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EDITED BY

Yafei Duan,
South China Sea Fisheries Research
Institute, China

REVIEWED BY

Jie Gong,
Nantong University, China
Xugan Wu,
Shanghai Ocean University, China

*CORRESPONDENCE

An Liu

✉ liuan@jmu.edu.cn

Haihui Ye

✉ hhye@jmu.edu.cn

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SIFa and its receptors play a possibly stimulatory role during ovarian development of the mud crab *Scylla paramamosain*

Sijia Chen, Yiwei Tang, Li Lu, Shaoming Gong, An Liu* and Haihui Ye*

State Key Laboratory of Mariculture Breeding, Fisheries College of Jimei University, Xiamen, China

SIFamide is an amidated neuropeptide with a conserved Ser-Ile-Phe structure at its C-terminal, which is crucial in insect courtship, sleep and feeding in insects. However, little information exists regarding its function in crustaceans. This study aimed to identify the cDNA of *Sp-SIFa* and its two putative receptors (*Sp-SIFaR1* and *Sp-SIFaR2*) in the mud crab *Scylla paramamosain*. The expression profiles of *Sp-SIFa* and its receptors signaling were investigated to explore their potential roles. The results indicated that *Sp-SIFa* was expressed primarily in the nervous tissues and the midgut, and *Sp-SIFaR1* was extensively expressed in the eyestalk ganglion, cerebral ganglion, hepatopancreas and the ovary whereas *Sp-SIFaR2* was restricted to the eyestalk ganglion and ovary. The *Sp-SIFa* expression in the cerebral ganglion was significantly increased at the late vitellogenic stage during the ovarian development. The expression patterns of *Sp-SIFaR1* and *Sp-SIFaR2* in the ovary were similar to *Sp-SIFa*, indicating a potential function in regulating ovarian development. Subsequently, *in vitro* and *in vivo* experiments were performed to further support this hypothesis. The expression of *Sp-Vg* and *Sp-VgR* was significantly induced by *Sp-SIFa* peptide *in vitro* and *in vivo*. Moreover, prolonged injection of the *Sp-SIFa* peptide caused a significant increase in *Sp-VgR* expression, oocyte diameter and gonadal development index, demonstrating a possible stimulatory effect on ovarian development. In conclusion, our results suggest that *Sp-SIFa* may regulate ovarian development by promoting Vg biosynthesis and oocyte uptake in the mud crab *S. paramamosain*.

KEYWORDS

SIFa, SIFa receptors, *Scylla paramamosain*, ovarian development, mud crab

1 Introduction

In arthropods, neuropeptides are important signaling molecules to regulate metamorphosis, ecdysis, growth, and reproduction (Charmantier et al., 1997). In crustaceans, past studies have predominantly focus on the neurohormones derived from the eyestalk ganglia, where the X-organ and sinus gland complexes play central roles in the regulation of gonadal development. It has been long known that vitellogenesis inhibiting hormone (VIH) plays an inhibitory role in ovarian development (Tsutsui et al., 2013). Recently, many neuropeptides have been identified in the nervous system of crustaceans using high-throughput sequencing technology; however, their functions in reproduction remain largely unexplored. Therefore, it is intriguing to identify gonadotrophic neuropeptides in crustaceans, which can be applied to the artificial breeding technology of economically significant species.

SIFa is an amidated neuropeptide that characterized by a conserved Ser-Ile-Phe structure in its C-terminal, with 12 amino acid residues in length (Verleyen et al., 2009). There are three isoforms of SIFa that have been identified in arthropods, which are distinguished by a single amino acid residue at the end of the amino (N) terminus: Ala1-SIFa, Gly1-SIFa and Val1-SIFa. AYRKPPFNGSIFamide (Ala1-SIFa), Gly1-SIFa, and Val1-SIFa were first identified in the grey fly *Neobellieria bullata* (Janssen et al., 1996), the giant tiger prawn *Penaeus monodon* (Sithigorngul et al., 2002), and the American lobster *Homarus americanus* (Dickinson et al., 2008), respectively. To date, a number of SIFa and its paralogs have been identified in insects and crustaceans through the application of mass spectrometry and next generation sequencing, for example, the Jonah crab *Cancer borealis* (Huybrechts et al., 2003) the red swamp crayfish *Procambarus clarkii* (Yasuda et al., 2004) and the mud crab *S. paramamosain* (Bao et al., 2015).

The findings regarding physiological effect of SIFa are primarily mediated by insects. In *Drosophila melanogaster*, SIFa is involved in the regulating various physiological processes, such as courtship (Sellami and Veenstra, 2015), sleep (Huang et al., 2021) and feeding (Martelli et al., 2017). Similarly, the role of SIFa in regulating feeding behavior has been reported in *Rhodnius prolixus* (Ayub et al., 2020). In *Rhyarobia maderae*, injection of SIFa delayed locomotor activity rhythms in a circadian time-dependently (Arendt et al., 2015). To date, few studies have documented the biological functions of SIFa in crustacean species. SIFa has been reported to regulate heartbeat in *C. borealis* and *Cancer irroratus* (Dickinson et al., 2019), and aggression and dominant behavior in the freshwater prawn *Macrobrachium rosenbergii* (Vázquez-Acedo et al., 2009).

The first SIFa receptor (SIFaR) was identified in *D. melanogaster* (Jørgensen et al., 2006). Subsequently, it was discovered in the genomes of the black-legged tick *Ixodes scapularis* (Šimo et al., 2013), silkworm *Bombyx mori* (Fan et al., 2010), red flour beetle *Tribolium castaneum* (Hauser et al., 2008) and bumblebee *Bombus terrestris* (Lismont et al., 2018). Two SIFa receptors exist in the American cockroach *Periplaneta americana* (Veenstra, 2021) and the brown planthopper *Nilaparvata lugens*

(Tanaka et al., 2014). It was observed that activation of its receptor by the SIFa neuropeptide mobilizes Ca^{2+} accumulation in the cytoplasm, which regulates the expression of downstream genes and induces corresponding physiological effects (Jékely, 2013). SIFa/SIFaR is homologous to the vertebrate gonadotropin inhibitory hormone (GnIH) and its receptor (GnIHR), which is conserved from fish to humans and play a key role in inhibiting reproduction (Ubuka and Tsutsui, 2014). consequently, we hypothesized that SIFa/SIFaR are involved in the regulation of reproduction in crustaceans was proposed in view of this similarity.

