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

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Summary statement: First report on morphotype transformation in adult *Macrobrachium rosenbergii* males from orange to blue claw on augmentation of insulin-like androgenic gland hormone.

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 ~60g 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 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 to 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 ones. 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.

1. Introduction

Giant freshwater prawn, *Macrobrachium rosenbergii* 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 (Karplus, 2005; New, 2002; Smith and Sandifer, 1975) that results in non-uniform harvest and makes its culture uneconomical. Size heterogeneity in this species occurs at all the stages of 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 (Karplus, 2005; Kuris et al., 1987; Lalrinsanga et al., 2012; New, 2002; Ra'anan and Sagi, 1985; Ranjeet and Kurup, 2002). These stages are known to undergo transformation from SM to OC to BC (Karplus, 2005). BC males, characterised by large blue colour claw comprising 10% of male population, are usually largest in size (up to 250 gm) in a community, sexually active and mate with females actively. Besides suppressing the growth of the SM, BC males are territorial, utilises most of the resources (viz. space, food etc) but cease to grow further. OC males comprise 40% of the male population, have higher growth rate than BC male and SM, weigh in the range of 50-150 gm, and are sexually less active. SM have thin translucent claw (50% of male population), weigh in the range of 5-15 gm, are sexually active and mate with females by sneaking behaviour. BC male are dominant over both OC male and small male, whereas OC male dominates over SM. When a

fast growing OC male becomes larger than the existing BC male in community, it transforms into BC male (Cohen et al., 1981; Karplus et al., 1991, 1992; Ra'Anan and Cohen, 1984; Ra'Anan and Sagi, 1985; Ra'Anan et al., 1991; Ranjeet and Kurup, 2002). Among the three male morphotypes of *M. rosenbergii*, OC males have the highest growth rate (Lalrinsanga et al., 2012; Ra'anan et al, 1991), and their relative proportion in harvest population determines the economic value of *M. rosenbergii* culture. This social control of growth, 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 (Aflalo et al., 2006; Barki et al., 2003; Nagamine and Knight, 1987; Nagamine et al., 1980a, b; Sagi et al., 1990). 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), *C. quardricarinatus* (Manor et al., 2007), and *P. monodon* (Mareddy et al., 2011). In blue crab *C. sapidus* it also expresses at a low level in hepatopancreas of males (Chung et al., 2011) and in the mud crab *S. 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 IAG levels increase significantly at stage-V when vitellogenin synthesis has ceased.

AG ablation in juvenile males of the *M. rosenbergii* also results in feminization (Nagamine et al., 1980a), whereas AG implantation effects masculinization (Nagamine et al., 1980b). Silencing Mr-IAG gene in *M. rosenbergii*

juveniles through repeated injection of Mr-IAG double stranded RNA lead to delayed regeneration of appendices masculinae (Ventura et al., 2009) and functional sex reversal (Ventura et al., 2012). Though Ventura et al. (2011) recorded a differential expression of *iag* between the male morphotypes with the least expression of IAG hormone in OC male in comparison to SM and BC, more information is required on the extend of its involvement in the 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. Further, administration of plasmid DNA can be achieved through immersion treatment, which is the least stressful to the animals (Chowdhury et al., 2014).

2. Materials and methods

2.1. *Primer designing*

Online software E-RNAi (http://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 V 5.2 software following the standard criteria for primer designing. Table 1 shows the list of primers used in this study along with the NCBI accession numbers for the target gene sequences used.

2.2. *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 (Table1) 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 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 PCR and further confirmed by restriction digestion and sequencing (Sup. Fig. 1).

Full length open reading frame (ORF) of *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).

2.3. *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 (1µg) using RevertAid cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's instructions. The nucleic acid preparations were quantified on Nanodrop2000/2000c spectrophotometer (Thermo Scientific, USA).

2.4. *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 striped snakehead fish whole fry cell line (SSN1). The cell line was tested for contamination and only healthy cell line was used for the experiment. SSN1 cells were grown for 24h in a 6-well plate (Growth area: 9 cm^2/well) in 1× L15 medium supplemented with L-glutamine, 10% foetal bovine serum and 1× antibiotic antimycotic solution (HiMedia, India). Transfections were done using Turbofect transfection reagent (Thermo Scientific, USA) following 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 buffer saline (PBS; pH 7.4) and harvested directly into 1 ml of Trizol reagent (Invitrogen, USA) for total RNA isolation. Silencing efficiency of the construct was studied by real-time PCR of *iag* transcripts using primers MR-IAG-RT-F/R normalised to β-actin gene using LR-β-actin-RT-F/R (Table 1).

