CHAPTER I
INTRODUCTION

1.1 General introduction

The demand of shrimp in world markets has increased. The number of farms has increased rapidly in recent years. The most important cultivated shrimp species are the black tiger shrimp, *Penaeus monodon*, the pacific white shrimp, *Litopenaeus vannamei*, and the Chinese white shrimp, *Fenneropenaeus chinensis* (Briggs et al., 2004). Thailand has been the world's leader exporter of cultured shrimp in terms of frozen and value-added product in several countries, e.g. USA, Japan and Canada since 1991. Shrimp is the number one seafood consumed in the USA. The all over frozen shrimp imported into USA reached 12,000 tons worth 3,000 million baht in 2005. The Japanese market is also important that the output reached 7,000 tons with a value of over 2,300 million baht in 2005 (Table 1.1).

Table1.1 Thailand's exports of *P. monodon* in various countries

Country	Year 2002		Year 2003		Year 2004		Year 2005	
	Quantity	Value	Quantity	Value	Quantity	Value	Quantity	Value
U.S.A.	36,609	13,690	37,700	12,156	20,593	5,878	12,222	3,008
Japan	17,131	6,476	15,237	5,380	11,671	3,961	6,980	2,353
Canada	4,748	1,760	5,452	1,766	3,345	1,109	1,973	580
Singapore	4,139	1,181	2,366	611	1,042	201	1,859	275
Korea	3,732	1,072	5,132	1,341	4,973	1,240	4,985	1,093
Australia	2,544	818	2,668	717	1,365	371	307	112
Total	**68,903**	**24,997**	**68555**	**21971**	**42,989**	**12,760**	**28,326**	**7,421**

Quantity = tons Value = million baht

(Source: The customs Department)

Penaeus monodon is farmed everywhere in Asia, e.g. India, Vietnam, Indonesia, Philipines, Malaysia, and Thailand. Extensive aquaculture has been practiced for many years in Thailand. In the 70's growing demand stimulated the use of supplementary feeds and a move to semi-intensive production. In the mid 80's a combination of technical and economic factors allowed the development of intensive systems using hatchery reared seed and formula feeds (Hambrey and Lin, 1996). The high demand for shrimp in overseas markets also changed traditional extensive farming practices along the coastal areas to intensive farming. Shrimp farms and hatcheries are expanded along the areas of Central, East regions and Gulf of Thailand. The intensive farming has been used for *P. monodon* farming activity resulting in the consistent increase in the outcome production.

Since 2005, production of black tiger shrimp was decreased due to several problems such as the outbreaks of infectious diseases, unfavorable weather, antibiotic residues, lack of broodstock, and water pollution. The shrimp species mainly cultured in Thailand has switched to the white shrimp, *Litopenaeus vannamei*. The black tiger shrimp production of Thailand fell down from 180,000 tons in 2004 to 19,000 tons in 2005 (Figure 1.1). In 2007, the prediction of black tiger shrimp production will be 12,480 tons while that of white shrimp will be 611,520 tons. There have been several reasons for the introduction and subsequent movement of *L. vannamei*: availability of specific pathogen free (SPF) stocks, perceived differences in susceptibility to White Spot Syndrome Virus (WSSV) from *P. monodon*, and the relative ease with which animals can be cultured and bred in captivity. There are, however, also disadvantages to the importation of *L. vannamei*, including its ability to act as a carrier of various viral pathogens exotic to Thailand, a lack of knowledge of culture techniques (particularly for broodstock development) in Thailand, and a smaller final size and hence lower market price than *P. monodon*. Since *L. vannamei* tends to be harvested at a relatively small size, this is creating new marketing challenges and also negatively affecting prices in the region. The larger-sized *P. monodon* compete in a different part of the market and often have a better market price. (Source: FAO Aquaculture Newsletter No. 30, 2007).

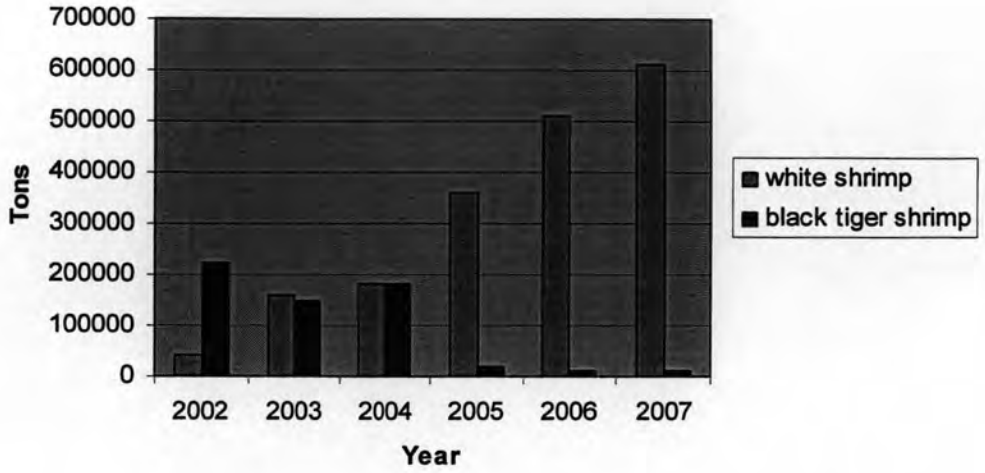

Figure 1.1 The shrimp production in Thailand between 2002 to 2006 and the prediction in 2007

(Source: http://www.shrimpcenter.com)

In addition, *L. vannamei* is a non-native species, thus, the broodstock of white shrimp have to be imported from aboard mainly from Hawaii. To maintain the production of *P. monodon*, the domestication and genetic improvement of the black tiger shrimp are urgently needed for sustainable shrimp culture. Domestication will provide captive broodstock while genetic selection could select traits of economically importance such as disease resistance, rapid maturation, tolerance of high stocking density, tolerance of low salinity and temperature, lower protein requirements, high survival during larval rearing, and some marketing advantages.

