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Potent ovarian development as being stimulated by cocktail hormone in the female *Scylla olivacea*

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The mud crab *Scylla olivacea* is widely cultured for its economic value, but reproduction issues limit its production. Vitellogenesis-inhibiting hormone (VIH), serotonin (5-HT), and gonadotropin-releasing hormone (GnRH) are important neurohormones that control reproduction in crustaceans. Mimicking crab hormone stimulation during reproduction has scarcely been reported. Comparison of the single hormone and multiple hormone approaches to crab hormonal control in *S. olivacea* is limited. *In situ* hybridization showed that injection of dsRNA-VIH could abolish its gene expression in neuronal clusters of female *S. olivacea* eyestalks, potentially reducing its inhibitory effects on ovarian maturation. This was confirmed by assessing the ovarian gonadosomatic index (GSI), hemolymph vitellogenin (Vg), an indicator of vitellogenesis, and gonad histology using dsRNA-VIH and 5-HT/GnRH combinations. Based on our findings, we demonstrated that administration of dsRNA-VIH significantly increased the gonadosomatic index (GSI) on days 14 and 28 post-treatment. The combination cocktail, however, consisting of 5-HT + GnRH + dsRNA-VIH on days 14 and 28, and GnRH + dsRNA-VIH on day 28, was the most efficacious in increasing GSI and enhancing crab ovarian maturation. Upregulation of hemolymph Vg levels was observed solely on the 28th day following treatment with dsRNA-VIH, 5-HT + GnRH + dsRNA-VIH, and GnRH + dsRNA-VIH. Differential gene expression analysis using quantitative RNA-sequencing of the neural tissues (brain and ventral nerve cord), revealed a significant upregulation of certain receptors (*5-HTR*, *GnRHR*, *LHR*, and *FSHR*), neuropeptides (*sNPF*, *NPF1*, *NPF2*, *SIFamide*, *AKH/Crz*, *CHH*, and *RPCH*), downstream reproductive-related genes (*FAMeT*, *ESULT*, *progesterone-like protein*), and prostanoid-related genes

(*phospholipase A and C, COX, Thromboxane A synthase, prostaglandin D, E, and F synthases*) following treatment, particularly dsRNA-VIH + GnRH and/or 5-HT-injected individuals. Upregulation of prostaglandin E synthase and estrogen sulfotransferase genes was confirmed by real-time PCR. Since the construction and propagation of dsRNA-VIH is costly, its lower dose application supplemented with synthetic GnRH and/or 5-HT may be an alternative approach to ensure that female *S. olivacea* attain sufficient reproductive fecundity in aquaculture. Furthermore, we propose that the administration of multiple hormones in crabs may better emulate the physiological conditions of crustaceans in their natural habitat.

KEYWORDS

dsRNA-VIH, GnRH, 5-HT, ovarian maturation, *S. olivacea*, sustainable development goals (SDGs)

1 Introduction

Comparative transcriptome analysis of the nervous system and gonads of aquaculture crustaceans at different reproductive stages has been beneficial for gaining a better understanding of their associated molecular components (Uawisetwathana et al., 2011; Suwansa-Ard et al., 2016). With regard to the neuroendocrine control of reproduction, several neurohormones and neurohormone-like molecules have been identified, which may now be explored to manipulate crustaceans in aquaculture (Alfaro-Montoya et al., 2019). However, a defined neuroendocrine pathway that facilitates crustacean reproduction has not yet been established.

The mud crab, *Scylla olivacea*, is one of the most valuable crustacean species, with high prices and demand in international markets, and lives mostly in the mangrove ecosystem of tropical and subtropical waters of the Indo Pacific region. Considering the high market demand for this species, the culture system must prioritize the sustainable long-term well-being of its population. In general, economic crustacean aquaculture practices always seek a more efficient approach to either enhance offspring production or sustainably retain captive broodstock. The technique of eyestalk ablation has been used to accelerate ovarian maturation in various crustacean species, including the Pacific white shrimp (Sultana et al., 2023), ridgetail white prawn (Jia et al., 2023), and mud crab (Muhd-Farouk et al., 2019); however, this practice has been criticized for being unethical (Tinikul et al., 2017). Recent practices have used the aforementioned key reproductive molecules, which are thought to trigger pathways that stimulate reproduction (Jayasankar et al., 2020). This study used the mud crab *S. olivacea*, an economically important crab in many areas of the world, including Thailand, as a model to demonstrate the effects of selected neurotransmitters and neurohormones (e.g., serotonin, gonadotropin-releasing hormone), as well as double-stranded RNA against the vitellogenin-inhibiting hormone (dsRNA-VIH). These outcomes were proposed to enhance the reproduction of mud crab aquaculture.

Serotonin (5-hydroxytryptamine; 5-HT) is considered a crucial neurotransmitter because substantial evidence indicates its role in promoting the reproduction of crustaceans. The presence of 5-HT in the central nervous system and/or gonads has been reported in *S. olivacea* (Khornchatrri et al., 2015), *Penaeus monodon* (Wongprasert et al., 2006), *Macrobrachium rosenbergii* (Soonthornsumrith et al., 2018), and *Portunus pelagicus* (Nakeim et al., 2020). Administration of 5-HT to numerous species of crustaceans, both *in vivo* and *in vitro*, demonstrated ovarian maturation (Meeratana et al., 2006; Wongprasert et al., 2006; Soonthornsumrith et al., 2018) and stimulation of ovarian steroid release (Soonthornsumrith et al., 2018). In addition, 5-HT positively modulates the mRNA expression of the red pigment concentrating hormone (*RPCH*) and crustacean hyperglycemic hormone (*CHH*) in *S. olivacea* (Kornthong et al., 2013; Duangprom et al., 2017).

A gonadotropin-releasing hormone (GnRH)-like peptide in crustaceans is difficult to identify; however, a single putative GnRH-like peptide was found in *M. rosenbergii* following *de novo* transcriptome assembly and analysis (Suwansa-Ard et al., 2016). Its mature peptide sequence contains 14 amino acids (pQQHFRTSHFRPDNVamide), which is distinct from other known GnRH core peptides. In addition, this GnRH had no effect on ovarian maturation when injected into prawns whereas the lamprey (I) GnRH-III injection stimulated ovarian maturation in *M. rosenbergii* (Ngernsoungnern et al., 2009; Suwansa-Ard et al., 2016). In crayfish, *Procambarus clarkii*, it was reported that GnRH contains 11 amino acids (pQSYHFSLGWKP-amide), and its effects were increases in the gonadosomatic index (GSI) and ovarian vitellin (Guan et al., 2014). In addition, a GnRH-like peptide was predicted in numerous species of crustaceans by immunohistochemistry using antibodies generated against tunicate (t) GnRH-I-, IGnRH-III-, and octopus (oct) GnRH-like peptides (Ngernsoungnern et al., 2008; Tinikul et al., 2011; Saetan et al., 2013). In addition, the administration of synthetic IGnRH-I, IGnRH-III, and octGnRH to various crustacean species has been positively correlated with ovarian maturation (Ngernsoungnern

et al., 2009; Saetan et al., 2013; Tinikul et al., 2014). However, to date, there is no evidence of GnRH function in *S. olivacea*.

