การโคลนและวิเคราะห์การแสดงออกของจีนในวิถีโปรตีนจีในกุ้งกุลาดำ *Penaeus monodon*

นางสาวพัชรี โยควิบูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLONING AND EXPRESSION ANALYSIS OF GENES IN THE G PROTEIN PATHWAY IN THE BLACK TIGER SHRIMP *Penaeus monodon*

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พัชรี โยควิบูล : การโคลนและวิเคราะห์การแสดงออกของ จีนในวิถีโปรตีนจีในกุ้งกุลาดำ *Penaeus monodon*. (CLONING AND EXPRESSION ANALYSIS OF GENES IN THE G PROTEIN PATHWAY IN THE BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร. เปี่ยมศักดิ์ เมนะเศวต, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. ศิราวุธ กลิ่นบุหงา, 153 หน้า.

การพิสูจน์เอกลักษณ์และลักษณะสมบัติของจีนและโปรตีนที่เกี่ยวข้องกับวิถีการส่งสัญญาณระหว่างการพัฒนารังไข่ มีความสำคัญต่อความเข้าใจของกลไกระดับโมเลกุลของการสมบูรณ์พันธุ์ของกุ้งกุลาดำ จึงหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของจีนที่เกียวข้องกับวิถีการส่งสัญญาณดังกล่าวของกุ้งกุลาดำ ประกอบด้วย *P. monodon GTP binding protein alpha subunit* G_o *(*$PmG_{\alpha o}$*), G protein gamma subunit (*PmG_{γ}*), calcitonin gene-related peptide-receptor component protein-like (PmCGRP-RCP)* and *downstream of receptor kinase (PmDrk)* พบว่ามี ORF ขนาด 1065, 204, 417 และ 672 คู่เบส ตามลำดับ นอกจากนี้ยังพบลำดับนิวคลีโอไทด์บางส่วนของจีน *GTP binding protein alpha subunit* G_q *(*$PmG_{\alpha q}$*), GTP binding protein alpha subunit* G_s *(*$PmG_{\alpha s}$*)* และ*G protein beta 1 subunit (*PmG_{β}*)*

ตรวจสอบการแสดงออกของจีนต่างๆ ในรังไข่ของแม่พันธุ์กุ้งจากธรรมชาติด้วยวิธี quantitative real-time PCR พบว่า $PmG_{\alpha q}$, $PmG_{\alpha s}$ และ *PmCGRP-RCP* มีระดับการแสดงออกเพิ่มขึ้นระหว่างการพัฒนารังไข่ของกุ้งปกติที่ไม่ตัดก้านตา ($P < 0.05$) ในขณะที่ *PmDrk* และ *PmSelM* มีระดับการแสดงออกระหว่างการพัฒนารังไข่ที่ไม่แตกต่างกันทางสถิติ ($P > 0.05$) อย่างไรก็ตามการตัดตาไม่ส่งผลต่อระดับการแสดงออกของจีน *PmDrk* ($P > 0.05$) แต่ส่งผลให้ $PmG_{\alpha q}$, $PmG_{\alpha s}$ และ *PmCGRP-RCP* มีการแสดงออกลดลงระหว่างการพัฒนารังไข่อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ซึ่งแตกต่างจาก *PmSelM* ในกุ้งที่ตัดก้านตามีการแสดงออกสูงขึ้นระหว่างการพัฒนารังไข่เมื่อเทียบกับกุ้งปกติที่ไม่ตัดก้านตาอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ผลการศึกษาบ่งชี้ว่า $PmG_{\alpha q}$, $PmG_{\alpha s}$, *PmCGRP-RCP* และ *PmSelM* มีหน้าที่สำคัญต่อการเจริญพันธุ์ โดยสามารถใช้จีนดังกล่าวเป็นเครื่องหมายโมเลกุลเพื่อตรวจสอบความสมบูรณ์พันธุ์ของแม่พันธุ์กุ้งกุลาดำ

ทำการฉีดกระตุ้นกุ้งกุลาดำที่ทำการเลี้ยงด้วยฮอร์โมนโปรเจสเตอโรน 17β-estradiol และ serotonin (5-HT) และตรวจสอบการแสดงออกของจีนด้วยวิธี quantitative real-time PCR พบว่า 5-HT (50 µg/g น้ำหนักตัว) ซึ่งเป็น neurotransmitter ที่มีรายงานว่าสามารถกระตุ้นการพัฒนารังไข่ของกุ้งกุลาดำได้ ส่งผลต่อรูปแบบการแสดงออกของจีน *PmCGRP-RCP* โดยมีระดับการแสดงออกเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติที่ 6-48 ชั่วโมงหลังการฉีดกระตุ้น ($P < 0.05$) ส่วนโปรเจสเตอโรน ส่งผลต่อการแสดงออกของ $PmG_{\alpha s}$ โดยทำให้มีการแสดงออกของจีนที่ลดลงในชั่วโมงที่ 24 หลังจากฉีดกระตุ้น ($P < 0.05$) แต่ไม่มีผลต่อการแสดงออกของ *PmDrk* ($P > 0.05$) ในขณะที่การฉีด 17β-estradiol ให้ผลกระทบในทางตรงกันข้าม โดยการฉีดสารดังกล่าวส่งผลให้ระดับการแสดงออกของ *PmDrk* ต่ำลงหลังจากการฉีดกระตุ้นเป็นเวลา 7 และ 14 วัน ($P < 0.05$) แต่ไม่ส่งผลต่อระดับการแสดงออกของ $PmG_{\alpha s}$ ในรังไข่ของกุ้งแม่พันธุ์ในภาวะเพาะเลี้ยง กระตุ้น ($P > 0.05$)

จากการศึกษาจีนที่เกี่ยวข้องกับวิถีการส่งสัญญาณระหว่างการพัฒนารังไข่ของกุ้งกุลาดำ บ่งชี้ว่าจีนดังกล่าวมีหน้าที่เกี่ยวข้องกับการพัฒนารังไข่ของกุ้งกุลำดำ ซึ่งสามารถนำความรู้ความเข้าใจที่ได้ไปช่วยควบคุมความสมบูรณ์พันธุ์ของกุ้งกุลาดำในภาวะเพาะเลี้ยงต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS *Penaeus monodon* / BLACK TIGER SHRIMP / OVARIAN MATURATION / G PROTEIN / SIGNAL TRANSDUCTION PATHWAY

PATCHARI YOCAWIBUN : CLONING AND EXPRESSION ANALYSIS OF GENES IN THE G PROTEIN PATHWAY IN THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR : PROF. PIAMSAK MENASVETA, Ph.D., CO-ADVISOR : SIRAWUT KLINBUNGA, Ph.D., 153 pp.

Identification and characterization of genes/proteins functionally involved in the signal transduction pathway during ovarian development in the black tiger shrimp (*Penaeus monodon*) are important for understanding molecular mechanisms of ovarian maturation in this economically important species. The full-length cDNA of *P. monodon GTP binding protein alpha subunit* G_o *(*$PmG_{\alpha o}$*), G protein gamma subunit (*PmG_{γ}*), calcitonin gene-related peptide-receptor component protein-like (PmCGRP-RCP)* and *downstream of receptor kinase (PmDrk)* were characterized. They were 2407, 514, 1182 and 1222 bp in length containing the ORFs of 1065, 204, 417 and 672 bp corresponding to the polypeptides of 354, 67, 138 and 223 amino acids, respectively. In addition, the partial cDNAs of *GTP binding protein alpha subunit* G_q *(*$PmG_{\alpha q}$*), GTP binding protein alpha subunit* G_s *(*$PmG_{\alpha s}$*), G protein beta 1 subunit (*PmG_{β}*)* were also isolated.

Quantitative real-time PCR indicated that the expression level of $PmG_{\alpha q}$, $PmG_{\alpha s}$, *PmCGRP-RCP* and *PmSelM* were significantly increased during ovarian development in wild intact broodstock ($P > 0.05$). Eyestalk ablation resulted in significant reduction of $PmG_{\alpha q}$, $PmG_{\alpha s}$ and *PmCGRP-RCP* during ovarian development of wild *P. monodon* ($P < 0.05$). In contrast, the expression level of *PmSelM* in each ovarian developmental stage of eyestalk-ablated broodstock was greater than that of the same ovarian stages in intact broodstock. Results indicated that these genes play the important role during development and maturation of *P. monodon* ovaries. Their expression profiles may be used as bioindicators for monitoring the progression of oocyte maturation in this species.

Effects of exogenous administration of serotonin (5-HT, 50 µg/g body weight), progesterone (0.1 µg/g body weight) and 17β-estradiol (0.01 µg/g body weight) on expression levels of genes functioned in oocyte signal transduction pathways were examined by quantitative real-time PCR. Results indicated that serotonin (5-HT) administration promoted the expression level of *PmCGRP-RCP* in ovaries of 18-month-old shrimp at 6-48 hours post injection (hpi, $P < 0.05$). Progesterone (P4) resulted in the reduction of $PmG_{\alpha s}$ expression at 24 hpi ($P < 0.05$) but had no effect on the expression level of *PmDrk* ($P > 0.05$). In contrast, 17β-estradiol injection had no effect on the expression of the former ($P > 0.05$) but resulted in a significant decrease of the expression level of the latter at 7 and 28 days post injection ($P < 0.05$).

Knowledge on the expression profiles of reproduction-related genes and molecular mechanisms of steroid hormone and neurotransmitter induction on oocyte development may lead to the possible ways to effectively induce ovarian maturation in captive shrimp in the future.

Field of Study : Biotechnology Student's Signature……

Academic Year : 2011 Advisor's Signature……

Co-advisor's Signature……...…...

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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
kD	kilodalton
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
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, *P. monodon* has dominated production of farmed shrimp along with the Pacific white shrimp (*Litopenaeus vannamei*) and is one of the most economically important penaeid species in South East Asia (Rosenberry, 2003). Reduced reproductive maturation of captive *P. monodon* females is found (Kenway et al., 2006; Preechaphol et al., 2007). Accordingly, breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality of larvae required by the industry. In Thailand, farming of *P. monodon* in Thailand relies almost entirely on wild-caught broodstock for supply of juveniles (Withyachumnarnkul et al., 1998; Klinbunga et al., 2001). The lack of high quality wild and domesticated broodstock has probably caused the reduction of aquacultural production of *P. monodon* since the last several years (Limsuwan, 2004).

Unilateral eyestalk ablation is used commercially to induce ovarian maturation of penaeid shrimp but the technique leads to an eventual loss in egg quality and death of the spawner (Benzie, 1998; Okumura, 2004; Okumura et al., 2006). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is an ultimate goal for the industry (Quackenbush, 2001).

The domestication and selective breeding programs of penaeid shrimp would provide a more reliable supply of seed stock and the improvement of their production efficiency. The use of selectively bred stocks having improved culture performance on commercially desired traits rather than the reliance on wild-caught stocks is a major mean of sustainability of the shrimp industry (Browdy, 1998; Coman et al., 2006).

Nevertheless, genetic improvement of *P. monodon* is slowing to the lack of the basic information related with ovarian development and maturation in penaeid

shrimp. An initial step toward understanding molecular mechanisms of ovarian and oocyte development in *P. monodon* is the identification and characterization of genes/proteins differentially expressed in different stages of ovaries in this economically important species (Preechaphol et al., 2007).

Oogenesis is the process for production of oocytes composing of both mitotic and meiotic cell divisions. It is comprised of two consecutive M-phases, meiosis I and meiosis II; as there is no intervening S-phase, haploid gametes are produced. In most animals, oocytes are arrested at prophase I during the growth period and meiosis is resumed near or at the end of growth. Oocytes arrested at prophase I are recognized the immature stage. The process since resumption of meiosis is called meiotic maturation. In many species, oocyte meiosis is arrested again at a certain stage a wait for fertilization (Masui, 1985; Kishimoto, 2003). Therefore, mechanisms underlying prophase I and the subsequent arrests and their release, in addition to those underlying the meiosis I to II transition in investigated species are of concern.

The meiotic maturation of animal oocytes is controlled by the maturation promotion factor (MPF), a complex of Cdc2 and Cyclin B. Typically, progesterone or its derivatives (collectively called progestins) which is recognized as the maturation inducing hormone (MIH) induced germinal vesicle breakdown (GVBD) of oocytes. Understanding how an immature oocyte transforms into an egg during oocyte maturation is critical for the knowledge of reproductive maturation of *P. monodon*. However, molecular mechanisms on the signal transduction control for meiotic maturation of *P. monodon* oocytes is not available at present.

The major obstacle in the development of shrimp maturation technology is the limited knowledge of the molecular events of ovarian maturation of shrimp (Benzie, 1998). Over the past few decades, there have been many studies on characterization of vitellogenin/vitellin and the elucidation of the process of vitellogenesis in penaeid shrimp as well as molecular endocrinology of shrimp reproduction, particularly on GIH and methylfarnosoate (MF) (Silva Gunawardene et al., 2001; Yamano et al., 2004). Although these studies begin to reveal a better picture of the endocrine control of ovarian maturation in shrimp, reproductive maturation of penaeid shrimp is still not well understood. Accordingly, knowledge of the molecular mechanisms and

functional involvement of reproduction-related genes in ovarian development of *P. monodon* is necessary for better understanding of the reproductive maturation of *P. monodon* to resolve the major constraint of this economically important species in captivity.

The heterotrimeric guanine nucleotide-binding proteins (G proteins) are signal transducers that communicate signals from several hormones and neurotransmitters for induction of embryonic and/or gonadal development (Neves *et al*., 2002). G proteins consist of three subunits; alpha (α), beta (β) and gamma (γ).

There have been reported that oocyte meiotic arrest in several species is maintained via constitutive activity of a stimulatory G protein (Gs) which stimulates adenylyl cyclase activity to elevate cAMP levels in oocytes (Eppig, 1991; Kalinowski *et al*., 2004). Inhibition of Gs activity in *Xenopus* and zebrafish oocytes was sufficient to induce GVBD in the absence of a MIS signal (Gallo *et al*., 1995; Kalinowski *et al*., 2003).

In rainbow trout, the activation of a pertussis toxin (PTX)-sensitive Gi protein is involved in the initiation of oocyte maturation, although microinjection of the oocytes with PTX, which inactivates Gi/o G-proteins, did not inhibit GVBD (Yoshikuni and Nagahama, 1994). Studies in starfish and Atlantic croaker oocytes also found that the activation of a Gi protein was necessary for maturation and microinjection of oocytes with PTX significantly inhibited MIS-induced GVBD in these species (Shilling *et al*., 1989; Thomas *et al*., 2002). In spotted seatrout (*Cynoscion nebulosus*) indicated that G-protein activation is necessary for MIS-mediated GVBD in oocyte (Pace and Thomas, 2005). Overexpression of $G_{\beta\gamma}$ inhibits both progesterone-induced maturation and the activation of MAPK pathway (Lutz *et al*., 2000). The functional involvement of G proteins in oocyte maturation in crustacean was unclear.

In this thesis, the molecular involvement of genes functional involvement in the signal transduction pathway of meiotic maturation of *P. monodon* oocytes were identified and characterized. Recombinant proteins will be produced and used for the production of polyclonal antibody. Expression patterns of various genes and proteins during ovarian development in wild and domesticated *P. monodon* broodstock will be

examined. Effects of eyestalk ablation (wild broodstock) and serotonin and 17β-estradiol (domesticated broodstock) on expression levels of various genes will also be examined, respectively.

1.2 Objectives of the thesis

The objectives of this thesis were isolation, characterization and expression analysis of *G protein subunits* (*α*, *β*, and *γ*), *CGRP-RCP* and *Drk* cDNAs during ovarian development of *P. monodon*.

1.3 General introduction

Shrimp farming is an important aquaculture activity. The total global production of farmed shrimp reached more than 1.6 million tons in 2003, representing a value of nearly 9 billion U.S. dollars. About three-quarters of farmed shrimp are produced in Asia countries, particular in China and Thailand. The United States of America is the main shrimp importer, followed by Japan (Table 1.1).

The black tiger shrimp, *P. monodon* has dominated production of farmed shrimp along with the Pacific white shrimp (*Litopenaeus vannamei*) and is one of the most economically important penaeid species in South East Asia (Rosenberry, 2003). FAO reported that the value of *P. monodon* ranked the seventh of world aquaculture production (Table 1.2). Although the production of *L. vannamei* was greater than that of *P. monodon*, the price of *L. vannamei* is quite low and broodstock used relies almost entirely on genetically improved stocks imported from different sources. This prevents 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.

Total aquaculture production of *P. monodon* increased gradually from 21,000 tons in 1981 to 200,000 tons in 1988; then it sharply increased to nearly 500,000 tons in 1993. Since then, the production has been quite variable, ranging from 480,000 tons in 1997 to 676,000 tons in 2001 (FAO Fishery Statistic, 2009). Owing to problems from diseases, the total production of farmed shrimp of *P. monodon* decreased gradually from 275,000 tons (279 Mkg) in 2005, 330,000 tons (336 Mkg) in 2006 and 413,000 tons (420 Mkg) in 2011 (Table 1.2).

In Thailand, marine shrimp farms and hatcheries are located along the coastal areas of Thailand where Nakorn Sri Thammarat and Surat Thani located in Peninsular Thailand are the major parts of shrimp cultivation. In addition, Chanthaburi (eastern Thailand), Samut Sakhon and Samut Songkhran (central region) also significantly contribute on the country production. The intensive farming system has resulted in consistent production of marine shrimp of Thailand. Thailand has been regarded as the leading shrimp producer of cultivated shrimp for over a decade.

Table 1.1 Exportation to key markets of the black tiger shrimp, Thailand during 2005-2010

Country	2005		2006		2007		2008		2009		2010	
	Quantity (Mkg)	**Value (MB)**	**Quantity (Mkg)**	**Value (MB)**	**Quantity (Mkg)**	**Value (MB)**	**Quantity (Mkg)**	**Value (MB)**	**Quantity (Mkg)**	**Value (MB)**	**Quantity (Mkg)**	**Value (MB)**
USA	157	39,221	196	15,0416	180	41,736	177	42,496	183	44,750	192	47,207
Japan	49	15,589	53	15,412	58	14,458	63	16,373	70	19,132	76	20,373
EU27	11	2,797	20	5,342	30	7,721	39	9,699	52	12,357	66	14,924
Canada	14	3,706	16	4,252	25	5,525	20	4,837	21	5,038	22	5,446
Australia	10	2,387	9	2,286	8	2,030	7	1,739	9	2,231	10	2,473
Korea	13	2,853	15	3,346	14	2,967	12	2,321	10	1,942	9	1,825
China	3	594	2	585	3	727	5	681	4	793	11	1,598
ASEAN10	5	1,127	5	1,039	7	1,193	7	1,112	10	1,484	9	1,527
Taiwan	3	818	2	600	3	638	5	936	5	993	6	1,070
Hong kong	3	987	4	1,249	4	1,142	3	905	3	874	3	835
OTHER	5	1,269	8	1,743	11	2,198	11	2,182	11	2,309	11	2,327
Total	**279**	**71,354**	**336**	**86,274**	**350**	**80,332**	**354**	**83,285**	**383**	**91,909**	**420**	**99609**

Sourec: http://www.fisheries.go.th/foreign/index.php

Table 1.2 World aquaculture production of fish, crustaceans, molluscs, etc., by principal species during 2001 - 2009

Q = t , V = USD 1000

Species		2001	2002	2003	2004	2005	2006	2007	2008	2009
World production	Q	34,612,407	36,784,066	38,912,283	41,905,109	44,291,547	47,280,697	49,921,691	52,928,359	55,680,738
	V	49,073,662	50,433,066	54,522,014	59,984,545	66,195,360	74,421,960	90,182,612	100,163,677	105,301,846
1. *Penaeus vannamei*	Q	269 412	475 363	984 624	1 305 730	1 650 255	2 090 115	2 317 134	2 265 346	2 327 534
	V	1460705	2295707	3444856	4511355	5872390	7508802	8711159	9070926	9217721
2. *Salmo salar*	Q	1030005	1086134	1147682	1261926	1267297	1318720	1378874	1451262	1440085
	V	2771299	2913563	3439156	4151639	4963662	6630208	7121324	7045650	6421910
3. *Ctenopharyngodon idellus*	Q	3021977	3136669	3261593	3236060	3382547	3473900	3617970	3774402	4159919
	V	2504453	2570200	2654014	2628706	2778535	2930226	4273314	4796965	5291468
4. *Hypophthalmichthys molitrix*	Q	3483023	3392243	3374747	3546167	3675551	3830005	3585501	3767905	4075115
	V	2885258	2849725	2831752	2990390	3148613	3331245	4224208	4771165	5221718
5. *Cyprinus carpio*	Q	2749571	2813373	2955955	2559301	2664074	2790759	2806686	3031597	3216203
	V	2720114	2457536	2572649	2430609	2417249	2586257	3328585	3759105	4186719
6. *Oreochromis niloticus*	Q	1033672	1115585	1271922	1458392	1659105	1890491	2145973	2338775	2542960
	V	1243930	1248034	1353774	1548691	1757182	2105715	2959955	3252069	3784193
7. *Penaeus monodon*	Q	673 012	631 471	723 882	707 422	665 488	641 270	593 639	720 064	769 219
	V	3935192	3495169	3360584	3360054	3071054	3045199	2863390	3347351	3648009
8. *Catla catla*	Q	484 691	564 891	574 140	1 175 652	1 305 390	1 376 246	2 114 429	2 354 981	2 418 821
	V	461 596	540 253	552 136	1 285 554	1 495 188	1 678 068	2 964 997	3 708 752	3 645 519
9. *Oncorhynchus mykiss*	Q	549 583	543 800	550 469	558 173	553 455	597 882	651 216	655 530	732 432
	V	1434569	1441761	1590990	1844632	1921108	2324456	2710911	2713275	3401057
10. *Hypophthalmichthys nobilis*	Q	1442184	1494571	1670891	1821284	1911938	2073173	2165041	2320176	2466578
	V	1244251	1287878	1437974	1567018	1661466	1843056	2606059	2975754	3162138
Top ten species	Q	12760432	13038575	13682790	13883130	14560512	15377048	15700045	16684117	17900860
	V	20199771	20559573	22685749	25033094	27591259	32305164	38798905	41732260	44334933
Other species	Q	21,851,975	23,745,491	25,229,493	28,021,979	29,731,035	31,903,649	34,221,646	36,244,242	37,779,878
	V	28,873,891	29,873,493	31,836,265	34,951,451	38,604,101	42,116,796	51,383,707	58,431,417	60,966,913

Source: http://www.fao.org/fishery/statistics/en

1.4 Penaeid shrimp biology

1.4.1 Taxonomy

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. This group of animals is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up of 42,000, predominantly aquatic species, that belong to 10 different classes. Within the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda (Figure 1.1). Taxonomical recognition of *P. monodon* is illustrated below.

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Order Decapoda
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1815
Genus *Penaeus* Fabricius, 1798
Subgenus *Penaeus*
Species *Penaeus monodon*

Figure 1.1 Taxonomy of the black tiger shrimp, *Penaeus monodon*, Fabricius, 1798 (Brusca and Brusca, 1990).

1.4.2 Distribution and life cycle

The black tiger shrimp is widely distributed throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward to Australia and westward to Africa. Penaeid shrimp life cycle include several distinct stages that are found in a variety of habitats (Figure 1.2). Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities, where mating and

reproduction takes place. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The eggs hatch into the first larval stage, which is the nauplius. The nauplii feed on their reserves for a few days and develop into the protozoeae. The protozoeae feed on algae and metamorphose into myses. The myses feed on algae and zooplankton and have many of the characteristics of adult shrimp and develop into megalopas, the stage commonly called postlarvae (PLs). Larval stages inhabit plankton-rich surface waters offshore, with a coastal migration as they develop.

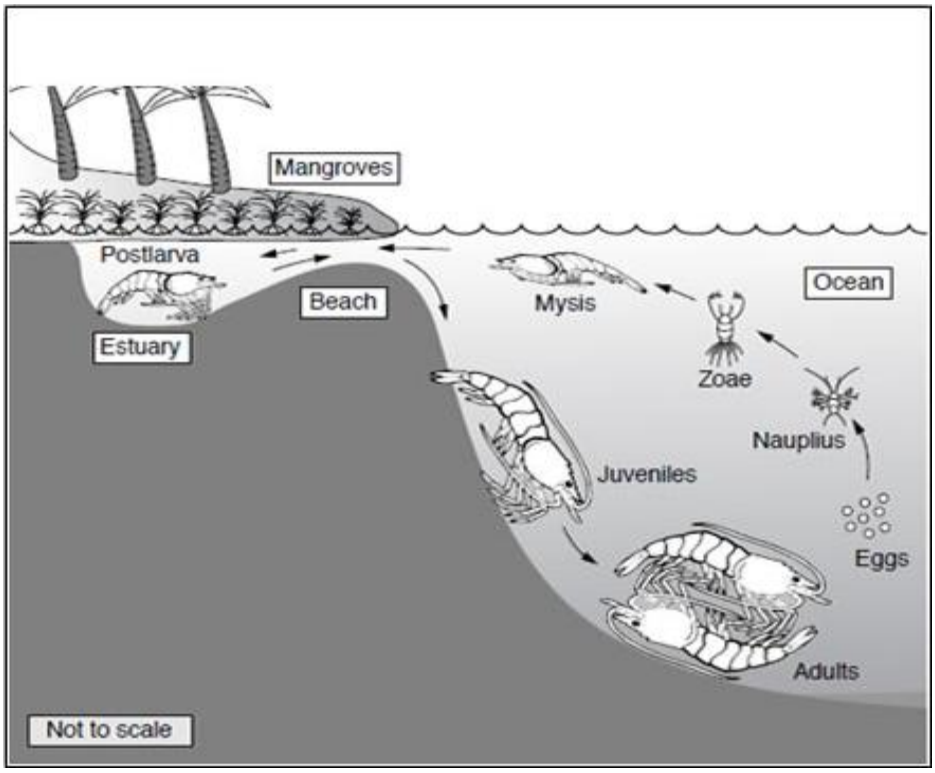


Figure 1.2 The life history of *Penaeus monodon* shrimp. Eggs hatch within 16 hours after fertilization. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called postlarvae. Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months (Motoh, 1984). The picture is not in proportion to actual size. (Source: Rosenberry, 2009).

P. monodon is the largest, reaching 330 mm or more in body length, and exhibits the highest growth rate of all cultured penaeid shrimps (Lee and Wickins, 1992). Generally, *P. monodon* can reach a market size up to 25-30 g. within 3-4 months after PL stocking in the culture ponds and tolerates a wide range of salinities (Rosenberry, 1997). Those facts together make *P. monodon* a leading species to culture.

1.5 Female reproductive system

1.5.1 Morphology of female reproductive system

The female reproductive system consists of paired ovaries, paired oviducts and a single thelycum (Figures 1.3 and 1.4). The first two are internal and the last is an external organ. The ovaries are partly fused, bilaterally symmetrical bodies extending in the mature female for almost its entire length, from the cardiac region of the stomach to the anterior portion of the telson. In cepharothorax region the organ bears a slender anterior lobe and five finger-like lateral projections (King, 1948). A pair of lobes, one from each ovary, extends over the length of the abdomen.

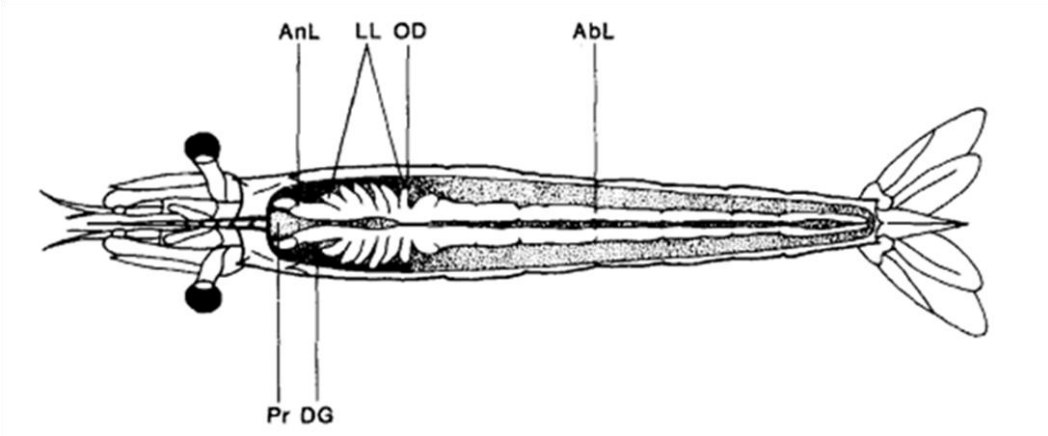
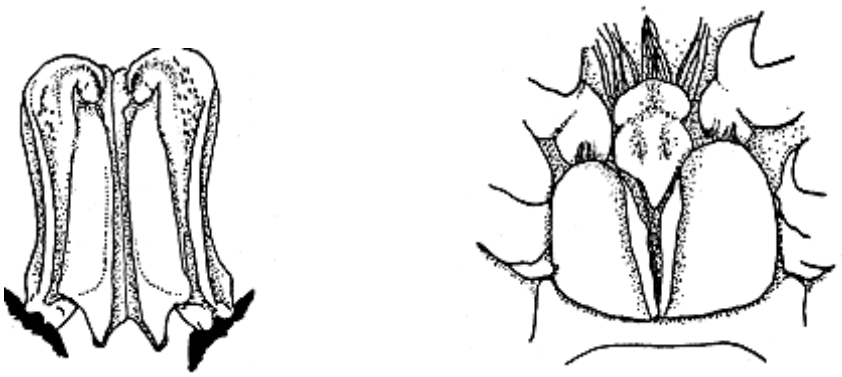


Figure 1.3 Female reproductive system of *P. monodon*, Abl, abdominal lobe of ovary; AnL, anterior lobe; LL, lateral lobes; OD, oviduct; Pr, proventriculus; DG, digestive gland.(Source: Primavera, 1990).



A **B**

Figure 1.4 External characteristics of sex organs of juveniles and broodstock of penaeid shrimp; petasma of males (A) and thelycum of females (B) (after King, 1998).

1.5.2 Ovarian development of *P. monodon*.

Generally, shrimp farmer visualize the ovarian development of female *P. monodon* broodstock by torchlight. These ovarian developmental phases correspond to the stages I to IV based on morphological characters (Rao *et al*., 1995; Figure 1.6).

The mature ovaries are paired organs, situated dorsally, extending from the base of the rostrum to the last abdominal segment. They are bilaterally symmetrical and partly fused. Each half of the ovary consists of three lobes, of which the slender anterior lobe occupies the anterior region. The middle lobe has 6 or 7 finger-like lateral lobules which entirely fill the area between the anterior region and the posterior border of the carapace. The posterior lobes extend the length of the abdomen. The two halves of the ovary are united by two commissures, one at the base of the anterior lobes and the other at the tip of the posterior lobes in the 6^{th} abdominal segment. Four maturation stages and spent-recovering of Penaeidae consist of stage I (immature stage), II (early maturing stage), III (late maturing stage) and IV (mature stage) (Roa, 1967).

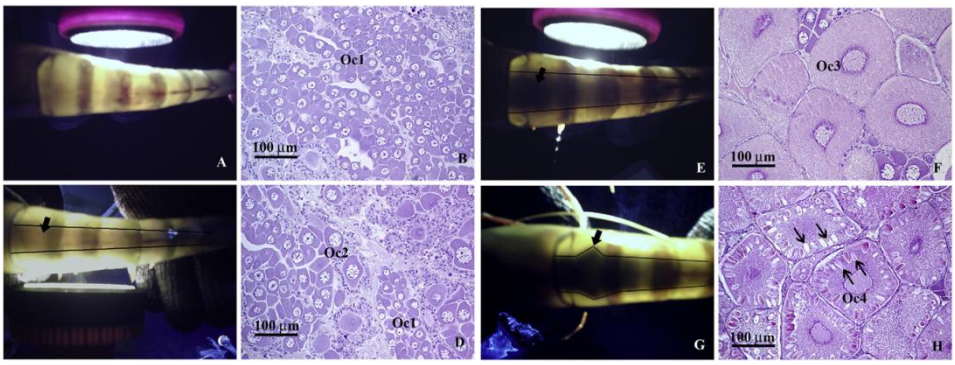


Figure 1.5 The external appearances shown by transmitted light and correlative histology of ovaries at various stages of development. (A) The external appearance of stage I ovaries, showing clear dorsal surface and (B) the histology, showing predominating step 1 oocytes (Oc1). (C) The external appearance of stage II ovaries as a thin dense midline (arrow). This stage of ovaries contained mostly step 2 oocytes (D, Oc2). (E) Stage III ovaries appeared as a thick band (arrow) and contained mostly step 3 oocytes (F, Oc3). (G) Stage IV ovaries appeared as a thick, broad band with wing-like structure representing lateral lobes (arrow), and it contained a large number of step 4 or mature oocytes (H, Oc4), with numerous cortical rods (arrows) in their peripheries (Ngernsoungnern *et al.*, 2008).

Immature stage: Ovaries of immature prawns are thin, translucent, unpigmented and confined to the abdomen. They contain oocytes and small spherical ova with clear cytoplasm and conspicuous nuclei.

Early maturing stage: Ovaries are increasing in size and the anterior and middle lobes are developing. The dorsal surface is light yellow to yellowish green. Opaque yolk granules are formed in the cytoplasm and partly obscure the nuclei. The developing ova are clearly larger than the immature stock.

Late maturing stage: Ovaries are light green and visible through exoskeleton. The anterior and middle lobes are fully developed. The maturing ova are opaque, due to the accumulation of yolk.

Mature stage: Ovaries are dark green and clearly visible through the exoskeleton. The ova are larger than in the preceding stage and the peripheral region becomes transparent. This stage is believed to be the last stage of maturity before actual spawning as the largest ova are encountered only in this stage.

Spent-recovering: It is probable that after the extrusion of eggs, the gonad revert almost immediately to the immature condition. The present stage is therefore distinguishable from that found in immature virgin females only on the size of the prawn (Vasudevappa and Suseelan, 1999).

15.3 Development of *P. monodon* ovaries

The average *P. monodon* broodstock varies according to geographic location. Female wild broodstock are normally range from 110 g to 160 g. However, large females, those over 150 grams, which are assumed to be older females, often do not perform well in hatcheries. The ovary lies dorsal to the gut and extends from the cephalothorax (head and thorax region) along the entire length of the tail as shown in Figure 4. The ovaries are paired, but partially fused in the cephalothorax region which consists of a number of lateral lobes. The ovarian development is divided in 4 phases according to its histological features and germ cell association as shown in Figure 1.7. It consists of ovary stage I (undeveloped phase or spent phase), stage II (proliferative phase), stage III (premature phase) and stage IV (mature phase).

The stage I ovaries (Figure 1.7A) are comprised of a connective tissue capsule surrounding a soft vascular area containing future eggs, called oogonia, and accessory cells, also called follicle or nurse cells. The undifferentiated oogonia exits the germinative zone of the ovaries and became oogonia that divided mitotically and enter the meiotic prophase.

The stage II ovaries (Figure 1.7B) contain the majority of previtellogenic oocytes characterized by accumulation of ribosomes and the development of rough endoplasmic reticulums. The developing eggs are increasing in size and they are not as yet producing yolk.

In the stage III ovaries (Figure 1.7C), the majority of oocytes are vitellogenic oocytes governed by the process of vitellogenesis in which yolk proteins (vitellin) are

recruited and made within the oocytes. Vitellin is the common form of yolk stored in oocytes and is a nutrient source for developing embryos. Vitellgenin is the precursor molecule of vitellin. Vitellgenin in crustacean was synthesized by fat body, hemocytes, ovaries or hepatopancreas. It is evidenced that vitellogenin fragment was cleaved into smaller size of vitellin fragment by protease function. At the end of the third phase, the oocytes become bright colored by the association of vitellin with carotenoids. By the end of vitellogenesis, the eggs develop cortical granules filled with a jelly-like substance destined to form part of egg shell membrane after ovulation.

In the stage IV ovaries (Figure 1.7D), the fully mature oocytes is composed of extracellular cortical rods. These cortical specializations are precursors of jelly layer (JL) of the egg. Spawning and direct contact of the spawned eggs with sea water leads to the release of extracellular cortical rods. Then, increasing vitellin envelope and formation of corona that is composed of a flocculent matrix around the egg consisting of jelly layer occur. The biochemical composition of the shrimp cortical rods and the nature of jelly layer still scarcely understood. Precursors isolated from mature ovaries comprised of approximately 70-75% protein and 25-30 % carbohydrate. Shrimp ovarian peritrophin (SOP) was demonstrated that it is a component of the cortical rod precursor of the jelly layer in shrimp eggs. It is glycosylated and binds chitin. The color of mature ovaries is characteristic dark green color as a result of deposition of carotenoid pigments.

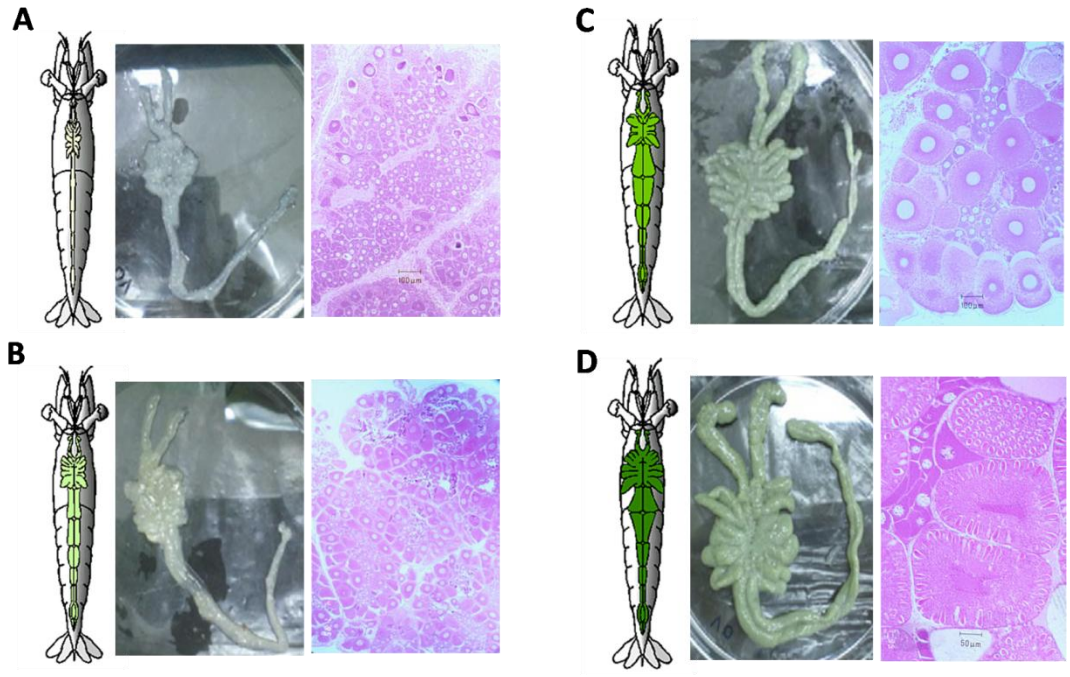


Figure 1.6 Different developmental stages of *P. monodon* ovaries and oocytes, A: previtellogenic ovaries (stage I), B: early vitellogenic ovaries (stage II), C: late vitellogenic ovaries (stage III), D: mature (stage IV) (Source: www.aims.gov.au/ /mdef/images/fig01-4a.gif,doi:10.1371/journal.pone.0024427.g001).

1.6 Hormones functionally involved in female reproduction of penaeid shrimp

1.6.1 Gonad-inhibiting hormone (GIH) and gonad stimulating hormone (GSH)

Female reproduction in crustaceans is controlled by an elaborate endocrine sytem, involves two endocine organs: the X-organ/sinus gland (XO/SG) complex located in the eyestalk ganglia and the Y-organ (YO) located the cephalothorax. Ovarian/oocyte maturation is regulated by key hormone from nueroendocrine organs, two antagonistic neuropeptides referred to as vitellogenesis-inhibiting hormone (VIH) or gonad- inhibiting hormone (GIH) synthesis and secreted from the XO/SG complex and gonad stimulating hormone (GSH), thought to be produced by the brain and thoracic ganglion (Table 1.3, Nagaraju, 2011).

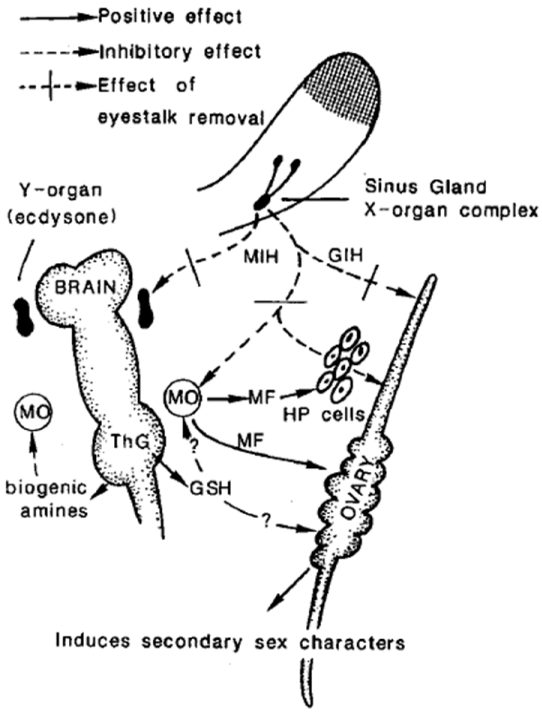


Figure 1.7 Major endocrine glands and their target tissues involve in crustacean female reproduction. Indicated with solid lines and arrows are stimulatory effects. Inhibitory interactions are indicated with dashed lines and arrows. Note that the eyestalk sinus gland x-organ complex can be removed by eyestalk ablation. These include both the inhibitory effects of the molt inhibitory hormone (MIH) on the Y-organ which produces ecdysones and the GIH, which according to the to the litalature may be similar or the same as the VIH and which may also inhibit the mandibular organ (MOIH) as well as other target tissues such as the ovary and hepatopancreas (HP). The brain and thorasic ganglion (ThG), according to some report, may also stimulate or inhibit reprodudtion, or do both. Biolgenic amine suchas serotonin and octopamine have been shown to inhibit the MO (Laufer and Homola, 1991).

Table 1.3 Effects of neuroendocrine and non-neuroendocrine hormones on target tissues and their physiological actions in crustacean (Nagaraju, 2010).

Hormone	Site of production	Target	Physiological action
Crustacean hyperglycemic hormone	X-organ–sinus gland of eyestalk	Many organs	Regulates glucose level Regulates reproduction Regulates growth
Gonad (vitellogenic) inhibiting hormone	X-organ–sinus gland of eyestalk	Gonads and HP	Inhibits gonad maturation
Molt inhibiting hormone	X-organ–sinus gland of eyestalk	Y-organ	Inhibits growth Stimulates vitellogenesis
Gonad stimulating factor	Brain, thoracic ganglia	Gonads and HP	Stimulates gonad development
Neurotransmitters: 5-HT, DA and OA	X-organ–sinus gland of eyestalk, brain, TG	Gonads, HP, brain, TG, etc.	Influences gonad development, growth and metabolism
Methyl farnesoate	Mandibular organ	Gonads, HP, Y-organ, brain and TG	Stimulates gonad development Stimulates ecdysteroid production
Farnesoic acid	Mandibular organ	Gonads and HP	Stimulates gonad development
Ecdysteroid	Y-organ	Eyestalk, gonads and HP	Stimulates growth Stimulates gonad development
Opioid peptides	Eyestalk	Brain, TG, ovary and HP	May inhibit or stimulate gonad maturation May stimulate molt
Prostaglandins		X-organ–sinus gland, brain, TG, ovary and HP	May inhibit or stimulate gonad maturation May stimulate molt
FSH, LH, HCG		Ovaries	Stimulates ovarian maturation
Estrogens, progesterone		Hemolymph and ovaries	May stimulate ovaries
Androgenic hormone	Androgenic hormone	Testis, HP, brain and TG	Masculine characteristics, spermatogenesis in the testis, secondary male characteristics

5-HT, serotonin; DA, dopamine; OA, octopamine; HP, hepatopancreas; TG, thoracic ganglia; FSH, follicle stimulating hormone; LH, luteinizing hormone; HCG, human chorionic gonadotrophin.

Neurosecretory structures in crustacean eyestalks are known to produce the crustacean hyperglycemic hormone (CHH), molting-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) of the CHH/MIH/GIH gene family (Chan *et al*., 2003).

Gonad inhibiting hormone (GIH) is secreted from the X-organ in the eyestalk, and inhibits the synthesis of vitellogenin in the ovary. The peptides also have an impact on the males, and hence it is called gonad inhibiting hormone instead of vitellogenesis inhibiting hormone (Huberman 2000).

Eyestalk ablation has been employed to induce reproductive maturation in crustacean. Eyestalks are the endocrine center for regulating many physiological mechanisms, such as molting, metabolism, sugar balance, heart rate, pigment and gonad maturation (Vaca and Alfaro, 2000). While the ablation can induce ovarian maturation, it also jeopardizes growth, shortens molting cycle, increases energetic demands and resulting in an eventual loss in egg quality and high mortality (Benzie, 1998).

Treerattrakool et al (2008) cloned and characterized GIH from cDNA obtained from the eyestalk of *P. monodon* (*Pem*-GIH), measured tissue expression, and performed a knockdown experiment of *Pem*-GIH using dsRNA interference. They discovered a cDNA encoding a polypeptide of 79 amino acids that was closely related to type II CHH. The *Pem*-GIH gene expression was observed in the eyestalk, brain, thoracic and abdominal nerve cords of adult *P. monodon*. Injection of dsRNA of *Pem*-GIH can reduce transcript levels in the eyestalk and in the abdominal nerve cord both *in vitro* and *in vivo*. *Pem*-GIH-knockdown shrimp showed increase vitellogenin gene expression.

