

Complete sequence of *Litopenaeus vannamei* (Crustacea: Decapoda) vitellogenin cDNA and its expression in endocrinologically induced sub-adult females

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Abstract

The gene that encodes vitellogenin (Vg), the precursor of the major yolk protein, vitellin, is expressed during vitellogenesis in decapod crustaceans. In this study, we sequenced the full-length cDNA from the Pacific white shrimp *Litopenaeus vannamei* Vg gene (*LvVg*). This is the first open thelycum penaeid shrimp Vg cDNA to be sequenced. The transcript encodes a 2587 amino acid polypeptide with up to 85% identity to Vg of different penaeid species. Peptide mass fingerprints (PMFs) of the vitelline polypeptides suggest that the predicted endoprotease cleavage site at amino acids 725–728 does indeed undergo cleavage. Five prominent high-density lipoprotein polypeptides of masses 179, 113, 78, 61, and 42 kDa were isolated from vitellogenic ovary, and their PMFs were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry. It is likely that these polypeptides are all products of the *LvVg* gene. Removal of the X-organ sinus gland complex (XO-SG), which secretes the neurohormones that control the endocrine system regulating molt and reproduction, can induce both these processes. During the course of a number of molt cycles in induced sub-adult females, periodic ovarian growth and resorption were observed. Ovary growth correlated with *LvVg* expression in both the hepatopancreas and the ovary. Expression in ovaries of induced intermolt–early premolt females was significantly higher compared to all other sub-groups. Expression in ovaries of induced females was significantly higher compared to hepatopancreas at all molt cycle stages. Periodicity of molt and vitellogenesis in endocrinologically induced sub-adult shrimps may serve as a model to study alternate regulation of gene expression during these two processes.

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1. Introduction

Molt and reproduction in decapods are both energetically demanding physiological processes. These two processes are regulated by two related endocrine axes that are governed by inhibitory neurohormones secreted

from the X-organ sinus gland complex (XO-SG) in the eyestalk (Huberman, 2000; Wilder et al., 2002). These neurohormones, the molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH), belong to the crustacean hyperglycemic hormone family (Böcking et al., 2002). Surgical extirpation of the eyestalks can thus induce both molting and vitellogenesis (Browdy and Samocha, 1985; Wilder et al., 2002). Vitellogenesis is the central event of egg maturation in all oviparous species, with vitellogenins (Vgs) being the major yolk

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protein precursors in most such animals including crustaceans. Vgs, produced in large amounts by female tissues, accumulate in the developing oocytes and are eventually used as a nutrient source for the developing embryos (Meusy and Payen, 1988). Vgs are members of a larger superfamily of molecules known as large lipid transfer proteins (Babin et al., 1999). Despite their low overall conservation across major taxonomic groups, their common function and disproportionate conservation of certain amino acids suggest a common phylogenetic ancestor (Chen et al., 1997; Sappington and Raikhel, 2002). Crustacean Vg is a high-molecular-weight protein associated with lipidic, glucidic, and carotenoid prosthetic groups. Vg is transported to the oocyte via the hemolymph, and internalized into the oocyte through receptor-mediated endocytosis (Warrier and Subramoniam, 2002; Wilder et al., 2002). In the oocyte, Vg undergoes further modifications to become vitellin.

In the past, crustacean vitellogenesis and the structure of Vgs have been studied by histological, immunological, electrophoretic, and radioactively labeled-amino acid incorporation techniques (Meusy and Payen, 1988; Wilder et al., 2002). The recent sequencing of decapod Vg cDNAs and their molecular characterization is extending, for the first time, previous biochemical studies to shed new light on unsolved questions, e.g., Vg gene sites of expression and Vg posttranslation modification. Currently, there are eight complete decapod Vg cDNA sequences available in the GenBank (Abdu et al., 2002; Avarre et al., 2003; Okuno et al., 2002; Tsang et al., 2003; Tsutsui et al., 2000; Tsutsui et al., 2004; data bank accession number AY499620) including the *LvVg* presented in this study (data bank Accession No. AY321153).

In pleocyemata species, the Vg gene is expressed exclusively in the hepatopancreas (Abdu et al., 2002; Okuno et al., 2002). In dendrobranchiata species, both the ovary and the hepatopancreas express the Vg gene (Avarre et al., 2003; Tsang et al., 2003; Tseng et al., 2001, 2002; Tsutsui et al., 2000). In decapods, the sub-epidermal adipose tissue is also believed to be a site of Vg synthesis (Han et al., 1994; Rani and Subramoniam, 1997; Tom et al., 1987), but this premise has not been supported by any of the recent Vg mRNA expression studies (Tsutsui et al., 2000; Yang et al., 2000).

The strategies by which molt and reproductive cycles are coordinated in adult decapod crustacean females may vary among the different species (reviewed by Adiyodi, 1985; Meusy and Payen, 1988; Nelson, 1991). A close link between the molt and vitellogenic cycles has been demonstrated in the female of the caridean species *Macrobrachium rosenbergii*, which carries a mature ovary through ecdysis and oviposits immediately thereafter (Meusy and Payen, 1988; Wickins and Beard, 1974). In some brachyuran (Cheung, 1969; Kurup and Adiyodi,

1981) and astacidean species (Nelson, 1991), reproductive cycle(s) is (are) completed within a single intermolt period with marked seasonality. In some astacidean (Aiken and Waddy, 1980; Barki et al., 1997), brachyuran (Cheung, 1969) and caridean species (Wickins and Beard, 1974), reproductive cycle or egg incubation may delay ecdysis and lengthen the molt cycle. In penaeids resources are more or less simultaneously utilized for weight gaining and reproduction throughout the molt cycle (Adiyodi, 1985). Penaeid females may spawn several times during a single molt cycle, but if they do not spawn, the oocytes are resorbed before molting takes place (Emmerson, 1980, 1983; Qunitio et al., 1993). Although numerous studies have described the stages of vitellogenesis, only a few have studied the precise coordination between vitellogenesis and the molt cycle (Aiken and Waddy, 1980; Anderson et al., 1985; Barki et al., 1997; Cheung, 1969; Emmerson, 1980, 1983; Jayasankar et al., 2002; Kurup and Adiyodi, 1981; Nelson, 1991; Okumura and Aida, 2000; Qunitio et al., 1993; Wickins and Beard, 1974).