1.2 Taxonomy of *Penaeus monodon*

Penaeus monodon, the black tiger shrimp, is a shrimp species that was classified into the largest phylum in the animal kingdom, the Arthropoda. The taxonomic definition of *Penaeus monodon* is as follow (Baily-Brook and Moss, 1992):

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

Species monodon

Scientific name: *Penaeus monodon* (Fabricius), 1798

Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu (Indonesia), Ushi-ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo(Philipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Timsa (Vietnam).

FA.O. Names: Giant tiger prawn, Crevette giante tigre, Camaron tigre gigante.

1.3 Morphology

From the external view, shrimp body is basically divided into three regions: head, thorax and abdomen (Figure 1.2). Each body region possesses appendages specialized for different functions. The head (five somites) and thorax (eight somites) are normally fused into a cephalothorax, which is completely covered by the carapace. The carapace protects internal organs and support muscle origins. Internal organs,

such as gills, digestive system, reproductive system and heart, located in thorax. Shrimp muscles concentrate in the abdomen. The pleura of the cephalothorax form the branchiostegite or gill cover. The carapace has characteristic ridges (carinae) and grooves (sulci). The rostrum is always prominent, with a high median blade bearing dorsal teeth and, in some genera, ventral teeth as well. The compound eyes are stalked and laterally mobile and the somites of the head bear, in order, pairs of antennules, antennae, mandibles, maxillae 1 and maxillae 2 (not visible in Figure1.2). The thorax has three pairs of maxillipeds and five pairs of pereopods (legs), the first three being chelate and used for feeding, and last two simple (non-chelate) are used for walking. The mouth is situated ventrally at the ventral surrounding with the cephalic appendages, plus the first and second maxillipeds and sometimes the third as well may be referred to collectively as the "mouth parts". The abdomen has the obvious segmentation of invertebrates. The abdomen consists of six somites, the first five with paired pleopods (walking legs) (Bell and Lightner, 1988; Baily-Brook and Moss, 1992) and the sixth with uropods. A tail fan comprises of a telson, which bears the anus. The anus is on the ventral surface of the telson towards its base (Dall et al., 1990)

The cuticle, secreted from an epidermal cell layer, consists of chitin and protein in which calcium carbonate and calcium phosphate have been deposited. While the old cuticle is moulted, the inner cuticle layer is detached from the epidermis and the epidermis begins to secrete a new cuticle. After moulting, the new cuticle is soft and stretched to accommodate the increased sized of the shrimp.

The black tiger shrimp has the following characteristic colorations: carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackish waters or cultured ponds, the color patterns are mostly changed to dark and blackish brown (Solis, 1988).

The internal morphology of penaeid shrimp is very well developed (Figure 1.3). Muscular, digestive, circulatory, respiratory, nervous, and reproductive systems

are all present. The movements of the body such as walking, crawling, burrowing, swimming, feeding, and breathing are controlled by the muscles. The digestive system is complex, in which parts of the tract are differentiated into a foregut, a midgut, and a hindgut. The circulatory system consists of a heart, dorsally located in the cephalothorax, with branching arteries conducting blood to the various organs. Gills are responsible for respiration process. The nervous system consists of two ventral nerve cords, a dorsal brain, and a pair of ganglia for each somite.

Hepatopancreas connects to the gastrointestinal tract via the primary duct. It occupies a large portion of the cephalothorax in penaeid shrimp and functions on absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leave the heart ends in the lymphoid organ where the haemolymph is filtered. This organ consists of two distinct lobes, each located ventro-lateral to the junction of the anterior and posterior stomach chambers. The lymphoid lobes are apposed slightly dorso-anterior to the ventral hepatopancreatic lobe. The haemocytes are produced in haematopoietic tissue. The haematopoietic tissue consists of densely packed lobules located at different parts of the shrimp anterior region. The first one is haematopoietic tissue surrounding the lateral arterial vessel, which joins the anterior recurrent artery at the base of the rostrum. The second one located within the 1st maxillliped. The third one is 2nd maxilliped haematopoietic tissue. The last one is epigastric haematopoietic tissue located dorsal to the anterior stomach chamber and just ventral to the dorsal cuticle.

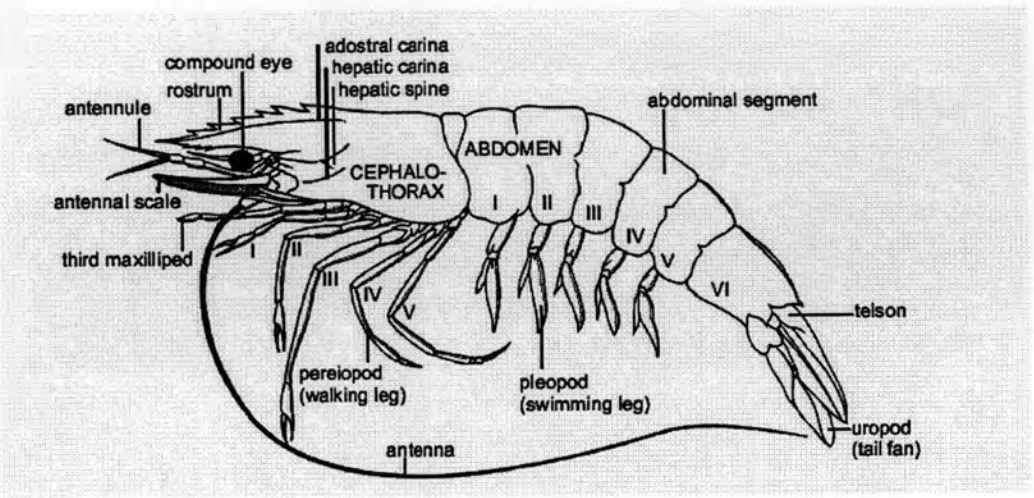

Figure 1.2 **Lateral view of the external morphology of *Penaeus monodon*** (Primavera, 1990)

