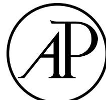
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The eyestalk–androgenic gland–testis endocrine axis in the crayfish *Cherax quadricarinatus*

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Abstract

In decapod crustaceans, a number of neurohormones regulating a variety of physiological processes, including reproduction, are to be found in the X-organ–sinus gland complex of the eyestalk. Bilateral eyestalk ablation was thus performed in mature males of the Australian red claw crayfish *Cherax quadricarinatus* with the aim of studying the role of eyestalk-borne hormones on spermatogenic activity in the testis and on the androgenic gland (AG). The latter gland controls the differentiation and functioning of male sexual characteristics in crustaceans. Eyestalk ablation caused hypertrophy of the AG, as indicated by an increase in gland weight (3.9 ± 0.44 mg vs < 0.1 mg in intact males) and by overexpression of AG polypeptides. In the testes of eyestalk-ablated males, empty spermatogenic lobules were common, while lobules containing primary spermatocytes were infrequent. These findings were reflected in decreased amounts of DNA in these testes and a consequent increase in the relative weights of the sperm ducts. Since it was found that eyestalk ablation affected both the AG and the reproductive system, *in vitro* experiments were conducted to study the direct effects of the sinus gland on the AG and testes and of the AG on the testes. Sinus gland extracts inhibited by 30% the incorporation of radiolabeled amino acids into AG polypeptides and almost totally inhibited the secretion of radiolabeled AG polypeptides into the culture medium. However, sinus gland extracts had no significant effects on testicular tissue. On the other hand, AG extracts affected the *in vitro* phosphorylation of a testicular polypeptide (of 28 kDa), in a time- and dose-dependent manner, suggesting a direct effect of AG-borne hormones on the testes. The above findings, together with the evidence for direct inhibition by the sinus gland on the AG, suggest an endocrine axis-like relationship between the sinus gland, the AG, and the male reproductive system in decapod crustaceans. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Androgenic gland; Sinus gland; Eyestalk ablation; Spermatogenesis; Reproductive system; Organ culture; Phosphorylation; Crustacea; Decapoda; *Cherax quadricarinatus*

1. Introduction

In decapod crustaceans—both male and female—the X-organ–sinus gland complex in the eyestalk produces the neurohormones that regulate various physiological processes (Keller, 1992; Sagi et al., 1997a; Tan-Fermin, 1991; Wilder et al., 1994). The effect of such neurohormones on male reproduction is less well known than that in the female. On the other hand, it is well known that the regulation of the male reproductive system is controlled by the androgenic gland (AG) (Charniaux-Cotton and Payen, 1988; Sagi and Khalaila, 2001; Sagi et al., 1997b), the initiation, completion, and intensity of spermatogenic activity being regulated by circulating AG hormone (Charniaux-Cotton and Payen, 1988). In certain decapod species, spermatogenesis starts only when the AGs are fully developed (Payen, 1973; Taketomi et al., 1996). In the male prawn *Macrobrachium rosenbergii* (Nagamine et al., 1980) and in intersex individuals of the Australian red claw crayfish *Cherax quadricarinatus* removal of the AG leads to cessation or regression of spermatogenesis (Khalaila et al., 1999). In contrast, in some decapod species removal of the X-organ–sinus gland complex by eyestalk ablation leads to hypertrophy of the AG, which then displays

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ultrastructural features related to hyperactivity (Demeusy, 1960; Hoffman, 1968; Payen et al., 1971). In the protandric shrimp *Pandalus platyceros*, eyestalk ablation leads to an increase in RNA synthesis in the AGs (Brockenbrough-Foulks and Hoffman, 1974). In some species, in addition to its effect on the AG, eyestalk ablation induces spermatogenesis in the testes (Demeusy, 1960; Payen et al., 1971), and in the hermaphrodite shrimp *Pandalus borealis* it delays transformation from the male to the female stage (Hoffman, 1968).

Several neurohormones are stored and released from the sinus gland, including molt inhibiting hormone (MIH) and mandibular organ inhibiting hormone (MOIH). The above neurohormones are part of the sinus gland–Y-organ endocrine axis and the sinus gland–mandibular organ endocrine axis, respectively (Aguilar et al., 1996; Boecking et al., 2002; Fingerman, 1995; Keller et al., 1999; Liu and Laufer, 1996; Terauchi et al., 1996; Wainwright et al., 1996). Although the inhibitory effect of the eyestalk on the male reproductive system has been documented in a number of species, the identity and mode of action of the factor actually responsible for this inhibition is not yet known.

It is believed that the secretory product(s) of the AG is proteinaceous in nature. Early support for this premise may be found in the considerable amount of protein in the cytoplasmatic secretory vesicles of the AG of the crab *Pachygrapsus crassipes* (King, 1964). Similarly, the ultrastructure of the AG of *Procambarus clarkii* supports the possibility of a proteinaceous secretion (Miyawaki and Taketomi, 1978; Taketomi, 1986). Recent histological evidence in *M. rosenbergii* supports the idea of a proteinaceous androgenic hormone (Awari and Kiran, 1999). As is the case for all peptide hormones, the target tissue is expected to have specific cell–surface receptors. Therefore, a presumably proteinaceous hormone such as the AG hormone should initiate multiple signal transduction pathways that alter the activation of kinases or phosphatases on specific proteins in its target organ, the testis.

