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Acceleration of Ovarian Maturation in the Female Mud Crab With RNA Interference of the Vitellogenesis-Inhibiting Hormone (VIH)

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In the present study, double strand RNA technology (dsRNA) was used to inhibit transcripts of vitellogenesis-inhibiting hormone (VIH) that mainly synthesized and secreted from the central nervous system in *Scylla olivacea* females. The results presented in this study clearly demonstrate the potential dsRNA-VIH was highly effective to inhibit VIH in the eyestalks of females injected with dsRNA-VIH on the 3rd, 7th and 14th day, respectively. The dsRNA-VIH injections were performed at 14-day intervals, a single dsRNA dose of 0.6 μg/gram body weight was enough to suppress *VIH* expression until 14th day after injection. The dsRNA-VIH injection significantly increased gonad-somatic index (GSI) and hemolymph vitellin level at day 14 and 28 when compared with control groups. The histological observation found that the number of oocyte step 4 in dsRNA-VIH group was significantly higher than that of the control group. Also, dsRNA-VIH has stimulatory function on other reproduction-related genes such as the *ScyoI-PGES* and *ScyoI-ESULT* that both genes gradually increased their expressions in brain and ventral nerve cord. In conclusion, the silence of *VIH* gene could reduce the production of *VIH* from eyestalk and brain that affected other downstream genes related to ovarian maturation in the mud crab.

Keywords: vitellogenesis-inhibiting hormone (VIH), double strand RNA, *Scylla olivacea*, ovarian maturation, reproductive-related genes

INTRODUCTION

The vitellogenesis-inhibiting hormone (VIH), also known as gonad-inhibiting hormone (GIH), is produced and secreted by neuroendocrine cells of the X-organ-sinus gland complex (XO-SG) in the eyestalk of various crustaceans (Treerattrakool et al., 2011; Feijo et al., 2016). This peptide hormone is a member of crustacean hyperglycemic hormone (CHH) family which their candidates include the CHH, GIH/VIH, molt-inhibiting hormone (MIH), and mandibular organ-inhibiting hormone

(MOIH) (Kegel et al., 1989; Hsu et al., 2006; Webster et al., 2012). Commonly, the CHH family peptides, as well as the VIH, contain six cysteine residues forming three intramolecular disulfide bonds in their molecules (Lacombe et al., 1999; Webster et al., 2012). Functional studies of this peptide hormone family revealed diverse physiological involvements in crustaceans include metabolism, osmoregulation, molting, and reproduction (Chen et al., 2014). Considering the VIH function, it was mostly recognized as a potent negative regulator of crustacean reproduction (Chen et al., 2014). It has been reported that VIH diminished the synthesis of vitellogenin (VTG) from the ovary (Tsukimura, 2015). The characterization of VIH have been reported in several crustacean species such as *Penaeus monodon*, *Rimicaris kairei*, *Litopenaeus vannamei, Scylla paramamosain* and *Scylla olivacea* (Treerattrakool et al., 2008; Qian et al., 2009; Chen et al., 2014; Liu et al., 2018; Kornthong et al., 2019). The white leg shrimp, *L. vannamei*, presented high rate of ovarian maturation (Kang et al., 2014) and increased hepatopancreatic VTG expression (Chen et al., 2014) after the eyestalk ablation. Moreover, dsRNA-mediated *GIH* silencing have been reported to increase ovarian maturation and eventual spawning in both domesticated and wild female broodstock, particularly with a comparable effect to eyestalk ablation in wild shrimp in *P. monodon* (Treerattrakool et al., 2011).

Ovarian maturation period of mud crabs (*Scylla* spp.) is a time-consuming process which takes approximately 55-60 days depending on the environmental conditions, i.e., hormonal control, water salinity, natural diet, biochemical compositions, and enzyme activities (Nagaraju, 2011; Amin-Safwan et al., 2016; Azra and Ikhwanuddin, 2016; Hidir et al., 2018; Muhd-Farouk et al., 2019; Hidir et al., 2021). The vitellin (Vn), a set of multiple cleaved products of VTG, is a key molecule of crustacean yolk proteins that plays a significant role in the embryonic development (Volz et al., 2002; Quackenbush, 2015). In the *Scylla serrata*, vitellogenesis takes place in both hepatopancreas and ovary while the cleaved Vn subunits being processed in hepatopancreas, and hemolymph was sequestered by the growing oocytes (Rani and Subramoniam, 1997; Subramoniam, 2011). Therefore, various researchers have suggested that Vn is the major egg yolk protein and a high density lipoglycoprotein frequently associated with carotenoid pigments and the common form of yolk stored in oocytes and a nutrient source for developing embryos. In many species, VTG, the precursor molecule to Vn, is transported through the hemolymph to developing oocytes in the marine shrimp *Penaeus semisulcatus* (Avarre et al., 2003). Previous studies have found that high hemolymph VTG levels result to late ovarian development as histologically demonstrated by the vitellogenic oocytes become larger and they are filled with yolk globules in *Pandalus hypsinotus* and *Marsupenaeus japonicus* (Okumura et al., 2004; Okumura, 2007).

Normal processes of crab reproduction consume long period to complete its cycle. The eyestalk ablation, therefore, was introduced to remove the reproductive inhibiting hormones in crustaceans, including CHH peptide family-the VIH and MIH (Okumura and Aida, 2001; Nagaraju, 2011; Kang et al., 2014). By microarray analysis, ablation of eyestalk positively increased the vitellogenesis, ovarian weight as well as altered gene expressions that participate in reproduction, immune response and calcium signaling transduction in the *Penaeus monodon* (Uawisetwathana et al., 2011). Therefore, this procedure has been used for ripening ovarian development and maturation of many crustaceans i.e., the *P. monodon*, *Procambarus clarkia* and *M. japonicus* (Tan-Fermin, 1991; Okumura et al., 2007; Guan et al., 2017). Recent study also showed that eyestalk ablation in the freshwater crab, *Barytelphusa lugubris*, was able to induce breeding by rapid ripening the ovary and shortening the molting cycle (Rana, 2018). Besides, an alternative technique to eyestalk ablation by neuropeptide and hormone administration such as the serotonin (5-HT) and dopamine antagonist was also noted (Alfaro-Montoya et al., 2004).

