# CHAPTER IV
# MATERIALS AND METHODS

## 4.1 Effects of serotonin, dopamine, gonadotropin-releasing hormones, and corazonin, on the androgenic gland of the giant freshwater prawn

### 4.1.1 Experimental animals

*M. rosenbergii* at the stage of small male (4 month olds) with average weight of 33 g were purchased from a manufactured farm in Chachoengsao province, Thailand. The 440 prawns were separated into 11 groups (n = 40) and maintained in circular concrete tanks with 0.75 m in radius and 0.8 m in depth. The photoperiod was a light: dark at 12:12 h, and the waste was removed every two days. The prawns were supplied with commercial pellets for broodstock (STC Feed Co., Phetchaburi, Thailand), and acclimatized before starting the experiments for two weeks.

### 4.1.2 Experimental design

Our experiment was designed following earlier studies of our group (Ngernsoungnern et al., 2008b; Ngernsoungnern et al., 2009; Poljaroen et al., 2011; Tinikul et al., 2008). The prawns were divided into graph and injected as follows (Figure 4.1):

**Group 1** (vehicle control group) treated with crustacean physiological saline (CPS; 0.496 M NaCl, 0.010 M KCl, 0.016 M $CaCl_2 \cdot 2H_2O$, 0.013 M $MgSO_4 \cdot 7H_2O$, 0.006 M $NaHCO_3$, 0.001 M $MgCl_2 \cdot 6H_2O$, and 0.019 M HEPES), pH 7.4

**Groups 2-5** treated with 2 doses ($2.5 \times 10^{-6}$ and $2.5 \times 10^{-7}$ mol/prawn) of the neurotransmitters 5-HT and DA (Sigma-Aldrich, St. Louis, MO, USA)

**Groups 6-11** treated with 2 doses (50 and 500 ng/g BW) of the three neurohormones (Table 4.1) synthesized by GenScript, Piscataway, NJ, USA.

**Table 4.1** Peptides sequences for bioassay

| Neurohormone | Peptide sequences |
|---|---|
| l-GnRH-III | pGlu-HWSHDWKPG-$NH_2$ |
| oct-GnRH | pGlu-NYHFSNGWHPG-$NH_2$ |
| Crz (Crustacean type) | pGlu-TFQYSRGWTN-$NH_2$ |

All doses had been successfully used in our group's other crustacean studies (Ngernsoungnern et al., 2008b; Poljaroen et al., 2011). The prawns were treated 4 times at 4-day intervals at day 0, 4, 8, and 12. Eight prawns in all groups on day 0, 4, 8, 12, and 16 were randomly selected, then injected with 5 mg/100 g BW of 5-bromo-2′-deoxy-uridine or BrdU (Roche, Mannheim, Germany) in distilled water, and then sacrificed after 8 h.

The AG and EB from one side of each prawn were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; 0.033 M $NaH_2PO_4 \cdot 2H_2O$, 0.067 M $Na_2HPO_4 \cdot H_2O$, and 0.145 M NaCl), pH 7.4, for histological and immunohistochemical studies (n = 8). The AG from another side of each prawn were removed and stored in liquid nitrogen for enzyme-linked immunosorbent assay (ELISA) (n = 8). An androgenic gland-somatic index (ASI) of each prawn was calculated using the formula (AG fresh weight/somatic body weight of each prawn ×100) (Phoungpetchara et al., 2011).

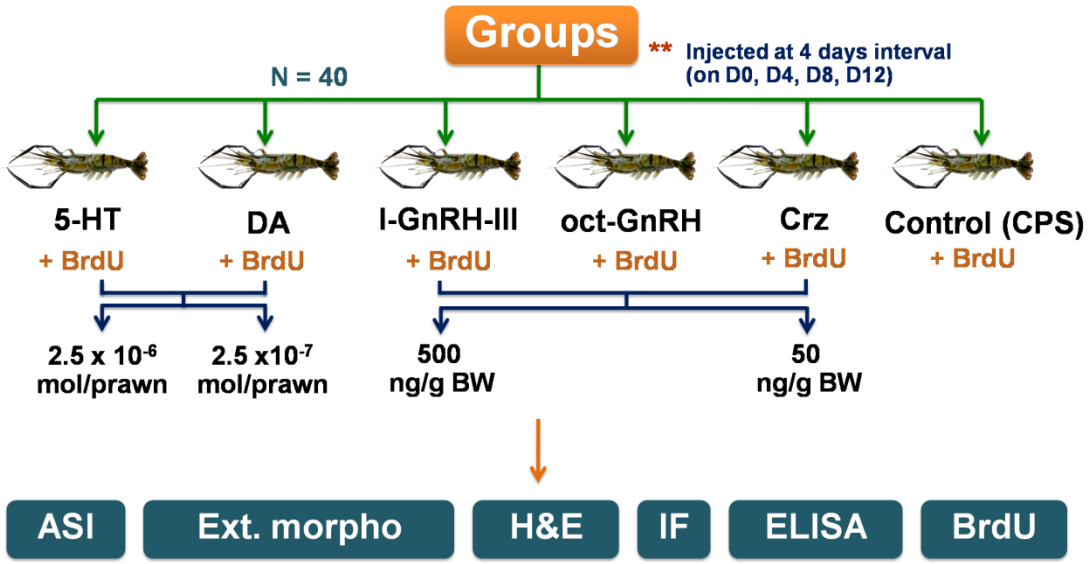


**Figure 4.1** Schematic diagram of the experimental design for studying the effects of 5-HT, DA, GnRHs, and Crz hormones.