The aim of this work was thus to determine—by *in vivo* and *in vitro* means—whether a sinus gland factor acts directly on the male gonad or via the mediation of the AG as part of an eyestalk–AG–testis endocrine axis. An understanding of this endocrine axis is particularly important since neither a sinus gland factor inhibiting the AG nor the AG hormone has yet been identified in decapod crustaceans.

2. Materials and methods

2.1. Animals and procedures

C. quadricarinatus males were collected at the Aquaculture Research Station Dor, Israel, approximately 9 months after hatching. The animals were held in our facility at Ben-Gurion University of the Negev for 1 month of acclimation prior to the experiment. In the first week of November, 100 males, with a carapace length of 41 ± 4.5 mm, were chosen for the experiment. Animals were held in a 2-m^3 container, each in an identical net cage ($20 \times 30 \times 10\,cm^3$, mesh $5 \times 5\,mm^2$). Water temperature was maintained at 25 ± 2 °C, and a photoperiod of 12L:12D was applied. Water quality was assured by circulating the water through a gravel biofilter. Animals were fed ad libitum with ground fresh vegetables, frozen fish flesh, and commercial crayfish pellets (30% protein). Seventy of the 100 animals were subjected to bilateral destalking, and the remaining 30 intact males served as control animals. Destalking was performed with a pair of scissors, followed by cauterization to prevent bleeding. Each week for a period of 1 month, approximately 10 destalked and 5 intact males were weighed (± 0.01 g), anesthetized in ice-cold water, and then dissected. Testes, sperm ducts, and AGs were separated out and weighed. Relative testicular weight was calculated as [(testes weight/body weight) $\times$ 100], and relative sperm duct weight as [(sperm duct weight/body weight) $\times$ 100]. The amount of DNA in the testes was measured by the diphenylamine reaction of Burton (1956). The amount of DNA (μg) relative to body weight was expressed as [(μg DNA in the testes/body weight)] $\times$ 100.

2.2. Electrophoresis

AGs obtained from male animals 1 month after eyestalk removal and those taken from intact males were homogenated separately in 0.05 M Tris–HCl, pH 7.4, in the presence of the following antiproteases: 0.8 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, and 1 mM EDTA. The samples were centrifuged at 10,800*g* for 15 min at 4 °C. Total protein in the supernatant was determined by the mini Bradford method (Bradford, 1976). The polypeptides (approximately 35 μg of protein per lane) were separated by 12.5% mini tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Schagger and Von-Jagow, 1987) and stained with Coomassie blue.

2.3. Histology

Testes obtained from two male animals 1 month after eyestalk removal and those from three intact animals were fixed in Bouin's solution for 24 h, embedded in Paraplast cross-sectioned at 5 μm, and stained with hematoxylin and eosin. In representative sections from destalked and intact individuals, lobules containing cells at different stages of spermatogenesis were counted in four randomly selected areas of 2 mm^2 each.

2.4. Preparation of sinus gland and muscle extracts

Forty eyestalks from 20 *C. quadricarinatus* males (carapace length, 48.1 ± 5.1 mm) were removed with an electric cauterizer. The sinus glands were immediately dissected from each eyestalk and homogenized in 50 µl of buffer containing 200 mM NaCl, 4.5 mM KCl, 2.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.35 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 5.5 mM glucose (pH 7.4, 426 mosmol). The homogenate was centrifuged twice (12,500*g*, 4 °C, 15 min), and the supernatant was collected. Muscle fragments, similar in size to the sinus gland fragments, were removed, and the muscle homogenate to be used as a control preparation was prepared as described above.

2.5. In vitro incubation of AG

Thirty hypertrophied AGs were collected from male animals 2 weeks after eyestalk ablation, a period of linear growth rate of the AG ($r^2 = 0.97$) (Table 1). Ten hypertrophied AGs were placed in each of three 35-mm plates. One plate was supplemented with sinus gland extract (equivalent to 12 glands), and the other two plates served as the controls, one being supplemented with muscle extract and the other solely with the buffer used in the preparation of the muscle extract. In a similar series of three plates, AGs were replaced with an equal amount of testis fragments. The contents of each of the plates were incubated in total volume of 1 ml of the above-described buffer in the presence of 4µCi of a ^{14}C-radiolabeled amino acid mixture (Amersham, England) at 28 °C for 24 h with gentle shaking. For each plate, the tissues were collected after the incubation and homogenized in 0.05 M Tris–HCl, pH 7.4, in the presence of the following antiproteases: 0.8 mM benzamidine, 0.1 mM PMSF, and 1 mM EDTA. The samples were then centrifuged (17,500*g*, 4 °C, 15 min), and the supernatants were collected. Total protein in each supernatant was determined by the mini Bradford technique. From each supernatant, 5 µl was loaded on a Whatman 3-mm disc and washed with 10% trichloroacetic acid (TCA), and radioactivity was counted in a Beckmann scintillation counter. The results, representing TCA-precipitated macromolecules from the cytosolic component of the AG or the testes, were expressed as cpm/µg protein. The culture medium in which AGs and testes were incubated was also collected to determine secretion of labeled polypeptides by these tissues. Twenty-five micrograms of protein of each homogenate and five AG equivalents of the culture media were loaded on a mini SDS–PAGE system (NuPAGE 4–12% Bis–Tris Gel NOVEX). The gel was stained with Coomassie blue, and the dried gel was exposed to Kodak BioMax film with an intensifying screen at −70 °C for autoradiography.