The Pacific white shrimp *Litopenaeus vannamei* is one of the most important shrimp species in aquaculture the world over. Thus, better understanding of the vitellogenic process and its genetic regulation and the relationship of these factors to the molt cycles and to their endocrine coordination in this species is of both basic scientific and applied importance. In the current study, we sequenced the *L. vannamei Vg (LvVg)* cDNA, which is the first open thelycum penaeid shrimp Vg transcript to be fully sequenced. We then analyzed the Vg cDNA products, and quantified Vg expression in the ovary and hepatopancreas at three major stages of the molt cycle in endocrinologically induced sub-adult females.

2. Materials and methods

2.1. Animals

Thirty-nine sub-adult, *L. vannamei* females, with body weights ranging from 18 to 31 g and ages ranging from 9 to 11 months, were obtained from a local inland brackish water shrimp farm. At the laboratory, they were introduced into closed seawater (~30 ppt) recirculating systems assembled from 130-L plastic tanks. Each tank held one or two animals. The animals were fed with polychaetes, squid, and commercial shrimp pellets to satiation. The water temperature was held between 27 and 29 °C, and a photoperiod regime of 14 light:10 dark was applied. After 10–17 days of acclimation, one eyestalk of each animal in one of the two systems was extirpated by ligation, after which the eyestalk fell off within a few days. The animals were tagged with a numbered colored ring around an eyestalk, and a colored patch was glued to their carapace, so the molt cycles and exu-

viations could be monitored precisely. Since the cuticle of the animal is partly translucent, ovarian development could be easily assessed from the anterior lobes of the ovary, which are located immediately beneath the carapace. The animals were observed carefully every day with the aid of an underwater flashlight so as to reduce manual handling to a minimum. All the animals underwent several molt cycles.

Animals were dissected only after the following two conditions had been met so that variability in weight and vitellogenic status parameters could be reduced to a minimum. First, the animal had to weigh between 29–35 g, and second, for induced females, at least one vitellogenic cycle had to be completed. If these conditions were met, late premolt animals were dissected by determination of molt stage using uropod pigment retraction and newly developed setae on uropods and pleopods (Dall et al., 1990; Robertson et al., 1987). Postmolt animals were dissected within 24 h of the exuviation, and intermolt/early premolt animals were dissected from the late-middle point of the molt cycle (based on the accumulated data for the specific individual and, in the case of induced animals, on the presence of a developed ovary). As a physiological marker of the vitellogenic stage of the animal, we used average oocyte diameter, rather than gonadosomatic index, which varies with body size (Palacios et al., 2003).

2.2. Sequencing and analysis of *Vg* cDNA

Total RNA was isolated from the ovaries of adult, secondary vitellogenic *L. vannamei* females with the aid of the TRIZOL Reagent (Invitrogen). First-strand cDNA was generated with oligo(d(T))₁₈ as primers using M-MLV reverse transcriptase H minus (Promega), according to the manufacturer's instructions. Sense and antisense primers were designed on the basis of the homology between two *Vg* cDNA sequences of closely related penaeid species, *Penaeus semisulcatus* and *Marsupenaeus japonicus* (Avarre et al., 2003; Tsutsui et al., 2000) and on the basis of the *LvVg* cDNA sequence itself as the sequencing progressed (GenBank entry AY321153). Amplifications were performed by the polymerase chain reaction (PCR) with 25 µl of reaction mixture containing 0.5 µM of each primer, 0.2 mM dNTP, 1× PCR buffer, and 0.2 U of Vent_R DNA Polymerase (New England BioLabs), under the following conditions: initial denaturation at 94 °C for 3 min; followed by 30 cycles at 94 °C for 1 min, variable annealing temperatures, depending on the composition and length of the primers, for 30 s–1 min and 72 °C for 1 min; ending with a final extension at 72 °C for 10 min. The PCR products were separated by 1.3% agarose gel electrophoresis, and the observed DNA band was excised, purified (QIAquick Gel Extraction kit, Qiagen), and then se-

quenced by means of the dideoxynucleotide chain termination method.

5' and 3' rapid amplification of cDNA ends (RACE) was carried out with the SMART RACE kit (BD Biosciences). PCR products were purified and cloned into a pGEM-T Easy Vector (Promega). Clones containing the inserts were isolated and allowed to grow overnight; plasmid DNA was purified (Qiagen Miniprep kit) and the inserts were sequenced. Assembly of all DNA sequence fragments was performed with the CAP (contig assembly program) EST (expressed sequence tags) Assembler at IFOM (Istituto FIRC di Oncologia Molecolare, <http://bio.ifom-firc.it/ASSEMBLY/assemble.html>). The Chromas original files of the sequenced fragments were consulted in each case of disagreement between sequenced fragments. Prediction of signal peptide cleavage site, putative endoprotease cleavage sites, and potential N-glycosylation and O-glycosylation sites was performed using algorithms from the CBS website (Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, www.cbs.dtu.dk). Basic sequence similarity searches and alignments were performed using the BLAST algorithm at the NCBI website.

