Immune defence of White Spot Syndrome Virus infected shrimp, *Penaeus monodon*

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Immune defence of White Spot Syndrome Virus infected shrimp, *Penaeus monodon*

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CHAPTER

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General introduction

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Penaeid shrimp biology

Taxonomy

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. Arthropods have a rigid cuticle that covers the whole animal, made largely of chitin and proteins, forming an exoskeleton that may or may not be further stiffened with calcium carbonate. The phylum takes its name from its distinctive jointed appendages (Hickman *et al.*, 2006).

The 42.000 species described today in the subphylum Crustacea include lobsters, crabs, shrimp, pill bugs, krill, barnacles, water fleas, brine shrimp (sea monkeys), copepods, ostracods. Shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda, which is part of the class Malacostraca (Hickman *et al.*, 2006). All farm-raised shrimp belong to the Penaeidae family and are referred to as penaeids (FAO, 2006).

Morphology

The body of penaeid shrimp is divided in two segments, the cephalothorax and the abdomen (figure 1.1). Most organs, such as gills, heart, hepatopancreas and stomach are located in the cephalothorax, while the gut and reproduction organs are in the muscled abdomen. The appendages of the cephalothorax are modified into different forms, including five pairs of walking legs (pereiopods), jaw-like structures, antennule and antennae. The latter two perform, together with the compound eye, the sensory functions. On the abdomen, five pairs of swimming legs (pleopods) are located (Bell *et al.*, 1988; Bailey-Brock *et al.*, 1992; Hickman *et al.*, 2006).

Arthopods have an open circulatory system, therefore the blood and the blood cells are called haemolymph and haemocytes, respectively. The heart and the main haemolymph vessels are located dorsally, together with the haemocoel. The heart pumps the haemolymph to the sinuses that are scattered throughout the body, were

exchange of gasses and nutrients takes place (e.g. oxygen in the gills). From the sinuses the haemolymph returns to the heart *via* the haemocoel.

In order to have an elevated exchange of substances with the external environment, the gills and the digestive tract have a very thin cuticle and, therefore, these organs can be a target for invading pathogens.

To enable growth, crustaceans periodically loosen their extracellular cuticle from the underlying epidermal layer to replace it for a new flexible, expandable exoskeleton that subsequently hardens. During and immediately after moulting, the animals are vulnerable to physical damage, predation and pathogens.

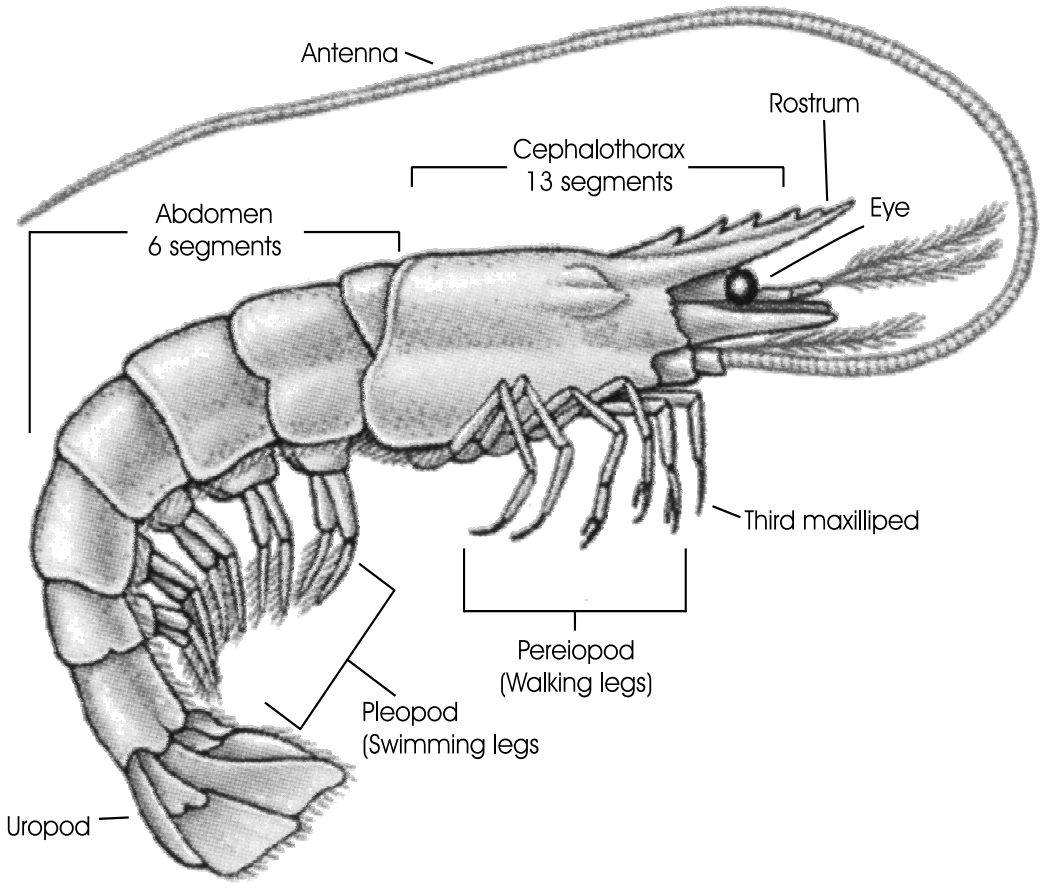

Figure 1.1 Lateral view of the external morphology of the giant tiger shrimp, *P. monodon* (adapted from Hickman *et al.*, 2006)

Life cycle

Mating and spawning of *Penaeus monodon* generally take place at night. The maximum number of eggs spawned at a time can be 50.000-1.000.000 per female. The eggs hatch within 16 hours after fertilisation. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days), and megalopa (6-35 days) stages (Figure 1.2). The next stage is the juvenile stage, where brackish water areas serve as nursery grounds. The megalopa and juvenile are called postlarvae.

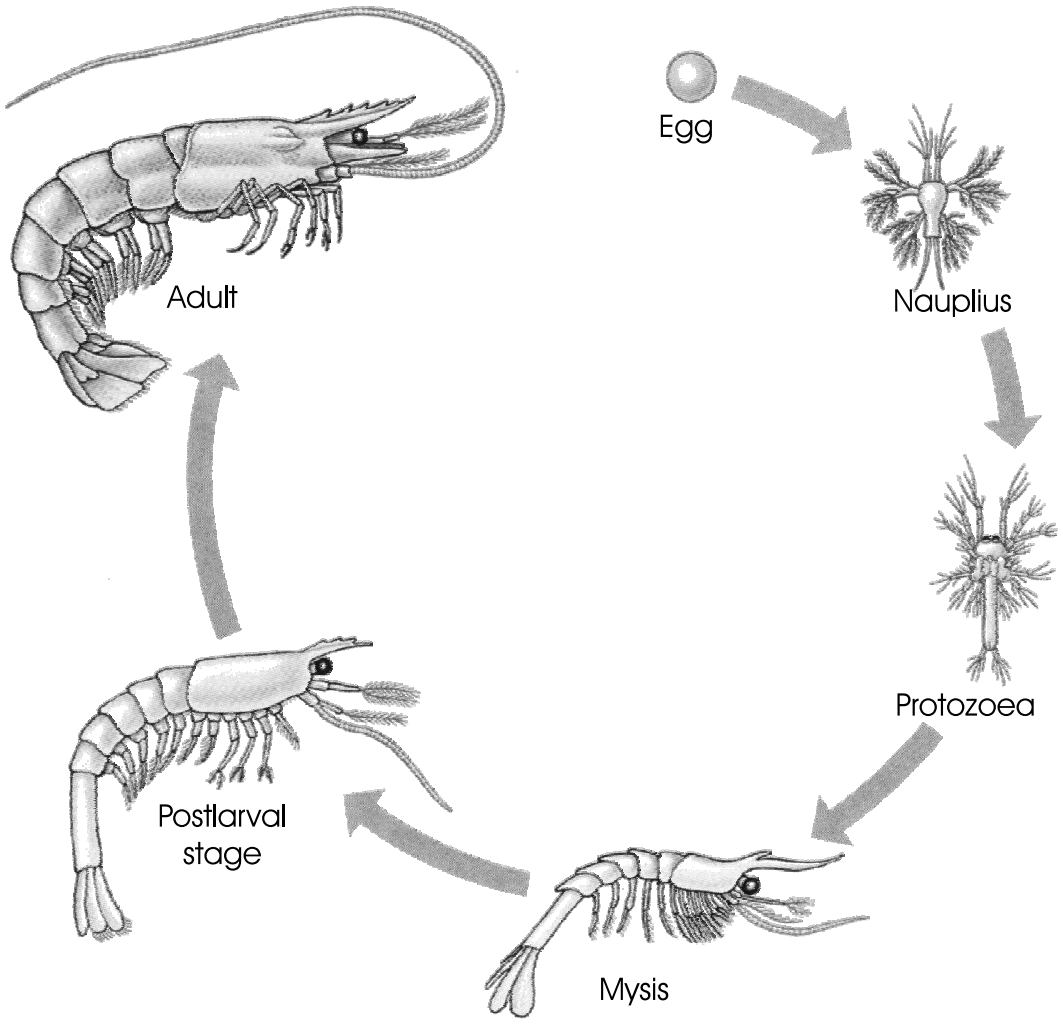

Figure 1.2 The life cycle of *P. monodon* shrimp. Eggs hatch within 16 h after fertilisation. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called postlarvae. Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months (Bailey-Brock *et al.*, 1992). Pictures are not in proportion to actual size.

In about 4 months juveniles develop into subadults. The subadult stage starts at the onset of sexual maturity when the shrimp migrate from nursery to spawning grounds. After 4 months the adult stage is reached, which is characterised by the completion of sexual maturity (Motoh, 1985).

Shrimp culture and viral diseases

Shrimp farming

Shrimp farming has its origin in Southeast Asia, where for more than a century ago farmers started to raise incidental crops of wild shrimp in tidal fish ponds (Rosenberry, 1994). An enormous growth in shrimp production was observed from the 1970's (collection of wild seed) and 1980's (advances in reproduction and hatchery technologies) onwards. In the 1990's the world's production of cultured *P. monodon* increased less dramatically and plateaud at around 600.000 metric tonnes per year (figure 1.3).

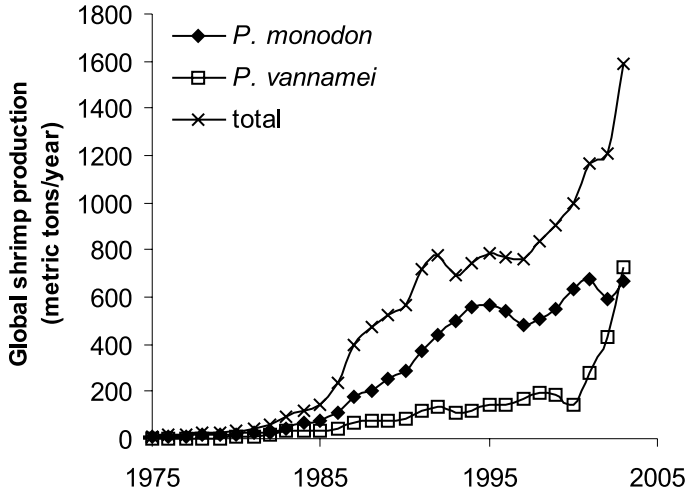

Figure 1.3 Shrimp production in metric tons from 1975 until 2003 (FAO, 2006).

Two extreme strategies of shrimp culture are practised: extensive and intensive culture, with different transitions inbetween. Extensive shrimp culture is carried out

in low densities (<25 PLs/m^2) in large ponds. Farmers depend almost entirely on natural conditions. In intensive culture, shrimp are stocked at high densities (>200 PLs/m^2) in intensively managed ponds with pumps, artificial food, fertilisers and other aquaculture chemicals to increase productivity.

The most commercially cultured shrimp species are *P. monodon* (giant tiger shrimp), *Litopenaeus vannamei* (whiteleg shrimp), *Marsupenaeus japonicus* (kuruma shrimp), *P. stylirostris* (blue shrimp) and *Fenneropenaeus chinensis* (fleshy shrimp) of which the whiteleg shrimp and the kuruma shrimp are mainly cultured on the Pacific coast of Central and South America (FAO, 2001).

Diseases

Shrimp can be affected by a wide variety of pathogens, including parasites, fungi, protozoa, rickettsiae, bacteria and viruses (Lightner *et al.*, 1998). The rapid intensification of shrimp farming leads to an increased environmental degradation, which make the shrimp more susceptible for diseases (Lightner, 1983; Johnson, 1989). Bacteria and viruses are the main cause of disease outbreaks. *Vibrio* spp. are by far the major bacterial pathogens and can cause severe mortalities, but can be controlled by antibiotics and probiotics. Nowadays, about 20 viruses have been described from cultured shrimp (FAO, 2006). The viruses with the most impact on shrimp culture are Monodon Baculovirus (MBV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHNNV), Taura Syndrome Virus (TSV), Yellow Head Virus (YHV) and White Spot Syndrome Virus (WSSV).

White Spot Syndrome Virus

WSSV has had the greatest impact on shrimp culture and at present still causes the major disease problem (Rosenberry, 2004). In cultured shrimp WSSV infection can cause a cumulative mortality of up to 100% within 3-10 days (Lightner, 1996).

In 1992, the first incidents with virus causing mass mortalities were reported in the Fuzhan and Quangzhou provinces in China (Nakano *et al.*, 1994). From there WSSV quickly spread through Asia in 1993 to Japan, Taiwan and Korea, in 1994 to Thailand and India and in 1995 to Indonesia. In 1995 the virus was detected in Texas, North America and one year later in South Carolina (Rosenberry, 1996). In early 1999 the virus reached Central and South America. In 2002, WSSV was also detected in France and Iran.

Since the first reports on this virus appeared, different names have been used to describe the same virus. Systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya *et al.*, 1995), Rod-shaped virus of *P. japonicus* (RV-PJ) (Inouye *et al.*, 1994), Penaeid rod-shaped DNA virus (PRDV) (Inouye *et al.*, 1996), Hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Huang *et al.*, 1995), Chinese baculovirus (CBV) (Nadala *et al.*, 1998) and White spot baculovirus (WSBV) (Chou *et al.*, 1995; Wang *et al.*, 1995). Nowadays, the official name for the virus is WSSV (Vlak *et al.*, 2005).

The acute phase of the disease is characterised by the presence of white spots on the inner surface of the exoskeleton from which the name is derived (Lo *et al.*, 1996). Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Wang *et al.*, 1999).

WSSV not only infects all shrimp species, but also a wide range of other aquatic crustaceans. Crustaceans, however, have a different susceptibilities to WSSV. For some species WSSV is not lethal and they can therefore serve as a virus reservoir and be an important source of infection in shrimp ponds (Lightner *et al.*, 1998).

WSSV is an enveloped, non-occluded ovoid-to-bacilliform shaped virus with a tail-like appendage at one end of the virion (Durand *et al.*, 1997). The virions contain a circular, supercoiled, double-standed DNA genome. Three WSSV isolates have been sequenced to date. The first one sequenced originated from Thailand and had

a genome size of 292,967 bp (AF369029) (van Hulten *et al.*, 2001a). Sequences of two other isolates originating from Taiwan and China revealed genome sizes of 307,287 bp (AF440570) and 305,107 bp (AF332093) (Yang *et al.*, 2001) respectively.

In situ hybridisation studies using WSSV specific DNA probes showed that tissues of ectodermal and mesodermal origin are the main targets for viral replication (Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Lo *et al.*, 1997). Early in infection, stomach, gills, cuticular epidermis and the connective tissue of the hepatopancreas are WSSV positive (Chang *et al.*, 1996; Lo *et al.*, 1997).

Penaeus monodon

The black tiger shrimp, *P. monodon*, is the largest species among penaeids, reaching 330 mm or more in body length. *P. monodon* is considered as exceptionally tough (e.g. it tolerates a wide range of salinities) and has a rapid growth (25-30 g within 3-4 months after post-larvae stocking) (Rosenberry, 1997). Therefore, the black tiger shrimp is a commercially interesting species for farming. However, in contrast with e.g. *L. vannamei*, broodstock of *P. monodon* has to be collected in nature. Culture of *P. monodon* occurs mainly in Southeast Asian waters. Its carapace and abdomen are transversely banded with red and white. When cultured in ponds, the color changes to blackish, hence this shrimp species is often referred to as black tiger shrimp (Motoh, 1985).

Immune functions in crustaceans versus insects

The innate immune system of crustaceans has been studied in some detail in only a limited number of species. There are some immune response systems that have been investigated intensively, such as the prophenoloxidase activating system (proPO) (Söderhäll *et al.*, 1998; Lee *et al.*, 2004), clotting (Chen *et al.*, 2005; Yeh *et al.*,

1999; Yeh *et al.*, 2006), and phagocytosis (Liu *et al.*, 2007). These investigations have led to unifying concepts based on molecular data from a variety of species. However, a comprehensive mechanistic approach to unravel the defence system of invertebrates is only possible when complete genomes, expressed genes and suitable disease challenge models are available.

The crustaceans belong to the Arthropoda, which includes the insects. Genomes of two representatives of the latter namely the fruit fly (*Drosophila melanogaster*) and the mosquito (*Anopheles gambiae*) have been completely sequenced (Adams *et al.*, 2000; Holt *et al.*, 2002). The availability of the complete genome now allows a genome-wide analysis of the immune responses in these insect species using standardised pathogen challenges (Irving *et al.*, 2001; Christophides *et al.*, 2002). Expression profiling of immune responses in insects may be instrumental in unravelling more expediently the immune functions in crustaceans, especially of *P. monodon*, as it has been suggested that this species is closely related to insects (Wilson *et al.*, 2000).

Identification of immune response genes in *Drosophila* and *Anopheles* has been performed in several ways. One approach uses cell lines, which are treated with LPS (Boutros *et al.*, 2002). RNA is extracted from the treated and untreated cell cultures at different time points. RNA is converted into cDNA and at the same time labelled with fluorescent dyes. The labelled cDNA pools are hybridized with a micro-array, containing all expressed genes of the organism used in the experiment. After hybridisation, the micro-arrays are washed and the relative fluorescence of each gene is determined. In this way gene expression can be determined as unaffected, up regulated or down regulated compared to the control treatment. The other makes use of *in vivo* challenges with pathogenic micro-organisms, such as Gram-positive, Gram-negative bacteria or a fungus (De Gregorio *et al.*, 2001; Irving *et al.*, 2001). These experiments have lead to the identification of different immune genes

involved in the responses to different pathogen challenges.

Expression profiling after challenge with gram-positive and gram-negative bacteria, and fungi identified two major pathways: Toll and Immunodeficiency (Imd) (Boutros *et al.*, 2002). These pathways in insects comprise four steps:

1. recognition, 2. regulation, 3. intracellular signal transduction and 4. cellular and humoral responses. These pathways, including the cellular and humoral immune responses have been reviewed in detail (Hultmark, 2003). The first step involves Pattern Recognition Receptors (PRR), which have been identified throughout the animal kingdom and play an important role as they discriminate between self and infectious non-self. These receptors recognize unique conserved molecular patterns, such as lipopolysaccharides and peptidoglycans, found only in bacterial and fungal pathogens. The regulation of the recognition is facilitated and controlled mainly by activating and inhibitory CLIP domain serine proteases. Intra-cellular signal transduction is highly conserved and comprises protein interactions similar to the NF-κB signal pathway found in vertebrates. The protein modifications and interactions will eventually lead to the release of transcription factors, which are translocated to the nucleus and drive the expression of immune response genes. Depending on the pathway used the response will either be the production of antimicrobial or antifungal peptides (humoral response) or activation of haemocytes (cellular response). However, to some degree, there is crosstalk between the two pathways.

Insect Immunity

Pattern Recognition Receptors

Studies in *Drosophila* have identified at least three important groups of pattern recognition receptors (PRRs): Peptidoglycan recognition proteins (PGRPs), β-glycan recognition proteins (β-GRPs) and Toll-like receptors (TLRs).

PGRPs are found in insects and mammals and bind to peptidoglycans, which are part of the cell wall of Gram-positive bacteria. In the genome of *Drosophila* 12 *PGRP* encoding genes have been identified of which five are up regulated after septic injury (Werner *et al.*, 2000; De Gregorio *et al.*, 2001). The *PGRP* genes encode different two types of proteins: short (PGRP-S) and long (PGBP-L). The PGRP-S are found only as extracellular proteins, while the PGRP-L can be found both in the cytoplasm and as a cell surface protein. The PGRPs may not to be directly involved in the recognition of fungal pathogens. The most interesting PGRPs are PGRP-SA, PGRP-LC, PGRP-SC1 and PGRP-SC2, where the PGRP-SA receptor has been demonstrated to be required for the activation of the Toll pathway after challenge with Gram-positive bacteria. PGBR-LC is implicated in the activation of the Imd pathway, not only by virtue of recognition of peptidoglycans, but also of LPS (Choe *et al.*, 2002). PGRP-SC1 and PGRP-SC2 control the intensity of the *Drosophila* IMD signaling pathway (Bischoff *et al.*, 2006).

The β-1,3 glucan induced activation of the phenoloxidase system was first described in the silkworm (Ochiai *et al.*, 1988) and was attributed to the presence of β-glucan receptor protein (β-GRP). This receptor binds both β-1,3 glucans of fungal origin and LPS from Gram-negative bacteria. It is hypothesised that this is a general characteristic of all β-GRPs. In the *Drosophila* genome three genes are found encoding β-GRPs and they have been renamed to Gram Negative Binding Proteins (GNBP1-GNBP3) (Kim *et al.*, 2000). These genes in *Drosophila* are not up regulated in response to infection. However, over expression of some *GNBP*-related genes have been induced by β-1,3 glucan and LPS and resulted in the induction of transcription of antimicrobial genes. This seems to suggest that the GNBPs are involved in defence mechanisms (De Gregorio *et al.*, 2001; Irving *et al.*, 2001).

The third group of receptors thought to be involved in recognition of infectious non-self are the Toll receptors. The first Toll receptor (Toll-1) was discovered by its role

in dorsoventral patterning. Subsequently, its importance as a PRR was revealed and confirmed by the fact that mammals possess Toll-like Receptors (TLR) implicated in the innate immune system by direct recognition of infectious non-self (Underhill *et al.*, 2002). The ligand of Toll-1 is Spätzle, the active form of which is the result of proteolytic cleavage by an extracellular serine protease. In addition to Toll-1, *Drosophila* has eight other Toll receptors (Toll-2 to Toll-9), some of them with a putative immune response function, such as Toll-5 (Imler *et al.*, 2002). Orthologous genes of *Drosophila* Tolls are present in the *Anopheles* genome, but some are lacking whilst others have expanded by gene duplication (Holt *et al.*, 2002). Thus, the repertoire of Toll receptors varies between insect species and as a consequence most likely also their immune functions.

In addition to the above three major PRR groups, there are other receptors which may play a role in the immune defence of insects such as C-type lectin domain containing proteins. However, none of the genes encoding these proteins were induced after infection (De Gregorio *et al.*, 2001).

Regulation of Immune Responses

Regulation of immune responses in insects occurs mainly through modulation and amplification of extracellular cascades of activating and inhibitory clip domain serine proteases (CLIPs) (De Gregorio *et al.*, 2001). One CLIP named Persephone activates the Toll pathway by cleaving Spätzle, producing the ligand for the Toll-1 receptor. Other activating CLIPs are associated with immune effector responses such as clotting and the phenoloxidase cascade, which leads to melanisation. Persephone is negatively regulated by an inhibitory serine protease, belonging to the serpin family, called Necrotic. There are many CLIP serine protease genes (41) found in the *Drosophila* genome, most but not all of them play a role in controlling immune responses (Adams *et al.*, 2000; De Gregorio *et al.*,

2001). Interestingly, no orthologs of Persephone and Necrotic have been found in the mosquito *Anopheles*, suggesting an independent evolution of CLIP serine proteases and serpins involved in immune responses (Christophides *et al.*, 2002). If this turns out to be a general observation it will be difficult to identify CLIP serine protease and serpins involved in the various immune response pathways in different arthropod species.

Signal Transduction Pathways

The recognition of infectious non-self by pattern recognition receptors will lead to transcriptional activation of immune response genes. In *Drosophila* using both bacterial and fungal challenges and *Drosophila* mutants of the *Toll* and *Imd* genes two major signal pathways have been described in great detail (Lemaitre *et al.*, 1995). In both pathways signal transduction involves members of the Rel family of transcription factors, which are similar to the mammalian transcription factor NF-κB. Toll signal transduction is initiated by the binding of the proteolytic cleaved ligand Spätzle to the extracellar part of the Toll-1 receptor. The intracellular part of this receptor contains a TIR domain, which interacts with homologs of NF-κB regulators, such as MyD88 and Traf2 (Horng *et al.*, 2001; Shen *et al.*, 2001). MyD88 is an intra-cellular protein consisting of a TIR domain and a death domain. Traf2 is a protein that contains a TRAF domain and is homologous to a human TRAF protein. Two other proteins Tube en Pelle are involved in the next signal transduction step by activation an unknown kinase through the kinase domain of Pelle. Eventually, this results in phophorylation of Cactus leading to its degradation and subsequent release of Dif, which is complexed with Cactus. Dif is a DNA-binding REL homology domain, which upon translocation to the nucleus acts as a transcription factor of a multitude of immune response genes (Boutros *et al.*, 2002).

