Aquaculture 435 (2015) 157–166

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

On the safety of RNAi usage in aquaculture: The case of all-male prawn stocks generated through manipulation of the insulin-like androgenic gland hormone

Yaara Lezer, Eliahu D. Aflalo, Rivka Manor, Omri Sharabi, Lihie Katzir Abilevich, Amir Sagi *

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

ARTICLE INFO

Article history:
Received 12 May 2014
Received in revised form 22 September 2014
Accepted 23 September 2014
Available online 2 October 2014

Keywords:
Gene silencing
Insulin-like androgenic gland hormone
Macrobrachium rosenbergii
Monosex culture
RNA interference
Sex reversal

ABSTRACT

The recent introduction of RNA interference (RNAi) based biotechnology in aquaculture has aroused public awareness regarding safety aspects related to the use of such temporal transcript manipulation. In the present study, we demonstrate use of this biotechnology in large-scale production of all-male freshwater prawn *Macrobrachium rosenbergii*, relying on a two-phased approach. First, the insulin-like androgenic gland hormone (*Mr-IAG*) was silenced by dsRNA injections, causing sex-reversal of males into 'neo-females' (genetic males with female phenotype, with 86% success in this study). The next step involved mating the neo-females with normal males to produce all-male progeny. The administered exogenous dsRNA was fully cleared from the prawn's tissues after 7 days. Gene silencing through ds*Mr-IAG* appeared to be temporary, with decay seen over time and nearly full expression of *Mr-IAG* (~82%) being restored after 28 days. Assessment of reproduction and fecundity in normal females versus neo-females revealed that in both groups, ~80% of the prawns were in a reproductive state and no significant difference was found in their reproductive output, either in terms of brood mass or number. Population structure, as indicated by the normal occurrence of male morphotypes, in both mixed-sex and all-male populations was examined in terms of weight and morphotype frequency distribution. The final harvest and marketing of the all-male prawn offspring occur about 19 months, i.e., one generation after the actual RNAi procedure. Thus, on top of the aquaculture yield advantage, the use of such RNAi procedure in crustacean aquaculture seems to be safe due to its temporary nature.

1. Introduction

Monosex culture offers numerous advantages over mixed-sex culture, in many sectors of agriculture and aquaculture. The potential benefits of monosex populations vary from enhancement of growth, reduction of sexual/ territorial behavior and prevention of environmental risks posed by establishment of escaped exotic populations (Sagi and Aflalo, 2005). Monosex culture has been established in the fish production sector (Bye and Lincoln, 1986; Mires, 1977; Tayamen and Shelton, 1978) and over the last 30 years, different strategies of achieving monosex culture have been attempted in the crustacean production sector as well (Curtis and Jones, 1995; Lawrence, 2004; Lawrence et al., 2000; Sagi et al., 1986; Siddiqui et al., 1997).

Such approaches would be beneficial for aquaculture of the giant freshwater prawn, *Macrobrachium rosenbergii*, one of the most important crustaceans in inland aquaculture production in many tropical and sub-tropical countries. Indeed, in 2011, the total world-wide farmed volume of *M. rosenbergii* reached more than 200,000 tons, with an estimated market value of more than $1 billion (FAO, 2011).

Many crustacean species present a dimorphic pattern of growth in which females are larger than males or vice-versa. In *M. rosenbergii*, males grow faster than females and reach higher weights (Sagi and Aflalo, 2005). Therefore, producing an all-male monosex population is economically valuable. One approach to create a monosex population is via sexual manipulation. In crustaceans, sexual differentiation is controlled by the androgenic gland (AG). This unique organ, found in male crustaceans, regulates the expression of primary and secondary sexual characteristics (Sagi and Khalaila, 2001). A crustacean AG hormone was first reported in 1987 in the isopod *Armadillidium vulgare*. Protein sequencing of this hormone and that from other isopod species showed similarity to members of the pro-insulin superfamily (Ohira et al., 2003). In 2007, the first insulin-like androgenic gland hormone (IAG) from the aquaculturally important decapod group was described (Manor et al., 2007), although its functionality remained unknown for several years.

Fire et al. (1998) observed that double-stranded RNA (dsRNA) in *Caenorhabditis elegans* was the source of sequence-specific inhibition of protein expression, a process they called RNA interference (RNAi).

* Corresponding author.
E-mail address: sagia@bgu.ac.il (A. Sagi).

http://dx.doi.org/10.1016/j.aquaculture.2014.09.040
0044-8486/

This discovery marked a breakthrough, allowing researchers to reveal novel genetic mechanisms and gene functions in metazoans, invertebrates and vertebrates (Couzin, 2002; Dorsett and Tuschl, 2004; Kamath et al., 2003; Robalino et al., 2005; Sagi et al., 2013). Indeed, RNAi-mediated silencing of the insulin-like androgenic gland hormone in *M. rosenbergii* (*Mr-IAG*) revealed the functionality of the gene (Ventura et al., 2009). Injections of ds*Mr-IAG* into males in early post-larval stages caused complete and functional sex-reversal into neo-females (i.e., genetic males with a functional female phenotype) (Ventura et al., 2012). Such complete sex-reversal enabled the large-scale production of all-male populations by a two-phase approach, as first presented by Aflalo et al. (2006). This two-phase biotechnology was since updated with the use of RNAi being introduced in the following scheme. In the first phase, the *IAG* is silenced through dsRNA injections into post-larval males, resulting in the production of neo-females. The second phase involves crossing the neo-females with normal males to produce all-male progeny. A prerequisite for successful application of these RNAi-based biotechnologies has been the use of a sex-specific marker in *M. rosenbergii* to enable the identification of genetic sex at early developmental stages when phenotypic differences are not identifiable (Ventura et al., 2011). All the above components have been integrated into a commercialized technology representing the first RNAi-based biotechnology applied in the entire field of aquaculture (Ventura and Sagi, 2012).

In the current study, we report a phase I sex-reversal success rate of 86% achieved through RNAi, a value that enables large-scale production of a monosex aquaculture population so as to increase yields. However, due to its novelty, uncertainties have been raised by stakeholders in the aquaculture sector regarding the following issues: a) possible transmission to further generations as found in other organisms (Grishok et al., 2000); b) the safety of use of dsRNA in terms of retention time and effect; and c) the normal development of the sex-reversed animals and their all-male progeny. We have addressed some of the above issues with respect to clearance of the transmitted factor from the organism and the decay of its effect at the transcript level, as well as from the points of view of broodstock reproductive output and next-generation offspring population performance.

2. Materials and methods

2.1. Clearance of injected dsRNA

To verify the clearance of an injected exogenous dsRNA, different sensitivity range methods were used, namely detection of exogenous non-targeting dsRNA via RT-PCR, quantitative RT-PCR and via dot blot. The techniques are described below.

2.2. Detection of dsRB via RT-PCR

2.2.1. Animals

M. rosenbergii juvenile males (1.96–7.2 g body weight) were collected from artificial ponds at Ben-Gurion University of the Negev, as previously described (Ventura et al., 2012). The males were used to study the retention time of an exogenous dsRNA in their bodies. Remebee (*RB*), a dsRNA formulated to silence the Israeli Acute Paralysis Virus (IAPV, accession no. NC_009025) affecting bees (Maori et al., 2009), supplied by Beeologics (Rehovot, Israel), was used as a non-targeting exogenous dsRNA. This ds*RB* sequence was chosen due to the fact that it does not align to any *M. rosenbergii* mRNA sequences and hence serves as a suitable control. Twenty-one individuals were injected with 5 μg/g body weight ds*RB* into the sinus at the base of their fifth walking leg. After injection, the males were maintained in 100-L tanks (each individual was maintained separately in a floating basket sized 30 × 20 × 10 cm with 0.5–1 cm^2 net pore size) for 3, 7 or 14 days. At each time-point, the animals were anesthetized on ice for 10 min, followed by surgical removal of the base of their fifth walking legs, including the presumed location of the AG, for total RNA extraction. Same-sized juvenile males served as negative and positive controls for the presence of ds*RB* in the hemolymph. T_0-negative controls included samples collected before ds*RB* injection and T_1-positive controls included samples taken one hour after ds*RB* injection. To increase sensitivity these samples were used for a quantitative real time RT-PCR as well (see Section 2.3).

