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# A Newly Established ELISA Showing the Effect of the Androgenic Gland on Secondary-Vitellogenic-Specific Protein in the Hemolymph of the Crayfish *Cherax quadricarinatus*

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A quantitative enzyme-linked immunosorbent assay (ELISA) was developed to monitor the onset of secondary vitellogenesis in *Cherax quadricarinatus* females and in intersex individuals (having both male and female reproductive systems) after removal of the androgenic gland (AG). As a prerequisite for the assay, the 106-kDa polypeptide was separated from newly laid *C. quadricarinatus* eggs by SDS–PAGE, and anti-106-kDa antibody was raised in rabbit. The specificity of the anti-106-kDa polypeptide for proteins specific for the hemolymph of secondary-vitellogenic females was confirmed by double immunodiffusion and immunoblot cross-reactivity tests. A characteristic standard ELISA curve, using egg high-density lipoprotein (HDL), showed linearity between 16 and 500 ng ($r = 0.953$) and was sensitive for amounts as low as 8 ng. The inter- and intraassay coefficients of variance were 14.8 and 7.2%, respectively. Only traces of egg HDL equivalents were detected in the hemolymph of male and primary-vitellogenic females (11 to 110 μg/ml), confirming the specificity of the assay, whereas high levels of such a protein (8–35 mg/ml) were detected in the hemolymph of secondary-vitellogenic females. Removal of the AG from intersex individuals leads to a significant increase in the concentration of vitellogenic-specific protein in the hemolymph (up to 2 mg/ml). Moreover, a significantly lower concentration was found in females subjected to AG transplant (79.3 μg/ml). The ELISA thus provided an accurate and sensitive tool to investigate the influence of the AG on the expression of a vitellogenic-specific protein in female and intersex *C. quadricarinatus*, confirming the central role of this gland in tuning sexual plasticity in this species. 

The most important sources of nutrients for the developing embryo are yolk proteins (Lee *et al.*, 1997). In crustaceans, the major yolk protein, vitellin, is a high-density lipoprotein (HDL) complex (Meusy and Payen, 1988). It has been suggested that yolk proteins or their precursors are synthesized in the ovaries, adipose tissue, and/or hepatopancreas of decapod crustaceans (Meusy, 1980; Paulus and Laufer, 1987; Rani and Subramoniam, 1997; Yano and Chinzei, 1987). In these animals, vitellogenesis—the process of egg yolk formation and accumulation—is regulated by a neuropeptide, known as gonad-inhibiting hormone (Adiyodi and Adiyodi, 1970; Meusy and Payen, 1988) or vitellogenesis-inhibiting hormone (Charniaux-Cotton and Payen, 1988). Extensive studies have been performed on the site of synthesis (Giulianini *et al.*, 1998; Rotlant *et al.*, 1993), the preprohormone (De-Kleijn *et al.*, 1994), and the mode of action (De-Kleijn *et al.*, 1998; Quackenbush and Keeley, 1988) of this hormone. Another, much less well known, vitellogenesis-inhibiting agent is a hormone secreted by the andro-

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genic gland (AG), which, when present, prevents ovarian maturation (Meusy and Payen, 1988; Taketomi and Nishikawa, 1996). Ablation of the AG (andrectomy) in gonochoristic species results in female differentiation (Charniaux-Cotton, 1964; Nagamine *et al.*, 1980; Sagi *et al.*, 1990). In species exhibiting sexual plasticity, such as protandric hermaphrodites, the AG degenerates to allow the development of the ovary and female secondary sexual characteristics. Delayed appearance of the AG in protogynic hermaphrodites allows female differentiation in early phases of life (Charniaux-Cotton, 1975). In protandric hermaphrodites the AG inhibits secondary but not primary vitellogenesis (Charniaux-Cotton and Payen, 1985).

