CHAPTER I

INTRODUCTION

1.1 Background information and objectives of this thesis

The giant tiger shrimp (*Penaeus monodon*) was an economically important cultured species in Thailand. The annual production was approximately 225,000 metric tons in 2002, but has significantly decreased owing to the outbreak of diseases since 2003. Farming of *P. monodon* in Thailand relies entirely on wild-caught broodstock for supply of juveniles because reproductive maturation success of captive *P. monodon* female is extremely low. The high demand on wild female broodstock leads to overexploitation of the natural populations of *P. monodon* in Thai waters (Klinbunga et al., 1999; Khamnamtong et al., 2005). The lack of high quality wild and/or domesticated broodstock of *P. monodon* has caused a significant decrease in its farmed production since the last several years (Limsuwan, 2004). Reduced degrees of reproductive maturation in captive *P. monodon* broodstock have limited the potential of genetic improvement resulted in remarkably slow domestication and selective breeding programs of *P. monodon* in Thailand (Withyachumnarnkul et al., 1998; Clifford and Preston, 2006; Preechaphol et al., 2007).

Several biotechnological areas including investigation of genetic variation (identification of stocks) and genome structure, controls of reproduction and growth, domestication of strains exhibiting required economically important traits (e.g. specific pathogen resistant, SPR and/or fast growing strains) are thought to have significant impact in the industry of this species. The domestication and selective breeding programs of *P. monodon* 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 (e.g. disease resistance and/or other commercially desired traits) rather than the reliance on wild-caught stocks is a major determinant of sustainability of the shrimp industry. Despite the potential benefits, the domestication of *P. monodon* has been

remarkably slow in Thailand and is still at the initial stage because the previous stocks (Withyachumnarnkul et al., 1998) were recently collapsed by WSSV infection.

The development of oocytes consists of a series of complex cellular events, in which different genes express to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu *et al.*, 2005). Different biotechnological approaches, for example; injection of vertebrate steroid hormones, neurotransmitters and ecdysteroids (Benzie 1998; Okumura, 2004) and the use of specially formulated feed (Harrison, 1990) have been applied to induce the ovarian maturation of female shrimp but results are inconsistent owing to limited knowledge on genetic and hormonal control of penaeid species (Okumura, 2004).

Maturation of female shrimp is controlled by several neuropeptides (Okumura, 2004). It has been proposed that ovarian and oocyte development is controlled by gonad stimulating hormone (GSH also called vitellogenin stimulating hormone, VSH) and gonad inhibiting hormone (GIH also called vitellogenin inhibiting hormone, VIH). In addition, ecdysteroids and methyl farnesoate represent two major endocrine signaling molecules in crustaceans that regulate many aspects of their development, growth, and reproduction.

O-methyltransfrase (OMT) is ubiquitously present in diverse organisms and plays an important regulatory role in growth, development, reproduction and defense mechanisms in plants and animals. Two kinds of OMT; farnesoic acid-O-methyltransferase (FAMeT) and catechol-O-methyltransferase (COMT) were identified according to their selectivity to methyl acceptors.

Methyl farnesoate (MF) is structurally related to the insect juvenile hormone and believed to be synthesized by the mandibular organ (MO). MF has been implicated in the regulation of crustacean development and reproduction in conjunction with eyestalk molt inhibiting hormones and ecdysteroids (Laufer et al., 1987; Lam Hui, 2008). The incubation of ovarian tissue with MF and the addition of MF to the diet stimulate ovarian development of *Procambarus clarkii* (Laufer et al.1998). However, no significant effects were detected in the Amercan lobster (*Homarus americanus*) and the giant freshwater

prawn (*Macrobrachium rosenbergii*) when MF was injected into the females (Tsukimura *et al.*, 1993; Wilder *et al.*, 1994) but the inhibitory effects were observed in tadpole shrimp, *Troops longicaudatus* (Tsukimura et al. 2006). Accordingly, the actual physiological function of MF on ovarian development of *P. monodon* is still not understood and should be examined.

Crustacean FAMeT catalyses methylation of farnesoic acid (FA) to produce isoprenoid methyl farnesoate (MF) at the final step of the MF biosynthetic pathway whereas COMT catalyses the transfer of the methyl group from *S*-adenosyl-methionine (SAM) to the hydroxyl group of catechol compounds. Thus, COMT plays an important role in the catabolism and *O*-methylation of endogenous catecholamines, such as dopamine and noradrenaline, in brains of animals.

In addition, ecdysteroids are known as the molting hormone in crustaceans and insects. In crustaceans, the inactive forms are secreted and converted to 20-hydroxyecdysone by the Y-organ. Ecdysteriods stimulate vitellogenesis in some insects. The broad-complex (BR-C), one of the ecdysteroid-responsive genes, is a key member of 20-hydroxyecdysone regulatory hierarchy that coordinates changes in gene expression during *Drosophila* metamorphosis (Bayer, et al., 1996). The BR-C gene family can be divided into 4 isoforms; BR-C Z1, Z2, Z3 and Z4 that share an amino-terminal common core domain alternatively spliced to four distinct carboxyl-terminal domains bearing pairs of zinc fingers of the C_2H_2 type (DiBello. et al.,1991; Bayer et al.,1996) and a highly conserved amino-terminal domain called the BTB/POZ domain. (Zollman et al., 1994)

Progress in genetic and biotechnology researches in penaeid shrimps have been slow because a lack of knowledge on fundamental aspects of their endocrinology and reproductive biology (Benzie, 1998). Therefore, molecular mechanisms and functional involvement of reproduction-related genes and proteins 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 which, in turn, provide the long term benefit for domestication and selective

breeding programs of this economically important species (Preechaphol et al., 2007; Klinbunga et al., 2009).

1.2 Objectives of this thesis

The objectives of this thesis were isolation and characterization of the full length cDNA and genomic DNA of *COMT*, *FAMeT* and *Br-c* genes in *P. monodon* and their expression levels of genes and/or protein during different stages of ovarian development of *P. monodon*. Localization of genes and/or protein during different stages of ovarian development of *P. monodon* is determined by *in situ* hybridization and/or immunohistochemistry.

1.3 General introduction

The giant tiger shrimp (*Penaeus monodon*) is one of the most economically important penaeid species in South East Asia. Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment.

In Thailand, *P. monodon* had been intensively cultured for more than two decades. Approximately 60% of the total shrimp production is from cultivation. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand where southern provinces (Nakorn Sri Thammarat and Surat Thani) are the majority and those in the east (Chanthaburi) and central regions (Samut Sakhon and Samut Songkhram) comprise the minority in terms of number. The intensive farming system had been used for *P. monodon* farming activity resulting in consistent increase in the production (Department of Fisheries, 1999).

The increased demand of *P. monodon* in world markets has elevated the expansion of shrimp industry and activity of this important species. The culture of *P. monodon* is basically a two-step process composed of a broodstock-hatchery phase for producing seed or postlarvae and a grow-out phase usually in earthen culture ponds for on-growing of fry to marketable size (where a nursery for rearing of postlarvae to larger juveniles is incorporated).

Table 1.1 Export of the giant tiger shrimp from Thailand during 2002-2007

Country	2002		2003		2004		2005		2006		2007	
	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)
USA	97681.81	36,011.41	89115.28	29,032.87	58365.2	17,206.75	29116.62	17,206.75	34537.23	8,847.42	7979.91	1,909.64
Japan	16644.6	13,813.33	33235.52	11,916.87	27977.27	9,586.59	20182.85	9,586.59	15,709.39	3,832.31	3711.32	1,067.25
Canada	6455.76	3,890.48	11216.47	3,412.09	6490.03	2,072.25	3249.37	2,072.25	2798.61	744.95	1762.16	462.68
Singapore	5251.66	3,138.86	3317.14	1,258.13	3383.18	537.88	1933.5	537.88	1580.11	236.31	401.47	63.53
Taiwan	4917.65	1,276.86	3051.77	799.44	2964.62	564.58	1673.65	564.58	607.7	170.12	692.69	194.78
Australia	4481.25	1,326.06	4817.5	1,252.31	2418.19	1,042.02	2097.76	1,042.02	1418.36	445.05	658.54	225.13
Hong Kong	1365.12	533.26	1437.54	340.42	1396.98	409.93	1026.84	409.93	921.88	256.91	1569	365.91
Chaina	1649.23	352.68	992.91	214.54	833.1	162.66	1003	162.66	710.7	85.65	1629.74	235.57
U. Kingdom	661.07	210.81	184.23	64.11	505.76	181.63	161.79	181.63	241.91	70.54	242.4	73.46
Total	180,615.81	63,822.73	160,986.48	51,524.10	118,343.12	16,629.05	69,168.96	16,629.05	64,565.41	16,178.85	23,933.1	5,922.11

Source: http://www.fisheries.go.th/foreign/doc/excel/export_backtiger.xls

The success of the shrimp industry in Thailand has resulted in the steadily increased income for the nation. This has also elevated the quality of life for Thai farmers. The reasons for this are supported by several factors including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils for pond construction.

Thailand has become the world's leader in shrimp exports. The largest export markets for Thai black tiger shrimp are the United States and Japan. The remaining important markets are Canada, Asian countries, Australia and others (Table 1.1). However, the industry has consistently encountered production losses from infectious diseases, particularly from white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio* sp., environmental degradation and overexploitation of high quality natural female broodstock for seed production (Browdy, 1998). As a result, Thai shrimp export decreased from 181,050 in 2002 to only 69,412 in 2005 (Table 1.2).

1.4 Taxonomy of *P. monodon*

Penaeid shrimps are taxonomically recognized as members 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 taxonomic definition of the giant tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992): a member of Phylum Arthropoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suborder Natantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Penaeidae Rafinesque, 1985; Genus Penaeus Fabricius, 1798; Subgenus *Penaeus*. The scientific name of this species is *Penaeus monodon* (Fabricius, 1798) and the common name is giant tiger prawn or black tiger shrimp.

1.5 Morphology

The external morphology of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rosthum, and by a segmented abdomen (Fig. 1.1). Most organs are located in cephalothorax, while the body muscles are mainly in the abdomen. The

internal morphology of penaeid shrimp is outlined by Fig. 1.2. Penaeids and other arthropods have an open circulatory system and, therefore, the blood and the blood cells are called hemolymph and hemocytes, respectively.

1.6 Female reproductive system

1.6.1 Morphology of female reproductive system

The female reproductive system consists of paired ovaries, paired oviducts and a single thelycum. 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. The anterior lobes lie close to the esophagus and cardiac region of the stomach. The lateral lobes are located dorsally in the large mass of the hepatopancreas and ventrally in the pericardial chamber. The abdominal extensions lie dorsa-lateral to the intestine and ventro-lateral to the dorsal abdominal artery. The oviducts origins at the tips of the sixth lateral lobes and descend to the external genital apertures hidden in the ear-like lobes of the coxopods of the third pair of pereiopods.

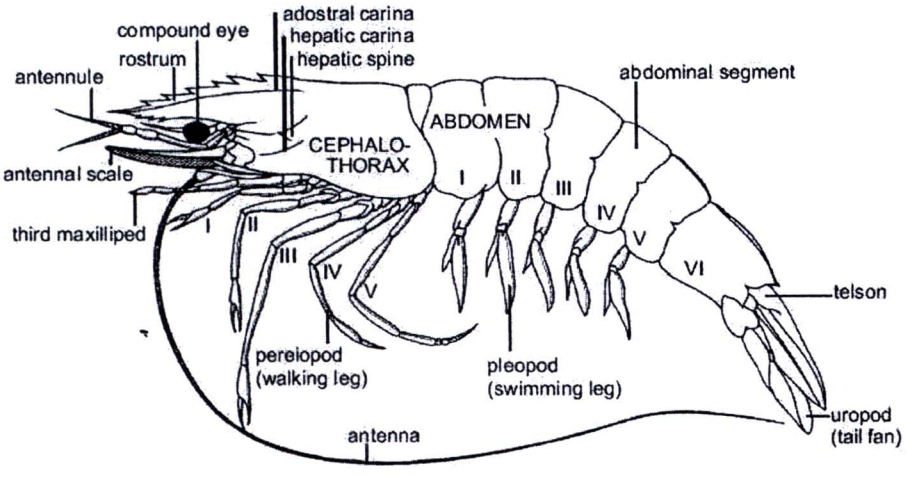

Figure 1.1 Lateral view of the external morphology of *P. monodon*. (Primavera, 1990)

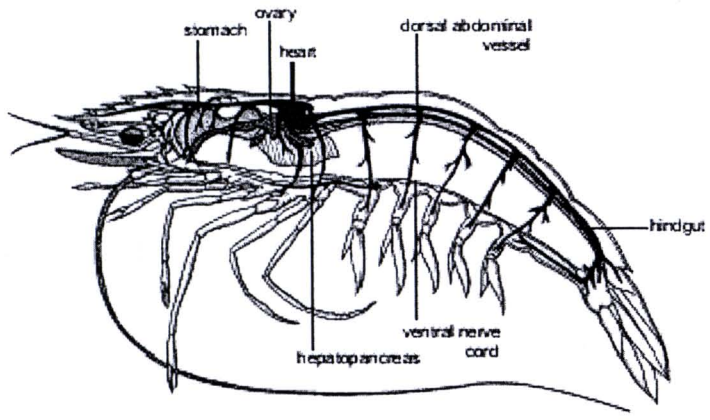

Figure 1.2 Lateral view of the internal anatomy of a female *P. monodon* (Primavera, 1990).

1.6.2 Ovarian development

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 1.3. It consists of stages I (undeveloped phase or spent phase), II (proliferative phase), III (premature phase) and IV (mature phase) ovaries.

The stage I ovaries (Figure 1.4A) 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 exists the germinative zone of the ovaries and became oogonia that divided mitotically and enter the meiotic prophase.

