รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของฟีโรโมนจากกุ้งเพศเมียต่อการแสดงออกของฮอร์โมนจาก androgenic gland ที่คล้าย insulin และ การกระตุ้นการกำหนดลักษณะทางเพศของกุ้งก้ามกรามเพศผู้ (*Macrobrachium rosenbergii*)

Effect of female sex-pheromone on the expression of insulin-like androgenic gland hormone (IAG) and acceleration of male morphotypic determination in the giant freshwater prawn, *Macrobrachium rosenbergii*

โดย

ดร. ธนพงศ์ เครื่องคำ

ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์

มหาวิทยาลัยมหิดล

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สัญญาเลขที่ MRG6080088

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ดร.ธนพงศ์ เครื่องคำ
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มหาวิทยาลัยมหิดล

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Investigator: ดร.ธนพงศ์ เครื่องคำ ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

E-mail Address: thanapong.kruangkum@gmail.com

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บทคัดย่อ

ฮอร์โมนจาก androgenic gland ที่คล้าย insulin (IAG) เกี่ยวข้องและมีบทบาทในการควบคุมการพัฒนาทางเพศแบบปฐมภูมิ และทุติยภูมิในเพศผู้ของสัตว์กลุ่มกุ้งและปู ในการศึกษาก่อนหน้านี้พบกว่าระดับการแสดงออกและการหลั่งฮอร์โมน IAG ในกุ้งก้ามกรามเพศผู้ สัมพันธ์กับสภาวะความสมบูรณ์ทางเพศและพฤติกรรมก้าวร้าว การเลี้ยงร่วมกับกุ้งเพศเมียในบ่อเลี้ยงที่เลี้ยงรวม พบว่ามีความแปรปรวนของขนาดในกุ้งเพศผู้ ซึ่งปรากฏการณ์นี้ไม่พบในรูปแบบการเลี้ยงแบบแยกเพศ ดังนั้นเราจึงมีสมติฐานว่าตัวกลางหนึ่งที่ถูกสร้างและปล่อยจากเพศเมีย (ฟีโรโมน) น่าจะมีผลต่อการแสดงออกของฮอร์โมน IAG ซึ่งเหนี่ยวนำให้เกิดความแตกต่างของขนาดในกุ้งเพศผู้ การศึกษาในครั้งนี้พบการแสดงออกของฮอร์โมน IAG สูงขึ้นอย่างมีนัยสำคัญทางสถิติ ในการเลี้ยงกุ้งเพศเมียที่อยู่ระยะดึงดูดทางเพศเพื่อการสืบพันธุ์ (ก่อนถึงหลังลอกคราบ) ร่วมกันกับกุ้งเพศผู้ทั้ง 3 ลักษณะ เป็นระยะเวลา 7 วัน อีกทั้งยังพบกระตุ้นการพัฒนาของอวัยวะสืบพันธุ์ การแสดงออกของฮอร์โมนดังกล่าวค่อยๆเพิ่มสูงขึ้นตั้งแต่วันแรกของการเลี้ยงร่วมซึ่งยืนยันด้วยผลการแสดงออกในระดับโปรตีนด้วยเทคนิค western blot การเลี้ยงร่วมกับกุ้งเพศเมียในระยะดึงดูดยังเพิ่มการแบ่งเซลล์ของเซลล์สืบพันธุ์ใน

อัณฑะ ในขณะที่กุ้งเพศผู้ในกลุ่มที่ตัดหนวดมีลักษณะที่ตรงข้ามกับผลดังกล่าว ดังนั้นจากการศึกษานี้จึงสรุปได้ว่ามีตัวกระตุ้นที่สร้างและปล่อยจากกุ้งเพศเมียในระยะเหนี่ยวนำทางเพศที่สามารถกระตุ้นการแสดงออกของฮอร์โมน IAG ในกุ้งเพศผู้ โดยรับผ่านอวัยวะรับกลิ่นที่อยู่บนหนวดคู่เล็กของกุ้งก้ามกราม