The Imd pathway is activated by the interaction of the PGRP-LC receptor with its

peptidoglycan or LPS ligand. The cytoplasmic region of the PGRP receptor interacts with the Imd molecule. This protein contains a death domain, which activates two pathways essential for the release of the transcription factor Relish from the REL-49/REL-68 complex (Georgel *et al.*, 2001; Boutros *et al.*, 2002). One pathway requires the product of the Dredd gene, a member of the caspase family of proteases, while the other pathway includes a kinase, which is able to phosphorylate the REL complex. The kinase is part of a complex known as IKK, the *Drosophila* homolog of IκB (Silverman *et al.*, 2000). Thus, both phosphorylation and proteolytic cleavage are a prerequisite for the release and translocation of Relish to the nucleus leading to transcription of a plethora of immune response genes, mostly involved in humoral immune responses.

The activation of the Toll and Imd pathways leads to translocation of transcription factors which drive the expression of immune defence genes, such as those encoding peptides which either behave as antibiotics, affect growing of bacteria, or kill fungi. This is only one of the three ways in which *Drosophila* responds to infections.

Effector mechanisms

Flies respond to infection with either bacteria or fungi with both cellular and humoral immune defences. The cellular responses are performed by haemocytes, which circulate through the haemolymph or are resident in certain tissues (Lanot *et al.*, 2001). Haemocytes can be morphologically distinguished and perform different functions. Some are involved in phagocytosis and encapsulation of pathogens, whilst others produce humoral factors such as antimicrobial peptides or contain phenoloxidase.

In the *Drosophila* genome at least 34 antimicrobial peptide encoding genes have been found (De Gregorio *et al.*, 2001). In general, four major structural classes of cationic antimicrobial peptides can be distinguished, 1. disulfide-bonded β-sheet peptides (e.g. defensins), 2. amphipathic α-helical peptides (e.g. cecropins),

3. extended peptides, which often have a single amino acid predominating (e.g. indolicidin), and 4. loop-structured peptides (e.g. bactenecin) (Bulet *et al.*, 1999). The expression of most of the antimicrobial peptides is induced by the presence of pathogenic micro-organisms, such as bacteria and fungi. The peptides are produced in cells of the fat body and are released into the haemolymph. The production of the peptides rises quickly after infection and can reach micromolar concentrations in a matter of hours. The modes of action of the different antimicrobial peptides are diverse and they are capable of killing a wide range of microbial pathogens. The antimicrobial peptides interact with the surface of pathogenic micro-organisms and are taken up by self-promoted uptake creating membrane channels. The latter process may induce death of the micro-organism due to disrupting the integrity of the cell wall or, alternatively, the peptides interact with cytoplasmic targets (Bulet *et al.*, 1999).

Melanisation is an important immune response in arthropods including insects (Söderhäll *et al.*, 1998). Proenzymes circulate in the haemolymph and upon activation by the CLIP domain serine proteases catalyse essential cascade steps in the synthesis of melanin. Melanin is a dark brown pigment that is involved in cuticle sclerotization, wound healing, and melanotic encapsulation of pathogens. The whole cascade is called the prophenoloxidase (proPO) system and will be discussed in detail below.

Other immune responses comprise clotting, complement-like proteins and lectin mediated opsonizing activity of phagocytes (Lee *et al.*, 2000; Irving *et al.*, 2001). These responses require both humoral factors and cells circulating in the haemolymph, so-called haemocytes. These responses have been studied in some measure of detail in Crustaceans.

Crustacean Immunity

Pattern Recognition Receptors

Several pattern recognition receptors (PRRs) have been identified in crustaceans, most of them related to β-glucan recognition proteins. In the freshwater crayfish *Pacifastacus leniusculus* a lipopolysaccharide- and β-1,3-glucan-binding protein (LBGP) have been isolated and characterised. LBGP has binding activity for LPS and β-1,3-glucans but not for peptidoglycans of Gram-positive bacteria. Experiments using a polyclonal antibody to block LBGP indicated that this protein plays a role in the activation of the proPO system (Lee *et al.*, 2000). In other penaeid shrimp a BGP (β-1,3-glucan-binding protein) and a LBGP have been reported. The BGP from *P. monodon* is not up regulated, whereas the LBGP from *P. stylirostris* is up regulated after bacterial infection (Sritunyalucksana *et al.*, 2002). Peptidoglycan recognition proteins and Toll receptors have not yet been found. However, we have recently isolated a partial sequence encoding a TIR domain in *P. monodon* (Arts *et al.*, 2007). The presence of this TIR domain suggests that a Toll receptor or intracellular signal transduction molecules such as MyD88 are present. Besides the three major PRR groups other receptors like α_2-microglobulin (Spycher *et al.*, 1987; Hergenhahn *et al.*, 1988), C-type lectins of penaeids (Ratanapo *et al.*, 1990; Fragkiadakis *et al.*, 1995; Gross *et al.*, 2001) and calcium independent lectins (Maheswari *et al.*, 1997; Kondo *et al.*, 1998; Cominetti *et al.*, 2002) have been reported.

Regulation of Immune Responses

Modulation and amplification of extracellular cascades occur mainly by serine proteases. These proteases are active in a large number of biochemical processes, and those that perform an immune function by cleaving a specific ligand that interacts with a receptor are difficult to identify. A cell adhesion protein, a mas-

like protein, from *P. leniusculus* shows homology with serine proteases, especially to the *Drosophila* Masquerade (Huang *et al.*, 2000) and may be such a candidate serine protease involved in immune responses. After binding of this masquerade-like protein to micro-organisms, a proteolytic enzyme cleaves this protein. The intact form of the masquerade-like protein has been extensively characterised and contains seven putative disulphide-knotted motifs and is implicated in the immune response to a bacterial infection (Lee *et al.*, 2001).

Effector mechanisms

In the crustacean cellular response three groups of cells can be recognised based on morphological differences. The first are hyaline cells, which phagocytes invading organisms. The second group is called the semi-granular group, which has a much broader function; encapsulation, (limited) phagocytosis, storage and release of proteins of the proPO system, and cytotoxicity. The latter two functions are also found in the third group, the granular cells (Huang *et al.*, 2000).

The first antimicrobial peptide characterised in crustaceans showed a high identity with bactenecin-7 (Schnapp *et al.*, 1996). The next group of antimicrobial peptides identified was the penaeidin family from the whiteleg shrimp *L. vannamei* (Destoumieux *et al.*, 1997). Although this family shows similarities to the insect proline-rich antimicrobial peptides, they do not have a strong activity against gram-negative bacteria (Bachère *et al.*, 2000). Callinectin, isolated from the haemocytes of the blue crab *Callinectes sapidus*, demonstrated activity against *E. coli* and possessed a proline-rich domain. The peptide does not show significant homology with any known peptide (Khoo *et al.*, 1999). Crustins, a relatively new family of antimicrobial peptides, also show no homology with other known antibacterial peptides, but do display sequence identity with proteinase inhibitory proteins (Bartlett *et al.*, 2002).

The prophenoloxidase (proPO) cascade-activating system is implicated in the immediate defence of shrimp against a variety of stimulatory conditions (Sritunyalucksana *et al.*, 1999). This system has been extensively studied. Phenoloxidase (PO; EC 1.14.18.1) is present in the haemolymph as an inactive pro-enzyme, proPO (Sanchez *et al.*, 2001). Common chemical components of bacteria and fungi, such as β-1,3-glucans, are reacting with β-glucan binding protein (BGBP) and this complex induces degranulation and the activation of proPO system (Sritunyalucksana *et al.*, 1999). This protein cascade is widely expressed and highly conserved in crustaceans. Proteins of the proPO system thus occupy a prominent position in non-self recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of haemocytes, the inactive proPO is converted into the active phenoloxidase (PO) by prophenoloxidase activating enzyme (ppA). This activation of proPO results in hydroxylation of phenols and oxidation of *o*-phenols to quinones, and after several intermediate steps the formation of melanin. During this formation antimicrobial factors are formed (Vargas-Albores *et al.*, 2000). Melanin is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. The melanisation process is observed in response to foreign intruders in the haemocoel and during wound healing.

An important factor that is associated with the proPO system is peroxinectin, which was cloned for *P. monodon* (Sritunyalucksana *et al.*, 2001). Peroxinectin displays cell-adhesion and peroxidase activity. Crayfish peroxinectin is synthesised in the haemocytes, stored in the secretory granules in an inactive form, released in response to stimuli and activated outside the cells (Holmblad *et al.*, 1999; Arala *et al.*, 2000; Bartlett *et al.*, 2002). Transmembrane receptors of the integrin family on the haemocytes play an important role in the cell adhesion function of peroxinectin. The cell-adhesion can lead to attachment, spreading, phagocytosis, encapsulation,

nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading micro-organisms (Holmblad *et al.*, 1999).

Conclusions

As a result of the molecular sequence information currently available on the expressed genes in insects, experiments have been performed adopting standardized challenge protocols either with bacteria, fungi or viruses to identify potential immune response genes. The problem with these experiments is that about 50 % of the genes that are modulated after the pathogen challenge have an unknown function (Irving *et al.*, 2001; Rojtinnakorn *et al.*, 2002; Dhar *et al.*, 2003). Nevertheless these experiments have led to the identification of a large number of proteins involved in the immune response, notably those of the Toll and Imd pathway. Some of the modulation observed might be due to wounding alone in the case of a bacterial challenge where the bacteria are introduced by pricking the flies with a septic needle. This issue needs to be resolved. The fungal challenge is a more natural challenge as spores are simply coated onto the flies and this procedure does not require rupture of the cuticle. The fungus has the capability to penetrate the cuticle by itself.
Given the relative close relationship between insects and crustaceans it is anticipated that the major immune signalling components and the full suite of effectors can be identified using the information from insects. However, this only applies for anti-bacterial and anti-fungal responses, but not for the immune response to pathogenic viruses. As far as we can ascertain little attention has been paid to the immune response to viruses, neither in insects nor in crustaceans. This could be due to the lack of appropriate viral challenge models. However, currently much research effort is devoted to the white-spot syndrome virus (WSSV). This virus has become a pandemic within a relative short time-span, and is particularly virulent in

P. monodon. The genome of the WSSV has been completely sequenced and turned out to be the largest animal viral genome of 292,967 base pairs with 184 open-reading frames (van Hulten *et al.*, 2001a). A recently developed 'vaccine' has shown to protect 2 *Penaeus* species against WSSV infection (Witteveldt et al., 2004/2006. To investigate the aetiology of the virulence and the immune response to a viral infection in crustaceans, adopting a similar approach as used in flies, namely expression profiling using micro-arrays, may provide clues which pathways, signalling cascades and effector mechanisms are involved.

Outline of the thesis

In the past decade, an increasing knowledge of WSSV and *P. monodon* led to the indentification of several genes of both the pathogen and its host (Lehnert *et al.*, 1999; van Hulten *et al.*, 2000a/b; van Hulten *et al.*, 2001b; Witteveldt *et al.*, 2001; Lehnert *et al.*, 2002). Recently, WSSV transcription profiling (Marks *et al.*, 2005) and a putative immune stimulating WSSV-protein was shown to decrease mortality of infected shrimp (Witteveldt *et al.*, 2004/2006). Infection challenges with WSSV revealed a number of genes involved in the defence response of *P. monodon*, but mainly late during the infection (1-3 days) (Di Leonardo *et al.*, 2005). Very limited information is available about the host response during the first hours post infection. The first focus has been on the haemocyte response of the shrimp upon an immersion infection as this mimics the most abundant route of natural infection (chapter 2). Immunocytochemistry and electron microscopy have been used to study the infection route of WSSV up to 72 hrs after immersion in gills and gut. Using a mouse haemocyte specific monoclonal antibody (WSH8) and a rabbit VP28 polyclonal antibody, double immunoreactivity could be observed. Different haemocyte characteristics in the gills and the midgut of *P. monodon* were determined.

Limited data is available about the immune response genes of *P. monodon* upon a viral infection. A combination of suppression subtractive hydridisation (SSH) and cDNA microarray analysis was used to enrich genes that are differentially expressed upon WSSV infection (chapter 3). The construction of SSH libraries and subsequently selection of differentially expressed genes is described. The selected clones were used to generate a focussed cDNA microarray.

The focus of chapter 4 is to determine the expression profile of the genes selected in chapter 3. By using the constructed microarray it was possible to follow a few hundred clones during the first day of infection. A putative vaccination strategy to protect shrimp against lethal WSSV infections has been developed previously (Witteveldt *et al*., 2004). The immune response of the shrimp upon 'vaccination' was studied with the generated microarray.

With information of *Drosophila* research available, it is possible to look more thoroughly into immune related genes. Toll receptors are known to play a substantial role in detecting pathogens, both in invertebrates as in vertebrates, where they are called Toll-like receptors. Therefore, in chapter 5, a putative Toll receptor from a crustacean was described for the first time and expression studies were performed in response to WSSV infection.

Finally, the results presented are summarised and discussed in chapter 6. The characterisation of a shrimp-specific Toll receptor is integrated with results from the microarray analysis and provide a integrative immune defence of *P. monodon* to exposure with WSSV. Moreover, the immunological background known so far is descrived with respect to the vaccination strategy for WSSV infection.

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CHAPTER

2

Haemocyte reactions in WSSV immersion infected *Penaeus monodon*

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Abstract

White spot syndrome virus (WSSV) has been a major cause of shrimp mortality in aquaculture worldwide in the past decades. In this study, WSSV infection (by immersion) and behaviour of haemocytes is investigated in gills and midgut, using an antiserum against the viral protein VP28 and a monoclonal antibody recognising all haemocytes (WSH8) in a double immunohistochemical staining and in addition transmission electron microscopy. More WSH 8^{+} haemocytes were detected at 48 and 72 hrs post infection in gills of infected shrimp compared to uninfected animals. Haemocytes in the gills and midgut were not associated with VP28-immunoreactivity. In the gills many other cells showed virus replication in their nuclei, while infected nuclei in gut cells were rare. Nevertheless, the epithelial cells in the midgut showed a clear uptake of VP28 and accumulation in supranuclear vacuoles (SNV) at 8 hrs post infection. However epithelial nuclei were never VP28-immunoreactive and electron microscopy suggests degradation of viral-like particles in the SNV. In contrast to the gills, the midgut connective tissue shows a clear increase in degranulation of haemocytes, resulting in the appearance of WSH8-immunoreactive thread-like material at 48 and 72 hrs post-infection. These results indicate recruitment of haemocytes upon immersion infection in the gills and degranulation of haemocytes in less infected organs like the midgut.

Introduction

White spot syndrome virus (WSSV) is an economically important viral pathogen occurring widely in shrimp culture worldwide (Flegel, 1997). In cultured shrimp, WSSV infection can reach a cumulative mortality of up to 100% within 3-10 days (Lightner, 1996). It affects penaeid shrimp such as Chinese shrimp (*Fenneropenaeus chinensis*), Kuruma shrimp (*Marsupenaeus japonicus*), black tiger shrimp (*Penaeus monodon*) and other crustaceans, such as salt, brackish and fresh water crayfishes, crabs and lobsters (Otta *et al.*, 1999; Supamattaya *et al.*, 1998; Zhan *et al.*, 1998). Tissues of ectodermal and mesodermal origin are the main targets for viral replication (Wongteerasupaya *et al.*, 1995). Early in infection, stomach, gills, cuticular epidermis and the connective tissue of the hepatopancreas are reported to be WSSV positive (Chang *et al.*, 1996; Lo *et al.*, 1997; Wang *et al.*, 1999). A significant reduction at 48 hrs after infection in the total haemocyte count is observed after shrimp are infected with WSSV (Jiravanichpaisal *et al.*, 2001; van de Braak *et al.*, 2002). WSSV can be transmitted by predation of diseased individuals, or by free virus particles released in the water. In this study the infection route of WSSV was followed up to 3 days after immersion in gills and midgut and in addition, the reaction of haemocytes upon the infection is studied by immunohistochemistry and transmission electron microscopy in these organs.

Materials and Methods

Animals and infection

Healthy *P. monodon* were imported as post-larvae from Malaysia and maintained in a recirculation system at “de Haar vissen” at Wageningen University. Each shipment was tested for the presence of WSSV, monodon baculovirus, yellowhead virus, Taura syndrome virus and infectious hypodermal and hematopoietic necrosis virus by a virus specific PCR. Shrimp of approximately 15 g were immersed in 6 µl

virus solution (as described by van Hulten *et al. 2001*)/500 ml seawater/shrimp for 4 hrs. This relative high dose was used to synchronise infection in the gills. After immersion, shrimp (n=18) were transferred to 180 litre aquaria and cultured for up to 72 hrs at 28°C and a salinity of 20 parts per thousand. WSSV infection in the shrimp was determined in the gills by PCR with VP26 (WSSV) and shrimp actin-specific primers (Marks *et al.*, 2003) on genomic shrimp DNA and WSSV DNA.

Immunohistochemistry

Gills and midgut were isolated from shrimp at 0, 4, 8, 24, 48 and 72 hrs after infection and immediately fixed for 24-48 hrs at room temperature (RT) in freshly prepared Davidson's fixative (Bell *et al.*, 1988) (29 ml Milli Q water, 29 ml 100% ethanol, 19 ml 37% neutral formalin and 10 ml 100% acetic acid). After fixation the organs were stored in 50% ethanol, subsequently dehydrated in ethanol series and embedded in paraffin. Sections of 5 μm were made and after deparaffination in xylol, endogenous peroxidase was inactivated in methanol and H_2O_2 for 30 min (at RT). After hydration, sections were treated with 10% normal goat serum (DAKO) followed by washing in PBS. Subsequently, the sections were incubated for 1 hr at room temperature with a rabbit VP28 polyclonal antibody (1:200; van Hulten *et al.*, 2001) and a mouse haemocyte specific mAb (WSH 8; 1:100; van de Braak *et al.*, 2000). After the first incubation, slides were washed with PBS-t (1 M PBS pH 7.2, 0.1% Triton) followed by a second incubation for 1 hr at room temperature with goat-anti-rabbit Ig conjugated to horse radish peroxidase (GAR-HRP: 1:200; DAKO) and goat-anti-mouse Ig conjugated to alkaline phosphatase (GAM-AP: 1:200; DAKO). After washing again, HRP was stained using 3-amino-9-ethyl-carbazole (260 mg/l Na-acetate buffer pH5) (Sigma; red) and AP was stained with 5-bromo-4-chloro-3-indolyl-phosphate (100 mg/ml; Sigma) and 4-nitro blue tetrazolium chloride (50 mg/ml) (Sigma; blue). All necessary controls were

performed and if not specifically mentioned found to be negative.

Transmission Electron Microscopy

Gills and midgut were fixed for 1 hr (at 4 °C) in 1% (w/v) $K_2Cr_2O_7$, 2% (v/v) glutaraldehyde and, after storage, in 1% (w/v) OsO_4 in 0,1M sodium cacodylate buffer (pH 7.2) and subsequently washed in double distilled water, dehydrated in ethanol followed by propylene oxide and embedded in Epon 812 (Electron Microscopy Science). Ultra-thin sections were cut on a Reichert Ultracut S (Leica) and stained with uranyl acetate and lead citrate. Sections were studied with a Philips EM208 electron microscope (FEI) equipped with a SIS digital camera (Soft Imaging System).

Results

Gills

Staining with the anti-VP28 antibody against an envelope protein (VP28) of WSSV showed that shrimp were infected with WSSV. Detection of WSSV by PCR with primers designed on another envelope protein (VP26) also confirmed infection (data not shown). With both methods first signs of infection could be detected between 24 and 48 hrs post infection. Based on observations without infection (Figure 2.1A) and at 48 hrs after infection (Figure 2.1B), the relative number of haemocytes in the gills was estimated using a semi-quantitative procedure, with haemocytes numbers varying from category 0-4, in which 0 means hardly haemocytes present and 4 abundant numbers of haemocytes (Figure 2.1C).

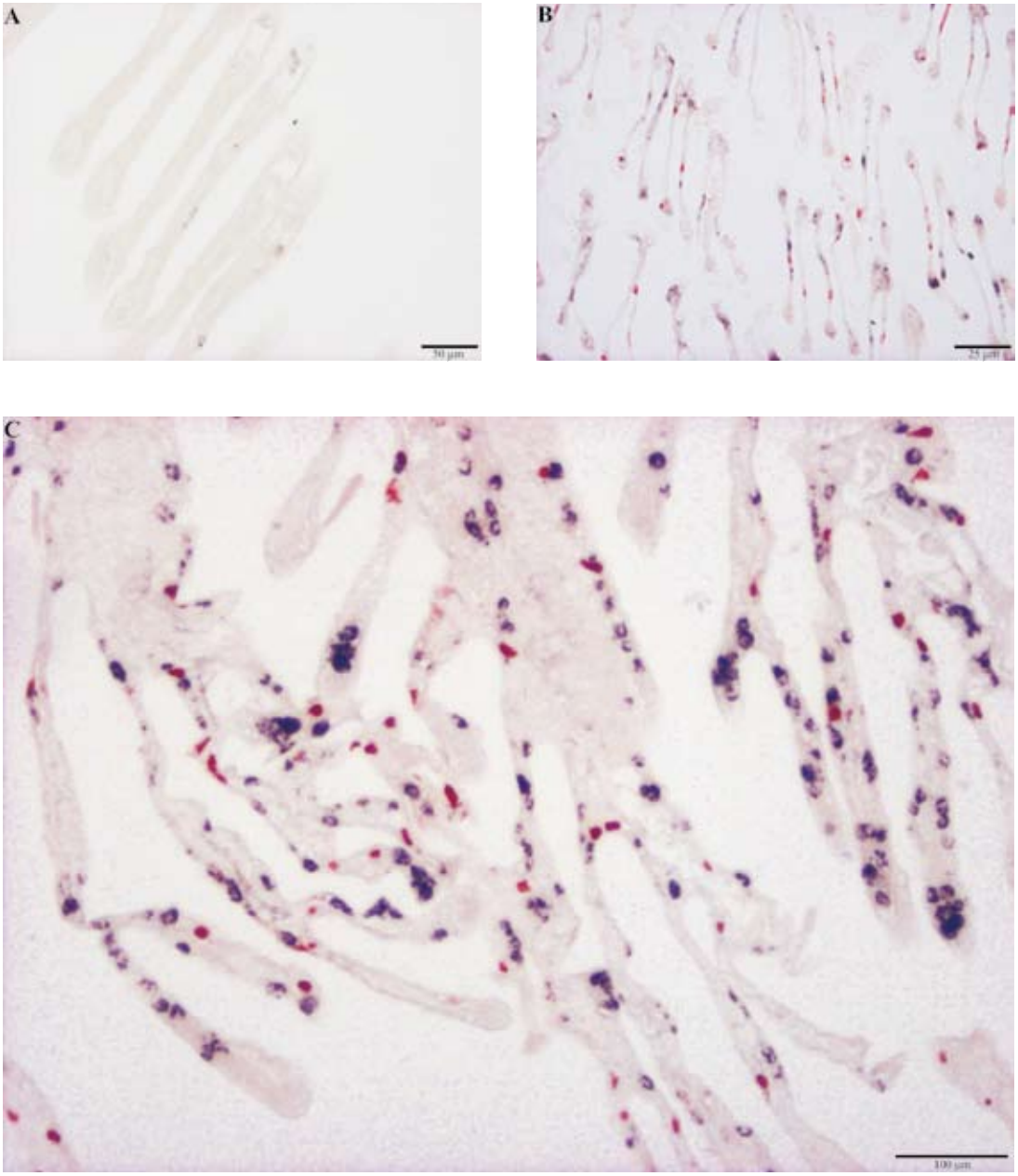


Figure 2.1 Light micrographs of histological sections of WSSV infected *Penaeus monodon* gills fixed 48 hrs after immersion. Haemocytes are immuno-stained with monoclonal antibody WSH 8 (GAM-AP; blue) and virus particles with polyclonal VP28 antibody (GAR-HRP; red).
A, haemocyte number category 0; B, haemocyte number category 4; C, higher magnification of category 4 (48 hrs post infection).

Directly after immersion (t=0) a short term increase of haemocytes is observed which stabilizes again between 12 and 24 hrs. At 48 and 72 hrs post infection more $WSH8^{+}$ haemocytes were detected in the gills of infected shrimp compared to the gills of uninfected shrimp (Figure 2.2).

