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Androgenic gland implantation promotes growth and inhibits vitellogenesis in *Cherax quadricarinatus* females held in individual compartments

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Summary

Androgenic glands (AGs) were implanted into young female red claw crayfish, *Cherax quadricarinatus*, with the aim of investigating the role played by the AG in the balance between growth and reproduction under conditions of minimal social interaction (individual compartments). The growth rate of the females with AG implants was significantly higher than that of the control non-implanted females (0.11 ± 0.03 g/day vs. 0.08 ± 0.02 g/day). This difference was attributed to the larger molt increments and slightly shorter molt intervals of the females with implants vs. the control females. At the end of the experiment (538 days), the mean weight of the implanted females was significantly higher than that of the control females (64.58 ± 18.24 g vs. 51.07 ± 12.71 g, respectively), a lead of 26.4% for the implanted females that started 91 days after implantation and became significant at 153 days after implantation. By that time, 55.5% of the implanted females had developed typical male secondary characters, such as the red patch on the propodus. The shift of energy from female reproduction to growth was further demonstrated by the level of expression of the vitellogenin gene in the hepatopancreas: gene expression was high in control females but lower by several orders of magnitude in the AG-implanted females, as shown by real time RT-PCR relative quantification. Confirmation of these findings was provided by an ELISA test, which showed that the level of vitellogenic cross-reactive protein in the hemolymph of AG-implanted females was significantly lower than that in intact females. The significant growth promotion in AG-implanted females was clearly not due to social interaction. It may be attributed to a direct growth factor — like the effect of androgens in vertebrates — in combination with an indirect effect, through the shift of energetic investment from reproduction and vitellogenesis to growth. Since the AG implant had a more marked effect on molt increment than on molt interval, it seems likely that the AG acts as a growth promoter rather than as a molt enhancer.

Key words: Crustacea, Decapoda, crayfish, red claw; *Cherax quadricarinatus*, androgenic gland, growth promotion, reproduction, vitellogenesis

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Introduction

Several crustacean species exhibit bimodal growth patterns, with males growing faster than females or vice versa. The red claw crayfish, *Cherax quadricarinatus,* is one such species. In a study of communal pond cultures, males were found to weigh more than females at the end of an experimental period (Curtis and Jones, 1995). In another study, a similar difference in weight was clearly manifested in a second grow-out season of over-wintered populations (Sagi et al., 1997). Since it is believed that social interaction influences the growth pattern in this species, an attempt was made to culture *C. quadricarinatus* in individual compartments designed to minimize social contact (Manor et al., 2001). In this separate-cell system, too, *C. quadricarinatus* males grew faster than females and reached greater weights. These results are in keeping with those of Hartnoll (1982), who reported that after puberty crustacean females usually molt less frequently, grow more slowly, and reach a smaller size than the males, probably because reproduction demands a large proportion of the available resources in females. Since our study showed that, for *C. quadricarinatus* housed in separate cells that prevented social interaction, males grew faster than females (Manor et al., 2001), it is likely that a direct physiological mechanism is involved in the differences in growth patterns. The physiological mechanism that confers an advantage in terms of growth is thought to be mediated by the androgenic gland (AG), which is responsible for male sexual differentiation and for control of sexual plasticity in crustaceans (Charniaux-Cotton and Payen, 1988; Sagi, 1988; Payen, 1990; Sagi et al., 1997). In the prawn *Macrobrachium rosenbergii*, for example, the AG has been shown to play a role in the mediation of growth (Sagi et al., 1997). This growth promotion (Sagi et al. 1986), mediated by the AG, could indeed be the result of a direct growth factor, such as an androgen, or it could take place via an indirect effect, i.e., through a shift of energetic investment in female reproduction and vitellogenesis to growth.

The AG has not been characterized in all species of Crustacea, but that of *C. quadricarinatus* has indeed been identified, and the effect of implanting AGs into immature females has been investigated (Khalaila et al., 2001). AG implantation causes the development of male secondary characteristics and the inhibition of female secondary characteristics and vitellogenesis, as indicated by a relatively low gonadosomatic index (GSI) and the small oocyte diameter (Khalaila et al., 2001). However, the exact effect on molt increment and molt interval and the inhibition of the vitellogenic process at the molecular level in AG-implanted females has not yet been investigated. The full-length *C. quadricarinatus* vitellogenin cDNA, which encodes for yolk protein subunits, has been cloned and sequenced and has been found to be expressed exclusively in the hepatopancreas of secondary vitellogenic females (Abdu et al., 2002). In the current study, we determined the expression of the vitellogenin gene by means of a real-time RT-PCR using RNA from the hepatopancreas of control and AG-implanted females as the template and primers based on the sequence of the *C. quadricarinatus* vitellogenin gene.

