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Co-culture of males with late premolt to early postmolt female giant freshwater prawns, *Macrobrachium rosenbergii* resulted in greater abundances of insulin-like androgenic gland hormone and gonad maturation in male prawns as a result of olfactory receptors

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ABSTRACT

Insulin-like androgenic gland hormone (*IAG*) controls development of primary and secondary male sex-characteristics in decapod crustaceans. In male giant freshwater prawns, *Macrobrachium rosenbergii*, the *IAG* concentration correlates with male reproductive status and aggressiveness. When female prawns are co-cultured with males this can result in male size variations while this variation does not occur when males are cultured in monosex conditions. It was hypothesized that pheromone-like factors from female prawns may affect the abundance of *IAG* mRNA and protein in co-cultured males which would affect the pattern of sexual maturation of these males. In the present study, late premolt to postmolt females co-cultured with males for 7 days had a greater abundance of *MrIAG* mRNA transcript in all male phenotypes as well as for the gonad-somatic indexes (GSI). The abundance of *MrIAG* mRNA gradually increased from days 1 to 7 and using Western blot procedures MrIAG protein also increased in a similar pattern. Furthermore, with use of BrdU labeling, there was an increased cell proliferation in the spermatogenic zone of testicular tubules and in the spermatic duct epithelium during the 1 to 7 day co-culture period when there were increases in *MrIAG* mRNA and protein. In contrast, these effects were negated if short lateral antennules of males were ablated. Thus, results of the present study provide evidence that there might be female-molting factors which function as important regulators of androgenic gland function and gonadal maturation that were perceived by males via their short lateral antennules which are the olfactory organs.

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1. Introduction

The giant freshwater prawns, *Macrobrachium rosenbergii*, is an important economic crustacean species that is of limited supply due to the large market demands for local consumption as well as for export and this demand can only be addressed by using aquaculture practices (New, 2005). In mixed cultures males vary in sizes from the largest blue clawed (BC), medium size orange clawed (OC), and smallest small clawed (SM) prawns (Sagi et al., 1986; Kuris et al., 1987; Karplus, 2005; Tidwell et al., 2015). Male size variation is affected by genetic, environmental, and social factors especially the hierarchical interaction among individuals in a male population in the same culture (Karplus et al., 1989, 2000; Karplus, 2005; Aziz et al., 2017). The dominant male morphotype (BC) is more aggressive than other subordinate morphotypes (OC, SM) and consumes most of the food resources (Karplus, 2005; Karplus et al., 1989). The presence of females in mixed-sex cultures results in induction of the morphotype transformation of OC and SM to the more mature BC (Tidwell et al., 2015), indicating females affect the maturation of males. Beside gaining more resources, the BC have greater mating activities compared to the OC males, whereas the immature SM males have been reported to be stealthy in copulating with molting females (Ra'anan and Sagi, 1985). In contrast, the OC males were reported to have less reproductive activity (Sagi and Ra'anan, 1988; Ventura et al., 2011). Physiologically, the male morphotype has testicular development in which the fully mature testes only develop in the BC males (Sagi et al., 1988), while the testes of SM are in transition for development into the OC morphotype and also have an abundant amount of spermatogenic activity (Sagi et al., 1988). These three stages of male morphotypes have markedly different growth patterns and reproductive behaviors as well as testicular development (Sagi et al., 1988; Sagi and Ra'anan, 1988; Ventura et al., 2011).

A primary factor involved with male morphotype transformation is the synthesis and release of the insulin-like androgenic gland hormone (MrIAG; Sagi et al., 1990; Ventura et al., 2009, 2011) from the paired androgenic glands (AG) located close to the terminal ampules of spermatic ducts. The MrIAG is reported to modulate male specific behaviors, including mating and aggressive displays (Barki et al., 2003, 2006; Ventura et al., 2011). Hence, the AG has been identified as the most important endocrine gland that controls male sex determination, growth, and secondary sex characteristics (Okumura and Hara, 2004; Ventura et al., 2011), and also the OC to BC transformation (Priyadarshi et al., 2017). The removal of this organ results in reduced aggression and onset of feminine characteristics, and in some cases sexual reversal and formation of ovaries instead of testes (Barki et al., 2006). There is an abundance of *MrIAG* mRNA transcripts with male BC and SM morphotypes having a greater abundance of MrIAG protein than the OC males (Ventura et al., 2011). The abundance of *MrIAG* mRNA transcript is suppressed by hormones released from the eyestalks (Khalaila et al., 2002) because the eyestalk-ablated prawns have AG hypertrophy, an increased GSI and spermatogenesis (Kim et al., 2002; Phoungpetchara et al., 2011). Interestingly, in mud crabs, *Scylla paramamosain*, the abundance of IAG also correlates with mating activity as its peak was detected when mating occurred (Zhang et al., 2014).

