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Three generations of cultured prawn without W chromosome

Nir Shpak, Rivka Manor, Eliahu D. Aflalo, Amir Sagi *

[a] Department of Life Sciences, Ben-Gurion University of the Negev, P.O.B. 653, Beer-Sheva 84105, Israel
[b] National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, P.O.B. 653, Beer-Sheva 84105, Israel

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ABSTRACT

The recent introduction into aquaculture of RNA interference (RNAi) for producing the preferred all-male monosex cultures, which give improved yields, has raised awareness of the need to investigate the consequences of such novel biotechnological manipulations. Here, we present meta-analysis style study on data from observations of three consecutive cultured all-male (ZZ) generations of the giant freshwater prawn *Macrobrachium rosenbergii* (De Man). Each consecutive generation comprised the progeny of RNAi-manipulated sex-reversed males. The manipulation was achieved through the administration of dsRNA encoding the insulin-like androgenic hormone into males (which transformed them into 'neofemales,' ZZ) of the previous all-male generation. Each generation was cultured in a separate earthen pond for a short (~4–5 months) growout period. At harvest of each of the generations, the typical *M. rosenbergii* population structure comprising three male morphotypes was obtained. An anatomical examination of the male reproductive system of a representative specimen of the third all-male generation showed normal reproductive outputs, even though the prawns had been grown without the presence of females (WZ) for three generations. At the molecular level, expression of vital male-specific genes in the third generation of all-male *M. rosenbergii* culture was demonstrated. Thus, the present study showing the lack of any overtly apparent long-term consequences of the RNAi-based biotechnology provides support for the responsible use of temporal RNAi in aquaculture. Finally, the absence of the W chromosome for three generations raises questions with regard to its role and content with respect to crustacean sexual determination and differentiation.

Contents

* Corresponding author at: Department of Life Sciences, Ben-Gurion University of the Negev, P.O.B. 653, Beer-Sheva 84105, Israel.
E-mail address: sagia@bgu.ac.il (A. Sagi).

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1. Introduction

In aquaculture, monosex culture provides a means for increasing yields and maintaining size uniformity of the animals at harvest (Curtis and Jones, 1995; Sagi et al., 1986; Trino et al., 1999). Additional advantages of monosex culture are a reduction in aggressive sexual and territorial behavior and the ecological benefit of decreased risk of escape from the exotic population of individuals, who then become invaders (Beardmore et al., 2001; Sagi and Aflalo, 2005). In the aquaculture industry, monosex culture was first established for fish and later for crustaceans through the application of various techniques, including sorting and surgical, physiological, endocrine or molecular interventions, according to the species of interest (Bye and Lincoln, 1986; Sagi and Aflalo, 2005; Tayamen and Shelton, 1978).

The giant freshwater prawn *Macrobrachium rosenbergii* (De Man) is one of the most important inland crustacean species in aquaculture in many tropical and subtropical countries. *M. rosenbergii* exhibits a typical dimorphic growth pattern in which some of the male morphotypes develop faster and reach a larger final size and weight at harvest than the females. The production of male monosex populations of *M. rosenbergii* thus has significant economic and ecological advantages (Aflalo et al., 2006; Nair et al., 2006).

Dimorphic differentiation in crustaceans is controlled by a specific male endocrine gland, termed the androgenic gland (AG), which regulates and maintains the sex differentiation and masculine secondary sexual characters. The activity of the AG is mediated by the AG hormone, which – when first discovered in a decapod crustacean (Manor et al., 2007) – was termed the insulin-like androgenic gland hormone (IAG). The *M. rosenbergii IAG* gene, *Mr-IAG*, was fully sequenced (Ventura et al., 2009), and subsequently its function was determined through temporal gene silencing via injection of dsRNA according to the IAG sequence. It was shown that inhibition of the hormone expression led to cessation of spermatogenesis and spermatophore development and to inhibition of the development of the appendix masculina, a secondary masculine sexual character in regenerated pleopods (Ventura et al., 2009, 2011). It was also shown that temporal gene silencing through dsRNA injection at a critical juvenile stage caused full and functional sex reversal of males into females, which are then known as 'neofemales.' Such 'neofemales' produced all-male progeny when crossed with normal males (Ventura et al., 2012).

The above-described sex reversal in *M. rosenbergii* to produce 'neofemales' is mediated by temporal RNA interference (RNAi) post-transcriptional gene silencing (Cogoni and Macino, 2000), through *in-vivo* administration of synthetic ds*Mr-IAG* to manipulate endogenous gene expression, as was first observed in *Caenorhabditi elegans* (Fire et al., 1998). It is believed that the administered ds*Mr-IAG* crosses the hemolymph into the cytosol, where it is processed by Dicer, a ribonuclease III-related enzyme into 21–22 fragments, known collectively as small interfering RNA (siRNA) (Elbashir et al., 2001). The siRNA guides an RNA-induced silencing complex (RISC) that recognizes the target mRNA and allows its digestion, thus preventing translation (Chendrimada et al., 2005) of the Mr-IAG peptide, presumably without causing a transmissible genetic modification.

