BIOLOGY OF REPRODUCTION **73,** 72–79 (2005)
Published online before print 2 March 2005.
DOI 10.1095/biolreprod.104.038554

Expression of the Reproductive Female-Specific Vitellogenin Gene in Endocrinologically Induced Male and Intersex *Cherax quadricarinatus* Crayfish[1]

Asaf Shechter,[3] Eliahu D. Aflalo,[3] Claytus Davis,[4] and Amir Sagi[2,3]

Departments of Life Sciences[3] and Molecular Genetics of Development,[4] Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

ABSTRACT

In oviparous females, the synthesis of the yolk precursor vitellogenin is an important step in ovarian maturation and oocyte development. In decapod Crustacea, including the red-claw crayfish (*Cherax quadricarinatus*), this reproductive process is regulated by inhibitory neurohormones secreted by the endocrine X-organ-sinus gland (XO-SG) complex. In males, the *C. quadricarinatus* vitellogenin gene (*CqVg*), although present, is not expressed under normal conditions. We show here that endocrine manipulation by removal of the XO-SG complex from male animals induced *CqVg* transcription. The *CqVg* gene was expressed differentially during the molt cycle in these induced males: no expression was seen in the intermolt stages, but expression was occasionally detected in the premolt stages and always detected in the early postmolt stages. Relative quantitation with a real-time reverse transcriptase-polymerase chain reaction showed that expression of *CqVg* in induced early postmolt males was an order of magnitude lower than that in reproductive females, a finding that was consistent with RNA in situ hybridization results. The SDS-PAGE of high-density lipoproteins from the hemolymph of endocrinologically induced early postmolt males did not show the typical vitellogenin-related polypeptide profile found in reproductive females. On the other hand, removal of the XO-SG complex from intersex individuals, which are chromosomally female but functionally male and possess an arrested female reproductive system, induced the expression, translation, and release of *CqVg* products into the hemolymph, as was the case for vitellogenic females. The expression of *CqVg* in endocrinologically manipulated molting males and intersex animals provides an inducible model for the investigation and understanding of the endocrine regulation of *CqVg* expression and translation in Crustacea as well as the relationship between the endocrine axes regulating molt and reproduction.

gene regulation, oocyte development, ovary, ovulatory cycle

INTRODUCTION

In all oviparous animals, the process of vitellogenesis includes the provision of proteins, lipids, carbohydrates, and other resources to the maturing oocytes for sustenance of the developing embryo [1, 2]. Most of the protein constituents of yolk form a high-density lipoprotein (HDL) known as vitellin [3, 4]. In female Crustacea, a yolk-protein precursor known as vitellogenin is produced in specific tissues during vitellogenesis. Vitellogenin often undergoes additional processing into smaller molecules and is subsequently accumulated in the developing oocytes as vitellin. In females of the red-claw crayfish (*Cherax quadricarinatus*), vitellogenin is synthesized in the hepatopancreas and then transferred to the hemolymph, where it is found as three major lipoprotein polypeptides of 208, 196, and 177 kDa, with the latter being termed LPII [5]. These polypeptides, which are specific indicators of female secondary vitellogenesis, are accumulated as vitellin in the developing oocytes [6].

Several vitellogenin cDNA sequences of different Crustacea have been obtained in recent years. Currently, eight complete decapod vitellogenin cDNA sequences are listed in the GenBank database [7–12] (accession nos. AY321153 and AY499620). The vitellogenin from *C. quadricarinatus* is encoded by a single gene (*CqVg*), the cDNA of which has been cloned and sequenced in our laboratory [8].

Although vitellogenin synthesis typically is specific to female reproduction, rare cases of male crustaceans synthesizing vitellogenin have been reported in the literature [13–15].

In decapod Crustacea, reproduction is controlled by the neurohormone gonad-inhibiting hormone (GIH) secreted by the X-organ-sinus gland (XO-SG) complex in the eyestalk [16]. This neurohormone is part of a multifunctional family of hormones related to the crustacean hyperglycemic hormone (CHH) that has been widely studied in several species [17, 18]. The inhibitory effect of GIH on crustacean reproduction [19, 20] was related to gonad maturation in both males and females [21], including inhibition of vitellogenin synthesis in extraovarian sites [22]. Removal of the XO-SG complex accelerated female reproductive processes, such as ovarian maturation [4] and vitellogenin production, that presumably are initiated by a decrease in GIH levels.

The XO-SG complex also regulates molting. Because molt-inhibiting hormone (MIH) inhibits the secretion of ecdysone by the Y-organ, removal of the XO-SG complex induces a rapid molt cycle [23]. In several crustaceans, calcium from the old exoskeleton is transported before molting through the hemolymph into calcium-storage organs known as gastroliths [24], which are located on both sides of the stomach. Following molting, the gastroliths collapse into the stomach, where they dissolve, and the stored calcium becomes available for production of the new cuticle. Thus, changes occurring in the size of the gastroliths during the molt cycle provide an accurate tool for determination of the molt stage [25]. In the present study, the gastrolith index (GI), which expresses the relationship of gastrolith weight to body weight, was used to determine the molt stage. Re-

[1]Supported in part by U.S.-Israel Binational Science Foundation (BSF) grant 2000116.
[2]Correspondence: Amir Sagi, Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel. FAX: 972 8 6472992; e-mail: sagia@bgu.ac.il

Received: 30 November 2004.
First decision: 27 December 2004.
Accepted: 25 February 2005.
© 2005 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. http://www.biolreprod.org

moval of the XO-SG complex caused male crayfish to enter an accelerated molt cycle through reduction in MIH levels.

Although the biochemistry and physiology of CHH and MIH have been studied extensively, very little is known about the effect of GIH on vitellogenin expression in extraovarian tissues, such as the hepatopancreas. In reproductive females, an additional factor, the vitellogenin-stimulating ovarian hormone (VSOH), is thought to stimulate expression of vitellogenin in extraovarian tissues [26], whereas the male androgenic gland (AG) [6] inhibits expression of *CqVg* [27] along with female characteristics.

The species *C. quadricarinatus* occasionally exhibits natural sexual plasticity in that intersex individuals that are functional males are also genetic females [28]. The unique reproductive system of these intersex animals consists of a functional male system and a permanently arrested female gonad [29]. Removal of the AG from such animals induces *CqVg* expression and a shift of the arrested female reproductive system toward the vitellogenic state [30], illustrating the inhibitory effect of the AG. A comparison between *C. quadricarnatus* males and intersex animals thus may constitute a useful model with which to study the possible involvement of factors from the XO-SG complex, ovary, and AG in the regulation of vitellogenin gene expression and translation.

