ผลของซีโรโทนิน โพรเจสเทอโรนและ 17บีตา เอสตราไดออลต่อการพัฒนารังไข่ของกุ้งกุลาดำ *Penaeus monodon*

นางสาวศรีภาพรรณ ตรีเจตน์

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EFFECTS OF SEROTONIN, PROGESTERONE AND 17ß-ESTRADIOL ON OVARIAN DEVELOPMENT OF THE BLACK TIGER SHRIMP *Penaeus monodon*

Miss Sripapan Treejate

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Thesis Title	EFFECTS OF SEROTONIN, PROGESTERONE AND 17ß-ESTRADIOL ON OVARIAN DEVELOPMENT OF THE BLACK TIGER SHRIMP *Penaeus monodon*
By	Miss Sripapan Treejate
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Padermsak Jarayabhand, Ph.D.
Thesis Co-advisor	Sirawut Klinbunga, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…… Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

…… Chairman
(Associate Professor Thaithaworn Lirdwitayaprasit, Ph.D.)

…… Thesis Advisor
(Associate Professor Padermsak Jarayabhand, Ph.D.)

…… Thesis Co-advisor
(Sirawut Klinbunga, Ph.D.)

…… Examiner
(Kittinan Komolpis, Ph.D.)

…… External Examiner
(Rachanimuk Hirunsuchalert, Ph.D.)

ศรีภาพรรณ ตรีเจตน์: ผลของซีโรโทนิน โพรเจสเทอโรนและ 17บีตา เอสตราไดออลต่อการพัฒนารังไข่ของกุ้งกุลาดำ *Penaeus monodon* (EFFECTS OF SEROTONIN, PROGESTERONE AND 17β-ESTRADIOL ON OVARIAN DEVELOPMENT OF THE BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เผดิมศักดิ์ จารยะพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ศิราวุธ กลิ่นบุหงา, 98 หน้า

การตรวจสอบผลของฮอร์โมนและสารสื่อประสาทเช่น 17บีตา เอสตราไดออล โปรเจสเตอโรนและซีโรโทนิน ต่อระดับการแสดงออกของจีนในแม่พันธุ์กุ้งกุลาดำที่ได้จากการเพาะเลี้ยงสามารถบ่งชี้ความเป็นไปได้ที่จะนำสารกระตุ้นที่เหมาะสมสำหรับเพิ่มความสมบูรณ์พันธุ์ของแม่พันธุ์กุ้งกุลาดำเพื่อทดแทนการตัดตาแม่พันธุ์กุ้งกุลาดำในอนาคต จึงนำจีนที่เกี่ยวข้องการพัฒนารังไข่ของกุ้งกุลาดำจากห้องสมุดจีนมาตรวจสอบการแสดงออกด้วยวิธีอาร์ทีพีซีอาร์ เพื่อศึกษาแนวโน้มของรูปแบบการแสดงออกที่แตกต่างกัน จากนั้นหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของจีน *Pm17β–HSD* ด้วยเทคนิค RACE-PCR พบว่าจีน *Pm17β–HSD* ประกอบด้วย ORF 768 คู่เบส แปลรหัสได้เป็นโปรตีนขนาด 255 กรดอะมิโน ตรวจสอบตำแหน่งการแสดงออกของจีน *Pm17β-HSD* ด้วยวิธี *in situ* hybridization พบว่ามีตำแหน่งการแสดงออกของ mRNA ในส่วนของไซโตพลาสซึมของเซลล์ไข่ระยะ previtellogenesis และได้สร้างโปรตีนลูกผสมของจีน *Pm17β-HSD* โดยโปรตีนลูกผสมมีขนาด 26.78 KDa ซึ่งมีการแสดงออกต่ำในรูปของ insoluble protein

ตรวจสอบการแสดงออกของจีนด้วยวิธี quantitative real-time PCR ในกุ้งกุลาดำแม่พันธุ์ธรรมชาติปกติและกุ้งกุลาดำที่ตัดก้านตา พบว่าจีน *PmATP/GTP* และจีน *Pm17β-HSD* มีระดับการแสดงออกที่คงที่ระหว่างการพัฒนารังไข่ โดยระดับการแสดงออกของจีนนี้ในระยะที่สองและสามของกุ้งที่ไม่ตัดก้านตามีระดับที่สูงกว่ากุ้งที่ตัดก้านตา ($P < 0.05$) จีน *PmInx1* และจีน *PmPHB2* นั้นมีการแสดงออกที่สูงขึ้นต่อเนื่องระหว่างการพัฒนารังไข่ โดยมีระดับการแสดงออกสูงสุดในรังไข่ระยะที่สี่ โดยการแสดงออกของจีนดังกล่าวในรังไข่ทุกระยะของกุ้งที่ตัดก้านตาสูงกว่ากุ้งแม่พันธุ์ปกติ ($P < 0.05$) สำหรับในกุ้งกำลาดำที่ได้จากการเพาะเลี้ยงอายุ 5, 9, 14 และ 19 เดือน พบว่าจีน *PmATP/GTP* มีระดับการแสดงออกในรังไข่ที่ไม่แตกต่างกัน ในขณะที่ *Pm17β-HSD* ในรังไข่ของกุ้งเลี้ยงอายุ 19 เดือน มีระดับการแสดงออกสูงกว่ากุ้งกลุ่มอื่นๆ ($P < 0.05$)

จากการตรวจสอบการแสดงออกของจีนในกุ้งกุลาดำที่ฉีดด้วย17บีตา เอสตราไดออล (0.01µg/g BW) โปรเจสเตอโรน (0.1 µg/g BW) และซีโรโทนิน (50 µg/g BW) พบว่า พบว่าการแสดงออกของจีน *Pm17β-HSD* เพิ่มขึ้นในวันที่เจ็ดของการฉีดกระตุ้นด้วย 17บีตา เอสตราไดออล ($P < 0.05$) ในขณะที่การฉีดด้วยโปรเจสเตอโรนมีผลให้ระดับการแสดงออกของจีน *PmATP/GTP* และ *Pm17β-HSD* ลดลงที่ 72 ชั่วโมง และ 24-72 ชั่วโมงหลังการฉีด ($P > 0.05$) ทั้งนี้ซีโรโทนินกระตุ้นการแสดงออกของจีน *PmATP/GTP* และ *Pm17β-HSD* ระหว่าง 6-48 ชั่วโมงหลังการฉีด ($P < 0.05$)

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....

ปีการศึกษา.....2554…… ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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SRIPAPAN TREEJATE. EFFECTS OF SEROTONIN, PROGESTERONE AND 17ß-ESTRADIOL ON OVARIAN DEVELOPMENT OF THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: ASSOCIATE PROF. PADERMSAK JARAYABHAND, Ph.D., CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D., 98 pp.

Knowledge on molecular mechanisms of steroid hormones and neurotransmitters on expression of reproduction-related genes may lead to the possible ways to effectively induce ovarian maturation in shrimp. RT-PCR was carried out to examine the expression patterns of several genes. In addition, the full-length cDNAs of *17β–hydroxysteroid dehydrogenase* (*Pm17β–HSD*) of the black tiger shrimp (*Penaeus monodon*) was isolated and it was 1400 bp in length containing an ORF of 768 bp corresponding to a polypeptide of 255 amino acids. *In situ* hybridization indicated that *Pm17β–HSD* mRNA was localized only in the cytoplasm of previtellogenic oocytes but not in more mature stages of oocytes. Recombinant Pm17β–HSD protein (26.78 kDa) was successfully expressed in the bacterial expression system. It was expressed at a very low level as the insoluble protein

Quantitative real-time PCR was carried out to estimate the expression of reproduction-related genes in *P. monodon*. The expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* were not differentially expressed during ovarian development in wild intact and eyestalk-ablated *P. monodon*. However, lower expression levels of these transcripts in stage II and III ovaries in eyestalk-ablated broodstock were found compared to those in intact broodstock ($P < 0.05$). The expression profiles of *PmInx1* and *PmPhb2* were similar as they were significantly increased in stages II-IV ovaries in intact broodstock ($P < 0.05$) and at stages III and IV ovaries in eyestalk-ablated broodstock ($P < 0.05$). Their expression levels in stages I, II, III and IV ovaries in eyestalk-ablated broodstock were greater than those in the same ovarian stages in intact broodstock ($P < 0.05$). In domesticated 5-, 9-, 14- and 19-month-old shrimp, the expression levels of *PmATP/GTP-BP* was not significantly different. In contrast, *Pm17β-HSD* in ovaries of 19 month-old shrimp was significantly greater than that in other groups of domesticated shrimp ($P < 0.05$).

Injection of 17β-estradiol did not affect the expression level of *PmATP/GTP-BP* but resulted in a significant increase of *Pm17β-HSD* at 7 days after injection ($P < 0.05$). In contrast, progesterone injection resulted in a lower expression of both *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 14-month-old shrimp at 72 hpi and 24-72 hpi, respectively ($P < 0.05$). Interestingly, serotonin injection promoted the expression level of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 18-month-old broodstock of *P. monodon* at 6-48 hpi ($P < 0.05$).

Field of Study :...Biotechnology.....	Student's Signature ……...……
Academic Year :……2011……	Advisor's Signature ……..……
	Co-advisor's Signature ……

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CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

Page

Page

LIST OF ABBREVIATIONS

bp	base pair
^{o}C	degree Celsius
DEPC	diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid (disodium salt)
EtBr	ethidium bromide
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pair
M	molar
mg	milligram
mRNA	messenger-ribonucleic acid
ml	millilitre
mM	millimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid

rpm	revolution per minute
RNase A	ribonuclease A
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* Fabricius 1798) is one of the economically important cultured crustaceans. Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment. Thailand has been regarded as the leading shrimp producer of cultivated shrimp for over a decade.

The production of *P. monodon* is largely constrained by the current dependency on wild-caught broodstock. The shrimp industry has faced several problems for the cultivation of this species including, difficulties in breeding of captive *P. monodon*, outbrake of diseases and the lack of high quality wild and/or domesticated broodstock. Accordingly, the farming of *P. monodon* has significantly decreased and was recently replaced by that of the Pacific white shrimp (*Litopenaeus vannamei*).

The domestication and selective breeding programs of *P. monodon* will provide a more reliable supply of seed stock and improvement of their production efficiency (Coman *et al*., 2006). The use of selective bred stocks having commercially on commercially desired traits such as ability to induce high quality eggs and high growth rate in domesticated females without the irreversible side-effects of eyestalk ablation is a major mean of sustainability of the shrimp industry (Browdy, 1998; Coman *et al*., 2006).

Unilateral eyestalk ablation is used commercially to induce ovarian maturation of *P. monodon* (Fingerman, 1997). It is generally believed that gonad inhibiting hormone (GIH) produced and secreted from the X-organ/sinus gland inhibits gonad development (Huberman, 2000; Okumura, 2004). While gonad stimulating hormone (GSH) is believed to be secreted from thoracic ganglion and brain, stimulates the ovarian maturation. Although this technique increases spawning activity of *P.*

monodon broodstock, the technique leads to many problems such as reduction in wild broostocks, deterioration in spawn quality over time, eventual loss in egg quality and death after spawning (Benzie, 1998).

Therefore, the hormonal and neurotransmitter studies are needed for maturation and spawning in captive of *P. monodon* without the use of eyestalk ablation. This issue is a long-term goal for a sustainable shrimp aquaculture (Quackenbush, 2001).

The presence of vertebrate-type steroids has been documented in almost all invertebrate groups including crustaceans (Lehoux and Sandor, 1970; Lafont, 1991; Cardosa *et al*., 1997). Progesterone (P4) and its derivatives are sex steroid hormones that play important roles in gametogenesis (Fingerman *et al*., 1993; Rodriguez *et al*., 2002; Miura *et al*., 2006). Progesterone and 17α-hydroxyprogesterone injection induced ovarian maturation and spawning in *Metapenaeus ensis* (Yano, 1985, 1987). The conversion of progesterone into estradiol- 17β was reported in *Marsupenaeus japonicus* (Summavielle *et al*., 2003). Estradiol-17β and progesterone levels in the hemolymph were shown to fluctuate closely with that of the serum vitellogenin level during ovarian maturation stages of *P. monodon* (Quinitio *et al*., 1994) implying its controlling role in vitellogenesis.

Gosh and Ray (1993) reported the 17β-estradiol stimulated biosynthesis of *17β-hydroxysteroid dehydrogenase* (*17β-HSD*) in the *Macrobrachium rosenbergii*. In *Cancer pagurus*, vitellogenic ovaries contained *17β-HSD* suggesting the ability to convert androstenedione to testosterone. Similarly, ESTs carrying the *17β-HSD* gene was identified in *P. monodon* (Preechaphol *et al*., 2007). Yano and Itakura, (1998) demonstrated the effects of 17β-estradiol on vitellogenesis of ovaries *in vitro*.

In female crustaceans, serotonin has been implicated in a wide variety of behaviors such as assertion, aggression and escape behavior. Serotonin was found to stimulate the release of several crustacean hormones including Red pigment dispersing hormone (RPDH), Molt inhibiting hormone (MIH) Crustacean hyperglycemic hormone (CHH), Neurodepressing hormone (NDH), and Gonad stimulating hormone (GSH) (Keller *et al*., 1985, Mattson and Spaziani, 1985 and Rao and Fingerman, 1970). Serotonin injection induced ovarian maturation in the crayfish,

Procambarus clarkii (Kullkarni *et al*., 1992; Sarojini *et al*., 1995) and the Pacific white shrimp, *Litopenaeus vannamei* (Vaca and Alfaro, 2000) at rates lower than unilateral eyestalk ablation. Alfaro *et al*. (2004) reported that injection of combined serotonin and dopamine antagonist, spiperone stimulated ovarian maturation and spawning in *L. stylirostris* and *L. vannamei*.

Previous studies illustrated that injection of serotonin clearly promoted the expression of various reproduction-related genes in ovaries of *P. monodon* for example, *Ovarian-Specific Transcript* (*PmOST1*) in cultured 5-month-old shrimp at 12 - 78 hpi ($P < 0.05$, Klinbunga *et al*., 2009), *adipose differentiation-related protein* (*PmADRP*) in domesticated 14-month-old shrimp at 48 hpt (Sittikankaew *et al*., 2010), and *farnesoic-O-methyltransferase* (*PmFAMeT*) in domesticated 18-month-old shrimp at 1 hpt (Buaklin, 2010).

To examine the molecular involvement of reproduction-related genes on ovarian development of *P. monodon*, the full-length cDNA of *P. monodon 17β-hydroxysteroid dehydrogenase* was isolated and characterized. Expression patterns of this gene and other reproduction-related genes (e.g. *innexin I* and *prohibitin 2*) during ovarian development in wild and domesticated *P. monodon* broodstock were examined. Effects of eyestalk ablation (wild broodstock) serotonin, progesterone and 17β-estradiol induction (domesticated broodstock) on expression levels of these genes were also examined.

1.2 Objectives of this thesis

The objectives of this thesis are isolation, characterization and expression analysis of reproduction-related genes in ovaries of *P. monodon*. Effects of neurotransmitters and steroid hormones (progesterone and 17β-estradiol) on the induction of ovarian development in *P. monodon* were examined.

1.3 Literature review

Shrimp farming is one of the most important food product industries of the world. The breeding of *P. monodon* was success of the first time at the Phuket Fisheries Station in 1972. The extensive and semi-intensive farms were commercially established in that year and in 1974, respectively. Subsequently, the ability to develop

formulated feed commercially led to the success development of the intensive culture system of this species.

By the late 1980s, the intensive farming system has resulted in consistent production of marine shrimp of Thailand and shrimp culture, mainly the black tiger shrimp became popular in Thailand. The rapid growth of shrimp farming has led to an economic boom especially in the coastal provinces of the Eastern and Southern regions (Herron, 2003). Accordingly, Thailand has been regarded as the leading shrimp producer of cultivated shrimp for over the last two decades.

Farming of *P. monodon* in Thailand relies almost entirely on wild-caught broodstock for supply of juveniles because reproductive maturation of cultured *P. monodon* female is extremely low. As a result, breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality of larvae required by the industry. The high demand on wild female broodstock leads to overexploitation of the natural populations of *P. monodon* in Thai waters (Klinbunga *et al*., 1999).

Table 1.1 Total shrimp production (in metric tons) from the aquaculture during 2004-2010

Country	2004	2005	2006	2007	2008	2009	2010
Thailand	360,000	380,000	500,000	530,000	495,000	563,000	640,000
China	352,000	380,000	400,000	480,000	523,000	560,000	600,000
Vietnam	106,000	115,000	150,000	170,000	200,000	200,000	224,000
Indonesia	205,000	230,000	260,000	210,000	230,000	180,000	140,000
India	100,000	100,000	103,000	110,000	870,000	100,000	120,000
Malaysia	28,000	32,000	42,000	62,000	68,000	92,000	105,000
Philippines	35,000	35,000	36,000	38,000	29,000	35,000	410,000
United state	275,000	304,000	395,000	495,000	397,000	412,000	387,000
Other	125,000	125,000	55,000	55,000	55,000	50,000	65,000
Total	**1,586,000**	**1,701,000**	**1,941,000**	**2,150,000**	**2,867,000**	**2,192,000**	**2,691,000**

(Source: http://www.thaiahpa.com/Feed5.pdf)

The production of *P. monodon* is largely constrained by the current dependency on wild-caught broodstock which varies in both quality and quantity. Recently, the farming of *P. monodon* in the region has significantly declined As a

result, *L. vannamei* has been introduced to Thailand as an alternative cultured species and become the main culture species at present (Table 1.1).

Nevertheless, *P. monodon* is a local species. Moreover, the price of *L. vannamei* is quite low and broodstock used relies almost entirely on genetic improved stocks brought from different sources. 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 open for Thailand because *L. vannamei* is not suitable for that market. Accordingly, *P. monodon* culture is currently promoted for increasing the production of this species.

1.3.1 Taxonomy of *P. monodon*

The black tiger shrimp is taxonomically classified as a member of the kingdom Animalia, Phylum Artrhpoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suborder Natantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Penaeidae, Rafinesque, 1985; Genus Penaeus, Fabricius, 1798 and Subgenus *Penaeus*. The scientific name of shrimp is *P. monodon* where the English common name is giant tiger shrimp or black tiger prawn (Baily-Brock and Moss, 1992).

1.3.2 Morphology

The external morphology of penaied shrimp is separated into 2 parts; cephalothorax is at the thorax and head are fused and covered with an abdomen and carapace (Figure 1.1). Most organs are located in the cephalothorax such as digestive system, gills and heart. In the abdomen has the muscles while, five pairs of p1eopods (swimming legs) (Baily-Brock and Moss, 1992).

The internal morphology of penaeid shrimp is showed in Figure 1.2. Penaeid shrimp have an open circulatory system. The blood and blood cells are called haemolymph and haemocytes, respectively. The muscular heart that is dorsally located in the cephalothorax.

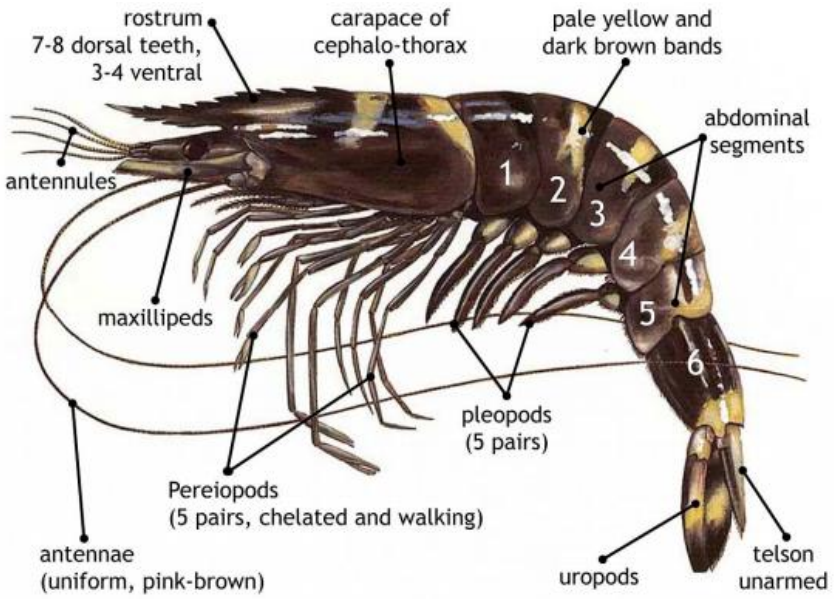


Figure 1.1 Lateral view of the external morphology of *P. monodon* (Altamirano, 2010).

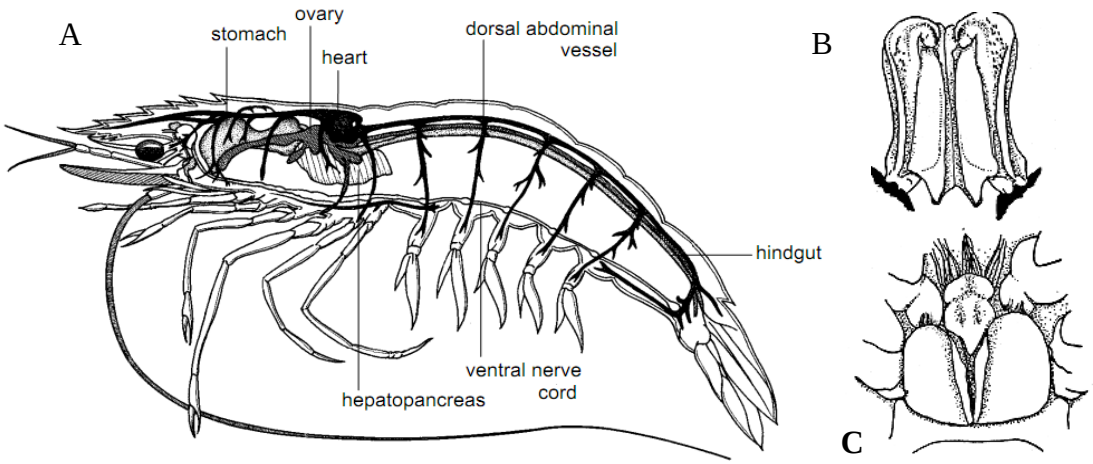


Figure 1.2 Lateral view of the internal anatomy of a female *P. monodon* (A). Sexes of juveniles and broodstok of penaeid shrimp can be externally differentiated by petasma of male (B) and thelycum of female (C)

1.3.3 Ovarian development in shrimp *P. monodon*

In penaeid shrimp, the major part of ovaries is found within the cephalothorax area. The ovaries are arranged in parallel pairs, but partially in cephalothoracic are fused and consist of a number of lateral lobes. The different the pigmentation of the

egg mass and density of the ovary affects the intensity of the shadow. The ovarian development of penaeid shrimp are classified to five different stages as shown in Figure 1.3 (Thurn and Hall, 1999).

Stage I (previtellogenic stage): The ovaries are composed of a connective tissue capsule surrounding a soft vascular area containing oogonia, and accessory cells, also called follicle or nurse cells (Figure 1.3A). The internal wall of the ovary capsule is lined with epithelial cells. Once the female is sexually mature, the germinal epithelium will produce oogonia by mitosis division throughout the reproductive life of the females. The ovaries either do not cast any shadow or a thin opaque line is seen along the length of the tail. The color of the ovarian lobules varies from white to cream

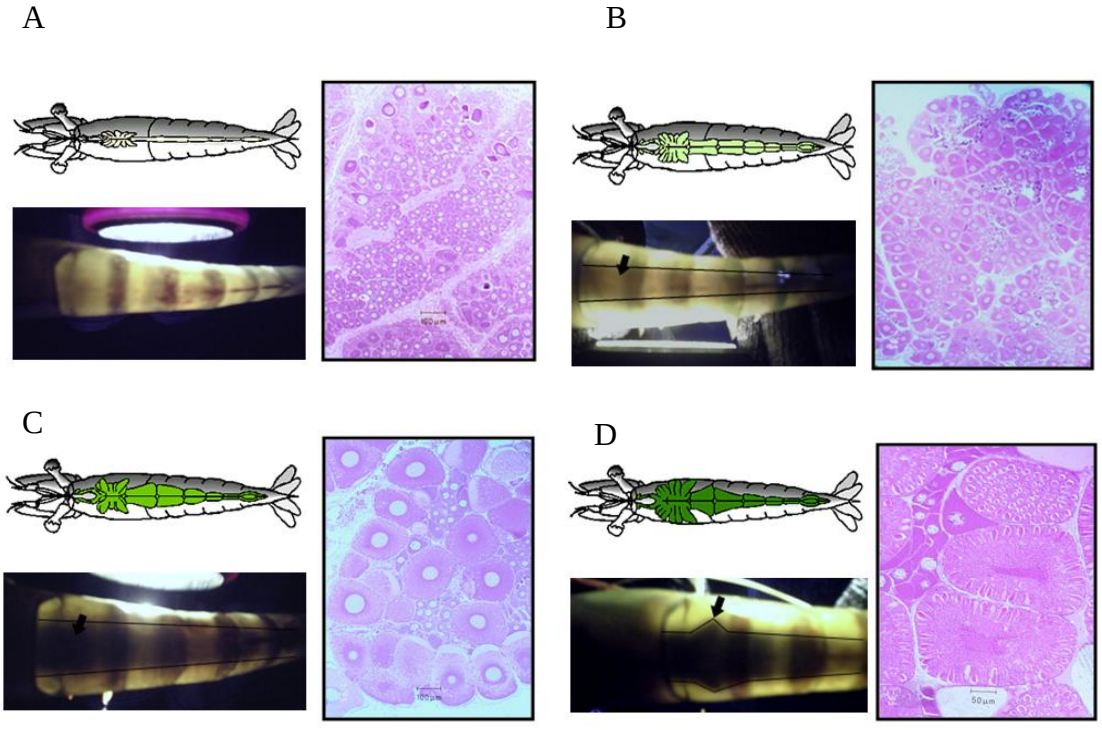


Figure 1.3 Ovarian developmental stages of *P. monodon*: (A) stage I, previtellogenic; (B) stage II, vitellogenic; (C) stage III, nearly-ripe and (D) stage IV, mature (Adapted from: www. aims.gov.au/.../mdef/images/fig01-4a.gif)

Stage II (vitellogenic stage): The eggs develop from oogonia as the oogonia increase in size and enter the first stage of meiotic divisions which are irreversibly destined to become haploid, with only one set of maternal chromosomes (Figure 1.3B). The color of the ovarian lobules varies from a light yellow to greenish yellow.

Stage III (nearly-rip stage): As the oocytes develop, they migrate out towards the margins of the ovarian lobes in preparation for ovulation. During this migration, follicle cells are attached to the periphery of each oocyte. It is believed that the follicle cells produce the yolk that is internal into the oocytes in a process called vitellogenesis. As vitellogenesis proceeds, oocytes are mature synchronously as yolk accumulates and develop a characteristic dark green (Figure 1.3C).

Stage IV (mature stage): The eggs develop cortical granules filled with a jelly-like substance destined to form part of the egg shell membrane after ovulation. At this time the shadow cast by the ovaries is large, resulting in a very distinct dark thick region extending the length of the abdomen, with an enlarged bulbous region directly behind the carapace, called the saddle (Figure 1.3D). The olive green to dark green ovaries is highly granular in texture.

1.3.4 Hormonal regulation in shrimp

Biological and physiological processes (body color, growth, reproduction and metabolism etc.) are hormonal controlled (Figure 1.4). Knowledge from shrimp endocrinology is necessary to develop the hormonal manipulation techniques in shrimp.

Neurosecretory cells in crustacean eyestalks produce the CHH, MIH and GIH (Chan *et al*., 2003). Eyestalk hormones play the important role for harnessing several physiological mechanisms such as sugar balance, metabolism, molting, heart rate, gonad maturation and pigments. It contains GIH that inhibits gonad maturation.