The aim of this study was to further elucidate the effect of the AG on the balance between growth and reproduction in *C. quadricarinatus* and the mechanism through which the AG promotes growth.

Materials and Methods

Animals

Forty young, sexually immature females (6.7± 1.6 g) were obtained from a normal population held at a commercial nursery (Parnes and Sagi, 2002). Each of 20 females was implanted with a hypertrophied AG, which had been dissected out of a large mature male that had been endocrinologically manipulated, according to the procedure described by Khalaila et al. (2001). The other 20 females served as controls. The females were randomly assigned to the two treatments. Each female was marked with a small piece of colored adhesive plastic tape on the carapace, which enabled the identification of a molting event. Each animal — implanted or control — was maintained individually for 406 days in a 400-cm^2 cell suspended in a 5-m^3 tank located in a greenhouse. Thereafter, the females were transferred to 1260-cm^2 cells (so that the size of the cell would not inhibit growth) until the end of the experiment, 538 days after implantation. Each cell contained a shelter and an individual feeding pipe. Food comprising shrimp pellets (Rangen, 30% protein) and wheat grains was supplied *ad libitum* through individual pipes to each cell three times a week. Water temperatures ranged between 20–30°C. Water quality was assured by circulating the whole volume of water through a biofilter. During the experiment the pH was 8.3±0.5, nitrite was less than 0.1 ppm, nitrate was less than 50 ppm, ammonium levels were negligible, and oxygen concentration exceeded 5 mg/l.

Approximately once a month females were weighed (±0.01 g), and the development of the red patch was followed. Molt intervals, i.e., the average times in days between molts, and molt increments, i.e., the average weight gains in grams per molt, were recorded for the AG-implanted and control females. Mature males of

approximately the same size that served as control for the RT-PCR and the claw image analysis procedures were collected from a 5-m^3 tank located in the same greenhouse and supplied with the same water system.

Morpho-anatomical measurements

Four hundred and thirty-eight days after implantation, the following morphometric measurements were performed with digital calipers (±0.05 mm): length and width of the propodus of the chela, widths of the endopod and exopod at the second segment, length of the carapace and width of the abdomen. Relative propodus width was expressed as propodus width/propodus length; relative endopod width, as endopod width/exopod width; and relative abdomen width, as abdomal width/carapace length. The percentage of simple (ovigerous) setae in a 1.25-mm internal endopod edge was calculated (Sagi et al., 1996). Same-sized males served as a reference group to compare the red patch that was developed in the implanted females to that of normal males. Lengths of the carapace and of the red patch were measured on a digitized image (Nicon D100) through image analysis using the ImageJ (NIH, Bethesda, MD) software. Relative red patch length was expressed as red patch length/carapace length.

Monitoring of vitellogenesis

An ELISA test using antibody raised against the specific 106-kDa vitellin polypeptide (Sagi et al., 1999) was used to monitor the amounts of secondary vitellogenic cross-reactive proteins in the hemolymph. For each animal, 10 μl of hemolymph was withdrawn and diluted in 490 μl of carbonate buffer 0.1 M, pH 9.6. To detect vitellogenin gene expression, small hepatopancreatic tissue fragments were sampled through a tiny hole that had been made in the cephalothorax. The biopsy was taken by an injector with a 2.0 × 45 mm venflon. RNA was extracted from each sample using the EZ-RNA Total RNA Isolation Kit (Biological Industries, Beit Haemek, Israel). The concentrations of all the produced RNAs were evaluated by spectrophotometry at 260 nm (GeneQuant Pro, Amersham Pharmacia Biotech). cDNA was obtained by a reverse transcriptase (RT) reaction from 1 μg of total RNA at 45°C for 1 h, in a volume of 20 μl, using 50 units of Expand Reverse Transcriptase (Roche, manufacturer's instructions) and polyT reverse primer at a final concentration of 2.5 μM. The cDNA was then amplified by PCR (one cycle at 95°C – 15 min; 35 cycles at 94°C – 1 min, 55°C – 1 min, 72°C – 2min; one cycle at 72°C –10 min). Each 25-μl reaction solution contained 0.625 units of HotStartTaq DNA Polymerase (QIAGEN), 0.2 mM of each d-nucleotide triphosphate, and 0.5 μM of primer concentrations, in buffer supplied with the DNA polymerase. The primers, based on the sequence of *C. quadricarinatus* vitellogenin gene (Abdu et al., 2002) were:

