RECENT ADVANCES IN CRUSTACEAN ENDOCRINOLOGY

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RECENT ADVANCES IN CRUSTACEAN ENDOCRINOLOGY

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EDITORIAL
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Editorial: Recent Advances in Crustacean Endocrinology

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Editorial on the Research Topic

Recent Advances in Crustacean Endocrinology

Crustacea constitute an important taxonomic group found throughout global aquatic ecosystems; their various physiological processes and life functions are regulated by the crustacean endocrine system working in concert with the nervous system. The objective of this Special Issue was to provide a forum for researchers to report upon cutting-edge research in Crustacean Endocrinology carried out using a variety of experimental models. This Research Topic contains 15 contributions, comprising eight original research articles, two brief research reports, and five reviews.

At the outset, crustacean eyestalk hormones are of great interest due to their key roles in controlling multiple physiological processes, among these, metabolic function, osmoregulation, molting, and reproduction. In this Special Issue, Meng et al. reported that in the swimming crab *Portunus trituberculatus*, ovarian proteome and miRNA profiles were altered following eyestalk ablation, and that miRNA-protein network analysis suggested that miRNAs are involved in promoting ovarian maturation by controlling the expression of proteins related to methyl farnesoate synthesis, calcium signaling, and energy metabolism. Further regarding *P. trituberculatus*, Jiang et al. found that the insulin receptor (Pt-IR) and the insulin-like growth factor-binding protein (Pt-IGFBP) may be the targets of eyestalk neuropeptides and thus respond to eyestalk ablation independently from insulin-like androgenic gland (IAG) hormone regulation. Kang et al. employed the whiteleg shrimp, *Litopenaeus vannamei*, and injected dsRNA corresponding to multiple sinus gland peptides (SGPs) into subadults; it was found that expression was significantly decreased for *SGP-G*, the most predominant isoform expressed in the eyestalks, while vitellogenin (Vg) gene expression in the ovaries and concentrations of Vg protein in the hemolymph were not changed by this treatment. In the mud crab *Scylla paramamosain*, Liao et al. investigated the transcriptional regulation of Vih (*SpVih*) and revealed that the binding site of Oct4/Sox9 transcription factor may be the key region for the positive regulation of the expression of *SpVih*. Also of interest, crustacean female sex hormone (CFSH) is a key regulator of crustacean sexual differentiation. Jiang et al. demonstrated that *Sp-CFSH* is expressed exclusively in the eyestalks in *S. paramamosain*, and that DNA methylation inhibits *Sp-CFSH* expression by blocking the binding of transcription factor Sp1. Sun et al. analyzed the eyestalk transcriptome of the oriental river prawn, *Macrobrachium nipponense* during salinity acclimation and found that 1,392 and 1,409 genes were differentially expressed in the eyestalks in response to conditions of low and high salinity. In a review by Chen et al. on the crustacean hyperglycemic hormone superfamily, the identification of the first receptors functioning for ion

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transport peptides (ITPs) in the silkworm as insect members of this superfamily was highlighted; this content is expected to provide impetus to other researchers to conduct further functional studies on these peptides in the near future.

In addition to eyestalk neurohormones, other hormones and factors are also addressed in this Special Issue. Toyota et al. investigated the *in vivo* physiological functioning of methyl farnesoate and 20-hydroxyecdysone during the larval stages of the kuruma prawn *Marsupenaeus japonicus*, shedding light not only on the ecotoxicological impacts of insect growth regulators (IGRs), but also on endocrine mechanisms underlying larval metamorphosis in benthic decapod crustaceans. Tsutsui et al. investigated gonadal peptide hormones and peptide hormone receptors by analyzing the transcriptome of the ovary of *M. japonicus*. The ovarian transcriptome data thus generated led to the identification of five candidate peptide hormones, including bursicon-α and -β, crustacean hyperglycemic hormone (CHH)-like peptide, insulin-like peptide (ILP), and neuroparsin-like peptide (NPLP). These results further suggested that various gonadal peptide hormones akin to those in vertebrates regulate reproductive physiology in crustaceans, although the actual substances differ. Bao et al. identified a total of 61 peptide and 40 G-protein coupled receptors transcripts from the peppermint shrimp *Lysmata vittata*. Among these, both IAG hormone and CFSH were each revealed to possess two unique mature peptides, and their transcripts showed higher expression levels in the male phase than in the euhermaphrodite phase. This suggested that these sexual differentiation hormones may be involved in producing sexual characteristics rather than promoting spermatogenesis or vitellogenesis. In relation to reproduction, Wang et al. investigated the Vg gene family in *L. vannamei*, suggesting that Vg as a substance may have some relation to growth and molt-related processes in addition to serving as a source of nutrients during the reproductive process. Furthermore, regarding this topic, Jayasankar et al. commented on the roles of wide-ranging vitellogenesis-stimulating factors, and their potential molecular functioning and associated pathways in decapod crustaceans. Levy and Sagi reviewed the crustacean IAG-switch, a unique crustacean endocrine mechanism, the existence of which has become clear based on earlier discoveries of the androgenic gland and IAG hormone and of more recent IAG-switch-based manipulations. These authors moreover discussed this unique, early pan-crustacean, insulin-based, sexual differentiation control mechanism in contrast to the extensively-studied mechanisms relating to vertebrate sex steroid function.

Also in this Special Issue, Mykles reviewed the extensive signaling pathways regulating the crustacean molting gland. The Y-organs transition through four physiological states over the molting cycle; these are sequentially mediated by molt-inhibiting hormone (MIH; basal state), mechanistic Target of Rapamycin complex 1 (mTORC1; activated state), Transforming Growth Factor-beta (TGFbeta)/Activin (committed state), and ecdysteroids (repressed state). Future research should focus on the interactions of such signaling pathways that integrate physiological status with assorted environmental cues, in order to obtain a fully holistic understanding of crustacean molt control.

Finally, relating to the field of endocrine disruption, Knigge et al. presented an extensive overview of the evolution of the crustacean endocrine system, highlighting known endocrine endpoints that are targets of chemical disruption, and identifying other components of endocrine signaling that may prove to be targets of disruption. This review highlights the fact that endocrine disruption in crustaceans needs to be more deeply evaluated with respect to their unique endocrine systems, at least for those of several model crustaceans used in ecotoxicology, as they differ considerably from those of vertebrate species.

In conclusion, as Guest Editors of this Special Issue, we would like to express our sincerest thanks to all of the authors for their valuable contributions to this Research Topic, to the many reviewers who generously gave their time allowing the research contributions to be presented in their best light, and to the regular editors serving *Frontiers in Endocrinology* and the relevant journal personnel for their guidance throughout this project. We hope that this Special Issue will not only serve as useful reference material but also as a stimulus to all those involved in research on Crustacean Endocrinology.

AUTHOR CONTRIBUTIONS

HY wrote the draft. MW, HD, and JC revised the text. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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DNA Methylation Inhibits the Expression of CFSH in Mud Crab

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Crustacean female sex hormone (CFSH) is a key regulator of crustacean sex differentiation. The expression of *Sp-CFSH* in the mud crab *Scylla paramamosain* showed a tissue-specific and gender-variant pattern. To explore the role of DNA methylation in *Sp-CFSH* expression, the 5′-flanking region of *Sp-CFSH* was cloned, and one CpG island containing 12 CpG sites was found. Results of sodium bisulfite sequencing and methylated DNA immunoprecipitation showed that CpG island methylation was stable in the eyestalk ganglion during ovarian development of the females, which was significantly lower than that in the muscle of the females and in the eyestalk ganglion of the males. Such results suggested that the involvement of DNA methylation in regulating *Sp-CFSH* expression followed an eyestalk ganglion-specific and gender-variant pattern. The analysis of CpG dinucleotide site methylation and activity of the site-directed mutation (SDM) reporter vector further demonstrated that methylation inhibited *Sp-CFSH* expression by blocking the binding of transcription factor Sp1. The finding suggested for the first time the involvement of CpG methylation in the regulation of *Sp-CFSH* expression.

Keywords: crustacean female sex hormone, promoter, DNA methylation, epigenetics, mud crab

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INTRODUCTION

The regulation of eukaryotic gene expression is complex and rigorous, and influenced by various levels, such as the genetic level of DNA regulation, epigenetic level of chromatin regulation, the post-transcription level of RNA regulation, the translation and protein processing regulation, phosphorylation and acetylation of protein, heat shock protein regulation, etc. (1–4). For a given gene, intergenic regions play an important role in the regulation (5–7). The eukaryotic promoter, a region with various regulatory elements, is located upstream of the gene and determines the level of expression via different regulatory factors (8, 9).

Methylation of genomic DNA, as a major epigenetic modification, generally modulates transcription by influencing the binding of regulatory factors to regulatory elements (10). In vertebrates, DNA methylation is throughout the genome and involved in silencing of gene expression during cellular differentiation and development (11–15). CpG island is the major target for methylation and plays a vital role in inhibiting gene expression (16). Dynamic methylation pattern of CpG island in a core promoter is involved in the regulation of gene expression (17). In insects, DNA methylation is restricted to the transcribed regions and primarily involved in behavioral plasticity and social behavior (17–22). Unlike mammals and insects, studies on DNA methylation are relatively rare in crustaceans and have so far been reported only in the water flea, *Daphnia magna* and *Daphnia pulex*. In *D. magna*, DNA methylation levels can be affected by Zn exposure and entailed at different histories (23, 24). In *D. pulex*, the 5-methyl-cytosine (5-mC) and

5-hydroxymethyl-cytosine (5-hmC) can inhibit the expression of the cullin-associated NEDD8-dissociated 2 (*Cand2*) gene, cytochrome C oxidase subunit IV (*Cox4*) gene, and juvenile hormone epoxide hydrolase 1 (*Ephx1*) gene (25).

Crustacean female sex hormone (CFSH), a key regulator of sex differentiation, has been shown to regulate reproductive processes such as the development of sexually dimorphic traits and expression of androgenic gland hormone (IAG) (26–28). The ontogenic of *CFSH* expression was detected in embryos at hatching stage in the blue crab, *Callinectes sapidus* (26). In the mud crab, *Scylla paramamosain*, *Sp-CFSH* expression shows tissue-specific and gender-variant pattern, which was exclusively expressed in the eyestalk ganglion and higher in mature females than in males (27). In addition, *Sp-CFSH* expression was dynamic during the development of the androgenic gland (AG) in *S. paramamosain*, which was maintained at high levels at the early stage (stages I and II) and then reduced significantly at the mature stage (stage III) (27).

To explore the regulatory mechanism of expression, the 5′-flanking region of *Sp-CFSH* was cloned and analyzed. Sodium bisulfite sequencing and methylated DNA immunoprecipitation were used to investigate the involvement of DNA methylation in the regulation of *Sp-CFSH* expression. Moreover, CpG dinucleotide site methylation and activity of the site-directed mutation (SDM) reporter vector were analyzed to demonstrate the regulatory mechanism that methylation inhibits *Sp-CFSH* expression.

TABLE 1 | The primers used in the present study.

Name	Sequence (5′→ 3′)	Application
SP1	CATGTGTCCTATGATGGAGGAACG	Tail-PCR
SP2	GCAAGAAATGCTGGACACGTGAAG	
SP3	AGGGAAGTTCTGTTCTGCTTCAT	
SP4	GTAGTAAATCCCAGGTGCGTAAAG	
SP5	GACAACCTACTCAGTAACATCG	
SP6	GCGAGCGACAAGGCACAGTAAT	
Sp-cfsh-QF	cggGGTACCAATCGGCATTTAGGTT TATTTGGTC	Cloning the 5′-flanking sequence
Sp-cfsh-QR	cgcGGATCCTGTAAGCCTTAGGG AAGTTCTGTT	
Sp-cfsh-F	CGTGTCCAGCATTTCTTGCAGTACC	qRT-PCR
Sp-cfsh-R	TCATGTGTCCTATGATGGAGGAACG	
Sp-ak-F	TTCCTCCACCCTGTCCAACC	
Sp-ak-R	GAAGCGGTCACCCTCCTTGA	
BSP-F	TTTTTAATTAAAATAAATATTTGATT	Determination of DNA methylation
BSP-R	ATAAAAATTTTAACATCCATCTCAC	
p*sp-cfsh*-F	cggGGTACCTATTTACATGAAGATG CAATGGGCT	SDM
p*sp-cfsh*-R	cccAAGCTTCCTTAGGGAAGTTC TGTTCTGCTTC	
m*sp-cfsh*-F	ACAAACACCTGACTCTACCGCGCTGGTT	
m*sp-cfsh*-R	AACCAGCGCGGTAGAGTCAGGTGTTTGT	

MATERIALS AND METHODS

Animal Sources

Mud crabs (*S. paramamosain*) were obtained in March from a local market in Xiamen, Fujian Province, China. They were reared in tanks (temperature: 27 ± 2°C; salinity: 26 ± 1 ppm) for a week and fed with the meat of the white Pacific shrimp, *Litopenaeus vannamei*. The female crabs (carapace width 4.9–12.5 cm, body weight 115–382 g) and male crabs at stage III of AG development ($n = 3$, carapace width 9.3–11.8 cm, body weight 230–372 g) (27, 29) were anesthetized; the muscle and eyestalk ganglion were dissected to prepare the genomic DNA. In addition, the eyestalk ganglion of female crabs ($n = 8$) were collected to analyze the *Sp-CFSH* expression. Ovarian development was distinguished according to the morphological characteristics and confirmed by histological observation (30, 31). All the animals used in this study have been approved by the Animal Ethics Committee of Xiamen University.

Cloning of the 5′-Flanking Sequence of *Sp-CFSH*

The genomic DNA was purified from muscle using the Universal Genomic DNA Extraction kit (TaKaRa, Japan). Tail-PCR was employed to clone the 5′-flanking region of *Sp-CFSH* according to the manufacturer's instructions (32, 33). The gene-specific primers (SP1-SP3 and SP4-SP6) (**Table 1**) were designed based on the sequence of *Sp-CFSH* (27) and used to clone the 5′-flanking region with random primers (AP1-4) in the Genome Walking kit (TaKaRa, Dalian, China).

Bioinformatics Analysis

NNPP (http://www.fruitfly.org/seq_tools/promoter.html) was used to predict the core promoter region and transcription initiation site with the minimum promoter score of 0.75.

AliBaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/index.html) with defaulted parameters was applied to predict the potential transcription factor binding sites.

MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) was applied to predict the CpG island, which is defined using the following criteria (34): island size with minimum length 200 bp, GC content <55%, and ratio CpG observed/expected <0.60.

Sp-CFSH Promoter Activity Analysis

The 5′-flanking sequence of *Sp-CFSH* was sequenced and cloned into pMD19-T vector (TaKaRa, Dalian, China). After digesting with KpnI and HindIII (TaKaRa, Dalian, China), the 5′-flanking sequence of *Sp-CFSH* was ligated to a promoterless enhanced green fluorescent protein (EGFP) report vector (pEGFP-1; YouBio, Changsha, China). The recombinant vector (pEGFP-pCFSH) was used to test promoter activity by transfecting HEK293FT cells. The HEK293FT cells were obtained from the China Center for Type Culture Collection, Wuhan. They were cultured in high-glucose DMEM (HyClone, USA) with 10% FBS (Gibco, USA), 1% 100 penicillin–streptomycin (Gibco, USA) and incubated at 37°C under 5% CO_2. Cells were plated on 24-well plates overnight, and then transient transfections were performed using Lipofectamine™ 2000 Reagent (Invitrogen, China) with

1 μg of pEGFP-pCFSH following the manufacturer's protocols. pEGFP-1 and pEGFP-N1 were used as the negative control and mock transfection, respectively. pEGFP-N1 was gifted by Dr. Kejian Wang of College of Ocean and Earth Sciences, Xiamen University, China.

Sp-CFSH Expression Analysis

Total RNA was extracted from eyestalks using TRIzol Reagent (Invitrogen, USA), and the quality was detected using NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific) according to the manufacturer's instructions. The cDNAs were synthesized using 1 μg of total RNA and TransScriptII One-step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen, Beijing, China) according to the manufacturer's protocol. The cDNAs were diluted four times for qRT-PCR analysis. The qRT-PCR was performed with 7500 Fast Real-Time PCR (Applied Biosystems), with the reaction volumes of 10 μl of 2× PCR Master Mix with SYBR Green, 2 μl of dilute cDNA, 0.8 μl of each primer (1 mM), 6.4 μl of water; the reaction was performed under the following conditions: 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. *Arginine kinase* (*AK*; GenBank accession number: JQ031765) was used as reference, and the primers used for qRT-PCR are listed in **Table 1**.

Determination of DNA Methylation Level Using Sodium Bisulfite Genomic Sequencing

The bisulfite modification of genomic DNA was performed using ZYMO EZ DNA Methylation-Gold kit (D5005, Zymo Research, USA) according to the manufacturer's instructions. PCR was carried out with the bisulfate-specific primers (**Table 1**), and products were purified and cloned into the pMD19-T vector. After transfection, 10 positive clones were selected and sequenced. The analysis was performed as previously described (17).

The rate of promoter methylation was calculated by the formula $I^{Me}/10$, where I^{Me} and 10 represent the number of the methylated promoters and sequenced promoters, respectively.

Average methylation of promoter was calculated by the formula $\sum_{i=1}^{N} Si/12/N$, where S_i, 12, and N represent the number of the methylated dinucleotides site, 12 sites of CpG island, and methylated promoters, respectively.

Average methylation levels of the CpG dinucleotide site was figured by the formula $S^{Me}/10$, where S^{Me} and 10 represent the number of methylated dinucleotides site and 10 dinucleotides site of sequenced promoters, respectively.

The results from at least three independent experiments were quantified and averaged.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP analysis was performed as previously described with minor modifications (35). The genomic DNA was sonicated (15 min on ice with 15 s on/off intervals; Branson Sonifier S-450D, USA) to yield DNA fragments from 200 to 500 bp in length. One microgram of fragmented DNA was heated and denatured to produce a single-stranded DNA, then immunoprecipitated with l μg of anti-5mC antibody (ab10805; Abcam, UK) or with 1 μg of normal mouse IgG (as negative control) at 4°C for 12 h. Pre-cleared Protein A/G PLUS-Agarose (sc-2003; Santa Cruz) immunoprecipitated antibody/DNA complexes were washed away unless specifically bound, and finally resuspended with 500 μl of digestive buffer containing proteinase K. The DNA fragments was purified with phenol chloroform extracting and ethanol precipitated, then resuspended in 50 μl of Tris buffer (10 mM Tris, pH 8.5). Finally, 2 μl of DNA fragments was used for analyzing the methylated rate of the 5′-flanking region by PCR.

Promoter Activity Analysis of the Binding Site of Sp1 With Site-Directed Mutagenesis (SDM)

SDM was achieved by overlap extension PCR reactions with primers containing the mutational bases and was used to identify the function of transcription elements. p*sp-cfsh*-F/m*sp-cfsh*-R and p*sp-cfsh*-R/m*sp-cfsh*-F (**Table 1**) were used to amplify p*sp-cfsh*-1 and p*sp-cfsh*-2 with procedure as follows: 94°C for 5 min; 34 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, followed by the final extension at 72°C for 10 min using the ABI 2720 Thermal Cycler (Applied Biosystems, USA). The second reaction was carried out with amplification system that contained 2.5 μl of 10× Ex Taq Buffer (TaKaRa, Dalian, China), 2 μl of dNTP, 0.25 μl of Ex Taq, 1 μl of p*sp-cfsh*-1, 1 μl of p*sp-cfsh*-2, and 16.65 μl of water, under the conditions 94°C for 5 min; 10 cycles of 94°C for 40 s, 57°C for 1 min, 72°C for 1 min, followed by 20°C for 5 min; Then, 0.8 μl of p*sp-cfsh*-F and 0.8 μl of p*sp-cfsh*-R were added to the amplification system and reaction under the conditions 35 cycles of 94°C for 40 s, 57°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min. The products were purified and digested with KpnI and HindIII (TaKaRa, Dalian, China), then inserted into pGL3-Basic vector. The reporter vectors were transiently transfected into HEK293FT cells, and the relative luciferase activity was evaluated by the Dual-Luciferase Reporter Assay System (Promage, USA). pRL-TK vector was co-transfected to normalize the transfection efficiency. The reporter vector with normal transcription element and pGL3-basic were employed as the control and the negative control, respectively.

Statistical Analysis

The data are presented as means ± standard error of mean (SEM) of three or six independent experiments. The statistical evaluation was performed in the GraphPad Prism 6 software package (San Diego, CA, USA). Statistical analysis among groups was conducted with one-way ANOVA test, and a value of $p < 0.05$ was considered statistically significant.

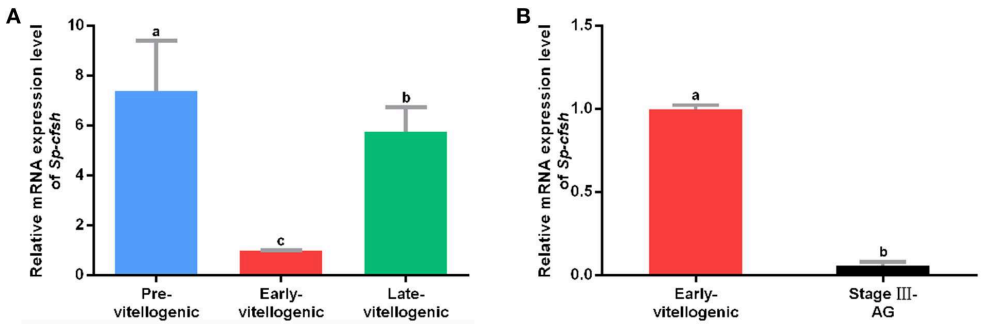

FIGURE 1 | Quantitative analysis of *Sp-CFSH* expression. **(A)** Expression of *Sp-CFSH* at different stages of ovarian development. **(B)** Expression of *Sp-CFSH* in two sexes. The eyestalk ganglion of females at pre-vitellogenic stage, early-vitellogenic stage, and late-vitellogenic stage are designated as Pre-vitellogenic, Early-vitellogenic, and Late-vitellogenic, respectively, and the eyestalk ganglion of males at stage III of AG development is designated as Stage III-AG. The data are presented as mean ± SEM ($n = 6$) with different letters indicating statistical significance at $p < 0.05$.

RESULTS

Quantitative Analysis of *Sp-CFSH* Expression

A previous study showed that the expression of *Sp-CFSH* was dynamic during the development of AG in males. It was high at the early stage (stages I and II) and significantly decreased at the mature stage (stage III). To examine the expression of *Sp-CFSH* during ovarian development, cDNA was derived and analyzed from the eyestalk ganglion at the pre-vitellogenic stage (**Figure S1A**), early-vitellogenic stage (**Figure S1B**) and late-vitellogenic stage (**Figure S1C**). The results showed that the expression of *Sp-CFSH* was significantly high at the pre-vitellogenic stage and late-vitellogenic stage compared with that of the early-vitellogenic stage (**Figure 1A**). Females at the early-vitellogenic stage and males at the mature stage were chosen to compare the expression of *Sp-CFSH* in the two sexes, and the results showed that the expression of *Sp-CFSH* in females was significantly higher than that in males (**Figure 1B**).

5′-Flanking Sequence of *Sp-CFSH*

A total of 1,250-bp 5′-flanking regions (GenBank accession number: MN938502) were obtained from the transcriptional start site (TSS). The core promoter was located between −40 bp and +10 bp and contained the TSS and a TATA box (21 bp upstream of the TSS). Forecast analysis identified some transcription factor binding sites, such as Sp1, GATA-1, Wt1, Sox-2, C/EBPalp, and c-Jun (**Figure 2**). One CpG island was found at −687 to −918 bp and contained 12 CpG sites (**Figure 3**), in which CpG-1 and CpG-2 were located in the binding site of the transcription factor Sp1.

Sp-CFSH Promoter Activity

The pEGFP-N1 contains the promoter of cytomegalovirus (CMV), as a positive control, which can effectively turn on the expression of EGFP and show a strong fluorescence (**Figure 4A**). **Figure 4B** showed the HEK293FT cells transfected with pEGFP-pCFSH, and the green cells demonstrated the promoter activity of the 5′-flanking sequence. The HEK293FT cells transfected with pEGFP-1 served as the negative control, and no fluorescence was detected (**Figure 4C**).

CpG Island Methylation

CpG island is known as the main target of methylation. In order to detect whether methylation is involved in the regulation of *Sp-CFSH* expression, sodium bisulfite sequencing was used to analyze the CpG island. The results showed that the level of CpG island methylation was similar in females at the stage between early-vitellogenic and late-vitellogenic, suggesting that CpG island methylation may be not involved in the regulation of *Sp-CFSH* expression during ovarian development. Compared with the eyestalk ganglion in females, CpG island methylation was significantly higher in the muscle of females and the eyestalk ganglion of males, suggesting that CpG island methylation may be involved in inhibiting *Sp-CFSH* expression in tissue-specific and gender-variant manners. The ratio of methylated CpG island in the eyestalk ganglion of females at the early-vitellogenic stage, late-vitellogenic stage, and muscle of females at the early-vitellogenic stage, as well as the eyestalk ganglion of males at the mature stage were 8.76, 8.85, 20.5, and 16.39% (**Figures 5A,C,E**), respectively, and the methylation levels of the methylated CpG island was 22.22, 24.99, 45.46, and 41.67% (**Figures 5B,D,F**), respectively.

MeDIP was carried out to further examine the effect of methylation on *Sp-CFSH* expression. While PCR of immunoprecipitated DNA fragments using normal IgG showed no amplified band, the immunoprecipitated DNA fragments using anti-5mC antibody were detected and showed that the amplified bands were more obvious in the muscle of females and the eyestalk ganglion of males than that in the eyestalk ganglion of females; in addition, the amplified bands of the DNA fragments were similar in three tissues (**Figure 6**). MeDIP further demonstrated that CpG island methylation was significantly higher in the muscle of females and the eyestalk ganglion of males than that in the eyestalk ganglion of females, suggesting that CpG island methylation may be involved in inhibiting *Sp-CFSH* expression in tissue-specific and gender-variant manners.

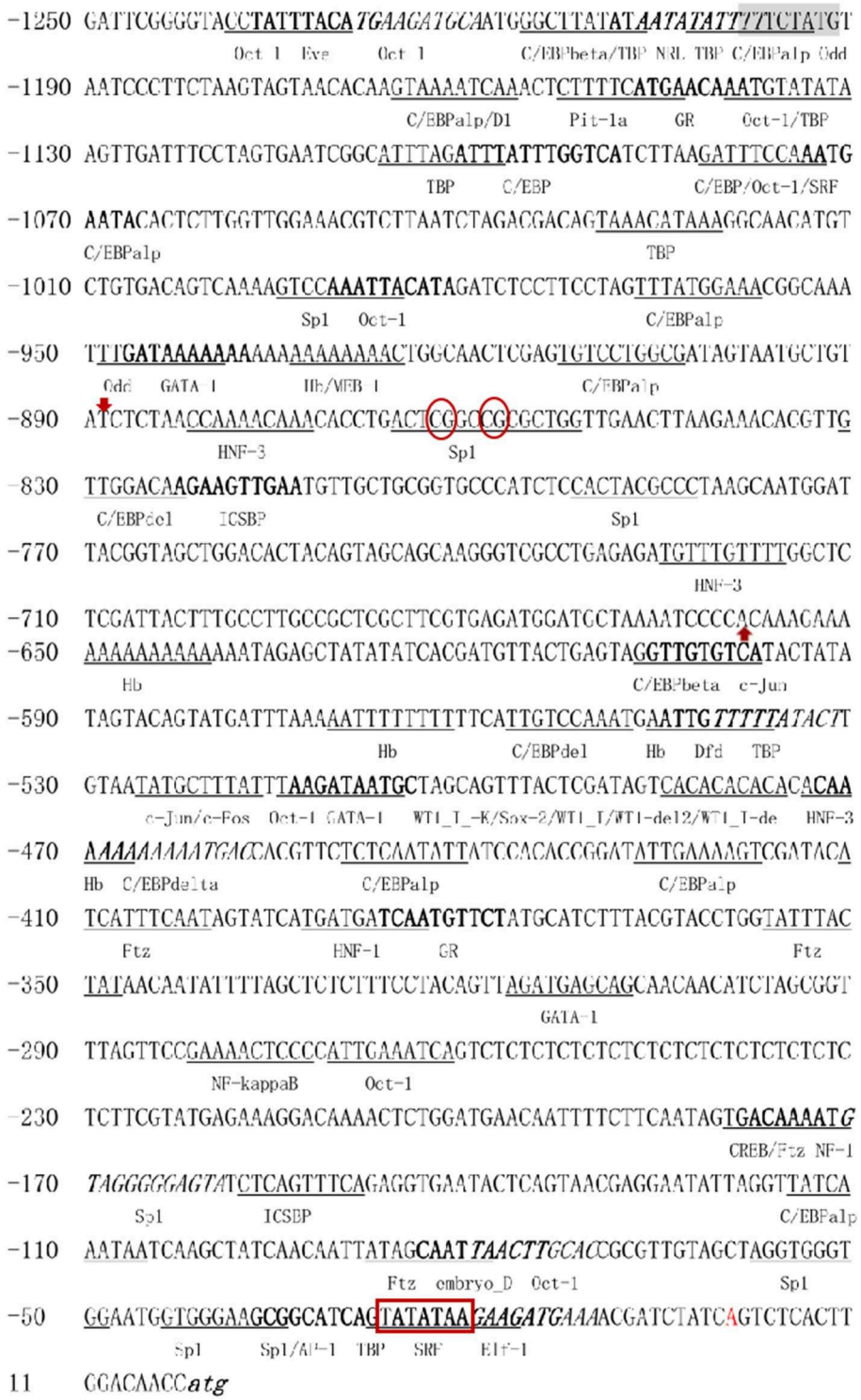

FIGURE 2 | The 5′-flanking region and putative *cis*-acting elements. The deduced transcriptional start site (**A**) is marked by red and defined as position 1. The nucleotide sequence is numbered on the left. The TATA box is marked in bold with red border. The putative transcription factor binding sites are marked by underlined, bold, or italic. Red arrows mark the beginning and end of CpG island, CpG-1 and CpG-2 are marked in red oval.

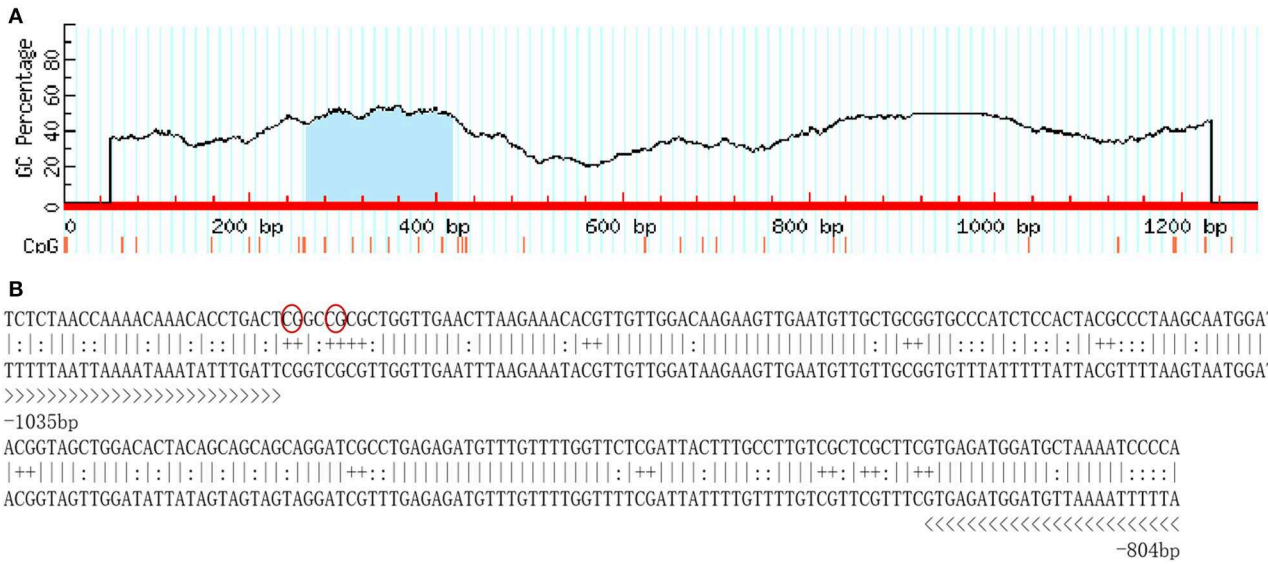

FIGURE 3 | The CpG island in the 5′-flanking sequence of *Sp-CFSH*. **(A)** CpG island predicted by MethPrimer (Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6). **(B)** The sequence of CpG island. Twelve CpG dinucleotides are marked with "+," and primers used for determination of DNA methylation are marked with ">." CpG-1 and CpG-2 are marked in red oval.

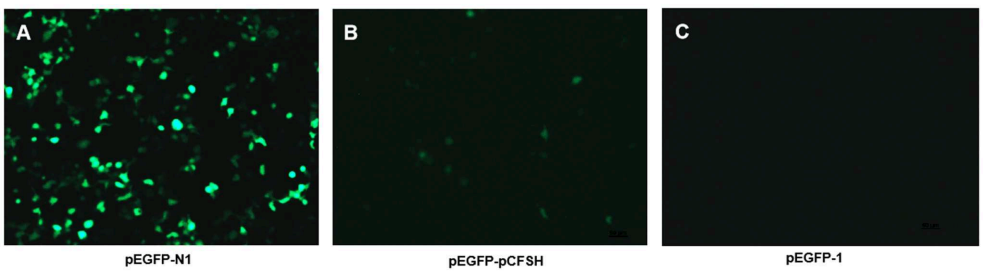

FIGURE 4 | The expression of green fluorescence (EGFP) in HEK293FT cells. EGFP was detected in HEK293FT cells transfected with pEGFP-N1 **(A)**, pEGFP-pCFSH **(B)**, and pEGFP-1 **(C)**. pEGFP-N1 and pEGFP-1 were used as positive control and negative control, respectively.

Methylation of CpG Dinucleotide Sites

The methylation of CpG dinucleotide sites was further studied to explore their regulation of *Sp-CFSH* expression in tissue-specific and gender-variant manners. The results showed that, compared with the eyestalk ganglion of females, there are seven CpG sites with high methylation in the muscle of females, including CpG-1, CpG-2, CpG-3, CpG-5, CpG-6, CpG-10, and CpG-12 (**Figure 7A**), and six CpG sites were found in the eyestalk ganglion of males with high methylation, including CpG-1, CpG-2, CpG-4, CpG-8, CpG-10, and CpG-12 (**Figure 7B**). Among them, CpG-1, CpG-2, CpG-10, and CpG-12 were shared between a tissue-specific manner and a gender-variant manner, suggesting that they may play an equally important role in inhibiting *Sp-CFSH* expression in two manners. Moreover, combined with the analysis of the 5′-flanking sequence of *Sp-CFSH*, CpG-1 and CpG-2 were found to be located in the binding site of the transcription factor Sp1.

Analysis of the Binding of Sp1 With the Site-Directed Mutation (SDM)

SDM was performed to investigate whether methylation of CpG-1 and CpG-2 inhibits *Sp-CFSH* expression by blocking the binding of transcription factor Sp1. The reporter vectors were named as p*sp-cfsh* (ACTCGGCCGCGCTGG) and p*sp-cfsh*-M (ACTCTACCGCGCTGG), respectively (**Figure 8A**), and results showed that the promoter activity of p*sp-cfsh*-M was significantly decreased ($p < 0.05$) compared with that of p*sp-cfsh* (**Figure 8B**), but both of them were significantly higher than that of the negative control (pGL3-basic).

DISCUSSION

To date, there are few reports on the epigenetic regulatory mechanism in crustaceans. In this study, DNA methylation was

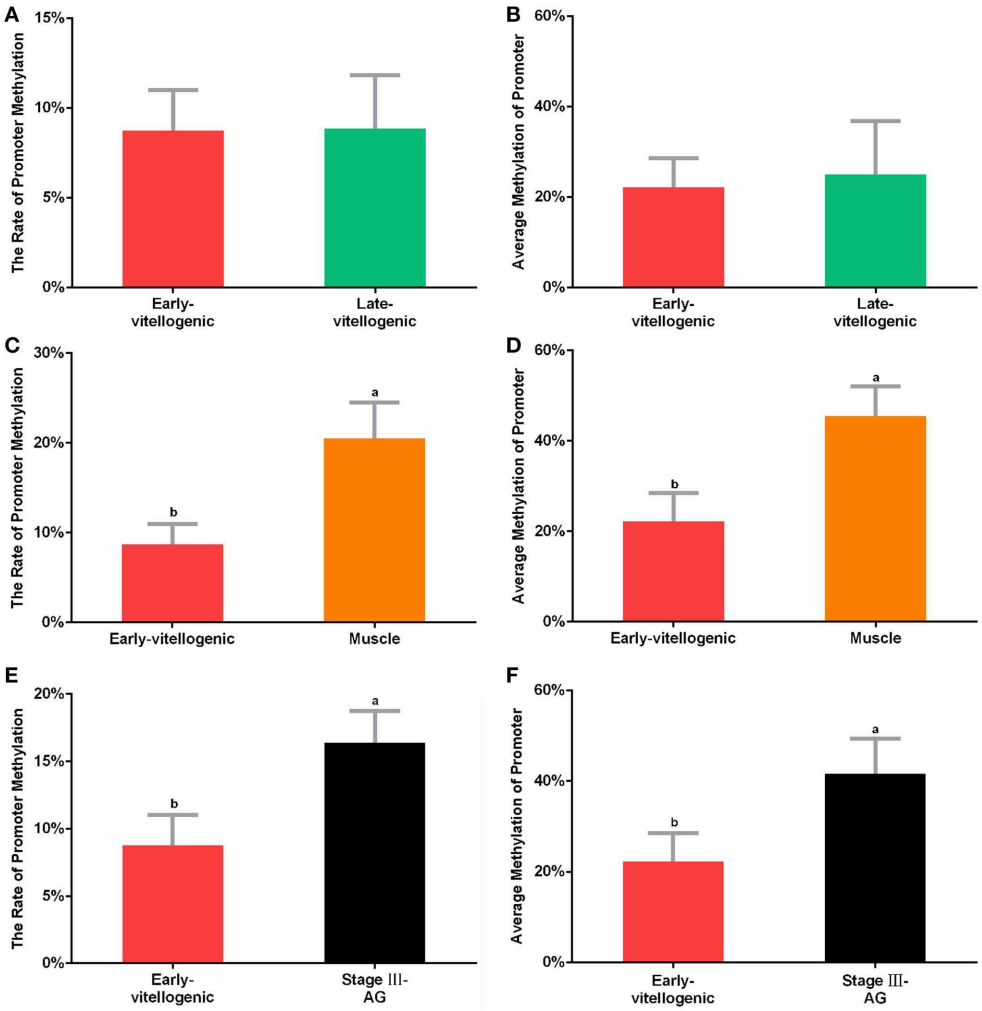

FIGURE 5 | Methylation of CpG island. The percentage of methylated CpG island in the eyestalk ganglion of females at early-vitellogenic stage compared with the eyestalk ganglion at late-vitellogenic stage **(A)**, the muscle of females **(C)**, and the eyestalk ganglion of males **(E)**. The average methylation of CpG island between the eyestalk ganglion of females at early-vitellogenic stage and the eyestalk ganglion at late-vitellogenic stage **(B)**, the muscle of females **(D)**, the eyestalk ganglion of males **(F)**. Different letters represent significant difference between groups ($p < 0.05$, one-way ANOVA followed by the Tukey's *post hoc* test). Each bar represents the mean ± SEM ($n = 3$).

shown to be involved in inhibiting *Sp-CFSH* expression in an eyestalk ganglion-specific and gender-variant pattern in the mud crab *S. paramamosain*.

To explore the regulatory mechanism of *Sp-CFSH* expression, the 5′-flanking region of *Sp-CFSH* was cloned and analyzed in this study. A total of 1,250-bp 5′-flanking regions were obtained and contained the core promoter with a TATA box, which is consistent with previous studies that there is only one transcription initiation site and a TATA box in the promoter (36, 37). Forecast analysis found some regulatory factor binding sites in the 5′-flanking regions of *Sp-CFSH*, such as GATA-1, Wt1, Sox-2, TBP, Sp1, C/EBPalp, and c-Jun. As we know, the eukaryotic promoter modulates the gene expression by the response to regulatory factor (8, 9).

In the present study, one CpG island was found in the 5′-flanking regions of *Sp-CFSH*, including 12 CpG sites, the presence of which suggests that *Sp-CFSH* expression may be regulated by DNA methylation. CpG island, as the major target for methylation, plays an important role in epigenetic regulation of gene expression (16). Most researches on DNA methylation has been done in vertebrates and plants, generally demonstrating that DNA methylation is involved in the silencing of gene expression (11–15). A study on DNA methylation in *S. paramamosain*, an important economic species of aquaculture, is completely lacking. In this study, CpG island methylation was analyzed, and the results showed that CpG island methylation was significantly higher in the muscle of females and the eyestalk ganglion of males compared to that in the eyestalk ganglion of females

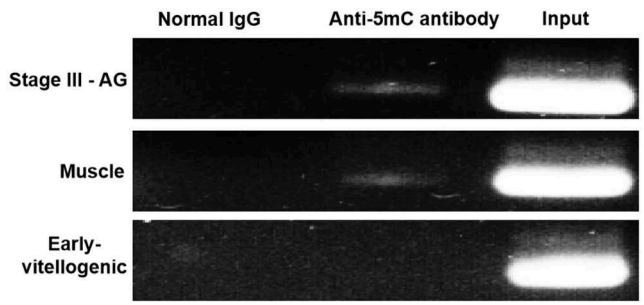

FIGURE 6 | MeDIP results of CpG island methylation. Normal IgG, the immunoprecipitated DNA fragments using normal IgG; Anti-5mC antibody, the immunoprecipitated DNA fragments using anti-5mC antibody; Input, the DNA fragments. Normal IgG and Input were used as control. PCR amplified using primer sets corresponding to CpG island. Products were separated on 2.0% agarose gels and visualized by staining with GelRed. Early-vitellogenic, the eyestalk ganglion of females at early-vitellogenic stage; Stage III–AG, the eyestalk ganglion of males at stage III of AG development; Muscle, the muscle of females at early-vitellogenic stage.

(**Figures 5**, **6**), and that was inversely correlated with the *Sp-CFSH* expression (27), suggesting that methylation may be involved in inhibiting *Sp-CFSH* expression in tissue-specific and gender-variant manners by reducing the activity of the promoter (38).

The methylation of CpG dinucleotide sites including CpG-1, CpG-2, CpG-4, CpG-8, CpG-10, and CpG-12 in the eyestalk ganglion of males and CpG-1, CpG-2, CpG-3, CpG-5, CpG-6, CpG-10, and CpG-12 in the muscle of females was significantly higher than that of the eyestalk ganglion of females, suggesting that they could play a vital role in the regulation of *Sp-CFSH* expression in gender-variant and tissue-specific manners, respectively. Moreover, CpG-1, CpG-2, CpG-10, and CpG-12 have high methylation in the muscle of females and the eyestalk ganglion of males, suggesting that they may play an equally important role in the regulation of *Sp-CFSH* expression in tissue-specific and gender-variant manners. CpG-1 and CpG-2 were found to be located in the binding site of transcription factor Sp1. In addition, our results of the activity analysis of the

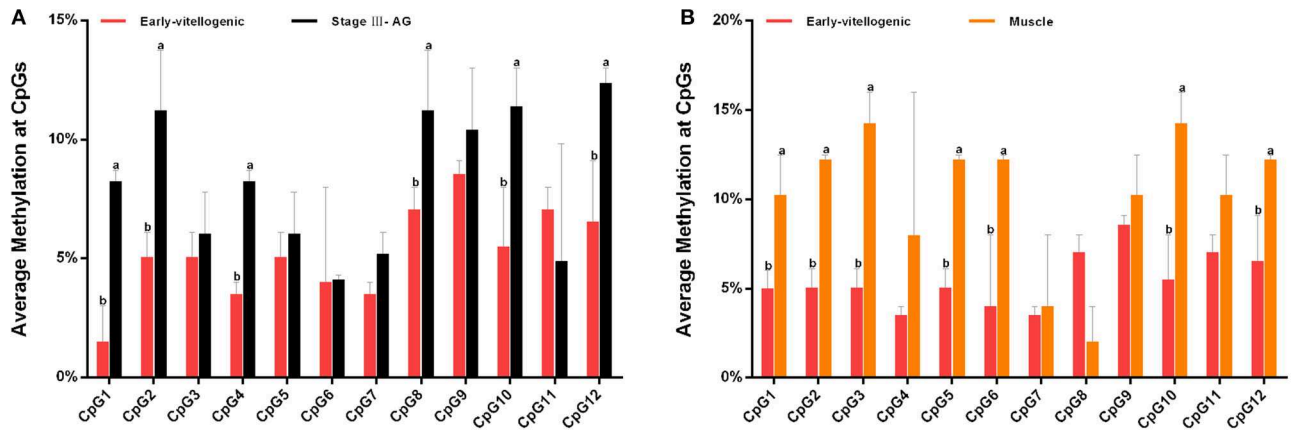

FIGURE 7 | Methylation of CpG dinucleotides sites. **(A)** Comparison of the methylation of CpG dinucleotides sites in the eyestalk ganglion between females and males. **(B)** Comparison of the methylation of CpG dinucleotide sites between the eyestalk ganglion and the muscle in females. Early-vitellogenic, the eyestalk ganglion of females at early-vitellogenic stage; Stage III–AG, the eyestalk ganglion of males at stage III of AG development; Muscle, the muscle of females at early-vitellogenic stage. Different letters represent significant difference between groups ($p < 0.05$, one-way ANOVA followed by the Tukey's *post hoc* test). Each bar represents the mean ± SEM ($n = 3$).

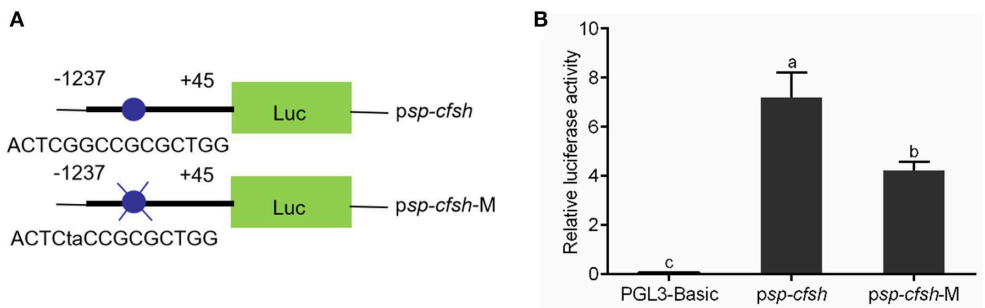

FIGURE 8 | Analysis of the binding site of transcription factor Sp1 with SDM. **(A)** Schematic diagram of reporter vector; p*sp-cfsh*-M was constructed by mutating the binding site of Sp1 from p*sp-cfsh*. **(B)** The analysis of relative luciferase activity; pGL3-basic was served as the negative control. The data are presented as mean ± SEM ($n = 3$) with diverse letters indicating significant differences at $p < 0.05$.

SDM reporter vector showed that SP1, as the transcriptional activator, plays a crucial role in promoter activity. Sp1 is a vital component of the eukaryotic cellular transcriptional machinery, which fine tunes cellular functions by regulating the gene expression with GC-rich promoters. Previous studies showed that DNA methylation interferes with the binding of Sp1 to *cis* sites (39–43). In the retinoblastoma gene, methylation of the CpG island directly inhibits the binding of Sp1 (44). In extracellular superoxide dismutase (EC-SOD) gene, methylation markedly decreased Sp1-/Sp3-driven promoter activity and was, at least in part, attributable to the competition of the methyl-binding protein MeCP2 with Sp1 for the same binding sites (45). Moreover, in the Synapsin I (SYN1) gene, methylation of Sp1 *cis* sites assist RE1-silencing transcription factor (REST) in the inhibition of SYN1 transcription (46). For these reasons, it was speculated that methylation of CpG-1 and CpG-2 may be involved in inhibition of Sp1 binding or the effectiveness of repressor complex, therefore leading to a reduced *Sp-CFSH* expression in tissue-specific and gender-variant manners.

In the present study, the difference between 22.22 and 45.46%/41.67% methylation levels of CpG island (**Figure 5**), and the difference of 1.5–7.05% vs. 8.25–14.25% methylation levels of CpG dinucleotide sites (**Figure 7**) have the significant consequence in *Sp-CFSH* expression, suggesting that *Sp-CFSH* expression was sensitive to slight methylation. It has been reported that the effect of methylation on gene expression depended on several parameters, including the location of CpG dinucleotide relative to the promoter (47), the local density of methylated CpG dinucleotide (48), the strength of the promoter (48), and the dependence of promoter function on transcription factors that are sensitive to methylated CpG dinucleotide (49). In this study, methylated CpG-1 and CpG-2 were located in the binding site of transcription factor Sp1, suggesting that Sp1 may contribute to the *Sp-CFSH* expression. Previous studies have already verified that subtle changes in methylation status can induce remarkable impacts at the organismal level. For example, methylation patterns can be modulated by environmental conditions in the California mussel, *Mytilus californianus*, and the change in methylation status of LIG4 gene can be largely attributed to a single CpG site (50).

The mud crab, *S. paramamosain*, is an annual animal and reproduces once in its lifetime. The ovarian development goes through three main stages: pre-vitellogenic stage, early-vitellogenic stage, and late-vitellogenic stage (30, 31). In this study, *Sp-CFSH* expression was also found to be different in females during ovarian development. This is not the only finding that shows that *Sp-CFSH* expression is linked to ovarian development. In kuruma prawn, *Marsupenaeus japonicus*, a CFSH isoform was also found to be highly expressed in the ovary and localized to oogonia and pre-vitellogenic oocytes in vitellogenic ovaries (51). Therefore, it is speculated that *Sp-CFSH* may also be involved in the regulation of ovarian development, especially in the stimulation of oocyte vitellogenesis. To exclude the influence of environment factors on methylation, female crabs from the same sea area and season were selected in this study. Although *Sp-CFSH* expression changed remarkably during ovarian development, CpG island methylation remained stable in the eyestalk ganglion. It showed that methylation was not involved in the regulation of *Sp-CFSH* expression during ovarian development. In addition to epigenetic regulation, the synthesis and release of neurohormone can also be regulated by some neurotransmitters in crustaceans (52–56), such as serotonin (5-hydroxytryptamine, 5-HT). It has been reported that 5-HT stimulates the release of neurohormones, including the crustacean hyperglycemic hormone, red pigment-dispersing hormone, neurodepressing hormone, molt-inhibiting hormone, and red pigment-concentrating hormone in the red swamp crayfish, *Procambarus clarkii* (57–59) and the white Pacific shrimp, *Litopenaeus vannamei* (60). Recent studies also demonstrate that 5-HT can promote ovarian *MroCFSHs* expression in the giant freshwater prawn, *Macrobrachium rosenbergii* (61). Therefore, the fluctuation *Sp-CFSH* expression during ovarian development may be influenced by other endocrine regulators, such as 5-HT. In addition, post-transcriptional regulation is another important way to affect the fate of mRNA, which can be realized by various modifications of mRNA to meet the needs of different physiological states (62). The regulatory mechanism of *Sp-CFSH* expression remains to be further explored.

In summary, the 5′-flanking region of *Sp-CFSH* was first cloned and analyzed in this study. Analysis of CpG island methylation proved that DNA methylation was involved in inhibiting *Sp-CFSH* expression in eyestalk ganglion-specific and gender-variant pattern in *S. paramamosain*. Analysis of CpG dinucleotide site methylation and activity of SDM reporter vector demonstrated that methylation inhibited *Sp-CFSH* expression by blocking the binding of transcription factor Sp1. The finding suggested, for the first time, the involvement of CpG methylation in the regulation of *Sp-CFSH* expression.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the National Center for Biotechnology Information, GenBank accession number: MF489232, MF489233.

ETHICS STATEMENT

The study was approved by Xiamen University animal care committee.

AUTHOR CONTRIBUTIONS

QJ and HY: conceptualization. QJ and DL: methodology, investigation, and visualization. QJ: software, validation, formal analysis, data curation, and writing—original draft. QJ, HY, and HH: writing—review and editing. HY: resources, supervision and project administration. HY and GW: funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00163/full#supplementary-material

Figure S1 | Hematoxylin and eosin (HE) staining in ovaries. **(A)** pre-vitellogenic ovaries, **(B)** early-vitellogenic ovaries, **(C)** late-vitellogenic ovaries. Og, oogonia; Oc, oocyte; FC, follicle cell; n, nucleus; nu, nucleolus; yg, yolk granules.

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Identification of Peptides and Their GPCRs in the Peppermint Shrimp *Lysmata vittata*, a Protandric Simultaneous Hermaphrodite Species

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Peptide hormones commonly binding with G-protein coupled receptors (GPCRs) achieve their function in reproduction. The peppermint shrimp *Lysmata vittata* popular in marine ornamental trade and is known to display protandric simultaneous hermaphrodite (PSH). Knowledge on reproductive biology of this commercial species is critical for resources management and aquaculture. This study employed Illumina sequencing and bioinformatics analysis to identify peptides and their candidate GPCRs from male phase (MP) and euhermaphrodite phase (EP) of *L. vittata*. A total of 61 peptide and 40 peptide GPCR transcripts derive from 44 peptide families and 13 peptide GPCR families were identified, respectively. Among them, insulin-like androgenic gland hormone and crustacean female sex hormone have two unique mature peptides, respectively, and their transcripts showed higher expression levels in MP than EP, which suggest that these sex differentiation hormones might be involved in sexual characters than spermatogenesis or vitellogenesis. Overall, the first study on identification of peptides and their GPCRs in the genus *Lysmata* extends our knowledge of peptidergic signaling in PSH species, and provides an important basis for development of aquaculture strategies.

Keywords: peptides, GPCRs, bioinformatics, protandric simultaneous hermaphrodite, *Lysmata vittata*

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INTRODUCTION

Peptide hormones play an important role in crustaceans reproduction. Crustacean hyperglycemic hormone superfamily (CHHs) are the typical crustacean peptide hormones. They are classified into type-I [CHH-type, comprised of crustacean hyperglycemic hormone (CHH) and ion transport peptide (ITP)] and type-II [MIH-type, comprised of molt-inhibiting hormone (MIH), mandibular organ-inhibiting hormone (MOIH), and vitellogenesis/gonad-inhibiting hormone (VIH/GIH)] peptides, involved in inhibiting ecdysteroid, methyl farnesoate, and vitellogenin synthesis (1). Insulin-like androgenic gland hormone (IAG) secreted by a crustacean male-specific androgenic gland (AG) is regarded as a peptidergic hormone and regulates male sex differentiation. IAG silencing in the giant prawn *Macrobrachium rosenbergii* (2) and red claw crayfish *Cherax quadricarinatus* (3) resulted in masculinization arrest and functional sex reversal, leading to the production of monosex populations (4). Moreover, IAG is not the sole sex differentiation hormone in crustaceans, and crustacean female sex hormone (CFSH), a specific hormone that plays critical

role in female reproductive phenotypes was recently isolated from the eyestalk of female blue crab *Callinectes sapidus* (5). Silencing CFSH impairs the mating and maternal care structures of females, such as absent or misplaced gonopores, sharper abdomens, as well as shorter and fewer setae on pleopods (5). Recent studies have found that several peptide hormones are also involved in crustacean reproduction. This includes the pigment-dispersing hormone (PDH) (6), neuroparsin (7), red pigment concentrating hormone (RPCH) (8), neuropeptide F (NPF) (9), short neuropeptide F (sNPF) (10), and allatostatin (AST) (11).

The colorful *Lysmata* shrimp collected from their natural environments is popular in marine ornamental trade, which are collected from their natural environments (12). Considering its growing demand in marine ornamental industry, it is important to study its reproductive biology for natural resources conservation and development of breeding techniques. Different from the gonochoristic reproductive system in most crustaceans, protandric simultaneous hermaphrodite (PSH) has been confirmed in all known species in genus *Lysmata* (13, 14). In PSH, the shrimp first develops as male (male phase, MP), and later become simultaneous hermaphrodite (euhermaphrodite phase, EP) which simultaneously produces sperms and eggs (13–15).

The peppermint shrimp *Lysmata vittata* is a small red-striped species, found in the coast of China, Japan, Philippines, Indonesia, and Australia (16–19). This species was also reported to have invaded the Atlantic Ocean, New Zealand and Brazil (12, 20, 21). Based on its histological features, four gonadal development stages (Stage I to IV) were defined for *L. vittata*. Among the four stages, stage I to III were defined as the male phase, during which testicular part of the gonads gradually develops and matures but the ovarian part remains immature. Stage IV was identified as euhermaphrodite phase, where both the testicular part and the ovarian part of the gonad mature (15). To date, study on the reproductive biology of *L. vittata* has mainly focused on: (1) the reproductive cycle of laboratory-reared (22), (2) ontogenetic development of gonads, and (3) external sexual characteristics (15). However, the reproduction molecular mechanisms, especially the information about peptide hormones is still unclear. Using Illumina sequencing and bioinformatics analysis, this paper tries to identify the peptide repertoire and their GPCRs in *L. vittata*, highlighting two sex differentiation peptide hormones, IAG and CFSH. This is useful for understanding specific PSH reproductive regulatory mechanism, and for supporting aquaculture to meet the emerging demand.

MATERIALS AND METHODS

Animals

L. vittata shrimps were cultured in the aquarium at Fisheries Research Institute of Fujian, Xiamen, China. Prior to dissections, the shrimps were anesthetized on ice for 5 min. Our study does not involve endangered or protected species.

Illumina Sequencing

Total RNA from mixed tissues of MP carapace (mixture of 5 individuals, body weight 86–100 mg, stage I and stage II) and EP carapace (mixture of 3 individuals, body weight 260–300 mg, stage IV), was extracted using Trizol Reagent (Invitrogen), followed by Illumina sequencing. Briefly, mRNA with poly (A) was isolated from total RNA using Oligo (dT) beads (Invitrogen). The mRNA was broken into short fragments (about 200 bp) using fragmentation buffer. These fragments were used as templates to synthesize the first-strand cDNA with random hexamers, after which a second-strand cDNA was synthesized. Adaptors were ligated onto the second-strand cDNA following by Illumina Hiseq sequencing (HiSeq 4000 SBS Kit (300 cycles), Illumina). The raw reads were quality controlled using Trimmomatic to generate clean reads, before performing *de novo* assembly through Trinity (v2.5.1). All clean reads were aligned with Bowtie2 (v2.3.4), followed by joint abundance estimation and RSEM to calculate transcripts per million (TPM) values.

Bioinformatics Analysis

Peptide and GPCR sequences were collected from the shrimp *de novo* assembly. To identify peptide, we used the well-established workflow (23). Signal peptide of the peptide precursors were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Prohormone cleavage sites prediction based on the standards were defined by Veenstra (24) and the peptide structures were predicted based on the established propeptide processing schemes (25–27). GPCRs identification was performed as our previous study (28). Deorphanized peptide GPCRs from insects and reported peptide GPCRs from crustaceans were used as reference sequences (28–37). A phylogenetic tree was built with related sequences of these GPCRs and *L. vittata* GPCRs transmembrane domains. Multiple sequence alignment was performed with ClustalX and the conserved sequence motifs were highlighted by LaTEX TexShade (38). The phylogenetic analysis was calculated using PhyML (SeaView software) (39) and the resultant phylogenetic tree was visualized with Figtree v1.4.3 and Photoshop CS 6.

RESULTS

The mRNA-sequencing and *de novo* assembly data are shown in **Table S1**. The assembled transcripts ($N = 71{,}009$) had a total size of 65,718,743 bp, an average size of 925.5 bp and N50 assembled transcripts with 1687 bp long. Using transcriptome mining, a total of 61 peptide and 40 peptide GPCR (34 belonging to A-family GPCRs (Lv-GPCR-A) and 6 belonging to B-family GPCRs [Lv-GPCR-B)] transcripts were predicted in *L. vittata*. These peptides included: adipokinetic hormone-corazonin-like peptide (ACP), agatoxin-like peptide, allatostatin-A (AST-A), AST-B, AST-C, AST-CC, AST-CCC, bursicon hormone, calcitonin, calcitonin-like diuretic hormone (DH31), CCHamide, CRF-like DH44, crustacean cardioactive peptide (CCAP), crustacean female sex hormone (CFSH), crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone/gonad-inhibiting hormone (MIH/GIH), CHH-MIH-like peptide, ecdysis triggering hormone (ETH), eclosion hormone (EH), EFLamide,

FLRFamide, glycoprotein-A2 (GPA2), glycoprotein-B5 (GPB5), Hyrg, insulin-like androgenic gland hormone (IAG), kinin, myosuppressin, natalisin, neuroparsin, neuropeptide F (NPF), orcokinin, pigment-dispersing hormone (PDH), proctolin, pyrokinin, red pigment-concentrating hormone (RPCH), RYamide, short neuropeptide F (sNPF), SIFamide, sulfakinin, tachykinin, terminal ampullae peptide (TAP), trissin and vasopressin. The peptide and GPCR transcripts source and their expression levels (TPM values) are summarized in Supplementary files (**Supplementary File 1** and **Table S1**). Most of the peptide transcripts TPM values (51 out of 61 transcripts) in MP are higher than those in EP (**Table S1**). Although the expression levels of GPCRs are generally lower than peptides, their expression patterns are similar, i.e., GPCR transcripts TPM values (33 out of 40 transcripts) in MP are higher than those in EP (**Table S1**).

Adipokinetic Hormone-Corazonin-Like Peptide (ACP)

Two transcripts putatively encoded complete ACP precursors of 104 and 99 amino acids (aa), respectively (**Supplementary File 1**). These precursors have the same mature peptide, pQITFSRSWVPQamide, a highly conserved decapod ACP peptide [e.g., (40–42)].

Agatoxin-Like Peptide

One transcript was identified to encode agatoxin-like peptide precursor of 111aa (**Supplementary File 1**). From this precursor, a 21aa signal peptide and three distinct peptides were predicted, one of which, WRSCIPRGGSCTHRPKSCCNSSSCR-CNLWGTNCRCQRMGLFQQLamide, shows 8 cysteine residues and amidated C-terminus associated with insect and decapod agatoxin-like peptides (43). Similarly, apart from toxic purposes, agatoxin-like peptide were identified in the neuroendocrine system of honey bee *Apis mellifera* and other insects (44).

Allatostatin (AST)

Three transcripts, a complete (AST-A2), a C-terminus (AST-A2) and a middle region (AST-A3) transcript with 334, 332 and 77aa, respectively that encode AST-A precursors were identified (**Supplementary File 1**). A total of 38 predicted peptides containing FGLamide were highly conserved motif derived from these precursors (**Figure 1**). Apart from SKSFSFGLamide, the rest of these peptides possess a conserved C-terminal motif YXFGLamide, e.g., SPGYAFGLamide, the signature of AST-A

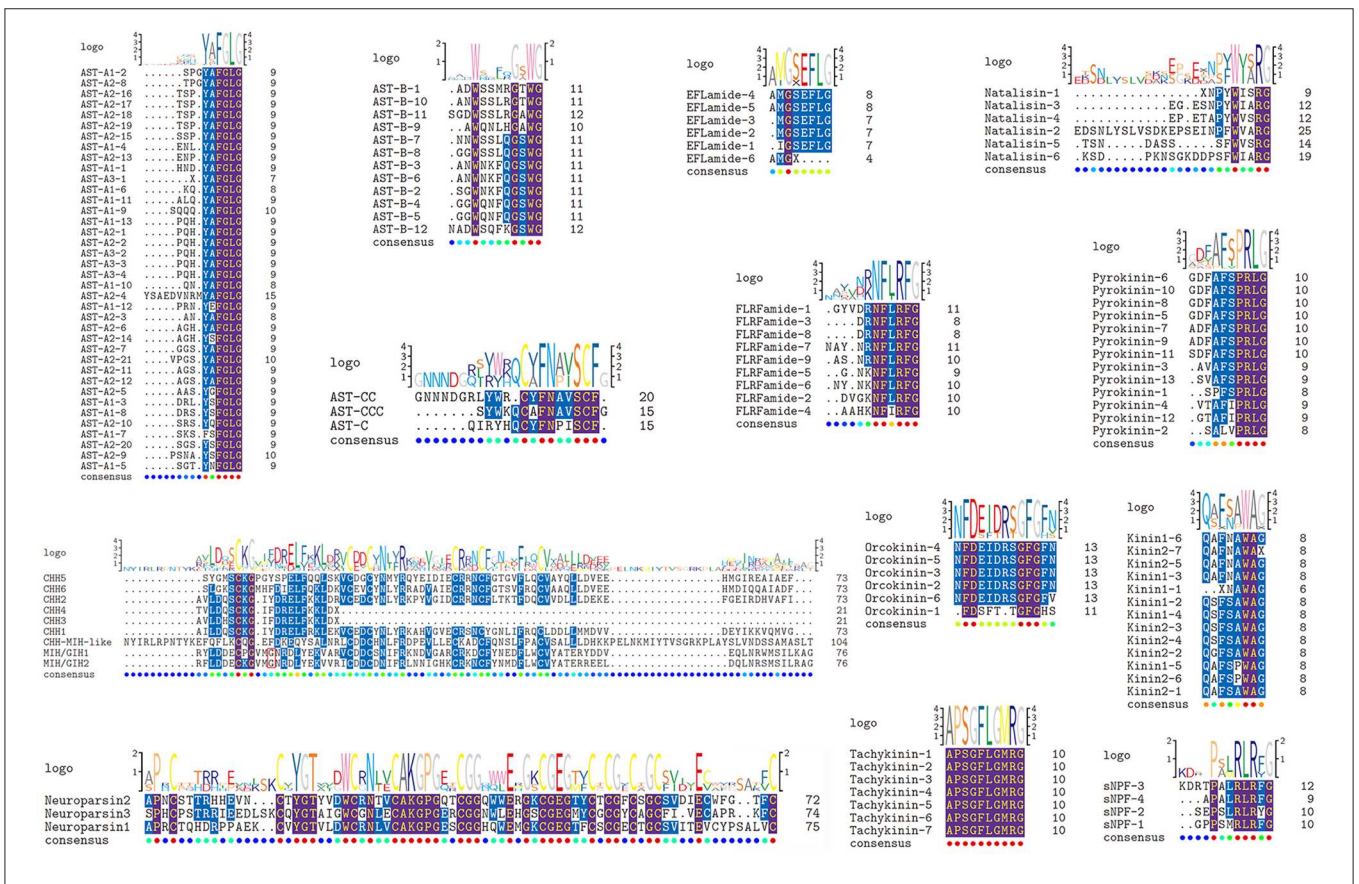

FIGURE 1 | Identification and characterization of mature peptides in *Lysmata vittata*. Schematic showing the mature peptides of ASTs, CHHs, EFLamide, FLRFamide, Kinin, Natalisin, Neuroparsin, Orcokinin, Pyrokinin, sNPF, and Tachykinin identified in *Lysmata vittata*. Logo is shown above alignments, where the height of each letter is proportional to the observed frequency of the corresponding amino acid in the alignment column.

family (45). One transcript putatively encoded the complete AST-B precursor with 350aa. This precursor has 12 predicted mature peptides with a X**W**XXXX**G**X**Wamide** conserved motif (**Figure 1**), e.g., ADWSSMRGTWGamide sequence, the signature of AST-B family (45). Three transcripts, two C-terminus partial regions (AST-C, AST-CC) and one full-length protein (AST-CCC), with 137, 192 and 108 aa, respectively that encode AST-C precursors were identified from the transcriptome assembly (**Supplementary File 1**). Each precursor possessed a predicted peptide with conserved motif X**C**X**FN**XX**SCF**X (**Figure 1**), i.e., pQIRYHQCYFNPISCF from AST-C, GNNNDGRLYWRCYFNAVSCF from AST-CC, and SYWKQCAFNAVSCFamide from AST-CCC (a disulfide bridge between cysteine residues in each peptide), previously reported decapod AST-C isoforms signature (46–49).

Bursicon Hormone

The heterodimeric peptide bursicon hormone alpha and beta subunit sequences were identified from the transcriptome assembly, encoding bursicon hormone alpha and bursicon hormone beta precursor of 148 and 136aa, respectively (**Supplementary File 1**). Both of these precursors start with a predicted signal peptide, followed by adjacent mature peptide with 11 cysteine residues.

Calcitonin

A single calcitonin transcript encodinng 164aa precursor was identified (**Supplementary File 1**). It comprised of 21aa signal peptide and three distinct peptides, one of which, T CYINAGLSHGCDYKDLVGAMAEKNYWDSLNSPamide (a disulfide bridge between two cysteine residues) is identical in structure to calcitonin from several decapod species, e.g., *M. rosenbergii*, the American lobster *Homarus americanus*, the crayfish *Procambarus clarkii* (43).

Calcitonin-Like Diuretic Hormone (DH31)

The predicted DH31 precursor was composed of 142aa with 23aa signal peptide and three distinct peptides (**Supplementary File 1**), where one of the peptides, GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPamide, possesses conserved motif XX**D**X**GL**X**RG**X**SG**XXX**AK**XXX XXXX**AN**XXX**GPamide**, the signature of DH31 family. Similar to calcitonin, *L. vittata* DH31 is identical in structure to DH31 from several decapod species, e.g., *H. americanus*, *M. rosenbergii* (43, 46, 50).

CCHamide

A single transcript encoding CCHamide precursor was identified (**Supplementary File 1**), starting with a 23aa signal peptide, followed by a C-terminal amidated peptide, i.e., VPKGGCLNYGHSCLGAHamide (a disulfide bridge between two cysteine residues), exhibiting conserved motif X**C**XX**W/Y/FG**XX**C**X**G**X**Hamide** of CCHamide (51).

CRF-Like DH44

A single transcript was identified to encode incomplete CRF-like DH44 precursor with 230aa (**Supplementary File 1**). This precursor has a 45aa mature peptide, i.e., NSGLSLSIDAS MKVLREALYLEMARKKQRQQMLRARHNQALLTTIamide, is similar to the previously described *M. rosenbergii* DH44 isoform, SSGLSLSIDASMKVLREALYLEMARKKQRQQMQRA RHNQELLTSIamide (43, 50).

Crustacean Cardioactive Peptide (CCAP)

A single transcript was identified to encode CCAP precursor of 141aa (**Supplementary File 1**). A 30aa signal peptide and five distinct mature peptides were predicted from CCAP precursor, one of which, PFCNAFTGCamide (a disulfide bridge between two cysteine residues), is identical to previously described authentic CCAP, a conserved arthropod peptide [e.g., (25, 45)].

Crustacean Female Sex Hormone (CFSH)

Two transcripts were identified to encode CFSH precursors representing the partial N-terminus (CFSH1a) and complete protein (CFSH1b) of 208 and 229aa, respectively. A 35aa signal peptide, a CFSH precursor-related peptide and a part of the 136aa mature peptide with 6 cysteines were predicted from CFSH1a. Similarly, the CFSH1b has a 33aa signal peptide, a CFSH precursor-related peptide and the 163aa mature peptide with 8 cysteines (**Supplementary File 1**). Both mature peptides (except two cysteine residues lacking in CFSH1a) showed similar cysteine residues with the other decapod CFSHs (43). The phylogenetic tree revealed that two *L. vittata* CFSHs clustered with previously described decapod CFSH1 isoforms (43), and were analogous to *M. rosenbergii* CFSH1a and CFSH1b respectively (**Figure S1**). Therefore, we arbitrarily named them as *L. vittata* CFSH1a and *L. vittata* CFSH1b in our study.

Crustacean Hyperglycemic Hormone Superfamily (CHHs)

Nine CHHs transcripts were identified from the transcriptome assembly. Phylogenetic analysis showed that the CHHs formed two major clades: type-I CHHs and type-II CHHs. Overall, type-I CHHs clade contained three subclades: the CHHs ortholog containing CHH1-4, the CHHs ortholog containing CHH5 and CHH6, and the CHH-MIH-like peptide ortholog. In type-II CHHs clade, MIH/GIH2, the oriental river prawn *Macrobrachium nipponense* GIH, *M. rosenbergii* SGP-B and the Antarctic shrimp *Chorismus antarcticus* MIH/VIH formed a subgroup, separate from the *M. nipponense* MIH ortholog containing MIH/GIH1 (**Figure 2**).

Crustacean Hyperglycemic Hormone (CHH)

Six transcripts were identified to encode four complete (CHH1, CHH2, CHH5, and CHH6), one N-terminus (CHH4) and one middle region (CHH3) CHH precursors. Altogether, these sequences have a CHH precursor-related peptide (CHH-PRP) between signal peptide and mature peptide. CHH1 precursor has a 26aa signal peptide, a 37aa CHH-PRP and a 72aa mature peptide with amidated C-terminus and 6 cysteines. CHH2 precursor has a 27aa signal peptide, a 43aa CHH-PRP and a 73aa mature peptide with 6 cysteines. CHH3 precursor has a partial signal peptide, a 44aa CHH-PRP and a part of mature peptide with 1 cysteine. CHH4 precursor

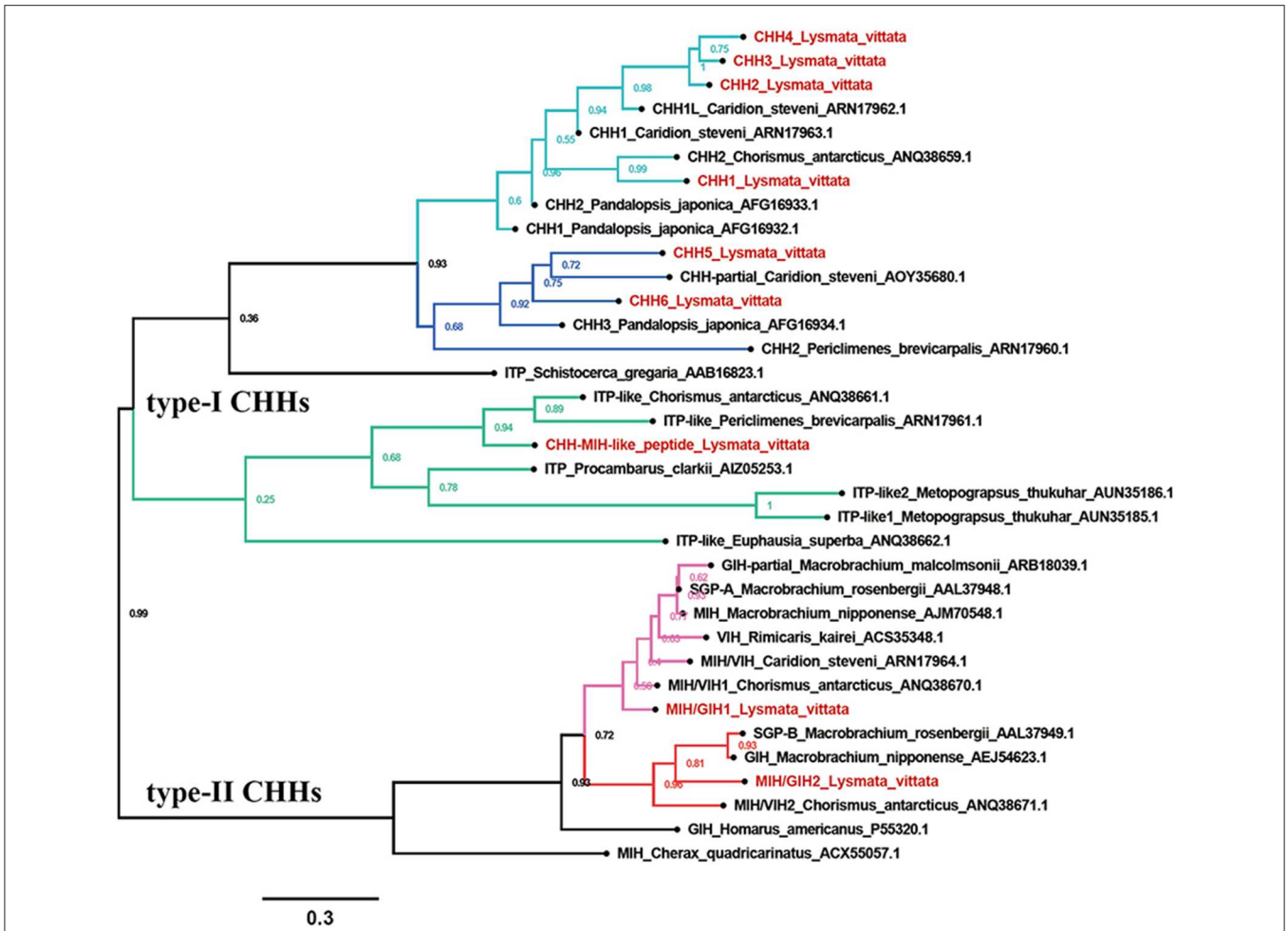

FIGURE 2 | Phylogeny of CHHs. Cladogram of CHHs showing connections in the clustermap of CHHs.

has a 21aa signal peptide, a 34aa CHH-PRP and a partial mature peptide (20aa) with 1 cysteine. CHH1-4 were shown to be highly conserved sequences at the N-terminus of their mature peptides (**Figure 1**). CHH5 precursor has a 29aa signal peptide, a 32aa CHH-PRP and a 73aa mature peptide with 6 cysteines. CHH6 precursor has a 28aa signal peptide, a 31aa CHH-PRP and a 73aa mature peptide with 6 cysteines (**Supplementary File 1** and **Figure 1**). Different from CHH1-4, CHH5, and CHH6 exhibited high sequence similarity with 3 Caridea CHHs, with a conserved C-terminus: **AIA**XX (**Figure 3**).

Molt-Inhibiting Hormone/Gonad-Inhibiting Hormone (MIH/GIH)

Two transcripts were identified to encode MIH/GIH precursors with 110 and 112aa, respectively. They lack CHH-PRP and have an additional specific glycine in position 12 of the mature peptide (Gly_{12}). The precursor MIH/GIH1 has a 32aa signal peptide and a 75aa mature peptide with amidated C-terminus. The precursor MIH/GIH2 has 34aa signal peptide and 75aa mature peptide with amidated C-terminus (**Supplementary File 1** and **Figure 1**).

CHH-MIH-Like Peptide

One transcript was identified to encode 133aa CHH-MIH-like peptide precursor with a 29aa signal peptide and a 104aa mature peptide with 6 cysteines (**Supplementary File 1** and **Figure 1**). This peptide defies the rules of CHH superfamily, i.e., lacks the CHH-PRP and Gly_{12}, and it is closer to the type-I CHHs than to type-II CHHs cluster (**Figure 2**). It exhibits similar characteristics of CHH-MIH-like peptide from several decapod species such as, *P. clarkii*, *E. sinensis* and the white shrimp *Litopenaeus vannamei* (43).

Ecdysis Triggering Hormone (ETH)

One transcript was identified from the transcriptome assembly to encode ETH precursor of 135aa (**Supplementary File 1**). A 19aa signal peptide and two distinct mature peptides were predicted from the ETH precursor, one of which,

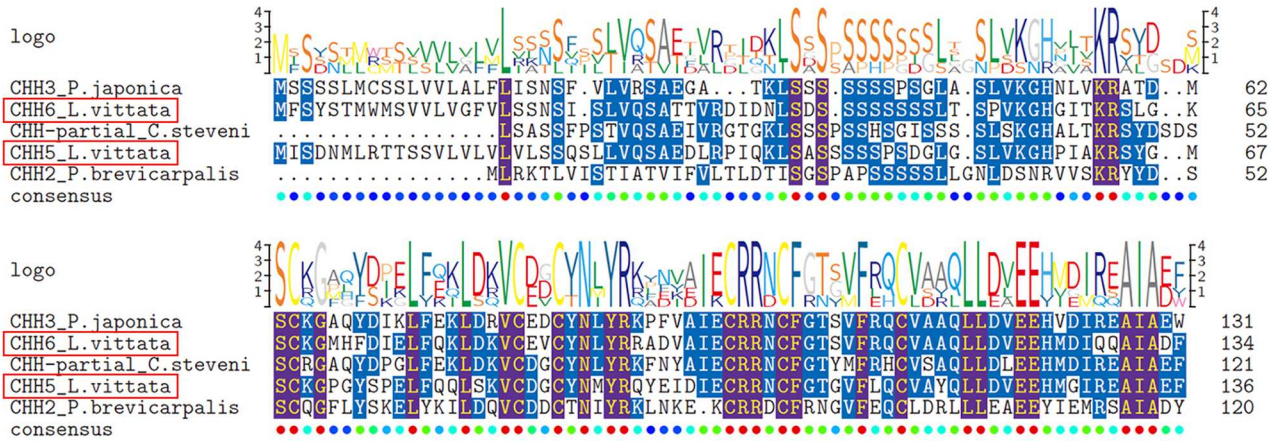

FIGURE 3 | Comparative sequence alignment of CHHs with "AIAXX" in different Caridea. Comparative sequence alignment of CHHs with "**AIA**XX" in *Lysmata vittata* with *Pandalopsis japonica*, *Caridion steveni* and *Periclimenes brevicarpalis*. Sequence logo is shown above alignments.

i.e., DAGHFFAETPKHLPRIamide, is identical in structure to decapod ETH isoforms [e.g., (43)].

Eclosion Hormone (EH)

One transcript was identified to encode EH precursor of 82aa, starting with a 26aa signal peptide, followed by a 52aa mature peptides with 6 cysteines, i.e., ASITSMCIRNCGQCKEMYGDYF HGQACAESCIMTQGVSIPDCNNPATFNRFL. This is identical in structure to EH from several decapod species, e.g., *M. rosenbergii*, *P. clarkii* (37, 43, 50).

EFLamide

One transcript was identified to encode the N-terminus EFLamide precursor of 210aa, starting with a 21aa signal peptide (**Supplementary File 1**). Eleven peptides were predicted from this precursor, five possessing the conserved motif GSEFLamide (**Figure 1**), e.g., IGSEFLamide, AMGSEFLamide, the signature of EFLamide (or called GSEFLamide) family [e.g., (43, 52)]. One of these predicted peptide with incomplete sequence, AMG, was predicted as the N-terminus of EFLamide isoform.

FLRFamide (FMRFamide)

One transcript was identified to encode FLRFamide precursor with 335aa, starting with a 19aa signal peptide (**Supplementary File 1**). Seventeen mature peptides were predicted from FLRFamide precursor, nine of which, are 7-10aa in length with conserved motif NFL/IRFamide (**Figure 1**), e.g., GYVDRNFLRFamide, and AAHKNFIRFamide, the signature of FLRFamide family [e.g., (45)].

Glycoprotein-A2 (GPA2)

The predicted GPA2 precursor has 18aa signal peptide and part of mature peptide with 4 cysteines (**Supplementary File 1**), i.e., FQHAWQTPGCHKVGHTRKISIPECVEFDITTNACRGYCE, which shows highly conserved sequence like previously described decapod GPA2 isoforms, e.g., it is 92% identical in protein sequence to *C. quadricarinatus* GPA2 isoform (53).

Glycoprotein-B5 (GPB5)

One transcript was identified to encode GPB5 precursor starting with a signal peptide with no N-terminus, followed by a 125aa C-terminal amidated mature peptide with 10 cysteines (**Supplementary File 1**). It shows a major sequence similarity to previously described decapod GPA5 isoforms, e.g., it is 87% identical/94% positives to GPA5 of *L. vannamei* (43).

Hyrg

Two transcripts were identified to encode Hyrg precursors with 60aa and 63aa. Each precursor is composed of a signal peptide and a mature peptide. The peptides, i.e., YPEPAVIVDGRPNM IPDGYIQAPRFHYRGFQKPIPKYDWS from Hyrg1, LPEAAVI VEGRPNRAPDDGYVQAAPPRFHYRGFQKFVPKYDWS from Hyrg2, possess conserved motif RFHYRGF, the signature of decapod Hyrg isoforms (43).

Insulin-Like Androgenic Gland Hormone (IAG)

Two transcripts were identified to encode IAG precursors with 146aa and 153aa (**Supplementary File 1**). IAG1 precursor has a 28aa signal peptide, a 30aa B chain, a 42aa C peptide, and a 45aa A chain. IAG2 precursor has a 27aa signal peptide, a 32aa B chain, a 41aa C peptide, and a 38aa A chain. Both IAGs have 8 cysteine residues located at B chain and A chain, and exhibits similar characteristics as previously described IAG isoforms (4). Different from other crustaceans, they have 9 and 4aa residues between Cys4 and Cys5 in A chain of IAG1 and IAG2, respectively (**Figure 4**).

Kinin

Two nucleotide sequences were identified from the transcriptome data putatively coding for two incomplete kinin precursors with 142 and 114aa (**Supplementary File 1**). Both have no signal peptide. Twelve peptides were predicted from kinin1 precursor, and the first peptide lacks N-terminus. Fourteen peptides were predicted from kinin2 precursor, and

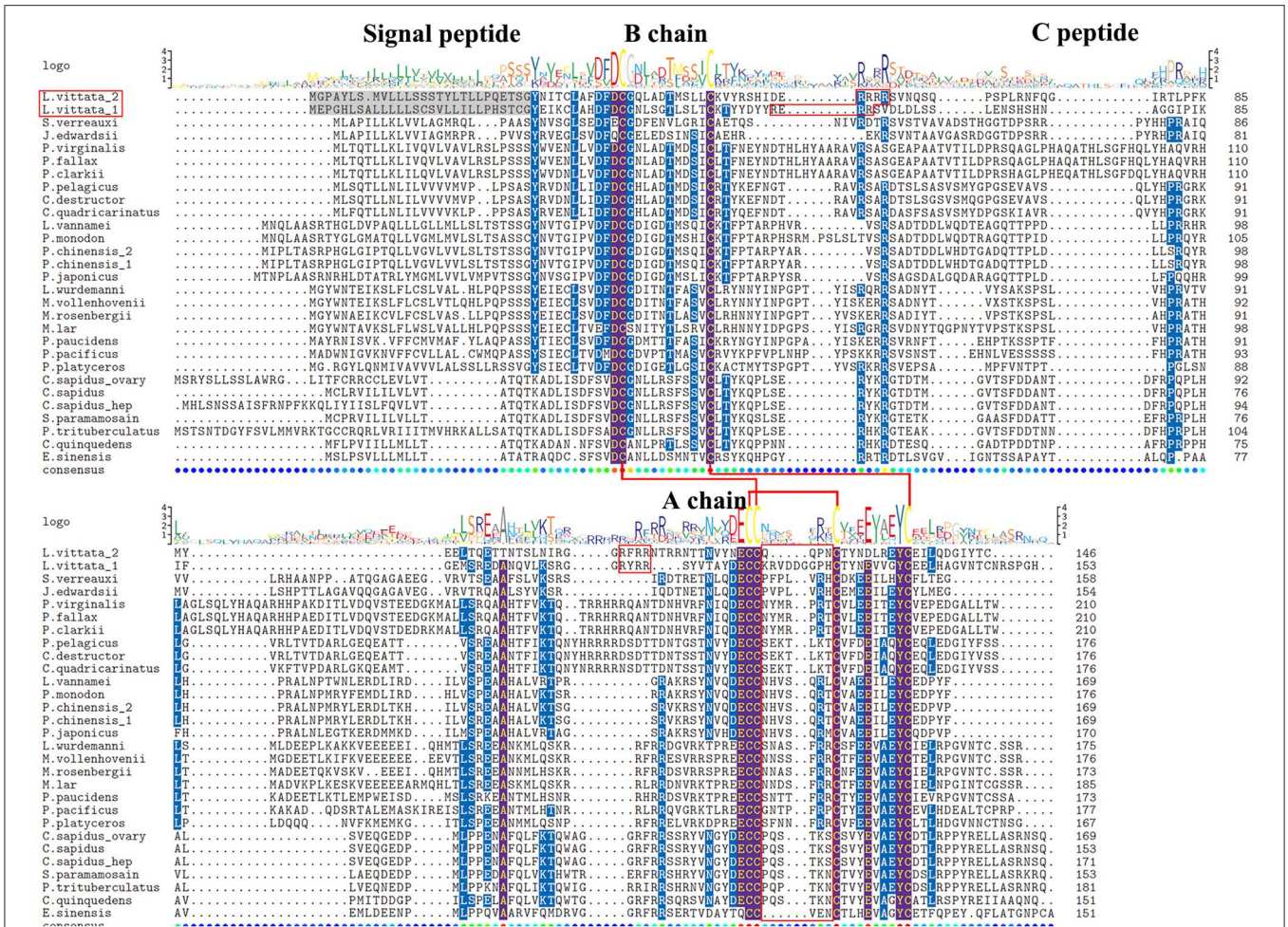

FIGURE 4 | Comparative sequence alignment of IAG in different decapod. Comparative sequence alignment of IAG precursors in *Lysmata vittata* with *Sagmariasus verreauxi*, *Jasus edwardsii*, *Procambarus virginalis*, *Procambarus fallax*, *Procambarus clarkii*, *Portunus pelagicus*, *Cherax destructor*, *Cherax quadricarinatus*, *Litopenaeus vannamei*, *Penaeus monodon*, *Penaeus chinensis*, *Penaeus japonicus*, *Lysmata wurdemanni*, *Macrobrachium vollenhovenii*, *Macrobrachium rosenbergii*, *Macrobrachium lar*, *Palaemon paucidens*, *Palaemon pacificus*, *Pandalus platyceros*, *Callinectes sapidus*, *Scylla paramamosain*, *Portunus trituberculatus*, *Chaceon quinquedens,* and *Eriocheir sinensis*. Sequence logo is shown above alignments. Six conserved cysteines are highlighted in the sequence logo shown above alignments and the putative disulfide bridges are drawn with red line.

the last peptide has no glycine, which is represent C-terminus amidation. Apart from these incomplete peptides, sequences of 11 mature peptides have conserved motif: XFX-A/P-WAamide (**Figure 1**), e.g., QSFSAWAamide, and QAFSPWAamide, the signature of kinin family (e.g., 45) (**Supplementary File 1** and **Figure 1**).

Myosuppressin

The predicted 102aa myosuppressin precursor has a 31aa signal peptide (**Supplementary File 1**). Three distinct peptides were predicted from this precursor, one of which is identical in structure to conserved decapod myosuppressin family, i.e., pQDLDHVFLRFamide [e.g., (45)].

Natalisin

The predicted incomplete natalisin precursor has 102aa (**Supplementary File 1**). Six predicted mature peptides were released from this precursor with conserved C-terminal WXXRamide (**Figure 1**), e.g., EDSNLYSLVSDKEPSEINPFW VARamide, EGESNPYWIARamide, the signature of natalisin family [also called WXXRamide, e.g., (43)].

Neuroparsin

Three transcripts were predicted to encode neuroparsin precursors with 99-101aa. These precursors are composed of signal peptide and the mature peptide with 12 aligned cysteines (**Supplementary File 1** and **Figure 1**). These mature peptides show highly conserved sequences as previously described neuroparsin isoforms, e.g., *L. vittata* neuroparsin1 is 63% identical/73% positive in protein sequence to *L. vannamei* neuroparsin [e.g., (43, 54)].

Neuropeptide F (NPF)

Two NPF transcripts were identified to encode 112 (NPF1) and 127aa (NPF2) precursors with 31aa and 29aa signal peptide (**Supplementary File 1**). Four distinct peptides were

predicted from NPF precursors, two of which possess the C-terminal motif RPRFamide, the hallmarks of NPF family members, i.e., ARTDNTAEVLQAMHEASLAGMLSSAEVPYP-SRPNVFKSPVELRQYLDALNAYYAIAGRPRFamide, KPDPTQLAAMADALKYLQELDKYYSQVSRPSPRSAPG-PASQIQALEKTLKFLQLQELGKLYSLRSRPRFamide (45).

Orcokinin

The predicted orcokinin precursor is composed of 140aa, and starts with a 22aa signal peptide (**Supplementary File 1**). Eight mature peptides were predicted from orcokinin precursor, where five adjacent mature peptides separated by "KR" cleavage sites possess N-terminal motif **NFDEIDR**X (**Figure 1**), the signature of orcokinin family members; and one (named orcokinin-1), i.e., FDSFTTGFGHS, an identified decapod orcomyotropin isoform (45).

Pigment-Dispersing Hormone (PDH)

Two transcripts were identified to encode PDH precursors with 83 and 79aa (**Supplementary File 1**). The precursor PDH1 has a 22aa signal peptide and mature octadecapeptide: NSELINSLLGLPKVMNDAamide, similarly, PDH2 has a 22aa signal peptide and the mature octadecapeptide: NSGMINSLLGIPKVMTDAamide. The two mature octadecapeptides exhibit highly conserved sequences as previously described decapod PDH isoforms (e.g., 43), e.g., *L. vittata* PDH1 is identical to the PDH predicted from *L. vannamei* PDH1 precursor (55).

Proctolin

The predicted 108aa proctolin precursor has a 22aa signal peptide (**Supplementary File 1**). Three distinct peptides were predicted from proctolin precursor, one of which, i.e., RYLPT, is identical to the authentic proctolin [i.e., (25, 45)].

Pyrokinin

One transcript was identified to encode 272aa pyrokinin precursor with a 18aa signal peptide (**Supplementary File 1**). Sixteen peptides were predicted from pyrokinin precursor, thirteen of which are 7-9aa in length with conserved motif PRLamide, e.g., SPFSPRLamide, GDFAFSPRLamide, the pyrokinin family signature [i.e., (45)].

Red Pigment-Concentrating Hormone (RPCH)

One transcript was identified to encode incomplete RPCH precursor with 91aa, starting with a 21aa signal peptide (**Supplementary File 1**). Three distinct peptides were predicted from RPCH precursor, one of which, i.e., pQLNFSPGWamide, is identical to the authentic RPCH [i.e., (45)].

RYamide

One transcript was identified to encode RYamide precursor representing a C-terminus region (**Supplementary File 1**). Two distinct peptides were predicted from this precursor, one of which is an incomplete peptide with conserved "RYamide" motif, i.e., SSPSQSELPEIKIRSSRFIGGSRYamide, the RYamide family signature [i.e., (45)].

Short Neuropeptide F (sNPF)

The 173aa sNPF precursor was identified from the transcriptome data, and starts with a signal peptide (**Supplementary File 1**). Nine distinct peptides were predicted from this precursor, four of which are 8-11aa long with **P**XX**RLRF/Yamide** conserved motif, i.e., GPPSMRLRFamide, SEPSLRLRYamide, KDRTPALRLRFamide, APALRLRFamide, the sNPF family signature [i.e., (45)].

SIFamide

One transcript was identified to encode 76aa SIFamide precursor, starting with a 27aa signal peptide (**Supplementary File 1**). Two distinct peptides were predicted from SIFamide precursor, one of which, GYRKPPFNGSIFamide, identical to Gly^1-SIFamide isoform [i.e., (45)].

Sulfakinin

One transcript was identified to encode 122aa sulfakinin precursor with a 21aa signal peptide (**Supplementary File 1**). Five distinct peptides were predicted from this precursor, where two adjacent mature peptides, pQFDEY$_{(SO3H)}$GHMRFamide and AGGDYDDY$_{(SO3H)}$GHLRFamide separated by carboxy-peptidase cleavage sites, possess conserved motif Y$_{(SO3H)}$GHM/LRFamide, the signature of sulfakinin family [i.e., (45)].

Tachykinin

The putative tachykinin precursor is comprised of 217aa, starting with a 26aa signal peptide. Twelve predicted peptides were released by two dibasic cleavage sites (RK, KK, RR) (**Supplementary File 1**). Seven peptides have the same sequence: APSGFLGMRamide (**Figure 1**), a broadly conserved decapod tachykinin isoform [i.e., (45)].

Terminal Ampullae Peptide (TAP)

One transcript encoding TAP precursor was found in transcriptome data. This precursor composed of a 18aa signal peptide and 70aa mature peptide with 8 cysteine residues (**Supplementary File 1**). This peptide has 77% identical/92% positive amino acid sequence compared to the TAP predicted from *M. rosenbergii* TAP precursor (56).

Trissin

The predicted 200aa trissin precursor has no signal peptide (**Supplementary File 1**). Two distinct peptides were predicted from trissin precursor, one of which is a partial C-terminus peptide, i.e., +EVSCGSCGLECQKACGTRNFRACCFNFQ. It has 89% identical/89% positive amino acid sequence compared to the trissin predicted from C. quadricarinatus trissin precursor (53).

Vasopressin

One transcript was found from transcriptome data to encode 148aa full-length vasopressin-neurophysin precursor starting with a 19aa signal peptide (**Supplementary File 1**). Three distinct peptides were predicted from vasotocin-neurophysin precursor, one of which, CFITNCPPGamide (with a disulfide bridge between two cysteine residues), is structurally identical

to vasopressin family (i.e., 42,43). One has a 100aa peptide with fourteen cysteine residues, and exhibits highly conserved sequence to previously described decapod neurophysin isoforms (23).

Peptide GPCRs

A total of 40 candidate peptide GPCR transcripts were predicted from *L. vittata*. To identify these GPCRs orthologs, hundreds of known peptide GPCRs from arthropod were collected for building phylogenetic tree. Phylogenetic analysis showed that 28 of these were clustered with known peptide receptor orthologs (**Figures 5**, **6**). Lv-GPCR-A1 and Lv-GPCR-A2 were clustered with the AST-A receptor ortholog. Lv-GPCR-A3 was clustered with CCAP receptor ortholog. Lv-GPCR-A4 was clustered with the FMRFamide receptor ortholog. Lv-GPCR-A5 and Lv-GPCR-A6 were clustered with the natalisin receptor ortholog. Lv-GPCR-A7 was clustered with the NPF receptor ortholog. Lv-GPCR-A8 and Lv-GPCR-A31 were clustered with the RYamide receptor ortholog. Lv-GPCR-A9, Lv-GPCR-A10, Lv-GPCR-A14, Lv-GPCR-A15, Lv-GPCR-A18, Lv-GPCR-A19, Lv-GPCR-A20, Lv-GPCR-A27 were clustered with the Moody &Tre ortholog. Lv-GPCR-A13 and Lv-GPCR-A30 were clustered with the ETH receptor ortholog. Lv-GPCR-A16 and Lv-GPCR-A17 were clustered with the GPA2/GPB5 receptor ortholog. Lv-GPCR-A23, Lv-GPCR-A25, Lv-GPCR-A29, and several putative CHH receptors were clustered with *Bombyx mori* BNGR-A34,

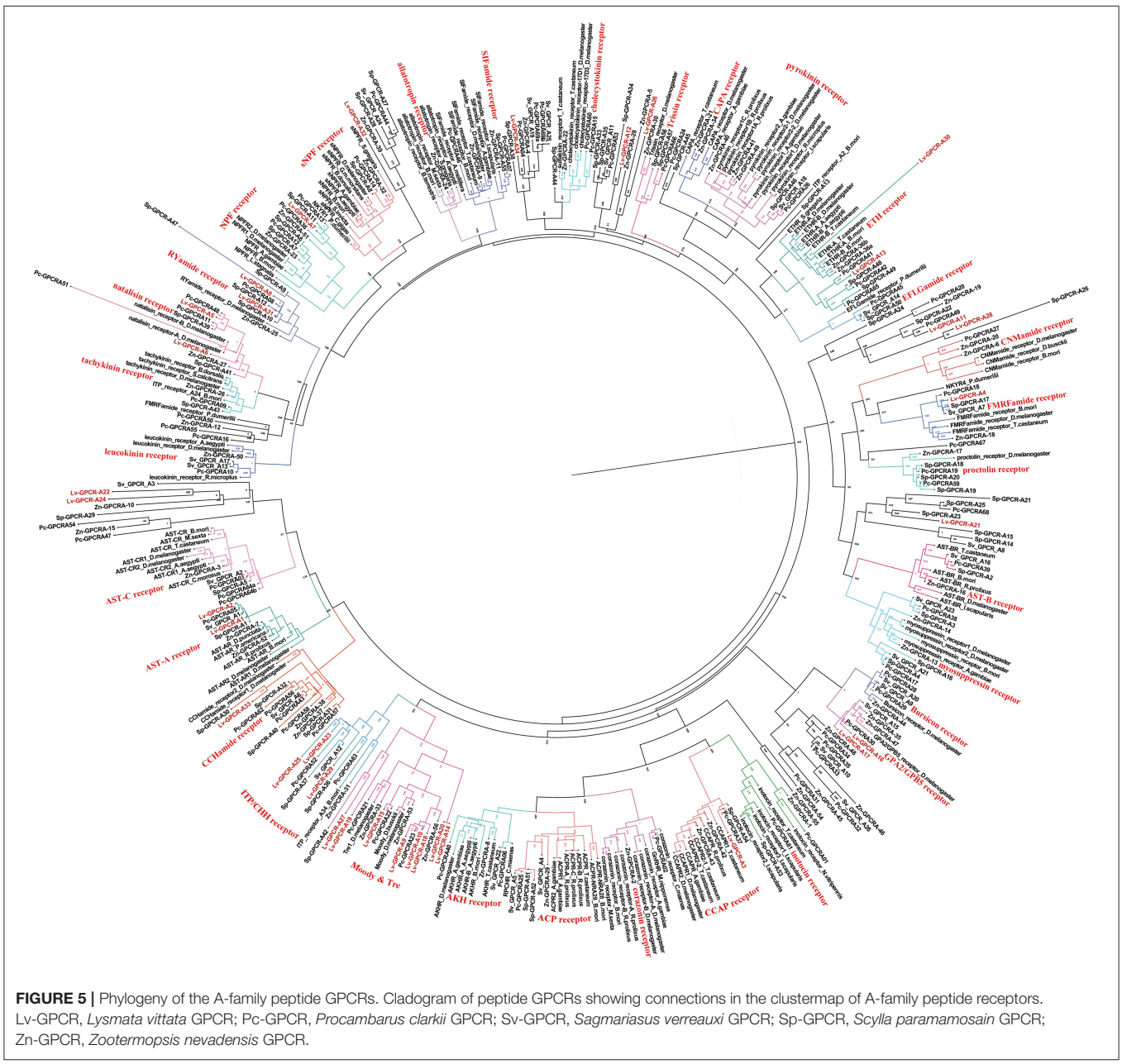

FIGURE 5 | Phylogeny of the A-family peptide GPCRs. Cladogram of peptide GPCRs showing connections in the clustermap of A-family peptide receptors. Lv-GPCR, *Lysmata vittata* GPCR; Pc-GPCR, *Procambarus clarkii* GPCR; Sv-GPCR, *Sagmariasus verreauxi* GPCR; Sp-GPCR, *Scylla paramamosain* GPCR; Zn-GPCR, *Zootermopsis nevadensis* GPCR.

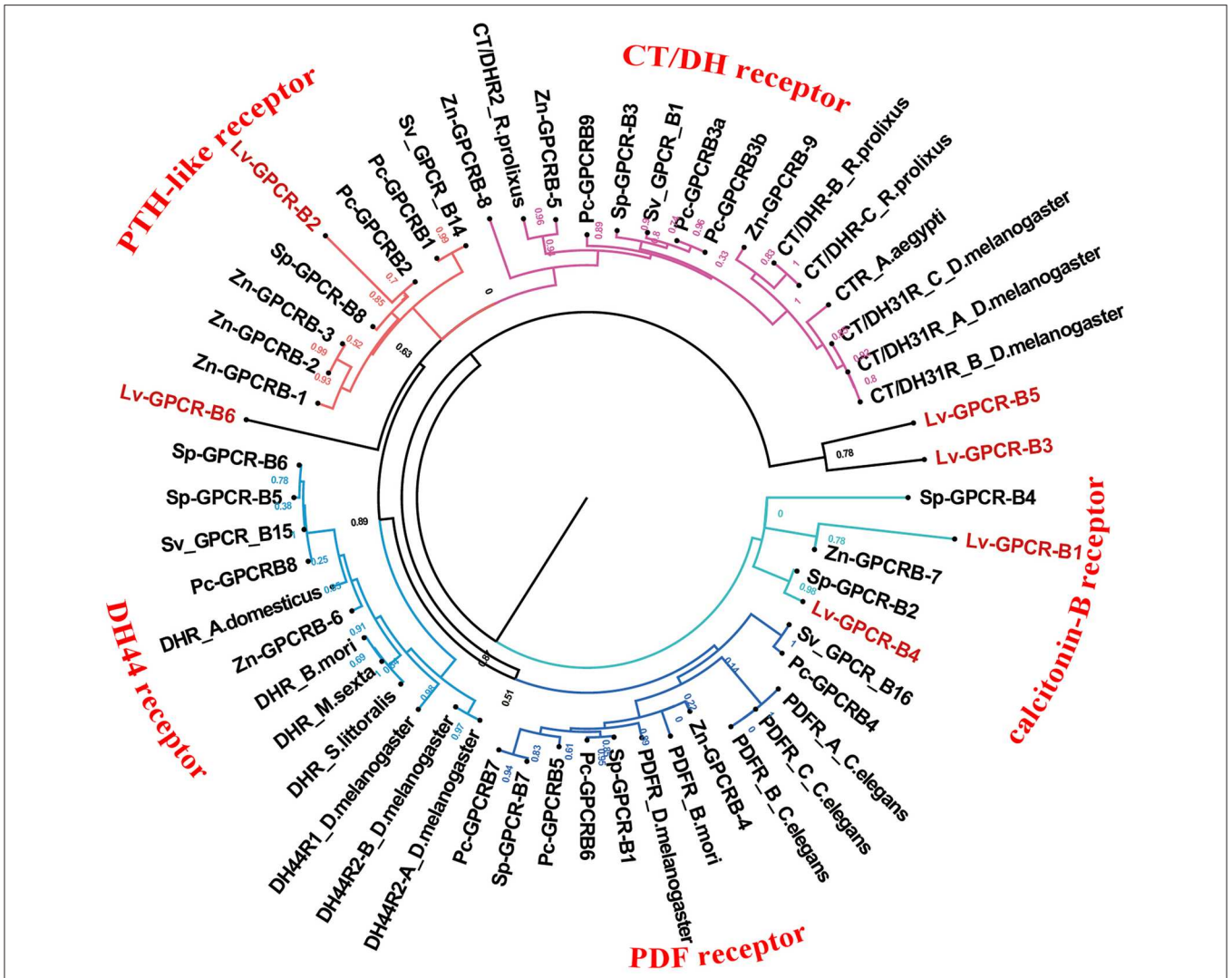

FIGURE 6 | Phylogeny of the B-family peptide GPCRs. Cladogram of peptide GPCRs depicting connections in the clustermap of B-family peptide receptors. Lv-GPCR, *Lysmata vittata* GPCR; Pc-GPCR, *Procambarus clarkii* GPCR; Sv-GPCR, *Sagmariasus verreauxi* GPCR; Sp-GPCR, *Scylla paramamosain* GPCR; Zn-GPCR, *Zootermopsis nevadensis* GPCR.

which has been defined as ITP receptor (57). Lv-GPCR-A33 was clustered with the CCHamide receptor ortholog. Lv-GPCR-B1 and Lv-GPCR-B4 were clustered with the calcitonin-B receptor ortholog. Lv-GPCR-B2 was clustered with the parathyroid hormone receptor (PTH)-like receptor ortholog.

