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The vitellogenesis-inhibiting hormone (SpVIH) of the crab *Scylla paramamosain* is not likely to have a vitellogenesis-inhibiting function in the ovary

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Abstract

The function and molecular characterization of the previously reported crab (*Scylla paramamosain*) vitellogenesis inhibiting hormone SpVIH is still unclear. In this experiment, SpVIH's bioinformatic and functional characterizations were analyzed. Sequence analysis showed SpVIH was clustered with several type II MIH/GIHs of the Brachyura. The expression of *SpVIH* in the eyestalk was low in the early ovarian developmental stage I and increased with the advancement of ovary maturation. Interestingly, the *SpVIH* transcript was still at a high level in the ovary of females at the middle to late maturation stage. SpVIH's function was studied according to the expression of vitellogenin (*Vg*) and vitellogenin receptor (*VgR*) in the ovary and hepatopancreas after incubation with SpVIH-dsRNA and recombinant SpVIH protein (rSpVIH). In vitro assay results indicated that rSpVIH has a significant stimulative effect on the expression of *Vg* in the hepatopancreas in the middle stage of vitellogenesis and in the ovary of each vitellogenesis stage. However, SpVIH-dsRNA has no significant effect on the expression of *Vg* when SpVIH-dsRNA is introduced to the ovary and hepatopancreas at the early, middle, and late stages of vitellogenesis. Furthermore, the co-incubation experiment result indicated that the eyestalk consists of factor(s) that can significantly up-regulate the expression of *Vg* in the ovary, with a further combination of dsVIH and eyestalk under incubation, the significant increase of Vg-mRNA expression levels were also found in the ovary of four ovarian development stages of *S. paramamosain*. Contrary to the previous reports, SpVIH does not possess a vitellogenesis-inhibiting function but can promote vitellogenesis in the ovary and hepatopancreas.

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Introduction

The crustaceans' X-organ sinus gland complex (XOSG) synthesizes and stores many neuropeptides, which are released into hemolymphs and transported to the target tissue (Jayasankar et al., 2020). Gonad development of female crustaceans is known to be regulated by chemical signals of the eyestalk XOSG(Hopkins, 2012). These neuropeptides include the chromatophore regulatory peptides, such as the red pigment concentrating hormone (RPCH)(Gade, 2009), pigment dispersing hormone (PDH)(Rao, 1992), and the crustacean hyperglycemic hormone family (CHHs) including the vitellogenesis-inhibiting hormone (VIH) or gonad-inhibiting hormones (GIH), crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), and mandibular organ-inhibiting hormone (MOIH)(Chen et al., 2020; Fanjul-Moles, 2006).

The CHH superfamily consists of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, stress response, molting, and reproduction (R, 1992). CHH-neuropeptides share a high degree of amino acid similarity. They consist of a divergent signal peptide region of variable sizes and a conserved mature peptide region with 72-83 amino acid residues (AAs). The mature peptide region all have 6 cysteine residues at conservative positions(Webster et al., 2012). Because of the variations in primary sequence, CHH family neuropeptides can further be divided into CHH subtype I and MIH/GIH subtype II. CHH-subtype I consists of a signal peptide, a precursor-related peptide, and a mature peptide comprising 72–73 AAs (Chen et al., 2005; Siu-Ming et al., 2003). CHH-subtype I neuropeptides include all CHHs and CHH-like members. CHH subtype II neuropeptides consist of neuropeptides that do not possess the CPRP but with an additional glycine at position 11 of the mature peptide and a longer mature peptide of 74-83 AAs. The subtype II members include the molt-inhibiting hormone（MIH）, vitellogenin-inhibiting hormone (VIH), and mandibular organ-inhibiting hormone (MOIH). Recently transcriptomic sequencing technique was applied to study eyestalk neuropeptides, and multiple CHH members have been identified in a single species(Liu et al., 2020). Transcription analysis of the central nervous system transcriptome of the eastern rock lobster *Sagmariasus verreauxi* reveals the complex nature of the putative neuropeptidome (Veenstra, 2016; Ventura et al., 2014). CHH family neuropeptides have been reported in many decapods in the GenBank database. Many CHH subtype II neuropeptides have been classified as either VIH or MIH through BLAST search analysis. However, the function of most of these neuropeptides is not properly confirmed. Early alignment study and evolutionary tree analysis results have been problematic as all members of this family have not been identified in a single species. For example, many so-called "*VIH or GIH*" cDNAs were classified as reproduction-related neuropeptides based initially on their similarity with the gonad-inhibiting hormone of the lobster *Homarus americanus*, but their functions have not been determined. Several *CHH* family polypeptides with VIH activity had been identified in *Litopenaeus vannamei* and classified as VIH (Tiu & Chan, 2007). It is reported that vitellogenin (Vg) production may be accelerated by adding putative factors that stimulate Vg synthesis (Chen et al., 2018; Chotigeat, 2011), and knocking down the *VIH* by RNAi can improve the expression level of *Vg* significantly (Kang, 2019; Tsutsui et al., 2007). In blue crab *Callinectes sapidus*, the hemolymph content of *MIH* is much higher in the middle stage of ovarian development than in the early stage (Zmora et al., 2009). MIH stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *C. sapidus. There* is an ovarian stage-dependent involvement and abundant *CsMIH* binding sites in the ovary. The phenomenon is consistent with the expression level of *MIH* (Zmora et al., 2009). *CsMIH* also stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *C. sapidus*, and the novel specific binding sites in hepatopancreas were analyzed. The study also indicated that cAMP is a second messenger in signal transduction. Similarly, in the sand shrimp *Metapenaeus ensis*, the expression of *MIH-B* in the eyestalk gradually increases with the development of the ovary. It reaches the maximum at the final mature stage(Gu et al., 2002). There were similar results from the characterization of an additional molt-inhibiting

hormone-like neuropeptide from the shrimp *M. ensis* (Tiu & Chan, 2007). *VIHs*, *GIHs and/or MIHs* have been cloned and identified in many crustacean species, including *Macrobrachium rosenbergii* (Yang & Rao, 2001) and the lobster *H. Gammarus* (Ollivaux et al., 2006). Most of VIH's mature peptides were 78-80 amino acids in size. However, the proposed SpVIH consists of a mature peptide with 103 amino acid residues, far longer than the 72-78 amino acids (AAs) characteristics of VIH/GIH mature peptides (Liu et al., 2018), which brings great uncertainty to its functional conservatism in the mud crab.

