การศึกษาพยาธิสภาพของเนื้อเยื่อและการแสดงออกของยีนที่เกี่ยวกับภูมิคุ้มกัน ในลำไส้ของกุ้งกุลาดำ *Penaeus monodon*

นางสาววิภาศิริ สุนทรชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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HISTOPATHOLOGICAL STUDY AND EXPRESSION OF IMMUNE-RELATED GENES IN INTESTINE OF BLACK TIGER SHRIMP *Penaeus monodon*

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Marine Science
Department of Marine Science
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Thesis Title	HISTOPATHOLOGICAL STUDY AND EXPRESSION OF IMMUNE-RELATED GENES IN INTESTINE OF BLACK TIGER SHRIMP *Penaeus monodon*
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วิภาศิริ สุนทรชัย : การศึกษาพยาธิสภาพของเนื้อเยื่อและการแสดงออกของยีนที่เกี่ยวกับภูมิคุ้มกันในลำไส้ของกุ้งกุลาดำ *Penaeus monodon* (HISTOPATHOLOGY STUDY AND EXPRESSION OF IMMUNE-RELATED GENES IN INTESTINE OF BLACK TIGER SHRIMP *Penaeus monodon*) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร. เผดิมศักดิ์ จารยะพันธุ์, อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. พิกุล จิรวาณิชไพศาล 126 หน้า.

ในการศึกษาการติดเชื้อของกุ้งที่ผ่านมาใช้วิธีฉีดเชื้อเข้าสู่ร่างกายของกุ้ง ซึ่งเป็นเทคนิคที่ไม่เป็นไปตามธรรมชาติของการติดเชื้อ ในการศึกษาครั้งนี้ อาศัยการแช่เชื้อซึ่งใกล้เคียงกับธรรมชาติของการติดเชื้อ เพื่อศึกษาการแสดงออกของการติดเชื้อในกุ้งกุลาดำ (*P. monodon*) เชื้อ *Vibrio harveyi strain 1526* เป็นเชื้อที่มีความรุนแรงต่อกุ้งวัยอ่อน (post-larvae shrimp) ในทางตรงกันข้ามกุ้งวัยรุ่นมีความทนทานต่อเชื้อแบคทีเรียในความเข้มข้นที่สูงกว่ากุ้งวัยอ่อน กุ้งวัยอ่อน *V. harveyi* เป็นสาเหตุของการทำลายโครงสร้างของลำไส้เป็นอย่างมาก โดยเฉพาะอย่างยิ่งบริเวณตับและลำไส้ส่วนต้น ในขณะที่การติดเชื้อเล็กน้อยสังเกตเห็นได้ที่บริเวณตับ และบางส่วนของลำไส้มีรอยซึ่งแสดงให้เห็นว่ามีการติดเชื้ออย่างหนัก จากการศึกษาลักษณะทางพยาธิสภาพของเนื้อเยื่อทั้งในกุ้งวัยอ่อนและกุ้งวัยรุ่นแสดงให้เห็นถึงกลไลการป้องกันของเซลล์ ได้แก่ การจับกินเชื้อโรค (phagocytosis) และ ระบบการกระตุ้นผ่านโปรฟีนอลออกซิเดส (prophenoloxidase activating system) ที่เข้าต่อสู้กับแบคทีเรีย และยังมีการศึกษาการแสดงออกของยีนในลำไส้ของกุ้งกุลาดำ โดยมีการตรวจสอบรูปแบบการแสดงออกของยีน *ALF2 ALF3 crustin penaeidin PrX proPO C-lectin BGBP MMR TL5a1 TL5a2 ficolin Lysozyme MnSOD* และ *mucin-like PM* ด้วยวิธีรีเวอร์สทรานสคริปชัน โพลีเมอเรสเชน รีแอกชัน (RT-PCR) และ เรียลไทม์ โพลีเมอเรสเชน รีแอกชัน (real-time PCR) ผลการศึกษาแสดงให้เห็นว่ามีการเปลี่ยนแปลงการแสดงออกของยีน *C-lectin* อย่างเด่นชัด ในขณะที่เปบไทด์ด้านจุลชีพ (antimicrobial peptides) (*ALF3 crustin* และ penaeidin) *TL5a1* และ *mucin-like PM* มีการเปลี่ยนแปลงเพียงเล็กน้อย และไม่มีผลต่อการแสดงออกของยีน *ALF2 PrX proPO BGBP MMR TL5a2 ficolin Lysozyme* และ *MnSOD* นอกจากนี้ยังพบว่ายีน *TL5a1* มีการแสดงออกมากในบริเวณลำไส้ส่วนท้าย ส่วนยีน *mucin-like PM* มีการแสดงออกมากในลำไส้ส่วนต้นและส่วนกลาง

ภาควิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต Wipasiri Soonthornchai

สาขาวิชา วิทยาศาสตร์ทางทะเล ลายมือชื่ออาจารย์ที่ปรึกษา

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WIPASIRI SOONTHORNCHAI : **HISTOPATHOLOGY STUDY AND EXPRESSION OF IMMUNE-RELATED GENES IN INTESTINE OF BLACK TIGER SHRIMP *Penaeus monodon*.** THESIS ADVISOR : ASSOC. PROF. PADERMSAK JARAYABHAND, Ph.D., THESIS COADVISOR : PIKUL JIRAVANICHPAISAL, Ph.D., 126 pp.

In all previous studies, to induce an infection in shrimp, the bacteria were directly injected into the shrimp body and as a consequence the initial step of a natural interaction was omitted. In this study, an immersion technique was used, which is a more natural way of establishing an infection, to study infection in black tiger shrimp (*Penaeus monodon*). *Vibrio harveyi strain 1526* is highly pathogenic to post-larvae shrimp (PL) but in contrast, juvenile shrimp can survive even at a higher dose of bacteria used to infect PL. In PL, *V. harveyi* causes a massive destruction of the digestive system, especially in the hepatopancreas and in the anterior midgut, while mild infection was observed in the hepatopancreas and only some part of the gut has lesions and signs of heavy infection. According to the histopathological results they show that both PL and juvenile have an efficient cellular defense mechanism including phagocytosis and the prophenoloxidase activating system to fight the infecting bacteria. In addition, the effects of infections were studied on immune gene expression in the intestine. Expression patterns of several genes (*ALF2*, *ALF3*, *crustin*, *penaeidin*, *PrX*, *proPO*, *C-lectin*, *BGBP*, *MMR*, *TL5a1*, *TL5a2*, *ficolin*, *Lysozyme*, *MnSOD* and *mucin-like PM*) were examined by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. Results indicated prominent alteration on expression of *C-lectin* whereas that of the antimicrobial peptides (AMPs; *ALF3*, *crustin*, and *penaeidin*), *TL5a1* and *mucin-like PM* was also slightly changed. The *V. harveyi*-immersion did not affected expression of *ALF2*, *PrX*, *proPO*, *BGBP*, *MMR*, *TL5a2*, *ficolin*, *Lysozyme* and *MnSOD*. Besides, the *TL5a1* transcript was highly expressed in the posterior part of the hindgut but the *mucin-like PM* transcript was highly expressed in the anterior and middle midgut.

Department.....Marine Science.....Student's signature.....Wipasiri Soonthornchai.....
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CONTENTS

PAGE

CHAPTER **PAGE**

CHAPTER **PAGE**

CHAPTER **PAGE**

LIST OF TABLES

TABLES **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

FIGURE **PAGE**

FIGURE **PAGE**

ABBREVATIONS

bp	base pair
ºC	degree Celcius
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EtBr	ethidium bromide
h	hour
L	litre
M	molar
mg	milligram
ml	millilitre
mM	millimolar
ng	nanigram
OD	optical density
R-F fixative	RNA-friendly fixative
RNA	ribonucleic acid
Tris	Tris (hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
UV	ultraviolet
vol	volume

CHAPTER I
INTRODUCTION

In Thailand, the giant tiger shrimp (*Penaeus monodon*) is one economically important cultured species. The annual production in 2002 reached over 200,000 metric tons, which made Thailand the leading exporter of this species. Nevertheless, the industry has suffered from a gradual decline in giant tiger shrimp production due to poor reproductive maturation in the domesticated female broodstocks, low quality of sperms from the captive male broodstocks and the outbreak of diseases. A world shrimp survey by the Global Aquacultue Alliance (GAA) reported an overall loss to disease of approximately 22% in 2001 (Tanticharoen et al., 2008). This indicates that disease control of the shrimp industry is urgently required. Moreover, the GAA survey reported that 60% of the losses could be attributed to virus and 20% to bacteria.

In *P. monodon*, vibriosis is the most predominant bacterial disease causing mass mortalities in both hatcheries and growout ponds. Vibriosis in giant tiger shrimp is commonly caused by several different vibrios such as *Vibrio harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. Among them, *V. hareyi* is the most virulent and prevalent pathogen of cultured penaeid shrimp (Lavilla-Pitogo et al., 1990; Jiravanichpaisal et al., 1994; Karunasagar et al., 1994). Luminous bacterial vibriosis was named after the luminous symptoms (glow in the dark) (Lavilla-Pitogo et al., 1990). Virulent factors in *V. harveyi* are involved in the attachment of bacteria and formation of biofilms, quorum sensing, secretion of various extracellular products (ECPs) including proteases, hemolysins, lipopolysaccharides, and interaction with bacteriophages (Austin and Zhang, 2006). ECPs of *V. harveyi* are harmful to shrimp (Liu et al., 1996), and a cysteine protease is one of the major exotoxins (Liu and Lee, 1999) which appears to be important as a virulence factor (Lee et al., 1999). Thus, vibriosis outbreak in shrimp farming leads to unaffordable financial losses.

Being an aquatic animal, shrimp are continuously encountered with the environment in their habitat, thus, they are constantly exposed to a variety of bacteria and viruses. While most of them are harmless bacteria, some of them can be

pathogenic. Shrimp and other crustaceans have several known defense mechanisms to protect themselves against pathogen invasion. The first line of defense is the external cuticle that provides an effective physical and chemical barrier against the attachment and penetration of pathogens. Moreover, pathogens can enter mouth and pass through shrimp digestive tract. To prevent pathogen colonization, shrimp digestive tract, foregut and hindgut, is partly lined with chitinous membranes where the acids and enzymes are produced to inactivate and digest many viruses and bacteria. In most cases, the cuticle and digestive tract are sufficient to protect against even highly virulent pathogens. However, the midgut is not lined by an external cuticle and therefore seems likely to be a site for penetration of pathogens carried in the water, food, and sediment (Ruby ey al., 1980, Jayabalan et al., 1982). Once pathogens penetrate host barriers and enter to the hemocoel of the host, they will encounter innate immune systems. The shrimp and crustacean innate immune system consists of cellular and humoral responses. The known mechanisms of shrimp innate immune response are phagocytosis, blood coagulation, nodule formation, and encapsulation of foreign cells. Hydrolytic enzymes are also involved in these processes (Somboonwiwat et al., 2006; Burge et al., 2007). Several innate immune response systems have been characterized in *P. monodon*: the prophenoloxidase activating system (proPO) is induced which in turn will lead to melanization and generation of factors for immune reactions such as the cell adhesion factor, peroxinectin (Jiravanichpaisal et al., 2006; Cerenius et al., 2008). Moreover, phagocytosis of hemocytes is another defense mechanism to trap microorganisms in hemocytic aggregation and nodule formations or to encapsulation for a larger microorganisms. Cytotoxic reactions are also triggered through the action of phenoloxidase (PO). During an infection, an array of inducible effector molecules, such as antimicrobial peptides (AMPs) and factors required for opsonization are produced (Jiravanichpaisal et al., 2006).

The mucosal epithelia in the gastrointestinal tract of all metazoans are in close contact with a large number of commensal microbiota (Hooper and Gordon, 2001). As a result, commensal bacteria are known to influence many aspects of the host gut physiology including innate immunity, development, and homeostasis (Koropatnick et al., 2004; Rakoff-Nahoum et al., 2004; Backhed et al., 2005; Macdonald and

Monteleone, 2005; Dale and Moran, 2006; Turnbaugh et al., 2006). Recently, intestinal immunity has been intensively studied in the fruitfly larvae (*Drosophila* sp.). Both foregut and trachea of *Drosophila* larvae are lined with a chitinous matrix and the gut lumen is hostile to microbial colonization due to its physical and physiological properties (Daffre et al., 1994; Hultmark, 1996). Local production of reactive oxygen species (ROS) and AMPs are inducible defense mechanisms in the gut of *Drosophila* (Ha et al., 2005a; Ha et al., 2005b; Ryu et al., 2006). Local production of AMPs in gut is the second line of defence following ROS production to fight against pathogenic infection such as diptericin or attacin. AMPs are produced by epithelial cells under the control of the Imd pathway upon recognition of peptidoglycan (PGN) that is released by Gram-negative bacteria (Bischoff et al., 2006; Zaidman-Remy et al., 2006).

Previously, most knowledge on the shrimp immune response has been elucidated from the analysis of host reactions after direct injection of bacteria into the body cavity of this animal (Sritunyalucksana et al., 2002; Hikima et al., 2003; Supungul et al., 2004; Supungul et al., 2004; Liu et al., 2005a; de-la-Re-Vega et al., 2006; Okumura, 2007; Kang et al., 2007; Tharntada et al., 2008; Yeh et al., 2009 Dong et al., 2009). Although this approach has been shown to be effective for identifying pathogen virulence factors and host defense mechanisms, it bypasses the natural entry of microbes through oral routes of infection and subsequent persistence within the organism (Vodovar et al., 2004). Therefore, to mimic a natural bacterial infection, in this study we choose to expose shrimp to *V. harveyi* indirectly by an immersion method. Using this method, the pathogenic bacteria entered through the mouth and then passed through the shrimp intestine.

The Objective

This study focuses on the morphology of intestine tissues affected by *V. harveyi* infection and examination of expression patterns of immune-related genes in response to the oral route of *V. harveyi* infection (i.e. by immersion-challenge). This basic information opens the possibility for better understanding of host defense mechanisms in this economically important species.

CHAPTER II
LITERATURE REVIEW

2.1 The importance of *P. monodon*

World production of the important species of shrimp farming industry, *P. monodon* and *Litopenaeus vannamei*, has increased exponentially since the early 1970s. The production of shrimp aquaculture in 2005 reached over 2.2 million metric tons (Tanticharoen et al., 2008). Such high demand for shrimp has resulted in high price and high profit of shrimp farming, and a significant source of income and employment.

Thailand has been regarded as the world leader in the export of farmed shrimp since mid-1990s, but it is not the leading producer of cultivated shrimp (Table 2.1). In the past, *P. monodon* was the major species for export to various countries. Recently, *L. vannamei* is, however, dominant species for export (Figure 2.1), because *P. monodon* is lacking of a appropriate selective breeding in programme, it has low quality of sperms from the captive male broodstock and then serious outbreaks of shrimp diseases (Tanticharoen et al., 2008). As a consequence the production of *P. monodon* has decreased dramatically since 2002 (Figure 2.2).

Recently, the sudden increase in cultivated shrimp production can be attributed to the widespread use of *L. vannamei* stocks. *L. vannamei* have the advantage of a rapid growth rate, high survival during larval rearing, tolerance of high stock density, low salinities and temperature, lower protein requirement and certain disease resistance if specific pathogen resistant stocks are used. However, *L. vannamei* is an exotic species in Thailand, they might act as a carrier of various exotic viral pathogens and the broodstocks must be imported from the strain stocking institute, mainly from the Hawaii Marine Institute. Therefore, *L. vannamei* contained pathogens such as *Baculovirus penaei* (BP), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Reo-like virus (REO) and Taura Syndrome virus (TSV) with the obvious possibility of spreading these pathogens to the native species such as *P. monodon* (Overstreet et al., 1997). Considering these potential and alarming

problems, shrimp farming of the local rather than the exotic shrimp species should be considered as crucial issue for aquaculture in Thailand.

Table 2.1 Thai shrimp export from 2005 to 2008. (Source: Thai Customs Department)

Country	2005		2006		2007		2008	
	Quantity	Value	Quantity	Value	Quantity	Value	Quantity	Value
United States	87,571	20,058	96,411	22,813	84,568	18,412	93,014	20,827
Japan	22,231	7,280	21,007	6,521	24,662	6,303	28,324	7,112
Canada	8,297	1,987	9,509	2,243	13,061	2,656	11,818	2,505
Korea	6,956	1,496	9,454	1,974	8,307	1,587	8,617	1,501
Australia	5,923	1,210	4,768	1,085	5,546	1,242	3,187	729
Hong Kong	3,077	733	3,008	871	3,107	782	3,055	700
China	2,927	556	2,556	529	3,411	636	5,006	638
Taiwan	2,155	537	1,076	320	2,239	470	4,158	781
Malaysia	1,366	373	1,389	286	1,347	309	850	162
France	1,851	269	1,868	352	1,421	275	2,655	597
Total	142,354	34,498	151,046	36,994	147,667	32,670	160,685	35,552

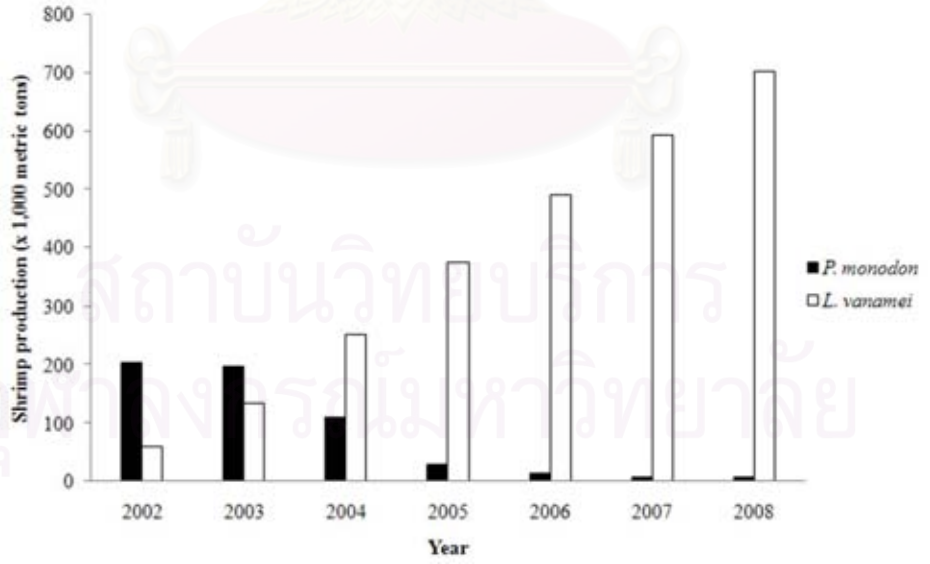

Figure 2.1 Production of *P. monodon* and *L. vannamei* in Thailand from 2002 to 2006 and the prediction in 2007 and 2008 (Source: FAO Fishstat, 2006).

A world shrimp survey by the Global Aquacultue Alliance (GAA) reported an overall loss due to diseases of approximately 22% in 2001 (Tanticharoen et al., 2008). This indicates that disease control to the shrimp industry is urgently required. Moreover, the GAA survey reported that 60% of the losses could be attributed to viruses, 20% to bacteria and 20% of others.

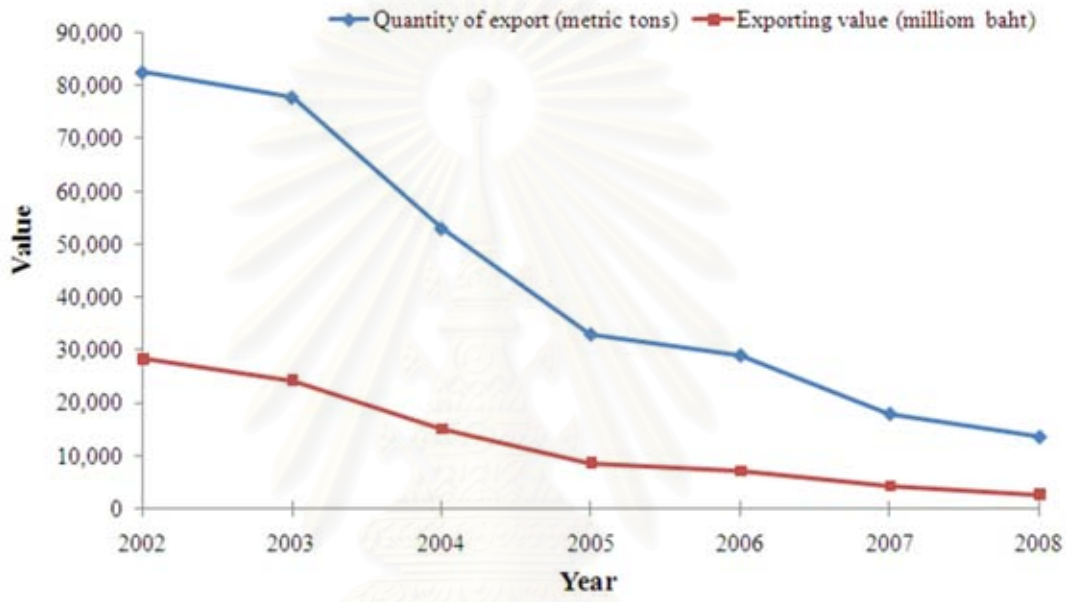

Figure 2.2 Exported *P. monodon* from Thailand during 2002 to 2008. (Source: Thai Customs Department).

2.2 Taxonomy of *P. monodon*

P. monodon, the black tiger shrimp, is classified in the 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* and the scientific name is *Penaeus monodon* (Fibricius, 1798). The common name is giant tiger shrimp (English) and Kung klula-dam in Thai. (Bailley-Brock and Moss, 1992).

2.3 Morphology

The shrimp body is basically divided into cephalothorax and abdomen (Figure 2.3). The cephalothorax is completely covered by a carapace that protects internal organs and supports muscle regions. The carapace has characteristic ridges (carinae) and grooves (sulci). The rostrum has 7-8 dorsal teeth and 3-4 ventral teeth and curves down very slightly. The appendages of the cephalothorax can be used in many functions. First, the antennules and the antennae form sensory functions. Secondly, the mandibles and the two pairs of maxillae form the jaw-like structures which help for feeding and burrowing. Finally, the maxillipeds are the first three pairs of appendages that are modified and used for food handing and five pairs are walking legs (pereiopods). The abdomen has five pairs of swimming legs (pleopods). The carapace and the abdomen have black bands giving a tiger-striped appearance to this species, the antennae are grayish brown, and the pereiopods and pleopods are brown with crimson fringing setae. In shallow brackish waters or cultured ponds, the color patterns are often changed following cultural environment. (Bailley-Brock and Moss, 1992; Bell and lightner, 1988; Moton, 1981: cited in Solis,1988).

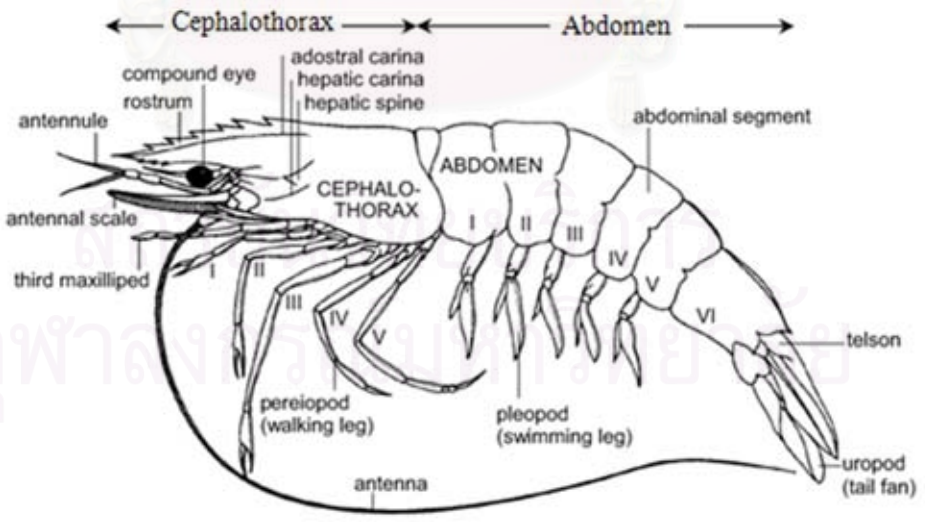

Figure 2.3 Lateral view of the external anatomy of a female *P. monodon* (Primavera, 1990).

The internal morphology of penaeid shrimp contains circulatory, muscular, respiratory, nervous, reproductive and digestive systems (Figure 2.4). The penaeid shrimp have an open circulatory system and have a heart that is dorsally located on the hepatopancreas. Many kinds of muscles control the body movements such as feeding, breathing, burrowing, crawling, walking and swimming. The gills are used for respiration. The nervous system consists of two ventral nerve cords, a dorsal brain, and a pair of ganglia. The ovary or testis is found on hepatopancreas in the cephalothorax area and a number of lateral lobes are seen along the length of the tail following intestine. A large part of the cephalothorax is occupied by hepatopancreas which connects to the gastrointestinal tract via the primary duct. The functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). The lymphoid organ consists of two distinct tiny lobes, and is located slightly dorsal-anterior to the ventral hepatopancreatic lobe. This organ is composed of a mass of anastomosing tubules where the haemolymph is being filtered. All tubules connect via a single vessel to the anterior aorta of the heart. The haematopoietic tissue, where the haemocytes are produced, is dispersed in the cephalothorax, but mainly present around the stomach and in the onset of the maxillipeds.

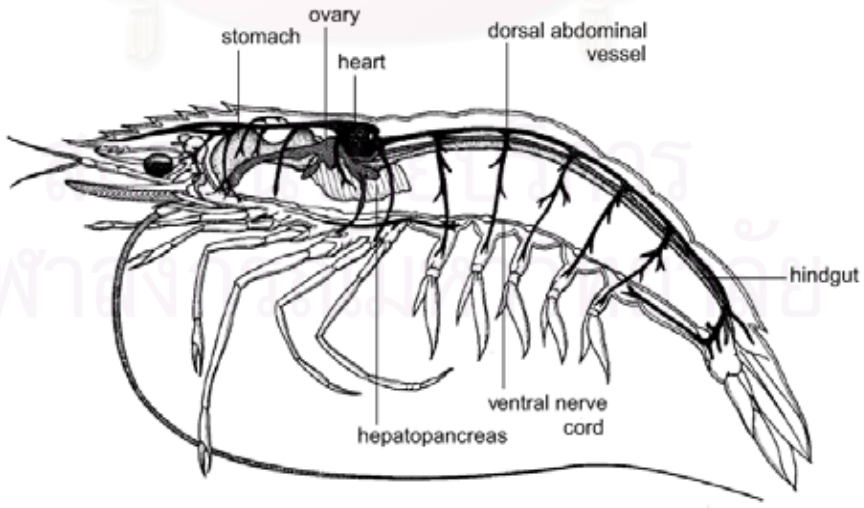

Figure 2.4 Lateral view of the internal anatomy of a female *P. monodon* (Primavera, 1990).

2.4 Digestive system

The digestive organ of decapod crustaceans is composed of foregut, oesophagus and stomach, which are lined with cuticle, then follows a cuticle-free midgut, hepatopancreas and a cuticle-lined hindgut.

The cuticle-lined foregut opens anteriorly at the mouthpart and is posteriorly followed by the midgut. The mouthpart consists of chitin and is divided into two parts including incisor process and molar process to cut, smash and grind food. The esophagus, lacking calcium, is a short tube covered with four cuticle layers: epicuticle, exocuticle, endocuticle and membranous layer. The function is receiving and transfering the feed from mouth to stomach. The stomach connecting esophagus to middle hepatopancreas is divided into two chamber of a cardiac (anterior) and a pyloric (posterior) chamber. The stomach is lined with a layer of non-calcified cuticle except for ossiles. The cardiac chamber has a gastric mill composed of superlateral teeth for mastication of consumed feed and mixes it with the digestive enzyme. The pyloric chamber is divided into a dorsal and ventral subchamber, with the gastric sieve, which is intimately associated with the primary hepatopancreatic ducts. The gastric sieve is a complex structure of cuticular setae and grooves and functions as a screening structure for masticated feed delivery to the hepatopancreas (Bell and Lightner, 1988). Normally, particles bigger than 50-100 nm are retained by the gastric sieve and transferred to the midgut for defaecation, while filtrate is transported into the hepatopancreas for absorption of the nutrients (Vogt, 1996).

The midgut is composed of the midgut tube, the dorsal anterior and posterior midgut caeca and the hepatopancreas. The midgut tube in penaeids is much loger than in freshwater crayfish. The end part is the hindgut which consist of rectum and anus (Vogt, 1996).

The midgut tube consists of mucosa and submucosa (Figure 2.5). The mucosa is a simple columnar epithelium with prominent nucleolus and has conspicuous basement membrane. At the top of the cells microvilli are covered with a peritrophic membrane (PM). The submucosa is thick of loose connective tissue with circular muscles and longitudinal muscles and the last layer is a fibrous connective

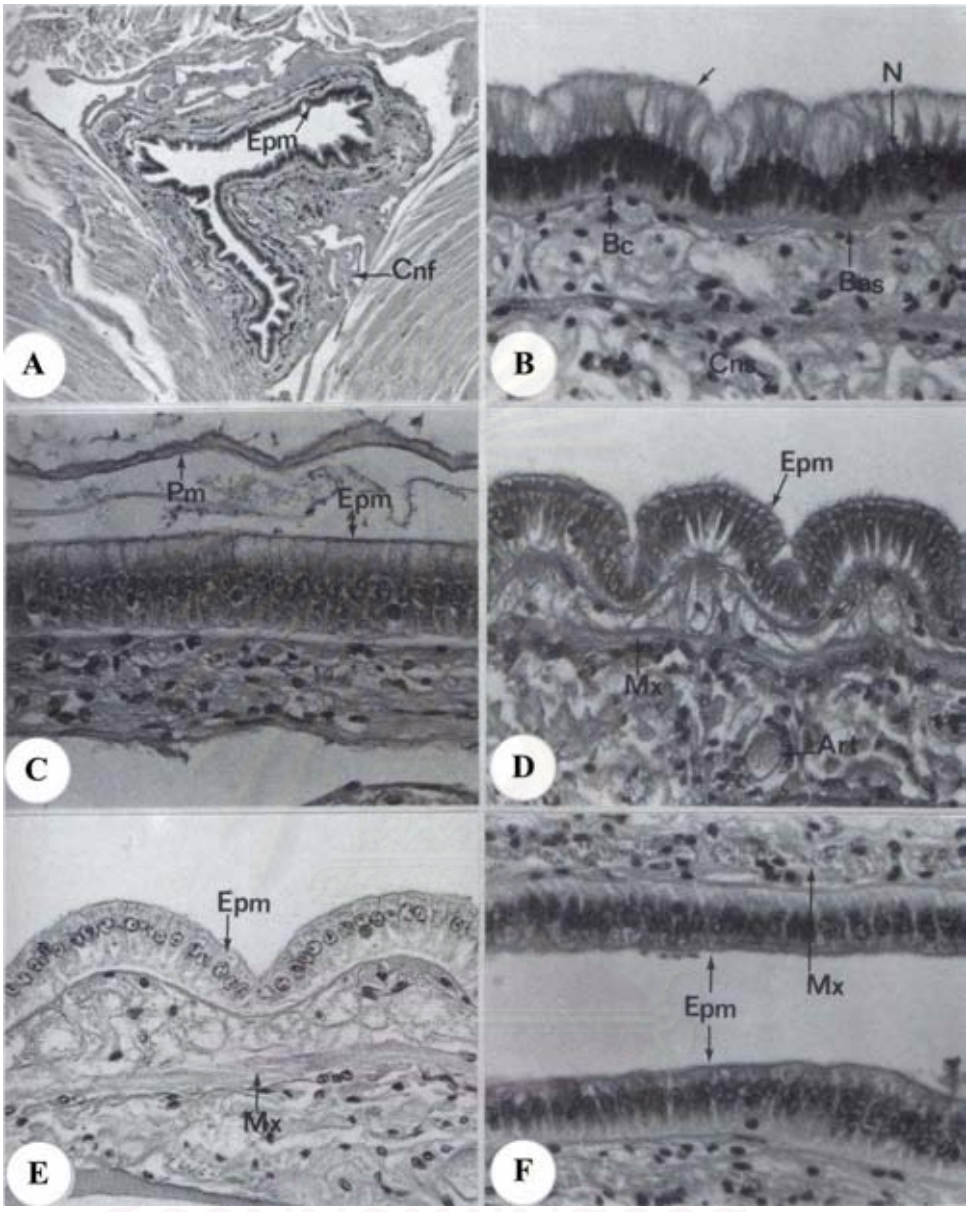

Figure 2.5 Histological study in intestine of one month *P. monodon*. (A) cross-sectioned view of the fifth segment. (B) cross section of epithelial intestine in hepatopanceas and lymphoid organ of broodstock. (C) longitudinal section of epithelial intestine in hepatopanceas and lymphoid organ. (D) longitudinal section of epithelial intestine in the first segment. (E) long section of epithelial intestine in the third segment. (F) long section of epithelial intestine in the fifth segment. Epm=epithelial membrane; Cnf=fibrous connective tissue; N=nucleus; Bc=basal cell; Bas=basement membrane; Cns=spongy connective tissue; Pm=peritrophic membrane; Mx= longitudinal muscles; Art=artery (Arkarajamon, 1991).

tissue called serosa. The PM are secreted in a lump on the pyloric stomach called anterior midgut caeca which is supported structure by a loose connective tissue. In addition, the last part of the intestine has posterior midgut caeca. From the posterior midgut caeca to the anus is hindgut which is a fold of simple epithelium in lumen called multi-chamber and it has many tegmental glands especially near the posterior midgut caeca (Arkarajamon, 1991).

The main functions of the midgut are apparently the formation of the PM which envelopeds the faeces, water uptake during moulting and absorb nutrient molecules. The anterior midgut caecum is the blindly-ended beginning of the midgut and has plenty of mitotic stages and delivers new cells to the midgut tube. For the posterior midgut caecum, the function is still unclear (Vogt, 1996). The hindgut serves for the transport of residual waste material to outer and perhaps also for ion transport (Icely and Nott, 1992).

The hepatopancreas is the most voluminous tissue of the digestive tract and consists of several hudread blind-ended tubules and each tubule is composed of a single-layered epithelium and is enveloped by a close-meshed muscle network. The epithelial cells have four cell types including E-cells (embryonic) at the tips of the tubules and mature R (resorotive)-, F (fibrillar)- and B (blisterlike)-cells along the tubules. R-cells are responsible for absorption and catabolism of the nutrients and storage of nutrient reserves such as glycogen and lipid. (Vogt, 1994). R-cells are believed to be involved in storage excretion of copper from both the haemocyanin metabolism and the environment (Vogt et al., 1989). F-cells are reported to be the site of synthesis of digestive enzymes such as the proteolytic enzymes astacin, trypsin and carboxypeptidase (Vogt et al., 1989). The function of B-cells is still unclear.

2.5 Bacterial Diseases

Bacterial diseases can be rapidly spread in the high-density environments of shrimp farming resulting in mass mortalities to growth retardation and periodic mortalities. Vibriosis is widespread in all marine crustaceans including shrimp. The mortalities caused by bacteria such as vibriosis usually occur when shrimp are stressed by environmental factors such as poor water quality, crowding, high water

temperature and low water exchange (Brock and Lightner, 1990). In *P. monodon*, vibriosis is the most predominant bacterial disease causing mass mortalities in both hatcheries and grow-out ponds including *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, and *Vibrio* sp. (Brock and Lightner, 1990; Ishimaru et al., 1995). Among them, *V. harveyi* is the most virulent and prevalent pathogen of cultured penaeid shrimp (Lavilla-Pitogo et al., 1990; Jiravanichpaisal et al., 1994). Characterization of the 172 bacterial isolates in the hepatopancreas of pond-reared *P. monodon* juvenile showed that most (90.12%) were *Vibrio* species dominated by *V. harveyi* (27.91%), *V. splendidus* II (13.37%) and *V. parahaemolyticus* (10.46%). Extreme losses of cultured *P. monodon* in hatcheries and shrimp farms result from *V. harveyi*. These bacteria outbreak causes mortalities of the affected shrimps up to 100%, whether they are larvae, post-larvae, juvenile, sub adults and adults (Lightner, 1983).