2.5. *Rearing of experimental animals*

Sexually mature *M. rosenbergii* males of one year age group (~60g) were collected from grow-out ponds of Powarkheda centre of 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 ton capacity

Fibre-reinforced plastic (FRP) tanks of cross-section area 1m^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; DO: 8.0±1.0 ppm; temperature: 28.0±1.3 °C; hardness (CaCO3): 50.2±2.0 ppm]. All males were OC males with smooth spineless claws. Animals were fed on commercial pelleted feed thrice a day and 40-60% 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), Government of India on care and use of animals in scientific research were followed to care and rare the animals used in the present study. The study was approved by the Board of studies and authorities of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai, India.

2.6. *Experimental design for IAG silencing in M. rosenbergii*

IAG silencing experiments were done both in isolation and community on OC males of ~60g size. All treated animals in the silencing studies received pcD-IAG-lh construct diluted with PBS @ 2 µg/g body weight while the control animals received PBS injections. The plasmid injections were given to a final volume of 100 µl per animal and administered directly into the sinus cavity ofthe animals between the third and fourth pereiopods. For rearing animals in isolation (Experiment 1; E1), total 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 & 2b). Experiment E1 was conducted in triplicate, where each tank had equal number of control and treated animals. For community rearing (Experiment 2; E2), the OC males were stocked in FRP tanks at a stocking density

of 5 animals/m^2 (Fig. 2c). The community rearing experiment was performed in triplicate. Shelters sufficient to hide all animals were provided.

2.7. *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 @ 1µg/g body weight as described above. The plasmid injections were given to a final volume of 100 µl per animal as explained in section 2.6. All animals in the control group were given PBS injections. Five OC males (~60g) that received the plasmid were kept along with one PBS injected larger OC male (~150g) in one FRP tank of bottom area $1m^2$. 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 hide outs.

2.8. *Data collection and tissue sampling*

Morphotype transformation events and molt events were observed and recorded daily. Morphotypes were differentiated on the basis of the key suggested by Kuris et al. (1987). For freshly molted animals, characters like death, loss of claw in territorial fight and claw colour after molt were observed (Sup. Fig. 2). For experiment 1 (E1; IAG silenced males in isolation), 3 animals each were sampled randomly from treatment and control groups on 4^{th}, 8^{th}, 12^{th}, 18^{th}, 21^{st} day and 4 animals on 25^{th} day post-injection. In the case of 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 four 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.

2.9. *In vivo plasmid persistence, distribution and expression*

For plasmid persistence studies, pleopod samples from both treatment and control group animals were collected on 4^{th}, 12^{th}, 18^{th}, & 25^{th} days, and stored in absolute ethanol for genomic DNA isolation. For plasmid distribution studies, pleopod, abdominal muscle (second segment), and gill tissues were sampled on 4^{th} day and stored in absolute ethanol for genomic DNA isolation. DNA samples from a particular day were pooled separately for treatment and control. To confirm 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 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 MR-IAG-EXP-F/R primer set (Table 1).

2.10. *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. *M. rosenbergii ef1α* gene was used as internal control in real-time PCR. Real-time PCR was performed on Roche 480 Light Cycler machine (Roche, Germany). Reaction mixture consisted of 50 ng cDNA, 2.5 pmole each of forward and reverse primers (MR-IAG-RT-F/R or MR-EF1α-RT-F/R; Table 1) and 5µl of 2× SYBR green master mix (Thermo Scientific, USA) in a final volume adjusted to 10µl. The real-time PCR program comprised 1 cycle of initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 20s, 60°C for 20s, 72°C for 30s, 1 cycle for melt curve analysis of 95°C for 5s, 65°C for 1 min followed by continuous signal acquisition until the temperature reached to 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 formula $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) after reference residual normalisation (Edmunds et al., 2014).

2.11. Histology

For assessment of the effect of different treatments on reproductive advancement in male *M. rosenbergii*, histology of testis was performed. On the last day of the experiment (day 25) 2 samples each of OC and BC were collected from each tank of E1, E2 & E3 for histological examination of testis. The testis for the preparation of histological slides were 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 DPX and

photographed using Zeiss Axiophot A1 digital fluorescence microscope (Carl Zeiss, Germany).

2.12. *Statistical analysis of the data*

Relative fold change values were normalised by log2 transformation for either student t-test or one-way ANOVA and analysed by Tukey's test using SPSS 16.0. Cox regression analysis and Fisher exact test were used to analyse morphotype transformation data of IAG silencing in isolation and community rearing, respectively. Molt data were analysed using Cox regression analysis in SPSS16.0 software package.

