

Male-like behavioral patterns and physiological alterations induced by androgenic gland implantation in female crayfish

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Accepted 26 February 2003

Summary

The androgenic gland (AG) has been shown to regulate male sexual differentiation and secondary male characteristics in Crustacea. This study presents for the first time in crustaceans evidence for masculinization effects of the AG on reproductive behavior, in addition to morpho-anatomical and physiological effects. AG implantation into immature female red claw crayfish *Cherax quadricarinatus* inhibited secondary vitellogenesis and development of the ovaries, as well as morphological traits that facilitate maternal egg brooding; it also caused the appearance of secondary male characteristics. However, primary male characteristics and a masculine reproductive system were not developed. In pair encounters, aggression was substantially lower in

interactions between AG-implanted and intact females than in interactions within AG-implanted or intact pairs. Moreover, elements of mating behavior, i.e. male courtship displays and false copulations, were exhibited by AG-implanted females in several encounters with intact females. In addition to known morpho-anatomical and physiological effects of the AG in crustaceans, the present study suggests that the AG has novel effects on the neural network that generates social behavior.

Key words: androgenic gland, androgenic hormone, agonistic behavior, aggression, fighting, mating behavior, crustacea, decapoda, crayfish, *Cherax quadricarinatus*.

Introduction

Social behavior patterns exhibited by males and females are commonly sexually dimorphic (Kelley, 1988), and the sexes usually differ in the frequency or intensity of behavioral acts (e.g. aggressive acts). The sexes may display completely different behavior patterns, however, as in reproductive behavior. The role of sex hormones in the regulation of sexually dimorphic behaviors and their mode of action in structuring and activating the neural networks underlying such behaviors have been studied extensively in vertebrates (Schulkin, 1999).

Numerous behavioral studies have been performed in crustaceans, mainly in decapods that have complex social behavior (Dingle, 1983; Hyatt, 1983), but only a few have addressed the hormonal regulation of social behavior. Correlations were found in the American lobster *Homarus americanus* between aggressiveness and molt stage (Tamm and Cobb, 1978) and levels of 20-hydroxyecdysone, the active form of the molting hormone ecdysone secreted by the Y-organ (Bolingbroke and Kass-Simon, 2001), and between reproductive behavior of male spider crabs *Libinia emarginata* and hemolymph concentrations of methyl-farnesoate, a juvenile-hormone-like compound secreted by the mandibular organ (Sagi et al., 1994). However, no study has addressed the

association between androgenic hormones and sexually dimorphic behavior patterns. One of the reasons for this gap is that specific sex hormones have yet to be identified in decapod crustaceans (Sagi and Khalaila, 2001).

Of the endocrine complex in crustaceans, the only gland that is specifically related to sexual functions is the androgenic gland (AG). The AG regulates male differentiation, inhibits female differentiation, and controls male primary (e.g. spermatogenesis) and secondary (e.g. external morphology) sexual characteristics (for reviews, see Charniaux-Cotton and Payen, 1988; Payen, 1990; Sagi et al., 1997; Sagi and Khalaila, 2001). Various morphological and physiological effects of the AG in crustaceans have been elucidated by hormonal manipulations at the whole-gland level (e.g. AG ablation or implantation). The extent of the hormonal influence seems to depend on the species and the developmental stage at which such manipulations were applied. Functional sex reversal has been obtained more readily in amphipod and isopod crustaceans, but has also been reported in gonochoristic decapods for AG-ablated males and AG-implanted females of the freshwater prawn *Macrobrachium rosenbergii*, manipulated at an early stage of differentiation (Nagamine et al., 1980a,b; Sagi and Cohen, 1990). However, most of the AG

implantations into immature females in decapods resulted in inhibition of secondary vitellogenesis, oocyte degeneration and development of various secondary male characteristics (Charniaux-Cotton and Payen, 1988; Sagi and Khalaila, 2001, and references therein).