RNAi biotechnological tools have become widely used both in the laboratory (in molecular and genes function research) and in the field, for example, in the monosex culture of *M. rosenbergii* (Ventura et al., 2012), where the use of RNAi for achieving sex reversal gave a success rate of up to 86% (Lezer et al., 2015). While the technology has become industrial, questions regarding the safety of using temporal gene silencing mediated by RNAi have been raised. In this context, the study of Lezer et al. (2015), showing the total disappearance of the introduced dsRNA from different tissues in the prawn, has indicated that RNAi usage is indeed safe. Furthermore, *Mr-IAG* silencing through ds*Mr-IAG* injection was shown to be transient, with the *Mr-IAG* transcript level returning to normal levels 28 days post injection. Lezer et al. (2015) also demonstrated a normal population structure in the all-male progeny, which exhibited the typical morphotypic differentiation of male *M. rosenbergii* populations described by Kuris et al. (1987), namely, male prawns with blue claws (designated BC), males with orange claws (designated OC), and small males with relatively unpigmented and translucent claws (designated SM).

Previous studies of all-male populations produced either by surgical manipulation (Sagi and Aflalo, 2005; Sagi et al., 1986) or by RNAi-based technologies (Lezer et al., 2015; Ventura et al., 2009, 2012) have not gone beyond one generation. Here, we report the feasibility of a sustainable culture of all-male populations lacking the W chromosome for several generations (F_1–F_3); a schematic presentation of the process over three consecutive generations is described in Fig. 1. Our study focused on the three issues described below, namely: 1) the population structure; 2) the masculine reproductive system and sperm morphology of third generation (F_3) all-male freshwater prawns, which do not have a W chromosome [with masculine traits being studied as described previously (Lynn and Clark, 1983; Okumura and Hara, 2004; Sagi et al., 1988) and 3) the expression of *Mr-IAG* and the related male reproductive gene, *Mr-Mrr*, which is believed to be involved in sperm capacitation and fertilization (Cao et al., 2006; Phoungpetchara et al., 2012).

2. Materials and methods

2.1. Animals

The *M. rosenbergii* brood stock currently being cultured at Ben-Gurion University of the Negev (BGU), Beer-Sheva, Israel was originally imported from Hawaii approximately three decades ago. This broodstock has been consecutively reproduced at the BGU aquaculture facilities throughout the years and is also grown at the Dor Aquaculture Research Station of the Ministry of Agriculture for short growout periods during the summer months. The *M. rosenbergii* BGU-line was thus established and maintained in these two facilities, as follows. The brood stock was held under the following conditions: food comprising shrimp pellets (Rangen Inc., Buhl, ID, USA; 30% protein) was supplied *ad libitum* three times a week. Water quality was assured by circulating the entire volume through a bio-filter, thereby maintaining all the water physicochemical parameters, as described previously (Khalaila et al., 2002). Each egg-berried female was moved to a separate closed 100-L tank containing 12–16 ppt seawater, circulated through a 100 μm mesh net. After the brood had hatched, the female prawn was removed from the tank. The newly hatched larvae were maintained according to a previously devised protocol (Uno and Kwon, 1969) and fed with *Artemia* nauplii daily until the larvae transformed to post-larvae (PLs). The PLs were stocked in earthen ponds at the Dor station for the summer period.

Mixed-sex progeny for the present study were obtained from crosses between males and normal females at the aquaculture facilities of BGU. The all-male progenies originating from crosses between males and 'neofemales' were supplied by the Tiran Group through its subcontractor, Colors Ltd., Hatzeva, Israel. This hatchery holds only genetic males of the BGU line, and genetic females have never been allowed on this farm.

2.2. Growth performance

Growth performance was evaluated using a meta-analysis style study in two representative mixed-sex populations and three consecutive generations (F_{1-3}) of all-male *M. rosenbergii*, with each population/generation being grown in a separate earthen pond. All the above populations were stocked at the Dor Research Station and grown for the summer period between May and October. The third generation (F_3) of the all-male population was stocked with ~1500 post larvae (~PL_{60}, stocking density 6 prawns/m^2). All the other populations were stocked with ~750 post larvae (~PL_{60}, stocking density 3 prawns/m^2). Details regarding the different stockings are given in Table 1.