DISCUSSION

The RNA-seq and bioinformatics analysis is highly effective for identifying peptides and their GPCRs (23, 26, 28, 37, 43, 58, 59). In this study, 61 transcripts derived from 44 peptide families and 40 transcripts derive from 13 peptide GPCRs were identified. This is the first study on genus *Lysmata* to characterize its peptide repertoire and their GPCRs.

Comparison of Peptide Sequences

Considering that the *L. vittata* peptide transcripts were computationally-generated from short reads and the mature peptide structures were predicted based on a bioinformatics workflow, the peptide repertoire from one Caridean shrimp, *M. rosenbergii* reported before was chosen for sequence comparison to provide increased confidence of the *L. vittata* peptide sequences/structures reported in present study (43, 50). Overall, sequence alignments of the predicted peptide amino acid sequences in *L. vittata* and full-length *M. rosenbergii* peptide precursors show that all peptides especially the mature peptides are conserved in the two species (**Supplementary File 2**). For example, the predicted mature peptides of calcitonin (TCYINAGLSHGCDYKDLVGAMAEKNYWDSLNSPamide), DH31 (GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPami

de), EH (ASITSMCIRNCGQCKEMYGDYFHGQACAESCIM TQGVSIPDCNNPATFNRFL), and a large number of relative short mature peptides (e.g., PQHYAFGLamide from AST-A, ADWSSMRGTWamide from AST-B, QIRYHQCYNPISCF from AST-C, DAGHFFAETPKHLPRIamide from ETH) from *L. vittata* are identical in amino acid sequences/structures to the corresponding mature peptides reported in *M. rosenbergii*. This suggests the putative peptide sequences in our study are mostly accurate.

In addition to the reported peptides data from *M. rosenbergii*, the peptide repertoire from another *Lysmata* species, a marine shrimp *Lysmata wurdemanni*, was chosen for sequence comparison. The *L. wurdemanni* peptide sequences from the only public *Lysmata* transcriptome data, i.e., the *L. wurdemanni* embryo and adult (brain and muscle) Sequence Read Archive (SRA) (SRR8715485, SRR8715486), was downloaded for peptide mining based on our bioinformatics workflow. A total of 78 transcripts derived from 45 peptide families were predicted from *L. wurdemanni*, mainly significant amino acid similar to those from *L. vittata* (**Supplementary File 2**). A large number of peptide families were identified from the two *Lysmata* shrimps, but a number of established peptide groups were not identified in each shrimp. No sequences encoding CNMamide, corazonin, elevenin, HanSolin, HIGSLYRamide, or RFLamide proteins were found in the *L. vittata* assembly. Similarly, no sequences encoding Bursicon, ETH, HanSolin, HIGSLYRamide, Hyrg, IAG, or RFLamide proteins were found in the *L. wurdemanni* assembly. Given the significant peptide sequence resemblance between the two *Lysmata* shrimps, we hypothesize that CNMamide, corazonin, and elevenin are likely to be found in *L. vittata*. On the other hand, Bursicon, ETH and IAG are likely to be found in *L. wurdemanni*. The hypothesis was verified when *L. wurdemanni* IAG was cloned from the testicular part of *L. wurdemanni* (60). In contrast, HanSolin, HIGSLYRamide and RFLamide families are likely to be absent in *L. vittata*, as these peptide families have not been identified from the current Caridean shrimps peptide data (43, 50, 61). Of which, HanSolin and RFLamide are recently-identified peptides from the stick insect, *Carausius morosus* (62), and subsequently were found in most Coleoptera species (63), and HIGSLYRamide have been identified only from brachyuran [e.g., (23, 40, 41)]. Therefore, additional transcriptome, peptidome, and/or a genome data can be created to clarify the presence/absence of these peptide families in *L. vittata*.

Peptides/GPCRs Expression

Given that the experimental design of each development stage single libraries comprised of pooled RNA samples, we did not investigate expression changes statistically. However, it is possible that the expression values presented provides valuable preliminary data to identify candidates for prospective study on PSH species peptides. Similar to expression patterns of previously reported peptides/GPCRs (23, 64–66), stage-specific expression of peptides/peptide GPCRs existed in *L. vittata*, i.e., TPM values of peptides/peptide GPCR transcripts in MP are higher than in EP. In *L. vittata* MP, higher expression levels of the peptidergic signaling promotes the growth of body size and development of testicular part of the gonad. For instance, NPFs, sNPF and IAGs have higher expression in MP, where NPF and sNPF were proved as the feeding behavior controllers in arthropod (67), and NPF has been shown to increase food intake in penaeid shrimp (68). Similar functions of NPF and sNPF are proposed in *L. vittata*. IAG is regarded as the regulator of male sex differentiation in crustaceans (2–4), and high IAG expression in MP suggest that IAG might be involved in promoting masculinization and developing testicular part of the gonad in *L. vittata*. In contrast, a number of peptides show different expression patterns, e.g., TPM values of two PDH transcripts in MP are lower than those in EP. PDH regulates pigment distribution controlling circadian rhythm (69), and also shows different expression levels during the mud crab *Scylla paramamosain* vitellogenesis (6), but the reproductive function of PDH is yet to be proved in crustaceans. TAP shows high expression in both MP and EP, i.e., TPM value of MP and EP is 24.3 and 29.9, respectively. In *M. rosenbergii*, TAP was distributed on terminal ampullae and sperms, as it participates in sperm proteolytic activity and plays a key role in sperm maturation (56). We hypothesized that TAP play similar functional roles in *L. vittata* sperm, in gonad of both MP and EP (15). Moreover, TPM values of neuroparsins and RPCH are higher than 15 in MP. They play a role in regulation in crustacean ovarian development (7, 8). These results imply that transcriptomes of different *L. vittata* gonadal stages should be analyzed to assess reproductive differences in peptidergic signaling in PSH species.

Key Peptides

Multiple members of the CHH superfamily contain 6 aligned cysteine residues, and nine transcripts of these peptides were found in this study. Given that CHH1-4 some regions in this gene have the same sequences, we speculated they are from different alternative splicing of one gDNA, and this phenomenon is common in type-I CHHs (1, 70). The C-terminal motif "**AIA**XX" of CHH5 and CHH6, members of type-I CHHs, seems to be more common in Caridean shrimps than other crustaceans. In type-II CHHs, MIH, and GIH show high similarity in sequences, with MIH participating in molting and GIH regulating reproduction process. These genes have been subdivided based on primary structures and motifs (71). However, this rule does not apply for type-II CHHs in Caridea, making it difficult to subdivide MIH and GIH based on primary structures in this species. Therefore, we named two *L. vittata* type-II CHHs as "MIH/GIH". These *L. vittata* MIH/GIHs were clustered into two subgroups, in which MIH/GIH2 was grouped with *M. nipponense* GIH. Type-II CHH was reported to inhibit ovary development (72). This suggests that MIH/GIH2 could be GIH. MIH/GIH (MIH/GIH1) was grouped with the predicted *M. nipponense* MIH, implying that may be MIH. CHH-MIH-like peptides were clustered with some predicted shrimp ITPs, but it is not appropriate to name these peptides as "ITP," because they neither have the typical CHH-PRP of ITP, nor are they similar to *L. vannamei* ITP, which modulate osmoregulation in shrimp (73).

Two unique mature peptides of IAGs were found in a species for the first time in this study. In the Chinese shrimp *Fenneropenaeus chinensis*, two IAG isoforms were identified

from one gene (74). Three *C. sapidus* IAG genes were identified from AG, hepatopancreas and ovary. However, mature peptides of these genes are identical (75–77). Only one IAG was cloned from *L. wurdemanni*, and this IAG showed higher sequence similarity with those of the genus *Macrobrachium* than with the two *L. vittata* IAGs (60). In this study, expression levels of two IAGs in MP shrimp were higher than in EP shrimp, of which, the TPM value of IAG1 was 76 in MP shrimp, but not expressed in EP shrimp. In *L. vittata*, it was reported that the male external sexual character had disappeared whereas testicular part had some degree of degeneration in EP shrimp (15), of which were ascribed to the down-regulation of IAGs. In *M. rosenbergii*, silencing of IAG not only arrested the degeneration of male secondary sexual characteristics and testis, but also prevented testicular spermatogenesis (2). *M. rosenbergii* IAG dsRNA injections canceled spermatozoa in the sperm duct and testis (2). In *L. vittata* EP, both ovarian and testicular parts were mature, testicular part being filled with many spermatozoa (15). It seems that spermatogenesis was unaffected following down-regulation of IAGs in *L. vittata*.

In many species, including in *L. vittata*, CFSH has one to three paralog genes (43). In this study, two CFSH transcripts were almost not expressed in EP, which is the ovarian mature stage. In female crabs with CFSH knock-down, the brooding and mating systems were abnormal at puberty (5). Analysis of expression pattern of *L. vittata* CFSH revealed that it might be involved in the development of female phenotypes at puberty, rather than the vitellogenesis and ovarian maturation.

Two sexual systems, two sex differentiation hormones, and two unigenes of these hormones exist in an individual, implying that a complicated sexual regulatory network exists in *L. vittata*. RNAi should be performed to reveal the discover bisexual mechanism regulated by sex differentiation hormones in *L. vittata*. This species is expected to be an ideal model for RNAi experiments because it is small, has a transparent body and short reproductive cycle (22).

SUMMARY

Total RNA was extracted from *L. vittata* mixed tissues and used to mine peptides. More than 60 peptide transcripts were identified. However, this method has the following limitation, i.e., the expression levels of some tissue specific (e.g., eyestalk) peptides may be too low to assemble long enough transcript encoding complete precursor. Here, 15 peptide transcripts encoded incomplete precursors. Some of them showed low expression levels, such as CFSH1a, CHH3, GPA2, and RYamide. Furthermore, the peptides contained several similar peptide paracopies making it difficult to assemble the complete CDSs encoding such precursors (43), as in the cases of AST-A, kinin, and natalisin in our study. Notably, 28 predicted Lv-GPCRs were grouped with known peptide GPCRs, including: AST-A receptor, CCAP receptor, FMRFamide receptor, natalisin receptor, NPF receptor, RYamide receptor, Moody & Tre, ETH receptor, GPA2/GPB5 receptor, CHH receptors, CCHamide receptor, calcitonin-B receptor, and PTH-like receptor. Together with the identified peptides in *L. vittata*, we speculate that AST-A, FLRFamide, natalisin, NPF, RYamide, ETH, GPA2/GPB5, CHHs, CCHamide, and calcitonin ligand-receptor pairs are expressed in *L. vittata*. In conclusion, complete peptide/GPCR genes remain to be cloned and confirmed through PCR experiments. Moreover, *in vitro* ligand-receptor binding tests are required to determine Lv-GPCRs. Nevertheless, identification of peptides and the associated GPCRs in *L. vittata* extends our knowledge on peptidergic signaling in PSH species, and provides experimental basis for further studies on the of function peptides in reproduction. This will promote aquaculture development of this shrimp.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the SRA accession: PRJNA561673.

ETHICS STATEMENT

The study was approved by Xiamen University animal care committee.

AUTHOR CONTRIBUTIONS

CB: conceptualization, data curation, software, writing-original draft. FL: Sample collection, data curation. YY: project administration, editing. QL: sample collection. HY: conceptualization, editing, supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00226/full#supplementary-material

Supplementary File 1 | Peptide precursors and their expression levels in male phase (MP) and euhermaphrodite phase (EP) of *L. vittata*.

Supplementary File 2 | Comparative sequence alignment of peptide precursor in *L. vittata* with *M. rosenbergii* and *L. wurdemanni*.

Table S1 | Supplementary information.

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Impacts of Methyl Farnesoate and 20-Hydroxyecdysone on Larval Mortality and Metamorphosis in the Kuruma Prawn *Marsupenaeus japonicus*

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Physiological functions of juvenile hormone (JH) and molting hormone have been demonstrated in insects. JH, molting hormone and their mimics (insect growth regulators, IGRs) show endocrine-disrupting effects not only on target pest insects but also on other arthropod species such as crustaceans. However, little is known about the endocrine-disrupting effects of IGRs on benthic crustaceans. In this study, laboratory experiments were conducted to investigate effects of representative innate JH in crustaceans (methyl farnesoate, MF) and molting hormone (20-hydroxyecdysone, 20E, active form of ecdysteroid) on larval stages of the kuruma prawn *Marsupenaeus japonicus*, which is a decapod crustacean living in warm seawater. Larval development of kuruma prawn progresses in the order of nauplius, zoea, mysis, and then post-larvae with molting and metamorphosis, but it is unknown whether both MF and 20E have crucial roles in metamorphosis and molting of this species. Treatments of either MF or 20E on shrimp larvae were attempted at each developmental stage and those effects were validated. In terms of EC_{50} values between mortality and metamorphosis, there were apparent differences in the transition from nauplius to zoea (MF: 7.67 and 0.12 μM; 20E: 3.84 and 0.06 μM in survival and metamorphic rates, respectively). In contrast, EC_{50} values in MF and 20E treatments showed high consistency in the transitions between zoea to mysis (EC_{50} values for survival; MF: 1.25 and 20E: 0.22 μM), and mysis to post-larvae (EC_{50} values for survival; MF: 0.65 and 20E: 0.46 μM). These data suggest that nauplius has strong resistance against exposure to MF and 20E. Moreover, both chemicals induced high mortality triggered by the disruption of molting associated with metamorphosis. To our knowledge, this is the first experimental evidence that investigates *in vivo* physiological functions of MF and 20E in the larval stages of kuruma prawn, shedding light on not only ecotoxicological impacts of IGRs released into nature, but also endocrine mechanisms underlying larval development with metamorphosis in benthic decapod crustaceans.

Keywords: crustacean, Penaeid, juvenile hormone, ecdysone, molting

INTRODUCTION

Recent advances in arthropod phylogeny have revealed that the Crustacean clade is not monophyletic, and can be divided into three extant clades (Ostracoda, Malacostraca, Branchiopoda). A current hypothesis supports that the clade of Hexapoda (insect species) is nested within the Crustacea, which forms a new clade known as Pancrustacea, although its details are still controversial (1, 2). This finding has provided impetus into the belief that the comparative analysis of crustaceans and insects is indispensable to understanding the evolutionary origin of a range of characteristics that are believed to be insect-specific. Indeed, both crustaceans and insects share various fundamental traits such as endocrine-driven developmental and reproductive processes, which are regulated primarily by juvenile hormone (JH) and molting hormone (ecdysteroids).

Methyl farnesoate (MF) is thought to be the equivalent of JH in crustaceans. Previous studies have demonstrated that MF may play a similar role to JH in insects, by regulating molting, sexual maturation, and reproduction in concert with ecdysteroids, the main active form being 20-hydroxyecdysone, or 20E (3–5). To date, the hormonal actions of MF and 20E are triggered by activation of the JH receptor (JHR) complex (methoprene-tolerant and steroid receptor coactivator) and ecdysone receptor complex (ecdysone receptor and ultraspiracle), respectively, which are the nuclear receptors responsible for transcriptional regulation of the aforementioned biological processes in crustaceans as well as insects (6–10). Based on those findings, endocrine-disrupting chemicals targeting the JHR and/or EcR have been designed and developed as insect growth regulators (IGRs) that disrupt metamorphosis and/or molting in pest arthropods resulting in the effective suppression of pest outbreaks (11, 12). However, due to highly conserved endocrine systems between insects and crustaceans, the environmental residues of these IGRs may also affect ecologically and economically important non-target species, such as aquatic crustaceans (e.g., prawns and crabs). Despite much earnest research to investigate the toxic effects of IGRs using tiny crustaceans such as water fleas, little is still known about endocrine-disrupting effects of IGRs on benthic crustaceans. This knowledge gap is largely due to a lack of established model crustacean species that can be applied for physiological and toxicological studies.

In Malacostracan crustaceans, the eyestalk neurosecretory system, which is referred to as the X-organ–sinus gland complex, plays a pivotal role in larval development associated with molting and metamorphosis. However, the endocrine mechanisms underlying larval development are still largely unknown. Generally, biosynthesis and secretion of ecdysteroids are negatively regulated by molt-inhibiting hormone, and those of MF are also suppressively controlled by mandibular organ-inhibiting hormone (MOIH) secreted from the X-organ–sinus gland complex in eyestalks, indicating that both endogenous ecdysteroids and MF titers increase when both eyestalks were ablated (13, 14). Based on this knowledge, numerous studies found that eyestalk ablation of larvae resulted in the formation of larval intermediates in the blue crab (*Callinectes sapidus*) and the American lobster (*Homarus americanus*), and took place in extra-larval stages causing a consequent delay in metamorphosis in the mud crab (*Rhithropanopeus harrisii*), shrimps (*Palaemon macrodactylus*, *Palaemonetes varians*), and in the swimming crab (*Portunus trituberculatus*) (15) reviewed in (4). However, the mechanisms by which these hormones exert their effects remain poorly understood.

Kuruma prawn, *Marsupenaeus japonicus*, is a member of the family Penaeidae (Class Malacostraca, Order Decapoda), and is widely distributed from Japan and Southeast Asia to Western Pacific Oceans (16). Due to the economic importance of this species, annual catches have declined sharply since the 1990s (17). To overcome this situation, research into seed production of kuruma prawn, which was reared from eggs to juveniles that were then released into sea water to maintain natural populations, was conducted (18). Based on a long history of seed production of kuruma prawn, its larval developmental process is well-described. Nauplius larvae hatch about 13–14 h after ovulation at 27–29°C, and repeat molting six times (stages I–VI) within 36 h, resulting in metamorphosis to the zoea stage. Likewise, the zoea molts three times (stages I–III) within 4 days and metamorphoses into the mysis stage. Finally, the mysis molts three times (stages I–III) within 3 days and metamorphoses into the post-larval stage (4). Although 20E-driven ecdysteroid signaling pathways are known to regulate molting in crustaceans, less is known about the regulation of larval development and metamorphosis by MF and 20E. In this study, we treated kuruma prawn larvae at each developmental stage with either MF or 20E and validated toxic effects such as rates of mortality and metamorphosis.

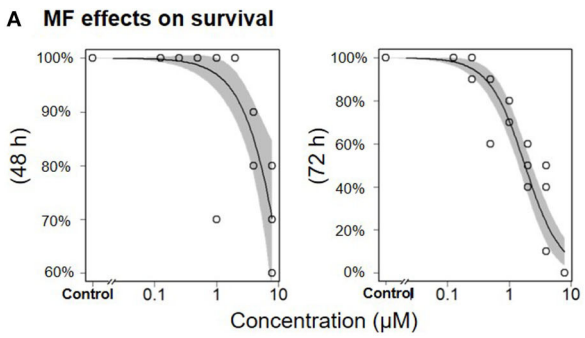
MATERIALS AND METHODS

Animals

Sexually matured female kuruma prawns (*M. japonicus*) were purchased from a local fishery shop at a fishing port of Isshiki in Nishio City, Aichi Prefecture, Japan, in April 2018. All prawns were transferred to the Mie Prefectural Fish Farming Center (Mie, Japan) and all experiments were conducted there. Prawns were maintained in a tank with natural seawater at 24°C under natural daylight and fed daily with polychaeta worms as raw bait for 1 day prior to treatment of eyestalk ablation that stimulates ovarian maturation and then spawning within a few days. Newly hatched nauplius larvae (*ca*. 10,000 individuals), which were obtained from seven females, were transferred to a 100 L black tank with natural seawater at constant 24°C under natural daylight. The culture feed series of kuruma prawn larvae was as follows: from egg to zoea stage I (3 days after beginning) was the commercial diatom *Chaetoceros gracilis* (Pacific Trading Co., Ltd., Fukuoka, Japan); from zoea stage 2 to mysis stage I was both commercial diatom and a prawn diet (Vitalprawn: Higashimaru Co., Ltd., Kagoshima, Japan); thereafter, both the commercial prawn diet and nauplius *Artemia* larvae were added until prawns grew to the post-larval stage.

Preparation and Treatment of Chemicals

A stock solution of 10 mg/mL MF (Echelon Bioscience, Salt Lake City, UT, USA) was dissolved in 100% ethanol (EtOH, Wako Pure



B EC_{50}s of MF on survival

Period	EC_{50} (μM)	Lower (95% CI)	Upper (95% CI)
48 h	7.67	6.20	9.14
72 h	1.74	1.33	2.15

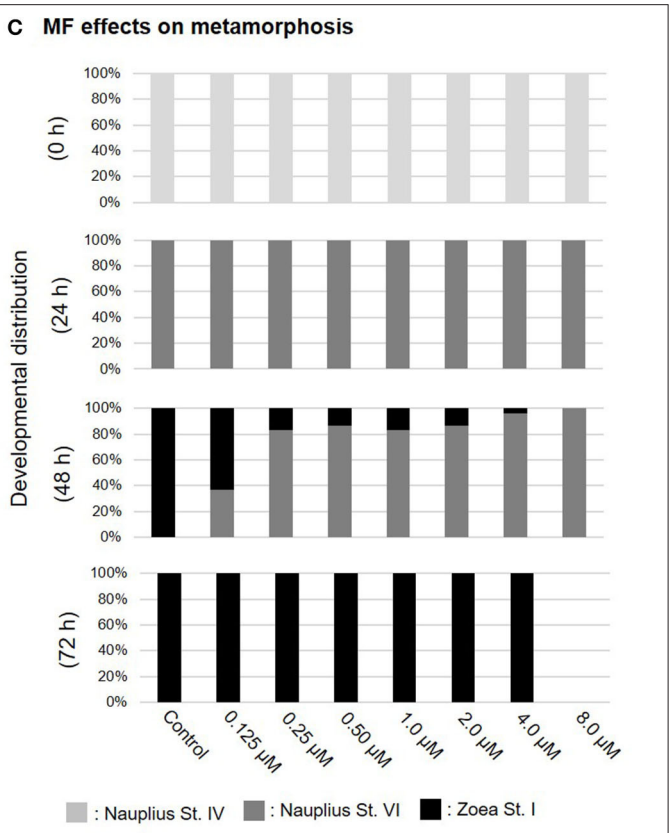


FIGURE 1 | Effects of MF treatment on nauplius stage IV. Regression curves of survival rate at 48 and 72 h **(A)**. Gray shade indicates 95% confidence interval. Each EC_{50} value of survival rate at 48 and 72 h **(B)**. Metamorphosis rates after exposure to MF at 0, 24, 48, and 72 h **(C)**.

Chemical Industries Ltd., Osaka, Japan) and kept at −20°C until use. Based on this stock solution, a dilution series was prepared, as follows: 5.0, 2.5, 1.25, 0.625, and 0.3125 mg/mL. These stock solutions were directly added to 500 mL of natural seawater containing 10 individuals each. Likewise, a stock of 100 mg/mL 20E (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100% EtOH and kept at −20°C until use. A second dilution series was prepared as follows: 50, 25, 12.5, 6.25, and 3.125 mg/mL. These stock solutions were directly added to 500 mL of natural seawater containing 10 individuals each. Beakers were prepared in triplicate for each condition.

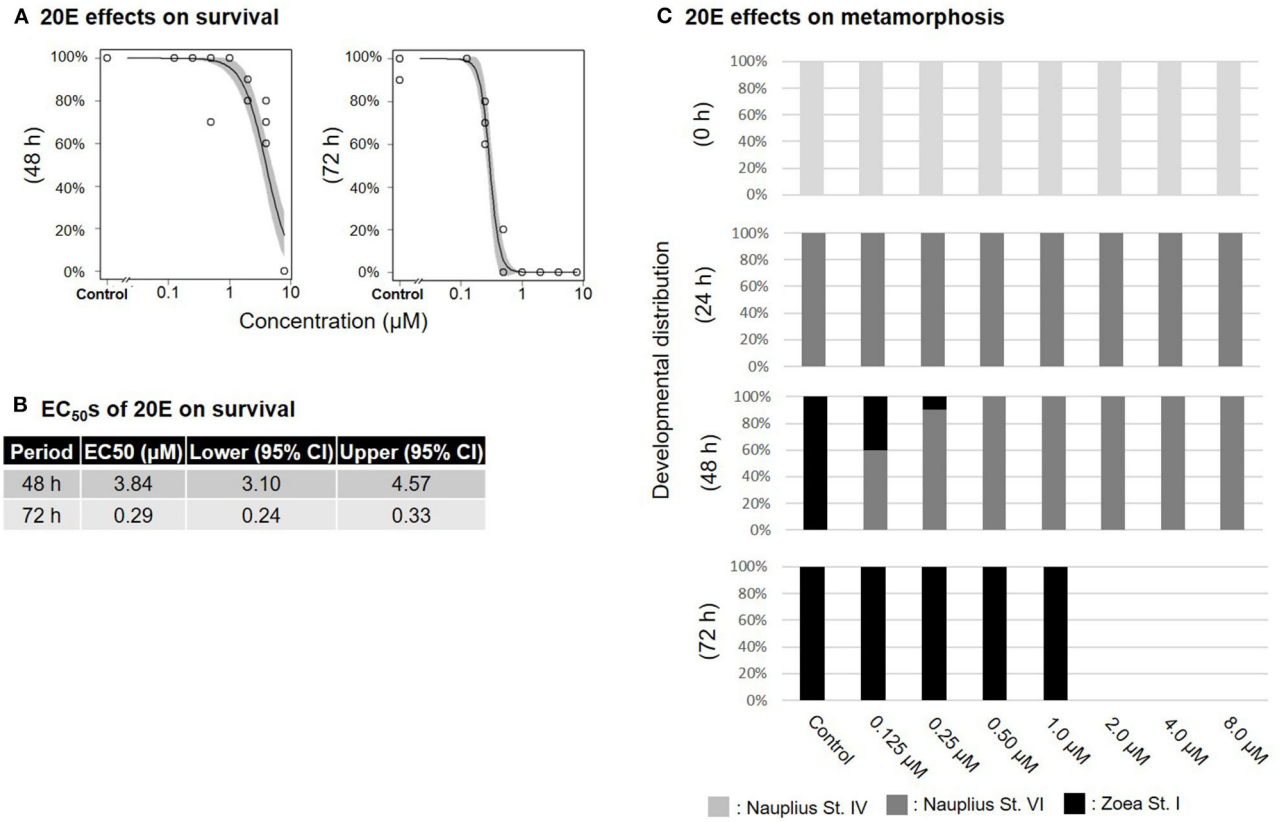
The final concentrations of MF for nauplius stage IV were 32.0, 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125 μM. Then, based on result of nauplius experiment, the range of concentrations of experiment of both zoea stage III and mysis stage III was decided as 2.0, 1.0, 0.5, and 0.25 μM. Similarly, the final concentrations of 20E for nauplius stage IV were 16.0, 8.0, 4.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 μM, and for both zoea stage III and mysis stage III, they were 2.0, 1.0, 0.5, and 0.25 μM.

After 24 h, starting from hatching, most prawns grow to nauplius stage IV. Those were exposed to MF or 20E, and mortality, molting, and metamorphosis (from nauplius stage VI to zoea stage I) were recorded for 72 h. The half maximal effective concentrations (EC_{50}) and 95% confidence interval (CI) of mortality and metamorphosis rates of tested chemicals were calculated using R software (19). All data is available upon request.

RESULTS

Effects of MF and 20E on the Nauplius Stage

Treatment of nauplius stage IV prawns with either MF or 20E caused a dose-dependent decline in survival rates, and EC_{50} values at 72 h were apparently lower (MF: 1.74 μM; 20E: 0.29 μM) than at 48 h (MF: 7.67 μM; 20E: 3.84 μM) (**Figures 1A,B**, **2A,B**). In the solvent control group, metamorphic transitions from nauplius stage VI to zoea stage I occurred within 48 h, but metamorphosis was delayed in a dose-dependent manner in both MF and 20E treatment groups (**Figures 1C**, **2C**). The EC_{50} values of metamorphosis for MF and 20E were 0.12 (95% CI: 0.05–0.20) and 0.06 (95% CI: 0.04–0.07) μM, respectively. No metamorphosis took place after exposure to 8.0 μM MF and >2.0 μM 20E. All individuals that died during this experiment were exclusively nauplius at stage VI. Moreover, transitions from nauplius stage IV to stage VI occurred at 24 h after initial exposure in all treatment groups (**Figures 1C**, **2C**).



B EC_{50}s of 20E on survival

Period	EC50 (μM)	Lower (95% CI)	Upper (95% CI)
48 h	3.84	3.10	4.57
72 h	0.29	0.24	0.33

FIGURE 2 | Effects of 20E treatment on nauplius stage IV. Regression curves of survival rate at 48 and 72 h **(A)**. Gray shade indicates 95% confidence interval. Each EC_{50} value of survival rate at 48 and 72 h **(B)**. Metamorphosis rates after exposure to 20E at 0, 24, 48, and 72 h **(C)**.

The results show that 20E was more toxic than MF to the nauplius stage in terms of the rate of mortality and metamorphosis.

Effects of MF and 20E on the Zoea Stage

When the zoea larvae at stage III were exposed to a concentration series of MF or 20E, survival ratios decreased sharply in a dose-dependent manner at 48 h (**Figures 3A,D**). The EC_{50} values of survivability of zoea larvae for MF and 20E were 1.25 (95% CI: 0.99–1.52) and 0.22 (95% CI: 0.10–0.33) μM, respectively (**Figures 3B,E**). Although the metamorphic transition from zoea to mysis occurs normally within 48 h, no metamorphic transition occurred in the 20E treatment at more than 0.5 μM, unlike in the MF treatment groups (**Figures 3C,F**), and all individuals that died were zoea at stage III, except for the 2.0 μM MF treatment group.

Effects of MF and 20E on the Mysis Stage

When mysis larvae at stage III were exposed to a concentration series of MF or 20E, survival ratios decreased dose-dependently at 48 and at 24 h, respectively (**Figure 4**). The EC_{50} values of 48 h survivability of mysis lavae for MF and 20E were 0.65 (95% CI: 0.46–0.83) and 0.46 (95% CI: 0.34–0.59) μM, respectively (**Figures 4B,E**). Even though the metamorphic transition from mysis to post-larvae occurs normally within 48 h, there was a dose-dependent decrease in metamorphic rate in response to MF treatments. In contrast, post-larvae were found in the 20E concentration series. No prawns survived exposure to 2.0 μM of either MF or 20E (**Figures 4C,F**). All individuals that died during this experiment were stage III mysis.

DISCUSSION

This study demonstrated that exogenous treatment of either MF or 20E to larval stages of kuruma prawn caused the retardation of molting associated with metamorphosis and a decline in survivability of larvae in a dose-dependent manner. Interestingly, EC_{50} values at 48 h after the exposure to these compounds indicated that 20E more effectively decreased survivability and delayed metamorphosis than MF (**Figure 5**). At the nauplius stage, there were the huge gaps in EC_{50} values between survival and metamorphosis in both MF and 20E treatments. However, each EC_{50} value between MF and 20E was very consistent when transitions occurred from zoea to mysis, and from mysis to post-larva (**Figure 5**). These findings suggest that nauplius larvae have higher tolerance against the lethal effect of MF and 20E than other stages (e.g., zoea and mysis), and that there are different endocrine cassettes regulating transition from nauplius to zoea and later metamorphosis (zoea to mysis,

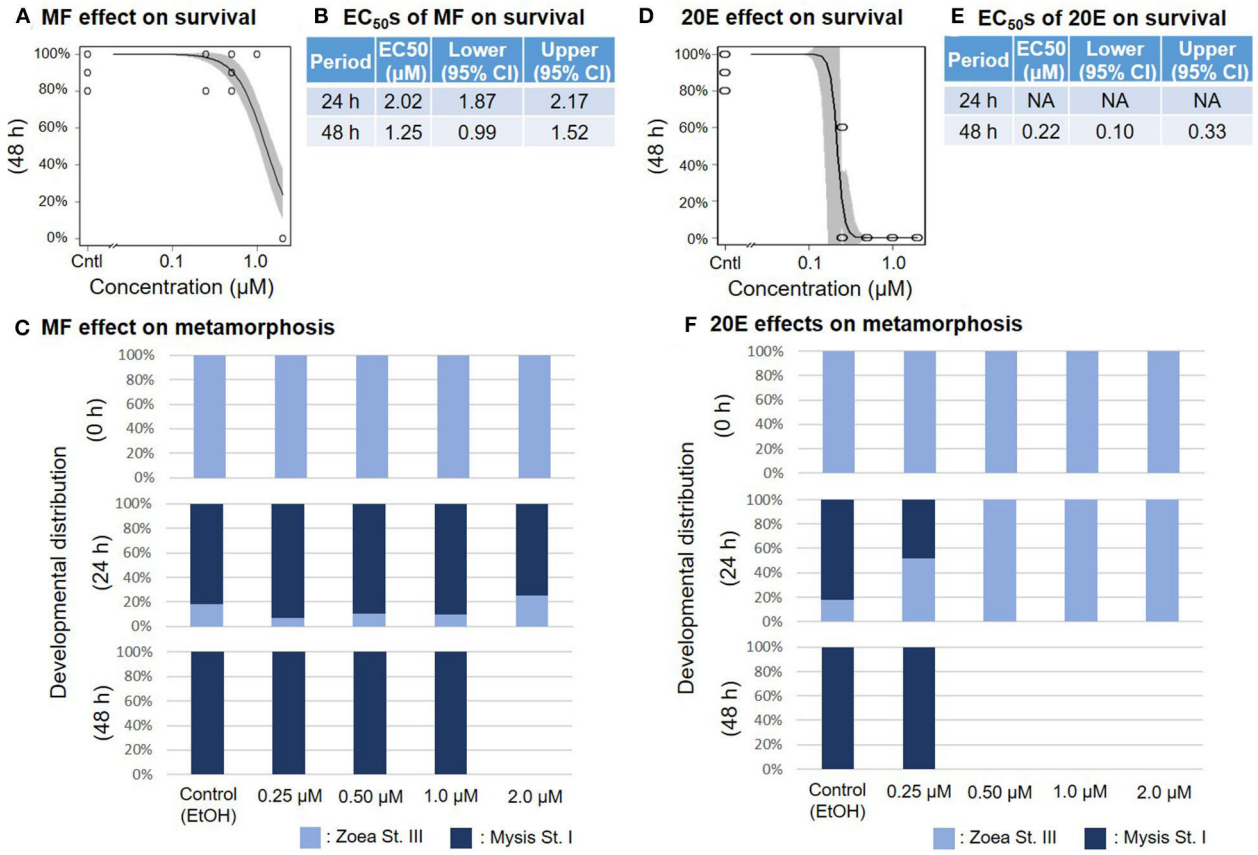


FIGURE 3 | Effects of MF and 20E treatments on zoea stage III. Regression curves of survival rate at 48 and 72 h in response to MF and 20E treatments **(A,D)**. Gray shades indicate 95% confidence intervals. EC_{50} values of MF and 20E treatments **(B,E)**. Metamorphosis rates after exposure to MF and 20E at 0, 24, and 48 h **(C,F)**.

and mysis to post larvae stages). In terms of the effects of MF on larval development and metamorphosis, some studies have reported similar results, such as the administration of MF, which delayed larval development and metamorphosis in freshwater prawn (*Macrobrachium rosenbergii*) (20, 21), or MF treatment, which induced precocious metamorphosis in the barnacle (*Balanus amphitrite*) (22, 23). Likewise, the precise regulation of the endogenous level of 20E plays a key role in the success of molting. Supporting evidence from various crustaceans consistently suggests that a pulse of the endogenous 20E titer is required for a complete molting cycle (12, 24).

Our data showed that either exogenous MF or 20E treatment to kuruma prawn larvae decreased the survival rate, and retarded larval development and metamorphosis. The EC_{50} values that we observed were similar to those in previous studies using the freshwater tiny crustacean (*Daphnia magna*), which is a well-known environmental indicator. For example, mortality in response to 20E treatment occurred with 5.119 μM (25), and males were induced by treatment with 0.278 μM MF (26). Moreover, using chemically-synthesized insecticides with MF- or 20E-like bioactivity, many studies investigated their endocrine-disrupting effects in various crustaceans. In the mud crab (*R. harrisii*), treatment with 0.159 μM fenoxycarb reduced survival and extended the duration of larval metamorphosis from zoea to megalopa (27). The LC_{50} (50% lethal concentration) values at 24 h for fenoxycarb and methoprene, two JH-mimicking chemicals, were 4.74 and 6.31 μM, respectively (at 48 h, values were 3.52 and 4.48 μM), in the cherry shrimp (*Neocaridina davidi*) (28). Natural concentrations of methoprene in freshwater have typically ranged from 3.0 to 30.0 nM (29, 30), suggesting that IGRs with JH-activity might have toxic effects on crustacean development in wild populations. Similarly, treatment with 3.37–33.7 μM of RH 5849 (1,2-dibenzoyl,l-t-butyhly drazine), which has IGR-bearing ecdysteroid activity, accelerated larval molting in the crab (*R. harrisii*) and enhanced attachment and metamorphosis in the barnacle (*B. amphitrite*) (31), while exogenous treatment with 0.5 μM 20E inhibited molting and ovulation in the water flea (*D. magna*) (32). Taken together with findings in other crustaceans, the present study demonstrates that larval development in the kuruma prawn may be highly sensitive to MF- and 20E-like chemicals. In addition to short-term assay, the long-term (chronic) assay will be more clearly demonstrated that impacts of those chemical exposure on larval development of kuruma prawn in the ecological point of view.

Recent advances in omics approaches enable the depiction of the expression pattern of many genes and to estimate the

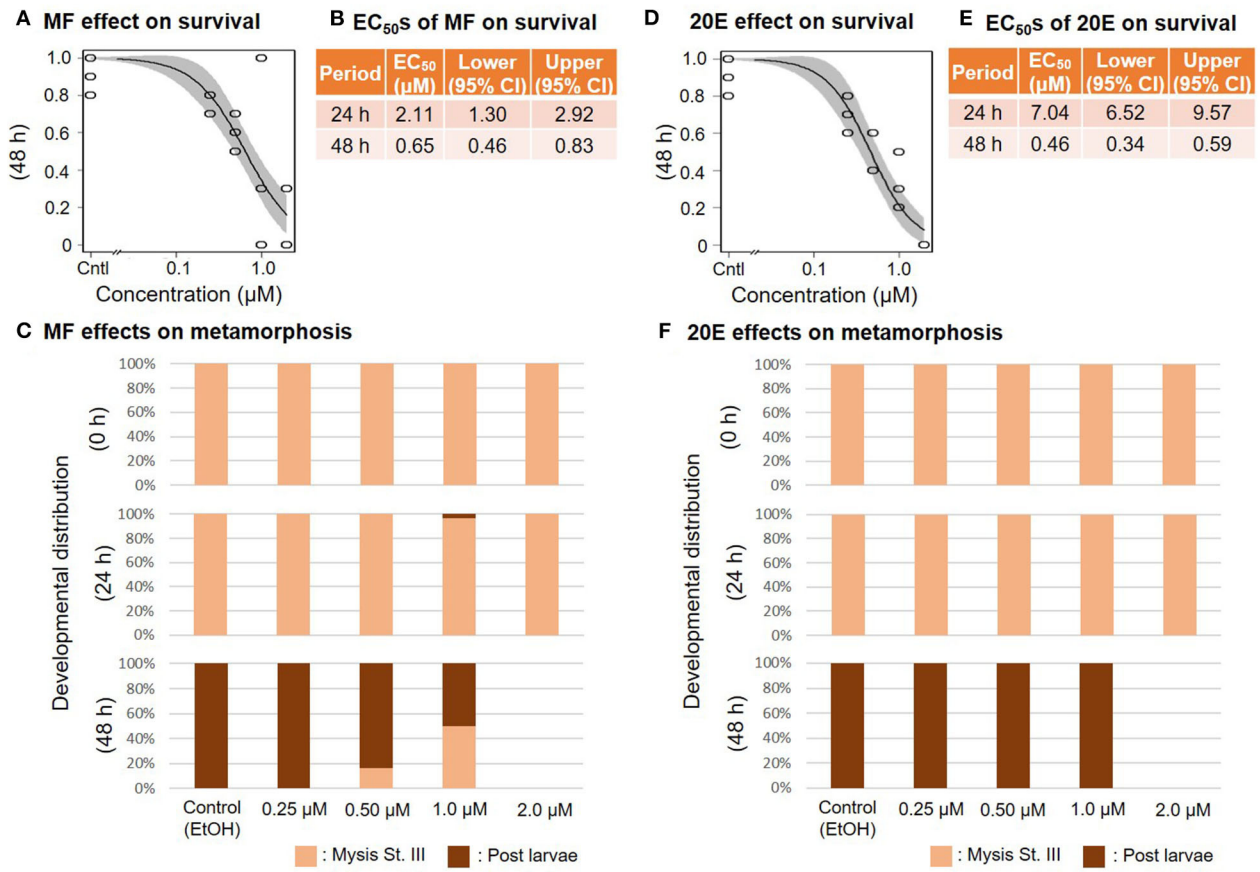


FIGURE 4 | Effects of MF and 20E treatments on mysis stage III. Regression curves of survival rate at 48 and 72 h in response to MF and 20E treatments **(A,D)**. Gray shades indicate 95% confidence intervals. EC_{50} values of MF and 20E treatments **(B,E)**. Metamorphosis rates after exposure to MF and 20E at 0, 24, and 48 h **(C,F)**.

regulatory interactions involved in metamorphosis in the prawn (*M. rosenbergii*) (33), the shrimp (*Neocaridina denticulata*) (34), and the spiny lobster (*Sagmariasus verreauxi*) (35, 36). Although the aforementioned transcriptome studies provided various new insights, the number of unannotated genes has hampered the completion of more comprehensive analysis due to the lack of publicly available genomes. To address this resource problem, the complete genome of the Pacific white shrimp (*Litopenaeus vannamei*) was decoded, providing a new hypothesis of the regulatory mechanisms underlying adult molting via sterol regulatory elements (SRE)-binding protein and opsin (37). In addition to next generation sequencing, mass spectrometry technology (e.g., LC- and GC-MS) has allowed the metabolite profiling (38), and quantification of endogenous juvenoid in hemolymph of freshwater prawn *M. rosenbergii* (39) and ecdysteroid titers in the extracts of tiny crustaceans (40, 41), enabling the monitoring of the pulse (rise and decline) of those hormones during metamorphosis. It will be necessary to elucidate the fluctuating dynamics of MF and 20E titers during larval development in kuruma prawn. Some advanced studies found that this can be successfully achieved by integrating the data acquired from *in vivo* pharmacological assays and omics approaches (28, 42, 43), suggesting that this approach will be applied for comprehensively understanding the mechanisms of diversified crustacean metamorphosis. Additionally, treatments of those inhibitors/antagonists will be useful for understanding their physiological function. Indeed, the fenarimol, which is an inhibitor of ecdysteroid synthesis, could be applied in the molting research, because it has been used in *Daphnia* (40). Although less is known about the inhibitor/antagonist of JH, some potential JH antagonists have been identified using the yeast two-hybrid system transformed with the mosquito JH receptor as a reporter system (44).

In conclusion, we conducted laboratory experiments to investigate the toxic effects of MF and 20E using larval stages of the kuruma prawn. We demonstrated that both MF and 20E induced high mortality caused by disruption of molting-associated metamorphosis, although the nauplius showed strong resistance to MF and 20E. This is the first experimental evidence of the *in vivo* physiological functions of MF and 20E in the larval stages of kuruma prawn, shedding light on not only the ecotoxicological impacts of IGRs released into nature, but also on the endocrine mechanisms underlying larval development with metamorphosis in benthic decapod crustaceans.

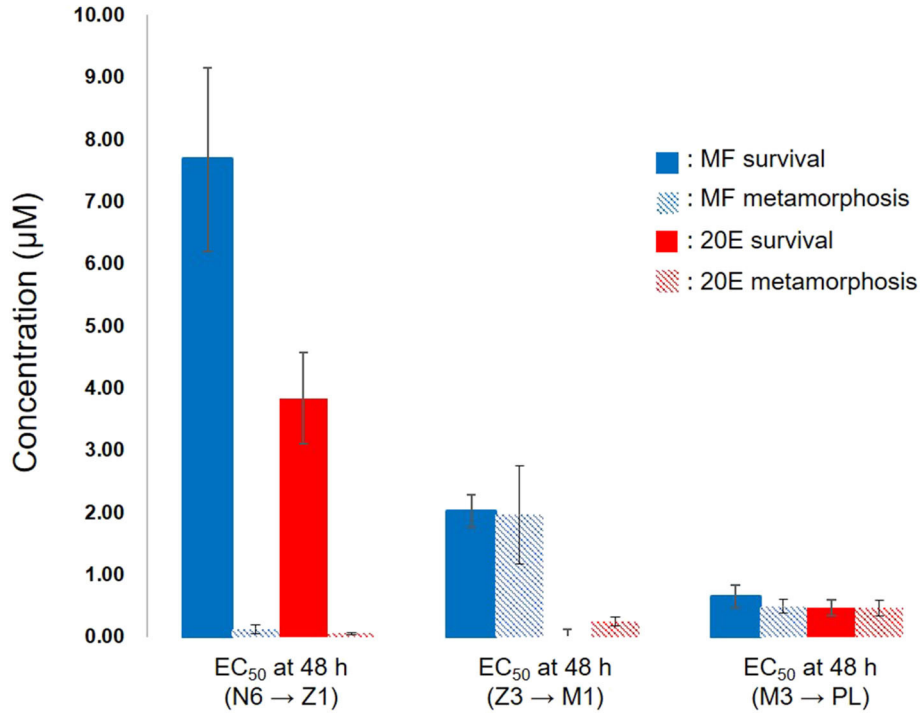


FIGURE 5 | Relationships between EC_{50} of survivability and metamorphosis at 48 h after exposure of MF and 20E. N6, nauplius stage VI; Z1, zoea stage I; Z3, zoea stage III; M1, mysis stage I; M3, mysis stage III; PL, post-larvae. Error bars indicate 95% confidence intervals. NA indicates that data is not available.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KT and TO designed all experiments in this study. KT and FY conducted all experiments and obtained all raw data. KT conducted all data analyses and prepared the first draft of the paper. All authors contributed to the article and approved the submitted version.

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Integrative Proteomic and MicroRNA Analysis: Insights Into Mechanisms of Eyestalk Ablation-Induced Ovarian Maturation in the Swimming Crab *Portunus trituberculatus*

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Meng X, Zhang M, Gao B, Lv J, Li J and Liu P (2020) Integrative Proteomic and MicroRNA Analysis: Insights Into Mechanisms of Eyestalk Ablation-Induced Ovarian Maturation in the Swimming Crab Portunus trituberculatus. Front. Endocrinol. 11:533. doi: 10.3389/fendo.2020.00533

Eyestalk ablation is the most common method to induce ovarian maturation in decapod crustacean aquaculture, but it jeopardizes broodstock survival and larvae production. It is important to understand the molecular basis underlying the maturation triggered by ablation and thereby develop an alternative measure for maturation manipulation. In this study, we investigate alterations of ovarian proteome and miRNA profile after ablation in a commercially important marine crab *Portunus trituberculatus*. Quantitative proteomic analysis using iTRAQ reveals that 163 proteins are differentially expressed following ablation, and modulation of methyl farnesoate metabolism and activation of calcium signaling may play important roles in the ovarian maturation induced by ablation. miRNA expression profiling identifies 31 miRNAs that show statistically significant changes. Integration of miRNA and proteome expression data with miRNA target prediction algorithms generates a potential regulatory network consisting of 26 miRNAs and 30 proteins linked by 71 possible functional associations. The miRNA-protein network analysis suggests that miRNAs are involved in promoting ovarian maturation by controlling expression of proteins related to methyl farnesoate synthesis, calcium signals, and energy metabolism. Experimental validation and temporal expression analysis indicate multiple miRNAs can act synergistically to regulate expression of Farnesoic acid O-methyltransferase and Calmodulin. Our findings provide new insights for elucidating the mechanisms underlying eyestalk ablation-induced ovarian maturation and could be useful for devising an alternative technique for manipulating reproduction in *P. trituberculatus* and other decapods.

Keywords: crab, eyestalk ablation, miRNA, proteome, ovarian development

INTRODUCTION

The eyestalk, where the X-organ/sinus gland (XO/SG) complex is located, is an important neuroendocrine system in crustaceans, which is the major site of production and storage for a variety of important neuropeptides, such as the gonad-inhibiting hormone (GIH), the molt-inhibiting hormone (MIH), the pigment-dispersing hormone (PDH), and the red pigment-concentrating hormone (RPCH) (1, 2). Among these neuropeptides, GIH is considered to play a key role in regulating female crustacean reproduction by repressing ovarian maturation and spawning (3). In decapod aquaculture practice, removal of GIH by eyestalk ablation (ESA) has been the most common method to induce ovarian maturation (4). However, in addition to GIH, secretion of other neuropeptides from the eyestalk is also impaired by this method, which results in significant hormonal imbalance and undesired impacts on not only the survival of broodstock, but also the quantity and quality of larvae produced (5). Hence, an alternative technique for maturation manipulation without ESA has been a long-term goal for the decapod culture industry.

To develop new techniques for triggering maturation, it is important to understand the molecular mechanisms of ablation-induced ovarian maturation. During the past decade, many efforts have been made to elucidate the molecular effects of ESA on ovarian development in decapods (6–10). With high-throughput transcriptome analysis, recent research provides a global picture of gene expression changes at the transcriptional level after ablation. Uawisetwathana et al. (11) find that eyestalk ablation activates several signal pathways important for ovarian development, such as the gonadotropin-releasing hormone (GnRH) signaling pathway and the progesterone-mediated oocyte maturation pathway, which promotes ovarian maturation in *Penaeus monodon*. Lee et al. (12) report that ESA can modulate gene expression in the hepatopancreas of *Litopenaeus vannamei*, upregulating genes related to metabolic processes as well as those associated with immunity and stress responses. These studies expand our knowledge on the transcriptional regulation that mediates ESA-induced maturation. Nevertheless, transcription change does not depict the changes of protein levels with complete accuracy because gene expression can also be modulated at the post-transcriptional and translational levels. To date, no information is available regarding gene expression regulation at post-transcriptional and translational levels following ESA.

MicroRNAs (miRNAs), a group of small non-coding RNAs, function as key post-transcriptional regulators of gene expression by complementary binding to 3′ untranslated regions of their target mRNA, leading to mRNA destabilization, or protein translation blockage (13, 14). They are involved in many fundamental biological processes, such as development, metabolism, cell proliferation, and signal transduction (15–17). Recent evidence has revealed that miRNAs are critical in ovarian development and maturation in decapod crustaceans. In *Eriocheir sinensis*, miR-2, and miR-133 are demonstrated to regulate meiotic oocyte maturation by modulating cyclin B expression (18). A number of miRNAs have been found to exhibit ovary-biased expression in *L. vannamei*, *Macrobrachium nipponense*, *Portunus trituberculatus*, and *Scylla paramamosain*, and many of the predicted target genes for these miRNAs are crucial in regulating ovarian development, indicating the important functions of miRNA in ovarian development (19–22). However, the roles of miRNA in ESA-induced ovarian maturation is still unknown.

Proteomic analysis is a tool that facilitates research on comprehensive protein expression profiling and identification of individual proteins involved in specific biological responses. It has been widely utilized in studying reproductive development in mammals, fish, and insects (23–25). In crustaceans, only a few proteomic studies have been conducted on ovarian development, and these studies all rely upon 2-D gel electrophoresis data (26–28), which cannot identify low abundant proteins, proteins with low or high molecular weights, and proteins that are excessively acidic or basic as well as hydrophobic proteins (29, 30). The isobaric tags for relative and absolute quantitation (iTRAQ) is a mass spectrometry–based proteomics technique established in recent years and has overcome some of the drawbacks of 2-D gel electrophoresis; it is sensitive and can detect large proteins and low-abundant proteins with high throughput and low experimental error (31). Hence, iTRAQ should be an ideal approach to study protein expression changes after ESA.

The swimming crab *P. trituberculatus* (Crustacea: Decapoda: Brachyura) is an important fishery and aquaculture species, widely distributed in the estuary and coastal waters of Korea, Japan, China, and Southeast Asia (32). This species supports a large aquaculture industry in China, and its farming developed quickly during the last decade. In 2017, the production of *P. trituberculatus* reached 119,777 t (China Fishery Statistical Yearbook, 2018). However, due to rapid expansion of crab farming and lack of an effective technique for reproductive manipulation, high-quality seed production cannot fulfill the demand, which restricts further development of the industry (33, 34). Therefore, it is crucial to understand the regulatory mechanisms of ovarian maturation and improve the means of artificial seed production. In this study, we investigate the alteration in proteome and miRNA transcriptome following ESA. The results can improve our understanding of molecular mechanisms underlying ESA-induced ovarian maturation and provide useful information for developing new reproduction manipulation techniques.

RESULTS

Protein Expression Altered by ESA

To identify proteins involved in ESA-induced ovarian maturation, iTRAQ was employed to assess ovarian protein expression changes at the fourth day after ESA. In total, 483,601 spectra were generated from the ovary of the eyestalk-intact (ESI) and ESA crabs. Based on the spectral data, 15,377 peptides and 3400 proteins were identified (cutoff: Mascot Percolator q-value <= 0.01). With the criteria of fold change $\geq$1.2 and $p < 0.05$, 163 proteins were identified to be differentially expressed (DE) between ESI and ESA crabs; a great majority (132 proteins, 80.98%) of the DE proteins showed upregulation in ESA individuals (**Table 1**).

TABLE 1 | List of the annotated proteins that exhibited differentially expression after ESA.

	NCBI Accession	Protein name	Fold change
1	XP_003702718.1	Peritrophin-1-like	3.66
2	XP_012151113.1	Fibrillin-2-like isoform X2	3.08
3	AAY56093.1	Death-associated protein-like	2.32
4	ACZ02405.1	Heat shock protein 70	2.28
5	XP_003248548.1	Clathrin light chain-like	1.95
6	XP_002431005.1	Ubiquitin-fold modifier 1 precursor	1.90
7	XP_002404909.1	Prefoldin	1.84
8	AFE88627.1	Thioredoxin 2	1.79
9	KDR08860.1	Tumor protein D54	1.79
10	XP_004922887.1	BolA-like protein	1.76
11	XP_012176731.1	Fibril-forming collagen alpha chain like	1.76
12	XP_974307.1	Similar to par-6 gamma	1.74
13	ADZ96217.1	JHE-like carboxylesterase 1	1.70
14	EFN74540.1	Sorting nexin-12	1.70
15	AFS60116.1	Selenoprotein M	1.69
16	NP_001037686.1	Aspartylglucosaminidase	1.67
17	BAJ22990.1	Cytochrome c	1.67
18	ACL26692.1	Farnesoic acid O-methyltransferase	1.67
19	XP_001865898.1	Antioxidant enzyme	1.66
20	AAY57406.1	Program cell death 5-like	1.66
21	ACU82846.1	Acyl-CoA-binding protein	1.64
22	ACO11851.1	RNA-binding protein 1	1.64
23	AAO73307.1	Ovary development-related protein	1.63
24	ACO11926.1	Charged multivesicular body protein 5	1.61
25	ACJ53746.1	Peroxiredoxin 6	1.61
26	XP_001942794.1	39S ribosomal protein L12, mitochondrial-like isoform 1	1.60
27	ACL13568.1	AMP-activated protein kinase alpha subunit	1.60
28	ACY66390.1	FK506-binding protein 1A	1.60
29	ABI98678.1	Ubiquitin-conjugating enzyme	1.59
30	XP_972770.2	Insulin receptor	1.58
31	ADE60733.1	Myosin essential light chain	1.57
32	NP_001103783.1	Tropomyosin-2 isoform 3	1.57
33	ABF55966.2	Cleavage stimulation factor 64-kDa subunit	1.56
34	EFN66390.1	PERQ amino acid-rich with GYF domain-containing protein 2	1.56
35	XP_796085.2	Transcription and mRNA export factor ENY2	1.56
36	XP_011300302.1	Prefoldin subunit 1	1.55
37	KDR23647.1	Protein phosphatase inhibitor 2	1.55
38	ACR56783.1	Small ubiquitin-like modifier-1	1.55
39	ACY66642.1	Thymosin beta	1.55
40	EGI57685.1	Non-specific lipid-transfer protein	1.54
41	KDR12501.1	Spondin-1	1.54
42	EFN86015.1	GS1-like protein	1.53
43	XP_003723328.1	ES1 protein homolog, mitochondrial-like	1.52
44	XP_011501070.1	Far upstream element-binding protein 1 isoform X3	1.52
45	XP_003445744.1	Methylmalonyl-CoA epimerase	1.52
46	EGW07359.1	Peptidyl-prolyl cis-trans isomerase, mitochondrial	1.52
47	XP_008470922.1	Verprolin-like	1.52
48	XP_003451145.1	Aminopeptidase W07G4.4-like	1.51
49	XP_012259502.1	DnaJ homolog subfamily B member 11	1.51
50	XP_001847373.1	Ubiquinol-cytochrome c reductase complex 14 kDa protein	1.51
51	ADW24146.1	Vesicle-associated membrane protein-associated protein	1.5
52	AAX94762.1	Vitellogenin	1.5
53	XP_003707900.1	28S ribosomal protein S36, mitochondrial-like	1.49
54	XP_002427853.1	Protein-L-isoaspartate O-methyltransferase	1.48
55	BAM18170.1	Prefoldin subunit	1.47
56	AGF39576.1	Double WAP domain-containing protein	1.46
57	XP_002413321.1	LIM domain-binding protein	1.46
58	XP_001660469.1	Low-density lipoprotein receptor	1.46
59	EGI66356.1	Prefoldin subunit 2	1.46
60	ELW71144.1	Calmodulin	1.45
61	KDR23803.1	Outer dense fiber protein 3	1.45
62	XP_001947263.2	Upstream activation factor subunit spp27-like	1.45
63	XP_008473520.1	Pumilio homolog 1-like	1.44
64	NP_001090127.1	Tubulin folding cofactor B	1.44
65	ACO36738.1	Ubiquitin carboxyl-terminal esterase L3	1.43
66	KMQ90936.1	Barrier-to-autointegration factor	1.42
67	NP_001156264.1	Calcium-regulated heat stable protein 1	1.42
68	XP_002423140.1	Charged multivesicular body protein 4C	1.42
69	XP_003690694.1	Synaptosomal-associated protein 29-like	1.42
70	ACI13851.1	Extracellular copper-zinc superoxide dismutase	1.41
71	XP_002425615.1	Methionyl-tRNA synthetase	1.41
72	XP_008199673.1	Nuclear protein MDM1 isoform X4	1.41
73	XP_003486756.1	Stress-induced-phosphoprotein 1-like isoform 1	1.41
74	ETN61219.1	Aldehyde oxidase	1.40
75	ADO00931.1	Calnexin	1.40
76	XP_002413938.1	Low-density lipoprotein receptor	1.40
77	KDR20387.1	Phosphoglycerate mutase 2	1.40
78	ACY66501.1	60S acidic ribosomal protein P2	1.39
79	XP_004065994.1	Cytosol aminopeptidase-like	1.39
80	XP_011416416.1	Histidine triad nucleotide-binding protein 2	1.39
81	EKC27215.1	Ras GTPase-activating protein-binding protein 2	1.39
82	EKC33829.1	Methenyltetrahydrofolate synthetase domain-containing protein	1.38
83	XP_011157802.1	Short-chain specific acyl-CoA dehydrogenase	1.38
84	XP_012150508.1	Multiple coagulation factor deficiency protein 2 homolog	1.37
85	XP_002409815.1	PDZ domain-containing protein	1.37
86	XP_001943654.1	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase	1.36
87	NP_001164152.1	Held out wings	1.36
88	ACR54112.1	Ribosomal protein P1	1.36

(Continued)

TABLE 1 | Continued

	NCBI Accession	Protein name	Fold change
89	KDR12892.1	Syntaxin-12	1.36
90	XP_003400244.1	Cyclin-dependent kinase 6-like	1.35
91	XP_005175277.1	ATP synthase subunit d	1.34
92	ACH88358.1	Cell division cycle 2	1.34
93	ADN52396.1	Triosephosphate isomerase	1.34
94	EFA07536.1	RAE1 RNA export 1 homolog	1.33
95	ACO14747.1	Calponin-3	1.32
96	NP_001020355.1	DnaJ homolog subfamily B member 9 precursor	1.32
97	ACZ06791.1	Eukaryotic translation initiation factor 5A	1.32
98	AET36895.1	Peroxiredoxin 2	1.32
99	AAC78141.1	Phosphopyruvate hydratase	1.32
100	AFC17961.1	O-methyltransferase	1.31
101	XP_002415663.1	Alternative splicing factor ASF/SF2	1.30
102	EKC42097.1	Cathepsin F	1.28
103	ACI46952.1	Cyclin B	1.28
104	KDR07772.1	Plastin-2	1.28
105	XP_012267954.1	Golgi resident protein GCP60	1.27
106	XP_972648.1	Similar to adaptin ear-binding coat-associated protein 2	1.25
107	ACY66506.1	Ubiquitin associated protein 2-like protein	1.25
108	KFM60612.1	DnaJ-like protein subfamily A member 2	1.24
109	XP_003705474.1	Aconitate hydratase	1.23
110	ADQ55791.1	Antimicrobial peptide hyastatin	1.23
111	CAA72032.2	Masquerade-like protein	1.22
112	XP_973346.1	Phosphoacetylglucosamine mutase	0.83
113	AAC64660.1	Pacifastin heavy chain precursor	0.82
114	AEF32710.1	Translationally controlled tumor protein	0.81
115	ACY66537.1	60S ribosomal protein L27	0.80
116	AAZ22828.1	Lymphoid organ expressed yellow head virus receptor protein	0.80
117	KFM60603.1	60S ribosomal protein L7a	0.79
118	ABQ10738.1	Cathepsin D	0.79
119	XP_011136175.1	Proteasome subunit alpha type-5	0.79
120	XP_972566.1	Succinate semialdehyde dehydrogenase, mitochondrial	0.79
121	AET34923.1	Peroxiredoxin 1	0.78
122	XP_002401133.1	Ribosomal protein S26	0.78
123	NP_001037263.1	Ribosomal protein S8	0.77
124	XP_003705948.1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase-like	0.75
125	XP_002423307.1	cAMP-dependent protein kinase catalytic subunit	0.73
126	ACN87221.1	Phenoloxidase activating factor	0.72
127	XP_007442568.1	Glutathione peroxidase 7-like	0.71
128	KFM75426.1	Protein canopy-like protein	0.71
129	ABX71209.1	Glycosyl-phosphatidylinositol-linked carbonic anhydrase	0.69
130	AAW57889.1	Hemocyanin subunit 1	0.69
131	AAW57890.1	Hemocyanin subunit 2	0.69
132	AAW57891.1	Hemocyanin subunit 3	0.65
133	AAA96966.2	Hemocyanin subunit 6	0.59
134	AAF64305.1	Hemocyanin subunit	0.56
135	EGI63299.1	Histone-lysine N-methyltransferase trr	0.24

(Continued)

The functional category of the DE proteins was analyzed against the Gene Ontology (GO) database using three sets of ontologies: biological process, molecular function, and cellular component (**Figure 1**). The most abundant proteins in the biological process category were related to metabolic process (GO:0008152, 37 proteins), followed by cellular process (GO:0009987, 35 proteins). In the cellular component category, the proteins associated with cells (GO:0005623, 29 proteins) and cell parts (GO:0044464, 29 proteins) were dominant. In the molecular function category, binding (GO:0005488, 37 proteins) was the most prominent, followed by catalytic activity (GO:0003824, 34 proteins).

miRNA Expression Modulated by Eyestalk Ablation

To decipher the miRNAs implicated in ovarian maturation induced by ESA, the global ovarian miRNA expression after ESA was investigated using miRNA high-throughput sequencing. Relatively strict criteria were used to identify known and novel miRNAs. Only the small RNAs that aligned to the miRNAs in miRBase with no mismatch were classified as known miRNAs, and only those identified by both miREvo and mirdeep2 software were considered as novel miRNAs. In total, 184 unique miRNAs were identified after eliminating low-abundance miRNAs (reads $\leq$ 20), among which 100 were novel. Despite stringent criteria used for identifying miRNAs, there is possibility that some of these miRNAs may be degraded products of other RNAs due to lack of reference genome for miRNA prediction.

miRNA profile analysis showed that 31 miRNAs exhibited differential expression (under the criteria of fold changes $\geq$ 2.0 and $p < 0.05$) between ESI and ESA crabs, and most of them (24 miRNAs, 77.42%) were downregulated in ESA individuals (**Figure 2** and **Table S1**).

In silico Correlation of Protein and miRNA and Experimental Validation

To uncover the potential crosstalk between specific miRNAs and proteins in response to ESA, we constructed a regulatory network based on miRNA target prediction algorithms and inverse correlation of protein and miRNAs expression (**Figure 3** and **Table 2**). The miRNA-protein network was composed of 26 miRNAs and 30 target proteins. Among the targets, several proteins related to methyl farnesoate (MF) synthesis [Farnesoic acid O-methyltransferase (FAMeT)], oocyte meiotic maturation (Cyclin B), calcium signal transduction [Calmodulin (CaM)], and energy metabolism [AMP-activated protein kinase (AMPK),

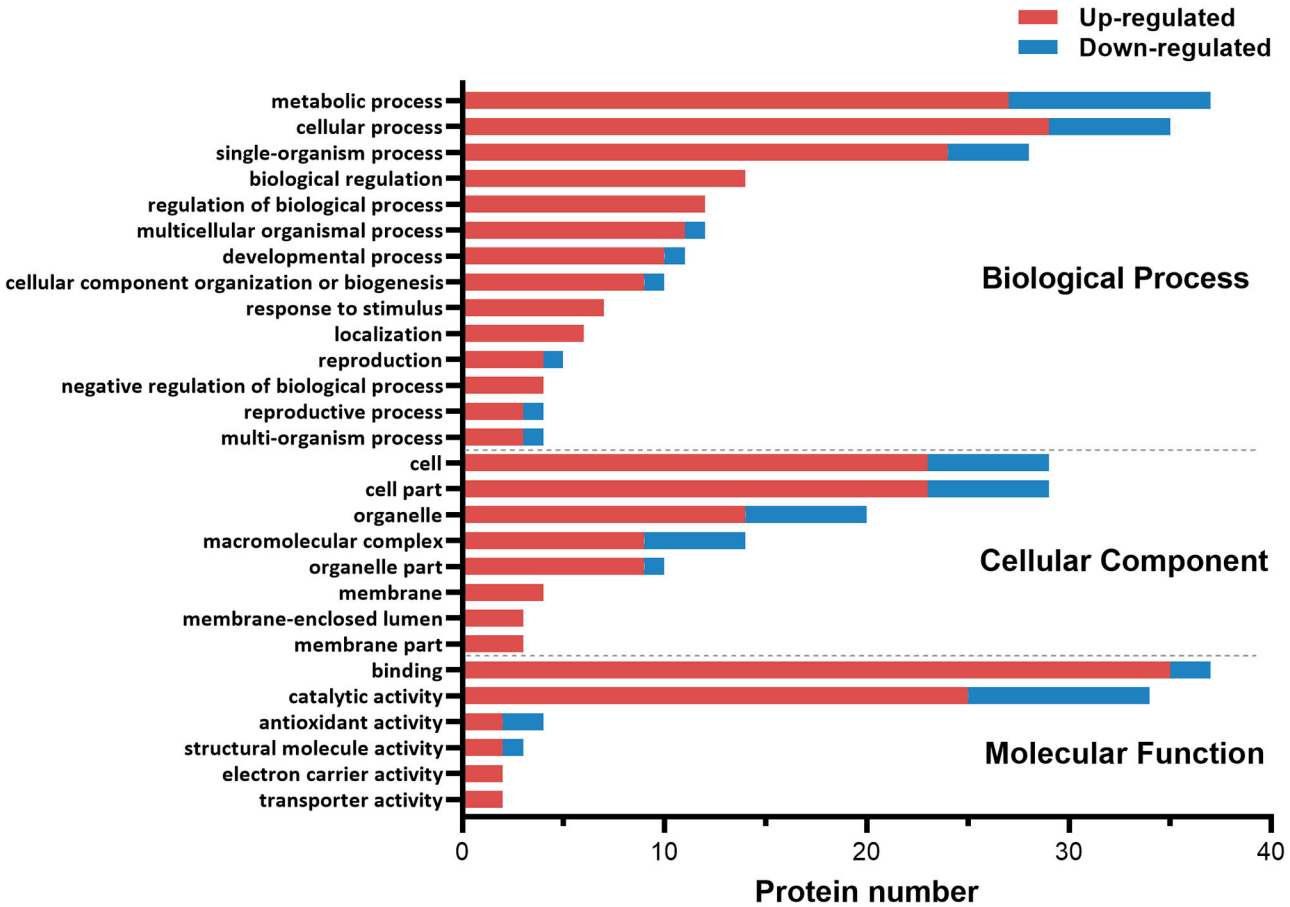

FIGURE 1 | Gene ontology (GO) classification diagram of differentially expressed proteins in the ovary of ESA and ESI crabs. Red and blue represent upregulated and downregulated proteins after ESA, respectively.

Phosphoglycerate mutase 2 (PGAM2), Triosephosphate isomerase (TPI), Phosphopyruvate hydratase (PPH), and Methylmalonyl-CoA epimerase (MCE)] were identified.

Using the dual luciferase reporter assay, we validated direct interaction of five miRNA-target pairs (miR-263a/FAMeT, miR-4171/FAMeT, miR-2b/CaM, miR-317/CaM, and miR-466f-3p/CaM) (**Figure 4**). The results show that the relative luciferase activity (firefly luciferase activity/Renilla luciferase activity) was significantly reduced after pmirGLO-FAMeT-3′UTR was cotransfected with miR-263a or miR-4171 and pmirGLO-CaM-3′UTR was cotransfected with miR-2b, miR-317, or miR-466f-3p, which indicates the interaction between the miRNAs and their targets.

To further validate the correlation between the miRNAs and their targets and investigate their temporal express pattern after ESA, we analyzed the levels of the miRNAs and their target genes at different times following ESA. The results show that expression of miR-263a, miR-4171, miR-2b, and miR-317 negatively correlates with that of their target genes (**Figure 5**) (miR-263a/*FAMeT*: $p < 0.01$; miR-4171/*FAMeT*: $p < 0.01$; miR-2b/*CaM*: $p < 0.01$; miR-317/*CaM*: $p < 0.01$), and there is no significant correlation between expression levels of miR-466f-3p and *CaM* ($p > 0.05$). *FAMeT* increased significantly from 72 h and maintained high expression until 168 h after ESA, whereas miR-263a and miR-4171 exhibited lower expression at those time points. *CaM* showed a significant upregulation from 48 to 168 h, and miR-2b and miR-317 were downregulated during these time periods. These results further confirm that miR-263a, miR-4171, miR-2b, and miR-317 are involved in regulating the expression of FAMeT and CaM after ESA.

DISCUSSION

Despite the undesirable effects, eyestalk ablation is currently the most effective method to induce ovarian maturation in the commercial hatchery of decapods. To date, the molecular mechanisms underlying the induced maturation have not been fully understood, which hinders development of new techniques for maturation manipulation. Previously, many studies have been focused on transcriptional regulation in response to ESA (35–37). Considering the fact that proteins and miRNAs form the cellular end point (proteins) of phenotypic features and their regulatory elements (miRNA), the present study concentrated on changes

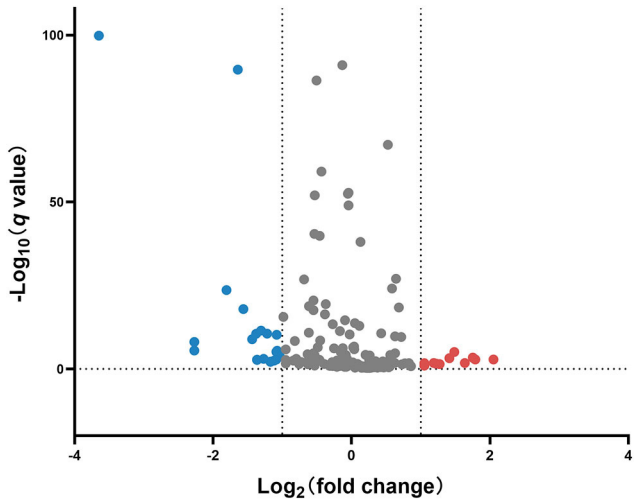

FIGURE 2 | Volcano plot of differentially expressed miRNAs in the ovary of ESA and ESI crabs. Gray, red, and blue dots represent non-significant, upregulated, and downregulated miRNAs after ESA, respectively.

that occur in protein and miRNA expression. The results here present for the first time extensive ovarian proteome and miRNA profiling reflecting the alterations induced by ESA. Proteomic analysis using iTRAQ shows that 163 proteins are differentially expressed in the ovary of intact and ablated crabs, and among them, proteins regulating vitellogenesis, oocyte meiosis, and energy metabolism are identified, indicating their involvement in promoting ovarian maturation after ESA. miRNA transcriptome results show that 31 miRNAs exhibit differential expression after ESA. By integrating bioinformatic algorithms with expression data of miRNA and protein, we identify 30 targets of the DE miRNAs and define the potential roles of miRNA in maturation induced by the ablation.

Vitellogenesis

Vitellogenin (Vg), the precursor of the major egg yolk proteins, is regarded as a reliable marker of ovarian maturation in decapods as its expression is positively correlated to ovarian maturation levels (11). Consistent with the results of Vg transcript levels in previous studies (38, 39), Vg protein level in the ovary was enhanced significantly after ESA in this study, confirming that ESA leads to increased ovarian vitellogenesis in the swimming crab. In crustaceans, various Vg synthesis sites have been reported in different species. For *P. trituberculatus*, vitellogenesis is demonstrated to occur both within the oocyte (endogenous vitellogenesis) and external to the oocyte (exogenous vitellogenesis), and exogenous Vg is the major source of Vg, which is synthesized in hepatopancreas, transported via hemolymph, and absorbed by oocytes or follicle cells through receptor-mediated endocytosis (40). In our study, an elevated level of Vg receptor (VgR) was observed in the ovary of the ablated crabs compared with that of the intact crabs. This result suggested that ESA can promote accumulation of exogenous vitellogenesis by upregulating VgR expression and facilitating uptake of Vg from hemolymph.

Methyl Farnesoate Metabolism

Methyl farnesoate (MF), a sesquiterpene compound, plays a crucial role in reproduction regulation of decapods (41). Previous studies demonstrate that there is a positive correlation between MF titer and ovarian maturation in decapods, and injection of MF stimulates oocyte growth and vitellogenesis (1, 42, 43). The biosynthetic pathway of MF is similar to the general mevalonate pathway for acyclic isoprenoids, and the final step is catalyzed by FAMeT (44–46). In this study, a significant upregulation of FAMeT in ovary was observed after ESA, indicating that ESA resulted in an increased rate of MF synthesis. This result is in accordance with previous studies that find the MF levels are negatively regulated by eyestalk neuropeptides, and removal of the eyestalk leads to elevated levels of MF (46). MF level is controlled by both anabolism and catabolism. Interestingly, a key enzyme in MF degradation, namely juvenile hormone esterase-like carboxylesterase (JHE-like CXE), was also found upregulated in ESA crabs, indicating an increase in MF catabolism in addition to MF anabolism. It is reported that a high level of MF has a deleterious effect on oocyte growth and development, resulting in oocyte degeneration (47). The increased MF catabolism in the ovary after ESA may represent a protective mechanism to regulate MF levels, avoiding the detrimental effects of excessive MF.

Calcium Signals

Calcium signals are demonstrated to be essential for oocyte maturation in mammals, fish, mollusks, and crustaceans (48–50). Elevated concentration of intracellular calcium in the oocyte can activate CaM, a molecular switch to regulate the network of calcium signaling and, in turn, trigger a cascade of downstream signaling, leading to activation of maturation-promoting factor (MPF), which is a heterodimeric complex composed of Cell division cycle 2 (Cdc 2) and Cyclin B and responsible for triggering G2/M-phase transition in oocytes (51–53). It is reported that ESA results in an increased level of CaM mRNA in the ovary of the red swamp crayfish *Procambarus clarkia*, and knockdown of CaM expression by RNA interference suppresses the ovarian maturation induced by ablation (54). In this study, CaM, as well as Cdc 2 and Cyclin B, were upregulated after ESA. These results together with previous findings suggest that ESA can stimulate meiotic maturation through calcium signaling. In mammals, GnRH signaling can trigger calcium signaling, thereby inducing oocyte maturation. Similarly, a recent study in the black tiger prawn *Penaeus monodon* finds that genes in the GnRH pathway exhibit an early induction pattern after ESA and speculates that GnRH signaling leads to the activation of calcium signaling (11). However, upregulation of the proteins in the GnRH signaling pathway was not observed in this study. It is possible that activation of GnRH signaling is transient at the onset of oocyte maturation after ESA. Further study is required to confirm the upstream signals triggering calcium-signaling pathways after ESA.

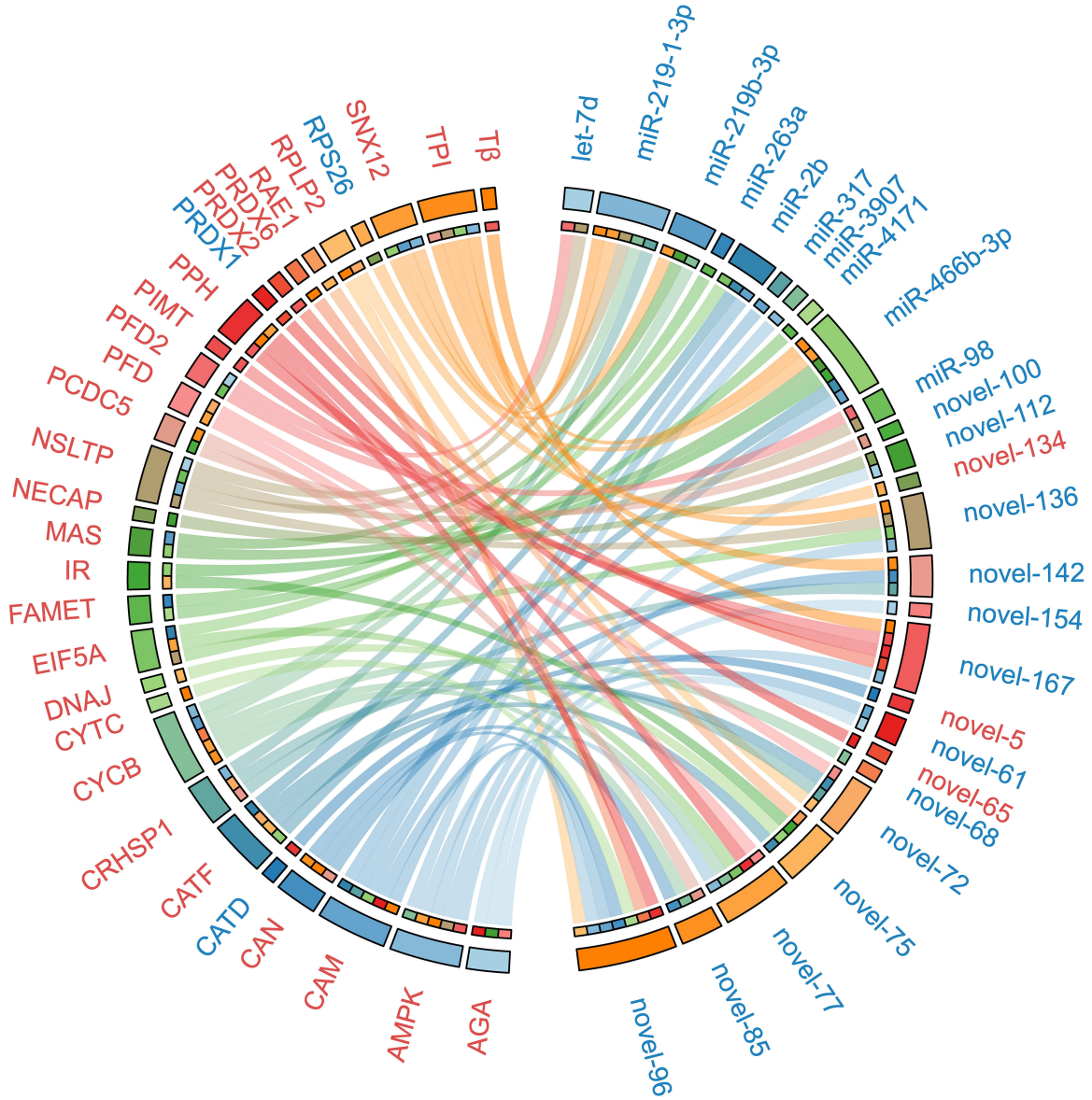

FIGURE 3 | The miRNA-protein regulatory network constructed on the basis of *in silico* miRNA target prediction and inverse correlation of protein and miRNAs expression after ESA. Upregulated miRNAs and proteins are represented in red, and downregulated miRNAs and proteins are represented in blue. The abbreviations in the figure: RPLP2, 60S acidic ribosomal protein P2; NECAP, Adaptin ear-binding coat-associated protein 2; AMPK, AMP-activated protein kinase alpha subunit; AGA, Aspartylglucosaminidase; CRHSP1, Calcium-regulated heat stable protein 1; CAM, Calmodulin; CAN, Calnexin; CATD, Cathepsin D; CATF, Cathepsin F; CATF, Cathepsin F; CYCB, Cyclin B; CYTC, Cytochrome c; DNAJ, DnaJ homolog subfamily B member 9 precursor; EIF5A, Eukaryotic translation initiation factor 5A; FAMET, Farnesoic acid O-methyltransferase; IR, Insulin receptor; MAS, Masquerade-like protein; NSLTP, Non-specific lipid-transfer protein; PRDX1, Peroxiredoxin 1; PRDX2, Peroxiredoxin 2; PRDX6, Peroxiredoxin 6; PPH, Phosphopyruvate hydratase; PFD, Prefoldin; PFD2, Prefoldin subunit 2; PCDC5, Program cell death 5-like; PIMT, Protein-L-isoaspartate O-methyltransferase; RAE1, RAE1 RNA export 1 homolog; RPS26, Ribosomal protein S26; SNX12, Sorting nexin-12; Tβ, Thymosin beta; TPI, Triosephosphate isomerase.

Metabolism

KEGG analysis shows that the proteins in metabolic pathways account for the largest proportion of DE proteins (22.70%), and they are mapped to a wide range of pathways related to metabolism, such as carbohydrate, amino acid, energy, lipid, and nucleotide metabolism, indicating that ESA has an extensive impact on the metabolism in the *P. trituberculatus* ovary. It is noteworthy that a significant upregulation of AMPK was observed after ESA. As a key regulator that maintains cellular energy homeostasis, AMPK reprograms cellular metabolism from anabolism to catabolism by controlling lipid and glucose metabolism when activated by falling energy status (55, 56). In accordance with AMPK upregulation, several important enzymes in glycolysis (PGAM2, TPI, and PPH), tricarboxylic

TABLE 2 | A list of significantly altered miRNAs and target proteins showing inverse correlation of expression after ESA.

miRNA	miRNA fold change	Target protein	Target protein fold change
let-7	0.39	Non-specific lipid-transfer protein	1.54
		Prefoldin subunit 2	1.46
miR-2b	0.41	Calmodulin	1.45
		Cathepsin F	1.28
		Eukaryotic translation initiation factor 5A	1.32
miR-98	0.39	Non-specific lipid-transfer protein	1.54
		Prefoldin subunit 2	1.46
miR-219-1-3p	0.43	Calcium-regulated heat stable protein 1	1.42
		Cyclin B	1.28
		Non-specific lipid-transfer protein	1.54
		Sorting nexin-12	1.70
		Triosephosphate isomerase	1.34
miR-219b-3p	0.40	Cyclin B	1.28
		Masquerade-like protein	1.22
		Sorting nexin-12	1.70
miR-263a	0.47	Farnesoic acid O-methyltransferase	1.67
miR-317	0.21	Calmodulin	1.45
miR-466b-3p	0.41	Calmodulin	1.45
		Cathepsin F	1.28
		Insulin receptor	1.58
		Masquerade-like protein	1.22
		Sorting nexin-12	1.70
		Triosephosphate isomerase	1.34
miR-3907	0.49	AMP-activated protein kinase alpha subunit	1.60
miR-4171	0.39	Farnesoic acid O-methyltransferase	1.67
novel-5	2.69	Cathepsin D	0.79
novel-61	0.29	Aspartylglucosaminidase	1.67
		Calmodulin	1.45
novel-65	2.80	Peroxiredoxin 1	0.78
novel-68	0.34	Cyclin B	1.28
novel-72	0.37	60S acidic ribosomal protein P2	1.39
		Calcium-regulated heat stable protein 1	1.42
		Cathepsin F	1.28
		Prefoldin	1.84
novel-75	0.47	Cathepsin F	1.28
		DnaJ homolog subfamily B member 9 precursor	1.32
		Insulin receptor	1.58
		RAE1 RNA export 1 homolog	1.33
novel-77	0.38	AMP-activated protein kinase alpha subunit	1.60
		Cyclin B	1.28
		Eukaryotic translation initiation factor 5A	1.32
		Phosphopyruvate hydratase	1.32
		Prefoldin	1.84
novel-85	0.21	Calnexin	1.40
		Cyclin B	1.28
		Program cell death 5-like	1.66
novel-96	0.44	60S acidic ribosomal protein P2	1.39
		AMP-activated protein kinase alpha subunit	1.60
		Calnexin	1.40
		Cytochrome c	1.67
		Peroxiredoxin 6	1.61
		Phosphopyruvate hydratase	1.32
		Calmodulin	1.45
novel-100	0.44	Program cell death 5-like	1.66
novel-112	0.32	Aspartylglucosaminidase	1.67
		Similar to adaptin ear-binding coat-associated protein 2	1.25
novel-134	3.45	Ribosomal protein S26	0.78
novel-136	0.46	AMP-activated protein kinase alpha subunit	1.60
		Eukaryotic translation initiation factor 5A	1.32
		Non-specific lipid-transfer protein	1.54
		Triosephosphate isomerase	1.34
novel-142	0.44	Calcium-regulated heat stable protein 1	1.42
		Calnexin	1.40
		Triosephosphate isomerase	1.34
novel-154	0.08	Aspartylglucosaminidase	1.67
novel-167	0.47	AMP-activated protein kinase alpha subunit	1.60
		Peroxiredoxin 2	1.32
		Phosphopyruvate hydratase	1.32
		Protein-L-isoaspartate O-methyltransferase	1.48
		Thymosin beta	1.55

acid cycle (ACO), and fatty acid catabolism (MCE) were also found upregulated following ablation. Previous studies in other decapods show that ESA can promote the transportation of glucose and lipid from the hepatopancreas to the ovary (57, 58). Those findings and our results together indicate ESA results in an increased energy requirement for the accelerated ovarian maturation. Hence, in aquaculture practice, the feeds for the broodstocks should be adjusted to meet the high energy demand after ablation.

The Roles of miRNA

Accumulating evidence suggests that miRNAs are critical in ovarian development of crustacean (59, 60). However, their functions in ESA-induced ovarian maturation are still unclear. In this study, 31 miRNAs were differentially expressed in ESA and ESI crabs. To uncover the functions of these miRNA during accelerated ovarian maturation induced by ESA, we integrated miRNA transcriptome with proteome data sets. Based on *in silico* miRNA target prediction and inverse correlation

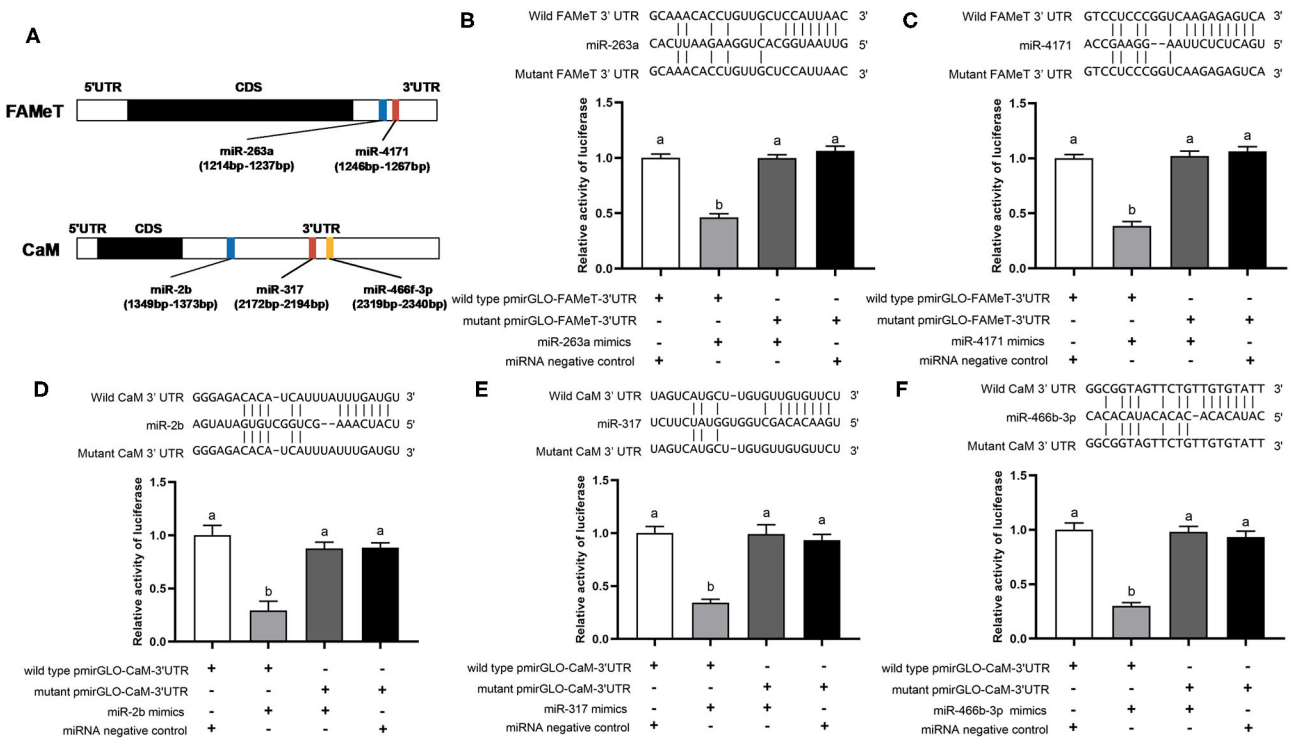

FIGURE 4 | Predicted binding sites of the miRNAs in the 3′ UTRs of their target **(A)** and validation of interactions between the selected pairs of miRNAs and their targets, including miR-263a/FAMeT **(B)**, miR-4171/FAMeT **(C)**, miR-2b/CaM **(D)**, miR-317/CaM **(E)** and miR-466b-3p/CaM **(F)**, via dual luciferase reporter assay. Above the histograms, nucleotide sequences of miRNAs and targets wild-type and mutant 3′ UTR regions are shown. The histograms present relative luciferase activity in HEK293T cells cotransfected with miRNA mimics or miRNA negative control, and pmirGLO vector containing wild-type or mutant 3′ UTR regions of miRNA targets. The relative luciferase activity was calculated by normalizing firefly luciferase activity to Renilla luciferase activity. Different letters on the bars indicate significant differences based on one-way ANOVA followed by Tukey's HSD multiple comparison test ($p < 0.05$).

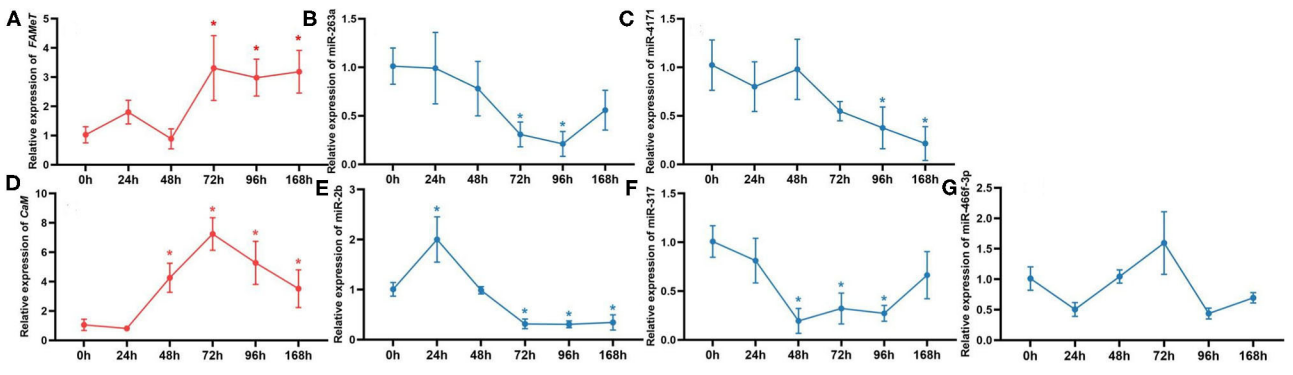

FIGURE 5 | The relative expression of *FAMeT* **(A)** and *CaM* **(D)** and their corresponding miRNAs, including miR-263a **(B)**, miR-4171 **(C)**, miR-2b **(E)**, miR-317 **(F)**, and miR-466f-3p **(G)** at 0, 24, 48, 72, 96, and 168 h after ESA. The asterisk indicates that the expression significantly differs from that at 0 h ($p < 0.05$).

of protein and miRNA expression, we generated a network encompassing 26 miRNAs, 30 target proteins, and 71 potential functional associations (**Figure 3**). Among the targets, a number of proteins mentioned above, including FAMeT, Cyclin B, CaM, AMPK, TPI, and PPH, were identified, suggesting that miRNAs may play important roles in ESA-mediating ovarian maturation through regulating proteins associated with MF synthesis, calcium signals, and energy metabolism. Within the miRNA-protein network, most miRNAs have multiple targets, and inversely, many proteins are targeted by several miRNAs, implying a complex post-transcriptional regulation between miRNAs and proteins in response to ESA. To experimentally validate the synergistic target regulation of the miRNAs, we tested FAMeT regulation by miR-263a and miR-4171, and CaM

regulation by miR-2b, miR-317, and miR-466f-3p, using the dual luciferase reporter assay. The results indicate that both miR-263a and miR-4171 directly regulate FAMeT expression, and miR-2b, miR-317, and miR-466f-3p repress expression of CaM. In addition, we investigated the expression pattern of the miRNAs and their target genes after ESA, and the results show significant negative correlation between miR-263a, miR-4171, and *FAMeT* and between miR-2b, miR-317, and *CaM*. Taken together, these data demonstrate that multiple miRNAs could function cooperatively to regulate FAMeT and CaM expression, and thereby participate in inducing ovarian maturation after ESA.

CONCLUSIONS

In summary, we characterize the changes in ovarian proteome and miRNA transcriptome in *P. trituberculatus* after ESA. The results suggest that alterations in MF metabolism and calcium signaling are crucial for promoting ovarian maturation, and ESA results in a higher energy production to meet the increased energy demand in the ovary. Furthermore, our study reveals a miRNA-mediated mechanism for inducing ovarian maturation. These findings improve our understanding of the molecular mechanisms of ESA-induced ovarian maturation and lay the foundation for developing an alternative technique for maturation manipulation without ESA in decapod crustaceans.

MATERIALS AND METHODS

Animal Collection and Eyestalk Ablation

All the experimental procedures involving the handling and treatment of the crabs used in this study were approved by the Institutional Animal Care and Use Committee of Yellow Sea Fisheries Research Institute prior to initiation of experiments. Female *P. trituberculatus* at 6-month age (198.65 ± 23.77 g) were collected from Haifeng Company, Weifang, China. The crabs were acclimated at laboratory conditions (temperature, 21°-23°C; salinity, 30–31) for 3 weeks. Then, three crabs were bilaterally eyestalk-ablated, and wounds were cauterized to minimize loss of hemolymph. Based on the results of our preliminary experiment, 96 h post ESA was selected as sample time; three eyestalk-ablated individuals (ESA) and three individuals with intact eyestalks (ESI) were placed in an ice bath until anesthetized, and the ovaries were collected and immediately frozen in liquid nitrogen and stored at −80°C.

During the period of the experiment, all the crabs were fed daily at 17:00 with live Manila clam *Ruditapes philippinarum*, and the feces and leftover feed were removed prior to feeding. Aeration was provided continuously, and the photoperiod was 12 h light:12 h dark. Seawater was filtered using a sand filter, and one third to one half of the rearing water was exchanged using fresh equi-temperature seawater. Water pH was around 7.5, and ammonia was <0.23 mg L^{-1}. Water salinity, pH, and ammonia were determined with a salinity refractometer (AIAGO, Japan), pH meter (WTW, Germany), and Hypobromite methods, respectively.

Protein Extraction, Digestion, and iTRAQ Labeling

iTRAQ analysis was performed at Beijing Genomics Institute (BGI, Shenzhen, China). The ovary of each crab from the ESI and ESA groups were disrupted in lysis buffer with enzyme inhibitors by TissueLyser (Qiagen, USA). The mixtures were centrifuged at 25,000 g for 20 min, and the supernatant was carefully removed and mixed with 5 volume of cold acetone and stored at −20°C for 2 h prior to centrifuging. The pellets were dissolved with lysis buffer, and 10 mM dithiothreitol (DTT) was added and maintained at 56°C for 1 h to reduce the disulfide bond of peptides. Then, 55 mM IAM was added to the solution and kept in a dark room for 45 min. After adding 5 volume of chilled acetone into the solution and kept at −20°C for 2 h, the solution was centrifuged again, and the pellet was dissolved with lysis buffer to get a protein solution. The protein concentration was determined using the Bradford method.

The protein solutions (100 μg) from each sample were digested with Trypsin gold (Promega, USA). After digestion, the peptides were vacuum centrifuged to dryness and dissolved with 0.5 M TEAB. The iTRAQ labeling of peptides was performed on the ESI and ESA groups using the iTRAQ Reagent 8-plex kit (Applied Biosystems, USA) according to the manufacturer's protocol.

Protein Identification, Quantification, and Functional Analysis

Raw LC-MS/MS data was converted into MGF format with the exported ProteoWizard tool (61). Proteins were identified with Mascot version 2.3.02 (Matrix Science, UK) against *P. trituberculatus* ovarian transcriptome. The proteins containing at least one unique set of spectra were used for the following quantification analysis with IQuant software (62). The proteomic data set was deposited in the iProX database under the accession number IPX0002268000. A q value $<= 0.05$ and foldchange $>= 1.2$ were set as the threshold for differentially expressed proteins (DEPs). The GO and KEGG databases were used to classify and group the DEPs.

RNA Extraction, Library Construction, and miRNA Sequencing

Total RNA was extracted from the ovary of the same crabs used for proteomic analysis with TRIzol reagent (Invitrogen, USA) and purified with a mirVana miRNA Isolation Kit following the manufacturer's protocol (Ambion, USA). Total RNA degradation and contamination was accessed on 1% agarose gels. Quantity and integrity of the RNA samples were determined using a Nano Photometer spectrophotometer (Implen, USA) and Bioanalyzer 2100 system (Agilent Technologies, USA). The RNA concentration was measured using the Qubit RNA Assay Kit in the Qubit 2.0 Flurometer (Life Technologies, USA).

For the ESI or ESA small RNA library, an equal amount of RNA samples from each replicate were pooled together. The

sequencing libraries were generated using NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, USA) according to the manufacturer's protocol, and index codes were added to attribute sequences to each sample. Library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA) using DNA High Sensitivity Chips. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumina, USA) according to the manufacturer's instructions. After cluster generation, the small RNA libraries were sequenced on an Illumina Hiseq 2500 platform at Novogene Company, Beijing, China. The small RNA sequencing data set was deposited in NCBI Sequence Read Archive (SRA) under the accession numbers PRJNA639350.

miRNA Data Analysis

After Illumina sequencing, clean reads were obtained by removing low-quality sequences, adapter-contaminated tags, and reads with poly N (where N represents unknown base). All the clean reads were searched against GenBank and Rfam databases to exclude known non-coding RNAs, including rRNAs, tRNAs, snRNAs, and snoRNAs. Any reads encoding proteins were also removed by blasting against the reference unigenes derived from the gonadal transcriptome data set of *P. trituberculatus*. Then, the remaining sequences were searched against the miRNAs from all the animals in miRBase to identify known miRNAs. Only the sequences that aligned to the miRNAs in miRBase without any mismatch were considered as known miRNAs. To predict novel miRNAs, the remainder unannotated small RNA sequences were analyzed with an integrated combination of miREvo (63) and mirdeep2 (64). The software identified novel miRNAs by exploring the secondary structure, the Dicer cleavage site, and the minimum free energy of the small RNA tags unannotated in the former steps, and the threshold score set as ≥ 50. The miRNAs predicted by the software were considered as novel miRNAs.

To analyze the expression profiles of the miRNAs in the ovary of ESA and ESI crabs, the read counts of miRNAs were normalized into TPM (transcript per million) through the normalization formula: Normalized expression = (Mapped reads/Total reads) $\times 106$ (65). Differential expression analysis of the two libraries was performed using the DEGseq R package (65). *P* values were adjusted using *q* value (66). The criteria of $q < 0.01$ and $|\log2(\text{fold-change})| > 1$ was set as the threshold for defining statistically different expression. Because genome information for the swimming crab is not available, 3′UTR sequences extracted from the *P. trituberculatus* gonad transcriptome data were used to predict putative targets of the identified miRNAs with miRanda software (67).

miRNA Target Validation

Five selected miRNA-target pairs (miR-263a/FAMeT, miR-4171/FAMeT, miR-2b/CaM, miR-317/CaM, and miR-466f-3p/CaM) were validated with dual luciferase reporter assay. The 3′ UTR sequences of FAMeT and CaM containing wild-type and mutant miRNA binding sites were artificially synthesized by General Biol Co. Ltd. (Hefei, China) and cloned into pmirGLO dual-luciferase reporter vector (Promega, USA) using *Sac*I and *Sal*I restriction sites. The plasmids were transformed into TOP10 *E. coli* cells and purified with a TIANprep Mini Plasmid Kit (Tiangen, China), and all the insertions were certified by DNA sequencing. The synthesized 3′ UTR sequences of FAMeT and CaM are shown in the supplementary materials (**Table S2**).

For the luciferase reporter assay, HEK293T cells were seeded in 24-well plates and transfected with 50 nM miRNA mimics or scrambled miRNA, and 1 μg luciferase reporter plasmid pmirGLO-wild type or pmirGLO-Mutant using Exfect 2000 Transfection Reagent (Vazyme, China). At 48 h after transfection, firefly and Renilla luciferase activities were determined using the dual luciferase reporter assay system (Promega, USA). The firefly luciferase signal was normalized to the Renilla luciferase signal. All experiments were performed in three replicates. The normalized firefly luciferase activity was compared between different groups using Tukey's HSD multiple comparison test ($p < 0.05$).

To further validate the expression correlation between the five selected miRNA target pairs, we analyzed levels of the miRNAs and their target at different time (0, 24, 48, 72, 96, and 168 h) after ESA. Because there is no proper antibody available, we measured mRNA levels of the targets instead. Total RNA of the samples was extracted using the RNAprep Pure Kit (Tiangen, China), and reverse transcription and RT-PCR of the target genes were performed with the FastKing RT Super Mix (Tiangen, China) and SuperReal PreMix Plus Kit (Tiangen, China), respectively, according to the manufacturer's protocol. The RT-PCR was programmed at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 30 s. miRNA was isolated using the miRcute miRNA Isolation Kit (Tiangen, China) following the manufacturer's instruction, and reverse transcription and RT-PCR of the miRNAs were performed using miRcute miRNA First-strand cDNA Synthesis Kit (Tiangen, China) and miRcute Plus miRNA qPCR Kit (Tiangen, China). The RT-PCR was programmed at 95°C for 15 min, followed by 40 cycles of 94°C for 20 s and 60°C for 34 s. Three biological replicates were measured at each time point, and each measurement was performed in triplicate. Relative levels of miRNAs and mRNAs were normalized to the U6 snRNA and β-actin, respectively, in each sample using the comparative C_T method (68). All the primers are shown in Supplementary Materials (**Table S3**). The correlation in expression between the miRNAs and their targets was determined using Spearman's correlation analysis.

DATA AVAILABILITY STATEMENT

The small RNA sequencing dataset was deposited in NCBI sequence read archive (SRA) under the accession numbers PRJNA639350.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Yellow Sea Fisheries Research Institute.

AUTHOR CONTRIBUTIONS

XM and PL: conceptualization, resources, supervision, project administration, and funding acquisition. MZ, XM and PL: methodology and investigation. XM, MZ, and JL: software, validation, formal analysis, data curation, and original draft. XM, MZ, and PL: writing, review, and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00533/full#supplementary-material

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Transcriptomic Analysis of the Kuruma Prawn *Marsupenaeus japonicus* Reveals Possible Peripheral Regulation of the Ovary

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Crustacean reproduction has been hypothesized to be under complex endocrinological regulation by peptide hormones. To further improve our understanding of the mechanisms underlying this complex regulation, knowledge is needed regarding the hormones not only of the central nervous system (CNS) such as the X-organ/sinus gland (XOSG), brain, and thoracic ganglia, but also the peripheral gonadal tissues. For example, in vertebrates, some gonadal peptide hormones including activin, inhibin, follistatin, and relaxin are known to be involved in the reproductive physiology. Therefore, it is highly likely that some peptide factors from the ovary are serving as the signals among peripheral tissues and central nervous tissues in crustaceans. In this work, we sought to find gonadal peptide hormones and peptide hormone receptors by analyzing the transcriptome of the ovary of the kuruma prawn *Marsupenaeus japonicus*. The generated ovarian transcriptome data led to the identification of five possible peptide hormones, including bursicon-α and -β, the crustacean hyperglycemic hormone (CHH)-like peptide, insulin-like peptide (ILP), and neuroparsin-like peptide (NPLP). Dominant gene expressions for the bursicons were observed in the thoracic ganglia and the ovary, in the CNS for the CHH-like peptide, in the heart for NPLP, and in the ovary for ILP. Since the gene expressions of CHH-like peptide and NPLP were affected by a CHH (*Penaeus japonicus* sinus gland peptide-I) from XOSG, we produced recombinant peptides for CHH-like peptide and NPLP using *Escherichia coli* expression system to examine their possible peripheral regulation. As a result, we found that the recombinant NPLP increased vitellogenin gene expression in incubated ovarian tissue fragments. Moreover, contigs encoding putative receptors for insulin-like androgenic gland factor, insulin, neuroparsin, and neuropeptide Y/F, as well as several contigs encoding orphan G-protein coupled receptors and receptor-type guanylyl cyclases were also identified in the ovarian transcriptome. These results suggest that reproductive physiology in crustaceans is regulated by various gonadal peptide hormones, akin to vertebrates.

