



Article

Function Analysis of Cholesterol 7-Desaturase in Ovarian Maturation and Molting in *Macrobrachium nipponense*: Providing Evidence for Reproductive Molting Progress

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Abstract: The Cholesterol 7-desaturase gene plays an important role in insect ecdysone synthesis, but its role in ovarian development has not been reported. In this study, characteristics and the phylogenetic relationship of Cholesterol 7-desaturase were identified by bioinformatics. qPCR showed that the *Mn-CH7D* gene was highly expressed in the ovary, which was much higher than that in other tissues, and the expression level of *Mn-CH7D* reached the highest level at the third stage of the ovarian development stage (O-III). During embryonic development, the *Mn-CH7D* gene expression was highest in the zoea stage. The function of the *Mn-CH7D* gene was explored by RNA interference. The experimental group was injected with *Mn-CH7D* dsRNA through the pericardial cavity of *M. nipponense*, while the control group was injected with the same volume of *dsGFP*. Statistical analysis of gonadal development and GSI calculation showed that the silencing of *Mn-CH7D* resulted in the suppression of gonadal development. In addition, the molting frequency of the experimental group was significantly lower than that of the control group during the second molting cycle after silencing *Mn-CH7D*. On the seventh day after silencing, ecdysone content in the experimental group was significantly reduced. These results demonstrated that the *Mn-CH7D* gene played a dual role in ovarian maturation and molting of *M. nipponense*.

Keywords: *Macrobrachium nipponense*; Cholesterol 7-desaturase; molting; ovarian maturation



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1. Introduction

Macrobrachium nipponense (Class: Crustacea, Order: Decapoda) is an economically important freshwater aquaculture species in China and the only indigenous species in large-scale aquaculture freshwater shrimp [1]. This species has the advantages of a short breeding cycle, small investment, less disease, strong adaptability, stable price, and so on [2]. In large-scale breeding, it was found that after the female prawns entered the breeding period (April to October), especially when the water temperature increased to more than 22 °C, the gonadal maturation cycle was greatly shortened, and the offspring were multiplied in large numbers in the pond, resulting in multi-generation reunion, high breeding density, large feed consumption, and large risk of hypoxia [3,4]. At the same time, the growth of the original female shrimp was affected, leading to a general miniaturization, and the market size of shrimp was greatly reduced; this phenomenon of rapid sexual maturation is very prevalent in shrimp [5,6]. It seriously affects the production and economic benefit of shrimp farming [7]. In response to this problem, the research team screened many genes related to rapid sexual maturity [8,9]. In the hepatopancreas transcriptome of female *M. nipponense* adult ovaries from the O-I to O-V stages of development, KEGG enrichment

revealed that the “insect hormone synthesis” signaling pathway was closely related to ovarian development. The Cholesterol 7-desaturase (*CH7D*) is significantly expressed in this pathway. Therefore, the role of cholesterol 7-desaturase in molt and ovarian maturation were further investigated.

Molting plays an extremely important role in the growth, development, reproduction, and survival of crustaceans and runs through their entire life cycle [10]. During the breeding period, female molting is interwoven with ovarian development, specifically manifested in the need to molt before holding eggs [11]. However, this phenomenon has rarely been confirmed, and the related genes have not been found. In crustaceans, ecdysis and gonadal development are two important physiological processes that are closely linked and interact with each other, both of which require a large amount of material energy and the regulation of endogenous hormone signaling pathways. During the breeding season, most of the female energy is adjusted for gonad development to meet the reproductive demand [12–14], so the energy mobilized for growth will reduce, resulting in slow growth, small specification, and failure to meet the market requirements, which affects production and economic benefit. Studies have shown that the levels of various ecdysones in insects were related to regulating physiological and developmental processes in the ecdysone synthesis pathway of insects [15,16]. Tiu S H K et al. [17] found that ecdysone could promote ovarian development, and similar results were found in *Acanthinix lunulatus* [18] and *Penaeus vannamei* [19]. The biosynthesis and metabolic pathways of ecdysone in insects have been well studied [20–22]. However, the biosynthetic pathways and the enzymes involved in ecdysteroid biosynthesis in crustaceans remain less well understood. In addition, previous studies on ecdysone mainly focused on growth, development, and morphological regulation [23,24]. The mechanism of regulation and coordination between ecdysogenesis and gonadal development is poorly understood. Therefore, it is of great significance to study the correlation between molting and gonad maturation of female *M. nipponense*, both in terms of technology and production.

In this study, sequence characteristics and phylogenetic relationships of *Mn-CH7D* were analyzed by bioinformatics. The expression patterns of the *Mn-CH7D* gene in different tissues, different stages of the ovary, and different stages of embryo development of *M. nipponense* were investigated by qPCR. The localization of *Mn-CH7D* mRNA in the ovary was detected by in situ hybridization (ISH). In addition, the regulation of the *Mn-CH7D* gene on molting and ovarian maturation of *M. nipponense* was studied using RNA interference technology (RNAi). After the *Mn-CH7D* gene was knocked down by RNAi, the function of *Mn-CH7D* in the molting and ovarian maturation of *M. nipponense* was confirmed by observing the molting number and ovarian development. Finally, the content of ecdysone in *M. nipponense* was detected by ELISA.

2. Results

2.1. Full-Length Sequence Analysis of *Mn-CH7D*

The full-length *Mn-CH7D* cDNA sequence determined by combining a subset of sequences from the *M. nipponense* hepatopancreas transcriptome library with the 3' race production was found to be 1225 bp (GenBank accession no. OQ553932). The open reading frame (ORF) was 876 bp long, encoding 291 amino acids, the 5' non-coding region (5'UTR) contained 162 bp, and the 3'UTR contained 187 bp. The cDNA sequence and amino acid sequence of the *Mn-CH7D* gene are shown in Figure 1. According to the analysis, the molecular weight (Mw) of the *Mn-CH7D* protein was 33,550.12 Da, and the theoretical isoelectric point was 9.12. The predicted full-length amino acid composition of *Mn-CH7D* showed that the serine (Valine, V) content was the highest (7.9%) while the cysteine (Cys, C) and methionine (Met, M) content was the lowest (1.4%). Among all the amino acids, there were 37 positively charged amino acids (Arg + Lys) and 31 negatively charged amino acids (Asp + Glu). Sequence analysis showed that *Mn-CH7D* contained two conserved structure domains involved in enzyme catalysis: the Rieske [2Fe-2S] domain and the non-heme iron

binding domain. In addition, the amino acid sequence of Mn-CH7D detected by Signal 5.0 analysis showed no signal peptide sequence.

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ccttctaccctaagtgttggtcgccatcgccgaatccaggaacgtggctgctggacaggaatccccggtaacgcctcg
gggaagccctggcagttttccgagggcactgatggagaagcccacgtggctgacgcctactgccccatctgggggccaac
1 ATGGCCGTTGGTGGGGTTCGTGAAGGGCAGCAGCCTGGAATGTCCCTTCCACGGATGGCAG
1 M A V G G V V K G S S L E C P F H G W Q
61 TTCGATGGGATCGATGGATCGTGTGTACACATTCTAATTGCGCTAAAGTCCCGAAGACA
21 F D G I D G S C V H I P N C A K V P K T
121 GCCAACGTGAGGACGTGGGAGGAGCCGCGAGATGAACGGCTTCATCTTCGTCTGGCACGA
41 A N V R T W E E P R D E R L H L R L A R
181 CGCAGAGGGCCGGGCGCCGATGTGGGAAATCACCGAAATCAAAGAGATTACCTCCACAAG
61 R R G P G A D V G N H R N Q R D Y L H K
241 TGGACTTTTCGGGGCCGCACTTCCCACGAGGTTCTGGCCCATATACAGGAAATTCGGAA
81 W T F R G R T S H E V L A H I Q E I P E
301 AACGGAGCTGATGTGGCCCATCTGCTGCACCTCCATCGTCCCAACATCTTCAAAGGGTCC
101 N G A D V A H L L H L H R P N I F K G S
361 GATCTCCGTGACGCCTTTCGAGACAATCAGGTTTTGGACGTGGCCACGACATCTGGGAC
121 D L R D A F A D N Q V L D V A Q H I W D
421 GGAGAGTGGAGGTCCCGGCCAGAGCCGGAGTCCACACGGCAGAACTGAAGGTGACCCAC
141 G E W R S R P E P E S H T A E L K V T H
481 ACATTCGCGCTCTTCGGTGGCAAGTTCAAGATCCTCACCATGACCGTCAAGGCAGAACAG
161 T F A L F G G K F K I L T M T V K A E Q
541 ATCGGGCCAGGCGTCTGTCACCTTGCACACAAGCCTAGGCTCCGGCGTCTCCTC
181 I G P G V V H L H F D T S L G S G V L I
601 CAAGTGGTCACCCAGTGGAGCCTCTGCGTCAGAAGATTGTGCATCAGTTCTATTTCGTCG
201 Q V V T P V E P L R Q K I V H Q F Y S S
661 TCAAGCTTCATCGCACCTTACGAAAATTCGTCCTGCTCTGTGAGGCACGCCATGTGGAG
221 S S F I A P Y A K F V L L C E A R H V E
721 AGAGACATCATGATCTGGAACAACAAGCAGTACAAAGCTCAGCCGATGTACGTCAAGGAA
241 R D I M I W N N K Q Y K A Q P M Y V K E
781 GACCGCTTCTGAAGAAGTTCCGCTCCTGGTACGCGCAGTTCTACTCTCAGAACAGCCAG
261 D R F L K K F R S W Y A Q F Y S Q N S Q
841 CAGTTCTCCTCAGGAACGAGACACTGAGCTGGTGA
281 Q F S F R N E T L S W *
aacgtagcaccagcgtggcgttagtgcgcaagcagctgcatgactgctcaggctgcatagcccgtagcatgcacacgtagcagataataacgacgtttgca
gagactctcgactcgactgtatagccagtggtgcccgcctgctgctagctagtagcaagcaataacgctgctcaaaaaaaaaa

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Figure 1. Full cDNA sequence and predicted amino acid sequence of *Mn-CH7D*. The start codon ATG and the stop codon TAG are marked with red. The gray shades are two conserved domains, in order: the Rieske [2Fe-2S] domain and the non-heme iron binding domain. The black box represents the tailing signal and poly(A). The stop codon TAG in the amino acid sequence is represented by an asterisk (*).