2.6. Phosphorylation assay

Testes from intact animals were homogenized in the following solution: 25 mM Tris–HCl (pH 7.4) containing 10% glycerol (v/v); 2 mM EGTA; 2 mM DTT; 25 mM NaCl; the protease inhibitors 10 mM leupeptin and 0.2 mM PMSF; the phosphatase inhibitors 50 mM NaF, 10 mM Na pyrophosphate, 2 mM Na_2VO_4; and with the phosphatase substrate *p*-nitrophenyl phosphate. Protein concentration was determined by the Bradford method. AG was extracted with 2 N glacial acetic acid and centrifuged at 17,000*g* for 15 min. The supernatant was dialyzed against PBS, pH 7.4, in snakeskin pleated dialysis tubing 3500 MW cutoff (Pierce).

The kinase assay was performed in safe-lock Eppendorf vials. The required equivalent of AG extract was totally lyophilized in each reaction vial (to reduce the volume of the reaction and to enable the loading of the sample volume into the gel). The assay mixture contained 5 µg of testicular proteins and the volume was made up to 15 µl with the following kinase buffer: 25 mM β-glycerol phosphate, pH 7.0; 2 mM DTT; 10 mM $MgCl_2$; 0.1 mg/ml bovine serum albumin; and 0.1 mM EGTA. The phosphorylation assay was initiated by the addition of 15 µl of kinase buffer containing 20 µM cold ATP and 10 µCi of [γ-^{32}P]ATP (Amersham, England). The phosphorylation reaction at 27 °C was terminated after 10 min by the addition of 10 µl of sample buffer (Laemmeli, 1970) followed by heating at 100 °C for 3 min. The samples were subjected to 13% SDS–PAGE, and the polypeptide profile was visualized by Coomassie blue staining. The gels were air dried between two sheets of cellophane, and the phosphorylated polypeptides were detected by autoradiography on Kodak X-ray films.

Table 1
Effect of eyestalk ablation on the weight (mg) of the androgenic glands

	Time after destalking (weeks)				
	0	1	2	3	4
Eyestalk ablated	<0.1	1.6 ± 0.25^{a}	2.4 ± 0.147^{ab}	2.9 ± 0.32^{b}	3.9 ± 0.44^{c}
Intact	<0.1	<0.1	<0.1	<0.1	<0.1

Note. Different letters represent significant differences by ANOVA–LSD test, $P < 0.05$.

2.7. Statistics

Results were analyzed using ANOVA, followed by the LSD test. Probabilities below 0.05 and 0.001 were considered significant and highly significant, respectively.

3. Results

Light microscopy showed the AG of C. *quadricarinatus* to be a long, thin cord-like organ situated beneath the articular membrane of the last thoracic sternum. The AG is connected to the proximal (ejaculatory) portion of the sperm duct on one side and to the cuticle of the last ventral thoracic sternum of the fifth walking leg on the other side (Fig. 1). Four weeks after eyestalk removal, the AG had become thickened and opaque in comparison with the transparent thin AG of the intact male (Fig. 1). The weight of the AG of destalked males increased gradually from the first week to the fourth week after destalking, the weight 3 weeks after eyestalk removal being significantly greater than that of 1 week after ablation (2.9 ± 0.32 vs 1.6 ± 0.25 mg) (Table 1). There was also a highly significant difference in the weight of the AG between the third and fourth weeks (3.9 ± 0.44 mg) after destalking (Table 1). There were clear differences in the polypeptide profiles of AGs removed from destalked and intact male animals: the polypeptides of 7, 8, 17, 20, 24, 28, 29, 36, 71, and 103 kDa were prominent in the AG profile of the destalked group but much less obvious or even absent in the profile of intact males (Fig. 2, black arrows). For the intact male, only one band of 83 kDa was prominent (Fig. 2, white arrow); this polypeptide was also present in the profile of the destalked animal, but it was much less dominant than the other polypeptides.

One week after eyestalk ablation, the relative testicular weight of destalked males was higher than that of intact males (0.309 ± 0.025 vs 0.242 ± 0.018). The relative testicular weight of destalked males remained high throughout the second week, but decreased sharply from the beginning of the third week to a minimum (0.197 ± 0.015) in the fourth week that was lower than the value for intact males (0.26 ± 0.033) (Fig. 3A). However, throughout the experimental period these differences were not statistically significant. Similarly, the relative amounts of DNA in the testes of intact and destalked males were not significantly different 1 week after eyestalk ablation (Fig. 3B), while, from the second week, the relative amount of DNA in the testes of destalked males became significantly lower than that of intact males ($P < 0.05$). A significantly ($P < 0.05$) sharp decrease in the relative amount of DNA was recorded in the testes of eyestalk-ablated males from the second week until the fourth week (Fig. 3B).