Crustacean VIH is known to inhibit reproduction. In *P. monodon*, immunohistochemical localization of a *Pem*VIH-like peptide was observed in the brain, ventral nerve cord, and commissural ganglion, whereas dsRNA-*Pem*VIH promoted ovarian vitellogenesis, suggesting that *Pem*VIH plays an inhibitory role in shrimp. The *lv*VIH identified in *Litopenaeus vannamei* belongs to the family of crustacean hyperglycemic hormones (CHHs) and is expressed in the brain and eyestalk. *lv*VIH suppresses the expression of vitellogenin in the hepatopancreas and eliminates the eyestalk ablation-induced vitellogenesis via the GC/cGMP-MAPK pathway (Chen et al., 2014; Chen et al., 2018). VIH was successfully identified in *S. olivacea* (*Scyol*-VIH). This hormone is produced in the eyestalk X-organ as well as in neuronal clusters of the brain and ventral nerve cord, as revealed by RT-PCR and *in situ* hybridization; its expression increases in response to dopamine administration (Kornthong et al., 2019). Recently, dsRNA-*Scyol*-VIH was successfully developed, as it effectively suppressed *Scyol*-VIH mRNA expression in the eyestalk (Duangprom et al., 2022). In addition, the administration of *Scyol*-VIH significantly enhanced both ovarian maturation and reproduction-related gene expression (Duangprom et al., 2022).

In the present study, we demonstrated the combined effects of 5-HT, GnRH, and VIH on vitellogenesis in *S. olivacea*. As this mud crab is considered an economically significant species, the application of this cocktail in aquaculture could lead to the development of a sustainable technique to increase *S. olivacea* production.

2 Materials and methods

2.1 Chemicals and *in vitro* synthesis of dsRNA-VIH

Serotonin (5-HT) was purchased from Sigma-Aldrich (USA). Synthetic gonadotropin-releasing hormone (GnRH: pQHWSHDWKPG-amide) lamprey III, *Petromyzon marinus*, GenBank accession no. P30948.1 was produced with 98% purity (GenScript, USA). *S. olivacea* double-stranded RNA-VIH (dsRNA-VIH) was synthesized using the method from Feijó et al. (2016) and Duangprom et al. (2022). Briefly, recombinant plasmids encoding the *Scyol*-VIH gene (GenBank accession no. MH882453) were linearized using XhoI and used as a template. dsRNAs were then generated using the SP6 RiboMAXTM Express Large-Scale RNA Production System (Promega, USA) according to the manufacturer's protocol. The remaining portion of the template was eliminated with RQ1 RNase-free DNase (Promega, USA), and dsRNA was extracted using TriPureTM Isolation reagent (Roche, Germany). The integrity of dsRNA was evaluated by using agarose gel electrophoresis, and the concentration was measured using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA).

2.2 Animals

Female *S. olivacea* were cultivated in Samut Sakhon, Thailand, at Samut Sakhon Coastal Aquaculture Research and Development Center under the Protocol Number 011/2562 approved by the Animal Care and Use Committee of Thammasat University, National Research Council of Thailand (NRCT), to ensure that animal suffering was kept to a minimum. To investigate the stimulation of ovarian maturation using a combination of hormones, experiments were carried out on mature female mud crabs during the intermolt stage (Kuballa et al., 2011; Xu et al., 2020). All crabs with an average weight of 135 g–145 g, gonad somatic index (GSI) <0.2, and a distance of 85 mm–92 mm between the two tips of the ninth spine of the anterolateral carapace (Amin-Safwan et al., 2016; Ghazali et al., 2017) were used to investigate the effect of hormone administration on ovarian maturation and reproductive gene expression.

2.3 Hormone administration

A total of 210 female *S. olivacea* were randomly assigned to seven groups, with each group comprising 30 individuals. Crabs were added to 100 µl of the solution. The doses of the tested molecules were selected based on previous studies (Saetan et al., 2013; Siangcham et al., 2013; Duangprom et al., 2022; and Tinikul et al., 2009; Tinikul et al., 2014). The treatment groups consisted of five different conditions, comprising (T1) dsRNA-VIH at 0.6 µg/g BW; (T2) 5-HT (3 µg/g BW) + dsRNA-VIH (0.2 µg/g BW); (T3) 5-HT (3 µg/g BW) + GnRH (50 ng/g BW); (T4) 5-HT (1.5 µg/g BW) + GnRH (25 ng/g BW) + dsRNA-VIH (0.1 µg/g BW), and (T5) GnRH (50 ng/g BW) + dsRNA-VIH (0.2 µg/g BW), respectively. The two control groups were administered (C1) 0.9% normal saline (NSS) and (C2) dsRNA-EGFP at 0.6 µg/g BW. The crabs were injected on days 0 and 14 post-injection, and on days 0, 14, and 28, five crabs from each group were randomly selected for hemolymph collection and subsequently sacrificed for further analysis. Following sacrifice, the central nervous system (CNS) and ovaries of each crab were obtained, and the GSI was determined based on the proportion of ovarian mass to body mass.

The brain and ventral nerve cord (VNC) were collected and frozen at –80°C for subsequent use in differential RNA-seq and quantitative real-time PCR. The eyestalk and ovary were fixed for 12 h–16 h in Davidson's fixative, rinsed, and then stored in 70% ethanol for *in situ* hybridization and histological analysis, respectively. Finally, hemolymph was obtained to quantify the vitellogenin (Vg) levels, as outlined in the subsequent section on Vg level measurement.

2.4 Gonadosomatic index (GSI) evaluation and histological examination

To determine the GSI: [ovary weight/crab body weight (BW)] * 100, the weight of each crab and ovary was measured and recorded

on days 0, 14, and 28 post-injections (Saetan et al., 2013; Kang et al., 2019). Routine histology was used to examine gonadal development cocktail hormone administration. Following sacrifice, the ovaries were fixed in Davidson's fixative for 12 h–16 h, washed, and processed using paraffin. Ovarian tissues were sectioned to a thickness of 7 μm and subsequently stained using hematoxylin (H) and eosin (E) solution (Saetan et al., 2017). To validate the histological differences between the groups, the number of oogonia (Og) and the four stages of oocyte development [oocyte step 1 (Oc1)–oocyte step 4 (Oc4)] from ovary samples collected on days 0, 14, and 28 were randomly enumerated from four fields of non-consecutive sections (at $\times 200$ magnification) taken from each crab ($n = 5$). The sections were examined and photographed using a Leica compound microscope connected to a digital camera (Leica, Germany).

2.5 *Scyol-VIH* transcript distribution by *in situ* hybridization

Following the treatment, the eyestalk was chosen as the site of interest to assess the inhibitory potential of dsRNA-VIH. The experimental design involved the selection of two groups, including the control group (NSS) and dsRNA-VIH group, to conduct *in situ* hybridization (ISH). To conduct a qualitative comparison of *Scyol-VIH* expression in the eyestalk of the dsRNA-VIH- and NSS-injected groups, *Scyol-VIH* mRNA was detected in consecutive sections. Sense and antisense probe labeling were performed using a DIG-oligonucleotide labeling kit SP6/T7 (Roche, Germany). The sections were imaged using Leica compound microscope equipped with a digital camera (Leica, Germany). The study referred to the protocol of *in situ* hybridization (ISH) in a comprehensive manner, as outlined by Duangprom et al. (2017); Duangprom et al. (2018); Kornthong et al. (2021).