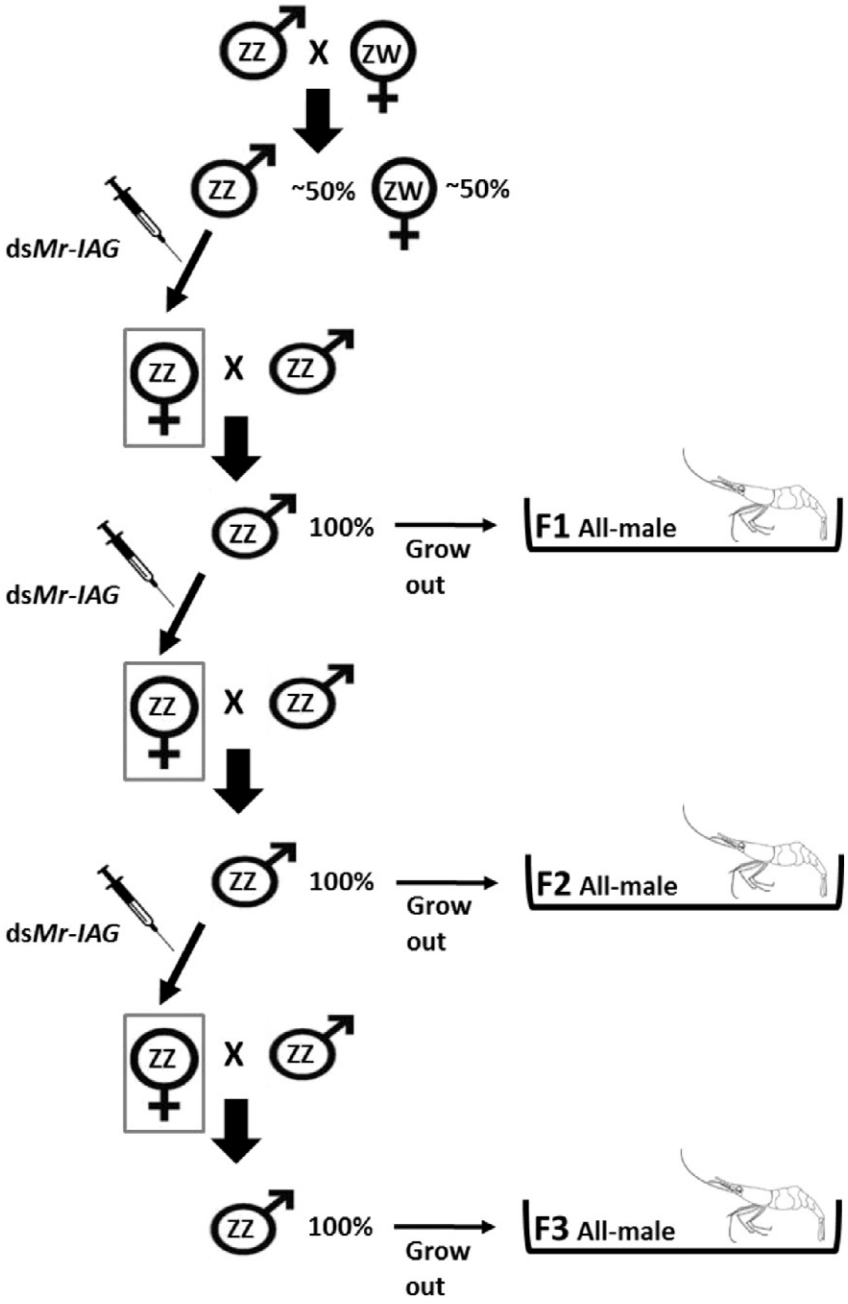

Fig. 1. Schematic demonstration of the production protocol for three consecutive generations of all-male populations through the use of 'neofemales' (rectangles) obtained with the ds*Mr-IAG* injection technology.

2.3. Social structure

For each pond, the prawns were collected at the end of the growout period. Weights were recorded, and male prawns were classified into the three morphotypes described above – BC, OC and SM (Kuris et al., 1987) – and large males without claws, which were designated no-claw (NC). Average weight and weight range were determined for each morphotype in each population.

Table 1
Ponds details including the type, duration and yield of the culture.

Type	Generation	Days of culture	Density (no. of prawns/m^2)	Stocking (no. of prawns)	Harvest (no. of prawns)
Mixed-sex population	F_1	102	3	750	605
	F_1	131	3	750	571
All-male population	F_1	102	3	750	551
	F_2	131	3	750	440
	F_3	122	6	1500	647

2.4. Histology

To monitor reproductive physiology performance, testis and terminal ampullae with their attached AGs from representative mature BC males from the mixed-sex and all-male F_3 populations were dissected. Prior to dissection, the prawns were anesthetized in ice-cold water. Tissue samples were fixed in 4% formalin for 48 h and dehydrated through a series of increasing alcohol concentrations. Tissues were cleared and embedded in Paraplast (Kendall) according to conventional procedures. Sections (thickness, 5 μm) were mounted onto saline-coated slides

Table 2
Growth performance of male morphotypes in mixed-sex and all-male populations.

Male morphotype	Mixed-sex population	All-male population		
		F_1	F_2	F_3
Average weight ± SE (g)				
SM	19.8 ± 1.8	10.8 ± 0.9	13.6 ± 0.9	6.5 ± 0.3
OC	65.4 ± 1.5	39.7 ± 1.1	74.9 ± 1.7	44.2 ± 0.7
BC	77.8 ± 1.6	74.8 ± 1.2	109.2 ± 1.8	60.9 ± 3.8
Weight range [min–max (g)]				
SM	3.8–61.9	2.3–58.2	3.0–46.0	1.5–30.0
OC	8.7–114.1	6.0–97.0	21.0–124.4	18.41–84.66
BC	31.2–127.0	43.0–99.3	46.0–135.0	47.22–80.96

SM — small males, OC — orange claw males, BC — blue claw males.

(Menzel-Gläser, Braunschweig, Germany) and stained with hematoxylin and eosin. The sections were observed and recorded with a Nikon ECLIPSE E600 microscope (Nikon Instruments, Melville, New York, USA).

