ผลของฮอร์โมนต่อการแสดงออกของจีนที่เกี่ยวข้องกับการสืบพันธุ์ของกุ้งกุลาดำ *Penaeus monodon*

นางสาวกิรติ์กานต์ กีรติวานิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF HORMONES ON EXPRESSION OF REPRODUCTION-RELATED GENES OF THE GIANT TIGER SHRIMP *Penaeus monodon*

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A Thesis Submitted in Partial Fulfillment of the Requirements
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กิรติ์กานต์ กีรติวานิชย์ : ผลของฮอร์โมนต่อการแสดงออกของจีนที่เกี่ยวข้องกับการสืบพันธุ์ของกุ้งกุลาดำ *Penaeus monodon*. (EFFECTS OF HORMONES ON EXPRESSION OF REPRODUCTION-RELATED GENES OF THE GIANT TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เผดิมศักดิ์ จารยะพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ศิราวุธ กลิ่นบุหงา, 141 หน้า.

องค์ความรู้เกี่ยวกับกลไกและหน้าที่ของจีนที่เกี่ยวข้องกับการพัฒนารังไข่มีความจำเป็นต่อความเข้าใจกระบวนการสมบูรณ์พันธุ์ของกุ้งกุลาดำในภาวะเลี้ยง จึงค้นหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของจีน *ring finger protein 121* (*PmRnf121*) ตรวจสอบผลของฮอร์โมนและสารสื่อประสาทต่อการแสดงออกของจีน *PmRnf121*, *non-receptor tyrosine kinase* (*PmnRTK*) และ *receptor tyrosine kinase* (*PmRTK*) โดยพบลำดับนิวคลีโอไทด์ที่สมบูรณ์ของ *PmRnf121* มีขนาด ORF ยาว 1023 คู่เบส สามารถแปรรหัสเป็นโปรตีนยาว 341 กรดอะมิโน นอกจากนี้ได้แยกลำดับนิวคลีโอไทด์ของ *PmnRTK* ซึ่งมีขนาด ORF บางส่วนยาว 587 คู่เบส แปรรหัสเป็น 195 กรดอะมิโน

ตรวจสอบระดับการแสดงออกของจีน *PmRnf121*, *PmnRTK* และ *PmRTK* ด้วยวิธี quantitative real-time PCR พบว่า *PmRnf121* มีระดับการแสดงออกที่ไม่แตกต่างกันระหว่างการพัฒนาของรังไข่ในกุ้งเต็มวัยธรรมชาติปกติ ($P > 0.05$) ในขณะที่ *PmnRTK* และ *PmRTK* มีระดับการแสดงออกที่แตกต่างกันระหว่างการพัฒนารังไข่ ($P < 0.05$) โดยการตัดก้านตาไม่ส่งผลต่อการแสดงออกของจีน *PmRnf121* ($P > 0.05$) แต่กระตุ้นการแสดงออกของ *PmnRTK* ในรังไข่ระยะที่ 4 และ *PmRTK* ในรังไข่ระยะที่ 4 และหลังวางไข่ ($P < 0.05$)

ศึกษาผลของการฉีดกระตุ้นด้วยฮอร์โมนและสารสื่อประสาท ต่อระดับการแสดงออกของจีนในกุ้งที่ปรับปรุงพันธุ์พบว่า การฉีดกระตุ้นด้วยซีโรโทนิน (50 มิลลิกรัม/กรัมน้ำหนักตัว) ไม่มีผลต่อระดับการแสดงออกของจีน *PmRnf121* ($P > 0.05$) แต่มีผลต่อระดับการแสดงออกที่เพิ่มขึ้นของจีน *PmnRTK* และ *PmRTK* อย่างมีนัยสำคัญทางสถิติในชั่วโมงที่ 6 และ 48 หลังจากการฉีดตามลำดับ ($P < 0.05$) ในขณะที่การฉีดกระตุ้นด้วยโปรเจสเตอโรน (0.1 ไมโครกรัม/กรัมน้ำหนักตัว) ไม่มีผลต่อระดับการแสดงออกของ *PmRnf121*, *PmnRTK* และ *PmRTK* ($P < 0.05$) แต่การฉีดกระตุ้นด้วย 17β-estradiol (0.01 ไมโครกรัม/กรัมน้ำหนักตัว) ส่งผลให้ *PmRnf121* มีระดับการแสดงออกสูงขึ้นในวันที่ 28 หลังจากได้รับการฉีดครั้งแรก เมื่อเปรียบเทียบการกลุ่มควบคุม ($P < 0.05$) โดยการฉีด 17β-estradiol ไม่ส่งผลต่อระดับการแสดงออกของจีน *PmnRTK* และ *PmRTK* นอกจากนี้พบว่ากุ้งที่ได้รับการตัดตามีระดับการแสดงออกของ *PmnRTK* และ *PmRTK* มากกว่ากลุ่มควบคุมในวันที่ 28 หลังจากการตัดตา ($P < 0.05$)

การให้อาหารผสมด้วย 17β-estradiol (1 และ 10 มิลลิกรัม/กิโลกรัมอาหาร) ส่งผลต่อระดับการแสดงออกของจีน *PmRnf121* และ *PmRTK* ในวันที่ 28 และ 35 หลังจากการทดลอง เมื่อเปรียบเทียบกับกลุ่มควบคุม ($P < 0.05$) แต่ทำให้ *PmnRTK* มีระดับการแสดงออกลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุม ในวันที่ 7 และ 35 หลังจากการทดลอง ($P > 0.05$)

ทำการสร้างรีคอมบิแนนท์โคลนสำหรับ *PmRnf121* และ *PmRTK* ไม่พบการแสดงออกของโปรตีนลูกผสม rPmRnf121 แต่พบการแสดงออกของ rPmRTK (23.83 kDa) ในรูปแบบของโปรตีนที่ไม่ละลายน้ำ ผลจากการทำ Western blot โดยใช้ anti-Rnf121 MAb จากการค้าให้แถบขนาด 58 kDa ซึ่งต้องทำการยืนยันความถูกต้องของผลการทดลองอีกครั้งด้วยวิธีแมสสเปคโตรเมทรี

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KIRAKARN KIRATIVANICH: EFFECTS OF HORMONES ON EXPRESSION OF REPRODUCTION-RELATED GENES OF THE GIANT TIGER SHRIMP *Penaeus monodon*. ADVISOR: ASSOC. PROF. PADERMSAK JARAYABHAND, Ph.D., CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D., 141 pp.

Knowledge on molecular mechanisms and functional involvement of reproduction-related genes in ovarian development are necessary for better understanding the reproductive maturation of *P. monodon* in captivity. Here is the full-length cDNA of *P. monodon ring finger protein 121* (*PmRnf121*). Effects of hormone and neurotransmitter administration on expression levels of *PmRnf121*, *non-receptor tyrosine kinase* (*PmnRTK*) and *receptor tyrosine kinase* (*PmRTK*) were also examined. The full-length cDNA of *PmRnf121* was characterized by RACE-PCR and it contained a complete ORF of 1023 bp in length deducing to a polypeptide of 341 amino acids. The partial cDNA sequence of *PmRTK* containing the ORF of 587 bp in length deducing to 195 amino acids was also isolated.

The expression levels of *PmRnf121*, *PmnRTK* and *PmRTK* during ovarian development were examined by quantitative real-time PCR. In intact broodstock, *PmRnf121* was not differentially expressed during ovarian development of wild *P. monodon* ($P > 0.05$) but *PmnRTK* and *PmRTK* were differentially expressed during ovarian development of wild *P. monodon* ($P < 0.05$). Eyestalk ablation did not affect the expression level of *PmRnf121* ($P > 0.05$) but promoted the expression level of *PmnRTK* in stages IV and post-spawning ovaries and that of *PmRTK* in the post-spawning ovaries ($P < 0.05$).

Effects of exogenous injection of hormones and neurotransmitters were determined. Serotonin injection (50 mg/g BW) did not affect the expression level of *PmRnf121* ($P > 0.05$) but resulted in up-regulation of *PmnRTK* (at 6 hpi) and *PmRTK* (at 6 and 48 hpi) ($P < 0.05$). Progesterone (0.1 μg/g BW) did not affect to expression levels of these genes ($P > 0.05$). Exogenous injection of 17β-estradiol (0.01 μg/g BW) increased the expression levels of *PmRnf121* at 28 dpi compare to the negative control ($P < 0.05$) but it did not affect *PmnRTK* and *PmRTK* expression ($P > 0.05$). Interestingly, the expression levels of *PmnRTK* and *PmRTK* in eyestalk-ablated group were significantly induced at 28 dpi ($P < 0.05$).

Moreover, shrimp were fed with diets supplemented with 17β-estradiol (1 and 10 mg/kg) and the diets affected the expression levels of *PmRnf121* and *PmRTK* at 28 and 35 days of treatment compared to the negative control ($P < 0.05$). In contrast, the expression level of *PmnRTK* was lower than that of the negative control at 7 and 35 days of treatment ($P > 0.05$).

Recombinant clones for expression of *PmRnf121* and *PmRTK* were constructed. The former was not expressed but the latter was expressed in the insoluble form in *E. coli* (23.83 kDa). Western blot analysis of total ovarian proteins was carried out against commercially available anti-Rnf121 MAb but results need to be confirmed by mass spectrometry of the positive immunoreactive band (58 kDa).

Field of Study: Biotechnology
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVATIONS

bp	Base pair
°C	Degree Celsius
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dpi	Days post injection
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
EtBr	Ethidium bromide
HCl	Hydrochloric acid
hpi	Hours post injection
IPTG	Isopropyl-thiogalactoside
kDa	Kilodalton
kg	Kilogram
M	Molar
mg	Milligram
mRNA	Messenger-Ribonucleic acid
ml	Millilitre
mM	Millimolar
ng	Nanogram
OD	Optical density

PCR	Polymerase chain reaction
pI	Isoelectric point
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
Tm	Melting temperature
Tris	Tris (hydroxy methyl) aminomethane
U	Unit
UV	Ultraviolet
w/v	Weight/volume
µg	Microgram
µl	Microlitre
µM	Micromolar

CHAPTER I
INTRODUCTION

1.1 Background information

The black tiger shrimp (*Penaeus monodon*) is the one of important economically culture species of Thailand. Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment. Farming of *P. monodon* relies almost entirely on wild-caught broodstock for supply of juveniles because of reproductive maturation of domesticated shrimp is quite low. The high demand on wild female broodstock leads to overexploitation of natural broodstock. The closed-life cycle culture is required for the sustainable aquaculture.

The shrimp industry in Thailand has faced the problem in cultivation including, lacking of high quality broodstock, diseases and slow growth of shrimp. Therefore, the production has continuously decreased in the last few years and the cultured shrimp species is almost entirely replaced by the Pacific white shrimp (*Litopenaeus vannamei*). Therefore, these problems should be resolved for sustainable aquaculture of *P. monodon* in Thailand.

The reproductive maturation of shrimp was regulated by the gonad inhibiting hormone (GIH) producing from the X-organ/sinus gland located at the eyestalk. Unilateral eyestalk ablation is used to induce ovarian maturation of *P. monodon* broodstock. Although the ovarian development of shrimp is activated, this technique leads to an eventual loss in egg quality and death of spawner. (Benzie, 1998; Okumura, 2004; Okumura *et al*., 2006) Therefore, the alternative method, using hormonal and neurotransmitter, without the use of eyestalk ablation is an ultimate goal for this industry (Quackenbush, 2001). Understanding the molecular functions of reproduction-related genes that are differentially expressed during ovarian maturation will be useful to increase the culture efficiency of this economically important species. During oogenesis in eukaryotes, oocytes are naturally arrested at

prophase I (Okano-Uchida *et al.*, 1998). Oocyte maturation is resumed by a specific hormone, such as progesterone in lower vertebrates that signals the oocyte to undergo germinal vesicle breakdown (GVBD). The signal transmission involving multiple pathways related to phosphorylation of cell cycle regulating proteins is a prerequisite for the occurrence of GVBD.

Ring finger 121 (Rnf121) functions as E3 ubiquitin protein ligases in the ubiquitination that is the post-translational modification process. Both non-receptor tyrosine kinase and receptor tyrosine kinase involve in phosphorylation that regulated several cellular mechanisms. To better understand molecular aspects of *P. monodon Rnf121*, non-receptor tyrosine kinase (*PmnRTK*) and receptor tyrosine kinase (*PmRTK*) in reproductive development and maturation of *P. monodon*, their cDNA sequence was characterized. The effect of eyestalk ablation, hormonal induction and neurotransmitter injection on expression levels of ovarian *PmRnf121*, *PmnRTK* and *PmRTK* in *P. monodon* broodstock was examined.

1.2 Objectives

1.2.1 Identification, characterization and expression analysis of reproductive-related genes of *P. monodon*

1.2.2 Determination of effects of hormone (progesterone and 17β-estradiol) and neurotransmitter (serotonin) injection and diets supplemented with 17β-estradiol on expression of reproductive-related genes of *P. monodon*

1.3 Literature review

1.3.1 General introduction

Thailand is one of the world's leaders in shrimp exports. Shrimp farms and hatcheries are located along the coastal areas of Thailand where Nakorn Sri Thammarat and Surat Thani are the major parts of shrimp cultivation. In addition,

Chanthaburi (eastern Thailand), Samut Sakhon and Samut Songkhram (central region) also significantly contribute on the country production.

In Thailand, *P. monodon* had been intensively cultured for more than two decades and formerly, had contributed approximately 60% of the total cultivated shrimp production. However, the production of *P. monodon* has been dramatically decreased since the last several years. Thai shrimp farmers have faced the outbreak of diseases. Much of decline in production of *P. monodon* could be attributed by yellow-head virus (YHV) and white spot syndrome virus (WSSV) diseases. Moreover, breeding of pond-reared *P. monodon* is difficult and rarely produced the sufficient amount and quality of larvae required by the industry. Therefore, farming of *P. monodon* relies almost entirely on wild-caught broodstock for the seed supply. This resulted in overexploitation of natural stock leading to the lack of high quality broodstock of *P. monodon*.

As a result, domesticated *L. vannamei* has been introduced to Thailand as a new cultured species and initially contributed approximately 20,000 MT of the cultured production in 2002 and dramatically increased to nearly 500,000 MT in 2006. At present, *L. vannamei* is the main cultured species in Thailand. The production of *P. monodon* and *L. vannamei* since January to October 2012 is shown by

Currently, *L. vannamei* accounts for approximately 98% of the total shrimp production in Thailand. However, the price of *L. vannamei* is quite low and broodstock used relies almost entirely on genetically improved stocks brought from different sources. In addition, the labor costs in Thailand are higher than other countries (e.g. Vietnam and China) preventing the advantage of competition for the world market. In contrast, the market of premium-sized *P. monodon* is still opened for Thailand because *L. vannamei* is not suitable for that market. Accordingly, *P. monodon* culture is currently promoted for increasing its farming production.

Table 1.1 Total production and value of *P. monodon* compare to *L.vannamei* during 2005-2011 of Thailand

Year	*P. monodon*		*L.vannamei*	
	Production unit (1,000 tons)	Value (million Baht)	Production unit (1,000 tons)	Value (million Baht)
2005	29.2	4982.0	374.5	41844.7
2006	17.4	2769.0	480.0	48962.6
2007	17.0	2369.3	508.5	46939.8
2008	7.8	1572.8	501.4	50176.8
2009	6.1	1311.9	571.2	60646.8
2010	7.1	1223.9	553.9	59086.0
2011	7.9	1500.9	603.2	75059.1

Source: Research and statistic analysis bureau, Information center of fisheries, Department of fisheries (Fisheries, 2009)

1.3.2 The biology of the giant tiger shrimp

The giant tiger shrimp is the biggest species in Penaeidae family, reaching 330 mm with a maximum total length of 336 mm. The body weight is approximately 130 grams. It generally dark brown colored, with the carapace and abdomen transversely banded with black and white. The rest of the body is variable, ranging from light brown to blue or red (Dore and Frimodt, 1987).

The external morphology is divided into a cephalothorax and a six-segmented abdomen. Most organs located in the cepharothorax region such as heart, gill and digestive system, also including spine called rostrum, one pair of eyes, two pairs of antennae, three pairs of maxillipeds for feeding and five pairs of walking legs. In the latter, five segments has a pair of fins called pleopods using for forward swimming, while the last segment called telson, help shrimp to kick or jump backward. (Figure 1.1)

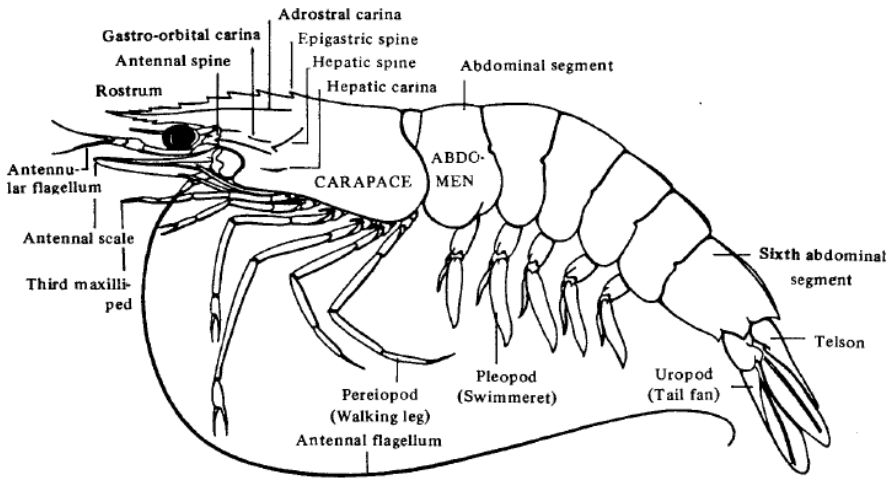


Figure 1.1 External anatomy of *P. monodon* (Motoh, 1981)

1.3.2.1 Distribution and life cycle

Penaeus monodon is found in the Indian Ocean and Indo-West Pacific and distributes to East and Southeast Africa, Northern and Eastern Australia, Japan, Pakistan, the Malay Archipelago and Thailand (Dore and Frimodt, 1987; Holthuis, 1980). It lives in mud and sand at 0 – 110 meters depth, water temperature around 18 – 34.5 °C and salinities of 5 – 45 ppt. (Branford, 1981; Chen, 1990).

1.3.2.2 Taxonomy

The giant tiger shrimp is a member of Superfamily Penaeoidae (de Freitas, 1987). Taxonomical recognition of the giant tiger shrimp is illustrated below.

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Order Decapoda

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1815

Genus *Penaeus* Fabricius, 1798

Subgenus *Penaeus*

Species *Penaeus monodon*

The life cycle of shrimp includes several distinct stages that are found in various habitats. Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities around 20 – 40 meters depth, where mating and reproduction takes place. Females produce as many as 500000 - 750000 eggs (Rosenberry, 1997). The maximum eggs were recorded is more than 1000000. The eggs hatch into the first larval stage, which is the nauplius. The nauplii have unsegmented spider-like body and they feed on their egg-yolk reserves. After undergoing six molting stages, nauplii metamorphose into protozoea. The protozoeae starting feed on algae and found distinct cephalothorax and feathery appendages. After 3 – 5 days, the protozoeae metamorphose into mysis that look similar to juveniles shrimp. They feed on algae and zooplankton around 3 – 4 days, and then metamorphose into post-larvae (PLs). The post-larvae move towards the coast, entering estuaries and mangrove swamps that serve as nursery grounds. They feed on zooplankton and detritus, and their pleopods are fully functions. All developing

steps are used for eight or nine days and were shown in Figure 1.2 (Jiravanichpaisal *et al.*, 2007).

A B

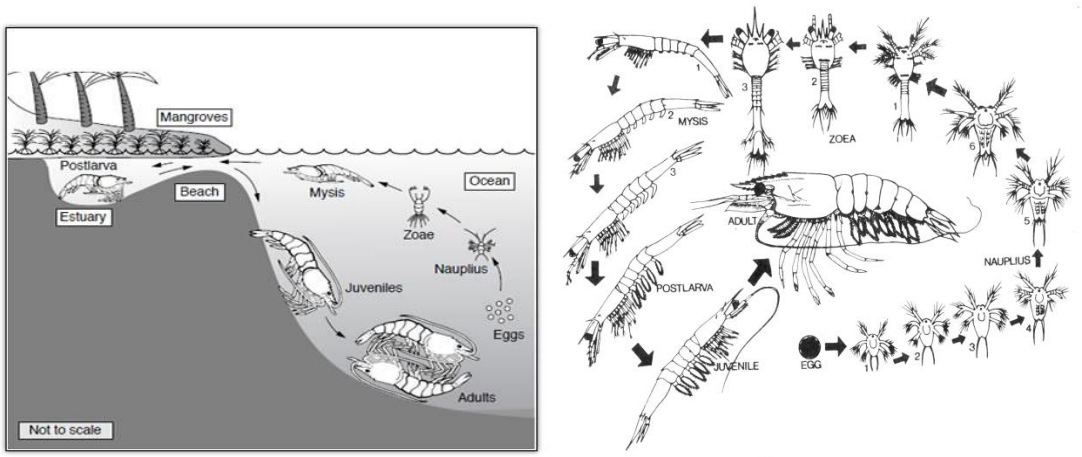


Figure 1.2 (A) The life cycle of *P. monodon* (Rosenberry, 2009). It started after the mating of adults at the deep offshore. The eggs hatch into larvae that metamorphose to nauplii, protozoea, mysis and post-larvae, respectively, and then move toward the estuaries for nursery. (B) The characterization of larvae metamorphosis composes of six nauplii, three protozoea and three mysis (Platon, 1978)

1.3.2.3 Ovarian development stage

Reproductive system of female *P. monodon* consists of pairs of ovaries, pairs of oviducts and thylecum. The thylecum is external organ and situates between the 4th and 5th pleopods. The oviducts are short and narrow tubes. The ovaries are found within the cephalothorax region with a slender anterior lobe, and five finger-like lateral projections and locate bilaterally symmetrical bodies extending in the mature female for almost its entire length, from the cardiac region of the stomach to the anterior portion of the telson (Figure 1.3). The anterior lobes locate closely to esophagus and cardiac region of the stomach (King, 1948). Ovarian development stages are classified to 5 stages depend on color changes and size that

the different pigment of egg mass and density of ovaries can visualize externally through the dorsal exoskeleton (Motoh, 1981; Santiago, 1977; Tan-Fermin and Pudadera, 1989).

The ovarian development of *P. monodon* is divided in 4 stages according to its histological features and germ cell association as shown in Figure 1.4. It consists of stages I, II, III and IV ovaries. The post-spawning stage may be recognized as stage V ovaries.

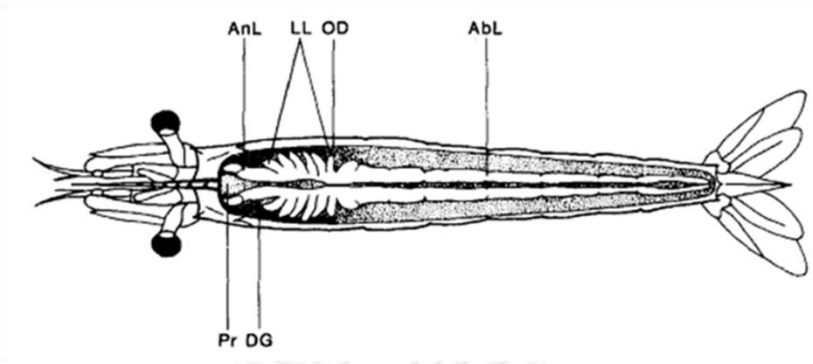
Stage I (Immature stage): The ovaries are thin, translucent, unpigmented and confined to the abdomen which they are invisible from dorsal exoskeleton. The ovary is composed of a central core of germinal cells, and a layer of sheath cells. The ovaries contain oocytes and small spherical ova. The ova cover with a layer of follicle cells and are small (Figure 1.4A).

Stage II (Developing stage): The ovaries appear thin and increase in size. The anterior and middle lobes are developing through the thoracic region. The dorsal surface is light yellow to yellowish green. Oogonial multiplication occurs at this stage. The developing ova are clearly larger than the immature and have yolk granule (Figure 1.4B).

Stage III (Nearly ripe stage): The ovaries are thicker and light green that are visible through the exoskeleton. The anterior and middle lobes are fully developed to posterior thoracic region. A butterfly shape can be seen at the anterior segment. The opaque ova are maturing (Figure 1.4C).

Stage IV (Mature stage): This is the final stage before spawning. The ovaries are dark green with butterfly shape expand entire the body captivity that they can clearly be seen from exoskeleton. The ovaries are firm and granular in texture with clumps of eggs. The ova are larger than other stages and are rod-like peripheral bodies that radiate from the opaque central region (Figure 1.4D).

A



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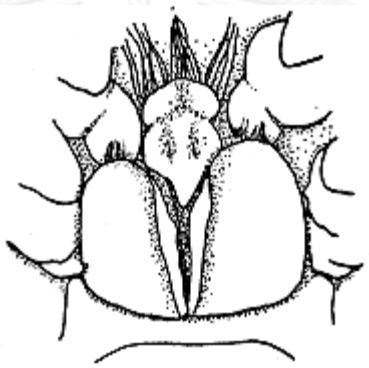


Figure 1.3 (A) Female reproductive system of *P. monodon*, Abl, abdominal lobe of ovary; AnL, anterior lobe; LL, lateral lobes; OD, oviduct; Pr, proventriculus; DG, digestive gland (Primavera, 1990). (B) The thelycum of female shrimp.

Stage V (Spent): The ovaries are thin, limp and look like the stage I but have dark areas in some regions. The gonad reverts almost immediately to the immature condition.

1.3.2.4 Development of oocytes

The oocytes of shrimp develop from mitotically dividing oogonia through meiotic cell division (Chen et al., 1999). The oocyte maturation stages are classified into 4 stages as shown in Figure 1.5 (Quinitio *et al*., 1993; Tan-Fermin and Pudadera, 1989).

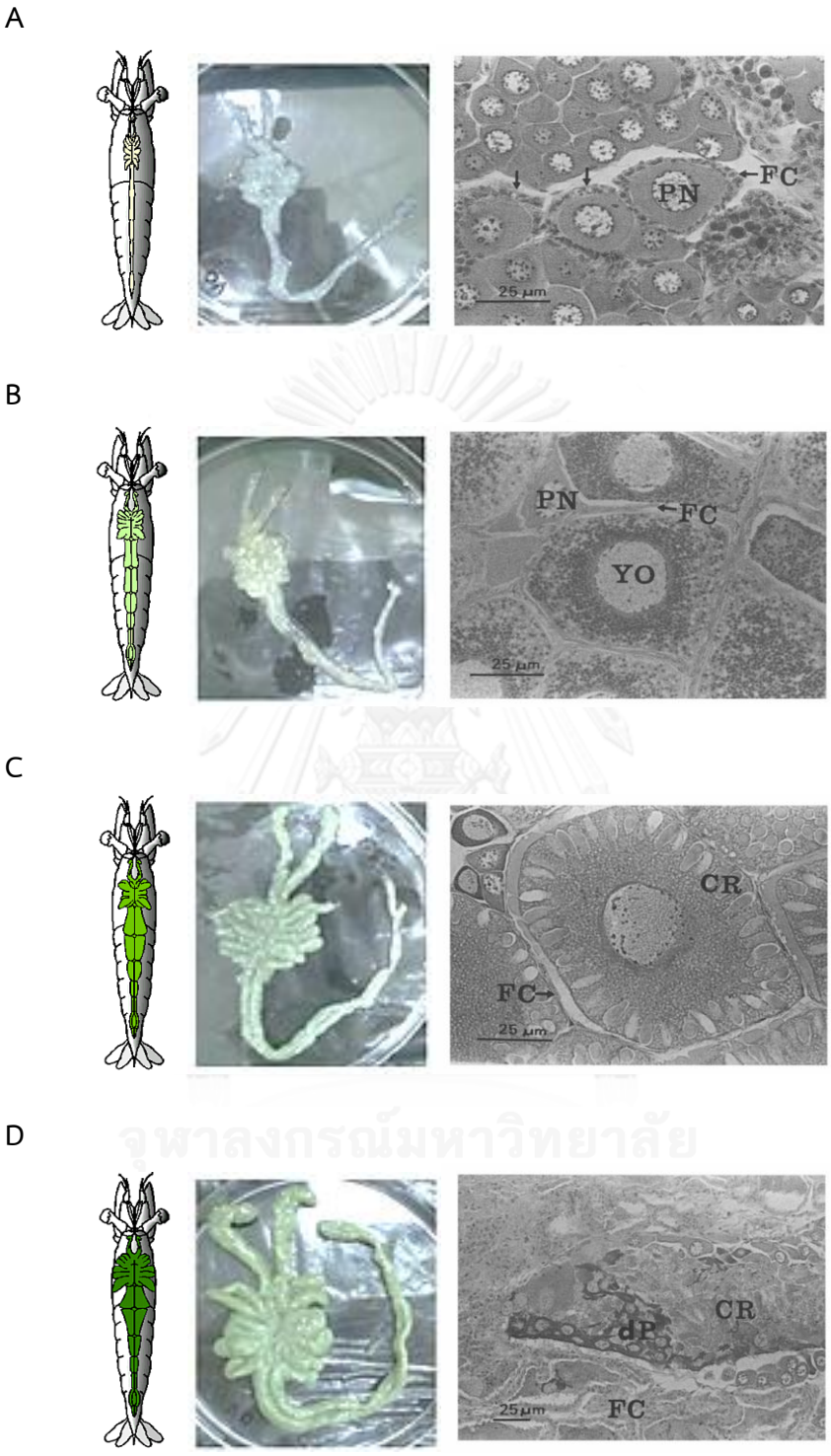


Figure 1.4 The characterization and schematic diagram of ovarian development stage of *P. monodon* (A) immature stage, (B) developing stage, (C) nearly ripe stage and (D) mature stage (Tan-Fermin and Pudadera, 1989; Uawisetwathana et al., 2011).

Previtellogenic stage (Stage I ovaries): This stage is characterized by the predominance of oogonia and primary oocytes in the chromatin nucleolus and/or perinucleolus stage which both stages have basophilic cytoplasm. Primary oocytes are quite small and without follicular cell layer that are rectangular or cubodial shape. The oocytes contain a few centrally-located nucleoli, and have prominent chromatin material in the nucleoplasm. The cytoplasm contains with large amount of ribosome. In late stage, the nucleoli size has enlarged (Figure 1.5A).

V**itellogenic stage (Stage II ovaries):** This stage is characterized by the presence of areas of mitotic activity and oocytes that started to accumulate yolky substrances in cytoplasm. Oogonia have developed into oocytes. The large round nucleus with nucleoli can be found in cytoplasm that is basophilic. Chromatin materials distribute in the nucleoplasm of the vitellogenic oocytes and follicle cells become flattened. The developing eggs are increasing in size (Figure 1.5B).

Early cortical rod stage (Stage III ovaries): This stage is characterized by the presence of oocytes with spherical or rod-like bodies near the peripheral cytoplasm and increasing of yolk proteins called vitellin. The spherical bodies elongate and extend towards the nucleus as maturation progresses. Follicle cells are spindle-shaped and hard to distinguish from the oocytes which they envelop. The eggs develop cortical granules filled with a jelly-like substance destined to form part of egg shell membrane after ovulation (Figure 1.5C).

Mature stage (Stage IV ovaries): This stage is characterized by the remaining of oocytes with yolky substances and cortical rods. The germinal vesicle breakdown (GVBD) is occurred. The follicle cells layers become thicker and darker (Figure 1.5D).

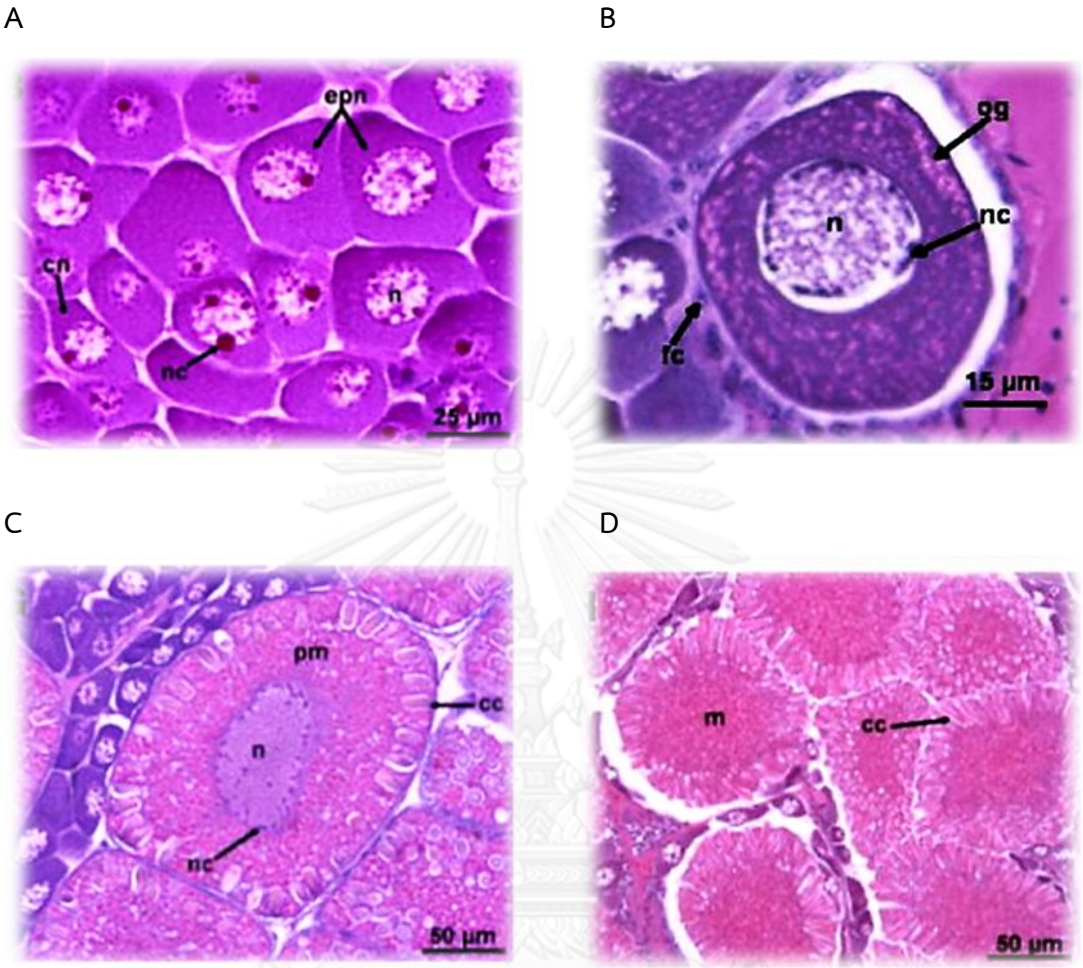


Figure 1.5 The characterization of *P. monodon* oocytes during the maturation stages (A) Previtellogenic stage; chromatin nucleolus oocyte (cn); early perinucleolus oocyte (epn); nucleus (n); nucleoli (nc), (B) Vitellogenic stage; oil globule oocyte (og); follicle cells (fc), (C) Early cortical rod stage; pre-maturarion oocyte (pm); cortical crypts (cc) and (D) Mature stage; maturation oocyte (m). (Arcos *et al.*, 2011)

1.3.3 Hormones functionally involved in female reproduction of penaeid shrimp

1.3.3.1 **Gonad-inhibiting hormone (GIH) and gonad stimulating hormone (GSH)**

The eyestalk of crustaceans is the endocrine center for regulating many physiological mechanisms, such as molting, metabolism, sugar balance, heart rate, pigments, and gonad maturation. Therefore, the eyestalk ablation can affect to all aspects of shrimp physiology (Vaca and Alfaro, 2000). For reproductive process, the ovarian development is regulated by the endocrine system from eyestalk as well. The vitellogenesis is induced by gonad-stimulating hormone (GSH) that is synthesized from brain and thoracic ganglia. Meanwhile, this process is negatively regulated by gonad-inhibiting hormone (GIH) from eyestalk (Sathapondecha *et al.*, 2011).

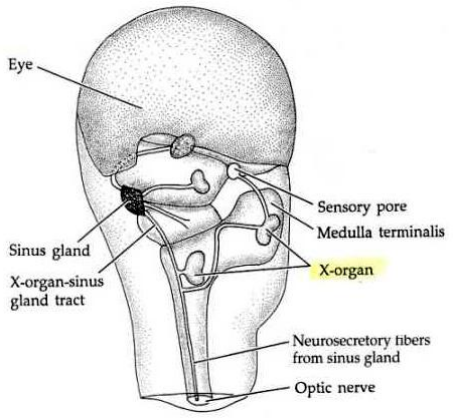
Yano (1988) found that vitellogenesis in female lobster, *Homarus americanus* can be stimulated by implantation of pieces of thoracic ganglion tissue. This result indicated that vitellogenesis could be stimulated by a vitellogenesis-stimulating hormone (VSH) secreted by neurosecretory cells of the thoracic ganglion of vitellogenic females and that lobster VSH was not species-specific in activity.