Abstract

The insulin-like androgenic gland hormone (IAG) controls the development of primary and secondary male sex-characteristics in decapod crustaceans. In male giant freshwater prawns, *Macrobrachium rosenbergii*, the IAG level correlates with male reproductive status and aggressiveness. Female prawns in co-cultured with males can result in size variation of the latter while this variation disappears in males cultured in monosex. We hypothesized that some pheromone-like factors from female prawns may influence the IAG expression in co-cultured males which affect their sexual maturation. In this study, we demonstrated that the late premolt to postmolt females co-cultured with males for seven days significantly increased MrIAG expression in all male phenotypes as well as their gonad-somatic indexes (GSI). The expression of MrIAG was gradually upregulated from days 1 to 7 as confirmed by Western blot of MrIAG protein, and increased cell proliferations were detected in spermatogenic zone of testicular tubules as well as in epithelium of spermatic duct by BrdU labeling. In contrast, these effects were negated if the short lateral antennules of males were ablated. Thus, our results suggested that there might be some certain female-molting factors which acted as key regulators of androgenic gland function and gonadal maturation that were perceived by males via their short lateral antennules which are the olfactory organs.

Keywords: Giant freshwater prawn; Premolt to postmolt female; Pheromone; Insulin-like androgenic gland hormone; Gonad maturation.

Executive Summary

This study mainly demonstrated the effects of late premolt to early postmolt females on the increase of MrIAG expression and gonadal cell proliferation in testes of all M. rosenbergii male morphotypes. From the basic fact that the mixed sex culture led to size-variation in male prawns, while this phenomenon absented in the male monosex culture. Moreover, our previous preliminary study demonstrated that the late premolt to early postmolt female prawns were sexually attractive to male prawns for mating induction. The IAG which controls primary and secondary male sex characteristics was found to specifically express with relation to female molting period. The increase of IAG in male prawns co-cultured with late premolt to early postmolt female prawns within 7-day assay might be caused by pheromone-like substance released by female prawns during molting period. Moreover, temporal expression of IAG showed gradually increase from day 1 to day 7 of assay. This study was confirmed by the expression of IAG revealed by western blot. The IAG also involved male germ cells as its high expression enhanced male gonad maturation as reflected by the GSI value and BrdU assay. In this study we suggested that the maturation of androgenic gland and gonad positively correlated with IAG level. These effects were possibly induced by some unidentified female factors during molting stages which were perceived via the male short lateral antennules. However, the molecular identity as well as mechanism of that pheromone-like substance is still not yet reported.

1.Introduction

The giant freshwater prawns, *Macrobrachium rosenbergii*, is an important economic crustacean species that has faced shortage due to high market demands for local consumption as well as for export that could only be met by aquaculture (New, 2005). In mixed culture males varies in sizes from the largest blue clawed (BC), the medium size orange clawed (OC), and the smallest size small male (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 male population in the same culture (Karplus et al., 1989; Karplus et al., 2000; Karplus, 2005; Aziz et al., 2017). The dominant male morphotype (BC) displays more aggressiveness than other subordinated morphotypes (OC, SM) and gains most of the food and supplemental resources (Karplus, 2005; Karplus et al., 1989). The presence of females in mixed-sex culture is able to induce the morphotype transformation of OC and SM to the more mature BC (Tidwell et al., 2015), suggesting that females affect the maturation of males. Beside gaining more resources, the BC has higher mating activities compared to those of OC, whereas the immature SM is reported to be sneakers in copulating with molting females (Ra'anan and Sagi, 1985). In contrast, the OC was reported to have less reproductive activity (Sagi and Ra'anan, 1988; Ventura et al., 2011). Physiologically, the male morphotype correlates with status of testicular development in which the fully mature testes only occur in the BC (Sagi et al., 1988), while the testes of SM in transition to become OC morphotype also show high spermatogenic activity (Sagi et al., 1988). Thus, these three stages of male morphotypes dramatically express different growth patterns and reproductive behaviors as well as state of testicular development (Sagi et al., 1988; Sagi and Ra'anan, 1988; Ventura et al., 2011).

