

## On intersexuality in the crayfish *Cherax quadricarinatus*: an inducible sexual plasticity model

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Received 30 October 2001; Accepted 2 April 2002

### Summary

Sexual differentiation is a plastic process. The plasticity may be manifested during embryogenesis, when one set of primordial reproductive ducts develops while the other degenerates. In adults, many normal (e.g., sequential hermaphroditism) and abnormal (e.g., endocrine disorders or exposure to endocrine disruptors such as estrogenic pollutants) cases are known in which sexual plasticity may be expressed as various degrees of feminization. In crustaceans, the androgenic gland (AG) regulates the development of male characteristics; its absence results in feminization, often including the onset of vitellogenesis. A unique model of intersexuality was found in the crayfish *Cherax quadricarinatus*, in which some degree of natural sexual plasticity is observed. Two to 14% of the population are intersex individuals, having both male and female genital openings. Intersex specimens always function as males but may also contain an ovary in a permanently arrested, pre-vitellogenic state. This sexual plasticity model was recently characterized and investigated with respect to the role of the AG and the onset of vitellogenesis. Removal of the AG in intersex individuals induced the reproductive system to shift from a permanently active male state to a female state. This shift included changes in morphology, cessation of spermatogenesis and onset of secondary vitellogenesis manifested by a change in the ovarian protein profile, translocation of protein kinase C (PKC) in the ovary and appearance of secondary vitellogenic high-density lipoprotein (HDL) in the hemolymph. The vitellogenin gene was found to be induced in the hepatopancreas of AG ablated intersex individuals suggesting that the AG represses transcription of this gene in intact intersex individuals. The experimentally inducible sex shift in the crayfish provides a unique and controlled model system for the study of sexual differentiation and plasticity at the physiological and molecular levels. The findings presented here also illustrate the central role of the AG in the regulation of sexual differentiation in sexually plastic as well as gonochoristic crustacean species.

**Key words:** Intersexuality, sexual plasticity, androgenic gland, vitellogenesis, Crustacea, Decapoda, crayfish, *Cherax quadricarinatus*

### Introduction

Intersexuality — a phenomenon in which an individual of a bisexual species has characteristics

intermediate between those of a female and a male — among crustaceans has mainly been documented as transitional forms in species that exhibit sequential

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hermaphroditism such as protandry (change of sex from male to female) or protogyny (change of sex from female to male). Protandry is predominant among hermaphrodite crustaceans, and within the Malacostraca it has been reported in nine families of Decapoda (Brooket *al.*, 1994). Cases of simultaneous hermaphroditism, in which female-phase individuals capable of mating and reproducing as either sex during the same reproductive period, have been reported (Bauer and Holt, 1998). Male and female gonopores are present in the same individual in such cases of true simultaneous hermaphroditism. However, cases of non-functional hermaphroditism in males of gonochoristic species were also described in malacostracans (Charniaux-Cotton and Payen, 1985).

Two types of hermaphroditism in which an individual possesses both male and female openings were described among decapod crustaceans. In one type, typically termed “gynandromorphism”, the individual exhibits a complete bilateral separation (e.g., *Nephrops norvegicus*); one half has male internal and external characteristics and the contralateral half only female characteristics (Farmer, 1972; Johnson and Otto, 1981; Chace and Moore, 1983). In the other type, observed in several species of Australian parastacides, intersex individuals possess both male and female openings, but all the other external characteristics are permanently masculine (Lake and Sokol, 1986; Sokol, 1988; Brummett and Alon, 1994). Intersex individuals may represent transitional forms of true hermaphrodites, in which the AG disappears to permit the expression of the female phase in protandric species (Charniaux-Cotton, 1958).

In this article we present a retrospect of the intersexuality phenomenon in the crayfish *Cherax quadricarinatus*, focusing on the central role of the AG in regulating the balance between maleness and femaleness in the intersex model. Experimentation using this model system contributes to our further understanding of sexual plasticity and vitellogenesis in crustaceans, and might be instrumental in efforts to identify the androgenic hormone of decapod crustaceans and to investigate its mode of action.

#### Intersexuality in *C. quadricarinatus*

The Australian red claw crayfish, *C. quadricarinatus* (von Martens), is a large, tropical freshwater crustacean that grows and reproduces successfully in temperate climates, attaining sexual maturity within 7 to 9 months (Rouse et al., 1991). It is a gonochoristic species with a bilaterally symmetrical reproductive