In the eyestalk, GIH or vitellogenesis-inhibiting hormone (VIH) is produced by the neurosecretory cells of the X-organ and transported to the sinus gland for storage and secrete. This is called X-organ/sinus gland complex (XO/SG) (Figure 1.6). GIH is the one of crustacean hyperglycemic hormone (CHH) family that hormones in this family contain six cysteine residues that is aligned in conserved position. GIH is known to modulate gonad maturation by inhibiting synthesis of vitellogenin (Vg), the precursor of yolk proteins (Treerattrakool *et al.*, 2008). The proposed model for ovarian development of penaeid shrimp is indicated by Figure 1.7.

Unilateral eyestalk ablation has been employed to induce the ovarian development of penaeid shrimp. This effect has been attributed to the

presence of GIH or VIH in X-organ/sinus gland complex. Therefore, the ovaries of eyestalk ablated shrimp are stimulated to mature stage faster and also related to growth, shorten molting cycle and increases energetic demands.

A



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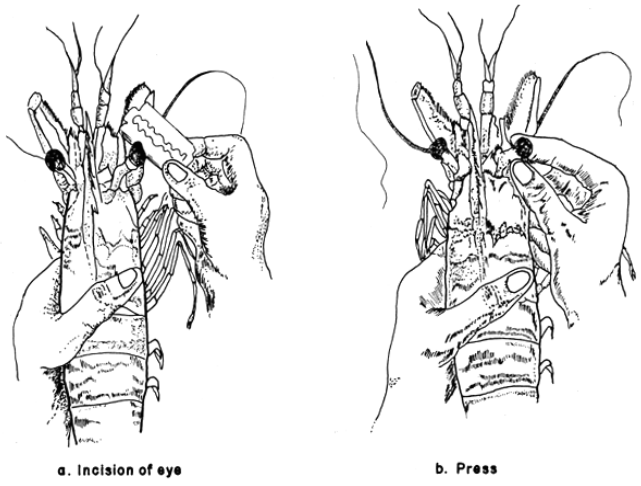


Figure 1.6 (A) The characterization of eyestalk of crustaceans. (B) The process to ablate the eyestalk of shrimp. (Left) Ablation is done by using a razor blade to cut the eye, then squeezing out the eyestalk from the base to the tip with the thumb and forefinger or (Right) using the fingers alone to break and squeeze the eye. (FAO, 1985)

Okumura *et al.* (2006) found that the synthesis of vitellogenin (VTG) and two types of cortical rod proteins (cortical rod protein; CRP and thrombospondin; MjTSP) in ovary were induced by the bilateral eyestalk ablation in immature female kuruma prawn, *Marsupenaeus japonicus*. The VTG synthesis was controlled at transcriptional level, while the CRP and MjTSP synthesizes were controlled at translational level, respectively.

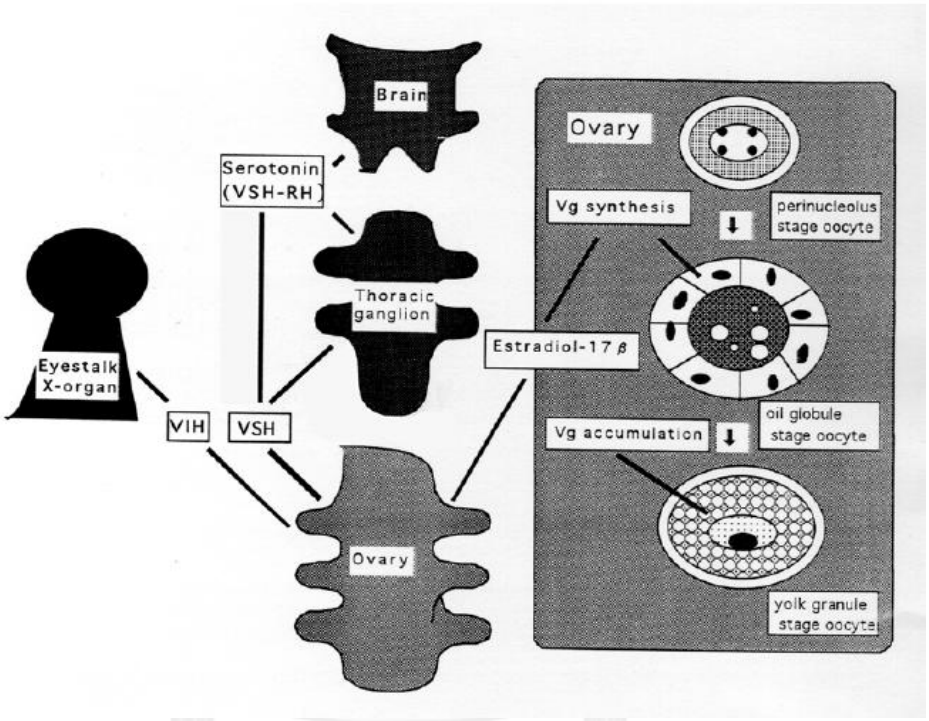


Figure 1.7 Proposed model for the hormonal control of vitellogenesis in penaeid shrimp. VSH-RH, vitellogenesis-stimulating hormone-releasing hormone; VSH, vitellogenesis-stimulating hormone;VIH, vitellogenesis-inhibiting hormone (Yano, 1998).

Choy (1987) studied the effect of the eyestalk ablation on growth and reproduction in *Penaeus canaliculatus* (Olivier, 1811). The results showed that eyestalk ablation has been enhanced the growth rate and induced gonad development and spawning. However, lower fecundity and hatching rate of ablated females shrimp resulted in lower larvae production.

1.3.3.2 Farnesoic acid O-methyl transferase (FAMeT)

Farnesoic acid O-methyl transferase (FAMeT) is enzyme involving in catalyses the final step of methyl farnesoate (MF) biosynthetic pathway in crustacean. FAMeT converts farnesoic acid (FA) to MF by catalyses the methylation of carboxylic acid group using S-adenosyl-L-methionine as a cofactor (Silva Gunawardene *et al.*, 2001). MF is the hormone regulated growth and reproduction in juvenile crustaceans (Figure 1.8).

FAMeT involved in physiological processes such as gametogenesis, oocyte maturation and development and metamorphosis of shrimp through MF biosynthesis. Silva Gunawardene *et al.* (2002) studied the function and localization of FAMeT in *Metapenaeus ensis* and found that, FAMeT localized in X-organ-sinus gland complex where may interacted with the eyestalk neuropeptides of the CHH/MIH/GIH family and affected to MF biosynthesis as a consequence of modulation in reproduction of shrimp. Moreover, the presence of FAMeT in juvenile shrimp suggested that it regulated the MF biosynthesis during the early developmental of shrimp as well.

Tsukimura *et al.* (2006) studied the effect of MF on the reproduction of the tadpole shrimp, *Triops longicaudatus*. The tadpole shrimp was fed with MF-incubated *Artemia* nauplii and MF-coated pellets. The results showed that, the low concentrations of MF had the inhibitory effects on gonadal development of tadpole shrimp. It suggested that MF may involve in ovarian development.

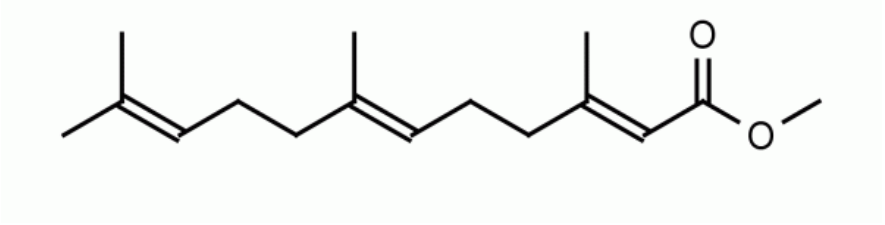


Figure 1.8 The structure of methyl farnesoate (Echelon, 1997)

1.3.3.3 Neurotransmitters

Neurotransmitters are the chemicals messenger that modulates signals between neurons and other cells in the body. They are released from the terminal of axon across synapse to reach the receptor site of the other cell or neuron. Neurotransmitters can be classified to 2 groups, excitatory and inhibitory neurotransmitters. The excitatory neurotransmitters increase the probability that the neuron will fire an action potential while, the inhibitory neurotransmitters decrease that possibility (Cherry, 2013; Neurogistics, 2013).

Serotonin (5-hydroxytryptamine; 5-HT) is the one of inhibitory neurotransmitters. Serotonin was found to stimulate the release of several crustacean hormones such as CHH, red pigment dispersing hormone (RPDH) or molt inhibiting hormone (MIH) (Meeratana *et al.*, 2006). Moreover, the action of serotonin is indirect stimulating release of the gonad-stimulating hormone (GSH) that is present in the brain and thoracic ganglia leading to stimulate ovarian development. Meanwhile, dopamine (DA) acts an inhibitor of ovarian development (Fingerman, 1997). Therefore, serotonin injection is used to induce the ovarian development.

Meeratana *et al.* (2006) investigated the effect of serotonin on the ovarian maturation of the giant freshwater prawn, *Macrobrachium rosenbergii* de Man. The stage I of ovarian cycle shrimps were injected with final volume 0.05 ml. of serotonin at dosage of 1, 5, 10, 20 and 50 μg/g body weights. The results found that serotonin 1 μg/g BW significantly increased the ovarian index compared to control and this increasing was generally lower when the dose of serotonin was higher. The ovaries of lower dose injection were developed into stage IV compared to control that ovaries were still at stage I or II. This suggested that serotonin can induce ovarian development and oocytes maturation in *M.rosenbergii*.

Santhoshi *et al.* (2009) studied the effect of serotonin on the ovarian maturation and the levels of vitellogenin in hemolymph in Indian white shrimp, *Fenneropenaeus indicus*. The shrimps were injected with final volume 10 μl of different doses serotonin 2.5×10^{6}, 2.5×10^{7} and 2.5×10^{8} mol/g live mass. Non-injected

shrimps and injected shrimps with sterile vehicle solution were control groups. The results showed that the serotonin injection increased the ovarian index and oocyte diameter compared to control. The levels of vitellogenin in hemolymph were increased in serotonin injected shrimps especially in dose at 2.5×10^{6} mol/g live mass. This study revealed the possible stimulatory role of serotonin on the ovarian maturation of *F.indicus* by increasing the vitellogenin levels.

The effect of serotonin on the ovarian maturation in *P. monodon* was also investigated. Wongprasert *et al.* (2006) investigated the effect of exogenous serotonin on the ovarian maturation and spawning of the black tiger shrimp *P. monodon*. The 50 μg/g body weight 5-HT solution was injected into domesticated *P. monodon*. The results showed that 5-HT injection can induced the ovarian maturation and spawning rate at the level comparable to that of unilateral eyestalk-ablated shrimp. Although both groups have equal number of eggs, but hatching rate of 5-HT injected shrimps was higher than the eyestalk-ablated shrimps. And, the former group also produced more nauplii than the latter. Moreover, they also found that 5-HT presence in follicular cells of previtellogenic oocytes, in the cytoplasm of early vitellogenic oocytes and on the cell membrane and cytoplasm of late-vitellogenic oocytes and 5-HT protein levels also continuously increased during the ovarian maturation stages.

From many studies, effect of serotonin on reproductive development are similar when compare to the use of eyestalk ablation. Therefore, using of exogenous serotonin injection might be the alternative method to induce the ovarian maturation in shrimp.

1.3.3.4 Steroid hormones

Steroid hormones are widely dispersed in animals which they act as regulators of many biochemical and physiology processes such as growth, development, reproduction and homeostasis. Steroid hormones molecules derived from four ring of cholesterol, except vitamin D that B-ring is broken (Figure 1.9). In vertebrate, steroid hormones are large groups including the different classes of

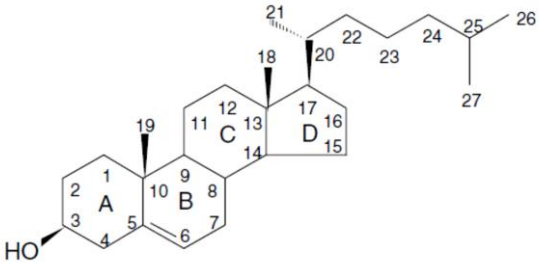
androgen, estrogen, progestins, glucocorticoids, mineralocorticoids and vitamin D derivatives. Meanwhile for invertebrate, ecdysteroids are the major of arthropod steroid hormone that called vertebrate-type steroids (Kohler *et al.*, 2007; Lafont and Mathieu, 2007; Marcinkowska and Wiedlocha, 2002).

There are many reports about the presence of vertebrate-type steroids in crustaceans, where estrogen was early found in *Homarus* ovary by Donahue in 1940. The vertebrate-type steroids composed of various types of steroids such as progesterone, 17α-hydroxyprogesterone, testosterone and 17β-estradiol (Lafont and Mathieu, 2007). They have importance roles in molting cycle and reproduction which involving in vitellogenesis. Quinitio *et al.* (1994) found the relation between progesterone and 17β-estradiol levels in hemolymph with the vitellogenin levels in serum during ovarian maturation of *P. monodon.* It implied that these vertebrate-type steroids can regulate vitellogenesis.

Coccia *et al.* (2010) investigated the effect of progesterone and 17β-estradiol on vitellogenin of the freshwater crayfish *Cherax albidus.* Three groups of female crayfish including Early-vitellogenic (EV), full-vitellogenic (FV), and non-vitellogenic (NV) were injected with 17β-estradiol (E), progesterone (P) and both steroids (E+P) 2 times a week for 4 weeks. Non-injected crayfish and crayfish injected with saline solution are the control. The results showed that 17β-estradiol increased the vitellogenin transcript in hepatopancreas of EV and FV females, whereas progesterone increased the vitellogenin concentration in hemolymph of EV and FV females. They concluded that progesterone and 17β-estradiol influenced to vitellogenin synthesis.

Progesterone (P4) and its derivatives, 17α-hydroxyprogesterone (17α-OHP), are sex steroid hormones that play important roles in gametogenesis. The progesterone actions are mediated through binding with nuclear progesterone receptor that is the member of steroid/thyroid hormone receptor superfamily (Preechaphol *et al.*, 2010). Fingerman *et al.* (1993) reported that progesterone related to the crustacean reproductive system.

A



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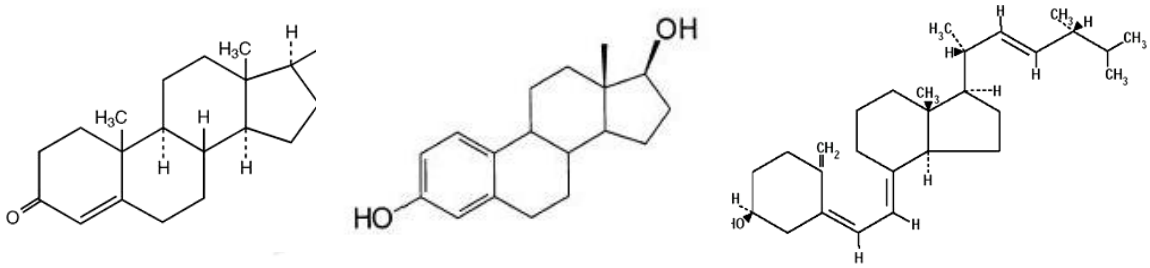


Figure 1.9 Structural characterization of steroid hormone. (A) The basic structure of cholesterol consisting of four linked hydrocarbon rings. (B) Progesterone (C) 17β-estradiol and (D) Vitamin D that B-ring is broken.

Yano (1985) investigated the effect of progesterone on induction of ovarian maturation and spawning in the greasyback shrimp, *Metapenaeus ensis*. The experiment groups were injected with 0.1 μg/g BW progesterone and absolute ethanol, while non-injected shrimp as control. The ovaries of shrimp injected with progesterone were advanced to stage III, IV and V compare to control which still remained at the early development stage. Two of fifteen injected females with progesterone can nocturnally spawned at 30 and 31 days after injection. Similarly in the derivatives of progesterone, 17α-hydroxyprogesterone, were studied in kuruma prawn, *Penaeus japonica.* In addition, significantly increased vitellogenin concentration in the sera of injected shrimp with 0.01 μg/g BW 17α-hydroxyprogesterone was observed (Yano, 1987). Results from these studies

indicated that progesterone and its derivative can induce ovarian maturation in shrimp.

17β-estradiol (E2) is the one of sex steroid hormone that was synthesized from progesterone in ovary (Summavielle *et al.*, 2003). It has two hydroxyl groups in its molecular structure. Several studies showed the importance of 17β-estradiol in vitellogenesis. Quackenbush (1992) investigated the effect of exogenous steroid hormones on the yolk synthesis of the marine shrimp, *Penaeus vannamei* ovarian fragment. It found that 17β-estradiol stimulated yolk protein synthesis.

Yano and Hoshino (2006) studied the effect of 17β-estradiol on induction of vitellogenin synthesis and oocyte development in kuruma prawn, *Marsupenaeus japonica.* The previtellogenic ovaries were incubated in Medium 199 at difference concentration of 17β-estradiol (3.6, 36.7, 367, 3671 nM). It found that, the experiment groups had significantly greater of vitellogenin concentration and oil globule stage of oocyte more than control. It revealed that 17β-estradiol can induced the vitellogenesis and appearance of prvitellogenic oocyte of immature prawn.

1.3.3.5 Ecdysteroid

Ecdysteroids are the arthropods steroid hormone that regulates molting, metamorphosis, reproduction and diapauses. The crustacean ecdysteroids (20-hydroxyecdysone; 20E) are converted from molting hormone in Y-organ into ecdysone and secreted to hemolymph by 20-hydroxylatse activity. The ecdysteroid are very polar, freely circulated and enter into cells by diffusion (Huberman, 2000).

There are evidences that ecdysteroids was related to reproduction of crustacean. Chaix and De Reggi (1982) found that ecdysteroids levels were increased during the beginning of vitellogenesis and once again as oocytes reach maturity as well as in the beginning and end of embryogenesis in the spider crab, *Acanthonyx lunulatus*. Spindler *et al.* (1987) also found that ecdysteroid concentrations were

increased at the appearance of Y-organ during the embryogenesis of shrimp, *Palaemon serratus*.

1.3.4 Nutrigenomics

Diets are the environmental factors affected to health and disease incident. The effects of nutrient deficiencies, imbalance of macronutrients or toxic concentrations of certain food compounds on health and physiological process have been reported. Other bioactive food constituents such as polyphenols, vitamins, carotenoids and terpenoids have significant beneficial effects for health promotion and disease prevention. The nutrition research has traditionally explored the importance between these relations. Therefore, the aims of advanced nutrition research have become more interesting in molecular level.

Many studies recognized that nutrient and food compounds can affect to gene expression in several ways by interacted with genes affecting transcription factors, protein expression and metabolite production. Consequently, nutrigenomics has emerged as a novel and multidisciplinary research field in nutritional science that study how diets affects the balance between health and disease by altering the expression of an individual's genetic makeup (García-Cañas *et al.*, 2010). Currently, nutrigenomics are increasingly applied in the industry of aquaculture. The use of specially formulated feed can be applied to induce ovarian maturation of female penaeid shrimp.

The studies of diets affected to physiological biology of commercial species were investigated. Jaya-Ram *et al.* (2008) investigated the effect of different levels of dietary highly unsaturated fatty acids (HUFAs) on reproductive performance and mRNA expression of desaturase and elongase genes in zebrafish, *Danio rerio*. The experiment diets were divided into 3 groups by different ratio of squid oil and linseed oil; 100% squid oil (SO), 1:1 squid oil:linseed oil (SLO) and 100% linseed oil (SO), respectively. The increasing levels of dietary linseed oil have become lowered deposition of HUFA. The results showed that the total egg, relative fecundity and

hatching rate were significantly increased in SLO and they showed the increasing trend of ovarian desaturase and elongase gene expression during low dietary HUFAs levels. It was concluded that HUFAs play the important role in reproductive performance of the zebrafish.

Montero *et al.* (2010) elucidated the effect of both total and partial substitutions of fish oil by vegetable oil on immunological parameters and expression of pro-inflammatory cytokines after exposure to the bacterial pathogen *Photobacterium damselae sp. Piscicida* in the gilthead sea bream, *Sparus aurata*. The experiments were divided into 5 groups such as only fish lipid source (FO), totally (100%) and partially (70%) substituted vegetable rich in n-3 or n-6 fatty acid (100L, 70L, 100S, 70S), respectively. After feeding 80 days, they were exposed with pathogen. The results showed that the immunological parameters were increased in the n-3 rich groups. The totally substituted groups can increased the expression level of cytokines. It found that, the different lipid source may affect to the imbalances of fish immune response.

Chen *et al.* (2013) determined the effect of honeysuckle (*Lonicera japonica*) on growth performance and the immune response in *P. monodon*. Shrimps were fed with individual five diets containing 0% (basal diet), 0.1%, 0.2%, 0.4% and 0.8% of honeysuckle, respectively, for 60 days. The results found that the growth performance of shrimps fed with honeysuckle were higher than control, while the 0.4% concentration group showed the highest rate. The shrimp fed 0.2% honeysuckle had highest of survival rate. The total antioxidant status of shrimps fed 0.2%, 0.4% and 0.8% honeysuckle were higher than basal diet and 0.1% concentration. They concluded that the dietary intake containing honeysuckle could enhance the growth performance and improve the immune response of *P. monodon*.

Understanding the induction mechanisms of reproduction-related genes during ovarian and oocyte maturation will be useful to develop methodologies that to effectively induce ovarian maturation in *P. monodon*. In this study, genes playing an important role in the signal transduction pathways including *P. monodon Rnf121*, *nRTK* and *RTK* were studied. Expression patterns of these transcripts during ovarian maturation of intact and eyestalk-ablated adults of wild *P. monodon* and those of domesticated shrimp under serotonin (5-HT) and steroid hormone (progesterone and 17β-estradiol) induction were examined by quantitative real-time PCR. In addition, the feeding trial of the diet supplemented with 17β-estradiol was carried out for determining its effects on gene expression.

CHAPTER II
MATERIALS AND METHODS

2.1 Experimental samples

For determination of gene expression during ovarian development, female adults were live-caught from the Andaman Sea (west of peninsular Thailand) and acclimated under the farm conditions for 2-3 days. The post-spawning group (stage V) was immediately collected after ovulation (*N* = 4). For the eyestalk-ablated group, wild shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2-7 days after the ablation to obtain stages I-V ovaries. The ovarian developmental stages of wild *P. monodon* were classified according to gonadosomatic indices (GSI, ovarian weight/body weight x 100): < 1.5, 2-4, 4-6 and > 6% for stages I (*N* = 5 and 4 for intact and eyestalk-ablated adults, respectively), II (*N* = 11 and 10), III (*N* = 5 and 6) and IV (*N* = 6 and 5) ovaries, respectively. The post-spawning group (stage V) of eyestalk-ablated shrimp was also collected (*N* = 8). In addition, juvenile shrimp (4-month-old) were collected from Broodstock Multiplication Center, (Burapha University, Chanthaburi Campus; *N* = 5). For tissue distribution analysis, various tissues of wild females and testes of wild males were collected.

In this thesis, cultured shrimp initially bred at Shrimp Genetic Improvement Center (SGIC) and maintained at Broodstock Multiplication Center (BMC, Burapha University, Chanthaburi Campus) were used in hormonal induction experiment. These samples were regarded as domesticated stock in this study.

To determine effects of serotonin on targets gene expression, eight groups of acclimated female shrimp for 4 days (18-month-old) were injected intramuscularly into the first abdominal segment with serotonin (50 μg/g body weight, *N* = 4 for each group). Specimens were collected at 0, 1, 3, 6, 12, 24, 48 and 72 hpi. Non-injected shrimp and those injected with normal saline (0.85% NaCl at 0 hpi) were included as the negative (NC) and vehicle (VC) controls, respectively. Ovaries of each shrimp were

sampled and immediately placed in liquid N_2. The samples were stored at -80°C until needed (Figure 2.1).

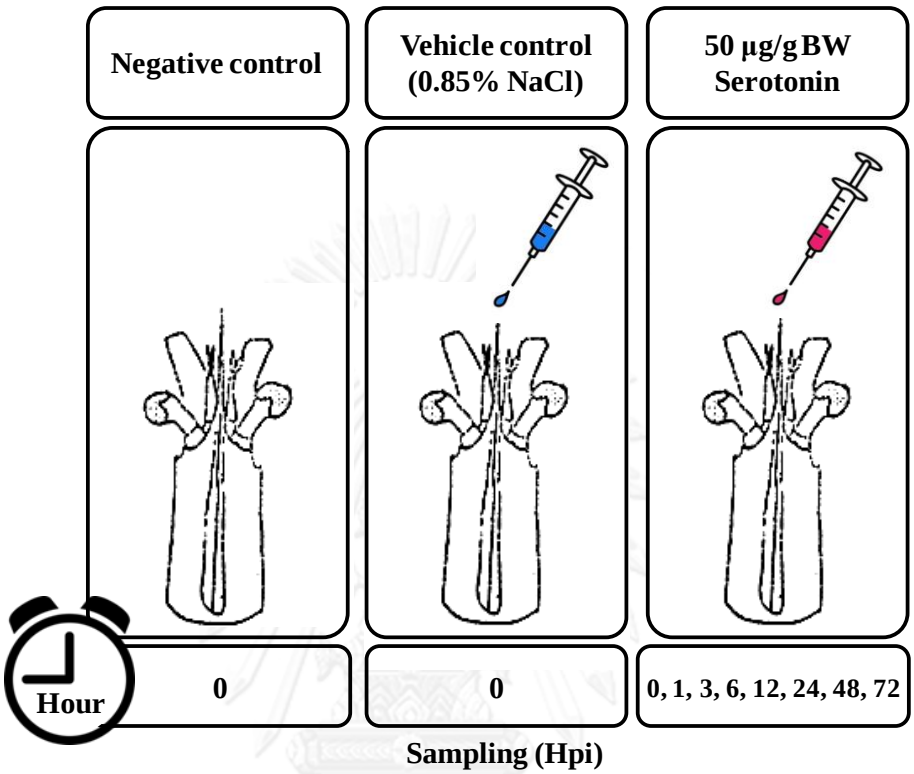


Figure 2.1 The experimental details for determined the effect of exogenous serotonin injection on expression of reproduction-related genes.

To determine effects of progesterone on targets gene expression, four groups of acclimated female shrimp for 4 days (14-month-old) were injected intramuscularly into the first abdominal segment with progesterone (0.1 µg/g body weight, *N* = 6 for each group). Specimens were collected at 12, 24, 48 and 72 hpi. Non-injected shrimp and those injected with absolute ethanol (at 0 and 12 hpi) were included as the negative (NC) and vehicle (VC) controls, respectively. Ovaries of each shrimp were sampled and immediately placed in liquid N_2. The samples were stored at -80°C until needed (Figure 2.2).

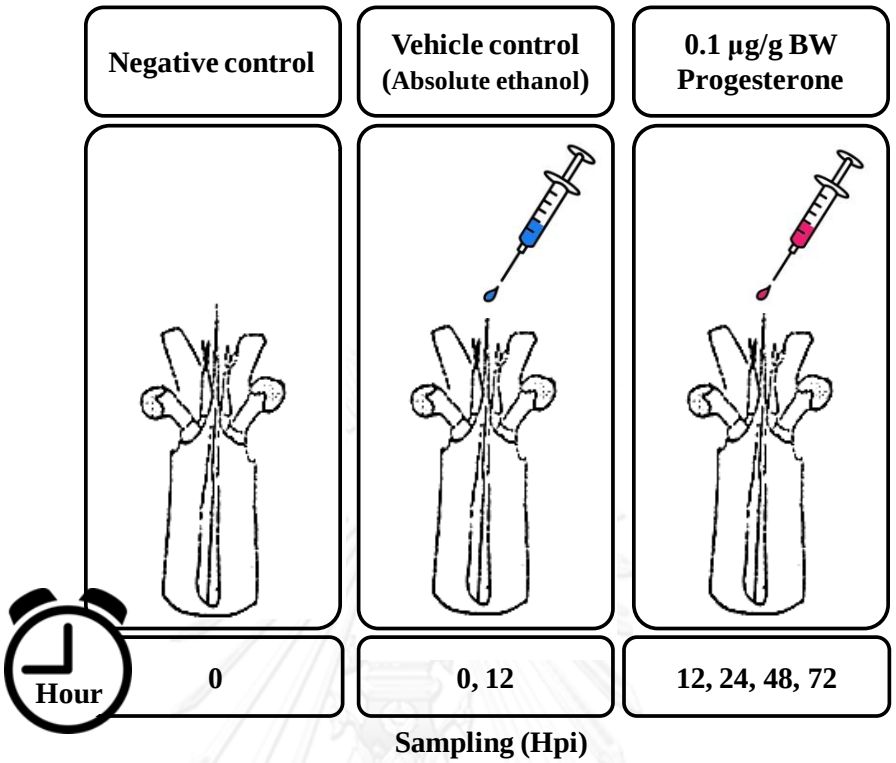


Figure 2.2 The experimental details for determination of effects of exogenous progesterone injection on expression of reproduction-related genes.

To determine effects of 17β-estradiol on targets gene expression, three groups of acclimated shrimp (14-month-old) were injected intramuscularly with 17β-estradiol (0.01 µg/g of body weight, *N* = 6 for each group) into the first abdominal segment of each shrimp. The injection was repeated with the original doses at 3 and 6 days post initial injection and specimens were collected at 7, 14 and 28 days post initial injection. Non-injected shrimp and those injected with 5% ethanol (at 7, 14 and 28 days after the initial injection; *N* = 4-6 for each group) were included as the negative (NC) and vehicle (VC) controls, respectively. In addition, unilateral eyestalk-ablated shrimp were collected at the same time intervals. Ovaries of each shrimp were sampled and immediately placed in liquid N_2. The samples were stored at -80˚C until needed (Figure 2.3).

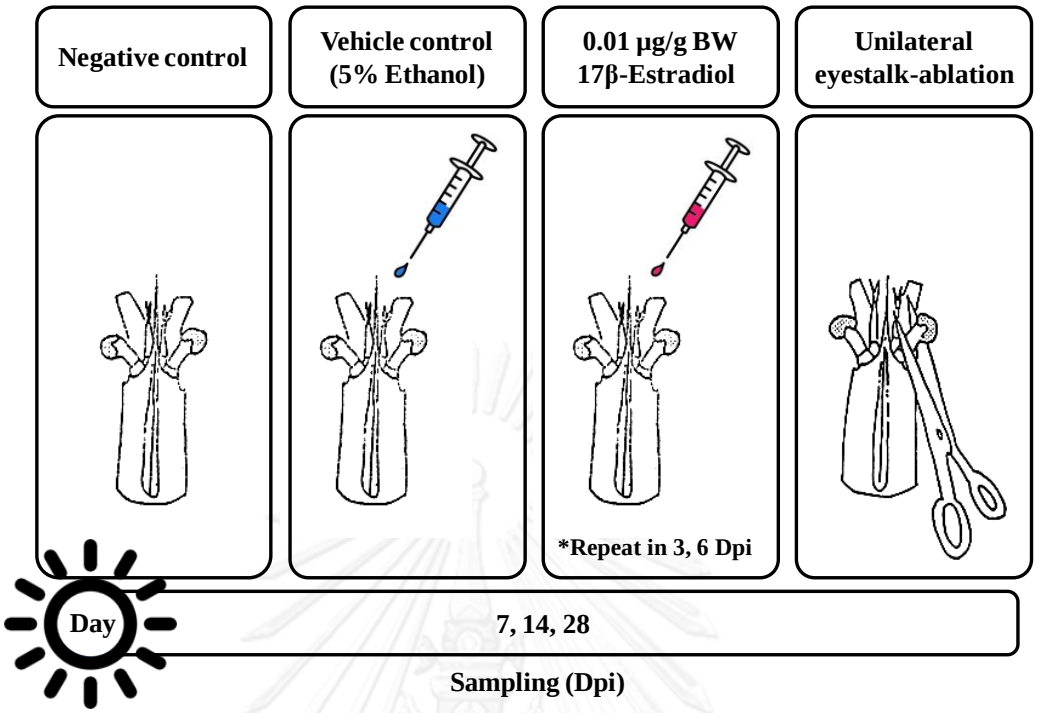


Figure 2.3 The experimental details for determination of effects of exogenous 17β-estradiol injection on expression of reproduction-related genes.

To determine the effect of the supplemented diets on the target genes, the feeding experiments were carried out for the duration of 35 days. The domesticated shrimp (approximately 12 month-old) were acclimated at the laboratory conditions (28±1 °C, 30 ppt seawater) in 1500 liter fish tanks for 1 week. Two groups of shrimp were fed 3 times daily (5% body weight) with the diets supplementary with 17β-estradiol (1 and 10 mg/kg, N= 8 for each group). Specimens were collected at 7, 14, 28 and 35 days after the initial treatment. Non-treated shrimps at 7, 14, 28 and 35 days were included as negative control. The unilateral eyestalk-ablated shrimps also were collected at the same time intervals. Ovaries of each shrimp were sampling and immediately placed in liquid N_2. The ovary samples were stored at -80 °C until use, and then were extracted RNA later (Figure 2.4).

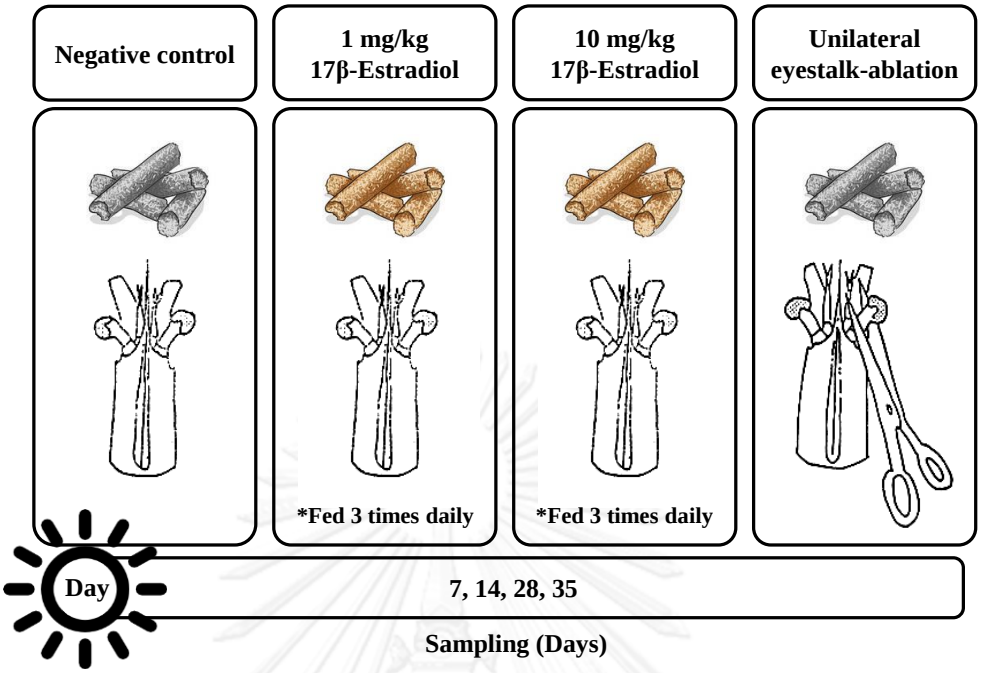


Figure 2.4 The experimental details for determination of effects of 17β-estradiol supplemented diets on expression of reproduction-related genes.

2.2 Total RNA extraction and the first-strand cDNA synthesis

2.2.1 Total RNA extraction

Total RNA was extracted from various tissues using TRI Pure. A piece of tissue was immediately placed in the mortar containing liquid nitrogen and ground to fine powder. The tissues powder 50-100 mg was transferred to 1.5 ml microcentrifuge tube containing 500 µl of TRI Pure and homogenized. Additional 500 µl TRI Pure was added. The homogenate was incubated at room temperature for 5 minutes and 200 µl of chroloform was added. The homogenate was vortexed and left for 15 minutes before centrifuged at 12000g for 15 minutes at 4 °C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase and the colorless upper aqueous phase. The aqueous phase that inclusively containing RNA was transferred to new microcentrifuge tube. Total RNA was precipitated by additional 500 µl of isopropanol and mix thoroughly. The mixture was left at room temperature for 10-15 minutes and centrifuged at 12000g for 10 minutes at 4 °C. The supernatant was

discarded. The RNA pellet was washed with 1 ml. of 75% ethanol then centrifuged at 12000g for 10 minutes at 4 °C. The 75% ethanol was discarded. The RNA pellet was air-dried for 5-10 minutes. The dried RNA was dissolved in DEPC-treated water for immediately used. Alternately, the total RNA was kept under absolute ethanol in -80 °C for longer storage.

2.2.2 Estimation of total RNA concentration by spectrophotometry

The concentration of extracted total RNA was spectrophotometrically measured the optical density at 260 nm (OD_{260}) and 280 nm (OD_{280}). An OD_{260} of 1.0 corresponds to a concentration of 40 µg/ml of single strand RNA, 50 µg/ml of double strand DNA and 33 µg/ml of single strand DNA (ssDNA) (Sambrook and Russell, 2001). The concentration of samples were estimated in µg/ml by multiplying an OD_{260} value with a dilution factor was 50, 40 and 33 for DNA, RNA and oligonucleotide, respectively, as follows

[DNA/RNA] = OD_{260} x dilution factor x 50 (40 or 33 for RNA or ssDNA, respectively)

The purity of samples can be evaluated from a ratio of $OD_{260/280}$. The appropriated ratios of DNA and RNA were 1.8 and 2.0, respectively (Sambrook and Russell, 2001).