MATERIALS AND METHODS

Animals

Female, male, and intersex *C. quadricarinatus* were grown in artificial ponds at Ben-Gurion University of the Negev. Food comprising shrimp pellets (30% protein; Rangen, Inc., Buhl, ID) and wheat grains were supplied ad libitum three times a week. The temperature was held at 27 ± 2°C, and a 14L:10D photoperiod was applied. Water quality was assured by circulating the entire volume of water through a biofilter. During the experiment, the pH of the water was 8.3 ± 0.5, the nitrite concentration less than 0.1 ppm, and the nitrate concentration less than 50 ppm. Ammonium levels were negligible, and oxygen exceeded 5 mg/L.

Vitellogenic females were collected during the reproductive period (April–November). Intact male and intersex crayfish were either dissected on ice or subjected to bilateral removal of the XO-SG complex through eyestalk ablation and held in individual cages for up to 19 days. Intact males with no apparent gastroliths were referred to as intermolt animals. The endocrinologically manipulated males were anesthetized on ice during the molt cycle and were classified as premolt or postmolt animals according to their GI values and the location of the gastroliths (i.e., on the stomach wall for premolt animals and inside the stomach for postmolt animals).

Molt Staging by GI

The GI, used as a measure of molt stage, was determined as

$$GI = (\text{gastrolith weight/body weight}) \times 100$$

The GI increased from 0 to 4.3 during the premolt stage and fell from 4.3 to 0 during the postmolt stage.

Amplification and Sequencing of CqVg

To detect vitellogenin gene expression, hepatopancreas and abdominal muscle fragments were sampled. The RNA was extracted (EZ-RNA kit; Biological Industries Ltd., Kibbutz Beit Haemek, Israel) from each sample, and first-strand cDNA was generated with oligo $(dT)_{18}$ as the primer by means of a reverse-transcriptase (RT) reaction (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany) and amplified by a polymerase chain reaction (PCR) using three different sets of primers based on the sequence of the *CqVg* gene [8]. The nucleotide sequences of the primers used for the PCR were

Primer 1-F: 5′-TGCTCCACIGAATGTCCCATC-3′
Primer 1-R: 5′-GCAACACCCTTYTTIAGGTTGAT-3′
Primer 2-F: 5′-CTTTCCGTGAATTCCTAAGGCGTCCG-3′
Primer 2-R: 5′-ATCATAAGTACCCAGAAGACCTGCAATGCG-3′
Primer 3-F: 5′-AACGAGAGCCAGTCTTTGTGGCTG-3′
Primer 3-R: 5′-CAGCTTGTAGCTGTATGGACTACCAAG-3′

where F indicates forward and R indicates reverse. The RT-PCR products were subjected to 1% agarose gel electrophoresis with Tris-borate EDTA buffer. Fragments were purified and sequenced by means of the dideoxynucleotide-chain termination method.

Quantitative Real-Time RT-PCR

First-strand cDNA was generated with random hexamers as primers by means of an RT reaction (Reverse-iT™-1st Strand Synthesis Kit; ABgene, Tamar Laboratory Supplies, Jerusalem, Israel) using hepatopancreas mRNA as the template. Relative quantitation of *CqVg* gene expression was performed using primer 4-QPCR-F (5′-GCTTCCCGGTGGTT AATCCT-3′), primer 4-QPCR-R (5′- GGGCGGCATGACACACATCT-3′), and SYBR Green PCR Master Mix (Applied Biosystems) with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) as follows: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min.

Histological Sample Preparation

Hepatopancreas and muscle tissue samples were stored in acetone at −70°C for 5 days. The acetone from each sample was replaced with fresh cold acetone each day. Tissue samples were fixed in modified Carnoy II for 24 h and then dehydrated gradually through a series of increasing alcohol concentrations. Tissues were cleared and embedded in paraplast (Paraplast Plus; Kendall, Tyco Healthcare, Mansfield, MA) according to conventional procedures. Sections (thickness, 5 μm) were cut onto silane-coated slides (Superfrost Plus; Menzel-Gläser, Braunschweig, Germany). Sections were stained with hematoxylin-eosin.

In Situ Hybridization

Digoxygenin (DIG)-labeled oligonucleotides for antisense and sense probes corresponding to nucleotides 3346–4100 of *CqVg* cDNA (GenBank accession no. AF306784) were synthesized using SP6 and T7 RNA polymerases. Slides with samples from the hepatopancreas and muscle were deparaffinized, rehydrated, and rinsed in diethyl pyrocarbonate (DEPC)-treated, double-distilled water. Slides were fixed in 4% paraformaldehyde in PBS (10 mM phosphate buffer Na_2HPO_4, 150 mM NaCl, pH 7.4) for 4 min and washed twice in fresh PBS containing 0.1% DEPC for 20 min. Samples were digested with 5 μg/ml of proteinase K (Roche Diagnostics GmbH) in 100 mM Tris-HCl and 50 mM EDTA (pH 7.6) for 10 min in 37°C, incubated in PBS containing 2 mg/ml of glycine for 10 min, and washed twice in PBS for 3 min. Slides were prehybridized overnight at 42°C in hybridization buffer (50% formamide, 2.25× saline sodium citrate, 10% dextran sulfate, 2.5× Denhardt solution, 5 mM dithiothreitol, 40 U/ml of RNase inhibitor, and 0.1 mg/ml of sheared and denatured salmon sperm DNA). Hybridization was performed with 0.1 μg/ml of antisense and sense probes in hybridization buffer (identical to prehybridization buffer). The slides were washed with 3× SSC (1× SSC: 0.15 M sodium chloride and 0.015 M sodium citrate) at room temperature for 5 min and then washed in NTE buffer (500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The slides were then incubated in NTE buffer containing 50 μg/ml of RNase-A for 30 min at 37°C, washed in 2× SSC at room temperature, and incubated for 1 h in 0.1× SSC at 57°C. Thereafter, the slides were washed with Tris-HCl buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 5 min at room temperature and blocked with blocking buffer (1% blocking reagent in Tris-HCl buffer; Roche Diagnostics) for 30 min at room temperature. Finally, slides were incubated with a diluted (1:200) anti-DIG-AP conjugate (Roche Diagnostics) for 120 min at room temperature. The DIG was visualized with colorimetric substrates NBT/BCIP (Roche Diagnostics) according to the manufacturer's instructions. Slides were mounted in 90% glycerol and observed under a light microscope.

Southern Blot Analysis

The DNA was isolated from the sperm cells of adult *C. quadricarinatus* by standard genomic methods. Genomic DNAs (20 μg each) were digested with *Not*I, *Mbo*I, *Hin*dIII, *Eco*RI, and *Bgl*II (New England Biolabs, Inc., Beverly, MA). The digested DNA fragments were separated by electrophoresis on a 0.7% agarose gel at 50 V for 20 h. The DNA fragments were transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany) and cross-linked by ultraviolet irradiation.

