BIOLOGY OF REPRODUCTION (2012) **86**(3):90, 1–6
Published online before print 30 November 2011.
DOI 10.1095/biolreprod.111.097261

Timing Sexual Differentiation: Full Functional Sex Reversal Achieved Through Silencing of a Single Insulin-Like Gene in the Prawn, *Macrobrachium rosenbergii*[1]

Tomer Ventura, Rivka Manor, Eliahu D. Aflalo, Simy Weil, Ohad Rosen, and Amir Sagi[2]

Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

ABSTRACT

In Crustacea, an early evolutionary group (~50 000 species) inhabiting most ecological niches, sex differentiation is regulated by a male-specific androgenic gland (AG). The identification of AG-specific insulin-like factors (IAGs) and genomic sex markers offers an opportunity for a deeper understanding of the sexual differentiation mechanism in crustaceans and other arthropods. Here, we report, to our knowledge, the first full and functional sex reversal of male freshwater prawns (*Macrobrachium rosenbergii*) through the silencing of a single IAG-encoding gene. These "neofemales" produced all-male progeny, as proven by sex-specific genomic markers. This finding offers an insight regarding the biology and evolution of sex differentiation regulation, with a novel perspective for the evolution of insulin-like peptides. Our results demonstrate how temporal intervention with a key regulating gene induces a determinative, extreme phenotypic shift. Our results also carry tremendous ecological and commercial implications. Invasive and pest crustacean species represent genuine concerns worldwide without an apparent solution. Such efforts might, therefore, benefit from sexual manipulations, as has been successfully realized with other arthropods. Commercially, such manipulation would be significant in sexually dimorphic cultured species, allowing the use of nonbreeding, monosex populations while dramatically increasing yield and possibly minimizing the invasion of exotic cultured species into the environment.

gene expression, insulin-like androgenic gland hormone, invertebrates, sex differentiation, sexual manipulation biotechnology

INTRODUCTION

The archaic group of Crustacea includes approximately 50 000 species inhabiting most ecological niches. The underlying mechanism regulating sexual differentiation in this varied group goes through the male-specific androgenic gland (AG) [1]. In the giant freshwater prawn (*Macrobrachium rosenbergii*), a full functional sex reversal has been achieved by removal [2] of the AG from juvenile males to obtain ''neofemales'' and transplantation [3] of the AG to juvenile females to obtain ''neomales.'' In a few other decapod species, as well as in some isopod and amphipod species, such surgical interventions caused a sexual shift [4].

The identification of AG-specific insulin-like factors (IAGs) [4] and genomic sex markers [5] in crustaceans offers an opportunity for a deeper understanding of the sexual differentiation mechanism in crustaceans and other arthropods. Previous findings revealed that *M. rosenbergii* IAG (Mr-IAG) is critical for spermatogenesis and the appearance of external sexual characteristics [6] through its silencing in mature males. These results placed Mr-IAG as a leading candidate for being an androgenic hormone. Since then, molecular sex markers identified for the first time in *M. rosenbergii* [5] enabled tracing of the genetically set gender before the appearance of any external sexual characteristics. This finding was followed by a detailed examination of the temporal expression pattern of *Mr-IAG*, revealing it starts to express approximately 20 days after metamorphosis (Postlarval Day [PLD] 20), before any documented sexual characteristic in crustaceans [7]. This result further supports the hypothesis according to which Mr-IAG is an AG hormone that not only governs the maintenance of male sexual characteristics during adulthood but also dictates early male sexual differentiation. Therefore, we aimed to achieve a full functional sex reversal of males into neofemales through silencing *Mr-IAG* earlier than the appearance of external sexual characteristics. This was feasible by applying genomic sex markers to identify juvenile males (PLD 20–30; see *Materials and Methods*).

Here, we report, to our knowledge, the first full functional sex reversal of males through the silencing of a single IAG-encoding gene. These neofemales produced all-male progeny, as proven by sex-specific genomic markers. This finding offers an insight regarding the biology and evolution of sex differentiation regulation, with a novel perspective for the evolution of insulin-like peptides. Our results demonstrate how temporal intervention with a key regulating gene induces a determinative, extreme phenotypic shift. Our results also carry tremendous ecological and commercial implications. Invasive and pest crustacean species represent genuine concerns worldwide without an apparent solution [8]. Such efforts might, therefore, benefit from sexual manipulations, as has been successfully realized with other arthropods [9]. Commercially, such manipulation would be significant in sexually dimorphic cultured species, allowing the use of nonbreeding monosex populations while dramatically increasing yield and possibly minimizing the invasion of exotic cultured species into the environment.

MATERIALS AND METHODS

Animals

Macrobrachium rosenbergii were maintained at Ben-Gurion University of the Negev under the following conditions: Food comprising shrimp pellets (30% protein; Rangen) was supplied ad libitum three times a week. Water quality was assured by circulating the entire volume through a biofilter maintaining all the required water physicochemical parameters as described previously [10]. Egg-bearing females were separately transferred to closed,

[1]Supported by grants from Tiran Ltd. through BGN and the National Institute for Biotechnology in the Negev (NIBN).
[2]Correspondence: Amir Sagi, Department of Life Sciences and the National Institute for Biotechnology, Ben-Gurion University of the Negev, POB 653, Beer-Sheva 84105, Israel. E-mail: sagia@bgu.ac.il

Received: 26 October 2011.
First decision: 16 November 2011.
Accepted: 23 November 2011.
© 2012 by the Society for the Study of Reproduction, Inc.
eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Downloaded from www.biolreprod.org.