system. In males, this consists of a pair of testes, sperm ducts, AGs and genital openings at the base of the fifth walking legs. Females have a pair of ovaries, oviducts and genital openings at the base of the third walking legs. Occasionally, intersex individuals with both male and female genital openings have been recorded (Thorn and Fielder, 1991). In cultured populations of *C. quadricarinatus*, various types of intersex individuals have been described, based on the observation of both male and female openings in the same individual (Medley and Rouse, 1993; Brummett and Alon, 1994). Seven different combinations of genital opening placements were found in the intersex crayfish (Sagi et al., 1996). Anatomically, all intersex individuals which possess a male opening also possess a testis and a sperm duct on that side. An AG is attached to the sub-terminal region of each sperm duct. However, not all visible female openings in intersex individuals indicate the presence of a female reproductive system. Individuals in which male and female openings are present on one side possess no ovary on that side. An ovary and an oviduct are found only in intersex individuals in which a female opening is present in the absence of a male opening on the same side (Sagi et al., 1996). The latter intersex individuals have male secondary sexual characteristics, including the red cuticular patch (a unique *C. quadricarinatus* male secondary sexual characteristic) on the propodus, and function as males. Their ovarian component is permanently arrested in a primary vitellogenic state, and none exhibits secondary female sexual characteristics such as ovigerous simple setae on the pleopods. Thus, in *C. quadricarinatus* intersexuality seems to represent a case of nonfunctional hermaphroditism (Sagi et al., 1996), in contrast to species such as the burrowing crayfish *Parastacus nicoleti* in which intersexuality is a manifestation of functional hermaphroditism (Rudolph, 1995).

#### The Role of the AG in Sex Differentiation and Sexual Plasticity

The AG plays an important role in the regulation of sex differentiation, including gonad development and function, in many malacostracan crustaceans (Charniaux-Cotton and Payen, 1988). Masculine differentiation of the genital apparatus and secondary sexual characteristics are under control of a hormone synthesized by the AG, which is separated from the gonads and connected to the distal portion of the sperm duct (Charniaux-Cotton and Payen, 1988). The AG also regulates spermatogenic activity in the testes

(Payen, 1973; Taketomi and Nishikawa, 1996) and inhibits ovarian maturation (Meusy and Payen, 1988; Taketomi et al., 1996). Ablation of the AG (andrectomy) results in female differentiation in isopods, amphipods and decapods (Charniaux-Cotton, 1964; Nagamine et al., 1980; Sagi et al., 1990).

In protandric hermaphrodites, the AG seems to be the driving force behind the male to female sexual shift. Degeneration of the AG allows the ovary and secondary sexual characteristics of the female to develop. Delayed appearance of the AG in protogenic hermaphrodites allows primary female differentiation (Charniaux-Cotton, 1975).

### Role of the AG in Controlling Balance between Maleness and Femaleness in Intersex *C. quadricarinatus*

#### Morphological shift

Removal of the AG caused an apparent morphological sex shift in intersex *C. quadricarinatus*. Several molts after AG ablation in intersex individuals, simple oosetae (a typical female secondary sexual characteristic) developed on the endopod edges. The ratio between the endopod and exopod widths became

feminine, replacing the masculine pleopod proportions of the intact intersex individuals (Sagi et al., 1996; Khalaila et al., 1999). The most typical male sex characteristic in this species, the red patch on the propodus, did not grow and even showed signs of degeneration several molts after AG ablation in intersex individuals. This was best seen if one claw was autotomized following the andrectomy. In such cases the red patch was not apparent on the regenerated propodus (Fig. 1 right) while it was clearly present in sham-operated control (Fig. 1 left). The degeneration of male secondary sexual characteristics and development of female secondary sexual characteristics following AG ablation in intersex individuals resembles the sex shift which occurs in true protandric hermaphrodites in which the AG degenerates spontaneously and the individual becomes a female with secondary sexual characteristics.

#### Anatomical and histological shift

Observations showed that AG ablation in intersex *C. quadricarinatus* individuals caused a decrease in the abundance of lobules containing primary and secondary spermatocytes in the testes (Khalaila et al., 1999). The majority of testicular lobules contained