In China, the aquaculture of the crab *S. paramamosain* has developed rapidly in recent years. Eyestalk removal is often used to promote the yolk development of some crustaceans in production. Still, it can lead to declining parental reproductive capacity, increasing mortality, and damaging animal welfare (Liu et al., 2020). Many studies are underway to find alternatives to eyestalk ablation(Browdy, 1998). This study analyzed the sequence characteristics and spatiotemporal expression distribution of vitellogenin inhibiting hormone of S. paramamosain. For *in vitro* experiments, ovaries and hepatopancreas from crabs at different ovarian development stages were collected, and these tissues were incubated with VIH-dsRNA and prokaryotic recombinant protein, respectively. Transcription levels of *Vg* and *VgR* in different groups were analyzed. This study aimed to identify the function of SpVIH on the development of yolk proteins and provide a theoretical reference for the development of ovarian maturation technology in *S. paramamosain*.

Materials and Methods

Animals and breeding

Healthy *S. paramamosain* (201-210 g) for the experiment were captured from the coastal area of Zhanjiang, Guangdong, China in July 2021. Crabs at different sizes (small, 2.3-3.6 g; juvenile, 70.3-79.5 g; adult, 201-210 g) and different ovarian development stages were collected and temporarily cultured in ponds (1 m^3) with sand-filtered sea-water at 28-30 °C with a salinity of 20‰ for at least one week and fed live oysters daily. All experimental protocols and animals used in this research were carried out following the Animal Care and Use Committee of Guangdong Ocean University, China.

Multiple sequence alignments and phylogenetic analyses

The VIH protein sequences of other species were obtained through the National Center for Biotechnology Information database (NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis and comparison were performed using the BLAST program and aligned using ClustalX2.0 software (http://www.ebi.ac.uk/tools/clustalw2). Analysis of the phylogenetic relationship among the different MIHs and VIHs was constructed with the neighbor-joining (NJ) method using MEGA 6.0 software and the reliability of the branching was estimated with 1000 bootstrap replications.

Tissue collection, RNA extraction and cDNA synthesis

For tissue distribution study of SpVIH, healthy crabs (n=10, 172.3-208.6 g) at early stage of ovarian or testicular development were selected. Tissues included the cerebral ganglia (CG), eyestalk (ES), thoracic ganglia (TG), stomach (ST), hepatopancreases (HP), testis (TE), ovary (OV), heart (HE), gill (GI), mandibular organ (MO), intestine (INT), muscle (MU) and blood (BL) were dissected for total RNA preparation. The ES from different vitellogenesis stages of ovary (small, 2.3-3.6 g; juvenile, 70.3-79.5 g; adult, 170-210 g, and intermolt stage (stage I, GSI = 0.25 ± 0.17); the early ovarian development stage (stage II, GSI =1.72 ± 0.55); the middle stage (stage III, GSI = 7.74 ± 1.69) and the late stage (stage IV, GSI = 10.02 ± 1.27)) were sampled for total RNA preparation followed by the analysis of *SpVIH* expression with primers qSpVIH-F and qSpVIH-R.

Total RNA from different tissues was purified with the TransZol Up Plus RNA extraction kit (TransGen Biotech, Beijing, China) using procedures according to the manufacture's

instructions. The concentration of total RNA was determined using a Nanodrop 2000 Spectrophotometer (Nanodrop 2000, Thermo Fisher, Waltham, USA). The quality and integrity of the RNA were confirmed with a 1.5% agar gel electrophoresis analysis. First-strand cDNA synthesis was performed with 1 μg of total RNA using the All-In-ONE RT MasterMix kits (ABM, Vancouver, Canada) with procedures from the manufacturer's instructions. All cDNA samples were stored at -20 °C for later gene cloning and tissue distribution experiments.

SpVIH-dsRNA synthesis

Primers flanked by the T7 promoter sequence were designed and used to synthesize the dsRNA for *SpVIH* (GenBank accession: KF006815.1). The dsRNA template for each gene was amplified (95℃ for 5 min, followed by 30 cycles of 95℃ for the 30s, 58℃ for the 30s and 72℃ for 30s, then 72℃ for 10min) with the above primers and purified using FastPure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China). After the concentration detection of DNA templates (Nanodrop 2000, Thermo Fisher Scientific, Inc., Waltham, MA, USA), dsRNA was produced with DNA templates under the OneScribe T7 synthesis Kit (ABM, Vancouver, Canada). The final dsRNA was diluted with water to the appropriate concentration (1μg/μL). Primer sequences (dsSpVIH-F/R) are listed in **Table 1**. The quality of dsVIH was detected by agarose gel electrophoresis (**Figure S1**).