100-L tanks containing 12–16 ppt salt water, which was circulated through a 100-μm mesh net. After hatching had occurred, the females were removed from the tanks, and the newly hatched larvae were maintained according to the protocol devised by Uno and Chin Soo [11] and fed with *Artemia nauplii* daily.

Double-Stranded RNA Preparation

The dsRNA of the *Mr-IAG* open reading frame was prepared with a MEGAscript T7 Kit (Ambion) according to the manufacturer's instructions. Quality of dsRNA was assessed on an agarose gel. The dsRNA was maintained at −20°C until used as described previously [6].

In Vivo Mr-IAG *Silencing*

For the time-dependent silencing experiment, young male prawns (wt, 2.49 ± 0.17 g [mean ± SEM]) were divided into four groups, of which one group (n = 5) was injected once with exogenous Remebee (RB) dsRNA (Beeologics) while the other three groups (n = 4 in each group) were injected once with *Mr-IAG* dsRNA. The dsRNA quantity was calculated as 5 μg dsRNA/g body wt. AGs from individuals of each of the three *Mr-IAG* dsRNA-injected groups were taken for RNA extraction 3 days, 1 wk, and 2 wk after the injection, parallel to AGs taken from RB dsRNA-injected individuals at each time.

For the dose-dependent silencing experiment, young male prawns (wt, 1.89 ± 0.21 g) were divided into four groups, of which one group (n = 7) was injected once with RB dsRNA while the other three groups (n = 6 in each group) were injected once with *Mr-IAG* dsRNA at different quantities as follows: 5, 1, and 0.1 μg dsRNA/g body wt. At 1 wk following injection, AGs were taken for RNA extraction from all individuals.

For the long-term in vivo injections experiment, *M. rosenbergii* males and females (wt, 30–70 mg; age, PLD 30) were selected according to the presence of the female-specific sex marker in females and its absence in males [5, 7]. The postlarval organisms were assigned to one of three treatment groups: *Mr-IAG* dsRNA-injected males (n = 19), water-injected control males (n = 15), and water-injected control females (n = 16) with no foreign dsRNA based on Ventura et al. [6]. Each animal was housed in a separate, floating, fenestrated plastic container (3 × 11 cm). Twice a week (over a period of 9 mo), each animal was injected with 5 μg dsRNA/g body wt or a similar volume of water (a total of 71 injections). Molts were recorded twice a week, and weight accumulation was documented. The appearance of male gonopores (MGPs) and appendix masculinae (AM) were viewed under a dissecting stereoscope.

RNA Extraction and Real-Time RT-PCR

The RNA was extracted from the AGs of males used in the time- and dose-dependency experiments. Total RNA was isolated with the EZ-RNA Total RNA Isolation Kit (Biological Industries) according to the manufacturer's instructions. First-strand cDNA was synthesized by means of reverse transcriptase reaction using the Verso cDNA Kit (Thermo Fisher Scientific, Inc.) with 1 μg of total RNA. Relative quantification of *Mr-IAG* transcript levels were obtained using the following primers: 5′-*GCCTTGCAGT CATCCTTGA*-3′ (IAG qPCR_F) and 5′-*AGGCCGGAGAGAAGAATGTT*-3′ (IAG qPCR_R) with the FastStart Universal Probe Master (Rox; Roche Diagnostics GmbH) and Universal ProbeLibrary Probe 144 (Roche). *Mr-18S* (GenBank accession no. GQ131934), which was used as a normalizing gene, was also quantified by means of real-time RT-PCR using the following primers: 5′-*CCCTAAACGATGCTGACTAGC*-3′ (q*Mr-18S*_F) and 5′-*TACCCCCGGAACTCAAAGA*-3′ (q*Mr-18S*_R) with the above-mentioned mix and Universal ProbeLibrary Probe 152 (Roche). Reactions were performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems).

Tissue Preparation

The AGs from mature male morphotypes were dissected together with the attached terminal ampullae. Tissue samples were fixed in modified Carnoy II for 48 h and dehydrated gradually through a series of increasing alcohol concentrations. Tissues were cleared and embedded in Paraplast (Kendall) according to conventional procedures. Sections (thickness, 5 μm) were cut and mounted onto silane-coated slides (Menzel-Gläser). These slides served for all histological procedures, including immunohistochemistry and in situ hybridization. Before the histological procedures, the slides were deparaffinized in xylene and gradually rehydrated through a series of decreasing alcohol concentrations.

Genomic DNA Extraction and Sex Markers

Genomic DNA was extracted from 2 to 10 mg of 70% ethanol-fixed tissue using a REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer's instructions. The genomic sex markers developed in the Israeli line (BGU line) were used as described previously [5].

PCR of Female-Specific and Positive-Control Sequences

The PCR was performed with 100 ng of genomic DNA, 1 μM forward primer and 1 μM reverse primer (primer sequences available upon request), 12.5 μl of REDTaq ReadyMix (Sigma), and water to a final volume of 25 μl. The PCR conditions used were as follows: 35 cycles of 30 sec at 94°C, 30 sec at 56°C, and 60 sec at 72°C. The PCR products were separated on a 2% agarose gel and visualized under ultraviolet light with ethidium bromide.