### 4.1.3 Morphological and histological changes of the AG

The external morphology of AG in each group was observed by using the paraformaldehyde fixed tissues, which were gently washed three times with 0.1 M PBS, then photographed using a stereomicroscope (Olympus, Japan).

For histological studies, the tissues were dehydrated with a serial dilution of ethanol i.e., 70%, 80%, 90%, and 100%, penetrated with melted paraplast, and then embedded in paraffin blocks. The embedded tissues were sectioned at 5 µm-thick with a rotary microtome (Leica RM2235), and then transferred to silane-coated slides (Sigma-Aldrich, Missouri, USA). The sections were firstly deparaffinized in xylene for three times, then rehydrated with 100%, 90%, 80%, 70% ethanol. The sections were washed with distilled water for 10 min, stained with hematoxylin for 1 min, washed with tap water for 15 min, and counterstained with eosin for 2 min, mounted in Permount (Bio-Optica, Milan, Italy), and then air dried at the room temperature. The H&E stained sections were examined under a Nikon E600 light microscope and images captured by a Nikon DXM digital camera using an ACT-1 program.

### 4.1.4 Cell proliferation assay using 5-bromo-2'-deoxyuridine (BrdU)

To investigate cellular proliferation in the AGs of treated prawns, each male was injected with 5 mg of BrdU per 100 g of body weight, and after 8 h the animals were sacrificed and the AG taken for paraffin embedding. The cell proliferation was estimated using the BrdU assay followed the instruction from the Detection Kit II (Roche, Mannheim, Germany). The sections were taken from the mid-region of the AG triangle shown by a dashed line in Figure 5.1 (top diagram). Sections of the BrdU injected prawns were deparaffinized with xylene, rehydrated with 100%, 90%, 80%, 70% ethanol, washed in 0.1 M PBS, incubated with 2 M HCl, at 37 °C for 1 h, washed in 0.1 M PBS, and treated with 1% glycine in 0.1 M PBS, at room temperature for 15 min. They were washed again in 0.1 M PBS and then incubated in blocking solution (4% bovine serum albumin (BSA) in 0.1 M PBS) for 30 min. The sections were then incubated with anti-BrdU (mouse monoclonal antibody, diluted 1:20 with 1% BSA in 0.1 M PBS), at room temperature overnight. The sections were washed, and then incubated with anti-mouse IgG linked to alkaline phosphatase (AP) (1:500 diluted with 1% BSA in 0.1 M PBS), at 37 °C for 2 h. After further washings in 0.1 M PBS, the sections were incubated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5). The color was developed with the substrate solution NBT/BCIP (Roche, Mannheim, Germany), then stopped by stop buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.1), for 10 min. The sections were mounted with glycerol buffer, and then photographed and counted under a Nikon E600 light microscope. Three non-consecutive sections (i.e., every fourth section) were taken from each AG of eight prawns (n = 8), then examined and photographed at 400 times magnification for the determination of the number of dividing cells which is expression mean ± S.E.

### 4.1.5 Specificity of rabbit antiserum against *Mr*IAG (anti-*Mr*IAG)

#### 4.1.5.1 Production of polyclonal antibody against *Mr*IAG in rabbit

A rabbit antiserum against *Mr*IAG (anti-*Mr*IAG), kindly provided by Dr. Ittipon Phoungpetchara (Phoungpetchara et al., 2011), was used in Dot blot and Western blot analyses, and then ELISA.

#### 4.1.5.2 Praparation of the AG crude extract

Fresh EBs with attached AGs from five intact prawns were dissected out and stored at -80ºC. The tissues were homogenized in a lysis buffer (2 M acetic acid), then sonicated and centrifuged at 8600*g* at 4 °C, for 15 min. The supernatants were collected and then filtered through a 0.45 μm nylon membrane. Isolated proteins were measured by Lowry's method, using serial dilutions of BSA as a standard. The protein concentrations were measured at 750 nm in an automatic spectrophotometer to determine the *Mr*IAG levels.

#### 4.1.5.3 Dot blot analysis

The synthetic peptides synthesized by GenScript, Piscataway, NJ, USA (Table 4.2), including l-GnRH-III, ELH (egg-laying hormone), RPCH (red pigment concentrating hormone), *Mr*IAG, and the AG crude extract and 1 μg BSA (1 μl each) were dotted onto nitrocellulose membranes. The membranes were dried at room temperature for 45 min, and then blocked with 10% normal goat serum (NGS) and 5% skim milk in PBST (0.1% Tween-20 in 0.1 M PBS) for 2 h. One strip was reacted with non-preadsorbed antibody in PBS at a dilution 1:400, the other with pre-adsorbed antibody with 20% BSA in 0.1 M PBS at a dilution 1:400, and a negative control using pre-immune serum. Then the membranes were washed with PBST for 3 times (5 min each) and incubated with anti-rabbit IgG linked to AP, diluted 1:2000 with 0.1 M PBS, for 1 h. After further washings in 0.1 M PBS, the membranes were incubated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5). The color was developed with the substrate solution NBT/BCIP (Roche, Mannheim, Germany), diluted in detection buffer, and then stopped by stop buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.1) for 10 min. The membranes were photographed using digital camera to observe cross reactivity.