The mud crab (*S. paramamosain*) is an important marine culture crustacean. It takes a long time for female crabs to develop into gonadally mature crabs, which results in high culture costs in aquaculture and constrains the development of mud crab culture industry. In this study, we aimed to investigate the putative role of *Sp*-SIFa and its receptors in the ovarian development of mud crabs. First, cDNA sequences of *Sp*-SIFa and its possible receptors were obtained by transcriptome sequencing and molecular cloning. Subsequently, their expression profiles were determined by reverse transcription polymerase chain reaction (RT-PCR) and Real-time Quantitative polymerase chain reaction (qPCR). Finally, *in vitro* and *in vivo* experiments were performed to explore the putative effects of *Sp*-SIFa on ovarian development.

2 Materials and methods

2.1 Animals

Wild-caught female mud crabs (*S. paramamosain*) were purchased from a local fish market in Xiamen city of Fujian Province, China. They were acclimated for one week at a temperature of $28 \pm 1^\circ\text{C}$ and a salinity of 28 ± 0.5 ppt. During this period, the crabs were fed with clams (*Ruditapes philippinarum*) and the seawater was refreshed daily.

2.2 cDNA cloning and sequences analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and 2% agarose gel electrophoresis, respectively. The first-strand cDNA was generated from 1 μg of total RNA using a PrimeScriptTM RT reagent Kit with a gDNA Eraser (Takara, Japan). From the transcriptome library, the sequences encoding *Sp*-SIFa and its two receptors were screened, and the complete coding sequences were subsequently obtained by PCR.

The online website (<https://www.ncbi.nlm.nih.gov/>) was used to predicted the open reading frame (ORF) of *Sp*-SIFa and its two putative receptors. Domains of the protein sequences were predicted using the online website SMART (<https://smart.embl-heidelberg.de/>). Multiple sequence alignments were performed using an online tool (<https://www.bioladder.cn/web/#/chart/29>). A

phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates using MEGA (version 11). Since the SIFa receptors (SIFaRs) were only identified in insects, thus, SIFaRs in fifteen insect species and two crustacean species (*Portunus trituberculatus* and *Penaeus indicus*) were used for phylogenetic analysis.

2.3 Expression profiles of *Sp-SIFa*, *Sp-SIFaR1* and *Sp-SIFaR2*

Tissue distribution analysis was performed by RT-PCR. 11 of tissues (eyestalk ganglion, cerebral ganglion, thoracic ganglion, Y-organ, gill, heart, middle gut, ovary, hepatopancreas, muscle and stomach) were collected from crabs at the early vitellogenic stage. The PCR was conducted using the primer pairs of *Sp-SIFa*-qF/-qR, *Sp-SIFaR1*-qF/-qR and *Sp-SIFaR2*-qF/-qR respectively. Additionally, the housekeeping gene β -actin was amplified as an internal control, and the amplification of deionized water was used as a negative control. PCR was performed with Ex-Taq[®] DNA polymerase (Takara) under the following program: 95°C for 3 min, 35 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 30 s and followed by 72°C for 10 min. Finally, the PCR products were subjected to 2% agarose gel electrophoresis analysis and imaged using a UV gel imager.

Vitellogenesis of the mud crab *S. paramamosain* was divided into pre-vitellogenic stage, early vitellogenic stage, and late vitellogenic stage based on the characteristics, including color, gonadal development index, and histological features of the ovary (Huang et al., 2014). At the pre-vitellogenic stage, the ovaries are off white to creamy white, then turn to pale or light yellow at the early vitellogenic stage and finally orange to reddish-orange at the late vitellogenic stage. In this study, the expression profile of *Sp-SIFa*, *Sp-SIFaR1* and *Sp-SIFaR2* of the mud crabs (n = 4) at three different stages were detected by qPCR. Primers used in this study were listed in Table 1.

2.4 In vitro effects of *Sp-SIFa* peptide on *Sp-Vg* and *Sp-VgR* expression

The mature *Sp-SIFa* peptide (GYRKPPFNGSIFamide) was synthesized (GL Biochem Ltd, Shanghai, China) with a purity of 98% for the subsequent experiments. The sequence identity of the synthetic *Sp-SIFa* peptide was determined by mass spectrometry.

The crabs at the early vitellogenic stage were used for the *in vitro* experiment. Crabs were placed on ice for 10 minutes to anesthetize, and the ovary samples and hepatopancreas tissues were dissected from the crabs. The samples were washed five times with crab saline containing penicillin G (300 IU/ml) and streptomycin (300 IU/ml). Subsequently, the tissues were cultured in 500 μ L of L-15 medium containing penicillin G and streptomycin in 24-well plates at 26°C. After 1 hour, the tissues were treated with synthetic *Sp-SIFa* peptide at concentrations of 0, 1, 10, and 100 nM. The *Sp-SIFa* peptide was prepared in an L-15 medium containing penicillin G and streptomycin and each treatment was repeated five times (n = 5).

TABLE 1 Summary of primers used in this study.

Primer	Primer sequence (5'-3')	Application
<i>Sp-SIFa</i> -F	CCCAGCTCGCTCCCTCGCTT	PCR
<i>Sp-SIFa</i> -R	ATGGGAGTTAGATGCTGTGAC	PCR
<i>Sp-SIFaR1</i> -F	ATGGCTTCCTCTGACGTCAACAG	PCR
<i>Sp-SIFaR1</i> -R	TCATACTAAGGTGCTACACCCGT	PCR
<i>Sp-SIFaR2</i> -F	ATGAATACCTTGGGGAACACC	PCR
<i>Sp-SIFaR2</i> -R	GTGTGGTGTTCCTCAAGGTATTC	PCR
RV-M	GAGCGCATAACAATTTACACA	PCR
M13-47	CGCCAGGGTTTCCAGTCACG	PCR
<i>Sp-SIFa</i> -qF	TGTCCATGCAGATGCGAGT	qRT-PCR
<i>Sp-SIFa</i> -qR	ATTTGCCGGGTTCGTACACA	qRT-PCR
<i>Sp-SIFaR1</i> -qF	GTATCCCACTCTGCTGCTC	qRT-PCR
<i>Sp-SIFaR1</i> -qR	CTACGTGTCTCGGCAGATCC	qRT-PCR
<i>Sp-SIFaR2</i> -qF	GCCATCTGGTTTCCCATGAA	qRT-PCR
<i>Sp-SIFaR2</i> -qR	GCCACATCTCAACACACACG	qRT-PCR
<i>Sp-Vg</i> -qF	CGCAACCGCCACTGAAGAT	qRT-PCR
<i>Sp-Vg</i> -qR	CCACCATGCTGCTCAGACT	qRT-PCR
<i>Sp-VgR</i> -qF	TTCTATACCAGGCCACTACC	qRT-PCR
<i>Sp-VgR</i> -qR	TTTTCCTCCAAGCACACTC	qRT-PCR
β -actin-qF	GAGCGAGAAATCGTTCTGTGAC	qRT-PCR
β -actin-qR	GGAAGGAAGGCTGGAAGAGAG	qRT-PCR