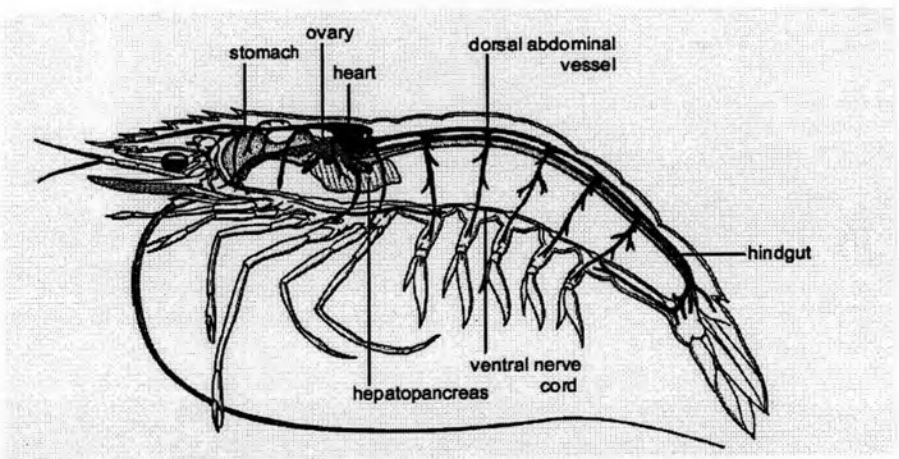

Figure 1.3 **Lateral view of the internal anatomy of a female *Penaeus monodon*** (Primavera, 1990)

1.4 Distribution

The black tiger shrimp, *P. monodon* is principally distributed in the major part of the Indo-West Pacific region. Northern part of Indo-Pacific region ranges from Japan and Taiwan. Eastern region distributes toward Tahiti. For southern and western part, it distributes to Australia and Africa, respectively. Generally, *P. monodon* is distributed from 30 °E. to 155 °E. longtitude and from 35°N. to 35°S. latitude (Motoh, 1985). The larvae, juvenile and adult stages of *P. monodon* inhabit inshore areas and mangrove estuaries. On the other hand, the adults basically inhabit offshore areas up to 160 meters in depth. *P. monodon* is a marine species that inhabits in mud or sands bottoms at all depths from shallows to 110 meters, so it can be caught from offshore or inshore as well as from tidal zones (or ponds) (Rosenberry, 1997).

1.5 Life cycle

The black tiger shrimp, *P. monodon* like all euryhaline crustaceans, have several distinct stages in various habitats (Figure 1.4). Development begins with a larva hatching from the fertilized egg to the first stage, nauplius (I-IV), followed by protozoa, mysis and post larval stages. These require the development times about 1 to 5 days, 5 days, 4 to 5 days and 6 to 15 days, respectively. After hatching, the eggs hatch into the nauplius which feed on their egg-yoke reserves for a few days and develop into the protozoeae. Around 4 to 5 days to metamorphosis, the protozoeae will metamorphose into mysis by feeding on algae. The mysis feed on algae and small zooplankton, worms, and larval stages of other aquatic invertebrates. Then, the mysis develop into early postlarvae, which the development time is 6 to 15 days (Solis, 1988). Transformation from newly hatched larvae to postlarvae depending upon food quantity and quality, temperature, and a variety of other water quality variables.

After metamorphosis to postlarvae, the shrimp behaviorally change from living suspended in the water column to principally bottom dwelling, crawling individuals. When they do swim, they move like adults with the dorsal (back) side uppermost and in a head-forward direction. Postlarvae are given a numerical suffix,

which indicates the time in days since metamophosis. They continue to moult as they grow. They migrate shoreward and settle in nursery areas close to shore or in estuaries, where they grow quickly to juvenile and subadults, tolerating the variable physico-chemical environment. Subadults migrate back to sea where they finally mature to mate and spawn. Penaeid shrimp are rarely older two years (Anderson, 1993; Solis, 1988).

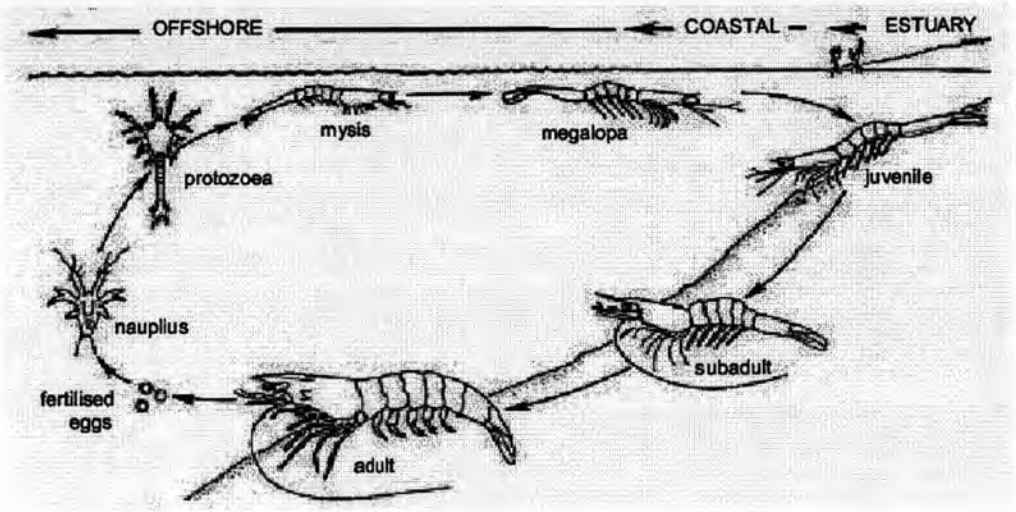

Figure 1.4 The life cycle of *Penaeus monodon* shrimp

(Baily-Brook and Moss, 1992)

1.6 Osmoregulation of crustaceans

Organisms living in estuaries and migrating to marine habitat as penaeid shrimp must be able to osmoregulate efficiently (Gilles, 1970; Gilles, 1979). Osmoregulation is one of the most important adaptive physiological processes permitting the successful establishment of a species in a given habitat (Charmantier, 1998).

