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Transcriptional silencing of vitellogenesis-inhibiting and molt-inhibiting hormones in the giant freshwater prawn, *Macrobrachium rosenbergii*, and evaluation of the associated effects on ovarian development

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ABSTRACT

In crustaceans, vitellogenesis-inhibiting hormone (VIH) plays a major regulatory role in vitellogenesis and the reproductive cycle, and molt-inhibiting hormone (MIH) controls both the molt cycle and the conditional pre-mating molt of mature *Macrobrachium rosenbergii* females occurring before every egg spawning event. In the current study, we investigated the effect of silencing the *VIH* gene alone or in combination with silencing the *MIH* gene on vitellogenesis and ovarian maturation in immature and mature *M. rosenbergii* females with the aim of determining the associated effects on ovarian development. The first step involved the construction of a dsRNA based on sequence alignment. Importantly, the sequence alignment and phylogenetic relationship between *M. rosenbergii* (*Mr*) *VIH* and *Mr-MIH* revealed that nucleotides 251–508 of *VIH* are almost identical to a sequence in *Mr-MIH*. However, nucleotides 9–250 – including the untranslated region (UTR; nucleotides 9–172) and the first 78 nucleotides of the open reading frame (ORF) - of *VIH* are completely different from the comparable *MIH* sequence. As a strategy to induce vitellogenesis and hence ovarian maturation, we used a single dsRNA retrieved from the above-mentioned conserved ORF (dsORF) sequence to silence *VIH* and *MIH* simultaneously. Another dsRNA retrieved from the distinct (dsDIS) sequence was used to silence *VIH* alone. Our results indicated that both dsRNAs are capable of inducing *VIH* silencing, as shown by a decline in mRNA levels. The results also indicated that *VIH* silencing is more effective in mature adults than in immature animals, leading to ovary maturation and vitellogenesis. Mature females injected with dsORF showed a decline in MIH levels. In addition, dsDIS also affected the expression of *MIH*, but the effect lagged behind that of dsORF by one week. We therefore concluded that dsDIS might be as effective as dsORF in silencing *VIH,* but without a marked influence on *MIH* and its mutual effects on reproduction and growth. The study thus lays down the foundation for the development of a unique novel tool for molecular manipulation of ovarian maturation and induction of the reproductive cycle in mature *M. rosenbergii* females. The suggested strategy might prove valuable for sustainable culture protocols not only in *M. rosenbergii* but also in other crustacean species. Further investigation is, however, needed to elucidate the regulation of the expression of VIH and MIH and how the neuroendocrine system controls growth molt, reproductive molt and reproduction in crustaceans.

1. Introduction

In the oviparous crustacean species, *Macrobrachium rosenbergii*, the giant freshwater prawn, the female carries the fertilized egg mass cemented to the setae of the abdominal pleopods until they hatch about three weeks after fertilization. Since the embryo develops outside the female body, the oocyte must contain sufficient yolk to support the development of the embryo (Blackburn, 1999). The yolk, composed of

Abbreviations: CHH, crustacean hyperglycemic hormone; GSI, gonadosomatic index; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptors; MIH, molt-inhibiting hormone; Mr-MIH, *M. rosenbergii* molt inhibiting hormone; Mr-VIH, *M. rosenbergii* vitellogenesis-inhibiting hormone; RME, receptor-mediated endocytosis; Vg, vitellogenin; VgR, vitellogenin receptor; VIH, vitellogenesis-inhibiting hormone.

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lipids, sugars, and lipoproteins, including the precursor yolk lipoprotein vitellogenin (Vg), is thus produced before oviposition in a process known as vitellogenesis and is stored in the oocyte (Valle, 1993). Ovary maturation occurs multiple times during the female life cycle, and this intensive reproduction, being metabolically demanding, competes with growth for energy sources. Therefore, crustacean female growth is halted during ovary maturation (Raviv et al., 2008).

Just as molt and reproduction are intertwined aspects of the crustacean life cycle, so, too, are vitellogenesis – during which the *Vg* gene is highly expressed – and ovary maturation tightly interwoven parts of the female reproductive cycle (Tsukimura, 2001). The ovary maturation process comprises five distinct stages, namely, a previtellogenic stage (stage I), three stages characterized by intensive vitellogenesis (stages II, III and IV), and a stage that culminates in a reproductive molt (stage V), which is followed by mating and egg spawning (Chang and Shih, 1995; Damrongphol et al., 1991). The regulation of vitellogenesis and, indeed, of female reproduction is orchestrated by a number of neuropeptides belonging to the crustacean hyperglycemic hormone (CHH)–neuroendocrine peptide superfamily, which includes, among others, CHH, molt-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIH) and mandibular organ-inhibiting hormone (Pamuru, 2019; Swetha et al., 2011; Webster et al., 2012). The CHH neuropeptides are produced in the X-organ in the eyestalk and are stored in the adjacent sinus gland. From there, they are secreted, via an axonal tract, into the hemolymph, and they circulate until they reach the target tissue (Webster et al., 2012). Among the above-mentioned CHH neuropeptides, this study focuses on VIH and MIH. VIH was first isolated and characterized in *M. rosenbergii* (Yang and Rao, 2001) and is known to be responsible for the inhibition of vitellogenesis. However, its mechanism of action in vitellogenesis is still to be elucidated. Among the suggestions that have been put forward are that it may act directly, by inhibiting the synthesis of Vg, or indirectly, by inhibiting vitellogenin uptake into the oocytes through restricting the synthesis of the vitellogenin receptor (VgR) or by inhibiting the release of gonad stimulating hormone (Pamuru, 2019). MIH is the major neuropeptide that inhibits ecdysteroid expression and thus controls the molt cycle (Webster et al., 2012). Importantly, it has been found that MIH stimulates vitellogenesis at advanced stages of ovary development (Zmora et al., 2009), but here, too, its mechanism of action in vitellogenesis remains to be investigated.