For total RNA extraction and cDNA synthesis, ovary and hepatopancreas samples were collected at the 2nd, 4th, and 6th hour post *Sp-SIFa* treatment. Finally, the expressions of *Sp-VgR* (GenBank accession number: KF860893.1) and *Sp-Vg* (GenBank accession number: FJ812090.1) were detected by qPCR to explore the potential effects of *Sp-SIFa* on vitellogenesis.

2.5 In vivo effect of *Sp-SIFa* peptide on ovarian development

After establishing the stimulatory effect of *Sp-SIFa* on vitellogenesis in *S. paramamosain*, we performed *in vivo* experiments to further explore its specific involvement in ovarian development. In the short-term experiment, mud crabs in the early vitellogenic stage were randomly divided into two groups, each has five individuals (n = 5). Crabs were then injected with *Sp-SIFa* peptide (15 ng/g body mass) prepared in 100 μ L crab saline, whereas the control group was received 100 μ L crab saline instead. Samples of ovary and hepatopancreas were dissected from the crabs at 12th h post the injection for *Sp-Vg* and *Sp-VgR* gene expression analysis.

Based on the results of the short-term experiment, a long-term experiment was conducted by prolonged injection of the *Sp-SIFa* peptide into mud crabs at the early vitellogenic stage. The crabs were randomly divided into three groups, with five individuals in

each group ($n = 5$). Before the injection, a group of crabs was sampled as a pre-injection control. Mud crabs were injected with *Sp-SIFa* peptide (15 ng/g body mass) prepared in 100 μ L crab saline once every five days, while the control group received 100 μ L crab saline instead. On the 16th day, approximately 24 h after the third injection, crabs were put on ice, and samples of ovary and hepatopancreas were collected for gene expression analysis. Moreover, the histological changes of the ovary in response to prolonged injection of *Sp-SIFa* were determined by hematoxylin and eosin staining and the index of gonadal development (GSI) was measured and calculated following a standard function, as follows: $GSI = (\text{gonad weight/body weight}) \times 100\%$.

2.6 qPCR assay

The qPCR was performed on a QuantStudio 5 Real-time PCR machine (Applied Biosystems) using PowerUpTM SYBRTM Green Master Mix (Thermo Fisher Scientific) in a total reaction volume of 20 μ L. The reaction mixture consisted of PowerUpTM SYBRTM Green Master Mix (10 μ L), 10-fold diluted cDNA (2 μ L), forward and reverse primers (1 μ L each), and deionized water (6 μ L). The reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and a subsequent melting curve analysis spanning 60 - 95°C.

2.7 Statistical analyses

The qPCR data were calculated using the $2^{-\Delta\Delta C_t}$ method and presented as mean \pm standard error of the mean (SEM). Statistical differences were analyzed using a one-way analysis of variance followed by Duncan's test or Student's t-test, using the Statistical Package for the Social Sciences software (version 26.0). Differences were considered statistically significant when $p < 0.05$.

3 Results

3.1 Cloning and sequence analysis of *Sp-SIFa* and its putative receptors

The ORF of *Sp-SIFa* (GenBank accession number: PQ519603) was 237 bp in length, encoding a 78 aa precursor (Figure 1A). The precursor contained a 27 aa signal peptide, a 12 aa mature peptide (GYRKPPFNGSIFamide), a dibasic cleavage site, and a related peptide (Figure 1B). Sequence alignment revealed that *Sp-SIFa* was highly homologous to SIFa in other crustaceans and insects (Figure 2).

In addition, two putative receptors for SIFa were screened from the transcriptome dataset, and further identified from the ovary of *S. paramamosian* in this study by PCR method. They differ in

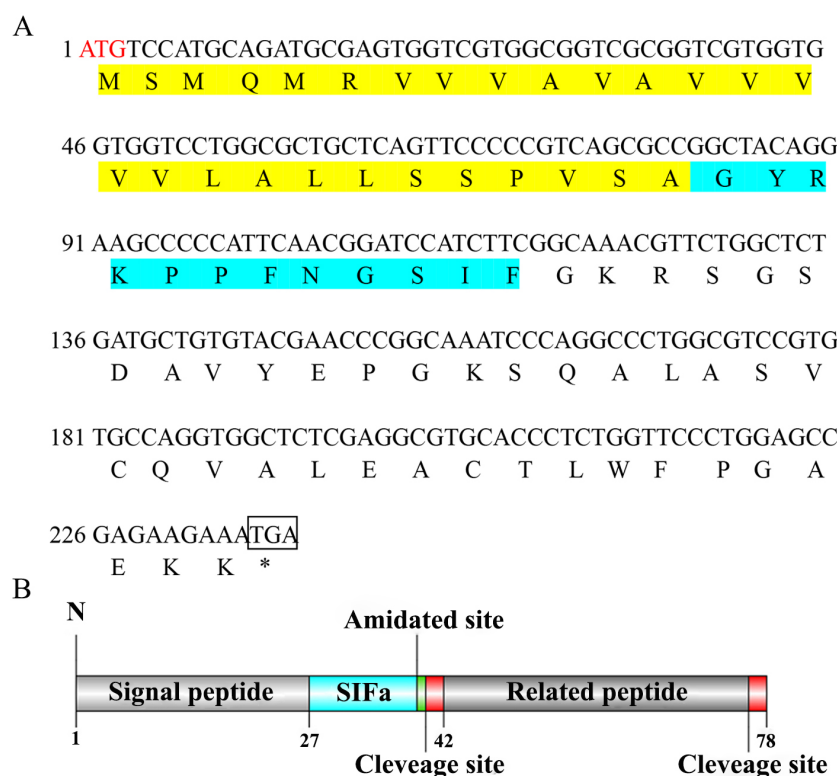
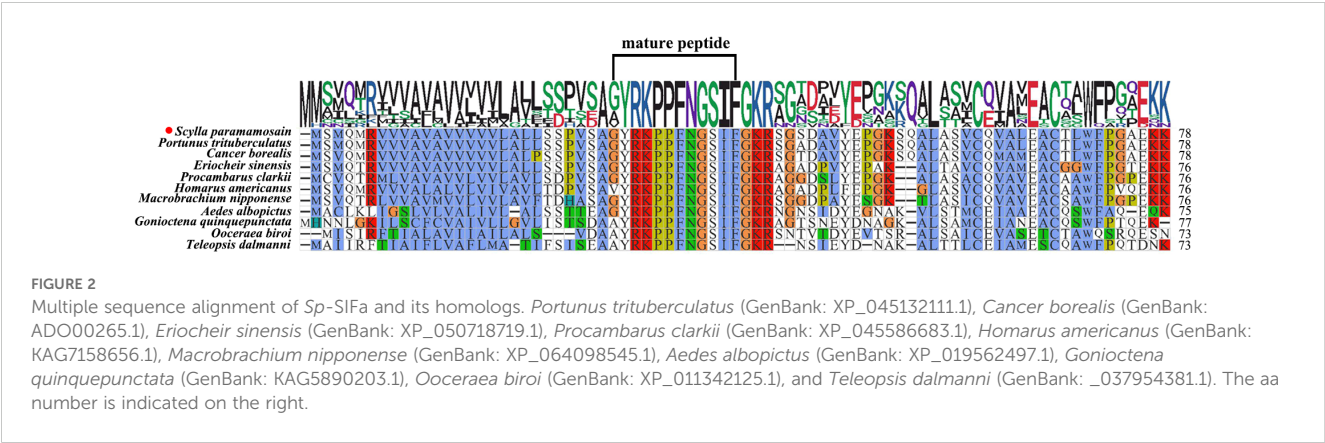


