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# Effects of Implantation of Hypertrophied Androgenic Glands on Sexual Characters and Physiology of the Reproductive System in the Female Red Claw Crayfish, *Cherax quadricarinatus*

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The role of the androgenic gland (AG), an organ unique to male Crustacea, in the development of sex characters and physiology of the reproductive system has not been fully documented in the red claw crayfish, *Cherax quadricarinatus.* To investigate the role of the AG in this species, the effect of implanting hypertrophied AGs into immature female animals was followed. Of the female animals with AG implants, 91.6% developed male-like propodi, including the red patch characteristic of males of this species. The development of female secondary sex characteristics such as a wider abdomen, a wider endopod, and simple setation was inhibited. At the end of the experiment, the ovaries of the AG-implanted females contained mostly lipid-stage oocytes, with a small number of oocytes at the early yolk stage. The gonadosomatic index of the AG-implanted females was significantly lower than that of the control (sperm duct-implanted or sham-operated) females, which had mature oocytes with a well-defined perinuclear zone and yolk globules. An immunohistochemical test using an antibody developed against a 106-kDa secondary vitellogenic polypeptide showed only slight immunoreactivity in the oocytes of AG-implanted females compared with abundant immunoreactivity in control ovaries. In the polypeptide profile of the high-density lipoprotein (HDL) from the hemolymph of AG-implanted females, the 206- and 79-kDa secondary vitellogenesis-specific polypeptides were not found, whereas they were present in the profile of control females. In contrast, the female-specific 177-kDa polypeptide was present in the polypeptide profile of hemolymph HDL of both AG-implanted females and control females. It seems therefore that while secondary sex characters were masculinized under the influence of the implanted AG, the process of vitellogenesis was suppressed but not fully eliminated in the AG-implanted females. 

*Key Words:* androgenic gland; sex differentiation; reproduction; vitellogenesis; sexual characters; Crustacea; Decapoda; crayfish.

The role of the androgenic gland (AG), an organ unique to male crustaceans, in regulating sex differentiation was first described by Charniaux-Cotton (1954, 1955) in the amphipod *Orchestia gammarella.* Since then, its role has been investigated in almost all superorders of malacostracan crustaceans. In the female of the isopod species *Armadillidium vulgare,* for example, AG implantation or injection of AG extracts at an early stage of differentiation resulted in the differentiation of testes and the appearance of secondary sexual characteristics (Katakura, 1961; Katakura and Hasegawa, 1983; Suzuki and Yamasaki, 1998). Similarly, AG implantation into female *Macrobrachium rosenbergii,* the decapod prawn, at an early stage of differentiation led to the differentiation of these ge-

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netic females to phenotypic reproductive males (Nagamine *et al.*, 1980b). The opposite situation—differentiation of genetic males into phenotypic reproductive females—resulted from AG ablation in an early stage of differentiation (Nagamine *et al.*, 1980a; Sagi and Cohen, 1990). In crayfish species, however, the role of the AG in sex reversal has not yet been successfully demonstrated. Recently, injection of AG extracts into juveniles of the crayfish, *Cherax destructor*, that were assumed to be females led to the development of male openings at the bases of their fifth walking legs (Fowler and Leonard, 1999). In the red claw crayfish, *Cherax quadricarinatus* (Parastacidae), the role of the AG was investigated in intersex individuals that had male characteristics and were reproductively active as males (Sagi *et al.*, 1996a). AG ablation in such animals resulted in the onset of vitellogenesis in the ovary, which is permanently arrested in intact individuals (Khalaila *et al.*, 1999). In the current study, the role of the AG in regulating male secondary characters in *C. quadricarinatus* was investigated by implanting hypertrophied AGs from mature males into immature female animals. The development of the unique red patch on the propodus, which is peculiar to mature *C. quadricarinatus* males, was studied in an attempt to develop a bioassay for identifying and characterizing decapod AG hormone(s), which have not yet been identified. In addition to a study of its effects on secondary sexual characters, a complete review of the anatomical and physiological effects of AG implantation into the female crayfish was undertaken.