2.6 Vitellogenin level evaluation by indirect enzyme-linked immunosorbent assay

In this study, the level of vitellogenin (Vg) in the hemolymph was measured using vitellin (Vn) antiserum and anti-*M. rosenbergii* Vn (anti-MrVn) using an indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Saetan et al., 2017; Tinikul et al., 2017; Duangprom et al., 2022). The specificity test for anti-MrVn was performed with *S. olivacea* ovaries using western blot analysis (Duangprom et al., 2022). Prior to sacrifice following the treatments, hemolymph samples from seven groups (T1–T5 and C1–C2) ($n = 5$ per group) were collected and mixed in equal volume ratio with an anticoagulant solution (0.45 M NaCl, 0.1 glucose, 30 mM tri-sodium citrate, 26 mM citric acid, and 10 mM EDTA at a pH of 4.6). The mixed samples were centrifuged at $7,000\times g$ at 4°C , and the supernatant was obtained to determine protein concentration using the BCA assay (Thermo Fisher, USA). Three replicates of 10

$\mu\text{g}/\text{ml}$ of protein (100 μl) from each sample were loaded into 96-well plates containing bicarbonate/carbonate-coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6) and incubated at 4°C overnight. Following washing, the plates were blocked with a solution consisting of 5% skim milk in Tris-buffered saline with 0.1% TweenTM 20 (TBST) for 1 h at room temperature. Subsequently, 100 μl of primary antibody: rabbit anti-MrVn antibody solution (1:1,000) (Duangprom et al., 2022) was applied to each well and incubated for an additional 2 h at 37°C . After thorough rinsing, 100 μl of secondary antibody:HRP-conjugated goat anti-rabbit IgG (Abcam, UK) at a dilution of 1:5,000 was applied to each well and incubated for 1 h at room temperature. The plates were washed and developed with 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate (Sigma, USA), and the absorbance at 492 nm was measured using a microplate reader (Thermo Fisher, USA). The hemolymph Vg concentration in each well was determined using a known standard Vn concentration, as outlined in the preparation section described by Duangprom et al. (2022).

2.7 mRNA profile detection by transcriptomic analysis

To investigate the effect of hormone potency on mRNA expression, five *S. olivacea* were randomly selected from each group (T1–T5 and C1) and sacrificed on day 14 following the hormone cocktail injections. Pooled brain and VNC samples were obtained and subjected to RNA isolation using the TriPureTM Isolation reagent (Roche, Germany). The concentration and purity of each sample were determined using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA). RNA samples were sent to BGI (Wuhan) for the evaluation of the RNA integrity number (RIN) using an Agilent 2100 Bioanalyzer (Agilent, CA, USA) and mRNA-seq using the BGISEQ-500 sequencing platform. Following read filtering, a *de novo* assembly was conducted using Trinity (Grabherr et al., 2011) on a set of clean reads. The TGICL software tool (Pertea et al., 2003) was employed to cluster transcripts, thereby eliminating abundance and generating unigenes for subsequent analyses.

2.8 Differentially expressed genes following treatments

Bowtie2 software (Langmead and Salzberg, 2012) was used to map all clean reads from each sample to *de novo* assembled unigenes. Gene expression levels were calculated and normalized to fragments per kilobase per transcript per million mapped reads (FPKM) using RSEM (Li and Dewey, 2011) based on the assembly results. Differentially expressed genes (DEGs) between the samples were identified based on gene expression levels. Bioinformatics service of BGI determined the significance of the DEGs using DESeq2 and PoissonDis, with the criteria of \log_2 fold-change ≥ 1.00 , p -value < 0.05 , and FDR ≤ 0.001 (Audic and Claverie, 1997; Love et al., 2014).

2.9 Real-time quantitative polymerase chain reaction of prostaglandin E synthase and estrogen sulfotransferase genes

The brain and VNC of *S. olivacea* ($n = 5$) were obtained on days 0 and 14 of the experimental period. The tissues were homogenized using TriPureTM Isolation Reagent (Roche, Germany) and subsequently stored at -80°C , in accordance with the guidelines provided by the manufacturer. Each crab RNA sample ($2\ \mu\text{g}$), was treated with DNase-I treatment to eliminate genomic DNA. Subsequently, cDNA synthesis was performed using a QuantiNova Reverse Transcription Kit (QIAGEN, United States) in accordance with the manufacturer's instructions. The investigation carried out real-time quantitative polymerase chain reaction (qPCR) was used to analyze the expression levels of prostaglandin E synthase (*Scyol-PGES*) and estrogen sulfotransferase (*Scyol-ESULT*) in the crab tissue samples collected on days 0 and 14. qPCR was conducted using specific *Scyol-PGES* primers (forward primer: 5' GCTGCTCGGTGTGGGT TTCGGT 3' and reverse primer: 5' GCAGCAAATGGGCAT GTCTGGTAC 3') and specific *Scyol-ESULT* primers (forward primer: 5' GCGTGGCAGAAGAGGCACCA3' and reverse primer: 5' TCCAGTCTCCCGTCTTGCCCT 3'). The β -actin gene was used as an internal control (forward primer: 5' GAGCG AGAAATCGTTCGTGACAT 3' and reverse primer: 5' CCGATG GTGATGACCTGGCCGT 3'). PCR conditions used in this study were based on the methodology described by Duangprom et al. (2022). The $2^{-\Delta\Delta\text{ct}}$ approach was employed to analyze the qPCR data and determine the relative expression levels of the two genes in relation to β -actin, as outlined by Livak and Schmittgen (2001).

2.10 Statistical analysis

The application of statistical methodologies was utilized in the assessment of the GSI value, hemolymph Vg, ovarian histological analysis, and the relative expression of *Scyol-PGES* and *Scyol-ESULT* genes in relation to the reference gene, β -actin. Graphs represent the mean values and standard error of the mean (SEM). One-way ANOVA with Tukey's *post hoc* analysis was employed to investigate disparities in mean values between the experimental and control groups. Statistical analysis was performed using Prism 9.5.1 software for Windows (GraphPad Software, USA). Statistical significance was determined at a significance level ≤ 0.05 , as denoted by the *p*-value (Duangprom et al., 2022).

3 Results

3.1 Localization of the *Scyol-VIH* transcript in eyestalk

An anatomical assessment of the eyestalk was conducted using H&E staining (Figures 1A, D), which showed three clusters containing clusters 1, 2, and 3. Individuals injected with NSS

exhibited the presence of *Scyol-VIH*-positive neurons within cluster 2 and X-organ (XO) (Figures 1B, E, H). Individuals that received dsRNA-VIH did not exhibit any neurons with positive results, indicating complete inhibition of *Scyol-VIH* mRNA production (Figures 1C, F, I). Finally, the control sections (using a sense riboprobe) exhibited no indication of positive staining (Figure 1G).