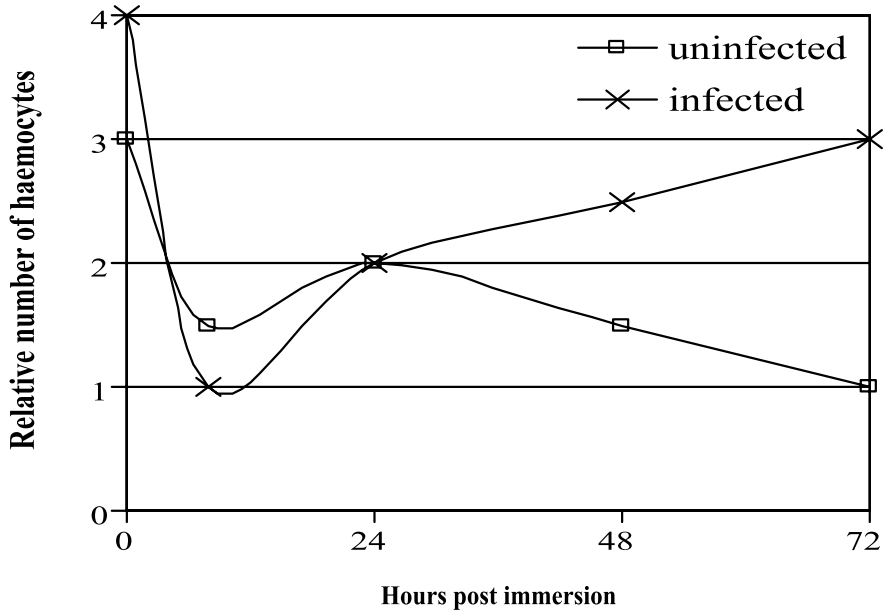
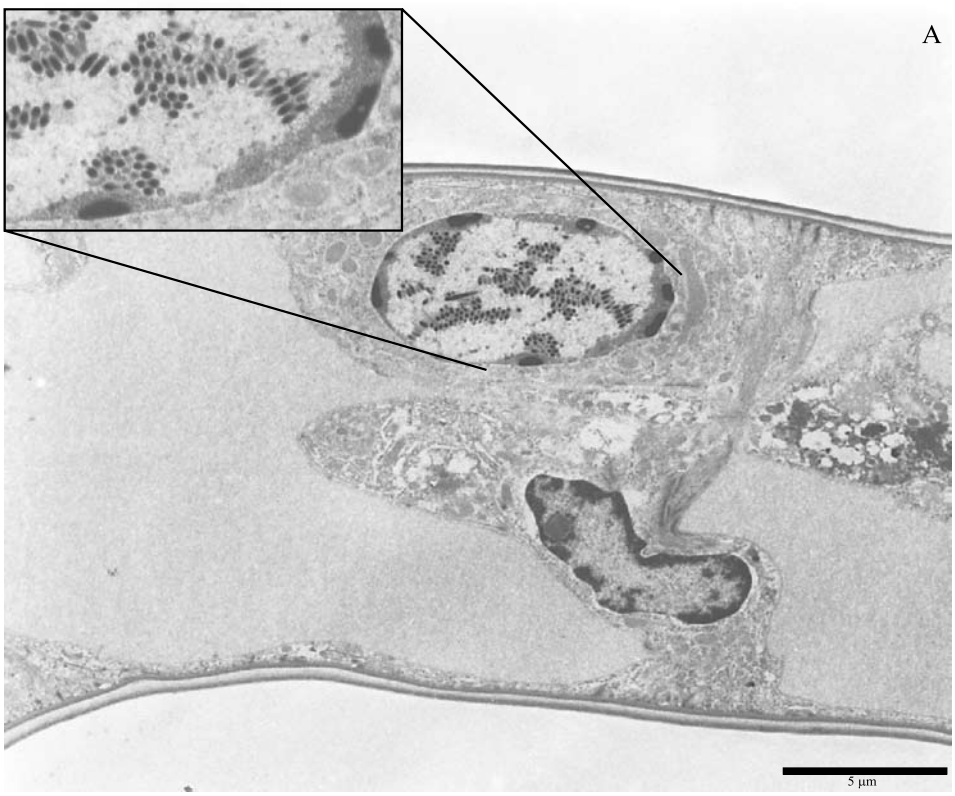


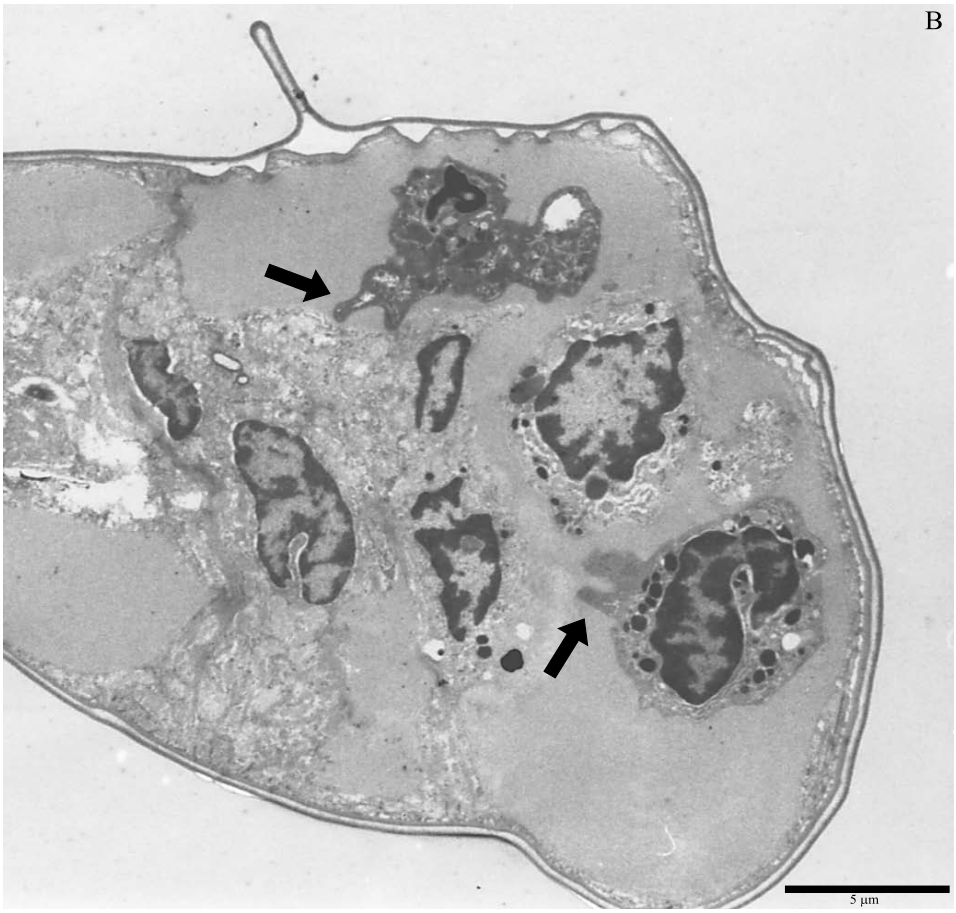
Figure 2.2 Semi-quantitative estimation of haemocytes in gills during WSSV infection by microscopic analysis of tissue sections stained for immunocytochemistry. In the midgut increase in haemocyte numbers was negligible.

Although immunohistochemistry does not clearly show whether the increase of $WSH8^+$ haemocytes is due to haemocytes in circulation or tissue, electron microscopy strongly indicates that these haemocytes are present in the haemolymph (Figure 2.3).

The extensions of haemocytes towards the vessel wall (Figure 2.3B) suggest that these cells were originally in contact with this wall. Histological observations in the gills showed that VP28-immunoreactivity was not associated with $WSH8^+$ haemocytes, but much more with the nuclei of other gill cells (Figure 2.1C), which was confirmed with electron microscopy (Figure 2.3A). In general, the viruses appear as electron dense round to rod-like structures, but more or less empty rods are occasionally found as well. In the present study, encapsulation of virus-infected haemocytes was not observed in the gills, despite the virus-induced influx of haemocytes.

Figure 2.3 next page Electron micrographs of gill lamellae 72 post-infection. A. an infected gill epithelial cell is shown clearly showing round to rod-like viral particles (inset); B. three haemocytes are shown in the hemolymph of which two have extensions (arrows) with the vessel wall.


A
5 µm


B
5 µm

Midgut

The microvilli border showed an increase in VP28-immunoreactivity between 0 and 4 hrs post-infection (not shown). Immunohistochemistry showed a clear uptake of VP28 by the epithelial cells (Figures 2.4B-C) and accumulation of VP28 in vacuole-like supranuclear structures (SNV) at 8 hrs (Figure 2.5). However, in the present study VP28-immunoreactivity was never observed in the epithelial nuclei of the midgut, neither in the epithelium nor in the underlying tissue. Electron microscopy consistently showed viral-like particles in SNV in the epithelium and underlying tissue cells, and thus strongly suggests that these SNV are fusion products of uptake vesicles and lysosomal structures (Figure 2.5).

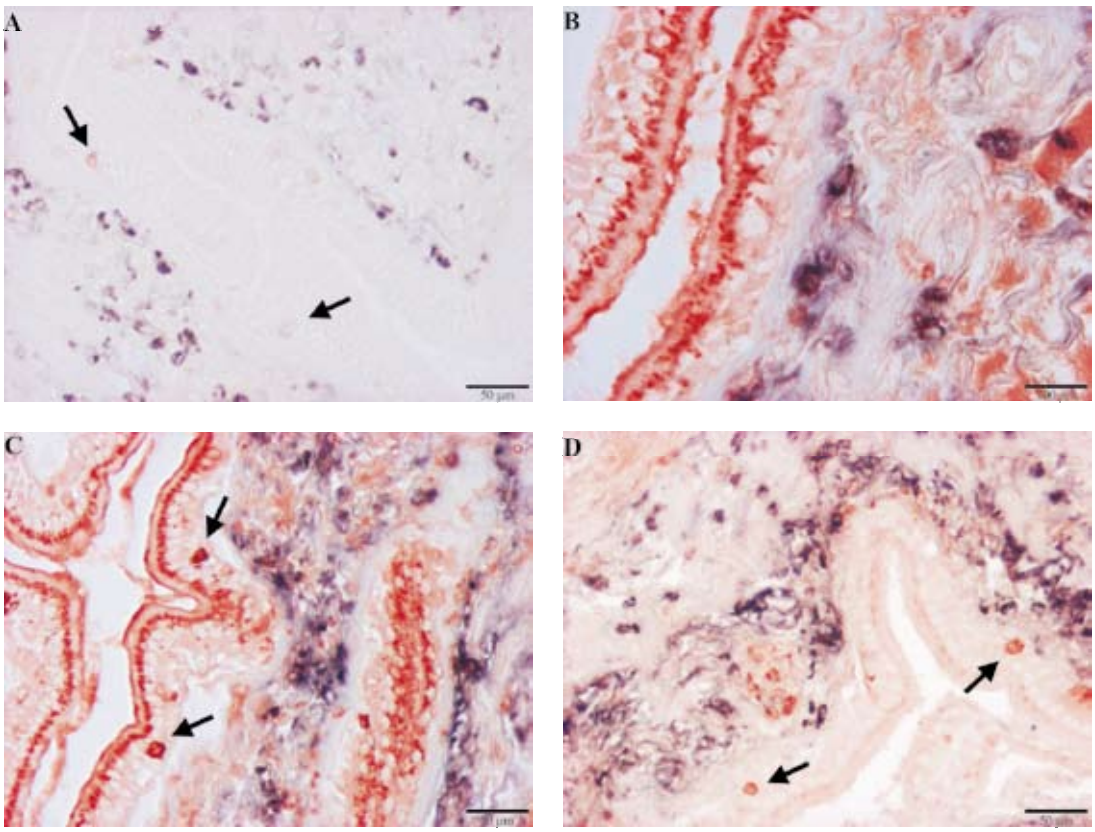


Figure 2.4 Histological sections of *Penaeus monodon* midgut, fixed 0 (A), 8 (B, C) and 72 hours (D) after a 4 hours immersion. Haemocytes are stained with WSH 8 (blue) and virus particles with anti-VP28 (red). Note the $VP28^{+}$ vacuole-like structures in the epithelial cells. The red basal cells (A: arrows) in the epithelium are stained due to cross-reactivity of anti-VP28, but a stronger staining is present after 8 and 72 hrs post-infection.

In contrast to the viruses in the gills, the viral-like particles in the SNV appear to be less electron dense to even empty (Figures 2.5: inset). At 72 hrs post-infection, VP28-immunoreactivity was hardly detectable anymore in the epithelial cells (Figure 2.4D).

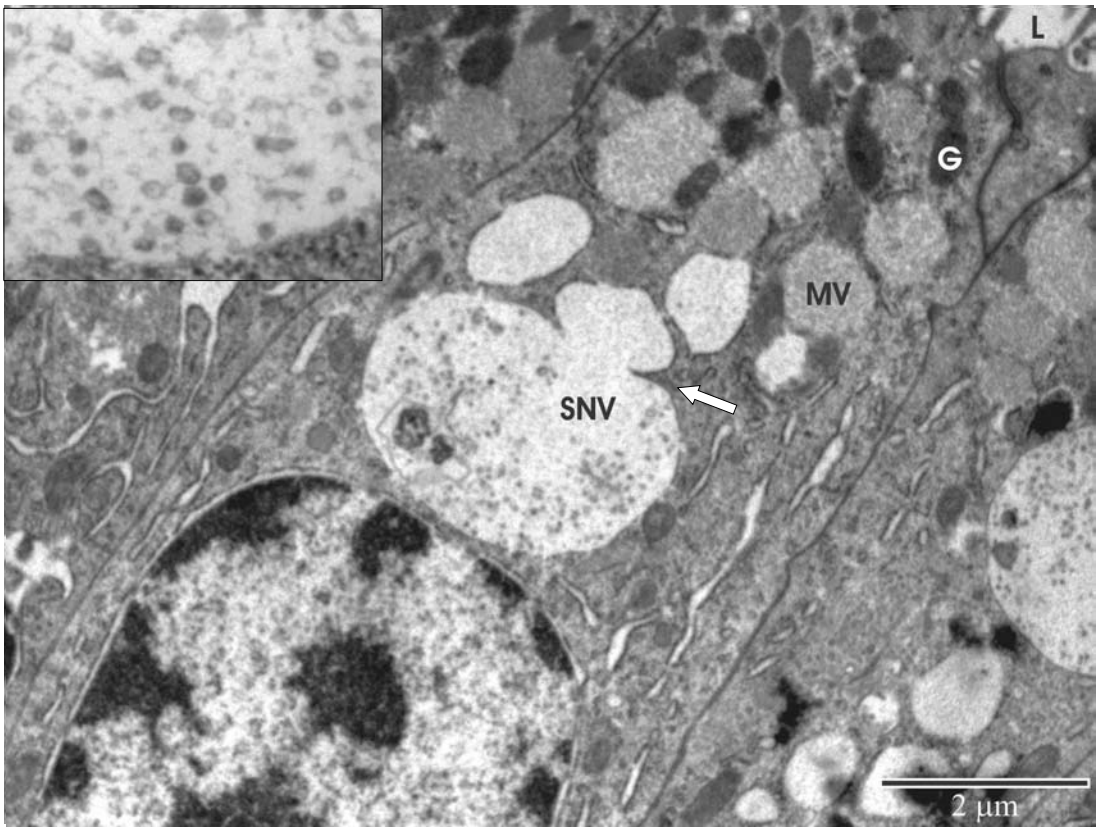


Figure 2.5 Electron micrograph of the apical part of the midgut showing the supranuclear part of the epithelial cells and a minor part of the lumen (L). Next to electron dense granular-like structures (G) , moderate dense minor vacuoles (MV) which clearly fuse with the large supranuclear vacuole (SNV). Note, the presence of viral particles in the SNV closely associated to the uninfected nucleus.

At all stages of infection, weak to moderate HRP^+ cells could be detected at the basal site of midgut epithelium. Controls with only the omission of the VP28 antibody did not show reactivity, implying a cross-reaction of the polyclonal VP28 serum with components present in the epithelial cells of the midgut (Figure 2.4). However, an increase in the reactivity of the basal epithelial cells indicates the presence of VP28 in these cells. Electron microscopy showed two types of basal epithelial cells (data not shown): round primitive cells and cells containing some granules, which may be

considered as hyalinocytes. On the other hand, the last cells were not visible with the WSH8 staining. (Semi-)granulocytes were only found outside the epithelium, close to the thickened basal membrane, with immunohistochemistry as well as electron microscopy. An increase in immunoreactive thread-like structures was detected in the gut but not in the gills from 48 to 72 hrs post-infection (Figure 2.4B-D).

Discussion

In this study the changes in haemocyte number in gills and midgut during an immersion infection with WSSV are studied. The increased number of haemocytes after immersion (t=0) observed in both infected and uninfected shrimp is probably a response to the stress of the handling and immersion. The most prominent stress factor is most likely the high density during the immersion in the virus solution, as shrimp are very sensitive to stocking density (Johnson, 1989; Lightner, 1983).

A clear increase in gill haemocytes numbers is found at 48 and 72 hrs post-infection. From immunohistochemistry it was not evident whether the increase of $WSH8^{+}$ cells was due to circulating haemocytes or tissue haemocytes. Electron microscopy indicates that most, if not all, haemocytes are in the haemolymph vessels. The decline in circulating haemocytes found during (oral) WSSV infection of *P. monodon* (van de Braak *et al.*, 2002) strongly suggest that haemocytes adhere to the vessel walls during the process of infection and the electron microscopy data seem to confirm this adherence. Immunohistochemistry as well as electron microscopy have shown that a considerable number of gill cell nuclei and not of haemocytes are infected at 48 and 72 hrs post-infection. Possibly these infected cells give signals to the haemolymph vessels to up-regulate adhesion molecules for the suggested attachment of haemocytes to the vessel walls.

The role of the enhanced number of haemocytes in the gills remains still unclear and more research is necessary to elucidate whether they are necessary for clearance

of virus-infected dead cells or play a role in the immune response against the virus. Although clear uptake of VP28 was found in the midgut, WSSV infected cells were not detectable in this organ, which was earlier suggested by others (Lo *et al.*, 1997; Wang *et al.*, 1999). As WSSV replication and virion assembly occur in the nucleus, leading to hypertrophied nuclei and chromatin margination (Lightner, 1996; Wang, *et al.*, 1999), we conclude that epithelial cells are not infected in the midgut, despite the presence of VP28 immunoreactivity and virions in SNV. Apparently the lysosomal micro-environment of the SNV blocks the virulence of the WSSV. Another unexplained phenomenon is the presence of some granule-containing haemocytes (hyalinocytes) in the midgut epithelium while they are not stained with WSH8. This is in contrast with observations by van de Braak *et al*. (2000). WSH8 reacts with granular molecules and is capable to visualise thread-like structures formed by the secretion of granules (van de Braak *et al*., 2000). In this study, non-secreting haemocytes were observed immediately after immersion (Fig. 4A). It is well known that crustacean haemocytes change shape and degranulate rapidly *in vitro* (Rodriguez *et al.*, 1995; Söderhäll *et al.*, 1983; van de Braak *et al.*, 2000). This study, however, shows that this phenomenon also occurs in the gut of shrimp during a viral infection *in vivo*. Whether this reaction has an anti-viral effect is not known yet. In any case this degranulation of haemocytes is not visible in the gills, while the number of infected cells is much higher there. However, this may be due to the presence of the haemocytes in the circulation, resulting in a immediate transport of the degranulated material. In any case, our results suggest that midgut haemocytes seem to react upon infection while infected cells are not in their direct neighbourhood. The signals responsible for this degranulation are still unknown. In conclusion, an invasion of haemocytes in the gills was observed in *Penaeus monodon* upon WSSV-infection, possibly caused by the adherence of haemocytes to the haemolymph vessels. Although many infected cells were found in the gills,

haemocytes were not WSSV-infected in this organ. Gills appear to be an important site of haemocyte invasion after immersion infection. In the gut, uptake of WSSV in the epithelium could be detected, however, infected nuclei of epithelial cells were not observed. In contrast to the gills, the gut connective tissue shows a clear increase in degranulation of haemocytes, resulting in the appearance of WSH8-immunoreactive thread-like material at later time points during the infection. The significance for the different reaction of haemocytes in both organs studied remains to be investigated further. The observation that haemocytes are not the main target for WSSV (this study; van de Braak *et al.*, 2002; Luna *et al.* 2002) which is in contrast with observations of others (Di Leonardo *et al.*, 2005) and this may be due to different infection routes used or less clear detection of haemocytes.

Acknowledgements

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CHAPTER

3

Construction of a dedicated cDNA microarray using suppressive-subtractive hybridisation of WSSV infected *Penaeus monodon*

Joop AJ Arts, Trudi Hermsen, René JM Stet & Huub FJ Savelkoul

Abstract

White spot syndrome virus (WSSV) is a large dsDNA virus infecting crustaceans. It is the most important viral pathogen of cultured penaeid shrimp worldwide. Despite the considerable progress made in the detection and molecular characterisation of WSSV the past decade, only limited information about shrimp genes that might be involved in WSSV pathogenesis is available. In the present study, we constructed a cDNA microarray based on suppressive-subtractive hybridisation (SSH). WSSV-infected shrimp and control shrimp were used to generate a SSH^+ library. Sequence analysis of the library revealed a high redundancy in mitochondrial and ribosomal sequences. Screening with mitochondrial and ribosomal probes and a reverse northern lead to a reduction in the redundancy of 30% in both procedures. Overall, the combination of suppressive-subtractive hybridisation and microarray analysis has resulted in a detection system of shrimp genes involved in the defence upon a WSSV infection.

Introduction

White spot syndrome virus (WSSV) is a major pathogen in shrimp that causes high mortality and major economic losses in shrimp aquaculture. In cultured shrimp WSSV infection can cause a cumulative mortality of up to 100% within 3-10 days (Lightner, 1996). Immune protection is not sufficient and needs to be improved by better knowledge of genes involved in early infection.

At present, there is little baseline information on the molecular biology of any crustacean species. In GenBank, only a few hundred crustacean entries are found, including many redundancies. Of these, only two hundred are from decapod crustaceans, the group to which most commercially important species belong.

The advent of rapid DNA sequencing technology has facilitated and enabled analyses of gene expression in organisms *via* the isolation of partial cDNA

sequences, termed expressed sequence tags (ESTs) (Adams *et al.*, 1991; Marra *et al.*, 1998). Despite the usefulness of EST sequencing and the relative ease with which large data sets can be generated, this approach has the disadvantage that many of the sequences obtained represent common 'housekeeping' genes (present in all developmental stages) and that many abundantly expressed genes are highly represented, thus generating relatively large amounts of redundant sequence information (Lehnert *et al.*, 1999). Also, genes expressed at low levels may not be detected.

Differential display (Liang *et al.*, 1992) is a direct PCR-based technique employed for isolating differentially expressed genes in tissues of organisms whether or not exposed to antigens, infections, stress conditions, etc. This approach has shown to be very useful in identifying specific genes not previously identified by EST sequencing of conventional cDNA libraries (Joshua *et al.*, 1995; Hartman *et al.*, 2001). However, in spite of its usefulness, the differential display approach has limitations in that it is laborious and time-consuming to carry out, and that frequently 'false positive' display products are isolated (Liao *et al.*, 2002; Stein *et al.*, 2002; Dhar *et al.*, 2003).

The technique of suppressive-subtractive hybridisation (SSH), first used in the mid-1960s (Bautz *et al.*, 1966) and improved by the ligation of linkers to cDNA to allow selective PCR amplification (Duguid *et al.*, 1990), was shown to be effective in isolating, normalising and enriching differentially expressed genes over 1.000-fold in a single round of hybridisation (Diatchenko *et al.*, 1996). SSH-PCR overcomes the limitations of differential display by effective removal of common (e.g. 'housekeeping' genes) genes from the RNA population of interest prior to library construction. It also has the advantage that rare transcripts are amplified (i.e. are enriched), which is not the case for the conventional EST sequencing approach. Despite its utility and effectiveness for isolating differentially expressed genes,

the SSH approach has not yet been applied widely to virus-infections in shrimp (Bangrak *et al.*, 2002). In the present study, we constructed with the use of SSH archives of White Spot Syndrome Virus (WSSV) infected *Penaeus monodon* ESTs, and constructed microarray glass-slides for future work on the functional aspects of genes differentially expressed between challenged and control shrimp.

Materials and methods

Shrimp culture

Healthy *P. monodon* shrimp were imported as postlavae from Malaysia and maintained in a recirculation system (pH 7.8-8.0, salinity of ±20 ppt, 0.3 mg/l NH_4^+, 0.1 mg/l NO_2^-, 200 mg/l NO_3^- at 28°C) at the facility "De Haar vissen" of the Wageningen University. Each shipment was tested for the presence of WSSV, Monodon baculovirus, yellow head virus, Taura syndrome virus and infectious hypodermal and hematopoietic necrosis virus by a virus specific PCR. Prior to each experiment, shrimp were transferred to 45- or 180-liter aquaria located at the laboratory of Virology (Wageningen University), each fitted with an individual filter system (Eheim; Fleuren & Nooijen Viskwekerij BV), heating (Schego) to 28 ± 1°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand.

