CHAPTER VI
DISCUSSION

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

In this study, we found new evidence that the AGs of *M. rosenbergii* undergo marked changes in histology and cell proliferation as well as in *Mr*-IAG production in response to the neurotransmitters, 5-HT and DA, and the neurohormones, GnRHs (l-GnRH-III and oct-GnRH) and Crz. In comparison with the control the AGs of prawns treated with 5-HT and l-GnRH-III increased in size, as shown by the ASI values. This is due in part to the increased proliferation of AG cells, as determined by BrdU labeling. Furthermore, there is an increased immunoreactivity against *Mr*IAG (*Mr*IAG-*ir*) in these AG cells, as well as *Mr*IAG production, as shown by ELISA. In contrast, the groups treated with DA and Crz showed opposite effects.

Previous studies have reported that 5-HT is stimulatory whereas DA is inhibitory to gonadal development in both sexes of decapods crustaceans, including *P. clarkii, U. pugilator, L. stylirostris, L. vannamei, P. monodon, and M. rosenbergii* (Kulkarni et al., 1992; Sarojini et al., 1993; Fingerman et al., 1994; Sarojini et al., 1994; Sarojini et al., 1995; Fingerman, 1997; Alfaro et al., 2004; Tinikul et al., 2008; Wongprasert et al., 2006; Poljaroen et al., 2011). In female *M. rosenbergii*, the 5-HT treated animals showed significantly increased ovarian-somatic index and shortening of the ovarian maturation cycle (Meeratana et al., 2006). Moreover, 5-HT injection also increased vitellogenin (Vg) concentration in the hemolymph of the treated prawns (Tinikul et al., 2008). Similarly, male *M. rosenbergii* treated with 5-HT exhibited increased testis-somatic index (TSI), diameter of seminiferous tubules (DST), and proliferation of germ cells in the seminiferous tubules (Poljaroen et al., 2011). In contrast, DA has opposite effects to 5HT in both males and females, suggesting that these two neurotransmitters play opposite roles in control during spermatogenesis and testicular maturation in males, as well as oogenesis and ovarian development in

females (Tinikul et al., 2008; Poljaroen et al., 2011). In the present study, we have also shown for the first time that 5-HT also affected the AGs positively in size, cell proliferation, and *Mr*IAG production, whereas DA had the opposite effects. It is possible that 5-HT suppresses gonad inhibiting hormone (GIH) in the eyestalk, allowing AG maturation and increased *Mr*IAG secretion, whereas DA has an opposite effect.

Although GnRH has not yet been identified in decapod crustaceans, there is strong evidence indicating that a homolog of this hormone may be present since GnRH-like immunoreactivity has been localized in the CNS and ovary of female *M. rosenbergii* (Ngernsoungnern et al., 2008), and isotypes of GnRH (i.e., l-GnRH-III and oct-GnRH) can also induce ovarian maturation in the same species (Ngernsoungnern et al., 2009). In male *M. rosenbergii*, l-GnRH-III and oct-GnRH administration can also increase TSI, DST, and germ cell proliferation (Poljaroen et al., 2011). In the present study, we found that injection of l-GnRH-III also increases the size of the AGs, cell proliferation in the AG, and *Mr*IAG production. However, we found that l-GnRH-III was more effective than oct-GnRH, so we hypothesize that the GnRH-like peptide in this species may be more similar to l-GnRH-III than oct-GnRH. It remains to be proved whether this hormonal factor exercises direct control over the testicular development and spermatogenesis, or acts through the AG which exercises control over these processes.

