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Sex reversal and growth performance in juvenile females of the freshwater crayfish *Cherax quadricarinatus* (Parastacidae): effect of increasing temperature and androgenic gland extract in the diet

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Abstract Monosex male culture of *C. quadricarinatus* is proposed for increasing yields because males reach a larger size at harvest than females. An experiment was done to evaluate the effects of androgenic gland extract and temperature on sex reversal and somatic growth in early juvenile females. Females were exposed to the following experimental conditions: C: control food and temperature maintained at 26 ± 1°C; HT (high temperature): control food and temperature maintained at 28.5 ± 1°C; VHT (very high temperature): control food and temperature maintained at 31 ± 1°C; AG: food with enriched with androgenic gland (1/10 dose for each juvenile per day) and temperature maintained at (26 ± 1)°C. The juveniles were weighed, sexed, and growth increment (GI) and growth rate (GR) were calculated twice a month. The HT and AG groups differed from the C group in GI and GR indicating a greater somatic growth. At the end of the experiment, the HT and AG groups had similar weight but only the HT group had enhanced oocyte diameter, with some vitellogenic oocytes compared to the C group. In the HT group, there was significant sex reversal, as indicated by development of male and intersex male characteristics in different individuals. Both temperature and AG diet have a high potential for culture of this species because of their enhancement of somatic growth; higher temperature increases the proportion of males, a desirable characteristic of cultured populations.

Keywords Androgenic gland · *Cherax quadricarinatus* · Red claw · Sex reversal · Temperature

Introduction

Cherax quadricarinatus, the "red claw" crayfish, is a large freshwater species native to north-west Queensland and the Northern Territory of Australia which is cultured

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commercially in many tropical and subtropical countries (Medley and Rouse 1993; Medley et al. 1994; Masser and Rouse 1997; Romero 1997; Lawrence and Jones 2002; Edgerton 2005). Yields, growth and feed efficiencies current production systems need to be improved (Rodgers et al. 2006; Pavasovic et al. 2007). In monosex culture, energy from reproduction is diverted to growth, resulting in larger size of cultured individuals. Monosex culture is a common practice in fish aquaculture, and many attempts have been made to apply this technology to crustacean aquaculture (see Aflalo et al. 2006 for revision).

In *C. quadricarinatus*, males grow faster and reach a larger size at harvest than females in communal pond cultures, as well as in separate cells (Curtis and Jones 1995; Manor et al. 2002, 2004; Rodgers et al. 2006). Monosex culture in crustaceans involves hand-sexing of juveniles to sort males from females (Sagi et al. 1986; Medley and Rouse 1993; Curtis and Jones 1995; Lawrence and Morrisy 1997; Rodgers et al. 2006). Recently, Aflalo et al. (2006) reported a large-scale microsurgical andrectomy assay in *Macrobrachium rosenbergii* for improving yields by manipulating the endocrine system, resulting in monosex progeny (Aflalo et al. 2006).

Another possible method for monosex culture is the manipulation of sex determination and/or sexual differentiation, but the mechanisms involved in crustacean sex determination/differentiation are not clearly understood. Genetic factors have been proposed, as well as environmental external factors, such as changes in the photoperiod duration and temperature, and factors related to the "social structure" of the population as modulators or even sex determinants in crustaceans (Ginsburger-Vogel and Charniaux-Cotton 1982; McCabe and Dunn 1997; Fielder 2002; Parnes et al. 2003; Calado and Dinis 2007, for revision). With respect to sexual differentiation, the androgenic gland is the only endocrine gland in crustaceans specifically related to male sexual function (Charniaux-Cotton and Payen 1988; Barki et al. 2003). The androgenic gland regulates the differentiation of the primary and secondary sexual characters, including reproductive behavior (Charniaux-Cotton and Payen 1988; Sagi 1988; Payen 1990; Sagi et al. 1997; Sagi and Khalaila 2001; Barki et al. 2003) and has a striking effect on both somatic growth and inhibition of vitellogenesis (Khalaila et al. 2001; Sagi et al. 2002; Barki et al. 2003; Manor et al. 2004). It is the exclusive source of the androgenic gland hormone (AGH) whose effects are considered similar to those of androgens in mammals (Sagi and Khalaila 2001; Manor et al. 2004). The biochemical nature of AGH is not clearly understood and is lipid or polypeptidic in nature (see Manor et al. 2004 for review). The recent discovery of an androgenic gland-specific gene expressed in males, structurally similar to the insulin/insulin-like growth factor, supports the hypothesis that insulin participates in sexual differentiation in crustaceans (Manor et al. 2007). Because of its determinant role in male differentiation, a biotechnological approach to induce monosex culture using AGE has been addressed (Cui et al. 2005; Sagi and Aflalo 2005).