Histological sections revealed that the number of spermatogenic lobules in the testes of destalked individuals differed from that in the testes of intact indi-

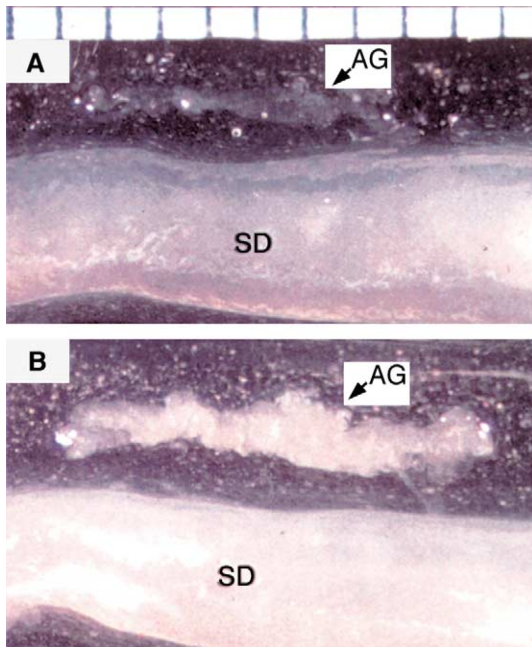

Fig. 1. Micrographs of androgenic glands (AG) of intact (A) and destalked (B) *C. quadricarinatus* males. SD, sperm duct. Each notch in the ruler at the top of the figure represents 1 mm.

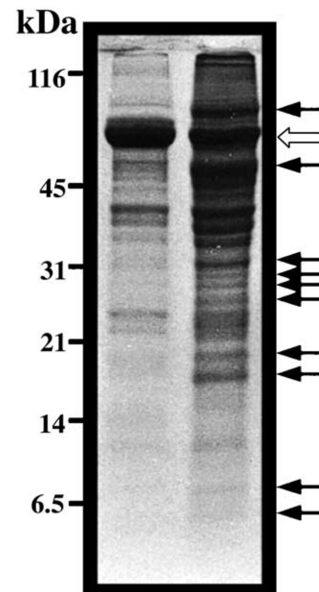

Fig. 2. SDS–PAGE of cytosolic polypeptides of the androgenic gland (AG) from intact (left lane) and destalked (right lane) *C. quadricarinatus* males. Black arrows represent polypeptides that are more prominent in the AG of destalked males. The open arrow represents the polypeptide that is more prominent in the AG of intact males. Numbers on the left indicate molecular weight standards.

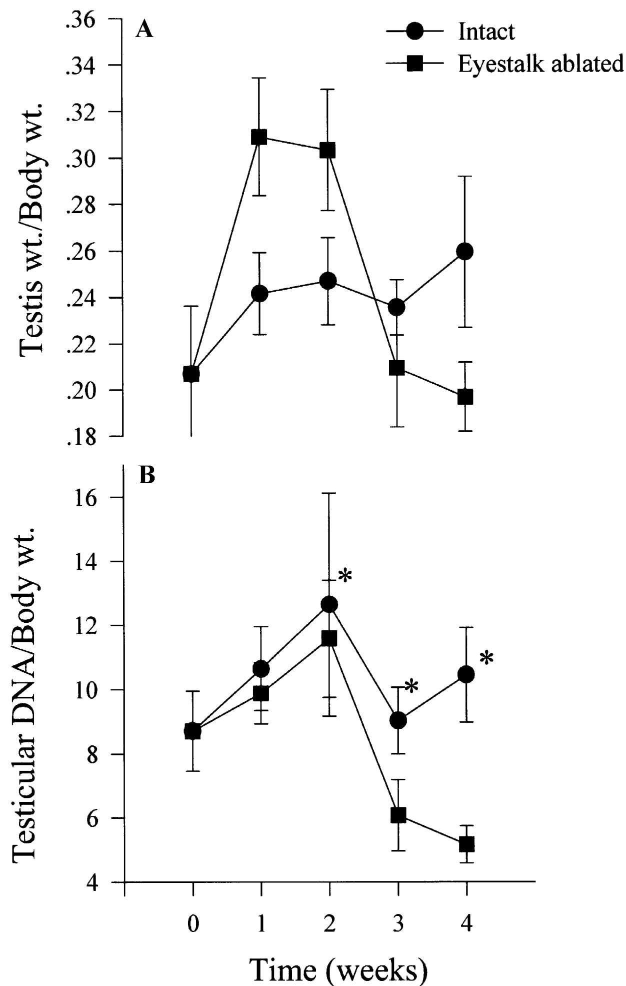

Fig. 3. Relative weight of the testis (A) and relative amount of testicular DNA (B) in intact vs destalked C. *quadricarinatus* males. Asterisks represent significant differences between treatments (ANOVA followed by LSD test, $P < 0.05$). Error bars represent SE.

viduals. The total numbers of lobules in 2-mm^2 areas of the relevant sections were significantly lower in destalked males than in intact animals (67 ± 4.0 vs 86 ± 3.1, $P < 0.05$). The abundance of lobules containing mostly primary spermatocytes was significantly higher in the testes of intact individuals than in the testes of the destalked individuals (25 ± 2.05 vs 10.59 ± 1.45, $P < 0.05$) (Figs. 4A and B). Empty lobules were significantly more abundant in the testes of destalked individuals than in those of intact males (33.72 ± 3.11 vs 11.55 ± 2.05, $P < 0.05$) (Figs. 4A and B). There were no significant differences in the abundance of lobules containing mostly secondary spermatocytes and lobules with spermatids in the testes of destalked and intact males (Figs. 4A and B).

