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Aquaculture 256 (2006) 468–478
Aquaculture
www.elsevier.com/locate/aqua-online

A novel two-step procedure for mass production of all-male populations of the giant freshwater prawn *Macrobrachium rosenbergii*

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Received 15 September 2005; received in revised form 22 January 2006; accepted 25 January 2006

Abstract

Males of the giant freshwater prawn *Macrobrachium rosenbergii* grow faster and reach higher weights at harvest than females a fact which makes the culture of all-male populations desirable. All-male populations were produced by mating sex-reversed males, i.e., neofemales, with normal males. Neofemales capable of mating and spawning were produced by removal of the androgenic gland (AG) from immature *M. rosenbergii* males. The main obstacle to developing a technology based on this type of manipulation is the difficulty of identifying males at a sufficiently early stage of development. To overcome this problem, we developed a novel two-step scheme for large-scale microsurgical andrectomy. Phase I post larvae were andrectomized at ages 25–60 days after metamorphosis (PL_{25-60}). A low success rate of functional sex reversal, resulting with all male progeny was obtained (1.3%). In the light of the low success rate and a number of cases of abnormal reproductive development, a second phase was introduced in which the progeny (presumed males) of neofemales from phase I were andrectomized at earlier ages (PL_{20-30}). This two-phase protocol enabled a large quantity of juvenile males to be andrectomized. In phase II there was a significant increase in the number of sex-reversed animals (developed ovaries) and a significant shortening of the time to maturation. Comparison of the all-male progeny with a normal mixed population showed higher growth performance of the all-male population. This advantage together with the shorter maturation time in phase II opens the possibility to scale-up the system to field conditions. Since sexual dimorphic growth patterns are common among decapod crustaceans, it is obvious that the results of this study will have applied significance for many aquacultured species.

Keywords: *Macrobrachium rosenbergii*; Decapoda; Crustacea; Androgenic gland; All male monosex culture; Sex differentiation; Sex reversal

1. Introduction

Monosex culture strategy has become a common practice in fish-based aquaculture (Beardmore et al., 2001; Devlin and Nagahama, 2002; Gomelsky, 2003), and attempts have been made to apply this aquatechnology to crustacean culture (Curtis and Jones, 1995; Lawrence, 2004; Lawrence et al., 2000; Sagi et al., 1997b; Siddiqui et al., 1997), since male and female crustaceans differ in terms of growth rates, behavior patterns and husbandry needs. By preventing of breeding activity in

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doi:10.1016/j.aquaculture.2006.01.035

the culture, such a management strategy probably results in the diverting of energy from reproduction to growth and thereby contributes to sustainable aquaculture.

Growth in aquaculture systems may be affected by a wide variety of factors, including the gender, sexual maturity and age of the animals (Aiken and Waddy, 1992; Botsford, 1985; Hartnoll, 1982). A number of crustacean species exhibit bimodal growth patterns, in which males exhibit superior growth to females or vice versa (Hartnoll, 1982). The first attempt to produce a male monosex culture of the giant freshwater prawn *Macrobrachium rosenbergii* was carried out by hand segregation in a small-scale, intensive, cage-culture system (Sagi et al., 1986). For a grow-out period of 150 days, the average weight of the prawns in the all-male population reached 473 g/m^2, whereas that for all-female and mixed populations was 248 and 260 g/m^2, respectively. An additional advantage of all-male culture was that the prawns reached market size at a faster rate, a factor that prolonged the fresh product marketing period and enabled the now-vacant pond to be used for further production (Sagi et al., 1986). Similar results were obtained when a male monosex prawn culture was tested under intensive monoculture conditions in earthen ponds. When the procedure was tested in polyculture ponds, all-male stocking yielded an 18% increase in net income (Hulata et al., 1988). It has thus become obvious that an efficient biotechnology for producing all-male prawn populations is required, especially in countries in which economically valuable crustaceans constitute an important source of income (Sagi and Aflalo, 2005).

It is widely accepted that sexual differentiation in crustaceans, based on their genetically determined predisposition, is mediated by the androgenic gland (AG) (Okumura and Hara, 2004; Sagi and Cohen, 1990; Sagi et al., 1997a). Charniaux-Cotton (1954) was the first researcher to suggest a regulatory role for the AG. She showed that bilateral AG ablation in *Orchestia gammarella* blocked the differentiation of secondary male characteristics and decreased spermatogenesis. Later, Touir (1977) described the effects of the AG on both primary and secondary male characteristics in a number of gonochoristic and hermaphroditic decapod crustaceans, and Taketomi et al. (1990) showed that injection of AG extracts into the crayfish *Procambarus clarkii* accelerated the appearance of the male reversed spines on the copulatory appendages. More recently, it was shown that AG implantation into immature females of the crayfish *Cherax quadricarinatus* inhibited vitellogenesis and promoted growth (Manor et al., 2004), and AG implantation into females of the mud crab *Scylla paramamosain* resulted in ovarian regression, with degeneration of the oocytes (Cui et al., 2005). In *M. rosenbergii*, an altered sex ratio showing more than 95% males was observed when juveniles were fed with *Artemia* nauplii enriched with 17 α methyl testosterone. However, no functional sex reversal was reported (Baghel et al., 2004).

