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Ecdysis Triggering Hormone, Eclosion Hormone, and Crustacean Cardioactive Peptide Play Essential but Different Roles in the Molting Process of Mud Crab, *Scylla paramamosain*

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Molting behavior in insects is controlled by the ecdysis triggering hormone (ETH), eclosion hormone (EH) and the crustacean cardioactive peptide (CCAP). At present, the regulation of molting behavior in crustaceans remains unclear. Here, we studied the roles of *ETH*, *EH,* and *CCAP* in the molt regulation of the crab, *Scylla paramamosain* from their expression pattern and *in vivo* assays. The results showed that transcripts of *ETH*, *EH,* and *CCAP* were mainly localized in thoracic ganglia and fluctuated periodically with the molting cycle. When *ETH* or *CCAP* was knockdown at early premolt stage (D0), molting of crabs was interrupted and all animals died at late premolt stage (D2). While the *EH* gene was knock-down, most crabs were dead before D2. Injection of synthetic peptide for ETH or CCAP rescued *ETH*- or *CCAP*-gene knock-down crabs separately. However, none of peptides could rescue *dsEH*-injected crabs. At D0 stage, knockdown of *ETH* down-regulated the transcriptions of *EH* and *CCAP*; while *ETH* was up-regulated when *EH* was knockdown. At D2 stage, *ETH* transcripts levels were reduced with the injection of *dsEH* but increased with the same dose of *dsETH* as crabs at D0 stage. Co-injection of ds*ETH* and ds*EH* down-regulated *ETH* at D2 stage. Results showed that *ETH*, *EH,* and *CCAP* play essential but different roles in molt regulation in mud crab. In summary, the result of this study contributes to the discovery of different molecular mechanisms between Insecta and Crustacea and may provide insight to develop fishery drugs that helps aquacultured crustaceans to molt successfully.

Keywords: *Scylla paramamosain*, Crustacea, ecdysis triggering hormone, eclosion hormone, crustacean cardioactive peptide, RNA interference, molt

INTRODUCTION

Insects and crustaceans share a number of similar hormones in the regulation of molting and reproduction. During the molting of insects, many neuropeptides related to molting were regulated by precise change of ecdysteroid titer to prepare for the event (Zitnan and Adams, 2012). These neuropeptides include the ecdysis triggering hormone (ETH), eclosion hormone (EH) and

crustacean cardioactive peptide (CCAP), etc. (Song et al., 2017). These hormones are released in large quantities, which in turn initiates the onset of molting behavior (Zieger et al., 2021). However, whether these neuropeptides have similar functions and regulation modes in crustaceans is still unknown. Molting in crustacean is under the tight endocrine control of many hormones. Among those hormones, the steroid hormone ecdysone is the major hormone known to initiate the molting process (Claeys et al., 2006). Therefore, an increase of ecdysteroid titer in the hemolymph is needed for the start of the molting process. A series of ecdysis-related genes are expressed under the influence of cyclical fluctuations of ecdysone, followed by the binding with nuclear receptors to transmit ecdysis signals, so that the crustaceans complete the pre-molting preparations on time and accurately (Zhao, 2020). When molting begin, the initiation of molting behavior occurs (Song et al., 2017). Previous studies have shown that arthropods mainly initiate molting behavior at the premolt stage (D) (Oliphant et al., 2018; Mykles and Chang, 2020). Even the initiation of the molting process has started, there is no guarantee that the animal will molt successfully as the fulfillment of the subsequent events must be straightly followed. In insects, neuropeptides that induce molting behavior include ecdysis-triggering hormone (ETH) (Shi et al., 2019a; Minh Nhut et al., 2020; Shen et al., 2021), eclosion hormone (EH) (Hull et al., 2009; Zhou et al., 2017; Scott et al., 2020), crustacean cardioactive peptide (CCAP) (Veelaert et al., 1997; Arakane et al., 2008; Jackson et al., 2009), etc.

