

## Intersex Red Claw Crayfish, *Cherax quadricarinatus* (von Martens): Functional Males with Pre-vitellogenic Ovaries

AMIR SAGI<sup>1</sup>\* ISAM KHALAILA<sup>1</sup>, ASSAF BARKI<sup>2</sup>,  
GIDEON HULATA<sup>2</sup>, AND ILAN KARPLUS<sup>2</sup>

<sup>1</sup>Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel; and <sup>2</sup>Department of Aquaculture, Institute of Animal Science, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet-Dagan 50250, Israel

**Abstract.** Intersex individuals, possessing both male and female genital openings, were assessed in two groups—7 and 19 months old—of Australian red claw crayfish (*Cherax quadricarinatus*). All intersex individuals investigated were functional males, as suggested by their male-like morphology and the presence of testes, sperm ducts, androgenic glands, and viable spermatozoa. When an ovary was present in an intersex individual from either group, the gonadosomatic index, the diameter of the oocytes, and the ovarian cytosolic polypeptide profile were similar to those of immature, pre-vitellogenic females. We conclude that intersexuality in *C. quadricarinatus* does not indicate a case of protandric sequential hermaphroditism, as previously suggested. The case of intersexuality described here presents a unique model for the study of the role of the androgenic gland in the regulation of sex differentiation in crustaceans.

### Introduction

Intersexuality among crustaceans has been documented mainly in species that exhibit either 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 (Brook *et al.*, 1994). Two types of cases in which an individual possesses both male and female openings were described among decapod crustaceans. In one type (*e.g.*, *Nephrops*

*norvegicus*), the individual exhibits a complete bilateral separation: half of the body has male internal and external characters, and the contralateral half has only female characters (Farmer, 1972; Johnson and Otto, 1981; Chace and Moore, 1983). In other cases, including several species of Australian parastacides, the intersex individual possesses both male and female openings, but all the other external characters are male-like (Lake and Sokol, 1986; Sokol, 1988; Brummett and Alon, 1994). Intersex individuals may represent cases of true hermaphroditism, in which the androgenic gland disappears to permit the expression of the feminine phase in protandric species (Charniaux-Cotton, 1958). However, some cases of non-functional hermaphroditism in males of gonochoristic species were also described in malacostracans (Charniaux-Cotton and Payen, 1985).

The Australian red claw crayfish, *Cherax 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, androgenic glands, 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 (Thorne 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). However, neither the sexual physiology

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\* Author to whom all correspondence should be addressed.

*Abbreviations:* GSI = gonadosomatic index; EW1 = endopod width index; AG = androgenic gland.

**Table I**

*Frequency and morphometric description of sexual types in a 7-month-old population of Cherax quadricarinatus*

Type	Total no. harvested	Sample size (n)	Body weight (g)	Carapace length (mm)	Propodus length (mm)	Red patch length (mm)
Male	531	50	53.1 ± 23.5 <sup>a</sup>	42.1 ± 6.0	48.1 ± 5.4 <sup>a</sup>	20.2 ± 19.9 (n = 46)
Female	514	50	43.3 ± 13.0 <sup>b</sup>	40.2 ± 4.2	42.1 ± 10.3 <sup>b</sup>	—
Intersex	13	13	41.9 ± 16.0 <sup>b</sup>	39.0 ± 5.3	43.2 ± 9.0 <sup>b</sup>	17.5 ± 7.5 (n = 12)

Values are means ± SD. Values sharing the same superscript letters are not significantly different.

of intersex crayfish nor the possibility of sequential hermaphroditism has been fully addressed.

