© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 4101-4108 doi:10.1242/jeb.163410

RESEARCH ARTICLE

Silencing and augmentation of IAG hormone transcripts in adult *Macrobrachium rosenbergii* males affects morphotype transformation

Himanshu Priyadarshi, Rekha Das, Annam Pavan-Kumar, Pathakota Gireesh-Babu, Hasan Javed, Sujit Kumar, Makesh Marappan, Somdutt, Gopal Krishna and Aparna Chaudhari*

ABSTRACT

Morphotypic differentiation is the external manifestation of dominance hierarchy in *Macrobrachium rosenbergii*. The intermediate morphotype orange claw (OC) male exhibits the highest growth rate and is subordinate in hierarchy to blue claw (BC) male while dominant on small male (SM). The present study was undertaken to examine the specific role of insulin-like androgenic gland (*iag*) hormone in morphotype differentiation of *M. rosenbergii*. To achieve this, RNAi mediated knockdown as well as augmentation of *iag* transcripts were effected in ~60 g OC males using plasmid-based constructs pcD-IAG-lh and pcD-IAGorf, respectively. The treatments were administered to animals maintained in isolation as well as in community. The knockdown plasmid construct that expresses *iag*-specific long hairpin RNA caused 16-fold reduction of *iag* transcripts in the SSN1 cell line *in vitro*. When injected into OC males living in a community, 2.3-fold *iag* knockdown was recorded, while in isolated OC males it was 4.2-fold initially, but returned to normal subsequently. Compared with the respective controls, OC to BC transformations in the *iag* silenced animals were significantly lower in the community-reared group, while no difference was observed in the isolated animals. It is reported here for the first time that *iag* augmentation in OC males resulted in significantly higher OC to BC transformations, when animals were reared in community. This plasmid-based IAG knockdown approach could be developed into a low stress, feed or immersion treatment for controlling heterogeneous individual growth of *M. rosenbergii* males in aquaculture.

KEY WORDS: Insulin-like androgenic gland hormone, Heterogeneous individual growth, Lh-RNA, Knockdown, Augmentation, Morphotype, Freshwater prawn

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* De Man 1879 is a decapod crustacean exhibiting excellent growth rate, market value and economical potential. These advantages are, however, overshadowed by the phenomenon of heterogeneous individual growth (HIG) exhibited by males of the species. HIG is a manifestation of social control of growth and reproduction (Smith and Sandifer, 1975; New, 2002; Karplus, 2005), which results in non-uniform harvest and makes its culture uneconomical. Size heterogeneity in this species occurs at all stages of the life cycle, but becomes apparent at the adult stage (Cohen et al., 1981). Adult males exhibit three subtypes (morphotypes), namely small male (SM) or runt, orange claw (OC), and blue claw (BC) or bull (Ranjeet and Kurup, 2002). They form a complex social hierarchy, in which they differ in morphological features, sexual activity, growth patterns and dominance (Ra'anan and Sagi, 1985; Kuris et al., 1987; New, 2002; Ranjeet and Kurup, 2002; Karplus, 2005; Lalrinsanga et al., 2012). These stages are known to undergo transformation from SM to OC to BC (Karplus, 2005). BC males, characterized by a large blue coloured claw and making up 10% of the male population, are usually largest in size (up to 250 g) in a community, sexually active and actively mate with females. Besides suppressing the growth of the SM, BC males are territorial, utilize most of the resources (such as space, food, etc.) but cease to grow further. OC males make up 40% of the male population, have higher growth rate than BC males and SM, weigh in the range of 50–150 g, and are sexually less active. SM have thin translucent claws (50% of the male population), weigh in the range of 5–15 g, are sexually active and mate with females by sneaking behaviour. BC males are dominant over both OC males and SM, whereas OC males dominate over SM. When a fast-growing OC male becomes larger than the existing BC male in community, it transforms into a BC male (Cohen et al., 1981; Ra'Anan and Cohen, 1984; Ra'Anan and Sagi, 1985; Karplus et al., 1991, 1992; Ra'Anan et al., 1991; Ranjeet and Kurup, 2002). Among the three male morphotypes of *M. rosenbergii*, OC males have the highest growth rate (Ra'anan et al., 1991; Lalrinsanga et al., 2012), and their relative proportion in harvest populations determines the economic value of *M. rosenbergii* culture. This social control of growth and sexual activities results in heterogeneous individual growth (HIG), cannibalism and poor survivability, which makes the culture of this species non-viable (Nair et al., 1999; New, 2002).

The androgenic gland (AG) has been demonstrated to play a crucial role in sexual and/or morphotypic differentiation in several crustaceans (Nagamine et al., 1980a,b; Nagamine and Knight, 1987; Sagi et al., 1990; Barki et al., 2003; Aflalo et al., 2006). Insulin-like androgenic gland (*iag*) hormone is reported to be a male hormone expressed specifically from AG of males in *M. rosenbergii* (Ventura et al., 2011), *Cherax quardricarinatus* (Manor et al., 2007) and *Penaeus monodon* (Mareddy et al., 2011). In blue crab *Callinectes sapidus* it is also expressed at a low level in the hepatopancreas of males (Chung et al., 2011), and in the mud crab *Scylla paramamosain* it is expressed in several tissues of both males and females including ovary (Huang et al., 2014). Huang et al. (2014) also noticed that *iag* and vitellogenin expression in the ovary are inversely related and that IAG levels increase significantly at stage V when vitellogenin synthesis has ceased.

ICAR-Central Institute of Fisheries Education, Mumbai-400061, India.

*Author for correspondence (achaudhari67@gmail.com)

P.G.-B, 0000-0001-6249-1624; A.C., 0000-0001-5253-8169

Received 24 May 2017; Accepted 22 August 2017

AG ablation in juvenile males of *M. rosenbergii* also results in feminization (Nagamine et al., 1980a), whereas AG implantation effects masculinization (Nagamine et al., 1980b). Silencing the *Mr-IAG* gene in *M. rosenbergii* juveniles through repeated injection of *Mr-IAG* double-stranded RNA led to delayed regeneration of appendices masculinae (Ventura et al., 2009) and functional sex reversal (Ventura et al., 2012). Although Ventura et al. (2011) recorded a differential expression of *iag* between the male morphotypes with the least expression of IAG hormone in OC males in comparison with SM and BC, more information is required on the extent of its involvement in morphotype differentiation.

Here we report the effect of specific silencing as well as augmentation of *iag* hormone gene on morphotype transformation in *M. rosenbergii* males using plasmid constructs delivered *in vivo*. The advantages of higher stability, economy, ease of handling and longer *in vivo* persistence of plasmid constructs, make this strategy a feasible option for managing HIG in grow-out systems. Furthermore, administration of plasmid DNA can be achieved through immersion treatment, which is the least stressful to the animals (Chowdhury et al., 2014).

MATERIALS AND METHODS

Primer designing

Online software E-RNA*i* (www.dkfz.de/signaling/e-rnai3/idseq.php) was used to identify an effective and specific target region in the *cds* of *M. rosenbergii iag* gene for designing the anti-Mr-IAG long hairpin (*lh*) insert. A BLASTn similarity search was performed for IAG lhRNA to avoid off-target effects over reported sequences from this species. All other primers used in the study were designed using Generunner version 5.2 software following the standard criteria for primer designing. Table 1 shows the list of primers used in this study along with the National Center for Biotechnology Information (NCBI) accession numbers for the target gene sequences used.