The need to elucidate the above-mentioned mechanisms is driven – among other considerations – by the importance of M. rosenbergii as an aquaculture species, with M. rosenbergii production in 2018 comprising about 2.5% of the global crustacean aquaculture volume (FAO, 2020). In aquaculture facilities, postlarvae are stocked during the spring and grown throughout the warm season. In Mediterranean areas, M. rosenbergii fecundity is significantly reduced during the autumn-winter period, which becomes a disadvantage in the spring when sufficient quantities of postlarvae are needed for stocking in aquaculture facilities. On a practical level regarding aquaculture operations, elucidation of the mechanisms of action of VIH and MIH is important, because techniques for endocrine manipulation as a means of enhancing reproduction have become essential tools in sustainable crustacean aquaculture (Nagaraju, 2011). One such technique is eyestalk ablation of mature *M. rosenbergii* females, with the procedure *reducing the production of the entire CHH neuropeptide family, including VIH and MIH. It thus* leads to increases in reproductive molt accompanied by the induction of ovary maturation and *vitellogenesis and* shortened durations of ovary development (Okumura and Aida, 2001). However, this technique has a number of drawbacks in that it induces growth molt in immature females (Choy, 1987; Pervaiz et al., 2011), and – being an aggressive procedure – may harm the females and reduce their survival rates (Okumura and Aida, 2001; Palacios et al., 1999; Pillai et al., 2010). More attractive alternatives appear to lie in biotechnologies based on loss of function through the administration of RNAi, which have proved to be harmless (Ventura et al., 2009) and safe (Lezer et al., 2015), as exemplified in the first such commercially implemented biotechnology in the field of aquaculture (Sagi et al., 2013). Some attempts have been made to stimulate ovary development by related procedures, the most relevant example being specific silencing of the *VIH* gene by dsRNA, leading to ovary maturation in crustacean species such as *Penaeus monodon*, *Litopenaeus vannamei* and *M. nipponense* (Feijó et al., 2016; Qiao et al., 2015; Treerattrakool et al., 2011). Bearing in mind the importance of *M. rosenbergii* as an aquaculture species and the close relationship between molt and reproduction, we sought to build on these previous studies by investigating the effects of silencing *M. rosenbergii VIH (Mr-VIH)* separately from and together with *Mr-MIH* on the vitellogenesis process and by determining whether the procedure had different effects in immature and mature *M. rosenbergii* females. To this end, we first elucidated the effect of silencing the conserved coding open reading frame (ORF) region of *Mr-VIH* versus the silencing of the distinct untranslated region (UTR), including the first 78 nucleotides, of the *Mr-VIH* ORF on vitellogenesis. Upon sequence homology analysis, the coding ORF of *Mr-VIH,* specifically the nucleotide sequence between 251 and 508, was found to be almost identical to that of *Mr-MIH*. However, the non-coding 5'-UTR of *VIH,* namely, nucleotides 9–172, including the first 78 nucleotides of the ORF (nucleotides 173–250), was found to be *VIH* specific. Therefore, we sought to show that dsRNA retrieved from ORF nucleotides 251–508 (dsORF) would silence not only *VIH* but also *MIH,* and that dsRNA retrieved from the distinct sequence (dsDIS) would silence only *VIH*. In this study, the efficacy of dsDIS *and* dsORF *in* silencing *VIH* or in simultaneously silencing *VIH* and *MIH* was investigated in immature and mature females.

2. Materials and methods

2.1. Animals

M. rosenbergii females obtained from the Aquaculture Station of the Ministry of Agriculture at Dor, Israel, were held in freshwater tanks at the aquaculture facility of Ben-Gurion University of the Negev at 27 °C and 12 h daylight. The animals were fed ad libitum (up to 7% body weight per day) with frozen marine water polychaeta (Ocean Nutrition Ltd., CA, USA) and shrimp pellets (Raanan Fish Feed, I.Z. Miluot, Israel, 40% protein). To minimize interactions and cannibalism, the tanks were provided with artificial black seaweed and PVC tubing to serve as hiding substrates.

2.2. Sequence alignment and phylogenetic analysis of crustacean CHH neuropeptides

To identify homologous conserved gene sequences within the CHH neuropeptide family, sequences were aligned by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) bootstrapped consensus. Phylogenetic trees ($N = 1000$) were generated by the neighbor-joining method of MEGAX computing platforms (Kumar et al., 2018).

2.3. dsDIS and dsORF synthesis

The distinct and ORF dsRNAs of Mr-VIH, designated dsDIS and dsORF, respectively, were synthesized separately in vitro in our laboratory. pGEM®-T Easy plasmids containing the *VIH* gene sequence served as templates for the dsRNA synthesis. The templates for the transcription reaction were amplified by PCR. The following primer pairs were used for PCR: the sense strand was synthesized using primer T7P forward vs. reverse primer, while the antisense strand was synthesized by T7P as the reverse primer vs. the forward primer (primers and T7 promotor sequences for dsRNA synthesis are given in Table 1). PCR amplicons were separated on a 1.3% agarose gel. DNA bands were visualized with SYBR Safe DNA Gel Stain (Invitrogen). Amplicons were purified with a PCR purification Kit (NucleoSpin Gel and PCR Clean-up, Machery-Nagel). The TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) was used to generate single-stranded RNA according

Table 1
Primers for dsRNA synthesis and real-time (RT)-PCR.

Gene and size	Orientation	Sequence (5′- > 3′)
dsMr-VIH DIS (242)	F	TGAAAGAGCAGGCAGAGACA
	R	TATTCCGAACACAGCCATCA
dsMr-VIH ORF (336)	F	TGGCATCTCGTCTAAACCAA
	R	ACTTGCGTCCGACTCGTATT
T7 promotor		TAATACGACTCACTATAGGG
QMr-VIH (probe #94)	F	TGTGTGTCAGCAAAACGTGTC
	R	GGGTCAACTGCGAATTCAGT
Q*Mr-MIH* (probe #113)	F	CGAGGAATGGTAAACCAGACA
	R	AGGGCTGTCACCAGGACTC
QMr-Vg (probe #144)	F	TTTGAAGTTAGCGGAGATCTGA
	R	TTCGAATTTGCGCAGTCTTT
QMr-VgR (probe #91)	F	GATAAGCAACCCGCAGGAG
	R	CTGAGGAACCTCGACTACGG
QMr-18S (probe #152)	F	CCCTAAACGATGCTGACTAGC
	R	TACCCCCGGAACTCAAAGA

to the manufacturer's instructions. Synthesized RNA molecules were purified with phenol:chloroform (1:1) and sodium acetate. After purification, RNA was precipitated with ethanol. Reconstituted sense and antisense RNA strands were hybridized by incubation at 70 °C for 15 min, 65 °C for 15 min and room temperature for 30 min. dsRNA quality was assessed on an agarose gel and diluted to 5 mg/ml. dsRNA was stored at −80 °C until being used.

2.4. Induction of vitellogenesis by dsORF and dsDIS in immature females

To investigate the effects of dsORF and dsDIS on vitellogenesis, 138 immature intermolt females (9.32 ± 0.93 g, 3 months old), each possessing a transparent (stage I) ovary, were divided into three groups; one group was injected with dsORF, the second group, with dsDIS (both 5 μg dsRNA/g body weight), and the third group, with saline in an amount equivalent to the volume of the treatments. All the animals received two injections, the first on day 0 and the second after 2 weeks. Each group was held in a separate 1-m^2 plastic tank (Plasson, Israel) at 27 °C. Every week for six weeks, starting on day 0, five females from each group were selected at random for the determination of the gonadosomatic index (GSI). For this purpose, each animal was weighed and anesthetized on ice, and its ovary was dissected out and weighed. The GSI was calculated as: $\mathrm{GSI} = \frac{\text{Ovary weight}}{\text{Animal weight}} \times 100$. In addition, a fresh thin slice was cut from the middle of the ovary of each animal, and the oocyte diameter was measured with a roller micrometer in the eyepiece of an Olympus light microscope.