2.5. Expression of endogenous Mr-IAG and Mr-Mrr

To study transcription aspects related to male-specific genes, AGs and sperm ducts from six representative BC males from a mixed-sex population and six from the all-male F_3 population (average weight 90 g) were dissected out, with the animals being anesthetized in ice-cold water. Total RNA was isolated using the EZ-RNA Total RNA Isolation kit (Biological Industries, Beit Haemek, Israel), according to the manufacturer's instructions. cDNA was prepared in a reverse-transcriptase reaction containing 1 μg total RNA using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA), according to the manufacturer's instructions. The cDNA was then amplified by PCR in a mixture containing 50 ng of cDNA, 0.5 μM of forward primer and 0.5 μM of reverse primer, 10 μl of Ready Mix REDTaq (Sigma) and water to a final volume of 20 μl. The PCR conditions were: 3 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 45 s at 72 °C. Two male specific transcripts, *Mr-IAG* (accession no. FJ409645.1) and *Mr-Mrr* (accession no DQ066890), were amplified through PCR reactions. *β-actin* (accession no. AF221096) served as the control using primers as described by Ventura et al. (2009). PCR products were electrophoresed in a 1.3% agarose gel and visualized by ethidium bromide staining and exposure to UV light.

3. Results

3.1. Male morphotype distribution

At the end of the growout period, prawn weights and male morphotypic differentiation were recorded for all the tested ponds – two ponds containing normal mixed sex populations and three ponds each containing a consecutive generation of an all-male population (F_1–F_3). The mean body weights of the different male morphotypes in the mixed-sex population are shown in Table 2. The females reached an average weight of 45.1 ± 0.6 g (Fig. 2a). The values for the prawns in the first (F_1), second (F_2) and third (F_3) all-male generations are shown in Table 2 and Fig. 3a, c and e, respectively.

For purposes of comparison of the distribution of morphotypes in the mixed-sex and all-male populations, the fraction of each morphotype in the mixed-sex populations was calculated as a percentage of the male fraction of those populations. In the latter populations, most of the males were OC (37%) and BC (31%) individuals, with SM (19%) and NC individuals (13%) comprising a smaller part of the male fraction (Fig. 2b). In the first (F_1) all-male generation pond, most of the prawns (57%) were OC males (vs SM 20%, BC males 13% and NC males 20%; Fig. 3b). Similarly in the second (F_2) and third (F_3) all-male generation ponds most of the prawns were OC males, i.e., 45% and 66%, respectively (Fig. 3d and f). The distribution of the other morphotypes for second and third generation ponds were, respectively: SM 23% and 30%, BC males 23% and 1%, and NC males 9% and 3% (Fig. 3d and f). Thus, all the populations showed the typical *M. rosenbergii* morphotypic differentiation; representative

