

## Intersexuality and behavior in crayfish: The de-masculinization effects of androgenic gland ablation

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### Abstract

In crustaceans, male differentiation and primary and secondary characteristics are regulated by the androgenic gland (AG). In gonochoristic crustaceans, the AG is also linked to intersexuality. Whereas the co-occurrence of various male and female characteristics has been demonstrated in intersex crustaceans, little is known regarding sexually dimorphic behavior patterns in such individuals. In the present study, we used an intersex crayfish model to investigate – for the first time in crustaceans – the agonistic and mating behavior of intersex individuals, and to explore the effects of AG ablation on behavior, morphology and physiology. As was the case for their morphological and physiological reproductive traits, intersex individuals – despite being genotypically females – generally resembled males in terms of behavior: they engaged in fighting with males and copulated with receptive females. However, fighting durations of intersex animals were intermediate between those of males and females, and the durations of the copulations were remarkably short. Adult intersex individuals that had been AG ablated at the juvenile stage were unlikely to engage in fighting with males (similar behavior to females) and did not exhibit any mating behavior with receptive females. AG ablation resulted in feminine morphological and physiological shifts in the treated intersex individuals and enabled vitellogenin gene transcription and the onset of secondary vitellogenesis. It thus appears that an as-yet-unknown AG-secreted factor(s) regulating maleness also seems to regulate the organization of male behavior in crustaceans.

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### Introduction

In sexually reproducing organisms, differences between the sexes have evolved by natural and sexual selection in morpho-anatomical, physiological and behavioral characteristics that are directly or indirectly optimizing the individual's capabilities for achieving successful fertilization and rearing of offspring (Darwin, 1871). However, intersex individuals that combine traits of both sexes have been reported for many gonochoristic species of diverse taxa. In crustaceans, intersexuality is evident throughout the gonochoristic Malacostraca, e.g., in isopods (Rigaud and Juchault, 1998), amphipods (Ford and Fernandes, 2005) and decapods (Sagi et al., 2002). There are a number of studies on intersex crustaceans dealing mainly with the

morphological and physiological traits (Dunn et al., 1994; Ford et al., 2005; Rudolph, 2002; Rudolph et al., 2001; Sagi et al., 1996; Zou and Fingerman, 2000), causative factors and fitness consequences (Dunn et al., 1990; Ford et al., 2003, 2004a; Kelly et al., 2004) associated with intersexuality. In contrast, the behavior of intersex individuals, specifically behavior patterns that are usually sexually dimorphic, such as aggressive and mating behavior, has rarely been investigated.

Sexual differentiation in crustaceans is hormonally controlled by the androgenic hormone(s) (AGH) secreted by the androgenic gland (AG) (Charniaux-Cotton, 1960; Charniaux-Cotton and Payen, 1985). During early development, AG primordia are expressed in genetic males, and they determine the development of masculine gametogenic (testes) and endocrine (AG) organs, which are distinct organs in crustaceans. Thereafter, the endocrine function of the AG regulates the development of male phenotypic characteristics (Charniaux-Cotton and Payen, 1988; Katakura,

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1989; Payen, 1990; Sagi et al., 1997). The AG has also been implicated in the mediation of intersexuality, although causative factors vary among crustacean species, and in some cases they are unclear. For example, in a number of amphipod species in which female sex differentiation is frequently under the control of feminizing microsporidian parasites, intersexuality results from incomplete feminization by these vertically transmitted sex-ratio distorters; it is believed that they act through prevention of AG differentiation and AGH production (Bulnheim, 1977; Kelly et al., 2004; Rodgers-Gray et al., 2004). In isopods, on the other hand, it has been proposed that intersex individuals that are functional males are the product of a delayed expression/action of AGH in genetic males (Azzauna et al., 2004). It has also been found that endocrine-disrupting pollutants cause de-masculinization and intersexuality in amphipods, possibly by interfering with the function of the AG (Ford et al., 2004b). Thus, it is evident that the AG, which is responsible for the control of male primary and secondary sexual characteristics (including behavior), is also a key factor in the formation of intersex individuals and sexual plasticity in crustaceans. Since intersex individuals may be particularly plastic with regard to sexually dimorphic behaviors and their responsiveness to sex-related hormonal manipulations, they offer a unique, inducible sexual plasticity model for the study of hormonal control of sexually dimorphic behaviors in crustacea (Sagi et al., 1996, 2002).

It is well known that androgens influence sexual and aggressive behavior in vertebrates (Hull et al., 2002; Kelley, 1988; Schulkin, 1999). In contrast, the study of the relationship between hormones and behavior in crustaceans is still in its infancy. Only a few studies have touched on the hormonal control of agonistic and reproductive behavior by the AG, but these studies have presented indirect evidence for the relationship between the presence of androgenic factors and male mating behavior in decapods (Gleeson et al., 1987; Sagi and Khalaila, 2001). Recently, we found for the first time in decapod crustaceans direct evidence for masculinization effects of the AG on agonistic and mating behavior (Barki et al., 2003; Karplus et al., 2003), in addition to its morphological, anatomical and physiological effects (Barki et al., 2003; Khalaila et al., 2001; Manor et al., 2004). Whereas our previous study explored the masculinization effects on behavior of implanting AGs into *Cherax quadricarinatus* females, the current study uses the intersex model to investigate the de-masculinization effects of AG ablation.