Engineering of the plasmid constructs

The plasmid construct expressing IAG lhRNA was prepared following the methods of Krishnan et al. (2009). Briefly, a 136 bp Mr-IAG fragment was amplified using primer pair MR-IAG-AS-F/R (Table 1) and AG cDNA as template. This was cloned into pcDNA3.1(+) vector in antisense orientation between *Xho*I and *BamH*I restriction sites downstream of cytomegalovirus (CMV) promoter. Thereafter, a 158 bp Mr-IAG fragment was amplified using primer pair MR-IAG-SE-F/R (Table 1) and cloned in this construct in sense orientation between *Nhe*I and *Hind*III sites upstream to the antisense fragment to obtain pcD-IAG-lh construct (Fig. 1A). The positive clone was selected by colony polymerase chain reaction (PCR) and further confirmed by restriction digestion and sequencing (Fig. S1).

The full-length open reading frame (ORF) of the *iag* gene (564 bp), harbouring a 25 bp 5′ UTR, translation initiation sequence and translation stop site was amplified using primer set MR-IAG-EXP-F/R (Table 1) and cloned between *Nhe*I and *Hind*III sites in pcDNA3.1(+) downstream of the CMV promoter. A positive clone was selected by colony PCR and further confirmed by sequencing. The confirmed construct was named pcD-IAGorf (Fig. 1B).

Nucleic acid isolation and cDNA preparation

For *in vitro* (SSN1 cell line) and *in vivo* studies, plasmid constructs were isolated in bulk using an endotoxin-free QIAGEN Plasmid Giga Kit (Qiagen, Germany). Genomic DNA was isolated from ethanol-preserved pleopod samples as per Sambrook and Russell (2001). Total RNA from AG and the transfected cells was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Complementary DNA (cDNA) was prepared from DNase I treated total RNA (1 µg) using a RevertAid cDNA synthesis kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The nucleic acid preparations were quantified on a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific).

In vitro assessment of silencing efficiency of the lhRNA construct

The silencing efficiency of lhRNA expressed from the pcD-IAG-lh plasmid was studied *in vitro* in the striped snakehead fish whole fry cell line (SSN1). The cell line was tested for contamination and only healthy cell lines were used for the experiment. SSN1 cells were grown for 24 h in a six-well plate (growth area: 9 cm^2 per well) in 1× L15 medium supplemented with L-glutamine, 10% fetal bovine serum and 1× antibiotic antimycotic solution (HiMedia, India). Transfections were done using TurboFect transfection reagent (Thermo Fisher Scientific) following the manufacturer's instructions after achieving 80% confluence. Plasmid constructs pcD-IAGorf and pcD-IAG-lh were co-transfected at 1:0 (positive control/PC) and 1:1 (silencing treatment/ST) ratios in three wells each in two plates. A total of 4 µg plasmid DNA was transfected per well. After 24 h of growth, the transfected cells were washed with phosphate-buffered saline (PBS; pH 7.4) and harvested directly into 1 ml of Trizol reagent (Invitrogen) for total RNA isolation. Silencing efficiency of the construct was studied by real-time

Table 1. Details of primers used in this study

SN	Primer	Sequence (5′ to 3′)	T_m (°C)	Target sequence	Size (bp)
1	MR-IAG-SE-F/R	AAAGCTAGCGAAACTCAGAAGGTATCTAAGG AAAAAGCTTCGTCTGAAAGAGGCGTTGTT	54.2 57.6	FJ409645	158
2	MR-IAG-AS-F/R	AAACTCGAGTGGAGGAGGAGATTCAGCAC AAAGGATCCCGTCTGAAAGAGGCGTTGTT	57.6 60.3	FJ409645	136
3	MR-IAG-EXP-F/R	AAAGCTAGCCGAAGTGAAACAAATCAAC AAAAAGCTTACCTCCTACCTGGAACTG	53.4 50.9	FJ409645	552
4	MR-IAG-RT-F/R	CGTTTCCAAAGAGCGACGATCTGC CATGTGCTGAATCTCCTCCTCCACC	54.9 53.6	FJ409645	148
5	MR-EF1α-RT-F/R	TGGACGTGTGGAGACTGGCATC ATCGCCTGGAACAGCCTCAGTC	50.1 50.5	KF228019	127
6	CMV-pro-F/R	AAAGCTAGCGAATCTGCTTAGGGTTAGG AAATCTAGAAATTTCGATAAGCCAGTAAGC	54.2 51.5	pcDNA3.1(+) vector	700
7	LR-β-actin-RT-F/R	GCCGAGAGGGAAATTGTCCGTGAC TTGCCAATGGTGATGACCTGTCCG	55.6 56.1	EU184877	146

SN, serial number; T_m, melting temperature.

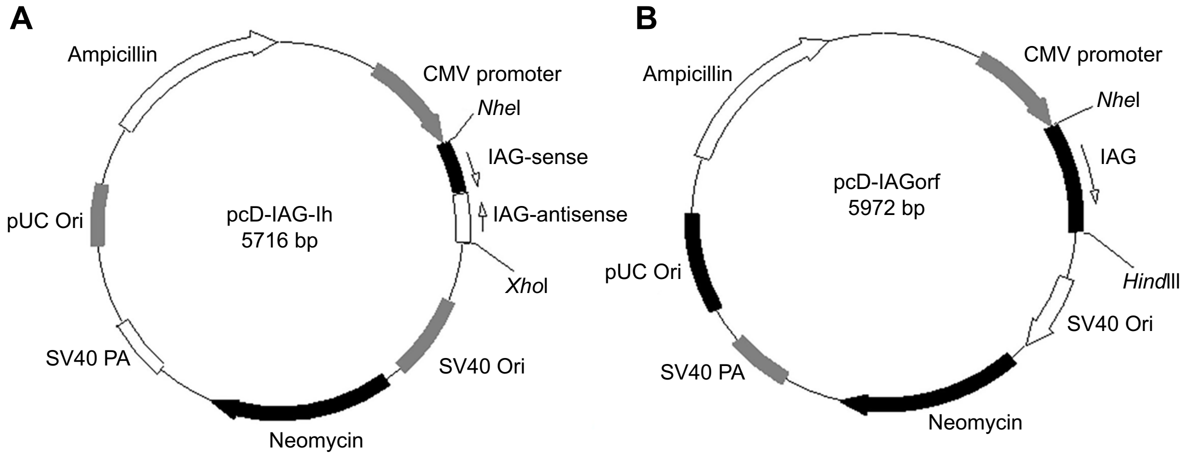


Fig. 1. Schematic sketch of plasmid constructs. (A) pcD-IAG-lh; (B) pcD-IAGorf.

PCR of *iag* transcripts using primers MR-IAG-RT-F/R normalized to β-actin gene using LR-β-actin-RT-F/R (Table 1).