2.3. Northern blot analysis

Total RNA was isolated from the ovary and hepatopancreas of an adult vitellogenic *L. vannamei* female. For each tissue, 7 µg RNA was electrophoresed through a formaldehyde 1% agarose gel, transferred to a nitrocellulose membrane, and UV-cross-linked. The blot was prehybridized overnight at 42 °C in 50% formamide, 5× saline sodium citrate (SSC), 0.05 M Na phosphate buffer, pH 6.6, 0.1 mg/ml denatured salmon sperm DNA, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 5 mM EDTA. A radiolabeled probe was prepared by adding dCTP together with a cDNA template isolated from a clone containing a *LvVg* cDNA insert (nt 5295–7658) to a random priming labeling mix (Biological Industries). The blot was incubated overnight in prehybridization buffer containing the above-described ³²P-labeled DNA. The membrane was washed twice in 2× SSC at 42 °C for 20 min, and then twice again in 1× SSC and 1% SDS at 65 °C for 20 min. To remove excess SDS, the membrane was rinsed in 0.1× SSC. The hybridized membrane was exposed to BioMax MS Kodak film with intensifying screens at –70 °C for 25 min. Ribosomal RNA was visualized with ethidium bromide.

2.4. Isolation of high-density lipoproteins from the ovary

High-density lipoprotein (HDL) isolation was performed as described by Lubzens et al. (1997). Adult

vitellogenic females were dissected, and the ovaries were pooled and homogenized in phosphate-buffered saline containing protease inhibitors. The homogenate was centrifuged, the supernatant was collected (after discarding cell debris), and HDLs were separated on a sodium bromide gradient by means of an ultracentrifuge. The uppermost orange fraction was collected and the protein concentration was determined by means of the Bradford method (Bradford, 1976). The sample was then separated by 4–12% gradient SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

2.5. Vitellin identification by matrix-assisted laser desorption ionization time-of-flight spectrometry

A 10- μ g sample of HDL from the ovary had been separated by 4–12% SDS–PAGE, the bands of interest were excised from the gel and washed thoroughly with double distilled water. In-gel digestion was carried out by modification of the protocol of Shevchenko et al. (1996). The polypeptide was reduced with 10 mM dithiothreitol for 30 min at 56 °C, followed by alkylation with 55 mM iodoacetamide for 20 min in the dark at room temperature. The polypeptide was digested overnight at 37 °C with 12.5 ng/ μ l trypsin (Roche Diagnostics GmbH, Mannheim, Germany, sequencing grade). Peptides extracted were collected and concentrated by vacuum centrifugation. Peptides originating from each polypeptide were characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry. The peptide mass fingerprints (PMFs) were compared to all known open reading frames (ORFs) in the GenBank by means of the ProFound algorithm (Chamrad et al., 2004; Zhang and Chait, 2000). Further analysis of the suggested peptide sequence distribution was used as a basis for postulating the location of the polypeptides on the *LvVg* deduced amino acid sequence.

2.6. Relative quantification of *Vg* mRNA by real-time PCR

Total RNA was isolated from the ovary, hepatopancreas, and muscle of each female by means of an EZ-RNA kit (Biological Industries). First-strand cDNA was generated with random hexamers as primers using AMV and M-MLV reverse transcriptases H plus (AB-gene), according to the manufacturer's recommendations. To compare *LvVg* expression in different samples, the relative abundance of *Vg* mRNA was normalized against the 18S subunit of rRNA as an endogenous reference. Although none of the current proposed normalization references for mRNA quantification is ideal, rRNA is considered a reliable reference (Bustin, 2002). Raw data were processed by means of the comparative C_t method by formula $2^{-\Delta\Delta C_t}$. The relative *Vg*

mRNA levels recorded in muscle, which does not express this gene, were used for calibration. Primers for *LvVg* cDNA amplified a 50-bp product (sense: nt 4753–4777, 5'-aatacaagaacgtgagggataggaa-3' and antisense: nt 4803–4775, 5'-aggcaatcacacttgatatttgtatttc-3'). Primers (sense: nt 563–583, 5'-tcggaaccgaggaatgattt-3' and antisense: nt 623–602, 5'-ctctagcgtcgagtagcgaatg-3') for 18S rRNA (GenBank entry AF186250) amplified a 60-bp product. Dissociation curve analysis indicated that the primers did not form a dimer structure. The PCR mixture consisted of 50 or 0.5 ng of a cDNA template for *Vg* cDNA amplification or for 18S rRNA amplification, respectively; 625 nM of each primer; and 10 μ l of 2 \times SYBR green Master mix (Applied Biosystems) in a final volume of 20 μ l. Amplification was carried out under the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C, and annealing followed by extension at 60 °C. Amplification of *Vg* cDNA and 18S rRNA cDNAs of the same sample was performed simultaneously in separate tubes in triplicate. Each plate contained repeated reference samples, and the pooled results for all plates were analyzed with the ABI Prism 7000 Sequence Detection System software version 1.1 (PE Applied Biosystems). Raw data for each plate were entered into a Microsoft Excel spreadsheet and the relative expression values of *LvVg* for all samples were calculated manually to confirm the results.

2.7. Statistical analysis

Data are presented as means \pm SD. Results were analyzed by one-way analysis of variance (ANOVA) using STATISTICA 6 software.

3. Results

3.1. *LvVg* cDNA sequencing and transcript size

The *LvVg* cDNA sequence was assembled from overlapping PCR fragments, each of which was amplified with different pairs of primers. Fragments were sequenced 2–10 times (Fig. 1), depending on the sequence quality. The assembled *LvVg* transcript is 7941 nucleotides long (Fig. 2), followed by a poly(A) tail.

Northern blot analysis (Fig. 3) further supported the sequenced *LvVg* transcript size, being approximately 8 kb and showing a single band both in the ovary and in the hepatopancreas, but not in the muscle tissue.