Statistical Analysis

The four groups in the real-time RT-PCR were analyzed using the Kruskal-Wallis test followed by the correction of a multiple pair-wise comparison (built-in within the Statistica 6.1 software [StatSoft]) as accepted. To evaluate the effect of dsRNA injections on the rate of MGP and AM appearance, a Cox proportional hazards regression model [12] was used. The model is expressed by $\mu(t,z) = \mu_0(t) \exp(\sum \beta_i z_i)$, where $\mu(t,z)$ is the MGP or AM appearance rate and $\mu_0(t)$ is the baseline hazard function that can change over time (t). The regression coefficient to be estimated (β_i) represents the independent effect of dsRNA injection on the MGP or AM appearance rate. These analyses were performed with S-PLUS 2000 software (Mathsoft). The effects of dsRNA injection on molt increment and the weight range associated with MGP appearance were statistically analyzed by the nonparametric Mann-Whitney *U*-test using Statistica software.

RESULTS

Mr-IAG-*Silencing: Determination of Effective Time and Dose*

Real-time RT-PCR performed on RNA extracted from AGs of individuals injected with *Mr-IAG* dsRNA (5 μg/g body wt) at different times following the injection showed that *Mr-IAG* transcript level was significantly reduced compared with the control group after 3 days and 1 wk. After 2 wk, *Mr-IAG* transcript level did not differ significantly from the control group (Fig. 1A). Dose dependency was assessed in the same manner. At 1 wk after injection with different doses of *Mr-IAG* dsRNA (0.1–5 μg/g body wt), real-time RT-PCR showed that 1 μg/g body wt significantly reduced *Mr-IAG* transcript level compared with the control group, whereas 0.1 μg/g body wt did not (Fig. 1B).

Mr-IAG-*Silencing in Juvenile Males Delays Appearance of Male Sexual Characteristics*

In the present study, *Mr-IAG* silencing caused a delay in the formation of male-specific AM. This delay became significant ($P = 0.043$, Cox plot hazards model followed by chi-square test) by PLD 128 (Fig. 2A). By PLD 220, all individuals from the control male group (n = 13, from a starting group of 15) had developed an AM, as compared with as few as 33% of the *Mr-IAG*-silenced male group (n = 10, from a starting group of 19). None of the control females developed an AM.

The appearance of MGPs was also delayed in the *Mr-IAG*-silenced group. By PLD 128, this delay became significant ($P = 0.032$, Cox plot hazards model followed by chi-square test) when compared with the control male group (Fig. 2B). By PLD 184, both groups included the maximum percentage of individuals with MGPs, and although all individuals developed MGPs in the control male group, only 60% did so in the *Mr-IAG*-silenced group of males. None of the control females developed MGPs. In control males, MGPs developed in individuals ranging in size from 236 to 546 mg and at times ranging from PLD 124 to PLD 180. In 6 of 10 *Mr-IAG*-silenced males, MGPs developed in individuals ranging in size from 208 to 271 mg and from PLD 145 to PLD 180. Whereas

Downloaded from www.biolreprod.org.