In male crustaceans, the endocrine and gametogenic functions are clearly separated into two distinct organs, the AG and the testis, respectively (Charniaux-Cotton and Payen, 1988; Ginsburger-Vogel and Charniaux-Cotton, 1982). Thus, sex differentiation can be manipulated through the removal of the AG, without damaging the gonads, and such manipulations can play a key role in producing monosex cultures. A study of maturing *M. rosenbergii* males that had been andrectomized at the youngest developmental stage indicated that these prawns exhibited a high degree of feminization, including initiation of oogenesis and development of oviducts and female gonopores (Nagamine et al., 1980a). Males andrectomized in later developmental stages were either partially feminized or not feminized at all (Nagamine et al., 1980a). A wide range of abnormalities in gonadal development was observed in andrectomized males, depending on the age at which the andrectomy was performed. In younger andrectomized males, the development of either gonads that were partly testicular and partly ovarian ("ovotestes") or abnormally lobulated was observed (Sagi et al., 1997a). Surgical removal of the AG from juvenile *M. rosenbergii* at an early developmental stage resulted in complete sex reversal, leading to the development of functional females capable of mating and producing progeny (Sagi et al., 1997a). In the latter case, all male progeny were obtained when fertile sex-reversed animals were crossed with normal prawns. It is expected that such crossings would result in all-male progeny, a premise supported by the homogametic male theory (Katakura, 1989; Malecha et al., 1992; Parnes et al., 2003; Sagi and Cohen, 1990). This premise was, in fact, confirmed under laboratory conditions, and attempts to scale up the methodology at the hatchery level are currently under way (Sagi and Aflalo, 2005).

In the current study, we established a feasible sex reversal procedure by using two phases of microsurgical AG ablation and mass production of 'neofemales' capable of producing all-male progeny (Fig. 1). These neo-females could then serve as broodstock for monosex culture.

2. Materials and methods

2.1. Animals

M. rosenbergii broodstock populations were collected from the wild in the Dong Nai river (Dong

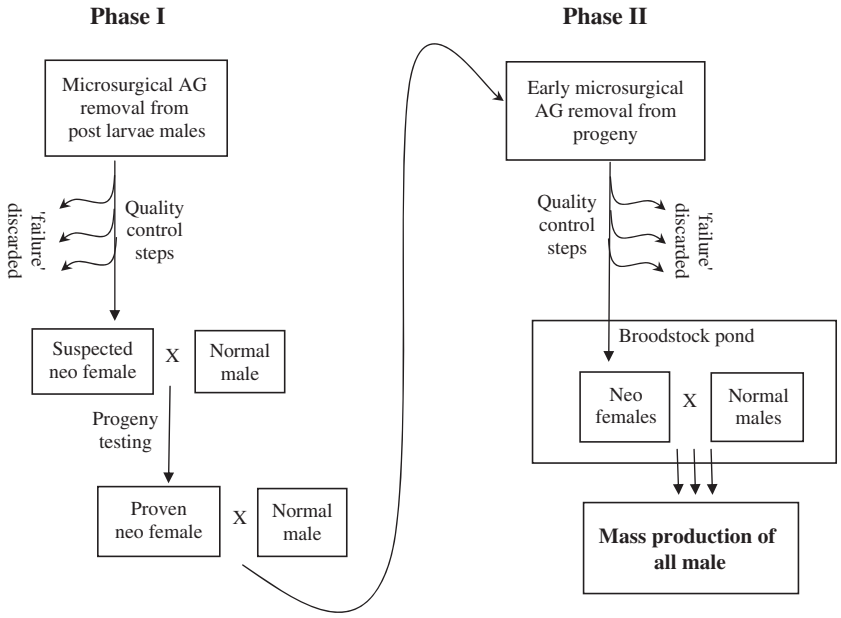


Fig. 1. Schematic representation of the novel two-phase procedure for mass production of all-male *M. rosenbergii* populations.

Nai Province, Vietnam) and kept in a flow-through water system in a research facility adjacent to one of the Saigon river streams. Progeny populations were reared in net cages at density of 100 post larvae (PL)/m^2 and fed with pellets containing 42% protein three times a day. During nursery rearing, juvenile males were identified by observation of their genital papillae on the fifth walking legs under a ×30 microscope. The males were selected for microsurgical AG ablation at the age of 25 to 60 days after metamorphosis ($PL_{25–60}$) when their weights ranged between 0.15 and 1 g.