One prime factor functionally involves with male morphotype transformation is 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; Barki et al., 2006; Ventura et al., 2011). Hence, the AG has been identified as the key 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 appearance of feminine characteristics, and in some cases sexual reversal and formation of ovaries in place of testes (Barki et al., 2006). The expression of *MrIAG* correlates with male morphotypes as a high level of MrIAG was detected in the BC and SM while a low level was detected in the OC (Ventura et al., 2011). The *MrIAG* is suppressed by hormones released from the eyestalks (Khalaila et al., 2002) as the eyestalk ablated prawns demonstrate AG hypertrophy, increased GSI and spermatogenesis (Kim et al., 2002; Phoungpetchara et al., 2011). Interestingly, in the mud crab, *Scylla paramamosain*, the level of IAG also correlates with mating activity as its peak was detected right after mating (Zhang et al., 2014).

During molting period, female prawns exhibit fully mature ovaries and respond positively to courtship and mating behaviors expressed by male prawns. It was 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 is still no study on the effect of molting females on male reproductive physiology in *M. rosenbergii*. In this study, the effects of late premolt, molting, and early postmolt females on the *MrIAG* expression in three different male morphotypes (BC, OC, and SM) as well as male gonad maturation were investigated by co-culture assay. We hypothesize that during the period of molting, females release certain pheromone-like substances that are perceived by male via their short lateral antennules which house 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 7-10 days before starting experiments. Prawns were fed with commercial food pellets (Betagro Co., Thailand) twice a day. All experimental animals were handled according to the procedure 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 summarized 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 kept in 100 L perforated basket which was placed centrally in each aquarium tank (Fig 1). Inducers (n = 7-8) included late premolt to early postmolt females, intermolt females, and male prawns. In this experiment, the BC, OC, and SM males were each divided into 4 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 according to 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 mentioned above and about 50% of cultured water was replaced twice. 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 2-3 days and later become early postmolt. Early postmolt inducers were used for only 2 days and replaced with equal number of new late premolt prawns. By this procedure, there were a total of 14-16 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 gonado-somatic index (GSI) (Siangcham et al., 2013). Half of the AG with the terminal ampule of each prawn was immediately dissected out and kept at -80ºC for biochemical examinations while the other half was fixed in Davison's fixative for histological examination. To investigate the temporal expression of *MrIAG* obtained from the first experiment, we designed the second phase of the experiment using immature SM prawn as a model. 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 under the same conditions as mentioned in 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) for 8-12 h before organ collections.

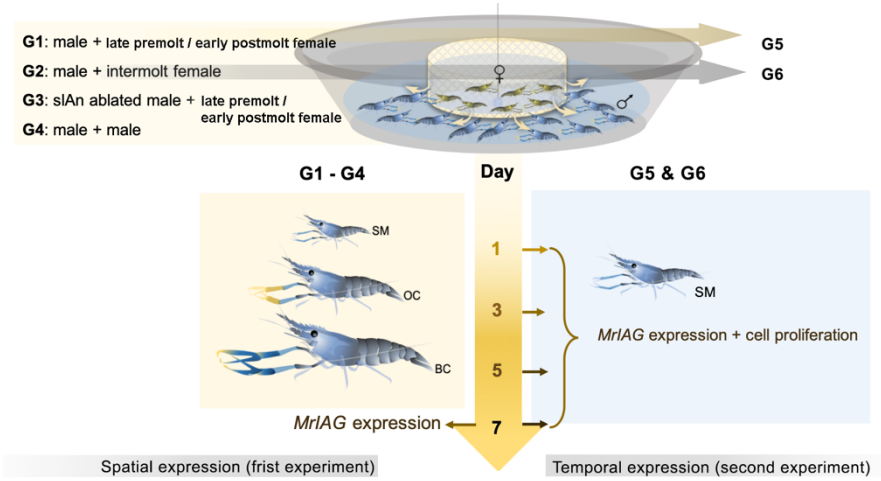

Fig.1. A schematic illustration of the experimental designs for co-culturing assays: on the left, co-cultures between all three male morphotypes *M. rosenbergii* with late premolt to early

postmolt females, intermolt females, corresponding males (G1, G2, G4), and between short lateral antennule-ablated males with late premolt to early postmolt females (G3). The *IAG* expression 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 expression of *IAG* and cell proliferation were determined and compared between these two groups every day from days 1 to 7.