In the second week after eyestalk ablation, the relative weight of the sperm ducts of destalked males increased sharply to become significantly greater ($P < 0.05$) than that of intact males (0.563 ± 0.064 vs 0.295 ± 0.012) and remained at that level for the next 2 weeks, i.e., until the end of the experiment (Fig. 5).

In experiments designed to measure the uptake of labeled amino acids into the AG and testes, basal amino acid incorporation into the AG tissue was significantly higher ($P < 0.001$) than that into testicular tissue (4920 ± 441 vs 1824 ± 195 cpm/μg of protein, Fig. 6A). Addition of muscle extract had no significant influence on amino acid incorporation into the AG or testes: it caused less than 13.4% of inhibition in both types of tissue (Fig. 6A). On the other hand, addition of sinus gland extract significantly inhibited amino acid incorporation into the AG vs the buffer control ($P < 0.05$). The mean incorporated amino acid (cpm/μg protein) for AG incubated in the presence of sinus gland extract was 3201 ± 197, which represents approximately 34.9% inhibition relative to the buffer control, whereas the value for testes plus sinus gland was 1711 ± 243, i.e., 6.2% inhibition (Fig. 6A).

Fig. 6B shows the inhibitory effect of the sinus gland extract on amino acid incorporation into specific polypeptides of the AG and its secretion. The same amounts of protein were loaded in all the lanes. This finding was confirmed by densitometry of the gel, which showed only a 4.5% difference between lanes 1 and 2 (Fig. 6B, lanes 1 and 2, SDS–PAGE). On the other hand, the densitometry of the autoradiogram showed that the sinus gland extract caused total inhibition of 41.2% of amino acid incorporation into AG proteins (Fig. 6B, lanes 1 and 2, autoradiogram). The AG polypeptides of 33, 36, 46, and 89 kDa were noticeably inhibited by the sinus gland extract (Fig. 6B, lanes 1 and 2, autoradiogram). No such effect of the sinus gland extract on amino acid incorporation into testicular polypeptides was detected (Fig. 6B, lanes 5 and 6, autoradiogram). The autoradiogram of the culture medium showed that polypeptides of approximately 7, 17, and 50 kDa were secreted by the AG. The radiolabeled secretion from the AGs was inhibited by the sinus gland extract (Fig. 6B, bottom, lanes 3 and 4, autoradiogram), whereas almost no labeled testicular secretion was detected (Fig. 6B, bottom, lanes 7, and 8, autoradiogram).

Among the testicular polypeptides found to be phosphorylated, one of the most prominent polypeptides had a molecular mass of 28 kDa (P^{28}). The dose–response effect of the AG extract (0.05–5 AG equivalents) on the phosphorylation of the testicular P^{28} is shown in Fig. 7A. A low basal phosphorylation level was detected in the control, which increased sharply to peak at 0.6 AG equivalents; from that point, there was a decline in the intensity to an undetectable level at 2 AG extract equivalents. The phosphorylation intensity of the testicular P^{28} exposed to 0.3 AG equivalents increased sharply with time and reached saturation within 45 min compared to the control, which showed a basal phosphorylation level at all times (Fig. 7B).

