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The Characteristic of Critical Genes in Neuroendocrine System and Their Regulation on Food Habit Transition and Metamorphosis of Veined Rapa Whelk *Rapana venosa* (Valenciennes, 1846)

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Metamorphosis is a critical developmental event in mollusks, and neuroendocrine system plays an essential role in this process. *Rapana venosa* is an economically important shellfish in China, but the artificial technology of *R. venosa* aquaculture is limited by metamorphosis. As a carnivorous gastropod, food habit transition makes the mechanism of *R. venosa* metamorphosis more complex. To investigate the changes in the neuroendocrine system and to reveal its role in regulating the food habit transition and metamorphosis of *R. venosa*, we cloned the cDNA sequences encoding 5-hydroxytryptamine receptor (*Rv-5HTR*), nitric oxide synthetase (*Rv-NOS*) and cholecystokinin receptor (*Rv-CCKR*), and investigated their expression by quantitative real-time PCR analysis, and explore the spatio-temporal changes of 5-HT protein expression using Immunohistochemical (IHC) analysis. The expression of the three geens was significantly increased in the early intramembrane veliger stage, which indicates that the three genes are related to the development of digestive system. Additionally, expression of the three genes was decreased after metamorphosis, while *Rv-NOS* and *Rv-CCKR* were increasingly expressed in competent larvae, which may help the larvae find suitable environments and promote digestive system development for metamorphosis, and the result of 5-HT IHC analysis also reflects the development of neuroendocrine system. Furthermore, results show that CCK can effect the expression of digestive enzyme, NOS and 5-HT receptor. Finally, based on the present results, we hypothesized that CCK and CCK receptor may be critical regulatory factors of food habit transition and metamorphosis. These results might provide information on the development of neuroendocrine system of *R. venosa*, and new insight into the regulation of the food habit transition and metamorphosis of gastropods.

Keywords: *Rapana venosa*, metamorphosis, neuroendocrine system, food habit transition, digestive system

INTRODUCTION

Metamorphosis is a critical developmental event in the biphasic life cycle in mollusks that is evolutionarily crucial because it develops independently in different clades (Hadfield et al., 2000; Huan et al., 2015). Extensive morphological and physiological changes occur during metamorphosis but are accompanied by high mortality (Harding, 2006; Huan et al., 2015). Therefore, premetamorphosis recruitment and postmetamorphosis survival control mollusk population dynamics. To date, the mechanism of metamorphosis has been most well studied in bivalves (Coon et al., 1985; Satuito et al., 1999; Yang et al., 2007; Gireesh and Cherukara, 2008; Yang B. et al., 2015; Niu et al., 2016; Moore and Bringolf, 2018), and in gastropods, studies are mainly focused on abalone and some herbivorous snails (Barlow and Truman, 2010; Li et al., 2018). Numerous neuroactive compounds, including g-aminobutyric acid (GABA), various catecholamines, serotonin (5-HT), and nitric oxide (NO) are associated with metamorphic processes in a variety of mollusks, which has been extensively studied (Leise et al., 2001), and the neuroendocrine system plays an important role in the regulation of metamorphosis.

The veined rapa whelk (*Rapana venosa*), a native to temperate Asian waters (Harding, 2006), is an economically important gastropod snail in China due to its high economic and medicinal value (Dolashka et al., 2011; Nesterova et al., 2011; Olga et al., 2015; Voelter et al., 2016). Unfortunately, *R. venosa* is highly threatened, and its population has dramatically declined in recent years (Wei et al., 1999; Yang et al., 2007). Although artificial aquaculture was started early (Yuan, 1992; Yang et al., 2007), the large-scale culture of this species is seriously limited by its high mortality during metamorphosis. As a carnivorous gastropod, *R. venosa* needs to undergo a food habit transition from herbivorous to carnivorous during metamorphosis, which is different from the metamorphosis of lifelong herbivory gastropods and makes the mechanism of metamorphosis more complex. Our group has performed some studies on the metamorphosis of *R. venosa*, including studies of the differences in transcriptome, proteome, metabolome, digestive enzyme and microorganisms before and after metamorphosis (Song et al., 2016a,b,c; Yang et al., 2020a,b), as well as the effect of bait induction on the rate of metamorphosis (Yu et al., 2020). Song et al. (2016a) mentioned that the expression of the NOS and 5-HT receptors decreased after metamorphosis (Song et al., 2016a). However, there is no further study on the regulation of the neuroendocrine system on the metamorphosis of *R. venosa*.

Bather (1921) showed that the presence of conspecific adults or useful food sources could trigger metamorphosis in marine invertebrates, and our previous study suggested that oysters can significantly improve the metamorphosis rate of larvae (Yu et al., 2020). In this process, oysters were inducers, as well as food sources; these results may suggest a link between the digestive system and metamorphosis and that the neuroendocrine system regulates metamorphosis by regulating the digestive system, which is very likely to occur in carnivorous gastropods. Many studies have indicated that food can increase digestive enzyme activity (Ou and Liu, 2007; Genodepa et al., 2018). Kinouchi et al. (2012) also indicated that the consumption of large protein molecules can promote the development of pancreatic digestive functions in rats, and cholecystokinin (CCK) is an important agent in this process. CCK is a brain-gut peptide with a variety of physiological functions that regulate the secretion of trypsin and the growth of the pancreas; as well as a neurotransmitter in the central and enteric nervous systems. Many studies have suggested the regulatory effect of CCK on the digestive system in the vertebrates, such as rats (Gibbs et al., 2012), porcines (Bugge et al., 2018), fish (Himick and Peter, 1994; Liu et al., 2014; Navarro-Guillén et al., 2017), and also a few studies in invertebrate, *Aplysia californica*, *Nereis diversicolor*, and *Crassostrea gigas* (Vigna et al., 1984; Dhainaut-Courtois et al., 1985; Schwartz et al., 2018). Furthermore, He et al. (2015) indicated that increased CCK expression after food habit transition might suggest a regulatory effect of CCK on the food habit transition of carp, and leptin, insulin and NOS were also described as being related to the food habit transition. These results may show that the regulation of the neuroendocrine system plays a vital role in digestive system development, food habit transition, and even metamorphosis in larvae. Meanwhile, NOS has been suggested to play an indispensable role in the regulation of feeding behavior in *Lymnaea stagnalis* (Kawai et al., 2011) and *Aplysia* (Jacklet, 1995). Moreover, Tripathi et al. (2015) have showed that the NOS signal transduction pathway is downstream of CCK receptor, and the NO has been indicated that can activate cGMP synthesis in the crab stomatogastric nervous system and regulate the digestive fuction in crab (Scholz et al., 1996). Additionally, NOS also plays a role in the regulation of 5-HT on metamorphosis, and 5-HT has also been indicated has effect on feeding behavior in goldfish, rat and chickens (Pedro et al., 1998; Vry and Schreiber, 2000; Zendehdel et al., 2012). Therefore, NOS, 5-HT and CCK are likely to be the key mediators of the process of the food habit transition oyster-induced metamorphosis of *R. venosa*.