Although the decapod crustacean *Cherax quadricarinatus* is regarded as a gonochoristic species, a certain degree of sexual plasticity is expressed in the form of intersex individuals, having both male and female openings and reproductive systems (Brummett and Alon, 1994; Medley and Rouse, 1993). Half of their reproductive system consists of a constantly arrested ovary at the primary-vitellogenic stage (Sagi *et al.*, 1996a). Following the removal of the AG, changes in the anatomy and structure have been described in the ovary of intersex individuals (Khalaila *et al.*, 1999), similar to those occurring in the normal female during the reproductive cycle (Sagi *et al.*, 1996b; Abdu and Sagi, 1998). To monitor these changes and the corresponding changes in the expression of proteins specific to secondary vitellogenesis *in vivo*, a sensitive noninvasive tool is needed.

We thus developed an enzyme-linked immunosorbent assay (ELISA) using an antibody (raised in rabbit) against the secondary-vitellogenic-specific 106-kDa polypeptide that was separated from newly laid eggs. This ELISA is capable of quantifying proteins specific for the hemolymph of secondary-vitellogenic *C. quadricarinatus* female. We then applied this assay to study the role of the AG in controlling the expression of proteins that are specific to the hemolymph of secondary-vitellogenic female and intersex *C. quadricarinatus.*

## MATERIALS AND METHODS

### *Animals*

*C. quadricarinatus* specimens were collected at the Aquaculture Research Station Dor, Israel. All individuals were held in our facility at the Ben-Gurion University of the Negev for at least 1 month for acclimation prior to the experiments.

Primary- and secondary-vitellogenic females were selected according to the GSI and to the diameter and color of the oocytes (Sagi *et al.*, 1996b; Abdu and Sagi, 1998). Primary-vitellogenic females had GSI values ranging from 0.10 to 0.35 and oocyte diameters from 180 to 380 μm. The values for secondary-vitellogenic females were 0.6 to 1.9 and 700 to 2000 μm, respectively. The selected crayfish were used for ovarian polypeptide characterization, testing the specificity of the antibody by double diffusion (Ouchterlony, 1949), and for quantification of vitellogenic-specific protein in the hemolymph by ELISA.

Several berried females were selected with the spermatophores still attached to their abdomens. Newly laid eggs were collected for separation of protein and HDL. Intersex *C. quadricarinatus* individuals were selected approximately 9 months after hatching; these animals had a female opening on the bases of both their third walking legs, but only one male opening was present at the base of one of the fifth walking legs (Sagi *et al.*, 1996a).

### *Polypeptide Separation*

Newly laid eggs and ovarian lobes of primary- and secondary-vitellogenic females were homogenized individually on ice with 0.05 M Tris–HCl buffer, pH 7.4, supplemented with the following antiproteases: 0.8 mM benzamidine (Sigma), 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 1 μg/ml leupeptine (Sigma), 1 μg/ml aprotinine (Sigma), and 1 mM ethylenediamine-tetraacetic acid (EDTA, Sigma). For HDL (at a density of 1.25 g/ml, adjusted with NaBr from a stock solution of 1.5 g/ml), egg homogenate was ultracentrifuged at 45,000*g* for 48 h at 12°C. The lipoprotein fraction was collected and dialyzed against PBS (10 mM sodium dihydrogen phosphate, 0.15 M sodium chloride, pH 7.4) for 24 h at 4°C, protein concentration was determined, and the dialysate was stored at −20°C. The ovarian samples were centrifuged at 10,800*g* for 15 min at 4°C, and the pellet was resuspended in the above-mentioned buffer supplemented with 1% $C_{12}E_9$ (polyoxethylene 9-lauryl ether polidocanol), allowed to stand for 30 min on ice, and then recentrifuged. Total protein was determined in