Peptides with gonad inhibiting properties was also cloned and characterized in whiteleg shrimp *L. vannamei* (Tsutsui et al. 2007),and lobster *Homarus americanus* (called VIH) (Ohiro et al. 2006). Both GIH from *L. vannamei* and *H. americanus* have shown *in vitro* to be inhibiting vitellogenin gene expression. No result from *in vivo* tests was reported in the literature.

For characterization of GSH, Tiu and Chan (2007) used recombinant protein and RNA interference approach to examine the gonad-stimulating property of the previously reported molt-inhibiting hormone, MeMIH-B, from *Metapenaeus ensis*. MeMIH-B can up regulate vitellogenin expression in hepatopancreas and ovary both *in vitro* and *in vivo*. Injection of shrimp with MeMIH-B dsRNA reduced the expression of MeMIH-B in the eyestalk and thoracic ganglion and vitellogenin expression in both the hepatopancreas and ovaries was reduced.

1.6.2 Vertebrate-type steroids

Estrogen-like compounds in invertebrates were first described in the ovaries of an echinoderm (Donahue and Jennings, 1937). In *P. monodon*, the titers of conjugated pregnenolone and unconjugated and conjugated dehydroepiandrosterone (DHEA) were found to be maximal at early and late vitellogenesis. Unconjugated progesterone was found in ovaries at the late vitellogenic and mature stages of ovarian development whereas conjugated testosterone was only detected in the mature ovaries (Fairs et al., 1990).

Progesterone, P4, and its derivatives (progestins) are sex steroid hormones that play important roles in gametogenesis (Miura et al., 2006). 17α-hydroxyprogesterone stimulated vitellogenin synthesis in *Marsupenaeus japonicus in vivo* (Yano, 1987). Progesterone stimulated ovarian maturation and yolk protein synthesis of penaeid shrimp (Yano, 1985; Quackenbush, 2001). It also promoted spawning of *Metapenaeus ensis* (Yano, 1985). Nevertheless, molecular mechanisms of vertebrate-like hormones have not been well established in penaeid shrimp at present.

Recently, progesterone and 17α-hydroxyprogesterone were extracted from the polychaetes. Their activity in comparison with the synthetic hormones (0.4, 0.7 and 1.0 ng/ml for P4 and 1.0, 2.0 and 3.0 ng/ml for 17α-OHP4) were *in vitro* tested against previtellogenic ovaries of *P. monodon* for 24 h. P4 was more effective in enhancing the final maturation of oocytes while 17α-OHP4 had more effects on vitellogenic oocytes. Interestingly, synthetic steroid hormones at equal hormone concentrations produced similar results to steroid hormones extracted from natural polychaetes (Meunpol et al., 2007). Nevertheless, receptors mediated the activity of progesterone and its derivatives have not been reported in penaeid shrimp.

In *Xenopus*, progesterone acts as a maturation-inducing hormone (MIH) resulting in meiotic resumption of oocytes from prophase-I arrest (Kishimoto, 2003). The presence of vertebrate-type steroids has been documented in almost all invertebrate groups including crustaceans (Lehoux and Sandor, 1970; Lafont and Mathieu, 2007). The conversion of progesterone into estradiol-17β was reported in *M. 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 their regulatory roles in vitellogenesis. Nevertheless, progesterone and other sex steroid receptors have not been reported in penaeid shrimp.

1.6.3 Neurotransmitters

Serotonin (5-HT), dopamine (DA) can have an indirect impact on reproduction by influencing the release of the relevant hormones or by modulating the responses of the target tissues to the hormones. These neurotransmitters can be a wide ranging effect on many physiological responses such as pigment regulation, energy metabolism, osmoregulation, and ion balance (Fingerman 1997). 5-HT stimulates the release of gonad-stimulating hormone (GSH) from the brain and thoracic ganglia in sand fiddle crab *Uca pugilator* and thus stimulate ovarian development. DA was reported to inhibit gonadal development (Fingerman 1997).

The effect of 5-HT in ovarian stimulation and its localization in the ovary of black tiger shrimp *P. monodon* were described in Wongprasert et al. (2006). They reported that 5-HT can stimulate ovarian maturation, and 5-HT presence in different tissues during different stage of ovarian development (visually determined): the follicular cells during pre-vitellogenic (ovarian stage I), cytoplasm during early vitellogenic (ovarian stage II), and cell membrane and cytoplasm during late vitellogenic (ovarian stage III). However, as was the case with other domesticated shrimp, the 5-HT stimulated ovary was not developed to ovarian stage IV like the ovary of fully matured wild brookstock (Wongprasert et al. 2006). Serotonin receptor was cloned and characterized from the ovary of *P. monodon* (Ongvarrasopone et al 2006). The expression of 5-HT receptor protein appeared to be stage-specific in the ovary. More precisely, the 5-HT receptor was found in the trabeculae of *P. monodon*

ovary during pre-vitellogenic and early vitellogenic stages (ovarian stage I and II – visually determined), and on the corticoid and oocyte membrane during late vitellogenic and mature stages (ovarian stage III and IV).

Meeratana et al (2006) examined the effect of serotonin injection on ovarian development of *M. rosenbergii*. They reported that at three injections of low concentration of serotonin (1µg g^{-1} body weight) can stimulate ovarian development to maturity in a 15 day trial. The culture medium from serotonin-stimulated thoracic ganglion was shown to stimulate the ovarian development.

Tinikul et al (2008) monitored the concentration of both serotonin and dopamine in the central nervous system and the ovary of giant freshwater prawn *M. rosenbergii*. They reported that serotonin concentration in the brain and thoracic ganglia gradually increased from ovarian stage I to reach a maximum at stage IV. In contrast, dopamine concentration in those two tissues peaked during ovarian stage II and dropped to the lowest at stage IV. The concentration of both serotonin and dopamine in the ovary followed similar trends as that in the brain and thoracic ganglian.

Serotonin injection was shown to increase vitellogenin concentration in the hemolymph of giant freshwater prawn *M. rosenburgii,* while dopamine had the opposite effect (Chan et al. 2003; Tinikul et al., 2008). Serotonin in combination with dopamine antagonist was shown to induce ovarian maturation and spawning in both wild *Litopenaeus stylirostris* and pond-reared *L. vannamei* (Alfaro et al. 2004).

The use of serotonin showed the potential to be an alternate to eyestalk ablation. Chen et al (2003) showed that the effect of serotonin and dopamine was similar in both intact and eyestalk-ablated prawn suggesting that both neurotransmitters do not exert their effect via the X-organ but rather via the thoracic ganglia and gonad stimulating hormones (GSH). Nonetheless, the failure of the ovary of domesticated shrimp to develop to their fully mature state (ovarian stage IV) in serotonin-stimulated shrimp suggested that serotonin is not the only factor affecting ovarian and oocyte maturation.

1.6.4 Signal transduction pathways associated with ovarian development

The actions of sex steroids are mediated through nuclear receptors as the classical pathway. The classical steroid receptors are members of the steroid/thyroid hormone receptor superfamily (Evans, 1988). They bind steroids in the nucleus or in the cytosol, dimerize, and migrate to the nuclear genome, where they act as transcription factors. This signal transduction pathway cannot account for the rapid cellular effects of steroids, and a number of different receptors may be involved (Mifsud and Bateman, 2002).

Subsequently, the distinct class of putative membrane-bound receptors have been reported in vertebrates; e.g membrane progestin receptors (mPR, subtypes α, β, γ). Effects of sex steroids are mediated by fast nongenomic mechanisms through membrane-associated receptors and signaling cascades. However, the signaling through these families of novel membrane proteins is still unclear.

Genomic mechanisms of steroid hormones act on new mRAN and protein synthesis that initiated by hormones diffuse past the cell membrane and bind to nuclear receptors located in the cytosol or nucleus of the cells. This causes a change in the conformation of the receptor, which, depending triggers a number of downstream events that eventually results in up or down regulation of gene expression.

Nongenomic mechanism actions mediate through the activation of intracellular signaling pathways, resulting in alterations in ion fluxes and intracellular free calcium concentrations occurring within second and of other second messengers. They are actions are initiated at or near the cell surface and evidence has accumulated that they are mediated by binding to specific receptor in plasma membrane of the target cell (Thomus, 2008).Steroid hormone response mediated via steroid membrane receptor, such as progestin can be induced oocyte maturation through progestin membrane receptors is via a nongenomic mechanism (Thomas *et al*., 2002) (Figure 1.8).

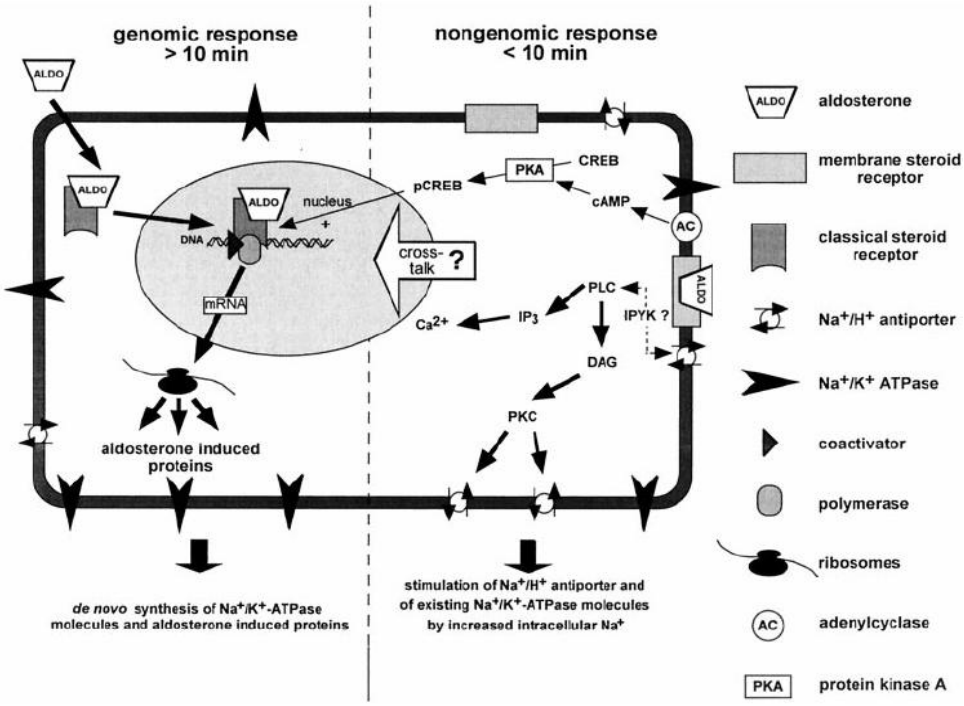


Figure 1.8 Shematic presentation of the two step model for steroid action comprising genomic and nongenomic action (Christ and Wehing, 1998)

Molecular mechanisms underlying meiotic maturation of oocytes and ovarian development of penaeid shrimp are still unknown. In many animals, the meiotic cell cycle of arrested oocytes is resumed for acquisition of fertilization. Maturation promoting factor (MPF), a complex of cyclin B and cdc2 (Cdk1) are activated and play essential roles during oocyte maturation (Yoshida et al., 2000; Kotani and Yamashita, 2002; Kishimoto, 2003).Typically, progesterone or its derivatives (collectively called progestins) which is recognized as the maturation inducing hormone (MIH) induced germinal vesicle breakdown (GVBD) of oocytes.

General mechanism of MPF formation during oocyte maturation by produced and activate in the oocyte cytoplasm which the sequential actions after the MIH signal received on the surface with the aid of GTP-binding protein (G-protein), signaling transducers. G protein or heterotrimeric guanine nucleotide-binding protein that couples to receptor communicate signals from a large number of hormones, neurotransmitters and other signaling factors (Reece and Campbell, 2002).

Signaling pathway is the oocyte meiotic maturation process of oocyte developed from immature oocyte into fertilizable egg, which is often induce by a specific hormone. Oocyte maturation of various signal transduction pathway that converge to activate maturation-promoting factor (MPF), it is a key activity that catalyses entry into M phase of meiosis I and meiosis II. The function of MFP in promoting oocyte maturation is ubiquitous in signaling pathway but the difference depending on species (Schmitt and Nebreda, 2002). The action of MPF involves an initial action of agonists at the oocyte surface.

The precise mechanism for the activation and/or stabilization of the maturation promoting factor (MPF) by the MIS appears to differ among species. For example, the MIS-receptor (MIS-R), which in fish is a mPR appears to be coupled to an inhibitory G protein (Gi) in fish species. In starfish Gi receptors was detected and functionally involves in germinal vesicle breakdown (GVBD) during meiotic maturation of oocytes. Oocytes injected with 1 μM of the adenosine A1 receptor (Gi-coupled receptor) RNA undergone GVBD within 20 - 45 min (Kalinowski *et al*., 2003). The $G_{\beta\gamma}$ subunit injection (≥1.8 μM) caused all of the oocyte to undergo GVBD (Jaffe *et al.*, 1993).

In spotted seatrout shown inhibitory G protein (G_i) is necessary for steroid-mediated oocyte maturation. The seatrout oocyte membrane were treated with various hormone and cAMP production was measure over incubation periods of 1 to 30 min. Addition of 290 nM 20β-S significantly reduced adenylyl cyclase activity within 1 min and for up to 5 min compared to the control with jno steroid added (Pace and Thomas, 2005).

Oocyte meiotic signal transduction involves two stages including a signaling molecule activates a specific receptor on the cell membrane causing a second messenger to continue the signal into the cell and involve a physiological responses. The signals associate with receptors that could be classified into three general classes.

1. Receptors that are found intracellularly and upon ligand binding migrate to the nucleus where the ligand-receptor complex directly affects gene transcription. Because this class of receptors is intracellular and functions in the nucleus as transcription factors they are commonly referred to as the nuclear receptors. Receptors

of this class include the large family of steroid hormone receptors. Nuclear receptors have a ligand-binding domain, a DNA-binding domain and a transcriptional activator domain.

2. Receptors that are coupled, inside the cell, to GTP-binding and hydrolyzing protein (termed G-proteins). G-proteins interacting receptors is characterized by 7 transmembrane spanning domains. These receptors are termed seven transmembrane receptors.

3. Receptor that penetrate the plasma membrane and have intrinsic enzymatic activity. These receptors are tyrosine kinases, tyrosine phosphotase, guanylated cyclases, and serine/threonie kinase. Receptors with intrinsic tysosine kinase activity are capable of autophosphorylation as well as phosphoryation of other substrates. Additionally, several families of receptors lacking intrinsic enzyme activity, yet are coupled to intracellular tyrosine kinases by direct protein-protein interactions.

1.7 Functionally important genes functional related to the signal transduction for ovarian development of *P. monodon* examined in this thesis

1.7.1 G protein subunits

G proteins are a family of proteins involved in transmitting chemical signals outside the cells, and causing physiological changes inside the cells. They communicate signals from many hormones, neurotransmitters and other signaling factor (Jane, 2002).

G proteins can refer to two distinct families of proteins. Heterotrimeric G protein, sometime referred to as the “large” G protein that are activated by G protein-coupled receptors and made up of subunits of G protein. Heterotrimeric G protein consist to three subunits are alpha (α), beta (β) and gamma (γ). There are also “small” G protein (20-25 kDa) that belong to the Ras superfamily of small GTPases. When signaling, they function in essence as dimmers because the signal is communicated either by the G_{α} subunit or the $G_{\beta\gamma}$ complex. Mechanisms of G protein subunit are activates or inhibits the cAMP-dependent pathways or by gating ion channels directly. $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha o}$ are involved hormonal regulation of adenylate cyclase activity where

$G_{\alpha s}$ activated, in contrast to that $G_{\alpha s}$ and $G_{\alpha i}$ and $G_{\alpha o}$ that inhibited the activity of adenylate cyclase.

Herterotrimeric G proteins are generally activated by ligand-bound seven transmembrane receptors by response to the conformation exchange of GDP for GTP and dissociate to activate other proteins in the pathway that specific mechanisms depending on type of G protein (Takesono et al., 1999; Hallak et al., 2000; Dalle et al., 2001; Voronina and Wessel, 2004; Figure 1.9). Steroid-mediated G protein signaling occurred during oocyte maturation in various eukaryotes.

In *Xenopus*, G proteins have been characterized including, $G_{\alpha s}$ (Gallo *et al*., 1995) and $G_{\beta\gamma}$ (Lutz *et al*., 2000) and overexpression of the latter inhibited oocyte maturation. The injection of $G_{\alpha s}$ antibody (0.3-0.2 μM) that inhibits $G_{\alpha s}$ activity stimulated GVBD similar to oocytes treated with progesterone (3 μM). Immunogold electron microscopy showed that $G_{\alpha s}$ is present in the yolk platelet membrane as well as the plasma membrane. The expression of $G_{\beta\gamma}$ in *Xenupus* oocytes significant attenuated progesterone-induced maturation. Incubation with higher concentration of progesterone overcame this inhibition indicating that the process of $G_{\beta\gamma}$-mediated inhibition and progesterone-mediated induction of maturation can compete with each other.

Experiments to determine whether the activation of receptors linked to $G_{\alpha i}$ or $G_{\alpha s}$ was sufficient to cause oocyte maturation was carried out in starfish. Interestingly, oocyte injected with RNA for the human adenosine A1 receptor, a $G_{\alpha i}$-couple receptor underwent GVBD within 20-45 min, a time course similar to that affected by 1-MA (Kalinowski *et al.*, 2003).

Jaffe et al. (1993) illustrated that G protein subunits ($G_{\alpha s}$,, $G_{\alpha i1}$,$G_{\alpha i2}$ and G_{β} in starfish were 44, 39 and 37 kDa in molecular mass, respectively. The $G_{\beta\gamma i}$-subunits from bovine transducin were injected into to starfish oocytes. The concentration in the cytoplasm $\geq$ 1.8 μM caused oocytes of the starfish to undergo GVBD.

In spotted seatrout, 20β-S acts through a PTX-sensitive G protein to decrease adenylyl cyclase activity. The cAMP production was measured in oocyte membrane pretreated with activated PTX. The pretreatment of oocyte membranes with activated

PTX slightly increased adenylyl cyclase activity when compared to untreated membrane. Results indicated that the $G_{\alpha i}$ protein is necessary for oocyte maturation by MIS-induced activation (Pace and Thomas, 2005).

Four families of G protein α subunit ($G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha s}$ and $G_{\alpha 12}$) were found in the sea urchin oocytes and eggs. Three of these ($G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha s}$) are present on the plasma membrane of oocytes, while $G_{\alpha 12}$ is located in the cytoplasmic vesicles. Upon oocyte maturation, these proteins remain in eggs, and continue to be expressed in embryonic tissues. Anti-$G_{\alpha i}$, and anti-$G_{\alpha q}$ antibody injection did not significantly affect oocyte maturation. In contrast, Anti-$G_{\alpha s}$ injection inhibited oocyte maturation. The information indicated that $G_{\alpha s}$ is functionally involved in the regulation of oocyte maturation in the sea urchin (Voronina and Wessel, 2004).

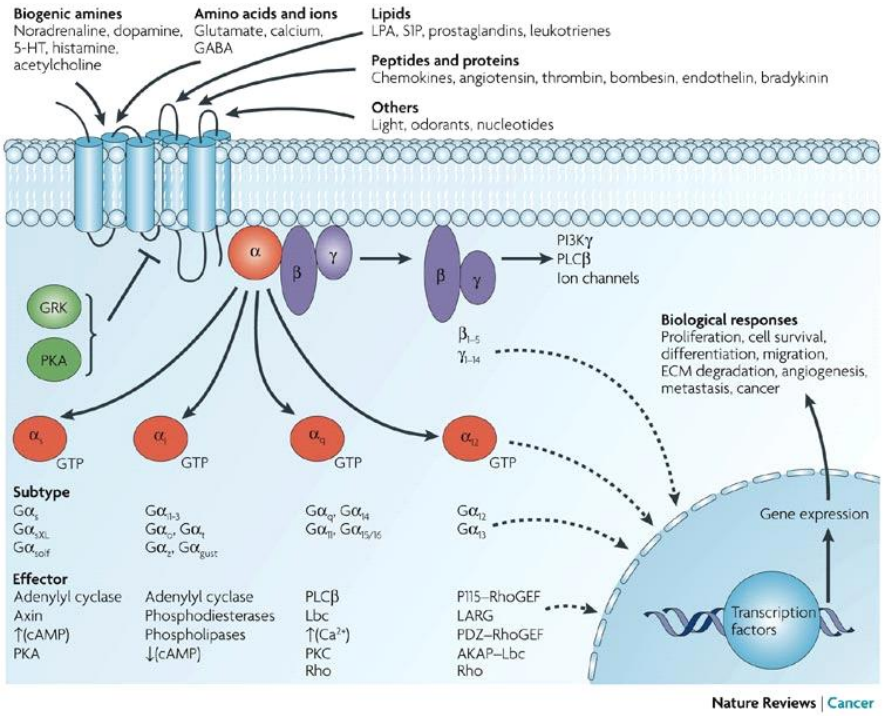


Figure 1.9 Different types of heterotrimeric G proteins share a common mechanism. They are activated in response to a conformation change in the G protein-coupled receptor and dissociate to activate other proteins in the signal transduction pathway. (Source: Dorsam and Gutkind *Nature Reviews Cancer* **7**, 79–94).

In addition, tyrosine-specific protein kinase is one mechanism of the signal transduction intracellularly. Tyrosine kinases catalyze the transfer of phosphate from ATP to a tyrosine residue of specific cell protein target. The major categories of tyrosine kinases are divided receptor- and non-receptor-dependent reactions. All receptor tyrosine kinases share a similar structure; a ligand-binding extracellular region, a hydrophobic transmembrane domain, and an intracellular (cytoplasmic) region. The latter region and the catalytic domain include regulatory sites (Wilks, 1993; Shawver *et al*., 1995).

Non-receptor protein tyrosine kinases, PTK, are intermediate conductors of diverse intracellular signal pathways. Many of them are associated with transmembrane receptors, such as hormone receptors, cytokines, growth factor receptors, etc. Non-receptor kinases are activated by means of association of receptors with extracellular ligands or cell adhesion components at particular phases of the cell cycle (Taniguchi, 1995; Bolen, 1993; Tatosyan and Mizenina,2000). The Src protein is a typical representative of the non-receptor tyrosine kinase that have the SH2 and SH3 domains play a key role in regulation of catalytic protein activity.

Downstream of receptor kinase (Drk) is one of the members of non-receptor protein tyrosine kinase. Immunofluorescence analysis of mouse oocyte and zygotes with an anti-phosphotyrosine antibody revealed that fertilization stimulated accumulation of P-Tyr-containing protein in the egg cortex and that their abundance was elevated in the region overlying the MII spindle. In addition, the poles of the MII spindle exhibited elevated P-Tyr level. As polar body extrusion progressed, P-Tyr-containing proteins were especially concentrated in the region of cortex adjacent to the maternal chromatin and the forming polar body (McGinnis *et al,* 2007).

In *Xenopus*, protein tyrosine phosphorylation plays an important role in the process of fertilization and early development. The active Src protein tyrosine kinase could accelerate oocyte maturation. The xSrc activity is localized mainly in plasma membrane-containing fraction of *Xenopus* oocyte and its activity transiently increased about 2-fold within 3 min after progesterone administration (Sato *et al*., 1998).

Boonyaratanakornkit et al. (2001) identified a specific polyproline motif in the amino-terminal domain of conventional progesterone receptor (PR) that mediates

direct progestin-dependent interaction of PR with SH3 domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases. Preliminary evidence for the biological significance of this PR signaling pathway through regulatory SH3 domains was show with respect to influence on progestin-induced growth arrest of breast epithelial cells and induction of *Xenopus* oocyte maturation.

In zebrafish, the first vertebrate egg in which Src-family PTK SH2 domains have been shown to suppress the sperm-induced calcium transient was shown (Kinsey *et al*., 2003). Detection of active SFKs in zebrafish eggs and zygotes by Western blot revealed the particulate fraction of unfertilized eggs and zygotes collected at 2.5 min post-insemination. Immunofluorescence results indicated that the initial response of Src-family PTK activation occurred in a diffuse region of the cortical cytoplasm immediately underlying the micropyle. That activation process was apparent as early as 30 s post-insemination. The region of elevated Src-family PTK activity then became restricted to the deeper cortical cytoplasm associated with the actin-rich cortical cytoskeleton and spread peripherally to involve the rest of the egg cortex (Sharma and Kinsey, 2006).

In addition, *calcitonin gene-related peptide (CGRP) receptor component protein* (*RCP*) is an intracellular membrane protein that is required for G-protein-couple signal transduction at receptors for the neuropeptide *CGRP* (Prado *et al*., 2002; Walker *et al*., 2010). Cacitonin (CT) secretion occurred as a consequent effect of progesterone in the TT cells. The levels of human CT release gradually increased with the incubation time in the TT cells. Adminstration of progesterone increased the level of human CT in medium from 10 nM to 1000 nM in a dose-dependent manner (Lu and Tsai, 2007). The human *CGRP-RCP* mRNA was expressed at the highest level in testis followed by ovaries, prostate, small intestine and spleen (Balkan et al., 1999).

Prado *et al* (2001) co-immunoprecipitated RCP with CRLR and RAMP2 proteins of the adrenomedullin receptor complex from NIH3T3 cell or guinea pig cerebellum suggesting that the functional adrenomedullin receptor consisted of three protein: CRLP, RCP, and RAMP2. RCP is expressed in many tissues including ovaries. To directly assess the role of RCP in CRLP-mediated signaling transduction indicated that loss of

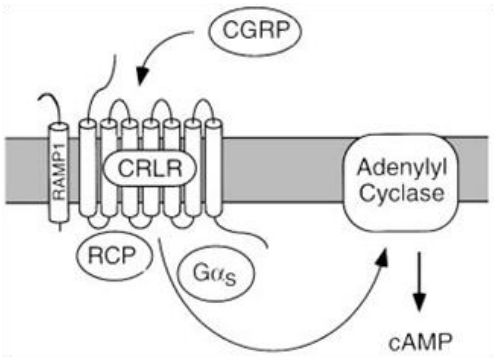


Figure 1.10 Model for functional CGRP receptor. The CGRP receptor complex is proposed to comprise a ligand-binding protein (CRLR), an accessory protein for trafficking and pharmacology (RAMP1), and an accessory protein for coupling to cellular signal transduction pathways (RCP) (Prado et al., 2002).

RCP is predicted to inhibit signaling by both adrenomedullin (55% reduction) and CGRP (75% reduction). The presence of RCP in membrane fraction from cell culture and tissue, combined with the co-immunoprocipitation with CRLR and the RAMP protein, suggests that RCP exerts its effects at the plasma membrane (Figure 1.10).

In this thesis, the full-length cDNA of these reproduction-related genes were identified and characterized. The expression levels of these genes during ovarian development of *P. monodon* in wild and domesticated shrimp were examined. Effects of serotonin and sex steroid like progesterone and 17-β estradiol on the expression of these genes were also evaluated.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

Wild male and female *P. monodon* broodstock were wild-caught alive from the Andaman Sea (west of peninsular Thailand). These specimens were used for RT-PCR analysis, the establishment of RACE-PCR template and quantitative real-time PCR.

Commercially cultured juveniles were purchased from local farms in Chachengsao, eastern Thailand. In addition, domesticated male and female *P. monodon* juveniles (4- and 6-month-old) and broodstock (10-, 14-, and 18-month old) were obtained from the Broodstock Mutiplication Center, (BMC) Burapha University (Chantaburi,Thailand). These specimens were used for RT-PCR and quantitative RT-PCR.

For RT-PCR, ovaries and testes were dissected out from juvenile and broodstock. For tissue distribution analysis, various tissues were dissected out from wild intact female broodstock, ovaries of juveniles and testes of male broodstock. The dissected tissues was immediately placed in liquid N_2 and kept at -80 °C until needed. Hemocytes were collected from each shrimp using 10% sodium citrate (1:1 v/v) as the anticoagulant. The mixture was centrifuged at 3,000 rpm for 5 min. The resulting pellet was used for RNA extraction

For quantitative real-time PCR analysis, female juveniles and broodstock from both wild and the domestication programe were sampled. Both intact and eyestalk-ablated broodstock were acclimated under the farm conditions for 2-3 days. Ovaries of normal broodstock (N = 22) were removed and weighed. Post-spawning intact broodstock were also collected at after spawning (N = 5). For the eyestalk ablation group, shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of ablated shrimp were collected at 2-7 days after ablation (N = 28). The gonadosomatic index (GSI, ovarian weight/body weight x 100) of each shrimp was

calculated. Ovarian developmental stages were divided to previtellogenic (stage I, GSI < 2.0 %), vitellogenic (stage II, GSI >2-4%), early cortical rod (stage III, GSI > 4-6%) and mature (IV, GSI >6%) ovaries, respectively.

To confirm developmental stages of *P. monodon* ovaries, conventional histology was carried out. Tissue sections of ovaries were stained by hematoxylin and eosin (Qiu and Yamano, 2005).

2.2 RNA extraction

2.2.1 total RNA extraction

Total RNA was extracted from ovaries (or other tissues) of each shrimp using TRI REAGENT® (Molecular Research Center). A piece of tissues was immediately placed in mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI-REAGENT® (Molecular Research Center) (1 ml/50-100 mg tissue) and homogenized. Additional 500 µl of TRI REAGENT® were added. The homogenate were left for 5 minutes, before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds and left at room temperature for 15 minutes before centrifuged at 12000g for 15 minutes at 4°C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase (inclusively containing RNA) was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10-15 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 5-10 minutes at 4°C. The ethanol was removed. The RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80 °C freezer for long storage.

Total RNA was also extracted from other tissues including testes, antennal gland, eyestalks, epicuticle, gills, heart, hemocytes, hepatopancreases, intestine,

lymphoid organs, pleopods, stomach and thorasic ganglion of *P. monodon* using the same extraction procedure.

2.2.2 Measuring concentrations of nucleic acid using spectrophotometry and gel electrophoresis

2.2.2.1 Estimation of nucleic acid concentration by spectrophotometry

The concentration of nucleic acid (RNA and DNA) samples is estimated by measuring the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 40 µg /ml of single stranded RNA , 50 µg/ml of double stranded DNA and 33 µg /ml of single stranded (ss) DNA (Sambrook and Russel, 2001). Therefore, the concentration of RNA or DNA samples were estimated in µg /ml by multiplying an OD_{260} value with a dilution factor was 50, 40 and 33 for DNA, RNA and oligonucleotide, respectively. The concentration of extracted nucleic acid could be estimated as follows,

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

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

2.2.2.2 Estimation of the amount of nucleic acid by gel electrophoresis

The quality of nucleic acid sample of verifying RNA integrity after extract was estimated from motion of RNA and DNA fragments pass through an agarose gel after electrophoresis. The nucleic acid can roughly estimated on the basic of the direct relationship between the amount of nucleic acid and the level of fluorescent dye (ethidium bromide) was visualized under a UV transilluminator. Nucleic acid was run in 1% agarose gel electrophoresis prepared in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.3). at 4 V/cm. After electrophoresis, the gel was stain with ethidium bromide (0.5 ug/ml). The quality of RNA extracted was estimated from the intensity of the fluorescent band and RNA fragment size by comparing with that of *Hind* III digested λDNA (λ-*Hind* III) was marker.

2.2.3 Agarose gel electrophoresis (Sambrook and Russell, 2001)

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

2.2.4 DNase I treatment of the extracted RNA

Fifteen micrograms of total RNA were treated with DNase I (0.5 U/µg of total RNA, Promega) at 37 °C for 30 minutes, before adding phenol:chloform:isoamylalcohol (25:24:1) and vortexed for 15 seconds and left at room temperature for 10-15 minutes before centrifuge at 12,000 g for 10 minutes at 4 °C. The upper aqueous phase was collected and repeated once extraction process with chloroform:isoamylalcohol (24:1) and one with chloroform. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube for precipitation by adding one-tenth final sample volume of 3 M sodium acetate (pH 5.2) and two point five volume of cold absolute ethanol and mixed thoroughly, whereat incubated mixture at -80 °C for 30 minutes before centrifuged at 12,000 g for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of cold 75% ethanol. The RNA pellet was air-dried for 5-10 minutes and dissolved in DEPC-treated H_2O for immediately used or kept under absolute ethanol in a -80 °C freezer for long storage.

2.2.5 First strand cDNA synthesis

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

2.3 Examination of expression patterns of genes related to ovarian development by RT-PCR and tissue distribution analysis

2.3.1 Experimental animal

Table 2.1 Shrimp samples used for RT-PCR and tissue distribution analysis

Sample	Sex and stage	*N*	Average body weight ± SD (g)
Wild shrimp	Male broodstock	5	135.51 ± 21.60
	Female broodstock	5	212.77 ± 43.33
Domesticated shrimp	Male juveniles	5	29.39 ± 5.18
(6-month-old)	Female juveniles	5	36.41 ± 5.17

2.3.2 Primer design

Forward and reverse primers of each gene were designed from nucleotide sequence obtained from ESTs database of *P. monodon* except the G protein beta

subunit (G_{β}) was designed from G protein beta subunit sequences of *Litopenaeus vanamei* (GenBank accession no. AY62793) using Primer Premier 5.0. (Table 2.2).

Table 2.2 Primer sequences and the expected size of the PCR product of gene homologue of *P. monodon* initially isolated by EST analysis

Gene/Primer	Sequence	Tm (°C)	Size (bp)
1. *GTP binding protein alpha subunit, $G_{\alpha o}$* (HC-H-S01-1003-LF)			
F:	5' –CGCAACAGATACCACTAACATTC -3'	66	287
R:	5' –ACTTCAGGACAGTGCCATTCTCG -3'	70	
2. ***GTP binding protein alpha subunit, $G_{\alpha q}$*** (HC-H-S01-0866-LF)			
F:	5'- GCATCCAACACTGCTATGATCGTCG -3'	76	121
R:	5'- AGAATATCCTGCTCGGTCGGTAAGA -3'	74	
3. *GTP binding protein alpha subunit, $G_{\alpha s}$* (HC-H-S01-0866-LF)			
F:	5'- CTTTCCCTGACTTTGCTCGGTATC -3'	72	129
R:	5'- CACCACTTGCCGTGCTTATCCTTAG -3'	76	
4. *G protein beta 1 subunit, G_{β}* (GenBank Accession No. AY62793)			
F:	5'- GACAAGAGAAGGCAATGTGAGAGTG -3'	74	364
R:	5'- GGCATCATCTGATCCCGTGGCAAAT -3'	76	
5. *G protein gamma subunit, G_{γ}* (HC-V-S01-0001-LF)			
F:	5′- CTCCAGCATGAACAGGAAGACTACC -3′	76	207
R:	5'- CACTTGGCTGAAACTTCCATTTGAGGCA -3'	82	
6. *Downstream of receptor kinase, Drk* (TT-N-S01-0189-W)			
F:	5'- CTCACGCACTCTTAGGGCGAATC -3'	72	227
R:	5'- CCGTCCAGCTCTGCTCTGAACCA -3'	74	
7. *Calcitonin gene-related peptide-receptor component protein-like*, CGRP-RCP (TT-N-ST02-0097-LF)			
F:	5′- GCCAATATCGCATTTGAAACCAT -3′	68	238
R:	5′- GCTCTACCAGCCCAATTAACTCT -3′	70	
8. *Selenoprotein M, SelM* (OV-N-ST02-0024-LF and OV-N-N01-0438-W)			
F:	5′- GACATCCCACTCTTCCATAAT -3′	60	240
R:	5′- TTTCATCTACAGTTCTTCCCTC 3′	62	
9. *Elongation factor-1α, EF-1α_{500}*			
F:	5′- ATGGTTGTCAACTTTGCCCC -3′	60	500
R:	5′- TTGACCTCCTTGATCACACC -3′	60	

2.3.3 RT-PCR analysis

The amplification reactions were performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25°C, 50 mM KCl and 0.1% Triton X-100, 2.0 mM $MgCl_2$, 100 μM each of dNTPs, 0.2 μM of each primer, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES) and 100-200 ng of first strand cDNA. The reaction thermal profile of each gene was shown in Table 2.3. Five microliters of the amplification products were electrophoretically analyzed though 0.8-1.5% agarose

gel. A total of 7 gene homogues were screened. Only differential expression patterns between states of ovaries were carried out.

2.3.4 Tissue distribution analysis

Total RNA extracted from antennal gland, eyestalks, epicutical, gills, heart, hemocytes, hepatopancreases, intestine, lymphoid organs, ovaries, pleopods, stomach, and thoracic ganglion of wild female broodstock and testes of wild male broodstock *P. monodon* was reverse-transcribed using the procedure described previously.

RT-PCR was performed in a 25 μl reaction mixture containing first strand cDNA of each tissue, 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 0.1% Triton X–100, 2.0 mM $MgCl_2$, 100 μM each of dNTPs, 0.2 μM of each primer and 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES). The reaction thermal profile of each gene was shown in Table 2.3. Five microlitters of the amplification products were electrophoretically analyzed though 1.8-2.0% agarose gel.*EF-1*α_{500} was included as the internal control.

Table 2.3 Amplification conditions for interesting gene expression level from RT-PCR and tissue distribution analysis.

Gene homologue	Amplification condition	1st cDNA (ng)
GTP binding protein alpha subunit Go ($G_{\alpha o}$)		
	94°C for 3 minutes	100
	28 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
GTP binding protein alpha subunit ($G_{\alpha i}$)		
	94°C for 3 minutes	200
	28 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
GTP binding protein alpha subunit ($G_{\alpha s}$)		
	94°C for 3 minutes	100
	28 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
G protein beta 1 subunit (G_{β})		
	94°C for 3 minutes	300
	25 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	

Table 2.3 (cont.)

Gene homologue	Amplification condition	1st cDNA (ng)
G protein gamma subunit (G_γ)		
	94°C for 3 minutes	300
	25 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
Downstream of receptor kinase (Drk)		
	94°C for 3 minutes	200
	30 cycles of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
Calcitonin gene-related peptide-receptor component protein-like (CGRP-RCP)		
	94°C for 3 minutes	200
	30 cycles of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
Selenoprotein M (SelM)		
	94°C for 3 minutes	100
	23 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	
Elonggation factor-1α (EF-1α_{500}) (control)		
	94°C for 3 minutes	100
	22 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 30 seconds and	
	72°C for 7 minutes	

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

2.4.1 Purification of mRNA

Total RNA was extracted from ovaries of wild broodstock-sized of female *P. monodon* using TRI-REAGENT®. Messenger (m) RNA was further purified using a QuickPrep *micro* mRNA Purification Kit (GE Healthcare).

Four hundred microliters of the extraction buffer were added to a microcentrifuge tube containing 25 µl of total RNA (150 µg) and mixed by pipetting. Two volumes (0.8 ml) of the elution buffer were added and mixed thoroughly. The mixture was centrifuged at 14,000g for 1 minute. Concurrently, the tube containing 1 ml of oligo(dT)-cellulose for each purification was centrifuged at the same speed for

1 minute. The supernatant was removed. The homogenate was transferred into the microcentrifuge tube containing the oligo(dT)-cellulose pellet. The tube was gently inverted to resuspend the oligo(dT)-cellulose for 3 minutes and centrifuged at 14,000g for 15 seconds at room temperature. The supernatant was carefully removed. The high salt buffer (1 ml) was added to a microcentrifuge tube and spun for 15 seconds at 14,000g. The supernatant was carefully removed. The pellet was washed repeated four more times, as described above. The low salt buffer (1 ml) was added to the oligo(dT)-cellulose pellet. The tube was inverted and spun at 350 g for 2 minutes. This wash was repeated once. The pellet from the final wash was resuspended in 0.3 ml of the low salt buffer. The slurry was transferred to a MicroSpin column and spun for 5 seconds. The flow-though solution was discarded. The low salt buffer (0.5 ml) was added and further spun for 10 seconds. This step was repeated twice. The column was then placed into a sterile 1.5 ml microcentrifuge tube and briefly centrifuged. The mRNA was eluted out by an addition of 0.2 ml of the pre-warmed elution buffer (65°C) to the top of column and centrifuged at 14,000g for 5 seconds. This step was repeated once before precipitated mRNA pellet by added 10 µl of Glycogen solution and 50 ul of K-acetate solution and two point five volume of cold absolute ethanol and mixed thoroughly, whereat incubated mixture at -20 °C for 30 minutes before centrifuged at 14,000 g for 5 minutes at 4 °C. The RNA pellet was washed with 1 ml of cold 75% ethanol and left on ice for 30 minutes before centrifugation at 14,000 g for 5 minutes. Alternatively, the mRNA pellet was kept under absolute ethanol in a -80 °C freezer (should not exceed 2 weeks).

2.4.2 Preparation of the 5′ and 3′ RACE template

Full length cDNAs of interesting gene homologues were characterized using a SMART™ RACE cDNA Amplification Kit (Clontech). RACE-Ready cDNA was prepared by combining 1.5 µg of ovarian mRNA with 1 µl of 5′-CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5′- RACE-PCR or 1.5 µg of ovarian mRNA with 1 µl of 3′-CDS primer 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 cooled on ice for 2 minutes. The reaction tube was spun briefly. After that, 2 µl of 5X First-Strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP

Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reactions were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom.

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

2.4.3 Primer designed for RACE-PCR

Gene-specific primers (GSPs) were designed from interesting transcripts obtained from ESTs database of *P. monodon* except G protein beta subunit was designed from G protein beta subunit sequences of *Litopenaeus vanamei* (GenBank Accession No. AY62793). The antisense and/or sense primers were designed for 5′- and 3′- RACE-PCR, respectively (Table 2.5). Internal forward and/or reverse primers were also desigjned for further sequencing of the internal regions of large RACE-PCR fragments (Table 2.5).

Table 2.4 Primer sequences for the first strand cDNA synthesis for RACE-PCR

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

Table 2.5 Gene-specific (GSPs) and internal primers used for characterization of the full length cDNA of functionally important gene homologues in *P. monodon* using RACE-PCR

Gene/Primer	Sequence	Tm (°C)
GTP binding protein alpha subunit Go ($G_{\alpha o}$)		
5′RACE	5'- CGCAACAGATACCACTAACATTC -3'	66
Internal 5'RACE	5'- TAGATGAGGCGGTGCAACTTAGTAGAGG -3'	84
3′RACE	5'- ACTTCAGGACAGTGCCATTCTCG -3'	70
GTP binding protein alpha subunit ($G_{\alpha q}$)		
5′RACE	5'- GCGACCAGGAAAATGATGGAGGTGACG -3'	84
3′RACE	5'- AGAATATCCTGCTCGGTCGGTAAGA -3'	74
GTP binding protein alpha subunit ($G_{\alpha s}$)		
3′RACE	5'- CACCACTTGCCGTGCTTATCCTTAG -3'	76
G protein beta 1 subunit (G_{β})		
5′RACE	5'- GACAAGAGAAGGCAATGTGAGAGTG -3'	74
3′RACE	5'- GGTCACACTGGTTACCTAAGTTGCT -3'	74
G protein gamma subunit (G_{γ})		
5′RACE	5′- CTCCAGCATGAACAGGAAGACTACC -3'	76
Downstream of receptor kinase (Drk)		
3′RACE	5'- GGGATTTGAACTTGACGACCCAGAGG -3'	80
Calcitonin gene-related peptide-receptor component protein-like (CGRP-RCP)		
3′RACE	5′- GCCAATATCGCATTTGAAACCAT -3′	68

2.4.4 RACE-PCR and cloning of the amplification products

The master mix which is sufficient for 5′- or 3′- RACE-PCR was prepared as described in Tables 2.6. The 5′- and 3′- RACE-PCR were set up as described in Table 2.7.

Table 2.6 Composition of 5′- and 3′- RACE-PCR

Component	5′-RACE Sample	3′-RACE Sample	GSP1+UPM (-Control)	GSP2+UPM (-Control)
5′-RACE-Ready cDNA	3.00 µl	-	-	-
3′-RACE-Ready cDNA	-	3.00 µl	-	-
UPM (10X)	2.5 µl	2.5 µl	2.5 µl	2.5 µl
GSP1 (10 µM)	1.0 µl	-	1.0 µl	-
GSP2 (10 µM)	-	1.0 µl	-	1.0 µl
10X BD adventage® 2 PCR Buffer	2.5 µl	2.5 µl	2.5 µl	2.5 µl
10 µM dNTP mix	0.5 µl	0.5 µl	0.5 µl	0.5 µl
50X BD Advantage® 2 polymerase mix	0.5 µl	0.5 µl	0.5 µl	0.5 µl
H_2O	Up to 25 µl	Up to 25 µl	Up to 25 µl	Up to 25 µl
Final volume	25µl	25µl	25µl	25µl

Table 2.7 The amplification conditions for RACE-PCR of various gene homologues of *P. monodon*

Gene homologue	Amplification condition
GTP binding protein alpha subunit Go ($G_{\alpha o}$)	
5′RACE	94 °C for 3 minutes
	28 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 2 min and 72 °C for 7 minutes
Internal 5′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes
3′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes
GTP binding protein alpha subunit ($G_{\alpha q}$)	
5′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes
3′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes
GTP binding protein alpha subunit Gs ($G_{\alpha s}$)	
3′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1 minutes and 72°C for 7 minutes
G protein beta 1 subunit (G_{β})	
5′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes
3′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes
	5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes
	28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes

Table 2.7 (cont.)