The dsRNA technique has been used to silence the hormonal target transcripts by RNA interference (Alfaro-Montoya et al., 2004; Treerattrakool et al., 2011; Kang et al., 2021). This method post-transcriptionally silenced the target gene using specific dsRNA (Meister and Tuschl, 2004; Pak and Fire, 2007; Kang et al., 2019; Kang et al., 2021). The administration of dsRNA to silence *P. monodon GIH* gene was reported giving the known function of GIH in inhibiting *VTG* gene expression (Treerattrakool et al., 2008). In addition, the injections of dsRNA of *CHH1* and *MIH1* reduced the hemolymph glucose level and days of molting period in the *P. monodon*. The dsRNA of *GIH* gene application also promoted the *VTG* gene expression in the same species (Sukumaran et al., 2017). Although eyestalk ablation in crab is quite limited since it provides physiological abnormalities and low survival rate (Sroyraya et al., 2010). Using dsRNA to silence the *VIH* gene is therefore an alternative method to stimulate vitellogenesis and ovarian maturation in the female *S. olivacea*.

Therefore, in this study we successfully used specific dsRNA to knockdown the *VIH* transcript of *S. olivacea* and demonstrated its effects in temporary silencing *VIH* gene. In addition, the dsRNA-VIH could significantly increase Vn level in hemolymph and enhance ovarian maturation as well as promote some reproduction-related gene expression in the central nervous system of the female mud crab.

MATERIAL AND METHODS

Animals

The female mud crabs, *S. olivacea*, were cultured in Coastal Aquaculture Research and Development Regional Center 2, Samut Sakhon, Thailand. Identification of maturity stage of each crab was followed the previous study (Overton and Macintosh, 2002). All procedures on experimental animals were approved by Animal Care and Use Committee of Thammasat University, National Research Council of Thailand (NRCT), Protocol Number 020/2561 in which all efforts were made to minimize the suffering of animals. Crabs were

maintained in the concrete tanks filled with approximately 1000 L natural seawater (30 ppt) at 26-28°C, under a 12 h light and 12 h dark photoperiod. Crabs were fed twice daily (at 8:00 AM and 4:00 PM) with commercial fresh food throughout this study. The intermolt female mud crabs (n = 60) with average weight of 45-55 g were used in the dsRNA optimization assay. The mature female mud crabs at the same molting stage with average weight of 135-145 g, together with gonadsomatic index (GSI) less than 0.2, and the distance between the two tips of the 9th spine of anterolateral carapace approximately 85-92 mm (Ghazali et al., 2017; Amin-Safwan et al., 2018), were used to study the prolonged inhibitory effect of *VIH* knockdown and effect of dsRNA on ovarian maturation.

Construction of Recombinant Plasmids

To engineer the recombinant plasmid for dsRNAs expression, the DNA fragments corresponding to *VIH* sequence (380 bp) with loop sequence (580 bp) were amplified from pUC57 containing synthetic *VIH* gene (GenBank accession no. MH882453). The enhanced green fluorescent protein (EGFP) fragments with loop (570 bp) and without loop sequences (380 bp) were amplified by PCR using pUC-EGFP as the template. The specific primers used in this study were shown in **Table 1**. The PCR condition was set as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by final extension at 72°C for 5 min. The PCR fragments (fragment with and without loop sequences) of *VIH* or *EGFP* were cloned into pET17b at *Xba*I and *Xho*I sites. The DNA fragments with and without loop sequence of each gene were linked together by using *Hind*III site to generate opposite direction. The ligated product was introduced into *Escherichia coli* DH5α for propagating the plasmids. The recombinant plasmids were digested with *Xba*I and *Xho*I and then confirmed the sequences by DNA sequencing. For template of dsRNAs production *in vitro*, both stem loop fragments were excised from pET17b and cloned into pDrive cloning vector at the *Xba*I and *Xho*I sites.

Production of DsRNA-VIH and DsRNA-EGFP

For *in vitro* dsRNA production, *Xho*I-linearized recombinant plasmids were used as the templates to produce the dsRNAs using SP6 RiboMAX^{TM} Express Large-Scale RNA Production System (Promega, USA) kit according to the manufacturer's protocol. The remaining template was removed by RQ1 RNase-free DNase (Promega, USA) treatment at 37°C for 15 min and dsRNA was extracted by TriPure Isolation reagent (Roche, Germany) as described by manufacturer's protocol. dsRNA pellet was solubilized by 150 mM NaCl and characterized by RNase III and RNase A digestion. dsRNA was verified the integrity by agarose gel electrophoresis and estimated the concentration by Nanodrop.

Dose Optimization of Double-Stranded RNAs (DsRNAs) and Prolonged Inhibitory Effect of DsRNAs

After acclimatization for 14 days, thirty-five crabs were divided equally into 7 groups (n = 5 each); (1) Vehicle control group, in which the animals were administered with 100 µl of 0.9% normal saline (NSS); (2-4) dsRNA-VIH groups in which the animals were administered with 100 µl of dsRNA-VIH at 0.2, 0.4, 0.6 µg/g BW dissolved in 0.9% normal saline; (5-7) dsRNA-EGFP groups in which the animals were administered with 100 µl of dsRNA-EGFP at 0.2, 0.4, 0.6 µg/g BW dissolved in 0.9% normal saline. Administration was performed intramuscularly at the base of the fifth walking leg. Animals were sacrificed at 24 h post injection, then the eyestalk and brain from individuals were collected for determining the *VIH* and *EGFP* genes expression. Prolonged inhibition effect of the dsRNAs was verified by the *VIH* and *EGFP* genes expression in both organs after 3rd day, 7th day and 14th day post injection.

VIH Gene Expression Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from eyestalk and brain using TriPure Isolation reagent (Roche, Germany), following the

TABLE 1 | Primers used for recombinant plasmids construction, RT-PCR and Real-time PCR.

Primer name	Size	Sequences
SO-dsVIH (580)-F (Xba)	580 bp	5′ACTGTCTAGAGATTTTTGTTCCTGTTCTGCATC 3′
SO-dsVIH (580)-R (Hin)		5′ATCGAAGCTTAGAAATGTCTCCTATTGGTGAGT 3′
SO-dsVIH-F (Xho)	380 bp	5′ATCGCTCGAGGATTTTTGTTCCTGTTCTGCATC 3′
SO-dsVIH-R2 (Hin)		5′ATCGAAGCTTACCCATTGCATCAGTTCCTTC 3′
dsEGFP-F (Xba)	570 bp	5′ACTGTCTAGAATGGTGAGCAAGGGCGAGGA 3′
dsEGFP-R (Hin)		5′ATCGAAGCTTGCCGATGGGGGTGTTCTGCT3′
dsEGFP-F (XhoI)	380 bp	5′ATCGCTCGAGATGGTGAGCAAGGGCGAGGA 3′
dsEGFP-R2 (Hin)		5′ATCGAAGCTTTTCAGCTCGATGCGGTTCACC 3′
β-actin-F	150 bp	5'GAGCGAGAAATCGTTCGTGACAT3'
β-actin-R		5'CCCATGGTGATGACCTGGCCGT3'
VIHq-F	153 bp	5'CACGTGGTGCATCAGCGCGA 3'
VIHq-R		5'GTACCGTCGTCAGCATGAGGGCG3'
PGE specific F:	166 bp	5'GCTGCTCGGTGTGGGTTTCGGT3'
PGE specific R:		5'GCAGCAAAATGGGCATGTCTGGTAC3'
ESULT-F	256 bp	5' GCGTGGCAGAAGAGGCACCA 3'
ESULT-R		5' TCCAGTCTCCCGTCTTGCCCT 3'

