CHAPTER II

LITERATURE REVIEW

2.1 Taxonomy of *P. monodon*

The taxonomic definition of the black tiger shrimp, *P. monodon* is as follow (Bailey-Brook and Moss, 1992):

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Order Decapoda
Infraorder Penaeidea
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Subgenus *Penaeus*
Species *monodon*

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

2.2 Morphology

Black tiger shrimp (*P. monodon*) is the largest commercial shrimp species. The body length can reach 330 mm or more. Morphology of adult *P. monodon* as described by Anderson (1993), is composed of 2 parts, thorax (head) and abdomen (Figure 2.1). The head is covered by a single, immobile carapace, which protects the internal organs and supports muscle origins. The rostrum, extending beyone the tip of the antennular peduncle, is sigmoidal in shape. There are 7-8 dorsal and 3-4 ventral teech and curves down slightly. Rostral ridge lacks a distinct groove behind it, and the hepatic ridge is long and curved. Telson has groove but dose not has lateral spines. The eye stalks, eyes and antennae arise rostrally. The walking legs are thoracic appendages. Gills are formed sac-like outgrowths of the base of the walking legs and situated in branchial chambers on either side of the thorax. The carapace extends laterally covering the gills completely. The abdomen has the obvious segmentation of invertebrates. A pair of swimming legs (or pleopods) arises from each of the six

abdominal seagments. A tail-fan containing a telson, which beard the anus, and two uropods attached to the last abdominal degments. For *P. monodon*, carapace and abdomen have black bands giving a tiger-striped appearance to this species. Pleoplods may appear red.

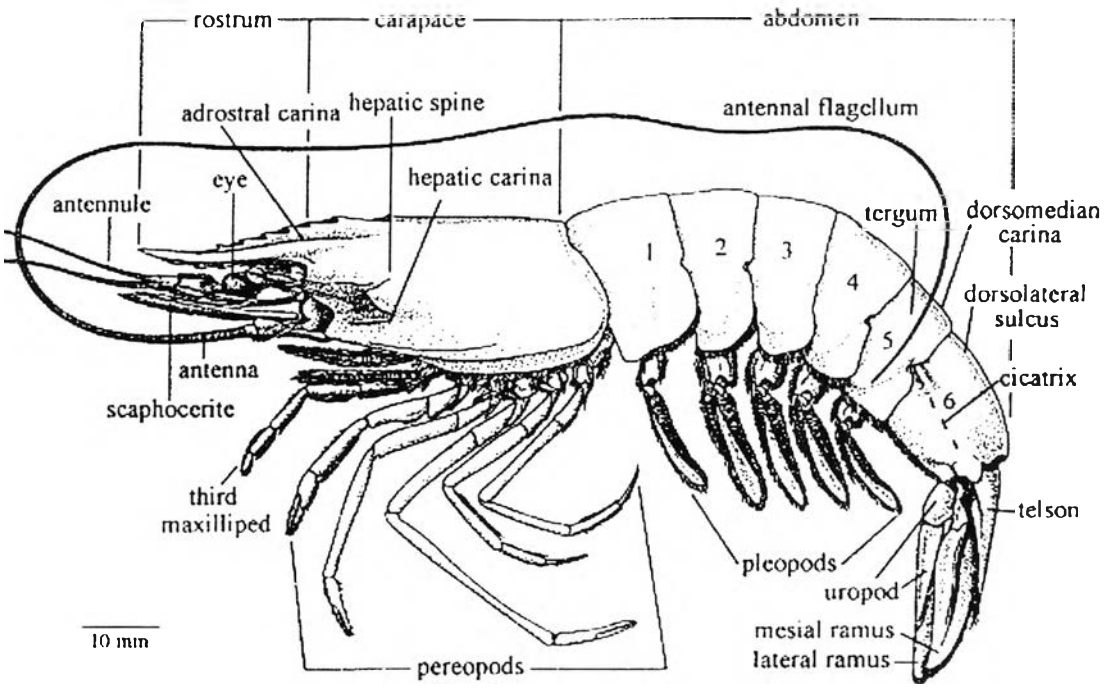

Figure 2.1 Lateral view of *P. monodon* showing important parts (Anderson, 1993)

2.3 Life cycle

Life cycle of *P. monodon* includes several distinct stages and is found in a variety of habitats. Juveniles live in brackish waters of estuaries and coastal wetlands while adults are usually found offshore with higher salinities and greater depths. Larvae inhabit plankton-rich surface waters offshore, with a subsequent onshore migration during the later stages of development (Figure 2.2). The development of *P. monodon* is complex. Larvae hatching from the fertilised eggs pass through a series of moults and metamorphic stages before becoming adult-like (juveniles). Juveniles continue growing, moulting and finally developing into adult (Bailey-Brock and Moss, 1992).

Generally, the nauplius hatches from a fertilised egg at approximately 12 hours after spawning. Larvae stages consist of three to six nauplii, three protozoea, and two or three mysis substages. The period of larval development varies depending on temperature and feeding level. It usually takes 10-14 days to complete all larval stages. The shrimps in post-larval stage have all the appendages and organs similar to adult shrimps. Shrimp larvae are naturally planktonic in behaviour. Swimming is possible using antennae in nauplii, antennae, and thoracic appendages in protozoa and thoracic appendages in mysis larvae. The first nauplii are about 0.3 mm long and are characterised by being totally planktonic and positively phototaxic. The larvae begin to feed at protozoea. They are filter-feeders and consume particles of the correct size at 8-200 μm. They are approximately 1 mm. in length, with a narrow elongate thorax and abdomen, and a loose-fitting carapace. Paired eyes, a rostrum and feeding appendages are present for the first time. The second metamorphic change is seen when the third protozoea moults into the first mysis stage. Mysids have five pairs of functioning periopods. The carapace at the third stage covers all the thoracic segments. The mysids swim in a more adult manner and actively seek out for phytoplankton and zooplankton. The final mysis larval stage metamorphoses to the post-larvae, where a full complement of functioning appendages is present (Anderson, 1993).