2.3. Estimation of MrIAG expression by RT-PCR

RNA from the AGs of all male morphotypes of four different subgroups were individually extracted by TriPure isolation reagent (Roche, IN, USA) following the manufacturer's protocol. Total RNAs were measured using a Nanodrop microvolume spectrophotometer (Thermo Scientific™, USA). One microgram of RNA from each individual was then treated with DNaseI (Invitrogen, CA, USA) prior to be used for the first-strand cDNA synthesis, using the SupersScript III Reverse Transcriptase kit (Invitrogen, CA, USA). RT-PCR of *MrIAG* (pooled RNA, n = 10) was done with specific primers (Table 1) designed from its corresponding sequence in GenBank NCBI (accession number: FJ409645.1). Thermocycling amplification was done 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). Non-templated PCR was performed as a negative control and PCR of β*–actin* or *16SrRNA* was performed as an internal control, respectively.

2.4. Estimation of MrIAG expression by Real-time PCR

In the first experiment, cDNA from each individual was used for estimating *MrIAG* expression by real-time PCR. Triplicate qPCR reactions were carried out for each sample, and each PCR reaction consisted of 0.5 µl of cDNA (except for Non-template control, 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.) under 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 were shown in Table 1. In the second experiment, cDNAs of AGs collected at day 1, 3, 5 and 7 from co-cultured males were used for determining *MrIAG* expression by real-time PCR using the same conditions as mentioned above. The relative expressions of *MrIAG* of all groups to that of group 2 and 6 for the first and second experiments, respectively, were calculated and presented as a mean of 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

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 measured by Bradford assay (BIO-RAD, USA), and hundred micrograms of crude extracts from each group were loaded onto 15% SDS-Tris-Glycine gels and separated with 110 V. Then, the separated proteins were transferred to PVDF membranes (Fluorotrans® W 3.3m Roll Pall BSP01 (Pall, China). 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 it was 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 1:5,000 dilution in blocking solution for 2 h. Then, the membranes were washed before developing signal using Pierce™ ECL Plus Western Blotting Substrate (Thermo Scientific, USA)] and hyper film (Hypercassette™, Amersharm). For internal control, the same membranes were immersed in stripping solution (10% SDS and 1% β-mercaptoethanol in 1M Tris-HCl buffer, pH 6.7) at 50 °C for 30 min. Then they were re-

probed with anti α-actin (Santa Cruz Biotechnology) at 1:3000 dilution followed by the remaining steps as mentioned above.

2.6. Determination of gonadal cell proliferation

Proliferation of gonadal cells was estimated by 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 day 1, 3, 5, and 7 for 8-12 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 six micron-thick sections were treated with 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 2N HCl at 37 °C for 1 h and washed three times by 0.5% Tween-20 in PBS solution (PBST). They 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 bindings. 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 under a light microscope (Leica DM750) with a digital camera (Leica ICC50 HD).

2.7. Statistical analysis

All data were analyzed and compared by one-way ANOVA with Tukey's multiple comparison post hoc-test (GraphPad Prism 5.01) with significant difference at the *P* value less than 0.5.

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		

3. Results

3.1. MrIAG expression in male prawns co-cultured with females for 7 days

The expressions of *MrIAG* in three different male morphotypes were investigated by semi-quantitative RT-PCR (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 significantly higher fold-change in *MrIAG* expression (BC_1 = 4.36 ± 0.77; OC_1 = 6.04 ± 2.91; SM_1 = 6.62 ± 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 short lateral antennules-ablated males co-cultured with late premolt to early postmolt females (G3), the *MrIAG* expression was lower than all male morphotypes in G1 [BC_3 = 0.64 ± 0.24, OC_3 = 0.97 ± 0.21, SM_3 = 1.44 ± 0.64] (Fig. 2B, D, F). Surprisingly, for the group of males co-cultured with their corresponding male morphotype (G4), the expression of *MrIAG* was found only in the SM_4 (7.12 ± 0.78) which was at the same level as seen in SM_1 (Fig. 2F). However, the BC_4 (1.82 ± 0.21) and OC_4 (2.29 ± 0.55) had low level of *MrIAG* expression when compared to BC_1 and OC_1 (Fig. 2B, D).