Gene homologue	Amplification condition
G protein gamma subunit (G_γ)	
5′RACE	5 cycles of 94°C for 30 seconds and 72°C for 1 minutes 5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 1 minutes 28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1 minutes and 72°C for 7 minutes
Downstream of receptor kinase (Drk)	
3′RACE	5 cycles of 94°C for 30 seconds and 72°C for 2 minutes 5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 2 minutes 28 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes and 72°C for 7 minutes
Calcitonin gene-related peptide-receptor component protein-like (CGRP-RCP)	
3′RACE	94 °C for 3 minutes 28 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 2 min and 72 °C for 7 minutes

2.4.4.1 Elution of RACE-PCR fragments from agarose gel

After electrophoresis, desired individual RACE-PCR bands were excised from agarose gels (200 - 300 mg) using a sterile scalpel. RACE-PCR product was extracted from the gel pieces using illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare). Three to five hundred microliters of the Capture buffer Type 3 was added to the sample. The mixture was incubated at 55-60 °C for 15-30 minutes until the gel slice was completely dissolved. During the incubation period, the tube was inverted every 3 minutes. An GFX MicroSpin column was placed in a collection tube and removed mixture was applied into the GFX MicroSpin column and inclubated at room temperature for 1 minute before centrifuged at 13,500 rpm for 30 seconds. The flow-through was discarded. The GFX MicroSpin column was placed back in the collection tube. The column was washed by the addition of 500 µl of the ethanol-added Wash buffer type 1 and centrifuged at 13,500 rpm for 30 seconds. After discarding the flow-through, the GFX MicroSpin column was centrifuged for 2 minutes at the full speed (14,000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 10-15 µl of the Elution buffer type 4 was added to the center of the column matrix. The column was left at room

temperature for 2 minutes before centrifuged for 2 minutes at the full speed to recover the gel-eluted DNA.

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

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

2.4.4.3 Preparation of competent cell

A single colony of *E. coli* JM 109 was inoculated in 3 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37 °C for 12-16 h. One milliliter of starting culture was then inoculated into 50 ml of LB broth and continued culture at 37 °C with vigorous shaking to OD600 of 0.4 to 0.6. The cells were briefly chilled on ice for 30 minutes and recovered by centrifugation at 3000 g for 15 minutes at 4 °C. The pellets were resuspended by pipetting in 30 ml of ice-cold $MgCl_2/CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and on ice for 45 min centrifuged as above. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and the suspension was divided into 100 µl aliquots. These competent cells was either used immediately or stored at -80 °C for subsequently used.

2.4.4.4 Transformation of the ligation product to *E.coli* host cell

The competent cells were thawed on ice for 5 minutes. Five microliters of the ligation mixture were added and gently mixed by pipetting. The mixture was left on ice for 30 minutes. During the incubation period, the ice box was gently moved forward and backward a few times every 5 minutes. The transformation reaction was heatshocked at 42 °C by water bath (without shaking) for exactly 45 seconds. The reaction tube was immediately placed on ice for 5 minutes. The mixture were removed from the tubes and added to a new tube containing 1 ml of pre-warmed SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with

shaking at 37 °C for 60-90 minutes. The mixture were centrifuged at 8,000 rpm for 20 seconds at room temperature. The pellet was resuspended in 100 µl of the SOC medium and spread onto a selective LB agar plates (containing 50 µg/ml of amplicillin, 20 µg/ml of X-gal and 25 µg/ml of IPTG) and further incubated at 37 °C for 12-16 h (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.4.4.5 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 15 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 mM of each dNTP, 2 mM $MgCl_2$, 0.1 µM both primer of pUC1 (5'- CCGGCTCGTATGTTGTGTGGA -3') and pUC2 (5'- GTGGTGCAAGGCGATTAAGTTGG -3'), 0.5 unit of Dynazyme™ DNA Polymerase (FINNZYMES). A colony was picked by a pipette tip or steriled toothpick and used as DNA template in the reaction. PCR was carried out in a thermocycler consisting of predenaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for suitable (1 kb of double DNA needed 1 minute). The final extension was carried out at the same temperature for 7 minutes. The colony PCR products were electrophoresed through a 1.2% agarose gel and visualized after ethidium bromide staining. The colony PCR products containing the insert were separately digested with *EcoR* I (Promega) in a 15 µl reaction volume containing 1X buffer (90 mM Tris-HCl, 10 mM $MgCl_2$ and 50 mM NaCl, pH 7.5 for *E.coR* I, 0.1 mg/ml BSA, 2 units of each enzyme and 4 µl of the colony PCR product. The reaction mixture was incubated at 37 °C overnight. The reaction was analyzed by 1.0-1.5% agarose gel electrophoresis.

2.4.4.6 Extraction of recombinant plasmid DNA

Plasmid DNA was isolated using plasmidPrep Mini Spin Kit (GE healthcare). A discrete white colony was inoculated into a sterile culture tube containing 3 ml of LB broth supplemented with 50 µg/ml of amplicillin and incubated with shaking (250 rpm) at 37 °C for 12-16 h. The culture was transferred into a sterile 1.5 ml 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 addition of 175 µl of the Lysis buffer type 8 and mixed immediately by gentle invertion (approximately 5 times) until solution clear. The Lysis buffer type 9 (350 µl) was added to neutralize the alkaline lysis step and and mixed immediately by gentle invertion until the precipitate was envenly dispersed. The mixture was then centrifuged at 14,000 rpm for 15 minutes at 15 °C. The supernatant was removed in new microcentrifuge tube before centrifuge repeated. The illustra^{TM} plasmid mini column was placed in a collection tube and the clear lysate was applied into the illustra^{TM} plasmid mini column and centrifuged at 13,500 rpm for 30 seconds. The flow-through was discarded. The illustra^{TM} plasmid mini column was placed back in the collection tube. The column was washed by the addition of 400 µl of the Wash buffer type 1 and centrifuged at 13,500 rpm for 30 seconds. After discarding the flowthrough, 400 µl of the ethanol-added Wash buffer was added and centrifuged as above. The illustra^{TM} plasmid mini column was further centrifuged for 2 minutes at the full speed (14,000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 30 µl of the Elution buffer type 4 was added at the center of the column matrix. The column was left at room temperature for 2 minutes before centrifuged for 2 minutes at the full speed to recover the purified plasmid DNA. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.4.4.7 DNA sequencing

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

2.4.4.8 The full length cDNA analysis

After RACE products have been characterized by partial DNA sequence from 5´- or 3´-RACE PCR sequencing. The overlapping of nucleotide sequences of RACE product with nucleotide sequence obtained from ESTs database of *P. monodon* was

assembly. The full length cDNA confirmed by compare nucleotide sequences were blasted in the GenBank (http://www.ncbi.nlm.nih.gov/blast) using Blast*X* (translated protein similarity against the nr database) and identified domain of protein using SMART analysis (http://smart.embl-heidelberg.de/).

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

Expression levels of several transcripts related to ovarian development were examined using quantitative real-time PCR analysis. *EF-1*α_{214} was used as the internal control.

2.5.1 Experimental animals

2.5.1.1 Wild and domesticated *P. monodon* used for expression analysis of various during ovarian development

Wlid female *P. monodon* broodstock were sampled and prepared as mentioned earlier; intact (*N* = 27, average body weight 217.07 ± 47.10 g) and eyestalk-ablated broodstock (*N* = 28, average body weight 209.97 ± 39.45 g). Ovaries of intact broodstock sample were divided to stage I, GSI < 2.0% (*N* = 4), stage II, GSI >2-4%, (*N* = 4), stage III, GSI >4-6% (*N* = 5), stage IV, GSI >6% (*N* = 9) and post-spawned normal broodstock shrimps (*N* = 5). For the eyestalk ablation group were divided to stage I (*N* = 5), stage II (*N* = 9), stage III (*N* = 9) and stage IV (*N* = 5) which GSI same in normal group.

The domesticated female *P. monodon* examined were juveniles and broodstock. Juvenile shrimp were 4-month-old (*N* = 6, average body weight approximately 20 g) and 6-month-old (*N* = 4, average body weight of approximately 30 g). Female bloodstock examined were 10-month-old (*N* = 6, average body weight = 46.68 ± 3.55 g and GSI < 0.5-1%), 14-month-old (*N* = 12, average body weight 64.06 ± 3.20 g and GSI = 0.5-1.5%) and 18-month-old (*N* = 4, average body weight 77.12 ± 3.10 g and GSI = 0.5-1.8%).

2.5.1.2 The domestication of female broodstock received treatment.

2.5.1.2.1 Effects of serotonin on expression of interesting genes in ovaries of *P. monodon*

Eighteen-month-old female of *P. monodon* (with the average body weight of 77.12 ± 3.10 g) were acclimated for 4 day before treated with serotonin (50 μg g^{-1} body weight). Eight groups of shrimp (6 for each group) are single injected intramuscularly into the first abdominal segment of each shrimp and specimens are collected at 0 h, 1, 3, 6, 12, 24, 48 and 72 h post injection. The normal saline control (0.85% at 0 hr, control A) is also included. Ovaries of each sample were sampling and immediately placed in liquid N_2. The samples were stored at -80°C prior to RNA extraction and first-stand cDNA synthesis.

2.5.1.2.2 Effects of progesterone on expression of interesting genes in ovaries of *P. monodon*

Fourteen-month-old female of *P. monodon* (average body weight of 64.06 ± 3.20 g) were acclimated for 4 days before treated with progesterone (0.1 μg/g body weight). Four groups of shrimp (*N* = 6 for each group) are single injected intramuscularly into the first abdominal segment of each shrimp and specimens are collected at 12, 24, 48 and 72 h post injection. The absolute ethanol was vehicle control (at 12 and 72 h). Ovaries of each sample were sampling and immediately placed in liquid N_2. The samples were stored at -80°C prior to RNA extraction and first-stand cDNA synthesis.

2.5.1.2.3 Effects of 17β-estradiol on expression of interesting genes in ovaries of *P. monodon*

Fourteen-month-old female broodstock of *P. monodon* (average body weight of 33.9 ± 6.40 g) were acclimated for 7 days (25 ppt seawater).Three group of female shrimp were injected with 17β-estradiol (0.01 μg/g body weight, *N* = 6 for each group) intramuscularly into the first abdominal segment. The second and third injection is carried out at 3 and 6 days post initial injection and the specimens were collected at 7, 14 and 28 days post first injection. The non-injected shrimp were collected at 0, 7, 14 and 28 days. The 5% ethanol was included as the vehicle control

and eyestalk-ablated shrimp were collected at 7, 14 and 28 days. Ovaries of each sample were sampling and immediately placed in liquid N_2. The samples were stored at -80°C prior to RNA extraction and first-stand cDNA synthesis and used for quantitative real-time PCR analysis**.**

2.5.2 Primers and construction of the standard curve

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 (Figure 2.1). A size of the expected PCR product size was approximately 100-250 bp (Table 2.8).

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

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

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

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

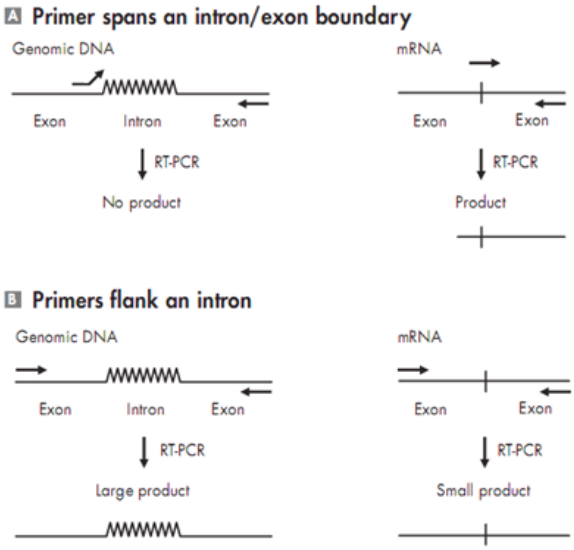


Figure 2.1 Two approaches of primer design to prevent amplification of the residual genomic DNA in the template for quantitative real-time RT-PCR based on primers spanning exon-intron boundaries (A) or primers flanking an intron (B).

2.5.3 Quantitative real-time PCR analysis

The target transcripts and the internal control *EF-1α*$_{214}$ 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 suitable concentration and reaction thermal profile for SYBR Green real-time PCR of each gene was shown in Table 2.9. The real-time RT-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 shrimp possessing different ovarian development (or treatment) were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

Table 2.8 Final concentration of primer and nucleotide sequences of primer use for quantitative real-time PCR analysis

Gene	Sequence primer	Tm (°C)	Size (bp)	Final conc. primer (μM)
PmG$_{\alpha q}$	F: 5'- GCGACCAGGAAAATGATGGAGGTGA CG -3'	84	121	0.2
	R: 5'- AGAATATCCTGCTCGGTCGGTAAGA -3'	74		0.2
PmG$_{\alpha s}$	F: 5'- CTTTCCCTGACTTTGCTCGGTATC -3'	72	109	0.3
	R: 5'- CACCACTTGCCGTGCTTATCCTTAG-3'	76		0.3
PmDrk	F: 5'- CTCACGCACTCTTAGGGCGAATC -3'	72	227	0.15
	R: 5'- CCGTCCAGCTCTGCTCTGAACCA -3'	74		0.15
PmCGPR-RCP	F: 5'- GCCAATATCGCATTTGAAACCAT -3'	64	238	0.2
	R: 5'- GCTCTACCAGCCCAATTAACTCT- 3'	70		0.2
PmSelM	F: 5′- GACATCCCACTCTTCCATAAT -3′	60	240	0.2
	R: 5′- TTTCATCTACAGTTCTTCCCTC 3′	62		0.2
EF-1α$_{214}$ **(control)**	F: 5´- GTCTTCCCCTTCAGGACGTC-3´	64	214	0.3
	R: 5´- CTTTACAGACACGTTCTTCACGTTG -3´	72		0.3

Table 2.9 Amplification condition for interesting gene expression level in ovaries of *P. monodon* by quantitative real-time PCR

Gene	1st cDNA (ng)	Amplification condition
PmG$_{\alpha q}$	100	95°C for 10 minutes 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds and at 72°C for 30 seconds. Melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds.
PmG$_{\alpha s}$	100	95°C for 10 minutes 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds and at 72°C for 20 seconds. Melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds.
PmDrk	200	95°C for 10 minutes 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds and at 72°C for 30 seconds. Melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds.

Table 2.9 (cont.)

Gene	1st cDNA (ng)	Amplification condition
PmCGPR-RCP	50	95°C for 10 minutes 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds and at 72°C for 30 seconds. Melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds.
PmSelM	50	95°C for 10 minutes 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds and at 72°C for 30 seconds. Melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds.
EF-1α$_{214}$ (control)	5	95°C for 10 minutes 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds and at 72°C for 30 seconds. Melting curve analysis was 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds.

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

2.6.1 Primers design

Forward and reverse primers were designed to amplify the full-length ORF of each gene (Table 2.9). The forward primer and reverse primer containing restriction enzyme recognized sites and six Histidine residues encoded nucleotides were illustrated in Table 2.9.

2.6.2 Construction of recombinant plasmids in cloning and expression vectors

The full-length ORF of interesting gene 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 0.5 μM of each forward primer and reverse primer were containing restriction enzyme recognize site and 6-repeated Histidine encoded nucleotides with reverse primer, 0.75-1.5 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 25-28 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, extension at 72°C for suitable and final extension at 72°C for 7 minutes.

The amplification product was analyzed by agarose gel electrophoresis and the gel-eluted product was digested with appropriate restriction enzymes. The digested DNA fragment was again analyzed by agarose gel electrophoresis and the gel-eluted product was ligated with pET-17b (for *PmDST*) 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 *PmDST* was subsequently transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL and *E. coli* BL21 (DE3)plysS competent cells, respectively.

Table 2.10 Nucleotide sequences of primers used for *in vitro* expression of *CGRP-RCP* and *DST* of *P. monodon*

Primer	Sequence
Full-length cDNA *PmCGRP-RCP*	F: 5'- GCAGCATCAACACAATCGTCCAGG- 3' R: 5'- GCAAGAACATCAAATACAATCAATA- 3'
Full-length cDNA containing *restriction* site and 6 repeated-Histidine encoded nucleotides	
PmCGRP-RCP- *Nde* I	F: 5'- CGGCATATGGAAGTGTTAAATTG -3'
PmCGRP-RCP- 6His and *Bam* HI	R: 5'- CGGGGATCCCTAATGATGATGATGATGATGTTTCC CTGGTGTA -3'
PmDrk-*Nde* I	F: 5'- CGGCATATGGAGGCGATAGCAAAAC -3'
PmDrk-6His and *Bam* HI	R: 5'- CGGGGATCCCTAATGATGATGATGATGATGGGTG TGGTAGGGAGCC -3'

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

2.6.3 Expression of recombinant proteins

A single colony of recombinant *E. coli* BL21-CodonPlus (DE3)-RIPL or *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 microlitres of the overnight cultured was transferred to 50 ml of LB medium containing 50 µg/ml ampicillin 50 µg/ml chloramphinical and further

incubated to an OD_{600} of 0.4-0.6. After IPTG induction (1.0 mM final concentration), appropriate volume of the culture corresponding to the OD of 1.0 was time-interval taken (0, 1, 2, 3, 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, 50 ml of the IPTG induced-cultured cells at the most suitable time-interval were taken (6 hours or overnight at 37°C or lower), harvested by centrifugation 5000 rpm for 15 minutes and resuspended in the lysis buffer (0.05 M Tris-HCl; pH 7.5, 0.5 M Urea, 0.05 M NaCl, 0.05 M EDTA; pH 8.0 and I mg/ml lysozyme). The cell wall was broken by sonication using Digital Sonifier® sonicator Model 250 (BRANSON). The bacterial suspension was sonicated 2-3 times at 15-20% amplitude, pulsed on for 15 seconds and pulsed off for 15 seconds in a period off 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.

2.6.4 Detection of recombinant proteins

Recombinant protein was analyzed in 15% SDS-PAGE. The electrophoresed proteins were transferred to a PVDF membrane (Hybond P; GE Healthcare) (Towbin, 1979). The membrane was washed three times with 1X Tris-buffer saline tween-20 (TBST; 25 mM Tris, 137 mM NaCl, 2.7 mM KCl and 0.05% tween-20) for 10 minutes, blocked with 20 ml of a blocking buffer (1.0 g of BSA in 20 ml of 1X TBST) and incubated for 1 hour at room temperature with gentle shaking. The membrane was washed three times in 1X TBST and incubated with diluted Mouse Anti-His antibody IgG2a (GE Healthcare; 1:5,000) in the blocking buffer for 1 hour. The membrane was incubated with diluted goat anti-mouse IgG (H+L) conjugated with alkaline phosphatase (Promega, U.S.A.; 1:10,000) in the blocking buffer for 1 hour. Visualization of immunoreactional signals was carried out by incubating the membrane in NBT/BCIP (Roche) as a substrate. The color reaction was stopped by transferring the membrane into water.

2.6.5 Purification of recombinant proteins

Recombinant protein was purified by using a His GraviTrap kit (GE Healthcare). Initially, 1 litre of IPTG-induced cultured at the optimal time and appropriate temperature was harvested by centrifugation at 7,500 rpm for 10 minutes. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), sonicated and centrifuged at 7,500 rpm for 45 minutes at 4 °C. The soluble and insoluble fractions were separated. Soluble fraction composed of the recombinant protein was loaded into column. The column was washed with 10 ml of binding buffer containing 20 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), 5 ml of the binding buffer containing 40 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) and 5 ml of the binding buffer containing 80 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 80 mM imidazole, pH 7.4). The recombinant protein was eluted with 6 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Each fraction of the washing and eluting step were analyzed by 15% SDS-PAGE and western blotting. The purified proteins were stored at 4^{o}C or -20^{o}C for long term storage.

2.6.6 Polyclonal antibody production

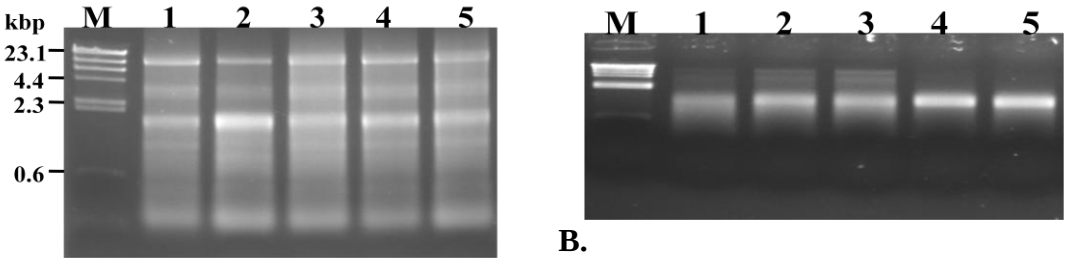
Polyclonal antibody against PmDrk was immunologically produced from the purified rPmDrk protein in a rabbit by Faculty of Associated Medical Sciences, Changmai University. The anti-PmDrk titer was examined direct enzyme-link immunosorbent assay (ELISA).

CHAPTER III

RESULTS

3.1. Total RNA extraction and first strand cDNA synthesis

Total RNA from ovaries, testes and other tissues of *P. monodon* were extracted. The quality and quantity of total RNA were determined by spectrophotometry and agarose gel electrophoresis (Figure 3.1). The ratio of OD_{260}/ OD_{280} of the extracted RNA was 1.8-2.0 indicating that its quality was acceptable for further applications. Agarose gel electrophoresis showed discrete ribosomal RNA bands reflecting good quality of total RNA. The first strand cDNA was successfully synthesized as illustrated by 1.0% agarose gel electrophoresis (Figure 3.1).



A. **B.**

Figure 3.1 A 1.0 % ethidium bromide-stained agarose gel showing the quality of total RNA extracted from ovaries of *P. monodon* broodstock (A) and the first stand cDNA synthesized from DNA-free total RNA of ovaries (B). Land M = λDNA-*Hind* III; Lanes 1-5 = total RNA (A) and the first strand cDNA from ovaries of different individuals of *P. monodon* broodstock.

3.2 Isolation and characterization of the full-length cDNA of reproduction-related transcripts of *P. monodon*

Several transcripts functionally involved in the signal transduction pathway of oocyte maturation were further characterized by RACE-PCR. These transcripts were

previously identified by EST analysis (http://.pmonodon.biotec.or.th/) and their matched gene homologues play the important roles in oocyte maturation.

3.2.1 The full-length cDNA of *PmG$_{\alpha o}$*

The partial cDNA sequence of *PmG$_{\alpha o}$* was initially obtained from EST analysis of the hemocyte cDNA library of *P. monodon* (Fig. 3.2A and B). It significantly matched *GTP binding protein alpha subunit Go* of *M. japonicas (E*-value = 1e-26). The primary 5'- and 3'RACE-PCR of *PmG$_{\alpha o}$* were further carried out for isolation of the full-length cDNA of this transcript. The positive amplification product of 1500 and 1000 bp in size were obtained (Fig. 3.3). The RACE-PCR fragments were cloned and sequenced for both directions (Fig. 3.4A and B). Nucleotide sequences of RACE-PCR products and the original EST were assembled (Fig. 3.4).

The full-length cDNA of *PmG$_{\alpha o}$* was 2407 bp in length containing an ORF of 1065 bp corresponding to a polypeptide of 354 amino acids. The 5' and 3'UTRs of *PmG$_{\alpha o}$* were 353 and 968 (excluding the poly A tail), respectively. The poly A addition signal (AATAA) was located at 233 nucleotides upstream from the poly A tail (Fig. 3.5A). The closest similarity to *PmG$_{\alpha o}$* was *GTP binding protein alpha subunit Go* of *M. japonicas (E*-value = 0.0, Fig. 3.5B). The calculated p*I* and molecular weight of the deduced PmG$_{\alpha o}$ protein was 5.09 and 40.47 kDa, respectively. The predict G protein alpha subunit (G_alpha) was found at positions 13-353 (*E* – value = 6.46e-220) of the deduced PmG$_{\alpha o}$ protein. Two predicted *N*-glycosylation sites were found at positions 152-154 (NDS) and 312-314 (NKS), respectively.

A.

TGAAGCAGCTGCTTACATCCAAGCCCAGTTTGAAGCAAAGAACAAATCAACGTCCAAAGAGATCTACTG
TCACATGACTTG*CGCAACAGATACCACTAACATTC*AGTTTGTATTTGATGCAGTAACAGATGTGATCAT
AGCAAACAACCTACGTGGATGTGGCCTCTAC**TAA**GTTGCACCGCCTCATCTAGATTGTGTGTTCAGTGT
AGAGAAAGATGGTACAGTCGTGGTCTAGCCTCCCACTCCAGATAATAATCTTGCCTCTCTTGCAAGGAA
TTAGTCAGGAGAATTGGCTAGTTGTACCTTGTACACCATTATCTCCTCTCCCTTTGGAGAGTTCTAGGC
CGAGAATGGCACTGTCCTGAAGTTTTATCATACGTGTTCATGGTGCGGTCATTGTCATACAGGAACGGA
TGCCATTAAAGTGATTTCTAGGTGCATAACTGACACAGACCGCTTTGGCCTTTGTAGCCACCCATTTCT
AGATCATGGAAGAATTGCATGAGTGATTATGAATTGAGCTTGTTAAAGACTTTCCAGCCAACCCCACTT
GTCTTGTATAGTACCAAGTTTAATTATCACTTCCTTACCAAATGAAGCCTTACAGTTCTCGTTATCTGG
TACAAGTTCGATAAAAAACAAATGTTGATGATTTTGAATCAGAAGGAAAAGCTTAAATTACTGGTTCAT
AGAGATGGAACATTAATGATTTTGAATTATTTGGTGCTTTCCATGCACTGATGAAGAAATGTACTCTAG
CTTTTGATTGATACCTTGAAGCTTTTGATAACCAAAATAAGACATAGCAGTTACCTACCTTAGTGTCTG
AGGGAAATGATATATTTTCCAAACCAGTTATATGTGCAGATGAGACGTGGATATCAATGAGGGCCCAAA
ACCTTAATAACAAAAATCCATTGAATGGCCAAACATTGGTGGCTTGGAGCAGTGAGTGTCATTCTTTCT
AGTCATGTCTTTAATCTTAGTCATTTGTCACAAAAGTAAGTGAGTGGAATACAACAATATATGTACACT
GGCAGAGTGTGGCGAGGTGATGTACAGATGTTGCCATTGCTGCTCTATTGTTTTTCTTCTGATATCTTG
ATTCTAATGGGCCAGGTTTCTAAGATCTC

B.

```
GTP binding protein alpha subunit Go [Marsupenaeus japonicus] Length=354 Score =  117
bits (293),  Expect = 3e-24 Identities = 56/56 (100%), Positives = 56/56 (100%), Gaps
= 0/56 (0%) Frame = +2
Query  2    EAAAYIQAQFEAKNKSTSKEIYCHMTCATDTTNIQFVFDAVTDVIIANNLRGCGLY  169
            EAAAYIQAQFEAKNKSTSKEIYCHMTCATDTTNIQFVFDAVTDVIIANNLRGCGLY
Sbjct  299  EAAAYIQAQFEAKNKSTSKEIYCHMTCATDTTNIQFVFDAVTDVIIANNLRGCGLY  354
```

Figure 3.2 Nucleotide sequence (A) and BlastX results (B) of an EST from the hemocyte cDNA library of *P. monodon* that significantly matched *M. japonicas* G_{ao}. The positions of 3' (underlined and italicized) and 5' RACE-PCR (underlined) primers are shown. The putative stop codon is illustrated in boldface.

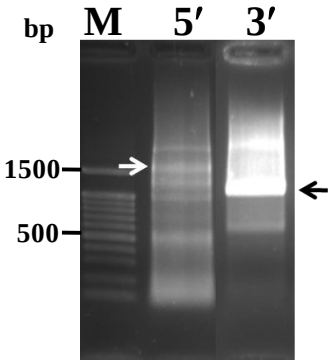


Figure 3.3 5'- and 3'RACE-PCR products of *Pm*G_{ao}. Arrows indicate RACE-PCR products those were cloned and sequenced. Lane M is 100 bp DNA ladder.

A.

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTGGGGGCAAGATATACAATGCTCAT
GTTGGAACTACATGTCATCAGTGCAGGCAGAAAACTGTCGATACTAAGACTGTATGTCGTTCTGGAGAG
TGTTGGTGTGCCGTGTGAGAAGTGAACTTGTAGCTCACTATGTAGTGGCCACTTGGTGTTACAGGTGGA
GAGCTTGATTAGCGGTGTGTGAAGAGATGATCATGCCGTCATAACAAGGTGGCAGTGTGCGTCATCGCG
TAGAAGCAGAGTGGCGCGTAGGCCTAAGGTGACTCGGAGTTCGTGTGAAATCTCCAGCAGGTGTCCGAG
GACCTGTGGACACCAGAATACCCGAGGGCGTGTGAGACATTAAAGCCAGCGCGATGGGGTGTACGATGT
CGATGGAGCAGAAGGCGGCCCTGGCCAAGAGTCGCCAGATCGACCTCATCCTCCAGGAGGACGGCGACC
GCATGAGAAGGGACGTTAAACTGCTGTTGCTAGGAGCGGGAGAGTCCGGCAAAAGTACCATCGTAAAAC
AAATGAAGATTATTCACGAGTCTGGGTTCACGAGCGAAGACTTCAAGCAGTACCGGCCGGTGGTGTATT
CCAACACCATACAGTCGTTGGTGGCCATTCTCCGCGCCATGCCCAACCTCGGCATATCCTTCGGTAACA
ACGAGCGGGAGCCCGACGCCAAGATGGTCTTCGATGTCATATCCCGTATGGAGGACACAGAGCCCTTCT
CCGAGGAGCTCCTGGCCGCCATGGAGCGTCTGTGGGCCGACACGGGCGTCCAGGAATGTTTCGGCCGCT
CCAACGAGTACCAGCTGAACGACTCCGCCAAGTATTTCTTGGACGATCTGGACCGCCTGGGAGCTAAGG
ACTATCAGCCAACCGAACAAGATATCCTACGAACTCGCGTCAAGACCACTGGTATCGTCGAGGTCCACT
TCTCTTTCAAGAACCTTAACTTTAAGTTATTTGATGTAGGAGGACAGCGCTCTGAACGAAAGAAGTGGA
TTCACTGTTTTGAAGATGTGACAGCCATTATCTTCTGTGTGGCCATGTCAGAATACGATCAAGTGCTGC
ATGAAGACGAGACTACGAATCGCATGCAAGAATCTCTTAAGTTATTTGACTCCATCTGCAACAACAAAT
GGTTTGGGGATACTTCTATTATTCTGTTCCTAAACAAGAAAGATCTTTTTGAAGAGAAGATAAAGAAGA
GTCCACTTACCATATGTTTCCAAGAATATTCAGGTGCCCAAGAGTATGGTGAAGCAGCTGCTTACATCC
AAGCCCAGTTTGAAGCAAAGAACAAATCAACGTCCAAAGAGATCTACTGTCACATGACTTGCGCAACAG
ATACCACTAACATTCAGTTTGTATTTGATGCAGTAACAGATGTGATCATAGCAAACAACCTACGTGGAT
GTGGCCTCTACTAAGTTGCACCGCCTCATCTAGATTGTGTGTTCAGTGTAGAGAAAGATGGTACAGTCG
TGGTCTAGCCTCCCACTCCAGATAATAATCTTGCCTCTCTTGCAAGGAATTAGTCAGGAGAATTGGCTA
GTTGTACCTTGTACACCATTATCTCCTCTCCCTTTGGAGAGTTCTAGGC**CGAGAATGGCACTGTCCTGA**
AGT

B.

CGAGAATGGCACTGTCCTGAAGTTTTATCATACGTGTTCATGGTGCGGTCATTGTCATACAGGAACGGA
TGCCATTAAAGTGATTTCTAGGTGCATAACTGACACAGACCGCTTTGGCCTTTGTAGCCACCCATTTCT
AGATCATGGAAGAATTGCATGAGTGATTATGAATTGAGCTTGTTAAAGACTTTCCAGCCAACCCCACTT
GTCTTGTATAGTACCAAGTTTAATTATCACTTCCTTACCAAATGAAGCCTTACAGTTCTCGTTATCTGG
TACAAGTTCGATAAAAAACAAATGTTGATGATTTTGAATCAGAAGGAAAAGCTTAAATTACTGGTTCAT
AGAGATGGAACATTAATGATTTTGAATTATTTGGTGCTTTCCATGCACTGATGAAGAAATGTACTCTAG
CTTTTGATTGATACCTTGAAGCTTTTGATAACCAAAATAAGACATAGCAGTTACCTACCTTAGTGTCTG
AGGGAAATGATATATTTTCCAAACCAGTTATATGTGCAGATGAGACGTGGATATCAATGAGGGCCCAAA
ACCTTAATAACAAAAATCCATTGAATGGCCAAACATTGGCGGCTTGGAGCAGTGAGTGTCATTCTTTCT
AGTCACGTCTTTAATCTTAGCCATTTGTCACAAAAGTAAGTGAGTGGAATACAACAATATATGTACACT
GGCAGAGTGTGGCGAGGTGATGTACAGATGTTGCCATTGCTGCTCTATTGTTTTACTTCTGATATCTTG
ATTCTAATGGGCCAGGTTTCTAAGATCTCCAATATCAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTT
GATACCACTGCTT

Figure 3.4 Nucleotide sequence of 5' (A) and 3'RACE-PCR fragment (B) of *PmG*$_{ao}$. The positions of sequencing primers are illustrated in boldface (RACE-PCR primer). Internal sequencing primer is underlined. UPM and nested UPM primers are illustrated with the broken lines.

A.

```
GGGGGCAAGATATACAATGCTCATGTTGGAACTACATGTCATCAGTGCAGGCAGAAAACT    60
GTCGATACTAAGACTGTATGTCGTTCTGGAGAGTGTTGGTGTGCCGTGTGAGAAGTGAAC   120
TTGTAGCTCACTATGTAGTGGCCACTTGGTGTTACAGGTGGAGAGCTTGATTAGCGGTGT   180
GTGAAGAGATGATCATGCCGTCATAACAAGGTGGCAGTGTGCGTCATCGCGTAGAAGCAG   240
AGTGGCGCGTAGGCCTAAGGTGACTCGGAGTTCGTGTGAAATCTCCAGCAGGTGTCCGAG   300
GACCTGTGGACACCAGAATACCCGAGGGCGTGTGAGACATTAAAGCCAGCGCG**ATG**GGGT   360
                                                    M  G  C     3
GTACGATGTCGATGGAGCAGAAGGCGGCCCTGGCCAAGAGTCGCCAGATCGACCTCATCC   420
  T  M  S  M  E  Q  K  A  A  L  A  K  S  R  Q  I  D  L  I  L    23
TCCAGGAGGACGGCGACCGCATGAGAAGGGACGTTAAACTGCTGTTGTTAGGAGCGGGAG   480
  Q  E  D  G  D  R  M  R  R  D  V  K  L  L  L  L  G  A  G  E    43
AGTCCGGCAAAAGTACCATCGTAAAACAAATGAAGATTATTCACGAGTCTGGGTTCACGA   540
  S  G  K  S  T  I  V  K  Q  M  K  I  I  H  E  S  G  F  T  S    63
GCGAAGACTTCAAGCAGTACCGGCCGGTGGTGTACTCCAACACCATACAGTCGTTGGTGG   600
  E  D  F  K  Q  Y  R  P  V  V  Y  S  N  T  I  Q  S  L  V  A    83
CCATTCTCCGCGCCATGCCCAACCTCGGCATATCCTTCGGTAACAACGAGCGGGAGCCCG   660
  I  L  R  A  M  P  N  L  G  I  S  F  G  N  N  E  R  E  P  D   103
ACGCCAAGATGGTCTTCGATGTCATATCCCGTATGGAGGACACAGAGCCCTTCTCCGAGG   720
  A  K  M  V  F  D  V  I  S  R  M  E  D  T  E  P  F  S  E  E   123
AGCTCCTGGCCGCCATGAAGCGTCTGTGGGCCGACACGGGCGTCCAGGAATGTTTCGGCC   780
  L  L  A  A  M  K  R  L  W  A  D  T  G  V  Q  E  C  F  G  R   143
GCTCCAACGAGTACCAGCTGAACGACTCCGCCAAGTATTTCTTGGACGATCTGGACCGCC   840
  S  N  E  Y  Q  L  N  D  S  A  K  Y  F  L  D  D  L  D  R  L   163
TGGGAGCTAAGGACTATCAGCCAACCGAACAAGATATCCTACGAACTCGCGTCAAGACCA   900
  G  A  K  D  Y  Q  P  T  E  Q  D  I  L  R  T  R  V  K  T  T   183
CTGGTATCGTCGAGGTCCACTTCTCTTTCAAGAACCTTAACTTTAAGTTATTTGATGTAG   960
  G  I  V  E  V  H  F  S  F  K  N  L  N  F  K  L  F  D  V  G   203
GAGGACAGCGCTCTGAACGAAAGAAGTGGATTCACTGTTTTGAAGATGTGACAGCCATTA  1020
  G  Q  R  S  E  R  K  K  W  I  H  C  F  E  D  V  T  A  I  I   223
TCTTCTGTGTGGCCATGTCAGAATACGATCAAGTGCTGCATGAAGACGAGACTACGAATC  1080
  F  C  V  A  M  S  E  Y  D  Q  V  L  H  E  D  E  T  T  N  R   243
GCATGCAAGAATCTCTTAAGTTATTTGACTCCATCTGCAACAACAAATGGTTTGGGGATA  1140
  M  Q  E  S  L  K  L  F  D  S  I  C  N  N  K  W  F  G  D  T   263
CTTCTATTATTCTGTTCCTAAACAAGAAAGATCTTTTTGAAGAGAAGATAAAGAAGAGTC  1200
  S  I  I  L  F  L  N  K  K  D  L  F  E  E  K  I  K  K  S  P   283
CACTTACCATATGTTTCCAAGAATATTCAGGTGCCCAAGAGTATGGTGAAGCAGCTGCTT  1260
  L  T  I  C  F  Q  E  Y  S  G  A  Q  E  Y  G  E  A  A  A  Y   303
ACATCCAAGCCCAGTTTGAAGCAAAGAACAAATCAACGTCCAAAGAGATCTACTGTCACA  1320
  I  Q  A  Q  F  E  A  K  N  K  S  T  S  K  E  I  Y  C  H  M   323
TGACTTGCGCAACAGATACCACTAACATTCAGTTTGTATTTGATGCAGTAACAGATGTGA  1380
  T  C  A  T  D  T  T  N  I  Q  F  V  F  D  A  V  T  D  V  I   343
TCATAGCAAACAACCTACGTGGATGTGGCCTCTAC**TAA**GTTGCACCGCCTCATCTAGATT  1440
  I  A  N  N  L  R  G  C  G  L  Y  *                          354
GTGTGTTCAGTGTAGAGAAAGATGGTACAGTCGTGGTCTAGCCTCCCACTCCAGATAATA  1500
ATCTTGCCTCTCTTGCAAGGAATTAGTCAGGAGAATTGGCTAGTTGTACCTTGTACACCA  1560
TTATCTCCTCTCCCTTTGGAGAGTTCTAGGCCGAGAATGGCACTGTCCTGAAGTTTTATC  1620
ATACGTGTTCATGGTGCGGTCATTGTCATACAGGAACGGATGCCATTAAAGTGATTTCTA  1680
GGTGCATAACTGACACAGACCGCTTTGGCCTTCGTAGCCACCCATTTCTAGATCATGGAA  1740
GAATTGCATGAGTGATTATGAATTGAGCTTGTTAAAGACTTTCCAGCCAACCCCACTTGT  1800
CTTGTATAGTACCAAGTTTAATTATCACTTCCTTACCAAATGAAGCCTTACAGTTCTCGT  1860
TATCTGGTACAAGTTCGATAAAAAACAAATGTTGATGATTTTGAATCAGAAGGAAAAGCT  1920
TAAATTACTGGTTCATAGAGATGGAACATTAATGATTTTGAATTATTTGGTGCTTTCCAT  1980
GCACTGATGAAGAAATGTACTCTAGCTTTTGATTGATACCTTGAAGCTTTTGATAACCAA  2040
AATAAGACATAGCAGTTACCTACCTTAGTGTCTGAGGGAAATGATATATTTTCCAAACCA  2100
GTTATATGTGCAGATGAGACGTGGATATCAATGAGGGCCCAAAACCTT*AATAA*CAAAAAT  2160
CCATTGAATGGCCAAACATTGGCGGCTTGGAGCAGTGAGTGTCATTCTTTCTAGTCACGT  2220
CTTTAATCTTAGCCATTTGTCACAAAAGTAAGTGAGTGGAATACAACAATATATGTACAC  2280
```

```
TGGCAGAGTGTGGCGAGGTGATGTACAGATGTTGCCATTGCTGCTCTATTGTTTTACTTC   2340
TGATATCTTGATTCTAATGGGCCAGGTTTCTAAGATCTCCAATATCAAAAAAAAAAAAAA   2400
AAAAAAA                                                        2407
```

B.

```
GTP binding protein alpha subunit Go [Marsupenaeus japonicus] Length=354 Score =  523
bits (1347),  Expect = 2e-178 Identities = 320/323 (99%), Positives = 323/323 (100%),
Gaps = 0/323 (0%) Frame = +3

Query  492   RDVKLLLLGAGESGKSTIVKQMKIIHESGFTSEDFKQYRPVVYSNTIQSLVAILRAMPNL  671
             +D+KLLLLGAGESGKSTIVKQMKIIHESGFTSEDFKQYRPVVYSNTIQSLVAILRAMPNL
Sbjct  32    KDIKLLLLGAGESGKSTIVKQMKIIHESGFTSEDFKQYRPVVYSNTIQSLVAILRAMPNL  91

Query  672   GISFGNNEREPDAKMVFDVISRMEDTEPFSEELLAAMERLWADTGVQECFGRSNEYQLND  851
             GISFGNNEREPDAKMVFDVISRMEDTEPFSEELLAAM+RLWADTGVQECFGRSNEYQLND
Sbjct  92    GISFGNNEREPDAKMVFDVISRMEDTEPFSEELLAAMKRLWADTGVQECFGRSNEYQLND  151

Query  852   SAKYFLDDLDRLGAKDYQPTEQDILRTRVKTTGIVEVHFSFKNLNFKLFDVGGQRSERKK  1031
             SAKYFLDDLDRLGAKDYQPTEQDILRTRVKTTGIVEVHFSFKNLNFKLFDVGGQRSERKK
Sbjct  152   SAKYFLDDLDRLGAKDYQPTEQDILRTRVKTTGIVEVHFSFKNLNFKLFDVGGQRSERKK  211

Query  1032  WIHCFEDVTAIIFCVAMSEYDQVLHEDETTNRMQESLKLFDSICNNKWFGDTSIILFLNK  1211
             WIHCFEDVTAIIFCVAMSEYDQVLHEDETTNRMQESLKLFDSICNNKWFGDTSIILFLNK
Sbjct  212   WIHCFEDVTAIIFCVAMSEYDQVLHEDETTNRMQESLKLFDSICNNKWFGDTSIILFLNK  271

Query  1212  KDLFEEKIKKSPLTICFQEYSGAQEYGEAAAYIQAQFEAKNKSTSKEIYCHMTCATDTTN  1391
             KDLFEEKIKKSPLTICFQEYSGAQEYGEAAAYIQAQFEAKNKSTSKEIYCHMTCATDTTN
Sbjct  272   KDLFEEKIKKSPLTICFQEYSGAQEYGEAAAYIQAQFEAKNKSTSKEIYCHMTCATDTTN  331

Query  1392  IQFVFDAVTDVIIANNLRGCGLY  1460
             IQFVFDAVTDVIIANNLRGCGLY
Sbjct  332   IQFVFDAVTDVIIANNLRGCGLY  354
```

Figure 3.5 The full-length cDNA and deduced amino acid sequences of *Pm*$G_{\alpha o}$ (A) and BlastX result (B) of *Pm*$G_{\alpha o}$ against previously deposited sequences in the GenBank. Start and stop codons are illustrated in boldface. The G alpha (positions 13-353) domain is highlighted and underlined. Polyadenylation additional signal (AATAA) is italicized and underlined.

3.2.2 The full-length cDNA of *PmG$_\gamma$*

The partial nucleotide sequence of *PmG$_\gamma$* was initially obtained from EST analysis of the lymphoid organs and hemocyte cDNA libraries. These sequences were assembled and its contig significantly matched *guanine nucleotide-binding protein subunit gamma 1* of *N. vitripennis (E*-value = 7e-23, Fig 3.6). 5'RACE-PCR of *PmG$_\gamma$* were further carried out. The positive amplification product of 500 bp in size was obtained (Fig. 3.7). The RACE-PCR fragment was cloned and sequenced. Nucleotide sequences of 5'RACE-PCR and original EST were assembled (Fig. 3.8).

The full-length cDNA of *PmG$_\gamma$* was 514 bp in length containing an ORF of 204 bp corresponding to a polypeptide of 67 amino acids with the 5' and 3'UTRs of 71 and 203 bp (excluding the poly A tail), respectively. The poly A additional signal (AATAAA) was located at 7 nucleotides upstream from the poly A tail (Fig. 3.8B) The closest similarity to this transcript was *guanine nucleotide-binding protein subunit gamma-1* of *A. mellifera* (*E*-value = 2e-22, Fig. 3.8C). The calculated p*I* and MW of the deduced PmG$_\gamma$ protein was 5.24 and 7.79 kDa, respectively. A predicted G protein gamma subunit-like motifs (GGL) domain was found at positions 5-67 (*E*-value = 6.89e-19) of the deduced PmG$_\gamma$ protein.

A.