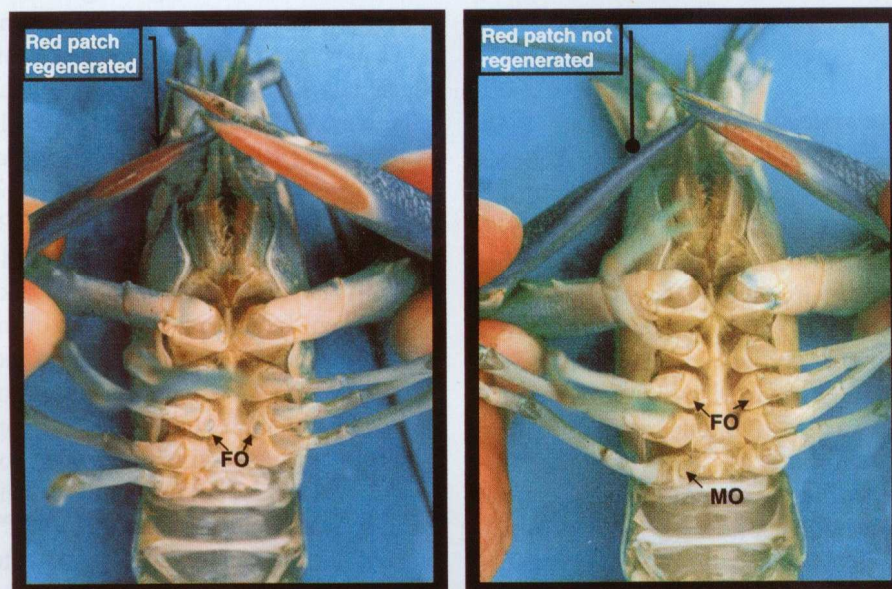


Fig. 1. Development of the red patch following autotomy and regeneration of the left claw in andrectomized and sham-operated intersex crayfish *C. quadricarinatus*. The red patch was regenerated in the sham-operated control crayfish (right) while the removal of the AG prevented the regeneration of the red patch in the andrectomized individual (left). The surgical manipulation was done with a diathermy surgical apparatus. Both intersex crayfish had two female (FO) and one male (MO) openings and were between 6–7 g at the time of AG removal. In the control intersex individual (left) the left fifth walking leg did not regenerate following the sham operation and thus is missing and so is the male opening at the base of this leg. The regenerated red patch on its propodus was apparent approximately 5 months after autotomy.

spermatids. This could be due to the lack of new cycles of spermatogenesis, i.e., an arrest of mitotic activity caused by the removal of the AG. It seems that the presence of the AG does not affect the conversion of primary spermatocytes to secondary spermatocytes and spermatids, but rather the rate of sperm release from the testis into the sperm duct. These results are in keeping with previous reports that the AG modulates spermatogenesis in the amphipod *Orchestia gammarella* and in the American crayfish *Cambarus bartonii bartonii* (Charniaux-Cotton, 1960; Puckett, 1964). Recent circumstantial evidence showing that spermatogenic activity is related to the development of the AG throughout the juvenile development in male *Procambarus clarkii* (Taketomi et al., 1996), supports the results on spermatogenesis found in AG-ablated intersex *C. quadricarinatus* (Khalaila et al., 1999). The latter show that the male component of the intersex reproductive system is arrested following AG ablation and the sexual physiology of the individual is feminized, including initiation of secondary vitellogenesis in the female component. The gonadosomatic index (GSI) and diameter of the oocytes increased and accumulation of yolk globules was observed histologically. These results are in keeping with the report of Charniaux-Cotton and Payen (1985) that in protandric hermaphrodites, the AG inhibits secondary, but not primary, vitellogenesis in the oocytes.

#### **Physiological and molecular observations**

In crustaceans, secondary vitellogenesis is accompanied by the accumulation of yolk (Charniaux-Cotton and Payen, 1988), which is composed of lipids, carbohydrates and proteins (Adiyodi and Subramoniam, 1983), the main protein being a HDL known as vitellin (Adiyodi and Subramoniam, 1983; Meusy and Payen, 1988). Three prominent polypeptides, 177, 150 and 106 (designated P<sup>106</sup>) kDa, which are predominant in ovaries of vitellogenic *C. quadricarinatus* females (Abdu et al., 2000), were found in the polypeptide profile of the AG-ablated intersex ovary. These three polypeptides are most probably major yolk polypeptides since they are less prominent in the ovaries of both immature females and of sham-operated intersex individuals (Khalaila et al., 1999). Moreover, P<sup>106</sup>, a negatively charged polypeptide, which is specific to secondary vitellogenesis (Sagi et al., 1999; Abdu et al., 2001), was present in the ovary of andrectomized intersex individuals following the sexual shift. Yet, this polypeptide was totally undetectable in immature and sham-operated-intersex

ovaries: thus, its presence in the ovaries of andrectomized intersex individuals was used as a marker for entry into secondary vitellogenesis. This is consistent with the results of recent studies reporting that implantation of AG in immature females of the crayfish *P. clarkii* and *C. quadricarinatus* inhibited vitellogenesis (Taketomi and Nishikawa, 1996; Khalaila et al., 2001).

During ovarian maturation in the crayfish *C. quadricarinatus*, changes in ovarian PKC isoenzymes take place in parallel with yolk accumulation (Soroka et al., 2000). Significant changes were recorded in the amounts of specific isoenzymes and in their distribution between the cytosol and the membranes. Among the isoenzymes tested, PKC $\alpha$  was the most clearly activated during ovarian maturation, as shown by significant translocation from the cytosol to the particulate fraction and the appearance of high-molecular-weight species. Moreover, a similar picture was obtained following a sex shift in the ovaries of intersex individuals upon induction of secondary vitellogenesis by AG ablation. In the induced intersex, PKC $\alpha$  activation took place in the ovary in parallel to the sex shift and the onset of the secondary-vitellogenic process (Soroka et al., 2000).

