Investigation of novel functions of three genes in oriental river prawn, *Macrobrachium nipponense*: Molecular Cloning, Expression, and *In situ* Hybridization Analysis

Shubo Jin[1], Hongtuo Fu[1,2]*, Yuning Hu[2], Shengming Sun[1], Sufei Jiang[1], Yiwei Xiong[1], Hui Qiao[1], Wenyi Zhang[1], Yongsheng Gong[1], Yan Wu[1]

1. Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, China

2. Wuxi Fisheries College, Nanjing Agricultural University, Wuxi 214081, China

*Corresponding author: Hongtuo Fu

Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences

9 Shanshui East Road, Wuxi 214081, Jiangsu Province, P. R. China

E-mail address: fuht@ffrc.cn

Tel.: +86.510 85558835

Abstract

Three genes were predicted to be potentially involved in the male sexual development in *M. nipponense*, including the Gem-associated protein 2-like isoform X1 (GEM), Ferritin peptide, and DNA polymerase zeta catalytic subunit (Rev3). In this study, we aimed to investigate their novel functions in depth. The full-length cDNA sequence of Mn-GEM was 1,018 bp, encoding 258 amino acids. The partial Mn-Rev3 cDNA sequence was 6,832 bp, encoding 1,203 amino acids. Tissue distribution indicated that all of these three genes have higher expression level in testis and androgenic gland, implying their novel functions in male sexual development. In situ hybridization analysis further confirmed the novel roles of these three genes. Rev3 promote the

23 testis development during the whole reproductive cycle, while GEM and ferritin only promote
24 the activation of testis development. Besides, these three genes play essential roles in funicular
25 structure development surrounding the androgenic gland cells, which promote and support the
26 formation of androgenic gland cells. The expression in hepatopancreas cells also suggested
27 their role in immune system in *M. nipponense*. This study advances our understanding of male
28 sexual development in *M. nipponense*, as well as providing the basis for further studies of male
29 sexual differentiation and development in crustaceans.

30 **Keywords**: *Macrobrachium nipponense*; Male sexual development; Gem-associated
31 protein 2-like isoform X1; DNA polymerase zeta catalytic subunit; Ferritin peptide; Expression
32 pattern; *In situ* hybridization.

33 Introduction

34 The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; Palaemonidae),
35 are widely distributed in freshwater and low-salinity estuarine regions of China and other Asian
36 countries [1-5]. It is a commercially important species with an annual aquaculture production
37 of 205,010 tons [6]. The growth performance of male *M. nipponense* and female *M. nipponense*
38 showed significant difference during the *M. nipponense* aquaculture. "Male prawns of *M.*
39 *nipponense* grow faster and reach larger size at the harvest time than their female counterparts"
40 [4-5]. The establishment of an artificial techniques to produce all male progeny on a
41 commercial scale is a long-term goal in *M. nipponense* aquaculture. Therefore, it is urgently
42 needed to fully understand the sex differentiation and determination mechanisms of *M.*
43 *nipponense*.

44 The androgenic gland is found in most crustaceans, producing hormones, which promote
45 the driving of male sexual differentiation, the establishment of male sexual characteristics, and

the development of the testes [7]. The ablation of androgenic gland from male *Macrobrachium rosenbergii* prawn results in the sex reversal to the "neo-females". All male progeny was generated when the "neo-females" were mated with normal male *M. rosenbergii* [7-9]. Thus, studies on androgenic gland is a hot topic on male sexual differentiation and development in crustacean species. The genes in the androgenic gland is of great importance to be fully understood, especially for those which may promote the male differentiation and development. The androgenic gland transcriptome and miRNA library have both been constructed for *M. nipponense* [10-11]. A series of genes identified in the androgenic gland transcriptome have been analyzed and proven to be involved in the sex-differentiation and determination mechanism of *M. nipponense* [12-15].

iTRAQ technique was used to perform the quantitative proteomic analysis of androgenic gland during the nonreproductive and reproductive season in *M. nipponense*. A total of 3 differentially expressed proteins (DEPs) showed highest expression level in androgenic gland, compared with those in testis and ovary, including Gem-associated protein 2-like isoform X1 (GEM), Ferritin peptide, and DNA polymerase zeta catalytic subunit (Rev3) [16]. According to the previous studies, ferritin peptide and Rev3 have been proven to be involved in the immune system maintenance. Ferritin peptide has been analysed in *M. nipponense*, which plays essential roles in its innate immune defence, especially for that in cellular and organismic iron homeostasis [17]. However, these 3 DEPs might play essential roles in male sexual development in *M. nipponense*, based on their higher expression levels in androgenic gland and the importance of the androgenic gland in male sex differentiation and sex determination in crustacean species. The roles in male sexual development might be the novel functions that did not report yet.

In this study, we aimed to further analyse their functions in *M. nipponense* in depth, especially for the important roles in male sexual roles. The full-length cDNA sequences from

71 *M. nipponense* were cloned and their structural characteristics were analysed. The mRNA
72 expression patterns in different tissues and reproductive cycle of testis were determined by
73 quantitative real-time PCR (qPCR), and their locations were further determined by *in situ*
74 hybridization. The results of this study provide the foundations for male sexual development
75 in *M. nipponense*, as well as that in other crustacean species.

76 **Materials and methods**

77 **Ethics Statement**

78 As described in detail previously [10], the prawns were obtained from the Tai Lake in Wuxi,
79 China. We got the permission from the Tai Lake Fishery Management Council. *M. nipponense*
80 is a normal species with huge production in China, which can be used for experimental
81 materials. All the experimental animal programs involved in this study were followed the
82 experimental basic principles, approved by committee of Freshwater Fisheries Research
83 Institute. MS222 anesthesia was used for each prawn when androgenic glands were collected,
84 in order to minimize suffering.