manufacturer's protocol, and kept at -80°C until use. The purity and quantity of RNA were measured by using a nanodrop spectrophotometer. Total RNA (2 μg) were synthesized the first-strand cDNA synthesis using QuantiNova Reverse Transcription Kit (Qiagen, Germany), following the manufacturer's protocol. To determine the inhibition effect of dsRNA-VIH and dsRNA-EGFP, the *VIH* gene expression was performed using VIHq-F and VIHq-R primers (**Table 1**). Thermocycling condition was set as follows: One cycle at 95°C for 5 minutes followed by 30 seconds at 94°C, 45 seconds at 55°C, and 30 seconds at 72°C for 35 cycles and a final extension of 10 minutes at 72°C. The PCR product was analyzed by electrophoresis with 1.5% agarose gels. The amplification of *β-actin* was used as internal control.

Effect of DsRNA-VIH on Ovarian Maturation Determining by Gonad Somatic Index (GSI) and Histological Analysis

Ninety crabs were divided equally into 3 groups (n = 30 each); (1) Vehicle control group in which the animals were administered with 100 μl of 0.9% normal saline (NSS); (2) dsRNA-VIH groups in which the animals were administered with 100 μl of dsRNA-VIH at 0.6 μg/g BW dissolved in 0.9% normal saline; (3) dsRNA-EGFP groups in which the animals were administered with 100 μl of dsRNA-EGFP at 0.6 μg/g BW dissolved in 0.9% normal saline. The injections were performed at day 0 and 14 of the experimental period, and ten crabs from each group were randomly collected and sacrificed at day 0, 14 and 28. Gonadal development was examined, based on external morphology (Stewart et al., 2007; Islam et al., 2010; Ghazali et al., 2017; Hidir et al., 2018) and routine histology (Saetan et al., 2017). To validate the histological differences between groups, the numbers of Oc1-Oc4 taken from ovary samples of day 0, 14 and 28 were randomly selected for four fields of non-consecutive sections (at a magnification of x200) taken from each crab (n=3). The sections were viewed and photographed under a Leica compound microscope equipped with a digital camera (Leica, Germany). Data on gonadosomatic index (GSI) was collected following the previous described protocol (Tinikul et al., 2016; Saetan et al., 2017). The eyestalk, brain, and ventral nerve cord (VNC) were collected for subsequent molecular analysis and the hemolymph was collected for Vn level measurement.

Standard Preparation of *S. olivacea* Vn

Different stages of *S. olivacea* ovaries (5 crabs each stage) were homogenized in 0.5 M Tris-HCl buffer containing 0.5 M NaCl, pH 7.5, at 4°C. Each homogenate was then centrifuged at 10,000 x g, for 30 min at 4°C, and the supernatant was retained for further purification. Western blot was used to test the specificity of anti-Vn which was raised against the *M. rosenbergii* Vn (anti-MrVn) in our laboratory (Soonklang et al., 2012). The 30 μg of ovarian total protein from stage 1 to stage 4 ovaries of ovary were loaded and run through the 12% tris-glycine gels for SDS polyacrylamide gel electrophoresis. Separated proteins were transferred to 0.45 μm nitrocellulose membrane and the membranes were blocked in 5% skim milk diluted in 0.1 M TBS containing 0.1% tween 20 (TBST) for 1 h and then were incubated in anti-MrVn (1:1000) diluted in blocking solution at 4°C for an overnight. After washing, the membranes were probed with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000) (Abcam, USA) at room temperature for 1 h. The immunoreactive protein bands were visualized by adding the ECL chemiluminescence substrate (Thermo Fisher Scientific Inc., Pittsburgh, PA) and all bands were finally visualized by Amersham Imager (GE Healthcare, Sweden).

To separate the Vn and use as standard in ELISA, the early- and late-stage 4 ovarian extracts were pooled and concentrated by ultrafiltration through the molecular weight-cut off concentrator (Ultra-30kDa, Amicon), followed by a final buffer exchange with 50-mM Tris-HCl buffer, pH 7.5, at 4 °C. The concentrated extract was then fractionated by fast protein liquid chromatography (FPLC) (GE Healthcare, Sweden) on an ion exchange column (HiScreen^{TM} Q HP columns, 100mm x 7.7 mm i.d., GE Healthcare, Sweden) equilibrated with 50-mM Tris-HCl buffer, pH 7.5, as described by (Chen and Kuo, 1998). The percentage of solution A (Tris-HCl) was gradually decreased from 100 to 0, while the percentage of solution B, elution buffer (Tris-HCl + 1N NaCl) was gradually increased from 0 to 100 at a flow rate of 1 mL/min, for 35 min with absorbencies monitored at 280 nm and 474 nm (Chen and Kuo, 1998). Presence of the *S. olivacea* Vn in each fraction was tested by dot blot. One hundred nanogram of each fraction collected from 22nd to 28th min was dotted onto a 0.45 μm PVDF membrane (Merck, Darmstadt, Germany). The membranes were blocked for non-specificity by incubating with 5% skim milk in TBST for 1 h at room temperature. The membranes were then incubated with anti-MrVn, at a 1:1000 dilution, for overnight at 4°C. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam, USA) was applied, at a 1:5,000 dilution, for 1 h at room temperature. An enhanced chemiluminescence kit (Thermo Fisher Scientific Inc., Pittsburgh, PA) was used to develop the membranes and the result was detected and photographed by Amersham Imager (GE Healthcare, Sweden).

Effect of DsRNA-VIH on Ovarian Maturation Determining by Vn Level in Hemolymph

The hemolymph Vn level of female *S. olivacea* was determined by an indirect ELISA (Tinikul et al., 2016; Saetan et al., 2017) with some modifications. Hemolymph was collected from the base of the fifth walking leg and transferred into a vial containing anticoagulant solution [0.45 M NaCl, 0.1 M glucose, 30 mM tri-sodium citrate, 26 mM citric acid and 10 mM EDTA (pH4.6)] at equal volume as hemolymph. The mixture was separated by centrifugation at 9000 x g, 4°C, 10 min and the supernatants were frozen at -20°C. One hundred microliters of all supernatant, and our purified Vn with known concentration, were diluted in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and coated on 96 well ELISA plate for overnight at 4°C. Each well was subsequently washed three times with 0.01 M PBS containing 0.05% tween 20 (PBST) and blocked non-specific binding with 0.25% bovine serum albumin (BSA) in PBST, at 37°C for 30 minutes. The primary antibody (anti-MrVn) diluted at 1:2000 in blocking buffer was

applied and incubated at 37°C for 2 h. The goat anti-rabbit IgG-HRP (Abcam, USA) diluted at 1:10000 in blocking buffer was added to each well and incubated at 37°C for 1 h. The plate was then washed in triplicate and the color was developed by adding TMB substrate for 15 min. The reaction was stopped by adding 1 N HCl. Then, the absorbance was measured by microplate spectrophotometer (Thermo Fisher, USA) at a wavelength of 450 nm. Calculation of the Vn level of each sample was based on the standard curve of purified Vn. Determination of hemolymph Vn level was performed in triplicate.