- Cherax mid-R 5′-
  CAGCTTGTAGCTGTATGGACTACCAAG-3′

and

- Cherax mid-F 5′-
  AACGAGAGCCAGTCTTTGTGGCTG-3′.

At the end of the experiment, the females were anesthetized in ice cold water, and the gonads were dissected out and weighed (± 0.001g). The 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 (±SD) for each female was calculated as described in Khalaila et al. (2001). Vitellogenin gene expression was evaluated by relative quantitative real time RT-PCR as follows: RNA was extracted as described above from each tissue fragment of the hepatopancreas sampled. First-strand cDNA was generated by an RT reaction (Reverse-iT™ 1st Strand Synthesis Kit – ABgene AB-0789) from 1 μg of total RNA at 47°C for 30 min with random hexamers as primers (20 ng/μl final concentration). Relative quantification of vitellogenin gene expression was performed using the following primers:

- Vit-Cher-QPCR-R 5′-
  GGGCGGCATGACACACATCT-3′

and

- Vit-Cher-QPCR-F 5′-
  GCTTCCCGGTGGTTAATCCT-3', each at 0.4 μM final concentration

and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, with an ABI Prism 7000 Sequence Detection System from Applied Biosystems (one cycle at 50°C – 2 min; one cycle at 95°C – 10 min; 40 cycles at 95°C – 15 s and 60°C – 1 min).

Statistical analysis

Data are expressed as mean ±SD. Statistical analysis was performed at each time point using one-way analysis of variance (ANOVA). P values <0.05 were considered statistically significant.

Results

From the 153rd day of the experiment, the mean weight (±SD) of the implanted females was

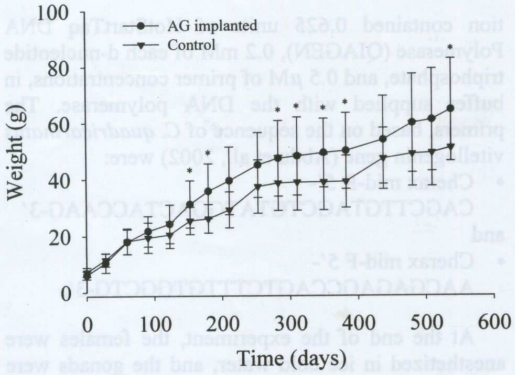


Fig. 1. Growth of AG implanted *C. quadricarinatus* females and control females over a period of 538 days in separate cells. Asterisk represents significant difference (p <0.05, one-way ANOVA).

significantly greater than that of the control females (p <0.05), with the values (on day 153) being 31.19 ± 8.31 g for the implanted females vs. 25.37 ± 4.84 g for the control females. At the end of the experiment, 538 days after implantation, the mean weights were 64.58 ± 18.24 g for the implanted females and 51.07 ± 12.71 g for the control females (Fig. 1), with maximum and minimum weights being 101.3 g and 35.4 g, respectively, for the implanted females, and 69.7 g and 34.1 g, respectively, for the control animals. The growth rate of the implanted females (0.11 ± 0.03 g/day) was significantly higher than that of the control females (0.08 ± 0.02 g/day).

Molt intervals of the implanted females were slightly shorter than those of the control females, but significant differences (p <0.05) were found only at 60, 91 and 180 days after implantation. At 180 days after implantation, molt intervals of the implanted and control females were 47.25 ± 14.39 and 58.0 ± 11.15 days, respectively (Fig. 2A). The molt increment of the implanted females became significantly greater than that of the control animals from the 91st day after implantation and remained so until the end of the experiment (p <0.05). At 180 days after implantation, molt increments of the implanted and control females were 12.23± 3.98 and 7.22 ± 2.28 g/molt, respectively (Fig. 2B).