3. Results

3.1. *Validation of silencing efficiency in vitro*

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

3.2. *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 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 (Sup. Fig. 3). The same primer set confirmed the presence of plasmid DNA in pleopod, muscle and gill tissue (Sup. Fig. 3).

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 (Sup. Fig. 4).

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

3.3.1. Morphotype transformation in animals reared in isolation

Morphotype transformation events were recorded daily over 25 days in experiment E1. There was not a single transformation event between 0 to 18 days in either treatment or control groups. Between 18 to 25 days, 3 OC to BC transformations occurred in the treatment group and 4 in the control group distributed over replicates (Fig. 2D). Cox regression analysis reveals that the rate of OC to BC transformation in IAG silenced and control group was similar ($P>0.05$). QPCR results show 4.2-fold lower expression of *iag* transcript in the androgenic gland of treated animals compared to controls ($P<0.05$) on the 4th day post-injection, although the expression recovered thereafter (Sup. Fig. 5).

3.3.2. *Morphotype transformation in animals reared in community*

By the end of the experiment E2 (25th day), 6 OC to BC transformations were recorded in control (2 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 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).

3.4. *Augmentation of IAG hormone gene expression promotes OC to BC transformation*

At the end of the experiment E3 (25th day), no transformation event was recorded in the control group, while the treated group had 5 conversions to BC distributed over replicates. The larger OC males of neither groups transformed. Fisher 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).

3.5. *Effect of IAG hormone level on molting*

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

3.6. Relative propodus length (RPL) and testicular histology

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. Besides, 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 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. On the other hand, 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, while Fig. 5b shows a single lobule from a newly transformed BC male in the control group of the same experiment. However, while 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.

4. 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 (Nair et al., 1999; Rahman et al., 2010; Sagi et al., 1990; Smith & Sandifer, 1975; Ventura et al., 2009 & 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 CMV promoter avoids repeated injection of dsRNA and associated stress (Das et al., 2015; Krishnan et al., 2009). The CMV promoter has been shown to demonstrate strong constitutive expression in fish and shrimps (Arenal et al., 2000; Chen et al., 2006; Yazawa et al., 2005). 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 (AG) 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 work, 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% *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 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 molting and the same was observed in this study with pooled data from E1 and E2. IAG augmentation in E3 appeared to promote molting, but the data was not significantly different from controls, most likely due to 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 hr) had reduced claw lengths compared with the untreated controls. Since 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 conformed to those of Okumura and Hara (2004), who detailed molt dependent variability in 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 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.

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Competing interests

The authors declare no competing interests

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Figures

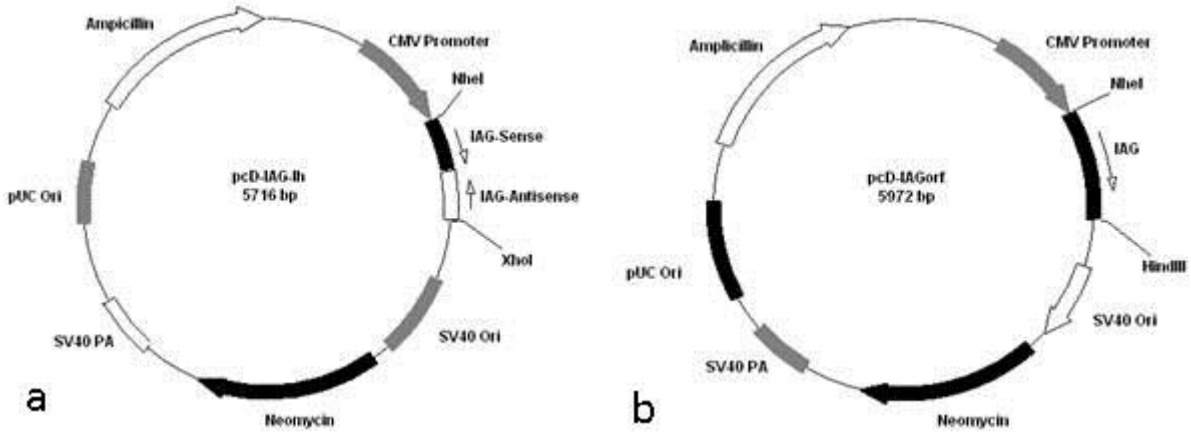


Fig. 1. Schematic sketch of plasmid constructs (a) pcD–IAG-lh (b) pcD -IAGorf

Fig. 2. Experimental set-up for rearing the animals and morphotype transformation studies (A): FRP tank holding perforated baskets caging both the treatment and control animals for isolated rearing experiments E1, (B): One of the experimental animals housed in the perforated basket, (C): Community rearing of uniformly sized OC males in Experiment E2, (D): OC male transformed to BC (left) & untransformed OC male (right) after experiment.