3.2 Effects of treatments on *S. olivacea* GSI

Injections of dsRNA-VIH (7.14 ± 0.92) and the combination of 5-HT + GnRH + dsRNA-VIH (8.01 ± 0.66) significantly increased *S. olivacea* GSI, compared to the control, NSS-injected group (3.48 ± 1.41) on day 14 post-injection. However, on day 28 post-injection, all groups except for the 5-HT + GnRH group exhibited a significant increase in the GSI value of *S. olivacea* in comparison to the control groups (Figure 2).

3.3 Effect of treatments on hemolymph vitellogenin level

The hemolymph Vg levels of crabs were measured on days 0, 14, and 28 of the experiment (Figure 3). No significant increase in Vg levels was observed in the hemolymph of any of the groups on days 0 and 14 post-injection. However, dsRNA-VIH ($1,428.170 \pm 124.010$), GnRH + dsRNA-VIH ($1,274.19 \pm 89.27$), and 5-HT + GnRH + dsRNA-VIH ($1,197.128 \pm 37.95$)-injected groups exhibited a significant increase in hemolymph Vg compared to the NSS-injected group (364.00 ± 52.68).

3.4 Effect of treatments on ovarian maturation

Ovary histology was investigated at days 0, 14, and 28 post-injection (Figure 4A). Comparative analysis of oocyte counts at various developmental stages was conducted across seven groups. On day 0, the ovaries of the treatment and control groups exhibited Og and Oc1–Oc3, as shown in Figures 4A, B. On day 14, the ovaries of the NSS-injected and dsRNA-EGFP-injected groups exhibited Oc1–Oc3, whereas the dsRNA-VIH, 5-HT + GnRH + dsRNA-VIH, and GnRH + dsRNA-VIH-injected groups predominantly contained Oc4 (Figures 4A, C). The 5-HT + GnRH-injected group exhibited a statistically significant increase in Oc3 expression (Figures 4A, C). On day 28, all the groups displayed predominantly mature ovaries. Oocyte counts revealed that dsRNA-VIH (96.12 ± 1.97), 5-HT + GnRH + dsRNA-VIH (97.25 ± 0.64), and GnRH + dsRNA-VIH (98.93 ± 1.06)-injected groups had significantly greater numbers of Oc4 than the NSS-injected group (85.07 ± 4.90) (Figures 4A, D). However, the 5-HT + dsRNA-VIH- and 5-HT + GnRH-injected groups also exhibited a high number of Oc4, which was not significantly different from the NSS-injected group (Figures 4A, D).

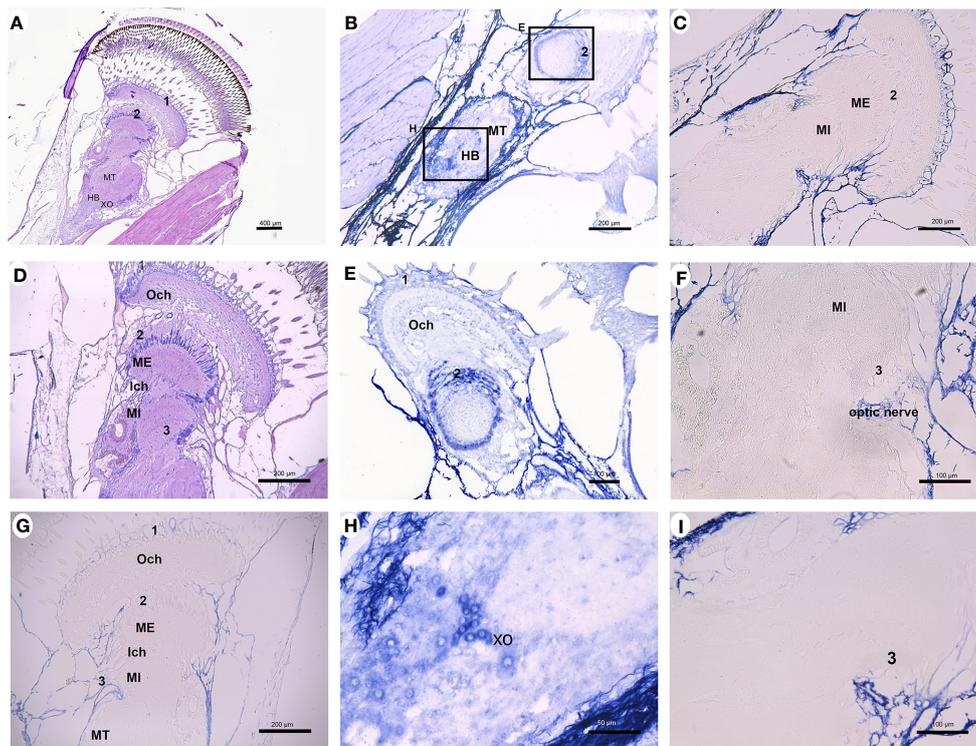


FIGURE 1

Localization of *Scyol-VIH* mRNA in the eyestalks of *S. olivacea* using *in situ* hybridization. (A) Sagittal section of the eyestalk stained with H&E showing the location of neuronal clusters (1, 2, 3) and neuropils with higher magnification shown in (D). (B) The NSS-injected group exhibited *Scyol-VIH* mRNA in neuronal clusters 2 and XO, as illustrated in (E, H) at a higher magnification. (C) The dsRNA-VIH-injected group exhibited no *Scyol-VIH* mRNA expression at higher magnifications (F, I). (G) Negative control sections probed with a DIG-labeled sense-strand *Scyol-VIH* riboprobe did not exhibit a positive signal. Abbreviations: LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; XO, X-organ; HB, hemielipsoid body; Och, outer optic chiasm; Ich, inner optic chiasm, 1, Cluster1; 2, Cluster 2; and 3, Cluster 3.

3.5 Differential gene expression analysis following treatments

The availability of data and materials Raw RNA-Seq reads of all treatments are accessible through the NCBI (Sequence Read Archive-SRA) (BioProject ID: PRJNA1006859 <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1006859>). DEG

analysis of the pooled fbrain and VNC of female *S. olivacea* was performed on day 14 post-treatment, showing that 30 genes were significantly upregulated and two genes were significantly downregulated. Hormone-related genes were targeted and classified into four distinct groups based on whether they encoded receptors, neuropeptides, downstream hormone-related, or prostanoid-related genes (Tables 1–4). Significant upregulation of 5-HT1 and GnRH receptors was observed following all treatments. The lutropin-choriogonadotropic hormone (LH) receptor was upregulated in the dsRNA-VIH, 5-

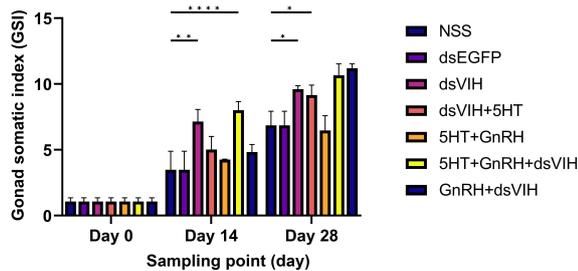


FIGURE 2

Graph showing the effect of treatments on female *S. olivacea* GSI on days 0, 14, and 28 post-injections. Bars indicate standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, and **** $p < 0.0001$ denoted level of significance.