Luminous bacterial vibriosis (LBV) is named after the luminous symptoms caused by this bacterium (Lavilla-Pitogo et al., 1990). *V. harveyi* is a rod shape, gram-negative bacterium with 0.5-0.8 µm width and 1.4-2.6 µm length. Presumptive diagnosis is based on clinical signs such as blood or hepatopancreas that is streaked on a *Vibrio*-selective or general marine agar plate. After incubation at room temperature overnight, colonies of *V. harveyi* grow and show strong luminescence in dim light. *Vibrio* species live in the cultured water, in oxygen-free environments such as in the gut of aquatic animals and sometimes found in the bottoms of shrimp ponds. Consequently, this bacteria can enter shrimp via the cuticle or sub-cuticle that also give the name shell disease or black/brown spot disease, puncture wounds so loss of limbs and cloudy musculature and the gut or hepatopancreas and/or general septicemia (Lightner, 1983). In Filipino hatcheries, the primary source of *V. harveyi* appears to be the midgut contents of female broodstock which are shed during spawning (Lavilla-Pitogo et al., 1992).

The infected shrimp show the milky white signs on the body and appendages, weakness, disoriented swimming, lethargy and loss of appetite. Postlarvae may display cloudy hepatopancreas and brown gills (Takahashi et al., 1985; Anderson et al., 1988). Finally, it leads to death. Histopathology of infected shrimp showed that epithelial cells of midgut and hepatopancreatic tubules are

commonly detached into the lumen (Lavilla-Pitogo et al., 1990). The cuticular colonisation is the cause of necrosis of the cuticular epithelium and the formation of melanised lesions. Septic hemocytic nodules are formed in the lymphoid organ, heart, connective tissues of the gills, hepatopancreas, antennal gland, nerve cord, telson and muscle (Anderson et al., 1988; Mohney et al., 1991; Jiravanichpaisal et al., 1994). In Thailand, vibriosis in *P. monodon* has the spheroids in the lymphoid organ (Nash et al., 1992). However, the pathogenicity mechanisms are still not fully understood and they are likely to involve an ability to attach and form biofilms, quorum sensing, various ECPs including proteases, hemolysins, lipopolysaccharides and interaction with bacteriophages (Austin and Zhang, 2006). ECPs of *V. harveyi* are harmful to shrimp (Liu et al., 1996), and a cysteine protease is one of the major exotoxins (Liu and Lee, 1999) which appears to be important as a virulence factor (Lee et al., 1999).

To control vibriosis, antibiotics have been used but they have caused problems of antibiotic resistance. Alternatively, probiotics are used to against shrimp pathogenic vibrios. A marine bacterial strain *Pseudomonas* I-2 has the properties to control shrimp pathogenic vibrios in shrimp hatcheries and farms. Therefore, it is an interesting strategy for controlling the shrimp pathogenic *Vibrio* spp. in cultured systems (Chythanya et al., 2002). Another probiotic bacterium is *Bacillus* S11. Shrimps fed these bacteria have survival rates higher than untreated shrimp when they were challenged with *V. harveyi* (Rengpipat et al., 2003). Another probiotic bacterium is *Bacillus subtilis* BT23 and cell free extracts of this bacterium can inhibit the growth of *V. harveyi* (Veseeharan et al., 2003).

2. 6 Systemic immune regulation

The immune system is classified into two types: innate and adaptive immunity. Invertebrates have only innate immunity and this is the first line defense to help and limit infection at an early stage. The model *Drosophila malanogaster* has been used to study invertebrate immunity as well as crayfish and some other insects. The recognition of infectious non-self by pattern recognition receptors in *Drosophila* occurs through two major signal pathways including Toll pathway and Imd (immune deficiency) pathway (Hoffmann and Reichhart, 2002; Tzou et al., 2002)

Tolls and Toll-like receptors (TLR) are evolutionary conserved transmembrane glycoproteins characterized by an extracellular domain containing several numbers of leucine-rich repeat (LRR) motifs and an intracellular signaling domain homologous to Toll/IL-1 receptor (TIR) domain. (Bowie and O'Neill, 2000). The Toll receptor was originally identified in *Drosophila* (dToll) and recently it was been from shrimp *L. vannamei* (lToll), *P. monodon* (PmToll), *Marsupenaeus japonicas* (MjToll) and *Fenneropenaeus chinensis* (FcToll) (Yang et al., 2007; Arts et al., 2007; Mekata et al., 2008; Yang et al., 2008). The dToll has the role in antifungal and anti Gram-positive bacteria. Furthermore, in vitro *MjToll* gene was up-regulated 76-fold as compared to a control in lymphoid organ stimulated with PGN at 12 h in *M. japonicas* (Mekata et al., 2008). The *FcToll* gene expression was significantly increased after 5 h post-*V. anguillarum* challenge but decreased immediately after white spot syndrome virus (WSSV) exposure (Yang et al., 2008). In the *P. monodon*, *PmToll* was constitutively expressed after WSSV challenge (Arts et al., 2007). Therefore, *Toll* gene may be involved in innate shrimp defense but so far no function has been shown for Toll. In fact a recent report showed that Toll was not involved in antiviral immunity in *L. vannamei* (Labreuche et al., 2009)

The Imd pathway is essentially activated by Gram-negative bacteria through the recognition of the diaminopimelic acid (DAP)-type peptidoglycan by specific peptidoglygan recognition proteins (PGRPs) (review by Vallet-Gely et al., 2008). Moreover, activation of *imd* in *Drosophila* larvae can be transformed by nitric oxide (Foley and O'Farrell, 2003) and this input is mediated by a Ca^{2+}-dependent phosphate calling calcineurin (Dijkers and O'Farrell, 2007). The first evidence of this pathway in *Drosophila* was identified as a mutation named *imd* gene that weakened the expression of several antimicrobial peptide gene. The Imd pathway shares some similarities with the vertebrate tumor necrosis factor receptor (TNF-R) pathway. However, the molecular organization of the Imd pathway in *Drosophila* is poorly defined because of difficulties in performing epistatic analyses and to the absence of data on the subcellular localization. Recently, there is an immune deficiency homolog (*Lv*IMD) from *L. vannamei*. This study shows that *Lv*IMD mRNA is expressed in most tissues and is induced in hepatopancreas and hemocytes after challenged by lipopolysaccharide (LPS)from *Escherichia coli* and Gram-negative *V. alginolyticus*.

However, *Lv*IMD mRNA is not induced in gill after challenge. In addition, the localization study showed that *Lv*IMD is localized in the cytoplasm (Wang et al., 2009).

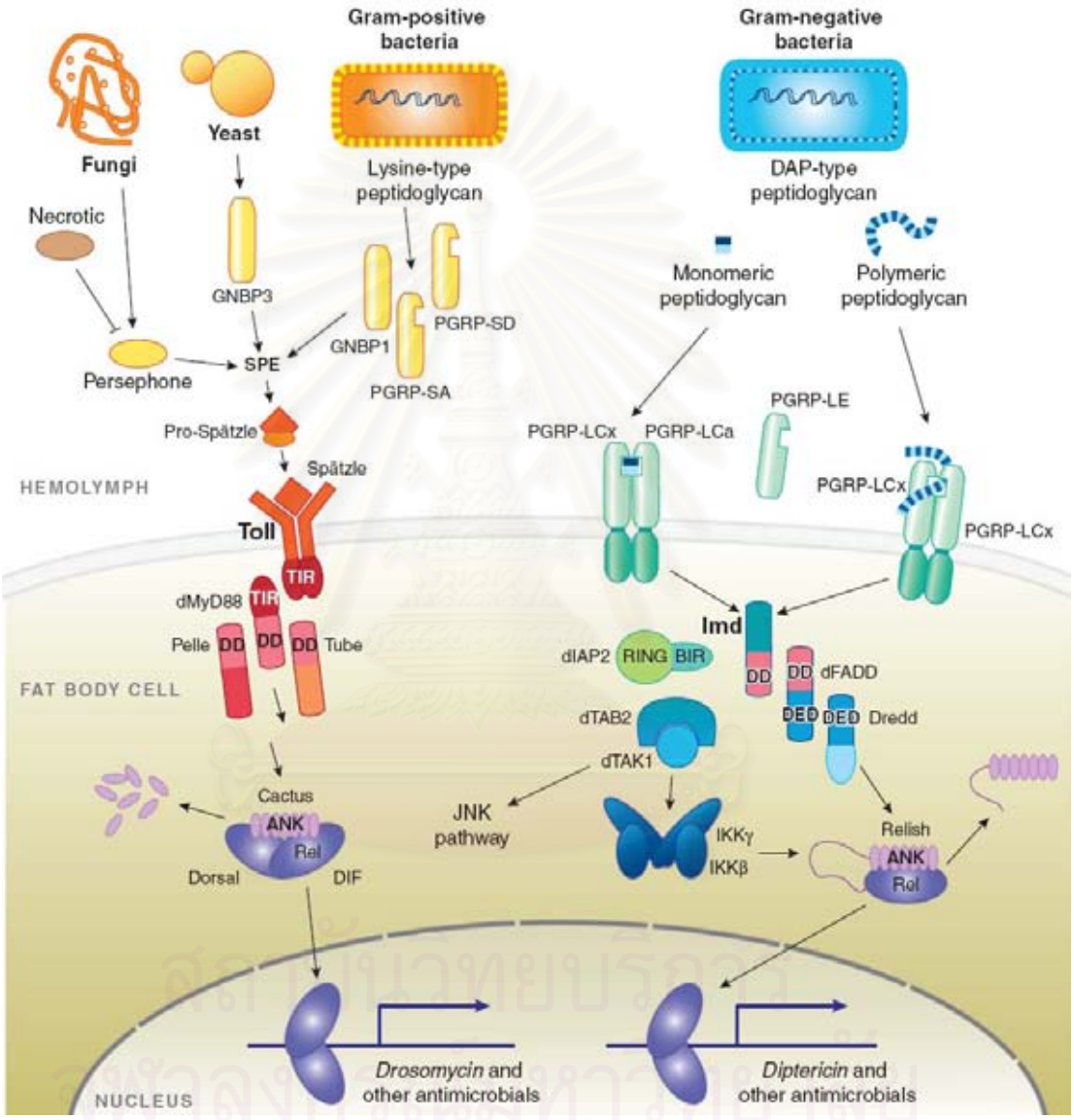

Figure 2.6 Schematic model of Toll and Imd pathway activation. AMP genes are regulated by a balance between two signaling pathways: the Toll pathway that is mainly activated by fungi and Gram-positive bacteria, and Imd pathway that is mainly activated by Gram-negative bacteria (Lemaitre and Hoffmann, 2007).

2.7 Epithelial immune defences

The epithelial surface must be protected with efficient systems for microbial recognition and control because the barrier epithelia are in constant contact with large numbers of microorganisms. *Drosophila* lives on decaying matter and feeds on fermenting medium. Both gut and trachea, two main routes of infection, are lined with a chitinous matrix. Moreover, the gut lumen is an hostile to microbial colonization due to its physical and physiological properties and the secretion of lysozymes (Daffre et al., 1994; Hultmark, 1996). In addition, local production of ROS and AMPs provide two complementary inducible defense mechanism in gut (review by Lemaitre and Hoffmann, 2007).

In *Drosophila*, natural gut infection has been associated with the rapid synthesis of ROS and the dynamic cycle of ROS generation and elimination appears to be vital, because flies that lack ROS removal capacity have an increased mortality (Dimarcq et al., 1994). The dual oxidase (Duox) proteins form a conserved family of molecules containing both the NADPH domain and N-terminal extracellular peroxidase domain (PHD) that can produce ROS in a regulated manner (Ritsick et al., 2004). These proteins can transform hydrogen peroxide (H_2O_2) into the highly microbicidal hypohalic acid (HClO). Excessive ROS production is prevented by immune responsive catalase (IRC) (Ha et al., 2005b) so ROS can be detoxified by IRC. The *IRC* and *Duox* phenotype demonstrate that a fine redox balanceis critical for control of microorganisms in the gut lumen. This ROS-dependent gut immunity is not affected by the Imd pathway and provides an additional barrier against ingested microorganisms (Ryu et al., 2006).

During phagocytosis of shrimp, the NADPH-oxidase is activated which in turn enhances the glycolytic reactions that will increase the consumption of oxygen and induce the production of ROS. These ROSs can kill foreign invaders and also play an important role in immune signal transduction (Schwarz, 1996; Bogdan et al., 2000; Xiang, 2001). However, the mass ROS production is detoxified by antioxidant enzymes including superoxide dismutase (SOD), catalase and other peroxidases. H_2O_2 is then converted to water and oxygen (O_2) by catalase providing innocuous

compounds to the cell (Pipe et al., 1993; Holmblad and Söderhäll, 1999: Devasagayam et al., 2004; Liochev and Fridovich, 2007)

Local AMP production in the gut has been suggested to be the second line of defence to fight pathogens after ROS production. The use of green fluorescent protein (GFP) reporter transgenes has revealed that AMP genes are expressed in several surface epithelia that are in contact with the external environment (Tzou et al., 2000). This AMP synthesis is referred to as the local immune response as opposed to the systemic response. One can distinguish between constitutive and inducible AMP expression in epithelia. Firstly, the AMP gene is expressed constitutively in a defined tissue, and its transcription is not up-regulated during microbial infection such as *Drosomycin* in salivary glands and in the female spermatheca, and for *Cecropin* in the male ejaculatory duct (Tzou et al., 2000). This constitutive expression is regulated by various tissue specific transcription factors such as the homeobox-containing protein Caudal (Ryu et al., 2004; Han et al., 2004). Secondly, there is the inducible local AMP gene expression. The second form is the inducible local AMP gene expression. This response is triggered upon natural infection by Gram-negative bacteria and is mediated by the Imd pathway (Tzou et al., 2000; Önfelt et al., 2001) such as *Drosomycin* and *Diptericin* induced in both trachea and gut via the Imd pathway in response to local infection by bacteria (Basset et al., 2000).

A central role in bacterial tolerance of the gut has been attributed to amidase PGRPs, as they scavenge PGN released by commensals (Bischoff et al., 2006). Because Gram-negative PGN is hidden in the periplasmic space underneath the outer LPS membrane and bacteria residing in the gut have a low division rate, commensals may well release only low amounts of PGN that can readily be hydrolyzed by amidase PGRPs. This implies the important concept of a threshold response for local immune activation in order to differentiate between commensal microorganisms and invading pathogens.

Being an aquatic animal, shrimp are constantly exposed to a variety of bacteria and viruses. While most of them are harmless bacteria, some of them can be pathogenic. Crustaceasns have several known defense mechanisms to protect themselves against pathogen invasion. The external cuticle provides an effective physical and chemical barrier against the attachment and penetration of pathogens

(Martin et al., 2004). Unlike foregut and hindgut the midgut (equivalent to intestine) is not lined by cuticle and therefore seems to be a likely favorite site for invasion of pathogens (Ruby et al., 1980; Jayabalan et al., 1982). In most cases, the defense mechanisms of the cuticle and that of the digestive tract are sufficient to protect against even highly virulent pathogens. However, when pathogens are able to break these defense barriers and enter into the body, they will encounter the innate immune systems. This system consists of cellular and humoral responses and the well-known mechanisms of these responses are phagocytosis, blood coagulation, nodule formation, and encapsulation (review by Jiravanichpaisal et al., 2006). Some hydrolytic enzymes are also involved in these processes such as lysozyme (Somboonwiwat et al., 2006; Burge et al., 2007). Several innate immune response systems have been characterized in crustaceans and *P. monodon*: the proPO is induced which in turn will lead to melanization and generation of factors for immune reactions such as the cell adhesion factor, peroxinectin (review by Jiravanichpaisal et al., 2006; Cerenius et al., 2008). Moreover, during an infection, an array of inducible effector molecules, such as AMPs, lectins and factors required for opsonization are produced (Figure2.7) (review by Jiravanichpaisal et al., 2006).

2.8 Hemocyte

The circulating hemocyte displays crucial roles in immune system protecting the animal against invading microorganisms by wounding and clotting system. In penaeid shrimp and crayfish, circulating hemocyte is divided 3 types; hyaline cell, semigranular cell and semigranular cell (Martin and Graves, 1985; Smith and Söderhäll 1983). This classification was based on granules.

Hyaline cells are the smallest, lack or few cytoplasmic granules and the ovoid nucleus filling much of the cell (Martin and Graves, 1985). The hyaline cells are involved in phagocytosis (Söderhäll et al., 1986) and coagulation (Omori et al., 1989). These hemocytes compose only 5-10 % of all hemocytes of penaeid shrimp (Martin and Graves, 1985).

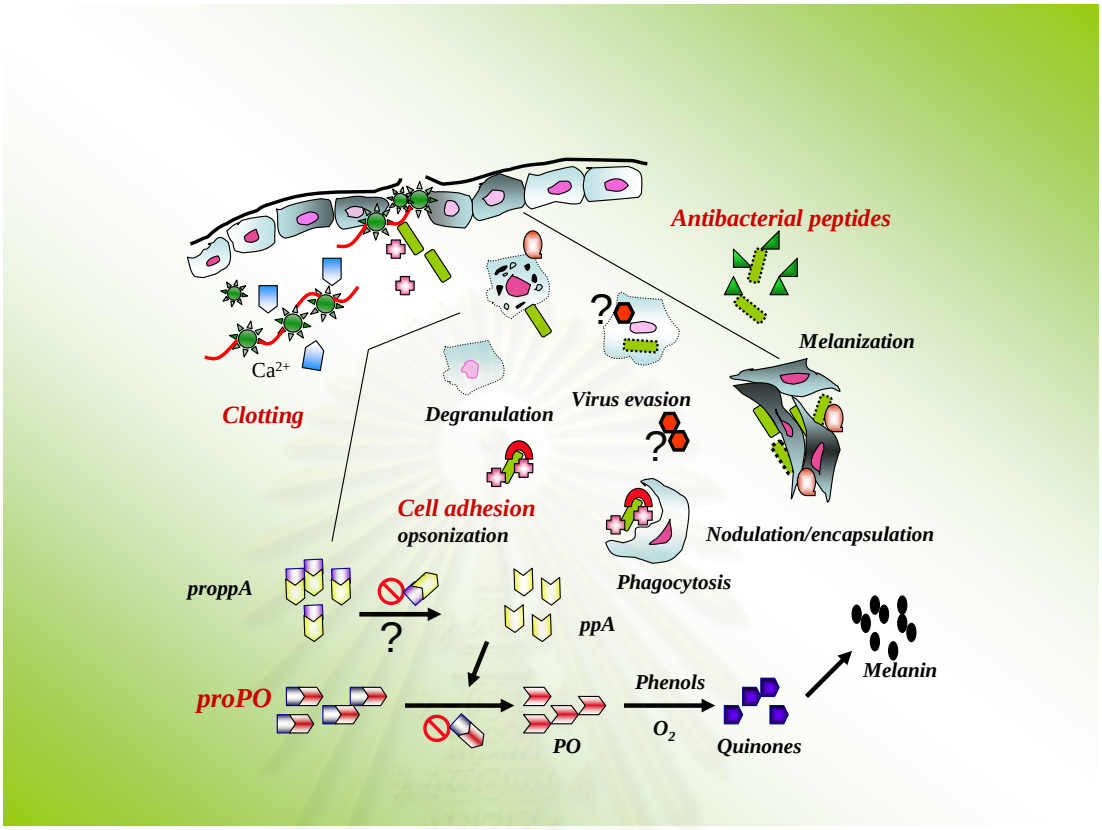

Figure 2.7 Diagrammatic view of innate immune response in crustacean.

Semigranular cells present of a variable number of small, black granules in the cytoplasm (Martin and Graves, 1985). The small eosinophilic granules contain the proPO system. Semigranular cells display some phagocytic capacities, would be specialized in particle encapsulation and the frist hemocyte type to defense to foreign particle (review by Jirevanichpaisal et al., 2006). These hemocytes are the most abundant type of hemocyte about 75% of all hemocytes of penaeid shrimp (Martin and Graves, 1985).

Granular cells are filled with large granules with darken the nucleus. The large granules are twice as large (0.8 pm diameter) as the granules in semigranular cells (Martin and Graves, 1985). They are a major storage of proPO system, and are capable of cytotoxic reaction but don't have phagocytosis function (Smith and Söderhäll 1983a). These hemocytes compose 10-20 % of the all hemocytes of penaeid shrimp (Martin and Graves, 1985).

2.9 Pattern recognition proteins (PRPs)

There are pathogen-associated molecular patterns (PAMPs) present on pathogenic microbes. Due to the absent of these molecules on host cells, they can serve as discriminate molecules between self and non-self (Janeway, 1989). In invertebrate, a number of soluble molecules can bind to PAMP of microbes have been reported and are called pattern recognition receptors (proteins) (PRRs, PRPs).

Interaction of PRPs and PAMPs triggers a series of immune responses, leading to the activation of the host-defense system (Medzhitov and Janeway, 2002; Janeway and Medzhitov, 2002). A non-self recognition system activated by component of PAMPs such as LPSs/PGNs from bacterial cell walls, lipoteichoic acids of bacteria, glycolipids of mycrobacteria , mannans of yeasts, β-1,3-glucan from fungal cell walls and double stranded RNA (dsRNA) of replicating viruses (Hoffmann et al., 1999, Söderhäll and Cerenius, 1998; Vargas-Albores et al., 1996). PRR is localized on the surface of cells and secreted into the hemolymph, ready to signal the presence of invading pathogen in every compartment. In the previous studies, PRRs such as LPSor/and β-1,3-glucan binding proteins (LBP, BGBP or LGBP), PGRPs, and lectins have been characterized in various invertebrates (Lee and Söderhäll, 2002).

It has been known that lectins are found in almost all living organisms and that they are PPRs, which recognize and bind to conserved molecules on the surface of invading microorganisms or PAMPs present on non-self pathogens. Lectins/agglutinins are sugar-binding proteins usually without catalytic activity. Many biological activities were found to be involved in the interaction between lectins and carbohydrates. In invertebrates, lectins have been reported to be involved in various defense mechanisms such as to exhibiting antimicrobial activity, (Tunkijjanukij and Olafsen, 1998; Schroder et al., 2003; Sun et al., 2008), enhancing phagocytosis (Kondo et al., 1992; Mercy and Ravindranath, 1994; Sierra et al., 2005), activating the proPO system (Chen et al., 1995; Yu et al., 1999; Yu et al., 2000) and nodule formation or encapsulation (Koizumi et al., 1999; Ma et al., 2008). Recently, lectins mainly C-type lectin (Calcium dependent lectins) have been increasingly studied in shrimp and the transcripts were detected exclusively in hemocytes (Gross et al., 2001;

Liu et al., 2007) or in the hepatopancreas (Gross et al., 2001; Liu et al., 2007; Luo et al., 2006; Ma et al., 2007, 2008; Sun et al., 2008). Some studies have shown that the expression of lectin changed in response to infection by WSSV after challenge by injection of the virus (Gross et al., 2001; Liu et al., 2007; Ma et al., 2007, 2008). Although, Pmlec did not have antibacterial activity or was involved in the activation of the proPO system (Liu et al., 2006), it functioned both as a PRP and an opsonin. Also, BGBP and LGBP have been reported to enhance activation of the proPO system (Cerenius et al., 1994; Ma and Kanost, 2000) and induction of AMPs (Kim et al., 2000) in invertebrates. Two other inducible immunolectins C-type lectin (Yu et al., 1999) and an LPS specific lectin (Yu and Kanost, 2000) were isolated and characterized in tobacco hornworm, *Manduca sexta*, and were both found to activate the proPO system.

Another lectins are tachylectins (TLs) which were first time cloned and characterized from horseshoe crab, *Tachypleus tridentatus* (Gokudan et al., 1999). TL5A and 5B have ability to agglutinate all types of human erythrocytes and Gram-positive and Gram-negative bacteria. TLs-5 posses a fibrinogen related domain (FRED) and these fibrinogen-related molecules and might play an important role as nonself-recognizing lectins. In the horseshoe crab, TL-5A was expressed in heart and intestine and very low expression was found in hepatopancreas and TL-5B was detectable only in hemocytes (Gokudan et al., 1999). So far, all tachylectins have never been syudied in shrimp.

2.10 Antimicrobial peptides

In all kingdoms, from bacteria to human, a wide variety of AMPs have been identified and characterized. Normally, AMPs contain less than 150-200 amino acid residues and have a wide variety and diversity in amino acid sequence, structure and range of activity. AMPs are active against a broad spectrum of microorganisms such as bacteria, filamentous fungi, virus and parasites (Hancock and Daimond, 2000; Pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1999). Depending on their tissue distribution, AMPs ensure either a systemic or local protection of the host against pathogen.

In arthropods, a spacious activity of AMPs against bacteria and filamentous fungi has been reported in insects, horseshoe crabs, and shrimps (Bachère et al., 2004; Bulet et al., 1999; Vizioli and Salz, 2002). In horseshoe crabs, AMP are mainly synthesized in hemocyte and stored within cytoplasmic granules. These cells are highly sensitive to LPS which is a major outer membrane component of Gram-negative bacteria and respond by degranulating granules after stimulation by LPS. In contrast, the fat body of insect is the main site for AMP symthesis (Hoffmann and Reichart, 1997). AMP gene transcription is induced by attacking bacteria resulting in their immediate synthesis and subsequent secretion into the blood circulation.

Many AMPs have been characterized from insects and chelicerates, but a few have been reported in crustaceans. In the last decade, however, several AMPs have been isolated from crab, crayfish and shrimp. There are AMPs such as penaeidin, astacidin1 and 2, parasin, hipsin, antilipopolysaccharide factor (ALF), crustin and hemocyanin. Of these the penaeidin, crustin and ALF families have been studied in more detail.

2.11 Coagulation system/the clothing system

Coagulation is important for limiting hemolymph loss and initiating wound healing that is the first line defense of the overall invertebrate immune system. And it is quickly forming a secondary barrier to infection and immobilizing bacteria. To date, two coagulation systems have been deciphered in detail namely crayfish and horseshoe crab. Noteworthy is that coagulation in insects is not known in any detail. In horseshoe crab, the clotting system is regulated by a proteolytic cascade that is linked with the release of antimicrobial substances (Iwanaga, 2002; Iwanaga and Lee, 2005). In another model the crayfish, the clotting system depends on a transglutaminase (TGase) dependent clotting reaction (Kopcek et al., 1993; Hall et al., 1999). In crustacean, coagulation is formed by the polymerization of a clotting protein in plasma catalyzed by a calcium ion dependent TGase, which is released from the hemocytes under foreign particle stimulate or tissue damage (Hall et al., 1999). The clotting reaction is very similar in crayfish and shrimp and TGase and

clotting protein are important molecules in shrimp coagulation (Maningas et al., 2007).

TGase, calcium ion dependent enzymes, catalyze calcium-dependent acyl-transfer reactions between glutamine residues and lysine residues in protein substrates in presence of calcium ion to form a solf gel at the wounding site. (Wang et al., 2001). TGase gene has been cloned and localized in crayfish *Pacifastacus leniusculus* (Wang et al., 2001) and two TGase gene including STG I and STG II are characterized from black tiger shrimp (Huang et al., 2004). Another type of TGase was found to be involved in the tiger shrimp *P. monodon* (Chen et al., 2005; Huang et al., 2004). Moreover, a biochemical study was utilized to investigate the involvement of TGase in coagulating plasma clotting protein (Yeh et al., 2006).

The clotting protein, a glycoprotein, has two physiological factions including crustacean coagulation and lipid transport (Hall et al., 1995). The regulation and localization of clotting protein are in the outer layer of stromal matrix cells of lymphoid organ (Yeh et al., 2007). Crustacean clotting proteins have been cloned and characterized from several species, and it was first cloned from the freshwater crayfish, *P. leniusculus* (Hall et al., 1999; Kopacek et al., 1993), and then was found in the tiger prawn, *P. monodon* (Yeh et al., 1999).

2.12 Melanization and adhesion

Melanization is a common defense mechanism among invertebrates such as wound healing, encapsulation, elimination of microorganism and the production of toxic intermediates that kill invading microorganism (review by Lemaitre and Hoffmann, 2007). Its reaction provides both toxic quinone substances also involved information of more long-lived products such as the melanin that physically encapsulates pathogen and other short-lived reaction intermediates in the melanin pathway participate in the wound healing process by the formation of covalent links in damaged tissues resulting in sclerotisation (review by Cerenius et al., 2008). Melanization requires the activation of PO which catalyses the oxygenation of monophenols to *o*-diphenols and further oxidation of *o*-diphenols to *o*-quinones and eventually the synthesis of melanin (review by Jiravanichpaisal et al., 2006). The

sticky protein PO can adhere to the surface of parasites that will lead to melanisation of the pathogen. The melanin and intermediates in the melanin formation can inhibit growth of microorganism. Therefore, the melanisation cascade is produced by proPO system. The melanin reaction also leads to production of ROS that may also combat infection (de Gregorio et al., 2001).

The proPO system is a part of innate immune defences in invertebrate and consists of several proteins involved in leading to melanin production, cell adhesion, encapsulation and phagocytosis (Söderhäll et al., 1998; Sritunyalucksana and Söderhäll, 2000).

In shrimp and crayfish proPO is synthesized in the hemocytes, and released into plasma by exocytosis after activation. After binding with PAMPs such as LPS, PGN and β-1,3-glucans, subsequently activation of proteinases in the proPO system. The proPO is proteolytically converted into active PO by an endogenous trypsin-like serine proteinase, the prophenoloxidase activating enzyme (ppA) and results in melanin production (Aspán et al., 1995). It was reported that a recombinant defensin-like peptide comprising the clip domain of ppA from crayfish has antibacterial activity against Gram-positive bacteria *in vitro*. This indicates that a multiple function of crayfish ppA (Wang et al., 2001).

A properoxinectin (proPXN), an intermediate proteinase and a myeloperoxidase homologue, directly is activated by ppA to change proPXN into peroxinectin (PXN). Pacifastin negatively regulates both PXN and ppA production. The proPXN lacks the cell adhesion, opsonin and encapsulation-promoting activities of PXN. Both proPXN and PXN, however, possess peroxidase activity. PXN has been shown to associate with an extracellular superoxide dismutase (eSOD) and to an integrin. Because PXN has peroxidase activity and mediates hemocyte-microbe binding such as opsonisation, this protein might produce hypohalic acid in the vicinity of the bound pathogen. An attractive hypothesis is that an active microbe-PXN-dismutase complex is phagocytosed and results in intracellular killing through the generation of reactive oxygen intermediates (HOCL) by NADPH oxidase. Independently of the proPO system cascade, plasma hemocyanin (Hcy) 2 may be proteolytically processed by a hemocyte-derived proteinase to a protein expressing PO-activity (Hcy 2-PO) (review by Cerenius et al., 2008)

2.13 Proteinase inhibitors

Proteinase cascades during the coagulation process or activation of the proPO systems have to be cautiously regulated by some process to prevent excessive activation of endogenous cascades and damage to host tissue. Proteinase inhibitors have been implicated in this control process. The activities have been detected in several invertebrates. The Kasal, Kunitz, serpins, α-macroglobulins and metalloproteinase inhibitors are well characterized families of proteinase inhibitors (review by Jiravanichpaisal, 2006). Injury and microbial infection in invertebrates lead to activation of the blood coagulation and proPO system which utilize cascades of serine proteinase to amplify an initial signal resulting in rapid and efficient responses to pathogen (O'Brien et al., 1993). Blood coagulation and PO activation can also be harmful to host if they are not limited as local and transient reaction. For this reaction the proteinase in these systems are tightly regulated by proteinase inhibitors.

PO can produce highly toxic intermediates, consequently, host cell can produce several proteinase inhibitors for preventing over-activation of ppA (Aspán et al., 1990) and a phenoloxidase inhibitor (POI) which can directly inhibit the activity of phenoloxidase (Sugumaran and Nellaiappan, 2000). In crayfish, the most efficient inhibitor of ppA is pacifastin. This molecule is held together by a peptide bond and forms a new class of proteinase inhibitor named pacifastin-like serine proteinase inhibitor that presents and inhibits the proPO system in many insect (Simonet et al., 2002).

Insect plasma contains serine protease inhibitors, including members of the serpin superfamily, which appear to regulate the pro-PO activation pathway. These proteins function as suicide-substrate inhibitors by forming stable covalent complexes with proteases after the cleavage of a scissile bond in the reaction center loop (Gettins, 2002; Silverman et al., 2001). From many studies about serpin indicate that it is required to restrict the PO activity to the site of injury or infection, preventing the insect from excessive melanization.

Next hemolymph proteases (HPs), HP-21 and two unidentified proteases were activated by Gram-positive bacteria as a result of the components of the proPO pathway associate to form a larger noncovalent complex which localizes the

melanization reaction to the surface of invading microorganisms. HP-1 and HP-6, were identified as target proteases of serpin-4 and serpin-5 forming covalent complexes after bacterial activation of the cascade. Also serpin-4 complexes with HP-21 and two unidentified proteases were unique to plasma treated with bacteria. Therefore, HP-1 and HP-6 may be components of the proPO activation pathway which are activated in response to infection and regulated by serpin-4 and serpin-5 (Tong et al., 2005).

2.14 Immune-related genes in crustacean

2.14.1 Antimicrobial peptides

ALFs are AMP originally isolated and characterized in hemocytes of the horseshoe crab *Limulus polyphenus* (Tanaka et al., 1982) and has been widely studied in many crustacean species including *L. setiferus* (Gross et al., 2001), *P. monodon* (Supungul et al., 2004), *F. chinensis* (Liu et al., 2005), *M. japonicus* (Nogishi et al., 2006) and *P. leniusculus* (Liu et al., 2006), *L. vannamei* (de la Vega et al., 2008), *Macrobrachium olfersi*, *Farfantepenaeus paulensis* and *L. schmitti* (Rosa et al., 2008). It is active against a broad spectrum of microorganisms such as Gram-negative bacteria, Gram-positive bacteria and fungi. In vivo *Vibrio*-challenged experiment of *P. monodon* and *F. chinensis*, the highest transcriptional level of ALF in hemocytes was observed at 6 h after injection (Supungul et al., 2004; Liu et al., 2005). There are five isoforms of ALFPms which were expressed in individual shrimp but the ALFPm2 and ALFPm3 were mainly expressed in hemocytes. These two isoforms were significantly increased after *V. harveyi*-challenged (Tharntada et al., 2008). Recently, localization of ALFPm3 was studied and shown in hemocytes which were increased after infection (Somboonwiwat et al., 2008). In crayfish, ALF was induced after WSSV challenge in crayfish (Liu et al., 2006) and silencing of ALF resulted in higher rates of WSSV replication both in vivo and in vitro studies. These suggested that ALF might involve in antiviral function against WSSV (Liu et al., 2006).

The crustin family initially was isolated and characterized in hemocytes of the shore crab *Carcinus maenas*. It is cystein-rich, hydrophobic and

contains specific activity against Gram-positive bacteria (Relf et al., 1999). Over 50 crustin sequence have been reported from various decapods including *L. vannamei* and *the L. setiferus* (Gross et al., 2001; Bartlett et al., 2002; Vargas-Albores et al., 2004). After that, the crustin was identified and characterized in many crustacean species including *P. monodon* (Supungul et al., 2002; Chen et al., 2004a; Supungul et al., 2004), *M. japonicus* (Rattanachai et al., 2004), *Panulirus argus* (Stoss et al., 2004), *Homarus gammarus* (Hauton et al., 2006), *P. leniusculus* (Jiravanichpaisal et al., 2007) and *F. chinensis* (Zhang et al., 2007a). In vitro study of Crus*Fc* and Crustin*Pm*1 have activities against only Gram-positive bacteria and have no inhibition on the growth of Gram-negative bacteria (Supungul et al., 2007). On the other hand, *V. harveyi*-challenged experiment of *P. monodon* showed that the expression of crustin was significantly decreased (Supungul et al., 2004). In addition, mRNA levels of crustin decreased at 4 h after injection of LPS into *L. vannamei* and the expression levels returned to initial levels by 72 h post-injection (Okumura, 2007). In a similar study in crayfish it was reported that Plcrustin1 was increased in both hemoytes and hematopoietic tissue as a response to challenge with Gram-negative non-pathogenic and pathogenic bacteria (Jiravanichpaisal et al., 2007).