2.2. Sex reversal microsurgery (phase I)

Microsurgical AG ablation was performed by removing the fifth pair of walking legs together with the AG. To ensure that the AGs had been completely removed, a significant part of the sperm ducts was also pulled off. The andrectomized males were stocked at a density of 100 PL/m^2 in net cages located in earthen ponds until the age of three months. Artificial substrate shelters covering about 60% of the net cages bottom, were provided. After 3 months, the andrectomized juveniles were sorted according to size ranges and transferred to round fiberglass tanks at a density of 15 prawns/m^2. Shelters covering 60% of the tank bottom area were provided. Water was recirculated through a biofilter.

2.3. Quality-control steps

2.3.1. Survival rate

The survival rate was calculated 24 h and 30 days after AG ablation.

2.3.2. Presence of the appendix masculina

Five days after AG ablation, the second left pleopod of the andrectomized juvenile (bearing the male sex character, the appendix masculina) was removed. Appearance of an appendix masculina on the regenerated leg at the subsequent checking times, i.e., after 30 and 90 days, served as an indicator of failed sex reversal. Such animals were discarded.

2.3.3. Gonad development

Starting 3 months after AG ablation, suspected neofemales were checked for gonad development every 2 weeks. These suspected neofemales, showing signs of ovarian development (the orange colored ovaries are visible through the transparent cuticle from a dorsal view), were placed separately in tagged $30 \times 40 \times 30\,cm^3$ baskets floating in the above-mentioned fiberglass tanks, and their gonad maturation and development was monitored visually. If and when ready for mating a normal sexually mature male was inserted into the basket following the pre-mating molt of the suspected neofemale.

2.3.4. Spawning

Berried suspected neofemales were monitored individually until egg color changed from orange to gray. The animals were then transferred to the hatchery and gradually acclimated to 12 ppt saline water. After hatching, the suspected neofemales were gradually acclimated back to fresh water and kept in individually marked floating baskets.

2.3.5. Progeny testing

To determine which of the suspected neofemales were indeed sex-reversed males, the progeny of each suspected neofemale were kept separately, and 70 days after metamorphosis the gender of the prawns in a sample of at least 100 individuals (usually 200–500 individuals) was determined.

2.4. Hatchery

After hatching, the larvae were transferred to a closed recirculation system composed of twenty 120 l fiberglass tanks connected to a biofilter. The circulating water had previously been treated with 30 ppm $Ca(OCl)_2$ for 48 h with aeration, followed by 25–27 ppm $Na_2S_2O_3$ for 6 h, and 10 ppm EDTA for 6 h before filtration through a 50 μm net. The tanks were stocked at a density of 60–80 larvae/l. During the few first hours after transfer, the larvae were kept at a low aeration rate, which was later increased. To calculate the spawn size, three samples of 300 ml each were taken, and the larvae were counted in each sample. Twenty-four hours after hatching, feeding with *Artemia nauplii* (3–5 *nauplii*/ml) was started. On the sixth day, the *Artemia* feed was replaced with an artificial feed comprising a mixture of chicken eggs, shrimps, milk powder and squid liver oil, which had been steamed. Temperature and pH were checked twice a day. Water temperature was maintained at 26–32 °C, and pH, at 7.6–8.1. NH_3 and NO_2 levels were checked every 3 days. Chemical oxygen demand was determined twice a week, and dissolved oxygen, twice a day on 2 days in each week. A 45 W bulb installed above each tank provided illumination during feeding time. Metamorphosis occurred 17–25 days after hatching.

2.5. Mass production of neofemales (phase II)

Only proven neofemales were used for this phase. The first spawn of each suspected neofemale was used for progeny testing to determine whether the suspected neofemale was indeed a functional female with a male genotype. The second spawn progeny of such proven neofemales were AG ablated at $PL_{20–30}$ when their weights ranged between 0.1 and 0.3 g.

2.6. Growth performance of all-male and mixed population progeny

Two groups of animals were used for this experiment, i.e., an all-male population which were the progeny of sex reversed males (neofemales), and a control population group with a male-to-female ratio of 1 : 1 (progeny of normal crossing). The parents of both experimental groups were taken from the same original population collected for the study as described above. Each group was examined in three replicates, each replicate comprising 60 individuals stocked in a 4 m^2 cage made of netting with a 1 mm^2 hole size. The cages were placed randomly in earthen ponds so as to provide the same conditions for all the groups. A total of 360 12-week-old juveniles, with an average weight of 3 g, from all-male and control populations were stocked into these cages. Shelters made of nylon ropes covered about 60% of the cage surface. The juveniles were fed three times a day with 42%-protein pellets for the first month and thereafter with 35%-protein pellets. The nets were cleaned twice a month to facilitate free water flow exchange in and out of the cages. Weight and body length