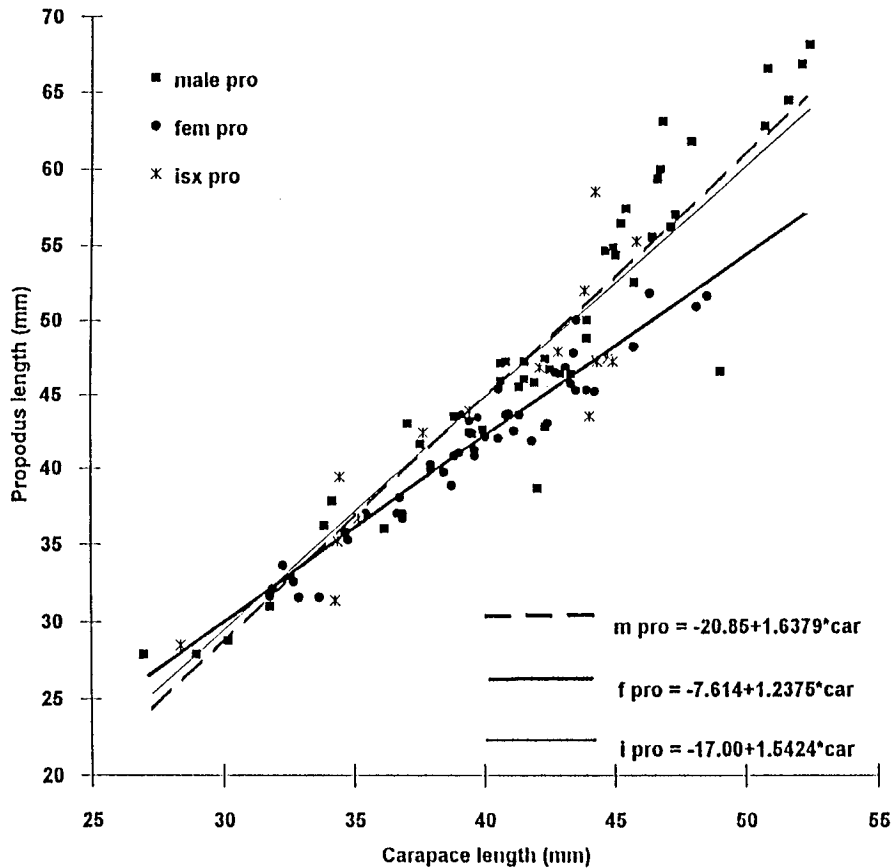
To shed more light (from a different angle) on the development of maleness and femaleness in decapod crustaceans, we examined intersex individuals from a cultured population of *C. quadricarinatus* to determine the status of their reproductive system and the presence of androgenic glands. Morphological, anatomical, and biochemical characteristics were compared among male, female, and intersex crayfish. For the study of possible sequential sex

changes, some intersex individuals were grown in the laboratory to 19 months of age.

**Materials and Methods**

*Animals*

Juvenile crayfish (*C. quadricarinatus*) were hatched at the Department of Aquaculture, Agricultural Research Organization, Bet-Dagan, Israel. At an average age of 1 month and a weight of 0.02–0.2 g, the juveniles were



**Figure 1.** Regression line for propodus length vs. carapace length in 7-month-old intersex, male, and female *Cherax quadricarinatus*.