During the molting period, female prawns have fully mature ovaries and respond positively to courtship and mating behaviors of male prawns. It has been suggested that the male behaviors towards molting females are modulated by pheromone-like substances released during female ecdysis (Bauer, 2011; Kruangkum et al., 2013). To our knowledge, there has been no study conducted on the effect of molting females on male reproductive physiology in *M. rosenbergii*. In the present study, the effects of late premolt, molting, and early postmolt females on the abundance of *MrIAG* mRNA transcript in three different male morphotypes (BC, OC, and SM) as well as male gonad maturation were investigated using a co-culture assay. It was hypothesized that during the period of molting, females release pheromone-like substances that are perceived by males via their short lateral antennules which contain olfactory receptors (Kruangkum et al., 2013).

2. Materials and methods

2.1. Experimental animals

Fully mature females and three male morphotypes i.e., blue claw (BC), orange claw (OC), and small male (SM) giant freshwater prawns, *Macrobrachium rosenbergii*, were obtained from a local farm in Suphan Buri province, Thailand. The prawns were maintained in fiberglass tanks with continuous aeration. Each male morphotype and female prawns were separately stocked in 500 l tanks at density of 20 prawns per tank for 7 to 10 days before starting experiments. Prawns were fed with commercial food pellets (Betagro Co., Thailand) twice a day. All experimental animals were managed in ways that were consistent with the procedures approved by the Experimental Animal Ethics Committee, Mahidol University, Thailand (MU-IACUC 2017/035).

2.2. Co-culture bioassays and tissue collections

Two experiments were performed as depicted in Fig. 1. In the first experiment, each male morphotype (BC, OC, or SM) depicted as testers were reared in the outer zone of a 500 l aquarium tank. Testers were co-cultured with inducers without direct contact while the inducers were housed in 100 l perforated baskets which were placed centrally in each aquarium tank (Fig. 1). Inducers (n = 7–8) included late premolt to early postmolt females, intermolt females, and male prawns. The BC, OC, and SM males were each divided into four groups (n = 10 each): Group 1 (G1) comprised males co-cultured with late premolt to early postmolt females; Group 2 (G2) comprised males co-cultured with intermolt females; Group 3 (G3) comprised males with ablated short lateral antennules (slAn) (ablation performed as previously described by Kruangkum et al., 2013) co-cultured with late premolt to early postmolt females; and Group 4 (G4) comprised males co-cultured with their corresponding male morphotypes. All experiments were conducted for 7 days with constant rearing conditions as previously described and about 50% of cultured water was replaced twice during the experiment.

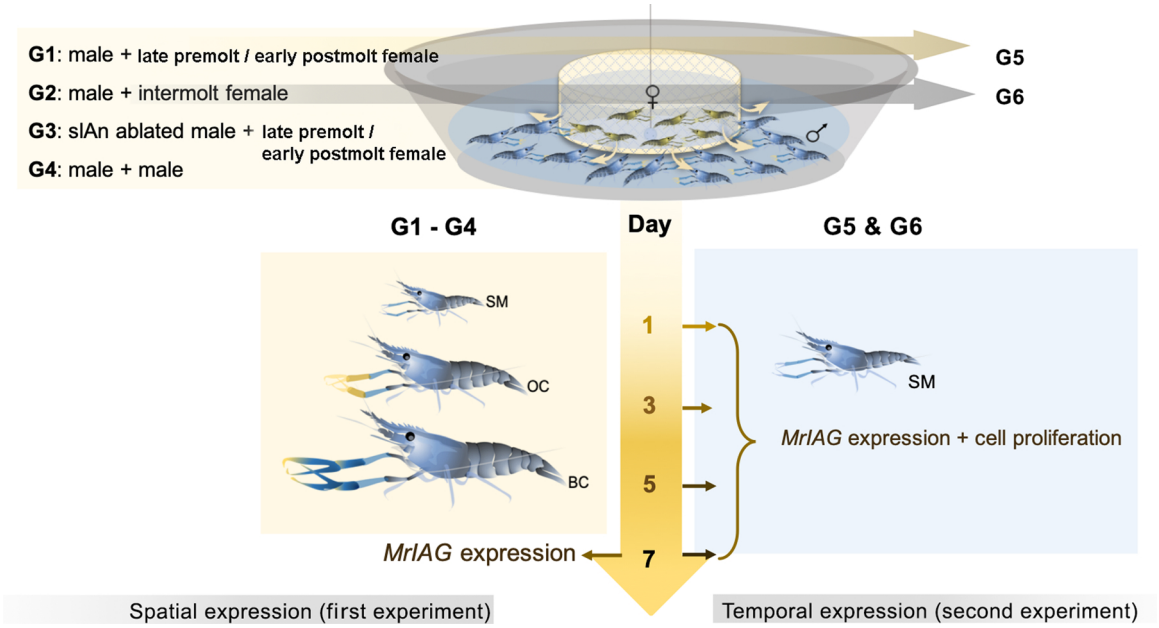

Fig. 1. Schematic illustration of the experimental designs for co-culturing assays: on the left, co-cultures between all three male morphotypes *M. rosenbergii* with late premolt to early postmolt females, intermolt females, corresponding males (G1, G2, G4), and between short lateral antennule-ablated males with late premolt to early postmolt females (G3); Relative abundance of *IAG* mRNA transcript and gonado-somatic indexes in all three male morphotypes were estimated at the end of day 7; On the right were the co-cultures between SM males with late premolt to early postmolt females (G5), and SM males with intermolt females (G6); Temporal differences in relative abundance of *MrIAG* mRNA transcript and cell proliferation were determined and compared between these two groups every day from days 1 to 7.