Table 1
Results obtained at quality control "check points" in phase I microsurgical androgenic gland removal and sex reversal of *M. rosenbergii* juvenile males

	Andrectomized male juveniles	Survival (time after andrectomy)		Suspected neofemales				Proven neofemales
		24 h	30 days	Appendix masculina absent	Developed ovary	Berried	Hatched	100% male progeny
#	1940	1554	1280	$225^a/177^b$	$222^a/127^b$	$37^a/1^b$	$37^a/1^b$	$25^a/1^b$
%	100	80.1	65.9	$11.59^a/9.12^b$	$11.44^a/6.54^b$	$1.9^a/0.05^b$	$1.9^a/0.05^b$	$1.28^a/0.05^b$

At andrectomy, the age and weight of the post larvae (PL) were 25–60 days after metamorphosis ($PL_{25–60}$) and 0.15–1 g, respectively.
[a] Designates absence of appendix masculina on both second pleopods.
[b] Designates absence of appendix masculina on the regenerated second pleopod.

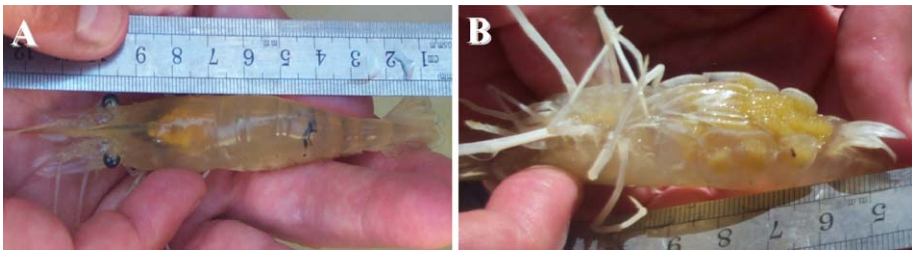


Fig. 2. Dorsal (A) and ventral (B) views of a successfully sex-reversed *M. rosenbergii* male developing an ovary and berried, respectively. Andrectomy was performed as early as PL_{34}.

of the prawns were measured twice a month. The experiment was stopped after 12 weeks, by which time most of the females in the mixed population had developed ovaries and some were carrying eggs.

2.7. Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed with two-way analysis of variance, followed by the least significant differences test for multiple comparisons using computer software (Statistica 6.0, Statsoft, Inc. Tulsa, OK). $P < 0.05$ was defined as a statistically significant difference.

3. Results

During phase I, 1940 juvenile males were selected for AG ablation. These animals were monitored throughout the experimental period according to the quality control steps described in Materials and methods (Table 1): 24 h after AG ablation, the survival rate was 80.1%: Almost 400 of the andrectomized prawns had died, most likely as a result of the microsurgical procedure. After 30 days of growth, the survival rate was reduced to 65.9%, the decrease reflecting the natural mortality and cannibalism among the andrectomized prawns due to the husbandry conditions (100 individuals/m^2, see Materials and methods). The regeneration of the second pleopod was checked at that time (after 30 days) and 60 days later: Out of 1280 prawns, 225 did not have appendices masculina on either the intact or the regenerated second pleopods [designated (^{a})], and 177 prawns did not have appendices masculina on the regenerated second pleopod [designated (^{b})]. The remainder of the prawns exhibited appendices masculina on both intact and regenerated second pleopods. The latter animals were considered a "failure" with respect to the AG ablation procedure and were discarded from the experiment. From that time onwards, the prawns were monitored twice a week for female sexual maturation: 222 (^{a}) and 127 (^{b}) prawns (11.44% and 6.54%, respectively) developed ovaries. These animals were housed in individual baskets floating in concrete tanks, a