Osmoregulation means the various strategies developed by the animal species to control their body water in the wide variety of possible environments which

they have been aggressively inhabited. It implies detailed study of all the processes at work in the control of the thermodynamic activity of water in biological fluids, either intracellular or extracellular, of the animal species considered. This includes analyses of any kind of transport processes and metabolic correlates involved in the adjustment and the control of the level of both inorganic and organic constituents (Charmantier and Charmantier-Daures, 2001; Martinez et al., 2005).

For many years the composition of the haemolymph has been studied in various groups of crustacean as a function of the salinity environment. Several generalizations for the most representative patterns discovered in animals living in a range of media from concentrated sea water to fresh water have been explored. The synthetic way of the haemolymph osmoregulation in aquatic crustaceans is shown in Figure 1.5.

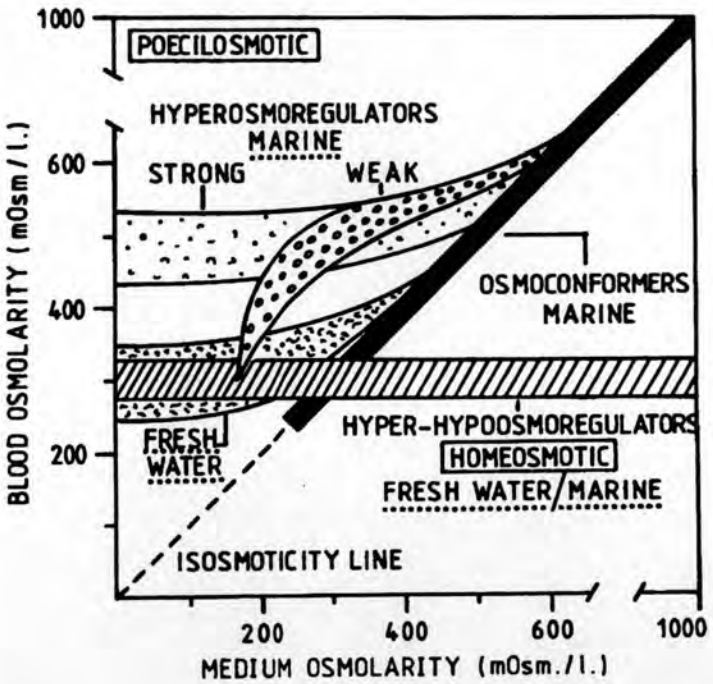

Figure 1.5 Illustration of the synthetic way of the haemolymph osmoregulation in aquatic crustaceans (Pequeux, 1995)

Classification of organisms according to their osmoregulation capabilities divides the organisms into three groups: osmoconformers, hyper-osmoregulators and hypo-osmoregulators. The osmoconformers regulate their ionic concentration in haemolymph such that it is always equal to the surrounding seawater. These animals keep their body fluids isotonic to the external environments The hyper-osmoregulators have higher ionic concentration in haemolymph than in surrounding medium while hypo-osmoregulators show lower ionic concentration in haemolymph than in external medium (Campbell and Jones, 1989; Castille and Lawrence, 1981; Charmantier and Charmantier-Daures, 2001; Gilles, 1970; Mantel and Farmer, 1983; Pequeux, 1995; Potts and Parry, 1964). Generally, *P. monodon* is a hypo-hyper osmoregulator. It exhibits hyperosmotic regulation to sea water at salinities below isosmotic concentrations and hypoosmotic regulation to those above (Wuthisuthimethavee et al., 2005). There are some other crustaceans that show an ability as hypo-hyper osmoregulation such as *Palemonetes varians*, and *Artemia salina* (Castille and Lawrence, 1981; Cheng and Liao, 1986; Ferraris et al., 1987; Knut, 1983). These organisms are extremely powerful osmoregulators and can cope with any fluctuation of salinity of the external medium that normally occurs in nature (Dall, 1985; Ferraris et al., 1986; Ferraris et al., 1987; Gilles, 1979; Motoh, 1981; Potts and Parry, 1964).

Salinity is one of the main environmental factors affecting growth, survival and development of the black tiger shrimp. The black tiger shrimp is naturally distributed over a wide range of salinities during its life cycle. The life cycle includes a marine phase during the first stages of development, then a brackish or estuarine phase for postlarvae and juvenile stages, after which they migrate towards marine water at preadult or adult stages. The matured shrimp naturally migrates from brackish water to the deep sea, a place with high salinity level and environmentally consistency, for mating. Spawning and the developing shrimp larva returns to the nutrition-rich in-shore sea or mangrove swamp for growing. Since the salinity of brackish water fluctuates more than that of marine sea water, it is likely that the degree of euryhalinity, possibility of supporting rapid salinity changes, varies with the

age of the shrimps. *P. monodon* is an euryhaline crustacean which is able to regulate their body fluid composition with regard to the ambient medium by modifying surface permeability, urine production, and ionic transport (Ferraris et al., 1987; Spaning-Pierrot et al., 2000).