White Spot Syndrome Virus stock

The virus isolate used in this study originates from infected *P. monodon* shrimp imported from Thailand in 1996 (van Hulten *et al.*, 2001). Crayfish *Orconectes limosus* were injected intramuscularly in the lateral area of the fourth abdominal segment with a lethal dose of WSSV using a 26-gauge needle (Microfine, B&D). After one week, haemolymph was withdrawn from moribund crayfish and mixed

with modified Alsever solution (Rodriguez *et al.*, 1995) as an anticoagulant. The virus particles were isolated by centrifugation at 58,500x g for 1.5 hours on a 20-45% sucrose gradient in TN (20 mM Tris, 400 mM NaCl, pH 7.4). The visible virus band was collected, two times diluted in TN and the virus particles were subsequently sedimented by centrifugation at 26,000x g at 4°C for 50 minutes. The virus pellet was resuspended in PBS (pH 7.4) and the virus integrity was checked by electron microscopy. The virus stock was stored at -80°C until use.

In vivo treatment

Since no crustacean cell lines are available, the WSSV stock was titered by *in vivo* infection experiments as described by van Hulten *et al.* (van Hulten *et al.*, 2001). *P. monodon* of approximately 15 gram were injected intramuscularly with 10 µl virus solution in 330 mM NaCl in the 4th or 5th tail segment with a 29 gauge needle (Microfine B&D). Alternatively *P. monodon* of approximately 15 gram were immersed in 6 µl virus solution (van Hulten *et al.*, 2001)/500 ml artificial seawater/ shrimp for 2 hours. In both procedures a relative high dose was used to synchronise infection in the gills. After immersion shrimp (n=5) were transferred to 60-liter aquaria. After 4 hours, gills were isolated, snap-frozen in liquid nitrogen and stored at -80°C until use in the generation of SSH libraries.

RNA extraction

Gill tissue was homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) followed by phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water (Chomczynski *et al.*, 1987). Concentrations were measured by spectrophotometry (Nanodrop Technologies); 1 µg was analysed on a 1% agarose gel to check integrity of the RNA, which was stored at -80°C for future use.

Construction of suppressive-subtractive hybridisation (SSH) libraries

Total gill RNA (4 h.p.i.) was used to synthesise cDNA (using the PCR-Select™ cDNA subtraction Kit; Clontech) for SSH according to the manufacturer's instructions. Briefly, cDNA was synthesised from challenged shrimp (tester-cDNA) and unchallenged shrimp (driver-cDNA) RNA with an oligo(dT)-containing primer. Tester- and driver-cDNA were separately digested with *Rsa*I to obtain shorter, blunt-ended molecules. Two tester populations were ligated to two different adapters (figure 3.1). The tester-cDNA was hybridised with an excess of driver-cDNA to remove common cDNA transcripts, generating the SSH^+ library. As control, tester-cDNA was hybridised with water instead of driver-cDNA to provide 'unsubtracted' cDNA. This was used for actin-specific PCR analysis (figure 3.2). cDNA was cloned into pGEM-T easy and used to transform JM109 cells. 1000 clones were picked and stored at -80°C. Of these 400 randomly selected clones were sequenced, using the BigDye Terminator Cycle Sequencing kit (ABI prism) and analysed using an ABI 377. Similarity searches were performed by comparing the shrimp SSH sequences to those in the GenBank database using BLASTX and BLASTP searches.

Preparation of driver cDNA probes and mitochondrial/ribosomal probes

Mitochondrial and ribosomal DIG labelled probes were made by PCR labelling according to the manufacture's instructions (Roche Diagnostics) using primers listed in table 3.1 and tester-cDNA as template. Driver-cDNA probes were made by PCR labelling accoding to the manufacture's protocol (Roche Diagnostics) using 5'PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3'; Smart™ cDNA Library Construction Kit, Clontech).

Screening of macro-DNA array filter

1536 independent recombinant colonies of the subtracted library were inoculated into 16 sterile 96-wells microtitre plates, each well containing 200 µl LB medium

and ampicillin. After overnight incubation at 37°C, each culture was transferred to a positively-charged nylon membrane (Roche Diagnostics) using a vacuum manifold. Duplicate membranes were denatured in 0.5M NaOH/1.5M NaCl, neutralised with 1.5M NaCl/1M Tris/HCl (pH 7.4), baked for 2 hours at 80°C to cross-link the DNA to the membrane and subsequently treated with proteinase K (3.75 mg/1.5 ml/ membrane) for 60 min at 37°C to remove proteins. The subtracted WSSV infected shrimp cDNAs arrayed on the membranes were prehybridised in hybridisation buffer (DIG Easy Hyb, Roche Diagnostics) for 2 hours at 37°C. The buffer was replaced with fresh hybridisation solution containing denatured DIG-labelled cDNA probes or mitochondrial/ribosomal probes (25 ng/ml). Hybridisation was performed overnight at 37°C. The membranes were washed at high stringency (0.1x SSC; 0.1% SDS at 50°C). Probe-target hybrids were visualised by chemiluminescent assay using CDP-star according to the manufacture's protocol (Roche Diagnostics) and Lumi-Film Chemiluminescent Detection film (Roche Diagnostics). Positive clones were removed from the library.

cDNA microarrays

Inserts from the suppressive-subtractive hybridisation libraries, cloned in pGEM-T easy, were amplified using vector primers in a colony PCR reaction. PCR reaction conditions: approximately 10 ng plasmid, 200 µM dNTP, 200 nM forward/reverse primer, 1 unit *Taq* DNA polymerase (Eurogentec), reaction buffer without $MgCl_2$, 1.5 mM $MgCl_2$, and H_2O in a final volume of 50 µL. PCR program: 94°C 10 min, (94°C 30 s, 55°C 30 s, 72°C 2 min) x 30, 72°C 7 min. QIAquick Gel Extraction kit (Qiagen) was used for gel extraction. 10 µg of the PCR product was dried to completion and dissolved in 12 µl 5x SSC. A total of 721 selected clones from the SSH^+ library, 11 housekeeping genes, 24 background (yeast), 24 reference (luciferase) clones (table 3.2) and 272 WSSV probes (Marks *et al.*, 2005) were

printed on GAPS amino-silane-coated glass slides (Corning) in double copies, using a PixSys 7500 arrayer (Cartesian Technologies) equipped with Chipmaker 3 quill pins (TeleChem International). Spotting volumes were 0.5 µl, resulting in a 120 µm spot diameter at a pitch of 160 µm. Microarrays were dried overnight, rehydrated with steam, snap-dried (95°C-100°C) and UV cross-linked (150 mJ). Subsequently the slides were soaked in 0.2% SDS (2 min), twice in MQ water (2 min) and boiling MQ water (2 min). After drying (5 min), the slides were rinsed 3 times in 0.2% SDS (1 min), once in MQ water (1 min), submerged in boiling MQ water (2 sec), and air-dried (Mercke *et al.*, 2004).

Results

SSH - cDNA

SSH-PCR was used to generate cDNA libraries enriched in infection-related putative differentially expressed sequences (figure 3.1). Each cDNA is restricted several times by *Rsa*I resulting in sequences with sizes between 100 to 500 bp as revealed by gel electrophoreses. These cDNAs were cloned into pGEM-T easy to generate libraries enriched for putative differentially expressed genes. This library should represent clones corresponding to transcripts upregulated by infection of tiger shrimp with White Spot Syndrome Virus at 4 hours post infection. The subtraction efficiency was evaluated by the constitutively expressed β-actin gene. In the subtracted library, β-actin was not detectable in contrast to the unsubtracted library (figure 3.2).

Sequence analysis

Sequencing of 400 randomly selected clones from the SSH^{+}-cDNA library showed that about 43% of the clones represented similarity with known transcripts. 24% of the sequenced clones showed similarity with mitochondrial and ribosomal transcripts.

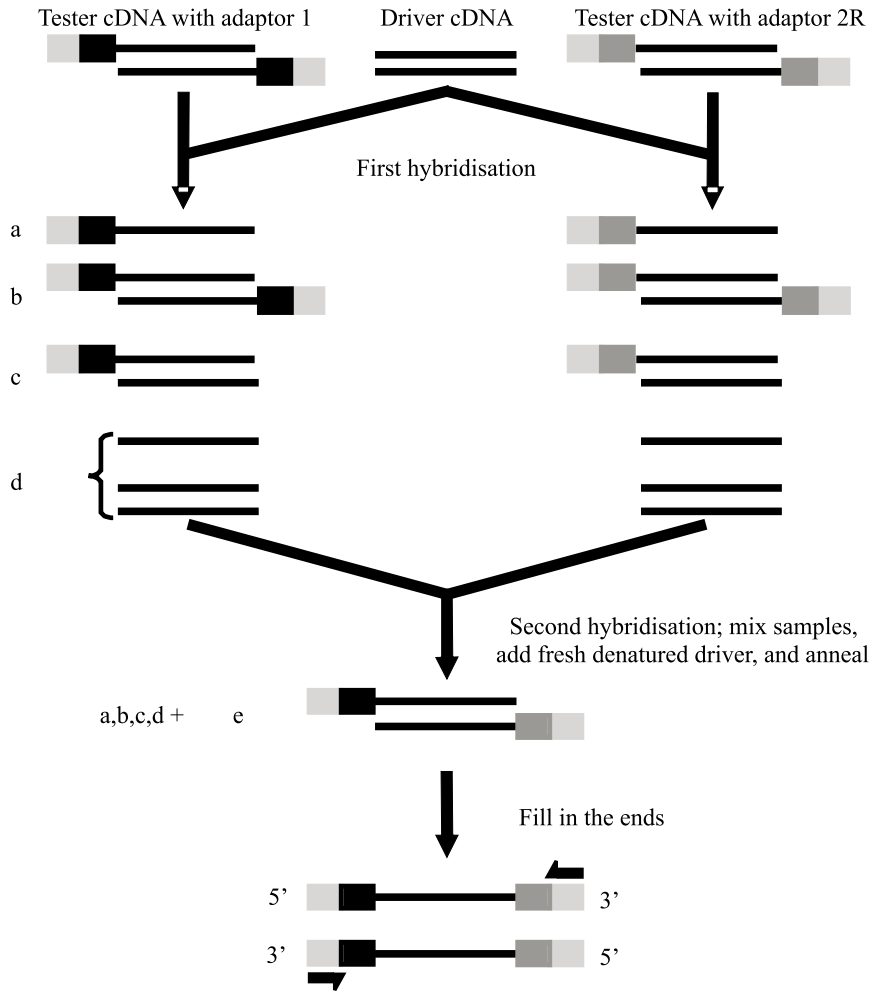

Figure 3.1 SSH procedure. Tester: challenged shrimp; driver: unchallenged shrimp. a, b, c, d and e: different possible hybridisations. cDNA with two different adaptors can be exponential amplified by PCR.

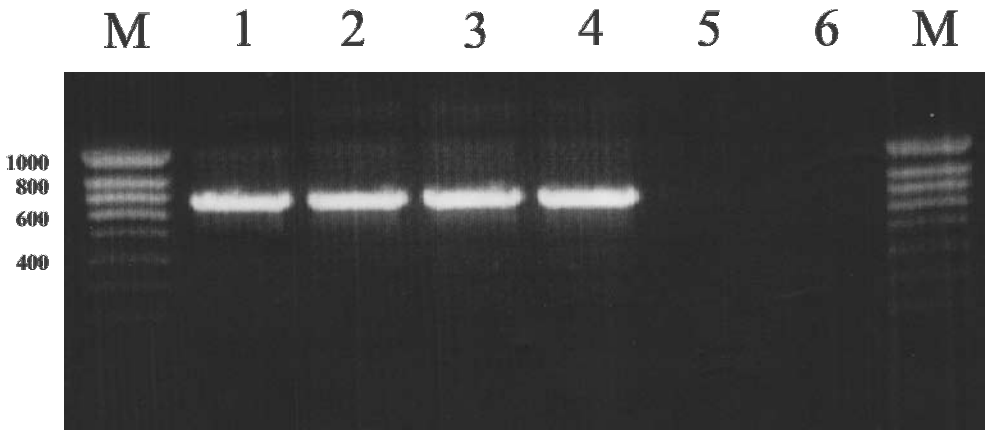

Figure 3.2 Evaluation of the subtraction efficiency. Lane 1-4 show Actin fragment in unsubtracted cDNA. Lane 5 and 6 show subtracted cDNA. M, marker

The limited size of the SSH$^+$-cDNAs hindered identification using GenBank, resulting in a large number of unknowns (Mercke *et al.*, 2004). To screen out mitochondrial and ribosomal genes, 768 clones were arrayed on nylon filters and screened with mitochondrial and ribosomal probes (table 3.1) leading to a reduction of 30%. Furthermore a reverse northern was performed, that is hybridising the filters with the control cDNA (driver). Products negative in reverse northern, approximately 30%, were used to generate a microarray (figure 3.3).

Table 3.1 Names and primer sequences of mitochondrial and ribosomal probes based on the sequenced mitochondrial genome (Wilson *et al.*, 2000).

PmCYTC1F1	cytochome c oxidase subunit 1	TCAACTGAAGCACCTGCGTGAG
PmCYTC1R1	cytochome c oxidase subunit 1	GTGCTCCAGATATGGCATTTCC
PmCYTC2F1	cytochome c oxidase subunit 2	TGTATGGACAGTACTTCCCGCTC
PmCYTC2R1	cytochome c oxidase subunit 2	GCTTTTACACCAAGGGCAGG
PmCYTC3F1	cytochome c oxidase subunit 3	GCTTTTACACCAAGGGCAGG
PmCYTC3R1	cytochome c oxidase subunit 3	TCAATATCAAGCGGCAGCTTC
PmNADH1F1	NADH dehydrogenase subunit 1	CCCTTTTGATTTCGCTGAGG
PmNADH1R1	NADH dehydrogenase subunit 1	GCTTATCATAACGGAAACGTGG
PmNADH2F1	NADH dehydrogenase subunit 2	CAATATTCCTCAGAAGCCGCTC
PmNADH2R1	NADH dehydrogenase subunit 2	TTAATCCCTCCGATTGCTCC
PmNADH3F1	NADH dehydrogenase subunit 3	TTGACCCTAAAGGATCCGCTC
PmNADH3R1	NADH dehydrogenase subunit 3	AAGCTCATTCTAGGGCTCCTTG
PmNADH4F1	NADH dehydrogenase subunit 4	GGTTGAGGTTATCAGCCCGAAC
PmNADH4R1	NADH dehydrogenase subunit 4	CCTAGTTTTAATAAAACGCCTGC
PmNADH5F1	NADH dehydrogenase subunit 5	TCATCTACTTTAGTTACAGCCGGAG
PmNADH5R1	NADH dehydrogenase subunit 5	ATAGCAGACTGTTAAACCCGTAGC
PmNADH6F1	NADH dehydrogenase subunit 6	CGTTTACTTCATCCCTTAGC
PmNADH6R1	NADH dehydrogenase subunit 6	TGATGATTGTTGTAGGGAAAGAGG
PmATP6F1	ATP synthase F0 subunit 6	TAATCATAACCCTCGCTCTTGC
PmATP6R1	ATP synthase F0 subunit 6	TCGAACAGCTAAGGTCCCTGG
PmsRNAF1	small subunit ribosomal RNA	CCTGGTTAAAATTTGTGCCAGC
PmsRNAR1	small subunit ribosomal RNA	CGCTTAAATTGACGAGAGCGAC
PmLRNAF1	large subunit ribosomal RNA	TATTATTTAAAGAAATCCCGAAAG
PmLRNAR1	large subunit ribosomal RNA	CAAAAGTAGTATTTGCCGAGTTCC
PmAPOF1	apocytochrome b	ATTATGGATGACTTCTCCGAACG
PmAPOR1	apocytochrome b	ATTACAGTGGCTCCCCAGAATG

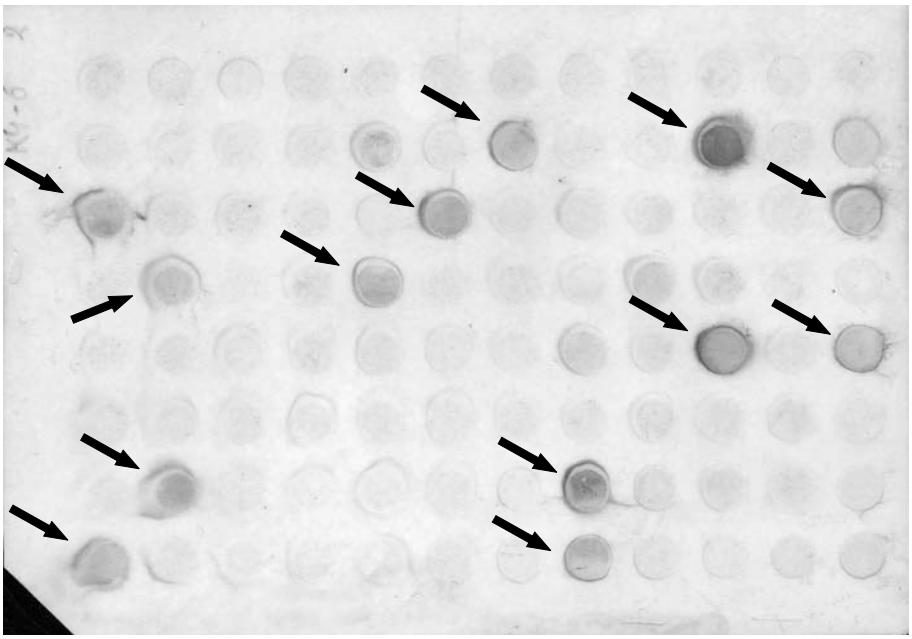

Figure 3.3 Dotblot analysis of SSH clones. Positive clones are hybridised with mitochondrial probes and subsequently removed from the library. Hybridisations with labeled driver cDNA showed similar results.

Microarray

To evaluate *P. monodon* gene expression during infection with WSSV a shrimp cDNA microarray was designed containing 721 clones from the SSH^+ library. In addition to the shrimp sequences, 11 putative constitutively expressed shrimp genes, 24 background (yeast: his, ade, hom), 24 reference (luciferase: total gene, 5' end, middle region, 3' end) clones (table 3.2) and 272 WSSV probes were spotted. Total RNA from uninfected and WSSV infected *P. monodon* gill tissue at 21 h.p.i. was isolated and labelled as described in chapter 4 and used to test the reproducibility and normalisation of the microarray. The gills are one of the primary target tissues of WSSV infection (Lo *et al.*, 2004).

Reproducibility: Due to the duplicates on the array, Cy3/Cy5 ratio for each gene was obtained in duplicate. The ratios of the duplicates were very similar (data not shown). Further analysis (chapter 4) was performed with the average of the duplicate. All hybridisations were repeated and data was consistent with this described experiment.

Table 3.2 Names and accessionnumbers of 11 shrimp housekeepinggenes

housekeeping genes	**(similar to) acc. no.**
Elongation faction 1α	AY117542
β-actin	AF100986
α-tubulin	AAM73790
Fructose-biphosphate aldolase	BE846454
Cytochrome C oxidase	AW497588
glutathione peroxidase	BI018091
Glyceraldehyde-3-phosphate dehydrogenase	CAB94909
NADH dehydrogenase subunit F	AF436051
6-phosphofructokinase	AAF58841
Tropomyosin	AAB31957
Ubiquitin	AW600779
background genes	
HIS3 gene (histidine biosynthetic pathway)	CAA99417
ADE2 gene (adenine biosynthetic pathway)	CAA99327
HOM3 gene (aspartate kinase)	NP_010972
GFP	CAA58790
reference genes	
Luciferase total gene	CAA59281
Luciferase 5' end	CAA59281
Luciferase middle	CAA59281
Luciferase 3' end	CAA59281

Normalisation: Despite the limited information on the shrimp transcription, we cloned 11 shrimp genes (table 3.2) that are presumably constitutively expressed and spotted these on the microarray. These shrimp genes were used to evaluate the normalisation procedure as described in material and methods. The overall expression of the housekeeping genes between time-points was within 20% of the average expression (set at 100%) and confirmed the normalisation procedure used for analysis of the time course (figure 3.4).

Probes synthesis control: 5'end and 3'end luciferase probes were spotted on the microarray to check the reverse transcription of the RNA. A PCR fragment containing the 3' region of a gene will result in a higher hybridisation signal than a fragment that contains the 5' region.

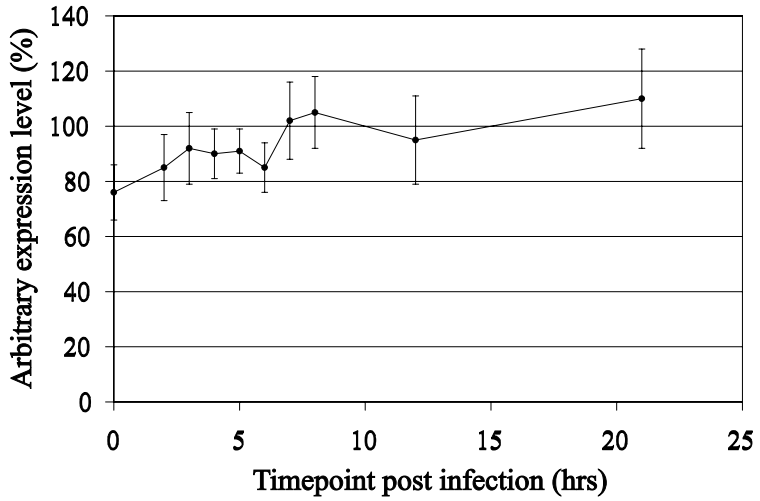

Figure 3.4 Housekeeping gene expression level in % of the 11 shrimp genes shown in table 3.2 (mean expression over the time course is set to 100%; standard deviations are indicated for each time point)

Discussion

In this study we attempted to increase the efficiency for isolating genes expressed in response to a WSSV infection in the shrimp *Penaeus monodon*. We used a combination of SSH, which has been reported to enrich for expressed genes (Diatchenko *et al.*, 1996) and cDNA microarray in this infection. SSH enriches gene expression, as is evaluated here by the results of β-actin specific PCR on tester (unsubtracted) and subtracted cDNAs (figure 3.2). Despite the manufacturer's claim that differentially expressed genes that are present in low abundance in the tester cDNA will be enriched more than differentially expressed genes that are present in high abundance, prevention of clone redundancy was not obtained. The most redundant cDNA clones in the unsubtracted libraries were also expressed at remarkably high levels in the subtracted libraries. Many low-expressing mRNAs were below the microarray detection level, in our case up to 50%. Similar observations were observed in mammalian systems when using SSH libraries (Hida *et al.*, 2000; Boeuf *et al.*, 2001).

Upon an infection most genes that are involved in a response against pathogens are upregulated rather than downregulated, as shown by earlier studies (Dhar *et al.*, 2003; Mercke *et al.*, 2004; Pan *et al.*, 2005). Therefore only a SSH^+ library, containing gene transcripts that are upregulated upon WSSV infection, was created and analysed.

The innate immune response is a rapidly responding system. In *Drosophila* many antimicrobial peptide genes show a maximum expression at 6 hours post microbial challenge (Irving *et al.*, 2001). Gene expression of genes involved in an immune response in shrimp was detected by Dhar *et al*. The response of *Penaeus stylirostris* upon a WSSV infection already starts at 2 hours post infection. Lipopolysaccharide and β-1,3 glucan binding protein (LGBP), serine protease, C-type lectin, macrophage mannose receptor and a lipoprotein receptor genes were overexpressed in WSSV-infected shrimp (Dhar *et al.*, 2003). Immune related genes like the proPO system (Söderhäll *et al.*, 1998) is a less rapidly (1-2 days) reacting system. LGBP serves as an elicitor of the prophenoloxidase cascade in insects and crustaceans (Söderhäll *et al.*, 1998).

Although SSH greatly enriches for differentially expressed genes, the subtracted sample will still contain cDNAs that are present in both the tester as the driver samples. To minimise background a differential screening can be performed. By labelling the tester sample and hybridising the subtracted library, positive clones that are differentially expressed can be detected. The disadvantage of this approach is that only cDNA molecules corresponding to highly abundant mRNAs will produce a detectable hybridisation signal (Wang *et al.*, 1991).

Random sequencing of the SSH^+-library showed that only 43% of the clones could be identified using GenBank. About 50% of the identified clones showed similarity with mitochondrial and ribosomal transcripts. Although mitochondrial and ribosomal genes can play a great role in the defense mechanism of the tiger shrimp, we decided

to screen them out with mitochondrial and ribosomal probes.

To increase the proportion of regulated target clones we used the subtractive cDNA library as a source for clone selection in the microarray preparation procedure.

In microarray analysis it is important to include several controls. Analysis and interpretation of microarray experiments depends on the quality of the obtained data.

Replication: Replication is a highly desirable feature of comparative microarray experiments. Printing cDNAs in duplicate on every slide provides quality information, as the degree of similarity between duplicate spot intensities is an indicator of quality. Duplicate spots were printed well spaced and not adjacent on the slides, providing a better view of the variability across the slide (Ball *et al.*, 2002).

Normalisation: Methods of normalisation based on intensity values of housekeeping genes may show sample-specific bias and do not address the issue of intensity-dependent dye biases. Designated housekeeping genes tend to be highly expressed and may not be representative of genes of interest that are expressed at lower levels and may be subject to an intensity-dependent bias (Ball *et al.*, 2002).

Probe synthesis: The luciferase probes (5' and 3') on the microarray make it possible to check the reverse transcription of the RNA. As reverse transcription was performed with an oligo(dT) primer, more 3' end of a gene will be produced compared to the 5' end. As a consequence: a PCR fragment containing the 3' region of a gene will result in a higher hybridisation signal than a fragment that contains the 5' region.