Penaeidins are a large family of AMPs initially isolated and characterized in hemocytes of *L. vannamei* (Destoumieux et al., 1997). Subsequently, the penaeidins had been detected in several shrimp including *L. setiferus* (Gross et al., 2001), *M. japonicus* (Rojtinnakorn et al., 2002), *P. monodon* (Supungul et al., 2002; Chen et al., 2004b), *F. chinensis* (Kang et al., 2004; Kang et al., 2007). It has strong antimicrobial activities against Gram-positive as well as fungi and modulate activitied against Gram-negative bacteria (Destoumieux et al., 2000; Li et al., 2005; Kang et al., 2007). Penaeidins have been divided into three subfamilies including penaeidin 2,3 and 4 based on amino acid sequence comparison and the position of specific amino acids (Bachère et al., 2004; Cuthbertson et al., 2002). The panaeidin subgroup3 was strongly decreased first 12 h and returned to normal levels at 48 h after microbial challenge in *L. vannamei* (Muñoz et al., 2002). *V. harveyi*-challenge caused significant decreases of penaeidin expression in challenged *P. monodon* within 3 hours after injection (Supungul et al., 2004). Morover, the mRNA levels of penaeidin 2, penaeidin 3, penaeidin 4 were decreased at 4 h post-injection of LPS and returned to

initial levels by 72 h post-injection (Okumura, 2007). Nevertheless, a new subfamily was recently reported in the penaeidin antimicrobial peptides (Penaeidin 5). The expression levels of penaeidin 5 increased after 24 h post-injection in all tissues especially in hemocyte, heart, gill, hepatopancreas, stomach and intestine (Kang et al., 2007).

2.14.2 Melanization and adhesion

The proPO, a major enzyme produced during proPO system activation, is necessary for the melanization. It had been cloned and characterised in *P. leniusculus* (Aspán and Söderhäll, 1991), *P. monodon* (Sritunyalucksana et al., 1999), *F. californiensis* (Gollas-Galván et al., 1999), *Penaeus semisulcatus* (AF521949), *M. japonicus* (AB065371), *L. vannamei* (Lai et al., 2005) and *M. rosenbergii* (Lu et al., 2006). Recently, a novel prophenoloxidase-II (proPO-II) cDNA was also cloned from haemocytes of *L. vannamei* (Yeh et al., 2009). The expression of proPO was significantly constant expression after injection with LPS (Okumura, 2007) and *V. alginolyticus* (Yeh et al., 2009) in *L. vannamei* and feeding with β-1,3-glucan (Wang et al., 2008). In contrast, proPOII expression significantly decreased after injection with *V. alginolyticus* 3 h, subsequently significantly increased after 12 h, and then no significant difference was detected 24 h (Yeh et al., 2009).

Peroxinectin, a cell adhesive protein associated with the proPO system, is a multifunctional protein including cell adhesion (Johansson and Söderhäll, 1988), degranulation (Johansson and Söderhäll, 1989), emcapsulation enhanment (Kobayashi et al., 1990), opsonin (Thörnqvist et al., 1994) and peroxidase activity (Johansson et al., 1995).The peroxinectin was first identified and characterized in *P. leniusculus* (Johansson et al., 1995), then in *P. monodon* (Sritunyalucksana et al., 2001), *L. vannamei* (Liu et al., 2004b) and *F. chinensis* (Dong et al., 2009). Peroxinectin is slightly down regulated after injection with laminarin and LPS in *P. monodon* (Sritunyalucksana et al., 2001). The expression of peroxinectin was significantly and constantly expression after injection with zymosan in *L. vannamei* (Liu et al., 2004b). In contrast, mRNA transcription increased significantly after injection with *V. alginolyticus* in *L. vannamei* (Liu et al., 2005a). The peroxinectin expression did

not significantly increase after injection with mixed heat-killed bacteria, *V. anguillarum* and *Staphylococcus aureaus.* 24 h but did significantly decrease after 72 h in *F. chinensis* (Dong et al., 2009).

2.14.3 Lectins

β-glucan binding proteins (BGBP) had been isolated in several shrimp including *P. leniusculus* (Duvic and Söderhäll, 1990; Cerenius et al., 1994; Lee et al., 2000), *F. californiensis* (Vargas-Albores et al., 1996; Yepiz-Plascencia, 1998), *L. stylirostris* (Vargas-Albores et al., 1997), *L. vannamei* (Vargas-Albores et al., 1997; Yepiz-Plascencia et al., 1998; Romeo-Figueroa et al., 2004), *P. monodon* (Sritunyalucksana et al., 2002), *F. chinensis* (Du et al., 2006b), *M. japonicus* (Lin et al., 2008). It is a plasma protein that after binding to β-glucan reacts with hemocyte surface (Cerenius et al., 1994). The BGBP strongly binds β-1,3-glucans which are structurally complex homopolymers of glucose, usually isolated from yeast and fungi.The BGBP was constitutively expressed in haemocytes of *P. monodon* within 12 hour of the *V. harveyi*-challenge (Sritunyalucksana et al., 2002).

C-type lectins or immulectins are capable of agglutinating bacteria and yeast in a calcium-dependent maner and are involved in immune recognition in invertebrates (Marques and Barrecco, 2000). Six shrimp C-type lectin genes have been discovered including *Pm*AV (Luo et al., 2003), *Pm*Lec (Luo et al., 2006), *Lv*LT (Ma et al., 2007), *Fc*lectin (Liu et al., 2007), *Pm*TL (Ma et al., 2008) and *Fc*-hsL (Sun et al., 2008). Both *Pm*AV and *Pm*Lec were found in *P. monodon* and contain a single cysteine rich domain (CRD domain) and *Pm*AV contributes to virus resistance, while *Pm*Lec serves as a PRR for Gram-negative bacteria. Recently, two CRD domains discovered in *Pm*TL was detected only in the hepatopancreas and the transcript level decreased initially and then gradually increased after WSSV-challenge but was not effected by bacteria. Another C-type lectin, *Lv*LT from *L. vannamei*, contains two putative CRD domains and may be involved with the WSSV infection in shrimp (Ma et al., 2007). Finally, C-type lectin from *F. chiensis*, Fclectin contains dual CRDs and is expressed in hemocytes in Chinese shrimp *F. chinensis*. Expression profiles of *Fc*lectin were greatly modified upon bacteria, LPS or WSSV challenge. In addition,

Fc-hsL contains a single CRD and its expression was up-regulated only in hepatopancreas and induced in stomach after bacteria or viral-challeng.

Five types of tachylectin had been identified in horseshoe crab *T. tridentatus* including four from hemocytes (tachylectins-1 to 4) and one (tachylectin-5) from plasma (Kawabata and Iwanaga, 1999). Tachylectin 1 is inhibiting the growth of Gram-negative bacteria and has no ability against the Gram-positive bacteria (Saito et al., 1995). Tachylectin 2 exhibits bacterial agglutinating activity against *S. saprophyticus* and recognises several kinds of LPS (Okino et al., 1995; Kawabata and Iwanaga, 1999). Tachylectin 3 is specific to the human blood group A antigen and recognises LPS of several Gram-negative bacteria (Inamori et al., 1999). Tachylectin 4 has a binding specificity for fucose and specifically recognises S-type of LPS from several Gram-negative bacteria (Saito et al., 1997). Finally, tachylectin 5 recognises microbes, has a broad specificity to *N*-acetetylated substances and is active against both Gram-positive and Gram-negative bacteria (Kawabata and Iwanaga, 1999). After that, a tachylectin-related gene identified and characterized from the colonial marine hydroid *Hydractinia echinata* was shown to be similar to horseshoe crab tachylectin 1. The transcripts showed a constant level for all time points after LPS-challenge (Mali et al., 2006). Recently, a tachylectin-related homolog was characterized in amphioxus *Branchiostoma belcheri*. It showed abundant expression in the hepatic caecum and hindgut and was significantly increased in expression in gut after LPS-challenge (Ju et al., 2009).

2.14.4 Enzymes

Lysozyme is one of the main enzymes that exist in the lysosome (Misra et al., 2006) that catalyzes the hydrolysis of bacterial cell walls and acts as an innate immunity molecule against the invasion of bacterial pathogens. It had been isolated in several rustaceans including the freshwater crayfish (Fenouil and Roch, 1991), *Artemia franciscana* (Stabili et al., 1999), *Pandalus borealis* (Haug et al., 2002), *L. vannamei* (Sotelo-Mundo et al. 2003), *M. japonicus* (Hikima et al., 2003), *P. monodon* (QingMei et al., 2004) and *F. chinensis* (Bu et al., 2008). The lysozyme displayed activities against bacteria pathogen including *Vibrio* spp.(Hikima et al.,

2003; de-la-Re-Vega et al., 2006; Bu et al.,2008). In *P. monodon*, lysozyme showed a constant level of expression after *V. harveyi*-challenge (Tyagi et al., 2007).

SOD is an enzyme that catalyses the dimutation of superoxide to hydrogen peroxide and oxygen and has role in the defense against oxidative stress (Scandalias, 1993). It was identified and characterized in many crustacean species including *Palinurus vulgaris* (Smith and Doolilttle, 1992), *P leniuscufus* (Johansson et al., 1999), *L. vannamei* (Gross et al., 2001), *Callinectes sapidus* (Brouwer et al., 2003), *M. rosenbergii* (Cheng et al., 2006a; Cheng et al., 2006b), *M. nipponense* (Yao et al., 2007) and *F. chinensis* (Zhang et al., 2007b; Zhang, 2008). The mRNA transcripts of SOD were down-regulated during 3 to 6 h and up-regulated to narmal expression after WSSV- injection (Gómez-Anduroa et al., 2006; Zhang et al., 2007b; Zhang et al., 2008) and bacterial injection (Cheng et al., 2006a; Cheng et al., 2006b).

2.14.5 Peritrophic membrane

The PM is a chitin and glycoprotein layer that lines the invertebrate midgut. It is functionally similar to the mucous secretions of the vertebrate digestive tract but structurally different. The functions are to protect the midgut epithelium from abrasive food particles, digestive enzymes, and pathogens infectious (review by Hegedus et al., 2009). It was identified and characterized in invertebrate including myiasis fly *Chrysomya bezziana* (Vuocolo et al., 2001), tsetse *Glossina morsitans morsitans* (Hao and Aksoy, 2002), Green-bottle *Lucilia cuprina* (Tellam et al., 2003), *F. chinensis* (Du et al., 2006a). In shrimp, there are many studies in expression of peritrophin during reproduction and only one study about immune defense. In fleshy prawn, the expression of peritrophin was up-regulated in hemocyte, heart, stomach, gill, intestine and testis and very strong in ovary (Du et al., 2006a).

2.15 Gene expression analysis

2.15.1 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a rapid and convenient method to analyze the level of gene expression. It is a direct method for examination of gene expression of known transcripts in the target species. The RNA can not serve as a template for PCR so

a first strand complementary DNA (cDNA) was used as a template in the amplication of reaction. It is necessary to reverse transcribe mRNA into the first strand cDNA. Therefore, reverse transcription can be performed with oligo(dT) or random primer using reverse transcriptase. This enzyme is RNA-dependent DNA polymerase that has been used predominantly to catalyze first strand synthesis but are also capable of synthesizing a DNA stand complementary to a primed single stand DNA. Consequently, RQ1 RNase-free DNase is required to remove chromosomal DNA contamination in total RNA. The product is the second strand using a gene specific primer. Moreover, RT-PCR can be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene specific primers from original species and the first strand cDNA of the interesting species as the template.

The semi-quantitative RT-PCR method is based on the use of an internal control that is comprised in the PCR with the target gene using the same template. The housekeeping gene expressed at a very high level that is assumed to be expressed at a constant level throughout all sample analyzed. Besides, it is assumed that the expression levels of RNA sample are not altered by experimental conditions. The common internal controls are *β-actin, elongation factor 1 α* and *18s rRNA*. The PCR products are separated with agarose gel electhophoresis, stained with ethidium bromide and analyzed to observe relative expression of the target transcript.

However, agarose gel results of semi-quantitative RT-PCR are obtained from the end point of the reaction. The detection is very time consuming so the result may not be obtained for days and agarose gel resolution is very poor about 10 fold. In addition, the end point is variable from sample to sample, while gels may not be able to resolve the variability in yield. Consequently, semi-quantitative RT-PCR has some of the problems including poor precision, low sentivity, short dynamic range<2 logs, low resolution, non-automated, size-based discrimination only and ethidium bromide for staining is not a quantitative method.

2.15.2 Real-time reverse transcription polymerase chain reaction (real-time PCR)

Real-time PCR is a highly sensitive method for the detection and quantification of gene expression levels. This method uses a variety of different

fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. The reactions are characterized by the PCR cycle where the target amplification is first detected. This value is usually referred to as cycle threshold (C_T), the time at which fluorescence intensity is greater than background fluorescence. There are many advantages of using real-time PCR over other method such as producing quantitative data with an accurate dynamic range of 7 to 8 log, no post-amplification manipulation, more sensitive and detectable a single copy of a specific transcript. On the other hand, it requires expensive equipment and reagents that is the disadvantage of real-time PCR.

PCR can be catagorized into four major phases: the linear ground phase, early exponential phase, log-linear phase, and plateau phase. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. The cycle at which this occurs is known as C_T which value is representative of the starting copy number in the original template and is used to calculate experimental results.

Two different methods of analyzing data from real-time PCR including absolute quantification and relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Another quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time course study. Absolute quantification is considered to be more labor-intensive than relative quantification because of the necessity to create standard curves for quantification and include these standard curves in every PCR. However, when performing relative quantification, the data C_T used for comparison are random values and only applicable to the samples run within the same PCR. To compare samples between two different PCRs, it is necessary to include a reference control in every plate or run. In cases where data compared are assayed on different days or in different laboratories, absolute quantification may be preferred because results are based on a constant. In terms of fold-change data, absolute and relative quantification methods produce comparable results.

Relative quantification, changes in sample gene expression are measured based on either an external standard or a reference sample known as a calibrator. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantification assays. Depending on the method employed, these can yield different results and thus discrepant measures of standard error such as standard curve method Livak, 1997 , comparative C_T $2^{-\Delta\Delta C_T}$ method Livak and Schmittgen, 2001, Pfaffl model 2004, Q-gene Muller et al., 2002, Gentle et al., 2001, Liu and Saint, 2002 and DART-PCR Peirson, 2003. (review by Wong and Medrano, 2005)

The comparative C_T method is convenient way to analyze the relative changes in gene expression. This method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample. While this method includes a correction for non ideal amplification efficiencies, the amplification kinetics of the target gene and reference gene assays must be approximately equal because different efficiencies will generate errors when using this method. Consequently, a validation assay must be performed where serial dilutions are assayed for the target and reference gene and the results plotted with the log input concentration for each dilution on the x-axis, and the difference in C_T (target-reference) for each dilution on the y-axis. If the absolute value of the slope of the line is less than 0.1, the comparative C_T method may be used. The PCR product size should be designed less than 150 bp and the reaction rigorously optimized. Because the comparative C_T method does not require a standard curve, it is useful when assaying a large number of samples since all reaction wells are filled with sample reactions rather than standards. The data were analyzed using equation (Livak and Schmittgen, 2001):

Amount of target = $2^{-\Delta\Delta C_T}$

Where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Cal.})_{Time\,x} - (C_{T,Target} - C_{T,Cal.})_{Time\,0}$

Time *x* is any time point

Time 0 is the 1x expression

$C_{T,Target}$ is C_T value of target gene

$C_{T,Cal.}$ is C_T value of caribator

The amplified products can be detected by fluorescent dyes that are specific for double stranded DNA (dsDNA) or by sequence-specific fluorescent oligonucleotide probes. Today, SYBR Green dye is an alternate method used to perform real-time PCR reaction. This dye can bind the Minor Groove of double stranded DNA and the intensity of the fluorescent emissions increases (Figure 2.8). The SYBR Green dye is excited at a 485 nm wavelength and the emission is measured at a 520-nm wavelength. Conversely, the disadvantage is that it can bind to any dsDNA as a result the specific product, non-specific product and primer dimmers are detected similarly well.

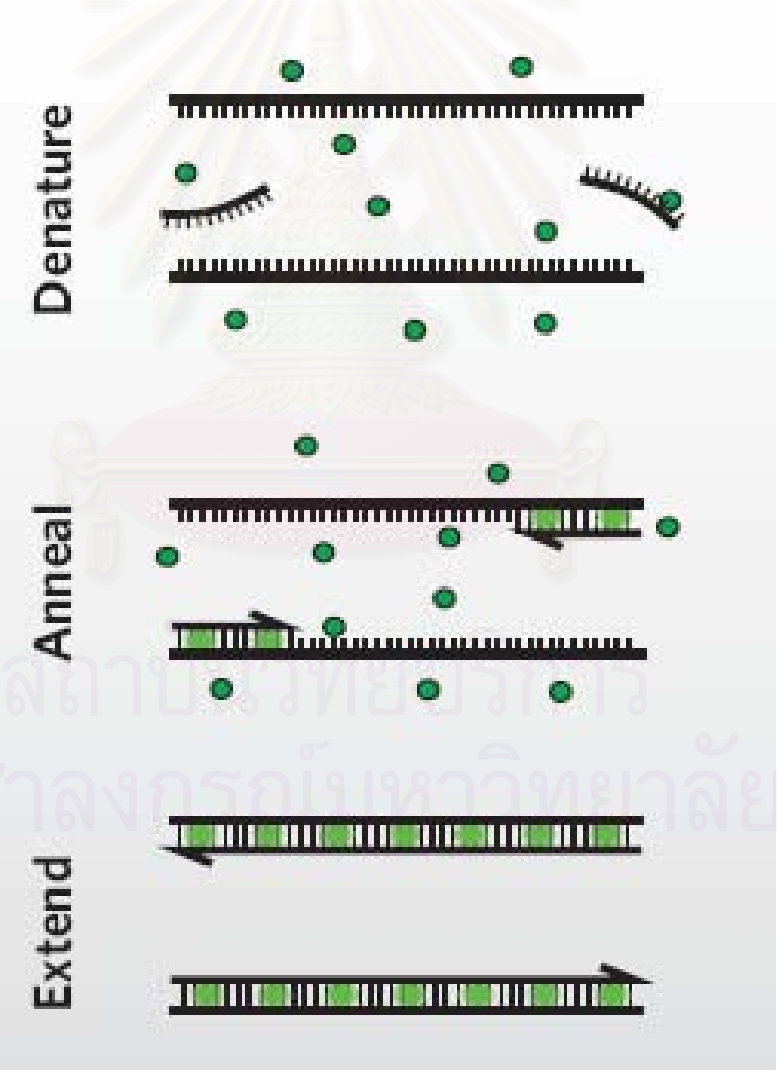

Figure 2.8 Detection of amplified products by SYBR Green. (http://www.the-scientist.com/article/display/36978/)

CHAPTER III
METHODOLOGY

3.1 Animals

Shrimp, *P. monodon*, post larvae (15 days old, PL15) and juveniles (approximately 3 month-old, 11-17 grams in body weight, and 11-13 cm in body length) were obtained from a hatchery in the Cha Cheung Sao province and shrimp farm from Nakhonsithammarat province, Thailand, respectively. They were transported to the Center of Excellence for Marine and Biotechnology (CEMB), Chulalongkorn University and maintained in tanks with running aerated water at 28±2 ºC, salinity at 20 parts per thousand (ppt). Prior to the initiation of the experiments only healthy shrimp and without contamination of bacteria, *V. harveyi*, were used and acclimated for 24 h where the experiment were to be performed.

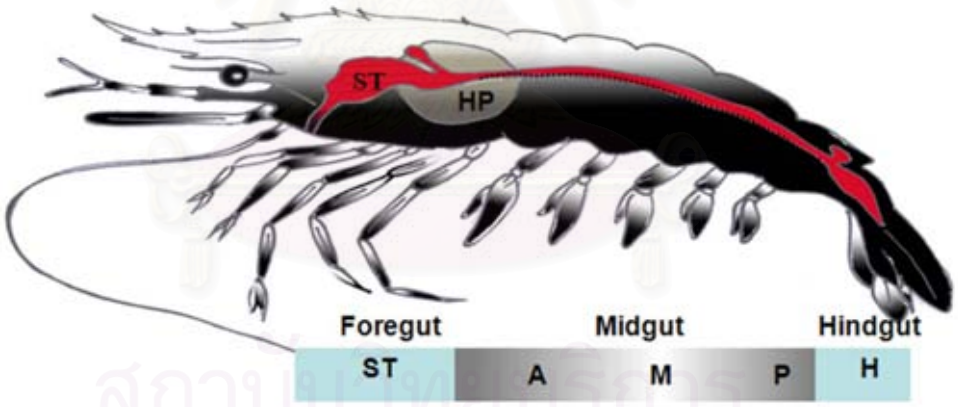

Figure 3.1 The different parts of the shrimp gut.

3.2 Infective experiments

3.2.1 Post larvae (PL) challenged by bacterial immersion

PL15 were randomly distributed in 2 sets of 6 rearing plastic boxes (2.5-L capacity), containing 1 L of seawater at a density of 100 PL per box. For histopathological study, the first set of experiment was designed including three

replicates of control (without *V. harveyi* immersion) and the other 3 replicates of *V. harveyi* immersion. The *V. harveyi* suspension was added into each box except the control boxes at the final concentration of approximately 3.2 x 10^5 CFU/ml. PL shrimps were no feeding during challenge period. Five PL were chosen at random from each box at 3, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 h post bacterial challenge and fixed in Davidson's fixative solution (Bell and Lightner, 1988). The second set of the experiment was designed in the same way as mentioned above for shrimp mortality observation only. The survival of the PL was recorded at 96 h post challenge.

3.2.2 Shrimp juvenile experiment

Ten juvenile shrimp (wet weight approximately 13.22 ± 1.90 grams) were placed in 12-L plastic boxes containing 10 L of concentrated bacteria solution (approximately 1x10^7 CFU/ml in 20 ppt salinity sea water). Three replicates were carried out. One shrimp was randomly collected the digestive system at 6, 12, 24, 48, 72 and 96 h after bacterial immersion and fixed in R-F fixative (Hasson et al, 1997) for 48 h and then the samples were changed to 70% ethanol and kept at 4 ºC for histopathological processing. Naïve shrimp, before infection (time 0) served as control.

3.2.3 Statistical analysis

Statistical analysis was performed on the SPSS 11.5 for Window. Data were expressed as mean ± SD. Significant difference of mortality between control and *V. harveyi*-challenge was determined by paired Student's t test (two-tail). A *P* value <0.05 was considered statistically significant for t test.

3.3 Morphological and histopathological studies in shrimp challenged with *V. harveyi* by immersion

3.3.1 Light microscopy (LM)

3.3.1.1 Sample

The samples were as mentioned in session 3.2.1 and 3.2.2.

3.3.1.2 Paraffin embedding and sectioning

The fixed digestive organs were dissected for parts of interest including stomach, hepatopancreas and intestine by a single edge razor blade. The stomach and hepatopancreas were longitudinally bisected and the intestine was dissected into three part (A, M and PH). All samples were placed into histological embedding cassettes and were dehydrated in the following solutions of ethanol series as 70% ethanol (two separate 1 h baths), 85% ethanol (two separate 1 h baths), 95% ethanol (two separate 1 h baths), 100% absolute ethanol (two separate 1 h baths), chloroform (two separate 1 h baths), and paraffin (Bio-Optica) (two separate 1.5 h baths). The successfully embedded tissues were placed in embedding molds which has melted paraffin to form blocks ready to be sectioned and then the embedding molds were placed on cold trays for about 20 minute. Finally, the infiltrated tissues were separated from embedding molds. The infiltrated tissue blocks were used for sectioning.

The tissue blocks were face-trimmed until the tissue can be seen. Each sample was cut into 5 µm thick sections. The ribbon of sections was placed on water bath at 42 ºC and the ribbon float and spread. The glass slides were used to collect the ribbon from water bath, and then excess water was removed, and then they were left to dry at room temperature and firmly fixed by placing on slide warmer at 45 ºC overnight. The slides were kept in slide box until use.

3.3.1.3 Staining

The tissues were sectioned using a modified method of Bell and Lightner (1988) and stained using hematoxylin and eosin (H&E; Humason, 1979). Briefly, all tissue sections were immersed in following solutions: xylene I 5 minutes, xylene II 5 minutes, absolute ethanol I 3 minutes, absolute ethanol II 3 minutes, 95% ethanol 3 minutes, 70% ethanol 3 minutes, slowly dripping tap water 5 minutes, hematoxylin staining (Mayer’s hematoxylin (Bio Optica)) 6 minutes, slowly dripping tap water 5 minutes, blue the hematoxylin with few drop of ammonia in tap water 3 minutes, slowly dripping tapwater 3 minute, 70% ethanol 2 minutes, 95% ethanol 2 minutes, eiosin staining (Eosin 1% aqueous solution (Bio-Optica)) 4 minutes, absolute ethanol I-3 minutes, absolute ethanol II - 4 minutes, absolute ethanol III - 4

minutes, xylene I - 3 minutes, xylene II - 3 minutes, xylene III - 3 minutes. Finally, the samples were mounted by mounting medium (Bio-Optica).

3.3.2 Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM) three parts of intestine including anterior midgut which adjacent to the stomach, middle of midgut and middle of hindgut were cut into short cylindrical segments with razor blades, and the segments were immersed in fixative (3 % glutalaldehyde in 0.1 M phosphate buffer (pH 7.4)) at 4 ºC for 24 h and the samples were changed into new fixative solution and kept at 4 ºC for further processing.

All samples were sent to Scientific and Technology Research Equipment Centre, Chulalongkorn University for analysis by transmission electron microscopy model JEM-2100.

3.4 Expression of immune-related genes in unchallenged shrimp by RT-PCR

3.4.1 Samples

Intestines were dissected from three healthy juvenile shrimp and then equally divided into three parts including anterior midgut (A), middle midgut (M) and posterior with hindgut (PH) (Figure 3.1). All samples were immediately frozen in liquid nitrogen and kept at -80 ºC until used.

3.4.2 Total RNA preparation

Each sample was homogenized in 1 ml of TriReagent® (Molecular Research Center, Inc.) (1 ml/50-100 mg tissue) and stored at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. The mixture was subjected to chloroform extraction, 200 µl of chloroform were added then vortexed for at least 15 seconds and incubated at room temperature for 15 minutes. After centrifugation at 12,000xg at 4 ºC for 15 minutes, the mixture was separated to the lower phenol-chloroform phase (red), the interphase and the upper aqueous phase (colorless). The aqueous phase containing total RNA was carefully transferred to a new microcentrifuge tube. Total RNA was precipitated with an equal volume of

isopropanol and incubated at room temperature for 10-15 minutes. The sample was centrifuged at 12,000xg at 4 ºC for 10 minutes. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol followed by centrifuged at 12,000xg at 4 ºC for 10 minutes. The ethanol was removed. The RNA pellet was briefly air-dried for 5-10 minutes. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated H_2O and was immediately used. Alternatively, the RNA pellet was kept in absolute ethanol at -80 ºC until used.

Chromosomal DNA contamination in total RNA samples was removed by treating 1 µg of total RNA with 0.5 unit of RQ1 RNase-free DNase (Promega) at 37 ºC for 30 minutes and purified by an equal volume of phenol, phenol/chloroform (1:1[vol/vol]) and chloroform, respectively. DNA-free RNA was precipitated with equal volume of isopropanol and 0.1 volume of 3 M sodium acetate ($Na_2C_2H_3O_2$).

The RNA concentration was determined by measuring the optical density at 260 nM (OD_{260}). One OD_{260} unit corresponds to approximately 40 µg/ml of RNA. As a result, the concentration of RNA were estimated in µg/ml using the following equation:

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40$$

Protein has a maximum absorption at 280 nM. Therefore, the purity of RNA could be estimated from a ratio of OD_{260}/OD_{280}. The ratios of purified RNA were appropriately 2.0 (Sambrook et al., 2001).

3.4.3 First strand synthesis

The first strand cDNA was synthesized from 1.5 µg of DNA-free RNA using an ImPromII™ Reverse Transcription System Kit (Promega). DNA-free RNA was combined with 0.5 µg of oligo (dT_{15}) and appropriated DEPC-treated H_2O in a final volume of 5 µl. The reaction was incubated at 70 ºC for 5 minutes and immediately chilled on ice. And then the reverse transcription reaction mixture (1x reaction buffer, 2 mM $MgCl_2$, 0.8 mM dNTP Mix, 20 unit Recombinant RNasin® Ribonuclease Inhibitor and 1 µl of ImProm-II™ Reverse Transcriptase) was added and gently mixed. The reaction was incubated at 25 ºC for 5 minutes and 42 ºC for 90 min. The reaction was terminated by incubated at 70 ºC for 15 minutes to stop reverse transcriptase activity.

3.4.4 Reverse transcription (RT)-PCR of immune gene

3.4.4.1 Primer design

PCR primers were designed from sequence of known genes in EST libraries or from Genbank. Each pair of forward and reverse primers had closely similar Tm values and they were checked for minimal hairpin self-dimer and hetero-dimer formation.

3.4.4.2 RT-PCR

RT-PCR was set up in a total volume of 25 µl containing 2-4 µl of a 1:20 dilution of cDNA template, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 2 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each appropriate forward and reward primers (Table 3.1) and 1 unit of DyNAzyme II DNA polymerase (Finnzymes). All reactions were carried out in Peltier Thermal Cycler (MJ Research) and cycling parameters were predenaturation step of 94 ºC for 3 minutes; followed by 22-35 cycles of denaturation at a 94 ºC for 30 seconds, annealing at a 55-60 ºC for 45 seconds and extension at a 72 ºC for 45 seconds and the final extension at a 72 ºC for 7 minutes. The housekeeping gene *elongation factor 1α* was used as an internal control for RT-PCR experiment.

3.4.4.3 Agarose gel electrophoresis

A proper percentage of agarose gel was prepared using 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.3). The slurry of agarose in TBE buffer was melted in a microwave oven to complete solubilization and allowed to cool lower than 60 ºC before it was poured into a casting tray with a well comb. The agarose gel was allowed to solidity. The comb was removed and the solid gel was submerged in a chamber containing an enough amount of TBE buffer covering the gel for approximately 0.5 cm.

Table 3.1 Primer sequence of immune-related genes for RT-PCR.

Gene	Gene Bank acession No.	References	Primer sequence (5' →3')	Product size (bp)
Antimicrobial peptides				
antilippopolysacharide factor isoform2 (***ALF2***)	BI784449	Tharntada et al., 2008	CAAGCGGTGCAGGACCTCC (F) TTAGTGCTCAAGCCAAATCCTGG (R)	297
antilippopolysacharide factor isoform3 (***ALF3***)	BI018071	Tharntada et al., 2008	CAAGGGTGGGAGGCTGTGG (F) TGAGCTGAGCCACTGGTTGG (R)	286
crustin	BI784446	Supungul et al., 2004	TCCCTGGAGGTCAATTGAGTG (F) AGTCGAACATGCAGGCCTATCC (R)	233
penaeidin	BI784459	Supungul et al., 2004	AGGATATCATCCAGTTCCTG (F) ACCTACATCCTTTCCACAAG (R)	243
Melanization and adhesion				
Peroxinactin (***PrX***)	AF188840	Sritunyalucksana et al., 2001	CGAAGCTTCTTGCAACTACCA (F) GCAGGCTGATTAAACTGGCTT (R)	547
Prophenoloxidase (***proPO***)	AF099741	Sritunyalucksanaet al., 1999	TGGCACTGGCACTTGATCTA (F) GCGAAAGAACACAGGGTCTCT (R)	590
Lectins				
C-type lectin (***C-lecitn***)	DQ078266	Luo, et al. 2006	AAAGTCACACCACCGAAGCAG (F) TCAAGGCAGTTCTCGTTTCC (R)	508
ß-1,3-Glucan Binding Protein (***BGBP***)	AF368168	Sritunyalucksanaet al., 2002	ACGAGATAACCATGTCCGGC (F) CATCGGCGAAGGAACCTGTA (R)	558
Macrophage mannose receptor 1 precursor homolog (***MMR***)	XP_001235106	Unpublished data	CCGCTGCTTATCTGGTCTTG (F) GCT TCG GTC TCC GCA CTT T (R)	439
Tachylectin 5a1 (***TL5a1***)	GlEp-N-N01-1607-LF	Unpublished data	GCCAGAGCCTGAACCAGATCC (F) TGTCCGTGCCAATCATACCAG (R)	350

Table 3.1 Cont.

Gene	Gene Bank acession No.	References	Primer sequence (5' →3')	Product size (bp)
Lectins				
Tachylectin 5a2 (***TL5a2***)	HC-N-N01-1087-LF, HC-N-N01-11852-LF	Unpublished data	GGCCTGCAGGAGATGAGAAT (F) AATGCCGGCCTTATCATCA (R)	386
ficolin 2 isoform b precursor homolog (***ficolin***)	HPa-N-N03-2032-LF	Unpublished data	GAAGACAACGAGAACATCAG (F) CTCGCAGTTGGTCTGGTTCG (R)	534
Enzymes				
Lysozyme	AF539466	Tyagi et al., 2007	GGCCTCCGTAAGGAACATTT (F) CTTGCTGTTGTAAGCCACCC (R)	460
Cytosolic manganese superoxide dismutase (***MnSOD***)	AY726542	Unpublished data	CAGTTCGCAAATGCAGCAGA (F) CAAGACCGAGCAATGGAACC (R)	460
Peritrophic membrane				
mucin-like peritrophic membrane (***Mucin-like PM***)	IN-N-S01-0247-LF	Unpublished data	ATTGGCAGCATCCTACCGAC (F) CGGATGAGGAATGTGGCAA (R)	653
Internal control gene				
Elongation factor 1α (***EF1α***)	DQ021452	Loongyai et al., 2007	ATGGTTGTCAACTTTGCCCC (F) TTGACCTCCTTGATCACACC (R)	500

Note to Table3.1

HC-H-S01 = Hemocyte-heat stressed library, GlEp-N-N01 = Gill-Epipodite-normalize library, HC-N-N01 = Hemocyte-normalilzed library,HPa-N-N03 = Hepatopancreas-normalized 3 library, IN-N-S01 = Intestine library

The 5 µl of PCR product were mixed with 2 µl of the 10x loading dry (0.25% bromophenal blue and 25% ficoll in water) and loaded into the well. A DNA ladder (100 bp marker) was used as standard DNA marker. Electrophoresis was carried out in TBE buffer at 100 volts until the bromophenol blue dry marker was moved about ¾ of the gel length.

The electrophoresed gel was stained in a 0.5 µg/ml ethidium bromide solution for 1 minutes and destained to remove unbound ethidium bromide from the gel by submerged in TBE buffer for 20 minutes. The amplicon of the PCR product was visualized under a transilluminator (BioRad) and photographed.

3.5 Expression of immune-related genes in *V. harveyi* challenged shrimp by RT-PCR

3.5.1 Dectection of *V. harveyi* by PCR

3.5.1.1 Sample

To investigate whether *V. harveyi* were presented in shrimp intestine or not, shrimp were randomly sampled for detection of contamination of bacteria, *V. harveyi*, in the intestine prior to the initiation of the experiments. Intestine samples were subjected to DNA extraction and using specific primer of *V. harveyi* for *V. harveyi* detection (Thaithongnum et al., 2006).