Random-primed ^{32}P-labeled (Random Primer DNA Labeling Mix; Biological Industries) *CqVg* cDNA was added for hybridization overnight at 42°C. Following hybridization, the membranes were washed once each with 2× SSC and 0.1% SDS at room temperature for 30 min and then twice each with 0.1× SSC and 0.5% SDS at 65°C for 30 min before exposure to x-ray film at −70°C.

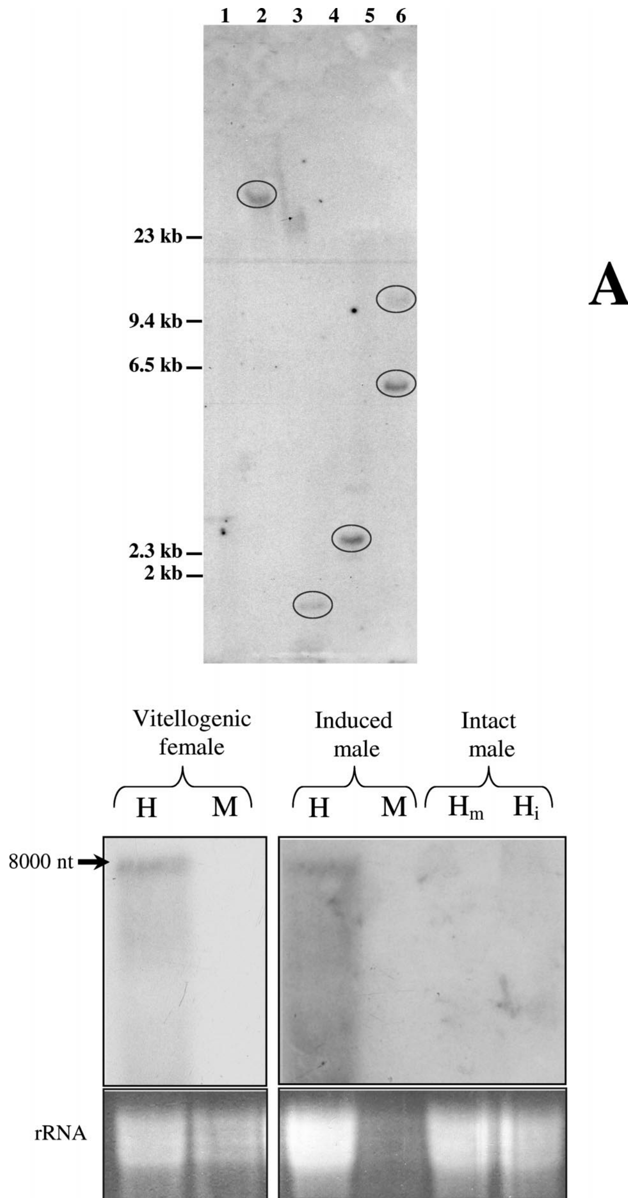

FIG. 1. Presence of a *CqVg* gene copy and its transcript size in the male *C. quadricarinatus.* **A**) Southern blot analysis. Male genomic DNA was cut with the restriction endonucleases indicated and electrophoresed through a 1% agarose gel. Following transfer to nitrocellulose, the blot was probed with a ^{32}P-radiolabeled probe, which covers nucleotides 3346–4100 of the 8000-nucleotide cDNA length. The positions of marker band fragments are indicated and their sizes listed in kilobase pairs (kb). Lane 1: uncut DNA; lane 2: *Not*1; lane 3: *Mbo*1; lane 4: *Hin*dIII; lane 5: *Eco*RI; lane 6: *Bgl*II. The ellipses are used to emphasize the bands. **B**) Northern blot analysis. Expression of the gene was examined using a radioactive *CqVg* gene probe in the hepatopancreas (H) and abdominal muscle (M) of a reproductive female (left) and an endocrinologically induced early postmolt male. Hepatopancreas samples from an intact intermolt male (H_i) and an intact molting male (H_m) also were examined. Longer exposure of hybridized membrane to film was needed for visualization in male samples for visualization; thus, this fraction is separated (right). Ribosomal RNA demonstrated RNA extraction.

Northern Blot Hybridization

The RNA from muscle and hepatopancreatic tissue of reproductive females and endocrinologically induced and intact males was extracted as described above. Hepatopancreatic tissue also was taken from intact molting and intermolt males. For each sample, 8 μg of RNA were separated on a formaldehyde 1% agarose gel, transferred to a nitrocellulose membrane, and ultraviolet cross-linked. Blot was prehybridized overnight at 42°C in 50% formamide, 5× SSC, 0.05 M sodium phosphate buffer (pH 6.6), 0.1 mg/ml of denatured salmon sperm DNA, 5× Denhardt solution, 0.5% SDS, and 5 mM EDTA. A *CqVg* probe was prepared using a random-priming labeling mix (Biological Industries), ^{32}P-radiolabeled deoxycytidine triphosphate, and 25 ng of cDNA template from nucleotides 3346–4100 of *CqVg* cDNA (GenBank accession no. AF306784). The membrane was incubated overnight in hybridization buffer containing the radiolabeled probe. The membrane was washed twice in 2× SSC at 42°C for 20 min and then twice each 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 x-ray film with intensifying screens at −70°C for 2 h for visualization of the female expression and 48 h for visualization of the male expression. Ribosomal RNA was visualized with ethidium bromide.

HDL Analysis

The HDL was separated from the hemolymph of endocrinologically induced males of different stages of the molt cycle (determined in terms of the GI), intact intermolt males, intact intersex animals, induced intersex animals, and reproductive females. For each crayfish, 2–4 ml of hemolymph were withdrawn from the sinus between the third and fourth walking leg, with a 2.5-ml syringe fitted with an 18-gauge needle. Both needle and syringe had been washed twice using 7% EDTA to prevent coagulation. Also, 100 μl/ml of 7% EDTA were added to the hemolymph samples, which were stored on ice. A saturated solution of sodium bromide was added to the hemolymph samples to bring the final density to greater than 1.22 g/ml. The HDL fraction from the hemolymph was separated by ultracentrifugation at 160 000 × *g* for 24 h at 4°C, collected as the supernatant and dialyzed against a cellulose tubular membrane (Sep T3) with a nominal molecular weight cutoff of 12 000–14 000 (Membrane Filtration Products, Inc., Seguin, TX) in PBS buffer overnight at 4°C. Protein concentration was determined using Bradford reagent. The isolated lipoprotein was subjected to 7% SDS-PAGE [31] in a steady current and changing voltage (from 80 to 120 V) for approximately 1.5 h using molecular mass markers of 200, 148, 98, 64, 50, and 36 kDa (Broad Range; Bio-Rad, Hercules, CA). The gel was stained with Coomassie brilliant blue R-250 for general protein staining.