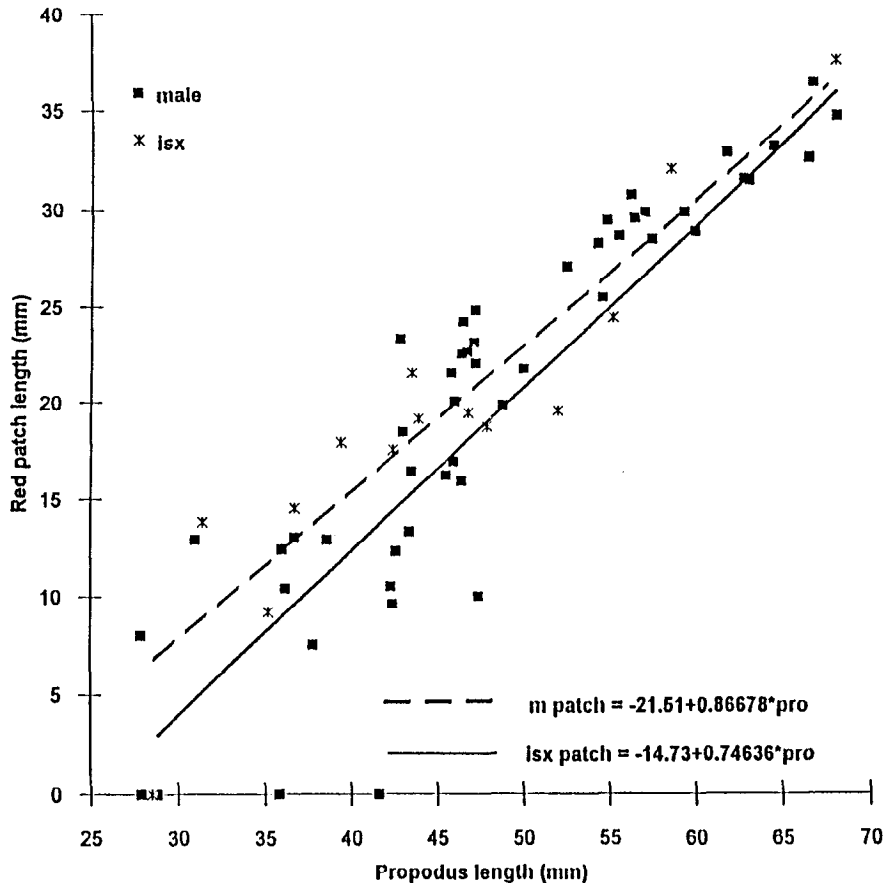


Figure 2. Regression line for red patch length vs. propodus length in 7-month-old intersex and male *Cherax quadricarinatus*.

moved to 400-m<sup>2</sup>, 1-m-deep earthen ponds at the Dor Fish Culture Research Station. The crayfish were harvested after 6 months: 1058 individuals were examined externally, and their sex was determined according to the presence or absence of male or female genital openings. Individuals that had both male and female openings (total of 13) were classified as intersex. Six intersex individuals, harvested from a different population grown under similar

conditions, were kept in aquaria at the Ben-Gurion University laboratory for an additional 12 months. These crayfish were fed daily with fresh ground fish flesh and vegetables, temperature in the aquaria was 27° ± 2°C, and water quality was maintained by recirculating the water through a gravel biofilter. At the age of 19 months, these animals were examined as described below.

#### Morphological observations

The following morphological variables were recorded at the time of harvest (age 7 months) for each intersex individual ( $n = 13$ ), for random control samples of females and males ( $n = 50$  of each sex) from the same population, and for the 19-month-old individuals ( $n = 6$ ): body weight ( $\pm 0.1$  g), carapace length, propodus length ( $\pm 0.1$  mm). The presence and length ( $\pm 0.1$  mm) of a red cuticular patch along the propodus was recorded. This patch is a typical male secondary character (Thorne and Fielder, 1991) that elongates with maturation.

Along with the process of maturation in *C. quadricarinatus* females, changes in the morphology of the pleopod occur that facilitate its role in holding the newly deposited

Table II

Morphological properties of *Cherax quadricarinatus* intersex individuals compared to males and immature and mature females

	Sample size (n)	Red patch	EWI	Setation
Intersex	16	+	1.13 ± 0.07 <sup>a</sup>	Plumose
Male	6	+	1.17 ± 0.08 <sup>a</sup>	Plumose
Immature Female	8	-	1.30 ± 0.13 <sup>b</sup>	Plumose
Mature Female	9	-	1.95 ± 0.23 <sup>c</sup>	Simple and Plumose

EWI = endopod width index ± SD. Values sharing the same superscript letters are not significantly different according to Sime's multiple test.