Table 1 Primers used in the present study

Primer name	Sequence 5′→3′
SpVIH-F	AGACGAGAAACATTATCCC
SpVIH-R	GTGGCTAGTAACAGTGAAAG
qSpVIH-F	CCTCAGTGTCCAGCGAACAG
qSpVIH-R	TGAATTCCTCGTTGTAGAAGCA
dsSpVIH-F	TAATACGACTCACTATAGGTGAGTGCCCGAACCTTAT
dsSpVIH-R	TAATACGACTCACTATAGGTTTCAGTGCCTCAGTAAGC
PrSpVIH-F	GGGGTACCAGGATGATCGACGATGAGTGCCC
PrSpVIH-R	CGGGATCCTCAGTAAGCAGTACCGTCGTCAGCA
CSpVg-F	CAGGAGCCTCCGTTACGACTA
CSpVg-R	TTCCACCAGTTCTTTGACCATC
CSpVgR-F	TTCTATACCAGGCCACTACC
CSpVgR-R	TTTTCACTCCAAGCACACTC
CSp18S-F	ACTCAACACGGGGAACCTCAC
CSp18S-R	CAAATCGCTCCACCAACTAAG

Production of recombinant VIH-his protein (PrVIH) production

The codingmature peptide sequence of *SpVIH* gene was amplified with restriction site linked (Kpn I and BamH I) primers rSpVIH-F and rSpVIH-R (**Table 1**). The amplified fragment and KpnI and BamH I digested vector pET-32a were DNA (Takara, Japan) ligated with T4 ligase (Takara, Japan). The recombinant expression vector (pET-His-VIH) was transformed into a competent BL21 (DE3) cell. After being cultured at 37 °C, the recombinant protein was induced by 1 mM IPTG for 6 h and collected by centrifugation (2500 × *g*, 30 min). The precipitated bacterial pellet was suspended in PBST solution (**Table 2**), further broken by ultrasonic, and centrifuged at 2500 × *g* for 30 min. The protein was expressed as inclusion bodies, stirred in washing buffer I and II by magnetic force for 2 h, and then centrifuged at 2500 × *g* for 30 min.

Table 2 Methods of solution preparation

PBST	10% Tween-20: 1×PBS=1: 99
Wash buffer Ⅰ	20 mM Tris-HCL、1 mM EDTA、1% TritonX-100
Wash buffer Ⅱ	20 mM Tris-HCL、1 mM EDTA、1% TritonX-100、4 M urea
Denatured buffer	20 mMβ-Mercaptoethanol、20 mM Tris-HCL 、8 M urea
Binding Buffer	20 mM Tris-HCl、500 mM NaCl、10 mM imidazole、8 M urea
Elution Buffer	20 mM Tris-HCl、500 mM NaCl、500 mM imidazol、8 M urea
Refolding buffer 1	20 mM Tris-HCL、1 mM GSH、0.2 mM GSSG、6 M urea
Refolding buffer 2	20 mM Tris-HCL、1 mM GSH、0.2 mM GSSG、4 M urea
Refolding buffer 3	20 mM Tris-HCL、1 mM GSH、0.2 mM GSSG、2 M urea
Refolding buffer 4	20 mM Tris-HCL、1 mM GSH、0.2 mM GSSG、0 M urea

After dissolving the inclusion bodies in the denaturation buffer and fully denatured by magnetic stirring at 4 °C for 12 h, recombinant protein purification was performed using a Ni-NTA column (Sangon, Shanghai, China) with procedures according to the manufacturer's protocol. The total amount of purified protein was quantified using the Bradford method using Bradford Protein Assay Kit (Sangon, Shanghai, China) and diluted to 100 μg/mL with 1 × PBS. The purified protein solution was dialyzed against PBS buffer containing 6 M, 4 M, 2 M, and 0 M urea separately at 4 °C for 12 h, and finally dialyzed against PBS solution for 24 h to obtain the renatured protein (rSpVIH).

The rVIH was verified by 12%SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB). One gel was stained by Coomassie brilliant blue as a control for Western blot. From another gel, after the protein bands were transferred to a 0.45 μm PVDF membrane (Beyotime, Shanghai, China) for WB, the membrane was immersed in blocking solution (Beyotime, Shanghai, China) for 10 min. Thereafter, the membrane was incubated in rabbit anti-6×His-Tag polyclonal antibody (1:1000 dilution; Servicebio, Wuhan, China) for 1 h and further incubated in HRP-labeled goat anti-rabbit IgG (H + L) antibody for 30 min. The protein bands were visualized using ECL luminescent solution (Applygen, Beijing, China) and a fully automatic chemiluminescence apparatus (Tanon, Shanghai, China).

In vitro incubation experiment

A female crab's tissue was placed in one sterile incubation plate, according to **Figure S2**. Each tissue block was about 50 mg, and each well in the plate contained 2 mL of sterile M199 culture medium and a 1:1000 ratio of prepared ampicillin solution. 10 *p*mol/mL dsVIH or 3.5 *p*mol/mL PrVIH was added to wells according to **Figure S2,** and tissues were collected separately after incubating for 3h at 28 °C. The control group was added with the same volume of 1 × PBS. Each group consisted of five replicates, and the samples were derived from one crab, and 5 crabs were used.

Semi-quantitative RT-PCR

According to the target gene sequence, specific primers were designed using Primer 5.0, and 18s rRNA was used as an internal reference gene (**Table 1)**. For the sequence amplification, the following program was used: 94 °C for 5 min followed by 32 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. RT-PCR reactions of 20 μL contained 1 μL of cDNA and each primer, 10 μL of 2 × PCR Master Mix (GenStar, Beijing, China), and 7 μL ddH_2O. Amplified products were electrophoresed on 1% agarose gel and imaged using Image J 1.51j8 software to perform data conversion.

Real-time quantitative PCR

Primers (qSpVIH-F/R) were designed using the Software Primer 5.0 (**Table 1**), and 18s rRNA was used as an internal control gene. 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 Real-Time PCR machines (Bio-Rad CFX Connect PCR, Bio-Rad, America). The following thermal cycling profile was applied: 95 ℃ for 2 min, followed by 39 cycles of 95 ℃ for 5 s and 57 ℃ 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 18S rRNA for each sample. The reaction was performed in triplicate for each sample.

Data statistics and analysis

Data of tests were determined and expressed as means ± standard deviation (SD). The expression of target genes between groups was normalized and subjected to one-way analysis of variance (ANOVA) using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, California, USA), followed by a post-hoc t-test with Welch's correction. Statistical significance was taken when $p < 0.05$.