RESULTS

In *C. quadricarinatus*, the female is the heterogametous sex. Therefore, the possibility existed that the *CqVg* gene, the expression of which had been observed previously only in females, would be located on the female-specific sex chromosome. The presence of the *CqVg* gene in males was confirmed using Southern blot hybridization with a *CqVg* specific probe (Fig. 1A). To determine whether transcription of the *CqVg* gene could be detected in both intact and endocrinologically induced males, RNA was extracted from the hepatopancreas of an induced postmolt male, an intact postmolt male, an intermolt male, and a vitellogenic female. The band observed at 8000 nucleotides in induced males (Fig. 1B, right) was the same size as that observed in the secondary vitellogenic female hepatopancreas (Fig. 1B, left) [8]. No band was observed in the intact male sample (Fig. 1B, right). Expression of *CqVg* also was detected using RT-PCR with two sets of primers generated from different ends of the known gene sequence (Fig. 2). Consistent with the Northern blot data, only the hepatopancreas of endocrinologically induced early postmolt males and reproductive females showed *CqVg* expression. Sections amplified by each primer set were sequenced and exhibited 100% homology to the corresponding regions of the *CqVg* gene shown to be expressed in reproductive females [8].

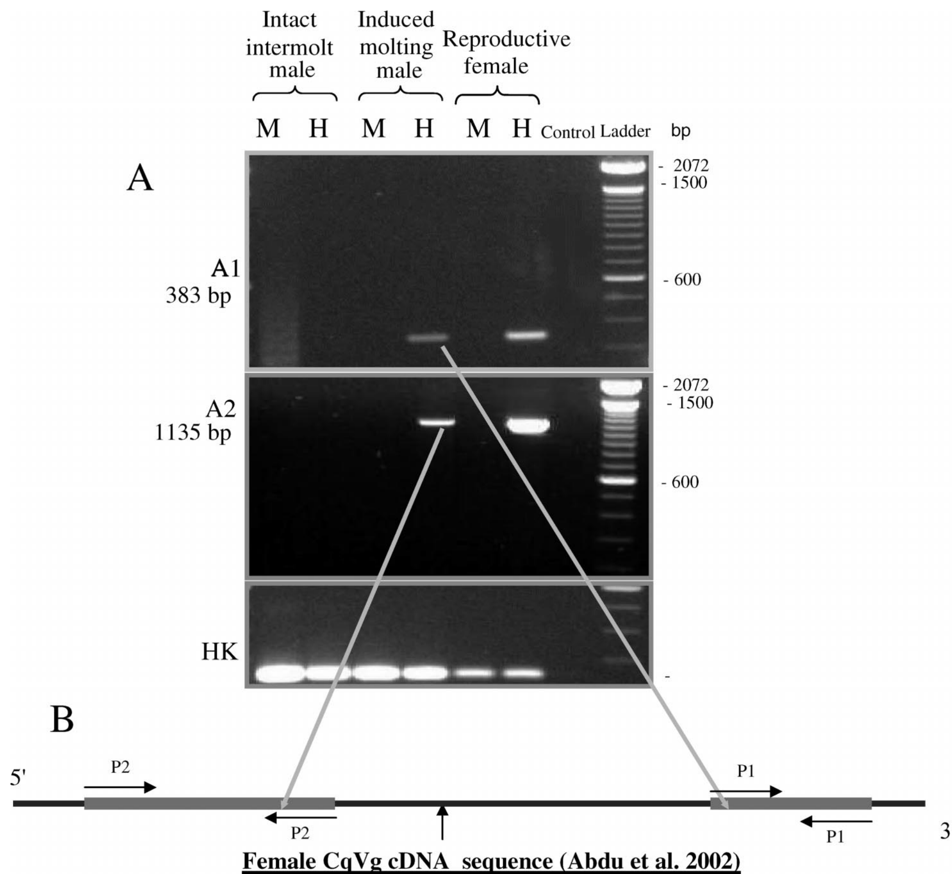

FIG. 2. Expression of the *CqVg* gene in endocrinologically induced male versus reproductive female. The reverse transcription-polymerase chain reaction (RT-PCR) products were amplified using two sets of primers generated from the *CqVg* gene 3′-end (primer 1, 383-base pair [bp] span; (**A**) and from the 5′-end (primer 2, 1135-bp span; (**B**). **A**) Agarose gel separation examining expression of the gene in the hepatopancreas (H) and abdominal muscle (M) of an intermolt intact male, an endocrinologically induced male (at an early postmolt stage) and a reproductive female. Housekeeping gene (HK) elongation factor 2 (Eft2) was used to reconfirm RNA extraction. Control for genomic contamination was used in all of the PCR. **B**) The two RT-PCR products obtained from mRNA of endocrinologically induced males were sequenced and aligned with the corresponding regions of the *CqVg* gene expressed in reproductive females, showing 100% homology.

Relative quantitation was performed by real-time RT-PCR of a total RNA template extracted from the hepatopancreas (Fig. 3). Vitellogenic females and induced intersex animals showed similar high levels of expression. Induced males showed a clear signal that was one-tenth as strong as that in vitellogenic females and induced intersex animals. No expression was evident for intact intersex animals and males.

The spatial and cellular distribution of vitellogenin expression in the hepatopancreas was determined by RNA in situ hybridization. The *C. quadricarinatus* hepatopancreas exhibits the lobular structure typical of an exocrine organ (Fig. 4). Adjacent sections were probed with *CqVg* sense and antisense probes. The antisense probe gave a strong signal in many, but not all, of the large cells lining the excretory lumen of the vitellogenic females. In contrast, the hepatopancreas of induced males showed less intense staining in a much smaller fraction of the same cells. The antisense probes did not bind to the hepatopancreas of intact males or to muscle tissue. No staining was observed with the negative-control, sense-strand probe.

The GI provides an accurate tool for determination of the molt stage (Fig. 5A). Removal of the XO-SG complex in males invariably induces a molt. During the premolt stage after the endocrine induction, an increase in GI was observed up to a peak at approximately 14 days later, when the crayfish molted. The molt was followed by a fast decrease in GI, which represents the postmolt stage. The expression of the *CqVg* gene in endocrinologically induced animals was followed by RT-PCR (Fig. 5B). Experiments to determine the differential expression of *CqVg* (Fig. 5B) revealed the following: In intermolt (GI of 0.2) intact males, no *CqVg* expression was observed. At the premolt stage (GI from 0.2 to 4.3), some cases of positive expression along with the negative cases were seen. In the early postmolt stage (GI from 4.3 to 1.15), positive expression was found exclusively, whereas at the late postmolt stage (GI from 1.15 to 0), negative cases reappeared, with a decrease in the GI.