Moreover, it is believed that GSH, methylfarnesoate (MF) and steroid hormone stimulate oocyte development in penaeid shrimp. GSH or vitellogenin stimulating hormone, VSH is believed to be secreted by the thoracic ganglia and supraesophageal has been proposed to have the opposite Effects of GIH (stimulates

the gonad maturation) of shrimp (Figure 1.5). However, this hormone has not been identified and characterized in any shrimp

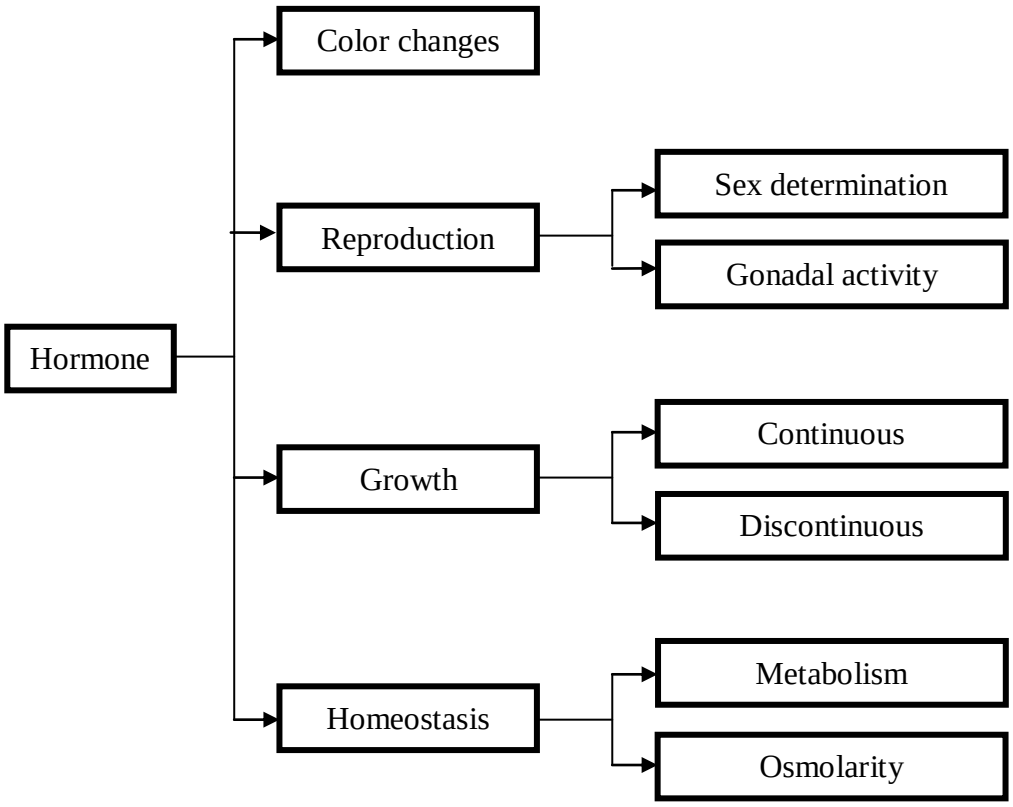


Figure 1.4 A diagram illustrating the hormonal controls of physiological processes of penaeid shrimp.

Reproduction of decapod crustaceans is regulated by various neurohormones that are synthesized and released from the X-organ/sinus gland complex (Chan *et al*., 2003) (Figure 1.6). The synthesis and release of these neurohormones are believed to be regulated by biogenic amines (Ridchardson *et al*., 1991)

Unilateral eyestalk ablation has been used to accelerate ovarian maturation and spawning in different shrimp species used as broodstock in aquaculture (Paran *et al*., 2010). The effect has been attributed to the presence of GIH or vitellogenin inhibiting hormone, VIH in the X- organ/sinus gland complex (Huberman, 2000).

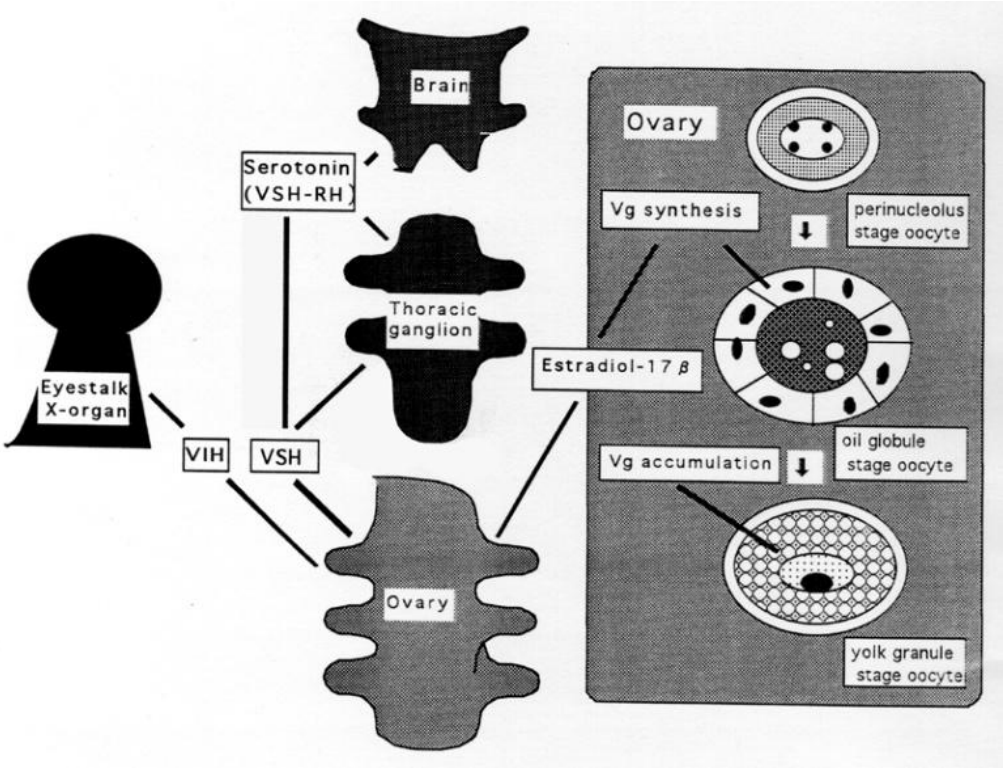


Figure 1.5 Proposed models for the hormonal control of vitellogenesis and final maturation in penaeid shrimp (Yano, 1998)**.**

1.3.5 Neurotransmitter

Neurotransmitters are chemicals that transmit messages from one cell to another. Neurotransmitters can be classified into several groups including amino acids, monoamines, soluble gases, acetylcholine and neuropeptides. Among these, the monoamine neurotransmitters can be further divided to two subclasses, are catecholamine and indolamine.

Several previous studies have reported the stimulatory effect of serotonin on gonad maturation in some decapods, including, *P. monodon* (Wongprasert *et al*., 2006), and *M. rosenbergii* (Meeratana *et al*., 2006). It was suggested that serotonin stimulates gonadal maturation by inhibiting the release of GIH and/or by enhancing the release of GSH.

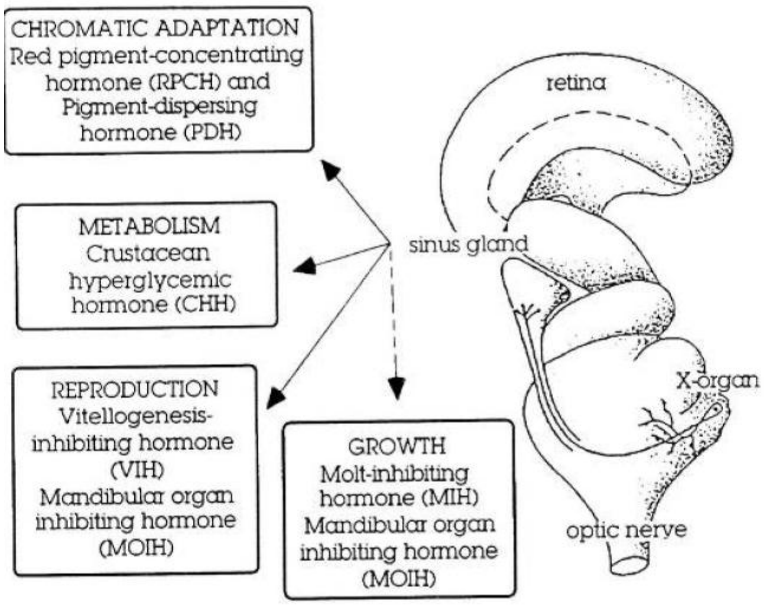


Figure 1.6 Localization and hormones that control several systems from the sinus gland/X-organ complex of *P. monodon* (Okumura, 2004)**.**

Serotonin also stimulates the release of other neurohormones, including the CHH, RPDH, MIH and neurodepressing hormone NDH (Sarojini *et al*., 1995). In *L. vanname*i, serotonin was injected into female wild broodstock at 15 and 50 ug/g BW. The result showed that serotonin induced ovarian maturation and spawning at both tested dose but at a later and shower rate of maturation and spawning than unilateral eyestalk ablation (Vaca. *et al*., 1999).

Zacharia *et al*. (2004) examined effects of serotonin on the histological structure of ovaries of the banana prawn (*Fenneropenaeus merguiensis*). The experimental females were given three injections of serotonin at 15 µg/g body weight on the first, fifth and tenth day. The control was injected with 100 µl of the normal saline. The experiment lasted for fifteen days. The ovarian index and oocyte diameter of females treated with serotonin were significantly increased compared to the controls reflecting by larger oocyte diameter and higher GSI of serotonin treated females. The conventional histology was carried out and ovaries of treated females were in the advanced second stage of maturation with nearly all oocytes were in the early phase of vitellogenesis.

In the *M. rosenbergii*, effects of serotonin on the ovarian development were investigated. Adult female prawns with the ovarian spent stage were injected with serotonin at 1, 5, 10, 20 and 50 μg/g BW on days 0, 5, 10 and 15. At low concentration (1 μg/g BW), serotonin induced a significant increase in the gonadosomatic index (5.79 ± 0.09%). The ovaries of the negative control and vehicle control prawns mostly developed to stage II (Meeratana *et al*., 2006).

1.3.6 Steroid hormones

Steroid hormones mediate a wide variety of vital physiological functions. Natural steroid hormones are generally synthesized from adrenal glands.and cholesterol in gonads. Sex steroids are hormones functionally involved in reproduction of organisms. Their effects are mediated by slow genomic mechanisms through the nuclear receptors as well as by fast non-genomic mechanisms through membrane-associated receptors (http://www.newworld encyclopedia.org). The steroid biosynthesis pathway is shown by Figure 1.7.

A positive correlation between vitellogenin circulating levels and hemolymph levels of progesterone and 17β-estradiol have been reported for crabs (Shih, 1997and Zapata *et al*., 2003) and shrimp (Quinitio *et al*., 1994; Yano, 2000). Couch *et al*. (1987) demonstrated that tissue and serum concentrations of endogenous 17β-estradiol and progesterone in American lobster, *Homarus americanus* change in relation to the condition of the ovaries.

An understanding the roles of steroid hormones on vitellogenesis may lead to the development of ways to induce ovarian maturation in decapod crustaceans. Progesterone has been shown to stimulate yolk protein synthesis in the ovary of white shrimp (Quackenbush, 2001) and ovarian maturation of penaeid shrimp (Yano, 1985). In addition, 17α-hydroxyprogesterone induces spawning in *P. stylifera* (Nagabhushanam *et al*., 1980), vitellogenin secretion into hemolymph of *M. japonicus* (Yano, 1987) and oocyte developments in and red swamp crayfish *P. clarkii* and *L. vannamei* (Rodriguez *et al*., 2002).

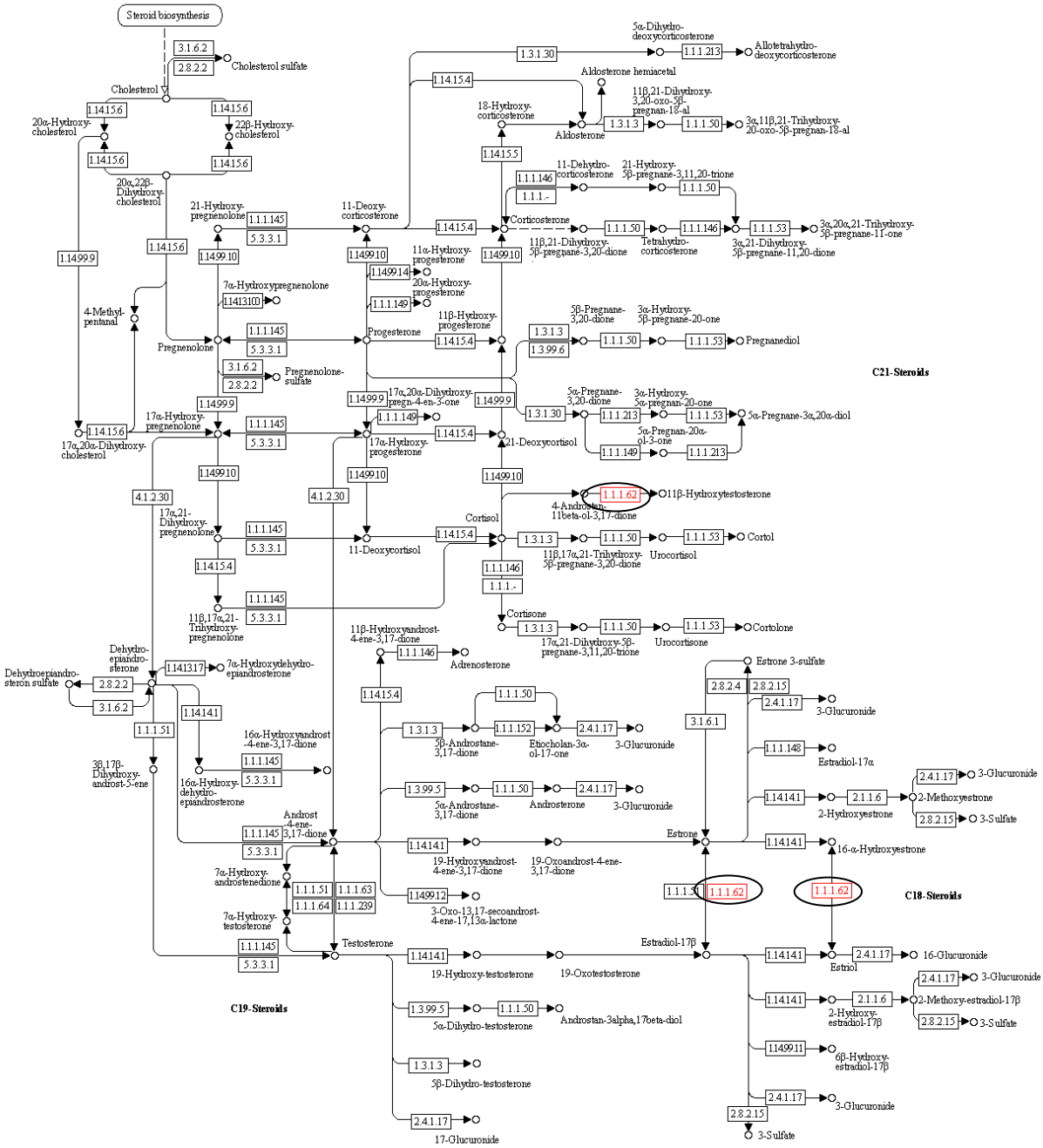


Figure 1.7 Steroid synthesis pathways. (Source: http://www.brenda-enzymes.org/). The actions of 17β-hydroxysteroid dehydrogenase on different substrates are marked by solid circles.

1.3.6.1 Progesterone and derivatives

The presence of vertebrate-type steroids has been documented in almost all invertebrate groups including crustaceans (Lehoux and Sandor, 1970; Lafont, 1991). Progesterone (P4) plays important roles in gametogenesis (Fingerman *et al*., 1993; Rodriguez *et al*., 2002; Miura *et al*., 2006).

The conjugated pregnenolone and unconjugated and conjugated dehydroepi-androsterone (DHEA) in ovaries of *P. monodon* were found to be maximal at stage II and III vitellogenesis. Unconjugated progesterone was found in ovaries at stages (III and IV of ovarian development whereas conjugated testosterone was only detected in stage IV ovaries (Fairs *et al*., 1990).

Effects of progesterone on ovarian maturation and spawning in *Metapenaeus ensis* (Yano, 1985) were reported where stages III, IV and V of ovarian development were observed after 1 month in the shrimp injected with progesterone at 0.1 μg/g BW compared to vehicle controls which showed only the early development stage II.

Moreover, effect of 17α-hydroxyprogesterone on the secretion of vitellogenin (Vg) in *M. japonicus* was examined *in vivo*. A significant increase in Vg concentration in the serum was observed after 48 hr in early vitellogenic prawn injected with 17α-hydroxyprogesterone at the concentration of 0.01 μg/g body weight compared to the control. The results indicated that 17α-hydroxyprogesterone stimulates Vg synthesis in prawns (Yano, 1987).

Molecular mechanisms of vertebrate-like hormones have not been well established in penaeid shrimps at present (Qui *et al*., 2005) and knowledge about steroid receptors and signaling is still limited.

1.3.6.2 17β-estradiol

Like other sex steroids, estradiol like other sex steroid is derived from cholesterol which is converted into androstenedione. Subsequently, androstendione is then either converted to testosterone by *17β-hydroxysteroid dehydrogenase* (*17β-HSD*) which in turn undergoes aromatization to estradiol by aromatase. Alternatively,

androstendione is aromatized to estrone which is converted to estradiol by *17β-HSD* (Figure 1.8).

The 17β-estradiol acts by binding to its nuclear receptors (i.e., ERα and ERβ) that then transactivate target genes. 17β-estradiol also induces rapid; nongenomic actions involving plasma membrane-associated signalling that require a membrane ER (Marino *et al*., 2002).

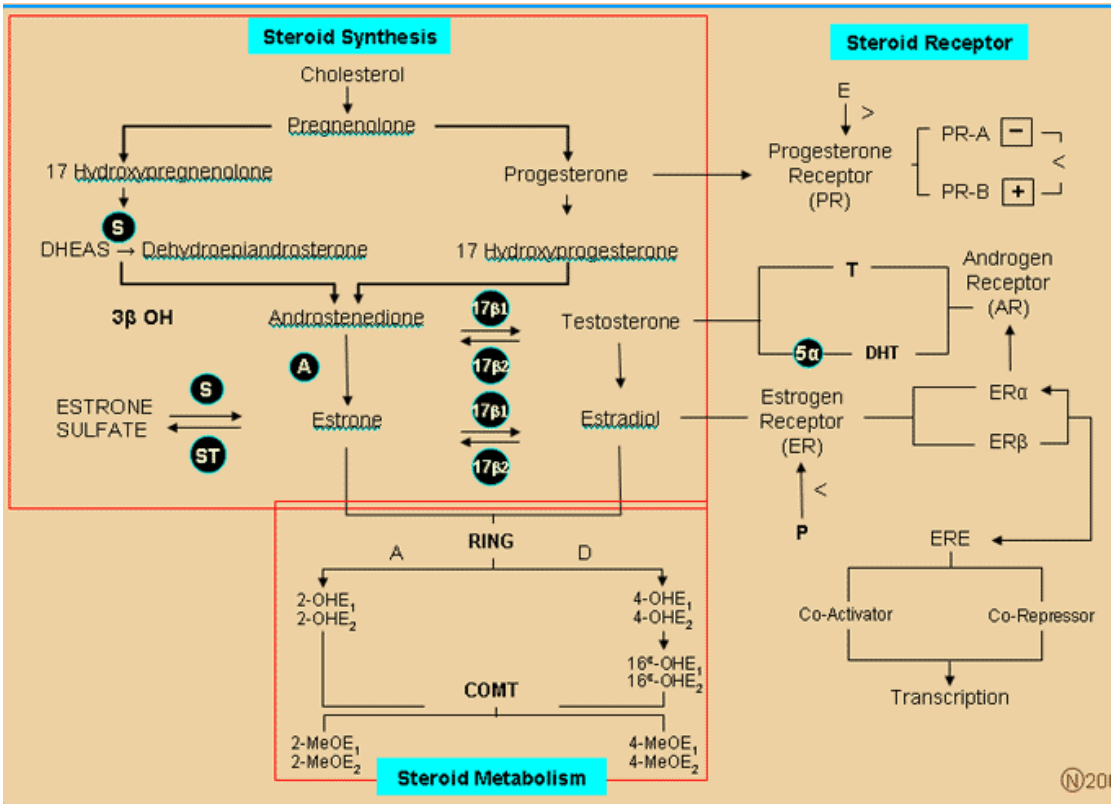


Figure 1.8 Pathways and enzymes in estrogen, progesterone, and testosterone biosynthesis, metabolism and steroid receptor activity (A = aromatase activity, S = sulfatase activity, ST = sulfotransferase activity, 17β1 and 17β2 = 17β-hydrohysteroid dehydrogenase, 3βOH = 3β-hydroxysteroid dehydrogenase, > = receptor up-regulation, < = receptor down-regulation, OHE1 = 2-hydroxyestrone, OHE2 = 2-hydroxyestradiol, MEOE1 = 2-methoxyestrone, MEOE2 = 2-methoxy-estradiol, 5α = 5α-reductase types 1 and 2, DHT = dihydrotestosterone, ERE = estrogen response element, RING = A-ring or D-ring metabolic pathway, COMT = catechol-O-methyltransferase). (http://www.medscape.com/viewarticle/523196_3)

In *P. monodon*, titers of conjugated 17β-estradiol and unconjugated and conjugated estrone in the ovaries peaked during vitellogenesis (Fairs *et al*., 1990). These observations and the fact that exogenous steroids can stimulate ovarian maturation and spawning may indicate that estradiol plays a part in the reproductive process in shrimp.

The presence of 17β-estradiol in ovaries or hemolymph has been documented in vitellogenic females of several decapod crustaceans (Quinitio *et al*., 1991). Quackenbush, (1992) demonstrated that 17β-estradiol stimulated *in vitro* yolk protein synthesis in the white shrimp.

The activity of 17β-hydroxysteroid dehydrogenase, a key enzyme in steroid metabolism, has been determined in the hepatopancreas and ovaries of *M. rosenbergii*. The enzyme activity was up-regulated by 17β-estradiol and higher in the hepatopancreas of maturing females (Ghosh and Ray, 1993). In *Procambarus clarkia*, 17β-estradiol resulted in a significant increase in the GSI compared to the control (Rodriguez *et al*., 2002).

Gunamalai (2005) estimated 17β-estradiol and progesterone, in the hepatopancrea, hemolymph and ovaries s of the mole crab (*Emerita asiatica*) during the reproductive and molt cycle stages by radioimmunoassay. The 17β-estradiol level in the hemolymph was high in crabs with mature ovaries, while those with quiescent ovaries were low or undetectable. Results suggested that ovaries may synthesize 17β-estradiol and release it into the hemolymph from where it may reach the hepatopancreas to stimulate vitellogenin synthesis.

1.3.7 Functions of genes related to ovarain development and maturation in this study

1.3.7.1 *17β -hydroxysteroid dehydrogenase*

The *17β-hydroxysteroid dehydrogenases* (*17β-HSDs*) are a group of alcohol oxidore-ductases which catalyses the dehydrogenation of 17-hydroxysteroids in steroidogenesis. The *17β-HSD* is the enzymes catalyzing stereospecific redox reactions at position C17 of the steroid scaffold, nowadays comprise a group of thirteen proteins. The *17β-HSD* catalyzes the reduction of estrone to estradiol with

NADPH as cofactor while it's capability to convert androstenedione to testosterone. The rodent *17β-HSD* 1 displays a very similar catalytic efficiency for both androgenic and estrogenic reactions (Mindnich and Adamski, 2007).

17β-HSDs are a group of intracellular isozymes catalyzing interconversions between estradiol (E2) and estrone (E1). In the last step of steroidogenesis, *17β-HSD* type 1 catalyzes the reduction and produces 17β-estradiol from estrone. The oxidative enzymes known as types 2, 4, and 8 are potent estrogen-inactivating enzymes that convert estradiol to estrone (Motohara *et al.*, 2010).

The family of *17β-HSD*, which by conversion at position 17 modulates biological potency of estrogens and androgens: keto-forms are inactive, whereas hydroxyl-forms are active and access the receptors (Figure 1.9).

In crustacean, *17β-HSD* activity was found in hepatopancreas and ovaries of the freshwater prawn in relation to the presence of estradiol at different life stages (Ghosh and Ray, 1993). The additionally intercomversion of estrone to estradiol and vice versa in the oyster *Crassostrea gigas* and the conversion of androstenedione to testosterone by *17β-HSD* has been demonstrated in the snail *Helix aspersa* (Belfond *et al*., 2001 and Guellec *et al*., 1987).

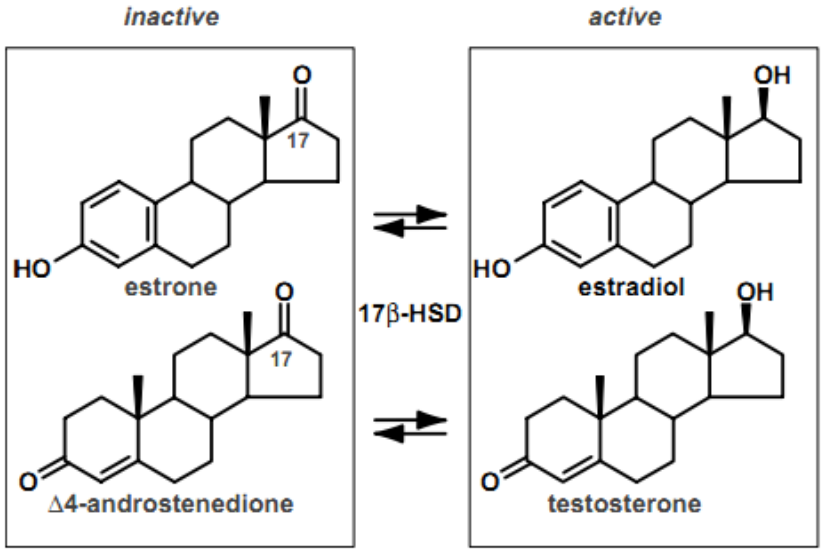


Figure 1.9 17β-hydroxysteroid dehydrogenases (*17β-HSDs*) modulate the biological potency of estrogens and androgens by the redox reactions at position 17.

1.3.8.2 *ATP/GTP binding protein*

ATP/GTP binding protein which is also recognized as cleavage and polyadenylation factor I (Clp1). Clp1 proteins are the essential components of the eukaryal mRNA cleavage polyadenylation machinery (jain and Shuman, 2009).

Clp1 is primarily localized at nucleolar organizing region and at the spindle pole body during interphase. During mitosis, Clp1 disperses throughout the nucleus and cytoplasm, concentrating at kinetochores, the mitotic spindle and the contractile ring, a highly dynamic structure that controls ingression of the division plane. Clp1 remains at the contractile ring during its constriction and then reconcentrates in the nucleolus (Clifford *et al*, 2008). Cells lacking Clp1 display strong negative genetic interactions with many cytokinesis mutants, including those which disrupt contractile ring assembly, septum formation, and cell wall synthesis (Trautmann *et al*., 2001).

In *P. monodon*, *ATP/GTP binding protein* (*ATP/GTP-BP*) was recently identified by EST analysis. It was abundantly expressed in ovaries whereas a lower expression was observed in other tissues. The full-length cDNA of *ATP/GTP-BP* was 1547 bp in length containing an ORF of 1263 bp encoding a polypeptide of 421 amino acids. The pre-mRNA cleavage complex II protein (Clp1) domain was found at positions 149 - 481 of the deduced protein. The calculated p*I* and molecular weight of the deduced protein was 5.98 and 46.52 kDa (*E*-value = 1e-155) (Sittikhankeaw, 2006). However, functions *ATP/GTP-BP* of during ovarian development of *P. monodo*n have not been reported.

1.3.7.3 *Innexin*

Gap junctions assure direct cell to cell communication via the exchange of ions and small metabolites within a tissue, an important prerequisite during development and homoeostasis (Nicholson, 2003). In vertebrates, the development of ovarian follicles and the production of fertile oocytes depend on gap junction mediated intercellular communication particularly between the oocyte and the surrounding granulosa cells. The granulosa cells infer the loss of gap junction interaction between the oocyte and granulosa cells as equivalent to ovulation thus, granulosa cells start to luteinize. This may represent that in normal follicles, inhibitory

signals are transmitted through gap junctions from the oocyte to granulosa cells to prevent luteinization until ovulation has occurred. Thus, communication via gap junctions seems to offer bidirectional signaling system to regulate follicle growth, oogenesis, ovulation and luteinization. The gap junctions are an important characteristic involved in folliculogenesis (http://php.med.unsw.edu.au/cellbiology/index.php?title=2011_Group_2_Project).

Meiosis in mammalian oocytes pauses in prophase until luteinizing hormone (LH) releases the arrest. It is suggested that LH does this phenomenon by closing the gap junctions between the somatic cells that surround the oocyte, thus blocking the transmission of a meiosis-inhibitory signal to oocytes (Dekel, 2005).

In invertebrates, gap junctions are formed by proteins that belong to the innexin family (Ducret *et al*, 2006). Innexins are a family of transmembrane proteins involved in the formation of gap junctions, specific intercellular channels. Innexins localize to gap junctions *in vivo* and are sufficient to form intercellular channels in *Xenopus* oocytes (Starich *et al*., 1996). Nevertheless, innexins as gap junction constituting proteins have been found indispensible for a number of developmental processes, for instance the establishment of the left-right neuronal asymmetry in nematode *Caenorhabditis elegans* (Chuang *et al*., 2007).