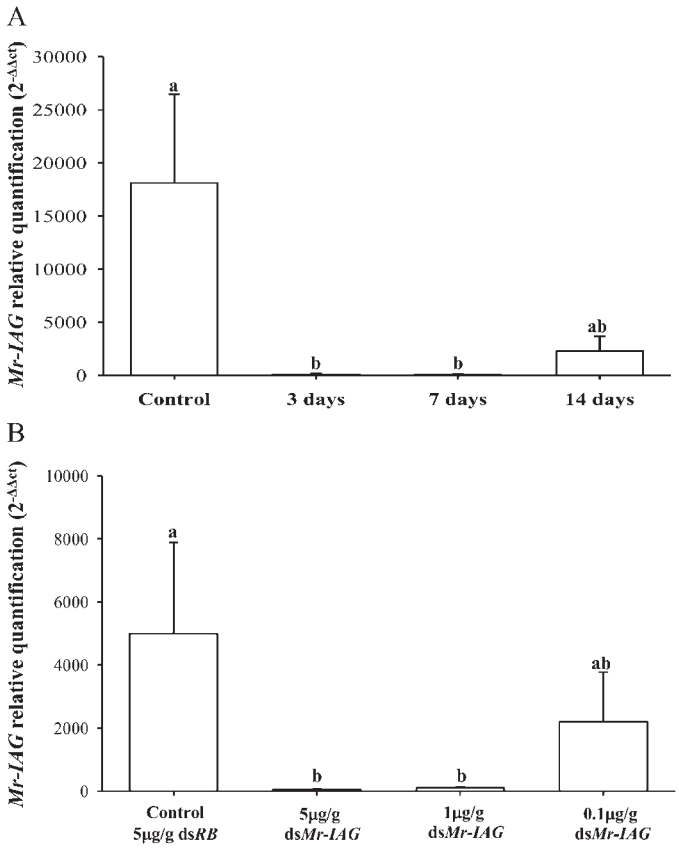

FIG. 1. Levels of *Mr-IAG* transcripts following a single dsRNA injection in male prawns. Relative *Mr-IAG* transcripts levels were quantified by real-time RT-PCR following a single injection of *Mr-IAG* dsRNA or exogenous dsRNA to the control groups. Different letters represent a significant difference, and error bars represent the SEM. **A**) Time dependency: Real-time RT-PCR was performed 3, 7, or 14 days after injection. Relative *Mr-IAG* transcripts levels were significantly reduced (Kruskal-Wallis test: H ($df = 3$, n = 17) = 12.16863, $P = 0.0068$) at 3 days (n = 4) and 1 wk (n = 4) after *Mr-IAG* dsRNA injection (5 µg/g body wt) compared with the control group (n = 5). **B**) Dose response: Real-time RT-PCR was performed 1 wk after injection of 5 (n = 6), 1 (n = 6), or 0.1 (n = 6) µg/g body wt. Relative *Mr-IAG* transcripts levels were significantly reduced (Kruskal-Wallis test: H ($df = 3$, n = 25) = 15.99912, $P = 0.0011$) at 1 wk after injection of 5 or 1 µg/g body wt.

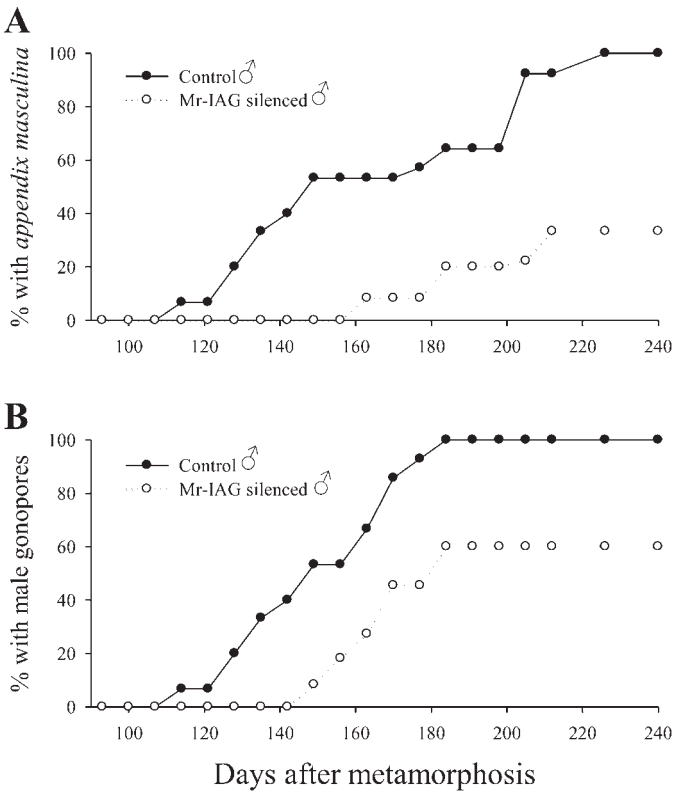

FIG. 2. *Mr-IAG* silencing inhibits the appearance of external sexual characteristics. **A**) Percentage of males displaying an AM in *Mr-IAG*-silenced and control populations over the period of *Mr-IAG* silencing. Significant delay in AM formation is noted in the *Mr-IAG*-silenced group by PLD 128 (Cox plot hazards model followed by chi-square test: $P = 0.043$). By the end of the experiment, all individuals from the control male group developed AM (n = 13), as compared to only 33% of the *Mr-IAG*-silenced male group (n = 10). **B**) Appearance of MGPs in the *Mr-IAG*-silenced and control males, showing significant delay in MGP appearance in the *Mr-IAG*-silenced group by PLD 128 (Cox plot hazards model followed by chi-square test: $P = 0.032$). Both groups reached the maximal percentage of individuals with MGPs by PLD 184. However, all individuals developed MGPs in the control group, whereas only 60% did so in the *Mr-IAG*-silenced group.

in *Mr-IAG*-silenced males the time of MGP formation was significantly delayed (Fig. 2B), it occurred in individuals covering a significantly ($P = 0.0033$, Mann-Whitney U-test) smaller weight range than seen in the male control group.

Unsuccessful sex reversal attempts were accompanied by delayed formation of male external sexual characteristics (Fig. 2) and hypertrophied AGs in accordance with previously reported results [6].

Mr-IAG-*Silencing in Juvenile Males Induces Ovarian Development*

One *Mr-IAG*-silenced individual (wt, ~800 mg) that did not develop an MGP was sampled for histological survey, whereas the others were injected until they reached a weight of 1 g, along with control males and control females. When the *Mr-IAG*-silenced individual with no MGP (Fig. 3, middle) was compared with individual male (Fig. 3, left) and female (Fig. 3, right) controls of the same weight, clear evidence for sex reversal was observed. The *Mr-IAG*-silenced male resembled the control female, with both having ovaries (*Mr-IAG*-silenced male and control female shown in Fig. 3, D and G, respectively, and at higher magnification in Fig. 3, E and H, respectively) and oviducts (*Mr-IAG*-silenced male and control female shown in Fig. 3, F and I, respectively), with no signs of testes, sperm ducts (as evident in the control male; testes shown in Fig. 3, A and B, and sperm duct in Fig. 3C), or AGs.