For inducers of G1 and G3, pooled late premolt (n = 4 each) and early postmolt (n = 3–4 each) female prawns, identified based on Peebles's report (1977), were used. The late premolt prawns started molting after 2 to 3 days and subsequently developed into early postmolt individuals. Early postmolt inducers were used for only 2 days and replaced with an equal number of new late premolt prawns. Using this procedure, there were a total of 14 to 16 female prawns used as inducers in G1 and G3 experiments. At the end of day 7, testes and male reproductive tracts with attached androgenic glands (AG) were dissected and collected from all male morphotypes after being anesthetized in ice-cold water. Masses of gonads were weighed for calculation of the gonado-somatic index (GSI) (Siangcham et al., 2013). Half of the AG with the terminal ampule of each prawn was immediately removed by dissection and stored at −80 °C for biochemical examinations while the other half of the tissue was fixed using Davison's fixative for histological examination. To investigate the temporal abundances of *MrIAG* mRNA transcript in the first experiment, the second phase of the experiment was designed using immature SM prawns. In the second experiment, the two groups comprised SM prawns co-cultured with either late premolt to early postmolt females (G5) or intermolt females (G6) which were reared in the same conditions as described for the first experiment (n = 7–8 each). The androgenic glands and male reproductive tracts were collected at days 1, 3, 5, and 7 and three SM prawns in each group were randomly injected with 5′ -bromo-2′-deoxy-uridine (BrdU) (Roche, Mannheim, Germany) 8 to 12 h before organ collections.

2.3. Estimation of MrIAG mRNA relative abundance using RT-PCR

The RNA from the AGs of all male morphotypes of four different subgroups was individually extracted using TriPure isolation reagent (Roche, IN, USA) following the manufacturer's protocol. Total RNAs were quantified using a Nanodrop microvolume spectrophotometer (Thermo Scientific™, USA). There was 1 µg of RNA from each individual that was treated with DNaseI (Invitrogen, CA, USA) prior to being used for the first-strand cDNA synthesis, utilizing the SupersScript III Reverse Transcriptase kit (Invitrogen, CA, USA). The RT-PCR of *MrIAG* (pooled RNA, n = 10) was conducted using specific primers (Table 1) designed from its corresponding sequence in GenBank NCBI (accession number: FJ409645.1). Thermocycling amplification was conducted at 95 °C for 3 min, followed by 28 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 45 s, and lastly at 72 °C for 5 min using KAPA2G Robust HotStart ReadyMix PCR Kit (Kapa Biosystems). The PCR was performed without inclusion of a template as a negative control and PCR of *β-actin* or *16SrRNA* was performed as an internal control, respectively.

2.4. Estimation of MrIAG relative abundance using real-time PCR

In the first experiment, the cDNA from each individual was used for estimating relative abundance of *MrIAG* mRNA transcript with real-time PCR. Triplicate qPCR reactions were conducted for each sample, and each PCR reaction consisted of 0.5 µl of cDNA

Table 1
Primer sequences for the RT-PCR and quantitative real-time PCR (qPCR).

Name	Primer sequence (5'-3')	Amplicon (bp)	Working purpose
MrIAG1 - F	GGGGCATATGGGATACTGGAATGCCGAGATC	558	RT-PCR
MrIAG1- R	GGGGCTCGAGTCAATGATGATGATGATGATGC-CTGGAACTGCAGGTGTTAACGC		
Mr-βactin -F	AAGTAGCCGCGTTGGTTGTA	457	RT-PCR
Mr-βactin - R	CCAGAGTCGAGCACGATACC		
MrIAG2 - F	TGTGTTGTTCTGCTCACTCGT	97	qPCR
MrIAG2- R	TATGTCGCCGCAGTCAAAGT		
16S rRNA - F	TGACCGTGCRAAGGTAGCATA	153	qPCR/ RT-PCR
16S rRNA- R	TTTATAGGGTCTTATCGTCCC		

(except for the control where no template was included, NTC), 9.5 µl SYBR Green PCR Master Mix (KAPA SYBR FAST qPCR Master Mix (2X) kit, KAPA Biosystems, U.S.A.), 0.2 µl of each forward and reverse primer (10 µM), 4.1 µl PCR grade water to a final volume of 10 µl (0.25 ng/µl in final concentration). The reaction was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, U.S.A.) with the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 3 s, 57 °C for 30 s and 65 °C for 5 s, followed by 95 °C for 30 s for melting temperature analysis. Primers used in this experiment are included in Table 1. In the second experiment, cDNAs of AGs collected at days 1, 3, 5 and 7 from co-cultured males were used for determining the relative abundance of *MrIAG* mRNA transcript with real-time PCR utilizing the same conditions as previously described. The relative abundances of *MrIAG* transcript of all groups was determined so there could be comparisons with Groups 2 and 6 of the first and second experiments, respectively, with abundances being calculated and presented as a mean fold change ± SEM (Comparative Ct Method = $2^{-\Delta\Delta CT}$, where $\Delta C_t = C_{t(target)} - C_{t(reference)}$).