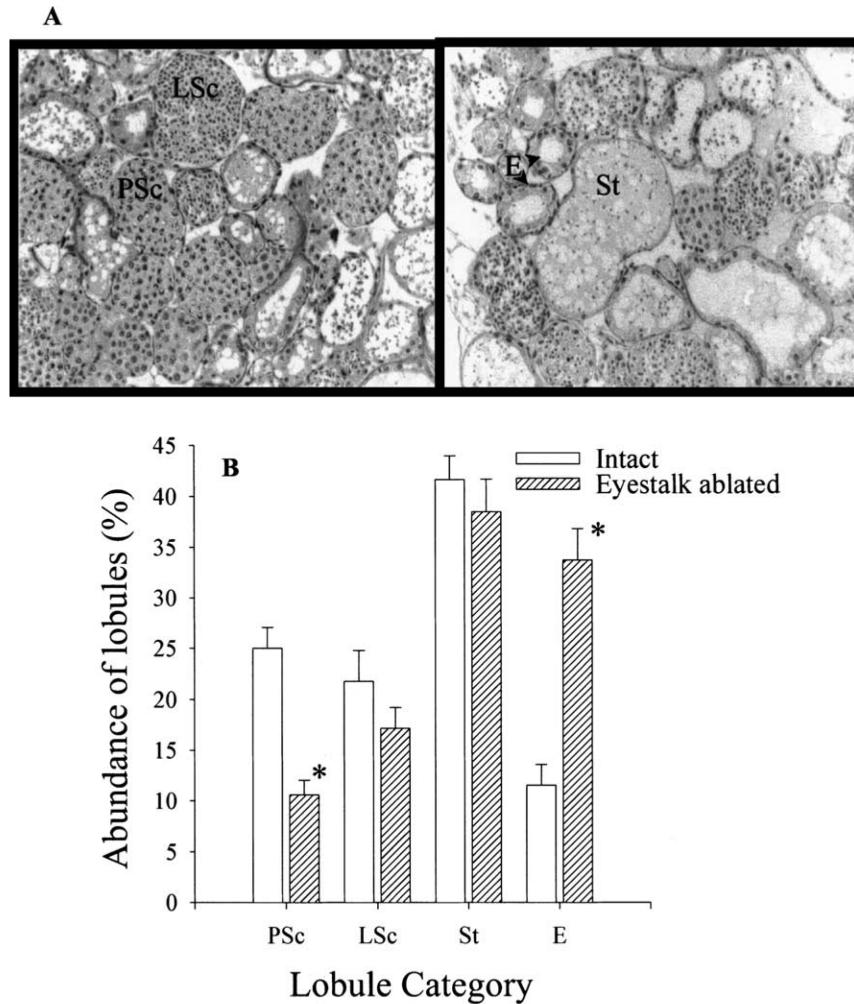

Fig. 4. Differences in the structure of the testis in intact vs destalked *C. quadricarinatus* males. (A) Cross-sections of the testes from intact (left) and destalked (right) animals. (B) Histogram representing the relative abundance in the testis of lobules at different spermatogenic stages. PSc, lobules containing mostly primary spermatocytes; LSc, lobules containing mostly secondary spermatocytes; St, lobules containing mostly spermatides; E, empty lobules. Asterisks represent highly significant differences between treatments (ANOVA followed by LSD test, $P < 0.05$).

4. Discussion

This study showed that eyestalk ablation of the mature *C. quadricarinatus* male resulted in hypertrophy of the AG, as indicated by increases in its size and weight (Fig. 1, Table 1) and by the enrichment of the polypeptide profile of the AG (Fig. 2). These results are consistent with previous findings in a number of decapod crustaceans of hypertrophy of the AG combined with hyperactivity and increase in RNA synthesis after eyestalk ablation (Adiyodi, 1984; Brockenbrough-Foulks and Hoffman, 1974; Hoffman, 1968; Kulkarni et al., 1984). Several polypeptides expressed in the AGs of eyestalk-ablated *C. quadricarinatus* males call for further study based on previous findings regarding the recently identified glycosylated AG hormone found in the isopod *Armadillidium vulgare* (Martin et al., 1999; Okuno et al., 1997; Okuno et al., 1999; Sagi and Khalaila, 2001), since in addition to the general effect of eyestalk ablation on protein synthesis, it could have been causing overexpression of specific polypeptides representing androgenic factors.

Eyestalk ablation also caused dynamic changes in the reproductive system of mature *C. quadricarinatus* males. To the best of our knowledge, descriptions of similar dynamic processes have not appeared in the literature on crustaceans. After eyestalk ablation, there was a gradual and significant increase in the weight of the sperm duct due to accumulation in the sperm duct of spermatophores ready for ejaculation. This accumulation is an indicator of the potency of the spermiation process following eyestalk removal. Additional evidence for the enhancement of spermiation was provided by the decrease in the amount of DNA in the testes from the second week onward and by the significantly higher number of empty spermatogenic lobules found in testicular sections of eyestalk-ablated males' testes vs those

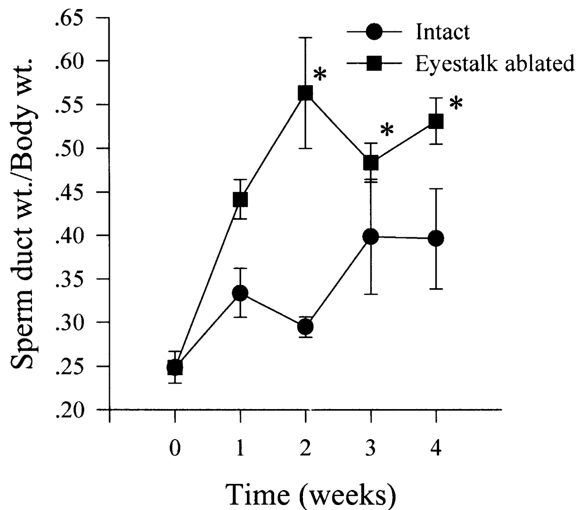

Fig. 5. Relative weight of the sperm duct in intact vs destalked *C. quadricarinatus* males. Asterisks represent highly significant differences between treatments (ANOVA followed by LSD test, $P < 0.05$). Error bars represent SE.

of intact animals. It is likely that the enhanced spermiation process subsequently leads to a decrease in the weight of the testes in the third and fourth weeks after eyestalk ablation, which is in keeping with our previous findings in *C. quadricarinatus* (Khalaila et al., 1999). This decrease in testis weight, coming after an increase in weight in the first and second weeks after eyestalk ablation, is consistent with previously reported findings in the prawn *Parapenaeopsis hardwickii* (Kulkarni et al., 1984). The preliminary increase probably represents the prespermiation enhancement effect of eyestalk ablation. The finding that the number of spermatogenic lobules containing primary spermatocytes was much lower in eyestalk-ablated males might indicate that eyestalk ablation elevated the miotic index and diminished the mitotic index in the testis. The latter decrease could explain why the sperm ducts became full of spermatophores, which might have caused feedback inhibition on the mitotic division from spermatogonia to primary spermatocyte. It is unclear whether this dynamic process in the testis is induced directly by eyestalk ablation or through the AG. The possibility of a direct control of testicular activity by the AG was investigated in the present study by the in vitro phosphorylation assay.