2.2.3 Agarose gel electrophoresis

The quality of nucleic acid sample of verifying RNA integrity after extract was estimated from motion of RNA and DNA fragments pass through an agarose gel after electrophoresis. The nucleic acid can roughly estimate on the basic of the direct relationship between the amount of nucleic acid and the level of fluorescent dye (ethidium bromide) was visualized under a UV transilluminator. The quality of RNA extracted was estimated from the intensity of the fluorescent band and RNA fragment size by comparing with that of *Hind* III digested λDNA (λ-*Hind* III) was marker.

Appropriate amount of agarose was weighed out and mixed with 1X TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The gel slurry was heated until complete solubilization in the microwave. The gel solution was left at room temperature to approximately 50-55 °C before poured into a gel mould. The comb inserted, the gel was allowed to solidify at room temperature for approximately 45 minutes. When needed, the gel mould was placed in the gel chamber and sufficient 1X TBE buffer was added to cover the gel for approximately 0.5 cm. The comb was carefully withdrawn. Amplification product was mixed with 2 µl or one-fourth volume of the gel-loading dye (0.25% bromophenol blue and 25% ficoll, MW 400,000 prepared in sterile deionized H_2O) and loaded into the well. λDNA digested with *Hind* III (λ-*Hind* III) was marker for comparing with RNA fragments. Electrophoresis was carried out at 100 volts for 30 minute. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 3-5 minutes and destained to remove unbound ethidium bromide by submerged in water for 15-30 minutes. The RNA fragments were visualized using a UV transilluminator, Molecular Imager® Gel Doc XR (Bio-rad laboratories).

2.2.4 DNAse I treatment

Fifteen micrograms of total RNA were treated with DNase I (0.5 U/µg of total RNA, Promega) at 37 °C for 30 minutes. An equal volume of phenol:chloform:isoamylalcohol (25:24:1) was added, vortexed for 15 seconds and left at room temperature for 10-15 minutes before centrifuge at 12,000 g for 10 minutes at 4 °C. The upper aqueous phase was collected and the extraction process was repeated once with chloroform:isoamylalcohol (24:1) and once with chloroform. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube for RNA precipitation by adding one-tenth final sample volume of 3 M sodium acetate (pH 5.2) and two and a half volume of cold absolute ethanol and mixed thoroughly. The mixture was incubated at -80 °C for 30 minutes before centrifuged at 12,000 g for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of cold 75% ethanol. The RNA pellet was air-dried for 5-10 minutes and dissolved

in DEPC-treated H_2O for immediately used or kept under absolute ethanol in a -80 °C freezer for long storage.

2.2.5 First strand cDNA synthesis

One and a half micrograms of DNase I-treated total RNA were reverse-transcribed to the first strand cDNA using an ImProm-II™ Reverse Transcription System Kit (Promega). Total RNA was combined with 0.5 µg of oligo dT_{12-18} and appropriate DEPC-treated H_2O in final volume of 5 µl. The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. Then 5X reaction buffer, $MgCl_2$, dNTP Mix, RNasin were added to final concentrations of 1X, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm-II™ Reverse transcriptase was add and gently mixed by pipetting. The reaction mixture was incubated at 25 °C for 5 minutes and at 42 °C for 90 minutes. The reaction mixture was incubated at 70 °C for 15 minutes to terminate the reverse transcriptase activity. Concentration and rough quality of the newly synthesized first strand cDNA was spectrophotometrically examined (OD_{260}/OD_{280}) and electrophoretically analyzed by 1.0% agarose gels, respectively. The first stranded cDNA was 10 fold-diluted and kept at 20°C until required.

2.3 RT-PCR and tissue distribution analysis

2.3.1 Primer design

Forward and reverse primers of each gene were designed from ESTs of *P. monodon* and NCBI database. *Elongation factor-1*α (*EF-1*α_{500}) was included as the positive control (Table 2.1).

Table 2.1 Primer sequences of reproduction-related genes and *EF-1*α_{500} melting temperature ™ and the expected product sizes

Gene/Primer	Sequence	Tm (°C)	Size (bp)
1. *RING finger protein 121* (OV-N-ST02-0220-LF)			
Forward	5' – CAGACGAGGAAAAATGGAGGTTAG -3'	70	248
Reward	5' – GGGCAGCCAGATGAAAATGA -3'	60	
2. *Non-receptor tyrosine protein kinase* (TT-N-S01-0724-W)			
Forward	5'- AGAATGAGTCTGAGAAGGAATCG -3'	66	289
Reward	5'- GTGTGCTCTGCCACCAAAAT -3'	60	
3. *Receptor tyrosine kinase* (GenBank GO081935.1)			
Forward	5'- TGGTTGCTTGATGTGGGAA -3'	56	221
Reward	5'- CATTGAGTAGGCTTTGTAGGTTG -3'	72	
4. *Elongation factor-1*α, *EF-1*α_{500}			
Forward	5′- ATGGTTGTCAACTTTGCCCC -3′	60	500
Reward	5′- TTGACCTCCTTGATCACACC -3′	60	

2.3.2 RT-PCR analysis

The amplification reactions were performed in a 25 µl reaction volume containing 1X buffer (10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl and 0.1% Triton X-100), 2.0 mM $MgCl_2$, 100 µM each of dNTPs, 0.2 µM of each primer, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES) and 100 ng of first strand cDNA. The thermal profiles were predenature at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 seconds, 53 °C for 45 seconds and 72 °C for 45 seconds. The final extension was carried out at 72 °C for 7 minutes. Five microliters of the amplification products were electrophoretically analyzed though 1.0-1.5% agarose gel. *EF-1*α_{500} was included as the internal control.

2.3.3 Tissue distribution analysis

The first strand cDNA of ovaries, testes, antennal gland, eyestalks, subcuticular epithelium, gills, heart, hemocytes, hepatopancreases, intestine, lymphoid organs, pleopods, stomach, and thoracic ganglion were used for tissue distribution analysis. The amplification was performed by using the same conditions as RT-PCR. The first strand cDNA template was stored at -20°C.

2.4 Isolation and characterization of the full-length cDNA using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE – PCR)

2.4.1 Preparation of the 5′ and 3′ RACE template

RACE-Ready cDNA was prepared by combining 1.5 µg of ovarian mRNA with 1 µl of 5′-CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5′- RACE-PCR or 1.5 µg of ovarian mRNA with 1 µl of 3′-CDS primer for 3′- RACE-PCR (Table 2.2). The components were mixed and spun briefly. The reaction was incubated at 70°C for 2 minutes and immediately cooled on ice for 2 minutes. The reaction tube was spun briefly. After that, 2 µl of 5X First-Strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reactions were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom.

The tubes were incubated at 42 °C for 1.5 hours in a thermocycler. The first strand reaction products were diluted with 125 µl of TE buffer and heated at 72 °C for 7 minutes. The first strand cDNA template was stored at -20 °C.

2.4.2 RACE-PCR primers design

Gene-specific primers (GSPs) were designed from the partial cDNA sequence of initial identified sequence in RT-PCR analysis. Typically, GSPs should have 23-28 nucleotides, 50-70 %GC and have melting temperature (T_m) more than 65 °C (or T_m > 70 °C for best results). The antisense and/or sense primers were designed for 5′- and 3′- RACE-PCR, respectively. Internal forward and/or reverse primers were

also designed for further sequencing of the internal regions of large RACE-PCR fragments (Table 2.3).

Table 2.2 Primer sequences for the first strand cDNA synthesis of RACE-PCR template

Primer	Sequence
BD SMART II™ A Oligonucleotide (12 µM)	5′- AAGCAGTGGTATCAACGCAGAGTACGCGGG -3′
3′-RACE CDS Primer A (3′-CDS; 12 µM)	5′- AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N -3′ (N = A, C, G or T; V = A, G or C)
5′-RACE CDS Primer (5′-CDS; 12 µM)	5′- $(T)_{25}$V N -3′ (N = A, C, G or T; V = A, G or C)
10X Universal Primer A Mix (UPM)	Long : 5′- CTAATACGACTCACTATAGGGCAAGCAGTGGTAT CAACGCAGAGT -3′ Short : 5′- CTAATACGACTCACTATAGGGC -3′
Nested Universal Primer A (NUP; 12 µM)	5′- AAGCAGTGGTATCAACGCAGAGT -3′

2.4.3 Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE – PCR)

RACE-PCR was carried out using SMARTer™ RACE cDNA Amplification Kit (BD Bioscience Clontech) following the manufacture's recommended protocol. The master mix for 5′- or 3′- RACE-PCR was prepared as described in Table 2.4 and the amplified condition of 5′- and 3′- RACE-PCR were set up as described in Table 2.5.

Table 2.3 Gene-specific (GSPs) and internal primers used for characterization of the full length cDNA of targets genes in *P. monodon* using RACE-PCR

Gene/Primer	Sequence	Tm (°C)
1. *RING finger protein 121*		
5′RACE	5'- CTTCTTCCAAGCCACGAGACCCACT -3'	80
3′RACE	5'- GCTGCCCTTCTCCTCCATCACCG -3'	76
2. *Non-receptor tyrosine protein kinase*		
5′RACE	5'- GCCACATCAGAGTCTTCCAGCCCGTT -3'	82
3′RACE	5'- GCAAGATGGAACGGGCTGGAAGACT -3'	78
3′Internal RACE	5'- GAGACCTCAGTTCATACCTCCAGCAG -3'	80
3. *Receptor tyrosine kinase*		
5′RACE	5'- GCCACTTCCCACATCAAGCAACCA -3'	74
5′Internal RACE	5'- AGCGTTTCCTCAGCGTGCCAGCG -3'	76
3′RACE	5′- CCTCCTTTGGTGGCGTAACTGTCATCC -3′	74

Table 2.4 Composition of 5′- and 3′- RACE-PCR

Component	5′-RACE Sample	GSP1+UPM (-Control)	3′-RACE Sample	GSP2+UPM (-Control)
5′-RACE-Ready cDNA	1.5 µl	1.5 µl	-	-
3′-RACE-Ready cDNA	-	-	1.5 µl	1.5 µl
UPM (10X)	5 µl	-	5 µl	-
GSP1 (10 µM)	1.0 µl	1.0 µl	-	-
GSP2 (10 µM)	-	-	1.0 µl	1.0 µl
10X BD adventage® 2 PCR Buffer	2.5 µl	2.5 µl	2.5 µl	2.5 µl
10 µM dNTP mix	0.5 µl	0.5 µl	0.5 µl	0.5 µl
50X BD Advantage® 2 polymerase mix	0.5 µl	0.5 µl	0.5 µl	0.5 µl
H_2O	Up to 25 µl	Up to 25 µl	Up to 25 µl	Up to 25 µl
Final volume	25µl	25µl	25µl	25µl

Table 2.5 The amplification conditions for RACE-PCR of various gene homologues of *P. monodon*

Gene homologue	Amplification condition
1. *RING finger protein 121*	
5′RACE	5 cycles of 94 °C for 30 seconds and 72 °C for 2 minutes 5 cycles of 94 °C for 30 seconds, 70 °C for 45 seconds and 72 °C for 2 minutes 20 cycles of 94 °C for 30 seconds, 68 °C for 45 seconds and 72 °C for 2 minutes and 72°C for 7 minutes
Semi-nested 5′RACE	94 °C for 3 minutes 20 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 2 min and 72 °C for 7 minutes
3′RACE	5 cycles of 94 °C for 30 seconds and 72 °C for 2 minutes 5 cycles of 94 °C for 30 seconds, 70 °C for 45 seconds and 72 °C for 2 minutes 20 cycles of 94 °C for 30 seconds, 68°C for 45 seconds and 72°C for 2 minutes and 72 °C for 7 minutes
2. Non-receptor tyrosine kinase	
5′RACE	5 cycles of 94 °C for 45 seconds and 72 °C for 6 minutes 5 cycles of 94 °C for 45 seconds, 70 °C for 45 seconds and 72 °C for 6 minutes 15 cycles of 94 °C for 45 seconds, 68 °C for 45 seconds and 72 °C for 6 minutes and 72 °C for 7 minutes
3′RACE	5 cycles of 94 °C for 45 seconds and 72 °C for 3.30 minutes 5 cycles of 94 °C for 45 seconds, 70 °C for 45 seconds and 72 °C for 3.30 minutes 20 cycles of 94 °C for 45 seconds, 68 °C for 45 seconds and 72 °C for 3.30 minutes and 72 °C for 7 minutes

Table 2.5 (Cont.) The amplification conditions for RACE-PCR of various gene homologues of *P. monodon*

3′Internal RACE	94 °C for 3 minutes 20 cycles of 94 °C for 45 seconds, 68 °C for 45 seconds and 72 °C for 3.30 min and 72 °C for 7 minutes
3. *Receptor tyrosine kinase*	
5′RACE	5 cycles of 94 °C for 30 seconds and 72 °C for 2 minutes 5 cycles of 94 °C for 30 seconds, 70 °C for 30 seconds and 72 °C for 2 minutes 20 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 2 minutes and 72 °C for 7 minutes
5′Internal RACE	94 °C for 3 minutes 20 cycles of 94 °C for 30 seconds, 66 °C for 45 seconds and 72 °C for 2 min and 72 °C for 7 minutes
3′RACE	5 cycles of 94 °C for 30 seconds and 72 °C for 3 minutes 5 cycles of 94 °C for 30 seconds, 70 °C for 45 seconds and 72 °C for 3 minutes 20 cycles of 94 °C for 30 seconds, 68 °C for 45 seconds and 72 °C for 3 minutes and 72 °C for 7 minutes

2.4.4 Elution of RACE-PCR fragments from agarose gel

The amplified fragment was electrophoretically analyzed. After that, the desired individual RACE-PCR bands were excised from agarose gels (200 - 300 mg) using a sterile scalpel and placed into microcentrifuge tube. They were extracted from the gel pieces using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Three to five hundred microliters of the Capture buffer Type 3 was added to the sample. The mixture was incubated at 60 °C for 15-30 minutes until the gel slice was completely dissolved. During the incubation period, the tube was inverted every 5-10 minutes. After spin down, the mixture was removed into the GFX

MicroSpin column that was prepared in a collection tube, and then incubated at room temperature for 1 minute before centrifuged at 13500 rpm for 30 seconds. The flow-through was discarded. The GFX MicroSpin column was placed back in the collection tube. The column was washed by the addition of 500 µl of the ethanol-added Wash buffer type 1 and centrifuged at 13500 rpm for 30 seconds. After discarding the flow-through, the GFX MicroSpin column was centrifuged for 2 minutes at the full speed (14000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 12 µl (60% total volumn) of the Elution buffer type 4 was added to the center of the column matrix. The column was left at room temperature for 2 minutes before centrifuged at the full speed for 2 minutes to recover the gel-eluted DNA.

2.4.5 Ligation of PCR products to the pGEM®-T Easy Vector

The DNA fragment was ligated to the pGEM-T easy vector (Promega) in a 10 µl reactions volume containing 3.5 µl of the gel-eluted DNA, 5 µl of 2X Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM MgCl2, 20 mM DDT, 2 mM ATP and 10% PEG8000), 25 ng of pGEM-T easy vector and 3 units of T4 DNA ligase. The reaction mixture was incubated at 4 ℃ overnight before transformed into *E. coli* stain JM 109.

2.4.6 Preparation of competent cell

A single colony of *E. coli* JM 109 was inoculated in 3 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37 ℃ for 12-16 hours. One milliliter of starting culture was then inoculated into 50 ml of LB broth and continued culture at 37 ℃ with vigorous shaking to OD_{600} of 0.4 to 0.6. The cells were briefly chilled on ice for 30 minutes and recovered by centrifugation at 3000 g for 15 minutes at 4 ℃. The pellets were resuspended by pipetting in 30 ml of ice-cold $MgCl_2/CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and on ice for 45 min before centrifuged at 3000 g for 15 minutes at 4 ℃. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and the suspension was divided into 100-150 µl aliquots. These competent cells was either used immediately or stored at -80 ℃ for subsequently used.

2.4.7 Transformation into *E. coli*

The competent cells were thawed on ice for 5 minutes. Five microliters of the ligation mixture were added and gently mixed by pipetting. The mixture was incubated on ice for 30 minutes. The transformation reaction was heat-shocked at 42 °C by water bath (without shaking) for exactly 45 seconds and immediately placed on ice for 5 minutes. The mixture were removed from tube and added to a new tube containing 1 ml of pre-warmed SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37 °C for 60-90 minutes. The mixture was placed to the new microcentrifuge tube and centrifuged at 8000 rpm for 1 minute at room temperature. The pellet was resuspended in 100 µl of the SOC medium and spread onto a selective LB agar plates (containing 50 µg/ml of amplicillin, 20 µg/ml of X-gal and 25 µg/ml of IPTG) and further incubated at 37 °C for 12-16 hours (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.4.8 Colony PCR

Colony PCR was performed in a 12.5 µl reaction mixture containing 1X buffer (10 mM Tris-HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% Triton X-100), 100 µM of each dNTP, 2 mM $MgCl_2$, 0.1 µM both primer of pUC1 (5'- CCGGCTCGTATGTTGTGTGGA -3') and pUC2 (5'- GTGGTGCAAGGCGATTAAGTTGG -3'), 0.5 unit of Dynazyme™ DNA Polymerase (FINNZYMES). A colony was picked by a pipette tip or steriled toothpick and used as DNA template in the reaction. PCR was carried out in a thermocycler consisting of predenaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 45 seconds and extension at 72 °C for suitable (1 kb of double DNA needed 1 minute). The final extension was carried out at the same temperature for 7 minutes. The colony PCR products were electrophoresed through a 1.0-1.5% agarose gel and visualized after ethidium bromide staining. The colony PCR products containing the insert were separately digested with *EcoR* I (Promega) in a 10 µl reaction volume containing 1X buffer (90 mM Tris-HCl, 10 mM $MgCl_2$ and 50 mM NaCl, pH 7.5 for *E.coR*I), 0.1 mg/ml

BSA, 5 units of each enzyme and 1 µl of the colony PCR product. The reaction mixture was incubated at 37 ℃ overnight. The reaction was analyzed by 1.0-1.5% agarose gel electrophoresis

2.4.9 Extraction of recombinant plasmid DNA

Plasmid DNA was isolated using Plasmid Prep Mini Spin Kit (GE healthcare). A discrete white colony was inoculated into a sterile culture tube containing 3 ml of LB broth supplemented with 50 µg/ml of amplicillin and incubated with shaking (250 rpm) at 37 ℃ for 12-16 hours. The culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 9000 rpm for 1 minute. The supernatant was discarded. The bacterial pellet was resuspended in 175 µl of the Lysis buffer type 7 containing RNaseA and thoroughly mixed by vortexed. The resuspended cells were lysed by the addition of 175 µl of the Lysis buffer type 8 and mixed immediately by gentle inversion (approximately 5-6 times) until solution clear. The Lysis buffer type 9 (350 µl) was added to neutralize the alkaline lysis step and mixed immediately by gentle inversion 5-6 times until the precipitate was evenly dispersed. The mixture was then centrifuged at 14000 rpm for 15 minutes at 15 °C. The supernatant was removed in new microcentrifuge tube before centrifuge repeated. The illustra™ plasmid mini column was placed in a collection tube and the clear lysate was applied into the illustra™ plasmid mini column and centrifuged at 13500 rpm for 30 seconds. The flow-through was discarded. The illustra™ plasmid mini column was placed back in the collection tube. The column was washed by the addition of 400 µl of the Lysis buffer type 9 and centrifuged at 13500 rpm for 30 seconds. After discarding the flow-through, 400 µl of the ethanol-added Wash buffer was added and centrifuged as above. The illustra™ plasmid mini column was further centrifuged for 2 minutes at the full speed (14000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 30 µl of the Elution buffer type 4 was added at the center of the column matrix. The column was left at room temperature for 2 minutes before centrifuged for 2 minutes at the full speed to recover the purified plasmid

DNA. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.4.10 Sequence assembly and analysis

Nucleotide sequences of recombinant plasmids were examined by automated DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing primer by MACROGEN (Korea). Nucleotide sequences were blasted against data in the GenBank (http://www.ncbi.nlm.nih.gov/blast) using Blast*X* (translated protein similarity against the nr database).

The overlapping of obtained nucleotide sequences from 5'RACE and 3'RACE PCR were assembled with the former nucleotide sequence from EST library. The full length cDNAs were confirmed by blasting once more in the GenBank using Blast*X*. The p*I* value and molecular weight of the deduced protein were examined using ProtParam (http://www.expasy.org/tools/protparam.html). The functional domain in the deduced proteins was predicted using SMART (http://smart.embl-heidelberg.de).

2.5 Preparation of 17β-estradiol-supplemented diets and Feeding trials

2.5.1 Preparation of diets

A Total of 4 kilograms of diets were prepared. The ingredients were ground and mixed together for 20 minutes. The mixed diets were pressed to pellet approximately 2 mm in diameter and 4 mm in length. The diets were streamed at 95 °C for 5 minutes and dried at 60 °C for 2 hours, then left to cool down. The diets size were screened by mesh and stored at 4 °C until use. The ingredients of artificial diets that also supplemented with 17β-estradiol were described in Table 2.6.

2.5.2 Feeding trials

The feeding experiments were carried out for the duration of 35 days. The domesticated shrimp (approximately 12 month-old) were acclimated at the laboratory conditions (28±1 °C, 30 ppt seawater) in 1500 liter fish tanks for 1 week.

Two experimental groups of shrimp were fed 3 times daily (5% body weight) with the diets supplementary with 17β-estradiol (1 and 10 mg/kg, *N* = 8 for each group). Non-treated shrimps and the unilateral eyestalk-ablated shrimps were fed with normal diets (no 17β-estradiol supplementation). These experimental shrimps were used for examined the expression analysis of target genes, as describe above in 1.5.

Table 2.6 The ingredients of artificial diets supplemented with 17β-estradiol

Component	Amount kg per 4kg diets
Fish meal	2.18
Shrimp shell powder	0.16
Soybean meal	0.40
Tuna fish oil	0.216
Chlorophyl pink	0.0008
Multiminerals	0.04
Multivitamin	0.04
Cholesterol	0.04
Lecithin	0.04
Wheat gluten	0.24
Vitamin C	0.0024
Vitamin A	0.0008
Vitamin E	0.24
17β-estradiol	1 and 10 mg/kg

2.6 Examination of expression levels of target genes in ovaries of *P. monodon* by quantitative real-time PCR

2.6.1 Primers and construction of the standard curve

The Primers were designed from cDNA sequence of each gene and used to PCR against genomic DNA as the template. The primers that amplified no product from genomics DNA were chosen to use. The expected size of PCR product was approximately 100-250 bp (Table 2.7).

For construction of the standard curve of each gene, the DNA segment covering the target PCR product and *EF-1*α were amplified from primers for quantitative real-time PCR. The PCR products were cloned. Plasmid DNA were extracted and used as the template for estimation of the copy number. A 10 fold-serial dilution was prepared corresponding to 10^3-10^8 molecules/µl. The copy number of standard DNA molecules can be calculated using the following formula:

X ng/µl DNA x 6.022x10^{23} / plasmid length in bp x 660 x 10^9 = Y molecules/µl

X is amount of plasmid dsDNA (ng/ul), 6.022x10^{23} is Avogadro's number (molecules/mole), 660 is average molecular weight of one mole of a bp weighs 650 g, 10^9 used convert to ng from g of dsDNA and Y is amount molecules of plasmid dsDNA per microliters,

The standard curves (correlation coefficient = 0.995-1.000 or efficiency higher than 95%) were drawn for each run. The standard samples were carried out in a 96 well plate and each standard point was run in duplicate. *EF-1*α_{214} was used as the internal control.

2.6.2 Quantitative real-time PCR analysis

The target transcripts and the internal control *EF-1*α_{214} of the synthesized cDNA were amplified in duplicate in volume of 10 µl containing 5 µl of 2x LightCycler 480 SYBR Green I Master (Roche), 0.3 µM each of the gene-specific primers and first strand cDNA template. The reaction thermal profile for SYBR Green real-time PCR of each gene was shown in Table 2.8. The real-time PCR assay was carried out in a 96 well plate and each sample was run in a LightCycler® 480 Instrument II system (Roche).

Table 2.7 Primer sequences and the expected sizes of target genes and *EF-1*α_{214} for quantitative real-time PCR analysis

Gene/Primer	Sequence	Size (bp)
1. ***RING finger protein 121***		
Forward	5' – CAGACGAGGAAAAATGGAGGTTAG -3'	248
Reward	5' – GGGCAGCCAGATGAAAATGA -3'	
2. ***Non-receptor tyrosine protein kinase***		
Forward	5'- TGGGAGACCTCAGTTCATACC -3'	160
Reward	5'- GTGTGCTCTGCCACCAAAAT -3'	
3. ***Receptor tyrosine kinase***		
Forward	5'- TGGTTGCTTGATGTGGGAA -3'	221
Reward	5'- CATTGAGTAGGCTTTGTAGGTTG -3'	
4. ***Elongation factor-1*** α***, EF-1*** α_{214}		
Forward	5′- GTCTTCCCCTTCAGGACGTC -3′	214
Reward	5′- CTTTACAGACACGTTCTTCACGTTG -3′	

A ratio of the absolute copy number of the target genes and that of *EF-1*α_{214} was calculated. The relative expression level between shrimp possessing different stages of ovarian development or treatment groups were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

Table 2.8 Amplification conditions for quantitative real-time PCR analysis of *PmRnf121*, *PmnRTK* and *PmRTK*

Gene	Amplification condition	1st cDNA (ng)
1. *RING finger protein 121*		
	95 ℃ for 10 minutes 40 cycles of 95 ℃ for 15 seconds, 56 ℃ for 30 seconds and at 72 ℃ for 30 seconds. Melting curve analysis was 95 ℃ for 15 seconds, 65 ℃ for 1 minute and at 98 ℃ for continuation and cooling was 40 ℃ for 30 seconds.	50
2. *Non-receptor tyrosine protein kinase*		
	95 ℃ for 10 minutes 40 cycles of 95 ℃ for 15 seconds, 53 ℃ for 30 seconds and at 72 ℃ for 30 seconds. Melting curve analysis was 95 ℃ for 15 seconds, 65 ℃ for 1 minute and at 98 ℃ for continuation and cooling was 40 ℃ for 30 seconds.	300
3. *Receptor tyrosine kinase*		
	95 ℃ for 10 minutes 40 cycles of 95 ℃ for 15 seconds, 58 ℃ for 30 seconds and at 72 ℃ for 30 seconds. Melting curve analysis was 95 ℃ for 15 seconds, 65 ℃ for 1 minute and at 98 ℃ for continuation and cooling was 40 ℃ for 30 seconds.	200
4. *Elongation factor-1α, EF-1α_{214}*		
	95 ℃ for 10 minutes 40 cycles of 95 ℃ for 15 seconds, 58 ℃ for 30 seconds and at 72 ℃ for 30 seconds. Melting curve analysis was 95 ℃ for 15 seconds, 65℃ for 1 minute and at 98℃ for continuation and cooling was 40℃ for 30 seconds.	5

2.7 *In vitro* expression of recombinant proteins using the bacterial expression system

2.7.1 Primers design

Forward and reverse primers were designed to amplify the full-length ORF of each gene (Table 2.9). The forward primer and reverse primer containing restriction enzyme recognized sites *Nde* I and *Bam* HI, respectively that have found only in primer. Moreover, reverse primer also has six Histidine residues encoded nucleotides.

2.7.2 Construction of recombinant plasmids in cloning and expression vectors

The full-length ORF of target genes were amplified by RT-PCR, ligated to pGEM®-T Easy vector and transformed into *E. coli* JM109. Plasmid DNA was extracted from a positive clone and used as the template for amplification using 0.5 μM of each forward primer and reverse primer, 0.5 units *Pfu* DNA polymerase (Promega) and 0.2 mM of each dNTPs. The thermal profiles were predenaturated at 95 ℃ for 3 minutes followed by 25 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 53 ℃ for 45 seconds, extension at 72 ℃ for suitable and final extension at 72 ℃ for 7 minutes.

The amplification product was analyzed by agarose gel electrophoresis and the gel-eluted product was digested with appropriate restriction enzymes. The digested DNA fragment was again analyzed by agarose gel electrophoresis and the gel-eluted product was ligated with pET29a, pET32a and pET43.1a expression vectors and transformed into *E. coli* JM109. Plasmid DNA of the positive clones was sequenced to confirm the orientation of recombinant clones. The corrected direction of plasmid DNA was subsequently transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells, respectively.

Table 2.9 Nucleotide sequences of primers used for *in vitro* expression of target genes of *P. monodon*

Gene/Primer	Sequence
1. *RING finger protein 121*	
Forward-*Nde* I	5'- CGCCATATGGGCCGAGATCTGGCCGAA -3'
Reward-6His-*Bam* HI	5'- GGATCCCTAATGATGATGATGATGATGTTGTAAGCCTAAATAGTT -3'
2. *Receptor tyrosine kinase*	
Forward-*Nde* I	5'- CGCCATATGGCAGAGAAAAATCGCAGG -3'
Reward-6His-*Bam* HI	5′- GGATCCCTAATGATGATGATGATGATCAATAAATTCAAGATG -3′

*underline = *Nde* I or *Bam* HI restriction site, Double underline = start or stop codon, bold line = Histidine usage codon

2.7.3 Expression of recombinant proteins

A single colony of recombinant *E. coli* BL21-CodonPlus (DE3)-RIPL carrying desired recombinant plasmid was inoculated into 5 ml of LB medium, containing 50 µg/ml ampicillin (using with pET32a and pET43.1a) or kanamycin (using with pET29a) and 50 µg/ml chloramphinical at 37 °C. Fifty microlitres of the overnight cultured was transferred to 50 ml of LB medium containing 50 µg/ml amplicillin or kanamycin and 50 µg/ml chloramphinical and further incubated to an OD_{600} of 0.4-0.6. After IPTG induction (1.0 mM final concentration), appropriate volume of the culture corresponding to the OD of 1.0 was time-interval taken (0, 1, 2, 3, 6, 9, 12 and 24 hours at 37 °C) and centrifuged at 12000g for 1 minute. The pellet was resuspended with 50 µl of 1X PBS buffer and examined by 15% SDS-PAGE (Laemmli, 1970).

In addition, 50 ml of the IPTG induced-cultured cells at the most suitable time-interval were taken (6 hours or overnight at 37°C or lower), harvested by centrifugation 5000 rpm for 15 minutes and resuspended in the lysis buffer (0.05

M Tris-HCl; pH 7.5, 0.5 M Urea, 0.05 M NaCl, 0.05 M EDTA; pH 8.0 and 1 mg/ml lysozyme). The cell wall was broken by sonication using Digital Sonifier® sonicator Model 250 (BRANSON). The bacterial suspension was sonicated 2-3 times at 10-15% amplitude, pulsed on for 15 seconds and pulsed off for 15 seconds in a period of 2-5 minutes. Soluble and insoluble portions were separated by centrifuged at 14000 rpm for 30 minutes. The protein concentration of both portions was measured using a dye-binding assay (Bradford, 1976). Expression of the recombinant protein was electrophoretically analyzed by 15% SDS-PAGE.

2.7.4 Detection of recombinant proteins

Recombinant protein was analyzed in 15% SDS-PAGE. The electrophoresed proteins were transferred to a PVDF membrane (Hybond P; GE Healthcare) (Towbin *et al.*, 1979). The membrane was washed three times with 1X Tris-buffer saline tween-20 (TBST; 25 mM Tris, 137 mM NaCl, 2.7 mM KCl and 0.05% tween-20) for 10 minutes each, blocked with 20 ml of a blocking buffer (1.0 g of BSA in 20 ml of 1X TBST) and incubated overnight at room temperature with gentle shaking. The membrane was washed three times in 1X TBST and incubated with diluted Mouse Anti-His antibody IgG2a (GE Healthcare; 1:5000) in the blocking buffer for 1 hour. The membrane was washed three times in 1X TBST before incubated with diluted goat anti-mouse IgG (H+L) conjugated with alkaline phosphatase (Promega; 1:10000) in the blocking buffer for 1 hour. After that, it was washed three times in 1X TBST. Immunoreactional signals were visualized by incubating the membrane in NBT/BCIP (Roche) as a substrate. The color reaction was stopped by transferring the membrane into water.

2.7.5 Purification of recombinant proteins

Recombinant protein was purified by using a His GraviTrap kit (GE Healthcare). Initially, 1 litre of IPTG-induced cultured at the optimal time and appropriate temperature was harvested by centrifugation at 7500 rpm for 10 minutes. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), sonicated and centrifuged at 7500 rpm for 45 minutes at 4 °C. The soluble and insoluble fractions were separated. Soluble or

insoluble fraction composed of the recombinant protein was loaded into column. The column was washed with 10 ml of binding buffer containing 20 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), 5 ml of the binding buffer containing 40 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) and 5 ml of the binding buffer containing 80 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 80 mM imidazole, pH 7.4). The recombinant protein was eluted with 7 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Each fraction of the washing and eluting step were analyzed by 15% SDS-PAGE and western blotting. The purified proteins were stored at 4 $^{\circ}$C or -20 $^{\circ}$C for long term storage.

2.7.6 Polyclonal antibody production

Polyclonal antibody was immunologically produced from the purified protein in a rabbit by Faculty of Associated Medical Sciences, Changmai University. The antibody titer was examined direct enzyme-link immunosorbent assay (ELISA).

2.8 Determination of expression of target proteins of *P. monodon* using Western blot analysis

2.8.1 Total Protein extraction

For extraction of total soluble proteins, approximately 0.5 g of frozen ovaries of *P. monodon* broodstock were ground to fine powder in the presence of liquid N_2 and suspened in 1.2 ml of the TCA–acetone extraction buffer: 10% TCA in acetone containing 0.1% DTT and complete protease inhibitor cocktail (Roche) and left at -20°C for 1 h. The mixture was centrifuged at 10000 g for 30 min at 4 °C and the supernatant was discarded. The pellet was washed three times with acetone before centrifuged at 10000 g for 30 min at 4 °C. The resulting pellet was air-dried and dissolved in the lysis buffer (30 mM Tris–HCl, 2 M thiourea, 7 M urea, 4% CHAPS, w/v). The amount of extracted total proteins was measured by a dye binding assay (Bradford, 1976).

2.8.2 Western blot analysis

Anti-Rnf121 MAb was purchased from Sigma-Aldrich, Inc. The antigen for raising the antibody was from *Homo sapiens* (NCBI reference sequence: NM_018320.4) which share 73.39% similarity with *PmRnf121*.

Total ovarian proteins were heated at 100 °C for 5 minutes and immediately cooled on ice for 5 minutes. Proteins were size-fractionated on a 15% SDS-PAGE (Laemmli, 1970). Electrophoretically separated proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin *et al.*, 1979) in 25 mM Tris and 192 mM glycine (pH 8.3) buffer containing 10% methanol at 100 volts for 90 minutes. The membrane was washed 3 times with 1X Tris Buffer Saline-Tween 20 (TBST; 50 mM Tris–HCl, 0.15 M NaCl pH 7.5 and 0.1% Tween 20) for 5 minutes each. The membrane was blocked by the blocking solution (5% BSA in 1X TBST) for 16 hours at room temperature with shaking. The membrane was washed 3 times with 1X TBST for 10 minutes each and incubated with primary antibody (1:300 Anti-Rnf121 MAb in blocking solution) for 1 hour at room temperature. The membrane was washed 3 times with 1X TBST for 10 minutes each and incubated with Anti-mouse IgG (1:3000 in blocking solution) for 1 hour at room temperature. Washing the membrane with 1X TBST and immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

CHAPTER III

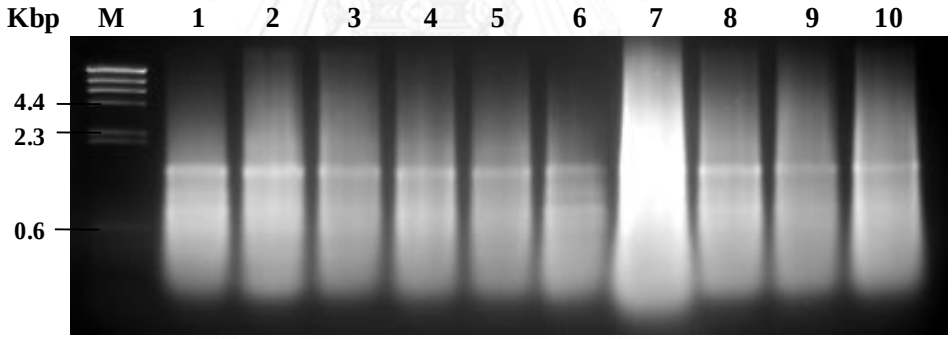
RESULTS

3.1 Isolation and characterization of *Ring finger 121 protein* and *Receptor tyrosine kinase*

3.1.1 Total RNA extraction and first strand cDNA synthesis

Total RNA from ovaries and other tissues of female broodstock and testes of male broodstock of *P. monodon* were extracted. The quality and quantity of total RNA were measured by spectrophotometry and the integrity was observed by agarose gel electrophoresis (Figure 3.1). The ratio of OD_{260}/ OD_{280} of the extracted RNA was 1.8-2.0 indicating that its quality was acceptable for further applications.