In *P. monodon*, the full-length cDNAs of *innexin1* was characterized and it was 2505 bp in length with an ORF of 1143 bp corresponding to a polypeptide of 380 amino acids. The full-length cDNA of *innexin2* was 1651 bp in length containing an ORF of 1077 bp corresponding to a protein of 358 amino acids. The closest similarity of these transcripts were *innexin1* of *Schistocerca americana* (*E*-value = 6e-120) and *innexin2* of *Homarus gammarus* (*E*-value = 7e-161), respectively. Deduced innexin1 and innexin2 proteins contained an innexin domain (positions 22^{th} -363^{th}, *E*-value = 3.30e-84 and positions 20^{th} -358^{th}, *E*-value = 9.90e-77, respectively). The expected MW and p*I* of *innexin1* were 44.0 kDa and 6.42, respectively and tthose of *innexin2* of *P. monodon* were 41.68 kDa and 6.37, respectively (Leelatanawit *et al*, 2008).

1.3.7.4 *Prohibitin*

Prohibitin is a growth-suppressive protein that has multiple functions in the nucleus and the mitochondria. Prohibitin has been shown to contribute to the folding and transport of mitochondrial proteins, which have cellular functions, including apoptosis, regulation of cell cycle, signal transduction and aging. Prohibitins have known to act as transcriptional repressor in the nucleus, suggesting that they function in both the organelles and couple nuclear-mitochondrial interaction (figure 1.12)

In human, prohibitin is expressed in the granulosa cell, oocytes, atretic follicles and corpus luteum. The increased levels of prohibitin correlate with the initial events of apoptosis. In this regard, the phosphorylation of prohibitin can be induced by estrogen and therefore it is involved in the ontogony of ovarian follicles and the regulation of granulosa cell proliferation. In atretic follicles, prohibitin is translocated from the cytoplasm to the nucleus and this event is associated with the atretic events (Hu *et al*., 2001).

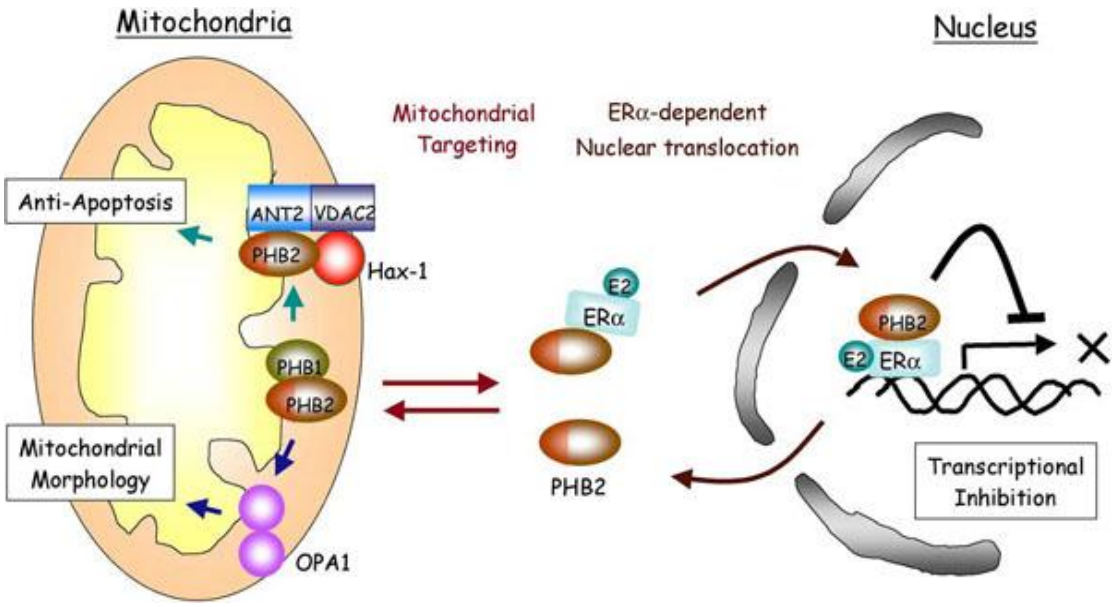


Figure 1.10 Summary of the localization and functions of Prohibitin2. (Source: http://www.jichi.ac.jp/biochem/kinou/research_E.html)

Prohibitin2 (also known as repressor of estrogen receptor activity, B-cell receptor associated protein BAP37, B-cell associated protein,REA and BAP37), have also been shown to regulate the transcription of other factors like the estrogen receptor (Park *et al*.,2005) and may also play a role in follicle development.

Park *et al*. (2005) prohibitin2 mRNA and protein levels in uteri of heterozygous animals were half that of the wild type, and studies with heterozygous animals revealed a greater uterine weight gain and epithelial hyperproliferation in response to estradiol and a substantially greater stimulation by estradiol of a number of estrogen up-regulated genes in the uterus. Even more dramatic in prohibitin2 heterozygous animals was the loss of down regulation by estradiol of genes in the uterus that are normally repressed by estrogen in wild-type animals.

Prohibitin2 is a significant modulator of estrogen responsiveness in vivo it normally restrains estrogen actions, moderating ER stimulation and enhancing ER repression of estradiol regulated genes.

In *P. monodon*, the full-length cDNA of *prohibitin 2* was composed of 1382 bp with the predicted ORF of 891 bp in length corresponding to a polypeptide of 296 amino acids. This sequence significantly matched that of *Tribolium castaneum* (E-value = 2e-128). The expected MW and p*I* of this deduced protein was 32.56 kDa and 9.73, respectively. A deduced prohibitin-2 protein contained a prohibitin domain (PHB, positions 39^{th} - 200^{th}, E-value = 1.25e-42) (Leelatanawit *et al.*, 2008).

Knowledge on molecular mechanisms of steroid hormonal induction on oocyte development may lead to the possible ways to effectively induce ovarian maturation in shrimp. In this thesis, effects of steroid hormone and serotonin on expression of genes from different pathways were examined. This basic information would allow better understanding of molecular mechanisms of ovarian development and reproductive maturation of *P. monodon*.

CHAPTER II
MATERIALS AND METHODS

2.1 Experimental animals

For gene expression analysis, female broodstock of *P. monodon* were wild-caught from the Andaman Sea and acclimated for 5 days. The post-spawning group was immediately collected after shrimp were ovulated and dissected out of ovaries from each broodstock and weighed. For the eyestalk ablation group, wild broodstock were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 3-5 days after ablation. The gonadosomatic index (GSI, ovarian weight/body weight x 100) of each shrimp was calculated. The ovarian developmental stages of wild shrimp were classified according to the GSI values (I= <1.5, II >2–4, III>4–6 and IV >6%). In addition, domesticated juveniles (4- and 6-months old) and domesticated broodstock (14- and 18-month-old) of *P. monodon* were collected from the Broodstock Multiplication Center (BMC), Burapha University Chanthaburi campus, Thailand and include in the experiments.

For tissue distribution analysis, various tissues of female, ovaries of juveniles and testes of male broodstock were collected, immediately placed in liquid N_2 and kept at -80°C until needed.

To examine effects of 17β-estradiol on expression of reproduction-related genes, domesticated shrimp (approximately 12 month-old with the average body weight of 42.47 ± 6.32 g) were acclimated (28-30ºC and 30 ppt seawater) in 500-liter tanks for 1 week. Six groups of shrimp were injected intramuscularly with different concentrations of 17β-estradiol (0.01, 0.02 and 0.05 µg/g of body weight, N = 6 for each group) and injected with 5% ethanol into the first abdominal segment of each shrimp. The injection was repeated with the original doses at 3 and 6 days post initial injection. Specimens were collected at 7 and 14 days post initial injection. Shrimp injected with 5% ethanol were included as the vehicle control.

Subsequently, domesticated shrimp (approximately 14 month-old with the average body weight of 48.99 ± 4.96 g) were acclimated as described previously.

Three groups of shrimp 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 are collected at 7, 14 and 28 days post injection. Non-injected shrimp (at 0, 7, 14 and 28 days after the initial injection; *N* = 6 for each group) and those injected with 5% ethanol (at 7, 14 and 28 days after the initial injection; *N* = 6 for each group) were included as the negative and vehicle control, respectively. In addition, shrimp were unilateral eyestalk-ablated and specimens were collected at the same time intervals. Ovaries of each shrimp were sampling and immediately placed in liquid N_2. The samples were stored at -80˚C until needed.

Moreover, eight groups of acclimated female shrimp (14-month-old, average body weight of 64.06 ± 3.20 g) were injected into the first abdominal segment with progesterone (0.1 µg/g body weight, *N* = 4) and collected at 12, 24, 48 and 72 hpi. Non-injected shrimp and those injected with absolute ethanol (at 0 hpi) were included as the normal (NC) and vehicle controls (VC), respectively.

To examine effects of serotonin (5-HT) on expression of interesting genes, 18-month-old domesticated *P. monodon* broodstock were sampled and acclimated for 7 days at the laboratory conditions (28-30ºC and 30 ppt seawater) in 500-liter fish tanks. Seven groups of female shrimp (18-month-old, average body weight of 77.12 ± 3.10 g) were injected into the first abdominal segment with serotonin (50 µg/g body weight, *N* = 4) and collected at 0, 1, 3, 6, 12, 24, 48 and 72 hpi. Shrimp injected with the 0.85% saline solution (at 0 hpi) were included as the VC.

2.2 RNA extraction and cDNA template preparation

2.2.1 Total RNA extraction

Total RNA was extracted from ovaries of *P. monodon* by using TRI Reagent® (Molecular Research Center). A piece of tissue was immediately placed in mortar containing liquid nitrogen and crushed to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI-REAGENT® and homogenized. Additional 500 µl of TRI Reagent® were added. The homogenate were incubated at room temperature for 5 minutes before adding 0.2 ml of chloroform and

then homogenized by vortexing for 15 seconds and incubated at room temperature for 2-15 minutes before centrifuged at 12000g for 15 minutes at 4°C.

The aqueous phase (inclusively containing RNA) was transferred to a new microcentrifuge tube. Total RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10 minutes and centrifuged at 12000g for 10 minutes at 4°C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12000g for 5 minutes at 4°C. The ethanol was removed. The RNA pellet was air-dried for 5-10 minutes. The dried RNA pellet was dissolved in DEPC-treated water for immediately used. Alternatively, the total RNA pellet was kept under absolute ethanol and stored in a -80 °C freezer for long storage.

2.2.2 Spectrophotometrically estimate of extracted total RNA concentration

The concentration of extracted RNA sample is estimated by spectrophotometry measuring 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 single stranded RNA and 33 µg/ml oligonucleotide (Sambrook and Russell, 2001). The concentrations of RNA sample were estimated in µg/ml by using the following equation;

[RNA] = OD_{260} x dilution factor x 40 (or 33 for single stranded DNA)

The purity of RNA sample can be evaluated from a ratio of $OD_{260}/OD_{280.}$ The ratios of purified RNA were appropriately 2.0 (Sambrook and Russell, 2001) indicates the good quality of the extracted RNA.

2.2.3 DNase I treatment of the extracted RNA

Fifteen micrograms of total RNA were treated with a RQ1 RNase Free DNaseI (Promega). DNaseI digestion reaction was set up as described in Table 2.1. The reaction mixture was left at 37°C for 30 minutes. After the incubation, the samples were gently mixed with the a sample volume of phenol:chloroform: isoamylalcohol (25:24:1) for 10 minutes. The sample was centrifuged at 12,000 rpm for 10 minutes at 4°C and the upper aqueous phase was collected. The extraction process was repeated once with chloroform:isoamylalcohol (24:1) and one with

chloroform. The final aqueous phase was mixed with one-tenth final sample volume of 3 M sodium acetate, pH 6. After that, RNA was precipitated by adding twice the sample volume of cold absolute ethanol. The mixture was incubated at -80 ℃ for 30 minutes and the precipitated RNA by centrifugation at 12,000 rpm for 10 minutes at 4 ℃. The RNA pellet was then washed twice with 1 ml of cold 75% ethanol. Alternatively, the RNA pellet was kept in absolute ethanol at -80 ℃ until required.

Table 2.1 DNase I digestion reaction for elimination of genomic DNA from total RNA

Component	Volume
RNA in DEPC water (15µg)	up to 28.5 µl
RQ1 RNase-Free DNase 10X Reaction Buffer	4 µl
RQ1 RNase-Free DNase (0.5 unit/µg RNA)	7.5 µl
Nuclease-free water	To a final volume of 40µl

2.2.4 Synthesis of the first strand cDNA

One and a half micrograms of DNase I-treated total RNA from various tissues of *P. monodon* were reverse transcribed to the first strand cDNA using an ImProm-II™ Reverse Transcription System Kit (Promega). Total RNA was merged with 0.5 µg of oligo dT_{12-18} and appropriate DEPC-treated water in final volume of 5 µl. The mixture was incubated at 70℃ for 5 minutes and immediately placed on ice for 5 minutes. Subsequently, 5x reaction buffers, $MgCl_2$, dNTP Mix and 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 added and gently mixed by pipetting. The reaction mixture was incubated at 25 ℃ for 5 minutes and at 42℃ for 90 minutes and incubated at 70 ℃ for 15 minutes to terminate the reverse transcriptase activity. Concentration and rough quality of the synthesized first strand cDNA was spectrophotometrically examined (OD_{260}/OD_{280}) and electrophoretically analyzed by 1.2% agarose gels. The first strand cDNA was kept at -20℃ until used.

2.3 Reverse transcription (RT)-PCR of reproduction-related genes in *P. monodon*

2.3.1 Primer design

Primers for amplification of 5 reproduction-related genes initially identified by EST analysis including *ATP/GTP binding protein*, *17β- hydroxysteroid dehydrogenase*, *Innexin 1*, *Innexin 2* and *B-cell receptor-associated protein 37* (*Prohibitin 2*) were designed. *EF-1*α_{500} was used as an internal control. The sequence of primers are illustrated in Table 2.2

Table 2.2 Gene homologue, primer sequences and the expected sizes of the PCR product from ESTs of *P. monodon*

Gene/Primer	Sequence	Tm (°C)	Size (bp)
1. *ATP/GTP binding protein*			
F:	5' TAGCAGTGTTCACCTGGCAT 3'	60	180
R:	5' GTAGGAAAGTCAACTCTGTGC 3'	62	
2. *17β hydroxysteroid dehydrogenase*			
F:	5' CCATTGTAGGCATGACACTTC 3'	62	225
R:	5' CTCCGTTCATCATTGGGTTTG 3'	62	
3. *Innexin 1*			
F:	5' CTCGCAAGAAGGTCATCATT 3'	58	288
R:	5' GTCATTCGTGGGAAGACATAGA 3'	64	
4. *Innexin 2*			
F:	5' GCTCAAAGTGGACTCCCTCA 3'	62	176
R:	5' TCCAGCAGTAGGTATCCATCA 3'	62	
5. *B-cell receptor-associated protein 37* (*Prohibitin 2*)			
F:	5' CTCACGCACTCTTAGGGCGAATC 3'	58	174
R:	5' CCGTCCAGCTCTGCTCTGAACCA 3'	58	
8. *Elonggation factor-1*α *(EF-1*α_{500}*)* (control)			
F:	5' GCCATCATCTTCAACCGTAT 3'	60	500
R:	5' CCTAAGGGAAATGTTCACCA 3'	60	

2.3.2 RT-PCR and tissue distribution analysis

For the target genes, 100 ng of the first strand cDNA from various *antennal gland*, epidermis, eyestalk, gills, hemocytes, heart, hepatopancreas, intestine, lymphoid organs, ovaries, pleopods, stomach, thoracic ganglion, testes and ovaries of female juvenile were used as the template in 25 µl reaction volume containing of 50 mM KCl,10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.2 µM of each primer, 1 unit of Dynazyme^{TM} DNA Polymerase

(FINNZYMES). *Elongation factor-1α (EF-1α$_{500}$)* was also amplified from the same template. The reactions were predenaturation at 94°C for 3 minutes followed by 28 cycles of denaturation at 94°C for 30 seconds, a 55°C annealing for 45 seconds and extension at 72°C for 45 seconds. The final extension was carried out at 72°C for 7 minutes. Five microlittres of the amplification products were electrophoretically analyzed though 1.5% agarose (Sambrook and Russell, 2001).

2.3.3 Agarose gel electrophoresis

An appropriate amount of agarose was weighed out and mixed with an desired volume of 1x TBE buffer (8.9 mm boric acid, 89 mM Tris-HC, and 2 mM EDTA, pH 8.3). Agarose gel was prepared by boiled in a microwave to complete solubilization and allowed to lower than 60 °C. A comb was inserted and poured into the gel mold on plastic tray. The agarose gel was incubated to solidify at room temperature for 45 minutes. When needed, the comp was removed and the gel was installed on to the electrophoresis tank containing enough amount of 1x TBE buffer to covering the gel for approximately 0.5 cm. The PCR product was mixed with 10x loading dye (0.25% bromophenol blue and 25% ficoll in water) at ratio 5:1 (DNA: loading dye) and loaded into the well. A 200 bp DNA ladder was used as the standard DNA marker. Electrophoresis was performing at 100 volts for 30 minute.

The electrophoresed gel was stained in ethidium bromide solution (0.5µg/ml) for 3-5 minutes and destained in tap water to remove unbound ethidium bromide from the gel for 15-30 minutes. DNA fragments were visualized under a UV transilluminator through Molecular Imager® Gel Doc XR (Bio-Rad Laboratories).

2.4 Isolation and characterization of the full-length cDNA of *17β-hydroxysteroid dehydrogenase* using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE – PCR)

2.4.1 Preparation of the 5′ and 3′ RACE template

Total RNA was extracted from ovaries of *P. monodon* broodstock using TRI-Reagent. Messenger (m) RNA was purified using a QuickPrep *micro* mRNA Purification Kit (Amercham Phamacia Biotech.) as described previously. The RACE cDNA template was prepared by combining 1 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 µg of ovarian mRNA with 1 µl of 3′ CDS primer A for 3′ RACE-PCR (Table 2.4). The components were mixed and spun briefly. The reaction was incubated at 70°C for 2 minutes and immediately placed 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 DDT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reactions were gently mixed by pipetting and centrifuged to collect the contents at the bottom of the tube.

Table 2.3 Primer sequence for the first strand cDNA synthesis and RACE-PCR

Primer	Sequence
SMART II A Oligonucleotide	5′-AAGCAG TGGTATCAACGCAGAGTACGC GGG-3′
3′ RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
5′ RACE CDS Primer	5′-$(T)_{25}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
10X Universal PrimerA Mix (UPM)	Long : 5′–CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGAGT-3′
	Short : 5′–CTAATACGACTCACTATAGGG C – 3′
Nested Universal Primer A (NUP)	5 – AAGCAGTGGTATCAACGCAGAGT -3′

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

2.4.2 Primer designed for RACE-PCR

Gene-specific primers (GSPs) were designed from an EST representing *17β hydroxysteroid dehydrogenase* (*17β-HSD*) from ESTs database of *P. monodon* (http://pmonodon.biotec.or.th). The antisense primer (5′-TCCATCCAGTCGCACAG TTTCTCCG-3′) was designed for 5′ RACE-PCR and the sense primer (CGGAGAAA CTGTGCGACTGGATGGA-3′) for the 3′ RACE-PCR.

2.4.3 RACE-PCR

The master mix for 5′ and 3′ RACE-PCR and the control reactions was prepared. For each 25 µl amplification reaction, 14.0 µl sterile deionized water, 2.5 µl of 10x Advantage® 2 PCR buffer, 0.5 µl of 10 uM dNTP mix and 0.5 µl of 50x Advantage® 2 polymerase mix were combined. The reaction was carried out for as described in Table 2.4. The primary 5′ and 3′ RACE-PCR product were electrophoretically analyzed through 1.0 – 1.2 agarose gels. If the discrete expected bands were not obtained from the primary amplification, semi-nested PCR was performed using the recipes illustrated in Tables 2.4. The primary PCR product was 50-fold diluted. The primary PCR product was executed using 5 µl of diluted PCR product as a template using the conditions described in Table 2.5.

Table 2.4 Composition of 5′- and 3′- RACE-PCR of *17β hydroxysteroid dehydrogenase*

Component	**5′-RACE**		**3′-RACE**	
	5′-RACE Sample	**GSP1 (Control)**	**3′-RACE Sample**	**GSP2 (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

2.4.4 Elution DNA fragments from agarose gels

After electrophoresis, the desired DNA fragment was excised from the agarose gel using a sterile scalpel and placed in a microcentrifuge tube and eluted out from the gel using a HiYield^{TM} Gel Elution Kit (RBC). Four hundred microlitres of the DF buffer was added to the sample. The mixture was incubated at 60 °C for 15 - 30 minute the gel was dissolved completely. During the incubation period, the tube was inverted every 5-10 minute. A DF column was placed in a collection tube and 800 µl of the sample mixture was applied into the DF column and centrifuged at 13,000 *g* for 1 minute and discarding the flow-through. The DF column was placed back in the collection tube and washed the column by the adding of 500 µl of the ethanol-added Wash Buffer and centrifuged at 13,000 *g* for 1 minute. After discarding the flow-through, the DF column was centrifuged for 2 min at 14,000 rpm to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 12 µl of the Elution Buffer was added to the center of the column matrix. The column was incubated at room temperature for 2 min before centrifuged for 2 minutes at 14,000 rpm to recover the gel-eluted DNA.

Table 2.5 The amplification conditions for RACE-PCR of *17β hydroxysteroid dehydrogenase* of *P. monodon*

Gene homologue	Amplification condition
5′ RACE	5 cycles of 94 °C for 30 s and 72 °C for 2 min 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min 20 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
Semi-nested	1 cycles of 94 °C for 3 min 23 cycles of 94 °C for 30 s, 68 °C for 30 s and 7 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE	5 cycles of 94 °C for 30 s and 72 °C for 2 min 5 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min 20 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min

2.5 Cloning of the PCR product

2.5.1 Ligation of the PCR product to the pGEM®-T Easy vector

The purified DNA were ligated to the pGEM®-T Easy vector (Promega) in a 10 µl reaction volume containing 3 µl of the gel eluted PCR product, 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 ligation mixture was gently mixed by pipetting and incubated overnight at 4 °C before transformed to competent *E. coli* stain JM109

2.5.2 Transformation of the ligation product to *E.coli* host cells

2.5.2.1 Preparation of competent cell

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (0.5% Bacto yeast extract, 1% Bacto tryptone and 0.5% NaCl, pH 7.0) with vigorous shaking at 37 °C overnight. The starting culture was then inoculated into 50 ml of LB broth and continued culture at 37 °C with shaking to OD_{600} of 0.5 to 0.8. The cells was briefly chilled on ice for 10 minutes and recovered by centrifugation at 2700 *g* for 10 minutes at 4 °C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2/CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged at 2700 *g for* 10 minutes at 4 °C. The cell pellet was resuspended with 2 ml of ice 0.1 M $CaCl_2$ and the cell suspension was divided into 200 µl aliquots. These competent cells was either used immediately or stored at -80°C for subsequently used.

2.5.2.2 Transformation

The competent cells were thawed on ice for 5 min. Four microlitres of the ligation mixture were added and gently mixed by pipetting. The mixture was incubated on ice for 30 minute. The transformation reaction was heat-shocked in a 42 °C (water bath, without shaking) for 45 seconds and immediately placed on ice for 5 minute. The mixture were removed from the tubes and added to a new tube containing 1 ml of 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 minute. The culture was transferred into a

sterile 1.5 ml microcentrifuge tube and centrifuged at 8,000 rpm for 1 minute at room temperature and resuspended in 100 µl of the SOC medium and spread onto a selective LB agar (containing 50 µg/ml of amplicillin, 20 µg/ml of X-gal and 25 µg/ml of IPTG for approximately 1 hr before using) and further incubated at 37 °C for 12-16 hour (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.5.2.3 Colony PCR and digestion of the amplified inserts by restriction endonucleases

After incubation for 12-16 hour, many white and blue colonies were observed. Choose the white colony approximately 40-50 clones on LB agar plate (containing 50 µg/ml of amplicillin, 20 µg/ml of X-gal and 25 µg/ml of IPTG) by a sterile toothpick. Colony PCR was performed in a 25 µl reaction mixture containing 50 mM $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 8.8, 100 mM of each dNTP, , 0.1% Triton X-100, , 2 mM $MgCl_2$, 0.1 µM each of pUC1 (5′-CCGGCTCGTATGTTGTGTGGA-3′) and pUC2 (5′-GTGGTGCAAGGCGATTAAGTTGG-3′), 0.5 unit of *Taq* Dynazyme™ DNA Polymerase (FINNZYMES). PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 second (35 cycles), annealing at 50°C for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 7 minutes. The colony PCR products were electrophoresed through a 1.5 % agarose gel and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russel, 2001).

2.5.2.4 Extraction of recombinant plasmid DNA

Plasmid DNA was isolated using a HiYield^{TM} Plasmid Mini Kit (RBC; Real Biotech Corporation). A discrete white colony was inoculated into a sterile culture tube containing 3 ml of LB broth (0.5% yeast extract, 1% tryptone, 1.0 % NaCl) supplemented with 50 µg/ml of amplicillin and incubated with shaking (200 rpm) at 37 °C for 12-16 hour. The culture was transferred into a sterile microcentrifuge tube and centrifuged at 8,000 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 added of 175 µl of the lysis buffer type 8 and mixed gently by inverting the tube for 5

times. After that, 350 µl of the lysis buffer type 9 was added to neutralize the alkaline lysis step and mixed gently swiftly by inverting the tube for 2 times. The mixture was then centrifuged at 13,000 rpm for 15 min at room temperature to separate the cell debris.

The supernatant was then applied into the illustraTM plasmid mini column and centrifuged at 13,000 rpm for 1 minute and the column by adding 400 µl of the wash buffer type 1 and centrifuged at 14,000 rpm for 1 minute. The flow-through was discarded and then 400 µl of the ethanol-added wash buffer was added and centrifuged at 13,000 rpm for 1 minute. The dried column was transferred onto a new 1.5 ml microcentrifuge tube. Approximately 30 µl of the Elution buffer type 4 was added into the centre of the column matrix. The column was placed for 1 minute at room temperature and centrifuged at 14000 rpm for 2 minutes. The concentration of extracted plasmid DNA was also spectrophotometrically measured.

2.5.2.5 DNA sequencing and data analysis

Plasmid DNA was unidirectional sequenced by automate DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing by MACROGEN (korea). The sequences were determined by comparing each sequence with the sequences in the GenBank using the BLAST*X* program (http://www.ncbi.nih.gov). Significant probabilities and numbers of matched proteins were considered when the *E*-values were less than 10^{-4}.

2.6 *In situ* hybridization (ISH)

2.6.1 Sample preparation

Ovaries of intact and eyestalk-ablated of *P. monodon* broodstock were excised to small pieces and fixed in 4% paraformaldehyde prepared in 0.1 M sodium phosphate buffered (pH 7.2) overnight at 4°C. The fixed was washed four times with PBS at room temperature and stored in 70% ethanol at 4°C until use. Tissues were histologically prepared, embedded in paraffin and conventional (5 µm) onto paraffin poly-L-lysine-coated slides.

2.6.2 Preparation of cRNA probes

2.6.2.1 Addition of RNA polymerase recognition sequence (RPRS) by PCR

The template of cDNA probes was synthesized from PCR of *P. monodon 17β-HSD*. The forward primers containing T7-sequence (TAATACGACTCACTA TAGGG) and the reverse primers containing SP6- sequence (ATTTAGGTGACAC TATAGAA) were designed (Table 2.6). Fifty nanograms of recombinant plasmid containing the complete ORF of target transcripts were used as the template in a 25 µl reaction volume (1.5 mM MgCl2, 1X Buffer, 0.2 µM of each primer, 0.2 mM of each dNTP and 1 unit of *Taq* polymerase). PCR was performed by predenaturation at 94°C for 2 minutes followed by denaturation at 94°C for 30 seconds 35 cycles, annealing at 60°C for 1 minute and a 72°C extension for 1 minute. The PCR product was putifiled using MinElute PCR purification Kit (Qiagen). The concentration of purified PCR product was estimated by comparing with the DNA marker after electrophoresis and spectrophotometry.

Table 2.6 Primer sequences for preparation of the templates for synthesis of *Pm17β-HSD* antisense and sense cRNA probes

Primer	
For sense cRNA probe	
T7-17HSD-F	5´-TAATACGACTCACTATAGGGCGCTGAAGGGTGTAGTGTCGC-3´
For antisense cRNA probe	
SP6-17HSD-R	5´-ATTTAGGTGACACTATAGAACCTCGCAATGGGAAGTGTCA-3´

2.6.2.2 Synthesis of cRNA probes

For synthesis of the cRNA probes, 1 µg of product was used as template for synthesis of antisense and sense cRNA probes**.** The mixture was left at 42°C for 2 hours for antisense probe and 42°C for 2 hours for sense prope. The synthesized cRNA was purified using an RNeasy Mini Elute Clean up Kit (Qiagen) and the probe concentration was spectrophotometrically measured. The cRNA probe was stored at -80°C until needed.