**Table 4.2** Peptides sequences for dot blot analysis

| Synthetic peptide | Peptide sequences |
|---|---|
| l-GnRH-III | pGlu-HWSHDWKPG-$NH_2$ |
| ELH | LSITNDLRAIADSYLYDQHKLRERQEENLRRRFLRL |
| RPCH | pGlu-LNFSPGW-$NH_2$ |
| *Mr*IAG | LASVCLRHNNYINPGPTYVSKE |

#### 4.1.5.4 Expression of r*Mr*IAG protein

A recombinant protein *Mr*IAG (*rMr*IAG) was produced in a prokaryotic system by inserting a full-length *Mr*IAG cDNA amplified by using primers designed from a full-length *Mr*IAG (Ventura *et. al*., 2009; GenBank accession no. FJ409645.1) (Figure 4.2), into a pET-30b Vector and transformed it into the host cells, *Escherichia coli* BL21 (DE3) (Changklungmoa et al., 2012). The colony were cultured in 5 ml high salt LB broth (10g Tryptone, 10 g NaCl, 5 g Yeast extract) contained 100 µl/ml kanamycin and then shaked overnight at 300 rpm, 37ºC for 14-16 hours. The cultured colony was separated into new culture tube and added new LB broth at the dilution 1:5.

The expression of *rMr*IAG was induced at 37 °C by isopropyl-β-D-thiogalactoside (IPTG) by adding 1 mM IPTG to the new culture tube. The colony was grown and shaked at 250 rpm, 37ºC, 3 hours, then centrifuged at 10000g at 4ºC, for 5 minutes. The pellet was stored at -20ºC until use.

#### 4.1.5.5 Analysis of *Mr*IAG protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Bacterial lysate and the AG extract were loaded at 10 µg into separate wells of two gels. The proteins were separated by 12.5% SDS-PAGE, after which one gel was stained with Coomassie blue to visualize proteins, and the other transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S, blocked with 10% normal goat serum (NGS) and 5% skim milk in PBST (0.1% Tween-20 in 0.1 M PBS) for 2 h, then incubated in anti-*Mr*IAG, diluted 1:2000 in 0.1 M PBS, for 1 h. The membrane was washed with PBST for 3 times (5 min each) and incubated with anti-rabbit IgG linked to AP, diluted 1:2000 with 0.1 M PBS, for

1 h. After further washings in 0.1 M PBS, the membrane was incubated in the detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5). The color was developed with the substrate solution NBT/BCIP (Roche, Mannheim, Germany), diluted in detection buffer, and then stopped by stop buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.1) for 10 min. The membrane was photographed using digital camera to observe anti-*Mr*IAG specific band.

#### 4.1.6 Detection of *Mr*IAG using immunofluorescence

Immunofluorescence detection was carried out using previous protocols (Phoungpetchara et al., 2011 and Tinikul et al., 2008) using rabbit antiserum against *Mr*IAG (anti-*Mr*IAG). Firstly, the anti-*Mr*IAG antiserum was pre-adsorbed with 20% BSA in 0.1 M PBS, pH 7.4, with gentle shaking at 4°C. AG sections were deparaffinized, rehydrated, and washed in 0.1 M PBS. They were then immersed in 1% glycine in 0.1 M PBS for 10 min, washed in PBS, and incubated with 10% NGS in PBST (0.1 M PBS with 0.4% Triton X-100), at room temperature, for 2 h. The sections were then incubated in BSA-adsorbed anti-*Mr*IAG (diluted 1:400 in 0.1 M PBS), at room temperature, overnight. In parallel, a negative control was performed by substituting the primary antibody with the pre-immune rabbit serum. All sections were further incubated with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, USA), diluted 1:500 in 0.1 M PBS, for 2 h. The nuclei of cells in the sections were counterstained with ToPro-3 (Molecular Probes), diluted 1:2000 in blocking solution, for 2 h. The sections were washed and mounted in Vectashield (Vector Laboratory, Burlingame, USA), and then viewed and photographed using an Olympus FV1000 confocal laser scanning microscope.