Cherax quadricarinatus is a gonochoric species with a variable proportion of intersex individuals both in farms and in natural populations (Sagi et al. 1996, 2002; Khalaila et al. 1999; Vazquez and López Greco 2007a). This sexual plasticity allows us to propose that sexual reversion from females towards males or intersex indiduals, both with the higher growth characteristic of fully male individuals (Sagi et al. 1996), is a possible strategy for male monosex culture with increasing yields. Sexual reversion, with a variable degree of masculinization depending on species and age of animals manipulated, has already been achieved by androgenic gland injection or implantation in females of isopods, amphipods, and decapods (Hasegawa et al.1993; Suzuki and Yamasaki 1998; Khalaila et al. 2001; Barki et al. 2003; Manor et al. 2004).

Temperature is an important abiotic factor that modulates many aspects of *C. quadricarinatus* biology including growth and reproduction (King 1994; Jones 1995a, b, 1997; Yeh and Rouse 1995; Zhao et al. 2000; García-Guerrero et al. 2003; Karplus et al. 2003). A recent study on sexually undifferentiated juveniles shows that it is possible to manipulate the sexual differentiation of early juveniles towards a higher proportion of males by means of increasing temperature (Vazquez et al. 2004).

The objective of this study is to evaluate the effects of dietary AGE and high temperature on sex reversion and somatic growth in early juvenile females of *C. quadricarinatus*.in order to advance the goal of monosex culture in this species.

Materials and methods

Experimental design

Three ovigerous females (average wet body weight 52.10 ± 11.05 g) from the reproductive stock of our laboratory were obtained from a commercial crayfish producer, San Mateo Farm, Entre Ríos, Argentina. These individuals were maintained in individual glass aquaria of 60 × 40 × 30 cm^3 containing 20 l of dechlorinated tap water (pH 7.4, hardness: 80 mg l^{-1}, as $CaCO_3$ equivalents), under continuous aeration, temperature of 26–27°C and 14:10 light:darkness photoperiod. They were fed daily ad libitum on *Elodea sp* (a freshwater weed widely used as aquarium vegetation) and commercial Tetradiskus granules until they became independent from the mother (Levi et al. 1999). At stage juvenile III, they were separated from the mother. During nursery rearing, juveniles were observed weekly under stereoscopic microscope to determine sex, using the presence/absence of the genital openings at the basis of the third (females), fifth (males) or both (intersex) pair of walking legs. When the juveniles could be sexed, 160 early female juveniles were selected and randomly assigned to each of the following experimental groups:

- control (C): control food and temperature maintained at 26 ± 1°C;
- high temperature (HT): control food and temperature maintained at 28.5 ± 1°C;
- very high temperature (VHT): control food and temperature maintained at 31 ± 1°C;
- androgenic gland enriched food (AG): enriched food and temperature maintained at 26 ± 1°C.