3.5.1.2 DNA extraction

Genomic DNA of shrimp intestine was extracted according to Xia et al. (2005). The sample was homogenized in 800 µl of extraction buffer (0.15 M NaCl, 0.1 M Na_2EDTA (pH 8.0) containing 15 mg of lysozyme/ml) and then 10 µl of proteinase K (10 mg/µl) were added into the mixture. The mixture was incubated and occasionally shaked (200 rpm) at 37 ºC for 30 minutes. After that 10 % sodium dodecyl sulfate (SDS) was added and incubated at 37 ºC for 2 hours. The sample was shaken every 15-20 minutes during the period of incubation. Afterward, the sample was centrifuged at 8,000xg for 10 minutes and the supernatant was transferred to the new microcentrifuge tube. The equal volume of chloroform-isoamyl alcohol (24:1[vol/vol]) was added to the supernatant and gradually mixed. The mixture was centrifuged at 8,000xg for 10 minutes. The supernatant was transferred to the new

microcentrifuge tube and 0.6 vol isopropanal was added. The sample was incubated at room temperature for 1 hour and centrifuge at 8,000xg for 10 minutes. The supernatant was removes and DNA pellet was washed with 1 ml of 75% ethanol. The sample was centrifuged at 8,000xg for 10 minutes and after that the ethanol was removed. The DNA pellet was dried and then added to an appropriate of TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). The DNA was incubated overnight at room temperature for a complete solution and kept at 4 ºC.

The DNA concentration was determined by measuring the optical density at 260 nM (OD_{260}). One OD_{260} unit corresponds to approximately 50 µg/ml of DNA. As a result, the concentrations of DNA were estimated in µg/ml using the following equation:

$$[DNA] = OD_{260} \times \text{dilution factor} \times 50$$

Protein has a maximum absorption at 280 nM. Therefore, the purity of DNA could be estimated from a ratio of OD_{260}/ OD_{280}. The ratios of purified DNA were appropriately 1.8 (Sambrook et al., 2001) and the quality of DNA was checked with 0.8% agarose electrophoresis and λDNA was maker.

3.5.1.3 PCR

PCR was set up in a total volume 25 µl containing 25 ng of DNA template, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of published specific primers A2: TCTAACTATCCACCGCGG and B3: AGCAATGCCATCTTCACGT TC (Thaithongnum et al., 2006) and 1 unit of DyNAzyme II DNA polymerase (Finnzymes). All reactions were carried out in Peltier Thermal Cycler (MJ Research) and cycling parameters were predenaturation step of 94 ºC for 3 minutes; followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 45 seconds and extension at 72 ºC for 45 seconds and the final extension at 72 ºC for 7 minutes. *V. harveyi* stain 1526 (Rengpipat et al. 2003) was served as positive control.

3.5.2 Preparation of *V. harveyi*

Vibrio harveyi from a glyceral stock culture was cultured in 25 ml tryptone soya broth (TSB) supplement with 2% [w/v] NaCl and were incubated with shaking at 250 revolutions min^{-1} at 28 ºC for 16 h. One ml was subsequently

inoculated in 25 ml of the same medium and cultured at the same condition. After 16 h bacteria were washed twice with 2 % NaCl by centrifugation at 3600xg for 10 minutes at 4 ºC. The number of *V. harveyi* is estimated by measuring the optical density at 600 nM (OD_{600}). An OD_{600} 1.0 corresponds to the number of *V. harveyi* approximaely $1.0x10^8$ CFU/ml.

Cell concentrations were also adjusted and verified by viable plate counts according to standard methods as described in Collins and Lyne (1976). The bacterial solution was diluted at final concentration of $1.0x10^7$ CFU/ml in 10 liters of 20 ppt sea water for immersion experiment. The number of *V. harveyi* in seawater was monitored when shrimp were collected at 0, 3, 6, 12, 24 and 48 h post immersion.

3.5.3 Samples

Ten juvenile shrimp were maintained in a plastic tank (43.5×27.5×25.5 cm) containing 10 liters of seawater for 24 h. Five replicates were carried out and *V. harveyi* solution was added into each tank at final concentation of approximately $1x10^7$ CFU/ml. One shrimp was randomly collected from each tank at 3, 6, 12, 24 and 48 h after bacterial immersion. Shrimp at time 0 without bacterial immersion were served as control. The intestine was dissected and divided into 2 parts including anterior and middle midgut (AM) as well as posterior with hindgut (PH) (Figure 3.1). Stomach and hepatopancreas were also dissected. All samples were immediately frozen in liquid nitrogen and kept at -80 ºC. To collect shrimp hemocytes, the hemolymph was bled from the ventral sinus of each shrimp using a 24 G/1/2 inch needle fitted into a 1.0 ml syringe preloaded with 500 µl of anticoagulant (10% sodium citrate, w/v). The samples were immediately centrifuged at 3,600xg at 4 ºC for 5 minutes to separate hemocytes from the plasma. The hemocyte pellet was resuspended in 1 ml of TriReagent® homogenized and kept at -80 ºC until used.

3.5.4 Total RNA preparation and first strand synthesis

Total RNA was extracted from 2 parts of intestine: (AM and PH), hemocytes, stomach and hepatopancreas using the same method as mentioned in session 3.4.2 and first strand synthesis as mentioned in session 3.4.3

3.5.5 Reverse transcription (RT)-PCR of immune genes and agarose gel electrophoresis

PCR primers were used as the same primer sequences in table 3.1. RT-PCR reaction and the condition of PCR were done as described previously in session 3.4.4.

3.6 Expression of immune-related genes in shrimp challenged with *V. harveyi* by real-time PCR

3.6.1 Sample

The samples of intestines in session 3.5.3 were used to confirmed the results of RT-PCR by real-time PCR.

3.6.2 Primer design

From our study of the expression of immune-related genes in *V. harveyi* challenged shrimp by RT-PCR, upregulated genes were selected for real-time PCR. PCR primers listed in Table 3.2 were designed from nucleotide sequence of the genes by using a free program on internet (www.idtdna.com). Each pair of primers should be considered to include amplicons in the size range between 100 to 220 bp, a GC content at least 50%, primer length at 18-21 bp and Tm at 59-60 ºC. and they were checked for hairpin, self-dimer and hetero-dimer formation.

3.6.3 Real-time RT-PCR

Real-time RT-PCR was performed in a total volume of 20 µl containing 0.5 µl of cDNA template, 10 µl of 2x iQ™ SYBR® Green Supermix (Bio-Rad) and 0.2 µM of each specific forward and reverse primers (Table 3.2). All reactions were carried out in iCycler (Biorad) and cycling parameters were 95 ºC for 3 minutes; 40 cycles of 95 ºC for 30 seconds, 59 ºC for 20 seconds, and 72 ºC for 30 seconds; final extension at 72 ºC for 1 minutes. The specificity of PCR products was analyzed by dissociation curve analysis which was performed at the end of realtime PCR cycle by continuously heating from 55 ºC to 95 ºC at the increment of 0.5 ºC.

The housekeeping gene *elongation factor 1α* was used as an internal control for all realtime PCR experiments.

Table 3.2 Primer sequence of immune-related genes for real-time PCR.

Gene	Primer sequence (5' →3')	Amplicon Size (bp)
ALF3	TCTCATCTCTCAACAGGAGGCCAA (F) GGTAGAGCTTCCATTGCCAACTGC (R)	103
crustin	AGTTCCTGGAGTTGGAGGTGGATT (F) ACCTCGTTCTGCAGTAATTGCACTC (R)	119
penaeidin	ACAGTCGTATTTGTCCCAGCAGGT (F) AACACCAACCACACACAGACCCAT (R)	111
C-lectin	AGTGCTGGACGAGTGCTTCTATCT (F) TTGAGAGCATAGACGTTCCTGGGT (R)	117
TL5a1	TTGGTGGTACACGAAATGTCACGC (F) AAGGAGTAATGGTGTCCGTGCCAA (R)	117
mucin-like PM	ACTGGAAACCGAAGGATGTTCCCT (F) TTGTTGCAGTCCTTGTGTGGCTTG (R)	123
EF1α	AGGCGTACTGGTAAGGAACTGGAA (F) AGAGGAGCATACTGTTGGAAGGTCTC (R)	123

3.6.4 Data analysis of real-time RT-PCR

The specificity of PCR products was detected by SYBR Green dye. When SYBR dye binds to double stranded PCR product, the intensity of fluorescent emissions increased and was analyzed by the data analysis software of iCycler iQ^{TM} Real-time Detection system (Biorad) using PCR base line Subtracted curve fit method. The level of detection at which a reaction reaches a fluorescent intensity above background was called threshold line. It was set in the exponential phase of the amplification. The cycle at which the sample reaches threshold line was called the cycle threshold (C_T). The values of cycle threshold were important for data analysis.

Relative quantification and the mathematical model described by Pfaffl (2001) was used to determine the relative expression. The change in gene expression was measured based on a calibrator which is the *elongation factor 1α* gene. Both target gene and calibrator are amplified in separate tubes so C_T values were averaged before performing the ΔC_T calculetion. The data were analyzed using equation.

Amount of target = $2^{-\Delta\Delta C_T}$

Where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Cal.})_{Time\ x} - (C_{T,Target} - C_{T,Cal.})_{Time\ 0}$

Time *x* is any time point

Time 0 is the 1x expression

$C_{T,Target}$ is C_T value of target gene

$C_{T,Cal.}$ is C_T value of caribator

3.6.5 Statistical analysis

Statistical analysis was performed on the Data Analysis tool of Microsoft Excel program (Microsoft Office 2007, Microsoft Inc., Seattle, WA). Data were expressed as mean ± SD. Significant difference of gene expression between each time after Vh-challenge was determined by paired Student's t test (two-tail). A *P* value <0.05 was considered statistically significant for t test. The t statistic to test whether the means are different can be calculated using equation.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_1 X_2} \cdot \sqrt{\frac{2}{n}}}$$

where $S_{X_1 X_2} = \sqrt{\frac{s_{X_1}^2 + s_{X_2}^2}{2}}$

Here $S_{X_1 X_2}$ is the grand standard deviation, 1=group one, 2=group two. For significance testing, the degrees of freedom for this test is 2n−2 where n is the number of participants in each group.

3.7 Tissue distribution of immune-related genes

3.7.1 Sample

The sample was collected from two juvenile shrimp: male and female. Epidermis, eye stalk, gill, heart, hemocyte, hepatopancreas, midgut, midgut and hindgut, lymphoid organ, muscle, abdominal ganglion, pleopod, stomach, torasic ganglion, testis and ovary were separately collected. RNA was extracted as mentioned in session 3.4.2 and synthesized first stand cDNA as mentioned in session 3.4.3.

3.7.2 End point RT-PCR

RT-PCR was set up in a total volume 25 µl containing 2-4 µl of a dilution of cDNA template from epidermis, eye stalk, gill, heart, hemocyte, hepatopancreas, midgut, midgut and hindgut, lymphoid organ, muscle, abdominal ganglion, pleopod, stomach, torasic ganglion, testis or ovary, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 2 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each appropriate forward and reward primers (Table 3.1) and 1 unit of DyNAzyme II DNA polymerase (Finnzymes). All reactions were carried out in Veriti 96 well Thermal Cycler (Applied Biosystems) and cycling parameters were predenaturation step of 94 ºC for 3 minutes; followed by 25 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55-60 ºC for 45 seconds and extension at 72 ºC for 45 seconds and the final extension at 72 ºC for 7 minutes. The housekeeping gene *elongation factor 1α* was used as an internal control for RT-PCR experiment.

CHAPTER IV
RESULTS

4.1 Pathogenicity of *V. harveyi* in shrimp

This bacterium was able to cause quite rapid death in PL. With a bacterial concentration of 3.2 x 10^5 CFU/ml, PL had a significantly higher mortality (85 %) than the control group (36.3%) (P<0.05) after 96 h post infection (Figure 4.1). In contrast, no mortality was observed in the juvenile shrimp even at a higher dose of bacteria such as at concentrations of 1x10^7 CFU/ml after 96 h post infection.

Infected or moribund shrimp had a lethargic and sluggish behavior in swimming, or were quiescence with or without leg movement.

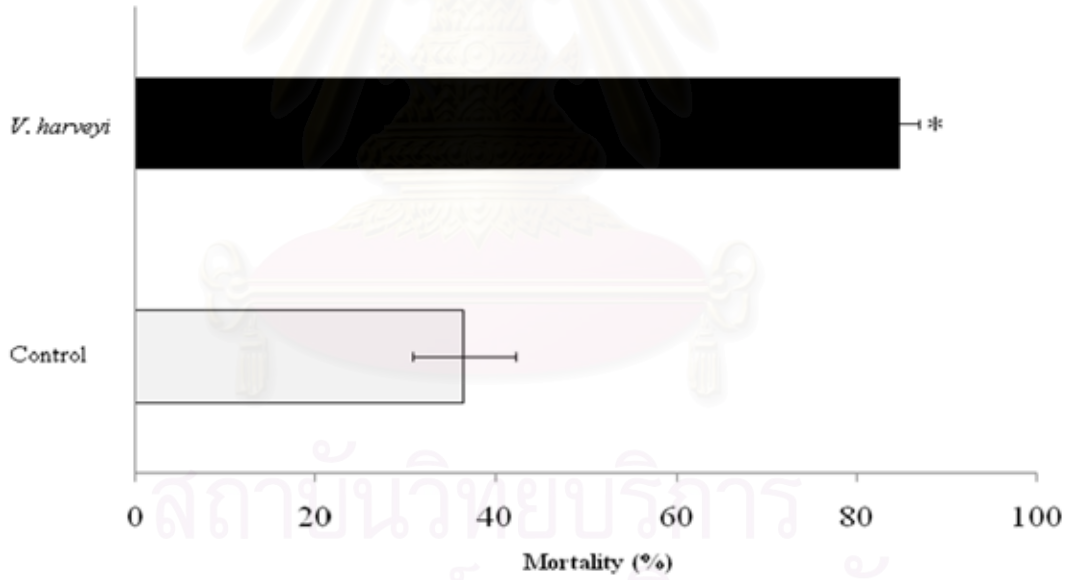

Figure 4.1 Mortality of PL15 challenged by immersion with *V. harveyi* after 96 h post bacterial immersion.

4.2 Morphological and histopathological studies in shrimp challenged with *V. harveyi* by immersion compared with normal healthy shrimp

4.2.1 Histology of normal shrimp

Histopathology by hematoxylin and eosin staining show normal tissues of digestive organs (Figure 4.2) of the control PL, in an overall longitudinal view of the hepatopancreatic lobe (HP) and its insertion in the gastrointestinal tract (ST). The primary duct (Hpd), connecting it to the gastrointestinal tract, lies between the posterior stomach chamber and the anterior end of the midgut (Mid) (Figure 4.2A). The hepatopancreas is a bilaterally bilobed brown-yellowish organ. The structure is formed by a mass of blind tubules, with scarce intertubular space. Each tubule consists of a cylindrical epithelial layer surrounded by a basal lamina and myoepithelial cells. Normally, four cellular types can be recognized in the hepatopancreas, which represent embryonic (E), fibrillar (F), resorptive (R) and blister-like (B) cells. Cross sections of hepatopancreatic tubules are shown in Figure 4.2B and the heart (H) is noted on the top of the hepatopancreatic lobe. A longitudinal view of the anterior region of the midgut, just posterior to its union with the stomach is shown in Figure 4.2C. The midgut is composed of the mucosal epithelium (Emp) with simple columnar cells. These are supported by the basement membrane (Bas), circular muscles and longitudinal muscle, which were not seen in this preparation (Figure 4.2C). The surface adjacent to the lumen (Lum) has a brush or microvillous border (MV). In Figure 4.2D, the peritrophic membrane (PM) is seen in the midgut. Also the hepatopancreas, midgut, and hindgut of uninfected juvenile shrimp are shown in Figure 4.3A, 4.3B, 4.3C and 4.3D, respectively. The embryonic (E), fibrillar (F), resorptive (R) and blister-like (B) cells are seen in the hepatopancreas in Figure 4.3A and the circular muscles (Msx) and longitudinal muscle (Msl) can be seen in Figure 4.3B.

Semithin cross sectional view of midgut and hepatopancreas (Figure 4.4A) and its enlarged view (Figure 4.4B), middle midgut (Figure 4.4C) and hindgut (Figure 4.4D) from the PL controls were observed under bright field microscopy. The results show that these tissues are still intact. Ultra-thin cross sections of anterior midgut (Figure 4.5A), middle midgut (Figure 4.5B) and hindgut (Figure 4.5C) from

the PL controls were observed by transmission electron microscopy. Epithelial cells of midgut and even the microvilli are seen and they have retained their integrity. In the hindgut (Figure 4.5C), a thin, non-calcified cuticle (Cu) lies adjacent to the hindgut lumen. Underlying the cuticle is the simple epithelia (Epm) and tegumental glands (Teg) occupying the majority of the hindgut folds.

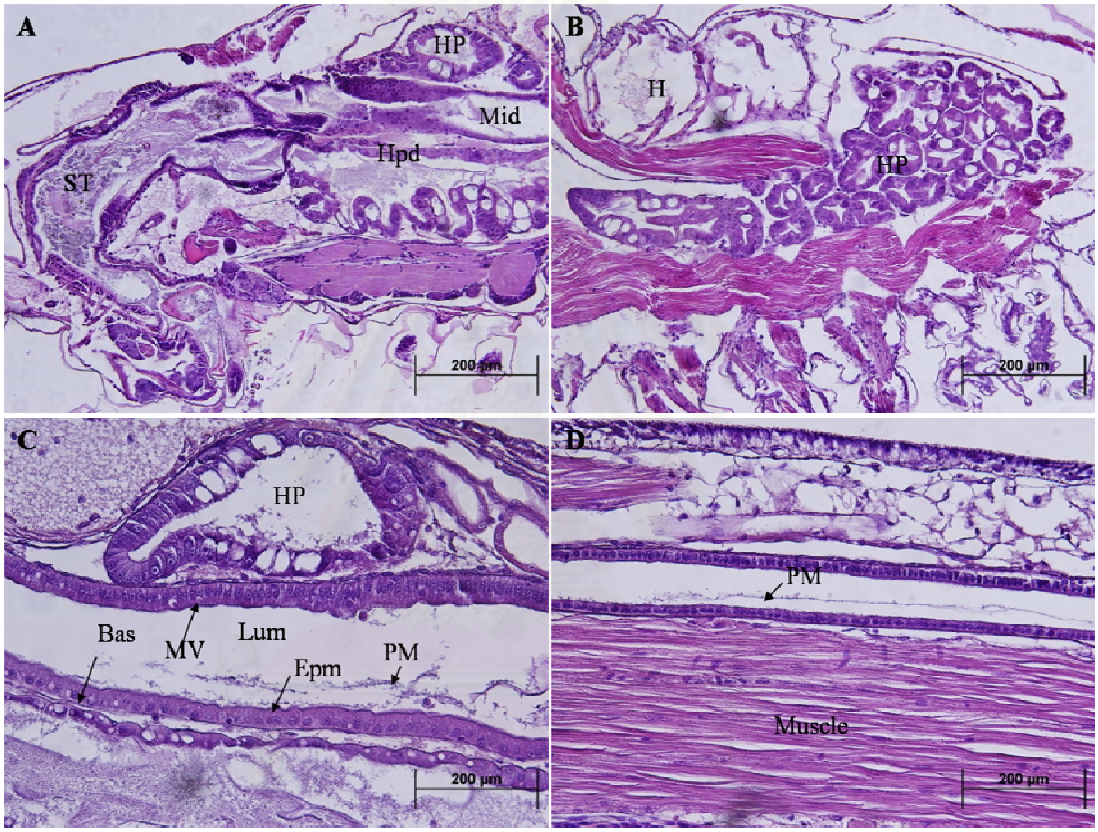

Figure 4.2 Post larval shrimp from the control group show normal appearance of the tissues. (A) Overall longitudinal view of the hepatopancreas and foregut. (B) Normal hepatopancreas. (C) Anterior midgut of control PL. (D) The middle midgut. HP= hepatopancreas; Hpd=hepatopancreatic duct; Mid=midgut; H=heart; Bas=basement membrane; Epm=epithelial membrane; Lum=lumen; PM=peritrophic membrane.

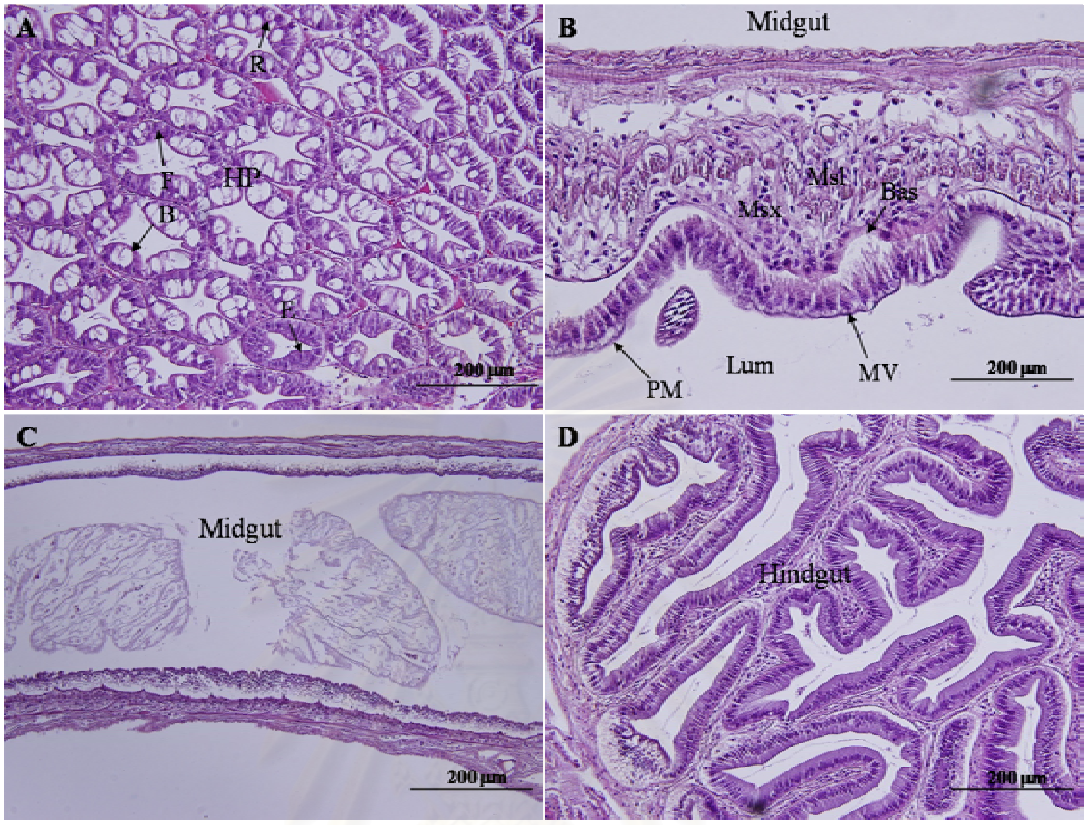

Figure 4.3 Juvenile shrimp from the control group show normal morphology of (A) the hepatopancreas, (B-C) the midgut and (D) the hindgut. HP= hepatopancreas; E=embryonic cells, F=fibrillar cells, R=resorptive cells; B=blister-like cells; Bas=basement membrane;; Lum=lumen; PM=peritrophin; MV=microvillous border; Msx=circular muscles; Msl=longitudinal muscles.

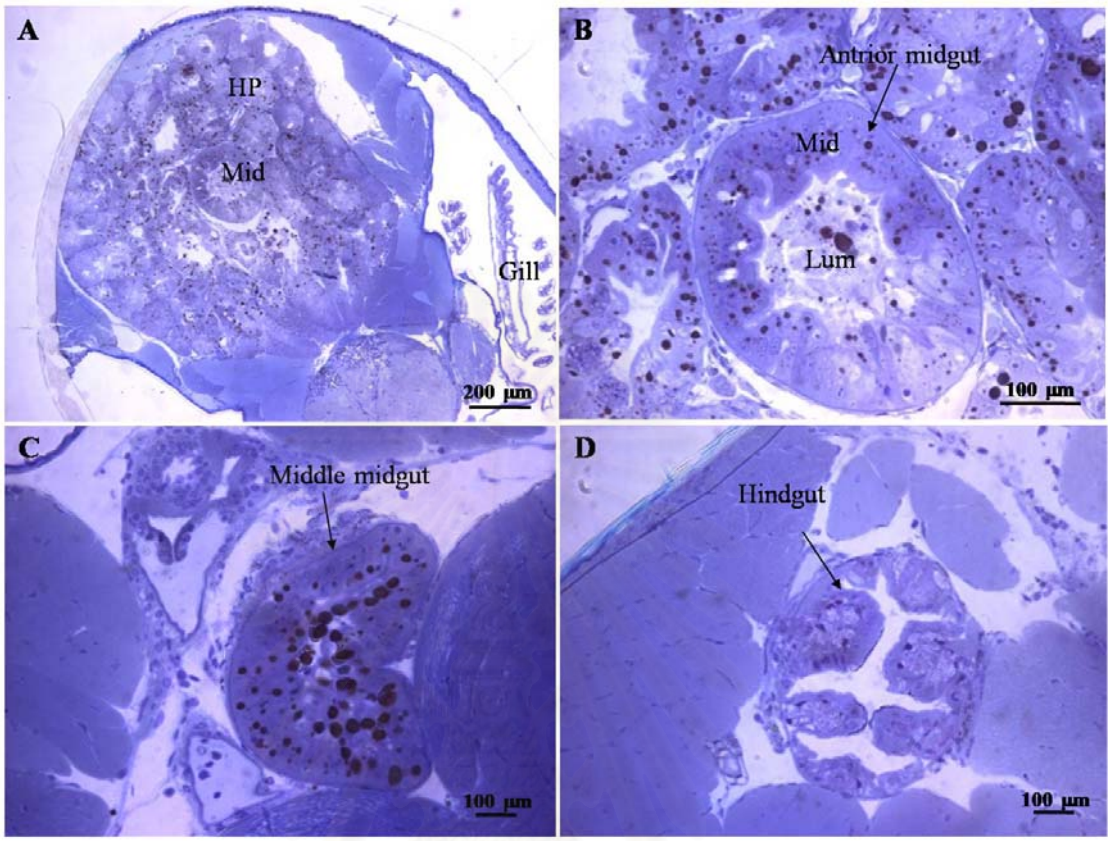

Figure 4.4 Semithin transversal sections of post larval shrimp from the control group. (A-B) Anterior midgut and the hepatopancreas. (C) Middle midgut. (D) Hindgut. HP= hepatopancreas; Mid=midgut; Lum= lumen.

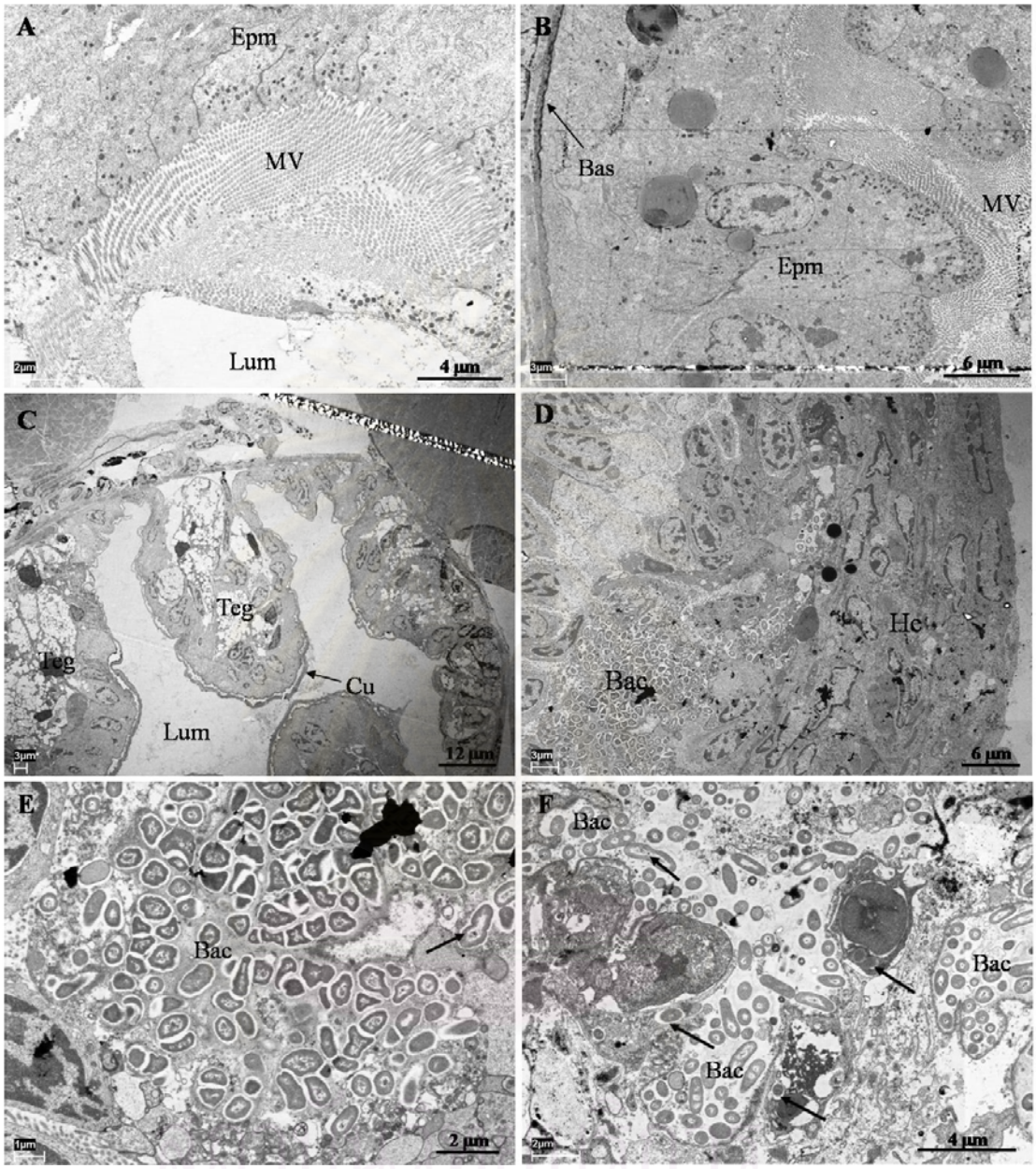

Figure 4.5 Electron micrographs showing normal and infected tissues. (A) Anterior midgut of normal shrimp. (B) Middle midgut of normal shrimp. (C) Hindgut of normal shrimp. (D) Large number of bacterial cells were seen among cell debris and these bacteria were deformed. (E) Binary fission of bacterial cells (Bac) was noted (arrow). (F) Some bacterial cells were phagocytosed by host cells in the infected region of the midgut (arrows). Epm=epithelial cells; Lum=lumen; MV=microvilli; Bas=basement membrane; Teg=tegumental glands; Cu=non-calcified cuticle; He=hemocytes; Bac=bacterial cells.

4.2.2 Histopathology of challenged shrimp

Infectivity trials of PL, demonstrated that most PL had bacterial infections mainly in the hepatopancreas, anterior midgut and middle midgut, whereas infection in the hindgut region was not found. The stages of lesions varied among sampled PL even at the same time. The degree of infection ranged from a normal appearance at an early infection as 3 h to heavy infection after 24 h post infection. At 12 h, in some PL the affected hepatopancreatic cells lining the tubules and epithelial cells in the midgut were sloughed from the basement membrane into the lumens and were necrotic (Figure 4.6B), while at 3 h most PL had normal appearance of the hepatopancreas and wavy epithelial layer of the midgut (Figure 4.6A). More PL with heavy infections were observed after 24-48 h post immersion, bacteria were most likely invading tubular lumens of the hepatopanrcreas. The hepatopancreatic cells lining the invaded tubules had completely disappeared from the basement membrane and this was accompanied by infiltration of hemocytes into the hepatopancreas (Figure 4.6C). Similar lesions were seen in the anterior part of the midgut, where the epithelial membrane was completely lost and infiltration of hemocytes occurred (Figure 4.6D).

Some PL had heavy infections only in the hepatopancreas but not in the midgut, while some PL was heavily infected only in the midgut but not in the hepatopancreas or both organs had an infection in some PL. The invaded tubules were walled off by multiple layers of semigranular cells fused to each other, and overlying fibrocyte-like cells that loosely disorganized arrangement around these foci to form encapsulated capsules (Figure 4.7A-B). Inside the capsules, the thickened basement membrane underwent inflamation-like reaction, and bacteria were retained in the cellular debris (Figure 4.7A-C). Developed capsules were walled up of multiple layers of flattened semigranular cells (Figure 4.7A-C; He), and the inner layers of necrotic hemocytes, basement membrane and the central enclosed mass (Figure 4.7A-C). At 48- 64 h after bacterial challenge, almost in all cases infected PL had melanization inside the capsules (Figure 4.7C; brown color). Old capsules consisted of densely semigranular capsules and a condensed central mass, and were walled off by a thin fibrous layer (Figure 4.7C; white arrows). Old capsules usually showed melanization, and bacteria were less commonly observed or absent from the central, condensed

mass probably due to melanisation reactions (Figure 4.7C). Electron micrographs showed that although multiplication of bacteria (B) was observed inside capsules, which is indicated by binary fission (arrow) (Figure 4.5E-F), most bacterial cells were deformed. This indicates that these bacterial cells were slowly killed by the host defense mechanism such as melanisation and perhaps also by antimicrobial peptides. In addition some bacterial cells were phagocytosed by host cells in the infected region of the midgut (Figure 4.5F; arrows).

In infectivity trials of juvenile shrimp, similar histopathological changes were also observed in the hepatopancreas and the midgut, but only some minor regions in these organs were infected. Infected juveniles showed that numerous hemocytes (He) were infiltrated into the hepatopancrease from 12- 48 h post immersion (Figure 4.8A) and many tubules showed detached epithelia from the basement membrane (Figure 4.8C; arrows) and in the midgut (Figure 4.9A), whereas no change was observed in the hepatopancreas (Figure4.3A), the midgut (Figure 4.3B-C) and the hindgut (Figure 4.3D) of control shrimp. Some juveniles collected at 48 h after immersion showed that *V. harveyi* infection provokes a strong perturbation of the shrimp gut. The epithelium layer was partially destroyed at the infected site of the hepatopancreatic duct (Figure 4.7B) and the midgut (Figure 4.9B) and was replaced by several layers of hemocytes (He), while the other region of the digestive organ such as anterior caecum (Figure 4.9C), the hindgut and posterior caecum (Figure 4.9D) were not infected. Electron micrographs of tranversal sections show that bacteria were seen among cell debris in the lumen at the infected site (Figure 4.10A-B; Bac). At 12 h after baterial immersion, some shrimp had no lesion in the midgut when examined under light microscopy (Figure 4.10C). However, to further investigate the gut of a normal appearance shrimp by TEM, we found that there were massive hemocytes infiltrated into the tissue under the epithelial layer of the midgut (Figure 4.10D).