2.2. Similarity Comparison and Phylogenetic Analysis

Using the DNAMAN 6.0 software, the amino acid sequences of the *Mn-CH7D* gene were homologous to more than 10 species, with multiple sequence comparisons performed. The results showed the gene similarity between *Mn-CH7D* and *Penaeus vannamei*, *Procambarus clarkii*, *Callinectes sapidus*, *Homarus americanus*, *Scylla paramamosain*, *Penaeus japonicus*, and *Eriocheir sinensis* of 64.65%, 65.19%, 60.88%, 64.85%, 61.90%, 65.32%, and 60.46%, respectively (Figure 2).

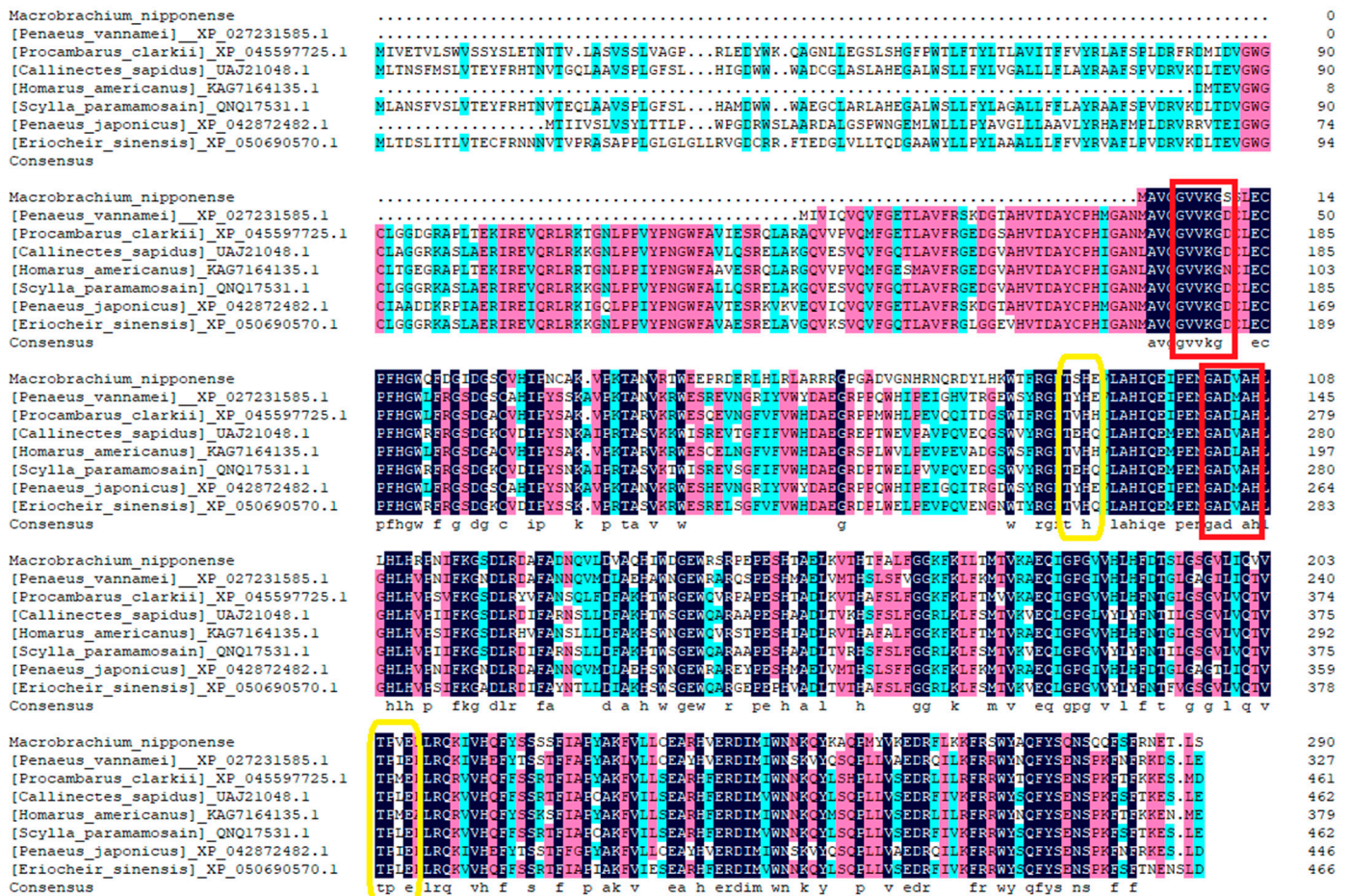


Figure 2. Alignment of the deduced amino acid sequence of *Mn-CH7D* with other species. N-myristoylation sites are marked by red boxes, and Casein kinase II phosphorylation sites are marked by yellow boxes.

Using the MEGA 7.0 software, the amino acid sequence of the *Mn-CH7D* gene and the amino acid sequence of the *Mn-CH7D* gene of other species were used for phylogenetic analysis. Phylogenetic tree analysis showed that *Mn-CH7D* first clustered with *Penaeus vannamei*, *Penaeus japonicus*, *Procambarus clarkii*, and other crustaceans and then clustered with insects, such as *Pieris napi*, *Bactrocera dorsalis*, *Anopheles merus*, and others (Figure 3).

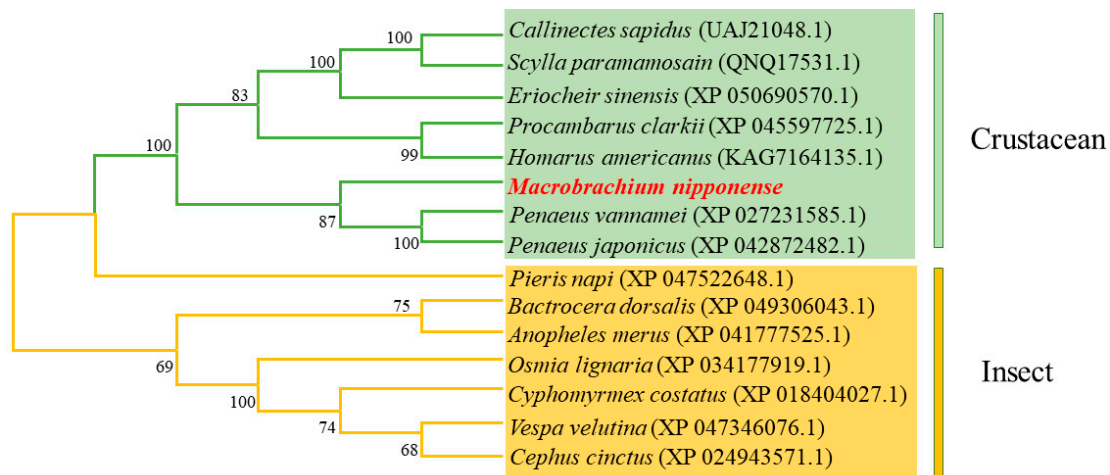


Figure 3. Phylogenetic tree connecting the *Mn-CH7D* amino acid sequence of *Macrobrachium nipponense* and other species. The numbers in brackets indicate the GenBank accession numbers. The numbers shown at the branches indicate the bootstrap values (%).

2.3. Tissue-Specific Gene Expression of *Mn-CH7D*

In the seven tissues of *M. nipponense*, the ovary had the highest expression, which was much higher than that in other tissues ($p < 0.05$). *Mn-CH7D* was weakly expressed in the muscle and gill. The relative expression levels of mRNA detected in the brain and hepatopancreas were the lowest (Figure 4A). In the ovary (Figure 4B), the expression level of *Mn-CH7D* increased significantly from stage O-I to stage O-III, reaching a maximum at the secondary vitellogenesis (O-III) stage ($p < 0.05$). The expression level of *Mn-CH7D* decreased sharply from stage O-III to stage O-IV, and there was no significant difference between O-IV and O-V or between O-V and O-I ($p > 0.05$).

Mn-CH7D was expressed in both the embryonic and metamorphic developmental stages (Figure 4C). At the early embryonic development stage, the expression level of *Mn-CH7D* was higher at the cleavage stage (CS) than at the blastula stage (BS) ($p < 0.05$). Subsequently, as the embryo developed, the expression level of *Mn-CH7D* reached the highest level in the zoea stage (ZS) ($p < 0.05$). During larval development, the expression level of the *Mn-CH7D* gene was higher on the 10th day (L10) after hatching ($p < 0.05$). After metamorphosis, the expression level of the *Mn-CH7D* gene was the highest at PL15, while there was no significant difference in other developmental stages ($p > 0.05$).