Relative Abundance of Reproductive-Related Genes Expression Following DsRNA-VIH Administration

To study the effect of dsRNA-VIH on gene expression, the *S. olivacea* reproduction genes were selected from previous reports (Kornthong et al., 2014; Duangprom et al., 2018). The brain and ventral nerve cord (VNC) were collected from day 0, 7 and 14 of the experimental periods. The cDNAs were then prepared as described in the previous section of this manuscript. Real-time PCR for amplification of reproduction related genes, including *S. olivacea prostaglandin E synthase* (*Scyol-PGES)* and *S. olivacea estrogen sulfotransferase (Scyol-ESULT)* was performed in duplicate for each sample (n = 10 per group). Amplifications were conducted on a CFX-96 (Bio-Rad, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad, USA). *Scyol-PGES* and *Scyol-ESULT* primers were shown in **Table 1**. Relative expression of *Scyol-PGES* and *Scyol-ESULT* against *β-actin* expression and a variance between individual treatments were performed, calculated, and statistically analyzed followed the methods described in (Kornthong et al., 2013; Kornthong et al., 2014; Duangprom et al., 2017; Duangprom et al., 2018).

Statistical Analysis

Values of the GSI, hemolymph Vn and relative gene expression levels of *Scyol-PGES* and *Scyol-ESULT* genes to *β*-actin, in different sample groups were tested by one-way analysis of variance (ANOVA) followed by Tukey test. All tests were performed using GraphPad Prism 9.0 software for Windows (GraphPad Software, USA). Mean values were identified as significantly different if the *p*-value was less than 0.05.

RESULTS

Determination of DsRNA-VIH and DsRNA-EGFP Production

The coding sequence of *S. olivacea VIH* (MH882453; nucleotide 167 to 537) were used as the template for dsRNA-VIH production. To determine the quality of dsRNA, a large fragment of the dsRNA-VIH (380 bp) genes and dsRNA-EGFP (380 bp) were amplified using RT-PCR. After purification, the non-digested, RNase A-digested and RNase III-digested dsRNAs were visualized by agarose gel electrophoresis (**Figure 1**). The non-digested dsRNA-VIH and dsRNA-EGFP were shown approximately 1200 bp in lane 1 and 4 (**Figure 1**), while those of RNase A-digested dsRNA-VIH and dsRNA-EGFP were shown approximately 600 bp in lane 2 and 5 (**Figure 1**). Lastly, there was no band detected for both RNase III-digested dsRNA-VIH and dsRNA-EGFP (**Figure 1**, lane 3 and 6).

Dose-Dependent Study of the DsRNA Induced *VIH* Knockdown and Prolonged Inhibitory Effect of *VIH* Knockdown

To determine the potent dose of dsRNA-VIH in sequestering the *S. olivacea* VIH expression, we administrated different doses of 0.2, 0.4, and 0.6 μg/g BW for the dsRNA-VIH and those of 0.2, 0.4 and 0.6 μg/g BW for dsRNA-EGFP and vehicle control (NSS) for injecting the female mud crabs. At 24 h post-injection, the VIH expressions of eyestalk and brain were inhibited by the 0.6 μg/g BW of dsRNA-VIH (**Figures 2A, B**). On the contrary, 0.2 and 0.4 μg/g BW of dsRNA-VIH failed to inhibit the VIH expressions in both organs (**Figures 2A, B**). According to these, the 0.6 μg/g BW of dsRNA-VIH was retained for further experiments. The selected dose of 0.6 μg/g BW dsRNA-VIH successfully displayed the prolonged inhibitory effect of *VIH* knockdown in the eyestalk for 3-, 7- and 14-days post-injection (**Figure 3**). However, at day 14 one of three crab was able to express VIH again while the remaining two ones were not (**Figure 3**). The irrelevant dsRNA-EGFP and vehicle control (NSS) had no effect on VIH inhibition (**Figures 2A, B**, and **3**).

Validation of the Anti-MrVn and Standard Vn Preparation

To determine the antibody reactivity between anti-MrVn and *S. olivacea* Vn, the western blot analysis was performed. The anti-MrVn was able to depict 2 molecular subunits of Vn comprising the 85 kDa and 105 kDa respectively. In stage 1 of ovary, the Vn subunits were not appeared. In stage 2-3 of ovary, the two subunits of Vn were slightly observed while they were mostly

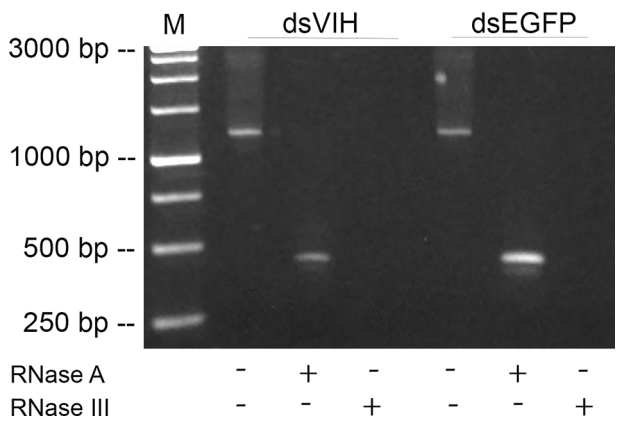

FIGURE 1 | Visualization of the dsRNA production. Lane M was the 3kb DNA ladder marker. Lanes 1 and 4 were shown approximately 1200 bp of non-digested dsRNA-VIH and dsRNA-EGFP. Lanes 2 and 5 were shown approximately 600 bp of RNase A-digested dsRNA-VIH and dsRNA-EGFP. Lanes 3 and 6 were the RNase III-digested dsRNA-VIH and dsRNA-EGFP revealed no product detected.