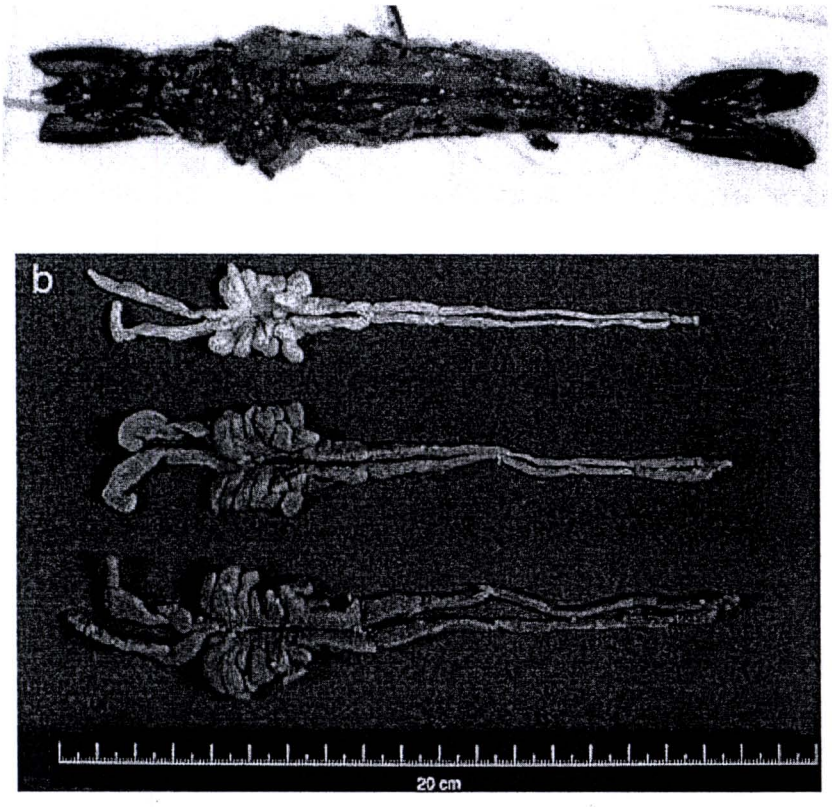

Figure 1.3 The illustration of ovaries that extend the entire length of shrimp (a) and complete ovaries of *P. monodon* females categorized as stages II to IV (b). Note the color changes are due to an increasing carotenoid content (http://www.aims.au/pages/reseach/mdef/mdef-00.html).

The stage II ovaries (Figure 1.4b) 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 1.4c), 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

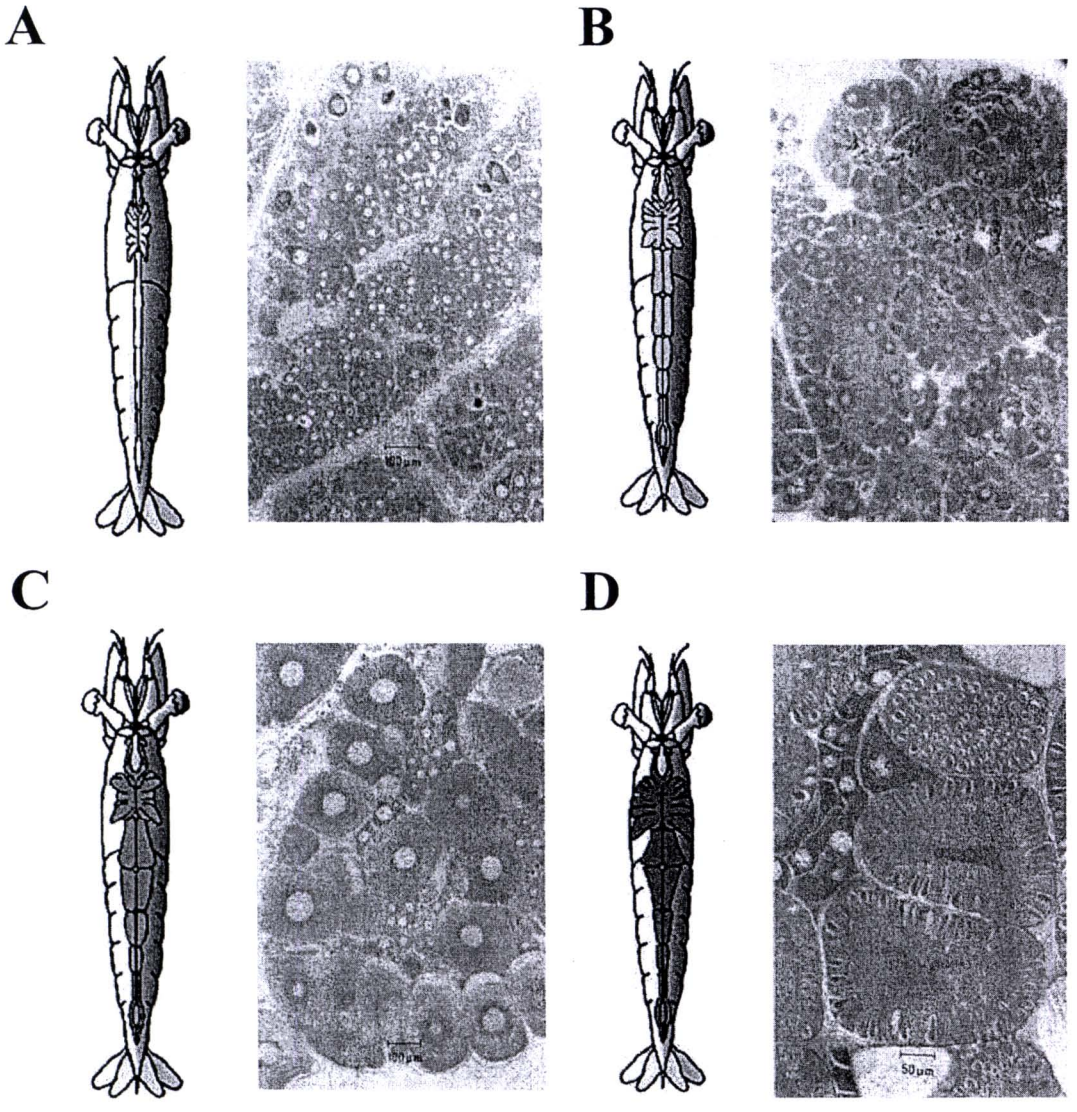

Figure 1.4 Different ovarian development stages of *P. monodon.* **Panel A.**, the underdeveloped ovaries (Stage I), **B.**, the developing stage (Stage II), **C.**, the nearly ripe stage (Stage III) and **D.**, the ripe stage (Stage IV) (www.aims.gov.au/.../mdef/images/fig01-4a.gif).

Table 1.2 A summary on the ovarian maturation stages in *P. monodon* based on histological studies (Tan-Fermin and Pudadera 1989)

Oocyte stage/component	Qualitative methods			
	Histology	Histochemistry		
		Mallory	AB-PAS	SB
P:previtellogenic				
a) oocyte	-oogonia and primary oocytes			
1) Nucleus	-chromatin nucleolus and/or perinucleolus stage; single to several nucleoli in nucleoplasm			
2) Cytoplasm	-clear	Basophilic	(-)	(-)
b) follicle cells	-rectangular or cuboidal in shape, if present in oocytes>55μm	Basophilic		
V:vitellogenic				
a) oocyte	-			
1) Nucleus	chromatin materials evenly distributed in nucleoplasm			
2) Cytoplasm	-as in stage P plus oocytes which contain yolky substances in cytoplasm	Red, blue	Magenta	Black
b) follicle cells	-flattened in shape		Light blue	
C; cortical rod				
a) oocytes	-as in stage P plus oocytes with yolky substances and cortical rods in cytoplasm			
1) nucleas	- chromatin materials evenly distributed in nucleoplasm			
2) cytoplasm	- yolky substances in granules	Red, blue	Magenta	Black
3) cortical rods	-spherical or elongated near periphery and extends towards nucleus	Red, blue, purple	Blue near periphery, red towards nucleus	(-)
b) Follicle cells	-spindle-shaped or not distinguishable			
S; spent				
a) oocytes	-remaining oocytes with yolky cortical rods -thicker layers of follicle cells retracted to one side -darkly-stained, irregularly-shaped primary oocytes			
b) follicle cells	-rectangular or cuboidal in shape when enveloping oocytes			

AB-PAS: alcian blue-periodic acid-Schiff. SB: Sudan black.

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 1.4d), 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.

1.7 Vitellogenesis and oocyte maturation

In crustaceans, oocytes grow during oogenesis through the process of vitellogenesis. During vitellogenesis, vitellogenin, the precursor of the major yolk protein, vitellin, is synthesized and is taken in by the oocytes. In the oocytes, vitellogenin is processed and accumulated. Vitellin is utilized as a nutritional source during embryogenesis. Vitellin and vitellogenin have been purified in several shrimps and determined to be large lipoprotein molecules (molecular weight, 280–700 kDa).

In decapod crustaceans, vitellogenesis is a necessary prerequisite for ovaries to reach full maturation (Serrano-Pinto et al., 2003). Vitellogenesis is comprised of vitellogenin (Vg) synthesis and its accumulation in ovarian oocytes as vitellin (Tsukimura et al., 2000; Ghekiere et al., 2004, 2005). Vg, a precursor of egg yolk protein, is synthesized by both ovaries and hepatopencreas (Lui et al., 1974; Eastman-Reks and

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Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989; Rankin et al., 1989; Browdy et al., 1990; Fainzilber et al., 1992; Khayat et al., 1994; Chen et al., 1999; Tseng et al., 2001). In decapod crustaceans, Vg has been identified electrophoretically and immunologically in the hemolymph of vitellogenic females of various species (Fielder et al., 1971; Wolin et al., 1973; Fyffe and O'Connor, 1974; Yano, 1987; Quinitio et al., 1989; Shafir et al., 1992; Jasmani et al., 2000; Vazquez-Boucard et al., 2002).

In *Marsupenaeus japonicus*, Yano et al. (1996) reported that electron dense materials showing the presence of Vg found in the irregular surface of yolk granule stage oocytes were incorporated into the micropinocytotic vesicles of the oocytes. This indicated that Vg is temporally transferred into hemolymph after its synthesis by ovarian tissue and is then incorporated into oocytes by pinocytosis.

It has long been suspected that vitellogenesis including Vg synthesis and its incorporation into oocytes in decapod crustaceans is controlled by stimulating and inhibiting antagonistic hormones. Several studies have shown that vitellogenesis is regulated by a vitellogenesis-inhibiting hormone (VIH) or gonad-inhibiting hormone (GIH) from the X organ–sinus gland complex of the eyestalk (Bomirski et al., 1981; Quackenbush and Herrnkind, 1983; Meusy et al., 1987; Rotllant et al., 1993; De Kleijn et al., 1998) (Figure 1.5). Knowledge of hormonal induction of Vg synthesis and secretion in crustaceans, however, is fragmentary. Understanding the roles of steroid hormones on vitellogenesis may lead to the development of ways to induce ovarian maturation in decapods crustaceans.

1.8 Hormonal control of shrimp and Female reproductive hormone

1.8.1 Hormonal control of shrimp

Closing the life cycle of aquaculture animal is crucial to the success and sustainability of the aquaculture industry. The increasing scarcity of high quality wild-caught broodstock for the giant tiger shrimp *P. monodon* brought attention to the need of captive broodstock. However, different sets of problems are associated with the captive broodstock. The development of oocyte in the ovary of captive females *P. monodon*

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showed a higher rate of regression than that of wild shrimp, and difference in the oocyte size were observed. Biological and physiological processes (growth, reproduction, body color, and metabolism etc.) in shrimp are hormonal controlled (Figure 1.6).

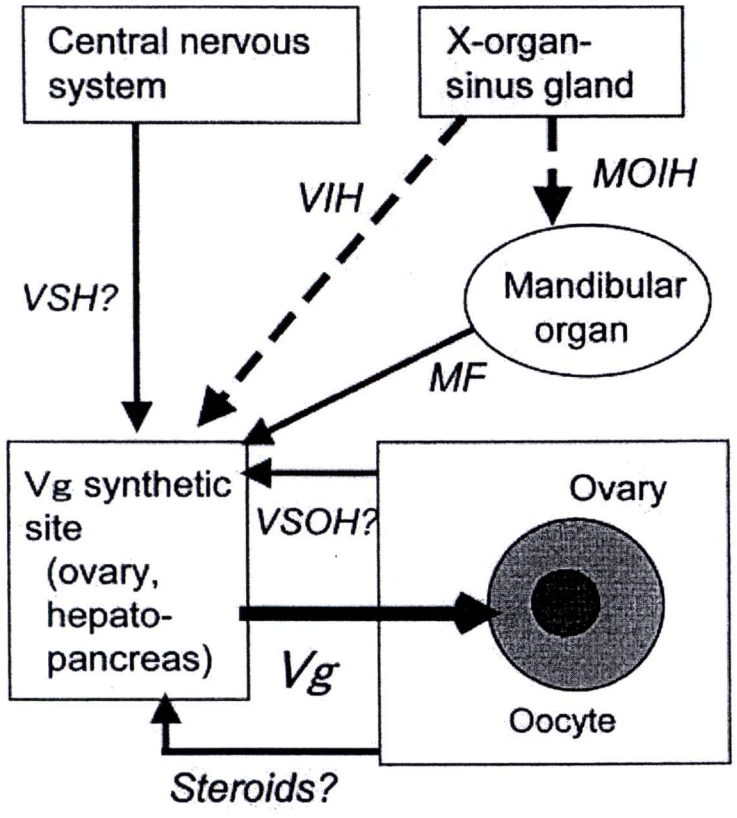


Figure 1.5 Schematic diagram of the endocrine control of vitellogenesis in shrimp, MF: methyl farnesoate, MOIH: mandibular organ inhibiting hormone, Vg: vitellogenin (Okumura et al., 2004).