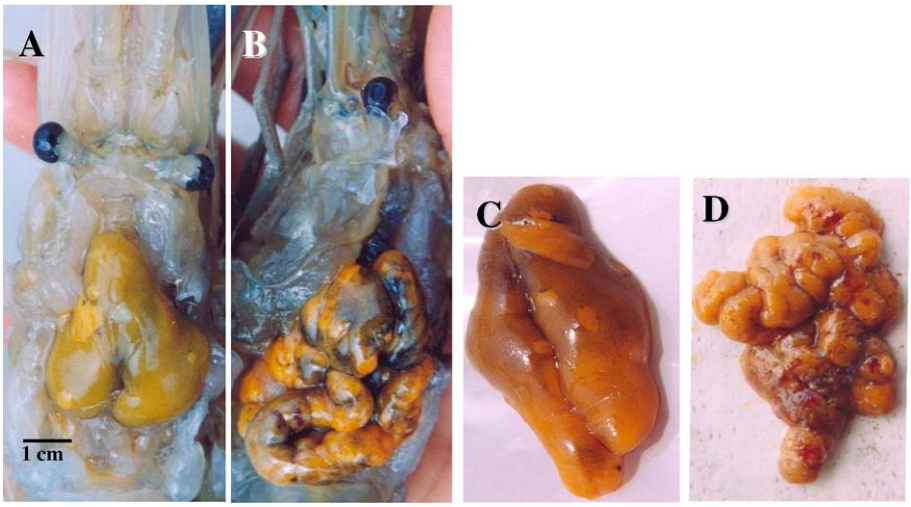


Fig. 3. Normal (A) versus abnormal (B) ovarian development in sex-reversed *M. rosenbergii* males. The dissected ovaries are presented in (C) and (D), respectively.

set-up that allowed easy and precise monitoring of the reproductive development of each individual prawn. Most of the suspected neofemales mated, but only 37 ([a]) animals and 1 ([b]) animal laid eggs (Berried, Table 1) and hatched progeny (1.9% and 0.05%, respectively). The progeny of the each suspected neofemale were kept separately for sex ratio determination (progeny testing): 25 ([a]) and 1 ([b]) suspected neofemales (1.28% and 0.05%, respectively) were found to be all-male-producing animals and were defined as proven neofemales.

During the quality-control process for phase I, development of the ovaries was observed through the transparent cuticle of the prawns. In specimens of sex-reversed *M. rosenbergii* males, normal female reproductive development was manifested in the development of two ovarian lobes and oviducts (Figs. 2A, 3A,C and 4A). Later inspection of the same prawn (Fig. 2A) from the ventral aspect (Fig. 2B) showed normal female sexual development, including eggs. Some cases of abnormal female sexual maturation were manifested morphologically as multi-lobulated ovaries (Fig. 3B and D). Cases of degenerated melanized oviducts were also observed (Fig. 4B). Most of the suspected neofemales showed female mating behavior, as suggested by spermatophore deposition (Fig. 5A, arrows), but in some cases, subcuticular egg laying was observed (Fig. 5B) due to abnormal or dysfunctional oviducts.

It was thus evident that a very small percentage of successfully sex-reversed *M. rosenbergii* males were obtained in phase I. The high degree of abnormalities in these animals was probably the result of late intervention in the sex differentiation process. Thus, a second-phase procedure for microsurgical sex reversal in *M. rosenbergii* was introduced (Fig. 1). In this procedure, the proven neofemales from phase I were used to produce all-male progeny. These progeny were AG ablated at an early developmental stage and age: the microsurgical manipulation at an earlier age than in phase I—in a presumably sexually undifferentiated stage—increased

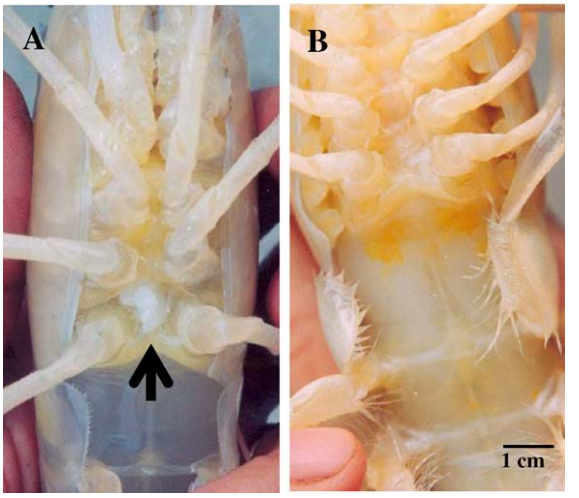


Fig. 5. Spermatophore deposition (↑) suggesting normal mating behavior (A) and abnormal, subcuticular, spawning (B) in sex-reversed *M. rosenbergii* males.

the success rate. In phase II, more males were andrectomized since no segregation of males or strict quality control steps were necessary. The main "check point" was the development of the appendices masculina, which indicated unsuccessful AG removal. In phase II, more than 4000 juvenile males, progeny of proven neofemales, were subjected to AG ablation. Survival rates of 80.92% and 65.69% were obtained 24 h and 30 days after AG ablation, respectively (Table 2). By day 30, 31.68% of the andrectomized prawns had not developed appendices masculina. Those that had developed appendices masculina were discarded from the experiment. Three months after AG ablation, some of the prawns started to develop ovaries. By the evaluation 12 months after AG ablation, 729 prawns (17.62%) were past the quality control steps, and most of these had developed ovaries. These suspected neofemales with developed ovaries were allowed to mate with normal males, and more than 157 were found to be berried. Samples of