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nt 1      GGTTATTCCAAGAGGGGCCAAGACTCTGGGATCACACCTCGAACGGCTCTG
nt 52     TCCCTTCCCCTCGTCCGTTTAACCGGTGTTTTCTAGCCACGCTCTCAACAC
nt 103    CTAAAAATTCCCTCTCTTGCTTTCTGGCCAGCCTTGCAGTCATCCTTGAAA
nt 154    TTCCCTCTTCCTTATATTTCGGGACATAACATTCTTCTCTCCGGCCTTTTC
nt 205    ATATCGAAGTGAAACAAATCAACTACAGAATGGGATACTGGAATGCCGAGA
aa 1                                  M  G  Y  W  N  A  E  I
nt 256    TCAAGTGTGTGTTGTTCTGCTCACTCGTAGCATCGCTTCTCCCTCAACCTT
aa 9        K  C  V  L  F  C  S  L  V  A  S  L  L  P  Q  P  S
nt 307    CTTCGAGCTATGAGATCGAATGCCTCTCCGTTGACTTTGACTGCGGCGACA
aa 26       S  S  Y  E  I  E  C  L  S  V  D  F  D  C  G  D  I
nt 358    TAACGAACACCCTTGCCTCCGTCTGCCTGAGACACAACAACTACATCAACC
aa 43       T  N  T  L  A  S  V  C  L  R  H  N  N  Y  I  N  P
nt 409    CAGGACCCACCTACGTTTCCAAAGAGCGACGATCTGCTGACATCTATACCG
aa 60       G  P  T  Y  V  S  K  E  R  R  S  A  D  I  Y  T  V
nt 460    TTCCTTCTACGAAGTCTCCATCGCTCGCCCACCCGAGAGCTACCCACTTGA
aa 77       P  S  T  K  S  P  S  L  A  H  P  R  A  T  H  L  T
nt 511    CCATGGCTGACGAAGAAACTCAGAAGGTATCTAAGGTGGAGGAGGAGATTC
aa 94       M  A  D  E  E  T  Q  K  V  S  K  V  E  E  E  I  Q
nt 562    AGCACATGACGCTGAGCAGGGAAGAAGCGAACAATATGCTGCATTCGAAGC
aa 111      H  M  T  L  S  R  E  E  A  N  N  M  L  H  S  K  R
nt 613    GTCGCTTCCGGAGGGACAGCGTGAGGAGAAGTCCAAGGGAGGAATGCTGCA
aa 128      R  F  R  R  D  S  V  R  R  S  P  R  E  E  C  C  N
nt 664    ACAACGCCTCTTTCAGACGCTGCAACTTCGAGGAAGTCGCCGAATATTGCA
aa 145      N  A  S  F  R  R  C  N  F  E  E  V  A  E  Y  C  I
nt 715    TCGAGCTGCGTCCCGGCGTTAACACCTGCAGTTCCAGGTAGGAGGTCTCAA
aa 162      E  L  R  P  G  V  N  T  C  S  S  R  *
nt 766    GGATCATCCCGTCCCTGTCCTATACTTGACAGGAGATGCTCAAAGTCAAAT
nt 817    CACCGTCTTCGAGTCATGATGTGGAATGACCTTCAGCTAAAGCTGCCTTTT
nt 868    GGCTTTCCTCACAGTCAACTAAAAACAATTTTTTTTATCCTACCGTTACCT
nt 919    TCAGATAAATTATTCCTTTGTCTCAGCTTTAATTTCGGCTAAAGCTTTTTT
nt 970    TTTTTTCTACCCATGCATTCAGCTAAAGCTTTCTTTTGTTTCGCCTTTAAA
nt 1021   TTCAACACTCCTCTGCCTTACCCTTATTTCAGCTAATGGCTTCTTTTTATT
nt 1072   TTACCATTACCATCCACAAAGCTTTGTTTTGTCTTACCCTCAGCTGAAACG
nt 1123   TTTGTTTGTCTCACCTTTACCCTCAGCTAAAACTTTCTTTTGTCTTCCCGC
nt 1174   TGCTTTAGTAAATGCTTTCTTCTGTCACACTTTTACTTTCAGCTAGGGATT
nt 1225   CTTTTTTTTTTTTTGCCACTTTTACCTTCAGCTAAAGGGTACTATTGTCTC
nt 1276   ACCCTTGCCTTCTGCTAAAGGTTCCTTTTGTCCCACCCTTGCCTTCAACTA
nt 1327   AAGGTTCCTTTTGTCTCACCCTTGCCTTCAGCTAAAGGTTCCTTTTGTCTC
nt 1378   ACCCTTGCCTTCAGCTAAAGGTTCCTTTTGTCTCACCCTTGCCTTCAGCCA
nt 1429   AAGGTTCCTTTTGTCTCACCCTTGCCTTCAGCTAAAGGTTCCATTTGTCTC
nt 1480   ACCTTTGCCTTCAGCTAAAGGTTCCTTTTGTCTCACCCTTGCCTTCAGCTA
nt 1531   AAGGTCCCTTTTGTCTCACCCGTGCATCCAACTAAAGGTTCCTTTTACCTC
nt 1582   TCTTTTATCTTTAACTAAAGTTTTTTGTTTTTGTATCCTTGCCTTCAGCCA
nt 1633   AACGTTCTTTTGTTTTATCTTTACACGCAACAACATCTAGACATTTCCAAA
nt 1684   CATTAAGCATATTGCATTATTATTGGTGATTCTTGTCGATGTTTCCGAAAA
nt 1735   ATTGTTTGATACATCAGTTATACGTCAAATAAATGCTTTTGAGAACCCGGA
nt 1786   AAAAAAAAAAGAAAAAAAAAAAAAAAAAAAAAAAAA
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**Figure 4.2** Full-length of *Mr*IAG gene (GenBank accession no. FJ409645.1), the bold letters represent amino acid sequence of IAG protein

### 4.1.7 Estimation of *Mr*IAG levels by ELISA

Fresh EBs with attached AGs from each group (n = 8) were homogenized in a lysis buffer (2 M acetic acid). The combined tissues were sonicated and centrifuged at 8600*g* at 4°C, for 15 min. The supernatants were collected and then filtered through a 0.45 μm nylon membrane. Isolated proteins were measured by Lowry's method, using serial dilutions of BSA as a standard. The protein concentrations were measured at 750 nm in an automatic spectrophotometer to determine the *Mr*IAG levels.