In conclusion, the approach to combine suppressive-subtractive hybridisation with microarray analysis has resulted in a read-out system for the detection of shrimp genes involved in the defence reaction upon a WSSV-infection. This approach has good potential for identifying genes involved in shrimp defences in the future. Further studies on these gene transcripts involved in the defence mechanism have to be initiated.

Acknowledgements

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CHAPTER

4

Suppressive-subtractive hybridisation coupled with microarray analysis to detect immune response genes in WSSV infected *Penaeus monodon*

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Abstract

WSSV, member of the virus family *Nimaviridae*, is a large dsDNA virus infecting shrimp and other crustaceans. These organisms protect themselves by mounting a rapid innate immune defence reaction. In order to characterise the early immune response genes involved, we infected *Penaeus monodon* by immersion with WSSV and by feeding with pellets containing recombinant VP28, a WSSV envelope protein, in order to vaccinate the shrimp. Analysis was performed by hybridising gill cDNA on microarrays made by differentially expressed genes from a SSH library of WSSV infected versus non-infected shrimp. We found different clusters of positivity depending on the WSSV or vaccine exposure. To evaluate the relevance of the genes identified by the microarray analysis, we performed real-time PCR on 11 genes representing the four clusters in response to infection challenge by immersion. The combination of host immune response genes and 'vaccination' can reveal the route of WSSV infection. Taken together, the present investigation demonstrates the application of a powerful approach of combining the high throughput technologies of SSH and microarray to study differential expression of genes in response to viral infection.

Introduction

White spot syndrome virus (WSSV) is the most important viral pathogen of cultured penaeid shrimp worldwide (Flegel, 1997). Since the initial discovery of the virus in Taiwan in 1992, it has spread to shrimp farming regions in Southeast Asia, the Americas, Europe and the Middle East causing major economic losses. The virus has a wide host range among crustaceans (Flegel, 1997) and induces distinctive clinical signs (white spots) in the exoskeleton of shrimps.

Recently, considerable progress has been made in the detection and molecular characterisation of WSSV (van Hulten *et al*., 2000, 2001a,b; Witteveldt *et al*., 2004). However, information about shrimp genes that might be involved in WSSV pathogenesis remains limited. Most studies on the immunity of shrimp have focused on bacterial and fungal pathogens (de Lorgeril *et al*, 2005) and little is known about how crustaceans respond to viral infections. Recent projects to characterise expressed sequence tags (ESTs) in *Penaeus monodon* revealed more defence related genes in WSSV-infected shrimp compared to healthy shrimp (Rojtinnakorn *et al.*, 2002). Antiviral activity has been detected in *Penaeus setiferus*, blue crab and crayfish and these antiviral molecules were able to bind to DNA and RNA viruses (Pan *et al.*, 2000). Since crustaceans lack antibody-based immunity, the humoral inhibitory antiviral activity is considered part of the innate immune response.

To gain more insight in which genes are involved in an antiviral response, transcriptional profiling experiments were carried out. We used dedicated cDNA microarray analysis (see chapter 3) to compare the kinetics of gene expression patterns in the gill tissues of healthy and WSSV-infected shrimp. The gills are one of the primary entry sites of natural WSSV infection (Lo *et al.*, 2004). Therefore, identification of genes whose expression is altered upon WSSV infection in these tissues may help to elucidate the pathways that are critical to the induction of a WSSV specific response.

Materials and methods

In vivo immersion

P. monodon of approximately 15 grams were immersed in 6 µl virus solution (as described in Chapter 3)/500 ml seawater/shrimp for 2 hours. A relative high dose was used to synchronise infection in the gills. After immersion shrimp were transferred to 180-liter aquariums and cultured for up to 21 hours. At 0, 2, 3, 4, 5, 6, 7, 8, 12 and 21 hours post infection, three animals were randomly selected, gills isolated, snap-frozen in liquid nitrogen and stored at -80°C.

Bacterial expression of VP28

A partial VP28 fragment (without the N-terminal hydrophobic region [amino acids 1-29]) was amplified from genomic WSSV DNA by PCR with primer VP28PF (3'-AAG GAT CCC ACA ACA CTG TGA CCA AG-5') and primer VP28PR (3'-TAG CGG CCG CAA AAG CAC GAT TTA TTT AC-5') and ligated into the *Bam*HI and *Not*I sites of the pET28a vector (Novagen). Expression of the pET28a-VP28 construct and the pET28a vector (control) was performed in BL21 cells as previously described by Witteveldt *et al.*, 2004 (figure 4.1).

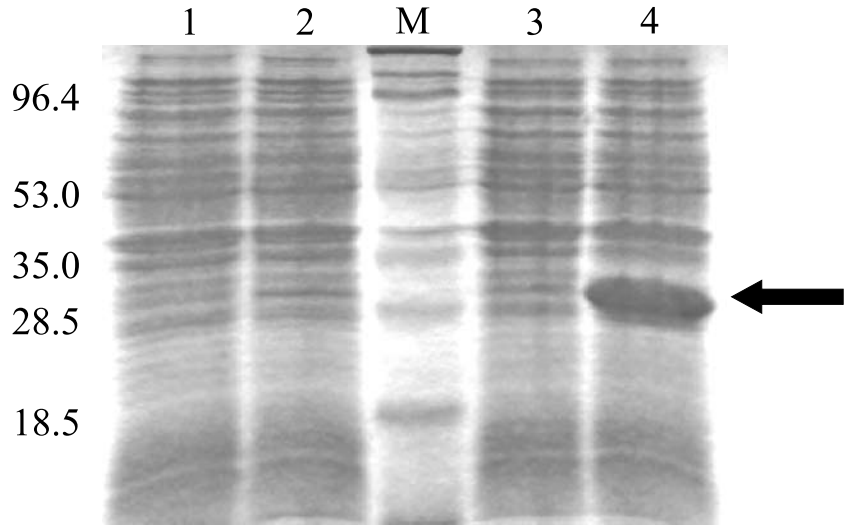

Figure 4.1 SDS-PAGE gel of *E. coli* expressing VP28 using the pET28a vector. Lane 1: empty pET28a vector without induction; lane 2: VP28-pET28a without induction; lane 3: empty pET28a vector after IPTG induction; lane 4: VP28-pET28a after IPTG induction. Numbers on the left side indicate size of the marker bands in kDa. Arrow indicates VP28 protein.

Protein production and inactivation

His_6-VP28 was overexpressed according to the manufacturer's instructions (Novagen) and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The bacterial concentration after inactivation was determined with a Beckman DU-7500 photospectrometer, where an optical density at 600 nm of 1 equaled 10^9 bacteria per ml. The bacteria were inactivated in 0.5% formaldehyde, incubated for 15 min at 20°C and stored at 4°C until further use.

Coating of food pellets

Commercial pellets weighing approximately 0.02 g (Coppens International) were coated with approximately 10^8 inactivated *E. coli* bacteria containing expressed VP28 or empty plasmid (control). The inactivated bacteria were centrifuged, washed twice in PBS, and resuspended in PBS. The bacteria were mixed with the food pellets, incubated on ice for 15 min to allow absorption of the bacterial suspension and subsequently coated with cod liver oil to prevent dispersion of the inactivated bacteria in the water (Witteveldt *et al*., 2004).

Vaccination experiments

In the vaccination experiments, 21 shrimp were vaccinated by feeding coated food pellets (3 food pellets/shrimp; twice a day) for 7 days and subsequently cultured for up to 14 days as decribed by Witteveldt *et al*., 2004. 3 shrimp were randomly collected at 2, 5, 7, 10, 14, 17 and 21 days after the beginning of the feeding with coated food pellets. At these time points the gills were isolated, snap-frozen and stored at -80°C for further use.

RNA extraction

Gill tissue was homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) followed by phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water (Chomczynski *et al.*, 1987). Concentrations were measured by spectrophotometry (Nanodrop Technologies); 1 µg was analysed on a 1% agarose gel to check integrity of the RNA. Equal amounts of RNA of 3 shrimp were pooled and stored at -80°C for future use.

Synthesis Cy3/Cy5 labelled cDNA

Total RNA preparations were labelled using a standard protocol for cDNA synthesis: 10 µg total *P. monodon* gill RNA (spiked with 2 ng luciferase mRNA (Promega) and 2.5 µg oligo$(dT)_{20}$ primer (Invitrogen) were heated to 70°C for 10 min and placed on ice for 5 min. RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) according to the protocol of the manufacturer, with the exception that 40% of dTTP was replaced with 5-(3-aminoallyl)-2'dUTP (AA-dUTP). After reverse transcription, the reaction was stopped with 0.45 M EDTA and 1 N NaOH for 15 min at 70°C. The solution was neutralised with 0.75 M HEPES (pH 7.4) and 0.75 M NaOAc (sodium acetate). Unincorporated AA-dUTP was removed using QIAquick Spin columns (Qiagen). Briefly, to the neutralised solution 5 volumes of Buffer PB (Qiagen) was added and the sample was loaded onto the column. After centrifugation the column was washed twice with 80% ethanol. The cDNA was eluted from the column with 20 µl prewarmed (70°C) MQ water and subsequently dried not to completion in a speed vac (Savant). cDNA was resuspended in 5 µl 0.1 M carbonate buffer (pH 9.3). Dyes were covalently bound to the incorporated amino-groups by adding 5 µl of 5 mM Cy3 or Cy5 reactive dyes (Amersham Pharmacia) in dimethyl-sulfoxide. This mixture was incubated at room

temperature in the dark for 1 hour. Unbound dyes were removed using a Sephadex™ G-50 fine (Amersham Pharmacia) column according to the manufacturer's protocol. Samples under study were labelled with Cy3; the reference sample was labelled with Cy5. Reference samples used were a pool of transcripts representing all the genes present in the time course.

Microarray hybridisations

After prehybridisation of the slides for 2 hours at 42°C in hybridisation solution (50% formamide, 5x Denhardt's reagent (142.5 mg Ficoll 400, 25 mg polyvinylpyrrolidone and 25 mg BSA fraction V in a total volume of 2.5 ml), 5x SSC, 0.2% SDS and 0.1 mg/ml denatured salmon DNA), the slides were washed with MQ water, followed by dipping in isopropanol. Slides were dried by centrifugation at 160x g for 1 min. Hybridisation occurred in a volume of 65 µl using a covered hybridisation frame (Gene Frame 15mm x 15mm (65 µl); ABgene). After heating 100 µl hybridisation solution containing both Cy3 and Cy5 labelled cDNA sample to 100°C for 2 min, it was loaded into the hybridisation chamber. The slides were hybridised during 24 hours at 42°C. Following hybridisation, the slides were washed in 1x SSC/0.1% SDS for 5 min, 0.1x SSC/0.1%SDS for 5 min and briefly rinsed in 0.1x SSC. Slides were dried by centrifugation at 160x g for 1 min.

cDNA microarray analysis

Slides were scanned for fluorescence emission with a ScanArray ExpressHT (Perkin Elmer) at 75% laser power and a resolution of 10 µm, using an attenuation of 65% (Cy3) or 60% (Cy5). The resulting Cy3 and Cy5 images were stored as TIFF files and individually processed. For each array element, the integrated optical density was determined within a defined circle, using AIS software (Imaging Research). Average background values, calculated from the hybridisation signals of the yeast

probes (table 3.2), were subtracted to correct for non-specific fluorescence. Next, Cy3 or Cy5 signals not reaching 0.5x background value were set to this cut-off (0.5x background value) for the respective dye. Elements for which both the Cy3 and Cy5 signal did not reach 0.5x background value were discarded from further analysis (Ball *et al*., 2002; Franssen-van Hal *et al.*, 2002).
Normalisation of the two samples in each hybridisation was done with the mean hybridisation signal of the luciferase probes. Finally, the Cy3/Cy5 ratio was calculated for each array element. The reference sample used on each slide was the same within the time series, allowing direct comparison of the different hybridisation experiments. Expression ratios for the on-array duplicates were calculated separately and the average of both values was used for further analysis. Improper duplicates were filtered out.

cDNA synthesis for RT-qPCR

cDNA was made by adding 1 µl 10× DNase I reaction buffer and 1 µl DNase I (Invitrogen Corporation) to 1 µg total RNA in a total volume of 10 µl and incubated at room temperature for 15 min. DNase I was inactivated by adding 25 mM EDTA. Synthesis of cDNA was adapted from Invitrogen's Superscript™ III First Strand Synthesis Systems for RT-PCR Systems. DNase I-treated RNA samples were mixed with 5× first strand buffer, 300 ng random primers, 10 mM dNTPs, 0.1 M DTT, 10 U/µl and RNase inhibitor, followed by incubation at RT for 10 min and at 37ºC for 2 min. One µl Superscript III Reverse Transcriptase (Invitrogen Corporation) was added and the mixture was incubated at 37ºC for 50 min. The reaction was stopped at 70ºC for 15 min. A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted 1:50 in nuclease-free water before use as template in real-time PCR experiments.

Relative quantification using Real-time Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed using RotorGene™ (Corbett Life Science) with Brilliant® SYBR® Green QPCR (Stratagene) as detection chemistry. PCR reactions (14 µl) were performed with 5µl cDNA, 2x Master SYBR Green I mix and 300 nM of each primer in a 100 µl tube. Cycling conditions were: denaturation at 95°C for 15 min, 40 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec and a final hold at 60°C for 1 min. Subsequently a melting curve was acquired with continuous fluorescence acquisition from 60°C to 99°C with a rate of 1°C/5 sec. *Penaeus monodon* specific primers (table 4.1) were used. A 82 bp elongation factor 2 fragment amplified with primers based on a tiger shrimp EF2 fragment (Lehnert *et al.*, 1999) (GenBank: AI253924: 5'-GATGGACAAGCGGAGGAA-3'; 5'-AAGGGAGTCGGTCAGGGTA-3') was used as a reference gene.

Table 4.1 Primers used for amplification of specific gene products by RT-qPCR.

clone ID	experiment		sequence (5' - 3')
imm1b4	infection	fw	CGCATTGTTGTGCTAGAGGTT
		rv	CGCCATGATGATTGTGTTTT
imm4f6	infection	fw	CTCCTCTTGGACGTTTTGCT
		rv	TGCCAGATTGTTCCTTCTTGT
imm4h9	infection	fw	CCTCACCTGCCACCTGTAA
		rv	TCGCCAAGGAAATGTATGAA
inj11a3	infection	fw	CTtGGAGGAACGTCAAACACT
		rv	CGACAAGGGAAGCCACAA
inj11a7	infection	fw	GCAAAGAGTTCCCCATTGTT
		rv	CTGGATGTTGGTGGCAGAG
inj11c3	infection	fw	GGGCAGCTCATTCATGGT
		rv	TCTCCGCTCGCAATGTCT
inj11c4	infection	fw	ACTGGAAGGCAAGAAGTTGG
		rv	ACTGGTGCAGCTTTGTTCAG
inj11d7	infection	fw	AATTGGATCACACTGGATTGG
		rv	AGAGTCGCCATGATTCTGCT
inj14d7	infection	fw	ACCAACTGGAGACTGCCAAC
		rv	AGACCCTTGAGGTCAGCAAA
inj7b2	infection	fw	CGAGTGGAATCTAGGAACGAAC
		rv	TGGCTTCATCCATCTTCTCC
inj7d11	infection	fw	GCGTCATCACAACACCTGAC
		rv	GCTGTCTGAAGGGAACACATT
inj8g9	infection	fw	CACCACCAAGAAAGGGTCAA
		rv	ATTCGGCAATACCACGAGAC
inj9b5	infection	fw	CACTAGCCCCCAAGTAAGCA
		rv	ACATCGAAAGGGATGAGAGG
inj9f10	infection	fw	GCAATCTTCGCCTTAATCGT
		rv	CTCCTTCGGTTGAATCTGCT
imm5a8	infection	fw	GGTGGTTCTTCGGTAGCAGT
		rv	CCGTCATTGTTCCCATTAGTTT
imm 4d7	vaccination	fw	TGATGCACTCTAGCACCTCTG
		rv	TGCAGCGAAGCAGTTTATATG
inj 7d8	vaccination	fw	AGCTGTTTGTTGGTGACGTTT
		rv	GTCTCTAAAACCACGGCACAG
imm 1g4	vaccination	fw	AATAACTTCCGTCCCGTCAA
		rv	GACATCCCTTGCTCCTGTG
inj 3h6	vaccination	fw	GTAAAACAAGAGCGTGGGTCA
		rv	GCCCCTCACACAAAGTGGT
imm 3a8	vaccination	fw	GCCTGATGAAGAGACCACAGA
		rv	CTTGGCTTCCTTTTGGGAAC
elongationfactor 1	reference	fw	GATGGACAAGCGGAGGAA
		rv	AAGGGAGTCGGTCAGGGTA

Results

Microarray analysis

In this paper we studied at the molecular level the immune response of shrimp after two types of immune challenge: an infection with WSSV under *in vivo* conditions and the induction of protective immunity by a potential vaccine. We thus used immersion challenge with WSSV or feeding with inactivated *E. coli* expressing VP28 coated food as treatments to obtain sources of probing material. RNA, extracted from gills at 2, 3, 4, 5, 6, 7, 8, 12 or 21 h after the beginning of the challenge or 7, 14 or 21 days after the beginning of the 'vaccination', was hybridised to a microarray consisting genes of relevance in early defence of shrimp against WSSV (chapter 3). Expression values were derived from the average of the on-array duplicates. HSP70, ATPsyntase, 16s ribosomal, phosphopyruvate hydratase are differentially regulated upon infection. Moreover, in all of the experiments, 11 putative housekeeping genes (Marks *et al.*, 2005) were analysed. The overall expression of the housekeeping genes between the different time-points showed a 20% difference and indicates the limited use of these genes as reference genes for baseline expression in the present context.

Pilot experiments revealed that the yeast probes (table 3.2), used as background controls, showed a minimal signal compared to the hybridisation of labelled cDNA of WSSV-infected and uninfected shrimp.

Immersion infection

In the immersion experiment at all evaluated time points, several different shrimp genes involved in the WSSV-specific immune response could be detected. On average, 15% of the probes present on the microarray could be detected (figure 4.2). Hierarchical clustering revealed shrimp gene clustering into four groups (figure 4.3). The genes in cluster 1 reached a minimum amount of mRNA at 12

h.p.i. Cluster 2 genes showed an upregulation up to 7 h.p.i. and then rapidly back to normal levels. Cluster 3 showed a maximum at 8 h.p.i. and cluster 4 showed a sigmoide curve with first upregulation, followed by downregulation. The genes in all four clusters showed normal expression levels at 20 h.p.i.

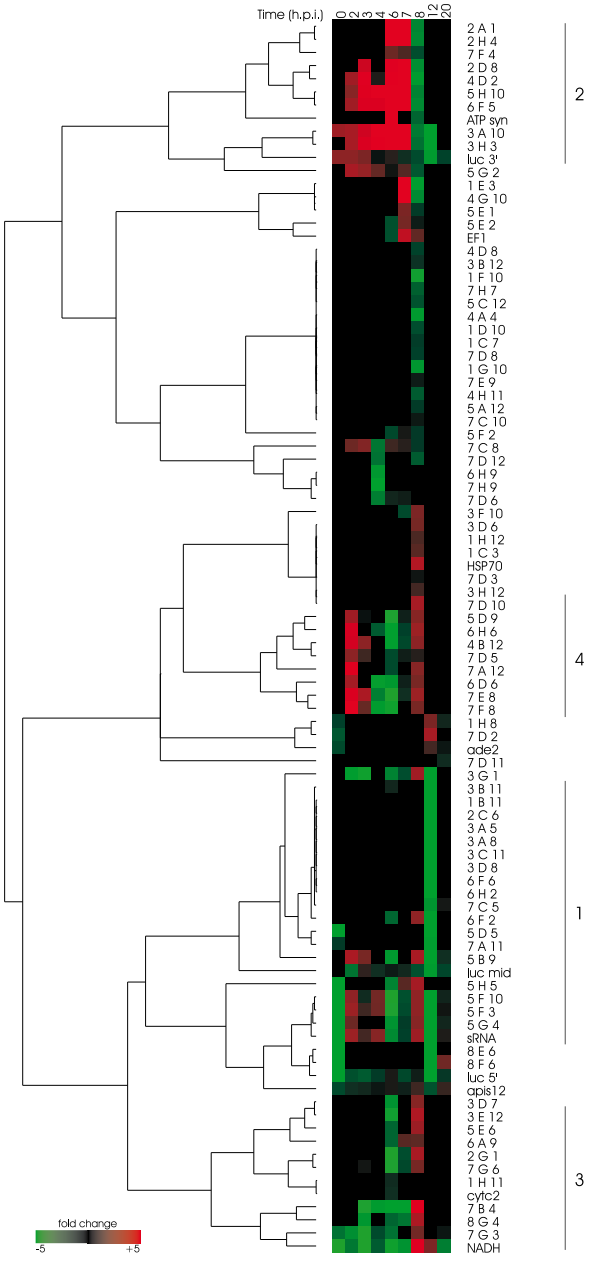

Figure 4.2 Hierarchical clustering (Euclidean distance clustering algorithm) of *P. monodon* gene expression profiles in WSSV infected gill tissues. Columns indicate separate time points (in hrs) and every row displays the expression profile of a single cDNA spotted on the microarray. The dendogram clusters the cDNAs based on the relatedness of their gene expression patterns.

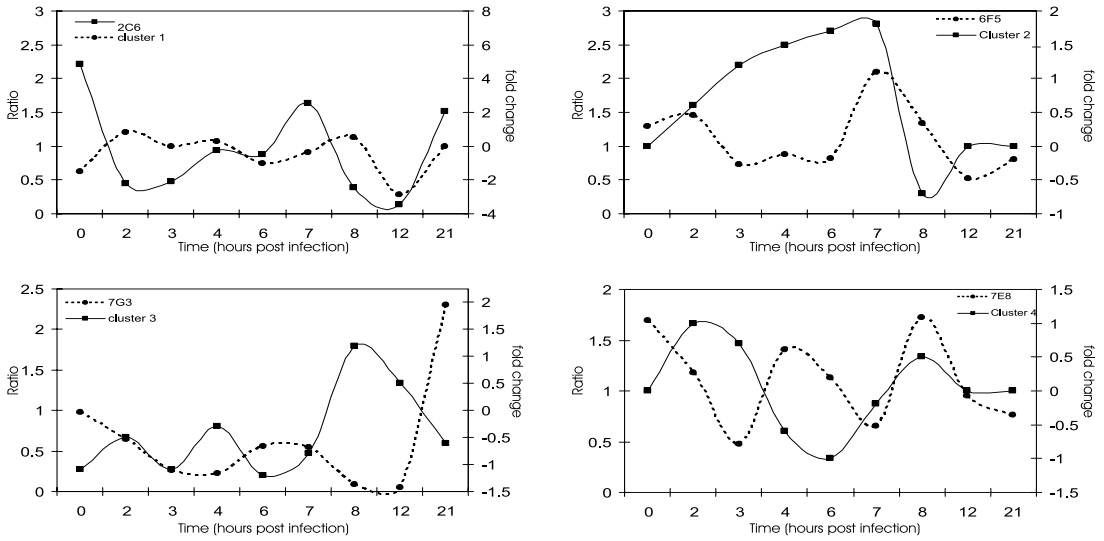

Figure 4.3 Transcription patterns of four different clusters (dashed line; righ y-axis) and RT-qPCR kinetics (solid line; left y-axis). Note the non-linear x-axis.

To evaluate the relevance of the genes identified by the microarray analysis we performed real-time PCR on 11 genes representing the four groups in response to infection challenge by immersion. Only one gene (2C6) showed a comparable expression profile in the evaluated time points as was found in the microarray analysis (figure 4.3).

Vaccination response

In the 'vaccination' experiment, shrimp genes involved in an immune response could be detected at all evaluated time points. About 100 gene transcripts (15%) did show a response, i.e. a ratio difference of >1, to VP28, one of the envelope proteins of WSSV (figure 4.4). RNA was isolated at 7, 14 and 21 days after the start of feeding the shrimp coated food pellets. Previous observations by Witteveldt et al., showed that the best protection against WSSV infection occured at 14 days after the beginning of the 'vaccination'.