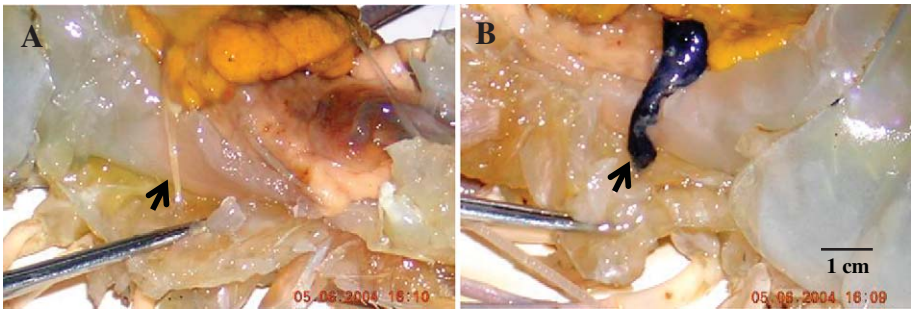
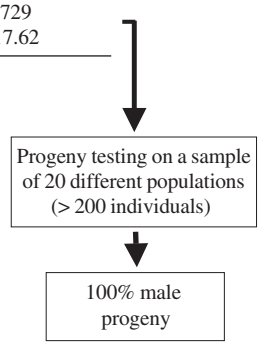


Fig. 4. Normal (A) and abnormal (B) oviduct development (arrows) in sex reversed *M. rosenbergii* males.

Table 2
Results obtained at quality control "check points" in phase II microsurgical androgenic gland removal and sex reversal of *M. rosenbergii* juvenile males

	Andrectomized male juveniles	Survival (time after andrectomy)		Appendix masculina absent	Suspected neofemales
		24 h	30 days		
#	4137	3348	2718	1311	729
%	100	80.92	65.69	31.68	17.62



These juveniles were collected from all male populations (progeny of neofemales from phase I). At andrectomy, the age and weight of the post larvae were 20–30 days after metamorphosis ($PL_{20–30}$) and 0.1–0.3 g, respectively.

20 progeny from different suspected neofemales were sexed, and all were found to be all-male animals, reflecting the success of phase II. A comparison of the average sexual maturation time of the neofemales from phase I (330 ± 17.9 days, $n=25$) and phase II (190 ± 7.3 days, $n=12$) indicated a significantly ($P<0.05$) shorter maturation time for the neofemales in phase II.

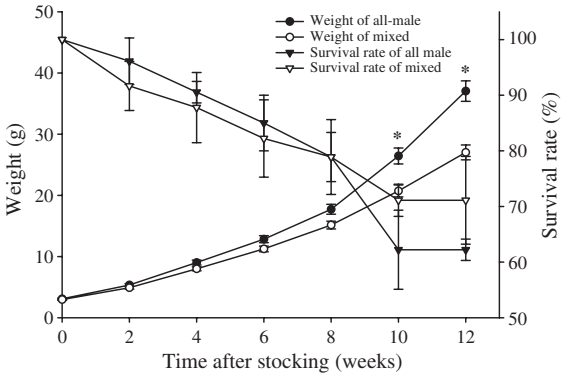


Fig. 6. Growth performances and survival rates of all male *M. rosenbergii* populations produced from crosses of neofemales and normal males compared with those for normal mixed populations. Three random blocks of all male groups (total $n=112$) and mixed groups (total $n=128$) comprised the experiment. Data are expressed as means ± SE. Asterisks represent statistically significant differences ($P<0.05$).

Differences in body mass between the all-male prawn population and the mixed population were noted from the fourth week of the experiment. Analysis of variance of the growth performance of males revealed that from the 10th week of the experiment the all-male groups weighed significantly more than the mixed groups ($P<0.05$) (Fig. 6). The final mean weights of the all-male and mixed groups reached 37 ± 1.7 and 27 ± 1.2 g, respectively. In all the groups, cannibalism and natural mortality appeared to be responsible for the gradual decline in the survival rate, with the rates reaching 62.2% and 71.1%, respectively (Fig. 6). Comparison of the growth of the all-male populations with the male fractions of mixed groups showed a significantly higher ($P<0.05$) mean weight for the all-male group, i.e., 37 ± 1.7 vs. 32 ± 2.3 g ($n=112$ and 55, respectively). The growth performance experiment was stopped at the end of the 12th week, because by that time most of the females had mated and had almost stopped growing.

4. Discussion

4.1. Bimodal growth, sex reversal and all male monosex culture

M. rosenbergii exhibits a bimodal growth pattern, in which males exhibit superior growth to females. A

pattern of this type constitutes a possible disadvantage in the growth management of sexually dimorphic crustacean species. Management of sexually dimorphic growth could be improved by providing the means for generating all-male broods. To date, a variety of methods have been used to increase aquaculture yields of *M. rosenbergii,* including improvement of environmental and nutritional conditions and manipulation of the population structure by selective stocking and harvesting (Hulata et al., 1988; Ra'anan and Sagi, 1985; Sagi et al., 1986). The present study presents, for the first time in crustaceans, a large-scale sex-reversal protocol to create broodstocks producing all-male-progeny, which could then be used for monosex culture of *M. rosenbergii*.