Results

Bioinformatic analysis of SpVIH

The SpVIH precursor protein comprises a signal peptide of 22-amino acid (AA) residues and a mature peptide of 103 AAs. The presences of six conserved cysteine residues and a glycine residue located at position 12 of the mature peptide indicate that the SpVIH precursor belongs to the CHH family subtype II. Sequence alignment analysis (**Figure 1**) shows that the core sequence of SpVIH protein is consistent with CHH-family neuropeptides of other species. However, the mature peptide of *S. paramamosain* is significantly longer than the 77-81 AAs, as exemplified by most traditional subtype II members. Phylogenetic tree analysis results show that SpVIH and MIHs of *Brachyura* were clustered, and VIHs and MIHs of Dendrobranchiata were clustered (**Figure 2**).

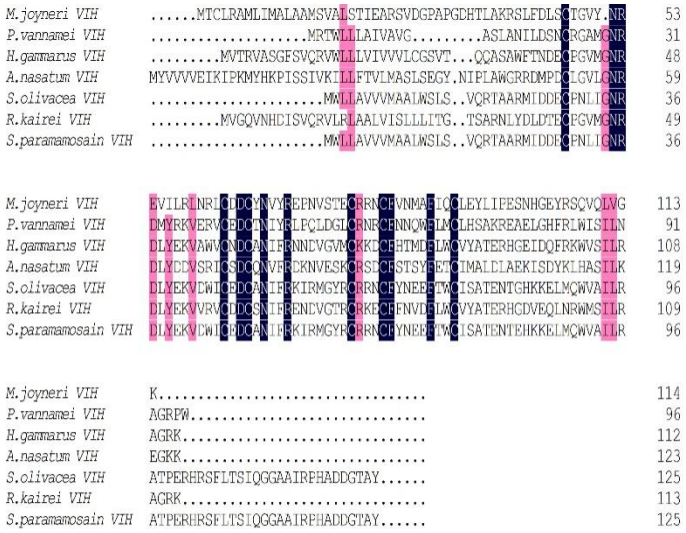

Figure 1 Sequence alignment of SpVIH from various crustaceans. Sequences used for alignment include: *Metapenaeus joyneri* VIH (GenBank accession no. BAN91404.1); *Penaeus vannamei* VIH (GenBank accession no. AGX26044.1); *Homarus gammarus* VIH (GenBank accession no. ABA42181.1); *Armadillidium nasatum* VIH (GenBank accession no. KAB7501390.1); *Scylla olivacea* VIH (GenBank accession no. AZF98733.1); *Rimicaris kairei* VIH (GenBank accession no. ACS35348.1); *Scylla*

paramamosain VIH (GenBank accession no. AHE40787.1). Highly conserved residues are highlighted in red or black.

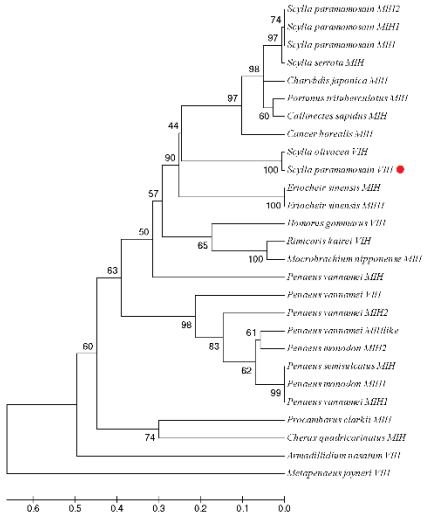

Figure 2 Phylogenetic tree analysis of VIHs/MIHs in *S. paramamosain* and other crustaceans. The various sequences are present in GenBank with the following accession numbers. *S. paramamosain* MIH2 (GenBank accession no. AFH36333.2); *S. paramamosain* MIH1 (GenBank accession no. AFM35653.1); *S. paramamosain* MIH (GenBank accession no. AFH36334.1); *Scylla serrata* MIH (GenBank accession no. AAL99355.1); *Portunus trituberculatus* MIH (GenBank accession no. ACF77140.1); *Callinectes sapidus* MIH (GenBank accession no. AAA69029.1); *Charybdis japonica* MIH (GenBank accession no. ACD11361.1); *Cancer borealis* MIH (GenBank accession no. QKO41649.1); *Scylla olivacea* VIH (GenBank accession no. AZF98733.1); *S. paramamosain* VIH (GenBank accession no. AHE40787.1); *Eriocheir sinensis* MIH (GenBank accession no. ABC68517.1); *Eriocheir sinensis* MIH1 (GenBank accession no. AAQ81640.1); *Homarus gammarus* VIH (GenBank accession no. ABA42181.1); *Rimicaris kairei* VIH (GenBank accession no. ACS35348.1); *Macrobrachium nipponense* MIH (GenBank accession no. AIP90070.1); *Penaeus vannamei* MIH (GenBank accession no. QBS36529.1); *Penaeus vannamei* VIH (GenBank accession no. AGX26044.1); *Penaeus vannamei* MIH2 (GenBank accession no. ABD73292.1); *Penaeus vannamei* MIHlike (GenBank accession no. XP_027237568.1); *Penaeus monodon* MIH2 (GenBank accession no. AAR89517.1); *Penaeus semisulcatus* MIH (GenBank accession no. BAN05500.1); *Penaeus monodon* MIH1 (GenBank accession no. ACS88073.1); *Penaeus vannamei* MIH1 (GenBank accession no. ABD73291.1); *Procambarus clarkii* MIH (GenBank accession no. QEM24161.1); *Cherax quadricarinatus* MIH (GenBank accession no. ACX55057.1); *Armadillidium nasatum* VIH (GenBank accession no. KAB7501390.1); *Metapenaeus joyneri* VIH (GenBank accession no. BAN91404.1).

Western blot verification of rSpVIH

The predicted molecular weight of pET-His-VIH is about 29 KDa. After expression and purification, SDS-PAGE (**Figure 3**) detected a clear band of the expected size. There was a band with a molecular mass of about 17 KDa in the pET-32a empty bacteria lane, and no target band existed in the non-induced VIH bacteria lane. And there was a target band of approximately 29 KDa in the IPTG-induced SpVIH bacteria lane, and a 29 KDa target band was found in the IPTG-induced sample. The SpVIH protein lane purified by the column showed a significant band at 29 KDa. These results were consistent with Western blot, proving that the purified protein is the SpVIH prokaryotic recombinant target protein.