3.2. Characterization of the *LvVg* full-length cDNA sequence

An ORF search revealed a 29-nucleotide long 5' untranslated region (UTR) and a 151-nucleotide long

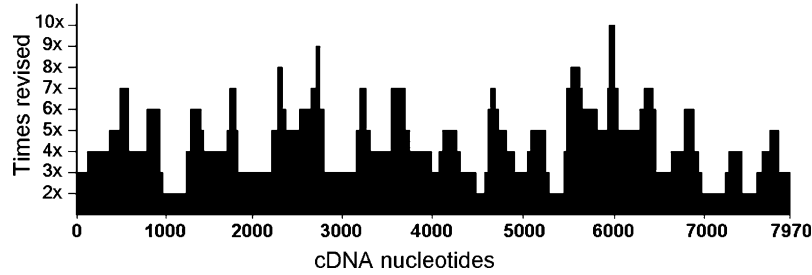


Fig. 1. Schematic representation of the *LvVg* cDNA sequencing process. The X-axis indicates the transcript size and the Y-axis indicates the number of times a certain segment has been sequenced (times revised).

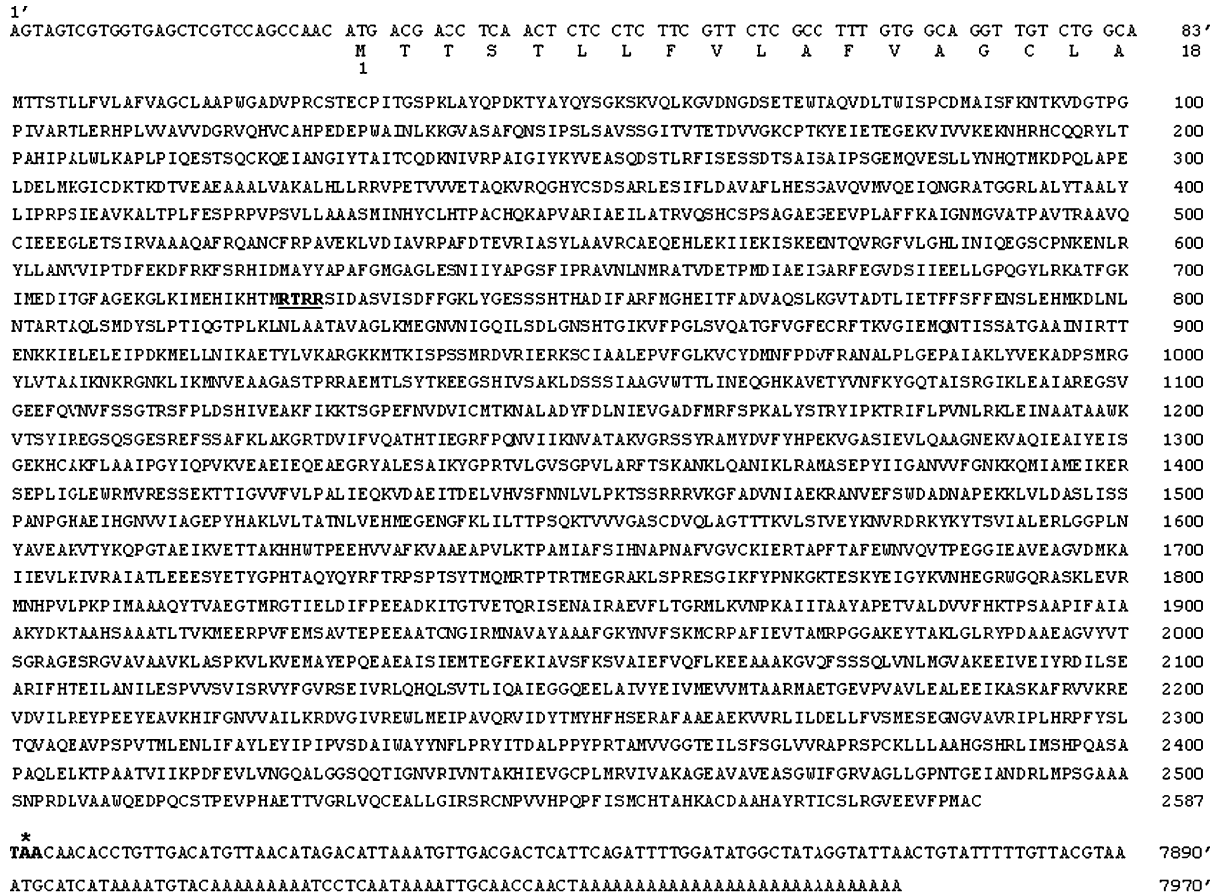


Fig. 2. Primary structure of the *L. vannamei Vg* deduced amino acid sequence and its 5' and 3' untranslated regions (UTRs). The first two lines show the 5' 83 cDNA nucleotides of *LvVg*, including the 5' UTR (nt 1–29) and the beginning of the ORF (nt 30–83) that putatively encodes the signal peptide (aa 1–18). The next 26 lines show the *LvVg* deduced amino acid sequence composed of 2587 amino acids with several potential consensus cleavage sites, out of which a single site (aa 725–728) that is believed to be cleaved is shown in underlined bold letters. The last two lines show the *LvVg* cDNA 3' UTR region (nt 7790–7970) begins with the ORF stop codon indicated in bold letters and marked with an asterisk. The sequence ends with the poly(A) sequence few nucleotides after its underlined signal.

3' UTR that includes a poly(A) tail signal (nt 7923–7929) (Fig. 2). The *LvVg* cDNA ORF encodes for 2587 amino acid residues with a predicted molecular mass of 283,462 Da and a *pI* of 6.55. The first 18 amino acids are hydrophobic and constitute a typical eukaryotic signal sequence (Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark), with a cleavage site located between Ala¹⁸ and Ala¹⁹.