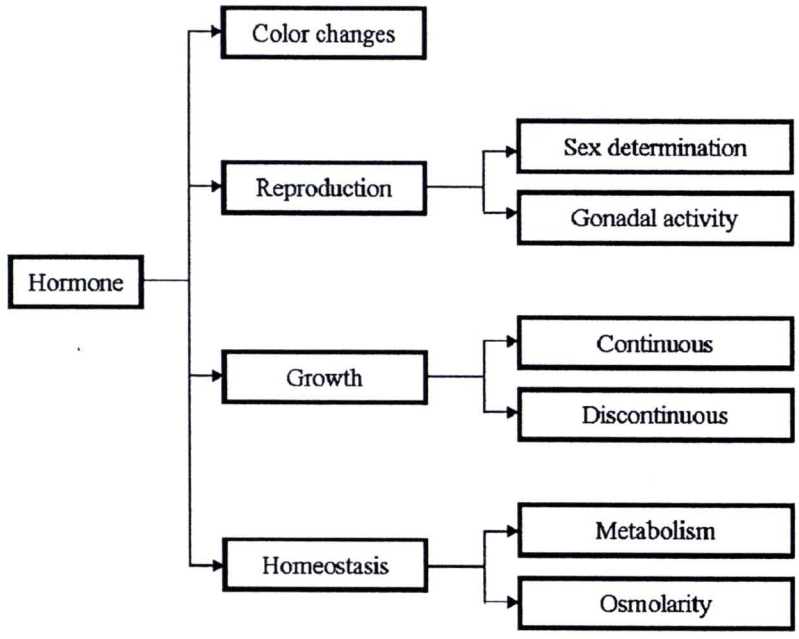


Figure 1.6 Diagram illustrating the hormonal controls of physiological processes of penaeid shrimp.

The current practice to stimulate ovarian development by eyestalk ablation is stressful to the animal, and it could lead to mortality. In order to avoid eyestalk ablation, different techniques to stimulate ovarian development such as maturation diet and hormone stimulation have been attempted. The use of these techniques to variable success reveals our lack of general understanding of oocyte development in crustacean. Therefore, the knowledge on hormonal factors influencing the ovarian and oocyte development in crustacean is necessary to develop the hormonal manipulation techniques in shrimp.

Eyestalk hormones play the important role for regulating several physiological mechanisms and unilateral eyestalk ablation is practically used for induction of ovarian development but this technique does not have the potential effects on testicular development of *P. monodon*. Therefore, the molecular mechanisms controlling testicular and ovarian maturation may be different.

1.8.2 Female reproductive hormone

Hormonal regulation is one of the several factors that control the female reproduction. The female reproductive hormone was produced from various tissues (Figure 1.7) including eyestalks, mandibular organ and Y-organ.

1.8.2.1 Eyestalk hormones

It is well-known that eyestalk ablation induces ovarian development and oviposition. This effect has been attributed to the presence of a vitellogenesis-inhibiting factor present in the MTXO-SG neurosecretory system (Figures 1.8 and 1.9). A group of neuropeptides that directly affect reproductive performances in crustacean have been identified. Many of these molecules belongs share a high degree of similarity with crustacean hyperglycemic hormone (CHH).

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).

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 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* (Treerattrakool et al. 2008). 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 (Treerattrakool et al. 2008).

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)(Ohira et al. 2006). Both GIH from *L. vannamei* and *H. americanus* have shown *in vitro* to be inhibiting vitellogenin gene expression.

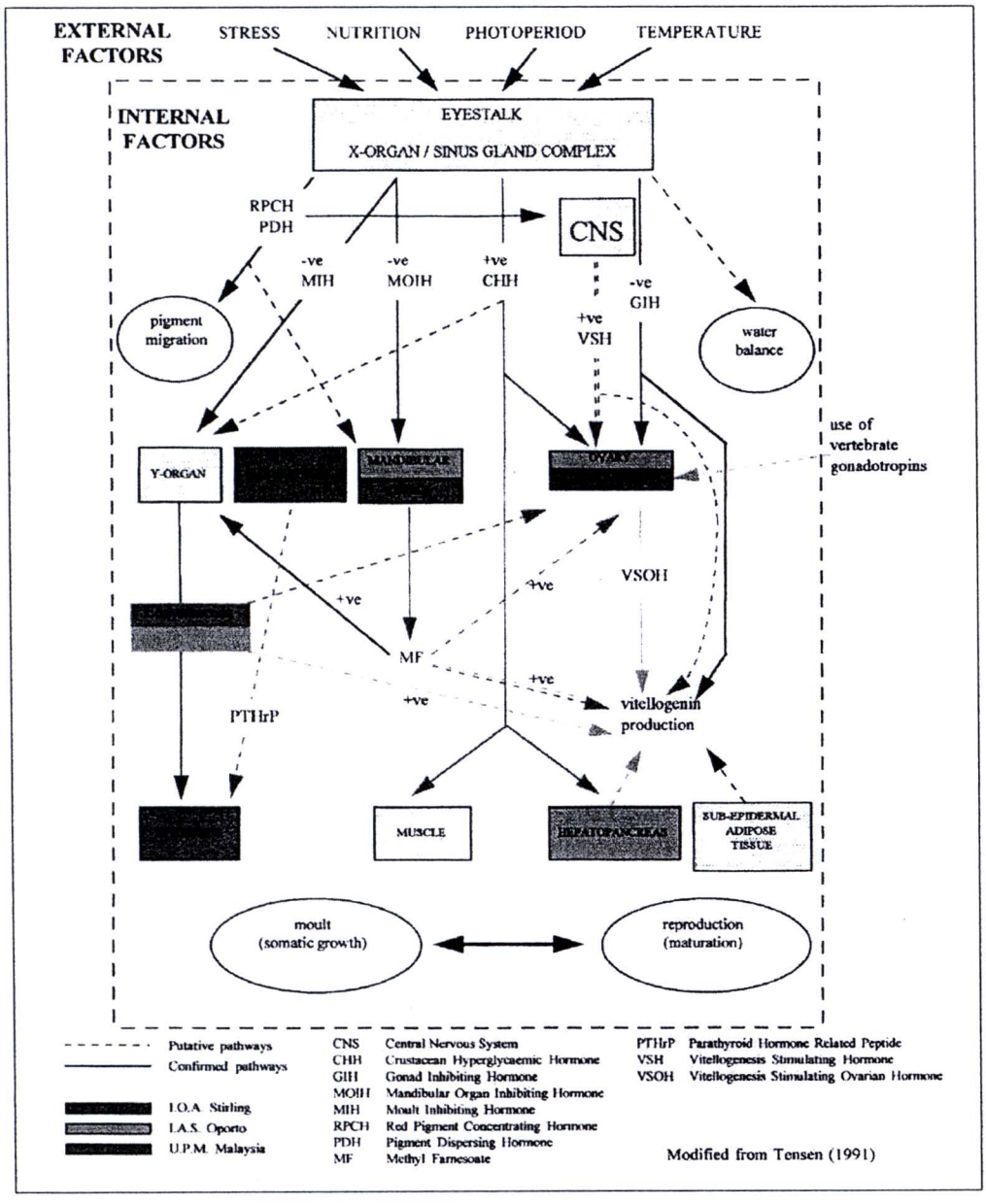


Figure 1.7 Diagram showing complexity of the neuroendocrine system controlling growth and maturation in female crustaceans. (Laufer et al., 1991).

There has been only one report in the literature 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* (Tiu and Chan, 2007). 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 ovary was lowered as a result. The secretion of GSH from the thoracic ganglion is confirmed in red claw crayfish *Cherax quadricarinatus* (Cahansky et al. 2008).

Two other hormones that can indirectly affect ovarian maturation are molt-inhibiting hormone (MIH) and mandibular organ inhibiting hormone (MOIH). MIH is secreted from the X-organ and has an effect on the production of ecdysteroids by the Y-organ. The role of ecdysteroids on ovarian development and reproduction has to do with the balance between growth and reproduction. Ecdysteroids' main role includes the control of molting, and therefore it is inevitably linked to reproduction as well. Molt inhibiting hormone (MIH) have been identified, cloned, and characterized in many organisms such as crab *Charybdis feriatus* (Chan et al. 1998), crab *Cancer magister* (Umphrey et al. 1998), shrimp *Metapenaeus ensis* (Gu et al. 2001), and whiteleg shrimp *Litopenaeus vannamei* (Lago-Lestón et al. 2007). In a review paper, Nakatsuji et al (2009) compared MIH structures, analyzed MIH protein and transcripts level during the molting cycle, and animal responses to MIH in crustaceans.

Only MOIH from crab *Cancer pagarus* and spider crab *Libinia emarginata* has been identified and characterized (Liu et al. 1997; Wainwright et al. 1996).

1.8.2.2 Ecdysteroids

Ecdysteroids primarily serves as molting hormones in crustaceans, a similar function as in other arthropods. Their roles in reproduction have been suspected. As reproductive development in crustacean often occurred at the same period of continuing somatic growth (molting), one cannot overlook the importance of the molting cycle when considering various aspects of crustacean reproduction. The roles of ecdysteroids in reproduction are difficult to generalize as each group of species has different reproductive strategies in relation to the timing between molting and reproductive development.

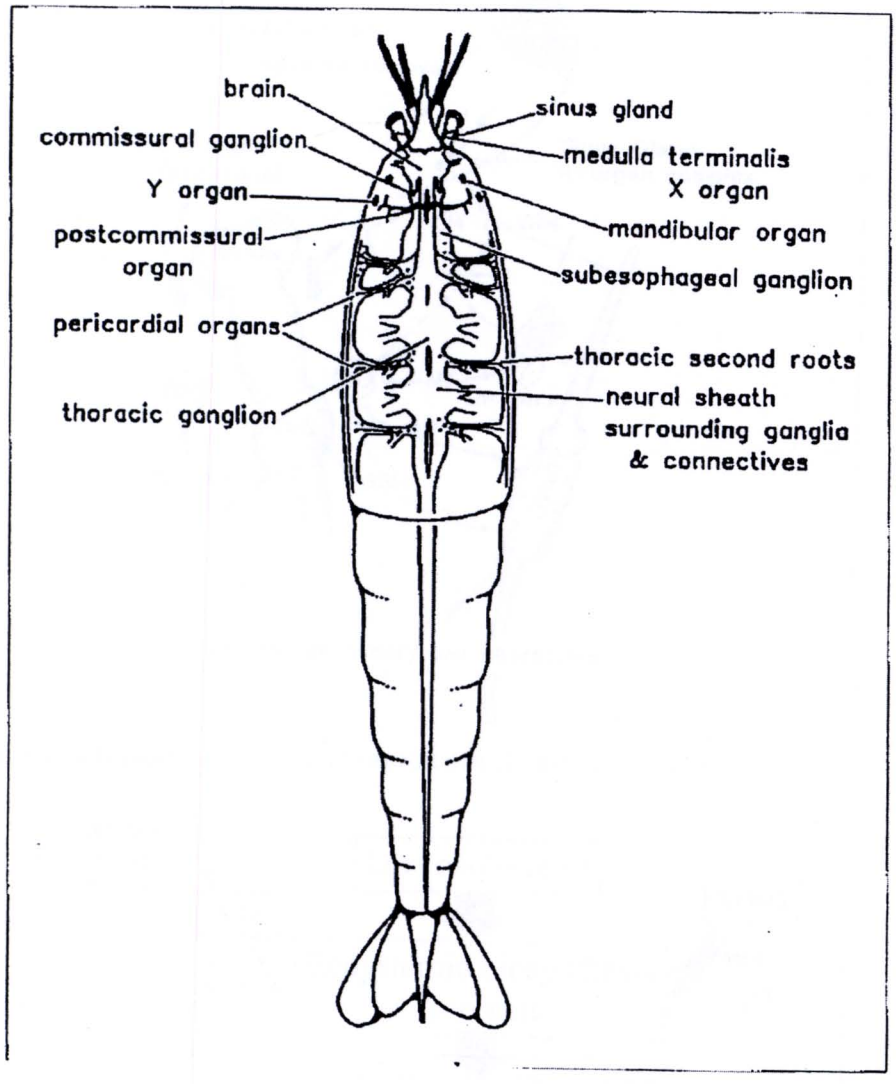


Figure 1.8 Major endocrine and neuroendocrine structures of generalized female crustacean. Included are the organs important for female reproduction, the eyestalk sinus gland x-organ, the mandibular organ, Y-organ, and thoracic ganglion (Laufer et al., 1991).

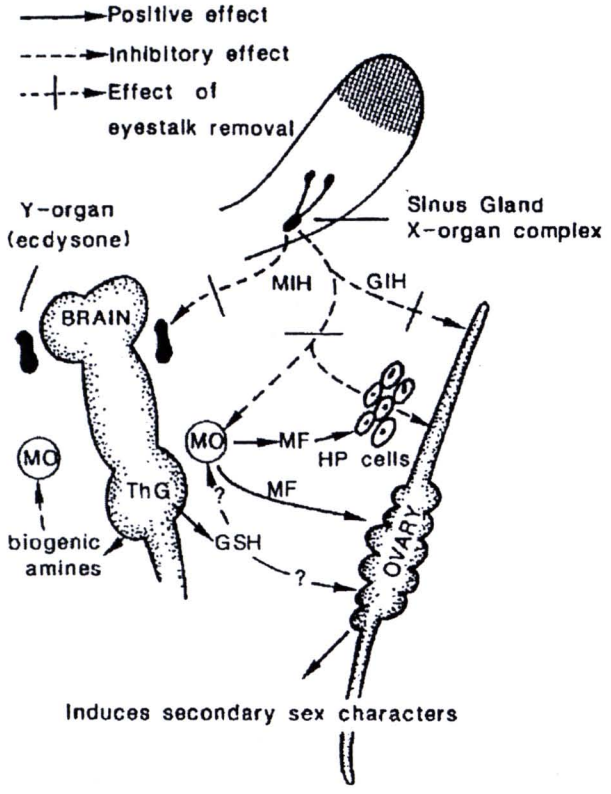


Figure 1.9 The reproduction cycle of crustacean (Laufer et al., 1991).