Unlike the GnRH homologs we tested, the neurohormone Crz negatively affected the AGs. Crz is classified as a member of the GnRH family because of the similarity of its amino acid sequence to other members (Cazzamali et al., 2002; Park et al., 2002; Christie et al., 2010; Kornthong et al., 2013). Initially, Crz was identified in insects and was suggested to have various functions, including control of heartbeat (Veenstra and Davis, 1993), and responses to stress (Boerjan et al., 2010). Recently, Crz has also been detected by MALDI-MS in the pericardial organ (PO) and commissural ganglia (CoG) of the Jonah crab, *C. borealis* (Ma et al., 2009) and in the brain of *L. vannamei* (Ma et al., 2010). Meanwhile, the functions of Crz in crustaceans remain unknown. We hypothesize that, Crz may exercise a direct inhibitory effect on the AGs, but this needs to be studied further. Furthermore, we found that

administration of Crz caused severe deterioration of health in prawns, as the animals treated with Crz died before day 16 of the experimental period.

In summary, we have found that 5-HT and l-GnRH-III have stimulatory effects on the AGs of small male *M. rosenbergii*, including increases of ASI, AGs cell proliferation and *Mr*IAG production. In contrast, DA and Crz have inhibitory effects on the AGs. From our results, we hypothesize that there may be two levels of controls over the AGs which control male reproduction in decapods crustaceans. The first level may be mediated by neurotransmitters including, 5-HT and DA, that may suppress or stimulate GIH synthesis and release in the eyestalk. The second level is that the GnRH-like factor and Crz that also exercise antagonist roles on the AG once the influence of GIH is removed. Whilst the l-GnRH-III can stimulate AG function, Crz may block the actions of endogenous reproductive stimulators, which include GnRH, to inhibit the AG function. This hypothesis needs to be studied further by measuring the gene expressions in direct responses to the stimulating hormones/factors.

6.2 Temporal expressions of *MrIAG* in larvae and post larvae, and the use this gene as a markers for selecting male offsprings

Male monosex culture is beneficial for freshwater prawn farming because male grows faster and larger than female (Sagi et al., 1986). Attempts have been made to obtain male prawn by AG manipulation, for example, the AG was removed (Charniaux-Cotton, 1962; Aflalo et al., 2006) or its IAG gene silenced by siRNA (Ventura et al., 2009; Ventura et al., 2012). Both methods cause sex reversal in male to become female which is called neo-female. Based on genetic knowledge of prawn, male contains two homologous sex chromosomes (ZZ) and female contained heterozygous sex chromosomes (ZW). This neo-female has ZZ genotype like normal male but it has female phenotypes. The cross breeding this neo-female (ZZ) with normal male (ZZ) were expected to yield all male offspring (Sagi and Cohen, 1990; Aflalo et al., 2006; Ventura et al., 2009; Ventura et al., 2012). However, both techniques are difficult to perform and, though theoretically sound, the results has not been uniformly successful and could still not be translated to practical usage. An alternative way is to use IAG gene as a male marker for selection of male PL prawns which is the aim of our study.

In Branchiopoda crustaceans such as *Daphnia magna*, environmental signals are more important than sex-specific genes or *Dsx1* and *Dsx2* (Kato et al., 2011). In contrast, malacostracan crustaceans, the sex-specific gene or *IAG* expressed by an AG is the most important factor in controlling male differentiation and it is known to be a male specific gene in most species including *Porcellio scaber*, *P. dilatatus*, *C. quadricarinatus*, *M. rosenbergii*, *P. monodon* (Ohira et. al., 2003; Manor et. al., 2007; Ventura et al., 2009; Mareddy et al., 2011). In Cherax spp., the expression of *CqIAG* was detected in male juvenile at 8 days after being released from their mother (Manor et. al., 2007). In *M. rosenbergii*, it has been reported that the earliest time of expression was in PL20 or 60 days after fertilization, the time which male or female differentiation were thought to be clearly defined (Ventura et al., 2011). In contrast to earlier reports, we found that the IAG gene started to express in PL1D1 (at the first day after flipping) or ~20-25 days after hatching (Figure 5.14). Therefore, we thought it might be useful for marking and selecting male prawns at very early stage during the first week of flipping. We found that the prawn of PL1D8