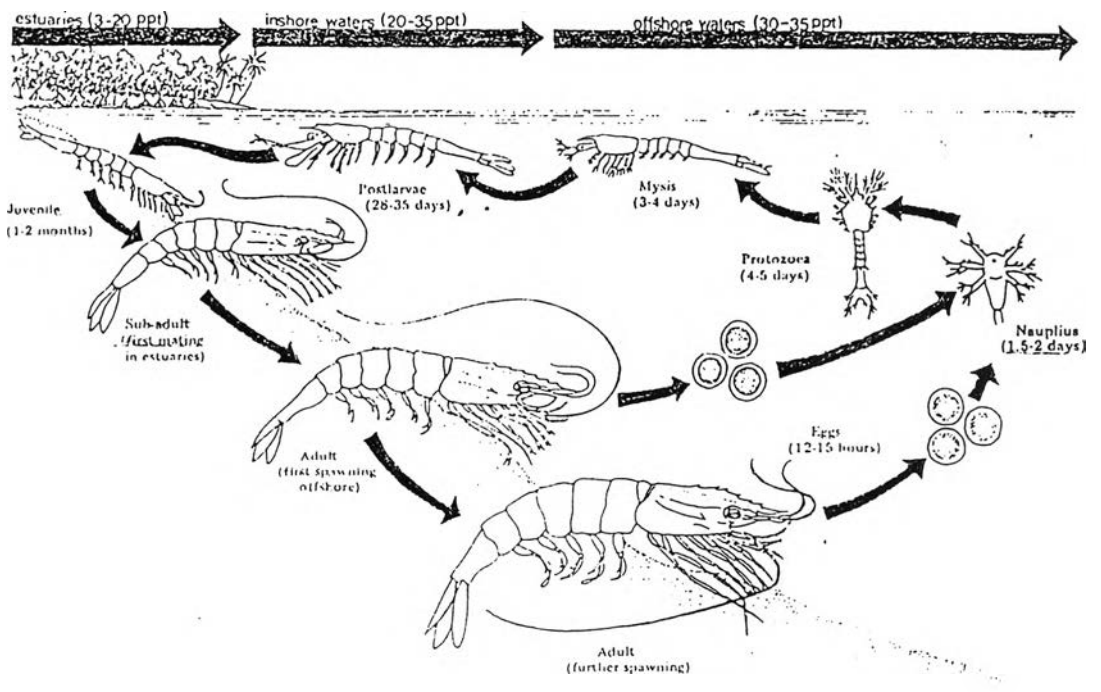

Figure 2.2 Life cycle of penaeid shrimp (Bailey-Brock and Mass, 1992)

2.4 Distribution

P. monodon is widely distributed over the major part of the Indo-West Pacific region. It is mainly found in the East and Southeast of Africa, through the Red Sea and Arabian Gulf, around the Indian subcontinent, and throughout the Malasian Archipelago to Northern Australia, and Japan (Anderson, 1993) (Figure 2.3). *P. monodon* can be caught offshore or inshore as well as from tidal zones. It is a local species in Thailand found in both sides of the Thai-Malaysian Peninsula (the Andaman and the South China Sea on the west and east coasts, respectely).

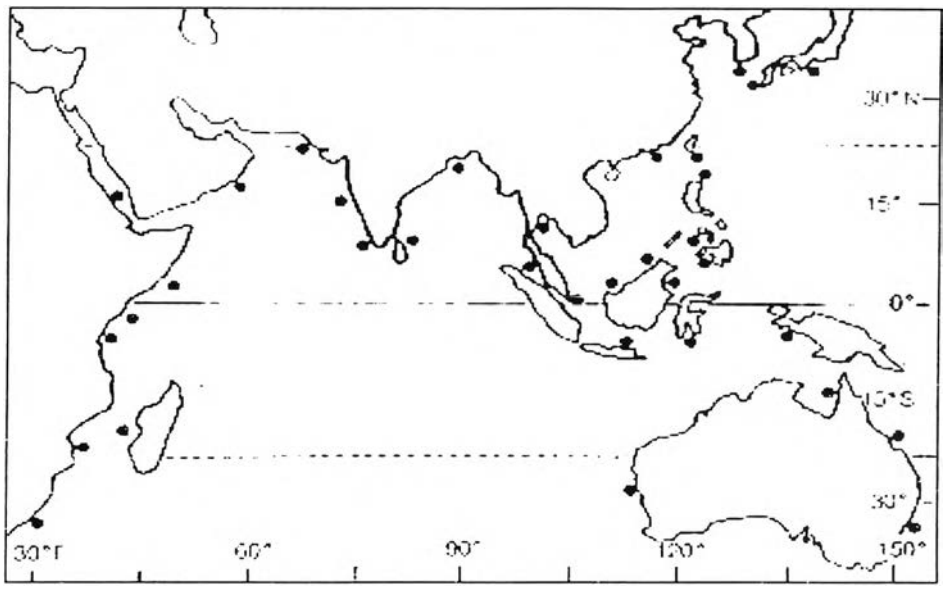

Figure 2.3 Geographic distribution of *P. monodon* (Anderson, 1993)
Black dot (•) represents main fishery areas

2.5 Diseases

Various diseases have been encountered and considerable looses have occurred because of the uncontrolled expansion of shrimp farms. Infectious disease is a primary limiting factor for the production of cultured shrimps. Various agents cause shrimp diseases including viruses, bacteria, fungi and parasites (Lighner, 1996). Among them, viruses and bacteria cause the most severe problems in Thailand and other Asian countries (Flegel *et al*, 1992). In 1996, the cost of lost production in Thailand because of virus-caused diseases was estimated to be 40% of total production (Alday-Sanz and Flegel, 1997).

2.5.1 Viral diseases

A number of important viral diseases caused loose in shrimp cultivation has been reported.

2.5.1.1 Yellow head virus (YHV)

YHV was first recognized and described from the shrimp farms in Thailand. It has been the main cause of massive losses of *P. monodon* culture in many countries (Lighner, 1996). The name "Yellow head virus" was given after the clinical occurrence of the light-yellow color appeared on the head of the infected shrimps due to the damage of the hepatopancreas. The mortality may reach as high as 100% of infected populations within 3-5 days (Limsuwan, 1991).

YHV was discovered by examination of the ultra thin sections of infected shrimp and disease-induced shrimps using electron microscopy. It was initially considered to be a granulosis-like DNA virus (Boonyyaratpalin *et al*, 1993), but latter study indicated that this virus was actually an RNA virus (Wongteerasupaya *et al*, 1995a). Based on morphology of negative stained virions and RNA content, YHV resembles rhabdoviruses (single strand plus RNA), coronaviruses or paramixovirus (single strand minus RNA). From the microscopic view, the mature virus particles are rod-shaped ranging from 150-170 nm in length and 40-50 nm in width. There is no drug, chemical or prevention method able to control this virus permanently.

2.5.1.2 White spot syndrome virus (WSSV)

WSSV is one of the virus causing serious disease outbreaks among shrimp culture in many Asian countries. It was first reported from the farmed specimens of *P. japonicus, P. monodon* and *P. penicillatus* in Taiwan in 1992 (Chou *et al*, 1995). It was then founded in China in 1993 (Flegel, 1997). In Thailand, white spot outbreaks were firstly found in the southern region where the yellow-head disease was earlier found and caused the heavily decreasing of shrimp production.

WSSV is a virus that can infect wide host species range. It has the ability to cause widespread acute epizootics within 2-7 days. The motility rate ranges from 10-70% and finally up to 100% with massive systemic pathology. Shrimp infected by WSSV showed rapid reduction in food consumption followed by general reddish coloration together with broken antennae and circumscribed whitish spots of 1-2 μm. or more in diameter in the cuticle or shell (Takahashi *et al*, 1994).