In male crayfish of the model species used in this study – the gonochoristic decapod *C. quadricarinatus* – the reproductive system consists of a pair each of testes, sperm ducts, androgenic glands and genital openings at the bases of the fifth pair of walking legs. Females have a pair each of ovaries, oviducts and genital openings at the bases of the third pair of walking legs. Intersexuality occurs both in wild (Curtis and Jones, 1995) and in cultured populations where intersex crayfish may constitute 1–14% of the population (Sagi et al., 2002). Intersex individuals are morphologically and functionally males but possess both male and female genital openings, a testis and sperm duct with attached AG in the lateral half displaying the male opening, and an arrested previtellogenic ovary in the contralateral half (Sagi et al., 1996). In intersex individuals, there is no vitellogenin gene expression in the hepatopancreas and no vitellogenin in the hemolymph (Sagi et al.,

2002). The results of crosses between females and either normal males or intersex individuals have suggested that intersex individuals are genotypically females (Parnes et al., 2003). The masculine phenotype with inhibited feminine characteristics of intersex animals has been attributed to the presence of the AG, but the primary cause of intersexuality in *C. quadricarinatus* is unclear.

The aims of the present study were to examine the agonistic and mating behavior patterns of intersex individuals in relation to those of male and female animals and to investigate the effect of AG ablation on the behavior and the morphological and physiological reproductive traits in intersex crayfish.

## Materials and methods

### AG ablation

From a population of sexually immature juvenile crayfish in a commercial nursery, we selected intersex individuals that possess one male genital opening coupled with a pair of female genital openings (the most common combination) or with one female genital opening on the contralateral side. AG ablation was performed by detaching the base of the fifth walking leg together with the male genital opening and gently pulling out, with a forceps, the distal part of the sperm duct with the attached AG, followed by the whole sperm duct. To destroy any remnant AG cells, the tissue surrounding the AG was cauterized by a diathermy. A sham ablation was performed by removing the fifth walking leg (on the opposite side) that did not possess a male genital opening and an AG.

AG ablations were performed at the juvenile stage [mean body mass of  $5.44 \pm 4.73$  (SD) g]. The AG-ablated individuals were reared in the laboratory for a period of at least 10 months before the experiment, during which time they reached sexual maturity. Average body mass at the time of the behavioral experiment was  $55.4 \pm 11.1$  (SD) g. Unsuccessfully AG ablated intersex individuals, i.e., animals whose various sexual characteristics were found to be similar to those of intact intersex individuals, were not used for the present study; such morphological features have proven most reliable for distinguishing between successful and unsuccessful cases of AG manipulation (Barki et al., 2003; Khalaila et al., 2001). All the crayfish used in the behavioral experiment were housed individually in separate aquaria in the laboratory for at least one month prior to the experiments.

### Behavior tests

#### Agonistic behavior

We investigated agonistic behavior in pair encounters of AG-ablated intersex crayfish ( $n = 11$ ), of intact intersex crayfish ( $n = 12$ ), of male crayfish ( $n = 15$ ) or of female crayfish ( $n = 15$ ) against size-matched male opponents. Size matching was based on claw length, since weapon size is considered the most reliable criterion for fighting ability in clawed crustaceans (Barki et al., 1992, 1997a; Sneddon et al., 1997), including crayfish (Rutherford et al., 1995). Chela length ratio (larger/smaller contestant) averaged  $1.01 \pm 0.01$  (SD), with no significant difference among encounter types ( $F_{3,50} = 0.9$ ,  $P > 0.4$ ). Encounters involving an individual that molted within a week after the behavioral trial were not considered in this study to minimize the effect of molt stage on behavior.

The pair encounters were conducted in large glass aquaria ( $50 \times 120 \times 50$  cm) divided into three sections by two opaque dividers. The two contestants were transferred simultaneously into the aquarium, one in each of the outside sections of the tank. After 6 min of acclimation, the dividers were lifted, and the encounter was video recorded. An encounter was terminated after one of the opponents had submitted and retreated in two consecutive interactions (to verify its subordination). Observations were stopped after a maximum of 30 min in situations where either no winner had yet emerged (to avoid excessive aggression), or where no fighting had ensued. The duration of the observation period was thus varied between pairs.

#### Mating behavior

We investigated mating behavior of AG-ablated and sham-ablated (control) intersex individuals paired with receptive females ( $n = 6$ ). We hypothesized that

if the AG was important for the expression of male mating behavior, then AG-ablated intersex crayfish would not exhibit mating behavior in the presence of receptive females and no copulation would occur. The reason for testing demasculinization of mating behavior in AG-ablated intersex (i.e., lack of response to receptive females) rather than feminization of their behavior (i.e., mating with a male) was that female receptivity occurs at an unpredictable point in time along the prolonged reproductive season and has no unequivocally detectable precedent signs (such as a premating molt which occurs in various decapods). Thus, a large stock of AG-ablated intersex animals would be required for a reasonable chance of detecting a receptive individual at a specific point in time.

Receptive females were obtained at the onset of the reproductive season (Barki et al., 1997b) from an all-female stock of 96 sexually mature females kept in four 600-l tanks with shelters (PVC pipes, 75 mm in diameter). To find receptive females, we introduced a large adult male into the tank and observed it exploring the tank for 10 min. A non-receptive female usually avoided the male or was evicted from the shelter by the male. In contrast, a receptive female sought contact with the male, whose reaction was non-aggressive, with antennae contact and restrained use of the chelipeds, which is typical of the initiation of mating. When we observed this behavior, we immediately caught the female and transferred it to a large aquarium with a sexually mature resident male for receptivity verification. The female was considered receptive if the stereotyped sequence of copulation commenced, i.e., ‘overturning of the male’, maneuvering the male into the copulatory position (Barki and Karplus, 1999). Copulation was then interrupted before the male could attach his spermatophore onto the female’s sternum.

Each receptive female served sequentially for testing an ablated and a sham-ablated intersex individual that were of similar age and size ( $52.8 \pm 7.5$  and  $56.5 \pm 15.6$  g, respectively). The encounters were conducted in the large aquaria described above. Each encounter lasted 30 min, with the AG-ablated trial always preceding the sham-ablated trial because the latter was expected to culminate in copulation and thus terminate the female’s receptivity.