The assay temperatures were selected from the range suggested by Zhao et al. (2000) for embryos and previously applied on stage III–IV juveniles of this species (Vazquez et al. 2004). The experimental period was 150 days and the experimental groups were run as duplicates (the total number of juveniles for each treatment was 40). Twice a month the juveniles were sexed, weighed (precision 0.01 mg) and the mortality was recorded. Each group was maintained in a 60 × 40 × 30 cm^3 glass aquarium filled with 20 l of dechlorinated tap water (as above) with continuous aeration. Little PVC tubes and onion bag mesh were used as shelter. The photoperiod 14:10 light:darkness was held constant throughout the experiment while temperature was maintained by ALTMAN water heaters (100 W, precision 1°C). In all cases, the total volume of water was renewed twice a week. The juveniles were daily fed with commercial Tetradiskus granules (approximate composition: min. crude protein 47.5%, min. crude fat 6.5%, max. crude fiber 2.0%, max. moisture 6.0%, min. phosphorus 1.5% and min. ascorbic acid 100 mg kg^{-1}) at 10% of their wet weight during the first 45 days, at 7% from 45 to 90 days and at 5% of their weight

from 90 up to 150 days. The AG group received twice a week, a food mixture based on the same commercial granules, enriched with androgenic gland extracts, and the rest of the days the normal food.

Isolation of androgenic gland for AG treatment

Sixty-four mature males (average wet body weight 82.54 ± 3.19 g) were purchased from San Mateo Farm, Entre Ríos, Argentina, to obtain both androgenic glands from the subterminal portions of the *vasa deferentia* (Khalaila et al. 1999, 2001). Androgenic gland extracts were prepared by homogenization in saline solution for crustaceans (Van Harreveld 1936). The extracts were kept in a freezer (−20°C) until needed. Every 2 weeks, the appropriate percentage of Tetradiskus granules was used to prepare the enriched food mix adjusted to the weight of the animals. Enriched pellets were prepared according to Zapata et al. (2003) with AGE-enriched food containing 1/10 of AGE for each juvenile per day. Previous assays with 1/25, 1/50, and 1/250 AGE per day had not shown any effect on inducing growth or sexual reversion (L.S. López Greco, unpublished data).

Morphological and histological examination

At the end of the assay, all animals were weighed (precision: 0.1 mg) and carapace length (CL) was measured (precision: 0.01 mm). After being cold-anaesthetized at −20°C for 15 min the carapace was removed and the gonads were removed, with observations recorded on their relative size, form and color. They were quickly dissected and fixed in Bouin's solution for 4 h at room temperature to estimate the mean oocyte diameter and to check the stage of the ovary according to the scale described by Abdu et al. (2000) and Vazquez et al. (2008). Gonads were then sequentially passed through 90% ethanol for 20 min, 96% ethanol for 20 min, 96% ethanol-buthylic alcohol (1:1 V/V) for 30 min, and buthylic alcohol for 30 min, and embedded in paraffin (López Greco et al. 1997). Sections, 5–6 μm thick, were stained with haematoxylin–eosin. At least three slides for each crayfish were inspected under light microscopy. Mean oocyte diameter (MOD) was measured by means of an 8× Zeiss microscopic ocular lens, calibrated against a Leitz Wetzlar plate with 10 μm spacing on a representative section of each ovary. All oocytes whose nuclei were visible were measured.

Growth rate (GR) was calculated from the equation, $GR = (W_t - W_i)/t$ where Wt, is the weight at time t (mg), Wi, is the initial weight (mg), and t is the time (days) (Manor et al. 2002). Growth increment (GI) was calculated from the equation: $((W_t - W_i)/Wi) \times 100$ according to Jones (1995b).

Statistical analysis

A one-way ANOVA (Zar 1999), followed by planned comparisons of each treatment against control, was done to test the null of hypothesis of no difference in final weight, CL, GI, GR, and MOD among treatments. GI and GR were calculated at specific time intervals. Tukey's test was used for posterior comparisons and Fisher exact test (Zar 1999) was used to compare the proportions of reverted animals. The AG group was only analyzed versus the C group. The results per treatment are presented as average ± SE according to Fotedar (2004). All analyses were conducted with STATISTICA (version 6.0) statistical package and the tests were carried out with a significance level of $P < 0.05$.