2.4. Localization of *Mn-CH7D* at Different Stages of Ovarian Development

The position of *Mn-CH7D* at different stages of ovarian development was located by ISH (Figure 5). The results showed obvious *Mn-CH7D* signals mainly distributed in intercellular space and cell membranes from the O-I stage to the O-IV stage. The signal of *Mn-CH7D* in the ovary was significantly enhanced during stage O-I to stage O-III and gradually weakened after stage O-IV. Moreover, the *Mn-CH7D* signal was detected in all five ovarian maturation stages, being strongest in O-III.

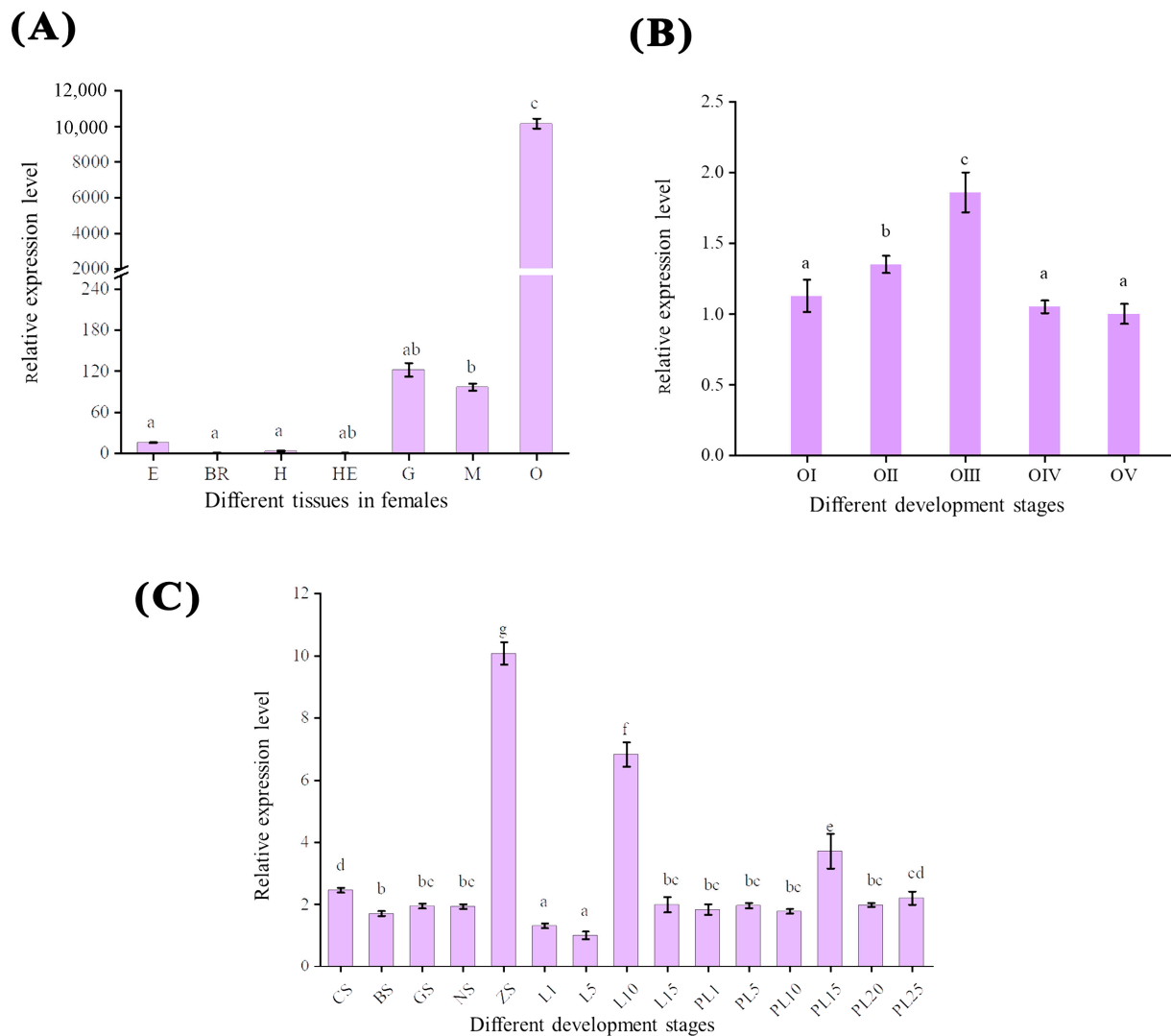


Figure 4. The expression pattern of the *Mn-CH7D* gene in different tissues (A), different stages of ovarian maturation (B), and developmental stages (C) of *Macrobrachium nipponense* were measured by qPCR. E: eyestalk, Br: cerebral ganglion, H: heart, He: hepatopancreas, G: gill, M: muscle, O: ovary; Data are presented as the mean \pm SD ($n = 6$). Different letters indicate significant differences. $p < 0.05$ was considered to be statistically significant.

2.5. Functional Analysis of *Mn-CH7D*

2.5.1. Interference Efficiency

To further explore the function of the *Mn-CH7D* gene in molting and ovarian maturation, RNAi technology was applied. The results in Figure 6 indicated that compared with the control group, the expression level of *Mn-CH7D* was down-regulated by 91.27% and 89.92% on days 4 and 7 after injection, respectively ($p < 0.05$).

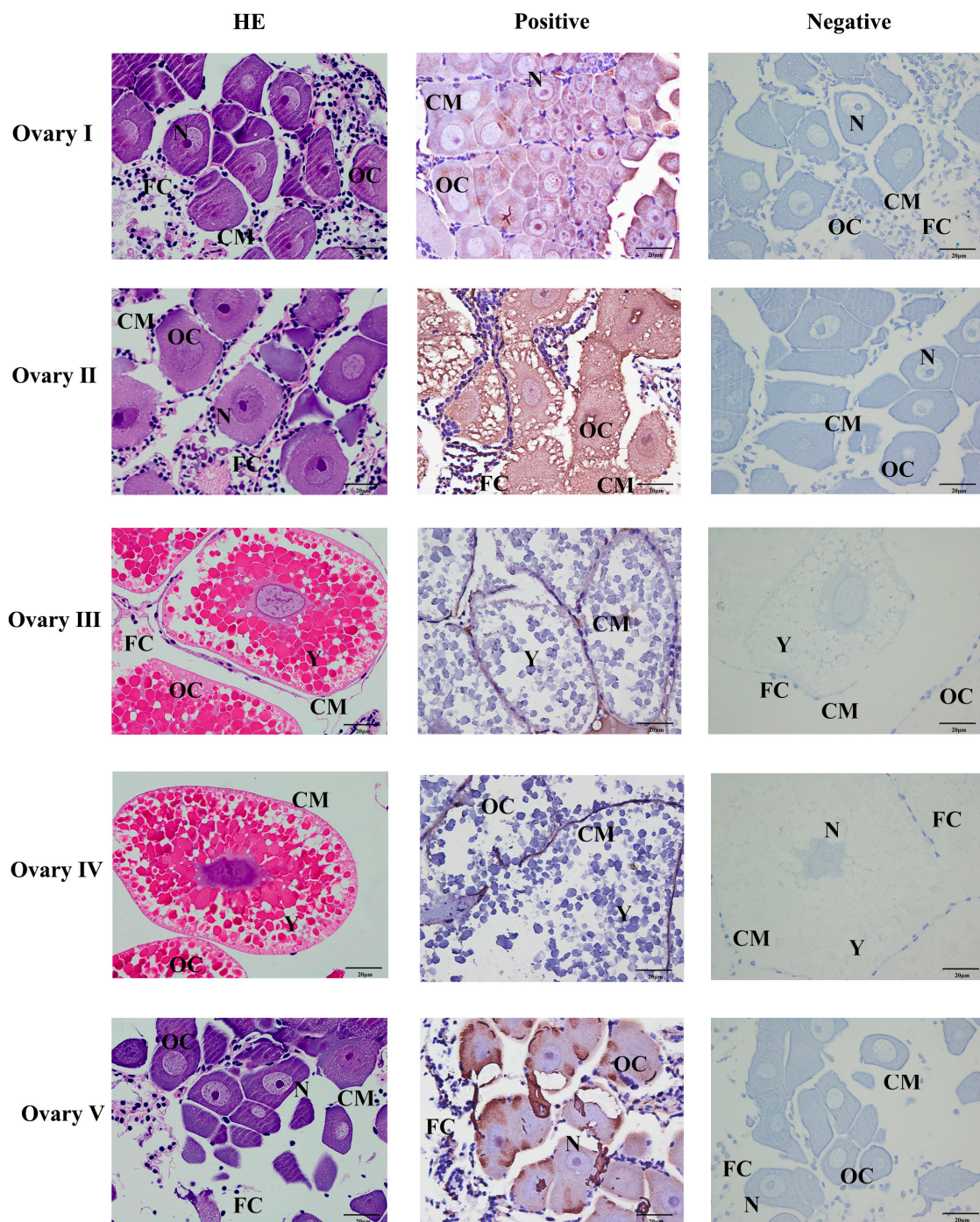


Figure 5. Localization of the expression of *Mn-CH7D* mRNA in *Macrobrachium nipponense* ovaries using in situ hybridization. OC: oocyte; N: nucleus; CM: cytoplasmic membrane; Y: yolk granule; FC: follicle cell; Scale bars: High magnification 400×.