Several AG ablated intersex individuals that had female-like morphometric characteristics (i.e., claws lacking a red patch with propodus width/length  $< 0.29$ , abdomen width/carapace length  $> 0.60$ ; Barki et al., 2003) were housed with males for at least 5 weeks during the reproductive season to examine their ability to reproduce as females.

### Behavior analysis

The most prominent component of contests is escalated fighting [for descriptions of fighting behavior, see Barki et al. (2003) and Karplus et al. (2003) for *C. quadricarinatus*; and Bruski and Dunham (1987), Pavay and Fielder (1996), Tierney et al. (2000) and Huber et al. (2002) for other species of crayfish]. To compare aggression among the different types of encounters, we used the following fighting-related parameters: (i) probability of fighting; (ii) total duration of fighting; (iii) time lapse between start of interaction (i.e., first approach of the opponents to within a distance of one body length) and start of escalated fighting and (iv) frequency and duration of crawling-over (in which one crayfish climbs over the other), standardized as means per minute of encounter because of the differing durations of the encounters. The latter parameter was added because not all encounters included fighting. These measures have previously been used in investigating behavioral influences of AG implantations into females and have proven useful for differentiating between male–male and male–female encounters (Barki et al., 2003; Karplus et al., 2003).

Mating behavior was analyzed in terms of the occurrence and duration of copulation (from overturning of male to the female disengagement by tail flipping) and the occurrence of ‘thrust’ (a typical male display using the chelipeds) and ‘overturning of male’ attempts. Following each encounter, the female was inspected for a spermatophore attached to its sternum to verify that copulation had been successful. In addition to the occurrence of copulation in the mating behavior test, the latency to first interaction, the duration of interaction (out of the 30-min encounter time) and the frequency of aggressive actions per min of interaction were quantified. The following behavior patterns were considered aggressive actions: Lunge, Push, Embrace, Extend, Strike and Grasp (Karplus et al., 2003).

### Morpho-anatomical and secondary vitellogenesis examinations

Crayfish were examined about 1 to 2 weeks after the behavioral trials. Morphological sexual characteristics were quantified on the basis of the

following measurements: orbital carapace length and abdomen, endopod and exopod widths at the second segment, measured with digital calipers ( $\pm 0.05$  mm). The percentage of simple (i.e., ovigerous) setae out of the total number of setae in 1.25 mm of the internal endopod edge was calculated. For anatomical examinations, the animals were anesthetized in ice-cold water, and the gonads (ovaries and/or testes) were removed and weighed to the nearest milligram. The gonadosomatic index (GSI) was calculated as the percentage of gonad mass to body mass. Mean oocyte diameter was calculated from a sample of 15 oocytes per ovary, measured under a light microscope.

Secondary vitellogenesis was quantified in terms of *C. quadricarinatus* vitellogenin gene (*CqVg*) expression in the hepatopancreas and cross-reactive vitellogenin proteins in the hemolymph. Vitellogenin gene expression was evaluated by relative quantitative real time RT-PCR as follows: RNA was extracted, using EZ-RNA Total RNA Isolation Kit (Biological Industries Beit Haemek, Ltd.), from small hepatopancreas fragments removed from crayfish anesthetized in ice cold water. The concentrations of all the produced RNAs were evaluated by spectrophotometry at 260 nm (GeneQuant Pro, Amersham Pharmacia Biotech). First-strand cDNA was generated by a RT reaction (Reverse-iT™ 1st Strand Synthesis Kit-ABgene AB-0789) from 1  $\mu$ g of total RNA at 47°C for 30 min with random hexamers as primers (20 ng/ $\mu$ l final concentration). Relative quantification of vitellogenin gene expression was performed with the following primers: Vit-Cher-QPCR-R 5'-GGGCGGCAT-GACACACATCT-3', Vit-Cher-QPCR-F 5'-GCTTCCCGGTGGTTAATCCT-3', each at 0.4  $\mu$ M final concentration, and SYBR Green PCR Master Mix (Applied Biosystems) used according to the manufacturer’s instructions (ABI Prism 7000 Sequence Detection System, 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). To present the data in units of  $\Delta\Delta$ ct, two sets of normalization were performed; the first with non-vitellogenic hepatopancreas from a male, and the second with a 18 s ribosomal housekeeping gene from the tested sample using the following primers: 18 s Cherax R 5'-CCGGAATCGAACCCTGATT-3' and 18 s Cherax F 5'-GGCGCTGTGTCTTTCAAGTG-3' at the same concentration as above.

Secondary vitellogenic cross-reactive proteins in the hemolymph were quantified by an enzyme-linked immunosorbent assay (ELISA), with an antibody raised against the specific 106-kDa vitellogenic polypeptide (Sagi et al., 1999). A 10- $\mu$ l hemolymph sample was withdrawn from the base of a swimming leg of each crayfish and diluted in 490  $\mu$ l of carbonate buffer 0.1 M, pH 9.6.

### Statistical analyses

Differences between group means were analyzed using one-way analysis of variance (ANOVA) at a significance level of  $P < 0.05$ . Where a significant difference was found, multiple comparisons were conducted by means of Tukey–Kramer honest significance difference (HSD) test to identify differences between specific groups. Data for which homogeneity of variances (Bartlett test) and normality (Shapiro–Wilk W test) were not confirmed were analyzed by means of the Kruskal–Wallis non-parametric test, followed by the Mann–Whitney *U* test at an adjusted significance level of  $P < 0.008$  (0.05/no. of possible pair-wise comparisons; Dunn, 1964). Proportion data were analyzed by means of the likelihood ratio Chi-square test. Differences between AG- and sham-ablated intersex individuals encountering the same receptive female were analyzed by means of paired *t* test (two tailed). All analyses were performed with the JMP 5.1 statistical software (SAS Institute, 2003, Cary, NC).