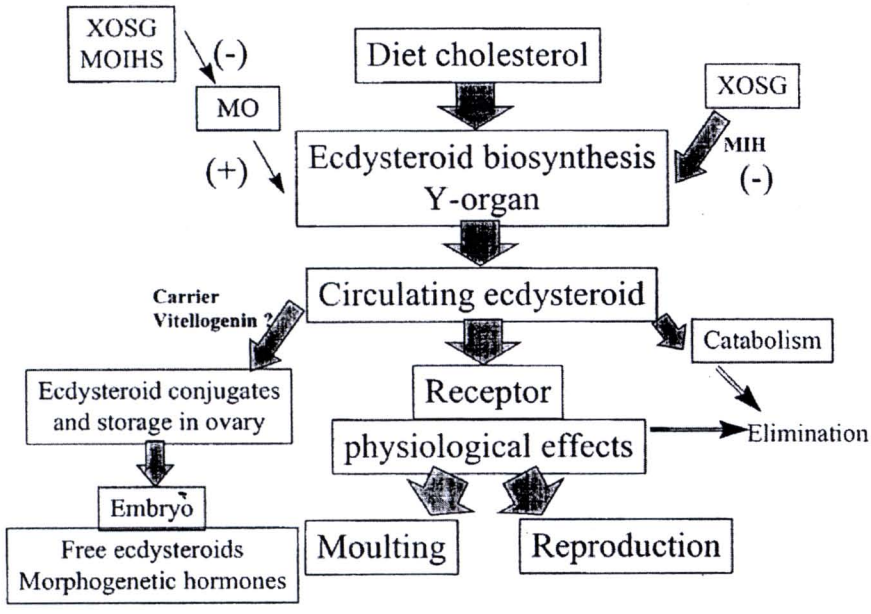


Figure 1.10 The effects of biologically active ecdysteroids on crustacean reproduction (Subramoniam., 2000)

For examples, active vitellogenesis and spawning in peneaid shrimp occurs during the prolonged premolt period before ecdysis, while *Macrobrachium* spp. alternates between reproductive molt and non-reproductive molt (Subramoniam 2000). Subramonium (2000) summarized the involvement of ecdysteroids in reproduction and embryogenesis in crustacean.

Ecdysteroids are synthesized by the Y-organs in crustacean, secreted into the hemolymph, and distributed to target tissues for conversion into active forms; 20-hydroxyecdysone (20E; also called crustecdysone, ecdysterone; Goodwin, 1978) (Subramoniam 2000). There is also evidence that ecdysteroids was also synthesized in the ovary and testis of crustaceans (Brown et al. 2009; Styrishave et al. 2008). Its production is negatively regulated by the molt-inhibiting hormone (MIH), secreted from the X-organ, and positively regulated by methyl farnesoate (MF). Important forms of ecdysteroids are 20-hydroxyecdysone (20E or 20HE) and ponasterone A (PoA) (Figure 1.11.

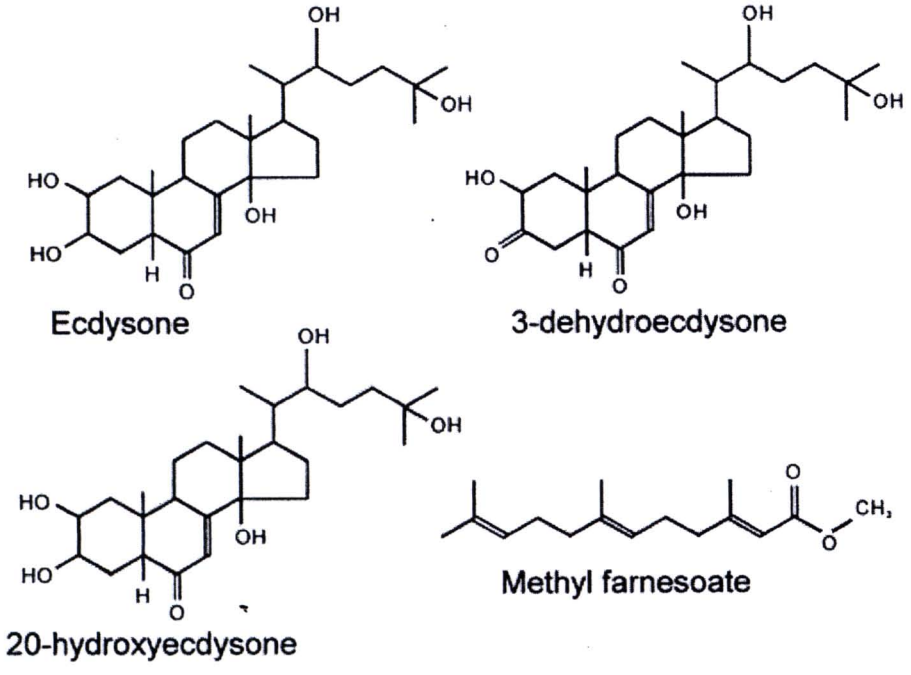


Figure 1.11 The chemical structures of ecdysteroids and methyl farnesoate (MF) (Okumura et al., 2004).

In the majority of the crustaceans, molting and hence somatic growth continue after the sexual maturity; with the result, Y-organ is active in adults also. Interestingly, the ovary in several crustacean species accumulates ecdysteroids for possible use during embryogenesis (Subramoniam et al., 1999).

In *P. monodon*, the predominant form of ecdysteroids in circulation is 20E (Kuo and Lin 1996). The peak concentration of ecdysteroids during *P. monodon* molting cycle coincides with stage D1 and D2 (proecdysis) followed by a rapid decline (Kuo and Lin 1996). Similar pattern of hemolymph ecdysteroid concentration was observed in *L. vannamei* (Chan 1995). The molting cycle was shown to override reproductive development in *P. monodon* (Quinitio et al. 1993) in that the shrimp would spawn or resorbed their eggs prior to molting.

Variation in ecdysteroids concentration during oogenesis was observed in shrimp *Palaemon serratus* Pennant (Azevedo et al. 2002). Cytochrome P450 enzymes was hypothesized to be involved with ecdysteroids synthesis and lipid storage in copepod *Calanus finmarchicus* (Hansen et al. 2008). The ecdysteroids are also packaged into oocytes to be used by the embryo. 20E was shown to accumulate in the ovary of *L. vannamei*, and its presence coincided with the inhibition of total protein synthesis in the ovary during secondary vitellogenesis (Stage II or III) (Chan 1995). Injection of 20E (10 ng/g body weight; similar normal to hemolymph concentration) did not lead to ovarian maturation in *L. vannamei* (Chan 1995). Other hormones are hypothesized to interact with circulating ecdysteroids to regulate vitellogenesis and ovarian maturation (Subramoniam 2000.

Ecdysteroids concentration was shown to be related to with both vitellogenesis and molting. (Gunamalai et al 2004) monitored the concentration of 20E in the hemolymph and ovary through the molting cycle of mole crab *Emerita asiatica,* and examined the effect of 20E injection at different molting stages on the molting cycle, ovarian development, and embryonic development. They reported that ovarian 20E concentration increased with ovarian development (intermolt), peaked at the mid-vitellogenic stage (*E. asiatic* ovarian Stage IV), and started to decline as the crab went into pre-molt stage (Gunamalai et al 2004). As the concentration of 20E in the ovary

decreased, the hemolymph 20E concentration sharply increased as the crab entered the pre-molt stage before it dropped post-molt (Gunamalai et al 2004). Injection of 20E at different stages of development had different effects on hatching and molting. The timing of injection in relation to molting or ovarian developmental stages could result in shorten molting time and, in some cases, no hatching (Gunamalai et al 2004).

In *Drosophila*, the balance between the concentration of 20E and juvenile hormones (JH) seems to play a significant role in the development of the oocytes (Gruntenko and Rauschenbach 2008; Richard et al. 1998; Soller et al. 1999). More precisely, 20E acts as a negative feedback by stimulating the resorption of yolk protein back into the hemolymph unless the condition (environment, nutrition, and mating status) were suitable (Soller et al. 1999). Thus, 20E prevents the developing oocytes from progressing beyond the control point (oocyte stage in *Dropsophila*) unless JH is present to modify the response of the oocyte to allow development beyond stage 9 (Soller et al. 1999). It is still unclear whether JH or 20E, alone or both, stimulates the early vitellogenesis in *Drosophila* (Richard et al. 2001; Richard et al. 1998). Gruntenko and Rauschenbach (2008) hypothesized dopamine and octopamine involvement in maintaining the balance of JH and 20E and their roles in reproduction of *Drosophila*.

1.8.2.3 Vertebrate-type steroid hormones

Vertebrate-type steroids have been found in many groups of invertebrate including crustacean (Lafont and Mathieu 2007). Steroids such as progesterone (PG), 17α-hydroxyprogesterone (17-OHP), testosterone, and 17β-estradiol (E2) are present in many crustacean species including kuruma prawn *M. japonicus* (Cardoso et al. 1997), giant freshwater prawn *M. rosenbergii* (Martins et al. 2007), black tiger shrimp *P. monodon* (Quinitio et al. 1994), mud crab *Scylla serrata* (Warrier et al. 2001).

Quinitio et al (1994) analyzed the profile of steroid hormones in relation to vitellogenin activity in female *P. monodon*. Progesterone and 17β estradiol were detected in the hemolymph only in shrimp with mature ovaries, while the level was low or undetectable in the hemolymph in those with immature ovaries (Quinitio et al. 1994) .

The concentration of progesterone showed a positive correlation with vitellogenesis (Quinitio et al. 1994).

Okumura and Sakiyama (2004) compared the hemolymph concentrations of several vertebrate-type steroids in kuruma prawn during both natural and induced (by eyestalk ablation) ovarian development. They observed no correlation and concluded that vertebrate-type steroids were not involved in ovarian development of kuruma prawn (Okumura and Sakiyama 2004).

Yano and Hoshino (2006) reported that 17β-estradiol induced vitellogenesis and oocyte development in previtellogenic ovary of kuruma prawn *in vitro*. They proposed that 17β-estradiol is an ovarian vitellogenesis-stimulating hormone (OVSH) in immature decapods crustaceans.

Gunamalai et al (2006) monitored the concentration of 17β-estradiol and progesterone in the hemolymph, ovary and hepatopancreas of mole crab *Emeriata asiatica* and freshwater prawn *M. rosenbergii.* Both 17β-estradiol and progesterone peaked in all tissues during the intermolt period of the reproductive molt cycle in freshwater prawn, and the basal level of 17β estradiol was detectable in the ovary and hepatopancreas during the non-reproductive molt cycle (Gunamalai et al. 2006).

Martins et al (2007) performed similar experiment to monitor the hemolymph concentration of 17α-hydroxyprogesterone, testosterone, and 17β-estradiol in female freshwater prawn *M. rosenbergii* and correlated the results with each stage of ovarian development. They reported high concentration of 17α-hydroxyprogesterone throughout the reproductive cycle and the concentration peaked during pre-vitellogenic (*M. rosenbergii* ovarian stage 2) and late vitellogenic/ mature (*M. rosenbergii* ovarian stage 5) (Martins et al. 2007). The concentration of the glucuronide-conjugated 17β-estradiol also peaked during the previtellogenis stage, while there was no significant variation in testosterone concentration (Martins et al. 2007).

Dietary source of vertebrate type steroids might play a role in ovarian development of penaeid shrimp. Meunpol et al (2007) extracted PG and 17α-OHP from

polychaetes *Perinereis* sp., a commonly used component in maturation diets for shrimp broodstock. They also reported that PG and 17α-OHP, both from the polychaetes extracts and synthetic, are capable of stimulating development of *P. monodon* oocytes from pre-vitellogenic stage to maturation (cortical rods) (Meunpol et al. 2007). It is possible the vertebrate steroids from polychaetes could stimulate the ovarian development in broodstock shrimp or supplement steroids produce by the shrimp.

Estrogen and androgen receptors were detected in the brain and thoracic ganglion of mud crab *Scylla paramamosain* using immunocytochemistry method (Ye et al. 2008). The presence of these receptors in the central nervous system suggested the possibility that estrogen and androgen may act as negative feedback in the endocrine system of crustacean (Ye et al. 2008).

Recently, the full-length cDNA of *progestin membrane receptor component 1* (*Pgmrc1*) of *P. monodon* was successfully identified and characterized. *Pgmrc1* was 2015 bp in length containing an ORF of 573 bp corresponding to a polypeptide of 190 amino acids. Northern blot analysis revealed a single form of Pgmrc1 in ovaries of P. monodon. Quantitative real-time PCR indicated that the expression level of *Pgmrc1* mRNA in ovaries of both intact and eyestalk-ablated broodstock was greater than that of juveniles ($P < 0.05$). *Pgmrc1* was up-regulated in mature (stage IV) ovaries of intact broodstock ($P < 0.05$). Unilateral eyestalk ablation resulted in an earlier up-regulation of Pgmrc1 since the vitellogenic (II) ovarian stage. Moreover, the expression level of Pgmrc1 in vitellogenic, early cortical rod and mature (II–IV) ovaries of eyestalk-ablated broodstock was greater than that of the same ovarian stages in intact broodstock ($P < 0.05$). *Pgmrc1* mRNA was clearly localized in the cytoplasm of follicular cells, previtellogenic and early vitellogenic oocytes. Immunohistochemistry revealed the positive signals of the Pgmrc1 protein in the follicular layers and cell membrane of follicular cells and various stages of oocytes.