A



B

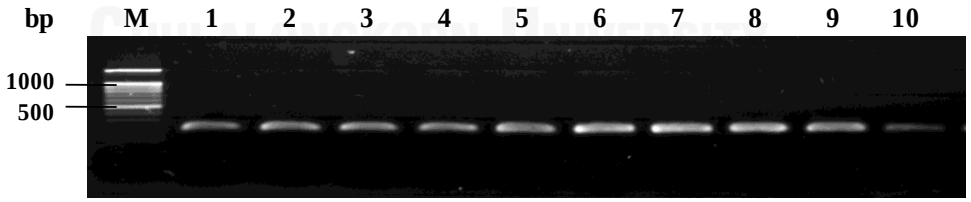


Figure 3.1 1.2% ethidium bromide-stained agarose gel showing (A) the quality of total RNA extracted from ovaries of *P. monodon* broodstock (Lane 1-10) and (B) the first strand cDNA synthesized from DNA-free total RNA ((Lane 1-10). Lanes M = λDNA/*Hind*III marker (A) and a 100 bp DNA ladder (B).

Agarose gel electrophoresis showed discrete ribosomal RNA band reflecting good quality of total RNA. Poor quality of total RNA is also observed (lane 7, A). The first strand cDNA was successfully synthesized from DNA-free total RNA as illustrated by agarose gel electrophoresis.

3.1.2 Isolation and characterization of the full-length cDNA of target genes of *P. monodon*

RACE-Ready cDNA templates were synthesized from ovarian mRNA. The gene-specific primers were design for 5' and 3' RACE-PCR. RACE-PCR was carried out for characterization of reproduction-related transcripts.

3.1.2.1 Ring finger 121 protein (PmRnf121)

The partial cDNA sequence of *PmRnf121* was initially obtained from an ovarian EST library (clone number: OV-N-ST02-0220-LF) of *P. monodon* (http:// pmonodon.biotec.or.th/). BlastX result showed that the closet similarity to this characterized sequence was RING finger protein 121 of *Tribolium castaneum* (*E*-value = 2e-31) (Figure 3.2).

The primary 5' and 3' RACE-PCR of *PmRnf121* were further carried out for isolation of the full-length cDNA of this transcript. The amplification product of 400 and 850 bp in size were obtained from 5' and 3' RACE-PCR (Figure 3.3). Gel-eluted RACE-PCR fragments were cloned and sequenced for both directions. The overlapping nucleotide sequences of RACE-PCR products and the original EST were assembled (Figure 3.4).

A

5’GGCCCACCACGGCCACCAGCACTTTCCCGGCGGCCACATCCCCAATCCGCTGGACGAGAAGC
TGCTCAGGATCATCAGGGAGAATCTGACAGATCAGGTGTCACTGTTATCAGACGAGGAAAAATG
GAGGTTAGAACACTTGAAACTTCACGAGAAACACAAAGGCCACGAAGACATGCATGCAGAAATG
GTCCTCATTCTGATGGTTGTGACGATCATTGCGCA**AGTGGGTCTCGTGGCTTGGAAGAAG**CACC
ATTATAGGTCTTATCAGTTGATAAGCATGTTGGGCGTGTGGCTGATTCCCTTCGTCCTGTGTCT
GCACAACCACTGGTGGAGATTCATTTTCATCTG**GCTGCCCTTCTCCTCCATCACCG**TCATTGTC
ATGAAGAAAGCGCTCGAGAAACCGCTCGATCCAACTACGCCAAGACAGG 3’

B

ref|XP_975438.1| PREDICTED: similar to RING finger protein 121 [*Tribolium castaneum*]
gb|EFA01904.1| hypothetical protein TcasGA2_TC007514 [*Tribolium castaneum*]
Length=317
GENE ID: 664336 LOC664336 | similar to CG15814-PA, isoform A [*Tribolium castaneum*] (10 or fewer PubMed links)

Score = 123 bits (309), Expect = 2e-31
Identities = 51/84 (61%), Positives = 69/84 (82%), Gaps = 0/84 (0%)
Frame = +2

```
Query  176  MHAEMVLILMVVTIIAQVGLVAWKKHHYRSYQLISMLGVWLIPFVLCLHNHWWRFIFIWL  355
            MHAEMVLIL++  ++AQV L+ WKK H RSY L+++LG+W+IP +LCLHN WWRFIFIWL
Sbjct  40   MHAEMVLILILTLVVAQVVLIEWKKRHSRSYLLVTLLGMWIIPLILCLHNRWWRFIFIWL  99

Query  356  PFSSITVIVMKKALEKPLDPTTPR  427
             FS +T +++ KA++KP+  TTPR
Sbjct  100  LFSCVTSLIVWKAMQKPITGTTPR  123
```

Figure 3.2 (A) Nucleotide sequence of an EST significantly matched (B) RING finger protein 121 of *Tribolium castaneum* (*E*-value = 2e-31). 5’- and 3’ RACE-PCR primers are bold-underlined and bold-double underlined, respectively. Arrows indicate the directions of primers.

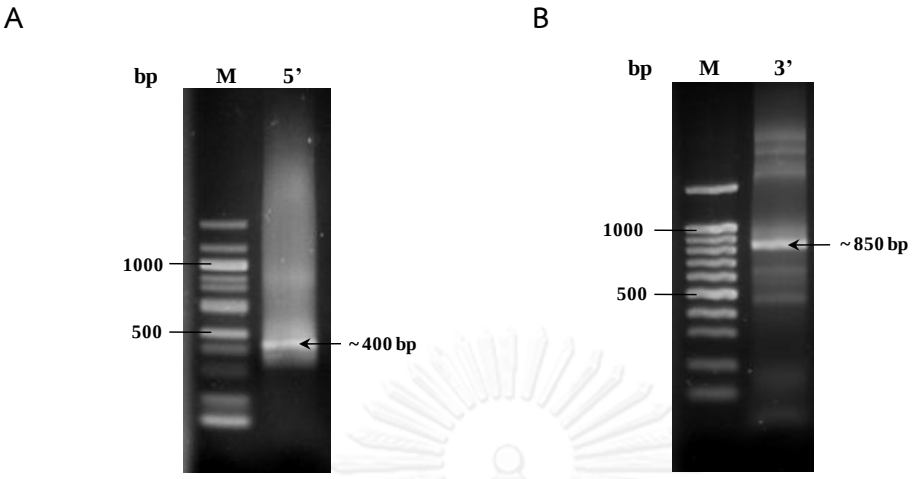


Figure 3.3 1.5% ethidium bromide-stained agarose gels show the amplification product of 400 and 850 from (A) 5'- and (B) 3' RACE-PCR. Lanes M = 100 bp DNA ladder

The full-length cDNA of *PmRnf121* was 1174 bp in length containing the open reading frame (ORF) of 1023 bp that deducing to a polypeptide of 341 amino acids. The 5' and 3' of untranslated regions (UTR) were 24 and 124 bp (excluding the poly A tail), respectively. The closet similarity to *PmRnf121* was RING finger protein 121 of *Tribolium castaneum* (*E*-value = 3e-149). The predicted RING domain was found at positions 240 – 289 of these deduced amino acids of *PmRnf121* (*E*-value = 2.98e-3). The calculated molecular weight and p*I* of the deduced PmRnf121 protein was 39.53 kDa and 8.34, respectively (Figure 3.5).

A

5′**CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT**AGTAACTGTGCCCACCC
GACCAAGATGGCGGCGCGCGAGGCCTACCACGGCCACCAGCACCTTCCCCGCGGCCACATCCCC
AATCCGCTGGACGAGAAGCTGCTCAGGATCATCAGGGAGAATCTGACAGATCAGGTGTCACTGT
TATCAGACGAGGAAAAATGGAGGTTAGAACACTTGAAACTTCACGAGAAACACAAAGGCCACGA
AGACATGCATGCAGAAATGGTCCTCATTCTGATGGTTGTGACGATCATTGCGCA**AGTGGGTCTC**
GTGGCTTGGAAGAAG 3′

B

5′ **GCTGCCCTTCTCCTCCATCACCG**TCATTGTCATGAAGAAAGCGCTCGAGAAACCGCTCGAT
CCAACTACGCCAAGACAGGTGTACAAGTGGTTCCTGCTGATCTACAAGATCTGCTGTGGCCTGG
GGGTGGTAGGCTACATCATCATAATGGTGACGATGATGGGATTCAACCTCCTGTTTGGTGCCAA
GCCCAACACCTGGATGGACTGCGGCCTGCTCTTCATTTTCTACGGCCTCTACTATGGGGTCCTA
GGCCGAGATCTGGCCGAAGTGTGTGCCGATAAGATGGCGTCGCATATCGGGTACTACACACCCC
ACGGCATGCCCACGCGTACCCTAGAGCCAGGCGTGTGTGTGGTGTGTGGCAACCCGCAGCTGGT
CAAGGAGGGGGAGGAAGGTATCATCGAAAACACATACCACCTCTCCTGCGGCCACACCTTCCAC
GAATTCTGCATCCGTGGCTGGTGTATTGTGGGCAAGAAGCAAACGTGCCCATACTGCAAGGAGA
AGGTTGACCTGAAGAAGATGTTCTGCAATCCGTGGGAGAAACCGCACGTGTTTTATGGGCAGTT
GTTAGACTGGATAAGATTACTGGTTGCCTGGCAGCCTGCCATTATATTTCTAGTCCAGGGAATC
AACAACTATTTAGGCTTACAATAGAGACAAGGACAATAGACATCTAGTCATGTTAATAGGAAAC
TTCTGCCTTTTTTGTAACTGAGGTTTACATGGAAATCCTATTGTAGTTTAAAAGAAAAAAAAAA
AAATCAAAAAAAAAAAAAAAGT**ACTCTGCGTTGATACCACTGCTT** 3′

Figure 3.4 Nucleotide sequences of (A) 5'- and (B) 3' RACE-PCR fragments of *PmRnf121*. RACE-PCR primers are boldfaced and highlighted. The overlapped nucleotide sequences of RACE-PCR products and the original EST are underlined. UPM and nested UPM primers are boldfaced and double-underlined.

A

```
AGTAACTGTGCCCACCCGACCAAGATGGCGGCGCGCGAGGCCCACCACGGCCACCAGCAC     60
                         M  A  A  R  E  A  H  H  G  H  Q  H      12
TTTCCCGGCGGCCACATCCCCAATCCGCTGGACGAGAAGCTGCTCAGGATCATCAGGGAG    120
 F  P  G  G  H  I  P  N  P  L  D  E  K  L  L  R  I  I  R  E     32
AATCTGACAGATCAGGTGTCACTGTTATCAGACGAGGAAAAATGGAGGTTAGAACACTTG    180
 N  L  T  D  Q  V  S  L  L  S  D  E  E  K  W  R  L  E  H  L     52
AAACTTCACGAGAAACACAAAGGCCACGAAGACATGCATGCAGAAATGGTCCTCATTCTG    240
 K  L  H  E  K  H  K  G  H  E  D  M  H  A  E  M  V  L  I  L     72
ATGGTTGTGACGATCATTGCGCAAGTGGGTCTCGTGGCTTGGAAGAAGCACCATTATAGG    300
 M  V  V  T  I  I  A  Q  V  G  L  V  A  W  K  K  H  H  Y  R     92
TCTTATCAGTTGATAAGCATGTTGGGCGTGTGGCTGATTCCCTTCGTCCTGTGTCTGCAC    360
 S  Y  Q  L  I  S  M  L  G  V  W  L  I  P  F  V  L  C  L  H    112
AACCACTGGTGGAGATTCATTTTCATCTGGCTGCCCTTCTCCTCCATCACCGTCATTGTC    420
 N  H  W  W  R  F  I  F  I  W  L  P  F  S  S  I  T  V  I  V    132
ATGAAGAAAGCGCTCGAGAAACCGCTCGATCCAACTACGCCAAGACAGGTGTACAAGTGG    480
 M  K  K  A  L  E  K  P  L  D  P  T  T  P  R  Q  V  Y  K  W    152
TTCCTGCTGATCTACAAGATCTGCTGTGGCCTGGGGGTGGTAGGCTACATCATCATAATG    540
 F  L  L  I  Y  K  I  C  C  G  L  G  V  V  G  Y  I  I  I  M    172
GTGACGATGATGGGATTCAACCTCCTGTTTGGTGCCAAGCCCAACACCTGGATGGACTGC    600
 V  T  M  M  G  F  N  L  L  F  G  A  K  P  N  T  W  M  D  C    192
GGCCTGCTCTTCATTTTCTACGGCCTCTACTATGGGGTCCTAGGCCGAGATCTGGCCGAA    660
 G  L  L  F  I  F  Y  G  L  Y  Y  G  V  L  G  R  D  L  A  E    212
GTGTGTGCCGATAAGATGGCGTCGCATATCGGGTACTACACACCCCACGGCATGCCCACG    720
 V  C  A  D  K  M  A  S  H  I  G  Y  Y  T  P  H  G  M  P  T    232
CGTACCCTAGAGCCAGGCGTGTGTGTGGTGTGTGGCAACCCGCAGCTGGTCAAGGAGGGG    780
 R  T  L  E  P  G  V  C  V  V  C  G  N  P  Q  L  V  K  E  G    252
GAGGAAGGTATCATCGAAAACACATACCACCTCTCCTGCGGCCACACCTTCCACGAATTC    840
 E  E  G  I  I  E  N  T  Y  H  L  S  C  G  H  T  F  H  E  F    272
TGCATCCGTGGCTGGTGTATTGTGGGCAAGAAGCAAACGTGCCCATACTGCAAGGAGAAG    900
 C  I  R  G  W  C  I  V  G  K  K  Q  T  C  P  Y  C  K  E  K    292
GTTGACCTGAAGAAGATGTTCTGCAATCCGTGGGAGAAACCGCACGTGTTTTATGGGCAG    960
 V  D  L  K  K  M  F  C  N  P  W  E  K  P  H  V  F  Y  G  Q    312
TTGTTAGACTGGATAAGATTACTGGTTGCCTGGCAGCCTGCCATTATATTTCTAGTCCAG   1020
 L  L  D  W  I  R  L  L  V  A  W  Q  P  A  I  I  F  L  V  Q    332
GGAATCAACAACTATTTAGGCTTACAATAGAGACAAGGACAATAGACATCTAGTCATGTT   1080
 G  I  N  N  Y  L  G  L  Q  *                                  341
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AAAGAAAAAAAAAAAAAATCAAAAAAAAAAAAAAA                            1174
```


B

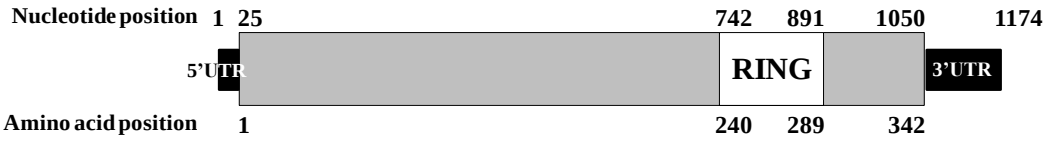


Figure 3.5 (A) The full-length cDNA and deduced amino acid sequences of *PmRnf121*. Start and stop codons are illustrated in boldface and underlined. The RING domain at amino acid positions 240 – 289 of the deduced protein is highlighted. (B) A schematic diagram illustrates the full-length cDNA and deduced amino acid sequences of *PmRnf121*.

3.1.2.2 Receptor tyrosine kinase (PmRTK)

The partial cDNA sequence of *PmRTK* was initially obtained from GenBank (accession no. GO081935.1; http://www.ncbi.nlm.nih.gov/). BlastX analysis showed that the closet similarity to this characterized sequence was tyrosine kinase receptor Cad96Ca-like of *Apis florea* (*E*-value = 7e-22) (Figure 3.6).

The primary 5' and 3' RACE-PCR of *PmRTK* were further carried out for isolation of the full-length cDNA of this transcript. The amplification product of 1.7 Kb and 800 bp in size were obtained 5' and 3' RACE-PCR (Figure 3.7). RACE-PCR fragments were excised from agarose gel, cloned and sequenced for both directions. The overlapping nucleotide sequences of RACE-PCR products and the original EST were assembled (Figure 3.8)

The partial cDNA sequence of *PmRTK* was obtained and it was 1387 bp in length containing the partial ORF of 587 bp deducing to a polypeptide of 195 amino acids. The 3’ UTR was 797 bp (excluding the poly A tail). The closet similarity to *PmRTK* was putative tyrosine-protein kinase Wsck-like of *Bombyx mori* (*E*-value = 1e-44). The predicted tyrosine kinase catalytic domain (STYKc) was found at positions 1 - 162 of these deduced amino acids of *PmRTK* (*E*-value = 5.44e-11). The calculated molecular weight and p*I* of the deduced PmRTK protein was 51.57 kDa and 8.97, respectively (Figure 3.9).

A

5′GCACGAGGTTGTGTTGATAGATAGCATACGTCCAAAGGTCACTGGGTTTGGACTCCTCCACT

ATCATAATGATTTGTATGTACCTGAGTATAAA**CGCTGGCACGCTGAGGAAACGCT**CCGGTCCAA

GGTGTCTGTTCCTAAGAGTGATGTTTGGTCATT**TGGTTGCTTGATGTGGGAAGTGGC**CACTCTT

GGGGGGACGCCCTATGCAGATGTAAAGACTGATGAGGTTCCTGGCAGGGTTATGCGAGGCCTGC

GACTGCCACAGCCTCAGTATGTTGGTGATGAGCTCTATCAACTGATGCTGAATTGCTGGCAGAT

GGACCGTGATGAGCGACCTACTTTCCAAGAGCTGGAGGGCAACCTACAAAGCCTACTCAATGAT

GATGTTACACCACATCTTCTCTTCTCTTTGTATCCATCATTCCAGTATGAGCACTATGCCCCCC

ATCTTGAATTTATTGATTAGAACCATACTGTA**CCTCCTTTGGTGGCGTAACTGTCATCC**ATGCT

CATCTCTATGAGCAAACATCTGTCTTGCCAGAACTCACATGCTTGAACATGTGGCAGATTTCTA

GGTGATTAGGTAAATCAATGTTGTTCAGAATTCTTGTGGCCAATAATTGAAACATGTGCCAATT

AAGCAGTATTGTTATGCACATGTTTTTCAAAGCACAAATTTTGAAAATTTATATTTGTTTTTAG

AAATACCTTATGAAATTGGTAAACTA 3′

B

ref|XP_003694975.1| PREDICTED: tyrosine kinase receptor Cad96Ca-like [*Apis florea*]
Length=175

GENE ID: 100868420 LOC100868420 | tyrosine kinase receptor Cad96Ca-like [*Apis florea*]

Score = 98.2 bits (243), Expect = 7e-22, Method: Compositional matrix adjust.
Identities = 55/139 (40%), Positives = 76/139 (55%), Gaps = 10/139 (7%)
Frame = +2

```
Query  11   VLIDSIR-PKVTGFGLLHYHN-DLYVPEYK--------RWHAEETLRSKVSVPKSDVWSF  160
            VL+D  +  K+  FG+  + + D  V E +         RW A E+L   +   K+DVWSF
Sbjct  24   VLVDHNKLCKIADFGMSRFADGDGEVIETRHGRNALPIRWMAPESLIYSLFTTKTDVWSF  83

Query  161  GCLMWEVATLGGTPYADVKTDEVPGRVMRGLRLPQPQYVGDELYQLMLNCWQMDRDERPT  340
            G LMWE+ TLG TPY D+   EV   V  G RL +P +   EL++++  CW  D D RP
Sbjct  84   GILMWEIVTLGSTPYPDMTAREVMRNVQNGYRLERPSHCRSELFRVISRCWHADPDRRPE  143

Query  341  FQELEGNLQSLLNDDVTPH  397
            FQ L  +L  LL D++  H
Sbjct  144  FQTLRRDLAQLLEDNMNGH  162
```

Figure 3.6 (A) Nucleotide sequence of a cDNA significantly matched (B) tyrosine kinase receptor Cad96Ca-like of *Apis florea* (*E*-value = 7e-22) retrieved from GenBank. 5'- and 3' RACE-PCR primers are bold-underlined. The 5' internal primer is bold-double underlined. Arrows indicate the directions of primers.

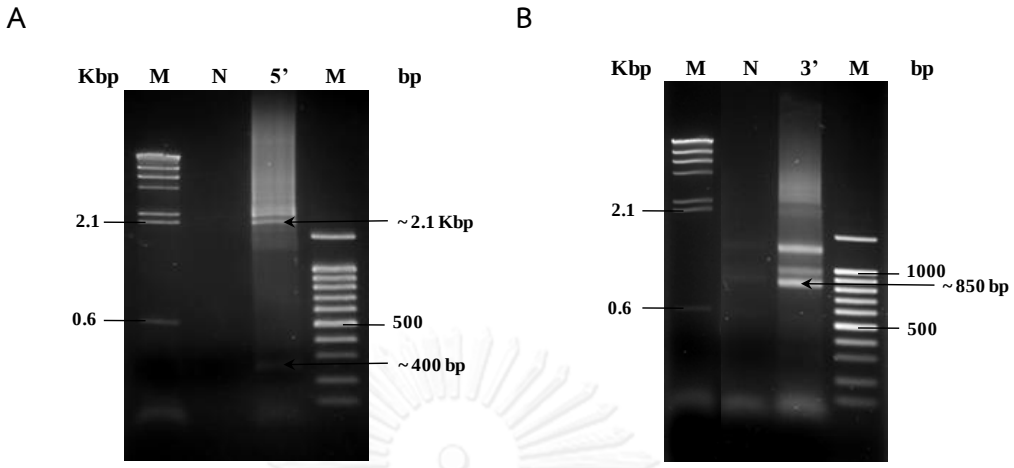


Figure 3.7 1.2% ethidium bromide-stained agarose gels showing the amplification products of 1.7 Kb and 800 bp of (A) 5'- and (B) 3' RACE-PCR. Lanes M = λDNA/*Hind*III marker (Left) and a 100 bp DNA ladder (Right), lanes N = negative control (without the cDNA template).

3.2 Expression patterns and tissue distribution analysis of reproduction-related genes in ovaries of *P. monodon* examined by RT-PCR

3.2.1 Expression patterns of reproduction-related genes in ovaries and testes of juveniles and broodstock of *P. monodon*

Three pairs of primers were designed. The amplification product of Ring finger 121 (*PmRnf121*), Non-receptor tyrosine kinase (*PmnRTK*) and Receptor tyrosine kinase (*PmRTK*) were examined in ovaries and testes of juvenile and wild broodstock of *P. monodon*.

A

5′**AAGCAGTGGTATCAACGCAGAGT**ATGCAGAGAAAAATCGCAGGTTTTCCACACTTCCAGAA
GGACAGGCCCTTGAAATCCTCCTTGGTGTTGCACGAGGTATGAATCATCTTGCCTCACTAAATG
TTACCCATGGTCAGCCCTGTGTCAGGAATGTTGTGTTGATAGATAGCATACGTCCAAAGGTCAC
TGGGTTTGGACTCCTCCACTATCATAATGATTTGTATGTACCTGAGTATAAA**CGCTGGCACGCT**
GAGGAAACGCT 3′

B

5′**CCTCCTTTGGTGGCGTAACTGTCATCC**ATGCTCATCTCTATGAGCAAACATCTGTCTTGCCA
GAACTCACATGCTTGAACATGCGGCGGATTTCTAGGTGATTAGGTAAATCAATGTTGTTCAGAA
TTCTTGTGGCCAATAATTGATACGTGTGCCAATTAAGCAGTATTGTTATGCACATGTTTTTCAA
AGCACAAATTTTGAAAATTAATATTTGTTTTTAGAAATACCTTATGAAATTGGTAAACTAAGAT
CTCCAGTCCATTCCACTAGATATAAACCAGATTCTTGTTTATATTGCTCAAGATTCTCTTGATA
CTTTGCAGCACAATAAATGCTTTTAGCATTTTCCTAGGAAAGTTTAGGCTATATTTTTTTCATT
TTTATGAATGGAAAACTTTCTGCTTTGTGATAATTAAGAATGTAAAATCAGGAATAGATTGCAA
ATTAATTACAGTGCACTCTCTGGTATCTTTTTTATTTTTATGTGATAGTTTTGTCAGAAGTGTC
TGCTAAAGAGTGTGCCAAAGATTATAGAAATTCCTTTTAGATACTTAAAAGTTCCTAGGTGAAA
ATGAAACAACTTGGCAGGCTTATTTTTCAGGTTACAGCCTTTAGGAACATAGTAACTTTTAGAT
GATGAGATGTCTAGTTATTAATGATATGCTTATGTGACTAAAGCATAATAAAAGATATTGTACT
TAATTTTTATATGATTTGTTTAATAGTATCAAATTTACAATAAAATTATGTAACCAAAAAAAAA
AAAAAAAAAAAAAAAAAGT**ACTCTGCGTTGATACCACTGCTT** 3′

Figure 3.8 Nucleotide sequences of (A) 5'- and (B) 3' RACE-PCR fragments of *PmRTK*. RACE-PCR primers are boldfaced and highlighted. The overlapping nucleotide sequences of RACE-PCR products and the original EST are underlined. A nested UPM primer is double-underlined.

A

```
ATGCAGAGAAAAATCGCAGGTTTTCCACACTTCCAGAAGGACAGGCCCTTGAAATCCTCC     60
   A  E  K  N  R  R  F  S  T  L  P  E  G  Q  A  L  E  I  L        19
TTGGTGTTGCACGAGGTATGAATCATCTTGCCTCACTAAATGTTACCCATGGTCAGCCCT     120
L  G  V  A  R  G  M  N  H  L  A  S  L  N  V  T  H  G  Q  P      39
GTGTCAGGAATGTTGTGTTGATAGATAGCATACGTCCAAAGGTCACTGGGTTTGGACTCC     180
C  V  R  N  V  V  L  I  D  S  I  R  P  K  V  T  G  F  G  L      59
TCCACTATCATAATGATTTGTATGTACCTGAGTATAAACGCTGGCACGCTGAGGAAACGC     240
L  H  Y  H  N  D  L  Y  V  P  E  Y  K  R  W  H  A  E  E  T      79
TCCGGTCCAAGGTGTCTGTTCCTAAGAGTGATGTTTGGTCATTTGGTTGCTTGATGTGGG     300
L  R  S  K  V  S  V  P  K  S  D  V  W  S  F  G  C  L  M  W      99
AAGTGGCCACTCTTGGGGGGACGCCCTATGCAGATGTAAAGACTGATGAGGTTCCTGGCA     360
E  V  A  T  L  G  G  T  P  Y  A  D  V  K  T  D  E  V  P  G     119
GGGTTATGCGAGGCCTGCGACTGCCACAGCCTCAGTATGTTGGTGATGAGCTCTATCAAC     420
R  V  M  R  G  L  R  L  P  Q  P  Q  Y  V  G  D  E  L  Y  Q     139
TGATGCTGAATTGCTGGCAGATGGACCGTGATGAGCGACCTACTTTCCAAGAGCTGGAGG     480
L  M  L  N  C  W  Q  M  D  R  D  E  R  P  T  F  Q  E  L  E     159
GCAACCTACAAAGCCTACTCAATGATGATGTTACACCACATCTTCTCTTCTCTTTGTATC     540
G  N  L  Q  S  L  L  N  D  D  V  T  P  H  L  L  F  S  L  Y     179
CATCATTCCAGTATGAGCACTATGCCCCCCATCTTGAATTTATTGATTAGAACCATACTG     600
P  S  F  Q  Y  E  H  Y  A  P  H  L  E  F  I  D  *               195
TACCTCCTTTGGTGGCGTAACTGTCATCCATGCTCATCTCTATGAGCAAACATCTGTCTT     660
GCCAGAACTCACATGCTTGAACATGCGGCGGATTTCTAGGTGATTAGGTAAATCAATGTT     720
GTTCAGAATTCTTGTGGCCAATAATTGATACGTGTGCCAATTAAGCAGTATTGTTATGCA     780
CATGTTTTTCAAAGCACAAATTTTGAAAATTAATATTTGTTTTTAGAAATACCTTATGAA     840
ATTGGTAAACTAAGATCTCCAGTCCATTCCACTAGATATAAACCAGATTCTTGTTTATAT     900
TGCTCAAGATTCTCTTGATACTTTGCAGCACAATAAATGCTTTTAGCATTTTCCTAGGAA     960
AGTTTAGGCTATATTTTTTTCATTTTTATGAATGGAAAACTTTCTGCTTTGTGATAATTA    1020
AGAATGTAAAATCAGGAATAGATTGCAAATTAATTACAGTGCACTCTCTGGTATCTTTTT    1080
TATTTTTATGTGATAGTTTTGTCAGAAGTGTCTGCTAAAGAGTGTGCCAAAGATTATAGA    1140
AATTCCTTTTAGATACTTAAAAGTTCCTAGGTGAAAATGAAACAACTTGGCAGGCTTATT    1200
TTTCAGGTTACAGCCTTTAGGAACATAGTAACTTTTAGATGATGAGATGTCTAGTTATTA    1260
ATGATATGCTTATGTGACTAAAGCATAATAAAAGATATTGTACTTAATTTTTATATGATT    1320
TGTTTAATAGTATCAAATTTACAATAAAATTATGTAACCAAAAAAAAAAAAAAAAAAAAA    1380
AAAAAGT                                                         1387
```

B

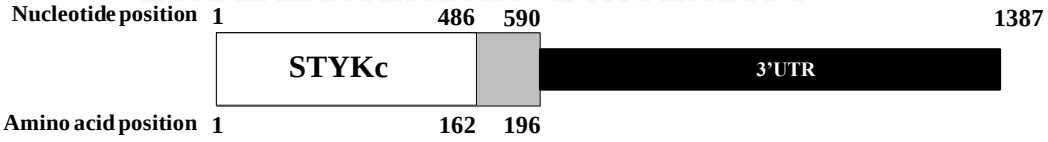


Figure 3.9 (A) The partial cDNA and deduced amino acid sequences of *PmRTK*. Stop codon is boldfaced and underlined and the predicted STYKc domain located at positions 1 - 162 of the deduced protein is highlighted. Polyadenylation additional signal (AATAA) is boldfaced and underlined (B) A schematic diagram illustrates the partial-length cDNA and deduced amino acid sequences of *PmRTK*.

3.2.1.1 Ring finger 121 protein (PmRnf121)

The expected size of 248 bp product was obtained for amplification of *PmRnf121*. RT-PCR indicated that *PmRnf121* was more abundantly expressed in ovaries than testes in both juveniles and wild broodstock (Figure 3.10).

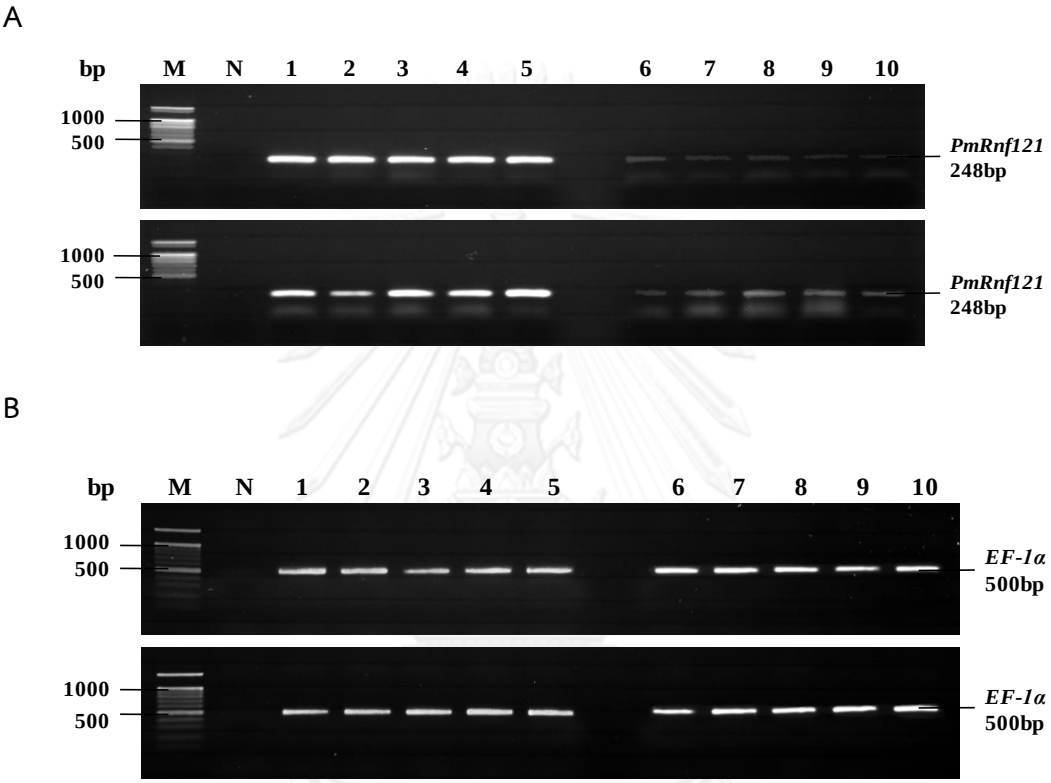


Figure 3.10 1.5% ethidium bromide-stained agarose gel shows the expression profiles of *PmRnf121* in ovaries (lanes 1-5, A) and testes (lanes 6-10, A) of juveniles (upper) and wild broodstock (lower) of *P. monodon*. (B) *EF-1α* was successfully amplified from the same template. Lanes M = 100 bp DNA ladder and Lane N = negative control (without cDNA templates).

3.2.1.2 Non-receptor tyrosine kinase (PmnRTK)

PmnRTK primers generated the expected amplification product of 289 bp in size. Like *PmRnf121*, *PmnRTK* was more abundantly expressed in ovaries than testes in both juvenile and wild broodstock (Figure 3.11).

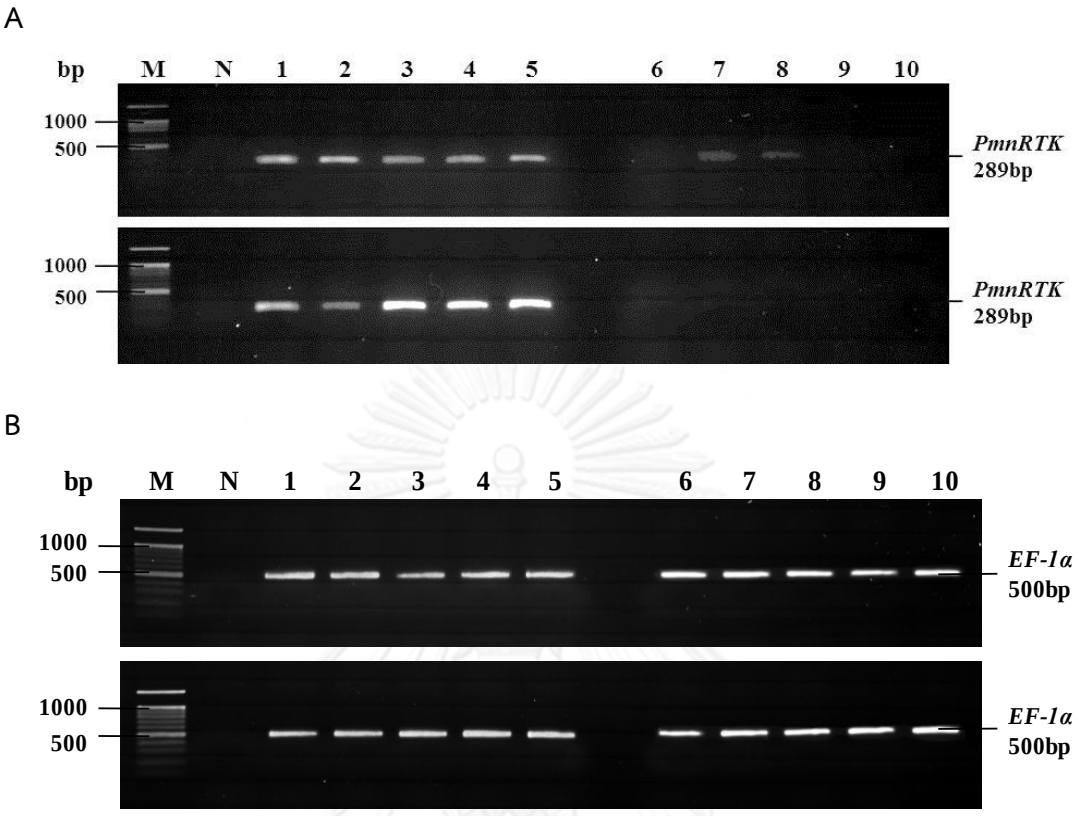


Figure 3.11 1.5% ethidium bromide-stained agarose gel shows the expression profiles of *PmnRTK* in ovaries (lanes 1-5, A) and testes (lanes 6-10, A) of juveniles (upper) and wild broodstock (lower) of *P. monodon*. (B) *EF-1α* was successfully amplified from the same template. Lanes M = 100 bp DNA ladder and Lane N = negative control (without cDNA templates).