## Results

### Behavior tests

#### Agonistic behavior

Fighting was more likely to occur in male–male and intact intersex–male encounters than in AG-ablated intersex–male and female–male encounters (likelihood ratio  $\chi^2 = 22.2$ ,  $P < 0.001$ ) (Fig. 1A). For the crayfish that did engage in fighting ( $n = 14, 12, 5$  and 6 for male, intact intersex, AG-ablated intersex and female animals, respectively), there was no significant difference among

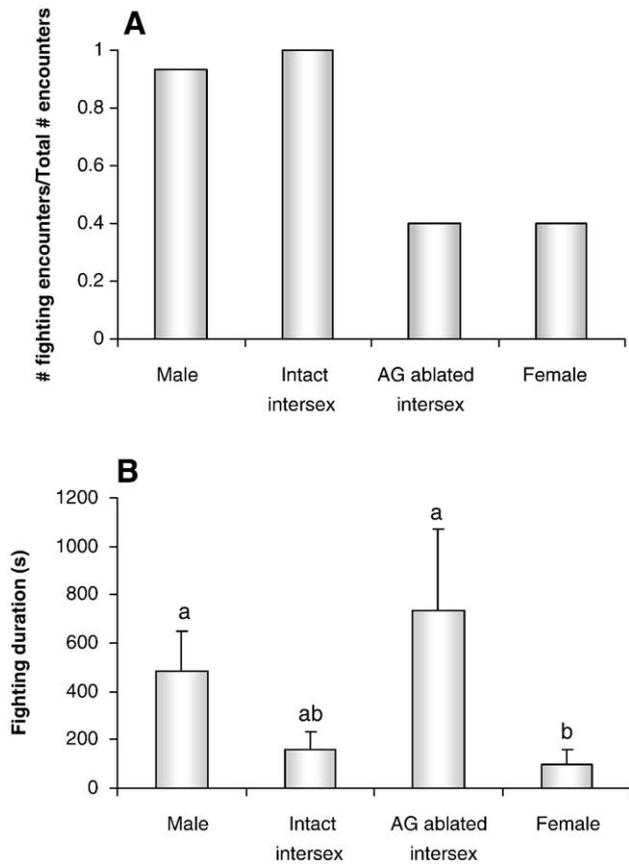


Fig. 1. Proportion of the occurrence of escalated fighting (A) and mean ( $\pm$  SEM) fighting duration (B) in pair encounters involving male, female, intact intersex or AG-ablated intersex animals against claw size-matched male opponents in *C. quadricarinatus* crayfish. Bars with different letters are significantly different (Tukey–Kramer HSD,  $P < 0.05$ ).

all the types of encounter in the time lapse from the start of the interaction to escalated fighting ( $F_{3,33} = 0.5$ ,  $P > 0.5$ ). However, the duration of fighting differed among encounter types ( $F_{3,33} = 5.2$ ,  $P < 0.01$ ): AG-ablated intersex fights and male fights had the longest mean duration but not significantly longer than encounters involving intact intersex individuals, whereas fights involving female animals were significantly shorter (Tukey–Kramer HSD,  $P < 0.05$ ) (Fig. 1B). The female crayfish did not win any of the encounters, the AG-ablated intersex animals emerged as the winners in one out of five encounters (in one of the encounters fighting was interrupted before decision), and the intact intersex animals triumphed in 6 out of 12 encounters. Within the encounters involving intact intersex animals, there was no significant difference in fighting duration between encounters won by the intersex animal and those won by the male opponent ( $t$  test,  $P > 0.1$ ).

Crawling-over was more likely to occur in female–male and AG-ablated intersex–male encounters than in intact intersex–male and male–male encounters (likelihood ratio  $\chi^2 = 7.62$ ,  $P = 0.05$ ) (Fig. 2A). The frequency and duration of crawling-over were highest in encounters involving females and lowest in encounters involving males. However, Kruskal–Wallis tests revealed a significant difference for the frequency ( $\chi^2 = 8.7$ ,  $df = 3$ ,  $P < 0.05$ ) and not for the duration variable ( $\chi^2 = 5.6$ ,  $df = 3$ ,  $P > 0.05$ ).

Pair-wise comparisons revealed no significant differences between specific encounter types (Mann–Whitney tests,  $P > 0.008$ ), suggesting an overall trend of an increase in crawling-over frequency from encounters involving males through those involving intersex individuals to those involving females (Fig. 2B).

Injuries (in the form of a sectioned antenna, walking leg or cheliped) were mostly evident in encounters involving males (in 5 out of 15 encounters) but also occurred in one each of the encounters involving intact and AG-ablated intersex individuals, and did not occur in encounters involving female animals. Typical mating behavior patterns were evident in 3 out of the 15 encounters involving females and 3 out of the 11 encounters involving AG-ablated intersex individuals. These included ‘thrust’ actions and attempts at ‘overturning of the male’. However, in none of the encounters was complete copulation accomplished, because it requires the active cooperation of the female (or of the AG ablated intersex), which was absent in all cases.

#### Mating behavior

Copulation occurred in all six encounters of sham-operated intersex with receptive females, and courtship displays were observed in five of these encounters (Table 1). Excluding one