Expression of the vitellogenin gene in induced males does not necessarily mean that the protein encoded is translated or, if translated, exported. The hemolymph HDL of

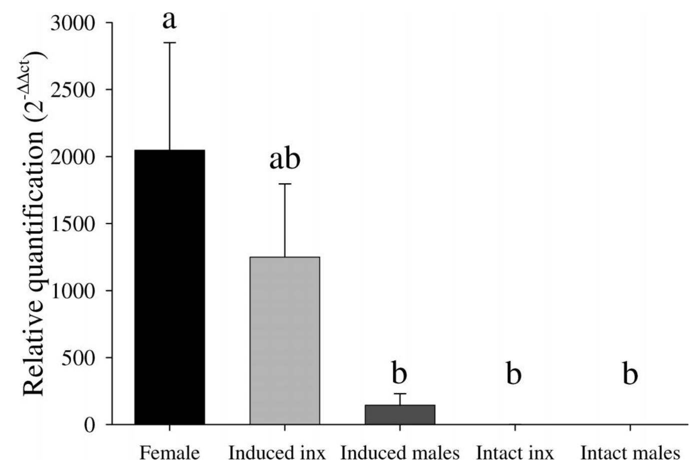

FIG. 3. Relative quantitation of *CqVg* gene expression measured using real-time reverse transcription-polymerase chain reaction (RT-PCR). Relative quantitation of *CqVg* gene expression in a vitellogenic female (n = 12), in induced intersex crayfish at the premolt and postmolt stages (n = 6), in induced molting males at the premolt and postmolt stages (n = 13), in intact intermolt intersex crayfish (n = 5), in intact early postmolt and intermolt males (n = 17). Error bars represent the SEM.

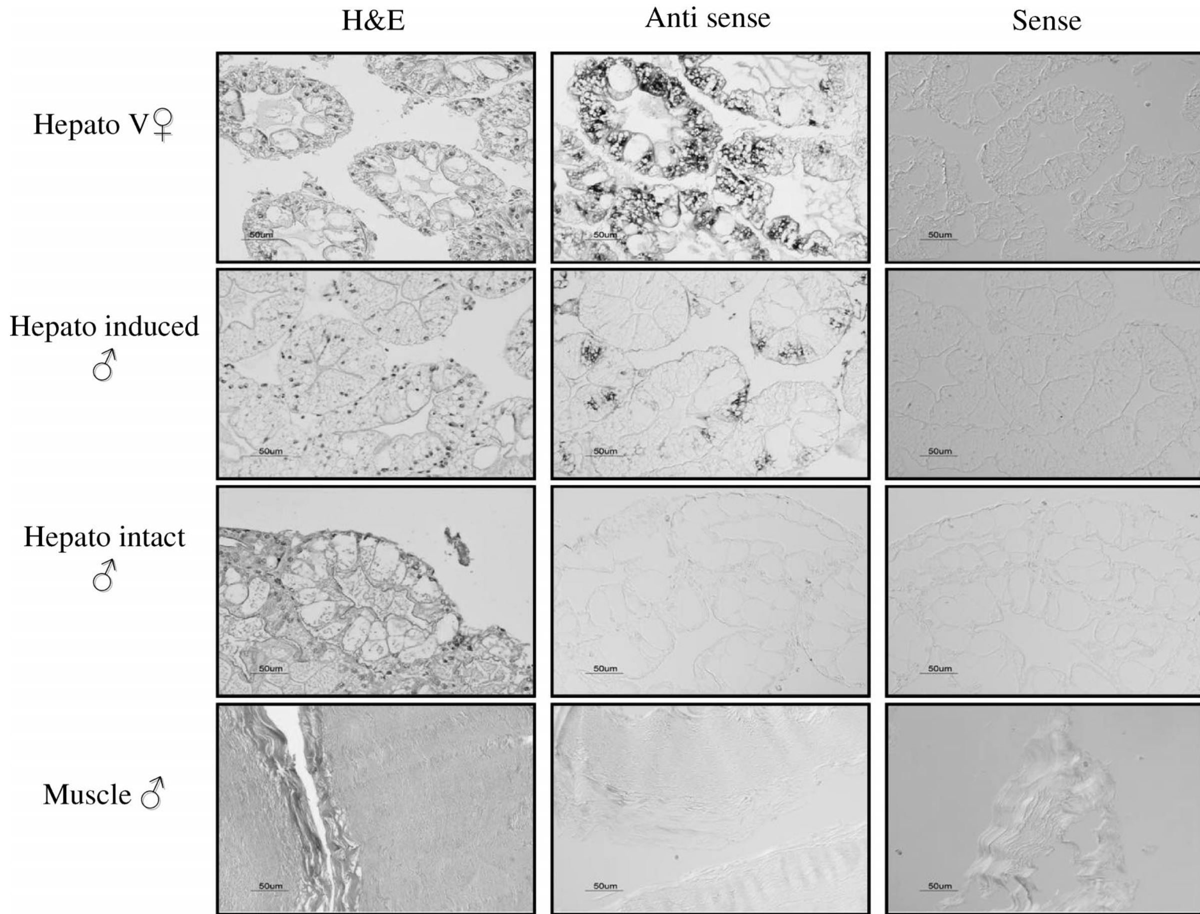

FIG. 4. Localization of *CqVg* gene expression by means of in situ hybridization. Expression of the *CqVg* gene was examined in the hepatopancreatic tissue of a reproductive female, an induced molting male, an intact molting male, and muscle; antisense, *CqVg* probe; H&E, hematoxylin-eosin staining; sense, *CqVg* probe. Bar = 50 μm.