In Thailand, WSV was firstly found and characterized from the infected *P. monodon* from the shrimp farms in 1995 (Wongteerasupaya *et al*, 1995b). The results from electron microscopic study and nucleic acid content indicated that this virus was a new non-occluded baculovirus consisting of double stranded DNA of approximately 168 kb in size. Based on morphology of negative stained virions from haemolymph samples, it was revealed that intact virions were cylindrical to elliptical or obovate whose size were 121±9 nm at the widest point and 276±26 nm in length, excluding the multifillament appendages. The appendages were attached to the end of virions (Wongteerasupaya *et al*, 1995a).

2.5.1.3 Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

IHHNV was first described as the cause of acute disease and high mortality in juveniles of the American blue shrimp, *P. stylirostris* (Bell and Lighner, 1987). No specific signs can be clearly seen on the body of the shrimps infected by IHHNV. The infected shrimps can be recognized by the reduction in food consumption, followed by changes in behavior and apperance (Lighner, 1996). By contrast, IHHNV cause pathogenomic signs of disease called "runting" in the American white shrimp, *P. vannamei*. The signs included retarded growth and deformities of the rostrum. The infectious hypodermal and hematopoietic necrosis virus is an unclassified, probable picornavirus. The virions are icosahedral and small, 17-28 nm, and the inclusions or polyhedra are eosinophilic, intranuclearm single and basophilic. About 80-90% of cumulative mortalities within 2 weeks from onset of IHHNV in 0.05-1.0 g. *P. monodon* have been reported (Lightner, 1983). This virus could cause epizootics in cultures prawn populations, particularly among post-larvae and juveniles

2.5.1.4 Penaeus monodon-type baculovirus (MBV)

MBV is widely distributed in penaeid shrimps of the Eastern hemisphere (Lighner, 1996). It was first described in Thailand in 1991. The disease agent was found to be a single enveloped, rod-shaped, presumed DNA virus of the genus *Baculovirus* subgroup A. MBV occurs free or within proteinaceous polyhedral occlusion bodies in the nucleus, with nucleocapcids measuring 75±7x324±33 nm (Lightner and Lewis, 1983b).

2.5.2 Bacterial diseases

Bacteria are always present in seawater and in the shrimp ponds where they act as opportunistic pathogens, either primary or secondary invaders, to attack shrimp under stressful conditions. A number of bacterial species have been reported to cause disease in shrimps (Table 2.1) (Lightner, 1996). The most frequently reported and the most serious cause of disease was *Vibrio* species (Anderson, 1975).

Vibriosis and luminous disease

There were many reports about the damage to shrimp stocks associated with bacterial diseases. It is a serious problem in Indonesia, Malaysia and the Philippines. The diseases are caused by the infection of *Vibrio* species. *Vibrio* sp. are common marine bacteria. They can cause shrimp disease at all stages of development. The mortalities of up to 80% in hatcheries have been caused mainly by *Vibrio* sp. (Lighner, 1996). Heavily infected larvae were moribund and luminescent in the dark due to the large numbers of motile bacteria in their tissues. *Vibrio* sp. can also be isolated from active larvae in low numbers. The mass mortalities appear to occur during heavy infections. In adult shrimps, *Vibrio* infections can cause black spots on the shell and gills (Ruangpan, 1987).

Table 2.1 Bacterial diseases of *P. monodon*

Disease	Agent	Stages Affected	Pathology/Signs
1. Necrosis of appendages	Bacteria	Zoea, mysis, and post larvae	Necrosis of appendages, twisted antennae or broken setae, "browning" of exoskeleton or tip of appendages, lique faction of gut contents in zoea
2. *Vibrio* disease	*Vibrio* *V. harveyi,*	Zoea	Heavy mortalities up to 80%
3. Luminous bacterial disease	*V. splendidus*	Nauplii, zoea, mysis, and post larvae	Prawn luminescent in the dark, heavy mortalties
4. Filamentous bacterial disease	*Leucothrix mucur*	Larvae and post larvae	Bacteria on gills, setae, appendages and body surface: mortalities due to hypoxia: and imparied molting
5. Shell disease	*V. alginolyticus, V. parahaemolyticus*	Juveniles,adults	Erosion of exoskeleton

Luminous bacteria isolated from the shrimp farms include *V. fischeri*, *V. harveyi*, *V. cholerae* biotype *albensis* and *Photobacterium leiognathi*. Of these, *V. harveyi* is often claimed to be the causative agent associated with shrimp mortality (Ruangpan and Kleechaya, 2002). In grow-out ponds, luminous disease ("kung ruangsang", in Thai) can cause mortality at all stages of shrimp rearing but can be especially serious with 2-3 months old stocks.

V. harveyi is a luminous, estuarine halophilic bacteria. It can exhibit luminescence via luciferase enzyme (Bassler *et al*, 1993). Its luminescence can be seen in dead and live shrimps or seawater in disease outbreak areas on very dark nights. The shrimps infected by *V. harveyi* will have milky white bodies, weakness, swimming disorders and loss of appetite, eventually leading to death.

Vibrio spp. were commonly isolated from healthy shrimps as well as from other invertebrates, fish, sedments and the water column (Ruangpan, 1987). The environmental factors such as temperature, crowding, organic pollution and other stresses are believed to be the main risk factors to increase the possibility of it causing disease in shrimp ponds. The strains of *V. harveyi* also vary considerably in virulence. It is not easy to eliminate *Vibrio* species from shrimp ponds. Therefore, prevention based on management practices should be the appropriate method for controlling the disease.

2.6 Immune system

Immune system has developed to protect multicellular organisms from foreign substances. During evolution, two types of immune systems have developed to detect foreign substances, namely innate (natural) immunity and adaptive (acquired) immunity.

2.6.1 Innate immunity

Innate immunity refers to antigen-nonspecific defense mechanisms that a host uses immediately or within several hours after exposure to an antigen (Bauchau, 1981). Most vertebrates obtain this immunity since they were born. It is the initial and immediate response by the body to eliminate microbes and prevent infection. There is no antigen-specific recognition system. It is designed to recognize a few highly conserved structures present in many different microorganisms. The structures recognized are called pathogen-associated molecular patterns. This includes lipopolysaccharide (LPS) from the gram-negative cell wall, peptidoglycan, lipotechoic acids from the gram-positive cell wall, the sugar mannose, bacterial DNA, double-stranded RNA from viruses, and glucans from fungal cell walls. Pathogen-associated molecular patterns can also be recognized by pattern-recognition receptors on most defense cells and a series of soluble pattern-recognition receptors in the blood that function as opsonins and initiate the complement pathways. Innate immune responses involve phagocytic cells, natural killer cells and molecules such as complement proteins, acute phase proteins, and cytokines.

2.6.2 Adaptive immunity

Adaptive or acquired immunity refers to antigen-specific defense mechanisms that take several days to become protective and are designed to remove a specific antigen (Sindermann, 1971). This kind of immunity has been developed throughout life. Antigen epitopes bind to at least one of the variety of epitope-specific receptor molecules on B-lymphocytes and T-lymphocytes. With this response, the body is able to recognize any conceivable antigen it may eventually encounter.

