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 WVD 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
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 lipophosphoproteins 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 protein (3, 7, 8). 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 crustaceans, it is well established that the eyestalk and nervous tissues such as brain and thoracic ganglion produce neuroendocrine 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

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**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) Intronic 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 \( L. \) \( \text{vannamei} \) 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 \( L. \) \( \text{vannamei} \) (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 \( L. \) \( \text{vannamei} \). 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 \( L. \) \( \text{vannamei} \) 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 \( L. \) \( \text{vannamei} \). 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 \( LvVg1 \), \( LvVg2 \), and \( LvVg3 \) 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 \( \beta \)-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 ddH2O 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 ddH2O as the blank control. The Cq values were obtained after the reactions were completed. 2^{ΔΔCt} 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; the amino acid sequence homology, the sizes of the deduced proteins, alignment results, and their homologies with potential phosphorylation sites, and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) 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 mm³. 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
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% sequence identity) was observed in two regions that we called >99.7% amino acid sequence identity). However, relatively low domain, DFU1934 domain, and von Willebrand domain (i.e. fi

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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.CL2883C1-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.

LvVg isofoms 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 Unigene9553 (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 Unigen19992 (Figure 3). Unlike the other two transcript groups, the deduced protein of Unigen19992 (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.
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#: QCYY01004146.1) (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, #QCYY0100515) 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
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
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. 

**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

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

| Comparison of LvVg1, LvVg2, and LvVg3 | LvVg1(CL2388) | LvVg2(Unigene9553) | LvVg3(Unigene15559) |
|--------------------------------------|--------------|--------------------|---------------------|
| LvVg1(CL2388) | 19 AA | 706 AA (77.2 kDa) | 731 AA (79.9 kDa) | 1126 AA (123.2 kDa) |
| LvVg2(Unigene9553) | 51.8 | 37.2 | 35.8 |
| LvVg3(Unigene15559) | 37.2 | 35.8 |

LvVg1 showed a higher level of similarity or identity 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 (%).

**Figure 6** | 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.
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,
brain, and thoracic ganglion consisted of factors that can stimulate the expression of \( \text{LvVg1} \) 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 \( \text{LvVg1} \) 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 \( \text{MeVg1} \) 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. \( \text{MeVg2} \)) was cloned and characterized. The \( \text{MeVg2} \) gene shared only a 50% amino acid sequence identity with the \( \text{MeVg1} \) 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 \( P. \) homarus, multiple vitellogenin transcripts have also been identified (data not
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 ofLvVg2 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,
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 and regulation of gene expression. We have previously reported 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

FIGURE 9 | Neuroendocrine regulation of LmVg1 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 LmVg1 gene.
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 precursor molecule and later processed into subunits in the hemolymph (23). This is why the full-length Vg 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 scarce. In the crab Potamon koolooenseis, 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, IS, 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

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.577745/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.