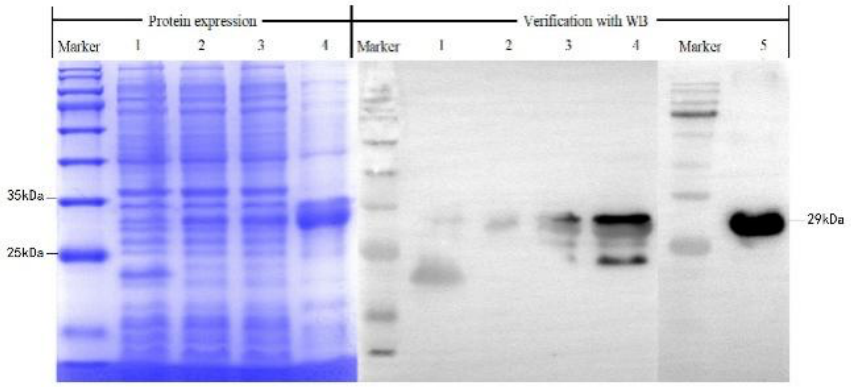

Figure 3 Detection of the expression and purification of SpVIH protein by SDS-PAGE and Western blot. Protein expression: SDS-PAGE analysis of expressing and purifying recombinant SpVIH protein. Lane 1, bacterial protein of vector pET-His; Lane 2, expressed protein of bacteria BL21 with recombinant pET-His-VIH induced by 0 mM IPTG at 37 °C; Lane 3, expressed protein of bacteria BL21 with recombinant pET-His-VIH induced by 1 mM IPTG at 37 °C; Lane 4 showed the purified protein of recombinant pET-His-VIH with Ni-NTA column. Lanes 1-4 of verification with WB corresponded to lanes 1-4 of protein expression. Lane 5 of verification with WB, Western blot analysis of the second purification of recombinant SpVIH protein with Ni-NTA column.

Tissue distribution of SpVIH

S. paramamosain at different stages of ovarian development (**Figure 4**) was chilled on ice and dissected for Es, Hp, and Ov. RT-qPCR was used to detect the expression of *SpVIH* in different tissues of females and different growth and development stages of *S. paramamosain*. The results show that *SpVIH* could be detected in the eyestalk of females. A low level of transcripts could be detected in ovaries (Figure 5A). In the eyestalks of females at different life cycle stages, the Sp*VIH* transcript level was the lowest in the small crab ($p < 0.05$), but a much higher level of *SpVIH* was detected in the subadult and the adult females ($p < 0.05$). With the development of the ovary, the transcript level of *SpVIH* in the eyestalks increases and reaches the highest level in the mid-late stage of the ovary and then returns to a low level at the post-spawning stage ($p < 0.05$) (**Figure 5B**).

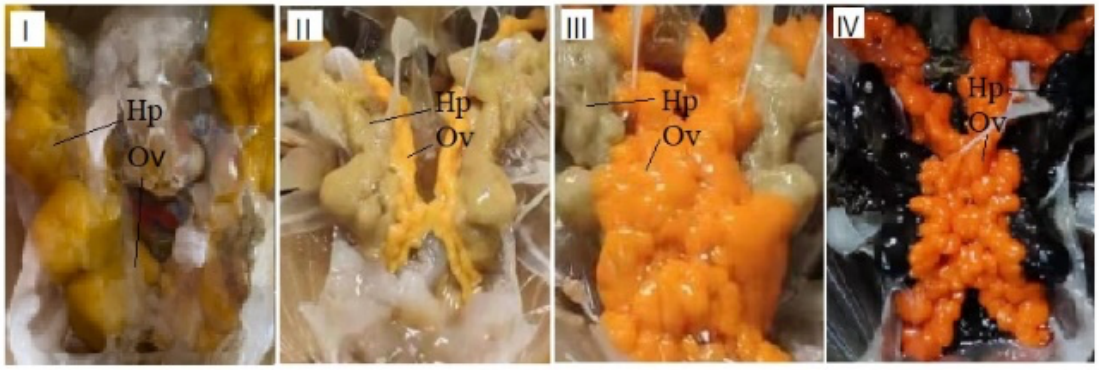

Figure 4 Ovary and hepatopancreas at different developmental stages. I: stage I of ovary development, GSI = 0.25±0.17; II: stage II of ovary development, GSI = 1.72 ± 0.55; III: stage III of ovary development, GSI = 7.74 ± 1.69; IV: stage IV of ovary development, GSI = 10.02 ± 1.27. Hp: hepatopancreas, Ov: ovary.

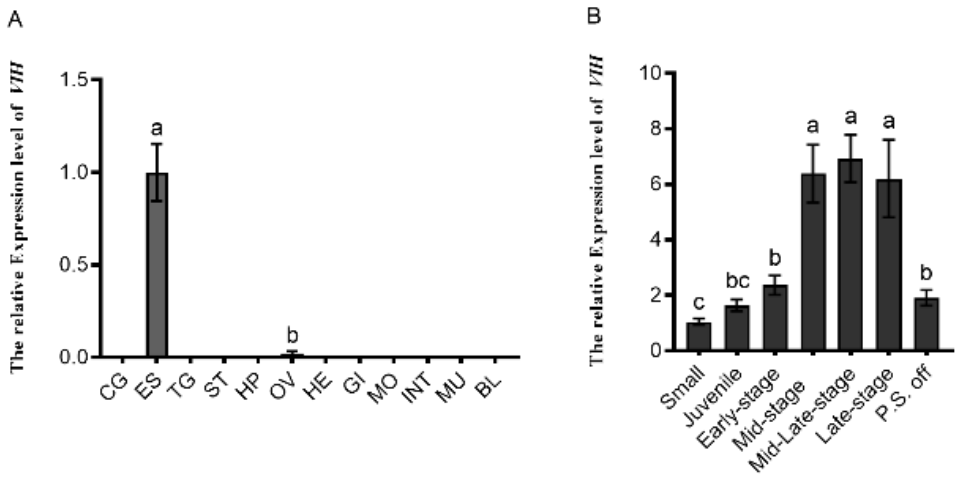

Figure 5 Tissue-specific relative expression of *SpVIH* in the eyestalk at early-stage of female crabs (**A**) and different development stages in crabs (**B**) by RT-qPCR. CG, cerebral ganglion; ES, eyestalk ganglion; TG, thoracic ganglion; St, stomach; Hp, hepatopancreas; TE, testes; OV, ovary; HE, heart, Gi, gill; MO, mandibular organ; INT, intestinal tract; MU, muscle; BL, blood. 18S was used as the internal control gene.