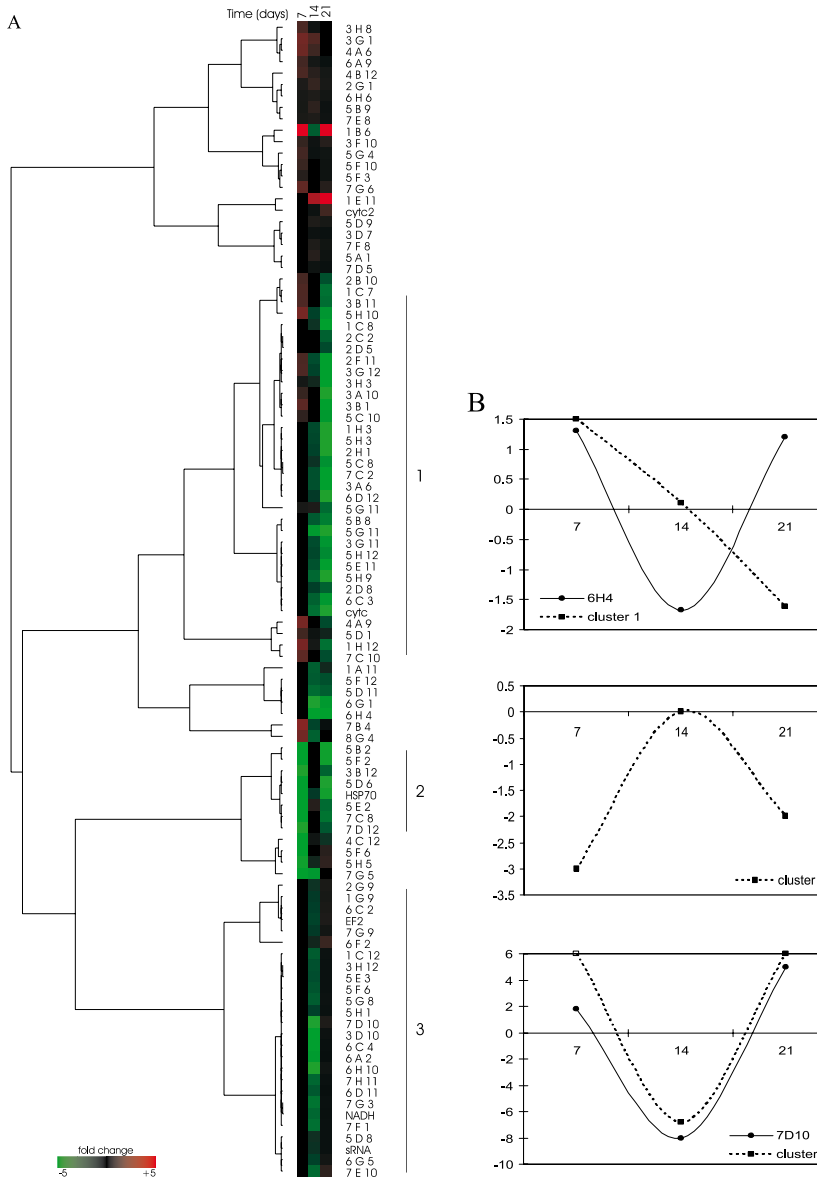

Figure 4.4 A:Hierarchical clustering (Euclidean distance clustering algorithm) of *P. monodon* gill gene expression profiles after "vaccination". Columns indicate separate time points (in days) and every row displays the expression profile of a single cDNA spotted on the microarray. The dendogram clusters the cDNAs based on the relatedness of their gene expression patterns. B: Transciption patterns of three different clusters (dashed line; left y-axis) and RT-qPCR kinetics (solid line; right y-axis).

Three major expression clusters could be determined that are profoundly different from the clusters obtained in the analysis after immersion challenge. These clusters showed downregulation upon 'vaccination'. Two genes within this cluster, 1B6 and 1E11, showed high levels of upregulation. Comparing the most profound gene expressions revealed in the infection experiment to those revealed in the 'vaccination' experiment showed 19 similar genes (table 4.2).

3G1	5B9	7F8	5F10	7B4
5F3	7D5	3D7	5D9	5G4
6A9	7E8	6H6	2G1	8G4
7G6	3B11	4B12	3F10	

Table 4.2 Genes showing differential expression in both infection and 'vaccination'.

Real-time PCR analysis performed on 5 genes clustered in two of the three clusters could confirm the data obtained from the microarray and 50% of the genes tested with RT-qPCR showed the same expression pattern as with the microarray (figure 4.4B).

Discussion

The present study describes the combined application of cDNA subtraction and microarray technologies in combination with RT-qPCR expression analysis to reveal information on changes in shrimp gene expression following infection with white spot syndrome virus.

SSH en microarray analysis

In our settings only 15% of the probes present on the microarray did reach the background threshold. It is possible that 85% of the transcripts that are picked up by SSH (Chapter 3) are not really immune response genes. Another explanation can be the detection limit of our microarray experiment, or inferior hybridisation properties of the probe spotted on the microarray (Franssen-van Hal *et al.*, 2002). We did not check whether the probes on the microarray were really expressed, because the SSH was made with oligo(dT) containing primers. Sequencing of the spotted clones could still reveal more information about the presence of poly(A)-signals on our spotted probes.

Expression analysis with microarray and RT-qPCR

Our data suggests that the kinetics of the shrimp immune response and relevant genes are regulated in, at least, four different ways. In the present study, it is not possible to describe a relation between genes involved in an immune response upon a WSSV infection and genes involved in a 'vaccination' response, since a profoundly different kinetics is occurring between these experiments. The infection experiment is monitored during the first hours post infection, while the results of the 'vaccination' experiment are evaluated at 7, 14 and 21 days after beginning of the 'vaccination'. Therefore, our evaluation does not permit such comparison as yet. However, there is some resemblance of genes both involved in an immune response and a 'vaccination' response. Genes upregulated the first 8 hours post infection, like heat shock protein 70 (HSP70), have the tendency to be downregulated after 'vaccination', suggesting a deviated normal immune response of the shrimp upon a WSSV infection. This finding underscores the importance of HSP70 in the induction of an immune defence against WSSV. The nature of this interaction remains to be elucidated.

Microarray analysis is a good tool to analyse large amounts of transcripts. However, to be able to determine differentially expressed genes that are statistically significant, especially in time-course experiments, many replicate slides are needed. As a consequence, a large amount of RNA and large numbers of experimental animals are needed.

RT-qPCR is a laborious technique for screening large numbers of different transcripts. However, only a limited amount of RNA is needed from every individual sample to carry out statistically relevant experiments. In the present study, we chose for non-statistical microarray analysis, combined with RT-qPCR analysis. The difference between expression profiles of the microarray and the RT-qPCR analysis can be caused by the fact that the microarray analyses were only repeated twice.

The fact that RT-qPCR is a more sensitive method to analyse gene expression and the absence of correspondence between RT-qPCR and microarray gene expression profiles suggests that repeating microarray analysis twice is not suitable for obtaining meaningful results from this type of experiments. Replicating microarrays and analysis of more genes by RT-qPCR can identify really differentially expressed genes.

In conclusion, the results obtained in this investigation provide insight into the previously unknown complexities of host-WSSV molecular interactions. The discovery of differential expression of genes in WSSV infected shrimp can allow the visualisation of several pathways and potential mechanisms that may play a role in WSSV pathogenesis. Identification of regulated genes in WSSV infected shrimp enabled the development of a model centered around differential gene expression profiles. This illustrates several ways in which host cells respond to infection. Gene expression changes also provided clues about the possible mechanisms involved in the development of pathological changes that are characteristic of the disease. Most importantly, the data obtained in this study identifies several genes whose mRNA is regulated on virus infection suggesting an array of hypotheses which could be tested to reveal their role in WSSV molecular pathogenesis.

This study also provides insight in 'vaccine'-host interactions. Microarray studies coupled with *in vivo* experiments obtain relevant data about the functionality of 'vaccines' in shrimp and invertebrates in general. The combination of host immune response genes and 'vaccination' can reveal the route of WSSV infection and may unravel the immune system of the giant tiger shrimp. Taken together, the present investigation demonstrates the application of a powerful approach of combining the high throughput technologies of SSH and microarray to study differential expression of genes in response to virus infection. SSH could be used for initial isolation of differentially expressed transcripts, a large-scale confirmation of which

can be accomplished very efficiently by microarray analysis. The detailed methods described herein could be potentially applied to any biological system.

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CHAPTER

5

Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon*

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Abstract

Invertebrates rely completely for their protection against pathogens on the innate immune system. This non-self-recognition is activated by microbial cell wall components with unique conserved molecular patterns. Pathogen-associated molecular patterns (PAMPs) are recognised by Pattern Recognition Receptors (PRRs). Toll and its mammalian homologs Toll-like receptors are cell-surface receptors acting as PRR and involved in the signalling pathway implicated in their immune response. Here we describe a novel partial Toll receptor gene cloned from a gill library of the giant tiger shrimp, *Penaeus monodon*, using primers based on the highly conserved Toll/IL-1R (TIR) domain. The deduced amino acid sequence of the *P. monodon* Toll (PmToll) shows 59% similarity to a Toll-related protein of *Apis mellifera*. LRRs of shrimp Toll contains no obvious PAMP-binding insertions. Phylogenetic analysis with the insect Toll family shows clustering with Toll1 and Toll5 gene products, and it is less related to Toll3 and Toll4. Furthermore, RT-qPCR shows that PmToll is constitutively expressed in gut, gill and hepatopancreas. Challenge with White Spot Syndrome Virus (WSSV) shows equal levels of expression in these organs. A role in the defense mechanism is discussed. In conclusion, shrimp possess at least one Toll receptor that might be involved in immune defense.

Introduction

The innate immune system is of crucial importance in host defence against pathogens of invertebrates. The non-self-recognising immune response cascade is triggered by receptors that recognise pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, peptidoglycans and mannans, through pattern recognition receptors (PRRs). Upon recognition these receptors activate signal transduction pathways leading to translocation of transcription factors to the nucleus and eventually control the expression of immune response genes. Depending on the pathway used the response will either be the activation of haemocytes (cellular response) or the production of antimicrobial peptides (humoral response) (Stet *et al.*, 2005). The clearest example of the cellular response is the strong phagocytic activities and encapsulation of pathogens by the hemocytes using the prophenol-oxidase (proPO) system. Granular hemocytes also produce a variety of defense molecules like clotting factors, proteinase inhibitors, lectins, and antimicrobial peptides. The activity spectra of these antimicrobial peptides are directed either against fungi (Drosomycins, Metchnikowin), Gram-positive (Defensin) or against Gram-negative (Attacins, Cecropins, Drosocin, Diptericins) bacteria (Bulet *et al.*, 1999; Hoffmann, 2003).

The recognition of infectious non-self by pattern recognition receptors in *Drosophila* occurs through two major signal pathways. Firstly, the Imd signal transduction is activated by the interaction of a putative transmembrane peptidoglycan recognition protein receptor with peptidoglycan or LPS as ligand (Gottar *et al.*, 2002; Choe *et al.*, 2002; Ramet *et al.*, 2002) and is primarily involved in defence against Gram-negative bacteria. Secondly, the Toll pathway is initiated by cleavage of Spätzle. Extracellular recognition proteins such as Peptidoglycan Recognition Proteins (PGRPs) and Gram Negative Binding Proteins (GNBPs) activate an extracellular serine protease, which cleaves the Spätzle protein. Binding of the proteolytic cleaved ligand Spätzle to the cell-surface receptor Toll leads to the activation of the intracellular signalling domain (Gobert *et al.*, 2003; Michel *et al.*, 2001).

The characteristics of Toll-like receptor proteins are extracellular amino-terminal leucine-rich repeat areas (LRR), one transmembrane domain (TM), and an

intracellular carboxyl terminal toll/interleukin-1 receptor domain (TIR), occasionally supplemented with an extracellular leucine-rich repeat C-terminal (LRR-CT). The extracellular part with its LRRs is involved in the binding of proteins like Spätzle. The intracellular part of this receptor, TIR domain, interacts with homologs of NF-κB regulators, such as MyD88 and Traf2 (Horng *et al.*, 2001; Shen *et al.*, 2001). MyD88 is an intracellular protein consisting of a TIR domain and a death domain. Traf2 is a protein that contains a TRAF domain and is homologous to a human TRAF protein. Two other proteins Tube and Pelle are involved in the next signal transduction step by activation of an unknown kinase through the kinase domain of Pelle. Eventually, this results in phophorylation of Cactus leading to its degradation and subsequent release of Dif, which is complexed with Cactus. Dif has a DNA-binding REL homology domain, which upon translocation to the nucleus acts as a transcription factor of a multitude of immune response genes (Boutros *et al.*, 2002). In *Drosophila*, the Toll pathway is important for anti-fungal, some anti-gram-positive bacterial and anti-viral responses by the synthesis of anti-microbial peptides (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002; Zambon *et al.*, 2005).
The immune system of crustaceans, including *P. monodon*, has been investigated in detail only for some response systems, such as the prophenoloxidase activating system (proPO) (Söderhäll *et al.*, 1998; Lee *et al.*, 2004) clotting (Chen *et al.*, 2005; Yeh *et al.*, 1999) and phagocytosis (Liu *et al.*, 2007).
Currently many research efforts are devoted to elucidate the immune response to white-spot syndrome virus (WSSV). This virus has become a pandemic within a relative short time-span, and is particularly virulent in *Penaeus monodon*. Given the relative close relationship between insects and crustaceans (Wilson *et al.*, 2000) it is anticipated that the major immune signalling components and effectors can be identified using the information from insects. However, this only applies for anti-bacterial and anti-fungal responses, but not for the immune response to pathogenic viruses.
Here we report the identification of a Toll homolog in the black tiger shrimp *Penaeus monodon* possibly involved in the defence against pathogens.

Materials and methods

Shrimp culture

Healthy *Penaeus monodon* shrimp were imported as postlavae from Malaysia and maintained in a recirculation system (pH 7.8-8.0, salinity of ±20 ppt, 0.3 mg/l NH_4^+, 0.1 mg/l NO_2^-, 200 mg/l NO_3^- at 28°C) at the facility "De Haar vissen" of the Wageningen University. Each shipment was tested for the presence of WSSV, Monodon baculovirus (MBV), yellow head virus (YHV), Taura syndrome virus (TSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) by PCR. Prior to each experiment, shrimp were transferred to 45- or 180-liter aquariums located at the laboratory of Virology at Wageningen University, each fitted with an individual filter system (Eheim, Fleuren & Nooijen Viskwekerij BV), heating (Schego) to 28 ± 1°C, and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand.

White Spot Syndrome Virus stock

The virus isolate used in this study originates from infected *Penaeus monodon* shrimp imported from Thailand in 1996. Crayfish *Orconectes limosus* were injected intramuscularly in the lateral area of the fourth abdominal segment with a lethal dose of WSSV using a 26-gauge needle to initiate infection. After approximately one week hemolymph was withdrawn from moribund crayfish and mixed with modified Alsever solution (Rodriguez *et al.*, 1995) as an anticoagulant. The virus particles were isolated by centrifugation at 58,500x g for 1.5 hours on a 20-45% sucrose gradient in TN (20 mM Tris, 400 mM NaCl, pH 7.4). The visible virus band was collected, two times diluted in TN and the virus particles were subsequently sedimented by centrifugation at 26,000x g at 4°C for 50 minutes. The virus pellet was resuspended in PBS (pH 7.4) and the virus integrity was checked by electron microscopy. The virus stock was stored at -80°C until use.

In vivo injection

Since no crustacean cell lines are available, the WSSV stock was titered by *in vivo* infection experiments as described by van Hulten *et al.* (van Hulten *et al.*, 2001). *P. monodon* of approximately 15 gram (n=3) were injected intramuscularly with 10 µl virus solution in 330 mM NaCl in the 4th or 5th tail segment with a 26 gauge needle. The shrimps were subsequently cultured for up to 24 hours until use in RNA extraction.

RNA extraction and cDNA synthesis

Organs for RNA isolation were harvested and snap-frozen in liquid nitrogen and stored at -80°C. Tissues were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) followed by phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water (Chomczynski *et al.*, 1987). Concentrations were measured by spectrophotometry at 260 nm (NanoDrop Technologies) and integrity was checked on a 1% agarose gel. RNA was stored at -80°C for future use. cDNA was made by adding 1 µl 10× DNase I reaction buffer and 1 µl DNase I (Invitrogen Corporation) to 1 µg total RNA in a total volume of 10 µl and incubated at room temperature for 15 min. DNase I was inactivated by adding 25 mM EDTA. Synthesis of cDNA was adapted from Invitrogen's Superscript™ III First Strand Synthesis Systems for RT-PCR Systems. DNase I-treated RNA samples were mixed with 5× first strand buffer, 300 ng random primers, 10 mM dNTPs, 0.1 M DTT, 10 U/µl and RNase inhibitor, followed by incubation at RT for 10 min and at 37ºC for 2 min. One µl Superscript III Reverse Transcriptase (Invitrogen Corporation) was added and the mixture was incubated at 37ºC for 50 min. The reaction was stopped at 70ºC for 15 min. A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted 1:50 in nuclease-free water before use as template in real-time PCR experiments.

Genomic organisation

Genomic DNA was isolated from tissue of the abdomen according to the "mouse

tail" Promega Wizard® Genomic DNA Purification Kit manual (Promega Corporation). Genomic fragments were amplified by standard PCR as described in section 2.6, using primers (Table 5.1) based on the partial cDNA sequence.

Table 5.1 Primer sequences used to amplify corresponding genomic fragments and expected sizes.

primer	sequence (5' → 3')	in exon	Intron length	Total genomic length
PmTIR10F	CTTATCAGCCACTACTCTTGACTACC	1	238	470
PmTIR15R	GAGAAGGAAAACTGTGATGAGCACGATT	2		
PmTIR31F	CCTTCTCCTGTTTGCTGTTC	2	152	283
PmTIR28R	GAGAATAGCTGATGAAGGCATC	3		
PmTIR33F	TGAGCTTCTATAAATACAAGCAAG	3	207	561
PmTIR17R	ACTATAATCCTGTTAGTTCTGTCCTG	4		
PmTIR20F	ATCTTGCAGAGTGTAGAGGACAGCC	4	303	477
PmTIR25R	GATGTACAGCCGTAACTTCTCG	5		
PmTIR22F	CAACCTGACTTTCTTATCAGCC	1	900	1644
PmTIR25R	GATGTACAGCCGTAACTTCTCG	5		

Cloning and sequencing

Approximately 10 ng of cDNA was used in a 50 µl PCR reaction carried out with degenerate primers: 5'-GAYAARGAYAARAARTTYGAYGC-3' and 5'-RTCCCARAACCANGGRTCNCCCCA-3' (Yamagata *et al.*, 1994). PCR reactions were performed with 0.2 µl Taq DNA polymerase (Goldstar, Eurogentec), 1.5 mM $MgCl_2$, 200 µM dNTPs and 400 nM of each primer. Cycling conditions were 94°C for 5 min, 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min for 35 cycles and 72°C for 7 min, using the GeneAmp PCR system 9700 (Applied Biosystems). PCR products were ligated in pGEM-T easy vector and used in transformation of competent JM-109 cells (Promega Corporation) according to the standard protocol. Products were sequenced, using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems), and analysed using an ABI 377 or 3730 DNA analyzer.

Nucleotide and amino acid sequence data were analysed for identity to other sequences using the GenBank database (Benson *et al.*, 2006). Searches for similar sequences within the database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997).

Construction of cDNA library

The cDNA for the library was prepared using the Smart™ cDNA library construction system (Clontech). Briefly, 1 µl containing 600 ng total gill RNA was transcribed into single strand cDNA using a Smart II A oligonucleotide, a 3' SMART CDS Primer II A (oligo-d$(T)_{30}$), and PowerScript™ reverse transcriptase (Clontech). The single strand cDNA was amplified in 17 cycles of PCR (95 °C for 15 s, 68 °C for 6 min) according to the manufacture's protocol (Clontech). The amplified cDNA was ligated into the vector pGEM-T easy.

Relative quantification using Real-time Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed using RotorGene™ (Corbett Life Science) with Brilliant® SYBR® Green QPCR (Stratagene) as detection chemistry. PCR reactions (14 µl) were performed with 2x Master SYBR Green I mix and 300 nM of each primer in a 100 µl tube. Cycling conditions were: denaturation at 95°C for 15 min, 40 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec and a final hold at 60°C for 1 min. Subsequently a melting curve was acquired with continuous fluorescence acquisition from 60°C to 99°C with a rate of 1°C/5 sec. *Penaeus monodon* specific primer (5'-GGAGTGAGCTGCCTTGAACT-3' and 5'-AGCCGTCGAACTATTGTGGT-3') were used resulting in a 81 bp TIR domain fragment. A 82 bp elongation factor 2 fragment amplified with primers based on a tiger shrimp EF2 fragment (Lehnert *et al.*, 1999) (GenBank: AI253924: 5'-GATGGACAAGCGGAGGAA-3'; 5'- AAGGGAGTCGGTCAGGGTA-3') was used as a reference gene.

Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the proportion of amino acid difference (p-distance) by the neighbour-joining and minimum evolution methods

with complete deletion of gaps (Saitou *et al.*, 1987) using MEGA software (Kumar *et al.*, 2004). Reliability of the tree obtained was assessed by bootstrapping, using 1000 bootstrap replications (Felsenstein, 1985). The following proteins were used in the alignment: Ag (*Anopheles gambiae*), Dm (*Drosophila melanogaster*), Am (*Apis mellifera*), Pm (*Penaeus monodon*), Ce (*Caenorhabditis elegans*), Tt (*Tachypleus tridentatus*) and Bm (*Bombyx mori*).
AgToll (AAL37901), AgToll6 (AAL37902), AgTrex (AAL37904), AmToll (XP_396158), Bm18W (BAB85498), CeToll (AAK37544), DmToll (AAQ64937), DmToll3 (AAF86229), DmToll4 (AAF86228), DmToll5 (AAF86227), DmToll6 (AAF86226), DmToll7 (NP_523797), DmToll8 (AAM49920), Dm18W (AAA79208), TtToll (BAD12073).

Results

Partial cDNA sequence of the Toll gene from P. monodon

A pair of degenerate primers based on the amino acid sequence of the Toll/ interleukin-1 receptor domain of *D. melanogaster* (Yamagata *et al.*, 1994) were used to amplify Toll-related genes from *P. monodon* gill cDNA libraries. A major band of 400 bp was obtained. Based on this sequence gene specific primers were developed and used in a 5'-RACE resulting in a sequence of in total 744 bp (Figure 5.1). Sequence analysis of this PCR product and subsequently a tBlastX (Altschul *et al.*, 1997) analysis revealed a 59% amino acid identity with *Apis mellifera* predicted Toll-related protein (GenBank XP_396158).
Different prediction programs, NCBI conserved domain search (LLR, LLR-CT) (Marchler-Bauer *et al.*, 2004), PFAM (TIR domain) (Bateman *et al.*, 2004) and PRED-TMR (transmembrane domain) (Pasquier *et al.*, 1999), were used to analyse *P. monodon* Toll.

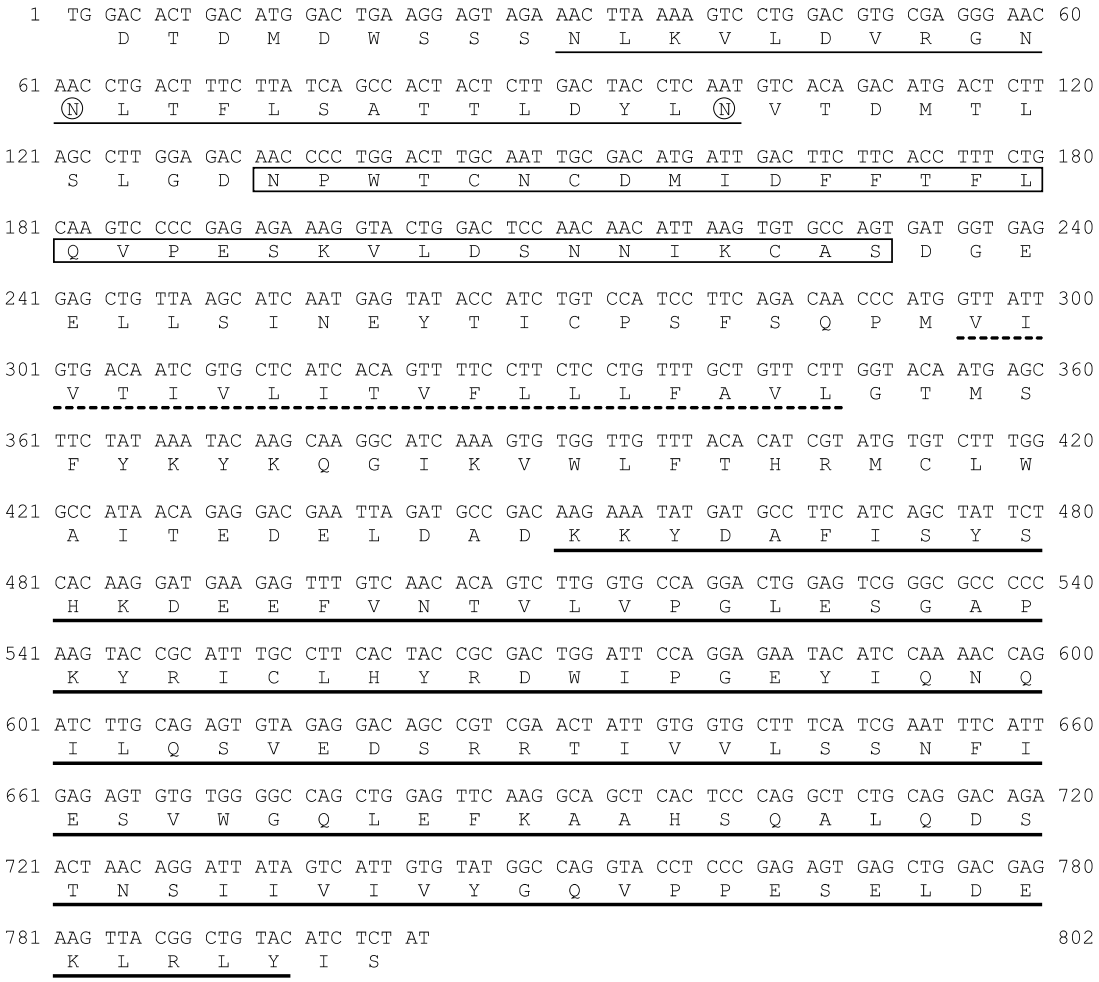

Figure 5.1 Partial nucleotide sequence and deduced amino acid sequence of PmToll. Detected domains: leucine rich repeat (solid line), leucine rich repeat C-terminal (boxed), transmembrane (dashed line) and Toll/Interleukin-1 receptor (bold solid line). Potential N-linked glycosylation sites in the extracellular domain are circled.