A previous study indicated that significant changes occur in the nervous system during the metamorphosis of *R. venosa*. The expression of the *NOS* and *5-HT receptor* was found to decrease after metamorphosis (Song et al., 2016a), but no further study has examined their roles in the metamorphosis of *R. venosa*, and whether the neuroendocrine system regulates metamorphosis by regulating the digestive system is unknown. In the present study, we aimed to examine the development of the neuroendocrine system by obtaining cDNA sequences and determining the expression profile of *5-HT receptor*, *NOS* and *CCK receptor* gene mRNA, and observed the spatiotemporal expression characteristics of 5-HT by immunohistochemical (IHC) analysis. Therefore, the present study can provide new insight into the mechanism of gastropod metamorphosis from the perspective of the neuroendocrine and digestive systems.

MATERIALS AND METHODS

Larval Rearing and Sample Preparation

A pair of adult *R. venosa* (2 year old) as parents, and the spawn, intramembrane larvae and planktonic larvae of *R. venosa* were cultured according to the methods described by Yang et al. (2007) at Blue Ocean Co., Ltd (Laizhou, Shandong, China).

Planktonic larvae were cultured in 3 m × 5 m × 1.5 m cement pools at 24–26°, at a density of 0.5/ml. The mixture of *Platymonas subcordiformis*, *Isochrysis galbana*, and *Chlorella vulgaris* was used as a diet (15 × 104 cell/ml, three or four times) for the Planktonic larvae. Intramembrane larval samples were collected from seven major developmental stages: the cleavage stage (c), the blastula stage (b), the gastrulae stage (g), the trochophore stage (t), the early intramembrane veliger stage (ev), the middle intramembrane veliger stage (mv), and the later intramembrane veliger stage (lv). Planktonic larval and postlarval samples were collected from five major developmental stages: the one-spiral whorl stage (V-I), the two-spiral whorl stage (V-II), the three-spiral whorl stage (V-III), the four-spiral whorl stage (competent larva, V-IV), and the postlarval stage (J). The samples were collected based on the methods described in our previous study (Yang et al., 2020a), and each developmental stage was sampled in triplicate and sextuplicate and stored at −80°C for qRT-PCR, respectively, and each sample contains 30 larvae. For the immunohistochemistry (IHC) assays, larvae at different developmental stages were washed in PBS, and 7.5% MgCl2 solution was slowly added to completely anesthetize the larvae. Then, the samples were fixed at room temperature for 2–6 h using 4% paraformaldehyde solution, washed again in PBS, transferred to 70% ethanol and stored at −20°C for use.

CCK Induction Assays

The larvae reached the four-spiral stage (shell height >1,250 μm, competent larvae) were used for the CCK induction assays (CCK peptide: T510159, Shanghai). The following controls and treatments were included in the assays: (1) control group without CCK polypeptide (control, C), and (2) treatment group with CCK polypeptide (1 mg/L) (CCK induction, K), each group with three biological repeats, each tank (393 mm × 282 mm × 223 mm) contained 80 larvae. We randomly collected three samples from each tank at 2 h (early stage, e) and 12 h (later stage, l) after treatment, and each sample contains 30 larvae, then washed them three times with PBS. We obtained a total of 12 samples, which were divided into four groups, namely Ce, Cl, Ke, and Kl, which were used to analyze the mRNA expression of critical genes related to metamorphosis, including carboxypeptidase, cellulase, 5-HTR, and NOS. All samples were stored at -80°C until use.

All procedures involved in the animal collection, rearing and dissection were conducted following the Guideline of Ethical Regulations of Animal Welfare of the Institute of Oceanology, Chinese Academy of Sciences (IOCAS 2013.3). Our study protocols were approved by the Animal Welfare Committee of the IOCAS.

Cloning of the Full-Length cDNA and Sequence Analysis

Total RNA was extracted from all the larval samples using the RNA Isolation Kit (Tiangen, China) following the manufacturer's protocol, and the quality was determined by a NanoDrop spectrophotometer (Thermo Scientific, United States) and gel electrophoresis. First-strand cDNA (5′- and 3′-RACE-ready cDNAs) synthesis and rapid amplification of the cDNA ends (RACE) were performed by using the SMARTer^{TM} RACE cDNA amplification kit (Clontech, United States). The primers for RACE were designed based on expressed sequence tags in the transcriptome library of *R. venosa* (Song et al., 2016a) using Primer Premier 5 software (**Supplementary Table 1**). The 5′ ends of the *5-HT receptor*, *NOS*, and *CCK receptor* genes were amplified with the primers 5HTR-5′-GSPn, NOS-5′-GSPn and CCKR-5′-GSPn, respectively, and a universal primer mix (UPM) (**Supplementary Table 1**). The 5′-RACE-ready cDNA was used as the template. The 3′ ends of the three genes were amplified using 5HTR-3′-GSPn and NOS-3′-GSPn, and UPM and 3′RACE-ready cDNA were used as the templates. Confirmation of the full-length cDNAs by polymerase chain reaction (PCR) was conducted as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s; 50–60°C for 30 s; 72°C for 1 min; and 72°C for 10 min (Eppendorf, Germany). The PCR products were inserted into the pMD19-T vector (Takara, Japan) and transformed into JM109 competent cells (Takara, Japan) according to the manufacturer's instructions, and ten positive clones were selected to confirm the nucleotide sequences by TsingKe Biological Technology (Beijing, China). The sequences were analyzed and assembled by DNAStar software to obtain the full-length cDNA sequence.