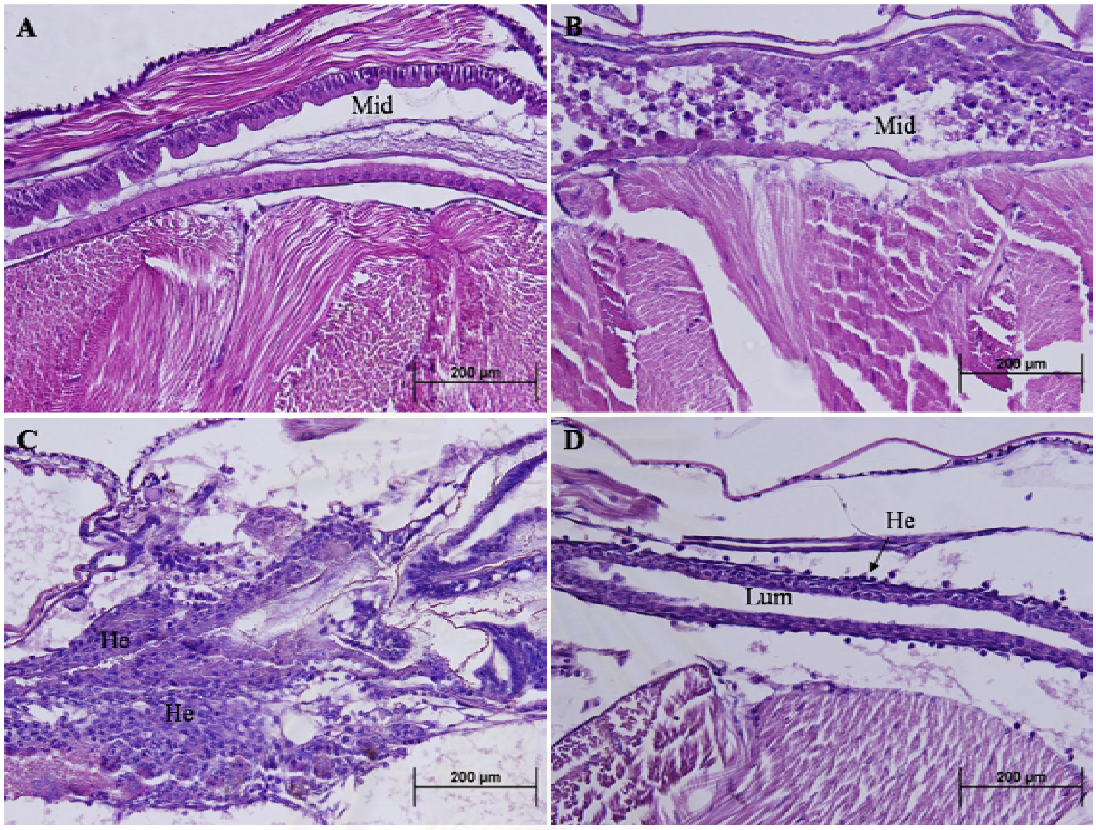

Figure 4.6 PL from the *V. harveyi*-immersion showed infection in the digestive tissues. (A) The midgut of PL at 3 h after bacterial immersion. (B) The midgut invaded by bacteria at 12 h, epithelial cells were necrotic and were separated from the basement membrane. (C) Heavy infection with infiltrated hemocytes in the hepatopancreas at 24 h after bacterial immersion. (D) The epithelial layer of the midgut of infected PL at 24 h completely disappeared and was replaced by several layers of hemocytes. Mid=midgut; He=hemocytes; Lum=lumen.

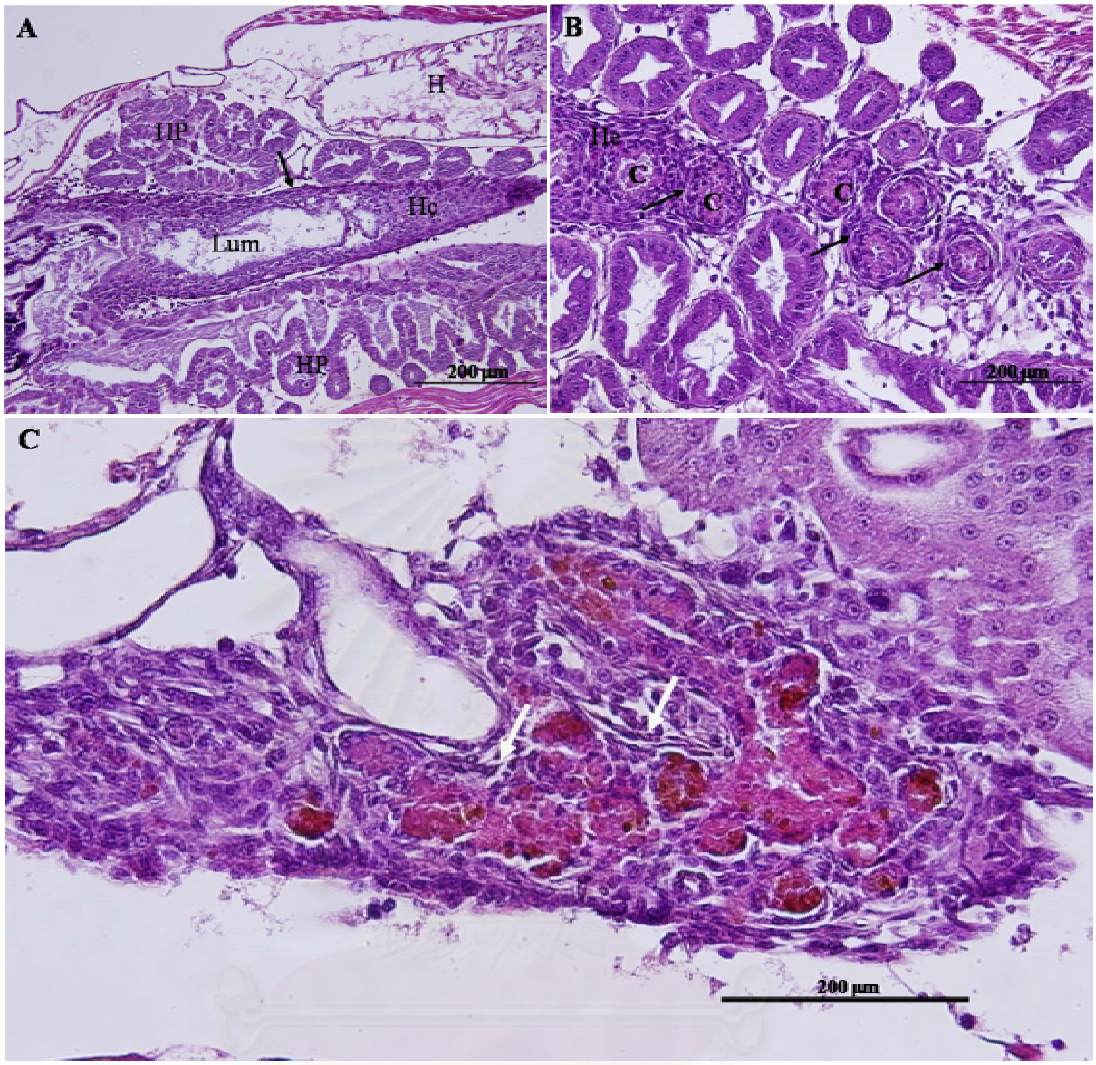

Figure 4.7 PL shrimp from the *V. harveyi*-immersion showed infection of tissues. (A) At 48 h some PL had heavy infection in the anterior midgut but had a mild infection in the hepatopancreas, (A-B) the developed capsules (arrows) were walled up of multiple layers of flattened hemocytes (He) and the inner layers of inflammatory response of hemocytes, basement membrane and the central enclosed mass. (C) At a late time point (64 h after bacterial challenge), almost in all cases infected PL had melanization (brown color) inside the encapsulated capsules and were walled off by a thin fibrous layer (white arrows).

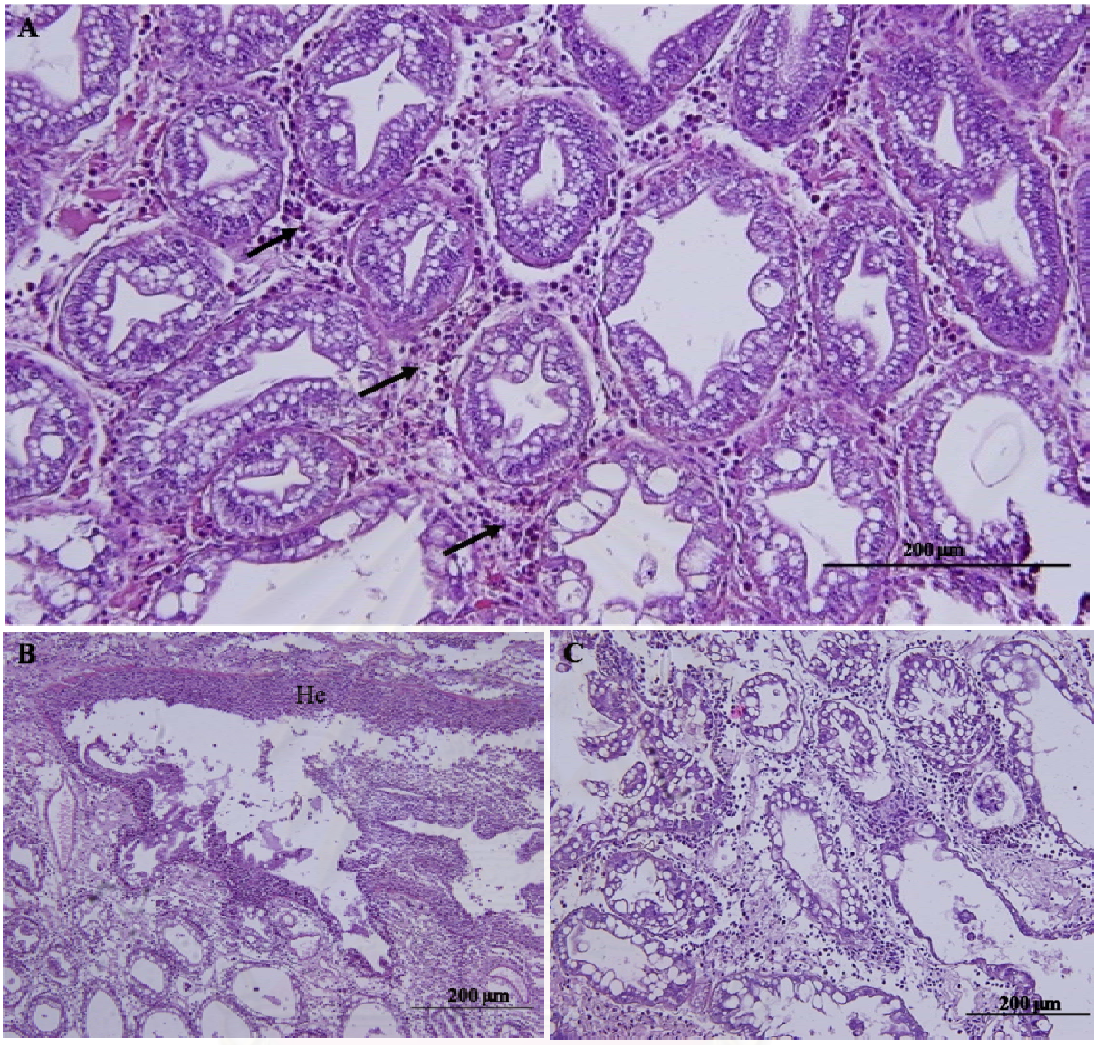

Figure 4.8 Juvenile shrimp from the *V. harveyi*-immersion showed infection of tissues. (A) At 12 h post bacterial immersion, juvenile shrimp showed partial infection in the hepatopancreas. (B) Infection in the hepatopancreatic duct. (C) Heavy infection was observed in the hepatopancreas and midgut of juveniles at 48 h post bacterial immersion. H=heart; HP=hepatopancreas; He=hemocytes; Lum=lumen; c=central enclosed mass.

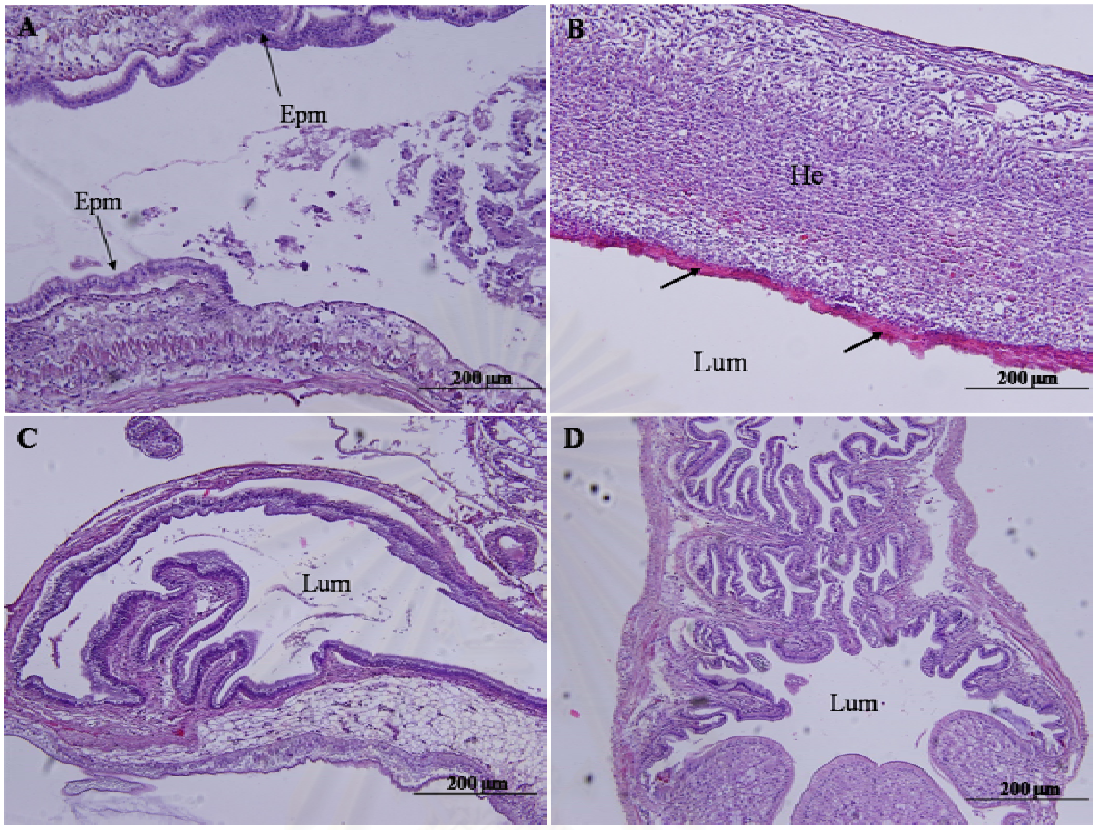

Figure 4.9 The juveniles from the *V. harveyi*-immersion showed infection of tissues. (A) Epithelial cells of some region in the midgut were detached from the basement membrane and were necrotic, while the other region was normal. (B) Accumulated hemocytes walled off the bacteria-invaded midgut and formed a capsule. Coagulation occurred in the thickened basement membrane (arrows). (C) No lesion was seen in the anterior caecum (D) No lesion was seen in the posterior caecum. Epm=epitherial membrane; He=hemocytes; Lum=lumen.

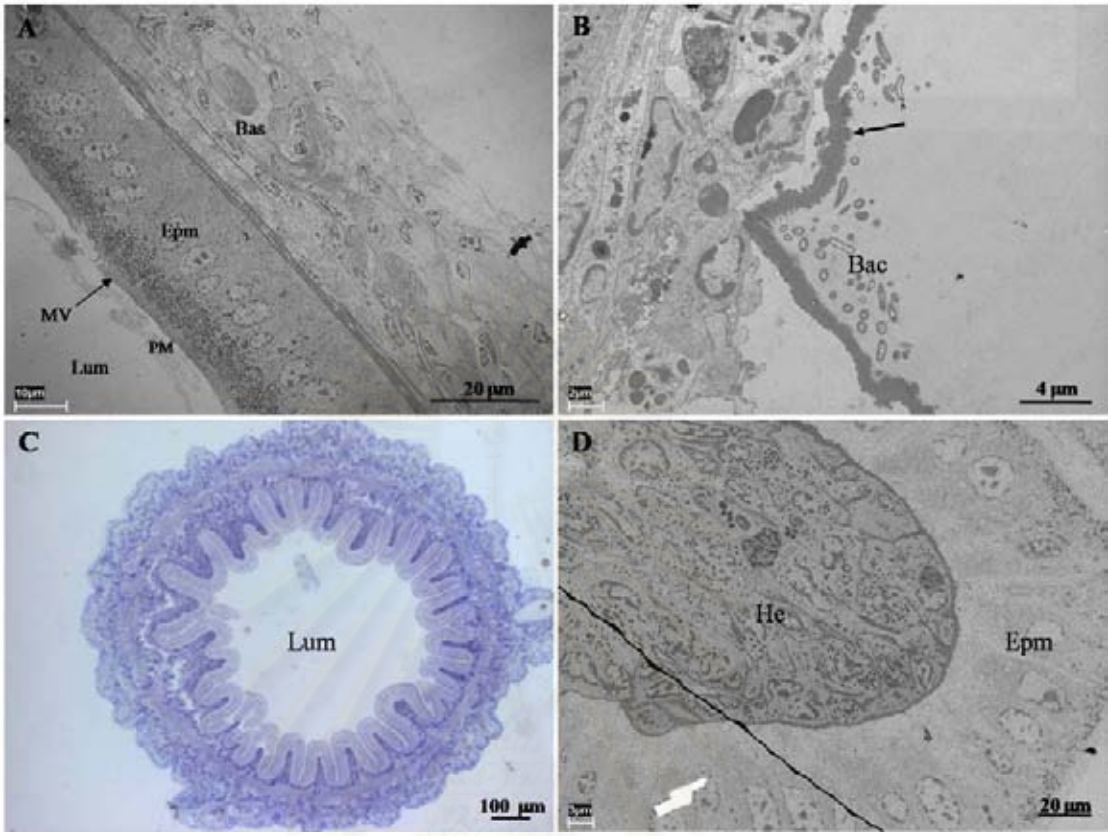

Figure 4.10 Electron micrographs (A, B, D) and light microscopy (C) of juvenile midgut. (A) Juvenile shrimp from the control group. (B) At 48 h after bacterial infection, juvenile midgut had similar lesion (arrow) as in the infected PL. (C) At the 12 h after bacterial immersion, semithin section observed under light microscope shows that the midgut of juvenile had normal apprearance. (D) At the 12 h after bacterial immersion, the intensively infiltrated hemocyted (He) were seen under the basement membrane when observed by TEM. Bas=basement membrane; Epm=epitherial membrane; MV=microvilli; PM=peritrophic membrane; Lum=lumen; Bac=bacterial cells; He=hemocytes.

4.3 Expression of immune-related genes in unchallenged shrimp by RT-PCR

4.3.1 RNA quality

The quality of the DNA-free RNA was electrophoretically determined using 1 % agarose gel that revealed a predominant band of 18S rRNA (1.9 kb) along

with smeared high molecular weight RNA. The $A_{260/280}$ ratio of DNA-free RNA which was 1.9-2.1 indicated acceptable quantity of DNA-free RNA. The first strand cDNA synthesized from this DNA-free RNA covered the large products representing the acceptable quantity of the first strand cDNA.

4.3.2 Expression of immune-related genes

The amplification products were 297, 286, 233, 243, 547, 590, 508, 558, 439, 340, 386, 534, 460, 460, 653 and 500 for *ALF2*, *ALF3*, *crustin*, *penaeidin*, *PrX*, *proPO*, *C-lectin*, *BGBP*, *MMR*, *TL5a1*, *TL5a2*, *ficolin*, *Lysozyme*, *MnSOD*, *mucin-like PM* and *EF1α*, respectively (Table 3.1). The tissue distribution analysis among these three different parts showed that all fifteen genes were expressed (Figure 4.11). Interestingly, *ALF2, ALF3, crustin, penaeidin, PrX, C-lectin, BGBP, MMR, TL5a2, Lysozyme, MnSOD* and *mucin-like PM* were expressed at moderate to high levels in all parts of the intestine of the three shrimp (N1-3) (Figure 4.11)

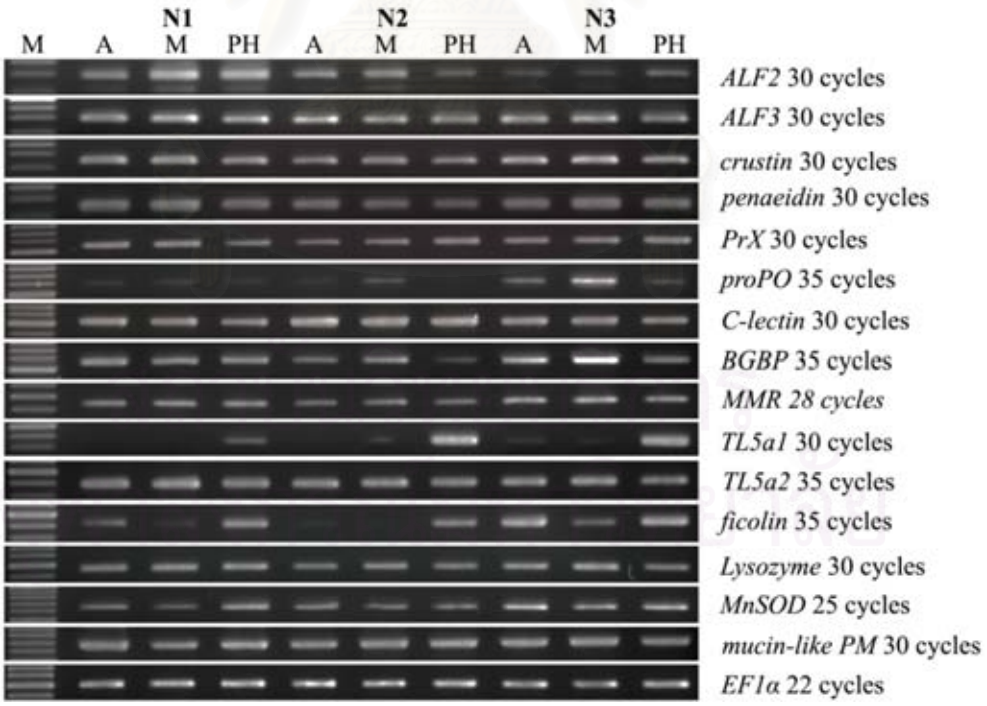

Figure 4.11 Expression profiling of fifteen immune-related genes in the intestine of three unchallenged shrimp (N1-3) in anterior midgut (A), middle midgut (M) and posterior midgut with hindgut (PH).

In contrast, the *TL5a1* transcript was highly expressed in the PH, but at a much lower level in A and M of the shrimp intestine. The *proPO* transcript was expressed at a very low level in all parts of the shrimp intestine. Inconsistent pattern of expression in different intestine regions was seen for *ficolin*. Finally, no obvious differences in expression of the tested genes between the anterior (A) and middle (M) parts of midgut so these two parts were combined into one part called anterior and middle midgut (AM) for further study.

4.4 Expression of immune-related genes in *V. harveyi* challenged shrimp by RT-PCR

4.4.1 Diagnosis of *V. harveyi*

The result showed that no PCR product was observed from amplification (Figure 4.12) indicating no contamination of *V. harveyi*, in the intestine of shrimp. As a result, these shrimp can be used for further studies.

Figure 4.12 PCR product amplified from genomic DNA of intestine from individual 5 shrimp prior to the initiation of the experiment. M: marker; P: positive control; N: negative control, 1-5: individual shrimp.

4.4.2 RNA quality

The quality of DNA-free RNA was monitored by running on 1% agarose gel that revealed a predominant band of 18S rRNA (1.9 kb) along with smeared high molecular weight RNA. The $A_{260/280}$ ratio of DNA-free RNA which was 1.9-2.1 indicating acceptable quality of DNA-free RNA. The first strand cDNA

synthesized from this DNA-free RNA covered the large products representing the acceptable quantity of the first strand cDNA.

4.4.3 Expression profiling of immune-related genes after *V. harveyi* challenged by immersion

The expression levels of *ALF3*, *crustin*, *penaeidin*, *C-lectin*, *TL5a1* and *mucin-like PM* were altered in their expression after 6 h of the bacterial challenge (Figure 4.13). Notably was that *TL5a1* was still mainly expressed in PH after the bacterial challenge (Figure 4.13). In contrast, the transcription levels of *ALF2*, *PrX*, *proPO*, *BGBP*, *MMR*, *TL5a2*, *ficolin*, *Lysozyme* and *MnSOD* were not obviously different after the *V. harveyi* exposure (Figure 4.14, 4.15).

In addition, the expression profiles of these six increased genes were characterized in three other tissues, circulating hemocytes, hepatopancreas and stomach of the same three challenged samples (Figure 4.16). The *AMP* transcripts were detected at very high levels in hemocytes (Figure 4.16). The expression levels of *ALF3*, *crustin*, *penaeidin*, *C-lectin*, and *TL5a1* were not affected in the circulating hemocytes. However, there was no detectable *mucin-like PM* transcript in the hepatopancreas and hemocytes but it was detected in stomach of control and bacteria-challenged shrimp (Figure 4.16).

In the hepatopancreas (Figure 4.16), two of three (N3 and N5) bacteria-challenged shrimp, *ALF3*, *crustin*, *penaeidin*, *C-lectin*, *TL5a1* and *proPO* transcript were induced at 24 h, and remained at a high level after 48 h exposure to *V. harveyi*. The expression level of the *C-lectin* was increased at 3 to 6 h, and then returned to control expression levels at 12 h.

In the bacteria-challenged shrimp, the expression levels of *ALF3*, *C-lectin*, and *TL5a1* were detected in the stomach tissue but no effect in their expression was observed by the *V. harveyi* challenge. However, in two of three shrimp, the *crustin* and the *penaeidin* transcript in stomach after the bacterial challenge increased slightly at 6 to 24 h and 6 to 12, repectively, and then decreased and returned to the non-stimulated levels or below it by 48 h post challenge.

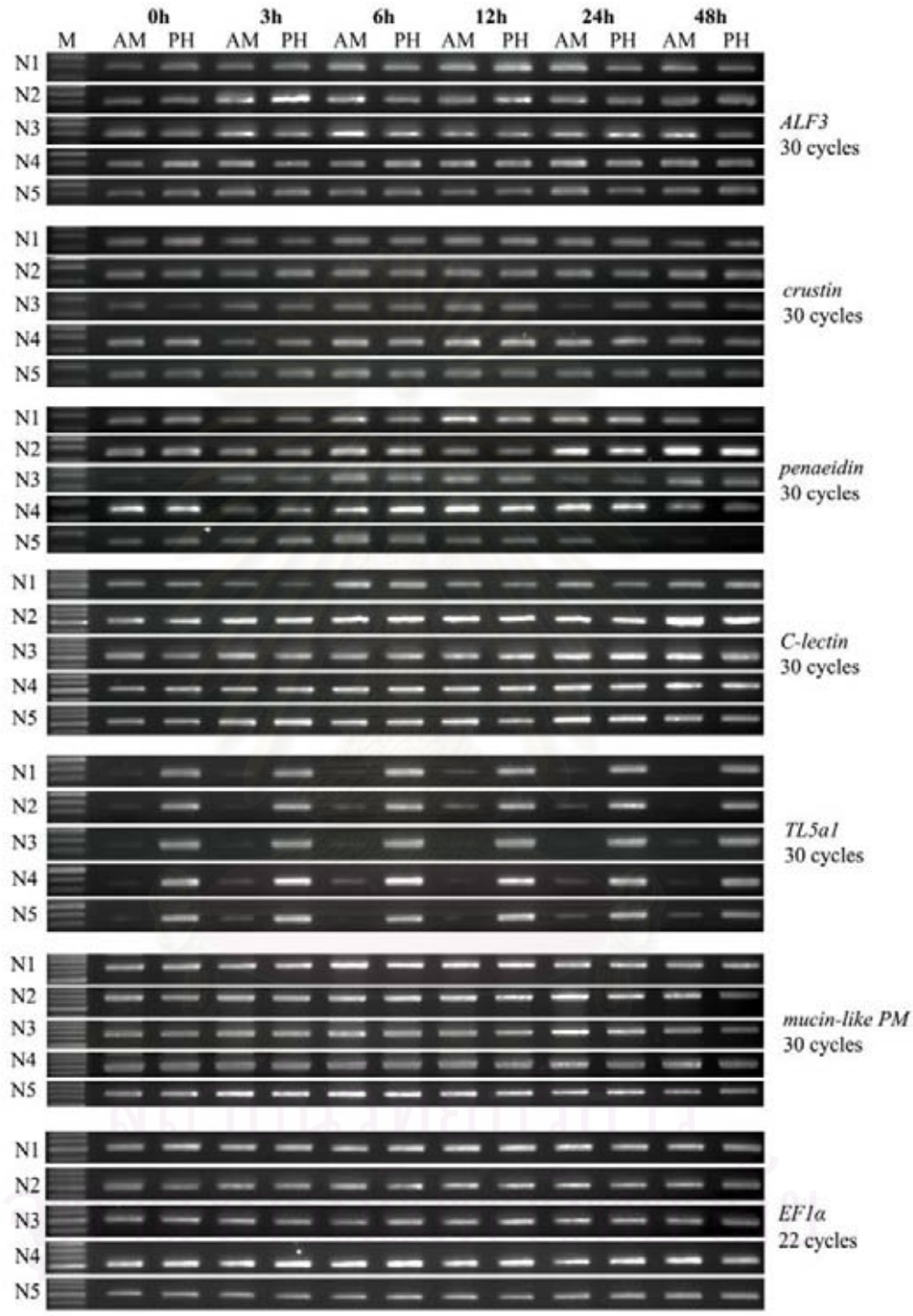

Figure 4.13 Time course study of the expression of six genes in the intestine of bacteria challenged shrimp (N1-5) in anterior and middle midgut (AM) and posterior with hindgut (PH). compared to the internal control, *EF1α*, after challenge by *V. harveyi*-immersion. The cycles of amplification depend on expression of genes.

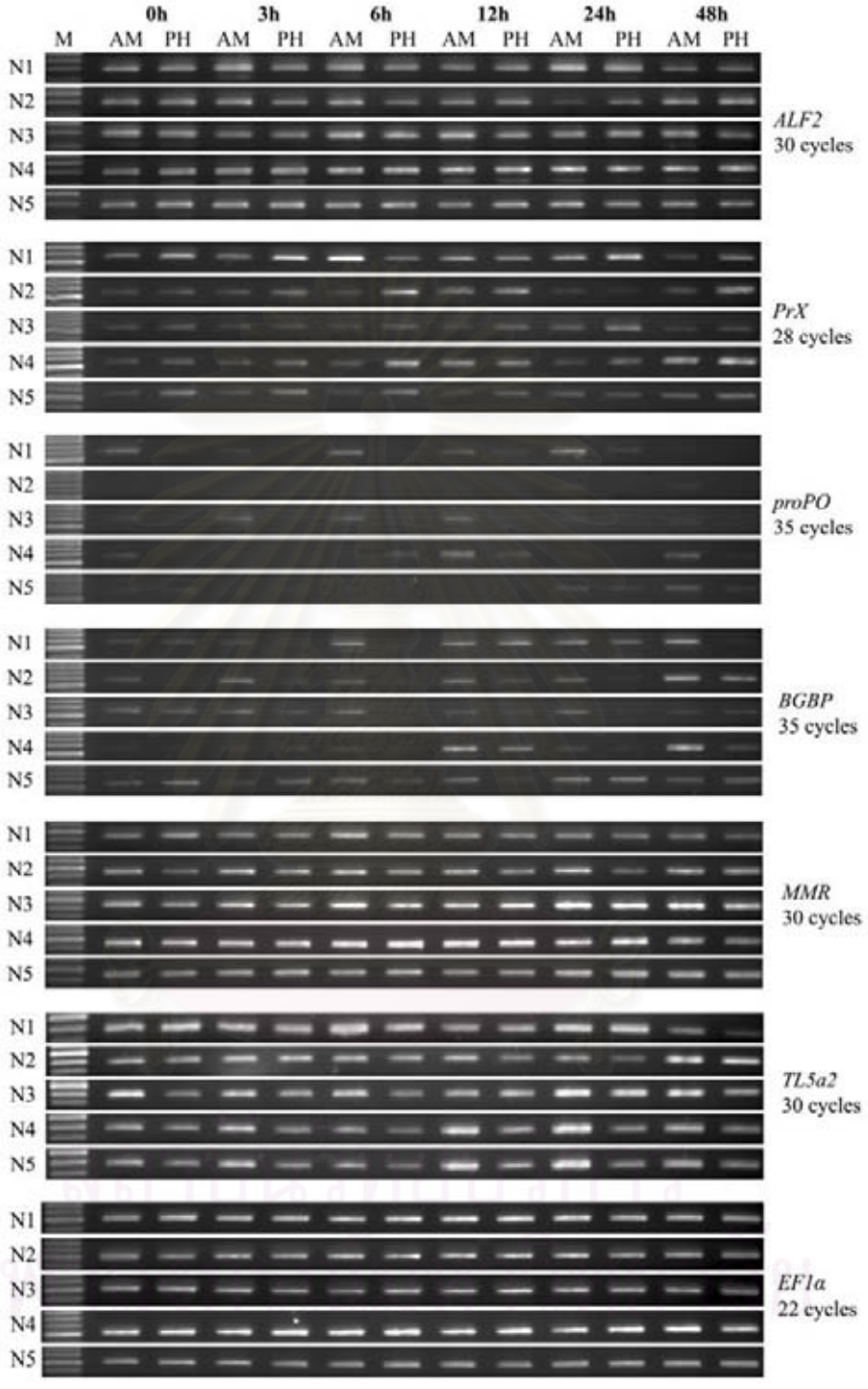

Figure 4.14 Time course study of the expression of six genes in the intestine of bacteria challenged shrimp (N1-5) in anterior and middle midgut (AM) and posterior with hindgut (PH). compared to the internal control, *EF1α*, after challenge by *V. harveyi*-immersion. The cycles of amplification depend on expression of genes.

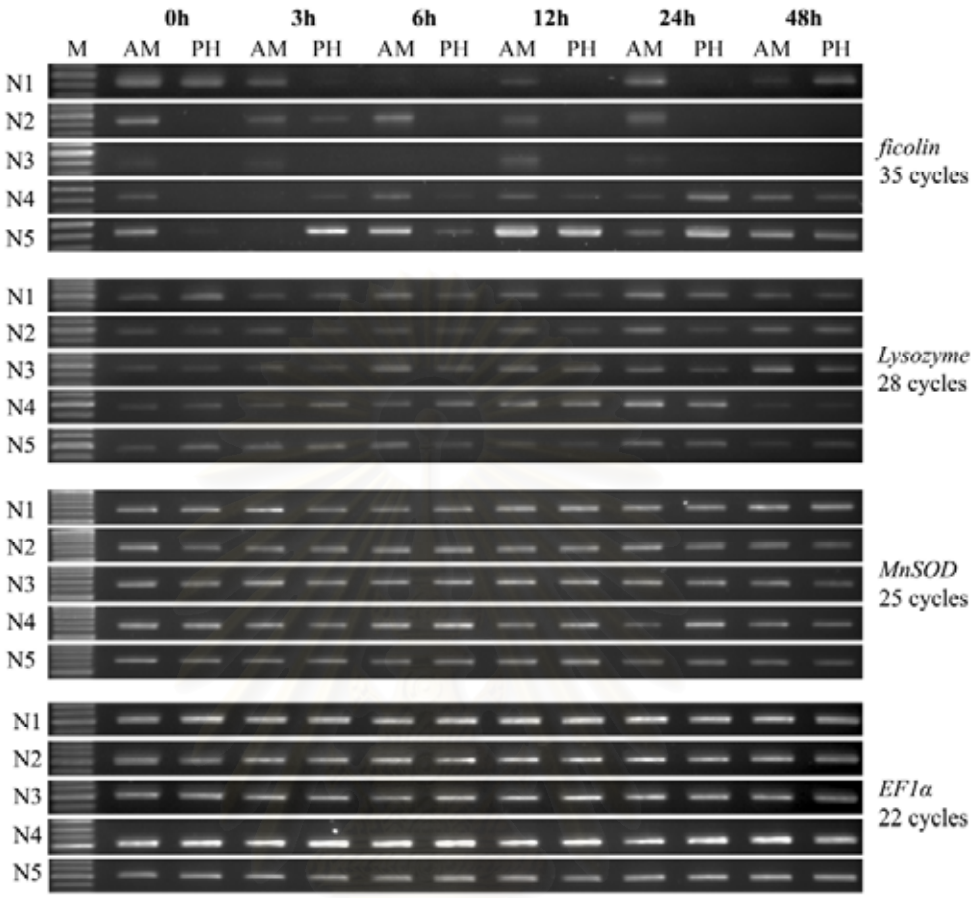

Figure 4.15 Time course study of the expression of three genes in the intestine of bacteria challenged shrimp (N1-5) in anterior and middle midgut (AM) and posterior with hindgut (PH) compared to the internal control, *EF1α*, after challenge by *V. harveyi*-immersion. The cycles of amplification depend on expression of genes.