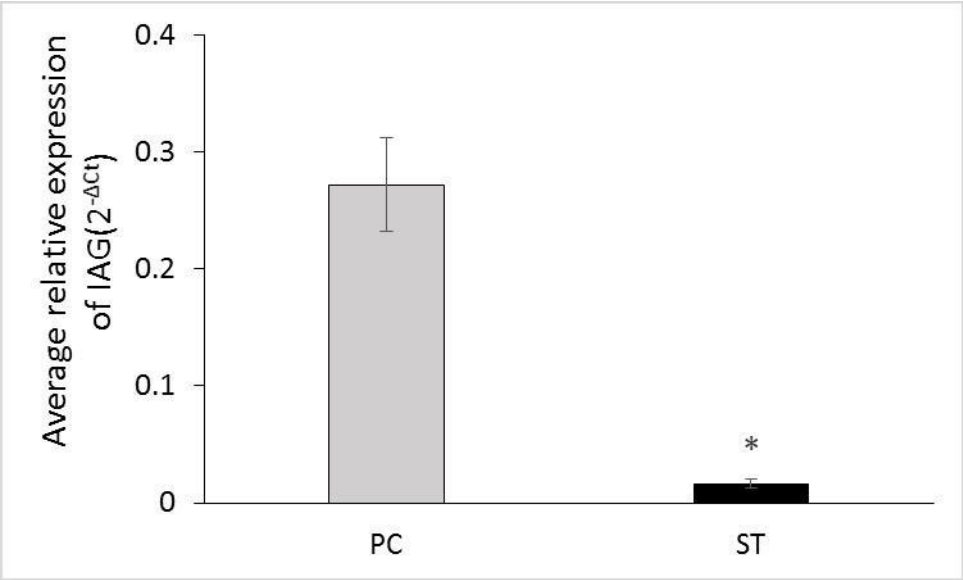


Fig. 3. Silencing efficiency of pcD-IAG-lh *in vitro.* The bars represent mean± SEM of expression of *iag* transcripts relative to that of β-actin in the transfected PC (pcD-IAGorf alone) and ST (co-transfected with pcD-IAGorf and pcD-IAG-lh in 1:1 ratio) cells. Asterisk over the bar represent the significant difference between treatment and control at $p<0.05$.

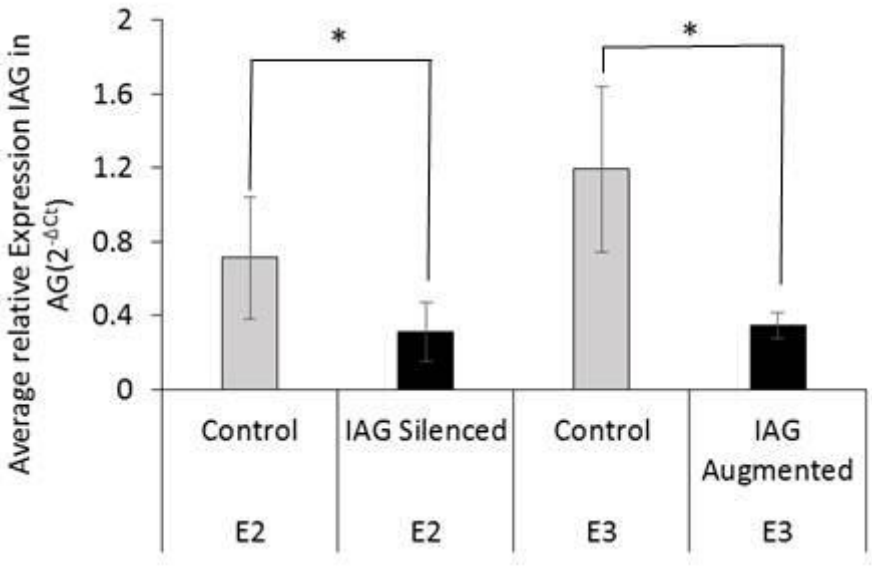


Fig. 4. Expression of iag in AG of iag silenced and augmented animals reared in community (E2 & E3). The bars represent mean±SEM of relative expression of IAG transcripts normalised to EF1α. Asterisks over the bar represent the significant difference between treatment and control at $p<0.05$.