The full-length cDNA and predicted protein sequences were analyzed using National Center for Biotechnology Information BLAST programs[1]. The molecular masses and theoretical isoelectric points of the putative proteins were predicted using ExPasy Compute pI/Mw software[2]. The transmembrane structure of 5-HT receptor was predicted by TMHMM. The sequences of the three genes from different species were obtained from GenBank databases. Then, DNAMAN software and the ClustalW sequence alignment program[3] were used to perform multiple alignments, and MEGA 7.0 was used to construct phylogenetic trees with the neighbor-joining method.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from all the larval samples using the RNA Isolation Kit (Tiangen, China) to analyze the mRNA expression of the *5-HT receptor*, *NOS*, *CCK receptor, carboxy* genes during early stages of development. The qRT-PCR primers were designed based on the full-length cDNA sequences of the three genes, and the 60S ribosomal protein L28 (RL28-F, RL28-R) was used as a housekeeping gene for within-experiment signal normalization (Song et al., 2017; **Supplementary Table 1**). First-strand cDNA was synthesized for qRT-PCR using reverse transcriptase (Takara). The SYBR Green real-time PCR assay (2 × SYBR Green qPCR Mix, Sparkjade) was used with an Eppendorf Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) for the qRT-PCR analysis. The 10-μl reaction mix volume was prepared, containing 10 μl of 2 × SYBR Green qPCR Mix (Sparkjade, China), 0.2 μl each of 10 pmol the forward and reverse primers, 0.6 μl (100 ng) of cDNA and 4 μl sterile deionized water. Amplifications were performed according to the manufacturer's instructions. Standard curves were generated

[1]http://www.ncbi.nlm.nih.gov/BLAST/

[2]http://www.expasy.org/tools/pi_tool.html

[3]http://www.ebi.ac.uk/clustalW

with cDNA template dilutions of 10, 10^2, 10^3, 10^4, and 10^5. qRT-PCRs were performed using the following thermocycler program: 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative gene expression was calculated using the $2^{-\Delta\Delta\ Ct}$ method.

Immunohistochemical (IHC) Analysis of 5-HT

The samples used for the immunohistochemical analysis of 5-HT in *R. venosa* were washed in PBS three times, for 10 min each time. An 8% EDTA solution was used to dissolve the shell of the larvae, and the soaking time varied from 1 to 12 h depending on the thickness of the larval shell at the different stages. The larvae were washed with PBS three times again, and then, blocking solution (0.25% BSA, 0.03% NaN_3, 1% Triton X-100 and PBS) was added overnight at 4°C. After that, the samples were incubated for 2 h at room temperature with primary antibodies (1:800 in PBS) and washed three times with PBS for 1 h each. Then, the samples were incubated with Alexa 594-conjugated goat anti-rat IgG and Alexa 488-conjugated goat anti-rabbit IgG (1:1,000 in PBS) for 12–24 h at 4°C and then washed three times with PBS for 10 min each. Next, a suitable amount of the samples was transferred to slides to generate slide specimens, which were sealed with 80% glycerol. The specimens were examined and imaged with a fluorescence microscope (Nikon ECLIPSE 90i), and the images were processed with Adobe Photoshop CS3. Negative controls for all the IHC tests were included. The primary antibodies used for IHC were 5-HT (Serotonin) Rabbit Antibody (ImmunoStar, 20080)[4] and anti-α-tubulin (Sigma, T6793). The secondary antibodies used for IHC analysis were Alexa 594-conjugated goat anti-rat IgG (Jackson Lab, United States) and Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Jackson Lab, United States). Negative control slides for all the IHC tests were included.

Data Analysis

The results of the qRT-PCR analysis of 5-HT receptor, NOS and CCK receptor were analyzed by using one-way ANOVA with Tukey's test and LSD test with a significance level of $P < 0.05$, and the normality and equality of variances were assessed by Levene's test before performing ANOVA. All the statistics were conducted using SPSS 19.0 software (SPSS Inc., United States), and the results were expressed in (Means ± SEM). Different superscript letters indicate significant differences ($P < 0.05$). The heatmaps in **Figure 3** were made using the pheatmap package in R with Euclidean distance.

RESULTS

Identification and Analysis of the 5-HT Receptor, NOS and CCK Receptor Genes

The full-length cDNA sequences of 5-HT receptor, NOS and CCK receptor were cloned from a cDNA library generated from an *R. venosa* larval developmental sample mixture and named *Rv-5HTR*, *Rv-NOS*, and *Rv-CCKR*, respectively. The full-length cDNA of *Rv-5HTR* is 1,741 bp in length, comprising a 47-bp 5′ UTR, a 212-bp 3′ UTR, and a 1,482-bp ORF encoding 493 amino acids (**Supplementary Figure 1A**), and contains a serotonin receptor subtype 7 domain (**Supplementary Figure 2**) (GenBank Accession No. MW383250). The calculated molecular mass and isoelectric point of the predicted *Rv-5HTR* protein are 54.26 kDa and 9.33, respectively. The full-length cDNA of *Rv-NOS* is 3,813 bp in length, comprising a 28-bp 5′ UTR, a 38-bp 3′ UTR, and a 3,747-bp ORF encoding 1248 amino acids, and contains a PDZ domain, a NOS eukaryotic oxygenase domain and a ferredoxin-reductase (FNR)-like C-terminal domain (**Supplementary Figure 1B**) (GenBank Accession No. MW383249). The calculated molecular mass and isoelectric point of the predicted *Rv-NOS* protein are 138.82 kDa and 6.12, respectively. Then, the full-length cDNA of *Rv-CCKR* is 3,630 bp in length, comprising a 105-bp 5′ UTR, a 2418-bp 3′ UTR, and a 1290-bp ORF encoding 429 amino acids, and contains a CCK receptor domain (**Supplementary Figure 1C**) (GenBank Accession No. MW383248). The calculated molecular mass and isoelectric point of the predicted *Rv-CCKR* protein are 48.86 kDa and 9.80, respectively.

Homology and Phylogenetic Analysis of the *Rv-5HTR*, *Rv-NOS*, and *Rv-CCKR* Genes

The protein sequences of *Rv-5HTR*, *Rv-NOS* and *Rv-CCKR* from different species were used for homology and phylogenetic analyses. A BLAST search showed that the predicted amino acid sequences of *Rv-5HTR* had the highest sequence identity with that from *Pomacea canaliculata* (XP_025094540.1, 79.08%), followed by that from *Aplysia californica* (XP_005102984.1, 67.43%), *Haliotis discus hannai* (QEZ90776.1, 63.79%), *Mizuhopecten yessoensis* (XP_021371611.1, 59.68%), *Octopus sinensis* (XP_036367596.1, 53.21%), *Bombyx mori* (AIZ66402.1, 42.28%), *Homo sapiens* (AAF35842.1, 41.48%), *Mus musculus* (NP_032334.2, 39.44%), and *Danio rerio* (NP_001116793.1, 41.10%) (**Figure 1A**). The *Rv-NOS* shared higher similarity with that from other species and had extremely high sequence identity with that from *Stramonita haemastoma* (CBV37021.3, 95.83%), followed by that from *P. canaliculata* (XP_025092594.1, 73.86%), *A. californica* (NP_001191470.1, 70.38%), *Crassostrea virginica* (XP_022339299.1, 58.20%), *Octopus vulgaris* (QHX41539.1, 62.99%), *Bos taurus* (XP_024833521.1, 50.16%), *H. sapiens* (NP_000611.1, 49.84%), *M. musculus* (NP_032738.1, 57.72%), and *D. rerio* (XP_005165110.1, 57.93%) (**Figure 1B**). *Rv-CCKR* had the highest sequence identity with that from *P. canaliculata* (XP_025088086.1, 67.37%), followed by that from *O. vulgaris* (XP_029642692.2, 51.52%), *M. yessoensis* (VDI47157.1, 52.70%), *C. virginica* (XP_034300900.1, 47.86%), *H. sapiens* (NP_795344.1, 41.69%), *M. musculus* (NP_033957.1, 40.48%), *Drosophila melanogaster* (NP_001097023.1, 48.46%), and *D. rerio* (XP_017213239.1, 42.09%) (**Figure 1C**). To further elucidate the evolutionary relationships of the three genes from *R. venosa* with those of other species, we chose >10 species for