Along the deduced amino acid sequence, seven cleavage sites might be recognized by enzymes of the subtilisin-like proprotein convertase (PCs) endoprotease family (Barr, 1991). Two of those cleavage sites correspond to the [R/K]-X-[R/K]-R consensus motif (aa 725–728 and aa 1008–1011) and the other five correspond to the R-X-X-R motif, the basic PC consensus motif (aa 943–946, aa 946–949, aa 1410–1413, aa 1732–1735, and aa 2373–2376). However, the artificial

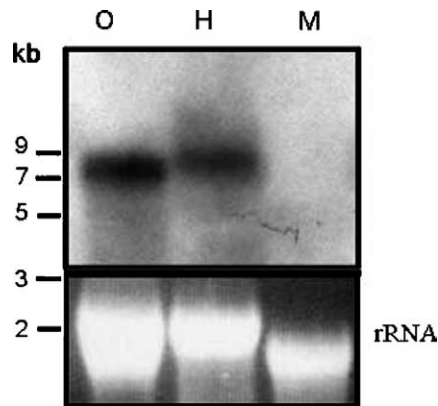


Fig. 3. Northern blot analysis of tissue-specific expression of *L. vannamei* Vg mRNA. Each slot was loaded with 7 μ g of total RNA purified from vitellogenic female tissue: ovary (O), hepatopancreas (H), and muscle (M). Bands of 7–9 kb, according to the RNA molecular size markers (left), appear in the lanes containing RNA from the ovary and the hepatopancreas. rRNA was visualized with ethidium bromide.

neural network ProP (Duckert et al., 2004) confirmed only one of the above-mentioned potential cleavage sites, i.e., that at the C-terminal of amino acid 728 immediately preceded by RTRR (Fig. 2B), and identified an additional site at the C-terminal of amino acid 2228, preceded by K-R, dibasic amino acids. The deduced amino acid sequence contains no potential N-glycosylation sites and only one potential O-glycosylation site, i.e., that at position 98 (Julenius et al., 2004).

3.3. Conservation of the *LvVg* ORF sequence

To study bioinformatically the conservation of the *LvVg* sequence, its ORF was divided into three segments: aa 1–1050 (which contains lipoprotein N-terminal domain and DUF1081, an apolipoprotein region with unknown function); aa 1050–2300; and from aa 2300 to the end of the sequence (which contains a von

Willebrand factor type D domain). Alignment of each of the three segments to its comparable segment in *Vgs* of the penaeids *P. semisulcatus* (Avarre et al., 2003), *M. japonicus* (Tsutsui et al., 2000), and *Fenneropenaeus merguensis* (data bank Accession No. AY499620) resulted in average amino acid identities of 88, 80, and 86% per segment, respectively (Table 1). However, applying the same alignment to *Vgs* of more distant decapod species, i.e., *Cherax quadricarinatus* (Abdu et al., 2002), *M. rosenbergii* (Okuno et al., 2002), and *Pandalus hypsinotus* (Tsutsui et al., 2004), gave average amino acid identities of 50, 31, and 46% per segment, respectively (Table 1). Although *Metapenaeus ensis* (Tsang et al., 2003) is a penaeid species, it gave intermediate identity values, i.e., 69, 56, and 73%, respectively. Among all decapod Vg sequences, the rate of amino acid substitution downstream to amino acid 1050 is higher than that for its upstream region. This difference in substitution rate suggests that the N-terminal region of polypeptides downstream to the conserved putative cleavage site around position 728 is conserved in decapod groups.

3.4. Identification of ovarian polypeptides encoded by *LvVg*

To map the empirically identified vitellin from the ovary onto the conceptual Vg sequence, HDLs were isolated from a vitellogenic ovary, separated by 4–12% SDS-PAGE (Fig. 4A), excised, and digested with trypsin. The masses of their peptide extracts (PMFs) were determined by MALDI-TOF analysis (Shevchenko et al., 1996). Five prominent HDL bands with approximate masses of 179, 113, 78, 61, and 42 kDa showed a statistically significant similarity ($P < 0.05$) to the PMF of a conceptual trypsin digest of the predicted *LvVg* ORF in a search against all GenBank ORFs using the ProFound algorithm (Zhang and Chait, 2000). Further analysis of each band's PMF data—considering the dis-

Table 1
Similarity to all known decapod *Vg* sequences of the three segments in *LvVg* ORF

Species	% identity to the <i>L. vannamei</i> vitellogenin sequence		
	aa 1–1050	aa 1050–2300	aa 2300–2587
<i>F. merguensis</i> (Data Bank Accession No. AY499620)	89	85	87
<i>P. semisulcatus</i> (Avarre 2003)	90	83	89
<i>M. japonicus</i> (Tsutsui 2000)	84	73	81
Average	88	80	86
<i>M. ensis</i> (Tsang 2003)	69	56	73
<i>C. quadricarinatus</i> (Abdu 2002)	52	35	48
<i>P. hypsinotus</i> (Tsutsui 2000)	51	30	47
<i>M. rosenbergii</i> (Okuno 2002)	47	27	43
Average	50	31	46