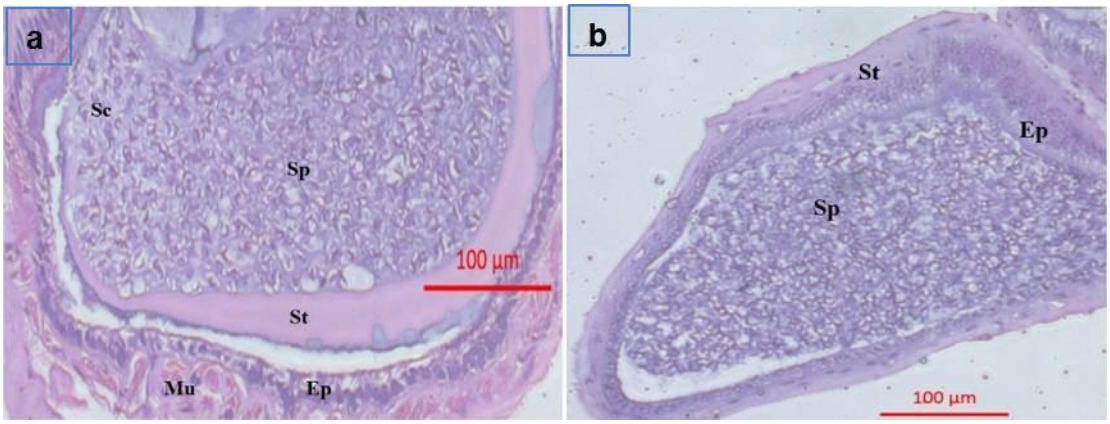


Fig. 5. Cross section through a single testicular lobule of *M. rosenbergii*. a) OC male (E2, treated): The featured lobule contains primarily mature sperm cells and spermatocytes but not spermatogonial cells; b) BC male (E2, control): The lobule virtually contains only mature sperms. The epithelial lining shows prominent presence of secretory vacuoles. Mu: Muscle; Ep: Epiththelial cells; St: Acellular Spermatotheca; Sp: Mature sperm cells; Sc: Spermatocytes.

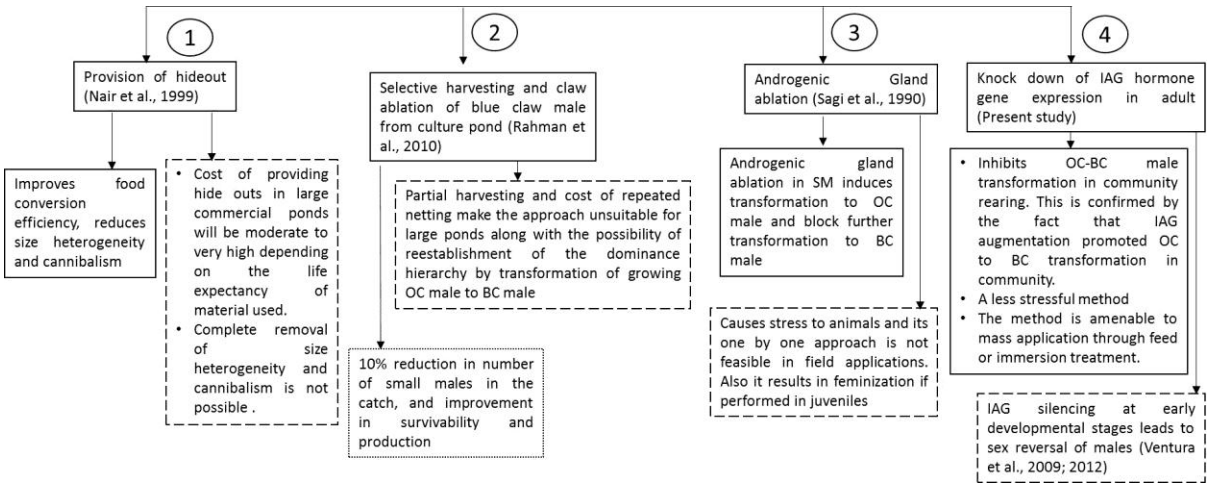
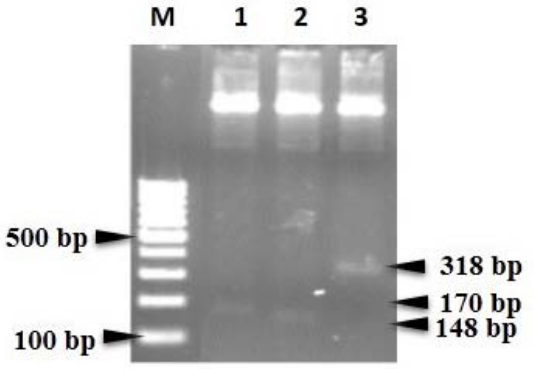


Fig. 6. Comparison of different approaches reported to reduce size heterogeneity in *M. rosenbergii* culture ponds. Solid box represents advantages/outcomes and dotted box represents shortcomings/challenges.