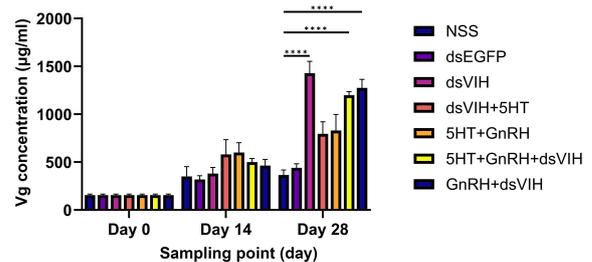


FIGURE 3

Hemolymph vitellogenin (Vg) levels in female *S. olivacea* on days 0, 14, and 28 post-injections. Values are presented as the term of mean \pm SEM and **** $p < 0.0001$ denotes the level of significance.

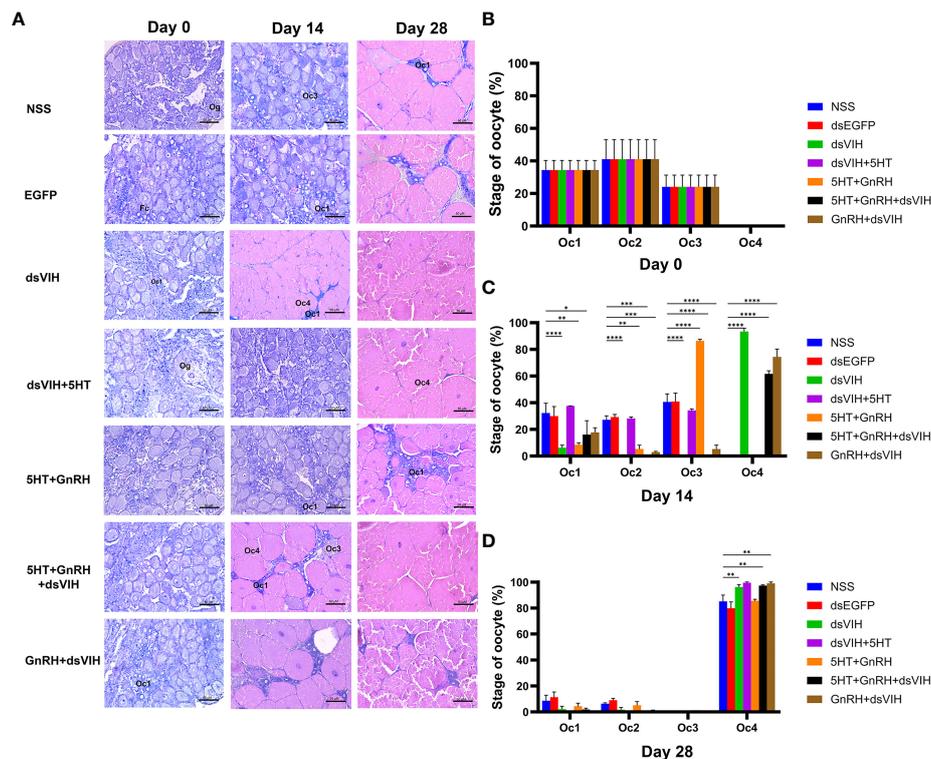


FIGURE 4 Histological examination and quantification of *S. olivacea* ovaries after treatment. (A) Representative images from each group on days 0, 14, and 28. On day 0, histological examination of the ovaries in all groups revealed a comparable number of Oc1–Oc3. On day 14, the ovaries of the dsRNA-VIH-HT + GnRH + dsRNA-VIH and GnRH + dsRNA-VIH-injected groups exhibited greater maturity, as evidenced by the presence of a higher number of Oc4, in contrast to the other groups that still displayed Oc1–Oc3. On day 28, the ovaries of all the groups reached maturity, exhibiting a higher quantity of Oc4. (B–D) Quantification of the oocyte on days 0, 14, and 28. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, and **** $p < 0.0001$ denoted level of significance. Abbreviations: Oc, oocyte; Og, oogonia; N, nucleus.

HT + GnRH + dsRNA-VIH, and GnRH + dsRNA-VIH groups. Additionally, the follicle-stimulating hormone (FSH) receptor was upregulated in all groups except the dsRNA-VIH + 5-HT group (Table 1). Transcriptomic profiles revealed significant upregulation of several neuropeptide hormone genes, such as red pigment-concentrating hormone precursor (RPCH), SIFamide (FMRFamide-related neuropeptides), and short neuropeptide F (sNPF) across all treatments. The expression levels of adipokinetic hormone/corazonin-related peptide (AKH/

Crz) and neuropeptide F (NPF-2) were upregulated in all treatments except for the dsRNA-VIH + 5-HT and 5-HT + GnRH treatments, respectively. The expression of CHH was upregulated in the dsRNA-VIH + 5-HT and 5-HT + GnRH groups but downregulated in the 5-HT + GnRH + dsRNA-VIH group. In contrast, NPF-F1 was upregulated only in the 5-HT +GnRH+ dsRNA-VIH group (Table 2).

This study identified upregulation of farnesoic acid O-methyltransferase (FAMeT) long and short isoforms, estrogen

TABLE 1 Summary of the differential expression of receptor genes compared with the control.

| Receptor gene | Transcript | Accession number | dsRNA-VIH (Log2) | dsRNA-VIH + 5-HT (Log2) | 5-HT + GnRH (Log2) | 5-HT + GnRH + dsRNA-VIH (Log2) | GnRH + dsRNA-VIH (Log2) |
|---|----------------|------------------|------------------|-------------------------|--------------------|--------------------------------|-------------------------|
| 5-HT1 receptor (5-HT1) | Unigene3640 | QDQ18757.1 | 4.10* | 1.82* | 1.97* | 3.28* | 4.10* |
| Gonadotropin-releasing hormone II receptor | Unigene15255 | TNN23789.1 | 5.22* | 3.28* | 3.58* | 5.30* | 5.22* |
| Lutropin-choriogonadotropic hormone (LH) receptor | Unigene23299 | QCE21330.1 | 2.19* | – | – | 2.71* | 2.19* |
| Follicle-stimulating hormone (FSH) receptor | CL3937.Contig2 | P35378 | 2.61* | – | 1.24* | 2.21* | 2.61* |

These significant differences are shown by asterisks. “–” indicates non-DEG.