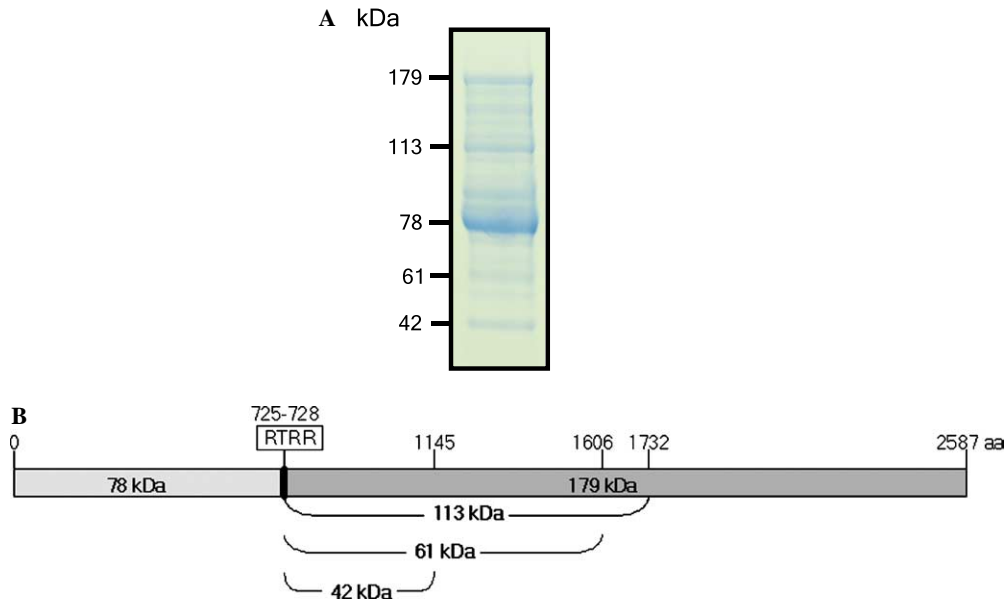


Fig. 4. (A) SDS-PAGE (4–12%) profile of HDLs from a vitellogenic ovary obtained by ultracentrifugation on a NaBr gradient. (B) Schematic presentation of the premature *Vg* protein and the postulated locations of its most prominent polypeptide products on the sequence, with a reference to the cleavage site motif at position 725–728.

tribution of their fitted sequences upon the *LvVg* ORF, the potential cleavage site (aa 725–728) and the ORF ends—enabled us to suggest a location for the five polypeptides upon the *LvVg* deduced amino acid sequence (Fig. 4B, Table 2).

All the tryptic peptides derived from the 78-kDa polypeptide that were identical to the *LvVg* ORF conceptual sequence matched it between deduced amino acids Leu⁴⁰ and Arg⁶⁹⁴; therefore, it is suggested that the 78-kDa polypeptide is encoded by the sequence between the N-terminal of the *LvVg* ORF and the putative cleavage site at the C-terminal of Arg⁷²⁸. The calculated size is consistent with the size observed following electrophoresis. Fourteen out of the 15 tryptic peptides derived from the 179-kDa polypeptide that were identical to the *LvVg* ORF conceptual sequences matched it between deduced amino acids Leu⁷⁴² and Arg²⁴⁹². Therefore, it is suggested that the 179-kDa polypeptide is

encoded by the sequence between Ser⁷²⁹, which immediately follows the putative cleavage site, and the C-terminal of the *LvVg* ORF, even though its calculated mass is approximately 204 kDa. It is also suggested that the other three polypeptides are cleavage products of the 179-kDa polypeptide and that the postulated N-terminal of the 113- and 42-kDa polypeptides is also Ser⁷²⁹ (Fig. 4B, Table 2), but their C-terminal boundary is uncertain. The 61-kDa PMF covers a sequence with a significantly higher calculated mass, therefore its location is thought to be somewhere between deduced amino acids 729 and 1606, but its exact boundaries remain unclear.

3.5. Vitellogenesis and the molt cycle in induced and intact sub-adult females

Following endocrinological induction of sub-adult females by unilateral eyestalk ablation, the initiation of ovarian development was noted in the first one to five postoperative molt cycles in 75% of the animals. The developed ovaries, which were visible through the carapace during the intermolt and early premolt stages, were resorbed one to 3 days before ecdysis. In most cases, the ovary redeveloped after ecdysis. Almost all the females, in which ovarian development was not observed after induction, died a few days to several molt cycles later. No ovarian development was observed in females from the intact group at any molt stage. Molt cycles of induced females were significantly shorter than those of the intact animals (12.7 ± 2 ($N = 23$) vs. 14.0 ± 2.0 ($N = 22$); $P < 0.05$).

Table 2

The prominent ovarian HDL polypeptides (Fig. 4A), which were identified as vitellin based on their peptide mass fingerprints, and their postulated location based on the matched peptide distribution upon the sequence

Approximate polypeptide mass (kDa)	Number of peptides matched to <i>Vg</i>	Postulated location on <i>LvVg</i> sequence		
		Number of peptides	Range (aa)	Expected mass (kDa)
78	10	10	19–728	77.689
179	14	13	729–2587	203.736
113	15	13	729–1732	110.137
61	16	12	729–1606	96.238
42	15	12	729–1145	45.462

3.6. Oocyte growth and relative quantification of *Vg* expression during the molt cycle

Relative levels of *Vg* mRNA in the ovary and hepatopancreas in intact and induced sub-adult *L. vannamei* females were determined by real-time PCR at three points of the molt cycle (postmolt, A; intermolt/early premolt, C–D₀–D₁, and late premolt, D₂–D₃) (Fig. 5). Body weight and oocyte diameter were recorded for these six groups of females (Table 3). The induced intermolt/early premolt group had a significantly ($P < 0.05$) larger oocyte diameter than those of the other groups. Expression in ovaries of induced intermolt–early premolt females was significantly higher compared to all other sub-groups ($P < 0.05$). In the induced group, the ovary contained significantly more *Vg* transcripts than the hepatopancreas at all molt cycle stages ($P < 0.05$). On the other hand, expression of *LvVg* in the ovary and hepatopancreas of intact animals exhibited no significant variation over the molt cycle.

