTRACT

Megalobrama pellegrini (Tchang, 1930) are endemic economic fishes in the upper reaches of the Yangtze River, and the current understanding of their early development in captivity is minimal. To enhance the breeding of *M. pellegrini* in a recirculating aquaculture system (RAS), this study examined the development of the digestive system, fluctuations in cholecystokinin (CCK) levels, and tryptic enzyme activity during the early developmental stages of *M. pellegrini*—additionally, the research aimed to explore the correlations among these variables. A combination of histological, biochemical, and molecular methods was employed to detect *M. pellegrini* larvae from hatching up to 50 days after hatching (DAH). At 4DAH, the oropharyngeal cavity was opened, and the larvae entered the exogenous nutrition stage at 6DAH. At 12DAH, the functional structures of the intestinal tract gradually developed, the hepatocytes became uniform in size, and the nuclei of the pancreatic cells were clearly visible. At 24DAH, the intestinal tract appeared coiled and curled, and the gyrus was clearly visible. At 42DAH, the digestive tract and hepatopancreas structures became perfect, and the larvae entered the post-larvae stage at 45DAH. A negative feedback regulation was identified between CCK content and trypsin activity; however, the secretion pattern was oscillated by the development of the digestive system structure. In addition, the distribution of CCK in the digestive system of *M. pellegrini* at 60DAH was determined by in situ hybridization, and it was found that CCK was mainly concentrated in the foregut. The findings of this study can help optimize feeding strategies for the early developmental stages of *M. pellegrini*. They provide theoretical and practical foundations for the aquaculture of *M. pellegrini* in RAS and contribute to the research on freshwater fish concerning CCK.

1. Introduction

Megalobrama pellegrini belongs to the order Cypriniformes, Cyprinidae, Cultrinae, and *Megalobrama*. *M. pellegrini*, an economically valuable fish endemic to the upper reaches of the Yangtze River, possesses high nutritional value and considerable potential for aquaculture. Its prospects for aquaculture are extensive. The recirculating aquaculture system (RAS) facilitates intensive, high-yield farming while ensuring long-term environmental stability. With the implementation of the Yangtze River 10-year fishing ban, it has become a major research hotspot to find out how high-quality aquatic products, such as *M. pellegrini*, can be cultured in an indoor recirculating water system to maintain production and increase income, and how to meet the growing consumer demand for high-value aquatic products.

One of the biggest challenges in the early developmental stages of *M. pellegrini* culture is to optimize feeding strategies. The digestive system of most fish at hatching is very rudimentary and has limited digestive capacity (Rønnestad et al., 2013). The early development of the digestive system is characterized by major developmental nodes (Khao et al., 2021a), and the developmental characteristics can be used to effectively judge the growth process of fish and adjust the culture management strategy promptly. The study of the anatomical configuration of the digestive system serves as a fundamental framework for comprehending and investigating the digestive physiology of fish. The exploration of the occurrence pattern of digestive enzymes and related metabolic factors in fish constitutes a pivotal study in the field of fish digestive physiology, particularly in the early stages of their life history. The secretion of various digestive enzymes is not entirely synchronized

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and varies according to the developmental differentiation of their main major secretory and absorptive organs (Ghasemi et al., 2020). Studies have shown that cholecystokinin (CCK) and trypsin, as digestive enzymes and hormones in the process of digestive metabolism, directly affect the feeding behavior of individuals (Tillner et al., 2013; Volkoff, 2006). Reports focusing on the physiological changes of CCK to explore the effects of fish development and immune response have gradually increased in recent years, e.g., Yuko Kamisaka investigated the distribution of cholecystokinin-immunoreactive cells in the digestive tract of larval teleost (Kamisaka et al., 2003); and Murashita examined the distribution of two isoforms of CCK in Atlantic salmon (Murashita et al., 2009). Trypsin is a highly specific protease and one of the most important digestive enzymes in the early developmental stages of fish. Researchers have demonstrated that there is a regulatory loop between CCK and tryptic enzyme activity (Tillner et al., 2014, 2013), and the loop can effectively respond to the physiological effects of feeding and growth of fish. Therefore, the combination of the two patterns of change and the observation of the structural development of the digestive system is more conducive to understanding the pattern of change in fish's digestive system. However, most studies on the early developmental stages, including CCK and trypsin secretion, have focused on marine fishes, and the distribution of CCK is still controversial.

In this study, we investigated the histological structure of the digestive system, CCK content, and tryptic enzyme activity in the early developmental stages of *M. pellegrini*, to understand the process of structural and functional improvement of the digestive system, the regulatory mechanism between CCK and trypsin, and to investigate the correlation between the structure of the digestive system, CCK content, and tryptic enzyme activity. We also used in situ hybridization to determine the distribution of CCK in the digestive system of *M. pellegrini*. The aim is to provide a theoretical basis for future research on the culture pattern and feeding strategy of *M. pellegrini* during early development in the RAS.

2. Materials and methods

2.1. Experimental fish and feeding trial

M. pellegrini larvae were obtained from artificial insemination broodstocks of Dongping Aquatic Products Co., LTD, Chongqing, China. After hatching, the larvae were cultured in the RAS (Fig. 1) randomly at 1DAH following treatment with 5000 ppm salt water for half an hour. Three parallel glass tanks were utilized, with an initial population of approximately 1200 larvae per tank (32 L).

During the experiment (1–50DAH), the following indicators were monitored once a week: dissolved oxygen was maintained at 5–8 mg/L, water temperature was controlled at 28 ± 0.5 °C, illumination was maintained at 1400 lx daily from 7:00–19:00 with LED tubes, ammonia nitrogen level was ≤ 0.1 mg/L, and nitrite nitrogen level was ≤ 0.02 mg/L. The larvae were fed to apparent satiation three times a day (9:00, 13:00, and 17:00). From 4DAH to 7DAH, the larvae were fed with fresh *Artemia salina* larvae and yolk. At 8–40DAH, *M. pellegrini* was fed with the mash feed (crude protein ≥ 40.0 , crude fat ≥ 3.5). At 41–50DAH, the mash feed was substituted for pelleted feed (crude protein ≥ 36.0 , crude fat ≥ 3.0). The remaining bait was removed after each feeding. Dead fish were cleaned up daily, and data was recorded.