2.2.2. RT-PCR

Total RNA was isolated from dissected fifth walking leg bases using the EZ-RNA Total RNA Isolation kit (Biological Industries, Beit Haemek, Israel), according to the manufacturer's instructions. Twenty μl of hemolymph also was extracted from juvenile males, diluted in 80 μl crayfish saline (194 mM NaCl, 5 mM KCl, 12 mM KH_2PO_4, 33 mM $NaHCO_3$, pH 7.5). 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 then was amplified by PCR in a mixture containing 50 ng of cDNA, 1 mM of forward primer and 1 mM of reverse primer, 10 μl of Ready Mix REDTaq (Sigma) and water to a final volume of 20 μl. The PCR conditions used were 30 s at 94 °C, 30 s at 61 °C and 30 s at 72 °C, followed for 34 cycles. *RB* detection was demonstrated through PCR amplification using forward (5′- GCATGAAGAGAGCGTTTTTAATAC CT -3′, nt 6266–6291) and reverse (5′- GGGCACCTCGACCATCACT -3′, nt 6478–6496) *RB*-specific primers. *Mr-actin*, accession no. AF221096, was utilized as a positive control using specific forward (5′-GAGACCTT CAACACCCCAGC-3′) and reverse (5′-TAGGTGGTCTCGTGAATGCC-3′) primers. RT-PCR products were electrophoresed in a 1.2% agarose gel and visualized by ethidium bromide staining and exposure to UV light.

2.3. Detection of dsRB via absolute real-time RT-PCR

RNA was extracted from 5th walking leg base of *M. rosenbergii* males 3, 7, and 14 days after ds*RB* injection. RNA also was extracted from 30 μl hemolymph samples prior to ds*RB* injection (T_0-negative control) and 1 hour after injection (T_1-positive control), as described above. Known doses of pGEM-T easy (Promega), which included insertion of *RB* (in a range of 100 ng–1 pg) were used as a standard curve. ds*RB* was first heated to 75 °C for 10 min to denature the strands as described by Garbutt et al. (2013). Then, first-strand cDNA was synthesized by means of a reverse transcriptase reaction using the Verso cDNA Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, using 1 μg of total RNA as template. qRT-PCR of *RB* was obtained using the following primers: RB qRT-PCR_F: 5′-ATTGACCGAGTATATGCAAA TGCG -3′ and RB qPCR_R: 5′- CACCTCGACCATCACTCTAAGC -3′ with the FastStart Universal Probe Master (Rox) and Probe 5'- TGGGTAAC CATAGAATGATGCCTCCAGAA -3′ (TIB MOLBIOL, Berlin, Germany).

The quantity of *RB* was calculated for each time point according to the linear curve fit. The data were analyzed using the Kruskal–Wallis test by STATISTICA software.

2.4. Detection of dsGFP via dot blot

2.4.1. Animals

Fourteen *M. rosenbergii* males (17.92 g, average body weight) were collected as described above. Thirty μl of hemolymph were extracted from each male. The prawns were injected (5 μg/g body weight) with dsRNA of green fluorescent protein (ds*GFP*) (720 bp, accession number U55762.1), synthesized in our laboratory using a TranscriptAid T7 High Yield Transcription kit (Thermo Fisher Scientific), according to the manufacturer's instructions. One hour after injection, 30 μl hemolymph was again collected from each prawn (T = 1 h, positive control) for RNA extraction. After injection, the males were maintained in isolation as described above for 3, 7, 14, 21 or 28 days. At each time point, 100 μl hemolymph were collected from one or two prawns which then were anesthetized on ice for 10 min, followed by surgical removal of their hepatopancreas, muscle and fifth walking legs for total RNA extraction.

2.4.2. RNA extraction

Total RNA was isolated from about 100 mg hepatopancreas, muscle, fifth walking leg bases and 30 or 100 μl hemolymph as described above at each time point (T = 0, T = 1 hour, and 3, 7, 14, 21 and 28 days after injection). At the end of the RNA extraction, samples were resuspended with 20 μl DDW DEPC (diethylpyrocarbonate) and stored at −20 °C until use.

2.4.3. Probe preparation

A dsRNA probe was synthesized for detection of ds*GFP*. The probe was targeted to anneal to *GFP* nucleotide positions 679 to 1398 using a TranscriptAid T7 High Yield Transcription kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Digoxigenin-11-UTP (Roche) was added to the reaction at a final concentration of 2.5 mM. The RNA product was purified by ethanol precipitation followed by hybridization of the two strands (70 °C for 15 min, 65 °C for 15 min and incubation at room temperature for 30 min). The dsRNA probe was subjected to alkaline hydrolysis for 33 min to obtain a probe length of ~200 bp, according to the DIG Application Manual for Nonradioactive *In Situ* Hybridization (Roche). The dsRNA probe was maintained at −80 °C until use.

2.4.4. Dot blot

Ten μl from the RNA samples was incubated at 68 °C for 10 min with loading buffer (containing 50% formamide, 16.6% formaldehyde and 10% morpholineopropanesulfonic acid, MOPS). Then the samples were chilled on ice for 1 min and spotted onto a nitrocellulose membrane, followed by UV crosslinking. The membrane was pre-hybridized with pre-warmed DIG Easy Hyb (Roche) at 68 °C for 30 min, and incubated overnight at 68 °C with DIG Easy Hyb containing the DIG-labeled denatured probe, as described in the DIG Application Manual for Filter Hybridization (Roche). The membrane was washed twice with 2× saline sodium citrate (SSC) solution containing 0.1% SDS at room temperature for 5 min and then twice more with 0.1xSSC containing 0.1% SDS at 68 °C for 15 min. The following steps were conducted at room temperature, based on the DIG Application Manual for Filter Hybridization. Briefly, the membrane was washed with washing buffer for 2 min, treated with blocking solution for 30 min and incubated with anti-DIG-alkaline phosphatase (1:10,000) in blocking solution for 30 min. The membrane was washed twice with washing buffer for 15 min and with detection buffer for 2 min. Ready-to-use CDP-Star (Roche) was added to the membrane and the signal generated was detected using a LAS-4000 chemiluminescence detection system (Fujifilm). Signals were quantified using ImageQuant TL V8.1.

2.5. Assessing the effect of dsRNA injection on gene expression over time

2.5.1. Animals

Forty-two *M. rosenbergii* males (2.0 − 7.2 g body weight) were collected as described above. These individuals were used to study the time-course of the knockdown of an endogenous *Mr-IAG* transcript. Twenty-one individuals were injected with ds*Mr-IAG* (treatment group, 5 μg/g body weight), while 21 individuals were injected with ds*RB* (5 μg/g body weight) to serve as a control group that included all the components of the treatment but the tested RNA.

After injection, the males were maintained separately in 100-L tanks as described above, for 3, 21 or 28 days. For each time-point, 7 individuals from each group were anesthetized on ice for 10 min, followed by surgical removal of their fifth walking legs, including the presumed location of the AG, for total RNA extraction.

2.5.2. RNA extraction and real-time RT-PCR

RNA was extracted as described above 3, 21 and 28 days after injection. First-strand cDNA was synthesized by means of a reverse transcriptase reaction using the Verso cDNA Kit (Thermo Fisher Scientific) with 1 μg of total RNA. Relative quantification of *Mr-IAG* transcript levels was obtained using the following primers: IAG qPCR_F: 5′-GCCTTGCA GTCATCCTTGA-3′ and IAG qPCR_R: 5′-AGGCCGGAGAGAAGAATGTT-3′ with the FastStart Universal Probe Master (Rox) and Universal Probe Library Probe #144 (Roche). *Mr-18S* RNA (accession no. GQ131934), which served as an expression of normalizing gene, was quantified by means of real-time RT-PCR using the primers qMr-18S_F: 5′ CCCTAA ACGATGCTGACTAGC-3′ and qMr-18S_R: 5′ TACCCCCGGAACTCAAAGA-3′ with the above-mentioned mix and Universal Probe Library Probe #152 (Roche). Reactions were performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems).

2.5.3. Calculation of relative Mr-IAG expression

The average relative level of expression of *Mr-IAG* was calculated for every group of animals separately. The average level of expression in each treatment group was divided by the average level of expression in its control group and the relative expression percentage was calculated. The data were analyzed using the Mann − Whitney *U* test ($P < 0.05$).