2.5. Induction of vitellogenesis by dsORF and dsDIS in mature females

To determine whether weight and age have an impact on ovary induction, 30 unmated mature females (14.23 ± 0.34 g, 6 months old), each possessing a transparent (stage I) ovary, were divided into three groups. One group was injected with dsORF, the second group, with dsDIS (both 5 μg dsRNA/g body weight), and the third group, with saline in an amount equivalent to the volume of the treatments. All the animals received a single injection at the beginning of the experiment. Each female was kept in a separate mesh plastic cage, and caged females of the same treatment were held floating together in a large tank. The same experimental design that had been used for the immature females was followed, with the following two exceptions: 1) the first analysis was performed for three females from each group on day 0, and the second for the remaining animals on day 21, and 2) RNA was also extracted from the ovaries. Six-month-old vitellogenic females ($n = 5$) were used as a positive control.

2.6. Relative mRNA expression of VIH, MIH, Vg and VgR

Total RNA was isolated from the eyestalk and hepatopancreas of each female and from the ovary for each mature female with the TRI-RNA isolation reagent (Sigma-Aldrich), according to the manufacturer's instructions. cDNA was prepared from 1 μg of the extracted RNA by a reverse-transcriptase reaction using a qScript cDNA synthesis kit (Quanta Biosciences) according to the manufacturer's instructions. Relative expression of *M. rosenbergii VIH*, *MIH*, *Vg and VgR* transcript levels was quantified using SensiFAST Probe Hi-ROX Mix (BIOLINE), specific primers and a Universal ProbeLibrary (Roche) (Table 1). *M. rosenbergii 18S* (GenBank accession number GQ131934) served as a normalizing gene. Reactions were performed using the ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, California, USA).

2.7. Determination of Vg and VgR expression levels by western blot

For determination of Vg levels, hemolymph was withdrawn with needle from the base of the fifth walking leg into a 7 M EDTA solution. To detect VgR expression, ovaries were collected, and ovary pieces in RIPA buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 0.5% protease and 1% phosphatase inhibitor cocktail) were sonicated in a Vibra-Cell™ sonic model VCX 130 (30% amplitude, 3 s pause interval and 4 s pulse for a total work time of 40 s). Hemolymph or sonicated ovary samples were centrifuged at 12,000*g* for 10 min, and the supernatant was collected. Total proteins in the supernatant were quantified with the Bradford reagent (Bio-Rad), and equal amounts of protein (25 μg protein/lane) were separated on 7% SDS-PAGE gel and transferred to nitrocellulose membranes. Following blocking with 5% skim milk in Tris- buffered saline (TBS) containing 0.1% Tween-20 (TBST), membranes were incubated overnight with anti-Vg or anti-VgR antisera at dilution of 1:375 or 1:2500 (*v*/v) respectively. After washing with TBST, membranes were incubated with HRP-conjugated mouse anti rabbit antibody (1:7500 v/v) (Santa Cruz). Chemiluminescence was monitored by ImageQuant (LAS4000, GE) after exposing the membrane to the HRP-substrate mixture (Western blotting detection kit, Advansta).

3. Results

3.1. Mr-VIH ORF is almost identical to Mr-MIH ORF

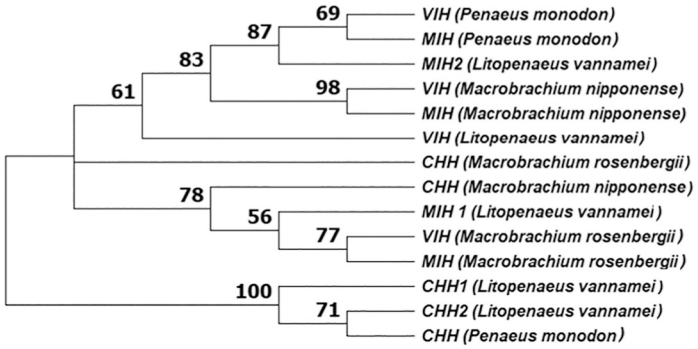
With the aim to design a *VIH*-specific RNAi sequence, the sequence homology between *VIH* and *MIH* of *M. rosenbergii* as well as with the same genes of other decapod species was performed by BLASTN alignment. The *Mr-VIH* mRNA sequence is composed of 1330 nucleotides, comprising 172 5'-UTRs, 336 ORFs, and 822 3'-UTRs. The mRNA sequence of *Mr-VIH* was aligned with *MIH* and *CHH* sequences of *M. rosenbergii*, *M. nipponense*, *L. vannamei* and *P. monodon*. In addition, the ORFs and 5'-UTRs were aligned separately (Fig. 1A). Pairwise alignment revealed that the ORFs of *VIH* and *MIH* sequences from the same species share high similarity, being 70% for *M. rosenbergii*, 55% for *M. nipponense*, 75% for L. *vannamei* and 68% for *P. monodon* (Fig. 1A). However, low or no similarity was found between the ORF sequences of *VIH* and *MIH* and the ORF sequence of the *CHH* of the same species. Significant sequence resemblance was also detected between the ORF sequences of *VIH* from *M. rosenbergii*, *M. nipponense*, *L. vannamei* and *P. monodon* (Fig. 1A). Moreover, high resemblance was also found when the ORF sequence of *M. rosenbergii VIH* was compared with the ORF sequence of *MIH* of the other species (Fig. 1A). In contrast, the 5'-UTR sequence was found to be conserved for the *VIH* of *M. rosenbergii* and *M. nipponense* and for the *VIH* of L. *vannamei* and *P. monodon*. Sequence similarity was also found for *MIH* of *M. rosenbergii* and *M. nipponense*, and *CHH* of *M. rosenbergii* and *M. nipponense*. No similarities were found between 5'-UTRs of *M. rosenbergii VIH* and the UTRs of *MIH* and *CHH* of the other species (Fig. 1A).

Three separate phylogenetic trees were generated for the full sequences, the ORF sequences and 5'-UTR sequences of *VIH*, *MIH* and *CHH*

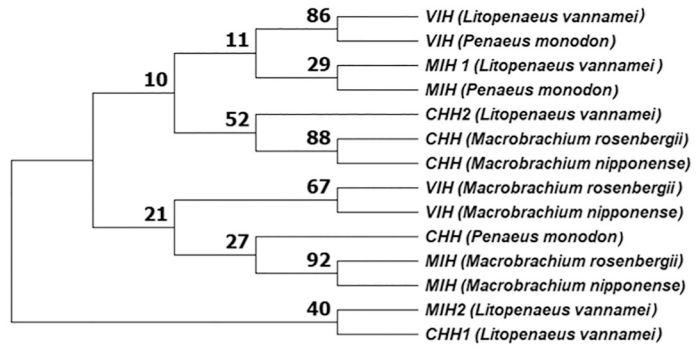
A

	VIH Mr	MIH Mr	CHH Mr	VIH Mn	MIH Mn	CHH Mn	VIH Lv	MIH1 Lv	MIH2 Lv	CHH1 Lv	CHH2 Lv	VIH Pm	MIH Pm	CHH Pm
VIH Mr		70%		100%	70%			4%	59%		3%		4%	
MIH Mr			25%	65%	98%	30%		28%	21%				27%	
CHH Mr					20%	74%				18%				
VIH Mn	93%				55%		10%	28%			2%		34%	
MIH Mn		94%				10%								
CHH Mn			100%							30%	18%			
VIH Lv								75%		12%		100%	75%	
MIH1 Lv									98%			62%	100%	
MIH2 Lv												63%	100%	
CHH1 Lv											48%	8%		
CHH2 Lv										46%				
VIH Pm							100%			5%				
MIH Pm								60%						
CHH Pm														