the supernatant (Bradford, 1976). The polypeptide profiles of the samples (approximately 35 µg of protein per lane) were characterized by 7% mini-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) and stained with Coomassie blue or with the cationic carbocyanine dye ''stains all'' (King and Morrison, 1976). For Western blot analysis, HDL from the hemolymph of *C. quadricarinatus* males and primary- and secondary-vitellogenic females were separated by SDS–PAGE as described above. Proteins were electroblotted to nitrocellulose for 2 h at 200 mA (Semi-Phor, Hoefer, San Francisco, CA) using 25 mM Tris, 0.192 M glycine buffer, pH 8.3, containing 0.5% SDS. After blocking with 3% skim milk in 10 mM Tris and 150 mM NaCl, pH 7.8 (TBS), containing 0.1% Tween-20, the membranes were incubated for 1 h with the prepared serum or with the preimmune serum at a dilution of [1:10,000]. The membranes were washed three times with TBS and then incubated for 1 h with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma). After washing, color was initiated by adding nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) as the substrate.

### *Preparation of Rabbit-Specific Antiserum*

The antigen for the preparation of polyclonal antibodies against the 106-kDa polypeptide was prepared as follows: (1) Newly laid eggs were collected from females and homogenized, and the 106-kDa polypeptide was separated as described above. (2) About 4 mg of the desired protein was collected from the Coomassie-blue-stained gels and electroeluted. (3) The eluted 106-kDa band was brought to a total volume of 10 ml with PBS, pH 7.4, and then stored for later use (for four separate injections, as described below. (4) A sample from the latter preparation was rechromatogramed to confirm the purity. The immune serum was produced in a rabbit as described by Harlow and Lane (1988). Immediately before immunization, blood was withdrawn from the marginal ear vein for preparation of preimmune serum. The rabbit was immunized, as described below, with a total of about 1 mg (in 2.5 ml) 106-kDa polypeptide diluted with 2.5 ml of PBS, pH 7.4, emulsified with an equal volume of Freund's complete adjuvant (Sigma, Israel). Three additional immunizations of 1 mg of antigen with incomplete Freund's adjuvant were given once every 4 weeks. The rabbit was injected subcutaneously (0.6 ml) in 10 different places on the back and intramuscularly (2 ml) in each hind leg muscle. A total of 50 ml of blood was withdrawn from the rabbit ear veins and allowed to stand at room temperature for 1 h. The clotted blood was separated from the walls of the tubes with a wooden spatula and incubated overnight at 4°C. The clotted blood was centrifuged at 16,000*g* for 10 min, and the serum (including the antibodies) was transferred to appropriate tubes and stored at −20°C. For immunoblot analysis, IgG fractions were further purified by Affi-Gel Protein A Gel Bio-Rad (Hercules, CA). Analysis of the immune serum by the double immunodifusion test was performed as follows: (1) an aliquot of 1:10 diluted antiserum or preimmune serum was added to the center of each 1% agarose gel Ouchterlony plate (Ouchterlony, 1949) and (2) hemolymph of either primary- or secondary-vitellogenic females was added to the peripheral wells.

### *ELISA*

For the quantification experiments, hemolymph samples (20 µl) were taken from the sinus at the base of the fifth walking leg of a number of primary- and secondary-vitellogenic females, males, and intersex individuals. The samples were diluted with 980 µl of carbonate buffer, 0.5 M, pH 9.6, and stored at −20°C until used in the assay. The polyclonal antibody against the 106-kDa polypeptide (anti-106-kDa) was used to develop a direct ELISA. In general, the assay was performed in high-binding 96-well plates (Costar) at 37°C with shaking. The antibody was diluted with carbonate buffer, 50 mM, pH 9.6. The antigen (standards and hemolymph samples) was coated on the plate and incubated overnight at 4°C. After washing with PBS, pH 7.4, the wells were incubated for 1 h with a blocking buffer (PBS, 1% skim milk and 0.05% Tween 20) at 37°C. The wells were washed, and anti-106-kDa was added to each well at a dilution of 1:1000, followed by incubation at 37°C for 1 h. Goat anti-rabbit IgG conjugated with alkaline phosphatase (A-3687, Sigma) was diluted to 1:34,000 and added to each well as the enzyme for color reaction. After incubation at 37°C for 1 h, the plates were washed, *p*-nitrophenylphosphate was added as the substrate, and the absorbance was read at 405 nm using an ELISA reader

SLT Spectra (SLT labinstruments, Spectra Readers, Austria).