Eclosion hormones (EH) is a neuropeptide that influences several aspects of pupal-adult ecdysis as well as larval-larval ecdysis (Hull et al., 2009; Scott et al., 2020). It was first characterized in the moth *Bombyx mori* as a brain neuropeptide (Truman and Riddiford, 1970). After its synthesis, EH was transported to the targets *via* hemolymph. The release of EH from the brain is controlled by a circadian clock in the brain and declining ecdysteroid titers (Truman and Riddiford, 1970). In the targets, signal transduction occurred *via* membrane receptors and through the action secondary messengers. As a result, the process of metamorphosis was initiated (Morton and Simpson, 2002). Although the crustaceans "EH" (decapod possess two of them, again entirely structurally conserved) has significant similarities to those of insects, homology with respect to function in arthropods is premature. ETH was first discovered in the moth *Manduca sexta*. It was produced from the secretion of the endocrine gland located in the trachea. ETH can directly stimulate ecdysis (Zitnan et al., 1996) and the larva responds much faster after they were injected as compared to EH. ETH is secreted by Inka cells of the trachea and it shared strong structural and functional conservation among a variety of insects (Adams and Zitnan, 1997; Mesce and Fahrbach, 2002; Park et al., 2002; Zitnan et al., 2003). Most insect *ETH* genes can express a variety of mature peptides through alternative splicing. The *Lepidoptera* can produce a pre-ecdysis triggering hormone (PETH) and ETH. PETH triggers early molt regulation, then ETH initiation of late molting regulation and molting behavior (Park et al., 1999, 2002); whereas, most other insects produce two mature peptides, ETH1 and ETH2, both of which are encoded by the same gene of *ETH*. For example, in *Drosophila melanogaster*, ETH1 is even more critical in the control of molting behavior (Zitnan et al., 1999). ETH plays a role by binding to its cell membrane receptor ETHR. The gene of *ETHR* can also encode two receptor subtypes ETHR-A and ETHR-B through alternative splicing and also with functional differentiation. In *Bactrocera dorsalis*, binding of ETH to the receptor ETHR-A will form a complex which in turn trigger ecdysis behavior. However, when ETH interact with ETHR-B, the complex trigger the process of reproduction regulation (Shi et al., 2017, 2019a). ETH in crustaceans, which was firstly identified as "carcikinin/ETH" in the crab *Carcinus maenas* (Oliphant et al., 2018), has some similarities with insect ETH, with respect to the C-terminal motif PRI/L-a. Through comparative transcriptomic study of *ETH* from different crustaceans, it was revealed that two splicing variants of *ETH* were present in the shrimp *Litopenaeus vannamei*. However, only one transcript was reported in the fresh water shrimp *Macrobrachium rosenbergii*, the crabs *Eriocheir sinensis* and *Scylla paramamosain*, the crayfish *Procambarus clarkii* so far. In crayfish *P. clarkii*, molting was delayed after they were injected with ETH (Minh Nhut et al., 2020), which was different to insects (Arakane et al., 2008; Lenaerts et al., 2017). Also, crustacean express ETH mainly in the nervous system rather than trachea, and its expression pattern increases dramatically in very late premolt (Veenstra, 2016; Oliphant et al., 2018). The situation differs quite markedly in insects where the authentic ETH is only expressed much earlier in the ecdysis program by non-neuronal Inka cells. The amino acid sequences of most decapod "carcikinin/ETH" is identical. To date, there is no published information on the neural architecture of either of these peptides, only expression patterns in the CNS. Also they are produced as secreted circulating neurohormones. Whether the functions of ETH in crustaceans undergo functional differentiation required more research. CCAP is mainly produced in the nervous tissues of both insects and crustaceans (Veenstra, 2016). It has been confirmed that they have different functions on various physiological processes in insects and crustaceans (Gammie and Truman, 1999; Kim et al., 2006; Fort et al., 2007). Whereas what function does *CCAP* act in the molting of crabs such as *S. paramamosain* is still unknown. In aquaculture of the crab *S. paramamosain*, failure of molting was commonly occured that result in an increase in mortality and causes losses to the aquaculture industry. To date, the release patterns, endocrine cascades and titers of the neurohormones such as ETH, EH, CCAP in crustaceans are still unclear. The detailed mechanism that *ETH*, *EH,* and *CCAP* affect molt behavior and the interrelationship of the hormones in the molt of *S. paramamosain* need further research, which may help to improve the economic benefits of aquaculture.

In this study, we have monitored the expression of several key molting-related genes *ETH*, *EH* and *CCAP* that induce the molting process the crab (Fort et al., 2007; Hull et al., 2009; Shen et al., 2021). Also, we have developed *in vivo* assays in juvenile crabs during the premolt stage of the molting crab. The irreplaceable role in *Scylla* molting and the mutual regulatory relationship have further verified that ETH and EH also have different regulatory effects on each other at different stages, which have different roles in different insects.

MATERIALS AND METHODS

Animals

Crabs were collected from coastal area of Zhanjiang, Guangdong Province, China. Individuals (i.e., 2.1–3.4 g) were selected and stocked in 1 m^3 concrete ponds. They were cultured at 24–27°C, salinity at 20‰ and fed daily with oyster. After a week, healthy crabs at D0 (i.e., 2.4–2.9 g) were selected for molting experiment, healthy crabs at D0 and D2 stages (Ong, 1966) were selected for dsRNA-injected experiments.

Tissue Collection, RNA Preparation and cDNA Synthesis

Crabs were chilled on ice before dissection for tissues. For every tissue that was analyzed, samples were collected in three different pools of five dissected animals each and stored at −80°C until further processing. Total RNA preparation was prepared using a column-based TransZol Up Plus RNA kit (TransGen Biotech, China). After elution, the concentration of total RNA was determined using the Thermo Fisher Scientific the NanoDrop 2000 (Waltham, MA, United States) and the quality of total RNA was tested by 1% agarose gel. For synthesis of first strand cDNA, 5 × All-In-One RT cDNA synthesis kit (ABM Inc., Richmond, BC, Canada) was used. The cDNAs were diluted tenfold for later analyses.

qT-PCR Determination of Transcript Contents

The open reading frame of ETH (SRR3086589, SRR3086590), EH (GeneBank accession No: KR078366.1) and CCAP (GeneBank accession No: MN923209.1) were obtained from NCBI Database (**Supplementary Figures 1–3**). Primers were designed using the Software Primer 5.0 (**Supplementary Table 1**) and 18s rRNA and β-actin were used as internal control genes (Chung and Lin, 2006; Liu et al., 2020). Each qPCR reaction contained 10 μL of SYBR qPCR Master Mix (Vazyme, Nanjing, China), 2 μL of cDNA, 7 μL of ultrapure water and 0.5 μL of each Forward and Reverse primer (10 μM). PCR was performed with the Real Time PCR machines (Bio-Rad CFX Connect PCR, Bio-Rad, United States). The following thermal cy-cling profile was applied: 95°C for 2 min, followed by 39 cycles of 95°C for 5 s and 55°C for 30 s, and then a melt curve analysis was performed to verify the specificity of the qRT-PCR reactions. The relative mRNA abundance of each gene was calculated by ΔΔCt method, then normalized by that of 18S and β-actin for each sample. The reaction was performed in triplicated for each sample. All primers used in the present study were given in **Supplementary Table 1**.