Results

Growth performance

After 150 days of experiment, the final mean weight of the HT group was significantly higher ($P < 0.05$) than the C and VHT groups. For the variables related to weight gain (GI and GR), the HT group differed ($P < 0.05$) from the C and VHT groups indicating a greater somatic growth of the crayfish exposed to high temperature treatment. The VHT group showed similar values to the C group in the variables related to growth performance, showing no net effect on somatic growth (Table 1). The AG group also differs from the C group in every variable related to growth that indicates a positive effect of AGE administration on somatic growth (Table 1).

Sex reversal

In all experimental groups, including the C group, the sexual reversion from female to intersex female (Vazquez and López Greco 2007a) was evident. Although this was the dominant type of reversion in all the treatments, only the HT, VHT and AG groups induced male or intersex male (Sagi et al. 1996) reversions with normal male gonads including the presence of spermatozoa in testes and normal *appendices masculinae* (Fig. 1a, b). When comparing the proportion of functional males (males + intersex males) the HT group had a higher proportion ($P < 0.05$) with respect to the C group (Table 2). Although at the end of the experiment the final weights of the HT and AG groups were similar (Table 1) the oocyte diameter was only significantly greater for the HT group (Table 2, $P < 0.05$), including the presence of oocytes in secondary vitellogenesis, while in the AG group the oocyte diameter was similar to the C group and no secondary oocytes were observed ($P > 0.05$, Fig. 1c, d). The sexual reversion of juvenile females of *C. quadricarinatus* throughout the experimental period showed that the reversion began very early (between 15 and 30 days) mainly in the HT and VHT group treatments.

Discussion

The present study demonstrates the possibility of achieving sexual reversion of early juvenile females of *C. quadricarinatus* towards males or intersex males; higher growth rates resulted from higher culture temperatures. The modulating effect of temperature on growth and reproduction has been previously reported in this species (Jones 1995a, b; Yeh and Rouse 1995; García-Guerrero et al. 2003) including its effect on early juveniles (King 1994; Jones 1997). Besides the known effect of temperature on early juvenile growth, no effects on juvenile sexual reversion have been reported until now.

The effects of higher temperature on early sexual differentiation, leading to a functional sexual reversion, is in accordance with previous results obtained in our laboratory when exposing sexually undifferentiated juveniles of *C. quadricarinatus* (stage III–IV, initial weight: 0.02 g) to greater temperatures (Vazquez et al. 2004). This thermolability in the phenotypic expression of sex has been previously reported in the sockeye salmon *Oncorhynchus nerka* showing the presence of a "temperature-sensitive window" in sex differentiation that can be manipulated to the desired sex (see Azuma et al. 2004 for review). It is not known how temperature manipulation is responsible for the shift from females to males in crustaceans. In fishes, the conversion of cortisol to androgens has been

Table 1 Growth performance in juveniles of *Cherax quadricarintaus* after 150 days for the experimental groups: *C* (control), *HT* (high temperature), *VHT* (very high temperature) and *AG* (androgenic gland)

Experimental group	*n* initial	IW (g)	*n* final	FW (g)	$GI_{(t0–t150)}$	$GR_{(t0–t150)}$ (mg day^{-1})	W60 (mg)	$GI_{(t60–t150)}$	$GR_{(t60–t150)}$ (mg day^{-1})	Final CL (mm)
C	40	0.18 ± 0.01	26	3.49 ± 0.42[a]	2,190.81 ± 161.29[a]	22.03 ± 3.14[a]	1.31 ± 0.14	180.40 ± 14.02[a]	25.25 ± 4.33[a]	24.72 ± 0.76[a]
HT	40	0.18 ± 0.01	19	4.61 ± 0.48[b]	3,292.41 ± 227.65[b]	30.56 ± 4.62[b]	1.37 ± 0.14	357.49 ± 16.96[b]	41.37 ± 6.98[b]	27.67 ± 1.10[b]
VHT	40	0.18 ± 0.01	30	3.20 ± 0.39[a]	1,848.94 ± 181.17[a]	20.35 ± 2,28[a]	1.21 ± 0.12	223.19 ± 13.50[a]	24.69 ± 3.08[a]	24.45 ± 0.86[a]
AG	40	0.19 ± 0.01	21	4.55 ± 0.43[b]	2,802.60 ± 179.46[b]	29.34 ± 3.08[b]	1.34 ± 0.14	324.59 ± 15.60[b]	39.34 ± 4.22[b]	26.99 ± 1.00[b]