Table 2.2 The distinguishing features of each type of immunity

Innate Immunity	Adaptive Immunity
1. Pathogen recognized by receptors encoded in the germline	1. Pathogen recognized by receptors generated randomly
2. Receptors have broad specificity, i.e., recognize many related molecular structures called PAMPs (pathogen-associated molecular patterns)	2. Receptors have very narrow specificity; i.e., recognize a particular epitope
3. PAMPs are essential polysaccharides and polynucleotides that differ little from one pathogen to another but are not found in the host	3. Most epitopes are derived from polypeptides (proteins) and reflect the individuality of the pathogen
4. Receptors are PRRs (pattern recognition receptors)	4. Receptors are B-cell (BCR) and T-cell (TCR) receptors for antigen
5. Immediate response	5. Slow (3–5 days) response (because of the need for clones of responding cells to develop
6. No memory of prior exposure	6. Memory of prior exposure

2.7 Haemocyte classification

Crustaceans have opened circulatory. The circulating haemocytes of crustacean are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Johansson and Söderhäll, 1989). The number of haemocytes can decrease dramatically during an infection. Haemocytes in crustaceans can be classified into 3 morphologically different types: hyaline, semigranular, and granular cells. These different haemocyte types carry out different functions in immunity (Johansson *et al*, 2000).

Hyaline cell

Hyaline cells are found only 1% of the total haemocytes (Iwanaga and Kawabata, 1998). No cytoplasmic granules are present. This type of haemocyte is involved in clotting process.

Semigranular cell

Semigranular cell is the most abundant type of haemocytes. It contains a variable number (1-40) of small (S) granules (0.4 μm diameter). This haemocyte responds to some phagocytosis and encapsulation (Persson *et al*, 1987). S-granules contain at least 6 proteins with molecular masses of less than 30 kDa, in addition to an antimicrobial peptide tachyplesin and its analogues (Shigenaga *et al*, 1993).

Granular cell

Granular cells contain a large number of secretory large (L) granules (0.8 μm diameter). This haemocytes are involved in phagocytosis, encapsulation and the clotting process. L-granules contain at least 24 proteins, a majority of which are clotting factors, serpins, and various lectins (Hose and Martin, 1989).

2.8 Immune system in crustaceans

Similar to most of Invertebrates, crustaceans comprise a diverse group of animals. Their internal defenses are believed to be the evolutionary precursors of those in the vertebrates. It was well documented that crustaceans do not possess adaptive immunity as in vertebrates. However, numerous species of crustacean success as survivors in pathogen-rich or hostile environments, indicating the capacity

of defense system to efficiently combat infectious organisms. Generally, 2 types of immune systems in crustaceans were classified. This includes cellular and humoral immunities (Smith and Chisholm, 1997).

2.8.1 Cellular immunity

The cell-mediated immunity of crustaceans involves a number of responses including haemolymph coagulation and clotting, phagocytosis, encapsulation and cytotoxic interactions (Ratchliffe, 1985).

2.8.1.1 Coagulation and blood cell clotting

This process occurs to prevent any invasion and the loss of body fluids once the physico-chemical barriers have been destroyed. During wound healing, the injury is sealed by a number of reaction including extrusion of the fat body or other organs, haemolymph coagulation and/or melanin deposition, followed by the haemocyte migration and aggregation at the wound site. Finally, a new cuticle, ectoderm, or epithelium can be formed. Coagulation or clotting mechanisms have been characterized into 2 systems in molecular detail; 1) the haemocyte-derived clotting cascade in horseshore crab, *Tachypleus tridentatus* (Kawabata *et al*, 1996), and 2) the transglutaminase (TGase)-dependent clotting reaction in crayfish, *Pacifastacus leniusculus* (Hall *et al*, 1999).

All the proteins participating in the horseshore crab clotting system reside in the haemocytes. Upon activation, they are released from the cytoplasmic L-granules into the haemolymph through rapid exocytosis (Kawabata *et al*, 1996). The microbial cell wall components activate factor C and G, respectively, which results in subsequent activation of proclotting enzyme and the resulting clotting enzyme catalyses the conversion of a soluble protein (coagulogen) into an insoluble aggregate (coagulin) (Iwanaga, 1993).

2.8.1.2 Phagocytosis and Encapsulation

Phagocytosis occurs by the internalization of pathogen into phagosomes. Phagocytosis is one of the most important cellular defense mechanisms in crustaceans. It is considered to be a non-specific cellular response, dependent upon recognition of physical differences between host cell and those of the pathogens. However, a certain degree of specificity in phagocytosis has been reported for many

invertebrates in that different types of foreign particles cause different rates of phagocytic action. Degranulation of granular cells occurred after phagocytois of foreign particles. The release of hydrolytic enzymes from the granular cells leads to break down of the phagocytosed particles. An *in vitro* study of shrimp haemocyte function in the ridgeback prawn, *Sicyonia ingentis* (Hose and Martin, 1989), showed that hyaline cells responded to foreign material by coagulation, while granulacytes responded by phagocytsis and encapsulation. Following the degranulation, hyaline cells become the majority in the circular system.

Encapsulation is a mechanism for a large foreign particle. It occurs when the invaders are too big to be ingested by a single blood cell. The process was initiated by a large number of haemocytes attaching to the foreign surface and forming a multicellular sheath to engulf the whole foreign body. Humoral factors are expected to been involved in the initiation process during the attachment. This non-specific recognition has also been demonstrated in the encapsulation process of the moth, *Heliothis virescens*. Thicker encapsulated layers of haemocytes were found around neutral beads. Thinner layers were found with weakly basic and weakly acidic beads.

Phagocytosis and encapsulation are the effective defenses in crustaceans. In lower animals without circulatory systems, the phagocytosis action of fixed cells seems to play an important role in defense (Lackie, 1981).

2.8.1.3 The prophenoloxidase (proPO) system

Cellular activities in crustaceans e.g. phagocytosis, coagulation, encapsulation and microbial killing appear to be influenced by products of the prophenoloxisase activationg (proPO) system (Söderhäll and Cerenius, 1998). These products comprise opsonic, cytotoxic, fungicidal and cell encapsulation promoting activities. There are some evidence that this system is involved in non-self recognition and cellular communication.

The proPO activating system consists of several proteins involved in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Sritunyalucksana and Söderhäll, 2000). It is an efficient immune system for non-self recognition and is initiated by recognition of lipopolysaccharides or peptideoglycans from bacteria and β-1,3-glucans from fungi. This system contains a proteinase cascade compose of pattern-recognition proteins

(PRPs), several zymogenic proteinases, and proPO. The activation of the proPO cascade is exerted by extremely low quantities of microbial cell wall components, resulting in limited proteolysis of proPO to the active phonoloxidase (PO). PO is a bifunctional copper-containing, also known as tyrosinase, catalyses two successive reaction: hydroxylation of a monophenol to o-diphenol (monophenoloxidase activity) and the oxidation of the o-diphenol to o-quinone (diphenoloxidase activity) (Decker and Tuczek, 2000). Production of o-quinones by PO is an initial step in the biochemical cascade of melanin biosynthesis. The production of melanin pigment can often be seen as dark spots in the cuticle of arthropods involving in the process of sclerotisation, wound healing and encapsulation of foreign materials. Several components and associated factors of the proPO system have been found to play several important roles in the defense reaction of the freshwater crayfish (Söderhäll and Cerenius, 1998).