2.6.2.3 Dot blot analysis

The quality of cRNA probes was determined before used for *in situ* hybridization by dot blot analysis. Plasmid DNA containing the target gene was 10 fold diluted to 10 ng/µl. One microliter of DNA solution was spotted on the membrane. The spotted double stranded cDNA was denatured by alkaline treatment, neutralized and fixed using UV cross linking (0.12 joules/cm^2) for 1 minute. The membranes were prehybridized with the DIG easyHyb solution (Roche) at 50°C for 30 minutes and hybridized with the DIG easyHyb solution containing 100 ng/ml of the denatured DIG-labeled cRNA probe at 50°C for 3 hours. The membranes were washed twice in 2X SSC (with 0.1% SDS) at room temperature for 5 minutes and followed by 0.1X SSC (with 0.1% SDS) once at room temperature and twice at 68°C for 15 minutes. The membranes were briefly washed with TBS with 0.1% tween 20. The dot bot signals were detected as described for *in situ* hybridization.

2.6.3 Hybridization and detection

The sections were dewaxed with xylene and dehydrated in absolute ethanol. The section were prehybridized in the hybridization buffer (2X SSC, 50% formamide, 1 µg/µl BSA, 1 µg/µl salmon sperm DNA, 1 µg/µl yeast tRNA and 10% dextran sulfate) (Wako) at 50°C for 30 minutes. The digoxigenin (DIG)-labeled sense or antisense cRNA probe was added to the hybridization buffer (pre-warmed at 50°C) and incubated overnight at 50°C. After hybridization, the sections were rinsed in 4X SSC twice at 50°C for 5 minutes, and 2X SSC containing 50% formamide at 50°C for 20 minutes. After equilibration in the RNase buffer (10mM Tris-HCl pH8.0; 0.5M NaCl, 1mM EDTA) at 37°C for 30 minutes and digested with 10 µg/ml of RNase A at 37°C for 30 minutes. The sections were washed four times with the RNase A buffer at 37°C for 10 minutes. The sections were rinsed with 2X SSC four times at 50°C for 15 minutes The section were washed twice with 0.2X SSC at 50°C for 20 minutes each. DIG was immunologically detected (anti-Digoxiginin-AP Fab fragment conjugated with alkaline phosphatase and NBT/BCIP) according to the manufacturer's instructions (Roche, Germany) (Qui and Yamano, 2005).

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

Expression levels of *ATP/GTP binding protein* (*PmATP/GTP-BP*), *17β-hydroxysteroid dehydrogenase* (*Pm17β-HSD*), *Innexin1* (*PmInx1*), *Innexin2* (*PmInx2*) and *B-cell receptor-associated protein 37*, *Prohibitin2* (*PmPhb2*) of *P. monodon* were examined using quantitative real-time PCR analysis.

2.7.1 Effects of 17β-estradiol, progesterone and serotonin on expression of reproduction-related genes in ovaries of domesticated *P. monodon* broodstock

2.7.1.1 Preparation of specimens and 17β-estradiol

Specimens for gene expression analysis were prepared as described previously. For preparation of 17β-estradiol stock solution, 17β-estradiol was weighed out (0.0025 g) and 125 µl of absolute ethanol was added to achieve the final concentration of 20 µg/µl. The solution was incubated on ice to facilitate dissolution. The stock solution was diluted by absolute ethanol to make the final concentration of 2 µg/µl and to 0.2, 0.4 and 1 µg/µl, respectively. Finally, the stock solutions were diluted with sterile deionized water to make the working solution (0.01, 0.02 and 0.05 µg g^{-1} body weight of 17β-estradiol in 5% ethanol) immediately before injection.

2.7.1.2 Progesterone

Progesterone was weighed out (0.001 g) and dissolved in absolute ethanol to achieve the final concentration of 1 µg/µl. The stock solution was diluted to the working concentration (0.1 µg/µl in 10% ethanol) immediately before used.

2.7.1.3 Serotonin (5-HT)

Serotonin was weighed out (0.1123 g) and resuspended in 5 ml of the sterile saline solution (0.85% NaCl) to achieve the final concentration of 22.5 µg/µl. The solution was incubated at 37°C to facilitate dissolution. The stock solution was diluted to the working solution (50 µg g^{-1} body weight) prior to injection into the acclimated shrimp.

2.7.2 Primer design.

The intron/exon structure of the target gene was characterized. Several primer pairs were designed from cDNA sequence of each gene and used to PCR against genomic DNA as the template. The PCR fragment was cloned and sequenced. The forward or reverse primer covering intron/exon boundaries or alternatively, a primer pairs sandwiching the large intron was designed as the same as RT-PCR. A size of the expected PCR product size was approximately 100 - 250 bp

For construction of the standard curve of each gene, the DNA segment covering the target PCR product and *EF-1*α_{214} were amplified from primers for quantitative real-time PCR. The PCR product 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 g/µl DNA / [plasmid length in bp x 660] x 6.022×10^{23} = Y molecules/µl

Table 2.7 Gene homologue, primer sequences and the expected sizes of the PCR product from ESTs of *P. monodon*

Gene/Primer	Sequence	Tm (°C)	Size (bp)
ATP/GTP binding protein			
	F: 5´-AAACCCCAATGGTTATGTAC-3´	56	149
	R:5´-GCACAGAGTTGACTTTCCTAC-3´	56	
17β hydroxysteroid dehydrogenase			
	F: 5´-ATGACACTTCCCATTGCGAGG-3´	64	214
	R:5´-CTCCGTTCATCATTGGGTTTG-3´	62	
Innexin 1			
	F: 5´-CTCGCAAGAAGGTCATCATT-3´	58	288
	R:5´-GTCATTCGTGGGAAGACATAGA-3´	64	
Innexin 2			
	F: 5´-GCTCAAAGTGGACTCCCTCA-3´	62	176
	R:5´-TCCAGCAGTAGGTATCCATCA-3´	62	
B-cell receptor-associated protein 37 (*Prohibitin 2*)			
	F: 5´-CTCACGCACTCTTAGGGCGAATC-3´	58	174
	R:5´-CCGTCCAGCTCTGCTCTGAACCA-3´	58	
EF-1α_{214} (control)			
	F: 5´-GTCTTCCCCTTCAGGACGT C-3´	58	214
	R:5´-CTTTACAGACACGTTCTTCACGTTG-3´	58	

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.

2.7.3 Quantitative real-time PCR analysis.

The target transcripts and the internal control *EF-1α*$_{214}$ (5´-GTCTTCCCCTTC AGGACGTC-3´ and R5: 5´-CTTTACAGACACGTTCTTCACGTTG-3´) of the synthesized cDNA were amplified in a reaction volume of 10 µl using 2X LightCycler® 480 SYBR Green I Master (Roche, Germany). The specific primer pairs were used at concentration of 0.2 µM. The thermal profile for SYBR Green real-time PCR was 95 °C for 10 minutes, 1 cycle followed by 40 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds. The melting curve analysis was carried out at 95 °C for 15 seconds, 65 °C for 1 minute and at 98 °C for continuation and cooling was 40 °C for 10 seconds. The real-time PCR assay was carried out in a 96 well plate and each sample was run in duplicate using a LightCycler® 480 Instrument II system (Roche).

A ratio of the absolute copy number of the target gene and that of EF-1α was calculated. The relative expression level between different group (treatment or ovarian development) were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple rang test. Significant comparisons were consulted when the *P value* was < 0.05.

2.8 *In vitro* expression of recombinant *Pm17β-HSD* protein using the bacterial expression system

2.8.1 Primers design

A primer pair was designed to amplify the complete ORF of *Pm17β-HSD*. The forward primer containing *Nde* I site, and reverse primer containing *Bam* HI site and six Histidine residues encoded nucleotides were illustrated in Table 2.7.

2.8.2 Construction of recombinant plasmid in cloning and expression vectors

The complete ORF of *Pm17β-HSD* was amplified by 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 0.2 µM of each primer, 0.75 unit *Pfu* DNA polymerase (Promega) and 0.2 mM of each dNTPs. The thermal profiles were predenaturation at 95°C for 2 minutes followed by 25 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 4 minutes and final extension at 72°C for 7 minutes.

Table 2.8 Nucleotide sequences of primers used for *in vitro* expression of *17β-hydroxysteroid dehydrogenase* of *P. monodon*

Primer	Sequence
Complete ORF	
Pm17β-HSD	F: 5′- CACGATGCTGAAGGGTGTAGT -3′
	R: 5′- TCCACCCCTTCTATGCTGTT -3′
Complete ORF containing *restriction* site and 6 repeated Histidine-encoded nucleotides	
Pm17β-HSD-*Nde* I	F:5′- CCGCATATGCTGAAGGGTGTAGTGTCG -3′
Pm17β-HSD-6His and *Bam* HI	R:5′- GGCGGATCCTCAATGATGATGATGATGATG GGCTGCATACGAATGG -3′

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

The amplification product was analyzed by agarose gel electrophoresis and the gel-eluted product was digested with *Nde* I and *Bam* HI. The digested DNA fragment was again analyzed by agarose gel electrophoresis and the gel-eluted product was ligated with pET-29a 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 of *DST* and was subsequently transformed into *E. coli* BL21 (DE3) plysS competent cells, respectively.

2.8.3 Expression of recombinant proteins

A single colony of recombinant *E. coli* BL21 (DE3)plysS carrying desired recombinant plasmid was inoculated into 3 ml of LB medium, containing 50 µg/ml ampicillin and 50 µg/ml chloramphinical at 37°C. Fifty microlittres of the overnight cultured was transferred to 50 ml of LB medium containing 50 µg/ml ampicillin and 50 µg/ml chloramphinical. Incubated until an OD_{600} was approximately 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, 4, 6 hours and overnight at 37°C) and centrifuged at 12000g for 1 minute. The pellet was resuspended in 1X PBS buffer and examined by 15% SDS-PAGE (Laemmli, 1970).

In addition, 20 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 I mg/ml lysozyme). The cell wall was broken by sonication using Digital Sonifier® sonicator Model 250 (BRANSON). The bacterial suspension was sonicated 3 times at 20-30% amplitude, pulsed on for 10 seconds and pulsed off for 10 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, 1972). Expression of the recombinant protein was electrophoretically analyzed by 15% SDS-PAGE.

CHAPTER III
RESULTS

3.1 Total RNA extraction

Total RNA from various organs (ovaries, antennal gland, epicuticle, eyestalk, gills, hemocytes, hepatopancreas, intestine, lymphoid organs, pleopods, stomach, and thoracic ganglion of female broodstock, testes of male broodstock and ovaries of female juveniles) were extracted. The quality and quantity of total RNA were determined by spectrophotometry and integrity of the total RNA was observed by agarose gel electrophoresis (Figure 3.1).

The ratio of the extracted RNA was 1.8 – 2.0. The first strand cDNA was successfully synthesized from those totals RNA (Figure 3.2). Results from the agarose gel electrophoresis showed discrete ribosomal RNA bands reflecting good quality of total RNA.

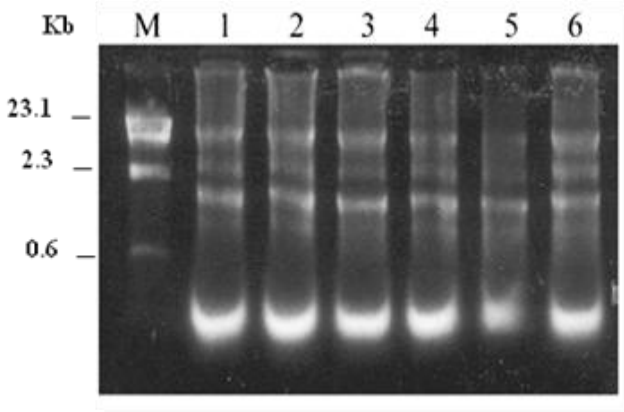


Figure 3.1 A 1.2% ethidium bromide-stained agarose gel showing the quality of total RNA extracted from ovaries of *P. monodon*. Lane M = a 100 DNA ladder; Lanes 1 - 7 = total RNA individually extracted from ovaries of each *P. monodon* broodstock.

3.2 RT-PCR and tissue distribution analysis

An EST representing the partial ATP/GTP binding protein cDNA was initially found from the ovarian cDNA library and the full-length cDNA of *P. monodon ATP/GTP binding protein* was further isolated by RACE-PCR. It was 1547 bp in length containing an ORF of 1266 bp deducing to a polypeptide of 421 amino acids. This sequence significantly matched *Pre-mRNA cleavage complex II protein clp1* of *Harpegnathos saltator* (*E*-value = 1e-155). The calculated p*I* and MW of the deduced ATP/GTP binding protein of *P. monodon* was 5.98 and 46.52 kDa. The pre-mRNA cleavage complex II protein (Clp1) domain was found at positions 149-481 of the deduced ATP/GTP binding protein (*E*-value = 5.50e-157) (Sittikhankeaw, 2006).

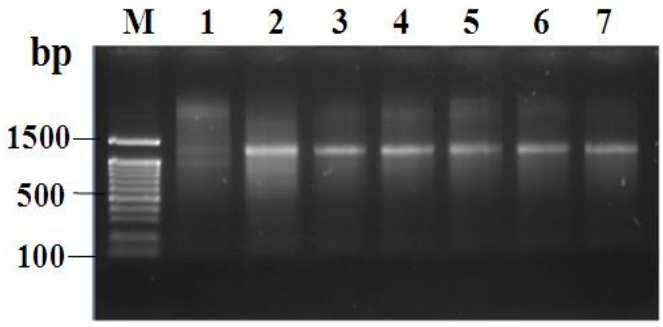
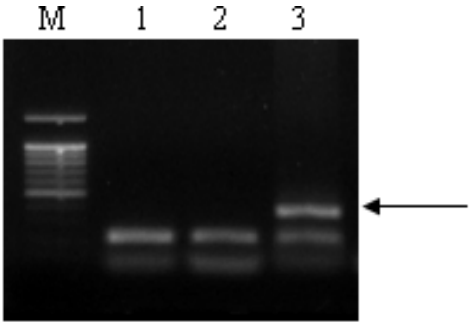


Figure 3.2 A 1.2% ethidium bromide-stained agarose gel showing the synthesized first strand cDNA from ovaries of *P. monodon*. Lane M = a 100 DNA ladder. Lanes 1 - 7 = the first strand cDNA from ovaries of *P. monodon* broodstock.

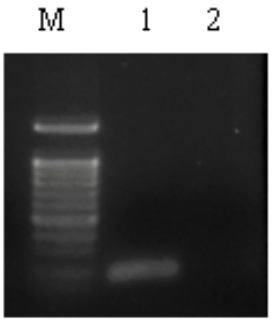
PCR was carried out for amplification of *PmATP/GTP-BP* against genomic DNA of *P. monodon*. The size of an amplified genomic DNA product (370 bp) was greater than that from the cDNA template (221 bp). After cloning and sequencing, an intron of 149 bp was found (Figure 3.3A). It was located between positions 251 and 252 of the amplified gene segment. A new pair of primers was designed to eliminate the possible amplification of contaminated genomic DNA. This primer pair was further used for RT-PCR (Figure 3.3B).

PmATP/GTP-BP was expressed in almost all of examined tissues. Its expression was not observed in hepatopancreas and testis of a male broodstock (Figure 3.4A).

A



C



B

CACAGCTATCGCCTCTTTCCTTCGGTTTATCATTGAAAATCCTAGAAAAGGTATACAGTGGATGCAAA**ATG**TCTGA
AAAGGAGTTCCGTTTGGACCCAGACAGCGAACTCAGGTTTG**AGGTTGAGGGCAAGCAGGAT**ACAGTTGATCTCACA
TTGGTAAATGGCAAAGCTGAAGTGTTTGGCACAGAACTAGCACCAGATAAGCCGTATACCTTCTTCCCTGGAGCAA
AAGTAGCAGTGT**TCACCTGGCATGGCTGTGTT**CTCAAGCTGTCTGGCCCAACAGAGGGAACATATGTGGCAA**AGGA**
AACCCCAATGGTTAGAAATTTATTTACTGATGATTCAACATTTGTTTACTTTTCTTTTATTACTGTTAATTTCTTA
CCATAATAACAAAATCACATACATTAATTTTGTTTGGACATCAATAAGAGATAACCTAATCATCTCATTTGCCTTC
TGATTAGGTTG**TGTACCTTAATACACA**TGCATGCTTGGAGCGGTTGCGCAGGCATGCAGATGAAGGTCTTAGCCGA
GGAGAGGATACCAGAGGACCAGTAACCATGGTTGTTGGTCCAGGAGATGTAGGAAAGTCAACTCTGTGCAGAA**TTC**
TCCTCAACTATGCTGTGCGGATGGGACGTCGGCCCATCTTTGTAGATTTGGATATTGGACAAGGATCCATTGCAAT
TCCAGGAACTATAGGTGCACTGCTTGTTGAGAGAGCTGCTGATGTTGGGGAAGGCTTTTCTCAGGAGGCTCCTCTT
GTGTACAACTTTGGGCACCTGAGTCCTGCATCCAACATGACTCTGTACAACATCCTTGTCTCACGCATGGCTGCCA
CCATTCAAGATAAGATGGTTGGCAATAGAAAAGTTGCTGCCTCAGGGGTGGTTATCAACACATGTGGCTGGATTAA
AAATGAAGGTTACAAGAGTCTGACACATGTTGCGCAGGCCTTTGAGGTTGACGTGATCATTGTCCTTGACCAGGAG
AGACTATATAATGAACTCGTCAGAGATATTCCCTTCATTAGGGTTGTTTTCTTGCCCAAGAGTGGAGGTGTGGTAG
AGAGAACTCAATCCATGAGGGCATCTGCTCGTGATGACCGCATCAGAGAATACTACTATGGACTGCGCACCAAGTA
CCACCCACACAGTTTTGAGGTGAAGATGAGCACGCTCCAGATATACAAGATTGGTGCTCCAGCCCTCCCAGATTCC
TGCATGCCTGCTGATATGAAGGTGGATGATCACATGACCAAACTTGTGCCTGTAGAGCCTAGTGTAAAGTTAAAGC
ATCACATGTTGGCTGTAAGTCTTGCAACAGAACCCGAGGACCTCTTGACCTCCAATGTCGCTGGATTTATTTGCGT
ACTTGACGTTGATGAAGACCTCAAAGTCATGAAGGTCTTGTCTCCACAACCAAAGCCACTCCCAAAAACAATTTTG
ATTTTGACGGAAATTCAGTTCATGGACTCATCG**TAA**AATGAAGAGTGGTTTTGTAATTTCTCGTAAATCATGTGGT
ATTACATTTTTTTTTTCTCTCTCTCTCTCTTCTCCTTTTTTCCTATATTTTCTTTGATATAGCAGTTATTTATAT
TGTGTAATTTTTGTATACAGATGATTTCTGCTGATTTTTGGTGTAACTTAAAATTTTTTATTAAATAAGTATTTTA
CAAGAAAAAAAAAAAAAAAAAAA

Figure 3.3 A. Agarose gel electrophoresis showing the PCR product of the *PmATP/GTP-BP* gene segment amplified from cDNA (lanes 1 and 2) and genomic DNA (lane 3) of *P. monodon*. B. The PCR product amplified from genomic DNA was cloned and sequenced. An intron of 149 bp was found C. And new pair of primers was designed and used for RT-PCR. The genomic DNA of *P. monodon* were illustrated in boldface and underlined.

Pm17β-HSD was expressed at low level in examined tissues and its expression in ovaries and lymphoid organ seemed to be greater than other tissues of female broodstock (Figure 3.4B).

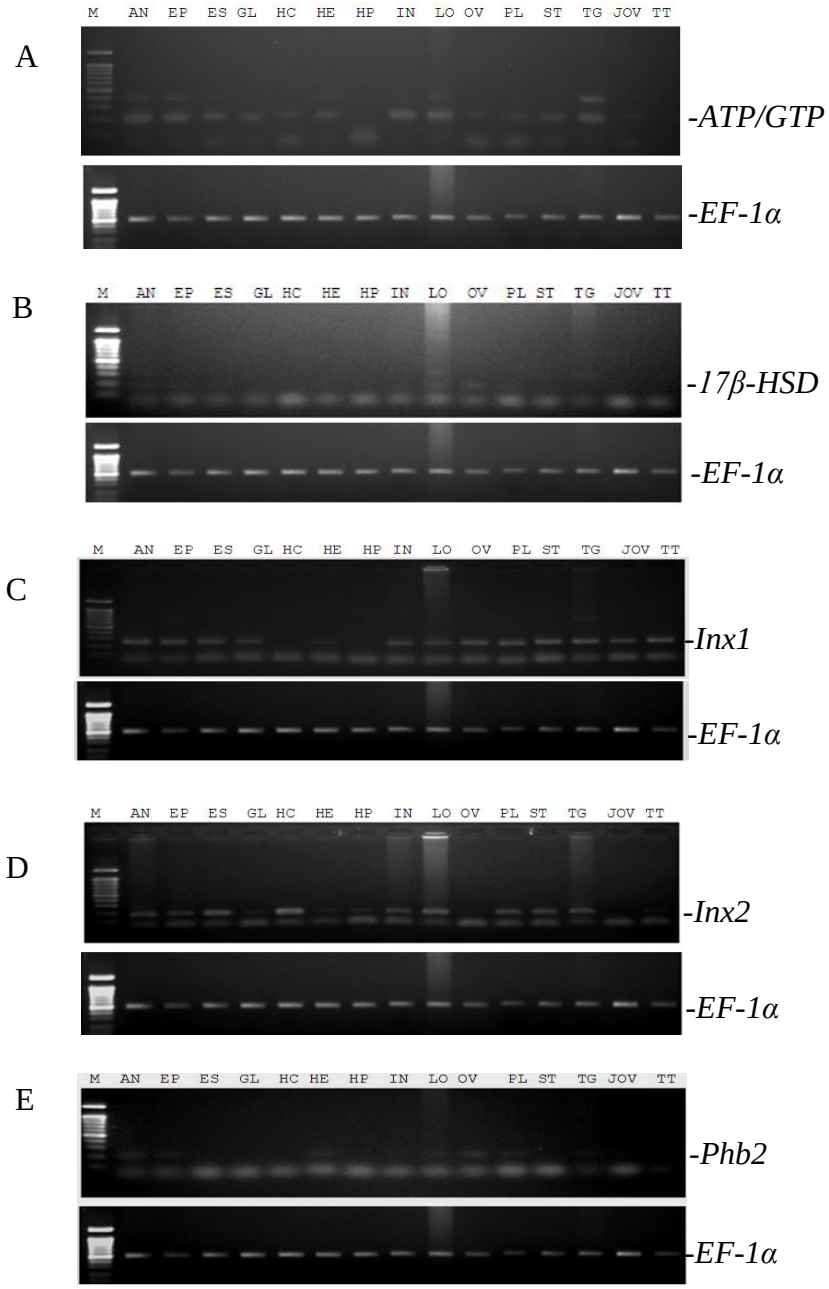


Figure 3.4 A 1.5% ethidium bromide-stained agarose gel showing results from tissue distribution analysis of *PmATP/GTP-BP(A)*, *Pm17β-HSD* (B), *PmInx1* (C) *PmInx2 (D)* and *PmPhb2* (E) transcripts using the cDNA template from *antennal gland* (AN), epicuticle (EP), eyestalk (ES), gills (GL), hemocytes (HC), heart (HE), hepatopancreas (HP), intestine (IN), lymphoid organs (LO), ovaries (OV), pleopods (PL), stomach (ST), thoracic ganglion (TG), ovaries of female juvenile (JOV) and testes (TT) of a male broodstock of wild *P. monodon*. *EF-1α* was included as the positive control. *EF-1α* was successfully amplified from the same template. Lanes M is a 100 bp DNA marker.

Table 3.1 Tissue expression analysis of *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1 PmInx2* and *PmPhb2* transcripts in *P. monodon*.

Gene	Tissue expression analysis														
	AN	**EP**	**ES**	**GL**	**HC**	**HE**	**HP**	**IN**	**LO**	**OV**	**PL**	**ST**	**TG**	**JOV**	**TT**
1. *ATP/GTP binding protein* (*PmATP/GTP-BP*)	++	++	++	++	+	++	-	+++	+++	+	+	+	+	+++	-
2. *17b-hydroxysteroid dehydrogenase* (*Pm17β-HSD,*)	+	+	+	-	-	-	-	-	++	++	+	-	-	-	-
3. *Innexin 1* (*PmInx1*)	+++	+++	+++	+++	-	++	-	+++	+++	+++	+++	+++	+++	+++	+++
4. *Innexin 2* (*PmInx2*)	++	++	+++	+	+++	+	+	++	+++	+	+++	+++	+++	-	+
5. *Prohibitin* (*PmPhb2*)	++	++	-	-	-	++	-	+	+	++	+	-	++	+	-

+++ = high expression level, ++ = moderate expression level, + = low expression level, - = no amplification in a particular tissue

PmInx1 was abundantly expressed in *antennal gland*, epicuticle, eyestalk, gills, intestine, lymphoid organs, ovaries, pleopods, stomach, thoracic ganglion of female broodstock, ovaries of female juvenile and testes of a male broodstock. It was expressed with a low level in hemocytes, heart and hepatopancreas (Figure 3.4C).

PmInx2 was abundantly expressed in lymphoid organs, hemocytes and eyestalk of female broodstock. It was expressed with a lower expression level in *antennal gland*, epicuticle, gills, intestine, pleopods, stomach and thoracic ganglion. *PmInx2* was rare expressed in heart, hepatopancreas, ovaries of female broodstock and ovaries of female juvenile and testes of a male broodstock (Figure 3.4D).

PmPhb2 was moderately expressed in ovaries. Low expression levels of *PmPhb2* were observed in other tissues of female broodtock, ovaries of juveniles and testes of male broodstock of *P. monodon* (Figure 3.4E).

3.3 Isolation and characterization of full-length cDNA of *Pm17β-HSD* using RACE-PCR

Total RNA from ovaries of *P. monodon* were extracted. The ovarian mRNA was purified and large amount of mRNA was obtained. The purified mRNA was subjected to the synthesis of the 5′ and 3′RACE template.

RACE-PCR was carried out and the amplification products of approximately 350 and 600 bp fragments obtained from the 5′ and 3′RACE-PCR were cloned and sequenced for both directions. The sequences obtained were assembled with that of the EST sequence.

The full-length cDNA of *P. monodon 17β-HSD* (*Pm17β-HSD*) was 1400 bp in length containing an ORF of 768 bp corresponding to a deduced protein of 255 amino acid and the 5′ and 3′ UTRs of 538 and 216 bp, respectively (Figures 3.5 and 3.6). The poly A additional signal (AATAAA) was located between 1192 - 1197 of the entire sequence (Figure 3.7). This sequence significantly matched hydroxyl-steroid (17-beta) dehydrogenase 10 of *Xenopus (Silurana) tropicalis* (*E*-value = 1e-122) analyzed by BLAST (Figure 3.9).

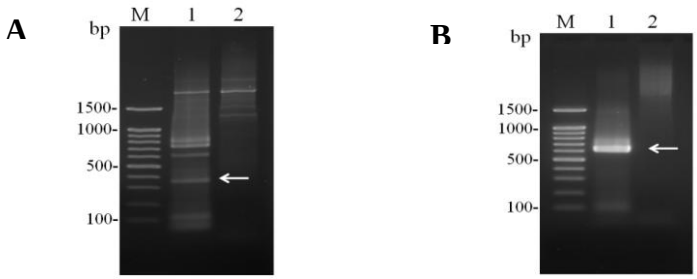


Figure 3.5 5' RACE-PCR (lane 1, A) and 3'RACE-PCR products (lane 1, B) of *Pm17β-HSD*. Arrows indicate RACE-PCR product that were cloned and sequenced. Lanes M and 2 are a 100 bp DNA ladder and the gene-specific primer (GSP) control, respectively

A.

GGCAAGTGTTGCGGCATTTGATGGGCAGATCGGTCAAGCTGCCTATTCTGCTAGCAAAGGAGCCATTGTAGGCATG
ACACTTCCCATTGCGAGGGATCTGGCACCTATAGGGATCAGAGTGTGCACTATTGCGCCAGGATTATTCAAGACCC
CCTTGCTGATGGCTCTCCCAGAGAAAGTGCAGAATTTCTTGGCCACGACTGTGCCCTTCCCCAAGAGACTAGGGGA
CCCAGATGAATATGCGCAGATGGTTCAGGCCATCATCACAAACCCAATGATGAA**CGGAGAAACTGTGCGACTGGAT**
GGAGCCATTCGTATGCAGCCTTAAGTGTAGACATAGTCAGTTGCACATACTTAGGAGTTTTGTGGCTATACTGTTT
GTGTCAAATGTTTTCAGAACTGAATTTAGGGCAGTTTTGTTTGAAGATCATGTTTACACAATATGGTTAAGAGTGA
AATATTCAGTTTGAAGTTTTTAAGGTGTTGTTGGCAGATCTGATGATGTAGCTATTTTGTCAGAGAATATTTACTA
GATTGTTGATACTGTATGTTAGGACTAACAGCATAGAAGGGGTGGATTTAACCTGCAGCTTATCATCATTATTCCG
AGTGTATAGTAATACATTATTATTTTGTAATTCTGAAC

B.