An indirect ELISA was used to determine the relative amount of *Mr*IAG in the AG extracts. These extracts were diluted in a coating buffer (0.050 M $NaHCO_3$ in 0.1 M PBS, pH 9.6) to the same protein concentration (1.25 μg/ml) and 100 μl loaded into triplicate wells of 96 well plates, which were then incubated at 4°C, overnight. After washing with 0.1 M PBST (0.05% Tween-20 in 0.1 M PBS), the plates were blocked with blocking reagent (5% skim milk in 0.1 M PBST), at 37°C for 30 min, washed 3 times with PBST, and then incubated in BSA-adsorbed rabbit anti-*Mr*IAG (diluted 1:500 in 0.1 M PBS), at 37°C for 2 h. After washings, the plates were incubated with goat-anti rabbit-HRP (Southern Biotech, Birmingham, USA), diluted 1:6000 in 0.1 M PBS, at 37°C, for 1 h. The plates were washed again in PBST, and the color developed by adding TMB substrate for 15 min, and the reactions stopped by adding 1 M HCl. The OD's were read with a spectrophotometer (Eppendorf, Hamburg, Deutschland) at 450 nm.

An ELISA standard curve was performed using a serial dilution of *Mr*IAG synthetic peptide diluted in 2 M acetic acid in sterile $ddH_2O$ at $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, $10^{-10}$, $10^{-11}$, $10^{-12}$, $10^{-13}$, $10^{-14}$, $10^{-15}$ g/ml, and anti-*Mr*IAG provided by Dr. Ittipon Phoungpetchara (Phoungpetchara et al., 2011), to verify that the experimental OD values were within the linear section of the standard curve.

### 4.1.8 Statistical analyses

The ASI, ELISA OD's, and cell proliferation value of each group were expressed as mean ± S.E., and the data of experimental groups were then compared with those of the control (VC) using a one-way ANOVA (MU-SPSS version 18) and a post hoc Duncan's test to verify the differences. A probability value of less than 0.05 ($P < 0.05$) indicated a significant difference.

## 4.2 Temporal expressions of *MrIAG* gene in larvae and postlarvae, and the use this gene as a marker for selecting male offsprings

### 4.2.1 Animals and experimental design

The larvae at stage XI of *M. rosenbergii* (n = 10,000) were obtained from a commercial farm in Suphanburi province, Thailand. Two thousand larvae were maintained until they reach the PL stage in a circular plastic tank (0.5 m in diameter with a depth of water approximately 0.2 m) that contained brackish water (12-14 ppt). The prawns were fed with blend egg tofu (until 1-week-old) and artemia (after 1-week-old of PL stage) twice a day. After metamorphosis, new PL prawns from each day were collected manually by hand, transferred and maintained in the new containers (Figure 4.3).

In this experiment, we used the code $PLn_1Dn_2$ to determine the stage and age of PL such as the number after PL ($n_1$) represented the larvae that transformed into PL, D and the number after D ($n_2$) represented the age of the prawn after transformed into PL. For example, PL1D1 represented the first lot of larvae that become PL at day 1, or PL2D8 represented the second lot of larvae that become PL at day 8.

Forty PL of each stage and day were randomly collected and put in to each sterile microcentrifuge tube, frozen immediately in liquid nitrogen, and then stored at -80˚C. Other PL prawns of each stage and day were maintained in the containers until 4 month olds estimate the proportion of male prawns by to observing the male gonopores present at the base of the fifth walking legs.