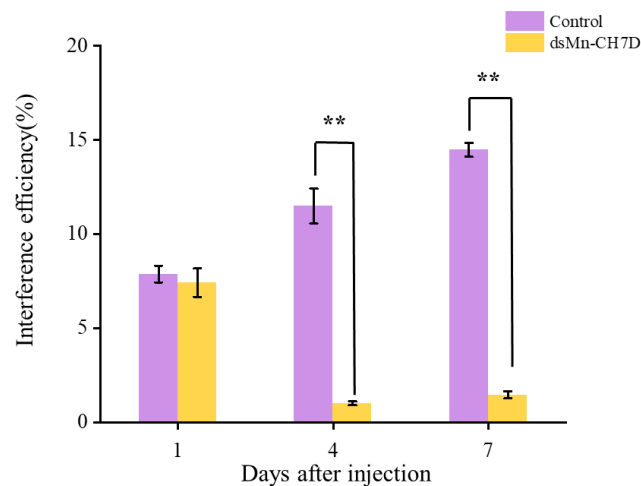


Figure 6. Expression levels of *Mn-CH7D* in ovaries of *Macrobrachium nipponense* after injection with *Mn-CH7D* dsRNA. Data are shown as mean \pm SD (n = 6). “***” indicates the significance of the differences ($p < 0.01$).

2.5.2. Effect of Mn-CH7D Knockdown on Ovarian Development of *M. nipponense*

The ovarian development of the experimental group and the control group is shown in Figure 7A. At the beginning of the experiment, shrimp in stage IV of ovarian development were selected. After 6 days, most of the ovaries of shrimp developed to stage II in both the experimental (85.72%) and control groups (96.74%). On the 14th day, the ovarian development in the control group reached stage IV (52.83%), while the experimental group remained in stage II (78.81%) ($p < 0.05$). After that, the percentage of developmental stages past stage III in the control group gradually declined. On the 16th day, part of the ovarian development in the control group entered the next round, while the experimental group remained in the previous round.

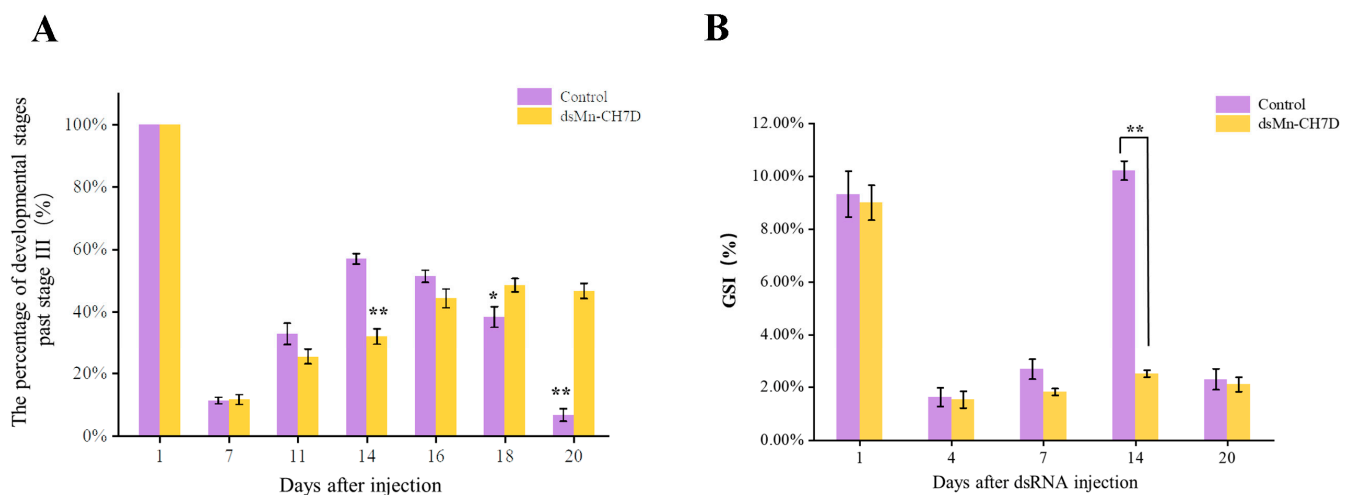


Figure 7. The percentage of development stages past stage III (A) and GSI (%) (B) of female *Macrobrachium nipponense* after injection with *Mn-CH7D* dsRNA. Data are shown as mean \pm SD (n = 6). “**” indicates the significance of the differences ($p < 0.05$). “***” indicates the significance of the differences ($p < 0.01$).

2.5.3. Effect of Mn-CH7D Knockdown on Gonadal Development Index of *M. nipponense*

The gonadal development index (GSI) data were consistent with the ovarian development data (Figure 7B). On the first day of injection, there was no significant difference in GSI between the experimental group and the control group ($p > 0.05$). On day 14, there

were significant differences in the GSI, 10.21% in the control group and 2.50% in the experimental group ($p < 0.05$). On day 20, the control group entered the next round of ovarian development, while the experimental group stagnated in stage II of the previous round.

2.5.4. Effect of Mn-CH7D Knockdown on Molting Frequency of *M. nipponense*

Figure 8A shows the molting frequency of *M. nipponense* in the control and experimental groups after the *Mn-CH7D* knock. During the first round of development, from the first day to the seventh, molting frequency showed no differences between both the experimental group and the control group ($p > 0.05$). However, the control group of *M. nipponense* began the second round of concentrated molting on the 14th day. In contrast, there was almost no molting in the experimental group in the second week ($p < 0.05$).

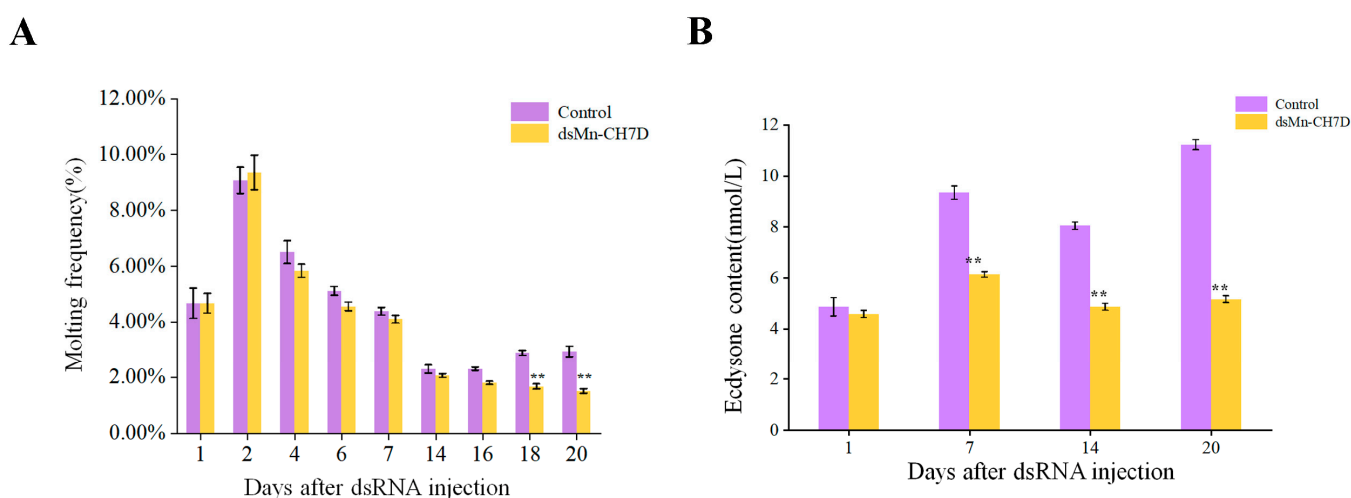


Figure 8. The molting frequency (A) and ecdysone content (B) of *Macrobrachium nipponense* after injection with *Mn-CH7D* dsRNA. Data are shown as mean \pm SD ($n = 6$). “***” indicates the significance of the differences ($p < 0.01$).

2.5.5. Effect of Mn-CH7D Knockdown on Ecdysis Hormone Content of *M. nipponense*

During the interference experiment, ecdysone content in *M. nipponense* was detected on day 1, day 7, day 14, and day 20 (Figure 8B). There was no significant difference between the control group and the experimental group on the first day after injection ($p > 0.05$). From the first day to the seventh day after injection, the content of ecdysone in both the experimental group and the control group increased and then decreased. From the seventh day, the ecdysone content of the experimental group was significantly lower than that of the control group ($p < 0.05$).

2.6. Tissue Section

On the last day of the experiment, tissue slices were taken from the ovaries of the shrimp. The results showed that the cell structure was unchanged, the nucleus was clearly visible, and there were no defects in the ovarian structure of the experimental and the female control shrimp (Figure 9).