AAGCAGTGGTATCAACGCAGAGTACTCGCTGATGCGTCGCGGCCGCTAGCAGACGCTTCGCTTCGAACCGGAAGGT
CTTCTGCAGGAGGTTTTACACGATGCTGAAGGGTGTAGTGTCGCTTGTCACCGGGGGAGCATCCGGCCTCGGAAGG
GCAACTGTGGAGAGAATTGTTAGAGAAGGTGGACGTGCTGTCATCTGCGATCTTCCCACCTCCCAGGGTGCCAAAG
TAGCCAGTGATTTAGGAGATAATGCCATTTTTGCCCCCACTGATGTATCATCAGCAGAGGATGTAGAGAAAGCCCT
TGCCATGTGCCAAGACAAATTTGGCAGGCTGGATGTGGCTGTCAACTGTGCTGGTATTGGCATTGCCGTGAAGACC
TACAACCCAAAGAAGAAATTGCCTCATTCTCTGGATGACTTTATGCGTGTTCAAAGGGTCAACTTGGGCGGCTCGT
TCAATGTGATCCGCCTGTCCTGTGGAGTGATGGCAGAGAATGAACCAAATGCCGACGGGCAGAGAGGAGTAATTGT
GAATACGGCAAGTGTTGCGGCATTTGATGGGCAGATCGGTCAAGCTGCCTATTCTGCTAGCAAAGGAGCCATTGTA
GGCATGACACTTCCCATTGCGAGGGATCTGGCACCTATAGGGATCAGAGTGTGCACTATTGCGCCAGGATTATTCA
AGACCCCCTTGCTGATGGCTCTCCCAGAGAAAGTGCAGAATTTCTTGGCCACGACTGTGCCCTTCCCCAAGAGACT
AGGGGACCCAGATGAATATGCGCAGATGGTTCAGGCCATCATCACAAACCCAATGATGAA**CGGAGAAACTGTGCGA**
CTGGATGGA

C.

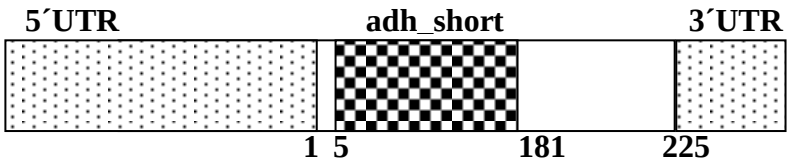
CGGAGAAACTGTGCGACTGGATGGAGCCATTCGTATGCAGCCTTAAGTGTAGACATAGTCAGTTGCACATACTTAG
GAGTTTTGTGGCTATACTGTTTGTGTCAAATGTTTTCAGAACTGAATTTAGGGCAGTTTTGTTTGAAGATCATGTT
TACACAATATGGTTAAGAGTGAAATATTCAGTTTGAAGTTTTTAAGGTGTTGTTGGCAGATCTGATGATGTAGCTA
TTTTGTCAGAGAATATTTACTAGATTGTTGATACTGTATGTTAGGACTAACAGCATAGAAGGGGTGGATTTAACCT
GCAGCTTATCATCATTATTCCGAGTGTATAGTAATACATTATTATTTTGTAATTCTGAACAAATCCTAATATAAAT
AGTAAACTGATTTCCTATTTTTCTTATTTGGTAAAATATTGAAAATGTTCAAGAAACTTTCACTAGTATTTTTATG
ATGTTTTATATATATTAAAAAGAAAATAGTTGCAATTTTTTTCCTAAAATGTAAAAGTAAAATTTCTCTGTATGTA
CATATATAATACTTTATATTTTCAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.6 Nucleotide sequences of EST (A), 5' RACE-PCR and 3' RACE-PCR (B) of *P. monodon 17β-HSD*. The positions of primers were illustrated in boldface and underlined.

The predicted molecular mass and pI of the deduced *Pm17β-HSD* protein was 26.78 kDa and 8.27, respectively. The adh_short domain was found at the amino acid positions 5 -181 (1.80e-26) (Figures 3.8 and 3.9).

```
AAGCAGTGGTATCAACGCAGAGTACTCGCTGATGCGTCGCGGCCGCTAGCAGACGCTTCG     60
CTTCGAACCGGAAGGTCTTCTGCAGGAGGTTTTACACGATGCTGAAGGGTGTAGTGTCGC   120
                                       M  L  K  G  V  V  S       7
TTGTCACCGGGGGAGCATCCGGCCTCGGAAGGGCAACTGTGGAGAGAATTGTTAGAGAAG   180
L  V  T  G  G  A  S  G  L  G  R  A  T  V  E  R  I  V  R  E      27
GTGGACGTGCTGTCATCTGCGATCTTCCCACCTCCCAGGGTGCCAAAGTAGCCAGTGATT   240
G  G  R  A  V  I  C  D  L  P  T  S  Q  G  A  K  V  A  S  D      47
TAGGAGATAATGCCATTTTTGCCCCCACTGATGTATCATCAGCAGAGGATGTAGAGAAAG   300
L  G  D  N  A  I  F  A  P  T  D  V  S  S  A  E  D  V  E  K      67
CCCTTGCCATGTGCCAAGACAAATTTGGCAGGCTGGATGTGGCTGTCAACTGTGCTGGTA   360
A  L  A  M  C  Q  D  K  F  G  R  L  D  V  A  V  N  C  A  G      87
TTGGCATTGCCGTGAAGACCTACAACCCAAAGAAGAAATTGCCTCATTCTCTGGATGACT   420
I  G  I  A  V  K  T  Y  N  P  K  K  K  L  P  H  S  L  D  D     107
TTATGCGTGTTCAAAGGGTCAACTTGGGCGGCTCGTTCAATGTGATCCGCCTGTCCTGTG   480
F  M  R  V  Q  R  V  N  L  G  G  S  F  N  V  I  R  L  S  C     127
GAGTGATGGCAGAGAATGAACCAAATGCCGACGGGCAGAGAGGAGTAATTGTGAATACGG   540
G  V  M  A  E  N  E  P  N  A  D  G  Q  R  G  V  I  V  N  T     147
CAAGTGTTGCGGCATTTGATGGGCAGATCGGTCAAGCTGCCTATTCTGCTAGCAAAGGAG   600
A  S  V  A  A  F  D  G  Q  I  G  Q  A  A  Y  S  A  S  K  G     167
CCATTGTAGGCATGACACTTCCCATTGCGAGGGATCTGGCACCTATAGGGATCAGAGTGT   660
A  I  V  G  M  T  L  P  I  A  R  D  L  A  P  I  G  I  R  V     187
GCACTATTGCGCCAGGATTATTCAAGACCCCCTTGCTGATGGCTCTCCCAGAGAAAGTGC   720
C  T  I  A  P  G  L  F  K  T  P  L  L  M  A  L  P  E  K  V     207
AGAATTTCTTGGCCACGACTGTGCCCTTCCCCAAGAGACTAGGGGACCCAGATGAATATG   780
Q  N  F  L  A  T  T  V  P  F  P  K  R  L  G  D  P  D  E  Y     227
CGCAGATGGTTCAGGCCATCATCACAAACCCAATGATGAACGGAGAAACTGTGCGACTGG   840
A  Q  M  V  Q  A  I  I  T  N  P  M  M  N  G  E  T  V  R  L     247
ATGGAGCCATTCGTATGCAGCCTTAAGTGTAGACATAGTCAGTTGCACATACTTAGGAGT   900
D  G  A  I  R  M  Q  P  *                                      255
TTTGTGGCTATACTGTTTGTGTCAAATGTTTTCAGAACTGAATTTAGGGCAGTTTTGTTT   960
GAAGATCATGTTTACACAATATGGTTAAGAGTGAAATATTCAGTTTGAAGTTTTTAAGGT  1020
GTTGTTGGCAGATCTGATGATGTAGCTATTTTGTCAGAGAATATTTACTAGATTGTTGAT  1080
ACTGTATGTTAGGACTAACAGCATAGAAGGGGTGGATTTAACCTGCAGCTTATCATCATT  1140
ATTCCGAGTGTATAGTAATACATTATTATTTTGTAATTCTGAACAAATCCTAATAAAAAT  1200
AGTAAACTGATTTCCTATTTTTCTTATTTGGTAAAATATTGAAAATGTTCAAGAAACTTT  1260
CACTAGTATTTTTATGATGTTTTATATATATTAAAAAGAAAATAGTTGCAATTTTTTTCC  1320
TAAAATGTAAAAGTAAAATTTCTCTGTATGTACATATATAATACTTTATATTTTCAAAAA  1380
AAAAAAAAAAAAAAAAAAAA                                           1400
```

Figure 3.7 The full-length nucleotide and deduced amino sequences of *P. monodon 17β-HSD* mRNA. The stop codon (TAA) is illustrated in boldfaced and underlined. The poly A tail is boldfaced. The short-chain dehydrogenases/reductases family (adh_short; 1.80e-26, positions 5^{th}-181^{th}) domains are highlighted. The poly A additional signal (AATAAA) is boldfaced and underlined.



Domain	Position	E-value
adh_short	5-181	1.80e-26

Figure 3.8 A diagram illustrating the full-length cDNA of *Pm17β-HSD*. The predicted adh_short domain was found in this transcript. The scale bar is 200 bp in length (underlined).

```
GENE ID: 549265 hsd17b10 | hydroxysteroid (17-beta) dehydrogenase 10
[Xenopus (Silurana) tropicalis] (10 or fewer PubMed links)
 Score = 369 bits (947),  Expect = 1e-122
 Identities = 181/254 (71%), Positives = 211/254 (83%), Gaps = 0/254 (0%)
 Frame = +3

Query  102  LKGVVSLVTGGASGLGRATVERIVREGGRAVICDLPTSQGAKVASDLGDNAIFAPTDVSS  281
            LKG+V +VTGGASGLGRATVER+VR+G  AVI DLP S+G  VA  LG+   F+PTDV+S
Sbjct  7    LKGLVGIVTGGASGLGRATVERLVRQGASAVILDLPKSEGKTVAESLGEKCAFSPTDVTS  66

Query  282  AEDVEKALAMCQDKFGRLDVAVNCAGIGIAVKTYNPKKKLPHSLDDFMRVQRVNLGGSFN  461
              DV+ AL + + KFGR+DV VNCAGI +A KTYN  K+LPHSL+DF RV  VN+ G+FN
Sbjct  67   EADVKNALELARTKFGRVDVVVNCAGIAVAAKTYNLNKQLPHSLEDFQRVINVNIAGTFN  126

Query  462  VIRLSCGVMAENEPNADGQRGVIVNTASVAAFDGQIGQAAYSASKGAIVGMTLPIARDLA  641
            VIRL+ G M +N+P+ DG RGVIVNTASVAAFDGQ+GQAAYSASKG IVGMTLPIARDLA
Sbjct  127  VIRLAAGEMGKNDPDEDGHRGVIVNTASVAAFDGQVGQAAYSASKGGIVGMTLPIARDLA  186

Query  642  PIGIRVCTIAPGLFKTPLLMALPEKVQNFLATTVPFPKRLGDPDEYAQMVQAIITNPMMN  821
            PIGIRV TIAPGLF TPLL+ LPEKV+NFLA  VPFP RLGDP EYA +VQ+I+ NPM+N
Sbjct  187  PIGIRVVTIAPGLFATPLLVGLPEKVRNFLAKQVPFPSRLGDPGEYAHLVQSIVENPMLN  246

Query  822  GETVRLDGAIRMQP  863
            GE +RLDGA+RMQP
Sbjct  247  GEVIRLDGALRMQP  260
```

Figure 3.9 Similarity search using BlastX indicates significantly matched between *P. monodon 17β-HSD* and that of *Xenopus (Silurana) tropicalis.*

3.4 Localization of *Pm17β-HSD* transcripts in ovaries of *P. monodon* broodstock

3.4.1 Quantification of the cRNA probes

The sense and antisense cRNA probes were synthesized from the PCR product (Figure 3.10). The amount of cRNA probes were estimated by dot blot analysis. The sensitivity of each probe was tested by dot blot analysis compared to the control RNA. The antisense and sense probes of *Pm17β-HSD* gave the signal from 10 pg - 100 ng (Figure 3.11). An appropriate amount of the cRNA probe of each transcript was applied for examination of transcriptional localization using *in situ* hybridization.

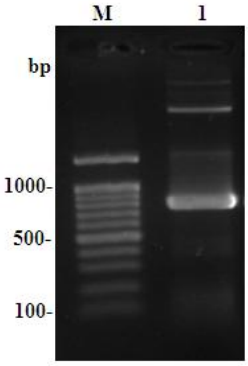


Figure 3.10 The amplification product (ORF overhang with *Nde* I-*Bam* HI-*6His* tag) for *Pm17β-HSD* (Lane 1). Lane M = a 100 DNA ladder.

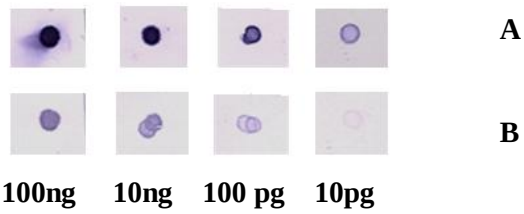


Figure 3.11 Dot blot hybridization using the antisense *Pm17β-HSD* probe (A) and sense *Pm17β-HSD* RNA probes (B) against different amounts of the RNA target.

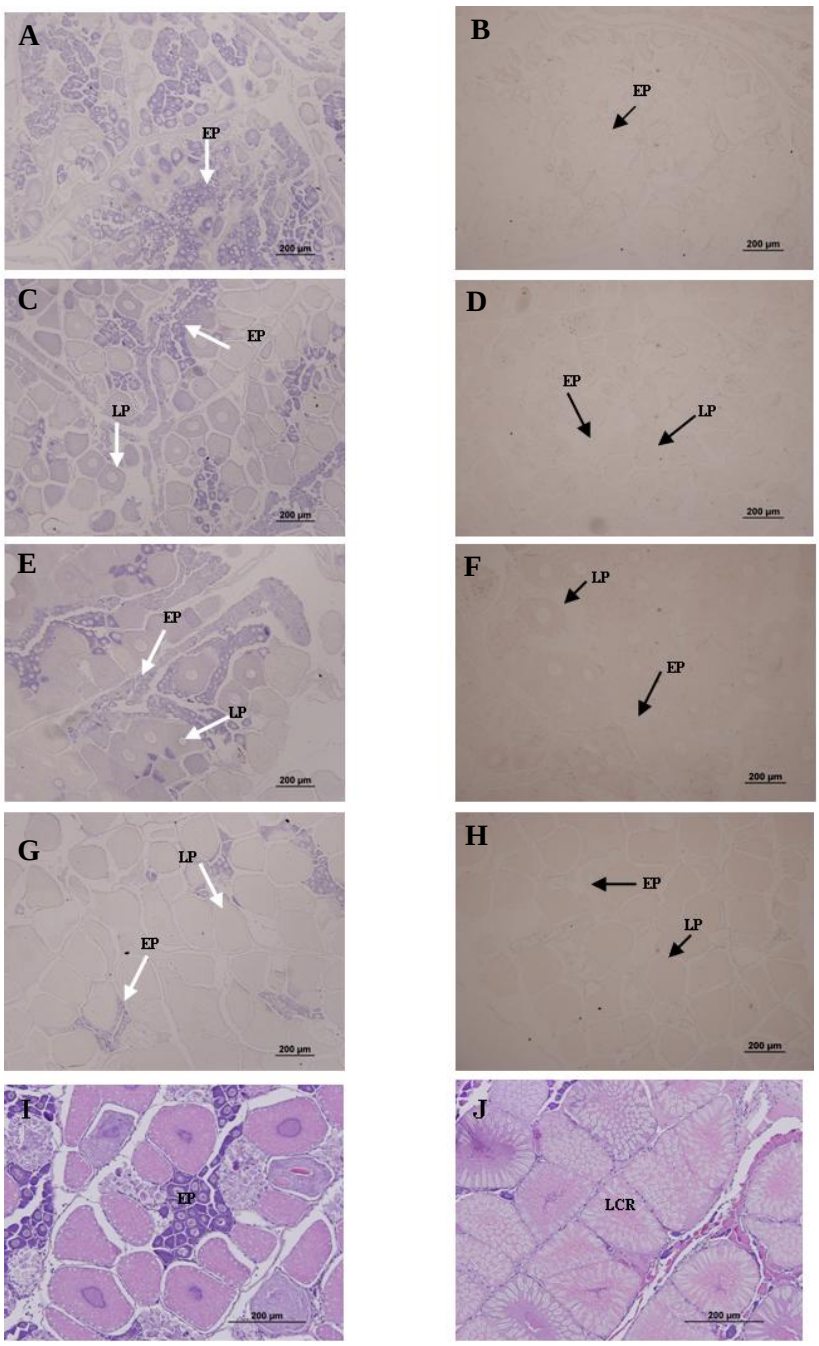


Figure 3.12 Localization of *Pm17β-HSD* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the antisense (A, C, E and G) and sense *Pm17β-HSD* RNA probes (B, D, F and H). Conventional HE staining was carried out for identification of oocyte stages (I and J). EP = early previtellogenic oocytes; LP = late previtellogenic oocytes and LCR = late cortical rod oocytes. (A and B = stage I, C and D = stage II, E and F = stage III, G and H = stage IV ovaries).

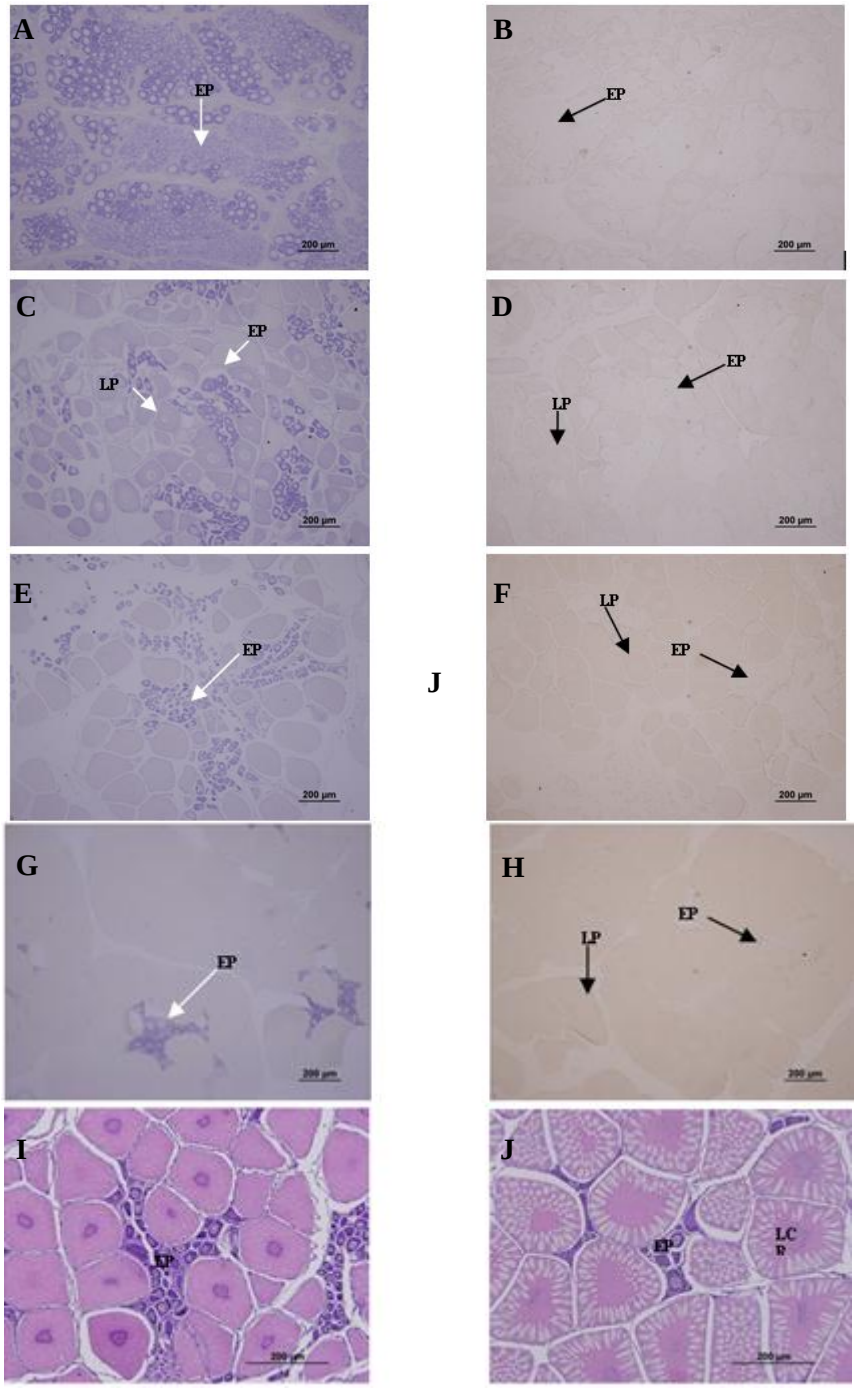


Figure 3.13 Localization of *Pm17β-HSD* transcript during ovarian development of eyestalk-ablated *P. monodon* broodstock visualized by *in situ* hybridization using the antisense (A, C, E and G) and sense *Pm17β-HSD* RNA probes (B, D, F and H). Conventional HE staining was carried out for identification of oocyte stages (I and J). EP = early previtellogenic oocytes; LP = late previtellogenic oocytes and LCR = late cortical rod oocytes. (A and B = stage I, C and D = stage II, E and F = stage III, G and H = stage IV ovaries).

3.4.2 *In situ* hybridization (ISH)

The subcellular localization of *Pm17β-HSD* transcripts in ovaries of *P. monodon* broodstock was determined by *in situ* hybridization. The positive signal was observed when the tissue sections were hybridized with the antisense probe of *Pm17β-HSD* (Figures 3.12 B, D, F, H) and no signal was observed with sense probe for all transcripts (Figures 3.13 B, D, F, H). The antisense of *Pm17β-HSD* probes gave a clear signal in cytoplasm of previtellogenic oocytes in different stages of ovarian development in both intact and eyestalk-ablated broodstocks (Figures 3.12 and 3.13). The localization of *Pm17β-HSD* was not observed in more mature oocytes.

3.5 *In vitro* expression of recombinant *Pm17β-HSD* protein using the bacterial expression system

Recombinant plasmid carrying the full-length ORF of *Pm17β-HSD* was prepared for *in vitro* expression of the corresponding protein was constructed. For the ORF of *17β-HSD* was characterized by RACE PCR prior. Initially, the primers for amplification of nucleotide sequence encoding the mature *Pm17β-HSD* protein were designed. The amplified full-length cDNA of *17β-HSD* was ligated to pGEM®-T easy vector and transformed into *E. coli* strain JM109. The recombinant plasmid of the positive clone was re-sequenced to confirm the correct oreintation. Plasmid DNA of the selected clone was used as the template for amplification using a forward primer containing *Nde* I restriction site and a reverse primer containing *Bam* HI restriction site and six-repeated Histidine (6His) encoded nucleotides by *Pfu* DNA polymerase (Figure 3.14).

The amplification product was digested with *Nde* I and *Bam* HI. The gel-eluted product was ligated with pET29a(+) expression vector and transformed into *E. coli* JM109. Plasmid DNA was extracted from a clone carrying the correct direction of *17β-HSD* gene and transformed into *E. coli* BL21 (DE3) plysS competent cells

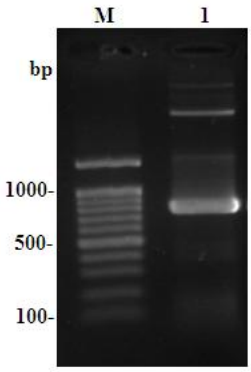
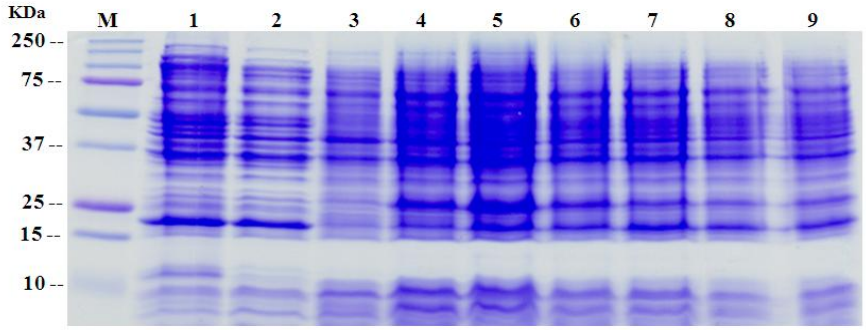


Figure 3.14 The amplification product (ORF overhang with *Nde* I-*Bam* HI-*6His* tag) for *Pm17β-HSD* (Lane 1). Lane M = a 100 DNA ladder.

A.



B.

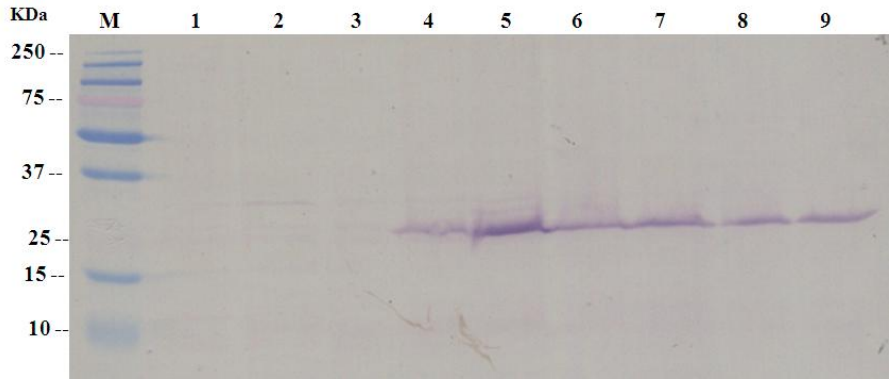


Figure 3.15 *In vitro* expression of recombinant *Pm17β-HSD* protein at 0, 1, 2, 3, 6, 12, and 24 hr after induced by 1 mM IPTG (lanes 3-9) using SDS-PAGE (A) and western blot analysis (B). *E. coli* BL21C+(DE3) plysS cells (lane 1) and *E. coli* BL21C+(DE3) plysS containing pET29a vector (lane 2) were included as the control.

Expression of the recombinant *Pm17β-HSD* was examined at 0, 1, 2, 3, 6, 12 and 24 hr after 1mMIPTG induction at 37°C (Figures 3.15). The lower expression levels of recombinant *Pm17β-HSD* was found at 1 hr after IPTG induction. A slightly greater level of expressed *Pm17β-HSD* was observed when the culture period was extended between 2 hr after induction. The expression of recombinant *Pm17β-HSD* was expressed at relative low level between 3-24 hr after IPTG induction.

Owing to the low expression level of recombinant *Pm17β-HSD* protein, polyclonal antibody adainst this recombinant protein was not further produced.

3.6 Examination of expression levels of interesting genes related with ovarian development of *P. monodon* by quantitative real-time PCR

The expression levels of *P. monodon ATP/GTP binding protein (PmATP/GTP-BP), 17β-hydroxysteroid dehydrogenase* (*Pm17β-HSD), Innexin1 (PmInx1)* and *prohibitin2 (PmPhb2*) in ovaries of wild intact and unilateral eyestalk-ablated broodstock were examined by quantitative real-time PCR. In addition, effects of 17β-estradiol, serotonin and progesterone on the expression of *PmATP/GTP-BP* and *Pm17β-HSD* were also determined.

The standard curves used for quantitative real-time PCR analysis of each target gene and the control (*EF-1α*) were constructed (Figure 3.16). High efficiency of amplification of the examined transcripts and limited errors were found. Therefore, these standard curves were acceptable to be used for quantitative estimation of the mRNA levels of examined genes.