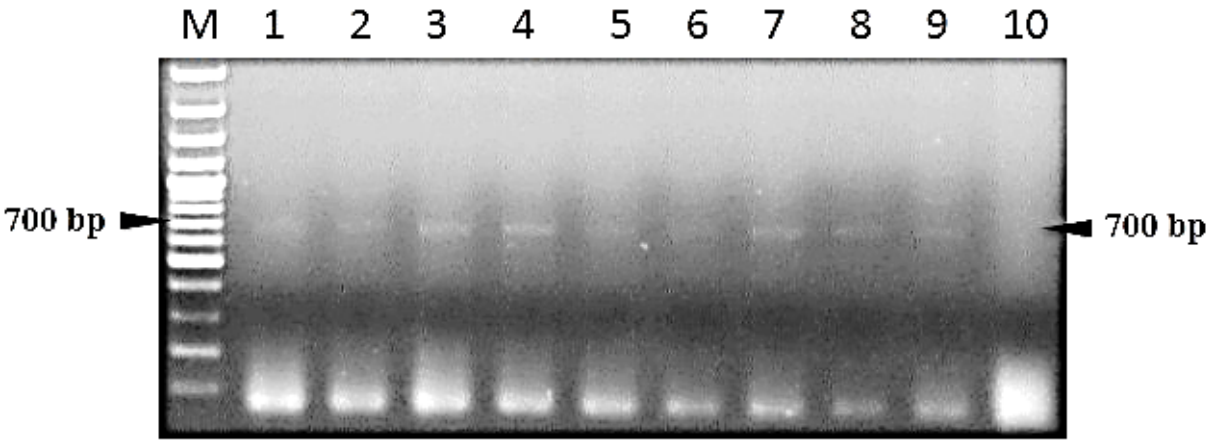
Table 1. Details of primers used in this study

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

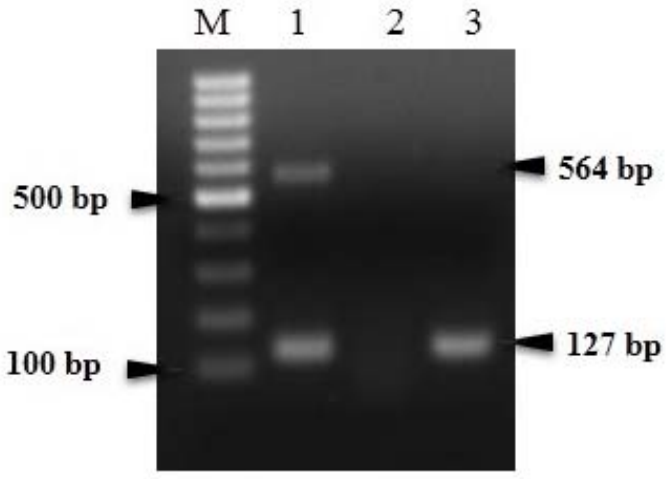


Sup. Fig. 1. Clone Confirmation of pcD-IAG-lh. Lane M: Generuler® 100 bp ladder (Thermoscientific, USA); Lane 1: *Nhe*I-*Hind*III digest releases sense fragment of 170bp; L 2: *Xho*I- *BamH*I digest releases antisense fragment of 148bp; L 3: *Nhe*I-*Xho*I digest releases the combined long hairpin fragment.

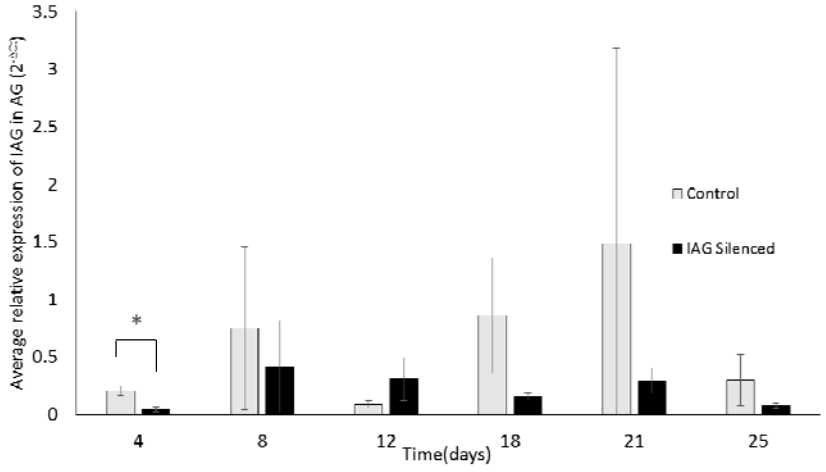
Sup. Fig. 2. An OC-BC Morphotype transformation in *M. rosenbergii.* The OC to BC transformation occurs in a single molt. The image shows a freshly molted male that has undergone transformation along with its shed exoskeleton. Solid arrow shows newly developed blue claw while the dotted arrow shows the orange claw that has been shed