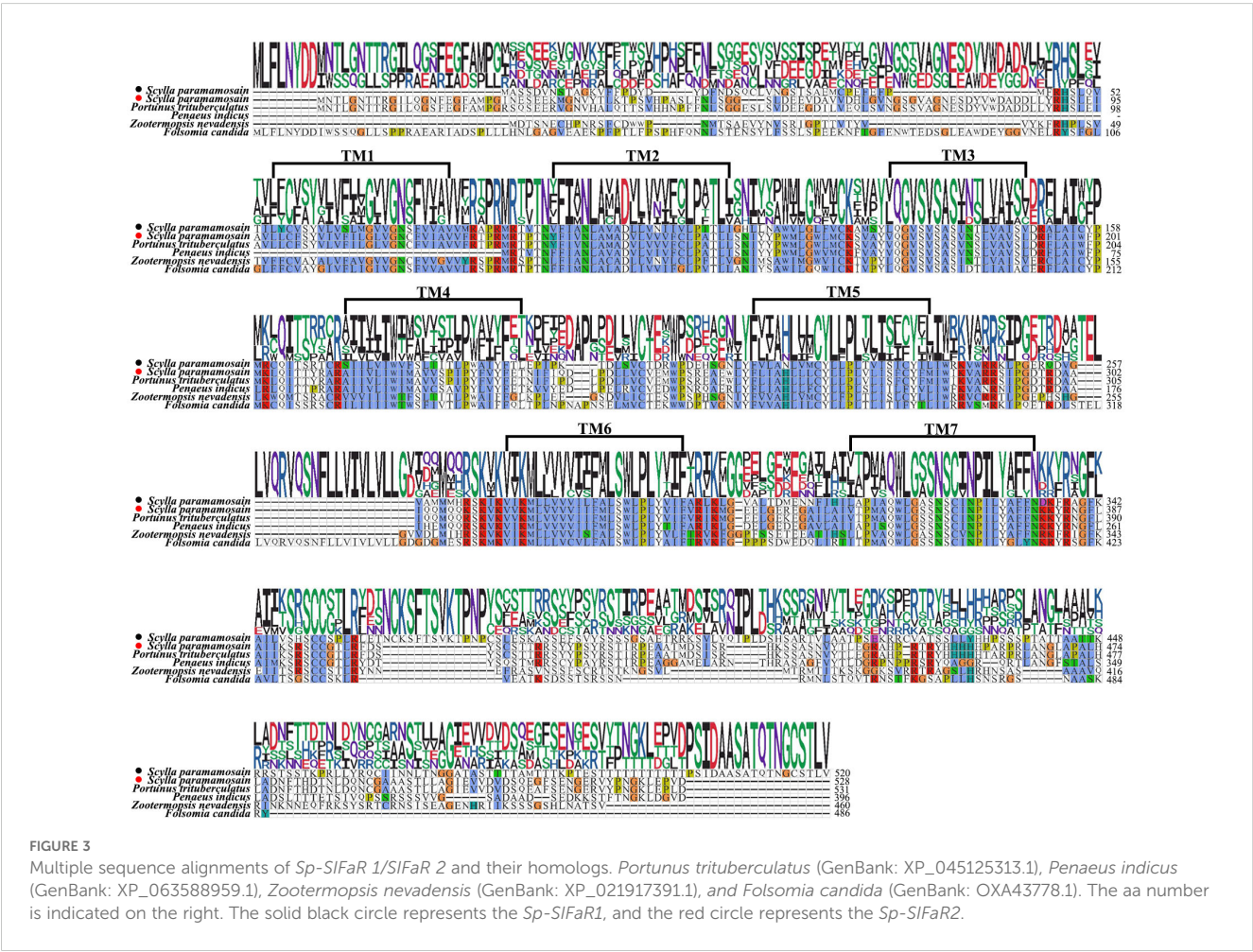
FIGURE 1

(A) Nucleotide and deduced aa sequence of *Sp-SIFa*. The ORF is indicated by a single-letter code below the nucleotide sequence. The signal and mature peptides are highlighted in yellow and green, respectively, and the initiation codon, amidated, and dibasic cleavage sites are indicated in red, gray, and blue, respectively. (B) Schematic representation of structural domains of *Sp-SIFa*.



sequence length, *Sp-SiFaR1* (GenBank accession number: PQ519601) was 1563 bp in length, which encodes a 520 aa protein, while *Sp-SiFaR2* (GenBank accession number: PQ519602) was 1587 bp in length, encoding a 528 aa protein. The sequence analysis indicated that these two receptors possess seven conserved transmembrane structural domains, exhibiting a

high similarity to the SiFaRs reported in other crustaceans and insects (Figure 3). However, *Sp-SiFaR1* and *Sp-SiFaR2* have low sequence similarity between them. The phylogenetic tree demonstrated that *Sp-SiFaR1* and *Sp-SiFaR2* were categorized into different branches. *Sp-SiFaR1* was clustered with SiFaRs in *N. lugens*, *Zootermopsis nevadensis*, *P. indicus*, and *Frankliniella*