1.7 Mechanisms of osmoregulation

Numerous studies on various decapod crustaceans have shown that various mechanisms of osmoregulation offer osmotic protection to cell during acclimation to the unsuitable salinity environment. These mechanisms are located in various tissues and organs (Khodabandeh et al., 2005a; Khodabandeh et al., 2005b).

1.7.1 Free amino acids (FAAs)

Several authors have studied the involvement of free amino acids (FAAs) in osmoregulation in crustacean species. The FAAs have a major role in the osmotic regulation of the intracellular fluids of aquatic invertebrates (Gilles, 1975), including the crustaceans (Schoffeniels and Gilles, 1970). Cobb et al. (1975) reported that in the white shrimp, *Penaeus stylirostris*, glycine, proline and alanine levels increased significantly in accordance with an increasingly saline environment. These authors further outlined that in penaeid shrimp, glycine and proline are main osmoeffectors under both increasing or decreasing salinity. Abe et al. (1999) reported that glycine, L-proline and D- and L-alanine increase significantly during artificial sea water acclimation in immature crabs, *Eriocheir japonicus*. Similarly Dalla Via (1989) has also studied the effects of salinity on free amino acid composition in the shrimp *Palaemon elegans*, revealing as well that highest concentrations of glycine, taurine, proline, arginine and alanine occur in seawater-adapted shrimp. In *Macrobrachium rosenbergii*, Tan and Choong (1981) reported that the transfer from dilute to more concentrated media causes a significant decrease in haemolymph protein content and increases in FAA concentration in muscle tissue.

1.7.2 Crustacean Hyperglycemic Hormone (CHH)

Numerous studies have shown that Crustacean Hyperglycemic Hormone (CHH) involve in osmoregulatory processes. CHH is synthesized in the eyestalk x-organ and stored prior to release from the sinus gland. The hormone not only plays its major role in controlling glucose level in the haemolymph, but is also significant to other processes such as edysteriod production (Yasuda et al., 1994), lipid metabolism (Santos et al., 1997), ovarian physiology (Khayat et al., 1998) and osmoregulation (Charmmantier-Daures et al., 1994; Serrano et al., 2003; Udomkit et al., 2004). The involvement of CHH in the control of osmoregulatory system has been suggested from in vivo experiment: ablation or ligation of eyestalks decrease haemolymph ionic concentration and/or osmotic pressure and increase water content, whereas reimplantation or injection of eyestalk extracts generally restores or enhances the osmotic and ionic regulation (Kamemoto and ono, 1969; Heit and Fingerman, 1975; Kamemoto, 1976; Charmantier et al., 1984; McNamara et al., 1991). Charmantier-Daures et al. (1994) demonstrated that CHH from sinus gland of *Homarus americanus* restored the ability to osmoregulate in destalked *H. americanus*.

1.7.3 Na^+/K^+-ATPase

Different enzymes are involved in crustacean osmoregulation. The Na^+/K^+-ATPase is a transmembrane protein composed of three subunits (α, β, γ) responsible for pumping three Na^+ out of animal cells in exchange for two K^+ or NH_4^+ per ATP hydrolyzed. It is generally involved in ionic transport and osmoregulation in aquatic animals (Goncalves et al., 2006; Maetz, 1974; Towle, 1981; Towle, 1984a; Towle, 1984b). The Na^+/K^+-ATPase not only mediates electrogenic transfer of Na^+ from the cytosol to the extracellular fluid and K^+ or NH_4^+ from the extracellular fluid to the cytosol but also establishes electrochemical gradients used by apical and basolateral transporters such as the Na^+/H^+ exchanger, $Na^+/K^+/2Cl^-$ cotransporter and various ion channels. Many studies have shown that the enzymatic activity of the Na^+/K^+-ATPase in gill is enhanced when euryhaline crustaceans are subjected to osmoregulatory stress following transfer from sea water to dilute salinities (Pequeux, 1995; Towle, 1990).

For example, the specific Na^+/K^+-ATPase activity in homogenates or microsomal fractions of posterior gills of *Callinectes sapidus* approximately doubles following transfer of the animals from 35 to 5 % salinity (Neufeld et al., 1980; Piller et al., 1995; Towle et al., 1976). The increased Na^+/K^+-ATPase activity is thought to drive the uptake of Na^+ and Cl^- across the gill epithelium, leading to hyperosmoregulation of the haemolymph in low-salinity environments.

1.7.4 Carbonic anhydrase (CA)

Carbonic anhydrase (CA) catalyses the reversible hydration of CO_2 and H_20 to HCO^{3-} and H^+. The presence of CA has been described in many tissues among the vertebrates and a smaller extent in invertebrate. CA has been found in different regions and involved in respiration, acid-base regulation and ionic transport (Henry and Cameron, 1983; Henry, 1984; Henry, 1987; Henry, 1988a; Henry, 1988b; Burnett et al. 1985). In decapod crustaceans, CA has been involved in all of the major gill functions such as CO_2 excretion, acid-balance and ionoregulation. High activities of CA are found in the gills of euryhaline crustaceans which are known to regulate their haemolymph osmolality when exposed to low salinities, in order to compensate for passive ion loss. In euryhaline species, CA activity is strongly dependent on environmental salinity as previously reported in *Callinectes sapidus* (crab) (Henry and Cameron, 1982a), *Carcinus maenas* (crab) (Botcher et al., 1990a; Botcher et al., 1990b) *Eriocheir sinensis* (Olsowoski et al., 1995) and *Pacifistacus leniusculus* (crayfish) (Wheatly and Henry, 1987). Its activity is increased in gills of crabs acclimated to diluted media (Henry and Cameron, 1982a; Henry and Cameron, 1982b; Wheatly and Henry, 1987).