3.2. Temporal MrIAG expression in SM males co-cultured with females from days 1 to 7

The temporal expression levels of *MrIAG* in SM males co-cultured with late premolt to early postmolt (G5) and with intermolt females (G6) were determined over the course of the experiment at days 1, 3, 5, and 7. It was found that the SM males in G5 exhibited higher expression of *MrIAG* than SM males in G6 (Fig. 3A). Relative *MrIAG* expression in SM of G5 to those of SM in G6 gradually increased from day 1 (1.17 ± 0.1) to day 5 (1.69 ± 0.2) and significantly reached the peak at day 7 (4.1 ± 1.2), respectively (Fig. 3B).

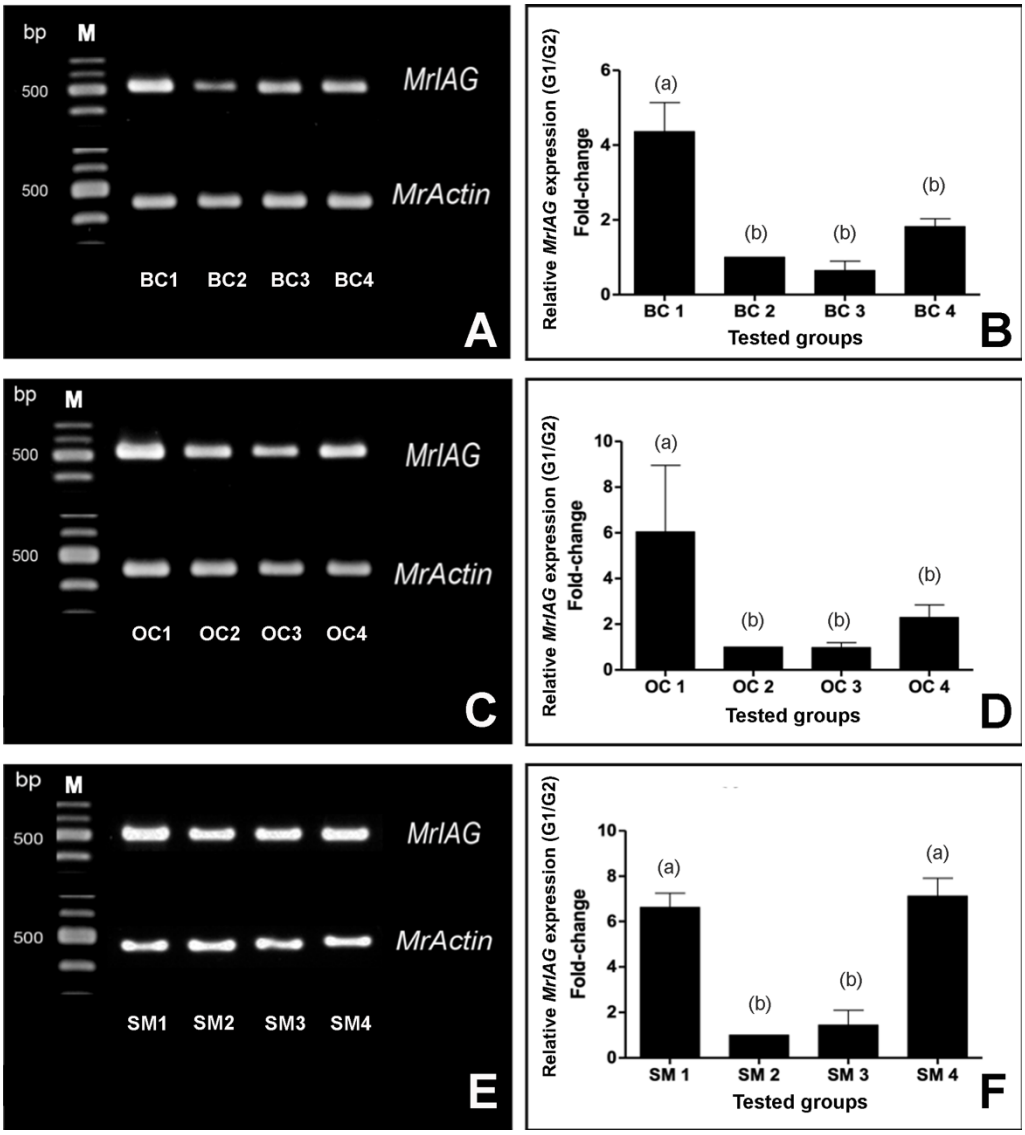

Fig.2. The expression of *IAG* in three male morphotypes in co-cultures as shown in Fig.1, i.e., blue claw (BC), orange claw (OC), and small male (SM) using semi-quantitative RT-PCR and quantitative real-time PCR. The RT-PCR and qRT-PCR of BC (A, B), OC (C, D), and SM (E, F) showed significant increase of *MrIAG* level in males of all morphotypes co-incubated with late premolt to early postmolt females (BC1, OC1, SM1) compared to other groups. However, the SM1 males did not show significantly difference from SM4 males as shown in F (a) and (b) indicated significant difference, $P<0.05$, when compared among groups. n = at least 4 in all groups.

3.3. Western blot of the MrIAG protein

Western blot of MrIAG protein of the SM of G5 and G6 was demonstrated in Fig. 3C. It appeared that a protein band at molecular mass of 14 kDa of SM male in G5 was greater than that of SM in G6. Western blot of α-actin, carried out as internal control, showed the same intensity (Fig. 3C).

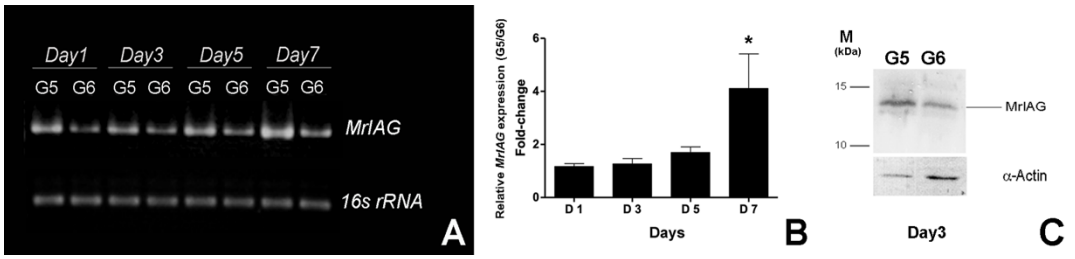