2.5. Western blot analysis of MrIAG protein

The AGs collected from males in the second experiment at day 3 co-culture were homogenized in lysis buffer, centrifuged and the supernatants were collected. Protein concentrations were quantified using the Bradford assay (Bio-Rad Laboratories, U.S.A.), and 100 µg of crude extracts from each group were loaded onto 15% SDS-Tris-Glycine gels and separated using 110 V conditions. The separated proteins were subsequently transferred to PVDF membranes (Fluorotrans® W 3.3 m Roll Pall BSP01 (Pall, China). The Western blot was performed using rabbit anti-MrIAG (Phoungpetchara et al., 2011). The membranes were first immersed in blocking solution (5% skim milk, 0.2% Tween-20 in PBS) for 2 h and then were covered with rabbit anti-MrIAG (at 1:1000 diluted in blocking solution) at 4 °C overnight with gentle agitation. After several washings, the membranes were immersed in HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a 1:5000 dilution in blocking solution for 2 h. The membranes were subsequently washed before developing a signal using Pierce™ ECL Plus Western Blotting Substrate (Thermo Scientific, USA)] and hyper film (Hypercassette™, Amersharm). For the internal control, the same membranes were immersed in stripping solution (10% SDS and 1% β-mercaptoethanol in 1 M Tris−HCl buffer, pH 6.7) at 50 °C for 30 min. These membranes were re-probed using anti α-actin (Santa Cruz Biotechnology) at 1:3000 dilution followed by the procedures previously described.

2.6. Determination of gonadal cell proliferation

Proliferation of gonadal cells was estimated using BrdU labelling (Roche, Mannheim, Germany). Three SM prawns from the second experiment were injected with BrdU at the dose of 5 mg/100 g BW on days 1, 3, 5, and 7 for 8 to 12 h before organ collections (Phoungpetchara et al., 2011; Thongbuakaew et al., 2016). The testes were fixed in Davison's fixative, washed and processed for paraffin sectioning. The 6 µm-thick sections were treated using the BrdU detection kit II (Roche, Mannheim, Germany). Briefly, the tissue sections were treated with 2% H_2O_2 in methanol for 30 min to block endogenous peroxidase. The sections were immersed in 2 N HCl at 37 °C for 1 h and washed three times using 0.5% Tween-20 in PBS solution (PBST). These were then treated with 1% glycine in PBST for 15 min and 5% normal goat serum in PBST for 1 h, respectively, to block non-specific binding. The sections were incubated in mouse-anti-BrdU (Roche, Mannheim, Germany) at a dilution of 1:100 in PBS, at 37 °C for 2 h. For negative control sections, the primary antibody was omitted. After several washings, sections were covered with the goat-anti-mouse-IgG H&L (HRP polymer; Abcam), at 37 °C for 1 h and the color was developed by treatment with 0.05% diaminobenzidine solution. All sections were counter-stained with hematoxylin, and dividing cells was examined and photographs were taken using a light microscope (Leica DM750) with a digital camera (Leica ICC50 HD).

2.7. Statistical analysis

All data were analyzed and compared using a one-way ANOVA with Tukey's multiple comparison *post hoc*-test (GraphPad Prism 5.01) with significant differences considered to exist when the *P* value was less than 0.5.