2.2. Growth and morphology

The larvae were sampled at 1–6, 9, 12, 15, 18, 21, 24, 27, 30, 35, 40, 45, and 50DAH regarding the table of postembryonic developmental stages and chronology of *M. pellegrini* (Table 1) (Jing et al., 2005). Sampling was completed prior to feeding in the morning, and individual samples were anesthetized with tricaine methanesulfonate (MS-222, 120 mg/L). At each sampling time point, 20 larvae were randomly collected. Before 12DAH, the total length (mm, from the tip of the maxilla to the end of the notochord) was measured by an SRE-1030 body microscope via Motic Images Plus 3.1 (x64) on the computer terminal. At 12–50DAH, the total length was measured by a vernier caliper. Throughout the experiment, the larvae were dissected and observed periodically using an SRE-1030 body microscope to obtain the

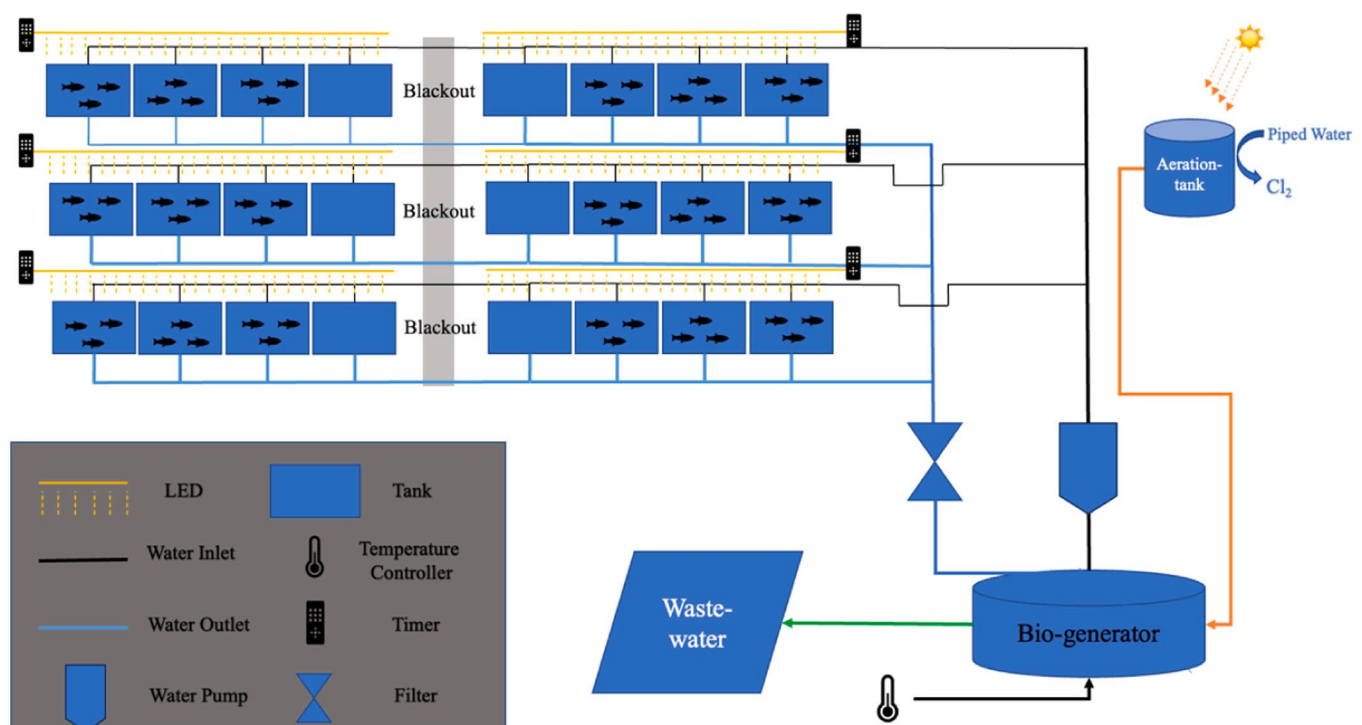


Fig. 1. The diagram of the recirculating water aquaculture system.

Table 1
Postembryonic development stage and schedule of *M. pellegrini*.

period	stage	time	water temp.
larvae	Hatching stage	0 h	24.5
	Rudiment of pectoral fin stage	12 h	24.5
	Gill arch stage	18 h	24.2
	Xanthic eye stage	34 h	23.2
	Melanoid eye stage	54 h	24.5
	Emergence of air bladder stage	80.5 h	24.8
	One chamber air bladder stage	89.5 h	24.8
	Exhaustion of yolk stage	115.5 h	25
post-larvae	Differentiation of dorsal fin stage	8.5d	24.5
	Caudal tip lifting stage	11.5d	24
	Two chamber air bladder stage	20.5d	24.5
	Formation of dorsal fin stage	25d	24.8
	Formation of anal fin stage	28d	24.8
	Formation of ventral fin stage	35d	24.2
juvenile	Emergence of scales stage	47d	23.8
	Scales formed stage	68d	24.1

epiphenomena of growth and development.

The growth parameters were calculated as follows:

$W = aL^b$ (the relationship of the body weight and the total length),

WRG (%), growth rate) = $(W_t - W_0) / W_0$,

SGR (%), specific growth rate) = $[(\ln W_t - \ln W_0) / t] \times 100$ %.

In these calculations, a is the determining coefficient, b is the index representing the isometric distance, W_t means weight at the time of measurement and W_0 means initial weight.

2.3. Histology and in situ hybridization analysis

Fifteen larvae were sampled randomly each sampling day before the morning feeding and individuals were anesthetized with MS-222 (120 mg/L). The samples were fixed in Bouin's solution for 12 h and transferred to the laboratory for subsequent histological processing. The samples were washed with 70 % ethanol, dehydrated through a graded ethanol series, and embedded in paraffin. Sagittal Section (5 μ m) were cut on a conventional slicer (RM2016, Shanghai Leica Instrument Co., Ltd.). The paraffin section was followed by hematoxylin-eosin (H-E) staining. Tissue sections were observed by a light biomicroscope (B302, Chongqing Aote Optical Instrument Co., Ltd.) and analyzed with a Leica four-camera digital camera and Image J.

Following the conclusion of the breeding trials, samples were collected randomly at 60DAH (the termination of the post-larval stage) for in situ hybridization to investigate the distribution of CCK in the digestive system. Mouse anti-Digoxin-labeled peroxidase (anti - DIG - HRP) was selected for CCK in situ hybridization, and the paraffin section DAB colorimetric in situ hybridization (CISH) kit (Sevier, Wuhan) was used. The hybridization probe was synthesized by Wuhan Sevier Biotechnology Co., Ltd, and the probe sequence was TGCGGTATGTACCTTGGTGGAGATGATTCTGG.