2.6. Comparing representative mixed-sex versus neo-female populations

2.6.1. Source of animals

M. rosenbergii broodstock populations comprising normal females and neo-females were maintained at Ben-Gurion University of the Negev as previously described (Ventura et al., 2011) and fed daily with *Artemia* nauplii. Containers were aerated vigorously using air stones on the bottom of the tank. Juvenile prawns (at ~PL_{60}) of the same hatching batch were obtained and reared from April to September in two separate earthen ponds at the Dor Research Station, Israel, at a density of 3/m^2.

2.6.2. Sex-reversal through RNAi

All-male progeny of the neo-female broodstock were treated for IAG gene silencing by injection of ds*Mr-IAG* (Ventura et al., 2009). The use of all-male progeny at this stage was made to ensure that the individuals to be manipulated are males and that all the resulting neo-females are indeed genetic males. After injection, ~250 PL males were maintained in a 100-L tank with artificial seaweed to provide shelter. dsRNA prepared from the *Mr-IAG* open reading frame was supplied by Beeologics (Rehovot, Israel) and stored at −20 °C until use. Molecular weight and lack of degradation were assessed by electrophoresis on an agarose gel.

2.6.3. Female reproductive stage measurement

For such measurements, two groups of animals were used, a neo-female population comprising successfully sex-reversed males and a control population with a male-to-female ratio of ~1:1 (i.e., progeny of normal crossing). The parents of both experimental groups were taken from the same original population collected for the study as described above. At the end of the grow-out period, the prawns were collected and measured. The following variables were recorded for each individual prawn: gender (male or female), body length (in mm), weight (in g) and female reproductive stage (Aflalo et al., 2006; Hulata et al., 1990), classified as either virgin (immature female with narrow and shallow brood chambers), developed gonad (female with developed ovary noticeable through the carapace), spent (mature female with deep and wide brood chamber not carrying eggs), or berried (female bearing eggs). The total number of prawns harvested from each pond was recorded to calculate relative survival rates and the number of neo-female prawns was recorded to calculate sex-reversal success rates.

2.6.4. Fecundity measurements

Sixteen representative berried females (eight for each group) were separately collected from the neo-female and normal population ponds. Total body weight of each female with eggs and egg mass were measured. After recording total body weight, the eggs were carefully removed from the female brood chamber, weighed and collected into a microfuge tube. Under magnification of 4× under an optical microscope,

egg diameter and the total number of eggs were recorded. From these parameters, the BSI (brood somatic index), corresponding to the ratio between prawn egg mass to body weight and eggs/g body weight, were calculated.

2.7. Comparison of a mixed-sex population versus a representative all-male population progeny of a neo-female

2.7.1. Growth performance of all-male and mixed-sex population progeny

Two groups of animals were used, an all-male population corresponding to the progeny of sex-reversed males (neo-females), 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. Both populations were grown from April to September in two separate earthen ponds at the Dor Research Station, Israel, at a density of 3/m^2. The following variables were measured for each individual prawn at the end of the rearing period: gender (male or female), body length (in mm), weight (in g), and male morphotype. Males were classified according to their morphotypic appearance as blue-claw (BC), orange-claw (OC) or small-claw males (SM), as described by Kuris et al. (1987). Large males without claws were classified as no-claw males (NC).

2.8. Statistics

For population experiments, an R × C test of independence (a contingency table test where R and C stand for the number of rows and columns in the frequency table, respectively, is assembled) was used to test for differences in the distribution of male morphotypes (BC, OC and SM) or female reproductive stages (virgin, developed ovary, spent and berried) between the mixed-sex and all-male populations and between normal and neo-female populations, respectively. The statistics used were $G = 2 \times \ln(\text{likelihood ratio})$, which can be approximated by the χ^2 distribution (i.e., the G-test of independence) (Sokal and Rohlf, 1995).

Pair-wise differences in prawn weight among the different male morphotypes and female reproductive stages of the corresponding experimental groups (i.e., mixed-sex vs. all-male or normal female vs. neo-female populations) were evaluated using a Bonferroni- corrected post-hoc test. Differences in average weight between the experimental groups were tested using nested analysis of variance with morphotypes nested within treatments followed by planned comparisons. $P < 0.05$ was defined as the statistically significant difference.

The two-sample Kolmogorov–Smirnov test was used to test differences in body-weight distribution for each male morphotype between the mixed-sex and all-male populations and of female reproductive stages between mixed-sex and neo-female populations. All analyses were performed using Statistica v9.0 (StatSoft Ltd., Tulsa, OK).

3. Results

3.1. Clearance of injected exogenous dsRNA

Assessment of the rate of clearance of the injected exogenous dsRNA from prawn hemolymph and tissues is presented in Fig. 1. The presence of ds*RB* was detected by RT-PCR in *M. rosenbergii* males, which showed a clear band in the hemolymph 1 h after ds*RB* injection. ds*RB* also was detected at the base of the 5th walking leg 3 days after injection in some cases. No ds*RB* was detected at the base of the 5th walking leg 7 or 14 days after injection. No ds*RB* was found in the hemolymph prior to ds*RB* injection (T_0) (Fig. 1A). ds*RB* was quantified by absolute real-time RT-PCR. This assay is more sensitive and is capable to detect amounts as low as 1 pg. The amount of ds*RB* in the hemolymph was approximately 480 pg 1 h after ds*RB* injection. Three days after injection, the amount of ds*RB* in the 5th walking leg base reached the background values (as in T = 0, before injection) and considered as zero. Similarly, seven and 14 days after injection, no ds*RB* was detected (Fig. 1B).

The clearance of injected exogenous dsRNA from the hemolymph and tissues also was tested by dot blot (Fig. 1C). The standard curve in the top panel shows that this assay is sensitive to detect amounts as low as 10 pg of ds*GFP*. ds*GFP* could be detected in *M. rosenbergii* males 1 hour after injection in the hemolymph and all other tissues. However, the signal was ~10 times stronger in the hemolymph (5.6 ng) than in other tissues (~0.5 ng), according to quantified image analysis. The presence of ds*GFP* in the hemolymph decreased approximately 20-fold after 3 days (0.25 ng) and could be detected in the hemolymph at very low amounts (~0.03 ng) up to 21 days after injection. No ds*GFP* was detected after 28 days. ds*GFP* was no longer detected in the hepatopancreas from 1 h after injection, while in muscle and at the base of the 5th walking leg, ds*GFP* was still detected 3 and 7 days later, but not after 14 days. Prior to injection, no ds*GFP* was detected in the hemolymph or in the other tissues considered.

3.2. The effect of dsRNA injection on gene expression over time

The efficiency of gene silencing following dsRNA injection was demonstrated by the injection of ds*Mr-IAG*, which caused a significant ($p = 0.02$) reduction in expression (by ~97%) after 3 days, as compared to the control injected with exogenous ds*RB* (Fig. 2). A recovery of approximately 30% of the expression was noted 21 days after the injection and by 28 days, nearly full recovery (~82%), which was not significantly different from the control, was observed.

3.3. Reproductive stages and fecundity in females of a mixed-sex population versus a neo-female population

To test the growth performance of neo-females produced through RNAi, the average weight of neo-females at each reproductive stage was compared to those of normal females (from a mixed-sex population). Mean body weights at the developed gonad, spent and berried reproductive stages were significantly higher in the neo-female population, than in the normal female population (developed gonad: 32.9 ± 0.5 g and 32.1 ± 0.4 g; spent: 36.8 ± 1.4 g and 29.9 ± 2.5 g; berried: 37.8 ± 0.8 g and 31.2 ± 0.4 g for neo-females and normal females, respectively; $P < 0.05$) (Table 1). According to the Kolmogorov–Smirnov two-sample test results, there were significant differences in the weight distributions of these reproductive stages as well. Two-sided probabilities were calculated for the spent and berried reproductive stage groups, with significant differences between the normal and the neo-female populations ($P < 0.05$), of which the neo-female population weight distribution included individuals with higher weights, whereas no normal females presented such values (Fig. 3A). Considering the percentages of each female reproductive stage within each population, a similar distribution was seen in both populations, with a slightly lower percentage of developed-ovary and higher percentage of berried-eggs in the neo-female group, than in the normal female population (Fig. 3B). In the neo-female population, 630 of 731 treated post-larval animals underwent a full and functional sex-reversal into neo-females, an 86% success rate. The 99 out of 731 who showed no sex-related phenotypic change following ds*Mr-IAG* injection were considered 'failures'. Notably, these individuals could mate as males with the neo-females, thereby giving rise to fertilized neo-females at the end of the experimental period, enabling us to test their reproductive performance (see below). In both groups, ~80% of the females observed were at an active reproductive state. The reproductive output of neo-females was similar to that of normal females in the mixed-sex population, both in terms of BSI (10.0 ± 1 and 13.3 ± 2.0 in the neo-females and normal females, respectively) and number of the eggs per gram body weight (731 ± 93 and 859 ± 147 in the neo-females and normal females, respectively) (Table 2).