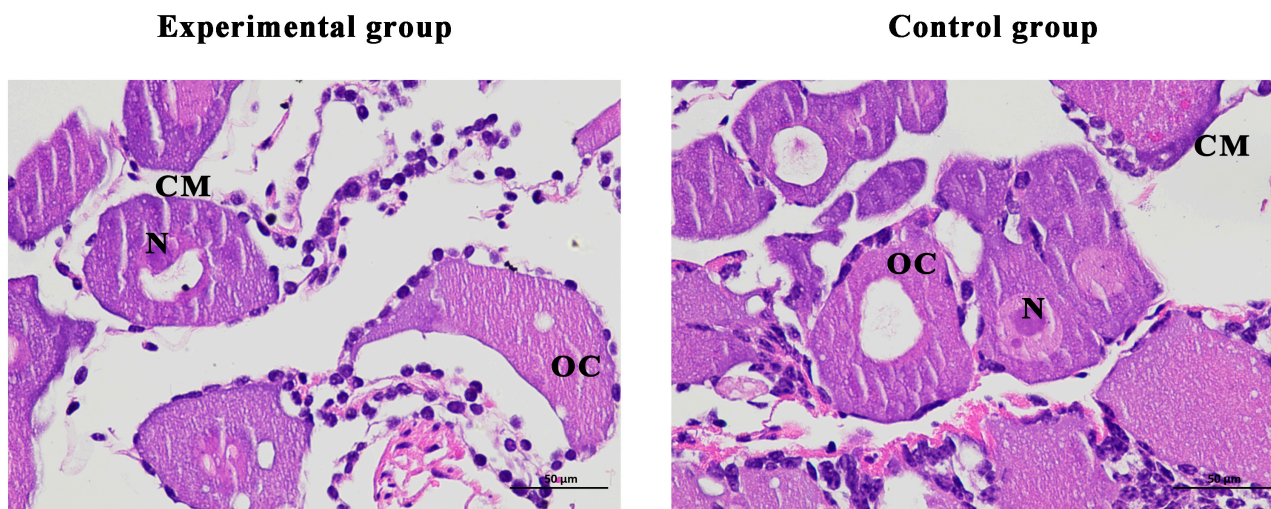


Figure 9. Histological observation of ovary of *Macrobrachium nipponense* after injection with *Mn-CH7D* dsRNA. OC: oocyte; N: nucleus; CM: cytoplasmic membrane; Scale bars: 400 \times .

3. Discussion

Ecdysteroids regulate many aspects of the developmental process and reproductive activity in insects and crustaceans [25–27]. Cholesterol and other sterols are indispensable precursors for the biosynthesis of steroid hormones, and the conversion of cholesterol to the next specific intermediate is an essential biochemical step across species [28,29]. In this study, we identified the Cholesterol 7-desaturase gene, a key gene in the first step of ecdysone synthesis, from the hepatopancreas transcriptome of *M. nipponense* and predicted that its coding region encodes 291 amino acids, including two conserved domains: the Rieske [2Fe-2S] domain and non-heme iron binding domain. The Rieske [2Fe-2S] domain and non-heme iron binding domain are involved in electron transfer and are essential for catalytic enzyme function [30]. It is, therefore, likely that *Mn-CH7D* itself has an enzymatic activity on sterols. *Mn-CH7D* can convert cholesterol into 7-dehydrogenated cholesterol, and many isoenzymes exist in insects and crustaceans, such as *DAF-36* [31] and *NVD* [32–34]. The mechanism of action is that cholesterol desaturase is used to convert cholesterol into 7-dehydrogenated cholesterol, and then through the action of a series of P450 enzymes, the active ecdysone with various structures is formed to act on target tissues and target cells, thus regulating and controlling the growth, deformation, molting, and development of insects and crustaceans. The phylogenetic tree showed that *Mn-CH7D* gene clustered into one branch in crustaceans and insects, indicating that *Mn-CH7D* is more conserved in the same class.

Tissue-specific analysis revealed that transcripts were detected at much higher levels in the ovaries of female adults than in other tissues, suggesting that the ovary of *M. nipponense* actively synthesized ecdysteroids, consistent with the previous reports [35]. The expression of *Mn-CH7D* peaked at O-III, which verified that *Mn-CH7D* was closely related to ovarian maturation [36]. Previous studies have shown that this period is the premolting stage of *M. nipponense*, suggesting that it is likely involved in the regulation of molting [37]. qPCR at different stages of the embryo showed that the expression of *Mn-CH7D* increased sharply from the nauplius stage (NS) to the zoea stage (ZS) and peaked at ZS. At the zoea stage, the embryonic development of *M. nipponense* was basically completed [38], indicating that *Mn-CH7D* played a critical role in hatching. Similar results were reported in the study of *Penaeoidean shrimp Sicyonia ingentis* by Chang E S et al., which found that in *Penaeoidean shrimp*, the content of molting steroid is very little after laying eggs, and it begins to increase in the embryonic development process. And it reached the maximum in the pre-hatching stage [39]. After membrane emergence, the expression level of *Mn-CH7D* began to increase until L10, and the expression level of L10 was the highest in

the whole process of larva development, indicating that the function of *Mn-CH7D* gene is closely related to ontogeny, especially larva development. After metamorphosis, the expression level of *Mn-CH7D* reached the highest on the 15th day after metamorphosis (PL15), and there was no significant difference in other stages. At PL15, the gonads began to differentiate, suggesting that *Mn-CH7D* was associated with gonad development.

To further understand the biological roles of *Mn-CH7D* in reproductive processes, we knocked down *Mn-CH7D* using RNAi. The expression of *Mn-CH7D* in the ovaries of *M. nipponense* was significantly reduced by injection of dsRNA on day 4 (Figure 6). After silencing of *Mn-CH7D* resulted in the suppression of gonadal development. Studies have found that ecdysteroids play an important role in *Vg* synthesis [40,41]. After *Mn-CH7D* silencing, ecdysteroid synthesis is blocked, so gonad development is inhibited. Similarly, the silencing of the *Nvd* gene, which was highly expressed in females, leads to failure of ovarian development and subsequent egg laying [34]. *Nvd* and *Mn-CH7D* are isoenzymes, so the results are reliable. During the RNAi experiment, the GSI was calculated to further demonstrate gonad maturation. On the 14th day, the GSI of the experiment group injected with *Mn-CH7D* dsRNA was significantly lower than that of the control group. By day 14, the ovarian maturation of the experimental group remained at O-II, whereas the ovarian maturation of the control group reached O-IV, and gonadal development was close to maturity. GSI indicates that injection of *Mn-CH7D* dsRNA can effectively inhibit ovarian maturation, confirming the important role of Cholesterol 7-desaturase in gonad maturation. In addition, the ovarian structure of the female shrimp injected with *dsCH7D* and *dsGFP* was free from defects through tissue sections, indicating that silencing this gene only delayed ovarian development.

After RNAi, the molting frequency of the experimental group was significantly lower than that of the control group on days 16–20 ($p < 0.05$) (Figure 8). Thus, successfully silencing *Mn-CH7D* in *M. nipponense* could significantly inhibit the molting of *M. nipponense*. This finding is consistent with the observations reported by Sumiya E et al., knockdown of *nvd* significantly resulted in the arrest of molting and growth by RNAi [33]. After the successful knockdown of *Mn-CH7D* in *M. nipponense*, we measured the content of ecdysone using ELISA. We found that the level of ecdysone was significantly decreased compared with the control group on days 7, 14, and 20 ($p < 0.05$). The level of ecdysone tends to increase on day 7, as the second molt begins on day 14, and the level of ecdysone will increase before molting, which has been demonstrated by previous studies [42,43]. These results suggested that the knockdown of the *Mn-CH7D* gene inhibited the synthesis of ecdysone, thereby affecting the molting of *M. nipponense*. Cholesterol is catalyzed to the active ecdysone by a series of P450 enzymes. Similar results were observed that the knockout of *Spook* and *CYP302a1* could inhibit molting and reduce the content of ecdysone [44,45]. It has also been described in other arthropods, including *Bemisia tabaci*, *Bombyx mori*, and *Locusta migratoria* [46–48].

These results strongly suggest that the *Mn-CH7D* gene is an ecdysone synthesis pathway gene, which is involved in ecdysone synthesis and plays an important role in the molting and ovarian maturation of crustaceans. This study demonstrated for the first time that Cholesterol 7-desaturase involved in ecdysone synthesis act as gonadotropin in *M. nipponense*, providing a theoretical basis for the artificial control of ecdysone. At the same time, it also provides strong evidence that this ecdysone is a key hormone in controlling reproductive ecdysone.

4. Materials and Methods

4.1. Experimental Prawns and Breeding Conditions

Healthy female *M. nipponense* (body weight 0.56 ± 0.13 g and body length 3.68 ± 0.22 cm) were obtained from Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi, Jiangsu Province, China. No endangered or protected species were involved in this experiment. All experimental protocols and methods were approved in September 2022 (Authorization no. 20220901002) by the Animal Care and Use Ethics Committee in

the Freshwater Fisheries Research Center (Wuxi, China). The prawns were raised in an indoor circulating aquaculture system (tanks, 1 m × 0.8 m × 0.5 m) to acclimate for one week. During the culture period, the water temperature was maintained at about 27 ± 1 °C. *M. nipponense* were fed a daily ration of paludina in the morning and evening. Each feeding amount depended on the weather, feeding conditions, timely adjustment, and clean food scraps once a day ensured water quality.

4.2. Sample Collection

Tissues of the eyestalk, brain, heart, hepatopancreas, gills, muscles, and ovaries of female *M. nipponense* were gathered, frozen immediately in liquid nitrogen, and then stored at −80 °C until use. Individuals of *M. nipponense* at different developmental stages (including embryonic developmental stage and larval stage) were also dissected, frozen, and stored at −80 °C separately for future experiments. The specific classification of ovarian stages and different stages of the embryo is listed in Table 1 based on the criteria studies previously [49].

Table 1. Different developmental stages of ovary and embryo of *M. nipponense*.