[4]https://www.immunostar.com/shop/antibody-catalog/5-ht-serotonin-rabbit-antibody/

phylogenetic analyses and constructed phylogenetic trees by N-J methods. The phylogenetic tree indicated that both *Rv-5HTR* and *Rv-CCK* were first clustered with those of *P. canaliculata*, while *Rv-NOS* was first clustered with that of *S. haemastoma* and was far from that of vertebrates. All three genes were clustered with those of mollusks, which are closely related to gastropods, and significantly separated from those of vertebrates (**Figure 2**).

Gene Expression of *Rv-5HTR*, *Rv-NOS*, and *Rv-CCKR* During Development

Quantitative real-time PCR was performed to detect the mRNA expression levels of the three genes at different developmental stages. The mRNA expression of *Rv-5HTR* was high in the cleavage stage (c) when the larvae had just hatched, then significantly decreased and was low in the early intramembrane larval stage (**Figure 3A**) ($P < 0.05$). The high expression reappeared in the early intramembrane veliger stage (ev) and into the planktonic larval stage, was maintained a high level throughout this stage, and a significant decrease occurred in the postlarval stage (J). Additionally, the expression of *Rv-NOS* greatly fluctuated throughout the whole early stage of development. *Rv-NOS* reached the first peak in the blastula stage (b), then significantly decreased in the gastrulae stage (g), significantly increased in the trochophore stage (t), significantly increased again in the early intramembrane veliger stage (ev) and reached the second peak. Then, the expression level slightly decreased and remained at this level until the two-spiral whorls stage (V-II), when it increased again, reached the third peak, sharply decreased in the three-spiral whorls stage (V-III), and increased again in the four-spiral layer (competent larva, V-IV), when it reached the fourth peak before significantly decreasing at postlarval stage (J) (**Figure 3B**). The expression of *Rv-CCKR* was low in the early intramembrane larval stage, then significantly increased in the early intramembrane veliger stage (ev), reached a peak in the middle intramembrane veliger stage (mv), continuously decreased until the four-spiral whorl stage (competent larva, V-IV), increased again and significantly decreased in the postlarval stage (J) (**Figure 3C**). The expression patterns of the three genes were slightly similar (**Figure 3D**), especially those of *Rv-5HTR* and *Rv-CCKR,* which were clustered, and all three genes first increased in the early intramembrane veliger stage (ev) and decreased in the postlarval stage (J) ($P < 0.05$).

Immunohistochemical (IHC) Analysis of 5-HT Throughout the Whole Early Developmental Stage

The results of the immunohistochemical analysis of 5-HT showed in **Figure 4**. We didn't observed positive immune signals in the cleavage stage (c), blastocyst stage (b) and gastrula stage (g) (**Figures 4a–c**). Two pairs of positive immune signals (**Figure 4d**, top organ, profile figure, only one signal is visible, as indicated by a white arrow) were observed at the base of the velum and the future eye site during the trochophore larval stage (t). The number of immune-positive signals increased to 5 in the early intramembrane veliger stage (ev), and these signals indicated the ganglia between the parietal and lateral ganglia of the parietal organs (**Figure 4e**, white arrow). The 5-HT immune-positive signal extended to both cerebral nerve fibers in the middle intramembrane veliger (mv) (**Figure 4f**, hollow arrow). Positive signals began to appear in the lower foot primordia of the later intramembrane veliger larvae (lv) (**Figure 4g**, white arrow), and a strong signal appeared at the velum (**Figure 4g**, white arrow). Furthermore, the immunohistochemical analysis of the four-spiral whorl stage larvae (competent larvae, V-IV) revealed a complex neural network in the mantle (**Figure 4h**, hollow arrow), foot (**Figure 4h**, white arrow) and velum (**Figure 4h**, blue arrow). The three major nerves in the velum (**Figure 4i**, white arrow) and the receptors in the base of the ciliate (**Figure 4i**, hollow arrow) were clearly connected in a complex nerve network.

Changes in Critical Gene Expression Induced by CCK

To assess the digestive and neuroendocrine systems of larvae induced by CCK, the mRNA expression of carboxypeptidase, cellulase, 5-HTR and NOS was detected (**Figure 5**). The expression of the carboxypeptidase gene was significantly increased in both the early and later stages in competent larvae induced by oysters, while cellulase was significantly decreased in the later stage ($P < 0.05$). The expression of *5-HT receptor* increased significantly, while the expression of NOS decreased significantly in both the early and later stage in competent larvae induced by oysters ($P < 0.05$).

DISCUSSION

Metamorphosis, the transition of free-swimming larvae to benthic and often sessile and attached juveniles, is one of the most distinctive life-changing events for many molluscan species, and the neuroendocrine system plays a key role in metamorphosis and has been extensively studied in herbivorous gastropods and bivalves. 5-HT and NO have been widely confirmed to regulate metamorphosis in *I. obsolete*, *Phestilla sibogae*, *Haliotis asinine* and a variety of bivalve species (Satuito et al., 1999; Leise et al., 2001; Croll, 2006; Yang et al., 2013; Ueda and Degnan, 2014; Zhu et al., 2020). However, the mec hanism by which metamorphosis is regulated in carnivorous gastropods may be more complicated due to the accompanying food habit transition, but little is known about this phenomenon. Therefore, to further understand the regulatory mechanism of *R. venosa*, the development and changes in the neuroendocrine system, especially those related to the digestive system during the early developmental stage, deserve more attention. This study elucidated the mechanism of neuroendocrine system development in *R. venosa* through an integrated approach of localization of the *Rv-5HTR* protein in the larva and examination of the molecular-metabolic responses of 5-HT, NOS, and CCK in order to further investigate the regulatory effect of the neuroendocrine system on the food habit transition and metamorphosis of *R. venosa*.