2.4. Analysis of CCK content and trypsin enzyme activity

The larvae were randomly sampled before the morning feeding and individuals were anesthetized with MS-222 (120 mg/L). The sample sizes were taken as shown in Table 2. Individual samples were analyzed for CCK and tryptic enzyme activity using a combination of highly

sensitive methods reported by Neda Gilannejad (Gilannejad 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 larvae excluding the head, back muscles, and tail. Due to the complexity of dissecting the digestive tract of the larvae at 1–15DAH, whole-body samples were taken for homogenization. After 18DAH, individuals were placed on ice trays, the head, tail, and dorsal musculature were cut off, and only the abdominal samples were taken.

The samples of enzyme activity were transferred to a 2 mL enzyme-free test tube, frozen with liquid nitrogen, and transferred to a -80 °C ultra-low temperature refrigerator, and stored until analyzed. Individual samples were homogenized in 0.05 M PBS (pH 7.4) at the ratio of 1:9 using a tissue grinder and analyzed by centrifugation at 2500 rpm for 20 min at 4 °C.

Total protein content and trypsin enzyme activity were determined using assay kits from Nanjing Jiancheng Bioengineering Institute following the manufacturer's protocols. Before the experiment, the diluted supernatant was diluted tenfold with 0.05 M PBS (pH 7.4). Total protein concentration was determined using the BCA assay, and absorbance was measured at 562 nm. Tryptic activity was calculated from the change in absorbance at 253 nm, normalized to total protein content.

The detection method was based on the practice of Neda Gilannejad (Gilannejad et al., 2021). CCK content was analyzed by Fish Cholecystokinin Elisa Assay Kit from Nanjing Jiancheng Bioengineering Institute. The kit used a competitive assay to detect CCK levels. After sample addition, incubation, and washing, the color was developed, and it had an absorption peak under 450 nm wavelength, and its absorbance was negatively correlated with the antigen density of the sample.

2.5. Statistical analysis

Data were expressed as mean $\pm$ SD. After demonstrating homogeneity and normality of variances, a one-way ANOVA was used to determine if significant differences existed, with $P < 0.05$ indicating significant differences. Duncan's test was used for multiple comparisons of changes in enzyme activity, and the independent samples t -test was used for analysis of variance between groups. All statistics were analyzed using IBM SPSS Statistics 26 and graphs were analyzed using Origin 2021. Histological images were processed with ImageJ.

3. Results

3.1. Growth and morphology

The average total length of the newly hatched larvae (1DAH) was 4.51 ± 0.15 mm and increased to 20.99 ± 3.14 mm at the end of the experiment (50DAH). The average body weight of the newly hatched larvae was 1.07 ± 0.04 mg and increased to 20.99 ± 3.14 mg at the end of the experiment. The growth equation for the total length and weight was $W = 0.006L^{3.053}$, the WGR was 116.10 %, and the SGR was 9.53 %. At 1–6DAH, a significant increase in the total length was observed, but no significant change in the body weight. At 6–30DAH, both the body weight and total length showed a more stable exponential upward trend. At 30–40DAH, significant increases in the body weight and total length curves were observed. At 50DAH, the body weight gain exceeded the total length (Fig. 2).

At 1DAH, the body of the larvae was transparent and the eye spots were observed (Fig. 3-A). At 3DAH, black pigmentation was observed on the body surface (Fig. 3-B). At 6DAH, the body color of the larvae deepened, and one chamber of the swim bladder was inflated (Fig. 3-C). At 9DAH, the structure of the eyeballs tended to be mature, the development of the dorsal spine skeleton tended to be perfect, and the caudal fin gradually differentiated and developed (Fig. 3-D). At 30DAH, the

Table 2
Sampling table for CCK content and trypsin activity measurements.

Sampling timepoint	Sample size
1–5DAH	200
6–21DAH	80
24–40DAH	30
45–50DAH	10

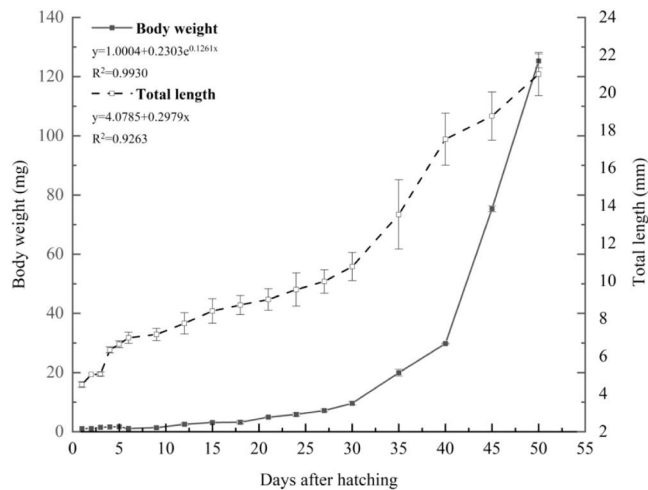


Fig. 2. The body weight (mg) and the total length of *M.pellegrini* at different days after hatching. Note. The upper left diagram shows the growth curve fitting equation of body weight and total length.

development of the fins and spindle skeleton in the individuals became increasingly refined. The second chamber of the swim bladder was inflated, signaling that its development was approaching maturity (Fig. 3-E). At 45DAH, scales began to emerge from the posterior margin of the gill cover and extend to the upper occipital epidermis. The skeleton of the appendages encased the inner portion of the vastus lateralis muscle, while the lateral line became distinctly visible. Overall, the morphology closely resembled that of adults, indicating that the larvae were transitioning into the post-larval stage of development at this stage (Fig. 3-F).

3.2. Histology

At 1DAH, the larvae were in an endogenous nutritional stage, and the volume of the yolk sac was substantial. The intestines were tubular, and the mucosal epithelium consisted of tightly packed monolayer columnar epithelial cells (Fig. 4-A). At 3DAH, the digestive tract started to differentiate. The intestinal lumen and the oesophagus were observed (Fig. 4-B, C). At 4DAH, the oropharyngeal cavity was opened to allow exogenous feeding, and the intestinal propria muscle layer was formed. At this time, slight undulation was observed on the intestinal epidermis (Fig. 4-D, E). At 6DAH, the yolk sac was almost completely absorbed and the larvae entered the exogenous feeding phase. The intestinal lamina propria appeared. The foregut and the midgut appeared wrinkled. Furthermore, the presence of goblet cells and supranuclear vacuoles in the hindgut was first observed (Fig. 4-F, G, H, I). At 12DAH, the intestinal basement membrane and the intestinal villi formation were observed (Fig. 4-J). At 16–18DAH, the cell surface of the foregut and midgut was specialised to form the supranuclear vacuoles (Fig. 4-K, L). At 20DAH, the striated margins appeared (Fig. 4-M). At 24DAH, the intestinal coiling appeared, and the supranuclear vacuoles in the foregut and midgut began to shrink and disappear (Fig. 4-N). At 34DAH, the supranuclear vacuoles had almost disappeared (Fig. 4-O). At 48DAH, the intestinal tract of *M.pellegrini* was similar to that of adults, and the measured indicators were maximized (Figs. 4-P, 5).