Rearing of experimental animals

Sexually mature *M. rosenbergii* males of one year age group (∼60 g) were collected from grow-out ponds of the Powarkheda centre of the Indian Council for Agricultural Research-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai, India. The experiments were conducted in the month of December when the ambient temperature was around 15°C. The collected animals were reared in 1-tonne capacity fibre-reinforced plastic (FRP) tanks of cross-section area 1 m^2 containing 250 litres of clear freshwater with constant aeration at a temperature of 28°C. The water quality parameters were checked regularly and maintained at optimum [pH 7.8±0.3; dissolved oxygen 8.0±1.0 ppm; temperature 28.0±1.3°C; hardness ($CaCO_3$) 50.2±2.0 ppm]. All males were OC males with smooth spineless claws. Animals were fed on commercial pelleted feed three times a day and 40–60% of the water was exchanged every alternate day. The guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division) and Government of India on care and use of animals in scientific research were followed to care for and rear the animals used in the present study. The study was approved by the Board of studies and authorities of ICAR-CIFE, Mumbai, India.

Experimental design for IAG silencing in *M. rosenbergii*

IAG silencing experiments were done both in isolation and community on OC males weighing ∼60 g. All treated animals in the silencing studies received pcD-IAG-lh construct diluted with PBS at 2 µg g^{-1} body weight while the control animals received PBS injections. The plasmid injections were given at a final volume of 100 µl per animal and administered directly into the sinus cavity of the animals between the third and fourth pereiopods. For rearing animals in isolation (experiment 1, E1), a total of 38 animals (19 each in control and treatment) were kept individually in round perforated cages (area 450 cm^2) and placed in FRP tanks to provide uniform conditions (Fig. 2A and B). Experiment E1 was conducted in triplicate, where each tank had equal numbers of control and treated animals. For community rearing (experiment 2, E2), the OC males were stocked in FRP tanks at a stocking density of five animals per square metre (Fig. 2C). The community rearing experiment was performed in triplicate. Shelters sufficient to hide all animals were provided.

Experimental design for IAG augmentation in OC males

In the IAG augmentation experiments (experiment 3, E3), the treatment group was injected with pcD-IAGorf diluted in PBS at 1 µg g^{-1} body weight as described above. The plasmid injections were given at a final volume of 100 µl per animal as explained above. All animals in the control group were given PBS injections. Five OC males (∼60 g) that received the plasmid were kept along with one PBS-injected larger OC male (∼150 g) in one FRP tank with bottom area 1 m^2. This was done to simulate the social hierarchy, and the large-sized untreated OC male was expected to either transform earlier or suppress the transformation of smaller OC males in untreated controls. The experiment was done in duplicate. The tanks were provided with sufficient hideouts.

Data collection and tissue sampling

Morphotype transformation events and moult events were observed and recorded daily. Morphotypes were differentiated on the basis of the key suggested by Kuris et al. (1987). For freshly moulted animals, characters such as death, loss of claw in territorial fight and claw colour after moult were observed (Fig. S2). For E1 (IAG silenced males in isolation), three animals each were sampled randomly from treatment and control groups on days 4, 8, 12, 18 and 21, and four animals on day 25 post-injection. In the case of the community rearing experiment (E2), sampling was done 4 days after a clear external manifestation of stable social hierarchy establishment was observed. Thus sampling was done 4 days after the stable morphotype transformation event (i.e. transformed BC male survived with intact claw) in the control and treatment groups, respectively, for IAG silencing and augmentation experiments. This mostly happened around 21 days post-injection. The margin of four additional days was included to give a fair chance for transformation to the counter group and to ensure that the recorded event was an actual effect of the treatment and not a chance happening. Relevant tissues were dissected aseptically during the sampling and stored appropriately for further analyses as outlined in the specific sections below. Carapace and propodus lengths were measured in each animal. Relative propodus length [propodus length (cm)/carapace length (cm)] was calculated for individual animals.

In vivo plasmid persistence, distribution and expression

For plasmid persistence studies, pleopod samples from both treatment and control group animals were collected on days 4, 12, 18 and 25, and stored in absolute ethanol for genomic DNA isolation. For plasmid distribution studies, pleopod, abdominal

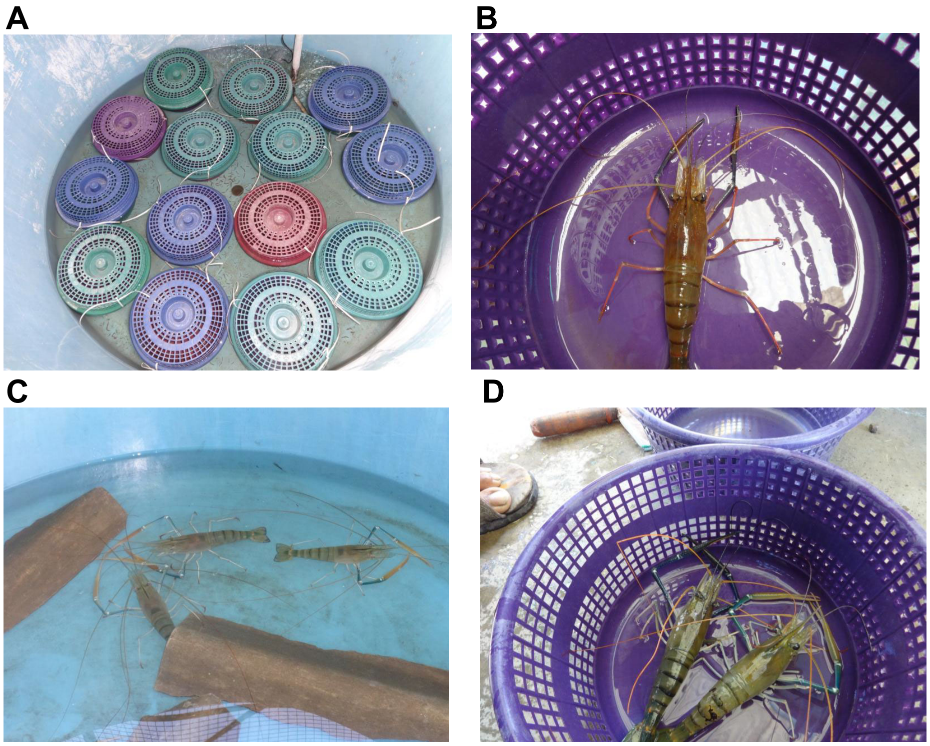


Fig. 2. Experimental set-up for rearing giant freshwater prawns *Macrobrachium rosenbergii* and morphotype transformation studies. (A) FRP tank holding perforated baskets caging both the treatment and control animals for isolated rearing experiment E1. (B) One of the experimental animals housed in the perforated basket. (C) Community rearing of uniformly sized orange claw (OC) males in experiment E2. (D) OC male transformed to blue claw (BC; left) and untransformed OC male (right) after experiment.

muscle (second segment) and gill tissues were sampled on day 4 and stored in absolute ethanol for genomic DNA isolation. DNA samples from a particular day were pooled separately for treatment and control. To confirm the presence of the plasmid in the animal body, PCR was performed on 100 ng pooled genomic DNA using vector-based primers CMV-pro-F/R (Table 1) following the PCR conditions of Chowdhury et al. (2014). In order to confirm the *in vivo* expression of IAG transcript from pcD-IAGorf, cDNA was prepared from abdominal muscle (second segment) tissue of pcD-IAGorf injected and control animals and subjected to PCR using a MR-IAG-EXP-F/R primer set (Table 1).