Keywords: peptide hormone, *Marsupenaeus japonicus*, ovary, reproduction, transcriptome, vitellogenesis

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INTRODUCTION

As a one of the most important aquaculture target worldwide, the production of penaeid shrimps/prawns has been steadily increased for over the past 30 years. The species occupying the majority of current shrimp aquaculture is Litopenaeus vannamei and Penaeus monodon. Their production has increased ∼430% from 1998 to 2008, and 190% from 2008 to 2018, achieving 5.7 million tons (Food and Agriculture Organization of the United Nations; www.fao.org/fishery/topic/16140/en). To enable sustainable penaeid shrimp production in future, efficient seed production technique is required. Information on the endocrine system governing reproductive physiology will help to have the similar efficient seed production technique as in other aquatic animals (1, 2).

A number of studies on endocrinological regulation of basic biological functions have so far focused on the central neurosecretory X-organ/sinus gland complex (XOSG) in the eyestalk among various crustacean species. It has been proved that various peptide hormones produced from the XOSG are regulating growth, metabolism, osmoregulation, and reproduction (3, 4), e.g., red pigment concentrating hormone (5), pigment dispersing hormone (6), crustacean hyperglycemic hormone (CHH) (7), molt-inhibiting hormone (MIH) (8), vitellogenesis- or gonad-inhibiting hormone (VIH/GIH) (9), mandibular organ-inhibiting hormone (MOIH) (10), and crustacean female sex hormone (CFSH) (11). Extensive works based on biological activity-oriented peptide purification and subsequent expansions of homologous cDNA cloning have been contributed to find the above peptide hormones. On the basis of these works, VIH is considered as a main regulator of reproductive process in terms of the inhibition of vitellogenin (VG, a major yolk protein precursor) synthesis. Furthermore, roles of peptide hormones from the central nervous system (CNS) other than XOSG, which includes the brain and thoracic ganglia, have been elucidated (12–14).

In Japan, the principal penaeid species is the kuruma prawn *Marsupenaeus japonicus* (former *Penaeus japonicus*), which is one of the most important aquatic resources. The reproductive processes of *M. japonicus* have also been extensively studied using vitellogenesis-related proteins and their genes (e.g., VG, cathepsin C, cortical rod protein, and thrombospondin) as indices of ovarian development (15–21). Among various peptide hormones which have been purified and characterized from the central XOSG (22–27), six type-I peptides of the crustacean hyperglycemic hormone (CHH) superfamily inhibit the expression of *VG* in the ovary (28, 29). Consequently, the six CHHs, called as *P. japonicus* sinus gland peptide-I (Pej-SGP-I), -II -III, -V, -VI, and –VII, have been hypothesized to be vitellogenesis-inhibiting hormones (VIHs), which explains why eyestalk-ablation acceralates ovarian development. In contrast to the hormones from central XOSG, only a few gender and reproductive organ-specific peripheral factors have been identified. An insulin-like androgenic gland factor of *M. japonicus* (Maj-IAG) is exclusively produced from the androgenic gland of the male gonad and supresses *VG* expression in the ovary (30, 31), which is presumed to control the development of male characteristics, like the orthologs in other decapods (32–34). An ovarian isoform of the crustacean female sex hormone of *M. japonicus* (Maj-CFSH-ov) is dominantly expressed in the ovary, but its function remains to be determined (35). Since some gonadal hormones, activin/inhibin (36–38), follistatin (39), and relaxin (40, 41), are known to be involved in the regulation of reproductive physiology in vertebrates, more attention should be paid for peptidergic factors from the gonad as well as the other peripheral tissues. Such factors may act as a feedback signal from the ovary to the CNS or as a signal that intermediate two VG synthetic site, the hepatopancreas and ovary, in female penaeid shrimps (42).

More recently, transcriptomic analysis has become an important tool for peptide/protein profiling in addition to the conventional approaches described above. The transcriptome data supports our comprehensive understanding of the mechanisms where target tissues are regulated. Indeed, many more transcripts encoding hormones homologous to those found in vertebrates or insects have been identified in various crustacean species (43–52). Some of the studies have shown the existence of transcripts for putative peptide hormones in the ovary: the red swamp crayfish *Procambarus clarkii* (47), the Australian red-craw crayfish *Cherax quadricarinatus* (49), and the giant freshwater prawn *Macrobrachium rosenbergii* (51). In addition to the peptide/protein hormone candidates, the transcriptomic data have been exploited for the identification and characterization of peptide hormone receptors (53, 54) and the elucidation of molecular pathways involved in the regulation of various biological functions (55, 56). Hence, transcriptomics is an essential tool in the quest to improve our understanding of reproductive biology underlying peptide hormones and their receptors in peripheral organs such as the gonads.

Herein, we performed next-generation RNA sequencing on *M. japonicus* ovary with the Illumina MiSeq system. The *de novo* assembled ovarian transcriptome data was searched for the putative peptide hormone precursors and peptide hormone receptors. Moreover, the effects of putative hormones on ovarian *VG* expression were examined using their recombinant peptides.

MATERIALS AND METHODS

Total RNA Extraction

Adult *M. japonicus* were purchased from a local fish market in Okayama Prefecture, Japan. For tissue-specific gene expression analysis, the brain, eyestalk, thoracic ganglia, heart, hepatopancreas, intestine, and gonad were dissected from both three male (20.7 g average body weitht) and three female prawns (23.0 g average body weight; 1.0% average gonadosomatic index, GSI). The prawns are determined to be in intermolt (C0–C1) and early premolt (D0) stages thorugh the observation of the setal development of the pleopods using a method modified from the previous report (57). Ovarian developmental stages of the female prawns were determined as previtellogenic by histlogical analysis (20, 35). Tissues were stored in RNA*later* solution (Thermo Fisher Scientific, MA, USA) at −20°C until further use. For RNA-sequencing, the ovary of two intermolt female prawns in previtellogenic stage (26.4 g body weight; 1.0% GSI) and early

exogenous vitellogenic stage (56.3 g body weight; 2.5% GSI) were dissected out and stored as described above. Total RNA was isolated using the illustra RNAspin mini RNA isolation kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Library Preparation and RNA-Sequencing

The concentration of the two total RNA samples isolated from the ovary was measured using the Qubit RNA BR assay kit (Thermo Fisher Scientific). The cDNA libraries were constructed with 1 μg of the total RNA using the NEBNext ultra directional RNA library prep kit for Illumina, NEBNext Poly(A) mRNA Magnetic Isolation Module, and NEBNext multiplex oligos for Illumina (index primers set 1; New England BioLabs, MA, USA). All protocols were performed according to the manufacturer's instructions with minor modifications, namely that the fragmentation of RNA was performed by 94°C followed by incubation for 7.5 min. The final library fragment size was estimated to be 200–870 bp (average of 520 bp) using the Agilent high sensitivity DNA kit (Agilent Technologies, CA, USA). The library was sequenced using the MiSeq with Reagent Kit v3 (Illumina, CA, USA) in the paired-end mode with a read length of 300 bases.

Data Processing and Bioinformatic Analyses

Bases with a quality score (QV $<$ 20) were trimmed from the 5′ and 3′ ends of each read, and reads containing $\geq$ 30% of low quality bases (QV $<$ 14) with $<$25 bp were removed before assembling. The preprocessed reads were assembled using the Trinity platform (58). The resultant contigs were analyzed using the Basic Local Alignment Search Tool + (BLAST+; version 2.3.0) to perform a homology search against the National Center for Biotechnology Information non-redundant (NCBI-nr) protein database (ver. 170504) with an E-value cutoff of 1×10^{-5}. The above-mentioned operations were executed using the DNA Data Bank of Japan (DDBJ) Read Annotation Pipeline and the supercomputer at the Research Organization of Information and Systems (ROIS), National Institute of Genetics (NIG), Japan. The BLAST output contained a maximum of 30 hits for each sequence which were used to assign the functional Gene Ontology (GO) terms to the protein sequences and for further GO Slim analysis on Blast2GO (59, 60). The signal peptide was predicted using SignalP 4.1 Server (61).

Molecular Cloning of Open Reading Frames of Hormonal Genes

Poly (A)+ RNA was prepared from 50 μg of the total RNA as described above using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs). First-strand cDNA was synthesized from the purified Poly(A)+ RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) and an anchored-oligo$(dT)_{18}$ primer. This first-strand cDNA was purified with AMPure XP (Beckman Coulter, IN, USA) and tailed with poly(A) using terminal transferase (Roche Diagnostics). The following PCRs were performed to obtain the correct open reading frame (ORF) of each target gene using the synthesized cDNA.

For 5′ -RACE, PrimeSTAR HS DNA polymerase or TaKaRa LA Taq DNA polymerase (Takara Bio, Shiga, Japan) were used with the cDNA on conventional PCR programs as per a previously described method (35). Adapter1 and adapter2, as forward primers, and bursA-R01 and -R02, as reverse primers, were used for the bursicon A subunit. Adapter1, adapter2, bursA-R01, and -R02 were used for the bursicon B subunit. Adapter1, adapter2, ilp-R01, -R07, -R10, R17, and R18 were used for the insulin-like peptide (**Supplementary Table 1**).

All PCR products were subcloned into the pGEM-T easy vector (Promega, WI, USA) after the addition of an adenine nucleotide at the 3′ ends. All plasmids were then sequenced on the 3730xl DNA analyzer (Applied Biosystems, CA, USA).

Construction of Plasmids for Recombinant Peptides

Expression plasmids for the *M. japonicus* CHH-like peptide and *M. japonicus* neuroparsin-like peptide (Maj-pCHH-B and Maj-NPLP, respectively), were prepared as per a previously described method (62). Both Maj-pCHH-B and Maj-NPLP cDNA fragments were amplified by PCR using the pchhbexF1/R1 and nplexF/R primer pairs, respectively (**Supplementary Table 1**). Each PCR product was mixed with the pET-44a(+) plasmid (Novagen, WI, USA), digested using *Sma* I and *Eco*R I (New England BioLabs), purified with AMPure XP, and then ligated. The thrombin protease cleavage site of the recombinant Maj-pCHH-B (rMaj-pCHH-B) expression plasmid was modified to a tobacco etch virus (TEV) protease cleavage site using the pchhbexF2/R2 primer pair (**Supplementary Figure 1**). Additionally, the thrombin protease cleavage site of the recombinant Maj-NPLP (rMaj-NPLP) expression plasmid was modified to a human rhinovirus 3C (HRV 3C) protease cleavage site using the nplexF2/R2 primer pair as per a previously described method (62). These modifications accompanied the substitution of N-terminal residues from Gln to Gly in rMaj-pCHH-B and from Ala to Gly in rMaj-NPLP (**Supplementary Figure 2**).

Expression and Purification of rMaj-pCHH-B and rMaj -NPLP

The transformation and culture of *E. coli* strain BL21(DE3) STAR (Thermo Fisher Scientific) with expression plasmids, induction of recombinant protein overexpression, and preparation of the soluble fraction of the cell lysate were performed as per previously described methods (62, 63).

The soluble fraction of the cell lysate containing the recombinant fusion protein, His-Nus-His-tagged rMaj-pCHH-B, was purified using the Ni Sepharose 6 Fast Flow resin (GE Healthcare). While the recombinant fusion protein was captured with the resin, the affinity tags were cleaved from rMaj-pCHH-B by protease digestion in a buffer containing 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.4 M urea, and ProTEV protease (Promega) at 20°C for 24 h. The untagged rMaj-pCHH-B was washed out from the resin and further purified by reverse phase high-performance liquid chromatography (RP-HPLC) on a Capcell Pak C18 SG300 column (150 × 6 mm; Shiseido, Tokyo, Japan)

using the following program: a 2-min hold at 5% acetonitrile (MeCN) in 0.05% trifluoroacetic acid (TFA), a 5-min linear gradient of 5–25% MeCN in 0.05% TFA, a 15-min gradient of 29–37% MeCN in 0.05% TFA, a 1.2-min gradient of 37– 85% MeCN in 0.05% TFA, and a 5-min hold at 85% MeCN in 0.05% TFA at a flow rate of 0.8 mL/min.

The soluble fraction containing recombinant His-Nus-His-tagged rMaj-NPLP was incubated with the Ni Sepharose 6 resin at 4°C for 20 h. The resin was then washed with washing buffer (20 mM phosphate buffer, 0.2 M NaCl, 50 mM imidazole, pH 7.4) and equilibrated with washing buffer without imidazole. For the cleavage of the tags, HRV 3C protease (Takara Bio) was added, and the resin slurry was incubated at 4°C for 3 days. Untagged rMaj-NPLP was eluted from the resin and further purified by RP-HPLC on the aforementioned column using the following program: a 1-min hold at 5% MeCN in 0.05% TFA, a 4-min linear gradient of 5–21% MeCN in 0.05% TFA, a 15-min gradient of 21–29% MeCN in 0.05% TFA, a 3.25-min gradient of 29–85% MeCN in 0.05% TFA, and a 5-min hold at 85% MeCN in 0.05% TFA at a flow rate of 0.8 mL/min.

The mass spectra of the purified recombinant peptides were measured on an Agilent 6,520 Accurate-Mass Quadrupole-TOF mass spectrometer with an electrospray ionization (ESI) interface (Agilent Technologies) as we have previously described (62).

Ex-vivo Ovarian Incubation

The effect of one of the CHH family of peptides (Pej-SGP-I) on the mRNA expression of the putative hormones was assessed using our *ex-vivo* ovarian incubation system (28, 29). The same system was also used to assess the effects of rMaj-pCHH-B and rMaj-NPLP on *vitellogenin* (*Maj-VG*) expression. The recombinant Pej-SGP-I (rPej-SGP-I) was prepared as per previously described methods (62–64). Adult female prawns (22.5 g average body weight; 0.9% average gonadosomatic index, GSI) acted as donors of the ovary. As shown in **Supplementary Figure 3**, the abdominal part of the ovary, where left and right ovarian lobes were sticking to each other, was dissected out and divided into two lobes, and one lobe was incubated as control lobe (medium only), whereas the other received the hormone treatment (experimental). The adjacent part of ovary was kept as initial sample (without incubation) and as sample for histological analysis to determine the vitellogenic stage. Only a single sample set of the ovary (initial, control, and experimental) was prepared from one prawn and counted as $n = 1$; total 30 prawns were used for the experiment in **Figure 5** (6 for each graph), 28 for **Figure 6A**, 24 for **Figure 6B**, and 12 for **Figure 6C**. All prawns used had immature ovary and were in intermolt to early premolt (C0, C1, D0, and D1) stages.

Following incubation, the ovarian tissue fragments were immersed in RNA*later* solution and stored at −20°C. Total RNA extraction was then performed as described above.

Quantitative Real-Time Reverse Transcriptase PCR

Quantitative real-time reverse transcriptase PCR (qRT-PCR) was used for the quantification of the putative hormone genes, *Maj-VG*, and *arginine kinase* (*Maj-AK*). The sequences of primers and TaqMan probes used in this study have been listed in **Supplementary Table 2**. The concentrations of total RNA prepared from various tissues were quantified using the Qubit RNA BR assay kit, and for each sample 8 ng RNA was used for qRT-PCR. The qRT-PCR reactions were carried out using the iTaq universal probes one-step kit (Bio-Rad, CA, USA), and the same methods were used for the real-time monitoring of the fluorescence signal on the CFX96 real-time PCR detection system (Bio-Rad) as has been previously described (35). For the quantification of gene expression levels, relative standard curve method was used. DNA templates were amplified with respective gene-specific primer sets (**Supplementary Table 2**) so that they include qRT-PCR amplicon sequence of respective target genes. RNA standards were synthesized by *in vitro* transcription using *in vitro* Transcription T7 Kit (Takara Bio) with the DNA templates. The synthesized RNA standards were purified using the illustra RNAspin mini RNA isolation kit and quantified using the Qubit RNA BR assay kit as described above. The standard curves were generated using each RNA standard ranging from 40 ng to 0.4 pg prepared by serial 10-fold dilutions, and arbitrary values ranging from 40,000 to 0.4 were assigned correspondingly. Relative gene expression levels were determined based on the threshold cycles using the standard curves. Each standard had almost the same length (371–426 nt, **Supplementary Table 2**), and similar amplification efficiencies were achieved (95.9–99.8%) in the qRT-PCR.

For the incubated ovary samples, the relative expression of the target genes were standardized to those of *Maj-AK*, and expressed as a percent change from the initial (**Figure 5**) or 0 nM group (**Figure 6**) samples (35) (**Supplementary Figure 3**). In contrast, for tissue-specific gene expression analysis (**Figure 4**), the relative expression of the target genes were standardized to the total RNA input in the qRT-PCR to account for variations in the *Maj-AK* expression between tissues.

Statistical Analysis

Gene expression levels have been represented as the mean ± standard error mean (SEM). Statistical differences in gene expression levels were analyzed using the Wilcoxon signed rank test or one-way analysis of variance (ANOVA) followed by the Dunnett's *post hoc* test in the GraphPad Prism version 4.0 for Windows (GraphPad Software, CA, USA).

RESULTS

RNA Sequencing and *de novo* Assembly

De novo assembly produced 98,509 contigs with a total size of ∼91.8 Mbp. The N50 of the transcriptome was 1,676 bp long, and the longest contig was 15,684 bp long. Redundant contigs were eliminated based on a sequence similarity. Among the remaining 47,026 contigs, 24,033 (51.1%) had a BLAST hit, and 13,839 (29.4%) were assigned at least one GO term (**Supplementary File**). Following bioinformatics analysis on the ovarian transcriptome, several transcripts encoding putative hormones were identified as described below.

Bursicon-α (Maj-burs-α)
MSGMFRTMVMLALALATQADECSLTPVIHILSYPGCNSKPIPSFACQGRCTSYVQVSGSKIWQTERSCMCCQESGEREASVTLSCPKARPGEPRLRKIL
TRAPIDCMCRPCTDVEEGTVLAQEIANFIEDSPMENVPFLK*

Bursicon-β (Maj-burs-β)
MMTRPLTVGACVAVTMAAVLVGGLAGPSRAHPYGSECETLPSTIHVAKEEFDDSGRLVRTCEEDLAVNKCEGACVSKVQPSVNTPSGFLKDCRCCRETH
LRARDVVLTHCYDGDGNRITGDNGKLTVKLREPADCQCFKCGNSIR*

Neuroparsin-like (Maj-NPLP)
MRTTTVTCILATCCLALLMQWATAAPRCKIHDRTSQDNCKYGVARDWCRNMVCAKGPGESCGGYRWENGKCGLGMICSCGRCSGCSIIDGTCSPHTMVC
VSN*

PutativeCHH-B (Maj-pCHH-B)
MNIKVTLVLIFSLIAALASSNGVHARSISEDVQELEAPPQERNMVAVRRRRQVFDASCKGVYDRGLWAKLNNACLDCQNIYRANPAIEGECRENCFGTE
IFYGCLKALKLPTKTYLYYGDLLRES*

Insulin-like peptide (Maj-ILP1)
MHAHQLLPPLLLLLAMGVECGRRDLVGQQVCGNQLVELLSLICRGRYYSPRERRIQEPATSRVRGPPPASNRFDRFDRSSRSPNRLQLPALIQSVFRDA
AKPKGVFAGYSEELEDAFREGSEEGATSRELEGPVGAAWMALLPRSAARDSYNGIPEDTSSSGSRKEERWDPPFLSRRSAFALLKTRERRGFTIVDECC
RLKACKLDELLAYCG*

FIGURE 1 | Amino acid sequences of putative hormones. Sequences were deduced from transcriptome analysis of *M. japonicus* ovary and additional cDNA cloning. Sequences shown in magenta and green represent the predicted signal sequences and cleavage sites, respectively. Underlined sequences represent CPRP in Maj-pCHH-B and C-peptide in Maj-ILP1, respectively.

Bursicon

Two contigs putatively encoding bursicon, a heterodimer composed of burs-α and burs–β, were identified in the ovarian transcriptome. Additional cDNA cloning and homology analysis revealed that these were the α and β subunits; Maj-burs-α and Maj-burs-β, respectively. The Maj-burs-α precursor contains a signal peptide (19 amino acid residues) which, once cleaved, gives a mature peptide comprising of 121 amino acid residues. Additionally, the Maj-burs-β precursor also contains a signal peptide (30 amino acid residues) and once processed results in a mature peptide composed of 115 residues (**Figure 1**). Mature Maj-burs-α and Maj-burs-β both showed ≥ 50% amino acid sequence identity with the known bursicon subunits, and they both contain 11 Cys residues, 9 of which are conserved in the cystine knot-like domain (smart00041 in Conserved Protein Domain Family, NCBI) of the transforming growth factor-β superfamily (**Supplementary Figure 4**).

Neuroparsin-Like Peptide

The ovarian transcriptome contained one contig encoding a precursor of Maj-NPLP which contains a 24 amino acid-long signal peptide and 78 amino acid-long mature peptide (**Figure 1**). We identified 12 conserved Cys residues, which were a characteristic of the crustacean neuroparsin family, in the mature Maj-NPLP peptide. Maj-NPLP shares 46% amino acid sequence identity with an neuroparsin in the greasyback shrimp *Metapenaeus ensis* (MeNPLP) which has been hypothesized to regulate ovarian maturation (65). Additionally, Maj-NPLP shares 32% amino acid sequence identity with the ovary ecdysteroidogenic hormone (OEH) in *Aedes aegypti*, the yellow fever mosquito (66) (**Supplementary Figure 5**).

CHH-Family Peptide

We found one contig encoding the precursor of a CHH-like peptide in the ovarian transcriptome. Additional cDNA cloning revealed that this precursor consisted of a 25 amino acid signal peptide, a 22 amino acid CHH precursor-related peptide (CPRP), an RXRR cleavage signal, and a 74 amino acid mature hormone (**Figure 1**). Considering the existence of CPRP and the absence of a single Gly residue 5 amino acids downstream of the first Cys residue, we considered this peptide to belong to the type-I subfamily of the CHH superfamily. On the other hand, there was no C-terminal amidation signal, a characteristic feature of type-I subfamily precursors. Additionally, there was a single-residue insertion seven amino acids downstream of the third Cys residue. Based on the primary structure nomenclature of the *M. japonicus* CHH-family peptides (25, 67), we categorized this peptide as a putative CHH-B (Maj-pCHH-B). Its mature peptide sequence showed 37–46% amino acid identity with the *Marsupenaeus* CHH-family peptides (**Supplementary Figure 6**). Phylogenetic analyses of the CHH superfamily characterized in the XOSG of *M. japonicus* so far (22, 23, 25, 26, 67) suggests that Maj-pCHH-B is diverse and most likely from the typical type-I and type-II subfamilies (**Figure 2**).

Insulin-Like Peptide

A putative insulin-like peptide precursor (Maj-ILP1) was identified in the ovarian transcriptome of *M. japonicas*. The full-length ORF of Maj-ILP1 was obtained by several rounds of 5′ -RACE. The precursor comprised a signal peptide composed of 20 residues, a B-chain consisting of 30 amino acid residues, an RXRR cleavage signal, a C-peptide composed of 131 amino acid residues, the other RXRR cleavage signal, and an A-chain composed of 24 amino acid residues (**Figure 1**). The primary

TABLE 1 | Putative hormone receptors found in the ovarian transcriptome.

Contig name	BLAST hit name[a]	Accession no.[a]	*E*-value[a]
G-protein coupled receptor homologs			
N13245	G-protein coupled receptor Mth2-like [*Hyalella azteca*]	XP_018019721	5.5×10^{-64}
N13763	Substance-P receptor-like, partial [*Limulus polyphemus*]	XP_013785397	1.0×10^{-94}
N14869	Dopamine D2-like receptor [*Centruroides sculpturatus*]	XP_023239863	5.0×10^{-105}
N16469	G-protein coupled receptor 143-like [*Frankliniella occidentalis*]	XP_026293244	6.0×10^{-81}
N21540	Parathyroid hormone-related peptide receptor-like [*Eufriesea mexicana*]	XP_015923473	5.0×10^{-14}
N28645	G-protein coupled receptor GRL101 [*Sagmariasus verreauxi*]	ARK36624	1.0×10^{-56}
N38792	Adenosine receptor A2b-like [*Copidosoma floridanum*]	XP_014203447	5.0×10^{-25}
Receptor guanylyl cyclase homologs			
N05354	Guanylate cyclase PcGC-M2 precursor [*Procambarus clarkii*]	AAQ74970	0
N33402	Receptor guanylyl cyclase [*Callinectes sapidus*]	AAX11210	3.0×10^{-13}
Insulin receptor homologs			
N06137	Insulin-like peptide receptor [*Orchesella cincta*]	ODM98443	1.0×10^{-169}
N15818	Insulin-like receptor [*Cryptotermes secundus*]	PNF35478	3.0×10^{-68}
N18043	Insulin-like receptor [*Trachymyrmex septentrionalis*]	KYN41515	1.0×10^{-123}
N35023	Insulin-like androgenic hormone receptor [*Penaeus chinensis*]	AVU05021	1.0×10^{-88}

aBLAST hit names, accession numbers, and E-values of some contigs are different from those in ***Supplementary File*** *because updated database was used for the BLAST search in this table.*

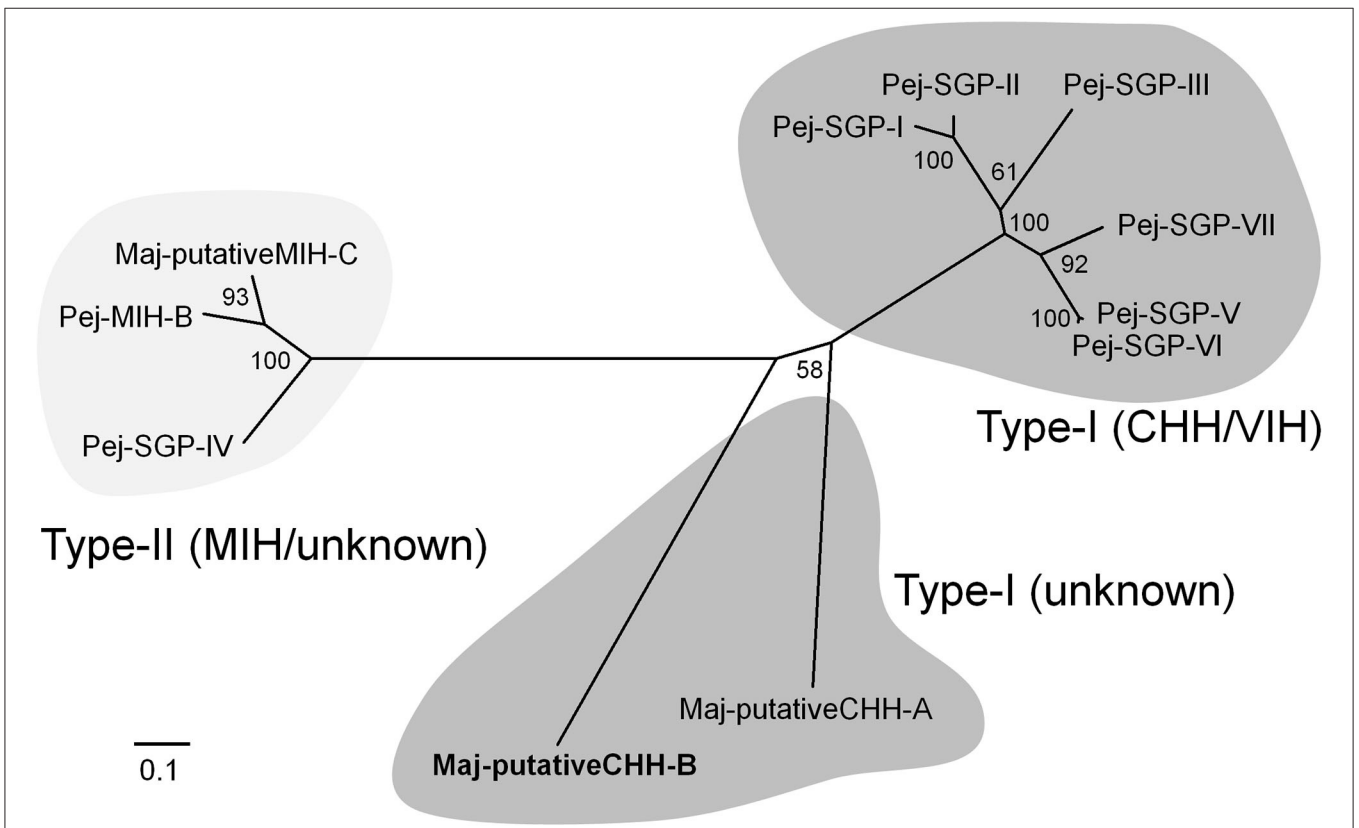

FIGURE 2 | Molecular phylogenetic tree of *M. japonicus* CHH-family peptides. This phylogenetic tree was constructed by the neighbor-joining method with computation of the evolutionary distances using the JTT matrix-based method. Values at the nodes represent the percentage of 1,000 bootstrap replicates. The scale bar shows the number of substitutions per site. Amino acid sequences have been cited from the previous reports (25, 26, 67).

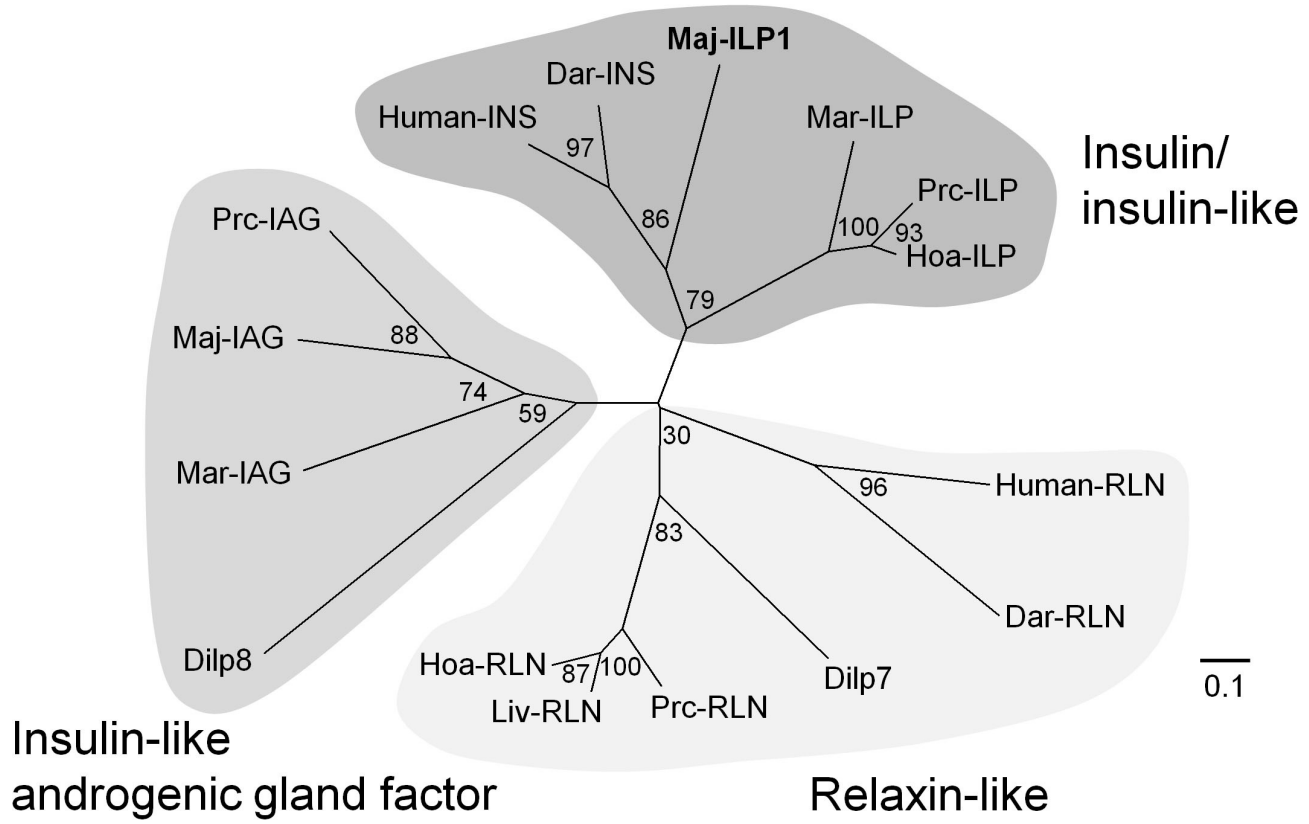

FIGURE 3 | Molecular phylogenetic tree of insulin-family peptides. This phylogenetic tree was constructed by the neighbor-joining method with computation of the evolutionary distances using Poisson correction. Values at the nodes represent the percentage of 1,000 bootstrap replicates. The scale bar shows the number of substitutions per site. Accession numbers of cDNA sequences used are as follows: human-INS, BC005255; human-RLN, A06846; Dar-INS, Dar-RLN, JN215212; Dilp7, NP_570070; Dilp8, NP_648949; Maj-IAG, BAK20460. The other sequences have been cited from a recent report (48).

structure of Maj-ILP1 was distinct from IAG in the same species (Maj-IAG); mature peptide (deduced B- and A- chains) of Maj-ILP1 shared only 28.6% amino acid sequence identity with that of Maj-IAG (**Supplementary Figure 7**). Phylogenetic analysis of known insulin/relaxin family shows that Maj-ILP1 is part of the insulin/insulin-related peptide group and not the IAG or relaxin groups (**Figure 3**).

Putative Hormone Receptors

Based on BLAST analysis, several contigs encoding the G-protein coupled receptor family, the receptor guanylyl cyclase family, and the insulin receptor family were found in the present ovarian transcriptome (**Table 1**). A full-length ORF of contig N13763 was obtained by additional cDNA cloning, and its seven-transmembrane domain showed 62% amino acid sequence identity to the neuropeptide Y (NPY) receptor in the Nevada dampwood termite *Zootermopsis nevadensis*. Additional cDNA cloning also revealed that the ORF of contig N28645 was composed of an N-terminal leucine-rich repeat domain and a seven-transmembrane domain that was highly similar to the transmembrane domain of the relaxin-family peptide receptors (cd15137 in Conserved Protein Domain Family). Furthermore, contigs N06137 and N35023 were the most similar to the receptor for OEH in *A. aegypti* (66) and to IAGR in the Chinese white shrimp *Fenneropenaeus chinensis* (53), respectively.

Tissue-Specific Expression of the Putative Hormones

Tissue-specific expression levels of the putative hormone genes were examined by qRT-PCR analysis (**Figure 4**). We found that *Maj-pCHH-B* was mainly expressed in the nervous system and in the intestine of both male and female prawns. Expression in the ovary was low compared to the nervous system, and we did not detect any significant sexual dimorphism in the expression pattern. Additionally, *Maj-NPLP* was expressed primarily in the heart. Apparent expression was also observed in the thoracic ganglia and in the intestine. *Maj-burs-α* and *Maj-burs-β* are primarily expressed in the thoracic ganglia of both sexes and in the ovary. The levels of *Maj-burs-α* and *-β* expression in the ovary were significantly higher compared to those in the testis. *Maj-ILP1* displayed a gender-specific expression pattern as it was expressed primarily in the ovary. Clear expression was also observed in the nervous system, while no expression was observed in the hepatopancreas of both sexes.

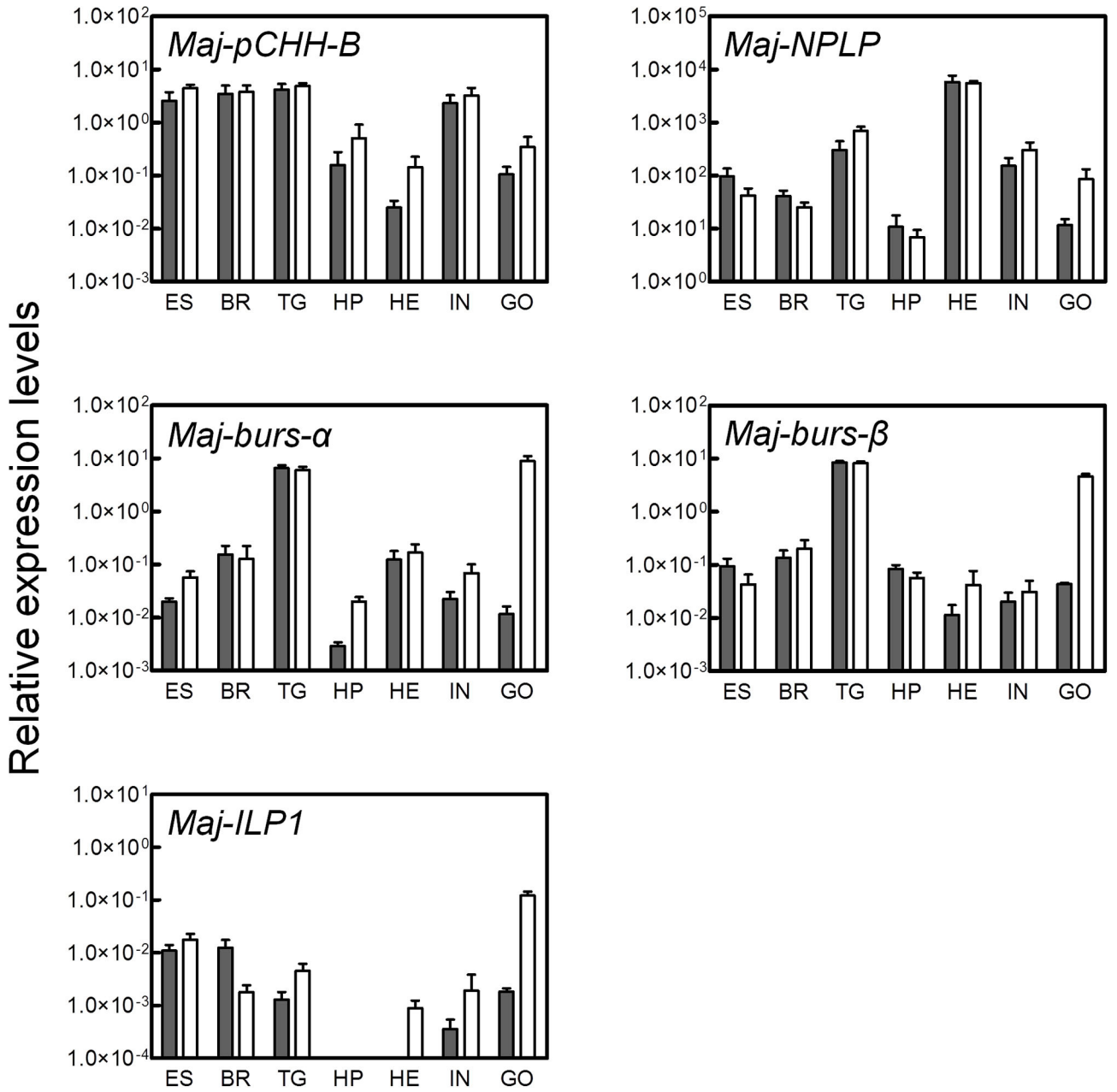

FIGURE 4 | Tissue-specific expression analysis of the putative hormones. Gene expression of the putative hormones were examined by qRT-PCR in various tissues (ES, eyestalk; BR, brain; TG, thoracic ganglia; HP, hepatopancreas; HE, heart; IN, intestine; GO, gonad). Relative expression levels per 8 ng of the total RNA have been represented as mean ± SEM ($n = 3$). Open and solid bars represent female and male prawns, respectively.

Effect of Eyestalk VIH on the Expression of the Putative Hormones

To investigate the potential involvement of the putative hormones in vitellogenesis, the effects of Pej-SGP-I, a VIH of *M. japonicus* (28), on hormone gene expression were first examined (**Figure 5**). In an *ex-vivo* ovarian incubation system, we found that *Maj-pCHH-B* mRNA level was significantly reduced by 50 nM rPej-SGP-I. *Maj-NPLP* expression was also reduced by rPej-SGP-I, but not changed ($p = 0.063$). *Maj-burs-α*, *Maj-burs-β*, and *Maj-ILP1* mRNA levels were not affected by rPej-SGP-I.

Preparation of rMaj-NPLP and rMaj-pCHH-B

Based on the above results, we further investigated potential involvement of Maj-NPLP and Maj-pCHH-B in vitellogenesis

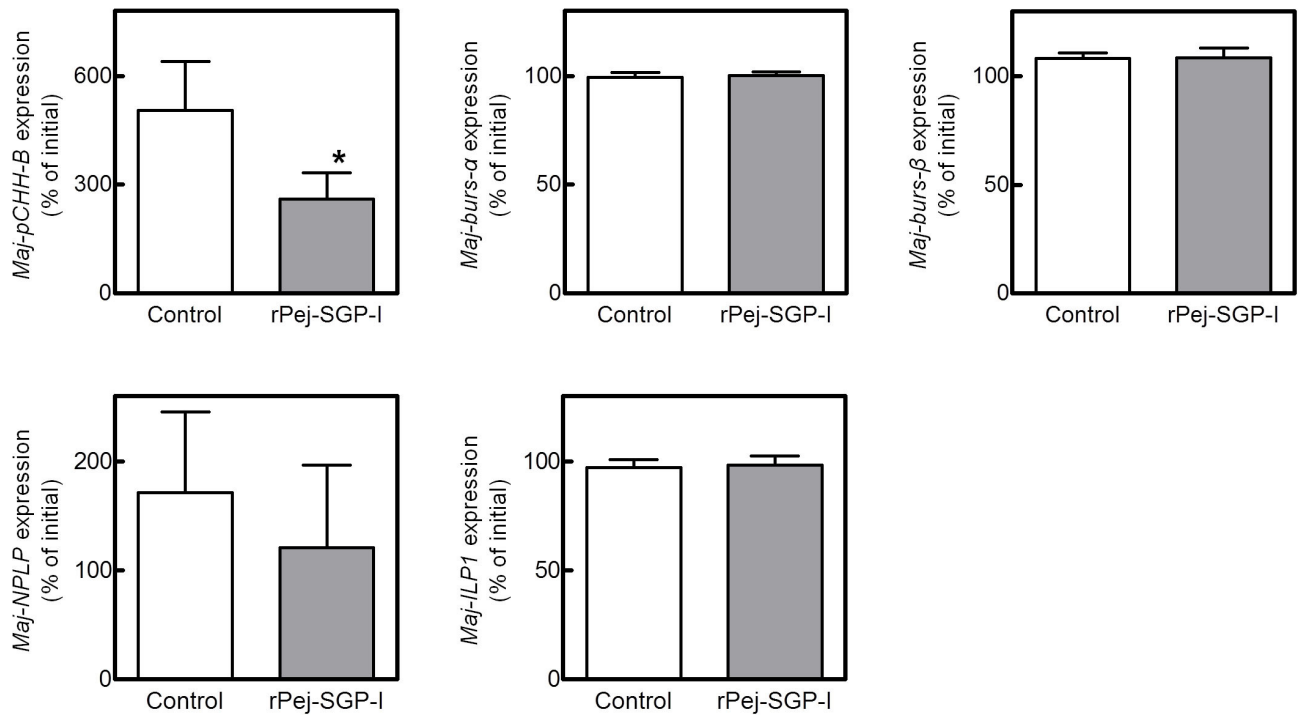

FIGURE 5 | Effect of rPej-SGP-I on mRNA levels of the putative hormones in the ovary. Relative expression levels have been shown as the percentage change relative to that of the initial expression (mean ± SEM, $n = 6$). Statistical differences between control and rPej-SGP-I-treated groups were analyzed by the Wilcoxon signed rank test (*$P < 0.05$).

using recombinant peptides. rMaj-NPLP and rMaj-pCHH-B were expressed both as Nus-tagged fusion proteins; they were mostly recovered in the soluble fraction of cell lysates and successfully purified using a Ni-sepharose resin and subsequent HPLC. The deconvoluted mass spectra of untagged and HPLC-purified recombinant peptides have been shown in **Supplementary Figure 8**. Electrospray ionization (ESI) mass spectrum of rMaj-NPLP revealed a molecular mass of 8,357.1 which agreed with the calculated value (8,368.7) minus 12 Da, suggesting the presence of 6 disulfide bonds in the structure. Similarly, the observed molecular mass of rMaj-pCHH-B was 8,391.7 which was similar to the calculated value (8,397.6) minus 6 Da, indicating the presence of 3 disulfide bonds in the structure.

Effect of rMaj-NPLP and rMaj-pCHH-B on Ovarian VG Expression

The effects of the recombinant peptides on *Maj-VG* expression were assessed in the ovarian incubation system. Although rMaj-NPLP did not affect *Maj-VG* expression at lower doses, *Maj-VG* expression increased at higher doses with significant changes observed in the ovary fragments treated with 20 nM rMaj-NPLP (**Figure 6A**). In comparison, rMaj-pCHH-B treatment did not affect the *Maj-VG* expression up to 200 nM (**Figure 6B**). When 200 nM rMaj-pCHH-B was co-incubated with 50 nM rPej-SGP-I, rMaj-pCHH-B acted neither cooperatively nor antagonistically on the vitellogenesis-inhibiting activity of rPej-SGP-I (**Figure 6C**).

DISCUSSION

The hypothalamus-pituitary-gonad is the main axis controlling vertebrate reproductive processes. In crustaceans, the XOSG and brain ganglia appear to correspond to the axis. Peptide hormones secreted from these niches, especially from the XOSG, have been well-characterized, and their involvements in reproductive processes have been studied. Conversely, there is little information on gonadal hormones. Most penaeid shrimps synthesize VG in the hepatopancreas and in the ovary (42). For example, in *M. japonicus*, the same VG transcript is present in both tissues, but their expression dynamics differ slightly during vitellogenesis (17, 20, 21). Therefore, some peptide factors from the ovary is thought to be serving as feedback signals among peripheral tissues and CNS. Additional studies on gonadal hormones will improve our understanding of the mechanisms behind crustacean reproduction. Thus, in this study, we analyzed the ovarian transcriptome of *M. japonicus* and identified Maj-burs-α, Maj-burs-β, Maj-NPLP, Maj-pCHH-B, and Maj-ILP1 (**Figure 1**) as some possible peptide hormones produced in *M. japonicus* ovary. Moreover, we examined functions of Maj-NPLP and Maj-pCHH-B. These data, in combination with previous work (35), suggest a peripheral regulation of the ovary as shown in **Figure 7** and below.

Regarding neuroparsin, its vitellogenesis-inhibiting activity in terms of the inhibition of the juvenile hormone system has been reported in the migratory locust *Locusta migratoria* (68).

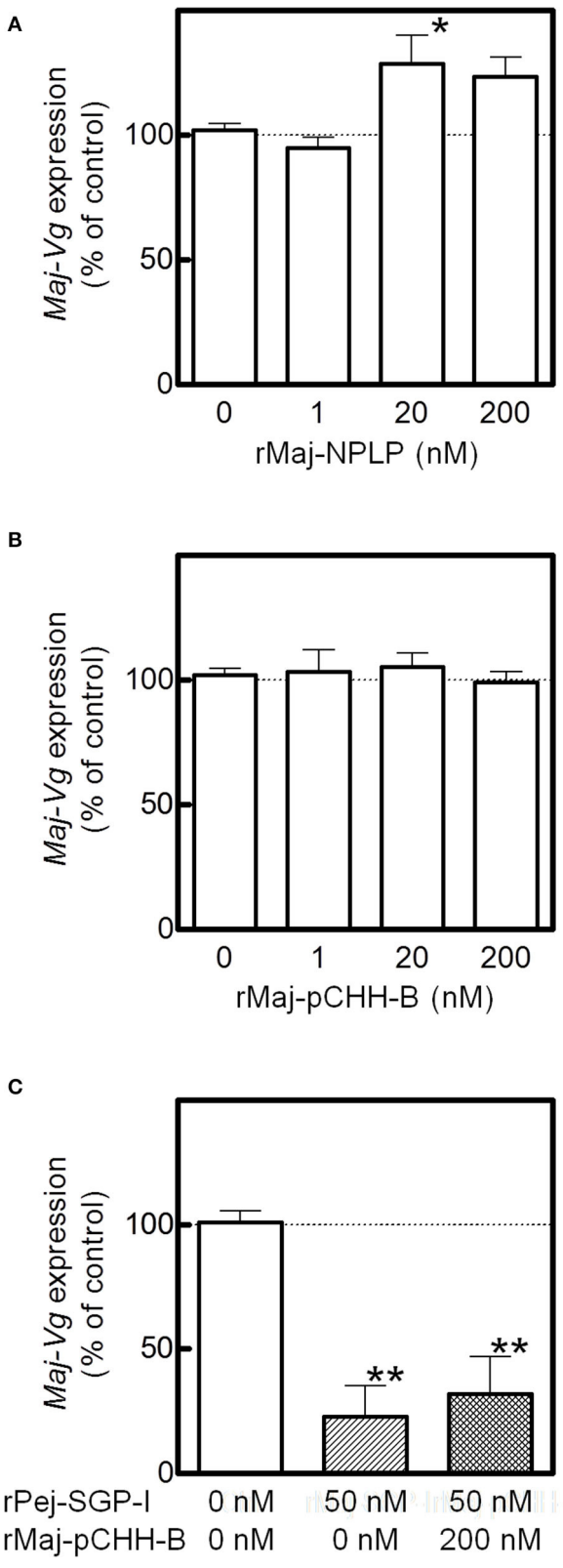

FIGURE 6 | Analyses of rMaj-NPLP and rMaj-pCHH-B functions. *Maj-VG* expressions in *ex-vivo* ovarian fragments incubated with **(A)** rMaj-NPLP and *(Continued)*

FIGURE 6 | (B) rMaj-pCHH-B are examined. Expression levels have been represented as the percentage change relative to those of 0 nM control groups. The differences between controls and the other groups are tested for significance using a one-way ANOVA followed by the Dunnett's post test (*$P <$ 0.05; $n = 4$–8 for rMaj-pCHH-B and 6–8 for rMaj-NPLP). **(C)** *Maj-VG* expression was also examined following incubation without hormones, with rPej-SGP-I alone, and with both rPej-SGP-I and rMaj-pCHH-B. The differences between controls and the other groups are tested for significance using a one-way ANOVA followed by the Dunnett's post test (**$P < 0.01$; $n = 4$).

In contrast, OEH of the neuroparsin family has a gonadotrophic effect in *A. aegypti* (66, 69); the OEH as well as several ILPs stimulate the ovarian ecdysteroid production, which induces VG synthesis in the fat body. In crustacean species, a neuroparsin-like peptide (MeNPLP) which is produced by the hepatopancreas and has been reported to induce *VG* expression in *M. ensis* (65). Transcriptomic analysis of *Fenneropenaeus merguiensis* (the banana shrimp) ovary shows that the expression of a neuroparsin precursor is higher in the vitellogenic stage compared to that in the non-vitellogenic stage (70). In the present study, we showed that Maj-NPLP has a stimulatory effect on VG synthesis in the ovary (**Figure 6A**). Although inhibitory effect of Pej-SGP-I on Maj-NPLP expression is not clear (**Figure 5**), it is likely that other five VIHs (Pej-SGP-II, -III, -V. -VI, and -VII) (28) regulate Maj-NPLP expression in the ovary. Taken together, we concluded that the NPLP is a regulator of reproductive process in arthropods. Multiple isoforms of NPLP are often found in a single crustacean species (47, 49, 65) with differing tissue-specific expression. The expression pattern of *Maj-NPLP* is similar to that of Mar-NP-2 in *M. rosenbergii* (51) which is expressed predominantly in the thoracic ganglia, heart, and gonads. Regarding the Maj-NPLP receptor, contig N06137 (**Table 1**) has significant sequence similarity to the OEH receptor (66) as well as a similar domain structure (i.e., extracellular venus fly trap domain, a single transmembrane domain, and intracellular tyrosine kinase domain), thereby suggesting that Maj-NPLP may act on the ovary through endocrine or autocrine/paracrine modes of action via this receptor. Such information may be used for further characterization of the role of NPLPs in *M. japonicus* vitellogenesis.

Bursicon, a heterodimer composed of burs-α (burs) and burs-β (pburs), regulates cuticle tanning and wing expansion after ecdysis in insects. Additionally, bursicon of the blue crab *Callinectes sapidus* (CasBurs) appears to be involved in the deposition and thickening of new cuticle as well as granulation of hemocytes (71). Reflecting the constitutive increased expression of the β subunit compared to the α subunit, the ββ homodimer as well as the αβ heterodimer are found in the pericardial organ of *C. sapidus*, but their intrinsic functioning is unknown. However, characteristics of bursicon, a member of the TGF-β superfamily (**Supplementary Figure 4**) and the dimerization patterns of the subunits, are analogous to those of the activin/inhibin family of proteins which participate in the regulation of reproductive physiology in mammals. Consequently, these data support our hypothesis of the role of bursicon in crustacean reproductive processes. In fact, bursicon has been reported to stimulate *VG* expression in the ovary of *Penaeus monodon*, also known as the

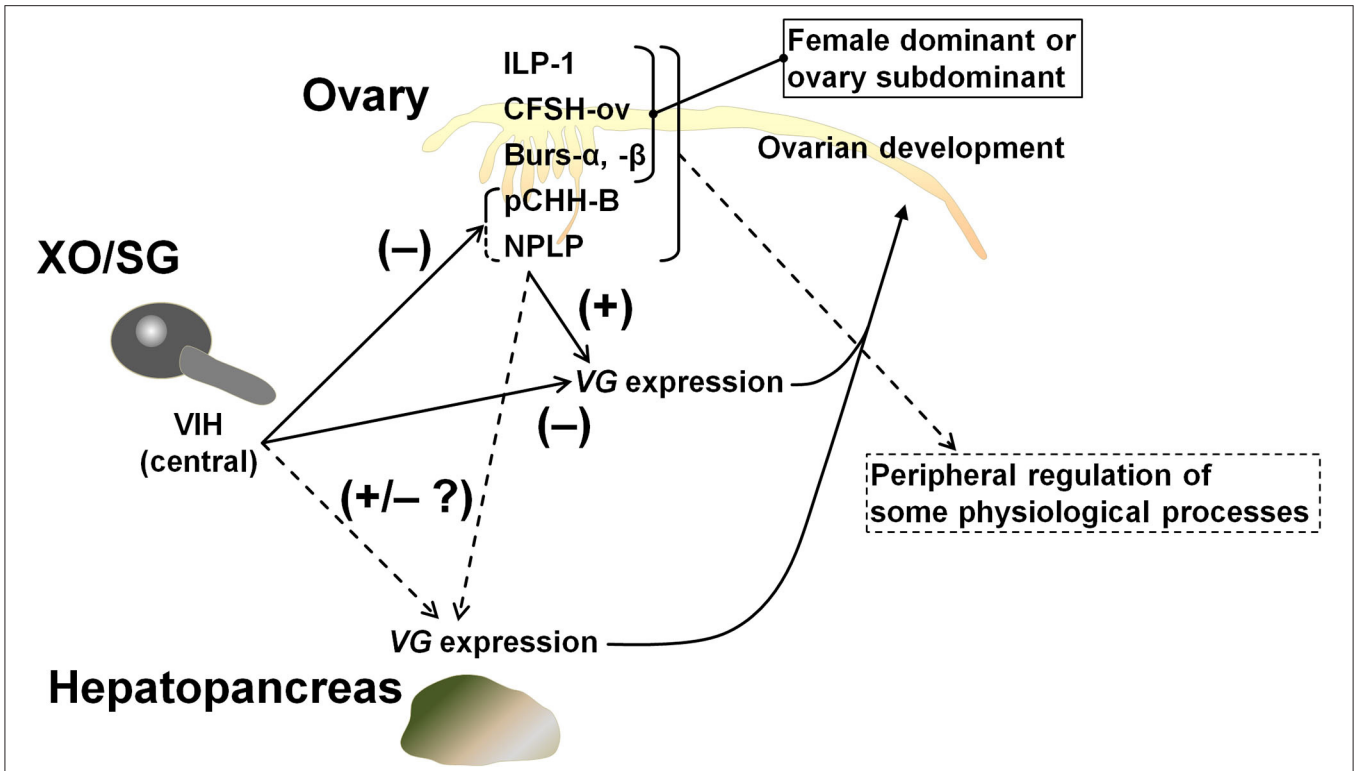

FIGURE 7 | Schematic model of peripheral regulation of the ovary. It is highly likely that Maj-NPLP is a vitellogenesis-regulating factor in *M. japonicus* considering its activity and the existence of a putative receptor in the ovary. The other female dominant or ovary subdominant hormones may play a role in some physiological processes. Maj-CFSH-ov has been previously characterized (35).

black tiger shrimp (13). However, only the heterodimer (i.e., Pmbursα and Pmbursβ subunits) exhibit such a stimulatory effect, whereas the αα and ββ homodimers do not. In contrast, the bursicon receptor ortholog DLGR2, which is encoded in the *rickets* gene in the fruit fly *Drosophila melanogaster* (72), is not found in our *M. japonicus* ovarian transcriptome. Although contig N28645 (**Table 1**) has a similar domain organization to DLGR2 in terms of the N-terminal leucine-rich repeat domain and the seven-transmembrane receptor domain, their overall sequence similarity is low.

Maj-pCHH-B expression was suppressed by a central VIH, suggesting its possible involvement in the regulation of vitellogenesis, but we were unable to show this experimentally using the recombinant peptide (**Figure 6B**). And Maj-pCHH-B acted neither antagonistically nor cooperatively on the vitellogenesis-inhibiting activity of Pej-SGP-I (**Figure 6C**). Considering the dominant gene expression pattern in the CNS including the eyestalk, Maj-pCHH-B may act as a neurotransmitter, much like the ion transport peptides (ITP and ITP-L) (73, 74), the ortholog of the CHH superfamily in insects (4, 75). Further functional analysis of Maj-pCHH-B, Mj-putativeCHH, and Mj-putativeMIH-C (25) are required to elucidate the diverse biological functions of the CHH superfamily in *M. japonicus*. As for the primary structure, the single-residue insertion seven amino acids downstream of the third Cys residue (**Supplementary Figure 6**) is also reported in a CHH from the Pacific white shrimp *Litopenaeus vannamei* (76). Although some venom peptides from spiders and centipedes, which are members of the CHH superfamily, have an unusual number of amino acid residues between the third and fourth or fourth and fifth Cys residues, they share the common tertiary CHH superfamily scaffold (62, 77). Thus, this suggests that Maj-pCHH-B also possess a similar backbone fold.

As shown by our previous studies, six CHH-family peptides from *M. japonicus* XOSG inhibit the ovarian *Maj-VG* expression (28, 29). Hence, the receptor for the CHH-family of peptides is most likely found in the ovary. However, the report of functional CHH-family receptor molecule, in which specific ligand-receptor interaction is proved, is currently very limited (78, 79). Contig N13763 (**Table 1**) shares 37% amino acid sequence identity with the ITPL receptor in the silkworm *Bombyx mori* (BNGR-A24). Since the ITPL receptor has lower but definite affinity to ITP, N13763 should be investigated as a potential CHH-family receptor in future. In contrast, the contig N13763 shows higher similarity with the NPY receptor. Interestingly, the existence of NPY/F in crustacean species has been reported in some transcriptome analyses (43–49, 51, 52), and NPF has been suggested to stimulate ovarian development in *M. rosenbergii* (80). Therefore, NPF may have a similar function in *M. japonicus*. In *L. vannamei*, it is suggested that a receptor guanylyl cyclase (LvGC) is CHH receptor and is involved in the regulation of *IAG* expression (78). Two contigs

encoding receptor guanylyl cyclase family found in the ovarian transcriptome in this study (N05324 and N33402, **Table 1**) do not contain the extracellular ligand domain, and their sequence similarities cannot be examined.

Recent advances in transcriptomic analysis have revealed multiple molecular species of an insulin family in a single crustacean species. For example, three ILPs (i.e., IAG, ILP, and relaxin-like) have been reported in *L. vannamei*, *M. rosenbergii*, and *P. clarkii*, respectively (48). Considering the present discovery of Maj-ILP1 and the result of phylogenetic analyses (**Figure 3**), there may be the third member of the relaxin-like molecular species in *M. japonicus*. Multiple ILPs have also been identified in insects such as *B. mori* (81), *D. melanogaster* (82), and the red flour beetle *Tribolium castaneum* (83). In *T. castaneum* ILP2 and ILP3 regulate *VG* expression in downstream juvenile hormone signaling (84), and in *A. aegypti* ILP3 controls egg production through the stimulation of yolk uptake and ecdysteroid production in the ovary (85). Arthropod ILP has been suggested to be a factor which links nutritional status and reproductive status (86, 87). Taken together, the female-specific and gonad-dominant Maj-ILP1 may also have such functions. Furthermore, our *M. japonicus* ovarian transcriptome contained a putative insulin receptor/IAGR (**Table 1**). Although *Fenneropenaeus* IAGR is not detected in any female tissues (53), the presence of IAGR homolog in *M. japonicus* ovary can account for the inhibitory effect of Maj-IAG on *Maj-VG* expression (31). The possibly important relationship between the insulin signaling pathway and reproduction should be studied in terms of the ligand as well as the receptor and downstream factors, such as the components of the signaling pathway which have been revealed to be conserved in *M. japonicus* by transcriptome analysis (**Supplementary Figure 9**).

In summary, we reported putative peptide hormones and receptors obtained through mRNA-sequencing analysis of the ovary of *M. japonicus*. Results of this study suggest a possible peripheral regulation by these hormones in the crustacean reproductive physiology (**Figure 7**). Factors involved in vitellogenesis regulation, ovary-specific expression patterns, and putative receptors for neuroparsin, ILP, and other peptide hormones are fascinating starting points for further detailed characterization. Above all, the effects of the ovarian hormones as well as central VIH on *Maj-VG* expression in the hepatopancreas should be addressed to outline the endocrine regulation of vitellogenesis. In addition, the transcriptome data generated in this work can also be utilized to further study hormonal functions. For example, target genes with expression patterns that are affected by VIH and other putative hormones can be efficiently searched using the combination with *ex-vivo* culture system and transcriptome analysis. Effective use of transcriptomic data from central and peripheral tissues will allow the comprehensive understanding of the regulatory mechanism of reproduction and other physiological processes in crustaceans.

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 below: https://www.ddbj.nig.ac.jp/, Data for DRA010103 is available in DDBJ Sequence Read Archive.

AUTHOR CONTRIBUTIONS

NT conceived the idea for the project, conducted most of the experiments, and analyzed the results. YK and KI analyzed the transcriptomic data. NT and TS prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00541/full#supplementary-material

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BRIEF RESEARCH REPORT
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Molecular Characterization of the Insulin-Like Androgenic Gland Hormone in the Swimming Crab, *Portunus trituberculatus*, and Its Involvement in the Insulin Signaling System

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The insulin-like androgenic gland hormone (IAG) is mainly produced in the androgenic gland (AG) of the male crustaceans and is a crucial regulator in male sexual differentiation. In the current study, the full-length cDNA of IAG in the swimming crab, *Portunus trituberculatus* (*Pt-IAG*), was cloned and characterized. Similar to other reported IAGs, the deduced amino acid sequence of Pt-IAG consists of signal peptide, B chain, C peptide, and A chain, containing six conserved cysteines that form two interchain disulfide bonds and one intra-B chain disulfide bond. Tissue distribution analysis suggested that the *Pt-IAG* cDNA was highly expressed in the AG and was slightly expressed in several other tissues. A short-term silencing of *PtIAG* with double-stranded RNA was found to reduce the transcript levels of insulin receptor (Pt-IR) and insulin-like growth factor-binding protein (Pt-IGFBP), suggesting the Pt-IAG might perform its biological function through the insulin family-based signaling system. Bilateral eyestalk ablation (ESA) induced the expression of *Pt-IAG* in the AG at 4 and 7 days after surgery, while the transcript levels of *Pt-IR* in the AG and testis and *Pt-IGFBP* in the muscle, testis, and thoracalia ganglia were significantly decreased from 1 day after surgery. The results suggested that the *Pt-IR* and *Pt-IGFBP* might also be the targets of eyestalk neuropeptides and responded to the ESA independent of IAG regulation.

Keywords: insulin-like androgenic gland hormone, cDNA clone, RNAi, eyestalk ablation, insulin signaling

INTRODUCTION

The insulin-like androgenic gland hormone (IAG) is an insulin-like hormone mainly produced in the androgenic gland (AG) of male crustaceans. As a crucial regulator in crustacean male sexual differentiation, IAG has been widely identified in various crustacean species, and its function has been firmly established by numerous studies, such as microsurgical removal or implantation of AG, injection of hypertrophic AG cells, and IAG silencing [see review in (1, 2)]. Besides its role in sexual development, IAG was also proposed to participate in the process of growth (3), glucose metabolism (4), and ovarian development (5).

Compared to the molecular characterization and functional analysis, the mechanisms on how IAG works were much less studied. Some early studies had noticed that eyestalk ablation (ESA) can lead to the hypertrophy and hyperactivity of AG, and it was eventually proposed that IAG production may be negatively regulated by the inhibitory neurohormone in the sinus gland of eyestalk through an eyestalk–AG endocrine axis (6). The hypothesis was further supported by several studies showing an inhibitory role of specific eyestalk neuropeptides on IAG expression, such as gonad-inhibiting hormone (GIH) and molt-inhibiting hormone (MIH) in *Macrobrachium nipponense* (7) and crustacean female sex hormone (CFSH) in *Scylla paramamosain* (8).

Khalaila et al. (6) also found that the AG secretory products can directly activate protein kinases and phosphatases of some testicular polypeptides. This suggests that the signal transduction of IAG may work resembling the insulin family-based signaling system, which was well-described in vertebrates. It has been revealed in some pioneer studies that IAG can act as an active ligand to the insulin receptor (IR) (9, 10), which is responsible for transducing the insulin or insulin-like peptide (ILP) signals from the intercellular to the intracellular environment (11). In a recent work, long-term knockdown of IR in *Macrobrachium rosenbergii* by injection siRNA successfully yielded neo-females, suggesting the essential role of IR in IAG functions (12). IAG also showed a potential in interacting with the insulin-like growth factor-binding protein (IGFBP), as demonstrated in studies involving the binding assays (13), RNAi (14), and *in vitro* studies (15). IGFBPs act as the "carriers" and "reservoirs" of IGFs, modulating their availability and activity (16). All the IGFBPs identified in crustaceans are structurally similar to the IGFBP-related proteins (IGFBP-rPs, also named IGFBP7) (15), that binds insulin with higher affinities. This would be compatible with the fact that the structure of IAG is more similar to insulin than IGFs.

The swimming crab, *Portunus trituberculatus*, is a commercial species that has been extensively artificially propagated and cultivated in Chinese water. Monosex culture is considered an attractive approach for gaining higher yields of commercial crustaceans during both breeding and sailing processes. Despite this, the basic knowledge of IAG in this species is still lacking. The present study reported the first cloning of the IAG gene in *P. trituberculatus* (*Pt-IAG*), in parallel to the determination of its mRNA levels in different tissues and developmental stages. To verify the involvement of *Pt-IAG* in the insulin-signaling system, a dsRNA-mediated RNAi of *Pt-IAG* was utilized to evaluate its regulation on the expression of putative *Pt-IR* and *Pt-IGFBP* genes. Furthermore, as the eyestalk–AG endocrine axis exists, ESA surgery was performed to investigate the response of the *Pt-IAG*, *Pt-IR*, and *Pt-IGFBP*.

MATERIALS AND METHODS

The full-length cDNA of the *Pt-IAG* was cloned using RT-PCR and rapid amplification of 5′ complementary DNA ends (RACE). Briefly, a fragment of *Pt-IAG* was firstly obtained using a pair of degenerate primers (**Table 1**). Then, the 3′ and 5′-ends of *Pt-IAG* were obtained according to the manufacturer's protocol of SMARTer^{TM} RACE cDNA Amplification Kit (Clontech). The sequencing results from the above fragments were spliced using Vector NTI 10.0 software. The accuracy of the splicing sequence was confirmed by a long PCR using a pair of primers covering all the predicted open reading frame (ORF) regions. The nucleotide sequences of *Pt-IAG* and its deduced amino acid sequences were compared to the known decapod IAGs from NCBI database using the Clustal W multiple sequence alignment program. A phylogenetic tree was generated with the neighbor-joining option of molecular evolutionary genetics analysis (MEGA) version 5.0 by multiple sequence alignment with 16 known sequences from the NCBI database. Bootstrap analysis of 1,000 replicates was carried out to determine the confidence of tree branch positions.

TABLE 1 | Primers used in this study.

Name	Sequence (5′-3′)	Purpose
DP-F	CCGACTTCTCCGTGGACTGYGGNAAYYT	RT-PCR
DP-R	GGGCCGCAGGGTGTCRCARTAYTC	RT-PCR
Pt-IAG-3F1	TTCGCAGATCCCACCGGAA	3′ RACE
Pt-IAG-3F2	AATGTTGCCCGCAGTCCAC	3′ RACE
Pt-IAG-5R1	CGGGCAACATTCGTCATA	5′ RACE
Pt-IAG-5R2	CTGCGAATCCTTCTTCCTATCC	5′ RACE
Pt-IAG-VF	GTCCTCACCAAGAATGTGCCTG	Long PCR
Pt-IAG-VR	CTTCCTCTTACTGCCTATTTCGGG	Long PCR
Pt-IAG-qF	TCTTATTAGCGACTTCTCCG	qPCR
Pt-IAG-qR	CCTCTGTCCCTCGTTTATGT	qPCR
Pt-IR-qF	AGAAGGTGCCCAGGAACTAAA	qPCR
Pt-IR-qF	AGGTGAGGTTGGATCGGAAT	qPCR
Pt-IGFBP-qF	TTACCACTATTGACGGCACCT	qPCR
Pt-IGFBP-qR	TCATTATC TGTACCCATCCTGTT	qPCR
β-Actin-F	CGAAACCTTCAACACTCCCG	qPCR
β-Actin-R	GATAGCGTGAGGAAGGGCATA	qPCR
Pt-IAG-IF	TAATACGACTCACTATAGGGTCTTATTAGCGACTTCTCCG	RNAi
Pt-IAG-IR	TAATACGACTCACTATAGGGCGTTGTCCTCATCCTCCT	RNAi
GFP-IF	TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT	RNAi
GFP-IR	TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGC	RNAi

The T7 promoter sequence was underlined.

For tissue distribution analysis, wild adult swimming crabs (four males with body weight of 164–209 g and carapace width of 13.4–15.1 cm and four females with body weight of 269–275 g and carapace width of 16.8–17.2 cm) were purchased from the local aquatic market in Zhenhai, Ningbo. Tissues including androgenic gland, testis, distal spermatic duct, ejaculatory bulb, gill, heart, eyestalk, Y-organ, mandibular organ, thoracic ganglion, hepatopancreas, ovary, muscle, brain, and intestine were dissected on ice and then stored in RNA preservation fluid (Cwbiotech) at −80°C.

For the RNAi experiment, the dsRNA was synthesized according to the instruction of MEGAscript T7 Kit (Ambion).

Briefly, 446 bp of Pt-IAG and 655-bp green fluorescent protein (GFP) amplicons were cloned into pMD18-T vector (Takara) and amplified by PCR with a T7 promoter linked primer (**Table 1**). The resultant DNAs were used as the templates to synthesize dsRNA in a 20-μl *in vitro* transcription system. Integrity of the dsRNA was checked on agarose gel, and the concentrations of dsRNA were determined using a NanoDrop 2000 UV Spectrophotometer (Thermo Fisher). For the *in vivo* dsRNA injection, healthy juvenile male crabs (C5 juveniles, body weight 0.8–1 g) were divided into two groups, the *Pt-IAG* dsRNA injection group and the GFP dsRNA injection group. Then, 3 μg of dsRNA was injected via the base of the last walking leg with 10-μl syringe. Five crabs in each injection group were sacrificed at 0, 12, 24, 48, and 96 h post-injection, respectively, and then placed into the RNA preservation fluid (Cwbiotech) at −80°C until used.

For the ESA experiment, male swimming crabs (50–80 g) were temporarily reared for 7 days before ESA. The crabs were divided into the initial control group, the concurrent control (non-surgery) group, and the ESA group. Initial and concurrent controls received no treatments, while the initial controls ($n = 4$) were sacrificed on the first day of the experiment. ESA group received bilateral ablation of their eyestalks using sharp sterile scissors. To minimize hemolymph loss and infection, the wounds were cauterized with flame-heated sharp spatula. Four crabs in each of the ESA and concurrent groups were sacrificed at 0, 12, 24, 48, and 96 h post-injection. Tissues including AG, testis, muscle, and thoracalia ganglia were dissected on ice and stored in RNA preservation fluid (Cwbiotech) at −80°C until used.

Gene expression levels in this study were determined using quantitative real-time PCR (qPCR). qPCR primers for *Pt-IAG*, *Pt-IR*, *Pt-IGFBP*, and the reference gene β-actin were listed in **Table 1**. PCR was carried out using the SYBR® Premix Ex Taq™ II kit (Takara) according to manufacturer's instructions. Relative mRNA expression levels normalized to β-actin were calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method (17). Transcript abundance in a representative replicate was set as the calibrator. All other quantities were expressed as an n-fold difference relative to the calibrator. The statistical significance in this study was analyzed using the SPSS 19.0 software. All data were subjected to the normality test using the Kolmogorov–Smirnov and Cochran tests prior to all statistical tests. Significant differences were accepted at $P < 0.05$ using one-way ANOVA followed by Student's *t*-test or Tukey test.

RESULTS

The full-length cDNA of the *Pt-IAG* (GenBank accession number: KX168425) was 1,126 bp in length, which encoded 153 amino acid (aa), consisting of a signal peptide of 19 aa, a B chain of 32 aa, a C peptide of 57 aa, and an A chain of 45 aa. Like other known IAGs, the deduced aa sequence of Pt-IAG contained a putative N-glycosylation site (NCT), two putative cleavage sites (RHKR and RIRR), and six conserved cysteine residues. The Pt-IAG was clustered into decapods

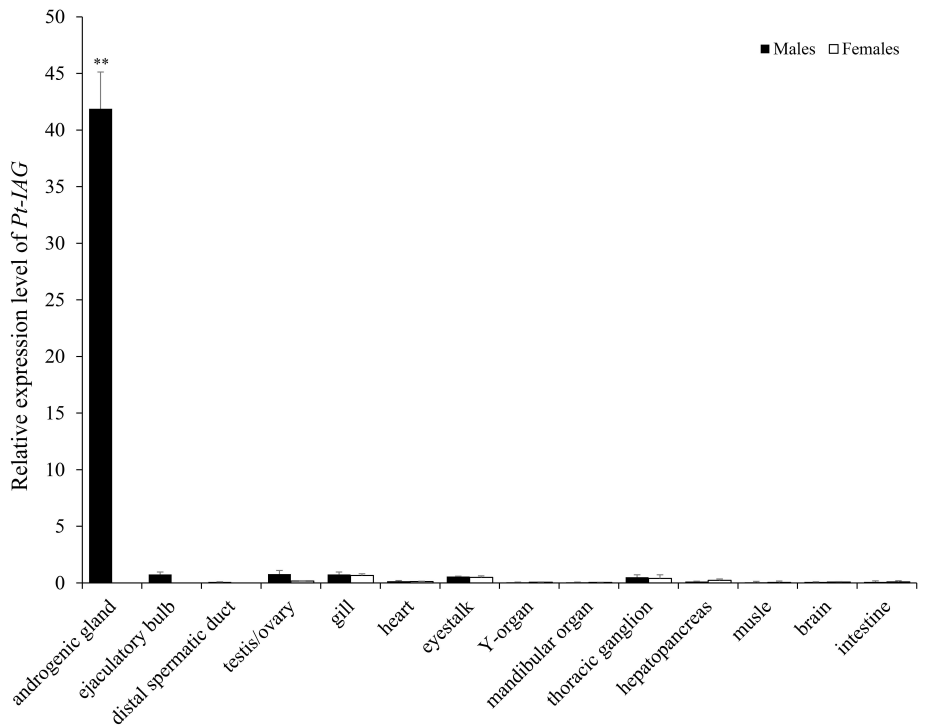

FIGURE 1 | Tissue distribution of the insulin-like androgenic gland hormone of *Portunus trituberculatus* (*Pt-IAG*) transcripts. Bars represent mean ± SEM ($n = 4$). **Values statistically different from other groups ($P < 0.01$, Student's *t*-test).

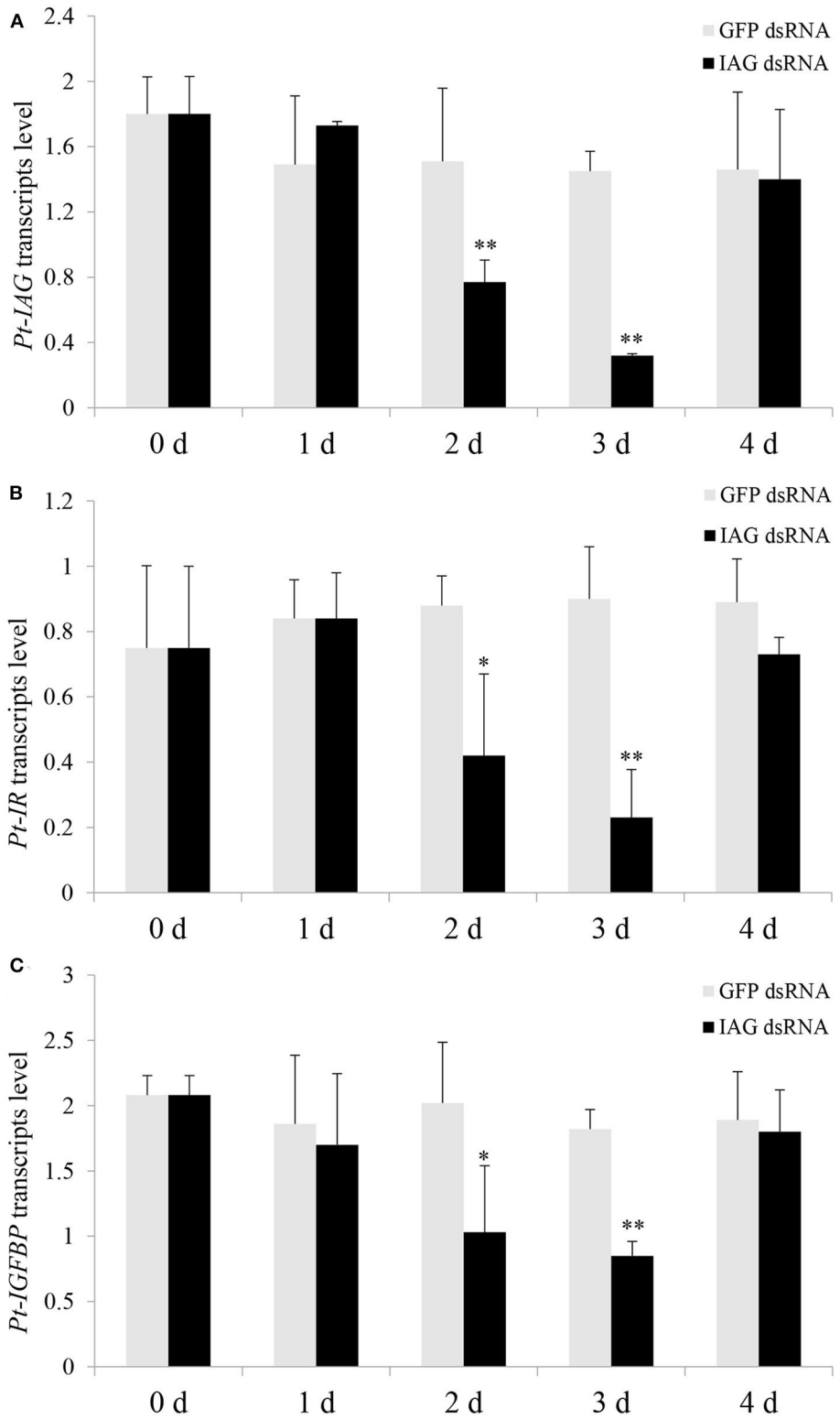

FIGURE 2 | Effects of insulin-like androgenic gland hormone (IAG) dsRNA injection on gene expression of *Portunus trituberculatus* (*Pt*)-*IAG* **(A)**, *Pt*-insulin receptor (*IR*) **(B)**, and *Pt*-insulin-like growth factor-binding protein (*IGFBP*) **(C)**. Bars represent mean ± SEM ($n = 5$). $^{**}P < 0.01$ or $^{*}P < 0.05$ values statistically different from those of the control groups (Student's *t*-test).

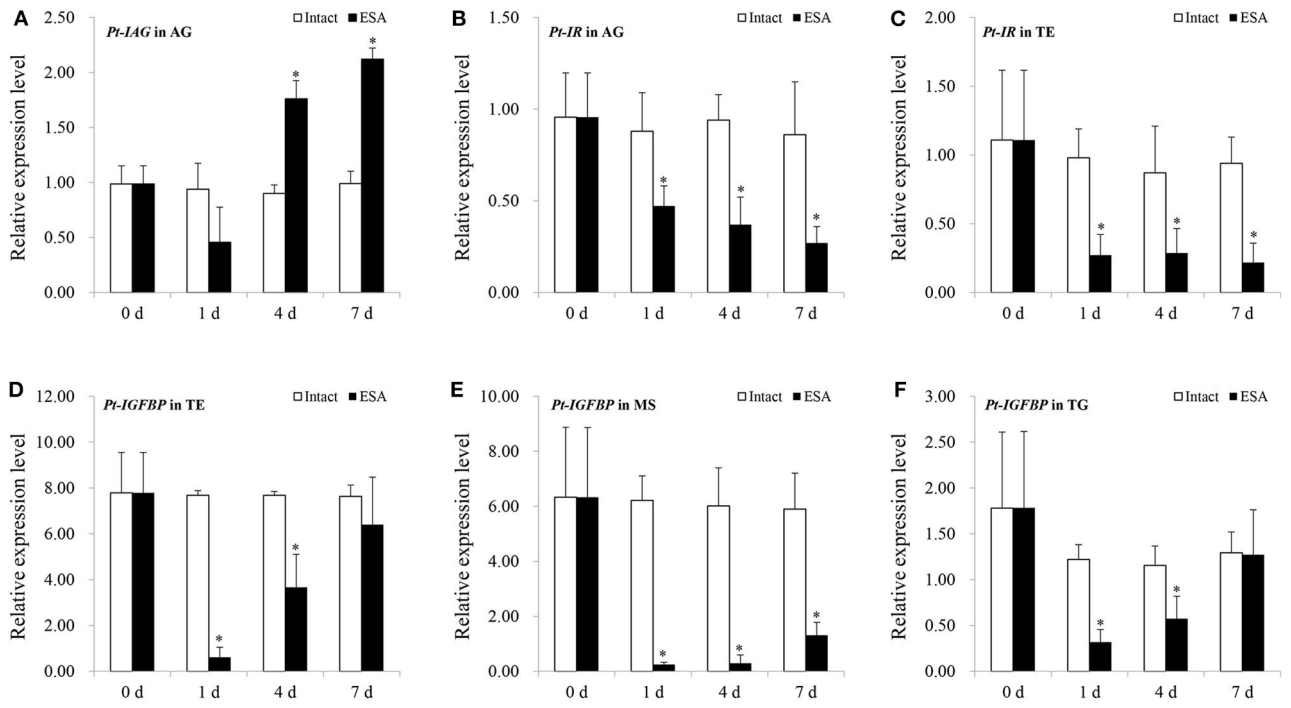

FIGURE 3 | Effects of eyestalk ablation (ESA) surgery on the gene expression of insulin-like androgenic gland hormone of *Portunus trituberculatus* (*Pt-IAG*) in the androgenic gland (AG) **(A)**, *Pt*-insulin receptor (*IR*) in the AG **(B)**, and testis **(C)**, and *Pt*-insulin-like growth factor-binding protein (*IGFBP*) in the testis **(D)**, muscle **(E)**, and thoracalia ganglia **(F)**. Bars represent mean ± SEM ($n = 4$). $^{**}P < 0.01$ represent values statistically different from those of the control groups (Student's *t*-test).

clade in the phylogenetic tree and was more closely related to Sp-IAG and Cs-IAG than to other IAGs. Tissue distribution analysis showed that the *Pt-IAG* mRNA was highly expressed in the AG and slightly expressed in the testis, ejaculatory bulb, gill, eyestalk, mandibular organ, thoracic ganglion, and hepatopancreas (**Figure 1**).

Injection of the *Pt-IAG* dsRNA caused a significant decrease in *Pt-IAG* expression at 2 and 3 days post-treatment compared to that in the GFP dsRNA group, indicating good RNAi efficiency (**Figure 2A**). Injection of *Pt-IAG* dsRNA also led to a similar reduction in the mRNA levels of *Pt-IR* and *Pt-IGFBP* at 2 and 3 days post-injection (**Figures 2B,C**). The expression of *Pt-IAG*, *Pt-IR*, and *Pt-IGFBP* recovered to normal levels at 4 days post-injection, suggesting that the silencing effects are transitory.

In the ESA experiment, the *Pt-IAG* expression in the AG was significantly induced from 4 to 7 days after the surgery (**Figure 3A**). By contrast, the expressions of *Pt-IR* and *Pt-IGFBP* were significantly decreased by ESA surgery. The *Pt-IR* expression in the AG and testis and the *Pt-IGFBP* expression in the testis, muscle, and thoracalia ganglia were examined. The test tissues were selected according to our preliminary data (unpublished), which were the main tissues of *Pt-IR* and *Pt-IGFBP* expression. For the *Pt-IR*, its mRNA level in the AG and testis decreased from 1 to 7 days after the surgery (**Figures 3B,C**). A similar pattern was also found for the *Pt-IGFBP* expression in the muscle. In the testis and thoracalia ganglia, the expression of *Pt-IGFBP* decreased from 1 to 4 days but recovered to normal level at 7 days after the surgery (**Figures 3D–F**).

DISCUSSION

The presented study reported the first study of the IAG gene in the swimming crab, *P. trituberculatus* (*Pt-IAG*). qPCR analysis showed a predominant expression of *Pt-IAG* in the AG, which is consistent with all the previous studies. Although the *Pt-IAG* transcripts were slightly expressed in the testis, ejaculatory bulb, gill, eyestalk, mandibular organ, thoracic ganglion, and hepatopancreas, it conforms to the recent consensus that the IAG is widely distributed among tissues, playing a broader role other than regulating sexual development. For instance, RNAi of IAG not only prevented the regeneration of male secondary sexual characteristics of *M. rosenbergii* but also led to a delay in molting and a reduction in growth (3). In the blue crab, *Callinectes sapidus*, *in vivo* silencing of hepatopancreas source IAG resulted in higher levels of hemolymph glucose than the control group, accompanied by significantly lower amounts of carbohydrate in the hepatopancreas, indicating a function of IAG in carbohydrate metabolism (4). Additionally, the IAG transcripts were also found in the ovary of *C. sapidus* and *S. paramamosain*, therefore being deduced as a regulator in ovarian development (5, 18).

To better understand the multiple functions of IAG, it is necessary to clarify the mechanisms of how IAG is transported to and is recognized by target tissues. An insulin family-based signaling system has been proposed based on the structure similarity of IAG to the insulin/IGF family. The hypothesis was further supported by several assays showing connections between IAG with the transmembrane IR (9, 10, 19) and the IGFBPs (13–15). The present study could provide another evidence for strengthening this hypothesis, concluded from the RNAi experiments showing a reduction in *Pt-IR* and *Pt-IGFBP* expression after injecting the *Pt-IAG* dsRNA. It is reasonable to speculate that the silencing in *Pt-IAG* might reduce the IAG signals, lessening the response of the insulin signaling system, thereby affecting the expression of *Pt-IR* and *Pt-IGFBP*.

ESA surgery has been extensively demonstrated to stimulate the IAG expression (7, 20–22), and this could also be verified by the ESA experiment in the present study. It was found that ESA also affected the expression of *Pt-IR* and *Pt-IGFBP*, which could be revealed by their decreased transcript levels in the tested tissues of surgery groups. The reduction in *Pt-IR* and *Pt-IGFBP* expression occurred earlier than the induction in *Pt-IAG* expression, suggesting that they responded to ESA independent of IAG regulation. In *M. rosenbergii*, IR silencing led to the hypertrophy of AG and the hyperactivity of Mr-IAG (10). In this respect, it is possible that the reduced expression of *Pt-IR* by ESA might in turn contribute to the subsequent increase in *Pt-IAG* expression.

In summary, the present study cloned the full-length cDNA of IAG in the swimming crab, *P. trituberculatus*. The Pt-IAG shared similarities in molecular characteristics and expression patterns to the IAGs that have been identified. *In vivo* silencing of the *Pt-IAG* reduced the transcript levels of *Pt-IR* and *Pt-IGFBP*, suggesting its involvement in the insulin family-based signaling system. Furthermore, the ESA experiments suggested that the *Pt-IR* and *Pt-IGFBP* might also be the targets of eyestalk neuropeptides and responded to the ESA independent of IAG regulation.

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.

AUTHOR CONTRIBUTIONS

DZ designed the study and wrote the manuscript. QJ contributed to the experimental work and integrated the data. HZ prepared the dsRNA and participated in tissue collection. LZ did the ESA surgery and participated in tissue collection. YW did the qPCR and participated in tissue collection. MW did the qPCR and ESA surgery. XX did the dsRNA injection and participated in tissue collection. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Identifying Neuropeptide and G Protein-Coupled Receptors of Juvenile Oriental River Prawn (*Macrobrachium nipponense*) in Response to Salinity Acclimation

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Neuropeptides and their G protein-coupled receptors (GPCRs) from the central nervous system regulate the physiological responses of crustaceans. However, in crustaceans, our knowledge regarding GPCR expression patterns and phylogeny is limited. Thus, the present study aimed to analyze the eyestalk transcriptome of the oriental river prawn *Macrobrachium nipponense* in response to salinity acclimation. We obtained 162,250 unigenes after *de novo* assembly, and 1,392 and 1,409 differentially expressed genes were identified in the eyestalk of prawns in response to low and high salinity, respectively. We used combinatorial bioinformatic analyses to identify *M. nipponense* genes encoding GPCRs and neuropeptides. The mRNA levels of seven neuropeptides and one GPCR were validated in prawns in response to salinity acclimation using quantitative real-time reverse transcription polymerase chain reaction. A total of 148 GPCR-encoding transcripts belonging to three classes were identified, including 77 encoding GPCR-A proteins, 52 encoding GPCR-B proteins, and 19 encoding other GPCRs. The results increase our understanding of molecular basis of neural signaling in *M. nipponense*, which will promote further research into salinity acclimation of this crustacean.

Keywords: ***Macrobrachium nipponense*, neuropeptides, salinity, GPCRs, eyestalk**

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INTRODUCTION

Crustacean culture provides high-quality food as well as huge economic benefits to farmers and the economy. Among them, the *Macrobrachium nipponense* is an economically important economic species in aquaculture, with a production of in excess of 250,000 tons and an output reaching 2 billion RMB per year in China (1). In the aquaculture industry, culturing seawater species for desalination and using freshwater crustacean species for saltwater acclimation are new trends (2). In the past two decades, large numbers of the genus *Macrobrachium* have invaded

freshwater habitats from the ancestral marine environment, and have exhibited high adaptability to slightly brackish and freshwater habitats (3–5). However, to date, few studies have investigated the mechanisms that regulate salinity adaptation in *M. nipponense*.

Salinity is an important environmental factor in estuarine and coastal systems, which affects the physiology of crustaceans and determines species distributions (6). There is a growing interest in improving prawn performance in aquaculture at low salinity. Previous studies have confirmed that a number of key neuropeptides participate in salinity stress responses of crustacean (7, 8). Neuropeptides mostly bind to G protein-coupled receptors (GPCRs) on the cell surface (9). GPCRs, as seven-pass integral membrane proteins, play key roles as transducers of extracellular signals across the lipid bilayer (10, 11), and act as salinity sensors in aquatic animal (12). Thus, the identification of neuropeptides and GPCRs represents an essential step to unraveling the roles of these molecules in response to salinity acclimation.

Rapid developments in RNA sequencing make it possible to use bioinformatics approaches to identify neuropeptides and their cognate GPCRs. Although neuropeptide sequences have been identified using *in silico* transcriptome analysis in many crustaceans (13–16), no information to date was provided to identify neuropeptides and GPCRs from eyestalk tissues of female *M. nipponense* during salinity acclimation, especially, knowledge of the GPCRs is limited in crustacean. In the present study, we aimed to perform gene expression profile analysis (control vs. low salinity group and control vs. high salinity group) to identify neuropeptides and GPCRs from eyestalk tissues of prawns responded to salinity stress. We also aimed to validate target transcripts encoding for neuropeptides and their cognate that might have important functions in *M. nipponense* salinity adaptation. The results will provide insights into salinity-mediated regulation of neuropeptide/GPCR signaling pathways in *M. nipponense*.

MATERIALS AND METHODS

Experimental Animals and Salinity Treatment

Juvenile *M. nipponense* specimens were obtained from a farm in Shanghai (Qingpu) and acclimated to laboratory conditions for 14 days in fresh water (temperature 22 ± 1°C, pH 7.7 ± 0.6, dissolved oxygen content 6.5 ± 0.5 mg/L). Thereafter, 360 healthy prawns (1.82 ± 0.46 g wet weight) were randomly and equally divided into 12 tanks (30 per tank), and the tanks were randomly assigned to three groups (three tanks per group). The salinity was gradually adjusted on the same day to reach the target salinity for each group: S0 = 0.4 (control group), S6 = 8 ± 0.2 (low salinity), S12 = 16 ± 0.2 (high salinity). Salinity and water quality were maintained as previously described (2), and the prawns provided with commercial feed (Zhejiang Tongwei Feed Group CO., Ltd) twice daily for 1 week at a ratio of 6–8% of their body weight.

Identification of Neuropeptides and Their Putative Cognate GPCRs

Total RNA extraction from nine prawns in each group, RNA-Seq library preparation and sequencing were carried out based on Illumina HiSeq™ 2500 paired-end sequencing technology, as previously described (17). Trinity was used to assemble a transcriptome data from eyestalk tissues and generated the unigenes. All unigenes were annotated based on the NCBI databases with a cut-off E value of 1.0×10^{-5}, Further, the BLAST2GO program was used for GO analysis (http://www.geneontology.org/), and Clusters of Orthologous Groups (COG) classification and signal pathway annotation of unigenes was performed by conducting BLASTx searches. EdgeR uses a negative binomial distribution method with pairwise test using Fisher for identified differentially expressed genes (DGE) between control and salinity treatment group. Subsequently, GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification of DEGs was carried out as previously described (17). The transcriptomic data (NCBI Sequence Read Archive: SRP251206) derived from eyestalk tissue were used to identify neuropeptides and receptors. To search for *M. nipponense* neuropeptides, the annotated sequences and the open reading frame (ORF) file were searched for keywords related to known neuropeptides and for conserved amino acid sequences, respectively (18, 19). Finally, the identified sequences were combined with a list of previously obtaining and characterized neuropeptides (**Supplementary Material 1**).

The Pfam-v27 module in CLC Genomics Workbench v9.5 (Qiagen, Hilden, Germany) was used to predict the structural domains in the GPCRs (intra/extracellular loops and seven transmembrane domains (7-TM). Bioinformatic analysis was also carried out on previously reported neuropeptide GPCRs from decapods (20, 21). Local BLAST was used to compare the GPCR sequences, followed by clustering analysis using BioLayout Express 3D (22) at an e-value cutoff of 1e-20. All GPCR sequences (those from our data and previously characterized receptors) were then combined into one list (**Supplementary Material 2**). Then, the GPCRs were multiply aligned using the CLUSTALW algorithm, imported into MEGA 7.0, and subjected to phylogenetic analysis (23, 24).

Quantitative Real-Time Reverse Transcription PCR

The identification and enrichment analysis of differentially expressed genes (DEGs) were performed according to our previously published methods (17). The cDNAs from salinity treatments of *M. nipponense* were synthesized from total DNA-free RNA (1 μg) using a Prime Script RT reagent kit (TaKaRa, Japan) following the manufacturer's instruction. The Bio-Rad iCycler iQ5 Real Time System (Biorad Inc., Berkeley, CA, USA) was used for qRT-PCR validation of DEGs expression, with the *Actb* gene as the internal control (25). The amplification efficiency and threshold were automatically generated by standard curves. The primer sequences are shown in **Supplementary Material 3**. The $2^{-\Delta\Delta CT}$

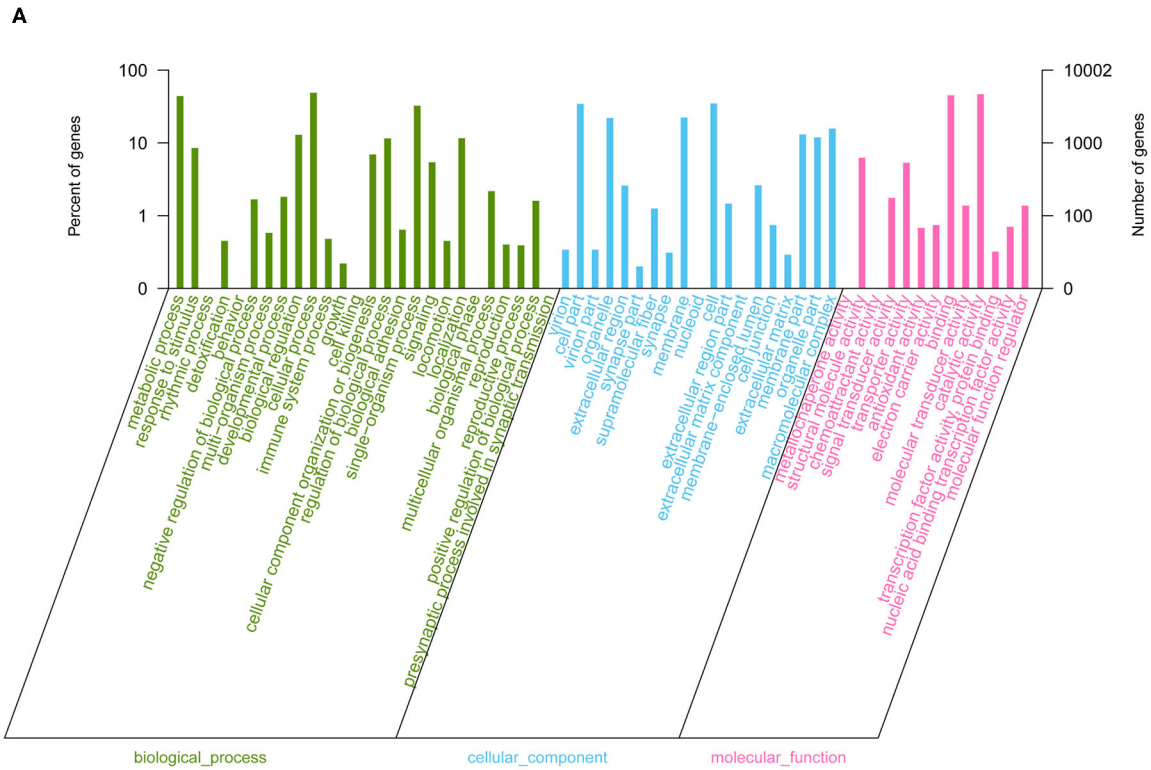

FIGURE 1 | The Gene Ontology **(A)** categorization and Clusters of Orthologous Groups **(B)** functional classification of assembled unigenes.

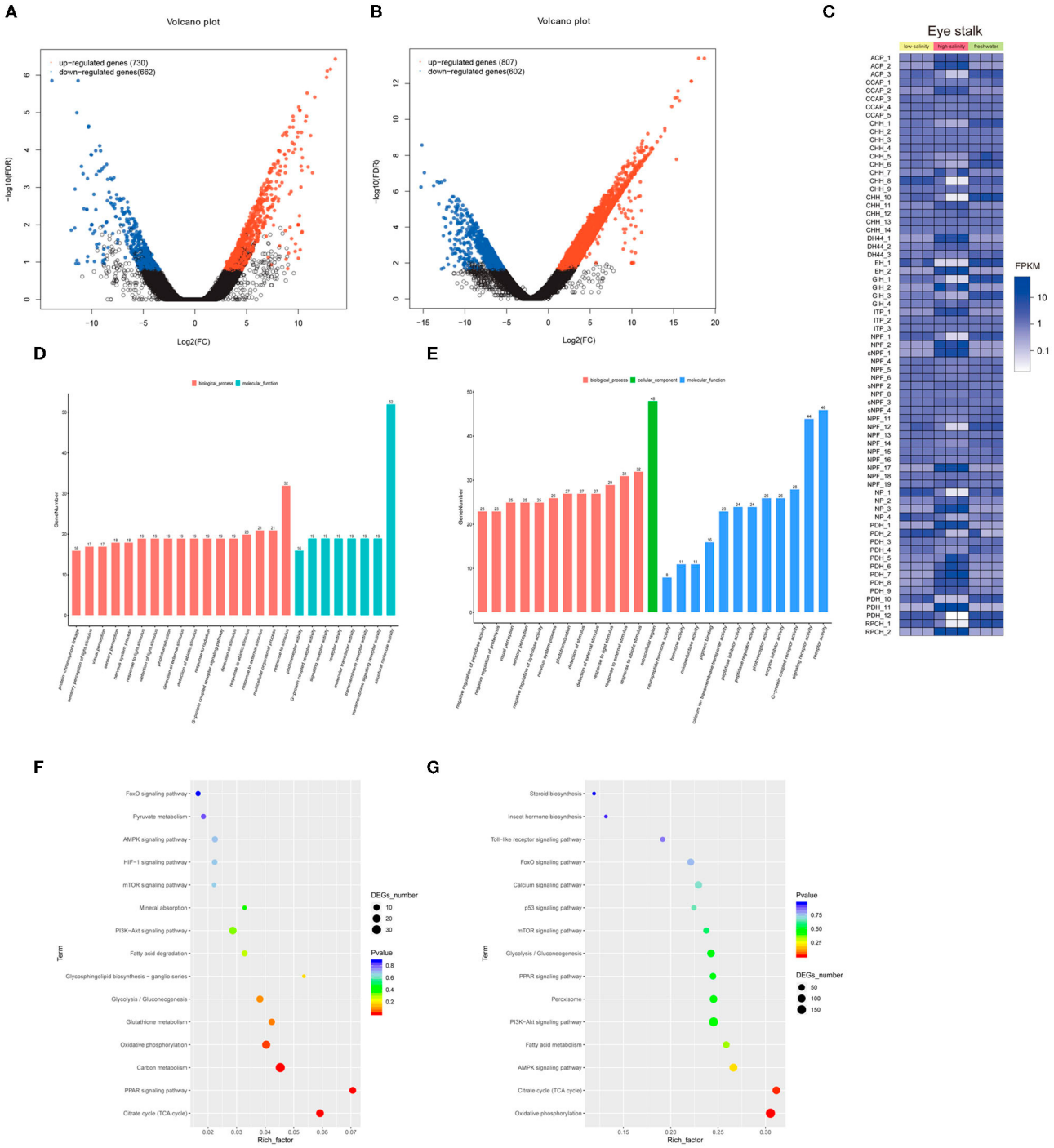

FIGURE 2 | Comparative transcriptome analysis explores neuropeptides from eyestalk in juvenile *M. nipponense* in response to salinity acclimation. Analysis of differentially expressed genes identified in the control vs. the low salinity group **(A)**, and the control vs. the high salinity group **(B)**. Notes: Upregulated and downregulated differentially expressed genes are shown in red and blue, respectively. The X- and Y-axes show the $\log_2$-fold change and the $\log_{10}$ *P*-value of the normalized expression level (Fragments Per Kilobase of transcript per Million mapped reads) of a gene between the two compared groups, respectively. Overview of putative neuropeptides identified in *M. nipponense.* **(C)**. Overview of identified differential expressed neuropeptides in *M. nipponense*. ACP, Adipokinetic hormone/Corazonin-related peptide; CCAP, Crustacean cardioactive peptide; CHH, Crustacean hyperglycemic hormone; EH, Eclosion hormone; GIH, Gonad inhibiting hormone; ITP, Ion transport protein; NP, Neuroparsin; NPF, Neuropeptide F; PDH, Pigment dispersing hormone; sNPF, short neuropeptide F. Top 25 GO classification analyses of DEGs in the control vs. the low salinity group **(D)**, the control vs. the high salinity group **(E)**. Top 15 significant KEGG pathway classifications of DEGs are shown for the control vs. the low salinity group **(F)**, the control vs. the high salinity group **(G)**.