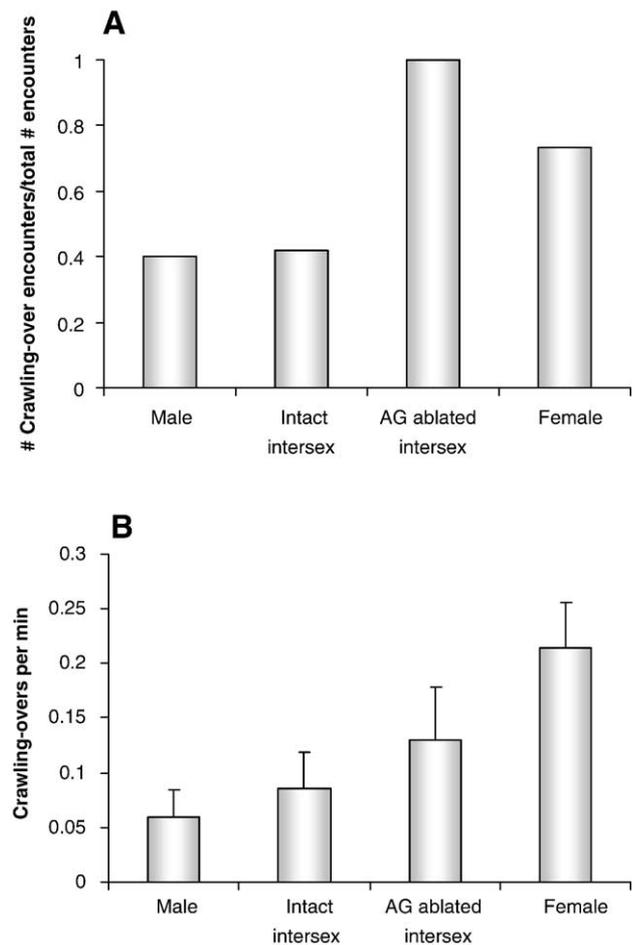


Fig. 2. Proportion of the occurrence (A) and mean ( $\pm$  SEM) frequency (B) of ‘crawling-over’ in pair encounters involving male, female, intact intersex or AG-ablated intersex animals against claw size-matched male opponents in *C. quadricarinatus* crayfish.

Table 1  
Mating behavior of AG-ablated and sham-ablated *Cherax quadricarinatus* intersex animals during 30 min encounters with receptive females

Intersex (n = 6)	Encounters with copulation	Encounters with courtship displays	Duration of interaction (min)	Aggressive actions (per min of interaction)
AG-ablated	0	0	12.1 ± 7.1	1.9 ± 1.1
Sham-ablated	6	5	18.9 ± 4.6	0.4 ± 0.3

case in which copulation lasted for 329 s, the copulations lasted for a short time, averaging  $10.8 \pm 8.6$  s. Copulation was terminated by the female's decision to disengage by tail flipping, probably immediately following the attachment of the spermatophore. In contrast, there were no copulations or any courtship displays in AG-ablated-receptive female encounters. The time (out of 30 min of an encounter) that the paired animals interacted (i.e., performed any kind of action within a distance of one body length) was longer in encounters involving sham-operated intersex animals than in those involving AG-ablated intersex animals (paired *t* test,  $P < 0.05$ ), but the frequency of aggressive acts towards the receptive female (per min of interaction) was higher in encounters involving AG-ablated intersex animals (paired *t* test,  $P = 0.05$ ) (Table 1).

#### Female reproductive characteristics

Morphological characteristics related to egg brooding, namely, relative abdomen and endopod widths and the percentage of simple (i.e., ovigerous) setae on the endopod's margins, were significantly different among male, female, intact-intersex and AG-ablated-intersex crayfish (Kruskal–Wallis test,  $\chi^2 > 26$ ,  $df = 3$ ,  $P < 0.001$ ) (Fig. 3). There was also a significant difference in the GSIs and in oocyte diameter among crayfish types (Kruskal–Wallis test,  $\chi^2 > 30$ ,  $df = 3$ ,  $P < 0.001$ ) (Fig. 4), as well as in the relative quantification of *CqVg* gene expression and the level of vitellogenin cross-reactive proteins in the hemolymph (Kruskal–Wallis test,  $\chi^2 > 17$ ,  $df = 3$ ,  $P < 0.001$ ) (Fig. 5). For all the above morphological, anatomical and physiological traits, intact intersex animals were similar to males (except for gonad anatomical traits, Fig. 4), and AG-ablated intersex animals were similar to females.

#### Fertilization and spawning in AG-ablated intersex crayfish

Several cases of spermatophore attachment on the abdomen of AG-ablated intersex individuals were observed. A unique case of one AG-ablated intersex animal that spawned twice was also observed. The eggs, however, had a pale-orange color (Fig. 6A). The eggs of the first spawning did not hatch. For the second spawning, the embryos hatched and started to develop (Figs. 6B, C), but their development was arrested, and no juveniles eventually emerged.

#### Discussion

Female and male *C. quadricarinatus* crayfish were clearly sexually dimorphic for all types of characteristics tested in this

study. This fact facilitated the examination of the study's central questions, namely, (i) where on the phenotypic scale from the male to the female extremes is the intersex individual situated with regard to various sexual characteristics, and (ii) how do endocrine manipulations at the whole-AG level influence the positioning on the scale. In keeping with previous studies (Sagi et al., 1996; Khalaila et al., 1999; Abdu et al., 2002), we