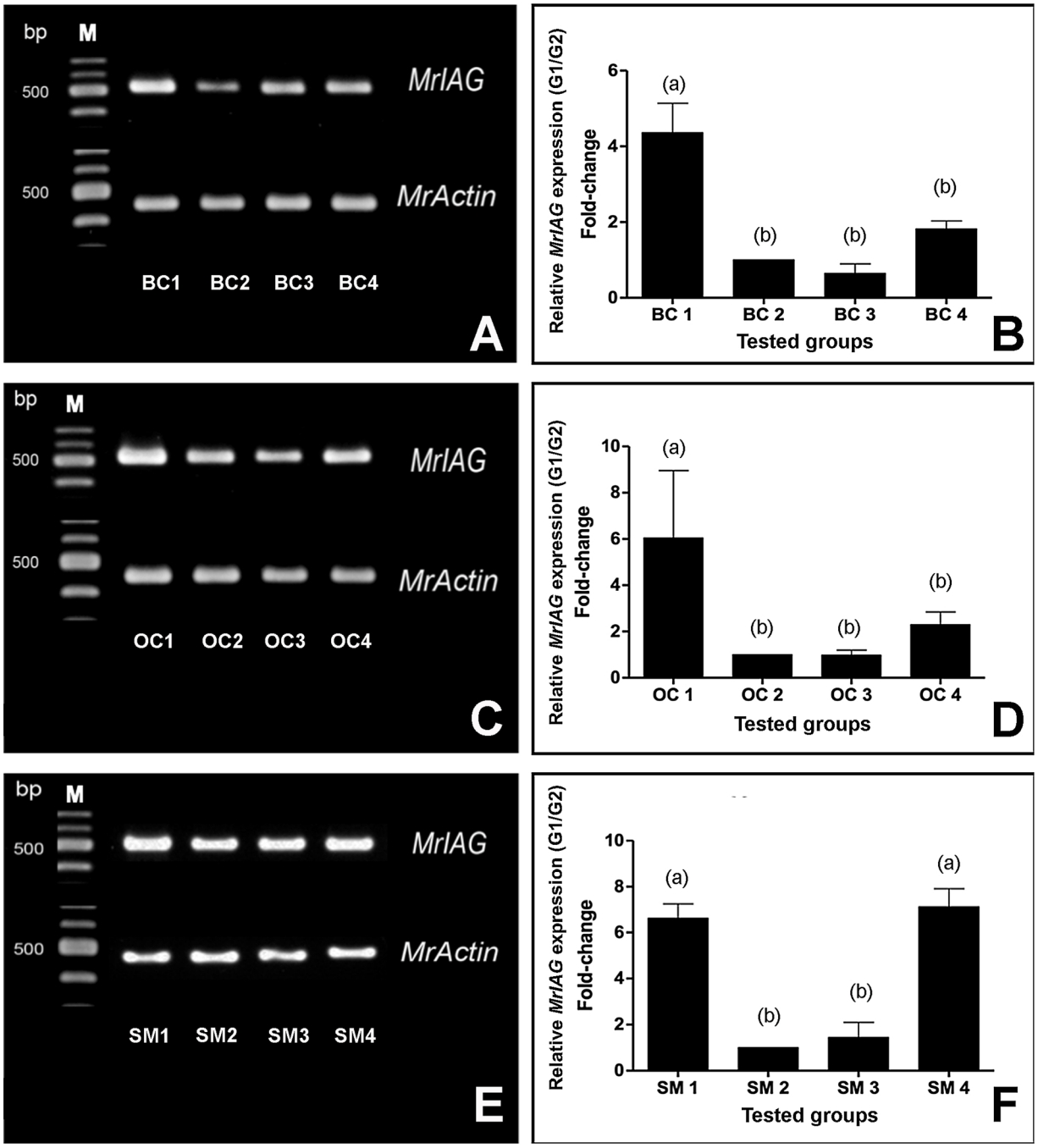

Fig. 2. Relative abundance of *MrIAG* mRNA in three male morphotypes in co-cultures as depicted in Fig. 1, i.e., blue claw (BC), orange claw (OC), and small male (SM) using semi-quantitative RT-PCR and quantitative real-time PCR; Results using RT-PCR and qRT-PCR of BC (A, B), OC (C, D), and SM (E, F) indicated there was an increase in relative abundance of *MrIAG* mRNA transcript in males of all morphotypes co-incubated with late premolt to early postmolt females (BC1, OC1, SM1) compared with other groups; Results for SM1 males, however, were not different from SM4 males as depicted in F (a) and (b) but they were difference, $P < 0.05$, when compared with those for SM2 and SM3 males; n = at least 4 in all groups.

3. Results

3.1. MrIAG mRNA transcript relative abundances in male prawns co-cultured with females for 7 days

The relative abundances of *MrIAG* in three different male morphotypes were investigated using semi-quantitative RT-PCR

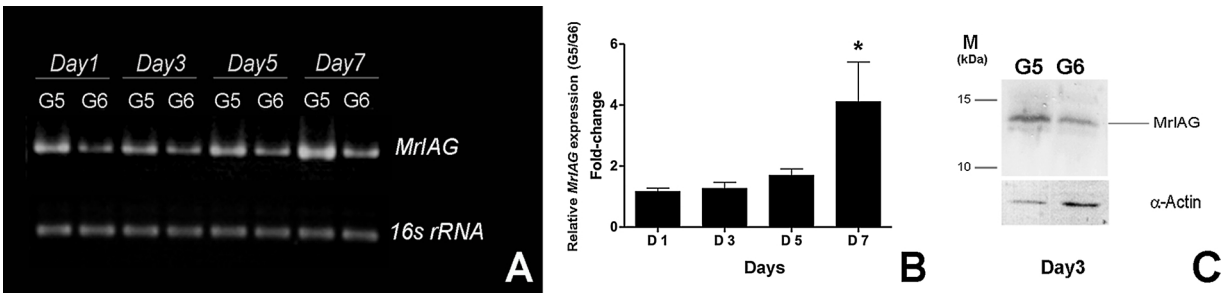

Fig. 3. (A) Temporal profiles for relative abundance of *MrIAG* transcript as determined using RT-PCR in SM males at days 1, 3, 5, and 7 of co-culturing by comparing results of G 5 and G6 (control) males; Relative abundance of *MrIAG* mRNA transcript in G5 males co-incubated with late premolt to early postmolt females with gradual increases from day 1 to day 7 compared with G6; (B) Results for relative abundance of *MrIAG* mRNA transcript (ratio of G5 to G6 as fold-change) as determined using qRT-PCR also indicated a gradual increase from day 1 to day 7 ($P < 0.05$), (Day 1 and 3, $n = 7$ each, day 5 and 7, $n = 6$ each); (C) Western blot detection of MrIAG protein in the blotted membranes with rabbit polyclonal antibodies against MrIAG and α-actin after 3-day of co-culture; Intensity of MrIAG staining in SM males in G5 was greater than in SM males in G6; (*) indicated differences, $P < 0.05$, when compared among groups.