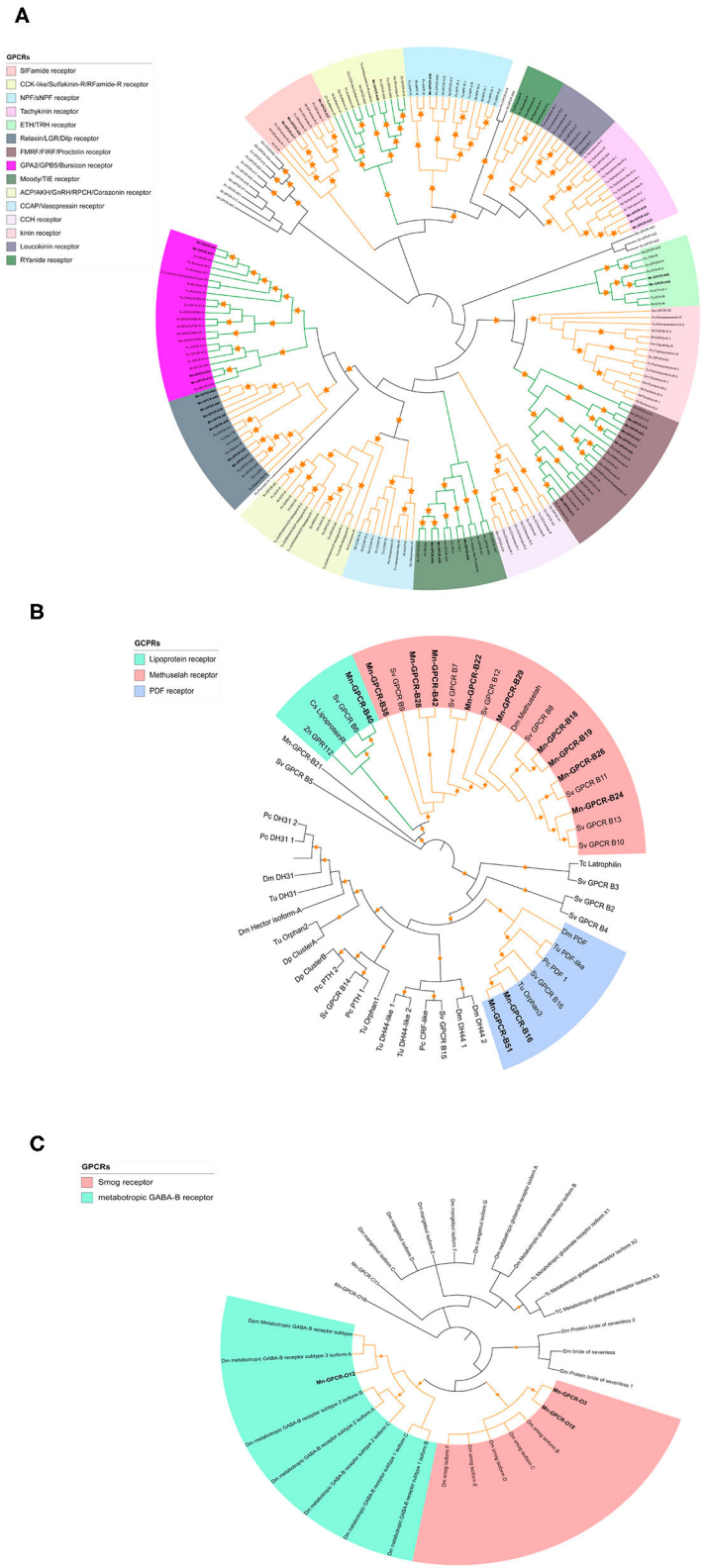

FIGURE 3 | Phylogenetic tree of Rhodopsin class GPCRs [GPCRs (GPCR-As, **(A)**], Secretin class GPCRs [(GPCR-Bs, **(B)**] and other GPCRs **(C)** of *M. nipponense* and other invertebrate species. Orange stars represent clades with a bootstrap value larger than 70. Green line: Clade annotated with high confidence. Yellow line: Clade annotated with low confidence. Red line: Unannotated clade. Bm, *Bombyx mori*; Dp, *Daphnia pulex*; Dm, *Drosophila melanogaster*; Nn, *Nephrops norvegicus*; Nl, *Nilaparvata lugens*; Pc, *Procambarus clarkii*; Sv, *Sagmariasus verreauxi*; Tu, *Tetranychus urticae*; Zn, *Zootermopsis nevadensis*; Tc, *Tribolium castaneum*.

comparative CT method (26) was used to calculate the relative transcript abundance.

RESULTS

Overview of the Transcriptomes

We generated nine eyestalk transcriptomes in prawns under the three experimental conditions in response to salinity acclimation, including freshwater, low salinity, and high salinity. Analysis using the BUSCO pipeline indicated that >92% of the arthropoda orthologs were present in the assembled transcriptome [Complete BUSCOs (C): 92.6%]. After removing adaptor sequences, ambiguous "N" nucleotides and low quality sequences, a total of 366,728,422 clean reads representing 54,659,786,418 clean nucleotides (nt) were shown in **Supplementary Material 4**. A total of 162,250 unigenes were obtained for the eyestalk transcriptome. In the GO analysis, 10,002 unigenes were enriched into 58 functional subgroups. Based on COG analysis, 8,755 of the unigenes were allocated to 25 COGs (**Figures 1A,B**).

DEGs Identification and Functional Analysis

We identified 1,392 and 1,409 genes that were differentially expressed under low salinity and high salinity, respectively (**Figures 2A,B**). The heat map of identified putative neuropeptide precursors and their RNA-seq FPKM expression levels, were compared between freshwater culture and salinity acclimation, such as CCAP, crustacean hyperglycemic hormone (CHH) and ion transport peptide (ITP), and so on (**Figure 2C**). The biological functions of the DEGs were determined using GO functional annotation (**Figures 2D,E**), which were significantly over-represented ($p < 0.05$, FDR < 0.01) as shown "G-protein

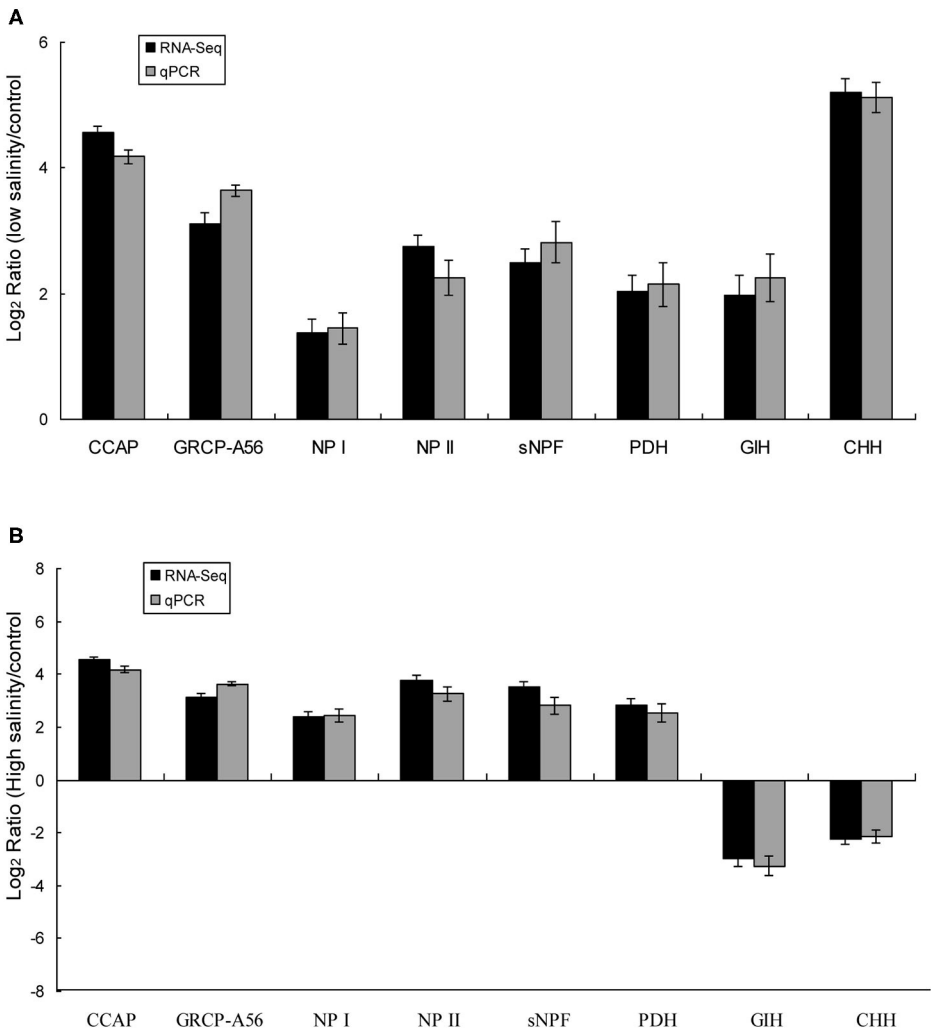

FIGURE 4 | Validation of the identified DEGs using qRT-PCR in the control vs. the low salinity group **(A)**, the control vs. the high salinity group **(B)**. The X-axis represents genes in the eyestalk tissue validated in this study; the Y-axis shows the Log_2 Ratio of expression in *M. nipponense* in response to salinity acclimatization. No significant differences were found between the qRT-PCR and Illumina data ($R = 0.95$).

coupled receptor signaling pathway" (GO:0007186), "response to external stimulus" (GO:0009605). In addition, KEGG pathway enrichment analysis identified the 15 most significant pathways ($Q < 0.05$) associated with salinity acclimation (**Figures 2F,G**), both including represented metabolism pathway "Glycolysis/Gluconeogenesis," "Citrate cycle," and "Fatty acid metabolism."

Bioinformatic Identification of Putative GPCRs

Clustering and phylogenetic analyses identified 223 putative GPCR genes based on the *de novo* nine transcriptome datasets. Phylogenetic analysis showed that 34 of the GPCRs could be classified as GPCR-A proteins (**Figure 3A**), which included receptors for red pigment concentrating hormone (RPCH), adipokinetic hormone-related neuropeptide/corazonin-related peptide (ACP), and CCAP. Forty-four of the putative GPCRs were classified as GPCR-B proteins (**Figure 3B**). Three putative GPCR families within the GPCR-B classification were identified using comparative phylogenetics with high-confidence, including the lipoprotein receptor, methuselah receptor, and pigment dispersing hormone (PDH) receptor. The third group comprised the remaining uncharacterized GPCR families (**Figure 3C**), for example the metabotropic GABA-B receptor and smog receptor.

Verification Neuropeptide Expression

Eight predicted significant DEGs encoding neuropeptides were identified, including those encoding isoforms of CCAP, CHH, short neuropeptide F (sNPF), PDH, gonad-inhibiting hormone (GIH), and neuroparsins (NP), as well as a CCAP receptor (GRCP-A56). The expression trends of the eight DEGs identified in eyestalk of prawns in response to salinity acclimation from the RNA-seq data were verified using RT-PCR (**Figures 4A,B**). The expression levels of the eight DEGs were significantly higher in the low salinity group compared with that in the control group. By contrast, two DEGs (encoding GIH and CHH) showed the opposite trend in the high salinity group compared with that in the control group. Additionally, DEGs encoding CCAP, GRCP-A56, sNPF, NP I, NP, II, and PDH were significantly upregulated in the high salinity group.

DISCUSSION

The assembled transcriptome contained sequences representing 52 different neuropeptide precursors, most of which are present in other crustacean species. Importantly, our study was the first to indicate that certain neuropeptides in prawns play an important role in response to salinity acclimation. Interestingly, some neuropeptide transcripts that were detected previously in other decapod crustacean species were not identified in this *M. nipponense* transcriptome, such as crustacean female sex hormone (CFSH) (27, 28). Notably, our previous *M. nipponense de novo* transcriptome assembly did include these neuropeptides, which partially disagrees with the results of the present study. A reasonable explanation is that differences in the identified neuropeptides were closely related to crustacean habitats (freshwater vs. estuary) and developmental stage (adult vs. larval). Data analysis predicted 148 different GPCRs, which is similar to the number predicted in *Chilo suppressalis* (29). A lack of close homologs of known function from related species made confident annotation of these GPCRs difficult. In addition, certain neuropeptide GPCRs identified previously in other arthropods (e.g., Crz, sulfakinin, and pyrokinin receptors) were not observed on the present phylogenetic analysis.

KEGG analysis identified energy metabolism pathways that were significantly affected by salinity, such as glycometabolism, which were similar to previous study in *Litopenaeus vannamei* (30), our further study will focus on the aspects of energy metabolism of prawns under salinity acclimation. Interestingly, GO functional annotation of the DEGs was associated with "G-protein coupled receptor signaling pathway" of prawns responded to salinity acclimation. Thus, we identified differentially expressed neuropeptides and GPCRs genes, which are plausibly related to salinity acclimation. The neuropeptides and their putative cognate receptors were analyzed using qRT-PCR. For example, CCAP is a C-terminal amidated non-apeptide hormone found in many crustacean species, such as blue crab (*Callinectes sapidus*) (31). In addition to its role in heartbeat regulation, direct evidence points to a role for CCAP in the regulation of homeostasis in *L. vannamei* (8), which was consistent with our results that CCAP and its receptor mRNA expression was upregulated under high- and low-salinity conditions in *M. nipponense*.

In agreement with the results of the present study, previous studies confirmed that salinity changes in crustaceans upregulated the transcript levels of peptide hormones (32, 33), such as CHH and ITP. The injection of purified CHH increased the Na+ concentration and osmolality in the hemolymph (34). Notably, crustacean CHHs showed high sequence homology to ITP (35). Our results indicated much higher levels of ITP transcripts in the high salinity and low salinity groups than in the control group, suggesting that ITP might function in ionic transport or osmo-regulation, or both, in prawns. GIH has an important function in crustacean ovarian maturation inhibition (36). The results of the present study showed that high salinity downregulated GIH expression. This indicated that salinity and gonadal development might correlate strongly in *M. nipponense*. Therefore, further study is required to gain a better understanding of the functions of these neuropeptides and their GPCRs associated with the effects of salinity on the prawn reproduction system.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SS and JL conceived, designed the experiments, and supervised the project. SS, MZ, FP, and JF carried

out the experiments and analyzed the data. SS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00623/full#supplementary-material

Supplementary Material 1 | Comparative list of neuropeptides detected in previous studies.

Supplementary Material 2 | All GPCRs sequences used in this study.

Supplementary Material 3 | The specific primers used for qRT-PCR.

Supplementary Material 4 | Summary of sequencing and functional annotation of unigenes.

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REVIEW
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The "IAG-Switch"—A Key Controlling Element in Decapod Crustacean Sex Differentiation

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The androgenic gland (AG)–a unique crustacean endocrine organ that secretes factors such as the insulin-like androgenic gland (IAG) hormone—is a key player in crustacean sex differentiation processes. IAG expression induces masculinization, while the absence of the AG or a deficiency in IAG expression results in feminization. Therefore, by virtue of its universal role as a master regulator of crustacean sexual development, the IAG hormone may be regarded as the sexual "IAG-switch." The switch functions within an endocrine axis governed by neuropeptides secreted from the eyestalks, and interacts downstream with specific insulin receptors at its target organs. In recent years, IAG hormones have been found—and sequenced—in dozens of decapod crustacean species, including crabs, prawns, crayfish and shrimps, bearing different types of reproductive strategies—from gonochorism, through hermaphroditism and intersexuality, to parthenogenesis. The IAG-switch has thus been the focus of efforts to manipulate sex developmental processes in crustaceans. Most sex manipulations were performed using AG ablation or knock-down of the *IAG* gene in males in order to sex reverse them into "neo-females," or using AG implantation/injecting AG extracts or cells into females to produce "neo-males." These manipulations have highlighted the striking crustacean sexual plasticity in different species and have permitted the manifestation of either maleness or femaleness without altering the genotype of the animals. Furthermore, these sex manipulations have not only facilitated fundamental studies of crustacean sexual mechanisms, but have also enabled the development of the first IAG-switch-based monosex population biotechnologies, primarily for aquaculture but also for pest control. Here, we review the crustacean IAG-switch, a unique crustacean endocrine mechanism, from the early discoveries of the AG and the IAG hormone to recent IAG-switch-based manipulations. Moreover, we discuss this unique early pancrustacean insulin-based sexual differentiation control mechanism in contrast to the extensively studied mechanisms in vertebrates, which are based on sex steroids.

Keywords: androgenic gland, IAG-switch, insulin-like androgenic gland hormone, monosex population, sex determination, sex differentiation, sexual plasticity

INTRODUCTION

To put the subject of this review into context, we start with a brief history of the discovery of the androgenic gland (AG) in crustaceans. In 1947, in the course of an anatomical/histological study of the male reproductive system in the blue swimming crab *Callinectes sapidus*, an "accessory" endocrine gland was found adjacent to the sperm duct (1). Some years later, this gland was termed the "androgenic gland" in light of its key role in crustacean masculine differentiation, as shown by functional experiments of AG ablation and implantation in the amphipod crustacean *Orchestia gammarella* (2). In later experiments, testis removal in males or implanting females with testicular tissues was found to be ineffective in causing sex reversal, thus suggesting that the vertebrate-like gonadal testosterone is probably not involved in crustacean masculine differentiation (3), and indeed, in 1964, it was first reported that the cells of the AG bore greater similarity to vertebrate protein-producing cells than to steroid-producing cells (4). Thereafter, some functional experiments involving the AG were performed not only by AG grafting but also by injections of AG extracts (5). However, it took a while until a specific AG hormone was first isolated from the terrestrial isopod *Armadillidium vulgare* (6, 7). In 2007, subsequent to the first transcriptomic identification of this hormone in a decapod—the redclaw crayfish *Cherax quadricarinatus*—further validation revealed the hormone structure to be that of an insulin-like peptide (ILP) family member, and the hormone was thus termed the "insulin-like androgenic gland" (IAG) hormone (8). It took about another 10 years before the first report appeared of the successful chemical synthesis of an IAG hormone—that of the giant freshwater prawn *Macrobrachium rosenbergii*, a commercially (9, 10) and environmentally (11) important species (12). Since its first discovery, the IAG hormone has been isolated and characterized in twenty-nine decapod species (**Table 1**), including prawns, shrimp, crayfish, lobsters and crabs, some of which are highly important for the aquaculture industry worldwide (13), and, as this review will show, the IAG-based sex differentiation mechanism is undoubtedly unique in the Pancrustacea, a diverse taxon that contains all crustaceans and hexapods.

TABLE 1 | IAG in decapod crustacean species.

Group	Family	Species	GenBank accession number
Prawn	Palaemonidae	*Macrobrachium rosenbergii*	FJ409645.1
		Macrobrachium nipponense	JX962354.1
		Macrobrachium vollenhovenii	KJ524578.1
		Macrobrachium lar	AB579012.1
		Palaemon paucidens	AB588013.1
		Palaemon pacificus	AB588014.1
Lobster	Palinuridae	*Sagmariasus verreauxi*	KF220491.1
		Jasus edwardsii	KF908794.1
Shrimp	Penaeidae	*Litopenaeus vannamei*	KX589057.1
		Fenneropenaeus chinensis	JQ388277.1
		Penaeus indicus	MG022137.1
		Litopenaeus occidentalis	KX589058.1
		Litopenaeus stylirostris	KX589059.1
		Marsupenaeus japonicus	AB598415.1
		Penaeus monodon	GU208677.1
	Pandalidae	*Pandalus platyceros*	KX619617.1
Crab	Varunidae	*Hemigrapsus sanguineus*	MH580760.1
		Eriocheir sinensis	KU724192.1
	Geryonidae	*Chaceon quinquedens*	KY497474.1
	Portunidae	*Portunus pelagicus*	HM459854.1
		Scylla paramamosain	JQ681748.1
		Callinectes sapidus	HM594945.1
		Portunus trituberculatus	MH119940.1
		Carcinus maenas	HM594946.1
Crayfish	Cambaridae	*Procambarus clarkii*	KT343750.1
		Procambarus virginalis	MF405195.1
		Procambarus fallax	KX619618.1
	Parastacidae	*Cherax quadricarinatus*	DQ851163.1
		Cherax destructor	EU718788.1

SEX DETERMINATION AND SEX DIFFERENTIATION IN CRUSTACEANS

In most organisms, sex is determined by chromosomes [i.e., genetic sex determination; GSD (14)] rather than by environmental factors [i.e., environmental sex determination; ESD (15)] (16). The most common GSD systems are the XX/XY and WZ/ZZ systems, in which females are homogametic and males are heterogametic in the former mode of inheritance, and vice versa in the latter (17). With some exceptions (18), most prawn, shrimp and crayfish species bear the WZ/ZZ sex determination system (13, 19–23), while some species of crabs and lobsters bear the XX/XY system (24–27). In the animal kingdom, there are only a few reports of sex-determining genes being associated with the W/Z sex chromosomes. Among them are the W-chromosome-associated *DM-W* gene, which is vital for ovarian development in the African clawed frog *Xenopus laevis* (28), and the Z-chromosome-linked *DMRT1* gene, whose dosage is assumed to control the sex determination process in the chicken *Gallus gallus domesticus* (29). In contrast to the sparse knowledge on the genes associated with the W/Z chromosomes (especially in crustaceans), the male sex-determining genes that are associated with the Y chromosome and that control masculinization in animals bearing the XX/XY system have been well-characterized. Among these genes, most mammals have the well-known *SRY* gene (30). Other examples include the DMY/Dmrt1bY gene, which is associated with the formation of the testis in the medaka fish (31), and the recently discovered *iDMY* gene, which is the male sex-determining factor during embryogenesis in the Eastern spiny lobster Sagmariasus verreauxi (25). To reveal the genetic content of the sex chromosomes, extensive karyotyping of different decapod crustacean species has been performed and published. However,

none of the available karyotypes can distinguish between the sex and autosomal chromosomes (32–38). Moreover, while genome sequencing using next generation techniques is common and genomes have been published for several decapod species (39–41), none, except that of *M. rosenbergii* (42), is a phased genome in which a certain scaffold could be attributed to a paternal or maternal origin. Therefore, verified sex-determining factors in decapod species, especially those with WZ/ZZ chromosomal content, are yet to be found.

For species in which sex is determined by sex chromosomes (43), the sex differentiation process starts with the expression of genes responsible for promoting masculinization or feminization during early developmental periods. Many such invertebrate genes have been well-studied, including the *mab-3* gene in the nematode *Caenorhabditis elegans* (44), the *transformer-2* (*tra-2*) gene in pancrustaceans (45), and the *doublesex* (*dsx*) gene in the fruit fly *Drosophila melanogaster*, which has alternative spliced variants yielding different sexes (46). Although the information on such sex differentiating genes in crustaceans remains limited, from the few studies that have been conducted, it is known that *dsx* is expressed in the branchiopod *Daphnia magna* (47), and the *dsx and mab-3 related transcription factor* (*DMRT*) is expressed in the testis of the decapod Eriocheir sinensis, the Chinese mitten crab (48). In *M. rosenbergii*, transcriptomic libraries obtained for different developmental stages—from the embryonic stage (49), through larvae and post-larvae, to adults (50, 51)—appear to contain homolog transcripts of the *dsx*, *tra-2,* and *DMRT* genes. Moreover, *IAG* silencing in *M. rosenbergii* resulted in significant decrease in the expression of two DMRTs and other sex related genes (52). However, the exact relationship of these genes to the sex differentiation mechanism—if such a relationship does indeed exist—has yet to be found.

Although in the next section we will describe the universal *IAG* gene as a master switch involved in crustacean sex differentiation, it is noteworthy that sex differentiation mechanisms in crustaceans are not only mediated by genes but also disrupted by external factors. For example, elevated bacterial dosage of *Wolbachia* reduces the functionality of insulin receptors in isopods which results in feminization (53). Additionally, some environmental pollutants serve as endocrine disrupting chemicals (EDCs) suggested to affect sex differentiation and sexual development in crustaceans (54). The latter concept was shown in various crustacean species from different orders. In daphnids, the exposure to DES, a synthetic estrogen, induced the development of secondary sexual characters like larger abdominal process in females of *D. magna*, while longer first antennae were observed in males exposed to the androgen androstenedione (55). Moreover, in *D. pulex*, exposure to methoprene, a juvenile hormone analog, yielded all-female broods, while gravid females exposed to 20-hydroxyecdysone, has resulted in all-male broods (56). In the amphipod *Gammarus pulex*, exposure to the xenoestrogen 17α-ethynylestradiol increased the females:males sex ratio (57) and in decapods, heavy metals such as cadmium and copper inhibited ovarian growth in the crabs *Uca pugilator* (58) and *Chasmagnathus granulata* (59), while the xenoestrogen 4-nonylphenol reduced testis weight in the crab *Carcinus maenas* (60). A correlation between endocrine disruptors and crustacean sex differentiation is also exemplified by EDCs discharge in polluted areas that increased the frequency of intersexuality in harpacticoid copepods where intersexuality is extremely rare (61), in amphipods (62) and in decapods (63). These findings raised major concerns regarding the impacts of pollutants on the reproductive success of many crustacean species.

THE IAG-SWITCH–A MASTER SEX CONTROLLING DEVICE IN CRUSTACEANS

As described above, in male crustaceans, the AG is a unique endocrine organ, secreting the IAG hormone, which serves as a master universal sex-differentiating switch abundant among crustaceans, thus termed the "IAG-switch" (13). A scheme describing the putative location of the IAG-switch from genotypic determination to sexual maturation in gonochoristic crustaceans is given in **Figure 1**. Residing within the eyestalk-AG-testis endocrine axis (64), the IAG-switch is controlled by upstream neuropeptides and interacts downstream with IAG receptors and binding proteins (51, 65–68). The neuropeptides that mediate growth and reproduction are produced in the X-organ (located in the eyestalk) and later accumulated in the adjacent sinus gland, from where they are secreted. It was found that eyestalk ablation in males caused hypertrophy and hyperplasia of the AG (64, 69) as well as over-expression of the IAG hormone (70) and of a membrane-anchored AG-specific factor (71). Therefore, it was suggested that some X-organ derived neuropeptides are upstream controlling elements of AG activity (64, 72, 73). Moreover, a reduction in the transcript levels of gonad-inhibiting hormone (GIH), molt-inhibiting hormone (MIH) and other eyestalk-derived neuropeptides (by using RNAi knock down) significantly increased IAG expression. On the basis of these findings, it was postulated that these neuropeptides exert an upstream function that controls AG activity (i.e., IAG secretion) (73). It has also been suggested that female molting factors, perceived by males via their short lateral antennules (functioning as olfactory organs), also contribute to the regulation of AG function and male gonadal maturation by increasing IAG expression, thus implying that the IAG-switch is also partially controlled by female reproductive activity (74).

Studying elements that are downstream to the AG within the insulin-like signaling pathway revealed several insulin receptors that interact with the IAG hormone. It was found that some receptors were neither sex specific nor tissue specific, but silencing their encoding genes resulted in AG hypertrophy and over-expression of the IAG hormone (51). In contrast, other receptors were male specific, and their knock down led to the arrest of most of the germ cells in the testes at the secondary spermatocyte stage (vs. those in the control group, which developed into sperm cells) (68). A study of the downstream signaling pathway of the IAG-switch also revealed an insulin-like binding protein (ILBP) that interacted with the IAG hormone, but whose expression was not AG specific (65). This finding indicated that the protein was perhaps synthesized in a location other than the AG, a

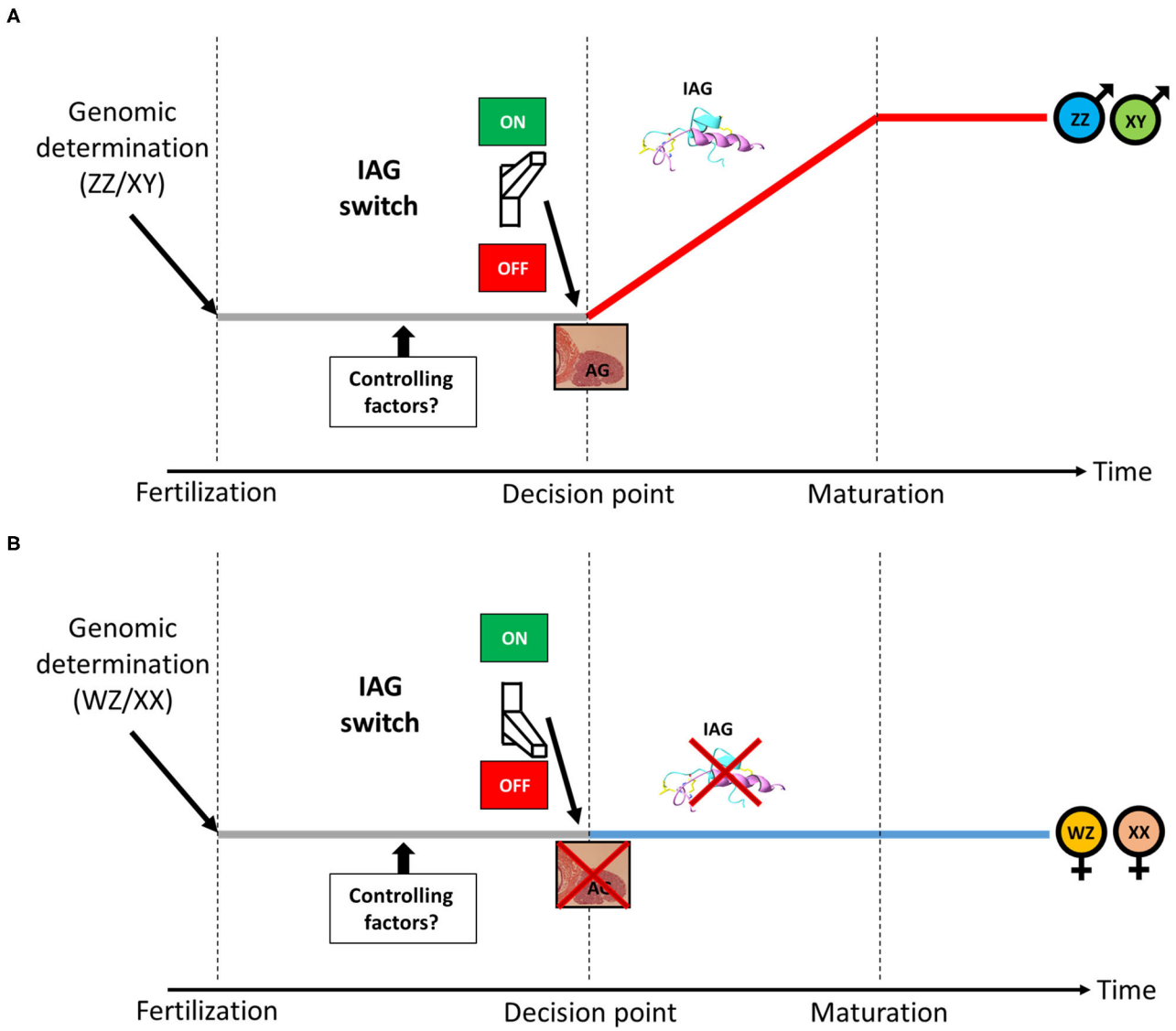

FIGURE 1 | The IAG-switch in gonochoristic crustaceans. **(A)** Following male genotypic determination (ZZ/XY), the IAG-switch initiates the formation of the IAG-secreting AG, leading to the development of a mature male. **(B)** Following female genotypic determination (WZ/XX), the IAG-switch inhibits the formation of the IAG-secreting AG, leading to the development of a mature female. The period in which putative upstream IAG-switch controlling factors are expressed is denoted.

premise later supported by a study showing that eight ILBPs characterized in a lobster were neither sex specific nor tissue specific (66). Nevertheless, while some studies have demonstrated that eyestalk-derived neuropeptides (e.g., GIH and MIH) are upstream elements to the IAG-switch and insulin receptors are downstream elements, to the best of our knowledge, specific ILBPs associated with the IAG-switch are still to be found.

The pivotal role of the IAG-switch in governing crustacean sexual differentiation has been under study ever since the discovery of the AG and is, in fact, best exemplified by functional experiments that were performed long before the discovery of the IAG. These studies are revisited here to exemplify the pivotal role of the IAG-switch and its universality among crustacean species (see also a summary of IAG-manipulation experiments performed to date in **Table 2**).

From an historical point of view, the first researcher to manipulate the IAG-switch, following the discovery of the AG in crustaceans (1), was Charniaux-Cotton (2). In her pioneering experiments on the amphipod *O. gammarellus,* she demonstrated that implantation of the AG into females induced the development of masculine characters and inhibited vitellogenesis, whereas the implantation of testicular tissue had no such effect (2, 3, 75). Later, studies on the terrestrial isopod *A. vulgare* showed that AG implantation (76) or injection of AG extract into females (5) induced partial masculinization,

TABLE 2 | Summary of experiments that included IAG-switch based manipulations in crustaceans.

Species	Order	Method	Results	References
Orchestia gammarellus	Amphipoda	AG implantation in females	Development of masculine characters and vitellogenesis inhibition	(2, 75)
Armadillidium vulgare	Isopoda	AG implantation in females	Transformation of gonads	(76)
Armadillidium vulgare	Isopoda	Injection of AG extracts into immature females	Transformation of gonads	(5)
Carcinus maenus	Decapoda	AG implantation in females	Development of male secondary characteristics	(77)
Lysmata seticaudata	Decapoda	AG implantation in females	Development of male secondary characteristics	(78)
Pandalus borealis	Decapoda	AG implantation in females	Development of male secondary characteristics	(79)
Rhithropanopeus harrisii	Decapoda	AG implantation in females	Development of male secondary characteristics	(80)
Palaemon varians	Decapoda	AG implantation in females	Development of male secondary characteristics	(81, 82)
Macrobrachium rosenbergii	Decapoda	AG ablation in males	Loss of masculine appendages and transformation of gonads	(83)
Macrobrachium rosenbergii	Decapoda	AG implantation in females	Development of masculine appendages and transformation of gonads	(84)
Macrobrachium rosenbergii	Decapoda	AG implantation in females	Full sex reversal of females to males	(20)
Macrobrachium rosenbergii	Decapoda	AG ablation in males	Full sex reversal of males to females	(85)
Macrobrachium rosenbergii	Decapoda	IAG knock-down using RNAi in males	Full sex reversal of males to females	(86)
Macrobrachium rosenbergii	Decapoda	AG cells transplantation in females	Full sex reversal of females to males	(22, 42)
Procambarus clarkii	Decapoda	AG implantation in females	Development of male secondary characteristics	(87)
Procambarus clarkii	Decapoda	AG implantation in females	Development of masculine characters and inhibition of vitellogenesis	(88)
Eriocheir japonicus	Decapoda	AG implantation in females	Development of masculine appendages	(89)
Cherax destructor	Decapoda	Injection of AG extracts into females	Development of male gonopores and inhibition of vitellogenesis	(90)
Cherax quadricarinatus	Decapoda	AG implantation in females	Development of masculine characters and inhibition of vitellogenesis	(91)
Cherax quadricarinatus	Decapoda	AG ablation in male-intersexuals	Loss of male secondary characteristics and induction of vitellogenesis	(92)
Cherax quadricarinatus	Decapoda	AG ablation in male-intersexuals	Loss of mating behavior with females and fighting behavior with males	(93)
Cherax quadricarinatus	Decapoda	IAG knock-down using RNAi in male-intersexuals	Vitellogenesis induction	(94)
Scylla paramamosain	Decapoda	AG implantation in females	Ovarian regression	(95)
Eriocheir sinensis	Decapoda	Injection of AG extracts from *S. paramamosain* and *E. sinensis* into females	Development of male gonopods	(96)
Procambarus virginalis	Decapoda	AG implantation from *P. clarkii*	Development of male secondary characteristics	(97)
Litopenaeus vannamei	Decapoda	AG ablation in males	Loss of masculine appendages and degradation of spermatids in the gonads	(98)
Litopenaeus vannamei	Decapoda	AG implantation in females	Partial development of male secondary characteristics	(99)

including the transformation of female reproductive organs into testes, sperm ducts and seminal vesicles.

Most IAG-switch manipulation experiments were performed in decapod crustaceans, including shrimp, prawns, crayfish and crabs (100). Among the earliest of such experiments in decapods were those performed on hermaphrodite species (100, 101); for example, AG implantation into females of the simultaneous hermaphrodite, the Monaco shrimp *Lysmata seticaudata* (78), and females of the sequential protandric Northern shrimp *Pandalus borealis* (79) resulted in the development of male secondary characteristics. In gonochoristic species, AG implantation caused episodic development of external male characteristics, as in the green shore crab Carcinus maenus (77) and the Harris mud crab Rhithropanopeus harrisii (80). In the Japanese mitten crab E. japonicus, AG-implanted females developed masculine appendages, even though all of them retained their oviducts (89), but in the mud crab Scylla paramamosain, ovarian regression occurred in female crabs implanted with AG (95). IAG-switch manipulations in crabs also suggested interspecies cross-activity of AG factors, as injection of an AG extract from S. paramamosain or E. sinensis males into E. sinensis females resulted in the development of male gonopods (96).

Many of the studies on the IAG-switch have been conducted in different species of crayfish. In C. quadricarinatus, implantation of hypertrophied AGs into females resulted in the development of masculine secondary sex characters, such as the typical red patch on the chela, and in a significant reduction in the gonadosomatic index and impairment of vitellogenesis (91). Particularly revealing studies were those conducted on intersex C. quadricarinatus animals. In this species, some animals in the population naturally exhibit intersexuality, in which individuals function as males and exhibit male secondary characters, but bear a mix of male and female gonopores and gonads (active testes on one side and a pre-vitellogenic ovary on the other) (102). In AG-ablated C. quadricarinatus intersexuals, there was a partial shift toward femaleness in that external male characters were not regenerated, but expression of the vitellogenin gene was induced (92). Moreover, examination of the agonistic and mating behavior of AG-ablated C. quadricarinatus intersexuals revealed that these animals did not exhibit typical mating behavior when exposed to females or the usual fighting behavior when confronted with males (93). Knocking down the IAG gene—by using RNAi through injection of dsCq-IAG—to C. quadricarinatus intersexuals caused elevation of vitellogenin expression to a level that did not differ from that in intact vitellogenic females (94). In a related species, the common yabby, C. destructor, a significant number of females (vs. a control group) injected with AG extract developed male gonopores at the base of the fifth pereiopod and showed inhibition of vitellogenesis (90). In some early experiments carried out in the red swamp crayfish *Procambarus clarkii*, implantation of AGs into females led to partial masculinization, as shown by the partial transformation of the first pair of pleopods into typical male gonopods (87) and by the inhibition of vitellogenesis in mature females (88). One of the most peculiar IAG-switch-based manipulations was performed on a crayfish species in which males do not exist—the marbled crayfish *P. virginalis*. This parthenogenetic species, in which a virginal form of reproduction gives rise to identical clones of all-female progeny (103, 104), nonetheless expresses the *IAG* gene (105). It was thus suggested that *P. virginalis* is a virginal form that diverged from the gonochoristic slough crayfish *P. fallax* (106), a premise supported by a report that both species retain a highly similar sequence of the *IAG* gene (105). Interestingly, implantation of *P. virginalis* females with AGs from *P. clarkii*, a related species, resulted in the appearance of masculine external characteristics, such as thickening of the first and second pairs of pleopods and the formation of reversed spines on the third and fourth pairs of pleopods (97). The above findings indicate not only that cross-activity of AG factors occurs between species, but surprisingly that in crustacean species, even those in which males do not exist, female animals are still susceptible to the effects of IAG-switch-based manipulations.

Studies on IAG manipulation in species of Palaemonidae have been reported from 1979/80 onwards. Among these, transplantation of AG grafts induced the development of external masculine characteristics in the common ditch shrimp *Palaemon varians* (81, 82), and the first partial sex reversal using IAG-switch manipulation that was undertaken in *M. rosenbergii*, the most extensively studied palaemonid. In the latter study, most andrectomized males did not regenerate the appendix masculina (AM), and a reduction of spermatogenic lobules in the testes was observed, while some AG-ablated males showed the development of female gonopores and oviducts and even the initiation of oogenesis (83). In a complementary study, it was shown that AG-implanted females developed masculine characters, including AMs and sperm ducts, and in some cases spermatogenesis was initiated in the gonads (84). That study also served to highlight the IAG-switch as a pivotal sex differentiating mechanism in crustaceans, since females implanted with sperm duct or testicular tissue were not masculinized, while ∼80% of AG-implanted females showed some degree of masculinization (84). In another important aquaculture species, the penaeid Pacific white shrimp *Litopenaeus vannamei*, AG ablation of males in various post-larval stages resulted in inferior development of the AMs and degradation of the spermatids in the gonads (98), while AG implanted females did not develop AMs and only the minority developed male-like claspers on the first endopods (99). However, we note that complete and functional sex reversal in this important species has not yet been achieved, despite the extensive attempts of various research groups around the world.

While the above studies have indeed demonstrated the crucial role of the IAG-switch in sexual differentiation in several orders and many species in the Crustacea, all the above-described cases of IAG-switch manipulations yielded various types of partial sex shifts but not fully functional sex reversal of one sex into the other. As shown in **Figure 1**, we assume that a putative decision point exists, in which an individual commences toward sexual maturation as a male or a female. It is hypothesized that IAG-switch controlling factors are accommodating this decision point. Functional experiments manipulating such factors will open a new window into IAG-switch upstream controlling mechanism and might achieve a complete shift between sexes. In order to find such IAG-switch controlling factors, advanced next generation sequencing (NGS) techniques may be employed to sequence the RNA of males and females at early developmental stages whose investigation might yield sexually biased genes that putatively control the IAG-switch. Those genes could be manipulated by knockdown techniques such as RNAi (107) or Morpholino oligos (108) and, if performed before the decision point, might lead to a functional shift between sexes. However, timing the decision point is species-specific and body size of the animal in such early developmental stage might be very small which makes the RNAi/Morpholino manipulation complicated. To overcome the size obstacle, a whole genome sequencing of the animal using latest NGS platforms could be used followed by CRISPR-Cas9 genome editing operations (109) that could be performed at the embryonic level and guarantee that the manipulation occurs before the decision point. To the best of our knowledge, CRISPR editing of IAG-switch related factors was never performed. In the next section, successful IAG-switch manipulation resulting with full sex reversal will be described.

IAG-SWITCH BASED BIOTECHNOLOGIES FOR PRODUCING MONOSEX POPULATIONS

The use of monosex populations is common in animal husbandry, since in many species males and females yield different agricultural products, particularly fish (110–112), poultry (113–115), and mammals (116). In crustaceans, monosex populations offer particular advantages in aquaculture (85, 117–119), since most decapod species exhibit dimorphic growth patterns, leading to variations in animal size at harvest. The dimorphic growth patterns, in turn, could be a result of different growth rates and behavioral patterns (120) and different food conversion ratio (FCR) values between the sexes (121, 122), or even cannibalism (96). Monosex populations of crustaceans can also be exploited in ecological applications, e.g., monosex prawn populations could be used as bio-control agents, serving as predators of the snails that damage rice crops (123) and that are vectors of parasites hazardous to humans (11, 124–127) and fish (128). Here, we should remember that introducing new species as bio-control agents into a given niche may result in devastating consequences to the ecosystem (129), and therefore monosex populations are preferable as biocontrol agents, since they are not able to reproduce and thus become invasive species.

In the exploitation of monosex aquaculture for yield improvement in crustacean species, the choice of sex will generally be guided by the optimal growth rates and size at harvest. Therefore, all-male aquaculture was proposed for species exhibiting male superiority, as is the case for most crayfish (130–132), lobsters (133), prawns (117, 120, 134) and crabs (135), while all-female aquaculture was suggested for shrimp species in which females are larger than males (118, 136). However, growth rates and size are not the only considerations in the choice of sex for monosex cultures; an additional consideration is the desired product: For example, for the edible female gonads of *E. sinensis* (137), the harvested animals would be vitellogenic females with developed ovaries, even if their body size is smaller than males. Additionally, even in some species in which males are larger than females, such as *M. rosenbergii* prawns (85), monosex female culture could improve the yield and profit in two possible ways: intensification of stocking densities permitted by the lack of aggressiveness of the females (138), and elimination of the need for costly size-selective harvests by virtue of the size uniformity of females (117, 139–141).

Traditionally, monosex aquaculture is achieved through manual sorting (117, 142), which is both time consuming and labor intensive and does not guarantee a 100% monosex population. Agro-biotechnologies are thus needed to replace this traditional method. To date, efforts to establish either all-male or all-female populations, for both WZ/ZZ and XX/XY sex heritability schemes, start with an initial sex reversal step of male to a female or vice versa, based on manipulating the IAG-switch during the sex differentiation process (see schemes in **Figure 2** which represent the methodology to achieve all-male and all-female populations in both WZ/ZZ and XX/XY systems). However, all the IAG-switch manipulations performed to date have resulted only in partial sexual shifts, with the exception of the fully functional sex reversal in the decapod species *M. rosenbergii* (20, 22, 42, 85, 86). All-female progenies of *M. rosenbergii* were achieved in the following way: Implantation of AGs in juvenile WZ females resulted in sex reversal to WZ "neo-males." When these neo-males were crossed with normal WZ females, a quarter of the progeny comprised viable WW females. Crossing of the WW females with normal ZZ males produced a monosex WZ female population (20) (**Figure 3A**). In contrast, to produce a monosex ZZ male population, the first step was AG ablation of juvenile ZZ males, which sex reversed them into ZZ "neo-females." Crossing these neo-females with normal ZZ males produced a monosex ZZ male population (85) (**Figure 3B**). Even though a single sex reversed animal may yield several monosex progenies of thousands of prawns, the complicated surgical procedure of AG ablation/implantation resulted in high mortality and low rates of fully sex reversed animals (20, 85), and the above sex reversal schemes were therefore not suitable for scaling up toward commercialization. The break-through was made with the development of the first RNAi-based biotechnology for *M. rosenbergii* monosex aquaculture, which relied on knock down of the *IAG* gene through a single injection of ds*Mr-IAG* into ZZ males at an early post-larval stage. This biotechnology successfully enabled mass production of ZZ neo-females and consequently of all-male aquaculture (86) (**Figure 3B**). This procedure has been commercialized and has already yielded several consecutive generations of W-free ZZ prawns (143).

For generating all-female populations, the complicated procedure of AG implantation into females was replaced with a single injection of AG cell suspension into WZ females at an early post-larval stage; this yielded WZ neo-males and subsequently WW females and all-female aquaculture (22) (**Figure 3A**). This biotechnology was later improved by using the same procedure of AG cell transplantation into WW females that were then sex reversed into WW neo-males. Crossing of the WW neo-males with WW females yielded all-female WW progenies, thereby making the production of all-female producing females much more efficient (42). We note that when this procedure was performed repeatedly, it, too, yielded consecutive generations of Z-free WW prawns (144).

The above studies on *M. rosenbergii* yielded both males and females of every possible genotype (ZZ, WZ and WW). This sexual plasticity further highlights the pivotal role of the IAG-switch in crustacean sexual differentiation, since even after the genotype is determined, manipulating the IAG-switch may alter the initial direction of sexual development toward maleness or femaleness, with a probable complete autosomal sex-differentiation toolkit for each gender, regardless of the presence or absence of either sex chromosome.

WHY INSULIN-LIKE?

The above review of the pivotal involvement of an ILP as a major factor in crustacean sex differentiation demands a broader evolutionary discussion of ILPs in the context of sex

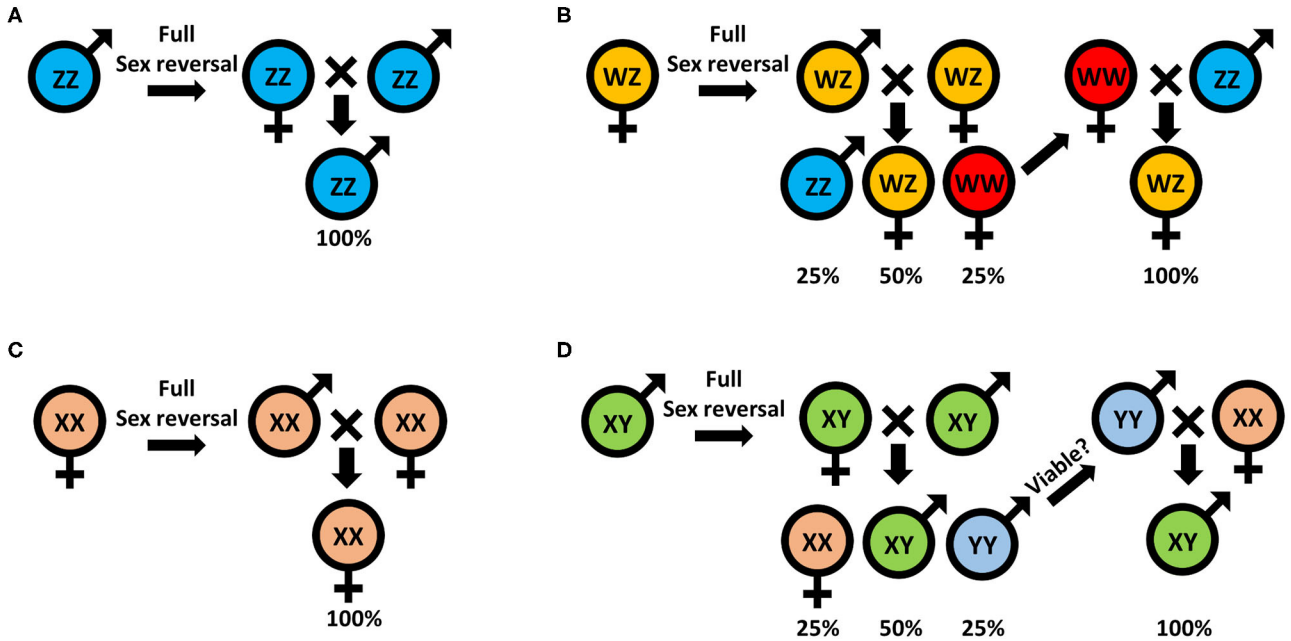

FIGURE 2 | Sex manipulation schemes for monosex populations: **(A)** all-male and **(B)** all-female in animals with the WZ/ZZ genotypic mode of inheritance; and **(C)** all-female and **(D)** all-male in animals with XX/XY genotypic mode of inheritance.

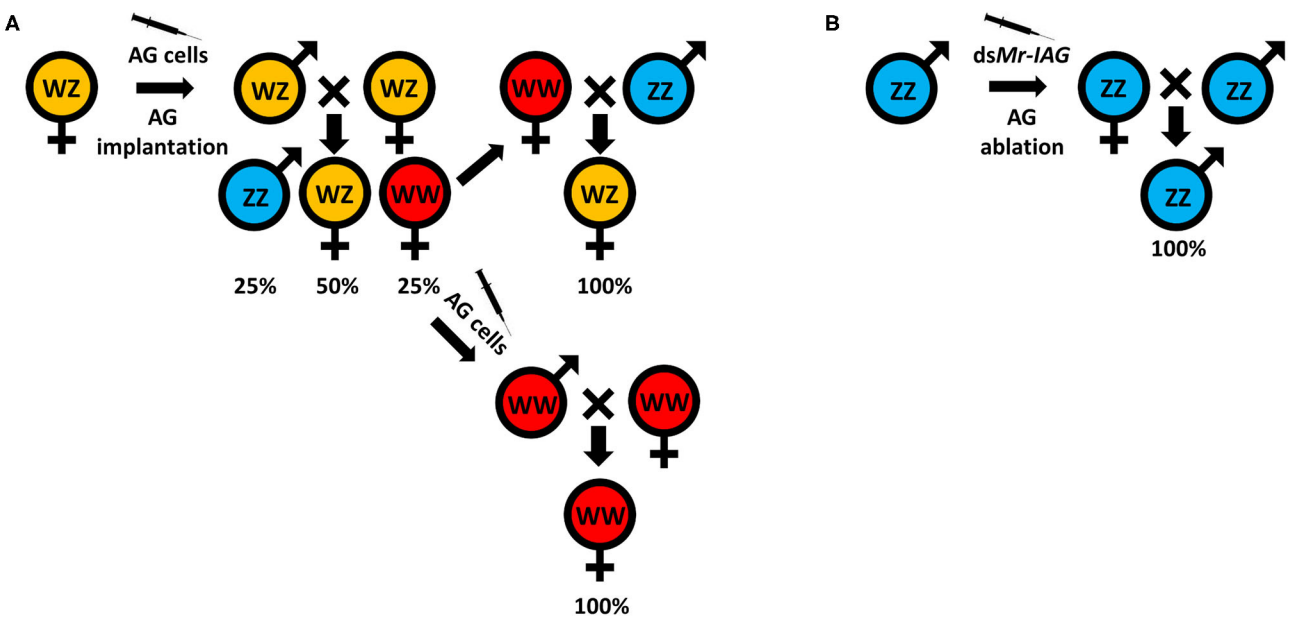

FIGURE 3 | Successful sex manipulations in *M. rosenbergii*. **(A)** A WZ female implanted with an AG (20) or injected with an AG cell suspension (22) inverted into a WZ "neo-male." The progeny, when crossed with normal WZ females, yielded 25% of WW females. These WW females can be crossed with normal ZZ males to produce all-female WZ populations, or they can be injected with an AG cell suspension (42) to produce WW neo-males that, when crossed with WW females, will give rise to all-female WW populations. **(B)** ZZ males that are AG ablated (85) or injected with ds*Mr-IAG* (86) inverted into ZZ "neo-females" that, when crossed with normal ZZ males, give rise to all-male ZZ populations.

regulation and reproduction in the animal kingdom, in which certain aspects of sexual differentiation are largely controlled by vertebrate-like sex steroids rather than ILPs. Indeed, similar to the IAG hormone, sex steroids (including androgens, estrogens and progestogens) mediate sexual development, secondary sex characters and dimorphic male/female physiological and

behavioral patterns (145, 146) in most vertebrates, including mammals, birds, reptiles, amphibians and fish (147). In aquaculture, this role for sex steroids finds application in the production of monosex fish populations by sex reversal of females into males or vice versa through 17α-methyltestosterone or estradiol-17β administration, as applicable (148–150).

An evolutionary survey of the animal kingdom reveals that vertebrate-type sex steroids (mostly estrogen, androgen and progestogen) and their related receptors and binding proteins are also found in aquatic invertebrates, including different classes of mollusks (151) [such as gastropods (152, 153), bivalves (154, 155), and cephalopods (156–158)] and echinoderms (159) [such as starfish (160) and sea urchins (161)]. Moreover, sex steroids are also found in flatworms (162), annelids (163), crustaceans (164), and cnidarians, such as corals (165). Their function in aquatic invertebrates is believed to be associated with reproduction (but not necessarily with sex differentiation) through the control of the levels of noradrenaline and dopamine, gonadic serotonin and catecholamine, and even cell metabolism and immunity (151). Moreover, as is the case for vertebrates (166), in invertebrates sex steroids are involved in growth processes. In crustaceans, and other arthropods, such growth processes depend on a periodic molt cycle in which the animal sheds its old extracellular cuticle and forms a new, larger cuticle (167). In crustaceans, steroid hormones (ecdysteroids) play a major role in molting and other developmental processes that are regulated by neuropeptides, such as MIH secreted from the Y-organ (168–170). Additionally, in some crustaceans, reproduction is linked to a pre-mating molt, which suggests some sort of coordination between molt-controlling steroid agents and gonad maturation (171). However, while the involvement of steroids in growth processes of crustaceans and other arthropods is clear, their function in controlling reproduction in crustaceans has been called into question. Nevertheless, it has been reported that vertebrate-like sex steroids could be involved in crustacean reproduction, as, for example, administration of progesterone induced ovarian maturation and spawning in the shrimp *Metapenaeus ensis* (172) and vitellogenesis in the shrimp *Penaeus japonicus* (173), while estradiol treatments promoted vitellogenesis in the crab *Portunus trituberculatus* (174). In contrast, administration of testosterone to female *Ocypoda platytarsis* crabs resulted in masculinization of the ovaries (175), and when administered to male *Parapenaeopsis hardwickii* shrimp, it even caused hypertrophy and hyperplasia of the AG (176). Moreover, administration of estradiol to entire populations of the freshwater amphipod *G. pulex* and of the decapod *L. vannamei* resulted in a clear female bias (57, 177). However, to the best of our knowledge, there are no reports of a fully functional sex reversal in crustaceans following the administration of vertebrate-like sex steroids, which implies that a different factor might be the main regulator in crustacean sexual differentiation. As described above, it is likely that an ILP, namely, the IAG hormone, is such a controlling element. Nonetheless, questions regarding the evolvement of an insulin-like factor, rather than a steroid, as the master sex controlling switch in crustaceans, and possible interactions between ILPs and steroids remain open.

As a step toward addressing these questions, let us examine the insulin superfamily. This group of proteins includes ILPs with a typical proteomic structure of B and A chains linked by disulfide bonds (178). Peptides of the insulin family are found in protozoans (179) and metazoans—both vertebrates and invertebrates (180). ILPs were first discovered in mammals and attracted extensive interest due to their involvement in many physiological processes (181). In vertebrates, they comprise a set of proteins including insulin, insulin-like growth factors (IGFs) and relaxins, which are essential in reproduction, growth, and developmental and metabolic pathways, such as carbohydrate and lipid metabolism (182–185).

In invertebrates, the first ILP was found in the clam *Mya arenaria* (186), and since then such proteins have been found in many species across different classes, including mollusks (187), annelids (188), flatworms (189), cnidarians (190), sponges (191), nematodes (192) and arthropods (193). A regulatory interaction between ILPs and steroids has indeed been found in insects in which the prothoracicotropic hormone (PTTH), a brain neuropeptide, controls the secretion of the ecdysteroids that regulate molting (194). Bombyxin, such a PTTH found in the silkworm *Bombyx mori* was found to be homologous to insulin (195). In addition to its affect on growth (196) and cell proliferation (197) in lepidopterans, bombyxin is also involved in ovarian development in dipterans (198). ILPs are also found in orthopterans, such as the migratory locust *Locusta migratoria*, in which a single copy of an ILP is expressed as two transcripts; one serving as a putative neurohormone is expressed in the brain, and the other serving as a putative growth factor is not tissue specific (199). In addition to their structural resemblance at the protein level, the conservation of invertebrate ILPs within vertebrates is best exemplified by the fact that an insulin-like protein extracted from the common fruit fly, *D. melanogaster*, showed cross reactivity between species by initiating insulin bioactivity in mice (200), while mammalian insulin was successful in activating *D. melanogaster* insulin receptors (201). Moreover, injection of recombinant human insulin into the shrimp *L. vannamei* led to increased levels of glucose in the hemolymph and of glycogen in the gills, thus suggesting that ILPs play a role in crustacean carbohydrate metabolism (202). Crustaceans are also known to possess ILPs that serve as growth factors (65) and some that regulate glucose metabolism and participate in the immune response against pathogens (203). However, while ILPs are generally not regarded as sex specific, crustaceans constitute a unique group in which a male-specific ILP (the IAG hormone) is the master factor in regulating sexual differentiation (8). This function of an ILP raises questions regarding the speciation of insulins into a variety of different physiological pathways during evolution, especially during the shift from invertebrates to vertebrates. However, while crustacean ILPs and vertebrate-like steroids share a physiological function as growth factors, the evolutionary processes regarding the alteration of the major sex differentiating mechanism from ILPs in early pancrustaceans (i.e., the crustacean IAG-switch) to sex steroids in vertebrates are still unknown.

CONCLUSIONS

The process of crustacean sexual development from the genotypic sex determination (WZ, ZZ, XX or XY), through the sexual differentiation process, to the final masculine or feminine maturation involves various sex controlling mechanisms that include factors such as ILPs or steroids. The IAG-switch is a unique crustacean endocrine-controlling mechanism involving an ILP that regulates sexual differentiation and function within the eyestalk-AG-testis endocrine axis. Despite earlier determination of the sexual genotype, the switch can be manipulated to induce either masculinization or feminization, thereby revealing striking sexual plasticity in crustaceans. It is this sexual plasticity that is often being exploited for sex manipulations for the establishment of monosex populations.

During the evolution of ILPs in the animal kingdom, numerous functions have evolved for such proteins in both invertebrates and vertebrates. A unique ILP function that evolved in the Crustacea is the IAG-switch mechanism, which constitutes the pivotal element in the sex differentiation processes. Nevertheless, to reveal the evolutionary pathways of sex differentiating controllers, i.e., ILPs in crustaceans and sex steroids in vertebrates, further evolutionary studies focusing on sex differentiation during the evolution of arthropods and the shift from invertebrates to vertebrates are required.

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REVIEW
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The Crustacean Hyperglycemic Hormone Superfamily: Progress Made in the Past Decade

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Early studies recognizing the importance of the decapod eyestalk in the endocrine regulation of crustacean physiology—molting, metabolism, reproduction, osmotic balance, etc.—helped found the field of crustacean endocrinology. Characterization of putative factors in the eyestalk using distinct functional bioassays ultimately led to the discovery of a group of structurally related and functionally diverse neuropeptides, crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) or vitellogenesis-inhibiting hormone (VIH), and mandibular organ-inhibiting hormone (MOIH). These peptides, along with the first insect member (ion transport peptide, ITP), constitute the original arthropod members of the crustacean hyperglycemic hormone (CHH) superfamily. The presence of genes encoding the CHH-superfamily peptides across representative ecdysozoan taxa has been established. The objective of this review is to, aside from providing a general framework, highlight the progress made during the past decade or so. The progress includes the widespread identification of the CHH-superfamily peptides, in particular in non-crustaceans, which has reshaped the phylogenetic profile of the superfamily. Novel functions have been attributed to some of the newly identified members, providing exceptional opportunities for understanding the structure-function relationships of these peptides. Functional studies are challenging, especially for the peptides of crustacean and insect species, where they are widely expressed in various tissues and usually pleiotropic. Progress has been made in deciphering the roles of CHH, ITP, and their alternatively spliced counterparts (CHH-L, ITP-L) in the regulation of metabolism and ionic/osmotic hemostasis under (eco) physiological, developmental, or pathological contexts, and of MIH in the stimulation of ovarian maturation, which implicates it as a regulator for coordinating growth (molt) and reproduction. In addition, experimental elucidation of the steric structure and structure-function relationships have given better understanding of the structural basis of the functional diversification and overlapping among these peptides. Finally, an important finding was the first-ever identification of the receptors for this superfamily of peptides, specifically the receptors for ITPs of the silkworm, which will surely give great impetus to

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the functional study of these peptides for years to come. Studies regarding recent progress are presented and synthesized, and prospective developments remarked upon.

Keywords: crustacean hyperglycemic hormone superfamily, structure diversity and evolution, biological functions, peptide structure, signaling pathway and receptor, Ecdysozoa

INTRODUCTION

It is generally considered that crustacean endocrinology began in the 1920s when a series of studies were published (1–4) confirming that color changes in crustaceans are under hormonal control, although some earlier observations (5–7) had already revealed evidence of endocrine regulation in molting, coloration, and secondary sex characteristics [(8); see (9) for a detailed historical account]. The following years witnessed significant developments indicating that many other physiological processes are also under hormonal regulation by eyestalk-derived factors. Thus, inferences made mainly through ablation and replacement experiments confirmed the presence in the eyestalk of a wide array of presumptive hormones. Physiological processes suggested to be regulated by the eyestalk hormonal factors included, in addition to migration of chromatophoric and retinal pigments, carbohydrate metabolism, reproduction, molting and growth, osmotic and ionic balance, cardiac activity (10). A number of studies (11–16) helped determine the source of these hormonal substances in the eyestalk, the X-organ-sinus gland (XO-SG) complex. The hormonal factors are stored in the sinus gland (11, 17), a neurohemal organ (18), which lies next to a blood sinus (hence the name), into which the hormones are released upon stimulation. The sinus gland is a bulbous cluster of neurosecretory axonal terminals, of which the majority of the axonal input originates from the cell bodies of a cluster of neurosecretory cells (the medulla terminalis X-organ) where the hormones are synthesized [see (18)].

Strikingly, most of the presumptive factors have since been biochemically purified and characterized by bioassays, as functionally defined from the earlier studies. These hormones include a group of sequence-related and functionally diverse neuropeptides—crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) or vitellogenesis-inhibiting hormone (VIH) (this hormone will be referred to as GIH in this review), and mandibular organ-inhibiting hormone (MOIH) that once constituted the entire CHH family (19–21). The peptides mainly consist of from 72 to more than 80 amino acids. The most remarkable characteristic is the presence of six particularly well-conserved cysteine residues forming three intra-molecular disulfide bridges, which has been shown to be an invariant signature for this superfamily, as it expands with the addition of new members. CHH from the shore crab *Carcinus maenas* is the first member peptide to be purified and its full amino acid sequence determined (22). This was followed by CHH isolated from the lobster *Homarus americanus*, which, interestingly enough, has both CHH and MIH activities (23), *C. maenas* MIH (24), CHH from the crayfish *Orconectes limosus* (19), and *H. americanus* GIH (25). Characterization of the sequences available revealed a new peptide family, with the realization that MIH and GIH are more similar to each other than each to CHH, in terms of sequence similarity, length, and modifications of the termini (MIH and GIH are free, whereas CHH is blocked, at both ends) (19, 20). MOIH was not characterized until a few years later when two biochemically MOIHs, differing from each other only by one amino acid, were purified and characterized from the crab, *Cancer pagurus*, with the sequences being more similar to MIHs than to CHHs (26). In the same year, three peptides from the spider crab *Libinia emarginata* were characterized and found to have MOIH as well as hyperglycemic activity (27). A cDNA encoding a CHH precursor protein demonstrated that the spider crab peptide with MOIH and CHH activities is indeed CHH as defined by sequence characteristics (28). The lobster CHH (23) and spider crab CHH (27), which respectively have MIH and MOIH activities, represent initial examples illustrating pleiotropy of these family members, which was frequently reiterated by later studies. The observations that some member peptides, in particular CHH, are pleiotropic and often overlap with biological activities of other members probably reflect the evolutionary history of the CHH family (29–31). Based on the structure of the genes and the hormone precursors, and the characteristics of the mature peptides, the CHH-family peptides were divided into two groups: Type I (CHH) and Type II (MIH/GIH/MOIH) (30, 32–34).

Increasingly, the CHH-family peptides have been identified more through nucleotide sequencing of transcripts (cDNA cloning or transcriptomics) than by the conventional approach of biochemical purification and peptide sequencing. In a recent report, *in silico* mining of transcriptome datasets from 112 crustacean species (representing three Classes: Malacostraca, Branchiopoda, Copepoda) resulted in a collection of 413 genes encoding CHH-family peptides (35). The CHH family has been expanded to the status of a superfamily as more member peptides are identified in non-crustacean species. The first non-crustacean member of the CHH-superfamily is the ion transport peptide (ITP), which was isolated from the corpora cardiaca of the desert locust *Schistocerca gregaria* and has structural characteristics that allow it to be integrated into the Type I group (36–38). The presence of the CHH-superfamily peptides is firmly established through *in silico* mining of data derived from several representative ecdysozoan clades [*e.g.*, (31, 39–42)]. Recently, the CHH superfamily has been expanded by addition of latrodectin peptides and HAND (helical arthropod-neuropeptide-derived) toxins present in the venoms of spiders and centipedes (43, 44). The fact that the CHH-superfamily peptides are expressed across the Ecdysozoa has singularly complicated our functional and

phylogenetic understanding of the different members of the superfamily. Nevertheless, this diversity now gives timely opportunities to better understand the evolution of the CHH superfamily. Indeed, the grouping of the CHH-superfamily peptides has been revised according to analyses accommodating the newly discovered peptides, thus creating two new types: Type III and Type IV (31, 45). Investigation of the structure-function relationships of the peptides can now benefit as more structural variants with novel functions are available for comparative study, as exemplified by the structural study of HAND toxins (43).

The existence of multiple copies of genes encoding the crustacean members of the CHH superfamily increases the structural diversity of the member peptides, although the physiological significance of such structural polymorphism has not been fully clarified (29, 30). Alternative splicing of RNA also contributes to the structural diversity of the Type-I peptides (37, 46–55). Thus, at the mature peptide level, two peptides are derived from a common transcript, a short-splice form (CHH or ITP) and a long-splice form (CHH-L or ITP-L), which share the same sequence for the first 40 residues from the N-terminal end but differ considerably after the 40th residue (56, 57). The peptides of the long-splice form have been far less characterized functionally than their short-splice counterparts. Recent studies utilizing RNA interference (RNAi), however, have shed some light on their functions (58–60). Structural diversity is further augmented by a post-translational isomerization of specific residues (Phe^3 in CHH and Trp^4 in GIH), resulting in a change in the configuration of the residue from L-form to D-form (61), which modifies the functionality of the peptides (62–66).

While the XO-SG complex in the eyestalk is the first tissue from which the crustacean members of the CHH superfamily were isolated and most likely the main source of these peptides, it has been shown that they are also expressed in other nervous and non-nervous tissues [*e.g.*, (47, 48, 50, 53, 55, 67–72)]. Similarly, ITP and ITP-L are widely expressed in the central and peripheral nervous system (49, 73, 74). The broad tissue expression of the CHH-superfamily clearly attests to their functional importance and might be closely related to the observed pleiotropy of these peptides. Physiologically adaptive roles of CHHs have been put to test using animal models that undergo life-history stages that presumably require regulation by CHH (75, 76). Recently, the metabolic effects of CHH on the muscle and hepatopancreas of the crayfish *Procambarus clarkii* were comprehensively characterized using a metabolomic analysis, revealing that the effects are more wide-ranging than previously realized and that the two tissues are differentially regulated by CHH (77, 78). The metabolic effects of CHH have also been implicated in the pathogenesis of diseases in infected crustaceans (77–81). On the other hand, several studies suggested that CHH modulates the immune functions (82–84), although the proposed immunomodulatory effects of CHH have not been thoroughly assessed in pathologically relevant conditions. It is entirely possible that an observed functional change in response to CHH treatment may be due directly to metabolic effects (especially energy metabolism) of CHH, as already suggested by several authors (70, 75, 85, 86). The fact that a given peptide member could be expressed in multiple tissues complicates physiological investigations. A case in point is the ecdysis-associated hemolymph surge of CHH that is released from gut endocrine cells, not the eyestalk XO-SG complex (68). The mode of action, endocrine or autocrine/paracrine, would be another issue that should attract the interest of physiologists working on the function of these peptides.

Structure-function relationships have been comprehensibly studied for MIH of *Marsupenaeus japonicus* (Pej-MIH), ITP of *S. gregaria* (Scg-ITP), and CHH of *Scylla olivacea* (Sco-CHH) using mutated recombinant peptides (87–90). Moreover, the three-dimensional structure of three crustacean member peptides (Pej-MIH, Pej-CHH-Gly, and Sco-CHHL) and that of a HAND toxin (Ta1a) are available for comparison (43, 91–93). Several functionally important residues, as assessed by the studies of the structure-function relationships are located in the parts of the structure that are in close proximity, which are likely involved in receptor binding and activation (87–90, 93). An exciting and important discovery in the field of study of the CHH superfamily is the identification of three orphan G protein-coupled receptors (GPCRs) as receptors for the silkworm *Bombyx mori* ITP and ITP-L (94), which subsequently led to identification of several crustacean GPCRs as the candidate receptors for crustacean member peptides of the superfamily, CHH or MIH (95–97). Identification of the receptors would give great impetus to many aspects of functional studies, including confirmation of target tissues, functional specificity (and overlap), and the cellular mechanism of action coupled to receptor activation.

This review is intended to highlight and discuss the progress that has been made in the past decade or so in the field of study of the CHH superfamily within the general framework of the knowledge accumulated since almost a century ago. Readers are also referred to reviews on similar topics that have been recently published (56, 98).

EVOLUTION AND STRUCTURAL DIVERSIFICATION OF THE SUPERFAMILY

Genomic Diversity

Isoforms of CHH were firstly characterized with regard to their specific functions. For example, CHHs *sensu stricto* are pleiotropic hormones mainly involved in the regulation of metabolism or water and ion balance, or may even have a negative effect on reproduction and molting. However, MIH/GIH/MOIH seemingly have, in the current state of our knowledge, more limited functions. These functional differences are obviously associated with structural differences which have enabled them to be distinguished and classified as distinct types, such as Type I and Type II, respectively (33). Type I brings together peptides with a cryptic sequence or PRP (precursor related peptide) and a dibasic cleavage site upstream of the mature peptide sequence. Type II is characterized by the absence of this PRP and has a glycine residue in position 5 after the first cysteine (33–35). It is important to keep in mind that if the first sequences were obtained from an identified biological

function, this is no longer the case for most member peptides identified subsequently, as most have been characterized mainly by high-throughput sequencing techniques.

CHH-superfamily members are clearly no longer restricted to crustaceans, since ITP (ion transport peptide) was found in locusts (37, 38). ITP has since been found not only in other hexapods but also in chelicerates (39–42, 44, 99–101) and nematodes (102). ITPs were initially believed to be specific to non-crustaceans until they were identified in non-malacostraca crustaceans such as phyllopods (31, 103, 104), copepods (105–107), and remipedes (108). In these species, the presence of ITP seems to be exclusive since no other CHH peptide has been characterized simultaneously. This isoform presents a precursor organization closer to that of CHHs than MIH/GIH/MOIH, *i.e.*, the presence of a PRP and a dibasic cleavage site, which has led to its inclusion in Type I (31). However, phylogenetic studies, carried out on the basis of sequences of the three isoforms, clearly place the ITPs, including those obtained from non-malacostracan crustaceans and hexapods, at the base of Types I and II. This observation led to the proposal to create a third group, Type III, which brings together all these ITP orthologues (31, 45).

The analysis of recent transcriptomes has added another level of complexity by identifying a new form of peptide belonging to the superfamily (45, 109, 110). These new sequences possess the six conserved cysteine residues, but do not have a PRP sequence, nor a dibasic cleavage site or the glycine residue, a signature of MIH (45). They are also significantly longer than other isoforms with more than 80 amino acids. Nevertheless, their BLAST hit clearly clusters them with ITP isoforms of insects and Cladocera crustaceans such as *Daphnia* rather than CHHs or MIH/GIHs. For this reason, they have been grouped together under the name ITP-like (45). Thus, inclusion of ITP-like sequences in a phylogenetic analysis conducted on more than 200 sequences of family members confirms their belonging to the same group and positions them as an independent group (45). The features of their sequences exclude them from the types currently recognized and once again question the dichotomous classification proposed by Lacombe et al. (33). It constitutes a new set of members in the same way as CHHs *sensu stricto* or MIHs and would thus form a fourth type (Type IV), if we refer to phylogeny.

The analysis of the different transcriptomes also addresses the number of isoforms, notably CHHs, present within each species. Information collected from these analyses revealed the presence of many additional isoforms within each species, which are growing more numerous as the number of tissues analyzed has increased. While there are so far only one or two isoforms of MIH/GIH (111–116), CHH peptides exhibit much greater variety with commonly four or more different isoforms (45, 117). The presence of multiple *chh* genes has been demonstrated in decapods, (up to nine) whereas only one copy of the *itp* gene is present in insects (29–31, 57). Similarly, only two *mih* genes have been identified in several species (111, 118). Some are clearly derived from recent duplications and show little divergence (119), while others differ substantially from the most represented isoforms and clearly demonstrate that diversity has been underestimated (45). It is very likely that additional transcriptomic analyses will uncover new isoforms, even in species that have already been well studied (119). This diversity supports the importance of this molecular family at the physiological level and could also explain the apparent pleiotropy of the CHHs.

Beyond this genomic diversity, there are mechanisms in some crustaceans that can produce new isoforms by post-transcriptional or post-translational modification, as detailed below.

Post-Transcriptional Modifications

Alternative splicing of the *chh* gene in pericardial organs of *C. maenas* (47) results in a CHH isoform that lacks the defining activity of CHH. Alternative splicing has since been demonstrated for several CHH genes in malacostracan crustaceans (47, 48, 53, 60, 72, 120) but also for insect ITPs (51, 52, 57, 59). These splicing patterns originate in the structure of the *chh* or *itp* genes, which have a minimum number of four exons. The splicing alternates two exons (exon 3 or 4) as the terminal part of the transcript, resulting in two transcript forms—the short-splice forms (exons 1, 2, 4) and the long-splice forms (exons 1, 2, 3, 4), in which the stop codon is located at the end of the third exon, leaving the fourth exon untranslated (**Figure 1**). This process generates two peptides from a single gene that differ in sequence after the 40th amino acid residue of the mature peptide. Exons 3 and 4 both encode peptide sequences of similar lengths, with two cysteine residues in the same positions, thus suggesting that alternative exon 4 appeared by tandem duplication of exon 3, or vice versa (31). The transcripts of the short-splice forms of CHH peptides were mainly found in neurons of the central nervous system (more precisely in the X-organ located in the eyestalk), whereas the long-splice forms were found in neurons of the peripheral nervous system and non-nervous tissues, thoracic ganglia, Y-organ, mandibular organ or hemocyte according the species (47, 48, 55, 60, 70, 121). Peptides encoded by the long-splice forms of CHH and ITP transcripts are slightly longer than peptides encoded by the short-splice transcripts and are never C-terminally amidated [see (56)]. They are named CHH-L and ITP-L, respectively. In addition to these genes with four exons, there are also genes with only three or even two exons, such as the majority of CHH isoforms of penaeid shrimps. MIH and MOIH are encoded by genes with three exons (29, 31, 122, 123). Alternative splicing mechanisms, as described here, have never been reported for these two- or three-exon genes of penaeid shrimps. It is also interesting to note that this splicing process can generate "truncated" forms of CHH as demonstrated in the hemocyte of the crayfish *P. clarkii* (72), resulting in a precursor, where the mature peptide sequence is truncated with only the first 40 residues.

Ubiquity of the CHH-Superfamily Peptides

The following examples underline the widespread ubiquity of the CHH superfamily members, which demonstrates the surprising

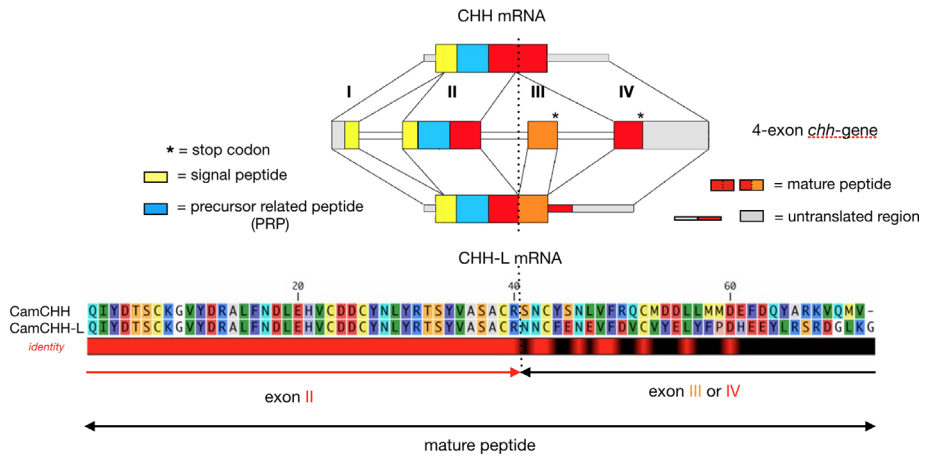

FIGURE 1 | RNA alternative splicing of the *chh* gene in the shore crab *Carcinus maenas*. *C. maenas chh* gene is a 4-exon gene. The long-splice form (CHH-L mRNA) consists of exon I, II, III, and IV, and the short-splice form (CHH mRNA) consists of exon I, II, and IV. The first 2 exons (exon I and II), which encode the signal peptide, the precursor-related peptide (PRP), and the first 40 residues of the mature peptide, are common to the two splice forms. The remaining sequence of the CHH-L and CHH mature peptide is encoded by exon III and IV, respectively. Note that both exon III and IV have a stop codon. The dashed line marks the splice site.

diversity of these peptide isoforms and their functions. Indeed, if the first isoforms were characterized at the level of neural ganglia, first in the XO-SG complex in the eyestalks and then followed by the thoracic ganglia, the detection of these peptides in other tissues is becoming increasingly common. The first evidence was provided by immunocytochemical analysis in *C. maenas*, in cells located at the fore and hindgut level, suggesting a mechanoreceptive function. These are "paraneurones" which secrete CHH into the hemolymph during ecdysis (68, 124). Since then, it has been shown that CHH-related peptides are expressed in a wide variety of tissues including the pericardial organ (120, 125), retina (126), gill (60, 127), spermatophore (71, 128), Y-organ (121, 129), hemocyte (70, 72), ovary (129, 130), stomach (130), and intestine (54).

Until recently, it was thought that MIH/GIH/MOIH peptides were only expressed in neuronal tissues of the XO-SG system. However, MIH expression has been identified in the heart of the shrimp *Litopenaeus vannamei* (131), and in the gill, ovary, and abdominal ganglion of *Macrobrachium nipponense*, though at relatively lower level than in the eyestalks (132).

Post-Translational Modifications

In parallel with gene multiplication or post-transcriptional processes such as alternative splicing, peptide modification in some taxa further increases the diversity of isoforms and functions of Type I or II members. This mechanism, hitherto restricted to Astacidea in crustaceans but present as well in other arthropods such as arachnids and mollusks and a few vertebrates, involves production of peptides in which the chiral form of a single amino acid has been modified, *i.e.* from the L-enantiomer to the D-enantiomer (61). Thus, lobsters and crayfish have CHH isomers that are differentiated by the configuration of the Phe^3, as either an L-form or a D-form. Both isomers exhibit the defining hyperglycemic activities and regulate energy metabolism, but the change in configuration results in modification of the biological activity of the peptide (64, 66, 133). In particular, the hyperglycemic response kinetics are delayed with D-Phe^3-CHH (3 to 4 h instead of 2 h for the L-isoform), and the amplitude of the hyperglycemic response is increased (10x higher for D-Phe^3-CHH). Additional functional changes for D-Phe^3-CHH were reported as having more potent inhibitory activity on the molting gland during ecdysteroidogenesis (65) or having a higher osmoregulatory activity (62, 63).

A similar modification from L-GIH to D-GIH has also been demonstrated in three Nephropidae species (American, European, and Norway lobsters). In this case, the modification is located at the level of the fourth N-terminal amino acid, a tryptophan (134, 135). However, the function of D-Trp^4-GIH is still unknown, as it does not display significantly inhibitory effect in a heterologous *in vivo* assay as its counterpart does.

The study of the maturation dynamics of the CHH isomers highlighted the different stages of the post-translational processing of the precursor (**Figure 2**). It appears that isomerization takes place after cleavage of the pro-peptide and before N-terminal cyclization (136, 137). The production of antibodies specifically directed against the two CHH isomers made it possible to monitor *in situ* their location during the maturation process in the XO-SG complex of eyestalks of the crayfish *O. limosus* and the lobster *H. americanus* (135, 136). Two cell types producing either L-CHH exclusively (L-CHH cell) or a mixture of both L- and D-isomers (D-CHH cell) were identified (**Figure 2**). Most of the transformation of the L-isomers seems to occur in the cytoplasm, before the granules penetrate the axons (138). A similar approach was conducted with GIH isomers. Differential localization of the two isomers in different cells was characterized, as for CHHs. In the same way, simultaneous monitoring of the four isomers showed co-

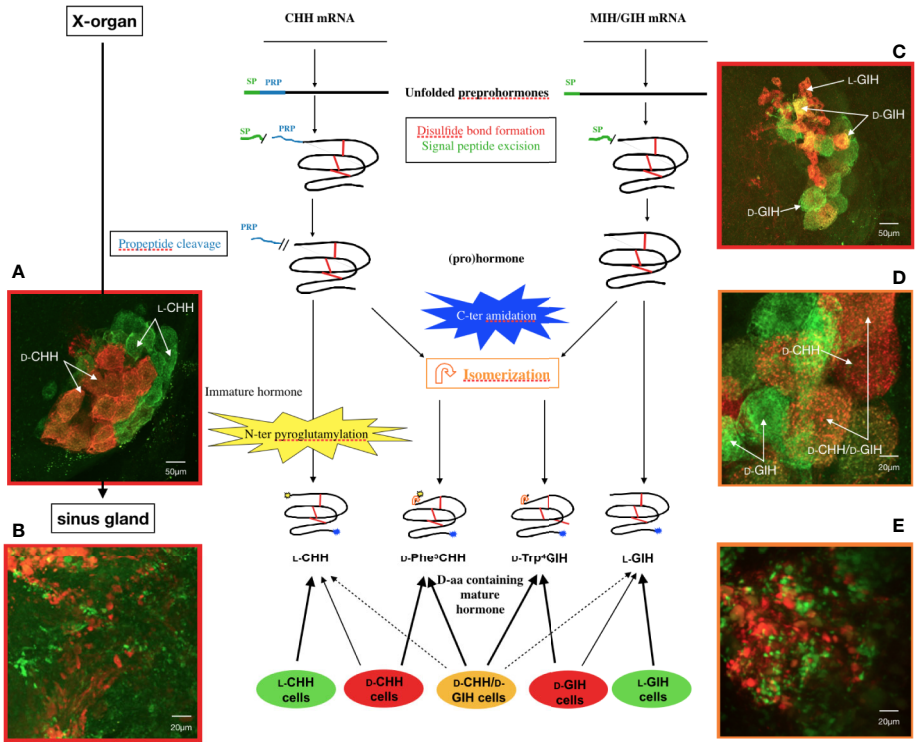

FIGURE 2 | State of the current understanding of L- to D- isomerization in *Homarus americanus*. General diagram of precursor processing of CHH and GIH isomers in relation to the different cell types in the X-organ–sinus gland complex and confocal micrographs of double labeled whole mounts of lobster *Homarus americanus*. Amidation can occur pre-, co- or post-cleavage of PRP. Cyclization of the N-terminus of CHH is optional (*i.e.*, N-terminus unblocked CHH can be released) and, similarly to isomerization, it occurs after PRP cleavage. GIH is not N-terminally cyclized (by pGlu). SP, signal peptide; PRP, precursor-related peptide. **(A)** Distribution of CHH cells in the X-organ showing green somata (L-CHH cells) and orange somata (D-CHH cells). **(B)** Axon terminals in the sinus gland showing secretory granules in L-CHH cells (green) and D-CHH cells (red). **(C)** General view of the X-organ visualizing small L-GIH cells (red) and larger D-GIH cells (green or yellow). L-CHH and L-GIH cells secrete exclusively CHH and GIH, respectively, whereas D-CHH and D-GIH cells release mainly the D-isomer of the respective hormone, in addition to a variable amount of L-isomer. **(D)** Immunolocalization of D-Trp^4 GIH and D-Phe^3 CHH in the X-organ where three cell types were observed: D-CHH cells (red), D-GIH cells (green) and D-cells producing both D-isomers (orange). D-cells secrete mainly the D-form of both CHH and GIH. Besides isomerization, the same posttranslational processes occur in every type of CHH or GIH cell. **(E)** Sinus gland axonal arborizations containing D-Trp^4 GIH (green) and D-Phe^3 CHH (red) (135).

localizations within the same cells. Only L-GIH and L-CHH could not be found together (135) (**Figure 2**).

These studies have highlighted a particularly original mechanism of post-translational modification and curiously, so far, this has been demonstrated only in Astacidea crustaceans. Because this L-to D-post-translational modification is subtle and not detectable by most sequence determination approaches, it cannot be excluded that it exists in other taxa, although it has been searched for in other crustaceans, without success. Nevertheless, this process is not specific to crustaceans. As mentioned above, it has not only been found in arachnids, but also in molluscs, tetrapods, and mammals (61).

Another post-translational modification, a C-terminal amidation event, seems particularly important to limit degradation of the peptides by carboxypeptidases (139). The C-terminal amidation concerns only CHH and ITP, while the long-splice form (CHH-L or ITP-L) do not present this modification. In addition to the protective role in resisting degradation, the amidation is also important for the activity of the peptides, since the amidated peptides, compared to the un-amidated ones, have more potent activities (139–141). This post-translational modification has also been infrequently observed in type-II peptides such as GIH or MIH, although the functional significance of the modification is not clear (132, 142, 143). Like the C-terminal extremity, the N-terminus has, again only for CHH and ITP, a post-translational conversion *via* cyclization of the terminal Glu or Gln residue into pyroGlu (56, 57). This modification would protect the N-terminus from degradation by aminopeptidases, but does not significantly change the biological activities of the peptide, as the N-terminally unblocked and blocked CHHs of *C. maenas* were shown to be almost distinguishable in the activities of elevating hemolymph glucose levels and of repressing ecdysteroid synthesis (144).

Weaponization of the CHH-Superfamily members?

Among the peptides characterized with D-amino acyl residues, it is interesting to note that there are many examples of these molecules

that have been extracted from venoms or the nervous system (145, 146). This link between members of the CHH superfamily and venom peptides was further strengthened by the discovery that certain peptides of low molecular weight identified in black widow spider venom (genus *Latrodectus*), e.g., the latrodectins, show sequence similarities with CHH, particularly in terms of size, around 70 residues, the signature of the six conserved cysteines including similar disulfide bond pairing, and a similar alpha-helical structure (99). Further analyses suggested that CHH-superfamily peptides might have been recruited for venom expression at least three times: once in Hymenoptera, once in scorpions, and at least once in spiders, each lineage independently evolving venom production (44). However, the functional role of the latrodectins remains unclear as they are not insecticidal or toxic to mice (99), nor do they produce hyperglycemic effects when injected into crabs, crayfish, or shrimps (147).

An exception was however first identified in the venom of common agelenid spider *Tegeneria agrestis*, the U1-agatoxin-Ta1a (148, 149), and then discovered in the venoms of members of the centipede genus *Scolopendra*, representing convergent recruitment of CHH-superfamily peptides into the toxin arsenals of these myriapods (43). Despite a weak sequence identity, the structure of MIH of the shrimp *M. japonicus* and CHH-L of the crab *S. olivacea* are surprisingly topologically similar to that of Ta1a. However, the N-terminal α-helix (α 5), present in both MIH and CHH-L, is absent from Ta1a [**Figure 3**; also see (43)] and the loss of this helix could be a key step in weaponization of CHH family peptides (43).

Recently, a proteomics approach of the venom components of the parasitoid wasp *Tetrastichus brontispae* resulted in the identification of, among a large number of diverse proteins or peptides, three ITP-L (ITPLn1-3) as abundant toxins (150). These ITP-Ls are distinct from ITPs by the implementation of a possible alternative splice allowing diversification of the gene products and a potential neo-functionalization that would lead to an evolution towards peptides of the CHHs family with venomous properties.

The mechanisms for recruiting members of the CHH superfamily as toxins are clearly varied and appear to have evolved independently in the different taxa. Nevertheless, the use of ITP/CHHs as evolutionary fodder for these transformations is a consensus among a growing number of species and is certainly not fortuitous. The structural properties (3 disulfide bridges which guarantee great stability), the multiplicity of genes and the post-transcriptional or even post-translational mechanisms are all processes that can lead to neo-functionalization, of which the acquisition of toxicity is one. In this context, one of the consequences of the acquisition of a D-amino acid peptide is a higher resistance to proteases and therefore a longer half-life for these peptides (146). In the future, it would not be surprising to find organisms with venomous ITP/CHHs carrying this type of modification.

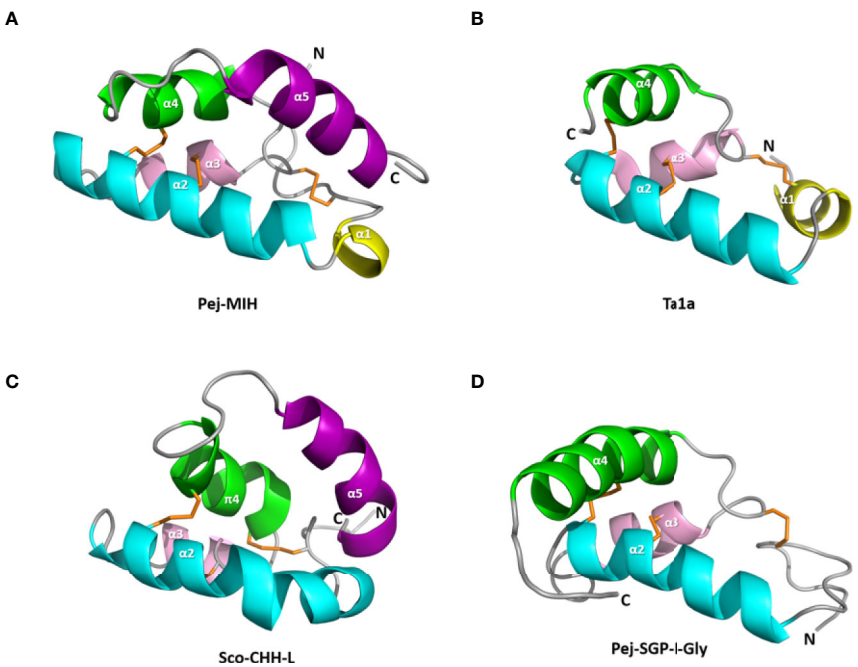

FIGURE 3 | Structure of the spider toxin Ta1a and representative crustacean member peptides. Ribbon structure of **(A)** Pej-MIH, **(B)** Ta1a, **(C)** Sco-CHH-L, and **(D)** Pej-SGP-I-Gly. The helices, and N- and C-terminus (N and C) are labeled. Note the absence of the terminal helix (α5), which is present in both MIH and CHH-L, from Ta1a and Pej-SGP-I-Gly. A π helix (π4), topologically equivalent to the α4 in other structures, is present in Sco-CHH-L. Protein Data Bank IDs for the structures are respectively 1J0T, 2KSL, 5XS1, and 5B5I.

BIOLOGICAL FUNCTIONS

Type-I and III Peptides

CHH and ITP were functionally defined by the hyperglycemic activity and stimulation of trans-epithelial Cl^- transport in the ileum, respectively, which formed the basis (by bioassay) for purification, chemical characterization, and eventual identification of the hormones (19, 22, 37, 38, 151, 152). Studies over the years have however revealed that the two peptides are more functionally conserved than the initial defining functions would have suggested.

Metabolism, Ionic and Water Homeostasis, and Development (CHH, ITP, CHH-L, and ITP-L)

A "diabetogenic factor", as the presumptive factor elevated hemolymph glucose levels, in the crustacean eyestalks was suggested more than 7 decades ago (153). It is generally accepted, based on early studies, that CHH mobilizes glycogen reserves in the CHH target tissues (*e.g.*, the hepatopancreas and muscle), leading to hyperglycemia *via* regulation of the amount and activity of the enzymes (glycogen synthase and glycogen phosphorylase) involved in glycogen metabolism (154–159). The increased availability of glucose for cells, resulting from the glycogen-mobilizing effect of CHH, may subsequently stimulate the glycolytic flux (160, 161). The stimulatory effects of CHH on the release of amylase from the hepatopancreas (162) and hemolymph levels of triacylglycerols and phospholipids (163) have been reported but not further characterized. Recently, the metabolic roles of CHH in the crayfish *P. clarkii* were more comprehensively characterized using an RNAi approach (double-stranded RNA) followed by profiling the hepatopancreas and muscle metabolomes (77, 78). The combined data indicated that CHH has more diverse effects than previously realized, and the two target tissues are differentially regulated. The main effects of CHH are stimulation of glycolysis and lipolysis in the hepatopancreas, and higher rate of utilization of carbohydrates (glucose and other sugars, including fructose, galactose, sucrose, and lactose) *via* glycolysis and TCA cycle (resulting in higher levels of ATP), stimulation of the pentose phosphate pathway (PPP) flux (leading to increased levels of nucleotide biosynthesis), and elevation of amino acid biosynthesis in the muscle (77, 78). Stimulation of the "Nicotinate and nicotinamide metabolism", which concerns the metabolism of two nicotinamide coenzymes (NAD^+ and $NADP^+$), is central to the metabolic effects of CHH in the muscle. Thus, the higher levels of NAD^+ (and higher NAD^+/NADH ratio) and $NADP^+$, respectively, drive these fluxes through glycolysis, the TCA cycle, and the PPP (77, 78). The tissue-specificity of CHH regulation is consistent with the results showing the transcript expression of carbohydrate metabolism-related enzyme genes were differentially regulated by CHH in the same two tissues of *M. japonicus* (159). A proposed scheme for the metabolic roles of CHH in the two target tissues is shown in **Figure 4**, based on combined data from several related studies.

A substantial amount of evidence has been accumulated indicating that CHH is involved in regulating osmotic homeostasis. It was first reported in the American lobster *H. americanus* that sinus gland extracts increased the osmoregulatory capacity of eyestalk-ablated animals kept at low salinity (164). A CHH variant, specifically D-Phe^3-CHH, when injected was able to significantly compensate the eyestalk ablation-induced decrease in hemolymph osmolality, Na^+ concentration, or both, in *H. americanus* (164) and the freshwater crayfish *Astacus leptodactylus* (63); CHH stimulated *in vitro* trans-

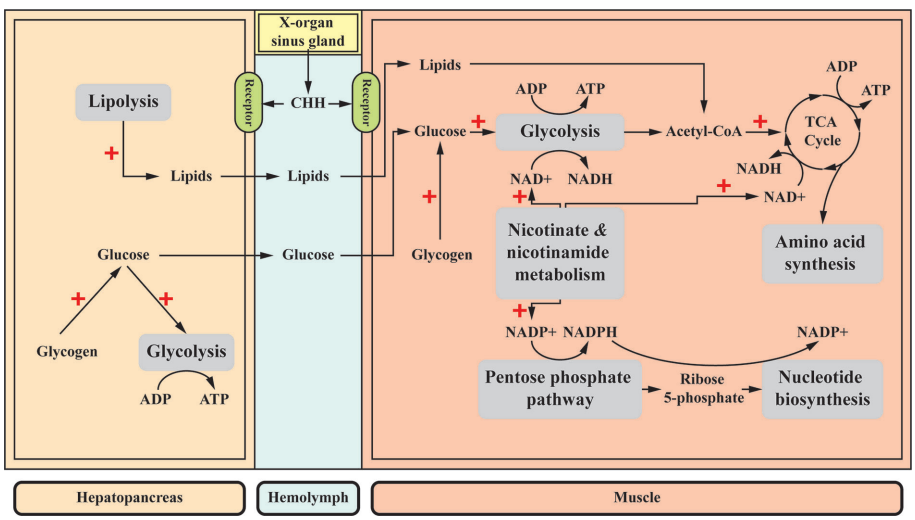

FIGURE 4 | A proposed scheme for the metabolic roles of CHH in the muscle and hepatopancreas. CHH differentially regulates its target tissues. CHH decreases glycogen accumulation in both target tissues, resulting in higher levels of glucose (154–158) that drives glycolytic flux (160, 161). Moreover, in the hepatopancreas, CHH enhances lipolysis (77, 163). Glucose and lipids are released into the hemolymph and taken up by the muscle where they are further metabolized *via* glycolysis and TCA cycle, respectively, for ATP production. In the muscle, central to the effects of CHH is a stimulated "Nicotinate and nicotinamide metabolism", which provides two nicotinamide coenzymes (NAD^+ and $NADP^+$) that drive glycolysis and TCA cycle, and the pentose phosphate pathway, respectively, resulting in increased ATP supply and biosynthesis of amino acids and nucleotides (77, 78). **+**, stimulatory effects.

epithelial electrical potential difference and Na^+ influx in the posterior gills in the crab *Pachygrapsus marmoratus* (165) and was effective in restoring stress-induced decrease in hemolymph Na^+ and K^+ levels to the pre-stress levels in the freshwater crayfish *Cherax quadricarinatus* (85). High-affinity CHH binding sites were identified in, among other tested tissues, the gill of *C. maenas* and *in vitro* treatment of the gill with CHH significantly increased both cGMP and glucose levels in the tissue (86). In a study exemplifying the physiological roles of CHH at specific life-history stages, a gut-derived CHH, which was massively released into hemolymph during late pre-molt and ecdysis, was suggested to be involved in stimulating water and ion uptake in the crab *C. maenas*, causing body swelling for successful ecdysis and subsequent increase in animal size during post-molt (68). While these studies strongly implicate CHH as an important factor for iono/osmoreguation, the molecular target(s) on which CHH acts to achieve its regulatory activity were not directly addressed by these studies. It is however relevant to note that CHHs of the Christmas Island blue crab *Discoplax celeste*, which stimulated Na^+ transport across the gill epithelia, had no effect on gill Na^+/K^+-ATPase or V-ATPase activity (75).

Characterization of the functional roles of CHH has led to the concept that CHH acts physiologically as a stress hormone (166). In various crustacean species, it has been shown that CHH mediates the stress-induced hyperglycemia in animals exposed to different stressors (extreme temperature, hypoxia, organic and inorganic pollutants, etc.), presumably metabolically acclimating animals to the stressful environment (50, 167–171). Interesting and insightful observations have been reported in studies working on crustaceans with distinct seasonal patterns of physiological demands (75, 76). The Christmas Island red crab (*Gecarcoidea natalis*) and blue crab (*D. celeste*) are two terrestrial brachyurans that undergo annual breeding migration towards the sea at the beginning of the wet season but remain inactive and fossorial during the dry season, and as such, were considered ideal species for investigating the roles played by CHH in metabolic and ionic homeostasis in an eco-physiological context as the endocrine status with respect to energy metabolism and osmoregulation should change seasonally (75, 76). One of the main findings derived from these studies is that in both species, while a 10-min extreme (forced) exercise stimulated a rapid and transient CHH release into the hemolymph followed by increases in hemolymph glucose levels, the hemolymph CHH levels in the migrating animals were actually significantly lower than those found in the animals during the dry season. This is in contrast to what would have been presumed based on the expected metabolic burden of the migration activity on the animals. It was further demonstrated that a negative feedback loop involving inhibition of CHH release by glucose existed only during the wet season but was uncoupled during the dry season. It was considered that the lower CHH levels in the wet season might be due to the inhibitory effect of high glucose levels, characteristic of migrating animals in the wet season when they continue to feed to conserve the glycogen stores that are to be utilized later for more strenuous activities, which deplete glycogen reserves upon the return migration. The significantly higher dry-season CHH levels, likely reflecting the lack of a functioning negative feedback loop at the time, might be metabolically advantageous when animals were fossorial with limited foraging activity (76). Alternatively, it was reasoned that the significantly higher CHH levels during the dry season, when conservation of water and ions would be crucial, might be closely related to the ionoregulatory activity of CHH (76). Indeed, experiments measuring branchial Na^+ flux indicated that CHHs affect sodium uptake in the gill in a season-dependent manner attesting to the importance of CHH for ionic and water homeostasis (75, 76). Data generated from these studies highlight the intricate and intriguing aspects of CHH regulation that must be shaped in ways to meet the physiological demands characteristic of particular stages of the life history of the animals. These studies underline the importance of such functional approaches both in the field and in the laboratory for improving our understanding of the adaptive significance of CHH.

While CHH has been functionally characterized rather extensively, the functional role of CHH-L has been far less studied. It is however known that CHH-L, unlike CHH, does not have either hyperglycemic or ecdysteroidogenesis-inhibiting activity (47, 172, 173), and co-injection of CHH and CHH-L did not change the pattern of hyperglycemic responses in *C. maenas* injected with CHH alone. This rules out the possibility that CHH-L functions as a negative regulator of CHH (47). A recent study suggested that Pt-CHH2, a CHH-L peptide of the crab *Portunus trituberculatus*, could be involved in regulating gill Na^+/K^+-ATPase and carbonic anhydrase activity, since CHH dsRNA treatment decreased Pt-CHH2 transcript levels and significantly reduced the enzyme activity in the gills (60). Given that CHH is involved in osmotic regulation and that CHH, at least in the Christmas Island blue crab *D. celeste*, has no effect on gill Na^+/K^{+-}ATPase or V-ATPase activity (75), it is tempting to suggest that CHH and CHH-L peptides are both physiologically relevant factors for regulating water and ionic balance. However, these peptides likely do so by acting on distinct molecular targets, perhaps in a concerted manner.

Ion transport peptide was first identified based on its antidiuretic activity in the ileum in the desert locust *S. gregaria* (36–38). ITP, released from the corpora cardiaca, stimulates the ileum to transport Cl^- ion from lumen to hemolymph, driving water reabsorption. It is tempting to propose that ITP or ITP-L are involved in regulating processes critical for successful molting, as has been shown for CHH in crustaceans (68), but the evidence so far has not been conclusive [see (57)]. Studies using an RNAi method targeting expression of *itp*/*itp-I* transcripts in the red flour beetle *Tribolium castaneum* indicated that these peptides are important for adult eclosion, but less important for larval–larval or larval–pupal molting, based on observations of developmental defects and mortality (58). RNAi targeting expression of *itp*/*itp-l* in the brown planthopper *Nilaparvata Lugens* resulted in increased cuticle melanism and failed wing expansion (59). If and how the

observed phenotypes during development are related to the water reabsorbing activity of ITP remain to be confirmed. In addition, because the phenotypes were mostly observed using dsRNA that simultaneously silenced *itp* and *itp-l*, the effects cannot be clearly assigned to individual peptides. Finally, functions of ITP are likely not limited to fluid reabsorption. In a recent study in *Drosophila melanogaster*, the roles of ITP have been extensively characterized as, not only enhancing water retention (estimated by the defecation rate), but also promoting thirst (estimated by the propensity to drink water and volume of water intake) and inhibiting intake of "dry food", which are thought to work collectively to protect the animals from dehydration (174).

Pathogenesis and Immune Regulation (CHH)

There have also been interesting developments with regard to the pathophysiological roles of CHH. It has been shown in various crustacean species that a sub-lethal dose of lipopolysaccharide (LPS), a major component of the capsule of Gram-negative bacteria, elicited a significant hyperglycemic response (175), with a significant increase of hemolymph CHH levels as early as 30 min after treatment (80).

In another example in which CHH is implicated in a pathological condition, hemolymph glucose levels in the patently infected Norway lobster *Nephrops norvegicus*, parasitized by dinoflagellate *Hematodinium* sp., were dramatically decreased, indicating that the parasite, acting as a "carbohydrate sink", was absorbing glucose from the hemolymph of the host, leading to a near depletion of the glycogen reserve in the hepatopancreas of the lobsters with late stages of patent infection (81). The observations that hemolymph CHH levels progressively increased as the severity of infection increased, while glycogen reserves were significantly decreasing, vividly highlight the glycogen-mobilizing effect of CHH. The opposite direction of changes in hemolymph glucose and CHH levels indicated that the negative feedback exerted by hemolymph glucose on CHH release is relieved in the patently infected hosts (81).

Similarly, in a study of white spot syndrome virus (WSSV) infection in *P. clarkii*, hemolymph CHH levels were significantly increased by WSSV. The virus-induced CHH release was rapid, commencing as early as 3 h post-infection, and long-lasting with the hemolymph CHH levels being significantly elevated for at least two days after the infection, leading to dramatic decreases in the CHH content in the eyestalk ganglia (79). However, hemolymph glucose levels in the infected hosts were not significantly higher than those in the uninfected animals, arguing for an enhanced glucose uptake by the host cells (79). Virus-induced alterations of metabolism, favoring viral replication and disease progression at the expenses of the host, have been reported in mammalian hosts [see (176)] and more recently also in crustaceans (*M. japonicus* and *Penaeus vannamei*) (177, 178). Thus, infection of WSSV alters the metabolism in the host cells, collectively known as the "invertebrate Warburg effect", including a higher rate of glycolysis, the pentose phosphate pathway, ribonucleotide biosynthesis, glutaminolysis, and amino acid biosynthesis (177, 178), which are largely in accordance with the metabolic effects of CHH, as revealed by two metabolomics studies (77, 78). In fact, analysis of the muscle metabolome using an enrichment analysis showed that the "Warburg Effect", among others, was significantly impacted when CHH expression was silenced (77). The combined data indicated that the virus-induced CHH release is at least partly responsible for inducing the Warburg effect (77–79). These studies illustrate how pathogens exploit the endocrine system of crustacean hosts, specifically increasing the CHH output and hence tilting the balance of host metabolism for the benefits of the pathogens. Silencing of *chh* gene expression was able to significantly decrease the viral load in the tissues and prolong the survival of the WSSV-infected *P. clarkii* (179).