GAAAACCAAAAACTCGGACAATCGCCTCCATTTTCCCCCATTTTCCGCCAAAATGCAGACCAACTTGCA
AGTCCAGAGCTCGATAGTGGAGCAGCTGCGCCGTGAAGCGAGCATCAAGCGTACTCCTGTGTCTGAGGC
CGTGGAGGACATCAAGCAGTTTATCCTCCAGCATGAACAGGAAGACTACCTCCTAATTGGGTTCTCCTC
CCAGAAGAACAACCCCTTCAGGGAAAAGTCC**TCCTGCGACATCCTCTAAATCATACG**TGCAGTTGCAAA
AACCCTTTTTGCAGTGTGGTTGGTGTTACAGTTCTGGGGCCAAGCATCTCATCGATCTCAGTATTTTGC
CTCAAATGGAAGTTTCAGCCAAGTGCTACTTCTAATTTTACTTATACACAAAATTGTACAAGTGGGGGA
AAGCTTTACTTTCTCCAAATAGCTTTGTATGTCAATAAAAATGTGAAAAAATAAAAAAAAAAAAAAAAA
AAAAAAAAAAAA

B.

```
similar to G protein gamma subunit [Nasonia vitripennis] Length=70 Score = 95.5 bits
(236),  Expect = 7e-23 Identities = 45/65 (69%), Positives = 56/65 (86%), Gaps = 0/65
(0%) Frame = +2

Query  59   TNLQVQSSIVEQLRREASIKRTPVSEAVEDIKQFILQHEQEDYLLIGFSSQKNNPFREKS  238
            +NLQ Q  + EQLRREA+IKR  VS+AVED+ +FI++HEQED LL+GFSSQK+NPFREKS
Sbjct  6    SNLQQQRQLTEQLRREAAIKRISVSKAVEDLMKFIIEHEQEDCLLVGFSSQKSNPFREKS  65

Query  239  SCDIL  253
            SC +L
Sbjct  66   SCTVL  70
```

Figure 3.6 Nucleotide sequence (A) and BlastX results (B) of an EST from lymphoid organs and hemocyte cDNA libraries of *P. monodon* that significantly matched *N. vitripennis Gγ*. The putative stop codon was illustrated in boldface and underlined.

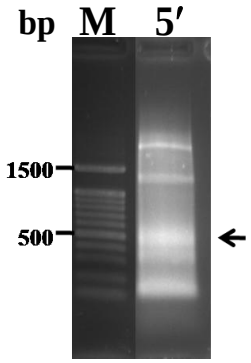


Figure 3.7 Agarose gel electrophoresis showing 5'RACE-PCR of PmG_{γ}. Lane M is a 100 bp DNA ladder.

A.

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTCGATTATTGATCAGCCTCAGAAAA
CCAAAAACTCGGACAATCGCCTCCATTTTCCCCCATTTTCCGCCAAAATGCAGACCAACTTGCAAGTCC
AGAGCTCGATAGTGGAGCAGCTGCGCCGTGAAGCGAGCATCAAGCGTACTCCTGTGTCTGAGGCCGTGG
AGGACATCAAGCAGTTCATCCTCCAGCATGAACAGGAAGACTACCTCCTAATTGGGTTCTCCTCCCAGA
AGAACAACCCCTTCAGGGAAAAGTCCTCCTGCGACATCCTCTAAATCATACGCGCAGTTGCAAAAACCC
TTTTTGCAGTGTGGTTGGTGTTACAGTTCTGGGGCCAAGCATCTCATCGATCTCAGTATTT**TGCCTCAA**
ATGGAAGTTTCAGCCAAGT

B.

```
CGATTATTGATCAGCCTCAGAAAACCAAAAACTCGGACAATCGCCTCCATTTTCCCCCAT    60
TTTCCGCCAAAATGCAGACCAACTTGCAAGTCCAGAGCTCGATAGTGGAGCAGCTGCGCC   120
           M  Q  T  N  L  Q  V  Q  S  S  I  V  E  Q  L  R  R     17
GTGAAGCGAGCATCAAGCGTACTCCTGTGTCTGAGGCCGTGGAGGACATCAAGCAGTTCA   180
  E  A  S  I  K  R  T  P  V  S  E  A  V  E  D  I  K  Q  F  I    37
TCCTCCAGCATGAACAGGAAGACTACCTCCTAATTGGGTTCTCCTCCCAGAAGAACAACC   240
  L  Q  H  E  Q  E  D  Y  L  L  I  G  F  S  S  Q  K  N  N  P    57
CCTTCAGGGAAAAGTCCTCCTGCGACATCCTCTAAATCATACGCGCAGTTGCAAAAACCC   300
  F  R  E  K  S  S  C  D  I  L  *                               67
TTTTTGCAGTGTGGTTGGTGTTACAGTTCTGGGGCCAAGCATCTCATCGATCTCAGTATT   360
TTGCCTCAAATGGAAGTTTCAGCCAAGTGCTACTTCTAATTTTACTTATACACAAAATTG   420
TACAAGTGGGGGAAAGCTTTACTTTCTCCAAATAGCTTTGTATGTCAATAAAAATGTGAA   480
AAAATAAAAAAAAAAAAAAAAAAAAAAAAAA                                514
```

C.

```
guanine nucleotide-binding protein subunit gamma-1 [Apis mellifera] (10 or fewer
PubMed links) Score = 94.4 bits (233),  Expect = 2e-22 Identities = 45/64 (70%),
Positives = 54/64 (84%), Gaps = 0/64 (0%) Frame = +3

Query  78   TNLQVQSSIVEQLRREASIKRTPVSEAVEDIKQFILQHEQEDYLLIGFSSQKNNPFREKS  257
            TNLQ Q  I EQLRREA++KR  VS+AVEDI ++I +HEQEDYLL+GFSSQK+NPFRE+S
Sbjct  6    TNLQQQRQITEQLRREAALKRITVSKAVEDIMKYITEHEQEDYLLVGFSSQKSNPFRERS  65

Query  258  SCDI  269
             C I
Sbjct  66   YCTI  69
```

Figure 3.8 A. Nucleotide sequence of the 5'RACE-PCR product of *PmG*$_{\gamma}$. The position of the sequencing primer is illustrated in boldface (RACE-PCR primer). The UPM primer is shown in the broken line. B. The full-length cDNA and deduced amino acid sequences of *PmG*$_{\gamma}$. Start and stop codons are illustrated in boldface. The predicted GGL domain is highlighted and underlined. The polyadenylation additional signal (AATAAA) is underlined. C. BlastX result of *PmG*$_{\gamma}$ against previously deposited sequences in the GenBank.

3.2.3 The full-length cDNA of *PmCGRP-RCP*

The partial nucleotide sequence of *PmCGRP-RCP* was initially obtained EST analysis of the testes cDNA library of *P. monodon*. This EST significantly match *calcitonin gene-related peptide-receptor component protein-like* of *S. kowalevskii (E*-value = 1e-21, Fig 3.9). 3'RACE-PCR was further carried out. The positive amplification product of 1100 bp was obtained (Fig. 3.10). The RACE-PCR fragment was cloned and sequenced for both directions (Fig. 3.11A). Nucleotide sequences of 3′RACE-PCR and the original EST were assembled.

The full-length cDNA of *PmCGRP-RCP* was 1182 bp in length containing an ORF of 417 bp corresponding to a polypeptide of 138 amino acids with the 5' and 3'UTRs of 203 and 536 (excluding the poly A tail), respectively (Fig. 3.13B). The poly A addition signal variant (ATTAAA) was located at 12 nucleotides upstream from the poly A tail. It significantly matched *calcitonin gene-related peptide-receptor component protein-like* of *S. kowalevskii* (*E*-value = 8e-22, Fig 3.13C). The calculated p*I* and MW of the deduced *PmCGRP-RCP* protein was 4.90 and 15.63 kDa, respectively. A predicted DNA-directed RNA-polymerase II subunit (RPOL4c) domain was found at positions 1-125 (*E*-value = 6.58e-30). The predicted *N*-linked glycosylation site was located at positions 133-135 (NGT). Hydrophilic analysis shown that is hydrophilic protein.

A.

GAACCTCGGTCGCTGAGCGGAAAAAACCTTCTCCGTGCTCTGGAAATAGAGAGTAAAGCTTGACATTAC
CTCCGAGGGATTGTTTGAGATACCGCAGAGGCTCGGTAAACCACAGGAGATCTATCGTTGTTTCTTCTG
GCCCTGCGTGGGAGAGGCGTGATACCGCACGTGGTGTAACTGCAGCATCAACACAATCGTCCAGGATGG
AAGTGTTAAATTGTAAAGGAG**CTGTTCTGAGCAACTGTGAG**GTTTATGCTCTCCTGCAGGATTTGTGTA
GGCAAGAAAAAGGCAGAAAAAATATGATGAAGACCCAAGCAGACCTTGCCAATATTGCATTTGAAACCA
TGAAATACCTTGAAAAAACAGCATGCAGAAATCAAAATGCTGAAGTAATACAAGGTTTCCTACAAGCTG
TGAAAGAATTCAAACTCACCAAAGCAGAAAAATTACAGTTAATCAACTTGCGGCCAACAACACCAGTGG
AAATGCAGCTTATAATTGAAGACAGCG

B.

```
calcitonin gene-related peptide-receptor component protein-like [Saccoglossus
kowalevskii] Length=139 Score =  106 bits (264), Expect = 1e-21 Identities = 53/102
(51%), Positives = 75/102 (73%), Gaps = 0/102 (0%) Frame = +3

Query  204  MEVLNCKGAVLSNCEVYALLQDLCRQEKGRKNMMKTQADLANIAFETMKYLEKTACRNQN  383
            ME+++   A+LSN EVY LL +L    KG++    +Q +LA I++ET+KYLEKT C  QN
Sbjct  1    MEIIDESAAMLSNYEVYTLLNELAVNIKGKRKANASQQNLATISYETIKYLEKTPCVEQN  60

Query  384  AEVIQGFLQAVKEFKLTKAEKLQLINLRPTTPVEMQLIIEDS  509
             EVI  FL+ ++ +KLTKAEK+QL+N RP + VE+QL+IE+S
Sbjct  61   EEVIGDFLKCLEPYKLTKAEKMQLLNSRPKSAVEIQLMIEES  102
```

Figure 3.9 Nucleotide sequence (A) and BlastX results (B) of an EST from the testes cDNA library of *P. monodon* that significantly matched *S. kowalevskii CGRP-RCP*. The position of RACE-PCR primer is illustrated in boldface and underlined.

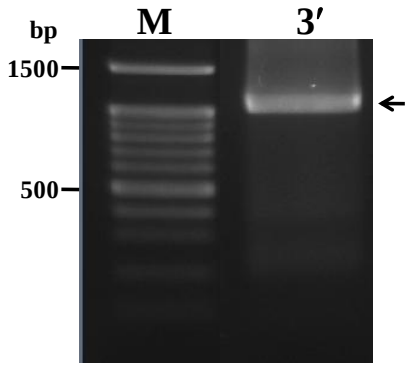


Figure 3.10 Agarose gel electrophoresis showing 3'RACE-PCR product of *PmCGRP-RCP*. Lane M is a 100 bp DNA ladder.

A.

GCAGCATCAACACAATCGTCCAGGATGGAAGTGTTAAATTGTAAAGGAGCTGTTCTGAGCAACTGTGAG
GTTTATGCTCTCCTGCAGGATTTGTGTAGGCAAGAAAAAGGCAGAAAAAATATGATGAAGACCCAAGCA
GACCTTGCCAATATCGCATTTGAAACCATGAAATACCTTGAAAAAAACAGCATGCAGAAATCAAAATGC
TGAAGTAATACAAGGTTTCCTTACAAGCTGTGAAAGAATTTCAAACTCACCCAAAGCAGAAAAATTACA
GTTAATCAACTTGCGGGCCAACAAACACCCAGTGGGAAATGCAGCTTATAATTGAAGACAGCGGAAGAA
AAGACTGAGTGAAGAACAAGTCGAAGAGTTAATTGGGCTGGTAGGAGCTCCATTTGCCAGACATTGATA
ACCCTCAAACATTAGTAAATGGTACACCCAGGGAAATAGTTGTTGCTGTCTGTTTTCTTTTCTTTTATT
ATTGTTATTATTAGATGGATATTAAGCCAATATAAGAGCATAGGATGTTATCACATTTAATTTATTTAG
TGAACTAGCTGTTACCAAAATAAATTATTGATTGTATTTGATGTTCTTGCCACTTGAGAGTATTTATGG
AAAGGAATTATTTTGAAAGTGAGGGTGATTAGACTACAGAAAAGAAATTCTGTATTATAATCTTTTAAA
GTTGTAGACATCTCTTTTTTTTCTATGTATAAACACTACTCTTATAAGCAGATAGGATCTACAGCTTAT
GATTATTGCAGTCTTGCATTCGGTGTAGAATACTTTATTCTTTTTTTGTCATGGAATTTAATATTTTAT
ACATTGATCTGTATGCAACAACTAGCAAGATGTTGCAGATGCAGGGCCTTTGGCTTGAGGCACAAACTG
AATACTCACTACCATTAAAGATGAGTGATATAAAAAAAAAAAAAAAAAAAAAAAAAAGTCTCTGCGTTG
ATACCACTGCTTGCCCTATAGTGAGTCGTATTAG

B.

```
GAACCTCGGTCGCTGAGCGGAAAAAACCTTCTCCGTGCTCTGGAAATAGAGAGTAAAGCT   60
TGACATTACCTCCGAGGGATTGTTTGAGATACCGCAGAGGCTCGGTAAACCACAGGAGAT   120
CTATCGTTGTTTCTTCTGGCCCTGCGTGGGAGAGGCGTGATACCGCACGTGGTGTAACTG   180
CAGCATCAACACAATCGTCCAGGATGGAAGTGTTAAATTGTAAAGGAGCTGTTCTGAGCA   240
                        M  E  V  L  N  C  K  G  A  V  L  S  N   13
ACTGTGAGGTTTATGCTCTCCTGCAGGATTTGTGTAGGCAAGAAAAAGGCAGAAAAAATA   300
  C  E  V  Y  A  L  L  Q  D  L  C  R  Q  E  K  G  R  K  N  M   33
TGATGAAGACCCAAGCAGACCTTGCCAATATTGCATTTGAAACCATGAAATACCTTGAAA   360
  M  K  T  Q  A  D  L  A  N  I  A  F  E  T  M  K  Y  L  E  K   53
AAACAGCATGCAGAAATCAAAATGCTGAAGTAATACAAGGTTTCCTACAAGCTGTGAAAG   420
  T  A  C  R  N  Q  N  A  E  V  I  Q  G  F  L  Q  A  V  K  E   73
AATTCAAACTCACCAAAGCAGAAAAATTACAGTTAATCAACTTGCGGCCAACAACACCAG   480
  F  K  L  T  K  A  E  K  L  Q  L  I  N  L  R  P  T  T  P  V   93
TGGAAATGCAGCTTATAATTGAAGACAGCGAAGAAAGACTGAGTGAAGAACAAGTCGAAG   540
  E  M  Q  L  I  I  E  D  S  E  E  R  L  S  E  E  Q  V  E  E  113
AGTTAATTGGGCTGGTAGAGCTCCACTTGCCAGACATTGATAACCCTCAAACATTAGTAA   600
  L  I  G  L  V  E  L  H  L  P  D  I  D  N  P  Q  T  L  V  N  133
ATGGTACACCAGGGAAATAGTTGTTGCTGTCTGTTTTCTTTTCTTTTATTATTGTTATTA   660
  G  T  P  G  K  *                                            138
TTAGATGGATATTAAGCCAATATAAGAGCATAGGATGTTATCACATTTAATTTATTTAGT   720
GAACTAGCTGTTACCAAAATAAATTATTGATTGTATTTGATGTTCTTGCCACTTGAGAGT   780
ATTTATGGAAAGGAATTATTTTGAAAGTGAGGGTGATTAGACTACAGAAAAGAAATTCTG   840
TATTATAATCTTTTAAAGTTGTAGACATCTCTTTTTTTTCTATGTATAAACACTACTCTT   900
ATAAGCAGATAGGATCTACAGCTTATGATTATTGCAGTCTTGCATTCGGTGTAGAATACT   960
TTATTCTTTTTTTGTCATGGAAATTTAATATTTTATACATTGATCTGTATGCAACAACTA   1020
GCAAGATGTTGCAGATGCAGGCCTTTGGGCTTGAGGCCATTGATCTGTATGCAACAACTA   1080
GCAAGATGTTGCAGATGCAGGCCTTTGGGCTTGAGGCACAAACTGAATACTCACTACCAT   1140
TAAAGATGAGTGATATAAAAAAAAAAAAAAAAAAAAAAAA                       1182
```

C.

calcitonin gene-related peptide-receptor component protein-like [Saccoglossus kowalevskii] Score = 98.6 bits (244), Expect = 8e-22 Identities = 49/96 (51%), Positives = 69/96 (72%), Gaps = 0/96 (0%) Frame = +3

```
Query  204  MEVLNCKGAVLSNCEVYALLQDLCRQEKGRKNMMKTQADLANIAFETMKYLEKTACRNQN  383
            ME+++   A+LSN EVY LL +L    KG++    +Q +LA I++ET+KYLEKT C  QN
Sbjct  1    MEIIDESAAMLSNYEVYTLLNELAVNIKGKRKANASQQNLATISYETIKYLEKTPCVEQN  60

Query  384  AEVIQGFLQAVKEFKLTKAEKLQLINLRPTTPVEMQ  491
             EVI  FL+ ++ +KLTKAEK+QL+N RP + VE+Q
Sbjct  61   EEVIGDFLKCLEPYKLTKAEKMQLLNSRPKSAVEIQ  96
```

D.

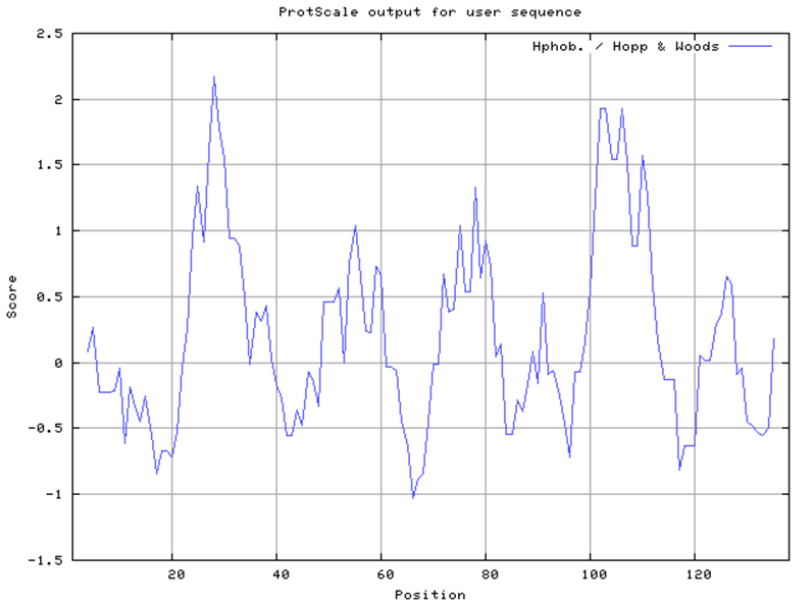


Figure 3.11 A. Nucleotide sequence of the 3'RACE-PCR of *PmCGRP-RCP*. The positions of the sequencing primer are illustrated in boldface (RACE-PCR primer) and the UPM primer is shown in the broken line. B. The full-length cDNA and deduced amino acid sequences of *PmCGRP-RCP*. Start and stop codons are illustrated in boldface. The RPOL4c domain is highlighted and underlined. Polyadenylation additional signal (ATTAAA) is underlined. C. BlastX result of *PmCGRP-RCP* against previously deposited sequences in the GenBank. D. Hydroplilic analysis.

3.2.4 The full-length cDNA of *PmDrk*

The partial nucleotide sequence of *PmDrk* was initially obtained EST analysis of the testes cDNA library of *P. monodon*. This EST significantly match *downstream of receptor kinase* of *A. mellifera (E*-value = 9e-70, Fig 3.12). 3'RACE was further carried out. The positive amplification product of 1000 bp in size was obtained (Fig. 3.13). The RACE-PCR fragment was cloned and sequenced for both directions (Fig. 3.14A). Nucleotide sequences of 3′RACE-PCR and the original EST were assembled.

A.

GCGCCTTCCACCCTCACGCACTCTTAGGGCGAATCTCGGGCGTTTTCACCCTTTGCCACGGCGAGAAAG
GGCGGCTCTCGCGGCGTGCGCTCCGGAGGGACAGATTTCACC**ATG**GAGGCGATAGCAAAACATGACTTT
AGCGCCACAGCTGAGGACGAGCTCAGTTTTAGGAAAGGGCAGATTCTTAAGGTACTAAATATGGAAGAT
GATATGAACTGGTTCAGAGCAGAGCTGGACGGCAGAGAAGGACTCATCCCTAGCAACTACATCGAGATG
AAGAGTCATGAATGGTATTATGGAAGGATAACTCGCG**CAGATGCGGAAAAACTCTTGCTT**AATAAACAC
GAAGGAGCGTTCCTCATCCGAGTTAGTGAGAGTTCTCCGGGAGATTTTACATTATCCGTCAAATGTGGA
GATGGTGTTCAGCACTTTAAGGTCTTGAGGGACACACAGGGCAAGTATAACCTCTGGGTCGTCAAGTTC
AAATCCCTAAATGAATTGGTGGAGTACACATACGGTCAGCGTCTGTGTCCCGGTCCCATGACATTAAGC
TCAAAGACATGACTCCAGAAGAATTCTTAGTGCAAGCCTATACGAC

B.

```
downstream of receptor kinase [Apis mellifera] (10 or fewer PubMed links) Score =  248
bits (632),  Expect(2) = 9e-70 Identities = 116/133 (87%), Positives = 127/133 (95%),
Gaps = 0/133 (0%) Frame = +1

Query  112  MEAIAKHDFSATAEDELSFRKGQILKVLNMEDDMNWFRAELDGREGLIPSNYIEMKSHEW  291
            MEAIAKHDF+ATAEDELSFR+ QILK+LNMEDDMNW+RAELD REGLIPSNYIEMK+H+W
Sbjct  1    MEAIAKHDFTATAEDELSFRRSQILKILNMEDDMNWYRAELDSREGLIPSNYIEMKNHDW  60

Query  292  YYGRITRADAEKLLLNKHEGAFLIRVSESSPGDFTLSVKCGDGVQHFKVLRDTQGKYNLW  471
            YYGRITRADAE+LL+NKHEGAFLIR+SESSPGDF+LSVKC DGVQHFKVLRD QGK+ LW
Sbjct  61   YYGRITRADAERLLMNKHEGAFLIRISESSPGDFSLSVKCSDGVQHFKVLRDAQGKFFLW  120

Query  472  VVKFKSLNELVEY  510
            VVKF SLNELVEY
Sbjct  121  VVKFNSLNELVEY  133
```

Figure 3.12 Nucleotide sequence (A) and BlastX results (B) of an EST from testes cDNA library of *P. monodon* that significantly matched *A. mellifera Drk*. The putative start codon was illustrated in boldface. The position of the RACE-PCR primer is illustrated in boldface and underlined.

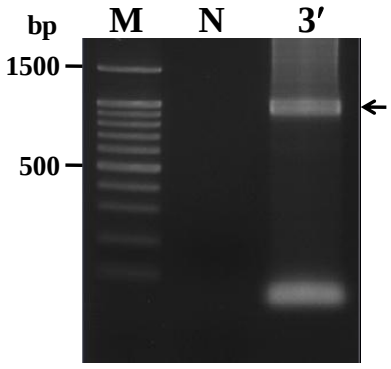


Figure 3.13 Agarose gel electrophoresis showing the result from 3'RACE-PCR of *PmDrk.* Lane M and N are a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

The full-length cDNA of *PmDrk was* 1222 bp in length containing an ORF of 672 bp corresponding to a polypeptide of 223 amino acids with the 5'- and 3'UTRs of 111 and 145 (excluding the poly A tail), respectively (Fig. 3.14B). The closest similarity to *PmDrk* was *downsteam of receptor kinase* of *G. morsitans (E*-value = 5e-126, Fig 3.14C). The calculated p*I* and molecular weight of the deduced PmDrk protein was 5.60 and 25.57 kDa, respectively. The predicted Src homology 3 (SH3) domain were found 2 domain at positions 1-57 (*E*-value = 4.73e-18) and 167-222 (*E*-value = 1.42e-21), respectively. And the predicted Src homology 2 (SH2) domains was found at 58-152 (*E*-value = 1.08e-32).

A.

CAGATGCGGAAAAACTCTTGCTTAATAAACACGAAGGAGCGTTCCTCATCCGAGTTAGTGAGAGTTCTC
CGGGAGATTTTTCATTATCCGTCAAATGTGGAGATGGTGTTCAGCACCTTAAGGTCTTGAGGGATACAC
AGGGCAAGTTCTTCCTCTGGGTCGTCAAGTTCAACTCCCTAAATGAATTGGTGGAGTACCATCGGTCAG
CGTCTGTGTCCCGGTCCCATGACATTAAGCTCAAAGACATGACTCCAGAAGAATTCTTAGTGCAAGCCC
TATACGACTTCACCCCTCAGGAGCAGGGCGAGTTGGAATTCAAGCGAGGTGATGTCATCACTGTCACAG
ACCGGTCAGACCCCCACTGGTGGAGCGGCGAAATGGGCAATCGCAGGGGGCTCCTTCCTGCCACCTACG
TGGCTCCCTACCACACCTAGATGCCCAGTGCAGGAGCTCCGCCTCGAGTACCACGTCATAACCGGAGTC
AGCAGCCATTCGTACCAGGAGGCTGCTCAAATAGTATCTTAACAGAAACAATGAAAGAGACCTTGTTGA
AAACAATGAATGGAAACTTGGCCAGGCTTAAGGGTGCTTTGGCCTACACACAGTGACAGACTGAGGGAG
GCCTTGCAGGAGATGAATAGTAGTTGGCTGGCACCCTATACAGTTTTTGGTTTGTGTTTCTGTGGCTTT
CACCCCAGTCAGTAATTGTGCCTACATCCTACTTTGATTTTGCCCCCATCCATTTATATTGGATTAGCC
AGTAAATATGTTTTATTATTGGTGCCATCTCATGCCTTTTCATCCTACATGGTTTTGTAAAGAATGACT
TTTTAAAAAAAAAGGAATTGCAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTT
GCCCTATAGTGAGTCGTATTAG

B.

```
GCGCCTTCCACCCTCACGCACTCTTAGGGCGAATCTCGGGCGTTTTCACCCTTTGCCACG   60
GCGAGAAAGGGCGGCTCTCGCGGCGTGCGCTCCGGAGGGACAGATTTCACC ATG GAGGCG   120
                                                  M  E  A      3
ATAGCAAAACATGACTTTAGCGCCACAGCTGAGGACGAGCTCAGTTTTAGGAAAGGGCAG   180
I  A  K  H  D  F  S  A  T  A  E  D  E  L  S  F  R  K  G  Q     23
ATTCTTAAGGTACTAAATATGGAAGATGATATGAACTGGTTCAGAGCAGAGCTGGACGGC   240
I  L  K  V  L  N  M  E  D  D  M  N  W  F  R  A  E  L  D  G     43
AGAGAAGGACTCATCCCTAGCAACTACATCGAGATGAAGAGTCATGAATGGTATTATGGA   300
R  E  G  L  I  P  S  N  Y  I  E  M  K  S  H  E  W  Y  Y  G     63
AGGATAACTCGCGCAGATGCGGAAAAACTCTTGCTTAATAAACACGAAGGAGCGTTCCTC   360
R  I  T  R  A  D  A  E  K  L  L  L  N  K  H  E  G  A  F  L     83
ATCCGAGTTAGTGAGAGTTCTCCGGGAGATTTTACATTATCCGTCAAATGTGGAGATGGT   420
I  R  V  S  E  S  S  P  G  D  F  T  L  S  V  K  C  G  D  G     103
GTTCAGCACTCATTATCCGTCAAATGTGGAGATGGTGTTCAGCACCTTAAGGTCTTGAGG   480
V  Q  H  S  L  S  V  K  C  G  D  G  V  Q  H  L  K  V  L  R     123
GATACACAGGGCAAGTTCTTCCTCTGGGTCGTCAAGTTCAACTCCCTAAATGAATTGGTG   540
D  T  Q  G  K  F  F  L  W  V  V  K  F  N  S  L  N  E  L  V     143
GAGTACCATCGGTCAGCGTCTGTGTCCCGGTCCCATGACATTAAGCTCAAAGACATGACT   600
E  Y  H  R  S  A  S  V  S  R  S  H  D  I  K  L  K  D  M  T     163
CCAGAAGAATTCTTAGTGCAAGCCCTATACGACTTCACCCCTCAGGAGCAGGGCGAGTTG   660
P  E  E  F  L  V  Q  A  L  Y  D  F  T  P  Q  E  Q  G  E  L     183
GAATTCAAGCGAGGTGATGTCATCACTGTCACAGACCGGTCAGACCCCCACTGGTGGAGC   720
E  F  K  R  G  D  V  I  T  V  T  D  R  S  D  P  H  W  W  S     203
```

```
GGCGAAATGGGCAATCGCAGGGGGCTCCTTCCTGCCACCTACGTGGCTCCCTACCACACC   780
G  E  M  G  N  R  R  G  L  L  P  A  T  Y  V  A  P  Y  H  T     223
TAGATGCCCAGTGCAGGAGCTCCGCCTCGAGTACCACGTCATAACCGGAGTCAGCAGCCA   840
*
TTCGTACCAGGAGGCTGCTCAAATAGTATCTTAACAGAAACAATGAAAGAGACCTTGTTG   900
AAAACAATGAATGGAAACTTGGCCAGGCTTAAGGGTGCTTTGGCCTACACACAGTGACAG   960
ACTGAGGGAGGCCTTGCAGGAGATGAATAGTAGTTGGCTGGCACCCTATACAGTTTTTGG   1020
TTTGTGTTTCTGTGGCTTTCACCCCAGTCAGTAATTGTGCCTACATCCTACTTTGATTTT   1080
GCCCCCATCCATTTATATTGGATTAGCCAGTAAATATGTTTTATTATTGGTGCCATCTCA   1140
TGCCTTTTCATCCTACATGGTTTTGTAAAGAATGACTTTTTAAAAAAAAAGGAATTGCAA   1200
AAAAAAAAAAAAAAAAAAAAA                                          1222
```

C.

```
downstream of receptor kinase [Glossina morsitans morsitans] Length=211 Score =  373
bits (958),  Expect = 5e-126 Identities = 178/223 (80%), Positives = 197/223 (88%),
Gaps = 12/223 (5%) Frame = +1

Query  112  MEAIAKHDFSATAEDELSFRKGQILKVLNMEDDMNWFRAELDGREGLIPSNYIEMKSHEW  291
            MEAIAKHDFSATA+DELSFRK QILK+LNMEDD NW+RAELDG+EGLIPSNYIEMK+H+W
Sbjct  1    MEAIAKHDFSATADDELSFRKNQILKILNMEDDSNWYRAELDGKEGLIPSNYIEMKNHDW  60

Query  292  YYGRITRADAEKLLLNKHEGAFLIRVSESSPGDFTLSVKCGDGVQHSLSVKCGDGVQHLK  471
            YYGRITRADAEKLL NKHEGAFLIR+SESSPGDF           SLSVKC DGVQH K
Sbjct  61   YYGRITRADAEKLLSNKHEGAFLIRISESSPGDF-----------SLSVKCPDGVQHFK  108

Query  472  VLRDTQGKFFLWVVKFNSLNELVEYHRSASVSRSHDIKLKDMTPEEFLVQALYDFTPQEQ  651
            VLRD QGKFFLWVVKFNSLNELVEYHR+ASVSRS D+KL+DM PEE LVQALYDF PQE
Sbjct  109  VLRDAQGKFFLWVVKFNSLNELVEYHRTASVSRSQDVKLRDMIPEEMLVQALYDFVPQES  168

Query  652  GELEFKRGDVITVTDRSDPHWWSGEMGNRRGLLPATYVAPYHT  780
            GEL+F+RGDVITVTDRSD +WW+GE+GNR+G+ P+TYV PYH+
Sbjct  169  GELDFRRGDVITVTDRSDENWWNGEIGNRKGIFPSTYVTPYHS  211
```

Figure 3.14 A. Nucleotide sequence of 3'RACE-PCR of *PmDrk*. The positions of sequencing primers are illustrated in boldface (RACE-PCR primers). The UPM primer is shown with the broken line. B. The full-length cDNA and deduced amino acid sequence of *PmDrk*. Start and stop codons are illustrated in boldface. The predicted SH3 and SH2 domains are underline and highlight, respectively. C. BlastX result of *PmDrk* against previously deposited sequences in the GenBank.

3.2.5 The partial cDNA sequence of $PmG_{\alpha q}$

The partial nucleotide sequence of $PmG_{\alpha q}$ was initially obtained EST analysis of the hemocyte cDNA library. This EST significantly match *heterotrimeric GTP-binding protein alpha subunit G-alpha-q* of *L. vannamei (E*-value = 3e-57, Fig 3.15). Both 5′- and 3′RACE-PCR of $PmG_{\alpha q}$ were further carried out. The positive amplification products of 500 and1000 in size were obtained (Fig. 3.16). The RACE-PCR fragment was cloned and sequenced for both directions (Figure 3.17). Nucleotide sequences of 3′RACE-PCR and the original EST were assembled.

Sequence similarity analysis revealed that the partial cDNA of *PmG*$_{\alpha q}$ (1200 bp) was obtained with the 3´UTR of 286 bp (excluding the poly A tail) in length, respectively (Fig. 3.18A). Its closest similarity was *GTP binding protein alpha subunit Gq* of *M. japonicus (E*-value = 0.0, Fig 3.18B). A predicted similarity G_alpha domain was found at positions 1-294 (*E* – value = 1.94e-127).

A.

GACAAACGAGGGTTCATCAAGCTGGTCTTCCAGAACATCTTCATGGCCATGCAGTCAATGATCAGGGCC
ATGGACCTTCTTCAAATATCTTATGGAGACTCTGCAAACAGTGAACACGCAGATCTGGTGCGAGCGGTG
GACTATGAGTCAGTCACAACGTTTGAGGAGCCATATGTAACTGCCATGAAAAGCTTATGGGCTGACACC
G**GCATCCAACACTGCTATGATCGTCG**CAGAGAGTACCAGCTCACAGATTCAGCAAAATATTATCTTGAT
GATTTGGAGCGTATCATATCATCGGACTTCTTACCGACCGAGCAGGATATTCTTAGGG

B.

heterotrimeric GTP-binding protein alpha subunit G-alpha-q [Litopenaeus vannamei]
Length=353 Score = 224 bits (571), Expect = 3e-57 Identities = 111/111 (100%),
Positives = 111/111 (100%), Gaps = 0/111 (0%) Frame = +1

```
Query  1    DKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANSEHADLVRAVDYESVTTFEEPYVT  180
            DKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANSEHADLVRAVDYESVTTFEEPYVT
Sbjct  65   DKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANSEHADLVRAVDYESVTTFEEPYVT  124

Query  181  AMKSLWADTGIQHCYDRRREYQLTDSAKYYLDDLERIISSDFLPTEQDILR  333
            AMKSLWADTGIQHCYDRRREYQLTDSAKYYLDDLERIISSDFLPTEQDILR
Sbjct  125  AMKSLWADTGIQHCYDRRREYQLTDSAKYYLDDLERIISSDFLPTEQDILR  175
```

Figure 3.15 Nucleotide sequence (A) and BlastX results (B) of an EST from hemocyte cDNA library of *P. monodon* that significantly matched *L. vannamei G*$_{\alpha q}$. The position of 3'RACE-PCR primer of *PmG*$_{\alpha q}$ is boldfaced.

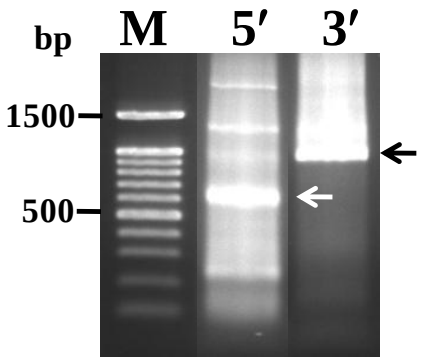


Figure 3.16 Agarose gel electrophoresis showing 5'- and 3'RACE-PCR of *PmG*$_{\alpha q}$. Arrows indicate RACE-PCR products those were cloned and sequenced. Lane M is a 100 bp DNA marker.

A.

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGATGCGTATTATCCACGGTGCAGGTTAC
AGCGATGATGACAAACGAGGGTTCATCAAGCTGGTCTTCCAGAACATCTTCATGGCCATGCAGTCAATG
ATCAGGGCCATGGACCTTCTTCAAATATCTTATGGAGACTCTGCAAACAGTGAACACGCAGATCTGGTG
CGAGCGGTGGACTATGAGTCAGTCACAACGTTTGAGGAGCCATATGTAACTGCCATGAAAAGCTTATGG
GCTGACACCGGCTTCCAACACTGCTATGATCGTCGCAGAGAGTACCAGCTCACAGATTCAGCAAAATAT
TATCTTGATGATTTGGAGCGTATCATATCATCGGACTTCTTACCGACCGAGCAGGATATTCTTAGGGCT
CGAGTACCAACCACTGGAATCATTGAGTACCCCTTTGATCTGGACTCAATCATCTTTAGAATGGTAGAT
GTCGGTGGTCAGCGATCTGAGCGACGGAAGTGGATTCATTGCTTCGAGAA**CGTCACCTCCATCATTTTC**
CTGGTCGC

B.

GCATCCAACACTGCTATGATCGTCGCAGAGAGTACCAGCTCACAGATTCAGCAAAATACTATTTAACAG
ACTTAGACCGCATAGCTGCCGAGGACTATGTTTCCACACTACAAGACATTCTAAGAGTGAGAGCACCCA
CAACAGGCATTATAGAATATCCCTTTGACCTAGAAGAAATCAGATTTAGAATGGTAGATGTCGGTGGTC
AGCGATCTGAGCGACGGAAGTGGATTCATTGCTTCGAGAACGTCACCTCCATCATTTTCCTGGTCGCAC
TTTCTGAGTATGATCAGATCTTGTTTGAGTCTGACAATGAGAACCGAATGGAAGAATCAAAGGCCCTGT
TCAAGACCATTATCACATACCCCTGGTTCCAGCACTCGTCTGTTATTCTCTTCCTTAACAAGAAGGATC
TATTGGAGGAGAAGATCATGTACTCACATCTGGTGGACTATTTCCCAGAATATGATGGCCCACAGAGGG
ATGCCATTGCAGCACGGGAGTTCATCCTACGTATGTTTGTAGAATTAAATCCTGATCCTGAGAAGATTA
TATGTTCACATTTCACATGCGCGACAGGTAAACAGCACCTTCTGTGGAAA**TGA**ACATGCATGATGACCA
TTCTTGACTGAATGTTCGGGAAGGCTGGAAATTGGAATGTACTTTTCTGCTGTCCAGTGTGCCAGTGCA
TGGTGGATTACCTTTATTATGTGTGCATTTGTCACCGATTTCTTGAAGTGTGGAAGCAAAGCGCTTCTT
GGCCTACTAGTGTCAAATTGGAAATCGGCACTTTTGTGTGACCGCTTTCTTTCTTCTGCCTTTTTCAGC
AAGCTTTTGGTACTTTATTTTTGTCTCTCCTGGACGAATGAATCACAAAAGGATGATGTGATGAAAAAA
AAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTTGCCCTATAGTGAGTCGTATTAGA

Figure 3.17 Nucleotide sequences of 5'- (A) and 3'RACE-PCR (B) of *Pm*G_{aq}. The positions of sequencing primers are boldfaced and underline (RACE-PCR primers). The UPM primer is indicated by the broken line**.** The putative stop codon is boldfaced.

A.

```
TCTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGATGCGTATTATCCACGG    60
 L  I  R  L  T  I  G  Q  A  V  V  S  T  Q  M  R  I  I  H  G     20
TGCAGGTTACAGCGATGATGACAAACGAGGGTTCATCAAGCTGGTCTTCCAGAACATCTT   120
 A  G  Y  S  D  D  D  K  R  G  F  I  K  L  V  F  Q  N  I  F     40
CATGGCCATGCAGTCAATGATCAGGGCCATGGACCTTCTTCAAATATCTTATGGAGACTC   180
 M  A  M  Q  S  M  I  R  A  M  D  L  L  Q  I  S  Y  G  D  S     60
TGCAAACAGTGAACACGCAGATCTGGTGCGAGCGGTGGACTATGAGTCAGTCACAACGTT   240
 A  N  S  E  H  A  D  L  V  R  A  V  D  Y  E  S  V  T  T  F     80
TGAGGAGCCATATGTAACTGCCATGAAAAGCTTATGGGCTGACACCGGCATCCAACACTG   300
 E  E  P  Y  V  T  A  M  K  S  L  W  A  D  T  G  I  Q  H  C    100
CTATGATCGTCGCAGAGAGTACCAGCTCACAGATTCAGCAAAATACTATTTAACAGACTT   360
 Y  D  R  R  R  E  Y  Q  L  T  D  S  A  K  Y  Y  L  T  D  L    120
AGACCGCATAGCTGCCGAGGACTATGTTTCCACACTACAAGACATTCTAAGAGTGAGAGC   420
 D  R  I  A  A  E  D  Y  V  S  T  L  Q  D  I  L  R  V  R  A    140
ACCCACAACAGGCATTATAGAATATCCCTTTGACCTAGAAGAAATCAGATTTAGAATGGT   480
 P  T  T  G  I  I  E  Y  P  F  D  L  E  E  I  R  F  R  M  V    160
AGATGTCGGTGGTCAGCGATCTGAGCGACGGAAGTGGATTCATTGCTTCGAGAACGTCAC   540
 D  V  G  G  Q  R  S  E  R  R  K  W  I  H  C  F  E  N  V  T    180
CTCCATCATTTTCCTGGTCGCACTTTCTGAGTATGATCAGATCTTGTTTGAGTCTGACAA   600
 S  I  I  F  L  V  A  L  S  E  Y  D  Q  I  L  F  E  S  D  N    200
```

```
TGAGAACCGAATGGAAGAATCAAAGGCCCTGTTCAAGACCATTATCACATACCCCTGGTT   660
 E  N  R  M  E  E  S  K  A  L  F  K  T  I  I  T  Y  P  W  F    220
CCAGCACTCGTCTGTTATTCTCTTCCTTAACAAGAAGGATCTATTGGAGGAGAAGATCAT   720
 Q  H  S  S  V  I  L  F  L  N  K  K  D  L  L  E  E  K  I  M    240
GTACTCACATCTGGTGGACTATTTCCCAGAATATGATGGCCCACAGAGGGATGCCATTGC   780
 Y  S  H  L  V  D  Y  F  P  E  Y  D  G  P  Q  R  D  A  I  A    260
AGCACGGGAGTTCATCCTACGTATGTTTGTAGAATTAAATCCTGATCCTGAGAAGATTAT   840
 A  R  E  F  I  L  R  M  F  V  E  L  N  P  D  P  E  K  I  I    280
ATGTTCACATTTCACATGCGCGACAGGTAAACAGCACCTTCTGTGGAAATGAACATGCAT   900
 C  S  H  F  T  C  A  T  G  K  Q  H  L  L  W  K  *             296
GATGACCATTCTTGACTGAATGTTCGGGAAGGCTGGAAATTGGAATGTACTTTTCTGCTG   960
TCCAGTGTGCCAGTGCATGGTGGATTACCTTTATTATGTGTGCATTTGTCACCGATTTCT   1020
TGAAGTGTGGAAGCAAAGCGCTTCTTGGCCTACTAGTGTCAAATTGGAAATCGGCACTTT   1080
TGTGTGACCGCTTTCTTTCTTCTGCCTTTTTCAGCAAGCTTTTGGTACTTTATTTTTGTC   1140
TCTCCTGGACGAATGAATCACAAAAGGATGATGTGATGAAAAAAAAAAAAAAAAAAAAA   1200
```

B.

```
GTP binding protein alpha subunit Gq [Marsupenaeus japonicus] Length=353 Score =  550
bits (1417),  Expect = 0.0 Identities = 272/278 (98%), Positives = 275/278 (99%), Gaps
= 0/278 (0%) Frame = +1

Query  1    RIIHGAGYSDDDKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANSEHADLVRAVDYE  180
            RIIHGAGYSDDDKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANSEHADLVRAVDYE
Sbjct  54   RIIHGAGYSDDDKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANSEHADLVRAVDYE  113

Query  181  SVTTFEEPYVTAMKSLWADTGIQHCYDRRREYQLTDSAKYYLTDLDRIAAEDYVSTLQDI  360
            SVTTFEEPYVTAMKSLWADTGIQHCYDRRREYQLTDSAKYYLTDLDRIAAEDYVSTLQDI
Sbjct  114  SVTTFEEPYVTAMKSLWADTGIQHCYDRRREYQLTDSAKYYLTDLDRIAAEDYVSTLQDI  173

Query  361  LRVRAPTTGIIEYPFDLEEIRFRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQIL  540
            LRVRAPTTGIIEYPFDLEEIRFRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQIL
Sbjct  174  LRVRAPTTGIIEYPFDLEEIRFRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQIL  233

Query  541  FESDNENRMEESKALFKTIITYPWFQHSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQ  720
            FESDNENRMEESKALFKTIITYPWFQHSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQ
Sbjct  234  FESDNENRMEESKALFKTIITYPWFQHSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQ  293

Query  721  RDAIAAREFILRMFVELNPDPEKIICSHFTCATGKQHL  834
            RDAIAAREFILRMFVELNPDPEKII SHFTCAT  +++
Sbjct  294  RDAIAAREFILRMFVELNPDPEKIIYSHFTCATDTENI  331
```

Figure 3.18 A. The partial cDNA and deduced amino acid sequences of *PmG$_{\alpha q}$*. B. BlastX result (B) of the partial cDNA of *PmGαq* against previously deposited sequences in the GenBank. Stop codon is illustrated in boldface. The G_alpha domain is highlighted and underlined.

3.2.6 The partial cDNA of *PmG$_{\alpha s}$*

The partial nucleotide sequence of *PmG$_{\alpha s}$* was initially obtained EST analysis of the antennal gland, hemocyte and ovary cDNA library. A contig was genated from these sequences and ti significantly match *GTP binding protein alpha subunit Gs of M. japonicas (E*-value = 2e-125, Fig 3.19). 3′RACE-PCR was further carried out and the positive amplification product of 600 bp was obtained (Fig. 3.20). The RACE-PCR fragment was cloned and sequenced for both directions (Fig. 3.21A). Nucleotide sequences of 3′RACE-PCR and the original EST were assembled (Fig. 3. 21B).