AG implantation caused an increase in the expression of male secondary sexual characteristics such as the red patch, and an inhibition of female characteristics. By the end of the experiment, all the implanted females displayed a red patch which was similar in length to that of same sized normal males (0.56 ± 0.03 and 0.58 ± 0.03, respectively), while control females did not display this characteristic. In addition,

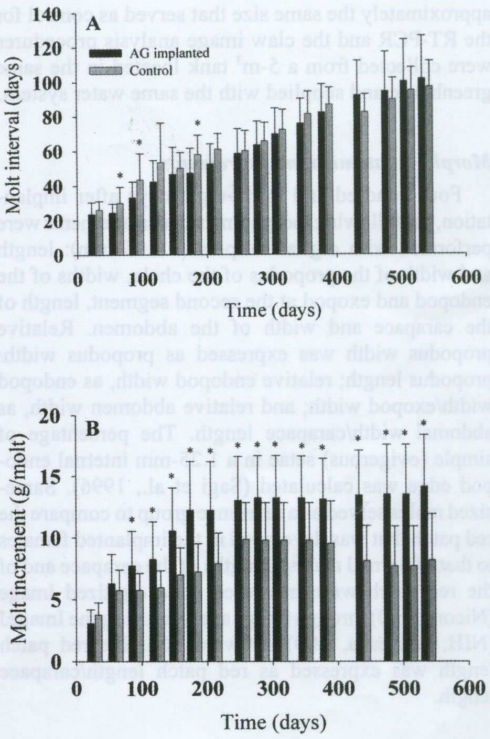
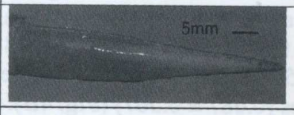
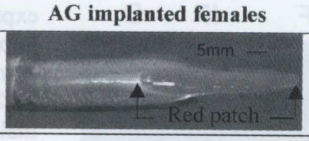
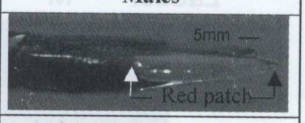


Fig. 2. Molt interval (A) and molt increment (B) of AG-implanted and control *C. quadricarinatus* females over a period of 538 days. Asterisk represents significant difference (p <0.05, one-way ANOVA).

comparing the claws to those of same sized control females showed that the implanted females developed a significantly (p <0.05) longer propodus similar to that of same sized normal males (Fig. 3A). Some implanted females developed the red patch as early as 28 days after implantation, and by 180 days after implantation 77.76% of the implanted females had already developed a red patch, while no instance of red patch development was detected in the control females (Fig. 3B). The earliest red patch appeared after the second post-implantation molt (28 days) and the latest, after the sixth post-implantation molt (383 days) (Fig. 3C). It is interesting to note that molt increments tended to be higher in the implanted females which developed a red patch compared to the same molt in implanted females that did not yet have a red patch. This tendency was significant after the first molt. The mean relative width of the propodus of AG-implanted females was significantly larger (p <0.05) than that of the control females (Table 1). Characteristics related to

A

Group	Females	AG implanted females	Males
Propodus Image			
Propodus length	51.18±3.68[a]	61.04±6.19[b]	57.21±4.88[b]
Carapace length	51.73±2.71	49.15±3.73	49.74±4.17
Relative red patch length	0	0.56±0.03	0.58±0.03