### *Androgenic Gland Experiments*

Thirty-two intersex individuals, carapace length 30–50 mm, were sorted by size into two groups. One group underwent sham operation (control) and the other andrectomy. Sham operation consisted of electrical cauterization of the coxa of the fifth walking leg on the side opposite to that of the male opening. Andrectomy was performed by cauterization through the articular membrane above the coxa of the fifth walking leg that had a male opening (Huxley, 1880). The duration of the experiment was 50 days. In addition to the above-described experiment, an experiment to test the effect of AG transplantation into females was conducted. Thirty-two females (age 3 months, carapace length 2.1 ± 0.3 cm) were subjected to AG transplant or sham operation (control). Glands used for the transplantation were hypertrophied due to previous destalking of the male donors. The implanted females were kept for about 8 months to assure gonad maturation. The crayfish were kept in glass aquaria, each 40 × 50 × 40 cm with polycarbonate partitions that formed four identical compartments, one for each individual. The temperature was maintained at 27 ± 2°C, and a photoperiod of 14L:10D was applied. Water quality was assured by circulating the water through a gravel biofilter. Crayfish food included corn, carrots, and potatoes, supplemented with commercial fish pellets (35% protein). It was supplied *ad libitum*.

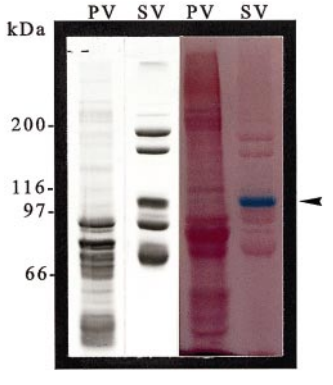


FIG. 1. 7% SDS–PAGE separation of polypeptides from primary- and secondary-vitellogenic *C. quadricarinatus* ovaries. The left lanes are stained with Coomassie blue and the right lanes are stained with "stains all." The arrow indicates the 106-kDa band. PV, primary-vitellogenic female (GSI 0.3; oocyte diameter 380 μm); SV, secondary-vitellogenic female (GSI 1.9, oocyte diameter 1360 μm).

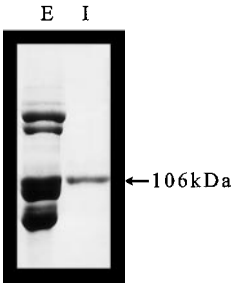


FIG. 2. 7% SDS–PAGE separation of a typical newly laid egg polypeptide profile (E) and a re-chromatogram of the isolated 106-kDa band (I, arrow).

## RESULTS

To select a secondary-vitellogenic-specific polypeptide, primary- and secondary-vitellogenic ovarian polypeptide profiles were compared by Coomassie blue and "stains all" staining of a 7% SDS–PAGE gel. The polypeptide profile of the ovarian extract of secondary-vitellogenic female (with an oocyte diameter of 1316 ± 23 μm) differed from that of a primary-vitellogenic female (with an oocyte diameter of 430 ± 25 μm); it showed three prominent polypeptides, with high molecular weights of approximately 177, 150, and 106 kDa, as revealed by Coomassie blue staining (Fig. 1, left). A negatively charged (confirmed by the "stains all" method) polypeptide (106 kDa) was evident only in the polypeptide profile of the secondary-vitellogenic ovaries (arrow, Fig. 1, right). Since this band was absent in the polypeptide profile of the primary-vitellogenic ovaries (Fig. 1, right), it was selected as a specific secondary-vitellogenic marker. This marker was also found in 7% SDS–PAGE from newly laid eggs (Fig. 2, lane E). The 106-kDa band was thus electro-eluted from the gel and re-chromatogramed to confirm the purity of its preparation as an antigen (Fig. 2, lane I, arrow).