Studies on shrimp proPO system have been carried out in Penaeid shrimps including *P. californiensis* (Vargas-Albores, 1993), *P. panlensis* (Perazzolo and Barracco, 1997), *P. stylirostris* and *P. monodon* (Sritunyalucksana *et al*, 1999). Shrimp proPO is synthesized in the haemocytes and not in the hepatopancreases. By comparison of amino acid sequences, shrimp proPO is more closely related to crayfish proPO than to the insect proPO. The conversion of inactive proPO to PO is by a serine protease named the prophenoloxidase activating enzyme (ppA). This enzyme has been isolated in several insects (Satoh *et al*, 1999) and from a crayfish haemocyte lysate. It was shown in crayfish that only ppA enzyme is sufficient for the activation of proPO.

2.8.2 Humoral immunity

Humoral defense factors have also been reported in crustaceans. Some reports showed that humoral factors could speed up and increase the effectiveness of phagocytic action in an individual animal (Cornick and Stewart, 1968a). Agglutinins have been widely investigated and have attracted most attention. These molecules are recognized to be lectin-like substances (Sharon and Lis, 1972). It is commonly agreed that invertebrates, including crustaceans do not posses immunoglobulins (Igs), the specific recognition molecules in vertebrate immunity. Nevertheless, it has long been demonstrated that crustaceans have the capability to discriminate foreigness from self

recognition. Humoral factors in the body fluid of crustaceans include agglutinins, clotting proteins, antimicrobial peptides and precipitins (Ratcliffe, 1985).

2.8.2.1 Antimicrobial proteins

Antimicrobial peptides are major components of innate immunity that have been conserved in evolution and found in different phyla of the plant and animal kingdom. Although these immune effectors share common characteristics and similarities in structural patterns or motifs (Bulet *et al*, 1999), one striking feature is their great diversity in term of amino acid sequences, antimicrobial activities and modes of action. Moreover, depending on their distribution, antimicrobial peptide expression appears to be regulated by different tissue-specific pathways and these effectors may consequently participate in either a local or a systemic reaction. Antimicrobial peptides are classified into 4 distinct groups base on amino acid sequences, secondary structures and functional similarities: i) linear basic peptides forming amphipathic α–helices which are devoid of cysteine residues including the cecropins; ii) peptides with one to six intramolecular disulfide bridges including the arthropod defensins, antifungal peptides from *Drosophila*, drosomycin and metchnikowin, thanatin from *Podisus*, anti-LPS factor, tachyplesin, big defensin and tachycitin from *Limulus*; iii) proline-rich peptides such as the apidaecins or drosocin; iv) glycine-rich peptides or polypeptides such as attacins, diptericin, and sarcotoxins (Barundes, 1988).

For many of these peptides, there was evidence indicated that one of the targets for the peptide was the lipid bilayer of the membrane. This was because these peptides could often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides were cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial menbranes. With regard to the mechanism by which the peptide breaks down the membrane permeabilty barrier, it is possible that the peptide induces complete lysis of the organism by rupture of the membrane or that it perturbs the membrane lipid bilayer which allows for leakage of certain cellular components as well as dissipating the electrical potential of the membrane (Kamiya *et al*, 1987).

2.8.2.2 Pattern recognition proteins

Pattern recognition proteins (PRPs) have been isolated and characterized in several invertebrates. These PRPs are recognition and response to microbial invaders by the presence of signature molecules on the surface of the intruders. Some of them contain common motifs for example, bacterial glucanase-like (Lee *et al*, 2001), bacteriophage lysozyme-like (Ochiai and Ashida, 1999), and immunoglobulin-like motif in their primary structures. Some of them are agglutinins or lectins that have the ability to bind to specific carbohydrates expressed on different cell surfaces (Vasgas-Albores *et al*, 1996). Due to the fact that they are in general at least bivalent, they can bind cells and an agglutination reaction occurs. Lectins have the ability to bind carbohydrate and promote the agglutination of different cells, such as bacteria and other invading pathogens. It is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self-recognition reactions. As with vertebrate immunoglobulins, they can agglutinate microorganisms and enhance their phagocytosis by mediating binding between the haemocyte surface and a foreign body, and are apparently synthesised by invertebrate immune cells.

The surface recognizing protein detected in arthropod plasma has the capability to react with β-1,3-glucan, and therefore, it is named β-glucan binding protein or BGBP. β-1,3-glucan is a major cell wall component of fungi. Although BGBPs have glucanase-like motif, none has been shown to contain glucanase activity suggesting that the BGBPs developed from a primitive glucanase and then evolved into proteins without glucanase activity but instead bind glucans. After binding, it operates as elicitors of defense responses. The activation of this zymogen triggers the clotting cascades, resulting finally in the conversion of coagulogen to an insoluble coagulin gel (Seki *et al*, 1994). Thus, the invaders in the haemolymph are engulfed and immobilized by the clot, and subsequently killed by antimicrobial substances that are also released from the two types of granules.

Table 2.3 Humural factor immune response in crustaceans (Smith and Chisholm, 1997)

Factor	Function	In crustraceans
Agglutinins	Aggregate foreigh particles. Include bacterial agglutinins, haemagglutinins and/or lectins	Appear to aid seqeustration of infective agents but little evidence for a role in recognition
Cytotoxic agents	Destroy cells	Crayfish granular cells are cytotoxic for normal and tumour vertebrate cells
Precipitins Cytokinase	Sequester soluble 'antigen' from blood Non-antibody proteins with diverse immunological and homeostatic functions. Produced by blood cells	Factor associated with proPO activation influence exocytosis, phagocytosis and cell adhesion in decapods
Modulators	Regulate the activity of immunological aggressive molecules	Macroglobulin cages proteinases and is found in crayfish plasma
Clotting factors	Prevent blood loss and seal wounds	Involve plasma gelation as well as cell aggregation
Recognition factors	Bind specifically to non-self molecules and trigger cell response	ProPo factors released from cells. β-1,3-glucan-binding factors found in crayfish

2.9 Stress

2.9.1 Stress condition

Aquatic and marine organisms are exposed to a series of variable factors, such as large alterations in temperature, availability of nutrients and water, and the presence of toxic molecules that originate from their abiotic and biotic environments. These factors can make their living conditions far from optimal. Survival in this changing environment requires a wide range of fast and adaptive responses. In the majority of cases, the response leads to transcriptional activation of genes whose

products cope with a given physico-chemical stress. Stress has been defined as the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state. Stress activation is regarded as an essential element in the total adaptive system of the organisms. There is a consensus that the stress response affects most other physiological systems, endocrine systems, autonomic systems, and immune systems, as well as the biochemistry of the brain.