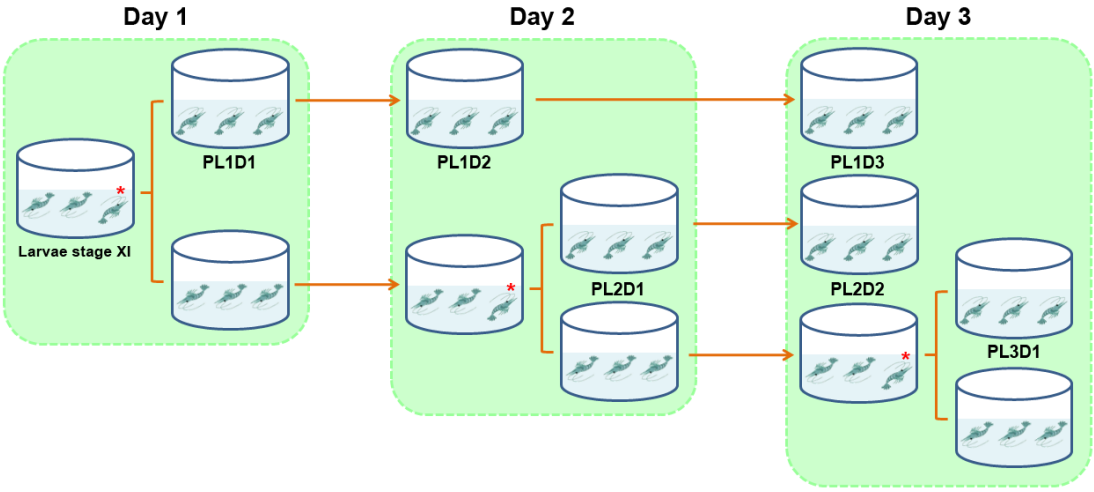


**Figure 4.3** A schematic diagram showing the grouping of PL prawns according to their flipping days. The number after PL represents the crop of larvae that transform into PL, the number after D represented the day (age) after flipping. * = flipping prawn.

### 4.2.2 RNA preparation

Both set of PL prawns, PL1D1-PL1D8 (n = 15) and PL1D8-PL4D8 (n = 15; 1 prawn per 1 reaction), were homogenized with 500 µl of Tripure reagent (Roche, Mannheim, Germany) at 4ºC, and then RNA prepared following Tripure reagent's protocol. After that, the dried RNA pellet was added with DEPC-treated water to 10 µl and stored at -80ºC until use. The amount and purity of RNA was measured by a Nanodrop ND 1000 (Nanodrop Technologies Inc., DE, USA). Then 1 µg of total RNA was treated with DNase I (Thermo Fisher Scientific, CA, USA) and converted into the cDNA with 100 ng of random hexamer primers (Thermo Fisher Scientific, CA, USA) by RevertAid Reverse Transcriptase (Thermo Fisher Scientific, CA, USA) following the recommended protocol.

### 4.2.3 Expression of *MrIAG* using RT-PCR

The cDNA were amplified using *MrIAG* or *β-actin* specific primers (Table 4.3) designed from the full sequences of *IAG* (GenBank accession no. FJ409645.1) (Figure 4.2) and *β-actin* (GenBank accession no. AY626840.1) genes of *M. rosenbergii* (Ventura et al., 2009; Zhu et al., 2005).

The PCR reaction mixture was composed of 17 µl $ddH_2O$, 2.5 µl 10X PCR buffer, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix, 1 µl forward primer, 1 µl reverse primer,1 µl cDNA, and 0.5 µl *Taq* DNA Polymerase (Thermo Fisher Scientific, CA, USA). The PCR cycle was started with 94ºC for 5 minutes, 40 cycles of 94ºC for 45 seconds, 55ºC for 45 seconds and 72ºC for 1 minutes, and finally 72ºC for 10 minutes and hold at 20 ºC. After that, the PCR products were separated with 1.5% agarose gel electrophoresis.

**Table 4.3** Primer sequences for RT-PCR

| Primer | Primer sequence |
|---|---|
| *MrIAG* | forward; 5′-GATCGAATGCCTCTCCGTTGACTT-3′<br>reverse; 5′-GCAATATTCGGCG ACTTCCTCGAA-3′ |
| *β-actin* | forward: 5′-GGTATCGTGCTCGACTCTGG-3′<br>reverse: 5′- CCAGAGTCGAGCACGAT ACC-3′ |

### 4.2.4 Gonopore complex observation

We cultured PL prawns, including PL1, Pl2, PL3, PL4 for 4 months to count the numbers of male and female within each lot. We separated each PL to 4 circular containers (0.5 m in diameter with a depth of water approximately 0.2 m) that contained freshwater. The prawns were fed with artemia (after 1-week-old of PL stage) until 1 month after which they were fed with commercial feed twice a day.

At the end of the experiment, we count the number of males by observing the gonopore complexes that appeared as small bulbs at the base of the fifth walking legs of male prawns, with naked eyes and took a picture by digital camera.

### 4.2.5 Statistical analysis

The percentage of male to female that is marked by *IAG* gene expression was showed as mean ± S.D., analyzed and compared to the controls by using Student's t-test, with significant difference at $P < 0.05$.

## 4.3 Changes of phosphatidylcholine and fatty acids in germ cells during testicular maturation in three developmental male morphotypes of *M. rosenbergii* revealed by imaging mass spectrometry

### 4.3.1 Animals and tissues preparation

The male giant freshwater prawns in each developmental morphotype (n = 30), namely SM, OC, and BC, were obtained from a commercial farm in Suphanburi province, Thailand. The prawns were anesthetized on ice for 2 min. The testes were dissected out and divided into two groups: (i) frozen immediately in liquid nitrogen and stored at -80˚C for lipid analysis, and (ii) fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; 0.033 M $NaH_2PO_4 \cdot 2H_2O$, 0.067 M $Na_2HPO_4 \cdot H_2O$, and 0.145 M NaCl), pH 7.4, embedded in paraffin for histological confirmation (5 μm).