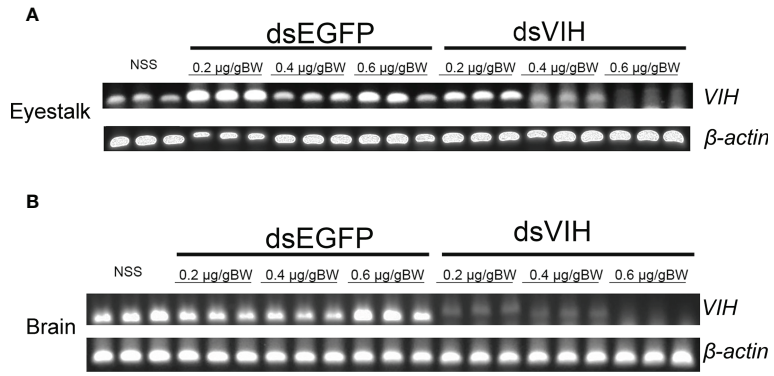

FIGURE 2 | Dose-dependent effects of dsRNA-VIH, dsRNA-EGFP, and vehicle control (NSS) in sequestering the *VIH* gene expression in **(A)** eyestalk and **(B)** brain. In both organs, the most potent dose of dsRNA-VIH to inhibit the *VIH* gene expression was 0.6 μg/g BW. No inhibitory effect on *VIH* gene expression was observed by the injection of either the dsRNA-EGFP and vehicle control (NSS).

intensely expressed in the stage 4 of ovary (**Figure 4A**). Fractionation of the *S. olivacea* Vn by ion exchange FPLC and dot blot with MrVn antibody revealed the immunoreactivity in the fractions of 24-28 min (**Figure 4B**) Therefore, these fractions were combined, measured the concentration and used as standard Vn in ELISA.

Effect of DsRNA-VIH on Ovarian Development of the *S. olivacea*

At day 14 and day 28 post injection, the ovarian development was determined by GSI value and hemolymph Vn level. The dsRNA-VIH treated crabs significantly produced higher GSI compared with the control group (**Figure 5A**). Moreover, the

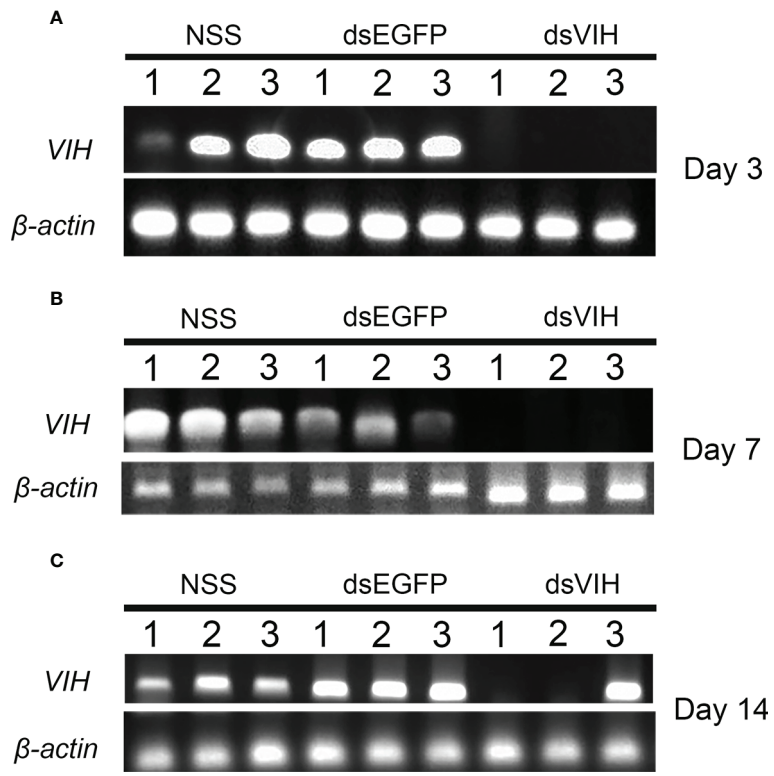

FIGURE 3 | Prolonged effect of the 0.6 μg/g BW of dsRNA-VIH on inhibiting the eyestalk *VIH* gene expression at day 3 **(A)**, day 7 **(B)** and day 14 **(C)** post-injection. No inhibitory effect on *VIH* gene expression was observed by the injection of either the dsRNA-EGFP and vehicle control (NSS).

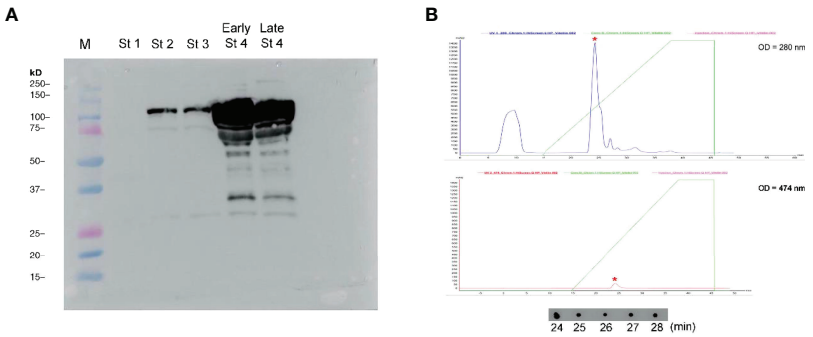

FIGURE 4 | **(A)** Validation of anti-MrVn in probing the Vn subunits of the *S. olivacea*, lane M referred to molecular ladder, St1-Late St4 referred to the ovary samples taken from stage 1 to late stage 4, respectively. **(B)** Chromatogram of ovarian extract of *S. olivacea* as fractionated by ion exchange FPLC on a HiScreen™ Q HP columns and monitored at wavelengths of 280 nm and 474 nm, respectively. Asterisk showed the immunoreactive peak whose fractions (24-28 min) were confirmed by dot blot with anti-MrVn.

hemolymph Vn level of the dsRNA-VIH treated crabs at day was significantly greater than that of the control crabs at the same day of comparison (**Figure 5B**).

Moreover, the histology of ovaries was observed at day 0, day 14 and day 28 of the experimental period. The histology of *S. olivacea* ovary, including oogonia and four steps of developing oocyte (Oc1-Oc4) was analyzed according to the previous reports (Stewart et al., 2007; Saetan et al., 2017). At day 0, ovaries in dsRNA-VIH and 0.9% normal saline (NSS) injected groups, showed three steps of oocyte (Oc1-Oc3) and oogonia (Og). The number of each stage of oocyte was similar between these two groups. (**Figures 6A, B**). At day 14, the NSS injected group showed Oc1-Oc3 in the ovaries, while the dsRNA-VIH injected group showed Oc4 that are fully mature oocyte (**Figures 6C, D**). At day 28, the NSS injected group showed Oc4 and some of Oc1-Oc2 in the ovaries, while the dsRNA-VIH injected group showed only Oc4 in the ovaries (**Figures 6E, F**). The number of Oc4 observed in the ovaries of dsRNA-VIH injected groups were significantly higher than those observed in the ovaries of females injected with NSS on the day 14, and 28 (**Figures 6G–I**).