2.9.2 Stressors

The potential stressors are grouped as being environmental, physical, or biological (Barton and Iwama, 1991). Many stressors are unique to certain species or geographic areas. There are differences in the generalized stress response among different species, and different stocks or races of the same species differ in their tolerance to applied stressors. The observed stress response is therefore an expression of both genetic and environmental factors such as season, rearing history, and nutritional state (Iwama, 1998).

Environmental stressors

Environmental stressors mainly include adverse physical and chemical conditions of the water. Extreme conditions or changes in water quality such as dissolved oxygen, ammonia, hardness, pH, gas content, partial pressures, and temperature can induce stresses. Metals (e.g., copper, cadmium, zinc, and iron) and other contaminants (e.g., arsenic, chlorine, cyanide, various phenols, and polychlorinated biphenyls) in the water can cause severe stress and death. Other potential environmental stressors include insecticides, herbicides, fungicides, and defoliants. Industrial, domestic, and agricultural activities add much of these contaminants to the environment that affect animals at all life stages. Natural changes in water quality, as occurs during low tide in tidepools, may stress the organisms that live in such environments.

Physiological stressors

Physiological stressors encompass a wide range of potential stressors including the physical disturbances such as handling, crowding, confinement, and transport. Other forms of physical disturbance to animals also have psychological

components. Many of these are practiced in the intensive culture of organisms for both wild stock enhancement, and for the commercial production of food. Chasing animals to exhaustion, or holding them in a net out of water for 30-60 seconds have been common protocols utilized to study acute stress responses (Aldo *et al*, 2000).

Pathogenic stressors

Pathogens and parasites can also be considered as biological stressors. Diseases, and outbreaks leading to massive mortalities, occur in nature as well as in cultured stocks. Plankton blooms can stress and kill animals in the wild as well as in aquaculture facilitiess. The plankton may kill directly by their toxins, irritate or severely damage the gill epithelium with their spines, or kill indirectly by hypoxic conditions by either lowering water oxygen levels directly or by increasing the diffusion distance between blood and water through the stimulation of mucus production on the gill surface.

2.9.3 Stress responses

There is also reasonable agreement that the response occurs whenever there is a discrepancy between what the organism is expecting, and what really exists. Therefore, stress response is an alarm of something missing or a homeostatic imbalance occurring (Levine and Ursin, 1991). The response is uncomfortable and drives the organism to provide specific solutions to abolish the source of the alarm, as well as the alarm itself. The stress response is an optimal physiological response, where physiological resources are mobilized to improve performance. The response is regarded as a positive and desirable alarm response.

The response to the stressors involves all levels of organization, from the cell to the individual organism and to the structure of the population (Barton and Iwama, 1991).

2.9.4 Behavioral stress response

Behavioral response is the immediate signs of a stress. Activities such as food acquisition, predator avoidance, prey capture, migration, and habitat preference are critical to the survival of the organism and thus the population. Alterations may take minutes to weeks to return to pre-stress conditions, depending on the nature and

magnitude of the stressor. Behaviors that are most important to the survival of the organism tend to return to normal in the shortest time (Schreck *et al*, 1997).

It may be intuitive that appropriate behavioral responses to the perception of a stressor will lessen the potential magnitude of that stressor and will increase chances of survival for that individual. The appropriate response may be to avoid the stressor. However, if avoidance or behavioral mitigation is not possible, induced changes in behavior may then reflect deleterious changes in how an animal senses and respond to its environment. Behavioral and physiological responses to a stressor are intimately related. The adaptive behavioral response to a stressor may lessen the energetic demand on the physiological systems that must respond to it. The physiological stress response serves to maintain, direct, and possibly limit the behavioral response (Iwama, 1998).

2.9.5 Physiological Stress Response

In response to a stressor such as handling or crowding, animals will undergo a series of biochemical and physiological changes in an attempt to compensate for the challenge imposed upon it and, thereby, cope with the stress. The stress response has been broadly categorized into the primary, secondary, and tertiary responses (Mazeaud *et al*, 1977 and Wedemeyer *et al*, 1990).

2.9.5.1 Primary Response

This initial response represents the preception of an altered state and initiates a neuroendocrine/endocrine response that forms part of the generalized stress response. This response includes the rapid release of stress hormones, such as catecholamines and cortisol, into the circulation. Adrenaline is released from the chromaffin tissue in the head kidney, and also from the endings of adrenergic nerves. In the plasma of salmonids, the levels of adrenaline concentration in the resting condition was less than 3 nmol/L and raised up to 20-70 nmol/L in stressed condition, while cortisol concentration in resting condition was less than 10 nmol/L and increased up to 40-200 nmol/L in stressed condition. A recent study showed that adrenocorticotrophic hormone (ACTH) might also stimulate adrenaline release, and that chronic cortisol treatment might affect catecholamine storage and release in trout (Reid *et al*, 1996). Catecholamines are released from the chromaffin tissue situated in the head kidney of teleosts and also from the endings of adrenergic nerves. Cortisol is released from the

interrenal tissue, located in the head (anterior) kidney, in response to several pituitary hormones, but most potently to ACTH. ACTH may also stimulate adrenaline release and that chronic cortisol treatment may affect catecholamine storage and release. The resting and stressed levels of adrenaline, cortisol, and glucose concentrations in the plasma of fish are used as an indicator of stressed states in fish. It is probably the most commonly measured secondary change that occurs during the stress response in fish.

2.9.5.2 Secondary Response

The seconday response comprises the several of biochemical and a physiological adjustment associated with stress, and is mediated to some extent by the stress hormones. Adrenaline and cortisol activate a number of metabolic pathways that result in alterations in blood chemistry and haematology. Stress is an energy demanding process and the animal mobilizes energy substrates to cope with stress metabolically. The production of glucose with stress assists the animal by providing energy substrates to tissues such as the brain, gills, and muscles, in order to cope with the increased energy demand. The stress hormones adrenaline and cortisol have been shown to increase glucose production in fish, by both gluconeogenesis and glycogenolysis, and they are likely to play an important role in the stress-associated increase in plasma glucose concentration. The rearing history of the fish, including nutritional state, can affect the stress response and glucose clearance rates. Thus, plasma glucose levels may or may not remain elevated despite the continued presence of the stressor.

2.9.5.3 Tertiary Response

This response represents whole-animal- and population-level changes associated with stress. If the fish is unable to acclimate or adapt to the stressor, whole-animal changes may occur as a result of energy repartitioning by diverting energy substrates to cope with the enhanced energy demand associated with stress and away from vital life processes such as reproduction and anabolic processes such as growth. Decreased recruitment and productivity may alter community species abundance and diversity.