1.8 Osmoregulatory organs

Among the few comprehensive histological studies of osmoregulatory structures in decapod crustaceans, most have concerned the gills of crabs (Drach, 1930; Chen, 1933; Smyth, 1942) and of shrimps such as *Palaemonetes varians* (Allen, 1892) *Crangon vulgaris* (Debaisieux, 1970) and *Penaeus aztecus* (Foster and Howse,

1978). Numerous studies hypothesize that the branchiostegite such as epipodite has an osmoregulatory function in *P. japonicus*. Moreover, previous studies found that the function of the antennal gland in penaeid shrimp has been associated with ion transport and osmoregulation. Therefore, these three organs were determined to elucidate genes involved in osmoregulation mechanism.

The antennal glands consist of the coelomosac, labyrinth, tubule and bladder, and their main function is urine production. By filtering the haemolymph, urine is produced and flows to the branchial chamber via the nephropore. In marine decapods, the antennal glands are involved in the control of haemolymph volume, hyporegulation of magnesium and sulfate in haemolymph, excretion of organic compounds and reabsorption of fluid, sugars and amino acids from the primary urine filtration (Riegel and Cook, 1975; Mantel and Farmer, 1983). The function of the antennal glands in penaeid shrimp have been associated with the differences of sodium and chloride concentrations between haemolymph and urine. Previously reports showed that antennal glands of *P. monodon* functioned to stabilize potassium concentrations by reabsorbing potassium from the primary urine and excreting sodium in exchange (Mantel and Farmer, 1983; Lin et al., 2000).

The epipodites, or mastigobranchs, are elongated, thin, biramous structures, attached to the coxopodites of some thoracic appendages. Previous study showed that the epipodites are probably involved in osmoregulatory mechanisms. The epithelial cells of epipodite contain numerous elongated mitochondrias which reveal the presence of the Na^+/K^+-ATPase. This is further supported by the high level of the Na^+/K^+-ATPase activity measured in epipodites of adult *P. japonicus* (Bouaricha et al., 1991) that the epipodite of *P. japonicus* could be involved in osmoregulatory mechanism. Moreover, previous study found that the excretion of salts in *Cladocera* species associated with epipodite cell.

The gills are specialized for several functions, including gas exchange, osmoregulation, acid-base balance and nitrogen excretion. In previous study reported that the gills are among the most permeable external surfaces of crustaceans, and they

are considered the primary site for ionic and osmotic regulation (Robertson, 1960; Lockwood, 1962; Lockwood, 1968; Gilles, 1975; Croghan, 1976; Kirschner, 1979; Pequeux and Gilles, 1981; Pequeux and Gilles, 1988; Towle, 1984a). Numerous studies reported that the gill of crab species consists of two kinds, anterior and posterior gills that are histologically different. In *Eriocheir sinensis* the anterior gills have a thin, little-differentiated epithelium, probably involved in respiration, while the posterior ones play a key role in osmotic and ionic regulation (Luquet et al., 2002; Pequeux and Gilles, 1981; Pequeux and Gilles, 1988; Schoffeniels and Gilles, 1970). In juveniles and adults of crustacean species, the epithelium of the gill containing mitochondrias can be involved in osmoregulation. This is supported by the increase in the size of epithelium in *Penaeus aztecus* that are transferred to low or high salinities. Similar epithelia have also been reported in the posterior gills of *E. sinensis* (Barra et al., 1983).

1.9 Significance of osmoregulation mechanism in the black tiger shrimp

As describe above, the black tiger shrimp is able to survive and grow in a wide range of salinity from 2 to 45 ppt salinities. However, extremely high or low salinity always causes more problems than the suitable salinities, which range from 15 to 25 ppt salinities. Culture in extremely high salinities over 30 ppt salinity may cause disease problems, particularly white spot or yellow head virus and luminescent bacteria. In low salinity at 3 to 8 ppt salinities, in fact that the shrimp can grow for a period of 4 months or a little bit more but no more growth appears to be significant transfer (Chanratchakool, 2003). In addition, the current domesticated broodstock after growing out of the broodstock-sized shrimp for gonad and sperm development still depends upon the stimulation of high salinity level before mating. Structural and functional characterization of salinity stress responsive genes has contributed to a better understanding of how shrimp response and adapt to salinity stress. An understanding of the osmoregulatory mechanism would be useful for the production of broodstock and shrimp farming. Moreover, genes controlling osmoregulatory

system in *P. monodon* can be applied for the selection of low-salinity tolerant shrimp strain.

1.10 Differential Display PCR (DD-PCR)

Differential display (DD-PCR) is a powerful technique that has been used to identify changes in gene expression that lead to certain phenotypes or arise in response to some external factor (Figure 1.6). Liang and Pardee first introduced differential display in 1992 and based their methodology on RAPDs. DD-PCR is an expression analysis method whereby mRNA from each sample is converted to cDNA. cDNA is then PCR-amplifed using a combination of oligo-dT and arbitrary primers. Oligo-dT primers have complementary sequences to the poly(A) tail of mRNA and the adjacent two nucleotides of the transcribed sequence. Under low stringency conditions, a secondary primer added in the reaction, resulted in amplification of double stranded cDNAs. The amplified cDNAs are separated by size comparison of mRNA expression profiles in a denaturing polyacrylamide gel and visualized using either autoradiography, silver staining or fluorography. The putative differentially expressed clones are further isolated, cloned, and sequenced to reveal identity.

The primary benefit of differential display is the sensitive mRNA display technique that enables comparative display of all transcribed genes in any cell or tissue types, and identification of cell or process-specific differentially expressed genes by comparison of reverse transcribed and arbitrarily amplified cDNAs from two or more cell or tissue types (Liang and Pardee, 1992; Liang and Pardee, 1995; McClelland et al., 1995). The use of differential display has been widespread and has revealed gene changes such as *Arabidopsis thaliana* (L.) Britton (Kreps et al., 2000), tobacco (Kimura et al., 2001), mosquito (Morlais and Severson, 2001), and humans (Doug et al., 200; Klumar et al., 2001).