Exposure of cells to proteinaceous hormones, neurotransmitters, or growth factors initiates a cascade of events facilitated by intracellular second messengers and mediated, in many cases, by protein kinases (PKs) or phosphatases (PPs). The level of phosphorylation at any instant reflects the relative activities of the PKs and PPs that catalyze the interconversion process. The subsequent covalent modification of the target proteins and the associated changes in their function account for diverse physiological responses and cellular processes, such as

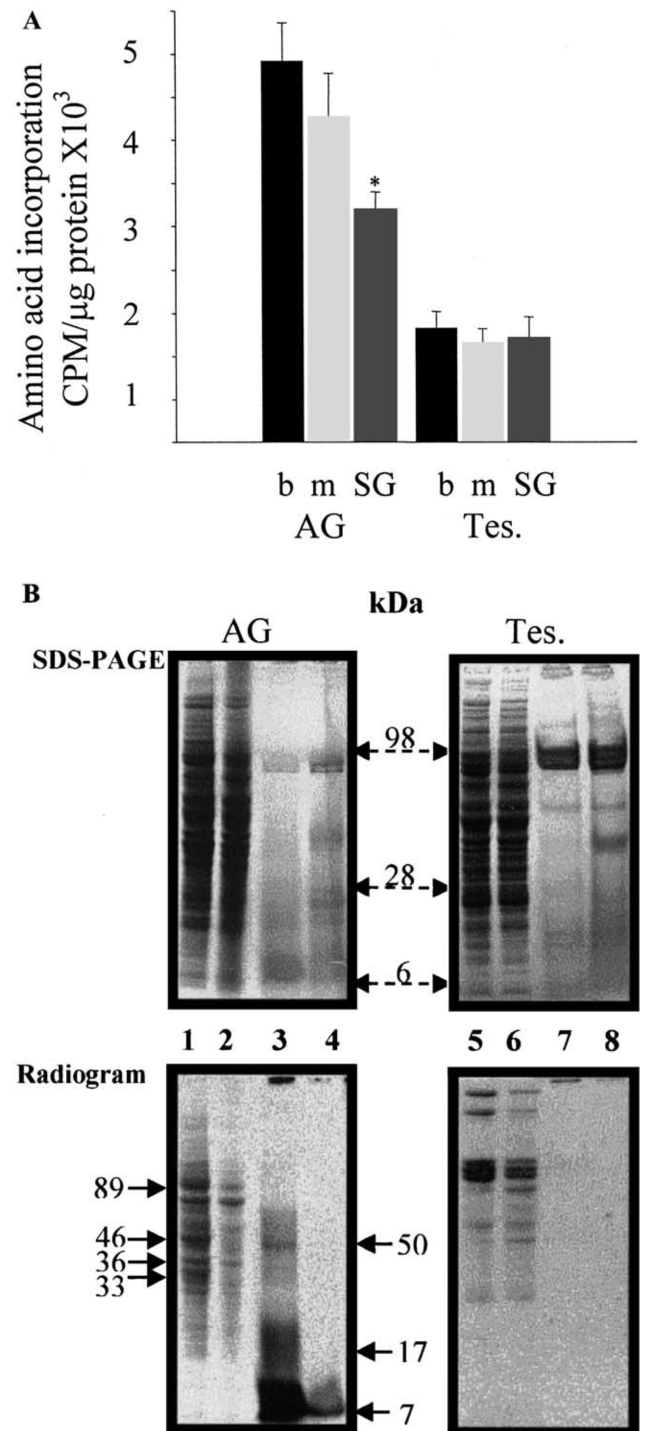

Fig. 6. Effect of sinus gland extract on amino acid incorporation into cultured androgenic gland (AG) and testis of C *quadricarinatus*. (A) Histogram representing incorporation into total protein of AG ($n = 4$, 10 AGs per n) and of testis ($n = 5$); b, buffer; m, muscle extract; SG, sinus gland extract. Asterisks represent significant differences ($P < 0.05$). (B) SDS–PAGE (top) and radiography (bottom) representing amino acid incorporation into specific polypeptides in the cytosol and culture medium. Cytosolic polypeptides of AG (lanes 1 and 2) and testis (lanes 5 and 6). Polypeptides from the culture medium of AG (lanes 3 and 4) and testis (lanes 7 and 8). All odd-numbered lanes are from control cultures and all even-numbered lanes are from incubations with sinus gland extract.