Fig.3. (A) Temporal expression profile of *MrIAG* determined by RT-PCR in SM males at days 1, 3, 5, and 7 of co-culturing by comparing between G 5 and G6 (control) males. The expression level of *MrIAG* in G5 males co-incubated with late premolt to early postmolt females showed gradual increase from day 1 to day 7 compared to G6. (B) The relative expression levels of *MrIAG* (ratio of G5 to G6 as fold-change) as determined by qRT-PCR also showed 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 co-culture. The intensity of MrIAG in SM males in G5 was more intense than in SM males in G6. (*) indicated significant difference, $P<0.05$, when compared among groups.

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

After day 7 co-culture, 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 significantly higher than those of males in G2 (BC_2 = 0.3 ± 0.09, SM_2 = 0.59 ± 0.09), G3 (BC_3 = 0.32 ± 0.09, SM_3 = 0.43

± 0.1), and G4 (BC_4 = 0.37 ± 0.03, SM_4 = 0.58 ± 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 found to be significantly lowest among the OC group (0.28 ± 0.05) (Fig. 4B). We then confirmed the result of GSI by observing the BrdU positive testicular cells in SM of G5 and SM of G6. At day 7 co-culture, BrdU-positive cells were predominantly found in the spermatogenic zone of the testicular tubules in the testes of SM of G5 (Fig. 4D) while fewer number of positive cells were detected in the testes of SM in G6 (Fig. 4E). The labeled cells were mostly spermatogenic cells and fewer nurse cells, and both were more numerous in testes of SM in G5 than SM of G6 (Fig. 4D, inset). Similar trend of BrdU positive testicular cells appeared at day 1, 3, and 5 of co-cultures (data not shown).