## MATERIALS AND METHODS

***Animals.*** Forty-eight immature females and sixteen *C. quadricarinatus* males, all of the same age, each with a carapace length of 20.8 ± 3.0 mm, were collected from artificial ponds at Ben-Gurion University of the Negev. The animals were divided into 16 groups, each group of four animals comprising one female from each of the three treatment types described below and one male. Eight mature males, carapace length 51.8 ± 3.9 mm, that had been bilaterally destalked 2 weeks earlier were anesthetized in ice-cold water and dissected, and their hypertrophied AGs were collected in physiological saline. Into each of 16 females, one hypertrophied AG was implanted under the articular membrane above the coxa of the left fifth walking leg, by means of a delicate forceps. The hole made in the membrane for the implantation was sealed with 5-s super glue. Another 16 females received implants of sperm duct (SD) fragments, each fragment being the same size as that of the hypertrophied AG. The remaining 16 females, which had been sham-operated, and the 16 males served as control groups. Each group of four animals was kept in a glass aquarium (40 × 50 × 40 cm) with polycarbonate partitions that formed four identical compartments, one for each individual. The water temperature was maintained at 27 ± 2°, and a photoperiod of 14L:10D was applied. Water quality was ensured by circulating the water through a gravel biofilter. Food was supplied *ad libitum*.

***Morphological and anatomical observations.*** Seven months after the beginning of the experiment, the manifestation of the red patch on the propodus of all the control males marked the end of the experiment. All the experimental specimens were examined externally and weighed, and carapace length and width were measured (±0.05 mm). Each animal was examined for the development of male secondary sexual characteristics, i.e., the red patch on the propodus, and female secondary sexual characteristics, such as increased abdominal and endopod width and development of simple setae on the endopod. In males only plumose setae are present while with the progress of female maturation simple (ovigerous) setae are developed (Sagi *et al.*, 1996b). The relative width of the propodus was calculated as the width of the propodus at the point underneath the chela divided by the length of the propodus. The relative width of the abdomen was calculated as width of the second abdominal segment divided by the length of the carapace, and the relative endopod width as the width of the endopod divided by the width of the exopod. For anatomical studies, the animals were anesthethized in ice-cold water, and the gonads were dissected out and weighed (±0.001 g). The gonadosomatic index (GSI) was calculated as the percentage of gonad weight to body weight. Oocyte diameter was measured under a light microscope, and the mean oocyte diameter (±SE) for each ovarian stage was calculated from a sample of 15 oocytes (from the large oocyte population) per ovary. The ovarian stage in the reproductive cycle was

determined in terms of GSI, mean oocyte diameter, and color of the gonad (Sagi *et al.*, 1996a).