$GI_{(t0–t150)}$ Growth increment (%) related to day 0, $GI_{(t60–t150)}$ growth increment (%) between day 60 and day 150 of experiment, $GR_{(t0–t150)}$ growth rate (mg day^{-1}) related to day 0, $GR_{(t60–t150)}$ growth rate (mg day^{-1}) between day 60 and day 150 of experiment, *PW* pleon weight (%), *CL* carapace length (mm), *IW* initial weight (g), *FW* final weight (g), W60 weight at day 60 (g)

Values in the same column having different superscripts are significantly different ($P < 0.05$) with respect to the C group. The AG group was only analyzed versus the C group. The results per treatment are expressed as average ± SE

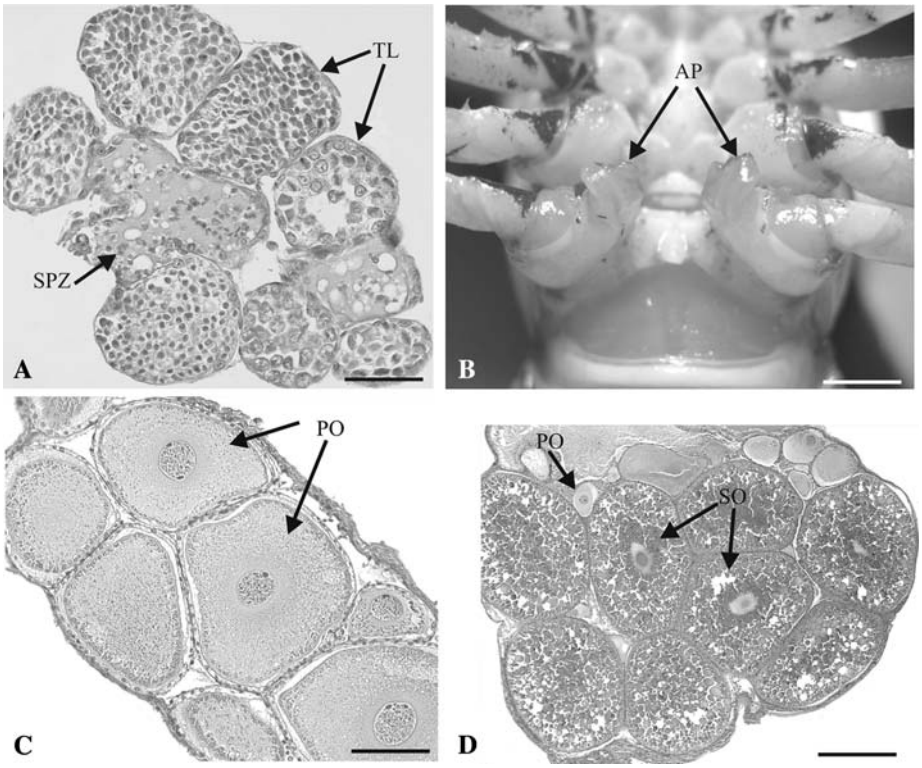

Fig. 1 Sex reversal in juvenile females of *Cherax quadricarintaus.* **a** Testicular lobes of normal males from the HT (high temperature) group (*scale bar* 110 μm); **b** *appendices masculinae* of males from HT (high temperature) group (*scale bar* 2.7 mm); **c** ovary with primary oocytes from a control female (*scale bar* 105 μm); **d** ovary with primary and secondary oocytes from *HT* group (*scale bar* 366.4 μm). *AP Appendices masculinae*, *PO* primary oocytes, *SO* secondary oocytes, *SPZ* spermatozoa, *TL* testicular lobes