Examination of *P. monodon*, named PmToll showed, besides the highly conserved cytoplasmic TIR domain (nt 452-793), a putative transmembrane domain (nt 294-348) and extracellular a leucine-rich repeat C-terminal (LRR-CT) domain (nt 132-229) and a domain composed of leucine-rich repeats (nt 33-101) (Figure 5.1 and 5.2).

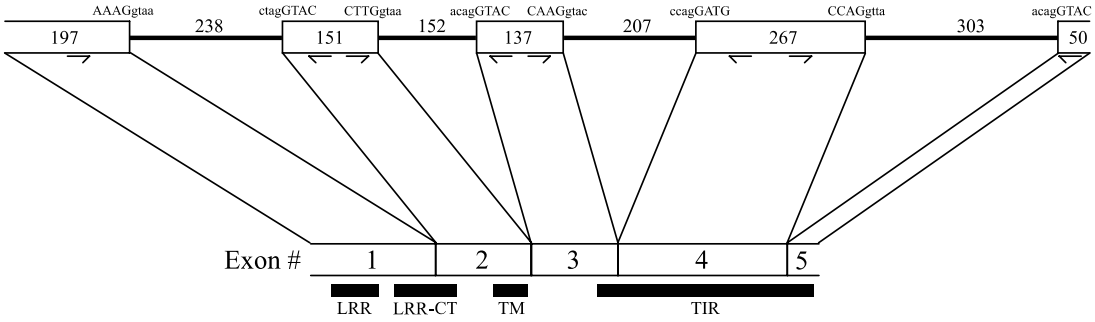

Figure 5.2 Genomic organization of the coding region of PmToll. Boxes represent exons and are drawn to scale. Exon lengths are indicated in nucleotides. Nucleotides surrounding each splice site are displayed, coding residues are represented by capitals.

Genomic organisation

Primers were designed based on the cDNA sequence obtained and were used to amplify the corresponding genomic fragment from *P. monodon* genomic DNA. The resulting fragments were cloned and sequenced. Comparison of the genomic fragment with the coding region fragment showed that PmToll is the splicing product of 5 exons and 4 introns (Figure 5.2 and 5.3) (Wheelan *et al.*, 2001).

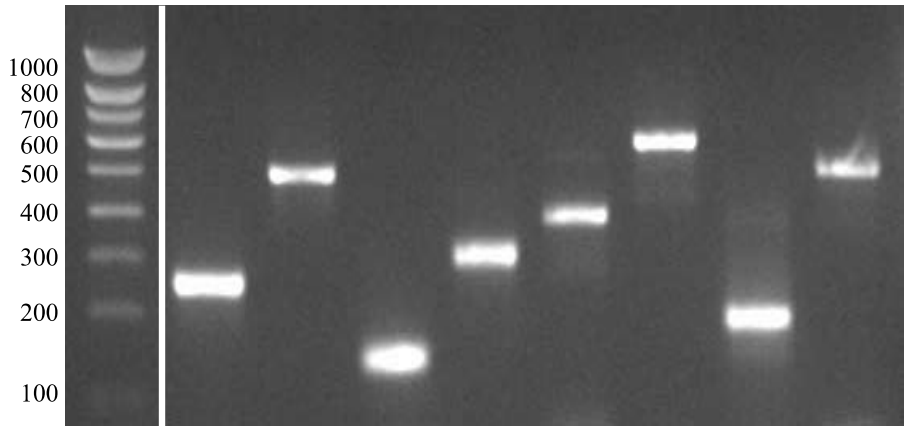

Figure 5.3 Genomic organization of the coding region of PmToll. Lanes 1, 3, 5 and 7 represent PCR products on cDNA and lanes 2 ,4 ,6 and 8 represent PCR products on genomic DNA all with primers flanking exons 1, 2, 3, and 4, respectively.

All tiger shrimp introns contain well-recognisable 5’ donor and 3’ acceptor (ag)

splice sites. LRR is located in exon 1, LRR-CT in exon 1 and 2, transmembrane domain also in exon 2 and the Toll/IL-1 receptor domain is scattered over exons 3, 4 and 5.

To address the relationship between *P. monodon* Toll and its invertebrates orthologues, phylogenetic trees were constructed using the neighbour-joining (data not shown) and minimum evolution method based on the LRR-TM-TIR domains (Figure 5.4) (Kumar *et al.*, 2004).

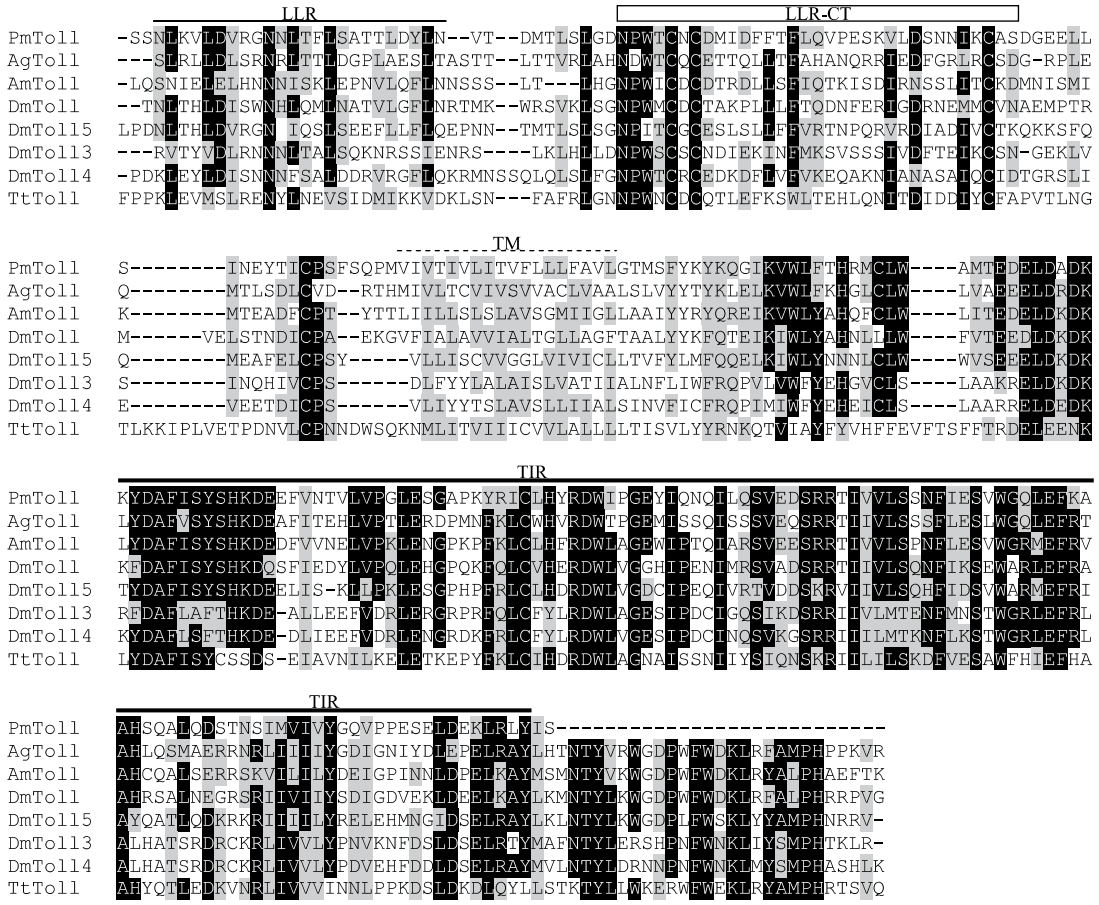

Figure 5.4 ClustalX alignment of LRR-TM-TIR domains. Ag (*Anopheles gambiae*), Dm (*Drosophila melanogaster*), Am (*Apis mellifera*), Pm (*Penaeus monodon*) and Tt (*Tachypleus tridentatus*) Accession numbers: DmToll: AAQ64937, DmToll5: AAF86227, AmToll: XP_396158, AgToll: AAL37901, TtToll: BAD12073, DmToll3: AAF86229, DmToll4: AAF86228.

Both the pair wise-deletion and the complete-deletion of gaps option was used to compute distances and showed a comparable phylogenetic tree. The overall topology of the tree showed clustering with *A. gambiae* Toll, *D. melanogaster* Toll and Toll5, and *A. mellifera* Toll whereas the marine *T. tridentatus* Toll seems to be more ancient. (Figure 5.5).

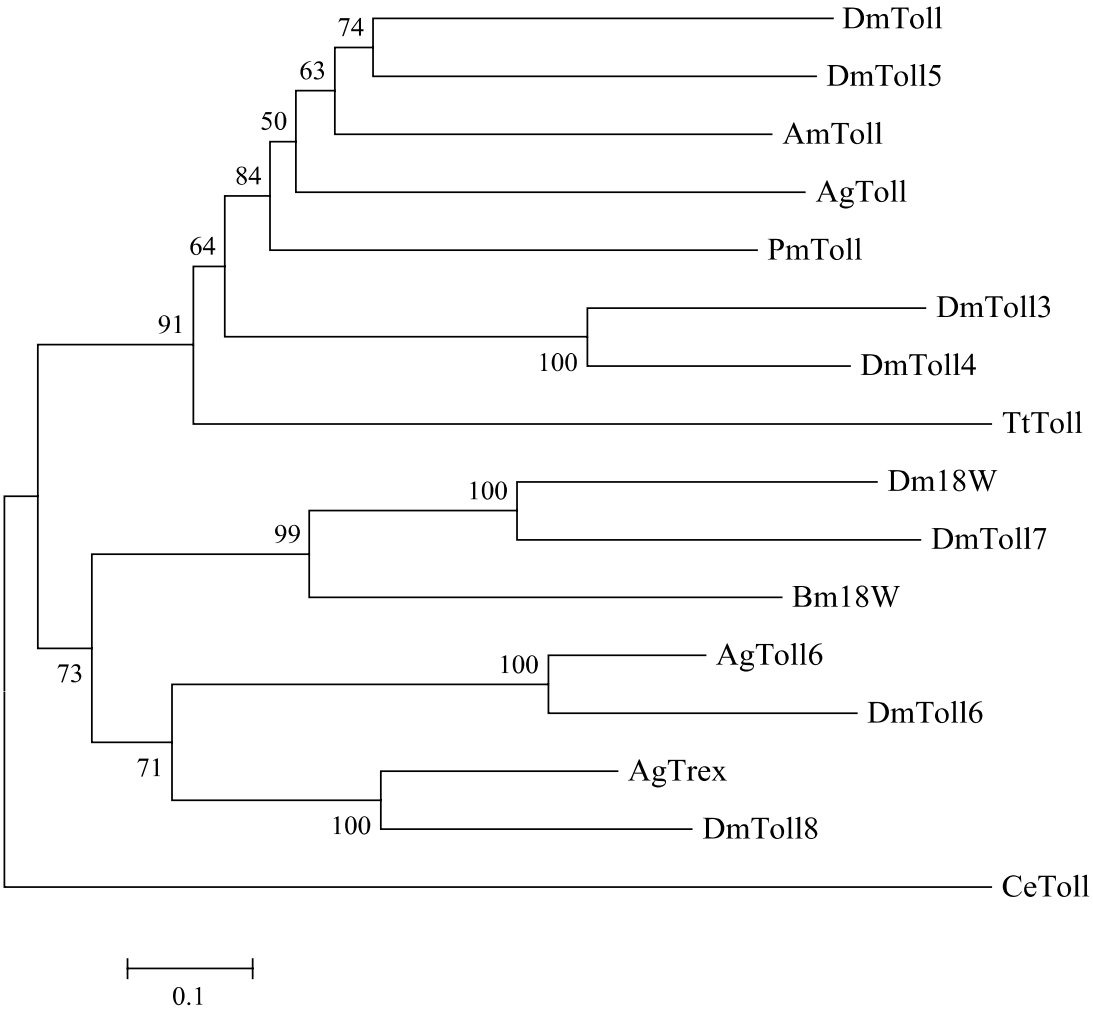

Figure 5.3 Neighbour-joining tree of the leucine rich repeat, transmembrane and Toll/interleukin-1 receptor (LRR-TM-TIR) domains. Numbers at branch nodes represent bootstrap confidence levels of 1000 bootstrap replications. Ag (*Anopheles gambiae*), Dm (*Drosophila melanogaster*), Am (*Apis mellifera*), Pm (*Penaeus monodon*), Ce (*Caenorhabditis elegans*), Tt (*Tachypleus tridentatus*) and Bm (*Bombyx mori*) Accession numbers: AgToll6: AAL37902, DmToll6: AAF86226, AgTrex: AAL37904, DmToll8: AAM49920, DmToll: AAQ64937, DmToll5: AAF86227, AmToll: XP_396158, AgToll: AAL37901, CeToll: AAK37544, TtToll: BAD12073, DmToll3: AAF86229, AeToll1: AAM97775, Bm18W: BAB85498, Dm18W: AAA79208, DmToll4: AAF86228, DmToll7: NP_523797.

Expression

The expression of PmToll was studied in the gut, gill and hepatopancreas of (unchallenged and WSSV-challenged) shrimp. No significant difference in expression levels in control shrimp was observed between the hepatopancreas, gut and gill (figure 5.6) nor in hemocytes and the compound eye (data not shown). Analysis of expression levels between the same organs of WSSV-challenged shrimp showed no significant difference.

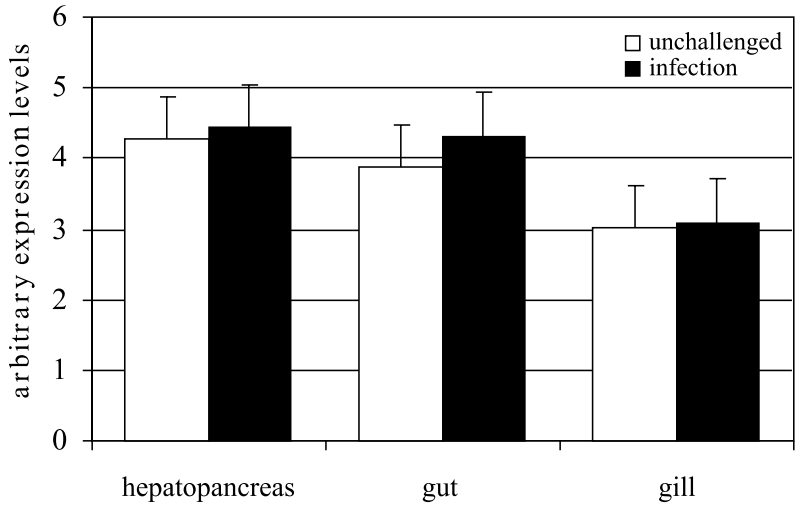

Figure 5.6 PmToll gene expression in hepatopancreas, gut and gill of unchallenged and WSSV challenged shrimp.

Expression levels of PmToll in the hepatopancreas, gut and gills of WSSV-challenged shrimp are comparable to the controls. Relative to the housekeeping gene the expression of PmToll is high. Analysis of the expression of PmToll during the first 24 h.p.i. in challenged, as well as in control shrimp, did not reveal any differential regulation of expression of PmToll.

Discussion

Here we describe, for the first time, the identification of a putative Toll receptor in tiger shrimp. Searches with BLAST for similar sequences within the GenBank database revealed significant hits with all known Toll genes of the phylum Arthropoda. Highest similarity (59% identity) was found with *Apis* Toll1. Other insect Toll1/5 genes showed high similarity (45-55% identity). In transfected cells DmToll1 and DmToll5 are drosomycin promotor activating receptors (Tauszig *et al.*, 2000). This similarity suggests that PmToll regulates the synthesis of proteins involved in the innate immunity in tiger shrimp.

Comparison of the PmToll functional domains with other Toll family members shows that PmToll consists of typical Toll receptor functional domains. That is, leucine-rich repeats, a C-flanking domain (LRR-CT), transmembrane domain and a cytoplasmic TIR domain. The fact that PmToll LRR domain is similar to other LRR domains of the Toll family suggests that PmToll can potentially recognise similar extracellular ligands. Insertions at position 10 or 15 in LRR arrays are widely considered to allow TLRs to function as PAMP binding receptor, not only in insects, but also in other arthropods like the horseshoe crab (Inamori *et al.*, 2004; Arbour *et al.*, 2000). Because only one LRR was detected without insertions no conclusions can be drawn as yet about the function of PmToll in respect to the binding of PAMPs. In the fruit fly a protein, Spätzle, is so far the only ligand for this receptor. The absence of insertions in the LRR and the different ligand in *Drosophila* together with the evolutionary distance between vertebrates and inverterbrates (Roach *et al.*, 2005), suggests that PmToll does not function as a pattern recognition receptor. The high degree of similarity of the TIR domain with respect to the Toll family indicates that the same signal transduction cascades are involved.

In the PmToll TIR domain two introns are detected whilst in most TIR domains of Toll and TLR no introns are found. In the Toll/TLR genes that do contain introns the locations and phases are different, which suggests that during the evolution the TIR domain has been subjected to many events of intron invasion (Kanzok *et al.*, 2004; Rogers, 1990). So PmToll, just like other invertebrate Toll and vertebrate TLR genes, was subject to intron invasion.

To study the phylogenetic relationship to known Toll sequences, a neighbour-

joining tree was constructed (Figure 5.5), from which it can be concluded that the tiger shrimp Toll sequence described in this study is closely related to Toll1 and Toll5. The hypothesis that an ancestral Toll1/5 appeared after the split between Hymenoptera and Coleoptera/Lepidoptera/Diptera by Kanzok *et al.* (Kanzok *et al.*, 2004) is unlikely in the view of our results. This gives rise to the conclusion that the common ancestor of the Toll gene is before the split between insects and crustaceans.

Innate immunity is the first-line defense of multicellular organisms. Invertebrates depend completely on its innate immune system. Selective pressures have made innate responses a rapidly reacting system. The observation that there is no regulation during the first 24 h.p.i. suggests that PmToll is not regulated in time during a viral challenge, not only in the first 24 hours, but also, due to the rapidity of the innate immune system, not in a later state of infection.

The presence of PmToll gene expression in different tissues such as gill, gut, hepatopancreas, hemocytes and the compound eye, suggests that all tested organs have the possibility to react upon an invasion of a pathogen. The observation that BmToll gene transcripts are expressed in different organs (Imamura *et al.*, 2002) supports the theory that PmToll is at a considerable genetic distance from BmToll. The absence of regulation in different organs upon a viral challenge suggests that PmToll is not directly involved in the defense against WSSV. However, the Toll pathway can be regulated at a higher level (PGRPs and GNBPs; extra cellular) and regulation of PmToll might not be necessary. Despite this absence of regulation we do not exclude a potential involvement of PmToll in the immune response to WSSV. Currently we are investigating the effect of bacterial challenges on the regulation of PmToll.

Acknowledgements

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CHAPTER

6

General discussion

General discussion

Aim of the thesis

Since the first incidence with mass mortalities in shrimp culture, WSSV has been identified as one of the main causative agents and WSSV is by now characterised in some detail. Although >90% of WSSV genes are still unknown, there is some information about WSSVs genetic organisation (see van Hulten *et al.*, 2001; Marks, 2005b). WSSV is a dsDNA virus and is the largest animal virus known to date, infecting shrimp and other crustaceans. It is a sole member of the virus family *Nimaviridae* (Vlak *et al.*, 2005). To date, three different WSSV isolates have been sequenced. Differences in virulence between these isolates have been studied and combined with comparison of the presumably complete genome sequences (van Hulten *et al.*, 2001; Marks *et al.*, 2005a). This comparison revealed a 19 kb genome difference between the various isolates, but the molecular basis for the difference in virulence is still enigmatic. The genetic basis for this difference could be based on the presence of a, as yet unknown, gene in this 19 kb fragment or the presence of regulatory sequences influencing the rate of transcription of other genes which ultimately result in a differential degree of virulence.

Sporadically, data becomes available about the molecular and cellular characteristics of the defence system of the giant tiger shrimp, or other crustaceans, upon an infection with a pathogen. The available information thus far largely concerns defence against infections with bacteria and parasites. Characteristics of defence of shrimp against viral infections, however, are largely unknown, but increasing (van de Braak *et al.*, 2002; Witteveldt, 2006).

In this thesis, we have explored the immune response of *P. monodon* upon a WSSV infection in further detail. We have focussed on the route of infection *via* the gills by an immunohistochemical and electron microscopical analysis (chapter 2) and on early immune response genes activated in response to the infection (chapters 3 and

4). Moreover, based on the paradigms of innate immune defence against infections a possible involvement of Toll receptors was predicted (chapter 1). Since this has not been described before for *P. monodon*, we characterised a Toll candidate molecule and analysed its role in WSSV infection (chapter 5).

General features of innate immune defence against infection in P. monodon

Invertebrates, including crustaceans already exist for over 300 million years, despite the lack of a true adaptive immune response. Since crustaceans rely completely on their innate immune defence, they are considered less sophisticated (Witteveldt, 2006). Nevertheless, this innate immune defence is 'sophisticated' enough to keep this species alive for such a long period in evolution.

The innate immune system in general has been ignored for decades, but is obviously very important: not only for the rapid initial immune defence but also for initiating antigen-specific adaptive immunity (Janeway *et al.*, 2001). The adaptive immune system is a very slow reacting mechanism, albeit being antigen-specific at the epitope level and able to greatly amplify the magnitude of the immune defence. Hence, the innate immune response plays an important and decisive role in the initiation of protective immunity upon vaccination. Recent progress has suggested an important role for Toll or TLR in the functional connection between innate and adaptive immunity (chapter 1). According to the central dogma in immunology, invertebrates, lacking the adaptive arm of the immune system, cannot be vaccinated against potentially lethal infections, including infection with WSSV. Recent studies however, have claimed that shrimp, like *P. monodon*, can mount responses against WSSV, protecting vaccinated individuals against lethal doses of the virus (Venegas *et al.*, 2000; Witteveldt *et al.*, 2004; Witteveldt *et al.*, 2006).

The fact that the WSSV surface protein VP28 induce an immune response suggests indeed a non-self recognition system. Without experiments showing a specific immunity only against WSSV, no conclusions can be drawn on the mechanism of

this ‘vaccination’. Moreover, specific immunity does not necessarily mean memory (Witteveldt, 2006).

All of this knowledge suggests that instead of looking for systems homologous to the vertebrate acquired immune system, we have to look further into the innate immune system. Considering the current knowledge, it is clear that the immune system of invertebrates, the innate immune system, is capable of much more defence responses than is always assumed and more research is needed to elucidate all the existing possibilities.

Evolutionary model of WSSV infection and shrimp innate immune defence

WSSV could be an existing virus with a long evolutionary history, possibly even with a different host specificity than shrimp. Due to a mutation or a stacking of several mutations, the modified virus could at some point in history now infect shrimp species and cause a lethal disease. In the beginning, high virulence and mortality are essential for viral spreading. The innate immune defence of the shrimp might be effective in killing many, but not all viral particles. In the initial phase the virus would become continuously more lethal as the pandemic spreads worldwide. Due to natural selection and ongoing variation and mutation, the virus later on might become less lethal when shrimp cultures are better protected due to the use of selection of more resistant shrimp phenotypes, potential use of medication (e.g. antibiotics) or by non-specific enhancement of immune function through feeding or altered housing conditions. New variants of the virus that appear less virulent and/or lethal ensure continuous spreading of the virus under such conditions. Such mechanisms have been described for the human immunodeficiency virus, but can be general to many different, if not all, viruses (Goudsmit, 1997; Jones, 2001). However, such conditions have not been met in shrimp culture as yet. Thus, the virus in its current state might still be very lethal to infected shrimp and the immune defence system is able to recognise the virus. The innate immune response seems

thus far not to be efficient enough to efficiently eliminate virus-infected cells.