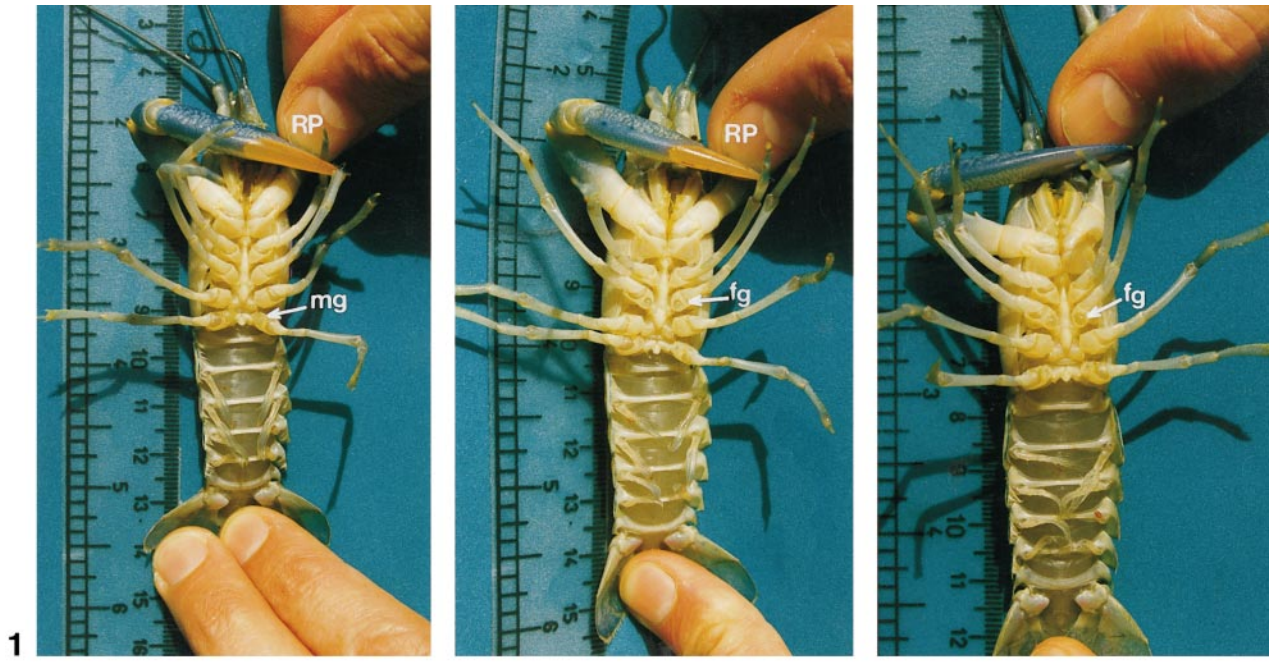
***Histology and immunolocalization of a vitellogenesis-specific polypeptide.*** Ovarian tissue was removed from the experimental and control females and fixed in Bouin's solution for 24 h. Thereafter, the tissue was dehydrated in an alcohol series and embedded in Paraplast (melting point, 60–61°). Sections of 5 $\mu$m were stained with hematoxylin and eosin. For immunohistochemistry, antibodies that had been raised against the 106-kDa yolk polypeptide, a secondary vitellogenic specific polypeptide, were used (Sagi *et al.*, 1999). Nonspecific antibody reactions were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 60 min, followed by incubation for 30 min with the 106-kDa antiserum (1:5,000) preabsorbed with male hemolymph, or with preimmune serum as negative control. After three washes with PBS, the sections were incubated for 30 min with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000; Sigma). After another three washes with PBS, sections were incubated with the alkaline phosphatase substrates, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

***Isolation and monitoring of hemolymph vitellogenic proteins.*** Hemolymph was withdrawn using a 2-ml syringe with an 18-gauge needle from the sinuses at the bases of the fifth walking legs of several experimental and control specimens. The hemolymph of each treatment was pooled in 10-ml tubes, each containing 1 ml of 7% EDTA, and was adjusted to reach 1% final concentration of EDTA. Sodium bromide was added to a final density of 1.22 g/ml, and the tubes were centrifuged at 41,000 rpm for 48 h at 12°C. The orange floating layer (<5 ml) containing the hemolymph high-density lipoproteins (HDLs) was collected and dialyzed overnight against 1 liter of 0.25 M NaCl–10 mM phosphate buffer (pH 7.4). Total protein content was determined by the method of Bradford (1976), with BSA as the standard. Aliquots of the dialysate containing hemolymph HDL were stored at −20° prior to 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) separation (Laemmli, 1970), for which high-molecular-mass (200, 116.5, 97.4, 66.2, 45, 31 kDa) markers (Sigma, St. Louis, MO) were used. To monitor the amounts of secondary vitellogenesis-specific proteins in the hemolymph, 20 $\mu$l of hemolymph was withdrawn and diluted in 480 $\mu$l carbonate buffer 0.1 M. An ELISA, using the above-described anti-106 kDa polyclonal antibody, was used (Sagi *et al.*, 1999).

## RESULTS

The characteristic male red patch developed on the propodi of 91.6% of the surviving AG-implanted females ($n = 12$) and all of the males ($n = 10$) (Fig. 1). None of the control females (sham-operated, $n = 15$, and SD-implanted females, $n = 8$) developed this character. The mean relative width of the propodus, a value reflecting "maleness," was significantly higher ($P \leq 0.005$) in AG-implanted females (0.31 ± 0.006) than in SD-implanted (0.29 ± 0.005) or sham-operated (0.29 ± 0.003) females and was identical in AG-implanted females and intact males (0.31 ± 0.006) (Fig. 2). Accordingly, the relative width of the abdomen (Fig. 2), a higher value of which reflects "femaleness," was significantly lower ($P \leq 0.005$) in AG-implanted females (0.90 ± 0.01) than in SD-implanted or sham-operated females (0.97 ± 0.20 and 0.96 ± 0.10, respectively) and similar in AG-implanted females and intact males (0.89 ± 0.008). The maternal care-related characteristics, such as simple setation of the pleopod and relative endopod width, were also affected by AG implantation. The relative endopod width of AG-implanted females was similar to that of intact males (1.08 ± 0.07 and 1.09 ± 0.05, respectively), both values being significantly lower (ANOVA followed by LSD test, $P \leq 0.005$) than those of SD-implanted and sham-operated females (1.85 ± 0.11 and 1.84 ± 0.06, respectively). The endopod of AG-implanted females did not contain simple setation.