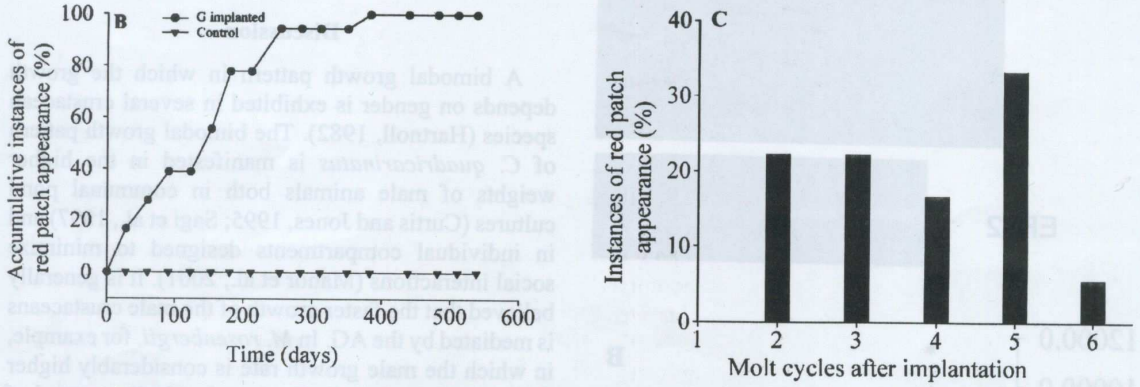


Fig. 3. Development of the masculine red patch in AG implanted *C. quadricarinatus* females. (A) Image analysis of the red patch of similar sized control females, AG implanted females and males. (B) Accumulating instances of red patch appearance along time in implanted versus control females. (C) Instances of red patch appearance in implanted females after each molt cycle.

Table 1. Morphological and physiological sex characteristics in AG implanted and control *C. quadricarinatus* females

Sex character	AG implanted	Control
Red patch on the propodus (% of females)	100	0
Relative propodus width (propodus. width/ propodus. length)	0.31 ± 0.02*	0.29 ± 0.02
Relative endopod width (endopod/exopod)	1.23 ± 0.18***	2.23 ± 0.18
Simple setae in 1.25 mm internal endopod edge patch (% simple setae out of total setae)	4.6± 8.8***	100
Relative abdomen width (abdomal width/carapace length)	0.57 ± 0.02***	0.61 ± 0.02
Vitellogenin cross reactive proteins in hemolymph (mg/ml)	0.59 ± 1.05***	37.56 ± 6.40
Oocyte diameter (mm)	0.37 ± 0.07**	1.431 ± 0.52
GSI (ovary mass/body mass × 100)	0.193 ± 0.08**	3.52 ± 1.61

***p <0.001, one-way ANOVA; **p <0.01, one-way ANOVA; *p <0.05, one-way ANOVA.

maternal brooding, namely, relative endopod widths, relative abdomen widths and percentage of simple setae, were significantly reduced by AG implantation (p <0.001) (Table 1).

The oocyte diameter and the GSI for the AG-implanted females were both significantly smaller than those for the intact females (p <0.05) (Table 1).

The expression of the vitellogenin gene was detected by RT-PCR in the hepatopancreas of control females, but not in the hepatopancreas of the AG-implanted females or of the negative control males (Fig. 4A). These results were confirmed by relative quantification of the vitellogenin gene by means of real time RT-PCR, which showed that the relative