B



C



D

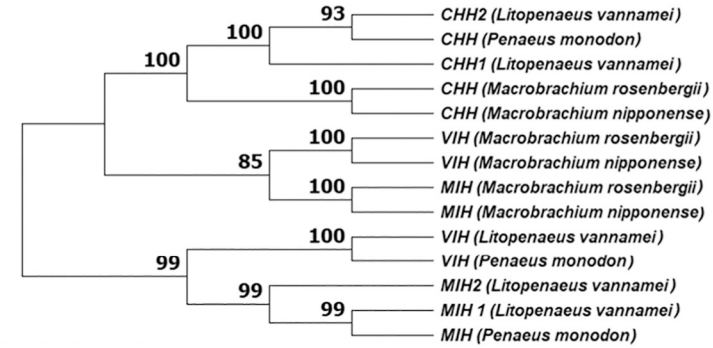


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Fig. 1. Conserved ORFs and UTRs of CHH superfamily sequences in decapod crustaceans. (A) BLASTN coverage percent of *VIH*, *MIH* and *CHH* ORF sequences (above black diagonal) and BLASTN coverage percent of *VIH*, *MIH* and *CHH* 5'-UTR sequences (below black diagonal), gray squares represent no similarity between the sequences. Alignment was performed between *VIH* from *M. rosenbergii* (*VIH-Mr*; AF432347.2), *MIH* from *M. rosenbergii* (*MIH-Mr*; AF432346.1), *CHH* from *M. rosenbergii* (*CHH-Mr*; AY466373.10), *VIH* from *M. nipponense* (*VIH-Mn*; HQ724326.1), *MIH* from *M. nipponense* (*MIH-Mn*; KF878973.1), *CHH* from *M. nipponense* (*CHH-Mn*; HQ724327.1), *VIH* from L. *vannamei* (*VIH-Lv*; KC962398.1), *MIH1* from *L. vannamei* (*MIH1-Lv*; AY425615.1), *MIH2* from *L. vannamei* (*MIH2-Lv*; AY425616.2), *CHH1* from *L. vannamei* (*CHH1-Lv*; AY425615.1), *CHH2* from *L. vannamei* (*CHH2-Lv*; MK732902.1), *VIH* from *P. monodon* (*VIH-Pm*; DQ643389.1), *MIH* from *P. monodon* (*MIH-Pm*; GQ221086.1) and *CHH* from *P. monodon* (*CHH-Pm*; GQ221085.1). (B, C, D) Phylogenetic analysis of *CHH* family mRNA full sequence, ORF and 5'-UTR, respectively. The diagram was generated by the neighbor-joining method using the MEGAX program. Bootstrapping replications were 1000.

of the different decapod species (Fig. 1 B—D). High homology was observed between the full sequences of the *MIH* and *VIH* genes. Interestingly, the highest homology was found between the full sequences of *VIH* and *MIH* of the same species, as they appear on the same phylogenetic branch (Fig. 1B). When comparing only the ORF sequences of the indicated neuropeptide genes, bootstrap homology value reached 100, the highest level between species of the same genus (Fig. 1C). The ORF sequence of *VIH* of *M. rosenbergii* is identical to that of *M. nipponense*. Moreover, the ORF of *MIH* was also found to be identical in these two Palaemonidae species. Identical sequences were also found for the *VIH* ORF of L. *vannamei* and *P. monodon*, as was the case for the *MIH* homology (Fig. 1C). It is thus clear that *MIH* and *VIH* belong to the same subfamily and are segregated from the subfamily of *CHH* genes (Fig. 1B & C). Low homology among species was found for the UTR phylogenetic trees. Again, the highest homology was observed between the sequences of the same *CHH* family members obtained from closely related crustacean species (Fig. 1D).

A 91% sequence homology was found when alignment was performed between the partial ORF sequences of *Mr-VIH* (nucleotides 251–508) and *Mr-MIH* (Supplementary Fig. 1). However, no resemblance was found when the non-coding 5'-UTR of *VIH* (nucleotides 9–172), and the first 78 nucleotides of the ORF (nucleotides 173–250), were compared with the corresponding sequence of *Mr-MIH*. Therefore, nucleotides 251–508 of *Mr-MIH* were used to prepare a dsRNA (dsORF)