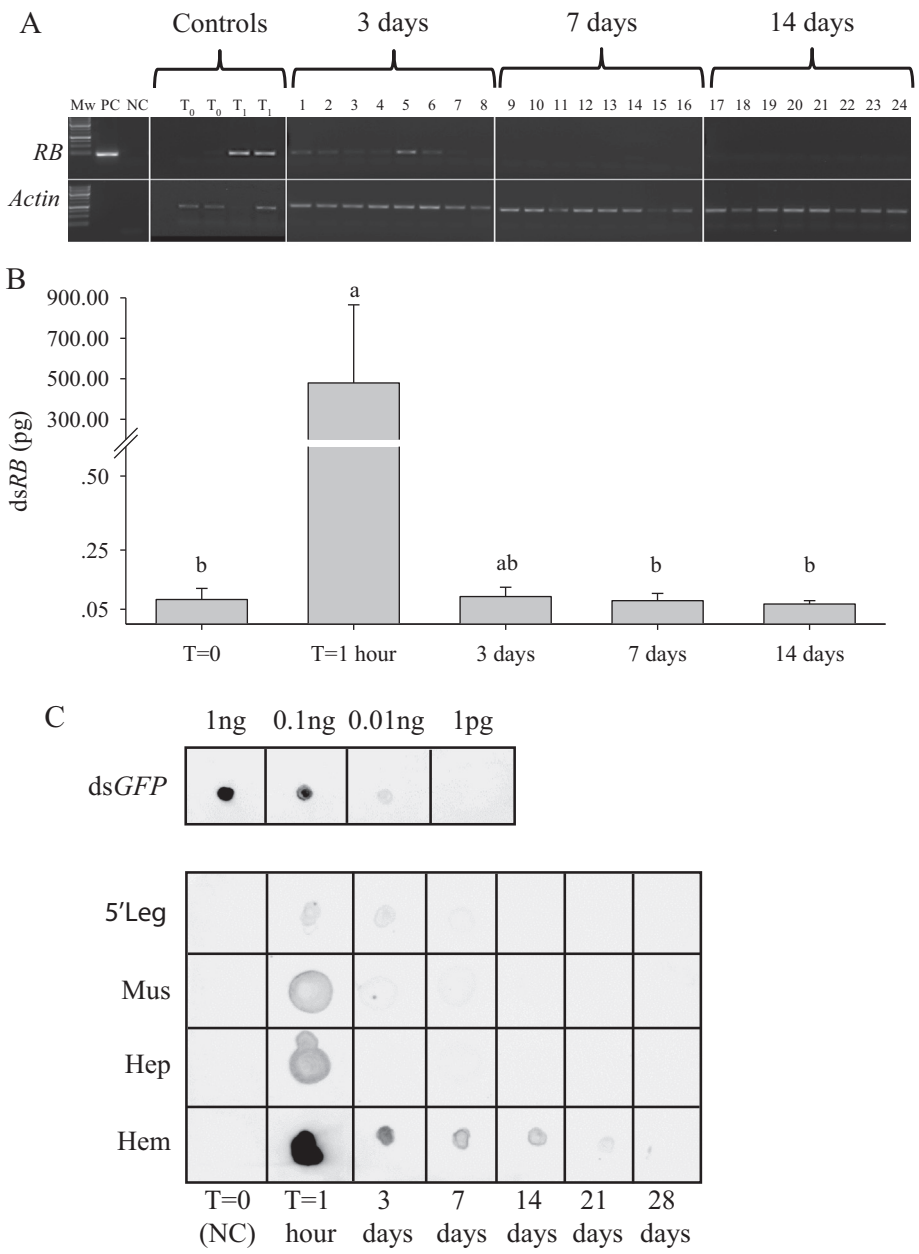

Fig. 1. Clearance of injected non-specific dsRNA in prawns. (A) Upper panels: RT-PCR detection of ds*RB*, bottom panels: positive control of actin as a housekeeping gene. From left to right: PC — positive control (clean exogenous ds*RB*) and NC — negative control (DDW). Detection of ds*RB* in the hemolymph of *M. rosenbergii* males prior to ds*RB* injection (negative controls T_0) and an hour after ds*RB* injection (positive controls T_1).The rest of the panels represent detection of ds*RB* in the 5th walking leg base 3, 7 and 14 days after ds*RB* injection. (B) Quantitative real-time RT-PCR of ds*RB* in *M. rosenbergii* males. In the hemolymph, prior to ds*RB* injection (negative control T = 0), an hour after ds*RB* injection (positive control T = 1) and in the 5th walking leg base 3, 7 and 14 days after ds*RB* injection. Bars represent SEM. Different letters represent significant differences (Kruskal–Wallis test: $H(4, N = 32) = 12.79329$; $p = 0.0123$). (C) Dot-blot detection of ds*GFP* in the hemolymph and other tissues of *M. rosenbergii* males. Prior to ds*GFP* injection (T = 0), an hour after ds*GFP* injection (T = 1 h) and at 3, 7, 14, 21 and 28 days after ds*GFP* injection. In the top panel, results of a dilution test demonstrating detection at concentration as low as 10 pg of ds*GFP*. NC — negative control, Hem — hemolymph, Hep — hepatopancreas, Mus — muscle, 5′Leg — 5th walking leg base.

3.4. Male morphotypes in a normal mixed-sex population versus a population comprising an all-male progeny of a neo-female

To test the performance of the all-male offspring of a neo-female, the weights of such males and their morphotypic distribution were compared to the male fraction of a representative mixed-sex population (Fig. 4). The comparison between the mixed-sex and all-male populations was confined to changes among the different male morphotypes in both groups, since no females were found in the all-male population. The mean body weights of SM and OC morphotypes was significantly higher in the mixed-sex population than in the all-male group (Fig. 4A) (SM: 18.1 ± 2.2 g and 10.7 ± 0.8 g; OC: 57.0 ± 1.4 g and 37.1 ± 1.0 g, respectively; $P < 0.01$). The mean body weight of the BC morphotype was marginally significantly higher in the all-male population, than in the mixed-sex group (Table 3). In both populations, the SM size distribution was confined to the lower range, with the SM in the mixed-sex population including significantly higher-weighing individuals. While the OC morphotype in the mixed-sex population followed the weight range of the BC group, the size distribution of the OC morphotype in the all-male population was broader with more individuals observed in the lower weight ranges. The BC size distribution was bimodal in the mixed-sex population with one peak around 45 g and

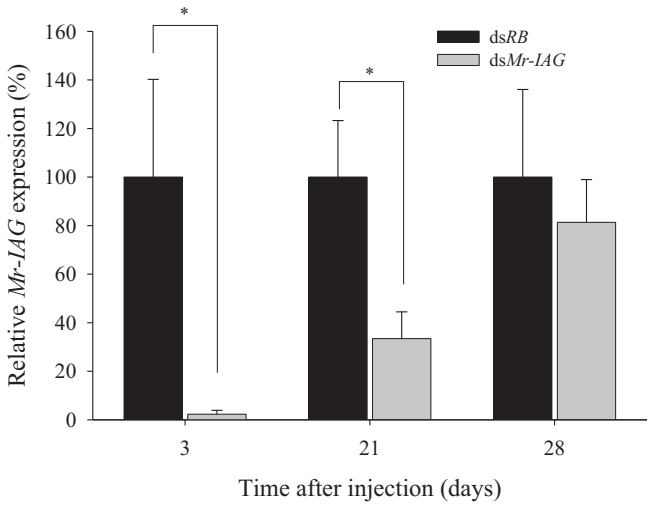

Fig. 2. The effect of a single injection of ds*Mr-IAG* on the expression of *Mr-IAG* in the androgenic gland after 3, 21 and 28 days. Controls represent expression levels of *Mr-IAG* in male individuals injected with exogenous ds*RB*. Bars represent SEM. Asterisks represent significant differences (Mann–Whitney U test, $P < 0.05$).

the other around 75 g. In contrast, the BC weight distribution in the all-male population was unimodal with a ~73 g average body weight (Fig. 4A).