Stages	Characteristic
O-I	undeveloped stage, transparent
O-II	developing stage, yellow
O-III	nearly-ripe stage, light green
O-IV	ripe stage, dark green
O-V	worn out stage, gray
CS	cleavage stage
BS	blastula stage
GS	gastrulation stage
NS	nauplius stage
ZS	zoea stage
L1	the 1st-day larvae after hatching
L5	the 5th-day larvae after hatching
L10	the 10th-day larvae after hatching
L15	the 15th-day larvae after hatching
PL1	the 1st day after metamorphosis
PL5	the 5th day after metamorphosis
PL10	the 10th day after metamorphosis
PL15	the 15th day after metamorphosis
PL20	the 20th day after metamorphosis
PL25	the 25th day after metamorphosis

4.3. Gene Cloning and Sequence Analysis of Cholesterol 7-Desaturase

The total RNA of *M. nipponense* at different developmental stages and different tissues was extracted using RNAiso Plus reagents (Takara, Japan) according to the manufacturer's instructions, and quality was assessed by 1% agarose gel electrophoresis. The first-strand cDNA was synthesized with RNA as a template using the M-MLV reverse transcriptase (Takara, Japan) kit at 42 °C for 2 min. The reaction was finished after 37 °C for 5 min and 85 °C for 30 s. Then, the synthesized cDNA was kept at −20 °C for subsequent quantitative real-time PCR (qPCR) reaction to detect the expression pattern of the *Mn-CH7D* gene in *M. nipponense*. EIF was used as the internal reference gene [50]. The content of *Mn-CH7D* mRNA was calculated by the $2^{-\Delta\Delta CT}$ method [51].

The full-length DNA sequence of Cholesterol 7-desaturase was cloned using a 3'RACE kit according to the manufacturer's instructions [52]. The full-length *Mn-CH7D* cDNA sequence determined by combining a subset of sequences from the *M. nipponense* hepatopancreas transcriptome library with the 3'race production was obtained. According to the Cholesterol 7-desaturase sequence, the primers used were designed by a prime designing tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>, accessed on 1 May 2022) (Table 2).

Domains in the protein sequence were determined via Conserved Domain Searches at NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 5 May 2022); amino acid sequence alignment was analyzed by DNAMAN 6.0 software; the open reading frame (ORF) of CH7D was predicted by the ORF Finder program (<http://ncbi.nlm.nih.gov/gorf/gorf.html>, accessed on 5 May 2022); signal peptide was predicted using Signal 5.0 Server (<http://www.cbs.dtu.dk/services/Signal/P>, accessed on 1 August 2022). Molecular weight (MW), the isoelectric point (PI), and amino acid composition were performed using ProtParam on the ExPASy website (<https://web.expasy.org/protparam/>, accessed on 1 August 2022). The phylogenetic tree was constructed based on the Cholesterol 7-desaturase protein and its orthologs in other species by the neighbor-joining (NJ) method using the MEGA 7.0 software.

Table 2. Primers for cDNA clone, qPCR analysis, and RNAi involved in this study.

Primer's Name	Sequence (5'-3')	Usage
CH7D F1	CTAATTGCGCTAAAGTCCCGAAG	ORF
CH7D R1	GGTCATGGTGAGGATCTTGAAC	ORF
CH7D F2	TTGCAGACAATCAGTTTTGGAC	ORF
CH7D R2	CTCACAGAGCAGGACGAATTTTG	ORF
CH7D F3	GTGCATCAGTTCTATTGTCGTC	ORF
CH7D R3	CTGAGCTTTGACTGCTTGTGT	ORF
CH7D 3'F1	GCTGAGAGTAGAACTGCGCGTAC	3'RACE
CH7D 3'F2	TCTCGTTCCGTAAGGAGAACTGC	3'RACE
CH7D F	TGGAACAACAAGCAGTACAAAG	qPCR
CH7D R	CTGGCTGTTCTGAGAGTAGAACT	qPCR
EIF F	CATGGATGTACCTGTGGTGAAAC	qPCR
EIF R	CTGTCAGCAGAAGGTCCTCATT	qPCR
CH7D dsF	TAATACGACTCACTATAGGGCGCTAAAGTCC CGAAGACAG	dsRNA
CH7D dsR	TAATACGACTCACTATAGGGACGAATTTTGC GTAAGGTGC	dsRNA
GFP dsF	TAATACGACTCACTATAGGGACGAAGACCTT GCTTCTGAAG	dsRNA
GFP dsR	TAATACGACTCACTATAGGGAAAGGGCAGAT TGTGTGGAC	dsRNA

4.4. In Situ Hybridization

Ovarian (stage I–V) samples fixed in 4% paraformaldehyde solution were used for in situ hybridization study. Based on the cDNA sequence of *Mn-CH7D*, the antisense and sense probes of Chromogenic in situ hybridization (ISH) with DIG signal were designed by Primer5 software and synthesized by Shanghai Sangon Biotech Company. More details of in situ hybridization have been described in previous studies [53]. The probe sequence of *Mn-CH7D* is as follows: 5'-CGGACCCTTTGAAGATGTTGGGACGATGGAG. The negative control uses the antisense chain of the sequence as the probe. The sequence is as follows: 5'-CTCCATCGTCCCAACATCTTCAAAGGGTCCG. HE represents the blank control groups with routine hematoxylin-eosin staining, and negative indicates the control groups poured with antisense probes. Positive suggests the experimental group with sense probes poured.

4.5. RNAi Experiment

4.5.1. Interference Efficiency Detection

The primers used to synthesize double-stranded RNA (dsRNA) were designed with the online software Snap Dragon (https://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl, accessed on 1 May 2022). The dsRNA of *Mn-CH7D* was synthesized using Transcript Aid™ T7 High Yield Transcription kit (Fermentas, Inc., Waltham, MA, USA), and its concentration was measured/determined at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). Expression levels of *Mn-CH7D* were measured by qPCR to determine the interference efficiency.

A total of 300 healthy female prawns in stage IV were randomly distributed into 6 aquaculture tanks in equal proportion, which were the experimental group and the control group. The experimental group was injected with *Mn-CH7D* ds RNA at a dose of 4 µg/g (calculated per gram of body weight) through the pericardial cavity of *M. nipponense* [54], while the control group was injected with the same volume of *dsGFP*. The frequency of injections was five days. The *Mn-CH7D* expression of the ovary was investigated to detect the interference efficiency by qPCR.

4.5.2. Ovarian Development and GONAD Somatic Index

The ovarian development was observed and recorded daily. The specific classification criteria are shown in Table 1. Three prawns were randomly selected from experimental and control groups on the 1st, 4th, 7th, 14th, and 20th day after injection, with 3 replicates per group. Among them, three shrimp were calculated for the gonad-somatic index (GSI). The prawns were weighed before and after the dissection of the ovaries. The GSI was calculated using the following formula: $GSI = \text{gonadal weight} / \text{body weight} \times 100\%$, as described previously [55].

4.5.3. Molting Frequency and Ecdysis Hormone Content

The number of molts was observed and recorded daily. Molting frequency is calculated using the following formula: $\text{Molting frequency} = (Nm/Ns)/D$, where *Nm* is the total number of molts, *Ns* is the number of prawns in the aquarium, and *D* is the number of experimental days.

Three prawns were randomly selected from experimental and control groups on the 1st, 7th, 14th, and 20th day after injection, respectively. These shrimp were tested for hormone contents. The contents of ecdysone in prawns were determined by enzyme-linked immunosorbent assay with the double antibody two-step sandwich method, according to the Shrimp EHELISA Kit instruction. Experiments were performed in triplicate.

4.6. Tissue Section

At the end of the experiment, the ovaries of two shrimp from the experimental group and the control group were collected and fixed with 4% paraformaldehyde solution for tissue section preparation. The tissue slides were deparaffinized and hydrated using standard procedures and stained with hematoxylin and eosin (H&E). The final observation is a cross-section of the tissue.

4.7. Data Analysis

All quantitative data were presented as mean ± standard deviation (Mean ± SD). SPSS Statistics 24.0 was used for data analysis of the experimental data. The significant differences between the control group and the treatment group were analyzed by one-way ANOVA and Duncan test for multiple comparisons. The significance level of data variance was set at 0.05. The relative mRNA expression levels of the genes were calculated according to the $2^{-\Delta\Delta C_t}$ comparative CT method.

5. Conclusions

In this study, the full-length cDNA sequence of Cholesterol 7-desaturase from *M. nipponense* was successfully cloned and analyzed. The expression characteristics of Cholesterol 7-desaturase in different tissues and different stages of ovary and embryo development of *M. nipponense* were investigated. Importantly, this study demonstrated for the first time that this gene involved in ecdysone synthesis act as gonadotropin in *M. nipponense*, providing a theoretical basis for the artificial control of ecdysone. It also provides some new insights and ideas to solve the production problem of rapid sexual maturation, which is of great significance to the development of the crustacean industry.