Immune serum raised against the 106-kDa polypeptide was characterized by double immunodiffusion and immunoblot cross-reactivity tests. The double immunodiffusion test for this new antiserum (pre-

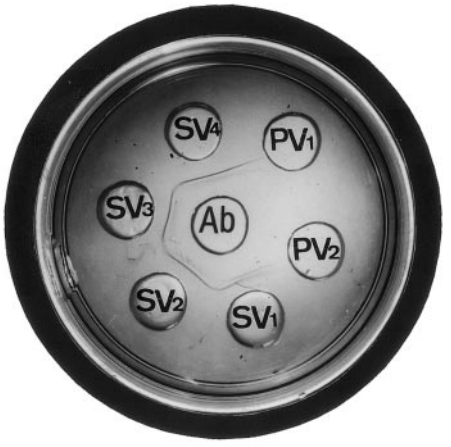


FIG. 3. Double immunodiffusion test for anti-106-kDa polypeptide from newly laid eggs, against hemolymph from various primary-vitellogenic and secondary-vitellogenic *C. quadricarinatus* females. PV, primary vitellogenic females 1 and 2 had GSI values of 0.25 and 0.21 and oocyte diameters of 430 ± 25 and 410 ± 30 µm, respectively, with uniform milky white shaded color. SV, secondary-vitellogenic females 1–4 had GSI values of 0.44, 0.49, 0.60, and 1.79 and oocyte diameters of 700 ± 40, 810 ± 25, 920 ± 30, and 1316 ±23 µm, respectively, with a yellow to orange color. AB, antiserum raised in rabbit against the 106-kDa polypeptide from newly laid eggs, was used in the central well.

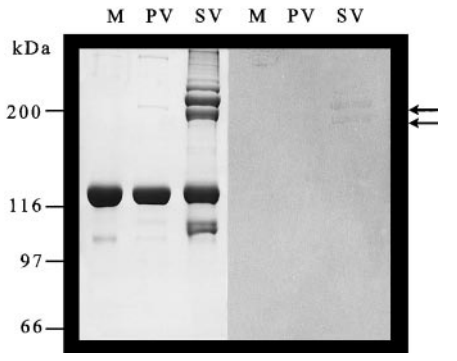


FIG. 4. Cross-reactivity test by immunoblot of HDL from hemolymph of *C. quadricarinatus* male, primary-vitellogenic female, and secondary-vitellogenic female. Anti-106-kDa polypeptide from newly laid eggs was used. The left lanes are Coomassie blue-stained profiles separated by 7% SDS–PAGE and the right lanes are the same, blotted onto a nitrocellulose membrane. M, male; PV, primary vitellogenic female (GSI value of 0.3, oocyte diameter of 356 ± 42 µm); SV, secondary vitellogenic female (GSI value of 1.93 and oocyte diameter of 1362 ± 23 µm).

pared from the hemolymph of six females with different GSI and oocyte diameters) showed cross-reactivity only with the hemolymph of secondary-vitellogenic females (Fig. 3, SV 1 to 4). No immunoprecipitation was observed against the hemolymph of primary-vitellogenic individuals (Fig. 3, PV 1 and 2). The specificity of the anti-106-kDa serum was confirmed by a Western blot analysis (Fig. 4). The Coomassie blue profile of a gel identical to the blot is presented in Fig. 4 (right lanes). The blot showed that antibodies in the immune serum specifically recognized polypeptides in the hemolymph of the vitellogenic female at molecular weight of about 170 kDa (Fig. 4, right). Moreover, these antibodies did not bind any polypeptide, in the male or in primary-vitellogenic female hemolymph (Fig. 4, left).