Mr-IAG-*Silencing in Juvenile Males Induces a Full Functional Sex Reversal into Neofemales*

The *Mr-IAG*-silenced genetic males with no MGP were raised to maturity and developed normal-appearing ovaries (Fig. 4A, top, arrow) as compared with control females (results not shown). These *Mr-IAG*-silenced genetic males were mated with normal males, and their reproductive behavior was normal and their copulation successful. Specifically, the spermatophore attached by the male onto the ventral cephalothorax of the *Mr-IAG*-silenced genetic males was maintained. These then spawned and incubated the eggs in their brood chambers, located ventrally on their abdomens (Fig. 4A, bottom, arrow). These egg-bearing neofemales were transferred to a hatching facility and, in parallel to control females, were hatched. The time of egg development until hatching was similar for the

Downloaded from www.biolreprod.org.

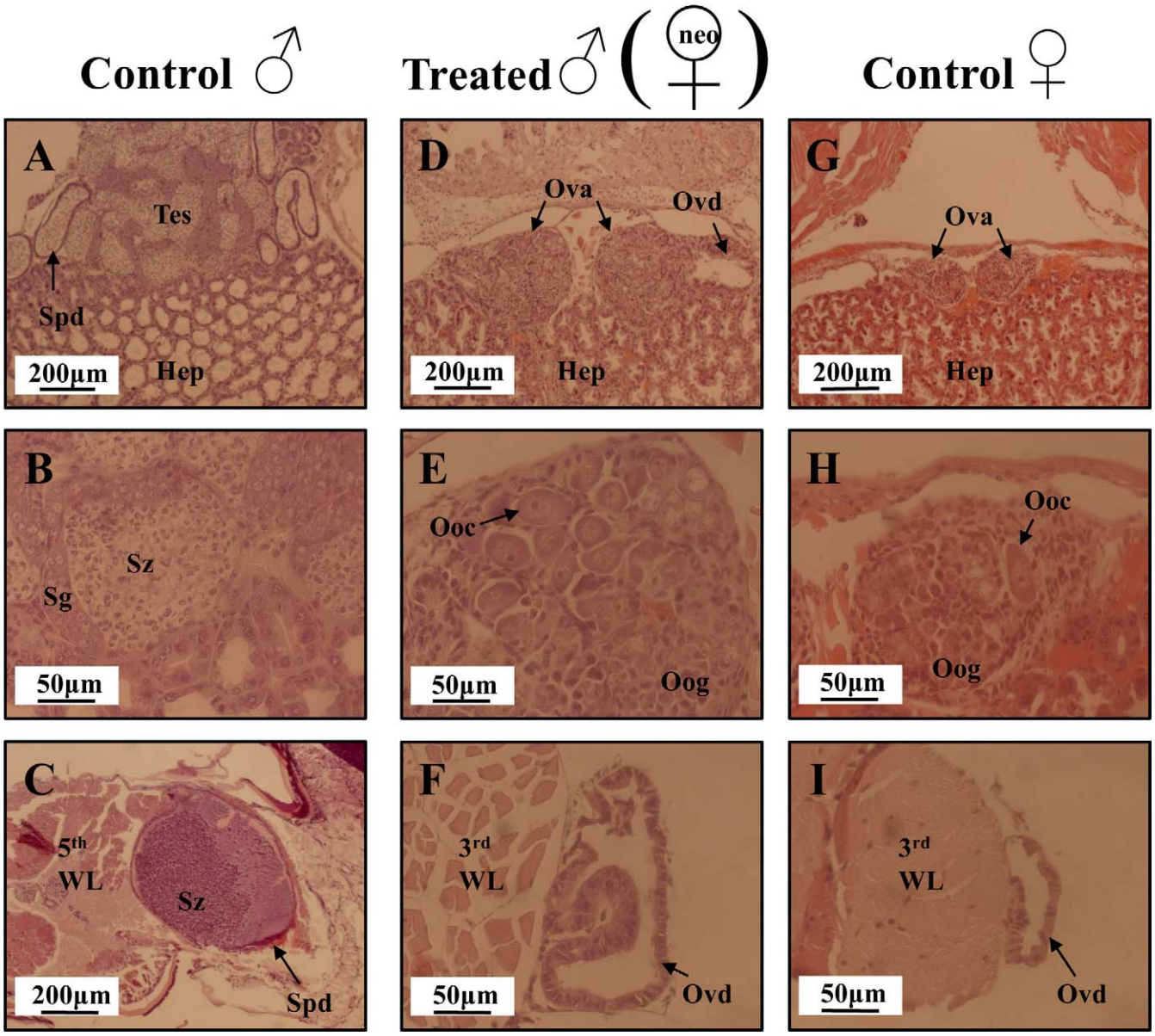

FIG. 3. *Mr-IAG* silencing induces an anatomical sex reversal. Longitudinal sections obtained from a control male (**A**–**C**) and a control female (**G**–**I**) showing testes (Tes; **A** and **B**) and sperm duct (Spd; **A** and **C**) in the male and ovaries (Ova; **G** and **H**) and oviduct (Ovd; **I**) in the female. In the *Mr-IAG*-silenced male gonads (**D** and **E**) and genital ducts (**F**) were feminine and resembled those of the control female. Hep, hepatopancreas; Ooc, oocytes; Oog, oogonia; Sg, spermatogonia; Sz, spermatozoa; WL, walking leg.