Typical *M. rosenbergii* male morphotypes could be distinguished in both populations according to the morphometric parameters described by Kuris et al. (1987). Thus, at no developmental stage were abnormal morphometric traits detected in the all-male population. In both the mixed-sex and all-male populations, the SM morphotype comprised ~20% of the male population. While the percentage of OC morphotype was relatively high in the all-male population, the percentage of the BC morphotype in the all-male population was slightly lower than in the mixed-sex population (OC: 57% compared with 42%; BC: 13% compared with 24% in the all-male and mixed-sex populations, respectively).

Regarding marketable yields (taken as ≤50 gr), the all-male population in this study had a higher proportion of marketable individuals than did the mixed-sex population (35% and 27.5%, respectively). Moreover, in the mixed-sex population, no female reached this threshold. The yield of the all-male population was also higher than that of the mixed-sex population (517 and 442 kg/ha^{-1}, respectively).

4. Discussion

RNAi is a gene-silencing phenomenon triggered by dsRNA and involves the generation of 21-26 nt RNA segments that guide mRNA destruction (Grishok et al., 2001). Since its discovery (Fire et al., 1998), RNAi has rapidly gained importance as a "reverse genetics" tool to silence the expression of targeted genes in plants and lower animals for diverse applications, including functional genomics and comparative

Table 1
Growth performance of female reproductive stages in a normal female population versus a neo-female population.

Reproductive stage	Weight range (g)		Average weight (g)	
	Normal population	Neo-female population	Normal population	Neo-female population
Virgin	10.0–40.4	6.0–35.5	30.3 ± 0.6	22.3 ± 0.5
Developed gonad	19.3–41.0	13.0–71.0†	32.1 ± 0.4	32.9 ± 0.5*
Spent	16.0–40.0	9.5–66.5†	29.9 ± 2.5	36.8 ± 1.4*
Berried	24.0–48.0	22–75.5†	31.2 ± 0.4	37.8 ± 0.8*

* Significantly different (nested ANOVA followed by planned comparison test, $P < 0.05$).
† Significanty different (two-sample Kolmogorov–Smirnov test, $P < 0.05$).

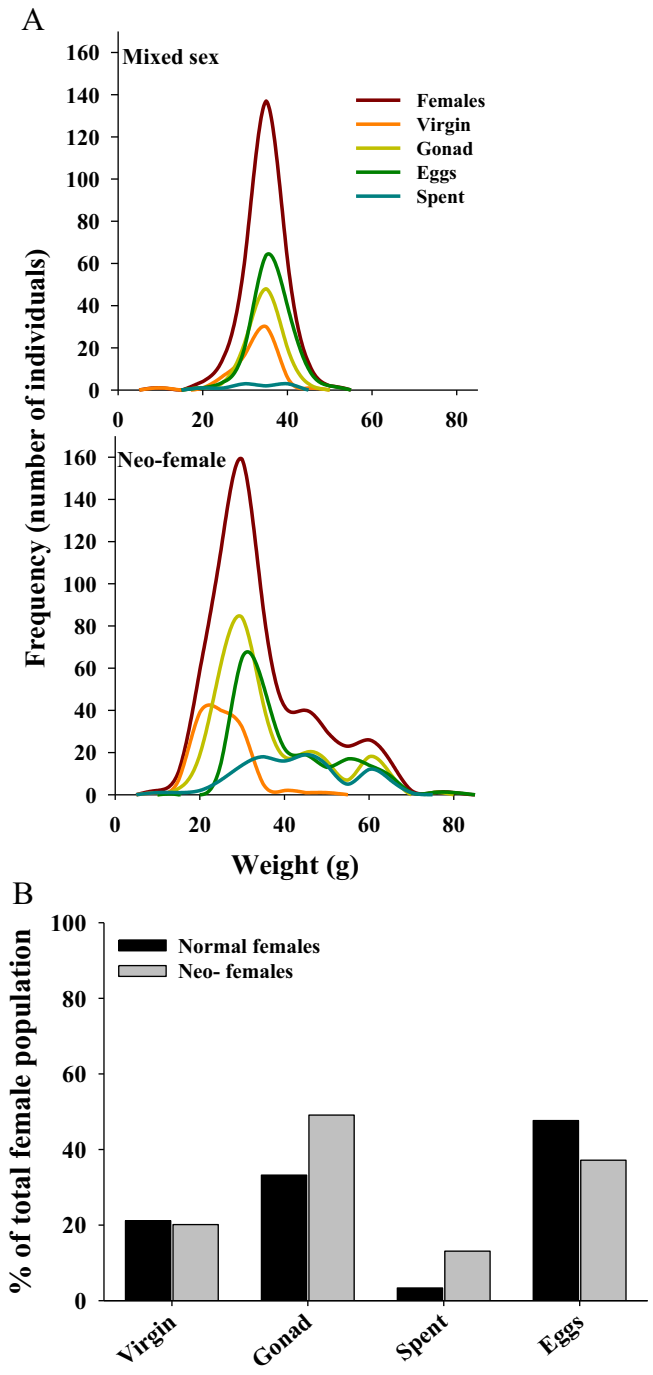

Fig. 3. Size distributions of female reproductive stages in representative mixed-sex and neo-female populations. (A) Distribution of female weights at different reproductive stages among the normal females (top) and neo-females (bottom) in ponds. (B) Frequencies of reproductive stages among females belonging to normal and neo-female populations. Total number of females (brown), virgin (orange), developed gonad (yellow), carrying eggs (green) and spent females, namely those who already experienced one or more round of carrying eggs (blue). Gray bars – normal females, Black bars – neo-females. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

development studies, as well as finding agriculture, pest control and therapeutic applications (Aflalo and Sagi, 2014; Huvenne and Smagghe, 2010; Kamath et al., 2003; Small, 2007; Vaishnaw et al., 2010). In recent years, RNAi-based research is gaining momentum in the study of crustaceans and is becoming an integral scientific component for the rapid and reliable study of crustacean gene functions (Copf et al., 2006; Kato et al., 2011; Sagi et al., 2013; White et al.,

Table 2
Reproductive output of normal females versus neo-females.

	Females	Neo-females
Body weight (g)	32.03 ± 0.28	41.31 ± 0.22
BSI (%)	13.3 ± 2.0	10.0 ± 1.0
Eggs/g BW	859 ± 147	731 ± 93

BSI — brood somatic index, BW — body weight. No statistical significance was found between the two groups for any parameter (Mann–Whitney U test, $P < 0.05$).

2011). The current study tested the first RNAi-based biotechnology in aquaculture introduced at the industrial scale designed to increase yields (Ventura and Sagi, 2012). This technology involves the administration of a dsRNA homologous to the *Mr-IAG* gene for post-transcriptional gene silencing. To better understand the potential impact of this technology, we produced a large population that enabled the study of sex-reversed *M. rosenbergii* neo-females and monosex progeny. The two-phase biotechnology used in the present study yielded 86% full and functional sex-reversal to neo-females. In former studies in which sex-reversal was achieved via ablation of the AG by microsurgery, far fewer cases of complete and functional sex-reversal from post-larval male to functional neo-female were observed (0.17–0.34%). Moreover, low survival rates due to difficulties in recovering from the surgery were observed (less than 50% survival rate in most cases) (Aflalo et al., 2006, 2012). Hence, the overall maturation rate to a neo-female capable of mating and raising progeny also was shortened significantly using the RNAi method, from twenty-two months following AG ablation approach to only 7 months in the current study.

4.1. On dsRNA injection and other genetic manipulations used in crustaceans and the issue of genetic modification

In agriculture, the stable and transmissible addition of foreign genes often has been used to generate plants able to produce novel proteins that prevent damage by pests or disease or to improve the chemical profile of food products (Anklam et al., 2002; Nature, 2013). In contrast to expression of a transgene, the suggested mechanism of RNAi involves the administration of dsRNA homologous to the gene of interest for temporal post-transcriptional gene silencing. This dsRNA is processed into approximately 21 nucleotide-long RNAs, known as small interfering RNAs (siRNAs), by the enzyme dicer. These siRNAs then serve as sequence-specific locators for the target RNA as part of the endonuclease activity of the RNA-induced silencing complex (RISC). The RISC targets homologous RNAs for degradation (Agrawal et al., 2003; Hannon, 2002; Qi and Hannon, 2005; Watson et al., 2005). We stress that all of the above steps do not involve any alteration of the genome of the targeted organism.