3.2.1.3 Receptor tyrosine kinase (PmRTK)

A 221 bp product was obtained for amplification of *PmRTK*. RT-PCR indicated that *PmRTK* was more abundantly expressed in ovaries than testes in both juveniles and wild broodstock (Figure 3.12).

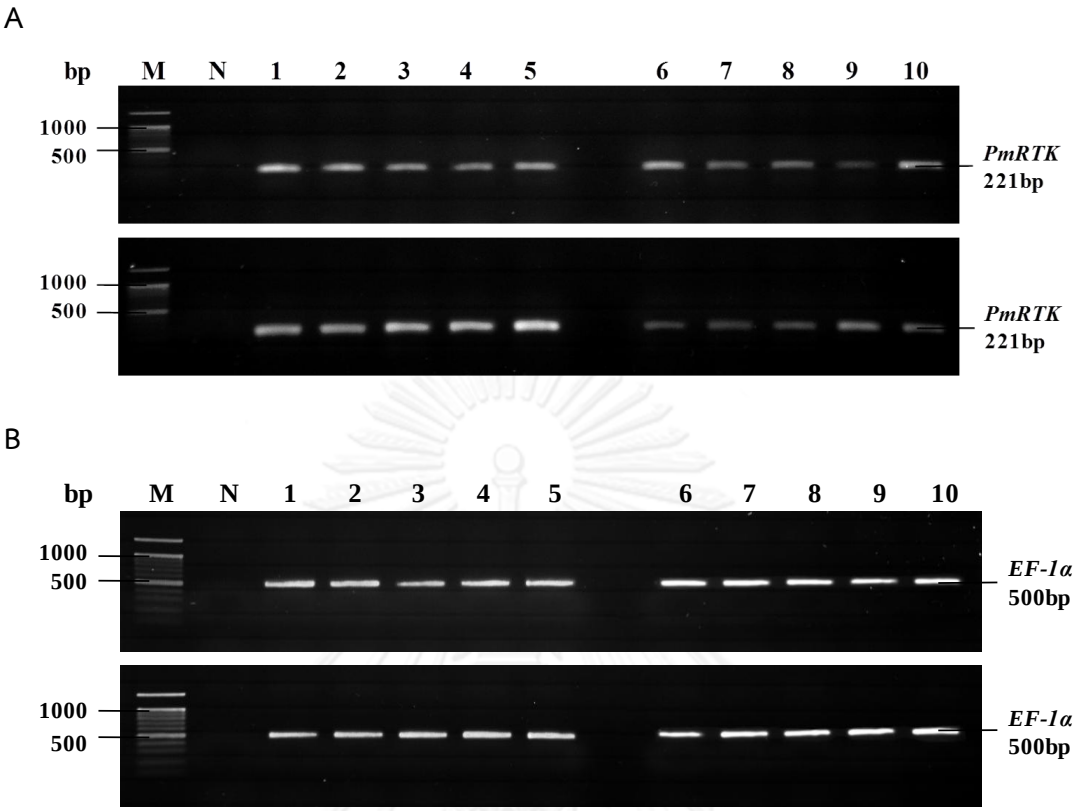


Figure 3.12 1.5% ethidium bromide-stained agarose gel shows the expression profiles of *PmRTK* (A) in ovaries (lanes 1-5, A) and testes (lanes 6-10, A) of juveniles (upper) and wild broodstock (lower) of *P. monodon*. (B) *EF-1*α was successfully amplified from the same template. Lanes M = 100 bp DNA ladder and Lane N = negative control (without cDNA templates).

3.2.2 Tissue distribution analysis

Expression of *PmRnf121*, *PmnRTK* and *PmRTK* in 14 tissues: ovaries, testes, antennal gland, eyestalks, subcuticular epithelium, gills, heart, hemocytes, hepatopancreases, intestine, lymphoid organs, pleopods, stomach, and thoracic ganglion.

3.2.2.1 Ring finger 121 protein (PmRnf121)

PmRnf121 was expressed in all tissues and the most abundant expression was observed in ovaries than other tissues. *PmRnf121* was moderately

expressed in hemocytes, gills and hepatopancreas. Low expression of *PmRnf121* was observed in testes, heart, stomach, intestine, eyestalk, antennal gland, subcuticular epithelium, lymphoid organs, pleopods, and thoracic ganglion (Figure 3.13 and Table 3.1).

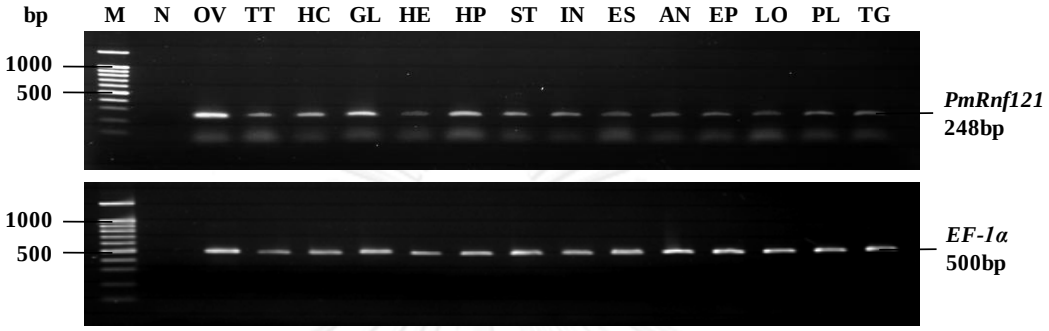


Figure 3.13 1.5% ethidium bromide-stained agarose gel showing tissue distribution analysis of *PmRnf121* in various tissues of wild broodstock of *P. monodon* against the first strand cDNA templates of ovaries (OV), testes (TT), hemocytes (HC), gills (Jiravanichpaisal et al.), heart (HE), hepatopancreas (Jiravanichpaisal et al.), stomach (ST), intestine (IN), eyestalk (ES), antennal gland (AN), subcuticular epithelium (EP), lymphoid organs (LO), pleopods (Platon), and thoracic ganglion (TG). *EF-1α* was successfully amplified from the same template. Lane M and N are 100 bp DNA ladder and negative control, respectively.

3.2.2.2 Non-receptor tyrosine kinase (PmnRTK)

PmnRTK was expressed in examined all tissues excepted in testes. Its expression in hemocytes and thoracic ganglion was greater than that of other tissues. A lower expression of *PmnRTK* were observed in ovaries, gills, heart, hepatopancreas, stomach, intestine, eyestalk, antennal gland, subcuticular epithelium, lymphoid organs and pleopods (Figure 3.14 and Table 3.1).

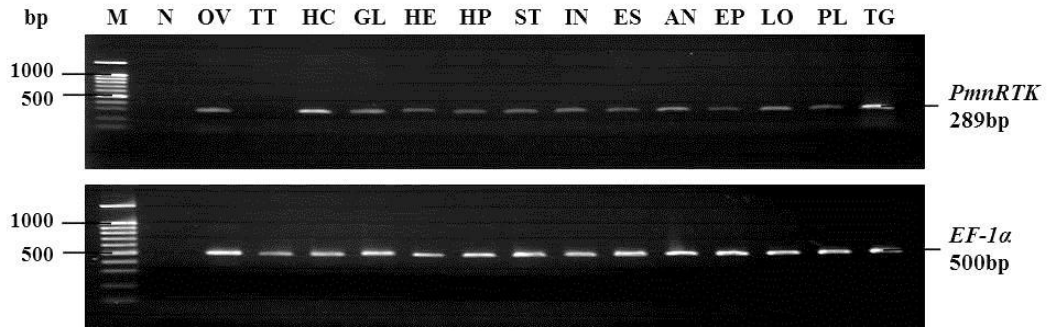


Figure 3.14 1.5% ethidium bromide-stained agarose gel showing tissue distribution analysis of *PmnRTK* in various tissues of wild broodstock of *P. monodon* against the first strand cDNA templates of ovaries (OV), testes (TT), hemocytes (HC), gills (Jiravanichpaisal et al.), heart (HE), hepatopancreas (Jiravanichpaisal et al.), stomach (ST), intestine (IN), eyestalk (ES), antennal gland (AN), subcuticular epithelium (EP), lymphoid organs (LO), pleopods (Platon), and thoracic ganglion (TG). *EF-1*α was successfully amplified from the same template. Lane M and N are 100 bp DNA ladder and negative control, respectively.

3.2.2.3 Receptor tyrosine kinase (PmRTK)

PmRTK was expressed in all tissues. Its expression in thoracic ganglion and ovaries was greater than other tissues. A lower expression of *PmRTK* was observed in gills, intestine, eyestalk, antennal gland and pleopods. Limited expressions of PmRTK were observed in testes, hemocytes, heart, hepatopancreas, stomach and subcuticular epithelium (Figure 3.15 and Table 3.1).

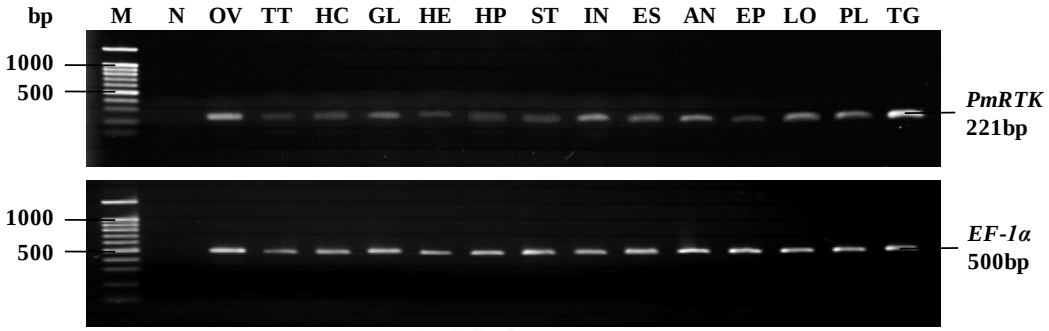


Figure 3.15 1.5% ethidium bromide-stained agarose gel showing tissue distribution analysis of *PmRTK* in various tissues of wild broodstock of *P. monodon* against the first strand cDNA templates of ovaries (OV), testes (TT), hemocytes (HC), gills (Jiravanichpaisal et al.), heart (HE), hepatopancreas (Jiravanichpaisal et al.), stomach (ST), intestine (IN), eyestalk (ES), antennal gland (AN), subcuticular epithelium (EP), lymphoid organs (LO), pleopods (Platon), and thoracic ganglion (TG). *EF-1α* was successfully amplified from the same template. Lane M and N are 100 bp DNA ladder and negative control, respectively.

Table 3.1 Expression profiles of *PmRnf121*, *PmnRTK* and *PmRTK* in various tissues of *P. monodon*

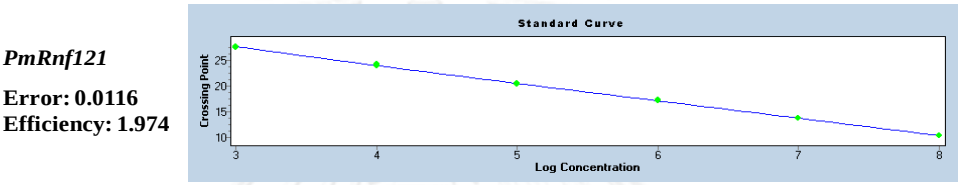
Gene	Various tissues of wild broodstock													
	OV	TT	HC	GL	HE	HP	ST	IN	ES	AN	EP	LO	PL	TG
PmRnf121	+++	+	++	++	+	++	+	+	+	+	+	+	+	+
PmnRTK	+	-	++	+	+	+	+	+	+	+	+	+	+	++
PmRTK	+++	+	+	++	+	+	+	++	++	++	+	+	++	+++

+ = low abundant expression, ++ = moderately abundant expression, +++ = high abundant expression

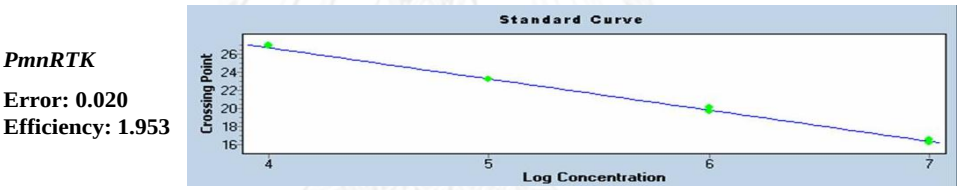
3.3 Examination of the expression levels of *PmRnf121, PmnRTK* and *PmRTK* during ovarian development of *P. monodon*

The standard curve of each target genes and the control *(EF-1α_{214})* were constructed from 10-fold dilutions covering 10^3- 10^8 copy numbers of dsDNA of these genes. High efficiency of amplification of the examined transcripts and limited errors were found (Figure 3.16). Therefore, these standard curves were acceptable to be used for quantitative estimation of examined genes.

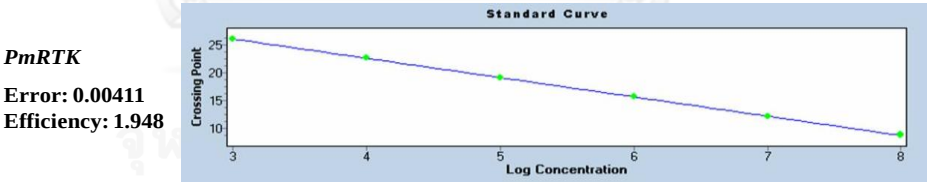
A



B



C



D

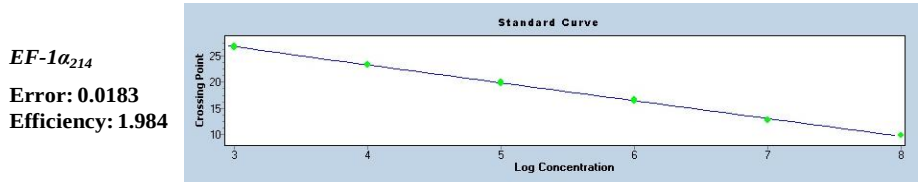


Figure 3.16 Standard curve of (A) *PmRnf121* (Efficiency = 1.974, R^2 = 0.999, Y = -3.464 * log(X) + 37.96), (B) *PmnRTK* (Efficiency = 1.953, R^2 = 0.999, Y = -3.521 * log(X) + 37.44), (C) *PmRTK* (Efficiency = 1.948, R^2 = 0.999, Y = -3.457 * log(X) + 36.39) and (D) *EF-1α_{214}* (Efficiency = 1.984, R^2 = 0.999, Y = -3.449 * log(X) + 38.74)

3.3.1 *Ring finger 121 protein* (*PmRnf121*)

Quantitative real-time PCR analysis indicated that the expression level of *PmRnf121* mRNA in 4-month-old juveniles was significantly lower than that in stages II, III and IV ovaries of intact broodstock ($P < 0.05$). Its expression level in stage II and IV of eyestalk-ablated broodstock was also significantly greater than that in juveniles ($P < 0.05$).

In intact broodstock, *PmRnf121* was not differentially expressed during ovarian development ($P > 0.05$). Eyestalk ablation did not affect the expression level of ovarian *PmRnf121* ($P > 0.05$, Figure 3.17).

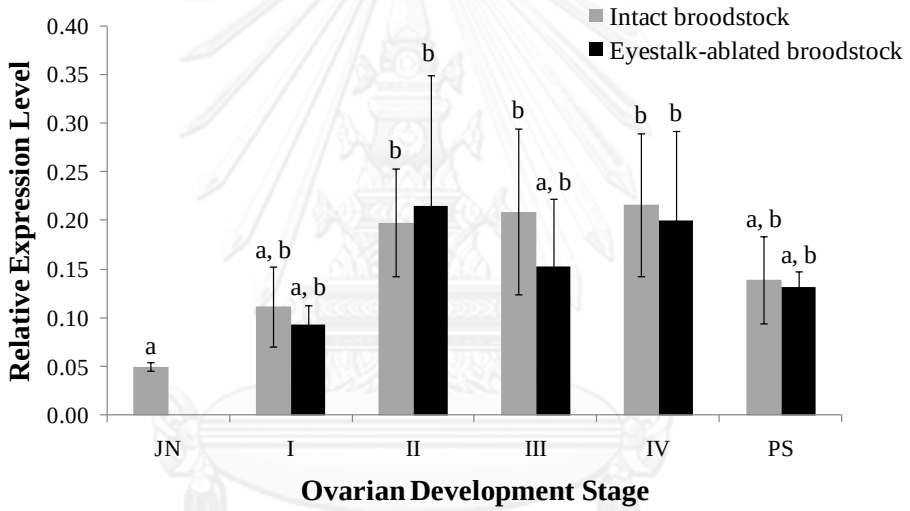


Figure 3.17 Histograms shows the relative expression profile of *PmRnf121* in ovaries of 4-month-old juveniles (JN) and different stages of ovarian development (I = previtellogenic, II = vitellogenic, III = early cortical rod, IV = mature ovaries and PS = post-spawning) of intact and unilateral eyestalk-ablated broodstock. Expression levels were measured as the absolute copy number of *PmRnf121* mRNA (50 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.3.2 *Non-receptor tyrosine kinase* (*PmnRTK*)

Quantitative real-time PCR analysis indicated that the expression level of *PmnRTK* mRNA in stage I - IV and after spawning in intact broodstock was significantly greater than that in juvenile ($P < 0.05$). Likewise, its expression level during ovarian development of eyestalk-ablated broodstock was also significantly greater than that in premature ovaries of juveniles ($P < 0.05$).

PmnRTK was not differentially expressed during ovarian development in intact broodstock ($P > 0.05$). The expression level in this transcript in post-spawning was significantly reduced from stage IV ovaries ($P < 0.05$). In eyestalk-ablated broodstock, *PmnRTK* was significantly up-regulated in stage IV ($P < 0.05$). The relative expression level of *PmnRTK* in stages IV ovaries was also significantly greater than that of the same stage in intact broodstock (Figure 3.18).

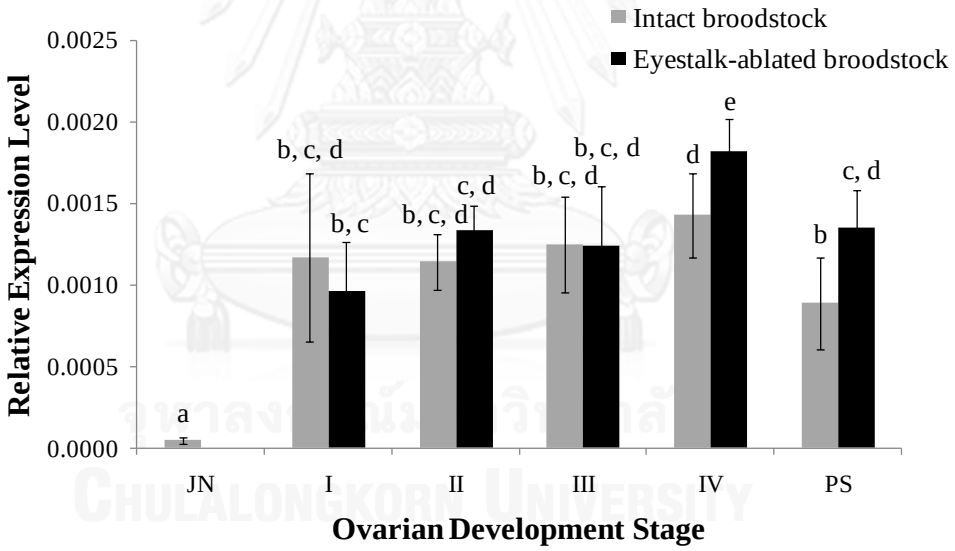


Figure 3.18 Histograms shows the relative expression profile of *PmnRTK* in ovaries of 4-month-old juveniles (JN) and different stages of ovarian development (I = previtellogenic, II = vitellogenic, III = early cortical rod, IV = mature ovaries and PS = post-spawning) of intact and unilateral eyestalk-ablated broodstock. Expression levels were measured as the absolute copy number of *PmnRTK* mRNA (200 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a

particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.3.3 *Receptor tyrosine kinase* (*PmRTK*)

Quantitative real-time PCR analysis indicated that the expression level of *PmRTK* mRNA in stage I - IV and post-spawning ovaries was significantly greater than that in premature ovaries of juvenile in intact broodstock ($P < 0.05$). Similarly, its expression level in stage II - IV of eyestalk-ablated broodstock was also significantly greater than that in juveniles ($P < 0.05$).

In intact broodstock, the expression levels of *PmRTK* were significantly increased in stage III relative to stage I ovaries. Its expression peaked in stage IV ovaries before reduced to threshold level after spawning ($P < 0.05$). In eyestalk-ablated broodstock, *PmnRTK* was significantly up-regulated in stage II and III relative to stage I ovaries and it was further increased in stage IV ovaries before reduced to the normal level after spawning ($P < 0.05$, Figure 3.19).

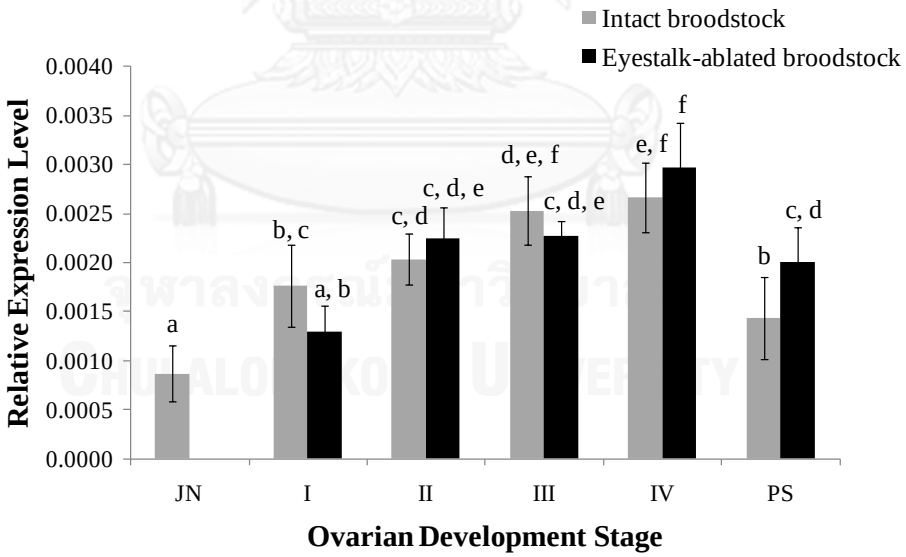


Figure 3.19 Histograms shows the relative expression profile of *PmRTK* in ovaries of 4-month-old juveniles (JN) and different stages of ovarian development (I = previtellogenic, II = vitellogenic, III = early cortical rod, IV = mature ovaries and PS = post-spawning) of intact and unilateral eyestalk-ablated broodstock. Expression levels were measured as the absolute copy number of *PmRTK* mRNA (100 ng template)

and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.4 Expression levels of *PmRnf121, PmnRTK* and *PmRTK* mRNA in domesticated shrimp injected with serotonin

3.4.1 *Ring finger 121 protein* (*PmRnf121*)

Quantitative real-time PCR analysis indicated that the expression level of *PmRnf121* mRNA in 18-month old domesticated shrimp injected with serotonin was not significantly different compared with the negative and vehicle control ($P > 0.05$). The relative expression profiles of *PmRnf121* in domesticated *P. monodon* broodstock were shown in Figure 3.20.

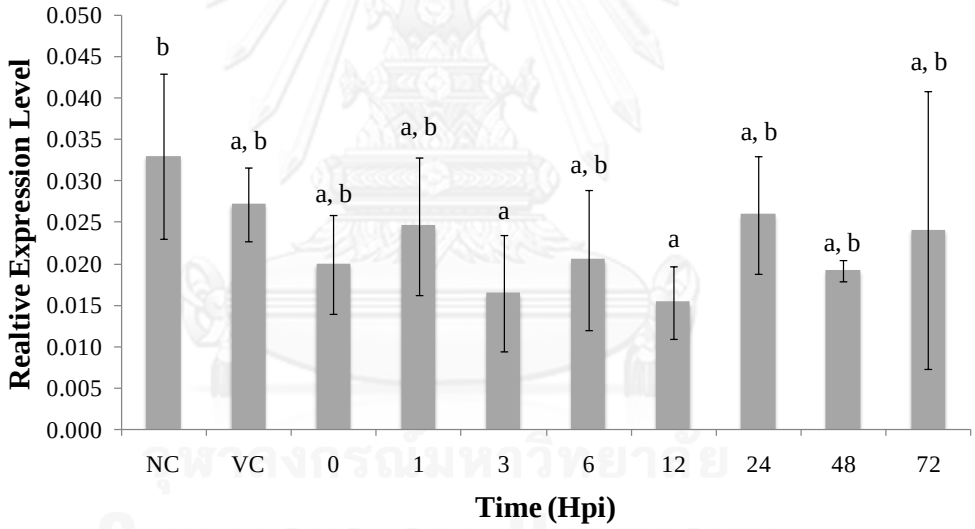


Figure 3.20 Histograms shows the relative expression profile of *PmRnf121* in domesticated shrimps injected with serotonin that collected at 0, 1, 3, 6, 12, 24, 48 and 72 hours after initial injection. NC and VC illustrate the negative control and vehicle control (0.85% NaCl at 0 hpi), respectively. Expression levels were measured as the absolute copy number of *PmRnf121* mRNA (50 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.4.2 *Non-receptor tyrosine kinase* (*PmnRTK*)

Quantitative real-time PCR analysis indicated that the expression level of *PmnRTK* mRNA in 18-month old domesticated shrimp injected with serotonin was significantly increased at 6–12 hours post injection (hpi) relative to the vehicle control ($P < 0.05$). Its expression returned to the normal level at 24-72 hpi as shown in Figure 3.21.

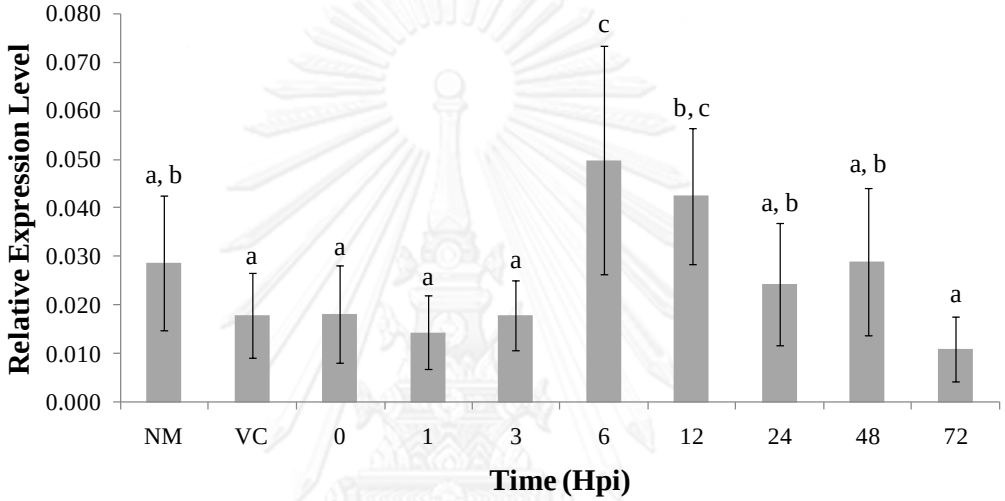


Figure 3.21 Histograms shows the relative expression profile of *PmnRTK* in domesticated shrimp injected with serotonin that collected at 0, 1, 3, 6, 12, 24, 48 and 72 hours after initial injection. NC and VC illustrate the negative control and vehicle control (0.85% NaCl at 0 hpi), respectively. Expression levels were measured as the absolute copy number of *PmnRTK* mRNA (300 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.4.3 *Receptor tyrosine kinase* (*PmRTK*)

Quantitative real-time PCR analysis indicated that the expression level of *PmRTK* mRNA in 18-month old domesticated shrimp injected with serotonin was

significantly up-regulated at 6 and 48 hpi compared to the vehicle control ($P < 0.05$) as shown in Figure 3.22.

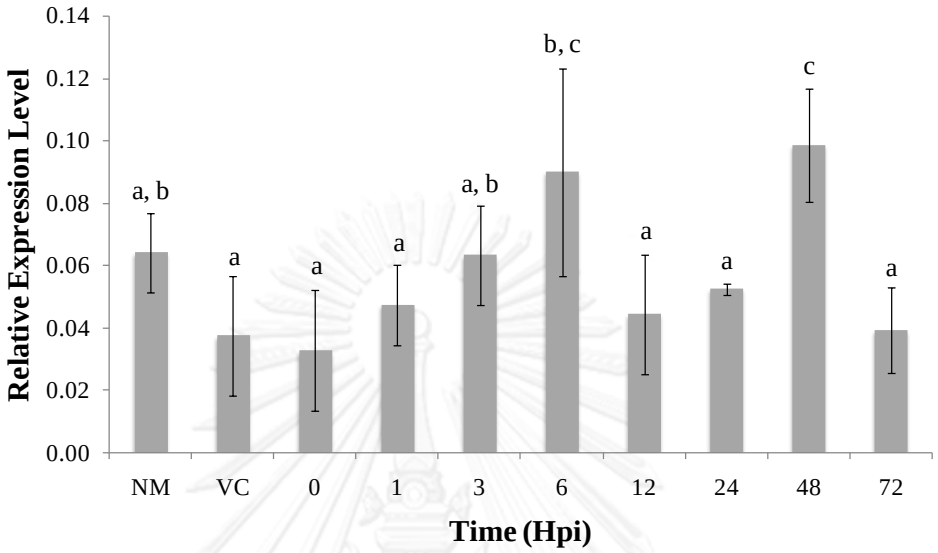


Figure 3.22 Histograms shows the relative expression profile of *PmRTK* in domesticated shrimp injected with serotonin that collected at 0, 1, 3, 6, 12, 24, 48 and 72 hours after initial injection. NC and VC illustrate the negative control and vehicle control (0.85% NaCl at 0 hpi), respectively. Expression levels were measured as the absolute copy number of *PmnRTK* mRNA (200 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.5 Expression levels of *PmRnf121, PmnRTK* and *PmRTK* mRNA in domesticated shrimp injected with progesterone

3.5.1 *Ring finger 121 protein* (*PmRnf121*)

An increasing expression level was observed in the vehicle control at 0 hpi and this should consider as the error of this experiment. The expression level of *PmRnf121* in the vehicle control at 12 hpi was not different from the un-injected control. Accordingly, comparison of gene expression upon progesterone injection was

considered at different time interval was determined by comparing with the vehicle control at 12 hpi. Quantitative real-time PCR revealed that progesterone did not affect to the expression level of *PmRnf121* mRNA in 14-month old injected shrimp ($P > 0.05$) as shown in Figure 3.23.

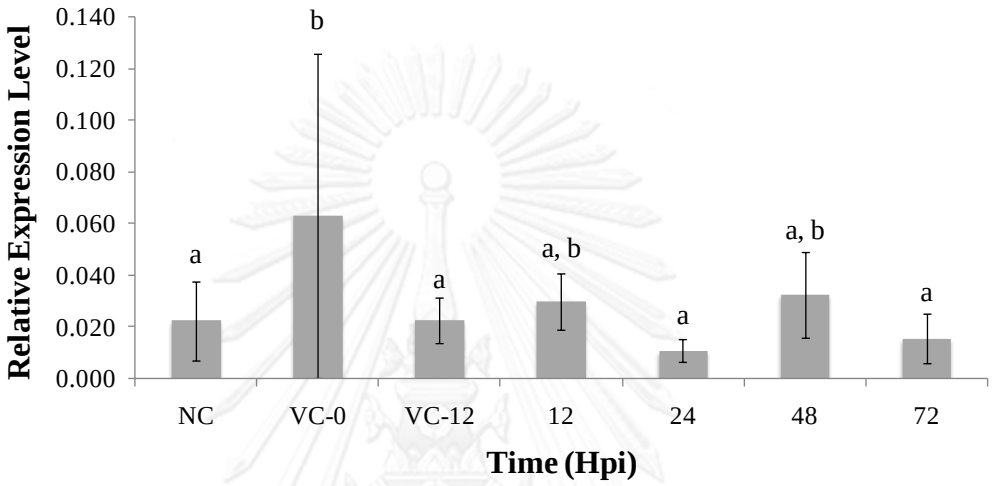


Figure 3.23 Histograms shows the relative expression profile of *PmRnf121* in domesticated shrimp injected with progesterone that collected at 12, 24, 48 and 72 hours after initial injection. NC, VC-0 and VC-12 illustrate the negative control, vehicle control (absolute ethanol) at 0 hpi and vehicle control at 12 hpi, respectively. Expression levels were measured as the absolute copy number of *PmRnf121* mRNA (50 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.5.2 *Non-receptor tyrosine kinase* (*PmnRTK*)

Progesterone seemed to affect the expression level of *PmnRTK* mRNA in ovaries of injected shrimp at 72 hpi. The result was not statistically significant from that of the vehicle control at 12 hpi ($P > 0.05$) but it was different from that of the negative control ($P < 0.05$) as shown in Figure 3.24.

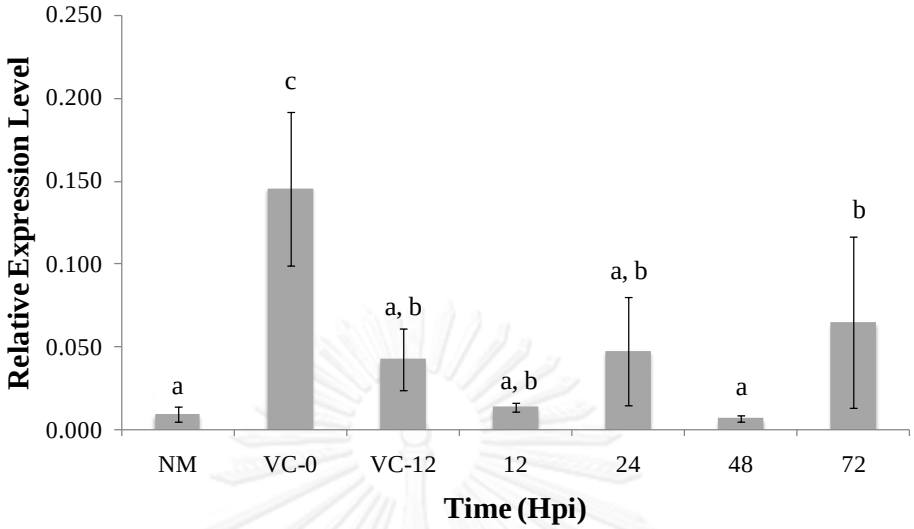


Figure 3.24 Histograms shows the relative expression profile of *PmnRTK* in domesticated shrimp injected with progesterone that collected at 12, 24, 48 and 72 hours after initial injection. NC, VC-0 and VC-12 illustrate the negative control, vehicle control (absolute ethanol) at 0 hpi and vehicle control at 12 hpi, respectively. Expression levels were measured as the absolute copy number of *PmnRTK* mRNA (300 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.5.3 *Receptor tyrosine kinase* (*PmRTK*)

The expression of *PmRTK* seemed to be increased at 48 and 72 hours after progesterone injection. However, the result was not significant due to large standard deviation between groups of samples ($P < 0.05$) as shown in Figure 3.25.

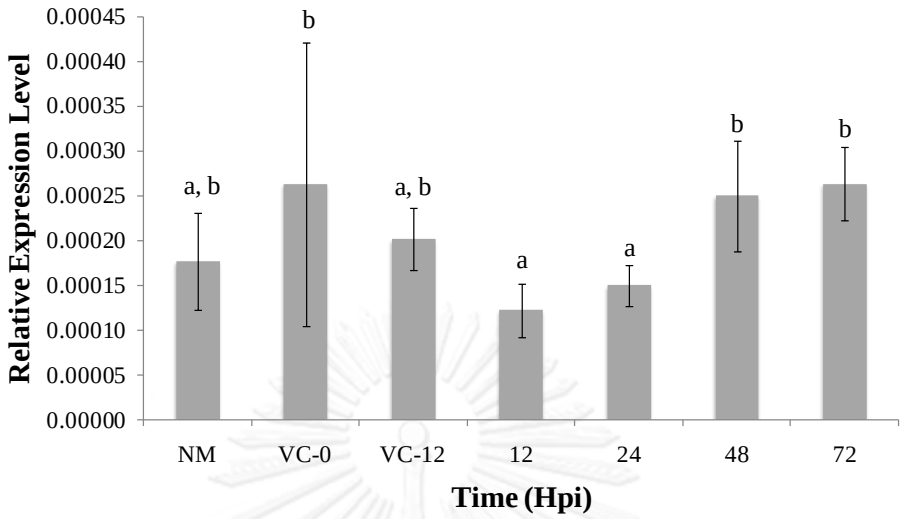


Figure 3.25 Histograms shows the relative expression profile of *PmRTK* in domesticated shrimp injected with progesterone that collected at 12, 24, 48 and 72 hours after initial injection. NC, VC-0 and VC-12 illustrate the negative control, vehicle control (absolute ethanol) at 0 hpi and vehicle control at 12 hpi, respectively. Expression levels were measured as the absolute copy number of *PmRTK* mRNA (300 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.6 Expression levels of *PmRnf121, PmnRTK* and *PmRTK* mRNA in domesticated shrimp injected with 17β-estradiol

3.6.1 *Ring finger 121 protein* (*PmRnf121*)

The expression level of *PmRnf121* mRNA in 14-month old domesticated shrimp injected with 17β-estradiol was significantly lower than that in the negative control, the vehicle control and eyestalk-ablated shrimp at 7 days post injection (dpi) ($P < 0.05$). Similar results were observed at 14 dpi with the exception that the expression level of *PmRnf121* in the vehicle control was lower than that of the negative control ($P < 0.05$). At 28 dpi, the expression levels of *PmRnf121* in ovaries of shrimp injected with 17β-estradiol was significantly greater than that of the

negative control ($P < 0.05$). Its expression level of the vehicle control was not different from that of the negative control and that in shrimp injected with 17β-estradiol ($P > 0.05$) as shown in Figure 3.26.