neofemales and the control female progeny (18–20 vs. 15–20 days, respectively), as was the time from hatching to metamorphosis (21–23 days). Larval staging [11] was similar throughout the hatching period. Thirty larvae of zoea stage 3 were sampled from each progeny for genetic sex determination using our molecular sex marker [5]. The genetic sex of the neofemales and control females that produced the progeny was reconfirmed, as was the genetic sex of intact male and female positive controls and a negative control (Fig. 4B, marked as ♂, ♀, and n.c., respectively). All controls and reconfirmations were as expected, revealing the female-specific sex marker only in the intact and control females. The progeny of the control females were found to comprise both males and females, as expected (Fig. 4B, top, F1), whereas the progeny of the neofemales comprised only males (Fig. 4B, bottom, F1).

Recently, numerous cases in our laboratory have confirmed that silencing the transcript encoding *Mr-IAG* in male individuals from PLD 20 caused complete and functional sex reversal into neofemales. When mated with normal males, the neofemale individuals produced all-male populations.

DISCUSSION

We report that silencing the transcript encoding the *Mr-IAG* in male individuals caused complete and functional sex reversal into neofemales. When mated with normal males, the neofemale individuals produced all-male populations. To our knowledge, this is the first report of a single gene manipulation inducing full functional sex reversal in crustaceans and beyond. Our results demonstrate how temporal intervention with the expression of a sexual differentiation key regulating gene in a certain time frame throughout postmetamorphic juvenile stages indefinitely alters a phenotype in a profound way. Using real-time RT-PCR, *Mr-IAG* dsRNA

Downloaded from www.biolreprod.org.