The partial cDNA of *PmG*$_{\alpha s}$ contained the ORF and completed 3′UTR was 939 and 266 bp (excluding the poly A tail) in length, respectively (Fig. 3.21B). The closest similarity to partial *PmG*$_{\alpha s}$ was *GTP binding protein alpha subunit Gs of M. japonicas (E*-value = 3e-158, Fig 3.21C). A predicted similarity G_alpha domain was found at positions 1-128 (*E* – value = 1.17e-53).

A.

AAGTATTTCCTCGACCGCGTACACATAGTCCGGCAGAACGACTACACCCCGACGGAACAGGATATTCTC
CGGTGTCGAGTCCTCACCCTAGGCATTTTTGAGACAAGATTTCAAGTAGATAAAGTTAATTTCCATATG
TTCGACGTGGGCGGTCAACGCGATGAGAGGCGGAAGTGGATCCAATGTTTCAATGACGTCACGGCCATC
ATCTTCGTCACCGCTTGCTCCTCTTATAACATGGTCCTTCGGGAAGACCCCAGTCAAAACCGGCTGCGG
GAGTCATTAGATCTCTTCAAAAGTATATGGAACAACAGGTGGCTACGCACAATCAGCATCATCCTGTTT
CTAAACAAGCAAGACCTCCTGGCCGAGAAGATCCGGGCTGGGAGGAGCAGGTTAGAAGATT**CTTTCCCT**
GACTTTGCTCGGTATCAGACCCCACTCGATGCTACCGTGGAACCAGGAGAAGATCCAGAGGTGGTGCGC
GCAAAGTACTTCATCAGGGATGAATTTCTAAGGATAAGCACGGCAAGTGGTGATGGCAAGCATTATTGC
TACCCTCACTTCACATGCGCCGTGGACACGGAAAACATCCGCCGAGTGTTCAATGACTGCAGGGACATA
ATACAAAGGATGCACCTCAGACAATATGAACTTTTG**TGA**TGGCCAGTANGTGTGGGTACTGCGGGCGGC
AACAGTATAGCTCCGTGCGAGCGAGCGAGGCAGGCAGGCTGGGTGGAATGGGGCGGGGGATCCGGGAGG
ACGGAGTGACCCTCCCCGTCTAGTCTCCTCAGAGGATGGTTCTCGCTCCCTCAGGACTTCCTCCCCCCC
TCTTCGCCCGCGAAAGCTTTTCCTGCGCCTCAAGCGTGGCACAACATCAACTTTCCTCAACCCAGTTTT
TGGTTTAGGTCCTCGTTTTGTTTTCTTACAAAAAAAA

B.

GTP binding protein alpha subunit Gs [Marsupenaeus japonicus] Length=379 Score = 453 bits (1165), Expect = 2e-125 Identities = 219/219 (100%), Positives = 219/219 (100%), Gaps = 0/219 (0%) Frame = +1

```
Query  1    KYFLDRVHIVRQNDYTPTEQDILRCRVLTLGIFETRFQVDKVNFHMFDVGGQRDERRKWI  180
            KYFLDRVHIVRQNDYTPTEQDILRCRVLTLGIFETRFQVDKVNFHMFDVGGQRDERRKWI
Sbjct  161  KYFLDRVHIVRQNDYTPTEQDILRCRVLTLGIFETRFQVDKVNFHMFDVGGQRDERRKWI  220

Query  181  QCFNDVTAIIFVTACSSYNMVLREDPSQNRLRESLDLFKSIWNNRWLRTISIILFLNKQD  360
            QCFNDVTAIIFVTACSSYNMVLREDPSQNRLRESLDLFKSIWNNRWLRTISIILFLNKQD
Sbjct  221  QCFNDVTAIIFVTACSSYNMVLREDPSQNRLRESLDLFKSIWNNRWLRTISIILFLNKQD  280

Query  361  LLAEKIRAGRSRLEDSFPDFARYQTPLDATVEPGEDPEVVRAKYFIRDEFLRISTASGDG  540
            LLAEKIRAGRSRLEDSFPDFARYQTPLDATVEPGEDPEVVRAKYFIRDEFLRISTASGDG
Sbjct  281  LLAEKIRAGRSRLEDSFPDFARYQTPLDATVEPGEDPEVVRAKYFIRDEFLRISTASGDG  340

Query  541  KHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL  657
            KHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL
Sbjct  341  KHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL  379
```

Figure 3.19 Nucleotide sequence (A) and BlastX results (B) of *PmG*$_{\alpha s}$ from an EST contig of *P. monodon* that significantly matched *M. japonicas G*$_{\alpha s}$. The position of 3'RACE-PCR primer of *PmG*$_{\alpha s}$ is boldfaced and underlined. The putative stop codon is boldfaced.

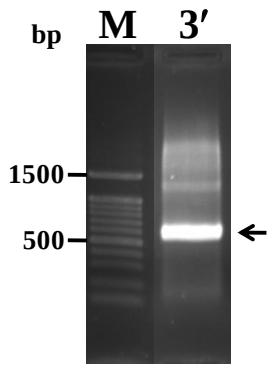


Figure 3.20 Agarose gel electrophoresis showing results from 3'RACE-PCR of *PmG*$_{\alpha s}$. Arrows indicate the RACE-PCR product that were cloned and sequenced. Lane M is a 100 bp DNA ladder**.**

A.

CTTTCCCTGACTTTGCTCGGTATCAGACCCCACTCGATGCTACCGTGGAACCAGGAGAAGATCCAGAGG
TGGTGCGCGCAAAGTACTTCATCAGGGATGAATTTCTAAGGATAAGCACGGCAAGTGGTGATGGCAAGC
ATTATTGCTACCCTCACTTCACATGCGCCGTGGACACGGAAAACATCCGCCGAGTGTTCAATGACTGCA
GGGACATAATACAAAGGATGCACCTCAGACAATATGAACTTTTG**TGA**TGGCCAGTAGGTGTGGGTACTG
CGGGCGGCAACAGTATAGCTCCGTGCGAGCGAGCGAGGCAGGCAGGCTGGGTGGAATGGGGCGGGGGAT
CCGGGAGGACGGAGTGACCCTCCCCGTCTAGTCTCCTCAGAGGATGGTTCTCGCTCCCTCAGGACTTCC
TCCCCCCCTCTTCGCCCGCGAAAGCTTTTCCTGCGCCTCAAGCGTGGCACAACATCAACTTTCCTCAAC
CCAGTTTTTGGTTTAGGTCCTCGTTTTGTTTTCTTGCAAAAAAAAAAAAAAGTACTCTGCGTTGATACCA
CTGCCTTGCCCTATAGTGAGTCGTATTAG

B.

```
AAGTATTTCCTCGACCGCGTACACATAGTCCGGCAGAACGACTACACCCCGACGGAACAG   60
K  Y  F  L  D  R  V  H  I  V  R  Q  N  D  Y  T  P  T  E  Q     20
GATATTCTCCGGTGTCGAGTCCTCACCCTAGGCATTTTTGAGACAAGATTTCAAGTAGAT  120
D  I  L  R  C  R  V  L  T  L  G  I  F  E  T  R  F  Q  V  D    40
AAAGTTAATTTCCATATGTTCGACGTGGGCGGTCAACGCGATGAGAGGCGGAAGTGGATC  180
K  V  N  F  H  M  F  D  V  G  G  Q  R  D  E  R  R  K  W  I    60
CAATGTTTCAATGACGTCACGGCCATCATCTTCGTCACCGCTTGCTCCTCTTATAACATG  240
Q  C  F  N  D  V  T  A  I  I  F  V  T  A  C  S  S  Y  N  M    80
GTCCTTCGGGAAGACCCCAGTCAAAACCGGCTGCGGGAGTCATTAGATCTCTTCAAAAGT  300
V  L  R  E  D  P  S  Q  N  R  L  R  E  S  L  D  L  F  K  S   100
ATATGGAACAACAGGTGGCTACGCACAATCAGCATCATCCTGTTTCTAAACAAGCAAGAC  360
I  W  N  N  R  W  L  R  T  I  S  I  I  L  F  L  N  K  Q  D   120
CTCCTGGCCGAGAAGATCCGGGCTGGGAGGAGCAGGTTAGAAGATTCTTTCCCTGACTTT  420
L  L  A  E  K  I  R  A  G  R  S  R  L  E  D  S  F  P  D  F   140
GCTCGGTATCAGACCCCACTCGATGCTACCGTGGAACCAGGAGAAGATCCAGAGGTGGTG  480
A  R  Y  Q  T  P  L  D  A  T  V  E  P  G  E  D  P  E  V  V   160
CGCGCAAAGTACTTCATCAGGGATGAATTTCTAAGGATAAGCACGGCAAGTGGTGATGGC  540
R  A  K  Y  F  I  R  D  E  F  L  R  I  S  T  A  S  G  D  G   180
AAGCATTATTGCTACCCTCACTTCACATGCGCCGTGGACACGGAAAACATCCGCCGAGTG  600
K  H  Y  C  Y  P  H  F  T  C  A  V  D  T  E  N  I  R  R  V   200
TTCAATGACTGCAGGGACATAATACAAAGGATGCACCTCAGACAATATGAACTTTTGTGA  660
F  N  D  C  R  D  I  I  Q  R  M  H  L  R  Q  Y  E  L  L  *   219
TGGCCAGTAGGTGTGGGTACTGCGGGCGGCAACAGTATAGCTCCGTGCGAGCGAGCGAGG  720
CAGGCAGGCTGGGTGGAATGGGGCGGGGGATCCGGGAGGACGGAGTGACCCTCCCCGTCT  780
```

```
AGTCTCCTCAGAGGATGGTTCTCGCTCCCTCAGGACTTCCTCCCCCCCTCTTCGCCCGCG   840
AAAGCTTTTCCTGCGCCTCAAGCGTGGCACAACATCAACTTTCCTCAACCCAGTTTTTGG   900
TTTAGGTCCTCGTTTTGTTTTCTTGCAAAAAAAAAAAAA                      939
```

C.

```
GTP binding protein alpha subunit Gs [Marsupenaeus japonicus] Length=379 Score =  453
bits (1165),  Expect = 3e-158 Identities = 219/219 (100%), Positives = 219/219 (100%),
Gaps = 0/219 (0%) Frame = +1

Query  1    KYFLDRVHIVRQNDYTPTEQDILRCRVLTLGIFETRFQVDKVNFHMFDVGGQRDERRKWI  180
            KYFLDRVHIVRQNDYTPTEQDILRCRVLTLGIFETRFQVDKVNFHMFDVGGQRDERRKWI
Sbjct  161  KYFLDRVHIVRQNDYTPTEQDILRCRVLTLGIFETRFQVDKVNFHMFDVGGQRDERRKWI  220

Query  181  QCFNDVTAIIFVTACSSYNMVLREDPSQNRLRESLDLFKSIWNNRWLRTISIILFLNKQD  360
            QCFNDVTAIIFVTACSSYNMVLREDPSQNRLRESLDLFKSIWNNRWLRTISIILFLNKQD
Sbjct  221  QCFNDVTAIIFVTACSSYNMVLREDPSQNRLRESLDLFKSIWNNRWLRTISIILFLNKQD  280

Query  361  LLAEKIRAGRSRLEDSFPDFARYQTPLDATVEPGEDPEVVRAKYFIRDEFLRISTASGDG  540
            LLAEKIRAGRSRLEDSFPDFARYQTPLDATVEPGEDPEVVRAKYFIRDEFLRISTASGDG
Sbjct  281  LLAEKIRAGRSRLEDSFPDFARYQTPLDATVEPGEDPEVVRAKYFIRDEFLRISTASGDG  340

Query  541  KHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL  657
            KHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL
Sbjct  341  KHYCYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL  379
```

Figure 3.21 A. Nucleotide sequence of 3'RACE-PCR of *PmG$_{\alpha s}$*. The positions of sequencing primers are boldfaced and underlined (RACE-PCR primer). The UPM primer is indicated by the broken line. B. The partial cDNA sequence of *PmG$_{\alpha s}$*. C. BlastX result of *PmG$_{\alpha s}$* against previously deposited sequences in the GenBank. Stop codon is illustrated in boldface. The G_alpha domain is highlighted and underlined.

3.2.7 Characterization of the partial cDNA of *PmG$_{\beta}$*

Primers were designed from nucleotide sequence of *L. vannamei G protein beta 1 subunit* in GenBank (Accession no. AY626793) for RT-PCR. The forward and reverse primers were also used for 3′ and 5′RACE-PCR of *PmG$_{\beta}$*. The positive amplification products of 600 bp of 5′RACE-PCR and 900 bp of 3′RACE-PCR were cloned and sequenced for both directions (Fig. 3.22). Only nucleotide sequence of 5′RACE-PCR was BlastX matched with G protein *G protein beta 1 subunit* of *L. vannamei* in GenBank, but nucleotide sequence of 3′RACE-PCR fragment were not matched G protein *G protein beta 1 subunit.* (Fig. 3.23).

After that, a 3′RACE-PCR primer was designed again from nucleotide sequence of *G protein beta 1 subunit* of *P. monodon.* The 3′RACE-PCR was carried

out. The positive amplification products of 1000 bp of 3′RACE-PCR was cloned and sequenced for both directions (Fig. 3.24A). Nucleotide sequences of 3′RACE-PCR (Fig. 2.24B) and the *PmG*$_{\beta}$ previously were assembled.

The partial cDNA of *PmG*$_{\beta}$ contained an incomplete ORF of 1148 bp and the 3′UTR was 1148 and 251 bp (excluding the poly A tail) in length, respectively (Fig. 3.25A). The closest similarity to this transcript was *G protein beta 1 subunit* of *L. vannamei (E*-value = 0.0, Fig 3.25B). A predicted seven WD40 domains were found in the deduced amino acid sequence of *PmG*$_{\beta}$

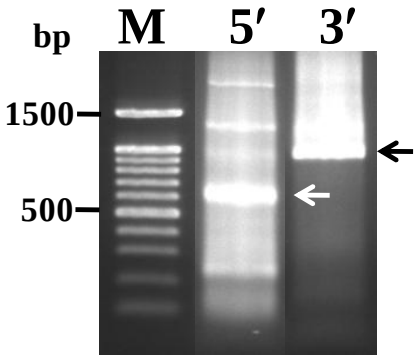


Figure 3.22 Agarose gel electrophoresis showing 5'- and 3'RACE-PCR of *PmG*$_{\beta}$. Arrows indicate RACE-PCR products that were cloned and sequenced. Lane M is a 100 bp DNA ladder**.**

A.

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGGACACTTAGCCAAAATA
TATGCCATGCACTGGGGGTCGGACTCTAGGAATTTGGTATCAGCATCTCAAGATGGCAAGCTCATAGTA
TGGGACAGTTACACTACGAACAAGGTGCATGCCATTCCCCTTCGGTCCAGCCGGGTCATGACCTGTGCC
TATGCTCCCTCGGGCAGTCACGTTGCCTGCGGTGGCCTTGATAACATCTGTTCTATATACAACCTAAAG
ACAAGAGAAGGAAATGTGAGAGTGAGTAGGGAGTTGCCCGGTCACACTGGTTACCTAAGTTGCTGTCGG
TTCCTAGACGACAACCAAATAGTCACAAGCTCGGGAGACATGACCTGTGCCCTCTGGGATATAGAGACG
GGTCAGCAGTACACGCAATTCACAGGCCATACAGGGGATGTGATGTCCCTGTCCCTGTGAACTGCGTGC
ACTGCTGACCCGCCTCTGCTAAGCTATGGGACATTCGTGATGGGATGTGCCGCCAGACCTTCCCAGGAC
ACGAATCTGACATCAATGCAGTTACATTCTTCCCCAATGGGCATGC**ATTTGCCACGGGATCAGATGATG**
CC

B.

gb|AAU12180.1| G protein beta 1 subunit [Litopenaeus vannamei] Length=340
Sort alignments for this subject sequence by: E value Score Percent identity Query start position Subject start position Score = 362 bits (930), Expect = 4e-125
Identities = 178/197 (90%), Positives = 182/197 (92%), Gaps = 6/197 (3%)
Frame = +1

```
Query  4    RGHLAKIYAMHWGSDSRNLVSASQDGKLIVWDSYTTNKVHAIPLRSSRVMTCAYAPSGSH  183
            RGHLAKIYAMHWGSDSRNLVSASQDGKLIVWDSYTTNKVHAIPLRSS VMTCAYAPSGS+
Sbjct  52   RGHLAKIYAMHWGSDSRNLVSASQDGKLIVWDSYTTNKVHAIPLRSSWVMTCAYAPSGSY  111

Query  184  VACGGLDNICSIYNLKTREGNVRVSRELPGHTGYLSCCRFLDDNQIVTSSGDMTCALWNI  363
            VACGGLDNICSIYNLKTREGNVRVSRELPGHTGYLSCCRFLDDNQIVTSSGDMTCALW+I
Sbjct  112  VACGGLDNICSIYNLKTREGNVRVSRELPGHTGYLSCCRFLDDNQIVTSSGDMTCALWDI  171
```

```
Query  364  ETGQQYTQFTCHTRDVMSLSL------YCMQS*RASAKLWDIRDGMCRQTFPGHESDINA  525
            ETGQQ TQFT HT DVMSLSL      +   +  ASAKLWDIRDGMCRQTFPGHESDINA
Sbjct  172  ETGQQCTQFTGHTGDVMSLSLSPNMNTFTSGACDASAKLWDIRDGMCRQTFPGHESDINA  231

Query  526  VTFFPNGHAFATGSDDA  576
            VTFFPNGHAFATGSDDA
Sbjct  232  VTFFPNGHAFATGSDDA  248
```

C.

GACAGAGAAGGCATGTGAGAGTGAGTTGCCTCCTCCACCGGACCCCAACACTTCCTGGAAAACTTCAAC
CGCGGGCGTAACTTTGAGCAGGGAGCCAATGACTACGCTGGCCAAAGAGGCCGAGACGCTGGGCGCAGC
AGATGGGGACGCAATGACCGAGATGACTACGGCCCAGCCAAGAGGGCCCGCTACTAGACTTGCTGTTTA
TAGGACTATTGCGTGCATGTAGCCTATAATGGTCCTTAGTCTGGAAGGCTCGCATAACATTGATGGAAA
ATCTTGTGTGCTAGAGTGGTTGGGCCTATAGTAGACAACACAGGGATGGCATAGTAAAGGTCTAATCCC
ATACTCTTATACAAGCACCTAATATATTAAGAGAAAAGAATTTCTAAAATTTTGGATACCCTCCATTGT
ATTTTATTGCTAGCAATGCCATAAGGTTTGCTCTTTAAGAACTTATTACACCATATGAATGTTACATAA
GGTACAATAATATTTCTGCTTATCTTTTCATTCTTGACTCCTAGTTTGATGACCTATTAATACATAAGA
TTAAGTTTCTTGCAGGTTAGAATCAGTAGTAGCTTGAAACATGTAAACTATATAACAATACAGTGTGAC
AAGTATTCCAATAATGGGTTTGATGAGTTAACCCGAGCTGCTTAGATTATAAGTGAAGATGTGAAGTGT
TTTATGTCAAATGGTACTAGATATTTTGGTAAGACTCAAGTTTTATTTTATATTATTGTGTAGTGGTAA
AGTTTTCATTTTTTATTGTCACAATTGTACAAAGTTTAAGTTTCTCTTCATAATTTGTATGGATTTTTC
TTGTCTTCATAATAAATGTCGATCTATTAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGA
TACCACTGCTTA

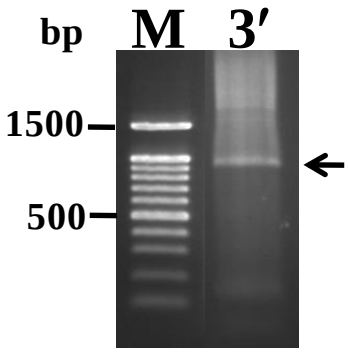
D.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
XP_002055377.1	GJ19335 [Drosophila virilis] >gb|EDW65578.1| GJ19335 [Drosophila v	38.5	38.5	23%	2.4	35%
EHB97108.1	serine/threonine phosphatase Ppp [Mycobacterium abscessus 47J26]	37.7	37.7	16%	3.5	42%
XP_003475532.1	PREDICTED: WD repeat-containing protein 73-like [Cavia porcellus]	37.0	37.0	13%	5.5	45%
EGB06618.1	hypothetical protein AURANDRAFT_65528 [Aureococcus anophagefferr	37.0	37.0	17%	7.4	38%
YP_001700793.1	serine/threonine phosphatase Ppp [Mycobacterium abscessus ATCC 1	36.6	36.6	16%	8.2	40%
EHS04310.1	CAAX amino terminal protease self- immunity [Staphylococcus lugdur	35.8	35.8	16%	9.3	29%

Figure 3.23 Nucleotide sequences of 5'- (A) and 3'RACE-PCR (C) of *PmG$_\beta$*. B and D BlastX results of nucleotide sequences of 5'- and 3'RACE-PCR, respectively. The positions of sequencing primers are boldfaced and underlined (RACE-PCR primers) The UPM primer is indicated by the broken line.

A.



B.

GGTCACACTGGTTACCTAAGTTGCTGTCGGTTCCTAGACGACAACCAAATAGTCACA
AGCTCGGGAGACATGACCTGTGCCCTCTGGGATATAGAGACGGGTCAGCAGTGCACG
TAATTCACAGGCCATACAGGGGATGTGATGTCCCTGTCCCTGTCACCGAACATGAAC
ACATTCACATCTGGTGCCTGTGACGCGTCTGCTAAGCTATGGGACATTCGTGATGGG
ATGTGCCGCCAGACCTTCCCAGGACACGAATCTGACATCAATGCAGTTACATTCTTC
CCCAATGGGCATGCATTTGCCACGGGATCAGATGATGCCACATGCCGCCTATTTGAC
ATTCGTGCAGACCAGGAGCTTGCCATGTACTCTCATGACAACATTATTTGTGGCATC
ACCTCAGTGGCATTCAGCAAGTCTGGCAGACTCCTGCTGGCTGGTTACGATGACTTT
AATTGTAACGTTTGGGACTCCATGAGGACAGAAAGAGCTGGTGTTCTGGCGGGCCAT
GACAACCGCGTCAGTTGCCTGGGTGTTACAGAAGATGGCATGGCAGTGGCCACAGGC
TCATGGGATAGCTTCCTCAAGATCTGGAAC**TAA**GCTTGTCATGCCACCACCACTCCA
GTATCAACATCAGTATCTTCCCAAGGAACCAAATTCCCTCTCTAGGATACACAGTCA
TGAAGCTTGGTGTTTGCATGCCTGCGTGTGTGTCTGTGCAGCCCACGCTTATGGTAA
CACCACTCTTTGCTTCATAGCCACCATTGATGTGGGTCACTTAAGTAGTTGTGTACC
GAGTGAGCATGCTTGCCGGACCTGAGTGGATCTTTCCTTTTCATTTTTTGATATGGA
AAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTT

Figure 3.24 A. Agarose gel electrophoresis showing 3'RACE-PCR of *PmG$_{\beta}$*. Arrows indicate RACE-PCR products that were cloned and sequenced. Lane M is a 100 bp DNA ladder**.** B. Nucleotide sequences of 3'RACE-PCR of *PmG$_{\beta}$*. The positions of sequencing primers are boldfaced and underlined (RACE-PCR primers) The UPM primer is indicated by the broken line. The putative stop codon is illustrated in boldface **.**

A.

```
ACGCGGGGACACTTAGCCAAAATATATGCCATGCACTGGGGGTCGGACTCTAGGAATTTG    60
T  R  G  H  L  A  K  I  Y  A  M  H  W  G  S  D  S  R  N  L      20
GTATCAGCATCTCAAGATGGCAAGCTCATAGTATGGGACAGTTACACTACGAACAAGGTG    120
V  S  A  S  Q  D  G  K  L  I  V  W  D  S  Y  T  T  N  K  V      40
CATGCCATTCCCCTTCGGTCCAGCCGGGTCATGACCTGTGCCTATGCTCCCTCGGGCAGT    180
H  A  I  P  L  R  S  S  R  V  M  T  C  A  Y  A  P  S  G  S      60
CACGTTGCCTGCGGTGGCCTTGATAACATCTGTTCTATATACAACCTAAAGACAAGAGAA    240
H  V  A  C  G  G  L  D  N  I  C  S  I  Y  N  L  K  T  R  E      80
GGAAATGTGAGAGTGAGTAGGGAGTTGCCCGGTCACACTGGTTACCTAAGTTGCTGTCGG    300
G  N  V  R  V  S  R  E  L  P  G  H  T  G  Y  L  S  C  C  R     100
TTCCTAGACGACAACCAAATAGTCACAAGCTCGGGAGACATGACCTGTGCCCTCTGGGAT    360
F  L  D  D  N  Q  I  V  T  S  S  G  D  M  T  C  A  L  W  D     120
ATAGAGACGGGTCAGCAGTGCACGCAATTCACAGGCCATACAGGGGATGTGATGTCCCTG    420
I  E  T  G  Q  Q  C  T  Q  F  T  G  H  T  G  D  V  M  S  L     140
TCCCTGTCACCGAACATGAACACATTCACATCTGGTGCCTGTGACGCGTCTGCTAAGCTA    480
S  L  S  P  N  M  N  T  F  T  S  G  A  C  D  A  S  A  K  L     160
TGGGACATTCGTGATGGGATGTGCCGCCAGACCTTCCCAGGACACGAATCTGACATCAAT    540
W  D  I  R  D  G  M  C  R  Q  T  F  P  G  H  E  S  D  I  N     180
GCAGTTACATTCTTCCCCAATGGGCATGCATTTGCCACGGGATCAGATGATGCCACATGC    600
A  V  T  F  F  P  N  G  H  A  F  A  T  G  S  D  D  A  T  C     200
CGCCTATTTGACATTCGTGCAGACCAGGAGCTTGCCATGTACTCTCATGACAACATTATT    660
R  L  F  D  I  R  A  D  Q  E  L  A  M  Y  S  H  D  N  I  I     220
TGTGGCATCACCTCAGTGGCATTCAGCAAGTCTGGCAGACTCCTGCTGGCTGGTTACGAT    720
C  G  I  T  S  V  A  F  S  K  S  G  R  L  L  L  A  G  Y  D     240
GACTTTAATTGTAACGTTTGGGACTCCATGAGGACAGAAAGAGCTGGTGTTCTGGCGGGC    780
D  F  N  C  N  V  W  D  S  M  R  T  E  R  A  G  V  L  A  G     260
CATGACAACCGCGTCAGTTGCCTGGGTGTTACAGAAGATGGCATGGCAGTGGCCACAGGC    840
H  D  N  R  V  S  C  L  G  V  T  E  D  G  M  A  V  A  T  G     280
TCATGGGATAGCTTCCTCAAGATCTGGAACTAAGCTTGTCATGCCACCACCACTCCAGTA    900
S  W  D  S  F  L  K  I  W  N  *                                 290
TCAACATCAGTATCTTCCCAAGGAACCAAATTCCCTCTCTAGGATACACAGTCATGAAGC    960
TTGGTGTTTGCATGCCTGCGTGTGTGTCTGTGCAGCCCACGCTTATGGTAACACCACTCT   1020
TTGCTTCATAGCCACCATTGATGTGGGTCACTTAAGTAGTTGTGTACCGAGTGAGCATGC   1080
TTGCCGGACCTGAGTGGATCTTTCCTTTTCATTTTTTGATATGGAAAAAAAAAAAAAAAA   1140
AAAAAAAA                                                       1148
```

B.

G protein beta 1 subunit [Litopenaeus vannamei] Length=340

```
 Score =  603 bits (1554),  Expect = 0.0
 Identities = 287/289 (99%), Positives = 288/289 (99%), Gaps = 0/289 (0%)
 Frame = +1

Query  4    RGHLAKIYAMHWGSDSRNLVSASQDGKLIVWDSYTTNKVHAIPLRSSRVMTCAYAPSGSH  183
            RGHLAKIYAMHWGSDSRNLVSASQDGKLIVWDSYTTNKVHAIPLRSS VMTCAYAPSGS+
Sbjct  52   RGHLAKIYAMHWGSDSRNLVSASQDGKLIVWDSYTTNKVHAIPLRSSWVMTCAYAPSGSY  111

Query  184  VACGGLDNICSIYNLKTREGNVRVSRELPGHTGYLSCCRFLDDNQIVTSSGDMTCALWDI  363
            VACGGLDNICSIYNLKTREGNVRVSRELPGHTGYLSCCRFLDDNQIVTSSGDMTCALWDI
Sbjct  112  VACGGLDNICSIYNLKTREGNVRVSRELPGHTGYLSCCRFLDDNQIVTSSGDMTCALWDI  171

Query  364  ETGQQCTQFTGHTGDVMSLSLSPNMNTFTSGACDASAKLWDIRDGMCRQTFPGHESDINA  543
            ETGQQCTQFTGHTGDVMSLSLSPNMNTFTSGACDASAKLWDIRDGMCRQTFPGHESDINA
Sbjct  172  ETGQQCTQFTGHTGDVMSLSLSPNMNTFTSGACDASAKLWDIRDGMCRQTFPGHESDINA  231

Query  544  VTFFPNGHAFATGSDDATCRLFDIRADQELAMYSHDNIICGITSVAFSKSGRLLLAGYDD  723
            VTFFPNGHAFATGSDDATCRLFDIRADQELAMYSHDNIICGITSVAFSKSGRLLLAGYDD
Sbjct  232  VTFFPNGHAFATGSDDATCRLFDIRADQELAMYSHDNIICGITSVAFSKSGRLLLAGYDD  291
```

```
Query  724  FNCNVWDSMRTERAGVLAGHDNRVSCLGVTEDGMAVATGSWDSFLKIWN  870
            FNCNVWDSMRTERAGVLAGHDNRVSCLGVTEDGMAVATGSWDSFLKIWN
Sbjct  292  FNCNVWDSMRTERAGVLAGHDNRVSCLGVTEDGMAVATGSWDSFLKIWN  340
```

Figure 3.25 A. The partial cDNA and deduced amino acid sequences of *PmG$_\beta$*. B. BlastX result of *PmG$_\beta$* against previously deposited sequences in the GenBank. Stop codon is illustrated in boldface. The WD40 domain is highlighted and underlined.

3.3 Expression patterns of reproduction-related genes in ovaries of *P. monodon* examined by RT-PCR

3.3.1 RT-PCR analysis

Six pairs of sequence-specific primers were designed from nucleotide sequences of cDNA libraries of *P. monodon*. In addition, a pairs of primers for amplification of *G protein beta 1 subunit* was designed from that of the Pacific white shrimp (*L. vannamei*, GenBank accession number AY626793.1).

Transcripts were non-quantitatively examined using the cDNA template of ovaries and testes of juvenile and broodstock. All primers generated the expected PCR products. These included *P. monodon GTP binding protein alpha subunit G$_o$ (PmG$_{\alpha o}$), GTP binding protein alpha subunit G$_q$ (PmG$_{\alpha q}$), GTP binding protein alpha subunit G$_s$ (PmG$_{\alpha s}$), G protein beta 1 subunit (PmG$_\beta$), G protein gamma subunit (PmG$_\gamma$), Calcitonin gene-related peptide-receptor component protein-like (PmCGRP-RCP), Downstream of receptor kinase (PmDrk)* and *Selenoprotein M (PmSelM)* were more abundantly expressed in ovaries than testes of both juveniles and wild broodstock of *P. monodon* (Fig. 3.26).

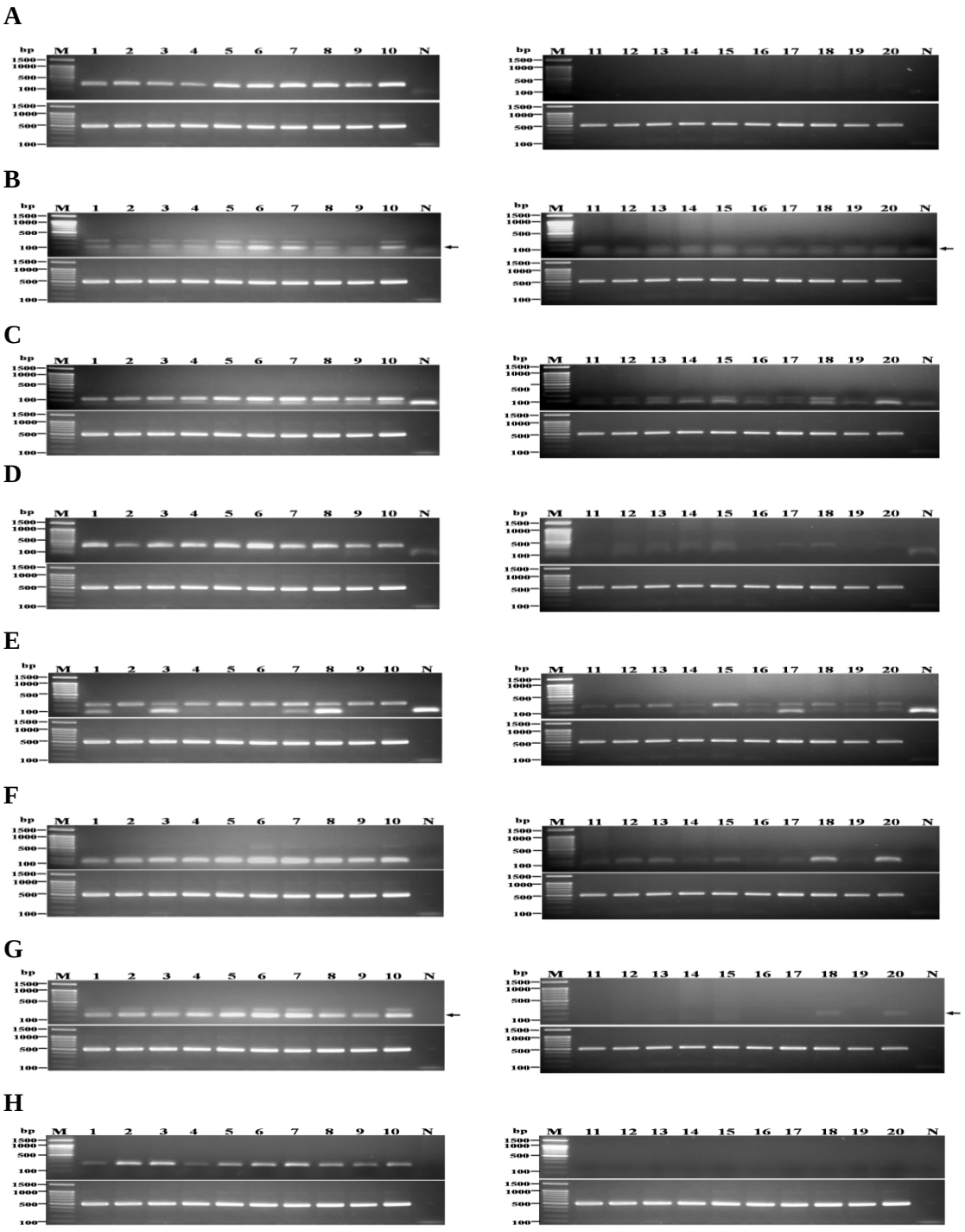


Figure 3.26 Agarose gel electrophoresis showing RT-PCR of *PmGαo* (A), *PmGαq* (B), *PmGαs* (C), *PmGbeta* (D), *PmGgam* (E), *PmCGRP-RCP* (F), *PmDrk* (G) and *PmSelM* (H) (top) and *EF-1α* (bottom) using the first stand cDNA from ovaries (lanes 1-10) and testes (lanes 11-20) of juveniles (lanes 1-5 and 11-15) and wild broodstock (lanes 6-10 and 16-20). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

Obvious differential expression patterns toward ovaries were observed for *PmG$_{\alpha o}$* and *PmDrk*. Generally, the expression of these transcripts in ovaries of wild broodstock was greater than that in ovaries of juveniles.

3.3.2 Tissue distribution analysis of reproduction-related genes

The expression of *PmG$_{\alpha o}$*, *PmG$_{\alpha q}$*, *PmG$_{\alpha s}$*, *PmG$_{\beta}$*, *PmG$_{\gamma}$*, *PmCGRP-RCP* and *PmDrk* in 13 tissues; 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), and thoracic ganglion (TG) of wild female broodstock, juvenile ovaries (JOV) and testes (TT) of wild male broodstock were examined using the conventional RT-PCR.

3.3.2.1 *PmG$_{\alpha o}$*

PmG$_{\alpha o}$ was constitutively expressed in almost examined tissues and more abundantly expressed in hemocytes than other tissues of wild female *P. monodon* broodstock. A lower expression of *PmG$_{\alpha o}$* was observed in epicuticle, eyestalk, gills, heart, intestine, lymphoid organs, ovaries, pleopods and stomach. The expression of this transcript in ovaries of broodstock was more abundant than that of juveniles and testes of wild male broodstock. *PmG$_{\alpha o}$* was not expressed in antennal gland and hepatopancreas (Fig. 3.27A and Table 3.1)

3.3.2.2 *PmG$_{\alpha q}$*

Like *PmG$_{\alpha o}$*, *PmG$_{\alpha q}$* was constitutively expressed in almost all of the examined tissues and it was more abundantly expressed in epicuticle, hemocytes and lymphoid organs and than other tissues of wild female *P. monodon* broodstock. Lower expression of this transcript was observed in antennal gland, heart, intestine, ovaries, intestine, pleopods and stomach of females and testes of male broodstock. It was not expressed in varies of juveniles (Fig. 3.27B and Table 3.1).

3.3.2.3 *PmG$_{\alpha s}$*

PmG$_{\alpha s}$ was expressed in all examined tissues and it was abundantly expressed in antennal gland, epicuticle, hemocytes, heart, intestine, pleopods, stomach, and thoracic ganglion of wild female broodstock and ovaries of juvenile. Lower

expression was found in eyestalk, gills, lymphoid organs and ovaries of wild female broodstock and testes of male broodstock. Rare expression was observed in hepatopancreas of female broodstock. The expression levels of this transcript in all tissues of females except hepatopancreas and thoracic ganglion were greater than that of testes of male broodstock (Fig. 3.27C and Table 3.1).

3.3.2.4 *PmG$_\beta$*

PmG$_\beta$ was constitutively expressed in all examined tissues and more abundantly expressed in epicuticle, hemocytes, heart, lymphoid organs, pleopods, stomach and thoracic ganglion of wild female broodstock of *P. monodon*. A lower expression was observed in antennal gland and intestine of wild female broodstock of *P. monodon*. Rare expression was observed in eyestalk, gills, hepatopancreas, ovaries and testes of broodstock. The expression of this transcript was not observed rare expressed in ovaries of juveniles (Fig. 3.27D and Table 3.1).

3.3.2.5 *PmG$_\gamma$*

PmG$_\gamma$ was more abundantly expressed in ovaries of juveniles than all tissues of female broodstock and testes of male broodstock. This transcript seemed to be expressed as a low level in *P. monodon* broodstock (Fig. 3.27E and Table 3.1).

3.3.2.6 *PmCGRP-RCP*

PmCGRP-RCP was abundantly expressed in testes of broodstock. It was not expressed in antennal gland, hepatopancreas and thoracic ganglion but expressed with a very low level in the remaining examined tissues of female broodstck and in juvenile ovaries (Fig. 3.27F and Table 3.1).

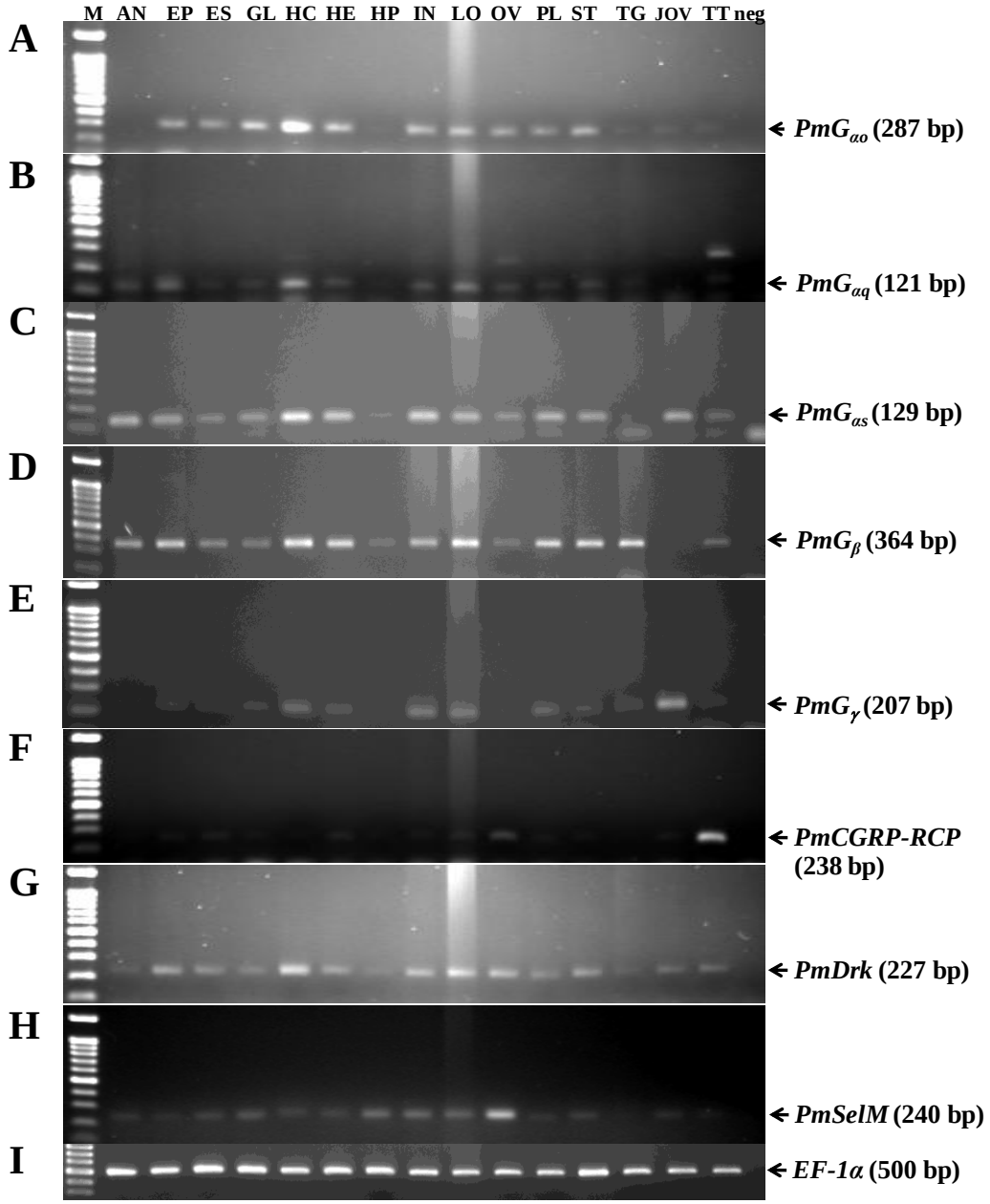


Figure 3.27 Tissue distribution analysis of $PmG_{\alpha o}$ (A), $PmG_{\alpha q}$ (B), $PmG_{\alpha s}$ (C), PmG_{β} (D), PmG_{γ} (E), *PmCGRP-RCP* (F), *PmDrk* (G), *PmSelM* (H) and *EF-1α* (I) was successfully amplified from the same template. The first stand cDNA template was from various tissues of wild female *P. monodon* broodstock: 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), and thoracic ganglion (TG), ovaries of juvenile (JOV) and testes (TT) of wild male broodstock. Lane M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

3.3.2.7 *PmDrk*

PmDrk was expressed in all examined tissues and it was more abundantly expressed in hemocytes, intestine, lymphoid organs, ovaries and stomach than other tissues of female and testes of male broodstock of *P. monodon*. Its expression in ovariesof juveniles was slightly lower than that of broodstock (Fig. 3.27G and Table 3.1).

3.3.2.8 *PmSelM*

PmSelM was expressed in all examined tissues except thoracic ganglion and it was abundantly expressed in ovaries of wild female *P. monodon* broodstock. A lower expression of *PmselM* was observed in gills, hepatopancreas, intestine and lymphoid organs. The expression of this transcript in ovaries of broodstock was more abundant than that of juveniles and testes of wild male broodstock. (Fig. 3.27A and Table 3.1)

Table 3.1 Expression profiles and tissue distribution analysis of various genes in *P. monodon*

Gene	Wild female broodstock													JOV	TT
	AN	EP	ES	GL	HC	HE	HP	IN	LO	OV	PL	ST	TG		
PmG$_{\alpha o}$	-	++	++	++	+++	++	-	++	++	++	++	++	+	+	+
PmG$_{\alpha q}$	++	+++	+	+	+++	++	++	++	+++	+	+	++	+	-	+
PmG$_{\alpha s}$	+++	+++	++	++	+++	+++	+	+++	++	++	+++	+++	+	++	++
PmG$_{\beta}$	++	+++	+	+	+++	+++	+	++	+++	+	++	+++	+++	-	+
PmG$_{\gamma}$	+	++	+	+	++	++	+	++	++	+	++	++	++	+++	+
PmCGRP-RCP	-	+	+	+	+	+	-	+	+	++	+	+	-	+	+++
PmDrk	+	+++	++	++	+++	++	+	+++	+++	+++	++	+++	+	++	++
PmSelm	+	+	+	++	+	+	++	++	++	+++	+	+	-	+	+

*** - = not expressed, + = low level of expression, ++ = moderate level of expression, +++ = abundant level of expression**

3.4 Examination of the expression levels of *PmG$_{\alpha q}$*, *PmG$_{\alpha s}$*, *PmCGRP-RCP* and *PmDrk* during ovarian development of *P. monodon* by quantitative real-time PCR

The expression levels of ovarian *PmG$_{\alpha q}$*, *PmG$_{\alpha s}$*, *PmCGRP-RCP* and *PmDrk* during ovarian development of *P. monodon* were examined by quantitative real-time PCR analysis. The standard curve of each target gene and the control *(EF-1α)* were constructed from 10-fold dilutions covering 10^3- 10^8 copy numbers of dsDNA of these genes. High R^2 vules and efficiency of amplification of the examined transcripts were found (Figure 3.28). Therefore, these standard curves were acceptable to be used for quantitative estimation of all genes.