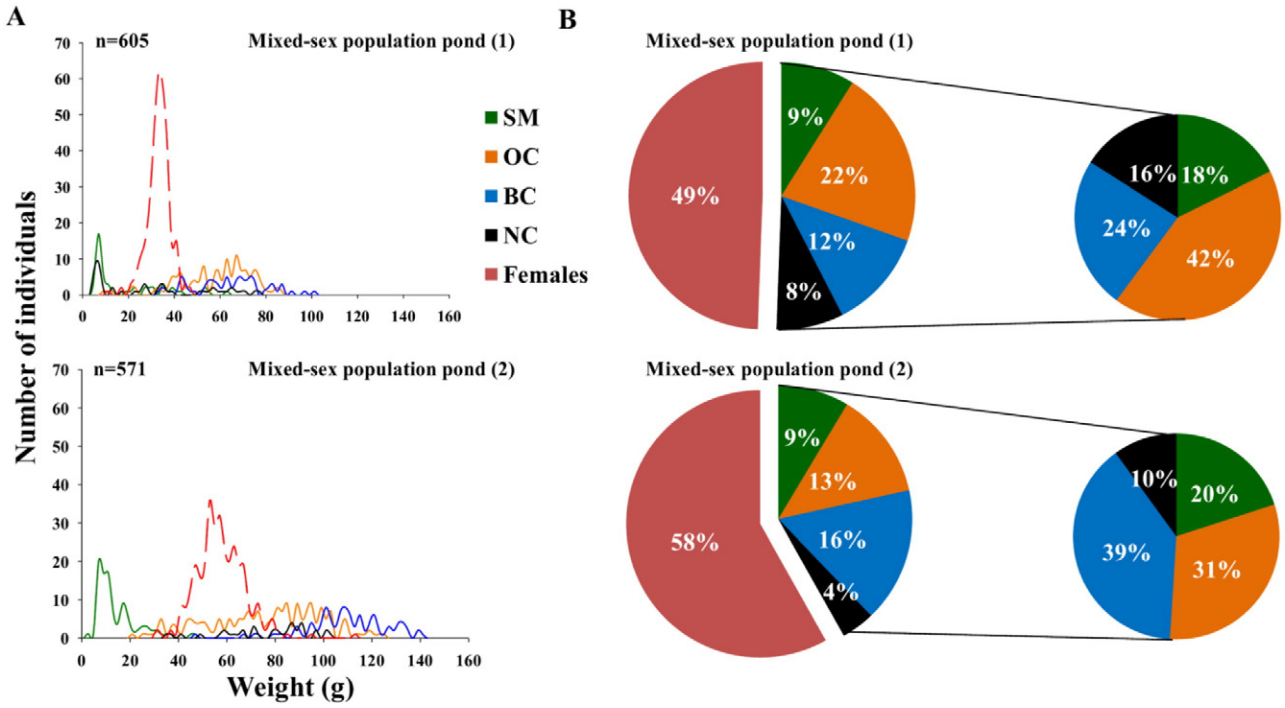

Fig. 2. Distributions of male morphotypes in representative mixed-sex population ponds. (A) Weight distribution of male morphotypes and females in two representative mixed-sex populations. (B) Frequency of male morphotypes and females in each population (left) and male morphotype frequency within the male fraction (right). SM — small male, OC — orange claw, BC — blue claw, NC — no-claw.

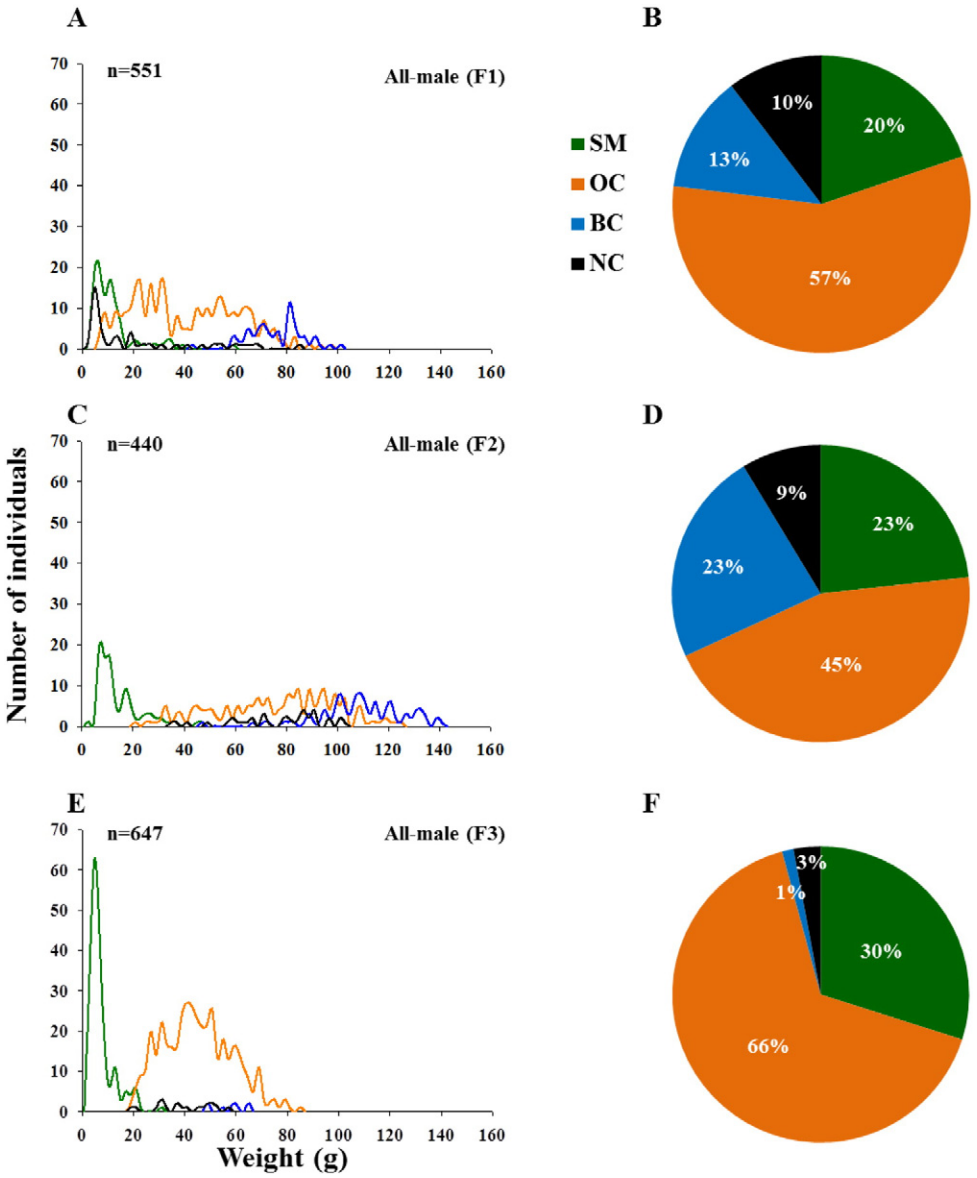

Fig. 3. Distributions of male morphotypes in three consecutive generations of all-male populations (F_1–F_3). Weight distribution (A) and frequency (B) of male morphotypes in F_1. Weight distribution (C) and frequency (D) of male morphotypes in F_2. Weight distribution (E) and frequency (F) of male morphotypes in F_3. SM — small male, OC — orange claw, BC — blue claw, NC — no-claw.