RNAi and Rescue Experiments

Firstly, primers flanked by the T7 promoter sequence were designed and used to synthesize the *dsRNA* for *EH*, *ETH*, *CCAP* and control gene (*GFP*). Secondly, the dsRNA template for each gene were amplified (PCR amplification procedure: 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s; then 72°C for 10 min) with above primers and purified by means of FastPure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China). After the concentration detection of DNA tem-plates (NanoDrop 2000, Thermo Fisher Scientific, Inc., Waltham, MA, United States). dsRNA was produced with DNA templates under the method of T7 RiboMAX^{TM} Express RNAi Synthesis kit (Promega, Beijing, China). The final dsRNA was diluted to appropriate concentration (1 μg/μL) with water. Synthetic peptides of ETH and CCAP (Oliphant et al., 2018) were synthesized by GenScript (Nanjing, China) and dissolved in water to 1 μg/μL.

RNA interference experiments (n = 50) were done with the injection of *dsEH, dsETH, dsCCAP,* and *dsGFP*, respectively. Each crab was injected in dsRNA at a dose of 5 μg/g. For the synthetic peptide compensation experiments, each of *dsEH, dsETH* and *dsCCAP* were co-injected with ETH and CCAP synthetic peptides, respectively, and each co-injected combination named a group (n = 50). Both dsRNA and synthetic peptide were injected at a dose of 5 μg/g, which was an over-added level of synthetic peptides in case of inadequate compensation to the negative effects of gene knockdown. Control group was injected with *dsGFP* following the same injected dose. Boost injections were given every 5 days to ensure a lasting affection of gene mRNA levels. The number of deaths and molts in each group were counted daily, also the stage of any deaths were observed by microscope. Crabs injected with *dsGFP* were served as control.

Crabs (n = 20) at D0 and D2 stages were collected and separately injected with *dsEH and dsETH* at a dose of 5 μg/g at each stage. Also, crabs (n = 20) at D2 stage were selected for co-injection of *dsEH* and *dsETH* at a dose of 5 μg/g for each dsRNA. After 48 h, the high-expression tissues of nerves were sampled and analyzed. Crabs injected with *dsGFP* were served as control.

Statistical Analysis

The relative transcriptional levels of *ETH*, *EH,* and *CCAP* were calculated using $2^{-\Delta\Delta Ct}$ method. All data were expressed as means ± standard deviation (SD) and analyzed by GraphPad Prism8.0.1 (GraphPad Software Inc., San Diego, CA, United States). Statistical analysis was performed using one-way ANOVA and followed by Tukey's analysis method with significant difference ($P < 0.05$). The differences on transcriptional level of genes between treatments and control groups were analyzed by Student's t test ($P < 0.05$). For the analyses of molting rates, the log-rank (Mantel-Cox) test was performed and followed by Student's t-test with Welch's correction ($P < 0.05$).

RESULTS

Expression Patterns of Ecdysis Triggering Hormone, Eclosion Hormone, and Crustacean Cardioactive Peptide in Juvenile Crab

Quantitative real-time PCR (qRT-PCR) results indicated that *ETH* expression level was the highest in the thoracic ganglion, followed by that in the eyestalks and brain of juvenile crabs (**Figure 1A**). *EH* transcripts were mainly localized in the

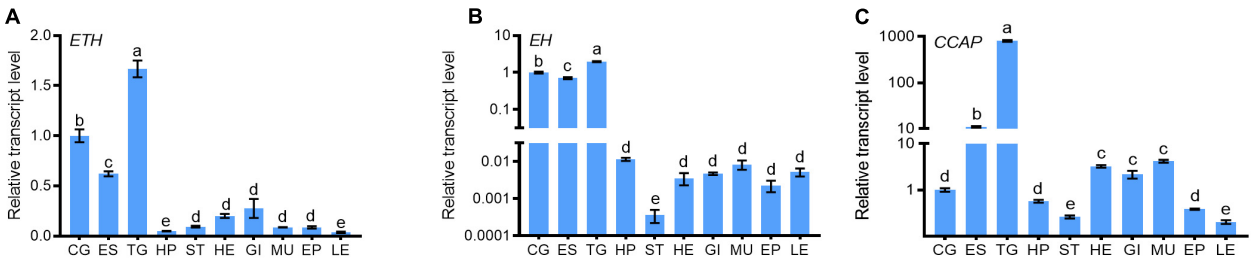

FIGURE 1 | Tissue expression patterns of *ETH* **(A)**, *EH* **(B)**, and *CCAP* **(C)** in *S. paramamosain*. Tissues include cerebral ganglion (CG), eyestalk (ES), thoracic ganglia (TG), hepatopancreas (HP), stomach (ST), heart (HE), gill (GI), muscle (MU), epidermis (EP), and leg (LE) of crabs at the intermolt stage. For each sample, five individuals were measured in triplicate using real-time quantitative PCR (qRT-PCR) analysis. The values were calculated using the $2-\Delta\Delta CT$ method. The relative transcript levels of CG were set as 1. The bars represented averages relative transcript levels and standard deviation (SD). Different letters indicated significant differences at $P < 0.05$ analyzed by ANOVA and the Tukey-Kramer test.