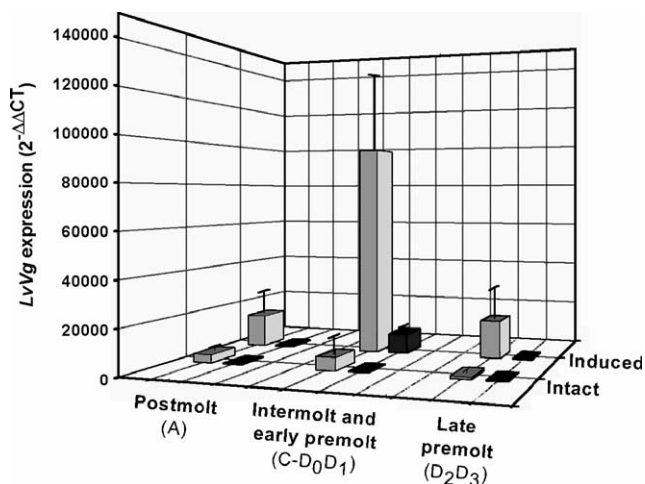


Fig. 5. Relative quantification using real-time PCR of *Vg* mRNA levels at different molt stages in sub-adult *L. vannamei* females. *Vg* gene expression of each sample was normalized to its 18S ribosomal RNA and then again to muscle mRNA according to the comparative C_t methods using the formula $2^{-\Delta\Delta C_t}$. The level of expression thus represents an average expression of four animals, except the column for the intermolt/early premolt induced group, which represents results of five animals. Results are presented as means \pm SD, dark gray columns, hepatopancreas; light gray columns, ovary. Expression in ovaries of induced intermolt–early premolt females (marked with an asterisk) was significantly higher compared to all other sub-groups. Expression in ovaries was significantly higher compared to hepatopancreas in the induced group at all molt cycle stages.

4. Discussion

In this study, we unravelled the complete *LvVg* transcript sequence and its differential expression both in the ovary and hepatopancreas at three molt stages. Like other known decapod *Vg* sequences, the *LvVg* transcript is approximately 8 kb in size, with an approximately 2600 amino acid deduced polypeptide encoded by its ORF.

The *LvVg* ORF sequence does not possess any potential N-glycosylation sites. Screening all known decapod *Vg* sequences for basic conditions for N-glycosylation motifs (i.e., Asn-X-Ser/Thr) suggests the following: *Vgs* of the penaeids *M. japonicus* and *F. merguensis* contain no potential N-glycosylation sites; those of *P. semi-sulcatus* and *M. ensis* contain 1 and 2 or 3 potential sites, respectively, and in sharp contrast to these dendrobranchiata species, the pleocyemata species *C. quadricarinatus*, *M. rosenbergii*, and *P. hypsinotus* contain 11, 11, and 10 potential N-glycosylation sites, respectively. In addition, experimental studies have confirmed three of the potential N-glycosylation sites in *C. quadricarinatus* (Khalaila et al., 2004). In the *LvVg* ORF, only one O-glycosylation site was predicted. However, in contrast to N-glycosylation, no O-glycosylation consensus sequence has yet been formulated, and the few prediction methods available rely on very little data, mainly from mammalian species (Julenius et al., 2004). Since glycan moieties may play a role in protein folding, processing, and transport, further investigation is thus required to elucidate the differences between the vitellogenic processes of different species and taxonomic groups in relation to the presence or absence of glycan moieties, in particular N-glycans.

The division of the *LvVg* ORF to three segments was performed out of considerations based on different degrees of conservation (Table 1). The intensity of the purifying selection is higher in segments 1 and 3. The latter is probably due to functional importance in receptor recognition, association of lipids, and/or binding of nutrients.

In this study, we identified five ovarian polypeptides encoded by the *Vg* gene (Fig. 4, Table 2). There are, however, a number of studies describing different ovarian polypeptide patterns from vitellogenic *L. vannamei* (Garcia-Orozco et al., 2002; Quackenbush, 1989; Tom et al., 1992; Vazquez-Boucard et al., 2003). This variability could be due to the use of different separation

Table 3
Average weight and oocyte diameter of induced and intact sub-adult females throughout the molt cycle

	Postmolt		Intermolt/early premolt		Late premolt	
	Intact ($N = 4$)	Induced ($N = 4$)	Intact ($N = 4$)	Induced ($N = 5$)	Intact ($N = 4$)	Induced ($N = 4$)
Weight (g)	30.7 \pm 1.5	32.8 \pm 1.3	30.8 \pm 1	31.9 \pm 2.1	31.5 \pm 2.1	30.5 \pm 1.3
Oocyte diameter (μm)	46 \pm 13	63 \pm 51	51 \pm 22	185 \pm 57	45 \pm 12	46 \pm 9

methods and the dissection of ovaries in different vitellogenic stages. Our repeated separations of ovarian HDL by SDS–PAGE revealed a stable and prominent band at approximately 78 kDa and several less prominent bands at different stain strengths. It seems that the appearance of the low-molecular-weight bands (i.e., 42 and 61 kDa) is at the expense of the high-molecular-weight bands (i.e., 113 and 179 kDa). Although we did not map the exact structure of vitellin polypeptides on the ORF deduced amino acids, the PMFs of all five examined bands showed an overall coverage of the sequence (Fig. 4B, Table 2). The use of PMF of each band in an attempt to locate the polypeptides on the ORF sequence was performed manually without statistical tests and without MS–MS confirmations, and therefore should be regarded as tentative.

The PC putative cleavage site consensus motif sequence at position 725–728 (Fig. 4B) is the most conserved putative PC site in all known decapod *Vg*s. This site received a high score in all decapod *Vg* sequences when the ProP algorithm was applied. Experimental studies have confirmed this cleavage site in the pleocyemate *M. rosenbergii* (Okuno et al., 2002) and the dendrobrachiate *P. semisulcatus* (Avarre et al., 2003). All the *LvVg* 78-kDa PMFs were located between positions Leu⁴⁰ and Arg⁶⁹⁴. The N-terminal boundary of all the other identified vitellin polypeptides is apparently located immediately downstream to the putative cleavage site at position Arg⁷²⁸. The N-terminal sequence of an *L. vannamei* ovarian 78-kDa polypeptide (Garcia-Orozco et al., 2002) was found to be similar to the N-terminals of other known decapod *Vg*s. All these findings suggest that the putative cleavage site at position Arg⁷²⁸ in *LvVg* ORF does indeed undergoes cleavage. The many ovarian HDL polypeptides and their partial correspondence to the sizes predicted from the potential subtilisin-like PC endoprotease sites suggests the existence of additional cleavage sites and perhaps the participation of an additional endoprotease family. If this is so, then we must postulate additional polypeptides encoded by the C-terminal of the *LvVg* ORF that were not empirically identified among the *L. vannamei* vitellins in this study.