The use of DD-PCR has some drawbacks. Most important among the shortcoming is the incidence of false positive (Liang et al., 1993). The false positive arise primarily as a result of the sensitivity of PCR. Because the amount of product

synthesized increase exponentially during PCR, small differences in quality or purity of the template and pipetting errors have large effects on amplification efficiency and produce large apparent difference in the yield of PCR products among samples. In order to avoid false positive, independent confirmation of differential expression patterns is necessary. DD-PCR is limited by the fact that it generates short expressed sequence tags, representing mainly the 3' untranslated region (UTR) of mRNAs. Sequencing of short non-coding 3' ends is predominantly uninformative for gene classification and prediction of function. Identification of coding sequence motifs requires screening of cDNA libraries and sequencing of full length cDNA (Liang et al., 1994; Lu et al., 2004; To, 2000).

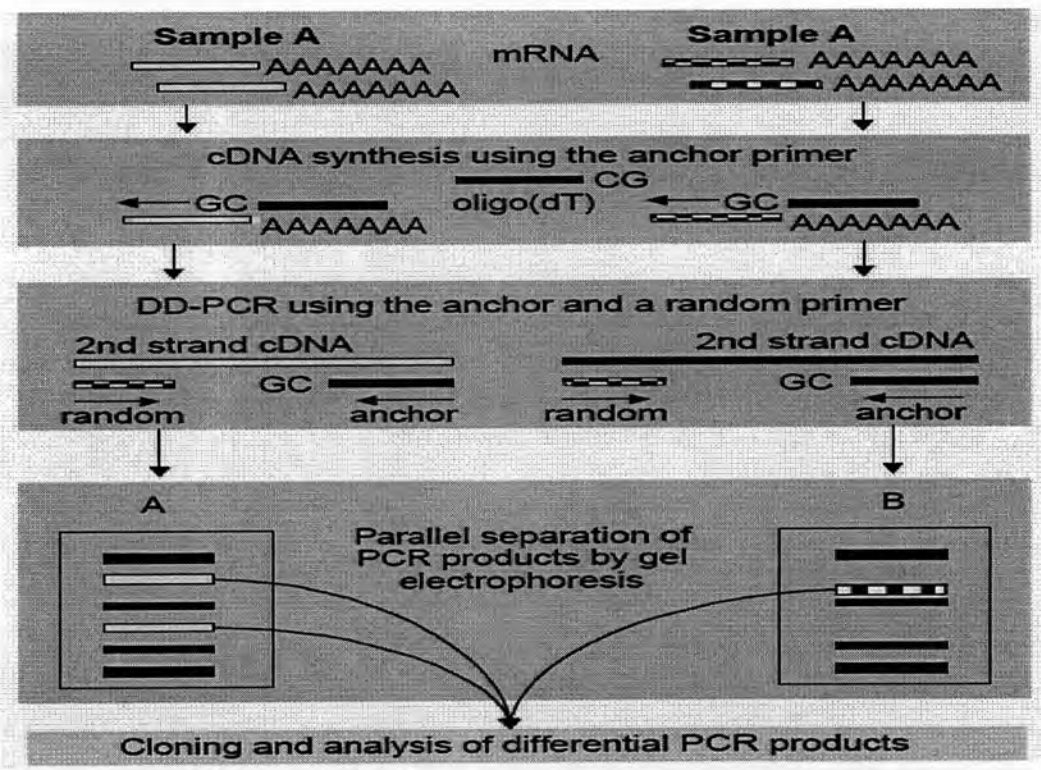

Figure 1.6 Illustration of differential display polymerase chain reaction (DD-PCR) to identify differentially expressed genes (Bals and Jany, 2001)

1.11 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a rapid and quantitative method in analyzing the level of expression of gene. It is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Carding et al., 1992; Marone et al., 2001). The RNA cannot serve as a template for PCR, reverse transcription was combined with PCR to make RNA into a complementary DNA (cDNA) suitable for PCR. The combination of both techniques is colloquially referred to as RT-PCR (Figure 1.7). The necessity to reverse transcribe mRNA into a cDNA prior to subjecting the RNA template to PCR is given by the fact that the polymerase used in PCR is a DNA-dependent polymerase. Reverse transcription of mRNA requires choosing a reverse transcriptase, a means of priming the mRNA to initiate polymerization and supplying optimal condition for the enzymatic reaction. Reverse transcriptase are RNA-dependent DNA polymerases which have been used predominantly to catalyze first strand synthesis (synthesis of a complementary DNA-cDNA), but are also capable of synthesizing a DNA strand complementary to a primed single stranded DNA. The RT-PCR can be used for semi-quantitative also known as relative or quantitative analysis.

The semi-quantitative RT-PCR method is based on the use of an internal control, which is included in the polymerase chain reaction with the gene specific primers. In the majority of cases, the internal control is a housekeeping gene expressed at a very high level, which is assumed to be expressed at a constant level throughout all samples analyzed. Also, it is assumed that the expression levels of the control RNA are not altered by the experimental conditions, thus acting as an experimental control. Common internal controls are β-actin and GAPDH mRNA and also 18s rRNA. The PCR products (including the internal control) are then separated with agarose gel electhophoresis, stained with ethidium bromide and analyzed to observe relative expression of the target transcript. However, Semi-quantitative RT-PCR is only able to tell you that one transcript is expressed at a higher level than the

other. The semi-quantitative RT-PCR method is one of generally methods that use for confirmation of differential expression from DD-PCR analysis. Since, this method is highly sensitive and specific method for the detection of rare transcript or for analysis of samples available in limiting amount.