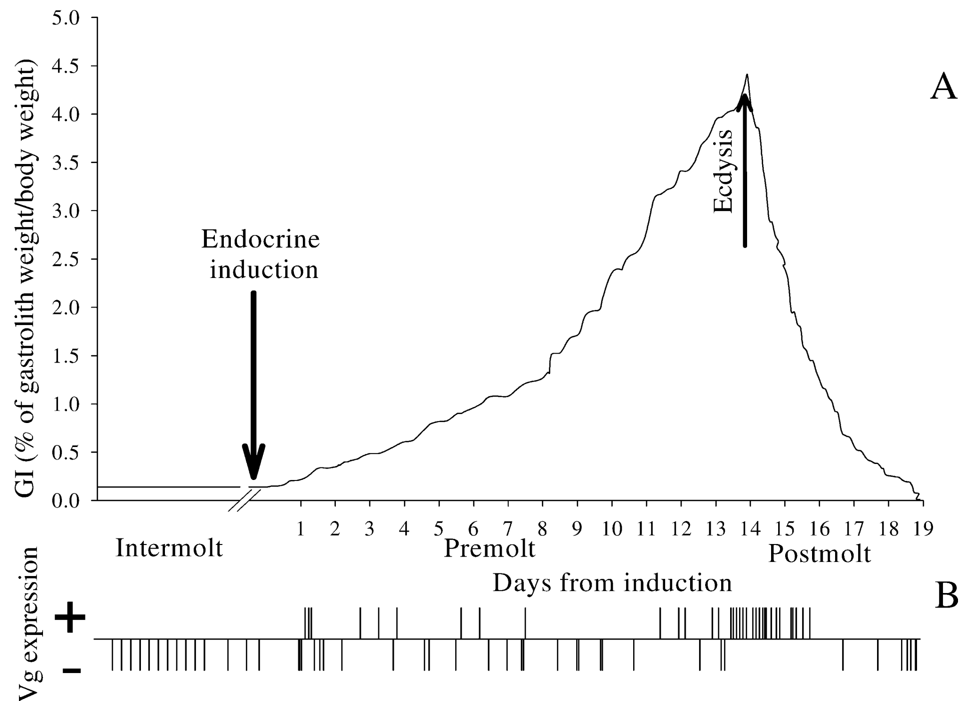

FIG. 5. Differential expression of the *CqVg* gene during the molt cycle of endocrinologically induced males. The relationship between the molt cycle of endocrinologically induced males represented by the gastrolith index (GI; **A**) and the differential expression of the *CqVg* gene in male *C. quadricarinatus* crayfish (**B**).

induced males therefore was examined at different stages of the molt cycle. The HDL profiles from the hemolymph of induced molting males in the different molt stages were similar to those of the reproductive female and the intact intermolt male (Fig. 6A). Although the HDL profile clearly changed in response to induction, little similarity was seen between the HDL profile of induced males and that of vitellogenic females (Fig. 6A) [32]. Only the 177-kDa polypeptide in the induced males corresponded to a similar-sized protein in the vitellogenic female. This polypeptide was observed in the early premolt stage and increased as the molt cycle progressed, with a peak in the postmolt stage. Mass spectroscopy (MS)-matrix-assisted laser desorption ionization time of flight (MALDI-TOF) data (not shown) indicated that this polypeptide was not generated from *CqVg*. A 177-kDa polypeptide, presumably the same as that mentioned above, also was observed in the intact male (Fig. 6B), supporting the MALDI-TOF findings. The profiles of induced male, intact male, and intersex animals showed no apparent resemblance to the profile of the vitellogenic female. On the other hand, the HDL pattern in induced intersex animals clearly was similar to the vitellogenic female profile.

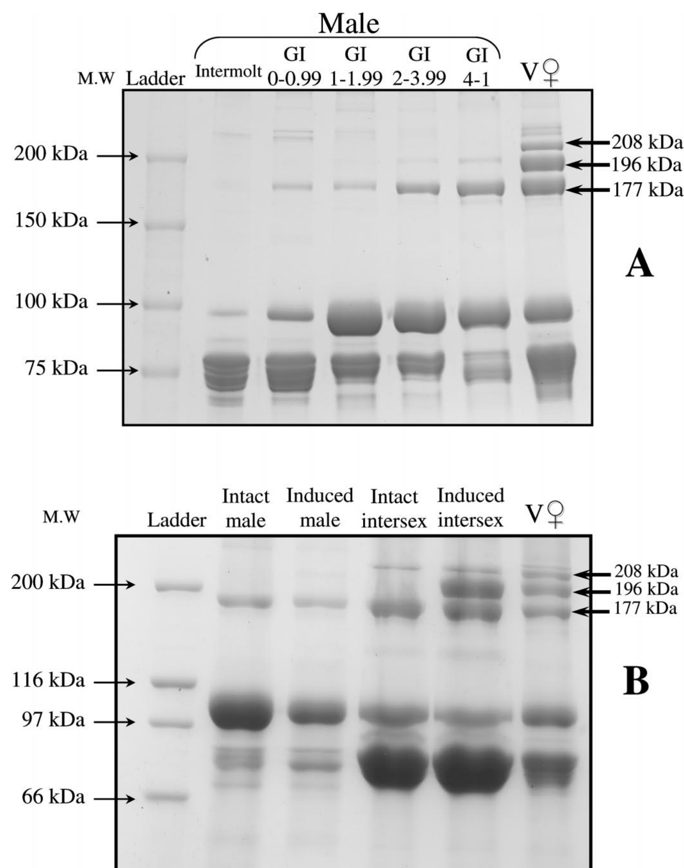

FIG. 6. SDS-PAGE of hemolymphatic high-density lipoproteins from endocrinologically induced *Cherax quadricarinatus*. **A**) Induced molting males during the molt cycle versus reproductive females and intact males. The lanes, from left to right, present the hemolymph profile from an intermolt intact male, a premolt induced male with a GI ranging between 0 and 1, a premolt induced male with a GI ranging between 1 and 2, a premolt induced male with a GI ranging between 2 and 4, a postmolt induced male with a GI ranging between 4 and 1, and a reproductive female. Three polypeptides from the profile of vitellogenic female (208, 196, and 177 kDa) serve as markers for the *CqVg* gene product, as confirmed by MALDI-TOF data peptide sequences. **B**) Induced intersex and male versus vitellogenic females, intact intersex, and males. The lanes, from left to right, present the hemolymph profile from an intermolt intact male, induced premolt and postmolt males (13 days from induction), an intact intermolt intersex animal, induced premolt and postmolt intersex animals (13 days from induction), and a vitellogenic female. Three polypeptides from the profile of the vitellogenic female (208, 196, and 177 kDa) serve as markers for the *CqVg* product.

DISCUSSION

Vitellogenesis, including expression and translation of the vitellogenin gene and the release of its proteinaceous products to the hemolymph, is specific to female reproduction in all oviparous organisms. Nevertheless, some cases of vitellogenin synthesis also have been reported in males (e.g., in male fish subjected to exogenous estrogenic stimulation [33, 34] and in male insects exposed to juvenile hormone and 20-hydroxyecdysone [35–38]). Two instances of vitellogenin synthesis in crustacean males have been reported in isopods, one in the fat body of andrectomized males [14] and the other in an in vitro-cultured fat body [13]. Only a single case of vitellogenin synthesis in male decapods, following removal of the XO-SG complex, has been reported in juvenile *Macrobrachium rosenbergii* prawns [15]. In *M. rosenbergii*, the molt cycle and vitellogenesis are closely related, and molt takes place at the end of the vitellogenic process [39]. In the present study, we have demonstrated, to our knowledge for the first time in a decapod crustacean, in which molt and reproduction are considered to be antagonistic [40], that vitellogenin expression could be induced in males if they were subjected to endocrine induction by removal of the XO-SG complex. This expression, which was accompanied by an accelerated molt cycle, demonstrates the regulatory role of the XO-SG complex in the control of the antagonistic balance between reproduction and molt. This premise is strengthened by the fact that intact males did not show *CqVg* expression during the same molt stages (Fig. 3).