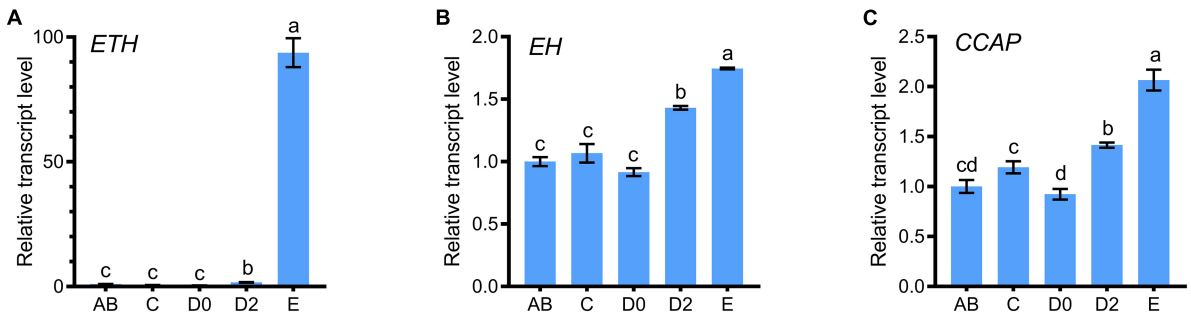

FIGURE 2 | Temporal expression patterns of *ETH* **(A)**, *EH* **(B)**, and *CCAP* **(C)** in *S. paramamosain*. The cDNA templates were derived from the post-molt stage (AB), intermolt stage (C), the early premolt stage (D0), the late premolt stage (D2) and the ecdysis stage (E). For each sample, three independent pools of five individuals were measured in triplicate using real-time quantitative PCR (qRT-PCR). The values were calculated using the $2-\Delta\Delta CT$ method. The relative transcript levels of AB were set as 1. The bars represented averages with vertical lines indicating standard deviation (i.e., SD). Different letters indicate significant differences at $P < 0.05$ computed with ANOVA and the Tukey-Kramer test.

neuronal tissues, and the level was the highest in the thoracic ganglia, followed by the brain and the eyestalks (**Figure 1B**). *CCAP* transcript level was the highest in the thoracic ganglia (**Figure 1C**). A progressive decrease of *ETH* transcript level was observed from post-molt stages (i.e., A and B) toward the early premolt stage (D0). However, a 100-folds increase in *ETH* transcript level occurred at the late premolt stage (D2) (**Figure 2A**). The transcript levels of *EH* and *CCAP* at A, B, C, and D0 stages have little changes until the sharp increase at D2 and reach the maximum level at the ecdysis stage (E) (**Figures 2B,C**).

Knockdown of *dsETH*, *dsEH* or *dsCCAP* in Crabs at D0 Stage Caused Molt Failure and Can Be Rescued by Synthetic Peptides Except *dsEH* Group

When juvenile crabs at the D0 stage were injected with *dsETH*, *dsEH* or *dsCCAP* (knockdown condition), the percent of crabs that molt normally was seriously affected compared to the *dsGFP*-injected group. For *dsETH* and *dsCCAP* groups, apolysis occurred in those crabs and most crabs could advance to the late premolt stage. Also, the old exoskeleton and the new epidermis for which could be separated from the new cuticle artificially (**Figures 3A,B**). However, more than half of the crabs failed to initiate the molting process and eventually death occurred at D2 stage in those crabs (**Figure 3C**). As a comparison, 58% of the crabs injected with *dsEH* died before D2 stage, which was significantly higher than that in other groups (**Figure 3C**).

To further verify the function of molt regulation between *ETH*, *EH*, and *CCAP*, synthetic peptides of ETH and CCAP were co-injected with these three dsRNAs separately. Injection of ETH increased the number of successful molts and survival rate in the *ETH*-knockdown group from 28 to 72%. However, this has no effect on the *EH*-knockdown and *CCAP*-knockdown groups (**Figure 3**). Injection of CCAP increased the number of successful molts and survival rate in the *CCAP*-knockdown group from 42 to 72%. However, it has no effect to the *EH*-knockdown group. However, injection of CCAP the number of successful molts and survival rate in *ETH*-knockdown group from 28 to 50% (**Figure 3**).

Transcript Levels of Molt-Related Genes in Injected Crabs at D0 Stage

To further elucidate the relationship between *EH* and *ETH* at different molting stages, transcriptional levels of genes in *dsETH*- and *dsEH*-injected crabs at D0 and D2 stages were analyzed. For crabs at the D0 stage, injection of *dsETH* caused