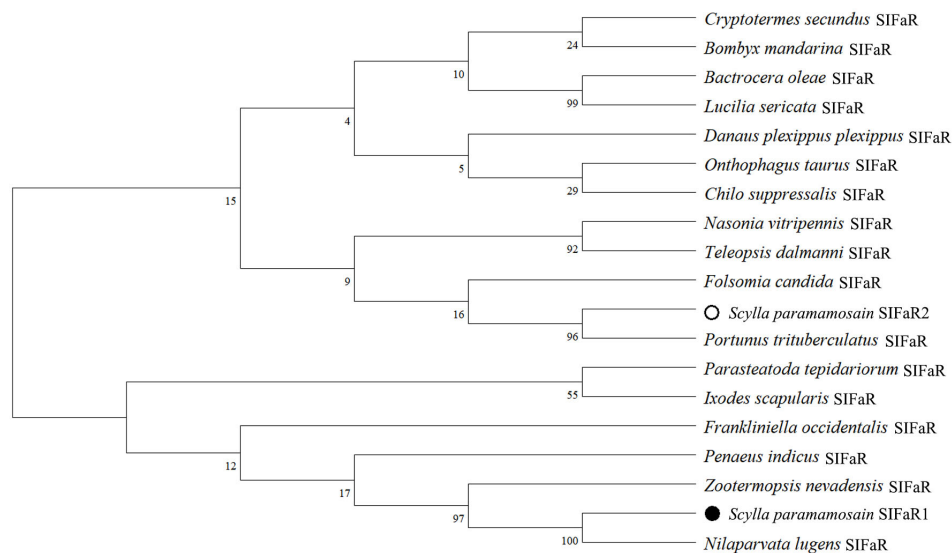


FIGURE 4

Phylogenetic analysis of SIFaR in crustaceans and insects. *Danaus plexippus* (GenBank: XP_032516043.1), *Chilo suppressalis* (GenBank: ALM88339.1), *Bombyx mandarina* (GenBank: XP_028044101.1), *Nasonia vitripennis* (GenBank: XP_0016000098.3), *Onthophagus taurus* (GenBank: XP_022919670.1), *Cryptotermes secundus* (GenBank: XP_023712423.2), *Nilaparvata lugens* (GenBank: BAO01053.1), *Lucilia sericata* (GenBank: XP_037808011.1), *Bactrocera oleae* (GenBank: XP_014089790.2), *Teleopsis dalmanni* (GenBank: XP_037955898.1), *Parasteatoda tepidariorum* (GenBank: XP_015924497.2), *Ixodes scapularis* (GenBank: XP_002406273.3), *Folsomia candida* (GenBank: OXA43778.1), *Zootermopsis nevadensis* (GenBank: XP_021917391.1) and *Frankliniella occidentalis* (GenBank: XP_052132360.1), *Portunus trituberculatus* (GenBank: XP_045125313.1), *Penaeus indicus* (GenBank: XP_063588959.1). The solid circle represents the *Sp-SIFaR1*, and the hollow circle represents the *Sp-SIFaR2*.

occidentalis. *Sp-SIFaR2* fit into a clade with SIFaRs in *P. trituberculatus*, *Folsomia candida*, *Teleopsis dalmanni*, and *Nasonia vitripennis* (Figure 4).

3.2 Expression profiles of *Sp-SIFa* and its putative receptors in female *S. paramamosain*

RT-PCR was conducted to determine the tissue expression of *Sp-SIFa* and its receptors in female *S. paramamosain*. *Sp-SIFa* exhibited high expression levels in the cerebral ganglion, followed by the eyestalk ganglion, middle gut, and thoracic ganglion. Additionally, *Sp-SIFaR1* was highly expressed in the cerebral ganglion and ovary and moderately expressed in the eyestalk ganglion and hepatopancreas. However, the *Sp-SIFaR2* expression was confined to the ovary and eyestalk ganglion (Figure 5A).

The expression profiles of *Sp-SIFa* and its two receptors at three different vitellogenic stages were determined using qPCR. *Sp-SIFa* was highly expressed in the eyestalk ganglion at the pre-vitellogenic stage, and its expression was significantly down-regulated at the late stage of vitellogenesis ($P < 0.001$; Figure 5B). However, an opposite expression profile was observed in the cerebral ganglion, which revealed that the level of *Sp-SIFa* transcript was low at the pre-vitellogenic stage and subsequently increased at the late stage of vitellogenesis ($P < 0.001$; Figure 5B). *Sp-SIFaR1* was highly expressed in the pre-vitellogenic hepatopancreas, and its expression significantly decreased in the early vitellogenic stage ($P < 0.01$; Figure 5C). In contrast, its expression level in the ovary was low at the pre-vitellogenic stage, significantly increased at the

early vitellogenic stage ($P < 0.05$), and subsequently reached the highest level at the late vitellogenic stage (Figure 5C). Additionally, the level of *Sp-SIFaR2* transcript in the eyestalk ganglion and ovary was low at the pre-and early vitellogenic stages but increased significantly in the late stage of vitellogenesis ($P < 0.05$ in the eyestalk ganglion; $P < 0.0001$ in the ovary; Figure 5D).

3.3 *In vitro* effects of *Sp-SIFa* peptide on *Sp-Vg* and *Sp-VgR* expression

To elucidate the potential role of *Sp-SIFa* in vitellogenesis, synthetic *Sp-SIFa* was applied to the hepatopancreatic and ovarian explants. Upon stimulation with *Sp-SIFa*, dynamic changes in the levels of *Sp-Vg* and *Sp-VgR* transcripts were detected using qPCR. The expression of *Sp-Vg* in the hepatopancreatic explants was significantly induced after 4 h of incubation with 10 nM *Sp-SIFa* peptide ($P < 0.01$; Figure 6B). The *Sp-VgR* transcript levels in the ovarian explants increased significantly when stimulated by *Sp-SIFa* peptide at 1 nM and 10 nM concentrations (Figure 6D) for 2 h ($P < 0.001$) and after treatment with *Sp-SIFa* for 4 h at concentrations of 10 nM ($P < 0.001$) and 100 nM ($P < 0.01$; Figure 6E).