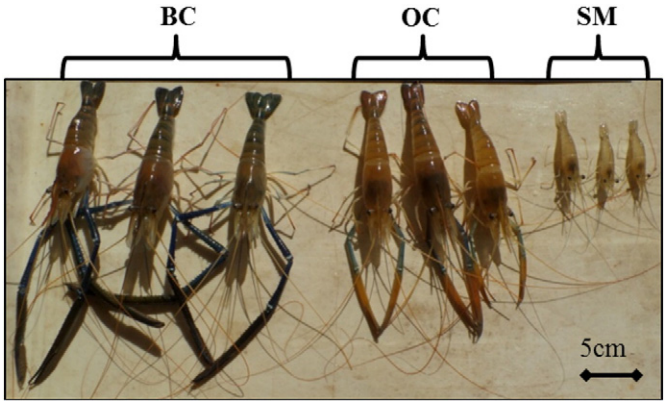

Fig. 4. Representative male morphotypes from the F_3 all-male population: SM — small male, OC — orange claw, BC — blue claw.

specimens of F_3 BC males, OC males and SM are shown in Fig. 4. In addition, it appeared that in the all-male populations OC was the most prevalent morphotype, whereas in the male fraction of the mixed population ponds the OC and BC morphotypes were the more prevalent morphotypes.

3.2. Reproductive characters

The male reproductive system of two representative BC males, one from a mixed-sex population and the other from a third generation all-male population, were examined histologically. In both, testis sections showed numerous testicular lobes. Spermatogonia – oval shaped cells with a nucleus consisting mainly of euchromatin – were present in the periphery of each lobe (Fig. 5). The cells in the center of each lobe towards the lumen comprised mainly mature spermatozoa (a considerable number) and sperm cells with an 'everted umbrella' appearance (Fig. 5). Sections of the terminal ampulla of the sperm duct with its adjacent AG obtained from the BC males from both types of population showed normal morphology, with the AG cells of the prawn from

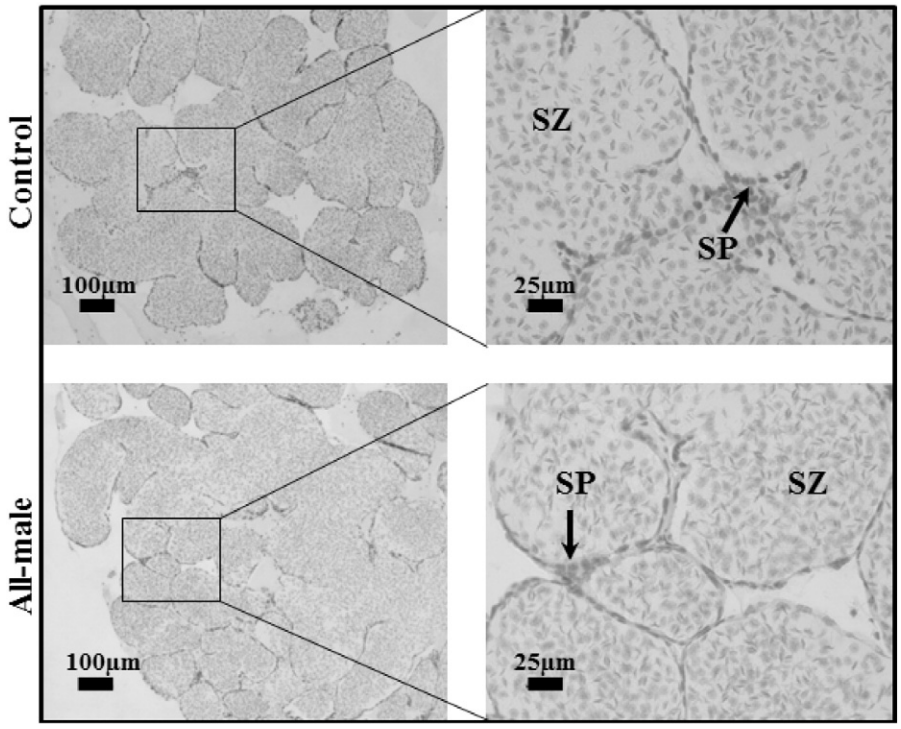

Fig. 5. Representative sections of testicular lobes of BC males from the mixed-sex population (control) and from the F_3 all-male population. SP — spermatogenic cells, SZ — mature spermatozoa.

the all-male population being slightly bigger and less condensed (Fig. 6). For both, the cellular organizations of the AGs were present in typical cord-like structures and the terminal ampulla contained the spermatophore with a large number of mature spermatozoa having typical morphology (Fig. 6).