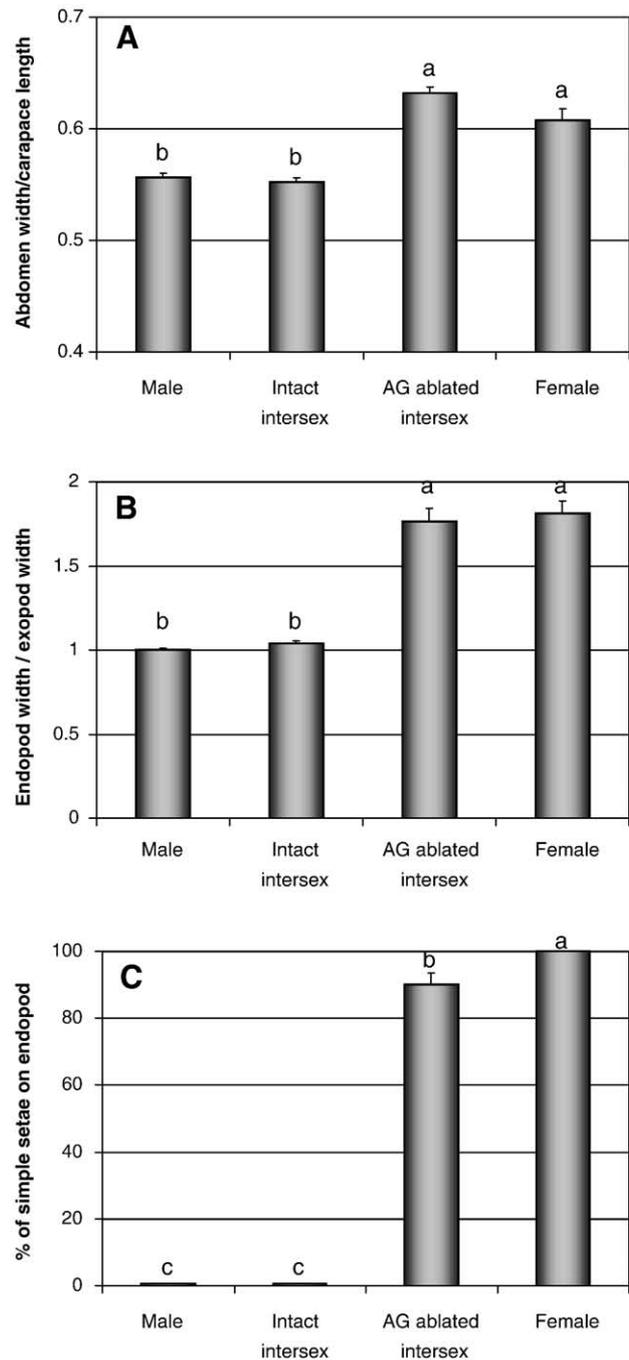


Fig. 3. Mean values ( $\pm$  SEM) of morphological characteristics in males, females, intact intersex and AG-ablated intersex in *C. quadricarinatus* crayfish: (A) relative abdomen width; (B) relative endopod width; (C) percentage of simple setae on a 1.25 mm fragment of the endopod's edge. Bars with different letters are significantly different (Kruskal–Wallis test,  $P < 0.05$ , followed by Mann–Whitney *U* test,  $P < 0.008$ ).

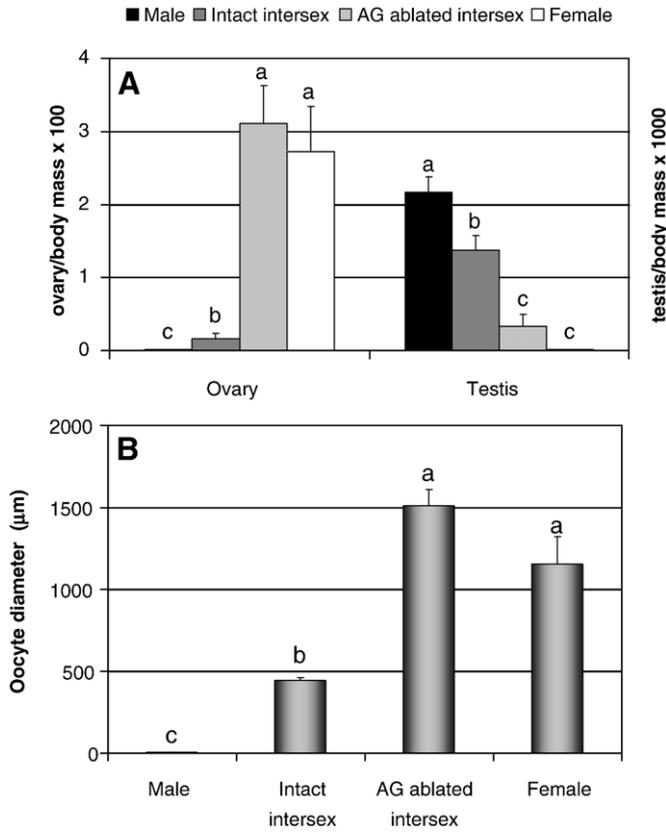


Fig. 4. Mean values ( $\pm$  SEM) of gonadal characteristics in males, females, intact intersex and AG-ablated intersex in *C. quadricarinatus* crayfish: (A) ovarian and testicular gonado-somatic indices (each index was multiplied by a different factor to bring the scales to a comparable level); (B) oocyte diameter. Bars with different letters are significantly different (Kruskal–Wallis test,  $P < 0.05$ , followed by Mann–Whitney  $U$  test,  $P < 0.008$ ).

demonstrated that *C. quadricarinatus* intersex individuals resemble males in secondary morphological characteristics and in physiological reproductive functions, but less so in the anatomical characteristics of the reproductive system, which consists of a single testis, sperm duct, an AG and papilla and a contralateral arrested ovary. In addition, we demonstrated, for the first time, that the aggressive and mating behavior patterns of intersex individuals generally resemble those of males. The main finding of the current study was the de-masculinizing influence of AG ablation on sexually dimorphic behavior patterns, indicating the involvement of AG-secreted androgens in the regulation of male behavior, as an additional element in the AG-modulated endocrine balance between male and female reproductive traits in intersex animals (Khalaila et al., 1999). This finding is in line with the opposing masculinization effect on behavior induced by AG implantation into female crayfish (Barki et al., 2003; Karplus et al., 2003).

### Behavior of intersex crayfish

Despite the male-like behavior of intersex individuals, as revealed in their absolute likelihood to engage in fighting with males and copulate with receptive females, some behavioral differences did emerge between intersex and male animals.