proposed as a possible mechanism triggered by thermic stress (Van den Hurk and van Oordt 1985). In *C. quadricarinatus,* changes in the profile of lipids, proteins, and carbohydrates levels have been demonstrated in early juveniles obtained from berried females exposed to different temperatures up to 31°C (García-Guerrero et al. 2003). Additionally, induction of heat shock in proteins while exposing crayfishes up to 33°C has been shown by Cimino et al. 2002. Clearly, growth can by sex reversal induced by higher culture temperatures in this species.

We recognize that the observed differences in mortality between treatments (greater mortality in the HT group) could affect the final sexual proportion (greater sexual reversion toward male or intersex males in this group), although in the VHT group with lower mortality, the same tendency toward sexual reversion is observed. According to King (1994), survival of 83% is observed in *C. quadricarinatus* hatchings (weight 40–46 mg) and, in concordance with our results, a greater growth rate was observed.

Another effect of higher culture temperature was that vitellogensis was induced earlier in juvenile females. The structure of the ovary and the mean size of oocytes was similar to that of mature females greater than 18 g (Vazquez et al. 2008) and similar to other freshwater crayfishes (Brian et al. 2001; Vazquez et al. 2008 for revision). Although the

Table 2 Sex reversal evaluation in juvenile females of *Cherax quadricarintaus* after 150 days for the experimental groups: *C* (control), *HT* (high temperature), *VHT* (very high temperature) and *AG* (androgenic gland)

Experimental groups	*n*	Sexual reversion (%)	Total number of males + intersex males	Final sexual proportion M:IM:IF:F	Ovarian stage				Mean oocyte diameter (μm)
					T	Y	O	G	
C	26	65.38[a]	0[a]	0:0:17:9	10	0	16	0	169.97 ± 6.10[a] (37.5–387.5) ($n = 189$)
HT	19	78.95[a]	6[b]	2:4:9:4	1	2	9	1	248.78 ± 15.69[b] (37.5–875.00) ($n = 148$)
VHT	30	90.00[b]	4[a]	1:3:23:3	12	4	10	0	156.55 ± 7.40[a] (37.5–375.0) ($n = 165$)
AG	21	85.71[a]	1[a]	1:0:17:3	5	1	14	0	162.78 ± 5.96[a] (37.5–375.0) ($n = 226$)

M Males, *IM* intersex males, *IF* intersex females, *F* females. For the ovary colors, showing degree of increasing maturation: *T* transparent, *Y* yellow, *O* orange, *G* green

Mean oocyte diameter is expressed as average ±SE. Values in the same column having different superscripts are significantly different ($P < 0.05$) with respect to the C group. The AG group was only analyzed versus the C group

experiment did not involve mating (150 days is not enough time to acquire full sexual maturity), these results indicate that the early exposure of juvenile females to high temperatures accelerates the beginning of the sexual maturity and thus perhaps might extend the brooding period in captivity. Recently, we have obtained precocious ovigerous females from early females exposed to high temperature under laboratory conditions (C. Tropea and L.S López Greco, unpublished). Although the positive effect of temperature on ovarian growth is a general trend within adult freshwater crayfishes (e.g., Portelance and Dube 1990; Carmona-Osalde et al. 2004) no studies starting so early in the sexual differentiation of the females have been previously reported for *C. quadricarinatus* or other Astacida.

In the males and intersex males of *C. quadricarinatus* obtained via temperature manipulation, normal male gonopores, *appendices masculinae* and testes (López Greco et al. 2007; Vazquez and López Greco 2007b) were observed, making high temperature an efficient tool for the sexual reversion of females towards functional males. Manipulation of temperature during early sexual differentiation could be a useful tool in *C. quadricarinatus* culture. Recently, it has been reported that the occurrence of precocious sexual phase change (male to simultaneous hermaphrodites) can be minimized by lower culture temperatures in the ornamental shrimp *Lysmata seticaudata* (Calado and Dinis 2007), as was previously demonstrated in *Lysmata wurdemanni* (Bauer 2002).