85 **Prawn and Tissue Preparation**

86 As described in detail previously [13], healthy adult *M. nipponense* with wet weight of 3.78-
87 5.26g were obtained from Tai Lake in Wuxi, China (120°13'44"E, 31°28'22"N). These
88 specimens were maintained in aerated freshwater under lab conditions at the temperature of
89 28°C for at least 72 h prior to tissue collection. A total of 6 tissues were collected from mature
90 prawns for qPCR analysis, including ovaries, testes, androgenic glands, heart, intestine and
91 hepatopancreas, in order to determine the mRNA expression levels in different tissues. An
92 additional androgenic gland was collected for Rapid Amplification of cDNA Ends (RACE)
93 cloning. The Olympus SZX16 dissecting microscope was used to extract the androgenic glands.
94 Testis in reproductive season at the temperature of 28°C and testis in non-reproductive season

95 at the temperature of 15°C were collected, in order to determine the expression levels in
96 reproductive cycle of testis. The samples were treated with phosphate buffer saline (PBS), and
97 immediately frozen in liquid nitrogen until used for RNA extraction to prevent total RNA
98 degradation.

99 **Rapid Amplification of cDNA Ends (RACE)**

100 As described in detail previously [13], total RNA was extracted from androgenic gland as
101 template using RNAiso Plus Reagent (Takara Bio Inc.), followed the protocol of the
102 manufacturer. The RNase-free DNase I (Sangon, Shanghai, China) was used to treat the
103 isolated RNA to eliminate possible genomic DNA contamination. BioPhotometer (Eppendorf,
104 Hamburg, Germany) was used to measure the concentration of the total RNA sample with the
105 A260/A280 in the range of 1.8-2.0. The RNA quality was then measured by 1% agarose gel.

106 As described in detail previously [13], a M-MLV reverse transcriptase was used to
107 perform the first strand 3′cDNA and 5′cDNA synthesis for gene cloning using the 3′-Full
108 RACE Core Set Ver.2.0 kit and the 5′-Full RACE kit (Takara Bio Inc., Japan), respectively
109 with the reaction conditions recommended by the manufacturer. The synthesized cDNAs were
110 kept at -20°C. 3′/5′-RACE PCR reactions were performed with the 3′ gene-specific primer
111 (GEM-3GSP1, GEM-3GSP2, Rev3-3GSP1, Rev3-3GSP2) or 5′GSP (GEM-5GSP1, GEM-
112 5GSP2) (Table.1). The partial unigene sequences were obtained from the *M. nipponense*
113 androgenic gland transcriptome, and the 3′GSP and 5′GSP of each gene were designed based
114 on the unigene sequence. 1% agarose gel was used to measure the PCR product.

115 As described in detail previously [13], the Gel Extraction kit (Sangon, shanghai,China)
116 was used to cut and purify the PCR products, following the manufacturer's instructions.
117 Amplified cDNA fragments were transferred into the pMD18-T vector (Takara Bio Inc., Japan).
118 Recombinant bacteria were identified by blue/white screening and confirmed by PCR. An

119 automated DNA sequencer (ABI Biosystem, USA) was used to determine the nucleotide
120 sequences of the cloned cDNAs. BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/)
121 was used to examine the nucleotide sequence similarities.

122 **Nucleotide Sequence and Bioinformatics Analyses**

123 As described in detail previously [13], the primer designing tool
124 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design all primers used in this
125 experiment. The 5′ and 3′ sequences from RACEs were assembled with the partial cDNA
126 sequence corresponding to each fragmental sequence by DNAMAN 5.0. The BLASTX and
127 BLASTN search program (http://www.ncbi. nlm.nih.gov/BLAST/) of GenBank was used to
128 analyse the sequences based on the nucleotide and protein databases using. The ORF Finder
129 tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict the open reading frame.
130 ClustalW1.81 was used to perform multiple sequence alignment. Molecular Evolutionary
131 Genetics Analysis, MEGA 5.1 was used to construct the phylogenetic trees based on the amino
132 acid sequences using the neighbor-joining method.

133 **Various Reproductive Cycle of Testis and Various Tissues Expression by qPCR**

134 As described in detail previously [13], qPCR was used to measure the relative mRNA
135 expression of Mn-GEM, Mn-Rev3 and Mn-Ferritin at various adult tissues and various
136 reproductive cycle of testis. Each tissue sample was dissected out from at least 3 mature prawns.
137 RNAiso Plus Reagent (TaKaRa) was used to exact and isolate the total RNA from various
138 tissues of adult prawns and different reproductive cycle of testis, following the manufacturer's
139 instructions. The concentration and quality of each total RNA sample was measured by
140 BioPhotometer (Eppendorf) with A260/A280 as 1.8-2.0, and 1% agarose gel. Experiments
141 were performed in triplicate. Approximately 1μg of total RNA from each tissue was used for
142 the first-strand cDNA synthesis using iScriptTM cDNA Synthesis Kit perfect Real Time (Bio-

Rad, CA, USA) and following the manufacturer's instructions. The synthesized cDNA template for qPCR was kept at -20°C. The qPCR primers of each gene were designed based on the open reading frame. The Bio-Rad iCycler iQ5 Real-Time PCR System (Bio-Rad) was used to carry out the SYBR Green RT-qPCR assay. β-actin was used as an internal reference to amplify for the same sample (the primer's sequences are shown in Table 1). Diethypyrocarbonate-water (DEPC-water) for the replacement of template was used as a negative control. All samples were run in triplicate (each duplicate for target gene and β-actin gene). The relative mRNA expression of each gene was calculated based on the $2^{-\Delta\Delta CT}$ comparative CT method. There are many advantages of the $2^{-\Delta\Delta CT}$ comparative CT method, including the ease of use and the ability of the data as "fold change" in expression. The amplification efficiency of target gene and β-actin were estimated by qPCR, using different concentrations of androgenic gland template. The androgenic gland templates include undiluted, two times diluted, four times diluted and eight times diluted sample. The slope of the Mn-GEM and β-actin at different concentrations of diluted samples were 1.393 and 1.411, respectively. The slope of the Mn-Rev3 and β-actin at different concentrations of diluted samples were 1.479 and 1.458, respectively. The slope of the Mn-Ferritin and β-actin at different concentrations of diluted samples were 1.576 and 1.583, respectively. Thus, the amplification efficiency between the target gene and β-actin are the same in this study. The tissue with lowest expression level was setted as 1 (a relative criterion), and other tissues were then compared with the relative criterion.