Previous interventions using anti-sense transcripts in cultured crustaceans were conducted in the context of viral defense and involved transgenic modifications with lasting effects. One such example is the transfection of an anti-sense DNA construct from a Taura syndrome virus (TSV) coat protein into *Litopenaeus vannamei* zygotes. This led to stable expression of an anti-sense transcript from the *TSV-CP* transgene (Lu and Sun, 2005). Other gene transfer-based attempts to cure prawn disease involved injections of foreign DNA into *M. rosenbergii* spermatophores (Li and Tsai, 2000). Introduction of a *Minos* transposable element was attempted for comparative developmental studies in crustaceans (Pavlopoulos and Averof, 2005). All of these genetic manipulations involved an artificial expression vector integrated into the host genome and thus able to affect later generations (i.e., yielding a genetically modified line). In the present study, we determined whether an introduced RNAi agent is stably expressed or cleared from the prawn. Using three different sensitivity ranged methods, we demonstrated that an exogenous dsRNA is cleared from the prawn tissues and could no longer be detected 28 days after introduction. Furthermore, the silencing effect of an endogenous dsRNA (ds*Mr-IAG*) was found to be temporary.

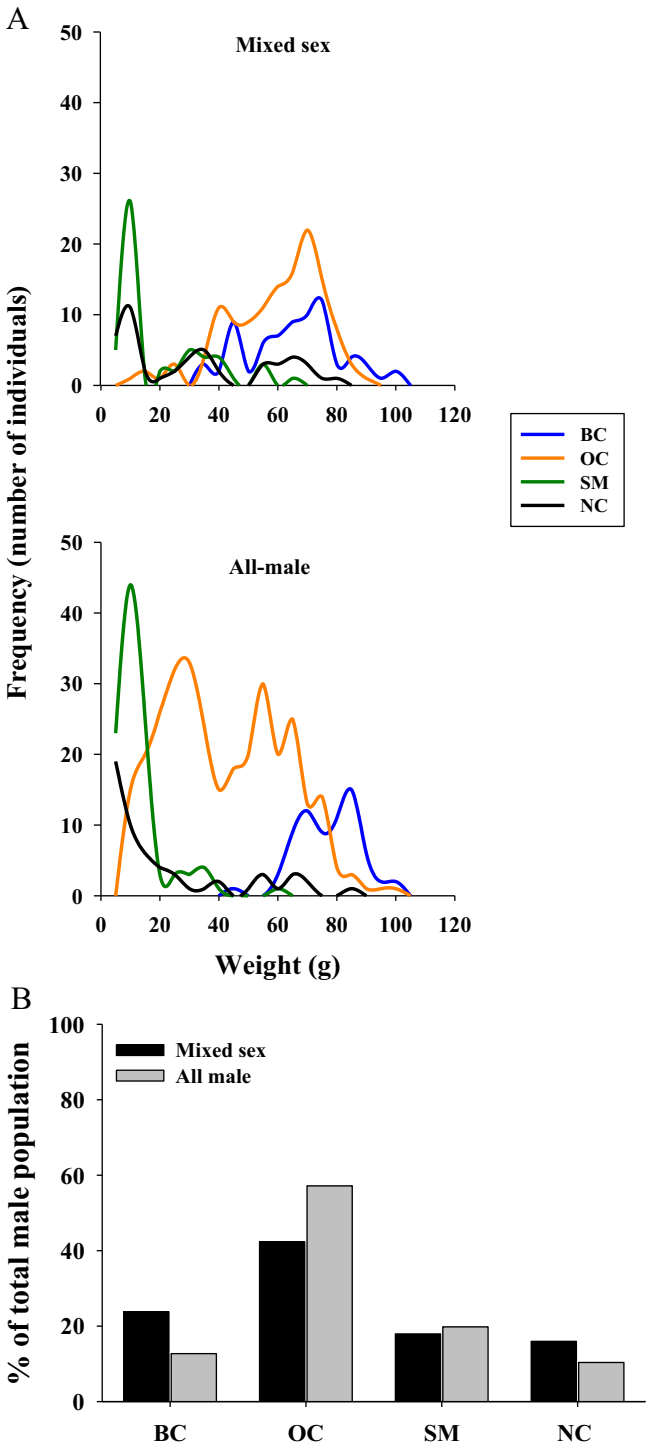

Fig. 4. Size distributions of male morphotypes in the male fraction of a representative mixed-sex population and a representative all-male population, progeny of a neo-female. (A) Distribution of male morphotypes weights in the male fraction of mixed-sex population (top) and in an all-male population (bottom). (B) Frequencies of morphotypes in males from mixed-sex (black bars) versus all-male (gray bars). BC – blue claw (blue), OC – orange claw (orange), SM – small male (green), and NC – no claw (gray). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Moreover, in the particular case of the two-phase biotechnology used for producing all-male prawn populations, dsRNA is injected only into first-generation post-larvae males. The resulting temporal silencing is enough to produce neo-females and the time from injection of a post-larva to its development into a fully matured neo-female is at least

Table 3
Growth performance of male morphotypes in a mixed population versus an all-male population.

Morphotype	Weight range (g)		Average weight (g)	
	Mixed-sex population	All-male population	Mixed-sex population	All-male population
SM	3.8–61.9	2.3–58.2††	18.1 ± 2.2**	10.7 ± 0.8
OC	8.7–85.6	6.0–97.0††	57.0 ± 1.4**	37.1 ± 1.0
BC	31.2–99.1	43.0–99.3††	62.7 ± 1.8	74.8 ± 1.2

SM — small male, OC — orange claw, BC — blue claw.
** Significantly different (nested ANOVA followed by planned comparison test, $P < 0.01$).
†† Significantly different (two-sample Kolmogorov–Smirnov test, $P < 0.01$).

8 months (i.e., 7 months after the silencing agent is no longer detected in the prawn; Fig. 5). Following crossing of the neo-female with a normal male, a second generation of all-male offspring is produced that are not injected with the silencing agent. The final harvest and marketing of the second generation (all-male offspring of the neo-female) occurs 19 months after the injection of RNAi into the first-generation animals. In other words, the RNA agent will have been cleared 8 months before neo-female broodstock selection, further arguing for the safety of the technology used here with respect to the transmission to subsequent generations. Still, transfer of an injected dsRNA to the germ line was reported in *C. elegans*, with the genetic interference effect being observed in the F_1 and F_2 generations (a generation time of approximately four days) (Grishok et al., 2000). In *M. rosenbergii* however, the dsRNA was cleared and the silencing effect reached complete decay within the injected individuals, and did not reach further generations (according to the findings of the current study). Furthermore, if the dsRNA was transmitted to the treated prawns' offspring in the present study, one would expect that instead of obtaining an all-male population, some percentage would have become neo-female like the treated neo-female broodstock, yet this was not the case (see below).

4.2. Comparisons of normal prawn populations with RNAi-manipulated neo-females and all-male populations

When checking the performance of neo-females as compared with a normal female population, we found that neo-females were able to mate with normal males and to carry viable eggs. Furthermore, the neo-females presented no significant fecundity changes relative to normal females in terms of the average number of eggs/g total body weight. In this respect, the results of the present study for both normal and neo-females were within the range reported by Habashy (2013), who showed a range of 435 to 3849 eggs/g and also within the values presented by Jee and Kok (1991), (1132 ± 484 to 745 ± 487 eggs/g body weight) in mature *M. rosenbergii* normal females. The brood somatic index (BSI) was very similar in both groups, implying a normal ratio between prawn egg mass to body weight of the neo-females. Both groups were similar in frequency of reproductive states, with virgin females comprising only 20% of both the normal and neo-female populations.