Since the anti-106-kDa serum recognized a specific protein in the hemolymph of secondary-vitellogenic females, it was used for vitellogenic protein titration by ELISA. A typical calibration curve is shown in Fig. 5 using newly laid egg HDL as the standard. The standard curve had a lag phase under 16 ng and reached a plateau over 500 ng. It showed linearity between 16 and 500 ng, with a correlation coefficient of $r = 0.953$. The sensitivity of the assay (i.e., the lowest quantity of HDL equivalents giving an O.D. significantly greater than the background) was 8 ng. The reproducibility of the assay was evaluated using low, medium, and high internal standards. The inter- and

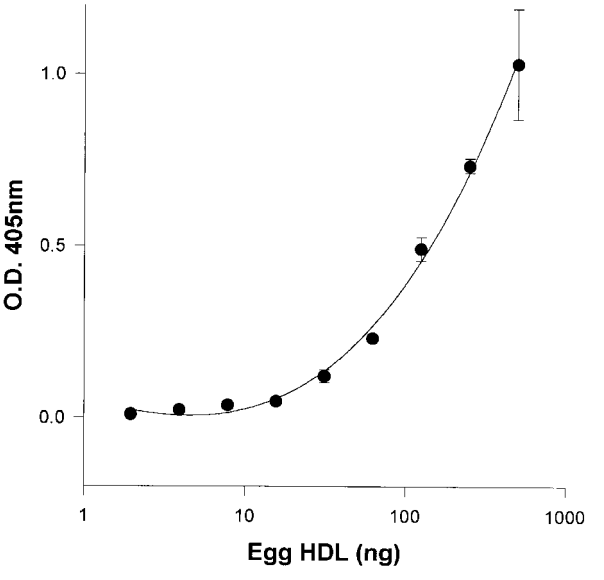


FIG. 5. A typical ELISA calibration curve obtained with newly laid egg HDL standards. Bars represent SE.

intraassay coefficients of variance were 14.8 and 7.2%, respectively.

To demonstrate the specificity of the assay, serially diluted hemolymph from males and primary- and secondary-vitellogenic females was assayed (Fig. 6). The newly established ELISA, using the anti-106-kDa serum, detected egg HDL equivalents in the hemolymph of secondary-vitellogenic females (Fig. 6). Moreover, the hemolymph of secondary-vitellogenic females, assayed quantitatively by serial dilutions, had levels of 20.4 ± 6.5 mg/ml equivalents. Only trace quantities of such a protein could be detected in the hemolymph dilution of both males and primary-vitellogenic females (22.0 ± 3.6 and 89.6 ± 21.0 μg/ml, respectively), as expected from the previous double immunodiffusion and immunoblot cross-reactivity tests (Figs. 6 and 7).

The amount of egg HDL equivalents (22.9 ± 6.2 μg/ml) detected in the hemolymph of intact intersex individuals was similar to that of normal males (Fig. 7). Yet, when intersex individuals were subjected to AG removal, significantly higher levels of secondary-vitellogenic-specific proteins (1.7 ± 0.1 mg/ml, $P < 0.001$, ANOVA followed by LSD test) were detected in their hemolymph. Furthermore, when AGs were transplanted into females, their egg HDL equivalent levels (79.3 ± 18 μg/ml) were significantly ($P < 0.001$) lower than those detected in the hemolymph of females that had undergone a sham operation (20.5 ± 3.8 mg/ml) or of secondary-vitellogenic females.