Several reports studying the immunoregulatory effects of CHH in the Pacific white shrimp *L. vannamei* showed that recombinant CHH increased pathogen clearance and survival rates in pathogen-infected shrimps (82), and elevated total hemocyte count and the phagocytic activity of hemocyte (180). CHH treatment also affected the expression of several immune effector protein genes, including superoxide dismutase, LvRelish, and anti-microbial peptides, suggesting protective roles of CHH through modulating the immune activity of the shrimps (84, 180). It would be informative to investigate CHH-modulated immune responses under pathogenic conditions, which are invariantly characterized by significantly higher CHH levels as has been previously reported (79–81, 175).

CHH transcripts encoding CHH and related peptides, including CHH and CHH-L peptides and a novel truncated CHH, have been reported in hemocytes of *P. clarkii* (70, 72). Production of CHH peptides was also demonstrated, with a cell type-specific expression pattern, and CHH was able to stimulate guanylyl cyclase activity in the hemocyte membrane preparations (70). Wu et al. (70) suggested that the hemocyte-derived CHH may act on hemocytes in an autocrine/paracrine manner, regulating carbohydrate metabolism in crustacean hemocytes as they have been shown to be an important site for carbohydrate storage and metabolism (181–183), or have direct roles in regulating immune responses of the hemocyte.

Activities Overlapping With Those of Type-II Peptides (CHH)

CHH has been implicated in regulating other biological functions, including activities that functionally define the Type-II peptides. Thus in various species, CHH has been found to inhibit ecdysteroid synthesis (23, 65, 172, 184, 185), although its activity in this regard is less potent than MIH (65, 184, 185), as well as methyl farnesoate synthesis (186). The physiological significance of CHH-regulated ecdysteroidogenesis or methyl farnesoate synthesis has not yet been fully characterized. The functional overlap of CHH with the Type-II peptides may be the result of an evolutionary scenario in which the current CHHs (Type-I lineage) retain a certain degree of the functional pleiotropy of an ancestral peptide, while the Type-II lineage evolved towards peptides with more specialized functions (31).

Type-II Peptides

Inhibition of Steroidogenesis in the Y-Organ by MIH

Eyestalk ablation has been known for a long time to shorten molt interval and induce molting in many crustacean species. Based on these observations, Zeleny (7) proposed the existence of a "molt-inhibiting" factor in the eyestalk. Follow-up studies in various species have repeatedly shown that eyestalk ablation leads to an increase in ecdysteroid production by the Y-organ and in hemolymph ecdysteroid levels (187–189). This factor has since been purified, sequenced, and named molt-inhibiting hormone (MIH) (20, 24, 184). Injection of recombinant MIH has been shown to prolong the inter-molt duration (190). Adding eyestalk extract or synthetic MIH to the incubation medium significantly diminished the secretion of ecdysteroids by the Y-organ *in vitro* (191, 192). Correspondingly, using ^{125}I-MIH as the ligand, the existence of MIH-specific binding sites was detected in Y-organ membrane preparations from *C. maenas* (193), *Callinectes sapidus* (194), and *M. japonicus* (195). Altogether, these data strongly suggest that MIH acts directly on Y-organs to suppress ecdysteroidogenesis.

According to the above findings, a model has been proposed that the Y-organ ecdysteroidogenesis is suppressed by MIH during much of the molt cycle, only to be relieved from the suppression during the pre-molt stage, when MIH levels are low [see (196)]. Normally, hemolymph MIH levels are rather low (typically at the levels of fmol/ml) and difficult to quantify precisely. Available data regarding hemolymph MIH titers throughout the molt cycle are limited to three species, *C. maenas*, *C. sapidus*, and *P. clarkii* (197–199). Data in line with this hypothesis mainly come from the variation of MIH transcript levels throughout the molt cycle. In *C. sapidus*, *L. vannamei*, *M. nipponense*, and *Scylla paramamosain*, MIH transcript levels were relatively high during the inter-molt stage (stage C), declined gradually in the pre-molt stage (stage D), and then returned to a level similar to that of the inter-molt after molting (stage A/B) (111, 132, 200, 201). The hemolymph ecdysteroid levels in *C. sapidus* and *L. vannamei* displayed a corresponding elevation during pre-molt stage when *mih* gene expression was low (111, 200). Unexpectedly, a significant drop in hemolymph MIH levels was observed only in *P. clarkii* (197), whereas those in *C. maenas* and *C. sapidus* remained unchanged during pre-molt stage (198, 199), which contradict the proposed hypothesis. Thus, the model is considered to be incomplete, mainly because the absence of the pre-molt drop in MIH levels, at least in certain species, is not accounted for. In this regard, Chung and Webster first noted that the effect of MIH on the Y-organ of *C. maenas* is molt stage-dependent. The inhibitory effect of MIH on ecdysteroidogenesis was the highest (~60%) during inter-molt stage. The effect then dropped significantly during pre-molt and post-molt stages (less than 10%), indicating that the Y-organ became refractory to MIH stimuli at these stages (185). Similar observations were also reported in *P. clarkii* that MIH significantly inhibited ecdysteroidogenesis during inter-molt stage (~80%), and this inhibitory effect became much weaker (less than 10%) during the middle-premolt stage (192, 202). Pharmacological studies showed that the activity of phosphodiesterase 1 (PDE1), a calcium/calmodulin-activated PDE isoform, is closely related to the decreased responsiveness of the Y-organ to the inhibition by MIH during the pre-molt stage (192).

Regardless of the inconsistencies in the results obtained from different species, silencing of MIH expression utilizing MIH dsRNA resulted in a significant acceleration of molt frequencies (132, 203, 204) and elevation of ecdysteroids in the hemolymph (203), indicating a definite role of MIH in the regulation of crustacean molting and growth.

Stimulation of Vitellogenesis by MIH

Vitellogenin (Vg), the precursor of vitellin, is synthesized in the ovaries, hepatopancreas, or both (205–207). It is cleaved into smaller subunits in the hemolymph, and then stored as vitellin in growing oocytes (208, 209). Therefore, levels of *vg* transcripts in target tissues and Vg in hemolymph are commonly used as indicators of the progression of female reproduction in crustaceans (210). More recently, a novel function of MIH in stimulating ovarian growth *via* inducing vitellogenesis has been reported in a few species. In *C. sapidus*, MIH is capable of inducing hepatopancreatic *vg* mRNA expression and Vg secretion at mid-vitellogenesis stage (211). The hemolymph MIH titers in female crab were about four-times higher at the mid-vitellogenesis stage than those at the pre-vitellogenesis stage. Additionally, MIH-specific binding sites have been identified on hepatopancreas membrane preparations (194), indicating that MIH might act directly on the hepatopancreas to increase Vg production. Interestingly, the number of MIH binding sites on the hepatopancreas of adult females shows an "ovarian stage-dependent" variation that is two-times higher at the mid-vitellogenesis stage than at the pre-vitellogenesis or early vitellogenesis stage (194). Similar findings were also reported in *Metapenaeus ensis* and *L. vannamei* in that one of the two MIH isoforms of each species (namely, MeMIH-B and Liv-MIH2) stimulated vitellogenesis and promoted ovarian growth. *In vitro* incubation of ovarian explants and/or hepatopancreas with recombinant MIH resulted in the upregulation of *vg* transcript levels in *M. ensis* and *L. vannamei* (114, 212). Injection of recombinant MIH increased Vg content in both hemolymph and the ovary in *M. ensis* and induced ovarian maturation in *L. vannamei*. Additionally, injection of dsRNA also reduces MeMIH-B transcript levels in the hepatopancreas and ovary, resulting in lower Vg levels in the hemolymph. These combined data demonstrate a stimulatory role for MIH on female reproduction (114, 194, 211, 212). Regarding crabs with a reproductive phase accompanied by a terminal anecdysis, Zmora et al. (211) concluded that MIH is a vital regulator coordinating the reciprocal antagonism of molt and reproduction by keeping animals at an anecdysial status and stimulating vitellogenesis when the animals are sexually maturing.

Inhibition of Ovarian Maturation by GIH

GIH is another member in the Type-II group. After its first identification in *H. americanus* (25), GIH has been identified in several other species mainly through molecular cloning (213–216).

Recombinant GIH decreased *in vitro vg* transcript levels in the ovary (216) and GIH dsRNA decreased *vg* transcript levels in the ovary of *P. monodon* and the hepatopancreas *of L. vannamei* (215, 216). A single injection of GIH dsRNA was capable of suppressing *gih* expression for at least 30 days, leading to ovarian maturation and spawning in *P. monodon* (217).

Inhibition of Methyl Farnesoate Synthesis by MOIH

Methyl farnesoate (MF), the sesquiterpenoid structurally related to insect juvenile hormone III (218), is a secretory product of the mandibular organ which participates in controlling growth and reproduction in crustaceans (219–222). The existence of an inhibitory factor that negatively regulates MF synthesis was proposed based on the observation that eyestalk ablation caused an increase in hemolymph MF levels in the hemolymph (223), while injection of eyestalk extract reversed the effect of eyestalk ablation (224).

In *C. pagurus*, two MOIHs (MOIH-1 and MOIH-2), which suppressed MF synthesis *in vitro*, have been purified from the sinus gland extract and characterized. MOIH-1 and MOIH-2, unblocked at both ends, exhibits significant sequence similarity with MIHs (26). Their gene structure and sequence similarity indicated that the *moih* and *mih* genes arose by divergence following a gene duplication event (118). An interesting issue regarding MOIH is the apparently restricted existence of MOIHs in the cancrid crabs (225), suggesting that the gene duplication event was relatively recent, probably not earlier than the divergence of the *Cancer* genus (118). In some non-cancrid brachyurans (*L. emarginata* and *C. maenas*), CHH inhibits MF synthesis (186, 226), probably assuming the role of MOIH, whereas in *C. pagurus*, functionally and structurally distinct CHH and MOIH are present (26). Whether *moih* genes are present and expressed in other brachyuran taxa is unknown.

PEPTIDE STRUCTURE, SIGNAL TRANSDUCTION PATHWAYS, AND RECEPTORS

Peptide Structure

The first structural model of the CHH superfamily peptide was that of MIH (Pej-MIH) from *M. japonicus* resolved by nuclear magnetic resonance (NMR) spectroscopy. It consists of a long N-terminal tail, followed by five alpha helices (α1–α5), and a C-terminal tail (92). Subsequently, two more structures of the crustacean member peptides have been elucidated: Pej-SGP-I-Gly, the glycine-extended precursor of a *M. japonicus* CHH by X-ray crystallography (91), and Sco-CHH-L, the CHH-L from the pericardial organ of *S. olivacea* by NMR spectroscopy (Protein Data Bank: 5XS1). Although topologically equivalent helices—α2, α3, and α4 (π4 in Sco-CHH-L)—are present in the core of the three structures, Pej-MIH and Sco-CHH-L are more topologically resemble one another than Pej-SGP-I-Gly (**Figure 3**). Thus, Pej-MIH and Sco-CHH-L additionally have a topologically similar C-terminal α5, which brings the C- and N-terminal ends sterically close each other (**Figure 3**). In contrast, Pej-SGP-I-Gly, which lacks an α5, has a relatively long α4 followed by a C-terminal tail that is kept away from the N-terminal end (**Figure 3**). Because of the limited availability of the C-terminal amidated Pej-CHH, Pej-SGP-I-Gly, a non-amidated precursor was used for structural determination (91). Post-translational amidation at the C-terminal increased the α-helical content of CHH (139, 140, 172), indicating that the C-terminal modification renders structural changes. Thus, while additional structures of the CHH-superfamily peptides (*e.g.*, amidated CHH and ITP) are needed for validation, it is likely that the folding pattern shared by Pej-MIH and Sco-CHH-L is a common theme for the crustacean and insect peptides of the superfamily. Functionally critical residues at the terminal regions of MIH, CHH, and ITP have been demonstrated using mutated recombinant peptides (87–90, 139, 140, 227). It is likely that parts of the structure, consisting of the sterically close C- and N-terminal regions of the peptide, where the functionally critical residues are located, play important roles in forming the binding site for receptor interaction and activation.

Signal Transduction Pathways and Receptors

Radiolabeled peptides (125-I) have been used in several studies to identify the potential target tissues of CHH, MIH, or CHH-L and to profile the binding characteristics of the presumed receptor (86, 193, 228, 229). Generally, each of the crustacean members of the superfamily binds to a distinct binding site (receptor) with high specificity. In the shore crab *C. maenas*, Y-organs clearly have separate and highly specific binding sites for CHH and MIH (193). Similarly, in the blue crab *C. sapidus*, displacement experiments in multiple tissues also revealed that CHH and CHH-L peptide each has discrete specific binding sites (229). Another aspect of CHH binding specificity was illustrated by studies on hepatopancreas membranes from *C. maenas* and *O. limosus* (228). Membranes derived from *C. maenas* had a much lower affinity for *O. limosus* CHH than for *C. maenas* CHH (228), reflecting the species-specificity of CHH in terms of hyperglycemic activity (152).

A wealth of literature has been accumulated over the years regarding the signal transduction pathways of the CHH-superfamily peptides, mostly concerning MIH, CHH, and ITP. These include studies in various species utilizing *in vitro* or *in vivo* assays with tissue preparations or hormones, cyclic nucleotide analogues, and pharmacological agents inhibiting or stimulating activity of enzymes involved in the signaling pathways. In general, the studies have concluded that cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), or both act as intracellular signaling messengers mediating the action of the CHH-superfamily peptides. However, experimental data are in several instances inconsistent and contradictory, particularly for studies involving MIH. Until recently, the nature (receptor guanylyl cyclase vs. adenylyl cyclase-activating G protein-coupled receptor) of the putative receptor for the CHH-superfamily peptides were only speculated upon based on data derived from the early studies. In

a more recent study of the silkworm *B. mori*, orphan G protein-coupled receptors (GPCRs) were determined to be receptors for ITPs (ITP and ITP-L peptides), the characterization of which provided data for building a model of the signaling transduction pathway of ITPs (94), which is in general similar to an earlier one suggested for MIH (230).

CHH and CHH-L

Early studies investigating CHH signal transduction pathways implicated both cyclic nucleotides as second messengers mediating the action of the hormone (156, 157). Thus, CHH preparations increased cGMP and cAMP levels *in vivo* in several target tissues, including the hepatopancreas and muscle of the crayfish, *O. limosus*. In *in vitro* incubations of the hepatopancreas, cGMP levels were significantly increased by CHH, which was followed by release of glucose into the tissue incubation media, whereas cAMP levels were already elevated during the incubation and not further increased by hormonal treatment (156). Further, injection of CHH into the eyestalk-ablated crayfish increased cGMP and cAMP levels and decreased glycogen synthase activity in the abdominal muscle. Cyclic nucleotide analogues mimicked the effect of CHH preparations in inhibiting synthase activity (157). In *M. japonicus*, bilateral eyestalk ablation decreased intracellular cGMP levels of the hepatopancreas, with little effect on cAMP levels; corroboratively, exposure of tissues to recombinant CHH significantly increased the levels of cGMP, but not those of cAMP (231). The CHH-induced increase in cGMP levels in the muscle of *H. americanus* (232) was due to stimulation of the membrane-bound guanylyl cyclase (mGC), as cGMP increased not only in intact tissue but also in the isolated membrane preparations (233). Involvement of soluble GC (sGC) in the CHH-induced cGMP increase was considered unlikely, as cytoplasmic sGC activity was not stimulated by CHH and the CHH-induced cGMP increase was not blocked by methylene blue, an inhibitor of the nitric oxide (NO)-activated sGC (233). These combined results favor a scenario in which CHH binds and activates an mGC, thus elevating intracellular levels of cGMP as a second messenger, while changes in cAMP, if present, could occur downstream to receptor activation and cGMP elevation. An mGC (PcGC-M2), which was identified by cDNA cloning in the muscle of *P. clarkii*, was found to contain the signature domains characteristic of receptor GC (rGC), including an extracellular ligand-binding domain, a single transmembrane domain, and intracellular kinase-like and cyclase catalytic domains (234). PcGC-M2 is widely expressed in several target tissues of CHH (234), although the binding ligand for PcGC-M2 has not yet been determined.

The signaling pathway activated by CHH-L is much less characterized. In the blue crab *C. sapidus*, CHH-L (pericardial organ-CHH) significantly increased cGMP production in several tissues including scaphognathites, heart, midgut, hindgut, and abdominal muscles (229).

MIH

Results obtained from studies of the MIH-mediated suppression of ecdysteroidogenesis in the Y-organs in different species are often contradictory. On the one hand, cGMP was shown to be an important second messenger of MIH in several species, including *O. limosus*, *C, sapidus*, and *P. clarkii*. Thus, Y-organ incubated with native or recombinant MIH resulted in an increase of cGMP, but not cAMP, in these species (192, 202, 235). In related experiments, cGMP invariably played a crucial role in the mediation of MIH action: a cGMP analogue (8-Br-cGMP) significantly suppressed ecdysteroid production by Y-organ of *C. sapidus*, but neither cAMP analogues (db-cAMP or 8-Br-cAMP) nor an activator of adenylyl cyclase (forskolin) had a detectable effect on ecdysteroidogenesis (236); addition of synthetic MIH to the incubation medium increased cGMP levels, but not cAMP levels, in *P. clarkii* Y-organs (192). Corroborative observations were also reported for *C. maenas* Y-organ in which treatment with purified MIH produced a large and sustained increase in intracellular cGMP levels (237). These results are consistent with a model in which cGMP functions as a second messenger in the cellular action of MIH and that the receptor for MIH is likely a rGC. On the other hand, studies in *Cancer antennarius* showed that adding eyestalk extract (which contains a wide variety of bioactive compounds, including MIH) to incubation of Y-organs resulted in an increase in cAMP levels (189). Additionally, db-cAMP and agents (forskolin and choleragen) that increased intracellular cAMP, each mimicked the inhibitory action of MIH, while 3',5'-cGMP did not. It was thus concluded that cAMP mediates MIH-induced suppression of ecdysteroid production (189).

Data supporting the notion that the MIH receptor is an mGC and that cGMP functions as a second messenger were obtained in follow-up studies in *C. sapidus*. Thus, a cDNA (*CsGC-YO1*) encoding an rGC was cloned from *C. sapidus* Y-organ (238). Immunohistochemical staining using a primary antibody raised against the extracellular domain showed that CsGC-YO1 is located on the Y-organ cell membrane. Y-organ preconditioned with the anti-extracellular domain antibody became refractory to the stimulation of MIH. In addition, sodium nitroprusside (an NO donor) failed to inhibit ecdysteroidogenesis in Y-organ of *C. sapidus*, suggesting that NO/sGC/cGMP signaling pathway is not involved in the MIH action (196). Finally, the transcript levels of *CsGC-YO1* in Y-organ of the inter-molt animals were four- and two-times higher than those in the pre-molt and post-molt Y-organs, respectively (239).

Alternatively, a model hypothesizing that the MIH receptor is a G protein-coupled receptor (GPCR) was proposed (230) based on data obtained mainly from the crab *Gecarcinus lateralis* (240–243). In combination with previous related studies (230, 243) and the transcriptomic analysis, the *G. lateralis* Y-organ was proposed to go through a four-stage transition (basal, activated, committed, and repressed stages) from inter-molt (stage C_4), early pre-molt (stage D_0), middle pre-molt (stage D_1, D_2) and late pre-molt stage (stage D_4), respectively (244, 245). The MIH signaling pathway plays a dominant role in the inter-molt and early pre-molt stage to suppress ecdysteroid production. According to the model, ligand activation of MIH receptor leads to, *via* a G protein, stimulation of adenylyl cyclase and subsequent cAMP-protein kinase A (PKA) pathway. Phosphorylation of enzymes by PKA in turn causes inhibition of ecdysteroidogenesis. Another phosphorylation event activated by

PKA leads to an increase in membrane calcium conductance, resulting in Ca^{2+} influx that activates NO-sensitive sGC pathways through Ca^{2+}/calmodulin activation of nitric oxide synthase. Increase in cGMP levels and subsequent activation of protein kinase G ultimately lead to inhibition of ecdysteroidogenesis. This working hypothesis is sustained by a series of experimental studies. First, analysis of *G. lateralis* Y-organ transcriptome indicated the expression of signaling components mentioned above (including adenylyl cyclases, PKA, PKG, calmodulin, NOS, NO-sensitive guanylyl cyclase (GC-I), and GPCRs) (95, 121, 246). Second, the production of ecdysteroids *in vitro* was repressed by NO donors (SNAP and SE175) and by cAMP and cGMP analogues (247, 248). Third, an adenylyl cyclase (AC) activator (forskolin) inhibited the production of ecdysteroids (248). Fourth, in combination with IBMX, an sGC activator (YC-1) inhibited ecdysteroid secretion in Y-organ (247). Finally, eyestalk ablation led to the up-regulation of NO-independent-NOS, GC-I, and GC-III mRNA levels, causing the Y-organ to be more sensitive to MIH stimuli, presumably a compensatory response to the removal of MIH (240).

GIH and MOIH

Information about GIH and MOIH signaling cascades are still limited. Effects of various pharmacological reagents have been tested in an *in vitro* assay using *vg* mRNA expression in ovarian explants from *M. japonicus* (249). Results showed that db-cAMP, db-cGMP, forskolin, and IBMX mimicked the inhibitory effects of GIH in reducing the levels of *vg* mRNA in a dose-dependent manner. Similar results were also obtained when A23187 (calcium ionophore) and PMA (an activator of protein kinase C) were added into the incubation medium. These results suggested that cyclic nucleotides, calcium, and protein kinase C are involved in regulating Vg transcription in the ovary (249). However, whether these signaling components were coupled to GIH activation has not been determined. Recently, a study on the mode of GIH action was carried out in *L. vannamei* using *vg* expression in the hepatopancreas as the bioassay. According to Chen et al. (250), *in vivo* injection and *in vitro* incubation of hepatopancreatic primary cells with recombinant GIH elevated the levels of intracellular cGMP, but not cAMP or nitric oxide, indicating that GIH exerts a cGMP-mediated inhibitory action. Pharmacological reagents were then tested to characterize the GIH signaling pathway. Results showed that GIH employs an rGC/cGMP/PKG signaling pathway in inhibiting *vg* expression; phosphorylation of c-Jun N-terminal kinases and upregulation of expression of p38MAPK (a mitogen-activated protein kinase) were implicated as signaling events downstream to PKG activation.

For MOIH, the only result has come from a study of *C. pagurus*, in which an increase in cAMP was observed with hormonal treatment and with cAMP analogues that mimicked the action of MOIH, implying cAMP as a second messenger for MOIH (251).

ITP/ITP-L

cAMP, cGMP, and agents that increase cAMP levels individually were able to mimic the effect of ITP on ileal short-circuit current. Additionally, synthetic ITP elevated intracellular levels of both cyclic nucleotides in a dose-dependent manner (36, 252), again implying the involvement of these two cyclic nucleotides in ITP signal transduction. Importantly, receptors of ITP and ITP-L peptides have recently been identified and characterized in the silkworm *B. mori* (94). *B. mori* orphan neuropeptide G protein-coupled receptors (BNGRs), obtained through *in silico* mining of the silkworm genome, were screened using a Ca^{2+}-imaging for activation by recombinant ITP and ITP-L peptides in HEK293T cells co-expressing a BNGR and a promiscuous mouse $G_{\alpha 15}$. Out of the 34 BNGRs tested, three Class-A BNGRs reacted positively to activation by ITPs, with BNGR-A2 and -A34 responding to ITP (EC_{50}: 1.1 x 10^{-8} M and 1.3 x 10^{-8} M, respectively) and BNGR-A24 to ITP-L (EC_{50}: 2.6 x 10^{-8} M). Interaction between ITPs and BNGRs was evaluated by an *in vitro* assay that showed co-localization of the ligand and BNGR at the cell membrane of CHO cells heterologously expressing the receptor; the interaction occurred in a ligand-receptor-specific manner, consistent with the data for the distinct receptor responses to ligand activation observed using Ca^{2+} imaging. Stimulation of *B. mori* ovary-derived (BmN) cells by either ITP or ITP-L significantly increased the intracellular cGMP levels. Coupling of the BNGRs to the ITPs-induced cGMP signaling was demonstrated by observations that simultaneous knockdown of *bngr-A2* and *-A34* significantly decreased the cGMP response to ITP in BmN cells, whereas knockdown of *bngr-A24* led to decreased cGMP responses to ITP-L; transient expression of *bngr-A24* potentiated the response of BmN cells to ITP-L. The involvement of mGC and sGC in the signaling of ITPs was examined in BmN cells using, respectively, dsRNA targeting BmGyc76c (a *B. mori* mGC) or ODQ (a selective and potent inhibitor of NO-sensitive sGC), showing that both GC forms are involved in signaling *via* ITPs through cGMP production (94). A model for the signaling pathway of ITPs in BmN cells was proposed (94), which is in general similar to the MIH model proposed by Chang and Mykles (230), except that it includes the involvement of both mGC and sGC (**Figure 5**). Thus, activation of BNGR receptors by their respective ligands leads to activation of adenylyl cyclase *via* G-protein coupling and to activation of mGC. Like the MIH model, one of the events downstream to activation of protein kinase A activation is the increase in membrane calcium conductance, leading to Ca^{2+} influx, which in turn stimulates NO-sensitive sGC by activating Ca^{2+}-calmodulin-dependent NO synthase. Increases in cGMP levels, due to the actions of both mGC and sGC, then activates protein kinase G, which together with protein kinase A, result in cellular responses (**Figure 5**).

Identification of receptor for insect ITPs not only represents an important breakthrough for insect studies but also provides an opportunity to identify the receptors for crustacean member peptides. Previous studies of molecular mass of the CHH- and MIH-binding proteins, respectively, in the hepatopancreas and Y-organ of *C. sapidus* and *M. japonicus* showed that the estimated values (51–70 kDa) were within the molecular mass range of typical GPCRs (194, 195). Given the concept that the peptide ligands co-evolve with their receptors, phylogenetic analysis of the crustacean and insect GPCRs has been performed searching for the crustacean orthologs that are phylogenetically clustered with the receptor for insect ITPs.

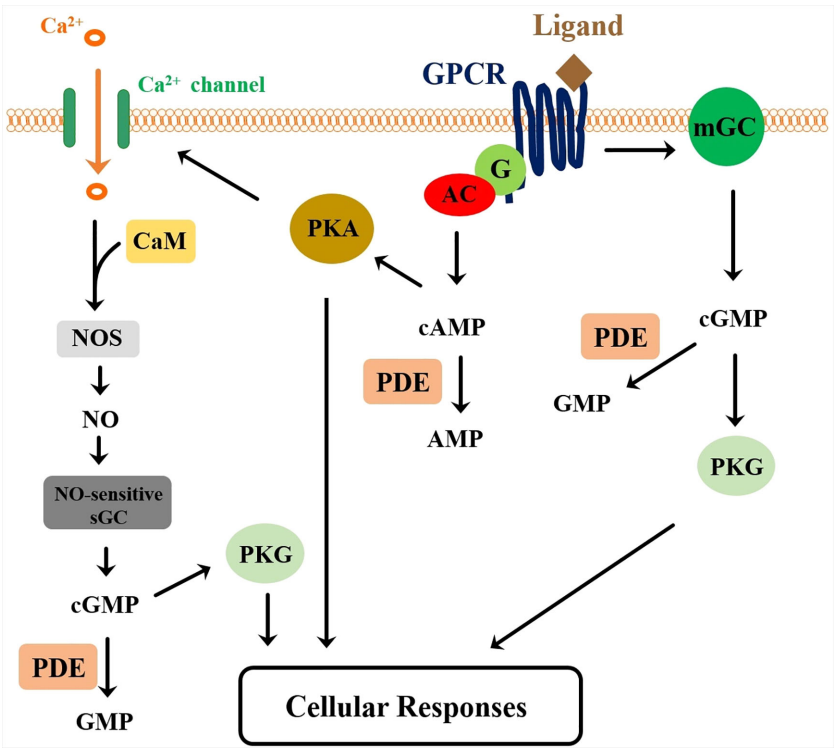

FIGURE 5 | A proposed signaling pathway for the crustacean and insect member peptides of the CHH-superfamily. A G protein-coupled receptor (GPCR) functions as the receptor for the peptide ligands. Ligand-receptor interaction leads to activation of the adenylyl cyclase (AC), *via* a coupling G protein (G), and of the membrane guanylyl cyclase (mGC), resulting respectively in the elevation of cAMP and cGMP levels. Activation of protein kinase A (PKA) by cAMP leads to the increase of intracellular Ca^{2+} levels, through phosphorylation of membrane calcium channel. Ca^{2+}/Calmodulin (CaM) complex stimulates nitric oxide synthase (NOS), increasing NO production, which in turn activates NO-sensitive soluble GC (sGC) for higher levels of cGMP production. Downstream events activated by the combined actions of PKA and PKG ultimately lead to cellular responses. Cyclic nucleotides are degraded by phosphodiesterases (PDEs). This model is a composite derived from Chang and Mykles (230) and Nagai et al. (94).

Thus, analysis of transcriptome data derived from *P. clarkii* tissues has uncovered GPCRs, *Procambarus* GPCRs A52, A53 and A63, that clustered with BNGR-A34, and *Procambarus* GPCR A9 with BNGR-A24, with A52 and A63 being abundantly expressed in the hepatopancreas, implying these GPCRs could be the crayfish CHH, MIH, or CHH-L receptor (97). In addition, analysis of the data derived from the spiny lobster *Sagmariasus verreauxi* revealed 2 annotated GPCRs (Sv-GPCRA11 and 12) that are phylogenetically clustered with BNGR-A34 (96). In the blackback land crab *G. lateralis* Y organ transcriptome 3 GPCRs (Gl-GPCRA9, Gl-GPCRA10, Gl-GPCRA12) were similarly identified using phylogenetic analysis as potential CHH-like receptors, as these sequences clustered into the putative CHH receptors clade (95). Expression of one of the GPCRs, Gl-GPCRA12, decreased in late pre-molt and post-molt stages, suggesting that it may be the MIH receptor (95). These results are promising and provide receptor candidates for testing of ligand binding and receptor activation to truly establishing the status of these receptors for the crustacean peptides.

The signaling model based on experimental data mainly derived from the studies of *G. lateralis* and *B. mori* (94, 230) is attracting and probably applicable to the crustacean and insect members of the CHH superfamily (**Figure 5**). Several major coupling events however need experimental verification, including coupling of GPCR activation to adenylyl cyclase, GPCR activation of membrane GC (as suggested in the ITPs signaling), PKA phosphorylation-induced Ca^{2+} influx and subsequent NO production. In addition, for the model to be applicable to the crustacean CHH-superfamily peptides, including CHH, the model would have to be refined, based on data from additional studies, to accommodate the apparently contradictory data derived from other studies. For example, in response to hormonal stimulation, the change in cAMP levels was usually small and insignificant (192, 235, 237) and manipulation of cAMP levels or adenylyl cyclase activity did not mimic the effect of hormonal treatment (192). Further, it is interesting note that the lobster CHH stimulated the guanylyl cyclase activity in isolated membrane (233). Thus, GPCR activation of mGC would occur within the membrane without signaling through the cytoplasm. A GPCR-activated mGC event within the membranes, if proven, would probably represent a novel mode of GPCR signaling mechanism. Finally, it is now feasible to conduct experiments for ligand-receptor interaction

and activation, with the availability of identified receptors and of information regarding ligand structure and structure-function relationship of several peptides, including MIH, CHH, CHH-L, ITP (87–93).

CONCLUSION AND PROSPECTIVE DEVELOPMENTS

The field of study of the CHH superfamily is now at a challenging and promising phase. With more research efforts, most likely through mining newly available genomes and transcriptomes from additional ecdysozoan taxa, it is certain that new members of the superfamily will be discovered. The phylogenetic profile would be updated as new members are admitted to the superfamily. The fact remains that the study of crustaceans suffers, at least at the level of the malacostracans, and in comparison with hexapods, from genomes of often enormous size, which constitutes an obstacle in technical as well as financial terms. The hypothetical multiplication of genomes of these taxa has certainly actively promoted diversity with the appearance of paralogues and processes of sub-functionalization.

Evolutionary recruitment of the CHH-superfamily peptides as venom toxins (43) elegantly illustrates how changes in the structural characteristics of the peptides during the course of evolution has led to the emergence of novel functions, which indeed carries significant implications for the structure-function relationships of the crustacean member peptides. In this context, we could also question the fact that, in crustaceans where isoforms of the superfamily members are the most numerous, no venomous forms have yet been identified (except for the remipede *Speleonectes tulumensis* (253) that has toxins unrelated to the CHH superfamily), whereas this is the case in other major arthropod phyla. On the other hand, post-transcriptional and post-translational mechanisms that are well known for structurally and functionally diversifying the superfamily peptides are expected (and have actually been suggested by recent studies) to be also working on the venom peptide-encoding genes and the peptides. We likely will see more instances where results gained from the study of one group of peptides of the superfamily "complement" or "echo" those from studies of others.

While the crustacean and insect member peptides of the CHH superfamily have been characterized and identified, each with a distinct functional assay, progress in the functional front has been limited, if compared to the number of publications devoted to identification of the peptides and genes. Technical resources are essential for functional studies. Recombinant protein production, RNAi, and various functional genomics methods are now readily available and have indeed been widely used by researchers in the field. In this context, one particular technical obstacle for the study of CHH and ITP is the limited availability of biologically active peptides, production of which usually relies on costly and tedious modifications (*e.g.*, the C-terminal amidation) and refolding of bacterially-produced peptides. The obstacle becomes almost prohibitively challenging for structural studies that require large amounts of peptide. Alternatives, *e.g.*, chemical synthesis (133, 254) and eukaryotic expression systems (88, 255), could be explored to optimize the production of biologically active peptides at larger scales. Advancements in the functional front are expected if more research efforts are to be made. A persuasive example is a sophisticated model for the regulation of steroidogenesis in the Y-organ built mainly based upon functional genomics data [see (245)]. Additional genetic resources enjoyed by the study of insects, especially those working in the fruit fly *D. melanogaster*, as exemplified by a study of ITP (174), would probably render a faster development for the study of insect member peptides. On the application side, initial attempts were made in developing methods for manipulation of ovarian maturation and control of viral diseases respectively through silencing of *gih* and *chh* genes by RNAi (179, 217), a technical approach that has been put to practical use in aquaculture industry in the case of manipulating a crustacean insulin-like androgenic gland hormone (256).

Functional studies would be greatly aided by the identification of receptors for the superfamily peptides, which was finally realized by the discovery of the receptors for the silkworm ITPs (94). Crustacean orthologues of the silkworm ITP receptors were suggested to be the receptors for the crustacean member peptides (95–97). The pressing issue would be to provide experimental data testing whether the ligands bind and activate the candidate receptors. With the receptor for the peptides being identified, confirmation of the target tissues of a given peptide and the signal transduction pathways coupled to receptor activation could be examined. Interesting and physiologically relevant questions could be asked, for instance, regarding a function shared by two superfamily peptides (functional overlap) or a multi-functional (pleiotropic) peptide. For example, whether the Y-organ cells express both MIH and CHH receptors, as suggested by the radiolabeled-ligand binding experiments? How are the respective signaling pathways activated by MIH and CHH orchestrated in regulating steroidogenesis in the Y-organ? The prospective developments for the functional study of the CHH superfamily in a post-receptor era are promising and the discoveries to be made will certainly be rewarding!

AUTHOR CONTRIBUTIONS

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REVIEW
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Insights on Molecular Mechanisms of Ovarian Development in Decapod Crustacea: Focus on Vitellogenesis-Stimulating Factors and Pathways

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Vitellogenesis in crustaceans is an energy-consuming process. Though the underlying mechanisms of ovarian maturation in decapod Crustacea are still unclear, evidence indicates the process to be regulated by antagonistically-acting inhibitory and stimulating factors specifically originating from X-organ/sinus gland (XO/SG) complex. Among the reported neuromediators, neuropeptides belonging to the crustacean hyperglycemic hormone (CHH)-family have been studied extensively. The structure and dynamics of inhibitory action of vitellogenesis-inhibiting hormone (VIH) on vitellogenesis have been demonstrated in several species. Similarly, the stimulatory effects of other neuropeptides of the CHH-family on crustacean vitellogenesis have also been validated. Advancement in transcriptomic sequencing and comparative genome analysis has led to the discovery of a large number of neuromediators, peptides, and putative peptide receptors having pleiotropic and novel functions in decapod reproduction. Furthermore, differing research strategies have indicated that neurotransmitters and steroid hormones play an integrative role by stimulating neuropeptide secretion, thus demonstrating the complex intertwining of regulatory factors in reproduction. However, the molecular mechanisms by which the combinatorial effect of eyestalk hormones, neuromediators and other factors coordinate to regulate ovarian maturation remain elusive. These multifunctional substances are speculated to control ovarian maturation possibly via the autocrine/paracrine pathway by acting directly on the gonads or by indirectly exerting their stimulatory effects by triggering the release of a putative gonad stimulating factor from the thoracic ganglion. Acting through receptors, they possibly affect levels of cyclic nucleotides (cAMP and cGMP) and Ca^{2+} in target tissues leading to the regulation of vitellogenesis. The "stimulatory paradox" effect of eyestalk ablation on ovarian maturation continues to be exploited in commercial aquaculture operations, and is outweighed by the detrimental physiological effects of this procedure. In this regard, the development of efficient alternatives to eyestalk ablation based on scientific knowledge is a necessity. In this article, we focus principally on the signaling pathways of positive neuromediators and other factors regulating crustacean reproduction, providing

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an overview of their proposed receptor-mediated stimulatory mechanisms, intracellular signaling, and probable interaction with other hormonal signals. Finally, we provide insight into future research directions on crustacean reproduction as well as potential applications of such research to aquaculture technology development.

Keywords: commercial aquaculture, crustacea, methyl farmesoate, red pigment-concentrating hormone, serotonin, vitellogenesis, vitellogenesis-stimulating hormone

INTRODUCTION

Female reproduction in decapod crustaceans is a complex and precisely regulated biological process controlled by an elaborate endocrine system. Vitellogenesis, the production and accumulation of vitellin in developing oocytes, is crucial to ovarian maturation (1–3) and relies on the coordinated actions of environmental cues and hormones along the hypothalamic-pituitary-gonadal axis in vertebrates (4, 5). Analogous to the vertebrate hypothalamic/neurohypophyseal system, in crustaceans, a neurosecretory tissue in the eyestalks, the X-organ/sinus gland (XO/SG) complex, along with the central nervous system, release several neuropeptides that exert responses in other distant target tissues (6–8). Though the antagonistic and multi-interlinked neuroendocrine cascades are not as well understood in crustaceans as in vertebrates, a bi-hormonal signaling axis consisting of neuropeptides [negatively-acting vitellogenesis-inhibiting hormone (VIH), also known as gonad-inhibiting hormone (GIH) and the putative vitellogenesis-stimulating hormone (VSH)] is considered to exist. Such substances are thought to be synthesized and released into the circulation, and may then bind G protein-coupled receptors (GPCRs) on target cell surfaces, thus activating downstream cascades as a central facet of crustacean reproductive endocrinology (1, 9, 10).

Among the known neuroendocrine factors regulating crustacean reproduction, the eyestalk neuropeptides of the crustacean hyperglycemic hormone (CHH)-family encoding multi-genes (crustacean hyperglycemic hormone (CHH), gonad/vitellogenesis-inhibiting hormone (GIH/VIH), molt-inhibiting hormone (MIH), and mandibular organ inhibiting hormone (MOIH)) have been studied intensively for their broad-spectrum roles (9–12). Amongst the CHH-family peptides, VIH has received much attention for its inhibitory effects on vitellogenesis in crustaceans whereby it suppresses vitellogenin production in the target tissues or inhibits protein uptake by the oocytes (13, 14). In the whiteleg shrimp, *Litopenaeus vannamei*, levels of VIH in the hemolymph were lower during vitellogenesis than at the immature and previtellogenic stages, in agreement with its inhibitory role in vitellogenesis (15). Silencing of VIH gene expression (16–18) and injection of VIH recombinant protein (19) has further provided confirmatory evidence for its inhibitory roles on ovary development. Other members of the CHH-family are reported to have various functions; the regulation of carbohydrate metabolism, inhibition of ecdysteroid synthesis at the Y-organs, and the suppression of methyl farnesoate synthesis at the mandibular organs are attributed to CHH, MIH, and MOIH, respectively (9–12). However, these substances also play crucial roles in regulating maturation in crustaceans. Isoforms of CHH and MIH were reported to have a stimulatory effect on the growth of oocytes and vitellogenesis in the American lobster, *Homarus americanus* and in *L. vannamei* (20, 21). The use of recombinant protein and RNAi has been useful in elucidating the roles of eyestalk hormones; for example, CHH was shown to regulate the gene expression of insulin-like androgenic gland hormone (IAG) in male *L. vannamei* (22) and such methodology provided supporting evidence for a stimulatory function of MIH in reproduction (23). In addition, several studies have proposed the existence of a gonad-stimulating factor, the so-called VSH, in the brain and thoracic ganglia of crustaceans, although its identity is still unknown.

Unilateral eyestalk ablation, a commonly practiced technique used to manipulate the endocrine system in order to stimulate gonadal maturation and spawning in captivity, is based on the assumption that eyestalk removal diminishes VIH production. However, this destructive technique alters the physiology of the animal, resulting in its offspring, or seed, having inferior quality and being of less quantity, with subsequent reduced performance and death of the parent broodstock (24). Consequently, developing alternative techniques to eyestalk ablation for controlling ovarian maturation in captivity is challenging, and attempts at resolving such issues have focused on the administration of exogenous compounds to stimulate vitellogenesis (25–29), and to reduce circulatory levels of inhibitory hormones via the use of VIH antibodies and RNA interference (RNAi) to achieve host-induced gene silencing of VIH (17–19).

In addition to eyestalk peptides, the neuroendocrine regulation of crustacean reproduction involves the interactions of several auxiliary factors including biogenic amine neurotransmitters (7, 30), as well as ecdysteroids (31) and the sesquiterpenoid methyl farnesoate (MF) (32) which are synthesized and released by the Y-organ and the mandibular organs, respectively. Thus, an intricate network, consisting of an array of chemically diverse molecules, harmonizes to establish a signaling cascade regulating gonad development in crustaceans. However, the molecular mechanisms by which these multifunctional neuromediators and other factors are involved in the upstream control of neuropeptide hormone release from the XO/SG complex, as well as from other neuroendocrine organs and secretory neurons, remains elusive. A simplified schematic diagram of the control of molting and reproduction in decapod Crustacea is shown in **Figure 1**. Various external factors

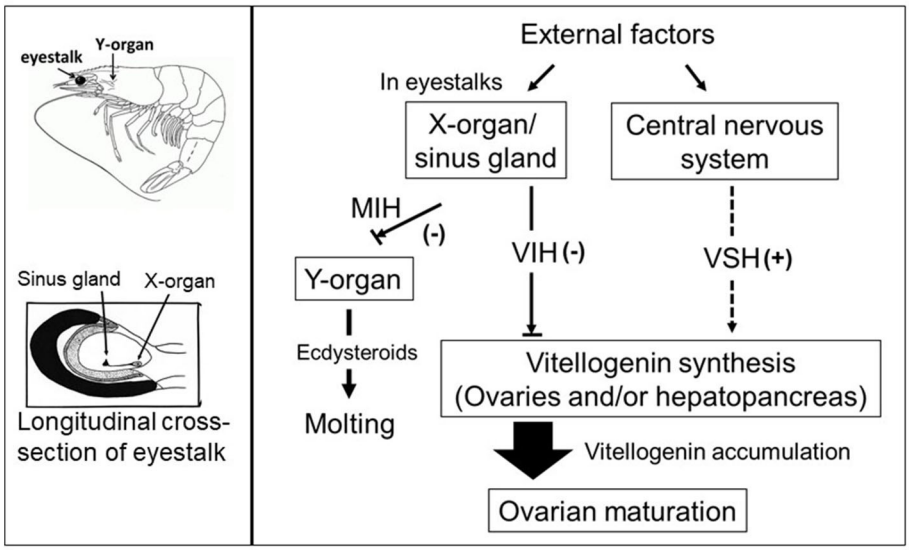

FIGURE 1 | Simplified schematic diagram of the control of molting and reproduction in decapod Crustacea (right-hand side). External factors such as water temperature, salinity, and pressure, season and daylength, and availability of nutrition, are thought to ultimately influence the X-organ/sinus gland (XO/SG) complex and central nervous system. It is well-established that the XO/SG complex is the source of vitellogenesis-inhibiting hormone (VIH) and molt-inhibiting hormone (MIH). In contrast, there are many potential pathways that fulfill the role of putative vitellogenesis-stimulating hormone (VSH) as described in the text. The locations of the eyestalks and Y-organs are indicated using a generic drawing of a shrimp (top left), and a close-up representation of the eyestalk is also presented (bottom left).

related to water temperature, salinity, and pressure, season and daylength, and availability of nutrition, are thought to ultimately influence the X-organ/sinus gland (XO/SG) complex and central nervous system. This figure has been abbreviated to show only the involvement of VIH and putative VSH; it is on the basis of this concept that eyestalk ablation is used in commercial hatcheries in order to diminish VIH levels and induce maturation/spawning.

More recently, advances in *in silico* neuropeptidome research and comparative genome analysis have led to the discovery of a large number of decapod hormones and neuropeptide-encoding transcripts in the crustacean eyestalk, cerebral ganglia, and also in extra-neural tissues (namely heart, midgut, gills, hepatopancreas, muscle, stomach, and ovaries) with pleiotropic and novel functions including reproduction in several crustacean species (33–35). Research findings also suggest that these neuropeptides harbor autocrine and/or paracrine functions, although the roles of many of them in crustaceans are yet unknown (9, 33, 35). Additionally, although a publicly available complete decapod genome is now available for *L. vannamei* (36) and the marbled crayfish, *Procambarus virginalis* (37), it remains difficult to interpret the roles of the majority of novel neuropeptides identified in crustaceans. Nevertheless, such information will be of help in interpreting the putative roles of identified novel neuropeptides. Therefore, in consideration of the above, while the negatively-controlled facets of reproduction are fairly-well understood, the stimulatory side of reproductive control offers scope for further research. This review therefore focuses on the possible signaling pathways of non-CHH-family neuromediators and other factors that are thought to promote gonadotrophic activity in female decapod crustaceans, with the aim of clarifying what is currently known, and possibly suggesting a means of developing viable technology for the control of female maturation in captivity. We thus provide an overview of reported receptor-mediated stimulatory effects of such substances, intracellular signaling, and their synergy with other hormonal signals, in order to underline both their known and putative roles in crustacean reproduction.

SEROTONIN

Serotonergic Pathways Regulate Ovarian Maturation in Crustaceans

The regulatory role of neurotransmitters in crustacean reproduction is well-documented (38). Among the identified major neurotransmitters in crustaceans (serotonin, dopamine, melatonin, and octopamine), serotonin (also referred to as 5-hydroxytryptamine, 5-HT), a biogenic amine derived from the amino acid tryptophan, has been reported to play a prominent role in crustacean reproduction. It is ubiquitously found in several microorganisms, and throughout the plant and animal world in significant amounts; it functions as a neurotransmitter in the brain and as a neurohormone in the periphery, regulating several important physiological functions both in vertebrates and invertebrates. In invertebrates, serotonin modulates multiple functions including circadian rhythms (39), neurogenesis (40), osmotic adjustment (41), growth (42), aggression (43), molting (44), and reproduction (27). Several reports have correlated the role of serotonin in the regulation of growth and reproductive development of vertebrates, rather than focusing on invertebrates. In fishes, it has been reported as a regulator of follicular growth (45). In invertebrates, the serotonin

system plays a unique role in the initiation and maintenance of reproductive function.

Emerging evidence suggests serotonin to be the upstream neurotransmitter controlling reproduction by acting at the eyestalks and the central nervous system in crustaceans (7, 30, 46, 47). Serotonergic neuromodulation of reproductive function resulted in a significant increase in oocyte diameter and enhanced ovarian maturation in *P. indicus* (27, 48), *Litopenaeus vannamei* (49), *Penaeus monodon* (50), *Fenneropenaeus merguiensis* (51), *Macrobrachium rosenbergii* (46, 52), and *Procambarus clarkii* (53). Injection of serotonin to the giant freshwater prawn *M. rosenbergii,* shortened the normal duration required for ovarian maturation in females and brought about a significant increase in the testis-somatic index in males (54). Furthermore, induced precocious reproduction was reported in the freshwater edible crab *Oziothelphusa senex senex* following administration of serotonin (55). Increasing levels of serotonin were observed in the ovaries of *P. monodon* (50), *L. vannamei* (56), and *M. rosenbergii* (52) during the early ovarian maturation stages, reaching maximum levels at the mature ovarian stages. In support of this observation, Soonthornsumrith et al. (57) also suggested that serotonin in both the central nervous system and ovary act in harmony to control oocyte maturation. A combination of spiperone (a dopamine antagonist) and serotonin was shown to induce ovarian maturation in *M. rosenbergii* (58) together with enhanced spawning in *L. vannamei* and *Litopenaeus stylirostris* (59) compared with the injection of serotonin alone. Serotonin also stimulated male reproductive parameters and testis development in the narrow clawed crayfish, *Pontastacus leptodactylus* (60) and the crayfish *P. clarkii* (61).

The endocrine pathway where serotonin is considered to stimulate downstream reproductive hormones is hypothesized to be performed mainly by the action of serotonin at the eyestalk and/or the central nervous system level. In more detail, serotonin may function to regulate the synthesis and release of endocrine factors such as VSH and/or VIH, or may also act upon red pigment-concentrating hormone (RPCH) to serve as an intermediary substance, causing its release from eyestalk neural tissue, which may, in turn, stimulate the release of the putative VSH (27, 38, 46, 47). A faster response in ovarian maturation observed in ablated females of *P. indicus and F. merguiensis* treated with serotonin suggests that lowering levels of inhibitory hormones is an essential prerequisite for serotonin to effectively induce maturation (27, 62). Tomy et al. (27) provided confirmatory evidence for serotonin-induced ovarian development to be significant but less effective than eyestalk ablation. Meeratana et al. (46) reported that serotonin-primed thoracic ganglion medium accelerated ovarian development in *M. rosenbergii*, indicating an indirect effect of serotonin on reproduction. The localization of serotonin-immunoreactive cells in the X-organ neurons and fibers innervating the sinus gland tissue and in other parts of the central nervous system of various decapod crustaceans (57, 63) support the suggested regulatory role of serotonin in the synthesis and release of other neurohormones from the XO/SG complex such as CHH (64, 65) and probably other neuropeptides of the CHH-family (56). However, the presence of serotonin immunoreactivity was also observed in crustacean gonads (46, 56, 57, 63) and its stimulatory effects on gonad maturation confirmed, thereby further advocating an autocrine/paracrine mode of receptor-mediated action for serotonin in the control of crustacean oocyte maturation.

In crustaceans, the serotonergic system constitutes a distinct signaling system that probably acts synergistically with other stimulatory factors forming an overarching system that regulates vitellogenesis and oocyte maturation. Contrary to the negative regulation of vitellogenesis performed by the CHH-family peptide VIH, serotonin is reported to regulate in a stimulatory manner, directly or indirectly, the action of other hormones and reproductive-related proteins in crustaceans such as RPCH (66), MIH (67), tachykinin and neuropeptide F (NPF; the invertebrate equivalent of neuropeptide Y) (68), and farnesoic acid O-methyltransferase (FAMeT) (29). Sathyanandam et al. (64) reported that serotonin injection resulted in hyperglycemia by triggering the release of CHH in *P. indicus*. A recent report by Soonthornsumrith et al. (57) indicated that serotonin enhanced the secretion of ovarian steroids (estradiol and progesterone) in mature ovarian explants from *M. rosenbergii* and suggested that serotonin-regulated ovarian maturation via the induction of female sex steroid hormone release in turn stimulated vitellogenesis.

Girish et al. (69) proposed that serotonin treatment increases the gene expression levels of retinoid X receptor (RXR) and ecdysteroid receptor (EcR) in the hepatopancreas and ovary of *Scylla serrata*, thereby upregulating methyl farnesoate and ecdysteroid synthesis, respectively. Furthermore, the upregulation of three reproductive-related genes, namely those of farnesoic acid O-methyltransferase (*FAMeT*), estrogen sulfotransferase (*ESULT*) and prostaglandin F synthase (*PGFS*) was evidenced by transcriptome data from the nervous tissue of female mud crab (*Scylla olivacea*) injected with serotonin; this is considered to provide additional supporting evidence of the stimulatory role of serotonin (47). Finally, serotonin was proposed to enhance the release of GnRH-like peptide from the nervous tissue with the result of stimulating reproduction in male *M. rosenbergii*; most likely, this occurred via a mechanism where the release of VIH from eyestalks is inhibited (70).

The serotonin system may also be modulated by reproductive hormones. In mammals, ovarian steroids such as progesterone and estrogen regulate the content of serotonin in the brain (71). Collectively, these results suggest that serotonin is a potent gonadotrophic agent in crustaceans, the actions of which are closely associated with other reproductive endocrine signaling pathways. However, there remain critical caveats in our understanding of the precise role of serotonin in crustaceans, specifically their potential interactive roles with other neurochemical systems; in this regard, a more complete holistic view of how crustacean reproduction is regulated, needs to be achieved.

Receptor-Mediated Action of Serotonin

Serotonin coordinates several physiological processes in both vertebrates and invertebrates through differential binding with specific cell-surface receptors (G protein-coupled receptors,

GPCRs or ligand-gated ion channels), that activate an intracellular second messenger cascade (including cAMP and protein kinase, PKA), to elicit a serotonergic response. RNAi-mediated gene silencing of the serotonin receptor has provided evidence for the receptor-mediated action of serotonin (72). Based on structure, signaling mechanisms, biochemical, and pharmacological properties, the vertebrate serotonin receptors have been assigned to seven receptor classes (5-HT1 to 5-HT7) consisting of six GPCRs (5HT1-5HT7), and a ligand-gated ion channel (5-HT3 receptor) (73).

The GPCRs are characterized by seven transmembrane domains, an extracellular N-terminus and the intracellular C-terminus and are associated with heterotrimeric G proteins (a polypeptide comprised of a Gα subunit which binds and hydrolyzes GTP, a Gβ and Gγ subunits), that are classified into four families (i.e., Gs, Gq, Gi/o, and G12/13) based on their specific type of Gα-subunit. The activated GPCR stimulates the dissociation of the interacting heterotrimeric G protein into a Gα subunit and a Gβγ complex, thereby coordinating the downstream signal pathway in accordance with the activation of the Gα subunit. Among the G-coupled protein serotonin receptors, 5-HT1 and 5-HT5 associate preferentially with Gαi subunit of the heterodimeric protein (Gi/o) and impede cAMP synthesis, whereas 5-HT4, 5-HT6, and 5-HT7 couple preferentially with the Gαs alpha subunit (Gs) of G protein, leading to increased cAMP production. Levels of protein kinase A (PKA), a cAMP-dependent protein kinase, enhances many functions in the cell. The Gq (Gq alpha subunit)-coupled 5-HT2 mediates the hydrolysis of inositol phosphates and cause a subsequent increase in cytosolic Ca^{2+}.

Blenau and Baumann (74) reported that the sequences of various serotonin receptors are conserved among vertebrates and invertebrates, indicative of their crucial functions across species. Orthologous vertebrate serotonergic GPCRs with conserved signaling pathways have been cloned and characterized in invertebrates, including crustaceans, and have been shown to belong to three major types of vertebrate GPCRs groups (5-HT1, 5-HT2, and 5-HT7) (75). An additional receptor, MOD-1, a serotonin-gated ion channel, was found in *C. elegans* and exhibited similarities to the mammalian 5-HT3 receptor, but with differences in function (76). Qi et al. (77) reported a novel receptor in the butterfly *Pieris rapae*, that was classified into a new family of receptors designated as 5-HT8. As in vertebrates, serotonin activates different downstream signaling pathways in order to control levels of second messengers, specifically adenylate cyclase activity and cAMP production (inhibited by Gi-coupled 5-HT1-like receptors, but stimulated by Gs-coupled 5-HT7-like receptors) or phospholipase C, and subsequently increases Ca^{2+} (stimulated by Gq-coupled 5-HT2-like receptors) (75), to exert both inhibitory and excitatory effects.

Serotonin and the Onset of Oocyte Germinal Vesicle Breakdown/Serotonergic Regulation of Oocyte Germinal Vesicle Breakdown

As a "universal" conserved principle, developing primary oocytes are arrested at prophase I in advance of the ensuing events of oocyte maturation (78). The resumption of oocyte meiotic maturation, germinal vesicle breakdown (GVBD), and final release of the mature egg from the ovary are essential processes in sexual reproduction. These events are triggered initially by substances referred to collectively as "maturation initiation hormones." More detailed discussion is beyond the scope of this review, but such maturation initiation hormones represent a variety of molecular identities, and have been reported in a range of species with the exception of mammals. This process is further orchestrated by maturation/M-phase promoting factors (an auto-regulated complex of cyclin-dependent kinase cdk1/cdc2 and its regulatory subunit cyclin B formed during oocyte maturation) and is highly conserved among animal species across the phylogenetic spectrum, thus establishing possible marker genes of oocyte developmental competence (79). The signal transduction pathway regulating maturation initiation hormone activity and subsequent process of maturation are highly complex. In vertebrates, active GPCRs coupled to Gs (e.g., Gs alpha subunit of G protein, a GTPase that acts as a cellular signaling protein) stimulate adenylate cyclase to maintain high intracellular second messenger cAMP concentrations in oocyte; this inhibits the resumption of prophase I arrest in many animal oocytes (80). Serotonergic receptors (Gq-coupled 5-HT2) are responsible for mobilizing Ca^{2+} levels in oocytes which in turn, leads to the hydrolysis of inositol phosphate and an increase in cytosolic Ca^{2+} levels (81). The maturation initiating hormone overrides this prophase arrest to initiate meiotic resumption and maturation by activating cdc2/cyclin B in oocytes, the mechanisms of which are yet unclear.

As discussed above, although the role of maturation initiation hormones during oocyte maturation has been well-studied in a wide variety of eukaryotic organisms, little is known regarding crustacean species. Cortical rod formation and GVBD are the hallmarks of oocyte maturation in penaeid shrimps (82). In naturally maturing penaeid females, elevated expression levels of reproductive genes (*vg*, *cdc2*, *cyclin B,* and *tsp)* (e.g., thrombospondin, a major component of cortical rods in penaeid shrimps) are seen with the advancement of ovarian maturation, and are considered to be indicators of the developmental competence of the oocytes (27, 83, 84). In addition, elevated expression of serotonergic receptors in invertebrate species seen at the time of maturity is considered to indicate an underlying function in promoting oocyte maturation (85). The direct receptor-mediated action of serotonin on oocytes in marine nemertean worms was associated with variation in intracellular Ca^{2+} and cAMP levels, activation of cdc2/cyclin B in oocytes, and the stimulation of meiotic resumption and maturation (86). Similarly, serotonin rapidly stimulated meiotic resumption and GVBD of oocytes in the mud crab, *Scylla paramamosain*, through receptor-mediated signaling activity downstream of cAMP pathways (85). This negative correlation between GVBD and cAMP levels was further confirmed by forskolin treatment which significantly blocked serotonin-induced GVBD (85). In the Chinese mitten crab, *Eriocheir sinensis,* cdc2 kinase and cyclin B were highly expressed in GVBD oocytes (84). Furthermore, microRNA (miR-2 and miR-133) was proposed to regulate oocyte meiosis in *E. sinensis* by inhibiting the translation or post-translation of cyclin B during meiosis (87).

Tomy et al. (27) reported that the action of serotonin resulted in the resumption of oocyte meiotic maturation in Indian white shrimp, *Penaeus indicus*, by stimulating the formation of a putative maturation initiation hormone which was more significant in ablated shrimps. As shown in **Figure 2**, previous work by this author revealed that ablated shrimp were in the advanced stages of ovarian development as noted by the presence of numerous eosinophilic yolk granules in the peripheral ooplasm. Serotonin-treated ablated shrimps exhibited even more pronounced changes, including the presence of yolk globules in the granular cytoplasm and distinct eosinophilic club-shaped cortical rods extending toward the nucleus of elongated oocytes. In addition, the differential expression of ovarian genes involved in vitellogenesis [vitellogenin (*vg*), vitellogenin receptor (*vgr*)] and meiotic maturation [cyclin-dependent kinase 2 (*cdc2*), cyclin B and thrombospondin (*tsp*)] was shown to increase significantly in these groups, indicating that post-vitellogenic meiotic resumption and maturation of the oocytes had occurred (**Figure 3**). Furthermore, based on cytological and molecular evidence, the authors corroborated the stimulatory effects of serotonin on ovarian maturation in *P. indicus*, and proposed the possibility of a dual regulatory role of serotonin in both vitellogenesis and oocyte maturation in penaeid shrimps (27). However, a delayed response was observed in shrimps with intact eyestalks treated with serotonin. This substantiated the hypothesis that the stimulatory impulse of serotonin to trigger final maturation is comparatively less effective in the presence of VIH/GIH from the eyestalks. Along the lines of the above, although a great deal has been learned about the effects of serotonin on crustacean maturation, the pathways through which serotonin exerts its gonadotropic influence remains to be fully determined. Additional studies employing RNAi, recombinant protein methodology and gene editing techniques, will aid in gaining a better understanding of the positively-controlled aspects of crustacean reproductive endocrinology.

RED PIGMENT-CONCENTRATING HORMONE (RPCH)

Involvement of RPCH in Crustacean Reproduction

A related, but distinct, peptide signaling system in crustaceans is constituted by red pigment-concentrating hormone (RPCH). It was the first invertebrate neuropeptide to be fully characterized (from the eyestalks of *Pandalus borealis*) (88) and is involved in the distribution of pigments in response to environment (89) and light-dark adaptation (90). The structural similarity among RPCH, adipokinetic hormone (AKH), corazonin (Crz), adipokinetic hormone/corazonin-related peptide (ACP) and gonadotropin-releasing hormone (GnRH) in various animals resulted in clustering them together into the GnRH-superfamily (91). In decapod crustaceans, RPCH is synthesized in the XO/SG complex of the eyestalks, but is also expressed in all major parts of the nervous system, where it is suggested to act as a neurotransmitter having dual endocrine and autocrine/paracrine function (66, 92, 93).

Based on its amino acid composition and chromatographic characteristics, the primary structure of mature RPCH as sequenced from several decapod crustaceans is known to be conserved; the molecule exhibits the same octapeptide sequence (pQLNFSPGWamide), blocked N-(pyroglutamate) and C-termini (carboxyamide) features, and presence of aromatic amino acids at positions 4 (phenylalanine) and 8 (tryptophan) in various species (66, 94). Such a blocked ligand was reported to be less susceptible to the action of exopeptidases in the hemolymph insects, thus necessitating a longer half-life of the peptide to achieve its hormonal effects (94). Recently, the existence of a variant isoform of RPCH (pQVNFSTSWamide) was identified in daphnids through transcriptome mining (95). Similarly, Christie (96) identified a structurally modified RPCH in the carp louse, *Argulus siamensis*.

RPCH plays multifunctional roles in crustaceans, regulating lipid and carbohydrate mobilization (97, 98), mediating circadian (39, 89), and swimmeret rhythms (99), modulating the stomatogastric nervous system in crustaceans (100) and stimulating MF production from the mandibular organs (101). Increased glucose levels could be elicited in the hemolymph of the isopod *Porcellio scaber* injected with synthetic RPCH, suggesting a novel role for this neuropeptide in carbohydrate mobilization by causing the release of crustacean hyperglycemic hormone (CHH) (102). Sathapondecha et al. (92) reported that the expression of RPCH was transiently stimulated upon hypersalinity change within 12 h in *P. monodon,* suggesting its osmoregulatory functioning. Furthermore, the study also revealed that injection of RPCH peptide increased gill Na^+/K^+ ATPase activity in 36–48 h after injection. Moreover, RPCH was reported to play a role in molting, probably by mediating hemolymph osmolality and ion transport enzymes during the late premolt stages.

RPCH has also been shown to have a potentially critical role in reproduction in crustaceans. RPCH transcripts were detected in ovaries and heart in addition to the neural tissue in *L. vannamei* (28), supportive of a role for this hormone in reproduction. *In vitro* co-incubation of ovary explant with nervous tissue caused significant oocyte growth when RPCH was added to the explant culture in several crustacean species (38, 103, 104). Fingerman (38) further confirmed that the combined effects of RPCH and thoracic ganglia on ovarian maturation was more significant compared to that with explants incubated with thoracic ganglia alone; this suggested that RPCH, as proposed for serotonin, acts as neurotransmitter stimulating the release of VSH, with calcium acting as a second messenger for RPCH. Contrary to the indirect role of RPCH in reproduction, Chen et al. (28) investigated the effects of synthetic RPCH on ovarian maturation in *L. vannamei* and reported higher vitellogenin mRNA expression in ovaries, increased protein levels in hemolymph, and enlarged oocyte area in treated shrimps (**Figure 4**); thus, a hypothesis was proposed where RPCH may have a more direct in vitellogenesis. Up-regulation of RPCH in eyestalks following serotonin-induced maturation in the whiteleg shrimp *L. vannamei* (28) and crab, *S. olivacea* (66) suggested

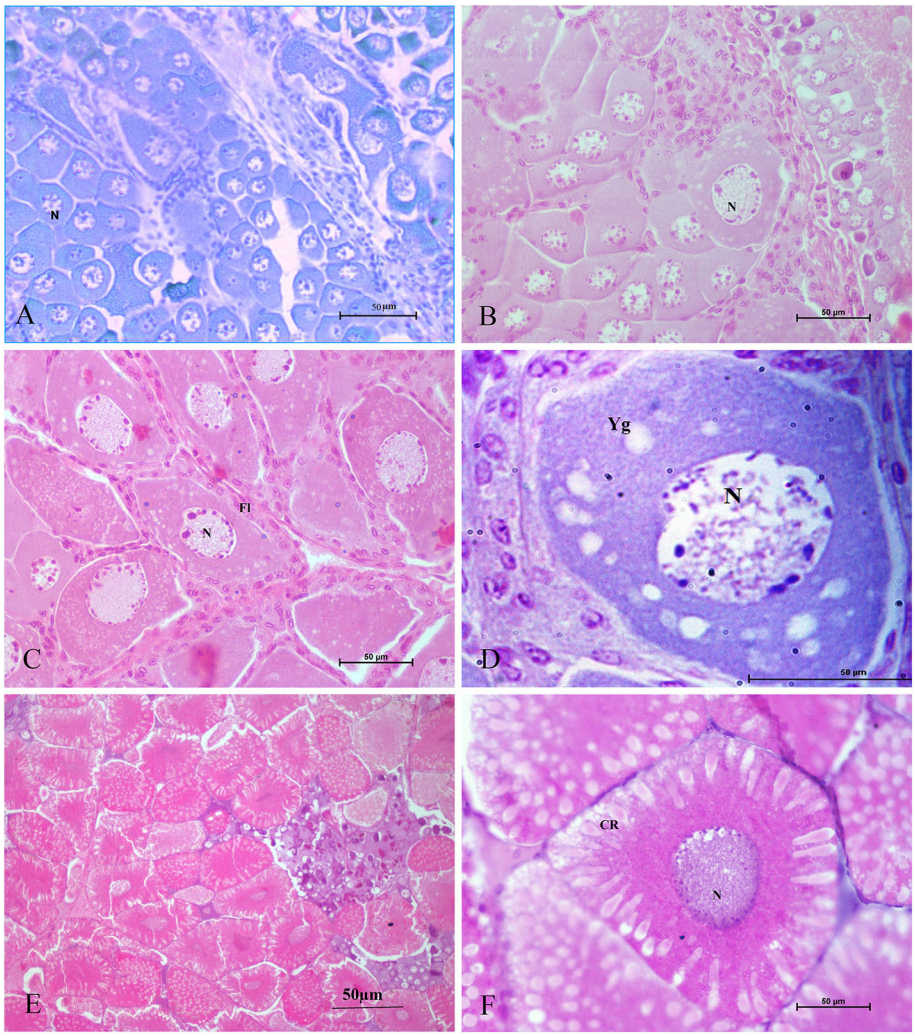

FIGURE 2 | Histological sections of ovarian tissues collected from *Penaeus indicus* 14 days after treatment and stained with hematoxylin and eosin. Ovarian tissues shown are as follows: **(A)** control (10×), **(B)** serotonin group (40×), **(C)** eyestalk-ablated group in the early vitellogenic stage with numerous eosinophilic yolk granules in the peripheral ooplasm; **(D)** eyestalk-ablated group with an early vitellogenic oocyte (100×); **(E)** serotonin + eyestalk-ablated group with oocytes having club-shaped cortical rods extending the nucleus (10×) and **(F)** mature oocyte with cortical rods (40×). CR: cortical rod; Fl: follicle layer; N: nucleus; Yg: yolk granules. Reproduced with permission from Tomy et al. (27).

that the downstream roles of serotonin in reproduction are mediated through RPCH. In red swamp crayfish, *P. clarkii*, RPCH promoted the synthesis and release of methyl farnesoate from the mandibular organs, which in turn regulates maturation (101). On the other hand, a significant decrease in RPCH levels in eyestalk and nervous tissue were suggested as the probable cause for ovarian degeneration in the freshwater shrimp *Macrobrachium nipponense* (105). Collectively, these findings indicate that RPCH plays a vital role in regulating reproductive functions in a similar pattern, as suggested for serotonin, via the autocrine/paracrine pathway and culminating in the release of VSH/GSH from the nervous tissues. Given the role of RPCH in reproduction, further studies utilizing different approaches such as dsRNA-mediated gene silencing or CRISPR-mediated gene editing, may lead to new insights on the role of RPCH in ovarian maturation.

Receptor-Mediated Action of RPCH

Like other neuropeptides, RPCH exerts its effects on target cells by binding to its receptors with high affinity. Since RPCH peptides identified in decapods to date harbor identical or near-identical sequence, it is assumed that the decapod RPCH receptor (RPCHR) is also conserved in terms of its ability to bind to RPCH/AKH ligands. Buckley et al. (106) proposed the identity of a putative RPCH-like receptor to be a G-protein coupled receptor based on *in silico* mining of transcriptomic data, with this receptor being expressed during the metamorphic molt of the spiny lobster, *Sagmariasus verreauxi*. Marco et al. (107) pharmacologically characterized the red pigment-concentrating hormone receptor (RPCHR) from the water flea, *Daphnia pulex*, and revealed it to be similar in terms of sequence to insect adipokinetic hormone (AKH) receptor. This was the first report

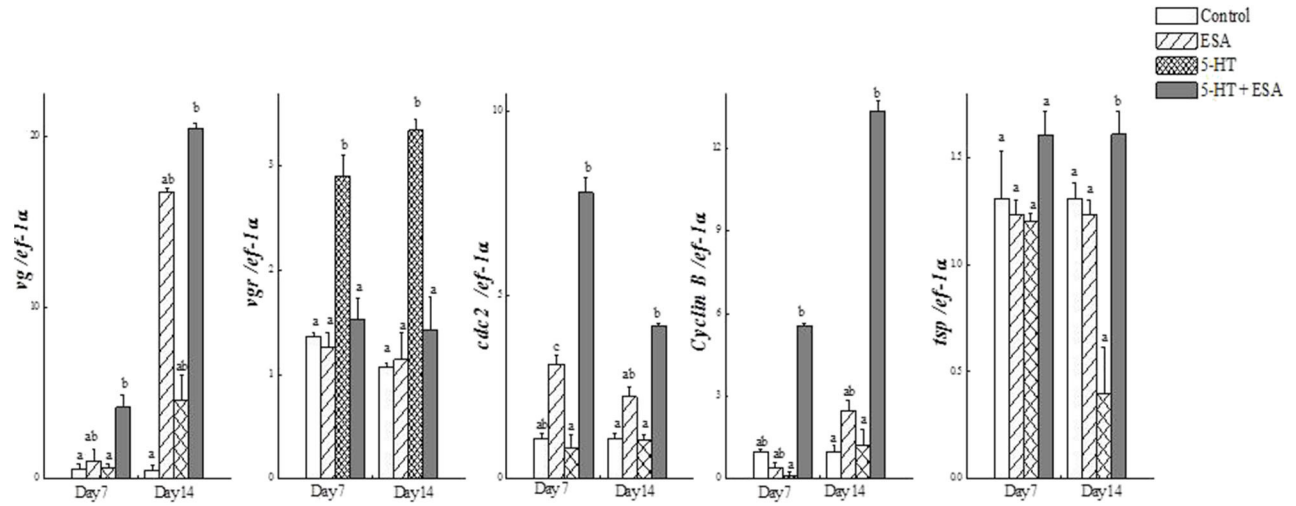

FIGURE 3 | Real-time PCR analysis of (a) *vg*, (b) *vgr*, (c) *cdc2*, (d) *cyclin B* and (e) *tsp* transcripts in ovaries of *Penaeus indicus* subjected to induced maturation 7 and 14 days after treatment. Each value represents the mean ± SE ($n = 10$ shrimp). Differing letters indicate significant differences ($P < 0.05$) according to one-way ANOVA followed by Duncan's multiple range test. Reproduced with permission from Tomy et al. (27).

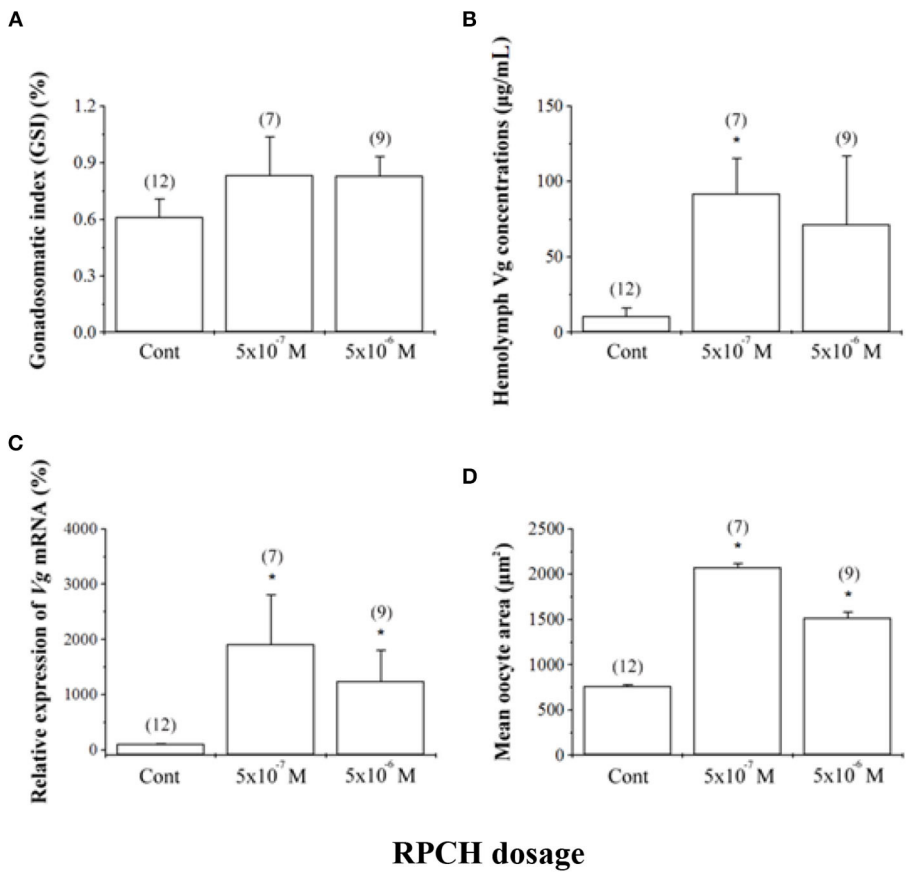

FIGURE 4 | Effects of red pigment-concentrating hormone (RPCH) on ovarian growth in the whiteleg shrimp, *Litopenaeus vannamei*. Animals received repeated injections of either sterile shrimp saline (Cont) or RPCH (100 μl of $5 \cdot 10^{-7}$ M or $5 \cdot 10^{-6}$ M). **(A)** Gonadosomatic index (GSI), **(B)** hemolymph vitellogenin (Vg) concentrations, **(C)** relative expression of *Vg* mRNA, and **(D)** mean oocyte area. Data are shown as the mean ± SE ($n = 7$ to 12). Asterisks indicate significant differences ($P < 0.05$) with respect to the control. Reproduced with permission from Chen et al. (28).

of a deorphanized neuropeptide G protein-coupled receptor (GPCR) in crustaceans. Recently, RPCHR has been deorphanized in *Carcinus maenas* and was seen to be expressed at high levels in the eyestalk neural tissue, but was also observed in the antennal gland and maturing ovary (93). The RPCHR had typical characteristics of rhodopsin-like GPCRs and interacted with RPCH with extremely high sensitivity (93).

Despite the lack of information on crustacean RPCHR sequences, investigations on the RPCH signaling cascade have been performed in several decapod crustacean species. The RPCH primarily functions in mediating pigment aggregation in crustaceans (89, 98), via the Ca^{2+}-activated cGMP signaling cascade (108). Milograna et al. (109) experimentally proved that RPCH lowered cAMP levels in the ovarian chromatophores of the freshwater shrimp, *Macrobrachium olfersi*.

METHYL FARNESOATE (MF)

Methyl Farnesoate-Mediated Ovarian Development in Crustaceans

Methyl farnesoate (MF), the major sesquiterpenoid synthesized and secreted from the mandibular organs in crustaceans, is under the negative control of MOIH from eyestalks (110). It was first characterized as a juvenile hormone-like factor in the spider crab *Libinia emarginata* (111), but was later unequivocally identified in several crustacean species. Due to the structural similarity between MF and the juvenile hormones (JH), MF is considered to be the crustacean homolog of JH. In crustaceans, farnesoic acid O-methyltransferase (FAMeT) is the rate-limiting enzyme catalyzing the methylation of farnesoic acid (FA) to MF. After release from the mandibular organs, MF is transported through the hemolymph to target tissues by MF-binding proteins (112).

MF is thought to be a key endocrine controller of several biological functions in crustaceans including molting (113), reproduction (29, 114, 115), morphogenesis (116), phenotypic plasticity (117), and osmoregulation (118). MF is capable of inducing the synthesis and release of ecdysteroids from the Y-organs (119, 120). Another significant function of MF seems to be the enhancement of reproductive maturation in both male and female crustaceans (29, 113, 114, 121, 122). A positive correlation between MF levels in hemolymph and stage of development of the ovaries was reported in *P. clarkii* (123) and in the freshwater rice field crab *O. senex senex* (124), *Portunus trituberculatus* (114), *S. paramamosain* (125), and estuarine crab *Neohelice granulate* (126), indicating a probable role in ovarian maturation in these species. Similarly, MF also stimulates testicular growth, and affects morphology and behavior in males, with MF levels being high in reproductively-active males compared to inactive males (10, 116, 121).

Elevated vitellogenin levels associated with ovarian maturation following MF administration was reported in *L. emarginata* (115), *L. vannamei* (32, 127), *P. indicus* (29), *Macrobrachium malcolmsoni* (122), *P. clarkii* (123, 128), *O. senex senex* (113), and *N. granulate* (126). MF secretion from the mandibular organs was highest during the vitellogenic stages of ovarian development (123), while MF levels were highest during the pre- and early vitellogenesis phases in crabs and shrimp (124, 127), suggesting a role for MF in relation to gonial proliferation and stimulation of vitellogenesis. In contrast, MF levels did not change with maturation in *M. rosenbergii*, but were correlated with molting (129) and MF was also demonstrated to inhibit late ovarian stage development with reduced fecundity levels in *P. monodon* (130). MF injection during the late vitellogenic phase had no significant effect on post-vitellogenesis or spawning in the freshwater crab *Travancoriana schirnerae* (131). Nevertheless, dietary inclusion of MF stimulated vitellogenesis in the crayfish *P. clarkii* and *P. monodon*, bringing about increased oocyte diameter, higher fecundity and egg fertility, and increased hatching rates (123, 132). In Daphnia, MF was suggested to act as a sex determinant (133).

The stimulatory effects of MF on ovarian maturation were observed to be pronounced in eyestalk-ablated animals, with a higher MF concentration in the hemolymph and elevated levels of FAMeT compared with eyestalk intact animals in the Indian white shrimp, *P. indicus* and the crab *O. senex senex* (29, 134). Buchi et al. (134) reported that MF caused more rapid ovarian maturation and also resulted in increased vitellogenin mRNA expression in hepatopancreas fragments incubated with MF in the crab *O. senex senex*; this suggested a direct action on vitellogenin expression at the hepatopancreas or indirect stimulation through other biologically-active molecules (such as ecdysteroids from the Y-organs). Similarly, up-regulation of vitellogenin gene expression was observed in *in vitro* hepatopancreas explant cultures from the red crab, *Charybdis feriatus* when MF was added at higher concentrations (135). On the contrary, Tiu et al. (136) reported that hepatopancreas explants from American lobster *H. americanus* at different vitellogenic stages treated with MF did not show any significant increases in expression of the vitellogenin gene. Furthermore, evidence for the stimulatory effects of MF on vitellogenin gene expression in crustaceans was obtained from experiments on ovarian explants with MF, where the addition of MF to culture media resulted in increased vitellogenin gene expression (29). Mandibular organ explants cultured in the presence of serotonin showed no significant increase in MF secretion levels, suggesting that serotonin-mediated MF synthesis is indirect, probably based on the inhibition of MOIH release (69). There is evidence that MF, in combination with vertebrate steroid hormones like 17-hydroxyprogesterone (126) and 17β-estradiol (128), regulates vitellogenesis in crustaceans. Swetha et al. (137) reported that prostaglandins mediated the induction of vitellogenesis in crabs *O. senex senex* by stimulating MF synthesis and consequent ecdysteroid production, and suggested that this phenomena is likely due to the inhibition of the release of MOIH and MIH from eyestalks, or based on the direct action of prostaglandins on the mandibular organs and/or the Y-organs.

Research on the intermediates of MF biosynthetic pathways have revealed the stimulatory effects of farnesoic acid (FA), the precursor of MF, on stimulating vitellogenin gene expression in hepatopancreatic and/or ovarian explants in the red crab *C. feriatus* (135), American lobster *H. americanus* (136) and penaeid shrimps *Metapenaeus ensis* (138) and *P. monodon* (139). These authors reported FA to be more potent than MF in stimulating

vitellogenesis. FAMeT levels were also demonstrated to be positively correlated with ovarian development in *P. indicus* (29) and *P. monodon* (140). Serotonin-stimulated ovarian maturation was associated with an increase in hemolymph MF levels in *F. merguiensis* (51), as well as the expression levels of retinoid-X receptors in crab (69) and also with a significant increase in FAMeT levels in the mud crab, *S. olivacea* (47), *P. monodon* (140), and *P. indicus* (29). Furthermore, higher levels of FAMeT were observed in ablated *P. indicus* treated with serotonin (29), suggesting that serotonin-mediated stimulation of vitellogenesis occurs by stimulating MF synthesis. In the shrimp, *M. ensis*, the observed co-localization of FAMeT with CHH and MIH in the neurosecretory cells together with the comparatively higher transcript levels of FAMeT, CHH, and MIH proteins supports the notion of a possible interaction between eyestalk neuropeptides and FAMeT (141). These lines of evidence taken overall, indicate a synergistic interaction among the serotonergic, neuropeptide and MF pathways in regulating crustacean ovarian maturation.

MF Signaling Pathways

A deeper understanding of the signaling pathways mediated by MF is necessitated in order to analyze the functional aspects of MF-regulated ovarian development in decapod Crustacea. However, many facets of this system remain unclear. For example, the characteristics of the MF receptor are unknown. In more detail, MF is thought to induce maturation through a mechanism based on ecdysteroid synthesis in the Y-organs. In this situation, MF acts as a ligand for retinoid-X receptors in synergy with ecdysteroids to stimulate the RXR–EcR heterodimer complex, initiating the expression of combined regulatory genes (E75 and E74) for both of these hormones in the hepatopancreas; this in turn induces vitellogenin gene expression (120, 142). Identification of two different RXRs in the ovary of green crab *C. maenas* and their expression levels at different vitellogenic stages indicates a role for retinoid-X receptors in crustacean ovarian development; this was further confirmed by experiments employing dsRNA-mediated silencing of retinoid-X receptors which lowered vitellogenic activity in this species.

MF can bind to membrane receptors and activate protein kinase C (PKC), and cause the subsequent modulation of potassium and calcium ion channels, resulting in a signal-transduction cascade that promotes vitellogenin uptake into the oocytes (143, 144). PKC is also well known to cause the activation of nuclear factor-kB (NF-kB) (145) and the mitogen-activated protein kinase pathway (MAP kinase) (146). On the contrary, the Ca^{2+}/PKC signaling cascade was reported to suppress vitellogenesis in marine penaeid shrimps, where a signaling cascade (GPCR-receptor tyrosine kinase (RTK)-phospholipase C (PLC)-inositol trisphosphate receptor (IP3R)-PKC), similar to JH-induced cascades reported in insects such as the locust *Locusta migratoria* (147), was proposed to regulate vitellogenesis (148, 149). In *L. vannamei*, Alnawafleh et al. (32) provided evidence of the involvement of a Ca^{2+}/PKC signaling cascade in vitellogenesis. On the contrary, Chen et al. (150) reported that PKC-α isoform may regulate ovarian growth in *L. vannamei* through a negative-based regulating mechanism. The functioning of Ca^{2+} signaling in ovarian development reported in different species is inconsistent, with strong evidence for fundamentally opposite (inducing and hindering) functions being attributed to it (15, 143, 148, 150, 151). These discrepancies relating to the actions of PKC in Crustacea appear to vary with the habitat of the species. Future experiments using PKC activators and inhibitors or CRISPR-mediated studies harbor much potential to shed light on the role of PKC in crustacean vitellogenesis.

VITELLOGENESIS-STIMULATING HORMONE (VSH)

The brain and thoracic ganglia have been suggested to release a putative vitellogenesis-stimulating hormone (VSH) that promotes vitellogenesis and ovarian development (1, 25, 152). Though its identity is still unclear, in early research, it was suggested to be a peptide that can be inactivated by trypsin (153). Some workers have proposed it to be an analog of GnRH, and would similarly be released from the nervous tissue to stimulate the release of a crustacean gonadotropin (154). The ability of implanted nervous tissue (brain and/or thoracic ganglion), or application of their extracts to stimulate ovarian development and enhance vitellogenesis, has been observed in different crustacean species; this ability seems be conditional upon the sourced animals being reproductively active (25, 152, 155). More specifically, for example, repeated implantation of the brain and thoracic ganglion into juvenile female freshwater field crab *Parathelphusa hydrodromus* increased oocyte size; however, effects were more pronounced in adult females (156). Yano et al. (25) suggested that the gonad-stimulating effects of brain and thoracic ganglion are not species-specific, as ovarian maturation was accelerated in *L. vannamei* by the implantation of lobster ganglion.

Several neuromediators in crustacean reproduction, including serotonin (27) and RPCH (103), and in addition the juvenoid substance MF (29) are reported to indirectly exert their stimulatory effects by inducing the release of putative VSH from the thoracic ganglion. In *M. rosenbergii,* serotonin-primed thoracic ganglion medium stimulated oocyte growth and ovarian maturation (46). Furthermore, *in vitro* and *in vivo* studies have demonstrated that the release of putative gonad-stimulating factors from the brain and thoracic ganglia could be hampered by the presence of copper and cadmium (157), while stimulated by the application of calcium ionophore (A23187) (104). However, whether the proposed VSH functions directly or indirectly is yet to be conclusively confirmed. Although there are many studies where VIH has been extracted and chemically characterized based on the extensive collection of sinus glands, such as that has been accomplished by Tsutsui et al. in *L. vannamei* (158), the equivalent type of experimentation remains difficult with respect to isolating and characterizing crustacean VSH. This is because the concept of a putative VSH is tied to the existence of different stage-specific factors that stimulate vitellogenesis; or alternatively, a situation where an actual substance does not actually exist, but rather is based on a negative feedback mechanism to which the XO/SG complex is central (159). Thus,

identifying and characterizing VSH in crustaceans remains a challenging research topic, the outcome of which could be of immense use in controlling the maturation of shrimp and other commercially-useful crustacean species in captivity, thus having important implications for the further development of the aquaculture industry.

POTENTIAL INVOLVEMENT OF OTHER FACTORS (ECDYSTEROIDS/VERTEBRATE STEROIDS, GONADOTROPIN-RELEASING HORMONE, KISSPEPTIN); FUTURE IMPLICATIONS FOR COMMERCIAL AQUACULTURE

Despite the significant economic importance of many decapod crustacean species, overcoming the problem of reproductive dysfunction seen in captive broodstock remains a major bottleneck in crustacean aquaculture. Understanding the dynamics of the complex crustacean neuroendocrine system and the precise interactions among multiple neuromediators and various hormonal substances having pleiotropic functional roles would be useful in this regard. Nevertheless, this topic remains challenging, although many studies have been carried out employing immunohistochemical, molecular, and biochemical methods. It is, however, clear that neuropeptides from the eyestalks functionally regulate other mediators/hormones, especially those originating from the brain and thoracic ganglion, achieving a coordinated regulation of ovarian maturation. Among these neuropeptides, eyestalk peptides of the CHH-family have been reviewed extensively (10) in context of their roles in crustacean development and reproduction. In addition to the eyestalk peptides, diverse factors including non-eyestalk peptide hormones, steroid hormones and neurotransmitters, as well as their receptors, have been shown to regulate ovarian development in various crustacean species (160).

Subramoniam (31, 160) has reviewed the role of ecdysteroids in crustaceans, and has put forth that after being synthesized at the Y-organs, they are accumulated in developing ovaries perhaps based on a mechanism that involves binding with vitellogenin; such ecdysteroids are then thought to serve as a reserve for use during embryogenesis (by the developing embryo enclosed in eggs brooded externally). Ecdysteroids are therefore considered to serve as important hormonal factors that are involved in not only molting, but also in reproduction, drawing similarities to certain species of insects. In this way, molting and reproduction are inextricably linked, but each species exhibits its own unique pattern of growth and reproductive strategy.

The presence of vertebrate-type steroid hormones (estradiol, testosterone, pregnenolone and progesterone) has been revealed in several crustacean species [reviewed by Subramoniam (160)], and a correlative fluctuation of these substances in the hemolymph, hepatopancreas, and ovaries with the reproductive cycle has suggested a regulatory function in reproduction analogous to that in vertebrates (31, 161, 162). Nevertheless, *in vitro* and *in vivo* studies employing the direct administration of these hormones in crustacean species has yielded contrasting results, ranging from the absence of effects (163) to positive regulation of vitellogenesis and ovarian maturation (26, 164–166). A possible explanation for the above discrepancies may be related to the ovarian stage-specific and dose-dependent effects of the examined substances. Merlin et al. (26) reported hormone levels in hemolymph to be higher in ablated shrimps, suggesting the influence of VIH on the synthesis and the release of the sex hormones. Coccia et al. (165) demonstrated E2 to be more effective than progesterone in stimulating Vg mRNA synthesis in hepatopancreas. The existence of sex steroids receptors is controversial in crustaceans, although estrogen and progesterone receptors have been identified in various crustacean species (167, 168).

Furthermore, recent reports on the existence of gonadotropin-releasing hormone (GnRH)/gonadotropin secretion, kisspeptins and vertebrate-like steroids in crustaceans have provided new vistas for exploring the presence of an evolutionarily-conserved neuroendocrine signaling mechanism in crustacean reproduction, similarly to vertebrates, with a gonadotropin-releasing hormone (GnRH)/gonadotropin-like axis regulated by kisspeptin-related factors. Gonad development/maturation and male-specific hormone production in the androgenic gland were stimulated by the administration of exogenous GnRH, suggesting the possible existence of a GnRH-mediated regulatory mechanism comparable to that in vertebrates (54, 70, 169, 170). Additionally, the existence of receptors for GnRH (GnRHR) was also reported in the ovaries of *M. nipponense* (170) and Chinese mitten crab, *E. sinensis* (171). The presence of gonadotropin-like substances in crustaceans as described by Ye et al. (172, 173) further provide evidence for the existence of GnRH/GtH-mediated regulation in the reproductive process.

The presence of kisspeptin-like peptides and their receptors was reported in the neural tissues of *M. rosenbergii* by Thongbuakaew et al. (174); the co-localization of Kiss-I and GnRH signals in the same neurons suggested that locally-synthesized kisspeptin plays a pivotal role in ovarian maturation and spawning by exerting autocrine and paracrine regulation on GnRH secretion from neural and ovarian tissue. Moreover, injection of exogenous kisspeptin was also shown to induce ovarian maturation and spawning in *M. rosenbergii* perhaps via action on GnRH or stimulation of E2 production in the ovary.

Returning to the main factors covered in this review, *in vitro* and *in vivo* experiments together with immunolocalization studies have demonstrated that serotonin and RPCH function as neurohormones and neurotransmitters, respectively, and exert receptor-mediated effects on vitellogenesis and oocyte maturation through an autocrine/paracrine regulatory mechanism. Moreover, serotonin has been suggested to exert its downstream effects on reproduction through RPCH in several crustacean species. In addition, the correlation of circulatory levels of MF and the intermediates in its biosynthetic pathway with vitellogenin gene expression and ovarian development, suggests a direct regulatory role in vitellogenesis via the hepatopancreas, or an indirect role through the stimulation of the synthesis of other biological molecules (such as ecdysteroids from the Y-organs). Serotonin treatment may also trigger a

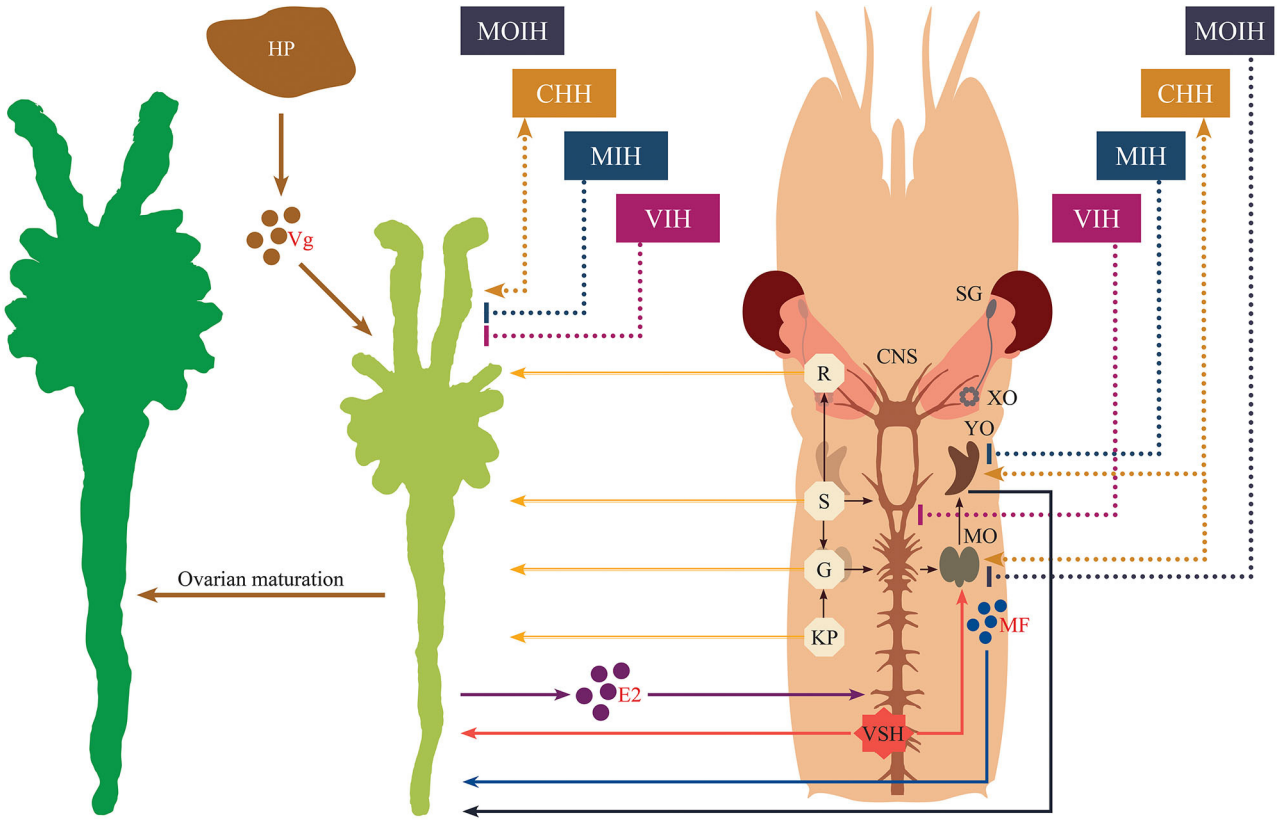

FIGURE 5 | Schematic representation of the endocrine regulation of crustacean maturation. Nervous tissue is diagrammatically depicted for the cephalothoracic region of a typical shrimp. Stimulatory effects are indicated by lines ending in arrowheads, while inhibitory effects are indicated by lines having blunted ends. Neuropeptides originating from the eyestalk are displayed with dashed lines. Solid lines correspond to all other factors or actions. The secretory products from hepatopancreas, ovaries and mandibular organs are represented as colored circles with their abbreviations (vitellogenin, Vg; 17β-estradiol, E2, and methyl farnesoate, MF, respectively). Other abbreviations shown in the diagram are given as follows. SG, sinus gland; XO, X-organ; CHH, crustacean hyperglycemic hormone; MIH, molt-inhibiting hormone; VIH, vitellogenesis-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; VSH, vitellogenesis-stimulating hormone; YO, Y-organ; MO, mandibular organ; R, red pigment-concentrating hormone; S, serotonin; G, gonadotropin-releasing hormone; KP, kisspeptin; HP, hepatopancreas.

significant increase in circulatory MF levels together with retinoid-X receptors expression in the hepatopancreas and ovary in crustaceans; RPCH may also stimulate MF synthesis in mandibular organs. Taken together, it is tempting to speculate that biogenic amine signaling, either alone or in combination with other factors including RPCH and MF, can potentially trigger the eyestalk neuropeptide signaling pathways to inhibit or stimulate the onset of ovarian maturation in an autocrine and/or paracrine manner.

Concurrently, with the recent advances in bioinformatic analysis of genomes and transcriptome and methods of mass spectrometry, our understanding of the functional roles of hormones/ neurotransmitters, other novel factors, and putative receptors involved in the complex regulatory network of reproduction has rapidly expanded. Of interest, recently much research attempting to elucidate the biological functioning of the various substances discussed in this review has been carried out based on approaches such as the application of recombinant or synthetic proteins and antibodies or utilization of dsRNAi/microRNAi techniques. Furthermore, RNAi harbors the potential to develop an artificial means of suppressing circulatory VIH levels, thus providing a partial replacement to eyestalk ablation currently used in hatcheries world-wide. For example, the reader is referred to Kang et al. (18) in which transcriptional silencing of VIH was achieved in *L. vannamei*. Gene-editing methodology may also yield new perspectives on the functional significance of various hormones in crustacean reproduction. Exploration of the possible use of gene-editing technologies such as CRISPR-Cas9 knock-ins or knock-outs will facilitate the acquisition of new knowledge on the regulatory roles of many neuromediators and other factors.

In conclusion, several hormones, neuromodulators and novel factors involved in the regulation of vitellogenesis are well-recognized; however, the cross-talk between them as well as their overlapping and multiple functions remain unclear. Another limitation is that information currently available on the functional roles of many neuromediators draws from research work performed on differing crustacean species; as noted above, functionality of hormonal substances and their related

mechanisms in terms of the stimulatory aspects of reproductive regulation may vary widely. This is why, until the present, it is still difficult to develop a comprehensive framework that fully explains crustacean reproduction in the way that this has been accomplished for vertebrates. Nevertheless, in recent years, a great deal of knowledge has accumulated regarding the stimulatory aspects of crustacean reproductive development that go much beyond the simple scheme presented in **Figure 1**. Our understanding of the synergistic effects of distinct regulatory signaling pathways involved in crustacean ovarian maturation has increased in recent years, and here we have made an attempt to integrate this previously-established knowledge and latest advances into a new schematic diagram (**Figure 5**). As delineated in the figure, analogously to the case of vertebrates, the ovaries may release sex steroids which are proposed to exert a stage-specific regulatory feedback along the nervous-gonadotropic axis.

The underlying mechanisms by which multifunctional neuropeptides, neurotransmitters/neuromediators and other factors switch their functional roles and act in concert together with other regulatory pathways merits further investigation. Such studies are expected to provide greater insight into reproductive mechanisms, and harbor potential applications in the development of maturation techniques that may be employed in crustacean aquaculture.

AUTHOR CONTRIBUTIONS

VJ: conceptualization and writing. ST: writing and data/manuscript curation. MNW: writing and editing.

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Transcriptional Regulation of *Vih* by Oct4 and Sox9 in *Scylla paramamosain*

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Mud crab (*Scylla paramamosain*) is one of the most economically-important marine crabs in China. However, research on mechanisms of reproductive regulation is not sufficient. Vitellogenesis-inhibiting hormone (VIH) is a member of the crustacean hyperglycemia hormones (CHH) family, which plays an essential role in the regulation of gonadal development and maturation in crustaceans, and current studies on the regulation of *Vih* transcription in crabs are relatively rare. Our previous studies on the transcriptional regulation of mud crab *Vih* (*SpVih*) have proved that the binding site of Oct4/Sox9 transcription factor may be the key region for positively regulating the expression of *SpVih*. In this study, the electrophoretic mobility shift assay (EMSA) experiment confirmed that the nuclear protein extracted from the eyestalk could bind to the key region of *SpVih* promoter, and these specific bindings were dependent on the presence of Oct4/Sox9 binding sites. Two specific binding complex bands were detected in the supershift group of EMSA supershift experiments by Oct4 and Sox9 antibodies, further confirming the specific recognition of these two transcription factors on the key regulatory region of *SpVih*. *In vitro*, *Oct4* and *Sox9* gene overexpression vectors and *SpVih* core promoter fragment vector were constructed and co-transfected into HEK293T cells. As a result, *SpVih* activity increased with the concentration of transcription factors. *In vivo*, when *Oct4* and *Sox9* dsRNA were injected into the eyestalks of mud crab, respectively, the expression level of *SpVih* decreased significantly after interference with *Oct4* or *Sox9*, and the expression level of *SpVtg* in the ovary and hepatopancreatic increased. Both *in vitro* and *in vivo* experiments showed that Oct4 and Sox9 had a positive regulatory effect on *SpVih*. The GST pull-down experiment was carried out by purified Oct4 and Sox9 proteins, and the results showed that there was an interaction between them. It was speculated that they regulated the expression of *SpVih* through the interaction.

Keywords: transcriptional regulation, *Vih*, Oct4, Sox9, *Scylla paramamosain*

INTRODUCTION

Vitellogenesis-inhibiting hormone (VIH), also known as gonad-inhibiting hormone (GIH), is a member of the crustacean hyperglycemia hormone (CHH) family secreted from the X-organ sinus gland complex (XO-SG) of eyestalk (1). VIH was first isolated from the eyestalk of *Homarus americanus*, and it has been described as a vitellogenesis inhibitor because it exerts a significant negative regulatory effect during gonad maturation (2, 3). Thus far, eyestalk ablation is commonly practiced in shrimp and crab to induce ovarian maturation in captivity. Nevertheless, there are a lot of drawbacks to this approach, such as increased parental mortality, lower egg quality, and lower hatching rate (4), etc. In order to solve this problem, many scholars began to try to find some reliable alternative methods. For example, Marins's group first used RNAi technology to silence GIH transcripts to develop an alternative approach to eyestalk ablation in captive shrimp *Litopenaeus vannamei* (5). Devaraj et al. (6) first used molecular signal intervention, a less invasive method than traditional eyestalk ablation, to suppress VIH expression via the MEK pathway to induce ovarian maturation in female *Penaeus monodon*. Treerattrakool et al. (7) reported the preparation of monoclonal antibody specific to Pem-GIH (anti-GIH mAb) and implication of antibody neutralization on the induction of ovarian maturation in *P. monodon*.

Mud crab (*Scylla paramamosain*) is one of the most important marine crabs in China, but the seedlings used in aquaculture operations are mainly obtained from wild sources (8). The shortage of seedling has severely hindered the development of mud crab aquaculture. In order to address the above problem, it is urgent to study the molecular regulation mechanism of gonadal development and maturation in mud crab. Transcription factors are essential molecules that control gene expression. They can activate or repress gene transcription through binding to specific sites of gene promoters, which are essential for a series of crucial cellular processes. The study of transcription factors controlling the expression of *SpVih* may also be one of the methods to develop alternative eyestalk ablation in the future. In the preliminary study on the transcriptional regulation of the *SpVih* gene in our laboratory, we found the key regulatory element in *SpVih* promoter and the key transcription factors, Oct4 and Sox9, that may regulate the expression of *SpVih* (9). Based on this research, this study will explore the regulation of *SpVih* expression by these two transcription factors.

Transcription factor Oct4 belongs to the Pou protein family, and its main function is to form and maintain pluripotent stem cells. In many vertebrates, the *Oct4* gene is highly or specifically expressed in early embryos, proving that it is indispensable for the early development of embryos. In terms of gonad development, Oct4 is highly expressed in primordial germ cells and mature gonads in a variety of vertebrates (10, 11). As a transcription factor, Oct4 participates in the regulation of various target genes. Jen et al. (12) found that Oct4 can activate the transcription of MALAT1, thereby promoting cell proliferation and movement. Wu et al. (13) found that the transcription factor Oct4 regulates the transcriptional activity of Dnmt1. In the mud crab, it was found in our previous studies that *Oct4* mRNA was highly expressed in the mature ovaries, and its protein could also be detected in the ovaries. Meanwhile, both Oct4 mRNA and protein were highly expressed in the eyestalk of female crabs (14).