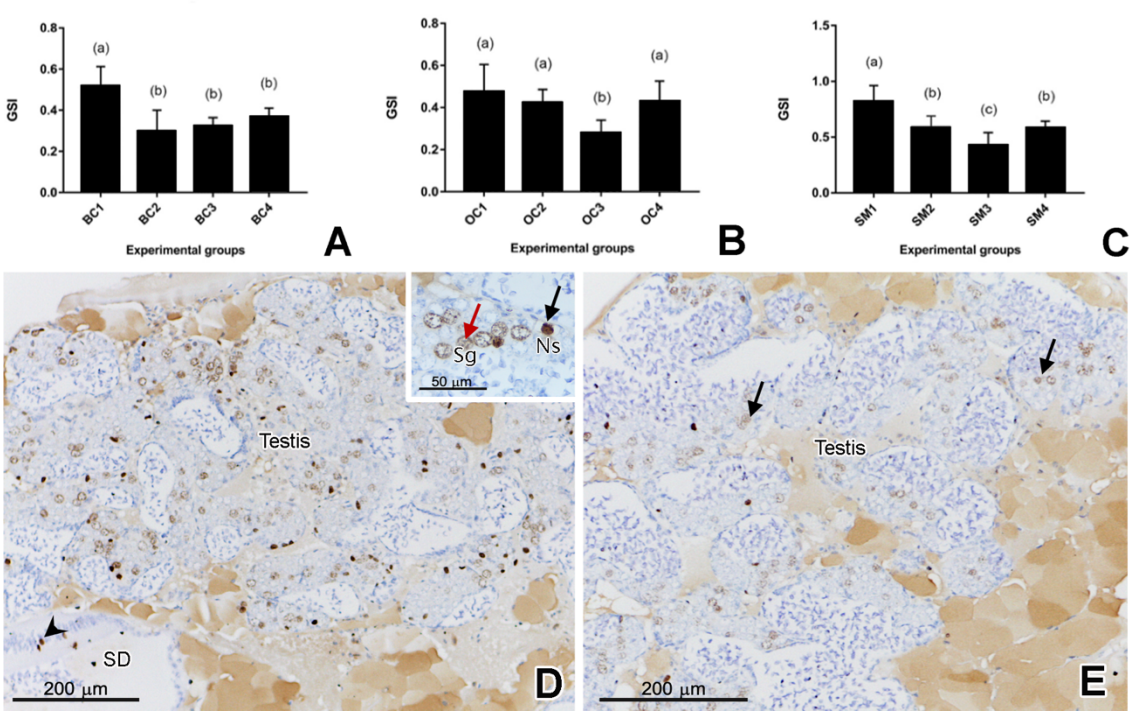

Fig.4. (A, B, C) Histograms of the gonado-somatic index (GSI) of three male morphotypes after being co-cultured for 7 days. BC1 OC1, and SM1 (co-cultured with late premolt to early postmolt females) showed more prominent GSI when compared with others, especially in SM1. The GSI of OC1 was not significant different compared to OC2 and OC4, but significantly different from OC3. $P<0.05$. n = at least 7 in all groups. (a), (b), (c) indicated

significant difference when the data were compared among all groups. (D) SM males in G5 showed 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) The positively labeled Sg cells were also detected in SM of G6 but at a much fewer number than in SM of G5, and there were even fewer positively labeled Ns cells.

4. Discussion

Mating pheromone is cocktail-liked substance which is secreted from one sex and received by the other. 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 *Telmessus cheiragonus* (Kamio et al., 2014; Yano et al., 2016). These chemicals have been proven to function as male-mating inducers which are predominantly released during female molting period. In this study, we demonstrated the effects of female undergo molting stages (late premolt to early postmolt) on IAG expression, androgenic gland cell proliferation as well as gonadal maturation in male *M. rosenbergii* by co-culture assays. It was further proposed that there might be pheromone-like substances being released during molting period by females into the water since the intermolt female prawns could not provide stimulatory effect on any male morphotypes. Late premolt to early postmolt female prawns typically have fully mature ovary and may release pheromones that can elicit courtship and mating responses in males (Kruangkum et al., 2013; Karplus and Barki, 2018). Our study indicated that the BC, OC, and SM prawns could perceived tentative female pheromone-like substances possibly via the short lateral antennules (slAn) which houses olfactory receptors (Kruangkum et al., 2013) as no such stimulatory effects could be detected in the ablated slAn males co-cultured with