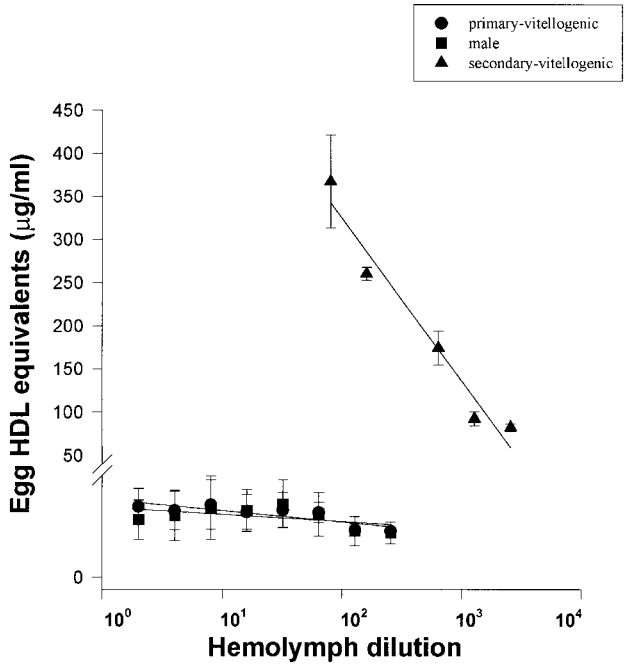


FIG. 6. Dilution curve of hemolymph from *C. quadricarinatus* male, primary-vitellogenic female, and secondary-vitellogenic female determined by ELISA. Values are presented as μg/ml equivalent of HDL from newly laid egg. Bars represent SE.

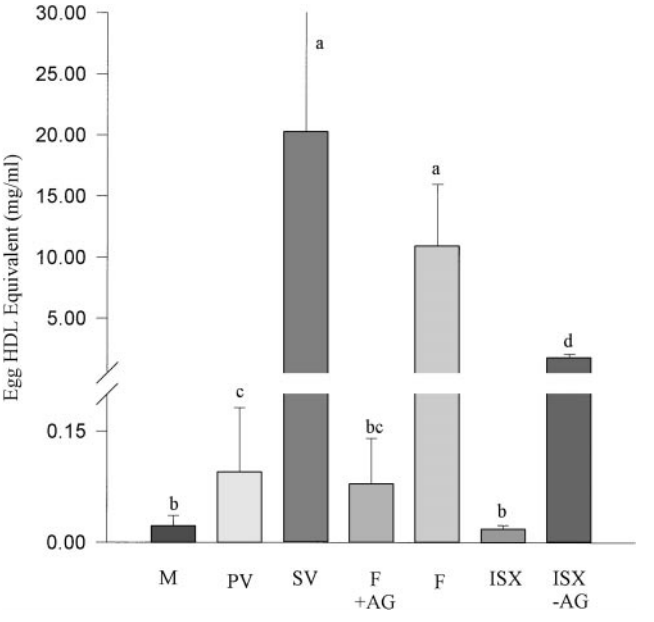


FIG. 7. Changes in level of secondary-vitellogenic-specific protein in the hemolymph of *C. quadricarinatus* following androgenic gland ablation or implantation. M, males; PV, primary-vitellogenic females; SV, secondary-vitellogenic females; F + AG, AG-implanted females; FSH, sham-operated females; ISX, intact intersex individuals; ISX − AG, AG ablated intersex individuals. Bars represent SE. Different letters represent significant difference, $P < 0.05$, according to an ANOVA followed by LSD test.

## DISCUSSION

Assays for vitellin and vitellogenin are required for a better understanding of the vitellogenic process. In the present study, an ELISA was established for *C. quadricarinatus* hemolymph using an antiserum prepared against a secondary-vitellogenic specific polypeptide (106 kDa), separated from newly laid eggs. This ELISA was capable of quantifying egg HDL equivalents in the