Given the broad influence of the AG on sexual characteristics, it is likely that this gland is also involved in the regulation of male behavior, which, in fact, is one of the sexually dimorphic traits of the animal. Effects on mating behavior were postulated for sex-reversed *M. rosenbergii* individuals that were capable of reproducing (Sagi and Khalaila, 2001). Gleeson et al. (1987) suggested that androgenic factors from the AG mediate the control of courtship behavior in male crabs *Callinectes sapidus*; the suggestion was based on the co-occurrence of spontaneous courtship displays and hypertrophy of the AGs within a few days following eyestalk ablation. However, none of these studies directly tested the role of the AG in the regulation of behavior.

In the present study we examined the effects of AG implantation on the behavior of *C. quadricarinatus* females, and focused on interactions between females. By contrasting AG-implanted and intact females across a range of morpho-anatomical, physiological and behavioral characteristics, we show directly that the aggressive and mating behaviors are among the sexually dimorphic traits that are modulated by the AG.

Materials and methods

Animals

Young sexually immature female crayfish *Cherax quadricarinatus* (von Martens) (mass 6.7 ± 2.0 g, mean \pm S.D.) obtained from a commercial nursery were implanted with hypertrophied androgenic glands from large mature males that had been eyestalk-ablated bilaterally according to Khalaila et al. (2001). After a 2-week recovery period, the implanted (IMP) females, together with non-implanted (NON) females, were transferred to 181 glass aquaria and maintained individually, in order to prevent cannibalism, for approximately 14 months prior to the behavioral testing. The mean mass of the IMP and NON females prior to the behavioral test was 67.3 ± 19.3 and 54.7 ± 7.8 g (mean \pm S.D.), respectively.

Behavior testing

The effects of AG implantation on the behavior of females were examined in pair encounters between size-matched females. Three types of pair encounters were conducted in two sessions. In the first session, encounters of IMP *versus* IMP ($N=10$) and NON *versus* NON ($N=9$) were conducted simultaneously. In the second session, which followed a period of at least 2 weeks in isolation to minimize the effects of prior experience, IMP and NON females that could be size-matched were used again for encounters of IMP *versus* NON ($N=8$). Size matching was based on the mean length of the left and right propodus of the chelipeds, since weapon size is a most

reliable criterion for fighting ability in clawed crustaceans (Barki et al., 1992, 1997; Sneddon et al., 1997), including crayfish (Rutherford et al., 1995). Mean chela length ratios (large/small contestant) were 1.02 ± 0.01 and 1.03 ± 0.04 for contests between IMP females and between NON females, respectively, and 1.00 ± 0.02 for contests between IMP and NON females (IMP/NON contestant). Molt staging was performed 3 days prior to the encounter to ensure that all females were in the intermolt stage.

The encounters were conducted in glass aquaria measuring $50 \text{ cm} \times 120 \text{ cm} \times 50 \text{ cm}$ placed inside an enclosure of black polyethylene to minimize disturbance. The bottom was covered with 3 cm layer of aquarium gravel, and each aquarium was divided into three sections by two opaque dividers that could be remotely lifted by a string. Each of the two outer sections contained a PVC tube ($75 \text{ mm} \times 220 \text{ mm}$, diameter \times length). The two contestants were transferred simultaneously to these sections and allowed 6 min without any physical or visual contact, for acclimation. The dividers were then lifted and the encounters were video-recorded from outside the enclosure.

Behavior, definitions and analysis

Behavior patterns observed in the encounters included typical elements of fighting and mating. The fighting behavior of *C. quadricarinatus* resembles that of other crayfish. Escalated fights begin when the two opponents face each other in the 'body up' position (Bruski and Dunham, 1987; Tierney et al., 2000), with the carapace elevated obliquely and antennae pointing upwards. In this position the opponents perform 'chela contact' (Tierney et al., 2000), usually with 'interlocked' chelae (Bruski and Dunham, 1987). During fighting the opponents engage in pushing against each other and grasping the antennae, pereopods and other anterior body parts (Bruski and Dunham, 1987; Pavey and Fielder, 1996). The fight is terminated by the loser retreating (either walking or tail flipping) or turning aside and lowering the body against the substrate. The copulatory behavior of *C. quadricarinatus* in aquaria was previously described in detail (Barki and Karplus, 1999): in general, it can be divided into three chronological phases differing in typical behavior patterns, namely, pre-copulation, copulation and post-copulation. In the copulation phase the mates perform stereotyped actions that reflect cooperation, whereas the pre- and post-copulation phases involve elements of male courtship and dominant-subordinate relationships, respectively.