The site of synthesis of vitellogenic-specific lipoproteins is a crucial issue in the role of the AG in sexual shift and the target organs of its hormones in intersex individuals. Both ovarian and extra-ovarian sites have been proposed for yolk protein synthesis in decapods (Lui and O'Connor, 1976; Eastman-Reks and Fingerman, 1985; Paulus and Laufer, 1987; Tom et al., 1987; Fainzilber et al., 1992). Females undergoing vitellogenesis have a specific hemolymphatic lipoprotein designated LP II (Lee and Puppione, 1988; Komatsu et al., 1993). LP II was found by HPLC in the hemolymph of andrectomized intersex individuals and was absent from the hemolymph of intact individuals (Yehezkel et al., 2000). Based on the above, a non-invasive ELISA test was developed to detect changes in the hemolymph of the intersex in order to elucidate the role of the AG in regulating the expression of sex specific polypeptides during sexual shift. This ELISA test showed high levels of anti-P<sup>106</sup> cross-reactive material in the hemolymph of andrectomized intersex individuals (Sagi et al., 1999). The appearance of cross reactive material in the hemolymph of AG-ablated intersex individuals is consistent with results described before (Sagi et al., 1999) in which AG-implanted females showed a low or undetectable level of P<sup>106</sup> cross reactive materials in their hemolymph.

Based on some P<sup>106</sup> peptide fragment amino acid sequence, the full length of the *C. quadricarinatus*

vitellogenin cDNA was cloned and sequenced (Abdu, 2000). The vitellogenin gene, which encodes for P<sup>106</sup> among other yolk protein subunits, was found to be expressed only in the hepatopancreas of secondary vitellogenic females. Northern blot analysis showed that the vitellogenin gene was also expressed in the hepatopancreas of intersex individuals only after AG ablation and following the sex shift (Abdu, 2000). This is best illustrated by RT-PCR reactions using RNA from ovaries, testes, hepatopancreas and muscle of different *C. quadricarinatus* animals as template (Fig. 2). The expression of the vitellogenin gene was detected in the hepatopancreas of the AG ablated intersex individuals, similar to the secondary vitellogenic female. When the sex shift was not induced in the intact intersex, the vitellogenin gene was not expressed, similar to the negative control male (Fig. 2A). The above suggests that AG-borne hormones suppress, either directly or indirectly, the expression of the vitellogenin gene in the hepatopancreas of intersex *C. quadricarinatus*.

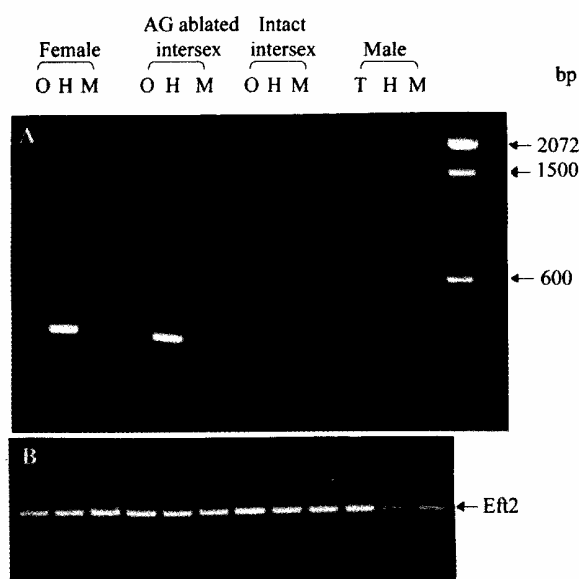


Fig. 2. Expression of the vitellogenin gene was induced in intersex individuals of the crayfish *C. quadricarinatus* following AG ablation and sex shift. The agarose gels show RT-PCR products demonstrating the expression of: (A) *C. quadricarinatus* vitellogenin gene (Abdu, 2000; GenBank accession number AF306784), and (B) a housekeeping elongation factor Eft2 (GenBank accession number A1253924). Template RNA was extracted from ovary (O), hepatopancreas (H) and abdominal muscle (M). A 100 bp DNA ladder marker (GIBCO-BRL) was used.

## Conclusions

The unique and novel model of sexual plasticity described in this article could serve as an instrumental tool for the study of several physiological processes related to important issues such as sex shift, onset of secondary vitellogenesis and the search for androgenic factors in crustaceans. The expression of the vitellogenin gene as part of a unique sexual plastic system that may be manipulated will enable, for the first time in decapod crustaceans, the study of the regulation of sexual processes at the molecular level.

## Acknowledgments

The study was supported in part by grants from the DFG (Ke 206/17-1) and the Israeli Ministry of Agriculture (857-0403-00). Recent molecular studies were supported by a grant from the BSF (200116).

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