3.4 *In vivo* effects of *Sp-SIFa* peptide on ovarian development

Experiments by injection of *Sp-SIFa* peptide into mud crab at stage III were performed to further confirm its role in ovarian

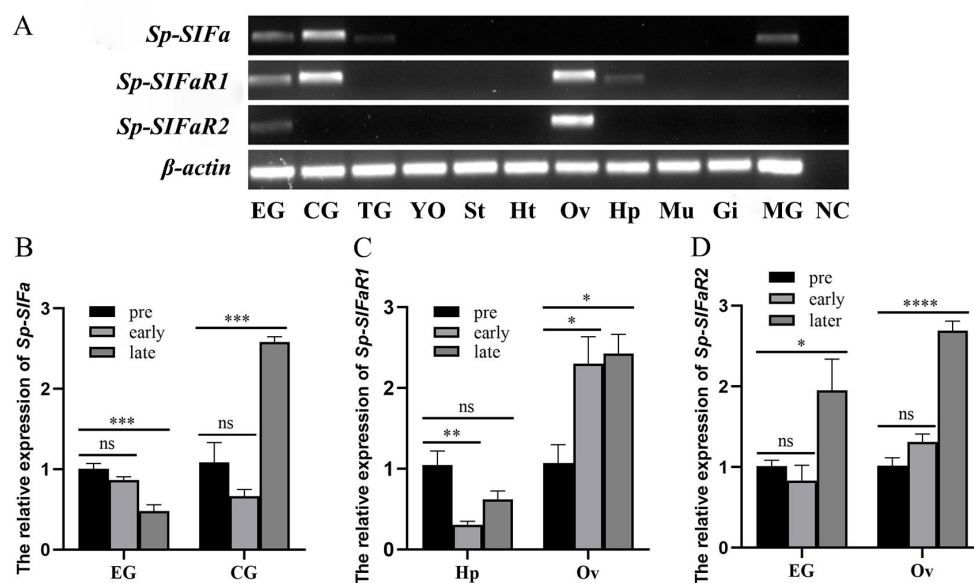


FIGURE 5

Expression profiles of *Sp-SIFa* and its putative receptors in female *S. paramamosain*. (A) Tissue distribution of *Sp-SIFa* and its putative receptors. β -actin was included as an internal reference. EG, eyestalk ganglion; CG, cerebral ganglion; TG, thoracic ganglion; YO, Y-organ; St, stomach; Ht, heart; Ov, ovary; Hp, hepatopancreas; Mu, muscle; Gi, gill; MG, middle gut; NC, negative control (amplification of deionized water). (B–D) The expression pattern of *Sp-SIFa* in the eyestalk ganglion and cerebral ganglion, *Sp-SIFaR1* in the hepatopancreas and ovary, and *Sp-SIFaR2* in the eyestalk ganglion and ovary during vitellogenesis. Pre: pre-vitellogenic stage; early: early vitellogenic stage; late: late vitellogenic stage. The data are presented as mean \pm SEM ($n = 4$). Asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) indicate significant differences from the pre-vitellogenic stage. ns indicates no significant difference from the pre-vitellogenic stage.

development. The results of the short-term experiment revealed that the expression of *Sp-Vg* in the hepatopancreas and *Sp-VgR* in the ovary was significantly induced by a 12-h injection of the *Sp-SIFa* peptide ($P < 0.05$; Figure 7).

In the long-term experiments, prolonged injection of *Sp-SIFa* significantly induced the expression of *Sp-VgR* ($P < 0.05$)

(Figure 8B) and increased the GSI ($P < 0.05$) of *S. paramamosain* (Figure 8C). However, the level of *Sp-Vg* expression in hepatopancreas did not change significantly at the sampling point (Figure 8A). Concurrently, histologic analysis further showed that oocyte diameter and the number of yolk granules were significantly increased ($P < 0.001$) (Figure 9). These results indicate that *Sp-SIFa*

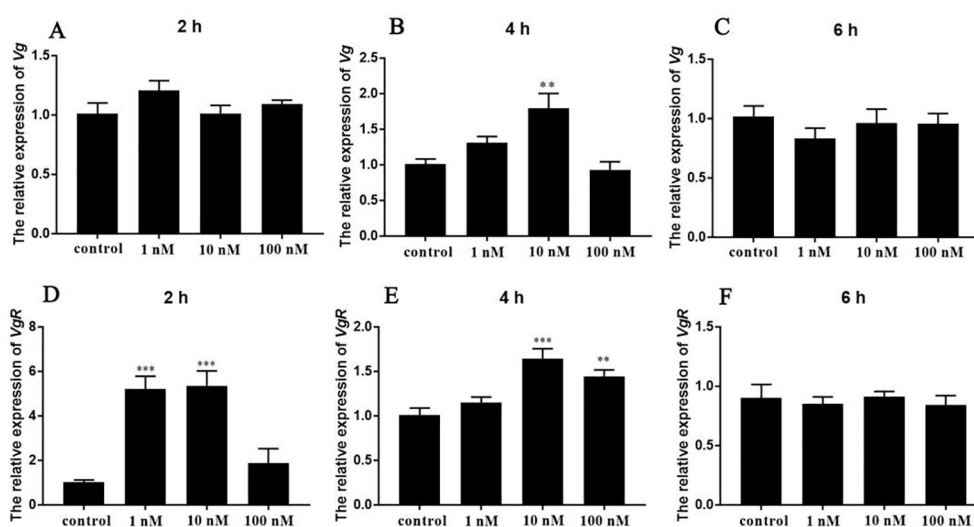


FIGURE 6

In vitro effects of synthetic *Sp-SIFa* peptide on the expression of *Sp-Vg* and *Sp-VgR*. (A–C) Relative expression of *Sp-Vg* in the hepatopancreas in response to the *Sp-SIFa* peptide. (D–F) Relative expression of *Sp-VgR* in the ovary in response to the *Sp-SIFa* peptide. Data are presented as mean \pm SEM ($n = 5$). Asterisks (** $P < 0.01$; *** $P < 0.001$) indicate significant differences from the control.