To avoid excessive aggression under our confinement conditions, we defined the termination of the encounters as the termination of fighting followed by at least one retreat by the loser, to verify its subordination. In cases of prolonged fights we stopped the encounter after 45 min of fighting. In cases where no fighting had occurred, we stopped the encounter 30 min after lifting the dividers (this criterion also included encounters with mating behavior patterns).

To compare aggression among the different types of encounters we quantified the following parameters: (i) total duration of fighting and mean duration per fighting bout; (ii)

latencies to start of interaction (i.e. first contact) and start of escalated fighting (response time from lifting the dividers); (iii) frequency and duration of crawling-over (a non-aggressive behavior pattern in which one crayfish climbs over the other). This behavior pattern was quantified since it has previously been found to differ among males, AG-implanted females and non-implanted females in confrontations with males (I. Karplus, A. Sagi, I. Khalaila and A. Barki, unpublished observations). Frequency and duration of crawling-over were standardized as means min^{-1} of encounter, because of the differing durations of the encounters. Non-parametric analysis of variance (Kruskal–Wallis test) was applied to these data at a significance level of $P < 0.05$. Where an overall significant difference was found, Mann–Whitney U -tests were performed for pairwise comparisons between specific types of encounters. Because multiple tests were performed, the critical significance level for this test was set at $P < 0.0167$, which is the quotient of 0.05 divided by the number of possible pairwise tests (3) (Dunn, 1964). Mating behavior was described quantitatively but no analysis was performed, because of the small number of encounters that involved this behavior pattern. All analyses were performed with the JMP 3.2 statistical software (SAS, 1997).

Morpho-anatomical and hemolymph vitellogenic protein examinations

18 IMP and 19 NON females were examined about a week after the behavioral tests. The propodus was checked externally for the development of the red patch. Morphometric measurements with a digital caliper (± 0.05 mm) included the length and width of the propodus of the chela, the length of the carapace, and the width of the abdomen, endopod and exopod at the second segment. The number of simple (ovigerous) setae in 1.25 mm of the internal endopod edge was counted. For anatomical studies, the animals were anesthetized in ice-cold water and the ovaries were removed and weighed to the nearest milligram. The gonadosomatic index (GSI) was calculated as the gonad mass expressed as a percentage of body mass. Mean oocyte diameter was calculated from a sample of 15 oocytes per ovary, measured under a light microscope. Secondary vitellogenic cross-reactive proteins in the hemolymph were examined by an enzyme-linked immunosorbent assay (ELISA), with an antibody raised against the specific 106 kDa vitellogenic polypeptide (Sagi et al., 1999). Differences between the IMP and NON females were statistically analyzed using the Mann–Whitney U -test.

Results

Behavioral test

Fighting occurred in all IMP *versus* IMP ($N=10$) and NON *versus* NON ($N=9$) encounters, but in only four out of eight IMP *versus* NON encounters.

Differences in the latencies to start of interaction and start of escalated fighting were statistically significant ($\chi^2=8.0$ and 7.9 , respectively, d.f.=2, $P < 0.05$). The first interaction