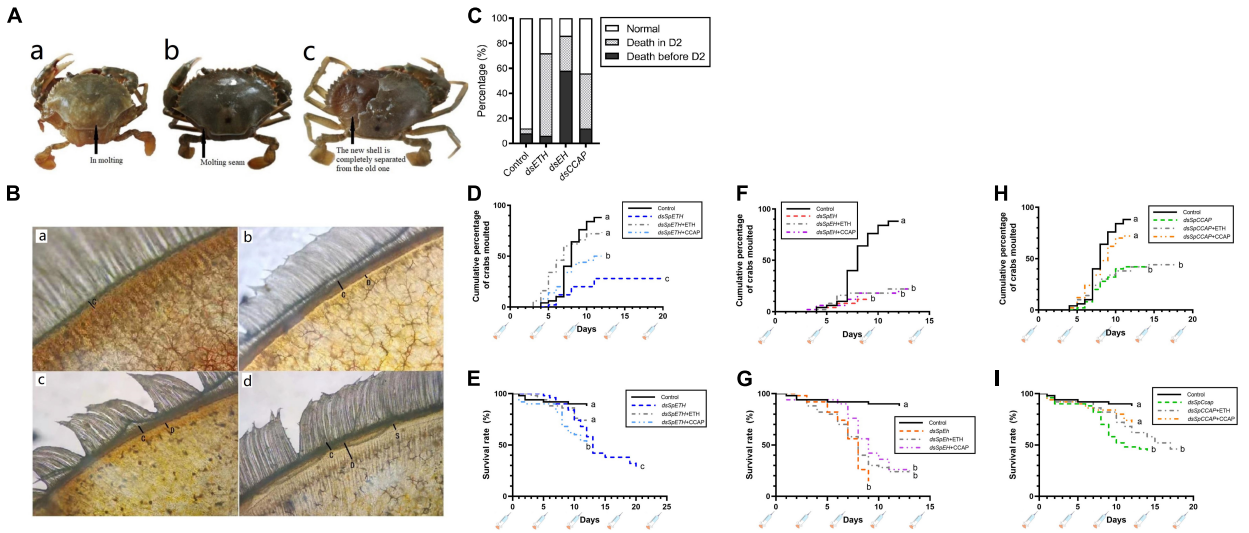

FIGURE 3 | Effect of *in vivo* dsRNA injection on crabs molting **(A,a)** Crab that started molting normally; **(A,b)** crab that unable to initiate molting and died; **(A,c)** crab died at D2 stage and the exoskeleton of could be peeled off easily. **(B)** Microscopic examination of a portion of the crab swimming legs at C **(B,a)**, D0 **(B,b)**, D1 **(B,c)**, and D2 **(B,d)**. The letter C in figures represented the old epidermis, letter D represented the new epidermis, letter S represented the ecdysial suture. **(C)** Death records of crabs at different stages for the four groups injected with *dsGFP*, *dsETH*, *dsEH*, and *dsCCAP*, respectively (n = 50). **(D)** The cumulative percentage of groups injected with *dsGFP*, *dsETH*, *dsETH* + ETH and *dsETH* + CCAP, respectively, at D0 stage. **(E)** The survival rate of groups injected with *dsGFP*, *dsETH*, *dsETH* + ETH and *dsETH* + CCAP respectively at D0 stage. **(F)** The cumulative percentage of groups injected with *dsGFP*, *dsEH*, *dsEH* + ETH and *dsEH* + CCAP respectively at D0 stage. **(G)** The survival rate of groups injected with *dsGFP*, *dsEH*, *dsEH* + ETH and *dsEH* + CCAP respectively at D0 stage. **(H)** The cumulative percentage of groups injected with *dsGFP*, *dsCCAP*, *dsCCAP* + ETH and *dsCCAP* + CCAP respectively at D0 stage. **(I)** The survival rate of groups injected with *dsGFP*, *dsCCAP*, *dsCCAP* + ETH and *dsCCAP* + CCAP respectively at D0 stage. Statistically significant differences (P) between the control and any knockdown conditions were found via a log-rank (Mantel-Cox) test and are indicated by different letters.

a significant reduction of *ETH* transcript level (−30.82%). The transcript levels of *EH* and *CCAP* also decreased significantly (**Figures 4A–C**). Injection of *dsEH* to juvenile crabs at stage D0 silenced the transcription of *EH* to a level of 61.92% (**Figure 4D**). However, the transcripts level of *ETH* increased sharply (+360%) (**Figure 4E**), but there was no change in the transcript level of *CCAP* (**Figure 4F**).

Transcriptional Levels of Genes in Injected Crabs at D2 Stage

When crabs at D2 stage were injected with the same amount of *dsETH* as at D0 stage, the transcripts level of *ETH* had a significant increase conversely and *CCAP* transcripts level was decreased significantly compared to control but the transcripts level of *EH* had no change (**Figures 5A–C**). When juvenile crabs at D2 stage were injected with the same amount of *dsEH* as at D0 stage, the transcripts level of *EH* was decreased to 23.22% compared to that of the controls (**Figure 5D**). In addition to the injection of d*sETH*, knock-down of *EH* could also reduce the transcripts level of *ETH* significantly (**Figure 5E**), in which the transcripts level of *CCAP* decreased significantly too (**Figure 5F**).

Co-injection of *dsETH* and *dsEH* to Crabs at D2 Stage

To verify the regulatory relationship between *ETH* and *EH*, co-injection of *dsETH* and *dsEH* were conducted to crabs at D2 stage. Results show that *ETH* expression was knock-down as compared to the controls, with a decrease of 68.06% (**Figure 6A**), and the transcriptional level of *EH* decreased by 77.19% (**Figure 6B**). Similarly, the transcriptional level of *CCAP* was significantly reduced than that of the control group (**Figure 6C**).

DISCUSSION

From post-larva onward, mud crab has to molt 18–20 times in its lifetime (Kim et al., 2006). The early larval molts were characterized by metamorphic transformation. From the juvenile stage onward, molting was characterized by size increment only. In the present study, *ETH*, *EH,* and *CCAP* were selected based on the knowledge that these genes are involved in insects molting (Fort et al., 2007; Hull et al., 2009; Jackson et al., 2009), and experiments were conducted under *in vivo* assays with juvenile crabs.

Transcripts of Ecdysis Triggering Hormone, Eclosion Hormone, and Crustacean Cardioactive Peptide Are Mainly Localized in Thoracic Ganglia and Fluctuate Periodically With Molt in *Scylla paramamosain*

In insects such as *Leptinotarsa decemlineata* and *Bactrocera dorsalis*, ETH is mainly produced by Inka epithelial cells of the endotracheal gland in the trachea. The ETH expression level is