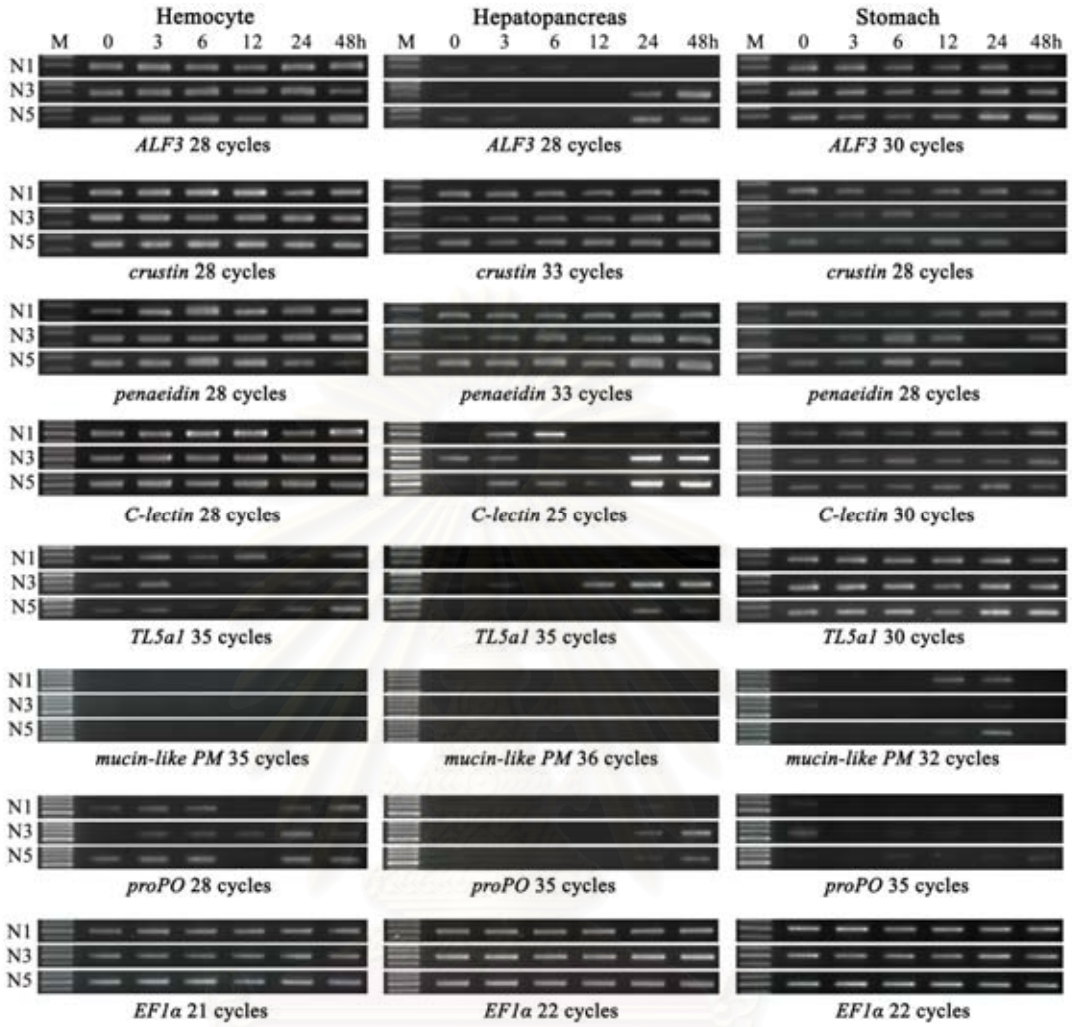

Figure 4.16 Time course study of RT-PCR of seven immune-related genes in circulating hemocytes, hepatopancreas and stomach compared to internal control, *EF1α*, after challenge by *V. harveyi*-immersion. The cycles of amplifing gene depend on each tissue expression.

4.5 Expression of immune-related genes in shrimp challenged with *V. harveyi* by real-time PCR

The *ALF3* expression showed a 7.1 and 7.7 fold significantly up-regulated in the AM ($P<0.05$) after 6 and 24 h post-challenge and significantly down-regulated to a 2.4 fold induction ($P<0.05$) at the 48 h time point (Figure 4.17A). In PH, the *ALF3* gene was 2.1 fold significantly induced ($P<0.05$) at 6 h post-challenge

and gradually decreased to 1.7 and 1.0 at 24 and significant at 48 h (P<0.05), respectively (Figure 4.17B). The expression of *crustin* was significantly up-regulated to a 1.5 and 2.1 fold induction (P<0.05) in the AM at 6 and 24 h post-challeng, repectively. In contrast, the *crustin* was no significantly induced (P<0.05) in its expression during the experimental period in PH. In AM, *penaeidin* was induced to its highest expression at the 6-h time point, and significantly decreased then down to ca. 2- and 3-fold (P<0.05) induction of transcripts at 24 and 48 h after the challenge and a similar trend was observed in PH.

The *C-lectin* gradually increased in the AM at 6, 24 and 48 h after challenge, where expression significantly up-regulatedly to 10.7 and 15.1 fold (P<0.05), at 24 and 48 h, respectively. This was the highest normalized level of expression observed for any tested gene in this study. Similarly, a dramatic difference was seen in PH at the 48-h time point, where the level reached 10.4 fold. *C-lectin* expression was the only gene among all genes tested which was highly induced in its expression during the experimental period following a *V. harveyi c*hallenge, with a significant induction as early as 6 h, and with a continuous increase to 48 h post challenge.

In AM, the *TL5a1* transcript level showed induction at 6 h (1.2 fold), whereas after 24 h exposure time, it was significantly induced 3 fold (P<0.05) and then decreased after a prolonged exposure to the pathogen for 48 h. In addition, there was increased to 2.0 fold at 6 h and then decreased to 1.2 and 1.3 fold after 24 and 48 h exposure time in PH. The *mucin-like PM* was the highest expression at 6 h and significantly down-regulated to 0.7 and 0.3 fold (P<0.05) at 24 and 48 h post-challenge in AM. Similarly, a dramatic difference was seen in PH. Interestingly, according to Log2 relative expression in AM/PH in all time points (Figure 4.18B), the expression level of *mucin-like PM* was much higher in AM than PH. This suggests that the *mucin-like PM* might play a crucial role for the upper part of the intestine since this part it is not lined by exoskeleton containing chitin and hence the mucin-like PM may provide an effective physical barrier to pathogens. On the other hand, *TL5a1* was expressed at a higher level in PH than AM (Figure 4.13, Figure 4.19). These observations suggest that shrimp might respond to a bacterial infection in a different way in different parts of the intestine.

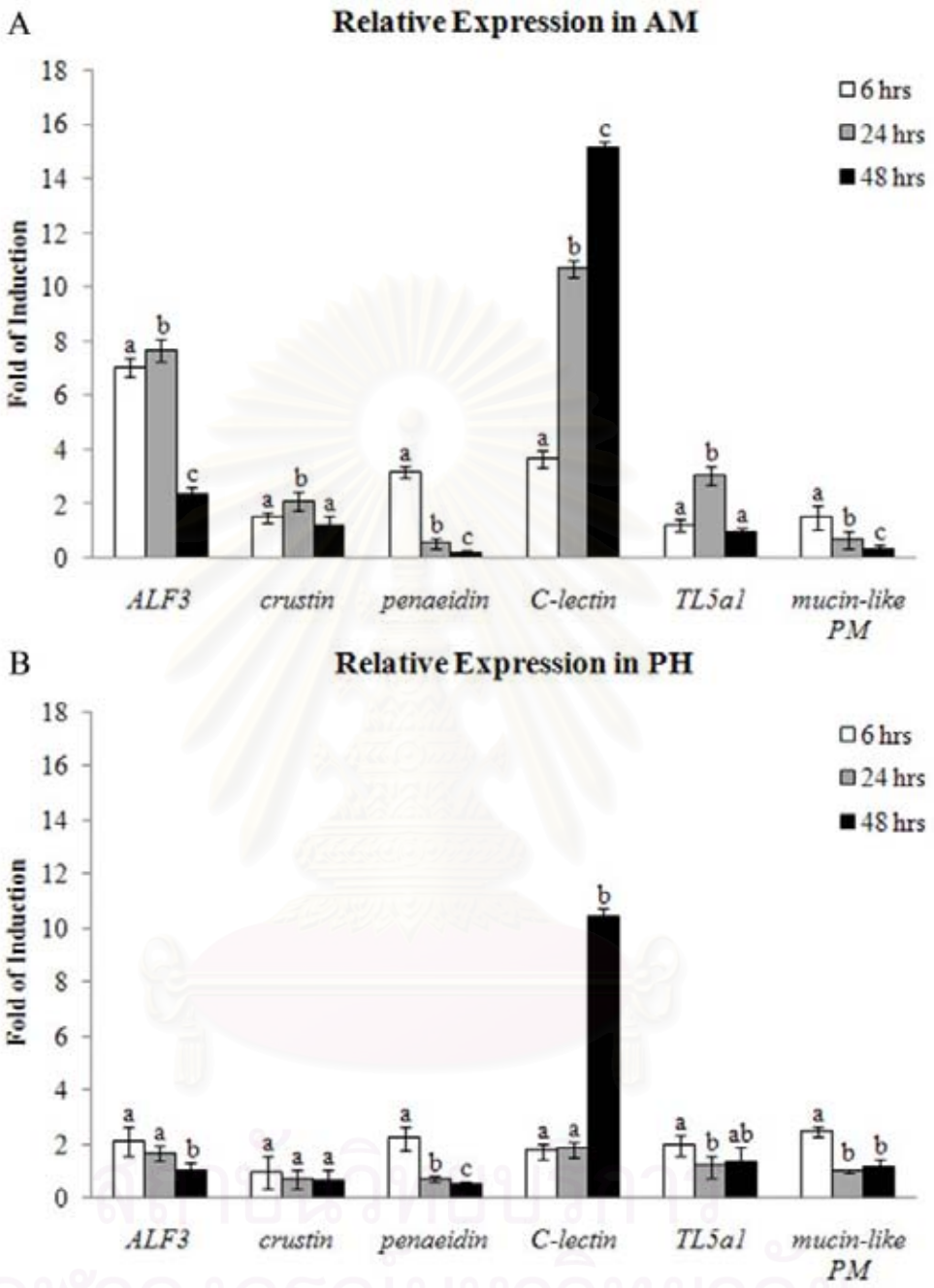

Figure 4.17 Time course study of expression of six genes by using real-time PCR in AM (A) or PH (B) after challenge by *V. harveyi*-immersion. The mRNA levels were determined by real-time PCR and are expressed relative to *EF1α* expression. Each histogram represents the mean fold change relative to the control±S.D. of five psudoreplicates. The same letters indicate that the expression levels were not significantly different (P>0.05)

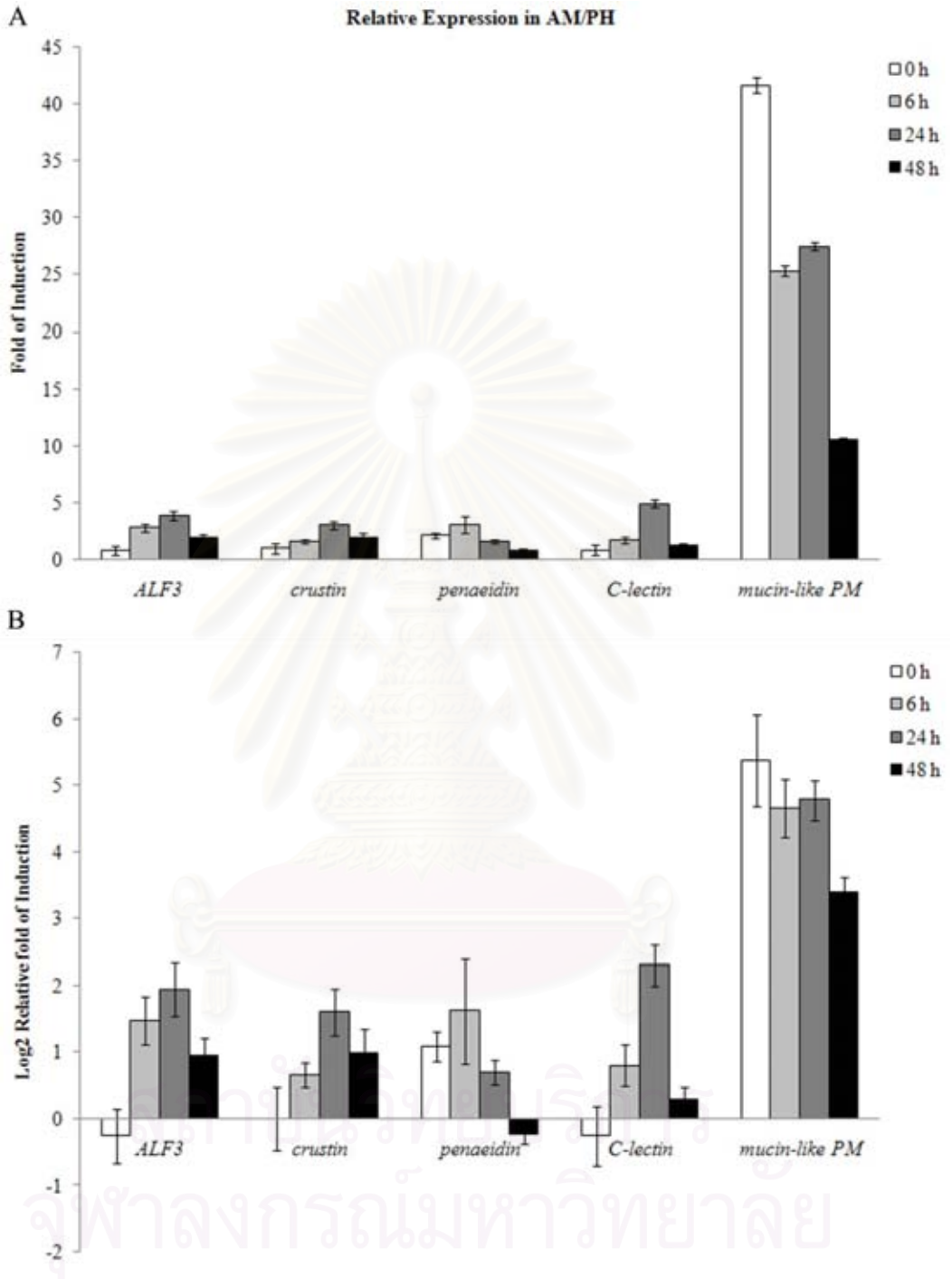

Figure 4.18 Time course study of relative expression in AM/PH of five genes after challenge by *V. harveyi*-immersion. The mRNA levels were determined by real-time PCR and are expressed relative to *EF1α* expression. Each histogram represents the mean fold change relative to the control±S.D. of five pseudoreplicates. (A) Fold of induction (B) Log2 relatice of fold induction.

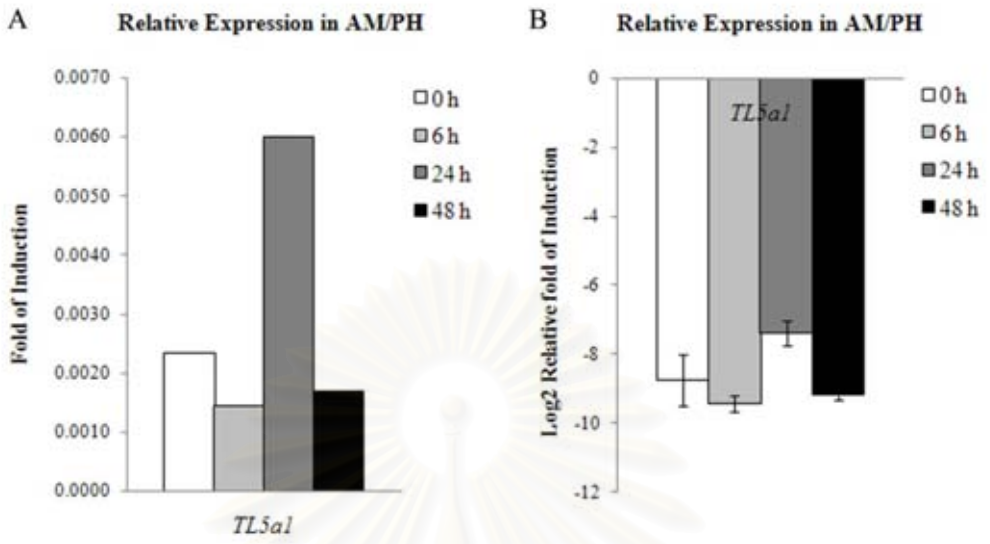

Figure 4.19 Time course study of relative expression in AM/PH of *TL5a1* after challenge by *V. harveyi*-immersion. The mRNA levels were determined by real-time PCR and are expressed relative to *EF1α* expression. Each histogram represents the mean fold change relative to the control±S.D. of five pseudoreplicates. (A) Fold of induction (B) Log2 relatice of fold induction.

4.6 Tissue distribution of immune-related genes

The *AMP* transcripts (*ALF3*, *crustin* and *penaeidin)*, were expressed highly in hemocytes and moderately in AM. A high level of expression of the *C-lectin* was found in the hepatopancreas, AM and PH, lesser expression in hemocytes and very low level in other tissues. Interestingly, not only was the *TL5a1* expression found in the stomach and the PH part of the digestive system, but it was also expressed at the highest level among the examined organs in epidermis and at a lower level in abdominal ganglion, pleopod and eyestalk. The *mucin-like PM* was expressed in limited number of tissues, the highest was found in AM and slightly lower levels in PH. The *proPO* expression level was specific to only the hemocytes (Figure 4.20).

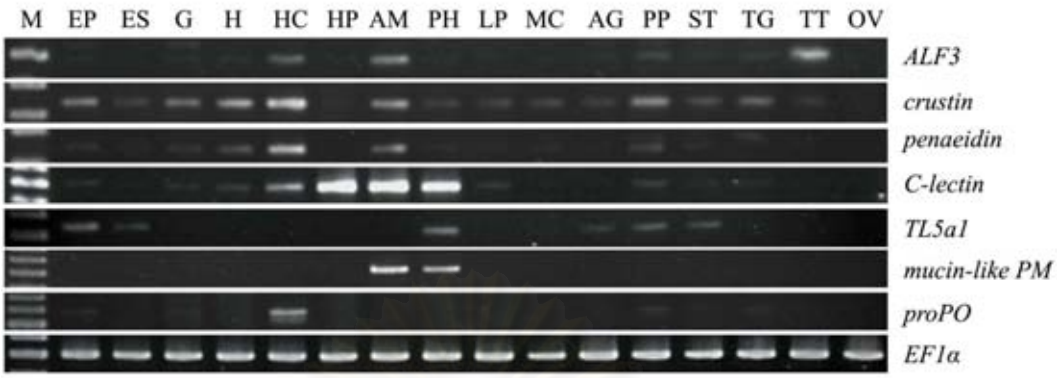

Figure 4.20 Expression of *ALF3*, *crustin*, *penaeidin*, *C-lectin*, *TL5a1*, *mucin-like PM* and *proPO* in sixteen different shrimp tissues. *EF1α* was used as a ubiquitously expressed control. EP=epidermis; ES=eye stalk; G=gill; H=heart; HC=hemocyte; HP=hepatopancreas; AM=middle midgut; PH=posterior midgut with hindgut; LP=lymphoid organ; MC=muscle; AG=abdominal ganglion; PP=pleopod; ST=stomach; TG=thoracic ganglion; TT=testis; OV=ovary.

CHAPTER V
DISCUSSION

This study shows a new approach in the type of study on bacterial infections in shrimp. To study pathogenicity and shrimp's defense mechanism against infections in a more natural context, the interactions between shrimp and a pathogenic bacterium was investigated in the natural route through intestinal tract.

To induce vibriosis in penaeid shrimp, it has been reported that regardless of the mode of infection, by either bacterial immersion or injecting bacteria, heavily infected shrimp typically appear lethargic, the blood loses its ability to clot, and degenerative changes in many tissues including the gills, lymphoid organ and digestive gland occur. Specific morphological changes in the digestive gland were tissue necrosis, loss of the epithelium and infiltration of hemocytes (Egusa et al., 1988; Chen et al., 1992, Sung et al., 1994, Hameed 1995, Esteve and Herrera, 2000; Lightner and Lewis 1975; Aruma 1989, de la Peña et al, 1993, Jiravanichpaisal et al., 1994, Lee et al., 1996). Martin et al. (2004) demonstrated that some morphological changes occurred in the midgut of a penaeid shrimp, *Sicyonia ingentis*, following immersion or injection with *Vibrio* (*V. parahaemolyticus* and *V. harveyi*). As a result, all shrimp immersed in *Vibrio* solution showed detachment of the epithelium in the midgut trunks (MGT), while by injection the epithelial detachment was common in diseased shrimp but not in apparently healthy shrimp.

In this study, *V. harveyi* was found to be highly pathogenic to PL. In contrast, juvenile shrimp can survive even at a higher dose of bacteria used to infect PL. In PL, *V. harveyi* causes a massive destruction of the digestive system, especially in the hepatopancreas and in the anterior gut, while mild or moderate infection was observed in the hepatopancreas and partial infection of the midgut. The histopathological changes due to this experimental infection by immersion were similar to those of diseased shrimp occurred in pond-cultured *P. monodon* juvenile. The disease usually occurs during the first month of culture (Lavilla-Pitogo et al., 1998). Luminous *Vibrio*, the causative agent of the disease, can invade the host through the hepatopancreas, a common target organ of most bacterial pathogens of shrimp (Chen, 1992; Frelier et al., 1992). Infection in juveniles younger than 45 days of culture had a

severe inflammation in and around the tubules of the entire hepatopancreas. The epithelial cells of the hepatopancreatic tubules were necrotic and dense clumps of bacteria were observed in the lumen. The intertubular sinuses were mainly congested with hemocytes and fiber cells. It was further found that smaller shrimp showed relatively more severe inflammatory responses (Lavilla-Pitogo et al., 1998).

Most infected PL or juveniles in the study had extensively affected hepatopancreas and the midgut, but showed well developed capsules around these infected organs and bacteria were usually completely confined inside these capsules (Figure 4.7A-C). As a consequence of these capsulses contain bacteria other visceral organs, including gills, heart, appeared normal. This indicates that there was no systemic infection occurring in the infected shrimp. Although PL have an effective cellular defense mechanism, high mortality was found in PL. This might be related to the malfunction of the digestive organ when it was completely destructed by pathogenic *V. harveyi*. In case of juveniles, they were able to survive after being exposed to the high dose of pathogenic bacteria because only a partial part of the organs were affected and because of well developed encapsulation reactions of the bacteria.

The early onset of mortalities (starting at 12 day) observed in rearing ponds has been reported (Lavilla-Pitogo et al., 1998). They suggest that a rapid progression of infection, where susceptibility may be depending on age and health status of post-larvae. Our study which shows that PL have a higher mortality by the oral route of infection than juveniles is consistent with the proposal made by Lavilla-Pitogo et al. 1998. This is most likely due to imperfect development of the digestive organs or local immune system in these organs in the PL.

The ingestion of bacteria is the most commonly reported about association between aquatic animals and gut microbes (Harris, 1993). These bacteria were able to multiply and colonize in the host digestive organ and become pathogenic (Colorni, 1985). Therefore, luminous *Vibrio* present in the environment can enter the host through ingestion of feeds and rearing water loaded both may contain high numbers of the pathogen. It was reported that the occurrence of mortalities in shrimp affected with luminous vibriosis is preceded by the dominance of *V. harveyi* in the rearing water. Therefore, continuous ingestion of these bacteria by the shrimp may initiate

the colonization of this pathogen in the digestive tract (Lavilla-Pitogo et al., 1998). Moreover, luminescent bacteria were found in the midgut content of *P. monodon* spawners and grow out pond juveniles (Lavilla-Pitogo et al., 1992).

Natural infections in shrimp are most likely due to infections through the mouth and then down through the intestine so the immunity of the digestive system is obviously of great importance for the well being of shrimp and calls for more intense studies. Therefore, studies on the immunity in intestine and its response to pathogens deserve and require more detailed studies.

For studying the intestinal immunity, the expression of immune-related genes was studied in the intestine of shrimp. The expression profiles of the fifteen immune-related genes in the intestine of shrimp challenged by the *V. harveyi* showed that the expression levels of *ALF3*, *crustin*, *penaeidin*, *C-lectin*, *TL5a1* and *mucin-like PM* were altered in their expression after the bacterial challenge. In addition, *TL5a1* was still mainly expressed in PH. In contrast, the transcription levels of *ALF2*, *PrX*, *proPO*, *BGBP*, *MMR*, *TL5a2*, *ficolin*, *Lysozyme* and *MnSOD* were not obviously different after the *V. harveyi* exposure.

Production of AMPs is one of the main defense mechanisms. In insect, for instance, *Drosophila* systemically responds by inducing the expression of AMP genes upon the *Pseudomonas entomophila* ingestion. Both *Diptericin* and *Drosomycin* transcripts were apparent 3 h after infection and expressed highest at 24 h in larvae. Also, natural *P. entomophila* infections induced a local immune response. *Diptericin* expression was detected in the anterior midgut (proventriculus), whereas expression of *Drosomycin* was observed in the trachea (Vodovar et al., 2005). In contrast, Liehl et al. (2006) revealed that the major contribution to the *Drosophila defense* against *P. entomophila* is provided by the local, rather than the systemic, immune response.

In decapod crustacean AMPs, ALFs (Somboonwiwat et al., 2005; Tharntada et al., 2008), crustins (Relf et al., 1999, Jiravanichpaisal et al. 2007, Supungul et al., 2007) and penaeidins (Destoumieux et al., 2000) and their tissue distribution have been studied in some detail (Relf et al., 1999). According to the tissue distribution analysis in this study, *ALF3*, *crustin* and *penaeidin* were detected in the midgut and expressed higher in the anterior and middle region than in the hindgut, and they all had a low expression in the hepatopancreas. After bacterial challenge, the expression

levels of all these *AMPs* were up-regulated by 24 h in AM and PH. Since these *AMP* transcripts were detected at very high levels in hemocytes (Figure 4.7; 4.10) and hemocyte infiltration in many tissues is predominant upon infection the increased expression of the *AMP* genes in the hepatopancreas and the midgut, is most likely a result of infiltrated hemocytes. The *proPO* gene is specifically expressed in the hemocytes; therefore, the *proPO* transcript was used as a hemocyte marker to monitor hemocyte infiltration into different tissues (Söderhäll et al., 2003). As suspected the *proPO* transcript was present in hepatopancreas and midgut and this result correlated well with the histopathological study that at 24 or 48 h infiltrating hemocytes were observed in these tissues of some shrimp. However, the expression profiles of *ALF3*, *crustin* and *penaeidin* in the midgut and hindgut did not correlate with *proPO* expression. In addition, the study of systemic immunity in circulating hemocytes by infection through injection showed that *ALF* was up-regulated after bacteria injection (Supungul et al., 2004, Somboonwiwat et al., 2005, Tharntada et al., 2008), the expression of *crustin* and *penaeidin* was significantly decreased in hemocyte after bacteria injection (Supungul et al., 2004). Therefore, these genes can play a role as local immune response in the gut and that they can be induced in their expression when a pathogen enters into the midgut.

Lectins are found in almost all living organisms. They are PPRs, which bind to conserved molecules on the surface of invading microorganisms or PAMPs present on non-self pathogens. Interaction of PRPs and PAMPs triggers a series of immune responses, leading to the activation of the host-defense system (Medzhitov and Janeway, 2002; Janeway and Medzhitov, 2002). In invertebrates, lectins have been reported to be involved in various defense mechanisms such as to exhibiting antimicrobial activity (Tunkijjanukij and Olafsen, 1998; Schroder et al., 2003; Sun et al., 2008), enhancing phagocytosis (Kondo et al. 1992; Mercy and Ravindranath, 1994; Sierra et al., 2005), activating the proPO system (Chen et al., 1995; Yu et al., 1999; Yu et al., 2000) and nodule formation or encapsulation (Koizumi et al., 1999; Ma et al., 2008). Recently, lectins have been increasingly studied in shrimp and the transcripts were detected exclusively in hemocytes (Gross et al., 2001; Liu et al., 2007) or in the hepatopancreas (Gross et al., 2001; Liu et al., 2007; Luo et al., 2006; Ma et al., 2007, 2008; Sun et al., 2008). Some studies have shown that the expression

of lectin changed in response to WSSV infection by injection method (Gross et al., 2001; Liu et al., 2007; Ma et al., 2007, 2008).

Among the examined genes, only the *C-lectin* was continuously increased until the end of the experiment (48 h after a bacterial challenge). The *C-lectin* in this study was previously cloned and characterized from *P. monodon* by Luo et al. (2006) and was named *Pmlectin* containing a single CRD domain. It was specific for bacterial LPS and binding was mainly mediated through the *o*-antigen. It has a strong hemagglutinating, bacteria-agglutinating activity and opsonic effect that enhance the efficiency of hemocytic phagocytosis (Luo et al., 2006).

Although Pmlec did not have antibacterial activity or was involved in the activation of the *proPO* system (Liu et al., 2006), it functioned both as a PRP and an opsonin. In this way, it can provide a local immune response and protect the digestive system in shrimp from a bacteria infection. Therefore, the presence of the C-type lectin can be one of the frontiers against pathogens in the digestive organs particularly in midgut and hepatopancreas, which are not covered with cuticle as in stomach and hindgut. Since shrimp contain several lectins and probably have different specificities for detecting a variety of pathogens (Kondo et al., 1992; Mercy et al., 1994; Sierra et al., 2005; Ma et al., 2007), more information of these lectins may give insights into the potential roles of lectins as a local immune response in the digestive system of shrimp.

Another *lectin* tested in our study was *Tachylectin 5a* (*TL5a*), which was first cloned and characterized from horseshoe crab, *T. tridentatus* (Gokudan et al., 1999). In the horseshoe crab, *TL5A* was expressed abundantly in heart and intestine and faintly in hepatopancreas whereas *TL5B* was detected only in hemocytes (Gokudan et al., 1999). In horseshoe crab, TL5A and 5B have ability to agglutinate all types of human erythrocytes and Gram-positive and Gram-negative bacteria. TLs-5 posses a FRED domain, and these fibrinogen-related molecules might play an important role as non self-recognizing lectins. Very recently, a *tachylectin-related homolog* was characterized in amphioxus (*B. belcheri*). It showed abundant expression in the hepatic caecum and hindgut and was obviously increased expression in gut after LPS-challenge (Ju et al., 2009). In present study, the expression of *TL5a1* in shrimp was detected by RT-PCR in gut (PH), epidermis, nerve, pleopod, stomach, and eyestalk

and hardly expressed in hemocytes. Surprisingly, in the gut the *TL5a1* transcript was expressed much higher in the hindgut than in the midgut (Figure 4.10). It was reported that the environment in the hindgut is a suitable site for bacterial colonization (Harris, 1993). As a consequence, the high constitutive expression of *TL5a1* in the hindgut might be involved in protection against bacterial colonization. However, the functions of *Tachylectins* need to be elucidated in shrimp.

Peroxinectin, a cell adhesive protein associated with the proPO system, is a multifunctional protein including cell adhesion, degranulation, encapsulation enhancement, opsonin and peroxidase (Johansson et al., 1998; Johansson and Söderhäll, 1989; Kobayashi et al., 1990; Thörnqvist et al., 1994; Johansson et al., 1995). Peroxinectin is slightly down regulated after injection with laminarin and LPS in *P. monodon* (Sritunyalucksana et al., 2001). In contrast, this result revealed it being constitutively expressed in intestine.

BGBP is a constitutive plasma protein that after binding to β-glucan reacts with hemocyte surface (Cerenius et al., 1994). It strongly binds β-1,3-glucans which are structurally complex homopolymers of glucose, usually isolated from yeast and fungi. The expression showed that it constitutively expressed in hemocytes of *P. monodon* within 12 hour of the *V. harveyi*-challenge (Sritunyalucksana et al., 2002). Moreover, this results also showed that *BGBP* is constitutively expressed in intestine of *P. monodon* after *V. harveyi*-immersion.

Lysozyme is one of the main enzymes that exist in the lysosome (Misra et al., 2004) that catalyzes the hydrolysis of bacterial cell walls and acts as an innate immunity molecule against the invasion of bacterial pathogens. The lysozyme displayed activities against bacteria pathogen including *Vibrio* spp.(Hikima et al., 2003; de-la-Re-Vega et al., 2006; Bu et al.,2008). In *P. monodon*, lysozyme showed a constant level of expression after *V. harveyi*-challenge (Tyagi et al., 2007). Similarly in this study, the expression of the *lysozyme* transcript was not distinctly altered in shrimp gut after bacterial immersion, the expression was found ubiquitously throughout the shrimp gut.

Superoxide dismutase (SOD) is an enzyme that catalyses the dismutation of superoxide to hydrogen peroxide and oxygen and has role in the defense against oxidative stress (Scandalias, 1993). In decapod crustaceans, the characterization of

SOD and its functions in immunomodulation have been reported (Brouwer et al., 1997; Johansson et al., 1999; Orbea et al., 2000; Brouwer et al., 2003; Cheng et al., 2006). *A cytosolic manganese SOD* (*cytMn-SOD*) was cloned from the hepatopancreas in freshwater prawn, *M. rosenbergii*. The study showed that the mRNA transcripts of *SOD* were down-regulated during 3 to 6 h and up-regulated to normal expression after WSSV- injection in *L. vannamei* and *F. chinensis* (Gómez-Anduroa et al., 2006; Zhang et al., 2007; Zhang et al., 2008) and bacterial injection in *M. rosenbergii* (Cheng et al., 2006a; Cheng et al., 2006b). Here this study show that the *MnSOD* transcript was abundant in the midgut, but no obvious change was observed following the immersion challenge.

Other defense mechanism like the PM is functionally similar to the mucous secretions of the vertebrate digestive tract but structurally different. The functions are to protect the midgut epithelium from abrasive food particles, digestive enzymes, and pathogens infectious (review by Hegedus et al., 2009). It is well documented in insects that the PM can protect the host from invasion of microorganisms and parasites in the midgut, which is not lined by cuticle. The PM is composed of a cellular material produced by the midgut epithelium and consists of proteins and glycosaminoglycans embedded in a chitinous matrix (see review by Tellam et al., 1999). Recently, several genes encoding PM constituents were reported to be up-regulated after an oral infection of *Pseudomonas* spp., confirming that the PM may play a defensive role by preventing contact between the bacteria and the gut epithelium (Vodovar et al., 2005). Also in a crustacean, a *peritrophin-like protein* was up-regulated in hemocytes, heart, stomach, gut, and gills following infection of bacteria by injection, but was constitutively expressed in the ovaries (Du et al., 2006). The study shows that the *mucin-like PM* expression was detected at very high level in the midgut. However, the expression of the *mucin-like PM* gene was slightly increased by 6 h and then declined at 24 h and 48 h after exposure to *V. harveyi.* This may indicate that *V. harveyi* and/ or its toxin perturbed the midgut epithelium and may have an affect on the constituent genes responsible for building the peritrophin-membrane such as the *mucin-like PM*.

Liehl et al., (2006) revealed that a major contribution to the *Drosophila* defense against *P. entomophila* is provided by a local, rather than a systemic immune

response which supports this results that the expression of five genes (*ALF3, crustin, penaeidin, C-lectin* and *TL5a*1) in the circulating hemocytes did not show any obvious changes during the experimental period whereas in the digestive system changes did occur. Also, no bacteria were re-isolated from the hemolymph of the treated shrimp, suggesting that no bacteria entered into hemolymph during these experiments. Therefore, under these conditions the bacterial infection may be confined only to the digestive system. This speculation was consistent with the histopathological observations that the midgut of infected shrimp showed complete destruction of the epithelial cells and it was replaced by intensely infiltrating hemocytes, which would provide a multi-layered barrier and assist in the defense of the pathogenic bacteria in this economically important species.

CHAPTER VI
CONCLUSIONS

1. A more natural way of establishing an infection in shrimp was performed by immersion shrimp in a suspension of a pathogen. *V. harveyi* invaded by an oral route in shrimp showed that PL have a higher mortality by this route of infection than juveniles even at the higher dose of *V. harveyi* used to infect PL.