group were over 80% male whereas PL2D8 and PL3D8 have 33.33% male. This result is in agreement with the earlier studies by Howlader and Kiortsis (1978) who found high percentage of male PL prawns collected at the initial stage of metamorphosis (Howlader and Kiortsis, 1978). We have verified the higher percentage of males among the early flippers by culturing the PL prawns collected at different flipping time until they reach young adult (up to 4 months) when their external male morphology could be clearly identified. The male gonopores and appendix musculina appear in male *M. rosenbergii* during PL20-PL120 (Ventura et al., 2011). Using these morphological criteria, we confirm that the percentage of male in the earliest flippers (PL1) was more than 80%, and all of these prawns have male gonopore and appendix musculina (Figure 5.17; Table 5.1). Therefore, these results supported our hypothesis that the early flipping prawns are mostly male. This could be because male PL may have stronger muscular activity than female which may help them to undergo flipping earlier than female PL prawns.

Our study offers alternative way to select male prawns by separating them early in the mixed culture system. We recommend that the prawns at PL1 and PL2 have the highest possibility to being male. This technique provides a simple and convenient way to preferentially collect male PLs for monoculture in the farm where farmers need no special skill.

6.3 Changes of phosphatidylcholine and fatty acids in germ cells during testicular maturation in three developmental male morphotypes of *M. rosenbergii* revealed by IMS and GC-MS

This study, using IMS and related techniques, is the first to show the localization and composition of PCs and FAs, especially HUFA and PUFA, in three maturing ST groups of the three male morphotypes of *M. rosenbergii*. We focused on the changes of PCs and FAs during ST maturation, and developmental stages of male morphotypes during maturation from young SM to mature BC (Poljaroen et al., 2011). We found that (1) STs of group B had higher amounts of PCs and FAs than groups A and C; (2) OC males contain higher amounts of PCs and FAs (except EPA) than the SM and BC; (3) SM males contain high ratios of HUFAs and PUFAs; (4) EPA is always higher than DHA in all ST groups, and in all male morphotypes; and (5) the PCs identified in the testes of this species were considerably higher in developing germ cells and the IT.

The composition of total lipids in each developmental morphotype of *M. rosenbergii* has been found to be different (Kumar, 1988). In particular, it was found that the total lipids in the hepatopancreas, a major energy storage organ in crustaceans, to be highest in OC males and lowest in mature BC males. This result supports our TLC results that showed trends of PC amounts in the three developmental morphotypes (Figure 5.19 B). The OC males are reproductively less active than BC males, but are growing more rapidly than young SM and mature BC males (Ra′anan and Sagi, 1985; Kuris et al., 1987; Sagi and Ra'anan, 1988). Surprisingly, in OC males the group B STs with differentiating Sts had higher levels of PCs than group C STs that contain only spermatozoa (Figure 5.19 A). It was reported that the decrease of lipid levels in the testes of BC males may relate to germ cell developmental processes in which there is an extrusion of numerous cytoplasmic components including lipids as they become mature spermatozoa (Ra′anan and Sagi, 1985; Kuris et al., 1987; Sagi and Ra'anan, 1988). This also supports our results as the lowest amounts of PCs and FAs were detected in the testes of BC males Figures 5.19 and 5.26.

IMS is a powerful technique to reveal the location of the lipids such as PCs, phosphatidylinositols, phosphatidylethanolamines, seminolipids, and TAGs in