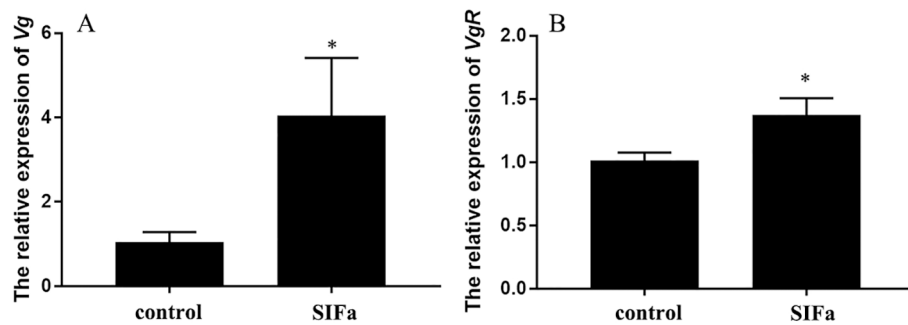


FIGURE 7

Relative expression of *Sp-Vg* (A) in the hepatopancreas and *Sp-VgR* (B) in the ovary after 12 h injection of *Sp-SIFa* peptide. Data are presented as mean ± SEM (n = 5). Asterisks (**P* < 0.05) on the error bar indicate significant differences from the control.

may promote ovarian development in mud crabs by facilitating oocyte uptake of Vg and oocyte growth.

4 Discussion

SIFa is extensively present in the central nervous systems of arthropods, and its conservation across various species suggests its potential significance (Verleyen et al., 2009). Leveraging advancements in high-throughput sequencing technology, SIFa has been widely recognized in several crustaceans (Sithigorngul et al., 2002; Dickinson et al., 2008; Huybrechts et al., 2003; Yasuda et al., 2004; Ma et al., 2009); however, its physiological functions remain largely unexplored. This study characterized SIFa and its two putative receptors and described their expression profiles in the mud crab *S. paramamosain*, illustrating a potential stimulatory role in ovarian development.

In the mud crab *S. paramamosain*, sequences encoding an *Sp-SIFa* precursor and its two potential SIFa receptors were identified. The *Sp-SIFa* precursor exhibited a characteristic similarity with the documented insect SIFa, comprising a signal peptide, mature peptide, and precursor-related peptide (Ayub et al., 2020). There was an amidated signal at the C-terminus of the mature peptide (Figure 1), suggesting it was an amidated hormone. Sequence alignment revealed that the crustacean SIFa precursor exhibited high sequence similarity to that in insects, suggesting that SIFa is highly conserved among arthropods (Figure 2). Previous research

has demonstrated that SIFaR is structurally related to the GnIHR; however, there is low similarity between their ligands SIFa and GnIH (Ubuka and Tsutsui, 2014; Elphick and Mirabeau, 2014). It has been proposed that GnIH/GnIHR and SIFa/SIFaR may be distinct from the ancestral Famide peptide system, which is a family of peptides that have independently co-evolved in vertebrates and insects, including FMRFamide, neuropeptide F, short neuropeptide F, sulfakin, myosuppressin and SIFa (Ubuka and Tsutsui, 2014). G protein-coupled receptors (GPCRs) are the largest family of receptors characterized by seven α -helical transmembrane structural domains, which are involved in regulating a wide variety of physiological processes (Horn et al., 1998; Pierce et al., 2002). The GPCRs are classified into six major classes, and SIFaR belongs to class A, Rhodopsin-like receptors (Šimo et al., 2013). Sequence alignment revealed that SIFa receptors from crustaceans and insects exhibited relatively high conservation in the seven transmembrane structural domains (Figure 3). In *S. paramamosain*, the two SIFaRs (*Sp-SIFaR1* and *Sp-SIFaR2*) exhibited low sequence identity (less than 32%), implying that they may be involved in a distinct physiological process.

In insects, SIFa is mainly expressed in the nervous system (Ayub et al., 2020). In crustaceans, including *H. americanus* and *P. clarkii*, SIFa is predominantly expressed in the stomatogastric nervous system and the middle gut (Dickinson et al., 2008). Similarly, the *Sp-SIFa* transcript is confined to nervous tissues (eyestalk, cerebral, and thoracic ganglion) and the middle gut of *S. paramamosain* (Figure 5A), suggesting that SIFa functions as a brain-gut hormone

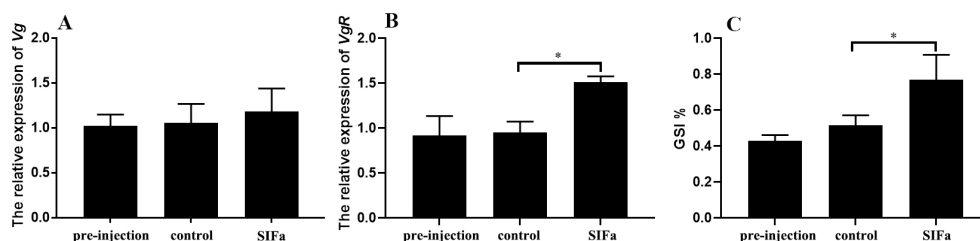


FIGURE 8

Relative expression of *Sp-Vg* (A) in the hepatopancreas, *Sp-VgR* (B) in the ovary, and GSI % (C) after 15 days of administration of *Sp-SIFa* peptide. Data are presented as mean ± SEM (n = 5). Asterisks (**P* < 0.05) on the error bar indicate significant differences from the control.