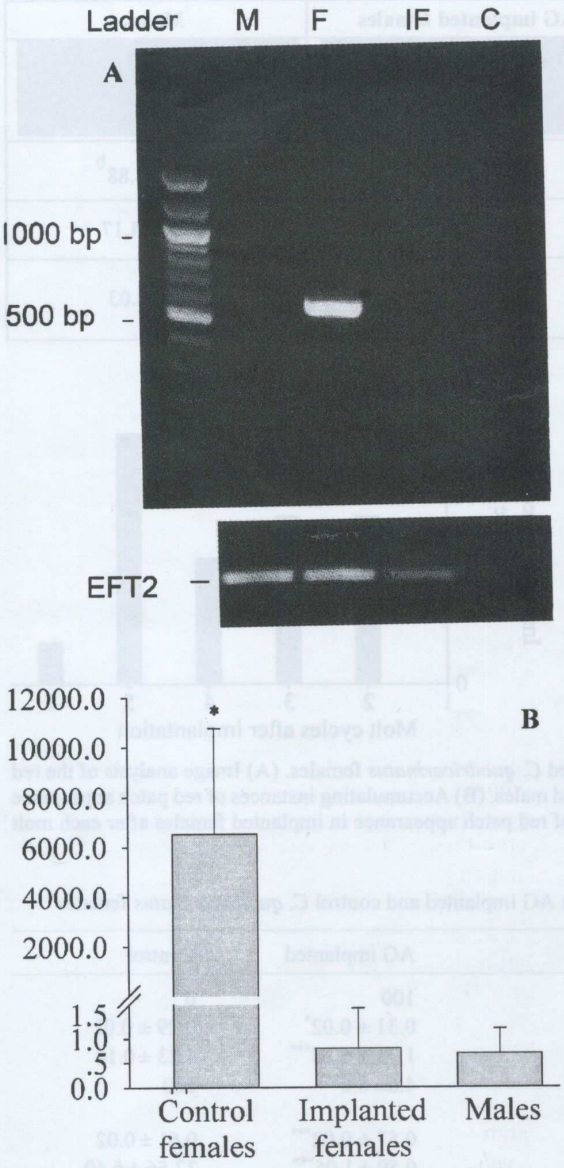


Fig. 4. Inhibition of vitellogenin gene expression in AG-implanted *C. quadricarinatus* females. (A) The agarose gels show RT-PCR products demonstrating the expression of *C. quadricarinatus* vitellogenin gene (Abdu et al., 2002; GenBank accession number AF306784), and a housekeeping gene (elongation factor (EFT2), GenBank accession number AI253924). Template RNA was extracted from hepatopancreases of a male (M), a control female (F) and an AG-implanted female (IF). Control for genomic contamination (C) was performed with RNA from the control female in the PCR reaction. A 100-bp DNA ladder marker (GIBCO-BRL) was used. (B) Real time RT-PCR shows the relative amounts of *C. quadricarinatus* vitellogenin gene in control females, AG-implanted females and males. Asterisk represents significant difference ($p < 0.05$, one-way ANOVA).

expression of the vitellogenin gene in the AG-implanted females was significantly lower ($p < 0.01$) than that in control females (0.7887 ± 0.836 vs.6530.4 ±4257.2) and similar to the relative expression in males (0.687 ± 0.507) (Fig. 4B).

The synthesis of vitellogenin was confirmed by an ELISA test showing that the level of vitellogenic cross-reactive protein in the hemolymph of AG-implanted females was significantly lower ($p < 0.001$) than that of such cross-reactive material in the hemolymph of intact females (Table 1).

Discussion

A bimodal growth pattern in which the growth depends on gender is exhibited in several crustacean species (Hartnoll, 1982). The bimodal growth pattern of *C. quadricarinatus* is manifested in the higher weights of male animals both in communal pond cultures (Curtis and Jones, 1995; Sagi et al., 1997) and in individual compartments designed to minimize social interactions (Manor et al., 2001). It is generally believed that the faster growth of the male crustaceans is mediated by the AG. In *M. rosenbergii,* for example, in which the male growth rate is considerably higher than that of the female (Sagi et al., 1986), removal of the AG reduces somatic growth to a level similar to that of normal females (Sagi et al., 1997). The 26.4% increase in growth of *C. quadricarinatus* females subjected to AG implantation may indeed be attributed to the action of the AG and not to social interaction (because the females were housed in individual compartments), in contrast to other crustacean species in which social interaction is involved in the regulation of growth. One such species in which growth is influenced by social conditions is *M. rosenbergii*: in populations of this species, particularly among the males, there is a wide range of sizes (Karplus et al., 2000). Social behavior patterns exhibited by males and females are sexually dimorphic (Kelley, 1988), and the sexes usually differ in the frequency or intensity of certain behavioral (e.g., aggressive) activities. It has been suggested that in *C. quadricarinatus* these social interactions are regulated by the AG, since AG implantation into females induced male-like aggressive and reproductive behavioral patterns (Barki et al., 2003; Karplus et al., 2003).

The slightly shorter molt intervals and the bigger molt increments in the implanted females suggest that the growth advantage of the implanted females is due mainly to a greater increase in weight per molt and not to molt promotion. The smaller molt increments in females in many crustacean species has been attributed

to reproductive demands for available resources in females, as has been illustrated in *Cancer magister*, for which a reduction in molt increments becomes apparent only at puberty (Hartnoll, 1982). This premise is in keeping with the development of maturation-related sex characters such as the red patch in *C. quadricarinatus*, as early as the second molt after implantation in the present study. In some crustacean species, a clear difference is often apparent after sexual maturity, with longer molt intervals in the females being mainly associated with egg bearing (Hartnoll, 1982). For example, in female *Hymenosoma orbiculare* with carapace widths between 13 and 22 mm, the mean intermolt period increased from 101 days in non-ovigerous females to 181 days in ovigerous animals (Broekhuysen, 1955). In the present study, the fact that differences in molt intervals contributed less to the advantage in weight of the implanted females could be explained by the fact that the females were isolated in separated cells and did not carry eggs.