Table III

*Different combinations of male and female gonopores in Cherax quadricarinatus intersex individuals and the anatomy of their reproductive systems*

Male gonopores	Female gonopores	<i>n</i>	Testis	Sperm duct	Ovary	Oviduct	Androgenic gland
Right	Right & left	10	Right	Right	Left	Left	Right
Left	Right & left	4	Left	Left	Right	Right	Left
Right	Left	1	Right	Right	Left	Left	Right
Right & left	Right	2	Right & left	Right & left	None	None	Right & left
Right & left	Left	1	Right & left	Right & left	None	None	Right & left
Right & left	Right & left	1	Right & left	Right & left	None	?	Right & left

\* On one side a rudimentary duct connecting the testicular tissue with the female genital opening was observed, in addition to the sperm duct.

eggs (Khalaila, unpub. data). The endopod of mature (vitellogenic) females is longer and wider than the exopod, and a mixture of plumose setae and long, thin simple (ovigerous) setae is present on the endopod. Pleopods were examined on all intersex individuals and on an additional sample of males (6), immature (pre-vitellogenic) females (8), and mature females (5) aged over 1 year. One of the third pair of pleopods was removed from each individual, and endopod and exopod width was measured with a pair of calipers to the nearest 0.1 mm. The relative growth of the two components of the pleopod, representing a female sex characteristic, was expressed as the endopod width index

$$(\text{EWI} = \text{Endo. Width}/\text{Exo. Width}).$$

The appearance of the setae (either plumose or a mixture of plumose and simple setae) attached to the endopod was studied under a light microscope.

#### *Anatomical observations*

To study the reproductive system, a sample of 11 (out of 19) intersex individuals of both age groups, 8 pre-vitellogenic females, 5 vitellogenic females (according to the pleopod features described above), and 6 mature males were dissected on ice. Their gonads, sperm ducts or oviducts, and androgenic glands were identified. The ovaries were removed and individually weighed after removal of excess water. The weights were used in calculating the gonadosomatic index

$$(\text{GSI} = (\text{Ovary Weight}/\text{Body Weight}) \times 100).$$

A random sample of at least 15 fresh oocytes per ovary was placed under a light microscope and the oocyte diameter was measured with an objective micrometer ( $\pm 10 \mu\text{m}$ ). A sample of the contents removed from the subterminal region of the sperm duct was spread in saline on a glass slide and examined microscopically for the presence of mature spermatozoa.

#### *Polypeptide profiles*

Ovarian tissue was removed from 9 intersex individuals, 8 pre-vitellogenic females, and 5 vitellogenic females, ho-

mogenized individually on ice with 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2, and centrifuged at  $10,800 \times g$  for 15 min at 4°C. The amount of total protein in the supernatant was determined (Bradford, 1976). Samples (8  $\mu\text{g}$  protein per lane) were separated by SDS-PAGE on 7% acrylamide gel (Laemmli, 1970) and stained with Coomassie blue.