Effect of DsRNA-VIH on the Upregulation of Reproductive-Related Genes

To study the effect of dsRNA-VIH, relative abundances of the *S. olivacea prostaglandin E synthase* (*Scyol-PGES)* and *S. olivacea estrogen sulfotransferase (Scyol-ESULT)* transcripts in brain and ventral nerve cord (VNC) were validated. The relative abundances of these two genes were normalized using abundance of *β-actin* transcript for both treatment and control groups. After dsRNA-VIH administration at day 7, relative abundance of the *Scyol-PGES* in the brain and VNC was greater when compared with those expressed in control crabs (**Figures 7A, B**). The relative abundance of *Scyol-ESULT* of dsRNA-VIH group in the brain and VNC were also significantly greater than those of control group at day 7 and day 14 post-injection (**Figures 7C, D**).

DISCUSSION

In this study, we demonstrated the use of dsRNA-VIH to promote ovarian development in the *Scylla olivacea*. As the reproduction of crustaceans was naturally promoted by groups of neurotransmitter (NT)/neurohormone (NH) i.e., the

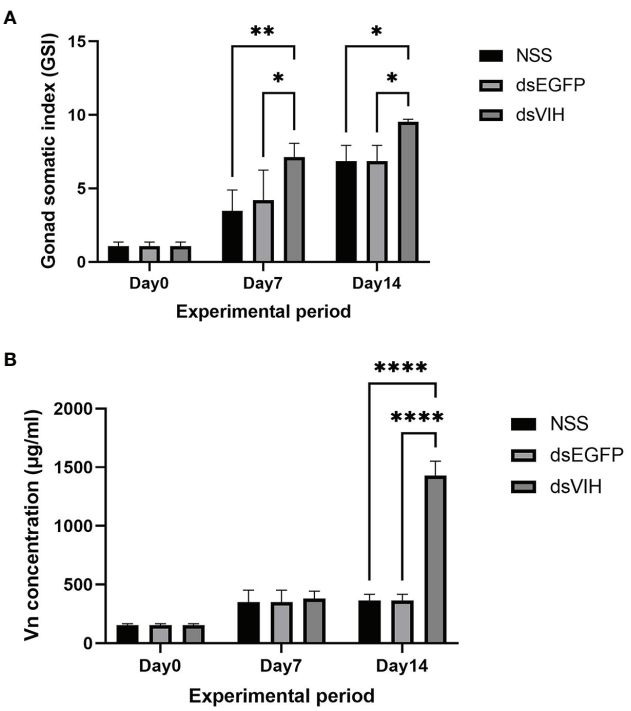

FIGURE 5 | The effects of dsRNA-VIH on **(A)** GSI and **(B)** hemolymph Vn level of the dsRNA-VIH treated crabs and control crabs at day 14 and day 28 post-injection. Bars represented the standard error of the mean (SEM). *$p < 0.05$ **$p < 0.005$, and ****$p < 0.0001$.