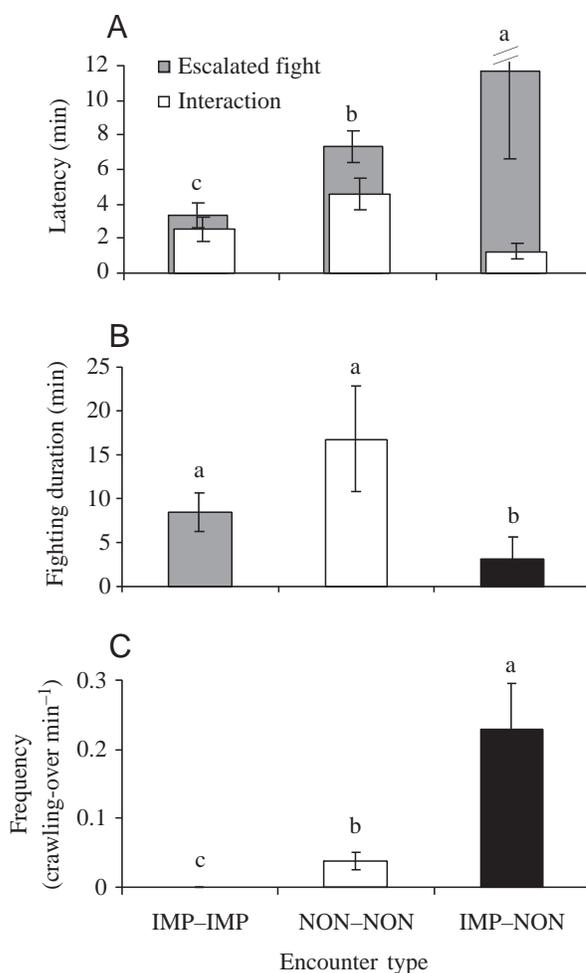


Fig. 1. Behavioral parameters in pair encounters involving androgenic gland (AG)-implanted (IMP) and intact (NON) *C. quadricarinatus* females in different combinations: IMP–IMP ($N=10$), NON–NON ($N=9$), IMP–NON ($N=8$). (A) Latency from lifting the dividers to first interaction (white bars) and escalated fight (gray bars). The difference between the gray and white bars denotes the time lapse from the start of interaction to escalated fighting (the adjacent letters refer to this difference). (B) Duration of escalated fighting. (C) Frequency of crawling-over. Values are means \pm S.E.M. Bars not sharing a letter are significantly different (Kruskal–Wallis test, $P < 0.05$, followed by Mann–Whitney U -test, $P < 0.0167$).

occurred soonest in IMP *versus* NON encounters, but the delay in these differed significantly only from NON *versus* NON and not from IMP *versus* IMP encounters (Fig. 1A). On the other hand, the first fight occurred latest in IMP *versus* NON encounters but the delay in this case differed significantly only from IMP *versus* IMP and not from NON *versus* NON encounters (Fig. 1A). Thus, the time lapses from the start of interaction to escalated fighting were also statistically different ($\chi^2=8.4$, d.f.=2, $P < 0.05$), being longest in IMP *versus* NON, shortest in IMP *versus* IMP interactions and intermediate in NON *versus* NON ($P < 0.0167$) (Fig. 1A).

Fighting duration (both total and mean per bout) differed among encounter types ($\chi^2=10.0$ and 11.7 , respectively, d.f.=2,

$P < 0.01$), being significantly lower in the IMP *versus* NON than in the other two types of encounters (Fig. 1B). Fighting duration in the IMP *versus* NON encounters was lowest also after excluding the four encounters in which there was no fighting from the analysis (8.4, 16.8 and 6.1 min, respectively to Fig. 1B), however non-significant statistically ($P > 0.05$).

Crawling-over was evident in seven of eight IMP *versus* NON encounters, five of nine NON *versus* NON encounters and in none of the IMP *versus* IMP encounters. The frequency (Fig. 1C) and duration of crawling-over were significantly different ($\chi^2 = 13.9$ and 14.7 , respectively, d.f. = 2, $P < 0.001$) and highest in the IMP *versus* NON encounters. The majority of the crawling-over actions recorded in the IMP *versus* NON encounters (52 of 55 climbs) were performed by the IMP female.

Characteristic mating behavior patterns were evident in three IMP *versus* NON encounters. In two pairs the IMP female performed 'thrusts' against the NON female, which is a typical male courtship pattern (Barki and Karplus, 1999). In the third encounter the mates exhibited the complete behavioral sequence of copulation twice, with the IMP female performing the male behavior pattern. The time durations of the main component of the copulation, namely 'freezing' – a motionless male-beneath-female position with the ventral surfaces brought face to face – were 74.9 and 77.9 s in these two copulations, which is within the range found previously for normal males and females under similar conditions (112 ± 81 s; Barki and Karplus, 1999), as were the time intervals from the first physical contact ('chela contact') to 'freezing' (12.9 and 14.3 s, respectively).