AGE included in the diet was also effective in inducing growth induction in *C. quadricarinatus*. Previously, the injection and/or implantation of the androgenic gland was used to induce growth (see Sagi et al. 1997; Manor et al. 2004 for review). Juvenile females implanted with androgenic gland showed sexual reversion involving inhibition of vitellogenesis (lower gonadosomatic index, oocyte diameter and secondary vitellogenic cross-reactive proteins in hemolymph), differentiation of the red patch and male-like behavior although no male gonopores were observed after 14 months (Barki et al. 2003). Similarly, in early juveniles of *Cherax destructor* injected with AGE, inhibition of vitellogenesis, differentiation of male gonopores and significant growth resulted after 80 days (Fowler and Leonard 1999). In our assays, we demonstrate that significant growth could be obtained in a similar manner by feeding early juvenile females with AGE-enriched pellets at a 1/10 dose twice a week. Thus, the inclusion of this endogenous hormone in food pellets could improve yields while avoiding negative effects of AGE injection or implantion of the androgenic gland.

When comparing the effects of high temperature and dietary androgenic gland, both factors produced the same effect on somatic growth, achieving similar final weights at 150 days (4.61 ± 0.48 g for the HT group and 4.55 ± 0.43 g for the AG group). However, their effects on ovarian growth were different. Individuals in the AG treatment showed no oocyte growth and no secondary oocytes were observed, presumably because of the inhibitory effect of androgenic gland on vitellogenesis (Fowler and Leonard 1999; Sagi et al. 1999; Khalaila et al. 2001; Barki et al. 2003; Cui et al. 2005). From a molecular point of view, the inhibitory effects of AGE on vitellogenesis of the crayfish should be tested by ELISA or western blot analysis to indicate the levels of vitellogenin in different groups of samples. However, AG treatment did not produce a significant sexual reversion from females to functional males (males and intersex males) as expected. We cannot disregard the fact that the assay dose may not have been sufficient to induce this kind of reversion, although one normal male was obtained. Future experiments including the recombinant androgenic hormone produced in vitro in the diet should be the most suitable method to improve the percentage of reversion towards functional males for long-term purposes.

The present results also show that the sexual differentiation-window occurred at a very juvenile stage (nearly 0.20 g) which corresponded to juvenile stage VI–VIII (Vazquez

et al. 2004). Other studies made on larger juvenile females showed no differentiation of male gonads or gonopores (see Manor et al. 2004 for revision). Future studies should attempt to identify more precisely the developmental window for manipulating young juveniles to produce the desired monosex culture.

In the present study, a great number of intersex females were obtained in all experimental groups including the control. The reversion of juvenile females to intersex females with normal female gonopores as well as male gonopores (but without other male secondary sexual characters) appears to be a result of the culture environment, i.e., captivity (Vazquez and López Greco 2007a). Intersex females have not been observed in nature while intersex males are reported both in farms and in the field (Medley and Rose 1993; Sagi et al. 1996 for review).

Finally, our results demonstrate that both experimental factors have a very high potential for implementation into aquaculture of the species because of their effect on inducing growth and, in the case of temperature, for increasing the proportion of functional males and advancing the beginning of the sexual maturity in females. The other advantage of AGE-enriched diet and higher temperature in culture is that these techniques are safe and environmentally friendly because the androgenic gland is the natural source of the androgenic gland hormone. The purification of AGH and its production at commercial levels for its inclusion on diets will be the key to induce greater somatic growth and probably to obtain a greater proportion of males in *C. quadricarinatus*. These treatments could be used alone, or combined with other methods, as recently proposed by Aflalo et al. (2006) for *Macrobrachium rosenbergii,* in order to increase yields in less time.

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