late premolt to early postmolt females. Further we found that males' co-culture with late premolt to early postmolt females expressed increased level of *MrIAG* from androgenic glands than males co-cultured with intermolt females, and the expression level was notably high in SM male compared to other male morphotypes. Our results were supported by others who demonstrated IAG expression peaks after mating in male mud crab (*Scylla paramamosain*) (Zhang et al., 2014). The IAG has been known as a key regulator of male sexual differentiation (Ventura et al., 2009). As well, Ventura et al. (2011) found higher IAG expression of BC and SM than that of OC, suggesting their stronger aggressiveness and reproductive activity. This is in contrast to our result which showed no difference of IAG expression level between BC and OC. Interestingly, the *IAG* spike in SM corresponded with the finding of Ventura et al. (2011) who suggested that this phenomenon is linked to SM behaving as sneakers during mating in the mix-sexed culture. However, the detailed mechanism remains unclear. It has been reported that in mixed male culture there was a greater number of SM transformed to OC when the BC were removed from the pond (Tidwell et al., 2015). Eventhough this male-male chemical interaction is poorly understood, this study could help explain why the SM in G4, without receiving 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 high IAG expression 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 posseses 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 a., 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). Our findings in the present study suggested that the effects of female pheromone-like substances on male *IAG* expression (G1) might be mediated by this neural pathway from slAn to ES. This 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 level 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 plays roles in male sex differentiation and growth in many crustaceans (Okumura and Hara, 2004; Ventura et al., 2009; Ventura et al., 2011). Sagi et al. (1990) demonstrated that androgenic gland-ablated SM could grow and develop to be the OC but not BC. In crayfish, *Cherax quadricarinatus*, the AG-implanted females could exhibit male-like behavior and expressed more aggressiveness 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 we also found that male prawns with higher IAG expression significantly had a higher 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 on female prawn can induce the formation of functional testis from ovary (Nagamine et al., 1980b). The AG extract was shown to be able to promote phosphorylation of testicular polypeptide in crayfish (*C. quadricarinatus*) (Khalaila et al., 2002). Using the recombinant IAG (rIAG), it was shown that the IAG promotes phosphorylation of insulin receptor 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 it predominantly expresses in testis and AG (Ebina et al., 1985). In *Fenneropenaeus chinensis*, the insulin-like receptor binds with the IAG (Guo et al., 2018). In this study, we demonstrated that late premolt to early postmolt female, through the induction of MrIAG, promoted testicular cell division in *M. rosenbergii* males. This might be mediated through insulin-like

receptor phosphorylation as demonstrated previously. The correlative effect of IAG on testicular cell proliferation is demonstrated in other crustaceans including the *Procambarus clarkii*, *C. quadricarinatus*, and *M. rosenbergii* (Taketomi et al., 1996; Khalaila et al., 2002; Ventura et al., 2009). By BrdU labeling, positive spermatogenic cells and nurse cells were detected in most area 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, we have demonstrated the effects of late premolt to early postmolt females in the increasing *MrIAG* expression, cell proliferation in testes of all *M. rosenbergii* male morphotypes. These effects might be initiated by pheromone-like substance released by females during molting period that were perceived by olfactory receptors on the slAn, and possibly mediated through the ES-AG-testis axis. The increased IAG expression from the AG accelerated testicular cell proliferation possibly by phosphorylation of the insulin-like receptor on the testicular cells. However, the identity of female pheromone-like substance is still lacking and its elucidation is essential for the understanding of molecular mechanism which underlines the male responses as demonstrated in this study.

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6. Suggestion for future research

- Identify the active compounds from molting water from late premolt and early postmolt females.
- Study the upstream neuropeptides or neurohormones which perform the main accelerators of androgenic gland control.
- Study the large-scale level of co-culture bioassay.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. Kruangkum, T., Saetan, J., Chotwiwatthanakun, C., Vanichviriyakit, R., Thongrod, S., Thintharua, P., Tulyananda, T., Sobhon, P. 2019. Late premolt to early postmolt female giant freshwater prawns, *Macrobrachium rosenbergii*, enhanced the expression of insulin-like androgenic gland hormone (MrIAG) and gonad maturation in male prawns through olfactory receptors. Animal Reproduction Science. (Resubmission for minor revision).