The largest reproductive female observed in the normal female population weighed ~55 g, while in the neo-female population, the largest animal weighed ~80 g. Both of these females carried eggs. The reproductive types (which are females with developed gonads, spent or eggs berried) of the neo-female population are shifted toward higher weights, where no normal female could be observed. The improved growth performance of the neo-females could be attributed to a sex-limited growth trait such as found in male penaeid species (Li et al., 2006), suggesting that growth ability could be dictated genetically. Thus, the neo-females produced in the present study, having original male chromosomes (ZZ), although acting as phenotypic females, might have been exhibiting growth ability dictated by their genomic background and grow larger than normal females (WZ). In *M. rosenbergii*, however, such mapping is not yet available, as the sex chromosomes are not morphologically distinguishable (Justo et al., 1991) and further research is needed to test this hypothesis. Moreover, the different growth rates could be attributed to the different ponds in which other environmental variables, including density, could have played a major role (Cohen and Ra'anan, 1983; Karplus et al., 1986; Sandifer and Smith, 1975). Furthermore, in the mixed-sex population, ~50% of the total population grown in the pond were males, which could contribute to the lower growth rate of the normal females (Sagi et al., 1986).

The final product of this RNAi-based biotechnology, an all-male population (offspring of neo-female), also was examined for phenotypic normality. The animals observed looked normal, exhibiting the typical morphometry found among adult *M. rosenbergii* male morphotypes (Kuris et al., 1987). The differences between groups showed higher frequency of OC males in the all-male population and a relatively lower proportion of BC males. Such alteration in morphotypic frequencies was reported earlier (Sagi et al., 1986) in other monosex population studies and could be explained by the fact that in all-male population there are no females with whom to mate, unlike the normal population. Therefore, individuals in the all-male population can invest in growth rather than in reproduction. Thus, the fast-growing OC males are more abundant at the expense of the SM and BC males which are considered as actively participating in mating and fertilization and investing little energy in somatic growth (Sagi et al., 1986). Moreover, the fastest-growing

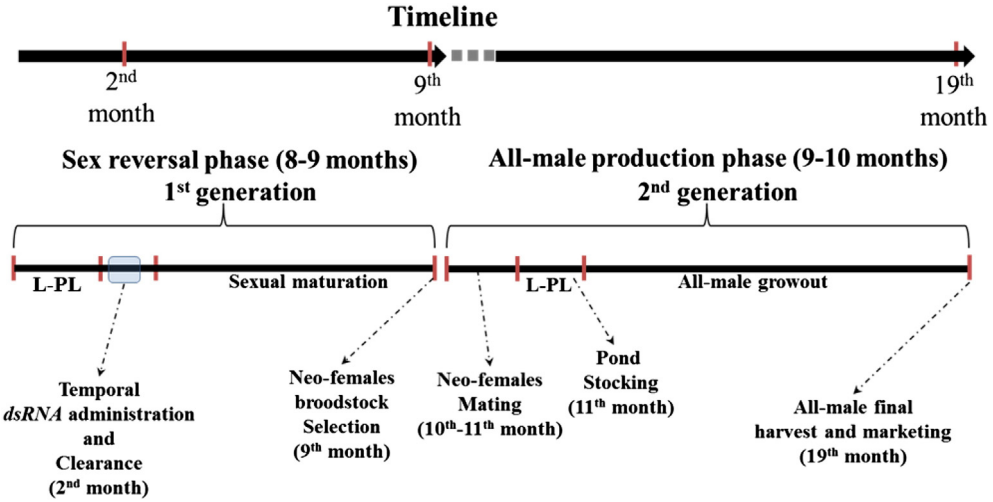

Fig. 5. Scheme of the timetable of all-male prawn production using RNAi-based biotechnology involving ds*Mr-IAG* injection.

OC males are less territorial than are the dominant BC males and hence, in many cases, are the most desirable for growers (Karplus et al., 1986). In accordance with previous studies (Aflalo et al., 2014; Nair et al., 2006; Sagi et al., 1986), regarding marketable yields, the all-male population in the present study proved to be superior with an increase of about 17% in yields compared to mixed-sex population. Moreover, in the mixed-sex population, no female reached the 50 g threshold set for marketable yields. To our estimation, the additional expenses for production of neo-female broodstock are insignificant compared to the increase in profitability relative to mixed-sex populations (+60%, Nair et al., 2006).

5. Conclusions

The results of our study of all-male prawn biotechnology suggest that apart from being not transmitted to subsequent generations, the RNAi achieved through dsRNA injection is safe for use due to its temporary effect and the normal development and performance of the broodstock and offspring. Moreover, the neo-females generated can be used as broodstock able to produce offspring of a single-sex which are unable to reproduce without females of the same species present, thus reducing the risk of establishment of invasive exotic populations. With the latter added value, we believe that with further research and development on challenging topics such as delivery and price, RNAi-based technologies will become pivotal practices in a wide range of aquaculture and environmental applications.

Acknowledgments

We thank Prof. Ofer Ovadia for his help with the statistical analysis, and Ms. Ayana Benet-Perlberg and her team at the Israel Ministry of Agriculture and Rural Development, Department of Fisheries and Aquaculture, Dor Research Center, for housing and maintaining the experimental prawns. This study was supported in part by the United States–Israel Binational Agricultural Research and Development Fund (BARD, Grant No. IS-4493-12), the National Institute for Biotechnology in the Negev (NIBN), and Tiran Shipping, Ltd.

References

Aflalo, E.D., Sagi, A., 2014. Sustainable aquaculture biotechnology using temporal RNA interference in crustaceans: the case of the insulin-like androgenic gland hormone and prawn monosex culture. In: Govil, J.N. (Ed.), Animal Biotechnology. Stadium Press LLC, Houston, Texas, USA, pp. 319–332.

Aflalo, E.D., Hoang, T.T.T., Nguyen, V.H., Lam, Q., Nguyen, D.M., Trinh, Q.S., Raviv, S., Sagi, A., 2006. A novel two-step procedure for mass production of all-male populations of the giant freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 256, 468–478.

Aflalo, E.D., Raju, D.V.S.N., Bommi, N.A., Verghese, J.T., Samraj, T.Y.C., Hulata, G., Ovadia, O., Sagi, A., 2012. Toward a sustainable production of genetically improved all-male prawn (*Macrobrachium rosenbergii*): evaluation of production traits and obtaining neo-females in three Indian strains. Aquaculture 338–341, 197–207.

Aflalo, E.D., Dandu, R.V.S.N., Verghese, J.T., Rao, N., Samraj, T.Y.C., Ovadia, O., Sagi, A., 2014. Neo-females production and all-male progeny of a cross between two Indian strains of prawn (*Macrobrachium rosenbergii*): population structure and growth performance under different harvest strategies. Aquaculture 428, 7–15.

Agrawal, N., Dasaradhi, P.V.N., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., Mukherjee, S.K., 2003. RNA interference: biology, mechanism, and applications. Microbiol. Mol. Biol. Rev. 67, 657–685.

Anklam, E., Gadani, F., Heinze, P., Pijnenburg, H., Van Den Eede, G., 2002. Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. Eur. Food Res. Technol. 214, 3–26.

Bye, V.J., Lincoln, R.F., 1986. Commercial methods for the control of sexual-maturation in rainbow-trout (*Salmo gairdneri* R). Aquaculture 57, 299–309.

Cohen, D., Ra'anan, Z., 1983. The production of the freshwater prawn *Macrobrachium rosenbergii* in Israel: III. Density effect of all-male Tilapia hybrids on prawn yeld characters in polyculture. Aquaculture 35, 57–71.

Copf, T., Rabet, N., Averof, M., 2006. Knockdown of spalt function by RNAi causes de-repression of Hox genes and homeotic transformations in the crustacean *Artemia franciscana*. Dev. Biol. 298, 87–94.

Couzin, J., 2002. Small RNAs make big splash. Science 298, 2296–2297.

Curtis, M.C., Jones, C.M., 1995. Observations on monosex culture of redclaw crayfish *Cherax quadricarinatus* von Martens (Decapoda: Parastacidae) in earthen ponds. J. World Aquacult. Soc. 26, 154–159.

Dorsett, Y., Tuschl, T., 2004. siRNAs: applications in functional genomics and potential as therapeutics. Nat. Rev. Drug Discov. 3, 318–329.

FAO, 2011. Aquaculture production 1950–2011. Fisheries and Aquaculture Information and Statistics Service. Food and Agriculture Organization of the United Nations, (Available at: http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp).

Fire, A., Xu, S.Q., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Garbutt, J.S., Belles, X., Richards, E.H., Reynolds, S.E., 2013. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence from *Manduca sexta* and *Blattella germanica*. J. Insect Physiol. 59, 171–178.

Grishok, A., Tabara, H., Mello, C.C., 2000. Genetic requirements for inheritance of RNAi in *C. elegans*. Science 287, 2494–2497.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., Mello, C.C., 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell 106, 23–34.