Expression analysis of *iag* transcript

Androgenic glands were dissected out from the sampled animals using sterile RNAse free tools and stored in RNAlater™ (Qiagen, Germany) for real-time PCR studies. Knockdown and augmentation effects of the respective constructs *in vivo* were ascertained by real-time PCR of *iag* hormone gene expression. *Macrobrachium rosenbergii ef1α* gene was used as internal control in real-time PCR. Real-time PCR was performed with a Roche 480 Light Cycler machine (Roche, Germany). Reaction mixture consisted of 50 ng cDNA, 2.5 pmol each of forward and reverse primers (MR-IAG-RT-F/R or MR-EF1α-RT-F/R; Table 1) and 5 µl of 2× SYBR Green Master Mix (Thermo Fisher Scientific) in a final volume adjusted to 10 µl. The real-time PCR programme consisted of one cycle of initial denaturation at 95°C for 10 min, 45 cycles at 95°C for 20 s, 60°C for 20 s, 72°C for 30 s, one cycle for melt curve analysis at 95°C for 5 s, and 65°C for 1 min followed by continuous signal acquisition until the temperature reached 97°C. Each cDNA was run in duplicate. Melting curve/peak analysis was done for all the genes after each qRT-PCR, which showed specific product amplification by each primer pair, namely *β-actin*, *ef1α* and *iag*. Relative fold change of IAG was obtained using the formula $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) after reference residual normalization (Edmunds et al., 2014).

Histology

For assessment of the effect of different treatments on reproductive advancement in male *M. rosenbergii*, histology of the testis was investigated. On the last day of the experiment (day 25), two samples each of OC and BC were collected from each tank of E1, E2 and E3 for histological examination of testis. The testis for the preparation of histological slides was dissected out aseptically and fixed using Davidson's fixative. The fixed testis tissues were washed with distilled water, dehydrated with ascending grades of alcohol and processed by standard protocols (Bell and Lightner, 1988). The paraffin embedded testis tissues were sectioned at 4 µm thickness using a rotatory microtome (Leica, Rm 2125RT) and stained with haematoxylin and eosin (H & E). The stained sections were mounted using D.P.X. and photographed using Zeiss Axiophot A1 digital fluorescence microscope (Carl Zeiss, Germany).

Statistical analysis of the data

Relative fold change values were normalized by log2 transformation for either Student's *t*-test or one-way ANOVA and analysed by Tukey's test using SPSS version 16.0. Cox regression analysis and Fisher's exact test were used to analyse morphotype transformation data of IAG silencing in isolation and community rearing, respectively. Moult data were analysed using Cox regression analysis in the SPSS 16.0 software package.

RESULTS

Validation of silencing efficiency *in vitro*

In vitro knockdown efficiency of pcD-IAG-lh was studied in the SSN1 cell line by real-time PCR. The knockdown construct resulted in 16-fold reduction in the expression of *iag* transcript *in vitro* compared with the positive control (Fig. 3).

Confirmation of IAG knockdown and augmentation *in vivo*

The *in vivo* persistence of the pcD-IAG-lh constructs throughout the experiment was confirmed by PCR using vector-specific primers

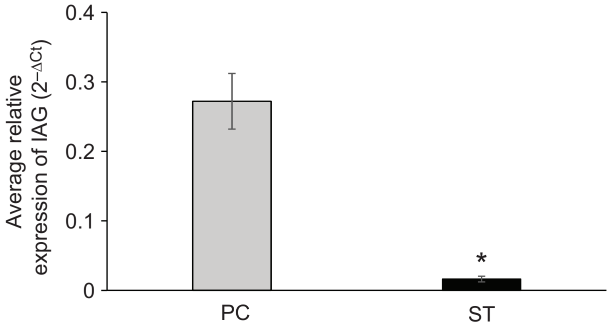


Fig. 3. Silencing efficiency of pcD-IAG-lh *in vitro*. The bars represent means±s.e.m. of expression of *iag* transcripts relative to that of β-actin in the transfected positive control (PC; pcD-IAGorf alone) and silencing treatment (ST; co-transfected with pcD-IAGorf and pcD-IAG-lh in 1:1 ratio) cells. *Significant difference between treatment and control at $P<0.05$.

and pleopod DNA as template. A specific band of 700 bp was amplified from pcD-IAG-lh injected animals, but not from PBS-injected animals (Fig. S3). The same primer set confirmed the presence of plasmid DNA in pleopod, muscle and gill tissue (Fig. S3).

Expression of *iag* transcripts from pcD-IAGorf was confirmed by performing PCR using *iag*-specific primers in cDNA prepared from tissues that do not express the gene naturally. An *iag*-specific 564 bp band was amplified from abdominal muscle (second segment) cDNA of augmented animals, but not from their DNase I-treated RNA or muscle cDNA of control animals. The housekeeping gene *ef1α* could be amplified from both treated and control groups (Fig. S4).

Silencing of IAG hormone gene inhibits OC to BC transformation in males

Morphotype transformation in animals reared in isolation

Morphotype transformation events were recorded daily over 25 days in E1. There was not a single transformation event between 0 and 18 days in either treatment or control groups. Between 18 and 25 days, three OC to BC transformations occurred in the treatment group and four in the control group distributed over replicates (Fig. 2D). Cox regression analysis revealed that the rate of OC to BC transformation in IAG silenced and control groups was similar ($P>0.05$). Quantitative PCR results show 4.2-fold lower expression of *iag* transcript in the androgenic gland of treated animals compared with controls ($P<0.05$) on the day 4 post-injection, although the expression recovered thereafter (Fig. S5).

Morphotype transformation in animals reared in community

By the end of E2 (day 25), six OC to BC transformations were recorded in control (two events per tank) while none were observed in the treated animals. In each control tank (triplicate) the first transformed BC male either died or lost its claws in territorial fight, but the subsequent transformed male survived, thus resulting in one BC survivor per tank. Fisher's exact test indicates that transformation of OC male to BC male in IAG silenced group was significantly less than the control group ($P<0.05$). *iag* transcript levels examined by real time PCR were 2.3-fold lower than controls in the AG on the final day of the experiment ($P<0.05$; Fig. 4).

Augmentation of IAG hormone gene expression promotes OC to BC transformation

At the end of E3 (day 25), no transformation event was recorded in the control group, while the treated group had five conversions to BC distributed over replicates. The larger OC males did not transform in either group. Fisher's exact test indicates that transformation of OC to BC males in the treatment group animals was significantly higher than control ($P<0.05$). Interestingly, the *iag* transcript level in androgenic gland of augmented males was 3.4-fold lower than controls ($P<0.05$; Fig. 4).

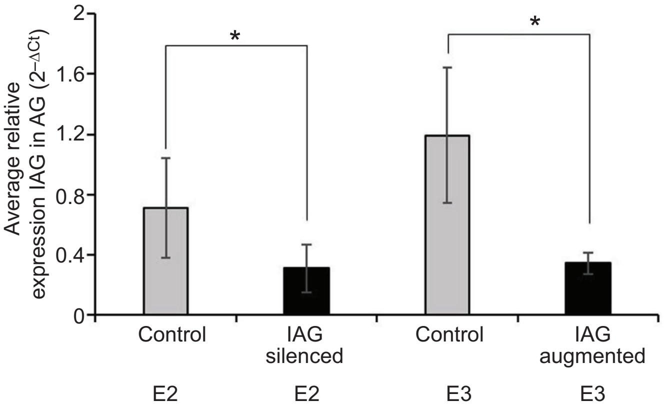


Fig. 4. Expression of *iag* in the androgenic gland of *iag*-silenced and augmented animals reared in community (E2 and E3). The bars represent means±s.e.m. of relative expression of IAG transcripts normalized to EF1α. *Significant difference between treatment and control at $P<0.05$.