In situ Hybridization of DEM, Rev3 and Ferritin peptide mRNA in testis, androgenic gland and hepatopancreas

As described in detail previously [13], 4% paraformaldehyde treated by DEPC water was used to fix the tissue samples for *in situ* hybridization study. Primer5 software was used to design the anti-sense and sense probes of CISH (Chromogenic in-situ hybridization) study with DIG

signal based on the cDNA sequence of each gene. The anti-sense and sense probes were then synthesized by Shanghai Sangon Biotech Company. Both of the anti-sense and sense probes were hybridized with the slide. The anti-sense probe and sense probe were prepared for the experimental group and control group, respectively. CISH study was performed on 4μ thick formalin fixed paraffin-embedded sections using Zytofast PLUS CISH implementation kit (Zyto Vision GmBH, Germany). As described in detail previously [13], a standard deparaffinization technique was performed by 10 min incubation in 3% H_2O_2. Following rinsing in deionized water (DW), target retrieval was achieved using pepsin digestion in a humidity chamber for 10 min. Slides were incubated in EDTA solution at 95 for 15 min after washing in DW. Slides were washed in DW and drained off; 20 μl of CISH anti-sense probe and sense probe were poured over each slide. Denaturation at 75 for 5 min was subsequently followed by hybridization at 37 for 60 min in the Thermobrite TM hybridization chamber (Vysis Inc., USA). Tris-buffered-saline (TBS) washing, at 55 and room temperature, each for five min was done concurrently. Mouse-anti-DIG (Zyto Vision GmBH, Germany) was poured drop-wise over each slide, and incubated in a humidity chamber at 37 for 30 min. Three washings, each for a minute with TBS was done, before and after incubating slides in anti-mouse-HRP-polymer for 30 min at room temperature. 3,3'-diaminobenzidine (DAB) solution was prepared as per guidelines (Zytofast PLUS CISH) and poured 50ul in each slide for 10 min at room temperature. After washing, a hematoxylin was used for counterstaining. Slides were dehydrated in graded alcohol solutions, air dried and mounted with mixture of distyrene, plasticizer and xylene (DPX). Slides were examined under light microscope for evaluation.

Statistical Analysis

Quantitative data were expressed as mean ± SD. Statistical differences were estimated by one-way ANOVA followed by LSD and Duncan's multiple range test. All statistics were measured

192 using SPSS Statistics 13.0. A probability level of 0.05 was used to indicate significance ($P <$
193 0.05).

194 **Results**

195 **Sequences analysis**

196 The full-length Mn-DEM cDNA sequence was 1,018 bp with an open reading frame of 777 bp,
197 encoding 258 amino acids. The 5′ and 3′ untranslated regions (UTRs) of Mn-DEM contained
198 147 bp and 94 bp, respectively. The partial Mn-Rev3 cDNA sequence was 6,832 bp with an
199 open reading frame of 3,612 bp, encoding 1,203 amino acids. The 3′ untranslated regions
200 (UTRs) of Mn-Rev3 contained 3220. The cDNA sequence of Mn-DEM and Mn-Rev3 have
201 been submitted to GenBank with the accession no.MH817847 and MH817848, respectively.
202 The relative information for Mn-Ferritin can be seen in previous study (Sun et al., 2014).

203 **Similarity comparison and phylogenetic analysis**

204 The species used for GEM amino acid sequence blast have been listed in Table 2. The identities
205 between Mn-GEM and GEM in other species was 42%-47% revealed by the BLASTP
206 similarity comparisons, while the query coverage reached to 98%. Mn-GEM has the highest
207 identity with GEM sequence from *Acanthaster planci*. MEGA 5.1 was used to construct a
208 condensed phylogenetic tree using the neighbour-joining method, in order to analyse the
209 evolutionary relationship between Mn-GEM and other well-defined GEM sequences, based on
210 their completed amino acid sequences deposited in NCBI. The phylogenetic tree generated two
211 main branches; one including amino acid sequences from different species, and another
212 separate branch only including *M. nipponense* (Figure 1-A). Mn-GEM has dramatically long
213 evolutionary relationship with those from other species.

214 The species used for Rev3 amino acid sequence blast have been listed in Table 3. The
215 identities between the Mn-Rev3 and Rev3 in other species was 39%-41%, and the query

coverage is only 35%. Mn-Rev3 has the highest identity with Rev3 sequence from *Orbicella faveolata*. Similar with that of Mn-GEM, *M. nipponense* is in a separate branch (Figure 1-B), revealed by phylogenetic tree using MEGA 5.1. Mn-GEM has dramatically long evolutionary relationship with those from other species.

Expression analysis in different tissues and reproductive cycle of testis

Tissue distribution may reflect the physiological function of a protein. qPCR was used to determine the tissue distributions of these three genes. According to the qPCR analysis, the mRNA expression of these three genes in testis and androgenic gland were higher than that in ovary (Figure 2). The expressions showed significant difference for Rev3 and ferritin peptide ($p<0.05$). Besides, the mRNA expression of Rev3 and ferritin showed high expression level in hepatopancreas, especially for that of Rev3. The expression was 22.1-fold higher than that in heart ($p<0.01$).

The mRNA expression of these three genes showed higher expression levels in reproductive season of testis than those in non-reproductive season and showed significant difference ($p<0.05$). The mRNA expressions of Mn-GEM, Mn-Rev3, and Mn-Ferritin in reproductive season were 2.31-fold, 2.97-fold and 2.67-fold higher than those in nonreproductive season (Figure 3).

In situ Hybridization analysis

To analyse the functions of these three genes in depth, the mRNA locations were determined in testis, androgenic gland and hepatopancreas by *in situ* hybridization. The fixed tissue samples were subjected to hematoxylin and eosin (HE) staining as well as in situ hybridization. According to the HE staining, mature testis includes spermatid, spermatocyte and sperms, whereas sperms were the dominant cells. Androgenic gland consisted of funicular structure and androgenic gland cells. Hepatopancreas includes the lipid granules and hepatopancreas cells.