(Fig. 2A, C, E) and real-time PCR (Fig. 2B, D, F). All male morphotypes co-cultured with late premolt to early postmolt females (G1) had a larger fold-change in relative abundance of *MrIAG* mRNA transcript ($BC_1 = 4.36 \pm 0.77$; $OC_1 = 6.04 \pm 2.91$; $SM_1 = 6.62 \pm 0.63$) when compared to other groups and males co-cultured with intermolt females (G2) (BC_2, OC_2, and SM_2) which were considered as the control groups (Fig. 2A, C, E). For the males in which the short lateral antennules were ablated that were co-cultured with late premolt to early postmolt females (G3), the relative abundance of *MrIAG* mRNA transcript was less than for all male morphotypes in G1 [$BC_3 = 0.64 \pm 0.24$, $OC_3 = 0.97 \pm 0.21$, $SM_3 = 1.44 \pm 0.64$] (Fig. 2B, D, F). Surprisingly, for the group of males co-cultured with the corresponding male morphotype (G4), the *MrIAG* mRNA transcript was only detected in the SM_4 (7.12 ± 0.78) which was at the same relative abundance as that in SM_1 (Fig. 2F). The BC_4 (1.82 ± 0.21) and OC_4 (2.29 ± 0.55), however, had a lesser relative abundance of *MrIAG* mRNA transcript when compared to BC_1 and OC_1 (Fig. 2B, D).

3.2. Temporal abundances of MrIAG mRNA transcript in SM males co-cultured with females from days 1 to 7

The temporal abundances of *MrIAG* mRNA transcript in SM males co-cultured with late premolt to early postmolt (G5) and with intermolt females (G6) were determined during the course of the experiment at days 1, 3, 5, and 7. The SM males in G5 had a greater relative abundance of *MrIAG* mRNA transcript than SM males in G6 (Fig. 3A). The relative abundance of *MrIAG* transcript in SM of G5 compared with those of SM in G6 gradually increased from day 1 (1.17 ± 0.1) to day 5 (1.69 ± 0.2) and peaked at day 7 (4.1 ± 1.2; Fig. 3B).

3.3. Western blot of the MrIAG protein

The Western blot of MrIAG protein of the SM of G5 and G6 is shown in Fig. 3C. The protein band with a molecular mass of 14 kDa in SM males in G5 appeared to be more dense than that of SM in G6. The Western blot with α-actin conducted as an internal control had the same intensity (Fig. 3C).

3.4. Testicular maturation in males co-cultured with females for 7 days

After day 7 of co-culturing, the gonado-somatic index (GSI) of the males in G1, especially the BC_1 (0.52 ± 0.09; Fig. 4A) and SM_1 (0.82 ± 0.14; Fig. 4C) were greater than those of males in G2 ($BC_2 = 0.3 \pm 0.09$, $SM_2 = 0.59 \pm 0.09$), G3 ($BC_3 = 0.32 \pm 0.09$, $SM_3 = 0.43 \pm 0.1$), and G4 ($BC_4 = 0.37 \pm 0.03$, $SM_4 = 0.58 \pm 0.05$). In contrast, the GSI of OC_1 (0.47 ± 0.11) was not different from those of OC_2 (0.42 ± 0.05) and OC_4 (0.43 ± 0.09) while the GSI of OC_3 was least among the OC group (0.28 ± 0.05) (Fig. 4B). There was confirmation of the results for the GSI by observing the BrdU positive staining testicular cells in SM of G5 and SM of G6. At day 7 of co-culturing, the BrdU-positive cells were predominantly in the spermatogenic zone of the testicular tubules in the testes of SM of G5 (Fig. 4D) while there were fewer positive cells in the testes of SM in G6 (Fig. 4E). The labeled cells were mostly spermatogenic cells and there were fewer nurse cells, and both were more numerous in testes of SM in G5 than SM of G6 (Fig. 4D, inset). There was a similar trend of BrdU positive testicular cells at days 1, 3, and 5 of co-culturing (data not shown).

4. Discussion

The mating pheromone is a mixture of substances secreted from one sex and perceived by the opposite sex. It is used in several taxa for intra-species communication especially with regards to sexual recognition and attraction (Bauer, 2011). The female pheromones have been characterized in several crustacean species. There are many chemicals reported as female pheromones, including uridine diphosphate (UDP) in *Carcinus maenas* (Hardege et al., 2011), *N*-acetylglucosamino-1,5-lactone in *Callinectes sapidus* and