TABLE 2 Summary of differential expression of neuropeptide reproductive-related genes compared with the control.

| Neuropeptide reproductive-related gene | Transcript | Accession number | dsRNA-VIH (Log2) | dsRNA-VIH + 5-HT (Log2) | 5-HT + GnRH (Log2) | 5-HT + GnRH + dsRNA-VIH (Log2) | GnRH + dsRNA-VIH (Log2) |
|--|----------------|------------------|------------------|-------------------------|--------------------|--------------------------------|-------------------------|
| Crustacean hyperglycemic hormones (CHH) | CL1611.Contig2 | ABP88270.1 | - | 3.28* | 1.49* | -1.59* | - |
| Red pigment concentrating hormone precursor (RPCH) | Unigene6049 | ADQ73633.1 | 6.37* | 3.87* | 5.13* | 6.65* | 6.37* |
| Adipokinetic hormone/corazonin-related peptide (AKH/Crz) | Unigene8722 | AVA26881.1 | 9.34* | - | 9.64* | 10.85* | 9.34* |
| SIFamide (FMRFamide-related neuropeptides) | Unigene20927 | ALQ28576.1 | 5.08* | 1.41* | 3.58* | 5.09* | 5.08* |
| Short neuropeptide F (sNPF) | Unigene26654 | ALQ28574.1 | 5.34* | 3.58* | 4.60* | 5.80* | 5.34* |
| Neuropeptide F1 (NPF-1) | CL3883.Contig1 | ALQ28586.1 | - | - | - | 1.88* | - |
| Neuropeptide F2 (NPF-2) | Unigene20912 | ALQ28587.1 | 2.56* | 1.23* | - | 3.46* | 2.56* |

These significant differences are shown by asterisks.

“-“ indicates non-DEG.

sulfotransferase (ESULT), and progesterone-like protein 2 across all treatments. The upregulation of estradiol 17-beta-dehydrogenase 8 was observed exclusively in the dsRNA-VIH+5-HT group, whereas upregulation of progesterone-like protein 1 was observed in both dsRNA-VIH and GnRH + dsRNA-VIH groups. In contrast, oxytocin/vasopressin-like peptide was significantly upregulated in the 5-HT + GnRH + dsRNA-VIH and GnRH + dsRNA-VIH groups (Table 3). Prostanoid-related genes have been shown to regulate reproductive capacity. Our results indicate that prostaglandin E synthase (PGES) was upregulated in all treatments, whereas prostaglandin F synthase (PGFS) was upregulated in all treatments, except for the dsRNA-VIH + 5-HT

group (Table 4). In addition, the supplementary data included the results of the BLAST search for each of the selected transcripts, as well as contig and unigene IDs, KEGG, KOG, InterPro, and GO annotation (Supplementary File S1).

3.6 Effects of hormones on the upregulation of estrogen sulfotransferase and prostaglandin E synthase genes

Changes in the expression of *Scyol-estrogen sulfotransferase (ESULT)* and *Scyol-prostaglandin E synthase (PGES)* in the brain

TABLE 3 Summary of the differential expression of downstream reproductive-related genes compared with the control.

| Reproductive-related gene | Transcript | Accession number | dsRNA-VIH (Log2) | dsRNA-VIH + 5-HT (Log2) | 5-HT + GnRH (Log2) | 5-HT + GnRH + dsRNA-VIH (Log2) | GnRH + dsRNA-VIH (Log2) |
|---|----------------|------------------|------------------|-------------------------|--------------------|--------------------------------|-------------------------|
| Farnesoic acid O-methyltransferase long isoform (FAME T long) | CL3641.Contig3 | AEE26196.1 | 1.78* | 1.01* | 1.08* | 1.99* | 1.78* |
| Farnesoic acid O-methyltransferase short isoform (FAME T short) | CL3641.Contig4 | AEE26197.1 | 3.69* | 1.95* | 2.56* | 4.21* | 3.69* |
| Estrogen sulfotransferase (ESULT) | CL1355.Contig5 | AJC52502.1 | 2.22* | 2.00* | 2.12* | 3.53* | 3.19* |
| Estradiol 17-beta-dehydrogenase 8 | Unigene21720 | ALO17565.1 | - | 1.07* | - | - | - |
| Progesterone-like protein 1 | Unigene14199 | AFV74661.1 | 1.06* | 2.32* | - | - | 1.06* |
| Progesterone-like protein 2 | CL3247.Contig2 | AFV74662.1 | 1.46* | 2.36* | 1.10* | 1.62* | 1.46* |
| Oxytocin/vasopressin-like peptide | Unigene27097 | AUT12056.1 | 1.25 | - | 1.49 | 1.88 | 1.25 |

These significant differences are shown by asterisks.

“-“ indicates non-DEG.

TABLE 4 Summary of the differential expression of prostanoid-related genes compared with the control.

| Prostanoid-related gene | Transcript | Accession number | dsRNA-VIH (Log2) | dsRNA-VIH + 5-HT (Log2) | 5-HT + GnRH (Log2) | 5-HT + GnRH + dsRNA-VIH (Log2) | GnRH + dsRNA-VIH (Log2) |
|---|----------------|------------------|------------------|-------------------------|--------------------|--------------------------------|-------------------------|
| Cytosolic phospholipase A2 | Unigene12165 | XP_027207890.1 | -2.68* | - | -2.17* | -2.55* | -2.27* |
| Secretory phospholipase A2 | CL3300.Contig3 | XP_027215735.1 | 3.24* | 1.85* | 1.78* | 3.45* | 3.24* |
| Phospholipase A2-like | Unigene4046 | XP_027210685.1 | 3.49* | 1.68* | 2.18* | 4.03* | 3.49* |
| Phospholipase C gamma | Unigene12272 | XP_027237383.1 | -2.49* | - | -1.30* | -2.30* | -2.49* |
| Phospholipase C class I and II | Unigene275 | XP_027237570.1 | 3.75* | 1.49* | 1.05 | 4.52* | 3.75* |
| Phospholipid phospholipase C beta | Unigene43090 | AAD32609.1 | 6.46 | - | - | 7.44* | 6.46 |
| Phospholipase C-like protein 1 | Unigene8075 | XP_027228698.1 | 3.08* | 2.21* | - | 3.39* | 3.08* |
| Phospholipase C-like protein 2 | Unigene12329 | Q9UPR0 | 2.37 | - | 1.03 | 2.41* | 2.37 |
| Cyclooxygenase | CL2412.Contig6 | ALG96667.1 | - | 3.31* | - | - | - |
| Hematopoietic prostaglandin D synthase | Unigene925 | XP_017730994.1 | 1.65* | - | - | 2.94* | 1.65* |
| Prostaglandin E synthase (PGES) | Unigene26325 | AVY03799 | 1.92* | 2.00* | 1.87* | 1.82* | 1.89* |
| Prostaglandin F synthase aldose reductase | CL2593.Contig2 | AFJ11397.2 | - | - | - | - | - |
| Prostaglandin F synthase (PGFS) | CL2109.Contig1 | AJC52503.1 | 2.00* | - | 1.31* | 2.22* | 2.00* |
| Thromboxane A synthase | Unigene5317 | ROT61358.1 | 1.96* | 1.93* | 1.29* | 2.39* | 1.96* |
| 15-hydroxyprostaglandin dehydrogenase | Unigene4166 | XP_027218216.1 | - | - | - | - | - |
| Prostaglandin reductase 1 | Unigene1144 | ROT74915.1 | 1.01* | 1.04* | - | 1.07* | 1.01* |
| Carbonyl reductase [NADPH] | Unigene14080 | KAB7494308.1 | - | - | - | - | - |

These significant differences are shown by asterisks. “-“ indicates non-DEG.

and VNC were determined between days 0 and 14 post-treatment. The administration of dsRNA-VIH and the combination of 5-HT + GnRH + dsRNA-VIH resulted in significant upregulation of *Scyol-ESULT* expression in the brain on day 14. In contrast, injection of dsRNA-VIH + 5-HT and 5-HT + GnRH exhibited a tendency to increase *Scyol-ESULT* expression, but the effect was not statistically significant. Only the combination of 5-HT + GnRH + dsRNA-VIH increased *Scyol-ESULT* expression in the VNC (Figures 5A, B). For *Scyol-PGES*, only the combination of 5-HT + GnRH + dsRNA-VIH injection significantly increased expression in both the brain and VNC on day 14 post-treatment (Figures 5C, D).