It has been hypothesized that in malacostraca, morphological sex differentiation is dependent upon the presence or absence of an androgenic hormone (Charniaux-Cotton, 1959, 1970). Thus, embryos destined to form males have the genetic capability to initiate androgenic hormone secretion, which, in turn, induces masculinization and inhibits feminization. However, embryos unable to initiate androgenic hormone secretion, autodifferentiate into females. Our data support this hypothesis, since young *M. rosenbergii* males released from the masculinizing effect of the AG by andrectomy underwent feminization, as indicated by changes in secondary sex characteristics, a decrease in growth rate and development of functional female gonads and reproductive tracts (Nagamine et al., 1980a; Sagi et al., 1990). Oogenesis is initiated only in males that have been andrectomized at the youngest developmental stage (Nagamine et al., 1980a). With an increase in size at the time of andrectomy, male prawns progressively lose their ability to develop female characteristics, i.e., they progressively become committed to their genetic gender (Nagamine et al., 1980a). The latter is one of the obstacles to mass production of sex-reversed males during phase I manipulations (Fig. 1 and Table 1).

4.2. Phase I

In the first set of microsurgical AG ablation experiments (phase I, Fig. 1), testing for successful sex reversal of andrectomized male post larvae ($PL_{25–60}$)—including a progeny test—showed that out of 1940 andrectomized males, 26 were completely feminized, including normal initiation of oogenesis and development of oviducts and female gonopores. These animals had thus become neofemales capable of reproducing all-male progeny. The results, showing only 1.3% of complete and functional sex reversal, are in accordance with the findings of Nagamine et al. (1980b), who reported several cases of complete sex reversal in *M. rosenbergii*, in which the degree of feminization depended on successful removal of the AG from juveniles at a certain age. Nagamine et al. (1980a) also showed that males andrectomized in later developmental stages were either partially feminized with the appearance of various gonadal abnormalities (Sagi et al., 1997a), or not feminized at all. These findings were mirrored in our first-phase results (Table 1, Figs. 2–4).

In phase I of AG ablation, we observed some cases in which the appendix masculina did not regenerate on the ablated pleopod and some cases in which appendices masculina did not develop on both second pleopods. Two possible reasons may account for the latter finding. The first one is incorrect identification of a juvenile prawn as male, which could have resulted in ablation of a genetically female animal. The second reason could be that the AG ablation had been performed prior to the differentiation of secondary sexual characteristics. These results are in keeping with those of Nagamine et al. (1980b) showing that *M. rosenbergii* male prawns andrectomized prior to differentiation of secondary sexual characteristics do not develop them later and that males andrectomized after differentiating secondary sexual characteristics do not lose them afterwards. However, when a sexually dimorphic appendage is lost, andrectomized males are unable to regenerate the masculine form of the appendage (Nagamine et al., 1980b). These findings were used as a quality-control tool for sex reversal, i.e., one of the second pleopods, bearing the appendix masculina was amputated, and the absence of the appendix masculina on the regenerated pleopod was taken as an indicator of sex reversal.

Three methods have been described in the literature for investigating the genetic mechanisms of sex determination in crustaceans: a) the use of sex-linked genes, b) karyotype analysis, which reveals the existence of heteromorphic pairs of sex chromosomes, and c) mating of sex-reversed individuals with normal individuals (Ginsburger-Vogel and Charniaux-Cotton, 1982). The use of the latter method (Malecha et al., 1992) is based on the hypothesis that the gender of prawns is determined chromosomally, with females being heterogamous (ZW) and males, homogamous (ZZ), as suggested by Katakura (1989). The above-described studies serve as the bases for our use of the population sex ratio as a validation tool for successful sex reversal in *M. rosenbergii* males. Out of 38 suspected sex-reversed males, 26 were found to be full and functional sex-reversed males (i.e., neofemales). The sex ratio of the progeny of these suspected neo-females provided

evidence supporting the male homogametic theory (Katakura, 1989; Malecha et al., 1992; Parnes et al., 2003) in this species. The progeny of the above-described 26 neo-females were all male, whereas the progeny of the other suspected neo-females exhibited a male-to-female sex ratio of 1 : 1. Thus, animals that were, in fact, normal females had probably been falsely selected as juvenile males at the early developmental stage and subsequently subjected to andrectomy. Sexual maturation of the 26 neo-females in phase I took significantly longer than that of normal females and is considered to be an effect of late intervention in the sexual differentiation process.