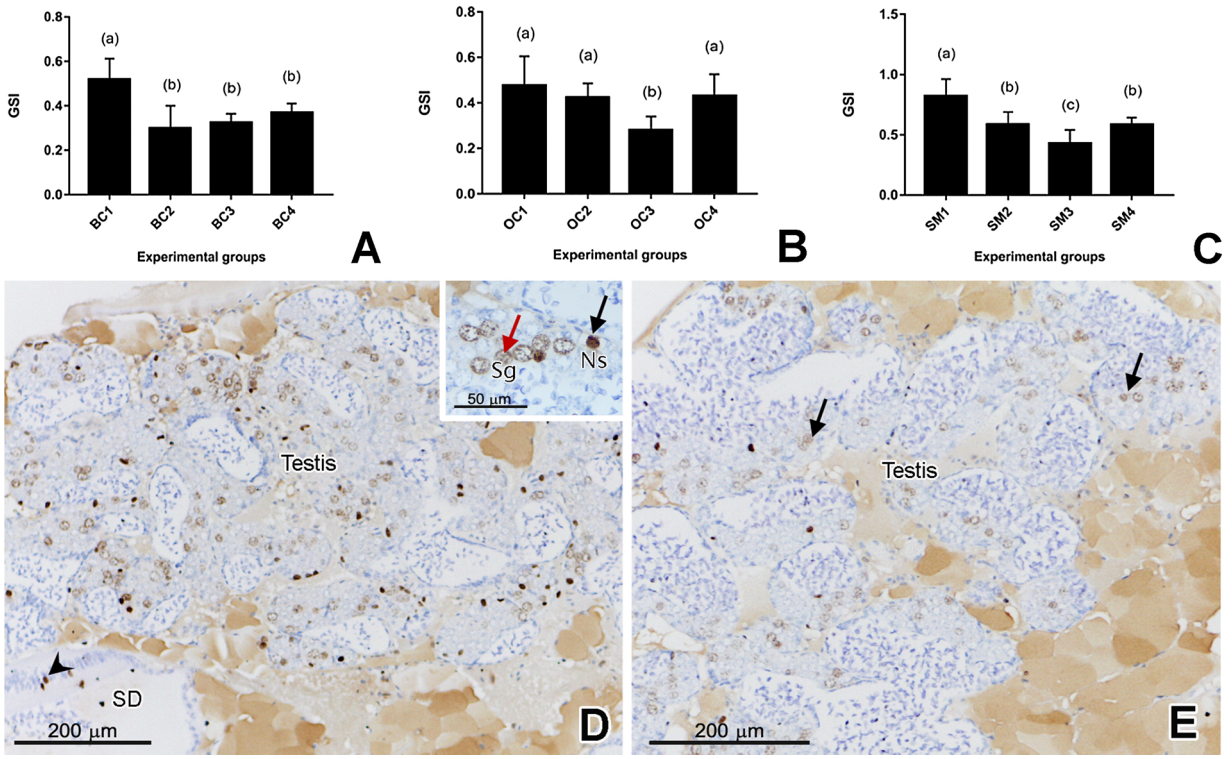

Fig. 4. (A, B, C) Histograms of the gonado-somatic index (GSI) of three male morphotypes after co-culture for 7 days; Results for BC1 OC1, and SM1 (co-cultured with late premolt to early postmolt females) indicated there was a greater GSI when compared with other groups, especially in SM1; GSI of OC1 was not different compared to OC2 and OC4, but was different from OC3; $P < 0.05$. $n =$ at least 7 animals in all groups; (a), (b), (c) indicated differences when the data were compared among all groups; (D) SM males in G5 had more BrdU-labeled cells in testicular tubules and spermatic duct epithelium (SD, arrow head) than those in SM of G6; There were two main populations of positively labeled cells, i.e., nurse cells (Ns, black arrow in inset) and spermatogenic cells (Sg, red arrow in inset); (E) Positively labeled Sg cells were also detected in SM of G6 but were much fewer in number than in SM of G5, and there were markedly less positively labeled Ns cells. (This figure will apprear in black and white for print version, and in color for online version.)

Telmessus cheiragonus (Kamio et al., 2014; Yano et al., 2016). These chemicals function as male-mating inducers which are predominantly released during the female molting period. In the present study, there were effects of females undergo molting stages (late premolt to early postmolt) on IAG abundance, and androgenic gland cell proliferation as well as gonadal maturation in male *M. rosenbergii* when assessed using co-culture assays. Furthermore, there might be pheromone-like substances released during the molting period by females into the water because the intermolt female prawns did not have a stimulatory effect on any male morphotypes. Late premolt to early postmolt female prawns typically have a fully mature ovary and may release pheromones that can elicit courtship and mating responses in males (Kruangkum et al., 2013; Karplus and Barki, 2018). Results of the present study indicated that the BC, OC, and SM prawns could perceive tentative female pheromone-like substances possibly via the short lateral antennules (slAn) which contain olfactory receptors (Kruangkum et al., 2013) because there were no such stimulatory effects detected in the ablated slAn males co-cultured with late premolt to early postmolt females. Furthermore, in the present study males' co-cultured with late premolt to early postmolt females had a greater abundance of *MrIAG* mRNA transcript from androgenic glands than males co-cultured with intermolt females, and the abundance was markedly greater in SM males compared to other male morphotypes. Results of the present study are supported by other results where there were peaks in the abundance of IAG after mating in male mud crabs (*Scylla paramamosain*; Zhang et al., 2014). The IAG is a key regulator of male sexual differentiation (Ventura et al., 2009). Furthermore, Ventura et al. (2011) reported that IAG abundance was greater in BC and SM than that of OC, indicating IAG actions may contribute to the aggressiveness and reproductive activity. This is in contrast to results of the present study where there was no difference in abundance of IAG between BC and OC. Interestingly, the peak in abundance of *IAG* mRNA in SM corresponded with the results reported by Ventura et al. (2011) where it was suggested that this phenomenon is linked to SM behaving in a stealthy manner during mating in the mix-sexed cultures. The detailed mechanism for these actions, however, remains unclear. It has been reported that in mixed male cultures there was a greater number of SM developing into OC when the BC were removed from the pond (Tidwell et al., 2015). Even though this male-male chemical interaction is poorly understood, the results from the present study could help explain why the SM in G4, without receiving an inhibitory signal from BC (Karplus et al., 1992), might rapidly and synchronously grow at a more rapid rate than usual and this phenomenon might be associated with a greater abundance of IAG in this group of SM.