3.4.1 *PmG$_{\alpha q}$*

Quantitative real-time PCR revealed that the expression level of *PmG$_{\alpha q}$* in ovaries of intact broodstock was greater than that of 4-month-old juveniles ($P < 0.05$). *PmG$_{\alpha q}$* was significantly up-regulated at the mature stage (IV) in intact broodstock ($P < 0.05$). Its expression level was decreased to the normal level after spawning ($P < 0.05$). The expression level of *PmG$_{\alpha q}$* in ovaries of eyestalk-ablated broodstock was comparable for all stages of ovarian development stage ($P > 0.05$). The expression level of *PmG$_{\alpha q}$* in stages III and IV ovaries of intact broodstock was significantly greater than that in eyestalk-ablated broodstock ($P < 0.05$).

In domesticated shrimp, the expression level of *PmG$_{\alpha q}$* in ovaries of domesticated 6-month-old juveniles was greater than that of 10-, 14- and 18-month-old shrimp ($P < 0.05$).

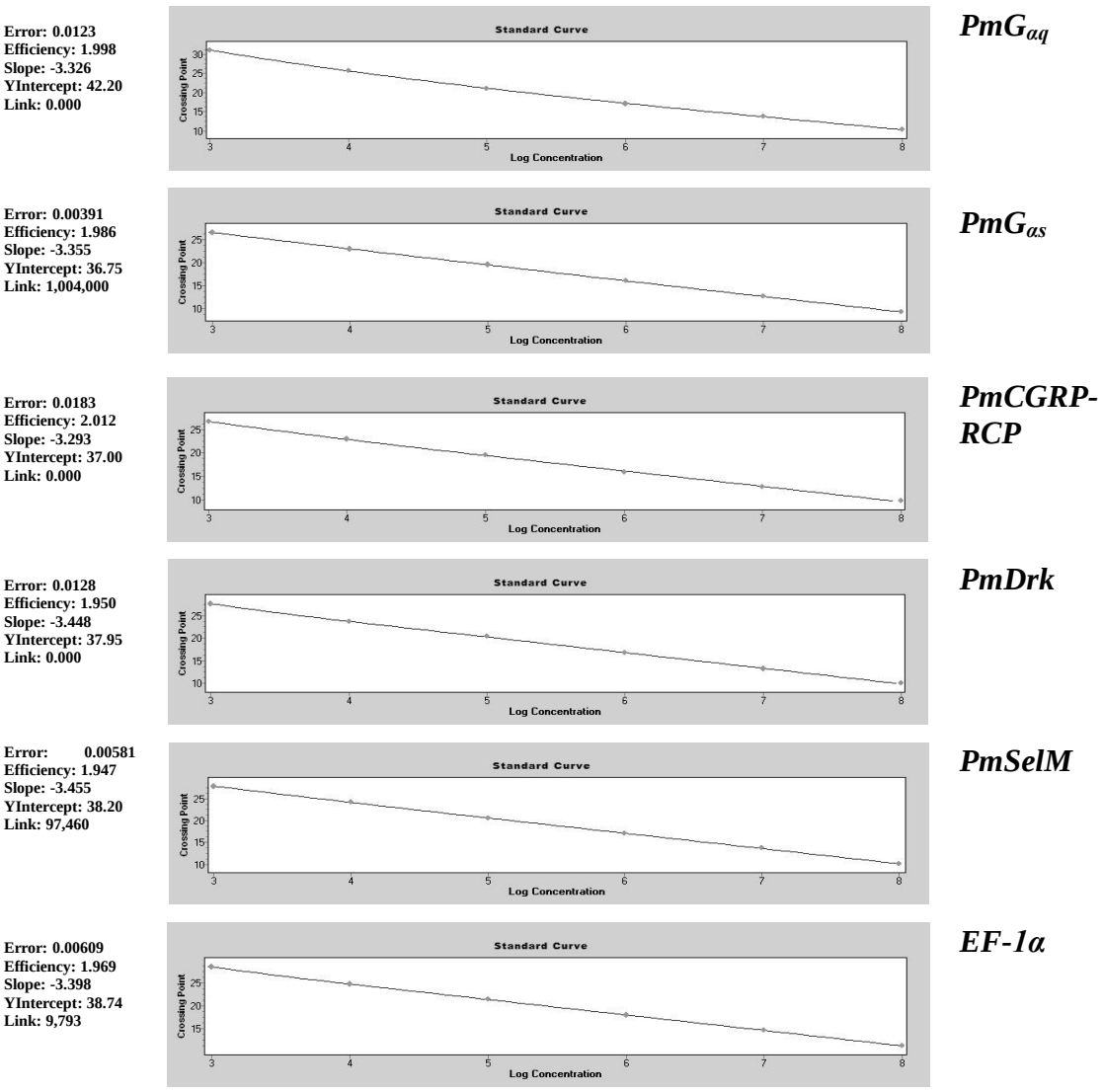


Figure 3.28 Standard curve of *PmG$_{\alpha q}$* (R^2 = 0.990, efficiency = 99.54% or ($\log_{10}$) 1.998 and equation; Y = -3.326 * log(X) + 42.20), *PmG$_{\alpha s}$* (R^2 = 0.999, efficiency = 96.83% or ($\log_{10}$) 1.986 and equation; Y = -3.355 * log(X) + 36.75), *PmCGRP-RCP* (R^2 = 0.998, efficiency = 102.80% or ($\log_{10}$) 2.012 and equation; Y = -3.293 * log(X) + 37.00), *PmDrk* (R^2 = 0.999, efficiency = 89.13% or ($\log_{10}$) 1.950 and equation; Y = -3.448 * log(X) + 37.95), *PmSelM* (R^2 = 0.990, efficiency = 88.51% or ($\log_{10}$) 1.947 and equation; Y = -3.455 * log(X) + 38.20) and *EF-1α* (R^2 = 0.999, efficiency = 93.11% or ($\log_{10}$) 1.969 and equation; Y = -3.398 * log(X) + 38.74).

3.4.2 *PmG$_{\alpha s}$*

Quantitative real-time PCR revealed that the expression level of *PmG$_{\alpha s}$* in ovaries of intact broodstock was greater than that of 4-month-old juveniles ($P < 0.05$). In intact broodstock, *PmG$_{\alpha s}$* was significantly up-regulated from stages I-III at the mature stage (IV) in intact broodstock ($P < 0.05$). Its expression level was decreased to the normal level after spawning ($P < 0.05$). The expression level of *PmG$_{\alpha s}$* in ovaries of eyestalk-ablated broodstock was also up-regulated at the mature stage (IV) of ovarian development ($P < 0.05$). The expression level of *PmG$_{\alpha s}$* in intact broodstock was significantly greater than that in eyestalk-ablated broodstock for late stages of ovarian development (III and IV, $P < 0.05$).

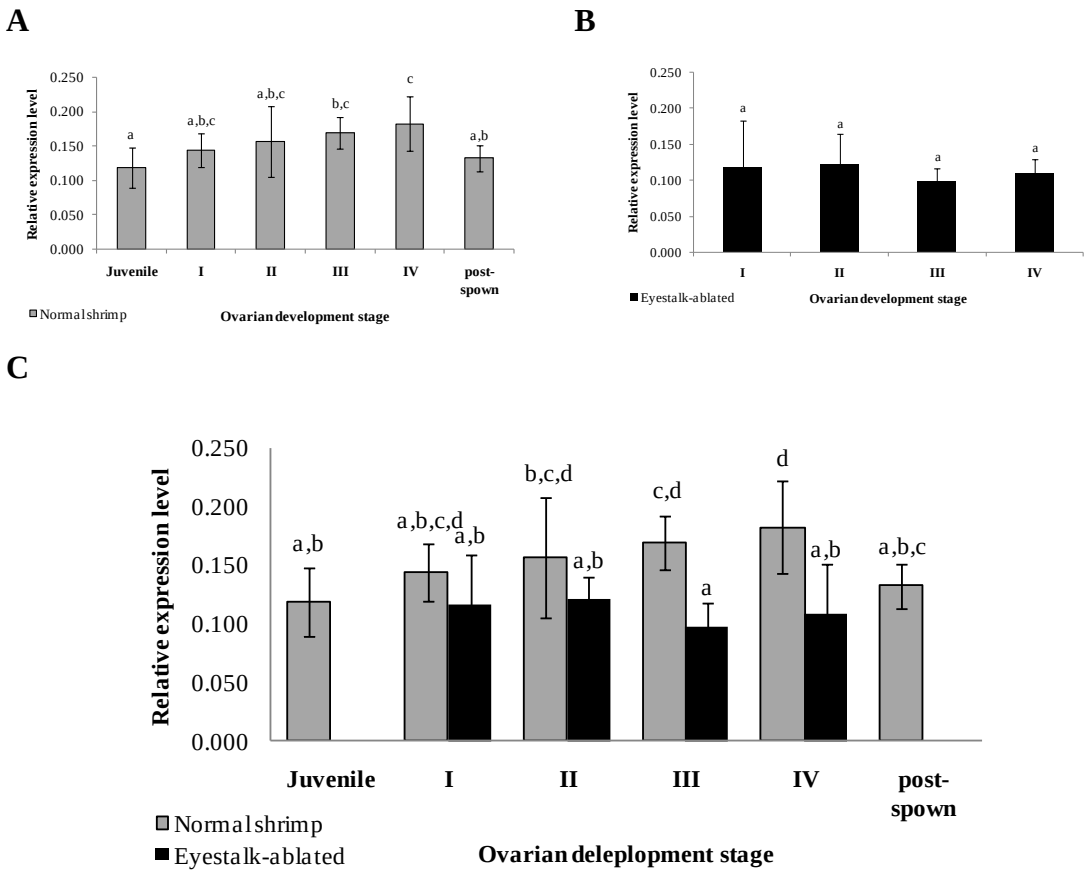
In domesticated shrimp, the expression level of *PmG$_{\alpha s}$* in ovaries of 5- and 9-month-old juvenile seemed to slightly greater than that of 14- and 19-month-old broodstock but results were not statistically significant owing to large standard errors between groups of samples ($P > 0.05$).

3.4.3 *PmDrk*

The expression level of *PmDrk* in ovaries of intact broodstock was comparable for all stage of ovarian development stage ($P < 0.05$), including that in juvenile ovaries (4-month-old, $P < 0.05$). In intact broodstock, *PmDrk* was not differentially expressed during ovarian development and after spawning ($P > 0.05$).

The expression level of *PmDrk* in ovaries of eyestalk-ablated broodstock was significantly up-regulated at the early cortical rod stage (III) of ovarian development ($P < 0.05$). The expression level of *PmDrk* in each developmental stage of intact and eyestalk-ablated broodstock was comparable ($P < 0.05$).

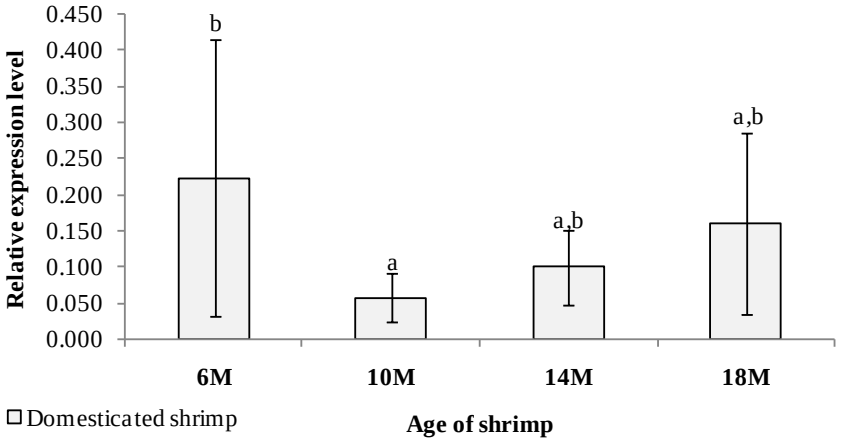
In domesticated broodstock, the expression level of *PmDrk* in ovaries of 4-month-old juveniles was significantly greater than that of domesticated 10-, 14- and 18-month-old broodstock ($P < 0.05$).



Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juvenile	0.1184 ± 0.029^{a}	6	-	-
Stage I (GSI < 1.5)	$0.1448\pm0.025^{a,b,c}$	4	0.1173 ± 0.066^{a}	5
Stage II (GSI < 2.0 - 4.0)	$0.1570\pm0.052^{a,b,c}$	4	0.1226 ± 0.042^{a}	13
Stage III (GSI < 4.0 – 6.0)	$0.1698\pm0.023^{b,c}$	5	0.0982 ± 0.018^{a}	14
Stage IV (GSI > 6.0)	0.1824 ± 0.040^{c}	8	0.1090 ± 0.020^{a}	5
Post-spowning (GSI 1.86 – 3.49)	$0.1328\pm0.019^{a,b}$	5	-	-

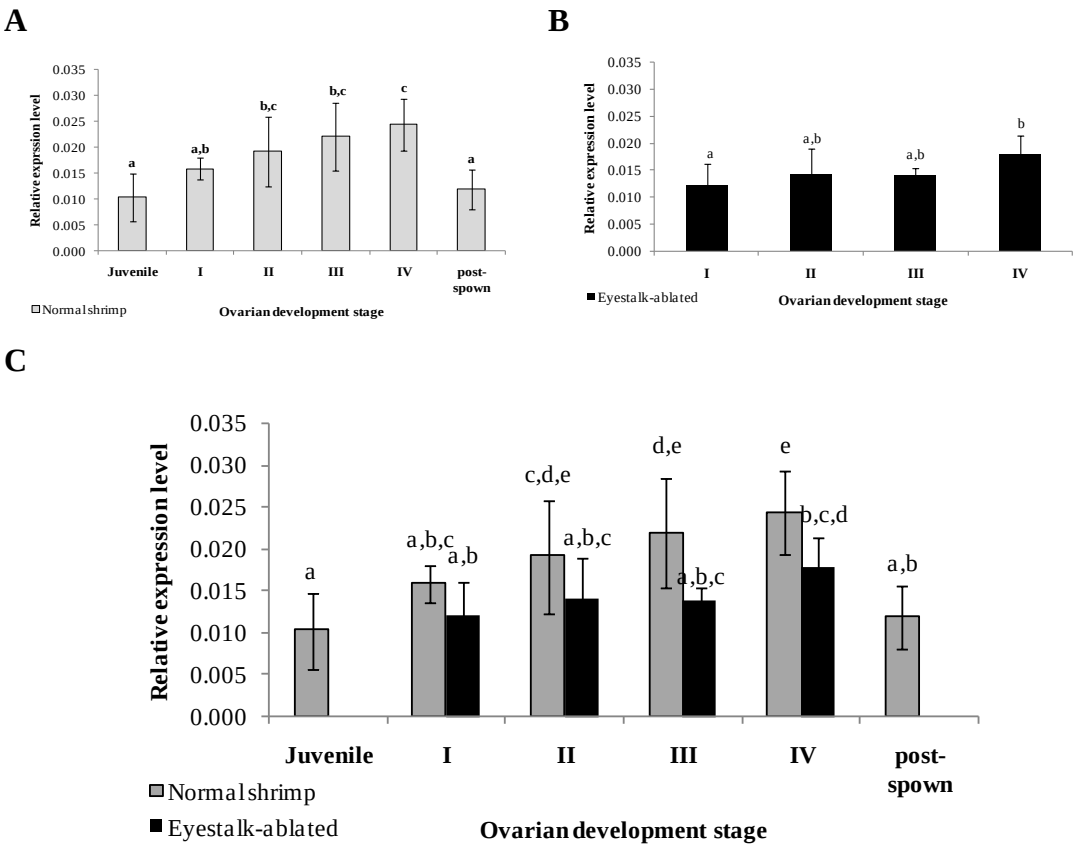
Figure 3.29 Histograms showing the relative expression profile of $PmG_{\alpha q}$ in ovaries of 4-month-old juveniles and different stages of ovarian development (stage I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spowning broodstock (A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of $PmG_{\alpha q}$ mRNA (100 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 ($P < 0.05$).



Age of shrimp	Relative expression level	*N*
6-month-old	0.2230 ± 0.192^{b}	5
10-month-old	0.0577 ± 0.033^{a}	6
14-month-old	$0.0998 \pm 0.052^{a,b}$	5
18-month-old	$0.1603 \pm 0.126^{a,b}$	5

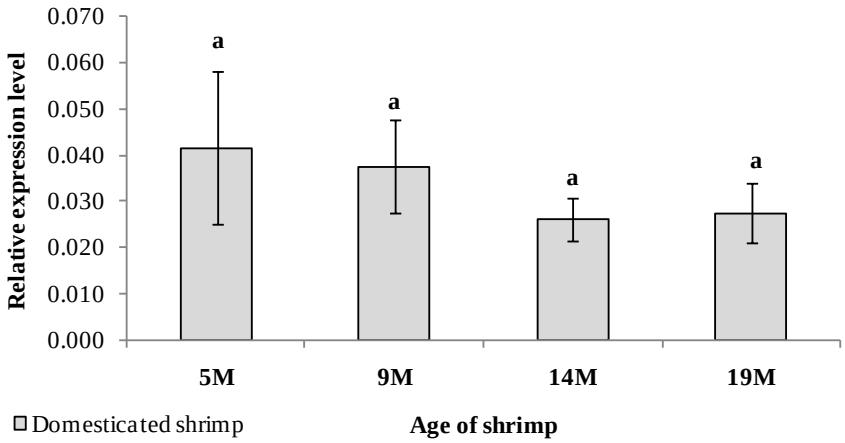
Figure 3.30 Histograms showing the relative expression profile of $PmG_{\alpha q}$ in ovaries of 6-, 10-, 14- and 18-month-old shrimp. Expression levels were measured as the absolute copy number of *PmGαq* mRNA (100 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P < 0.05$).



Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juvenile	0.0103 ± 0.005^{a}	6	-	-
Stage I (GSI < 1.5)	$0.0159\pm0.002^{a,b}$	4	0.0122 ± 0.004^{a}	4
Stage II (GSI < 2.0 - 4.0)	$0.0192\pm0.007^{b,c}$	3	$0.0141\pm0.005^{a,b}$	9
Stage III (GSI < 4.0 – 6.0)	$0.0220\pm0.007^{b,c}$	5	$0.0139\pm0.002^{a,b}$	8
Stage IV (GSI > 6.0)	0.0244 ± 0.005^{c}	8	0.0180 ± 0.003^{b}	5
Post-spowning (GSI 1.86 – 3.49)	0.0119 ± 0.004^{a}	5	-	-

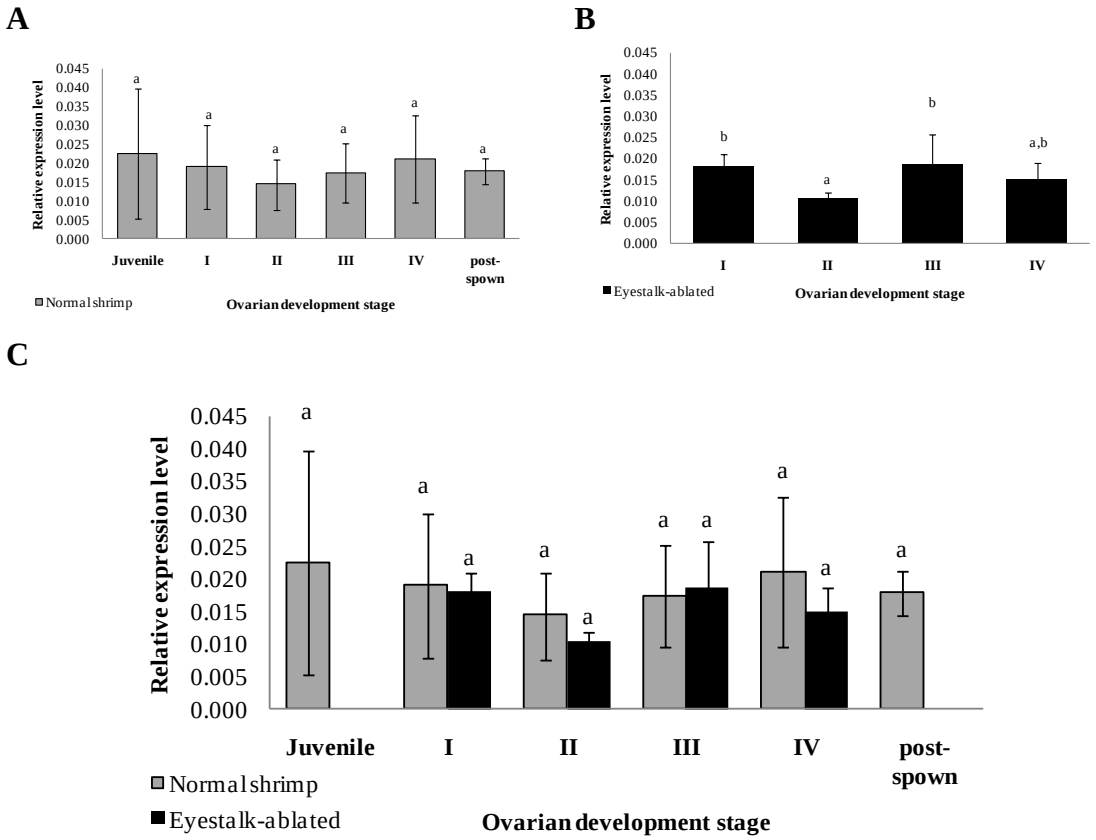
Figure 3.31 Histograms showing the relative expression profile of *PmG$_{\alpha s}$* in ovaries of 4-month-old juveniles and different stages of ovarian development (stage I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spowning broodstock (A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmGαs* mRNA (100 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 ($P < 0.05$).



Age of shrimp	Relative expression level	*N*
5-month-old	0.0416±0.037[a]	5
9-month-old	0.0376±0.027[a]	7
14-month-old	0.0261±0.013[a]	8
19-month-old	0.0275±0.015[a]	5

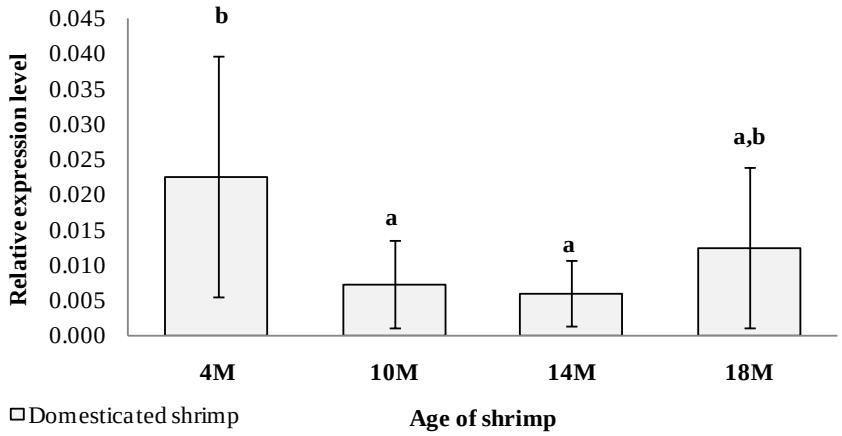
Figure 3.32 Histograms showing the relative expression profile of *PmGαq* in ovaries of 5-, 9-, 14- and 19-month-old shrimp. Expression levels were measured as the absolute copy number of $PmG_{\alpha s}$ mRNA (100 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P < 0.05$).



Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juvenile	0.0226±0.017[a]	4	-	-
Stage I (GSI < 1.5)	0.0190±0.011[a]	6	0.0181±0.003[b]	4
Stage II (GSI < 2.0 - 4.0)	0.0144±0.007[a]	7	0.0106±0.001[a]	5
Stage III (GSI < 4.0 – 6.0)	0.0175±0.008[a]	6	0.0187±0.007[b]	8
Stage IV (GSI > 6.0)	0.0212±0.012[a]	7	0.0150±0.004[a,b]	6
Post-spowning (GSI 1.86 – 3.49)	0.0179±0.003[a]	4	-	-

Figure 3.33 Histograms showing the relative expression profile of *PmDrk* in ovaries of 4-month-old juveniles and different stages of ovarian development (stage I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmDrk* mRNA (200 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 ($P < 0.05$).



Age of shrimp	Relative expression level	*N*
4-month-old	0.0226 ± 0.017^{b}	4
10-month-old	0.0072 ± 0.006^{a}	6
14-month-old	0.0060 ± 0.005^{a}	7
18-month-old	$0.0124 \pm 0.011^{a,b}$	4

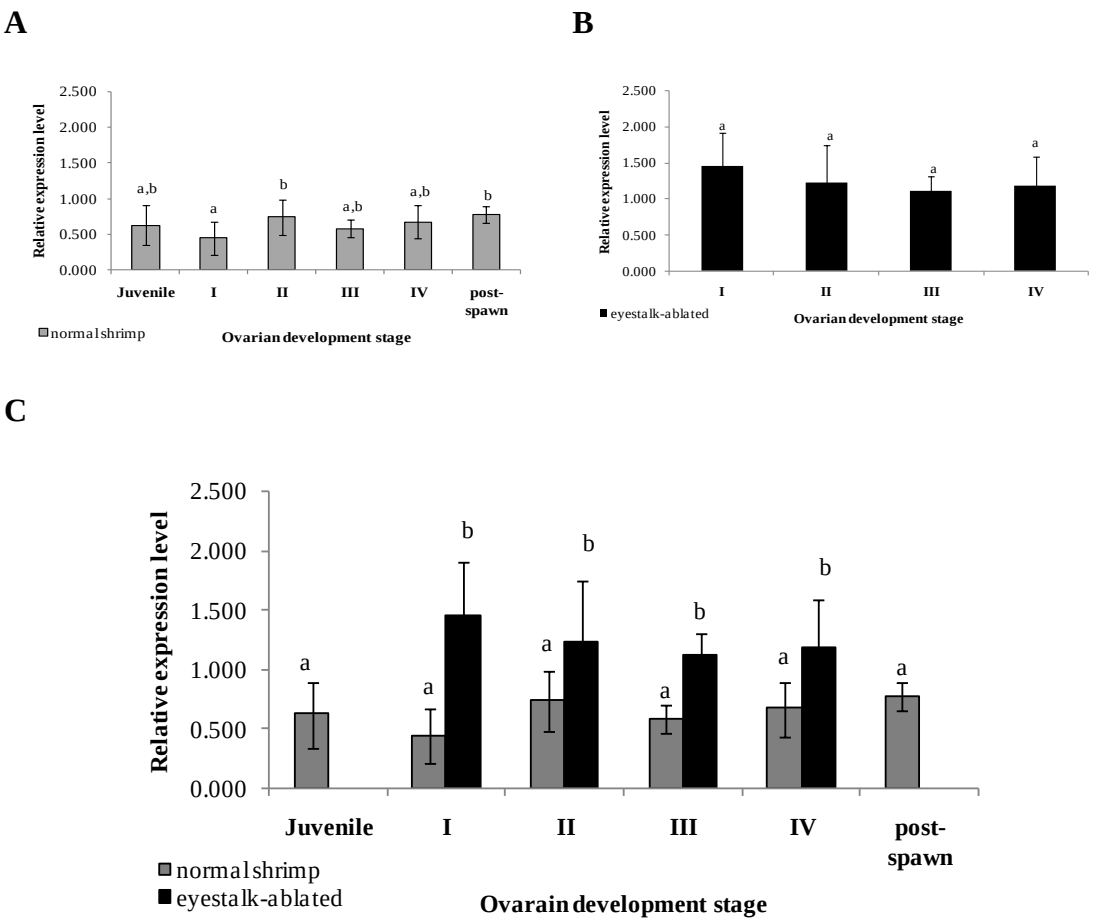
Figuer3.34 Histograms showing the relative expression profile of *PmDrk* in ovaries of 4-month-old juveniles and 10-, 14- and 18-month-old broodstock. Expression levels were measured as the absolute copy number of *PmDrk* mRNA (200 ng template) and normalized by that of *EF-1α* mRNA (5 ng template). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P < 0.05$).

3.4.4 *PmSelM*

Quantitative real-time PCR revealed that the expression level of *PmSelM* in premature ovaries of juveniles and various ovarian stages of intact broodstock was not significantly different ($P < 0.05$). In intact broodstock, its expression was up-regulated in vitellogenic (II) ovaries before returned to the basal level in early cortical rod (III) and mature (IV) ovaries and the highest level was observed in ovaries of post-spawning shrimp (V). The expression level of *PmSelM* in different ovarian stages of eyestalk-ablated broodstock was comparable. When data were

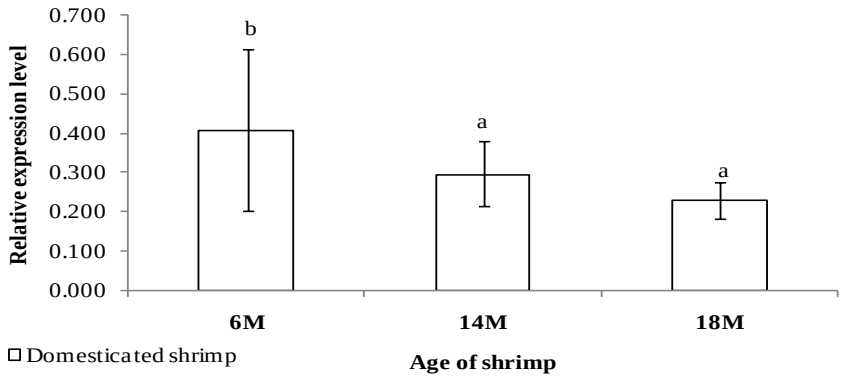
analyzed together, its expression in eyestalk-ablated broodstock was significantly greater than that in intact broodstock for all stages of ovarian development ($P < 0.05$).

In addition, the *PmSelM* mRNA level in domesticated 14- and 18-month-old broodstock was significantly lower than that of 6-month-old juveniles ($P < 0.05$, Fig. 3).



Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juvenile	0.6268±0.277[a,b]	6	-	-
Stage I (GSI < 1.5)	0.4488±0.233[a]	8	1.4590±0.453[a]	4
Stage II (GSI < 2.0 - 4.0)	0.7415±0.254[b]	7	1.2263±0.525[a]	7
Stage III (GSI < 4.0 – 6.0)	0.5842±0.119[a,b]	7	1.1169±0.197[a]	10
Stage IV (GSI > 6.0)	0.6732±0.232[a,**b**]	9	1.1919±0.401[a]	10
Post-spowning (GSI 1.86 – 3.49)	0.7753±0.117[**b**]	6	-	-

Figure 3.35 Histograms showing the relative expression profile of *PmSelM* in ovaries of cultured 4-month-old juveniles and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmSelM* 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 ($P < 0.05$).



Age of shrimp	Relative expression level	*N*
6-month-old	0.4079±0.207[b]	3
14-month-old	0.2955±0.083[a]	23
18-month-old	0.2296±0.046[a]	5

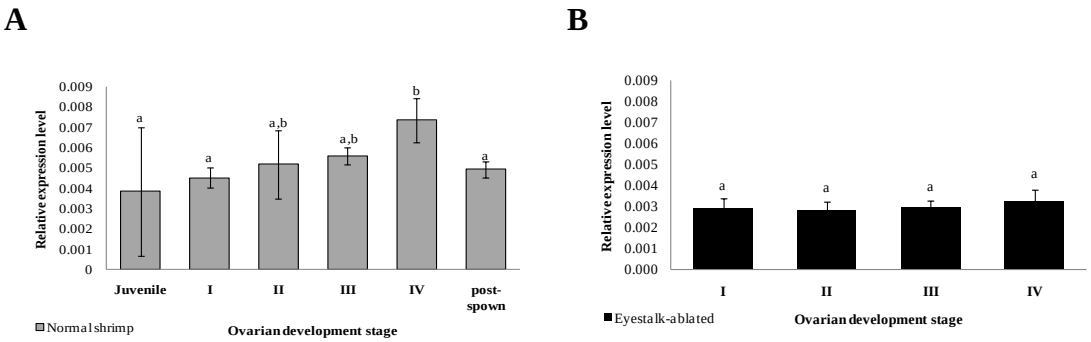
Figuer3.36 Histograms showing the relative expression profile of *PmSelM* in ovaries of 6-month-old juveniles and 14- and 18-month-old broodstock. Expression levels were measured as the absolute copy number of *PmSelM* mRNA (50 ng template) and normalized

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 ($P < 0.05$).

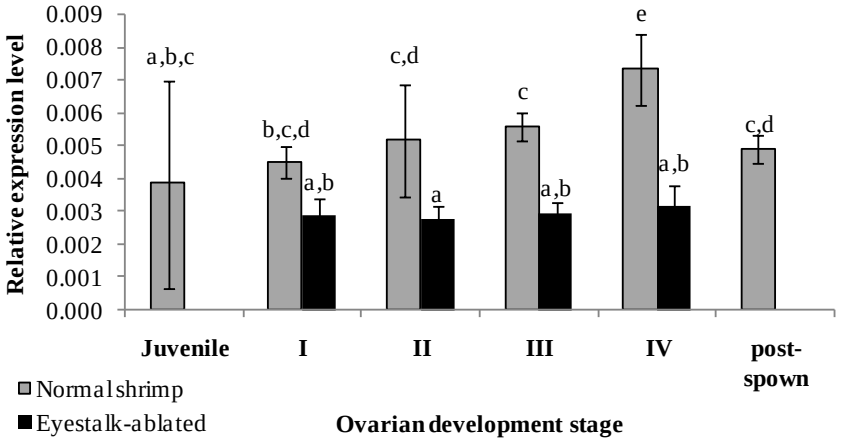
3.4.5 *PmCGRP-RCP*

Quantitative real-time PCR revealed that the expression level of *PmCGRP-RCP* in ovaries of intact broodstock was greater than that of 4-month-old juveniles ($P < 0.05$). In intact broodstock, *PmCGRP-RCP* was significantly up-regulated from stages I-III in the mature stage (IV) ($P < 0.05$). Its expression level was decreased to the normal level after spawning ($P < 0.05$). The expression level of *PmCGRP-RCP* in ovaries of eyestalk-ablated broodstock was comparable at all stages of ovarian development ($P < 0.05$). The expression level of *PmCGRP-RCP* in stages II-IV in intact broodstock was significantly greater than that in the same stages in eyestalk-ablated broodstock ($P < 0.05$).

In domesticated broodstock, the expression level of ovarian *PmCGRP-RCP* in 6-month-old juveniles was significantly greater than that in 10-, 14- and 18-month-old shrimp ($P < 0.05$).

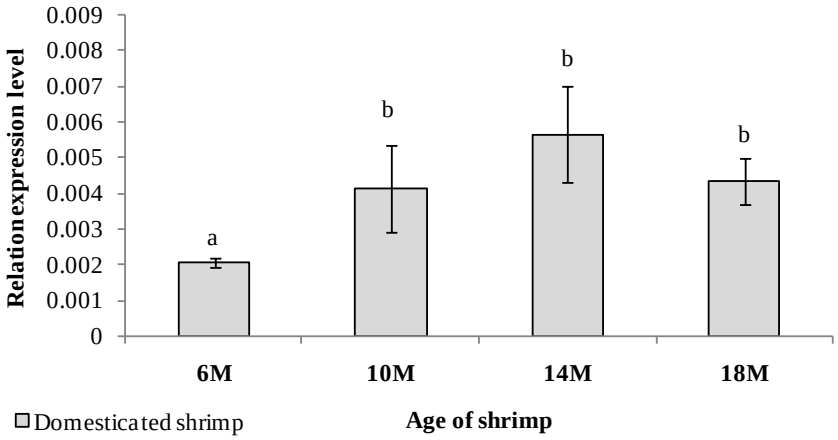


C



Ovarian stage	Relative expression level	*N*	Relative expression level	*N*
	Intact broodstock		**Eyestalk-ablated broodstock**	
Juvenile	0.0038±0.003[a]	6	-	-
Stage I (GSI < 1.5)	0.0045±0.000[a]	4	0.0029±0.000[a]	5
Stage II (GSI < 2.0 - 4.0)	0.0052±0.002[a,b]	4	0.0028±0.000[a]	9
Stage III (GSI < 4.0 – 6.0)	0.0056±0.000[a,b]	5	0.0030±0.000[a]	9
Stage IV (GSI > 6.0)	0.0074±0.001[b]	8	0.0032±0.001[a]	5
Post-spowning (GSI 1.86 – 3.49)	0.0049±0.000[a]	5	-	-

Figure 3.37 Histograms showing the relative expression profile of *PmCGRP-RCP* in ovaries of cultured 4-month-old juveniles and different stages of ovarian development (stage I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Expression levels were measured as the absolute copy number of *PmCGRP-RCP* 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 ($P < 0.05$).

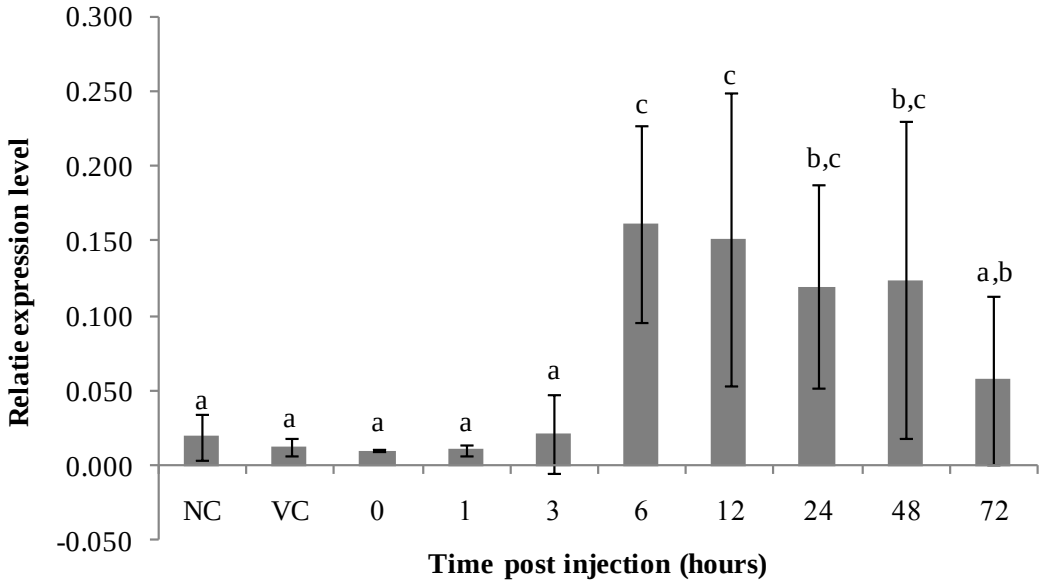


Age of shrimp	Relative expression level	*N*
6-month-old	0.0021 ± 0.000^{a}	4
10-month-old	0.0041 ± 0.001^{b}	6
14-month-old	0.0056 ± 0.001^{b}	12
18-month-old	0.0043 ± 0.001^{b}	4

Figure 3.38 Histograms showing the relative expression profile of *PmCGRP-RCP* in ovaries of cultured 6-, 10-, 14- and 18-month-old shrimp. Expression levels were measured as the absolute copy number of *PmCGRP-RCP* 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 ($P < 0.05$).

3.5 Effects of serotonin (5-HT) on expression of *PmCGRP-RCP*

Effects of exogenous administration of 5-HT (50 μg/g body weight) on the expression of *PmCGRP-RCP* in ovaries of 18-month-old *P. monodon* was examined. 5-HT resulted in up-regulation of *PmCGRP-RCP* at 6-48 hr after injection (hpi) ($P < 0.05$). Its expression was reduced to the normal level at 72 hpi ($P > 0.05$).



Group	Relative expression level	*N*
NC (negative contral)	0.0198 ± 0.015^{a}	5
VC (vehicle control)	0.0134 ± 0.006^{a}	4
0 hpi (control)	0.0106 ± 0.001^{a}	4
1 hpi	0.0110 ± 0.003^{a}	4
3 hpi	0.0218 ± 0.026^{a}	3
6 hpi	0.1619 ± 0.066^{c}	4
12 hpi	0.1521 ± 0.098^{c}	4
24 hpi	$0.1203\pm0.068^{b,c}$	4
48 hpi	$0.1249\pm0.105^{b,c}$	3
72 hpi	$0.0581\pm0.056^{a,b}$	3

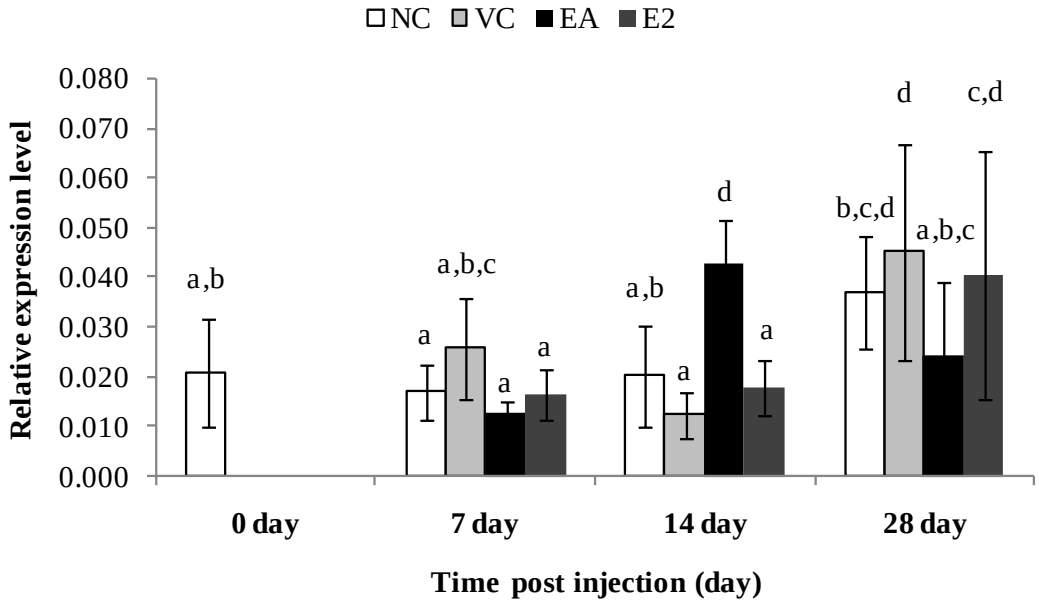
Figure 3.39 Time-course relative expression levels of *PmCGRP-RCP* in ovaries of 18-month-old shrimp after serotonin administration (50 ug/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpi). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

3.6 Effects of 17β-estradiol (E2) on expression of *PmG$_{as}$* and *PmDrk*

Effect of exogenous administration of 17β-estradiol (0.01 μg/g body weight) on the expression of *PmG$_{as}$* and *PmDrk* in ovaries of 14-month-old *P. monodon* was examined.

3.6.1 *PmG$_{as}$*

E2 resulted expression level of *PmG$_{as}$* were normal level at all treatment of experiment after injection (dpi) ($P < 0.05$).

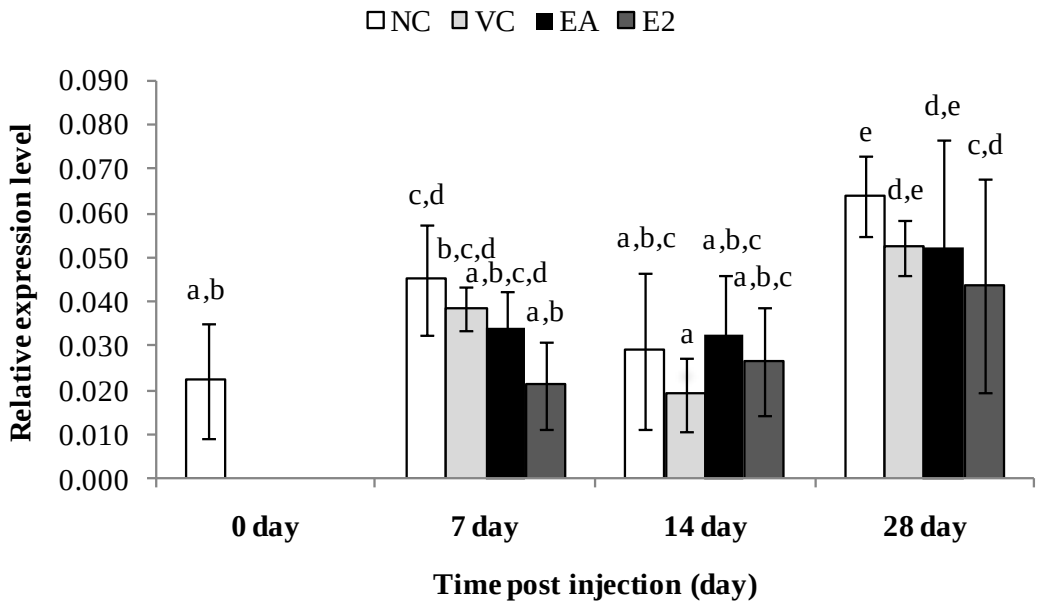


Group	Relative expression level	*N*
0 day		
NC (negative control)	0.0209±0.011[a,b]	6
7 days		
NC	0.0170±0.005[a]	5
VC (vehicle control)	0.0257±0.010[a,b,c]	5
EA (eyestalk-ablated)	0.0128±0.002[a]	5
E2 injection	0.0165±0.005[a]	7
14 days		
NC	0.0202±0.010[a,b]	4
VC	0.0123±0.005[a]	5
EA	0.0426±0.009[d]	5
E2 injection	0.0180±0.006[a]	6
28 days		
NC	0.0369±0.011[b,c,d]	5
VC	0.0452±0.022[d]	6
EA	0.0245±0.015[a,b,c]	7
E2 injection	0.0404±0.025[c,d]	6

Figure 3.40 Time-course relative expression levels of *PmG*$_{\alpha s}$ in ovaries of 14-month-old shrimp after 17β-estradiol administration (0.01 ug/g body weight) at 7, 14 and 28 days post injection (dpi). Shrimp injected with ethanol solution at 7, 14 and 28 dpi were included as the vehicle control.