Effect of IAG hormone level on moulting

The cumulative moult frequencies between treated and control groups of E1, E2 and E3 over the entire experimental period were not significantly different ($P>0.05$).

Relative propodus length and testicular histology

The relative propodus length (RPL) of each animal was measured at the end of the experiment and compared between all the groups. The mean RPL of the newly transformed BC males did not differ significantly ($P>0.05$) from that of the animals that remained OC at the time of sampling. In addition, the mean RPL of treated animals of experiments E1, E2 and E3 did not differ from that of their respective controls ($P>0.05$).

The histological appearance of the testes of the sampled individuals differed with respect to their morphotype. The lobules sampled from untransformed OC males of E1, E2 and E3 contained gametes at variable stages of spermatogenesis. However, lobules of every transformed BC male contained mature spermatozoa and a small zone of spermatogonia, as expected. Moreover, the epithelial lining of BC males featured secretory vacuole-like structures, which were absent in the OC males. Fig. 5A details a single testicular lobule of a treated OC male from E2, and Fig. 5B shows a single lobule from a newly transformed BC male in the control group of the same experiment. However, although histological appearance of testes of animals differed from each other on the basis of morphotype, they did not differ between the treated and control animals of similar morphotype in any of the experiments.

DISCUSSION

Morphotype differentiation in males is a major stumbling block in the culture of *M. rosenbergii*. Several control measures have been suggested so far with limited efficiency (Smith and Sandifer, 1975; Sagi et al., 1990; Nair et al., 1999; Ventura et al., 2009; Rahman et al., 2010; Ventura et al., 2012). In the present study, the effect of *iag* gene silencing through a plasmid construct expressing lhRNA on morphotype differentiation in male *M. rosenbergii* was tested. The use of a plasmid-based knockdown construct regulated by

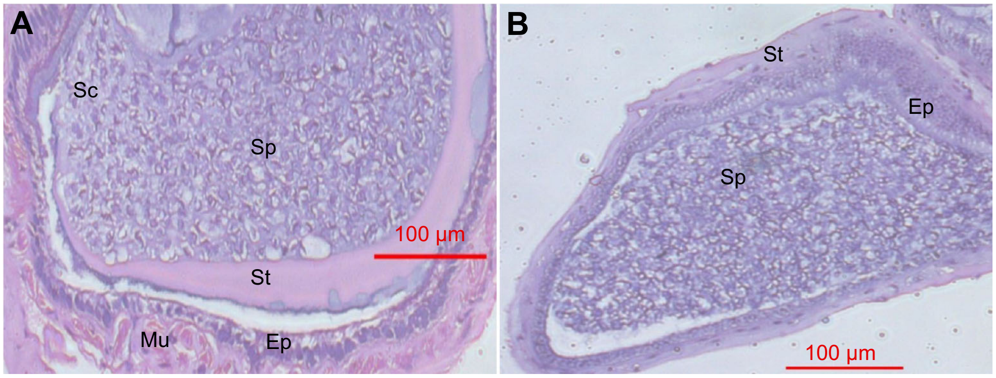


Fig. 5. Cross-section through a single testicular lobule of *M. rosenbergii*. (A) OC male (E2, treated); the featured lobule contains primarily mature sperm cells and spermatocytes but not spermatogonial cells. (B) BC male (E2, control); the lobule contains virtually only mature sperm. The epithelial lining shows prominent presence of secretory vacuoles. Mu, muscle; Ep, epiththelial cells; St, acellular spermatotheca; Sp, mature sperm cells; Sc, spermatocytes.

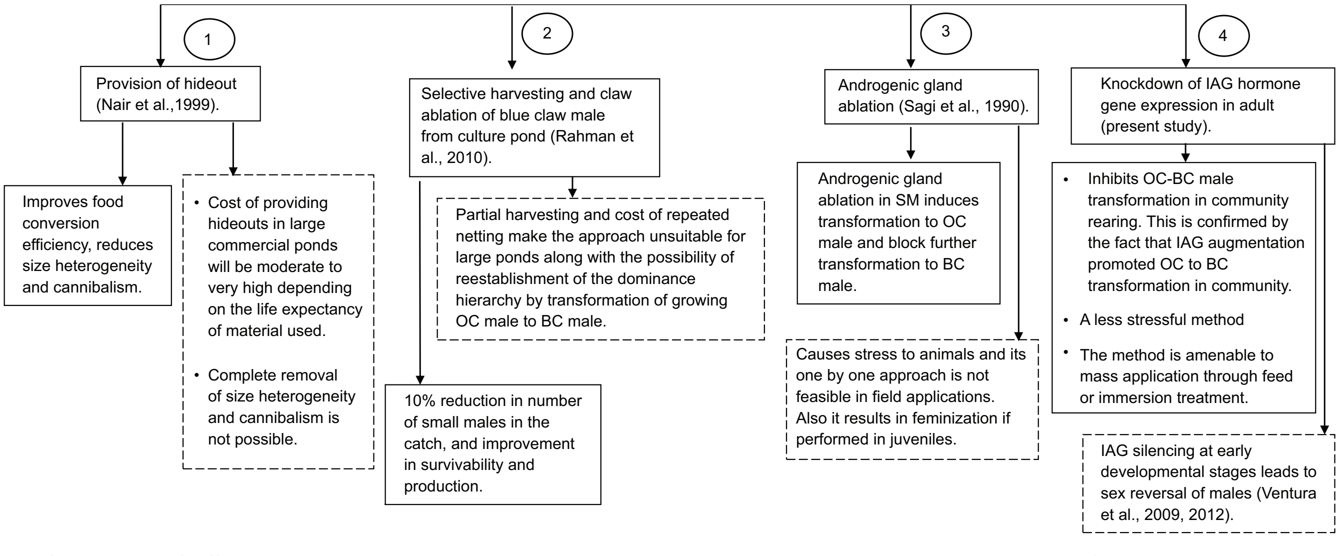
CMV promoter avoids repeated injection of dsRNA and associated stress (Krishnan et al., 2009; Das et al., 2015). The CMV promoter has been shown to demonstrate strong constitutive expression in fish and shrimps (Arenal et al., 2000; Yazawa et al., 2005; Chen et al., 2006). This approach resulted in knockdown of the targeted gene both *in vitro* (≥90%) and *in vivo* (≥75%) as estimated in E1 animals on day 4 by real-time PCR analysis. Fig. 6 provides an update on the reports available in controlling the issue of morphotype differentiation in *M. rosenbergii* culture ponds.

Here we report for the first time that knockdown of *iag* transcript in OC males being reared in community (experiment E2) completely inhibited OC to BC transformation. As confirmed by real-time PCR, the level of *iag* transcript in AG of treated animals was significantly lower than controls on the last day of the experiment. However, these results are in contrast with E1, where OC to BC transformations happened equally in control and treated groups, and *iag* transcript level returned to normal after initial silencing recorded on day 4. In both E1 and E2, the presence of pcD-IAG-lh *in vivo* was confirmed by PCR up to the last day of the experiment.