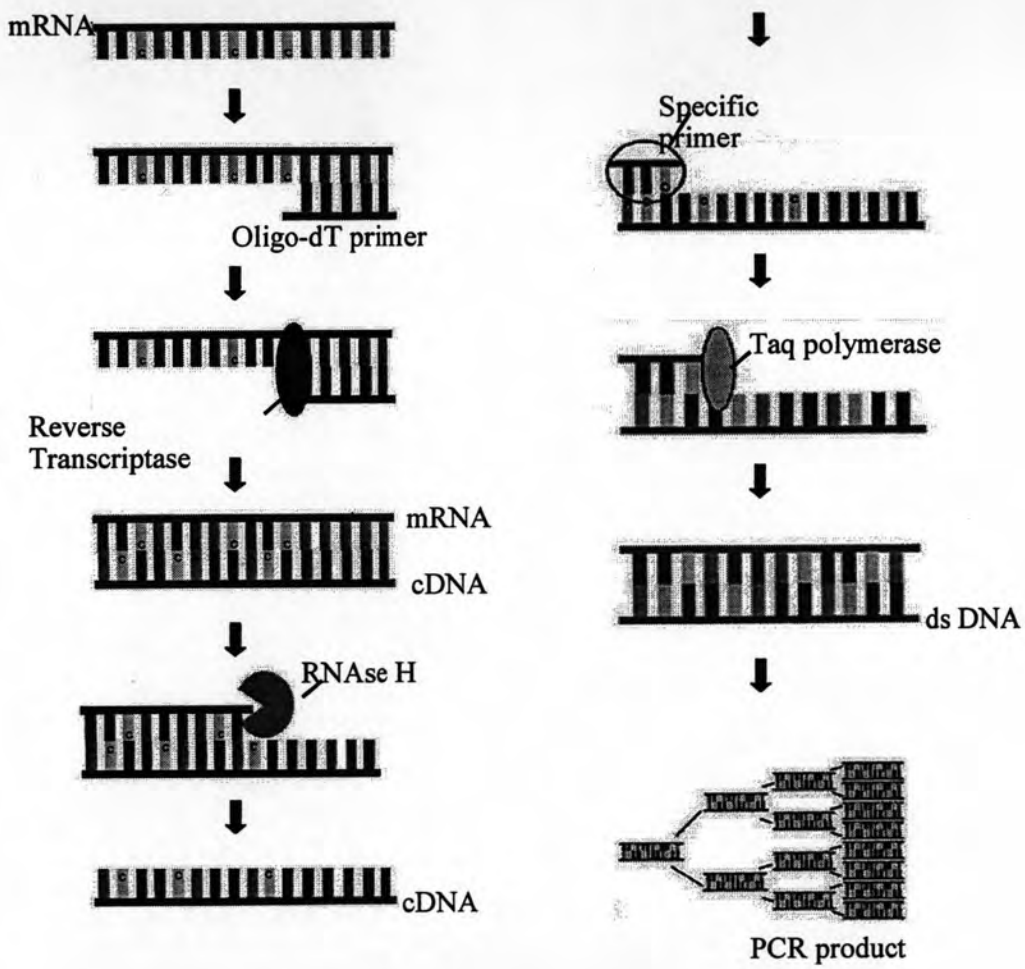

Figure 1.7 Overview of the RT-PCR technique

(http://ccm.ucdavis.edu/cpl/Tech%20updates/RT-PCR%20folder/RT-PCRweb.jpg)

1.12 Prediction of domain proteins

Predicting the function of a protein from its sequence can be a laborious process that is fraught with pitfalls associated with sequence divergence, non-identical multidomain architectures and non-equivalent functions of homologues. SMART is a simple modular architecture research tool and database that provides domain identification and annotation on the WWW (http://coot-embl-heidelberg.de/SMART). The tool compare query sequences with its database of domain sequences and multiple alignments. For each protein in the database, intrinsic features such as transmembrane region, coiled-coils, signal peptide and internal repeats are now included. TMHMM2 analytical method is now being used to predict domain transmembrane sequences, since this method demonstrates 97 to 98% accuracy for transmembrane prediction (Ponting et al., 1999; Schultz et al., 1998; Schultz et al., 2000)

Hydrophobicity (or hydrophilicity) plots are designed to display the distribution of polar and apolar residues along a protein sequence. Most commonly, such analysis has the goal of predicting membrane-spanning segments (highly hydrophobic) or regions that are likely exposed on the surface of proteins (hydrophilic domains) and therefore potentially antigenic.

To generate data for a plot, the protein sequence is scanned with a moving window of some size. At each position, the mean hydrophobic index of the amino acids within the window is calculated and that value plotted as the midpoint of the window. Several scales of hydrophobicity have been developed, most of which were derived from experimental studies on partitioning of peptides in apolar and polar solvents. One of the most commonly used hydrophobicity scales is The Kyte and Doolittle hydrophobicity/hydrophilicity plot. Kyte-Doolittle scale: Hydropathic regions achieve a positive value. Setting window size to 5-7 is suggested to be a good value for finding putative surface-exposed regions, whereas a window size of 19-21 yields a plot in which transmembrane domains stand out sharply, with values of at least 1.8 at their centers (Kyte and Doolittle, 1982).

1.13 Objectives of the thesis

The aim of this study was to identify and characterise changes in gene expression within the major osmoregulatory tissues of the black tiger shrimp, *Penaeus monodon* which enable these shrimp to make the physiological adaptations required for culture under unsuitable salinity levels. For this purpose, we employed the DD-PCR method and identified several cDNA fragments whose expressions were highly responded by salinity stress. Genes corresponding to these cDNAs fragments were confirmed by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to be expressed differentially in response to salinity stress. The salinity responsive genes identified from this study may be postulated to be involved in osmoregulation of *P. monodon*.