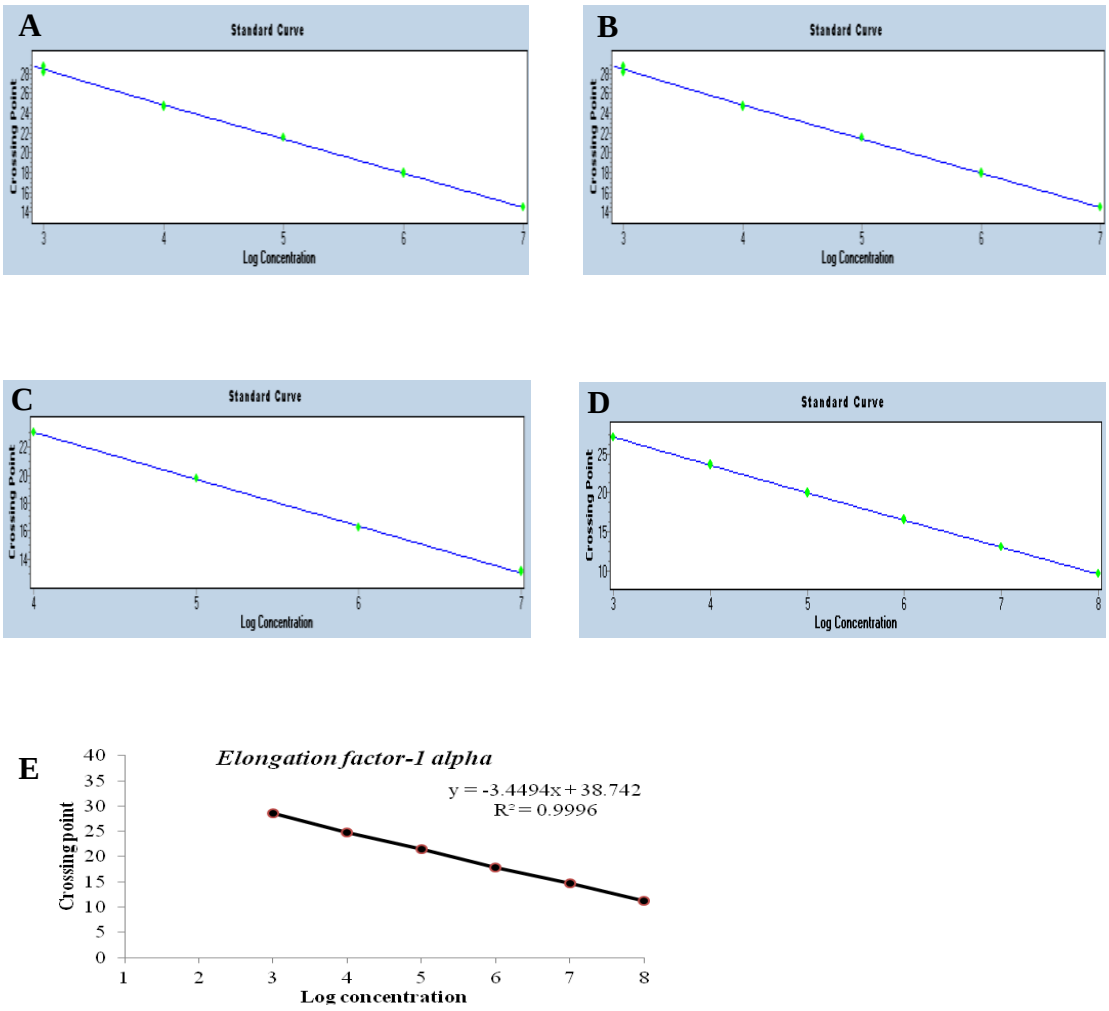


Figure 3.16 Standard curves of *PmATP/GTP-BP*; efficiency = 1.956 and equation; Y= -3.432* log(X) + 38.72 (A), *Pm17β-HSD*; efficiency = 1.963 and equation; Y= -3.428* log(X) + 38.64 (B), *PmInx1*; efficiency = 1.990 and equation; Y = -3.347* log(X) + 36.42 (C), *PmPhb2*; efficiency = 1.946 and equation; Y = -3.458 * log(X) + 37.58 (D) and *EF-1α*; efficiency = 1.984 and equation; Y = -3.45 * log(X) + 38.74 (E)

3.6.1 Expression profile of *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2*

The expression level of *PmATP/GTP-BP* mRNA in ovaries of juveniles and wild broodstock was not significantly different spawning ($P > 0.05$). In intact broodstock, its expression was comparable during ovarian development but up-regulated after spawning ($P > 0.05$). Likewise, the expression level of *PmATP/GTP-BP* was not significantly different during the rapid ovarian development in eyestalk-ablated broodstock. The expression level of *PmATP/GTP-BP* in stages II and III ovaries in intact broodstock was significantly higher than that in the same developmental stages in eyestalk-ablated shrimp ($P < 0.05$) (Figure 3.17 and Table 3.2).

The expression level of *Pm17β-HSD* mRNA in ovaries of cultured juveniles and broodstock was not significantly different ($P > 0.05$). In intact broodstock, *Pm17β-HSD* was not differentially expressed during ovarian development ($P > 0.05$) but was up-regulated after spawning ($P < 0.05$). The mRNA levels of *Pm17β-HSD* in eyestalk-ablated broodstock were not significantly different throughout ovarian development of *P. monodon* ($P > 0.05$, Figure 3.20). Like *PmATP/GTP-BP*, the expression level of *Pm17β-HSD* in stages II and III ovaries in intact broodstock was significantly higher than that in the same developmental stages in eyestalk-ablated shrimp ($P < 0.05$) (Figure 3.18 and Table 3.3).

The expression level of *PmInx1* mRNA in ovaries of cultured juveniles was significantly lower than that of broodstock ($P < 0.05$). In intact broodstock, *PmInx* was initially up-regulated from stages I in stages II and III ovaries ($P < 0.05$) and its expression peaked in stage IV ovaries ($P < 0.05$). The expression of this gene was returned to similar as stages I ovaries after spawning.

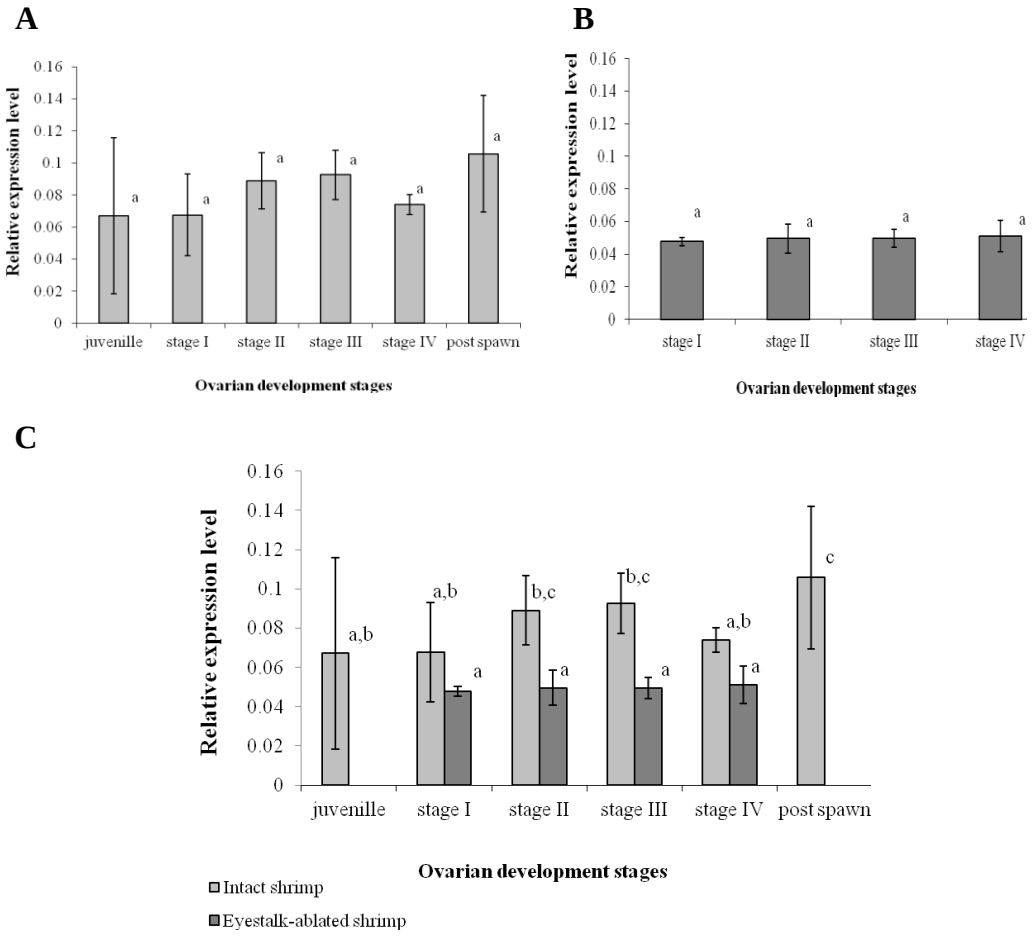


Figure 3.17 Histograms showing the relative expression profiles of *PmATP/GTP-BP* during ovarian maturation of wild intact (A) and unilateral eyestalk ablated (B) *P. monodon* broodstock. Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmATP/GTP-BP* mRNA (50 ng template) and normalized by that of *EF-1α* mRNA (5 ng template) Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.2 Relative expression levels of *PmATP/GTP-BP* in different ovarian stages of *P. monodon* female broodstock.

Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		Eyestalk-ablated broodstock	
Juveniles	$0.0671\pm0.049^{a,b}$	3	-	-
Stage I (GSI < 1.5%)	$0.0676\pm0.025^{a,b}$	8	0.0476 ± 0.003^{a}	3
Stage II (GSI = 2.0 – 4.0%)	$0.0890\pm0.018^{b,c}$	5	0.0496 ± 0.009^{a}	5
Stage III (GSI >4.0 - 6.0%)	$0.0926\pm0.015^{b,c}$	7	0.0495 ± 0.006^{a}	6
Stage IV (GSI > 6.0%)	$0.0740\pm0.006^{a,b}$	6	0.0511 ± 0.010^{a}	7
Post-spawning (GSI = 1.86 – 3.49)	0.1057 ± 0.036^{c}	5	-	-

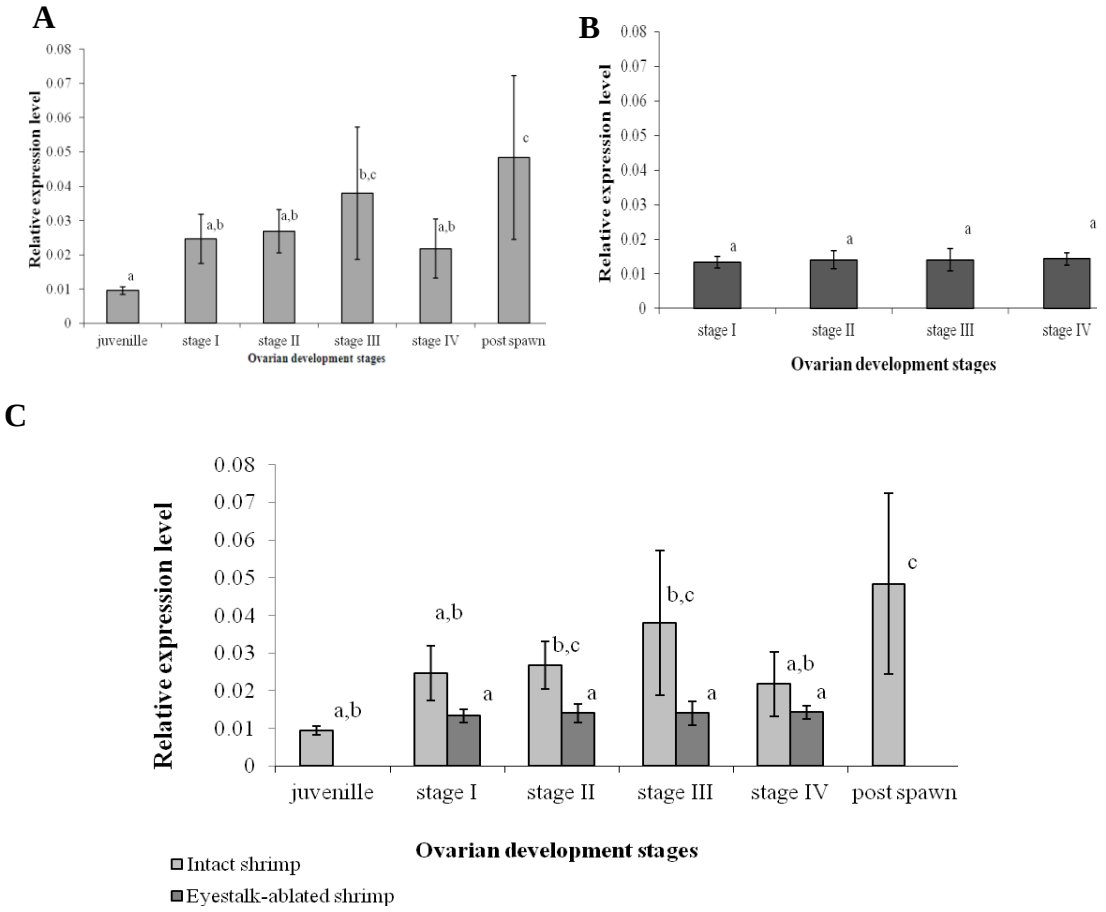


Figure 3.18 Histograms showing the relative expression profiles of *Pm17β-HSD* during ovarian maturation of wild intact (A) and unilateral eyestalk ablated (B) *P. monodon* broodstock. Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *Pm17β-HSD* mRNA (50 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.3 Relative expression levels of *Pm17β-HSD* in different ovarian stages of *P. monodon* female broodstock.

Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juveniles	$0.0096\pm0.001^{a,b}$	3	-	-
Stage I (GSI < 1.5%)	$0.0247\pm0.007^{a,b}$	4	0.0133 ± 0.002^{a}	3
Stage II (GSI = 2.0 – 4.0%)	$0.0268\pm0.006^{b,c}$	6	0.0140 ± 0.003^{a}	5
Stage III (GSI >4.0 - 6.0%)	$0.0380\pm0.019^{b,c}$	3	0.0140 ± 0.003^{a}	5
Stage IV (GSI >6.0%)	$0.0218\pm0.009^{a,b}$	5	0.0143 ± 0.002^{a}	6
Post-spawning (GSI = 1.86 – 3.49)	0.0484 ± 0.024^{c}	4	-	-

In eyestalk-ablated broodstock, the expression level of *PmInx1* was significantly increased from stages I and II ovaries in more mature stages of ovarian development (III and IV ovaries). Interestingly, the expression level of *PmInx1* in stages I, II, III and IV ovaries of eyestalk-ablated broodstock was significantly greater than that of the same developmental stages in intact broodstock ($P < 0.05$, Figure 3.19 and Table 3.4).

Similarly, the expression level of *PmPhb2* mRNA in ovaries of cultured juveniles was significantly lower than that of broodstock ($P < 0.05$). In intact broodstock, *PmPhb2* was sequentially up-regulated in stages II and III ovaries ($P < 0.05$) and its expression peaked in stage IV ovaries ($P < 0.05$). The expression of *PmPhb2* was slightly reduced to as similar as stages I-III ovaries after spawning.

In eyestalk-ablated broodstock, the expression level of *PmPhb2* was significantly increased from stages I and II ovaries in more mature stages of ovarian development (III and IV ovaries). The expression level of *PmPhb2* during ovarian development (stages I-IV ovaries) of eyestalk-ablated broodstock was significantly greater than that of the same developmental stages in intact broodstock ($P < 0.05$), Figure 3.20 and Table 3.5).

3.6.2 Expression profiles of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of domesticated *P. monodon*.

Quantitative real-time PCR was carried out for determination of the expression level of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of culture 5-month-old juveniles and domesticated 9-, 14- and 19 month-old broodstock of *P. monodon*.

The expression level of *PmATP/GTP-BP* in ovaries of 5 month-old juveniles seemed ti be greater than that in domesticated broodstock (9-, 14- and 19-month-old) but results were not statistically significant owing to large standard deviations between different groups of samples ($P > 0.05$, Figure 3.21A).

In contrast, the expression level of *Pm17β-HSD* in ovaries of 19 month-old domesticated broodstock was significantly greater than that in ovaries of 5-month–old juveniles and 9- and 14-month-old domesticated broodstock ($P < 0.05$, Figure 3.21B).

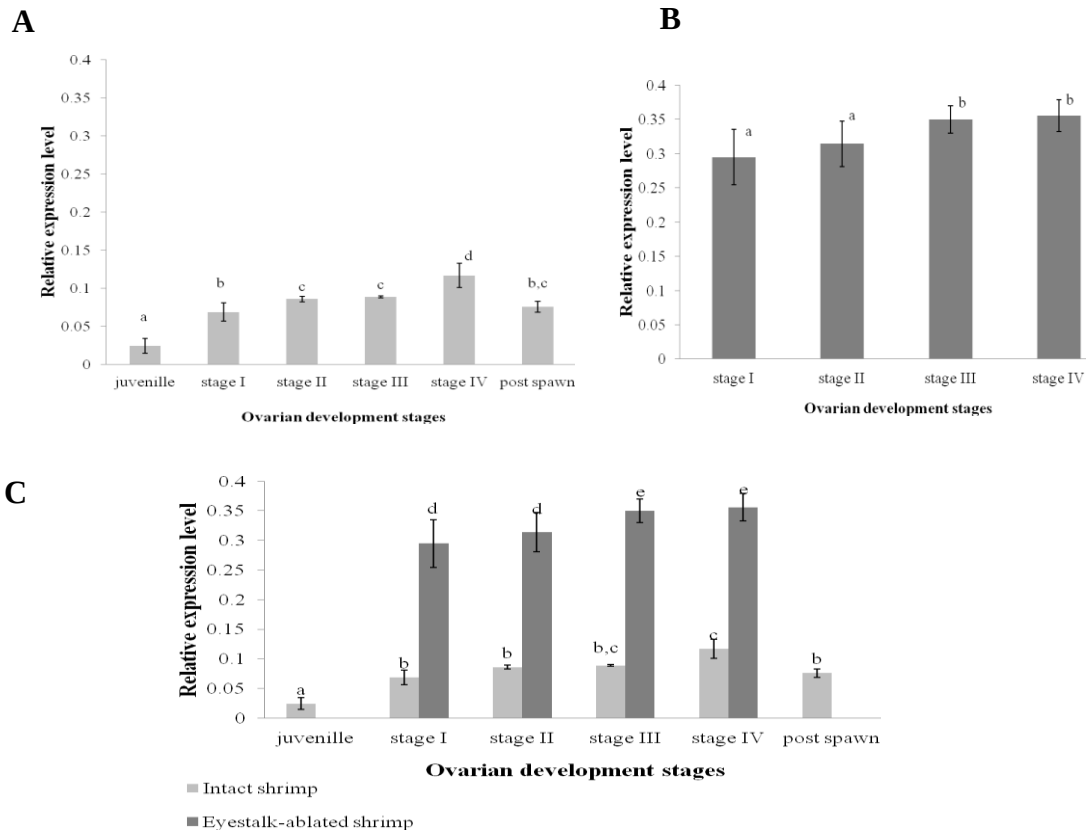


Figure 3.19 Histograms showing the relative expression profiles of *PmInx1* during ovarian maturation of wild intact (A) and unilateral eyestalk ablated (B) *P. monodon* broodstock. Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmInx1* mRNA (50 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.4 Relative expression levels of *PmInx1* in different ovarian stages of *P. monodon* female broodstock.

Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juveniles	0.0244 ± 0.010^{a}	5	-	-
Stage I (GSI < 1.5%)	0.0687 ± 0.012^{b}	4	0.2948 ± 0.040^{a}	3
Stage II (GSI = 2.0 - 4.0%)	0.0860 ± 0.003^{b}	3	0.3144 ± 0.033^{a}	10
Stage III (GSI >4.0 - 6.0%)	$0.0888\pm0.001^{b,c}$	4	0.3497 ± 0.020^{b}	11
Stage IV (GSI > 6.0%)	0.1169 ± 0.016^{c}	7	0.3557 ± 0.023^{b}	5
Post-spawning (GSI = 1.86 – 3.49)	0.0759 ± 0.007^{b}	4	-	-

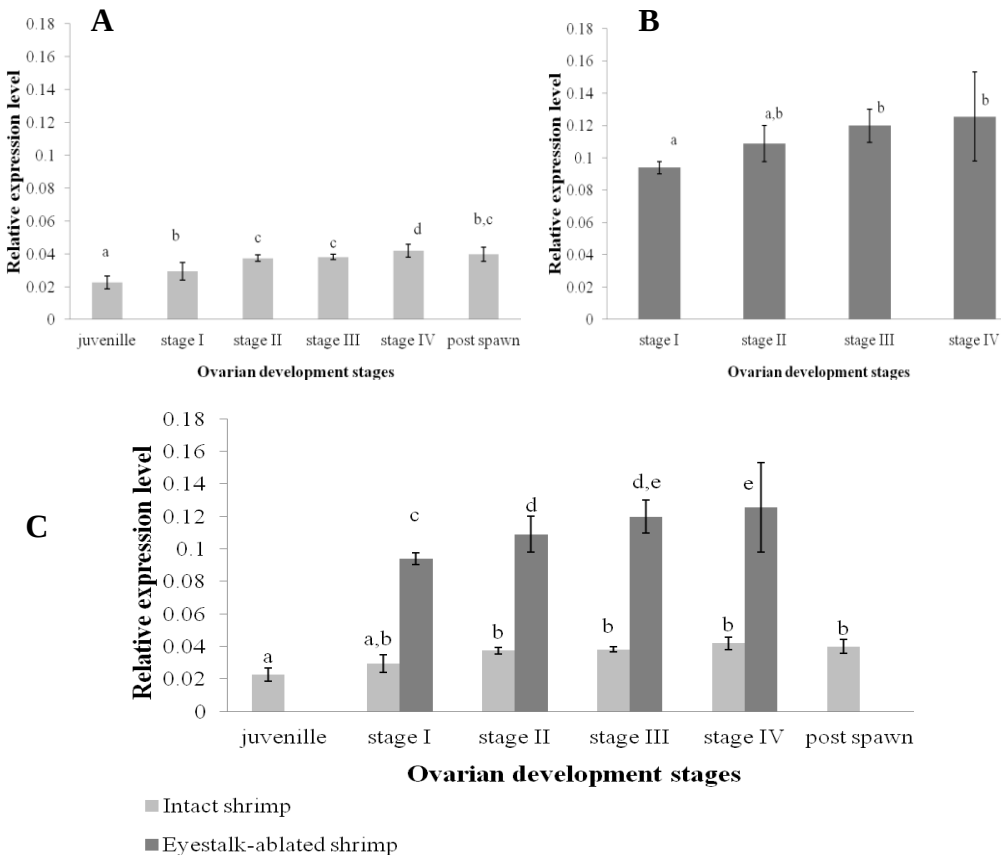
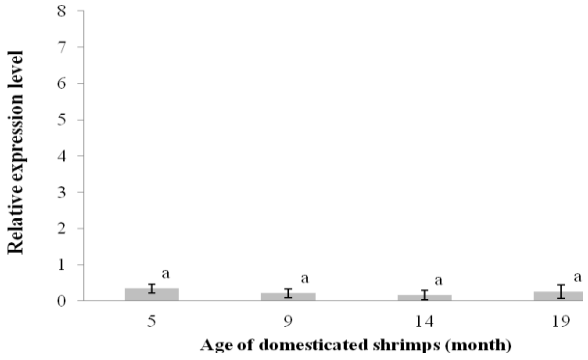


Figure 3.20 Histograms showing the relative expression profiles of *PmPhb2* during ovarian maturation of wild intact (A) and unilateral eyestalk ablated (B) *P. monodon* broodstock. Data of intactl and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmPhb2* mRNA (50 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.5 Relative expression levels of *PmPhb2* in different ovarian stages of *P. monodon* female broodstock.

Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juveniles	0.0226 ± 0.004^{a}	6	-	-
Stage I (GSI < 1.5%)	0.0293 ± 0.005^{b}	4	0.0939 ± 0.004^{a}	3
Stage II (GSI = 2.0 – 4.0%)	0.0373 ± 0.002^{c}	3	$0.1089\pm0.011^{a,b}$	11
Stage III (GSI >4.0 - 6.0%)	0.0381 ± 0.001^{c}	4	0.1198 ± 0.010^{b}	11
Stage IV (GSI > 6.0%)	0.0419 ± 0.004^{d}	7	0.1255 ± 0.027^{b}	5
Post-spawning (GSI = 1.86–3.49%)	0.0398 ± 0.004^{bc}	5	-	-

A



B

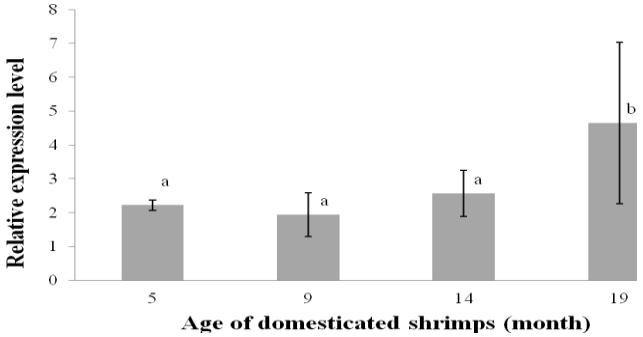


Figure 3.21 Histograms showing the relative expression profiles of *Pm ATP/GTP-BP* (A) and *Pm17β-HSD* (B) in ovaries of domesticated juvenile (5 month–old) and broodstock (9, 14 and 19 month–old) *P. monodon*. Expression levels were measured as the absolute copy number of *Pm ATP/GTP* and *Pm17β-HSD* mRNA (300 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.6 Relative expression levels of *Pm ATP/GTP-BP* and *Pm17β-HSD* in different ovarian stages of *P. monodon* female broodstock.

Age of shrimp	Relative expression level of *Pm ATP/GTP*	*N*	Relative expression level of *Pm17BHSD*	*N*
5 months old	0.3416±0.118[a]	6	2.2185±0.146[a]	4
9 months old	0.2151±0.122[a]	4	1.9414±0.651[a]	4
14 months old	0.1613±0.130[a]	4	2.5670±0.679[a]	5
19 months old	0.2594±0.180[a]	5	4.6460±2.389[b]	4

3.6.3 Effects of 17β-estradiol on expression of reproduction-related genes in ovaries of domesticated *P. monodon*

3.6.3.1 Preliminary experiments

Different doses of 17β-estradiol (0.01. 0.02 and 0.05 μg/g BW) was injected 12-month-old shrimp. Specimens were collected at 7 and 28 days post injection.

Quantitative real-time PCR revealed that the expression level of *Pm17β-HSD* seemed to be marginally increased as a result of exogenous injection of 17β-estradiol at 0.01 µg /g BW at 7 days after injection. The experimental error was observed at 28 days post injection as the gene expression level was highest in the vehicle control group. Accordingly, the appropriate concentration selected for stimulation of *Pm17β-HSD* expression was 0.01 µg/g BW.

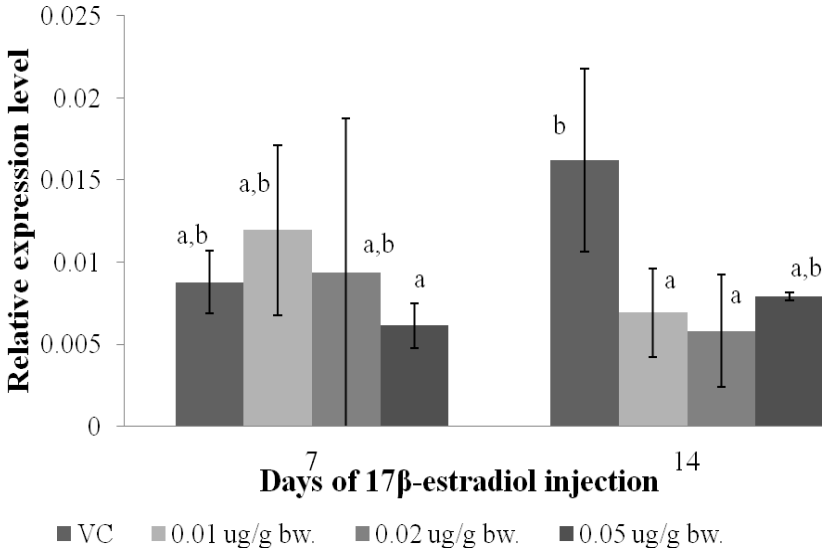


Figure 3.22 Histograms showing the relative expression profiles of ovarian *Pm17β-HSD* in domesticated broodstock injected with 17β-estradiol. Expression levels were measured as the absolute copy number of *Pm17β-HSD* mRNA (300 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.7 Relative expression levels of *Pm17β-HSD* in different group of *P. monodon* female broodstock.