In isolated rearing conditions similar to those used in this study, Sagi et al. (1990) achieved SM to OC transformation by bilateral androgenic gland ablation, but the same treatment given to OC could not inhibit advancement to BC, even though the latter morphotype is associated with higher IAG expression that is not possible in AG-ablated animals. To explain their observations, Sagi et al. (1990) proposed the likelihood of a 'commitment stage' beyond which the transformation becomes independent of IAG levels. However, this 'commitment' was not observed in community-reared E2 animals where the OC morphotype continued in all *iag*-silenced animals in contrast with controls that progressed to BC.

It appears that the isolated rearing condition is a significant common factor between the recovery of *iag* transcript levels in E1 males (this study) and BC transformations of AG-ablated OC males (Sagi et al., 1990). The results hint at a higher controlling mechanism that regulates OC to BC transformation in response to social environment either through upregulating IAG (as in silenced E1 males) or through an IAG-independent pathway (as in ablated animals). A related observation reported earlier is that the proportion of BC males is high at lower stocking densities (Karplus et al., 1986; Ranjeet and Kurup, 2011), suggesting that reduced interaction results in more OC males converting to BC.

In this study, we have also tested for the first time the hypothesis that augmentation of IAG levels in OC males should result in transformation to BC. Almost 50% of *iag*-augmented E3 animals transformed, while no changes were recorded in controls or the larger OC males kept with treated animals. This confirmed that the



Provision of hideout (Nair et al.,1999).

Improves food conversion efficiency, reduces size heterogeneity and cannibalism.

- Cost of providing hideouts in large commercial ponds will be moderate to very high depending on the life expectancy of material used.
- Complete removal of size heterogeneity and cannibalism is not possible.

Selective harvesting and claw ablation of blue claw male from culture pond (Rahman et al., 2010).

Partial harvesting and cost of repeated netting make the approach unsuitable for large ponds along with the possibility of reestablishment of the dominance hierarchy by transformation of growing OC male to BC male.

10% reduction in number of small males in the catch, and improvement in survivability and production.

Androgenic gland ablation (Sagi et al., 1990).

Androgenic gland ablation in SM induces transformation to OC male and block further transformation to BC male.

Causes stress to animals and its one by one approach is not feasible in field applications. Also it results in feminization if performed in juveniles.

Knockdown of IAG hormone gene expression in adult (present study).

- Inhibits OC-BC male transformation in community rearing. This is confirmed by the fact that IAG augmentation promoted OC to BC transformation in community.
- A less stressful method
- The method is amenable to mass application through feed or immersion treatment.

IAG silencing at early developmental stages leads to sex reversal of males (Ventura et al., 2009, 2012).

Fig. 6. Comparison of different approaches reported to reduce the size heterogeneity in *M. rosenbergii* culture ponds. Continuous boxes represent advantages/outcomes and dashed boxes represent shortcomings/challenges.

observed OC to BC transformations were an outcome of IAG augmentation. Although the *iag* transcript was reduced in AG itself, perhaps on account of feedback inhibition (Rosenthal et al., 1986), its expression from the pcD-IAG-ORF plasmid present in other tissues of treated prawns is expected to have caused this transformation. Ventura et al. (2011) have shown that IAG is specifically expressed in only the AG of *M. rosenbergii*.

Ventura et al. (2009) reported that the silencing of IAG in juvenile males delayed moulting and the same was observed in this study with pooled data from E1 and E2. IAG augmentation in E3 appeared to promote moulting, but the data were not significantly different from controls, probably owing to the small sample size.

Although claw colour is the primary basis of morphotype discrimination, RPL is reported to be higher in BC compared with OC in a naturally established hierarchy (Okumura and Hara, 2004). No significant differences were observed in the present study, possibly because the experiment terminated shortly after transformation. In a recent study, Banu et al. (2015) observed that BC males developing in a population grown from cold shock-treated juveniles (18°C for 24 h) had reduced claw lengths compared with the untreated controls. As our experimental animals were retrieved from a low temperature (15°C), the possibility of an effect of water temperature on the RPL in the animals in our study cannot be ruled out. Histological observations validated our morphotype transformation observations. The testis samples of OC males exhibited variable stages of spermatogenesis, which was expected. Our observations agreed with those of Okumura and Hara (2004), who detailed moult-dependent variability in the testicular histology of *M. rosenbergii* morphotypes.

The present study confirms the role of IAG in OC–BC morphotype transformations, not only through knockdown but also by augmentation of the *iag* transcript. The fact that *iag* gene silencing using a plasmid knockdown construct could completely inhibit transformation of adult OC males reared in community to BC, has possible applications in aquaculture. Moreover, in terms of field applicability, plasmid DNA can be administered by immersion, which is the least stressful mode of delivery to the animals. However, the effect of this treatment on growth rate remains to be seen. Further work is required to determine the suitable developmental stage at which such a treatment would be effective in maintaining a larger proportion of the OC morphotype in an aquaculture pond. In addition, it could be possible in future to administer the IAG augmentation construct to immature animals for obtaining an all-male population for culture.

Acknowledgements

The authors are grateful to the Director and Vice-Chancellor, ICAR-CIFE, Mumbai, for providing necessary facilities. The first author thanks Professor S. N. Puri, Former Vice-Chancellor, Central Agricultural University, Imphal, for granting permission to pursue a PhD at ICAR-CIFE, Mumbai, and to ICAR, New Delhi, for a fellowship.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.C.; Methodology: A.P.-K., P.G.-B., S.K., M.M., S., G.K., A.C.; Software: A.P.-K.; Validation: A.P.-K., S.K.; Formal analysis: A.P.-K., M.M., A.C.; Investigation: H.P., R.D.; Resources: A.C.; Data curation: H.P., R.D., H.J.; Writing - original draft: H.P., R.D.; Writing - review & editing: P.G.-B., A.C.; Visualization: H.J.; Supervision: P.G.-B., M.M., S., G.K., A.C.; Project administration: A.C.; Funding acquisition: A.C.

Funding

The present work was funded by the Indian Council of Agricultural Research, New Delhi, India.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.163410.supplemental

References

Aflalo, E. D., Hoang, T. T. T., Nguyen, V. H., Lam, Q., Nguyen, D. M., Trinh, Q. S., Raviv, S. and 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.

Arenal, A., Pimentel, R., Guimarais, M., Rodríguez, A., Martínez, R. and Abad, Z. (2000). Gene transfer in shrimp (*Litopenaeus schmitti*) by electroporation of single-cell embryos and injection of naked DNA into adult muscle. *Biotechnol. Appl.* **17**, 247-250.

Banu, M. R., Christianus, A., Ikhsan, N. F. M. and Rajaee, A. H. (2015). Effect of cold shock and hormone on growth and male morphotypes of freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Aquacult. Res.* **47**, 3740-3746.

Barki, A., Karplus, I., Khalaila, I., Manor, R. and Sagi, A. (2003). Male-like behavioral patterns and physiological alterations induced by androgenic gland implantation in female crayfish. *J. Exp. Biol.* **206**, 1791-1797.

Bell, T. A. and Lightner, D. (1988). *A Handbook of Normal Penaeid Shrimp Histology*. Baton Rouge, LA: World Aquaculture Society.

Chen, W., Liu, M., Jiao, Y., Yan, W., Wei, X., Chen, J., Fei, L., Liu, Y., Zuo, X. and Yang, F. (2006). Adenovirus-mediated RNA interference against foot-and-mouth disease virus infection both *in vitro* and *in vivo*. *J. Virol.* **80**, 3559-3566.