2. A histopathological study showed that both PL and juveniles have massive destruction of the digestive system and they have efficient cellular defense mechanisms including phagocytosis and the prophenoloxidase activating system to fight the infecting bacteria.

3. Almost all of the immune related genes including *ALF2, ALF3, crustin, penaeidin, PrX, C-lectin, BGBP, MMR, TL5a1, TL5a2, Lysozyme, MnSOD* and *Mucin-like PM* were expressed at high levels in intestine of normal shrimp, except *proPO* which had a very low expression in all part of the intestine and *ficolin* which was expressed in varying in different region of the intestine.

4. The effects of *V. harveyi* immersion on expression of the 15 immune-related genes, nearly all of these genes were found to be constitutively expressed at high levels and only six of them were slightly affected in their expression. The expression level of *C-lectin* was obviously changed. Other examined genes such as antimicrobial peptides (AMPs; *ALF3, crustin*, and *penaeidin*), *TL5a1* and *mucin-like PM* was slightly changed. In addition, the transcription levels of *ALF2, PrX, proPO, BGBP, MMR, TL5a2, ficolin, Lysozyme* and *MnSOD* were not obviously different after *V. harveyi*-immersion.

5. Besides the digestive organ, the *AMP* transcripts (*ALF3, crustin* and *penaeidin),* were expressed highly in hemocytes and moderately in AM. A high level of expression of the *C-lectin* was found in the hepatopancreas, AM and PH, lesser expression in hemocytes. Interestingly, not only was the *TL5a1* expression found in the stomach and the PH part of the digestive system, but it was also expressed at the highest level among the examined organs in epidermis and at a lower level in nerves, pleopods and eyestalk. The *mucin-like PM* was expressed in a limited number of tissues, the highest was found in AM and slightly lower levels in PH.

REFERENCES

Anderson, I.G., Shamsudin, M.N., Shariff, M. and Nash, G. 1988. Bacterial septicemia in juvenile tiger shrimp, *Penaeus monodon*, cultured in Malaysian brackishwater ponds. Asian Fisheries Science 2: 93-108.

Arkarajamon, A. 1991. Histology of tiger shrimp (*Penaeus monodon* Fabricius). Master of Science (Fisheries Science) Fisheries Science Kasetsart University.

Arts, J.A.J., Cornelissen, F.H.J., Cijsouw, T., Hermsen, T., Savelkoul, H.F.J. and Stet, R.J.M. 2007. Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon*. Fish and Shellfish Immunology 23: 504-513.

Aruma, C. 1989. Determining the lethal dose (LD50) of *Vibrio* and *Pseudomonas* bacteria for marine shrimp. Pacific Science 43: 186.

Aspán, A., Huang, T.S., Cerenius, L., Söderhäll, K. 1995. cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proceedings of the National Academy of Sciences USA 92: 939-943.

Aspán A., and Söderhäll, K. 1991. Purification of prophenoloxidase from crayfish blood cells, and its activation by an endogenous serine proteinase. Insect Biochemistry 21: 363-373.

Aspán, A., Sturtevant, J., Smith, V.J. and Söderhäll, K. 1990. Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. Insect Biochemistry 20: 709-718.

Austin, B. and Zhang, X-H. 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Letters in applied microbiology 43:119-124.

Bachère, E., Gueguen, Y., Gonzalez, M., de Lorgeril, J., Garnier, J., and Romestand, B. 2004. Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. Immunological Reviews 198: 149-168.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. 2005. Host-bacterial mutualism in the human intestine. Science 307: 1915-1920.

Bailey-Brock, J.H. and Moss, S.M. 1992. Penaeid taxonomy, biology and zoogeography. In Marine culture: Principles and practices (Fast A. W. and Lester L. J., Eds). Elsrvier Science Publishers, Amterdam.

Bartlett, T.C., Cuthbertson, B.J., Shepard, E.F., Chapman, R.W., Gross, P.S. and Warr, G.W. 2002. Crustins, homologues of an 11.5-kDa antimicrobial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*. Marine Biotechnology 4: 278-293.

Basset, A., Khush, R.S., Braun, A., Gardan, L. and Boccard, F. 2000. The phytopathogenic bacteria, *Erwinia carotovora*, infects *Drosophila* and activates an immune response. Proceedings of the National Academy of Sciences 97: 3376-3381.

Bell, T.A. and Lightner, D.V. 1988. A handbook of normal penaeid shrimp histology. World Aquaculture Society, USA.

Bischoff, V.,Vignal, C., Duvic, B., Boneca, I.G., Hoffmann, J.A. and Royet, J. 2006. Downregulation of the *Drosophila* immune response by peptidoglycan-recognition proteins SC1 and SC2 . PLoS Pathogen 2: e14.

Bogdan, C., Röllinghoff, M. and Diefenbach, A. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Current Opinion in Immunology 12: 64-76.

Bowie, A. and O'Neill, L.A.J. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. Journal of Leukocyte Biology 67(4): 508-514.

Brock, J.A. and Lightner, D.V. 1990. Chapter 3: Diseases of Crustacea. In: Diseases of Marine Animals Vol. 3 (Kinne O., Ed). Biologische Anstalt Helgoland, Hamburg. pp. 245-424.

Brouwer, M., Brouwer, T.H., Grater, W. and Brown-Peterson, N.B. 2003. Replacement of a cytosolic copper/zinc superoxide dismutase by a novel cytosolic manganese superoxide dismutase in crustaceans that use copper (haemocyanin) for oxygen transport. Biochemistry Journal 374: 219-228.

Brouwer, M., Brouwer, T.H., Grater, W., Enghild, J.J. and Thogersen, I.B. 1997. The paradigm that all oxygen-respiring eukaryotes have cytosolic CuZn-superoxide dismutase and that Mn-superoxide dismutase is localized to the

mitochondrion does not apply to a large group of marine arthopods. Biochemistry 36: 13381-13388.

Bu, X., Du, X., Zhou, W., Zhao, X. and Wang, J. 2008. Molecular Cloning, Recombinant Expression, and Characterization of Lysozyme from Chinese Shrimp *Fenneropenaeus chinensis*. Chinese Journal of Biotechnology 24: 723-732.

Bulet, P., Hetru, C., Dimarcq, J-L. and Hoffmann, D. 1999. Antimicrobial peptides in insects; structure and function. Developmental and Comparative Immunology 23: 329-344.

Burge, E.J., Madigan, D.J., Burnett, L.E. and Burnett, K.G. 2007. Lysozyme gene expression by hemocyte of Pacific white shrimp *Litopenaeus vannamei*, after injection with *Vibrio*. Fish and Shellfish Immunology 22: 327-329.

Cerenius, L., Lee, B.L. and Söderhäll, K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends in Immunology 29(6): 263-271.

Cerenius, L., Liang, Z., Duvic, B., Keyser, P., Hellman, U. and Tapio-Palva, E. 1994. Structure and biological activity of a 1,3-beta-D-glucan-binding protein in crustacean blood. Journal of Biological Chemistry 269: 29462-29467.

Chen, C., Durrant, H.J., Newton, R.P. and Ratcliffe, N.A. 1995. A study of novel lectins and their involvement in the activation of the prophenoloxidase system in *Blaberus discoidalis*. Biochemistry Journal 310: 23-31.

Chen, J.Y., Pan, C.Y. and Kuo, C.M. 2004a. cDNA sequence encoding an 11.5-kDa antimicrobial peptide of the shrimp *Penaeus monodon*. Fish and Shellfish Immunology 16: 659-664.

Chen, J.Y., Pan, C.Y. and Kuo, C.M. 2004b. Molecular cloning and sequencing of shrimp (*Penaeus monodon*) penaeidin-5 cDNA. Fish and Shellfish Immunology 16: 665-670.

Chen, M.Y., Hu, K.Y., Huang, C.C. and Song, Y.L. 2005. A second type of transglutaminase is involved in shrimp coagulation. Developmental and Comparative Immunology 29:1003-1016.

Chen, S.N., Huang, S.L. and Kou, G.H. 1992. Studies on the epizootiology and pathogenicity of bacterial infections in cultured giant tiger prawns, *Penaeus monodon* in Taiwan. In: Diseases of cultured penaeid shrimp in Asia and the

United States (Fulks W. and Main K.L., eds). The Oceanic Institute, Honolulu, pp. 195-205.

Cheng, W, Tung, Y-H, Chiou, T-T and Chen, J-C. 2006a. Cloning and characterisation of mitochondrial manganese superoxide dismutase (mtMnSOD) from the giant freshwater prawn *Macrobrachium rosenbergii*. Fish and Shellfish Immunology 21(4): 453-466.

Cheng, W., Tung, Y-H, Liu, C-H. and Chen J-C. 2006b. Molecular cloning and characterisation of copper/zinc superoxide dismutase (Cu,Zn-SOD) from the giant freshwater prawn *Macrobrachium rosenbergii*. Fish and Shellfish Immunology 21: 102-112.

Chythanya, R., Karunasagar, I. and Karunasagar, I. 2002. Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. Aquaculture 208: 1-10.

Collins, H. and Lyne,P.M. 1976. Microbiologicd Methods. 4th ed. Butterworths, London. pp. 200-201,169-180.

Colorni, A. 1985. A study on the bacterial flora of giant prawn, *Macrobrachium rosenbergii*, larvae fed with *Artemia salina* nauplii. Aquaculture 49: 1-10.

Cruciani R.A., Barker, J.L., Zasloff, M., Chen, H.C. and Colamonici, O. 1999. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proceedings of the National Academy of Sciences of the United States of America 88(9): 3792-3796.

Cuthbertson, B.J., Shepard, E.F., Chapman, R.W. and Gross, P.S. 2002. Diversity of the penaeidin antimicrobial peptides in two shrimp species. Immunogenetics 54: 442-445.

Daffre, S., Kylsten, P., Samakovlis, C. and Hultmark, D. 1994. The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract. Molecular and General Genetics 242: 152-162.

Dale, C. and Moran, N.A. 2006. Molecular interactions between bacterial symbionts and their hosts. Cell 126: 453-465.

de Gregorio, E., Spellman, P.T., Rubin, G.M. and Lemaitre, B. 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide

microarrays. Proceedings of the National Academy of Sciences USA 98: 12590-12595.

de la Peña, L., Tamaki, T., Monoyama, K., Nakai, T. and Muroga, K. 1993. Characteristics of the causative bacterium of vibriosis in the kuruma prawn, *Penaeus japonicus*. Aquaculture 115: 1-12.

de-la-Re-Vega, E., Garcia-Galaz, A., Diaz-Cinco, M.E. and Sotelo-Mundo, R.R. 2006. White shrimp (*Litopenaeus vannamei*) recombinant lysozyme has antibacterial activity against Gram negative bacteria: *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio cholerae*. Fish and Shellfish Immunology 20: 405-408.

de la Vega, E., García-Galaz, A., Díaz-Cinco, M.E. and Sotelo-Mundo, R.R. 2006. White shrimp (*Litopenaeus vannamei*) recombinant lysozyme has antibacterial activity against Gram negative bacteria: *Vibrio alginolyticus, Vibrio parahemolyticus* and *Vibrio cholerae*. Fish and Shellfish Immunology 20: 405-408.

de la Vega, E., O'Leary, N.A., Shockey, J.E., Robalino, J., Payne, C., Browdy, C.L., Warr, G.W. and Gross, P.S. 2008. Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): A broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Molecular Immunology 45: 1916-1925.

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J. and Bachere, E. 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). Journal of Biological Chemistry 272: 28398-28406.

Destoumieux, D., Munoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M. and Bachere, E. 2000. Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. Journal of Cell Science 113: 461-469.

Devasagayam, T.P., Tilak, J.C., Boloor, K.K., Sane, K.S., Ghaskadbi, S.S. and Lele, R.D. 2004. Free radicals and antioxidants in human health: current status and future prospects. Journal of Association of Physicians of India 52: 794-804.

Dijkers, P. and O'Farrell, P. H. 2007. *Drosophila* calcineurin promotes induction of innate immune responses. Current Biology 17: 2087–2093.

Dimarcq, J.L., Hoffmann, D., Meister, M., Bulet, P. and Lanot, R. 1994. Characterization and transcriptional profiles of a *Drosophila* gene encoding an insect defensin. A study in insect immunity. European Journal of Biochemistry 221: 201-209.

Dong, B., Liu, F., Gao, H., Wang, B. and Xiang, J. 2009. cDNA cloning and gene expression pattern following bacterial challenge of peroxinectin in Chinese shrimp *Fenneropenaeus chinensis*. Molecular Biology Reports (in press).

Du, X-J., Wang, J-X., Lui, N., Zhao, X-F., Li, F-H. and Xiang, J-X. 2006a. Identification and molecular characterization of a peritrophin-like protein from Freshy Prawn (*Fenneropenaeus chiensis*). Molecular Immunology 43: 16633-1644.

Du, X-J., Zhao, X-F. and Wang, J-X. 2006b. Molecular cloning and characterization of a lipopolysaccharide and β-1,3-glucan binding protein from fleshy prawn (*Fenneropenaeus chinensis*). Molecular Immunology 44: 1085-1094.

Duvic, B. and Söderhäll, K. 1990. Purification and characterization of a β-1,3-glucan binding protein from plasma of crayfish *Pacifastacus leniusculus*. Journal of Biological Chemistry 265: 9327-9332.

Egusa, S., Takahashi, Y., Itami, T. and Momoyama, K. 1988. Histopathology of vibriosis in the Kurama prawn, *Penaeus japonicus*. Fish Pathology 23: 59-65.

Endo, Y., Matsushita, M. and Fujita, T. 2007. Role of ficolin in innate immunity and its molecular basis. Immunobiology 212: 371-379.

Endo, Y., Takahashi, M. and Fujita, T. 2006. Lectin complement system and pattern recognition. Immunobiology 211: 283-293.

Esteve, M. and Herrera, F.C. 2000. Hepatopancreatic alterations in *Litopenaeus vannamei* (Boone, 1939) (Crustacea: Dacapoda: Penaeidae) experimentally infected with a *Vibrio alginolyticus* strain. Journal of Invertebrate Pathology 76: 1-5.

Fenouil, E. and Roch, P. 1991. Evidence and characterization of lysozyme in six species of freshwater crayfishes from Astacidae and Cambaridae families. Comparative Biochemistry and Physiology 99: 43-49.

Foley, E. and O'Farrell, P. H. 2003. Nitric oxide contributes to induction of innate immune responses to Gram negative bacteria in Drosophila. Genes and development 17: 115–125.

Frelier, P.F., Sis, R.F., Bell, T.A. and Lewis, D.H. 1992. Microscopic and ultrastructural studies of necrotizing hepatopancreatitis in Pacific white shrimp (*Penaeus vannamei*) culture in Texas. Veterinary Pathology 29: 269-277.

Gettins, P.G.W. 2002. Serpin structure, mechanism, and function. Chemical Reviews. 102: 4751-4803.

Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoe, Y., Wai, S.N., Iwanaga, S. and Kawabata, S-I. 1999. Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. Proceedings of the National Academy of Science 96: 10086-10091.

Gollas-Galván, T., Hernández-López, J. and Vargas-Albores, F. 1999. Prophenoloxidase from brown shrimp (*Penaeus californiensis*) hemocytes. Comparative Biochemistry and Physiology 122: 77-82.

Gómez-Anduroa, G.A., Barillas-Mury, C-V., Peregrino-Uriartea, A.B., Guptab, L., Gollas-Galvána, T., Hernández-Lópezc, J. and Yepiz-Plascencia, G. 2006. The cytosolic manganese superoxide dismutase from the shrimp *Litopenaeus vannamei*: Molecular cloning and expression. Developmental and Comparative Immunology 30(10): 893-900.

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapman, R.W. and Warr, G.W. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and the Atlantic white shrimp, *L. setiferus*. Developmental and Comparative Immunology 25: 565-577.

Ha, E.M., Oh, C.T., Bae, Y.S. and Lee, W.J. 2005a. A direct role for dual oxidase in *Drosophila* gut immunity. Science 310: 847-850.

Ha, E.M., Oh, C.T., Ryu, J.H., Bae, Y.S. and Kang, S.W. 2005b. An antioxidant system required for host protection against gut infection in *Drosophila*. Developmental Cell 8: 125–132.

Hall, M., Vanheusden, M.C. and Söderhäll, K. 1995. Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. Biochemical and Biophysical Research Communications 216: 939-946.

Hall, M., Wang, R., Antwerpen, R.V., Sottrup-Jensen, L. and Söderhäll, K. 1999. The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proceedings of the National Academy of Sciences 96: 1965-1970.

Hameed, A.S. 1995. Susceptibility of 3 *Penaeus* species to a *Vibrio campbellii*-like bacterium. Journal of the World Aquaculture Society 26: 315-319.

Hancock, R.E.W. and Daimond, G. 2000. The role of cationic antimicrobial peptides in innate host defences. Trends in Microbiology 8: 402-410.

Han, S.H., Ryu, J.H., Oh, C.T., Nam, K.B. and Nam, H.J. 2004. The moleskin gene product is essential for Caudal-mediated constitutive antifungal Drosomycin gene expression in *Drosophila* epithelia. Insect Molecular Biology 13: 323-327.

Harris, J.M. 1993. The presence, nature and role of gut microflora in aquatic invertebrates: a synthesis. Microbial ecology 25: 195-231.

Hasson, K.W., Hasson, J., Aubert, H., Redmana, R.M. and Lightner, D.V. 1997. A new RNA-friendly fixative for the preservation of penaeid shrimp samples for virological detection using cDNA genomic probes. Journal of Virological Methods 66: 227-236.

Haug, T., Kjuul, A.K., Stensva, K., Sandsdalen, E. and Strvold, O.B. 2002. Antibacterial activity in four marine crustacean decapods. Fish and Shellfish Immunology 12: 371-385.

Hauton, C., Brockton, V. and Smith, V.J. 2006. Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria. Molecular Immunology 43(9): 1490-1496.

Hegedus, D., Erlandson, M., Gillott, C. and Toprak, U. 2009. New Insights into Peritrophic Matrix Synthesis, Architecture, and Function. Annual Review of Entomology 54: 285-302.

Hikima, S., Hikima, J-i., Rojtinnakorn, J., Hirono, I. and Aoki, T. 2003. Characterization and function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio* species. Gene 316: 187-195.

Hoffmann, J.A., Kafatoa, F.C., Janeway, Jr.C.A. and Ekekowitz, R.A. 1999. Phylogenetic perspectives in innate immunity. Science 248(5418): 1313-1318.

Hoffmann, J.A. and Reichart, J-M. 1997. *Drosophila* immunity. Trends in Cell Biology 7: 309-316.

Holmblad, T., and Söderhäll, K. 1999. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. Aquaculture 172: 111-123

Holmskov, U., Thiel, S. and Jensenius, J.C. 2003. Collections and ficolins: humoral lectins of the innate immune defense. Annual Review of Immunology 21: 547-578.

Hooper, L.V. and Gordon, J.I. 2001. Commensal host-bacterial relationships in the gut. Science 292: 1115-1118.

Huang, C.C., Sritunyalucksana, K., Söderhäll K. and Song, L.Y. 2004. Molecular cloning and characterization of tiger shrimp (*Penaeus monodon*) transglutaminase. Developmental and Comparative Immunology 28: 279-294.

Hultmark, D. 1996. Insect lysozymes. In: Lysozymes: Model Enzymes in Biochemistry and Biology (Jollès P., Ed.). Birkhäuser Veriag Basel, Switzerland. pp. 87-102.

Humason, G.L. 1979. Animal tissue techniques. 4th ed. WH Freeman, San Francisco.

Icely J.D., Nott, J.A. 1992. Digestion and absorption: digestive system and associated organs. In: Microscopic anatomy of invertebrates: Decapod, Crustacea. Vol. 10 (Harrison F .W. and Humes A.G., Eds.). Wily-Liss Inc., N.Y. pp. 147-201.

Inamori, K., Saito, T., Iwaki, D., Nagira, T., Iwanaga, S., Arisaka, F. and Kawabata, S. 1999. A newly identified horseshoe crab lectin with specificity for blood group A antigen recognizes specific O-antigens of bacterial lipopolysaccharides. Journal of Biological Chemistry 274: 3272-3278.

Ishimaru, K.M., Akagawa-Matsushita, M. and Muroga, K. 1995. *Vibrio penaeicida* sp. nov., a pathogen of Kuruma shrimps (*Penaeus japonicus*). International Journal of Systematic Bacteriology 45 (1): 134-138.

Iwanaga, S. 2002. The molecular basis of innate immunity in the horseshoe crab. Current Opinion in Immunology 14: 87-95.

Iwanaga, S. and Lee, B.L. 2005. Recent advances in the innate immunity of invertebrate animals. Journal of Biochemistry and Molecular Biology 38: 128-150.

Janeway, C.A. 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor Symposia on Quantitative Biology 54: 1-13.

Janeway, Jr.C.A. and Medzhitov, R. 2002. Innate immune recognition. Annual Reveiw Immunology 20: 197-216.

Jayabalan, N., Chandran, R., Sivakumar, V. and Ramamoorthi, K. 1982. Occurence of luminescent bacteria in sediment. Current science 51: 710-711.

Jiravanichpaisal, P., Lee, B.L. and Söderhäll, K. 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211(4): 213-236.

Jiravanichpaisal, P., Miyazaki, T. and Limsuwan, C. 1994. Histopathology, biochemistry and pathogenicity of *Vibrio harveyi* infecting black tiger *prawn Penaeus monodon*. Journal of Aquatic Animal Health 6: 27-35.

Jiravanichpaisal, P., Puanglarp, N., Petkon, S., Donnuea, S., Söderhäll, I and Söderhäll. K. 2007. Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. Fish and Shellfish Immunology 23: 815-824.

Johansson, M.W., Holmblad, T., Thörnqvist, P.O., Cammarata, M. and Parrinello, N. 1999. A cell-surface superoxide dismutase is a binding protein for peroxinectin, a cell-adhesive peroxidase in crayfish. Journal of Cell Science 112: 917-925.

Johansson, M.W., Lind, M., Holmblad, T., Thörnqvist, P.O. and Söderhäll, K. 1995. Peroxinectin, a novel cell adhesion protein from crayfish blood. Biochemical and Biophysical Research Communications 216: 1079-1087.

Johansson, M.W. and Söderhäll, K. 1988. Isolation and purification of a cell adhesion factor from crayfish blood cells. Journal of Cell Biology 106: 1795-1803.

Johansson, M.W. and Söderhäll, K. 1989. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. Insect Biochemistry 19: 183-190.

Johnson P.T. 1980. Histology of the blue crab, *Callinectes sapidus*: A model for the decapoda. Praeger Publishers, New York.

Ju, L., Zhang, S, Liang, Y. and Sun, X. 2009. Identification, expression and antibacterial activity of a tachylectin-related homolog in amphioxus *Branchiostoma belcheri* with implications for involvement of the digestive system in acute phase response. Fish and Shellfish Immunology 26: 235-242.

Kang, C-J., Wang, J-X., Zhao, X-F., Yang, X-M., Shao, H-L. and Xiang, J-H. 2004. Molecular cloning and expression analysis of the *ch*-penaedin, an antimicrobial peptide from Chinese shrimp, *Fenneropenaeus chinensis*. Fish and Shellfish Immunology 16: 513-525.

Kang, C-J., Xue J-F., Liu, N., Zhao, X-F. and Wang J-X. 2007. Characterization and expression of a new subfamily member of penaeidin antimicrobial peptides (penaeidin 5) from *Fenneropenaeus chinensis*. Molecular Immunology 44: 1535-1543.

Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128: 203-209.

Kawabata, S-I. and Iwanaga, S., 1999. Role of lectins in the innate immunity of horseshoe crab. Developmental and Comparative Immunology 23: 391-400.

Kim, Y.S., Ryu, J.H., Han, S.J., Choi, K.H., Nam, K.B., Jang, I.H., Lemaitre, B., Brey, P.T. and Lee, W.J. 2000. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. Journal of Biological Chemistry 275: 32721-32727.

Kobayashi, M., Johansson, M.W. and Söderhäll, K. 1990. The 76 kDa cell-adhesion factor from crayfish haemocytes promote encapsulation in vitro. Cell and Tissue Research 260: 113-118.

Koizumi, N., Imamura, M., Kadotari, T., Yaoi, K., Iwahana, H. and Sato, R. 1999. The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains. FEBS Letters 443: 139-143.

Kondo, M.H., Matsuyama, H. and Yano, T. 1992. The opsonic effect of lectin on the phagocytosis by hemoytes of kuruma prawn, *Penaeus japonicus*. Fish Pathology 27: 217-222.

Kopacek, P., Hall, M and Söderhäll, K. 1993. Characterization of a clotting protein, isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*. European Journal of Biochemistry 213: 519-597.

Koropatnick, T.A., Engle, J.T., Apicella, M.A., Stabb, E.V., Goldman, W.E. and McFall-Ngai, M.J. 2004. Microbial factor-mediated development in a host-bacterial mutualism. Science 306: 1186-1188.

Labreuche, Y., O'Leary, N.A., de la Vega, E., Veloso, A., Gross, P.S., Chapman, R.W., Browdy, C.L. and Warra, G.W. 2009. Lack of evidence for *Litopenaeus vannamei* Toll receptor (lToll) involvement in activation of sequence-independent antiviral immunity in shrimp. Developmental and Comparative Immunology 33: 806-810.

Lai, C.Y., Cheng, W. and Kuo, C.M. 2005. Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, *Litopenaeus vannamei*. Fish and Shellfish Immunology 18: 417-430.

Lavilla-Pitogo, C.R., Albright, L.J., Paner, M.G. and Sunaz, N.A. 1992. Studies on the sources of luminescent *Vibrio harveyi* in *Penaeus monodon* hatcheries. In Diseases in Asian Aquaculture 1 (Shariff M., Subasinghe R.P. and Authur J.R., Eds). Fish Health Section, Asian Fisheries Society, Manila, Philippines. pp. 157-164.

Lavilla-Pitogo, C.R., Baticados, C.L., Cruz-Lacierda, E.R. and de la Pena, L. 1990. Occurrence of luminous bacteria disease of *Penaeus monodon* larvae in the

Philippines. Aquaculture 91: 1-13.

Lavilla-Pitogo, C.R., Leaño, E.M. and Paner, M.G. 1998. Mortalities of pond cultured juvenile shrimp, *Penaeus monodon*, associated with the dominance of Luminescent *Vibrio harveyi* in the rearing water. Aquaculture 164: 337-349.

Lee, K-K, Chen, Y-L. and Liu, P-C. 1999. Hemostasis of tiger prawn *Penaeus monodon* affected by *Vibrio harveyi*, excellular products, and a toxic cysteine protease. Blood Cells, Molecules, and Diseases 25(13): 180-192.

Lee, S.Y. and Söderhäll, K. 2002. Early events in crustacean innate immunity. Fish and Shellfish Immunology 12: 421-437.

Lee, S.Y., Wang, R. and Söderhäll, K. 2000. A lipopolysaccharide- and *β*-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leninusculus*. Purification, characterization, and cDNA cloning. Journal of Biological Chemistry 275: 1337-1343.

Lemaitre, B. and Hoffmann, J. 2007. The Host Defense of *Drosophila melanogaster*. Annual Review of Immunology 25: 697-743.

Li, L., Wang, J-X., Zhao, X-F., Kang, C-J., Liu, N., Xiang, J-H., Li, F-H., Sueda, S. and Kondo, H. 2005. High level expression, purification, and characterization of the shrimp antimicrobial peptide, *Ch*-penaeidin, in *Pichia pastoris*. Protein Expression and Purification 39: 144-151.

Liehl, P., Blight, M., Vodovar, N., Boccard, F. and Lemaitre, B. 2006. Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. PLoS Pathogen 2: 0551-0561.

Lightner, D.V. 1983. Diseaese of penaeid shrimp. In Handbook of mariculture vol.1 Crustacean Aquaculture (McVey J., Ed). Boca Raton: CRC Press. pp. 289-320.

Lightner, D.V. and Lewis, D.H. 1975. A septicemic bacterial disease syndrome of penaeid shrimp. Marine Fisheries Review 37: 25-28

Lin, Y-C., Vaseeharan, B. and Chen J-C. 2008. Identification and phylogenetic analysis on lipopolysaccharide and β-1,3-glucan binding protein (LGBP) of kuruma shrimp *Marsupenaeus japonicus*. Developmental and Comparative Immunology 32: 1260-1269.

Liochev, S.I. and Fridovich, I. 2007. The effects of superoxide dismutase on H_2O_2 formation. Free radical biology and medicine 42: 1465-1469.

Liu, C-H., Cheng, W and Chen, J-C. 2005a. The peroxinectin of white shrimp *Litopenaeus vannamei* is synthesised in the semi-granular and granular cells, and its transcription is up-regulated with *Vibrio alginolyticus* infection. Fish and Shellfish Immunology 18: 431-444.

Liu, C-H., Cheng, W., Hsu, J-P. and Chen, J-C. 2004a. *Vibrio alginolyticus* infection in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing. Diseases of Aquatic Organisms 61: 169-174.

Liu, C-H., Cheng, W., Kuo, C.-M. and Chen, J.-C. 2004b. Molecular cloning and characterization of a cell adhesion molecule, Peroxinectin from the white shrimp *Litopenaeus vannamei*. Fish and Shellfish Immunology 17: 13-26.

Liu, F., Liu, Y. Li, F., Dong, B. and Xiang, J. 2005b. Molecular Cloning and Expression Profile of Putative Antilippopolisaccharide Factor in Chinese shrimp (*Fenneropenaeus chinensis*). Marine Biotechnology 7: 600-608.

Liu, H., Jiravanichpaisal, P., Soderhall, I., Cerenius, L. and Soderhall, K. 2006. Antilipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish *Pacifastacus leniusculus*. Journal of virology 80: 10365-10371.

Liu, P-C. and Lee, K-K. 1999. Cysteine protease is a major exotoxin of pathogenic luminous *Vibrio harveyi* in the tiger prawn, *Penaeus monodon*. Letters in Applied Microbiology 28: 428-430.

Liu, P.C., Lee, K.K. and Chen, S.N. 1996. Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. Letters in Applied Microbiology 22: 413-416.

Liu, Y.C., Li, F.H., Dong, B., Wang, B., Luan, W., Zhang, X.J., Zhang, L.S. and Xiang, J.H. 2007. Molecular cloning, characterization and expression analysis of a putative C-type lectin (*Fc*lectin) gene in Chinese shrimp *Fenneropenaeus chinensis*. Molecular Immunology 44: 598-607.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the Ct $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408.

Loongyai, W., Phongdara, A. and Chotigeat, W. 2007. Cloning and expression of a TCTP homolog from the ovaries of banana prawn. Marine Biology 150 (3): 455-462.

Lu, K-Y., Huang, Y-T., Lee H-H. and Sung H-H. 2006. Cloning the prophenoloxidase cDNA and monitoring the expression of proPO mRNA in prawns (*Macrobrachium rosenbergii*) stimulated in vivo by CpG oligodeoxynucleotides. Fish and Shellfish Immunology 20: 274-284.

Luo, T., Yang, H., Li, F., Zhang, X. and Xu, X. 2006. Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. Developmental Comparative Immunology 30: 607-617.

Luo, T., Zhang, X., Shao, Z. and Xu, X. 2003. *Pm*AV, a novel gene involved in virus resistance of shrimp *Penaeus monodon*. FEBS Letter 551: 53-57.

Ma, C. and Kanost , M.R. 2000. A β,1-3-glucan-recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. Journal of Biological Chemistry 275: 7505-7514.

Ma, T.H., Tiu, S.H., He, J.G. and Chan, S.M. 2007. Molecular cloning of a C-type lectin (*Lv*LT) from the shrimp *Litopenaeus vannamei*: early gene down-regulation after WSSV infection. Fish and Shellfish Immunology 23: 430-437.

Ma, T.H-T., Benzie, J.A.H., He, J-G and Chan, S-M. 2008. *Pm*LT, a C-type lectin specific to hepatopancreas is involved in the innate defense of the shrimp *Penaeus monodon*. Journal of Invertebrate Pathology 99: 332-341.

Macdonald, T.T. and Monteleone, G. 2005. Immunity, inflammation, and allergy in the gut. Science 307: 1920-1925.

Mali, B., Soza-Ried, J., Frohme, M. and Frank, U. 2006. Structural but not functional conservation of an immune molecule: a tachylectin-like gene in *Hydractinia*. Developmental and Comparative Immunology 30: 275-281.

Maningas, M.B.B., Kondoa, H., Hironoa, I., Saito-Taki, T. and Aokia, T. 2007. Essential function of transglutaminase and clotting protein in shrimp immunity. Molecular Immunology 45: 1269-1275.

Marques, M.R.F. and Barracco, M.A. 2000. Lectins, as non-self-recognition factors, in crustaceans. Aquaculture 191: 23-44.

Martin, G.G. and Grsves, B.L. 1985. Fine Structure and Classification of Shrimp Hemocytes. Journal of Morphology 185: 339-348.

Martin, G.G., Rubin, N. and Swanson, E. 2004. *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*. Diseases of Aquatic Organisms 60: 21-29.

Matsushita, M. and Fujita, T. 2001. Ficolins and the lectin complement pathway. Immunological Reviews 180: 78-85.

Matsushita, M. and Fujita, T. 2002. The role of ficolins in innate immunity. Immunobiology 205: 490-497.

Medzhitov, R. and Janeway, Jr. C.A. 2002. Decoding the patterns of self and nonself by the innate immune system. Science 296: 298-300.

Mekata, T., Kono, T., Yoshida, T., Sakai, M and Itami, T. 2008. Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus*. Fish and Shellfish Immunology 24: 122-133.

Mercy, S.P.D. and Ravindranath, H. 1994. Hemolysis and clearance of erythrocytes in *Scylla serrata* are related to the agglutination by the native sialic acid-specific lectin. Comparative biochemistry and physiology 109: 1075-1083.

Misra, C.K., Das, B.K., Pradhan, J., Pattnaik, P., Sethi, S. and Mukherjee, S.C. 2006. Changes in lysosomal enzyme activity and protection against *Vibrio* infection in *Macrobrachium rosenbergii* (De Man) post larvae after bath immunostimulation with β-glucan. Fish and Shellfish Immunology 17: 389-395.

Mohney, L.L., Lightner, D.V. and Bell, T.A. 1991. An epizootic due to *Vibrio* spp. in pond-reared *Penaeus vannamei* in Ecuador. World Aquaculture Meeting, Book of Abstracts, Puerto Rico. pp. 45.

Muñoz, M., Vandenbulcke, F., Saulnier, D. and Bachere, E. 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte

reactions in microbial challenged shrimp. European Journal of Biochemistry 269: 2678-2689.

Nagoshi, H., Inagawa, H., Morii, K., Harada, H., Kohchi, C. and Nishizawa, T. 2006 Cloning and characterization of a LPS-regulatory gene having an LPS binding domain in kuruma prawn *Marsupenaeus japonicus*. Molecular Immunology 43: 2061-2069.

Nash, G. Nithimathachoke, C., Tungmandi, C., Arkarjamorn, A., Prathanpipat, P. and Ruamthaveesub, P. 1992. Vibriosis and its control in pond-reared *Penaeus monodon* in Thailand. In: Diseases in Asian Aquaculture 1 (Shariff M., Subasinghe R.P. and Authur J.R., Eds). Fish Health Section, Asian Fisheries Society, Manila, Philippines. pp. 143-155.

O'Brien S.J., Womack J.E., Lyons L.A., Moore K.J., Jenkins N.A. and Copeland N.G. 1993. Anchored reference loci for comparative genome mapping in mammals. Nature Genetics 3: 103-112.

Okino, N., Kawabata, S., Saito, T., Hirata, M., Takagi, T. and Iwanaga, S. 1995. Purification, characterization, and cDNA cloning of a 27-kDa lectin (L10) from horseshoe crab hemocytes. Journal of Biological Chemistry 270: 31008-31015.

Okumura, T. 2007. Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*. Fish and Shellfish Immunology 22: 68-76.

Omori, S.A., Martin, G.G. and Hose, J.E. 1989. Morphology of hemocyte lysis and clotting in the ridgeback prawn, *Sicyonia ingentis*. Cell and Tissue Research 255(1): 117-123.