No report of progesterone, estrogen and androgen receptors in other crustacean species at present. The presence of both receptors in the central nervous system of crustacean indicates that vertebrate type steroids might play a role in crustacean

endocrine system. Assessing the role of vertebrate steroids in crustaceans could have many applications. The effect of dietary vertebrate steroids on ovarian maturation suggested potential uses of these steroids to stimulate reproductive development.

1.8.2.4 Prostaglandins and other eicosanoids

It has been proposed that prostaglandins play a role in ovarian maturation in crustacean. Prostaglandins are derived from fatty acids such as arachidonic acid or eicosapentaenoic acid. Prostaglandins as a group have many physiological functions in many animals. In invertebrates, prostaglandins were found in sponges, cnidarians, nematodes, platyhelminthes, mollusks, annelids, crustaceans, acari, urochordates, and insects ((Rowley et al. 2005; Stanley 2006). Their known functions in invertebrates are diverse including immunity, homeostasis, feeding, larval settlement, and reproduction (Rowley et al. 2005).

Tahara and Yano (2003) monitored the hemolymph concentration of prostaglandins during ovarian maturation in kuruma prawn *M. japonicus*. They reported the concentration of prostaglandins ($PGF_{2\alpha}$ and PGE_2) in the hemolymph peaked during early vitellogenic stage and declined during the later developmental stages ((Tahara and Yano 2003).

Tahara and Yano (2004) analyzed total lipids, fatty acids, and prostaglandins in the ovaries of the kuruma prawn. Total lipid concentration increased with gonado-somatic index, and arachidonic acid concentration was lower at the early stages of development (pre-vitellogenic and early vitellogenic) than at the maturation stage (Tahara and Yano 2004). Prostaglandins concentration in the ovary peaked during the pre-vitellogenic stage and decreased at the later stage (Tahara and Yano 2004). The conconcentration peaked in the ovary before the hemolymph.

Spaziani et al (Spaziani et al. 1993) observed an increasing trend of prostaglandins ($PGF_{2\alpha}$ and PGE_2) concentrations in the ovary of Florida freshwater crayfish *Procambarus paeninsulanus* during the late vitellogenic stage with the concentration peaking just prior to ovulation. They also reported a gradual increase of PGE_2 as vitellogenesis progresse (Spaziani et al. 1993). Spaziani et al (1995) also

reported that $PGF_{2\alpha}$ can induce ovarian contraction (cAMP-mediated) and suggested that $PG_{2\alpha}$ may be involved with ovulation.

The reproduction-related functions of prostaglandin in other animals appeared to occur at several stages of oocyte maturation (Rowley et al. 2005; Stanley 2006). So far the concentration profile of prostaglandins in crustacean during ovarian development suggested its importance at the end of oocyte maturation. This hypothesis still needs to be confirmed with more research. Since precursor of prostaglandins is fatty acids, dietary manipulation could have some effects on prostaglandins in crustacean.

1.9 Molecular technique used for studies in this thesis

1.9.1 PCR

The introduction of the polymerase chain reaction (PCR) by Mullis et al. (1987) has opened a new approach for molecular genetic studies. This method is a molecular biology technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast and is a method using specific DNA sequences by the two oligonucleotide primers, 17-30 nucleotides in length. Million copies of the target DNA sequence can be synthesized from the low amount of starting DNA template within a few hours.

The PCR reaction components are composed of DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by heat-stable DNA polymerase. The cycle is repeated for 30-40 times (Figure 1.12). The amplification product is determined by agarose or polyacrylamide electrophoresis.

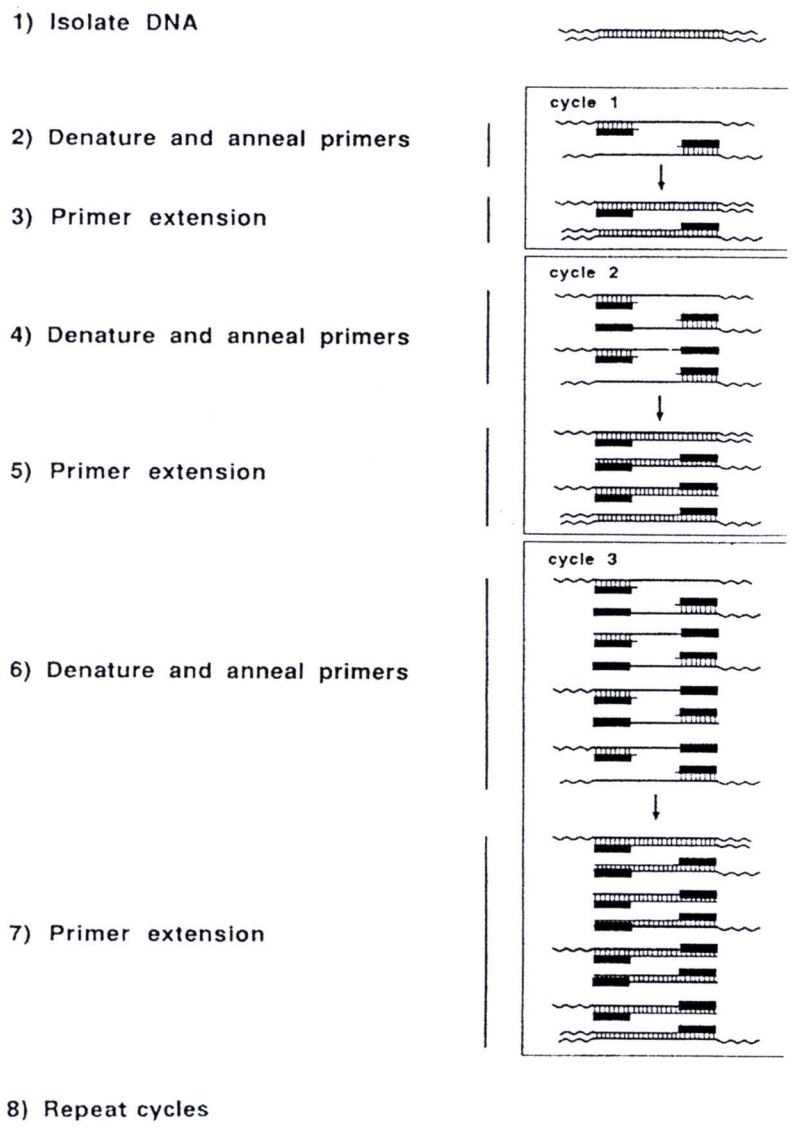


Figure 1.12 General illustration of the polymerase chain reaction (PCR) for amplifying DNA

1.9.2 Reverse Transcription-polymerase chain reaction (RT-PCR) and semiquantitative RT-PCR

RT-PCR is the method that was used to amplify, isolate or identify a known sequence transcripts. This method is a comparable method of conventional PCR but the

first strand cDNA template rather than genomic DNA was used as the template in amplification. This method contained 2 steps, in the first step first strand cDNA was synthesis using reverse transcriptase, which is made from a messenger RNA (mRNA). After that the cDNA was amplified using specific primer as same as amplified from genomic DNA (Fig. 1.13).

RT-PCR is a comparable method of conventional PCR but the first strand cDNA rather than genomic DNA used as the template in the amplification reaction. It is a basic technique for determination of gene expression in a particular RNA population. The template for RT-PCR can be the first stranded cDNA synthesized from total RNA or poly A^+ RNA. Reverse transcription of total RNA can be performed with oligo(dT) or random primers using a reverse transcriptase. The product is then subjected to the second strand synthesis using a gene specific primer. The resulting product is used as the typical PCR.

Semiquantitative RT-PCR is a semiquantitative approach where the target genes and the internal control (e.g. a housekeeping gene) are separately or simultaneously amplified using the same template. The internal control (such as β-actin, elongation factor EF-1α or G3PDH) is used under the assumption that those coding genes are transcribed constantly and independently from the extracellular environment stimuli and that their transcripts are reverse transcribed with the same efficiency as the product of interesting transcript.

1.9.3 Rapid Amplification of cDNA Ends-polymerase chain reaction (RACE-PCR)

RACE-PCR is an approach used for isolation of the full length of characterized cDNA. This method generates cDNA fragments by using PCR to amplify sequences between a single region in the mRNA and either the 3'- or the 5'-end of the transcript. To use RACE it is necessary to know or to deduce a single stretch of sequence within the mRNA. From this sequence, specific primers are chosen which are oriented in the 3' and 5' directions, and which usually produce overlapping cDNA fragments (Primrose. 1998).

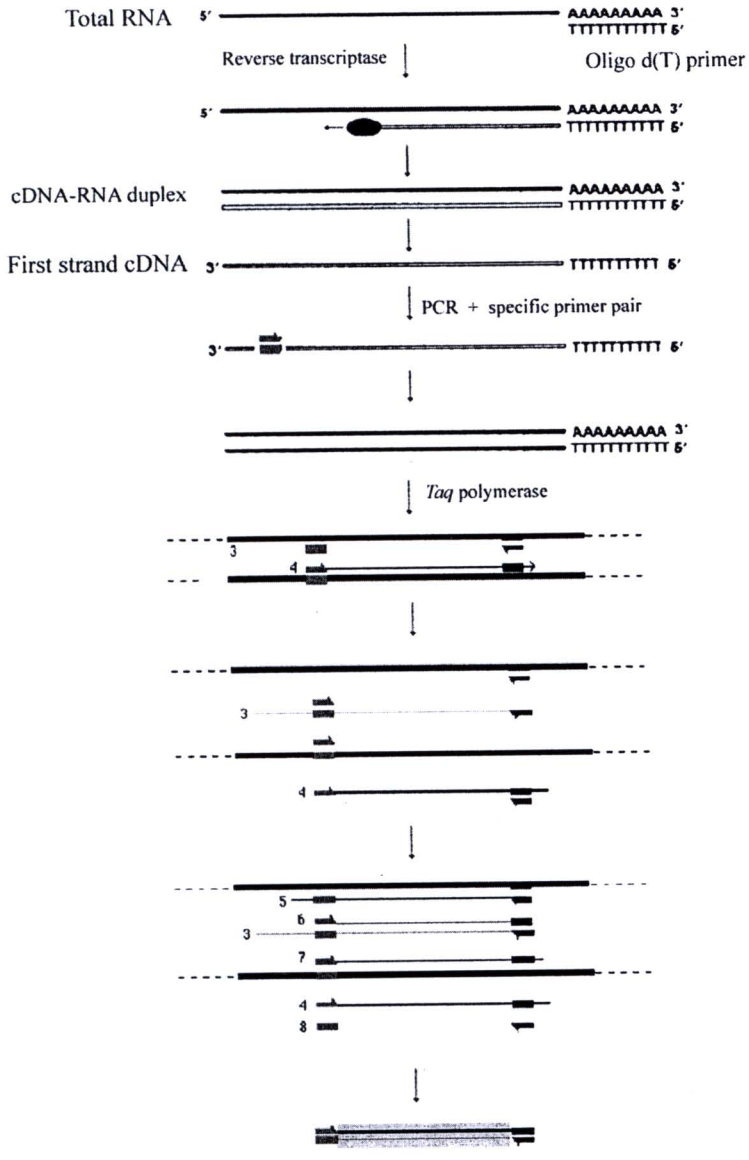


Figure 1.13 Overall concepts of the RT-PCR procedure. During first-strand cDNA synthesis an oligo d(T) primer anneals and extends from sites present within the total RNA. Second strand cDNA synthesis primed by the 18 - 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA fragments serve as templates for PCR amplification.

Using SMART (Switching Mechanism At 5' end of RNA Transcript) technology, terminal transferase activity of Powerscript Reverse Transcriptase (RT) adds 3-5 nucleotides (predominantly dC) to the 3' end of the first strand cDNA. This activity is harnessed by the SMART oligonucleotides whose terminal stretch of dG can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase. A complete cDNA copy of original mRNA is synthesized with the additional SMART sequence at the end (Fig. 1.14).

The first strand cDNA of 5' and 3' RACE is synthesized using a modified oligo (dT) primers and serve as the template for RACE-PCR reactions. Gene specific primers (GSPs) are designed from interested gene for 5'-RACE PCR (antisense primer) and 3'-RACE PCR (sense primer) and used with the universal primer (UPM) that recognize the SMART sequence. RACE products are characterized. Finally, the full length cDNA is isolated.

1.9.4 Genome walk analysis

Genome walk analysis is a method for identifying unknown genomic regions flanking a known DNA sequences. Initially, genomic DNA is separately digested with different blunt-end generating restriction endonucleases (usually, *Hae* III, *Dra* I, *Pvu* II and *Ssp* I). The digested genomic DNA in each tube was then ligated to the adaptor. The ligated product is used as the template for PCR amplification.

PCR was carried out with the primer complementary to the adaptor (AP1) and the interesting gene (gene specific primer; GSP). The resulting product is amplified with nested primers (AP2 and nested GSP). The nested PCR products were cloned and characterized (Fig. 1.15). This technique allows isolation of the promoter region of interesting genes and 3' and 5' Un-translated region (UTR) that required further characterization of SNPs at 3' and 5'UTR.