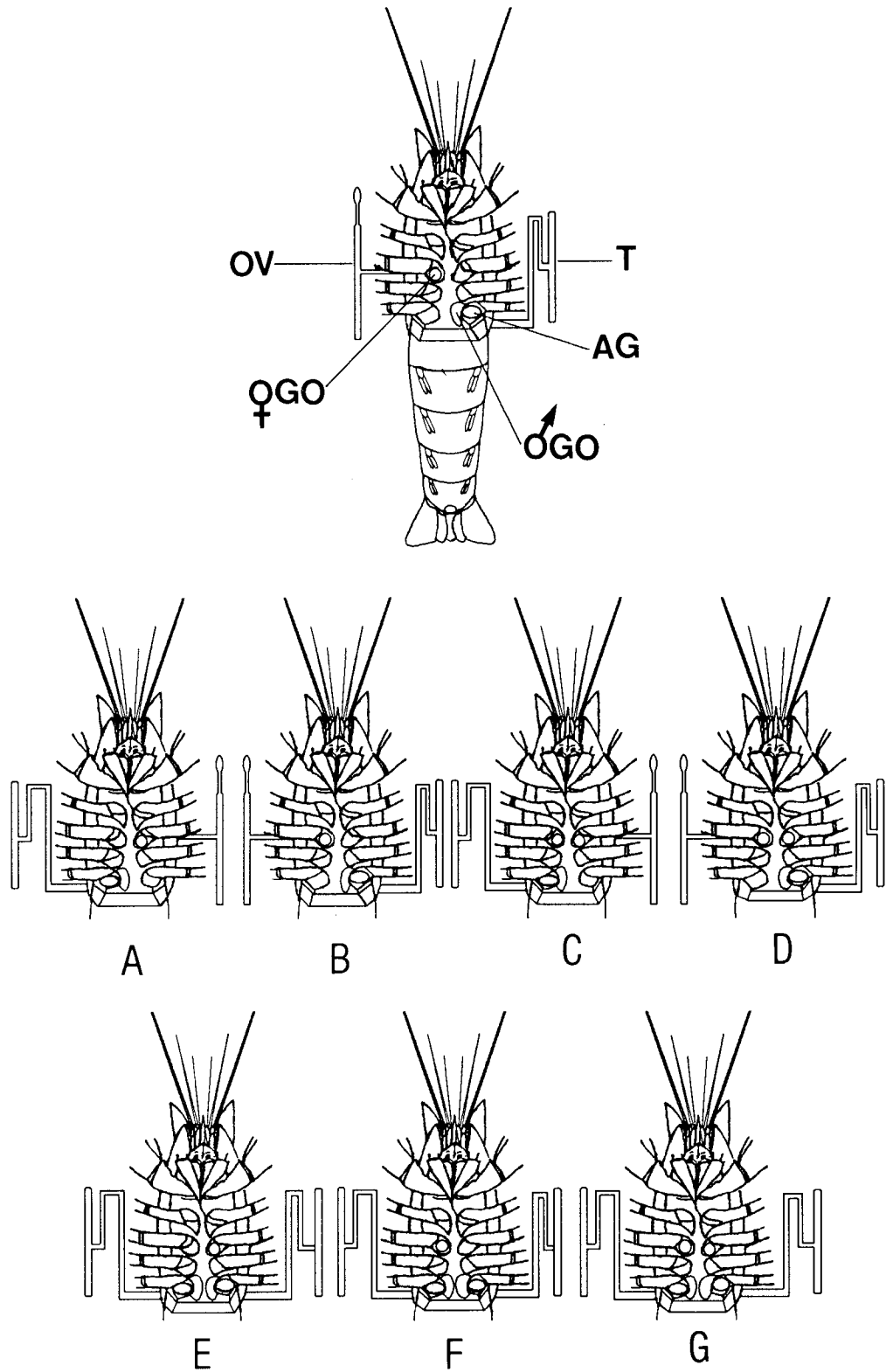
#### *Statistical analyses*

Data were analyzed using ANOVA, followed by Duncan's multiple range test, except for EWI and GSI. The latter are ratios, and their sample size was rather small, hence the Kruskal-Wallis nonparametric test was used for testing significance of difference among groups, followed by Simes's (1986) procedure for multiple tests. Probabilities below 0.05 and 0.01 were considered significant and highly significant, respectively.

## Results

#### *Morphology of intersex individuals*

The population investigated—1058 crayfish grown at the Fish Culture Research Station—included 1.2% intersex individuals. All but one of the 13 intersex individuals developed cuticular red patches, a male sexual characteristic (Thorne and Fielder, 1991), on the propodus. Male crayfish were significantly heavier and had a longer propodus than did females and intersex individuals (Table I). No significant differences between males and intersex individuals were found in the carapace length or in the length of the cuticular red patch. The latter was missing in four males and one 7-month-old intersex individual. A highly significant regression of propodus length vs. carapace length was found for all three sex types ( $r = 0.945, 0.967, \text{ and } 0.919$  for males, females, and intersexes, respectively). The slope of the regression line for males was significantly different from that for females (Fig. 1). The slope of the regression line for intersexes was not significantly different from that for males or females (Fig. 1). The regression of the cuticular red patch length vs. propodus length was signifi-



**Figure 3.** Different types of *Cherax quadricarinatus* intersex individuals. T = testis; OV = ovary; GO = genital opening; AG = androgenic gland.