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References

1. Zhu, J.; Fu, H.; Qiao, H.; Jin, S.; Zhang, W.; Jiang, S.; Gong, Y.; Xiong, Y. Expression and functional analysis of cathepsin L1 in ovarian development of the oriental river prawn, *Macrobrachium nipponense*. *Aquac. Rep.* **2021**, *20*, 100724. [[CrossRef](#)]
2. Jin, S.; Zhang, W.; Xiong, Y.; Fu, H. Recent progress of male sexual differentiation and development in the oriental river prawn (*Macrobrachium nipponense*): A review. *Rev. Aquac.* **2022**, *15*, 305–317. [[CrossRef](#)]
3. Li, F.; Qiao, H.; Fu, H.; Sun, S.; Zhang, W.; Jiang, S.; Gong, Y.; Xiong, Y. Identification and characterization of opsin gene and its role in ovarian maturation in the oriental river prawn *Macrobrachium nipponense*. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **2018**, *218*, 1–12. [[CrossRef](#)]
4. Zhou, Z.; Fu, H.; Jin, S.; Qiao, H.; Zhang, W.; Jiang, S.; Xiong, Y.; Gong, Y.; Hu, Y.; Gu, X.; et al. Function analysis and molecular characterization of cyclin A in ovary development of oriental river prawn, *Macrobrachium nipponense*. *Gene* **2021**, *788*, 145583. [[CrossRef](#)]
5. Qiao, H.; Fu, H.; Xiong, Y.; Jiang, S.; Zhang, W.; Sun, S.; Jin, S.; Gong, Y.; Wang, Y.; Shan, D.; et al. Molecular insights into reproduction regulation of female Oriental River prawns *Macrobrachium nipponense* through comparative transcriptomic analysis. *Sci. Rep.* **2017**, *7*, 12161. [[CrossRef](#)]
6. Li, X.; Yang, H.; Gao, X.; Zhang, H.; Chen, N.; Miao, Z.; Liu, X.; Zhang, X. The pathogenicity characterization of non-O1 *Vibrio cholerae* and its activation on immune system in freshwater shrimp *Macrobrachium nipponense*. *Fish Shellfish Immunol.* **2019**, *87*, 507–514. [[CrossRef](#)] [[PubMed](#)]
7. Jin, S.; Hu, Y.; Fu, H.; Sun, S.; Jiang, S.; Xiong, Y.; Qiao, H.; Zhang, W.; Gong, Y.; Wu, Y. Analysis of testis metabolome and transcriptome from the oriental river prawn (*Macrobrachium nipponense*) in response to different temperatures and illumination times. *Comp. Biochem. Physiol. Part D Genom. Proteom.* **2020**, *34*, 100662. [[CrossRef](#)] [[PubMed](#)]
8. Qiao, H.; Xiong, Y.; Zhang, W.; Fu, H.; Jiang, S.; Sun, S.; Bai, H.; Jin, S.; Gong, Y. Characterization, expression, and function analysis of gonad-inhibiting hormone in Oriental River prawn, *Macrobrachium nipponense* and its induced expression by temperature. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2015**, *185*, 1–8. [[CrossRef](#)]
9. Qiao, H.; Xiong, Y.; Jiang, S.; Zhang, W.; Xu, L.; Jin, S.; Gong, Y.; Wu, Y.; Fu, H. Three neuroparsin genes from oriental river prawn, *Macrobrachium nipponense*, involved in ovary maturation. *3 Biotech* **2020**, *10*, 537. [[CrossRef](#)]
10. Qi, H.; Cao, H.; Zhao, Y.; Cao, Y.; Jin, Q.; Wang, Y.; Zhang, K.; Deng, D. Cloning and functional analysis of the molting gene CYP302A1 of *Daphnia sinensis*. *Front. Zool.* **2023**, *20*, 2. [[CrossRef](#)]
11. Qiao, H.; Jiang, F.; Xiong, Y.; Jiang, S.; Fu, H.; Li, F.; Zhang, W.; Sun, S.; Jin, S.; Gong, Y.; et al. Characterization, expression patterns of molt-inhibiting hormone gene of *Macrobrachium nipponense* and its roles in molting and growth. *PLoS ONE* **2018**, *13*, e0198861. [[CrossRef](#)] [[PubMed](#)]
12. Delgado, M.; Camacho, A.P. Influence of temperature on gonadal development of *Ruditapes philippinarum* (Adams and Reeve, 1850) with special reference to ingested food and energy balance. *Aquaculture* **2007**, *264*, 398–407. [[CrossRef](#)]
13. Suzuki, T.; Sakurai, S.; Iwami, M. Juvenile hormone delays the initiation of rectal sac distention by disrupting ecdysteroid action in the silkworm, *Bombyx mori*. *Pestic. Biochem. Physiol.* **2010**, *97*, 199–203. [[CrossRef](#)]
14. Zhu, Y.; Wu, J.; Leng, X.; Du, H.; Wu, J.; He, S.; Luo, J.; Liang, X.; Liu, H.; Wei, Q. Metabolomics and gene expressions revealed the metabolic changes of lipid and amino acids and the related energetic mechanism in response to ovary development of *Chinese sturgeon* (*Acipenser sinensis*). *PLoS ONE* **2020**, *15*, e0235043. [[CrossRef](#)] [[PubMed](#)]
15. Bond, N.D.; Nelliott, A.; Bernardo, M.K.; Ayerh, M.A.; Gorski, K.A.; Hoshizaki, D.K.; Woodard, C.T. ssFTZ-F1 and Matrix metalloproteinase 2 are required for fat-body remodeling in *Drosophila*. *Dev. Biol.* **2011**, *360*, 286–296. [[CrossRef](#)]

16. Dou, X.; Chen, K.; Brown, M.R.; Strand, M.R. Multiple endocrine factors regulate nutrient mobilization and storage in *Aedes aegypti* during a gonadotrophic cycle. *Insect Sci.* **2022**, *30*, 425–442. [[CrossRef](#)] [[PubMed](#)]
17. Tiu, S.H.-K.; Chan, S.-M.; Tobe, S.S. The effects of farnesoic acid and 20-hydroxyecdysone on vitellogenin gene expression in the lobster, *Homarus americanus*, and possible roles in the reproductive process. *Gen. Comp. Endocrinol.* **2010**, *166*, 337–345. [[CrossRef](#)] [[PubMed](#)]
18. Chaix, J.C.; De Reggi, M. Ecdysteroid Levels during Ovarian Development and Embryogenesis in the Spider Crab *Acanthonyx lunulatus*. *Gen. Comp. Endocrinol.* **1982**, *47*, 7–14. [[CrossRef](#)] [[PubMed](#)]
19. Chan, S.-M. Possible roles of 20-hydroxyecdysone in the control of ovary maturation in the white shrimp *Penaeus vannamei* (Crustacea: Decapoda). *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* **1995**, *112*, 51–59. [[CrossRef](#)]
20. Chen, Y.; Fan, B.; Yassen, A.; Zhu, J.; Wang, M.; Shen, X. YTHDF3 Is Involved in the Diapause Process of Bivoltine *Bombyx mori* Strains by Regulating the Expression of Cyp307a1 and Cyp18a1 Genes in the Ecdysone Synthesis Pathway. *Biomolecules* **2022**, *12*, 1127. [[CrossRef](#)] [[PubMed](#)]
21. Kamiyama, T.; Niwa, R. Transcriptional regulators of ecdysteroid biosynthetic enzymes and their roles in insect development. *Front. Physiol.* **2022**, *13*, 85. [[CrossRef](#)]
22. Rewitz, K.F.; Gilbert, L.I. *Daphnia* Halloween genes that encode cytochrome P450s mediating the synthesis of the arthropod molting hormone: Evolutionary implications. *BMC Evol. Biol.* **2008**, *8*, 1–8. [[CrossRef](#)] [[PubMed](#)]
23. Dai, T.-H.; Sserwadda, A.; Song, K.; Zang, Y.-N.; Shen, H.-S. Cloning and expression of ecdysone receptor and retinoid X receptor from *Procambarus clarkii*: Induction by eyestalk ablation. *Int. J. Mol. Sci.* **2016**, *17*, 1739. [[CrossRef](#)]
24. Shen, H.; Ma, Y.; Hu, Y.; Zhou, X. Cloning of the ecdysone receptor gene from the Chinese Mitten Crab, *Eriocheir sinensis*, and sexually dimorphic expression of two splice variants. *J. World Aquac. Soc.* **2015**, *46*, 421–433. [[CrossRef](#)]
25. Tian, H.; Yang, C.; Yu, Y.; Yang, W.; Lu, N.; Wang, H.; Liu, F.; Wang, A.; Xu, X. Dietary cholesterol level affects growth, molting performance and ecdysteroid signal transduction in *Procambarus clarkii*. *Aquaculture* **2020**, *523*, 735198. [[CrossRef](#)]
26. Yang, F.; Wang, Y.; Lu, W.; Zong, W.; Zhu, Q.; Cheng, J. The Comparative Survey of Coordinated Regulation of Steroidogenic Pathway in Japanese Flounder (*Paralichthys olivaceus*) and Chinese Tongue Sole (*Cynoglossus semilaevis*). *Int. J. Mol. Sci.* **2022**, *23*, 5520. [[CrossRef](#)] [[PubMed](#)]
27. Leyria, J.; Benrabaa, S.; Nouzova, M.; Noriega, F.G.; Tose, L.V.; Fernandez-Lima, F.; Orchard, I.; Lange, A.B. Crosstalk between Nutrition, Insulin, Juvenile Hormone, and Ecdysteroid Signaling in the Classical Insect Model, *Rhodnius prolixus*. *Int. J. Mol. Sci.* **2022**, *24*, 7. [[CrossRef](#)] [[PubMed](#)]
28. Zhu, T.; Jin, M.; Xie, S.; Guo, C.; Luo, J.; Zhang, X.; Shen, Y.; Sun, P.; Jiao, L.; Zhou, Q. Transcriptome and targeted metabolomics revealed that cholesterol nutrition promotes ovarian development by regulating steroid hormone metabolism in swimming crab. *Aquac. Rep.* **2022**, *27*, 101396. [[CrossRef](#)]
29. Zeng, J.; Kamiyama, T.; Niwa, R.; King-Jones, K. The Drosophila CCR4-NOT complex is required for cholesterol homeostasis and steroid hormone synthesis. *Dev. Biol.* **2018**, *443*, 10–18. [[CrossRef](#)]
30. Bak, D.W.; Elliott, S.J. Alternative FeS cluster ligands: Tuning redox potentials and chemistry. *Curr. Opin. Chem. Biol.* **2014**, *19*, 50–58. [[CrossRef](#)]
31. Wollam, J.; Magomedova, L.; Magner, D.B.; Shen, Y.; Rottiers, V.; Motola, D.L.; Mangelsdorf, D.J.; Cummins, C.L.; Antebi, A. The Rieske oxygenase DAF-36 functions as a cholesterol 7-desaturase in steroidogenic pathways governing longevity. *Aging Cell* **2011**, *10*, 879–884. [[CrossRef](#)]
32. Sathapondecha, P.; Panyim, S.; Udomkit, A. An essential role of Rieske domain oxygenase *Neverland* in the molting cycle of black tiger shrimp, *Penaeus monodon*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2017**, *213*, 11–19. [[CrossRef](#)]
33. Sumiya, E.; Ogino, Y.; Toyota, K.; Miyakawa, H.; Miyagawa, S.; Iguchi, T. *Neverland* regulates embryonic moltings through the regulation of ecdysteroid synthesis in the water flea *Daphnia magna*, and may thus act as a target for chemical disruption of molting. *J. Appl. Toxicol.* **2016**, *36*, 1476–1485. [[CrossRef](#)]
34. Yoshiyama, T.; Namiki, T.; Mita, K.; Kataoka, H.; Niwa, R. *Neverland* is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. *Development* **2006**, *133*, 2565–2574. [[CrossRef](#)]
35. Uryu, O.; Ameku, T.; Niwa, R. Recent progress in understanding the role of ecdysteroids in adult insects: Germline development and circadian clock in the fruit fly *Drosophila melanogaster*. *Zool. Lett.* **2015**, *1*, 32. [[CrossRef](#)]
36. Zhou, X.; Ye, Y.-Z.; Ogihara, M.H.; Takeshima, M.; Fujinaga, D.; Liu, C.-W.; Zhu, Z.; Kataoka, H.; Bao, Y.-Y. Functional analysis of ecdysteroid biosynthetic enzymes of the rice planthopper, *Nilaparvata lugens*. *Insect Biochem. Mol. Biol.* **2020**, *123*, 103428. [[CrossRef](#)] [[PubMed](#)]
37. Yuan, H.; Qiao, H.; Fu, Y.; Fu, H.; Zhang, W.; Jin, S.; Gong, Y.; Jiang, S.; Xiong, Y.; Hu, Y. RNA interference shows that Spook, the precursor gene of 20-hydroxyecdysone (20E), regulates the molting of *Macrobrachium nippon*. *J. Steroid Biochem. Mol. Biol.* **2021**, *213*, 105976. [[CrossRef](#)] [[PubMed](#)]
38. Chen, Y.; Zhu, Q.; Chen, H.; Zhu, X.; Cui, Z.; Qiu, G. The morphological and histological observation of embryonic development in the oriental river prawn *Macrobrachium nipponense*. *J. Shanghai Ocean Univ.* **2012**, *21*, 33–40.
39. Chang, E. Reproductive endocrinology of the shrimp *Sicyonia ingentis*: Steroid, peptide, and terpenoid hormones. *NOAA Tech. Rep. NMFS* **1992**, *106*, 1–6.
40. Dong, S.Z.; Ye, G.Y.; Guo, J.Y.; Hu, C. Roles of ecdysteroid and juvenile hormone in vitellogenesis in an endoparasitic wasp, *Pteromalus puparum* (Hymenoptera: Pteromalidae). *Gen. Comp. Endocrinol.* **2009**, *160*, 102–108. [[CrossRef](#)] [[PubMed](#)]