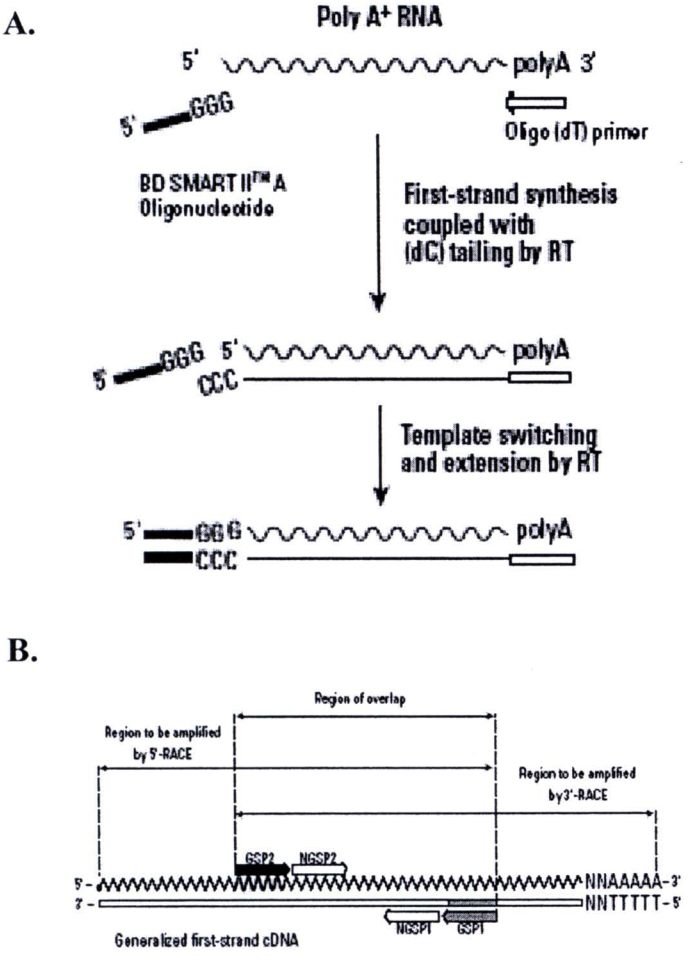


Figure 1.14 (A) Mechanism of a SMART™ technology cDNA synthesis. First-strand synthesis is primed using a modified oligo (dT) primer. After Powerscript reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The SMART II A oligonucleotide anneals to the tail of the cDNA and serves as an extended template for PowerScript RT. (B) The relationship of gene-specific primers to the cDNA template. This diagram shows a generalized first strand cDNA template.

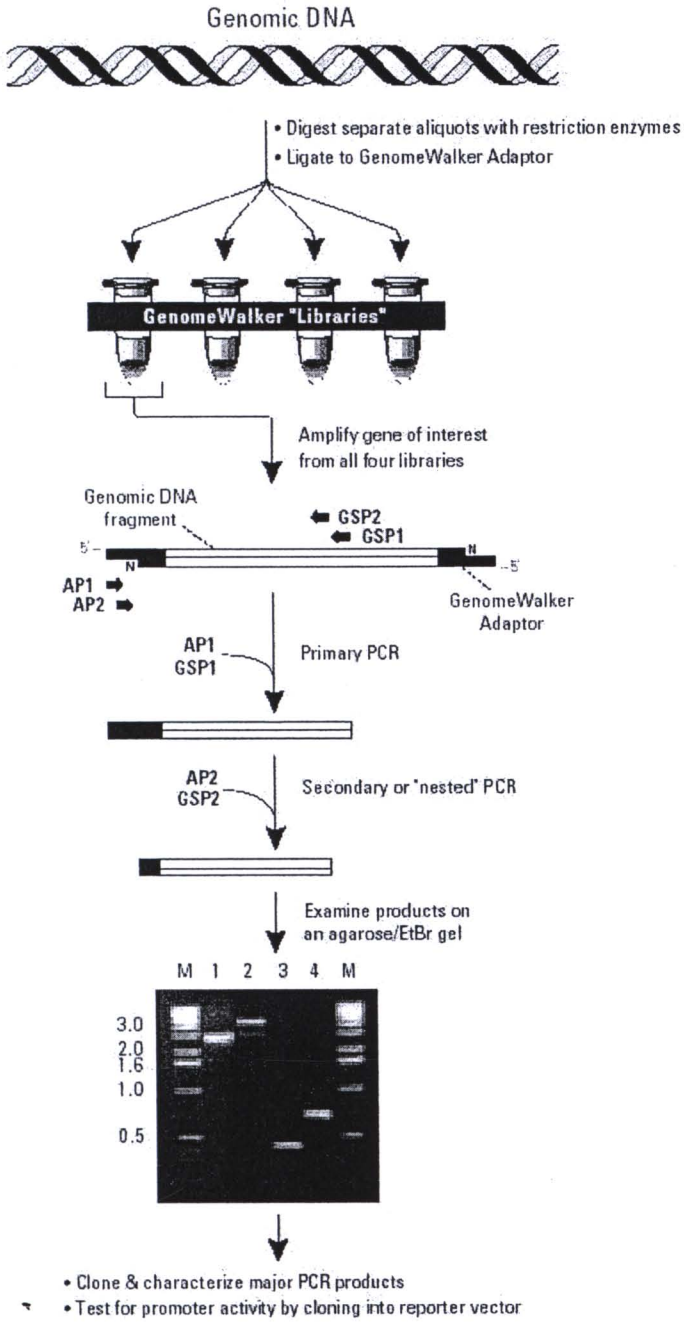


Figure 1.15 A flow chart illustrating the GenomeWalk analysis protocol.

1.9.5 DNA sequencing

DNA sequencing is the process of determining the exact order of the bases (A, T, C and G) in a piece of DNA. In essence, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by size, and the bases at the end are identified, recreating the original sequence of the DNA. There are two general methods for sequencing of DNA segments: the "chemical cleavage" procedure described by Maxam and Gilbert, 1977 and the "chain termination" procedure was described by Sanger, 1977. Nevertheless, the latter method is more popular because chemical cleavage procedure requires the use of several hazardous substances. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.

DNA sequencing is the molecular biology technique for determined sequence of a piece of DNA. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced and commonly used (Figure 1.15). This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.9.6 Quantitative real-time PCR

Real-time polymerase chain reaction, also called "quantitative real-time polymerase chain reaction" (Q-PCR/qPCR) or "kinetic polymerase chain reaction", is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a target DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample (Figure 1.16).

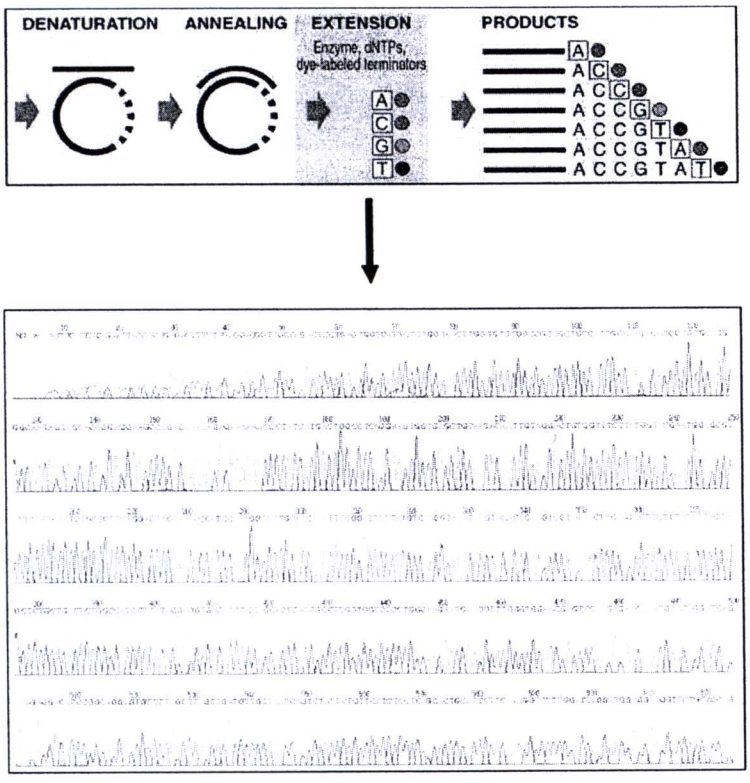
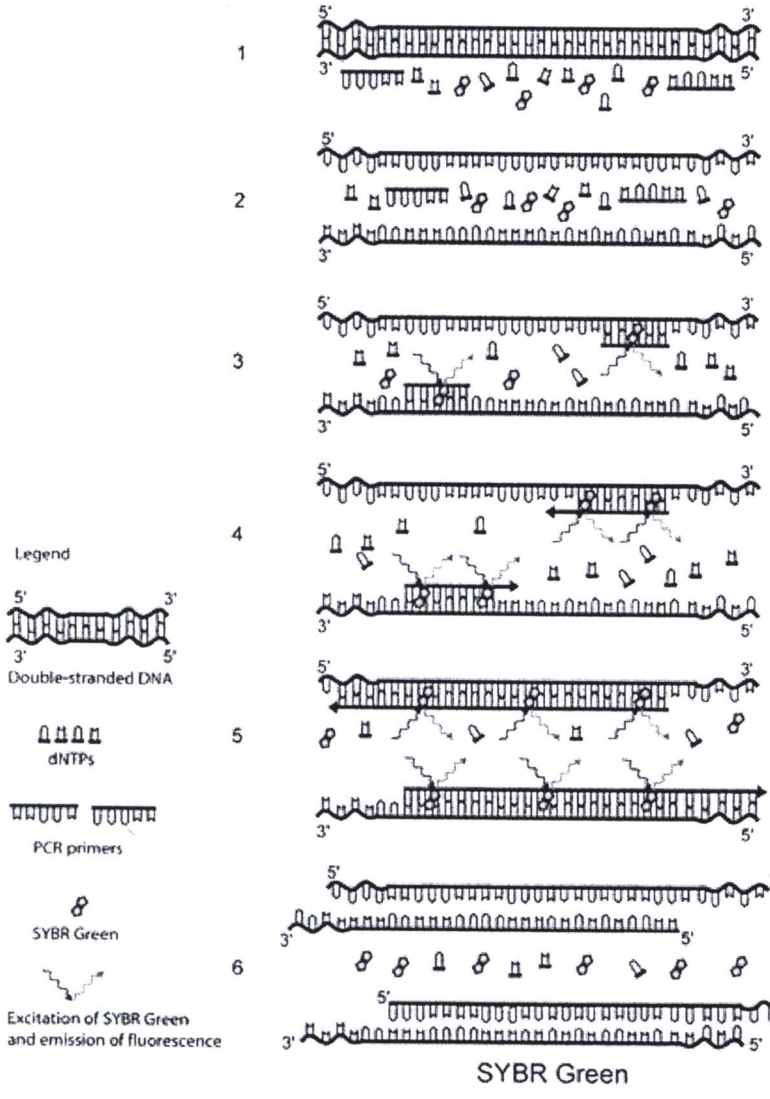


Figure 1.16 A schematic diagram illustrating principles of automated DNA sequencing.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real tine after each amplification cycle (Figure 1.17). Two common methods of quantification are: (1) the use of fluorescent dyes that intercalate with double-stranded DNA, and (2) modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA (VanGuilder *et al.*, 2008)

Typically, the reaction is prepared as usual, with the addition of fluorescent dsDNA dye. The reaction is run in a thermocycler and after each cycle, the levels of fluoresce are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the



Source: www.dpd.cdc.gov

Figure 1.17 The principle of SYBR Green detection in real-time. The fluorescent dye SYBR Green is added to the PCR mixture. SYBR Green is a DNA binding dye that fluoresces strongly when bound to double-stranded DNA. At the start of the reaction, very little double stranded DNA is present, and so the fluorescent signal detected by the thermocycler is low. As the reaction proceeds and PCR product accumulates, the amount of double-stranded DNA increases and with it the fluorescence signal. The signal is only detectable during annealing and extension, since the denaturation step contains predominantly single-stranded DNA.

PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluoresce of the dye. An increase in DNA product during PCR therefore leads to an increase in fluoresce intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, SYBR Green binds to all dsDNA PCR products, including nonspecific PCR products (such as "primer dimmers"). This can potentially interfere with or prevent accurate quantification of the intended target sequence.

1.9.7 *In situ* hybridization

In situ hybridization allows specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. In combination with immunocytochemistry, *in situ* hybridization can relate microscopic topological information to gene localization at the DNA, mRNA, and protein level. The technique was originally developed by Pardue and Gall (1969). At that time radioisotopes were the only labels available for nucleic acids, and autoradiography was applied of detecting hybridized sequences. Furthermore, as molecular cloning was not possible in those days, *in situ* hybridization was restricted to those sequences that could be purified and isolated by conventional biochemical methods (e.g., mouse satellite DNA, viral DNA, ribosomal RNAs).

At present, non-radioactive labeling using the digoxigenin (DIG) system id commonly applied for *in situ* hybridization. Digoxygenin is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms (Figure 1.18). The DIG-labeled nucleotides may be incorporated, at a defined density, into nucleic acid probes by DNA polymerases (such as *E. coli* DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, Transcriptase, and Taq DNA Polymerase) as well as RNA Polymerase (SP6, T3, or T7 RNA Polymerase), and Terminal Transferase.

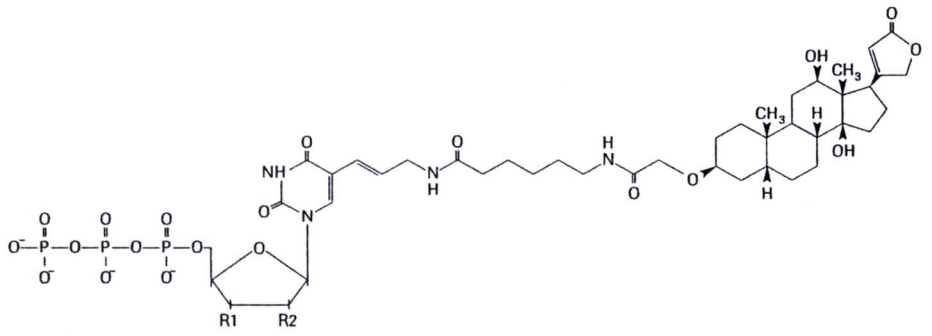


Figure 1.18 Digoxigenin-UTP/dUTP/ddUTP, alkali-stable. Digoxigenin-UTP (R1 = OH, R2 = OH) Digoxigenin-dUTP (R1 = OH, R2 = H) Digoxigenin-ddUTP (R1 = H, R2 = H)

DIG label may be carried out by radom primed labeling, nick translation, PCR, 3'-end labeling/tailing, or *in vitro* transcription. Hybridized DIG-labeled probes may be detect with high affinity anti-digoxigenin (anti-DIG) antibodies that are conjugated to alkaline phosphatase, peroxidase, fluorescein, rhodamine, or colloidal gold. Alternatively, unconjugated anti-digoxigenin antibodies and conjugated secondary antibodies may be used.