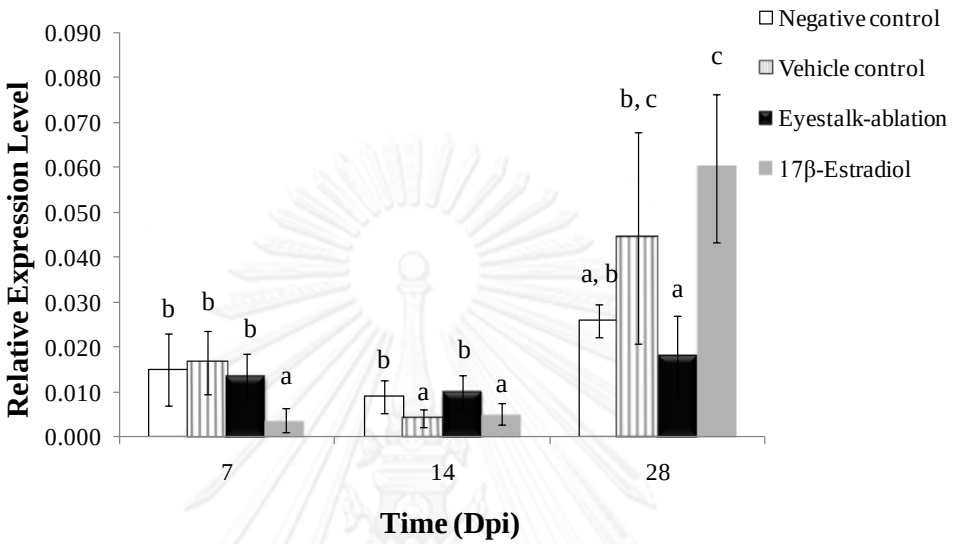


Figure 3.26 Histograms shows the relative expression profile of *PmRnf121* in domesticated shrimp injected with 17β-estradiol that collected at 7, 14 and 28 days after initial injection. The graphs ordering in each day permute the negative control, vehicle control (5% ethanol), eyestalk-ablated shrimp and shrimp injected with 17β-estradiol, respectively. Expression levels were measured as the absolute copy number of *PmRnf121* mRNA (50 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.6.2 *Non-receptor tyrosine kinase* (*PmnRTK*)

Quantitative real-time PCR analysis indicated that 17β-estradiol injection was not affected to the expression level of *PmnRTK* mRNA in 14-month old domesticated shrimp ($P > 0.05$). As the positive control, the expression level of *PmnRTK* in eyestalk-ablated shrimp was significantly greater than that of the controls at 28 dpi ($P < 0.05$) as shown in Figure 3.27.

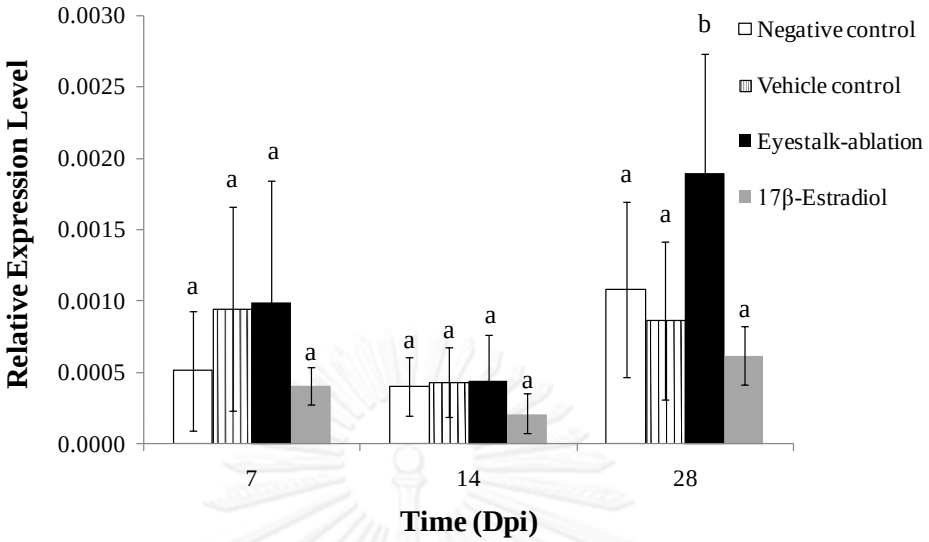


Figure 3.27 Histograms shows the relative expression profile of *PmnRTK* in domesticated shrimp injected with 17β-estradiol that collected at 7, 14 and 28 days after initial injection. The graphs ordering in each day permute the negative control, vehicle control (5% ethanol), eyestalk-ablated shrimp and shrimp injected with 17β-estradiol, respectively. Expression levels were measured as the absolute copy number of *PmnRTK* mRNA (300 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.6.3 *Receptor tyrosine kinase* (*PmRTK*)

Like *PmnRTK*, 17β-estradiol injection was not affected to the expression level of *PmRTK* mRNA in 14-month old domesticated shrimp during the treatment period ($P > 0.05$) However, the expression level of *PmRTK* in eyestalk-ablated shrimp was significantly greater than that of the controls at 28 dpi ($P < 0.05$) as shown in Figure 3.28.

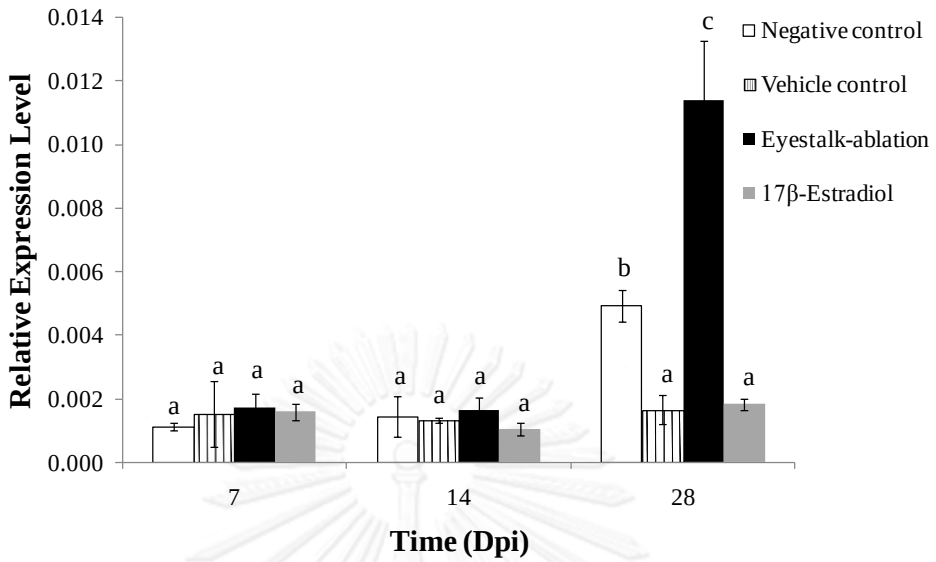


Figure 3.28 Histograms shows the relative expression profile of *PmRTK* in domesticated shrimp injected with 17β-estradiol that collected at 7, 14 and 28 days after initial injection. The graphs ordering in each day permute the negative control, vehicle control (5% ethanol), eyestalk-ablated shrimp and shrimp injected with 17β-estradiol, respectively. Expression levels were measured as the absolute copy number of *PmRTK* mRNA (200 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.7 Expression levels of *PmRnf121, PmnRTK* and *PmRTK* mRNA in domesticated shrimp fed with supplemented diets with 17β-estradiol

The residual 17β-estradiol in diets was kindly analyzed by the Hormonal Analysis Laboratory in Wildlife, Chaingmai Zoo. Based on competitive ELISA, 17β-estradiol was not found in the control diet while 29.9% and 40.9% were found in diets originally supplemented with 1 and 10 mg/kg 17β-estradiol, respectively.

Effects of 17β-estradiol-supplemented diets on expression of *PmRnf121, PmnRTK* and *PmRTK* in 18-month-old domesticated *P. monodon* were examined at 7, 14, 28 and 35 days of treatment.

3.7.1 *Ring finger 121 protein* (*PmRnf121*)

The expression level of *PmRnf121* mRNA in 12-month old domesticated shrimp after feeding with the diet supplemented with 10 mg/kg of 17β-estradiol for 7 days of treatment were significantly lower than that of the control ($P < 0.05$). However, results were not significantly different at 14 days of treatment. Interestingly, prolonged treatment of oral-administrated 17β-estradiol significantly affected the expression level of this gene regarding doses of 17β-estradiol. At 28 days, the expression level of *PmRnf121* in shrimp fed with diets supplemented with 10 mg/kg 17β-estradiol was greater than that of the control ($P < 0.05$).

In addition, significantly greater expression of *PmRnf121* was observed in shrimp fed with diets supplemented with 1 mg/kg of 17β-estradiol compared to the control at 35 days of treatment ($P < 0.05$). In eyestalk-ablated shrimp, the expression level of *PmRnf121* was not significantly different than that of the control throughout the experiment ($P > 0.05$) as shown in Figure 3.29.

3.7.2 *Non-receptor tyrosine kinase* (*PmnRTK*)

The expression level of *PmnRTK* mRNA in 12-month old domesticated shrimp after feeding with the diet supplemented with 1 and 10 mg/kg of 17β-estradiol for 7 days was significantly lower than that of the control in a dose dependent manner ($P < 0.05$). Results were not significantly difference among the 17β-estradiol-supplemented diets and the control at 14 and 28 days of treatment. Both diets supplemented with 1 and 10 mg/kg of 17β-estradiol resulted in a reduction of *PmnRTK* transcriptional levels at 35 days of treatment ($P < 0.05$). Eyestalk ablation resulted in a lower expression level of this gene relative to the control at 7 and 35 days of treatment ($P > 0.05$). The expression level of *PmnRTK* in eyestalk-ablated shrimp seemed to be greater than other treatment at 14 days after ablation but the result was not statistically significant due to a large standard deviation among specimens in this treatment group (Figure 3.30).

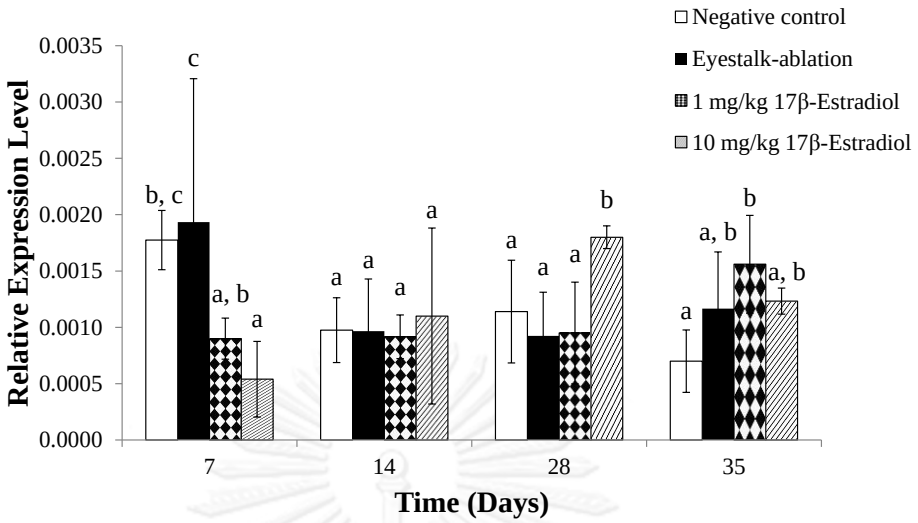


Figure 3.29 Histograms shows the relative expression profile of *PmRnf121* in domesticated shrimp were fed with supplemented diets of 17β-estradiol that collected at 7, 14, 28 and 35 days of treatment. The graphs ordering in each day permute the negative control, eyestalk-ablated shrimp, shrimp were fed with 1mg/kg and 10 mg/kg of 17β-estradiol, respectively. Expression levels were measured as the absolute copy number of *PmRnf121* mRNA (50 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.7.3 *Receptor tyrosine kinase* (*PmRTK*)

The expression level of *PmRTK* after feeding with the diet supplemented with 1 and 10 mg/kg of 17β-estradiol for 7 and 14 days was not significantly different from that of the control ($P > 0.05$). However, an increased expression level of *PmRTK* relative to the control was observed at 28 (10 mg/kg of 17β-estradiol) and 35 (both 1 and 10 mg/kg of 17β-estradiol) days of treatment ($P < 0.05$). Eyestalk ablation resulted in an increased expression level of this gene at 35 days after ablation ($P < 0.05$) as shown in Figure 3.31.

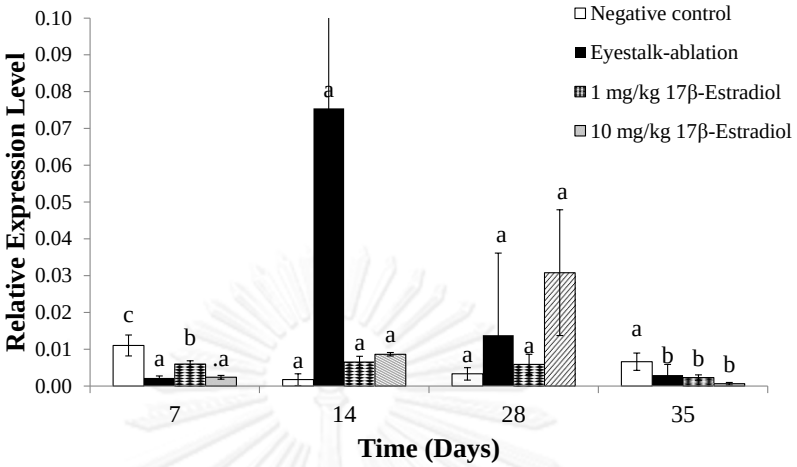


Figure 3.30 Histograms shows the relative expression profile of *PmnRTK* in domesticated shrimp were fed with supplemented diets of 17β-estradiol that collected at 7, 14, 28 and 35 days of treatment. The graphs ordering in each day permute the negative control, eyestalk-ablated shrimp, shrimp were fed with 1mg/kg and 10 mg/kg of 17β-estradiol, respectively. Expression levels were measured as the absolute copy number of *PmnRTK* mRNA (300 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

3.8 *In vitro* expression of recombinant proteins using the bacterial expression system.

3.8.1 Construction of recombinant *PmRnf121* and *PmRTK* plasmids

3.8.1.1 Ring finger 121 protein (PmRnf121)

Recombinant plasmids carrying the entire ORF of *PmRnf121* 1023 bp were prepared for *in vitro* expression of the corresponding proteins. The amplification product (*Pfu* DNA polymerase) was digested with *Nde* I and *Bam* HI and the product

was electrophoresed (Figure 3.32). The gel-eluted product was ligated with pET29a and pET43.1a expression vectors and transformed into *E. coli* JM109.

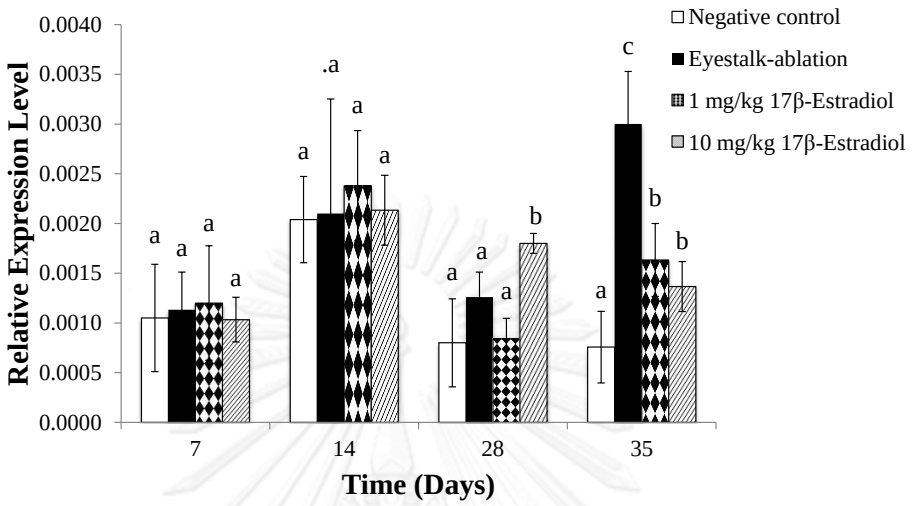


Figure 3.31 Histograms shows the relative expression profile of *PmRTK* in domesticated shrimp were fed with supplemented diets of 17β-estradiol that collected at 7, 14, 28 and 35 days of treatment. The graphs ordering in each day permute the negative control, eyestalk-ablated shrimp, shrimp were fed with 1mg/kg and 10 mg/kg of 17β-estradiol, respectively. Expression levels were measured as the absolute copy number of *PmRTK* mRNA (200 ng template) and normalized by that of *EF-1*α_{214} mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

The positive clone was sequenced for both directions to confirm the orientation of the insert. Plasmid DNA was extracted from a clone carrying the correct direction and transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells.

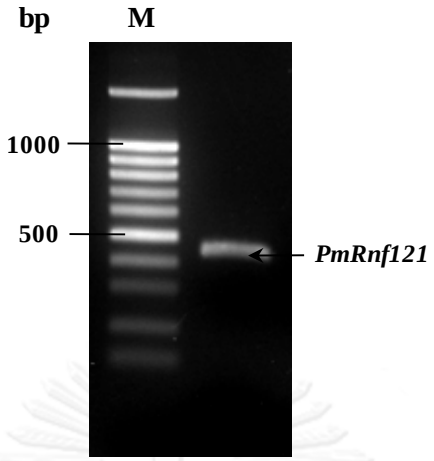


Figure 3.32 1.2% ethidium bromide-stained agarose gel shows the amplification product of *PmRnf121* was digested with *Nde* I and *Bam* HI. Lane M is 100 bp DNA ladder.

3.8.1.2 Receptor tyrosine kinase (PmRTK)

Recombinant plasmids carrying the partial ORF of *PmRTK* 587 bp were prepared for *in vitro* expression of the corresponding proteins. The target product was amplified using *Pfu* DNA polymerase and digested with *Nde* I and *Bam* HI (Figure 3.33). The product was eluted and ligated with pET29a, pET32a and pET43.1a expression vectors and cloned as described above.

3.8.2 *In vitro* expression of recombinant proteins

The recombinant clone was selected and the expression profile of the corresponding recombinant protein was examined at 37 ℃ for 0, 1, 2, 3, 6, 9, 12 and 24 hours after induced by 1 mM IPTG.

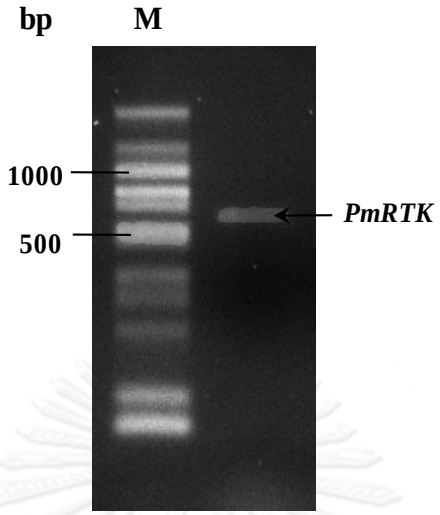


Figure 3.33 1.2% ethidium bromide-stained agarose gel shows the amplification product of *PmRTK* was digested with *Nde* I and *Bam* HI. Lane M is 100 bp DNA ladder.

3.8.2.1 Ring finger 121 protein recombinant protein (rPmRnf121)

The expected molecular mass of rPmRnf121 protein was 16.63 kDa. However, overexpression of the target protein band was not observed in both of pET29a and pET43.1a expression vectors after recombinant clones were induced by 1mM IPTG (Figure 3.34).

3.8.2.2 Receptor tyrosine kinase recombinant protein (rPmRTK)

The recombinant clone of PmRTK protein was approximately 23.83 kDa. Overexpression of the target protein band was observed in pET29a expression vector but not in pET32a and pET43.1a expression vectors after induced by 1mM IPTG (Figure 3.35). The rPmRTK protein was expressed at 1- 24 hours after IPTG induction (from SDS-PAGE). Its expression level seemed to be gradually increased during 2-12 hours after IPTG induction (Western blot analysis). A slightly larger protein band (25 kDa) was also expressed in all vector types after induction with IPTG. However, Western blot analysis did not give the positive signal of this band when hybridized with anti-6X His MAb.

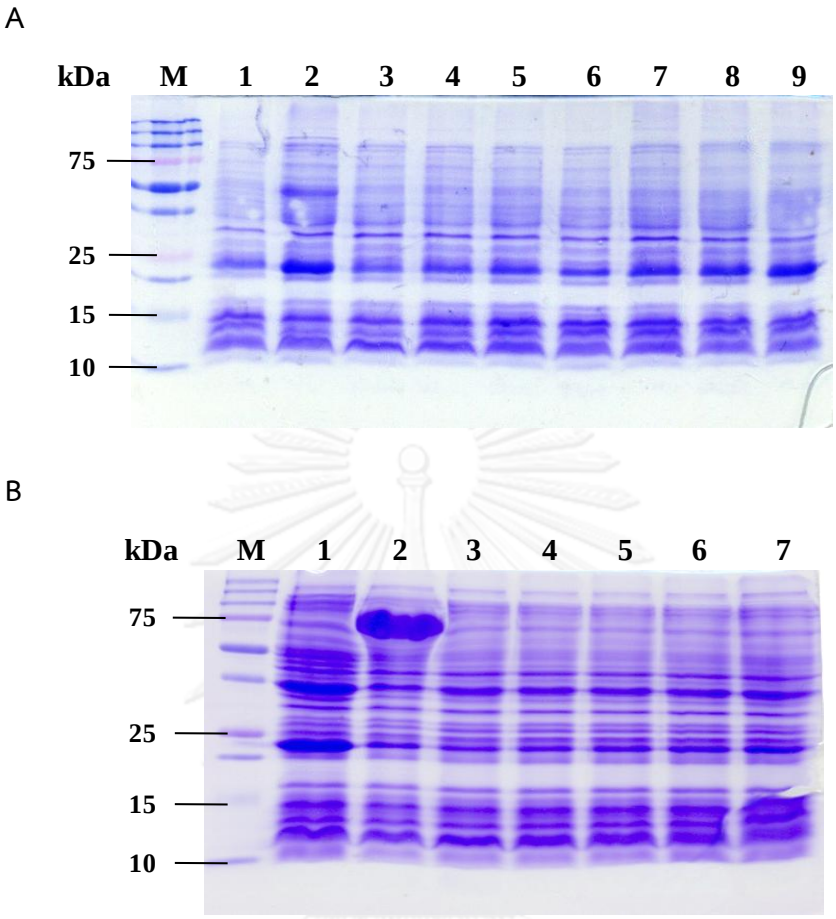


Figure 3.34 15% SDS-PAGE shows the *In vitro* expression profile of rPmRnf121 protein in (A) pET29a and (B) pET43.1a expression vector in *E. coli* BL21-CodonPlus (DE3)-RIPL after induction with 1 mM IPTG for 0, 1, 2, 3, 6, 12 and 24 hours. Lane 1 is *E. coli* BL21-CodonPlus (DE3)-RIPL. Lane 2 is (A) pET29a and (B) pET43.1a expression vector in *E. coli* BL21-CodonPlus (DE3)-RIPL. Lanes 3-9 are the protein profiles of recombinant PmRnf121 clone at 0, 1, 2, 3, 6, 12 and 24 hours after induction with 1 mM IPTG, respectively. Lane M is protein standard marker.

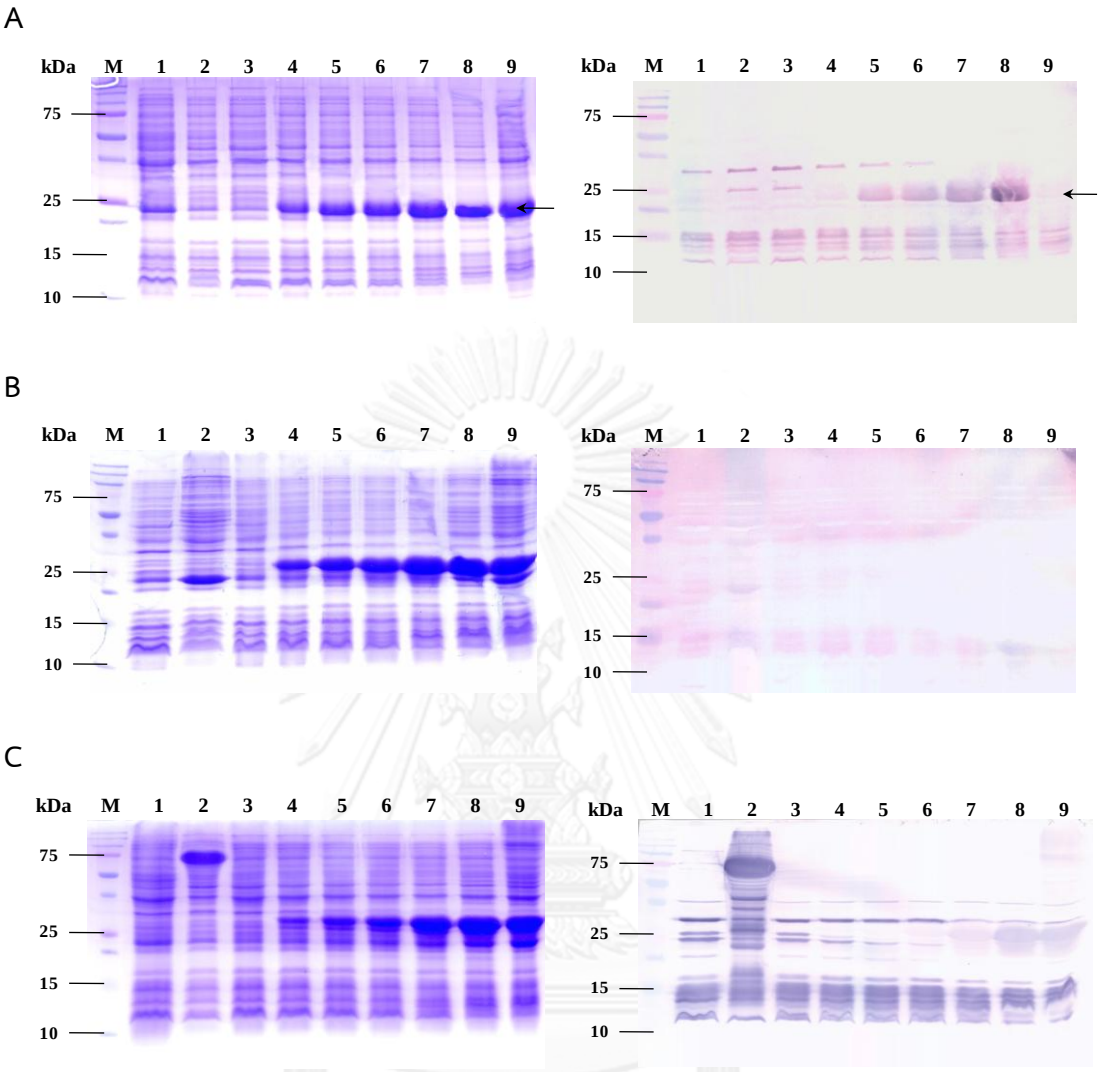


Figure 3.35 15% SDS-PAGE (Left) and Western blot analysis (Right) show the in vitro expression profile of rPmRTK protein in (A) pET29a, (B) pET32a and (C) pET43.1a expression vector in E. coli BL21-CodonPlus (DE3)-RIPL after induction with 1 mM IPTG for 0, 1, 2, 3, 6, 12 and 24 hours. Lane 1 is *E. coli* BL21-CodonPlus (DE3)-RIPL. Lane 2 is (A) pET29a, (B) pET32a and (C) pET43.1a expression vector in *E. coli* BL21-CodonPlus (DE3)-RIPL. Lanes 3-9 are the protein profiles of recombinant PmRTK protein at 0, 1, 2, 3, 6, 12 and 24 hours after induction with 1 mM IPTG, respectively. Lane M is protein standard marker.

The rPmRTK clone in pET29a was cultured at 37 ℃ for 6 hours after IPTG induction. The soluble and insoluble protein fractions were analyzed by 15% SDS-PAGE. Results indicated that the rPmRTK protein was expressed in an inclusion bodies (Figure 3.36).

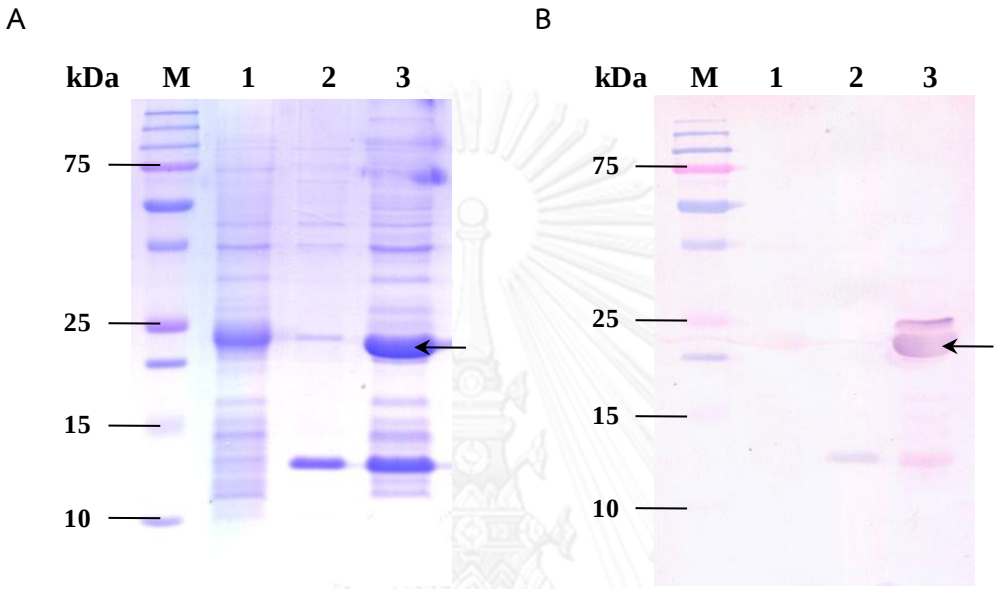


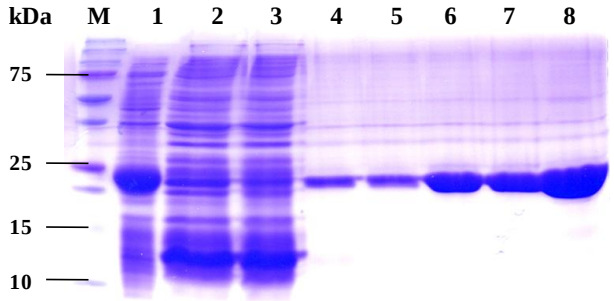
Figure 3.36 (A) 15% SDS-PAGE and (B) Western blot analysis show showing localization of rPmRTK protein expression when cultured at 37 ℃ for 6 hours after IPTG induction. Lane 1 is *E. coli* BL21-CodonPlus (DE3)-RIPL. Lanes 2-3 are whole cell in soluble protein fraction and insoluble protein fraction, respectively. Lane M is protein standard marker.

3.8.3 Purification of recombinant proteins

3.8.3.1 Receptor tyrosine kinase recombinant protein (rPmRTK)

The rPmRTK protein was purified under denaturing conditions. The purified rPmRTK protein was size-fractionated by 15% SDS-PAGE (Figure 3.37). Due to limitation of time, the production of anti-PmRTK PAb is not further carried out.

A



B

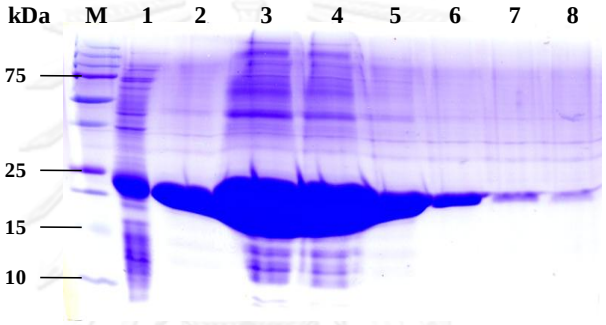


Figure 3.37 15% SDS-PAGE shows the purified rPmRTK protein. (A) Each collected fractions of recombinant protein was washed by binding buffer containing 20, 40 and 80 mM imidazole with urea. Lane 2 is the flow-through of recombinant protein. Lanes 3-4 are fraction 1 and 10 washed with 20 mM imidazole, lanes 5-6 are fraction 1 and 5 washed with 40 mM imidazole and lanes 7-8 are fraction 1 and 5 washed with 80 mM imidazole. (B) Each collected fractions of recombinant protein was eluted by elution buffer containing 500 mM imidazole. Lanes 2-8 are fraction 1-7 eluted with 500 mM imidazole. Lane M and 1 are protein standard marker and *E. coli* BL21-CodonPlus (DE3)-RIPL, respectively.

3.9 Determination of expression of target proteins of *P. monodon* using Western blot analysis

3.9.1 Ring finger 121 protein recombinant protein (rPmRnf121)

The expression profile of PmRnf121 during ovarian development of *P. monodon* was preliminary studied using commercially available anti-Rnf121 (similarity between Rnf121 antigen and PmRnf121 = 73.39%). The positive-immunoreactive signal of approximately 60 kDa was observed in stage I ovaries but not in other stages of eyestalk-ablated broodstock. Surprisingly, the positive signal was not observed in various ovarian stages in intact broodtock (Figure 3.38).

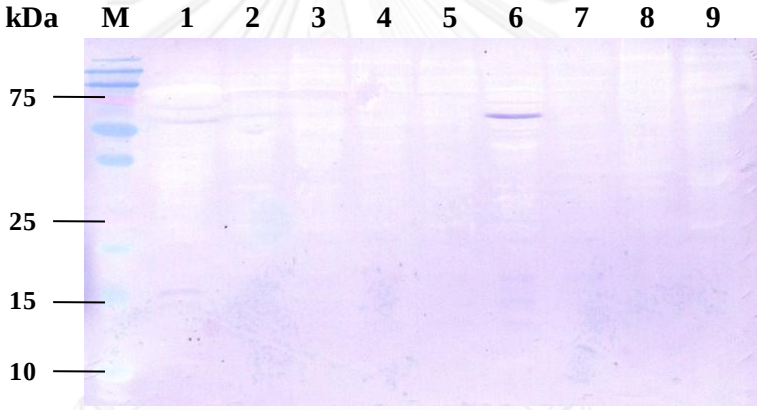


Figure 3.38 Western blot analysis shows the signals of PmRNF121 protein in each ovarian development stage of *P. monodon*. Lane 1 is 30 ug of total ovarian protein from juvenile. Lanes 2-5 are 30 ug each of total ovarian protein from stage I, II, III and IV of intact broodstock, respectively. Lanes 6-9 are 30 ug each of total ovarian protein from stage I, II, III and IV of eyestalk-ablated broodstock, respectively. Lane M is protein standard marker.

CHAPTER IV
DISCUSSION

Farming of *P.monodon* has faced several problems in cultivation and management including lack of high quality broodstock, reduction of degrees of ovarian maturation and spawning potential in captive shrimp. The sustainable cultural was required for the shrimp industry in Thailand. Therefore, understanding molecular mechanisms involving in ovarian maturation of shrimp is necessary for sustainable activity of this industry.

4.1 Isolation and characterization of the full-length cDNAs of genes functionally involved in the signal transduction of oocytes of *P. monodon*

The ubiquitin–proteasome pathway involved in several control mechanisms of gametogenesis (Sakai *et al.*, 2004; Sutovsky *et al*., 1999; 2001). Ubiquitin conjugating enzymes play the important role in the sumoylation pathway for which small ubiquitin modifier 1 (SUMO1) plays an important role in diverse reproductive functions and modulation of steroid receptor activity. SUMO is transferred to substrate lysine residues through the thioester cascade of ubiquitin activating enzyme E1 and ubiquitin conjugating enzyme E2 (UBE2), and SUMO ligase E3 functions as an adaptor between E2 and each substrate (Takahashi and Kikuchi, 2005).

In this thesis, the full-length cDNAs of *PmRnf121* transcripts was successfully characterized by RACE-PCR. In addition, the partial cDNA sequence of *PmRTK* also obtained. The full-length cDNA of *PmRnf121* contained an ORF 1023 bp that deducing to a polypeptide of 341 amino acids. *PmRnf121* contained the predicted RING domain that played the important role in E3 ubiquitin-ligase activity and linking towards E2 ubiquitin-conjugating enzyme.