**Table IV**

Properties of the gonads and their products in *Cherax quadricarinatus* intersex individuals compared to those of males, and pre-vitellogenic (P) and vitellogenic (V) females

	Sample size (n)	Ovarian index (GSI)	Oocyte diameter ( $\mu\text{m}$ )	Sperm
Intersex	4	$0.12 \pm 0.05^a$	$292 \pm 148^a$	Present
Male	6	—	—	Present
Female (P)	8	$0.42 \pm 0.25^a$	$287 \pm 87^a$	Absent
Female (V)	5	$2.15 \pm 0.28^b$	$1020 \pm 292^b$	Absent

Statistical comparisons among groups were done by Simes's multiple test (for GSI) and Duncan's multiple range test for oocyte diameter. Values marked with the same superscript letters are not significantly different. Oocyte diameter represents mean of 15 oocytes per ovary.

icant in both male and intersex crayfish ( $r = 0.904$  and  $0.888$ , respectively), but the slopes of the lines were not significantly different (Fig. 2).

In intersex crayfish, the endopod of each pleopod was nearly equal in size to the exopod ( $\text{EWI} = 1.13 \pm 0.07$ ); a similar endopod/exopod ratio was found in males ( $\text{EWI} = 1.17 \pm 0.08$ ). In females the endopods were longer and wider than the exopods, and the EWIs of immature ( $1.30 \pm 0.13$ ) and mature ( $1.95 \pm 0.23$ ) females were significantly different from each other and from those of males and intersex individuals (Table II). In intersex male and immature female crayfish, each endopod and exopod had two distinct rows of delicate plumose setae. A mixture of plumose setae and long, thin simple setae was present in the endopod of mature females. All intersex individuals, from both age groups, possessed morphological properties, such as EWI and setation, that resembled the characteristics of males (Table II).

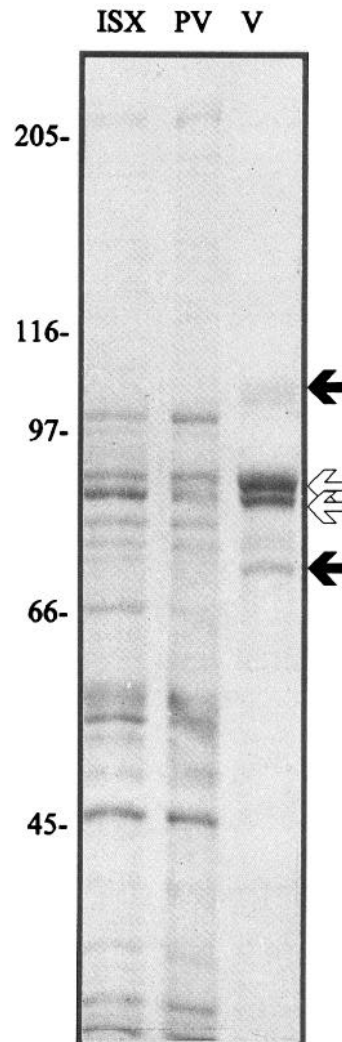
#### Male and female genital openings and the reproductive system of the intersex individuals

Seven combinations of genital-opening placement were found in the intersex crayfish (Table III). Anatomically, all dissected intersex individuals (from both age groups) that possessed a male opening also possessed a testis and a sperm duct on that side (Fig. 3). An androgenic gland was attached to the subterminal region of each sperm duct. Not all visible female openings indicated the presence of a female reproductive system, however. Individuals (from both age groups) in which a male and a female opening were present on one side possessed no ovary on that side (Fig. 3, C through G). In one case a rudimentary duct connecting the testicular tissue with a female genital opening was observed, in addition to the sperm duct. The identification of this duct was difficult because it was very thin and inconspicuous. An ovary with an oviduct was found only in cases in which a female opening was present

in the absence of a male opening on the same side (Fig. 3, A through D).