Expression of SpVIH with in vitro experiment

After 3 h incubation with dsSpVIH, the *SpVIH* transcript levels in the eyestalks at all gonadal development stages decreased significantly ($p < 0.05$), especially for females at the early stage ($p < 0.01$) (**Figure 6A**). In contrast, *SpVIH* was not detected in Hp of all stage groups. The results in Ov fragments (**Figure 6B**) show that compared with the blank incubation well in the sub-adults group, the expression of *SpVIH* had no significant change after dsSp*VIH* or rSpVIH or ES single added ($p > 0.05$). At the same time, *SpVIH* levels were significantly increased after the incubation with Es+dsSpVIH ($p < 0.05$). In the early stage, *SpVIH* expression in Ov was higher than in the sub-adults and on an increasing trend after treatment with Es+dsVIH ($p > 0.05$). In the mid-stage, the SpVIH transcription level was significantly decreased after adding only dsSpVIH and significantly increased after incubating with Es+dsVIH ($p < 0.05$), but there was no significant change in other pores ($p > 0.05$). In late stage, *SpVIH* expression was significantly decreased when rSpVIH was added, and similarity was seen in the well incubated with Es+dsSpVIH ($p < 0.05$), but there was no significant change in samples from other wells ($p > 0.05$).

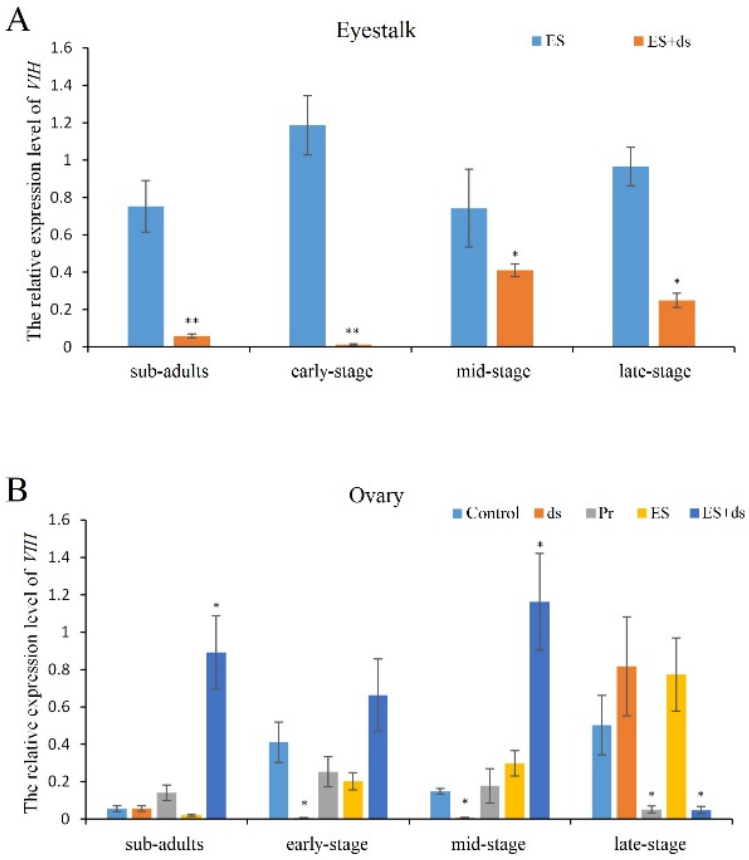

Figure 6 *SpVIH* expression in the eyestalks (A) and ovaries (B) after incubation with ds*VIH*. 0 means the group incubating alone, ds means the group incubating with ds*VIH*, Pr means the group incubating with recombinant protein SpVIH, ES means the group incubating with eyestalk, ES+ds means the group incubating with eyestalk and ds*VIH*. Results are expressed as the mean ± SD (n=5). Significant differences between the control group and treatment groups in the same stage are indicated as * (p < 0.05) or ** (p < 0.01).

In vitro study of SpVIH on Vg expression

The results (**Figure 7A**) showed that there was no significant difference in the Vg transcript level of Hp fragments in all incubation wells at the sub-adults (p > 0.05). In early-stage group, after incubation with Es+dsSp*VIH*, the transcription level of *Vg* in Hp was significantly increased (p < 0.05), and the changes in other tests were not significant (p > 0.05). In middle-stage, *Vg* transcript level in Hp was increased by adding recombinant protein or co-incubating with Es+dsSpVIH (p < 0.05) but decreased by only incubating with Es (p < 0.05). There was no significant change in the *Vg* transcription level of Hp tissue in all incubation wells in late stage (p > 0.05). In Ov fragments, there was no significant change (p > 0.05) in *Vg* at each stage treated with dsSpVIH compared to different incubation wells at each development stage. After incubation with Es, the *Vg* transcription level was significantly increased in the sub-adults stage, early-stage, and late-stage groups compared with the control (p < 0.05) (Figure 7B). After incubation with Es+dsSpVIH, the *Vg* transcription level in each group was significantly increased compared with the control (p < 0.05), and groups at the sub-adults stage, mid-stage, and late-stage were significantly higher than groups incubated with Es (**Figure 7B**).