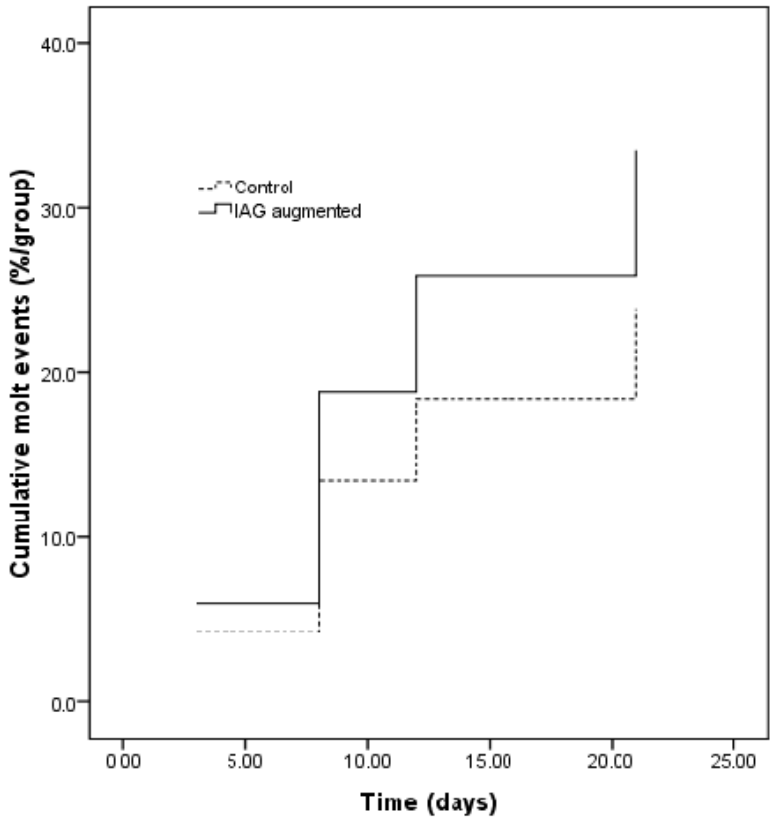
Sup. Fig. 3. Distribution and persistence of plasmid constructs in OC males. Lane M: Generuler ® 100 plus ladder; L 1 to 4: 700 bp band amplified from pleopods of pcD-IAG-lh injected animals on days 4, 12, 18, & 25 post-injection; L 5 & 6: 700 bp band amplified from muscle and gill tissues of pcD-IAG-lh 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).



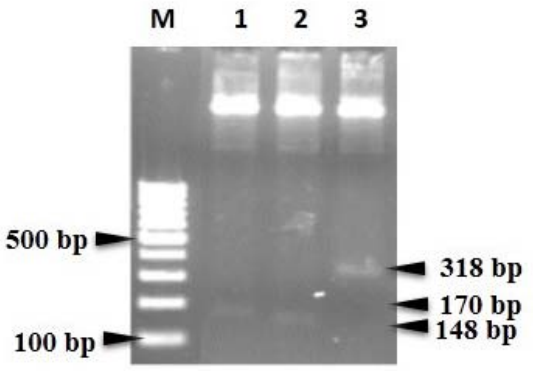
Sup. Fig. 4. Expression of *iag* transcripts in abdominal muscle tissue. Lane 1: 564 bp *iag* fragment amplified from muscle cDNA of animals injected with pcD-IAGorf; L 2: No amplification from DNAse I treated abdominal muscle total RNA used for cDNA preparation; L 3: 127 bp ef1α fragment amplified from muscle cDNA of PBS injected animal. Lane M: Generuler® 100 bp ladder (Thermoscientific, USA).



Sup. Fig. 5. 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).



Sup. Fig. 6. Effect of *iag* augmentation on cumulative molt frequency in E3. Cumulative molt events of pcD-IAGorf (solid line, n=10) and PBS injected (dotted line, n=10) groups in the experiment. Molt events are represented as a percentage for the respective groups. Cox regression analysis indicates that the molt rate was similar in both the groups ($p>0.05$).