3.6.2 *PmDrk*

E2 resulted in down-regulation of *PmDrk* at 7, 14 and 28 day after injection (dpi) ($P < 0.05$) when compared of expression level in normal and eyestalk-ablated of *P. monodon.*



Group	Relative expression level	*N*
0 day		
NC (negative control)	$0.0222\pm0.013^{a,b}$	5
7 days		
NC	$0.0450\pm0.013^{c,d}$	6
VC (vehicle control)	$0.0384\pm0.005^{b,c,d}$	5
EA (eyestalk-ablated)	$0.0344\pm0.008^{a,b,c,d}$	6
E2 injection	$0.0213\pm0.010^{a,b}$	11
14 days		
NC	$0.0289\pm0.018^{a,b,c}$	5
VC	0.0192 ± 0.008^{a}	5
EA	$0.0327\pm0.013^{a,b,c}$	6
E2 injection	$0.0264\pm0.012^{a,b,c}$	6
28 days		
NC	0.0369 ± 0.009^{e}	5
VC	$0.0524\pm0.006^{d,e}$	4
EA	$0.0525\pm0.024^{d,e}$	5
E2 injection	$0.0438\pm0.024^{c,d}$	5

Figure 3.41 Time-course relative expression levels of *PmDrk* in ovaries of 14-month-old shrimp after 17β-estradiol administration (0.01 ug/g body weight) at 7, 14 and 28

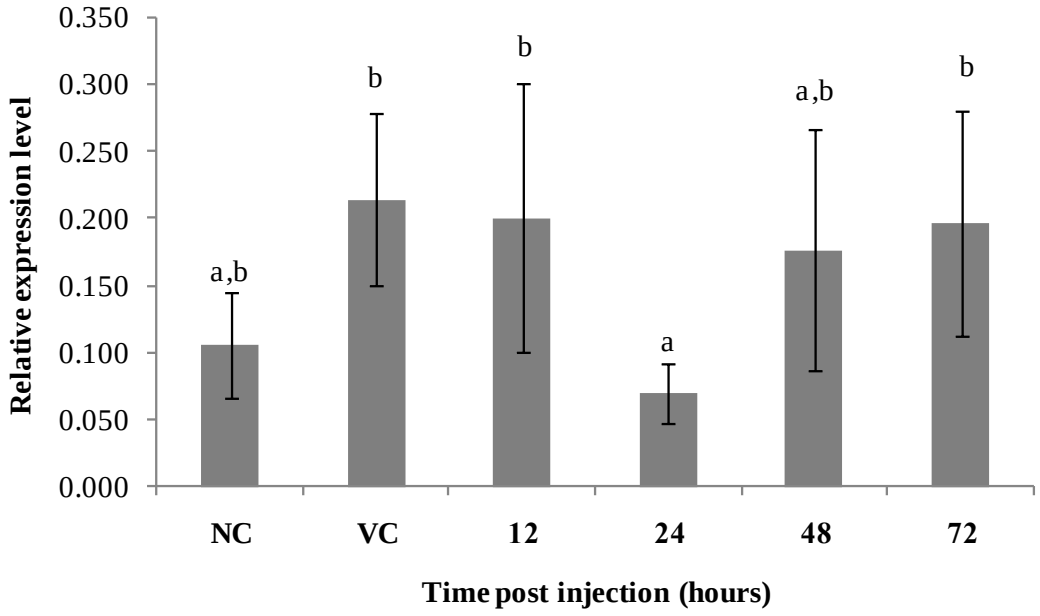
days post injection (dpi). Shrimp injected with ethanol solution at 7, 14 and 28 dpi were included as the vehicle control.

3.7 Effects of progesterone (P4) on expression of $G_{\alpha s}$ and *PmDrk*.

Effect of exogenous administration of progesterone (P4) (0.01 μg/g body weight) on the expression of $PmG_{\alpha s}$ and *PmDrk* in ovaries of 14-month-old *P. monodon* was examined.

3.7.1 $PmG_{\alpha s}$

P4 resulted expression level of $PmG_{\alpha s}$ were down-regulated 24 hours after P4 injected with compared normal level ($P < 0.05$). The expression level of $PmG_{\alpha s}$ were up-regulated at 12, 48 and 72 hour after P4 injection. The E12 was vehicle control, EtOH 12 hours post injection.

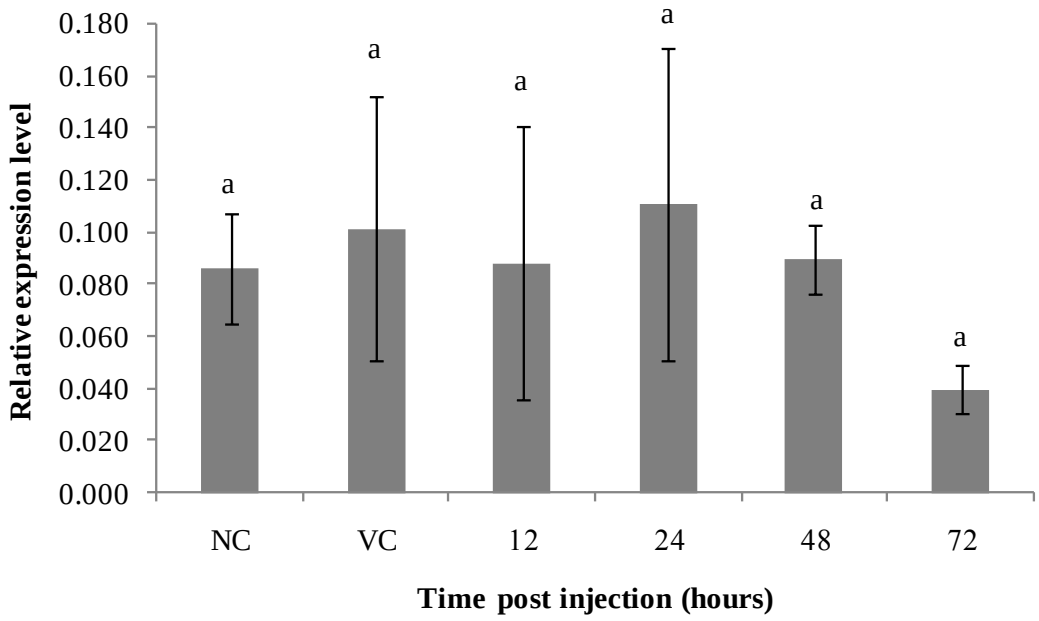


Group	Relative expression level	*N*
NC (negative contol)	$0.1062\pm0.040^{a,b}$	4
VC (EtOH, vehicle control)	0.2143 ± 0.064^{b}	4
12 hpi	0.2007 ± 0.100^{b}	3
24 hpi	0.0701 ± 0.022^{a}	4
48 hpi	$0.1770\pm0.089^{a,b}$	4
72 hpi	0.1968 ± 0.084^{b}	4

Figure 3.42 Time-course relative expression levels of $PmG_{\alpha s}$ in ovaries of 14-month-old shrimp after P4 administration (0.01 ug/g body weight) at 12, 24, 48 and 28 hours post injection (hpi). Shrimp injected with ethanol solution at 12 hpi were included as the vehicle control.

3.7.2 *PmDrk*

P4 resulted expression level of *PmDrk* were up-regulated 24 hours after P4 injected with compared normal level ($P < 0.05$). The expression level of $PmG_{\alpha s}$ were down-regulated at 72 hour after P4 injection ($P < 0.05$). The expression level of *PmDrk* at 12 and 48 hours were comparable with normal levels ($P < 0.05$).



Group	Relative expression level	*N*
NC (normal shrimp)	0.0864±0.021[a]	3
VC (EtOH, vehicle control)	0.1017±0.050[a]	5
12 hpi	0.0883±0.053[a]	4
24 hpi	0.1108±0.060[a]	3
48 hpi	0.0900±0.013[a]	4
72 hpi	0.0398±0.009[a]	3

Figure 3.43 Time-course relative expression levels of *PmDrk* in ovaries of 14-month-old shrimp after P4 administration (0.01 ug/g body weight) at 12, 24, 48 and 28 hours post injection (hpi). Shrimp injected with ethanol solution at 12 hpi were included as the vehicle control.

3.8 *In vitro* expression of recombinant PmCGRP-RCP and PmDrk proteins using the bacterial expression system.

3.8.1 Construction of recombinant plasmids

Recombinant plasmids carrying the entire ORF of *PmCGRP-RCP* and *PmDrk* were prepared for *in vitro* expression of the corresponding proteins. A primer pair was designed to amplify each cDNA. The amplified full-length cDNA of *PmCGRP-RCP* and *PmDrk* was ligated to pGEM®-T easy vector and transformed into *E. coli* JM109. Plasmid DNA of the selected clone was used as the template for the amplification using a forward primer containing *Nde* I restriction site, and a reverse primer containing *Bam* HI restriction site and six-repeated Histidine encoded nucleotides by *Pfu* DNA polymerase (Figure 3.40). The amplification product was digested with *Nde* I and *Bam* HI and the product was electrophoresed. The gel-eluted product was ligated with pET-17b, 29a and 32a(+) (for *CGRP-RCP*) or pET-17b (for *DST*) expression vectors and transformed into *E. coli* JM109.

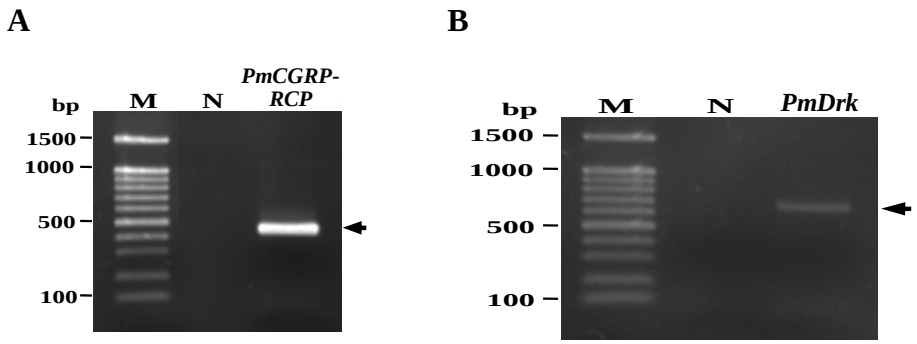


Figure 3.44 RT-PCR of the mature transcript of *PmCGRP-RCP* (A) and *PmDrk* (B) Fragments were cloned and sequenced. A hundred-fold dilution of plasmid DNA was used as the template to amplify the ORF overhang with *Nde* I-*Bam* HI of each gene. Lane M is a 100 bp DNA marker.

Plasmid DNA of the positive clone was sequenced to confirm the orientation of the recombinant clones. Plasmid DNA was extracted from a clone carrying the correct direction of each gene and transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL and *E. coli* BL21 (DE3)plysS competent cells, respectively.

3.8.2 *In vitro* expression of recombinant proteins

Expression of recombinant clones of PmCGRP-RCP (15.63 kDa) and PmDrk (25.57 kDa) proteins was selected and the expression profile of the corresponding recombinant protein was examined at 37°C for 0, 1, 2, 3, 4, 12 and 24 hours after induced by 1 mM IPTG.

No up-regulated protein was observed in case of PmCGRP-RCP (Fig. 3.45). Nevertheless, two induced protein bands of PmDrk were observed since 1 hour post IPTG induction (Fig. 3.46). The recombinant Drk protein was stably expressed at 1-24 hours post induction but its expression level seemed to be comparable during 6-24 hours post IPTG induction (Figures 3.46).

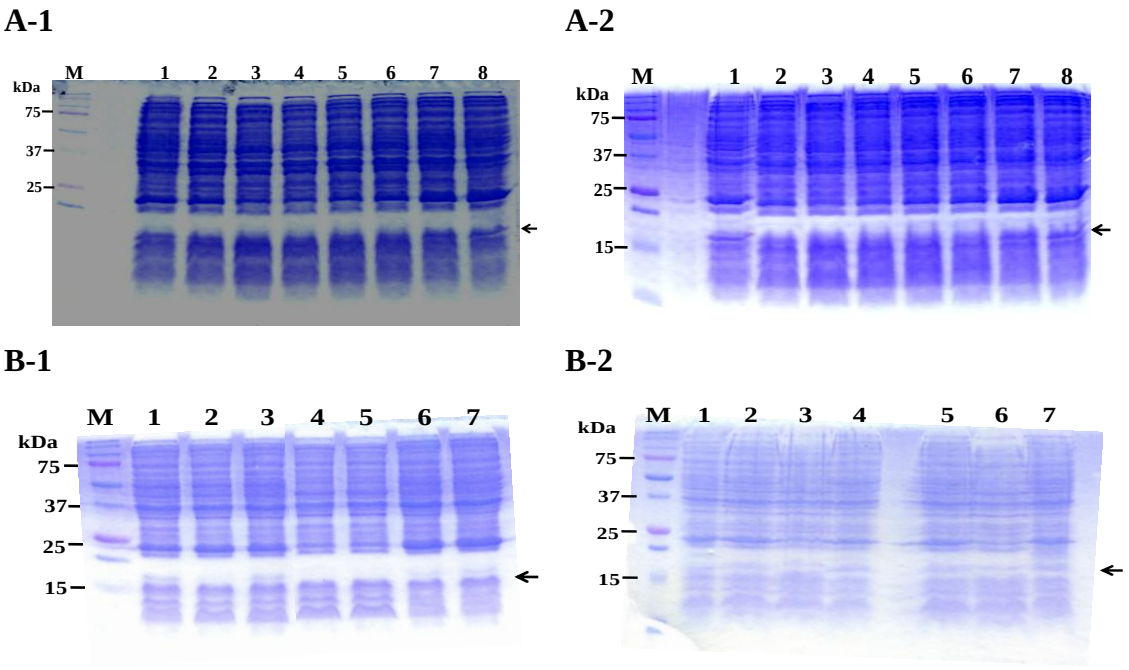


Figure 3.45 *In vitro* expression of *P. monodon CGRP-RCP* after induction with 1 mM IPTG analyzed by 17% SDS-PAGE . The *CGRP-RCP* in pET-17b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (A-1) and *E. coli* BL21-pLySS (DE3)-RIPL (A-2), lanes 3-8 were *CGRP-RCP* at 0, 1, 2, 3, 6 and 12 hours after induction with 1 mM IPTG. Lane 1 and 2 (A-1), The *E. coli* BL21-CodonPlus (DE3)-RIPL and pET-17b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL, respectively. Lane 1 and 2 (A-2), The *E. coli* BL21-pLySS(DE3) –RIPL and and pET-17b vector in *E. coli* BL21-pLySS(DE3) –RIPL, respectively. B-1: lane 1, the *E. coli* BL21-CodonPlus (DE3)-RIPL is control. Lane 2 and 3, the pET-29a and 32a(+) vector in *E. coli* BL21-CodonPlus (DE3)-RIPL. The *CGRP-RCP* in pET29a vector (lanes 4 and 6) and *CGRP-RCP* in pET32a(+) vector (lanes 5 and 7) in *E. coli* BL21-CodonPlus (DE3)-RIPL at 0 and 6 hours after induction with 1 mM IPTG. In B-2: lane 1, the *E. coli* BL21-pLySS (DE3)-RIPL is control. Lanes 2 – 4 were pET-29a vector, *CGRP-RCP* in pET29a vector in BL21-pLySS (DE3)-RIPL at 0 and 6 hours after induction with 1 mM IPTG. Lanes 5-7 were pET-32a(+) vector, *CGRP-RCP* in pET32a(+) vector in BL21-pLySS (DE3)-RIPL at 0 and 6 hours after induction with 1 mM IPTG.

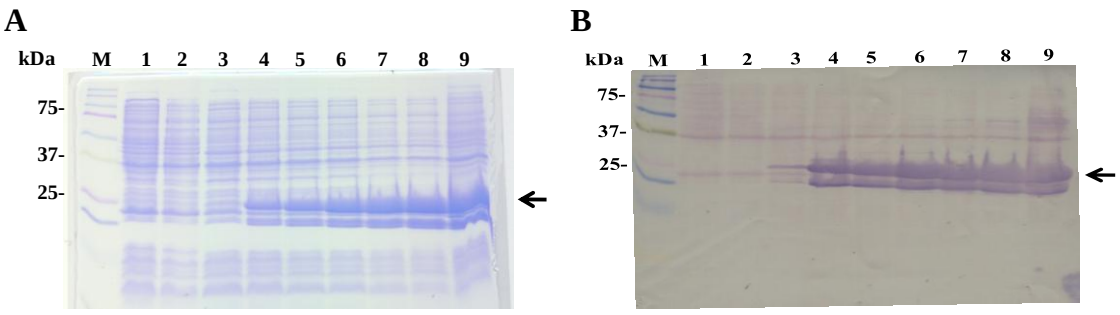


Figure 3.46 *In vitro* expression of *P. monodon Drk* at 0, 1, 2, 3, 6, 12 and 24 hours after induction with 1 mM IPTG (lanes 3-9) analyzed by 15% SDS-PAGE (A) and Western blot analysis (B). A pET-17b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lanes 1; A and B) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lanes 2; A and B) were included as the control

For determination of expressed proteins, an aliquot of the IPTG-induced culture (OD = 1) of the PmDrk protein was collected. The soluble and insoluble protein fractions were analyzed by 15% SDS-PAGE. The recombinant PmDrk cultured at 37°C for 6 hours post IPTG induction was mainly expressed in an insoluble form and a lower level of the recombinant PmDrk protein was expressed in the soluble form (Figure 3.47).

3.8.3 Purification of recombinant proteins

The recombinant PmDrk protein was purified from the insoluble fractions (Fig. 3.48). The eluted fractions were kept at 4°C.

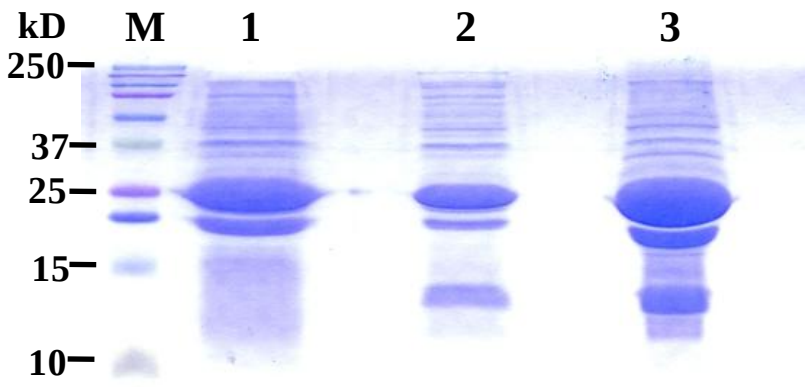


Figure 3.47 A 15% SDS-PAGE showing the expression of rDrk when a recombinant clone was cultured at 37°C for 6 hours after IPTG induction (1 mM) Arrowheads indicated the expected protein products. Lanes 1, 2 and 3 are whole cells (OD_{600} = 1.0), a soluble protein fraction (20 µg protein), and an insoluble protein fraction (20 µg protein), respectively.

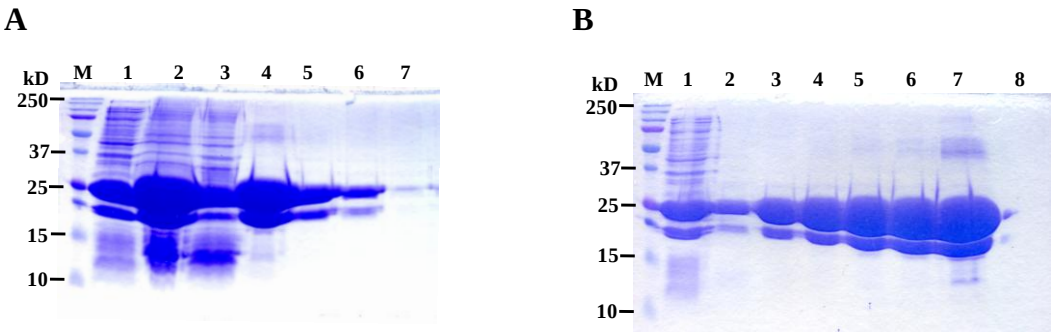


Figure 3.48 The rPmDrk protein was purified and examined by a 15% SDS-PAGE. Panel A, lane 1 is the whole cells cultured at 37 °C for 6 hours, lanes 2-3 are the first washing fractions 1 and 10, lanes 4-5 are the second washing fractions 1 and 5, lanes 6-7 are the third washing fractions 1 and 5, respectively. Panel B, lanes 1 and 2 are the insoluble fraction before and after pass through the column, lanes 3 - 8 are eluted protein fractions 1 - 6, respectively.

The purified recombinant PmDrk protein from the second fraction was concentrated and size-fractionated by 15% SDS-PAGE. After electrophoresis, the expected PmDrk was excised from the gel and electroeluted. The purified

recombinant PmDrk protein (2 mg) was sent to Faculty of Associated Medical Sciences, Chiengmai University, for production of the polyclonal antibody in rabbit.

3.8.4 Direct enzyme-linked immunosorbent assay (ELISA)

The production of antibodies against rPmDrk in rabbit was fail. ELISA resulted indicated that in Table 3.2.

Table 3.2 ELISA of Serum rabbit anti-rDrk

Dilution of serum	**Pre-immunised serum**	**Uncoated pre-immunised**	**Immunised serum**	**Uncoated immunised**
1:500	0.017	0.055	0.017	0.024
1:2000	0.008	0.016	0.009	0.013
1:8000	0.007	0.008	0.007	0.008
1:32000	0.003	0.006	0.005	0.007
	0.003	0.005	0.000	
	Conjugate control		Blank	
Positive control: Serum rabbit anti-subtilisinA (1:2000)				
Coated	1.874			
uncoated	0.063			
conjugate control	0.004	0.007		

CHAPTER IV

DISCUSSION

Closing the life cycle of cultured *P. monodon* is crucial to the sustainability of the shrimp industry. However, reduced spawning potential and degrees of maturation of *P. monodon* in captivity crucially prohibits the ability to improve the culture and management efficiency through domestication and selective breeding programs of this species (Withyachumnarnkul et al., 1998). Molecular mechanisms and functional involvement of genes and proteins in ovarian development of *P. monodon* is necessary for better understanding of the reproductive maturation *P. monodon* in captivity to resolve the major constraint and may lead to the possible ways to effectively induce ovarian maturation in this economically important species.

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

Heterotrimeric G-protein complexes form by association of three subunits: α, β and γ (Neer, 1995; Hamm, 1998). Typically, the G_α subunit contains two functional domains; the GTPase domain (the catalytic subunit) and the alpha-helical domain while β and γ lack the enzymatic activity and form a dimer that only dissociates under denaturing conditions. The inactive state of G-proteins represents a heterotrimeric complex with an α subunit in a GDP-bound form. The G-protein complex generally activated by ligand-bound seven transmembrane receptors by exchanging G_α-bound GDP for GTP (Strathmann and simon, 1991; Takesono et al., 1999; Hallak et al., 2000; Dalle et al., 2001, Voronina and Wessel, 2004).

Upon activation, the GDP of the α subunit is exchanged for GTP, and the α dissociates from the βγ complex. Free GTP-α and free βγ diffuse in the plane of membrane and regulate the activity of their respective effectors. Upon hydrolysis of GTP by the α subunit, it returns to the inactive GDP-conformation and reassociates with βγ, thus concluding the activity cycle. Different G_α subunits have distinct sets of intracellular targets and some G_α proteins have opposing effects on the same targets.

For example, $G_{\alpha s}$ and $G_{\alpha i}$ both target adenylate cyclase, however $G_{\alpha s}$ activates this enzyme while $G_{\alpha i}$ inhibits it (Voronina and Wessel, 2004).

In this thesis, the full-length cDNAs of four transcripts including *PmG$_{\alpha o}$*, *PmG$_{\gamma}$*, *PmCGRP-RCP* and *PmDrk* were successfully characterized by RACE-PCR. In addition, the partial cDNA sequence of *G$_{\alpha q}$*, *G$_{\alpha s}$* and *G$_{\beta}$ G$_{\gamma}$* were also obtained.

PmG$_{\alpha o}$ contained the predicted G_{α} domain and small GTPase domain typically found in the G_{α} protein of previously reported in various species. The partial cDNA sequence of *PmG$_{\alpha q}$* and *PmG$_{\alpha s}$* also contained an incomplete G_{α} domain. Importantly, the concensus sequence found in different classes of the G_{α} protein (Itoh et al. 1986) were also found in *PmG$_{\alpha o}$* (N-K-K-D), *PmG$_{\alpha q}$* (N-K-K-D) and *PmG$_{\alpha s}$* (N-K-Q-D).

Seven repeats of the WD40 domain were found in *PmG$_{\beta}$*. Repeated WD motifs in G_{β} form a circularized seven-fold beta propeller. The conserved cores of these motifs are a scaffold for displaying their more variable linkers on the exterior face of each propeller blade (Wall et al., 1995). *PmG$_{\gamma}$* contained the GGL domain. This functional domain is found in several RGS (regulators of G protein signaling) proteins (Sondek and Siderovski, 2001).

G protein-coupled receptors are proposed to act as monomer receptors that bind ligand and then interact with G proteins to initiate the signal transduction. The calcitonin gene related peptide (CGRP)-receptor component protein (RCP) is an intracellular peripheral membrane protein that required for CGRP (neuropepetide) signaling transduction (Luebke et al., 1996; Naghashpour et al., 1997; Pardo et al., 2001). The *CGRP-RCP* was successfully cloned in *Xenopus laevis* oocytes. It is a hydrophilic 146 amino acid protein and does not contain the signal peptide (Luebke et al., 1996).

In this study, the full-length cDNA of *PmCGRP-RCP* was successfully isolated and reported for the first time in crustacean. Like that previously characterized in various species, the deduced PmCGRP-RCP was hydrophilic and did not contain the signal peptide sequence. A predicted RPOL4c domain was found in *PmCGRP-RCP* as previously reported in *CGRP-RCP* of *Rattus norvegicus* (GenBank accession no. AY626793.1), *Mus musculus* (GenBank accession no. NM007761 and

AF118271), *Ciona intestinalis* (GenBank accession no. NM001032603 and AB081313), *Cavia porcellus* (GenBank accession no. CPU50188 and NM001172933) and *Osmerus mordax* (GenBank accession no. BT074516 and BT074545).

The full-length cDNA of *PmDrk* in ovaries of *P. monodon* was also isolated. The deduced PmDrk was composed of a central SH2 domain flanked between an N-terminal SH3 (nSH3) domain and a C-terminal SH3 (cSH3) domain. The SH3 and SH2 domains are conserved for the Src-family kinases (Kinsey et al., 2003).

Fertilization triggers the activation of Src-family kinases in eggs of various species including marine invertebrates and lower vertebrates. The SH2 and SH3 domains play a key role in regulation of catalytic activity of the Src family kinases (Tatosyan and Mizenina, 2000) and as the ligand-binding surface of both the SH2 and SH3 domains are involved in the intramolecular interaction (Erpel et al., 1995). The SH2 domain is able to bind to phosphorylated tyrosines on receptore tyrosine kinases (RTKs) and their auxiliary docking proteins. The SH3 domains interact with proline-rich sites on numerous signaling proteins which the most notable is the scaffolding protein, son of sevenless (SOS) which is pivotal for RTK-mediated ras-MAP kinase activation (Ahmed et al., 2010).

Expression analysis of functionally important genes in the signal transduction pathways in various tissues of *P. monodon*

RT-PCR was used to determine differentially expressed genes during ovarian maturation of *P. monodon*. Expression analyses of $PmG_{\alpha o}$, $PmG_{\alpha q}$, $PmG_{\alpha s}$, PmG_{β}, PmG_{γ}, *PmCGRP-RCP*, *PmDrk* and *PmSelM* were carried out and they were more abundantly expressed in ovaries than testes in both juveniles and wild broodstock. This indicated that these genes should play an important role on reproductive development of female shrimp.

Tissue distribution analysis indicated that $PmG_{\alpha q}$, $PmG_{\alpha s}$, PmG_{β}, PmG_{γ} and *PmDrk* expressed in all tissue of wild female broodstock including ovaries of juveniles and testes of wild male broodstock. $PmG_{\alpha o}$ was not expressed in antennal gland and hepatopancreas while *PmCGRP-RCP* was not expressed in those tissues and thoracic ganglion was not expressed only of *PmCGRP-RCP*. *PmSelM* was not

expressed in thoracic ganglion. Generally, heterotrimeric G-proteins are universal signaling molecules present in most of the tissues. Results from tissue expression analysis further indicated that the proteins of these transcripts should play multi-functions in several biological pathways.

Expression levels of *PmG$_{\alpha q}$*, *PmG$_{\alpha s}$*, *PmCGRP-RCP* and *PmDrk* are related with ovarian development of *P. monodon*

Quantitative real-time PCR indicated that *PmG$_{\alpha q}$* was significantly increased at the mature stage (IV) of ovarian development in intact broodstock and was decreased to the normal level after spawning. Therefore, the *PmG$_{\alpha q}$* gene products should be involved in oocyte development and maturation in *P. monodon*. Results from the present study were concordant with those from the Northern blots on the detection of *G$_{\alpha q}$* mRNA in mature eggs in the sea urchin (Voronina and Wessel, 2004) and *Xenopus* (Rizzoti et al., 1998).

Levels of the *PmG$_{\alpha q}$* mRNA in eyestalk-ablated *P. monodon* broostock were comparable during ovarian development of *P. monodon*. Its expression in stages III and IV ovaries was significantly lower than that in the same stages in intact broodstock. The expression profiles suggested that sufficient *PmG$_{\alpha q}$* mRNA was transcribed for multiple translations during ovarian development in eyestalk-ablated broodstock of *P. monodon*. However, greater levels of this transcript were required in intact broodstock where much slower ovarian development was fond in comparison with eyestalk-ablated shrimp.

In cultured shrimp, the expression level of ovarian *PmG$_{\alpha q}$* in juveniles was significantly greater than that in 10-month-old shrimp ($P < 0.05$) but was not significantly different from that in 14- and 18-month-old shrimp. Results critically suggested that *PmG$_{\alpha q}$* play an important role in a non-reproductive pathway in juveniles.

The $G_{\alpha q}$ family is activated by calcium-mobilizing hormone and stimulates phospholipase C-β (PLC-β) to produce the intracellular messages and the release of Ca^{2+} (Lee et al., 1992; Smrcka and Sternweis, 1993; Boyer et al., 1994; Gallo et al., 1996; Neves et al., 2002). During oocyte maturation, the proceeds from meiotic

prophase to second metaphase, and in parallel, there is a dramatic increase in the ability to release intracellular Ca^{2+} in response to sperm (Schlichter and Elinson, 1981). Ca^{2+} release at fertilization initiates events of egg activation. In *Xenopus*, these events include the prevention of polyspermy and completion of meiosis (Kline, 1988, Gallo et ai., 1996). The expression profiles of $PmG_{\alpha q}$ during ovarian development strongly suggested that its gene/protein should promote the ovarian maturation of *P. monodon.*

The expression profile of $PmG_{\alpha s}$ was similar as that of $G_{\alpha q}$.for intact and eyestalk-ablated broodstock. Nevertheless, this transcript was not differentially expressed in cultured shrimp ($P > 0.05$).

Typically, $G_{\alpha s}$ activates the cAMP-dependent pathway by stimulating the production of cAMP from ATP. This is accomplished by direct stimulation of the membrane-associated enzyme adenylate cyclase which acts as a second messenger that continues interacting with and activated protein kinase A (PKA). The above finding are compatible with the hypothesis that oocyte maturation in shrimp is also dependent on the intraoocyte cAMP levels and several signaling pathways are activated in the oocyte to either decrease cAMP levels or to override the cAMP inhibitor effects (Conti et al., 2002).

$G_{\alpha s}$ has been proposed in the arrest of *Xenopus* oocyte and inhibition of the oocyte $G_{\alpha s}$ by injection of its inhibitory antibody made against the C terminus of the $G_{\alpha s}$ subunit, causes oocyte maturation, indicated that a constitutively active $G_{\alpha s}$ keep the oocyte arrested (Gallo et al., 1995). In mouse (Mehlmann et al, 2002) and zabrafish (Kalinowski et al., 2004), the injection of a $G_{\alpha s}$ inhibitory antibody into oocyte also causes the meiotic resumption of oocyte. The most likely target activated by Gs in this cell is adenylate cyclase. It produces cAMP, a second messenger that can sustain meiotic arrest of the oocytes.

In contrast, maturation of pig, sheep and rabbit oocytes require a transient increase rather than a decrease in cAMP levels. Similarly, treatments that increase cAMP levels can induce oocyte maturation in jellyfish (Schmitt and Nebreda 2002; Takeda et al., 2006). Currently, there has been no reported on the cAMP levels and the progression of oocytes in paneid shrimp.

The expression profile of *PmCGRP-RCP* in wild broodstock was similar with both $PmG_{\alpha q}$ and $PmG_{\alpha s}$ with the exception that eyestalk ablation resulted in an earlier lower expression level since stage II ovaries. Like $PmG_{\alpha q}$, its expression in domesticated juveniles was significantly greater than that of 10-, 14 and 18-month-old broodstock. Based on the resuts from this thesis, it is suspected that the increasing levels of $PmG_{\alpha q}$ during ovarian development should promote rather than inhibit the ovarian maturation of *P. monodon.*

CGRP-RCP is an intracellular peripheral membrane protein that associates with the CGRP receptor. CGRP-RCP expression typically correlates with CGRP responsiveness in many tissues of human tissue (such as testes and ovaries) and mouse uterus (Balkan et al., 1999). Its structure suggests that rather than directly binding CGRP, CGRP-RCP, in combination with the endogenous CGRP receptors enables *Xenopus* oocytes responding to CGRP via a stimulation of cAMP and/or an increase in the PKA activity (Guilemare et al., 1994; Luebke et al., 1996; Balkan et al., 1999). As a result, the increasing expression levels of ovarian *PmCGRP-RCP* in intact broodstock may be involved in the stimulation of cAMP during oocyte development of *P. monodon*.

The signal transduction pathway activated in eggs of invertebrate and lower vertebrate species involves one or more Src-family protein tyrosine kinase (PTKs) (Runft et al., 2002; Sato et al., 2000a and 2000b). However, the expression level of *PmDrk* mRNA was comparable during ovarian development in both intact and eyestalk-ablated shrimp. Like *Xenopus*, the expression level of *Src* mRNA was not significantly different during oocyte maturation. Different expression levels of xSrc protein in various species (including xSrc protein) implied that the meiotic maturation of oocytes is regulated at the translational levels (Tokmakov et al., 2005).

The association of selenoprotein deficiency with decreased male and female fertility has been widely documented (Maiorino et al., 1999). The expression level of *PmSelM* mRNA of eyestalk-ablated shrimp was significantly up-regulated. The results suggest that the expression level *PmSelM* can be used as a biomarker to indicate degrees of reproductive maturation of domesticated *P. monodon* female in captivity.

Effects of neurotransmitters (5-HT), progesterone and 17β-estradiol on expression of reproduction-related genes in ovaries of *P. monodon*

Effect of exogenous 5-HT on the reproductive performance of shrimp were reported. 5-HT has been reported to induce ovarian maturation and spawning in the *P. clarkii, L. vannamei, P. vannamei, M. rosenbergii* and *P. monodon* dose dependently (Vaca and Alfaro, 2000; Wongprasert et al., 2006).

Likewise, 5-HT induced ovarian development of *P. monodon* (Wongprasert et al., 2006) and *M. rosenbergii* (Meeratana et al., 2006) dose dependently. Several previous studies illustrated that administration of 5-HT 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). Nevertheless, the effect of 5-HT on the expression levels of gene involved the signal transduction pathways in ovaries of *P. monodon* broodstock has not been reported.

Molecular effects of 5-HT on expression of *PmCGRP-RCP* in ovaries of domesticated 18-month-old shrimp was examined in the present study and its expression levels in ovaries of domesticated *P. monodon* broodstock were significantly induced following 5-HT administration at 6-48 hpi ($P < 0.05$). The information further confirmed molecular effects of 5-HT on transcription of reproduction-related genes in *P. monodon* and critically suggested that 5-HT affects both the G-protein signal transduction and, in turn, resulted in changes of the cAMP levels in *P. monodon* oocytes.

Results in the present study suggested that eyestalk ablation and 5-HT administration promoted ovarian development and further support that the gonad stimulating hormone/gonad inhibiting hormone (GSH/GIH) pathway is a primary control mechanism in *P. monodon* females.

Progesterone (P4) and 17β-estradiol (E2) are sex steroid hormones that play important roles in gametogenesis (Miura et al., 2006). 17α-hydroxyprogesterone stimulated vitellogenin synthesis in *Marsupenaeus japonicus in vivo* (Yano, 1987). Progesterone stimulated ovarian maturation and yolk protein synthesis of penaeid shrimp (Yano, 1985; Quackenbush, 2001). It also promoted spawning of *Metapenaeus ensis* (Yano, 1985). The conversion of progesterone into estradiol-17β was reported in *M. 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 their regulatory roles in vitellogenesis.

In this study, effects of progesterone on expression of reproduction-related genes in ovaries of 14-monnth-old *P. monodon* were examined. The injection of progesterone resulted in a significant decrease of $PmG_{\alpha s}$ mRNA at 24 hpi. The results were concordant with the expression level of this transcript as a consequence effect of eyestalk ablation. This implies that the reduced levels of $PmG_{\alpha s}$ allow the progression of oocyte/ovary development.

The injection of progesterone did not affect the expression level of *PmDrk*. Similar expression profiles of this gene during ovarian development of intact and ablated-eyestalk *P. monodon* were observed. It is interesting to examine whether the expression of PmDrk protein was regulated at the translational level or not.

In addition, effects of 17β-estradiol on expression of gene in ovaries of 14-monnth-old *P. monodon* were also examined. The injection of 17β-estradiol did not affect the expression of *PmDrk*. Likewise, eyestalk ablation did not significantly alter the expression level of $PmG_{\alpha s}$ in captive shrimp. In contrast, 17β-estradiol admintration resulted in a significant decrease of the expression level of *PmDrk* at 7 and 28 days post injection ($P < 0.05$) but eyestalk ablation did not result in changes of the expression level of *PmDrk*.

Results in this study clearly suggested that administration of different sex steroid may affect different signal transduction pathways. Progesterone and 17β-estradiol may directly enhance maturation through vitellogenesis stimulation or act as

a precursor of the ovarian vitellogenesis stimulating hormone (VSOH) (Fingerman et al., 1993; Yano and Hoshino, 2006).

In this thesis, the expression profile of G proteins and other genes functionally related with the signal transduction of *P. monodon* oocytes were examined in different stages of ovarian development of intact and eyestalk-ablated broodstock of wild *P. monodon* and domesticated shrimp. Effects of both progesterone and 17β-estradiol on expression of $PmG_{\alpha s}$ and *PmDrk* were also carried out. The expression profile of this gene in ovaries of different groups of *P. monodon* implied that it seems to contribute in ovarian development of *P. monodon*. Molecular mechanisms of genes functionally contribution in the signal transduction pathways on meiotic oocyte maturation and ovarian development can be further carried out and applied for the control of reproductive maturation of female *P. monodon* in captivity.

CHAPTER V

CONCLUSIONS

1. The full-length cDNAs of *PmG$_{\alpha o}$*, *PmG$_{\gamma}$*, *PmCGRP-RCP* and *PmDrk* were successfully characterized. They were 2407, 514, 1182 and 1222 bp in length containing the ORFs of 1065, 204, 417 and 672 bp corresponding to the polypeptides of 354, 67, 138 and 223 amino acids, respectively.

2. Expression patterns of *PmG$_{\alpha o}$*,*PmG$_{\alpha q}$*, *PmG$_{\alpha s}$*, *PmG$_{\beta}$*, *PmG$_{\gamma}$*, *PmCGRP-RCP*, *PmDrk* and *PmSelM* were examined by RT-PCR. These transcripts were more abundantly expressed in ovaries than testes of both juveniles and wild broodstock of *P. monodon*.

3. Quantitative real-time PCR indicated that the expression level of *PmG$_{\alpha q}$*, *PmG$_{\alpha s}$*, and *PmCGRP-RCP* were significantly up-regulated during ovarian development in intact broodstock. Eyestalk ablation resulted in the reduction of these transcripts at stage III and IV for *PmG$_{\alpha q}$*, *PmG$_{\alpha s}$* and at stages II-IV for *PmCGRP-RCP* compared to intact broostock of *P. monodon*. In contrast, the expression level of *PmSelM* in each ovarian developmental stage of eyestalk-ablated broodstock was greater than that of the same ovarian stages in intact broodstock ($P < 0.05$).

4. Serotonin injection promoted the expression level of *PmCGRP-RCP* in ovaries of 18-month-old shrimp at 6-48 hpi.

5. Progesterone injection resulted in the reduction of *PmG$_{\alpha s}$* expression at 24 hpi ($P < 0.05$) but had no effect on the expression level of *PmDrk* ($P > 0.05$).

6. Exogenous injection of17β-estradiol had no effect on the expression of *PmG$_{\alpha s}$* ($P > 0.05$) but resulted in a significant decrease of the expression level of *PmDrk* at 7 and 28 days post injection ($P < 0.05$).