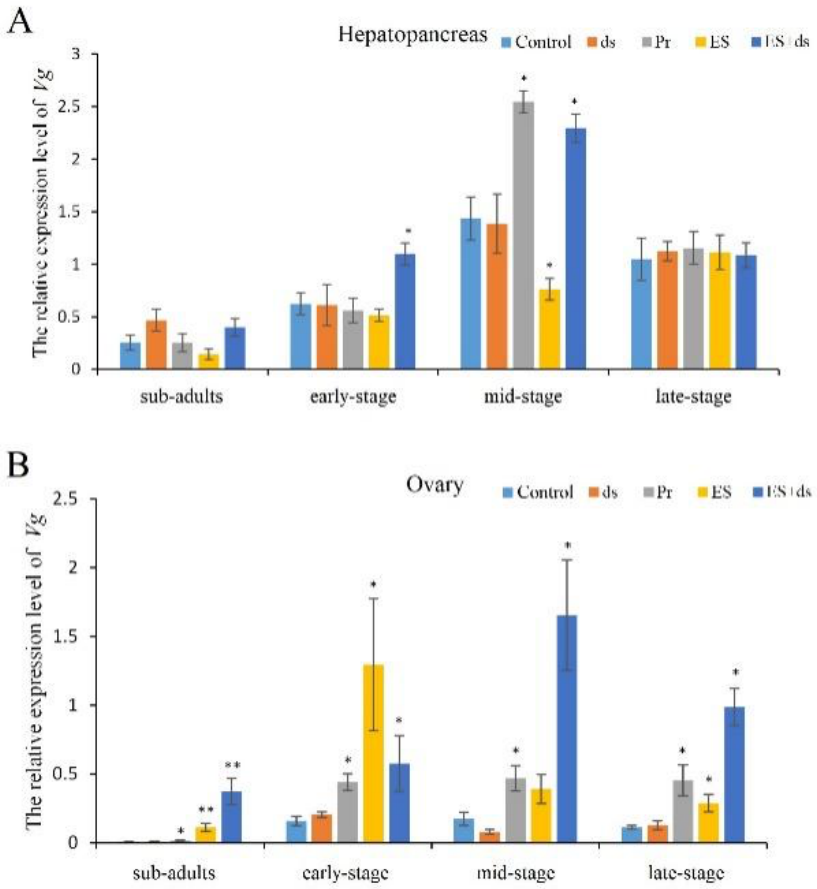

Figure 7 Expression of *Vg* in hepatopancreas (A) and ovaries (B) after exposure to different compounds. Control means the group incubating alone, ds*VIH* means the group incubating with ds*VIH*, Pr-VIH means the group incubating with recombinant protein SpVIH, ES means the group incubating with eyestalk, ES+ds means the group incubating with eyestalk and dsVIH. Results are expressed as the mean ± SD (n=5). Significant differences between the control group and treatment groups in the same stage are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Expression of VgR with in vitro experiment

The expression level of *VgR* in Ov was low at the early stage of ecdysis, increased gradually with ovarian development, reached the highest level at the middle stage of ovarian development, and then began to decrease. There was no significant change in *VgR* transcription level in Ov after *in vitro* incubation with different treatments at different developmental stages (**Figure 8**).

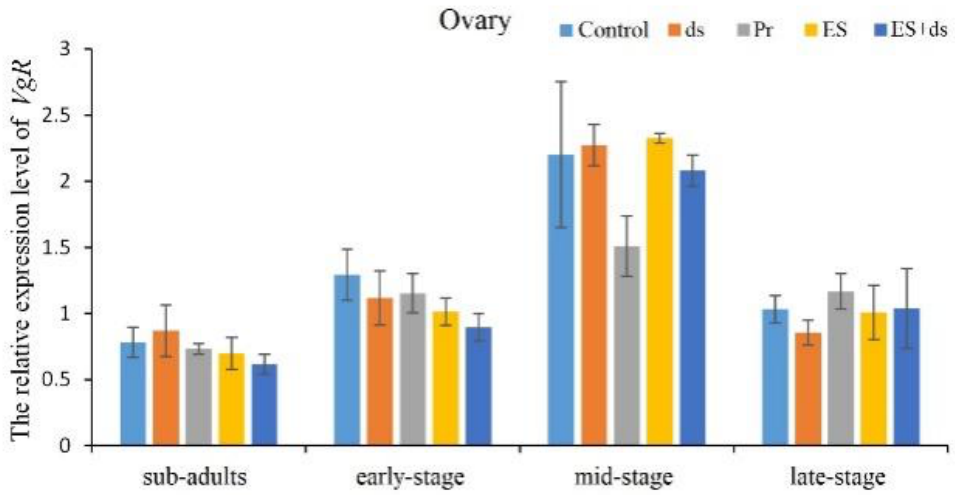

Figure 8 Expression of *VgR* in ovaries after different incubation. 0 indicates the blank control group, ds indicates the ds*VIH* group, Pr indicates the treated group incubating with recombinant protein SpVIH, ES indicates the group incubating with eyestalk, ES+ds indicates the group incubated with eyestalk and ds*VIH*. Results are expressed as the mean ± SD (n=5). Significant differences between the control group and treatment groups in the same stage are indicated as * ($p < 0.05$) or ** ($p < 0.01$)