According to the *in situ* hybridization analysis, strong signals for Rev3 mRNA in mature testis were observed in spermatid, spermatocyte and sperm in mature testis (Figure 5), while strong signals for GME and ferritin peptide were only observed in spermatid, no signals were observed in spermatocyte and sperms (Figure 4, Figure 6). In androgenic gland, strong signals were observed in funicular structure surrounding the androgenic gland cells for all of these three genes, while no signal was directly observed in androgenic gland cells. Strong signals were observed in hepatopancreas cells for all of these three genes, rather than that in lipid granules (Figure 4; Figure 5; Figure 6). No signals were observed when sense RNA probe was used.

Discussion

Three DEPs were identified from the quantitative proteomic analysis of androgenic gland from *M. nipponense* during non-reproductive and reproductive season, which showed the expressions with highest mRNA level in androgenic gland, compared with those in testis and ovary, including GEM, Rev3 and ferritin peptide. According to the important functions of androgenic gland in male sexual differentiation and development, these 3 DEPs were predicted to be strong candidate novel genes in the male sexual development in *M. nipponense*. According to the previous studies, ferritin peptide and Rev3 play essential roles in immune system maintenance [17-19]. In this study, we aimed to investigate their functions in *M. nipponense*, especially for the potentially novel roles in male sexual development. The full-length cDNA sequence of Mn-GEM was 1,018 bp with an open reading frame of 777 bp, encoding 258 amino acids. Mn-GEM has the highest identity with GEM sequence from *Acanthaster planci*, while the identity was only 41%. According to the phylogenetic analysis, Mn-GEM has dramatically long evolutionary relationship with those from other species. These suggest considerable evolutionary divergence between *M. nipponense* and other species in terms of GEM, consistent with BLASTP analysis. The partial cDNA sequence of Mn-Rev3 was 6,832 bp with an opening reading frame of 3,612 bp, encoding 1203 amino acids. The full-

length cDNA sequence of Mn-Rev3 was hard to obtain by using 5'RACE cloning and homologous cloning because the absent cDNA sequence at 5'-terminal is too long and the degenerate primers were hard to designed due to long evolutionary relationship with the well-defined Rev3 sequences in other species. The full-length cDNA sequence of Mn-Rev3 will be obtained when the middle fragment is long enough. As the same as that of Mn-GEM, the similarity comparison analysis and phylogenetic analysis also showed a considerable evolutionary relationship between the Mn-Rev3 and the other well-defined sequences of Rev3 in other species. A reasonable explanation for the long evolutionary relationship with well-defined sequences is that the GEM and Rev3 sequences were only identified and isolated from limited species, and to the best of our knowledge, no previous researches related to GEM and Rev3 were found in crustacean species.

To the best of our knowledge, the functions of GEM have not been well defined and analysed. Rev3 and ferritin peptide have been proven to play essential roles in the immune system maintenance, based on the previous studies. In cultured human fibroblasts, Rev3 decrease the UV-induced mutagenesis through carrying out translesion DNA synthesis [18-19]. Ferritin peptide protect the cells from damage by excess iron through regulating the cellular and organism-wide iron homeostasis [20-23]. In addition, ferritin peptide has been also proven to play vital roles in development, cell activation, and angiogenesis [24-27]. Ferritin peptide showed the expression with highest mRNA level in hepatopancreas in *M. nipponense*, and proved to play critical roles in its innate immune defence, especially for those in cellular and organismic iron homeostasis [17]. In this study, the mRNA expression of Rev3 was the highest in hepatopancreas, and showed significant expression difference with that in other tissues ($P<0.01$). Rev3 also showed high expression in androgenic gland and testis. The dramatic high expression of Rev3 in hepatopancreas implies its potential roles in immune system in *M. nipponense*, which is similar with the previous studies. The ferritin peptide

showed the highest mRNA expression in testis, followed by in androgenic gland and hepatopancreas. However, there is no significant expression difference between the testis, androgenic gland, and hepatopancreas. A reasonable explanation for the different from previous study is that the tissues samples may collect at different season and have individual difference. The mRNA expression of GEM was the highest in androgenic gland, followed by testis and ovary. The high expression levels of these 3 DEPs in testis and androgenic gland suggested their potentially novel functions in male sexual development of *M. nipponense*, which have not yet been identified in any species. Besides, the mRNA expressions of these 3 DEPs in reproductive seasons were dramatically higher than those in nonreproductive season, which also suggested their potential roles in testis development.

The *in situ* hybridization of ferritin peptide has been performed in several species. The *in situ* hybridization analysis in *Branchiostoma belcheri* showed that ferritin homolog is ubiquitously expressed [28]. Ferritin mRNA from *Pinctada fucata* is highly expressed at the mantle fold, revealed by the *in situ* hybridization analysis. The mantle fold is a region, playing essential roles in metal accumulation and contributing the metal incorporation into the shell [29]. In iron-loaded rats with up-regulated levels of L-ferritin mRNA, L-ferritin mRNA was localized in many organs, including colonic crypt, villus epithelial cells, small intestinal crypt and surface epithelial cells [30]. To the best of our knowledge, no previous studies focused on the *in situ* hybridization of GEM and Rev3 in any species. In this study, strong signals for Rev3 mRNA were detected in spermatid, spermatocyte and sperm in mature testis, while strong signals for GME and ferritin peptide were only detected in spermatid. These results indicated that Rev3 promotes the testis development during the whole reproductive cycle of testis, while GME and ferritin peptide only promote the activation of testis development. Strong signals were detected in the funicular structure surrounding the androgenic gland cells for all of these three genes, while no signal was directly detected in androgenic gland cells. The histological

observation during the post-larval developmental stages of *M. nipponense* indicated that androgenic gland was developed at the post-larval day 10 (PL10) with the formation of funicular structure, then the androgenic gland cells were formed into the funicular structure, and the androgenic gland was matured at PL19 [31]. No signal in androgenic gland cells indicated that these three genes were not directly secreted by the androgenic gland, while the strong signals in funicular structure suggested their essential roles in the development of funicular structure, which promote and support the formation of androgenic gland cells. The strong signals in hepatopancreas cells suggested that important roles in immune system in *M. nipponense*. The similar expression pattern of these three genes in *M. nipponense* implies some relationship between each other.