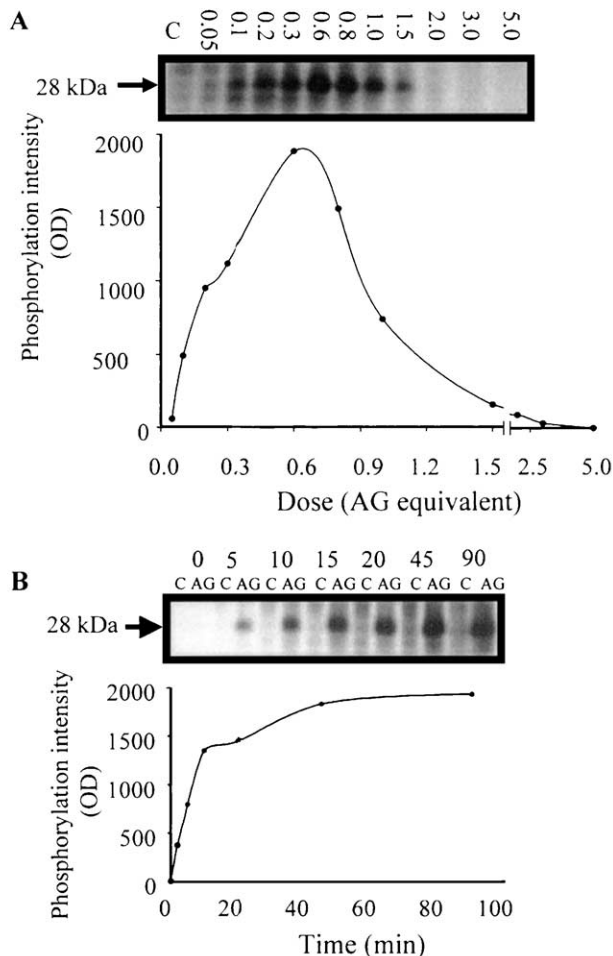

Fig. 7. Phosphorylation intensity of the 28-kDa testicular polypeptide (A) exposed to increasing doses of AG extract for 10 min (the numbers on the top side of the autoradiograph indicate AG equivalent at each dose; C, control) and (B) exposed to 0.3 equivalents of AG extract for increasing times. The numbers on the top side of the autoradiograph indicate the phosphorylation time in minutes. The different lanes represent control (C) and AG-treated (AG) at each time. Data represented by the plots indicate the optical density calculated from the autoradiograph after subtraction of the control value.

motility, metabolism, growth, and differentiation (Shenolikar, 1988; Zolnierowicz and Bollen, 2000). It is well known that the onset of the spermatogenic process in the testis of the crayfish is AG dependent, as has been demonstrated in the *C. quadricarinatus* model (Khalaila et al., 1999) and in the crayfish *P. clarkii* (Taketomi et al., 1996). The current study constitutes the first report of the effect in crustaceans of the AG—or AG extract and/or secretory products—on phosphorylation of testicular polypeptides in a time- and dose-dependent manner. It seems that AG secretory products activate PKs and PPs to directly enhance the phosphorylation of some of the testicular polypeptides and to reduce the phosphorylation of others. The above phosphorylation assay could provide evidence for the presence of testicular receptors that recognize the AG secretory product, which in turn controls cell proliferation and the differentiation of sperm cells in the testes. This evidence is in keeping with our previous work on the response to AG ablation, which leads to retardation of the testes in intersex individuals (Khalaila et al., 1999).

In keeping with previous work (Adiyodi, 1984; Brockenbrough-Foulks and Hoffman, 1974; Hoffman, 1968; Kulkarni et al., 1984), it was shown here that eyestalk ablation leads to hypertrophy and hyperactivity of the AG. Although *in vitro* experiments have shown the inhibitory effect of sinus gland factors in decapod endocrine axes, such as the eyestalk–Y-organ (Sefiani et al., 1996) and the eyestalk–mandibular organ (Liu and Laufer, 1996; Wainwright et al., 1996), this is the first in vitro report of direct inhibition by sinus gland extracts on polypeptide synthesis in the AG. The inhibitory effect of the sinus gland extract was evident in the autoradiograms not only of the polypeptide profile of cytosolic components, but also of the AG culture medium, supposedly inhibiting AG secretion. Such effects were not detected in the testis despite the significant changes in the testis following eyestalk ablation *in vivo*. Moreover, direct evidence for AG influence on the phosphorylation of a specific polypeptide in the testis was shown by the phosphorylation assay. These findings suggest an endocrine axis-like effect, mediated through the AG, of eyestalk factors on the reproductive system, probably as a consequence of the hypertrophy and hyperactivity of the AG after eyestalk ablation. It is noteworthy that sinus gland inhibitory factors inhibiting ecdysone synthesis in the Y-organ and methyl farnesoate synthesis in the mandibular organ have been identified and characterized (Landau et al., 1989; Liu and Laufer, 1996; Sefiani et al., 1996; Tang et al., 1999; Wainwright et al., 1996). The roles of Y-organ secretion and mandibular organ secretion on the reproductive system of decapod crustaceans have been discussed in the last few years (Jo et al., 1999; Subramoniam, 2000; Tang et al., 1999). It is possible that some of the effects on the male reproductive system are caused by these endocrine glands and their products.

The current work suggests that eyestalk neuroendocrine factors control the reproductive system in decapod crustaceans via an endocrine axis—the sinus gland–AG–testicular axis. Although the effects of the AG on testicular activity in decapod crustaceans have been widely described in the literature, the AG inhibiting factor(s) produced by the sinus gland and the AG hormone itself still remain to be characterized.

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