the reproductive organs of mice and shrimps, without contamination that may be introduced with embedding media (Chansela et al., 2012; Goto-Inoue et al., 2009). Recently, Goto-Inoue (2012) reported that lipids changed during testis maturation in mice, especially lipids in the positive ion mode detected in the range of *m/z* 700-900, with substantial signals corresponding to PCs. The highest intensity of these was found at *m/z* 798.5 [PC (16:0/18:1) + K]$^+$ (Goto-Inoue et al., 2012). Moreover, Chansela (2012) reported that there were relatively large amounts of PCs in the ovary of *P. merguensis* (Chansela et al., 2012). Our IMS results of the testis of *M. rosenbergii* showed numerous signals corresponding to PCs, which included HUFA-containing PCs with *m/z* 820.5 [PC (16:0/20:4 (ARA)) + K]$^+$, *m/z* 826.5 [PC (18:2/20:5 (EPA)) + Na]$^+$, *m/z* 828.5 [PC (16:0/22:6 (DHA)) + Na]$^+$, *m/z* 832.5 [PC (18:0/20:4 (ARA)) + Na]$^+$, *m/z* 844.5 [PC (16:0/22:6 (DHA)) + K]$^+$, *m/z* 846.5 [PC (18:0/20:5 (EPA)) + K]$^+$, *m/z* 870.5 [PC (18:1/22:6 (DHA)) + K]$^+$, and *m/z* 872.5 [PC (18:0/22:6 (DHA)) + K]$^+$. These signals were present in the IT and developing germ cell areas, including Sg, Sc, and St, but not in spermatozoa (Figures 5.23, 5.24, 5.25). However, the compositions of PLs in each type of developing male germ cells were found to be different. In mammals, PUFA and the major HUFA (namely DHA) accumulated at highest levels in cell membranes of male germ cells, and were essential for male fertility (Martinez and Morros, 1996; Oresti et al., 2010). In rats, the spermatids contain more docosapentaenoic acid (DPA)-containing phospholipids than spermatocytes (Beckman et al., 1978), indicating that there are species differences in the types of lipids, and qualities of developing male germ cells, implying their importance during differentiation.

Our GC-MS analysis showed the highest amount of all FAs, including 14:0, 15:0, 16:0, 17:0, 18:0, 16:1, 18:1, 18:2 (linoleic acid), 20:1, and 20:2, in the STs of OC males. Furthermore, we showed that the EPA level was higher than that of DHA in all male morphotypes, of *M. rosenbergii*. Notable increase of HUFA including DPA also occur during the maturation of testes in cattle (Ahluwalia and Holman, 1966), rats (Ewing et al., 1966), hamster (Bieri and Prival, 1965), mouse (Bieri and Prival, 1965), guinea pig (Bieri and Prival, 1965), dog (Bieri and Prival, 1965), boars (Evans and Setchell, 1979a), rams (Evans and Setchell, 1979b), and monkeys (Lin et al., 2004), as this may be related to sperm high mobility facilitated by

the more fluid membrane. The levels of FAs in *M. rosenbergii* testes were considerably lower compared with the ovaries of *M. rosenbergii* (Cavalli et al., 2001) and *P. merguensis* (Chansela et al., 2012). We suggest that these different levels of FAs may be related to a greater lipid requirement by oogenesis. Furthermore, the levels of HUFA in the male of this species are decreasing in the testes of the blue claw males which contained mostly mature sperm cells with small numbers of early germ cells (Sg, Sc). In SM and OC, the testes contain large amounts of developing germ cells in the spermatogenic zone (in STs of groups A, B), which is highly active in spermtogenesis (Sagi et al., 1988). After maturation, the testes of BC males contain much thinner spermatogenic zone, but lager area of mature Sz, thus the STs function at this stage is more in the storage of Sz rather than producing Sz (Sagi et al., 1988). In addition the Sz of this prawn are immobile due to the lack of tail and the nuclear chromatin is totally decondensed (Poljaroen et al., 2011). They are thus relatively inert compared to the mammalian sperm. It is possible that their membranes are less fluid and need much less HUFA when they reach complete maturity as compared to mammalian sperm.

Finally, we recommend that diets containing lipids with high levels of HUFA, PUFA, especially EPA and DHA, should be given to the SM males for improving germ cell development and increase energy accumulation to shorten their developmental processes. This knowledge could be useful in formulating suitable nutrition to each male morphotype broodstock of *M. rosenbergii*, which is important commercial species in freshwater prawn farming countries.