3.3. Expression levels of male-specific transcripts

Mr-IAG and *Mr-Mrr* expression were examined in representative BC males from the mixed-sex and third-generation all-male populations. *Mr-IAG* and *Mr-Mrr* transcripts were shown by RT-PCR to be expressed

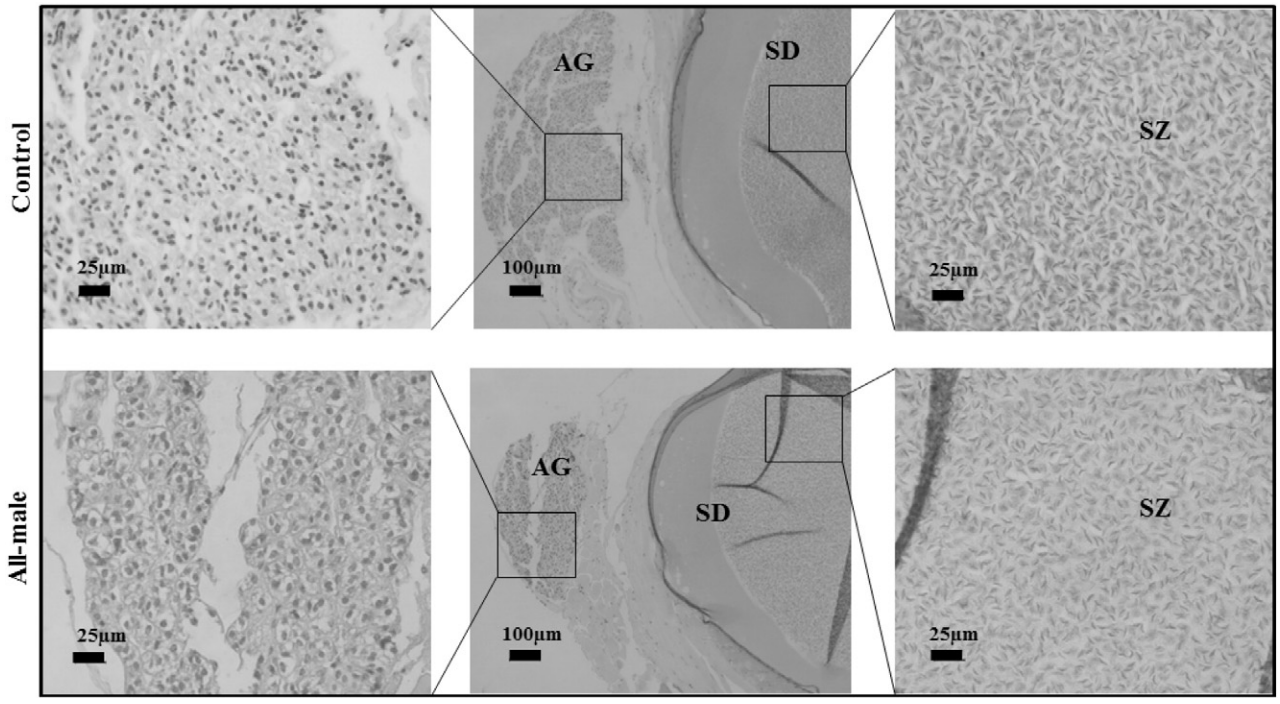

Fig. 6. Representative sections of the androgenic gland (AG) and terminal ampulla of the sperm duct (SD) of BC males from the mixed-sex population (control) and from the F_3 all-male population.

in all the RNA samples from the AG and sperm duct, respectively, in both BC males compared to the positive control, *Mr-β-actin* (Fig. 7).

4. Discussion

The present study constitutes the first investigation of a third-generation pure homogametic (ZZ) all-male cultured crustacean population. This all-male population was obtained by sex reversal through the production of RNAi manipulated 'neofemales' (genetic males) (Ventura et al., 2012) in each consecutive generation (see Fig. 1). The findings showed that this long-term reproductive process, occurring in the absence of the W chromosome, yielded the typical *M. rosenbergii* male morphotypes in all consecutive all-male progenies ($F_1 - F_3$), namely, an adult population that is highly heterogeneous in terms of prawn size and that includes the main three male morphotypes with the unique morphological claw features described by Kuris et al. (1987). In comparison with the mixed-sex cultures, in which the fractions of BC and OC males were similar, in the all-male populations the largest fractions of males were the fast-growing OC males, with lower numbers of BC males. This observation is supported by all-male growout experiments in India that showed the same trend of a high frequency of OC males and a low frequency of BC individuals in all-male populations, i.e., 63.5–79.9% and 0.7–7%, respectively (Aflalo et al., 2014). However, differences in density (as seen in F_3 versus F_{1-2}) could affect morphotypic differentiation.

Sex heritability studies have suggested that the size variation among *M. rosenbergii* males is mainly environmentally mediated – and less so genetically – being driven by social factors within the population (Malecha et al., 1984). Therefore, the absence of females in the all-male populations could be a factor driving the males toward somatic growth rather than spending energy on reproduction. This notion is supported by the fact that the percentage of the fast-growing OC males in the all-male populations was higher than that in the male fractions of the mixed populations in our study. This tendency constitutes an advantage for aquaculture farmers in that OC males have the highest growth rate potential in the population and, as described previously, are less territorial than the BC males (Barki et al., 1991, 1992; Ra'anan and Sagi, 1985).

In a study focusing on the temporal effect of dsRNA in the silenced tissues together with the population structure of the first generation, Lezer et al. (2015) provided supporting evidence for the safety of the usage of RNAi for establishing all-male cultures. The current study differs from that of Lezer et al. (2015) in that we investigated an F_3 generation of all-male vis-à-vis males from a conventional mixed-sex population. In addition, we focused on the male sex organs (testis, terminal ampulla) and on the male-specific gene expression (*Mr-IAG* and *Mr-Mrr*) in BC males, which is indicative of normal reproductive activity.