#### Gonads and their products

The GSI of the ovarian component of intersex individuals ( $\times 2$ , since they possess only one ovarian lobe) ( $0.12 \pm 0.05$ ) was not statistically different from that of pre-vitellogenic females ( $0.42 \pm 0.25$ ) and was significantly smaller than that of vitellogenic females ( $2.15 \pm 0.28$ ) (Table IV). The diameter of the oocyte in the ovarian component of the intersex individuals ( $292 \pm 148 \mu\text{m}$ ) was similar to that of immature, pre-vitellogenic females ( $287 \pm 87 \mu\text{m}$ ) and significantly smaller than that of mature, vitellogenic females ( $1020 \pm 292 \mu\text{m}$ ,  $n = 4$  ovaries) (Table IV). As in normal males, in intersex individuals the sperm duct contained viable spermatozoa.



**Figure 4.** SDS-PAGE separation of polypeptides from a cytosolic extract of ovaries from a 19-month-old *Cherax quadricarinatus* intersex (ISX) compared to a pre-vitellogenic female (PV) and a vitellogenic female (V).  $\Leftrightarrow$  = Polypeptides present in all lanes.  $\blackleftarrow$  = Polypeptides present only in the vitellogenic ovary.

The polypeptide profile of the cytosolic component of the ovary of intersex individuals (from both age groups) was similar to that of pre-vitellogenic female ovary (Fig. 4: lanes 1 and 2, representing an intersex of 19 months and a pre-vitellogenic female, respectively). The Coomassie-blue-stained polypeptides with molecular weights of about 204, 199, 106, 85, 59, and 43 kDa were the most prominent polypeptides in both the intersex ovary and the female pre-vitellogenic ovary. These polypeptides were not noted in the vitellogenic ovary. The polypeptides with molecular weights of about 93 and 90 kDa were present and pronounced in all three types of ovary (lanes 1, 2, and 3, Fig. 4, white arrows). The most prominent polypeptides in the vitellogenic ovary had molecular weights of about 111 and 76 kDa (lane 3, Fig. 4, dark arrows). These polypeptides were present in the vitellogenic ovary and were detected neither in the pre-vitellogenic female ovary nor in the ovary of intersex individuals.

### Discussion

Intersexuality is the occurrence in a protandric, protogynic, or normally gonochoristic species of an individual with both male and female characteristics. These characteristics may be limited to the external morphology or they may extend to gonadal differentiation. The frequency of intersex individuals in cultured populations of *C. quadricarinatus* has been reported to range from 2%–4% (Brummett and Alon, 1994) and from 4% (Thorne and Fielder, 1991) up to 17% (Medley and Rouse, 1993). The 1.2% frequency of intersex individuals presented in this study is relatively low. This frequency was consistent for the same breeding population over several years (unpub. data): it was 1.3% in 1993 (Karplus *et al.*, 1995). Recently, based on external observations, Medley and Rouse (1993) described five types of intersex individuals in cultured populations of *C. quadricarinatus*, including one animal with a complete testis on one side and what appeared to be undeveloped ovarian tissue on the other side. Our results confirm these observations in that they show that intersex *C. quadricarinatus* individuals that have a male genital opening also have a testis, a sperm duct, and an androgenic gland on that side (Fig. 3). On the other hand, we observed an ovary only in cases in which a female genital opening was present in the absence of a male genital opening on the same side (Fig. 3, A through D).

Medley *et al.* (1994) observed a normal testis and a pre-vitellogenic ovary in a single *C. quadricarinatus* intersex individual, and described it on the basis of histological examinations as a case of “true hermaphroditism.” They reported one intersex individual that had a right-side male genital opening and two female genital openings and functioned as a male, siring a batch of offspring. Our results agree with the observation that intersex *C. quadricarinatus* (from both age groups) are functional males.