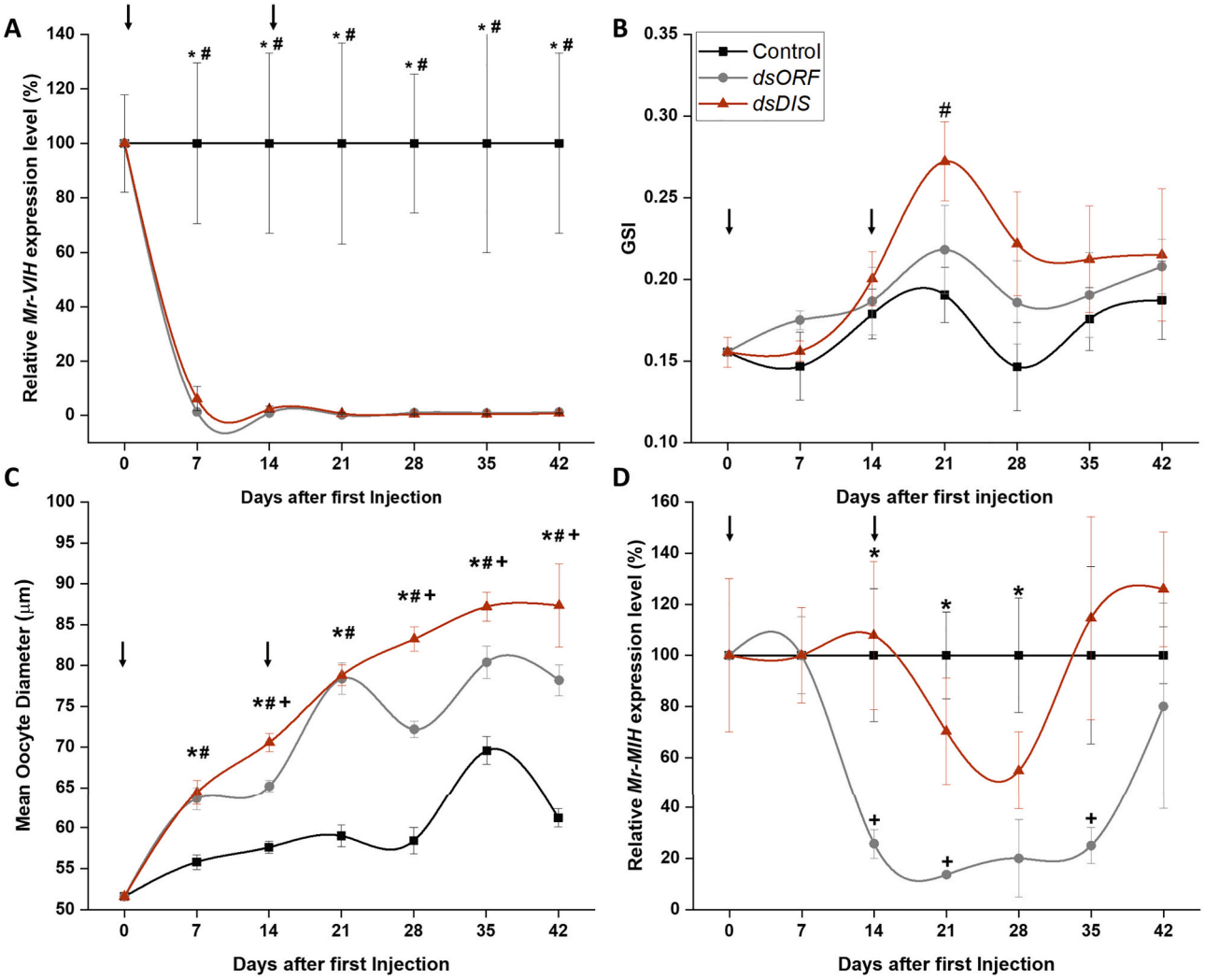

Fig. 2. *Mr-VIH* gene silencing leads to the onset of vitellogenesis. Three groups of female animals were used for the *Mr-VIH* silencing experiment: dsORF-, dsDIS- and saline (control)-injected groups, with 5 females being taken from each group each week. (A) PCR analysis of the relative quantities of *Mr-VIH*. (B) Changes in GSI. (C) Changes in mean oocyte diameter. (D) Relative q-PCR analysis of *Mr-MIH*. Arrows indicate injection days. (*) represents significant difference between the dsORF and control groups ($P < 0.05$); (#) represents significant differences between the dsDIS and control groups ($P < 0.05$); (+) represents significant differences between the dsORF and dsDIS groups. Comparisons of the experimental data were analyzed by SPSS *t*-tests.

to simultaneously silence both *Mr-VIH* and *Mr-MIH*. In addition, the 5'-UTR (nucleotides 9–172), including nucleotides 173–250, of the ORF of *VIH* were used to prepare a dsRNA (dsDIS) to specifically silence *Mr-VIH* (Supplementary Fig. 1 and 2).

3.2. dsORF and dsDIS induce ovary development

Mr-VIH dsRNAs complementary to the ORF (dsORF) or to the VIH distinct region (dsDIS; Supplementary Fig. 2) were synthesized with the aim to test whether they would elicit different responses of the ovaries of *M. rosenbergii* females. In separate experiments, the dsRNA (either dsORF or dsDIS) was injected into 3-month-old immature primary vitellogenic females (9.32 ± 0.93 g). One week after the dsRNA injections, the mRNA levels of *Mr-VIH* dropped drastically (down to 0%) in the dsORF- and dsDIS-injected females (Fig. 2A). The level of *Mr-VIH* remained low for six weeks after dsRNA injection. To test whether ovary development had been induced, two parameters were used, the GSI and the oocyte diameter (Fig. 2B & C, respectively). The GSI increased gradually from the second to the third week after the injection but plateaued out or dropped in the fourth to sixth weeks (Fig. 2B). On day 21, the GSI was significantly higher in the dsDIS-injected group than in the control group. Starting one week after the dsRNA injection and continuing until the end of the experiment, the oocyte diameter of the dsORF- and dsDIS-injected groups was significantly higher than that in the control group. In addition, in the second, fourth, fifth, and sixth weeks after first injection, the oocyte diameter of the dsDIS-injected group was significantly higher than that in the dsORF-injected group (Fig. 2C). An additional finding was that the levels of *Mr-MIH* in the dsORF-injected group were significantly lower in the third week and slightly lower throughout the remaining period compared to the dsDIS and control groups (Fig. 2D).

3.3. VIH silencing effect is more pronounced in mature females

To elucidate whether age has an effect on vitellogenesis induction, dsDIS and dsORF were injected into six-month-old females (14.23 ± 0.34 g). As in the experiment with the immature females, the mRNA levels of *Mr-VIH* could hardly be detected and dropped drastically (down to 0%) in the dsORF- and dsDIS-injected females at 21 days post injection (Fig. 3A). The positive control vitellogenic females had the same *Mr-VIH* expression level as the control females injected with saline (Fig. 3A). As for the immature females, *Mr-MIH* relative expression levels were also determined. The expression level of *Mr-MIH* was slightly lower in both dsORF- and dsDIS-injected females compared to the control group (Fig. 3B). Interestingly a significant difference was found between dsDIS-injected immature and mature females in both *Mr-VIH* and *Mr-MIH* expression levels. *Mr-VIH* and *Mr-MIH* expression levels values of the saline-injected group were similar to those of the previtellogenic animals, i.e., the values that were determined on the first day of the experiment.

The GSI and the oocyte diameter of dsORF- and dsDIS-injected females were significantly higher than the values for the control females (Fig. 4A & B, respectively). Vitellogenic females (mature) had significantly the higher mean GSI (2.69 ± 0.79) and oocyte diameter (303.6 ± 9.7 μm) values than the other control groups and the group of the previtellogenic females (immature) (Fig. 4A & B). A higher GSI and a larger oocyte diameter were obtained in mature females compared to immature females for both dsORF or dsDIS treatments. The GSI and oocyte diameter values of the saline-injected group were similar to those of the control female group that was tested at beginning day of the experiment.