Chowdhury, L. M., Gireesh-Babu, P., Pavan-Kumar, A., Babu, P. S. and Chaudhari, A. (2014). First report on vertical transmission of a plasmid DNA in freshwater prawn, *Macrobrachium rosenbergii*. *J. Invertebr. Pathol.* **121**, 24-27.

Chung, J. S., Manor, R. and Sagi, A. (2011). 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.* **173**, 4-10.

Cohen, D., Raanan, Z. and Brody, T. (1981). Population profile development and morphotypic differentiation in the giant freshwater prawn *Macrobrachium rosenbergii* (de Man). *J. World Aquacult. Soc.* **12**, 231-243.

Das, R., Krishna, G., Priyadarshi, H., Gireesh-Babu, P., Pavan-Kumar, A., Rajendran, K., Reddy, A., Makesh, M. and Chaudhari, A. (2015). Captive maturation studies in *Penaeus monodon* by GIH silencing using constitutively expressed long hairpin RNA. *Aquaculture* **448**, 512-520.

Edmunds, R. C., McIntyre, J. K., Luckenbach, J. A., Baldwin, D. H. and Incardona, J. P. (2014). Toward enhanced MIQE compliance: reference residual normalization of qPCR gene expression data. *J. Biomol. Tech.* **25**, 54-60.

Huang, X., Ye, H., Huang, H., Yang, Y. and Gong, J. (2014). An insulin-like androgenic gland hormone gene in the mud crab, *Scylla paramamosain*, extensively expressed and involved in the processes of growth and female reproduction. *Gen. Comp. Endocrinol.* **204**, 229-238.

Karplus, I. (2005). Social control of growth in *Macrobrachium rosenbergii* (De Man): a review and prospects for future research. *Aquacult. Res.* **36**, 238-254.

Karplus, I., Hulata, G., Wohlfarth, G. W. and 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.

Karplus, I., Barki, A., Israel, Y. and Cohen, S. (1991). Social control of growth in *Macrobrachium rosenbergii*. II. The ‘leapfrog’ growth pattern. *Aquaculture* **96**, 353-365.

Karplus, I., Hulata, G. and Zafrir, S. (1992). Social control of growth in *Macrobrachium rosenbergii*. IV. The mechanism of growth suppression in runts. *Aquaculture* **106**, 275-283.

Krishnan, P., Babu, P. G., Saravanan, S., Rajendran, K. V. and Chaudhari, A. (2009). DNA constructs expressing long-hairpin RNA (lhRNA) protect *Penaeus monodon* against White Spot Syndrome Virus. *Vaccine* **27**, 3849-3855.

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

Lalrinsanga, P., Pillai, B. R., Patra, G., Mohanty, S., Naik, N. K. and Sahu, S. (2012). Length–weight relationship and condition factor of giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879) based on developmental stages, culture stages and sex. *Turk. J. Fish. Aquat. Sci.* **12**, 917-924.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402-408.

Manor, R., Weil, S., Oren, S., Glazer, L., Aflalo, E. D., Ventura, T., Chalifa-Caspi, V., Lapidot, M. and 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.

Mareddy, V. R., Rosen, O., Thaggard, H. B., Manor, R., Kuballa, A. V., Aflalo, E. D., Sagi, A., Paterson, B. and Elizur, A. (2011). Isolation and characterization of the complete cDNA sequence encoding a putative insulin-like peptide from the androgenic gland *of Penaeus monodon*. *Aquaculture* **318**, 364-370.

Nagamine, C. and Knight, A. W. (1987). Induction of female breeding characteristics by ovarian tissue implants in androgenic gland ablated male freshwater prawns *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae). *Int. J. Inver. Rep. Dev.* **11**, 225-234.

Nagamine, C., Knight, A. W., Maggenti, A. and Paxman, G. (1980a). Effects of androgenic gland ablation on male primary and secondary sexual characteristics in the Malaysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae), with first evidence of induced feminization in a nonhermaphroditic decapod. *Gen. Comp. Endocrinol.* **41**, 423-441.

Nagamine, C., Knight, A. W., Maggenti, A. and Paxman, G. (1980b). Masculinization of female *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) by androgenic gland implantation. *Gen. Comp. Endocrinol.* **41**, 442-457.

Nair, K., Branislav, M., Rosenthal, H., Jayalakshmy, K. and Nost, J. (1999). Experimental studies on the cannibalistic habit of *Macrobrachium rosenbergii* (de Man). 1996 The Fourth Indian Fisheries Forum Proceedings, Kochi, pp. 227-232.

New, M. B. (2002). *Farming Freshwater Prawns: A Manual for the Culture of the Giant River Prawn (Macrobrachium rosenbergii)*, pp. 207. Rome: FAO.

Okumura, T. and Hara, M. (2004). Androgenic gland cell structure and spermatogenesis during the molt cycle and correlation to morphotypic differentiation in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Zool. Sci.* **21**, 621-628.

Ra'anan, Z. and Cohen, D. (1984). Characterization of size distribution development in the freshwater prawn *Macrobrachium rosenbergii* (De Man) juvenile populations. *Crustaceana* **46**, 271-282.

Ra'anan, Z. and Sagi, A. (1985). Alternative mating strategies in male morphotypes of the freshwater prawn *Macrobrachium rosenbergii* (De Man). *Biol. Bull.* **169**, 592-601.

Ra'anan, Z., Sagi, A., Wax, Y., Karplus, I., Hulata, G. and Kuris, A. (1991). Growth, size rank, and maturation of the freshwater prawn, *Macrobrachium rosenbergii*: analysis of marked prawns in an experimental population. *Biol. Bull.* **181**, 379-386.

Rahman, S. M., Wahab, M., Islam, M., Kunda, M. and Azim, M. (2010). Effects of selective harvesting and claw ablation of all-male freshwater prawn (*Macrobrachium rosenbergii*) on water quality, production and economics in polyculture ponds. *Aquacult. Res.* **41**, e404-e417.

Ranjeet, K. and Kurup, B. (2002). Heterogeneous individual growth of *Macrobrachium rosenbergii* male morphotypes. *Naga, Naga ICLARM Q.* **25**, 13-18.

Ranjeet, K. and Kurup, B. M. (2011). Density dependent variations on the production and population structure of *Macrobrachium rosenbergii* reared in the wetland polders of South India. *Braz. J. Aquat. Sci. Technol.* **15**, 55-62.

Rosenthal, S. M., Hulse, J. A., Kaplan, S. L. and Grumbach, M. M. (1986). Exogenous growth hormone inhibits growth hormone-releasing factor-induced growth hormone secretion in normal men. *J. Clin. Invest.* **77**, 176-180.

Sagi, A., Cohen, D. and Milner, Y. (1990). Effect of androgenic gland ablation on morphotypic differentiation and sexual characteristics of male freshwater prawns, *Macrobrachium rosenbergii*. *Gen. Comp. Endocrinol.* **77**, 15-22.

Sambrook, J. and Russell, D. (2001). *Molecular Cloning: A Laboratory Manual (3 volume set)*. New York: Cold Spring Harbour Laboratory Press.

Smith, T. I. J. and Sandifer, P. A. (1975). Increased production of tank-reared *Macrobrachium rosenbergii* through use of artificial substrates. *J. World Aquacult. Soc.* **6**, 55-66.