### 4.3.2 Histology of the seminiferous tubules

The fixed tetes of the three male morphotypes were dehydrated with a serial dilution of ethanol i.e., 70%, 80%, 90%, and 100%, penetrated with melted paraplast, and then embedded in paraffin blocks. The embedded tissues were sectioned at 5 μm-thick with a rotary microtome (Leica RM2235), and then transferred to silane-coated slides (Sigma-Aldrich, Missouri, USA). The sections were firstly deparaffinized in xylene for three times, then rehydrated with 100%, 90%, 80%, 70% ethanol. The sections were washed with distilled water for 10 min, stained with hematoxylin for 1 min, washed with tap water for 15 min, and counterstained with eosin for 2 min, mounted in Permount (Bio-Optica, Milan, Italy), and then air dried at

the room temperature. The H&E stained sections were examined under a Nikon E600 light microscope and the images captured by a Nikon DXM digital camera using an ACT-1 program.

The frozen testes were divided into two equal parts for cryosection and lipid extraction (Figure 4.4). One part of each frozen tissue was attached to a specimen plate at the bottom using OCT compound (Optimum Cutting Temperature, Sakura Finetek 4583, Sakura, Tokyo, Japan), then sectioned (at ~10 µm) with a cryostat, CM 1950, (Leica Microsystems, Wetzler, Germany), after which sections were transferred to silane-coated slides (Sigma-Aldrich, Missouri, USA) for characterizing the stages of seminiferous tubules by hematoxylin and eosin (H&E) staining. The sections were dried using a hair dryer and stained with Mayer's hematoxylin solution for 10 min, washed with tap water, counterstained with eosin, and mounted by Permount (Bio-Optica, Milan, Italy). They were then examined under a Nikon E600 light microscope (Nikon, Tokyo, Japan), and images were captured by a Nikon DXM digital camera using an ACT-1 program.

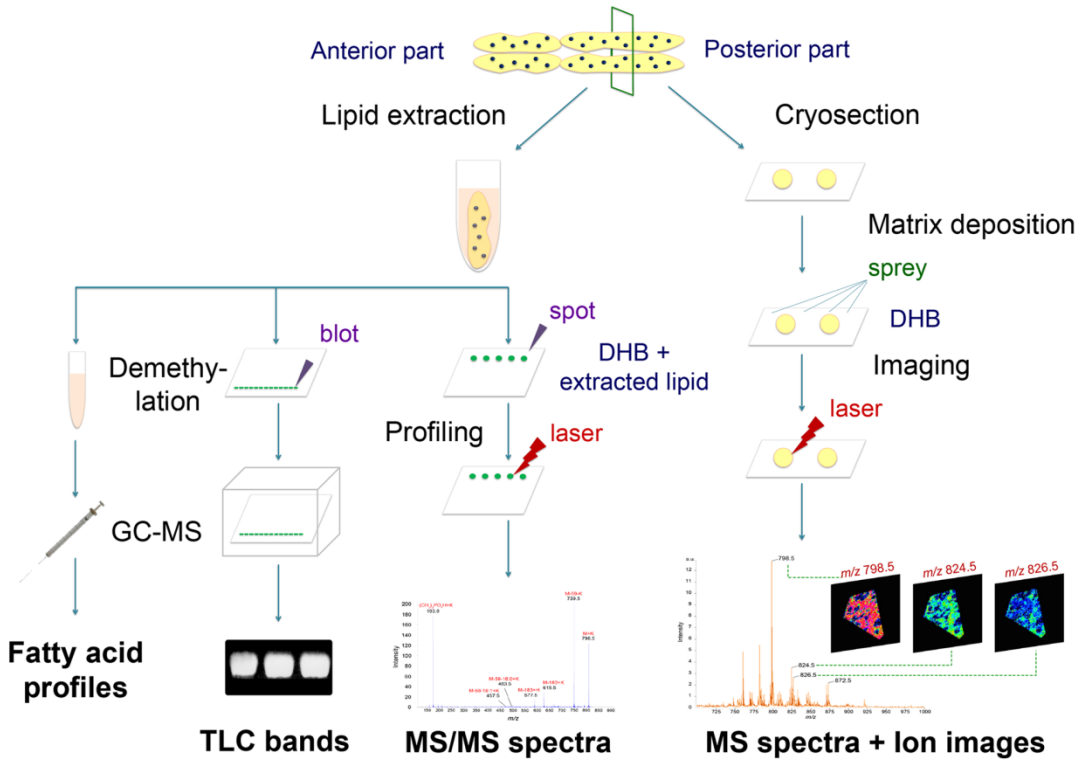


**Figure 4.4** Schematic diagram of the experimental design for lipid analysis.

### 4.3.3 Lipid extraction

The frozen testes of each group and stage were weighted, pulverized, and extracted with 0.1 g/ml of extraction solution (chloroform: methanol, 2:1 v/v) following the method described earlier (Goto-Inoue et. al., 2009; Chansela et. al., 2012). The samples were then sonicated for 10 s and stopped for 5 s, and this procedure was repeated 10-15 times using a Microson, Ultrasonic Cell Disruptor XL-2000 (Wakenyaku Co. Ltd., Kyoto, Japan). The glass tubes containing the sonicated tissues were tightly wrapped with parafilm, and then incubated overnight at room temperature. The samples were centrifuged at 3000 x*g* for 5 min to separate the tissue residues, and the solutions containing lipid were collected and transferred to new glass tubes, wrapped, and stored at -80˚C until being analysed.