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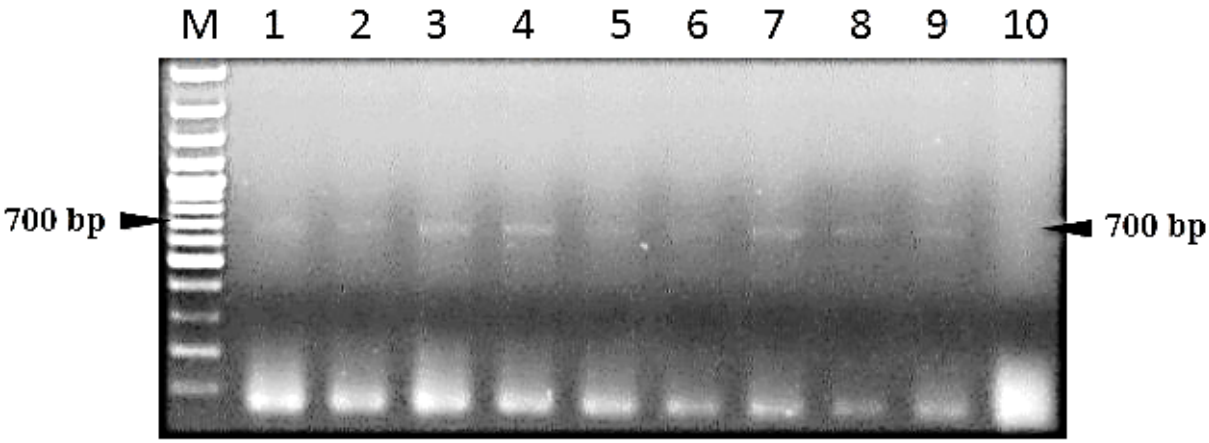

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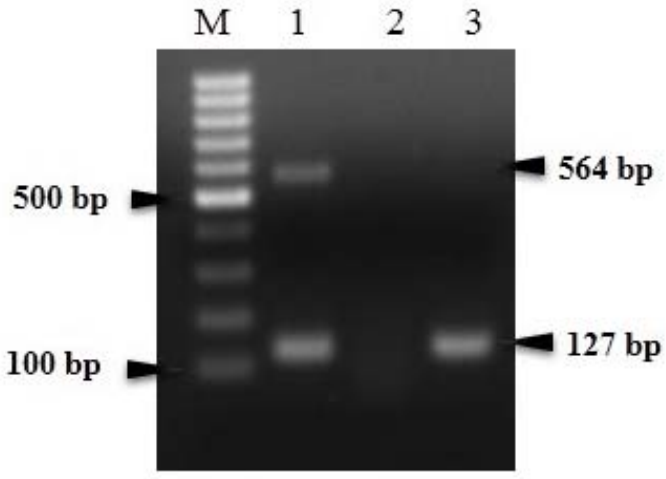

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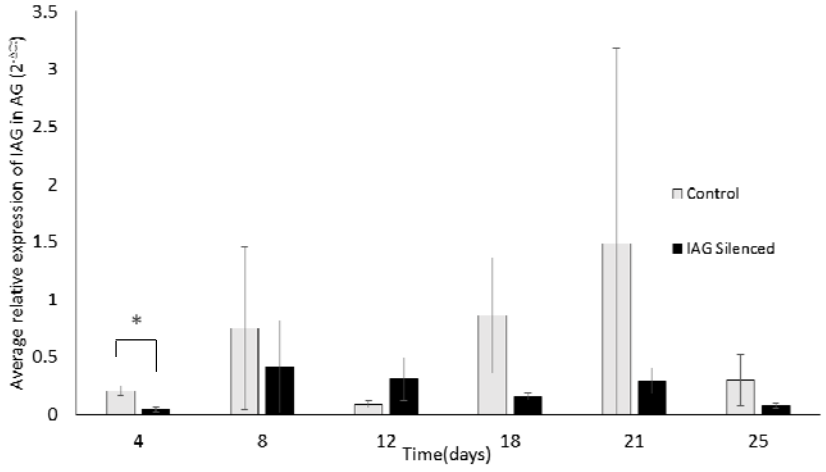

Fig. S5. IAG expression in AG of E1 samples. The bars represent average relative expression of endogenous *iag* transcripts normalized with EF1α. Vertical lines above the bar represent SEM. N=3 for each group at each time point. Asterisk denotes statistically significant difference in mean values ($P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