In *Marsupenaeus japonicus*, *ubiquitin-conjugating enzyme E2* (*UBE2*) was expressed at a higher level in testes than in ovaries. The expression at the stage I (GSI = 0.33 ± 0.004, N = 5) was significantly lower than that of the stage II (GSI = 0.45 ±

0.12, N = 5) but comparable to that of the stage III (GSI = 0.57 ± 0.006, N = 5) of testes. *UBE2* in ovaries was up-regulated since the stage III of ovaries. This suggested that *UBE2* has an important role in spermatogenesis and oogenesis of *M. japonicus* (Shen *et al.*, 2009).

Although the full-length cDNA of *PmRTK* was not obtained, the predicted protein kinase domain (STYKc) that acted as one of enzyme in phosphotransferases group was found in the partial cDNA characterized. RTKs catalyzed the phosphate group and transferred to protein or other molecule (Phosphorylation process). The phospholyration played the role in regulated cellular mechanism. Qiu *et al.* (2008) characterized Elongation factor 2 (EF 2) that is essential for polypeptide chain elongation steps in protein synthesis. The phosphorylated EF 2 reduced elongation rates that regulated cellular protein level. EF 2 functions was an important regulatory step in gene expression. *PmRTK* contained a STYKc domain suggesting that it should functionally involve in the signal transduction during ovarian development of *P. monodon*.

4.2 Expression levels of *PmRnf121*, *PmnRTK* and *PmRTK* during ovarian development stage of *P. monodon*

RT-PCR is used to detect the gene expression. The gene expression and tissue distribution analysis are important and provide the basic information to set up the priority for further analysis of functional genes. Based on the fact that a particular genes may express in several tissues and possesses a different function in different tissues.

The expression analyses of *PmRnf121, PmnRTK* and *PmRTK* were more abundant expressed in ovaries than testes in both juvenile and wild broodstock. Therefore, *PmRnf121, PmnRTK* and *PmRTK* may play more important role in oogenesis than spermatogenesis of *P. monodon*.

In addition, tissue distribution analysis showed that *PmRnf121, PmnRTK* and *PmRTK* were expressed in various tissues of wild broodstock. *PmRnf121* was abundantly expressed in ovaries similar to *PmRTK.* Meanwhile, *PmnRTK* was more

expressed in thoracic ganglion and hemocytes than ovaries. This suggested that *PmRnf121, PmnRTK* and *PmRTK* are multi-functional proteins playing different roles in different tissues.

In various animals, a wide variety of maternal mRNA is generally transcribed at the early oogenesis stage, to then be stored in oocytes and carried into fertilized eggs (Nishimura *et al.*, 2009; Qiu *et al.*, 2008). Several reproduction-related genes that are up-regulated during the ovarian development of *P. monodon*, for example, *Ovarian-Specific Transcript 1* (*Pm-OST1*) and *cyclin B* (*PmCyB*), have been previously reported (Klinbunga *et al.*, 2009; Visudtiphole *et al.*, 2009). To address the functional involvement of various genes during ovarian development of *P. monodon*, the expression profiles of *PmRnf121, PmnRTK* and *PmRTK* were examined by quantitative real-time PCR analysis. The control gene (*EF-1*α) was comparably expressed in all the groups of samples examined, thereby inferring its acceptability for use in normalizing target gene expression.

The expression level of *PmRnf121* in stages II-IV ovaries of broodstock was greater than that of juvenile suggesting that the transcribed mRNA level of this gene was sufficient for rapid translation during vitellogenesis and final maturation of ovarian development. Its transcriptional level was not affected by eyestalk ablation implying that GIH did not affect the expression of *PmRnf121*.

Likewise, the expression level of *PmnRTK* during ovarian development (stages I-IV ovaries) was significantly greater than that of juvenile. Its expression level was reduced in the post spawning stage. Eyestalk ablation resulted in a greater level of *PmnRTK* during the mature (stage IV) and post-spawning (stage V) stages. Accordingly, this transcript should involve the final maturation of ovarian development in *P. monodon*.

The expression level of *PmRTK* in juveniles was lower than that in broodstock. Its expression was gradually increased from previtellogenin in vitellogenic ovaries and peaked in mature ovaries. The expression profiles of *PmRTK* in eyestalk-

ablated broodstock were similar with the exception that its expression in ablated broodstock was greater than non-ablated broodstock at the post-spawning stage.

4.3 Effects of serotonin, progesterone and 17ß-estradiol injection on expression of *PmRnf121*, *PmnRTK* and *PmRTK* in ovaries of *P. monodon*

Effects of exogenous serotonin on the reproductive performance of shrimp were reported (Vaca and Alfaro, 2000). Serotonin induced ovarian development of *P. monodon* (Wongprasert *et al.*, 2006) and *M. rosenbergii* (Meeratana *et al.*, 2006) dose dependently. Exogenous injection of serotonin clearly promoted expression of *P. monodon* Ovarian-Specific Transcript (*Pm-OST1*) in ovaries of 5-month old shrimp. *Pm-OST1* was up-regulated at 12-78 hpi ($P < 0.05$), with the highest expression level observed at 48 hpi ($P < 0.05$) (Klinbunga *et al*., 2009). In this study, effects of serotonin on expression of *PmCOMT* in ovaries of 18-month-old *P. monodon* were examined. The injection of 5-HT resulted in increasing of *PmCOMT* expression at 72 hpt suggesting its late response effects on the transcription of *PmCOMT* in oocytes of *P. monodon*.

Quantitative real-time PCR was used to determine effects of exogenous 5-HT injection on the expression of reproduction-related genes in this thesis. The expression level of *PmRnf121* was not significantly different following the injection of 5-HT. In contrast, the expression levels of *PmnRTK* and *PmRTK* were significantly promoted by 5-HT injection. The results further confirmed effects of 5-HT on the expression of various reproduction-related genes in different pathways (i.e. stimulation of genes in the tyrosine kinase pathway but not the pathway involving *PmRnf121*) of *P. monodon*. Appropriate doses and the number of repeated injection of 5-HT for inducing reproductive maturation of *P. monodon* should be further carried out for the possible replacement of eyestalk ablation with 5-HT injection at the laboratory scale.

Progesterone (P4) and 17α-hydroxyprogesterone (17α-OHP4) stimulated ovarian maturation and yolk protein synthesis of penaeid shrimp *in vivo* (Quackenbush, 2001; Yano, 1985; 1987). The conversion of progesterone into 17ß-

estradiol was reported in the kuruma prawn, *Marsupenaeus japonicus* (Summavielle *et al.*, 2003). Levels of 17β-estradiol in the hemolymph were shown to fluctuate closely with that of the serum vitellogenin levels during ovarian maturation stages of *P. monodon* (Quinitio *et al.*, 1994).

Progestins (progesterone and derivatives) acts as the maturation inducing factor resulting in the resumption of meiotic maturation of oocyte (Kishimoto, 1999; 2003) whereas estrogens, especially 17β-estradiol (E2) are the principal stimulator of ovarian vitellogenesis in shrimp. Comparing with information described above that progesterone did not significantly promote the expression level of *PmRnf121*, *PmnRTK* and *PmRTK* mRNA in this study. These genes should not play a role in the progesterone signal transduction pathway.

Effects of 17β-estradiol on induction of vitellogenin synthesis were recently reported *in vitro* in previtellogenic ovaries (gonadosomatic index, GSI = 0.72-0.87%) of *M. japonicus* incubated with M199 containing 3.6 nM, 36.7 nM, 367 nm and 3671 nM 17β-estradiol (Yano and Hoshino, 2006). In contrast, publications of no effect of 17β-estradiol on ovarian development of penaeid shrimp were also observed. Koskela *et al*. (1992) did not observe the ovarian development of *Penaeus esculentus* after treated with 17β-estradiol alone or in combination with prostaglandin. Tsukimura and Kamemoto (1991) showed that 17β-estradiol did not affect the oocyte diameter of *Penaeus vannamei*.

The injection of 17β-estradiol did not affect the expression of *PmnRTK* and *PmRTK* but significantly increased the expression level of *PmRnf121* at 28 day post injection relative to negative control. This indicated the late effects of 17β-estradiol on expression of *PmRnf121*. Interestingly, eyestalk ablation resulted in increasing the expression level of *PmnRTK* and *PmRTK* but not significantly altered the expression level of *PmRnf121* at 28 day post injection. The circumstance suggested that eyestalk ablation and 17β-estradiol functionally affect expression of genes in different pathways. The induction of ovarian development of *P. monodon* by hormonal and/or neurotransmitter injection is suitable for application in small scale breeding programs of *P. monodon*.

4.4 Feeding effects of diets supplemented with 17β-estradiol on expression of *PmRnf121*, *PmnRTK* and *PmRTK* genes in ovaries of *P. monodon*

The feeding experiment using diets supplemented with 1 and 10 mg/kg of 17β-estradiol was carried out. The remaining 17β-estradiol in the diets was analyzed and 25.9% and 40.9% resulting in the actual amount of 0.259 and 4.09 mg/kg diets were found.

The feeding experiment with diets supplemented with 17β-estradiol increased the expression level of *PmRnf121* at 28 days of the treatment with 10 mg/kg of 17β-estradiol and at 35 days of the treatment of the treatment with 1 mg/kg of 17β-estradiol. It is likely that the response of *PmRnf121* to 17β-estradiol-supplemented diets depended on doses and treatment periods. Like the previous experiment on injection of 17β-estradiol, eyestalk ablation (the positive control) did not affect the expression of this gene.

Feeding of shrimp with the diet supplemented with 17β-estradiol for 1 mg/kg resulted in the decrease of *PmnRTK* expression levels at 7 and 35 day of treatment. Eyestalk ablation seemed to promote the expression level of *PmnRTK* but the result was not significant owing to a large standard variation of this sample group.

Expression of *PmRTK* was significantly increased in dose-dependent and time-dependent manners following the feeding experiment with diet supplemented with 17β-estradiol. Like results from the 17β-estradiol injection experiment, the expression of *PmRTK* in eyestalk-ablated shrimp was increased after ablated for 35 days.

Generally, results from injection and diet supplementation of 17β-estradiol on expression of *PmRnf121*, *PmnRTK* and *PmRTK* were not similar. However, *PmRnf121* and *PmRTK* in shrimp were more abundantly expressed after fed with diet supplemented with 17β-estradiol. This suggested that effects of 17β-estradiol on stimulation of gene expression largely dose-dependent and better stimulating effects of ovarian development of *P. monodon* should require continuously treatment rather than a single dose injection of 17β-estradiol. Therefore, the most appropriate concentration of 17β-estradiol on stimulating expression of these genes (leading to

the most potential way for induction of ovarian development) in a particular administration method should be further examined. The other important issue for the application of maturation inducing diets supplemented with 17ß-estradiol is the possible contamination of a sex steroid in the environments. This risk can be minimized by operation of the treatment in appropriate aquaria with good laboratory practice on wastewater treatment.

In this study, genes that play the important roles in the signal transduction during development and maturation of oocytes/ovaries of *P. monodon* were characterized. Expression of these genes during normal ovarian development and under the induction condition by eyestalk ablation in wild broodstock was examined. In addition, effects of proposed stimulators on crustacean reproductive development including 5-HT, progesterone and 17ß-estradiol in captive shrimp were examined. The knowledge gained provided better understanding on molecular reproductive development of *P. monodon*.

CHAPTER V
CONCLUSION

1. The full-length cDNA of *PmRnf121* was successfully characterized (1174 bp in length containing an ORF of 1023 bp deducing to 341 amino acids). In addition, the partial cDNA of *PmRTK* was characterized and it was 1387 bp in length containing ORF 587 bp deducing to a polypeptide of 195 amino acids.

2. *PmRnf121* was not differentially expressed during ovarian development of wild *P. monodon* ($P > 0.05$). Serotonin and progesterone injection affected its expression level in domesticated shrimp ($P > 0.05$). 17β-estradiol induced the expression level of *PmRnf121* in domesticated shrimp in both the injection and oral administration ($P < 0.05$).

3. *PmnRTK* was not differentially expressed during ovarian development of wild *P. monodon* ($P > 0.05$). Serotonin but not progesterone injection induced its expression level in domesticated shrimp ($P < 0.05$). 17β-estradiol injection did not induce the expression level of *PmnRTK* ($P > 0.05$) but oral administration resulted in the reduction of its expression level in ovaries of domesticated shrimp ($P < 0.05$).

4. The expression level of *PmRTK* was gradually increased during ovarian development of wild *P monodon* ($P < 0.05$). Like *PmnRTK*, serotonin but not progesterone injection induced its expression level in domesticated shrimp ($P < 0.05$). 17β-estradiol injection reduced the expression level of *PmRTK* ($P < 0.05$) but oral administration resulted in the promotion of its expression level in ovaries of domesticated shrimp ($P < 0.05$).

5. Serotonin could be applied for induction of reproductive maturation of *P. monodon*. The appropriate derivative form of progesterone should be further evaluated for better induction effects. The effects of 17β-estradiol on stimulation of gene expression were largely dose-dependent. Therefore, the most appropriate concentration of 17β-estradiol on stimulating reproductive maturation of *P. monodon* should be further examined.

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APPENDICES

APPENDIX A

Expression of reproduction-related genes during ovarian development of *P. monodon*

Table A1 The relative expression level of *PmRnf121* during ovarian development of wild intact and eyestalk-ablated broodstock of *P. monodon*

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
Juvenile	BU5 OV13	1.54E+04	2.90E+05	0.05303	0.05036	0.00477
	BU5 OV14	1.12E+04	2.50E+05	0.04485		
	BU5 OV17	4.86E+03	9.14E+04	0.05319		
N-BD-Stage I	BFN OV32	9.49E+04	9.10E+05	0.10428	0.11160	0.04115
	BFN OV22	4.11E+04	5.51E+05	0.07461		
	WFN OV9	7.11E+04	4.56E+05	0.15592		
N:BD-Stage II	WFN OV2	6.59E+04	4.27E+05	0.15443	0.19805	0.05554
	WFN OV27	7.62E+04	3.64E+05	0.20960		
	WFN OV14	3.04E+04	3.74E+05	0.08130		
	WFN OV5	1.18E+05	5.32E+05	0.22113		
	WFN OV3	9.45E+04	4.75E+05	0.19888		
	WFN OV15	5.68E+04	2.49E+05	0.22838		
	WFN OV16	7.22E+04	2.93E+05	0.24623		
	WFN OV23	7.66E+04	3.13E+05	0.24443		
N:BD-Stage III	WFN OV13	5.52E+04	3.32E+05	0.16637	0.20967	0.08524
	WFN OV12	3.52E+04	3.00E+05	0.11742		
	WFN OV21	6.45E+04	3.93E+05	0.16413		
	WFN OV11	3.62E+04	1.25E+05	0.29000		
	WFN OV22	7.28E+04	2.34E+05	0.31043		
N:BD-Stage IV	WFNOV19	9.63E+03	6.18E+04	0.15589	0.21631	0.07354
	WFNOV8	1.85E+04	9.88E+04	0.18677		
	WFNOV6	5.46E+03	4.32E+04	0.12652		

Table A1 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
	WFNOV20	8.85E+04	3.88E+05	0.22830		
	WFNOV18	7.00E+04	2.25E+05	0.31100		
	WFNOV10	4.34E+04	1.50E+05	0.28940		
N:BD Post-	WFNOV1	3.51E+04	3.68E+05	0.09517	0.13940	0.04464
spawning	WFNOV4	5.04E+04	3.90E+05	0.12927		
	WFNOV7	4.78E+04	3.63E+05	0.13163		
	WFNOV17	1.46E+05	7.26E+05	0.20152		
EA:BD-Stage I	WFEA OV4	1.25E+04	1.08E+05	0.11593	0.09308	0.02057
	WFEA OV6	1.09E+04	1.43E+05	0.07603		
	WFEA OV2	2.68E+04	3.08E+05	0.08728		
EA:BD-Stage II	WFEA OV1	9.79E+03	1.23E+05	0.07978	0.21525	0.13499
	WFEA OV5	1.13E+04	1.09E+05	0.10355		
	WFEA OV28	1.22E+04	1.26E+05	0.09672		
	WFEA OV21	8.39E+04	2.38E+05	0.35267		
	WFEA OV20	3.12E+04	1.03E+05	0.30386		
	WFEA OV19	8.25E+04	2.32E+05	0.35490		
EA:BD-Stage III	WFEA OV10	6.99E+03	7.87E+04	0.08876	0.15360	0.06880
	WFEA OV25	1.60E+04	9.46E+04	0.16934		
	WFEA OV12	1.33E+04	7.68E+04	0.17367		
	WFEA OV8	3.61E+03	4.22E+04	0.08547		
	WFEA OV9	1.16E+04	4.64E+04	0.25073		
EA:BD-Stage IV	WFEA OV13	1.02E+04	3.51E+04	0.28978	0.20103	0.09129
	WFEA OV11	5.99E+03	4.90E+04	0.12231		
	WFEA OV15	4.69E+03	3.84E+04	0.12226		
	WFEA OV14	1.77E+04	6.55E+04	0.26977		
EA:BD Post-	WFEA OV30U	8.53E+03	3.71E+04	0.22962	0.13230	0.01523
spawning	WFEA OV31U	3.36E+03	6.45E+04	0.05207		
	WFEA OV32U	6.51E+03	2.44E+04	0.26622		

Table A1 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
	WFEA OV30L	5.34E+03	6.00E+04	0.08898		
	WFEA OV32L	2.87E+03	2.03E+04	0.14160		
	WFEA OV31L	4.83E+03	3.42E+04	0.14120		

Table A2 The relative expression level of *PmnRTK* during ovarian development of wild intact and eyestalk-ablated broodstock of *P. monodon*

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
Juvenile	JN OV1	2.05E+03	7.03E+07	0.00003	0.00005	0.00002
	JN OV6	9.13E+02	1.24E+07	0.00007		
	JN OV7	1.12E+03	2.44E+07	0.00005		
N-BD-Stage I	BFN OV32	6.48E+03	1.01E+07	0.00064	0.00117	0.00052
	BFN OV22	7.08E+03	5.95E+06	0.00119		
	WFN OV9	7.46E+03	4.45E+06	0.00167		
N:BD-Stage II	WFN OV2	4.15E+03	4.33E+06	0.00096	0.00114	0.00017
	WFN OV3	5.45E+03	5.15E+06	0.00106		
	WFN OV27	4.57E+03	3.91E+06	0.00117		
	WFN OV15	3.46E+03	2.57E+06	0.00135		
	WFN OV14	3.94E+03	4.31E+06	0.00091		
	WFN OV16	3.97E+03	3.32E+06	0.00119		
	WFN OV 23	4.84E+03	3.60E+06	0.00135		
N:BD-Stage III	WFN OV13	4.09E+03	3.42E+06	0.00120	0.00125	0.00029
	WFN OV12	4.06E+03	3.33E+06	0.00122		
	WFN OV11	1.66E+03	1.01E+06	0.00164		
N:BD-Stage IV	WFN OV22	2.16E+03	2.31E+06	0.00093	0.00143	0.00026
	WFN OV20	4.56E+03	4.14E+06	0.00110		

Table A2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
	WFN OV18	3.13E+03	2.24E+06	0.00139		
	WFN OV10	2.26E+03	1.52E+06	0.00149		
	WFN OV8	1.59E+03	9.20E+05	0.00173		
N:BD Post-	WFN OV1	2.64E+03	3.96E+06	0.00067	0.00089	0.00028
spawning	WFN OV4	4.60E+03	4.09E+06	0.00113		
	WFN OV7	4.42E+03	3.87E+06	0.00114		
	WFN OV17	4.46E+03	7.17E+06	0.00062		
EA:BD-Stage I	WFEA OV4	1.19E+04	1.05E+07	0.00114	0.00096	0.00031
	WFEA OV33	1.41E+03	1.24E+06	0.00113		
	WFEA OV6	1.13E+04	1.87E+07	0.00061		
EA:BD-Stage II	WFEA OV1	4.06E+03	2.87E+06	0.00141	0.00134	0.00015
	WFEA OV21	5.15E+03	4.18E+06	0.00123		
	WFEA OV29	2.76E+03	1.98E+06	0.00139		
	WFEA OV5	2.32E+03	2.11E+06	0.00110		
	WFEA OV20	3.88E+03	3.04E+06	0.00128		
	WFEA OV24	3.02E+03	2.33E+06	0.00130		
	WFEA OV18	3.00E+03	2.32E+06	0.00129		
	WFEA OV28	1.51E+04	9.22E+06	0.00164		
	WFEA OV19	8.05E+03	5.41E+06	0.00149		
	WFEA OV26	2.93E+03	2.36E+06	0.00124		
EA:BD-Stage III	WFEA OV10	6.09E+03	4.35E+06	0.00140	0.00124	0.00037
	WFEA OV17	3.00E+03	2.81E+06	0.00107		
	WFEA OV25	3.13E+03	1.74E+06	0.00180		
	WFEA OV9	1.53E+03	1.47E+06	0.00104		
	WFEA OV12	2.55E+03	2.90E+06	0.00088		
EA:BD-Stage IV	WFEA OV11	2.34E+03	1.32E+06	0.00178	0.00182	0.00020
	WFEA OV14	2.30E+03	1.13E+06	0.00204		
	WFEA OV15	1.66E+03	1.01E+06	0.00164		

Table A2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
EA:BD Post-spawning	WFEA OV27U	3.71E+03	3.20E+06	0.00116	0.00135	0.00023
	WFEA OV27L	3.57E+03	3.67E+06	0.00097		
	WFEA OV30U	2.81E+03	1.87E+06	0.00150		
	WFEA OV30L	2.59E+03	1.82E+06	0.00143		
	WFEA OV32U	2.55E+03	1.81E+06	0.00141		
	WFEA OV32L	2.38E+03	1.28E+06	0.00187		
	WFEA OV31U	2.14E+03	1.58E+06	0.00136		
	WFEA OV31L	1.39E+03	1.22E+06	0.00114		

Table A3 The relative expression level of *PmRTK* during ovarian development of wild intact and eyestalk-ablated broodstock of *P. monodon*

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
Juvenile	JN OV1	3.95E+03	6.02E+06	0.00066	0.00087	0.00029
	JN OV2	4.94E+03	5.39E+06	0.00092		
	JN OV3	4.53E+03	6.23E+06	0.00073		
	JN OV4	2.98E+03	3.34E+06	0.00089		
	JN OV5	2.43E+03	1.73E+06	0.00140		
	JN OV8	3.36E+03	5.72E+06	0.00059		
N-BD-Stage I	BFN OV32	1.36E+04	1.01E+07	0.00135	0.00177	0.00042
	BFN OV22	1.14E+04	5.95E+06	0.00191		
	BFN OV9	9.24E+03	4.45E+06	0.00207		
N:BD-Stage II	WFN OV5	1.13E+04	4.96E+06	0.00229	0.00204	0.00026
	WFN OV2	8.16E+03	4.33E+06	0.00188		
	WFN OV3	9.77E+03	5.15E+06	0.00190		
	WFN OV27	7.57E+03	3.91E+06	0.00194		
	WFN OV15	6.29E+03	2.57E+06	0.00245		

Table A3 (Cont.)

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmRTK	*EF-1α*			
	WFN OV14	7.06E+03	4.31E+06	0.00164		
	WFN OV16	6.87E+03	3.32E+06	0.00207		
	WFN OV23	8.03E+03	3.60E+06	0.00223		
N:BD-Stage III	WFN OV13	9.83E+03	3.42E+06	0.00288	0.00253	0.00035
	WFN OV12	7.38E+03	3.33E+06	0.00221		
	WFN OV22	5.88E+03	2.31E+06	0.00255		
N:BD-Stage IV	WFN OV20	9.58E+03	4.14E+06	0.00232	0.00267	0.00035
	WFN OV18	6.76E+03	2.24E+06	0.00301		
	WFN OV10	4.08E+03	1.52E+06	0.00268		
N:BD Post-	WFN OV1	4.47E+03	3.96E+06	0.00113	0.00143	0.00042
spawning	WFN OV7	7.26E+03	3.87E+06	0.00188		
	WFN OV17	9.56E+03	7.17E+06	0.00133		
EA:BD-Stage I	WFEA OV4	1.57E+04	1.05E+07	0.00149	0.00130	0.00026
	WFEA OV33	1.79E+03	1.24E+06	0.00144		
	WFEA OV6	1.91E+04	1.87E+07	0.00102		
EA:BD-Stage II	WFEA OV1	7.47E+03	2.87E+06	0.00261	0.00225	0.00031
	WFEA OV21	7.83E+03	4.18E+06	0.00187		
	WFEA OV29	3.80E+03	1.98E+06	0.00191		
	WFEA OV5	4.77E+03	2.11E+06	0.00226		
	WFEA OV20	7.69E+03	3.04E+06	0.00253		
	WFEA OV18	5.36E+03	2.32E+06	0.00231		
	WFEA OV19	1.43E+04	5.41E+06	0.00265		
	WFEA OV26	4.46E+03	2.36E+06	0.00189		
EA:BD-Stage III	WFEA OV10	9.12E+03	4.35E+06	0.00210	0.00227	0.00015
	WFEA OV17	6.79E+03	2.81E+06	0.00242		
	WFEA OV25	3.91E+03	1.74E+06	0.00225		
EA:BD-Stage IV	WFEA OV11	3.62E+03	1.32E+06	0.00274	0.00297	0.00046
	WFEA OV14	4.01E+03	1.13E+06	0.00355		

Table A3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
EA:BD Post-spawning	WFEA OV15	2.79E+03	1.01E+06	0.00275	0.00200	0.00036
	WFEA OV30U	4.60E+03	1.87E+06	0.00246		
	WFEA OV30L	3.99E+03	1.82E+06	0.00220		
	WFEA OV32U	3.98E+03	1.81E+06	0.00219		
	WFEA OV32L	2.64E+03	1.28E+06	0.00207		
	WFEA OV31U	2.57E+03	1.58E+06	0.00163		
	WFEA OV31L	2.03E+03	1.22E+06	0.00166		

APPENDIX B

Expression of reproduction-related genes in domesticated *P. monodon* following exogenous serotonin injection

Table B1 The relative expression level of *PmRnf121* mRNA of domesticated *P. monodon* injected with serotonin

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
Negative	NM18 OV2	1.90E+05	5.26E+06	0.03611	0.03297	0.00994
control	NM18 OV4	5.97E+04	2.73E+06	0.02185		
	NM18 OV6	1.41E+05	3.44E+06	0.04097		
Vehicle	NS18F OV1	2.89E+03	1.25E+05	0.02308	0.02719	0.00446
control	NS18F OV2	9.51E+03	3.58E+05	0.02658		
	NS18F OV4	1.52E+05	4.76E+06	0.03192		
0 hpi	5HT18F OV0-1	1.87E+04	9.29E+05	0.02007	0.01993	0.00595
	5HT18F OV0-2	8.08E+03	5.80E+05	0.01393		
	5HT18F OV0-3	1.64E+04	9.23E+05	0.01772		
	5HT18F OV0-4	4.25E+04	1.52E+06	0.02801		
1 hpi	5HT18F OV1-1	7.84E+03	2.17E+05	0.03608	0.02458	0.00833
	5HT18F OV1-2	3.25E+03	1.96E+05	0.01656		
	5HT18F OV1-3	8.23E+03	3.36E+05	0.02452		
	5HT18F OV1-5	2.85E+04	1.35E+06	0.02115		
3 hpi	5HT18F OV3-1	5.90E+03	2.99E+05	0.01970	0.01644	0.00702
	5HT18F OV3-2	6.31E+03	7.52E+05	0.00839		
	5HT18F OV3-3	1.33E+04	6.28E+05	0.02124		
6 hpi	5HT18F OV6-2	6.10E+04	2.19E+06	0.02779	0.02048	0.00847
	5HT18F OV6-3	1.32E+04	1.18E+06	0.01120		
	5HT18F OV6-4	2.52E+04	1.12E+06	0.02245		
12 hpi	5HT18F OV12-1	6.09E+04	2.84E+06	0.02148	0.01533	0.00440
	5HT18F OV12-2	1.42E+04	1.28E+06	0.01112		

Table B1 (Cont.)

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmRnf121	*EF-1α*			
	5HT18F OV12-3	4.53E+03	3.27E+05	0.01383		
	5HT18F OV12-4	1.83E+04	1.23E+06	0.01490		
24 hpi	5HT18F OV24-2	2.01E+04	6.96E+05	0.02895	0.02591	0.00715
	5HT18F OV24-3	2.42E+04	1.36E+06	0.01774		
	5HT18F OV24-4	1.40E+05	4.52E+06	0.03103		
48 hpi	5HT18F OV48-1	3.80E+04	1.95E+06	0.01944	0.01914	0.00130
	5HT18F OV48-2	2.24E+04	1.09E+06	0.02055		
	5HT18F OV48-5	1.34E+04	7.67E+05	0.01741		
	5HT18F OV48-6	2.08E+04	1.09E+06	0.01916		
72 hpi	5HT18F OV72-1	5.79E+03	6.87E+05	0.00842	0.02407	0.01682
	5HT18F OV72-3	3.80E+04	9.07E+05	0.04186		
	5HT18F OV72-4	5.71E+03	2.61E+05	0.02193		

Table B2 The relative expression level of *PmnRTK* mRNA of domesticated *P. monodon* injected with serotonin

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmnRTK	*EF-1α*			
Negative	NM18 OV1	2.36E+02	2.07E+04	0.01145	0.02869	0.01382
control	NM18 OV2	2.55E+03	5.25E+04	0.04866		
	NM18 OV3	1.25E+03	3.65E+04	0.03433		
	NM18 OV5	7.31E+02	3.04E+04	0.02401		
	NM18 OV6	7.71E+02	3.08E+04	0.02500		
Vehicle	NS18F OV1	7.78E+01	5.97E+03	0.01303	0.01784	0.00872
control	NS18F OV2	3.30E+02	2.62E+04	0.01259		
	NS18F OV3	1.18E+03	4.21E+04	0.02792		
0 hpi	5HT18F OV0-1	8.54E+02	3.80E+04	0.02247	0.01819	0.01002
	5HT18F OV0-2	1.80E+02	2.16E+04	0.00831		

Table B2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
	5HT18F OV0-3	1.13E+03	3.73E+04	0.03020		
	5HT18F OV0-4	1.09E+03	9.21E+04	0.01179		
1 hpi	5HT18F OV1-1	1.83E+02	1.05E+04	0.01735	0.01442	0.00747
	5HT18F OV1-2	6.49E+01	9.75E+03	0.00666		
	5HT18F OV1-3	1.88E+02	1.84E+04	0.01023		
	5HT18F OV1-5	1.70E+03	7.26E+04	0.02341		
3 hpi	5HT18F OV3-1	4.46E+02	1.93E+04	0.02313	0.01785	0.00723
	5HT18F OV3-2	3.75E+02	2.76E+04	0.01359		
	5HT18F OV3-3	8.85E+02	3.57E+04	0.02477		
	5HT18F OV3-4	2.37E+02	2.39E+04	0.00990		
6 hpi	5HT18F OV6-2	4.92E+03	8.44E+04	0.05827	0.04980	0.02354
	5HT18F OV6-3	1.13E+03	4.85E+04	0.02320		
	5HT18F OV6-4	5.69E+03	8.38E+04	0.06793		
12 hpi	5HT18F OV12-1	7.50E+03	1.33E+05	0.05660	0.04247	0.01406
	5HT18F OV12-2	1.76E+03	6.19E+04	0.02848		
	5HT18F OV12-4	2.28E+03	5.37E+04	0.04234		
24 hpi	5HT18F OV24-1	2.08E+03	5.60E+04	0.03706	0.02431	0.01253
	5HT18F OV24-3	1.58E+03	6.61E+04	0.02386		
	5HT18F OV24-5	3.21E+02	2.68E+04	0.01201		
48 hpi	5HT18F OV48-2	1.20E+03	6.55E+04	0.01833	0.02905	0.01517
	5HT18F OV48-5	9.81E+02	4.38E+04	0.02241		
	5HT18F OV48-6	3.14E+03	6.77E+04	0.04641		
72 hpi	5HT18F OV72-1	1.59E+02	3.59E+04	0.00443	0.01091	0.00663
	5HT18F OV72-2	3.01E+02	2.61E+04	0.01153		
	5HT18F OV72-3	8.19E+02	4.12E+04	0.01984		
	5HT18F OV72-4	1.19E+02	1.51E+04	0.00783		

Table B3 The relative expression level of *PmRTK* mRNA of domesticated *P. monodon* injected with serotonin

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
Negative	NM18 OV1	1.13E+03	2.07E+04	0.05467	0.06410	0.01253
control	NM18 OV3	2.68E+03	3.65E+04	0.07335		
	NM18 OV5	2.33E+03	3.04E+04	0.07642		
	NM18 OV6	1.61E+03	3.08E+04	0.05204		
Vehicle	NS18F OV1	1.63E+02	5.97E+03	0.02735	0.03740	0.01925
control	NS18F OV2	6.62E+02	2.62E+04	0.02526		
	NS18F OV3	2.51E+03	4.21E+04	0.05962		
0 hpi	5HT18F OV0-1	2.04E+03	3.80E+04	0.05378	0.03273	0.01930
	5HT18F OV0-2	3.44E+02	2.16E+04	0.01590		
	5HT18F OV0-4	2.62E+03	9.21E+04	0.02849		
1 hpi	5HT18F OV1-1	6.54E+02	1.05E+04	0.06204	0.04740	0.01289
	5HT18F OV1-3	6.92E+02	1.84E+04	0.03759		
	5HT18F OV1-5	3.09E+03	7.26E+04	0.04265		
3 hpi	5HT18F OV3-1	1.26E+03	1.93E+04	0.06552	0.06323	0.01592
	5HT18F OV3-2	1.28E+03	2.76E+04	0.04628		
	5HT18F OV3-3	2.78E+03	3.57E+04	0.07791		
6 hpi	5HT18F OV6-2	1.03E+04	8.44E+04	0.12177	0.09003	0.03329
	5HT18F OV6-3	2.69E+03	4.85E+04	0.05540		
	5HT18F OV6-4	7.78E+03	8.38E+04	0.09288		
12 hpi	5HT18F OV12-1	7.19E+03	1.33E+05	0.05422	0.04443	0.01912
	5HT18F OV12-2	3.51E+03	6.19E+04	0.05668		
	5HT18F OV12-3	5.53E+02	2.47E+04	0.02239		
24 hpi	5HT18F OV24-1	2.91E+03	5.60E+04	0.05186	0.05257	0.00180
	5HT18F OV24-3	3.38E+03	6.61E+04	0.05117		
	5HT18F OV24-4	9.36E+03	1.72E+05	0.05457		

Table B3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
48 hpi	5HT18F OV48-1	1.22E+04	1.03E+05	0.11825	0.09843	0.01816
	5HT18F OV48-5	3.61E+03	4.38E+04	0.08249		
	5HT18F OV48-6	6.40E+03	6.77E+04	0.09465		
72 hpi	5HT18F OV72-1	1.08E+03	3.59E+04	0.02997	0.03930	0.01384
	5HT18F OV72-2	8.54E+02	2.61E+04	0.03274		
	5HT18F OV72-3	2.28E+03	4.12E+04	0.05519		

APPENDIX C

Expression of reproduction-related genes in domesticated *P. monodon* following exogenous progesterone injection

Table C1 The relative expression level of *PmRnf121* mRNA of domesticated *P. monodon* injected with progesterone

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
Negative	NM14 OV3	5.82E+02	5.38E+04	0.01082	0.02252	0.01533
control	NM14 OV4	1.14E+03	7.20E+04	0.01589		
	NM14 OV5	2.38E+03	1.30E+05	0.01832		
	NM14 OV7	7.36E+02	1.63E+04	0.04503		
Vehicle	PG14 E0 OV1	8.61E+02	5.13E+04	0.01678	0.06333	0.06282
control 0 hpi	PG14 E0 OV2	4.92E+03	1.28E+05	0.03842		
	PG14 E0 OV3	5.80E+02	4.30E+03	0.13478		
Vehicle	PG14 E12 OV1	1.70E+03	6.31E+04	0.02701	0.02272	0.00887
control 12 hpi	PG14 E12 OV3	4.41E+03	3.00E+05	0.01469		
	PG14 E12 OV4	4.27E+03	1.63E+05	0.02624		
	PG14 E12 OV5	3.19E+03	9.59E+04	0.03330		
	PG14 E12 OV6	7.41E+02	6.00E+04	0.01235		
12 hpi	PG14 OV12-2	1.16E+03	2.85E+04	0.04066	0.02995	0.01073
	PG14 OV12-4	6.38E+03	1.88E+05	0.03391		
	PG14 OV12-5	8.78E+02	5.74E+04	0.01529		
	PG14 OV12-6	5.59E+02	1.86E+04	0.02996		
24 hpi	PG14 OV24-1	8.65E+02	1.46E+05	0.00594	0.01089	0.00455
	PG14 OV24-2	1.30E+03	1.03E+05	0.01266		
	PG14 OV24-3	6.14E+02	3.76E+04	0.01632		
	PG14 OV24-5	9.10E+02	1.05E+05	0.00865		
48 hpi	PG14 OV48-1	1.00E+03	3.34E+04	0.03000	0.03265	0.01658
	PG14 OV48-3	3.79E+03	6.70E+04	0.05650		

Table C1 (Cont.)