3.4. Mr-VIH silencing influences Vg and VgR expression profiles

To test whether *Mr-VIH* silencing affects *Vg* and *VgR* expression levels, qPCR analysis was performed. In vitellogenic females, the expression of *Vg* in the hepatopancreas was two million-fold higher than that in the pre-vitellogenic females (Fig. 5A). The expression level of *Vg* in the hepatopancreas of dsORF-injected females was significantly higher than the control previtellogenic females. Similar results were obtained for dsDIS-injected females (Fig. 5A). *Vg* expression in the ovary of dsORF-injected females and vitellogenic females was more than twofold that in the control previtellogenic females. Interestingly, *Vg* expression in the ovaries of dsDIS-injected females was significantly lower than that expressed in previtellogenic females (Fig. 5B). Additionally, western blot analysis revealed that the Vg levels in the hemolymph were higher in the dsORF- and dsDIS-injected females than that of the control previtellogenic females (Supplementary Fig. 3, top). *VgR* expression in the ovary was also tested, with *VgR* expression being

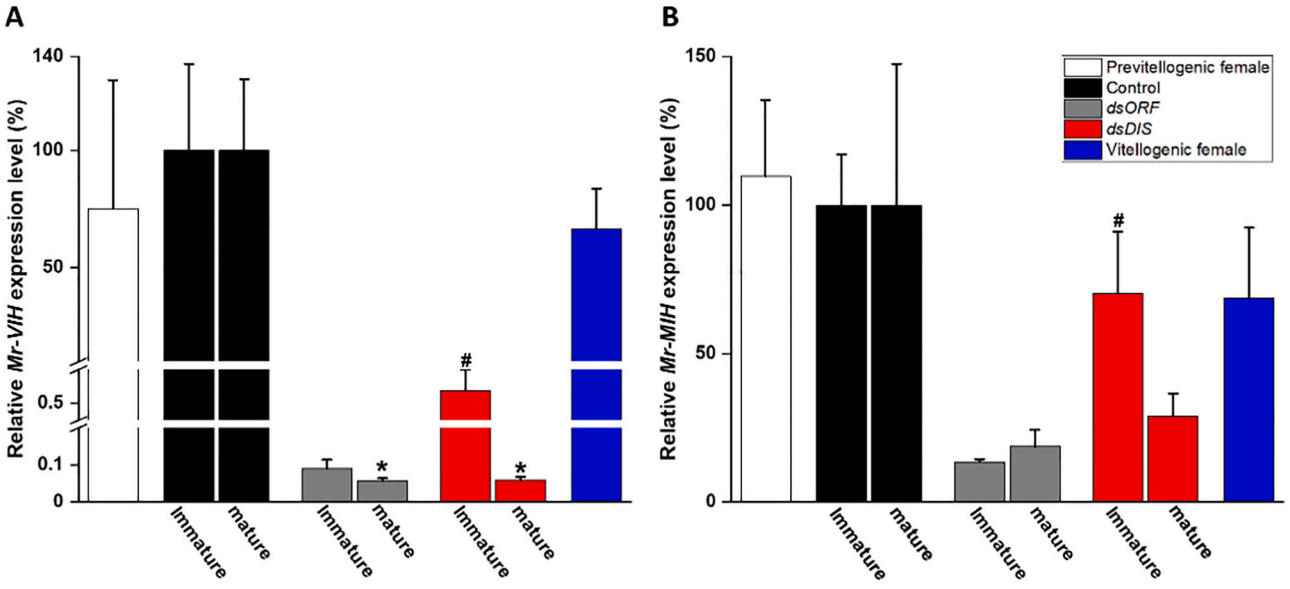

Fig. 3. Effect of *Mr-VIH* gene silencing on *Mr-MIH* expression in 3-month-old (immature) and 6-month-old (mature) females. The *Mr-VIH* silencing experiment was performed on three groups of females: dsORF-, dsDIS- and saline (control)-injected groups ($n = 5$). (A) PCR analysis of *Mr-VIH* relative quantities. (B) Relative q-PCR analysis of *Mr-MIH*. (*) represents significant differences between the mature experiment group and the mature control group, and (#) represents significant differences between mature and immature females in the same experimental group ($P < 0.05$). Comparisons of the experimental data were analyzed by SPSS t-tests.

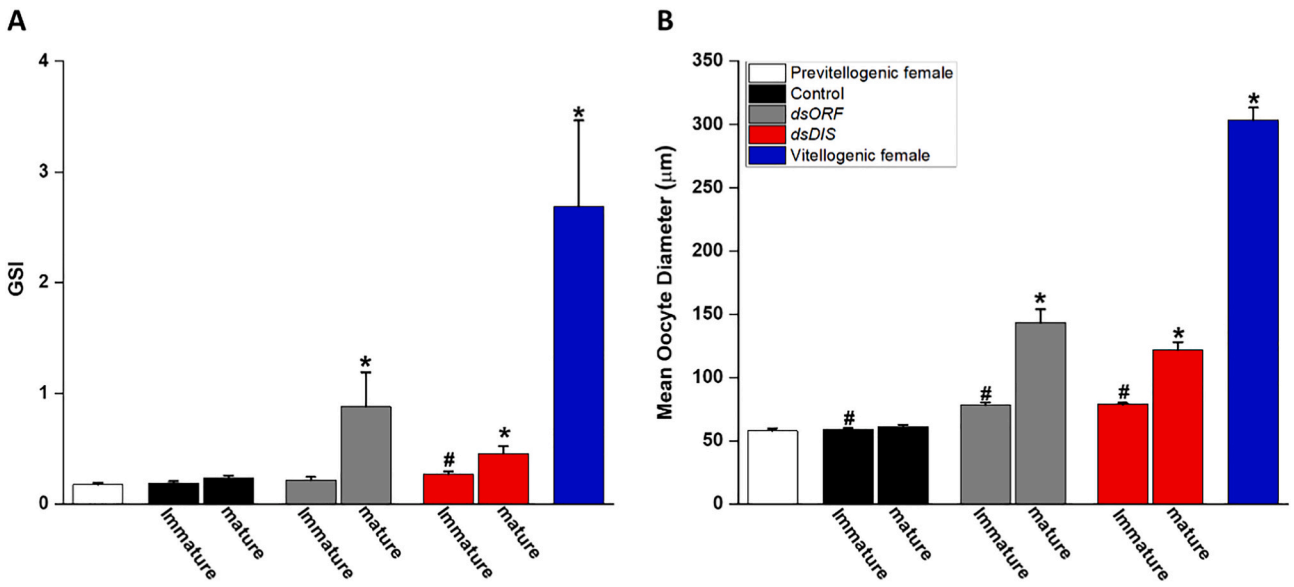

Fig. 4. Effect of *Mr-VIH* gene silencing in 3-month-old (immature) and 6-month-old (mature) females. The *Mr-VIH* silencing experiment was performed on three groups of females: dsORF-, dsDIS- and saline (control)-injected groups (n = 5). Females with previtellogenic ovaries and females with highly vitellogenic ovaries were used as control groups. (A) Changes in GSI. (B) Changes in mean oocyte diameter. (*) represents significant differences between the mature experiment group to the mature control group and (#) represents significant differences between mature and immature females in the same experimental group ($P < 0.05$). Comparisons of the experimental data were analyzed by SPSS t-tests.

higher in previtellogenic females than in vitellogenic females. *VgR* expression was similarly low in all three groups of vitellogenic females (Fig. 5C). However, for VgR, protein levels were higher in the dsORF- and dsDIS-injected females than in the vitellogenic females of the control group, but different from those of the control previtellogenic group, which exhibited low expression levels (Supplementary Fig. 3, bottom).