Removal of the XO-SG complex in males coupled the expression of *CqVg* to the molt cycle. Changes in the gastroliths during the molt cycle (Fig. 5A) provided an accurate tool for molt staging, which is crucial for investigation of the differential expression of *CqVg* (Fig. 5B) at specific stages of this coupled process. That during the premolt stage some cases of *CqVg* expression were found along with cases in which the gene was not expressed and during the early postmolt stage only positive expression was found suggests the involvement of additional molt cycle-related moderation of *CqVg* expression, which could not be explained simply by the absence of inhibitory neuropeptides from the XO-SG complex, which had been removed in the induction process.

The weaker expression in the endocrinologically induced males compared to that in reproductive females (Figs. 3 and 4) suggests the involvement of an additional non-XO-SG complex-derived regulatory factor in the control of hepatopancreatic *CqVg* expression. One potential source is the male-specific AG. The inhibitory effect of the AG on *CqVg* expression has, indeed, been demonstrated in both AG-implanted females [27] and andrectomized intersex *C. quadricarinatus* [30]. Nevertheless, removal of the XO-SG complex in the intersex model induced high *CqVg* expression despite the presence of the AG. Hypertrophy of the AG caused by XO-SG complex removal has been illustrated in the past [41], which suggests that the inhibitory effect of the AG should be enhanced by removal of the XO-SG complex. The present study demonstrates that although the AG is in a hypertrophied state, caused by removal of the XO-

SG complex, *CqVg* expression was detected both in male and intersex crayfish.

Another explanation for the lower expression of *CqVg* in induced males could be the absence of a stimulatory factor. The existence of such a factor was hypothesized by Junera et al. [26], who termed it VSOH. This factor is believed to be secreted from the ovaries but has never been isolated. In the present study, we used intersex animals, which have both a male reproductive system and an ovary, as a model for investigation of the existence of this hypothesized stimulatory effect. Endocrine induction by removal of the XO-SG complex in the intersex crayfish induced a shift of the arrested ovary toward the vitellogenic state, a finding that was illustrated by the higher expression of *CqVg* in the hepatopancreas (Fig. 3), despite the presence of a hypertrophied AG. The HDL hemolymph profile of these induced intersex individuals showed a high resemblance to that found in vitellogenic females (Fig. 6B), suggesting that translation and secretion of the *CqVg* product had occurred. On the other hand, in males, the translation of the *CqVg* transcript or its secretion into the hemolymph was doubtful (Fig. 6A). Only one hemolymphatic polypeptide (177 kDa) correlated with the differential expression of *CqVg* in endocrinologically induced males resembled the known hemolymph pattern of vitellogenin in females. Unlike the three typical vitellogenic markers (208, 196, and 177 kDa) (Fig. 6), the induced male 177-kDa polypeptide was not detected as a vitellogenin product by the MS-MALDI-TOF analysis (data not shown). These results further support the hypothesized involvement of a stimulatory ovarian factor not only in the hepatopancreatic expression but also in the translation and secretion of *CqVg* products.

Because the weaker *CqVg* expression in the male (unlike the intersex) crayfish was manifested in a system characterized by the lack of ovarian components, the present results indicate a need for further work on the possible stimulatory effect of the ovary on *CqVg* expression and translation in males. Nevertheless, the known inhibitory effect of XO-SG complex neuropeptides and AG factors on the expression of *CqVg* in male and intersex crayfish provides an inducible model for the investigation of vitellogenesis regulation in Crustacea. The use of quantitative *CqVg* expression could elucidate the regulatory roles and interactions between XO-SG neuropeptides, AG factors, ovarian factors, and other hormones along the endocrine axes, taking into consideration the complexity of this regulatory system in different developmental stages. The vitellogenin gene could be regarded as a leading gene for multigene studies of the molt and reproduction processes along their regulatory axes in this oviparous model.

ACKNOWLEDGMENTS

The technical assistance and animal husbandry by Nadav Ron, Alon Zinger, and Dudu Azulay are highly appreciated. We would like to thank Inez Mureinik for styling the manuscript.