Ventura, T., Manor, R., Aflalo, E. D., Weil, S., Raviv, S., Glazer, L. and 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., Manor, R., Aflalo, E. D., Weil, S., Khalaila, I., Rosen, O. and Sagi, A. (2011). Expression of an androgenic gland-specific insulin-like peptide during the course of prawn sexual and morphotypic differentiation. *ISRN endocrinology* **2011**, 476283.

Ventura, T., Manor, R., Aflalo, E. D., Weil, S., Rosen, O. and 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**, 90.

Yazawa, R., Watanabe, K., Koyama, T., Ruangapan, L., Tassanakajon, A., Hirono, I. and Aoki, T. (2005). Development of gene transfer technology for black tiger shrimp, *Penaeus monodon*. *J. Exp. Zool. A Comp. Exp. Biol.* **303A**, 1104-1109.

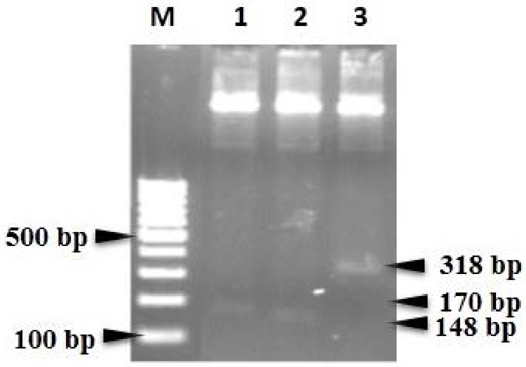


Fig. S1. Clone Confirmation of pcD-IAG-lh. Lane M: Generuler® 100 bp ladder (Thermoscientific, USA); Lane 1: *Nhe*I-*Hind*III digest releases sense fragment of 170bp; L 2: *Xho*I- *BamH*I digest releases antisense fragment of 148bp; L 3: *Nhe*I-*Xho*I digest releases the combined long hairpin fragment.

Fig. S2. An OC-BC Morphotype transformation in *M. rosenbergii.* The OC to BC transformation occurs in a single molt. The image shows a freshly molted male that has undergone transformation along with its shed exoskeleton. Solid arrow shows newly developed blue claw while the dotted arrow shows the orange claw that has been shed

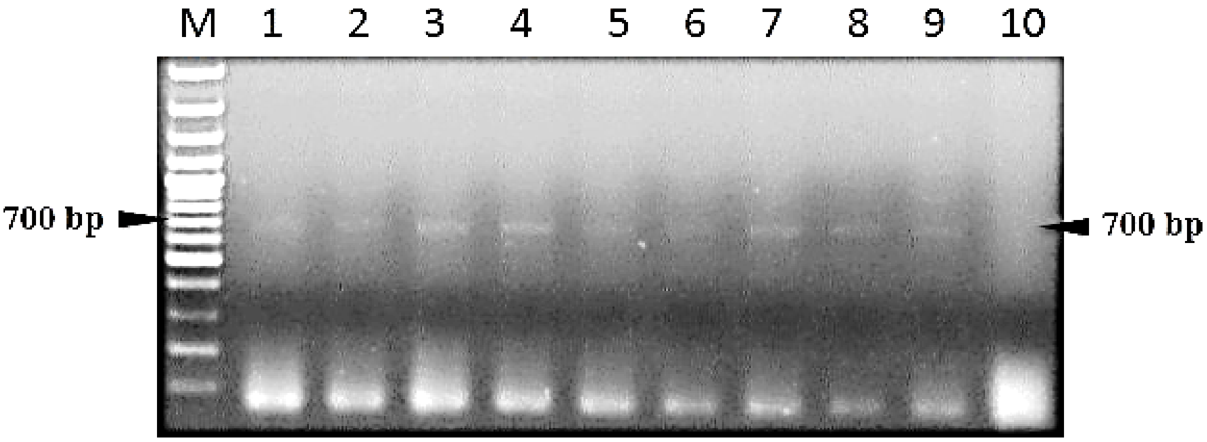


Fig. S3. Distribution and persistence of plasmid constructs in OC males. Lane M: Generuler ® 100 plus ladder; L 1 to 4: 700 bp band amplified from pleopods of pcD-IAG-lh injected animals on days 4, 12, 18, & 25 post-injection; L 5 & 6: 700 bp band amplified from muscle and gill tissues of pcD-IAG-lh injected animals of day 4; L 7 to 9: 700 bp band amplified from pcD-IAG orf injected animal on 25th day in E3; L 10: PBS injected animal (negative control).

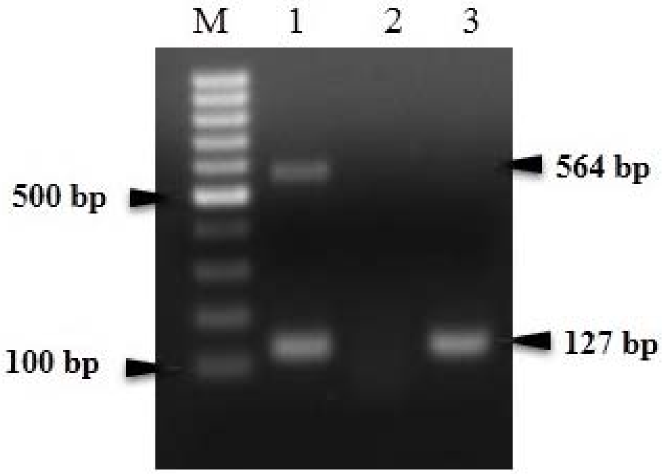


Fig. S4. Expression of *iag* transcripts in abdominal muscle tissue. Lane 1: 564 bp *iag* fragment amplified from muscle cDNA of animals injected with pcD-IAGorf; L 2: No amplification from DNAse I treated abdominal muscle total RNA used for cDNA preparation; L 3: 127 bp ef1α fragment amplified from muscle cDNA of PBS injected animal. Lane M: Generuler® 100 bp ladder (Thermoscientific, USA).

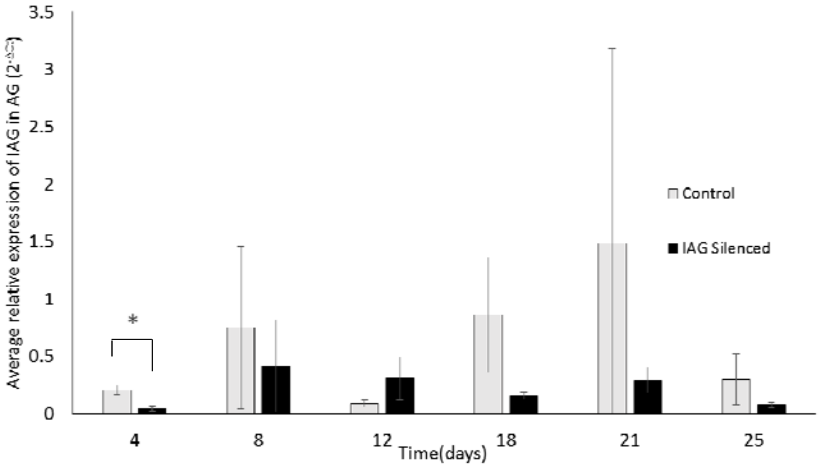


Fig. S5. IAG expression in AG of E1 samples. The bars represent average relative expression of endogenous *iag* transcripts normalized with EF1α. Vertical lines above the bar represent SEM. N=3 for each group at each time point. Asterisk denotes statistically significant difference in mean values (P<0.05).