Morpho-anatomical and hemolymph examinations

The specific growth rate of the IMP females in the isolated aquaria was higher ($0.62 \pm 0.11\%$ day⁻¹) than that of the NON females ($0.46 \pm 0.07\%$ day⁻¹) (Mann-Whitney *U*-test, $P < 0.001$).

AG implantation caused an increase in the expression of male secondary sexual characteristics and an inhibition of female characteristics. The red patch, a secondary male characteristic located on the outer surface of the chela propodi,

developed in all the AG-implanted females whereas none of the intact females developed this characteristic. The mean relative width of the propodus of AG-implanted females was significantly greater ($P < 0.001$) than that of the intact females (Table 1). Maternal brooding-related characteristics, namely relative abdomen and endopod widths and the number of simple setae, were significantly reduced by AG implantation ($P < 0.001$) (Table 1).

The gonads of AG-implanted females did not show any sign of testis-like development and a sperm duct. The ovaries were white yellowish in color compared with the green brownish ovaries of the intact females. The oocytes in the ovaries of AG-implanted females were smaller in diameter and had lower gonadosomatic index (GSI) than those of the intact females ($P < 0.001$) (Table 1). An ELISA (enzyme-linked immunosorbent assay) test showed that the level of vitellogenic cross-reactive protein in the hemolymph of AG-implanted females was significantly lower ($P < 0.001$) than that in the hemolymph of the intact females (Table 1).

Discussion

This study found evidence for an array of masculinization effects of the androgenic gland in *C. quadricarinatus*: AG implantation into immature females brought about morpho-anatomical, physiological and behavioral alterations. For the first time in decapod crustaceans, we directly showed the influence of the AG on mating behavior. The impact of the AG on behavior was dramatic, since it caused a qualitative inversion of female mating behavior into a completely different male pattern, in addition to its quantitative effects on agonistic behavior. The effect of producing male-like behavior seems to be uniquely attributable to the presence of the AG, since no other components of the male reproductive system were developed.

Morpho-anatomical and physiological effects of the AG

AG implantation inhibited the development of the ovaries and of morphological traits that facilitate maternal egg

Table 1. External sex characteristics and female-specific anatomical and physiological characteristics in AG-implanted and intact *C. quadricarinatus* females

Characteristic	AG-implanted (<i>N</i> =18)	Intact (<i>N</i> =19)
Red patch (% of females)	100	0
Relative propodus width (propodus width/length)	$0.30 \pm 0.03^{***}$	0.28 ± 0.02
Relative endopod width (endopod/exopod)	$1.37 \pm 0.34^{***}$	2.29 ± 0.21
Number of simple setae in 1.25 mm internal endopod edge	$5.33 \pm 5.37^{***}$	33.16 ± 4.70
Relative abdomen width (abdomen width/carapace length)	$0.56 \pm 0.02^{***}$	0.62 ± 0.02
GSI (ovary mass/body mass $\times 100$)	$0.46 \pm 0.004^{***}$	5.0 ± 0.02
Oocyte diameter (mm)	$0.73 \pm 0.34^{***}$	2.33 ± 0.66
Secondary vitellogenic cross-reactive proteins in hemolymph (mg ml ⁻¹)	$0.021 \pm 0.010^{***}$	14.42 ± 20.45

AG, androgenic gland; GSI, gonadosomatic index.

Values are means \pm S.D.