At 3DAH, the hepatocyte clusters appeared near the intestine (Fig. 6-A). At 5DAH, the gallbladder was observed to appear. At 6DAH, the hepatocyte interstitial cavities increased in size, and a small number of pancreatic cells were observed (Fig. 6-B). At 8DAH, the size of the liver increased significantly, and the pancreatic cells began to aggregate and set in the liver in a looser arrangement (Fig. 6-C, D). At 16DAH, the volume of the pancreatic cell clusters increased significantly, and the embryonic islet cell clusters appeared (Fig. 6-E). At 20DAH, the lipocyte vacuoles in the liver increased in size, and the lipids were accumulated (Fig. 6-F). At 24DAH, the islet cell clusters were embedded in the liver (Fig. 6-G). At 30DAH, the boundaries of the gallbladder wall were clear,

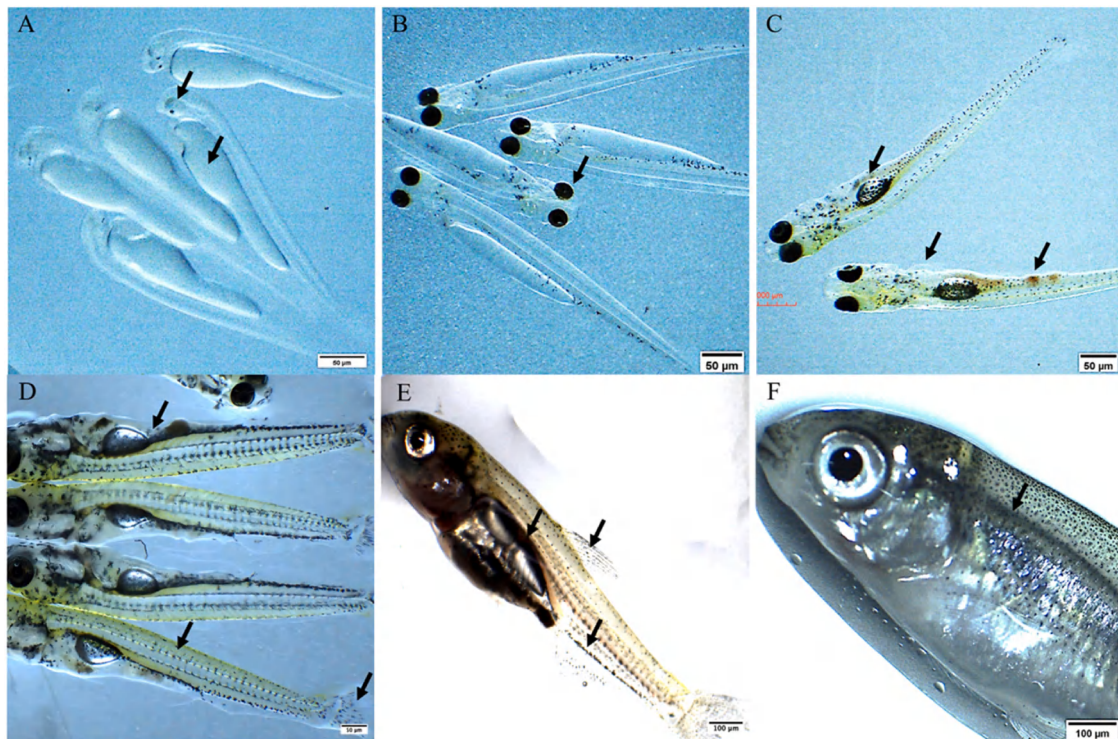


Fig. 3. Morphological observation of the early developmental stage of *M. Pellegrini* (A/B/C/D×10 , E/F×4). Note: The arrow indicates the melanin deposition point, A is 1DAH, B is 3DAH, C is 6DAH, D is 9DAH, E is 30DAH, and F is 40DAH.