Scheduling of vitellogenesis within the molt cycle has been studied in a variety of species, mainly in the ecological and physiological context (Aiken and Waddy, 1980; Anderson et al., 1985; Cheung, 1966, 1969; Emmerson, 1980, 1983; Kurup and Adiyodi, 1981; Nelson, 1991; Qunitio et al., 1993; Rice and Armitage, 1974; Webb, 1977; Wickins and Beard, 1974). It has already been demonstrated in some brooding species that egg incubation (Barki et al., 1997; Cheung, 1969; Scudamore, 1948) or vitellogenesis and ovarian development (Aiken and Waddy, 1980; Wickins and Beard, 1974) might delay ecdysis. Ovarian growth is associated with intermolt stages

but females could possibly molt while having full-size matured ovaries (Cheung, 1966) or intermediate-stage ovaries, for example, in *C. quadricarinatus* (unpublished findings from our laboratory), in some lobsters (Aiken and Waddy, 1980), and in the caridean prawn *M. rosenbergii* (Okumura and Aida, 2000; Wickins and Beard, 1974). In penaeid females, whether intact or induced, several vitellogenic cycles could be observed during a single molt cycle, even though their ovaries will always be resorbed prior to ecdysis (Anderson et al., 1985; Emmerson, 1980, 1983; Qunitio et al., 1993). In many studies of the interrelationship between molt and vitellogenesis, the information presented is not adequate for drawing definite conclusions, since data on molt and vitellogenic stages (e.g., number of spawns, gonadosomatic index, average oocyte diameter, ovary pigmentation, etc.) were not taken from the same individuals or for each sampling only one parameter was documented from single animals (Barki et al., 1997; Cheung, 1969; Kurup and Adiyodi, 1981; Rice and Armitage, 1974; Webb, 1977). Individual documentation of molt stage and of a precise upstream vitellogenic parameter could shed further light on the interrelationship between the molt cycle and vitellogenesis. Jayasankar et al. (2002) described the *Vg* gene expression pattern along the molt cycle of the prawn *M. rosenbergii*. In that study, *Vg* mRNA levels started to increase as the molt cycle progressed but decreased sharply prior to the molt event. This pattern could be clearly seen in the induced *M. rosenbergii* group, but it is not clear whether it also represented the intact *M. rosenbergii* group. In the same species, Okumura and Aida (2000) described a decrease in *Vg* levels in the hemolymph, which started at molt stage D₂ and continued until oviposition after ecdysis. Avarre et al. (2003) shows, in *P. semisulcatus*, that the postmolt *Vg* gene expression level was lower than that in the intermolt state in previtellogenic females. However, interestingly, *Vg* gene expression levels in the ovaries of previtellogenic and vitellogenic females were the same.

The current work describing the differential expression of the *Vg* gene with precise relation to distinct time points of the molt cycle is the first of its kind in penaeids. The present study shows significantly higher levels of *Vg* mRNA in the ovary than in the hepatopancreas in endocrinologically induced females (Fig. 5). In contrast, ovarian and hepatopancreatic *Vg* mRNA levels in naturally matured *M. japonicus* and *P. semisulcatus* females were shown to be similar (Avarre et al., 2003; Tsutsui et al., 2000). The difference between our results and the latter studies may be related to differences between endocrinologically induced and intact animals or between mature and immature animals or they may be related to species differences. Although removal of one source of MIH/GIH secretion disrupts normal hormon-

al control, *Vg* gene expression along the molt cycle in the current study seems to follow the known vitellogenesis pattern in penaeids (Emmerson, 1980): *Vg* gene expression was high in the intermolt/early premolt stages and was downregulated immediately before and after ecdysis (Fig. 5). In the postmolt and late premolt stages, *Vg* mRNA levels were higher in the induced group than in the intact group. This difference is probably due to the endocrine induction. Molt cycle lengths in all treatment groups spanned 8–20 days. Since vitellogenesis and *Vg* gene expression are closely linked to the molt cycle, future studies on vitellogenesis must refer to the molt cycle.

The observed ovarian resorption in *L. vannamei* females between 1 and 3 days before ecdysis (at the approximate molt stage of D₂–D₃) and the redevelopment of the ovaries approximately 1 day after ecdysis (a time point that corresponds to molt stage B) are in keeping with the quantities of *Vg* mRNA measured at these periods. Dall (1986) showed that metabolic rates during the molt cycle of *Penaeus esculentus* did not change until the late premolt stage (3 days prior to ecdysis), when it increased by 55%, and then fell to previous levels after 1 day. Presumably, there is a transition point at D₂–D₃ at which the hormonal balance causes an extreme change in the physiological programming of the animal. At this point, expression of a set of genes causes the animal to undergo ecdysis, and another set of genes, among them the *Vg* gene and probably other vitellogenesis-related genes, is downregulated. *Vg* gene could serve as a leading gene for determining a more precise timing of this transition point and for identifying additional factors involved in these processes.

In summary, we sequenced and characterized the complete length of the *L. vannamei* *Vg* transcript. We showed that *Vg* gene expression is significantly higher in the ovary than in the hepatopancreas in induced sub-adult females and the pattern of *Vg* expression has an inverse relationship with the molt event, i.e., *LvVg* is downregulated in proximity to molt event and its level rises again in the intermolt/early premolt stages. The findings of this study could be used as guidelines for studies focusing on the interrelationship between molt and vitellogenesis processes at the molecular level using the *Vg* gene as a leading gene.

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