Histological analysis of WSSV infection in P. monodon

Studies with *in situ* hybridisations (ISH) using viral specific DNA probes suggest WSSV infection of midgut epithelium and haemocytes (van de Braak *et al.*, 2001; Martin *et al.*, 2003). Although ISH has a higher specificity compared to specific antibodies, the higher background of these DNA probes makes it more difficult to detect the exact position of the virus within the cell. In this study (chapter 2), a clear uptake of viral-like particles was observed in the midgut. Despite VP28 immunoreactivity in the SNV in epithelial cells. WSSV infected cells were not observed, as was suggested by others (Lo *et al.*, 1997; Wang *et al.*, 1999). In the gills, however, a massive infection can be observed at 72 hrs post infection as evidenced by large numbers of viral particles within and between cells. This includes the presence of resident and infiltrating haemocytes. The role of haemocytes in WSSV infection is still not clear, as described above. In contrast to the present study, there are reports that haemocytes can be infected or can be WSSV positive (Martin *et al.*, 2003). These haemocytes could be positive because they remove infected and/or dead cells, while not being able to actively kill the virus infected host cells (as described above). On the other hand, it is equally possible that haemocytes occasionally become infected by WSSV.

The fact that an epithelial layer is only present in the midgut and not in the gills has an important consequence for the open circulation of haemolymph in invertebrates. The observation that during the first days after infection the epithelium in the midgut is not infected, suggests that the epithelial cells are involved in preventing or spreading of infection. The absence of such an epithelial layer in the gills, makes this organ extra susceptible for infection. The influx of haemocytes in the gills suggests the presence of a (chemo-attractive) danger signal released by infected cells, but

the absence of encapsulation of infected cells shows the lack of immune reactivity (e.g. killing) of infiltrating and resident haemocytes towards virus infected cells. In the midgut, a clear degranulation of haemocytes can be observed. We interpret this as evidence that the midgut is reacting to the virus, while such a response is absent in the gills. This despite the fact that the virus is present in the gills and even could induce a chemotactic signal for infiltrating haemocytes.
Experiments with oral infection by WSSV infected food definitively showed a WSSV related mortality. Although this route of infection is not comparable to immersion infection applied here and the observations of WSSV infected cells in the posterior stomach, it is still possible that infection occers through the gills by infected tissue of cannibilised animals piling up in the gills.

Microarray generation and application in the analysis of infection
The rapid development of DNA sequencing technology has facilitated and enabled analyses of ESTs (expressed sequence tags) (Adams *et al.*, 1991; Marra *et al.*, 1998). The main disadvantage of this random sequencing is the presence of a large amount of housekeeping genes and a high redundantce of sequence information caused by the fact that many abundantly expressed genes are highly represented (Lehnert *et al.*, 1999). With this method it may thus difficult and laborious to find genes that are specifically involved in the defence response against infection and may be in low abundance. Other tools to identify differentially expressed transcripts between two mRNA populations are: differential display (Liang *et al.*, 1992; Sokolov *et al.*, 1994), representational difference analysis (Lisitsyn *et al.*, 1993), enzymatic degradation subtraction (EDS) (Zeng *et al.*, 1994), techniques involving physical removal of common sequences (Akopian *et al.*, 1995), serial analysis of gene expression (Velculescu *et al.*, 1995), linker capture subtraction (Yang *et al.*, 1996) and differential analysis of library expression (DazLE) (Li *et al.*, 2004).

The technique of suppressive-subtractive hybridisation (SSH) has been shown to be effective in isolating, normalising and enriching differentially expressed genes in a number of immune responses in several different animal species (Diatchenko *et al.*, 1996; Pan *et al.*, 2005; Dr. B. Jordan, personal comm.). The resulting data, however, show often inconstant duplo results (Dr. C. Waalwijk, PRI, personel comm.) or have a clear need to repetitive SSH in order to reveal useful results (Fujiki *et al.*, 2000). A SSH approach coupled with microarray analysis applied in the present study (chapter 4) has been used successfully before (Villaret *et al.*, 2000; Mercke *et al.*, 2004; Cottee *et al.*, 2006). Careful analysis of SSH clones with the proper controls (as outlined in chapter 3) can lead to the isolation of genes that are genuinely related to the treatment. However, as SSH analysis comprises a PCR amplification step, care has to be taken in order to prevent a preferential amplification of short gene fragments.

Worldwide, cDNA microarrays have become a common tool to analyse gene expression profiles in response to stimulation. Generally, these analyses are used for genome-wide screening or for screening large numbers of immune response genes spotted on dedicated microarrays. Compared to Northern blot, RT-PCR, and Dot blot assays, microarray analysis has advantages. A large number of genes (> 10.000) can be analysed simultaneously and multiple dyes can be used on the same microarray slide at the same time (Murphy, 2002). Main disadvantages of microarray analysis are its limited sensitivity compared to e.g. RT-qPCR and the inability to examine expression patterns of alternatively or multiple spliced mRNA (Paulose-Murphy *et al.*, 2001; Murphy, 2002). The cDNA microarray system is rather noisy at the individual gene level. The determination of differentially expressed genes between two treatments and at the same time have the assurance that the determinations are not false positives, needs replication of microarray analysis (Lee *et al.*, 2000). Different forms of replication to be used in an experiment affect the precision and

impacts on the generalisability of the results obtained. In many cases the source of RNA affects the amount of RNA. In these cases it can be necessary to reduce the number of replicate slides, use of a different type of replication or even pool mRNA (Ball *et al.*, 2002). When only a limited amount of mRNA is available, microarray analysis could be used for initial identification of relevant genes, but not for statistical analysis of e.g. expression studies.

Toll receptor and WSSV-specific defence in P. monodon

The invertebrate host defence consists of both humoral and cellular components (Hoffmann, 2003). The cellular response is mediated by phagocytic cells, like haemocytes (chapter 2). An essential feature of the humoral response is the production of antimicrobial peptides and secretion in the haemolymph (Bulet *et al.*, 1999). The expression of these genes is controlled by at least two different pathways, the Imd and the Toll pathway (Khush *et al.*, 2001; Hoffmann, 2003; chapter 1). Activation of the Toll pathway upon infection is dependent on the cleavage of Spätzle, into an extracellular protein. Subsequently, soluble Spätzle is a ligand for the Toll receptor (chapter 1). The cleavage is controlled by activated circulating recognition proteins (e.g. PGRP-SA and GNBP1) in the haemolymph of *Drosophila*, which play an important role in activation of the Toll signaling pathway (Michel *et al.*, 2001; Gobert *et al.*, 2003; chapter 1). HSP70 was suggested to be an important ligand for TLR 2 and 4, which underscores the importance of such heat shock proteins as early immune defence factors. Indeed, in our microarray analysis (chapter 4), we found HSP70 as one of the differentially expressed genes upon WSSV infection in the gills of shrimp. Moreover, the same gene was upregulated in response to oral exposure with the putative vaccine, recombinant VP28 (Figure 4.3). These findings may show that HSP70 is critically involved in both types of immune response and underscores the relevance of this family of proteins in the induction of

rapid innate immune defence. Indeed, HSP70 has been shown to induce the release of proinflammatory cytokines from phagocytic cells (Tsan *et al.*, 2004).
A new Toll receptor, PmToll, was partially cloned, sequenced and characterised for the first time in a crustacean, *P. monodon*. The sequence obtained is not yet full length, despite the fact that we have tried multiple times by 3' RACE to obtain such a full sequence. Whether this was due to an excessive length of the transcript, peculiarities in secondary or tertiary structure of the transcript or technical problems, we do not know. The sequence obtained does contain the TIR domain and, importantly, also a TM and several extracellular LRR parts and is therefore a genuine Toll receptor. Other studies have suggested the presence of SARM orthologs in invertebrates and their role in inhibiting and/or killing invading pathogens (Couillault *et al.*, 2004). These orthologs are characterised by the presence of a TIR domain protein, encoded by the *tir-1* gene. We consider PmToll a distinctly different gene encoding a protein with a different function. Nevertheless, our microarray analysis revealed also a differential upregulation of a ATP synthase gene. The same gene has been implicated in innate defence against invading pathogens in invertebrates like *C. elegans* (Couillault *et al.*, 2004). Recently, cDNA microarray analysis was performed on WSSV infected *F. chinensis* shrimp (Wang *et al.*, 2006). Also, this study revealed differential regulation of HSP70 and ATP synthase in response to infection. The use of different shrimp species, different infection routes and analysis of different organs makes comparison of the results very difficult. Further work has to be awaited in order to arrive at more general conclusions regarding the molecular basis of the innate immune response to WSSV infection in shrimp.

Conclusions

Collectively, results from this thesis show that WSSV infection modulates gene

expression of several genes that are implicated in early recognition and subsequent downstream signalling through transduction pathways leading to the induction of an innate immune defence response in shrimp. This defence comprises several candidate genes, like Toll genes, that are known from other invertebrate species and challenges with different pathogens. However, Toll expression was not affected upon infection or 'vaccination' (4 h.p.i.). Moreover, our studies show the involvement of a similar set of genes in the induction of a putative protective vaccination response in shrimp. Further work has to reveal the underlying mechanisms of the innate immune response and the regulation thereof in WSSV infected *P. monodon*.

We found a significant upregulation of several apparently important genes upon WSSV infection and a downregulation of the same genes upon vaccination. We hypothesized that the vaccine does not induce a new type of immune response, but rather corrects a potentially harmful defence reaction of the infected shrimp. By downregulating this defence response the shrimp might now survive this potentially lethal infection. Normally the shrimp will respond to infection by the infiltration of haemocytes into infected organs, like the gills, that may harm the health status of the individual. Rather than inducing a protective immune response, shrimp seem to respond to infection by clearing cells that were killed by the virus. This activity of the haemocytes does not prevent the spreading of the infection throughout the animal which results in a lethal outcome. Vaccination could downregulate genes important in virus recognition or binding, induction of chemotactic signals for haemocytes, improved killing of the virus and/or viral infected cells, *et cetera* resulting in increased survival of the infected shrimp. Further challenge studies of vaccinated shrimp have to provide the proper answers to this hypothesis.

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Summary

White spot syndrome virus (WSSV) is the most important viral pathogen of cultured penaeid shrimp worldwide. Since the initial discovery of the virus in Taiwan in 1992, it has spread to shrimp farming regions in Southeast Asia, the Americas, Europe and the Middle East causing major economic losses. The virus has a wide host range among crustaceans and induces distinctive clinical signs (white spots) on the inner surface of the exoskeleton of penaeid shrimps. Limited data is available about the immune response genes of *P. monodon* upon a WSSV infection. This thesis describes the results of our study into the generation of tools, like the generation of a dedicated microarray enabling the analysis of induction and regulation of (innate) immune defence genes in the host that are activated upon infection. Moreover, a putative vaccination strategy to protect shrimp against lethal WSSV infections has been developed previously. We have also analysed the induction of protective vaccination for induction and regulation of gene expression using this microarray.

The first focus had been on the haemocyte response of the shrimp upon an immersion infection (chapter 2). Immunocytochemistry and electron microscopy has been used to study haemocyte behaviour in gills and midgut up to 3 days after WSSV immersion infection. Using a mouse haemocyte specific monoclonal antibody (WSH8) and a rabbit VP28 polyclonal antibody, double immunoreactivity could be observed. Differential haemocyte characteristics in the gills and the midgut of *P. monodon* were determined. An invasion of haemocytes in the gills was observed in *Penaeus monodon* upon WSSV-infection, possibly caused by the adherence of haemocytes to the haemolymph vessels. Although many infected cells were found in the gills, haemocytes were not WSSV-infected in this organ. Gills appear to be an important site of haemocyte invasion after immersion infection. In the midgut, uptake of WSSV in the epithelium could be detected, however, infected

nuclei of epithelial cells were not observed. In contrast to the gills, the midgut connective tissue shows a clear increase in degranulation of haemocytes, resulting in the appearance of WSH8-immunoreactive thread-like material at later time points during the infection. The significance for the different reaction of haemocytes in both organs studied remains to be investigated further. The observation that haemocytes are not the main target for WSSV suggests that free virions circulating in the haemolymph lead to systemic infection *in vivo.*

A combination of suppression subtractive hybridisation (SSH) and cDNA microarray analysis was used to enrich for those genes that are differentially expressed upon a WSSV infection (chapter 3). The construction of SSH libraries and subsequent selection of differentially expressed genes is described in detail. The selected clones were used to generate a dedicated WSSV infection-related cDNA microarray comprising 750 differentially expressed genes. The approach to combine suppressive subtraction hybridisation with microarray analysis has resulted in a read-out system for the detection of shrimp genes involved in the defence reaction upon a WSSV-infection. This approach has good potential for identifying genes involved in shrimp defences in the future. Further studies on these gene transcripts involved in the defence mechanism have to be initiated.

The focus of chapter 4 is to determine the expression profile of the genes selected in chapter 3. By using the generated microarray it was possible to follow a few hundred clones during the first day of infection. In addition, the immune response of the shrimp upon ‘vaccination’ was studied with the microarray. The results obtained in this investigation provide insight into the previously unknown complexities of host-WSSV molecular interactions. The discovery of differential expression of genes in WSSV infected shrimp can allow the visualisation of several pathways and potential mechanisms that may play a role in WSSV pathogenesis. Identification of regulated

genes in WSSV infected shrimp enabled the development of a model depicting several ways in which host cells responds to infection. Gene expression changes also provided clues about the possible mechanisms involved in the development of pathological changes that are characteristic of the disease. Most importantly, the data obtained in this study identifies several genes whose mRNA is regulated on virus infection suggesting an array of hypotheses which could be tested to reveal their role in WSSV molecular pathogenesis. This study also provides insight in 'vaccine'-host interactions. Microarray studies coupled with *in vivo* experiments obtain relevant data about the functionality of 'vaccines' in shrimp and invertebrates in general. The combination of host immune response genes and 'vaccination' can reveal the route of WSSV infection and may unravel the immune system of the giant tiger shrimp. Taken together, the present investigation demonstrates the application of a powerful approach of combining the high throughput technologies of SSH and microarray to study differential expression of genes in response to virus infection. SSH could be used for initial isolation of differentially expressed genes, a large-scale confirmation of which can be accomplished very efficiently by microarray analysis. The detailed methods described herein could be potentially applied to any biological system. With information available of *Drosophila* research it is possible to look more thoroughly into immune related genes. Toll receptors are known to play a substantial role in detecting pathogens, both in invertebrates as well as in vertebrates, where they are called Toll-like receptors (TLR). Therefore, in chapter 5, a new Toll receptor was identified and described and expression studies upon WSSV infection were performed. The absence of regulation in different organs upon a viral challenge suggests that PmToll is not directly involved in the defence against WSSV. However, the Toll pathway can be regulated at a higher level (PGRPs and GNBPs; extra cellular) and regulation of PmToll might not be necessary. Currently we are investigating the effect of bacterial challenges on the regulation of PmToll.

Finally, the results presented, are summarised and discussed in chapter 6. We provide an evolutionary framework for the virus-host response and describe the relevance of differentially expressed and regulated innate immune response genes. We integrate the characterisation of a shrimp-specific Toll receptor with results from the microarray analysis and provide a integrative immune defence of *P. monodon* to exposure with WSSV. Moreover, we describe the immunological background known so far with respect to the vaccination strategy for WSSV infection.

Samenvatting

White spot syndrome virus (WSSV) is het belangrijkste wereldwijd voorkomende virale pathogeen van gekweekte *penaeid* garnalen. Sinds de eerste ontdekking van het virus in Taiwan in 1992, heeft het zich verspreid over de garnalenkweekregio's in Zuid Oost Azië, Noord-, Midden- en Zuid-Amerika, Europa en het Midden Oosten, waar het enorme economische verliezen veroorzaakt. Het virus heeft veel verschillende gastheren onder crustaceeën en leidt tot duidelijk waarneembare klinische symptomen (witte vlekken) op de binnenkant van het exoskelet van de garnalen. Er is relatief weinig bekend over de rol van immuunresponsgenen van *Penaeus monodon* bij de bescherming tegen WSSV infectie. Dit proefschrift beschrijft de resultaten van ons onderzoek naar de ontwikkeling van een betrouwbare *microarray*, die de analyse van de inductie en de regulatie van aangeboren (innate) immuungenen in de gastheer, geactiveerd door infectie, mogelijk maakt. Bovendien, is eerder een mogelijke vaccinatiestrategie ontwikkeld om garnalen tegen een dodelijke WSSV infectie te beschermen. We hebben ook de inductie van een beschermende vaccinatie voor de inductie en regulatie van genexpressie geanalyseerd met deze *microarray*.

Ten eerste is de haemocytenrespons van garnalen tegen WSSV tijdens een immersie-infectie bestudeerd (hoofdstuk 2). Om gedrag van haemocyten in de kieuwen en de middendarm te bestuderen, hebben we tot drie dagen na WSSV infectie door immersie, immunocytochemie en electronenmicroscopy gebruikt. Dubbele immuunreactiviteit werd waargenomen door gebruik te maken van muis haemocyten-specifieke monoklonale antilichamen (WSH8) en WSSV-specifieke VP28 polyklonale antilichamen uit een konijn. We vonden verschillen in de haemocytenkarakteristieken tussen de kieuwen en de middendarm van *P.*

monodon. Een grote invasie van haemocyten in de kieuwen in WSSV geïnfecteerde *P. monodon* werd waargenomen, mogelijk veroorzaakt doordat haemocyten zich hechten aan de haemolymphvaten. Hoewel veel geïnfecteerde cellen in de kieuwen werden gevonden, waren deze haemocyten op basis van electronenmicroscopie niet door WSSV geïnfecteerd. Kieuwen lijken een belangrijke plaats te zijn van haemocyteninvasie na infectie door WSSV bij immersie-infectie. In de middendarm werd een duidelijke opname van WSSV in het epitheel waargenomen, hoewel in deze geïnfecteerde epitheelcellen geen virusdeeltjes in de kernen werden waargenomen. In tegenstelling tot de kieuwen, laat het aangrenzende weefsel van de darmen een duidelijke toename in de degranulatie van de haemocyten zien, resulterend in de verschijning van WSH8-positive draadachtige structuren op latere tijdstippen gedurende infectie. De betekenis voor de verschillende reacties van haemocyten, zoals wij die in beide organen hebben gevonden, dient verder onderzocht te worden. De observatie dat haemocyten niet het primaire doel van de WSSV infectie lijken te zijn, suggereert dat vrije virusdeeltjes kunnen circuleren in het haemolymph en zo kunnen leiden tot een systemische *in vivo* infectie

Om de immuunrespons die in de garnaal optreedt na WSSV infectie te bestuderen is het noodzakelijk de betrokken genen te kennen en hun expressiepatroon in relatie tot infectie zichtbaar te maken. Een combinatie van de *suppressive subtractive hybridisation* (SSH) en cDNA *microarray* analyse werd gebruikt om de genen die, tijdens WSSV infectie differentieel tot expressie komen, te verrijken. De constructie van SSH banken en vervolgens de selectie van differentieel tot expressie gekomen genen is tot in detail beschreven in hoofdstuk 3. De geselecteerde klonen werden gebruikt om een WSSV infectie gerelateerde cDNA microarray te genereren, bestaande uit 750 differentieel tot expressie komende genen. Hiervoor werd cDNA gebruikt dat afkomstig was van RNA dat op 4 en 8 uur na WSSV infectie

werd geïsoleerd uit de kieuwen van de via immersie geïnfecteerde garnalen. De combinatie van *suppressive substraction hybridisation* met microarray analyse heeft geresulteerd in een uitleessysteem voor de detectie van vroege immuunresponsgenen van de garnaal, betrokken in de afweerreactie tegen een WSSV infectie. Deze benadering heeft een goede potentie om in de toekomst meerdere genen te identificeren die betrokken zijn bij de afweer van garnalen. Verder onderzoek naar deze gen transcripties betrokken in het afweermechanisme moet nu geïnitieerd worden.

In hoofdstuk 4 lag de focus op het vaststellen van het expressieprofiel van de geselecteerde genen op basis van de microarray-analyse zoals beschreven in hoofdstuk 3. Door gebruikmaking van de gegenereerde *microarray* was het mogelijk om honderden klonen gedurende de eerste dagen na WSSV infectie te volgen in diverse organen. Daarnaast is de immuunrespons van garnalen na WSSV-specificke 'vaccinatie' bestudeerd met de *microarray*. Bij deze analyse blijkt dat 2 genen een opregulatie laten zien (1E11 en 1B6), de functie van deze genen is onbekend. Daarnaast laten veel genen minder afgelezen worden, waaronder bijvoorbeeld HSP70 (heat shock protein 70). Van HSP70 is bekend dat het betrokken is in immuunafweer.

De verkregen resultaten van dit onderzoek leveren nieuw inzicht in de voorheen onbekende complexiteit van moleculaire gastheer-WSSV interacties. De ontdekking van differentieel tot expressie komende genen in WSSV geïnfecteerde garnalen maken de visualisatie van verschillende *pathways* en potentiële mechanismen mogelijk, die een rol spelen in de WSSV pathogenese. Identificatie van gereguleerde genen in WSSV geïnfecteerde garnalen maken bovendien de ontwikkeling van een model mogelijk, dat verschillende manieren laat zien hoe gastheercellen kunnen reageren op infectie. Nog belangrijker is dat de gegevens die in dit onderzoek zijn verkregen, verschillende genen identificeren van welke het mRNA gereguleerd

wordt door virusinfectie, en die een reeks hypothesen suggereren welke getest kunnen worden om de rol van deze genen in de WSSV pathogenese te onthullen. Dit onderzoek geeft ook inzicht in 'vaccin'-gastheer interacties. *Microarray* onderzoek gekoppeld aan *in vivo* experimenten gaven relevante gegevens over de functionaliteit van 'vaccins' in garnalen en invertebraten in het algemeen. De combinatie van gastheer immuunresponsgenen en 'vaccinatie' kunnen de route van WSSV infectie onthullen en kunnen het immuunsysteem van de tijgergarnaal ontrafelen.

Met de beschikbare informatie van *Drosophila* is het mogelijk om immuun gerelateerde genen diepgaander te bekijken. Het is bekend dat Toll receptoren een substantiële rol spelen in het detecteren van pathogenen, zowel in invertebraten als in vertebraten, waar ze *Toll-like receptors* (TRL) worden genoemd. Daarom is in hoofdstuk 5 een nieuwe Toll receptor geïdentificeerd en beschreven en zijn expressiestudies verricht.

De afwezigheid van de regulatie van de PmToll expressie in verschillende organen tijdens een virale infectie suggereert dat PmToll niet direct betrokken is in de afweer tegen WSSV. Echter, de Toll *pathway* kan op een hoger niveau (PGRPs en GNBPs) gereguleerd worden en regulatie van PmToll zelf is mogelijk niet noodzakelijk. Momenteel onderzoeken we het effect van bacteriële infecties op de regulatie van PmToll.

Samenvattend, het huidige onderzoek laat de toepassing zien van een sterke benadering van de combinatie van de *high throughput technologies* zoals SSH en *microarray,* om differentiële expressie van genen in respons tot virusinfectie te bestuderen. SSH kan gebruikt worden voor de initiële isolatie van differentieel tot expressie gekomen genen, een grootschalige bevestiging die tot stand gebracht kan worden door efficiënte *microarray* analyse. De gedetailleerde methoden die hier beschreven zijn kunnen mogelijk worden toegepast in elk biologisch systeem. Uiteindelijk worden de gepresenteerde resultaten samengevat en bediscussieerd in

hoofdstuk 6. In dit hoofdstuk beschrijven wij een evolutionair raamwerk voor de virusgastheer respons en beschrijven de relevantie van de differentieel tot expressie komende genen en gereguleerde innate immuunresponsgenen. We integreren de karakteristieken van een garnaalspecifieke Toll receptor met resultaten van de *microarray* analyse en voorzien in een integratieve immuunrespons van *P. monodon* tegen de blootstelling met WSSV. Bovendien beschrijven we de immunologische achtergrond, voor zover bekend, met in acht neming van de vaccinatiestrategie tegen WSSV infectie.

‘Dank!’

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Joop Arnoldus Johannes Arts, geboren te Vierlingsbeek op 24 december 1973, het jaar van de autoloze Zondagen.

In 1992 heeft hij zijn VWO diploma behaald aan het Elzendaal college te Boxmeer, waarna hij de studie Moleculaire Wetenschappen aan de toenmalige Landbouw Universiteit Wageningen startte. Het eerste afstudeervak heeft hij gevolgd bij de vakgroep Microbiologie. Het onderwerp van studie betrof PCE reductase in *Desulfitobacterium* sp. PCE1. Zijn tweede afstudeervak volgde hij bij de vakgroep Biochemie waar hij het prismaan-reductase van *E. coli* bestudeerde. Vervolgens bestudeerde Joop ENOD-40 in het semi-afstudeervak “Toolbox”, de herkomst van zijn liefde voor moleculaire biologie. Na een rondreis door Portugal studeerde Joop in november 1999 af.

Diverse banen volgden bij onder andere Plant Research International waar hij het fumonisine cluster van *Fusarium proliferatum* heeft gesequenced. De promotiebaan bij de vakgroep Celbiologie en immunologie deed zich voor in 2001, waar hij in maart van dat jaar kon starten. Vijf jaar onderzoek resulteert in de afronding van dit proefschrift, wederom voorafgegaan door een reis in Portugal.

In juli 2006 is Joop gestart met een postdoc baan bij Celbiologie en Immunologie (Wageningen Universiteit). Het onderzoek is gericht op stimulatie van het immuunsysteem van garnalen door middel van electromagnetische velden (EMF).