4. Discussion

As stated above, either a reduction or a complete halt in ovary maturation and egg spawning is common during the autumn-winter period in *M. rosenbergii* females or under conditions of captivity in the major aquaculture crustacean species L. *vannamei* and *P. monodon* (Feijó et al., 2016). Techniques to control the timing and enhancement of reproduction are essential for sustainable crustacean aquaculture (Nagaraju, 2011). In the past, eyestalk ablation was one of the techniques commonly used to achieve ovarian maturation. However, this aggressive manipulation leads to a reduction in female survival rates (Okumura and Aida, 2001; Palacios et al., 1999; Pillai et al., 2010). Moreover, although eyestalk ablation promotes oocyte development, the resulting oocytes are not always properly developed (Nagaraju, 2011). Therefore, stimulation of ovary development by silencing of inhibitory factors produced in the eyestalk rather than removing the whole organ seems to be a better approach.

With the current availability of the *M. rosenbergii* genome (Levy et al., 2019), such an approach has indeed become feasible. As a step towards developing an efficient biotechnology, this study set out to delineate the differences between the ORF and UTR of *Mr-VIH* and to elucidate their roles in the maturation of the ovary. The study commenced with sequence alignment of the full mRNA across crustacean neuropeptides. The alignment indicated high sequence similarity between *VIH* and *MIH*. This similarity between *VIH* and *MIH* in sequences and alignment and their location on the same branch of the phylogenetic tree indicates evolutionary conservation and suggests related or cross-functional activities. The classification of VIH and MIH into the same subfamily of neuropeptides (Chan et al., 2003; Webster et al., 2012), both assembled in one evolutionary group, may be taken as support for roles of the two neuropeptides in ovarian maturation in *M. rosenbergii*. These results are indeed in line with previous studies describing roles for VIH and MIH in ovarian maturation and reproduction in other decapod crustaceans (Pamuru, 2019; Tsutsui et al., 2007; Yang and Rao, 2001). In *M. rosenbergii*, the female reproductive cycle includes a pre-mating molt that occurs upon completion of stage V of ovary development (Chang and Shih, 1995). It is currently held that in intact females MIH declines sharply upon completion of stage IV. However, our results indicate that the *Mr-MIH* expression level in control vitellogenic females was higher than the levels in the dsORF- and dsDIS-treated females. These results are in keeping with the findings of Zmora et al. (2009) showing that MIH plays a role in the regulation of vitellogenesis. Past attempts to exploit RNAi have typically targeted the ORF region of the mRNA (Ebenzer, 2020; Hung and Kumar, 2004; Knorr et al., 2018), but the similarity between the ORF sequences of the *VIH* and *MIH*, described here, suggests that dsRNA obtained from the conserved ORF sequence of *Mr-VIH* would also affect *Mr-MIH*. Indeed, our results indicate that dsORF of *Mr-VIH* led to silencing and hence to the decline in *Mr-MIH* expression. However, the significant decline in MIH expression appeared only on day 14. The fact that dsORF of MrVIH has 70% similarity to MrMIH ORF may explain the delay in silencing MIH. Aa additional reason for the difference in silencing could be a result of different expression levels of VIH and MIH. Based on the sequence alignment and the phylogenetic tree analysis, we posited that dsRNA retrieved from the distinct region of *Mr-VIH* would not lead to *Mr-MIH* silencing. However, a slight decrease in *Mr-MIH* expression was observed when dsDIS was injected into *M. rosenbergii* females. This decline in *MIH* expression could be attributed to a feedback effect, in which VIH regulates the expression of MIH at a specific reproduction stage, and therefore silencing of *VIH* would lead to a decline in the expression of *MIH*. Support for the regulatory effect of VIH on MIH is indeed provided by the significant decline in *MIH* expression in dsDIS-injected mature females (Fig. 3B). The suggested feedback scenario is supported by previous studies showing that MIH levels are regulated throughout ovarian maturation and the mass cemented (Huang et al., 2015; Nagaraju, 2011). However, a non-target effect could not be ruled out.

The results obtained in this study of dsRNA silencing show that both dsORF and dsDIS of *Mr-VIH* were able to silence *Mr-VIH* and reduce *Mr-VIH* mRNA to barely detectable levels. Injection of dsRNA led to the

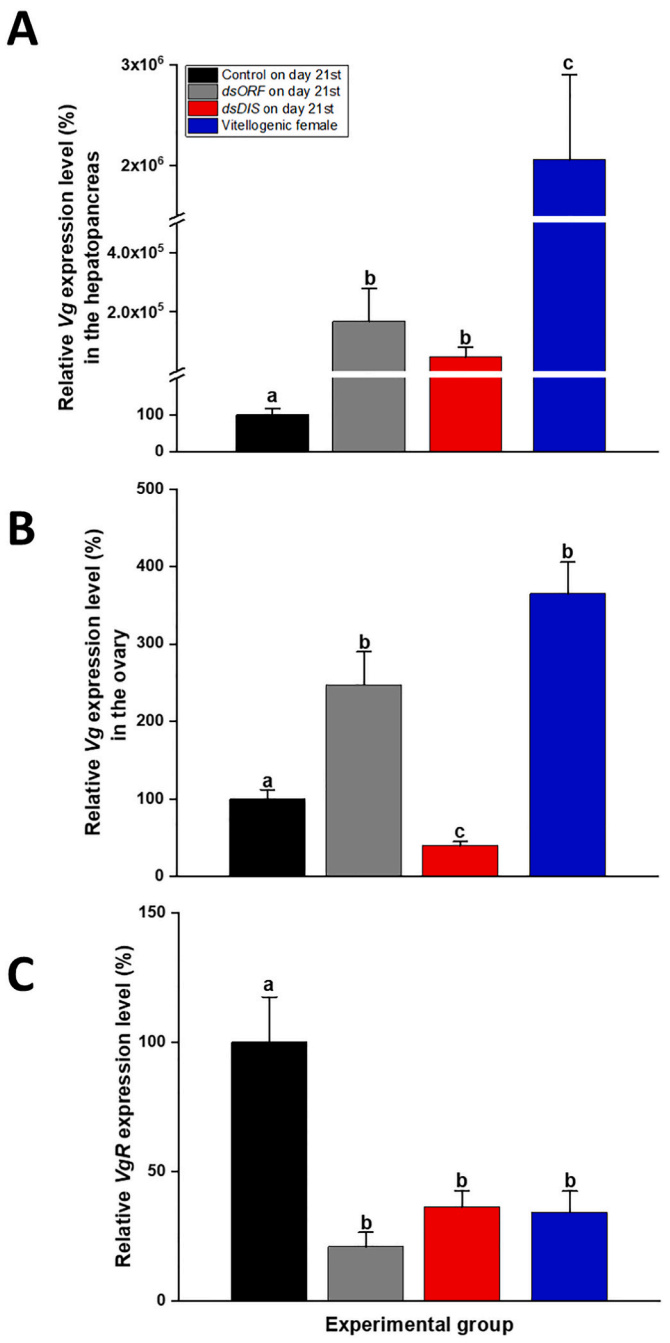

Fig. 5. *Mr-VIH* silencing affects *Vg* and *VgR* expression profiles. (A-C) Relative q-PCR analysis of *Vg* expression in the hepatopancreas and ovary and *VgR* expression in the ovary (n = 5). Different letters represent significant differences ($P < 0.05$). Comparisons of the experimental data were analyzed by SPSS t-tests.