Sox9 belongs to the SoxE subfamily of the Sox gene family. It is a critical factor in the sex determination and sex differentiation process of many animals. In mammals, it is a key gene in the testicular determination pathway (15, 16), similar effects have been found in some amphibians and reptiles (17, 18). Sox9 was found to be expressed at high levels or specifically in the testis in many bony fishes, such as *Pelteobagrus fulvidraco* (19), *Betta splendens* (20), *Pelteobagrus fulvidraco* (21), *Acipenser baerii Brandt* (22), and *Oncorhynchus mykiss* (23), etc. In addition to being involved in testis development, Sox9 has been found to be expressed in oocytes. It carries an additional function in the posttranscriptional processes in some amphibians such as *Pleurodeles waltl*, *Xenopus laevis,* and *X. tropicalis* (24). Researches on invertebrate *Bombyx mori* (25), *Drosophila melanogaster* (26), and *Apis florea* (27) have also found that homologous genes of Sox9 are indispensable for their sex differentiation and gonadal development. As a transcription factor, Sox9 is also involved in the regulation of multiple target genes. In mice, embryo knockout and cell transfection experiments confirmed that Sox9 and SF1 could upregulate the expression of *Cyp26b1* during gonadal development to maintain the development of germ cells in males (28). Minerva et al. (29) found that Sox9 can also enhance the activity of the *Catsper1* promoter, together with the transcription factor Sox5. In the tissues of mature mud crab, Sox9 was only expressed in the gonads, eyestalk, and cerebral ganglia, suggesting that it may play a role in the development of gonads (14).

MATERIALS AND METHODS

Animal and Tissue Collection

All mud crabs with good vitality were purchased from the Jimei market, Xiamen city, Fujian province. The eyestalk of immature female crabs weighing about 100 g was used to isolate nucleoproteins. Female crabs weighing about 250 g were temporarily kept in the seawater field of Jimei University for a week before the RNA interference experiment. After the experiment, the eyestalks, gonads, and hepatopancreas of the crabs were sampled and frozen immediately in liquid nitrogen, then stored at −80°C for total RNA extraction. All animal experiments were conducted following the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Jimei University.

Electrophoretic Mobility Shift Assay (EMSA)

In order to detect whether there is a specific binding site for Oct4 or Sox9 at the core regulatory region of *SpVih*, EMSA experiments were performed. Briefly, according to the manufacturer's protocol for the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA), biotin-labeled and unlabeled double-stranded DNA probes containing intact core recognition element (CRE) for Oct4 and Sox9

TABLE 1 | Primers used in this study.

Primer name	Sequence (5′–3′)	Usage
*Sp*TF-wt-F	GGCATGGTTCCGGGCAGAAATATAAAGTGCATGAGATTCTGGTGAAGGAAGGCTTAAGTA	EMSA
*Sp*TF-wt-R	TACTTAAGCCTTCCTTCACCAGAATCTCATGCACTTTATATTTCTGCCCGGAACCATGCC	EMSA
*Sp*TF-mut-F	GGCATGGAACCGGGCAGAAATATAAAGTGCATGAGATTCTGGTGAAGGAAGGCTTAAGTA	EMSA
*Sp*TF-mut-R	TACTTAAGCCTTCCTTCACCAGAATCTCATGCACTTTATATTTCTGCCCGGTTCCATGCC	EMSA
*Sp*Vih-4-F	cggGGTACCTGGTTCCGGGCAGAAATATAAAGT	Cell transfection
*Sp*Vih-4-R	atttGCGGCCGCGACGAAACTCAATGAACAC	Cell transfection
*Sp*Sox9-ORF-F	cggGGTACCAACTCCATCACCAACTACATTCATT	Cell transfection
*Sp*Sox9-ORF-R	atttGCGGCCGCCCGACTGCGGTGTAGAGGAG	Cell transfection
*Sp*Oct4-ORF-F	cggGGTACCATGGCTACAACAACTTACATCGCAT	Cell transfection
SpOct4-ORF-R	atttGCGGCCGCGGTTGAGCGACCATCAGTGGGAGAC	Cell transfection
Oct4-RNAi-F	AGACCACCATCTGCAGGTTC	dsRNA synthesis
Oct4-RNAi-R	ACACGCACCACCTCTTTCTC	dsRNA synthesis
Sox9-RNAi-F	AGGAATGGGGTCACGTTGG	dsRNA synthesis
Sox9-RnAi-R	ATCGGTTCTCGCCTGTTGC	dsRNA synthesis
EGFP-RNAi-F	GGTGAACTTCAAGATCCGCC	dsRNA synthesis
EGFP-RNAi-R	CTTGTACAGCTCGTCCATGC	dsRNA synthesis
*Sp*Oct4-RT-F	AGACCACCATCTGCAGGTTC	qRT-PCR
*Sp*Oct4-RT-R	TCCGCTTTTTACGTTTCCTG	qRT-PCR
*Sp*Sox9-RT-F	TCCACGTGAAGAGGCCAATG	qRT-PCR
*Sp*Sox9-RT-R	CATGCCCCTCCATATCCTGC	qRT-PCR
*Sp*Vih-RT-F	AGGAGGAACTGCTTCTACAACGAGG	qRT-PCR
*Sp*Vih-RT-R	GAGTGAATAATGTGAGATGTGGCTA	qRT-PCR
18S-rRNA-RT-F	ATGATAGGGATTGGGGTTTGC	qRT-PCR
18S-rRNA-RT-R	AGAGTGCCAGTCCGAAGG	qRT-PCR
*Sp*Vtg-RT-F	CGTACCGGACATCTTCCGAG	qRT-PCR
*Sp*Vtg-RT-R	ACGAGCCCACTACAGAGACT	qRT-PCR
*Sp*Oct4-F	cgcGGATCCATGGCTACAACAACTTACATCGCAT	Prokaryotic expression
*Sp*Oct4-R	ccgCTCGAGGGTTGAGCGACCATCAGTGGGAGAC	Prokaryotic expression
*Sp*Sox9-F	ccgCTCGAGAACTCCATCACCAACTACATTCATT	Prokaryotic expression
*Sp*Sox9-R	cccAAGCTTCCGACTGCGGTGTAGAGGAG	Prokaryotic expression

The underline indicates the primer cleavage site, lowercase English letters indicate protective bases.

were prepared. For competitive analysis, the mutant probe was synthesized. Nuclear proteins were extracted from the eyestalk of immature female crabs using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific). In the competition experiment, we set up a 100-fold amount of mutation probe group and a 10- and 100-fold amount of unlabeled probe group, respectively. Supershift group added Oct4 or Sox9 antibodies (Abcam, Cambridge, UK, ab19857 and ab3697). Protein-DNA complexes were separated on 6% polyacrylamide gels in 1×TBE via electrophoresis at 100 V for 1 h and transferred onto nylon membranes. Chemiluminescence detection was carried out according to the Chemiluminescent Nucleic Acid Detection Module kit (Thermo Scientific).

Plasmid Construction, Cell Culture, Transfection, and Dual-Luciferase Reporter Assay

For overexpression studies, the *SpVih* core promoter fragment was amplified by PCR, and then cloned into the Kpn I-Xho I site of the pGL3-basic vector. The full-length ORF of Oct4 and Sox9 was cloned into the pcDNA3.1 vector, respectively. All plasmid constructs were verified by sequencing analysis. The primers with the restriction enzyme cutting sites were listed in **Table 1**.

HEK293T cell line was cultured in the DMEM medium supplemented with 10% FBS (fetal bovine serum, Gibco, Waltham, USA) and 1% (v/v) antibiotic (penicillin/streptomycin, Gibco, Waltham, USA) at 37°C in a humidified incubator with 5% CO_2. The medium was changed upon the state of cells.

Transfection of plasmids in HEK 293FT cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. pRL-TK plasmid (10 ng) was used as an internal control. The transfection reagent and plasmids (Oct4 and Sox9 at 50 ng, 100 and 200 ng, respectively, or 25 ng Oct4 + 25 ng Sox9, 50 ng Oct4 + 50 ng Sox9, and 100 ng Oct4 + 100 ng Sox9) were mixed and co-transfected into the HEK293T cell lines. At 3–6 h post-transfection, the transfection mixture was replaced with DMEM containing 10% FBS. The pEGFP-N1 plasmid was employed as a positive control. After continuing culture for 24 h, the

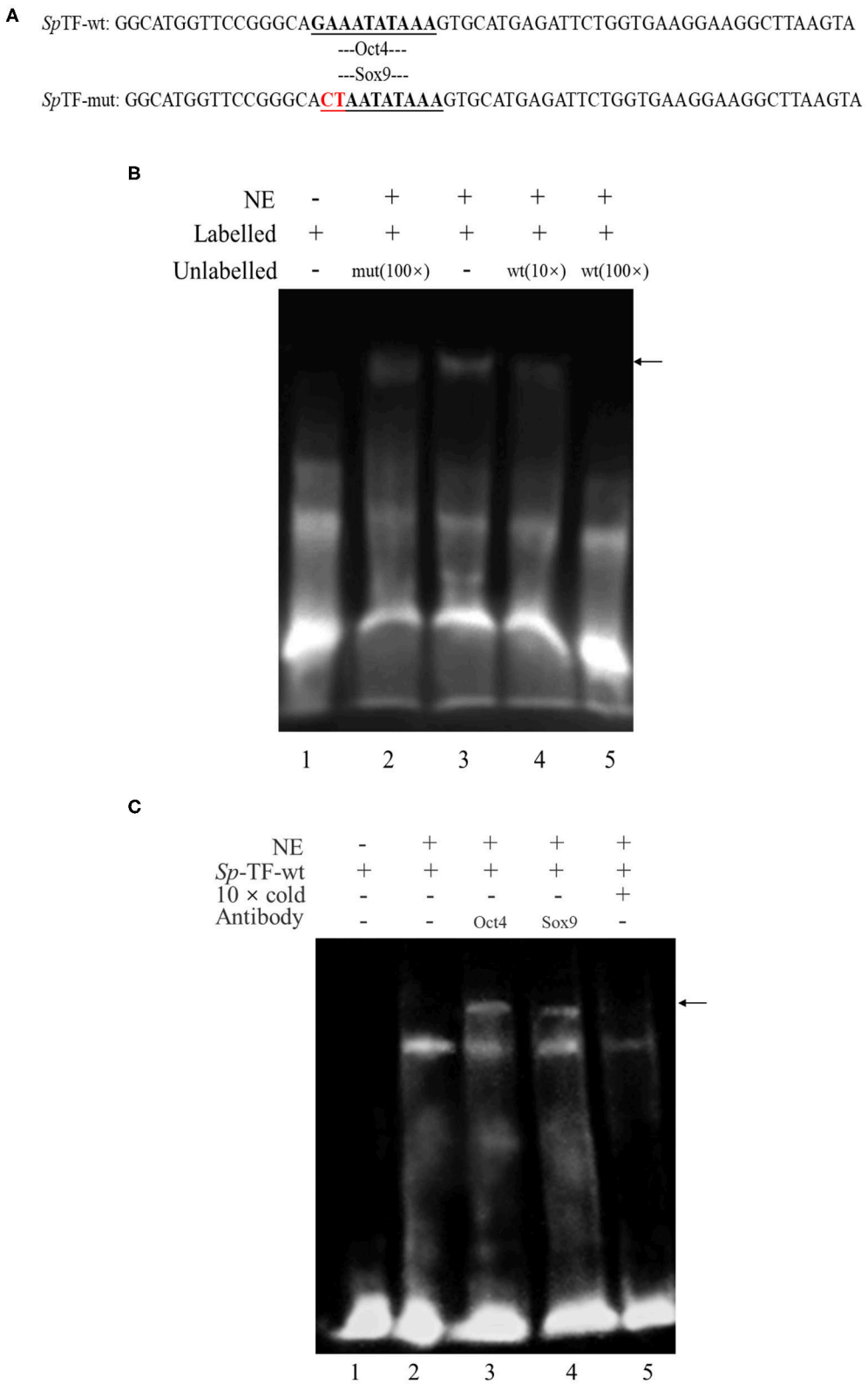

FIGURE 1 | Electrophoretic mobility shift assay (EMSA) analysis of the binding of Oct4 and Sox9 to specific CRE in the *SpVih* promoter. **(A)** The sequence of the wild-type probe and the mutant probe. **(B)** EMSA. Biotin-labeled and unlabeled double-stranded DNA probes containing complete CRE of Oct4 and Sox9 as well as mutated probes that mutated the binding sites of Oct4 and Sox9 were prepared. Lane 1 was the free probe. The *Sp*TF-wt could combine with the eyestalk nuclear

(Continued)

FIGURE 1 | extract to form a DNA-protein complex (lane 3). The formation of the complex was not affected when 100-fold of unlabeled *Sp*TF-mut and *Sp*TF-wt co-existed (lane 2). When the cold probe was added, the binding band will be competitively bound by the cold probe (lane 4-5). **(C)** EMSA supershift. Biotin-labeled and unlabeled double-stranded DNA probes containing Oct4 and Sox9 intact CRE were prepared. Lane 1 was the free probe. The *Sp*TF-wt could combine with the eyestalk nuclear extract to form a DNA-protein complex (lane 2). The unlabeled intact probe could compete for the binding of Oct4 and Sox9 to the labeled intact probe (lane 5). After Oct4 and Sox9 antibodies were added, a supershift band appeared (lane 3-4), and the black arrow indicates the supershift band. NE, eyestalk nuclear extract.

cells were harvested. The firefly and renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's protocol. The promoter activity was assessed by the average of firefly luciferase activity normalized as a ratio to the renilla luciferase activity. Each experiment was conducted for three independent samples in triplicate.

RNA Interference

In *in vivo* experiments, the 286 and 275 bp-length ORF of Oct4 and Sox9, respectively, were chosen as the templates for synthesizing gene-specific dsRNA. Enhanced green fluorescent protein (EGFP) gene was used as a negative control group, and its 229 bp-length ORF was selected as a template for synthesizing EGFP dsRNA. These dsRNAs were synthesized from the linearized template by using the T7 RiboMAXTM Express RNAi System (Promega). These dsRNAs were uniformly diluted to 1 μg/μL, and each crab was injected with 10 μL of dsRNA on the base of each eyestalk. The blank control group was injected with the same volume of stroke-physiological saline solution (SPSS). After continuous injection for 3 days, the eyestalks, gonads, and hepatopancreas were dissected on the fourth day and placed in RNA later and then transferred to −20°C overnight for RNA extraction.

RNA Extraction and Reverse Transcription

Total RNA was extracted from different tissues using Total RNA Extraction Kit (Promega, Shanghai, China) according to the manufacturer's protocol. Total RNA quality was assessed by agarose gel electrophoresis and Nanodrop 2000 (Thermo Scientific). The complementary DNA (cDNA) was synthesized in a 20 μL reaction system including 1 μg total RNA (previously treated with DNase I), 2 μL random primers (10 mM), 4 μL 5× First-strand Buffer, 1 μL dNTP mix (10 mM), and 1 μL M-MLV reverse transcriptase (200 U/μL) (Promega, Shanghai, China). The synthesized cDNA was diluted and stored at −20°C until use.

Quantitative Real-Time PCR (qRT-PCR) Analysis

qRT-PCR was carried out in a LightCycler480 Roche Realtime Thermal Cycler (Mannheim, Baden-wurttemberg, Germany) in accordance with the manual with a 20 μL reaction volume containing 9 μL of 1:100 diluted original cDNA, 10 μL SYBR Green Master Mix (Promega, USA), 0.5 μL of the forward primer and 0.5 μL of reverse primer (10 mM). Primer sequences are shown in **Table 1**. *18s rRNA* was used as the reference gene. The cycling conditions for PCR reaction were set as follows: 1 min at 95°C, followed by 40 cycles at 95°C for 15 s, 59°C for 1 min. Quantitative measurements were performed using the ΔΔCt method. Each sample had at least 4 biological replicates. Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) using Statistical Package for the Social Sciences 20.0 (SPSS 20.0) software (IBM Corporation, New York, NY, USA). The statistically significant differences were shown at $p < 0.05$; the most significant differences were shown at $p < 0.01$.

Recombinant Expression and Purification

According to the full-length cDNA sequences of Oct4 and Sox9, two primer pairs were designed to amplify the sequences that encode their corresponding fragments, respectively (**Table 1**). The amplified fragment of Oct4 was digested by BamHI and XhoI, and then inserted into a pGEX-4T-1 vector. Meanwhile, the Sox9 fragment was ligated into a pET30a expression vector after being cut by NdeI and HindIII. Two recombinant expression vectors, namely pGEX-4T-Oct4, pET30a-Sox9, were transformed into competent *Escherichia coli* Rosetta (DE3) host cells. Isopropyl-b-D-thio-galactoside (IPTG) was added to induce protein expression. The recombinant Oct4 protein with a glutathione S-transferase (GST) tag was purified using glutathione Sepharose 4B chromatography (Detai Biologics, Nanjing, China) according to the manufacturer's instructions. Sox9 protein with an His-tag was harvested by His Bind resin chromatography (Detai Biologics) according to the manufacturer's instructions.

GST Pull-Down Assay

Approximately 15 μg of purified recombinant Oct4 was incubated with glutathione sepharose for 1 h at 4°C and then washed with binding buffer (1× PBS, pH = 7.4). Afterward, ∼15 μg of purified recombinant Sox9 was added and then incubated for 1 h at 4°C. After being washed thoroughly with binding buffer (1× PBS, pH = 7.4), the proteins were eluted with elution buffer (30 mM GSH, 50 mM Tris, 0.1% Triton X-100) and then finally analyzed by SDS-PAGE and Western blot. GST protein was used as the negative control in this assay.

RESULTS

Confirmation of Oct4 and Sox9 Binding to *SpVih* Promoter by EMSA

To determine whether the transcription factor Oct4/Sox9 can recognize and bind to the corresponding site of *SpVih* promoter, the EMSA experiment was conducted after incubation with eyestalk nuclear extract and probe. The results showed that the wild-type probe *Sp*TF-wt (**Figure 1A**) could combine with the eyestalk nuclear extract to form a DNA-protein complex (**Figure 1B**, lane 3, arrow marked). The formation of this complex can be competitively bound by cold probe (wild type

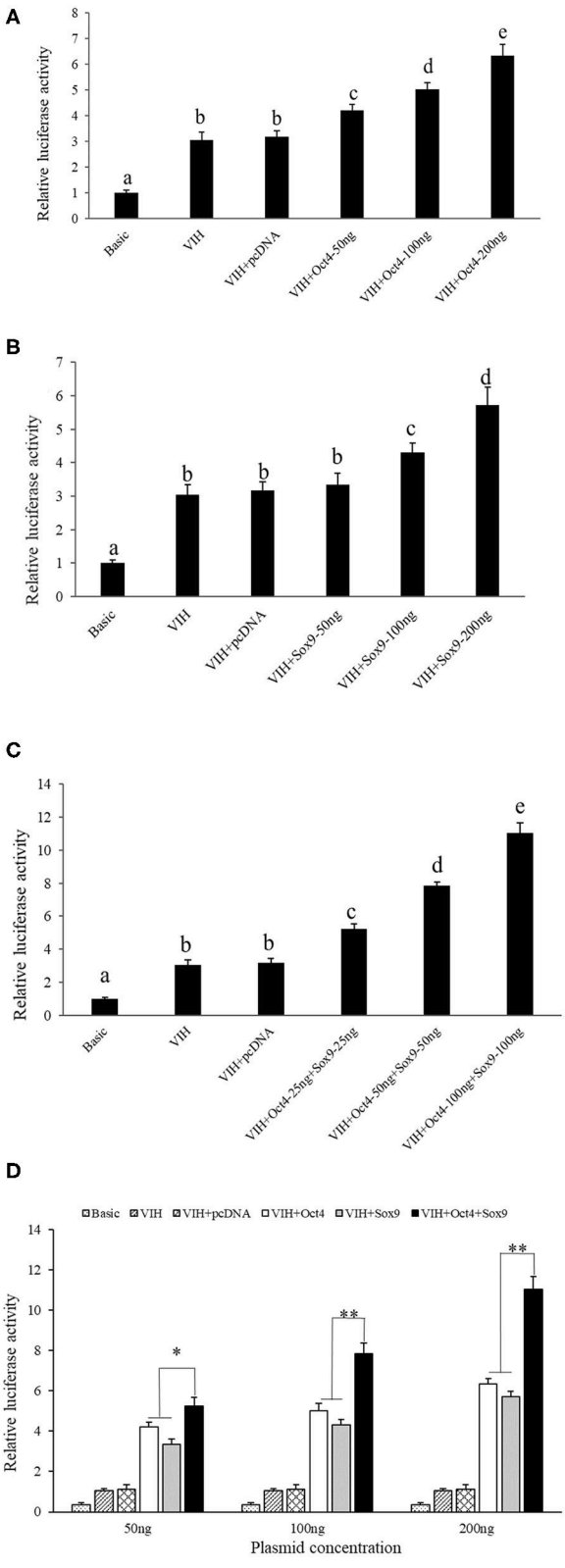

FIGURE 2 | Overexpression of transcription factors can promote the activity of *SpVih* core promoter in HEK293T cell. **(A)** Effect of *Oct4* gene overexpression on *SpVih* core promoter activity. **(B)** Effect of *Sox9* gene overexpression on *(Continued)*

FIGURE 2 | *SpVih* core promoter activity. **(C)** Effect of the co-overexpression of *Sox9* and *Oct4* genes on *SpVih* core promoter activity. **(D)** Effect of *Sox9* or/and *Oct4* overexpression plasmids, respectively, at different concentrations on the activity of *SpVih* core promoter. Bar with different letters indicates significant differences ($P < 0.05$). * Represents significant difference ($P < 0.05$), ** represents extremely significant difference ($P < 0.01$).

unlabeled *Sp*TF-wt). The binding band became shallow when it combined with 10-fold cold probe. When the amount of cold probe was 100-fold, the DNA-protein complex binding band is entirely undetectable (**Figure 1B**, lane 5), indicating that this complex is specific to *Sp*TF-wt. When 100-fold unlabeled *Sp*TF-mut (mutated the binding site of Oct4 and Sox9) and *Sp*TF-wt coexisted, the complex formation was not affected, indicating that the mutated probe *Sp*TF-mut did not participate in competitive binding.

To confirm the Oct4 and Sox9 binding to the promoter of *SpVih*, EMSA supershift was carried out using both biotin-labeled and unlabeled DNA probes containing intact CRE for Oct4 and Sox9. EMSA supershift with crab eyestalk nuclear extract demonstrated a DNA-protein complex formation with a biotin-labeled probe containing Oct4 and Sox9 motifs (**Figure 1C**, lane 2). Further, Oct4 and Sox9 antibody's addition resulted in the supershift of protein–DNA complex (**Figure 1C** lanes 3–4, arrow marked).

Transcription Regulation of *SpVih* by Oct4 and Sox9

Different concentrations of Oct4 and Sox9 overexpression plasmids were co-transfected with the *SpVih* core promoter-reporter plasmid *Sp*Vih-4 into the HEK293T cell line, respectively. PGL3-basic was used as a negative control, while pRL-TK was used as the reference vector. The cells at 24 h after transfection were collected to detect reporter gene carrier activity. The results showed that the addition of either 50 ng Oct4 overexpressed plasmid or 100 ng Sox9 overexpressed plasmid could significantly promote the activity of *SpVih*, and the *SpVih* activity increased with the increment of the overexpressed plasmid concentration (**Figures 2A,B**). When HEK293T cells were co-transfected with two overexpression plasmids and *SpVih* fragment reporter plasmid at the same time, the activity of *SpVih* increased significantly. It showed a dose-effect with the concentration of transcription factors (**Figure 2C**). Compared with a single transcription factor overexpression plasmid group, co-transfection with two overexpression plasmids can significantly upregulate the activity of *Sp*Vih (**Figure 2D**).

Effect of RNA Interference Experiment on *SpVih*

To further verify the regulation of *SpVih* by Oct4 and Sox9 in cell experiments, we designed an *in vivo* RNA interference experiment. After 3 consecutive days of dsOct4 and dsSox9 injections, respectively, compared with the control group, the relative expression of *Oct4* or *Sox9* in the eyestalk of the experimental groups was significantly reduced

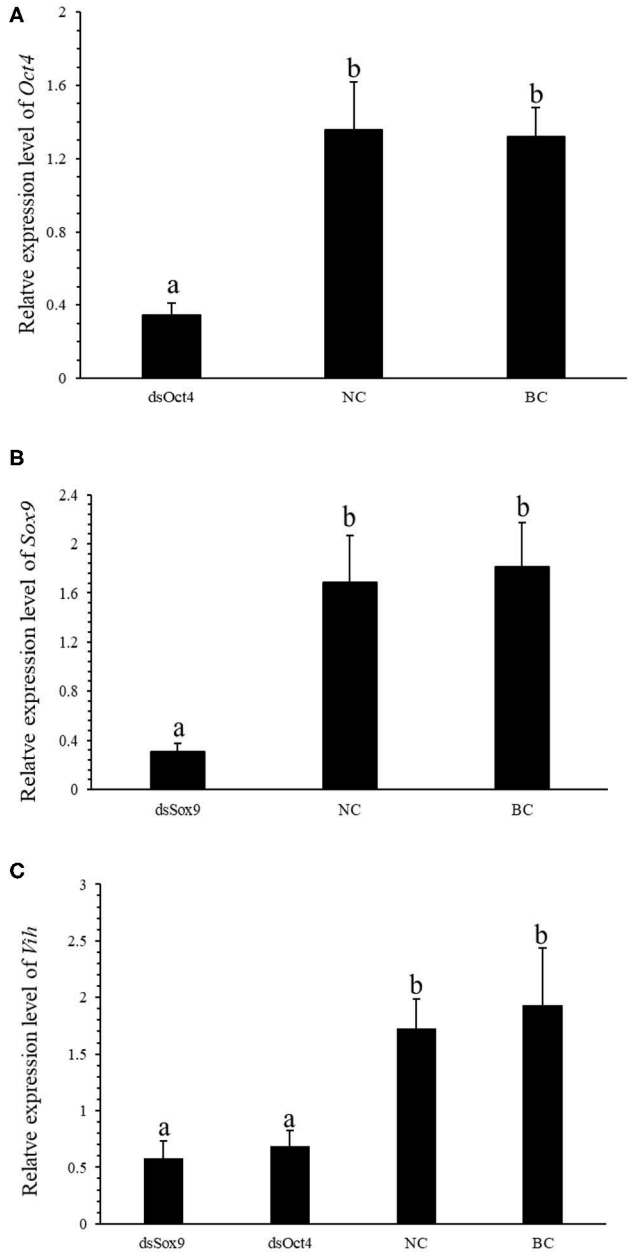

FIGURE 3 | *Oct4* or *Sox9* RNA interference reduces the expression level of *SpVih*. **(A)** Relative expression of *Oct4* in the eyestalk after injection of dsOct4, dsEGFP, and SPSS. **(B)** Relative expression of *Sox9* in the eyestalk after injection of dsOct4, dsEGFP, and SPSS. **(C)** The relative expression of *SpVih* in the eyestalk after injection of dsSox9, dsOct4, dsEGFP, and SPSS. NC, negative control group; BC, blank control group; Bar with different letters indicates significant differences, $P < 0.05$.

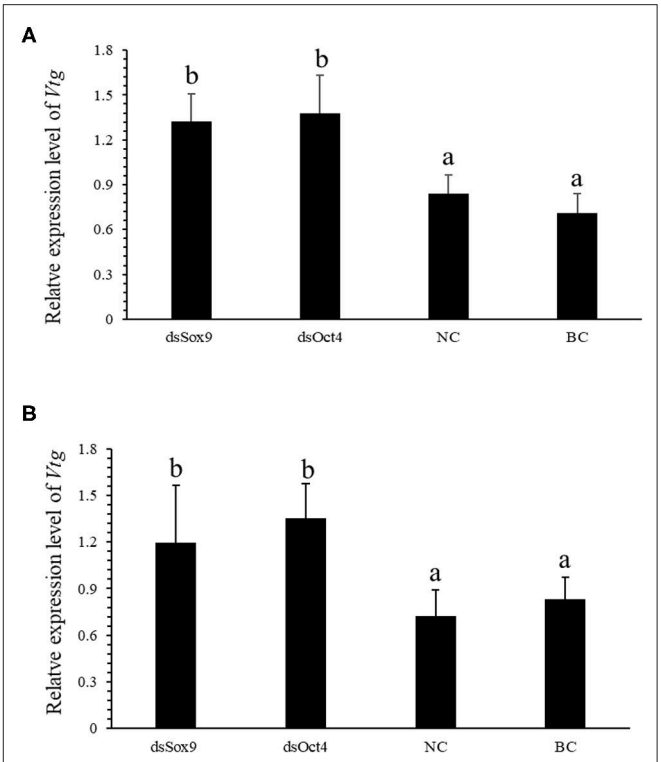

FIGURE 4 | *Oct4* or *Sox9* RNA interference promotes the expression level of *SpVtg*. **(A)** Relative expression of *SpVtg* in the ovary after injection of dsSox9, dsOct4, dsEGFP, and SPSS. **(B)** The relative expression of *SpVtg* in hepatopancreas after injection of dsSox9, dsOct4, dsEGFP, and SPSS. NC, negative control group; BC, blank control group; Bar with different letters indicates significant differences, $P < 0.05$.

(**Figures 3A,B**), indicating that the experiment successfully interfered with the expression of Oct4 or Sox9 in the crabs. Compared with the blank control group and the negative control group, the expression of *SpVih* of the eyestalk in the experimental group injected with dsOct4 or dsSox9 was significantly decreased (**Figure 3C**), indicating that interference with Oct4 or Sox9 can inhibit *SpVih* expression in the crab.

Effect of RNA Interference Experiment on *SpVtg*

Vitellogenin (Vtg) is the precursor of Vitellin (Vn or Vt) and is the main component in the yolk of oviparous animals. VIH in crustaceans can inhibit the production of Vtg and regulate ovarian development and maturation. The qRT-PCR experiment was conducted to investigate the expression of *SpVih* in the eyestalk and the expression of *SpVtg* in ovary and hepatopancreas after the injection with dsOct4 and dsSox9. The experimental results showed that, compared with the blank control group and the negative control group, when dsOct4 and dsSox9 were injected into the eyestalk of the crab, the expression of *SpVih* in the eyestalk decreased significantly while the relative expression of *SpVtg* in the ovary and hepatopancreas had a significant rise (**Figure 4**).

Recombinant Expression and Purification

Oct4 was expressed as a soluble protein after IPTG induction. It was conveniently purified by glutathione sepharose 4B chromatography. The purified protein comprised of an

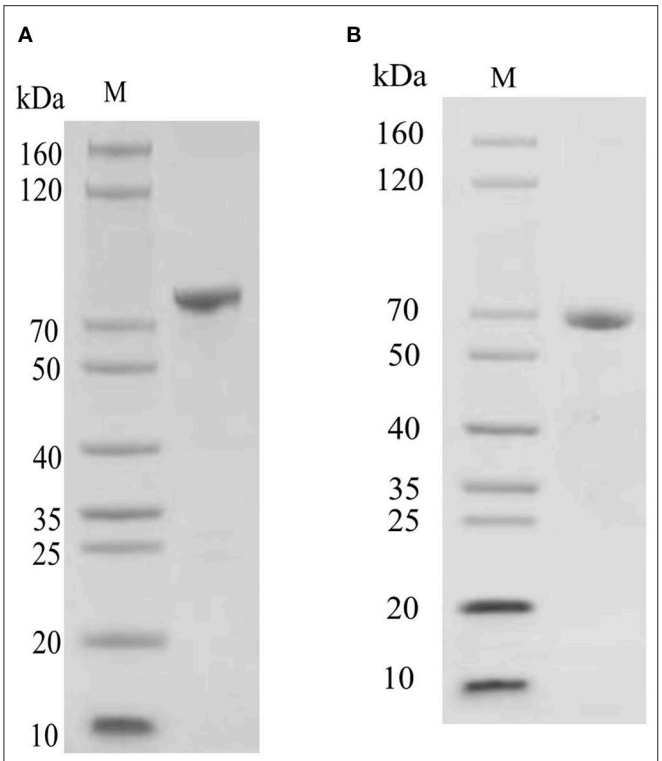

FIGURE 5 | SDS-PAGE detection of recombinant protein. **(A)** SDS-PAGE detection of pGEX-4T-Oct4 protein. **(B)** SDS-PAGE detection of pET30a-Sox9 protein. M, Marker.

~44 kDa Oct4 protein and a nearly 26 kDa GST tag expressed by the plasmid pGEX4T1, which was roughly consistent with the size (about 70 kDa) of the unique band in the purified protein lane (**Figure 5A**). Sox9 was purified by His-bind resin chromatography. Because it contained an additional His Tag, the Mw values of the purified Sox9 was larger than its theoretical 53 kDa, which was approximately consistent with the SDS-PAGE result (**Figure 5B**).

Oct4 Interacts With Sox9

The binding activity of Oct4 with Sox9 was examined by a pull-down assay. The results are shown in **Figure 6A**. In the SDS-PAGE electrophoresis detection, lane 3 is the control group with GST instead of pET30a-Sox9, and only two protein bands are GST and GST-Oct4; and the lane 4 is the experimental group with pET30a-Sox9, three protein bands are GST, GST-Oct4, and His-Sox9. GST-Oct4 and His-Sox9 were detected by anti-his polyclonal antibody in Western blot. The eluent showed that bands of the same size as His-Sox9 were detected in the experimental group in which pET30a-Sox9 was added (**Figure 6B**). Both results illustrated that pGEX-4T-Oct4 can interact with pET30a-Sox9 *in vitro*, while the negative control GST cannot bind to pET30a-Sox9.

DISCUSSION

In this study, the interaction between the two selected transcription factors and the critical promoter region of *Sp Vih* was verified by the EMSA experiment. EMSA is a common method for studying transcription factors *in vitro*, which can be used to detect the interaction of DNA and protein sequences. Through EMSA experiment, Li et al. (30) confirmed that Sox9 could directly bind to the COL10A1 gene promoter and activate its transcription, and Card et al. (31) found that Oct4 and Sox2 bind to a conserved promoter region of miR-302, a cluster of eight microRNAs expressed specifically in ESCs and pluripotent cells. In this experiment, a specific binding band can form when eyestalk nucleoprotein combined with the *Sp*TF-wt probe. However, the binding band became shallow when it combined with the 10-fold cold probe. At the same time, the binding band completely disappeared when the 100-fold cold probe was added, indicating that the cold probe can compete with the *Sp*TF-wt probe to bind certain components of eyestalk nucleoprotein. When 100-fold of *Sp*TF-mut probe (Oct4/Sox9 binding site was mutated) was added to the experimental system containing *Sp*TF-wt, the *Sp*TF-mut does not affect the formation of binding band, indicating that *Sp*TF-mut does not participate in the competition and proving that the formation of binding band is dependent on the presence of Oct4/Sox9 site. In the EMSA supershift experiment, when Oct4 or Sox9 antibody was added, the supershift band (DNA-Antigen-Antibody complex) appeared, indicating that Oct4 and Sox9 can directly and specifically bind to the key promoter region of *Sp Vih*.

In order to understand how Oct4 and Sox9 regulate *Sp Vih* expression, we constructed Oct4 and Sox9 overexpressed plasmids and *Sp*Vih core promoter fragment reporter vector. After they were co-transfected into HEK293T cell line, it was confirmed by double luciferase reporter gene that both transcription factors can promote *Sp Vih* expression and have a concentration effect. To verify the authenticity of the cell experiment results, we obtained Oct4 and Sox9 dsRNA by *in vitro* transcription. Then we injected the dsRNA into the eyestalks to interfere with the expression of two transcription factors in the crabs. The results showed that both Oct4 and Sox9 in the two experimental groups were successfully inhibited compared with the control group. The expression level of *Sp Vih* in the eyestalk decreased accordingly. Both the cell overexpression experiment and the *in vivo* RNA interference experiment showed that the transcription factors Oct4 and Sox9 could positively regulate *Sp Vih*.

Vitellogenin (VTG) is a precursor of vitellin and the main component of egg yolk proteins in oviparous animals. In the mud crab, it mainly exists in the gonads and hepatopancreas and can promote ovarian development and maturation (32). VIH can play a negative regulatory role in the expression of VTG (33). In the *S. olivacea*, VIH in the eyestalk is involved in inhibiting the production of VTG (34). Type II VIH in *P. vannamei* has the activity of inhibiting its vitellogenesis, and it can effectively inhibit the expression of Vtg mRNA in the hepatopancreas of the shrimp (35). In order to further explore the influence of Oct4 and Sox9 on *Sp Vtg*, we tested the expression

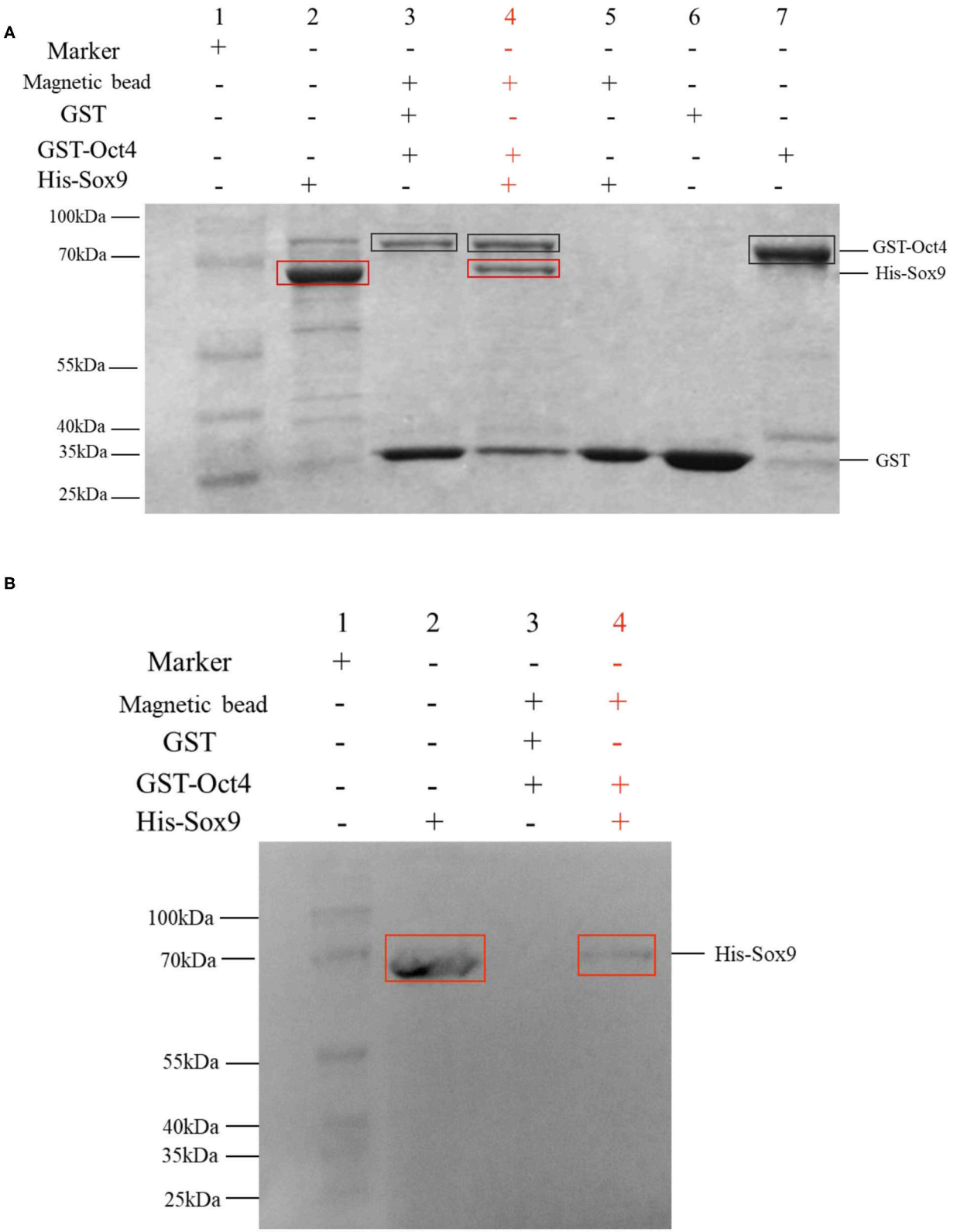

FIGURE 6 | GST pull-down assay to determine the interaction between Oct4 and Sox9. Recombinant His-Sox9 was incubated with recombinant GST-Oct4. **(A)** Western blotting was performed using antibodies against His tags. **(B)** SDS-PAGE detection. The experimental group was shown in red. In the figures, the GST-Oct4 protein was marked with a black box, and His-Sox9 protein was marked with a red box.

level of *SpVtg* in ovary and hepatopancreas of mud crab after Oct4 and Sox9 interference. It was found that compared with the control group, as the *SpVih* expression level of the eyestalk in the RNA interference group decreased, the *SpVtg* expression level in the ovary or hepatopancreas increased significantly.

As transcription factors, Oct4 and Sox9 often interact with other transcription factors to achieve regulatory functions. Oct4 and Sox2 directly regulate the expression of another pluripotent transcription factor Zfp206 in embryonic stem cells (36). In Xenopus, Oct4 homolog Oct91 can bind to each type of SoxB1 (Sox1, Sox2, Sox3) protein and had different effects, Oct91 cooperates with Sox2 to maintain neural progenitor marker expression (37). During chondrogenesis, the direct binding of Sox9 and ap-1 promotes the activity of multiple genes, for example, the interaction of Sox9 and AP-1 synergistic activates the Col10a1 enhancer (38). Masuda and Esumi (39) found that Sox9 can regulate the expression of BEST1 in retinal pigment epithelium by interacting with the microphthalmia-associated transcription factors MITF and OTX2. In the cell transfection experiment, a group that simultaneously transfected two transcription factors with *SpVih* into the cell was set up, and their activity of *SpVih* was significantly enhanced compared to the group with only one transcription factor. It is speculated that there may be some interaction between the two transcription factors to strengthen the regulation of *SpVih*. In order to verify whether there is a real interaction between the two transcription factors, we obtained the Oct4 and Sox9 recombinant proteins with the GST tag and His tag through prokaryotic expression, respectively. GST pull-down analysis showed that there was indeed a direct interaction between Oct4 and Sox9.

In conclusion, the transcriptional regulation of *SpVih* by the transcription factors Oct4 and Sox9 in mud crab was further elaborated through cell overexpression, *in vivo* RNA interference, and GST pull-down. These results will help us to better understand the regulatory mechanism of *SpVih* gene, and therefore we hope that it will be possible to achieve the goal of promoting the development and maturity of crab ovaries by controlling key factors and finally apply the research results to production practice in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Jimei University.

AUTHOR CONTRIBUTIONS

JL, ZZh, and YW: conceptualization and writing—review and editing. JL, XJ, ZZo, and KL: methodology, investigation, and visualization. JL: software, validation, formal analysis, data curation, and writing—original draft. YW: resources, supervision, and project administration. YW and ZZh: funding acquisition. All authors contributed to the article and approved the submitted version.

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Investigation of Gene Sequence Divergence, Expression Dynamics, and Endocrine Regulation of the Vitellogenin Gene Family in the Whiteleg Shrimp *Litopenaeus vannamei*

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In this report, we studied the vitellogenin gene family in the whiteleg shrimp *Litopenaeus vannamei* by transcriptomics, bioinformatics, and molecular biology methods. At least three moderately homologous vitellogenin (Vg) genes (i.e. *LvVg1*, *LvVg2*, and *LvVg3*) were identified in the genome. The deduced LvVg proteins consisted of a vitellogenin_N domain, a DUF1943 domain, and a VWD domain typical of most vitellogenins from oviparous animals. *LvVg1* was the most abundant *Vg* expressed in the hepatopancreas and ovary of maturing females. Furthermore, multiple isoforms of *LvVg1* were evolved presumably due to the need for rapid Vg production during the rapid phase of vitellogenesis. *LvVg* transcripts were detected in different larval stages, juveniles, and subadults. During the non-reproductive cycle, *LvVg* expression in the hepatopancreas peaked at the intermolt stages. During the female vitellogenesis cycle, a two-phase expression pattern of *LvVg1* gene was observed in the hepatopancreas and ovary. Moreover, the eyestalk optic nerve, brain, and thoracic ganglion consisted of factors that differentially regulated the expression of the three *Vg* genes. In addition to their reproduction-related roles, Vg may also be involved in growth and molt-related processes. Phylogenetic analysis revealed the early expansion and separation of these *Vg* genes, and it is most likely correlated with the expansion of *Vg*'s function. In conclusion, the evolution of multiple *LvVg1* isoforms and the acquisition of different *Vg* genes (i.e. *LvVg2* and *LvVg3*) may occur universally in most decapods. Full information on the total number of *Vg* genes and precise knowledge on the expression pattern and endocrine regulation of each *Vg* during all life cycle stages are crucial for us to understand the roles of this emerging gene family in the control of shrimp reproduction and other non-reproductive processes.

Keywords: vitellogenin, shrimp, expression dynamics, gene divergence, ovary

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INTRODUCTION

Growth and reproduction are two energy-requiring processes important for the species' continuation. During female maturation of shrimp, the ovary undergoes rapid vitellogenesis from the synthesis of a large quantity of the major egg yolk protein or vitellogenin (Vg). As in other oviparous animals, shrimp vitellogenins (Vgs) are large lipophospoproteins with several conserved domains including the N-terminal LNP domain, domain of unknown function DUF1940, and the von Willebrand factor type D domain (VWD) (**Figure 1A**). Vg is defined as a storage molecule with the main function to supply nutrients, energy, and raw materials to developing oocytes for embryonic and larval growth (1, 2). It was initially known to be expressed only in females (5, 6). After synthesis from its site(s), Vg is cleaved into several subunits and then transported to the ovary for uptake. Research on Vg in decapod crustaceans began with the purification of Vg from maturing ovary, followed by amino acid sequence determination of the protein (3, 7, 8). After that, degenerated primers were designed to clone a partial fragment of the Vg gene by RT-PCR. Eventually, the RACE and RT-PCR cloning approaches were used to obtain the full-length sequence of Vg (3, 7). Previous studies using molecular cloning approaches resulted in the identification of one shrimp vitellogenin gene. In subsequent studies based on genomic PCR cloning, genome walking and genomic library screening, the vitellogenin genes of several decapods have been reported (**Figure 1B**) (3, 4, 8–10).

Historically, the site of Vg synthesis in decapods has been an issue of debate as there are many conflicting results. Several tissues/organs have been reported as the sites of Vg synthesis in crustaceans. These sites include the sub-epidermal adipose tissue, the hepatopancreas, and the ovary (11–16). While the idea that Vg is synthesized by the sub-epidermal adipose tissues has not gained support in recent years due to the lack of further evidence, there are still back-and-forth arguments for the ovary and/or hepatopancreas as the site of Vg synthesis. Recently, increasing evidence has indicated the existence of multiple Vg genes. For example, in the sand shrimp *Metapenaeus ensis*, two forms of *Vg* gene were reported (2, 3); in the fresh-water prawn *Macrobrachium rosenbergii*, four different *Vg* genes have been identified (10). Other decapods that have been reported to have more than one *Vg* gene include the whiteleg shrimp *Litopenaeus vannamei* and the red crab *Charybdis feriatus* (9, 16, 17). The controversy in the source of Vg synthesis might be due to stage-specific expression pattern of *Vg* genes. In short, without full knowledge of the total number of vitellogenin genes, it would be difficult to obtain precise expression information of *Vg*(s).

Concerning the endocrine regulation of vitellogenesis in crustacean, it is well established that the eyestalk and nervous tissues such as brain and thoracic ganglion produce neuro-endocrine factors that regulate vitellogenesis. For example, eyestalk ablation can cause precocious ovary maturation in shrimp and the brain of shrimp may consist of factors that can stimulate oogenesis.

Recently, the role of vitellogenin as a reproduction-related protein has been expanded. Some studies reported that, as in vertebrates, Vg can also be detected in male individuals under environmental stress. Furthermore, some studies also reported the expression of *Vg* in non-reproductive tissues and these *Vgs*

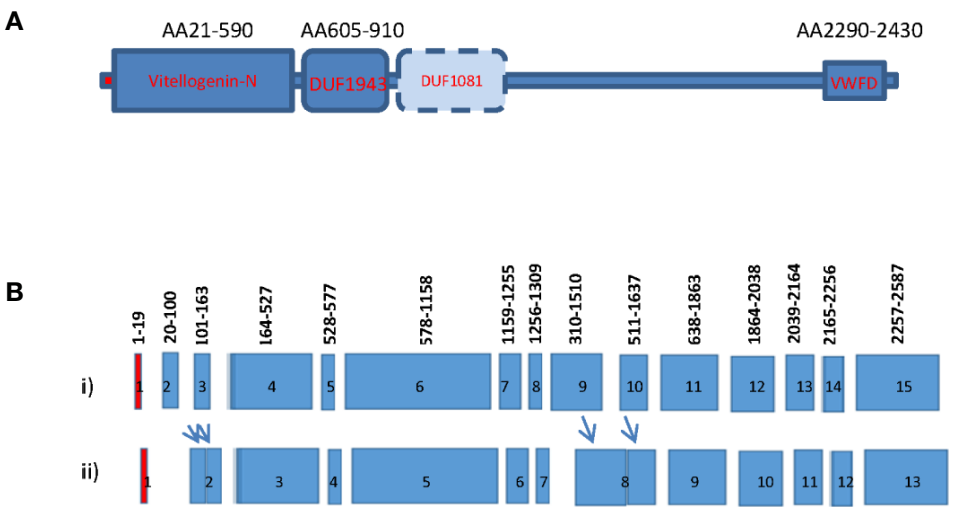

FIGURE 1 | Gene structure and domain organization of vitellogenin of decapod crustaceans. **(A)** Vitellogenin gene structure of decapod crustaceans. The *Vg* of crustacean consists of the N-terminal vitellogenin-N domain, a domain of unknown function 1943 (DUF1943), occasionally a domain of unknown function 1081 (DUF1081), and a C-terminal vertebrate Von Willebrand D domain (VWFD) (1–2); **(B)** Intron and exon gene organization of shrimp vitellogenin. Exons are indicated by the colored boxes and introns are represented by gaps between neighboring exons. Most crustacean *Vg* genes consist of 15 exons interrupted by 14 introns (i). Only a few Vg genes consist of less than 15 exons (ii). The red boxes represent exon1 that encodes for the signal peptide of the *Vgs*. The numbers above each exon indicate the position of the amino acid encoded by the exons. The relative position of these intron/exon boundaries are also conserved in many reported *Vg* genes (3–4). Arrows indicate fusion of the exons to form a larger exon.

may have an antimicrobial function or immune-related functions (18–21). For example, in the crab, a *Vg2* cDNA was detected in the hemocyte and the function of the *Vg2* appeared to be immunity-related (17, 18). Knowledge of the structure, organization, and promoter sequence information of vitellogenin is important for us to understand the evolution and regulation of *Vg* genes (10).

The whiteleg shrimp *L. vannamei* is the dominant shrimp species cultured worldwide. The production of shrimp fry from the hatchery is hampered by the lack of information on the endocrine regulation of female vitellogenesis and reproduction (16). Therefore, manipulation of female maturation has not been successful and shrimp hatcheries have to rely on the undesirable and harmful technique of eyestalk ablation to stimulate female maturation (2). Unilateral eyestalk ablation is used to remove the source of a gonad inhibition hormone leading to the stimulation of gonad maturation or rapid vitellogenesis. The drawback of eyestalk ablation is the removal of other endocrine factors associated with the eyestalk X-organ sinus gland complex. As a result, the quality of the egg produced is inferior (17). Although a *LvVg* cDNA has already been reported in the whiteleg shrimp *L. vannamei* (16), the finding that this *Vg* represents only a member of the *Vg* gene family leads us to reconsider its contribution in vitellogenesis and the evolution of different members involved in gonad maturation. In this study, we used transcriptomic, bioinformatic, and molecular biology approaches to characterize several vitellogenin genes from the whiteleg shrimp *L. vannamei*. The genomic structures and phylogenetic relationships of various *Vg* genes were analyzed, and expression patterns of these *Vg* genes during ovary maturation, at different ontogenic stages or during the molting cycle were investigated. Potential regulation of these *Vg* genes by the eyestalk, brain, and thoracic ganglion was also examined, with discussion on the roles of this emerging gene family in the control of shrimp reproduction and other non-reproductive processes.

MATERIALS AND METHODS

Animals

Larval, juvenile, and adult whiteleg shrimp were obtained from the Donghai Island shrimp breeding and research center of Guangdong Ocean University (GDOU). Subadult (22–28 g) and adult (45–49 g) whiteleg shrimp *L. vannamei* were either obtained from our own culture facility or from local sea-food markets. All animals were cultured in indoor culture tank with natural lighting, in flow-through seawater at a temperature of 27–29°C and a salinity of 32–34‰. They were fed with either pellet diet (Yuehai Feed Group) or maturation diet (fresh squid and polychaetes) three times daily at a rate of 10% body weight.

RNA Extraction, Transcriptome Sequencing, and *Vg* Homologs Identification

We have performed a transcriptome sequence analysis of the eyestalk, brain, hepatopancreas, and ovary of the whiteleg shrimp *L. vannamei*. Total RNAs were prepared by an RNA extraction kit (Trizol, Invitrogen Life Technology, CA, USA). Each shrimp was considered a separate sample and three samples were taken as biological replicates for each group, and an equal amount of RNA from three individuals was pooled to make a sample for library construction. RNA library construction and paired-end sequencing was carried out at the Gene Denovo Biotechnology Company (Guangzhou, China) using Illumina HiSeq™ 4000. After data filtering, the clean sequencing reads were subjected to transcriptome assembly using the Trinity software and associated packages. Sequences in the assembly homologous to vitellogenin genes were identified by Blast tools and used in following studies.

Genomic PCR

Genomic DNA from individual shrimp was prepared either from muscles or hepatopancreas using a spin column based Genomic DNA preparation kit (Tiangen, China). After elution from the column, the concentration of the genomic DNA was determined by OD measurement with a Nano plus spectrophotometer (Thermo, Grand Island, NY, USA). For PCR, gene specific primers were designed. To avoid the designed primer being located in the junction of the intron/exon, we choose primers from the 5' region of the exon-intron boundary.

RT-PCR and *LvVg* cDNA Cloning

To validate the assembled sequences of the cDNA of *LvVg1*, *LvVg2*, and *LvVg3*, RT-PCR and cDNA cloning was performed. Based on *LvVg* gene sequences from the transcriptome data, specific primers were designed for the amplification of the *LvVg* cDNA. PCR was performed using 2× Taq PCR Master Mix (Tiangen, Beijing, China) for the validation of *LvVg1*, *LvVg2*, and *LvVg3* fragments. PCR reactions condition was as follows: Denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. PCR products were further extended at 72°C for 5 min. For the purification and recovery of PCR products, a PCR product purification kit was used (Thermos, USA). The PCR product was then ligated to pMD20 vector (Invitrogen, USA). Afterwards, the transformation of Trans5a competent cells was conducted according to the manufacturer's instructions (Tiangen, China). The positive clones were verified by PCR and sequenced, and overlapping fragments of the *Lv1Vg*, *Lv2Vg*, and *Lv3Vg* genes were obtained.

Expression Patterns of *LvVgs*

To investigate the tissue specificity of *LvVg* expression, total RNAs were prepared from different tissues (including the epidermis, hepatopancreas, eyestalk, ovary, brain, nerve cord, thoracic ganglion, and muscle) from female adults. To examine gene expression changes during ontogeny, RNA was also extracted from the nauplius, zoea, mysis, as well as tissues of the juvenile and adult shrimp. Total RNAs were reversely transcribed to cDNA. Both β-actin and the elongation factor (EF-1a) were used as the internal references for the reverse transcription-polymerase chain reaction (RT-PCR) analysis. PCR reaction conditions were as follows: denaturation at 94°C

for 3 min, followed by 34 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Finally, extension at 72°C for 5 min was conducted to complete the reactions. The PCR products were analyzed by 1% agarose gel electrophoresis.

In addition, total RNA was extracted from the hepatopancreas and ovaries of females at different maturation stages (gonadosomatic index [GSI] from 1–12%) and at various molt cycle stages (including A, B, C1–C3, D, and E). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using the Bio-Rad and SYBR Premix Ex Taq II (TaKaRa, Tokyo, Japan) to investigate the expression patterns of *LvVgs* at different developmental stages, using β-actin and LvEF-1a as the internal control. The primers for the qRT-PCR are shown in **Supplement Table 1**. The PCR amplification system contained 1 μg cDNA, 10 μl 2× SYBR mix, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), and ddH_2O was added to make a total volume of 20 μl. The conditions for the qRT-PCR reactions were as follows: pre-denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Next, 1 cycle of 95°C for 1 min, 65°C for 30 s, and 95°C for 1 s was conducted to obtain the melting curve, which was used to verify the specificity of the qPCR primers. The reactions were repeated for three times for each sample, using sterilized ddH_2O as the blank control. The Cq values were obtained after the reactions were completed. $2^{-\Delta\Delta}$Ct method was used to calculate the relative expression of *LvVgs* at different developmental stages and different molt cycle stages.

Bioinformatic Analysis of the Vitellogenin Gene Family

Vg and *Vg* related transcripts were identified from the transcriptome assembly mentioned above, and their sequences were further validated by molecular cloning techniques. For sequence comparison of these transcript, BLAST search analysis was performed initially to determine if they were homologous to *Vg* gene of a specific crustacean species. Multiple sequence alignment was performed by CLUSTALW (https://www.genome.jp/tools-bin/clustalw); the amino acid translation was performed using http://molbiol.ru/eng/scripts/01_13.html; and Expasy MW/pI tool (http://web.expasy.org/compute_pi/) was used to obtain theoretical molecular weight and isoelectric point. National Center for Biotechnology Information open reading frame (ORF) finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the ORF. SignaIP 5 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to identify the signal peptides, NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict the potential phosphorylation sites, and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) were used to predict the N- and O-linked glycosylation sites. BLASTP search (https://blast.ncbi.nlm.nih.gov/) was used for the homology comparisons of *LvVgs* sequences with other crustaceans. The Fast Tree software was adopted to construct the phylogenetic tree, using the Neighbor-Joining method. The bootstrap test was used for statistical analysis of each branch, with a repetition time of 1,000.

Neuroendocrine Factors Regulating the Expression of *Vg* Genes

Eyestalk optic nerve and other neuronal tissues have been implicated to consist of factors that can stimulate or inhibit vitellogenesis. To investigate the endocrine factors from various neuronal tissues that may differentially regulate the three *Vg* genes, an *in vitro* explants culture assay was conducted. Hepatopancreas and ovary fragments were incubated with either optic nerve of the eyestalk, brain, or thoracic ganglion. To optimize the study, we used shrimp that were at the early to middle stages (GSI 3–4%; N = 5) of gonad maturation for the test. Ovary and hepatopancreas were dissected and cut into fragments of ≤8 mm^3. They were placed together in a well of the culture plate containing 2 ml of nutrient Medium 199 (Sigma, St. Louis, MO, USA). The dissected brain, eyestalk optic nerve, and thoracic ganglion were placed separately into different wells that consisted of the hepatopancreas and ovary fragments as described above. The culture plates were incubated in a gentle rocking/shaking device for 4 h. At the end of the experiment, all the tissues in the well were harvested and extracted for total RNA. The total RNAs were reverse transcribed to cDNA and used for RT-PCR detection of *LvVg1*, *LvVg2*, and *LvVg3* expression.

Statistical Analysis

Relative gene expression was recorded in expression study and statistical analysis of the gene expression with mean normalized ratios (± SD) between the copy number of target genes and the mean copy number of the reference genes. All values were analyzed using one-way analysis of variance (ANOVA), and then the Turkey (B) multiple-range test was used for comparisons in SPSS statistical package version 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Transcriptomic and Molecular Identification of Vg Gene Family

A total of 15 transcripts encoding for full-length or partial *Vg* or *Vg-like* cDNAs were identified from the hepatopancreas and ovary transcriptomes of the whiteleg shrimp *L. vannamei*. Most of the larger transcripts were full-length cDNAs encoding for Vg proteins and the smaller transcripts encoded for partial and truncated cDNA of *Vgs*. We focused on analysis of the larger transcripts. These transcripts can be divided into three groups based on the amino acid sequence homology, the sizes of the deduced proteins, alignment results, and their homologies with other vitellogenins from the GenBank BLASTX search results. The first group of vitellogenin consisted of five transcripts (i.e. CL2883C1-C5) that we named as *LvVg1a* to *LvVg1e* (**Figure 2A**, **Supplement Figure 1**). BLASTX search analysis of these *LvVgs* revealed that they shared high sequence identity (i.e. 99.1–99.4%) with the previously reported *Vg* gene (Genbank# XP027235402.1), followed by the *Vg-like* gene (Genbank# AAP76571.2, 93.8%) of whiteleg shrimp *L. vannamei*. All the

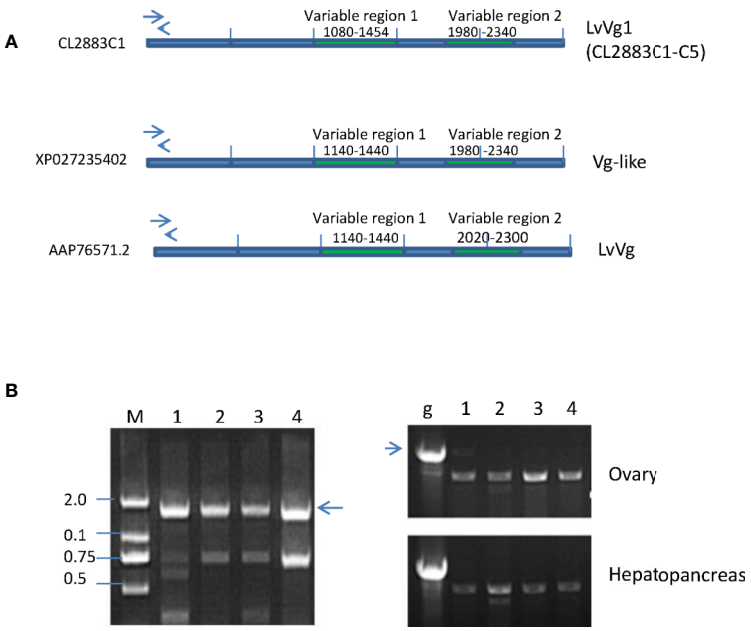

FIGURE 2 | Comparison and confirmation of *LvVg1* genes isolated from the transcriptomic study with previously reported *Vg* (Genbank# XP027235402) and *Vg*-like (GenBank# AAP76571) genes in the whiteleg shrimp *L. vannamei*. **(A)** The LvVg1 transcripts (i.e.CL2283C1-C4) shared high degree of amino acid sequence identity (>97%) in the Vitellogenin-N domain, DUF1943, and VWB domain but only low degree of identity (50–56%) in the VR1 and VR2 regions. Arrows indicate location of the primers used to distinguish different *LvVg1* isoforms. **(B)** Left panel: Genomic PCR detection of multiple *LvVg1* genes from hepatopancreas derived gDNA of a single female. Lane M: 2 kb size marker; lanes 1–4 are gDNA amplified by primers specific to *LvVg1a*, *LvVg1b*, *LvVg1c*, and *LvVg1d*, respectively. The arrow indicates the expected genomic DNA amplified by the specific primers. Right panel: RT-PCR amplification of ovary and hepatopancreas cDNA. Lane g shows the PCR result of the genomic DNA (arrow) using one of the primer-pairs mentioned above. Lanes 1–4 are RT-PCR result using the corresponding four pairs of gene specific primers. All transcripts (C1 to C4) can be detected in the ovary and hepatopancreas, confirming that at least four copies of *LvVg1* genes are present in the shrimp genome.

five *Vg* sequences shared high sequence identity in the LPN domain, DFU1934 domain, and von Willebrand domain (i.e. >99.7% amino acid sequence identity). However, relatively low sequence identity was observed in two regions that we called variable regions 1 (62% aa identity) and variable region 2 (65.4% aa identity). Since the transcriptomes were constructed using cDNAs from more than one shrimp and the presence of seven transcripts (together with the previously published *LvVgs*) of *LvVg1* suggested that multiple *LvVg1* isoforms may exist in the whiteleg shrimp *L. vannamei*. Therefore, to demonstrate the existence and expression of multiple *LvVg1* isoforms, genomic DNA PCR and RT-PCR approaches were used to confirm the existence of multiple *LvVg1* genes in a single whiteleg shrimp *L. vannamei* (**Figure 2B**). When genomic PCR was performed using specific primers for *LvVg1a-LvVg1e* genes spanning the first and second exon of *LvVg1*, positive amplifications were obtained **(Supplementary Table 1)**. Also, the RT-PCR results confirmed the presence of multiple isoforms of *LvVg1* (**Figure 2B).** Therefore, these highly similar *LvVg1* cDNAs were derived from different genes of a single shrimp. When *LvVg1a* was compared with *Vgs* of other penaeidaes, it shared high sequence identity with *Vg* of the black tiger shrimp *P. monodon* (82%), the banana shrimp *F. merguiensis* (83%), and the fleshy shrimp *Fenneropenaeus chinensis* (84%). When other *LvVg* isoforms were used for the BLASTX search comparison, similar sequence identity results were observed (data not shown).

The second group of vitellogenins was represented by the transcript Unigene9553 (**Figure 3**). The deduced protein of this transcript (i.e *LvVg2*) consisted of only 2,560 amino acid residues. However, BLASTX search analysis did not return any homologous mRNA sequence from *L. vannamei*. Instead, two genomic sequences were identified to share high sequence identity with Unigne9553 (see text described below). The top decapod sequence that showed high sequence identity with *LvVg2* was the *MeVg2* gene of the sand shrimp *Metapenaeus ensis*, with 63% aa sequence identity. Other penaeidae *Vgs*, however, shared only ~50% identity. For example, *LvVg2* shared only 52% aa sequence identity with the *Vg* of the kuruma shrimp *Penaeus japonicus* (BAD98732), the fleshy shrimp *Penaeus chinensis* (ABC86571), and the banana shrimp *F. merguiensis* (#Q6RG02). However, a transcript (i.e. ROT77685) identified from the whiteleg shrimp *L. vannamei* genome project shared the highest sequence identity (i.e. 97%)

The third group of *LvVgs* consisted of the transcript Unigene19992 (**Figure 3**). Unlike the other two transcript groups, the deduced protein of Unigene19992 (*LvVg3*) encoded for a smaller *Vg* like protein with only 2,087 amino acids. Furthermore, *LvVg3* appeared to be a truncated Vg that lacked the signal peptide

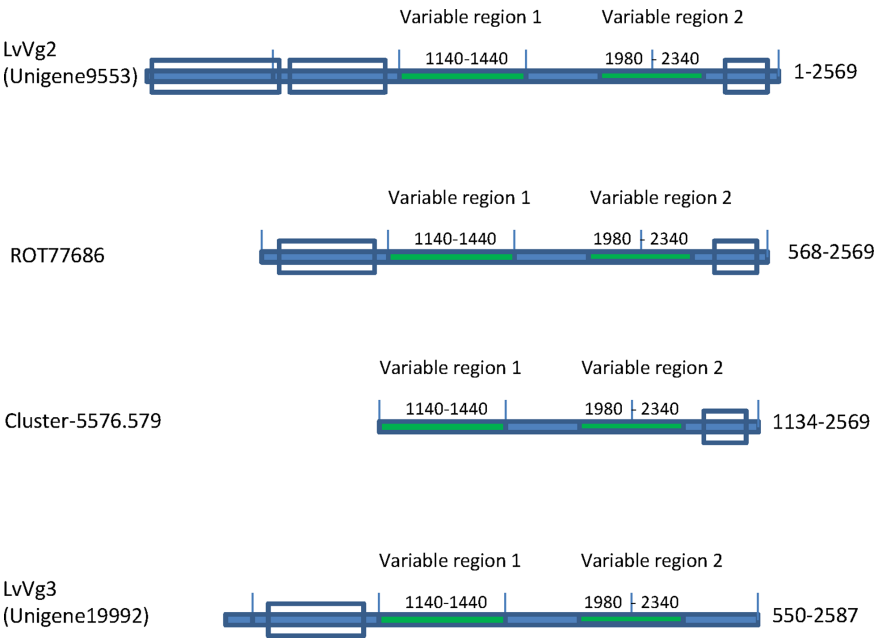

FIGURE 3 | Presence of two other *Vg* transcripts (i.e. *LvVg2* and *LvVg3*) in the whiteleg shrimp *L. vannamei*. Domain structures of *LvVg2* and *LvVg3* isolated from the transcriptome are shown. Unigene 9553 was most similar (>99% aa identity) to the deduced protein from the *L. vannamei* genome project (ROT77686), followed by *MeVg2* of the sand shrimp *M. ensis* (XP027235402) (i.e. 56% aa identity) and another reported *Vtg* (Genbank# AAP76571). Cluster-5576.579 was a transcript identified from the hepatopancreas of *Vibrio hervyei* challenged *L. vannamei*. Unigene19992 was a partial transcript for *LvVg3* as it encoded a truncated *Vg* with coding sequence from AA550-2587 as compared to *LvVg1*.

and the N-terminal LPD domains as present in the *LvVg1* and *LvVg2*. BLASTX search analysis of *LvVg3* revealed that it was most similar to the Vg of *P. japonicus* (Genbank # BAB01568) with only 37% aa sequence identity in the overlapping region.

Bioinformatic Analysis of *LvVg* Gene Family

The recent release of genome information of the whiteleg shrimp *L. vannamei* allows us to search for the vitellogenin gene from the GenBank *L. vannamei* genome database. Using *LvVg* cDNAs as a query to BLAST search the *L. vannamei* genome, several *LvVg* related genes were identified (**Figure 4**). The *LvVg1* sequence shared high sequence identity to a portion of the *Penaeus vannamei* Kehai breed No.1 LVANscaffold_4148, whole genome shotgun sequence (GenBank#: QCYY0100 4146.1) (**Figure 4**). Within this scaffold, four *LvVg1* like genes were identified (since these genes may not be identical to *LvVg1a–LvVg1d*, we tentatively named them as *LvVg1w*, *LvVg1x*, *LvVg1y*, and *LvVg1z*). These four *Vg* genes showed a high degree of amino acid identity (>99% aa identity) with the transcripts (CL2883C1-C5) obtained from this study. However, only *LvVg1w* and *LvVg1z* isoforms were full-length sequences. These two genes consisted of 15 exons interrupted by 14 introns and they all showed the same orientation. The intergenic region between the *LvVg1a* and *LvVg1b* genes was approximately 3 kb. Similarly, the intergenic region between *LvVg1c* and *LvVg1d* was also 3 kb. The intergenic region between the *LvVg1b* and *LvVg1c* was 30kb. These four genes were located at approximately 270,000–295,000, 290,000–300,000, 580,000–595,000, and 625,000–645,000 region of the scaffold (**Figure 4A**). In addition, another scaffold (i.e. Scaffold LVAN515, #QCYY0 1000515) also consisted of two gene sequences that shared high sequence identity with the *LvVg1* gene (**Figure 4B**). These two genes, named as *LvVg1n* and *LvVg1p*, only contained partial sequences and encoded for only a portion of the expected LvVg1 protein.

For the *LvVg2* gene, two corresponding genes were identified from an unplaced genomic scaffold of the whiteleg shrimp *L. vannamei* (i.e., ASM378908v1 LVANscaffold_1494, NCBI Reference Sequence: NW_020868836.1). The two *LvVg2* genes were located at the 260,000–340,000 and 475,000–525,000 regions of the scaffold. They were separated by an intergenic region of 13 kb and the genes were arranged in the same orientation (**Figure 4C**). BLASTX sequence search confirmed that the *LvVg2* gene consisted of 14 introns. Further analysis revealed that the second *LvVg2* gene was truncated and consisted of only 8 exons. As for the Unigene19992, BLASTX search did not return any gene sequence that shared significant homology from the whiteleg shrimp *L. vannamei* genome.

As the first step to study the regulation of *LvVg* genes, we analyzed the promoter regions of these genes. We have retrieved the ~2 kb upstream region of the *LvVg1w*, *LvVg1x*, *LvVg2a*, and *LvVg2b* genes from the GenBank. The alignment was performed and the result indicated that promoter regions of *LvVg1w*/*LvVg1x* or *LvVg2a*/*LvVg2b* shared >80% sequence identity. Between *LvVg1* and *LvVg2* gene promoters, they shared a

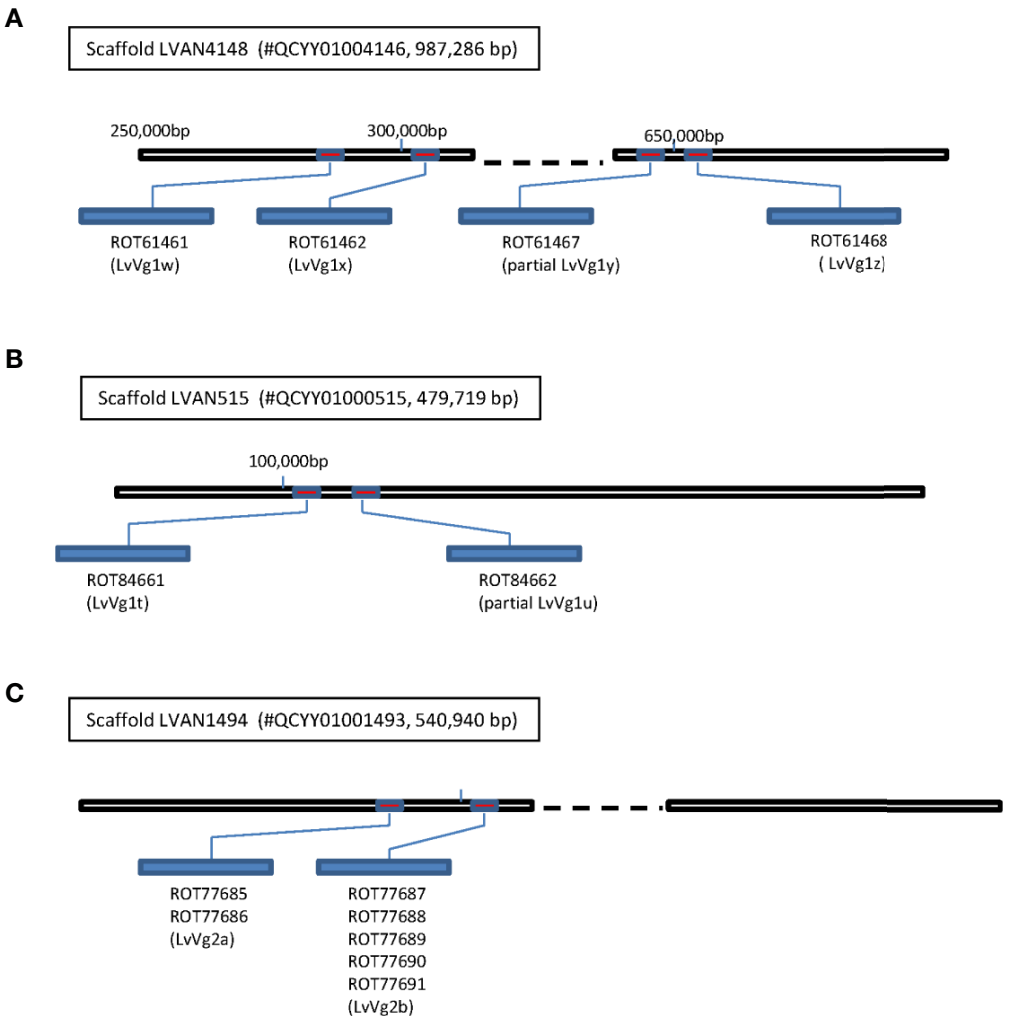

FIGURE 4 | Clustering of vitellogenin/vitellogenin-like genes in the whiteleg shrimp *L. vannamei* genome. Three *Vg* gene clusters were identified from the *L. vannamei* genome sequencing project reported by Xiang's group (22). The draft genome sequence was retrieved from GenBank database with an accession number PRJNA438564 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA438564). **(A)** Four *LvVg1* related genes (red boxes) were identified from Scaffold LVAN4148 (#QCYY010004146); **(B)** The Scaffold LVAN515 (#QCYY01000515) consisted of another two *LvVg1* related genes; **(C)** The Scaffold LVAN1494 (#QCYY01001493) consisted of a cluster of two *LvVg2* genes (red boxes). The deduced *Vgs* from these genes are shown in the blue elongated bars with GenBank accession numbers shown below the bars.

much lower sequence similarity except for the proximal region (i.e. <250 bp). In the more distal region of the promoter, most of the *Vg* members consisted of stretches of poly(AT)n rich region. This poly(AT)$_n$ repeat was highly homologous between the *MeVg2* and *LvVg2* genes as they shared >80% nt identity in this region (**Supplement Figure 2**, Promoter comparison).

Shrimp vitellogenins are synthesized as large precursor proteins and later processed into different subunits (23). SignalP 5.0 result showed that the first 18 amino acids constituted the signal peptide and all shrimp shared a conserved sequence of APW after the hydrophobic cleavage site. We have aligned some selected decapod Vg sequences and the result revealed that some conserved regions of Vg consisted of potential cleavage sites (i.e. RX(R/K)R) for the Ca^+ dependent subtilisin-like proprotein convertases (PCs). Also, several potential PC cleavage sites (i.e. Arg-Arg, Lys-Arg) can be identified in scattered locations of the Vg precursors, and two conserved cleavage sites can be detected in the conserved region of the Vg precursors (**Figure 5**).

Amino acid sequence comparison among *LvVg1*, *LvVg2*, and *LvVg3* revealed that *LvVg1a* shared 51.8 and 37.2% similarities with *LvVg2* and *LvVg3* respectively. Furthermore, the similarity between *LvVg2* and *LvVg3* was 35.8% only (**Table 1**). In addition to the lowering of amino acid similarities in the three conserved domains, the degree of amino acid similarity also decreased in the VR1 or VR2 regions.

Because of the economic interest in shrimp aquaculture, more research studies have focused on *Vg* sequences of shrimp. There is a lot less *Vg* sequence information on other decapods. Of all the decapod *Vgs*, the penaeid *Vg* sequences are most reported. The phylogenetic tree revealed that decapod *Vgs* can be divided into

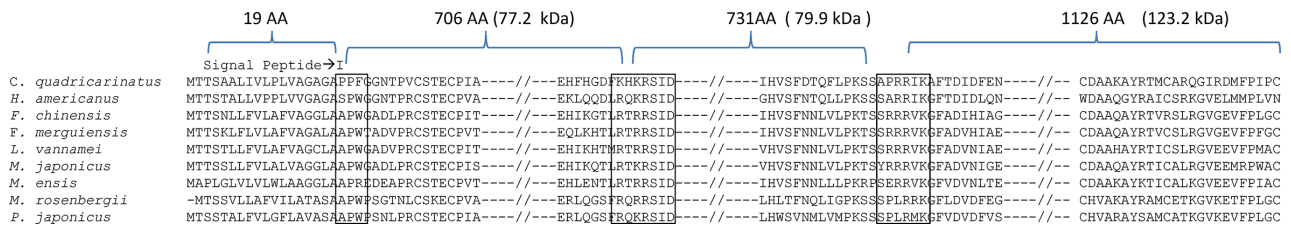

FIGURE 5 | Alignment of selected decapod vitellogenin sequences. The open boxes marked regions consisting of conserved amino acid sequence for peptide cleavage sites. The symbol "—//—" indicates omission of other regions for simplification of the alignment. With reference to *L. vannamei*, the *LvVg* precursor was predicted to produce subunits of 72.2, 79.9, and 123.2 kDa.

TABLE 1 | Comparison of LvVg1, LvVg2, LvVg3, and other Vg homologs.

		LvVg1(CL2388)	LvVg2(Unigene9553)	LvVg3(Unigene15559)
Comparison of LvVg1, LvVg2 and LvVg3	LvVg1(CL2388)		**71.2**	**58.2**
	LvVg2(Unigene9553)	51.8		**56.9**
	LvVg3(Unigene15559)	37.2	35.8	
		Shrimp	Lobster	Crab
Comparison of LvVgs and Vgs from other species	LvVg1(CL2388)	84.2-87.3	40.1-43.2	35.1-36.6
	LvVg2(Unigene9553)	52.0-62.2	39.1-39.6	35.2-36.4
	LvVg3(Unigene15559)	36.1-37.3	31.9-33.1	31.9-33.2

LvVg1 shared a higher level of similarity or identify with LvVg2 than LvVg3. LvVg1 showed the highest degree of sequence conservation in different groups of crustaceans. The three Vg transcripts of L. vannamei were generally more similar to Vg homologs from shrimp compared with those from lobsters and crabs. Bold numbers in the table indicate sequence similarity (%) while other numbers stand for sequence identity (%).

three major clusters according to their taxonomic divisions (**Figure 6**). The first group consisted of freshwater prawns such as *M. rosenbergii*, while the second group was represented by marine penaeid shrimp and the third group was formed by crabs and lobsters. *Vg* from the freshwater prawns shared a common ancestral gene with *Vgs* of the marine shrimp and lobster/crab groups. The *LvVg1* of the whiteleg shrimp *L. vannamei* clustered together with all the *Vgs* from other marine penaeid Vgs reported. Within marine shrimp, three to four phylogenetically different subgroups can be identified. The first group consisted of *Vgs* from the kuruma shrimp *P. japonicus* and the black tiger shrimp *P. monodon*, *MeVg1* from the sand shrimp *M. ensis*, and other *LvVg1* related sequences described in this study. The second group consisted of *LvVg2*, *MeVg2* of *M. ensis* and a *Vg* from *P. monodon* (identified from the transcriptome of *P. monodon* in our different project). The third group consisted of *LvVg3*. The degree of similarity between members of the same group was very consistent. For example, all members of the Vg1 or Vg1-like group shared 80% similarity. However, members of the second group (i.e. *LvVg2* and *MeVg2*) shared only 56% aa sequence identity.

Expression Study of *LvVg1*, *LvVg2*, and *LvVg3*

In this study, q-PCR primers were designed from regions that can amplify the isoforms, and tissues from females at the early vitellogenin stage were used. Tissue-specific analysis results showed that all three *LvVgs* were expressed in the hepatopancreas and ovaries with different intensities (**Figure 7A**), while *LvVg1* was the major Vg expressed in the hepatopancreas and ovary. In addition to the hepatopancreas and ovary, *Vg* transcripts can also be detected in the brain, eyestalk, and thoracic ganglion. The *LvVg1* transcript abundance in the eyestalk was low during early reproductive stages but increased rapidly during the active phase of vitellogenesis (data not shown).

At different developmental stages, *LvVgs* transcripts can be detected in the whole nauplius, zoea, and mysis (**Figure 7B**). In the hepatopancreas, these three transcripts can be detected at the juvenile stage, then starting to decrease and consolidating at a relatively low level at the sub-adult stage. At different molt cycle stages, *LvVg1*, *LvVg2*, and *LvVg3* can be detected with the maximum level at the intermolt stage (**Figure 7C**).

At different stages of female ovary maturation (GSI = 0 to >10), a unique pattern of *LvVg* expression in the hepatopancreas was observed (**Figure 8**). In the hepatopancreas, vitellogenesis begins as the shrimp enters from early postmolt to the early intermolt stage. The expression level of *LvVg1* increased rapidly at the early intermolt stage. The expression rate maintained a steady increase towards the mid-intermolt stage. Thereafter, the expression rate decreased towards the early premolt stage. The expression of *LvVg2* lagged behind *LvVg1* and increased to the maximum towards the mid intermolt stage. Expression levels of both *LvVg1* and *LvVg2* increased steadily towards the end of intermolt. For *LvVg3*, its expression was initiated at the end of the maturation cycle (i.e. from GSI = 8–10, **Figure 8A**). For the ovary, a progressive increase in expression of *LvVg1* occurred from GSI = 0.3 to the maximum level towards GSI = 10%. The

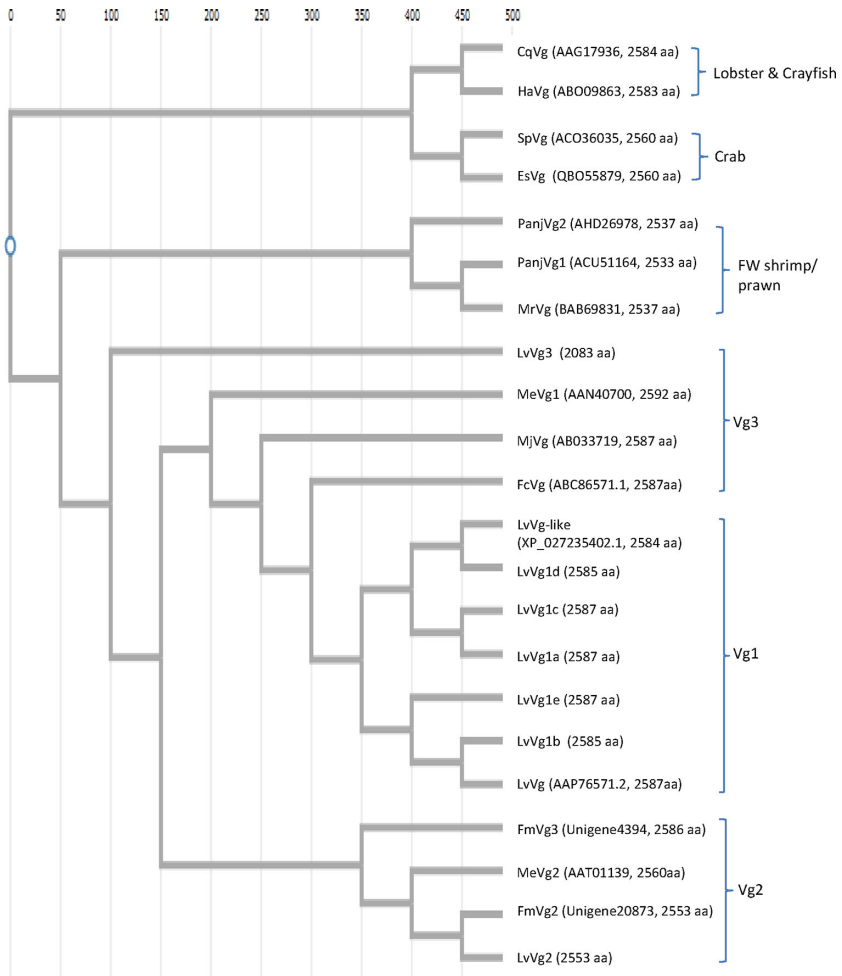

FIGURE 6 | Phylogenetic analysis of selected *Vg* genes from different decapods. Nucleotide sequences were aligned first with ClustalW software (https://www.genome.jp/tools-bin/clustalw). Phylogenetic tree reconstructions were performed using the function "build" of ETE3 v3.1.1 as implemented on the GenomeNet (https://www.genome.jp/tools/ete/). The tree was constructed using fast tree program with slow NNI and MLACC = 3 (24). The GenBank accession numbers and full-length amino acid sizes of the *Vg* are shown. These include: the red claw crayfish *Cherax quadricarinatus CqVg* (#AAG17936.1), the American lobster *Homarus americanus HaVg* (ABO09863.1), the mud crab *Scylla paramomosian SpVg* (ACO36035), and Chinese mitten crab *Eriocheir sinensis EsVg* (QBO55879.1); the freshwater shrimp *Pandalopsis japonica PanjVg1* (AHD26978) and *PanjVg2* (ACU511645), the giant river prawn *Macrobrachium rosenbergii MrVg* (BAB69831); the whiteleg shrimp *Litopenaeus vannamei LvVg*-like (XP_027235402) and *LvVg* (AAP76571), the sand shrimp *Metapenaeus ensis MeVg1* (AAN40700) and *MeVg2* (AAT01139), the kuruma shrimp *Marsupenaeus japonicus MjVg* (ABC33719), the Chinese shrimp *Fenneropenaeus chinensis FcVg* (ABC86571), and the banana shrimp *Fenneropenaeus merguiensis FmVg3* and *FmVg2* (Unigene4394 and Unigene20873 in our unpublished data). Other *L. vannamei Vg* genes identified from the current study included *LvVg1a-e* (CL2883.Contig1-5), *LvVg2* (Unigene9553), and *LvVg3* (Unigene19993).

expression level decreased rapidly after spawning. At the end of the intermolt, the GSI of the females reached 9–10% and the shrimp is ready to spawn (**Figure 8B**). Spawning usually occurs during the late intermolt when the GSI is >9%. During the post-spawn phase, the GSI of the female dropped to 2–3% and a low level of *LvVg1* transcript can still be detected in the hepatopancreas.

It was evident that the ovary and hepatopancreas appeared to respond very differently to the treatment of the nervous tissues. For example, the hepatopancreas fragments all responded to the stimulating factors in the optic nerve, brain, and thoracic ganglion to different degrees. Compared to the ovary, the hepatopancreas fragment was highly active with the *LvVg1* transcript levels at least five times higher than that of the ovary fragments (**Figure 9**). The response of the ovary, however, appeared to lag behind the hepatopancreas as most of the stimulation occurred at 3 to 4 h after incubation. Moreover, the effect of the thoracic ganglion or brain on *LvVg1* induction in the ovary fragments was much larger than that of the eyestalk optic nerve. Compared with *LvVg1*, *LvVg2*, and *LvVg3* exhibited quite variable expression profiles (data not shown) following incubation with the nervous tissues, and no specific patterns can be discerned. In summary, the results indicated that the eyestalk,

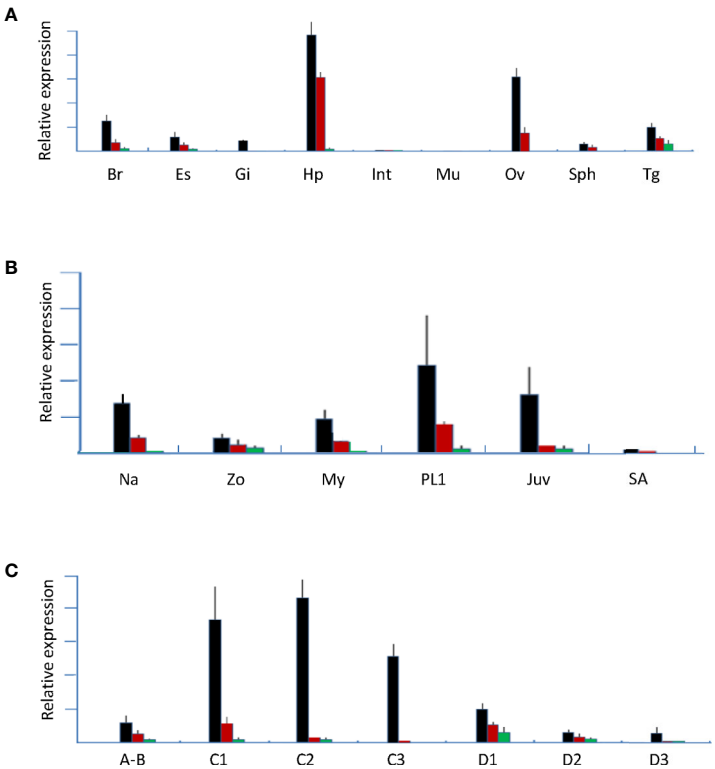

FIGURE 7 | Gene expression analysis of *LvVg1*, *LvVg2*, and *LvVg3*. The black bars are for *LvVg1*, the red bars are for *LvVg2*, and the green bars are for *LvVg3* (N = 3). **(A)** Tissue specific expression of *Vgs*. Brain (Br), eyestalk (Es), gill (Gi), hepatopancreas (Hp), intestine (Int), muscle (Mu), ovary (Ov), spermatophore (Sph), and thoracic ganglion (Tg); **(B)** Expression of different *LvVg* transcripts in the nauplius (Na), zoea (Zo), mysis (My), post-larvae (PL1), juvenile (Juv), and subadult (SA); **(C)** Molt cycle expression pattern in the hepatopancreas. Postmolt (A, B); early-mid intermolt (C1–2), late intermolt (C3), early premolt (D1), middle premolt (D2), and late premolt (D3).

brain, and thoracic ganglion consisted of factors that can stimulate the expression of *LvVg1* gene (**Figure 9**) and the responses of hepatopancreas and ovary to the neuronal factors were different.

DISCUSSION

Next generation sequencing (NGS) methods including the RNA-Seq technique have provided us important tools for gene discovery, gene structure analysis, gene evolution research, and population genetic studies (25, 26). RNA-seq and transcriptome analysis is also important for gene expression regulation studies (25). In recent years, the number of vitellogenin genes identified from decapod species increases with the growth in data from genome sequencing, transcriptomic and proteomic projects (26). In this study, we have identified several *Vg* transcripts from a transcriptome assembly of *L. vannamei*. Sequences of these assembled *Vg* transcripts were validated with molecular cloning techniques. Expression patterns of these genes in different tissues, at different ovarian developmental stages, or during the molting cycle were examined. Potential regulation of the *Vg* genes by major nervous organs was also investigated. Moreover, bioinformatic approaches were also utilized to dissect the phylogenetic relationships of *Vg* homologues, and data-mine information about *Vg* genomic sequences of *L. vannamei* from the public database.

In this study, we identified many Vg or Vg-like transcripts from the transcriptome of the whiteleg shrimp *L. vannamei*. These Vg-like transcripts can be divided into three major groups with the *LvVg1* being the most abundant type. The first report for the existence of multiple *Vg* genes in marine shrimp was in the sand shrimp *M. ensis*. In the study, genomic southern blot analysis using a probe spanning a large *MeVg1* cDNA had identified many DNA fragments hybridized to the probe (2). It was proposed that at least three to four Vg genes were present in the genome. Subsequently, a vitellogenin like cDNA (i.e. *MeVg2*) was cloned and characterized. The *MeVg2* gene shared only a 50% amino acid sequence identity with the *MeVg1* gene. The presence of multiple vitellogenin genes was also reported in the freshwater prawn *M. rosenbergii* (10). In our analysis of transcriptomes from the hepatopancreas and ovary of the banana shrimp *F. merguiensis*, the black tiger shrimp *P. monodon*, and the lobster *Panilus homarus*, multiple vitellogenin transcripts have also been identified (data not

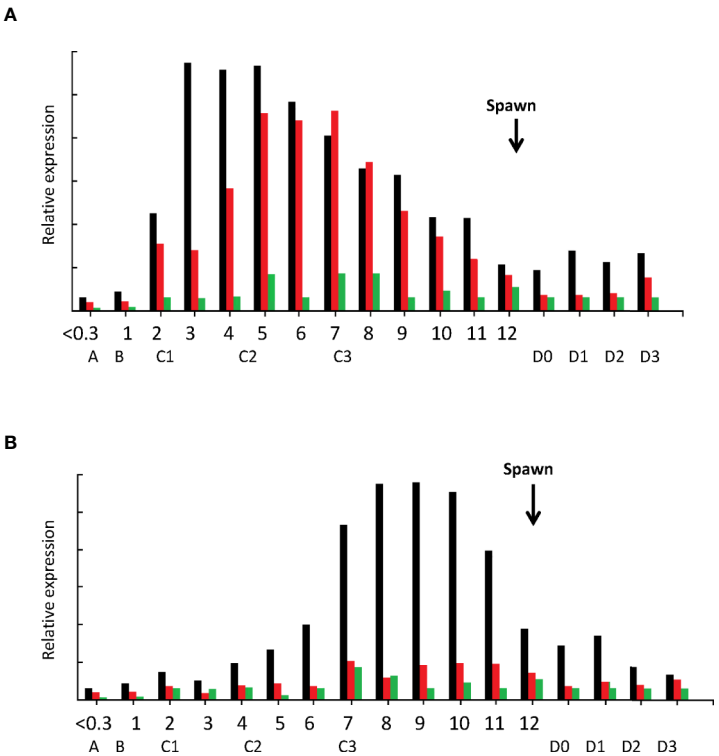

FIGURE 8 | Expression analysis of *LvVg1*, *LvVg2*, and *LvVg3* in the hepatopancreas **(A)** and ovary **(B)** during female maturation cycle. The black bars are for *LvVg1*, the red bars are for *LvVg2*, and the green bars are for *LvVg3*. The gonadosomatic index (GSI) was from 0 to 12%; the molt cycle stage was from A to D3. The arrow indicates the time of immediate post-spawn. Postmolt (A, B), early-Mid intermolt (C1–2), late intermolt (C3), early premolt (D0–1), middle premolt (D2), and late premolt (D3).

shown, but available upon request). Therefore, we have concluded that multiple vitellogenin genes also exist in most decapod crustaceans. Concerning the different *LvVg* transcripts identified in the whiteleg shrimp *L. vannamei*, the *LvVg1* transcript constituted the major RNA species and further study demonstrated that four to five *LvVg1* isoforms (ortholog) existed. The presence of multiple isoforms of *LvVg1* suggested the need for production of a large amount of protein for rapid ovary maturation. The production of multiple vitellogenin gene isoforms has been reported in many fish species (25–27). Since shrimp produces a nutrient poor egg (i.e. oligolecithal), we speculate that the evolution of multiple *Vg1* genes also occurred in other decapods for the production of a much larger content of yolks.

LvVg2 was the second most abundant vitellogenin gene member identified from the whiteleg shrimp *L. vannamei*. Genbank BLASTP search analysis has identified two *LvVg2* gene sequences (>99.8% amino acid identity) from the published *L. vannamei* genome (**Figure 4C**). It was most similar to the *MeVg2* gene of *M. ensis*, sharing 52% amino acid sequence identity. The full-length coding/amino acid sequence of *LvVg2* was relatively short (i.e. 2,560 aa) which is similar to the *Vg2* identified in *M. ensis* (i.e. 2,553 amino acid). In the hepatopancreas and ovary transcriptomic dataset of the shrimp *F. merguiensis*, a second form of *Vg2* (i.e. *FmVg2*) also existed and it shared the highest sequence homology with *LvVg2* (data not shown but can be provided upon request). *FmVg2* was a shorter *Vg* sequence similar to that of the whiteleg shrimp *L. vannamei* and the sand shrimp *M. ensis*. Therefore, we believe that homologous genes for *Vg2* also exist in other shrimp and the size of these *Vg* genes is also shorter. Despite the claim that a second *Vg* gene was present in the mud crab *Scylla paramamosain*, closer inspection of the two sequences indicated that these two genes can be considered to be *Vg* isoforms derived from two different genes (19). However, in our crab hepatopancreas and eyestalk transcriptomic database (unpublished), we have identified a second *Vg* like gene in the mitten crab *Eriocheir sinensis*. Sequence alignment of the transcriptomic database derived *EsVg2* with *EsVg1* from the published data revealed that they only shared 54% similarity to each other. Moreover, full length sequence of *EsVg2* was much shorter than that of *EsVg1* (2,560 *vs.* 2,525). Although *Vg* sequence information in crabs is far from complete as compared to shrimp, it is logical to speculate that crabs also consist of multiple vitellogenin genes. Based on the above information, we speculate that other forms of the *Vg* gene, such as *Vg2* (shorter in length) and *Vg3*, may also occur in other decapods including lobsters and crayfish. In other words,

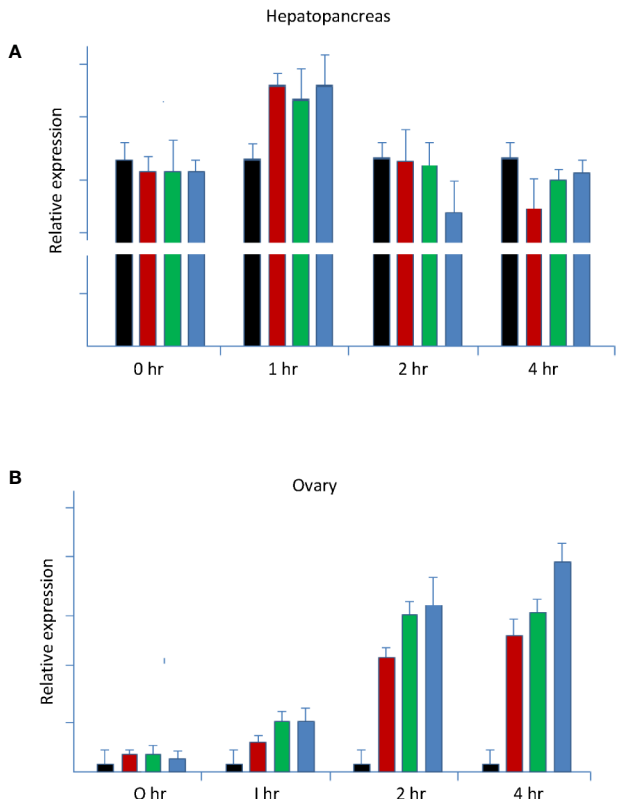

FIGURE 9 | Neuroendocrine regulation of *LvVg1* expression in *in vitro* cultured hepatopancreas and ovary (N = 5). The tissues were incubated with explants of the eyestalk optic nerve (Es; red bar), brain (Br; green bar), or thoracic ganglion (Tg; blue bar). Hepatopancreas **(A)** and ovary fragments **(B)** were incubated in a medium containing one of the above tissues. The control tissues (black bar) were incubated without addition of the nervous tissues. Samples were taken at the 0, 1, 2, and 4 h time points following incubation. Q-PCR was performed to determine expression levels of the *LvVg1* gene.

the presence of multiple vitellogenin genes may universally occur during the early evolution of decapods.

Understanding the structure and organization of *Vg* genes may provide information for the evolution of this gene family and regulation of gene expression. We have previously investigated the *Vg* gene structure of shrimp (2), crab (9), and lobster (28) and the results indicated that the organization of *Vg* in decapods was highly conserved. Most of the decapod *Vg* genes consist of 15 exons interrupted by 14 introns (4). In the sand shrimp *M. ensis*, the *MeVg2* consists of 13 exons because of the fusion of exons 6–7 and 7–8 (3). Here, from bioinformatic analysis, it was found that all the full-length *LvVg* genes identified consisted of 15 exons interrupted by14 introns and the exon-intron junction were also conserved in all the genes. In the red crab *Charybdis feriatus*, the *CfVg1* gene also consists of 14 introns (9). Although the mud crab ***S.*** *paramamosain* has a second *Vg* that consists of 11 introns, closer inspection of this *SpVg2* gene revealed that it was most likely a partial *SpVg1* gene. The situation is similar to the truncated *LvVg* gene discussed in the *L. vannamei* genome study above. Therefore, more sequence information is needed to confirm the conservation or divergence of *Vg* gene organization in decapods. Since the proximal promoter region of the *LvVg1* and *LvVg2* genes shared a significantly high degree of identity, the basic regulation mechanism of *Vg* may be conserved in shrimp.

Many genes are known to be arranged along the chromosomes in groups of related gene. These groups are called gene clusters. Related genes may be arranged in more than one physical cluster and a whole set of related genes is called a gene family. This is the first report for the vitellogenin gene family in shrimp and multiple *Vgs* exist as a cluster in the genome. It is common for the evolution of multiple copies of highly expressed gene. For example, the CHH/MIH/GIH gene family is also known to be arranged in clusters. In *M. ensis*, the CHH/MIH/GIH gene family represents an important group of neuropeptide hormones for growth and reproduction control. Members of the same family may have different gene functions. Two clusters of CHH-family neuropeptides have been identified. In many fish, several vitellogenin genes have been identified and the vitellogenin gene family members are also arranged in clusters. Gene clusters and gene families vary in importance in different taxonomic groups. In *L. vannamei*, three different Vg gene clusters were identified similar to the CHH family clusters, but it is still unknown whether these *Vg* genes in *L. vannamei* are located on the same chromosome.

There are many examples for *Vg* gene duplication and gene clustering in other vertebrates (27, 29). Although it is not known whether the three clusters of *Vg* genes identified in the whiteleg shrimp *L. vannamei* are linked, we have proposed an evolution model for the formation of the *LvVg* gene family based on the transcriptomic sequences and available genomic data from public database. In this model, *LvVg1* and *LvVg2* genes were derived from a common ancestor gene. After the separation from *LvVg1*, only a few mutations occurred in *LvVg2* and a more recent gene duplication event occurred to produce the two highly similar *LvVg2a* and *LvVg2b* genes. *LvVg3* was derived from the same lineage as *LvVg1* at a later time but remained relative stable throughout evolution. However, ancestor of *LvVg1* may undergo two or more rounds of gene duplications and give rise to the current four to five *LvVg1* isoforms.

The lack of complete sequence information for all *Vgs* is the major reason for the discrepancy in identifying the major sites of vitellogenin synthesis reported in different crustaceans (30). This discrepancy may be a result of using primers that are not common to amplify other vitellogenin gene members in PCR. Therefore, to obtain a precise spatial and temporal expression profile of different *Vg* genes, gene specific primers must be employed in RT-PCR or qPCR assay. Because of the lack of additional *Vg* gene sequences, results reported in many previous studies in decapod may not be accurate (16).

In *L. vannamei*, it was confirmed that both the hepatopancreas and ovary are the major synthetic sites of vitellogenins and vitellogenin expression follows a bi-phasic expression pattern for the completion of vitellogenesis in shrimp. The hepatopancreas is the major synthesis site in the initial phase and ovary will become

the major site at the later stage of vitellogenesis. The differential expression patterns of the three vitellogenin genes during ovary development further indicate their functional diversification, which merits further in-depth studies. It is also obvious that the amino acid profiles of these *Vgs* are different as they may fulfill different functions at different developmental stages.

The expression of *LvVg1*, *LvVg2*, and *LvVg3* at different life cycle stages were investigated. The results indicated that all these transcripts can be detected in the nauplius, zoea, mysis, postlarvae, juveniles, and subadult. In the expression study, the detection of *Vg* transcript in early larva such as the nauplius suggested that some of the transcripts could be maternal. During early stage of embryonic development, when the transcriptional process is not fully functional, the reservation of these maternal vitellogenin transcripts would be important for successful embryo development. As a nutrient molecule, vitellogenin can be processed into small peptide and amino acid. As shrimp produce a nutrient poor egg, nutrient from the maternal part can supply all energy requirement for the nauplius (28). Feeding only begins when the nauplius metamorphoses into the zoea. The presence of *Vg* transcripts in free swimming nauplius, mysis, and post-larvae suggests that *Vg* also has its function in larval development. As the larvae begins to assume active feeding from predation, the expression of *Vg* is reduced at the later juvenile and subadult stages.

As a nutrient molecule, *Vg* is first produced as a large precursor molecule and later processed into subunits in the hemolymph (23). Many biochemical studies reported the different sizes of *Vg* subunits in the hemolymph (23). This is probably due to the presence of several cleavage sites (RR, KR or RK). Cleavage at these sites may further process the *Vg* into smaller peptides of different sizes. During the transcriptomic screening of *Vg*, many smaller *Vg*-related transcripts were identified. Some of these transcripts may represent degrading gene products. However, some transcripts should represent alternative splicing products of the full-length gene or transcript from a partial vitellogenin gene. For example, the deduced transcripts for the partial *LvVg1y* and *LvVg1u* gene can produce transcripts of ROT614614 (GenBank#) and ROT84662 (GenBank#). Similarly, in the sand shrimp *M. ensis*, Northern Blot analysis revealed the probe hybridized to many mRNA of different sizes (3). Therefore, some of those transcripts may produce truncated *Vg* proteins. These small *Vg*-like transcripts can be processed to produce smaller *Vg*-like protein and supply sufficient amino acid or raw materials for growth.