Group	Relative expression level	*N*	Relative expression level	*N*
	7 days		**14 days**	
Vehicle control	$0.0088\pm0.0019^{a,b}$	3	0.0162 ± 0.0056^{b}	3
0.01µg/g BW	$0.0119\pm0.0052^{a,b}$	3	0.0069 ± 0.0027^{a}	3
0.02µg/g BW	$0.0093\pm0.0094^{a,b}$	3	0.0058 ± 0.0034^{a}	3
0.05µg/g BW	0.0061 ± 0.0014^{a}	3	$0.0079\pm0.000^{a,b}$	3

3.6.3.2 Effects of 17β-estradiol on expression of *PmATP/GTP-BP* and *Pm17β-HSD*

Effects of exogenous 17β-estradiol injection on expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 14-month-old *P. monodon* were examined. Injection of 17β-estradiol did not affect the significant difference in expression of *PmATP/GTP-BP* at different time intervals of the experiment ($P > 0.05$).). Likewise, eyestalk ablation did not result in significant difference in the expression of this gene ($P > 0.05$, Figure 3.23A and Table 3.8).

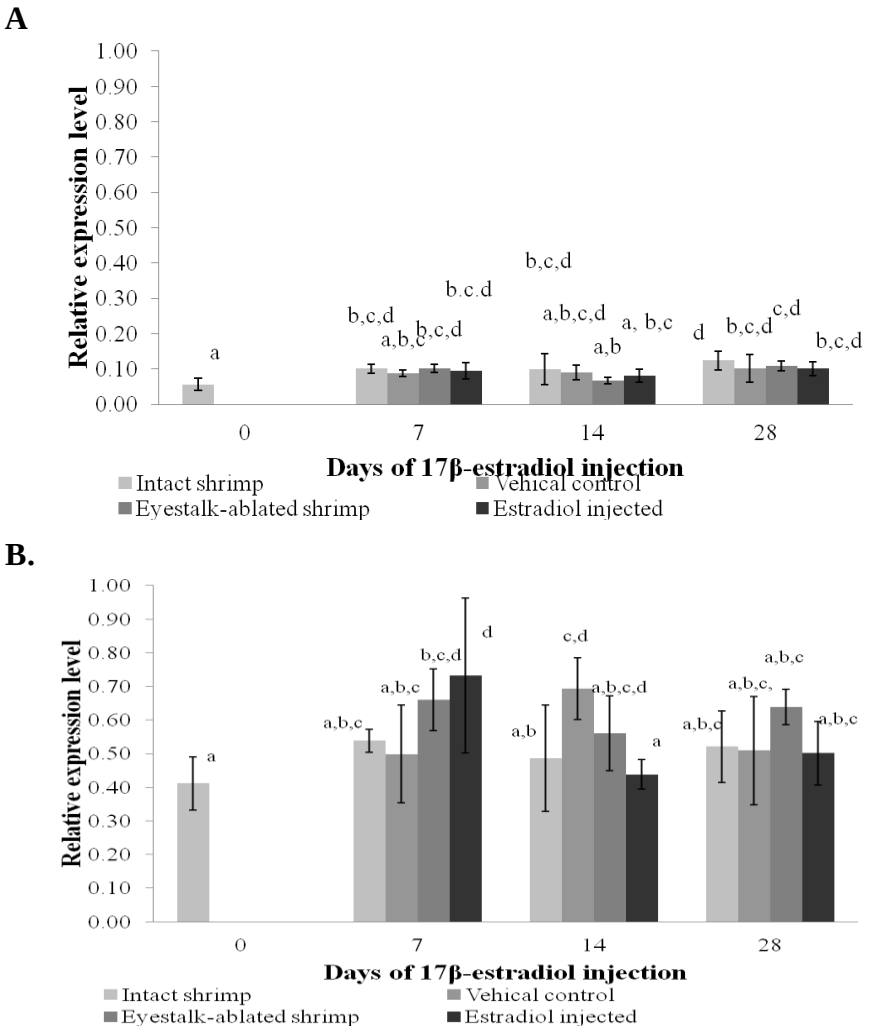


Figure 3.23 Histograms showing the relative expression profiles of *PmATP/GTP-BP* (A) and *Pm17β-HSD* (B) in ovaries of domesticated *P. monodon* (0.01µg/g BW) at 0, 7, 14 and 28 days after 17β-estradiol injection. Expression levels were measured as the absolute copy number of *Pm ATP/GTP-BP and Pm17β-HSD* mRNA (300 ng template) and normalized by that of *EF-1α* mRNA (5 ng template Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

In contrast, the injection of 17β-estradiol resulted in significant increase of *Pm17β-HSD* at 7 days after injection compare to non-injected and vehicle controls ($P < 0.05$) (Figure 3.23B). At this period of the treatment, eyestalk-ablated domesticated shrimp showed a trend of a slightly greater level of *Pm17β-HSD* than the controls but results were not statistically significant ($P > 0.05$). Nevertheless, no significant difference between the treatment and the controls was observed at 14 and 28 days post injection ($P > 0.05$, Table 3.8).

Table 3.8 Time-course relative expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries, of *P. monodon* female broodstock treated with 17β-estradiol (0.01µg/g bw.).

Group	Relative expression level of *PmATP/GTP-BP*	*N*	Relative expression level of *Pm17β-HSD*	*N*
Negative control, 0 day	0.0565 ± 0.018^{a}	4	0.3933 ± 0.080^{a}	5
Negative control, 7 days	$0.1010\pm0.013^{b,c,d}$	4	$0.5074\pm0.075^{a,b,c}$	5
Negative control, 14 days	$0.0988\pm0.043^{b,c,d}$	4	$0.4861\pm0.158^{a,b}$	4
Negative control, 28 days	0.1235 ± 0.026^{d}	4	$0.4935\pm0.093^{a,b,c}$	5
Vehicle control, 7 days	$0.0870\pm0.009^{a,b,c}$	4	$0.4987\pm0.145^{a,b,c}$	5
Vehicle control, 14 days	$0.0900\pm0.020^{a,b,c,d}$	4	$0.6932\pm0.091^{c,d}$	4
Vehicle control, 28 days	$0.1013\pm0.039^{b,c,d}$	4	$0.5090\pm0.161^{a,b,c}$	4
Eyestalk-ablated, 7 days	$0.1012\pm0.012^{b,c,d}$	5	$0.5968\pm0.093^{b,c,d}$	5
Eyestalk-ablated, 14 days	$0.0665\pm0.009^{a,b}$	4	$0.5081\pm0.118^{a,b,c,d}$	6
Eyestalk ablated, 28 days	$0.1080\pm0.014^{c,d}$	7	$0.5861\pm0.092^{a,b,c}$	6
17β-estradiol, 7 days	$0.0950\pm0.023^{b,c,d}$	6	0.6563 ± 0.179^{d}	4
17β-estradiol, 14 days	$0.0803\pm0.018^{a,b,c}$	4	0.4381 ± 0.044^{a}	4
17β-estradiol, 28 days	$0.1008\pm0.019^{b,c,d}$	4	$0.5012\pm0.095^{a,b,c}$	4

3.6.4 Effects of progesterone on expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of domesticated *P. monodon* broodstock

Effects of progesterone injection on the expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 14-month-old *P. monodon* were examined at 12, 24, 48 and 72 hour after injection. The result showed that *PmATP/GTP-BP* was down-regulated at 72 hours post injection ($P < 0.05$; Figure 3.26A and Table 3.9).

Likewise, significant decrease in the expression of *Pm17β-HSD* was observed in shrimp injected with progesterone (0.1 µg/g BW) for 12 - 72 hours ($P < 0.05$; Figure 3.26B and Table 3.9).

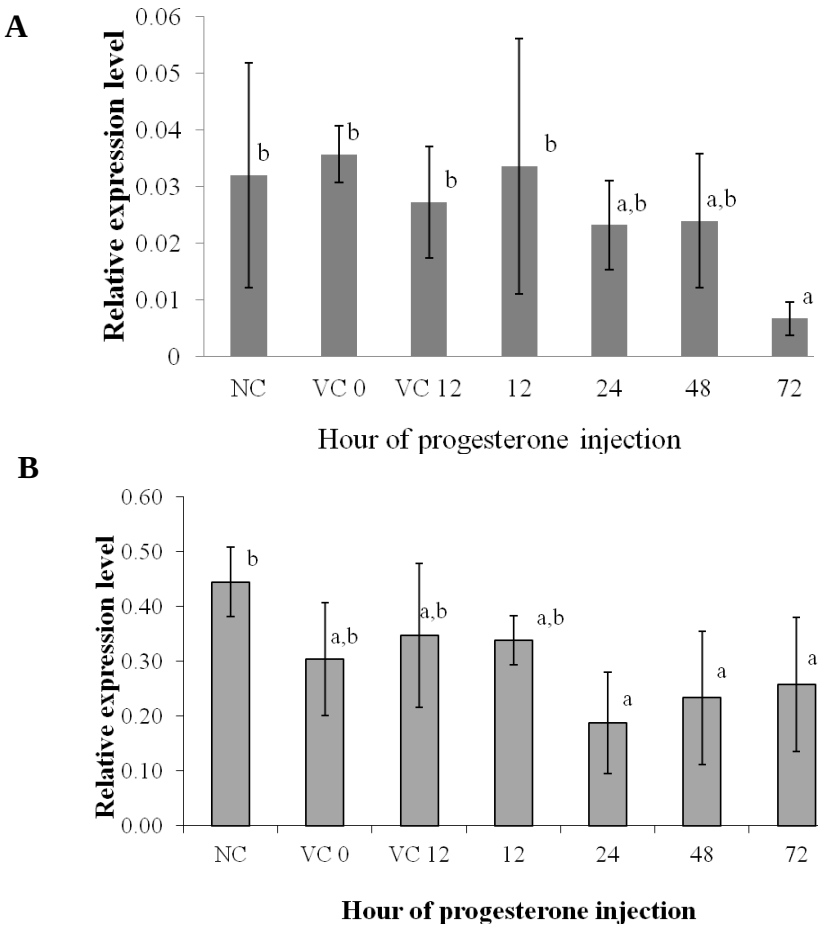


Figure 3.24 Histograms showing the relative expression profiles of *PmATP/GTP-BP* (A) and *Pm17β-HSD* (B) in ovaries of domesticated *broodstock P. monodon* after progesterone injection (0.1 µg/g BW) at 12, 24, 48 and 72 hour post injection. Expression levels were measured as the absolute copy number of *PmATP/GTP-BP* and *Pm17β-HSD* mRNA (50 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).(NC = Normal control, VC = Vehicle control.)

Table 3.9 Time course relative expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of *P. monodon* female broodstock treated with progesterone (0.1µg/g BW.).

Group	Relative expression level of *PmATP/GTP-BP*	*N*	Relative expression level of *Pm17β-HSD*	*N*
Negative control	0.0319 ± 0.020^{a}	5	0.4450 ± 0.064^{a}	4
Vehicle control, 0 hr	0.0356 ± 0.005^{a}	4	$0.3038\pm0.103^{a,b}$	3
Vehicle control, 12 hr	0.0272 ± 0.010^{a}	5	$0.3479\pm0.131^{a,b}$	5
Progesterone, 12 hr	$0.0335\pm0.023^{a,b}$	4	$0.3384\pm0.045^{a,b}$	4
Progesterone, 24 hr	$0.0232\pm0.008^{a,b}$	4	0.1875 ± 0.092^{b}	3
Progesterone, 48 hr	0.0239 ± 0.012^{b}	5	0.2333 ± 0.122^{b}	5
Progesterone, 72 hr	$0.0067\pm0.003^{c,d}$	4	0.2582 ± 0.122^{b}	5

3.6.5 Effects of serotonin on expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of domesticated *P. monodon* broodstock

Effects of serotonin on the expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 14-month-old *P. monodon* were examined. The injection of serotonin resulted in increasing of *PmATP/GTP-BP* between 6-48 hpi for approximately 20-40 folds ($P < 0.05$) before returned to the normal level at 72 hpi. The peak level was observed at 12 hpi ($P < 0.05$; Figure 25, Table 3.10).

Similar induction effects on the expression of *Pm17β-HSD* were also observed and serotonin resulted in the increase about 20-25 times during 6-48 hpt where the peak level was found at 12 hpi ($P < 0.05$, Figure 3.25 and Table 3.10).

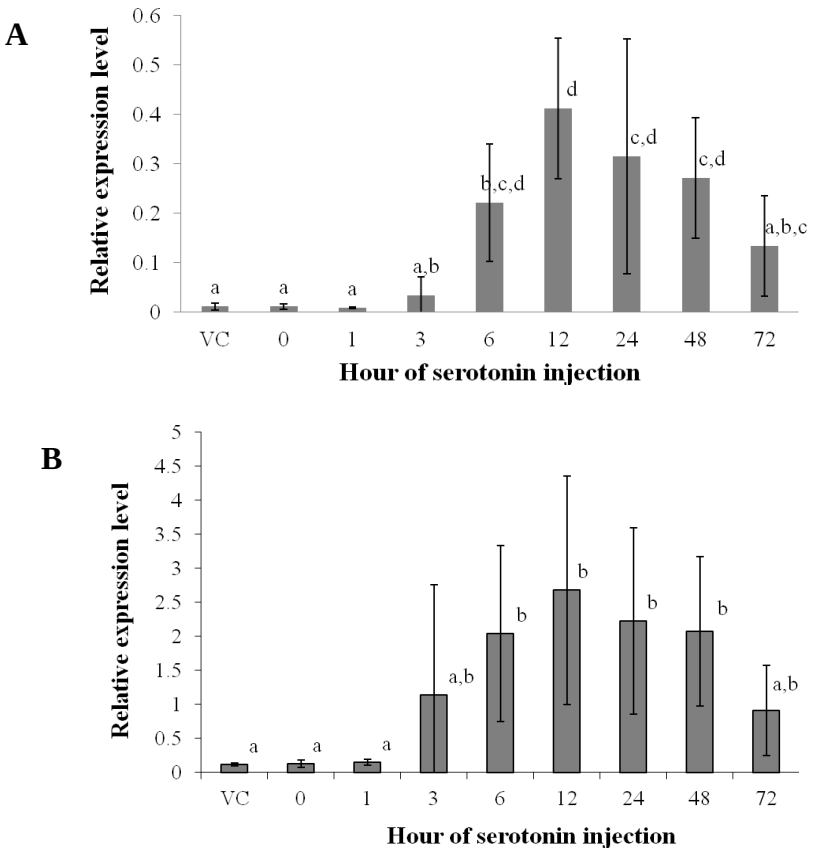


Figure 3.25 Histograms showing the relative expression profiles of *PmATP/GTP-BP* (A) and *Pm17β-HSD* (B) in ovaries of domesticated *broodstock P. monodon* after serotonin injection (50µg/g BW) for 1, 3, 6, 12, 24, 48 and 72 hours. Expression levels were measured as the absolute copy number of *Pm ATP/GTP-BP* and *Pm17β-HSD* mRNA (50 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$). (VC = Vehicle control.)

Table 3.10 Time-course relative expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of *P. monodon* female broodstock induced by serotonin (50µg/g BW).

Group	Relative expression level of *Pm ATP/GTP-BP*	*N*	Relative expression level of *Pm17BHSD*	*N*
Vehicle control	0.0114±0.007[a]	4	0.1119±0.023[a]	4
5-HT, 0 hr	0.0114±0.005[a]	4	0.1245±0.057[a]	4
5-HT, 1 hr	0.0086±0.001[a]	3	0.1485±0.045[a]	3
5-HT, 3 hr	0.0344±0.036[a,b]	3	0.3459±0.360[a,b]	3
5-HT, 6 hr	0.2206±0.119[b,c,d]	4	2.0351±1.292[b]	4
5-HT, 12 hr	0.4117±0.142[d]	4	2.6742±1.674[b]	4
5-HT, 24 hr	0.3150±0.237[c,d]	4	2.6779±1.258[b]	3
5-HT, 48 hr	0.2709±0.122[c,d]	4	2.0709±1.093[b]	4
5-HT, 48 hr	0.1335±0.101[a,b,c]	3	0.9036±0.662[a,b]	3

CHAPTER IV
DISCUSSION

The knowledge on molecular events of ovarian maturation of shrimp and the control of shrimp reproduction are limited (Benzie, 1998). Different biotechnological approaches, for example; injection of neurotransmitters and hormones and the use of specially maturation feed (Harrion, 1990) may be applied for promoting maturation and spawning of captive *P. monodon* without the irreversible side-effects of eyestalk ablation. In ovaries, transition from mitosis to meiosis is regulated by the signals from somatic cells. Oogonia differentiate into primary oocytes when they begin meiosis by replicating their DNA, and arresting in prophase of first meiosis.

During oogenesis, oocytes accumulate an extensive collection of RNAs, proteins, and organelles, such as cortical granules, yolk vesicles, ribosomes, and mitochondria (Voronima and Wessel, 2003). Knowledge on molecular mechanisms and functional involvement of proteins and reproduction-related genes in ovarian development of *P. monodon* is essential for progressive understanding the reproductive maturation of captive *P. monodon* to correct the major confinement of this species (Preechaphol *et al.*, 2007).

Isolation and characterization of the full length cDNA of genes expressed in ovaries of *P. monodon*

The full-length cDNA of *17β-hydroxysteroid dehydrogenase (Pm17β-HSD)* of *P. monodon* was characterized for the first time in penaeid shrimp. It was 1400 bp (with an ORF of 768 bp and encoding a polypeptide of 255 amino acids). *Pm17β-HSD* exhibits a relatively high degree of sequence similarity with hydroxyl-steroid (17-beta) dehydrogenase 10 of *Xenopus (Silurana) tropicalis*. The deduced *Pm17β-HSD* protein contained short-chain dehydrogenases/ reductases family (adh_short) domain which is necessary for its activity in the steroid biosynthetic pathway.

Summavielle *et al.,* 2003 examined metabolites in cultured ovarian and hepatopancreatic tissues of *M. japonicus* using radioimmunoassay (RIA). It was possible to infer the enzymatic activity of the steroid biosynthetic pathway by

detection of several metabolites. The progesterone is conversion into 17β-estradiol were reported as 17α-hydroxylase, C17-C20 lyase, *17β-HSD* and aromatase were detected in ovaries of *M. japonicus*

Localization of *Pm17β-HSD* transcripts was carried out. *In situ* hybridization signals from the *Pm17β-HSD* transcript in late previtellogenic vitellogenic oocytes.early lower than previtellogenic oocytes No localization of *Pm17β-HSD* was observed in the more mature (vitellogenic, early and late cortical rod) stages of oocytes and follicular cells. This further indicates cell-type specific expression of *Pm17β-HSD* in ovaries of *P. monodon* broodstock. Contradictory results from quantitative real-time PCR (see below) and in-situ hybridization on the disappearance of *Pm17β-HSD* hybridization signals from the ooplasm in oocytes at later stages may have been due to a significant increase in oocyte size as oogenesis proceeded.

Examination of expression patterns of genes functionally related to ovarian development of *P. monodon* by RT-PCR

Understanding how an immature oocyte transforms into an egg during oocyte maturation is critical for the knowledge of shrimp reproduction. Protein synthesis patterns change significantly during the transition from an oocyte to a mature egg. Certain mRNAs are translationally activated while others become repressed. Therefore, expression profiles of reproduction-related genes during ovarian development of *P. monodon* are the first step toward understanding sex differentiation cascades and molecular mechanisms of ovarian maturation in *P. monodon*. In addition, gene expression and tissue distribution analysis are important and provide the basic information to set up the priority for further analysis of functional genes. In this thesis, the expression profiles of *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2* in ovaries of wild broodstock of *P. monodon* were examined.

RT-PCR analysis across various tissues and testes of female and male broodstock indicated that *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2* were not especially expressed in ovaries or gonads but were observed in a variety of tissues of *P. monodon* broodstock suggesting that they should be multifunctional proteins playing the role in several physiological and biological processes.

Examination of expression levels of *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2* during ovarian development of *P. monodon*

The functional involvement of various genes during ovarian development of *P. monodon*, the expression profiles of *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2* were examined by quantitative real-time PCR.

ATP-GTP binding protein is also recognized as cleavage and polyadenylation factor I (Clp1). Clp1 Cytoplasmic polyadenylation is one of the translational regulation mechanisms for maternal mRNAs during oocyte maturation (Tremblay *et al.*, 2005). This suggests the possible involvement of *PmATP/GTP-BP* in ovarian development in *P. monodon*. *17β-hydroxysteroid dehydrogenases* (*17β-HSDs*) are enzymes involved in both the inactivation and activation of estrogens.and androgens.

PmATP/GTP-BP and *Pm17β-HSD* were not differentially expressed during ovarian development in both intact and eyestalk-ablated broodstock of wild *P. monodon*. This indicated that the steady-state of *PmATP/GTP-BP* and *Pm17β-HSD* mRNA may be adequate to maintain the translational product throughout oogenesis in non-ablated shrimp and the up-regulation of *PmATP/GTP-BP* and *Pm17β-HSD* is not necessary for maturation of *P. monodon* ovaries.

Eyestalk ablation resulted in a significant decrease of these transcripts at stages II-III ovaries. Accordingly, lower levels of *PmATP/GTP-BP* and *Pm17β-HSD* gene products may be necessary for the development and maturation of *P. monodon* oocytes. In addition, the expression level of *Pm17β-HSD* also implied that 17β-estradiol may not play the positive effects on ovarian development in *P. monodon* The findings facilitate the conceivable use of RNA interference (RNAi) for study their functional involvement in *P. monodon* ovarian development.

This study illustrated that the expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* in eyestalk-ablated broodstock of wild *P. monodon* were significantly lower than those in intact broodstock. This implied that the lower expression of these genes will result in the better ovarian development of *P. monodon*. This circumstance was in agreement of their expression profiles in intact broodstock where reduced expression levels were observed in the mature stage.

Gap-junctional communication plays an important regulatory role for oocyte maturation. In mammals, hormones targeting oocyte development exert their action on somatic cells of the follicle, which then relay the signal to the oocyte. Gap junctions appear to transmit both inhibitory and stimulatory signals between follicle cells and the oocyte (Voronima and Wessel, 2003).

In invertebrates, gap junctions are formed by proteins that belong to the innexin family (Ducret et al, 2006). Innexin 1 as in embryonic epithelia is predominantly localized to the baso-lateral domain of follicle cells, while innexin 2 is positioned apico-laterally, apically between follicle cells, oocyte and germ-line cells.

Leelatanawit et al (2008) examined the expression of *PmInx2* by RT-PCR. *PmInx2* did not reveal differential expression between ovaries and testes of *P. monodon* ($P > 0.05$). Tissue distribution analysis indicated that *PM-Inx2* was more abundantly expressed in testes than ovaries.. Therefore, *PmInx2* may play the important role in spermatogenesis but not in oogenesis. However, the functional importance of *PmInx1* has not been reported.

The expression levels of *PmInx1* and *PmPhb2* in ovaries of intact and eyestalk-ablated shrimp were significantly greater than that of juveniles and were sequentially up-regulated during ovarian development. The expression level of *PmInx1* and *PmPhb2* in stage I-IV ovaries of intact broodstock was significantly lower than that of the same stages in eyestalk-ablated broodstock ($P < 0.05$). This critically suggested that gonad inhibiting hormone (GIH) may suppress the transcription of these genes.

On the basis of expression analysis of *PmATP/GTP-BP, Pm17β-HSD, PmInx1* and *PmPhb2* in intact and eyestalk-ablated broodstock, the expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* were significantly reduced following unilateral eyestalk ablation. In contrast, eyestalk ablation resulted in significant increases of the expression levels of *PmInx1* and *PmPhb2* throughout the ovarian development of *P. monodon*. The findings facilitate the possible use of RNA interference (RNAi) for studying functional involvement of *PmATP/GTP-BP* and *Pm17β-HSD* in *P. monodon* ovarian development. Therefore, molecular effects of serotonin, progesterone and 17β-estradiol on expression levels of these genes were further examined.

Examination of expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* in ovarian of *P. monodon* following the injection of 17β-estradiol, progesterone and serotonin

Quinitio et al. (1994) analyzed the profiles of steroid hormones in relation to vitellogenesis in female *P. monodon*. 17β-estradiol was detected in the hemolymph only in shrimp with mature ovaries, while the level was low or undetectable in hemolymph of immature shrimp.

Yano and Hoshino (2006) determined the effects of 17β-estradiol in immature *M. japonicus* on induction oocyte development and vitellogenin synthesis were reconnoitered in vitro by incubation of previtellogenic ovaries of incubated with Medium 199 supplemented with 17β-estradiol. After three days incubation of the ovarian tissue, Vg concentrations in ovary incubated in media containing 3.6 nM, 36.7 nM, 367 nm and 3671 nM 17β-estradiol were significantly greater than that of the control ($P < 0.01$). The results suggested that 17β-estradiol induces appearance of primary vitellogenic oocyte and Vg synthesis in the ovaries of immature *M. japonicus*.

Exogenous injection of 17β-estradiol did not affect the significant difference in expression of *PmATP/GTP-BP*. In contrast, the injection of 17β-estradiol resulted in significant increase of *Pm17β-HSD* at 7 days after injection compared to non-injected and vehicle controls. Its expression showed a tendency decrease on 14 days after injection. Eyestalk ablation did not result in significant difference in the expression of *PmATP/GTP-BP* and *Pm17β-HSD* in domesticated shrimp at any time interval. This implied the difficulties for induction reproductive maturation of captive *P. monodon* even by the use of a conventionally effective method like eyestalk ablation. Therefore, it is proposed that 17β-estradiol injection, at least at the concentration used in this study, may induce ovarian development of *P. monodon*.

In *Metapenaeus ensis* 17α-hydroxyprogesterone and progesterone induced ovarian maturation and spawning (Yano, 1985, 1987). Estradiol and progesterone levels in the hemolymph were shown to fluctuate closely with that of the serum vitellogenin level during ovarian maturation stages of *P. monodon* (Quinitio *et al.*,

1994) The conversion of progesterone into 17β-estradiol was reported in *M. japonicus* (Summavielle *et al.*, 2003).

Effects of progesterone injection on the expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 18-month-old *P. monodon* were also examined in this study. The expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* was significantly reduced as a result of progesterone injection for 72 hpi and 12-72 hpi, respectively. Apparently, the expression profiles of these transcripts resulted from progesterone injection was similar as those from eyestalk ablation of wild broodstock. This further evidences that the reduction of *PmATP/GTP-BP* and *Pm17β-HSD* should be related to the progress of ovarian development and maturation in *P. monodon*.

Unilateral eyestalk ablation is used in practice to induce ovarian maturation in penaeid shrimp, but this technique leads to the eventual loss of egg quality and the death of spawners (Okumura, 2004). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is a long term objective of the shrimp industry (Quackenbush, 2001).

Biogenic amines (e.g., dopamine, epinephrine and serotonin or 5-HT) and peptide neuroregulators are known to modulate the release of neuropeptide hormones from the sinus gland (Sarojini *et al.*, 1995; Fingerman, 1997; Okumura, 2004). Concurrent injections of serotonin (25 μg/g BW) induced ovarian maturation and spawning in wild pond-reared *L. vannamei* and *L. stylirostris* (Alfaro *et al.*, 2004).

The effects of serotonin on reproductive performance in *P. monodon* were reported. Domesticated shrimp injected with serotonin (50 μg/g BW) exhibited ovarian maturation and spawning rates comparable to those in eyestalk-ablated shrimp. Interestingly, the hatching rate and the quantity of nauplii produced per brooder were significantly higher in the serotonin injected shrimp ($P<0.05$) (Wongpraset *et al.*, 2006). Serotonin also induced ovarian maturation and spawning in *L. vannamei* (Vaca and Alfaro, 2000). Accordingly, the effects of serotonin on the expression levels of reproduction-related genes and/or proteins in ovaries of penaeid shrimp should be carried out.

Eyestalk ablation affects an increase in the mRNA levels of *vitellogenin* and *cortical rod protein* in ovaries of *M. japonicus* (Okumura *et al.,* 2006). Likewise, the decrease in *PmATP/GTP-BP* and *Pm17β-HSD* mRNA during ovarian development in eyestalk-ablated female broodstock suggests that GIH affects the transcription of these genes (Meusy and Payen, 1988).

Similarly, exogenous injection of serotonin clearly promoted the expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of *P. monodon* at 6-48 hpt. The molecular effects of serotonin on the expression of genes implied the possible use of serotonin to induce ovarian maturation of *P. monodon* in place of eyestalk ablation.

Owing to the expression profiles of *PmATP/GTP-BP* and *Pm17β-HSD* following serotonin and progesterone injection, the synergistic effects of the neurotransmitter and sex steroid on inducing reproductive maturation of *P. monodon* may be obtained by the initial injection of serotonin (50 μg/g BW) followed by the injection of progesterone (0.1 μg/g BW).

Considering all information, *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2* likely play the main role in the ovarian development and maturation of *P. monodon*. The expression levels and localization of their protein during ovarian development and/or oogenesis should be further examined for an unambiguous conclusion on the functions of these gene products. The basic knowledge obtained allows functional characterization of *PmATP/GTP-BP*, *Pm17β-HSD*, *PmInx1* and *PmPhb2* gene products on ovarian development for better understanding of the reproductive maturation of female *P. monodon* in captivity.