*** $P < 0.001$, Mann-Whitney *U* test.

brooding, namely, wide abdomen, large endopods of the abdominal appendages (pleopods) and simple (ovigerous) setae specialized for egg attachment. The low GSI and small oocyte diameter and the low level of a secondary vitellogenin-specific polypeptide in the hemolymph indicated that secondary vitellogenesis – the accumulation of yolk protein in the ovary – was arrested in AG-implanted females. Concomitantly, AG implantation led to the development of secondary male characteristics in the form of large claws possessing a soft red patch. In fact, the AG-implanted females resembled intersex individuals in their external appearance and inhibition of primary female characteristics. Such individuals occur naturally in *C. quadricarinatus* populations (Thorne and Fielder, 1991; Medley and Rouse, 1993). Intersex individuals are morphologically and functionally males but possess both male and female genital openings, a testis and sperm duct with attached AG on the lateral half displaying the male opening, and an arrested pre-vitellogenic ovary at the contralateral half (Sagi et al., 1996). However, the AG-implanted females differed from intersex crayfish in that they lacked a male reproductive system and genital openings. The present findings are in keeping with those of recent studies of AG implantation into immature *C. quadricarinatus* females (Sagi et al., 1999; Khalaila et al., 2001), as well as with results reported for other crayfish (Nagamine and Knight, 1987; Taketomi et al., 1990; Taketomi and Nishikawa, 1996; Fowler and Leonard, 1999). The fact that the AG-implanted females had higher growth rates than intact females reflects the broad influence of the AG, which extends beyond reproductive processes. A similar growth-enhancing effect of AG implantation in females was described for the crayfish *C. destructor* (Fowler and Leonard, 1999), and the growth-inhibiting effect of AG removal in males was reported in the freshwater prawn *M. rosenbergii* (Sagi et al., 1990, 1997). This effect may be a direct result of the involvement of the androgenic hormone in somatic growth processes, or an indirect result of shifts in allocation of resources between ovarian and somatic growth, because of the inhibition of the ovaries by the AG.

Behavioral effects of the AG

The current results, coupled with previous observations in *C. quadricarinatus* (Levi, 1997; I. Karplus, A. Sagi, I. Khalaila and A. Barki, unpublished observations), indicate that both males and females fight vigorously in same-gender contests, using the same agonistic behavioral acts. Thus, the effect of AG implantation on the agonistic behavior was reflected mainly in the substantially lower aggressive motivation of both AG-implanted and intact females when they encountered one of the other rather than one of their own types. This was reflected in lower probability for, and shorter duration of, escalated fighting, and higher frequency of tolerance-indicative behavior (i.e. crawling-over) in the AG-implanted *versus* intact females' contests. Recently, we have examined the effects of AG implantation on behavior in the context of male–female interactions. The agonistic behavior of AG-implanted females was compared with that of males and non-implanted females

in interactions with dominant males (I. Karplus, A. Sagi, I. Khalaila and A. Barki, unpublished observations). Aggression measures (i.e. duration of fighting periods and grasps) were found to be lower, and tolerance measures (i.e. duration of crawling-over periods) higher, in male–non-implanted female interactions compared with male–male interactions, and intermediate values were found for male-implanted–female interactions. In this regard, encounters between AG-implanted and intact females in the present study resembled those between males and females. These results suggest a masculinization effect of the AG on the agonistic behavior of implanted females.

The most striking evidence for the masculinization effect of the AG came from the complete reversal of the mating behavior of females into typical male behavior following AG-implantation, in spite of the absence of any other component of a masculine reproductive system. In the present study, a pair comprising an AG-implanted and an intact female exhibited normal sequence and timing of copulatory behavior, with the implanted female performing the male behavior, and in two other pairs the AG-implanted females exhibited male courtship displays. This finding is astounding considering that we did not detect any sign of mating behavior in male–female encounters under similar conditions (I. Karplus, A. Sagi, I. Khalaila and A. Barki, unpublished observations). Mating behavior is rarely observable in *C. quadricarinatus* under experimental conditions of single short encounters, because of the difficulty in predicting receptivity of females (Barki and Karplus, 1999). The exceptionally large oocyte diameter in intact females in the current study (2.33 mm, comparing with approximately 1.5 mm reported by Khalaila et al., 2001) suggested that these females were more likely to be receptive. During mating, the male and female follow a coordinated sequence of behavior patterns that requires cooperation (Barki and Karplus, 1999). Thus, in addition to the effect on behavior, AG implantation into immature females led to alterations in male-specific factors that stimulate other females to respond receptively and less aggressively.