### 4.3.4 Separation and quantification of lipids by thin layer chromatography (TLC)

The extracted lipids were separated with TLC using the method described earlier by our group (Goto-Inoue et. al., 2009; Chansela et. al., 2012). The solution containing extracted lipids (3 µl per sample) and the PC standard (Sigma-Aldrich, Missouri, USA) were spotted (with each spot being 5 x 1 mm in size) onto high performance thin layer chromatography glass plates (HPTLC silica gel 60 with the size 100 x 100 mm-Merck, Darmstadt, Germany), and dried at room temperature. Each HPTLC plate was immersed in a TLC chamber containing separation buffer (methylacetate, n-propanol, chloroform, and 0.25% KCl in the ratio 25:25:10:9 v/v/v/v). Each HPTLC plate was air-dried after separation, and then was sprayed with primuline reagent (Nacalai Tesque, Inc., Kyoto, Japan) composed of 1 mg of primuline in 100 ml of 80% acetone in water. After drying, the PC bands were visualized and photographed under UV light (FAS-III, Toyobo Co. Ltds, Osaka, Japan). The intensities of the bands were analysed by ImageJ software (http://rsbweb.nih.gov/ij/).

### 4.3.5 Identification of lipids by tandem mass spectrometry (MS/MS)

The extracted lipids from testicular tissues of each maturation group of STs and male morphotypes were thoroughly mixed at 1:1 v/v with matrix solution (20 mg/ml DHB in 70% methanol and 0.1% TFA). Aliquots of 1 µl of the solutions were applied manually to a stainless plate and air-dried with a hair dryer. A calibration process was performed using 10 pmol/µl bradykinin and 10 pmol/µl human angiotensin-II as standard peptides. The MS/MS analyses were performed using a QSTAR Elite high-performance, hybrid quadrupole TOF mass spectrometer (Applied Biosystems/MSD Sciex, Foster City, CA). The extracted lipids were ionized in the positive ion mode and fragmented with collision energy between 30-40 V. After being analysed, the precursor ions were identified based on neutral losses in the product ion spectra and confirmed by using Metabolite MS Search (http://www.hmdb.ca/spectra/ms/search).

### 4.3.6 Distributions of phosphatidylcholine by imaging mass spectrometry (IMS)

A part of each frozen testis (used for histology) was sectioned at 10 µm of thickness with a cryostat (CM 1950, Leica Microsystems). The sections were thaw-mounted onto indium tin oxide (ITO)-coated slides (Bruker Deltonics, Bremen, Germany), dried and then kept at -30˚C until IMS analysis. Before IMS analyses, the sections were dried at room temperature and then sprayed with matrix solution using a 0.2-mm nozzle caliber airbrush (Procon Boy FWA Platinum, Tokyo, Japan). The matrix used was 2,5-dihydroxybenzoic acid (DHB) (Bruker Daltonics), and it was firstly dissolved to reach a concentration of 50 mg/ml in 70% methanol and 0.1% trifluoroacetic acid (TFA). A calibration process was performed using 10 pmol/µl bradykinin and 10 pmol/µl human angiotensin-II as standard peptides by applying on to the sprayed area out of the tissue sections. The sprayed sections were then analysed in the positive ion mode using an ultraflex II MALDI TOF/TOF mas nics). The mass spectra were obtained in the mass ranges between *m/z* 500-1000. The settings of laser spectrometer (Bruker Deltonics) irradiation were 200 Hz in frequency and a raster width at 20 µm. After IMS analyses, ion images were obtained using FlexImaging 2.1 software (Bruker Daltonics). Finally, the analysed sections were stained with H&E to confirm the histology of the areas of interest.

### 4.3.7 Analyses of fatty acids by gas chromatography-mass spectrometry (GC-MS)

These analyses followed the methods described earlier by our group (Chansela et. al., 2012). The extracted lipids were spiked with an internal control (0.4 mg/ml arachidic acid (20:0) diluted in chloroform:methanol at a ratio of 2:1), and then dried by nitrogen gas using a TurboVap LV Evaporation System (Caliper Life Sciences, Hopkinton, MA, USA). After being completely dried, the lipids were methylated using a fatty acid methylation kit (Nacalai Tesque, Inc., Kyoto, Japan), and then purified using a fatty acid methyl ester purification kit (Nacalai Tesque, Inc.). The purified FAs were stored at -30˚C until analysed by GC-MS.

The purified FAs from testes of *each* morphotype were separately injected (1 µl per sample) into a GC-MS QP-2010 Plus (Shimadzu Co., Kyoto, Japan), applied to a DB-5MS column (3060.25 mm I.D., 0.25 mm; D.F., Agilent technologies, CA, USA). The purified FAs were analysed under a column temperature of 210˚C and column pressure between 110 kPa-380 kPa at 7 kPa/min. After analyses, the FAs were identified and the amount calculated using the internal controls as a reference.

### 4.3.8 Statistical analyses

The intensity of each band from TLC analyses and FAs amount of each testis stage and male morphotype from GC-MS analyses were expressed as a mean ± S.D. and the data was then compared using a Student's *t*-test to determine differences. A probability value of less than 0.05 ($P<0.05$) indicated a significant difference.