The female pheromone-like substances might be perceived by males via the short lateral antennules (slAn). In *M. rosenbergii*, the

slAn possess aesthetasc, a special seta containing olfactory neurons (ORNs) that mediate mating behavior (Kruangkum et al., 2013). Neuronal fibers from slAn terminate bilaterally in the olfactory neuropils (ON) where the interneurons (cluster 9, 11) and projection neurons (cluster 10) are jointly located, and these neurons are then linked to the eyestalk (ES), a secondary olfactory center of crustaceans, via the olfactory globular tract (OGT) (Saetan et al., 2013; Derby and Schmidt, 2017). In the ES, the OGT terminates in several neuropils including the X-organ sinus gland complex where gonad- and molt-inhibiting hormones are produced (Rodríguez et al., 2007; Suwansa-ard et al., 2015; Qiao et al., 2018); and these hormones, in turn, control the IAG production and release from the AG (Khalaila et al., 2002). Findings in the present study indicate the effects of female pheromone-like substances on male abundance of *IAG* mRNA transcript (G1) might be mediated by this neural pathway from slAn to ES. This rationale is compatible with suggestions that pheromone perception in crustaceans are possibly mediated through the ES–AG–testis axis (Khalaila et al., 2002). Similarly, in mammals blood testosterone concentrations increased in male mice and rats following the perception of female pheromones (Amstislavskaya and Popova, 2004), and this phenomenon is thought to be mediated through the hypothalamic-pituitary-gonadal axis (Maruska and Fernald, 2011)

The AG has an important function in male sex differentiation and growth in many crustaceans (Okumura and Hara, 2004; Ventura et al., 2009, 2011). Sagi et al. (1990) reported that androgenic gland-ablated SM could grow and develop to be OC but not BC. In crayfish, *Cherax quadricarinatus*, the AG-implanted females could express male-like behaviors and were more aggressive than other females (Barki et al., 2003). The relationship between the AG and testicular development in crustaceans is well documented (Nagamine et al., 1980a, 1980b). In the present study, male prawns that had a greater abundance of IAG had a greater rate of testicular cell proliferation and gonado-somatic index (GSI). In *M. rosenbergii*, ablation of AG results in testis atrophy and reduced spermatogenesis (Nagamine et al., 1980a) while implantation of AG into female prawns can induce the formation of a functional testis from ovarian tissues (Nagamine et al., 1980b). The AG extract promotes phosphorylation of a testicular polypeptide in crayfish (*C. quadricarinatus*; Khalaila et al., 2002). Using the recombinant IAG (rIAG) for assessments, the IAG was detected to promote phosphorylation of insulin receptors in testicular cells of *Sagmariasus verreauxi, C. quadricarinatus*, *M. rosenbergii*, and *M. australiense* (Aizen et al., 2016). The insulin-like receptor belongs to a receptor tyrosine kinase subfamily and is predominantly expressed in testis and the AG (Ebina et al., 1985). In *Fenneropenaeus chinensis*, the insulin-like receptor binds with the IAG (Guo et al., 2018). In the present study, the late premolt to early postmolt females, through the induction of MrIAG, promoted testicular cell division in *M. rosenbergii* males. This might be mediated through insulin-like receptor phosphorylation as previously described. The correlative effect of IAG on testicular cell proliferation occurs in other crustaceans including the *Procambarus clarkii*, *C. quadricarinatus*, and *M. rosenbergii* (Taketomi et al., 1996; Khalaila et al., 2002; Ventura et al., 2009). Using BrdU labeling, positive spermatogenic cells and nurse cells were detected in most areas of testicular lobules of G1 males while a fewer number of spermatogenic cells were observed in G2 males at the same experimental days.

5. Conclusion

In present study, there were effects of late premolt to early postmolt females in the increasing the abundance of *MrIAG* mRNA transcript, and cell proliferation in testes of all *M. rosenbergii* male morphotypes. These effects might be initiated by a pheromone-like substance released by females during the molting period that were perceived by olfactory receptors on the slAn, and possibly mediated through the ES-AG-testis axis. The increased IAG abundance from the AG accelerated testicular cell proliferation possibly by phosphorylation of the insulin-like receptor of the testicular cells. The identity of female pheromone-like substance, however, is still lacking and its elucidation is essential for the understanding of molecular mechanism which underlies the male responses that occurred in the present study.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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