Conclusion

We cloned and characterized GEM, Rev3 and ferritin peptide from the androgenic gland of *M. nipponense*. qPCR analysis of different tissues and reproductive cycles of testis suggested that these three genes may have additional functions in male sexual development in *M. nipponense*, which is the novel functions of these three genes and has not been reported in any species yet. In situ hybridization analysis further confirmed their important roles in male sexual development in *M. nipponense*.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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455 Table 1. Universal and specific primers used in this study

Primer name	Nucleotide Sequence(5′→3′)	Purpose
GEM-3GSP1	AGTATGGGAGGCTCGTCAGT	FWD first primer for GEM 3′ RACE
GEM -3GSP2	ATGCCCAGTGGCTAGAAGTG	FWD second primer for GEM 3′ RACE
GEM -5GSP1	AATGCTCCAGTTCCTTTGCCT	RVS first primer for GEM 5′ RACE
GEM -5GSP2	CCTGTCGGATATGCTCCGTC	RVS second primer for GEM 5′ RACE
Rev3-3GSP1	CCATCTACTTCCCATGGTATGT	FWD first primer for Rev3 3′ RACE
Rev3 -3GSP2	ATCCATTGACTGCCCTATCATT	FWD second primer for Rev3 3′ RACE
3′RACE OUT	TACCGTCGTTCCACTAGTGATTT	RVS first primer for 3′ RACE
3′RACE IN	CGCGGATCCTCCACTAGTGATTTCACTATAGG	RVS second primer for 3′ RACE
5′RACE OUT	CATGGCTACATGCTGACAGCCTA	FWD first primer for 5′ RACE
5′RACE IN	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	FWD second primer for 5′ RACE
GEM-RTF	ATGCCCAGTGGCTAGAAGTG	FWD primer for GEM expression
GEM-RTR	GCAGAATCCAGCGATGCTCT	RVS primer for GEM expression
Rev3-RTF	AGTGACAGCAACGCTAGTGG	FWD primer for Rev3 expression
Rev3-RTR	GGCCAAACAACTCTGTCAGC	RVS primer for Rev3 expression
Rev3-RTF	CCGAAATCCGCCAGAACTAC	FWD primer for Ferritin expression
Rev3-RTR	GCTTATCGGCATGCTCTCTC	RVS primer for Ferritin expression
β-actinF	TATGCACTTCCTCATGCCATC	FWD primer for β-actin expression
β-actinR	AGGAGGCGGCAGTGGTCAT	RVS primer for β-actin expression
GEM anti-sense Probe	GCACTGACGAGCCTCCCATACTACATGCTGAAGATAC	Probe for GEM ISH analysis
GEM sense Probe	GTATCTTCAGCATGTAGTATGGGAGGCTCGTCAGTGC	Probe for GEM ISH analysis
Rev3 anti-sense Probe	CATCTAATCCCTGCGAAGAGCCTGAAGGAACTTGTGAG	Probe for Rev3 ISH analysis
Rev3 sense Probe	CTCACAAGTTCCTTCAGGCTCTTCGCAGGGATTAGATG	Probe for Rev3 ISH analysis
Fer anti-sense Probe	GCTGGCATACAATTCCATGTTGATCTGCTTGTTAATG	Probe for Ferritin ISH analysis
Fer sense Probe	CATTAACAAGCAGATCAACATGGAATTGTATGCCAGC	Probe for Ferritin ISH analysis

456 Table 2. Amino acid sequence used for phylogenetic analysis of GEM

Species	Accession Number
Macrobrachium nipponense	
Acanthaster planci	XP_022103928.1
Xenopus laevis	XP_018087535.1
Stylophora pistillata	XP_022804116.1
Odobenus rosmarus divergens	XP_004399834.1
Pomacea canaliculate	XP_025106566.1
Centruroides sculpturatus	XP_023219163.1
Biomphalaria glabrata	XP_013071285.1
Aplysia californica	XP_005105416.1
Mizuhopecten yessoensis	XP_021367489.1
Xenopus tropicalis	NP_001096228.1
Xenopus laevis	NP_001087945.2
Mus musculus	NP_079932.2
Mus caroli	XP_021034811.1

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468 Table 3. Amino acid sequence used for phylogenetic analysis of GEM

Species	Accession Number
Macrobrachium nipponense	
Kryptolebias marmoratus	XP_024866820.1
Chaetura pelagica	KFU85727.1
Falco cherrug	XP_014140871.1
Podiceps cristatus	KFZ50056.1
Merops nubicus	KFQ35536.1
Fulmarus glacialis	XP_009579679.1
Sinocyclocheilus graham	XP_016087315.1
Poecilia Mexicana	XP_014866147.1
Falco peregrinus	XP_013160015.1

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Fig. 1: The construction of phylogenetic tree from different organisms based on amino acid sequence comparisons. Species names are listed on the right of the tree. A indicated the phylogenetic tree of GEM; B indicated the phylogenetic tree of Rev3.

Fig. 2：Expression characterizations in the various adult tissues were revealed by real-time quantitative PCR. The amount of mRNA was normalized to the β-actin transcript level. Data are shown as mean ±SD (standard deviation) of tissues in three separate individuals. Capital letters indicate expression difference of Mn-Foxl2 in different adult tissues. A indicated the expression characterization of GEM; B indicated the expression characterization of Rev3; C indicated the expression characterization of ferritin peptide.

Fig. 3：Expression characterizations at various reproductive cycle of testis were revealed by real-time quantitative PCR. The amount of mRNA was normalized to the β-actin transcript level. Data are shown as mean ± SD (standard deviation) of tissues from three separate individuals. Capital letters indicate expression difference of testes from control group.

Fig. 4: Location of GEM gene was detected in testis, androgenic gland and hepatopancreas of M. nipponense by in situ hybridization. Testis, androgenic gland and hepatopancreas were sampled at reproductive season. AG: androgenic gland; ST: spermatid; SC: spermatocyte; SP: sperm; M: muscle; C: androgenic gland cell; FS: Funicular structure; He: hepatopancreas; LG: lipid granules; HC: hepatocytes. Scale bars = 50 μm.