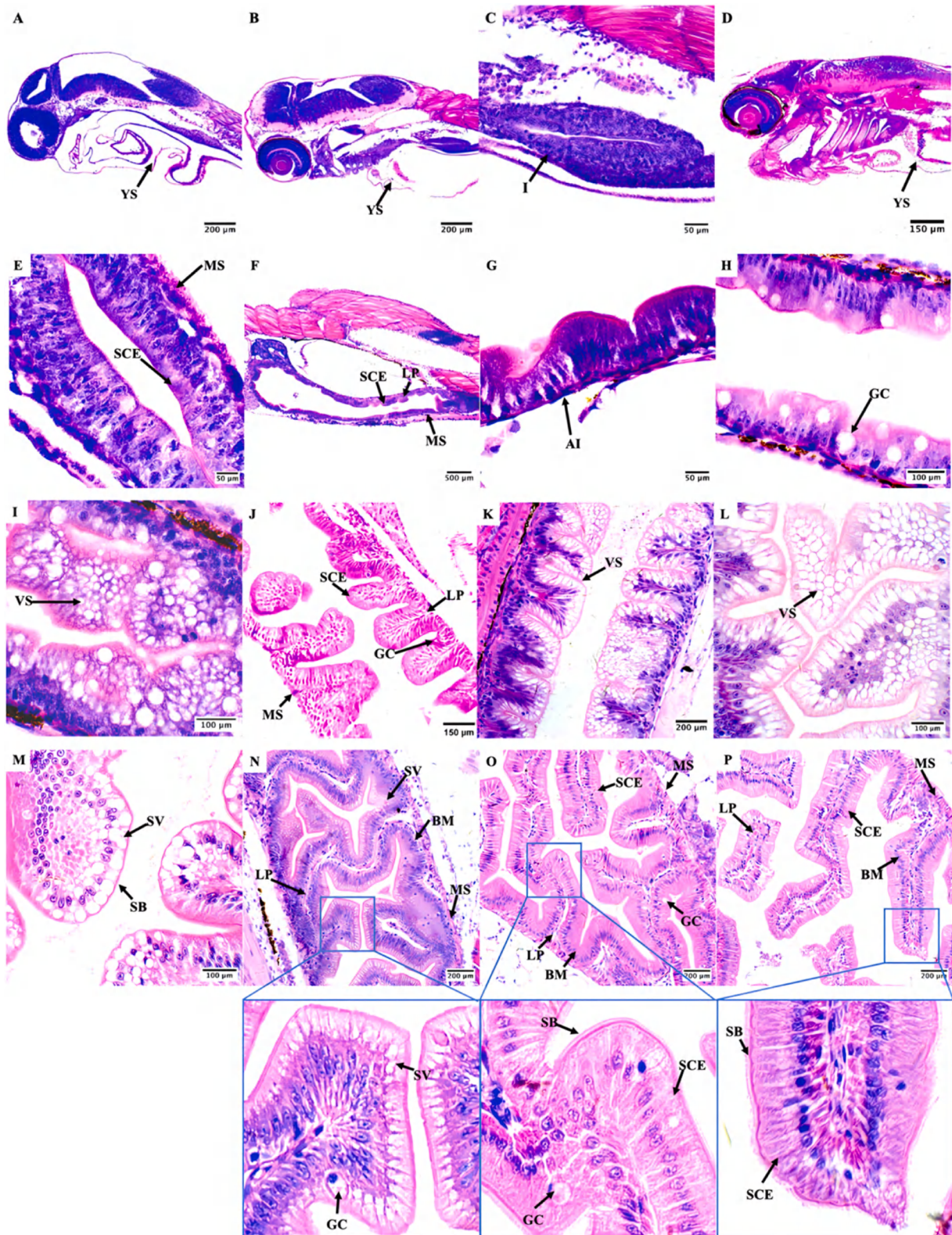


Fig. 4. The digestive tract development of *M. pellegrini* at different days after hatching. A-longitudinal section of the yolk sac and the oropharyngeal cavity at 1DAH ($\times 40$), B-longitudinal section of the yolk sac and the oropharyngeal cavity at 3DAH ($\times 40$), C-longitudinal section of the intestinal tract at 3DAH ($\times 100$), D-longitudinal section of the yolk sac at 4DAH ($\times 40$), E-longitudinal transection of the foregut at 4DAH ($\times 100$), F-longitudinal section of the intestinal tract at 6DAH ($\times 40$), G-longitudinal section of the foregut at 6DAH ($\times 100$), H-longitudinal section of the hindgut at 6DAH ($\times 100$), I-longitudinal section of the hindgut at 6DAH ($\times 100$), J-longitudinal section of the intestinal tract at 12DAH ($\times 40$), K-longitudinal section of the midgut at 16DAH ($\times 40$), L-longitudinal section of the foregut at 18DAH ($\times 100$), M-longitudinal section of the intestinal tract at 20DAH ($\times 40$), N-longitudinal section of the intestinal tract at 24DAH ($\times 40$), O-longitudinal section of the intestinal tract at 34DAH ($\times 40$), P-longitudinal section of the intestinal tract at 48DAH ($\times 40$). YS: yolk sac, I: intestine, SCE: single-layer columnar epithelium, MS: muscle layer, AI: anterior intestine, LP: lamina propria, GC: goblet cell, SV: supranuclear vacuole, BM: basement membrane, SB: striate margin.

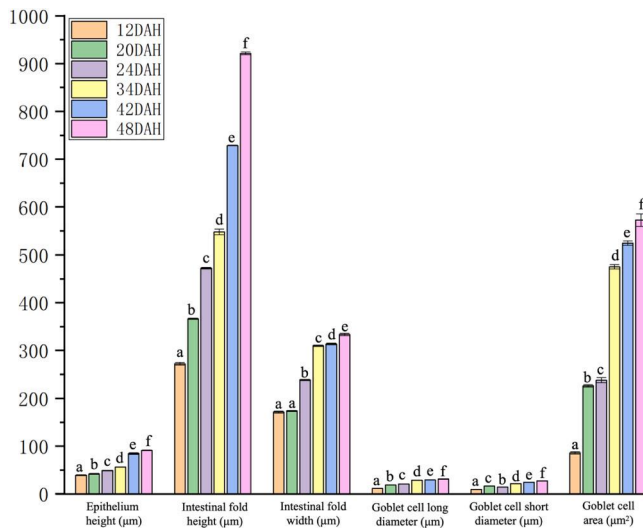


Fig. 5. Growth data on the intestinal histological structure of *M. pellegrini* on different days after hatching. Note: Those with different lowercase letters in the same column indicate significant differences between groups ($P < 0.05$), and those marked with the same lowercase letters indicate no significant difference between groups ($P > 0.05$).

and the gallbladder developed to approximate the state of adult fish (Fig. 6-H). At 42DAH, the hepatopancreas nearly became the adult fish state (Fig. 6-I).

3.3. Localization of CCK secretion sites

The site of CCK mRNA expression was mainly concentrated in the monolayer of columnar epithelial cells in the intestine (especially the proximal part of the intestine) of *M. pellegrini* (Fig. 7-A, B). The interstitial space of the muscular propria cells exhibited a weak positive signal (Fig. 7-C). A subtle hybridization signal was observed on the surface of hepatocytes and in the surrounding interstitial areas (Fig. 7-D). The pancreatic cells were densely clustered, with a small amount of hybridization signal accumulation present in the intercellular spaces (Fig. 7-E), and the interstitial spaces of the pancreatic cell clusters set between hepatocytes were infiltrated by weakly positive yellow signal (Fig. 7-F).

3.4. Analysis of CCK content and tryptic enzyme activity

The CCK content was 715.70 ± 49.98 ng/L at 1DAH (4.51 ± 0.15 mm, TL) and $12,902.27 \pm 4734.84$ ng/L at 50DAH (20.99 ± 3.14 mm, TL). At 1–3DAH, the CCK content continued to increase, and then the CCK content decreased at 4DAH. The first peak occurred at 12DAH. At 12–30DAH, the CCK content showed a decreasing and then increasing trend, ultimately reaching a peak at 30DAH. The CCK content at 30DAH was significantly higher than the previous level ($P < 0.05$). At 30–40DAH, the CCK content decreased slightly. At 45 DAH, the CCK content decreased significantly to the level of the pre-existing period ($P < 0.05$). Moreover, the CCK content rebounded significantly at 50DAH ($P < 0.05$) but was lower than the CCK content at 40DAH (Fig. 8).