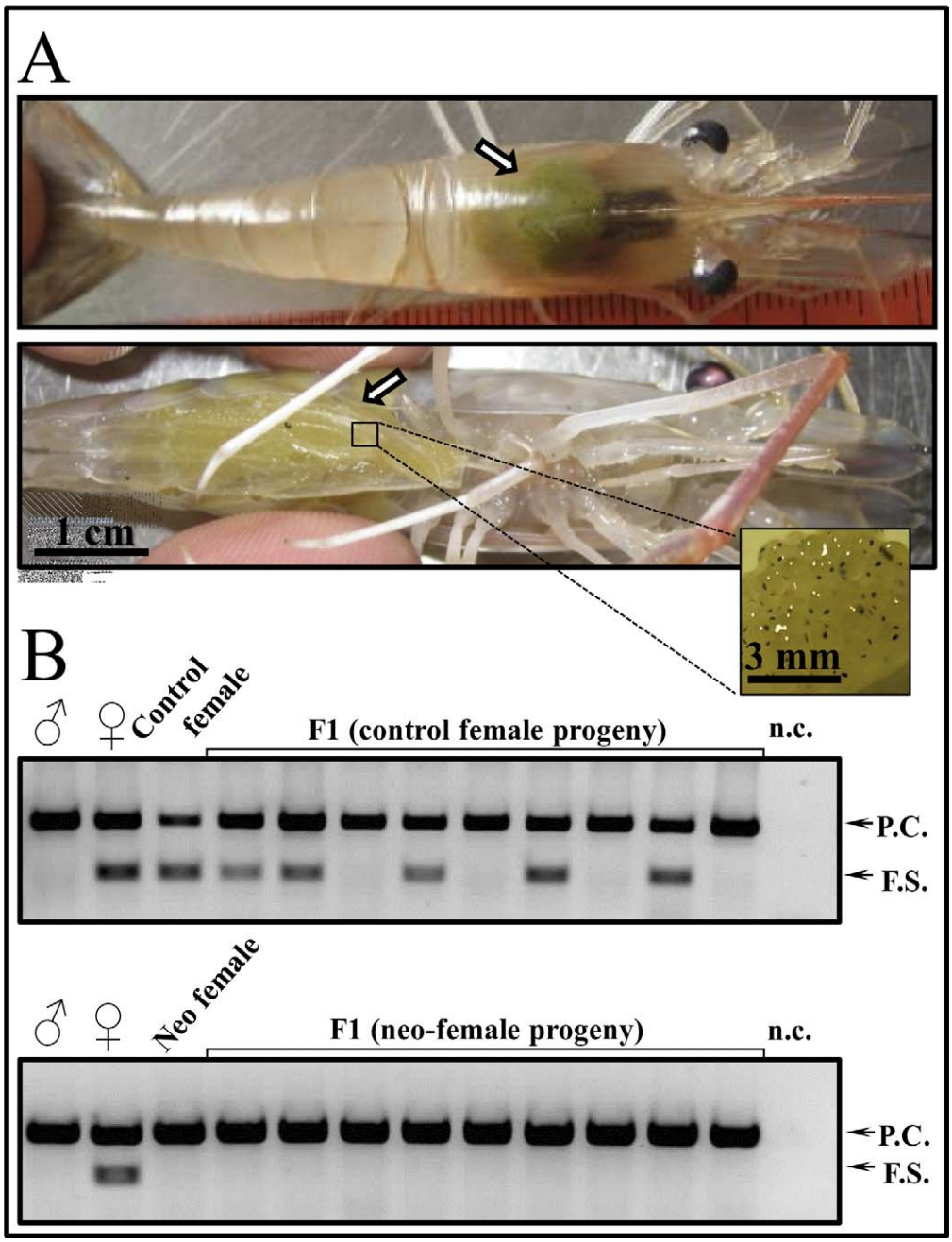

FIG. 4. *Mr-IAG*-silencing induces full-functional sex reversal of males into all-male-progeny-producing neofemales. **A**) Ventral view of a *Mr-IAG* silenced neofemale incubating eggs in its brood chamber (arrow, with a few eggs magnified). **B**) Genomic validation of the neofemale and broodstock maleness. PCR products generated from intact male and intact female control individuals and from a control female and individuals from its progeny (**top**) as templates, as well as the neofemale and individuals from its progeny (**bottom**), were separated on an agarose gel. F.S., female-specific marker; n.c., negative control; P.C., positive control.

injection down to the dose of 0.1 μg dsRNA/g body wt was shown to effectively silence *Mr-IAG* transcript up to 1 wk (Fig. 1), longer than the practiced bi/tri-weekly injection regimes with higher doses that were commonly used [6]. Previous reports also show that *Mr-IAG* silencing inhibits growth [6], which implicates *Mr-IAG* as being involved in regulation of metabolism. This result, which was reconfirmed by the present study (data not shown), might explain, in part, the sexually dimorphic growth pattern observed in *M. rosenbergii*, in which males grow larger and faster than females.

Our results are unique compared with those of other single-gene interventions in the cascade of sexual differentiation, which were performed at earlier stages and did not yield full functional sex reversal [13, 14]. Our results place an insulin-like peptide (Mr-IAG) as a key regulator of sexual differentiation in a crustacean.

In other taxonomic groups, different mechanisms, which do not involve male-specific insulin-like peptides, prevail in the regulation of sexual differentiation. In all vertebrates, an array of peptide and steroidal sex hormones govern the process of sexual differentiation, but with distinctions between and within phyla [15–18]. Our results suggest an additional viewpoint concerning the evolution of insulin-like peptides, which might

Downloaded from www.biolreprod.org.

have emerged in the context of sexual differentiation in the archaic invertebrate group of crustaceans.

Evolutionarily, crustaceans are among the earliest arthropods [19, 20]. In other arthropods, such as insects, mechanisms of sexual differentiation are under debate as to whether those organisms possess any sex hormones at all. The elaborated studies of sexual determination and differentiation mechanisms in *Drosophila* sp. favor the belief that these processes rely solely upon autonomous cellular differentiation mechanisms governed by the cascade of three genes. Contrary to the above is the steroidogenic effect of the gonadotropins of two locust species, the mosquito *Aedes* and the male gypsy moth *Lymantria* [21]. Thus, the absence of an underlying common effector for arthropods that triggers sexual differentiation and male reproductive physiology is puzzling.

Because *M. rosenbergii* is a commercially valuable commodity, the molecular novelties of the present study could result in a new crustacean monosex biotechnology. Our results provide a proof of concept for a biotechnology of crustacean monosex population culture based on AG-specific factor manipulation, primarily the IAG, which was recently identified in representative species of all four major cultured crustacean groups [22–24], as well as on genomic sex markers that are also available for several species [5, 25]. Indeed, many of the gonochoristic cultured crustacean species are sexually dimorphic, making monosex culture a preferred technology [9, 26–28] to increase yields in this industry, which had an estimated annual value of more than $24 billion in 2009 [28].

In terms of ecological significance, monosex population culture can serve as the first line of defense against dissemination of newly introduced species; because monosex populations are inherently nonbreeding, slippage of specimens to the wild will result in a limited, single-generation impact. Many crustacean species inflict ecological hazard as invasive species, cause losses to the economy, and damage human health and well-being due to their high invasiveness, and efforts to manage these species are yet to prove successful [8]. Sexual manipulation is a promising route, as has been successfully realized with eradication of insects where sterile male dissemination minimized the impact of the detrimental species [9]. Adopting sexual differentiation manipulation schemes through research avenues aiming to eradicate ecologically detrimental crustacean species [29] might, therefore, be a promising area of research for this field.

ACKNOWLEDGMENT

We thank Mr. Amit Savaya for prawn maintenance and Dr. Ofer Ovadia for advice regarding statistical analysis.