The androgenic gland hormone

We have shown for the first time a complete picture of the effect of the androgenic gland in crustaceans, including behavioral changes, which accompany the physiological process. However, the basic findings of this study raise three main questions. First, what is the nature of the androgenic hormonal factor that controls male behavior in crustaceans? Recently, the androgenic gland hormone (AGH) from the isopod crustacean *Armadillidium vulgare* was purified and characterized on the basis of a morphological bioassay, and the complete amino acid sequence was determined (Okuno et al., 1997, 1999; Martin et al., 1998, 1999). The isopod AGH is a glycosylated protein composed of two peptide chains connected each to the other by two disulfide bridges. Attempts to identify the decapod AGH are currently underway (for a review, see Sagi and Khalaila, 2001). The link between the AG and male behavior could offer a behavioral assay suitable for

studies aimed at the identification of androgenic factors from the AG in decapods.

Secondly, what is the mode of action of this androgenic factor? Hormonal effects on behavior are mediated by the nervous system. Although the effect of AG implantation was evident on the adult behavior, the hormonal manipulation was performed in young immature females. Thus, similarly to its action on secondary male characteristics, it is possible that, at the early stage at which the AG was implanted, the AGH induced male structural reorganization of neural circuits underlying the generation of sexually dimorphic behaviors. The AGH may also fill a role in modulating variations in behavior, as in the case of the aggressive motivation during interactions. Extensive research in this regard in crustaceans has been devoted to biogenic amines, in particular serotonin (5-hydroxytryptamine), that function as neuromodulators in the nervous system (for recent reviews, see Kravitz, 2000; Huber et al., 2001). Increased levels of serotonin generally correlate with increased aggression and dominance in crustaceans. Serotonin and octopamine elicit opposite postures that resemble those of dominant and subordinate individuals, respectively (Livingstone et al., 1980; Antonsen and Paul, 1997). Levels of serotonin, octopamine and dopamine differed between winners and losers among shore crabs, after fighting (Sneddon et al., 2000). Infusion of serotonin into crayfish altered the timing of the decision to withdraw, which caused fights to last longer (Huber and Delago, 1998). However, as with elevations of serotonin, reduced levels of this amine increased fighting duration in naïve juvenile lobsters (Doernberg et al., 2001). Furthermore, the modulatory effect of serotonin on the tail-flip circuit responsible for the rapid escape reaction of crayfish depends on the social status of the individual (Yeh et al., 1996, 1997). It is believed that several other substances, such as hormones, are important in agonistic behavior and may function in similar ways, possibly in dynamic balance with these neuromodulators (Kravitz, 1988, 2000). For example, the molting hormone 20-hydroxyecdysone (20E) appears to act through a rapid, nongenomic mechanism that reduces synaptic efficacy in crayfish neuromuscular junctions, and this effect can be reversed by application of serotonin (Cooper and Ruffner, 1998). 20E has been shown to exert differential effects on the abdominal phasic flexor muscles (related to the escape response) and the dactyl closer muscle of the claw (related to fighting) (Cromarty and Kass-Simon, 1998). This correlates to the variation in the aggressive motivation with 20E (i.e. the tendency of the lobster to flee or fight) over the molt cycle (Bolingbroke and Kass-Simon, 2001).

Third, and finally, what stimulated females to respond in a similar way to AG-implanted females as to a male? Some change must have occurred in the AG-implanted females that provided them with male stimulus signals and/or eliminated female stimulus signals. AG implantation might have caused changes in urine-borne chemical cues (perhaps the AGH itself or its derivatives) used in recognition of sex and dominance (e.g. lobsters and crayfish, Ameyaw-Akumfi and Hazlett, 1975;

Atema and Voigt, 1995; Dunham and Oh, 1996; Bushman and Atema, 1997). Alternatively, the stimulating cue might have been visual, namely the male-specific soft red patch that had developed on the claws of the AG-implanted females, or the male-like behavior of the AG-implanted females.

In conclusion, the results presented here strongly suggest causal relationships between an androgenic hormone derived from the AG and male behavior. Important questions remain concerning the mechanisms linking complex behavior, the nervous system and the androgenic hormone. Since androgenic hormones have not been identified yet in decapod crustaceans, further research is needed on their nature and the hormonal mechanism underlying variations in reproductive behavior.

The study was supported by grants from the Chief Scientist of the Israeli Ministry of Agriculture and Rural Development (857-0403-00) and the DFG (Ke 206/17-1).

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