Discussion

SpVIH was found and identified in a previous study. Its mature peptide contains 103-AAs, which is much longer than other CHHs. SpVIH is specifically expressed in the eyestalk and is potentially regulated by the transactivator of Sox9/Oct4/Oct1(Liu et al., 2018). Further studies indicated that several transcription factors might regulate the expression of *SpVIH*(Liao et al., 2020). Studies have shown that VIH mature *Scylla olivacea* and *S. paramamosain* peptides are identical, and the expression level of *SpVIH* can be regulated by dopamine(Kornthong et al., 2019). The molecular characterization of the mud crab *S. olivacea* vitellogenesis-inhibiting hormone (VIH) and temporal changes in the abundance of VIH mRNA transcripts during ovarian maturation and after neurotransmitter administration was studied. However, the regulatory relationship between SoVIH and vitellogenesis has not been studied. As in other VIHs or GIHs, the core sequence of SpVIH mature peptide was conserved, but the C-terminal end of SpVIH was significantly lengthened. Phylogenetic analysis shows that SpVIH is more like Brachyura's MIHs than VIH of *Dendrobranchiata*. Like multiple CHHs, multiple subtype II also exist in crabs, the type II CHH-family members (Rotllant et al., 2018). Multiple type II members also exist in the identification of female gonad-stimulating factors. Therefore, the existence of highly similar *SpVIH* and *SpMIH* may lead to difficulties in their functional differentiation. Whether *SpVIH* truly has, a vitellogenesis inhibiting function still needs more study. Like most CHH-family neuropeptides, *SpVIH* is mainly expressed in the eyestalks of female adult crabs. The temporal expression pattern in reproductive females indicates that *SpVIH* probably does not inhibit but promotes ovarian development. In the blue crab *C. sapidus*, studies have shown that the content of a Cs*MIH* is much higher in the middle stage of ovarian development than in the early stage (Zmora et al., 2009). Also, CsMIH stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab. The abundance of *CsMIH* binding sites in the ovary is consistent with the expression level of the *MIH* (Zmora et al., 2009). CsMIH stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *C. sapidus*. Similarly, results were also found in the shrimp *M. ensis.* The expression of *MIH-B* in the eyestalk gradually increased with the development of the ovary and reached the maximum at the final mature stage, and the recombinant protein

and RNA interference experiment further confirmed the vitellogenin stimulation function of MeMIH-B. Considering that VIHs and MIHs are too similar to be distinguished in crustaceans, and some MIH-types also have vitellogenin stimulating properties, the specific function of SpVIH (VIH or VSH or another subtype of MIH) is difficult to determine only from sequence alignment and the distribution and/or expression characteristics during gonadal development stages, and further experimental confirmation is necessary.

The XOSG complex of crustaceans synthesizes and stores many known and unknown neuropeptides, which are involved in a wide range of physiological processes in crustaceans. The regulation mechanisms among neuropeptides are universal and complicated (Hopkins, 2012). Furthermore, for the highly conserved sequences of the CHHs family, it is possible to not only trigger the mutual regulation between neuropeptides by conventional injection of dsRNA but also easy for other highly homologous CHHs to be wrongly knocked down by the same dsRNA, which ultimately leads to the failure of the experiments to reflect the true function of the target gene. To avoid the off-target effect, SpVIH-dsRNA and rSpVIH protein were included in separate hepatopancreas and ovary fragments in this study. Moreover, the addition of eyestalks incubated alone or co-incubated *in vitro* can explore the effect of *SpVIH* on the vitellogenesis of *S. paramamosain*. The results show that dsSpVIH could successfully knock down the SpVIH in the ovary in different stages, but the *Vg* expression level in the ovary was not affected. When rSpVIH was incubated with hepatopancreas and ovaries in different stages, the expression of *Vg* was significantly increased in the hepatopancreas at the middle stage of ovarian development, and no significant change in other stages. In contrast, the expression of *Vg* in the ovary increased significantly at all developmental stages. Similarly, in blue crab *C. sapidus*, co-incubation of hepatopancreas with MIH *in vitro* decreased Vg transcription and protein levels at the second developmental stage and significantly increased in the third stage development (Zmora et al., 2009). Also, CsMIH stimulates vitellogenesis at advanced ovarian developmental stages. In *M. ensis*, when recombinant MIH-B was added to the hepatopancreas and ovary incubated *in vitro*, *Vg* expression in both tissues was observed (Liu et al., 2018). The use of recombinant protein and RNA interference approaches to study the reproductive functions of a gonad-stimulating hormone was first reported in the shrimp *M. ensis*. These results suggested that SpVIH, similar to the MIH of *C. sapidus* and *M. ensis*, may promote the development of hepatopancreas and ovary in the middle developmental stage of mud crabs.

When the hepatopancreas or ovary was co-incubated with eyestalks, the expression of *Vg* in the hepatopancreas decreased at the middle stage of development. There was no significant change in the expression of Vg at other stages compared with incubation alone. In contrast, in the ovary, the *Vg* expression was higher than in the control group and significantly increased in every stage of development (except the middle stage). The results may be due to various neuropeptides in the eyestalk, which have certain specificity for hepatopancreas and ovarian function, leading to inconsistent performance between the two during co-incubation. When dsSpVIH was added to incubated with hepatopancreas, ovary, and eyestalk, the expression of *Vg* in hepatopancreas in the early and middle stages of development was significantly increased. The expression of *Vg* in ovaries at all developmental stages was significantly higher than in the ovaries incubated alone. Probably because there are not only vitellogenesis-stimulating factors in the eyestalks but also other 'vitellogenesis-regulating factors' with sequence similarity to the CHH-family member or SpVIH. These factors and *SpVIH* may be knocked down by dsRNA simultaneously and promote the expression of *Vg* in ovaries by interacting with promoting factors. A similar result was observed in *M. rosenbergii*, where dsVIH ORF simultaneously silenced VIH and MIH expressions (Cohen et al., 2021). The expression level of *VgR* was the highest in the ovary at the middle stage of development, but there was no significant effect on *VgR* in Ov by adding dsVIH, PrVIH or eyestalk. The result indicated that many vitellins were transferred from the hepatopancreas to the ovary at the middle stage of vitellogenesis, and SpVIH and other neuropeptides in eyestalk did not regulate the vitellin receptor in the ovary.

In conclusion, the results show that contrary to the previous reports, SpVIH is not likely to have a vitellogenesis-inhibiting function in the ovary. It can promote the expression of *Vg* during the middle stages of maturation in the ovary and hepatopancreas. Given SpVIH's high similarity with MIH and its spatiotemporal expression distribution, whether SpVIH has a certain regulatory effect on the reproductive molt of mud crabs needs further study.

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