4 Discussion

4.1 Suppression of *Scyol-VIH* transcript with supplemented dsRNA-VIH, GnRH, and 5-HT for promoting ovarian development

Ovarian maturation in crustaceans requires the cooperation of molecules from multiple pathways (Rotllant et al., 2018; Jayasankar et al., 2020). Captivity-induced stress in marine invertebrate broodstock, such as handling, overcrowding, diseases, and other inappropriate culture conditions, can interfere with these ovarian maturation-related pathways, which is problematic for their

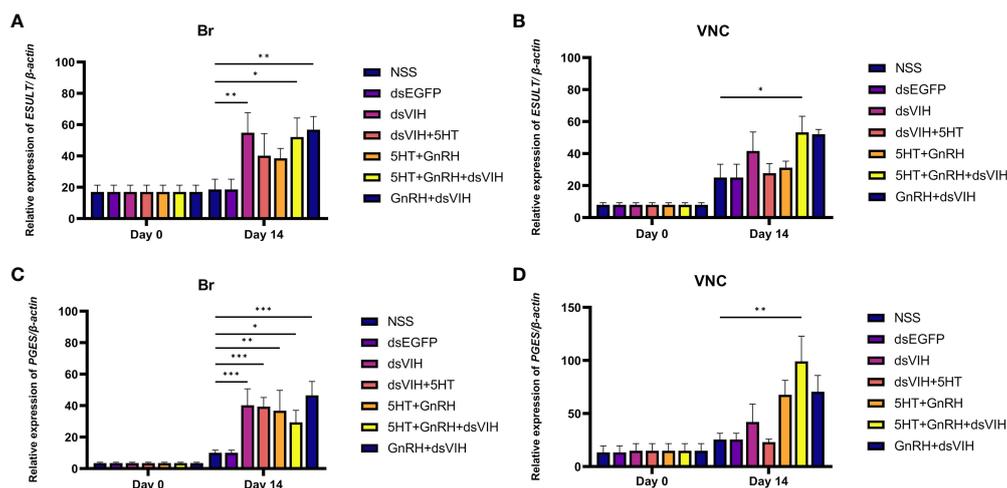


FIGURE 5

The effect of hormones on the expression of *Scyol-ESULT* and *Scyol-PGES* in the brain (Br) and ventral nerve cord (VNC) using quantitative RT-PCR. (A, B) Histograms showing the expression levels of *Scyol-ESULT* in the Br and VNC groups on days 0 and 14 post-treatment. (C, D) Histograms showing the expression levels of *Scyol-PGES* in the Br and VNC groups on days 0 and 14 post treatment. Bars represent standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.001$ denoted level of significance.

sustainable culture (Gianasi, 2017). In some cultured crustaceans, reproductive dysfunction may be overcome by eyestalk ablation, which stimulates maturation, but it is associated with a high mortality rate (Gianasi, 2017; Jin et al., 2021; Asmat-Ullah et al., 2023; Jia et al., 2023). The use of dsRNA-VIH and reproduction-associated neurohormones has shown promise as a replacement for eyestalk ablation, and is highly effective in enhancing reproductive performance in several crustaceans (Cohen et al., 2021; Kang et al., 2021; Duangprom et al., 2022). In this study, we initially confirmed the role of dsRNA-VIH in the female crab *S. olivacea*, and showed that combinatorial administration of dsRNA-VIH, 5-HT, and GnRH, may provide the most efficient approach for manipulating reproductive maturation in captive broodstock.

Scyol-VIH is expressed in the X organ of the eyestalk, brain, and VNC of *S. olivacea* (Kornthong et al., 2019). Its expression in the eyestalk was significantly promoted by dopamine, a potent reproductive inhibitor; however, no significant effect was noted for *Scyol-VIH* in the brain and VNC (Kornthong et al., 2019). Therefore, we expected the eyestalk *Scyol-VIH* to be the target of our dsRNA-VIH. In this study, this transcript was in the X-organ cluster and its expression was abolished by dsRNA-VIH treatment, as shown by *in situ* hybridization which corresponded to our previous dsRNA-VIH efficiency shown by RT-PCR (Duangprom et al., 2022). As described elsewhere, dsRNA-VIH potentiates reproductive enhancement in *S. olivacea* and other crustaceans. Crustacean GnRH-like molecules have been successfully identified in *P. clarkii* (Guan et al., 2014) and *M. rosenbergii* (Suwansa-Ard et al., 2016). LGnRH-III and 5-HT have long been known to promote reproduction in crustaceans (Ngernsoungern et al., 2008; Tinikul et al., 2009; Poljaroen et al., 2011; Saetan et al., 2013; Siangcham et al., 2013). However, the effect of GnRH on the reproduction of *S. olivacea* has not yet been reported. In this study, we combined various doses of dsRNA-VIH, GnRH, and 5-HT to lower them compared to the previous successful doses reported to

promote reproduction (Poljaroen et al., 2011; Saetan et al., 2013; Duangprom et al., 2022). We believe that these three molecules might work together, and this might be closer to their physiological conditions in mud crabs. Our present study provided evidence that either dsRNA-VIH itself (T1) or a combination of dsRNA-VIH and GnRH with or without 5-HT (T4 and T5) significantly increased the GSI and hemolymph Vg, and possessed more advanced stage of ovary maturation than the control group. This indicated the importance of dsRNA-VIH as the key molecule to promote reproduction, whereas GnRH and 5-HT probably showed supportive effects, especially in the presence of low concentrations of dsRNA-VIH. Duangprom et al. (2022) reported that 0.6 $\mu\text{g/g}$ BW dsRNA-VIH was most effective at abolishing brain and eyestalk *Scyol-VIH* expression, while dosages below this concentration partially inhibited brain expression but not eyestalk expression. This could explain our results that a combination of GnRH and 5-HT with a low dose of dsRNA-VIH acceptably promoted mud crab reproduction at the same level as that seen in the 0.6 $\mu\text{g/g}$ BW dsRNA-VIH. Noticeably, the low dose of dsRNA-VIH, 5-HT (T2), GnRH, and 5-HT (T3) did not strongly elevate the hemolymph Vg, and their ovarian histology progressed slowly to meet maturation as seen on day 14. This corresponds with evidence in *P. indicus* that lower 5-HT efficiency involving reproduction was reported in the presence of VIH (eyestalk-intact animals) (Tomy et al., 2016). Therefore, we suggested the combination of 5-HT and GnRH with low dose of dsRNA-VIH (not lower than 0.1 $\mu\text{g/g}$ BW) was recommended for mud crab reproduction stimulation.