In many transcriptomic datasets that we have on-hand, there are many *Vg* or *Vg*-like transcripts containing sequences with deleted N-terminal LPD domain or deleted C-terminal coding regions. These transcripts could be derived from alternative splicing of the *Vg* genes. For example, a search of the Genbank for the whiteleg shrimp *L. vannamei* genome project revealed a *Vg*-like gene encoding for a partially deleted vitellogenin (i.e. Genbank#: ROT61467). Transcription of this mRNA will result in a N-terminally truncated Vg protein without a signal peptide sequence. Therefore, these truncated *Vg*-like molecules may not be a secreted product but will only function in an intracellular manner. In the mud crab *S. paramamosain*, a second vitellogenin gene has been cloned, and the expected cDNA of this *Vg* gene also lacks the signal peptide and is therefore truncated (19).

Vitellogenesis in decapods is known to be controlled by many hormones (31). In shrimp aquaculture, unilateral eyestalk ablation is widely used to induce female gonad maturation, as eyestalk ablation removes the source of the gonad inhibiting hormone (GIH) which is a member of the CHH/MIH/GIH family neuropeptides (32). Meanwhile, there are many reports for the presence of a gonad stimulating hormone (GSH) in the brain, thoracic ganglion, or other neuronal tissues in crustacean, but information on the identity of this GSH is scared. In the crab *Potamon kooloonenseis*, extract of thoracic ganglion induced oocyte growth and precocious vitellogenesis with an increase in the ovarian weight or gonad index and oocyte diameter (31). However, the brain extract injection did not produce marked changes in the immature ovary. The results suggest that the ovarian activity, particularly growth and vitellogenesis, may depend on neurosecretion of the thoracic ganglion (32–34).

In this study, when hepatopancreas and ovary fragments were co-incubated with eyestalk optic nerve and other neuronal tissues, different responses were observed for the ovary and hepatopancreas. Stimulatory effects of the three neuronal tissues on *LvVg1* expression were all detected. The hepatopancreas fragment was highly active with the *LvVg1* transcript induced to higher levels only at the 1 h time point, while response of the ovary lagged behind the hepatopancreas as most of the stimulation occurred at 3 to 4 h after incubation. Moreover, the effect of the thoracic ganglion or brain on *LvVg1* induction in the ovary fragments was much larger than that of the eyestalk optic nerve, a phenomenon not observed in the hepatopancreas fragment. More in-depth study is needed to explain the different responses and to identify the causal factors in these neuronal tissues (33).

In conclusion, this is the first comprehensive study of multiple vitellogenin genes in a decapod crustacean. The clustering of multiple vitellogenin genes in the whiteleg shrimp *L. vannamei* suggests that evolution of the *Vg* genes is the result of several gene duplication events. The results presented in this study demonstrates that two phases of vitellogenin gene expression are needed for the completion of vitellogenesis: the extra-ovarian hepatopancreas phase followed by the intra-ovarian phase undertaken by the ovary. Because the responses of hepatopancreas and ovary to eyestalk, brain or thoracic ganglion stimulation are different, vitellogenin expression in these two tissues may be under control of different endocrine factors. In addition to its nutrition-providing role in reproduction, shrimp vitellogenin genes are also likely to be involved in growth and molt cycle regulation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material** and other "data not shown" results mentioned in the text are available upon request.

ETHICS STATEMENT

The animal studies described in this report were conducted under the guideline of animal research ethics approved by the Committee for animal research of the Guangdong Ocean University.

AUTHOR CONTRIBUTIONS

Paper writing: SC, WW, BL. Data analysis: SC, WW, BL, LS, TZ. Performing experiments: TZ, CW, LS, AK, SC. Securing funding support: SC, WW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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A Crab Is Not a Fish: Unique Aspects of the Crustacean Endocrine System and Considerations for Endocrine Toxicology

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Crustaceans—and arthropods in general—exhibit many unique aspects to their physiology. These include the requirement to moult (ecdysis) in order to grow and reproduce, the ability to change color, and multiple strategies for sexual differentiation. Accordingly, the endocrine regulation of these processes involves hormones, receptors, and enzymes that differ from those utilized by vertebrates and other non-arthropod invertebrates. As a result, environmental chemicals known to disrupt endocrine processes in vertebrates are often not endocrine disruptors in crustaceans; while, chemicals that disrupt endocrine processes in crustaceans are often not endocrine disruptors in vertebrates. In this review, we present an overview of the evolution of the endocrine system of crustaceans, highlight endocrine endpoints known to be a target of disruption by chemicals, and identify other components of endocrine signaling that may prove to be targets of disruption. This review highlights that crustaceans need to be evaluated for endocrine disruption with consideration of their unique endocrine system and not with consideration of the endocrine system of vertebrates.

Keywords: endocrine disruption, neuroendocrine disruption, ecdysteroid signaling, color change, sexual differentiation

Abbreviations: AG, androgenic gland; AGH, androgenic gland hormone; CCAP, crustacean cardioactive peptide; CHH, crustacean hyperglycemic hormone; Cyp19, aromatase; EcR, ecdysone receptor; EDC, endocrine disrupting chemical; EH, eclosion hormone; ETH, ecdysis triggering hormone; FAMeT, farnesoic-O-methyl transferase; HMGCR, 3-hydroxy-3methyl-glutaryl coenzyme A reductase; IAG, insulin-like androgenic gland factor; MET, methoprene tolerant; MF, methyl farnesoate; MfR, methyl farnesoate receptor; MIH, moult inhibiting hormone; NE, norepinephrine; NMDAR, N-methyl-D-aspartate receptor; PCBs, polychlorinated biphenyls; PDH, pigment dispersing hormone; PoA, ponasterone; RPCH, red pigment concentrating hormone; RPCHR, red pigment concentrating hormone receptor; RNAi, RNA interference; RXR, retinoid-X-receptor; SRC, steroid receptor coactivator; US EPA, United States Environmental Protection Agency; XO/SG, X-organ/sinus gland; 5-HT, 5-hydroxytryptamine, serotonin; 20E, 20-hydroxyecdysone.

INTRODUCTION

Over the last two decades, both the scientific community and the lay public have become increasingly aware of the risk associated with endocrine disruption. Environmental authorities in Europe, the United States and many other countries are preoccupied with the identification of Endocrine Disrupting Chemicals (EDCs) and their effects on human health and wildlife. In 2012, a report on the state of endocrine disruption was composed under the auspices of the United Nations Environment Program (UNEP) and the World Health Organization (WHO). This report stated that in spite of numerous reports of reproductive disorders, skewed sex ratios and intersex phenomena, the understanding of endocrine disruption in invertebrates—including crustaceans—was limited (1). Since then some progress has been made, but knowledge about the effects of EDCs on the endocrine system of crustaceans remains scarce and concerns mainly pesticides designed to disrupt moulting in insects (2, 3). Among the vast array of chemical compounds listed on the Toxic Substances Control Act (TSCA) inventory (US EPA)—and many others not regulated under the TSCA—there will certainly be many other chemicals that have the potential to interfere with the crustacean endocrine system.

With about 68,000 extant species of crustaceans described—and still more to be discovered—these predominantly aquatic invertebrates form a large and very diverse arthropod taxon (4–6). As predators, scavengers, or filter feeders, they take important positions within the aquatic ecosystems at various levels of the food web. They also provide high-value fishery products and contribute with over 14 million tons—of which half are wild stock captures—to about 8% of the worldwide seafood resources (7).

This ecological and economical wealth is, however, endangered by habitat loss, climate change, pollution, overexploitation, invasive species and other anthropogenic stressors (8). In the early nineties, drastic declines of amphipods were reported in some of the Great Lakes (9, 10). Similarly, populations of the amphipod *Gammarus lacustris* significantly declined in the Selenga River delta, the main tributary to Lake Baikal (11). Although pollution, such as polychlorinated biphenyls (PCBs) or pulp mill effluents, respectively, were given consideration, the actual cause-effect relationships were complex and difficult to establish. Massive decreases in blue crab populations of Chesapeake Bay occurred in the nineties, where spawning stock abundance declined by about 80% (12). Similarly, catches of the edible crab, *Cancer pagurus*, in the English Channel were halved from 2012 to 2018 with no clear cause for this decline (13). Even if overfishing, increasing temperatures, and ocean acidification are contributing causes, reproduction impairment due to endocrine disruption could be adding to the declines, or hampering the recovery of stocks. A contribution of EDCs in these declines of marine and freshwater crustaceans is difficult to discern. Firstly, it is generally challenging to determine the quantitative contribution of individual challenges to population sustainability. Secondly, it is difficult to identify endocrine disruption without knowing how to measure it. In any case, no prominent example of endocrine disruption, comparable to imposex in prosobranch gastropods, is known for crustaceans. Examples of intersex that appear to be related to pollution have been reported for different crustacean species (14–19). But no cause-effect relationships have been established.

The assessment of endocrine disruption in crustaceans can be approached either from the perspective of the molecules suspected to cause endocrine disruption, *i.e.*, effects of established EDCs, or from the perspective of the endocrine targets of EDCs, *i.e.*, crustacean endocrinology. Environmental chemicals that have been classified as EDCs, are, for the most part, compounds that interfere with the vertebrate hormone system. Strenuous effort has been made to demonstrate possible endocrine disrupting effects of such EDCs in crustaceans (20–30). Although effects on growth and reproduction are often reported, most studies failed to demonstrate that endocrine mechanisms were involved. The generally high concentrations that are necessary to produce negative consequences for growth or reproduction suggest that the observed effects were merely a result of overt toxicity (*e.g.*, (31, 32). More specific assays are being developed that use elements of the crustacean endocrine system, such as reporter assays for the ecdysteroid and methyl farnesoate receptors, measurement of 20-hydroxyecdysone (20E) titers, or assays for chitobiase activity (33–38). Nevertheless, establishing reliable testing protocols for endocrine disruption in crustaceans remains challenging and widely ignores the importance of neurohormonal regulation for the control of many physiological functions in crustaceans.

This review argues in favor of a more arthropod specific approach to endocrine disruption in crustaceans.

EVOLUTION OF THE INVERTEBRATE ENDOCRINE SYSTEM

Endocrine systems are key features of evolution reflecting metazoan diversification (39). Metazoan endocrine systems have evolved from a common bilaterian ancestor before the divergence of protostomes and deuterostomes more than 600 million years ago (40, 41). Specialized neurosecretory cells were already present in the pre-bilaterian cnidarians and neurohormonal signaling persists as a major endocrine component in both, the protostome and deuterostome lineages (42–44). The arthropods diverged within the protostome lineage some 500 million years ago (45). Unique aspects to the physiology of the arthropods, including crustaceans, required the development of unique endocrine pathways to regulate these physiological processes. For example, arthropods lost the capacity to synthesize cholesterol (41, 46). This loss of cholesterol synthesis may have limited opportunities for the evolution of steroid hormones, which utilize cholesterol as a precursor. However, this loss may have also promoted the evolution of methyl farnesoate (MF) in crustaceans and juvenile hormone in insects, which do not utilize cholesterol, into functional hormones (41). The divergence of the arthropod endocrine system has been deepened further by the evolution of an

exoskeleton in arthropods, which requires moulting for growth and reproduction (47). Endocrine signaling processes were required to regulate moulting and coordinate this process with those operative in growth and reproduction.

While the endocrine systems of the two major arthropod taxons, crustaceans and insects, share many commonalities, divergences also occurred. For example, both groups possess the capacity to produce MF. However, insects possess a cytochrome P450 monooxygenase, which epoxidates MF to form juvenile hormone III (48). This latter hormone regulates many of the processes in insects that are under the control of methyl farnesoate in crustaceans. Crustaceans produce a family of hormones known as crustacean hyperglycemic hormones (CHHs) (49, 50). These hormones regulate a myriad of processes including aspects of moulting, reproduction, and energy generation. Only a single member of this family, the ion transport peptide, is known to exist in insects. This insect hormone controls ion transport (51, 52).

The presence or absence of nuclear receptors, *i.e.*, ligand-regulated transcription factors, reflects the separation of steroid hormone signaling within the endocrine systems of crustaceans and vertebrates. The evolution of these receptors was shaped by whole genome duplications and losses combined with the evolution of neofunctionalization or subfunctionalization among duplicate receptors (39, 53–55). Importantly, arthropods lack receptors of the steroid receptor subfamily 3, which include the estrogen and androgen receptors. This may represent the loss of the progenitor of this subfamily (56, 57). However, subfamily 3 receptors are also absent in the ascidian *Cionia intestinalis*, a deuterostome invertebrate distantly related to vertebrates (55, 58). This latter observation suggests that subfamily 3 nuclear receptors evolved later in chordate evolution, long after protostomes and deuterostomes diverged (59, 60). This hypothesis is supported by the appearance of sex steroid receptors, including the estrogen receptor, in the cephalochordate *Amphioxus* (61, 62). The ecdysteroid receptor (EcR) of arthropods does not belong to the subfamily 3 nuclear receptors, but is an ortholog of the vertebrate liver X and farnesoid X receptors, which are members of the subfamily 1 nuclear receptors (63).

The loss and gain of functions and elements of the endocrine system, such as nuclear receptors or neuropeptide hormones in arthropods is likely to be related to the invention of a new body plan with an exoskeleton (64). The exoskeleton constrains growth and the impermeable cuticle limits gas and ion exchange with the environment. Moulting solves the first problem, while the second problem requires organs for gas (and ion) exchange, such as the gills in crustaceans or the tracheae in insects, both of which have similar organogenetic origins. Sanchez-Higueras et al. (65), demonstrated that trachea develop as serial homologues to the ectodermal prothoracic gland and the *corpora allata*, which have their counterparts in the Y-organ and the mandibular organ of crustaceans (see also (43)). These endocrine glands produce ecdysone and juvenile hormone/MF, respectively, *i.e.*, insect/crustacean hormones related to moulting and metamorphosis. Hence, these findings link the evolution of arthropod hormones and endocrine organs with the evolutionary innovation of an exoskeleton. As a matter of consequence, the homology of endocrine organs and gills provides evidence for the integration of environmental cues into physiological responses, such as respiration, osmoregulation and growth by an endocrine system specific to arthropods.

Crustaceans and vertebrates share the principal concept of connecting neural, neurosecretory, and endocrine components (58). Yet, the endocrine systems of arthropods and vertebrates have evolved independently and differently since more than 540 million years ago when crustaceans and deuterostomes appeared in the early Cambrian (66, 67). As a matter of consequence, they have little in common. The fundamental difference between arthropod and vertebrate endocrine systems has implications for determining endocrine disruption. Exogenous substances that alter functions of the endocrine system are, for the most part, likely to be different for crustaceans and vertebrates. Substances designed to interfere with the insect endocrine system (*i.e.*, insect growth regulating insecticides) by their design have significant potential to interfere with the crustacean endocrine system, but are much less likely to interfere with the endocrine system of vertebrates. Conversely, steroid hormone functional analogs are highly likely to disrupt endocrine regulated processes in vertebrates, but are much less likely to disrupt endocrine signaling in crustaceans. The most conserved elements associated with the endocrine regulation of physiological functions shared by both, arthropods and vertebrates are the upstream control of neurosecretory processes by biogenic amines. Besides their function as neurotransmitters and neuromodulators, biogenic amines, such as serotonin, can also serve as neurohormones circulating in the blood stream and affecting peripheral organs like ovaries.

ENDOCRINE REGULATION OF MOULTING

The monophyly of arthropods is based on a sclerotized exoskeleton (68). To allow for growth, the cuticle has to be shed and renewed periodically. Moulting, *i.e.*, ecdysis, is a distinctive characteristic of the crustacean life cycle (69), which involves a complex interplay of numerous neuropeptide and steroid hormones (**Figure 1**), thereby covering key features of the endocrine system of crustaceans. As such it is an endpoint par excellence for endocrine disruption in crustaceans. In many cases, moulting in crustaceans is also related to reproductive periods by alternating cycles of moulting and reproduction, as in *Daphnia* (80, 81), so that perturbations of ecdysis, may also affect reproductive phases.

The crustacean moult cycle is divided into five major stages comprising numerous substages [**Figure 1**; (69, 82)]. During the intermoult stage (C_4), the neuropeptide moult-inhibiting hormone (MIH) represses ecdysteroid synthesis. The proecdysial period begins with the apolysis of the old exoskeleton, during which the membrane layer and the endocuticle are degraded and their building materials are partially resorbed (D_1–D_3). Simultaneously, the new exoskeleton is synthesized. A surge of ecdysone during premoult triggers downstream events that lead to the extrication of

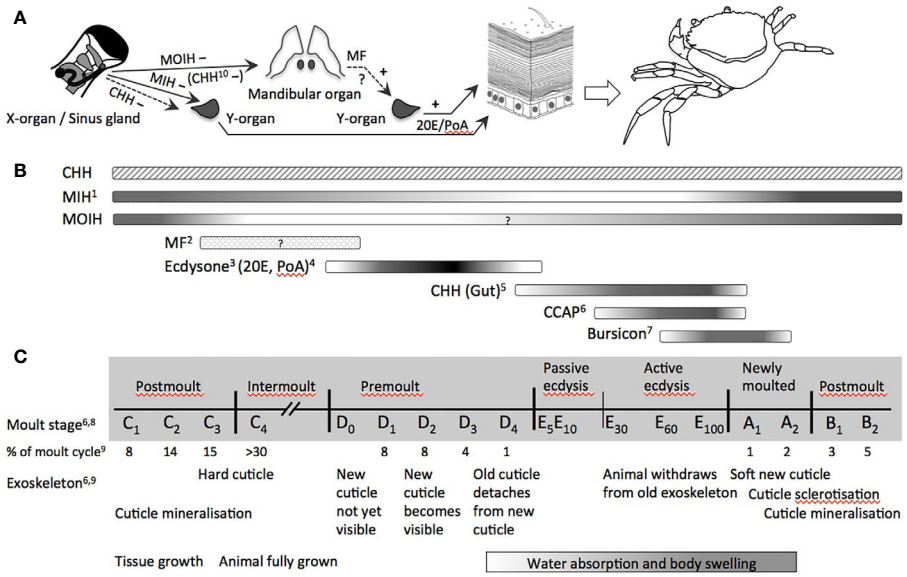

FIGURE 1 | Endocrine control of moulting in decapod crustaceans (Malacostraca). **(A)** Organs directly involved in the control of moulting (release of CCAP and bursicon by the central nervous system and/or the pericardial organ as well as release of CHH by paraneurons of the fore- and hindgut not shown): MIH and CHH from the X-organ/sinus gland-complex negatively inhibit the synthesis of ecdysone by the moulting gland (Y-organ); MOIH (and in some species CHH) negatively inhibit the synthesis of MF, the stimulatory activity for ecdysteroid synthesis has been reported in several studies; ecdysteroids of the major forms 20E and PoA stimulate the epidermis *via* its corresponding EcR to decalcify and to lyse the membrane layer and the old cuticle by proteases, chitinase and chitobiase, and to synthesize material for the new cuticle. **(B)** Dynamic hemolymph titres of (neuro-)hormones involved in the control and/or physiological processes during moulting and ecdysis over an entire moult cycle. **(C)** Moult stages and main modifications of exoskeleton and growth (non-proportional presentation with respect to duration of each period). Abbreviations: CCAP, crustacean cardioactive peptide; CHH, crustacean hyperglycemic hormone; EcR, ecdysteroid receptor; MF, methyl farnesoate; MIH, moult-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; 20E, 20-hydroxyecdysone; PoA, ponasterone **(A)** [1] (70); [2] (71); [3] (72); [4] (73); [5] (74); [6] (75); [7] (76); [8] (77); [9] (78); [10] (79).

the animal from its carapace (E). Despite the long history of endocrine research in crustaceans, the hormonal regulation of these events still lacks precise knowledge of some factors, such as homologs of ecdysis-triggering hormone (ETH) and eclosion hormone (EH). The function of these neuropeptides is well described for insects, but awaits further elucidation in crustaceans. More recent work also suggests a yet unknown role for corazonin in the control of moulting (83). Eventually, CHH and crustacean cardioactive peptide (CCAP) are tightly linked, respectively, with ecdysial water uptake and the onset of the behavioral motor program by which the animal extricates from its carapace. Tanning and sclerotization of the new cuticle are under the control of bursicon, another neuropeptide hormone. At large, the complex control of moulting described in the following, which involves many other hormones beyond MIH and ecdysone, provides various targets for endocrine disruption in crustaceans.

Neuro-Endocrine Regulation

Almost 70 years after Passano (84) proposed the existence of MIH secreted by the major neurohemal organ in decapod crustaceans, the X-organ/sinus gland (XO/SG) complex of the eyestalk (reviewed in (85, 86)) and the characterization of the respective neuropeptide by Webster and Keller in 1986 (87), the main features of neuroendocrine moult control are generally accepted for decapods. According to this regulatory model, the activity of the moulting gland (*i.e.*, Y-organ) is inhibited by MIH during intermoult, thus subjecting ecdysteroid signaling to the negative control by neuropeptides from the XO/SG (**Figure 1**) (69, 72, 88–90). Evidence is provided by experiments removing eyestalks or XO/SGs, which results in shortened moult intervals and higher moult frequency (72). This inhibition of ecdysis in eyestalk/XO/SG-ablated animals can be restored with extracts from the SG. Dynamic variations of MIH during the moult cycle as well as decreases of MIH stored in the SGs and circulating MIH in the hemolymph during pro- and postecdysis further corroborated the role of MIH in neuro-endocrine regulation of ecdysis (70, 88, 90, 91). Furthermore, Techa and Chung (70) suggest a feedback control by which elevated ecdysteroid concentrations in the hemolymph stimulate *mih*-expression, but inhibit MIH-secretion. As a matter of consequence, high amounts of MIH are stored in the SG during ecdysis and released during post-moult, resulting in low ecdysteroid titers. Hence, any perturbation of synthesis, storage and release of MIH from the XO/SG could result in modifications of Y-organ inhibition, ecdysone synthesis and the resulting circulation of ecdysteroid levels (*e.g.*, (92)).

MIH is not the only factor controlling ecdysteroid secretion from the Y-organ. During proecdysis, the Y-organ becomes progressively less sensitive toward MIH-mediated inhibition of ecdysteroid production (88, 89, 93, 94), most likely due to modifications in intracellular signaling cascades following binding of MIH to its G-protein coupled receptor. Little

attention has been given to the role of CHH in the inhibition of ecdysone synthesis. Albeit, CHH is 10-fold less effective in inhibiting the Y-organ, its levels are roughly 10-fold higher than those of MIH. Chung and Webster (88), therefore, argued that CHH could have an equivalent role to MIH in the negative control of ecdysteroid synthesis. The situation is further complicated by the implication of other neurohormones in moulting (**Figure 1**). Notably, CCAP displays considerable dynamics during ecdysis (75) and increases massively during late ecdysis when the animals actively exuviate. Phlippen et al. (75) explain this surge of CCAP by its mytropic action, thus potentiating muscle contraction while the animals withdraw from their old exoskeleton, and by its effect on cardiovascular functions regulating hemolymph flow and pressure. Interestingly, CHH released from paraneurons of the fore- and hindgut rises about 100-fold during ecdysis, followed by the surge of CCAP, both reaching their maximum at late ecdysis and decreasing rapidly in newly moulted animals (74) (**Figure 1**). Injections of CHH at physiological concentrations were able to initiate ecdysis through swelling as a result of isosmotic water uptake by the hindgut, suggesting a role of CHH in ion and water uptake during moulting, similar to the function of the homologous ion transport peptides in the hindgut of insects (74). Eventually, bursicon, another neuropeptide hormone produced by the central nervous system overlapping with CCAP is massively released from the pericardial organ into the hemolymph when ecdysis is completed and remains significantly elevated during early postmoult (76) (**Figure 1**). Its consistent presence throughout the moult cycle at about one quarter of its concentration during ecdysis gives rise to further cuticle-associated functions of bursicon during moulting (95).

Interestingly, the nature of hindgut CHH, CCAP, and bursicon and their role in ecdysis bear resemblance to neuropeptides involved in the ecdysis of insects. Because any spontaneous release of, notably, CCAP could be excluded, the existence of yet non-identified ecdysis-inducing factors similar to ETH and EH of insects would complete the overall picture of ecdysis in crustaceans (75). Indeed, analyses of the *Daphnia pulex* genome, suggest that such factors may exist in crustaceans (96, 97). More recently, carcikinin, an ortholog of ETH was identified in *Carcinus maenas* (98) and ETH genes were found in several crustacean transcriptomes [see (99) for references]. Injection of ETH into the crayfish *Cherax quadricarinatus* prolonged the moult period, suggesting a role in the control of the moult cycle in decapod crustaceans (99). Similarly, EH was identified in the shrimp *Exopalaemon carinicauda*, and displayed highest expression levels during premoult, while gene-silencing lead to a delay in moulting and reduced rates of ecdysis (100). Furthermore, the virtually exclusive expression of a G-protein coupled receptor for corazonin in the Y-organ indicates a role for this neuropeptide in the control of ecdysis (83). Despite these promising results, the roles of ETH, EH, and corazonin in crustacean ecdysis await further characterization.

Ecdysteroids

Ecdysteroids are the predominant hormones responsible for moulting and other processes in crustaceans. Ecdysteroid metabolism is relatively well understood owing to advances in insect biochemistry and conserved pathways between insects and crustaceans. While crustacean ecdysteroids and their nuclear receptors are similar to those of insects, they differ in the number of hormones and in the number and structure of the receptor isoforms (101).

The concentrations of ecdysteroid hormones circulating within crustaceans vary during the moult cycle, and depending on the species, either gradually or quite rapidly spike to start a period of ecdysis or moulting (**Figure 1**) (102). Ecdysteroids and the enzymes responsible for breakdown of the chitin skeleton have been measured through various molecular and cellular assays targeting gene expression and/or enzyme synthesis, respectively.

Enzyme Synthesis and Inactivation

Ecdysteroid biosynthesis is divided into two stages (103). The first stage involves the conversion of cholesterol, derived from diet, to 5β-diketol and the second stage converts 5β-diketol to secreted products. Depending on the species, four major secreted products are ecdysone, 3-dehydroecdysone (3DE), 25-deoxyecdysone (25dE), and 3-dehydro-25-deoxyecdysone (3D25dE) (103). A large number of ecdysteroidogenic enzymes and associated genes have been identified in insects and other arthropods (104) with a large degree of conservation in the crustaceans (105, 106). For example, orthologs of *nmg*/*sro*, *spo*, *phm*, *dib*, *sad*, and *shd* have been identified in the *D. pulex* genome (46, 107, 108).

Both the increase and subsequent rapid decline in ecdysteroid titers are critical to moulting. The cytochrome P450 hydroxylase cyp18a1 is primarily responsible for the inactivation of the hormone, rendering it susceptible to further modification and elimination (103, 109).

The inhibition of cytochrome P450s by chemical compounds is a widespread mechanism (110), which could interfere with the multiple hydroxylation reactions catalyzed by the cytochrome P450s encoded by the "Halloween genes" (104). For instance, several classes of fungicides function by inhibiting cytochrome P450-mediated demethylation of sterols that are critical components of fungal cell membranes. Some of these compounds are also capable of inhibiting cytochrome P450s of non-target organisms that are involved in steroid biosynthesis. Exposure of *Daphnia magna* to the demethylase inhibiting fungicides fenarimol, pyrifenox, prochloraz, triadimefon, and propiconazole delayed moulting and/or caused developmental abnormalities in neonates (111, 112). Mechanistic studies revealed that fenarimol exposure reduced ecdysteroid levels in daphnids and that co-exposure with 20E protected against the delay in moulting and developmental abnormalities caused by this fungicide (113). These results are consistent with the hypothesis that fenarimol elicited toxicity by inhibiting cytochrome P450s involved in ecdysteroid synthesis.

Receptor-Mediated Activation

Ecdysteroid hormones, such as the major forms 20E and ponasterone A (PoA), bind to the ligand-binding domain of the EcR and activate the expression of primary early ecdysteroid responsive genes, such as E75 and E74 and early late genes such

as HR3 and HR4 (114, 115) (see also reviews by (116, 117)). These transcriptional regulators drive the "late genes" responsible for metamorphosis, moulting, and/or ovarian development (114, 118–120). For ligand binding, the EcR forms a heterodimer with the retinoid-X-receptor (RXR), the ortholog of the insect ultraspiracle (121). The RXR contributes to DNA-binding and helps to stabilize the EcR-ligand binding pocket and allows for flexibility in ligand binding (122, 123). Unliganded EcR-RXR is a repressor of transcription (123).

An account of the transcriptional activities of some of these late genes during premoult and ecdysis is given by Li et al. (124). Notably, genes involved glucosamine synthesis, corresponding to the formation of material for a new cuticle, were upregulated during the premoult, whereas genes encoding chitin synthase and several chitinases were upregulated during ecdysis and postmoult stages. Consequently, enzymes such chitobiase (125), N-acetyl-β-glucosaminidase (126) or chitin synthase (127) can be used as biomarkers for EcR-induced late genes provided that the xenobiotic concentrations are low and act *via* EcR-signaling rather than inhibiting the enzyme itself or exerting non-specific effects on gene-expression. Furthermore, enzyme-expression and activity have to be put in the perspective of the precise moulting stage (127, 128).

Disruption of Ecdysteroid Signaling

Several targets exist at which environmental chemicals might interfere with ecdysteroid signaling. These include disruptions in ecdysteroid synthesis, ecdysteroid inactivation, and interactions with the EcR. The neuroendocrine regulation of ecdysteroid signaling also may provide targets of disruption, though few definitive examples of such disruption exist. Overall, the XO/SG-Y-organ-EcR axis of malacostracan crustaceans offers an endocrine signaling cascade similar to the mammalian hypothalamus-pituitary-gonadal-ER axis.

Modulation of Ecdysteroid Levels

Studies have reported on the ability of environmental chemicals to alter the expression of ecdysteroid biosynthetic or biotransformation enzymes. For example, exposure of the copepod *Tigriopus japonicas* to 20 $mg{\cdot}L^{-1}$ atrazine resulted in a reduction of mRNA transcripts for several enzymes involved in ecdysteroid biosynthesis and biotransformation (129). Unfortunately, impacts on ecdysteroid levels were not established. A generalized reduction in relevant gene expression levels suggest that atrazine may have disrupted common neuro-endocrine control of these genes, or that the high concentration of atrazine used resulted in overt toxicity presented as an overall decrease in transcription.

Exposure of the Chinese mitten crab, *Eriocheir sinensis*, to the pharmaceutical carbamazepine reduced hemolymph ecdysteroid levels and epidermal chitobiase activity. Carbamazepine exposure also increased *chh* and *mih*-expression; while, decreasing EcR and RXR mRNA levels. Taken together, these effects suggest that carbamazepine may have perturbed the neuroendocrine control of ecdysteroid synthesis resulting in a decrease in ecdysteroid levels and a down-regulation of ecdysteroid-regulated genes. These perturbations in the ecdysteroid-signaling pathway also resulted in delayed moulting (130).

Ecdysteroid Receptor Agonists/Antagonists

Plants produce compounds with ecdysteroidal activity, presumably to serve as an endocrine-disrupting defense against invading insects (*e.g.,* (131)). Similarly, EcR-agonist activity has been exploited as a mode of action of some next-generation non-steroidal insecticides (132). The insecticidal EcR-agonist tebufenozide only weakly activated the *D. magna* EcR in a reporter two-hybrid assay (133). Similarly, tebufenozide, along with the related diacylhydrazines halofenozide and methoxyfenozide were weak agonists in a reporter assay containing the shrimp *Neocaridina davidi*-EcR (34). De Wilde et al. (33), however, could not confirm accommodation of tebufenozide into the ligand binding pocket of the shrimp *Neomysis integer* and found no effect of 100 μg tebufenozide$\cdot L^{-1}$ on nymphal development and moulting. Taken together, these results are consistent with the manufacturer's report that the diacylhydrazine insecticides exhibit low specificity for EcRs of non-target arthropods (132). They may, however, displace 20E and/or PoA from the binding site by competitive binding (34).

A recent *in silico* study by (134) identified 274 potential non-steroidal EcR-ligands. Furthermore, the screening of 8795 compounds listed in the US EPA's ToxCast chemical library revealed 34 potential agonists including the diacylhydrazines insecticides and numerous pharmaceuticals, such as non-steroidal anti-inflammatory drugs containing pyrazolone derivatives, or members of the amphenicol antibiotic family. Using the *Drosophila melanogaster* B_{II} cell assay, Dinan et al. (135) detected no EcR-agonist activity among 80 environmental chemicals. Bisphenol A, diethylphthalate, some polycyclic aromatic hydrocarbons, naphthalenes, pesticides, and pharmaceuticals were weak antagonists in this assay. Notably, estradiol, progesterone, and testosterone as well as synthetic steroids neither displayed agonist, nor antagonist activity, except for two compounds, 4-androstene-3,17-dione and 17α-ethinylestradiol, which were weak antagonists.

Despite the lack of anti-ecdysteroidal activity associated with testosterone reported by Dinan et al. (135), weak EcR-antagonist activity of testosterone was reported in daphnids (136). Exposure of *D. magna* to micromolar concentrations of testosterone caused a concentration-dependent delay in moulting and an increase in developmental abnormalities among neonates. Co-exposure with 20E protected against this toxicity of testosterone. Testosterone did not lower endogenous 20E-levels, but rather appeared to antagonize the EcR, based upon competition assays between testosterone and 20E in ecdysone-responsive *Drosophila* Kc cells. While these results suggest that testosterone is anti-ecdysteroidogenic in daphnids, the results have little environmental relevance to environmental androgens due to the high concentrations required to elicit a response.

Gene Product Changes

Several studies have reported on the impacts of exposure to pollutants, pharmaceuticals, and vertebrate hormones on expression profiles of mRNAs or proteins along the ecdysteroid signaling pathway. Expression of *ecr* was elevated from exposure of prawns, *Macrobrachium potiuna*, to glyphosate-based herbicides,

ethinylestradiol (estrogen), 4-hydroxytamoxifen (anti-estrogen), 17α-methyltestosterone (androgen), and cyproterone acetate (anti-androgen) (30, 137). Exposure of the intertidal mud crab *Macrophthalmus japonicas* to bisphenol A and di-(2-ethylhexyl) phthalate significantly elevated *ecr* expression levels (138). Studies, such as these are indicative of exposure to potential endocrine disrupting chemicals. However, whether the exposure actually results in apical disruption remains equivocal in the absence of demonstrated consequences of the molecular alterations.

Conversely, several studies have reported on effects of chemical exposure on apical endpoints, such as moulting. Zou (139) identified 33 compounds that have been shown to delay, impede, or advance moulting in crustaceans. While such studies inform on the toxicity of the chemicals to crustaceans, they do not provide insight on whether the effects elicited are actually due to endocrine disruption.

Neurohormone Activity Modulation

The negative control of ecdysteroid synthesis by MIH and by CHH is under the control of biogenic amines, notably, serotonin (5-hydroxytryptamine, 5-HT). Evidence suggests that the hyperglycemic action of 5-HT is due to the direct stimulation of CHH neurons (50, 140, 141). Indeed, 5-HT-immunopositive efferent axons to the *medulla terminalis* and the XO-neuropile have been demonstrated (142). The excitatory role of 5-HT on these XO-neurons was shown by Sáenz et al. (143). Therefore, serotoninergic stimulation of the release of neurohormones from the XO/SG-complex is likely to be a general phenomenon that applies to CHH as well as to MIH or mandibular organ inhibiting hormone (MOIH). The effects of fluoxetine on ecdysteroid levels in *C. maenas* demonstrated that fluoxetine, a selective serotonin reuptake inhibitor, significantly decreased 20E-levels at 0.5 and 0.75 μM after 8 and 4 h, respectively (92). Because, this effect was even more rapid and more pronounced with 0.5 μM 5-HT, but no effect of 5-HT or fluoxetine on 20E-levels could be observed in eyestalk-ablated animals, it was concluded that the mechanism leading to reduced 20E would be the inhibition of ecdysteroid synthesis by 5-HT-stimulated release of MIH. Interestingly, low ecdysteroid levels appeared to relate to an increased *mih*-expression in this study.

To date, only few studies examined the effects of pollutants on neuroendocrine processes that control moulting in crustaceans and only recently has MIH been proposed as a biomarker of endocrine disruption in crustaceans (30). This may be explained by the difficulties to quantify MIH-levels in the hemolymph, due to its 10 times lesser concentrations as compared to CHH and because of the pulsatory release of these neuropeptides from the SG (88, 144). Thus, *mih*-expression has been used to evaluate the effects of EDCs on the neuroendocrine regulation of moulting (30, 92, 137). Gismondi (30) and de Melo et al. (137) found an over-expression of *mih* in response to estrogen agonists and antagonists as well as antiandrogenic and androgenic compounds and a glyphosate-based herbicide. Because 20E equally increased *mih*-expression in the study of Gismondi (30), probably by the feedback control described by Techa and Chung (70), it was concluded that EDCs interfering with vertebrate steroid hormone signaling could affect *mih*-expression *via* an ecdysteroid related pathway, but without any further mechanistic explanation. The fact that very different compounds, which either activate or block the estrogen receptor or interact with other steroid receptors and even glyphosate all stimulated *mih*- as well as *ecr*-expression rather points to other, non-specific effects. Nevertheless, increased expression of *mih* may lead to an increased synthesis and release of this neuropeptide with the potential to modulate ecdysis.

Vertebrate-Type Sex Steroids

Whether or not crustaceans utilize estrogen, androgen, and progestogen signaling pathways has been debated for decades (145). Evidence in support of these signaling pathways is based largely upon observational studies; while, evidence against the existence of these signaling pathways is supported by genomic investigations and evolutionary biology. Interaction with the EcR is often cited as a mechanism by which vertebrate-type sex steroids function in crustaceans. However, as discussed above such interactions typically occur at high, non-physiologic levels.

Evidence in Support

Presence of the Hormones

The detection of vertebrate-type sex steroids in crustaceans was often cited in earlier literature as evidence that these hormones are of physiological significance in these organisms (146, 147). These include 17β-estradiol and testosterone in amphipods (148) and crayfish (149), pregnenolone in brine shrimp (150), and progesterone in shrimp (151). Several studies report that vertebrate-type steroid levels vary with the ovarian development cycle suggesting some functionality related to this process (discussed in (152)). However, the mere presence of a hormone in an organism does not indicate that the chemical possesses a signaling role in that organism. The hormone may be present as a consequence of dietary uptake or as a non-functional intermediate or metabolite of a biosynthetic pathway (153–156).

Responses to Exogenous Steroids

Many studies have demonstrated physiological responses of crustaceans to exogenously administered steroid hormones. For example, administration of 17β-estradiol advanced ovarian development (29) and stimulated vitellogenesis in female decapods (28). This compound also suppressed vitellogenesis-inhibiting hormone gene-expression, which was presumably responsible for the effects on ovarian development (28). While 17β-estradiol administration to crayfish stimulated vitellogenin-mRNA accumulation in the hepatopancreas, progesterone administration increased vitellogenin protein levels in the hemolymph of crayfish (157).

The provision of exogenous 17β-estradiol and progesterone support suggestions of a role for these hormones in crustacean reproduction. Some studies, however, indicate that estrogens downregulate monoamine oxidase activity (158–160) and may, therefore, increase 5-HT-levels that directly influence ovarian development. Furthermore, administration of testosterone has largely resulted in detrimental effects. Administration of testosterone to water fleas suppressed embryo development (136) and decreased lipid storage (161). These effects

were attributed to the ability of testosterone to elicit anti-ecdysteroidal activity.

Responses to Endocrine Disruptors

Several investigators have reported on the negative effects of estrogenic and anti-androgenic compounds on crustaceans. Estrogenic compounds, such as diethylstilbestrol, endosulfan, Aroclor 1242, and diethylphthalate delayed moulting in *D. magna* (162, 163). Studies that have shown neuroendocrine disruption by some of these compounds [**Table 1**; (172, 173, 176–178)] may provide an explanation for these observations and would situate their effects upstream of ecdysteroid signaling.

The anti-androgen cyproterone acetate severely reduced growth of *D. magna* without eliciting any discernible effect on moult frequency (145). Effects of several anti-androgens (cyproterone acetate, linuron, vinclozolin, p,p'-DDE) on the reproductive system of copepods revealed varied effects, although consistent among the treatments were degeneration of spermatocytes and deformed spermatophores (163).

Evidence Against

Lack of a Critical Enzyme for Estrogen Biosynthesis

Aromatase (CYP19) is responsible for the metabolic conversion of androstenedione to estrone and testosterone to estradiol. It is thus critical to the synthesis of estrogens. CYP19 is a product of chordate evolution (187) and has not been detected among the protostome invertebrates. Notably, CYP19 is absent from the *D. pulex* genome (159, 188). While it is possible that estrogens are synthesized in crustaceans *via* an alternative metabolic pathway, we are aware of no support for this premise.

Lack of Sex Steroid Receptors

Arguably, the greatest evidence against a role of vertebrate-type sex steroids in crustaceans and other Ecdysozoans is the lack of sex steroid hormone receptors (see Section 2). Immunochemical studies have suggested the presence of estrogen receptor α in *Gammarus fossarum* (27), androgen and estrogen receptors in the mud crab (189), and progesterone and estrogen receptors in crayfish (190). However, immunochemical assays are prone to false positive results due to cross-reactivities or non-specific binding to abundant proteins (191). In the latter studies, putative estrogen receptor co-localized with the other receptor evaluated (progesterone receptor in crayfish and androgen receptor in crab) indicating that antibodies in the same studies may have all been binding to the same abundant protein. Further, results from these studies were inconsistent with estrogen receptor detected in the cytosol from crayfish and membranes of the crab.

We are aware of no reports of the identification of high-affinity sex-steroid binding proteins, indicative of receptors, in crustaceans. Importantly, no sex steroid receptor genes were found in the genome of *D. pulex* (57). Similarly, the sequenced genomes of the ecdyzoans *D. melanogaster* and *Caenorhabditis elegans* revealed no androgen, estrogen, or progesterone receptors [discussed in (27)]. The dominant consensus among researchers is that sex steroid receptors were lost in the lineage leading to the evolution of arthropods (54) and are not present in crustaceans.

High Exposure Concentrations of Hormones and EDCs Are Typically Required to Elicit a Response

Steroid, and similar acting, hormones regulate physiological processes such as development, growth, metabolism, and reproduction. Thus, the action of these chemicals, *via* receptor-mediated signal transduction, typically does not result in rapid, overt responses by the organism (*e.g.*, acute responses). Rather, acute responses to these chemicals are largely the consequence of some ancillary response to high exposure concentrations of the chemical (*e.g.*, membrane disruption). In contrast, receptor-mediated responses to the chemical present as long-term consequences, such as alterations in development or reproduction (*e.g.*, chronic responses). These chronic responses are often, though not always, elicited at exposure concentrations significantly below those that elicit acute responses. The magnitude of the difference between concentrations of a chemical that elicit acute *versus* chronic responses is a function, in part, of the binding affinity of the agonist to responsive receptor protein. Hormones bind their receptors with high affinity and, thus, chronic responses to the hormone are typically elicited at concentrations orders-of-magnitude below concentrations that elicit acute toxicity (*e.g.*, high acute/chronic ratio; see **Table 2**, 20E).

Compounds known to be endocrine active in crustaceans typically elicit an acute/chronic ratio of 10–1,000 (**Table 2A**). The chronic responses listed in **Table 2A** are due to disruption of ecdysteroid and MF-signaling. Compounds known to act in vertebrates *via* estrogen and androgen signaling pathways, typically elicit acute/chronic ratios in crustaceans of <10 (**Table 2B**). The latter suggests that chronic responses of crustaceans to these vertebrate EDCs are not elicited through interaction with a hormone receptor.

The strongest evidence for the susceptibility of crustaceans to vertebrate sex steroid agonists are those studies that have shown effects of 17β-estradiol on reproductive system development. Studies cited above reported stimulatory effects of 17β-estradiol on crustacean vitellogenesis (28, 157). However, 17β-estradiol treatments also have been shown to have a negative effect or no effect on crustacean vitellogenesis (194). Exposure of daphnids to the estrogens diethylstilbestrol and bisphenol A had no effect on vitellogenin mRNA levels (195). 17β-Estradiol has been shown to interact with the ecdysteroid receptor at sufficiently high concentrations (196), thus some effects of estrogen injection, the common mode of administration in these studies, may have been the consequence of low affinity interaction of the estrogenic hormone with the ecdysteroid receptor.

ENDOCRINE REGULATION OF COLOR CHANGE

Neuro-Endocrine Regulation

Active color changes are termed "morphological" in the case of slow color changes established over weeks and months, whereas the rapid type that can take place in minutes to hours is termed "physiological" (197, 198). In arthropods, coloration and

TABLE 1 | Disruption of neuroendocrine pathways in crustaceans.

Species	Chemical	Concentration*	Effect	Endpoint	Reference
Procamburus clarkii	$CdCl_2$	5 mg·L^{-1}	Stimulation of CHH release; reduction of CHH responsiveness	Glycaemia	(164)
Uca pugilator	$CdCl_2$	10 mg·L^{-1}	Inhibition of PDH synthesis	Color change	(165)
U. pugilator	$CdCl_2$	10 mg·L^{-1}	Reduction of NE-mediated PDH release from SG	Distal pigment migration	(166)
P. clarkii	$CdCl_2$ $HgCl_2$	0.5 µg/g body weight (injection)	Inhibition of 5-HT mediated VSH release	Ovarian growth	(167)
U. pugilator	$CdCl_2$	1 mg·L^{-1}	Increase of VIH secretion from SG	Ovarian growth	(168)
Chasmagnathus granulata	$CdCl_2$ $CuCl_2$	0.5 mg·L^{-1} 0.1 mg·L^{-1}	Reduction of VIH secretion from SG	Ovarian growth	(169)
Palaemon elegans	$CuCl_2$	0.1 + 0.5 mg·L^{-1}	5-HT mediated increase of CHH release from SG	Glycaemia	(170, 171)
Barytelphusa guerini	DDT	2 mg·L^{-1} (injection)	Increase of CHH release from SG	Glycaemia	(172)
U. pugilator	PCB	8 µg·L^{-1} (Aroclor1242)	Reduction of NE-mediated PDH release from SG	Color change	(173)
Oziotelphusa senex senex	Fenitrothion	0.1 mg·L^{-1}	Increase of VIH secretion from SG	Ovarian growth	(174)
		0.5, 1, 2 mg·L^{-1}	Increase of CHH release from SG	Glycaemia	(175)
U. pugilator	Naphthalene	10 mg·L^{-1}	Inhibition of VSH release	Ovarian growth	(176)
U. pugilator	Naphthalene	2.54, 7.83, 9.98 mg·L^{-1}	Inhibition of NE-mediated melanin dispersion	Color change	(177, 178)
U. pugilator	Fluoxetine Fluvoxamine	20 µg/animal 20 µg/animal	Increased 5-HT mediated red pigment dispersion, reduced red pigment concentration	Color change	(179)
U. pugilator	Reserpine Bretylium	20 µg/animal 20 µg/animal	Increased NE-mediated melanin concentration, reduced melanin dispersion	Color change	(180)
U. pugilator, *P. clarkii*	Opioids	10^{-10} –10^{-8} mol/animal	Increase of VIH secretion from SG Inhibition of VSH release	Ovarian growth	(181, 182)
Daphnia magna	Fluoxetine	40 µg·L^{-1}	Increase of offspring production under limiting food conditions *via* 5-HT signaling	Reproductive output	(183)
Carcinus maenas	Fluoxetine	0.5 nM (injection)	Stimulation of CHH (and MIH) release from SG	Glycaemia, ecdysteroids	(92)
C. maenas	Fluoxetine	0.5–1nM (inject.)	5-HT mediated activation of heart and-scaphognathites	Cardioventilatory activity	(184)
Erichoeir sinensis	Carbamazepine	0.01–10 µg·L^{-1}	Increase of *chh* and *mih*-expression	Ecdysteroids, moulting	(130)
Daphnia pulex	Fluoxetine Citalopram	1 µM 1 µM	Increase of male production under short-day photo-period *via* glutamate/monoamine signaling	Male sex determination	(185)
Crangon crangon	Fluoxetine	0.1, 10, 100 ng·L^{-1}	Increased 5-HT mediated red pigment dispersion	Color change	(186)

**All concentrations correspond to waterborne exposures unless otherwise stated.*

CHH, crustacean hyperglycemic hormone; MIH, moult inhibiting hormone; NE, norepinephrine; PDH, pigment dispersing hormone; SG, sinus gland; VIH, vitellogenesis inhibiting hormone; VSH, vitellogenesis stimulating hormone; 5-HT, 5-hydroxytryptamine, serotonin.

reversible color change have evolved together with the development of an exoskeleton (197). Hence, color may be produced by pigments embedded in the pigmented layer of the endocuticle, or by pigment-containing cells in the epidermis (199). Accordingly, morphological color changes are related to moulting, notably, in terms of ontogenetic color changes (200, 201), whereas physiological color changes are produced by chromatophores.

Rapid color changes in most crustaceans rely on the dispersion and aggregation of pigments within stellate cells containing pigment granules. Generally, monochromatic chromatophores, *i.e.*, black-brown melanophores, red erythrophores, yellow xanthophores, and white leucophores, are intimately arranged in clusters called chromatosomes so as to produce a wide variety of colors (197, 198, 202–204). The dispersion of pigment granules, *i.e.*, their migration from the cell center into the ramifications of the chromatophores renders the coloration more intense, whereas the opposite is the case when the pigment granules aggregate within the center of the cell. Dispersion and aggregation of pigment granules can be completed within half an hour in *Crangon* up to 2 h in *Carcinus* or *Macrobrachium* (83, 205–208). Aggregation and dispersion are regulated by an antagonistic system of neuropeptide hormones composed of red pigment concentrating hormone (RPCH, or simply PCH) and pigment dispersing hormone (PDH) with its isoforms α- and β-PDH (198, 203). Notably, RPCH represents a highly conserved neuropeptide, which, in all decapods so far investigated, has an identical sequence (209, 210).

The antagonistic neurosecretory control of rapid color changes belongs to the best-studied hormonal systems in crustaceans [reviewed in (198, 202, 203)]. Indeed, RPCH and PDH have been the first neuropeptides to be characterized in crustaceans (211, 212). It has been established long ago that the eyestalk of decapod crustaceans is the source of hormones regulating color change in crustaceans (202). This was confirmed by Mangerich et al. (213), who localized the main perikarya of RPCH-secreting cells adjacent to or within the XO of *C. maenas* and *Orconectes limosus*, respectively. More recently, Alexander et al. (83) confirmed the presence of about 30 perikarya located in the XO, which project into the SG. A similar situation was shown for PDH, with the majority of PDH-perikarya located between the *medulla interna* and the

TABLE 2 | Acute and chronic toxicity values for crustaceans exposed to **(A)** compounds that disrupt ecdysteroid or methyl farnesoate signaling and **(B)** compounds that disrupt estrogen or androgen signaling.

Species	Chemical	EC50 ($mg \cdot L^{-1}$)[1]	MATC ($mg \cdot L^{-1}$)[1]	Acute/chronic ratio	Chronic endpoint
A. Endocrine active chemicals in crustaceans					
Water flea (*Daphnia magna*)	Tributyltin	1.67	0.14	11.9	Reproduction
Opossum shrimp (*Americamysis bahia*)	Tributyltin	2.2	0.37	5.9	Growth/reproduction
Water flea (*Daphnia magna*)	Methoprene	340	15.7	21.6	Development
Opossum shrimp (*Neomysis integer*)	Methoprene	320	10	32	Moulting
Water flea (*Daphnia magna*)	Pyriproxyfen	80	0.070	1,143	Reproduction
Opossum shrimp (*Neomysis integer*)	Pyriproxyfen	65	–		
Scud (*Gammarus fossarum*)	Pyriproxyfen	–	1.5	43[2]	Reproduction
Water flea (*Daphnia magna*)	Ponasterone	175[3]	<12.5[3]	>14	Moulting
Water flea (*Daphnia magna*)	20-Hydroxyecdysone	2,457[3]	88[3]	28	Moulting
Water flea (*Daphnia magna*)	Azadirachtin	680	82	8.3	Reproduction
Water flea (*Daphnia magna*)	Tebufenozide	17,370	62	280	Reproduction
Opossum shrimp (*Americamysis bahia*)	Tebufenozide	10,000	138	>72	Growth
B. Endocrine active chemicals in vertebrates					
Water flea (*Daphnia magna*)	Bisphenol A	1,336	540	2.5	Reproduction
Water louse (*Asellus aquaticus*)	Bisphenol A	9,500	224	42	Moulting
Water flea (*Daphnia magna*)	Diethylstilbestrol	1,550[4]	350[5]	4.4	Moulting
Water flea (*Daphnia magna*)	4-Nonylphenol	130	35	3.7	Reproduction
Scud (*Hyalella azteca*)	4-Nonylphenol	38	7.0	5.4	Survival
Water flea (*Daphnia magna*)	Atrazine	54,000	6,900	7.8	Reproduction
Copepod (*Amphiascus tenuiremis*)	Atrazine	>1000	86	>11.6	Reproduction
Water flea (*Daphnia magna*)	Cyproterone acetate	–	353[6]	–	Growth/reproduction
Water flea (*Daphnia magna*)	Butyl benzyl phthalate	3,700	444	8.3	Reproduction
Opossum shrimp (*Americamysis bahia*)	Butyl benzyl phthalate	900	113	8.0	Reproduction

[1]*Acute (EC50) and chronic toxicity values derived from the EPA EcoTox database (https://cfpub.epa.gov/ecotox/) unless indicated otherwise in comments. Chronic values (CVs) represent the square root of the no observed effect concentration (NOEC) X the lowest observed effect concentration (LOEC)). The NOEC or LOEC was used as a surrogate for the CV where both values were not available.* [2]*Value was determined using EC50 with Neomysis and maximum acceptable toxicant concentration (MATC) with Gammarus.* [3] *(192),* [4] *(193),* [5] *(20),* [6] *(145)*

medulla lateralis of the eyestalks of *C. maenas* and *O. limosus* (214), the axon terminals of which may project into the SG. Hence the major neurosecretory structures of RPCH and PDH are located within the eyestalk from where these neurohormones are released into hemolymph that transports them to the respective epithelial target cells. Because pigment dispersion and aggregation in eyestalk ablated animals could be observed, extra-eyestalk sources of RPCH and PDH have been considered. Indeed, RPCH-cells were found in small numbers in the brain, the thoracic ganglia and the circumoesophageal commissure as well as PDH-cells in the thoracic and connective ganglia (83, 213, 215). However RPCH and PDH of some of these cells may rather serve as a neurotransmitter instead of being implied in color change [*e.g.,* (216)].

A model for signal transduction and intracellular signaling cascades upon binding of RPCH to a G-protein coupled receptor has been proposed for the freshwater shrimp *Macrobrachium olfersii* (207, 217). In this model, RPCH activates cyclic guanosine monophosphate (cGMP) and Ca^{2+} second messenger cascades, which in turn stimulate a protein kinase to phosphorylate a myosin II molecular motor. As a result, pigment aggregation is effectuated by the movement of pigment granules along actin filaments in the chromatophore [for details see (198, 218)]. More recently, highly specific RPCH receptors (RPCHR) have been cloned and functionally deorphanized in *D. pulex* and *C. maenas* (83, 210). The RPCHR of *C. maenas* bound RPCH at doses lower than 0.001 nM (EC50 0.02nM). A dose of as low as 0.1 pmol effectively induced pigment aggregation in erythrophores *in vivo* within 5 min and the effect was stronger and longer lasting when the concentrations of RPCH were increased to 1 and 10 pmol, respectively (83). The RPCHR of *D. pulex*, on the other hand, bound *Daphnia*-RPCH at an EC_{50} of 0.065 nM, but binding of crustacean RPCH was at least two orders of magnitude less efficient. Insect adipokinetic hormone did equally activate the *Daphnia*-RPCHR in a dose-dependent and only slightly less efficient manner than *Daphnia*-RPCH (210).

Potential Sites of Endocrine Disruption

The capacity to change color and to adapt to the surrounding luminance may be impaired by pollutants as different as metals, such cadmium or mercury (165), organic chlorine compounds, like PCBs and naphthalene (173, 177), or drugs that affect the levels of biogenic amines (179, 180, 219) (**Table 1**). In spite of the high concentrations that were employed in these studies, the authors could exclude toxicity and plausibly demonstrate that the respective compounds caused neuroendocrine disruption by affecting the neurotransmitters responsible for the release of, notably, PDH, or the synthesis of the latter. For instance, Aroclor 1242 appeared to reduce norepinephrine (NE) titers in the XO-neuropile (173), thereby reducing the dispersion of black pigment in the chromatophores. Similar observations were made for naphthalene (177). In both studies, the authors took care to verify that neither the chromatophores were affected, which were still able to respond to extracts from the eyestalk containing PDH, nor was the neural tissue damaged. In the case

of cadmium, rather the amount of PDH stored in the SG was affected, putatively by inhibiting the synthesis of PDH (165). Fingerman et al. also showed that drugs that deplete monoamine levels, such as reserpine or bretylium, hamper pigment dispersion when fiddler crabs, *Uca pugliator*, were transferred from a white background with concentrated pigments to a black background, whereas fluoxetine enhanced pigment dispersion by increasing 5-HT-levels (179, 180, 219). Therefore, these early studies pointed to the possibility of psychoactive drugs targeting monoamine levels to interfere with the neurohormonal regulation of color change. This was confirmed by recent studies using more environmentally realistic concentrations of waterborne antidepressants (186). Color change in the sand shrimp, *Crangon crangon*, was affected by fluoxetine in the range of 10–1,000 $ng{\cdot}L^{-1}$ when exposed for 1 day or 1 week (186), suggesting enhanced dark adaptation following fluoxetine exposure (**Table 1**).

ENDOCRINE REGULATION OF SEXUAL DIFFERENTIATION

Vertebrates typically utilize a variety of genetic sex-determining strategies including sex-determining genes assembled on sex-chromosomes where females are the heterogametic sex, and sex-determining genes assembled on sex-chromosomes where males are the heterogametic sex (220), and autosomal sex-determining genes whose expression are environmentally controlled (221). Common to these sex-determining strategies, sex steroids (androgens, estrogens) are ultimately responsible for sexual differentiation. Indeed, androgens and estrogens from exogenous sources can sometimes circumvent genetic sex-determination (222).

Similarly, crustaceans possess diversity in sex determining mechanisms. Some decapods utilize sex chromosome where female are the heterogametic sex [*Penaeus monodon* (223); *Penaeus japonicus* (224)], while male are the heterogametic sex in others [*Orchestia cavimana* and *Orchestia gammarellus*, (225)]. Among branchiopods, clam shrimp *Eulimnadia texana* consists of monogametic males and heterogametic hermaphrodites (226), and brine shrimp *Artemia franciscana* consist of monogametic males and heterogametic females (227). In contrast, the female and male genomes of *D. pulex* are identical (228). A major distinction between vertebrates and crustaceans is that crustaceans have evolved strategies for sexual differentiation that do not involve steroidal androgens and estrogens.

Malacostracans

Evidence for an underlying genetic component to sex-determination in malacostracan crustaceans has come from a series of ablation/implantation experiments followed by cross breeding. The chromosomal system for these crustaceans is often referred to as ZW males and WW females. Using isopods (*Armadillidium vulgare*), Suzuki and Yamasaki (229) were able to transform males into functional females through the ablation of the androgenic gland (AG) and females into functional males through the implantation of the AG. The reciprocal crosses of "genetic" males with converted females and "genetic" females with converted females results in single sex broods. Similar experiments have been done with a variety of other crustaceans including prawns, crayfish, and hermit crabs (230–232). Further evidence of a genetic and potential chromosomal basis to sex-determination has come from breeding experiments with intersex crayfish. When intersex crayfish, *Cherax quadricarinatus*, that are functionally males were crossed with females the result was a 1:3 (male:female) sex ratio (232). Subsequent crossbreeding between female (WW) crayfish with normal males resulted in an all-female progeny 0:1 (male: female), leading the authors to conclude that the intersex specimens must have been genetic females (WZ). While the evidence for a genetic component to sex- determination remains strong, sex determination in Crustacea can also show degrees of plasticity. Therefore, it is most likely controlled also by epigenetic factors including environmental variables such as light and temperature (233, 234), parasites (235), and even diet (236).

Androgenic Gland Hormone

Male secondary sex characteristics in malacostracan crustaceans are under the control of androgenic gland hormone (AGH), which is produced by the ductless AG (237–239). The AG is usually situated on the paired testes or *vas deferens* in crustaceans. Its important role was discovered through a series of ablation and implantation experiments in the 1950's (240–243). Removal of AG results in the cessation of spermatogenesis and the demasculinization of male secondary sexual characteristics [(244) and references within]. Complete andrectomy in some species leads to the conversion of testicular to ovarian tissues that have the capacity to accumulate yolk proteins (245). Similarly, implantation of AGs into female crustaceans results in the conversion from ovarian to testicular tissues and the development of male sexual characteristics. Suzuki (230) was also able to demonstrate through a series of these ablation and implantation experiments at different maturation stages within the isopods that AGH was a sex- differentiating, but not a sex-determining factor in these organisms.

First purifications by Hasegawa et al. and Martin et al. have identified the AGH (246–248). The full insulin like peptide structure, consisting of B chain, A chain, and C peptide and the gene sequence of AGH have been characterised in the late 1990s for the isopod *A. vulgare* (249–251). Immunohistochemistry has shown that antibodies raised from AGH-peptides display relatively strong species specificity (252), which is not surprising as sexual characteristics are under strong selection pressures. Unfortunately, this makes developing immuno-histochemistry based bioassays for endocrine disrupter studies more problematic. The cDNA sequence for the insulin-like androgenic gland (IAG) gene has now been reported by several species including crayfish, and several prawn/shrimp and crabs (253). This allows for RNA interference (RNAi) techniques to be used to demasculinize and sex reverse aquaculture species with an AG-specific IAG peptide-encoding transcript (254, 255).

Potential Targets of Endocrine Disruption

Currently, it is not known whether environmental pollutants can impact the development of the AG development, or the synthesis of AGH. A number of studies correlated pollutants with increased incidences of intersexuality in crustaceans or male crustaceans displaying certain degrees of feminization or de-masculinization (18). These phenotypic changes in field collected animals mirror the physiological changes caused by feminizing parasite infection, AG ablation or RNAi silencing the AG leading authors to hypothesize whether chemicals can directly or indirectly interfere with the AG or AGH (18, 244). These hypotheses require further testing. In the light of an endocrine axis between the XO/SG, the AG and the male reproductive system, which has been confirmed for decapods (256), a disruption of the neuroendocrine regulation of AGH synthesis and spermatogenesis is conceivable. Indeed, specific CHH-isoforms appear to regulate AGH-expression (257) and its has been demonstrated that metal and organic pollution has the potential to affect CHH-synthesis or -secretion (92, 164, 170–172) (**Table 1**).

Branchiopods

Sexual differentiation in branchiopods has been extensively studied in *Daphnia*. Daphnids do not possess sex chromosomes (258, 259) and sexual differentiation of offspring is regulated by environmental cues (32). Under environmental conditions that favor rapid population growth, daphnids reproduce parthenogenetically (diploid oocytes) with all offspring being largely female (260). Maternal organisms produce broods, often consisting of dozens of offspring, every few days. These female offspring then mature in a matter of days and begin producing broods of female offspring. As a result, the population expands at an exponential rate. Under conditions that foretell adversity to population sustainability (exhaustion of resources, impending temperature extremes associated with summer or winter), females introduce male offspring to the population. Males mate with females that are producing haploid oocytes (260). The resulting embryo has undergone genetic exchange, which helps to purge deleterious mutations (261). The embryo is in a resting state of diapause to wait out the period of adversity, and is encased in a protective ephippium, which withstands desiccation and freezing. The ephippium is also hydrophobic, which facilitates transport on transient biota (*e.g.*, aquatic birds) or dispersal in air currents. This facilitates dispersal of the organisms to new habitats (262).

Environmental Regulation

The role of photoperiod and temperature in male sex differentiation of daphnids has been well characterized. Photoperiod and temperature function in concert to regulate sex ratios in *D. pulex* and *D. magna* populations (263). Under a long-day, summer-like photoperiod, daphnids produced only female offspring, regardless of temperature (range evaluated was 16-22°C). However, under a short-day, autumn-like photoperiod, daphnids became susceptible to temperature-dependent sex-determination. The different species exhibited different temperature optima for male sex-determination, probably relating to the geographic locations at which the populations used in the study were originally derived. Other environmental factors that have been implicated in male sex-determination include food restriction (264) and crowding (265).

Neuroendocrine Regulation

Exposure of daphnids to environmental conditions that stimulate the production of male offspring resulted in increased mRNA levels for various components of glutamate signaling based upon gene ontology (GO) terms (266). Further, Camp et al. (185) demonstrated that environmental stimulation of male sex determination resulted in increased mRNA levels of the subunit 2 of the N-methyl-D receptor (NMDAR) while having no effect on the NDMAR-a subunit. This change in the abundance of a single subunit of the receptor would result in alterations in subunit composition of the receptors, which has been shown to be responsible for plasticity in receptor function in vertebrates (267, 268). Conceivable, a reduction in the NMDAR-a/NMDAR-b subunit ratio may prompt glutamate signaling leading to male sex differentiation.

A role for the NMDAR in male sex-differentiation was further indicated by the observation that exposure of maternal daphnids to the NMDAR antagonists MK-801 and desipramine significantly increased the number of male offspring (185). Toyota et al. (266) also observed an effect of MK-801 on male sex differentiation; however, these investigators reported that the NMDAR antagonist suppressed male offspring production (266). Differences in results between these two research teams may reflect differences in experimental design. Where Camp et al. (185) reported the number of male and female offspring produced per female over six broods, Toyota et al. (266) reported the percentage of 30-day-old females that produced males, presumably in a single brood.

MK-801 also inhibits 5-HT, NE, and dopamine reuptake transporters, while desipramine inhibits noradrenergic reuptake transporters (269, 270). Therefore, Camp et al. (185) investigated the potential role of these neurotransmitter-signaling pathways in male sex differentiation. The mRNA levels of the serotonin reuptake transporter *SERT-a* and the α-adrenergic-like octopamine receptor *OctaR-A* were significantly elevated in daphnids reared upon a short-day photoperiod as compared to those reared under a long-day photoperiod. Two selective serotonin reuptake inhibitors fluoxetine hydrochloride and citalopram hydrobrimide increased offspring male sex determination, although the effect of fluoxetine hydrochloride was not statistically significant ($p=0.08$). These results suggest that in addition to glutamate signaling other neurotransmitters may be operative in male sex differentiation.

Methyl Farnesoate

Farnesyl units (C15), derived from acetate serve as building blocks for several important biomolecules, such as cholesterol and steroid hormones. In crustaceans, and other arthropods, farnesyl units also are used for the synthesis of farnesoic acid. Crustaceans utilize farnesoic acid as the substrate for MF. MF is a major sesquiterpenoid hormone in crustaceans, akin to juvenile hormone in insects (271).

Male-sex differentiation depends on MF (272, 273). During late stages of maturation MF programs oocytes to differentiate

into males. In the absence of methyl farnesoate, offspring differentiate into females (272). MF is responsible for the induction of the doublesex gene (*dsx1*) during oocyte susceptibility to sex differentiation (274). The doublesex gene product is transcriptionally upregulated in males and is responsible for orchestrating male sex-differentiation (274, 275). Sexually dimorphic expression of the double sex gene also has been shown in other Branchiopod crustaceans including *D. magna*, *Ceriodaphnia dubia*, and *Moina macrocopa* (276).

Synthesis and Degradation

In insects, MF is a product of the mevalonate biosynthetic pathway (277), as is likely the case in crustaceans. Two enzymes along this pathway were identified in lobster that were induced commensurate with MF synthesis (278). 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) activity increased within 24 h of eye-stalk ablation, which increased MF hemolymph levels, while farnesoic acid O-methyl transferase (FAMeT) activity was increased substantially two weeks after ablation. The reduction of HMGCR is the final step in the biosynthesis of mevalonate; while, methylation of farnesoic acid is the final step in the synthesis of MF. The authors surmised that increased production of mevalonate *via* heightened HMGCR reductase activity was responsible for the immediate increase in MF-production following eyestalk ablation, while prolonged increased synthesis of MF was due to elevation in FAMT activity.

In insects, MF is susceptible to metabolism and inactivation through ester hydrolysis and conjugation to polar molecules (279). These inactivation processes are operative in crustaceans as well (276, 280).

The location of synthesis in branchiopod crustaceans has not been established. In decapod crustaceans, MF is synthesized in the mandibular organ, which is under the negative control of mandibular organ inhibiting hormone (281, 282) (**Figure 1**). MF functions in some aspects of masculinization in decapods, such as the development of the male reproductive morphotype of the spider crab, *Libinia emarginata*. Abraded males (have not moulted in about a year or more) characteristically have high hemolymph MF-levels, large reproductive organs, and aggressive mating behavior (283).

Receptor-Mediated Activation

The regulatory activity of MF is mediated primarily through interaction with the bHLH-PAS protein methoprene-tolerant (MET) (284, 285). MET derives its name from the discovery that resistance of *Drosophila* to the insecticidal MF-analog, methoprene, was associated with a functional mutation in this gene (286). MF-activated MET recruits the bHLH-PAS protein, steroid receptor coactivator (SRC) (37). Together, this MET-SRC complex comprises the activated MF- receptor (MfR) in crustaceans.

Other suggested receptors for MF in crustaceans include the RXR and hormone receptor 97g (HR97g). RXR is a member of the nuclear receptor superfamily that has been identified in several crustacean species including the American lobster *Homarus americanus* (287), the fiddler crab *Uca pugilator* (288), the tropical land crab *Gecarinus lateralis* (289), the crayfish *Procambarus clarkii* (290), the amphipod *G. fossarum* (291) and the water fleas (*D. magna, D. pulex*) (58, 292). While MF was unable to activate daphnid RXR in a luciferase reporter assay, its co-administration with 20E to a reporter system consisting of RXR and EcR, resulted in activation greater than that observed with 20E alone (293). This apparent synergistic interaction between activated EcR and activated RXR was also observed *in vivo* using tributyltin as the RXR agonist (294). These results suggest that MF, through interaction with RXR, may function in concert with 20E to regulate crustacean moulting.

Nuclear receptor HR97g, isolated from *D. pulex*, was mildly activated by MF and the MF-analog pyriproxyfen in a luciferase reporter assay (295). This crustacean receptor was first identified in *D. pulex* (57), and has since been identified in the spiny lobster *Panulirus ornatus* (296). The physiological significance of the receptor as a ligand-activated regulator of crustacean physiology remains unknown.

Known Targets of Disruption

The MfR is the best-demonstrated target of disruption of this regulatory pathway by environmental chemicals. Compounds that elicit insecticidal activity as juvenile hormone analogs also typically bind and activate the MfR (37, 285). This activity is responsible for the high sensitivity of crustaceans to this class of insecticides (273, 297, 298). However, the MfR appears to have high ligand recognition specificity. We are aware of no demonstrations that compounds, other than juvenile hormone analogs, are capable of activating the MfR at environmentally relevant exposure levels (37).

Potential Targets of Disruption

Alterations in MF levels due to toxicant-mediated effects on biosynthetic or inactivating hormones is a plausible mechanism of endocrine disruption. The herbicide atrazine reportedly increased male offspring production in *Daphnia pulicaria* (299). However, atrazine did not interact with the MfR (37), suggesting that if atrazine did indeed activate this pathway, it may have been due atrazine increasing endogenous MF. Such an effect may have been the consequence of competition between atrazine and MF at a site of inactivation or elimination. Alternatively, atrazine disrupts endocrine function in mammals by increasing dopamine and reducing NE levels in the hypothalamus (300). These neuromediators are operative in the regulation of MF and sex determination in daphnids (185). Thus, disruption of these upstream signaling components may be responsible for the effects of atrazine on the MF-signaling pathway.

FUTURE DIRECTIONS OF RESEARCH ON ENDOCRINE DISRUPTION IN CRUSTACEANS

In 1998, a workshop was held in the Netherlands to address the issue of endocrine disruption in invertebrates sponsored by The Society of Environmental Toxicology and Chemistry (SETAC).

The proceedings of this workshop were subsequently published (301) and made a number of recommendations. Recently, Ford and LeBlanc conducted a survey of experts to reflect on the progress made in endocrine disruptor studies with invertebrates over the past few decades (302). The majority of participants in that survey believed endocrine disruption was an issue for invertebrates that needed to be addressed, but were mixed over the relative progress that had been made. Strikingly, many of the recommendations provided in this recent survey mirrored those made back in 1998 indicating that the field had not significantly progressed.

A major impediment to advancing research on endocrine disruption in crustaceans has been attempts to detect endocrine effects using chemicals known to be endocrine disruptors in vertebrate species along with endpoints known to be indicative of endocrine disruption in vertebrates. This conclusion has recently been acknowledged by several researchers (303, 304). For example, many studies focus upon the effects of estrogens on crustaceans (18) and use biomarkers of feminization, such as vitellogenin induction, in crustaceans (305). Elevated vitellogenin levels following chemical exposure or in field-collected samples has often been interpreted as an estrogenic effect (306). However, unlike vertebrates, vitellogenin is not regulated by estrogens in crustaceans (195). Despite existing knowledge on the endocrine regulation of vitellogenesis in crustaceans, the precise molecular mechanisms by which vitellogenesis and ovarian maturation are controlled require further elucidation (307). Given the lack of clear understanding of seasonal or developmental fluctuations in normal vitellogenin levels and overall susceptibility of vitellogenin production to non-endocrine stressors, associations between altered vitellogenin levels and endocrine disruption in crustaceans are tenuous at best.

One area where progress has been made is the onset of more affordable "omic" technologies allowing for the high-throughput sequencing of genomes, transcriptomes, peptidomes, and metabolomes (19, 139, 304). These omics-techniques offer a rich opportunity for discovering conserved molecular and biochemical pathways, which can applied to the development of adverse outcome pathways (308). These in turn provide opportunities for further advancing our mechanistic understanding of crustacean endocrinology from which to develop appropriate biomarkers of disruption. Zou (139) in a recent review identified over 30 compounds that have either inhibited or stimulated moulting in crustaceans. However, in the absence of mechanistic linkages between exposure and effect, endocrine disruption cannot be invoked as responsible for these effects on moulting. The identification of appropriate biomarkers will facilitate establishing whether an effect on moulting, or some other endocrine-regulated process, is due to specific disruption of the endocrine system or due to some non-specific toxicity.

We hope to have advocated strongly that a "crab is not a fish" and therefore toxicity evaluations using crustaceans require appropriate endpoints to determine whether current and newly licensed chemical compounds might target endocrine processes in this ecologically important group. While significant progress has been made in our understanding of crustacean endocrinology, application of this knowledge to the study of endocrine disruption in crustaceans is lagging. Where, in the past, we were limited in our ability to develop the tools to confirm whether a substance was an endocrine disruptor, these limitations have been largely overcome with affordable omics-technologies. We now have the ability to develop high throughput screenings using key crustacean-relevant endocrine targets. Given the number of crustacean species incorporated into national toxicity programs, such tools are sorely needed.

AUTHOR CONTRIBUTIONS

TK, GL, and AF have developed the concept of this review and have contributed to the writing. All authors contributed to the article and approved the submitted version.

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Assessment of the Effects of Double-Stranded RNAs Corresponding to Multiple Vitellogenesis-Inhibiting Hormone Subtype I Peptides in Subadult Female Whiteleg Shrimp, *Litopenaeus vannamei*

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Vitellogenesis-inhibiting hormone (VIH) negatively regulates reproduction in shrimp and other decapod crustaceans. In order to assess the effects of transcriptional silencing by multiple VIH subtype I sinus gland peptides (SGPs) on ovarian maturation in female whiteleg shrimp, *Litopenaeus vannamei*, we synthesized five dsRNAs targeting *Liv-SGP-A*, *–B*, *–C*, *–F*, and *–G* and injected them into subadults. The following treatments were employed: sgpG-dsRNA (targeting *Liv-SGP-G*), sgpC-dsRNA (targeting *Liv-SGP-C*), and mixed-dsRNA (targeting *Liv-SGP-A*, *–B*, and *–F*). The expression of *Liv-SGP-G* in eyestalks was significantly decreased at 10, 20, and 30 days after the injection of sgpG-dsRNA In addition, it was significantly decreased at 10 and 30 days after the injection of mixed-dsRNA. The expression of vitellogenin (Vg) gene expression in the ovaries, and concentrations of Vg protein in the hemolymph, were not changed by the administration of any dsRNA treatment (the ovaries remained immature in all treated individuals and contained mostly oogonia and previtellogenic oocytes). Although the administration of dsRNAs corresponding to multiple VIHs did not promote ovarian maturation, this is the first report of the co-transcriptional repression of *Liv-SGP-G* by the injection of dsRNA for homologous genes (*Liv-SGP-A*, *–B*, and *–F*). These results indicate that subadults can respond to the techniques of transcriptional silencing.

Keywords: double-stranded RNA, *Litopenaeus vannamei*, RNA interference, vitellogenesis-inhibiting hormone, vitellogenin

INTRODUCTION

The hormonal regulation of ovarian maturation in decapod Crustacea is more well-understood in relation to inhibitory factors than to stimulatory factors. Eyestalk ablation is frequently employed as an artificial means of promoting ovarian maturation in economically important shrimp species, and is frequently used in commercial hatcheries to induce spawning (1–3). For this reason, it had long been assumed that the eyestalk harbors a maturation-inhibiting factor, now referred to as vitellogenesis-inhibiting hormone (VIH; also gonad-inhibiting hormone, GIH). There now exist

many reports regarding its physiological functioning, including information on its identification and characterization (4–7). VIH is expressed and synthesized at the X-organs and stored in the sinus glands in the eyestalks; these tissues are collectively referred to as the X-organ/sinus gland complex. VIH thus synthesized is secreted into the hemolymph and negatively regulates ovarian maturation. In *Litopenaeus vannamei* and other penaeid shrimp species, VIH comprises a set of peptide hormones that belong to the crustacean hyperglycemic hormone (CHH) family. CHH-family neuropeptides also include CHH, VIH, molt-inhibiting hormone (MIH), and mandibular organ-inhibiting hormone (MOIH). Most CHH-family peptides are synthesized at and secreted predominantly from the X-organ/sinus gland complex, but some are synthesized at other tissues (8–11). Mature CHH-family peptides all have six conserved cysteine positions, and are divided into two subtypes depending on the absence (subtype I) or presence (subtype II) of a glycine residue at position 12. Generally, CHH is of subtype I, and VIH, MIH, and MOIH are of subtype II; however, subtype I VIH has also been reported in the penaeid shrimps *Penaeus japonicus* and *L. vannamei* (4–7).

Penaeid shrimp species are commercially important in shrimp farming worldwide; in particular, the whiteleg shrimp, *L. vannamei*, accounts for over 70% of farmed shrimp (12). Eyestalk ablation is routinely performed in adult females in commercial hatcheries in order to induce ovarian maturation, but the procedure often promotes adverse effects on the animals, lowers reproductive efficiency, and poses concerns for animal welfare (13). Thus, alternative methods based on the physiological functioning of VIH should be sought. The use of RNA interference (RNAi) has been considered as an alternative to eyestalk ablation where the administration of double-stranded RNA (dsRNA) silences the transcription of the vitellogenesis-inhibiting hormone gene *vih*; such work has been carried out in *Penaeus monodon* and *L. vannamei* (11, 14–16).

Both VIH subtype I and II are present in *L. vannamei*. As delineated in Tsutsui et al. (7), subtype I includes several peptides identified from the sinus glands, referred to as sinus gland peptides (SGPs)-A to −G, six of which (except SGP-D) possess VIH activity. We subsequently conducted the molecular characterization of five of these (*Liv-SGP-A, −B, −C, −F, −G*) and investigated their expression levels in relation to molting and eyestalk ablation (17, 18). In addition, we achieved the knockdown of the most predominant VIH subtype I (SGP-G) in adult *L. vannamei via* VIH-dsRNA injection into adult females, but this did not lead to an increase in vitellogenin (Vg) gene expression in the ovary and levels of Vg in the hemolymph even 20 days after injection (16). On the other hand, in Chen et al. (10) a gene for a VIH subtype II in *L. vannamei* was cloned from the eyestalks and brain, and recombinant peptide corresponding to VIH subtype II inhibited *vg* mRNA expression (10). Feijo et al. (15) reported the knockdown of GIH (VIH subtype II) transcription *via* GIH-dsRNA injection, with *vg* mRNA expression in the ovary being increased 37 days after injection (15).

Previously, we analyzed gene expression levels for VIH subtype I peptides in the eyestalks following unilateral eyestalk ablation in adult and subadult *L. vannamei*, and found a significant decrease only in subadults; specifically, the expression of *Liv-SGP-A*, *−C*, and *−G* decreased 10 or 20 days after eyestalk ablation (18). The question still remained as to why a significant reduction of *vih* expression following eyestalk ablation was shown only in subadults and for only three among five of the VIH species (e.g., Liv-SGP-A, -B, and -G as listed above).

In *L. vannamei*, subadult females can also be induced to undergo ovarian maturation using eyestalk ablation; therefore, such females are often used as experimental material in order to study the mechanisms of ovarian maturation (10, 19, 20). In this study, we employed subadult females in order to study in more detail the action of VIH on shrimp ovarian maturation at a very basic level in the absence of complications that may be observed in the adult system. We therefore suggest that if the inhibiting action of VIH on ovarian maturation is more powerful in subadults than in adults, the knockdown of VIH expression may be more effective in subadults. Therefore, here we have selected subadult female *L. vannamei* in order to assess the effects of multiple dsRNAs on the transcriptional silencing of *vih* genes, and to shed further light on the dynamics of *vih* transcription in early-stage shrimp, with the aim of better understanding the phenomenon of ovarian maturation. Therefore, in regard to the above, we aimed to examine the co-transcriptional repression of *Liv-SGP-G* based on the administration of dsRNA corresponding to multiple VIH subtype I peptides having differing degrees of similarity; this is first report of this kind for a decapod crustacean species.

MATERIALS AND METHODS

Animals

Subadult female *L. vannamei* were purchased from IMT Engineering Inc. (Niigata Prefecture, Japan) and were gradually acclimated to recirculated natural seawater (31–33‰ salinity) after which they were kept in 600-L tanks at 28°C under a 13-h light/11-h dark photoperiod for 2 weeks before use in experimentation. All shrimp were fed with a commercial diet (Goldprawn; Higashimaru Co., Kagoshima, Japan) at a rate of 2–3% body weight per day until use. The treatment of all animals complied with institute regulations and Japanese policy on animal use (21).

Preparation of Double-Stranded RNA

dsRNA targeting *vih* genes in *L. vannamei* (*Liv-SGP-A*, *−B*, *−C*, *−F*, and *−G*) and the gene for green fluorescent protein (GFP) as a negative control were prepared as previously elaborated (16). T7 promoter-linked linear DNA for each gene was amplified by PCR using the following T7 promoter-linked gene-specific primers: T7-sgpC-L (5′-TAATACGACTCACTATAGG GAGACT- CGCTCTTCGACCCTTCC-3′) and T7 promoter (5′-TAATACGACTCACTATAGGG-3′) for *Liv-SGP-A*; T7-sgpB-L2 (5′-TAATACGACTCACTATAGGGAGAC GCAGCATATCCTTCGACTCGT-3′) and T7 promoter for

Liv-SGP-B; T7-sgpC-L and T7-sgpC-R (5′-TAATACGACTCACTATAGGGAGACTATTTCCCGACCATCTGG-3′) for *Liv-SGP-C*; T7-sgpF-L (5′-TAATACGACTCACTATAGGGAGAAAGCGCTCCCTCTTCGACC-3′) and T7-sgpF-R (5′-TAATACGACTCACTATAGGGAGACTTTATTTGCCGACGGTCTGCAGG-3′) for *Liv-SGP-F*; and T7-VIH-L (5′-TAATACGACTCACTATAGGGAGAAAGCGAGCAAACTTCGAC-3′) and T7-VIH-R (5′-TAATACGACTCACTATAGGGAGACTACTTGCCCACCGTCTG-3′) for *Liv-SGP-G*. DNA fragments that encoded each mature peptide were purified and used to synthesize each dsRNA (sgpA-dsRNA for *Liv-SGP-A*, sgpB-dsRNA for *Liv-SGP-B*, sgpC-dsRNA for *Liv-SGP-C*, sgpF-dsRNA for *Liv-SGP-F*, sgpG-dsRNA for *Liv-SGP-G*, and GFP-dsRNA for *GFP*), following the protocol described in Kang et al. (16) using the MEGAscript RNAi kit (Ambion, Thermo Fisher Scientific, Tokyo, Japan) according to the manufacturer's instructions.

Injection of Double-Stranded RNA and Tissue Collection

sgpG-dsRNA, sgpC-dsRNA, and GFP-dsRNA were diluted with elution buffer (TE buffer: 10 mM Tris-HCl [pH 7], 1 mM EDTA) to 0.75 μg μl^{-1}. Mixed dsRNA was prepared using three separate dsRNA mixtures having equivalent concentrations of sgpA-dsRNA, sgpB-dsRNA, and sgpF-dsRNA (each dsRNA concentration was 0.25 μg μl^{-1}). This was diluted with TE buffer to yield 0.75 μg μl^{-1} total dsRNA. We next randomly selected 80 female shrimp having a body weight of above 15 g and injected 18 of them each with one of the above four dsRNA preparations at 3 μg g^{-1} body weight as in our previous report (16). In brief, all female shrimps were injected once only in the first abdominal segment intraperitoneally using a BD Ultra-Fine Insulin Syringe (0.3 ml, 29 G × 12.7 mm; BD Biosciences, Tokyo, Japan). Four dsRNA treatment groups were designed as follows: a single injection of either GFP-dsRNA (GFP-dsRNA group), sgpG-dsRNA (sgpG-dsRNA group), sgpC-dsRNA (sgpC-dsRNA group), or mixed-dsRNA (mixed-dsRNA group). All injected shrimps were maintained under the conditions delineated in the *Animals* section. We sampled six individuals from each treatment at 10, 20, and 30 days after injection and collected samples of hemolymph, eyestalk, ovary, and hepatopancreas as described previously (16). Body weight in each group are shown in **Table 1**. As the initial control, six non-treatment shrimps were also collected (body weights, mean ± SEM: 23.0 ± 1.23 g, n = 6). The gonadosomatic index (GSI) was calculated as gonad weight (g)/body weight (g) × 100 and expressed in terms of percentage. Since the number of shrimps that can be reared simultaneously under our recirculating rearing system poses certain limitations, in order to compare each treatment in an equivalent manner, six individuals for each treatment was the maximum number that could be employed under our rearing system. Statistically, n = 5 is generally considered acceptable, and in this way, we believe that our methodology is valid.

TABLE 1 | Average body weight of treated shrimps (g, mean ± SEM, *n* = 6) at 0, 10, 20, and 30 days after treatment with each dsRNA preparation.

dsRNA prep.	10 days	20 days	30 days
GFP-dsRNA	21.2 ± 0.88	26.1 ± 1.90	28.8 ± 0.91
sgpG-dsRNA	23.5 ± 0.72	26.8 ± 0.96	27.4 ± 0.67
sgpC-dsRNA	23.6 ± 1.15	26.4 ± 1.20	27.7 ± 1.77
mixed-dsRNA	23.0 ± 0.89	25.4 ± 1.18	28.3 ± 1.08

Quantitative Real-Time PCR for *vih* and *vg*

Total RNA was purified from eyestalks, ovaries, and hepatopancreas tissue using an RNeasy Mini kit with DNase I digestion according to the manufacturer's protocol (Qiagen, Tokyo, Japan). In order to maximize the quality of RNA for subsequent qPCR analysis, the purified eyestalk total RNA was purified once more using an RNeasy MinElute cleanup kit (Qiagen) according to the manufacturer's protocol. The relative expression of *Liv-SGP-G* was quantified by two-step qPCR as reported previously (16). In brief, the twice-purified eyestalk total RNA was used for reverse-transcription (RT) with a High Capacity RNA-to-cDNA kit (Applied Biosystems, Thermo Fisher Scientific, Tokyo, Japan) in accordance with the manufacturer's protocol, and the synthesized cDNA was used as a template for qPCR. qPCR for *Liv-SGP-G* and *beta-actin* (*actb*, as an internal control) was performed with TaqMan Fast Universal PCR Master Mix (2×, No AmpErase UNG; Thermo Fisher Scientific) on a Model 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously reported (16, 18), using the primer pair LvsgpG-Fw and LvsgpG-R3 and the TaqMan probe LvsgpG-Prb for *Liv-SGP-G*; and the primer pair Lvact_F01 and Lvact_R02 and the TaqMan probe Lvact_Prb for *actb*. The standard curves for *Liv-SGP-G* and *actb* were linear over five orders of magnitude of serially diluted cDNA libraries (eyestalk cDNA for *Liv-SGP-G*, testis cDNA for *actb*) (16). Expression was quantified in duplicate, and the mean of *Liv-SGP-G* was normalized to that of *actb*.

The relative expression of *Liv-SGP-A*, *-B*, and *-C* was also quantified by two-step qPCR as reported previously (18). In brief, the synthesized cDNA from twice-purified eyestalk total RNA was used as a template for qPCR. qPCR for *Liv-SGP-A*, *-B*, *-C* and *beta-actin* (*actb*, as an internal control) was performed using the same protocols of qPCR for *Liv-SGP-G*, but using the following gene-specific primers and TaqMan probes: SgpA-Fw, SgpA-Rv, and SgpA-Prb for *Liv-SGP-A*; SgpB-L2, SgpB-Rv, and SgpB-Prb for *Liv-SGP-B*; and LvsgpC-Fw, LvsgpC-Rv, and LvsgpC-Prb for *Liv-SGP-C*. The standard curves for *Liv-SGP-A*, *-B*, and *-C* were linear over seven orders of magnitude of serially diluted 4 ng of each plasmid (18). Expression was quantified in duplicate, and the mean of each gene expression data point was normalized to that of *actb*.

The expression of *vg* in the ovary and hepatopancreas was quantified by one-step real-time RT-PCR using a QuantiFast Probe RT-PCR + ROX Vial Kit (Qiagen) with purified total RNA as the template. The expression of *vg* and *actb* mRNAs was quantified on a Model 7500 Fast Real-Time PCR System as previously reported (16, 19), using the primer pair vg-qF01 and vg-qR01 and the TaqMan probe vg-Prb for *vg*; and the primer pair Lvact_F01 and Lvact_R02 and the TaqMan probe Lvact_Prb for *actb* (16). The standard curves for *vg* and *actb* were linear over five orders of magnitude of serially diluted total RNA from

mature ovary. Expression was quantified in duplicate, and the mean of *vg* expression was normalized to that of *actb*.

Measurement of Vitellogenin Concentrations in Hemolymph

Hemolymph Vg was measured by time-resolved fluoroimmunoassay (TR-FIA) as previously reported (19). In brief, each hemolymph sample was directly diluted 1:2,000 with 0.1 M carbonate buffer (CB, pH 9.6), and 100 µl of diluted sample was dispensed into the wells of 96-well plates (Delfia Yellow; Perkin Elmer, Waltham, MA, USA). To construct standard curves, purified vitellin from *L. vannamei* was serially diluted in negative buffer (male hemolymph diluted 1:2,000 in CB) to 50.1 to 0.10 ng per well for the TR-FIA Vg assay. For sample-coating, the 96-well plates were incubated at 4°C overnight with 100 µl of sample or standard, and then the wells were blocked with 1% BSA in assay buffer (Perkin Elmer). After blocking, vitellin antiserum from *Penaeus japonicus* (anti-PjVn) was diluted 1:10,000 in assay buffer for use as the primary antibody. Secondary antibody was Delfia Eu-N1-labeled anti-rabbit antibody (Perkin Elmer), diluted 1:2,000 in assay buffer. Plates were incubated following each step for 2 h at 24°C, and the wells were washed five times for 3 min at a time with 0.3 ml of washing buffer (50 mM Tris·HCl, pH 7.8, 150 mM NaCl, 0.05% Tween 20) in an ImmunoWash 1575 Microplate Washer (Bio-Rad) each time between steps. Finally, enhancement solution (Perkin Elmer) was added to the plate, and the fluorescence was measured at 615 nm on a time-resolved fluorometer (Wallac 1420 ARVOsx-d; Perkin Elmer).

Histological Analysis

A small piece of ovary from each sampled individual was fixed in Davidson's fixation solution (formalin, 220 ml L^{-1}; EtOH, 330 ml L^{-1} 95%; acetic acid, 115 ml L^{-1}) for 16–20 h and then held in 70% ethanol at room temperature before processing for histological analysis. Tissues were dehydrated through a graded series of ethanol and lemosol (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan), and then embedded in paraffin. Tissues were sectioned to 5 µm, hydrated through a graded ethanol series, stained with hematoxylin and eosin, and again dehydrated through a graded ethanol series and xylene. Oocyte developmental stage, determined according to previous reports, was observed under a light microscope (19).

Statistics

Significant outliers among each group were identified by Grubbs's test for outliers ($\alpha = 0.01$) on the GraphPad website (https://www.graphpad.com/quickcalcs/Grubbs1.cfm) and omitted. All results are expressed as the mean ± SEM. Significant differences were assessed by one-way ANOVA using SigmaPlot v. 11 software (Hulinks Inc., Tokyo, Japan). The significance between *vg* expression levels and Vg concentrations in all groups was tested by multiple comparisons between the untreated (initial) group and treatment groups by Dunnett's method. The significance of expression levels of *Liv-SGP-G* among individuals was tested by multiple comparisons between the initial group and treatment groups by Kruskal–Wallis ANOVA on ranks using Dunn's method.

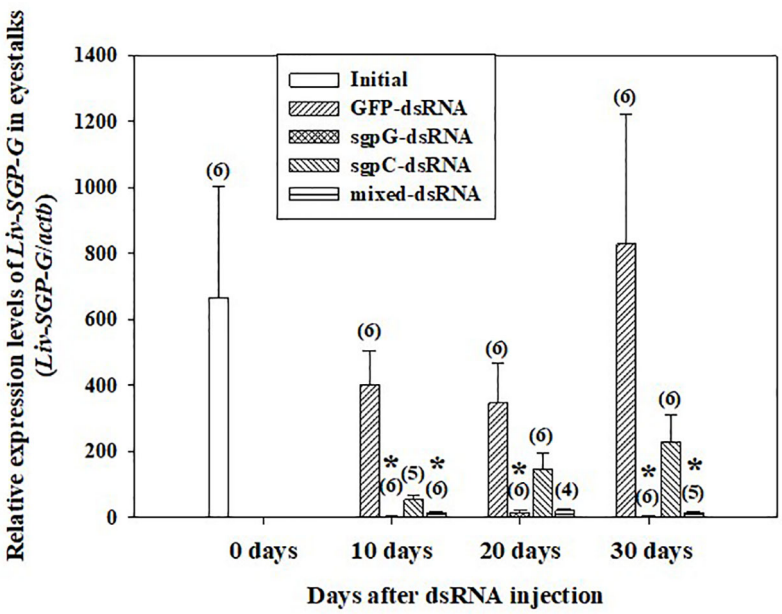

FIGURE 1 | Levels of *Liv-SGP-G* expression in eyestalks following a single injection of GFP-dsRNA, sgpG-dsRNA, sgpC-dsRNA, mixed-dsRNA, or no injection (initial). Results are expressed as the mean ± SEM. Asterisks indicate significant difference ($P < 0.05$) from the initial group. Values in parentheses indicate numbers of shrimp analyzed in each group. *Beta-actin* is indicated as *actb*. The content of each treatment group is indicated in the legend box that appears above the bar graph.

RESULTS

Effects of the Injection of Multiple Double-Stranded RNAs on the Expression of *Liv-SGP-G*

Figure 1 shows relative *Liv-SGP-G* expression levels in the eyestalk. In shrimps injected with GFP-dsRNA, *Liv-SGP-G* expression was high at 10 (*Liv-SGP-G* levels 400.7 ± 104.0) to 30 days (828.9 ± 393.6) after injection, with no significant difference from the initial group (664.0 ± 337.9). In the sgpG-dsRNA group, expression was significantly lower than that of the initial group at 10 days (3.011 ± 1.413) to 30 days (3.052 ± 0.943). In the sgpC-dsRNA group, expression was lower, but not significantly, than that in the initial group at 10 days (54.08 ± 13.14), and then increased gradually from 20 days (145.7 ± 51.31) to 30 days (229.0 ± 83.46). In the mixed-dsRNA group, expression was significantly lower than that in the initial group at 10 days (14.91 ± 2.379), lower (but not significantly) at 20 days (20.89 ± 5.447), and then significantly lower at 30 days (14.21 ± 2.830).

Dynamics of *vg* Expression and Concentrations Following Double-Stranded RNA Injections

Figure 2A shows relative *vg* mRNA expression in the ovary. In the initial group, expression was weak (four out of six shrimps) or easily detectable (two out of six shrimps) (*vg* mRNA levels 220.6 ± 147.8). In the GFP-dsRNA group, expression was weak at 10 days (15.16 ± 14.08) and 20 days (1.408 ± 0.578), and then strong at 30 days (343.1 ± 193.1), but with no significant difference compared to the initial group owing to high variation in measured values. In the other groups, *vg* mRNA expression was weak. Expression of *vg* mRNA in the hepatopancreas was weak or not detectable in all treatment groups, including the initial group (data not shown).

Figure 2B shows Vg levels in the hemolymph. In the initial group, levels were low (8.56 ± 1.82 μg ml^{-1}). In the GFP-dsRNA group, levels were low at 10 days (3.76 ± 0.801 μg ml^{-1}) and 20 days (7.51 ± 0.573 μg ml^{-1}), with no significant difference observed from the initial group, but were significantly increased at 30 days (80.7 ± 40.6 μg ml^{-1}). Levels in the other groups from 10 to 30 days were not significantly different from those of the initial group.

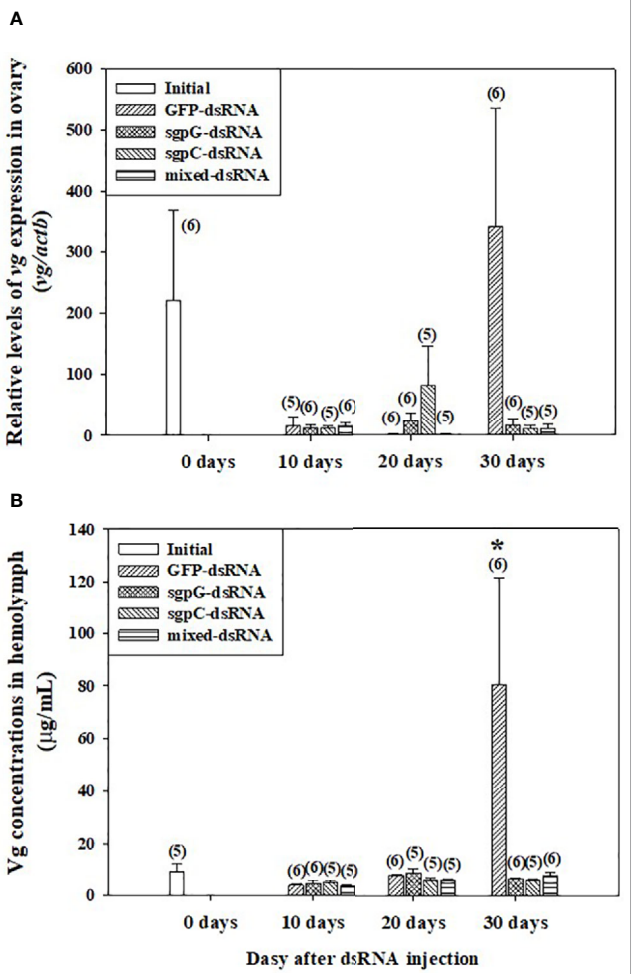

FIGURE 2 | Levels of **(A)** *vg* mRNA in ovary and **(B)** Vg in hemolymph following a single injection of GFP-dsRNA, sgpG-dsRNA, sgpC-dsRNA, mixed-dsRNA, or no injection (initial). Results are expressed as the mean ± SEM. The asterisk indicates significant difference ($P < 0.05$) from the initial group. Values in parentheses indicate numbers of shrimp analyzed in each group. Abbreviations are as follows. Vg, vitellogenin; actb, beta-actin. The content of each treatment group is indicated in the legend box that appears above each bar graph.

Gonadosomatic Index and Oocyte Development

GSI was low (<2%) from 10 to 30 days in all individuals (**Figure 3**), with no significant difference observed compared to the initial group (0.80 ± 0.23%). GSI tended to gradually increase from 10 days to 30 days in the GFP-dsRNA injected groups. In the sgpG-dsRNA injected group, GSI increased slightly from 10 days to 20 days and then decreased at 30 days.

Figure 4 shows histological images of oocyte development from representative individuals exhibiting a GSI close to the mean of each group. In the initial group, oocytes in five of the six shrimps were in the previtellogenic stage, and contained mostly oogonia and previtellogenic oocytes (GSI = 0.94%, **Figure 4A**); only one shrimp exhibited an ovary in the primary vitellogenic stage (GSI = 1.9%). In the GFP-dsRNA group, ovaries were in the previtellogenic stage in all six shrimps at 10 days (GSI = 0.33%, **Figure 4B-1**) and 20 days (GSI = 0.70%; **Figure 4B-2**), and in five of the six shrimps at 30 days, two of which also had a few early-stage endogenous vitellogenic oocytes (GSI = 1.3%, **Figure 4B-3**); ovaries in the other shrimp were in the primary vitellogenic stage, containing endogenous vitellogenic oocytes (GSI = 1.9%).

In the sgpG-dsRNA group, ovaries were mostly in the previtellogenic stage in all six shrimps at 10 days (GSI = 0.69%, **Figure 4C-1**), 20 days (GSI = 1.1%, **Figure 4C-2**), and 30 days (GSI = 0.73%, **Figure 4C-3**). In the sgpC-dsRNA group, ovaries were mostly in the previtellogenic stage in all six shrimps

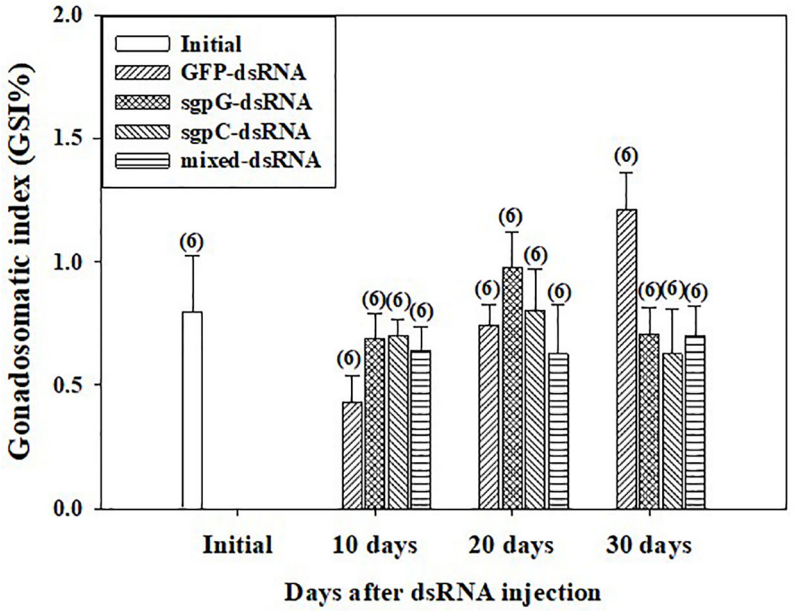

FIGURE 3 | Changes in gonadosomatic index (GSI) following a single injection of GFP-dsRNA, sgpG-dsRNA, sgpC-dsRNA, mixed-dsRNA, or no injection (initial). Results are expressed as the mean ± SEM. Values in parentheses indicate numbers of shrimp analyzed in each group. The content of each treatment group is indicated in the legend box that appears above the bar graph.

at 10 days (GSI = 0.64%, **Figure 4D-1**), at 20 days (GSI = 0.82%, **Figure 4D-2**), one of which also possessed a few early-stage endogenous vitellogenic oocytes, and in all six shrimps at 30 days (GSI = 0.65%, **Figure 4D-3**). In the mixed-dsRNA group, ovaries were mostly in the previtellogenesis stage in all six shrimps at 10 days (GSI = 0.64%, **Figure 4E-1**), at 20 days (GSI = 0.77%, **Figure 4E-2**), one of which also had a few early-stage endogenous vitellogenic oocytes, and in all six shrimps at 30 days (GSI = 0.62%, **Figure 4E-3**).

Expression Levels of *Liv-SGP-A*, *-B*, and *-C* Following Double-Stranded RNA Injections

The expression of *Liv-SGP-A*, *-B*, and *-C* was low or not detectable in the eyestalks of subadults (**Figure 5**). In experimental animals 10 days after dsRNA treatment (**Figure 5A**), *Liv-SGP-A* expression were relatively high in the GFP-dsRNA group; however, it was low or not detectable in the other dsRNA treatment groups. Regarding *Liv-SGP-B* expression, levels were relatively higher in the GFP-dsRNA and sgpC-dsRNA groups than in the sgpG-dsRNA and mixed-dsRNA groups. Regarding *Liv-SGP-C* expression, levels were relatively high in the GFP-dsRNA group compared with other dsRNA treatment groups. In experimental animals 20 days after dsRNA treatment (**Figure 5B**), expression levels of *Liv-SGP-A* were relatively high in the GFP-dsRNA group; however, they were low or not detectable in the other dsRNA treatment groups. Regarding *Liv-SGP-B* expression, relatively higher levels were observed in the sgpC-dsRNA group compared to the GFP-dsRNA group (treated as baseline data in this experiment) (**Figure 5B**). Regarding *Liv-SGP-C* expression, levels were relatively high in the GFP-dsRNA group and in the mixed-dsRNA group. In experimental animals 30 days after dsRNA treatment (**Figure 5C**), the expression of *Liv-SGP-A*, and *-C* was relatively high in the GFP-dsRNA group and somewhat high in the sgpG-dsRNA group; however, they were low or not detectable in all other groups. Regarding *Liv-SGP-B* expression, levels were low or not detectable in all dsRNA-treated groups. In this way, *Liv-SGP-A* and *-C* exhibited a similar expression profile, which was to be expected as their corresponding peptides possess a high degree of similarity in terms of amino acid sequence. In general, expression levels of *Liv-SGP-C* were relatively high compared to those of *Liv-SGP-A* and *-B*.