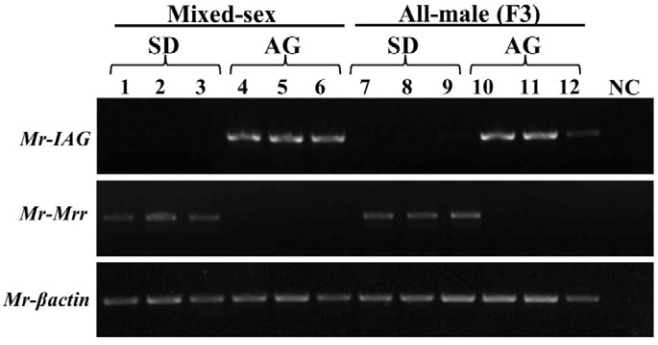

Fig. 7. Male reproductive system-specific gene expression. *Mr-IAG* and *Mr-Mrr* expression in sperm ducts (SD) from three BC males from a mixed-sex population (1–3) compared to three all-male BC males (7–10) and in AG cells from three BC males from a mixed-sex population (4–6) compared to three all-male BC males (10 − 12). All samples were compared to a positive control of endogenous β-actin.

In the present study, the examination of sexual morphology and reproductive output was performed on BC males, because BC males represent the culmination of male development: these terminally molted males – as the dominant males – exhibit the highest mating success and thus their reproductive system is highly developed and active (Barki et al., 1992; Ra'anan and Sagi, 1985; Ventura et al., 2011). We found that the morphology of the testis in F_3 BC males is the same as that of BC males in the mixed-sex populations. Testes from both types of BC males consisted of testicular lobes that exhibited regular spermatogenesis, spermatogonia in the peripheral part of each lobe and toward the center of the lobe, both contained considerable amounts of 'everted umbrella' spermatozoa, as described elsewhere (Lynn and Clark, 1983; Okumura and Hara, 2004; Sagi et al., 1988). The morphology of the terminal ampulla of the sperm duct and the adjacent AG seemed similar, with typical organization (cord-like structures), in both the F_3 BC male and the BC male from a mixed-sex population, in accordance with previous studies (Okumura and Hara, 2004; Ventura et al., 2011).

As mentioned above, the morphology of the AG is mostly dependent on the expression of *Mr-IAG* (Ventura et al., 2009), with *Mr-IAG* being highly expressed in the dominant BC males (Ventura et al., 2011). Moreover, similar expression patterns of this male specific-gene were demonstrated in both the F_3 BC male and the BC male from a mixed-sex population. In addition, the expression of the *Mr-Mrr* gene, uniquely expressed in the epithelial cells of the vas deferens and terminal ampulla, was investigated. Phoungpetchara et al. (2012) suggested that Mr-Mrr is transferred to sperm and becomes the main component of the anterior spike baseplate, a structure known to be important for the acrosome of mature spermatozoa. Thus, the role of *Mr-Mrr* in sperm capacitation and fertilization establishes it as a marker for tracing abnormal changes during the production of all-male progenies from RNAi-manipulated 'neofemales'. The current study showed similar expression of *Mr-Mrr* in both a representative F_3 male and a BC specimen from a mixed-sex population. However, the relationship between *Mr-IAG* and *Mr-Mrr* is yet to be examined.

Most publications refer to the chromosomal sex determination system in decapod crustaceans as the W/Z system (Katakura, 1989). It is noteworthy that, to date, this sexual heritability scheme is supported solely by progeny testing studies (Katakura, 1989; Parnes et al., 2003; Ventura et al., 2012). In our study, this scheme was once again confirmed in *M. rosenbergii* by several crosses between sex-reversed animals (Fig. 1). Unlike in other animal groups, the W/Z system has never been confirmed visually in decapod crustaceans by tracing the different sex chromosomes through karyotyping. It seems that the lack of karyotype characterization is largely due to technical problems resulting from the large numbers and small size of the chromosomes in crustaceans (e.g., our model organism *M. rosenbergii*, which seems to have 59 chromosome pairs) (Choudhary et al., 2013; Damrongphol et al., 1991; Justo et al., 1991; Mlinarec et al., 2016; Morelli et al., 1998). Assuming that the sexual heritability model is indeed W/Z, our results that demonstrate the production of three generations without the W chromosome raise important questions that need to be addressed; for example: Which sex-determining genes reside within the W and Z sex chromosomes? What are the gene targets of such sex switching regulators? And what is the importance of maternal effects when raising consecutive generations without true genetic females?

Here, we demonstrated normal morphology and function of all-male cultures along three generations; these findings provide support for the safety of the use of the RNAi-based biotechnology in prawn aquaculture for yield improvement through monosex culture. Such culture relies on a genetically responsible use of a combined approach comprising independent selective breeding programs towards best performing males of selected strains, in parallel with sex reversal of males. According to this approach, sex-reversed males (neofemales) of one of the selectively

bred strains should be crossed with selected males from a reciprocal strain and the all-male progeny will be used for grow out. Such a combined approach is aimed at genetic improvement of the selected strains while preventing inbreeding that could result from using neofemales and males from the same strain (Aflalo et al., 2012). Such technology could be adopted for various additional aquaculture and environmental applications.

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