The trypsin activity was 100.71 ± 1.26 U/mgprot at 1DAH (4.51 ± 0.15 mm, TL) and 862.64 ± 55.64 U/mgprot at 50DAH (20.99 ± 3.14 mm, TL). The trypsin activity increased at 1–3DAH, then fell back to the initial level at 5DAH. The trypsin activity increased slightly at 6DAH, and fluctuated within a relatively stable range up to 24DAH. The trypsin activity increased to 552.79 ± 90.40 U/mgprot at 27DAH, and maintained at this level until 35DAH. At 35DAH, the trypsin activity significantly increased ($P < 0.05$) and peaked at 40DAH. At 45DAH, the

trypsin activity decreased to the level of the 27–35DAH phase—the trypsin activity rose again to a near peak at 50DAH (Fig. 8). The trend of unfavorable correlation fluctuation between the CCK content and trypsin activity of the digestive system in this experimental phase was observed at 4DAH, 8DAH, 18DAH, 33DAH, and 39DAH. However, the feedback mechanism was not stable throughout the developmental stage (Fig. 8).

4. Discussion

The early developmental stages of *M. pellegrini* have obvious phases, successively going through the endogenous nutrient stage, mixed nutrient stage, exogenous nutrient stage of the larval stage, the post-larvae stage, and the juvenile stage. The present experiment focused on the growth of the postembryonic developmental stage (within 50 days after hatching). The results of the experiment demonstrated a significant increase in total length of the larvae at 1–6DAH, with both body weight and total length exhibiting exponential growth after 6DAH. The optimal growth curve fitting equation obtained shows that both the total length and body weight have a strong correlation with the day after hatching. The fitting equation can be utilized in seedling breeding to determine the age of larvae based on their total length and body weight. This information allows us to adjust feeding strategies for various developmental stages, including the selection of bait and the frequency of feeding, among other factors.

Early development of the digestive organs is also an important process in fish, determining the type of open-top bait and influencing the choice of environment and survival rate of larvae. In the experiment, the oropharyngeal cavity was opened at 4DAH, at the same time as *Sparus aurata* L. (Elbal et al., 2004), one day later than *Paralichthys olivaceus* (Khoja et al., 2021b), and two days later than *Seriola lalandi* (Chen et al., 2006) and *Paralabrax maculatofasciatus* (Peña et al., 2003). At 6DAH, the yolk sac was largely gone and the larvae entered the exogenous trophic phase, at a time point similar to *Seriola rivoliana* (Pacheco-Carlón et al., 2024). In addition, the goblet cells appeared. The goblet cells are mucus-secreting, and the mucus can promote food digestion, help the body absorb nutrients as well as defend against pathogens, and play an important role in maintaining intestinal homeostasis (Gipson, 2016; Specian and Oliver, 1991). We found that the first functional goblet cells in larvae emerge with the first exogenous feeding, similar to the *Umbrina cirrosa* L research results (Zaiss et al., 2006). At 12DAH, the intestinal villi were formed. The functions of intestinal villi are mainly to increase the surface area of the small intestine, absorption and transportation of nutrients, and defense mechanisms. At 24DAH, the intestinal tract appeared coiled and curled, and the coiled intestine provided an effective surface area for digestion and absorption (Holt et al., 2011; Lazo et al., 2011). Overall, the development of the digestive system of *M. pellegrini* was in a relatively stable early stage, and the digestive system was developing smoothly towards the adult state, synchronizing with the development of the digestive system of *Katsuwonus pelamis* (Khoja et al., 2021a) and *Spinibarbus sinensis* (Hong-yu, 2010). The complete disappearance of the supranuclear vacuoles at 34DAH indicates that the intestinal structure enters a mature developmental stage at this point. At 42DAH, the development of the digestive system of the larvae was close to the structure of adult fish.

During fish's early developmental stages, the digestive system is not well developed and supranuclear vacuoles are important digestive and metabolic sites for the uptake of nutrients such as lipids and proteins (Hamlin et al., 2000). As the digestive system's structure improves, the vacuoles disappear. The decrease of supranuclear vacuoles is usually considered an indication of a shift from intracellular to extracellular digestion modes in fish larvae (Chen et al., 2022). At 6DAH, the supranuclear vacuoles were observed for the first time in the hindgut, consistent with the results of the supranuclear vacuoles in the hindgut of *Pampus argenteus* (Zheng et al., 2023) just entering the exogenous nutritional stage. At 16–18DAH, the supranuclear vacuoles were visible,