REFERENCES

1. Charniaux-Cotton H. Androgenic gland of crustaceans. Gen Comp Endocrinol 1962; 1:241–247.
2. Sagi A, Cohen D. Growth, maturation and progeny of sex-reversed *Macrobrachium rosenbergii* males. World Aquacult 1990; 21:87–90.
3. Malecha SR, Nevin PA, Ha P, Barck LE, Lamadrid-Rose Y, Masuno S, Hedgecock D. Sex-ratios and sex-determination in progeny from crosses of surgically sex-reversed freshwater prawns, *Macrobrachium rosenbergii*. Aquaculture 1992; 105:201–218.
4. Ventura T, Rosen O, Sagi A. From the discovery of the crustacean androgenic gland to the insulin-like hormone in six decades. Gen Comp Endocrinol 2011; 173:381–388.
5. Ventura T, Aflalo ED, Weil S, Kashkush K, Sagi A. Isolation and characterization of a female-specific DNA marker in the giant freshwater prawn *Macrobrachium rosenbergii*. Heredity 2011; 107:456–461.
6. Ventura T, Manor R, Aflalo ED, Weil S, Raviv S, Glazer L, Sagi A. Temporal silencing of an androgenic gland-specific insulin-like gene affecting phenotypical gender differences and spermatogenesis. Endocrinology 2009; 150(3):1278–1286.
7. Ventura T, Manor R, Aflalo ED, Weil S, Khalaila I, Rosen O, Sagi A. Expression of an androgenic-gland-specific insulin-like peptide during the course of prawn sexual and morphotypic differentiation. ISRN Endocrinol 2011; 2011:1–11.
8. Gherardi F, Aquiloni L, Diéguez-Uribeondo J, Tricarico E. Managing invasive crayfish: is there a hope? Aquat Sci 2011; 73:185–200.
9. Schetelig MF, Wimmer EA. Insect transgenesis and the sterile insect technique. In: Vilcinskas A (ed.), Insect Biotechnology, vol 2, 1st ed. Heidelberg: Springer Netherlands: 2011:169–194.
10. Khalaila I, Manor R, Weil S, Granot Y, Keller R, Sagi A. The eyestalk-androgenic gland-testis endocrine axis in the crayfish *Cherax quadricarinatus*. Gen Comp Endocrinol 2002; 127:147–156.
11. Uno Y, Chin Soo K. Larval development of *Macrobrachium rosenbergii* (de Man) reared in the laboratory. Tokyo University of Fisheries 1969; 55:179–190.
12. Kleinbaum DG, Klein M. Survival Analysis: A Self-Learning Text, 2nd ed. New York: Springer; 2005.
13. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 2009; 461:267–271.
14. Zarkower D. Establishing sexual dimorphism: conservation amidst diversity? Nat Rev Genet 2001; 2:175–185.
15. Rey R, Josso N, Sexual differentiation. In: Degroot L (ed.), Endotext.org, New M, Series ed., Pediatric Endocrinology, Chapter 7. S. Dartmouth, MA: MDTEXT.COM, Inc.; 2009. World Wide Web (URL: http://www.endotext.org/pediatrics/pediatrics7/pediatrics7.pdf). (October 1, 2010).
16. Bull JJ. Sex determination in reptiles. Q Rev Biol 1980; 55:3–21.
17. Lance VA. Sex determination in reptiles: an update. Am Zool 1997; 37:504–513.
18. Scholz S, Kluver N. Effects of endocrine disrupters on sexual, gonadal development in fish. Sex Dev 2009; 3:136–151.
19. Glenner H, Thomsen PF, Hebsgaard MB, Sørensen MV, Willerslev E. The origin of insects. Science 2006; 314:1883–1884.
20. Lukhtanov V, Kuznetsova V. What genes and chromosomes say about the origin and evolution of insects and other arthropods. Russian Journal of Genetics 2010; 46:1115–1121.
21. De Loof A, Huybrechts R. ''Insects do not have sex hormones'': a myth? Gen Comp Endocrinol 1998; 111:245–260.
22. Chung JS, Manor R, Sagi A. Cloning of an insulin-like androgenic gland factor (IAG) from the blue crab, *Callinectes sapidus*: implications for eyestalk regulation of IAG expression. Gen Comp Endocrinol 2011; 173:4–10.
23. Mareddy VR, Rosen O, Thaggard HB, Manor R, Kabulla AV, Aflalo ED, Sagi A, Paterson B, Elizur A. Isolation and characterization of the complete cDNA sequence encoding a putative insulin-like peptide from the androgenic gland of *Penaeus monodon*. Aquaculture 2011; 318:364–370.
24. Manor R, Weil S, Oren S, Glazer L, Aflalo ED, Ventura T, Chalifa-Caspi V, Lapidot M, Sagi A. Insulin and gender: an insulin-like gene expressed exclusively in the androgenic gland of the male crayfish. Gen Comp Endocrinol 2007; 150:326–336.
25. Staelens J, Rombaut D, Vercauteren I, Argue B, Benzie J, Vuylsteke M. High-density linkage maps and sex-linked markers for the black tiger shrimp (*Penaeus monodon*). Genetics 2008; 179:917–925.
26. Sagi A, Milstein A, Eran Y, Joseph D, Khalaila I, Abdu U, Harpaz S, Karplus I. Culture of the Australian red-claw crayfish (*Cherax quadricarinatus*) in Israel, II. Second growout season of overwintered populations. Israeli Journal of Aquaculture (Bamidgeh) 1997; 49:222–229.
27. Nair CM, Salin KR, Raju MS, Sebastian M. Economic analysis of monosex culture of giant freshwater prawn (*Macrobrachium rosenbergii* De Man): a case study. Aquaculture Research 2006; 37:949–954.
28. Food and Agricultural Organization of the United Nations. Global aquaculture production. World Wide Web (URL: http://www.fao.org/fishery/statistics/global-aquaculture-production/en). (August, 2011).
29. Aquiloni L, Becciolini A, Berti R, Porciani S, Trunfio C, Gherardi F. Managing invasive crayfish: use of x-ray sterilization of males. Freshwater Biology 2009; 54:1510–1519.

Downloaded from www.biolreprod.org.