Firstly, the durations of fights of intersex individuals were intermediate between those of males and females in contests against males, and less injurious than those between males. Fighting duration is determined by the loser’s decision to retreat and can thus be used as a measure of the aggression persistence of the eventual loser. However, in the encounters involving intersex animals, the intersex opponent was the loser in only half of the contests (unlike the females, who were invariably subordinate to the male opponents), and there was no significant difference between the persistence time of intersex and male losers. Thus, the intermediate fighting duration of intersex individuals does not lend credence to the possibility that intersex animals are competitively inferior or inherently less aggressive than male crayfish. Secondly, the duration of copulation of intersex individuals with females was much shorter (by an order of magnitude) than that previously reported for males (Barki and Karplus, 1999). This difference might, however, be the result of the different experimental designs of the two studies. Alternatively, the rapid copulation process might suggest that intersex animals employ a mating tactic that often typifies individuals that are competitively inferior and/or less preferred by females (e.g., in crustaceans, Shuster, 1989; Telecky, 1984; Thiel and Correa, 2004).

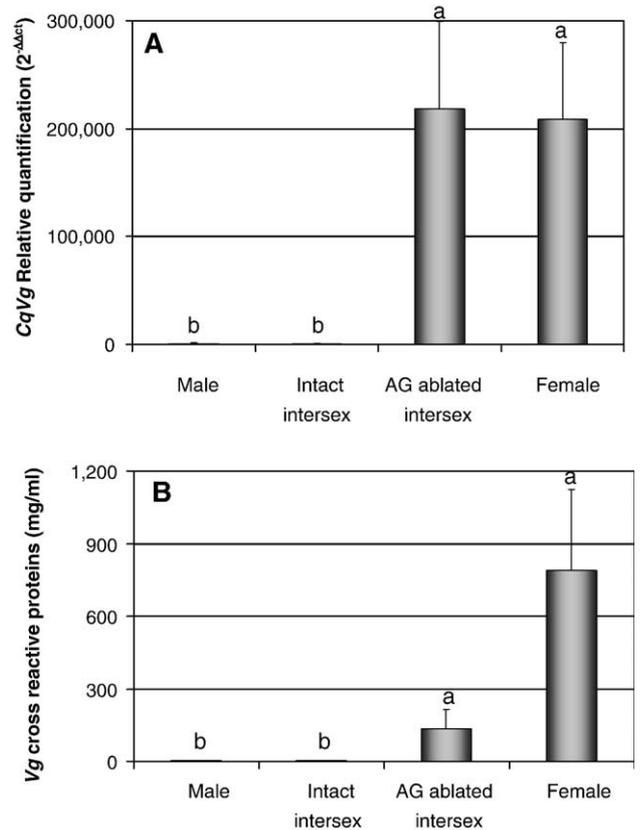


Fig. 5. Mean values ( $\pm$  SEM) of vitellogenic characteristics in males, females, intact intersex and AG-ablated intersex in *C. quadricarinatus* crayfish: (A) relative quantification of *CqVg* gene expression monitored by real time RT-PCR; (B) levels of vitellogenic cross-reactive proteins in the hemolymph monitored by ELISA. Bars with different letters are significantly different (Kruskal–Wallis test,  $P < 0.05$ , followed by Mann–Whitney  $U$  test,  $P < 0.008$ ).

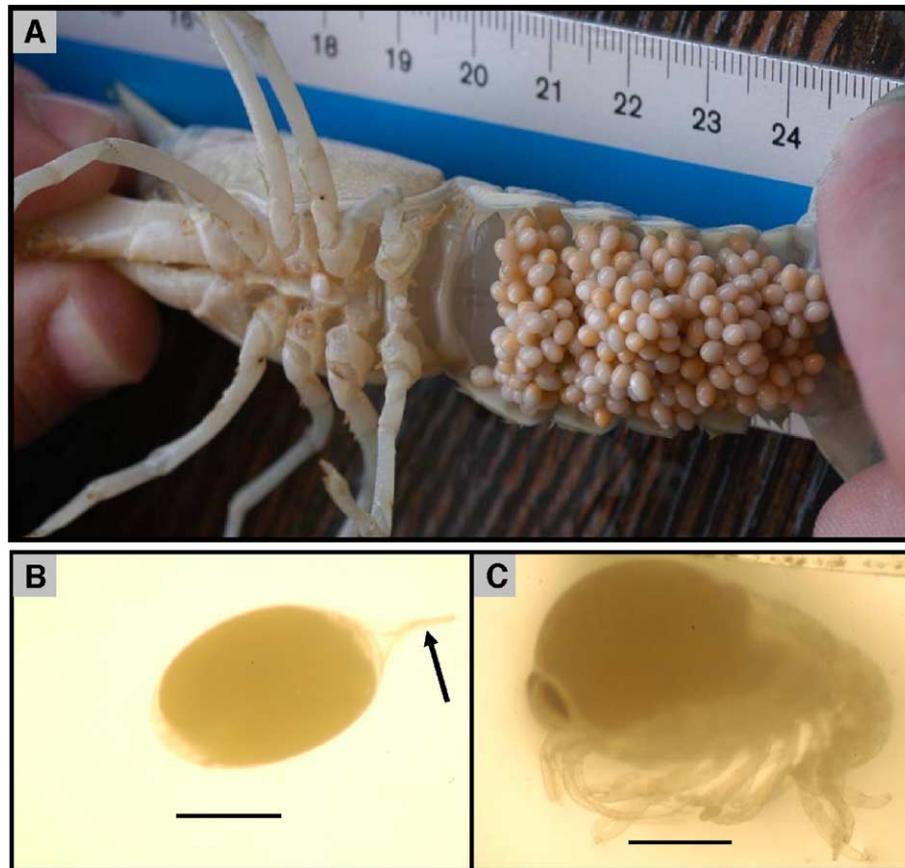


Fig. 6. An AG-ablated intersex individual carrying eggs. (A) The first batch of eggs that had a pale orange color and did not hatch. (B) An egg from the second batch, 17 days after spawning. The arrow shows the side connecting the egg to the simple seta on the mother's pleopod. (C) A hatched embryo, 35 days after spawning. The scale bars in panels B and C represent 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Notwithstanding these differences, it is still unclear whether there is indeed any fitness cost associated with intersexuality in crayfish and whether the behavioral differences can account for it. Fitness cost may be incurred in intersex animals through a reduction in reproductive output or through behavioral alterations. In amphipod crustaceans, fitness cost in terms of reduced fecundity and fertility has been demonstrated mainly in intersex individuals that are functional females (Ford et al., 2003, 2005; Kelly et al., 2004) and to a lesser extent in male intersex animals (McCurdy et al., 2004). Cost-incurring behavioral alteration in intersex amphipods has also been postulated on the basis of a reduced probability for their occurrence in precopula pairs compared with pure-gender individuals (Dunn et al., 1990). However, to the best of our knowledge, no study has directly examined the behavioral performance and the consequences for competitive ability and mating success of intersex compared to pure-gender individuals.