Anatomically, the gonads of the AG-implanted females were yellowish white in color in comparison with the brownish green ovaries of the sham-operated females (Fig. 3). The oocyte diameter for the AG-implanted females (551 ± 32 $\mu$m) was significantly smaller ($P \leq 0.005$) than that for the SD-implanted or sham-operated females (1725 ± 310 and 1480 ± 119 $\mu$m, respectively) (Fig. 4). The GSI of the AG-implanted females (0.63 ± 0.09) was significantly lower ($P \leq 0.005$) than that of SD-implanted or sham-operated females (4.37 ± 1.27 and 2.94 ± 0.42, respectively) (Fig. 4).

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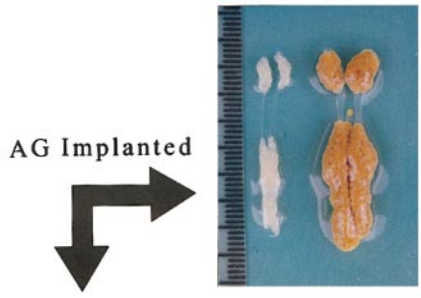


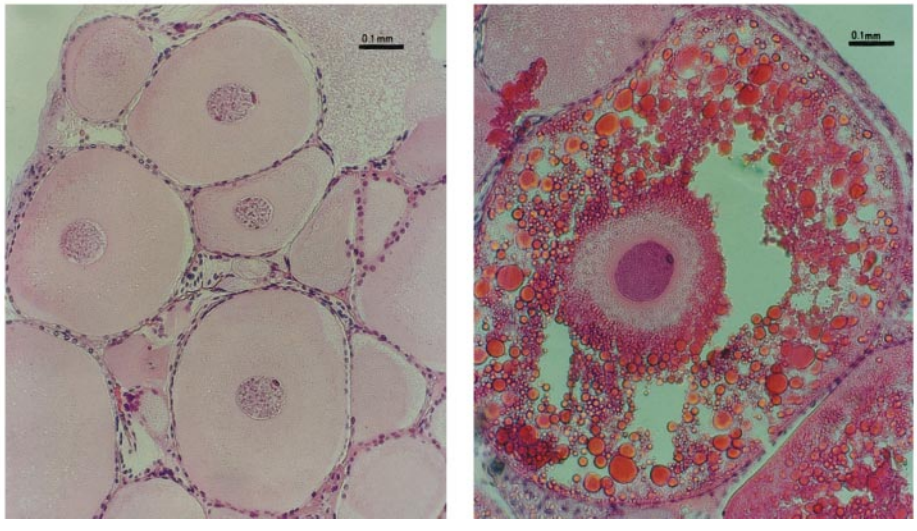


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FIG. 1. Effect of AG implantation on the development of the red patch on the propodus of *C. quadricarinatus* females. Ventral and propodus view. Left, intact male; middle, AG-implanted female; and right, intact female. RP, red patch; mg, male genital opening; fg, female genital opening.

FIG. 3. Ovaries of an AG-implanted *C. quadricarinatus* female (left) and a sham-operated female (right) and the respective hematoxylin and eosin-stained transverse sections. Bars = 0.1 mm.