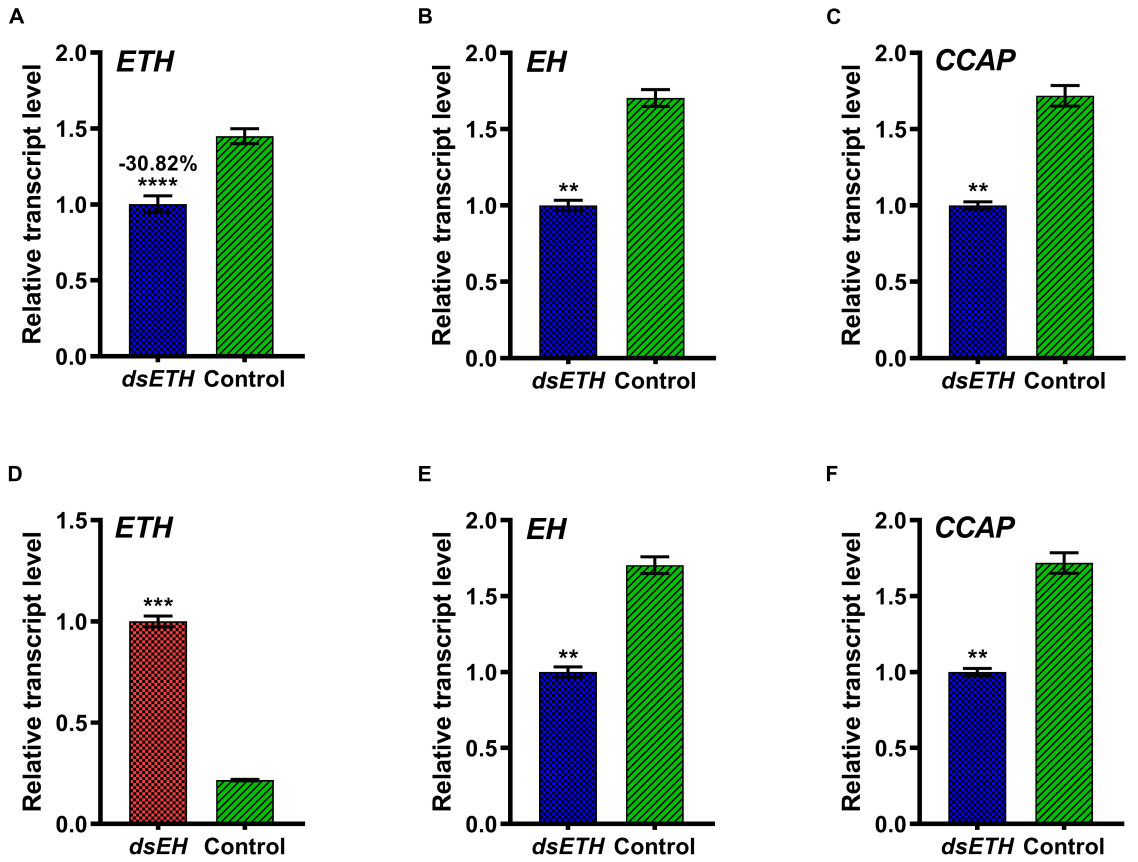

FIGURE 4 | Relative transcript levels of *ETH* **(A)**, *EH* **(B)**, and *CCAP* **(C)** in thoracic ganglia of *dsGFP* and *dsETH*-injected crabs at D0 stage were measured. Also, relative transcript levels of *ETH* **(D)**, *EH* **(E)**, and *CCAP* **(F)** in the thoracic ganglia of *dsGFP* and *dsEH*-injected crabs at the D0 stage were measured. These data represented the mean ± SD. For each sample, three independent pools of five individuals were measured in technical triplicate using real-time quantitative PCR (qRT-PCR). Statistically significant differences between the measurements were found *via* a *t*-test with Welch's correction and are indicated by asterisks (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

the highest at the late premolt stage (Shi et al., 2017; Shen et al., 2021). In *Drosophila*, EH is expressed in the neuronal tissues and trachea, which participate in the regulation of molting behaviors (Scott et al., 2020). Although the crustaceans did not evolve a tracheal system, Both the *Insecta* and *Crustacea* shared a large number of homologous neuropeptides that are highly conserved in structure and functions (Veenstra, 2016; Oliphant et al., 2018). In this study, *ETH*, *EH* and *CCAP* are mainly expressed in thoracic ganglion in *S. paramamosain* (**Figures 1A–C**), which indicates that crab nerve tissue assumes part of the similar endocrine regulation function as the insect tracheal tissue. In *S. paramamosain*, the transcriptional level of *ETH* fluctuated dramatically with the ecdysis cycle, which indicated that *ETH* might be the major gene initiating molting behavior (**Figure 2A**). Unlike *ETH*, the transcripts of *EH* and *CCAP* at D2 and E stages increased gradually (**Figures 2B,C**). At the initial premolt stage of insects such as *Manduca sexta*, ETH is at a low level and could regulate the initiation of the entire molting behavior (Gammie and Truman, 1999). In the crab, the transcription level of *ETH* is also the lowest at D0 stage, it's interesting to know if crab has a similar ETH regulatory mode.

Ecdysis Triggering Hormone, Eclosion Hormone, and Crustacean Cardioactive Peptide Play Different but Critical Roles to Molt in *Scylla paramamosain*

Results show that *ETH*, *EH,* and *CCAP* are essential for the regulation of molting behavior in mud crabs. In insects, mortality occurred when *ETH*, *EH* or *CCAP* was interrupted. For example, *EH* null mutant *Drosophila* and *EH*-interrupted *Tribolium*, pre-ecdysis behaviors were disrupted and mass mortality occurred before molting (Arakane et al., 2008; Kruger et al., 2015). Similarly in the red flour beetles *Tribolium*, the *dsETH*- or *dsCCAP*-injected animals have completed material accumulation, water re-absorption, but eventually died instead of shedding the old exoskeleton (Arakane et al., 2008). Interestingly, for *dsEH*-injected crabs, pre-ecdysis behaviors were disrupted and crabs were unable to advance to D2 stage and died (**Figures 3C,F,G**). Both ETH and CCAP synthetic peptides could rescue the molting of crabs when their genes were silenced, which suggests they play key roles in crab molting. Compared to *ETH* and *CCAP*, *EH* appears to have a specific