41. Kamruzzaman, A.S.M.; Mikani, A.; Mohamed, A.A.; Elgendy, A.M.; Takeda, M. Crosstalk among Indoleamines, Neuropeptides and JH/20E in Regulation of Reproduction in the American Cockroach, *Periplaneta americana*. *Insects* **2020**, *11*, 155. [[CrossRef](#)] [[PubMed](#)]
42. Gunamalai, V.; Kirubakaran, R.; Subramoniam, T. Hormonal coordination of molting and female reproduction by ecdysteroids in the mole crab *Emerita asiatica* (Milne Edwards). *Gen. Comp. Endocrinol.* **2004**, *138*, 128–138. [[CrossRef](#)] [[PubMed](#)]
43. Mykles, D.L.; Chang, E.S. Hormonal control of the crustacean molting gland: Insights from transcriptomics and proteomics. *Gen. Comp. Endocrinol.* **2020**, *294*, 113493. [[CrossRef](#)] [[PubMed](#)]
44. Hafeez, M.; Li, X.; Chen, L.; Ullah, F.; Huang, J.; Zhang, Z.; Zhang, J.; Siddiqui, J.A.; Zhou, S.-X.; Ren, X.-Y. Molecular characterization and functional analysis of cytochrome P450-mediated detoxification CYP302A1 gene involved in host plant adaptation in *Spodoptera frugiperda*. *Front. Plant Sci.* **2022**, *13*, 1079442. [[CrossRef](#)] [[PubMed](#)]
45. Rewitz, K.F.; Rybczynski, R.; Warren, J.T.; Gilbert, L.I. Identification, characterization and developmental expression of Halloween genes encoding P450 enzymes mediating ecdysone biosynthesis in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **2006**, *36*, 188–199. [[CrossRef](#)]
46. Fujinaga, D.; Gu, J.; Kawahara, H.; Ogihara, M.H.; Kojima, I.; Takeshima, M.; Kataoka, H. Twenty-hydroxyecdysone produced by dephosphorylation and ecdysteroidogenesis regulates early embryonic development in the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* **2020**, *127*, 103491. [[CrossRef](#)]
47. Han, P.; Han, J.; Fan, J.; Zhang, M.; Ma, E.; Li, S.; Fan, R.; Zhang, J. 20-Hydroxyecdysone activates PGRP-SA mediated immune response in *Locusta migratoria*. *Dev. Comp. Immunol.* **2017**, *72*, 128–139. [[CrossRef](#)]
48. Liu, S.; He, C.; Liang, J.; Su, Q.; Hua, D.; Wang, S.; Wu, Q.; Xie, W.; Zhang, Y. Molecular characterization and functional analysis of the Halloween genes and CYP18A1 in *Bemisia tabaci* MED. *Pestic. Biochem. Physiol.* **2020**, *167*, 104602. [[CrossRef](#)]
49. Cheng, D.; Zhang, W.; Jiang, S.; Xiong, Y.; Jin, S.; Pan, F.; Zhu, J.; Gong, Y.; Wu, Y.; Qiao, H. Cathepsin D Plays a Vital Role in *Macrobrachium nipponense* of Ovary Maturation: Identification, Characterization, and Function Analysis. *Genes* **2022**, *13*, 1495. [[CrossRef](#)]
50. Hu, Y.; Fu, Y.; Jin, S.; Fu, H.; Qiao, H.; Zhang, W.; Jiang, S.; Gong, Y.; Xiong, Y.; Wu, Y. Comparative transcriptome analysis of lethality in response to RNA interference of the oriental river prawn (*Macrobrachium nipponense*). *Comp. Biochem. Physiol. Part D Genom. Proteom.* **2021**, *38*, 100802. [[CrossRef](#)]
51. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]
52. Xu, L.; Yang, M.; Fu, H.; Sun, S.; Qiao, H.; Zhang, W.; Gong, Y.; Jiang, S.; Xiong, Y.; Jin, S. Molecular cloning and expression of MnGST-1 and MnGST-2 from oriental river prawn, *Macrobrachium nipponense*, in response to hypoxia and reoxygenation. *Int. J. Mol. Sci.* **2018**, *19*, 3102. [[CrossRef](#)] [[PubMed](#)]
53. Jin, S.; Fu, H.; Jiang, S.; Xiong, Y.; Qiao, H.; Zhang, W.; Gong, Y.; Wu, Y. RNA interference analysis reveals the positive regulatory role of ferritin in testis development in the oriental river prawn, *Macrobrachium nipponense*. *Front. Physiol.* **2022**, *13*, 204. [[CrossRef](#)] [[PubMed](#)]
54. Wang, Y.; Jin, S.; Fu, H.; Qiao, H.; Sun, S.; Zhang, W.; Jiang, S.; Gong, Y.; Xiong, Y.; Wu, Y. Identification and characterization of the DMRT11E gene in the oriental river prawn *Macrobrachium nipponense*. *Int. J. Mol. Sci.* **2019**, *20*, 1734. [[CrossRef](#)] [[PubMed](#)]
55. Jiang, S.; Xiong, Y.; Zhang, W.; Zhu, J.; Cheng, D.; Gong, Y.; Wu, Y.; Qiao, H.; Fu, H. A novel legumain-like proteases in *Macrobrachium nipponense*: Identification, characterization and function analysis in ovary maturation. *Front. Endocrinol.* **2022**, *13*, 460. [[CrossRef](#)]

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