The cases of full and functional sex reversal and the wide range of partial feminization found in phase I of the study strongly support the hypothesis that males andrectomized during an undifferentiated stage will not develop male secondary sexual characteristics and will eventually autodifferentiate to females (Charniaux-Cotton, 1959, 1970). Functional sex reversal was achieved only in few cases. Thus, to overcome the very low success rate of phase I, a second phase was introduced.

4.3. Phase II

To address the challenge of producing all-male progeny, we performed a second step of sex reversal (phase II) in which proven neofemales from phase I were mated to produce all-male progeny. The second phase eliminated the need for male segregation from mixed populations, allowing a large quantity of juvenile males to be andrectomized. The procedure could be performed at an early, presumably undifferentiated, developmental stage ($PL_{20–30}$). The phase II andrectomy experiments included the control step of examination of the second pleopods of the andrectomized males. Approximately one third of the andrectomized juvenile males developed female-like second pleopods (bearing only the appendix interna). The remainder, which developed male-like second pleopods with appendices masculina, were regarded as andrectomy failures. In the successfully andrectomized animals, female sexual characteristics, like ovary development, mating and spawning, started to appear earlier than in phase I, namely, three months after andrectomy, and the first spawning events occurred at similar ages to those for normal females. These phase II findings support the hypothesis that males andrectomized at an undifferentiated stage will autodifferentiate into females (Charniaux-Cotton, 1959, 1970; Nagamine et al., 1980a; Sagi et al., 1997a). The female autodifferentiation hypothesis was further supported by the absence of partial feminization among the phase-II andrectomized animals. Progeny testing of a sample of 20 phase II animals showed 100% male progeny, confirming the higher frequency of complete and functional sex reversal in the second phase.

The proposed two-step scheme raises some concerns regarding the long duration of the whole process as a result of the quality control steps, and the potential loss of genetic variability due to the potential "bottle neck" in phase I of the scheme. Proper management of broodstocks is required to prevent founder effects, which could lead to inbreeding and/or genetic drift, as pointed out by Tave (1999). Phases I and II should be designed so as to preserve a large portion of the genetic variation available in the original population, as suggested by Chevassus (1989).

4.4. Growth performances of all-male progeny of neofemales

Water quality parameters such as temperature, transparency, dissolved oxygen, carbonate alkalinity and pH were in the standard aquaculture range throughout the culture period (Boyd and Zimmermann, 2000), and it is therefore assumed that these growth conditions had no adverse influence on growth and survival of *M. rosenbergii*. The all-male groups grew faster than the controls. This is the first time that an all-male progeny has been tested for growth performance, and the results are in keeping with those for hand-segregated all-male monosex cultures of freshwater prawns (Cohen et al., 1988; Sagi et al., 1986; Siddiqui et al., 1997). Males of the all male populations grew significantly faster than the male fraction of the mix population possibly due to the presence of females which resulted in divergence of energy from somatic growth to reproductive effort, i.e., competition for mates (Lawrence et al., 2000). It is also possible that a larger fraction of males in the mix population remained smaller due to the biological advantage of small males in reproduction (Ra'anan and Sagi, 1985). The total production obtained in the present study from all-male population was 37% higher than that from the mixed population at a stocking density of 15 individuals/m^2. Earlier studies also recorded higher production of all-male culture than for mixed cultures (Hulata et al., 1988; Sagi et al., 1986; Siddiqui et al., 1997). Cohen et al. (1988) reported a smaller yield difference of 7.8% between an all-male stocking and a mixed population when the prawns were grown in earthen ponds at a stocking density of 9 individuals/m^2. It is now necessary to scale-up the two-phase protocol to field dimensions and conditions to check profitability under such conditions.

5. Summary and conclusions

This study offers a new feasible way of improving *M. rosenbergii* yields by manipulating the endocrine system—which regulates growth and development—resulting in monosex progeny. The advantages of such a broodstock are the higher weights of the male prawns, the shorter times to maturation, the saving of resources that would have been expended on growing unmarketable females and the obviation of the problems associated with reproduction in grow-out ponds. Future research is needed to evaluate the genetic implications of the suggested scheme and to shorten its duration. Since many cultured decapod crustaceans exhibit sexual dimorphic growth patterns, it is obvious that tremendous applied significance lies in the understanding of the AG and sexual differentiation processes. Elucidation of these processes could result in new ways to achieve monosex culture in additional species.

Acknowledgments

Our study on sex reversal was supported by the German Agro Action, German-Israeli Fund for Research and International Development (GIFRID). We thank Mr. Yoki Lotan and Prof. Amram Oshri for their support, encouragement and help. We also thank Prof. Gideon Hulata for useful discussions on the genetic implications of our two-phase sex reversal scheme. We would like to thank Ms. Inez Mureinik for editing the manuscript.

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