Habashy, M.M., 2013. On the breeding behaviour and reproduction of the freshwater prawn, *Macrobrachium rosenbergii* (de Man 1879) (Decapoda-Crustacea) under laboratory conditions. Aquac. Res. 44, 395–403.

Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.

Hulata, G., Karplus, I., Wohlfarth, G.W., Halevy, A., 1990. Effects of size and age of juvenile freshwater prawns *Macrobrachium rosenbergii* at stocking on population structure and production in polyculture ponds. J. World Aquacult. Soc. 21, 295–299.

Huvenne, H., Smagghe, G., 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J. Insect Physiol. 56, 227–235.

Jee, A.K., Kok, L.Y., 1991. Fecundity changes in *Macrobrachium rosenbergii* (de Man) during egg incubation. Aquac. Res. 22, 1–6.

Justo, C.C., Murofushi, M., Aida, K., Hanyu, I., 1991. Karyological studies on the freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 97, 327–334.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421, 231–237.

Karplus, I., Hulata, G., Wohlfarth, G.W., Halevy, A., 1986. The effect of density of *Macrobrachium rosenbergii* raised in earthen ponds on their population structure and weight distribution. Aquaculture 52, 307–320.

Kato, Y., Kobayashi, K., Watanabe, H., Iguchi, T., 2011. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a *doublesex* gene in the sex-determining pathway. PLoS Genet. 7, e1001345.

Kuris, A.M., Ra'anan, Z., Sagi, A., Cohen, D., 1987. Morphotypic differentiation of male Malaysian giant prawns, *Macrobrachium rosenbergii*. J. Crustac. Biol. 7, 219–237.

Lawrence, C.S., 2004. All-male hybrid (*Cherax albidus* × *Cherax rotundus*) yabbies grow faster than mixed-sex (*C-albidus* × *C-albidus*) yabbies. Aquaculture 236, 211–220.

Lawrence, C.S., Cheng, Y.W., Morrissy, N.M., Williams, I.H., 2000. A comparison of mixed-sex vs. monosex growout and different diets on the growth rate of freshwater crayfish (*Cherax albidus*). Aquaculture 185, 281–289.

Li, S.S., Tsai, H.J., 2000. Transfer of foreign gene to giant freshwater prawn (*Macrobrachium rosenbergii*) by spermatophore-microinjection. Mol. Reprod. Dev. 56, 149–154.

Li, Y.T., Dierens, L., Byrne, K., Miggiano, E., Lehnert, S., Preston, N., Lyons, R., 2006. QTL detection of production traits for the Kuruma prawn *Penaeus japonicus* (Bate) using AFLP markers. Aquaculture 258, 198–210.

Lu, Y.N., Sun, P.S., 2005. Viral resistance in shrimp that express an antisense Taura syndrome virus coat protein gene. Antivir. Res. 67, 141–146.

Manor, R., Weil, S., Oren, S., Glazer, L., Aflalo, E.D., Ventura, T., Chalifa-Caspi, V., Lapidot, M., Sagi, A., 2007. Insulin and gender: an insulin-like gene expressed exclusively in the androgenic gland of the male crayfish. Gen. Comp. Endocrinol. 150, 326–336.

Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., Sela, I., 2009. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Mol. Biol. 18, 55–60.

Mires, D., 1977. Theoretical and practical aspects of the production of all-male *Tilapia* hibrids. Isr. J. Aquacult. Bamidgeh 29, 94–101.

Nair, C.M., Salin, K.R., Raju, M.S., Sebastian, M., 2006. Economic analysis of monosex culture of giant freshwater prawn (*Macrobrachium rosenbergii* De Man): a case study. Aquac. Res. 37, 949–954.

Nature, 2013. GM crops: a story in numbers. Nature 497, 22–23.

Ohira, T., Hasegawa, Y., Tominaga, S., Okuno, A., Nagasawa, H., 2003. Molecular cloning and expression analysis of cDNAs encoding androgenic gland hormone precursors from two Porcellionidae species, *Porcellio scaber and P. dilatatus*. Zool. Sci. 20, 75–81.

Pavlopoulos, A., Averof, M., 2005. Establishing genetic transformation for comparative developmental studies in the crustacean *Parhyale hawaiensis*. Proc. Natl. Acad. Sci. U. S. A. 102, 7888–7893.

Qi, Y.J., Hannon, G.J., 2005. Uncovering RNAi mechanisms in plants: biochemistry enters the foray. FEBS Lett. 579, 5899–5903.

Robalino, J., Bartlett, T., Shepard, E., Prior, S., Jaramillo, G., Scura, E., Chapman, R.W., Gross, P.S., Browdy, C.L., Warr, G.W., 2005. Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? J. Virol. 79, 13561–13571.

Sagi, A., Aflalo, E.D., 2005. The androgenic gland and monosex culture of freshwater prawn *Macrobrachium rosenbergii* (De Man): a biotechnological perspective. Aquac. Res. 36, 231–237.

Sagi, A., Khalaila, I., 2001. The crustacean androgen: a hormone in an isopod and androgenic activity in decapods. Am. Zool. 41, 477–484.

Sagi, A., Ra'anan, Z., Cohen, D., Wax, Y., 1986. Production of *Macrobrachium rosenbergii* in monosex population: yield characteristics under intensive monoculture conditions in cages. Aquaculture 51, 265–275.

Sagi, A., Manor, R., Ventura, T., 2013. Gene silencing in crustaceans: from basic research to biotechnologies. Genes 4, 620–645.
Sandifer, P.A., Smith, T.I.J., 1975. Effects of population density on growth and survival of *Macrobrachium rosenbergii* reared in recirculating water management system. Proceedings of the Annual Meeting — World Mariculture Society 6, pp. 43–53.
Siddiqui, A.Q., Al-Hafedh, Y.S., Al-Harbi, A.H., Ali, S.A., 1997. Effects of stocking density and monosex culture of freshwater prawn *Macrobrachium rosenbergii* on growth and production in concrete tanks in Saudi Arabia. J. World Aquacult. Soc. 28, 106–112.
Small, I., 2007. RNAi for revealing and engineering plant gene functions. Curr. Opin. Biotechnol. 18, 148–153.
Sokal, R.R., Rohlf, F.J., 1995. Biometry: The Principles and Practice of Statistics in Biological Research, 3rd ed. W.H. Freeman, New York.
Tayamen, M.M., Shelton, W.L., 1978. Inducement of sex reversal in *Sarotherodon niloticus* (Linnaeus). Aquaculture 14, 349–354.
Vaishnaw, A.K., Gollob, J., Gamba-Vitalo, C., Hutabarat, R., Sah, D., Meyers, R., de Fougerolles, T., Maraganore, J., 2010. A status report on RNAi therapeutics. Silence 1, 1–13.
Ventura, T., Sagi, A., 2012. The insulin-like androgenic gland hormone in crustaceans: from a single gene silencing to a wide array of sexual manipulation-based biotechnologies. Biotechnol. Adv. 30, 1543–1550.
Ventura, T., Manor, R., Aflalo, E.D., Weil, S., Raviv, S., Glazer, L., Sagi, A., 2009. Temporal silencing of an androgenic gland-specific insulin-like gene affecting phenotypical gender differences and spermatogenesis. Endocrinology 150, 1278–1286.
Ventura, T., Aflalo, E.D., Weil, S., Kashkush, K., Sagi, A., 2011. Isolation and characterization of a female-specific DNA marker in the giant freshwater prawn *Macrobrachium rosenbergii*. Heredity 107, 456–461.
Ventura, T., Manor, R., Aflalo, E.D., Weil, S., Rosen, O., Sagi, A., 2012. Timing sexual differentiation: full functional sex reversal achieved through silencing of a single insulin-like gene in the prawn, *Macrobrachium rosenbergii*. Biol. Reprod. 86, 1–6.
Watson, J.M., Fusaro, A.F., Wang, M.B., Waterhouse, P.M., 2005. RNA silencing platforms in plants. FEBS Lett. 579, 5982–5987.
White, A.J., Northcutt, M.J., Rohrback, S.E., Carpenter, R.O., Niehaus-Sauter, M.M., Gao, Y.P., Wheatly, M.G., Gillen, C.M., 2011. Characterization of sarcoplasmic calcium binding protein (SCP) variants from freshwater crayfish *Procambarus clarkii*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 160, 8–14.