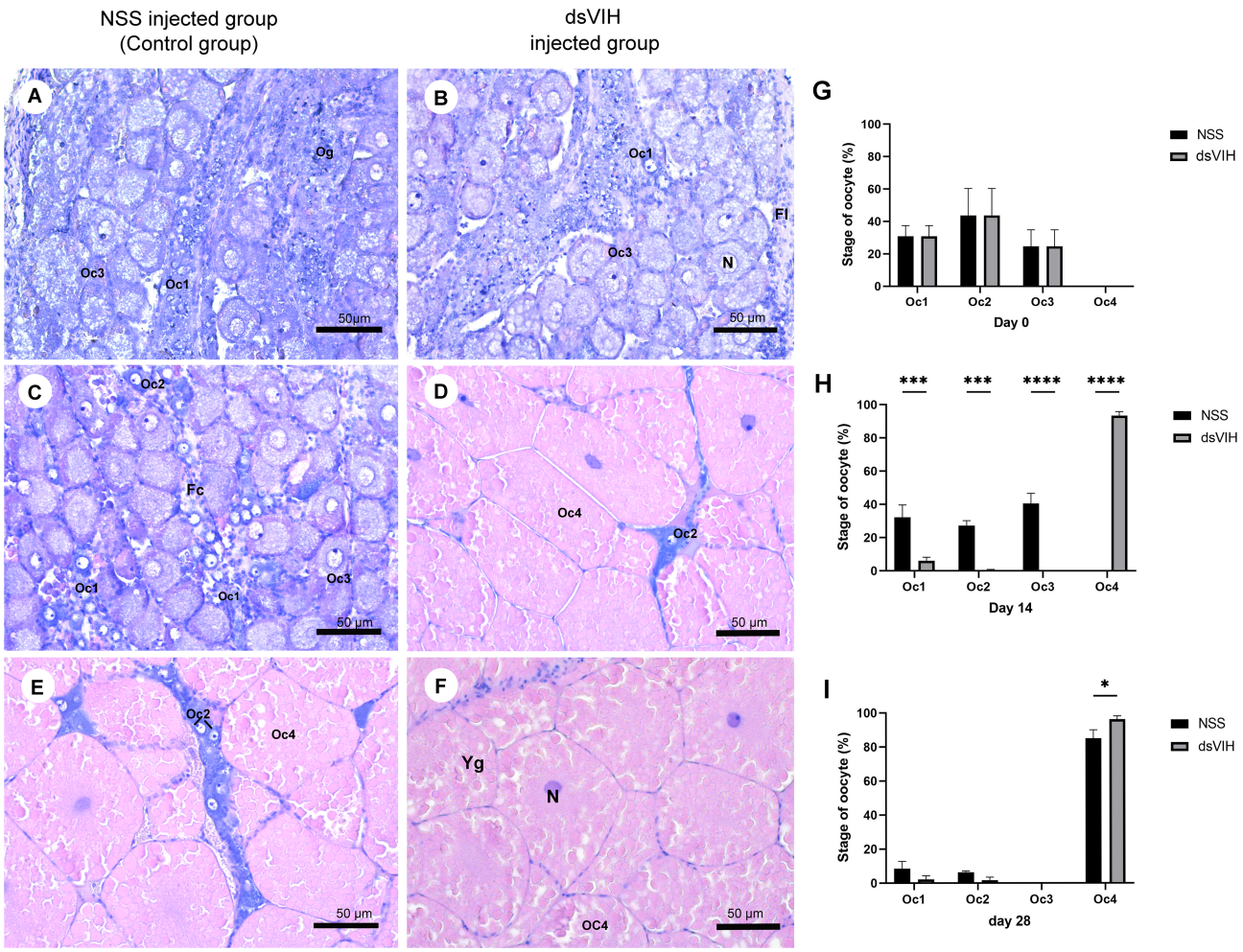

FIGURE 6 | Histological analysis of the ovaries of dsRNA-VIH and NSS treated crabs. A representative sample from each group is shown. **(A, B)** At day 0, ovaries in both group showed oogonia (Og) and three steps of oocyte (Oc1-Oc3) **(C, D)** At day 14, NSS injected group and dsRNA-VIH injected group showed Oc1 that clearly displayed euchromatic nucleus, Oc2 that was oval and was surrounded by follicular cells, Oc3 that was oval and it contained mostly euchromatin and prominent nucleolus with small lipid droplets in cytoplasm, and Oc4 that was fully mature oocyte with large spherical ovoid shape and heavy lipid droplets in cytoplasm. **(E, F)** At day 28, the dsRNA-VIH injected group showed more Oc4 than that of the NSS injected group while the latter also showed some pre-mature stage (Oc1-Oc2). **(G–I)** Quantification of the oocyte stage on day 0, 14 and 28. Asterisks indicate significant differences compared to the NSS injected group and dsRNA-VIH injected group with $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Oocyte developmental features are indicated as Yg, yolk granule oocyte; Oc, oocyte, Og, oogania; Fc, foccicle cell; N, nucleus.

serotonin (Tinikul et al., 2016), the GnRH-like peptide (Guan et al., 2014; Bao et al., 2015), the RPCH (Zeng et al., 2016; Jayasankar et al., 2020) while it was together suppressed by some particular NT/NH, i.e., the dopamine (Alfaro-Montoya et al., 2004; Tinikul et al., 2016), oxytocin/vasopressin-like peptide (Saetan et al., 2018; Lin et al., 2020; Saetan et al., 2021) and the VIH (Kang et al., 2021). In the mud crab, the *VIH* gene was characterized and demonstrated its expression in the eyestalk, brain, and ventral nerve cord (VNC) (Kornthong et al., 2019) while this gene expressed specifically in the eyestalk of the *S. paramamosain* (Liu et al., 2018). The expression of *VIH* gene detected in eyestalk, brain, VNC as well as in other peripheral organs was also mentioned in the *L. vannamei* (Chen et al., 2014). Since there is no direct evidence of VIH in inhibiting vitellogenesis in this species, however, the VIH inhibited hepatopancreas vitellogenesis was reported in the *L. vannamei* (Chen et al., 2014). Referring the common practice for enhancing crustacean reproduction, as reported in the *L. vannamei* (Chen et al., 2014; Kang et al., 2014; Feijo et al., 2016), *Penaeus monodon* (Treerattrakool et al., 2008) and *Barytelphusa lugubris* (Rana, 2018), is the unilateral eyestalk ablation which resembled the removal of eyestalk GIH/VIH synthesis and release, this practice may cause significant hormonal imbalance and severe injury to the animal that affect the quantity and quality of larvae (Okumura, 2007; Uawisetwathana et al., 2011). Hence, based on the *S. olivacea* VIH sequence (GenBank accession no. MH882453.1), we successfully produced the dsRNA-VIH with expectation to silence the endogenous

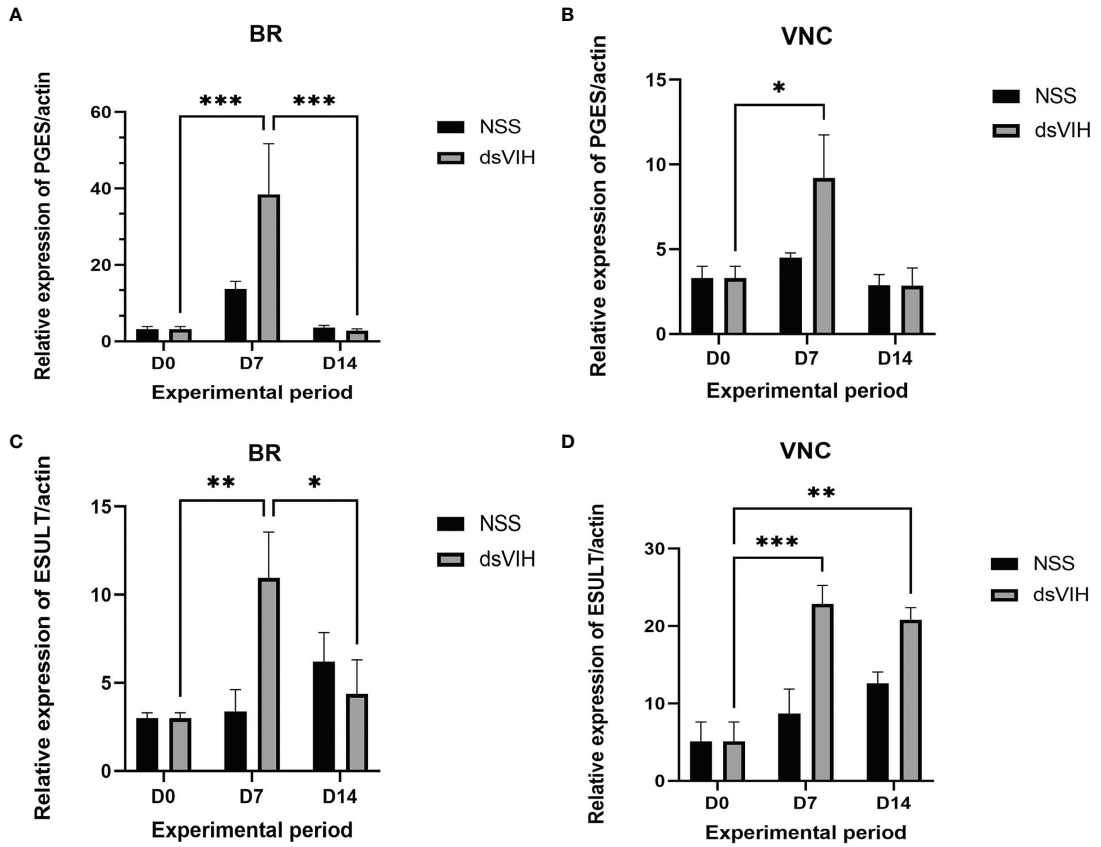

FIGURE 7 | Effect of dsRNA-VIH on the *Scyol-PGES* and *Scyol-ESULT* transcript abundances using quantitative RT-PCR. **(A, B)** The histograms showed the level of *Scyol-PGES* which significantly expressed higher in brain and VNC after dsRNA-VIH administration for 7 days. **(C, D)** The histograms showed the level of *Scyol-ESULT* which significantly expressed higher in brain and VNC after dsRNA-VIH administration for 7 and 14 days. BR, brain; VNC, ventral nerve cord. Bars represent the standard error of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.005$ and $^{***}p < 0.0005$.

eyestalk and brain *VIH* expression that in turn helped promoting mud crab reproduction without torturing the animals and hence, can sustainably employ them in aquaculture.