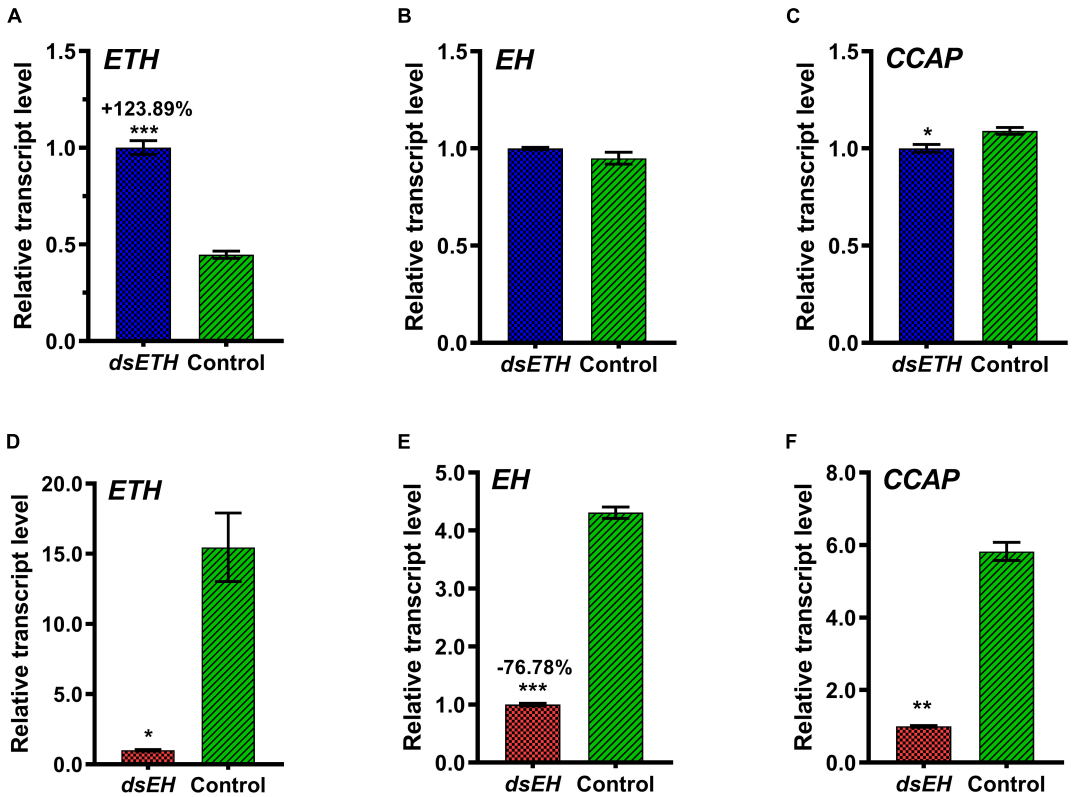

FIGURE 5 | Relative transcript levels of *ETH* **(A)**, *EH* **(B)**, and *CCAP* **(C)** in thoracic ganglia of *dsGFP* and *dsETH*-injected crabs at D2 stage were measured. Also, relative transcript levels of *ETH* **(D)**, *EH* **(E)**, and *CCAP* **(F)** in the thoracic ganglia of *dsGFP* and *dsEH*-injected crabs at D2 stage were measured. These data represented the mean ± SD. For each sample, three independent pools of five individuals were measured in technical triplicate using real-time quantitative PCR (qRT-PCR). Statistically significant differences between the measurements were found *via* a *t*-test with Welch's correction and are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

function on the formation of new epidermis at premolt stage as synthetic peptides for ETH and CCAP can't offset the gene knock-down effect of *EH* (**Figures 3D–I**). Also, our results show that *dsETH*- or *dsCCAP*-injected crabs developed to D2 stage smoothly but failed to molt, at which crabs had completed the formation of new epidermis (**Figure 3A**). The results were similar to that of the red flour beetles. Remarkably, for the *dsETH*-injected crabs, injection of CCAP rescued half of them from failed molt, whereas same result didn't see in the *dsCCAP*-injected crabs under the rescue of ETH synthetic peptide (**Figures 3D,E,H,I**). Considering that *CCAP* may be a downstream gene of *ETH* and can replace part of the role of *ETH* in ecdysis behaviors.

The Transcriptional Level of Eclosion Hormone and Crustacean Cardioactive Peptide Were Effected by Ecdysis Triggering Hormone at D0 Stage

In *Manduca sexta*, 20-E level at early premolt stage is high, which can activate the low-level expression of ETH in the body (Zitnan et al., 2007). The release of ETH then activates the synthesis and release of EH in the brain neurons by binding to its receptor ETHR-A (Gammie and Truman, 1999; Kim et al., 2006). Similarly, the *ETH* and *EH* transcriptional levels of *S. paramamosain* at D0 stage were low (**Figures 2A,B**), the transcriptional levels of *EH* and *CCAP* were increased when *ETH* was knockdown (**Figure 4**). In *Drosophila*, *CCAP* null mutants did not affect the normal development and molting of the worms (Clark et al., 2004). Whereas the knockdown of *CCAP* caused failure of molt and eventually died in *Tribolium* (Arakane et al., 2008). Studies found that *CCAP* was regulated by 20E in *Bactrocera dorsalis* (Shi et al., 2019b), which means *CCAP* is involved in molting-related regulation in *B. dorsalis*. The above reports indicate that *CCAP* has functional differentiation in some species such as *Drosophila* in *Insecta*. In the present study, the strong dependence of the transcript levels of *EH* and *CCAP* on *ETH* indicated that *EH* and *CCAP* have positive correlations with *ETH* in *S. paramamosain* at D0 stage.

Increase of Ecdysis Triggering Hormone Transcripts Was Affected by Eclosion Hormone and an Unknown Factor at D2 Stage

At late premolt stage of *Tribolium castaneum* (Arakane et al., 2008), ETH acts on the ventral neurons to release EH into hemolymph, and EH then acts on the ETH storage cells