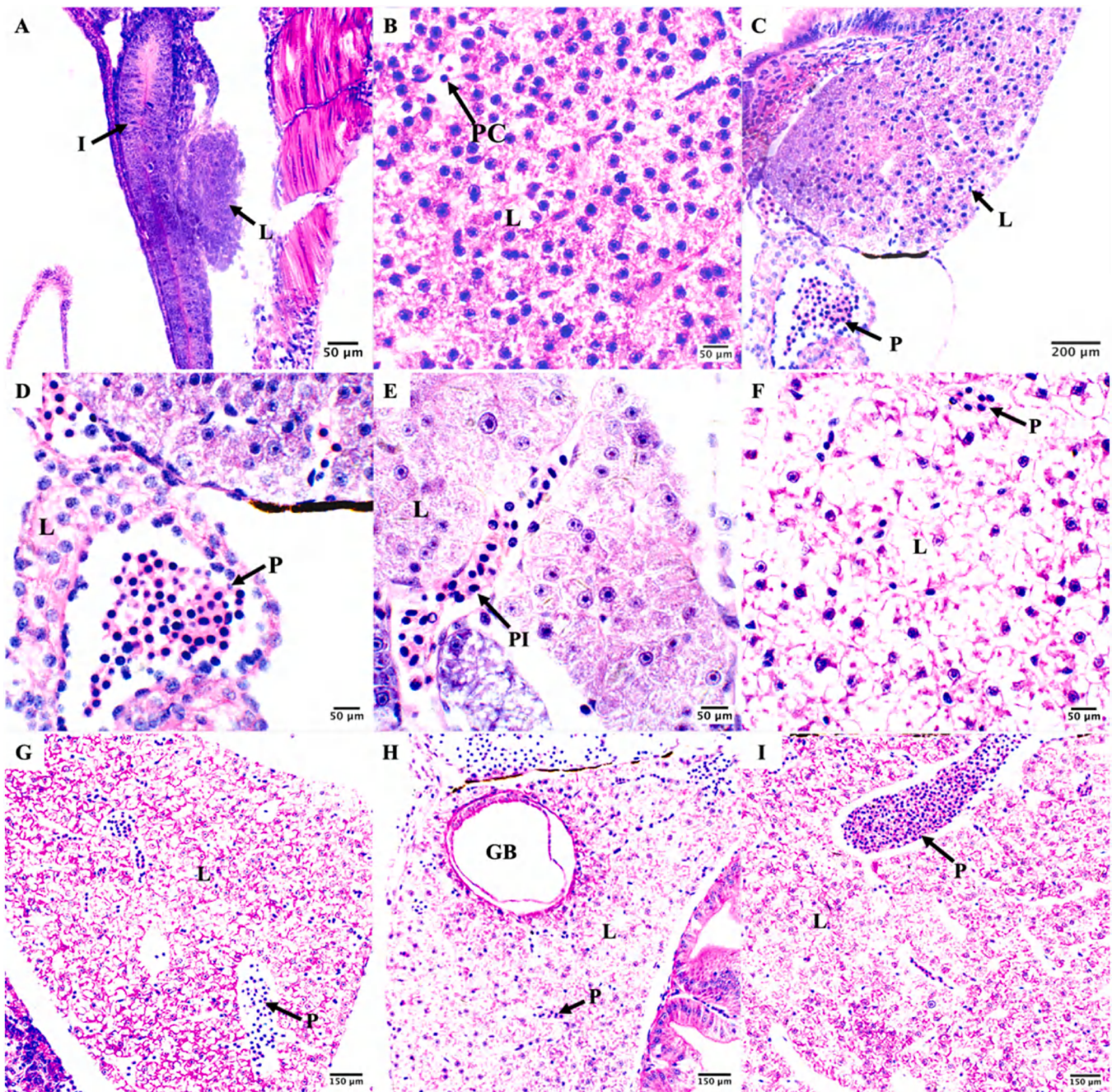


Fig. 6. Hepatopancreatic development of *M.pellegrini* at different days after hatching. A-longitudinal resection of the liver cell mass at 3DAH ($\times 100$), B-longitudinal resection of the liver cell mass and pancreatic cells at 6DAH ($\times 100$), C-longitudinal resection of the hepatopancreas at 8DAH ($\times 40$), D-longitudinal resection of the hepatopancreas at 16DAH ($\times 100$), E-longitudinal resection of the hepatopancreas at 20DAH ($\times 100$), F-longitudinal resection of the hepatopancreas at 24DAH ($\times 100$), G-longitudinal resection of the hepatopancreas at 30DAH ($\times 40$), H-longitudinal resection of the hepatopancreas at 36DAH ($\times 40$), I-longitudinal resection of the hepatopancreas at 42DAH ($\times 40$). I: intestine, L: liver; PC: pancreatic cell, P: pancreas, GB: gall bladder, PI: pancreatic islet.

indicating that the intestinal cellular-induced protein absorption was highly active at this stage (Shen et al., 2018). The number of vacuoles significantly decreased at 24DAH when the goblet cells tended to be well developed, suggesting a transformation of the protein digestive pattern. The disappearance of the structures indicates that the intestinal tissue function enters a mature developmental stage at this time.

Among the numerous gastrointestinal hormones implicated in the process of digestion, CCK assumes a pivotal role in the contraction of the gallbladder, the peristaltic movement of the intestines, and the delayed emptying of the stomach. Additionally, it is a paramount regulator of pancreatic enzyme secretion (Tillner et al., 2013). CCK also signals

satiety in the brain. However, there is still much controversy over the distribution of CCK in fish. According to existing studies, the distribution of CCK in the gastrointestinal tract is related to the feeding habits of fish (Lustosa do Carmo et al., 2023). The gastrointestinal tract of most carnivorous and omnivorous fish consists of the stomach, pyloric caecum, and short intestine, and these features are opposite to those of herbivorous fish. Therefore, most carnivorous fish have low levels of CCK in their intestinal tracts and high levels in the pyloric caecum, e.g., red-bellied piranha (Volkoff, 2014) and tambaqui (Lustosa do Carmo et al., 2023), possibly since the pyloric caecum has endocrine organ that regulates gastric and pancreatic secretion during digestion (Small,

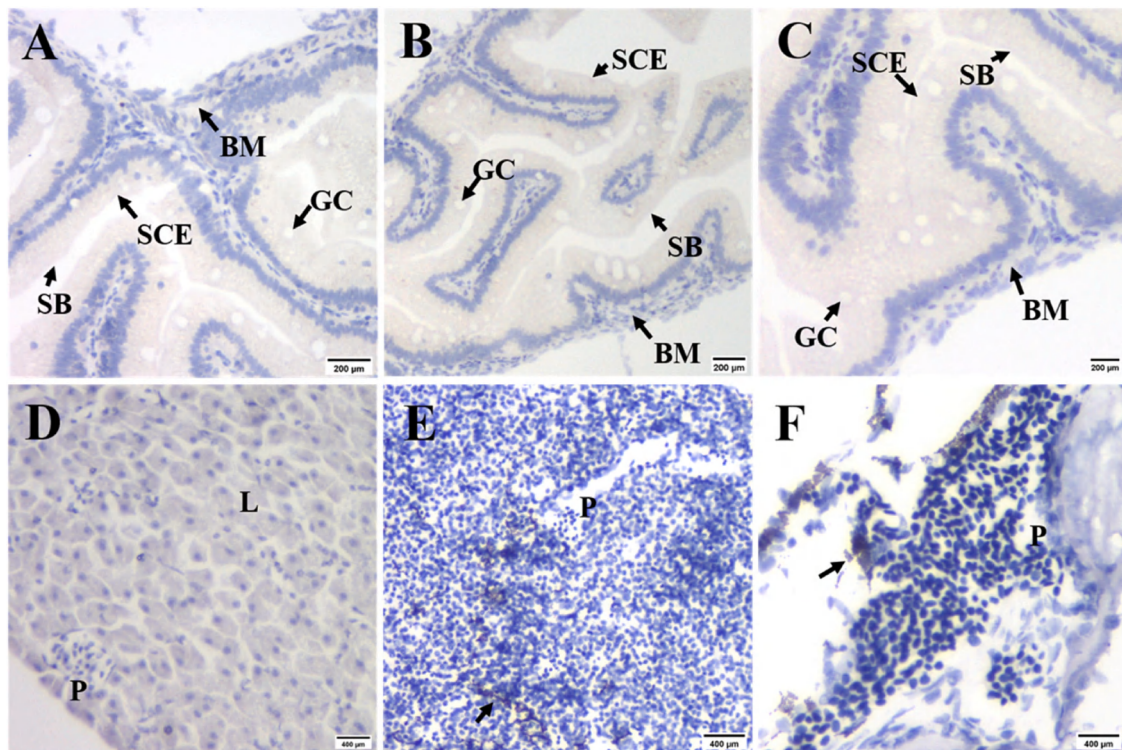


Fig. 7. The digestive system CCK mRNA in situ hybridization of *M. pellegrini*. SCE: single columnar epithelium, BM: basement membrane, SB: striated border, GC: goblet cells, P: pancreatic cells; L: Hepatocytes. Note: The arrow indicates the positive hybridization signal.