2.9.6 Energy Metabolism and Stress

Stress is an energy-demanding process. Animal has to mobilize energy substrates to metabolically cope with stress. The most common indicator of metabolic effects due to stress is the increase in plasma glucose concentration. The plasma glucose concentration in circulation is dependent on glucose production and its clearance from the circulation. The production of glucose with stress assists the animal by providing energy substrates to tissues such as brain, gills and muscles in order to cope with the increased energy demand. Liver is the main source of glucose production and is achieved by glycogenolysis and/or gluconeogenesis. Adrenaline and cortisol increase glucose production in fish and play an important role in the stress-associated increase in plasma glucose concentration. Adrenaline is cleared rapidly from circulation after a stressor (<30 minute), but plasma glucose remains elevated for longer periods of time. Cortisol, which remains elevated for a longer period of time, plays a role in the long-term maintenance of glucose post-stress in fish.

2.9.7 Cellular stress response

The cellular stress response is characterized by the reversible increase in the concentration of a family of highly conserved proteins, referred to as the heat shock proteins, in the stressed cells. Gene regulators respond to specific signals (such as environmental and cellular signals) by stimulating or inhibiting transcription, translation or some other event in gene expression, so that the rate of synthesis of gene products is appropriately modified.

2.9.8 Stress or heat shock proteins

The major responses of organisms to acute heat shock (and other physical stressors) are well known to involve changes in gene transcription and translation of stress proteins or heat shock proteins (HSPs). The precise temperature required to initiate this heat shock response varies considerably from organism to organism but is usually 5 to 10°C above the appropriate ambient temperature. Analysis of the HSP genes shows that there are 3 major families in addition to several minute or species. This includes HSP90 (85-90 kDa), HSP70 (68-72 kDa), HSP60 (GroEL, 60 kDa), and a low molecular weight series of HSP20-30 (20-30 kDa) (Feder and Hofmann, 1999). In most organisms, HSP90, HSP70, and HSP60 predominate while a single low molecular weight or group of low molecular weight proteins (HSP20-30) is also

commonly found. Ubiquitin, a protein of 80 kDa, is also a HSP. The HSP70 genes form a multigene family with several closely related members displaying varying degrees of constitutive synthesis, heat-enhancibility and heat inducibility. The structures and sequences of the HSP90, HSP70 and HSP60 show strong evolutionary conservation while the low molecular weight proteins are less well conserved. In many cases, non-HSP synthesis is suppressed at the elevated temperature. HSPs may be the only proteins synthesised for several hours post-heat shock. Thus both transcriptional and translational control mechanisms operate to produce dramatic changes in the patterns of gene expression and protein synthesis at elevated temperatures.

HSPs act as molecular chaperones, promoting the initial folding of other proteins at the ribosome and the refolding of unfolded proteins when they are partially denatured (Nover *et al*, 1984). Environmental stresses, such as changes in temperature (Hofmann and Somero, 1996), hypoxia (Ropp *et al,* 1983), salinity (Gonzalez and Bradley, 1994), and metal ion concentration (Steinert and Pickwell, 1988, Ryan and Hightower, 1994), can all induce the synthesis of HSP that act to prevent protein aggregation and to maintain functional conformations. A wide range of organic compounds, purportedly causing cellular oxidative stresses, may also induce HSPs level changes in many organisms. The recovery from heat shock and other stress events requires the protein folding abilities of HSPs in all eukaryotes (Feder and Hofmann, 1999). Members of the HSP70 and HSP90 stress protein families (and possibly others) are also involved in the stabilization of different types of intracellular receptors. For instance, both HSP70 and HSP90 are required for stable steroid hormone receptor complexes (Hutchinson *et al*, 1994). HSP90 also binds to and stabilizes the aryl hydrocarbon receptor protein in a state capable of interacting with its substrates (Whitelaw *et al*, 1995).

Thermal stress causes damage to most macromolecular structures e.g. DNA (depurination), membranes (changes in fluidity) and proteins (denaturation). Quantitatively, the unfolding and disruption of proteins and protein-containing structures are thought to be the most important clues to the function of the HSPs which have come from immunological and sequence comparisons between them and proteins of known functions. A constitutive, non-heat inducible relative of the HSP70 proteins called HSC70 (heat shock cognate 70) has been found to be identical to uncoating ATPase, a protein which dismantles the clathrin-coated vesicles produced

by receptor-mediated endocytosis (Ungewickell, 1985, Chappell *et al*, 1986). HSP70 is also closely related to a glucose-regulated protein GRP78, which seems to be responsible for preventing the aggregation of nascent under-glycosylated proteins in the endoplasmic reticulum under conditions of glucose starvation. This latter protein is itself identical to a protein that binds to immunoglobulin heavy chains in pre-β cells to prevent self-association (Munro and Pelham, 1986). Equivalents of the prokaryotic HSPs (GroE) act to promote the correct assembly of multisubunit protein structures in mitochondria and chloroplasts. They have been termed 'chaperonins' (Hemmingsen *et al*, 1988). Hence the function of at least one group of heat shock related proteins is in the assembly and/or disassembly of protein complexes in unstressed cells. It has, therefore, been suggested that the heat-induced proteins are the responses to an increased requirement for the disruption and subsequent correct refolding of protein aggregates which spontaneously form from denatured polypeptide chains, i.e. they are part of a protein-repair mechanism. One area where this seems to be of particular importance is in the nucleolus, the site of assembly of nascent ribosomes and the site of accumulation of HSP70 after heat shock (Pelham, 1984).

The increased requirement for the other HSPs in stressed cells is still unclear. HSP90 is a component of most steroid receptors and may be involved in the transport or regulation of certain protein tyrosine kinases. The low molecular weight HSPs have a predominantly cytoplasmic location in proximity to the golgi apparatus before heat shock but form large nuclear aggregates after stress. It has been suggested that they may play a role in protein trafficking or secretion (Arrigo *et al*, 1988).

2.9.9 Heat shock proteins in crustaceans

Until recently, an evaluation of the role of the heat shock response and HSPs in the survival of crustaceans during cyclical temperature variations could not properly be made since no such studies had been reported on crustaceans in the literature. However, comparative studies of the thermotolerance and heat shock responses of the stress-resistant embryonic cysts and nauplius larvae of the brine shrimp *Artemia* have now indicated that, this crustacean at least exhibits a fairly classical heat shock response and possesses HSPs typical of the major classes described above (Miller and McLennan, 1986, 1987, 1988 a, b).

One-dimensional SDS-polyacrylamide gel analysis of radiolabelled proteins extracted from heat shock larvae of various developmental stages shows a temperature dependent increase in the synthesis of two major protein species, HSP89 and HSP68, between 28 and 40°C. A 5 minute heat shock at 40°C was sufficient to enhance the expression of both proteins, particularly HSP89, with maximal induction occurring after 15 minutes at this temperature. Longer period (e.g. 60 minutes) at 40°C caused complete repression of non-HSP synthesis. Embryonic cysts show higher constitutive levels of HSP89 and HSP68 synthesis at 28°C compared to larvae but enhance this synthesis up to 40°C. However, normal protein synthesis in cysts was not repressed relative to HSP synthesis until 47°C. This behaviour wass reminiscent of what was observed in cells with acquired thermotolerance and reflected the natural thermotolerance of cysts relative to larvae. Two-dimensional SDS-polyacrylamide gel analysis of these extracts coupled to immunoblotting with an anti-chick HSP70 antibody shows that HSP68 comprises inducible HSP68 and HSP70 isoforms and constitutive HSC70. The HSP68 isoforms were absent from cysts but were induced upon heat shock. Larvae constitutively express some HSP68 isoforms but these were enhanced considerably by heat. The HSP70 proteins showed a variable pattern of stage specific and heat activation. A low molecular weight HSP, HSP31, was constitutively synthesised by cysts but is strictly heat-inducible in larvae. The behaviour of this protein most closely reflects the constitutive and inducible thermotolerance of cysts and larvae respectively and it might possibly have a role to play in this phenomenon. The existence of these proteins in *Artemia* made it extremely likely that all other crustaceans possessed them too and were, therefore, capable of mounting a comparable response to elevated temperatures.