#### Effects of the AG on behavior

Using the intersex model, we demonstrated that the AG plays an important role in the expression of male behavior in crayfish. This was most strikingly evident in the mating behavior of intersex animals: in the absence of an AG, intersex individuals

did not exhibit male mating behavior (in contrast to sham-operated AG-intact intersex individuals). Thus, removal of the AG caused a shift in the behavior of the intersex animal towards the female end of the behavioral phenotypic scale. However, for behavior to be completely feminized, a female mating pattern leading to successful insemination by males should be evident in AG-ablated intersex animals, in addition to the lack of sexual responsiveness towards receptive females observed in this study. In three encounters in the agonistic behavior experiment, the male exhibited some elements of mating behavior towards the AG-ablated intersex individual, but the latter did not respond receptively. This finding, in itself, cannot serve as conclusive evidence for inappropriate female behavior in AG-ablated intersex animals, because these individuals were perhaps merely non-receptive at that particular time. To date, we have rarely observed an AG-ablated intersex crayfish with an attached spermatophore (indicating successful copulation) or eggs, and in the rare cases that such animals were observed, the embryos did not develop. Although the vitellogenic activity in AG-ablated intersex individuals was similar to that in females, as revealed by quantitative physiological markers such as *CqVg* gene expression and vitellogenin cross reactive proteins in the hemolymph, complete feminization of all physiological reproductive functions following AG ablation was not accomplished.

Likewise, AG-ablation of intersex individuals might not (or rarely) lead to complete feminization of behavior. This supposition is supported by the results of the agonistic behavior experiment; despite being as unlikely as females to engage in fighting with males, some of the AG-ablated intersex animals that did engage in fighting were extremely aggressive, as reflected in the duration of fighting, which was at least as long as that of males. We have no clear explanation for this finding, but it certainly highlights the non-complete female-like behavior of some AG-ablated intersex individuals.

In decapod crustaceans, male and female reproductive functions are down-regulated by neurohormones secreted from the X-organ sinus gland complex in the eyestalk (Chang, 2001). Eyestalk neuropeptides act directly on the female ovaries (Charniaux-Cotton and Payen, 1988; Fingerman, 1995), whereas in males they modulate the stimulatory action of the AG on the testes (Khalaila et al., 1999, 2002). Removal of this complex had different effects on male and intersex *C. quadricarinatus* crayfish: in both it induced the expression of *CqVg*, but in intersex animals it also induced translation and release of *CqVg* products into the hemolymph (Shechter et al., 2005) and consequently maturation of the ovary (Khalaila et al., 1999). A possible explanation for this difference is the presence in the genetically female intersex animal of a female stimulatory factor (Shechter et al., 2005), such as a gonad stimulating hormone (GSH), which is believed to be released from the brain and the thoracic ganglia (Fingerman, 1997). The release of this stimulating hormone is, in turn, stimulated by serotonin (Fingerman, 1997), which is also an important neuromodulator of the neural circuits underlying agonistic behavior in decapods (Huber, 2005; Kravitz, 2000). Within this complex dynamic interaction between hormones, neurohormones, neuromodulators and reproductive functions, it is possible that the female hormone may also be involved in the regulation of sexual behavior. Comparison of the effects of AG ablation on the behavior of males (which unlike intersex animals lack any female component) with that found in intersex animals could possibly provide a clue to whether a female factor is involved in the sexual/behavioral plasticity observed in AG-ablated intersex individuals.

While it is clear that AG-secreted bioactive materials are responsible for the masculinization of sexually dimorphic behaviors in decapod crustaceans, the nature of the androgenic hormone(s) – ranging from lipid substances (Berreur-Bonnenfant and Lawrence, 1984) to proteins (King, 1964; Martin et al., 1998, 1999; Miyawaki and Taketomi, 1978; Ohira et al., 2003; Okuno et al., 1999; Taketomi, 1986) – and their modes of action are still to be elucidated. We have demonstrated the effects of the AG on behavior in *C. quadricarinatus* crayfish by manipulating the AG at the juvenile stage and testing for effects on behavior at the sexually mature stage. The male-like behavior in AG-implanted females (Barki et al., 2003) coincided with the development of various male primary and secondary characteristics, and the female-like behavior in AG-ablated intersex animals was accompanied by a lack of these characteristics. Thus, it is possible that the AG manipulation at the early stage induced organizational alterations in neural circuits in sensory,

central or motor pathways controlling the generation of sexually dimorphic behaviors. Nevertheless, we cannot rule out the possibility that the presence of androgens from the AG is also necessary for the activation of male behavior patterns in the adult. Gleeson et al. (1987), for example, demonstrated the co-occurrence of spontaneous courtship displays and hypertrophy of the AGs within a few days following eyestalk ablation in adult male crabs (*Callinectes sapidus*). Further research involving manipulations of the AG in the adult crayfish is needed to clarify the role of the AG in the activation of male behavior in crustaceans. Furthermore, bioactive materials from the AG could be tested using a behavioral assay, as suggested by the present study, to elucidate their direct effect on adult behavior rather than their indirect effect through early differentiation processes.

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