Önfelt, T.T., Roos, E. and Engstrom, Y. 2001. The imd gene is required for local Cecropin expression in *Drosophila* barrier epithelia. The European Molecular Biology Organization Journal 2: 239-243.

Orbea, A., Fahimi, D. and Cajaraville, M.D. 2000. Immunolocalization of four antioxidant enzymes in digestive glands of mollusk and crustacean and fish liver. Histochemistry and Cell Biology 114: 393-404.

Overstreet, R.M., Lightner, D.V., Hasson, K.W., McIlwain, S. and Lotz, J.M. 1997. Susceptibility to taura syndrome virus of some penaeid shrimp species native to the gulf of Mexico and the southeastern United States. Journal of Invertebrate Pathology 69(2): 165-176.

Pan, J., Kurosky, A., Xu, B., Chopra, A.K., Coppenhaver, D.H., Singh, I.P. and Baron, S. 2000. Broad antiviral activity in tissues of crustaceans. Antiviral Research 48: 39-47.

Pipe, R., Porte, C. and Livingstone, D. 1993. Antioxidant enzymes associated with the blood cells and haemolymph of the mussel *Mytilus edulis*. Fish and Shellfish Immunology 3: 221-233.

Primavera, J. H. 1990. External and internal anatomy of adult penaeid prawns/shrimps. SEAFDEC. Aquaculture Department. The Philippines.

QingMei, Z., Xing, Y., JunJie, B., RuiQuan, W. and JianRen, L. 2004. Isolation and characterization of the lysozyme-encoding gene from the black tiger shrimp, *Penaeus monodon*. Acta Hydrobiologica Sinica 28: 417-419.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg ,S. and Medzhitov, R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118: 229-241.

Rattanachai, A., Hirono, I., Ohira, T., Takahashi, Y. and Aoki, T. 2004. Cloning of kuruma prawn *Marsupenaeus japonicus* crustin-like peptide cDNA and analysis of its expression. Fisheries Science 70: 765-771.

Relf, J.M., Chisholm, J.R.S., Kemp, G.D., Smith, V.J. 1999. Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from thegranular haemocytes of the shore crab, *Carcinus maenas*. European Journal of Biochemistry 264: 350-357.

Rengpipat, S., Tunyanun, A., Fast, A.W., Piyatiratitivorakul, S. and Menasveta, P. 2003. Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. Diseases of Aquatic Organism 55: 169-173.

Ritsick, D.R., Edens, W.A., McCoy, J.W. and Lambeth, J.D. 2004. The use of model systems to study biological functions of Nox/Duox enzymes. Biochemical Society symposium 71: 85-96.

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y. and Aoki, T. 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. Fish and Shellfish Immunology 13: 69-83.

Romeo-Figueroa, M.G., Vargas-Requena, C., Sotelo-Mundo, R.R., Vargas-Albores, F., Higuera-Ciapara, I. and Söderhäll, K. 2004. Molecular cloning of a *β*-glucan pattern-recognition lipoprotein from the white shrimp *Penaeus* (*Litopenaeus*) *vannamei*: correlations between the deduced amino acid sequence and the native protein structure. Developmental and Comparative Immunology 28: 713-726.

Rosa, R.D., Stoco P.H. and Barracco, M.A. 2008. Cloning and characterisation of cDNA sequences encoding for anti-lipopolysaccharide factors (ALFs) in Brazilian palaemonid and penaeid shrimps. Fish and Shellfish Immunology 25: 693-696.

Ruby, E.G., Greenberg, E.P. and Hastings, J.W. 1980. Planktonic marine luminous bacteria: species distribution in the water column. Applied and Environmental Microbiology 39: 302-306.

Ryu, J.H., Ha, E.M., Oh, C.T., Seol, J.H. and Brey, P.T. 2006. An essential complementary role of NF-κB pathway to microbicidal oxidants in *Drosophila* gut immunity. The European Molecular Biology Organization Journal 25: 3693–3701.

Ryu, J.H., Nam, K.B., Oh, C.T., Nam, H.J. and Kim, S.H. 2004. The homeobox gene Caudal regulates constitutive local expression of antimicrobial peptide genes in *Drosophila* epithelia. Molecular and cellular biology 24: 172-185.

Saito, T., Hatada, M., Iwanaga, S. and Kawabata, S. 1997. A newly identified horseshoe crab lectin with binding specificity to O-antigen of bacterial lipopolysaccharides. Journal of Biological Chemistry 272: 30703-30708.

Saito, T., Kawabata, S., Shigenaga, T., Takayenoki, Y., Cho, J., Nakajma, H., Hirata, M. and Iwanaga, S. 1995. A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antibacterial activity. Journal of Biochemistry 117: 1131-1137.

Sambrook, J. E. and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 8.18-8.96

Scandalias, J.G. 1993. Oxygen stress and superoxide dismutase. Plant Physiology 101: 7-12.

Schroder, H.C., Ushijima, H., Krasko, A., Gamulin, V., Thakur, N.L. and Diehl-Seifert, B. 2003. Emergence and disappearance of an immune molecule, an antimicrobial lectin, in basal metazoan. A tachylectin-related protein in the sponge *Suberites domuncula*. Journal of Biological Chemistry 278: 32810-32817.

Schwarz, K.B. 1996. Oxidative stress during viral infection: a review. Free Radical Biology and Medicine 21: 641-649.

Sierra, C., Lascurain, R., Pereyra, A., Guevara, J., Martinez, G. and Agundis, C. 2005. Participation of serum and membrane lectins on te oxidative burst regulation in *Macrobrachium rosenbergii* hemocytes. Developmental and Comparative Immunology 29: 113-121.

Silverman, A., Bird, P.I., Carrell, R.W., Church, F.C., Coughlin, P.B., Gettins, P.G.W., Irving, J.A., Lomas, D.A., Luke, C.J., Moyer, R.W., Pemberton, P.A., Remold-O'Donnell, E., Salvesen, G.S., Travis, J. and Whisstock, J.C. 2001. The Serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Journal of Biological Chemistry 276: 33293-33296.

Simonet, G., Claeys, I. and Broeck, J.V. 2002. Structural and functional properties of a novel serine protease inhibiting peptide family in arthropods. Comparative Biochemistry and Physiology 132: 247-255.

Smith, M.W. and Doolilttle, E.F. 1992. A comparison of evolutionary rates of the two major kinds of superoxide dismutase. Journal of Molecular Evolution 34: 175-184.

Smith, V.J. and Söderhäll, K. 1983. Induction and degranulation and lysis of hemocytes in the freshwater crayfish, *Astacus astacus* by components of the prophenoloxidase activating system in vitro. Cell and Tissue Research 233(2): 295-303.

Söderhäll, I., Bangyeekhun, E., Mayo, S. and Söderhäll, K. 2003. Hemocyte production and maturation in an invertebrate, proliferation and gene expression in hematopoeitic stem cells of the freshwater crayfish, *Pacifastacus leniusculus*. Developmental and Comparative Immunology 27: 661-672.

Söderhäll, K. and Cerenius, L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Current Opinion in Immunology 10: 23-28.

Söderhäll, K., Smith, V.J. and Johansson, M.W. 1986. Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell and Tissue Research 245(1): 43-49.

Solis N.B. 1988. Biology and ecology. In: Biology and culture of *Penaeus monodon* (Taki Y., Premavara J.H. and Lobrera J., Eds). SEAFDEC. Aquaculture Department. The Philippines.

Somboonwiwat, K., Bachère, E, Rimphanitchayakit, V. and Tassanakajon, A. 2008. Localization of anti-lipopolysaccharide factor (ALF*Pm*3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties. Developmental and Comparative Immunology 32: 1170-1176.

Somboonwiwat, K., Marcos, M., Tassanakajon, A., Klinbunga, S., Aumelas, A. and Romestand, B. 2005. Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. Developmental and Comparative Immunology 29: 841-851.

Somboonwiwat, K., Supungul, P., Rimphanitchchayakit, V., Aoki, T., Hirono, I. and Tassanakajon, A. 2006. Differentially expressed genes in hemocytes of *Vibrio harveyi*-challenged shrimp *Penaeus monodon*. Journal of Biochemistry and Molecular Biology 39: 26-36.

Sotelo-Mundo, R.R., Islas-Osuna, M.A., de-la-Re-Vega, E., Hernández-López, J., Vargas-Albores, F. and Yepiz-Plascencia, G. 2003. cDNA cloning of the lysozyme of the white shrimp Penaeus vannamei. Fish and Shellfish Immunology 15: 325-331.

Sritunyalucksana, K., Cerenius, L. and Söderhäll, K. 1999. Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. Developmental and Comparative Immunology 23: 179-186.

Sritunyalucksana, K. and Söderhäll, K. 2000. The proPO and clotting system in crustaceans. Aquaculture 191: 53-69.

Sritunyalucksana, K., Lee, S.Y. and Söderhäll, K. 2002. A β-1,3-glucan binding protein from the black tiger shrimp, *Penaeus monodon*. Developmental and Comparative Immunology 26: 237-245.

Sritunyalucksana, K., Wongsuebsantati, K., Johansson, M.W. and Söderhäll, K. 2001. Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. Developmental and Comparative Immunology 25: 353-363.

Stabili, L., Miglietta, A.M. and Belmonte, G. 1999. Lysozyme-like and trypsin-like activities in the cysts of *Artemia franciscana* Kellog, 1906. Is there a passive immunity in a resting stage?. Journal of Express Marine Biology and Ecology 237: 291-303.

Stoss, T.D., Nickell, M.D., Hardin, D., Derby, C.D. and McClintock, T.S. 2004. Inducible transcript expressed by reactive epithelial cells at sites of olfactory sensory neuron proliferation. Journal of Neurobiology 58(3): 355-368.

Sugumaran, M. and Nellaiappan, K. 2000. Characterization of new phenoloxidase inhibitor from the cuticle of Manduca sexta. Biochemical and Biophysical Research Communications 268: 379-383.

Sun, Y-D., Fu, L-D., Jia, Y-P., Du, X-J., Wang, Q., Wang, Y-H., Zhao, X-F., Yu, X-Q. and Wang, J-X. 2008. A hepatopancreas-specific C-type lectin from the Chinese shrimp *Fenneropenaeus chinensis* exhibits antimicrobial activity. Molecular Immunology 45: 348-361.

Sung, H.H., Kou, G.H. and Song, Y.L. 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). Fish Pathology 29: 11-17.

Supungul, P., Klinbunga, S., Pichyangkura, R., Hirono, I., Aoki, T. and Tassanakajon, A. 2004. Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. Diseases of Aquatic Organism 61: 123-135.

Supungul, P., Klinbunga, S., Pichyangkura, R., Jitrapakdee, S., Hirono, I., Aoki, T. and Tassanakajon, A. 2002. Identification of immune-related genes in

hemocytes of black tiger shrimp (*Penaeus monodon*). Marine Biotechnology 4(5): 487-494.

Supungul, P., Tang, S., Maneeruttanarungroj, C., Rimphanitchayakit, V., Hironoc, I., Aokic,T. and Tassanakajon, A. 2007. Cloning, expression and antimicrobial activity of crustin*Pm*1, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. Developmental and Comparative Immunology 32: 61-70.

Takahashi, Y., Shimoyama, Y. and Momoyama, K. 1985. Pathogenicity and characteristics of *Vibrio* sp. isolated from cultured Kuruma Prawn *Penaeus japonicus* Bate. Bulletin of the Japanese Society of Scientific Fisheries 51(5): 721-730.

Tanaka, S., Nakamura, T., Morita, T. and Iwanaga, S. 1982. *Limulus* anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of *Limulus* coagulation system. Biochemical and Biophysical Research Communications 105(2): 717-723.

Tanticharoen, M., Flegel, T.W., Meerod, W., Grudloyma, U. and Pisamai, N. 2008. Aquacultural biotechnology in Thailand: the case of the shrimp industry. International Journal of Biotechnology 10(6): 588-603.

Tellam, R.L., Vuocolo, T., Eisemann, C., Briscoe, S., Riding, G., Elvin, C. and Pearson, R. 2003. Identification of an immuno-protective mucin-like protein, peritrophin-55, from the peritrophic matrix of *Lucilia cuprina* larvae. Insect Biochemistry and Molecular Biology 33: 239-252.

Tellam, R.L., Wijffels, G. and Willadsen, P. 1999. Peritrophic matrix proteins. Insect Biochemistry and Molecular Biology 29: 87-101.

Thaithongnum, S., Ratanama, P., Weeradechapol, K., Sukhoom, A. and Vuddhakul, V. 2006. Detection of *V. harveyi* in shrimp postlarvae and hatchery tank water by the Most Probable Number technique with PCR. Aquaculture 261: 1-9.

Tharntada, S., Somboonwiwat, K., Rimphanitchayakit,V. and Tassanakajon, A. 2008. Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci. Fish and Shellfish Immunology 24: 46-54.

Thörnqvist, P.O., Johansson, M.W. and Söderhäll, K. 1994. Opsonic activity of cell adhesion proteins and β-1,3-glucan-binding proteins from two crustaceans. Development and Comparative. Immunology 18: 3-12.

Tong, Y., Jiang, H. and Kanost, M.R. 2005. Identification of plasma proteases inhibited by Manduca sexta serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. Journal of Biological Chemistry 280: 14932-14942.

Tunkijjanukij, S. and Olafsen, J.A. 1998. Sialic acid-binding lectin with antibacterial activity from the horse mussel: further characterization and immunolocalization. Developmental and Comparative Immunology 22: 139-150.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R. and Gordon, J.I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444: 1027-1031.

Turner, M.W. 1996. Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunology 17: 532-540.

Tyagi, A., Khushiramani, R., Karunasagar, I. and Karunasagar, I. 2007. Antivibrio activity of recombinant lysozyme expressed from black tiger shrimp, *Penaeus monodon*. Aquaculture 272: 246-253.

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M. and Reichhart, J.M. 2000. Tissuespecific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. Immunity 13:737-748.

Vallet-Gely, I., Lemaitre, B. and Boccard, F. 2008. Bacterial strategies to overcome insect defences. Nature Reviews Microbiology 6: 302-313.

Vargas-Albores, F., Jiménez-Vega, F. and Söderhäll, K. 1996. A plasma protein isolated from brown shrimp (*Penaeus californieneis*) which enhances the activation of prophenoloxidase system by 1,3-glucan protein. Developmental and Comparative Immunology 20: 299-306.

Vargas-Albores, F., Jiménez-Vega, F. and Yepiz-Plascencia, G.M. 1997. Purification and comparison of β-1,3-glucan binding protein from white shrimp (*Penaeus vannamei*. Comparative Biochemistry and Physiology 116: 453-458.

Vargas-Albores, F., Yepiz-Plascencia, G., Jimėnez-Vega, F. and Ávila-Villa, A. 2004. Structural and functional differences of *Litopenaeus vannamei* crustins. Comparative Biochemistry and Physiology 138: 415-422.

Veseeharan, B. and Ramasamy, P. 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtillis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Letters in Applied Microbiology 36: 83-87.

Vizioli, J. and Salz, M. 2002. Antimicrobial peptides from animals: focus on invertebrates. Trends in Pharmacological Sciences 23: 494-496.

Vodovar, N., Acosta, C., Lemaitre, B. and Boccard, F. 2004. *Drosophila*: a polyvalent model to decipher host-pathogen interactions. Trends in Microbiology 12: 235-242.

Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., Boccard, F. and Lemaitre, B. 2005. *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. Proceedings of the National Academy of Sciences 102(32): 11414-11419.

Vogt, G. 1994. Life-cycle and functional cytology of the hepatopancreatic cells of *Astacus astacus* (Crustacea, Decapoda). Zoomorphology 114: 83-101.

Vogt, G. 1996. Morphology and physiology of digestive epithelia in *Decapod crustaceans*. Pflügers Archiv European Journal of Physiology 431: R239-R240.

Vogt, G., Stöcker, W., Storch, V. and Zwilling, R. 1989. Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish. Histochemistry 91: 373-381.

Vuocolo, T., Eisemann, C.H., Pearson, R.D., Willadsen, P. and Tellam, R.L. 2001. Identification and molecular characterisation of a peritrophin gene, peritrophin-48, from the myiasis fly *Chrysomya bezziana*. Insect Biochemistry and Molecular Biology 31: 919-932.

Wong, M.L. and Medrano, J.F. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39: 75-85.

Wang, P-H., Gu, Z-H., Huang, X-D., Liu, B-D., Deng, X-X., Ai, H-S, Wang, J., Yin, Z-X., Weng, S-P., Yu, X-Q. and He, J-G. 2009. An immune deficiency homolog from the white shrimp, *Litopenaeus vannamei*, activates antimicrobial peptide genes. Molecular Immunology (in press).

Wang, R., Lee, S.Y., Cerenius, L., Söderhäll, K. 2001a. Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. European Journal of Biochemistry 268: 895-902.

Wang,R., Liang, Z., Hall, M. and Söderhäll, K. 2001b. A transglutaminase involved in the coagulation system of the freshwater crayfish, *Pacifastacus leniusculus*, Tissue localization and cDNA cloning. Fish and Shellfish Immunology 11: 623-637.

Wang, Y-C., Chang, P-S. and Chen H-Y. 2008. Differential time-series expression of immune-related genes of Pacific white shrimp *Litopenaeus vannamei* in response to dietary inclusion of β-1,3-glucan. Fish and Shellfish Immunology 24: 113-121.

Xiang, J.H. 2001. Disease occurrence and control strategies of Mariculture organisms. Ocean Press, Beijing. pp. 1–4, 78–83.

Yan, J., Cheng, Q., Narashimhan,S., Li, C-B. and Aksoy, S. 2002. Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans morsitans*. Insect Biochemistry and Molecular Biology 32: 979-989.

Yang, C., Zhang, J., Li, F., Ma, H., Zhang, Q., Priya, T.A.J., Zhang, X. and Xiang, J. 2008. A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to Vibrio anguillarum infection. Fish and Shellfish Immunology 24: 564-574.

Yang, L-S., Yin., Z-Y., Liao, J-X., Huang, X-D., Guo, C-J., Weng, S-P., Chan, S-M., Yu, X-Q. and He, J-G. 2007. A Toll receptor in shrimp. Molecular Immunology 44: 1999-2008.

Yao, C-L., Wang, A-L., Wang, Z-Y., Wang, W-N. and Sun, R-Y. 2007. Purification and partial characterization of Cu, Zn superoxide dismutase from haemolymph of Oriental river prawn *Macrobrachium nipponense*. Aquaculture 270 (1-4): 559-565.

Yeh, M.S., Huang, C.J., Cheng, J.H. and Tsai, I.H. 2007. Tissue-specific expression and regulation of the hemolymph clottable protein of tiger shrimp (*Penaeus monodon*). Fish and Shellfish Immunology 23: 272-279.

Yeh, M.S., Huang, C.J., Leu, J.H., Lee, Y.C. and Tsai, I.H. 1999. Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (*Penaeus monodon*). European Journal of Biochemistry 266: 624-633.

Yeh, M.S., Kao, L.R., Huang, C.J. and Tsai, I.H. 2006. Biochemical characterization and cloning of transglutaminases responsible for hemolymph clotting in *Penaeus monodon* and *Marsupenaeus japonicus*. Biochimica et biophysica acta. Proteins and proteomics 1764: 1167-1178.

Yeh, M-S., Lai, C-Y., Liu, C-H., Kuo, C-M. and Cheng, W. 2009. A second proPO present in white shrimp *Litopenaeus vannamei* and expression of the proPOs during a *Vibrio alginolyticus* injection, molt stage, and oral sodium alginate ingestion. Fish and Shellfish Immunology 26: 49-55.

Yepiz-Plascencia, G., Vargas-Albores, F., Jimenz-Vega, F., Ruiz-Verdugo, L.M. and Romo-Figueroa, G. 1998. Shrimp plasma HDL and β-glucan binding protein (BGBP): comparison of biochemical characteristics. Comparative Biochemistry and Physiology 121: 309-314.

Yu, J., Angelin-Duclos, C., Greenwood, J., Liao, J. and Calame, K. 2000. Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. Molecular and Cellular Biology 20: 2592-2603.

Yu, X.Q., Gan, H. and Kanost, M.R. 1999. Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. Insect Biochemistry and Molecular Biology 29: 585-597.

Yu, X.Q. and Kanost, M.R. 2000. Immuletin-2, a lipopolysaccharide-specific lectin from an insect, Manduca sexta, is induced in response to gram-negative bacteria. Journal of Biological Chemistry 275: 37373-37381.

Zaidman-Remy, A., Herve, M., Poidevin, M., Pili-Floury, S. and Kim, M.S. 2006. The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. Immunity 24: 463-473.

Zhang, J., Wang, F., Li, Z. and Xiang, J. 2007a. Cloning and recombinant expression of a crustin-like gene from Chinese shrimp, *Fenneropenaeus chinensis*. Journal of Biotechnology 127(4): 605-614.

Zhang, Q., Li, F., Zhang,X., Dong, B., Zhang, J., Xie, Y. and Xiang, J. 2008. cDNA cloning, characterization and expression analysis of the antioxidant enzyme gene, catalase, of Chinese shrimp *Fenneropenaeus chinensis*. Fish and Shellfish Immunology 24(5): 584-591.

Zhang, Q., Lia, F., Wang, B., Zhang, J., Liu,Y., Zhou, Q. and Xiang, J. 2007b. The mitochondrial manganese superoxide dismutase gene in Chinese shrimp *Fenneropenaeus chinensis*: Cloning, distribution and expression. Developmental and Comparative Immunology 31(5): 429-440.

APPENDIX

APPENDIX A

Table A.1 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *ALF3* in AM.

Sample	h	$ALF3 C_T$	$EF1\alpha C_T$	Average $ALF3 C_T$±SD	Average $EF1\alpha C_T$±SD	$\Delta\Delta C_T$±SD	Average fold change±SD
AM1	0	18.3	14.8	18.48±1.30	14.74±0.26		
AM2	0	20.7	15.1				
AM3	0	17.6	14.6				
AM4	0	17.5	14.4				
AM5	0	18.3	14.8				
AM1	6	14.6	15.6	16.14±1.30	15.22±0.67	-2.82±1.46	7.06±0.36
AM2	6	16.7	15.0				
AM3	6	17.4	16.2				
AM4	6	17.1	14.6				
AM5	6	14.9	14.7				
AM1	24	15.2	15.0	15.72±1.26	14.92±0.38	-2.94±1.31	7.67±0.40
AM2	24	17.7	15.1				
AM3	24	16.1	14.9				
AM4	24	14.4	15.3				
AM5	24	15.2	14.3				
AM1	48	18.2	14.7	17.06±1.69	14.56±0.69	-1.24±1.83	2.36±0.28
AM2	48	19.1	15.1				
AM3	48	17.2	15.3				
AM4	48	14.9	13.7				
AM5	48	15.9	14.0				

Table A.2 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *ALF3* in PH.

Sample	h	$ALF3C_T$	$EF1\alpha C_T$	Average $ALF3C_T$±SD	Average $EF1\alpha C_T$±SD	$\Delta\Delta C_T$±SD	Average fold change±SD
PH1	0	17.2	14.0	17.74±1.24	14.26±0.69		
PH2	0	19.6	14.9				
PH3	0	18.4	14.4				
PH4	0	16.9	13.2				
PH5	0	16.6	14.8				
PH1	6	15.5	14.4	16.54±0.85	14.14±0.29	-1.08±0.89	2.11±0.54
PH2	6	16.4	14.4				
PH3	6	17.8	14.1				
PH4	6	16.2	13.7				
PH5	6	16.8	14.1				
PH1	24	14.6	15.0	16.68±1.58	13.94±1.00	-0.74±1.87	1.67±0.27
PH2	24	19.0	15.0				
PH3	24	16.7	13.4				
PH4	24	16.2	12.8				
PH5	24	16.9	13.5				
PH1	48	19.1	14.5	17.96±1.65	14.52±0.27	-0.04±1.67	1.03±0.31
PH2	48	19.2	14.5				
PH3	48	19.2	14.8				
PH4	48	16.1	14.1				
PH5	48	16.2	14.7				

Table A.3 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *crustin* in AM.

Sample	h	*crustin* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				crus C_T±SD	*EF1α* C_T±SD		
AM1	0	20.7	14.8	22.10±1.06	14.74±0.26		
AM2	0	22.4	15.1				
AM3	0	23.6	14.6				
AM4	0	21.7	14.4				
AM5	0	22.1	14.8				
AM1	6	21.2	15.6	21.98±2.34	15.22±0.67	-0.60±2.44	1.52±0.18
AM2	6	21.3	15.0				
AM3	6	21.1	16.2				
AM4	6	26.1	14.6				
AM5	6	20.2	14.7				
AM1	24	19.9	15.0	21.20±1.44	14.92±0.38	-1.08±1.49	2.11±0.36
AM2	24	21.5	15.1				
AM3	24	23.4	14.9				
AM4	24	19.9	15.3				
AM5	24	21.3	14.3				
AM1	48	22.8	14.7	21.64±1.23	14.56±0.69	-0.28±1.41	1.21±0.38
AM2	48	19.7	15.1				
AM3	48	21.6	15.3				
AM4	48	22.6	13.7				
AM5	48	21.5	14.0				

Table A.4 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *crustin* in PH.

Sample	h	*crustin* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				crus C_T±SD	*EF1α* C_T±SD		
PH1	0	19.5	14.0	21.62±1.78	14.26±0.69		
PH2	0	22.8	14.9				
PH3	0	24.0	14.4				
PH4	0	20.8	13.2				
PH5	0	21.0	14.8				
PH1	6	21.8	14.4	21.56±0.67	14.14±0.29	0.06±0.73	0.96±0.60
PH2	6	22.2	14.4				
PH3	6	22.1	14.1				
PH4	6	21.0	13.7				
PH5	6	20.7	14.1				
PH1	24	20.7	15.0	21.82±1.14	13.94±1.00	0.52±1.52	0.70±0.35
PH2	24	22.7	15.0				
PH3	24	23.1	13.4				
PH4	24	20.6	12.8				
PH5	24	22.0	13.5				
PH1	48	23.0	14.5	22.58±1.02	14.52±0.27	0.70±1.05	0.62±0.48
PH2	48	20.8	14.5				
PH3	48	22.7	14.8				
PH4	48	23.1	14.1				
PH5	48	23.3	14.7				

Table A.5 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *penaeidin* in AM.

Sample	h	*penaeidin* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				pen C_T±SD	*EF1α* C_T±SD		
AM1	0	17.7	15.4	19.00±2.07	15.77±0.64		
AM2	0	17.4	16.7				
AM3	0	22.5	15.1				
AM4	0	18.4	15.6				
AM5	0	19.1	16.1				
AM1	6	16.8	16.2	17.44±2.10	15.88±0.22	-1.67±2.11	3.19±.23
AM2	6	17.4	15.7				
AM3	6	17.2	15.9				
AM4	6	20.8	16.0				
AM5	6	15.0	15.7				
AM1	24	16.9	15.8	19.61±2.33	15.53±0.70	0.852.43	0.55±0.19
AM2	24	18.8	16.1				
AM3	24	21.5	14.7				
AM4	24	18.4	16.2				
AM5	24	22.5	14.9				
AM1	48	20.2	15.1	20.65±2.92	15.13±0.34	2.29±2.94	0.20±0.23
AM2	48	17.1	15.6				
AM3	48	18.8	15.4				
AM4	48	23.1	14.8				
AM5	48	24.1	14.8				

Table A.6 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *penaeidin* in PH.

Sample	h	*penaeidin* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				pen C_T±SD	*EF1α* C_T±SD		
PH1	0	18.0	14.6	19.02±1.88	14.70±0.70		
PH2	0	17.7	15.6				
PH3	0	22.3	13.9				
PH4	0	18.2	14.2				
PH5	0	18.9	15.2				
PH1	6	18.2	14.7	18.11±1.09	14.93±0.31	-1.15±1.13	2.21±0.46
PH2	6	18.0	15.5				
PH3	6	17.8	14.7				
PH4	6	19.8	14.9				
PH5	6	16.8	14.9				
PH1	24	17.0	15.3	19.64±2.28	14.87±0.71	0.45±3.22	0.73±0.11
PH2	24	18.8	15.8				
PH3	24	21.5	14.1				
PH4	24	18.4	14.3				
PH5	24	22.5	14.8				
PH1	48	18.4	14.3	20.30±3.10	15.01±0.54	0.97±3.15	0.51±0.11
PH2	48	17.1	15.7				
PH3	48	18.8	15.4				
PH4	48	23.1	14.8				
PH5	48	24.1	14.8				

Table A.7 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *C-lectin* in AM.

Sample	h	*C-lectin* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				C-lectin C_T±SD	*EF1α* C_T±SD		
AM1	0	19.4	14.8	20.64±1.13	14.74±0.26		
AM2	0	20.7	15.1				
AM3	0	19.6	14.6				
AM4	0	21.8	14.4				
AM5	0	21.7	14.8				
AM1	6	16.5	15.6	19.24±1.56	15.22±0.67	-1.88±1.70	3.68±0.31
AM2	6	19.6	15.0				
AM3	6	20.3	16.2				
AM4	6	19.7	14.6				
AM5	6	20.1	14.7				
AM1	24	18.1	15.0	17.40±1.63	14.92±0.38	-3.42±1.67	10.70±0.31
AM2	24	19.9	15.1				
AM3	24	15.8	14.9				
AM4	24	16.5	15.3				
AM5	24	16.7	14.3				
AM1	48	17.2	14.7	16.54±2.20	14.56±0.69	-3.92±2.31	15.14±0.20
AM2	48	14.6	15.1				
AM3	48	14.2	15.3				
AM4	48	17.1	13.7				
AM5	48	19.6	14.0				

Table A.8 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *C-lectin* in PH.

Sample	h	*C-lectin* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				C-lectin C_T±SD	*EF1α* C_T±SD		
PH1	0	19.0	14.0	19.90±0.70	14.26±0.69		
PH2	0	20.9	14.9				
PH3	0	19.9	14.4				
PH4	0	20.1	13.2				
PH5	0	19.6	14.8				
PH1	6	15.9	14.4	18.96±1.77	14.14±0.29	-0.82±1.79	1.77±0.29
PH2	6	19.7	14.4				
PH3	6	19.7	14.1				
PH4	6	19.1	13.7				
PH5	6	20.4	14.1				
PH1	24	19.1	15.0	18.72±1.60	13.94±1.00	-0.86±1.89	1.82±0.27
PH2	24	21.1	15.0				
PH3	24	16.7	13.4				
PH4	24	18.2	12.8				
PH5	24	18.5	13.5				
PH1	48	17.9	14.5	16.78±1.76	14.52±0.27	-3.38±1.78	10.41±0.29
PH2	48	15.5	14.5				
PH3	48	14.9	14.8				
PH4	48	16.4	14.1				
PH5	48	19.2	14.7				

Table A.9 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *TL5a1* in AM.

Sample	h	*TL5a1* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				TL5a1 C_T±SD	*EF1α* C_T±SD		
AM1	0	27.1	14.8	26.68±0.36	14.74±0.26		
AM2	0	26.4	15.1				
AM3	0	26.3	14.6				
AM4	0	26.6	14.4				
AM5	0	27.0	14.8				
AM1	6	27.4	15.6	26.88±2.01	15.22±0.67	-0.28±2.12	1.21±0.23
AM2	6	24.4	15.0				
AM3	6	26.6	16.2				
AM4	6	26.1	14.6				
AM5	6	29.9	14.7				
AM1	24	26.3	15.0	25.24±1.49	14.92±0.38	-1.62±1.54	3.07±0.34
AM2	24	24.3	15.1				
AM3	24	27.1	14.9				
AM4	24	25.1	15.3				
AM5	24	23.4	14.3				
AM1	48	28.1	14.7	26.54±2.87	14.56±0.69	0.04±2.95	0.97±0.13
AM2	48	29.8	15.1				
AM3	48	24.7	15.3				
AM4	48	27.5	13.7				
AM5	48	22.6	14.0				

Table A.10 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *TL5a1* in PH.

Sample	h	$TL5a1 C_T$	$EF1\alpha C_T$	Average $TL5a1 C_T$±SD	Average $EF1\alpha C_T$±SD	$\Delta\Delta C_T$±SD	Average fold change±SD
PH1	0	17.7	14.0	17.46±0.53	14.26±0.69		
PH2	0	18.0	14.9				
PH3	0	16.8	14.4				
PH4	0	17.0	13.2				
PH5	0	17.8	14.8				
PH1	6	17.5	14.4	16.36±1.42	14.14±0.29	-0.98±1.45	1.97±0.37
PH2	6	16.4	14.4				
PH3	6	15.2	14.1				
PH4	6	14.7	13.7				
PH5	6	18.0	14.1				
PH1	24	18.2	15.0	16.88±0.78	13.94±1.00	-0.26±1.27	1.20±0.41
PH2	24	16.6	15.0				
PH3	24	16.5	13.4				
PH4	24	16.2	12.8				
PH5	24	16.9	13.5				
PH1	48	18.7	14.5	17.30±0.83	14.52±0.27	-0.42±0.88	1.34±0.54
PH2	48	17.4	14.5				
PH3	48	16.9	14.8				
PH4	48	16.6	14.1				
PH5	48	16.9	14.7				

Table A.11 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *mucin-like PM* in AM.

Sample	h	*mucin-like PM* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				mucin C_T±SD	*EF1α* C_T±SD		
AM1	0	16.3	14.8	16.24±0.47	14.74±0.26		
AM2	0	16.4	15.1				
AM3	0	15.5	14.6				
AM4	0	16.8	14.4				
AM5	0	16.2	14.8				
AM1	6	17.2	15.6	16.14±0.97	15.22±0.67	-0.58±1.18	1.49±0.44
AM2	6	17.1	15.0				
AM3	6	15.6	16.2				
AM4	6	15.8	14.6				
AM5	6	15.0	14.7				
AM1	24	18.8	15.0	16.98±1.69	14.92±0.38	0.56±1.74	0.68±0.30
AM2	24	14.8	15.1				
AM3	24	16.4	14.9				
AM4	24	18.6	15.3				
AM5	24	16.3	14.3				
AM1	48	17.4	14.7	17.84±2.09	14.56±0.69	1.78±2.20	0.29±0.22
AM2	48	15.9	15.1				
AM3	48	16.1	15.3				
AM4	48	18.9	13.7				
AM5	48	20.9	14.0				

Table A.12 Data analysis using the $2^{-\Delta\Delta C_T}$ method of *mucin-like PM* in PH.

Sample	h	*mucin-like PM* C_T	*EF1α* C_T	Average		$\Delta\Delta C_T$±SD	Average fold change±SD
				mucin C_T±SD	*EF1α* C_T±SD		
PH1	0	21.1	14.0	21.14±2.98	14.26±0.69		
PH2	0	26.3	14.9				
PH3	0	19.7	14.4				
PH4	0	19.3	13.2				
PH5	0	19.3	14.8				
PH1	6	21.9	14.4	19.72±2.51	14.14±0.29	-1.30±2.52	2.46±0.17
PH2	6	22.0	14.4				
PH3	6	20.6	14.1				
PH4	6	17.3	13.7				
PH5	6	16.8	14.1				
PH1	24	22.8	15.0	20.78±3.64	13.94±1.00	-0.04±3.78	1.03±0.07
PH2	24	26.2	15.0				
PH3	24	19.0	13.4				
PH4	24	18.4	12.8				
PH5	24	17.5	13.5				
PH1	48	22.6	14.5	21.20±1.56	14.52±0.27	-0.20±1.58	1.15±0.33
PH2	48	21.9	14.5				
PH3	48	21.9	14.8				
PH4	48	18.6	14.1				
PH5	48	21.0	14.7				

BIOGRAPHY

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Publication related with this thesis

1. **Soonthornchai**, W., Klinbunga, S., Jarayabhun, P. and Jiravanichpaisal, P. 2009. Expression of immune-related genes in intestine of black tiger shrimp (*Penaeus monodon*). The 4th Conference on Science and Technology for Youth (2009), 20-21 January 2009, Bangkok International Trade and Exhibition Centre (BITEC Bangna), Banhkok, Thailand (Oral presentation).