Detection sensitivity depends upon the method used to visualize the anti-DIG antibody conjugate. For instance, when an anti-DIG antibody conjugated to alkaline phosphatase is visualized with colorimetric (NBT; blue tetrazolium chloride and BCIP; 5-Bromo-4chloro-3-indolyl phosphate, toluidine salt) or fluorescent (HNPP) alkaline phosphatase substrates, the sensitivity of the detection reaction is routinely 0.1 pg of the target on a Southern blot.

1.10 Effects of *O*-methyltransferase and ecdysteroids on ovarian development and/or molting of crustaceans

1.10.1 *O*-methyltransferase

O-methyltransfrase (OMT) is ubiquitously present in diverse organisms and plays an important regulatory role in growth, development, reproduction and defense mechanisms in plants and animals. In animal two kinds of OMT; catechol-*O*-methyltransferase (COMT) and farnesoic acid-*O*-methyltransferase (FAMeT) were identified according to their selectivity to methyl acceptors.

1.10.1.1 Cathecol-*O*-methyalation (COMT)

Catechol-*O*-methyltransferase (COMT) functionally transferred of the methyl group from S-adenosine-methionine to one of the hydroxyl group of catechol compounds (Figure 1.19). Accordingly, COMT plays an important role in the catabolism and *O*-methylation of endogenous catecholamines with hormonal and meurotransmission activities such as dopamine, noradrenaline, epinephrine, cathecholestroge) and their metabolites, ascorbic acid (Guldberg and Marsden. 1975, Mannisto et al. 1992., Kopin. 1985 and Filipenko et al., 2001), some indolic intermediates of melanin metabolism (Smit et al., 1990) and xenobiotic cathechols and carcinogenic catechol-containing flavonoids (Zho and Liehr. 1994).

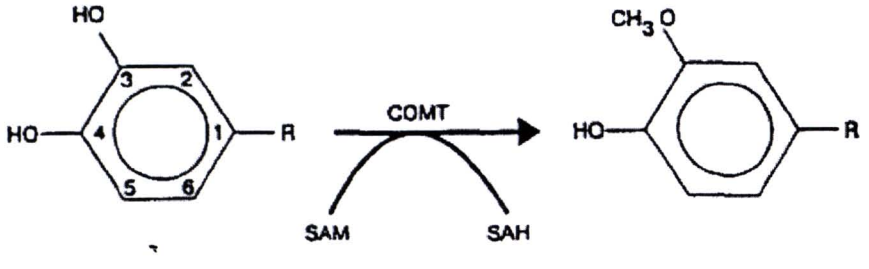


Figure 1.19 The *O*-methylation of the catechol substrate catalysed by COMT. (Lundstrom et al. 1995)

Interestingly, 17β-estradiol can be hydroxylated at 2- and 4- carbons of ring A by specific hydroxylases (estrogen-2/4-hydroxylases). The hydroxyestrogens can be *O*-methylated by COMT to form methoxyestrogens (Figure 1.20; Ball et al. 1983, Fishman 1983). In the mammalian ovaries, catecholestrogens have been demonstrated as potent autocrine/paracrine regulators of ovarian functions. They stimulate progesterone synthesis, cAMP and β-adrenergic receptors (Spicer and Hammond, 1989). Therefore, COMT should also involve the steroidogenetic pathway in shrimp and may play the important role on ovarian development of *P. monodon*.

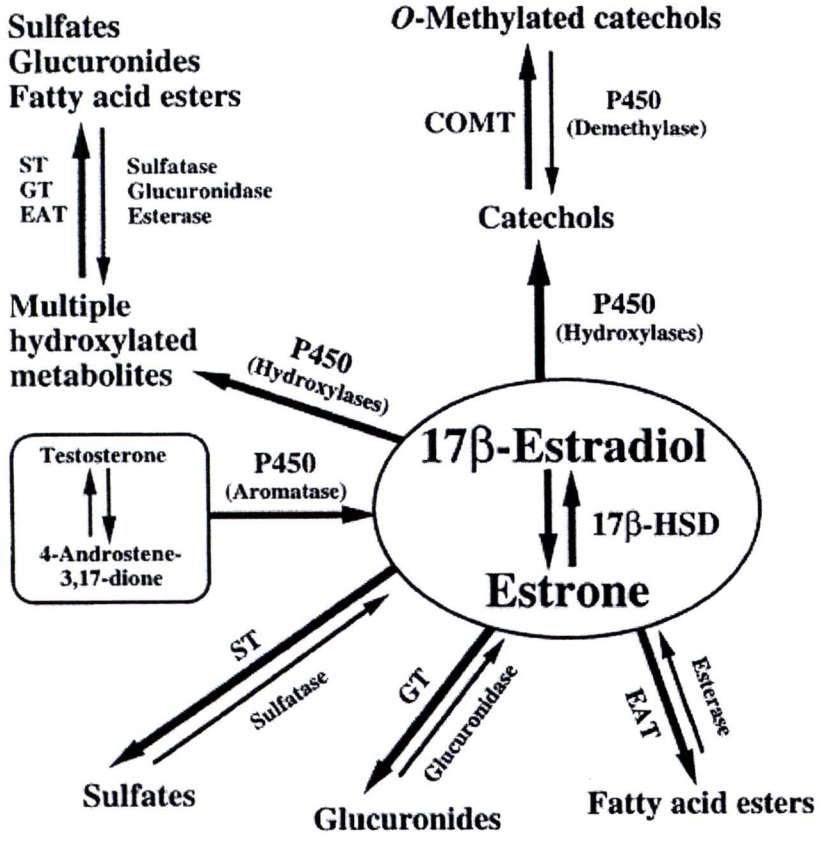


Figure 1.20 Complexities of estrogen metabolism. Abbreviations used. ST: sulfotranferase, GT: glucurosyltransferase, EAT: estrogen acyltransferase, 17β-HSD: 17βhydroxysteroid dehydrogenase, COMT: cathechol-*O*-methyltransferase and P450: cytochrome P450 (Zho and Conney., 1998).

COMT has an important role in the metabolism of catecholestrogens which are 2- and 4-hydroxylated products of estrogens. The competition with catecholamines for the metabolism through COMT locally in tissues (e.g. breast, ovaries and uterus) has been noticed *in vitro* (Ball et al. 1972). Catecholestrogens seem to have importance at least in early pregnancy and in the initiation of some estrogen-dependent tumors (Männistö et al. 1992b; Cavalieri et al. 1997; Weisz et al. 1998; Zhu and Conney 1998). Expression of COMT is regulated by estrogens (Xie et al.1999). The role of COMT and catecholestrogens *in vitro* and *in vivo* has not been clarified (Männistö and Kaakkola 1999).

Li et al. (2006) isolated the full length cDNA of *O*-methyltransferase in *Fenneropenaeus chinensis* from the hemocytes of bacteria-infected shrimp by suppression subtractive hybridization (SSH) coupled with the SMART cDNA method. The phylogenetic analysis indicated that *F. chinensis OMT* was not a member of *FAMeT* but recognized as a new member of *COMT*.

More recently, the recombinant COMT protein of *F. chinensis COMT* (Fc-COMT) was expressed *in vitro*. The rFc-COMT was found in the soluble form in *E. coli* lysate. Two types of methyl products of DHBAc (VA and IVA) were detected in the enzymatic reaction mixtures with rFc-COMT by HPLC-MS. The rFc-COMT has the catalytic activity for transferring the methyl group from SAM to the 3′- or 4′hydroxyl group of the benzyl ring of DHBAc (Li et al., 2010).

1.10.1.2 Farnesoic acid-*O*-methyltransferase (FAMeT)

Methyl farnesoate (MF) is structurally similar to the insect juvenile hormones (JH III, Figure 1.21). MF synthesized in mandibular organ (MO) from farnesoic acid (FA) by the action of farnesoic acid *O*-methyltransferase (FAMeT) in the presence of *S*-adenosyl methionine (Nagaraju, 2007). From the MO, MF is secreted into the hemolymph and distributing to the target organ. MF has an effect on several organs including the Y-organ and ovaries.

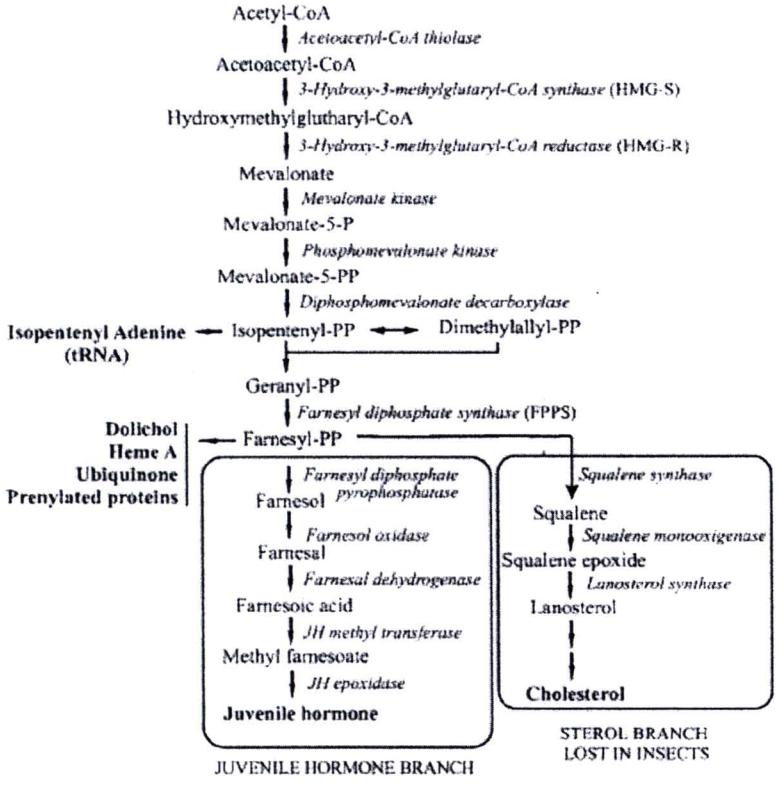


Figure 1.21 The malonate and juvenile hormone biosynthetic pathways in insects (Belles et al. 2005).

MF was first isolated from the hemolymph of the spider crab *Libinia emarginata* and is believed to regulate growth and reproduction in crustaceans (Huberman, 2000). It has been reported that MF maintain juvenile morphology and, therefore, inhibits gonadal development in juveniles but enhances reproductive maturation in adults (Laufer et al., 1987; Borst and Laufer, 1990; Laufer and Sagi, 1991; Laufer and Biggers, 2001; Nagaraju et al., 2003).

Methyl farnesoate has been shown to stimulate ovarian development during pre-vitellogenic and vitellogenic stages in several species of crustacean. Laufer et al (1998) studied the effect of orally administered MF on ovarian maturation in red swamp crayfish

Procambarus clarkii at different stages of ovarian development. Both previtellogenic (immature) and vitellogenic ovaries of the crayfish was stimulated, as measured by ovarian index, after 30 days of feeding diets with MF supplement (1-2 μg $indiv^{-1}$ day^{-1}; Laufer et al. 1998). Similar results also obtained on a longer feeding trial, 60 days (Laufer et al. 1998).

Rodriguez et al (2002) examined oocyte development of *P. clarkii* after the injection of MF in combination with other hormones: twice a week with MF (2.5 μg $crayfish^{-1}$; 10^{-8} mol) alone or in combination with other hormones (JH III, 17β-hydroxyprogesterone, or 17β-estradiol; 10^{-7} mol each), and during the 3 week experiments. Results indicated that injections of MF and MF in combination with 17β-estradiol increased gonadosomatic index (GSI > 1.0 at the end of the trial) of crayfish during middle of the vitellogenic stage.

Nagaraju et al (2003) examined the effect of MF injection on ovarian development of adult freshwater prawn *Macrobrachium malcolmsonii.* MF at physiological concentration (2 ng mL^{-1} hemolymph; twice 7 days apart) was shown to increase ovarian index and mean oocyte diameters of *M. malcolmsonnii.* After the duration of their trial (14 days), the oocytes of the MF injected shrimp had progressed from the previtellogenic stage (clear) at the beginning of the trial to the vitellogenic stage (dark brown in color) at the end of the trial, while there was little change in the control groups. The evidence of ovarian stimulation by MF injection was also documented for previtellogenic freshwater crab *Oziotelphusa senex* (Reddy et al. 2004).

Nagaraju et al. (2006) indicate that MO of the freshwater south Indian rice field crab *Oziotelphusa senex senex* secreted terpenoid hormone (methyl farnesoate, MF). The secretory rate of MF by the MO isolated during premolt and vitellogenesis was significantly greater compared to the secretory rate of MO isolated from intermolt and previtellogenesis stage crabs. Accordingly, the regulation of the crustacean molt and reproduction is complex and also involves MF, besides steroid (ecdysteroid) and peptide (sinus gland peptide) hormones.

Kalavathy et al. (1999) studied the relations between the MF levels and gonad index and body weight in *O. senex senex* Fabricius. MF stimulates testicular growth in the freshwater crab as evidenced by increased testicular weight, testicular index and testicular follicle diameter in MF-injected crabs. Therefore, MF may act as a male reproductive hormone in crustaceans.

Nagaraju et al. (2003) also found the relation to body weight, sex, molt and reproduction in this crab. The weight of the MO exhibited a positive correlation with the body weight. The weight of the MO increased with ovarian index, molting and as the crab progressed towards reproduction and the MF content of the MO increased with increase in the organ weight. The results presented strongly support a potential role of the MO in regulating both molting and reproduction in this crab species.