Indeed, all but one of the intersex individuals examined developed the red cuticular patch (a male secondary sexual characteristic), but none developed ovigerous simple setae (a female sexual characteristic). True hermaphroditism, in which both male and female reproductive systems are functional, either simultaneously or sequentially, was not observed in the present study. Clarification of the function of the female component of the reproductive system in intersex crayfish requires further investigation. The intersex phenomenon seems to be a stable state rather than a transient one since no changes occurred in intersex individuals monitored from 7 up to 19 months of age.

With respect to the role of the androgenic gland (AG) in regulating crustacean sex differentiation, Nagamine and Knight (1987a) stated that “knowing that the AG can masculinize genotypic females makes the presence of a bilateral gynandromorph a paradox since the AG at the male half should be capable of masculinizing the contralateral female half.” The authors suggest an explanation in which the feminized half of the bilateral gynandromorph consists of “AGH receptor-minus cells.” That explanation may apply to the presence of bilateral gynandromorphs such as those described in the Bay prawn *Nephrops norvegicus* (Farmer, 1972). In this species, an individual possessing both male and female genital openings was described as a rare case (1:40,000), presenting a complete bilateral asymmetry in which one side had male primary and secondary sex characters while in the other half only female characters were expressed. The expression of intersexuality in *C. quadricarinatus*, in which the secondary external characters were masculine on both sides (red patch, pleopod morphology, etc.), may call for a different explanation. It better conforms with the hypothesis that male differentiation in decapods is mediated by a substance that is secreted from the androgenic gland primordia and diffuses along the genital tract (Charniaux-Cotton and Payen, 1988). This may explain the presence of a male reproductive system and the absence of a female system on the same side, *i.e.*, the side on which the androgenic gland exerts its local effect through diffusion; on the other side, in the absence of an androgenic gland, differentiation of an ovary is permitted (Charniaux-Cotton, 1959).

Our findings showed that intersex individuals of both age groups possessed an androgenic gland and an active testis, but the ovarian component was always pre-vitellogenic. The latter observation was confirmed by the polypeptide profile of the intersex ovarian component; the profile was similar to that of an ovary from a pre-vitellogenic female and did not contain the specific polypeptides typical of the vitellogenic ovary (Fig. 4). These findings are in agreement with reports in the literature that oogenesis can proceed in the testes of hermaphrodites until the end of the primary vitellogenesis (Charniaux-Cotton, 1965; Charniaux-Cotton and Payen, 1985). In-

hibition of vitellogenesis by androgenic hormones has also been reported (Berreur-Bonnenfant and Lawrence, 1984). The authors of that study suggest that the androgenic gland exerts an effect on remote target organs, via the circulation, in addition to its effect, by local diffusion, on the differentiation process.

Mature *C. quadricarinatus* females possessed vitellogenic ovaries and ovigerous setae; intersex individuals and immature *C. quadricarinatus* females possessed pre-vitellogenic ovaries but did not have ovigerous setae. The development of ovigerous setae in crustaceans is regulated by an ovarian factor (Nagamine and Knight, 1987b). We suggest that this factor is not synthesized by the ovaries of intersex individuals and immature females. Suzuki and Yamasaki (1991) found that female secondary sex characteristics were induced by the presence of vitellogenic ovaries, whereas pre-vitellogenic ovaries had no effect. The absence of ovigerous setae in intersex individuals (possessing an androgenic gland) may be explained by the antagonistic effect of the androgenic hormone on the ovarian factor (Suzuki and Yamasaki, 1991).

In these respects, intersex *C. quadricarinatus* individuals present a unique model for the study of the various regulatory roles of androgenic hormones and ovarian factors in sex differentiation and gonadal function in decapod crustaceans.

#### Acknowledgments

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