hemolymph of vitellogenic females, confirming the cross-reactivity of this serum with hemolymph proteins associated with secondary vitellogenesis, as was also evident by both immunodifusion and immunoblot tests. All previous studies in which such an ELISA was developed in crustaceans (Chi-Ying and Watson, 1994; Lee and Chang, 1997; Okumura *et al.,* 1992; Derelle *et al.,* 1986), including the present study, assumed similarity between vitellogenin and vitellin in their biochemical characteristics. Indeed, such a similarity in the amino acid sequences was recently demonstrated in *Macrobrachium rosenbergii* (Lee *et al.,* 1997). When the cytosolic component of *C. quadricarinatus* oocytes was subjected to SDS–PAGE, the protein was denatured into a number of polypeptides, most of which were dominant in the ovaries of secondary-vitellogenic females and less so in those of primary-vitellogenic females (Abdu, personal communication). In the present study, a dissociated PAGE preparation of the 106-kDa polypeptide, exclusively related to the secondary-vitellogenic process in the maturing ovary of *C. quadricarinatus,* was used for the first time for generating an antibody. This antibody recognized polypeptides with higher molecular weight in the hemolymph of secondary-vitellogenic females (Fig. 4), which in the case of an extraovarian site of vitellin synthesis might represent vitellin precursors. This recognition was the basis for the development of a highly reliable and sensitive assay, enabling the detection of small quantities (as little as 8 ng) of yolk HDL equivalents in the hemolymph. Previous studies using polyclonal antibodies have described a reliable ELISA for vitellogenin in the crab, *Callinectes sapidus* (Chi-Ying and Watson, 1994), and in the prawns *M. rosenbergii* (Lee and Chang, 1997) and *M. nippponense* (Okumura *et al.,* 1992). Derelle *et al.* (1986) used monoclonal antibodies in *M. rosenbergii* for such an ELISA. Unlike the present study, in all the above-mentioned species, antiserum was prepared against native preparations of vitellin.

Although the crayfish *C. quadricarinatus* is a gonochoristic species, partial sexual plasticity is expressed in the form of intersex individuals (Medley and Rouse, 1993; Sagi *et al.,* 1996a) having an active AG and exhibiting male secondary characteristics and behavior (Sagi *et al.,* 1996a). An ovary with an oviduct is found in such intersex individuals in which a female opening is present in the absence of a male opening on the same side. In such cases, the ovary has always been found at an arrested primary-vitellogenic stage. Secondary vitellogenesis was permitted in such ovaries only following surgical removal of the AG (Khalaila *et al.,* 1999). Only then was the 106-kDa polypeptide, which is specific to secondary vitellogenesis, present in the ovary of an intersex individual. This polypeptide was totally undetectable in ovaries of intact or sham-operated intersex individuals (Khalaila *et al.,* 1998). The presence of such polypeptides in the ovaries and of their cross-reactive component in the hemolymph of andrectomized intersex individuals clearly indicates the occurrence of secondary vitellogenesis permitted by the removal of the AG. Moreover, the present study demonstrates clearly that transplantation of the AG into female *C. quadricarinatus* totally prevented secondary vitellogenesis. Similarly, in other crustaceans, the AG, when present, is known to inhibit ovarian maturation (Meusy and Payen, 1988; Taketomi and Nishikawa, 1996), while removal of the AG resulted in female differentiation in isopods, amphipods, and decapods (Charniaux-Cotton, 1964; Nagamine *et al.,* 1980; Sagi *et al.,* 1990).

The ELISA developed in this study, using the anti-106-kDa polypeptide, detected marked differences in the content of hemolymphatic cross-reactive protein between AG-transplanted and control females and between andrectomized and control intersex individuals. It provides strong evidence for the central role of the AG in controlling the balance between maleness and femaleness in *C. quadricarinatus* intersex individuals. The ELISA described here presents a simple, noninvasive diagnostic tool by which the endocrine control of the expression of vitellogenic-specific proteins could be studied *in vivo.* Thus far, the bioassays utilized to investigate the effects of the AG hormone in female malacostracan Crustacea were based on its effect on external morphology or gonadal structure (Katakura and Hasegawa, 1983; Martin *et al.,* 1990; Okuno *et al.,* 1997; Taketomi and Nishikawa, 1996). The new assay could be instrumental in future studies on the nature and mode of action of the AG hormone in regulating sexual plasticity in crustaceans, thus replacing the classical morphological *in vivo* bioassays previously used for such studies. This new tool may have even greater significance in the light of the fact that the nature of the AG hormone has, thus far, not been fully

elucidated in decapod crustaceans. Moreover, the only androgenic substances purified thus far from the androgenic gland of decapod crustaceans were lipidic in nature, while a major body of supporting evidence suggests that the androgenic hormone may be a protein or a polypeptide (Sagi *et al.*, 1997 for review).

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