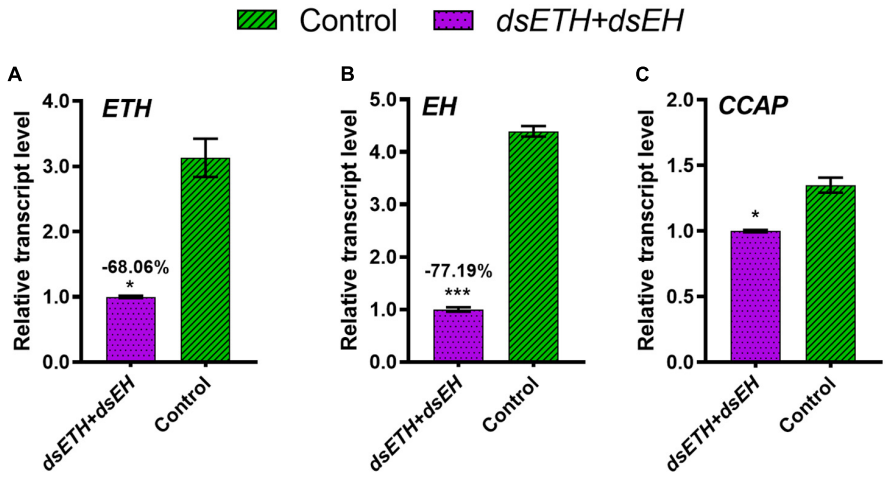

FIGURE 6 | Relative transcript levels of *ETH* **(A)**, *EH* **(B)**, and *CCAP* **(C)** in thoracic ganglia of *dsGFP* and co-injected crabs at D2 stage were measured. These data represented the mean ± SD. For each sample, three independent pools of five individuals were measured in technical triplicate using real-time quantitative PCR (qRT-PCR). Statistically significant differences between the measurements were found *via* a *t*-test with Welch's correction and are indicated by asterisks (*$P < 0.05$; ***$P < 0.001$).

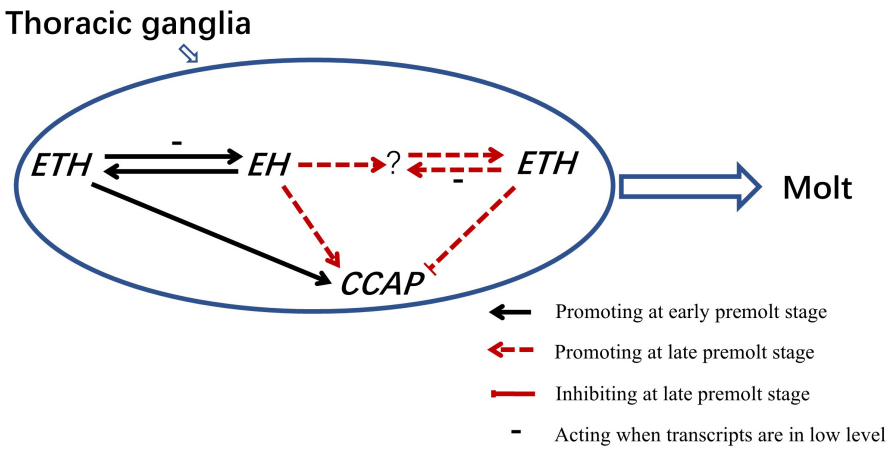

FIGURE 7 | A model of *ETH*, *EH*, and *CCAP* genes in the regulation of molting behavior in *Scylla paramamosain*.

to stimulate large quantities release of ETH. The positive feedback regulation further activates a series of molting-related neuronal genes such as *CCAP* and bursicon, and initiates molting. However, in *Drosophila*, the release of ETH has no correlation with EH (Clark et al., 2004), which indicates that the positive feedback regulation mode between ETH and EH is not conserved in *Insecta*. Our study shows that when crabs developed to D2 stage, *ETH* and *EH* transcriptional levels rose rapidly (**Figures 2A,B**). A strong positive of *ETH* may exist at D2 stage to ensure its continuous and massive expression. The obvious change in *dsETH*-injected crabs didn't cause a fluctuation of *EH* (**Figures 5A,B**), which suggests that the gene that directly regulates the retaliatory increase of *ETH* is not *EH*. The expression of *ETH* and ecdysis of crabs were significantly inhibited with the knockdown of *EH* at D2 stage (**Figures 5D,E**), which suggesting that although not a direct regulatory gene to *ETH* at D2 stage, *EH* plays an important role in the massive expression of *ETH*. To further verify the relationship between *ETH* and *EH* at D2 stage, co-injection of *dsETH* and *dsEH* to crabs at D2 stage were down (**Figures 6A–C**). The results indicated that when *EH* was disturbed, the unknown factor required for *ETH*'s surge was also inhibited, and *EH* may indirectly regulate the increase of *ETH* at this stage by regulating the unknown factor. Also, the transcription levels of *ETH, EH,* and *CCAP* are highest at E stage, whereas the higher levels of mRNA for *EH* and *CCAP* should not to be coincident with the highest levels of ETH because stage E lasts just a few minutes in crabs, and it would be very difficult to envisage how an increased levels of *ETH* or *EH* might influence transcription/translation of *CCAP* or *ETH* in such a short time frame. Therefore, the correlation of these three genes might not be a direct regulation but an inter-relationships with each other.

CONCLUSION

Following with recent climate anomalies, environmental water pollution and increased using of pesticide, failure of molt has become more frequent in wild and farmed crustaceans (Verslycke et al., 2004; Soin and Smagghe, 2007; Zitnan et al., 2007). In this study, *ETH* is the upstream regulatory gene of *CCAP*, and *ETH*, *EH*, and *CCAP* had different but essential roles in crab's molt. Also, *ETH* and *EH* have different interactional relationships at D0 and D2 stages. As a result, we proposed a model for the molting behavior regulation of *Scylla paramamosain* (**Figure 7**). The complicated molting regulation mode of arthropods is highly susceptible to fluctuations by external interference, chemical disturbances, and habitat deterioration. The study of molting process may reveal novel molecular targets for the discovery of different molecular mechanisms between *Insecta* and *Crustacea*, and may contribute to develop strategy to reduce molting failure in aquaculture crabs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

S-FC and Y-FZ: conceptualization, writing, review, and editing. S-FC, Y-FZ, and C-GW: methodology. Y-FZ and Q-QW: software and data curation. C-MA, Q-QW, and WW: validation. Y-FZ and WW: formal analysis. S-FC, Y-FZ, and WW: investigation. C-GW: resources. Y-FZ: writing – original draft preparation. C-MA and L-LS: visualization. S-FC: supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.855391/full#supplementary-material

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