Fig. 5: Location of Rev3 gene was detected in testis, androgenic gland and hepatopancreas of M. nipponense by in situ hybridization. Testis, androgenic gland and hepatopancreas were sampled at reproductive season. AG: androgenic gland; ST: spermatid; SC: spermatocyte; SP: sperm; M: muscle; C: androgenic gland cell; FS: Funicular structure; He: hepatopancreas; LG: lipid granules; HC: hepatocytes. Scale bars = 50 μm.

Fig. 6: Location of ferritin peptide gene was detected in testis, androgenic gland and hepatopancreas of *M. nipponense* by in situ hybridization. Testis, androgenic gland and

hepatopancreas were sampled at reproductive season. AG: androgenic gland; ST: spermatid; SC: spermatocyte; SP: sperm; M: muscle; C: androgenic gland cell; FS: Funicular structure; He: hepatopancreas; LG: lipid granules; HC: hepatocytes. Scale bars = 50 μm.

Figure 1

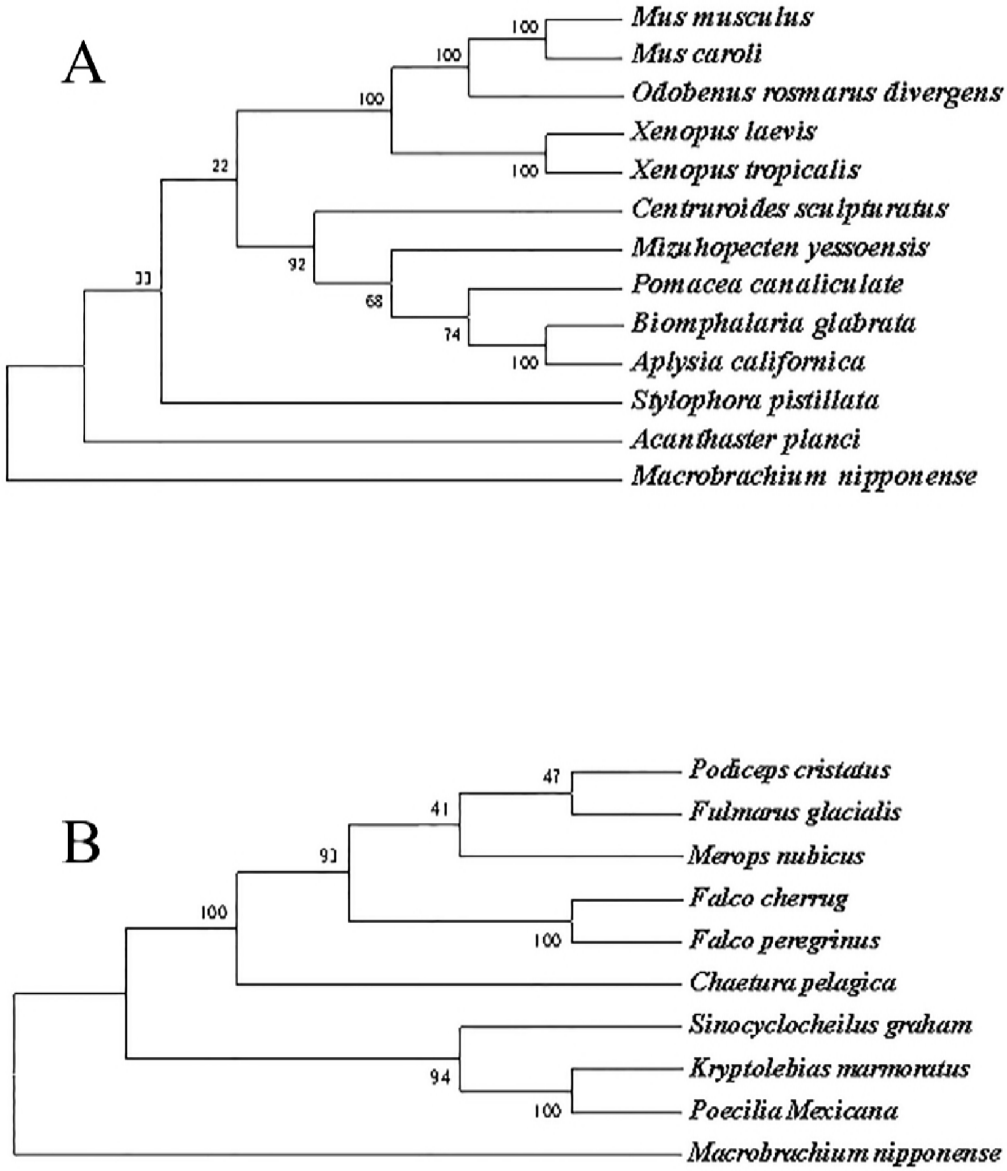


Figure 2

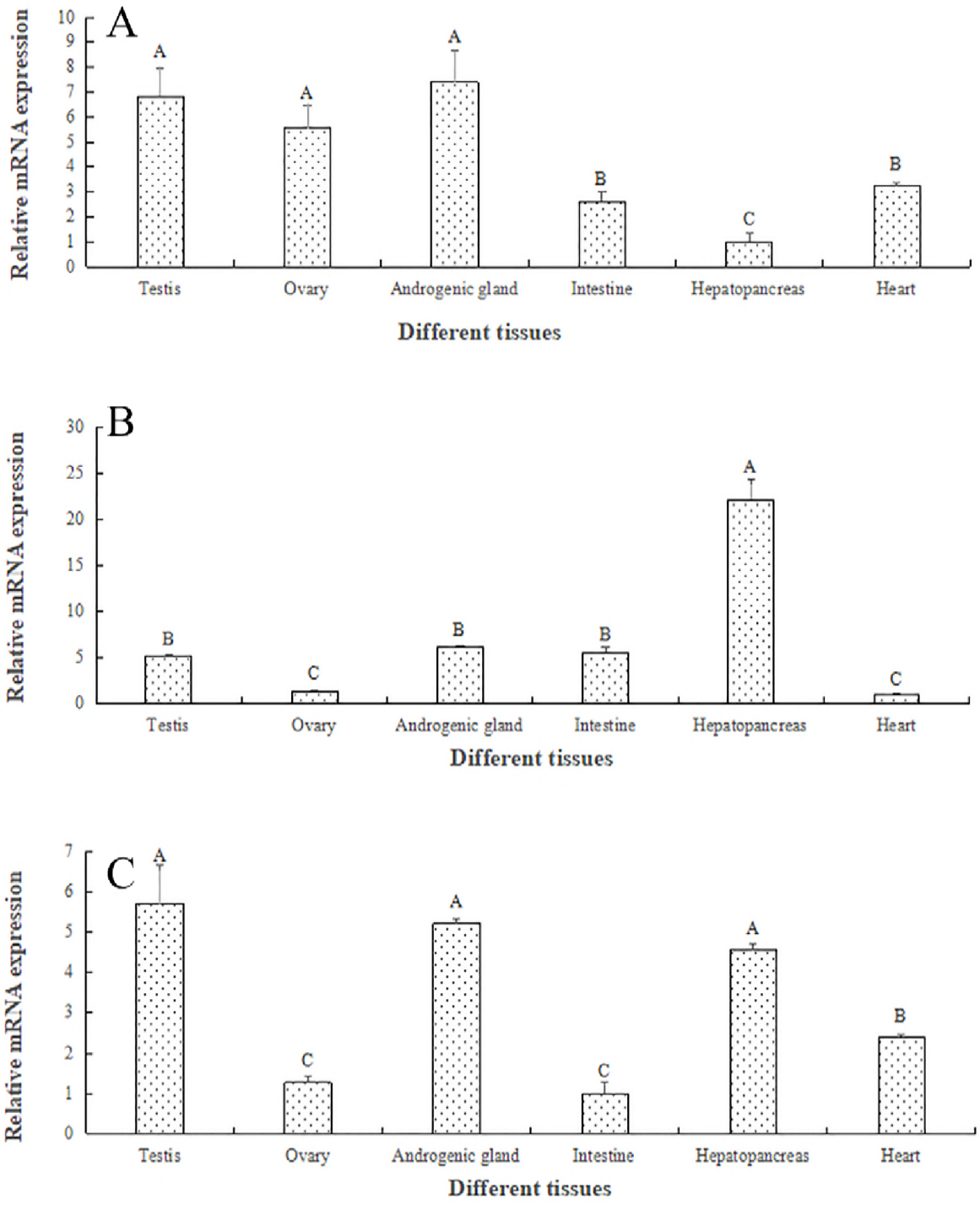
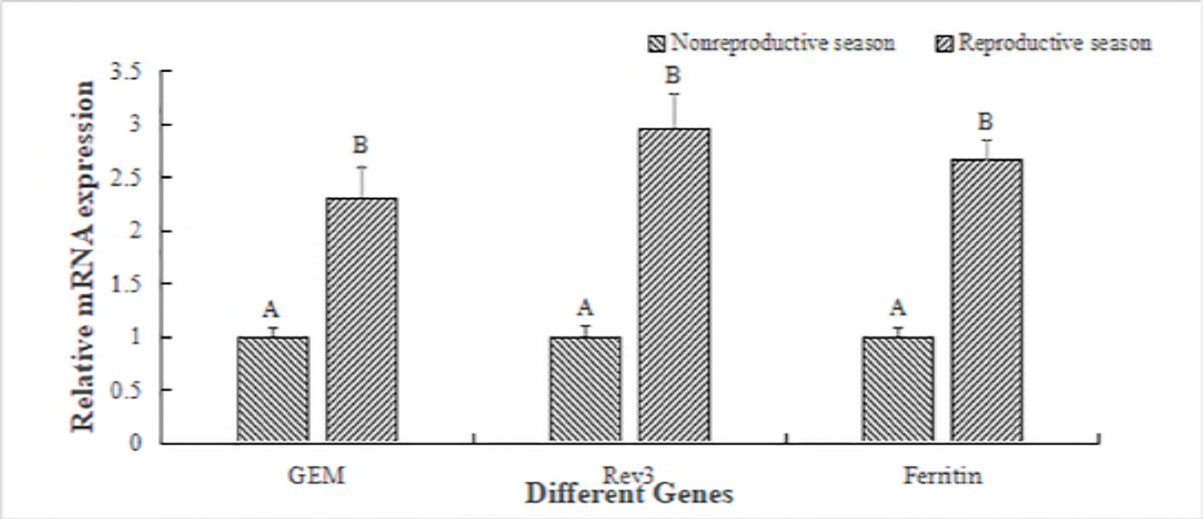