onset of ovary development, and its effect was consistent and constant for at least 28 days after the last injection. Subsequently, the oocyte diameter of the dsRNA-injected females became significantly larger than that of the control females. Yet, dsRNA injection into immature females proved to be insufficient for full ovary maturation, as only stage II (opaque white oocytes of diameter 110 μm) of ovarian development was reached six weeks after the injection. Similar treatment in *M. nipponense* females led to an increased spawning rate (Qiao et al., 2015). Previous work in L. *vannamei* indicated that *VIH* dsRNA injection into females led to the onset of vitellogenesis but only up to an early stage of ovary development (Feijó et al., 2016). In that study, a comparison of *VIH* dsRNA-injected females with eyestalk-ablated females revealed ovary maturation and spawning only in the latter group (Feijó et al., 2016). In a different study, higher rates of spawning events were observed in wild *P. monodon* females injected with dsRNA of *VIH*. The authors attributed differences in silencing efficiency to differences in animal size, age or ambient conditions, such as water temperature, nutrition and culture density (Treerattrakool et al., 2011). Paulraj et al. (2008) showed that even small (7.6 g) 4-month-old *M. rosenbergii* females could show the ovarian color and cell arrangement of a mature female. Their findings taken together with our results may indicate that female age is a critical factor in the induction of ovary development by *Mr-VIH* dsRNA. Therefore, to investigate how VIH levels and reproduction system development are correlated with female age, six-month-old females (14.23 ± 0.34 g, almost twice the age of the females used in the first experiment) were injected with dsRNA. The higher GSI values and the larger oocyte diameters recorded in this older female group (6 months old) indicate that age is indeed a factor of significant importance for vitellogenesis and ovary development in *M. rosenbergii* females. In contrast to the younger animals (3 months old) whose ovary development did not advance beyond stage II, six-month-old females injected with dsORF or dsDIS reached advanced ovary development stages.

To test whether *Mr-VIH* silencing affects ovary maturation through a direct effect on *Vg* expression, the levels of Vg in the hepatopancreas were determined. *Vg* mRNA levels in dsORF- and dsDIS-injected females appeared to be elevated but were not significantly higher than the level in previtellogenic females. However, the ovaries of the treated groups were significantly more developed and reached stages III-IV. The lack of a significant change in Vg expression in the hepatopancreas could be attributed to the high standard deviation in the levels of Vg expression between females of the same group.

The Vg of *M. rosenbergii* is mostly synthesized in the hepatopancreas, but previous studies have found minor expression in the ovary (Jayasankar et al., 2002; Okuno et al., 2002). Therefore, the *Vg* expression level in the ovary was also determined. The results show that in the dsORF group and the vitellogenic group *Vg* expression in the ovary was threefold higher than that in the control group. However, *Vg* expression in the ovary of dsDIS group was significantly lower than that in the other experimental groups. Previous studies in L. *vannamei* showed that VIH inhibits *Vg* mRNA levels in the hepatopancreas and in the ovary (Chen et al., 2014; Feijó et al., 2016), but, to the best of our knowledge, the current study is the first to show a correlation between *VIH* and *Vg* expression in the ovary of *M. rosenbergii* females.

VIH may also affect other processes contributing to ovary maturation. One such suggested VIH-based mechanism of action is the inhibition of Vg uptake into oocytes through the downregulation of the expression of VgRs or inhibition of the release of gonad-stimulating factors (Pamuru, 2019). In contrast to the Vg expression profiles, the VgR expression levels in *M. rosenbergii* appeared to be highest in previtellogenic females and to decrease to the lowest expression levels in mid and late vitellogenic females (Roth and Khalaila, 2012). The explanation for this finding may lie in the fact that VgR belongs to the family of low-density lipoprotein (LDL) receptors (LDLRs), which have a recycling mode of action (Davis et al., 1987). Upon interaction of LDLRs on the cell membrane with LDLs, the LDL-LDLR complexes are internalized into the cells by a receptor-mediated endocytosis (RME) process. Inside the cells, the LDLs are released at low endosomal pH, and the LDLRs are recycled to the cell membrane for additional cycles of interaction and RME (Brown et al., 1997). The maximum VgR expression at early vitellogenic stages (stages I and II) and its decline at late vitellogenic stages (stages III and IV) revealed in the current study confirms and fits with the timing of vitellogenesis. Upon the onset of vitellogenesis – when the VgRs have reached an appropriate level – VgR expression halts, and Vg expression in the hepatopancreas and ovary increases gradually. It seems that high expression of VgR at the pre-vitellogenic stage prepares the oocytes for the massive accumulation of Vg during vitellogenesis.

The silencing effect of dsORF on *Mr-MIH* appears to have contributed significantly to Vg expression in the ovary. The explanation for this finding may lie in the natural lower levels of Mr-MIH in vitellogenic females as a factor preparing the female for reproductive molt. However, the effect of dsORF on other genes with sequence homology cannot be ruled out. In contrast, specific *Mr-VIH* silencing by dsDIS did not appear to increase ovarian Vg expression levels. This finding can be regarded as additional circumstantial evidence for the importance of MIH in ovary development and maturation. It is also possible that there are two different vitellogenesis routes in *M. rosenbergii* females—one, controlled by VIH, that takes place in the hepatopancreas, and the other, significantly affected by MIH, that takes place in the ovary.

The current research indicates that both dsORF and dsDIS *Mr-VIH* silencing protocols could be used to induce vitellogenesis and ovary development in six-month-old females. Both silencing agents affect the relative expression of Mr-MIH, but the effect of dsORF is more pronounced and partially resembles eyestalk ablation. It appears that dsDIS causes a reduction in *Mr-MIH* expression, which could result from a regulatory effect of low VIH levels. Our study presents the first evidence of the effect of VIH dsRNA on Mr-MIH expression. Due to the fact that molt and reproduction are coupled in the prawn, further investigation is needed to elucidate the exact interactions between VIH and MIH. Understanding the exact roles of the sinus gland neuropeptides would enable the development of powerful tools for controlling the reproduction cycle in crustaceans, thereby contributing to sustainable crustacean aquaculture.

Authors' contributions

SC conceived the study, carried out the lab work and data analysis, participated in the design of the study and drafted the manuscript. OI carried out the quantitative PCR and western blot analyses. RM designed and coordinated the work with dsRNA, its cloning and synthesis. AS contributed to the experiment design and manuscript draft. IK, designed the work, coordinated the molecular and biochemical study, carried out animal dissections, statistical analyses and helped draft the manuscript. All authors gave final approval for publication.

Declaration of Competing Interest

We have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736540.

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