5-HT is one of the evolutionarily oldest neurotransmitters and performs its various physiological functions through seven 5-HT receptor subfamilies (5-HT 1–7) (Hannon and Hoyer,

FIGURE 1 | Multiple alignment based on *Rv-5HTR* **(A)**, *Rv-NOS* **(B)**, and *Rv-CCKR* **(C)** deduced amino acid sequences from *Rapana venosa* and other species. *Rv-5HTR*: serotonin receptor subtype domain 7, denoted in green box; *Rv-NOS*: PDZ domain denoted in pink box, NOS eukaryotic oxygenase domain denoted in green box and ferredoxin-reductase (FNR) like C-terminal domain denoted in yellow box; *Rv-CCKR*: CCK receptors domain denoted in green box. Additionally, the logo shows how conservative motif is in each position. The higher the letter, the more conservative the position. Different amino acids in the same position will scale according to its frequency. (*5-HT receptor*: GenBank Accession No. MW383250; *NOS*: GenBank Accession No. MW383249; *CCK receptor*: GenBank Accession No. MW383248) (The proteins used in the analysis were showed in **Supplementary Table 2**).

2008); 5-HT exerts a significant effect on the development and metamorphosis of marine invertebrate larvae (Leise et al., 2001; Sahoo and Khandeparker, 2018). In the present study, *Rv-5HTR*, which may belongs to serotonin receptor subtype 7, one of the 14 vertebrates serotonin receptors, is a member of the class A GPCRs and is activated by the neurotransmitter 5-HT. We found that the different subtypes of 5-HTR evolved independently in vertebrates, which has been not clear in invertebrates since the few studies. Previous studies indicated that the most prominent 5-HT cells during larval development were the five apical cells,

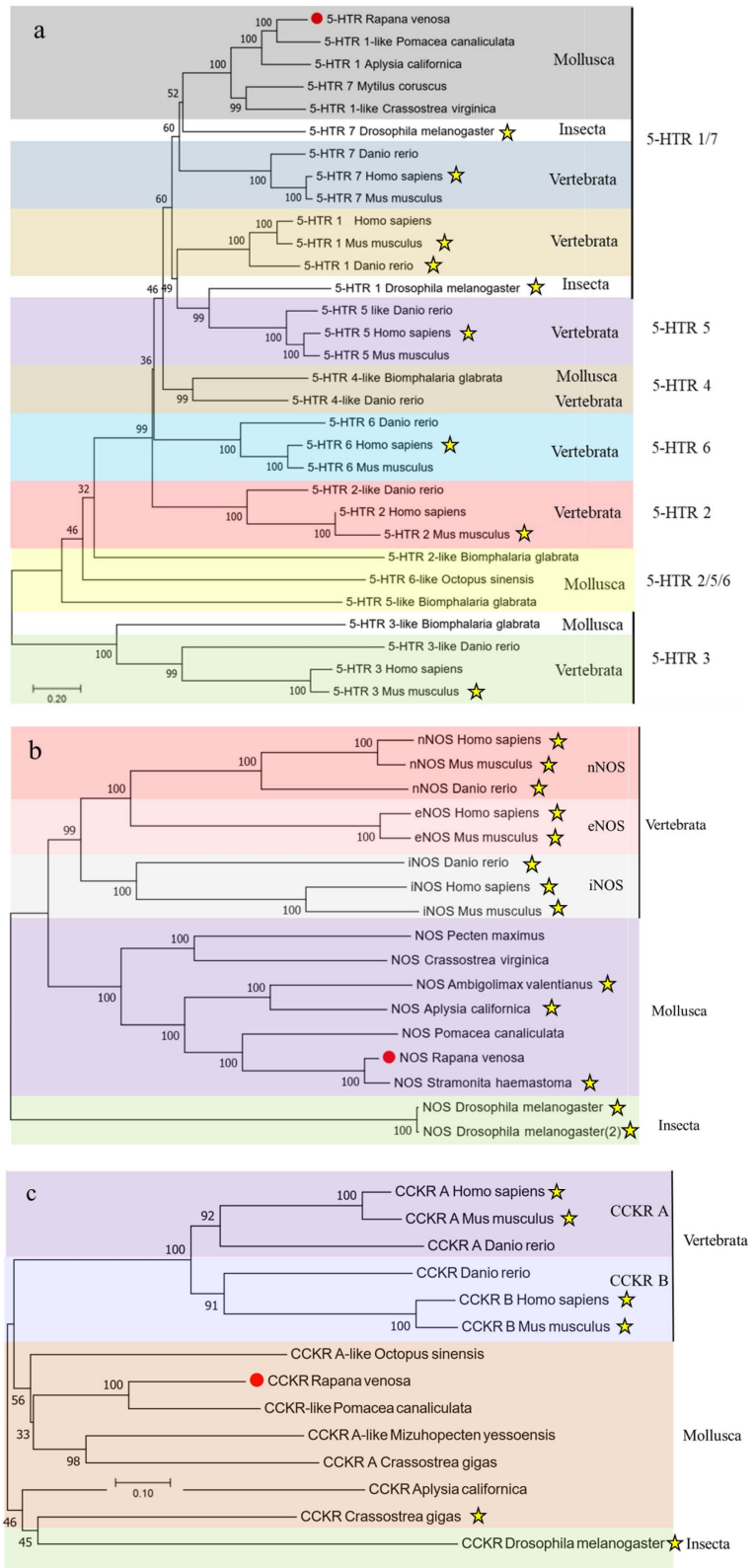

FIGURE 2 | Phylogenetic trees based on *Rv-5HTR* **(a)**, *Rv-NOS* **(b)**, and *Rv-CCKR* **(c)** deduced amino acid sequences from *Rapana venosa* and other species. The trees were constructed based on the multiple sequence generated by Clustal X and aligned using the neighbor-joining method in MEGA 7.0. The genes which have been pharmacologically proven were highlighted with asterisk, which has been listed in **Supplementary Table 3**.