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmRnf121	*EF-1α*			
	PG14 OV48-4	7.36E+02	2.89E+04	0.02551		
	PG14 OV48-5	4.18E+03	2.25E+05	0.01859		
72 hpi	PG14 OV72-1	8.05E+02	7.94E+04	0.01013	0.01565	0.00947
	PG14 OV72-2	1.07E+03	1.04E+05	0.01022		
	PG14 OV72-3	7.41E+02	2.79E+04	0.02658		

Table C2 The relative expression level of *PmnRTK* mRNA of domesticated *P. monodon* injected with progesterone

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmnRTK	*EF-1α*			
Negative	BU14 OV5	7.03E+03	4.45E+05	0.01581	0.00933	0.00476
control	BU14 OV6	1.93E+03	4.23E+05	0.00456		
	BU14 OV13	3.32E+03	4.49E+05	0.00740		
	BU14 OV34	3.81E+03	4.01E+05	0.00949		
Vehicle	PG14 E0 OV2	4.83E+04	5.13E+05	0.09416	0.14550	0.04633
control 0 hpi	PG14 E0 OV3	8.92E+04	5.65E+05	0.15802		
	PG14 E0 OV5	1.05E+05	5.67E+05	0.18426		
Vehicle	PG14 E12 OV1	7.60E+04	2.57E+06	0.02958	0.04227	0.01850
control 12 hpi	PG14 E12 OV3	1.77E+05	2.78E+06	0.06350		
	PG14 E12 OV4	1.00E+05	2.98E+06	0.03373		
12 hpi	PG14 OV12-4	3.97E+04	2.54E+06	0.01560	0.01347	0.00244
	PG14 OV12-5	6.54E+04	6.07E+06	0.01077		
	PG14 OV12-6	9.53E+03	6.83E+05	0.01395		
24 hpi	PG14 OV24-1	9.74E+04	1.24E+06	0.07834	0.04723	0.03237
	PG14 OV24-3	1.34E+04	9.77E+05	0.01370		
	PG14 OV24-5	3.29E+04	6.61E+05	0.04972		

Table C2 (Cont.)

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmnRTK	*EF-1α*			
48 hpi	PG14 OV48-2	2.24E+04	3.26E+06	0.00687	0.00660	0.00187
	PG14 OV48-4	5.58E+04	6.69E+06	0.00834		
	PG14 OV48-6	1.83E+04	3.95E+06	0.00464		
72 hpi	PG14 OV72-1	7.86E+04	1.46E+06	0.05389	0.06447	0.05177
	PG14 OV72-2	1.66E+04	1.38E+05	0.12073		
	PG14 OV72-5	4.16E+03	2.21E+05	0.01881		

Table C3 The relative expression level of *PmRTK* mRNA of domesticated *P. monodon* injected with progesterone

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmRTK	*EF-1α*			
Negative	BU14 OV5	7.51E+01	4.45E+05	0.00017	0.00018	0.00005
control	BU14 OV6	7.78E+01	4.23E+05	0.00018		
	BU14 OV13	4.68E+01	4.49E+05	0.00010		
	BU14 OV19	5.28E+01	4.56E+05	0.00012		
	BU14 OV27	4.33E+02	2.11E+06	0.00021		
	BU14 OV33	3.78E+02	1.91E+06	0.00020		
	BU14 OV34	1.04E+02	4.01E+05	0.00026		
Vehicle	PG14 E0 OV2	2.53E+02	2.67E+06	0.00009	0.00026	0.00016
control 0 hpi	PG14 E0 OV3	7.04E+00	1.76E+04	0.00040		
	PG14 E0 OV5	5.80E+01	1.96E+05	0.00030		
Vehicle	PG14 E12 OV1	4.71E+02	2.57E+06	0.00018	0.00020	0.00003
control 12 hpi	PG14 E12 OV2	3.56E+02	2.02E+06	0.00018		
	PG14 E12 OV3	6.94E+02	2.78E+06	0.00025		
	PG14 E12 OV4	6.89E+02	2.98E+06	0.00023		
	PG14 E12 OV5	3.20E+02	2.04E+06	0.00016		
	PG14 E12 OV6	3.44E+01	1.95E+05	0.00018		

Table C3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
	PG14 E12 OV7	7.28E+02	3.19E+06	0.00023		
12 hpi	PG14 OV12-1	2.26E+02	2.42E+06	0.00009	0.00012	0.00003
	PG14 OV12-2	2.28E+01	1.82E+05	0.00013		
	PG14 OV12-4	4.18E+02	2.54E+06	0.00016		
	PG14 OV12-6	7.19E+01	6.83E+05	0.00011		
24 hpi	PG14 OV24-1	2.18E+02	1.24E+06	0.00018	0.00015	0.00002
	PG14 OV24-2	9.09E+01	6.79E+05	0.00013		
	PG14 OV24-3	1.67E+02	9.77E+05	0.00017		
	PG14 OV24-4	1.05E+02	8.16E+05	0.00013		
	PG14 OV24-5	8.85E+01	6.61E+05	0.00013		
	PG14 OV24-6	4.59E+01	2.86E+05	0.00016		
48 hpi	PG14 OV48-3	1.31E+02	7.30E+05	0.00018	0.00025	0.00006
	PG14 OV48-4	1.67E+03	6.69E+06	0.00025		
	PG14 OV48-5	2.99E+01	1.25E+05	0.00024		
	PG14 OV48-6	1.30E+03	3.95E+06	0.00033		
72 hpi	PG14 OV72-1	3.21E+02	1.46E+06	0.00022	0.00026	0.00004
	PG14 OV72-2	3.77E+01	1.38E+05	0.00027		
	PG14 OV72-5	6.60E+01	2.21E+05	0.00030		

APPENDIX D

Expression of reproduction-related genes in domesticated *P. monodon* following exogenous 17β-estradiol injection

Table D1 The relative expression level of *PmRnf121* mRNA of domesticated *P. monodon* injected with 17β-estradiol

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
7 dpi: Negative control	NM7D OV1	8.52E+03	1.24E+06	0.00687	0.01508	0.00806
	NM7D OV2	2.08E+04	1.83E+06	0.01140		
	NM7D OV3	6.22E+04	2.62E+06	0.02374		
	NM7D OV4	3.33E+04	2.02E+06	0.01653		
	NM7D OV5	9.97E+03	1.45E+06	0.00689		
	NM7D OV6	3.92E+04	1.57E+06	0.02505		
7 dpi: Vehicle control	EtOH7D OV2	2.01E+04	1.33E+06	0.01510	0.01667	0.00708
	EtOH7D OV3	1.93E+04	8.83E+05	0.02190		
	EtOH7D OV4	1.41E+04	1.54E+06	0.00915		
	EtOH7D OV5	2.31E+04	8.92E+05	0.02589		
	EtOH7D OV6	6.74E+03	5.97E+05	0.01129		
7 dpi: Eyestalk ablation	EA7D OV1	9.91E+03	9.69E+05	0.01024	0.01354	0.00499
	EA7D OV2	3.44E+04	2.25E+06	0.01526		
	EA7D OV3	3.53E+04	1.87E+06	0.01887		
	EA7D OV4	2.19E+04	1.91E+06	0.01148		
	EA7D OV5	5.36E+04	2.84E+06	0.01889		
	EA7D OV6	9.10E+03	1.40E+06	0.00651		
7 dpi: 17β-Estradiol	ESA7D OV1	1.14E+03	6.72E+05	0.00170	0.00382	0.00261
	ESA7D OV2	3.12E+03	8.08E+05	0.00387		
	ESA7D OV3	2.59E+03	5.70E+05	0.00455		
	ESA7D OV4	4.56E+03	5.29E+05	0.00862		
	ESA7D OV5	2.13E+03	1.04E+06	0.00204		

Table D1 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
	ESA7D OV6	4.98E+03	2.33E+06	0.00214		
14 dpi: Negative	NM14D OV1	2.48E+03	5.30E+05	0.00468	0.00913	0.00368
control	NM14D OV2	7.21E+03	6.82E+05	0.01057		
	NM14D OV3	9.46E+03	6.55E+05	0.01445		
	NM14D OV4	6.09E+03	8.44E+05	0.00721		
	NM14D OV6	6.15E+03	7.05E+05	0.00873		
14 dpi: Vehicle	EtOH14D OV1	2.19E+03	5.97E+05	0.00367	0.00413	0.00194
control	EtOH14D OV2	7.46E+03	9.96E+05	0.00749		
	EtOH14D OV4	2.68E+03	8.72E+05	0.00308		
	EtOH14D OV5	2.72E+03	7.14E+05	0.00380		
	EtOH14D OV6	1.85E+03	7.13E+05	0.00259		
14 dpi: Eyestalk	EA14D OV1	4.94E+03	3.98E+05	0.01240	0.01006	0.00382
ablation	EA14D OV2	3.63E+03	2.33E+05	0.01558		
	EA14D OV3	3.51E+03	3.57E+05	0.00983		
	EA14D OV4	2.83E+03	3.10E+05	0.00912		
	EA14D OV5	2.99E+03	7.29E+05	0.00410		
	EA14D OV6	2.38E+03	2.55E+05	0.00933		
14 dpi:	ESA14D OV1	3.26E+03	6.72E+05	0.00485	0.00521	0.00233
17β-Estradiol	ESA14D OV2	3.16E+03	8.08E+05	0.00391		
	ESA14D OV3	1.40E+03	3.41E+05	0.00410		
	ESA14D OV4	2.54E+03	3.68E+05	0.00691		
	ESA14D OV5	3.61E+03	4.03E+05	0.00896		
	ESA14D OV6	7.28E+02	2.86E+05	0.00255		
28 dpi: Negative	NM28D OV2	6.36E+03	2.13E+05	0.02988	0.02596	0.00355
control	NM28D OV3	5.24E+03	2.25E+05	0.02329		
	NM28D OV4	8.29E+03	2.96E+05	0.02804		
	NM28D OV5	5.82E+03	2.57E+05	0.02263		

Table D1 (Cont.)

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmRnf121	*EF-1α*			
28 dpi: Vehicle	EtOH28D OV1	6.59E+03	2.08E+05	0.03175	0.04442	0.02363
control	EtOH28D OV2	2.20E+04	2.85E+05	0.07733		
	EtOH28D OV3	9.92E+03	2.21E+05	0.04492		
	EtOH28D OV4	4.46E+03	1.89E+05	0.02366		
28 dpi: Eyestalk	EA28D OV2	4.18E+03	2.52E+05	0.01661	0.01795	0.00917
ablation	EA28D OV3	1.18E+04	3.97E+05	0.02981		
	EA28D OV4	1.03E+04	4.17E+05	0.02463		
	EA28D OV5	7.15E+03	3.34E+05	0.02142		
	EA28D OV7	4.33E+03	7.79E+05	0.00556		
	EA28D OV8	7.02E+03	7.24E+05	0.00969		
28 dpi:	ESA28D OV1	1.78E+04	2.39E+05	0.07464	0.06002	0.01646
17β-Estradiol	ESA28D OV3	8.48E+03	1.95E+05	0.04358		
	ESA28D OV4	9.37E+03	1.95E+05	0.04814		
	ESA28D OV6	1.79E+04	2.43E+05	0.07371		

Table D2 The relative expression level of *PmnRTK* mRNA of domesticated *P. monodon* injected with 17β-estradiol

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmnRTK	*EF-1α*			
7 dpi: Negative	NM7D OV1	1.61E+03	5.62E+06	0.00029	0.00030	0.00003
control	NM7D OV2	2.41E+03	8.27E+06	0.00029		
	NM7D OV4	1.12E+03	3.33E+06	0.00034		
7 dpi: Vehicle	EtOH7D OV1	2.80E+03	7.91E+06	0.00035	0.00060	0.00024
control	EtOH7D OV3	2.70E+03	4.36E+06	0.00062		
	EtOH7D OV5	4.18E+03	4.98E+06	0.00084		
7 dpi: Eyestalk	EA7D OV1	4.51E+03	1.14E+07	0.00039	0.00080	0.00027
ablation	EA7D OV2	6.39E+03	7.22E+06	0.00089		

Table D2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
	EA7D OV4	3.11E+03	4.65E+06	0.00067		
	EA7D OV6	1.82E+03	1.86E+06	0.00098		
	EA7D OV8	3.23E+03	2.98E+06	0.00108		
7 dpi:	ESA7D OV1	1.82E+03	5.67E+06	0.00032	0.00036	0.00007
17β-Estradiol	ESA7D OV2	1.45E+03	4.00E+06	0.00036		
	ESA7D OV3	2.53E+03	8.82E+06	0.00029		
	ESA7D OV4	1.53E+03	3.41E+06	0.00045		
14 dpi: Negative	NM14D OV1	1.56E+03	3.67E+06	0.00043	0.00040	0.00020
control	NM14D OV2	5.71E+02	2.28E+06	0.00025		
	NM14D OV4	6.70E+02	2.69E+06	0.00025		
	NM14D OV5	2.40E+03	3.55E+06	0.00068		
14 dpi: Vehicle	EtOH14D OV1	6.35E+02	2.44E+06	0.00026	0.00049	0.00021
control	EtOH14D OV3	2.77E+03	7.24E+06	0.00038		
	EtOH14D OV4	2.51E+03	3.40E+06	0.00074		
	EtOH14D OV5	2.26E+03	3.27E+06	0.00069		
	EtOH14D OV6	2.47E+03	6.34E+06	0.00039		
14 dpi: Eyestalk	EA14D OV1	9.37E+02	4.19E+06	0.00022	0.00025	0.00013
ablation	EA14D OV2	8.94E+02	5.56E+06	0.00016		
	EA14D OV3	8.51E+02	4.61E+06	0.00019		
	EA14D OV5	2.10E+03	4.79E+06	0.00044		
14 dpi:	ESA14D OV1	5.72E+02	2.58E+06	0.00022	0.00024	0.00014
17β-Estradiol	ESA14D OV3	2.56E+03	5.74E+06	0.00045		
	ESA14D OV4	6.60E+02	5.53E+06	0.00012		
	ESA14D OV6	1.77E+03	9.59E+06	0.00018		
28 dpi: Negative	NM28D OV1	1.24E+03	9.29E+05	0.00133	0.00120	0.00053
control	NM28D OV2	6.51E+02	1.06E+06	0.00062		
	NM28D OV6	1.23E+03	7.40E+05	0.00166		

Table D2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
28 dpi: Vehicle control	EtOH28D OV1	1.46E+03	2.24E+06	0.00065	0.00051	0.00013
	EtOH28D OV2	1.05E+03	2.20E+06	0.00048		
	EtOH28D OV3	6.66E+02	1.72E+06	0.00039		
28 dpi: Eyestalk ablation	EA28D OV4	2.86E+03	1.25E+06	0.00230	0.00247	0.00024
	EA28D OV5	3.99E+03	1.65E+06	0.00243		
	EA28D OV6	4.50E+03	1.88E+06	0.00240		
	EA28D OV7	2.38E+03	8.22E+05	0.00289		
	EA28D OV8	1.52E+03	6.49E+05	0.00235		
28 dpi: 17β-Estradiol	ESA28D OV1	7.89E+02	1.34E+06	0.00059	0.00070	0.00007
	ESA28D OV2	1.96E+03	2.66E+06	0.00074		
	ESA28D OV3	1.37E+03	2.06E+06	0.00067		
	ESA28D OV4	1.06E+03	1.35E+06	0.00078		
	ESA28D OV5	9.64E+02	1.31E+06	0.00073		

Table D3 The relative expression level of *PmRTK* mRNA of domesticated *P. monodon* injected with 17β-estradiol

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
7 dpi: Negative control	NM7D OV1	6.31E+03	5.62E+06	0.00112	0.00113	0.00012
	NM7D OV2	1.03E+04	8.27E+06	0.00125		
	NM7D OV3	1.56E+04	1.53E+07	0.00102		
7 dpi: Vehicle control	EtOH7D OV1	8.62E+03	7.91E+06	0.00109	0.00154	0.00103
	EtOH7D OV3	3.53E+03	4.36E+06	0.00081		
	EtOH7D OV5	1.35E+04	4.98E+06	0.00271		
7 dpi: Eyestalk ablation	EA7D OV1	1.37E+04	1.14E+07	0.00119	0.00172	0.00046
	EA7D OV2	1.54E+04	7.22E+06	0.00213		

Table D3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
	EA7D OV4	6.91E+03	4.65E+06	0.00149		
	EA7D OV8	6.22E+03	2.98E+06	0.00208		
7 dpi:	ESA7D OV2	6.24E+03	4.00E+06	0.00156	0.00158	0.00025
17β-Estradiol	ESA7D OV3	1.08E+04	8.82E+06	0.00123		
	ESA7D OV4	6.21E+03	3.41E+06	0.00182		
	ESA7D OV5	3.49E+03	2.05E+06	0.00170		
14 dpi: Negative	NM14D OV2	2.90E+03	2.28E+06	0.00127	0.00145	0.00062
control	NM14D OV4	2.54E+03	2.69E+06	0.00094		
	NM14D OV5	7.61E+03	3.55E+06	0.00214		
14 dpi: Vehicle	EtOH14D OV1	3.47E+03	2.44E+06	0.00142	0.00133	0.00008
control	EtOH14D OV3	9.48E+03	7.24E+06	0.00131		
	EtOH14D OV6	8.05E+03	6.34E+06	0.00127		
14 dpi: Eyestalk	EA14D OV1	7.28E+03	4.19E+06	0.00174	0.00163	0.00042
ablation	EA14D OV3	5.41E+03	4.61E+06	0.00117		
	EA14D OV5	9.52E+03	4.79E+06	0.00199		
14 dpi:	ESA14D OV1	3.29E+03	2.58E+06	0.00128	0.00104	0.00021
17β-Estradiol	ESA14D OV3	5.39E+03	5.74E+06	0.00094		
	ESA14D OV5	4.50E+03	4.93E+06	0.00091		
28 dpi: Negative	NM28D OV1	4.40E+03	9.29E+05	0.00474	0.00495	0.00049
control	NM28D OV5	2.04E+03	4.45E+05	0.00459		
	NM28D OV6	4.07E+03	7.40E+05	0.00551		
28 dpi: Vehicle	EtOH28D OV1	3.64E+03	2.24E+06	0.00163	0.00167	0.00046
control	EtOH28D OV3	2.11E+03	1.72E+06	0.00123		
	EtOH28D OV6	1.29E+04	6.01E+06	0.00214		
28 dpi: Eyestalk	EA28D OV4	1.39E+04	1.25E+06	0.01117	0.01139	0.00190
ablation	EA28D OV5	1.47E+04	1.65E+06	0.00891		
	EA28D OV7	1.11E+04	8.22E+05	0.01345		
	EA28D OV8	7.82E+03	6.49E+05	0.01204		

Table D3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
28 dpi:	ESA28D OV1	2.21E+03	1.34E+06	0.00165	0.00182	0.00018
17β-Estradiol	ESA28D OV2	4.50E+03	2.66E+06	0.00169		
	ESA28D OV3	4.17E+03	2.06E+06	0.00202		
	ESA28D OV5	2.49E+03	1.31E+06	0.00190		

APPENDIX E

Expression of reproduction-related genes in domesticated *P. monodon* following the feeding trial with diets supplemented with 17β-estradiol

Table E1 The relative expression level of *PmRnf121* mRNA of domesticated *P. monodon* were fed with supplemented diets with 17β-estradiol

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
7 days: Negative control	7C BUF OV1	1.89E+04	9.97E+06	0.00189	0.00178	0.00026
	7C BUF OV2	1.05E+04	5.28E+06	0.00199		
	7C BUF OV3	8.70E+03	4.84E+06	0.00180		
	7C BUF OV6	5.60E+03	4.10E+06	0.00137		
7 days: Eyestalk ablation	7C BUF EA OV1	5.48E+03	5.01E+06	0.00109	0.00193	0.00127
	7C BUF EA OV2	1.45E+04	4.29E+06	0.00339		
	7C BUF EA OV3	6.54E+03	5.01E+06	0.00131		
7 days: 1 mg/kg	7 ES1 OV1	1.52E+04	1.45E+07	0.00105	0.00090	0.00018
	7 ES1 OV4	8.80E+03	8.85E+06	0.00099		
	7 ES1 OV5	2.89E+03	3.61E+06	0.00080		
	7 ES1 OV6	1.26E+03	1.84E+06	0.00068		
7 days: 10 mg/kg	7 ES10 OV1	1.85E+03	4.97E+06	0.00037	0.00040	0.00014
	7 ES10 OV2	1.79E+03	5.86E+06	0.00030		
	7 ES10 OV4	2.77E+03	4.88E+06	0.00057		
	7 ES10 OV6	1.57E+03	5.16E+06	0.00031		
14 days: Negative control	14C BUF OV3	6.08E+02	4.47E+05	0.00136	0.00098	0.00029
	14C BUF OV4	3.23E+02	4.04E+05	0.00080		
	14C BUF OV5	7.01E+02	8.67E+05	0.00081		
	14C BUF OV6	6.99E+02	8.04E+05	0.00087		
14 days: Eyestalk ablation	14C BUF EA OV1	5.05E+02	7.23E+05	0.00070	0.00097	0.00046
	14C BUF EA OV2	1.51E+03	1.00E+06	0.00151		
	14C BUF EA OV3	7.77E+02	1.08E+06	0.00072		

Table E1 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRnf121	*EF-1α*			
14 days: 1 mg/kg	14 ES1 OV1	9.63E+02	8.04E+05	0.00120	0.00092	0.00019
	14 ES1 OV2	1.01E+03	9.36E+05	0.00108		
	14 ES1 OV3	3.59E+02	4.51E+05	0.00080		
	14 ES1 OV4	3.65E+02	4.17E+05	0.00087		
	14 ES1 OV5	5.22E+02	6.35E+05	0.00082		
	14 ES1 OV6	2.67E+02	4.06E+05	0.00066		
14 days: 10 mg/kg	14 ES10 OV1	3.44E+02	4.60E+05	0.00075	0.00110	0.00078
	14 ES10 OV3	3.00E+02	5.29E+05	0.00057		
	14 ES10 OV5	1.73E+03	8.56E+05	0.00202		
28 days: Negative	28C BUF OV1	4.26E+02	5.50E+05	0.00078	0.00098	0.00031
control	28C BUF OV4	1.97E+03	2.85E+06	0.00069		
	28C BUF OV5	6.90E+02	7.03E+05	0.00098		
	28C BUF OV6	4.59E+03	3.18E+06	0.00144		
28 days: Eyestalk	28C BUF EA OV1	3.23E+03	6.79E+06	0.00048	0.00093	0.00039
ablation	28C BUF EA OV2	1.08E+03	8.63E+05	0.00125		
	28C BUF EA OV3	3.46E+03	4.97E+06	0.00070		
	28C BUF EA OV5	7.90E+02	6.23E+05	0.00127		
28 days: 1 mg/kg	28 ES1 BUF OV1	2.19E+03	3.41E+06	0.00064	0.00073	0.00015
	28 ES1 BUF OV3	4.00E+02	4.32E+05	0.00093		
	28 ES1 BUF OV5	2.37E+03	3.52E+06	0.00068		
28 days: 10 mg/kg	28 ES10 BUF OV3	4.58E+02	2.72E+05	0.00169	0.00180	0.00010
	28 ES10 BUF OV4	7.31E+02	3.81E+05	0.00192		
	28 ES10 BUF OV6	5.21E+02	2.83E+05	0.00184		
35 days: Negative	35C BUF OV1	9.71E+02	2.74E+06	0.00035	0.00070	0.00028
control	35C BUF OV2	9.08E+02	1.24E+06	0.00073		
	35C BUF OV3	1.02E+03	1.24E+06	0.00082		
	35C BUF OV4	8.71E+02	2.67E+06	0.00033		
	35C BUF OV6	1.92E+03	2.09E+06	0.00092		

Table E1 (Cont.)

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmRnf121	*EF-1α*			
	35C BUF OV7	1.37E+03	1.29E+06	0.00107		
	35C BUF OV8	1.72E+03	2.30E+06	0.00075		
35 days: Eyestalk	35C BUF EA OV1	1.76E+03	1.61E+06	0.00109	0.00117	0.00050
ablation	35C BUF EA OV2	2.92E+03	1.73E+06	0.00168		
	35C BUF EA OV3	1.76E+03	2.58E+06	0.00068		
35 days: 1 mg/kg	35 ES1 BUF OV2	2.64E+03	1.30E+06	0.00203	0.00145	0.00041
	35 ES1 BUF OV3	1.50E+03	1.53E+06	0.00098		
	35 ES1 BUF OV4	5.19E+03	3.60E+06	0.00144		
	35 ES1 BUF OV5	2.80E+03	2.05E+06	0.00137		
35 days: 10 mg/kg	35 ES10 BUF OV2	3.77E+03	3.29E+06	0.00114	0.00123	0.00012
	35 ES10 BUF OV4	2.02E+03	1.52E+06	0.00132		
	35 ES10 BUF OV5	2.34E+03	1.80E+06	0.00130		

Table E2 The relative expression level of *PmnRTK* mRNA of domesticated *P.monodon* were fed with supplemented diets with 17ß-estradiol

Sample		Mean conc.		Ratio (target / EF-1α)	Average	SD
		PmnRTK	*EF-1α*			
7 days: Negative	7C BUF OV2	4.20E+04	5.28E+06	0.00794	0.01100	0.00285
control	7C BUF OV3	6.55E+04	4.84E+06	0.01353		
	7C BUF OV5	1.03E+05	8.88E+06	0.01159		
7 days: Eyestalk	7C BUF EA OV1	7.52E+03	5.01E+06	0.00150	0.00217	0.00058
ablation	7C BUF EA OV2	1.06E+04	4.29E+06	0.00246		
	7C BUF EA OV3	1.27E+04	5.01E+06	0.00253		
7 days: 1 mg/kg	7 ES1 OV4	5.24E+04	8.85E+06	0.00592	0.00597	0.00090
	7 ES1 OV5	1.85E+04	3.61E+06	0.00512		
	7 ES1 OV6	1.27E+04	1.84E+06	0.00691		
7 days: 10 mg/kg	7 ES10 OV1	1.23E+04	4.97E+06	0.00247	0.00237	0.00051

Table E2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
	7 ES10 OV3	6.49E+03	3.68E+06	0.00177		
	7 ES10 OV4	1.38E+04	4.88E+06	0.00283		
14 days: Negative	14C BUF OV1	5.56E+02	1.13E+06	0.00049	0.00173	0.00157
control	14C BUF OV2	8.52E+02	5.59E+05	0.00152		
	14C BUF OV3	4.21E+02	4.47E+05	0.00094		
	14C BUF OV6	3.25E+03	8.04E+05	0.00405		
14 days: Eyestalk	14C BUF EA OV1	1.91E+03	7.23E+05	0.00264	0.07543	0.11863
ablation	14C BUF EA OV2	2.23E+05	1.00E+06	0.22264		
	14C BUF EA OV3	1.43E+04	1.08E+06	0.01316		
14 days: 1 mg/kg	14 ES1 OV1	3.74E+03	8.04E+05	0.00465	0.00650	0.00159
	14 ES1 OV3	3.22E+03	4.51E+05	0.00715		
	14 ES1 OV5	4.88E+03	6.35E+05	0.00768		
14 days: 10 mg/kg	14 ES10 OV1	3.76E+03	4.60E+05	0.00817	0.00863	0.00045
	14 ES10 OV3	4.79E+03	5.29E+05	0.00906		
	14 ES10 OV5	7.34E+03	8.56E+05	0.00858		
28 days: Negative	28C BUF OV1	2.14E+03	5.50E+05	0.00388	0.00330	0.00168
control	28C BUF OV3	2.27E+03	4.91E+05	0.00463		
	28C BUF OV4	3.92E+03	2.85E+06	0.00137		
28 days: Eyestalk	28C BUF EA OV2	5.41E+04	8.63E+05	0.06268	0.01380	0.02230
ablation	28C BUF EA OV3	1.38E+04	4.97E+06	0.00278		
	28C BUF EA OV6	6.76E+03	7.75E+04	0.08720		
28 days: 1 mg/kg	28 ES1 BUF OV1	1.61E+04	3.41E+06	0.00472	0.00587	0.00274
	28 ES1 BUF OV4	1.14E+03	2.94E+05	0.00388		
	28 ES1 BUF OV5	3.17E+04	3.52E+06	0.00903		
28 days: 10 mg/kg	28 ES10 BUF OV3	1.08E+04	2.72E+05	0.03969	0.03080	0.01709
	28 ES10 BUF OV5	4.51E+04	4.06E+06	0.01111		
	28 ES10 BUF OV6	1.18E+04	2.83E+05	0.04156		

Table E2 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmnRTK	*EF-1α*			
35 days: Negative	35C BUF OV2	7.71E+03	1.24E+06	0.00620	0.00660	0.00235
control	35C BUF OV3	9.16E+03	1.24E+06	0.00738		
	35C BUF OV7	1.19E+04	1.29E+06	0.00921		
	35C BUF OV8	8.35E+03	2.30E+06	0.00363		
35 days: Eyestalk	35C BUF EA OV1	9.14E+02	1.61E+06	0.00057	0.00299	0.00290
ablation	35C BUF EA OV3	2.53E+04	2.58E+06	0.00981		
	35C BUF EA OV4	1.87E+03	4.27E+05	0.00438		
35 days: 1 mg/kg	35 ES1 BUF OV2	3.96E+03	1.30E+06	0.00305	0.00227	0.00074
	35 ES1 BUF OV5	4.04E+03	2.05E+06	0.00197		
	35 ES1 BUF OV6	6.18E+03	3.62E+06	0.00171		
35 days: 10 mg/kg	35 ES10 BUF OV3	6.40E+02	1.44E+06	0.00044	0.00060	0.00035
	35 ES10 BUF OV4	1.56E+03	1.52E+06	0.00102		
	35 ES10 BUF OV5	7.04E+02	1.80E+06	0.00039		

Table E3 The relative expression level of *PmRTK* mRNA of domesticated *P. monodon* were fed with supplemented diets with 17β-estradiol

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
7 days: Negative	7C BUF OV1	8.19E+03	9.97E+06	0.00082	0.00105	0.00054
control	7C BUF OV2	3.64E+03	5.28E+06	0.00069		
	7C BUF OV3	4.89E+03	4.84E+06	0.00101		
	7C BUF OV4	9.11E+03	7.02E+06	0.00130		
	7C BUF OV5	4.83E+03	8.88E+06	0.00054		
	7C BUF OV6	8.07E+03	4.10E+06	0.00197		
7 days: Eyestalk	7C BUF EA OV1	6.84E+03	5.01E+06	0.00137	0.00113	0.00038
ablation	7C BUF EA OV2	5.42E+03	4.29E+06	0.00126		
	7C BUF EA OV3	3.64E+03	5.01E+06	0.00073		

Table E3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
7 days: 1 mg/kg	7 ES1 OV1	1.26E+04	1.45E+07	0.00087	0.00120	0.00058
	7 ES1 OV2	3.80E+03	4.91E+06	0.00077		
	7 ES1 OV3	3.85E+03	4.90E+06	0.00079		
	7 ES1 OV4	8.20E+03	8.85E+06	0.00093		
	7 ES1 OV5	5.83E+03	3.61E+06	0.00161		
	7 ES1 OV6	4.12E+03	1.84E+06	0.00223		
7 days: 10 mg/kg	7 ES10 OV1	5.30E+03	4.97E+06	0.00107	0.00103	0.00023
	7 ES10 OV2	4.40E+03	5.86E+06	0.00075		
	7 ES10 OV3	4.09E+03	3.68E+06	0.00111		
	7 ES10 OV4	4.73E+03	4.88E+06	0.00097		
	7 ES10 OV5	1.24E+03	8.95E+05	0.00139		
	7 ES10 OV6	3.89E+03	5.16E+06	0.00075		
14 days: Negative	14C BUF OV2	1.13E+03	5.59E+05	0.00202	0.00204	0.00043
control	14C BUF OV3	1.14E+03	4.47E+05	0.00256		
	14C BUF OV4	8.85E+02	4.04E+05	0.00219		
	14C BUF OV5	1.73E+03	8.67E+05	0.00200		
	14C BUF OV6	1.12E+03	8.04E+05	0.00139		
14 days: Eyestalk	14C BUF EA OV1	2.48E+03	7.23E+05	0.00343	0.00210	0.00115
ablation	14C BUF EA OV2	1.66E+03	1.00E+06	0.00166		
	14C BUF EA OV3	1.30E+03	1.08E+06	0.00120		
14 days: 1 mg/kg	14 ES1 OV1	2.32E+03	8.04E+05	0.00288	0.00238	0.00055
	14 ES1 OV2	1.72E+03	9.36E+05	0.00183		
	14 ES1 OV3	1.06E+03	4.51E+05	0.00235		
	14 ES1 OV4	1.25E+03	4.17E+05	0.00300		
	14 ES1 OV5	1.17E+03	6.35E+05	0.00185		
14 days: 10 mg/kg	14 ES10 OV1	9.87E+02	4.60E+05	0.00214	0.00213	0.00035
	14 ES10 OV3	1.34E+03	5.29E+05	0.00253		
	14 ES10 OV5	1.57E+03	8.56E+05	0.00183		

Table E3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
28 days: Negative control	28C BUF OV1	8.35E+02	5.50E+05	0.00152	0.00080	0.00044
	28C BUF OV2	3.21E+02	3.31E+05	0.00097		
	28C BUF OV3	1.73E+02	4.91E+05	0.00035		
	28C BUF OV4	1.49E+03	2.85E+06	0.00052		
	28C BUF OV5	3.16E+02	7.03E+05	0.00045		
	28C BUF OV6	3.27E+03	3.18E+06	0.00103		
28 days: Eyestalk ablation	28C BUF EA OV1	6.07E+03	6.79E+06	0.00089	0.00126	0.00025
	28C BUF EA OV2	1.35E+03	8.63E+05	0.00156		
	28C BUF EA OV3	5.79E+03	4.97E+06	0.00116		
	28C BUF EA OV5	8.21E+02	6.23E+05	0.00132		
	28C BUF EA OV6	9.78E+01	7.75E+04	0.00126		
28 days: 1 mg/kg	28 ES1 BUF OV1	2.35E+03	3.41E+06	0.00069	0.00084	0.00021
	28 ES1 BUF OV2	3.28E+01	4.64E+04	0.00071		
	28 ES1 BUF OV3	3.65E+02	4.32E+05	0.00085		
	28 ES1 BUF OV4	2.24E+02	2.94E+05	0.00076		
	28 ES1 BUF OV6	2.83E+02	2.30E+05	0.00123		
28 days: 10 mg/kg	28 ES10 BUF OV3	4.75E+02	2.72E+05	0.00175	0.00180	0.00010
	28 ES10 BUF OV4	7.21E+02	3.81E+05	0.00189		
	28 ES10 BUF OV6	5.09E+02	2.83E+05	0.00180		
35 days: Negative control	35C BUF OV1	1.91E+03	2.74E+06	0.00070	0.00076	0.00036
	35C BUF OV2	4.35E+02	1.24E+06	0.00035		
	35C BUF OV3	9.76E+02	1.24E+06	0.00079		
	35C BUF OV4	2.37E+03	2.67E+06	0.00089		
	35C BUF OV5	2.32E+03	3.04E+06	0.00076		
	35C BUF OV7	1.76E+03	1.29E+06	0.00137		
	35C BUF OV8	8.63E+02	2.30E+06	0.00037		
35 days: Eyestalk ablation	35C BUF EA OV1	4.58E+03	1.61E+06	0.00284	0.00300	0.00053
	35C BUF EA OV2	4.44E+03	1.73E+06	0.00256		

Table E3 (Cont.)

Sample		Mean conc.		Ratio (target / *EF-1α*)	Average	SD
		PmRTK	*EF-1α*			
	35C BUF EA OV4	1.52E+03	4.27E+05	0.00356		
35 days: 1 mg/kg	35 ES1 BUF OV1	1.45E+03	6.93E+05	0.00209	0.00163	0.00037
	35 ES1 BUF OV2	1.98E+03	1.30E+06	0.00153		
	35 ES1 BUF OV3	2.19E+03	1.53E+06	0.00143		
	35 ES1 BUF OV4	7.01E+03	3.60E+06	0.00195		
	35 ES1 BUF OV5	3.70E+03	2.05E+06	0.00181		
	35 ES1 BUF OV6	3.93E+03	3.62E+06	0.00108		
35 days: 10 mg/kg	35 ES10 BUF OV2	5.11E+03	3.29E+06	0.00155	0.00137	0.00025
	35 ES10 BUF OV3	1.99E+03	1.44E+06	0.00138		
	35 ES10 BUF OV4	1.64E+03	1.52E+06	0.00107		

VITA

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Publications from this thesis

Kirativanich, K., Sittikankaew, K., Yocawibun, P., Jarayabhand, P. and Klinbunga, S., 2013. Isolation and expression analysis of Ring Finger Protein 121 in ovaries of the giant tiger shrimp Penaeus monodon. The 39th Congress on Science and Technology of Thailand “Innovative Sciences for a Better Life”, October 21-23, BITEC, Bangkok, THAILAND (oral presentation).