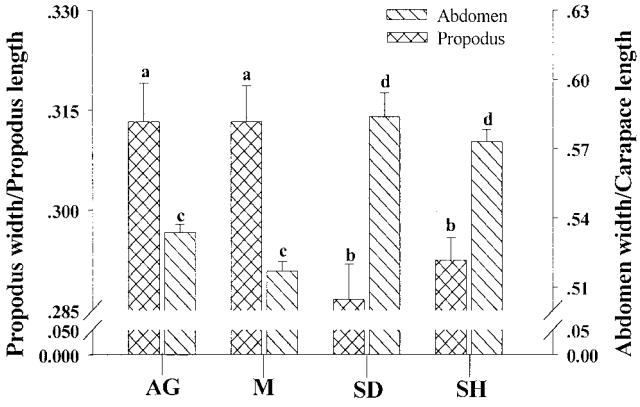


FIG. 2. Effect of AG implantation on the manifestation of male and female secondary sex characters in *C. quadricarinatus* females. Mean relative width of the propodus (propodus width / propodus length) and mean relative width of the abdomen (abdomen width / carapace width) represent male and female sex characters, respectively. AG, AG-implanted females; M, intact males; SD, sperm duct-implanted females; SH, sham-operated females. Different letters represent significant difference between groups (ANOVA followed by LSD test, $P \leq 0.005$).

Transverse sections of the ovaries showed that the majority of the oocytes of AG-implanted females were at the lipid stage, with a small number of oocytes at the early yolk stage in which yolk accumulation begins (Fig. 3). In contrast, ovaries of the SD-implanted and sham-operated females had matured oocytes, having a perinuclear zone and yolk globules (Abdu *et al.*, 2000) (Fig. 3). The immunohistochemical study with the anti-106-kDa polyclonal antibody showed significant cross-reactivity in the oocytes of sham-operated females (Fig. 5, SH) versus negligible cross-reactivity in those of AG-implanted females (Fig. 5, AG).

The ELISA test with the antibody raised against the specific 106-kDa vitellogenic yolk polypeptide showed that the level of cross-reactive material in the hemolymph of the AG-implanted females was similar to that of intact males ($0.149 \pm 0.176$ and $0.054 \pm 0.072$ mg / ml, respectively) but significantly different ($P \leq 0.005$) from levels of SD-implanted and sham-operated females ($11.157 \pm 3.355$ and $17.139 \pm 1.473$ mg / ml, respectively). SDS–PAGE of hemolymph HDL gave a polypeptide profile for the AG-implanted females that differed from that of the males and from that of the sham-operated females (Fig. 6). Two polypeptides with approximate molecular masses of 206 and 79 kDa were prominent in the polypeptide profile of the sham-operated females but absent from the profile of AG-implanted females. However, the female-specific 177-kDa polypeptide was present in the profiles of both AG-implanted and sham-operated females but not in the profile of intact males. The 96-kDa polypeptide was present in the polypeptide profiles of intact males, AG-implanted females, and sham-operated females (Fig. 6).

## DISCUSSION

The red patch—the bright soft uncalcified tissue on the outer edge of the propodus—constitutes a unique male secondary sexual feature in mature males of *C. quadricarinatus.* The function of the red patch is not known; it is thought to be used for sexual attraction or as a mating device (Thorn *et al.*, 1989). This study showed, for the first time, that secretion from AGs implanted into immature *C. quadricarinatus* females resulted in the development of the red patch on the propodus of 91.6% of the females (Fig. 1). In natural or aquacultured populations, the development of the red patch on the propodus of females is a rare occurrence, although it has been described in intersex individuals

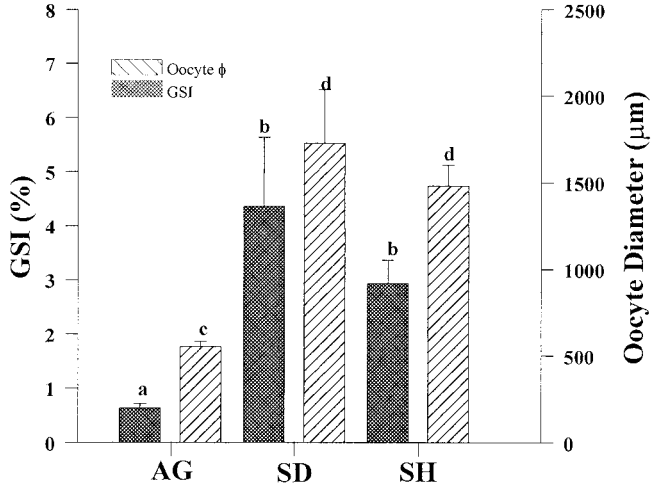


FIG. 4. Effect of AG implantation on the mean GSI and the mean oocyte diameter in *C. quadricarinatus* females. AG, AG-implanted females; SD, sperm duct-implanted females; SH, sham-operated females. Different letters represent significant difference between groups (ANOVA followed by LSD test, $P \leq 0.005$).