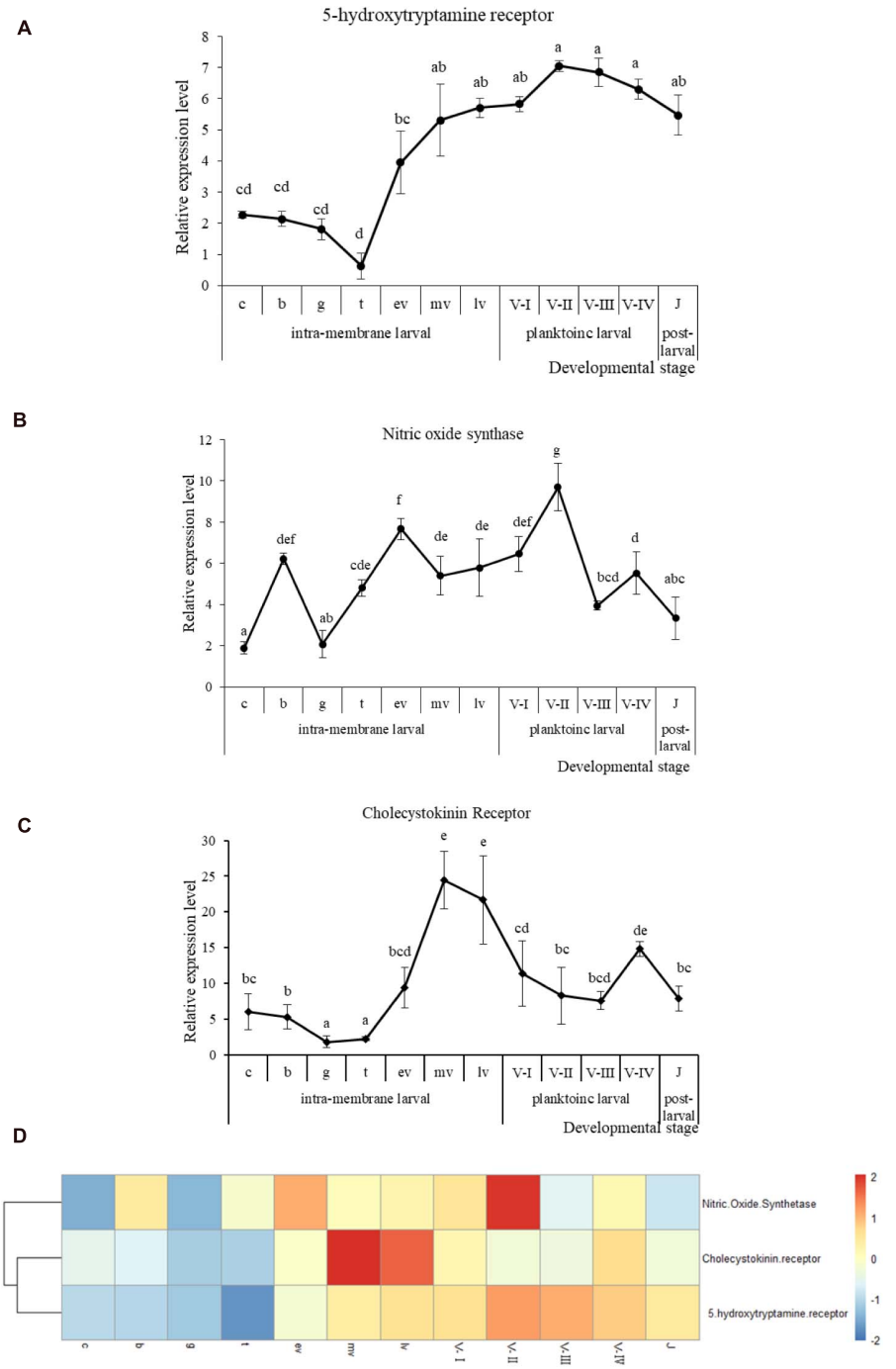

FIGURE 3 | qRT-PCR analysis of *Rv-5HTR* **(A)**, *Rv-NOS* **(B)**, and *Rv-CCKR* **(C)** mRNA expression and the heatmap **(D)** of three genes expression during *Rapana venosa* larval development (mean ± SEM, $n = 3$). Different superscript letters indicate significant differences ($P < 0.05$). [the cleavage stage (c), the blastula stage (b), the gastrulae stage (g), the trochophore stage (t), the early intramembrane veliger stage (ev), the middle intramembrane veliger stage (mv), and the later intramembrane veliger stage (lv). the one-spiral whorl stage (V-I), the two-spiral whorl stage (V-II), the three-spiral whorl stage (V-III), the four-spiral whorl stage (competent larva, V-IV), and the postlarval stage (J)].

and Hadfield et al. (2000) found that 5-HT was first expressed in apical organs during the development of gastropod larvae and was the main neurotransmitter in the apical organs. Our results showed that the 5-HT increased in the early intramembrane veliger stage (ev) both in the results of qRT-PCR and IHC, which may be the critical stage in the initial development of the digestive system in *R. venosa*. These results may reveal the regulation of 5-HT in the early development of *R. venosa* and suggest a