In view of the simultaneous development of thermotolerance and the induction of the HSPs which occurs in the majority of cases, it has always been tempting to conclude that the HSPs have an important role to play in this phenomenon. However, there has been no convincing evidence to implicate any of the major classes of HSPs in the increased survival of the thermotolerant cell after heat shock (Landry *et al*, 1987). Most compelling of all are the demonstrations that mutants of all the major yeast HSPs classes show no alterations in the kinetics of thermotolerance induction (Craig and Jacobsen, 1984). These and other results suggest that the primary role of the HSPs is the recovery of both the cell and the whole organism from thermal damage, such as the recovery of DNA replication and the repair or removal of heat

denatured proteins and protein complexes, rather than in the protection of the cell from further damage. Nevertheless, HSPs appear to be important to the thermotolerant cells when aspects other than simple survival at elevated temperatures are considered, e.g. stabilisation of the translational apparatus (Mizzen and Welch, 1988).

2.9.10 Enhancement of Stress tolerance

Different taxa of organisms have different tolerances to stress. This implies that for a particular stressor, severity may vary depending on the species to which it was applied. Species may differ in the nature of their physiological response and reproductive consequences to stressors. Strategies for coping with stress affect reproductive fitness either in terms of gamete or progeny quality. The physiology associated with maturation and spawning appears tightly coupled with stress physiology. Environmental variables, particularly nutrition, are ultimately important in affecting gamete quality and reproductive timing. The physiological response to stressors is also quite polymorphic, within and between species. For example, the circulating concentration of the primary stress response factor cortisol varies greatly among resting and among stressed rainbow trout stocks.

Immunocapacity can be influenced by stress, reducing reproductive fitness of broodstocks. Effects of nutritional stressors are moderated by the effects on timing of first maturity or subsequent reproductive events and/or by maintenance the quality of some eggs via atresia of others. Barriers to vertical transmission of numerous pathogens seem to exist, while maternally derived immune protection is provided to assist with disease prevention of pathogenic organisms acquired from parents or by direct post-spawning infection.

Heat-induced protein denaturation and aggregation are supposed to be the cause of heat-induced cell death (Kampinga, 1993, Parsell and Lindquist, 1993). Cells exposed to a priming heat shock become more resistant to a challenging heat shock. This thermotolerant state correlates with an increase in the expression of HSPs (Parsell and Lindquist, 1994, Li *et al*, 1995). The best evidence for a link between (reduced) protein damage, HSPs expression, and thermotolerance comes from the data in yeast. A functional HSP104 was found to be essential for thermotolerance and repair of protein damage (Parsell *et al*, 1994, Lindquist and Kim, 1996).

In mammalian cells, protection against protein damage by HSPs and cellular resistance to heat also seem to be linked. Overexpression of HSP70 attenuates heat

induced protein damage (Stege *et al*, 1994) and increased cell survival after heat shock (Li *et al*, 1991, Stege *et al*, 1994, Nollen *et al*, 1999). Interestingly, inhibition of protein synthesis by cycloheximide (CHX) and puromycin (PUR) also conferred heat resistance but without an increase in the expression of HSPs (Lee *et al*, 1987).

2.10 Molecular techniques for the determination of gene expression

2.10.1 Differential Display Polymerase Chain Reaction

Differential display-polymerase chain reaction (DD-PCR) was first described by Liang and Pardee in 1992 as a method for analyzing gene expression in eukaryotic cells and tissues. DD-PCR has been widely applied to study changes in mRNA expression induced by temporal development, diseases, and various cellular factors. This simple and powerful technique simultaneously screens for both up-regulated and down-regulated transcripts in multiple cell populations. To understand the advantages of DD-PCR, it is helpful to briefly review some other gene expression analysis methods. DD-PCR is powerful methods that allow the comparison of similar cells or tissue types and the identification and isolation of differentially expressed genes (Welsh *et al*, 1992).

This method produces visually appealing results that allow side-by-side comparison of gene expression levels. Gene fragments from the differentially expressed genes can be excised from the DD-PCR gel, identified and used to prepare gene tags for the study of gene expression levels.

2.10.2 RNA fingerprinting by arbitrarily primed PCP (RAP-PCR)

RNA fingerprinting by RAP-PCR is a powerful tool for the temporal and spatial analysis of differential gene expression. Many biological situations exist where differential gene expression results in distinguishable phenotypes, including, for example, tissue and cell types, responses to hormones, growth factors, stress, and the heterozygous expression of certain genes. There are several methods for detecting differential gene expression and cloning differentially expressed genes that do not rely on a biological assay of phenotype. Most of these methods fall into two general categories: subtractive hybridization and differential screening. RAP-PCR offers numerous advantages over these methods, including its simplicity and its ability to compare the fluctuations in gene expression between multiple samples simultaneously using minute amounts of RNA. In addition, RAP-PCR can yield information on the

overall patterns of gene expression between different cell types or between different physiological conditions of the same cell type. Comparison of the RAP-PCR fingerprints from these different experimental groups permits one to draw inferences regarding the overall cellular states of gene expression and the interrelation between gene transcripts belonging to the same or different regulatory pathways. Hypotheses regarding signal transduction pathways can be obtained using this information.

RAP-PCR offers applications in cancer research in the detection of tumor-specific alterations in gene expression, providing a bountiful source of tumor markers. The pleiotropic impact of oncogene activation, tumor suppressor gene inactivation, and mutator mutations, in gene regulation, can be readily assessed by RAP-PCR in model systems both *in vitro* and *in vivo* (Welsh *et al*, 1992).

2.10.3 Quantitative analysis of induced genes

Numerous techniques have been developed to measure gene expression in tissues and cells. These include Northern blots, the reverse transcription polymerase chain reaction (RT-PCR), Rnase protecting assay insitu hybridization, and dot blot hybridization. Of these methods, RT-PCR is the most sensitive in obtaing quantitative information of mRNA levels because it enables to amplify the individure RNA molecules. To achive this, RNA must be first converted to cDNA using the enzyme reverse transcriptase whose products will act as templates for the PCR reaction. The technique can be used to clone cDNA products without the necessity of constructing and screening a cDNA library to determine the presence or absence of a transcript, and to estimate gene expression levels.