Figure 3



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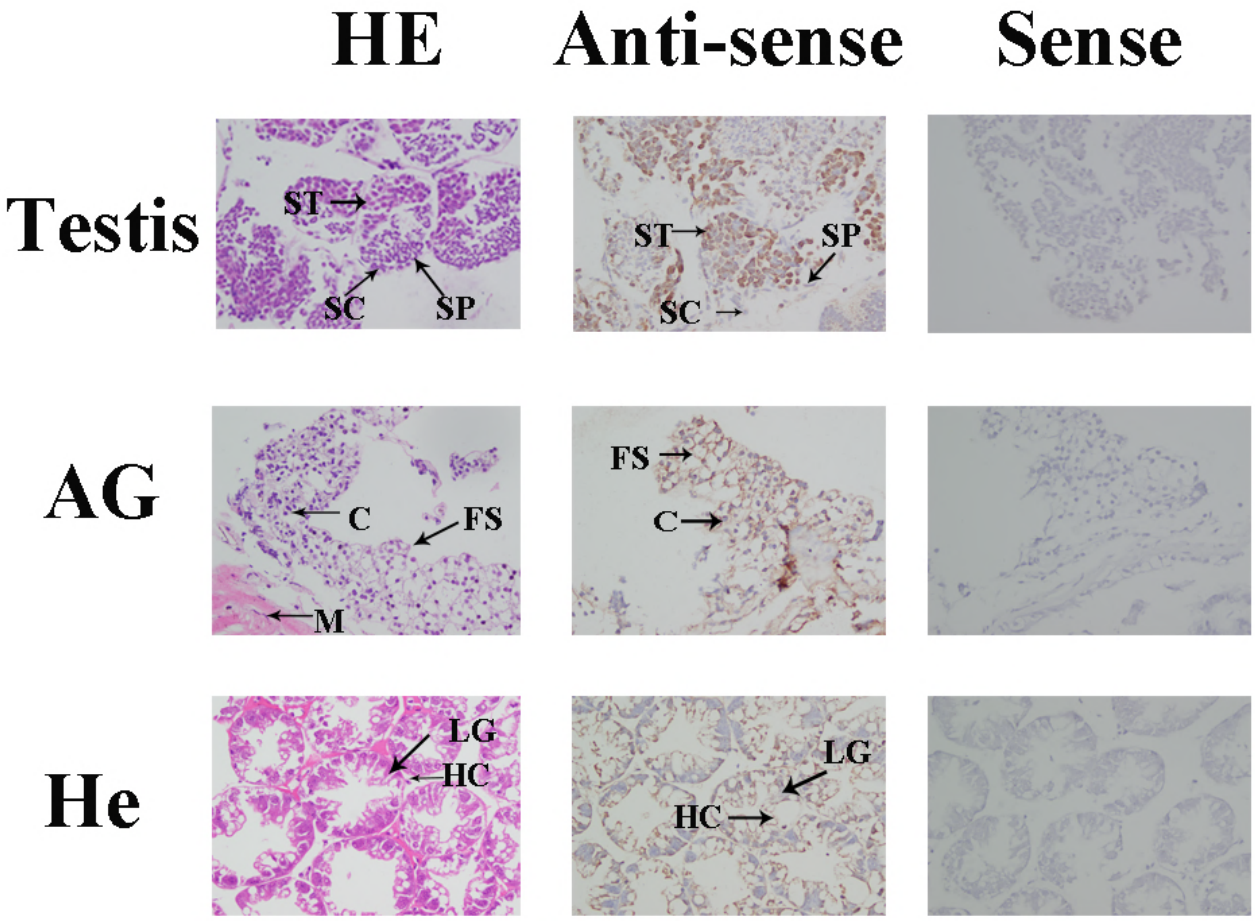

Figure 5

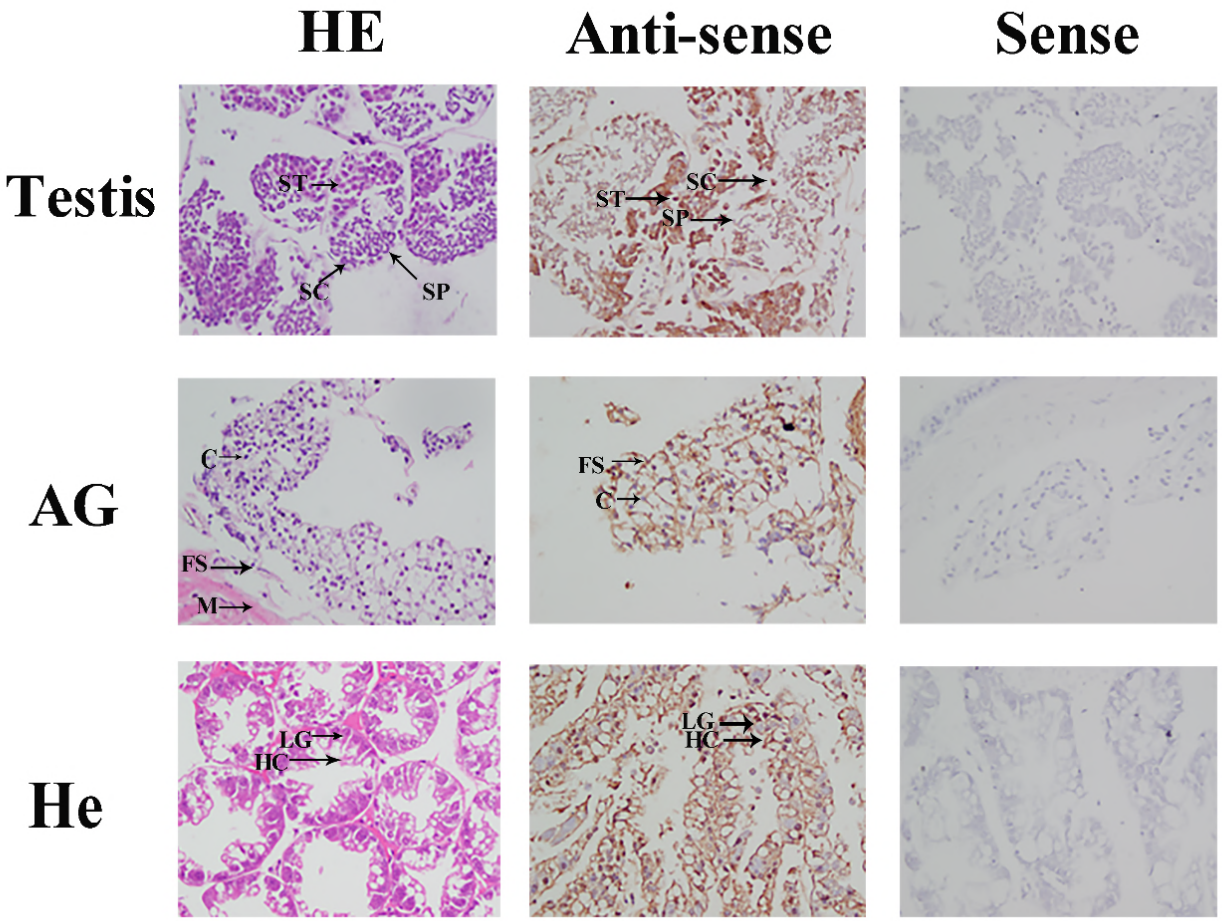

Figure 6