REFERENCES

1. Valle D. Vitellogenesis in insects and other groups—a review. Memorias Do Instituto Oswaldo Cruz 1993; 88:1–26.
2. Chen JS, Sappington TW, Raikhel AS. Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. J Mol Evol 1997; 44:440–451.
3. Meusy JJ, Payen GG. Female reproduction in malacostracan crustacea. Zool Sci 1988; 5:217–265.
4. Adiyodi RG, Subramoniam T. Oogenesis, oviposition, and oosorption. In: Adiyodi KG, Adiyodi RG (eds.), Reproductive Biology of Invertebrates, vol. 1. New York: John Wiley and Sons; 1983:443–495.
5. Yehezkel G, Chayoth R, Abdu U, Khalaila I, Sagi A. High-density lipoprotein associated with secondary vitellogenesis in the hemolymph of the crayfish *Cherax quadricarinatus*. Comp Biochem Physiol B Biochem Mol Biol 2000; 127:411–421.
6. Charniaux-Cotton H, Payen G. Crustacean reproduction. In: Laufer H, Downer RGH (eds.), Endocrinology of Selected Invertebrate Types. New York: Alan R. Liss; 1988:279–303.
7. Tsutsui N, Kawazoe I, Ohira T, Jasmani S, Yang WJ, Wilder MN, Aida K. Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. Zool Sci 2000; 17:651–660.
8. Abdu U, Davis C, Khalaila I, Sagi A. The vitellogenin cDNA of *Cherax quadricarinatus* encodes a lipoprotein with calcium binding ability, and its expression is induced following the removal of the androgenic gland in a sexually plastic system. Gen Comp Endocrinol 2002; 127:263–272.
9. Okuno A, Yang WJ, Jayasankar V, Saido-Sakanaka H, Huong DTT, Jasmani S, Atmomarsono M, Subramoniam T, Tsutsui N, Ohira T, Kawazoe I, Aida K, Wilder MN. Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. J Exp Zool 2002; 292: 417–429.
10. Avarre JC, Michelis R, Tietz A, Lubzens E. Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. Biol Reprod 2003; 69:355–364.
11. Tsang WS, Quackenbush LS, Chow BKC, Tiu SHK, He JG, Chan SM. Organization of the shrimp vitellogenin gene: evidence of multiple genes and tissue specific expression by the ovary and hepatopancreas. Gene 2003; 303:99–109.
12. Tsutsui N, Saido-Sakanaka H, Yang WJ, Jayasankar V, Jasmani S, Okuno A, Ohira T, Okumura T, Aida K, Wilder MN. Molecular characterization of a cDNA encoding vitellogenin in the coonstriped shrimp, *Pandalus hypsinotus* and site of vitellogenin mRNA expression. J Exp Zool 2004; 301A:802–814.
13. Gohar M, Souty C. Demonstration in vitro for a synthesis and release of vitellogenin by the fat-body in the male of a crustacean isopoda, *Porcellio-Dilatatus*, (Brandt). Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences 1983; 297:145–148.
14. Suzuki S, Yamasaki K, Katakura Y. Vitellogenin synthesis in andrectomized males of the terrestrial isopod, *Armadillidium-vulgare* (Malacostraca, Crustacea). Gen Comp Endocrinol 1990; 77:283–291.
15. Wilder MN, Okumura T, Suzuki Y, Fusetani N, Aida K. Vitellogenin production induced by eyestalk ablation in juvenile giant fresh-water prawn *Macrobrachium-Rosenbergii* and trial methyl farnesoate administration. Zool Scie 1994; 11:45–53.
16. Chang ES. Crustacean hyperglycemic hormone family: Old paradigms and new perspectives. American Zoologist 2001; 41:380–388.
17. Keller R. Crustacean neuropeptides—structures, functions, and comparative aspects. Experientia 1992; 48:439–448.
18. Bocking D, Dircksen H, Keller R. The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. In: Wiese K (ed.), The Crustacean Nervous System. Berlin-Heidelberg: Springer-Verlag; 2002:84–97.
19. Marco HG, Avarre JC, Lubzens E, Gade G. In search of a vitellogenesis-inhibiting hormone from the eyestalks of the South African spiny lobster, *Jasus lalandii*. Invert Reprod Dev 2002; 41:143–150.
20. Aguilar MB, Quackenbush LS, Hunt DT, Shabanowitz J, Huberman A. Identification, purification, and initial characterization of the vitellogenesis-inhibiting hormone from the Mexican crayfish, *Procambarus-Bouvieri* (Ortmann). Comp Biochem Physiol B Biochem Mol Biol 1992; 102:491–498.
21. Azzouna A, Philippe M, Jarry T, Greve P, Martin G. Localization of crustacean hyperglycemic and vitellogenesis-inhibiting hormones in separate cell types in the protocerebrum of the woodlouse *Armadillidium vulgare* (Crustacea, Isopoda). Gen Comp Endocrinol 2003; 131: 134–142.
22. Greve P, Sorokine O, Berges T, Lacombe C, Van Dorsselaer A, Martin G. Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). Gen Comp Endocrinol 1999; 115:406–414.
23. Brown FA, Cunningham O. Influence of the sinus gland of crustaceans on normal viability and ecdysis. Biol Bull 1939; 77:104–114.
24. Travis DF. The deposition of skeletal structures in the Crustacea. 1. The histology of the gastrolith skeletal tissue complex and the gastro-

lith in the crayfish, *Orconectes* (*cambaus*) *verilis* Hagen (Decapoda). Biol Bull 1960; 16:137–149.
25. Pavey CR, Fielder DR. Use of gastrolith development in molt staging the fresh-water crayfish *Cherax cuspidatus* Riek, 1969. Crustaceana 1990; 59:101–105.
26. Junera H, Zerbib C, Martin M, Meusy JJ. Evidence for control of vitellogenin synthesis by an ovarian hormone in *Orchestia gammarella* (*Pallas*), (Crustacea, Amphipoda). Gen Comp Endocrinol 1977; 31:457–462.
27. Manor R, Aflalo DE, Segall C, Weil S, Azulay D, Ventura T, Sagi A. Androgenic gland implantation promotes growth and inhibits vitellogenesis in *Cherax quadricarinatus* females held in individual compartments. Invert Reprod Dev 2004; 45:151–159.
28. Parnes S, Khalaila I, Hulata G, Sagi A. Sex determination in crayfish: are intersex *Cherax quadricarinatus* (Decapoda, Parastacidae) genetically females? Genet Res 2003; 82:107–116.
29. Sagi A, Khalaila I, Barki A, Hulata G, Karplus I. Intersex red claw crayfish, *Cherax quadricarinatus* (von Martens): functional males with previtellogenic ovaries. Biol Bull 1996; 190:16–23.
30. Sagi A, Manor R, Segall C, Davis C, Khalaila I. On intersexuality in the crayfish *Cherax quadricarinatus*: an inducible sexual plasticity model. Invert Reprod Dev 2002; 41:27–33.
31. Laemlli UK. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature 1970; 227:680–685.
32. Abdu U, Yehezkel G, Sagi A. Oocyte development and polypeptide dynamics during ovarian maturation in the red-claw crayfish *Cherax quadricarinatus*. Invert Reprod Dev 2000; 37:75–83.
33. Bowman CJ, Kroll KJ, Hemmer MJ, Folmar LC, Denslow ND. Estrogen-induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). Gen Comp Endocrinol 2000; 120:300–313.
34. Schultz IR, Orner G, Merdink JL, Skillman A. Dose–response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17α-ethinylestradiol. Aquat Toxicol 2001; 51:305–318.
35. Adams TS, Filipi PA, Kelly TJ. Effect of 20-hydroxyecdysone and a juvenile-hormone analogue on vitellogenin production in male houseflies, *Musca domestica*. J Insect Physiol 1989; 35:765–769.
36. Briers T, Huybrechts R. Control of vitellogenin synthesis by ecdysteroids in *Sarcophaga bullata*. Insect Biochemistry 1984; 14:121–126.
37. Deitsch KW, Chen JS, Raikhel AS. Indirect control of yolk protein genes by 20-hydroxyecdysone in the fat body of the mosquito, *Aedes aegypti*. Insect Biochem Mol Biol 1995; 25:449–454.
38. Lamy M. Vitellogenesis, vitellogenin, and vitellin in the males of insects—a review. Int J Invert Reprod Dev 1984; 7:311–321.
39. Jayasankar V, Tsutsui N, Jasmani S, Saido-Sakanaka H, Yang WJ, Okuno A, Hien TTT, Aida K, Wilder MN. Dynamics of vitellogenin mRNA expression and changes in hemolymph vitellogenin levels during ovarian maturation in the giant freshwater prawn, *Macrobrachium rosenbergii*. J Exp Zool 2002; 293:675–682.
40. Barki A, Levi T, Hulata G, Karplus I. Annual cycle of spawning and molting in the red-claw crayfish, *Cherax quadricarinatus*, under laboratory conditions. Aquaculture 1997; 157:239–249.
41. Khalaila I, Manor R, Weil S, Granot Y, Keller R, Sagi A. The eyestalk-androgenic gland-testis endocrine axis in the crayfish, *Cherax quadricarinatus*. Gen Comp Endocrinol 2002; 127:147–156.