4.2 Expressions of reproductive genes as a result of combined hormonal treatments

Differential gene expression analysis of the mixed brain and VNC of female crabs following individual or combined dsRNA-

VIH, 5-HT, and GnRH treatments, led to the identification of molecular factors that may explain their role in ovarian development. The upregulation of 5-HTR and GnRHR could be attributed to the increased abundance of their ligands. In addition, due to peptide structure similarity, we propose that injected GnRH (lGnRHR-III) could activate crab adipokinetic hormone/corazonin-related peptide (ACP) receptor expression. ACP has been identified in *M. rosenbergii* and postulated to play a role in lipid metabolism (Suwansa-Ard et al., 2016). Similarly, 5-HT treatment stimulated higher levels of *RPCH*, *CHH*, and *FAMeT* expression, as previously described (Kornthong et al., 2013; Kornthong et al., 2014; Duangprom et al., 2017).

Our study is the first to report the potential of VIH, 5-HT, and GnRH in upregulating the expression of *short neuropeptide F* (*sNPF*), *NPF-1*, *NPF-2*, and *SIFamide*. In *M. rosenbergii*, both short and long NPFs have been reported to be involved in spermatogenesis and ovarian maturation (Tinikul et al., 2017; Thongrod et al., 2019). In addition, the expression of *NPF-1*, *NPF-2*, *sNPF*, and *SIFamide* transcripts in the cerebral ganglia of the crab *Scylla paramamosain* was high in pre- and early vitellogenic individuals (Bao et al., 2015), suggesting a possible role in reproduction. Oxytocin/vasopressin-like peptide was not upregulated following our treatments, correlating with the non-reproductive function of this peptide reported in *P. pelagicus* (Saetan et al., 2018) and *S. paramamosain* (Lin et al., 2020).

In vertebrates, the LH, FSH, and their cognate receptors contribute to the reproductive steroid hormone activation (Casarini and Crépieux, 2019). In this study, *LHR* and *FSHR* gene upregulation in hormonally injected groups correlated with the upregulation of steroid-related genes, progesterone-like protein, and *ESULT*, the latter of which was confirmed by real-time qPCR. However, the association of *LHR/FSHR* with the steroid pathway or reproduction has not been reported in crustaceans. A possible relevance to mammalian LH and FSH is the glycoproteins *GPA2/GPB5* identified together with its receptor, *LGR1*, in invertebrates, including crustaceans (Rocco and Paluzzi, 2016; Wahl et al., 2022). The *GPA2/GPB5* heterodimer neither activated *FSHR* nor *LHR* (Rocco and Paluzzi, 2016), and it served as a gonad inhibiting factor in the female *M. rosenbergii* (Wahl et al., 2022). The pathway associated with the prostanoid-related gene product has been described in the transcriptome data of *M. rosenbergii* (Thongbuakaew et al., 2021), and it has also been suggested that this gene is regulated in terms of reproductive potential (Di Costanzo et al., 2019). Upregulation of *phospholipase A* and *C*, *COX*, and *prostaglandin D, E, and F synthases* was detected after treatment, and real-time qPCR confirmed the upregulation of *Scyol-PGES*. Numerous studies have indicated that these genes are involved in reproduction of crustaceans. For example, in *M. rosenbergii*, the immunoreactivities of *PGE2*, *COX1*, and *PGES* and the level of *PGE2* were abundant in early vitellogenic ovaries and the effects of *PGE2* positively stimulated several reproductive parameters (Sumpownon et al., 2015). This gene is upregulated following eyestalk ablation, which correlates with the progression of ovarian development (Thongbuakaew et al., 2021). In addition, 5-HT injection increased *PGFS* and *ESULT* expression in the CNS and ovaries of *S. olivacea* (Kornthong et al., 2014).

Most reproductive-related genes associated with GSI and hemolymph Vg levels in this study were stimulated by either the injection of dsRNA-VIH (0.6 µg/g BW) or lower doses of dsRNA-VIH (0.2 µg/g and 0.1 µg/g BW) combined with GnRH and/or 5-HT. However, the receptor-related reproductive genes (*5-HTR*, *GnRHR*, *LHR*, and *FSHR*) were significantly more highly expressed in the dsRNA-VIH (T1), 5-HT + GnRH + dsRNA-VIH (T4), and GnRH + dsRNA-VIH (T5)-injected groups. The peptide hormone genes *sNPF*, *NPF1*, *NPF2*, *ACP*, *CHH*, and *RPCH* were highly expressed in the cocktail hormone-injected groups. Finally, other downstream reproductive-related genes, such as *FAMeT*, steroid-, and prostanoid-related genes, were shown to be more abundant in the multiple hormonal treatment groups (especially T4) than in the dsRNA-VIH-treated group. However, the effects of the combined hormonal treatment were significantly more powerful in resolving aquaculture issues. As discussed, dsRNA-VIH, GnRH, and/or 5-HT significantly modified the expression of numerous reproduction-related genes, inducing ovarian maturation and vitellogenesis. The precise signaling pathways activated by these hormones and dsRNA-VIH in crustaceans are not yet known and require further investigation. In contrast to the use of synthetic lGnRH-III and 5-HT, dsRNA-VIH production is expensive (Duangprom et al., 2022). Therefore, we propose that a combination of low-dose dsRNA-VIH with synthetic GnRH and/or 5-HT would be an effective solution for enhancing reproduction in captive broodstocks.

In conclusion, this study demonstrated the effects of dsRNA-VIH, GnRH, and 5-HT on the reproduction of the mud crab, *S. olivacea*. These three molecules are known to stimulate reproduction in crustaceans. In the case of mud crab, *S. olivacea*, we suggested a combination of the dsRNA-VIH at dose not lower than 0.1 µg/g BW with either lGnRH-III at dose of 25 ng/g–50 ng/g BW or 5-HT at dose of 1.5 µg/g BW, or with both molecules to effectively boost ovarian development.

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.

Ethics statement

The animal study was approved by The Animal Care and Use Committee of Thammasat University, National Research Council of Thailand (NRCT) which ensured that animal suffering was minimal under authorized Protocol Number 011/2562. The study was conducted in accordance with local legislation and institutional requirements.

Author contributions

JS: Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing, Writing – original

draft. SD: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. SS: Formal analysis, Investigation, Methodology, Writing – original draft. PA: Formal analysis, Investigation, Methodology, Writing – original draft. TP: Formal analysis, Investigation, Methodology, Writing – original draft. PSu: Formal analysis, Investigation, Methodology, Writing – original draft. CS: Formal analysis, Investigation, Methodology, Writing – original draft. MT: Formal analysis, Investigation, Methodology, Writing – original draft. SS-A: Supervision, Writing – review & editing. SC: Supervision, Writing – review & editing. PSo: Supervision, Writing – review & editing. NK: Writing – review & editing, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2023.1286789/full#supplementary-material>

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