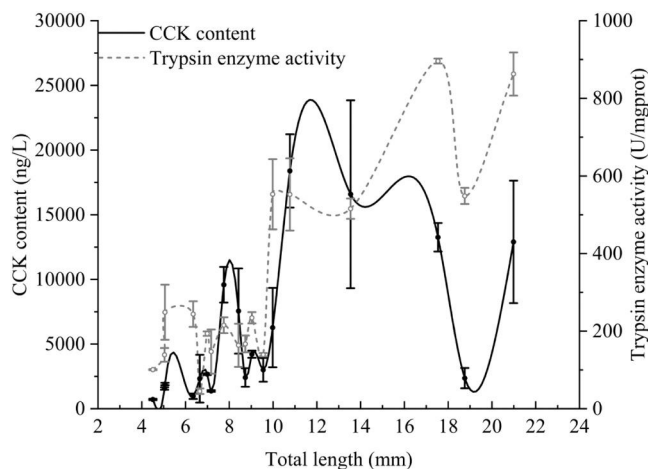


Fig. 8. The CCK content and the trypsin enzyme activities of *M. pellegrini* at different total lengths.

2022). The distribution of CCK in the gut varies for different fish species. Fish with a rectal gut have CCK predominantly in the midgut, e.g., *Oncorhynchus mykiss*. In contrast, fish with a rotary gut have CCK predominantly in the anterior part of the midgut, e.g., *Thunnus thynnus* (Rønnestad et al., 2007). In addition, some species, such as rainbow trout, dourado, and pirapitinga also have high CCK expression levels in the gills. This is because CCK contributes to gill flake perfusion, which improves oxygen delivery and metabolic rate, especially under stressful conditions such as hypoxia and elevated temperatures (Lustosa do Carmo et al., 2023). In this experiment, CCK was primarily found in the foregut of *M. pellegrini*. This distribution may be due to the nature of *M. pellegrini* as a non-gastric, omnivorous herbivorous fish with a rotary gut. The CCK content of *M. pellegrini* was detected on the day of initial hatching, and the same results were found in studies for *Paralichthys*

olivaceus (Khoa et al., 2021b), *Clupea harengus* (Kamisaka et al., 2005) and *Anguilla anguilla* (Parmeggiani et al., 2020). From an individual developmental point of view, the likely reason for this is that CCK, an enterohormone, is similar to trypsin in that the timing and amount of its production are genetically preprogrammed at the yolk sac stage and then progressively influenced by exogenous nutrients in the gut (Rønnestad et al., 2007). There were no significant changes in CCK levels during the period of mixed nutrition. Because CCK levels are only affected by the nutrients supplied to the tissues by the yolk sac during the mixed-nutrition period, independent of the food in the intestine, the intestinal CCK-secreting cells produce meal-responsiveness only when the nutrients in the yolk sac are depleted (Kamisaka et al., 2013). In this experiment, the larvae entered the stage of exogenous nutrition at 6DAH, and there was a significant increase in the CCK level and trypsin activity. This is because the gallbladder was formed, the pancreas was largely differentiated at this time, and the bile and trypsin were secreted into the intestines under the stimulation of food, increasing the CCK levels and trypsin activity. Overall, CCK levels are genetically pre-programmed and unrelated to exogenous food prior to the exhaustion of the yolk sacs of *M. pellegrini* larvae; however, after the larvae enter the exogenous nutritional phase, CCK levels are gradually influenced by exogenous food conditions.

It has been demonstrated (Tillner et al., 2014, 2013) that negative feedback regulation between trypsin and CCK during early developmental stages of fish, such as Japanese eel (Kurokawa et al., 2004) and *Oreochromis niloticus* (Drossou et al., 2006), and the same phenomenon was found in this experiment at 4, 8, 18, 33 and 39DAH. However, Robert found a negative but unstable feedback regulation between CCK and trypsin in *Gadus morhua* (Tillner et al., 2013) during early developmental stages, the same phenomenon was found in *Dicentrarchus labrax* (Tillner et al., 2014) larvae and *Solea senegalensis* (Navarro-Guillén et al., 2017) larvae. It can be concluded that the negative feedback regulation is not stable during the early developmental stages of fish. In this experiment, combined with the results of histological sections, it was found that negative feedback regulation was

not detected at a number of critical growth and developmental nodes, such as at 12DAH when hepatopancreatic single-cell intercellular structures were relatively stable, at 30DAH when bones and muscles were rapidly developing, and at 45DAH when larvae and post-larvae staging were at the turning point. This phenomenon may be attributable to the immature regulation of trypsin secretion by CCK during the initial developmental phases of fish. CCK is genetically programmed, and its secretion is subject to changes in response to developmental progress, leading to the spontaneous release and re-synthesis of CCK in endogenous rhythms, irrespective of trypsin activity. In addition, other gut hormones as well as neurological factors play a role in digestive regulation and may change with developmental progression, thereby influencing the negative feedback regulatory mechanism of CCK and trypsin during the early developmental stages of fish. For example, neuropeptide Y (NPY)-related peptides, also produced by enteroendocrine cells, act antagonistically to CCK in controlling pancreatic secretion (Walsh, 1993). The CCK content declined during the total length of 18–20 mm, probably due to the fact that at this time, *M. pellegrini* began to enter the early juvenile stage. The fish body's morphology and the digestive system's structure had qualitative changes during this time. In the process of structural changes, the function has not yet been adapted to the structural changes, which will lead to a decrease in the level of secretion, which is consistent with the same fluctuating tendency as early juveniles into the post-larvae stage. Little is known about the negative feedback regulation of CCK and trypsin during the early developmental stages of fish and further studies are needed.

In conclusion, the present study demonstrated that the digestive system of *M. pellegrini* exhibited a structure that was similar to that of adult fish at 42DAH and transitioned to the post-larvae stage at 45DAH. We also found a negative feedback regulation between CCK content and trypsin activity in the larvae's early developmental stage, but the secretion pattern oscillated with the development of the digestive system structure. CCK was primarily found in the foregut of the digestive system during the early developmental stages of *M. pellegrini*. This study offers a robust theoretical foundation for cultivating *M. pellegrini* in RAS and contributes to advancing research on CCK in freshwater fish.

CRediT authorship contribution statement

Tang Hongyu: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Li Wenyu:** Investigation, Formal analysis, Data curation. **Lin Xiaogang:** Writing – original draft, Investigation, Data curation. **Li Qin:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ma Feifan:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

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Data availability

Data will be made available on request.

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