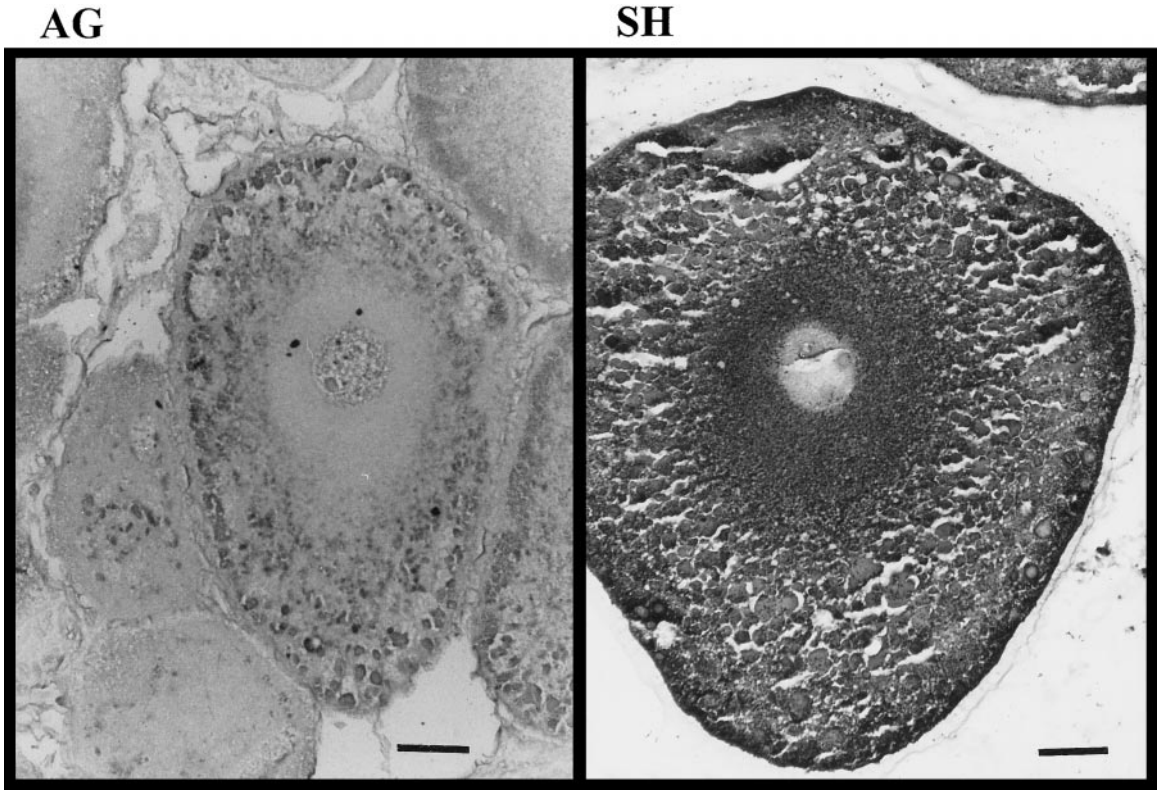


FIG. 5. Immunohistochemistry showing the effect of AG implantation on the abundance of cross-reactive material with antibody raised against the 106-kDa secondary vitellogenic-specific yolk polypeptide in *C. quadricarinatus* oocytes. AG, ovarian tissue of AG-implanted females; SH, ovarian tissue of sham-operated females. Bars = 0.1 mm.

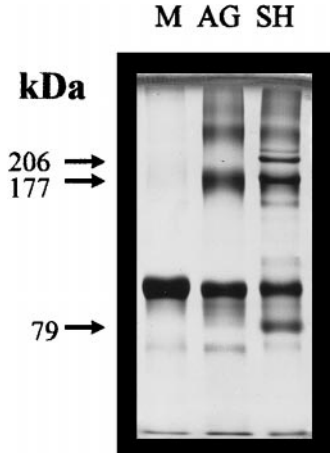


FIG. 6. Coomassie blue-stained 7% SDS–PAGE of HDL from the hemolymph of AG-implanted *C. quadricarinatus* females (AG), sham-operated females (SH), and intact males (M). Arrows indicate major yolk polypeptides.