As the *VIH* in *S. olivacea* was demonstrated to express in the eyestalk, brain and VNC, and the treatment of dopamine was found to promote the eyestalk *VIH* gene expression in this species (Kornthong et al., 2019). In this study, injection of dsRNA-VIH potentially inhibited the eyestalk and brain *VIH* transcripts for 24 h, with prolonged effect for 3 to 14 days for eyestalk *VIH* suppression. In *P. monodon*, the dsRNA-GIH, once being applied into the animal, could suppress the *GIH* gene expression for 24 h to 60 h (Sukumaran et al., 2017). The use of RNAi against the *GIH* in the same species displayed longer suppressing effect up to 30 days post-injection (Treerattrakool et al., 2011). Moreover, in *L. vannamei* injection of the various dsRNA-VIHs to the animals could suppress multiple VIH candidates for 10 to 30 days (Kang et al., 2019; Kang et al., 2021). Since the suppressing effect of dsRNA-VIH on its target gene was varied among species, however, it could say that the dsRNA-VIH produced in this study was efficient to silence the *VIH* transcript in mud crab.

The effect of dsRNA-VIH on the *VTG* expression was assumed by the stage of ovarian maturation and level of hemolymph Vn. The VTG, a key protein of yolk protein, has been used as indicator for determining ovarian maturation in many crustaceans (Soonklang et al., 2012; Guan et al., 2014), in this study, we fractionated the Vn from crab ovary and used it as standard protein in our Vn indirect-ELISA (Tinikul et al., 2016; Saetan et al., 2017). However, the anti-MrVn which was produced against the Vn of *Macrobrachium rosenbergii* was used (Soonklang et al., 2012). We, therefore, validated the antibody specificity by probing it against the *S. olivacea* ovarian proteins. The anti-MrVn was able to depict 2 Vn subunits which sizes were about 85 and 111 kDa and this result corresponded with previous report of 2 Vn subunits in the *S. olivacea* (Chen et al., 2007). Moreover, the well-organized standard curve of Vn in ELISA (data not shown) also conformed the ability of anti-MrVn to effectively probe the mud crab Vn in our study.

A single dose of 0.6 μg/g BW of dsRNA-VIH was enough to suppress *VIH* gene expression, without the repeat injection of dsRNA-VIH until day 14 after injection. Expectedly, the dsRNA-VIH injected crabs significantly had higher GSI value at day 28

with ovarian histology showing more mature oocytes. As similar with Vn level, the dsRNA-VIH injected crabs significantly had higher Vn level in hemolymph at day 28 compared to the control group. This was in concerted with other studies that the dsRNA-VIH injection can trigger vitellogenesis and ovarian maturation for example, in the *P. monodon* (Treerattrakool et al., 2008); *L. vannamei* (Feijo et al., 2016) and *M. rosenbergii* (Cohen et al., 2021). In contrast, the dsRNA-VIH injected in *L. vannamei* showed no significant elevation of either hemolymph Vg or ovarian Vg transcripts (Kang et al., 2019; Kang et al., 2021), while the authors suggested that the synthesized VIH was probably kept in the eyestalk and released during the time the *VIH* gene was silenced (Kang et al., 2021). In addition, we purposed that the dsRNA-VIH itself might not fully exert the stimulatory effects on *S. olivacea* reproduction in this time since other unidentified factors might probably affect. Removal of VIH by dsRNA-VIH might change their expression thresholds that in turn possibly decelerated some reproductive parameters in the *S. olivacea*. However, combination of dsRNA-VIH with other known stimulating agents, i.e., serotonin (Tinikul et al., 2016); or spiperone (Alfaro-Montoya et al., 2004) might be more effective in promoting female mud crab reproduction.

The *S. olivacea prostaglandin E synthase* (*Scyol-PGES)* and *S. olivacea estrogen sulfotransferase (Scyol-ESULT)* were identified in our previous studies (Kornthong et al., 2014; Duangprom et al., 2018). The *PGES* functioned in biogenesis of PGE_2 which was reported in participating ovarian maturation in many crustaceans (Sarojini et al., 1988; Reddy et al., 2004; Sumpownon et al., 2015). The *Scyol-PGES* was highly expressed in stage 4 ovary and abundantly found in the small- and medium-sized neurons of the brain (Duangprom et al., 2018). In this study, the absence of *VIH* by our dsRNA-VIH administration enhanced the expression of *Scyol-PGES* in brain and VNC at day 7 post-injection. These findings provide the first insight into a relationship between the *VIH* and *Scyol-PGES* in a crustacean related with ovarian maturation. As well, the dsRNA-VIH could enhance expression of the *Scyol-ESULT* which presumably functioned in solubility of estradiol in hemolymph (Cole et al., 2010). The ESULT plays an essential role by adding a sulfate group to estradiol which play role in gonadal maturation in the *S. serrata* and *P. monodon* (Quinitio et al., 1994; Warrier et al., 2001). Therefore, this gene has also been a potential candidate for reproduction in mud crab and the *Scyol-ESULT* expressed in the brain and VNC was negatively regulated by the *VIH*. Since the direct connection between *VIH* and these two genes was not reported yet in any crustaceans, however, it can be assumed that the lack of VIH by the dsRNA-VIH turned other stimulating molecules, i.e., serotonin, to positively regulated the expression of these two genes, and probably of others. For example, the serotonin injection was able to increase the *Scyol-ESULT* expression in both brain and ovary of the mud crab (Kornthong et al., 2014). Since the serotonin injection had no effect on the *VIH* expression in the same species (Kornthong et al., 2019), we could therefore not set any direct link between these two molecules in the mud crab.

In conclusion, the present study was successful in synthesizing the dsRNA-VIH for applying in the *S. olivacea*. The dsRNA-VIH was proved to last inhibit the VIH synthesis in eyestalk and brain of the mud crabs. Injection of the dsRNA-VIH at the dose of 0.6 μg/gram body weight to the crabs could promote many reproductive parameters as well as the expression of *Scyol-PGES* and *Scyol-ESULT* which reflected the effectiveness of dsRNA-VIH. However, to get better reproductive stimulation, the combination of the dsRNA-VIH with other stimulating neuropeptides/neurohormones was suggested.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SD, TP, SS, JS, MT and NK designed experiment, analyzed data and wrote manuscript. SD, SS, TP, PSu, and MT performed experiments. SD, JS, PSo, and NK conceptual designed the experiment, provided experimental tools and made manuscript revisions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.880235/full#supplementary-material

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