Jo et al (1999) noticed matured oocyte degradation in the spider crab *Libinia emarginata* after several weeks following bilateral eyestalk ablation and the increase in MF concentration in the hemolymph. Another example of MF inhibiting effect on ovarian development was observed in juvenile tadpole shrimp *Triops longicaudatus*. Tsukimura et al (2006) reported that juvenile tadpole shrimp receiving orally administered MF (regardless of delivery vector) had smaller the number of oocytes, and lower ovarian weight. However, MF has no effect on adult tadpole shrimp and MF functions as a juvenilizing agent rather than inhibitor of ovarian development (Tsukimura et al. 2006).

In *P. monodon*, FA had no effects on expression of *vitellogenin* in ovaries (GSI = 3-4%) *in vitro*. However, treatment of hepatopancreas with FA (1.3 and 13 nM) resulted in a rapid expression of the *vitellogenin* gene after 3 h. Nevertheless, higher concentration of FA (i.e. at >130 nM) inhibited to transcription of the vitellogenin encoding gene (Tiu et al., 2006). This strongly support the hypothesis that FA functions as a hormone in crustacean with the final conversion to MF occurring in the target tissues (Nagaraju 2007).

Marsden et al (2008) studied the effect of orally-administered MF on ovarian development and fecundity in *P. monodon*. They monitored ovarian development (by

visual inspection) and fecundity (number of spawns, number of eggs, hatch rate, zoea 1 survival, and mean zoea 1 output) between eyestalk-ablated *P. monodon* (Stage 0, previtellogenic) fed moist artificial diet containing in 5.5 μg MF g^{-1} diet and those shrimp fed the same diet without additional MF during a 14 day feeding trial (Marsden et al. 2008). No histological analysis of oocyte was described. The ovarian development of shrimp fed MF-supplemented feed was arrested at stage III (late vitellogenic) at a higher percentage than that of shrimp fed diet without MF supplement, and that the number of spawns per shrimp and relative fecundity were lower in shrimp fed MF-supplemented feed compared to that of shrimp fed diet without MF (Marsden et al. 2008).. Shrimp from both experimental dietary group performed worse (higher % arrested Stage III ovary) than the control group fed mussel and squid mantel (Marsden et al. 2008).

The results from several experiments such Marsden et al (2008) Jo et al (1999), and Tsukimura et al (2006) hinted at the possible drawback of MF stimulation of ovarian development. The acceleration of oocyte development by MF stimulation might lead to a developmental arrest if subsequent developmental steps were not occurring or the timing was not suitable for the animals. The difference between species, method of MF delivery, and the animal physiological state could potentially affect the results. It appears that the effect of MF on crustacean reproduction has more nuance than simply stimulation or inhibition of reproductive process. Understanding its mechanisms, its timing, and its interaction with other hormones could better clarify its roles in crustacean reproduction.

Kuballa et al. (2006) isolated multiple isoforms of putative FaMeT from six crustacean species. The portunid crabs *Portunus pelagicus* and *Scylla serrata* code for three forms. Two isoforms (short and long) were isolated from the penaeid shrimp *P monodon* and *F. merguiensis* and the scyllarid *Thenus orientalis* and parastacid *Cherax quadricarinatus*. Putative FAMeT sequences were also amplified from the genomic DNA of *P. pelagicus* and compared to the putative FAMeT transcripts expressed. Each putative FAMeT cDNA isoform was represented in the genomic DNA, indicative of a multi-gene family. Various tissues from *P. pelagicus* were individually screened for putative FAMeT expression using PCR and fragment analysis. Each tissue type expressed all three isoforms of putative FAMeT irrespective of sex or moult stage. Protein domain analysis

revealed the presence of a deduced casein kinase II phosphorylation site present only in the long isoform of putative FAMeT.

Ruddell et al. (2003) characterized a putative FAMeT in the female edible crab *Cancer pagurus*. A full length cDNA was identified from the MO of the female crab by cDAN library screening and RT-PCR. A high degree of sequence identity was found between this and other putative crustacean FAMeT. The conceptual translation and protein sequence analysis suggested that phosphorylation could occur at multiple sites in the FAMeT. This finding is consistent with the recent observation that endogenous FAMeT activity in the MO extracts can be regulated by phosphorylation *in vitro*. They demonstrated that the recombinant FAMTase could be expressed as a LacZ-fusion protein in *Escherichia coli* and have undertaken its partial purification from inclusion bodies. In an established assay system, the rFAMeT lacked activity. Northern blotting demonstrated widespread expression of an approximately 1250-nucleotide FAMeT transcript in female *C. pagurus* tissues. Levels of FAMTase transcripts in MO of female *C. pagurus* were found to fluctuate during vitellogenesis and embryonic development. An HPLC-based method was used to measure hemolymph MF titers ($N > 70$) and specimeens were classified into "high MF" and "low MF" groups. The high MF titers, which occurred before or during early vitellogenesis, coincided with, or were preceded by the elevated levels of putative FAMeT mRNA in the MO.

Silva Gunawardene et al (2001) isolated and characterized FAMeT from the greasyback shrimp *Metapenaeus ensis* and analyzed its expression from many shrimp tissues. The highest expression of FAMeT in the central nervous system and constant expression in the ventral nerve cord of mature females during reproductive cycle. Another study on the function and localization of FAMeT in the same species showed that FAMeT localized in the neurosecretory cells of the X-organ-sinus gland complex of the eyestalk and was expressed in all molting stages (Silva Gunawardene et al. 2002).

Hui et al (2008) analyzed FAMeT expression and its functions in *L. vannamei*. Similar to that observed in *M. ensis*, LvFAMeT was widely expressed in many shrimp tissues. LvFAMeT also expressed throughout the larval stages of shrimp (whole animal

assay) with high expression during the nauplius, zoea, and mysis stage. A variation of LvFAMeT level was observed during the molt cycle in both male and female shrimp. The function of LvFAMeT on molting was examined by a gene knock down experiment using LvFAMeT dsRNA injection. The results showed that the knockdown of LvFAMeT affected the regulation of two molt-relating genes, *cathepsin* and *hemocyanin*, resulting in failure to molt and mortality (Hui et al. 2008).

The role of FAMeT in regulation of MF production and its relevance to ovarian development is yet to be examined. FAMeT wide distribution in shrimp tissues suggested that the gene's role might not be limited to only reproduction and molting-related functions, and that their effect might be extensive.

1.10.2 *Broad-Complex* (*Br-c*)

In *Drosophila*, the *Broad-Complex* (*Br-c*) is a key member of the 20-hydroxyecdysone regulatory hierarchy that coordinates changes in gene expression during metamorphosis. The family of transcription factors encoded by the *Br-c* share a common amino-terminal domain which is fused by alternative splicing to one of four pairs of C2H2 zinc-finger domains (Z1, Z2, Z3, and Z4) (Bayer et al., 1996). The common core region contains a highly conserved 120 amino acids, called the BTB or POZ domain which appears to be involved in protein–protein interaction (Bardwell & Treisman 1994, Zollman et al. 1994). The BR isoforms are critical mediators of the ecdysteroid hierarchy because they are required in the regulation of intermolt, early and late gene activities in Drosophila (Belyaeva et al. 1980, Karim et al. 1993).

The relation between *Br-c* and metamorphosis was reported (Fletcher and Thummel, 1995). The *Br-c* and *E74* are induced directly by ecdysone and encode families of transcription factors that regulate ecdysone primary- and secondary-response genes. Genetic analyses have revealed that mutations in the *Br-c* and *E74* are lethal during metamorphosis and that these mutations cause some similar lethal phenotypes and alterations in secondary-response gene transcription. Representative alleles from each *Br-c* and *E74* complementation group were combined and results indicated that the functions of *Br-c* and *E74* to regulate ecdysone-inducible taget gene transcription

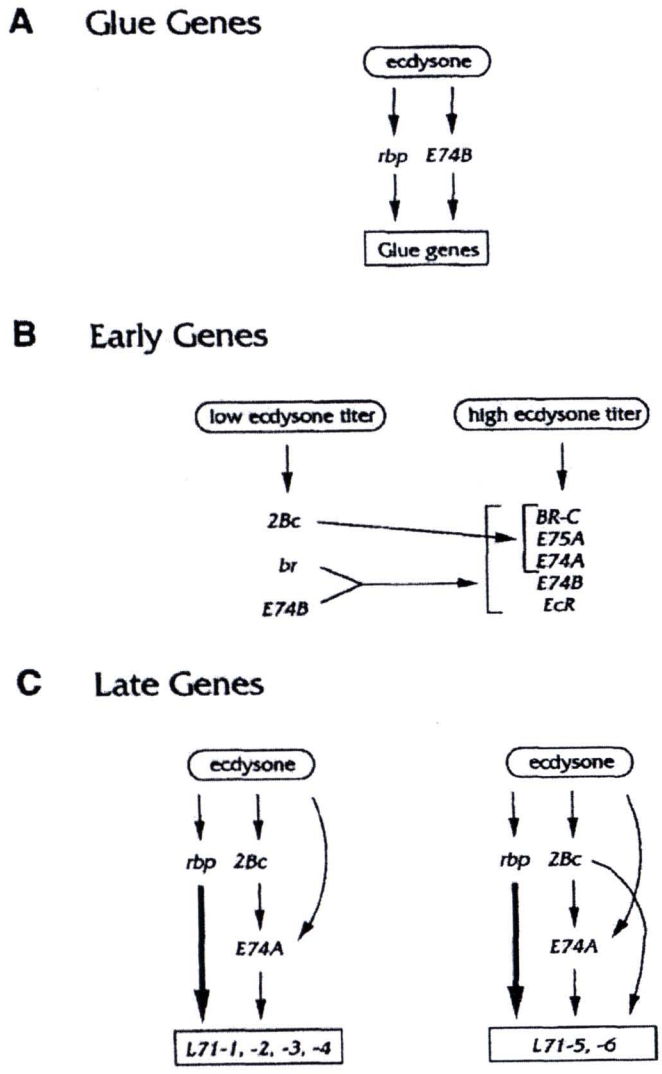


Figure 1.22 Models for the regulation of glue, early, and late gene transcription by the BR-C+ and E74' functions. (A) The rbp+ and E74B+ functions act together to regulate glue gene transcription in mid third instar larvae. The rbp+ is required for the proper timing of glue gene induction while E74B' is required for maximal levels of glue gene transcription. In the absence of both functions, glue mRNA is almost undetectable. (B) The BR-C and E74B have redundant functions in up-regulating early gene transcription in late third instar larvae. It has been shown previously that the early induction of the 2Bc' function, apparently by a low titer ecdysone pulse, is required for the maximal induction of the BR-C, E74A, and E75A early mRNAs by the high titer late larval ecdysone pulse

together (Figure 1.22). Analysis of the morphological and molecular phenotypes of the double-mutant animals reveals that *Br-c* and *E74* alleles act together to produce both novel and synergistic effects. The *Br-c* and *E74* share functions in puparium formation, pupation and early gene induction. These transcription factors may directly interact to regulate the expression of salivary gland glue and late genes. This data is supported the combinations of ecdysone primary-response genes regulate common morphogenetic pathways during insect metamorphosis (Karim et al., 1993).

In both *Drosophila* and *Manduca*, *Br-c* induction only occurs in the final larval instar. Br-c protein activates the pupal program and suppresses both the larval and adult programs. JH application at the onset of the adult molt causes re-expression of *Br* and the formation of a second pupal cuticle, suggesting that Br-c is sufficient to mediate the action of JH (Zhou and Riddiford 2002). The *Br-c* gene is expressed in a dynamic pattern during oogenesis. It is activated by ecdysteroid in follicle cells at stage 6 of oogenesis, where it is essential for the control of endoreplication and chorion gene amplification. *Br* is also involved in cell migration, as well as morphogenesis of chorionic.

RNAi knock-down of *Br-c* in the silkmoth *Bombyx mori* results in the failure of animals to complete the larval–pupal transition or in later morphogenetic defects (Uhlirova *et al.* 2003).

Chen et al. (2004) found that the *Br-c* gene is involved in the 20E-regulatory hierarchy controlling vitellogenesis in the mosquito, *Aedes aegypti*. Unlike *E74* and *E75*, early gene expression of *Br-C* was activated in previtellogenic females during a JH-dependent period. The levels of Z1, Z2 and Z4 mRNAs were elevated in the fat body of 2-day-old females after *in vitro* exposure to JH III. However, JH III repressed 20E activation of *Br-c* in 3- to 5-day-old females, indicating a switch in hormonal commitment. The results suggested that *BR* isoforms are essential for proper activation and termination of the *Vg* gene in response to 20E.

The role of *Br-c* in embryogenesis was studied. The Z1-Z6 isoforms of Br-c was found in cockroach. The temporal-expression patterns indicate that *BgBR-c* isoforms are

present through out the embryogenesis of *B. germanica*, although with weak fluctuations. Silencing all *BgBr-c* isoforms in the embryo through parental RNAi elicited adiversity of phenotypes. These phenotypes suggest roles for *BgBR-C* indifferent in embryogenesis processes of *B. germanica* (Piulachs et al., 2010)..

Nishita and Takiya (2004) studied the gene encoding a *Br-C* homolog in *B. mori.* Four isoforms of *Br-c* were found in this species and expression patterns of the *Bm Br-c* isoforms during late larval to pupal development were observed in in the epidermis, fat body and silk gland. During the metamorphic transformation, the epidermis and silk gland are completely histolyzed; however, the fat body survives into the adult phase. Expression patterns of BmBr-*c* during development differed extensively between the histolyzed group and the survival group. The BmBr-c expression patterns in silk glands also differed between the anterior and other areas (the middle and posterior silk glands).

Clearly, elucidating the mechanism controlling the expression of the *Br-c* homolog and its function in *P. monodon* would provide insight into how ecdysone controls metamorphosis and larval ecdysis in shrimp.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