(Thorn *et al.*, 1989). This finding is in keeping with previous studies showing that all intersex individuals have active AGs (Khalaila *et al.*, 1999; Sagi *et al.*, 1996a).

AG implantation into immature females induced the development of a number of male-like secondary sexual characteristics, as reflected in the relative width of the propodus, the relative width of the abdomen (Fig. 2), and the structure of the pleopods. These findings are in keeping with previous studies on the prawn, *M. rosenbergii*, in which AG implantation led to the development of secondary male characters such as the appendix masculina and male chelipeds, but inhibition of the development of female characteristics such as structure of the pleopods and brood chambers (Nagamine *et al.*, 1980b). Similarly, AG implantation experiments in the crayfish, *Procambarus clarkii*, induced the development of male-like abdominal appendages in implanted females (Nagamine and Knight, 1987; Taketomi and Nishikawa, 1996). Development of oosetae was also prevented in immature *C.*

*destructor* females after the injection of aqueous extracts of AG (Fowler and Leonard, 1999).

In the present study, neither differentiation of the gonad to testes nor development of the male genital opening was detected in AG-implanted females. These results differ from those previously obtained for *M. rosenbergii*, in which AG implantation at an early stage of differentiation induced testicular development and the appearance of male genital openings at the bases of the fifth walking legs (Nagamine *et al.*, 1980b). The differences between the results of these two studies may be due to the timing of the intervention. However, in keeping with the present results, AG implantation into *P. clarkii* females (2–3 months old, 7–15 mm in carapace length) did not induce male genital openings, even though masculinization of secondary sexual characters did take place (Nagamine and Knight, 1987; Taketomi *et al.*, 1990; Taketomi and Nishikawa, 1996). In contrast, Fowler and Leonard (1999) reported the appearance of male genital openings at the bases of the fifth walking legs of *C. destructor* (carapace length 11.94 ± 2.23 mm) following an aqueous AG extract injection into individuals that were assumed to be females according to the presence of gonopors. However, the possibility that some of the injected animals might have been intersex individuals cannot be ruled out, since the phenomenon of intersexuality in which male and female openings are present in the same individual is common in the genus *Cherax* (Sagi *et al.*, 1996a).

In this study, AG implantation into immature *C. quadricarinatus* females inhibited vitellogenesis, as indicated by the relatively low GSI and small oocyte diameter. These results are in keeping with those results of a previous study in *P. clarkii*, in which the development of the oocytes was inhibited after AG implantation (Taketomi and Nishikawa, 1996). Similarly, in *C. destructor* the gonads were inhibited and vitellogenesis was arrested in AG extract-injected females (Fowler and Leonard, 1999).

In *C. quadricarinatus*, unique polypeptides in the ovary and in the hemolymph serve as markers for secondary vitellogenesis (Abdu *et al.*, 2000; Yehezkel *et al.*, 2000). These markers include a secondary vitellogenic-specific 106-kDa polypeptide, which accumulates in the oocytes of vitellogenic females (Sagi *et al.*, 1999). The presence of this polypeptide in the oocytes and ovaries of mature *C. quadricarinatus* females was shown by immunohistochemical means using the anti-106-kDa polypeptide antibody developed in rabbit (Sagi *et al.*, 1999). This technique showed that the 106-kDa polypeptide accumulated in significant amounts in the oocytes of control female ovaries but not in the ovaries of AG-implanted females. Polypeptides with molecular masses of 206 and 79 kDa in the hemolymph HDL of *C. quadricarinatus* vitellogenic females, which are indicative of the progress of the secondary vitellogenic process (Yehezkel *et al.*, 2000), were not found in the polypeptide profiles of hemolymph HDL from AG-implanted females. These novel results of the effect of the AG on hemolymphatic HDL polypeptides together with the immunohistochemical study of the ovary emphasize the fact that the AG affects the expression of secondary vitellogenesis-specific polypeptides and / or the accumulation of secondary vitellogenesis-specific polypeptides in the oocytes. Further experimentation is required to determine whether the AG exerts its effect directly on the site of synthesis, as a target organ, or via intermediary organ(s). Since the AG hormone of decapod Crustacea has yet to be characterized, the finding of the current study would acquire greater significance if applied in the construction of bioassay systems for the identification and characterization of the AG hormone.

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