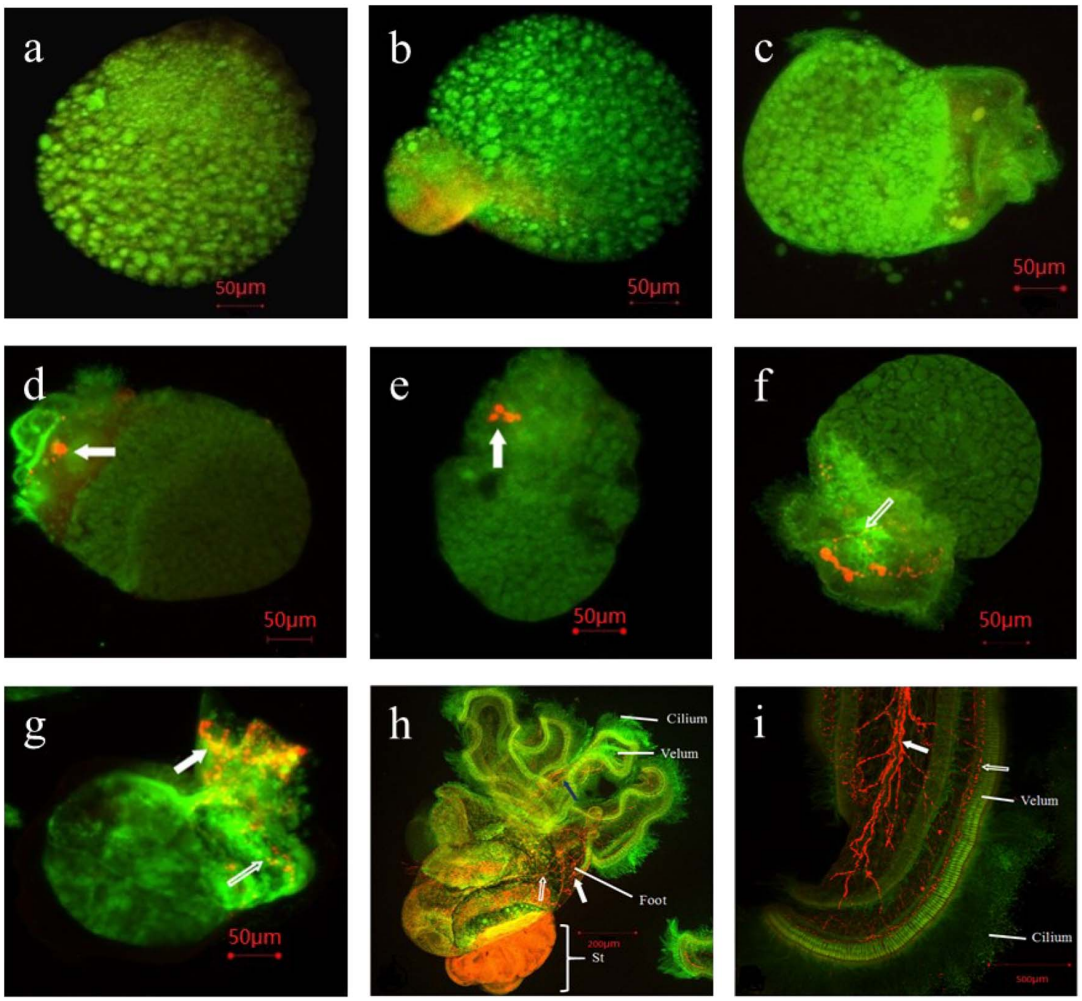

FIGURE 4 | Immunoreactivity of 5-HT and α-tublin in early development stage of *Rapana venosa*. [**(a)**. cleavage; **(b)**. blastula; **(c)**. gastrulae; **(d)**. late trochophore; **(e)**. early intra-membrane veliger; **(f)**. middle intra-membrane veliger; **(g)**. later intra-membrane veliger; **(h)**. competent larval stage; **(i)**. velum of competent larvae]. No positive immune signals in the cleavage stage **(a)**, blastula stage **(b)** and gastrulae stage **(c)**. Two pairs of positive immune signals in late trochophore stage [**(d)**, top organ, profile figure, only one signal is visible, as indicated by a white arrow]. Immune-positive signals increased to 5 in early intra-membrane veliger stage **(e)**. The 5-HT immune-positive signal extended to both cerebral nerve fibers in the middle intramembrane veliger stage [**(f)**, hollow arrow]. Positive signals began to appear in the lower foot primordia of the later intramembrane veliger larvae, and a strong signal appeared at the velum [**(g)**, white arrow]. The immunohistochemical analysis revealed a complex neural network in the mantle in four-spiral whorl stage larvae (competent larvae, V-IV) [**(h)**, hollow arrow], foot [**(h)**, white arrow] and velum [**(h)**, blue arrow]. The three major nerves in the velum [**(i)**, white arrow] and the receptors in the base of the ciliate [**(i)**, hollow arrow] were clearly connected in a complex nerve network.

relationship between 5-HT and the digestive system; a previous study also reported that 5-HT and its receptors play important roles in the regulation of gastrointestinal and endocrine functions (Berger et al., 2009).

Furthermore, 5-HT has been reported to significantly increase the rate of metamorphosis in *Ilyanassa obsoleta* by soaking or injection (Couper and Leise, 1996; Leise et al., 2001). Hadfield et al. (2000) indicated that 5-HT and 5-HTR in the apical organ can facilitate metamorphosis by sensing external chemical signals, and 5-HT is the key factor of signal transduction and metamorphosis initiation. We also observed the complex neural network based on the localization of the 5-HT protein in the competent larval stage of *R. venosa* (**Figure 4h**) and the velum of competent larvae (**Figure 4i**), and the receptors in the base of the ciliate network were particularly clear. Croll (2006) indicated that early development of serotonergic pedal neurons may reflect the need to regulate the activities of large numbers of ciliated cells found on the foot, which has been demonstrated in adults of a variety of gastropod species (Audesirk et al., 1979; Syed and Winlow, 1989). On the other hand, Glebov et al. (2014) reported that the expression level of 5-HT receptor gene was downregulated during the transition from the premetamorphic to metamorphic stages in *Helisoma trivolvis*; similarly, the expression level of *Rv-5HTR* was decreased after metamorphosis in the postlarval stage (J) in the present