DISCUSSION

The use of RNAi to silence hormonal transcripts of factors that negatively regulate reproduction offers an opportunity to develop alternatives to eyestalk ablation in shrimp. This is because it is generally known that vitellogenesis-inhibiting hormone (VIH) is produced in the eyestalks and the removal of the source of VIH by eyestalk ablation allows ovarian maturation to occur. In previous work of this laboratory, several sinus gland peptides (SGP) were purified from the eyestalks of subadult *Litopenaeus vannamei*, and six of these were shown to inhibit vitellogenin (VG) gene expression levels using of *Marsupenaeus japonicus* ovary as the *in vitro* incubation system (7). Thereafter, the full-length cDNA of the predominant subtype I VIH in *L. vannamei* was reported, and the ability of recombinant Liv-SGP-G to

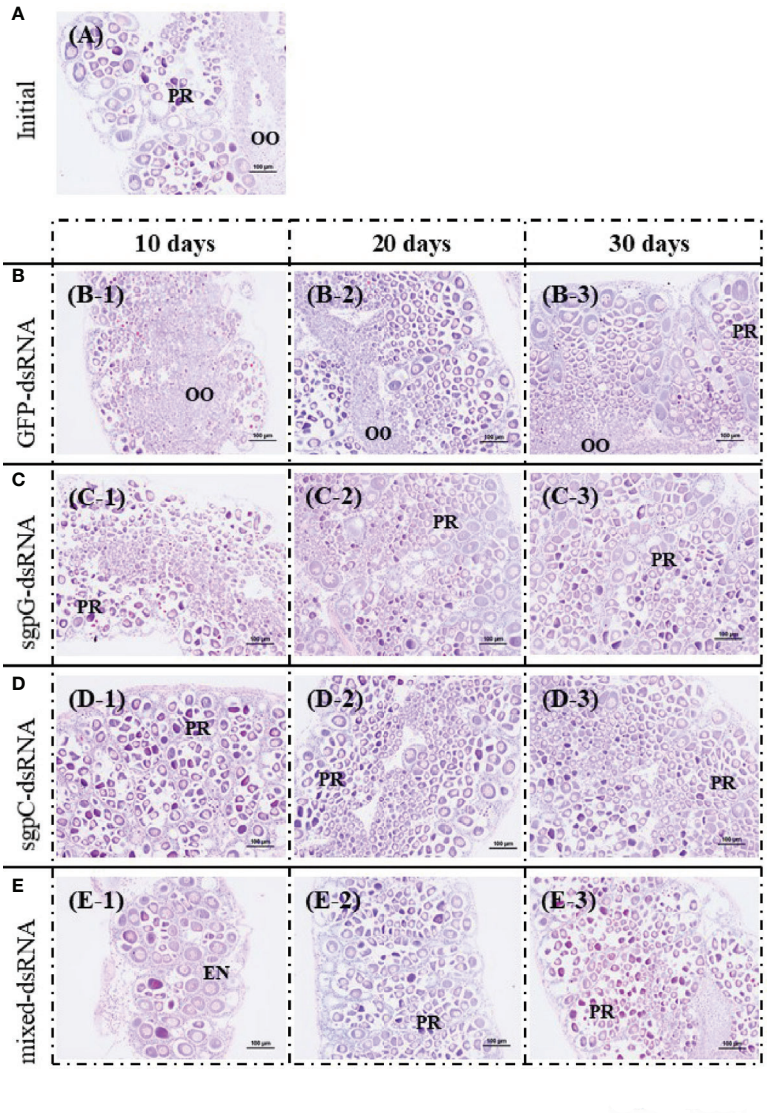

FIGURE 4 | Histological images of oocyte development from representative individuals exhibiting a GSI close to the mean of each group, stained with hematoxylin and eosin. **(A)** Initial (no injection); A, gonadosomatic index (GSI) = 0.94%. **(B)** GFP-dsRNA; B-1, 10 days, GSI = 0.33%; B-2, 20 days, GSI = 0.70%; B-3, 30 days, GSI = 1.3%. **(C)** sgpG-dsRNA; C-1, 10 days, GSI = 0.69%; C-2, 20 days, GSI = 1.1%; C-3, 30 days, GSI = 0.73%. **(D)** sgpC-dsRNA; D-1, 10 days, GSI = 0.64%; D-2, 20 days, GSI = 0.82%; D-3, 30 days, GSI = 0.65%. **(E)** Mixed-dsRNA; E-1, 10 days, GSI = 0.64%; E-2, 20 days, GSI = 0.77%; E-3, 30 days, GSI = 0.62%. Oocyte developmental features: OO, oogonium; PR, previtellogenic oocytes; EN, endogenous vitellogenic oocytes. Bars = 100 μm.

suppress *vg* expression *in vitro* was demonstrated (17). Thus following, we reported genetic information for all VIH subtype I peptides (Liv-SGP-A, -B, -C, -F, and -G) and the dynamics of their transcription in relation to molting and eyestalk ablation (18). As shown in **Figure 6A**, the molecular structure for VIH subtype I peptides consists of a signal peptide, CHH precursor-related peptide (CPRP), and mature peptide. All VIH subtype I peptides are cleaved at a conserved cleavage site (Lys-Arg) yielding a 74-residue mature peptide having a C-terminal amidation site (Gly-Lys). All mature VIH peptides, which were used as the target region for the preparation of dsRNA synthesis in this study, showed high similarity in terms of deduced amino acid sequence; *Liv-SGP-A*, *-B*, *-C*, and *-F* showed 64, 56, 71, and 74% similarity with *Liv-SGP-G*, respectively (**Figure 6A**). Since these peptides are perceived as isoforms that exhibit the same biological functioning, it is possible that they act in a coordinated fashion, although in terms of their endogenous action, it is unclear whether multiple VIHs act alternately or simultaneously in suppressing ovarian maturation. It is also unclear as to why multiple forms of VIH subtype I exist simultaneously, but if they do have coordinated functioning, it remains necessary to investigate their overall effects on the regulation of ovarian maturation. In this regard, the current study is the first report in shrimp in which transcriptional

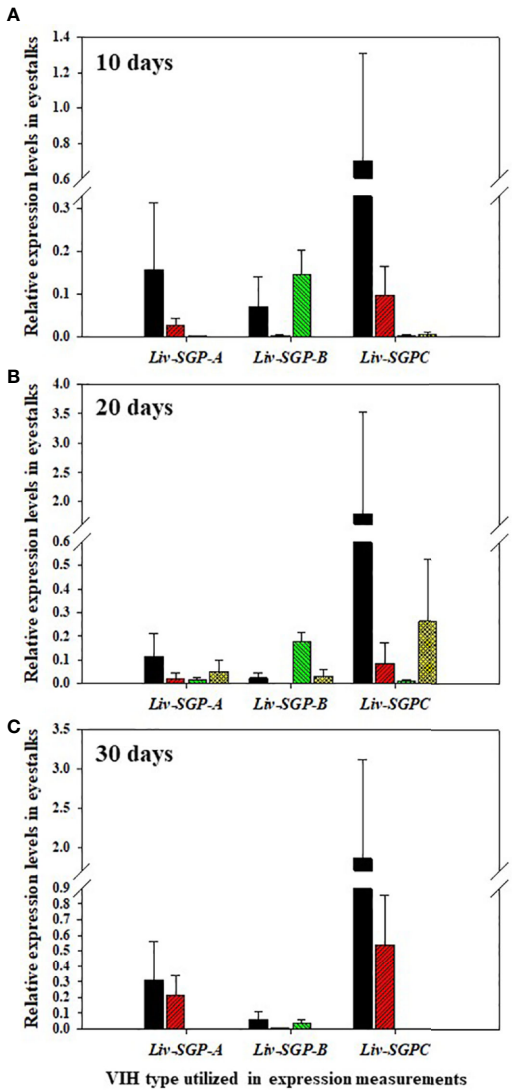

FIGURE 5 | Relative expression levels of *Liv-SGP-A*, *-B*, and *-C* in eyestalks. **(A)** 10 days following a single injection of GFP-dsRNA (n = 5), sgpG-dsRNA (n = 6), sgpC-dsRNA (n = 6), or mixed-dsRNA (n = 6). **(B)** 20 days following a single injection of GFP-dsRNA (n = 6), sgpG-dsRNA (n = 6), sgpC-dsRNA (n = 6), or mixed-dsRNA (n = 5). **(C)** 30 days following a single injection of GFP-dsRNA (n = 4), sgpG-dsRNA (n = 6), sgpC-dsRNA (n = 4), or mixed-dsRNA (n = 4). Results are expressed as the mean ± SEM. Results for each treatment groups are expressed in the bar graphs using the follows colors. Black: GFP-dsRNA group; red: sgpG-dsRNA; green: sgpC-dsRNA; yellow: mixed-dsRNA.

silencing of a major VIH could be silenced by the administration of dsRNA corresponding to multiple VIH genes.

In this study, a single injection to subadults of sgpG-dsRNA (for *Liv-SGP-G*) silenced the transcription of *Liv-SGP-G* in the eyestalks for at least 30 days. Although we did not include treatment groups for eyestalk ablation and a vehicle control, the observance of positive effects in eyestalk-ablated animals is a well-documented fact (for example, see previously cited studies 19–20); negligible effects can be inferred from the results of the GFP-dsRNA group, the treatment for which was prepared using the same vehicle buffer. As expected, eyestalk ablation induces ovarian maturation and *vg* mRNA expression in subadult females (19, 20), but administration of sgpG-dsRNA did not increase *vg* mRNA expression in this investigation. Injection with multiple dsRNAs corresponding to other VIH subtype I peptides (Liv-SGP-A, –B, –C, –F) also did not promote ovarian maturation. Interestingly, however, the administration of mixed-dsRNAs (for *Liv-SGP-A*, *–B*, and *–F*) significantly reduced the expression of *Liv-SGP-G* (**Figure 1**). These results show for the first time that administration of dsRNAs can inhibit transcription not only of their specific target genes, but also of homologous genes in shrimp. Perhaps the usage of relatively longer dsRNA in RNAi may achieve the co-transcriptional repression of the specific gene and additionally other homologous genes having high similarity to the basic dsRNA sequences. The possibility of co-suppression by dsRNA was first suggested in the nematode, *Caenorhabditis elegans* (22). Evidence of such a phenomenon was reported in arthropods, specifically in insects [for example, the African malaria mosquito (23)], and as well was observed in shrimp as detailed in this study. Transcriptional silencing generally occurs through long-dsRNA (>200 bp), which is processed into short interfering RNAs (siRNA of 20–30 bp) by endogenous Dicer (an RNase-III-like enzyme) in cells; the resultant siRNA represses transcription of the target gene (24, 25). Therefore, the endogenously produced siRNA may disrupt the expression of homologous genes with short-matched sequences. In *L. vannamei*, the primary structure and encoded sequences have high similarity among the various VIH subtype I peptides; the deduced amino acid sequence of *Liv-SGP-G* has high identity with the other SGP's as described above. Moreover, CHH-family peptides in general harbor similarity that reflects taxonomic classification; thus, *Penaeus japonicus* and *L. vannamei* show a high degree of similarity, and both species possess several isoforms of VIH subtype I peptide (6, 7). In this way, cross-species effects on VIH activity have been revealed, as Liv-SGP-G peptide inhibited *vg* mRNA expression in the ovary of *P. japonicus* (7). With this in mind, the engineering of a synthetic universal siRNA that targets multiple VIHs in several shrimp species could lead to the development of technology that is valid for implementing artificial maturation in a suite of penaeid shrimp species as a future alternative to eyestalk ablation.

With the above in mind, in this study, we focused on the effects of multiple dsRNA administration on the expression of *Liv-SGP-G*, which is predominant in the experimental species, *L. vannamei*. According to our previous study (18), among the VIH subtype I peptides originating from the eyestalks, corresponding gene expression levels were highest for *Liv-SGP-G* and moderate for *Liv-SGP-C*. On the other hand, the expression of *Liv-SGP-A*, *-B*, and *-F* was low or not detectable throughout molt cycle in adults (of note: *Liv-SGP-E* has been shown to correspond to a peptide identical to Liv-SGP-G, and the peptide translated from

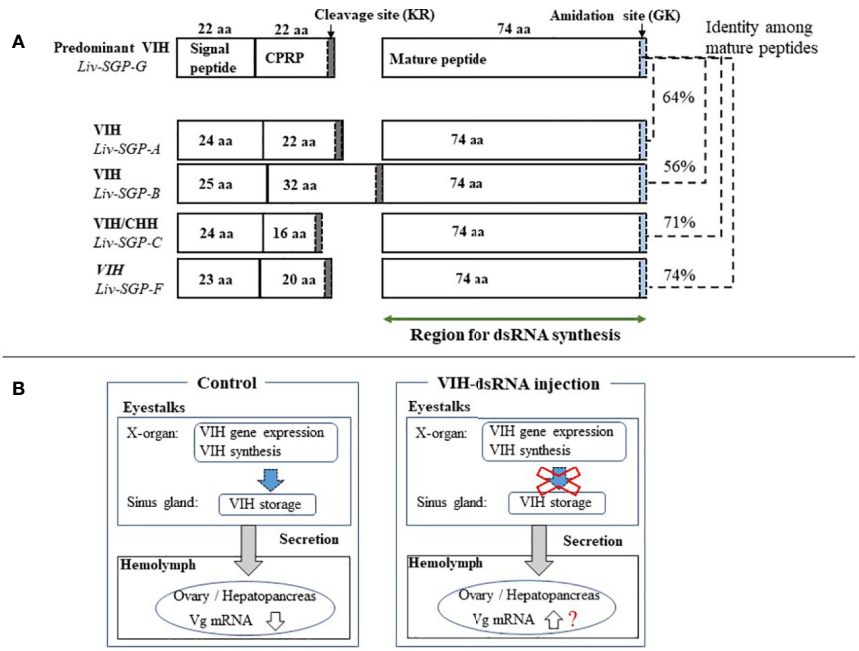

FIGURE 6 | **(A)** Schematic representation of the molecular structure of VIH subtype I peptides in *L. vannamei*. Genetic information for all VIH subtype I peptides (*Liv-SGP-A*, *-B*, *-C*, *-F*, and *-G*) from our previous study was used to construct the drawing (18). The number of amino acid residues (aa) for each corresponding region is shown within each box or above the box in the case of *Liv-SGP-G*. Respective parts are shown as signal peptides, CPRP (CHH precursor-related peptide), and mature peptides. Cleavage sites are indicated by gray shading. Amidation sites are indicated by blue shading. The target region for dsRNA synthesis is indicated by a green arrow. Identity between amino acid sequences corresponding to mature peptide are shown in terms of percentage. **(B)** Schematic representation of VIH regulation on ovarian maturation. VIH that is synthesized and secreted from the eyestalks is thought to fluctuate, thus alternatively suppressing ovarian maturation and allowing it to proceed in nature. Therefore, the suppression of VIH synthesis could be artificially lifted by transcriptional silencing, thus promoting ovarian maturation; however, the knockdown of *vih* transcription alone cannot induce oocyte maturation.

Liv-SGP-D does not possess VIH activity; hence, there were not examined further) (18). Although expression levels of most of the VIH genes other than *Liv-SGP-G* were low or not detectable in the eyestalks of subadults, their expression profiles yielded useful information on the effects of multiple dsRNA injection as shown in **Figure 5**. These results have revealed that dsRNA corresponding genes other than *Liv-SGP-G*, can also cause the knockdown of their respective genes, and that some of these can also inhibit the transcription of homologous genes (see the **Table 2** for these results represented schematically). In this way, the administration of multiple dsRNA administration is an efficient means of inhibiting a range of VIH expression. These results are expected to be scalable to use in adult animals, and will serve as an important basis for the further development of technology to control reproduction in an artificial environment.

Previously, the administration of dsRNA with the aim of promoting ovarian maturation, has been used in two of the world's most important farmed species, *Penaeus monodon* and *L. vannamei* (11, 14–16). In *P. monodon*, a single injection into adult females with GIH-dsRNA at 3 μg g^{-1} body weight knocked down GIH (subtype II) transcription for at least 30 days (11). Nevertheless, it did not promote an increased rate of spawning to the extent that eyestalk ablation did, although it did seem to have some efficacy in wild females as opposed to in domesticated females (14). Results were similar in a study by Feijo et al. (15) using subtype II VIH adult female *L. vannamei*; a single injection with GIH-dsRNA at 2.8 μg g^{-1} body weight knocked down GIH for 37 days, but females did not spawn, and ovarian maturation and increasing *vg* mRNA expression were observed only at 37 days (15). In our previous study, injection with VIH-dsRNA (for *Liv-SGP-G*) at 3 μg g^{-1} body weight suppressed transcription, but did not promote ovarian maturation or increases in endogenous Vg gene expression or concentration even after 20 days (16). Previous work by these and other authors suggests that RNAi could offer an alternative to eyestalk ablation, but with less stimulatory effect than eyestalk ablation. In other words, results obtained thus far have revealed that the knockdown of *vih* transcription alone cannot induce oocyte maturation and spawning in the same manner as eyestalk ablation. Thus, this suggests to us that VIH after being synthesized, is collectively retained in the sinus glands; even after gene expression is suppressed by the administration of dsRNA VIH likely remains in the eyestalks, and can still be secreted into the hemolymph for some time, thereby continuously suppressing ovarian maturation. As we have illustrated in **Figure 6B**, although we believe that endogenous VIH that is synthesized and secreted from the eyestalks fluctuates to thus alternatively suppress ovarian maturation and allow it to proceed in nature, this does not necessarily occur in an artificial environment. Therefore, the suppression of VIH synthesis could be

TABLE 2 | Schematic representation of the effects of dsRNA administration on the expression levels of *Liv-SGP-A*, *-B*, and *-C* in eyestalks of subadult individuals.

dsRNA treatment type	Levels of Liv-SGP-A			Levels of Liv-SGP-B			Levels of Liv-SGP-C		
	10 days	20 days	30 days	10 days	20 days	30 days	10 days	20 days	30 days
GFP									
sgpG									
sgpC									
mixed									

Color for each treatment group corresponds with that utilized in ***Figure 5****. Levels of gene expression for each group are shown with the following symbols. GFP-dsRNA group (basal levels): black rectangles; decreased expression: downward-facing solid arrows; increased expression: upward-facing shaded arrow (red, green or yellow rectangle: no change from basal levels).*

artificially lifted by transcriptional silencing, thus promoting ovarian maturation in a hatchery situation, for example. Perhaps the blocking of actual VIH secretion is also necessary in order to release the suppression of ovarian maturation. Nevertheless, it is still not well-understood how multiple VIHs act to control ovarian maturation, and how one might change their threshold levels artificially. Considering the results obtained thus far, the regulation of ovarian maturation is complicated and is based on the actions of many factors, not only on that of VIH. One of these factors is considered to be the putative vitellogenesis-stimulating hormone (VSH). Although the identity of VSH in shrimp is not clear, various hormonal factors seem to be able to stimulate ovarian maturation (2, 26–29). Hence, the use of RNAi combined with the application of various stimulatory factors may eventually lead to the goal of being able to accelerate ovarian maturation. In *L. vannamei*, injection of synthetic red pigment-concentrating hormone (27), serotonin (2), or serotonin/spiperone (26) can stimulate ovarian maturation, but several injections are required. However, fully matured ovaries that are ready for spawning were not obtained in such studies; this is likely due to the presence of endogenous VIH remaining in the eyestalks. We believe that simultaneous *vih* knockdown and hormonal stimulation may be a means of accelerating ovarian maturation in shrimp. In addition, the engineering of more of potent siRNAs targeting multiple VIHs and the assessment of the effects of combined treatments on ovarian maturation should be investigated in subsequent research.

According to our results, it appeared as though GFP-dsRNA injection positively affected ovarian maturation, because Vg levels in hemolymph were increased at 30 days after the injection of GFP-dsRNA, but not of VIH-related-dsRNA. In actuality, high levels of hemolymph Vg were observed in two individuals after 30 days of the GFP-dsRNA injected group, but there was a large variation in standard error (**Figure 2B**). Perhaps increasing body weight could have caused individual differences in the maturation of subadult females. However, a significant increase in hemolymph Vg levels was shown only at 30 days after GFP-dsRNA injection. We found a similar result in adult female *L. vannamei* in our previous study; injection of adult females with sgpG-dsRNA knocked down *Liv-SGP-G*, but injection with GFP-dsRNA significantly increased GSI only 20 days after (16). Perhaps the surge of VIH in the hemolymph after the knockdown of *vih* transcription delayed ovarian maturation. These results also revealed that subadults have the ability to respond to transcriptional silencing in the same manner as adults. Although the observed levels were low and the effects on ovarian maturation insignificant, *vg* expression levels in the sgpG-dsRNA and sgpC-dsRNA groups were higher than in the GFP-dsRNA group at 20 days after injection. Perhaps this is the result of a *vih* knockdown effect appearing earlier in subadults than in adults. Often, it is the case that ovaries having a large number of immature oocytes will have a similar GSI compared with ovaries having a smaller number of developed oocytes. As in our previous study (19), subadult females that have undergone eyestalk ablation may exhibit developed oocytes even while showing low GSI values. In order to ascertain the effects of each dsRNA treatment on ovarian maturation, we considered it necessary to conduct histological examination of oocyte development as shown in **Figure 4**. Nevertheless, there were no large differences in oocyte development among the various treatments, but there did seem to be more early-stage endogenous vitellogenic oocytes in the mixed-dsRNA group at 10 days as shown in **Figure 4E-1**. On the other hand, RNAi techniques using dsRNA have been used to increase immunity in shrimps; injection of dsRNA for non-specific genes improved the survival of diseased shrimp affected with white spot syndrome virus (25, 30). In our case, injection of GFP-dsRNA may have eventually improved shrimp health conditions in *L. vannamei*. Although we focused only on ovarian maturation, further research focusing not only on ovarian maturation, but also on the immune system, will be valuable for understanding the physiological effects of dsRNA.

In conclusion, we investigated the dynamics of the expression of *Liv-SGP-G* in the eyestalks after injection with dsRNAs corresponding to VIH subtype I peptides (Liv-SGP-A, –B, –C, –F, and –G) and analyzed their effects on ovarian maturation. These results will help improve the current understanding of the biological functioning of VIH subtype I peptides, and in the future may lead to the development of alternatives to eyestalk ablation.

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 below: https://www.ncbi.nlm.nih.gov/genbank/, LC278950; https://www.ncbi.nlm.nih.gov/genbank/, LC278952; https://www.ncbi.nlm.nih.gov/genbank/, LC278953.

AUTHOR CONTRIBUTIONS

BJK and MNW designed the experiments. BJK and ZS performed the experiments and collected raw data. All authors contributed to the article and approved the submitted version.

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REVIEW
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Signaling Pathways That Regulate the Crustacean Molting Gland

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A pair of Y-organs (YOs) are the molting glands of decapod crustaceans. They synthesize and secrete steroid molting hormones (ecdysteroids) and their activity is controlled by external and internal signals. The YO transitions through four physiological states over the molt cycle, which are mediated by molt-inhibiting hormone (MIH; basal state), mechanistic Target of Rapamycin Complex 1 (mTORC1; activated state), Transforming Growth Factor-β (TGFβ)/Activin (committed state), and ecdysteroid (repressed state) signaling pathways. MIH, produced in the eyestalk X-organ/sinus gland complex, inhibits the synthesis of ecdysteroids. A model for MIH signaling is organized into a cAMP/Ca^{2+}-dependent triggering phase and a nitric oxide/cGMP-dependent summation phase, which maintains the YO in the basal state during intermolt. A reduction in MIH release triggers YO activation, which requires mTORC1-dependent protein synthesis, followed by mTORC1-dependent gene expression. TGFβ/Activin signaling is required for YO commitment in mid-premolt. The YO transcriptome has 878 unique contigs assigned to 23 KEGG signaling pathways, 478 of which are differentially expressed over the molt cycle. Ninety-nine contigs encode G protein-coupled receptors (GPCRs), 65 of which bind a variety of neuropeptides and biogenic amines. Among these are putative receptors for MIH/crustacean hyperglycemic hormone neuropeptides, corazonin, relaxin, serotonin, octopamine, dopamine, allatostatins, Bursicon, ecdysis-triggering hormone (ETH), CCHamide, FMRFamide, and proctolin. Contigs encoding receptor tyrosine kinase insulin-like receptor, epidermal growth factor (EGF) receptor, and fibroblast growth factor (FGF) receptor and ligands EGF and FGF suggest that the YO is positively regulated by insulin-like peptides and growth factors. Future research should focus on the interactions of signaling pathways that integrate physiological status with environmental cues for molt control.

Keywords: Y-organ, molting (control of), mTOR - mammalian Target of Rapamycin, ecdysteroid, neuropeptide, insulin, growth factor, G protein coupled receptor (GPCR)

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INTRODUCTION

The progression of decapod crustaceans through the molt cycle depends on ecdysteroids synthesized by the Y-organ [YO; reviewed in (1)]. The molt cycle is unidirectional, progressing from the intermolt stage through premolt, ecdysis, and postmolt stages to the next intermolt stage [reviewed in (2, 3)]. Molting encompasses the preparatory processes during the premolt stage,

culminating with the actual shedding of the exoskeleton (ecdysis), followed by restorative processes during the postmolt stage. Rising titers of ecdysteroids in the hemolymph initiate and coordinate premolt processes, such as synthesis of the new exoskeleton, degradation and resorption of the old exoskeleton, claw muscle atrophy, and limb regenerate growth (2, 4, 5). A precipitous drop in hemolymph ecdysteroids at the end of premolt triggers ecdysis (1). The low ecdysteroid titer during postmolt allows claw muscle growth and completion of exoskeleton synthesis and its calcification. Intermolt can last from weeks to years in adult decapods.

Molt stage transitions are determined by phenotypic changes in the activity and properties of the YO. In the intermolt stage (stage C_4), inhibitory neuropeptides produced in the X-organ/sinus gland complex, such as molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH), maintain the YO in the basal state (**Figure 1**). A proposed model for MIH signaling couples a cAMP/Ca^{2+}-dependent triggering phase with a NO/cGMP-dependent summation phase [reviewed in (2)]. The prolonged activation of a calmodulin-dependent NO synthase and NO-dependent guanylyl cyclase (GC-I) represses ecdysteroidogenesis between MIH pulses (2, 6–9). The decision to molt, or enter premolt, is determined by integration of environmental and physiological cues by the central nervous system that are not completely understood (10). A decrease in MIH release by the X-organ/sinus gland complex, which can be experimentally induced by eyestalk ablation (ESA), triggers YO activation and entry into early premolt (stage D_0) (2, 3, 11). Multiple limb autotomy (MLA) also induces molting, as limb regenerates only become functional appendages when extended at ecdysis (4, 12, 13). It is hypothesized that MLA-induced molting is mediated by a stimulatory factor, designated limb autotomy factor – anecdysis (LAF_{an}), produced by the developing limb buds (3). YO activation requires mechanistic Target of Rapamycin Complex 1 (mTORC1) activity, as rapamycin inhibits YO ecdysteroidogenesis *in vitro* and prevents YO activation *in vivo* (14, 15). mTORC1-dependent protein synthesis drives the initial increase in ecdysteroid synthesis by the YO. The activated YO remains sensitive to MIH, CHH, and other factors, giving the animal the flexibility to suspend or delay molting when conditions turn unfavorable (2).

A critical decision point occurs at the end of early premolt, when the animal becomes committed to molt. The transition of the YO from the activated to the committed state is mediated by transforming growth factor beta (TGFβ)/Activin signaling, as SB431542, an inhibitor of Activin receptor signal transduction, prevents progression of animals from early premolt to mid-premolt (stage D_1; **Figure 1**) (15). mTORC1 activity affects the mRNA levels of thousands of genes, including those in the mTORC1 and TGFβ/Activin signaling pathways (**Figure 1**) (16). An invertebrate Myostatin (Mstn)-like factor, first described in scallop and crustacean muscles (17–19), appears to be the ligand for the Activin receptor. It is highly expressed in the YO and its mRNA levels are highest in the activated YO (15, 20, 21). The committed YO increases ecdysteroid synthesis, resulting in increasing ecdysteroid titers in the hemolymph during mid-premolt and reaching a peak in ecdysteroid titer at the end of late premolt (1). The committed YO also becomes

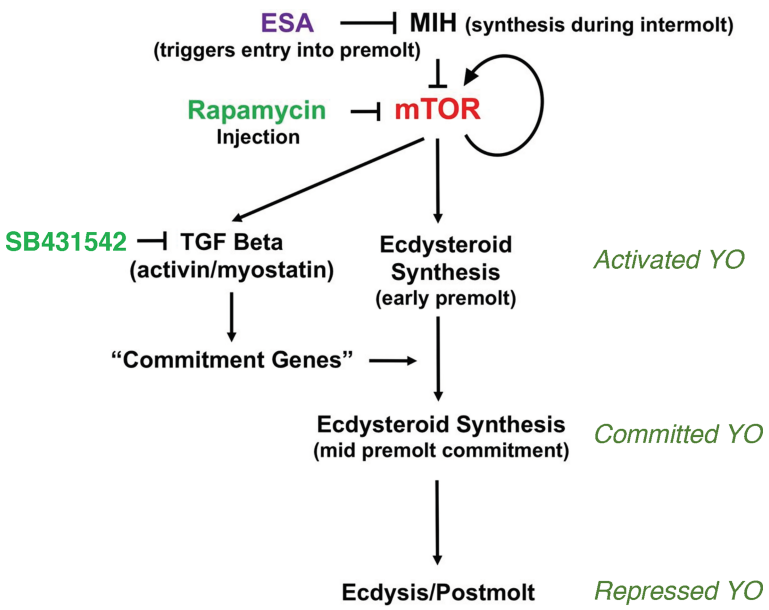

FIGURE 1 | Organization of the signaling pathways mediating YO phenotype transitions over the molt cycle. Cyclic nucleotide-mediated MIH signaling maintains the YO in the basal state by inhibiting mTOR signaling. Reduction in MIH, such as by eyestalk ablation (ESA), stimulates mTOR activity, which is inhibited by rapamycin. mTOR stimulates ecdysteroid synthesis and up-regulates mTOR and TGFβ/Activin signaling genes, and down-regulates MIH signaling genes. Activin/myostatin signaling, which is inhibited by SB431542, up-regulates mTOR signaling genes and controls expression of commitment genes that determine the committed phenotype. High ecdysteroid titers in late premolt may trigger the repressed phenotype in postmolt. From (2).

insensitive to MIH and CHH (2). Limb bud autotomy, which suspends molting processes in early premolt, is no longer effective in mid- and late premolt animals (2, 12, 13).

The signaling mechanisms controlling the transition of the committed YO to the repressed YO at the end of late premolt and the transition of the repressed YO to the basal YO at the end of postmolt are not well understood. It is hypothesized that the large peak in hemolymph ecdysteroid titer triggers the transition to the repressed phenotype (**Figure 1**), as the YO expresses the ecdysteroid receptor (EcR/RXR) and ecdysteroid-responsive genes (2). The repressed YO has low ecdysteroid synthetic activity, which results in low hemolymph ecdysteroid titers during postmolt (1). Most of the 478 differentially-expressed genes assigned to signal transduction pathways are down-regulated to their lowest levels during the postmolt stage (21). Among these are critical components of the MIH, mTORC1, and TGFβ/Activin signaling pathways (21). These data suggest that the YO is not inhibited by MIH during postmolt and that repression of the YO involves transcriptional regulation that prevents premature reactivation of the YO until exoskeleton synthesis and calcification are completed (2). The model assumes that normal MIH control is not restored until the YO returns to the basal state in intermolt.

Transcriptomics and proteomics have revolutionized crustacean physiology (22, 23). These approaches have shown that the YO undergoes molt stage-specific changes in phenotype that differ quantitatively and qualitatively in mRNA and protein levels (2, 16, 21, 24). mTORC1 activity plays a critical role in controlling ecdysteroid synthesis at the transcriptional and translational levels (2, 14, 16). Transcriptomics and proteomics can also be tools for discovery. Analysis of the MLA *Gecarcinus lateralis* YO transcriptome identified 878 unique contigs assigned to 23 KEGG signaling pathways, including those for MIH/CHH, mTOR, and TGFβ/Activin [**Table 1**; (21)]. The YO also expresses MAP kinase, AMP kinase, ErbB, Hedgehog, HIF-1, Jak-STAT, Hippo, NF-kappa B, Notch, TNF, and Wnt signaling pathway genes among others, raising the possibility that ecdysteroidogenesis is regulated by a great many factors (20, 21). Proteomic analysis has revealed that anti-radical oxygen species, cytoskeletal, vesicular secretion, immune response, protein homeostasis proteins contribute to *G. lateralis* YO function (24). This review presents the current knowledge of the signaling pathways that control ecdysteroid synthesis by the YO and identifies areas for future research. It includes relevant research on signaling mechanisms that control the insect prothoracic gland.

G PROTEIN-COUPLED RECEPTOR-MEDIATED SIGNALING

Transcriptomic analysis has revealed that a large number of G protein-coupled receptors (GPCRs) are expressed in decapod crustacean tissues. GPCRs are characterized by seven transmembrane domains, an external N-terminal domain, and a C-terminal cytosolic domain (25). These are divided among three large classes: rhodopsin-like (Class A), which represents the largest number of GPCRs; secretin-like (Class B); and metabotropic glutamate (Class C) (25–44).

All three GPCR classes are expressed in the YO, but represent a subset of those cataloged in decapod tissues (25, 29). In green shore crab *Carcinus maenas*, 62 contigs encoding GPCRs were identified in the central nervous system (29). The YO expresses 37 GPCRs annotated to 17 ligand clusters (**Table 2**). Thirty-two contigs are Class A and 5 contigs are in Class B; no Class C

TABLE 1 | Number of total and differentially expressed annotated contigs in the *G. lateralis* YO transciptome assigned to KEGG signal transduction pathways. From (21).

Signaling Pathway	Pathway ID	Number of annotated contigs	Number of DE contigs (percentage)
AMPK signaling pathway	k04152	86	53 (62%)
Calcium signaling pathway	k04020	92	49 (53%)
cAMP signaling pathway	k04024	104	65 (63%)
cGMP-PKG signaling pathway	k04022	94	51 (54%)
ErbB signaling pathway	k04012	48	24 (50%)
FoxO signaling pathway	k04068	79	46 (58%)
Hedgehog signaling pathway	k04340	36	21 (58%)
HIF-1 signaling pathway	k04066	50	26 (52%)
Hippo signaling pathway	k04390	95	52 (55%)
Jak-STAT signaling pathway	k04630	26	12 (46%)
MAPK signaling pathway	k04010	106	66 (62%)
mTOR signaling pathway	k04150	92	54 (59%)
NF-kappa B signaling pathway	k04064	33	16 (48%)
Notch signaling pathway	k04330	28	17 (61%)
Phosphatidylinositol signaling system	k04070	86	47 (55%)
Phospholipase D signaling pathway	k04072	83	50 (60%)
PI3K-Akt signaling pathway	k04151	128	69 (54%)
Rap1 signaling pathway	k04015	115	63 (55%)
Ras signaling pathway	k04014	105	57 (54%)
Sphingolipid signaling pathway	k04071	98	60 (61%)
TGF-beta signaling pathway	k04350	32	18 (56%)
TNF signaling pathway	k04668	40	23 (58%)
Wnt signaling pathway	k04310	79	51 (65%)

TABLE 2 | Classification and number of contigs encoding G protein-coupled receptors in the *Gecarcinus lateralis* and *Carcinus maenas* Y-organ transcriptomes.

Predicted receptor	*G. lateralis*	*C. maenas*
Class A (Rhodopsin-like)		
Adenosine	1	0
Allatostatins	3	4
CCAP	1	3
CCHamide	2	3
CHH/ITP	3	2
Corazonin	2	2
Dopamine	0	1
ETH	1	2
FMRFamide	2	1
HPR1	4	0
Leucine-rich repeats family		
Type A (GPA2/GPB5)	2	3
Type B (Lgr2; Bursicon)	1	3
Type C1 (Lgr3; Dilp8)	1	3
Type C2 (GRL101-like)	1	0
Moody	1	2
Myosuppressin	0	1
Octopamine	1	0
Peropsin	1	0
Proctolin	1	2
Prostaglandin	4	0
Serotonin (5-HT)	2	0
sNPF	1	1
Tre-1 (formerly TIE)	1	0
TRH	1	0
Class B (Secretin-like)		
DH31	1	4
DH41	1	0
Latrophilin	3	0
Lipoprotein	4	0
Methuselah	12	0
Parathyroid	1	0
PDF	3	1
Class C (Metabotropic glutamate)		
Boss	1	0
Mangetout	1	0
Metabotropic glutamate	1	0

CCAP, crustacean cardioactive peptide; CHH, crustacean hyperglycemic hormone; DH, diuretic hormone; ETH, ecdysis triggering hormone; HPR1, protein receptor in hepatopancreas 1; 5-HT, 5-hydroxytryptamine; ITP, ion transport peptide; PDF, pigment dispersing factor; sNPF, short neuropeptide F; Tre-1, trapped in endoderm-1; TRH, thyrotropin-releasing hormone. Data from (25, 29, 45).

contigs were identified in the *C. maenas* YO (**Table 2**) (29). Eleven GPCRs are enriched in the YO compared to the epidermis; these were identified as gonadotropin-releasing hormone receptor, tachykinin-like R86C, relaxin R1, two rhodopsin G0-coupled receptors, two methuselah-like R1, dopamine D2-like receptor, opsin UV-sensitive receptor, serotonin R4, and GPCR161 (29). In addition, seven GPCRs are differentially expressed over the molt cycle; these include short neuropeptide F, Bursicon R2, CHHa R1, relaxin R3, ITPR-like, Moody-like, and Ast-B/MIP-R1 (29). Deep high throughput RNA sequencing and *de novo* assembly of the intermolt *G. lateralis* YO identified 99 putative GPCRs, 65 of which were annotated to 32 ligand clusters (**Table 2**) (25). The ligands are mostly neuropeptides, but also include biogenic amines, such as dopamine, octopamine, and serotonin (**Table 2**). These data suggest that the YO can potentially respond to a wide variety of ligands. The possible roles of some of these GPCRs are discussed in the sections below.

Surprisingly, the YO expresses a variety of peptide hormones. In *C. maenas*, contigs encoding 19 full-length peptides were identified (29). The six peptides that are expressed at the highest levels are Neuroparsin-1, -3, and -4; CHH-1, inotocin/vasopressin, and Eclosion Hormone-2 (29). Further research should determine if the transcripts are translated into peptides and the peptides are secreted into the hemolymph. If so, it would provide compelling evidence that the YO has endocrine functions beyond that of ecdysteroid production.

Putative G Protein-Couple Receptors for CHH Family Neuropeptides

Peptides in the CHH family are divided into two types that differ in the amino acid sequences of the precursor proteins. Members of this family, which includes insect ion transport peptide (ITP), have a 66-amino acid "CHH family motif" in the mature peptide with six conserved cysteines that form three intramolecular disulfide bridges to stabilize the structure of the native protein (46–50). Type I peptides, which include CHH and ITP, are characterized by a signal peptide sequence followed by CHH/ITP precursor-related peptide (CPRP) and mature peptide sequences. Type II peptides, which include MIH, gonad-inhibiting hormone (GIH), and mandibular organ-inhibiting hormone (MOIH), lack a precursor-related sequence and have a glycine inserted between residues #11 and #12 and an invariant valine at position #20 in the mature peptide (46). Recently, a comprehensive phylogenetic analysis of crustacean ITPs proposed that ITPs be assigned to a third group (Type III) distinct from Type I CHHs (48, 50). The solution structures of MIH and CHH are similar, except that a short alpha-1 helix at positions #10 through #13 in MIH is lacking in CHH (49, 51). It is thought that the Gly12 contributes to the formation of the alpha-1 helix in Type II peptides (51). The surface structures of the N- and C-terminal regions confer specificity of binding to distinct receptors in the YO membrane (47, 49, 51–54).

The identification and characterization of the MIH receptor has remained elusive for more than three decades (46, 55). It is hypothesized that the receptors for the CHH family are GPCRs, given the similar native structures of Type I and Type II peptides (49, 51) and signal transduction mediated by cyclic nucleotide second messengers (56). In insects, studies of silk moth GPCRs (*Bombyx* neuropeptide G-protein coupled receptors, or BNGRs) identified BNGR-A2 and -A34 as ITP receptors, and BNGR-A24 is an ITP-like receptor (57). Based on this discovery, two full-length contigs (Pc-GPCRA52 and A53) and one partial contig (Pc-GPCRA63) from the transcriptome of adult *Procambarus clarkii* were identified as putative CHH-like receptors (CHHRs) (58). Subsequently, CHHR orthologs from more than ten other decapod crustacean species have been identified (25, 27, 29).

Three putative CHHRs, designated Gl-GPCR-A9, -A10, and -A12, are expressed in the *G. lateralis* YO transcriptome (25). Two CHH/ITP-like receptors were identified in the *C. maenas*

YO transcriptome (29). Further phylogenetic analysis showed that the arthropod CHH/ITP GPCRs formed three clusters, designated CHHR1, CHHR2, and CHHR3/ITP-like R/tachykinin R (**Figure 2**). As Gl-GPCR-A9 and -A10 were grouped in the CHHR1 cluster, they are renamed Gl-CHHR-1A and -1B, respectively; Gl-GPCR-A12 is renamed Gl-CHHR-2 (**Figure 2**). Modeling the 3-dimensional structures of the *G. lateralis* CHHRs gave a highly conserved outcome of a predicted cleft at the N-terminus, suggesting a common role in binding CHH family hormones (**Figure 2**). End-point PCR showed that neither *Gl-CHHR-1A* or *-2* are exclusively expressed in the YO, as would be expected for the MIH receptor; *Gl-CHHR-1B* was not examined (25). Interestingly, the YO is the only tissue to express both CHHRs. Eyestalk ganglia, thoracic ganglion, gill, heart, and midgut only express *Gl-CHHR-1A*; testis, hindgut, and hepatopancreas only express *Gl-CHHR-2*; and claw muscle does not express either CHHR (25). The three *G. lateralis* CHHRs are differentially expressed in the YO over the molt cycle, which suggests altered sensitivities to CHH neuropeptides associated with YO phenotypic changes. *Gl-CHHR-1A* has higher expression in intermolt and decreases during premolt stages; *Gl-CHHR-1B* is expressed during intermolt with higher expression during premolt stages; and *Gl-CHHR-2* is expressed at high levels at late premolt (25). None of the *Gl-CHHRs* are expressed during postmolt (25). One of the CHH/ITP-like receptors in the *C. maenas* YO is differentially expressed over the molt cycle, with highest expression in late premolt (29). Future research must use a functional assay to establish which, if any, of the GPCR candidates is the MIH receptor.

Corazonin Receptor

Corazonin (CRZ) is a conserved 11- amino acid neuropeptide with an amidated C-terminus and pGlu at the N-terminus. The sequence (pQTFQYSRGWTNa) is highly conserved among insect and crustacean species, although variants with single amino acid substitutions occur in some insects (59, 60). In *Drosophila melanogaster*, CRZ neurons modulate prothoracicotropic hormone (PTTH) action on basal ecdysteroidogenesis by the prothoracic gland (PG), thus controlling larval growth without affecting metamorphosis (61). *C. maenas* corazonin receptor (*Cm-CRZR*) is primarily expressed in the YO, suggesting that CRZ plays a role in regulating ecdysteroidogenesis (45). However, CRZ peptide (50 nM), which is produced in the eyestalk ganglia and other areas of the central nervous system, has only a small stimulatory effect on YO ecdysteroidogenesis in postmolt (stages A-B) *C. maenas* (45).

Leucine-Rich Repeat Receptor and Insulin-Like Peptides

Tissue loss or injury delays molting, allowing time for regeneration or regrowth of tissues or organs prior to the next ecdysis. Molting delay by limb bud autotomy (LBA) in crustaceans and injury to imaginal discs in insects allows time for tissue regeneration, while growth of remaining or undamaged

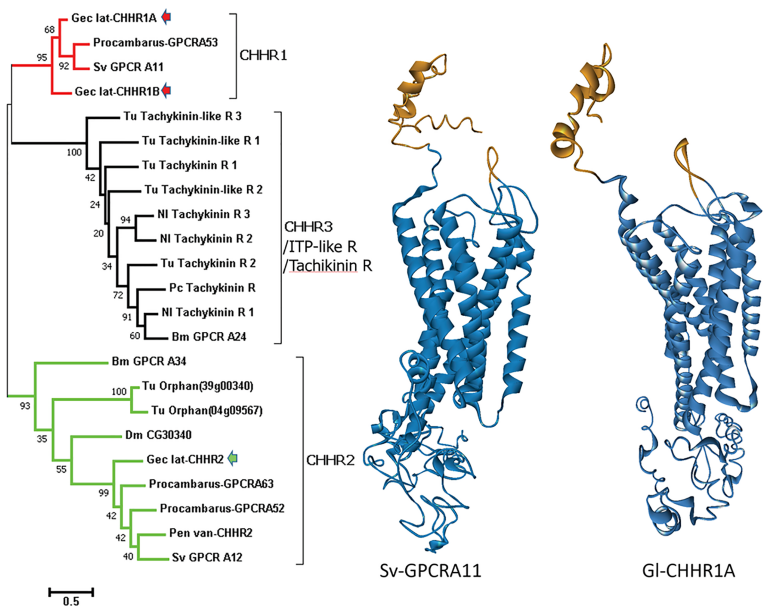

FIGURE 2 | Comparison of decapod putative CHH and insect and mite ITP-like/tachykinin GPCRs. Left: A phylogram (left) of arthropod neuropeptide GPCRs showing three clusters designated CHHR1 (red), CHHR2 (green), and CHHR3/ITP-like R/tachykinin R (black). *G. lateralis* (Gec_lat) CHHR1A and 1B, originally designated Gl-GPCR-A9 and -A10, respectively (25), are within the CHHR1 cluster (red arrows) and CHHR2, originally designated Gl-GPCR-A12 (25), is within the CHHR2 cluster (green arrow). Species: Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Nl, *Nilaparvata lugens*; Pen van, *Penaeus* (*Litopenaeus*) *vannamei*; Procambarus, *P. clarkii*; Sv, *S. verreauxi*; and Tu, *Tetranychus urticae* (red spider mite). Right: Structural models comparing the putative CHHR1 proteins from two decapod species predicted by GPCR-I-TASSER (27): *S. verreauxi* (Sv-GPCRA11) and *G. lateralis* Gl-CHHR1A. Gold indicates the N-terminal neuropeptide binding cleft in the extracellular domain. Blue indicates the transmembrane domain containing the 7 α-helices characteristic of GPCRs and the C-terminal intracellular domain. Figure provided by Dr. Tomer Ventura.

tissues slows or stops (12, 13, 62–66). In crustaceans, LBA during early premolt (stage D_0) suspends premolt two to three weeks by lowering hemolymph ecdysteroid titers, so that animals can regain a full set of functional claws and legs at ecdysis (13, 67). In *G. lateralis*, secondary LBs produce a factor, designated Limb Autotomy Factor – proedysis (LAF_{pro}), that lowers hemolymph ecdysteroid (3, 12, 13). In insects, regenerating imaginal discs produce a factor, identified as *Drosophila* insulin-like peptide 8 (Dilp8) in *D. melanogaster*, that delays metamorphosis by lowering ecdysteroid synthesis by the prothoracic gland (PG) (64, 68–70). Dilp8 also delays molting by activating Lgr3 neurons in the brain, which inhibit PTTH synthesis in PTTH neurons (71–74).

The LAF_{pro} signaling pathway has not been fully characterized, but parallels with the action of Dilp8 on the insect PG suggest a common mechanism. Dilp8 is one of eight insulin-like peptides (ILPs) in *D. melanogaster* (74–78). The ILP superfamily consists of insulin, insulin-like growth factors (IGFs), and relaxin-like peptides (38, 71). Dilp 1 to 6 are in the insulin/IGF clade and bind to receptor tyrosine kinase receptors; Dilp 7 and 8 are in the relaxin-like clade and bind to leucine-rich repeat GPCRs (71, 74, 75). LAF_{pro} is a peptide that is distinct from MIH. MIH is resistant to boiling in deionized water or weak acids (see (13) for references). LAF_{pro} is stable when boiled 15 min in deionized water, but is inactivated by boiling in 0.1 M acetic acid (pH 2.9) and by proteinase K digestion (13). Dilp8 action is mediated through relaxin receptor Lgr3 and activation of NOS (73, 74, 79). NO donors inhibit ecdysteroidogenesis in both insect and crustacean molting glands (80–82). Dilp8 binding to Lgr3 stimulates production of cAMP in *Drosophila* cells (71). Up-regulation of *Dilp8* delays expression of Halloween genes *disembodied* (*Dib*) and *phantom* (*Phm*) in the PG (68, 69). Moreover, targeted tissue damage or NOS overexpression in the PG lowers the expression of Halloween genes *spookier* (*Spok*) and *Dib* (79). These data suggest that Dilp8-induced NO inhibition of ecdysteroid synthesis is mediated by the down-regulation of cytochrome P450 enzymes, but it is unclear how NO represses gene transcription. A Lgr3-like GPCR is expressed in the *G. lateralis* and *C. maenas* YO transcriptomes (**Table 2**) (25, 29). An ILP2 is an ortholog of Dilp8 that is primarily expressed in nervous tissue, such as brain and eyestalk ganglia of Eastern spiny lobster (*Sagmariasus verreauxi*); brain and thoracic ganglion of red-claw crayfish (*Cherax quadricarinatus*); and in eyestalk ganglia, brain (males), thoracic ganglion (males), and sperm duct in ornate spiny lobster (*Panulirus ornatus*) (42, 83). It is not known if ILP2 is expressed in limb regenerates, and if there is higher expression in secondary than primary regenerates, as only 2° regenerates have LAF_{pro} activity (13). Taken together, these data suggest that LAF_{pro} is an Dilp8-like peptide that binds to Lgr3, activating the MIH signaling pathway to inhibit YO ecdysteroidogenesis (**Figure 3**).

Ca^{2+}/Diacylglycerol/Protein Kinase C Signaling

In the canonical pathway, ligand binding to a GPCR activates phospholipase C (PLC) *via* a G_q protein anchored in the cell membrane. PLC converts phosphatidylinositol to diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG and IP3-initiated release of Ca^{2+} from smooth endoplasmic reticulum activate protein kinase C (PKC), which phosphorylates downstream targets to effect metabolic changes. All these components are represented in the KEGG calcium and phosphatidylinositol signaling pathways (**Table 1**) (21). Moreover, the YO has PKC activity (85).

Activation of PKC stimulates ecdysteroid synthesis and secretion by the YO. Studies using pharmacological reagents show that the pathway activating PKC is distinct from the MIH signaling pathway (**Figure 3**). A DAG analog or phorbol 12-myristate 13-acetate (PMA) stimulate PKC activity and ecdysteroid secretion by the *Cancer antennarius* YO *in vitro* (85). PMA counters the inhibitory effects of reagents that stimulate MIH signaling, such as forskolin, dibutyryl cAMP, and cyclic nucleotide phosphodiesterase inhibitor IBMX, and has no effect on cAMP levels (85). By contrast, PMA has the opposite effect on crayfish YO by inhibiting ecdysteroid secretion (86). PLC inhibitor U-73122 has no effect on crab and crayfish YO ecdysteroid production *in vitro*, and there are no changes in IP3 and DAG contents of YOs from intact and eyestalk-ablated crabs (86), suggesting that PKC is not involved in YO activation in early premolt. The downstream targets of PKC are unknown. mTORC1 signaling is likely involved, as PMA stimulates protein synthesis in the YO (84, 85, 87). A potential target of PKC is Akt in the mTOR signaling pathway (**Figure 3**).

The ligand and GPCR for the PKC pathway have not been identified. An intriguing possibility is that PKC is activated by serotonin (5-hydroxytryptamine) and other biogenic amines. The YO expresses serotonin, dopamine, and octopamine GPCRs (**Table 2**) (25, 29). Serotonin, dopamine, and octopamine function as neurotransmitters and neuromodulators in the crustacean central nervous system, but they may also act as neurohormones (46, 60, 88–91). Interestingly, there is evidence that the YO can synthesize serotonin (92). Much of the research of biogenic amines functioning as neurohormones has been focused on their roles in regulating decapod reproduction. For example, serotonin stimulates ovarian maturation, whereas octopamine delays gonadal development and inhibits ovarian maturation (93–95). Serotonin stimulates YO ecdysteroid production *in vitro* in mud crab (*Scylla serrata*) (96). In insects, serotonergic neurons directly innervate the PG and stimulate ecdysteroidogenesis (97–99). Octopamine acts as an autocrine factor that enhances PG ecdysteroidogenesis (100), but its effect on YO ecdysteroidogenesis is unknown. In *C. maenas*, dopamine D2-like and 5-hydroxytryptamine receptor 4 are up-regulated in the YO relative to their levels in the epidermis, although the receptors are not differentially expressed in the YO over the molt cycle (29). In *G. lateralis* YO, two serotonin receptors, designated *Gl-GPCR-A30* and *-A32*, and an octopamine receptor, designated *Gl-GPCR-A34*, show different patterns of expression over the molt cycle (dopamine receptor was not identified in the *G. lateralis* YO transcriptome) (25). *Gl-GPCR-A30* shows higher expression in early premolt and no expression in postmolt animals, while *Gl-GPCR-32* is expressed in all five molt stages with higher expression in postmolt (25). *Gl-GPCR-A34* is expressed in all molt stages, with higher expression during premolt (25). These data suggest that serotonin and octopamine are

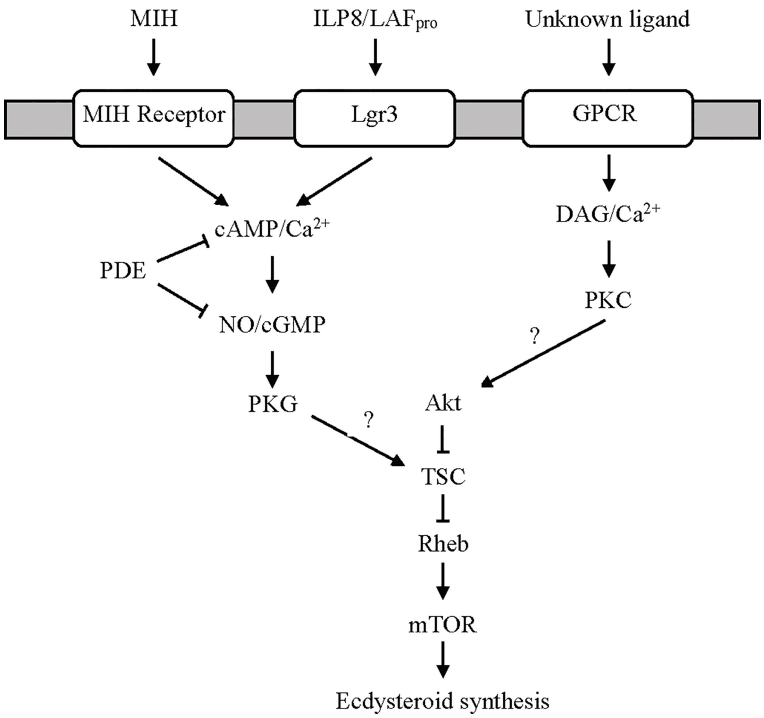

FIGURE 3 | Proposed G protein-coupled receptor-mediated signaling pathways regulating ecdysteroidogenesis in the YO. MIH and limb autotomy factor - proecdysis (LAF$_{pro}$) activate cyclic nucleotide/NO-dependent signaling *via* distinct receptors. It is hypothesized that LAF$_{pro}$, secreted by secondary limb regenerates during early premolt, is an insulin-like peptide similar to dILP8 in *Drosophila* that binds to Lgr3. Cyclic phosphodiesterase (PDE) activity inhibits MIH and LAF$_{pro}$ signaling by hydrolyzing cAMP and cGMP to AMP and GMP, respectively. An unknown ligand, possibly serotonin or other biogenic amines (see Ca2+/Diacylglycerol/ Protein Kinase C Signaling Section), binds a GPCR to activate the Ca^{2+}/diacylglycerol (DAG)/protein kinase C (PKC) pathway. Both pathways converge on mTOR signaling, possibly by phosphorylation of tuberous sclerosis complex (TSC) by protein kinase G (PKG) to inhibit ecdysteroid synthesis or by phosphorylation of Akt by PKC to stimulate ecdysteroid synthesis (2, 12, 50, 84).

tropic factors in the YO. However, the signaling pathways activated by biogenic amines differ between insects and crustaceans. In the PG, serotonin and octopamine increase cAMP (97, 98), while in the YO, serotonin and octopamine action may be mediated by Ca^{2+}/DAG (**Figure 3**). Future research should be directed to establishing the mode of action of biogenic amines on the YO.

RECEPTOR TYROSINE KINASES

The receptor tyrosine kinase (RTK) superfamily regulates animal development and homeostasis (101). There are about 58 RTKs in 20 subfamilies in mammals, with fewer in arthropods. *D. melanogaster*, for example, has 20 RTKs distributed among 14 subfamilies (76). RTKs have an extracellular ligand-binding domain, a single-pass transmembrane domain, and an intracellular tyrosine kinase domain (TKD). Most RTKs are heterodimers with each subunit consisting of a single polypeptide. By contrast, the insulin receptor is a heterotetramer, consisting of heterodimers of alpha and beta chains linked by disulfide bonds; the α-chain is completely extracellular and, together with the extracellular domain of the β-chain, binds ligand (101). Ligand binding activates RTK activity; autophosphorylation of the TKD activates MAPK, PI3K/Akt, PLCγ-PKC, JAK/STAT, or Rac-Rho signaling cascades (76, 101). The YO expresses both ILP receptors and growth factor receptors (**Table 3**).

Insulin-Like Peptide Receptor Signaling

In insects, ILPs are among the many factors that coordinate organismal growth and organ size and determine the timing of molting and metamorphosis (74, 78, 98, 102–105). A target of ILPs is the PG. Insulin-producing neurons in the brain secrete ILPs, in particular Dilp2, 3 and 5, that stimulate ecdysteroid production by the PG (74, 76, 102, 106). ILP/insulin-like protein receptor (InsR) signaling in the PG is mediated by PI3K/Akt/mTOR (74, 97, 106, 107). The role of ILP/InsR signaling in development and growth of crustaceans is not well understood, but it is likely that it has similar actions. Much of the research on crustacean ILP/InsR signaling has focused on reproduction (108, 109).

Insulin/ILPs are synthesized as a single polypeptide with an N-terminal signal peptide sequence, followed by B, C, and A chains (75, 77, 109). Proteolytic processing removes the signal peptide and excises the C chain, producing a B chain/A chain heterodimer stabilized by inter- and intra-chain disulfide bonds between conserved cysteines (75, 77, 109). In some ILPs, the C chain is not completely removed, producing a polypeptide, in

TABLE 3 | Tyrosine receptor kinases and ligands expressed in *G. lateralis* MLA or ESA Y-organ transcriptomes (16, 20, 21).

Identity	Contig ID	Transcriptome	ORF (aa)	Partial/Full Length	Top Hit	% Identity
EGF	c202604_g1_i1	ESA	197	Partial	*P. monodon*	60%
FGF	c189642_g1_i1	MLA	202	Full	*L. vannamei*	91%
EGFR	c267955_g1_i3	MLA	1491	Full	*C. opilio*	90%
InsR	contig_69766	MLA	1297	Full	*L. vannamei*	29%
FGFR	c219909_g2_i1	ESA	746	Full	*L. vannamei*	75%

Sequences were identified using a reciprocal BLAST and locating conserved domains with NCBI conserved domain search tool. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; InsR, insulin-like receptor β subunit; ORF, open reading frame (amino acids). Species: C. opilio, Chionoecetes opilio; L. vannamei, Litopenaeus vannamei; and P. monodon, Penaeus monodon.

which part of the C chain is retained (77). ILPs have been identified in decapod crustacean transcriptomes (28, 32, 33, 38, 83, 110–114). One of the best characterized ILP is the insulin-like androgenic gland hormone (IAG); it is expressed primarily in the androgenic gland and determines adult male characteristics (42, 83, 108, 109, 115, 116). In *Portunus trituberculatus*, *Pt-IAG* is expressed at very low levels in the YO (116). Other crustacean ILPs are expressed in most tissues, but at differing levels. In the oriental river prawn, *Macrobrachium nipponense*, *Mn-ILP* is expressed in brain, eyestalk ganglia, nerve cord, gonads, hepatopancreas, and muscle in adults (117). *Mn-ILP* expression is highest during the rapid growth stage in younger individuals and during the intermolt stage in older individuals (117). *Sv-ILP1* and *Cq-ILP1* are relaxin-like ILPs that are expressed in brain, antennal gland, gonads, and hepatopancreas (females only) (83, 113).

It is generally accepted that decapod crustaceans, like most invertebrates, express a single functional insulin receptor (InsR) (118). InsR has been biochemically characterized in gill, muscle, and hepatopancreas (119–121). InsR β-chain is expressed in many tissues, including the androgenic gland of male *Macrobrachium rosenbergii* (*Mr-IR*) (122), *Fenneropenaeus chinensis* (*Fc-IAGR*) (123), and *S. verreauxi* (*Sv-TKIR*) (124). Orthologs of *Mr-IR* have been identified in the neuropeptidomes of six other decapod species (33). Interestingly, silencing of *Mr-IR* led to androgenic gland hypertrophy and increased *Mr-IAG* production, but had no effect on somatic growth or sex determination, suggesting that molting and sexual differentiation are not solely dependent on the insulin receptor (122). *S. verreauxi tyrosine kinase insulin receptor* (*Sv-TKIR*), when expressed in a COS-7 cell reporting system, is activated by recombinant Sv-IAG and, to a lesser extent, recombinant human insulin, followed by recombinant Mr-IAG and Cq-IAG (124). In the YO, ESA results in a down-regulation of *G. lateralis-InsR*, which is blocked by rapamycin, suggesting that mTORC1 activity represses *Gl-InsR* expression (16).

Remarkably, there are no studies on the effects of insulin or ILPs on YO ecdysteroidogenesis. However, studies of other crustacean tissues indicate that insulin/ILP action is mediated through PI3K/Akt or MAPK/ERK signaling. Bovine insulin increases *Sp-vitellogenin (Sp-Vtg)* mRNA levels in hepatopanceas explants from *Scylla paramamosain* (125). Relatively high concentrations of bovine insulin were needed to elicit a response (>200 ng/ml). The insulin-induced increase in *Sp-Vtg* is blocked by PI3 kinase inhibitor (LY294002) and mTORC1 inhibitor rapamycin (125). Their respective recombinant IAGs increase MAPK/ERK phosphorylation in *M. rosenbergii*, *S. verreauxi*, and *Cherax quadricarinatus* testis explants *in vitro* (124). In insects, ILPs (e.g., Dilps1-6 in *D. melanogaster* and Bombyxin in *Bombyx mori*) stimulate PI3K/Akt signaling and mTORC1-dependent ecdysteroidogenesis in the insect PG (78, 97, 106, 107, 126–129). Based on these data, a model for ILP signaling in the YO is proposed (**Figure 4**). Binding of ILPs, such as ILP2, to InsR activates PI3K/Akt signaling, leading to mTOR activation and increased ecdysteroid synthesis. Alternatively, or perhaps in conjunction with PI3K/Akt signaling, ILP activates MAPK/ERK signaling (**Figure 4**). Both pathways converge on mTOR in animal cells (130, 131).

Growth Factor Receptor Signaling

Growth factor signaling is mediated by the Ras/Raf/MAPK and PI3K/PDK1/Akt pathways (**Figure 4**) (132). In insects, growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor, (PDGF) and vascular endothelial growth factor (VEGF), serve many functions critical for embryogenesis, molting, and metamorphosis (76). Activation of the Ras/Raf/MAPK pathway stimulates ecdysteroidogenesis in the insect PG (97, 106). Although not a growth factor, prothoracicotropic hormone (PTTH) activates this pathway by binding to an RTK encoded by *Torso* (97, 98, 102, 106, 133). PTTH is the primary factor that initiates larval molts in most insects. However, a recent study has shown that EGF receptor (EGFR) signaling supports PG ecdysteroidogenesis during the 3rd larva to pupa transition in *D. melanogaster* (134). The function of growth factors in YO ecdysteroidogenesis is unknown. Growth factor signaling pathways are well represented in the YO. These include the ErbB, MAPK, PI3K/Akt, and Ras KEGG signaling pathways (**Table 1**). Contigs can be assigned to two or more of the KEGG pathways (**Figure 4**) (21). The *G. lateralis* YO transcriptome has contigs encoding 106 MAPK signaling components, 66 of which are differentially expressed over the molt cycle (**Table 1**) (21). Forty-eight contigs assigned to the ErbB pathway, which includes EGFR signaling, have been identified (**Table 1**).

Knowledge of growth factors and their functions in crustaceans is limited. Unfortunately, there are no studies determining the effects of EGF, FGF, or VEGF on YO ecdysteroidogenesis. Immunohistochemical analysis indicated that eyestalk ganglia express a VEGF-like protein and VEGF receptor (135). The broad scale application of transcriptomics has aided the identification of growth factors and their receptors in decapod crustacean tissues. VEGFR is expressed in the

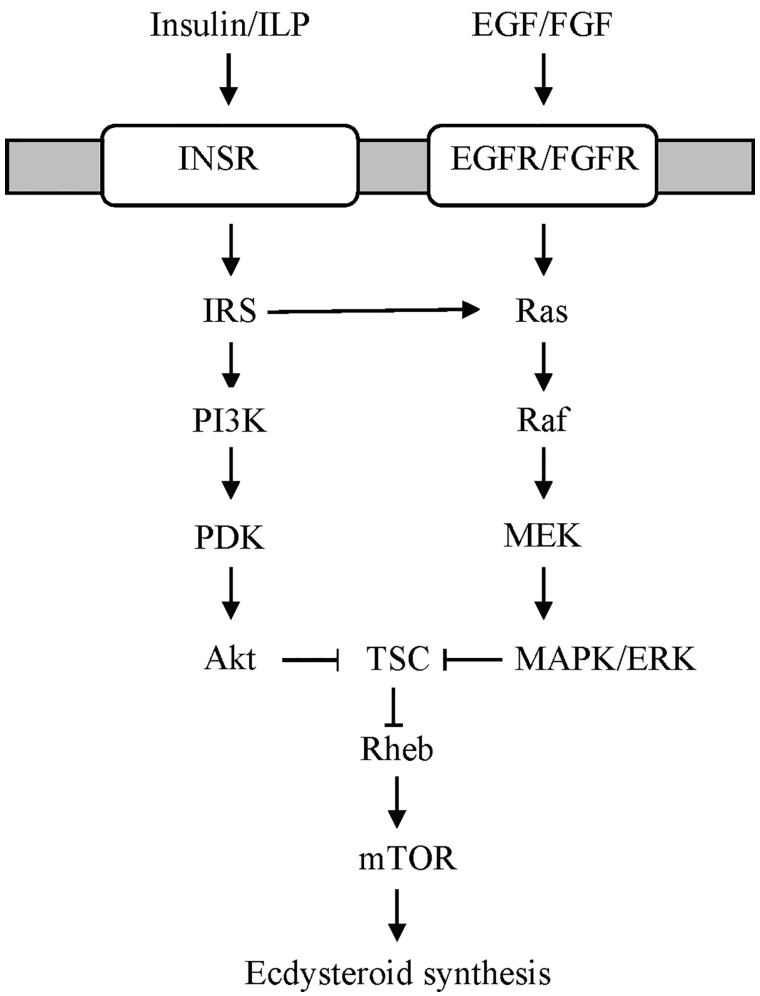

FIGURE 4 | Proposed receptor tyrosine kinase (RTK)-mediated signaling pathways stimulating ecdysteroidogenesis in the YO based on data from the insect prothoracic gland (97, 98). YOs express both types of RTKs (**Table 3**). Growth factors, such as epidermal growth factor (EGF) or fibroblast growth factor (FGF), bind to EGF or FGF receptors, respectively, to activate the Ras/Raf/MEK/ERK signaling pathway. Insulin/insulin-like peptide (ILP) binds to insulin receptor (INSR) to activate PI3K/PDK/Akt and/or Ras/Raf/MEK/ERK signaling. Both pathways converge on mTOR signaling by phosphorylation of tuberous sclerosis complex (TSC) by Akt or ERK, respectively. ERK, extracellular signal-regulated kinase; IRS, insulin receptor substrate; MEK, MAPK/ERK kinase; PDK, protein 3-phosphoinositide-dependent protein kinase; PI3K, phosphoinositide 3-kinase (PI3K).

embryos of *Macrobrachium olfersi* (136). The VEGF signaling pathway is enriched in hemocytes from pathogen-infected *Eriochier sinensis*, suggesting that VEGF is involved in mounting an immune response (137). *Lv-EGF*, *Lv-EGFR*, and *Lv-VEGFR* are expressed in embryos and larvae of *Litopenaeus vannamei* (138). In *M. rosenbergii*, *Mr-EGFR* is expressed in most tissues, with higher expression in thoracic ganglion, ovary, and testis (139). Knockdown of *Mr-EGFR* slows accumulation of mass in juvenile male *M. rosenbergii*, but has no effect on ecdysis frequency (139). Using transcriptomic data, a cDNA encoding the complete EGFR sequence was cloned from *S. parmamosain* ovary; *Sp-EGFR* is expressed in most tissues, with higher expression in YO, ovary, stomach, heart, and gill (140). Human EGF (1 nM and 10 nM) caused a transient increase in *Sp-Vtg receptor* and *Sp-Cyclin B* mRNA levels in ovary explants; the increases were blocked by pretreatment with EGFR tyrosine kinase inhibitors AG1478 and PD153035 (140). The *G. lateralis* YO expresses *Gl-EGF*, *Gl-FGF*, *Gl-EGFR*, and *Gl-FGFR* (**Table 3**) (16, 20). These data suggest that EGFR functions in a wide variety of tissues, including the YO (**Table 3**). The expression of *Gl-EGF* and *Gl-FGF* suggests that EGF, and perhaps FGF, act as autocrine factors in the YO as EGF does in the PG (134).

TRANSFORMING FACTOR β/ACTIVIN SIGNALING

TGFβ signaling plays essential roles in animal cell differentiation and homeostasis, and dysregulation of TGFβ signaling contributes to many diseases including cancer (141–143). In the

canonical pathway, a ligand binds to TGF receptor 2 (R2) homodimer, which associates with TGFR1 homodimer to form the active heterotetramer receptor complex by TGFR2 autophosphorylation and phosphorylation of TGFR1 and regulatory (R)-Smad (144, 145). Two phospho-R-Smads bind to one Co-Smad and the R-Smad/Co-Smad complex translocates to the nucleus to regulate gene transcription (141, 144, 145). Several proteins inhibit TGFβ signaling. Inhibitory (I)-Smads block TGFβ signaling by either preventing R-Smad phosphorylation by TGFR2 or preventing binding of Co-Smad to phospho-R-Smad. FK-506 binding protein 1A (FKBP12) binds to TGFR1 and prevents phosphorylation of TGFR1 by TGFR2 (141, 146). BMP and activin membrane bound inhibitor (BAMBI) acts as a TGFR1 pseudo receptor, as it binds ligand, but lacks the protein kinase domain for signal transduction. TGFβ signaling cross-talks with many other signaling pathways, such as MAPK, Akt, PKC, CAMKII, GSK3, JAK, JNK, Wnt, Notch, and Hedgehog (145).

TGFβ ligands include bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), Activin, anti-Müllerian hormone, nodal, and TGFβs (143, 147). They often function as autocrine factors, acting on the same tissue in which they are synthesized and secreted. TGFβ/Activin signaling, in particular, determines the competency of insect and crustacean molting glands to respond to neuropeptides (2, 97, 103). Insects express three Activins: Activin-β (Actβ), Myoglianin (Myo)/Myo-like, and Dawdle (Daw) (148, 149). In *D. melanogaster*, knocking out Activin signaling by targeting R-Smad *dSmad2*, Type I receptor *Baboon* (*Babo*), Type II receptor *Punt*, or Co-Smad *Medea* in the PG leads to 3rd instar arrest and failure of larvae to metamorphose and down-regulation of signaling genes *Torso* and *InR* and Halloween genes *Dib* and *Spok* in the PG (150). In the German cockroach *Blatella germanica*, an increase in *Bg-Myo* mRNA levels in the PG is associated with increased ecdysteroid synthesis at the end of the 5th instar (151). These data indicate that Activin signaling is necessary for the stimulation of ecdysteroidogenesis by PTTH and ILP. Interestingly, knocking out any one of the three Activin ligands Actβ, Myo, or Daw has no effect on *D. melanogaster* molting and metamorphosis (148). All three ligands must be knocked out in the PG in order to manifest the developmental arrest phenotype, suggesting some degree of redundancy between the three ligands and their Babo receptors (148). Adult decapod crustaceans express a single myostatin (Mstn)-like/GDF11 that is related to mammalian Mstn/GDF8 and GDF11 (2, 18). TGFβ/Mstn signaling drives the transition of the YO to the committed state, resulting in the YO becoming less sensitive to MIH and CHH in mid-premolt and late premolt (2, 15, 152). Recently, a cDNA encoding a Dawdle-like factor was characterized in *S. paramansosain*. *Sp-Daw* is expressed primarily in embryos and larvae, suggesting that it plays a role in developmental processes (153). It may also be involved in the innate immune response in adults (153).

Mstn is expressed in most crustacean tissues, with generally higher levels in the YO, heart, and muscle (15, 18, 154–161). Most studies have focused on the role of *Mstn* as a negative regulator of muscle growth, which contributes to organismal growth (5). In Chinese shrimp *F. chinensis*, *Fc-Mstn* mRNA levels are inversely correlated with growth traits among individuals from different genetic lineages (162). It appears that Mstn slows muscle growth by inhibiting mTORC1-dependent protein synthesis. In *G. lateralis* claw muscle, increased protein synthesis during premolt is correlated with decreased expression of *Gl-Mstn* and increased expression of *Gl-Rheb*, the activator of mTORC1 (17, 163). Several studies have attempted to knock down *Mstn* expression as a means to promote growth in aquacultural species. Surprisingly, in several cases, *Mstn* ds-RNA injection has just the opposite effect: an increase in molt interval and/or decrease in growth rate in *Penaeus monodon*, *L. vannamei*, and *Fenneropenaeus merguiensis* (155, 159, 164). These studies did not consider off-target effects. Reduced expression of *Mstn* in the YO could have blocked or delayed the progression from early premolt to mid-premolt, thus lengthening the interval between ecdyses. The effects on YO *Mstn* mRNA levels were not examined in these studies. However, injection of *Es-Mstn*-dsRNA or *Es-Activin receptor IIB* (*Es-ActRIIB*) dsRNA into juvenile *E. sinensis* and injection of *Fc-Mstn*-dsRNA into juvenile *F. chinensis* accelerated molting and growth when compared to a control group (154, 165, 166). However, control animals were injected with RNase-free water or phosphate-buffered saline, rather than an unrelated dsRNA construct (154, 164–166). Thus, one cannot rule out a nonspecific response to dsRNA injection. Only two of the studies used dsRNA products of unrelated sequences as controls, and both those showed molt inhibition (155, 159).

Molting alters TGFβ/Activin signaling gene expression in the *G. lateralis* YO. Analysis of RNAseq data of MLA-induced animals shows increases of *Gl-Activin RI*, *Gl-Smad2/3* (*Gl-R-Smad*), and *Gl-Smad4* (*Gl-Co-Smad*) in early premolt to mid-premolt, while TGFβ signaling inhibitors, *Gl-Smad6* (*Gl-I-Smad*) and *Gl-BAMBI*, are down-regulated during premolt (21). By contrast, ESA decreases *Gl-Activin RI*, *Gl-Smad2/3*, *Gl-Smad4*, *Gl-Smad6*, and *Gl-BAMBI* expression (16). The differences between the MLA and ESA transcriptome results are attributed to the ESA study focusing on initial YO activation as the animals did not transition to mid-premolt (16). Neither MLA nor ESA had a significant effect on the relative expression of *Gl-Mstn* in the *G. lateralis* YO transcriptomes (16, 21), although qPCR showed that *Gl-Mstn* mRNA level increases by three days post-ESA (15). Future research should use qPCR to establish the precise timing of the effects of ESA ± SB431542 on the expression of TGFβ/Mstn signaling genes.

mTOR SIGNALING

mTOR is a serine/threonine PI3-related protein kinase that allocates energy in response to nutrients, growth factors, and stress in eukaryotic cells at transcriptional and post-translational levels (167–169). mTOR associates with other proteins to form two complexes: mTOR Complex 1 (mTORC1) and Complex 2 (mTORC2) (169). mTORC1 contains Raptor and controls protein translation, lipid and nucleotide synthesis, and autophagy (169, 170). mTORC2 contains Rictor and controls cytoskeletal remodeling, ion transport, and cell survival and proliferation

(169, 170). mTORC1 is inhibited by rapamycin, mediated by FKBP12, while mTORC2 is insensitive to rapamycin (167, 169). mTORC1 is activated by GTP/Ras homolog enriched in brain (Rheb). Rheb, in turn, is controlled by the tuberous sclerosis complex (TSC), a heterotrimeric protein composed of Hamartin (TSC1), Tuberin (TSC2), and TBC1 domain family member 7 (TBC1D7) (169). TSC is a GTPase activating protein (GAP) that inactivates GTP/Rheb by promoting the hydrolysis of GTP to GDP (170). Growth factor signaling pathways converge on the TSC to promote cellular growth (**Figure 4**) (170). Phosphorylation of TSC by Akt, ERK, p90 ribosomal S6 kinase 1 (RSK1), and other protein kinases inhibit the GAP activity to prevent inactivation of GTP/Rheb, which leads to mTORC1 activation (169). Phosphorylation of ribosome subunit 6 kinase (S6K) and 4E-binding protein (4E-BP) by mTORC1 increases translation of mRNA to protein.

mTORC1 is required for increased ecdysteroidogenesis in the arthropod molting gland. PTTH stimulates mTORC1-dependent protein synthesis, which increases ecdysteroid synthesis of the insect PG (126, 128, 171–178); see (179) for earlier references). In *D. melanogaster*, *Rheb* overexpression or *TSC2* knockdown in the PG reduces the developmental delay in food-limited 3rd-stage larvae, resulting in lower adult weights (180). Additionally, *Rheb* overexpression under food-limiting conditions increases transcription of Halloween genes *Phm* and *Dib* (180). Comparable studies on decapod crustaceans, such as knockdown of *Rheb* and *TSC2* with dsRNA constructs, have not been done. Incubation of hepatopancreas explants with *Sp-Akt*-dsRNA reduced the insulin-induced increase in *Sp-Vtg* mRNA level, but had no effect on *Sp-Rheb* mRNA level (125). Rapamycin inhibits YO ecdysteroid synthesis and secretion *in vivo* and *in vitro* and blocks entry of intermolt animals into premolt and delays the transition from early premolt to mid-premolt in eyestalk-ablated *G. lateralis* (14, 15).

Molting up-regulates many of the mTOR signaling components in the YO. In *G. lateralis*, *Gl-mTOR* and *Gl-Akt* mRNA levels increase in mid-premolt and late premolt stages in MLA animals and *Gl-mTOR*, *Gl-Akt*, and *Gl-S6K* mRNA levels increase by three days post-ESA in eyestalk-ablated animals (14, 15). *Gl-elongation factor 2* (*Gl-EF2*) mRNA level is also increased by MLA and ESA, which is consistent with increased protein synthesis in the YO (14, 15). By contrast, molt stage (intermolt, early premolt, and late premolt) has no effect on *Cm-EF2*, *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, and *Cm-S6K* mRNA levels in *C. maenas* (14). SB431542 injection blocks the ESA-induced increases in *Gl-EF2*, *Gl-mTOR*, and *Gl-Akt* mRNA levels and decreases *Gl-Rheb* mRNA level, which suggest that TGFβ/Activin signaling is required for sustained up-regulation of mTOR signaling during premolt (15). RNAseq data expands on the results from qPCR analysis. *Gl-mTOR*, *Gl-Raptor*, *Gl-Rictor*, *Gl-S6K*, and *Gl-Akt* are expressed at high levels during intermolt and early premolt and at their lowest levels during postmolt (21). *Gl-Rheb* expression increases during early premolt and mid-premolt stages (21). ESA increases expression of *Gl-mTOR*, *Gl-Raptor*, *Gl-mLST8*, *Gl-Rheb*, *Gl-Akt*, *Gl-S6*, *Gl-S6K*, *Gl-EIF4E*, and *Gl-EF2* (16). These increases are inhibited by rapamycin, suggesting a positive feedback mechanism in which mTORC1 activity up-regulates expression of mTOR signaling genes (**Figure 1**) (16).

Temperature affects metabolic processes in crustaceans, including molting, and it is likely that mTORC1 activity contributes to the response of the YO and other tissues to temperature. Within normal physiological ranges, increasing temperature stimulates molting and growth of decapod crustaceans (3, 181–183). However, when an animal reaches its upper thermal limit, molting is inhibited, either directly on the YO or indirectly by inhibitory neuropeptide, such as CHH, secreted by the X-organ/sinus gland (183–186). The effects of temperature on survival, molting, and mTOR signaling gene expression in YO, eyestalk ganglia, and heart were determined in juvenile Dungeness crab, *Metacarcinus magister*. Animals at three different molt stages (12, 19, or 26 days post-ecdysis; these intervals spanned stages C to $D_{2\text{-}3}$) were transferred from ambient temperature (~15°C) to 5, 10, 15, 20, 25, or 30°C for 14 days (187). None of the animals transferred to 25 and 30°C survived (187). *M. magister* molt successfully at 21°C, but the growth increment is less than that at 14°C (188); see (187) for additional references). These results indicate that the upper temperature limit for *M. magister* molting success is between 21°C and 25°C. Low temperature (5°C) inhibits molting (187). Between 10°C and 20°C, molt stage progression increases with temperature (187). Gene expression in YO, eyestalk ganglia, and heart is affected by temperature and molt stage, but there is little or no interaction in gene expression between temperature and molt stage (187). In eyestalk ganglia, *Mm-MIH*, *Mm-CHH*, *Mm-Rheb*, *Mm-AMP kinase α subunit (AMPkα)*, and *Mm-Akt* mRNA levels decrease with increasing temperature, particularly at 20°C; *Mm-S6K* mRNA level is not affected by temperature (187). In heart, mRNA levels of *Mm-Rheb*, *Mm-S6K*, *Mm-AMPKα*, *Mm-Akt*, and *Mm-mTOR* are higher at 10°C than at 15°C and 20°C. Of the six genes quantified in the YO, only *Mm-Rheb* expression is affected by both molt stage and temperature (**Figure 5**). *Mm-Rheb* mRNA level is higher in premolt stages (**Figure 5**) and is positively correlated with hemolymph ecdysteroid titers at all three temperatures (187), which suggests that Rheb stimulates mTORC1-dependent ecdysteroid synthesis. *Mm-Rheb* mRNA level decreases with increasing temperature at most molt stages (**Figure 5**). It is noteworthy that only the mRNA level of *Mm-Rheb* is negatively correlated with temperature in all three tissues (**Figure 6**). It appears that the down-regulation of mTOR signaling serves as a compensatory mechanism for higher metabolic rates at higher temperatures, so that energy allocation to protein synthesis is maintained at relatively constant levels (**Figure 6**). Taken together, the data suggest that *Rheb* expression can be used as a proxy to assess the effects of molting and temperature on mTORC1 activity in crustacean tissues.

CONCLUSIONS AND FUTURE RESEARCH

The control of molting involves the integration of a variety of signals that affects the ecdysteroidogenic capacity and activity of

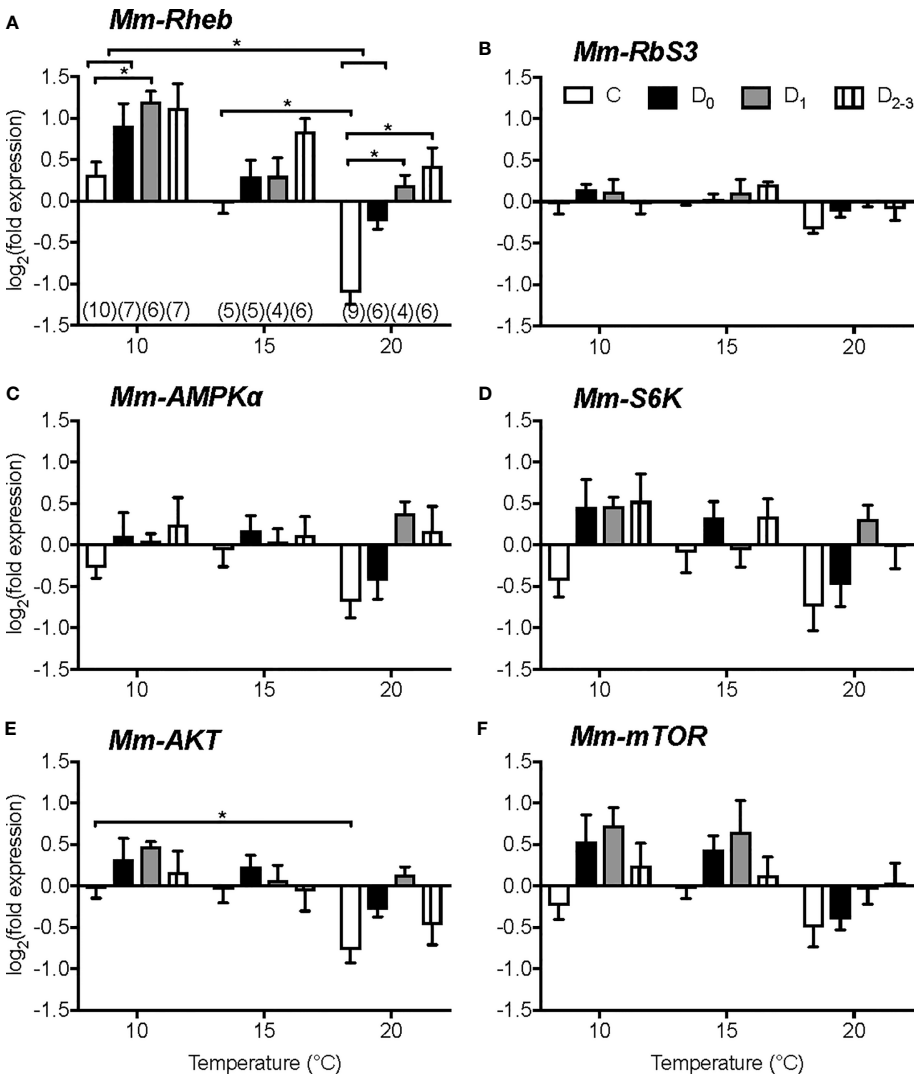

FIGURE 5 | Effects of temperature and molt stage on gene expression in Y-organs of juvenile Dungeness crab, *Metacarcinus magister*. mRNA levels of *Mm-Rheb* **(A)**, *Mm-ribosome subunit 3* (*RbS3*) **(B)**, *Mm-AMPKa* **(C)**, *Mm-S6K* **(D)**, *Mm-AKT* **(E)**, and *Mm- mTOR* **(F)** after 14 days at 10°C, 15°C or 20°C of juveniles in intermolt (C, white), early premolt (D_0, black), mid-premolt (D_1, grey), or late premolt (D_{2-3}, lines). Data are normalized to the mean absolute mRNA copy numbers in stage C at 15°C. Asterisks denote significant differences at $P < 0.05$. Sample size (*n*) given in brackets below columns in A also apply to the other genes. Data are presented as mean ± 1 S.E.M. *Mm-Rheb* expression is affected by temperature and molt stage. *Mm-Rheb* mRNA level increases during premolt stages and decrease with increasing temperature. From (187).

the arthropod molting gland (2, 62, 78, 106). This is reflected by the diversity and actions of the factors involved. In the insect PG, tropic factors, such as PTTH, ILPs (e.g., dILP1-6, Bombyxin), growth factors (e.g., EGF and VEGF), biogenic amines (e.g., serotonin and octopamine), and FXPRLamide peptide, stimulate ecdysteroidogenesis (97, 98, 105). Static factors, such as dILP8, prothoracicostatic peptides, Bommo-myosuppressin, and FMRFamide-related peptide, inhibit ecdysteroidogenesis (97, 98, 105). By contrast, the neuropeptides MIH and CHH are the only known ligands identified for YOs in crustaceans (2, 50, 55, 189, 190). Transcriptomic analysis has revealed that the YO expresses receptors for ILPs, growth factors, biogenic amines, and neuropeptides (**Tables 2**, **3**) (25, 29). The large number of GPCRs in particular indicates that the YO resembles the insect PG in being able to integrate a large number of signals to coordinate organ growth, development, and molting. MIH, CHH, LAF_{pro}, and perhaps FMRFamide act as static factors on the YO. Drawing on comparisons with the insect PG, ILPs, EGF, VEGF, corazonin, and LAF_{an} may act as tropic factors. The effects of these and other ligands (e.g., serotonin, octopamine, FGF, dopamine, pigment dispersing factor, allatostatins, ecdysis-triggering hormone, crustacean cardioactive peptide (CCAP), CCHamide, diuretic hormones DH31 and DH44, and Bursicon) on YO ecdysteroidogenesis remain to be determined.

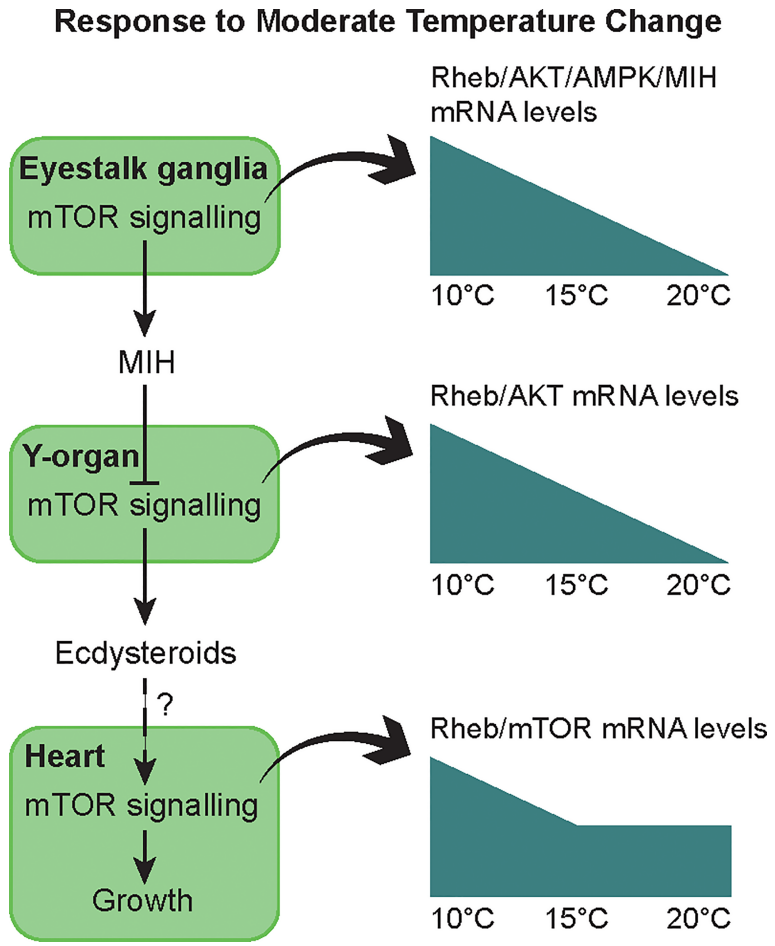

FIGURE 6 | Summary of the effects of moderate thermal stress on molting and growth through the mTOR signaling pathway in juvenile Dungeness crabs. In intermolt, MIH keeps the YO in a basal state with low ecdysteroid secretion by inhibiting mTOR. Low levels of ecdysteroid may stimulate heart muscle growth *via* mTOR signaling. Moderate temperature change (10 to 20°C range) allows acclimation of the animals, at least with respect to some physiological functions. After 14 days, thermal compensation is observed in molt control, i.e. similar ecdysteroid titer across temperatures throughout the molt cycle (187). Mechanisms include up-regulation or down-regulation of *Mm-MIH* and mTOR signaling genes (*Mm-Rheb*, *Mm-Akt*, *Mm-AMPK*) during cold (10°C) or warm (20°C) exposure in the eyestalk ganglia and YO. In the heart, thermal compensation of metabolism is incomplete, as oxygen demand and heart activity increase with temperature. A sustained mRNA level of *Mm-Rheb* and *Mm-mTOR* indicates a greater allocation of energy to maintaining cardiac capacity during warm exposure. From (187).

PTTH and MIH are the primary neuropeptides controlling molting in insects and crustaceans, respectively (2, 50, 105, 106). It is remarkable that the control of a process as critical as ecdysis is to organismal growth would have evolved diametrically opposite mechanisms in these two major arthropod groups. PTTH activates the PG, while MIH inhibits the YO. Thus, molting in insects is initiated by the release of PTTH from neurosecretory neurons in the brain, while molting in crustaceans is initiated by reduced MIH release from neurosecretory neurons in the eyestalk X-organ/sinus gland complex (2, 11, 105). In *D. melanogaster*, PTTH stimulates PG ecdysteroidogenesis by binding to the Torso RTK and activating the Ras/Raf/MAPK signaling pathway (106). In lepidopterans (*Manduca sexta* and *Bombyx mori*), a PTTH-induced Ca^{2+} influx activates both Ras/Raf/MAPK signaling and cAMP-dependent signaling and both contribute to a large increase in ecdysteroid synthesis (97, 105, 179). MIH inhibits YO ecdysteroidogenesis by binding to a putative GPCR and activating a cyclic nucleotide-dependent signaling pathway (2, 50). A cAMP/Ca^{2+}-dependent triggering phase is linked to a NO/cGMP-dependent summation phase to inhibit the YO between MIH pulses (2, 50, 55). RTKs in the YO most likely function to stimulate ecdysteroidogenesis, as they do in the PG (76, 97, 98).

The YO undergoes phenotypic changes over the molt cycle. The molt cycle is unidirectional, with YO transitions occurring at critical checkpoints that determine progression to the next molt stage. **Figure 7** presents a working model for the signaling pathways that control YO basal (intermolt stage), activated (early premolt stage), and committed (mid- and late premolt stages) phenotypes. The most important decision is to initiate

molting, which is determined by the integration of external cues, most likely mediated by the brain and eyestalk ganglia, which control the release of MIH from the X-organ/sinus gland complex, and internal cues, such as nutritional status, organ size, and limb regeneration, that act directly on the YO (2, 3, 11). The signaling pathways converge on Rheb/mTORC1 to regulate ecdysteroidogenesis (**Figure 7**). Activation of GPCR signaling by static factors (e.g., MIH and CHH) inhibits Rheb/mTORC1, maintaining the YO in the basal state. Activation of RTK signaling by tropic factors (e.g., ILPs and growth factors) stimulates Rheb/mTORC1. Although RTK signaling can potentially activate the YO, MIH release prevents molt initiation until environmental conditions are met. YO activation is mediated post-translationally by mTORC1-dependent increased global translation of mRNA to protein, resulting in increased ecdysteroid synthesis and secretion (**Figure 7**, dashed lines). The rising ecdysteroid titers in the hemolymph mark the entry into early premolt (1). The activated YO remains sensitive to static factors, as ecdysteroidogenesis is inhibited by MIH, CHH, and LAF_{pro} (2). During early premolt, the YO synthesizes and secretes Mstn-like factor, which binds to Activin receptors to activate Smad transcription factors, leading to down-regulation of MIH signaling genes and up-regulation of Rheb/mTORC1 and Halloween genes in the committed YO (**Figure 7**, solid lines).

Although much progress has been made over the last ten years, many questions remain and research efforts should be directed at answering them:

1. What is the identity of the MIH receptor? The evidence indicates that the MIH receptor is a GPCR and several potential candidates have been identified from *in silico* analysis of YO transcriptomes (**Figure 2**) (25). Moreover, there is evidence from studies of lobster muscle that the CHH receptor is a membrane receptor guanylyl cyclase (GC-II) (8, 56, 179, 191). A heterologous reporting system in COS-7 cells holds promise as a functional assay for quantifying the specificities of candidate GPCRs and GC-II to recombinant MIH and CHH (124).

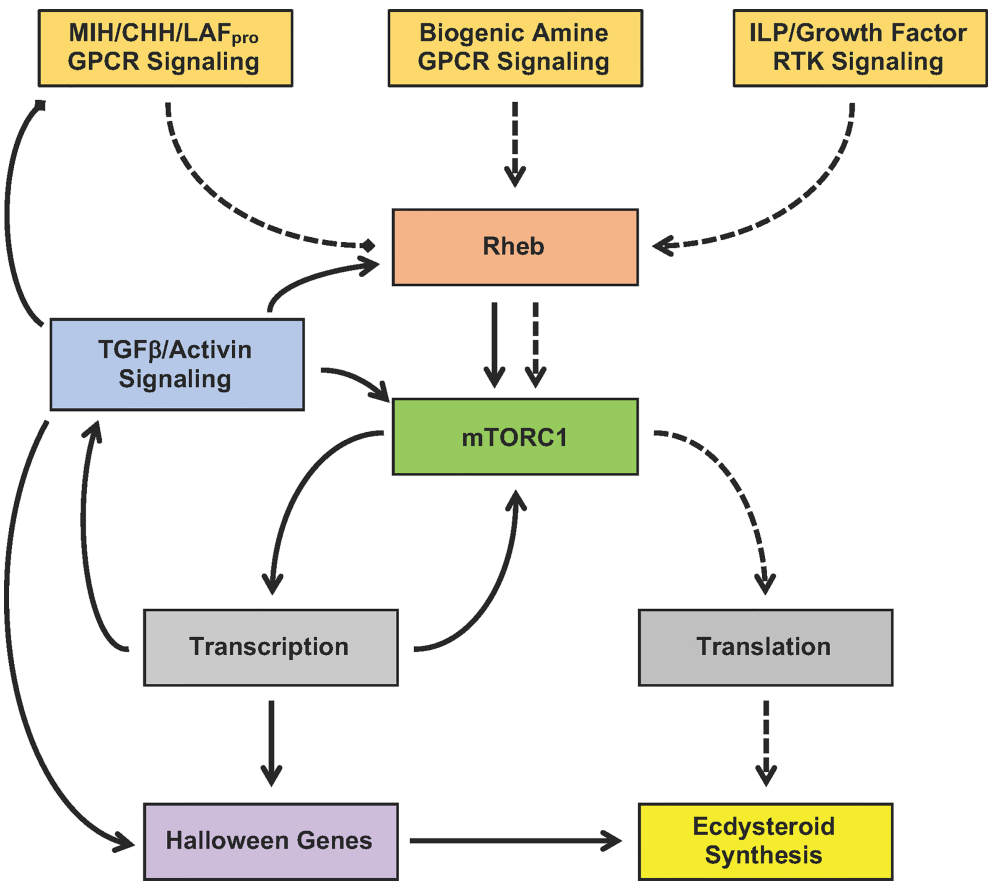

FIGURE 7 | Proposed model for the signaling pathways mediating YO activation (dashed lines) and commitment (solid lines). Signaling pathways converge on mTORC1 by controlling Rheb activity. MIH/CHH/LAF_{pro} GPCR signaling inhibits mTORC1 by inactivating Rheb, while biogenic amine GPCR signaling and ILP/growth factor RTK signaling stimulates mTORC1 by activating Rheb. YO activation during early premolt requires mTORC1-dependent global translation of mRNA to protein, which leads to increased ecdysteroid synthesis. YO commitment involves mTORC1-dependent changes in gene transcription, resulting in up-regulation of TGFβ/Activin, Rheb/mTORC1, and Halloween genes and down-regulation of MIH/CHH/LAF_{pro} GPCR signaling genes.

2. How does MIH signaling inhibit mTORC1? The current thinking is that MIH inhibition of ecdysteroidogenesis is mediated by PKG (2, 56). The downstream substrates of PKG are unknown. A possible target is TSC (**Figure 3**), in which phosphorylation stimulates GAP activity, although it is not known if TSC is phosphorylated by PKG (192). Proteomic analysis using liquid chromatography-tandem mass spectrometry now provides the technology to identify and quantify phosphoproteins in the YO in response to rMIH and PKG inhibitors. A similar approach was used to show that NO synthase is phosphorylated in the activated YO (7).
3. What is the identity of LAF_{pro}? LAF_{pro} is a peptide factor produced by secondary limb buds to delay molting (13). As discussed in Section 2.3, the discovery of an Lgr3 GPCR in the YO transcriptome suggests that LAF_{pro} is a Dilp8-like peptide that inhibits ecdysteroidogenesis *via* the MIH signaling pathway (**Figure 3**). Like the YO, inhibition of PG ecdysteroid synthesis by Dilp8 is through the activation of NO synthase (73, 79). This suggests that the inhibition of ecdysteroidogenesis by NO/cGMP/PKG is conserved in arthropod molting glands.
4. How does mTORC1 control gene expression? mTORC1 activity alters the mRNA levels of thousands of genes, including those for mTOR, TGFβ, and MIH signaling and ecdysteroidogenesis (16), presumably by altering the activities of transcription factors and co-factors. In *D. melanogaster*, the transcription factors Krüppel homolog 1 (Kr-h1), seance, ouija board, molting defective, ventral veins lacking, and Knirps are linked to Halloween gene expression (193–195). *Kr-h1* is a critical component of the juvenile hormone (JH)/methyl farnesoate (MF) signaling pathway in insects (196–198), and recent studies indicate that *Kr-h1* plays a role in crustacean development and reproduction (199–203). The YO expresses *Kr-h1* and other MF signaling components, suggesting that it also has a role in molt regulation (204). One potential function is in the down-regulation of Halloween gene expression when the YO transitions to the repressed state in late premolt.
5. What are the gene targets of TGFβ/Activin signaling? TGFβ/Activin drives the transition of the YO from the activated to the committed state. It is hypothesized that Smad transcription factors, activated by Mstn-like factor, up-regulate the expression of commitment genes that determine the phenotypic properties of the committed YO (**Figure 1**), such as low sensitivity to MIH, CHH, and LAF_{pro} and high ecdysteroid production. Possible targets are Rheb/mTORC1, MIH signaling genes, and Halloween genes (**Figure 7**). These and other gene targets can be identified by determining the effects of ESA ± SB431542 on the YO transcriptome and proteome.
6. What are the mechanisms mediating the transitions of the YO from the committed to repressed state and from the repressed to basal state? The repressed YO is transcriptionally inactive and has very low ecdysteroid synthesis, leading to low hemolymph ecdysteroid titers during the postmolt stage. It is hypothesized that the ecdysteroid peak at the end of premolt triggers the repressed state, mediated by ecdysteroid receptor (EcR/RXR) and ecdysone-response proteins. Even less is known about what causes the YO to return to basal state at the end of the postmolt stage. Perhaps a signal from the integument, upon completion of exoskeleton synthesis marked by the deposition of the membranous layer, is involved.
7. What is the role of RTKs in regulating ecdysteroidogenesis? Our understanding of RTKs and their ligands in the YO is largely based on inferences from research on the insect PG. It is hypothesized that ILPs and growth factors stimulate ecdysteroidogenesis (**Figure 4**), but their effects are dampened or nullified by MIH. The YO expresses EGF and FGF, suggesting that both have an autocrine function. *In vitro* assays can determine the effects of recombinant insulin, EGF, and FGF on YO ecdysteroid synthesis and secretion.
8. Is ecdysteroidogenesis regulated by biogenic amines and neuropeptides other than MIH and CHH? The YO expresses a large number and diversity of GPRCs. Of the 99 GPCRs in the *G. lateralis* YO, 65 are assigned to known receptors (25). Of particular interest are GPCRs for corazonin, serotonin, and octopamine, which stimulate ecdysteroidogenesis (**Figure 3**; Sections *Corazonin Receptor* and *Ca^{2+}/Diacylglycerol/Protein Kinase C Signaling*). However, other ligands involved in molting, such as ecdysis triggering hormone and Bursicon, should also be investigated.
9. What are the roles of Wnt, Hedgehog, Notch, and Hippo signaling pathways in the YO? These pathways are implicated in controlling ecdysteroidogenesis in the insect PG (74, 97, 98, 205) and are well represented in the YO transcriptome (**Table 1**). Decapods express a large number of Wnt ligands (206). *Wnt4* is implicated in having roles in limb regeneration and the immune response in decapods (207, 208). *Gl-Wnt5* and *Gl-Wnt7* are expressed at their highest levels in late premolt, suggesting that these ligands are involved with the ecdysteroid peak and transition of the YO to the repressed state (21). This constitutes an entirely new area of research in the coming years.

Transcriptomics and proteomics have revealed the complexities of the regulation of the arthropod molting gland. These approaches have been successfully applied to insect PG and complement functional genetic studies on *D. melanogaster* (176, 205, 209–215). Transcriptomic and proteomic analysis of the YO has revealed that the PG and YO are more similar than they are different. The PG and YO express the same KEGG signaling pathways. Many of these signaling pathways converge on mTORC1, which plays a central role in regulating ecdysteroid synthesis in both endocrine organs. A second shared property is that TGFβ/Activin signaling alters the ligand sensitivity of the molting gland. In insects, Activins increase the sensitivity of the PG to PTTH in preparation for the large ecdysteroid peak prior to the metamorphic molt, whereas Mstn/Activin decreases the sensitivity of the YO to MIH and CHH in mid- and late premolt. The great diversity in GPCRs indicates that the YO, like the PG, can respond to a variety of ligands, some of which are inhibitory and others are stimulatory. As RTKs stimulate ecdysteroidogenesis in the PG, it seems reasonable to postulate that RTKs have the same

function in the YO. The study of insect molting and metamorphosis has informed research on crustaceans, but this does not mean that one can fully understand the control of molting in crustaceans by studying *Drosophila*. There are fundamental differences in evolutionary history and life history between insects and decapod crustaceans and even between insect orders. The lineages that gave rise to insects and crustaceans have been separated for more than 500 million years, allowing time for the evolution of divergent ligands and signaling pathways to become dominant (216, 217). Unlike insects, most decapod species continue to molt as adults, enabling them to grow to larger sizes. The larger size is a distinct advantage, as one can obtain the amount of YO tissue needed for transcriptomics from two or three individuals (16, 20, 21, 29) and proteomics (24, 218). This allows for increased sample sizes for statistical analysis and potentially more experimental treatments and time points for the study of molting gland function. Thus, crustacean models complement insect models for achieving a broader understanding of how arthropods integrate growth control with external and internal cues.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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