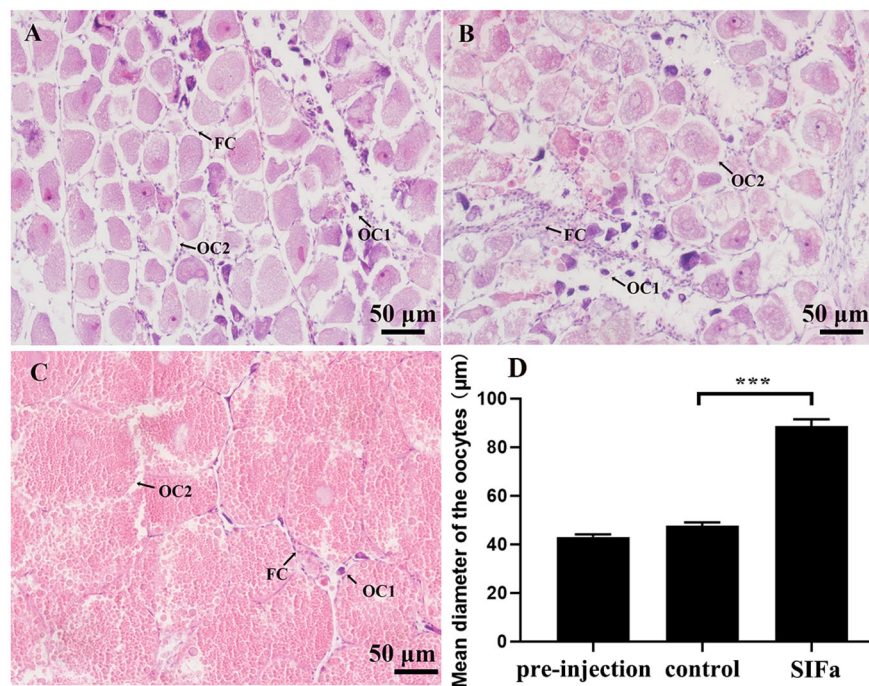


FIGURE 9

Histological changes in *S. paramamosain* ovary at the early vitellogenic stage in response to administration of synthetic *Sp-SIFa* peptide. (A) Pre-injection control. (B) Crab saline treatment (saline control). (C) *Sp-SIFa* peptide treatment. OC1, pre-vitellogenic oocyte; OC2, early vitellogenic oocyte; FC, follicular cell. Scale bars: 50 μm. (D) Mean oocyte diameter. Asterisk (*** $P < 0.001$) indicates significant differences from the control.

in most crustaceans. SIFa is present in the nerve fibers of the olfactory lobes of certain insect species, suggesting a possible neuromodulatory role for SIFa in the olfactory system (Verleyen et al., 2004). In *Drosophila*, SIFa is expressed in only four medial neurons of the brain (Terhzaz et al., 2007), which can inhibit sexual behavior (Dickson, 2008), promote sleep (Park et al., 2014), and induce appetitive and feeding behaviors (Martelli et al., 2017). In *R. prolixus*, SIFa is released by the interneurons and dorsal vessel, where it also has a role in facilitating ingestion and inducing heart rate in a dose-dependent manner (Ayub et al., 2020). Similarly, SIFa functions as a cardioactive peptide in *C. borealis* and *C. irroratus* to promote cardiac output, however, this regulation differs from that of *R. prolixus*. SIFa appears to be synthesized in the middle gut and delivered to the heart to regulate *C. borealis* and *C. irroratus* (Dickinson et al., 2019). In this study, *Sp-SIFa* was highly expressed in the cerebral ganglion, implying that it may be involved in similar physiological activities, including reproduction, sleeping, and feeding, which was further supported by the wide expression of *Sp-SIFaR1* (Figure 5A). *Sp-SIFaR1* was extensively expressed in the eyestalk ganglion, cerebral ganglion, ovary, and hepatopancreas, whereas the *Sp-SIFaR2* expression was restricted to the eyestalk ganglion and ovary (Figure 5A). This outcome is broadly comparable to the tissue distribution of *SIFaR* in insects (Sellami and Veenstra, 2015; Lismont et al., 2018; Veenstra, 2021). Moreover, the *Sp-SIFa* expression in the eyestalk and cerebral ganglia was significantly altered during vitellogenesis, implying its possible role in ovarian development (Figure 5B). The presence of two *Sp-SIFa* receptors in the ovary of *S.*

paramamosain further supports their significance in regulating ovarian development. *Sp-SIFaR1* levels was significantly elevated in the early and late vitellogenic stages (Figure 5C), while *Sp-SIFaR2* was significantly increased at the late vitellogenic stage (Figure 5D). These results indicated that *Sp-SIFaR1* and *Sp-SIFaR2* may involve in different regulation during ovarian development, which warrants future study. Two receptors for SIFa in *I. scapularis* collaborate to regulate salivary secretion (Guerrib et al., 2023).

Vitellogenesis, the process by which Vg synthesized by the hepatopancreas and ovary is endocytosed into the oocyte mediated by the Vg receptor, is essential for ovarian development in crustaceans (Subramoniam, 2010). Although vitellogenesis in crustaceans has been extensively studied, the mechanisms underlying its regulation remain incompletely comprehended. Multifunctional neuropeptides, neurotransmitters, neuromodulations, and other factors also regulate ovarian development in crustaceans (Subramoniam, 2010). VIH, crustacean hyperglycaemic hormone (CHH), and molt-inhibiting hormone (MIH) are eyestalk peptides of the CHH family. VIH inhibits the Vg production in the hepatopancreas and the Vg uptake by oocytes during vitellogenesis (Chen et al., 2014; Qiao et al., 2015). In contrast, CHH and MIH may act as positive regulators of vitellogenesis and ovarian development (de Kleijn et al., 1998; Luo et al., 2015). 5-HT is a neurotransmitter that significantly contributes to ovarian development by modulating downstream endocrine factors, hormones, and reproduction-associated proteins (Jayasankar et al., 2020). The present study revealed the role of *Sp-SIFa* in the vitellogenesis of mud crabs by detecting the effects of *Sp-SIFa* on the expression of *Sp-Vg* and *Sp-VgR*. The *in vitro* results demonstrated that

Sp-SIFa significantly induced *Sp-Vg* expression in the hepatopancreas and *Sp-VgR* expression in the ovary (Figure 6). Short-term injection of SIFa into female crabs consolidated its stimulatory effects on vitellogenesis by promoting *Sp-Vg* and *Sp-VgR* expression (Figure 7). A similar effect was observed with the addition of hEGF protein, which can induce *Sp-VgR* expression (Lu et al., 2020). In the long-term experiment, the *Sp-Vg* level did not change significantly (Figure 8), which may have been caused by the sampling point. However, the level of *Sp-VgR* (Figure 8), GSI, and oocyte diameter (Figure 9) were significantly increased by the prolonged injection of *Sp-SIFa*, suggesting a stimulatory role in ovarian development.

In conclusion, this study isolated *Sp-SIFa* and its two putative receptors from mud crabs. It delineated their expression patterns in females, offering insight into their physiological activities. This study further confirmed the stimulatory role of *Sp-SIFa* in ovarian development by promoting Vg biosynthesis and oocyte uptake.

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 studies were approved by Animal Care and Use Committee of the Fisheries College of Jimei University (Approval Code: 2021-04; Approval Date: January 22, 2021). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SC: Formal analysis, Investigation, Writing – original draft. YT: Formal analysis, Investigation, Writing – original draft. LL: Formal

analysis, Investigation, Writing – original draft. SG: Investigation, Writing – original draft. AL: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. HY: Conceptualization, Funding acquisition, Writing – review & editing.

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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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