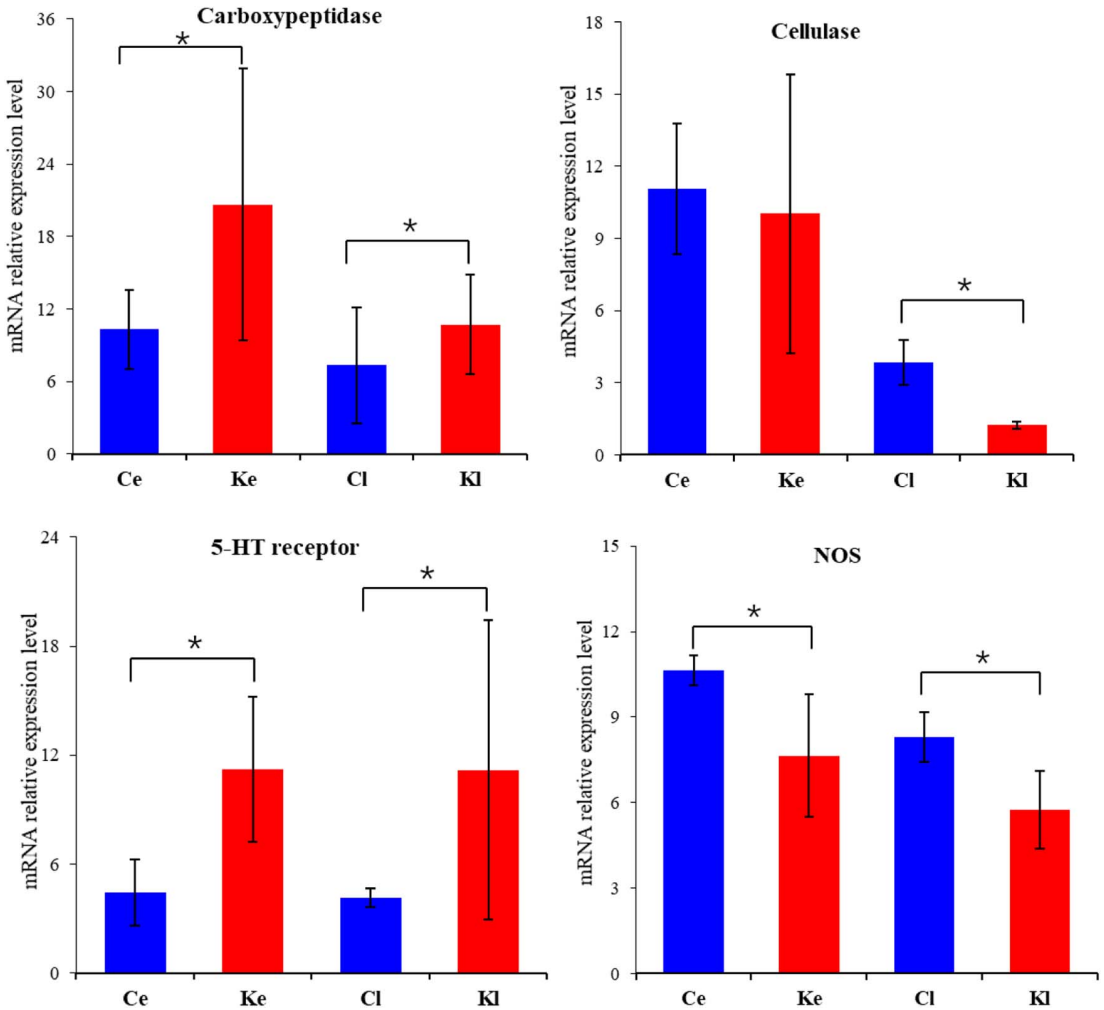

FIGURE 5 | mRNA expression of critical genes (Carboxypeptidase, Cellulase, 5-HT receptor and NOS) in Competent larva induced by CCK (mean ± SE, $n = 3$). (Different superscript letters indicate significant differences, $^*P < 0.05$). [Ce: Control (early, 2 h), Cl: Control (later, 12 h), Ke: CCK Induction (early, 2 h), Kl: CCK Induction (later, 12 h)].

study, and Yang Z. et al. (2015) indicated that 5-HT did not significantly induce the metamorphosis of *R. venosa*. These results may suggest that 5-HT and *Rv-5HTR* may not be positive regulators of metamorphosis in *R. venosa*. Therefore, the regulatory mechanism of 5-HT and *Rv-5HTR* requires further exploration.

Nitric oxide is an important molecular messenger that plays a critical role in the nervous systems of both vertebrates and invertebrates (Snyder and Bredt, 1992; Colasanti and Venturini, 1998), and Leise et al. (2001) indicated that NO played an antagonistic role in the 5-HT mediated pathway of larval metamorphosis, and that NO was produced by NOS catalyzing the five-electron heme-based oxidation of the guanidine nitrogen of L-arginine to L-citrulline. In the present study, the *Rv-NOS* gene contains a PDZ domain, a NOS eukaryotic oxygenase domain and a ferredoxin-reductase (FNR)-like C-terminal domain. The PDZ domain is often found in a variety of eumetazoan signaling molecules and may be responsible for specific protein-protein interactions. The NOS eukaryotic oxygenase domain also has a C-terminal electron-supplying reductase region, which is homologous to cytochrome P450 reductase and binds to NADH, FAD, and FMN. We found that *Rv-NOS* was tightly clustered with the gene from *S. haemastoma* in the phylogenetic tree (**Figure 2b**), and the sequence similarity of homologous alignment was as high as 95.83%; this result indicated that the two species were closely related. In *S. haemastoma*, the mRNA transcripts mainly expressed in the central nervous system and peripheral structures which involved in sensory organs, such as the osphradium, cephalic tentacles, and buccal/esophageal tissues (Brown et al., 2004; Cioni et al., 2011); this result has also been confirmed in *A. californica* (Moroz, 2010). The present results showed that the expression of *NOS* was increased in both trochophore larvae (t) and the early intramembrane veliger stage (ev), which are critical periods for the development of velum and the digestive system. These results may suggest that NOS plays an important role in

the olfactory and digestive systems in *R. venosa* and that NOS dramatically changes during the metamorphosis of *R. venosa* (Song et al., 2016a; Yang et al., 2020a,b). In addition, we found that both the mRNA expression levels of NOS were decreased in the postlarval stage (J) compared to the competent larval stage (V-IV), which may further confirm the hypothesis that NOS plays a negative regulatory role (Froggett and Leise, 1999). Additionally, Sahoo and Khandeparker (2018) indicated that the inhibitory effects of NO and NOS may enhance the probability of locating a suitable substratum by delaying metamorphosis and the dispersal capability of the larvae to increase gene flow and enhance individual vigor, which may explain the increase in the mRNA expression of *Rv-NOS* in the competent larvae (V-IV) in our results. However, Ueda and Degnan (2014) found that NOS and NO may be positive regulators of the initiation of metamorphosis in some gastropods, such as *H. asinine*. The signaling pathways of NOS and NO have extensive functions, and their regulation during the metamorphosis of *R. venosa* requires further study.

Tripathi et al. (2015) demonstrated that CCK stimulates NOS, which has also been confirmed by Moustafa et al. (2011), while we found the inhibition of CCK on NOS in *R. venosa*. The *Rv-CCKR* gene contains a CCK receptor domain and is a member of the class A family of seven-transmembrane G-proteins, which are abundant in pancreatic acinar cells. CCK is a gastrointestinal regulatory hormone that is implicated in the regulation of digestion and appetite control by stimulating pancreatic enzyme secretion, gallbladder contraction, and gut motility satiety and inhibit acid secretion from the stomach via CCK receptor (Tripathi et al., 2015; Rehfeld, 2017). In the present result, we also found that CCK can decreased the expression of Carboxypeptidase in *R. venosa*, and increased the expression of Cellulase, which may suggest that CCK can promote the food habit transition of *R. venosa*. In addition, CCK is also known to stimulate pancreatic growth *in vitro* and *in vivo* in mice, rats, or hamsters (Cordelier et al., 1999), which may potentially explain the increased expression of *Rv-CCKR* in the early intramembrane veliger stage (ev), which is the critical stage of digestive system development in *R. venosa*. Additionally, Schwartz et al. (2018) indicated that plausible role of Cragi-CCK signaling in the regulation of feeding in in the oyster *Crassostrea gigas*. He et al. (2015) reported that CCK was increasingly expressed in grass carp after food habit transition, while in the present study, the expression of *Rv-CCKR* was significantly decreased, the difference may due to the different type of food habit transition, and the significant changes suggest that *Rv-CCKR* and CCK are involved in food habit transition during metamorphosis. Additionally, the CCK can increase the expression of *5-HT receptor*, and Cooper and Dourish (1990) have also indicated that the 5-HT and CCK are synergistic and interdependent in the regulation of feeding.

CONCLUSION

Here, we show the changes of 5-HT and NOS during food habit transition in metamorphosis of *R. venosa*. Moreover, we found CCK is an important factor that regulates the development and function of the digestive system, which may further suggest a close relationship between food habit transition and metamorphosis. By exploring the changes in critical genes during the early stages of larval development, the developmental process of the neuroendocrine system was initially identified; by further exploring the regulation of CCK, we reveal the relationship between digestive system and neuroendocrine system. This study provides new insight for studying the process of metamorphosis in carnivorous gastropods.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

TZ conceived and designed the experiments. M-JY and HS conducted the experiments. M-JY, JF, and HS analyzed the data. Z-LY, ZH, CZ, PS, JL, and X-LW contributed reagents, materials, and analytical tools. M-JY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.690282/full#supplementary-material

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