It is well known that in *C. quadricarinatus* the AG inhibits the development of the female reproductive system (Khalaila et al., 2001; Sagi et al., 2002) and the relatively slower growth of females may be attributed to the energetic investment in female physiological activity, such as ovarian development and yolk protein synthesis (Abdu et al., 2000). In the present experiment, the lead of the AG-implanted females in terms of growth became evident when the females weighed about 20 g, the weight threshold at which in *C. quadricarinatus* females begin to invest in female reproductive development and males invest in secondary male characters, such as the red patch on the propodus and a wider propodus. It seems that in *C. quadricarinatus* growth promotion by the AG is a combination of a direct effect of an androgen — as manifested by a higher molt increment — with an indirect effect on the inhibition of the energetic investment of females in reproduction — manifested by the inhibition of vitellogenin gene expression. These effects might be exerted through direct silencing of genes related to vitellogenesis and reproduction in females or through activation of genes related to higher weight and wider propodus in males. From the comparison of the red patch of implanted females vs. same-sized normal males, it is clear that the implanted females developed typical male secondary characters. However, primary sex characters such as testes or sperm duct were not developed probably due to the limited sexual plasticity in this species and the fact that differentiation of primary sex characters occurs earlier in development.

It is believed that the AG produces an AG hormone (AGH) that can act directly as a growth promoter or indirectly as an inhibitor of female energetic reproductive investment. Such a hormone has, however, yet to be characterized in decapods, but the results of the current study suggest that the effects of such an AGH are similar to those of androgens in mammals in that they stimulate the growth, maturation, and maintenance of the male reproductive system and accessory sex tissues (Lehninger, 1982). Similar to the above androgens, the only androgenic substance purified thus far from the AGs of decapods is lipidic in nature: a lipoidal substance from the AG of the crab, *Carcinus maenas,* inhibited vitellogenesis upon injection into a sexually active *Orchestia* female and caused secondary male characteristics to appear in *Talitrus* females (Berreur-Bonnenfant et al., 1973). The active molecule, characterized as farnesylacetone (Ferezou et al., 1978), was shown to be synthesized by the AG, to be active at low concentrations in a rapid and organ-specific manner with no species specificity, and to affect protein and RNA synthesis in its target organs (Berreur-Bonnenfant and Lawrence, 1984).

In contrast to the findings described above, the ultrastructure of the AG in several decapod crustaceans and the presence of considerable amounts of protein in the secretory vesicles of the cytoplasm suggest that the androgenic hormone may be a protein or a polypeptide (Miyawaki and Taketomi, 1978; Taketomi, 1986). Complete amino acid sequencing of the AGH of the isopod *Armadillidium vulgare* showed a structure that seems to be a pro-AGH in the form of a protein containing three peptide chains — B chain, C peptide, and A chain, in that order — being analogous to the proinsulin superfamily of peptides (Martin et al., 1998; Martin et al., 1999; Okuno et al., 1999). Chain A and chain B each contain a single intra-chain disulfide bridge, and the two chains are linked by two inter-chain disulfide bridges, while the C peptide links chains A and B in the pro-hormone. The amino acid sequence of the A and B chains, which comprise the mature AGH peptide, was highly conserved among *A. vulgare* and two other isopod species, *Porcellio scaber* and *P. dilatus,* while that of the C peptide showed only low sequence similarity (Ohira et al., 2003). The analogy between the AGH from these species and the insulin superfamily of hormones might support the hypothesis that the AGH acts as a direct growth promotor, known for the proinsulin superfamily of peptides in vertebrates and in arthropods (Claeys et al., 2002).

The results of the current study on the effect of the AG on growth, together with the molecular study on vitellogenin gene expression, demonstrate that the AG both promotes growth and inhibits female reproduction (as was shown by the inhibition of the expression of

the vitellogenin gene). Further experimentation is required to determine whether the AG exerts its effect directly on growth in a growth-factor-like manner or whether growth promotion is a secondary effect resulting from the reduction of investment in female reproduction. In either case the mechanism, either direct or through intermediary organs, has yet to be elucidated. The findings of the current study could be instrumental in the construction of bioassays for the identification and characterization of AGHs in decapod Crustacea.

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