CHAPTER V
CONCLUSIONS

1. The full-length cDNAs of *Pm17β-HSD* was successfully isolated and it was 1400 bp in length containing an ORF of 768 bp and deducing of 255 amino acids. The deduced *Pm17β-HSD* protein contained a short-chain dehydrogenases/reductases family domain.

2. Tissue expression analysis of *P. monodon ATP/GTP binding protein (PmATP/GTP-BP), 17β- hydroxysteroid dehydrogenase (Pm17β-HSD), Innexin 1 (PmInx1)* and *Prohibitin 2* (*PmPhb2)* was examined and these transcripts were differentially expressed among exmined testes of male and tissues of female broodstock.

3. *In situ* hybridization revealed that *Pm17β-HSD* was localized only in the cytoplasm of previtellogenic oocytes of different stages of ovaries.

4. Quantitative real-time PCR indicated that the expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* were not differentially expressed during ovarian development in both wild intact and eyestalk ablated shrimp,. In eyestalk-ablated broodstock, the expression levels of these transcripts were lower in stage II and III ovaries than those in intact broodstocks *P. monodon* ($P < 0.05$).

5. The expression profiles of *PmInx1* and *PmPhb2* were similar. These transcripts were significantly increased in stages II, III and IV ovaries in intact broodstock ($P < 0.05$) and at stages III and IV ovaries in eyestalk-ablated broodstock ($P < 0.05$). Eyestalk ablation resulted in greater expression levels of these transcripts in stage I, II, III and IV ovaries compared to those in intact broodstock ($P < 0.05$).

6. In 5, 9, 14 and 19 month-old domesticated shrimp, the expression levels of *PmATP/GTP-BP* was not different ($P > 0.05$). In contrast, the expression level of *Pm17β-HSD* in ovaries of 19 month-old domesticated broodstock was significantly higher than that in other groups of domesticated shrimp ($P < 0.05$).

7. Exogenous injection of 17β-estradiol did not affect the expression level of *PmATP/GTP-BP* but resulted in a significant increase of *Pm17β-HSD* at 7 days after

injection compare to negative and vehicle controls ($P < 0.05$) while progesterone injection resulted in a lower expression of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 14-month-old shrimp at 72 and 24-72 hpi, respectively ($P < 0.05$).

8. Exogenous injection of serotonin promoted the expression levels of *PmATP/GTP-BP* and *Pm17β-HSD* in ovaries of 18-month-old broodstock of *P. monodon* at 6-48 hpi ($P < 0.05$).

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APPENDICES

APPENDIX A

Table A1 The percentage of GSI and other data of wild *P. monodon* broodstock used in this thesis

No.	Body weight (g.)	Gonad weight (g.)	GSI (%)	Total length (cm.)	Original codes	Gonad colour	Remark
1	115.28	0.32	0.27	23.5	BUFOV03	light white	Stage I
2	76.37	0.47	0.61	23.0	BUFOV06	light white	Stage I
3	105.70	0.70	0.66	24.5	BUFOV04	light white	Stage I
4	99.96	0.77	0.77	23.5	AGYLOV03	White	Stage I
5	170.28	1.46	0.86	-	PMBF2	White	Stage I
6	112.35	1.00	0.89	23.0	BUFOV07	light yellow	Stage I
7	82.71	0.90	1.08	21.5	BUFOV05	turbid white	Stage I
8	157.33	1.73	1.10	27.0	AGYLOV01	white + light pink	Stage I
9	104.97	1.18	1.12	23.0	AGYLOV04	White	Stage I
10	120.58	1.60	1.33	23.5	AGYLOV02	White	Stage I
11	186.69	2.69	1.44	26.0	BFNOV32	Yellow	Stage I
12	188.30	2.76	1.47	-	PMBF1	light yellow	Stage I
13	218.71	4.70	2.15	28.0	BFNOV38	light green + yellow	Stage II
14	205.67	4.61	2.24	27.5	BFNOV33	light yellow	Stage II
15	128.74	2.25	2.25	24.5	ASPOV10	light yellow	Stage II
16	205.05	5.29	2.58	28.0	BFNOV35	light yellow	Stage II
17	149.64	2.68	2.68	26.7	ASPOV06	light yellow	Stage II
18	208.54	6.16	2.95	26.0	BFNOV31	light green	Stage II
19	181.30	6.00	3.31	28.5	BFNOV04/1	yellow + green	Stage II
20	159.80	6.40	4.01	27.7	BFNOV07	light green	Stage III
21	187.10	8.27	4.42	30.0	BFNOV18	green + yellow	Stage III
22	173.40	8.00	4.61	28.0	BFNOV03	Green	Stage III
23	164.50	7.60	4.64	27.5	BFNOV04	Green	Stage III
24	230.93	12.12	5.28	32.0	BFNOV23	green + yellow	Stage III
25	235.98	12.69	5.37	33.0	BFNOV24	Green	Stage III
26	172.30	9.90	5.75	28.0	BFNOV05	Green	Stage III
27	172.60	10.20	5.91	28.0	BFNOV01	Green	Stage III
28	136.40	8.40	6.16	26.5	BFNOV09	dark green	Stage IV
29	133.20	8.30	6.23	26.0	BFNOV08	Green	Stage IV
30	176.20	12.90	7.32	29.0	BFNOV06	dark green	Stage IV
31	272.20	20.30	7.46	32.0	BFNOV02	Green	Stage IV
32	152.20	12.80	8.42	27.0	BFNOV14	Green	Stage IV
33	139.90	13.10	9.36	25.5	BFNOV10	dark green	Stage IV
34	162.20	16.20	9.99	28.0	BFNOV12	dark green	Stage IV
35	166.90	16.70	10.01	27.5	BFNOV11	light green	Stage IV
36	239.86	24.98	10.41	33.0	BFNOV21	Green	Stage IV
37	207.40	23.20	11.19	30.5	BFNOV15	dark green	Stage IV
38	232.57	26.08	11.21	30.0	BFNOV20	Green	Stage IV
39	156.10	18.20	11.66	27.0	BFNOV16	dark green	Stage IV
40	252.11	31.30	12.41	30.0	BFNOV17	Green	Stage IV
41	158.60	19.90	12.55	27.5	BFNOV13	dark green	Stage IV
42	300.12	10.47	3.49	32.5	BFNOV30	light green + yellow	post-spawn

Table A1 (cont.)

No.	Body weight (g.)	Gonad weight (g.)	GSI (%)	Total length (cm.)	Original codes	Gonad colour	Remark
43	194.49	3.61	1.86	27.5	BFNOV36	light yellow + light green	post-spawn
44	256.40	8.39	3.27	29.5	BFNOV34	Light yellow	post-spawn
45	264.70	7.66	2.89	30.0	BFNOV37	Light yellow	post-spawn
46	285.97	8.30	2.90	32.0	BFNOV39	Yellow	post-spawn
47	200.79	5.18	2.58	28.0	BFNOV40	Light yellow	post-spawn
48	236.51	1.79	0.76	27.50	BFEAOV18	White	Eyestalk-ablated; Stg I
49	111.00	1.00	0.90	24.50	YLBOV01	White + light yellow	Eyestalk-ablated; Stg I
50	163.00	2.00	1.22	25.00	YLBOV06	White	Eyestalk-ablated; Stg I
51	272.20	3.71	1.36	30.00	BFEAOV15	Yellow	Eyestalk-ablated; Stg I
52	125.00	2.00	1.60	24.50	YLBOV05	White + light yellow	Eyestalk-ablated; Stg II
53	118.00	2.00	1.69	24.50	YLBOV08	White	Eyestalk-ablated; StgII
54	173.37	4.72	2.72	25.50	BFEAOV19	Light green + yellow	Eyestalk-ablated;Stg II
55	252.03	7.16	2.84	29.50	BFEAOV17	Green + yellow	Eyestalk-ablated;Stg II
56	151.00	5.00	3.31	25.00	YLBOV07	Light green	Eyestalk-ablated;Stg II
57	291.39	10.31	3.54	30.50	BFEAOV16	Green	Eyestalk-ablated;Stg II
58	164.00	6.00	3.66	26.00	YLBOV04	Light green	Eyestalk-ablated;Stg II
59	193.65	8.82	4.55	27.00	BFEAOV21	Dark green	Eyestalk-ablated;Stg III
60	153.00	7.00	4.57	25.50	YLBOV02	Light green	Eyestalk-ablated;Stg III
61	125.00	6.00	4.80	25.00	YLBOV03	Light green	Eyestalk-ablated;Stg III
62	118.80	5.90	4.97	24.50	BFEAOV08	Green	Eyestalk-ablated;Stg III; BIOTEC shrimp
63	186.50	9.40	5.04	27.50	BFEAOV05	Light green	Eyestalk-ablated;Stg III
64	196.90	10.00	5.08	29.50	BFEAOV02	Light green + yellow	Eyestalk-ablated;Stg III
65	96.20	4.90	5.09	23.30	BFEAOV11	Green	Eyestalk-ablated;Stg III; BIOTEC shrimp
66	182.70	9.40	5.15	28.00	BFEAOV03	Yellow + little green	Eyestalk-ablated;Stg III
67	278.23	14.37	5.16	29.50	BFEAOV20	Dark green + little yellow	Eyestalk-ablated;Stg III
68	197.40	10.80	5.47	29.50	BFEAOV04	Light green + yellow	Eyestalk-ablated;Stg III
69	229.60	14.60	6.36	30.00	BFEAOV01	Green + yellow	Eyestalk-ablated; Stg IV
70	220.10	14.00	6.36	28.50	BFEAOV07	Light green + yellow	Eyestalk-ablated; Stg IV
71	170.20	11.60	6.82	27.00	BFEAOV06	Light green + yellow	Eyestalk-ablated; Stg IV
72	365.38	25.08	6.86	33.00	BFEAOV24	Green	Eyestalk-ablated; Stg IV
73	116.40	8.00	6.87	25.50	BFEAOV10	Dark green	Eyestalk-ablated; Stg IV; BIOTEC shrimp
74	128.70	8.90	6.92	25.20	BFEAOV12	Dark green	Eyestalk-ablated; Stg IV; BIOTEC shrimp

Table A1 (cont.)

No.	Body weight (g.)	Gonad weight (g.)	GSI (%)	Total length (cm.)	Original codes	Gonad colour	Remark
75	188.20	13.80	7.35	27.50	BFEAOV13	Dark green	Eyestalk-ablated; Stg IV
76	167.54	14.33	8.55	25.00	BFEAOV22	Dark green	Eyestalk-ablated; Stg IV
77	256.34	22.41	8.74	29.50	BFEAOV23	Dark green	Eyestalk-ablated; Stg IV
78	249.50	22.30	8.94	31.50	BFEAOV09	Light green	Eyestalk-ablated; Stg IV

APPENDIX B

Table B1 Relative expression levels of *PmATP/GTP* in different ovarian developmental stages of *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups		Mean conc.		Ratio
		PmATP/GTP	***EF-1α***	**(target / EF-1α)**
Juveniles	JNOV05	1.98E+04	3.21E+05	6.16E-02
	JNOV06	4.50E+04	4.40E+05	1.02E-01
	JNOV07	4.63E+04	4.44E+05	1.04E-01
N-BD-Stage I	BUFOV03	5.28E+04	1.24E+06	4.26E-02
	BUFOV04	4.87E+04	1.03E+06	4.75E-02
	AGYLOV03	4.69E+02	9.69E+05	4.84E-04
	BUFOV07	2.32E+04	2.21E+05	1.05E-01
	BUFOV05	1.88E+04	2.38E+05	7.87E-02
	AGYLOV01	7.24E+04	4.06E+05	1.79E-01
	AGYLOV04	3.32E+02	1.22E+06	2.72E-04
	AGYLOV02	6.42E+04	1.00E+06	6.41E-02
N-BD-Stage II	ASPOV10	6.98E+04	6.66E+05	1.05E-01
	ASPOV06	7.78E+04	8.64E+05	9.01E-02
	BFNOV35	3.93E+04	2.75E+05	1.43E-01
	BFNOV31	4.66E+04	4.82E+05	9.68E-02
	BFNOV4/1	1.92E+04	2.99E+05	6.43E-02
N-BD-Stage III	BFNOV18	1.27E+04	1.16E+05	1.09E-01
	BENOV03	2.72E+04	2.62E+05	1.04E-01
	BFNOV04	2.14E+04	2.28E+05	9.39E-02
	BFNOV23	2.49E+04	3.82E+05	6.50E-02
	BFNOV24	1.42E+04	1.49E+05	9.52E-02
	BFNOV05	2.41E+04	2.03E+05	1.19E-01
	BFNOV01	1.45E+04	1.63E+05	8.87E-02
N-BD-Stage IV	BFNOV14	1.23E+04	1.75E+05	7.05E-02
	BFNOV10	1.25E+04	2.30E+05	5.46E-02
	BFNOV12	1.32E+04	1.63E+05	8.11E-02
	BFNOV21	1.08E+04	1.57E+05	6.84E-02
	BFNOV15	1.09E+04	1.35E+05	8.07E-02
	BFNOV16	7.75E+03	1.12E+05	6.95E-02
N-BD-Stage PS	BFNOV17	8.17E+03	3.07E+04	2.66E-01
	BFNOV30	2.67E+04	3.86E+05	6.92E-02
	BFNOV36	2.10E+04	3.75E+05	5.59E-02
	BFNOV34	2.72E+04	1.91E+05	1.42E-01
	BFNOV39	2.72E+04	2.57E+05	1.06E-01

Table B1 (cont.)

Sample Groups		Mean conc.		Ratio
		PmATP/GTP	***EF-1α***	**(target/*EF-1α*)**
EA-BD-Stage I	BFEAOV18	2.71E+04	5.55E+05	4.88E-02
	YLBOV01	9.48E+04	2.12E+06	4.47E-02
	YLBOV06	2.18E+04	4.40E+05	4.94E-02
EA-BD-Stage II	BFEAOV15	3.06E+04	5.28E+05	5.81E-02
	YLBOV08	6.39E+04	1.08E+06	5.93E-02
	BFEAOV17	2.08E+04	4.55E+05	4.58E-02
	YLBOV07	1.85E+04	4.01E+05	4.61E-02
	YLBOV04	3.22E+04	8.38E+05	3.85E-02
EA-BD-Stage III	BFEAOV21	3.06E+04	6.21E+05	4.92E-02
	BFEAOV05	4.01E+04	9.04E+05	4.43E-02
	BFEAOV02	2.53E+04	4.46E+05	5.67E-02
	BFEAOV11	2.19E+04	4.98E+05	4.41E-02
	BFEAOV20	1.30E+04	2.33E+05	5.55E-02
	BFEAOV04	2.16E+04	4.57E+05	4.72E-02
EA-BD-Stage IV	BFEAOV07	3.17E+04	7.70E+05	4.11E-02
	BFEAOV06	1.41E+04	3.25E+05	4.35E-02
	BFEAOV24	2.22E+04	3.68E+05	6.03E-02
	BFEAOV10	2.72E+04	5.21E+05	5.23E-02
	BFEAOV13	3.30E+04	6.50E+05	5.07E-02
	BFEAOV22	1.22E+04	1.82E+05	6.69E-02
	BFEAOV14	4.87E+03	1.13E+05	4.31E-02

Table B2 Relative expression levels of *Pm17β-HSD* in different ovarian developmental stages of *P. monodon* based on quantitative real-time PCR analysis

Sample Groups		Mean conc.		Ratio
		Pm17β-HSD	***EF-1α***	**(target/*EF-1α*)**
Juveniles	JNOV05	2.79E+03	3.21E+05	8.71E-03
	JNOV06	4.01E+03	4.40E+05	9.11E-03
	JNOV07	4.86E+03	4.44E+05	1.09E-02
N-BD-Stage I	BUFOV06	4.39E+03	1.67E+05	2.63E-02
	BUFOV04	2.44E+04	1.03E+06	2.38E-02
	AGYLOV01	1.34E+04	4.06E+05	3.30E-02
	AGYLOV04	1.89E+04	1.22E+06	1.55E-02
N-BD-Stage II	ASPOV10	1.70E+04	6.66E+05	2.56E-02
	ASPOV06	2.10E+04	8.64E+05	2.43E-02
	BFNOV38	1.58E+04	5.70E+05	2.77E-02
	BFNOV33	7.15E+03	1.85E+05	3.88E-02
	BFNOV35	5.67E+03	2.75E+05	2.06E-02
	BFNOV31	1.16E+04	4.82E+05	2.40E-02
N-BD-Stage III	BFNOV18	7.00E+03	1.16E+05	6.02E-02
	BFNOV24	3.73E+03	1.49E+05	2.51E-02
	BFNOV01	4.72E+03	1.63E+05	2.88E-02
N-BD-Stage IV	BFNOV14	3.00E+03	1.75E+05	1.71E-02
	BFNOV21	5.55E+03	1.57E+05	3.53E-02
	BFNOV15	1.91E+03	1.35E+05	1.41E-02
	BFNOV20	1.81E+03	1.04E+05	1.75E-02
	BFNOV16	2.80E+03	1.12E+05	2.51E-02
N-BD-Stage PS	BFNOV34	1.31E+04	1.91E+05	6.87E-02
	BFNOV37	8.83E+03	5.00E+04	1.77E-01
	BFNOV39	1.61E+04	2.57E+05	6.27E-02
	BFNOV40	1.25E+04	2.66E+05	4.70E-02
EA-BD-Stage I	BFEAOV18	7.86E+03	5.55E+05	1.42E-02
	YLBOV01	2.39E+04	2.12E+06	1.13E-02
	YLBOV06	6.41E+03	4.40E+05	1.45E-02
EA-BD-Stage II	BFEAOV15	7.61E+03	5.28E+05	1.44E-02
	YLBOV08	1.15E+04	1.08E+06	1.07E-02
	YLBOV07	4.92E+03	4.01E+05	1.23E-02
	BFEAOV16	6.30E+03	3.92E+05	1.61E-02
	YLBOV04	1.40E+04	8.38E+05	1.67E-02

Table B2 (cont.)

Sample Groups		Mean conc.		Ratio
		PmATP/GTP	***EF-1α***	**(target/*EF-1α*)**
EA-BD-Stage III	BFEAOV21	6.31E+03	6.21E+05	1.02E-02
	BFEAOV08	9.56E+03	7.40E+05	1.29E-02
	BFEAOV02	5.61E+03	4.46E+05	1.26E-02
	BFEAOV20	3.78E+03	2.33E+05	1.62E-02
	BFEAOV04	8.36E+03	4.57E+05	1.83E-02
EA-BD-Stage IV	BFEAOV07	8.65E+03	7.70E+05	1.12E-02
	BFEAOV06	4.83E+03	3.25E+05	1.49E-02
	BFEAOV24	5.46E+03	3.68E+05	1.48E-02
	BFEAOV10	6.80E+03	5.21E+05	1.31E-02
	BFEAOV12	5.54E+03	3.47E+05	1.60E-02
	BFEAOV13	1.03E+04	6.50E+05	1.58E-02

Table B3 Relative expression levels of *PmInx1* in different ovarian developmental stages of *P. monodon* based on quantitative real-time PCR analysis

Sample Groups		**Mean conc.**		**Ratio**
		PmInx1	***EF-1α***	**(target/*EF-1α*)**
Juveniles	JNOV4	5.68E+04	2.26E+06	2.52E-02
	JNOV5	2.83E+04	1.26E+06	2.24E-02
	JNOV6	4.18E+04	2.02E+06	2.07E-02
	JNOV7	9.49E+04	2.38E+06	3.99E-02
	JNOV14	3.39E+04	2.43E+06	1.39E-02
N-BD-Stage I	BU14OV8	1.83E+05	2.56E+06	7.15E-02
	BU14OV15	1.02E+05	1.94E+06	5.25E-02
	BU14OV18	1.46E+05	2.11E+06	6.90E-02
	BFNOV22	1.28E+05	1.57E+06	8.18E-02
N-BD-Stage II	BFNOV25	8.91E+04	9.90E+05	9.00E-02
	BFNOV31	1.47E+05	1.75E+06	8.40E-02
	BFNOV33	1.19E+05	1.41E+06	8.39E-02
N-BD-Stage III	BFNOV1	4.78E+04	5.43E+05	8.81E-02
	BFNOV7	4.82E+04	5.51E+05	8.75E-02
	BFNOV23	8.20E+04	9.21E+05	8.90E-02
	BFNOV24	7.69E+04	8.48E+05	9.07E-02
N-BD-Stage IV	BFNOV10	2.55E+04	2.21E+05	1.15E-01
	BFNOV11	3.79E+04	2.80E+05	1.35E-01
	BFNOV14	3.60E+04	3.46E+05	1.04E-01
	BFNOV15	2.79E+04	2.59E+05	1.08E-01
	BFNOV16	2.99E+04	3.17E+05	9.44E-02
	BFNOV17	3.47E+04	2.56E+05	1.35E-01
	BFNOV20	3.38E+04	2.68E+05	1.26E-01
N-BD-Stage PS	BFNOV30	1.31E+05	1.86E+06	7.04E-02
	BFNOV34	1.65E+05	2.39E+06	6.91E-02
	BFNOV39	1.95E+05	2.33E+06	8.37E-02
	BFNOV40	1.13E+05	1.41E+06	8.04E-02
EA-BD-Stage I	WFEA4	8.86E+04	3.14E+05	2.82E-01
	WFEA3	8.90E+04	3.40E+05	2.62E-01
	WFEA33	4.94E+04	1.45E+05	3.40E-01

Table B3 (cont.)

Sample Groups		Mean conc.		Ratio
		PmInx1	***EF-1α***	**(target/*EF-1α*)**
EA-BD-Stage II	WFEA27U	1.04E+05	3.21E+05	3.24E-01
	WFEA27L	1.31E+05	4.49E+05	2.92E-01
	WFEA6	7.06E+04	2.41E+05	2.93E-01
	WFEA1	7.38E+04	2.54E+05	2.91E-01
	WFEA21	1.20E+05	3.44E+05	3.48E-01
	WFEA29	5.68E+04	2.10E+05	2.71E-01
	WFEA20	9.38E+04	2.57E+05	3.65E-01
	WFEA24	7.42E+04	2.20E+05	3.37E-01
	WFEA30U	6.20E+04	1.80E+05	3.44E-01
	WFEA30L	4.83E+04	1.73E+05	2.79E-01
EA-BD-Stage III	WFEA18	6.89E+04	1.83E+05	3.76E-01
	WFEA19	8.97E+04	2.35E+05	3.81E-01
	WFEA28	7.06E+04	2.02E+05	3.50E-01
	WFEA26	6.89E+04	1.93E+05	3.57E-01
	WFEA10	7.42E+04	2.12E+05	3.50E-01
	WFEA32U	3.89E+04	1.15E+05	3.38E-01
	WFEA32L	3.04E+04	8.49E+04	3.57E-01
	WFEA25	5.88E+04	1.78E+05	3.30E-01
	WFEA17	5.76E+04	1.64E+05	3.51E-01
	WFEA31U	3.94E+04	1.28E+05	3.09E-01
	WFEA31L	2.75E+04	7.93E+04	3.46E-01
EA-BD-Stage IV	WFEA13	3.44E+04	9.77E+04	3.52E-01
	WFEA11	4.29E+04	1.30E+05	3.29E-01
	WFEA14	4.34E+04	1.10E+05	3.93E-01
	WFEA16	5.51E+04	1.56E+05	3.53E-01
	WFEA15	2.81E+04	8.01E+04	3.51E-01

Table B4 Relative expression levels of *PmPhb2* in different ovarian developmental stages of *P. monodon* based on quantitative real-time PCR analysis

Sample Groups		Mean conc.		Ratio
		PmPhb2	***EF-1α***	**(target/*EF-1α*)**
Juvenile	JNOV4	4.45E+04	2.26E+06	1.97E-02
	JNOV5	2.31E+04	1.26E+06	1.83E-02
	JNOV6	3.98E+04	2.02E+06	1.97E-02
	JNOV7	5.94E+04	2.38E+06	2.50E-02
	JNOV12	5.36E+03	1.92E+05	2.79E-02
	JNOV14	6.13E+04	2.43E+06	2.52E-02
N-BD-Stage I	BU14OV8	7.11E+04	2.56E+06	2.77E-02
	BU14OV15	4.49E+04	1.94E+06	2.31E-02
	BU14OV18	6.32E+04	2.11E+06	2.99E-02
	BFNOV22	5.68E+04	1.57E+06	3.63E-02
N-BD-Stage II	BFNOV25	3.60E+04	9.90E+05	3.64E-02
	BFNOV31	6.95E+04	1.75E+06	3.97E-02
	BFNOV33	5.09E+04	1.41E+06	3.60E-02
N-BD-Stage III	BFNOV7	2.10E+04	5.51E+05	3.80E-02
	BFNOV18	3.49E+04	9.68E+05	3.61E-02
	BFNOV23	3.58E+04	9.21E+05	3.89E-02
	BFNOV24	3.34E+04	8.48E+05	3.94E-02
N-BD-Stage IV	BFNOV10	8.46E+03	2.21E+05	3.83E-02
	BFNOV11	1.27E+04	2.80E+05	4.52E-02
	BFNOV14	1.61E+04	3.46E+05	4.65E-02
	BFNOV15	1.04E+04	2.59E+05	4.02E-02
	BFNOV16	1.13E+04	3.17E+05	3.57E-02
	BFNOV17	1.13E+04	2.56E+05	4.42E-02
	BFNOV20	1.16E+04	2.68E+05	4.31E-02
N-BD-Stage PS	BFNOV30	6.76E+04	1.86E+06	3.63E-02
	BFNOV34	1.03E+05	2.39E+06	4.31E-02
	BFNOV37	7.98E+04	1.97E+06	4.06E-02
	BFNOV39	8.04E+04	2.33E+06	3.45E-02
	BFNOV40	6.27E+04	1.41E+06	4.46E-02

Table B4 (cont.)

Sample Groups		Mean conc.		Ratio
		PmPhb2	***EF-1α***	**(target/*EF-1α*)**
EA-BD-Stage I	WFEA4	2.98E+04	3.14E+05	9.51E-02
	WFEA3	3.05E+04	3.40E+05	8.98E-02
	WFEA33	1.41E+04	1.45E+05	9.69E-02
EA-BD-Stage II	WFEA27U	3.46E+04	3.21E+05	1.08E-01
	WFEA22	4.68E+04	4.45E+05	1.05E-01
	WFEA6	2.30E+04	2.41E+05	9.55E-02
	WFEA2	3.92E+04	3.89E+05	1.01E-01
	WFEA1	2.95E+04	2.54E+05	1.16E-01
	WFEA21	4.26E+04	3.44E+05	1.24E-01
	WFEA29	1.94E+04	2.10E+05	9.25E-02
	WFEA20	3.20E+04	2.57E+05	1.25E-01
	WFEA24	2.32E+04	2.20E+05	1.05E-01
	WFEA30U	2.18E+04	1.80E+05	1.21E-01
	WFEA30L	1.81E+04	1.73E+05	1.05E-01
EA-BD-Stage III	WFEA18	2.25E+04	1.83E+05	1.23E-01
	WFEA19	3.00E+04	2.35E+05	1.27E-01
	WFEA28	2.30E+04	2.02E+05	1.14E-01
	WFEA26	2.21E+04	1.93E+05	1.14E-01
	WFEA32U	1.44E+04	1.15E+05	1.25E-01
	WFEA32L	1.04E+04	8.49E+04	1.23E-01
	WFEA25	1.90E+04	1.78E+05	1.07E-01
	WFEA17	1.82E+04	1.64E+05	1.11E-01
	WFEA31U	1.37E+04	1.28E+05	1.08E-01
	WFEA31L	1.00E+04	7.93E+04	1.26E-01
	WFEA12	2.06E+04	1.47E+05	1.40E-01
EA-BD-Stage IV	WFEA13	1.69E+04	9.77E+04	1.73E-01
	WFEA11	1.40E+04	1.30E+05	1.07E-01
	WFEA14	1.20E+04	1.10E+05	1.08E-01
	WFEA16	1.93E+04	1.56E+05	1.24E-01
	WFEA15	9.19E+03	8.01E+04	1.15E-01

BIOGRAPHY

Miss Sripapan Treejate was born on May 13, 1983 in Phra Nakhon Si Ayutthaya. She graduated with the degree of Bachelor of Science from the Faculty of Marine Technology, Burapha University, Chanthaburi Campus in 2004. She has studied for the Degree of Master of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University since 2009.

Publication related with this thesis

Treejate S, Sittikhankeaw K, Hiransuchalert R, Yuvanatemiya V, Klinbunga S and Jarayabhand P., 2012. Molecular cloning and expression analysis of the *ATP/GTP binding protein* gene in the giant tiger shrimp *Penaeus monodon*. The 23nd Annual Meeting of the Thai Society for Biotechnology "International Conference on Systems Biotechnology: Quality and Success", 1-2 February, Bangkok, Thailand (oral presentation).