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APPENDICES

APPENDICES

Table 1 Relative expression levels of $G_{\alpha q}$ in different ovarian developmental stages of *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups		Mean conc.		Ratio (target / *EF-1α*)	Average	STDEV
		$G_{\alpha q}$	*EF-1α*			
Juvenile	JNOV4	4.57E+04	5.56E+06	0.1053	0.1184	0.029
	JNOV5	2.67E+04	3.09E+06	0.0919		
	JNOV6	3.48E+04	4.96E+06	0.0823		
	JNOV7	6.04E+04	5.86E+06	0.1440		
	JNOV12	9.93E+03	4.56E+05	0.1522		
	JNOV14	5.83E+04	6.00E+06	0.1347		
N-BD-Stage I	BU14OV8	7.23E+04	6.33E+06	0.1685	0.1448	0.025
	BU14OV15	4.26E+04	4.77E+06	0.1120		
	BU14OV18	5.38E+04	5.20E+06	0.1396		
	BU14OV22	4.71E+04	3.84E+06	0.1592		
N:BD-Stage II	BFNOV25	3.99E+04	2.41E+06	0.2033	0.1570	0.052
	BFNOV31	5.29E+04	4.29E+06	0.1654		
	BFNOV33	4.72E+04	3.46E+06	0.1766		
	BFNOV38	4.19E+04	6.29E+06	0.0830		
N:BD-Stage III	BFNOV1	2.40E+04	1.31E+06	0.1874	0.1698	0.023
	BFNOV7	2.53E+04	1.33E+06	0.1981		
	BFNOV18	3.00E+04	2.35E+06	0.1418		
	BFNOV23	3.29E+04	2.24E+06	0.1687		
	BFNOV24	2.88E+04	2.06E+06	0.1528		
N:BD-Stage IV	BFNOV8	1.36E+04	7.09E+05	0.1547	0.1824	0.040
	BFNOV10	1.25E+04	5.26E+05	0.1851		
	BFNOV11	1.88E+04	6.68E+05	0.2601		
	BFNOV14	1.90E+04	8.28E+05	0.2133		
	BFNOV15	1.28E+04	6.16E+05	0.1638		
	BFNOV16	1.52E+04	7.58E+05	0.1709		
	BFNOV17	1.37E+04	6.10E+05	0.1812		
	BFNOV20	1.12E+04	6.40E+05	0.1298		
N:BD Post-spawning	BFNOV30	4.53E+04	4.57E+06	0.1270	0.1328	0.019
	BFNOV34	5.75E+04	5.89E+06	0.1344		
	BFNOV37	5.74E+04	4.84E+06	0.1636		
	BFNOV39	4.98E+04	5.74E+06	0.1144		
	BFNOV40	3.61E+04	3.44E+06	0.1246		
EA:BD-Stage I	YLBOV6	5.53E+04	3.22E+06	0.2338	0.1173	0.066
	BFEAOV15	3.34E+04	4.84E+06	0.0797		

	BFEAOV18	1.78E+04	1.84E+06	0.0878		
	WFEAOV4	3.62E+04	4.38E+06	0.0982		
	WFEAOV33	2.00E+04	2.20E+06	0.0866		
EA:BD-Stage II	WFEAOV27U	3.81E+04	4.86E+06	0.0946	0.1226	0.042
	WFEAOV6	3.37E+04	3.58E+06	0.1089		
	WFEAOV1	6.43E+04	3.55E+06	0.2575		
	WFEAOV29	2.87E+04	2.82E+06	0.1109		
	WFEAOV5	2.73E+04	2.88E+06	0.1017		
	WFEAOV20	3.43E+04	3.71E+06	0.1078		
	WFEAOV24	3.41E+04	3.24E+06	0.1222		
	WFEAOV30U	1.93E+04	2.65E+06	0.1241		
	WFEAOV30L	1.72E+04	2.53E+06	0.1113		
	WFEAOV27L	2.85E+04	5.11E+06	0.1090		
	WFEAOV21	3.18E+04	5.08E+06	0.1263		
	WFEAOV2	3.16E+04	5.86E+06	0.1089		
	WFEAOV22	3.03E+04	5.51E+06	0.1095		
EA:BD-Stage III	WFEAOV18	1.78E+04	2.98E+06	0.0991	0.0982	0.018
	WFEAOV19	2.51E+04	3.81E+06	0.1231		
	WFEAOV28	1.82E+04	2.94E+06	0.1031		
	WFEAOV9	1.30E+04	1.79E+06	0.1065		
	WFEAOV12	1.44E+04	2.56E+06	0.0862		
	WFEAOV26	1.57E+04	3.03E+06	0.0822		
	WFEAOV31U	1.23E+04	1.82E+06	0.0971		
	WFEAOV31L	1.03E+04	1.35E+06	0.1021		
	WFEAOV32U	1.23E+04	1.60E+06	0.1102		
	WFEAOV8	5.23E+04	9.96E+06	0.0510		
	WFEAOV10	2.10E+04	3.08E+06	0.1204		
	WFEAOV17	1.59E+04	2.42E+06	0.1044		
	WFEAOV32L	9.98E+04	1.26E+06	0.1041		
	WFEAOV25	1.43E+04	2.50E+06	0.0874		
EA:BD-Stage IV	WFEAOV11	1.31E+04	1.85E+06	0.1039	0.1090	0.020
	WFEAOV13	1.24E+04	1.64E+06	0.1086		
	WFEAOV14	1.21E+04	1.71E+06	0.1008		
	WFEAOV15	9.42E+03	1.36E+06	0.0890		
	WFEAOV16	1.79E+04	2.09E+06	0.1422		
6-month-old	JN6OV1	3.87E+03	5.79E+06	0.1439	0.2230	0.192
	JN6OV2	3.73E+04	4.77E+05	0.1665		
	JN6OV3	3.52E+04	5.54E+05	0.1327		
	JN6OV4	1.85E+04	2.89E+05	0.1079		
	JN6OV5	2.00E+03	6.15E+05	0.5644		
10-month-old	BU10OV13	1.14E+03	1.08E+06	0.0393	0.0577	0.033

	BU10OV14	8.98E+03	1.09E+06	0.1035		
	BU10OV15	7.05E+03	1.31E+06	0.0606		
	BU10OV17	1.54E+03	9.48E+04	0.0754		
	BU10OV19	3.52E+03	4.51E+05	0.0615		
	BU10OV20	1.99E+03	2.06E+06	0.0054		
14-month-old	BU14OV19	1.27E+04	1.82E+06	0.1011	0.0998	0.052
	BU14OV30	1.81E+03	2.50E+06	0.1203		
	BU14OV41	2.96E+04	3.36E+06	0.1737		
	BU14OV42	1.61E+03	6.19E+06	0.0417		
	BU14OV54	2.66E+03	8.18E+06	0.0622		
18-month-old	NM18OV2	3.43E+04	5.64E+06	0.1260	0.1603	0.126
	NM18OV3	2.66E+04	4.42E+06	0.1200		
	NM18OV4	1.85E+04	1.70E+06	0.1819		
	NM18OV5	9.01E+03	7.21E+06	0.0157		
	NM18OV6	2.89E+04	1.15E+06	0.3577		

Table 2 Relative expression levels of $G_{\alpha s}$ in different ovarian developmental stages of *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups		**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
		$G_{\alpha s}$	***EF-1α***			
Juvenile	JNOV4	4.50E+04	5.56E+06	0.0081	0.0103	0.005
	JNOV5	2.37E+04	3.09E+06	0.0077		
	JNOV6	3.62E+04	4.96E+06	0.0073		
	JNOV7	6.29E+04	5.86E+06	0.0107		
	JNOV12	8.82E+03	4.56E+05	0.0193		
	JNOV14	5.31E+05	6.00E+06	0.0089		
N-BD-Stage I	BU14OV8	1.01E+04	6.33E+06	0.0160	0.0159	0.002
	BU14OV15	6.51E+04	4.77E+06	0.0136		
	BU14OV14	9.71E+04	5.20E+06	0.0187		
	BFNOV22	5.78E+04	3.48E+06	0.0151		
N:BD-Stage II	BFNOV25	6.03E+04	2.41E+06	0.0250	0.0192	0.007
	BFNOV31	5.06E+04	4.19E+06	0.0118		
	BFNOV33	7.15E+04	3.46E+06	0.0207		
N:BD-Stage III	BFNOV1	4.03E+04	1.31E+06	0.0308	0.0220	0.007
	BFNOV7	3.52E+04	1.33E+06	0.0265		
	BFNOV18	3.41E+04	2.35E+06	0.0145		
	BFNOV23	4.42E+04	2.24E+06	0.0198		
	BFNOV24	3.38E+04	2.06E+06	0.0185		
N:BD-Stage IV	BFNOV8	1.76E+04	7.09E+05	0.0248	0.0244	0.005
	BFNOV10	1.55E+04	5.26E+05	0.0295		

	BFNOV11	2.09E+04	6.68E+05	0.0313		
	BFNOV14	2.18E+04	8.28E+05	0.0263		
	BFNOV15	1.38E+04	6.16E+05	0.0224		
	BFNOV16	1.45E+04	7.58E+05	0.0192		
	BFNOV17	1.57E+04	6.10E+05	0.0257		
	BFNOV20	1.04E+04	6.40E+05	0.0163		
N:BD Post-spawning	BFNOV30	4.58E+04	4.57E+06	0.0100	0.0119	0.004
	BFNOV34	6.98E+04	5.89E+06	0.0118		
	BFNOV37	7.48E+04	4.48E+06	0.0155		
	BFNOV39	3.76E+04	5.74E+06	0.0066		
	BFNOV40	5.31E+04	3.44E+06	0.0155		
EA:BD-Stage I	YLBOV6	4.91E+04	3.22E+06	0.0153	0.0122	0.004
	WFEAOV4	6.44E+04	4.38E+06	0.0147		
	WFEAOV33	2.72E+04	2.20E+06	0.0123		
	BFEAOV18	1.20E+04	1.84E+06	0.0065		
EA:BD-Stage II	WFEAOV27U	4.03E+04	4.86E+06	0.0083	0.0141	0.005
	WFEAOV1	4.03E+04	3.55E+06	0.0113		
	WFEAOV5	7.48E+04	2.88E+06	0.0260		
	WFEAOV6	5.34E+04	3.58E+06	0.0149		
	WFEAOV29	3.75E+04	2.82E+06	0.0133		
	WFEAOV20	5.25E+04	3.71E+06	0.0142		
	WFEAOV24	4.10E+04	3.24E+06	0.0126		
	WFEAOV30U	3.47E+04	2.65E+06	0.0131		
	WFEAOV30L	3.40E+04	2.53E+06	0.0134		
EA:BD-Stage III	WFEAOV12	3.86E+04	2.56E+06	0.0151	0.0139	0.002
	WFEAOV18	4.61E+04	2.98E+06	0.0155		
	WFEAOV19	4.96E+04	3.81E+06	0.0130		
	WFEAOV26	3.69E+04	3.03E+06	0.0122		
	WFEAOV28	4.12E+04	2.94E+06	0.0140		
	WFEAOV32U	1.19E+04	1.60E+06	0.0199		
	WFEAOV31U	2.36E+04	1.82E+06	0.0130		
	WFEAOV31L	2.18E+04	1.35E+06	0.0161		
EA:BD-Stage IV	WFEAOV11	3.09E+04	1.85E+06	0.0167	0.0180	0.003
	WFEAOV13	3.15E+04	1.64E+06	0.0192		
	WFEAOV14	2.21E+04	1.71E+06	0.0129		
	WFEAOV15	2.93E+04	2.76E+06	0.0216		
	WFEAOV16	4.12E+04	2.38E+06	0.0197		
5-month-old	BU5OV4	2.16E+03	2.09E+05	0.0103	0.0416	0.037
	BU5OV13	1.66E+04	4.90E+05	0.0339		
	BU5OV14	1.40E+04	4.82E+05	0.0291		

	BU5OV15	1.11E+04	1.06E+05	0.1054		
	BU5OV17	7.79E+03	2.62E+05	0.0297		
9-month-old	BU9OV2	4.03E+03	1.00E+05	0.0402	0.0376	0.027
	BU9OV4	2.96E+03	2.44E+05	0.0121		
	BU9OV8	6.04E+03	5.00E+05	0.0121		
	BU9OV11	9.38E+03	2.22E+05	0.0423		
	BU9OV14	1.33E+04	1.46E+05	0.0914		
	BU9OV18	1.74E+04	6.79E+05	0.0256		
	BU9OV20	2.82E+04	7.20E+05	0.0392		
14-month-old	BU14OV5	5.84E+03	2.10E+05	0.0278	0.0260	0.013
	BU14OV6	5.96E+03	2.77E+05	0.0215		
	BU14OV19	1.09E+04	2.66E+05	0.0412		
	BU14OV25	9.23E+03	1.98E+05	0.0467		
	BU14OV27	6.29E+03	8.14E+05	0.0077		
	BU14OV33	1.85E+04	6.92E+05	0.0267		
	BU14OV34	1.04E+04	7.52E+05	0.0138		
	BU14OV62	1.48E+04	6.42E+05	0.0230		
19-month-old	BU19OV1	1.06E+04	2.03E+05	0.0523	0.0275	0.015
	BU19OV4	9.26E+03	3.93E+05	0.0236		
	BU19OV5	1.06E+04	2.03E+05	0.0523		
	BU19OV6	9.26E+03	3.93E+05	0.0236		
	BU19OV7	1.06E+04	2.03E+05	0.0523		
	BU19OV8	9.26E+03	3.93E+05	0.0236		

Table 3 Relative expression levels of *CGRP-RCP* in different ovarian developmental stages of *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups		**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
		CGRP-RCP	***EF-1α***			
Juvenile	JNOV4	5.56E+04	5.56E+06	0.0026	0.0038	0.003
	JNOV5	7.52E+03	3.09E+06	0.0024		
	JNOV6	1.28E+04	4.96E+06	0.0026		
	JNOV7	1.48E+04	5.86E+06	0.0025		
	JNOV12	4.71E+03	4.56E+05	0.0103		
	JNOV14	1.55E+04	6.00E+06	0.0026		
N-BD-Stage I	BU14OV8	2.84E+04	6.33E+06	0.0045	0.0045	0.000
	BU14OV15	2.14E+04	4.77E+06	0.0045		
	BFNOV22	1.97E+04	3.84E+06	0.0051		
N:BD-Stage II	BFNOV25	1.77E+04	2.41E+06	0.0073	0.0052	0.002
	BFNOV31	2.05E+04	4.29E+06	0.0048		

	BFNOV33	1.86E+04	3.46E+06	0.0054		
	BFNOV38	2.04E+04	6.29E+06	0.0032		
N:BD-Stage III	BFNOV1	8.00E+03	1.31E+06	0.0061	0.0056	0.000
	BFNOV7	7.80E+03	1.33E+06	0.0059		
	BFNOV18	1.19E+04	2.35E+06	0.0050		
	BFNOV23	1.27E+04	2.24E+06	0.0057		
	BFNOV24	1.08E+04	2.06E+06	0.0053		
N:BD-Stage IV	BFNOV8	4.33E+03	7.09E+05	0.0061	0.0074	0.001
	BFNOV10	3.69E+03	5.26E+05	0.0070		
	BFNOV11	5.78E+03	6.68E+05	0.0086		
	BFNOV14	6.60E+03	8.28E+05	0.0080		
	BFNOV15	7.73E+03	6.16E+05	0.0061		
	BFNOV16	4.86E+03	7.58E+05	0.0064		
	BFNOV17	5.02E+03	6.10E+05	0.0082		
N:BD Post-spawning	BFNOV30	2.09E+04	4.57E+06	0.0046	0.0049	0.000
	BFNOV34	2.89E+04	5.89E+06	0.0049		
	BFNOV37	2.65E+04	4.84E+06	0.0055		
	BFNOV39	2.57E+04	5.74E+06	0.0045		
	BFNOV40	1.78E+04	3.44E+06	0.0052		
EA:BD-Stage I	YLBOV6	9.30E+03	3.22E+06	0.0029	0.0029	0.000
	BFEAOV15	1.77E+04	4.84E+06	0.0036		
	BFEAOV18	5.38E+03	1.84E+06	0.0029		
	WFEAOV4	1.05E+04	4.38E+06	0.0024		
	WFEAOV33	5.94E+03	2.20E+06	0.0027		
EA:BD-Stage II	WFEAOV27U	1.10E+04	4.86E+06	0.0023	0.0028	0.000
	WFEAOV6	8.57E+03	3.58E+06	0.0024		
	WFEAOV1	1.06E+04	3.55E+06	0.0030		
	WFEAOV29	1.04E+04	2.82E+06	0.0037		
	WFEAOV5	7.74E+03	2.88E+06	0.0027		
	WFEAOV20	1.05E+04	3.71E+06	0.0028		
	WFEAOV24	8.24E+03	3.24E+06	0.0025		
	WFEAOV30U	7.83E+03	2.65E+06	0.0030		
	WFEAOV30L	7.01E+03	2.53E+06	0.0028		
EA:BD-Stage III	WFEAOV18	8.89E+03	2.98E+06	0.0030	0.0030	0.000
	WFEAOV19	1.10E+04	2.81E+06	0.0029		
	WFEAOV28	8.92E+03	2.94E+06	0.0030		
	WFEAOV9	5.27E+03	1.79E+06	0.0029		
	WFEAOV12	7.99E+03	2.56E+06	0.0031		
	WFEAOV26	7.45E+03	3.03E+06	0.0025		
	WFEAOV31U	5.43E+03	1.82E+06	0.0030		
	WFEAOV31L	3.76E+03	1.35E+06	0.0028		

	WFEAOV32U	5.81E+03	1.60E+06	0.0036		
EA:BD-Stage IV	WFEAOV11	5.60E+03	1.85E+06	0.0030	0.0032	0.001
	WFEAOV13	5.99E+03	1.64E+06	0.0037		
	WFEAOV14	4.87E+03	1.71E+06	0.0028		
	WFEAOV15	3.41E+03	1.36E+06	0.0025		
	WFEAOV16	8.28E+03	2.09E+06	0.0040		
6-month-old	JN6OV1	1.10E+04	5.79E+06	0.0019	0.0021	0.000
	JN6OV2	9.87E+03	4.77E+06	0.0021		
	JN6OV3	1.21E+04	5.54E+06	0.0022		
	JN6OV6	1.19E+04	5.58E+06	0.0021		
10-month-old	BU10OV3	9.95E+02	3.53E+05	0.0028	0.0041	0.001
	BU10OV14	6.01E+03	1.09E+06	0.0055		
	BU10OV15	6.75E+03	1.31E+06	0.0052		
	BU10OV18	1.77E+03	6.48E+05	0.0027		
	BU10OV19	1.70E+03	4.51E+05	0.0038		
	BU10OV20	9.95E+03	2.06E+06	0.0048		
14-month-old	BU14OV33	2.84E+04	3.46E+06	0.0072	0.0056	0.001
	BU14OV08	2.05E+04	3.06E+06	0.0067		
	BU14OV35	3.38E+04	7.07E+06	0.0048		
	BU14OV42	3.04E+04	6.19E+06	0.0049		
	BU14OV54	2.88E+04	8.18E+06	0.0035		
	BU14OV05	1.72E+04	2.93E+06	0.0059		
	BU14OV20	2.31E+04	2.72E+06	0.0085		
	BU14OV30	1.29E+04	2.50E+06	0.0052		
	BU14OV41	1.43E+04	3.36E+06	0.0042		
	BU14OV19	9.32E+03	1.82E+06	0.0051		
	BU14OV24	1.64E+04	2.90E+06	0.0056		
	BU14OV34	2.44E+04	3.98E+06	0.0061		
18-month-old	BU18OV2	2.24E+04	5.64E+06	0.0040	0.0043	0.001
	BU18OV3	1.80E+04	4.24E+06	0.0043		
	BU18OV4	9.00E+03	1.70E+06	0.0053		
	BU18OV5	2.78E+04	7.21E+06	0.0039		

Table 4 Relative expression levels of *SelM* in different ovarian developmental stages of *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups		**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
		SelM	***EF-1α***			
Juvenile	JNOV1	1.88E+05	4.85E+05	0.3878	0.6268	0.277
	JNOV2	5.84E+05	1.02E+06	0.5742		

	JNOV3	5.02E+05	1.87E+06	0.2678		
	JNOV4	4.22E+05	5.44E+05	0.7756		
	JNOV5	1.05E+06	1.02E+06	1.0321		
	JNOV6	1.02E+06	1.41E+06	0.7234		
N-BD-Stage I	BUFOV3	5.28E+05	1.71E+06	0.3098	0.4488	0.233
	BUFOV4	5.98E+05	1.51E+06	0.3970		
	BUFOV7	2.66E+05	3.14E+05	0.8466		
	BUFOV5	2.04E+05	2.73E+05	0.7477		
	AGYLOV1	9.39E+05	3.77E+06	0.2491		
	AGYLOV4	6.17E+05	2.92E+06	0.2114		
	AGYLOV2	5.72E+05	1.71E+06	0.3343		
	AGYLOV32	5.96E+05	1.21E+06	0.4947		
N:BD-Stage II	ASPOV10	8.00E+05	8.89E+05	0.8998	0.7415	0.254
	ASPOV6	1.01E+06	1.50E+06	0.6752		
	BFNOV38	5.28E+05	6.02E+05	0.8769		
	BFNOV33	2.37E+05	8.57E+05	0.2766		
	BFNOV35	4.79E+05	5.25E+05	0.9115		
	BFNOV31	5.97E+05	6.02E+05	0.9908		
	BFNOV4/1	2.92E+05	5.22E+05	0.5595		
N:BD-Stage III	BFNOV18	1.37E+05	2.99E+05	0.4594	0.5842	0.119
	BFNOV3	2.26E+05	3.01E+05	0.7511		
	BFNOV4	2.82E+05	4.97E+05	0.5688		
	BFNOV23	2.40E+05	5.30E+05	0.4535		
	BFNOV24	1.91E+05	2.90E+05	0.6584		
	BFNOV5	2.61E+05	5.21E+05	0.5011		
	BFNOV1	3.07E+05	4.40E+05	0.6973		
N:BD-Stage IV	BFNOV2	1.20E+05	1.06E+05	1.1264	0.6732	0.232
	BFNOV14	1.82E+05	2.45E+05	0.7421		
	BFNOV10	1.80E+05	3.11E+05	0.5803		
	BFNOV12	1.39E+05	3.51E+05	0.3966		
	BFNOV21	1.51E+05	3.89E+05	0.3887		
	BFNOV15	1.10E+05	2.01E+05	0.5472		
	BFNOV20	1.13E+05	1.54E+05	0.7297		
	BFNOV16	9.24E+05	1.35E+05	0.6861		
	BFNOV13	1.64E+05	1.91E+05	0.8620		
N:BD Post-spawning	BFNOV30	3.76E+05	6.27E+05	0.5996	0.7753	0.117
	BFNOV36	4.99E+05	5.58E+05	0.8941		
	BFNOV34	4.70E+05	5.17E+05	0.9079		
	BFNOV37	3.88E+05	4.91E+05	0.7889		
	BFNOV39	4.96E+05	6.52E+05	0.7604		
	BFNOV40	4.26E+05	6.08E+05	0.7011		

EA:BD-Stage I	YLBOV1	2.49E+06	2.31E+06	1.0788	1.4590	0.453
	YLBOV6	5.31E+05	5.02E+05	1.0579		
	BFEAOV15	1.02E+06	5.34E+05	1.9081		
EA:BD-Stage II	YLBOV5	1.83E+06	2.67E+06	0.6842	1.2263	0.525
	YLBOV8	1.85E+06	1.32E+06	1.3997		
	BFEAOV19	4.74E+05	7.20E+05	0.6593		
	BFEAOV17	6.60E+05	5.06E+05	1.3037		
	YLBOV7	5.10E+06	4.74E+05	1.0749		
	YLBOV4	7.28E+05	3.91E+05	1.2460		
EA:BD-Stage III	YLBOV2	5.59E+05	9.29E+05	1.1099	1.1169	0.197
	BFEAOV8	1.07E+06	6.12E+05	1.2772		
	BFEAOV5	1.05E+06	5.04E+05	1.2430		
	BFEAOV2	6.17E+05	4.97E+05	1.2394		
	BFEAOV11	5.18E+05	4.29E+05	1.2751		
	BFEAOV4	5.89E+05	5.33E+05	1.1058		
EA:BD-Stage IV	BFEAOV1	4.77E+05	6.93E+05	0.6886	1.1919	0.401
	BFEAOV7	9.64E+05	9.25E+05	1.0421		
	BFEAOV6	5.43E+05	4.11E+05	1.3211		
	BFEAOV24	5.63E+05	4.72E+05	1.1933		
	BFEAOV10	6.20E+05	5.90E+05	1.0504		
	BFEAOV12	4.78E+05	4.05E+05	1.1815		
	BFEAOV13	5.59E+05	7.04E+05	1.2194		
	BFEAOV9	2.26E+05	2.00E+05	0.6126		
	BFEAOV14	1.38E+05	8.40E+05	1.6442		
6-month-old	JN6OV1	2.75E+05	9.53E+05	0.2889	0.4079	0.207
	JN6OV3	1.82E+05	1.05E+06	0.1737		
	JN6OV5	6.38E+05	1.82E+06	0.3494		
14-month-old	BU14OV13	9.51E+05	4.84E+06	0.1967	0.2955	0.083
	BU14OV27	2.10E+06	7.98E+06	0.2628		
	BU14OV33	7.55E+05	2.90E+06	0.2604		
	BU14OV54	1.51E+06	4.80E+06	0.3137		
	BU14OV66	1.04E+06	3.78E+06	0.2747		
	BU14OV36	1.66E+06	6.00E+06	0.2758		
	BU14OV14	1.30E+06	3.59E+06	0.3630		
	BU14OV48	9.88E+05	5.52E+06	0.3560		
	BU14OV22	1.96E+06	3.89E+06	0.2539		
	BU14OV47	1.05E+06	3.40E+06	0.3079		
	BU14OV32	1.67E+06	6.54E+06	0.2556		
	BU14OV55	9.83E+05	3.88E+06	0.2531		
	BU14OV42	1.68E+06	5.42E+06	0.3103		
	BU14OV41	1.04E+06	4.31E+06	0.2426		

	BU14OV15	9.00E+05	3.00E+06	0.3000		
	BU14OV21	1.13E+06	4.72E+06	0.2388		
	BU14OV17	1.11E+06	4.70E+06	0.2358		
	BU14OV30	4.83E+05	1.74E+06	0.2777		
	BU14OV11	1.12E+06	3.26*E*+06	0.3437		
	BU14OV19	1.81E+06	6.62E+06	0.2741		
	BU14OV24	1.15E+06	5.19E+06	0.2209		
	BU14OV34	1.22E+06	3.34E+06	0.33639		
18-month-old	BU18OV6	9.49E+05	4.84E+06	0.1960	0.2296	0.046
	BU18OV3	5.07E+05	2.71E+06	0.1869		
	BU18OV2	1.53E+06	4.83E+06	0.3178		
	BU18OV5	2.45E+05	1.11E+06	0.2199		
	BU18OV1	4.34E+05	1.84E+06	0.2354		
	BU18OV4	5.34E+05	2.41E+06	0.2220		

Table 5 Relative expression levels of *Drk* in different ovarian developmental stages of *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups		**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
		Drk	***EF-1α***			
Juvenile	JNOV3	6.32E+04	5.58E+06	0.0113	0.0226	0.017
	JNOV4	7.84E+04	1.63E+06	0.0482		
	JNOV9	7.83E+04	4.55E+06	0.0172		
	JNOV10	9.34E+04	6.81E+06	0.0137		
N-BD-Stage I	BUFOV3	1.44E+04	5.09E+06	0.0283	0.0190	0.011
	BUFOV4	4.89E+04	4.50E+06	0.0109		
	BUFOV7	3.38E+04	9.38E+06	0.0361		
	AGYLOV1	1.09E+05	1.13E+07	0.0096		
	AGYLOV4	9.12E+05	8.73E+06	0.0104		
	AGYLOV2	9.39E+04	5.11E+06	0.0184		
N:BD-Stage II	ASPOV10	5.34E+04	2.66E+06	0.0201	0.0144	0.007
	ASPOV6	3.48E+04	4.48E+06	0.0086		
	BFNOV38	1.27E+04	1.18E+06	0.0071		
	BFNOV33	1.91E+04	2.56E+06	0.0075		
	BFNOV35	3.27E+04	1.57E+06	0.0208		
	BFNOV31	3.99E+04	1.80E+06	0.0221		
	BFNOV4/1	2.29E+04	1.56E+06	0.0147		
N:BD-Stage III	BFNOV18	1.62E+04	8.93E+05	0.0181	0.0175	0.008
	BFNOV3	2.90E+04	9.01E+05	0.0321		
	BFNOV4	2.64E+04	1.49E+06	0.0178		
	BFNOV23	2.02E+04	1.58E+06	0.0127		

	BFNOV5	1.71E+04	1.56E+06	0.0110		
	BFNOV1	1.71E+04	1.32E+06	0.0130		
N:BD-Stage IV	BFNOV2	1.01E+04	3.18E+05	0.0316	0.0212	0.012
	BFNOV14	1.16E+04	7.34E+05	0.0158		
	BFNOV12	5.62E+04	1.05E+06	0.0054		
	BFNOV20	8.63E+04	4.62E+05	0.0187		
	BFNOV16	5.13E+04	4.02E+05	0.0128		
	BFNOV17	8.77E+03	2.22E+05	0.0394		
	BFNOV13	1.39E+04	5.69E+05	0.0244		
N:BD Post-spawning	BFNOV30	2.54E+04	1.87E+06	0.0136	0.0179	0.003
	BFNOV36	2.80E+04	1.67E+06	0.0168		
	BFNOV37	3.17E+04	1.47E+06	0.0215		
	BFNOV39	3.80E+04	1.95E+06	0.0195		
EA:BD-Stage I	BFEAOV18	2.62E+04	1.67E+06	0.0157	0.0181	0.003
	YLBOV1	1.35E+05	6.81E+06	0.0199		
	YLBOV06	2.32E+04	1.48E+06	0.0157		
	BFEAOV15	3.34E+04	1.57E+06	0.0212		
EA:BD-Stage II	YLBOV05	8.50E+04	7.87E+06	0.0108	0.0106	0.001
	YLBOV08	4.91E+04	3.90E+06	0.0126		
	BFEAOV19	1.95E+04	2.12E+06	0.0092		
	BFEAOV17	1.36E+04	1.49E+06	0.0091		
	BFEAOV16	1.29E+04	1.15E+06	0.0111		
EA:BD-Stage III	BFEAOV21	2.56E+04	1.81E+06	0.0142	0.0187	0.007
	YLBOV02	2.27E+04	1.49E+06	0.0153		
	YLBOV03	2.08E+04	1.71E+06	0.0122		
	BFEAOV8	5.78E+04	2.50E+06	0.0231		
	BFEAOV2	3.07E+04	1.47E+06	0.0209		
	BFEAOV11	2.50E+04	1.27E+06	0.0198		
	BFEAOV03	3.66E+04	3.25E+06	0.0113		
	BFEAOV20	2.52E+04	7.74E+05	0.0326		
EA:BD-Stage IV	BFEAOV24	2.18E+04	1.39E+06	0.0157	0.0150	0.004
	BFEAOV10	2.61E+04	1.74E+06	0.0150		
	BFEAOV12	2.14E+04	1.19E+06	0.0180		
	BFEAOV22	1.18E+04	5.89E+05	0.0200		
	BFEAOV23	6.58E+03	5.80E+05	0.0113		
	BFEAOV09	1.07E+04	1.09E+06	0.0098		
10-month-old	BU10OV17	4.04E+02	9.48E+04	0.0043	0.0072	0.006
	BU10OV19	1.63E+04	4.51E+05	0.0036		
	BU10OV22	3.49E+04	2.01E+06	0.0017		
	BU10OV13	5.40E+02	1.08E+05	0.0050		

	BU10OV14	2.04E+04	1.09E+06	0.0187		
	BU10OV15	1.27E+04	1.31E+06	0.0098		
14-month-old	BU14OV35	4.99E+04	7.07E+06	0.0071	0.0060	0.005
	BU14OV66	2.14E+04	1.13E+07	0.0019		
	BU14OV34	3.36E+04	3.98E+06	0.0084		
	BU14OV11	1.83E+02	7.41E+04	0.0025		
	BU14OV41	4.35E+04	3.36E+06	0.0129		
	BU14OV30	2.33E+04	2.50E+06	0.0093		
18-month-old	NM18OV2	5.51E+04	5.64E+06	0.0151	0.0124	0.011
	NM18OV3	2.76E+03	4.24E+06	0.0065		
	NM18OV4	1.57E+04	1.70E+06	0.0092		
	NM18OV6	4.85E+04	1.58E+06	0.0306		

Table 6 Relative expression levels of *CGPR-RCP* in the time-course for 12, 24, 48 and 72 hours post treatment of 5-HT in bloodstock *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups	**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
	CGRP-RCP	***EF-1α***			
NC18OV2	6.16E+04	5.64E+06	0.0136	0.0198	0.015
NC18OV3	4.39E+04	4.24E+06	0.0128		
NC18OV4	3.71E+04	1.70E+06	0.0268		
NC18OV5	1.96E+04	7.21E+06	0.0033		
NC18OV6	5.43E+04	1.58E+06	0.0426		
VC0-1	5.53E+03	2.78E+05	0.0216	0.0134	0.006
VC0-2	1.58E+04	1.48E+06	0.0130		
VC0-3	2.63E+04	3.28E+06	0.0097		
VC0-4	1.25E+04	1.69E+07	0.0093		
5HT0-1	2.54E+04	2.81E+06	0.0110	0.0106	0.001
5HT0-2	1.26E+04	1.43E+06	0.0103		
5HT0-3	2.12E+04	2.32E+06	0.0110		
5HT0-4	4.19E+04	5.20E+06	0.0099		
5HT1-1	7.65E+03	7.85E+05	0.0109	0.0110	0.003
5HT1-2	6.50E+03	7.65E+05	0.0094		
5HT1-3	6.61E+04	1.22E+06	0.0157		
5HT1-5	3.10E+04	4.76E+06	0.0079		
5HT3-1	6.53E+03	9.13E+05	0.0079	0.0218	0.026
5HT3-2	9.71E+03	2.10E+06	0.0053		
5HT3-4	7.24E+03	1.54E+05	0.0523		
5HT6-1	1.28E+04	2.08E+05	0.0717	0.1619	0.066
5HT6-2	3.72E+04	2.90E+05	0.1577		

5HT6-3	1.55E+04	8.16E+04	0.2244		
5HT6-4	2.74E+04	1.71E+05	0.1942		
5HT12-1	3.82E+04	1.61E+05	0.2892	0.1512	0.098
5HT12-2	1.92E+04	1.60E+05	0.1426		
5HT12-3	6.77E+03	1.26E+05	0.0564		
5HT12-4	2.22E+04	2.28E+05	0.1174		
5HT24-2	1.45E+04	2.30E+05	0.0738	0.1203	0.068
5HT24-3	1.87E+04	1.52E+05	0.1465		
5HT24-4	5.22E+04	3.19E+05	0.2029		
5HT24-5	1.17E+04	2.36E+05	0.0574		
5HT48-2	1.44E+04	2.25E+05	0.0753	0.1249	0.105
5HT48-5	2.89E+04	1.43E+05	0.2464		
5HT48-6	1.97E+04	4.41E+05	0.0534		
5HT72-1	4.17E+03	1.99E+05	0.0220	0.0581	0.056
5HT72-3	1.96E+04	1.91E+05	0.1227		
5HT72-4	7.55E+03	2.88E+05	0.0293		

Table 7 Relative expression levels of $G_{\alpha s}$ in the time-course for 7, 14 and 28 days post treatment of E2 in bloodstock *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups	**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
	$G_{\alpha s}$	***EF-1α***			
NC0-1	8.38E+03	6.83E+05	0.0123	0.0209	0.011
NC0-2	8.28E+03	5.00E+05	0.0166		
NC0-3	1.67E+04	5.06E+05	0.0330		
NC0-4	9.04E+03	8.28E+05	0.0109		
NC0-5	1.64E+04	4.51E+05	0.0363		
NC0-6	7.30E+03	4.50E+05	0.0162		
NC7-2	3.83E+04	1.83E+06	0.0210	0.0170	0.005
NC7-3	5.23E+04	2.62E+06	0.0200		
NC7-4	3.43E+04	2.02E+06	0.0170		
NC7-5	1.10E+04	1.45E+06	0.0076		
NC7-6	3.04E+04	1.57E+06	0.0194		
VC7-2	2.72E+04	1.33E+06	0.0204	0.0257	0.010
VC7-3	1.99E+04	8.83E+05	0.0225		
VC7-4	3.09E+04	1.54E+06	0.0200		
VC7-5	1.94E+04	8.92E+05	0.0217		
VC7-6	2.63E+04	5.97E+05	0.0441		
EA7-1	1.16E+04	9.65E+05	0.0120	0.0128	0.002
EA7-2	2.29E+04	2.25E+06	0.0101		
EA7-3	2.38E+04	1.87E+06	0.0127		

EA7-4	2.54E+04	1.91E+06	0.0133		
EA7-5	4.58E+04	2.84E+06	0.0161		
E2-7-1	5.39E+03	4.25E+05	0.0127	0.0165	0.005
E2-7-2	9.13E+03	3.25E+05	0.0260		
E2-7-3	7.56E+03	5.70E+05	0.0133		
E2-7-4	7.10E+03	5.29E+05	0.0134		
E2-7-5	9.13E+03	4.36E+05	0.0209		
E2-7-6	7.86E+03	6.10E+05	0.0129		
E2-7-7	1.37E+04	8.54E+05	0.0160		
NC14-1	5.89E+03	5.30E+05	0.0111	0.0202	0.010
NC14-2	1.40E+04	6.82E+05	0.0206		
NC14-4	2.25E+04	6.55E+05	0.0343		
NC14-6	1.05E+04	7.05E+05	0.0149		
VC14-1	5.18E+03	5.97E+05	0.0087	0.0123	0.005
VC14-2	1.87E+04	9.96E+05	0.0188		
VC14-3	9.60E+03	8.72E+05	0.0110		
VC14-4	1.08E+04	7.14E+05	0.0151		
VC14-6	5.52E+03	7.13E+05	0.0077		
EA14-1	1.92E+04	3.98E+05	0.0483	0.0426	0.009
EA14-2	1.12E+04	2.33E+05	0.0483		
EA14-3	1.23E+04	3.57E+05	0.0344		
EA14-4	9.83E+03	3.10E+05	0.0317		
EA14-6	1.28E+04	2.55E+05	0.0502		
E2-14-1	1.05E+04	6.72E+05	0.0157	0.0180	0.006
E2-14-2	9.41E+03	8.08E+05	0.0117		
E2-14-3	7.29E+03	3.41E+05	0.0214		
E2-14-4	8.49E+03	3.68E+05	0.0231		
E2-14-5	9.73E+03	4.03E+05	0.0241		
E2-14-6	3.49E+03	2.86E+05	0.0122		
NC28-1	1.43E+04	2.91E+05	0.0493	0.0369	0.011
NC28-2	7.84E+03	2.08E+05	0.0378		
NC28-3	5.39E+03	1.95E+05	0.0277		
NC28-4	6.9E+03	2.85E+05	0.0235		
NC28-5	1.02E+04	2.21E+05	0.0462		
VC28-1	6.92E+03	3.09E+05	0.0224	0.0452	0.022
VC28-2	1.96E+04	2.50E+05	0.0785		
VC28-3	1.38E+04	2.13E+05	0.0650		
VC28-4	9.00E+03	2.25E+05	0.0400		
VC28-5	9.55E+03	2.96E+05	0.0323		
VC28-6	8.50E+03	2.57E+05	0.0331		
EA28-1	1.60E+04	2.92E+05	0.0549	0.0245	0.015
EA28-2	4.32E+03	2.52E+05	0.0172		

EA28-3	9.26E+03	3.97E+05	0.0233		
EA28-4	1.03E+04	4.17E+05	0.0246		
EA28-5	8.92E+03	3.34E+05	0.0267		
EA28-7	1.10E+04	7.79E+05	0.0141		
EA28-8	7.62E+03	7.24E+05	0.0105		
E2-28-1	1.34E+04	2.39E+05	0.0562	0.0404	0.025
E2-28-2	1.00E+04	9.15E+05	0.0110		
E2-28-3	7.84E+03	1.95E+05	0.0403		
E2-28-4	1.46E+04	1.95E+05	0.0749		
E2-28-5	1.21E+04	9.21E+05	0.0132		
E2-28-6	1.14E+04	2.43E+05	0.0470		

Table 8 Relative expression levels of *Drk* in the time-course for 7, 14 and 28 days post treatment of E2 in bloodstock *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups	**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
	$G_{\alpha s}$	***EF-1α***			
NC0-1	1.07E+04	6.83E+05	0.0156	0.0222	0.013
NC0-2	1.06E+04	5.00E+05	0.0212		
NC0-4	1.08E+04	8.28E+05	0.0131		
NC0-5	2.01E+03	4.51E+05	0.0446		
NC0-6	7.36E+03	4.50E+05	0.0164		
NC7-1	3.89E+04	1.24E+06	0.0313	0.0450	0.013
NC7-2	9.54E+04	1.83E+06	0.0522		
NC7-3	1.31E+05	2.62E+06	0.0498		
NC7-4	1.00E+05	2.02E+06	0.0496		
NC7-5	4.02E+04	1.45E+06	0.0278		
NC7-6	9.32E+04	1.57E+06	0.0595		
VC7-2	5.24E+04	1.33E+06	0.0393	0.0384	0.005
VC7-3	3.97E+04	8.83E+05	0.0450		
VC7-4	5.05E+04	1.54E+06	0.0327		
VC7-5	3.66E+04	8.92E+05	0.0410		
VC7-6	2.04E+04	5.97E+05	0.0342		
EA7-1	3.89E+04	9.65E+05	0.0402	0.0344	0.008
EA7-2	7.97E+04	2.25E+06	0.0354		
EA7-3	6.56E+04	1.87E+06	0.0351		
EA7-4	8.10E+04	1.91E+06	0.0424		
EA7-5	9.35E+04	2.84E+06	0.0329		
EA7-6	2.83E+04	1.40E+06	0.0202		
E2-7-1	5.92E+03	4.25E+05	0.0139	0.0213	0.010
E2-7-2	9.87E+03	3.25E+05	0.0281		

E2-7-3	1.34E+04	5.70E+05	0.0235		
E2-7-4	1.91E+04	5.29E+05	0.0360		
E2-7-5	1.26E+04	4.36E+05	0.0121		
E2-7-6	1.74E+04	4.36E+05	0.0400		
E2-7-7	1.89E+03	1.79E+05	0.0106		
E2-7-8	1.84E+04	9.44E+05	0.0195		
E2-7-9	1.17E+04	6.10E+05	0.0191		
E2-7-10	1.18E+04	1.07E+06	0.0110		
E2-7-11	1.76E+04	8.54E+05	0.0206		
NC14-1	9.23E+03	5.30E+05	0.0174	0.0289	0.018
NC14-2	2.33E+04	6.82E+06	0.0342		
NC14-3	3.04E+04	2.91E+06	0.0105		
NC14-4	3.71E+04	6.55E+05	0.0566		
NC14-6	1.83E+04	7.05E+05	0.0260		
VC14-1	1.21E+04	5.97E+05	0.0203	0.0192	0.008
VC14-2	3.22E+04	9.96E+05	0.0323		
VC14-3	1.20E+03	8.72E+05	0.0138		
VC14-4	1.38E+04	7.14E+05	0.0193		
VC14-6	7.36E+03	7.13E+05	0.0103		
EA14-1	1.90E+04	3.98E+05	0.0478	0.0327	0.013
EA14-2	8.55E+03	2.33E+05	0.0368		
EA14-3	1.43E+04	3.57E+05	0.0400		
EA14-4	9.80E+03	3.10E+05	0.0316		
EA14-5	6.54E+03	7.29E+05	0.0090		
EA14-6	7.88E+04	2.55E+05	0.0309		
E2-14-1	1.17E+04	6.72E+05	0.0175	0.0264	0.012
E2-14-2	9.53E+03	8.08E+05	0.0118		
E2-14-3	8.71E+03	3.41E+05	0.0256		
E2-14-4	1.58E+04	3.68E+05	0.0430		
E2-14-5	1.56E+04	4.03E+05	0.0387		
E2-14-6	6.25E+03	2.86E+05	0.0219		
NC28-1	2.23E+04	2.91E+05	0.0766	0.0369	0.009
NC28-2	1.42E+04	2.08E+05	0.0686		
NC28-3	1.01E+04	1.95E+05	0.0521		
NC28-4	1.76E+04	2.85E+05	0.0619		
NC28-5	1.33E+04	2.21E+05	0.0603		
VC28-1	1.41E+04	3.09E+05	0.0457	0.0524	0.006
VC28-3	1.28E+04	2.13E+05	0.0600		
VC28-4	1.21E+04	2.25E+05	0.0537		
VC28-5	1.48E+04	2.96E+05	0.0500		
EA28-1	2.74E+04	2.92E+05	0.0938	0.0525	0.024
EA28-2	9.83E+03	2.52E+05	0.0390		

EA28-3	1.78E+04	3.97E+05	0.0448		
EA28-4	2.21E+04	4.17E+05	0.0529		
EA28-5	1.06E+04	3.34E+05	0.0318		
EA28-8	1.09E+04	7.24E+05	0.0151		
E2-28-2	1.71E+04	9.15E+05	0.0187	0.0438	0.024
E2-28-3	1.17E+04	1.95E+05	0.0599		
E2-28-4	1.38E+04	1.95E+05	0.0710		
E2-28-5	1.69E+04	9.21E+05	0.0183		
E2-28-6	1.23E+04	2.43E+05	0.0509		

Table 9 Relative expression levels of $G_{\alpha s}$ in the time-course for 12, 24, 48 and 72 hours post treatment of P4 in bloodstock *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups	**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
	$G_{\alpha s}$	***EF-1α***			
NC-1	1.20E+04	1.53E+05	0.0781	0.1062	0.040
NC-4	1.92E+03	2.17E+04	0.0882		
NC-5	6.26E+03	3.81E+04	0.1646		
NC-7	4.78E+02	5.11E+03	0.0935		
VC-1	3.53E+03	1.83E+04	0.1929	0.2143	0.064
VC-2	9.67E+03	5.36E+04	0.1805		
VC-3	1.25E+03	2.19E+03	0.5698		
PG12-2	2.53E+03	1.10E+04	0.2310	0.2007	0.100
PG12-4	5.87E+03	6.59E+04	0.0891		
PG12-6	1.40E+03	4.96E+03	0.2818		
PG24-1	3.03E+03	5.83E+04	0.0519	0.0701	0.022
PG24-2	3.80E+03	4.10E+04	0.0926		
PG24-3	1.59E+03	1.86E+04	0.0858		
PG24-5	2.19E+03	4.37E+04	0.0501		
PG48-2	3.22E+03	2.67E+05	0.1205	0.1770	0.089
PG48-3	8.58E+03	2.78E+04	0.3084		
PG48-4	2.90E+03	1.80E+04	0.1614		
PG48-5	1.54E+04	1.30E+05	0.1185		
PG72-1	3.38E+03	2.52E+04	0.1341	0.1968	0.084
PG72-2	4.63E+03	2.87E+04	0.1609		
PG72-3	1.84E+03	1.08E+04	0.1707		
PG72-4	2.37E+03	7.38E+03	0.3206		

Table 10 Relative expression levels of *Drk* in the time-course for 12, 24, 48 and 72 hours post treatment of P4 in bloodstock *P. monodon* based on Quantitative real-time PCR analysis

Sample Groups	**Mean conc.**		**Ratio (target / *EF-1α*)**	**Average**	**STDEV**
	Drk	***EF-1α***			
NC-1	1.44E+04	1.53E+05	0.0939	0.0864	0.021
NC-4	1.35E+03	2.17E+04	0.0623		
NC-5	1.24E+04	3.81E+04	0.3262		
NC-7	5.25E+02	5.11E+03	0.1027		
VC-1	2.36E+03	1.83E+04	0.1290	0.1017	0.050
VC-2	7.44E+03	5.36E+04	0.1389		
VC-3	4.58E+02	2.19E+03	0.2093		
VC-4	4.32E+02	1.94E+03	0.2222		
PG12-2	9.69E+02	1.10E+04	0.0883	0.0883	0.053
PG12-4	6.93E+03	6.59E+04	0.1052		
PG12-6	7.10E+02	4.96E+03	0.1432		
PG24-2	1.19E+03	4.10E+04	0.0905	0.1108	0.060
PG24-3	7.78E+03	1.86E+04	0.0640		
PG24-5	2.19E+03	4.37E+04	0.1782		
PG48-2	2.36E+04	2.67E+05	0.0885	0.0900	0.013
PG48-3	2.48E+03	2.78E+04	0.0892		
PG48-4	1.35E+03	1.80E+04	0.0751		
PG48-5	1.40E+04	1.30E+05	0.1071		
PG72-1	1.14E+03	2.52E+04	0.0450	0.0398	0.009
PG72-2	8.43E+02	2.87E+04	0.0293		
PG72-3	4.87E+02	1.08E+04	0.0452		

BIOGRAPHY

Miss Patchari Yocawibun was born on September 12, 1981 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of biotechnology, Ramkhamhaeng University in 2004. She has enrolled a Master degree program at the program in Biotechnology, Chulalongkorn University since 2009.

Publications related with this thesis

Yocawibun, P., Hiransuchalert, R., Klinbunga S., and Menasveta, P., 2011. Full-length cDNA cloning and expression analysis of the *selenoprotein M* gene during ovarian development of the giant tiger shrimp *Penaeus monodon*. The 23rd Annual Meeting of the Thai Society for Biotechnology *"Systems Biotechnology: Quality & Success"*, February 1-2, Bangkok, THAILAND (oral presentation).