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Antibacterial and antiviral activity of different haemocyte subpopulations of *Litopenaeus vannamei*

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List of Abbreviations

βGBP	β-glucan-binding protein
ALPF	Anti-lipopolysaccharide factors
ANOVA	analysis of variance
AMPs	Antimicrobial peptides
ARC	Artemia Reference Center
CFU	Colony forming units
CpG	Cytosine-phosphate-Guanine
CsCl	Caesium chloride
CS	Chen's salts
DNA	Deoxyribonucleic acid
DW	Distilled water
EDTA	Ethylene diaminetetra acetic acid
EMA	Ethidium monoazide bromide
EMS	Early Mortality Syndrome
FITC	Fluorescent isothiocyanate
FC	Flow cytometry
FACS	Fluorescence activated cell sorting
FAO	Food and Agriculture Organization of The United Nations
FCS	Foetal calf serum
FSC-H	Forward scatter height
GCs	Granular cells
GFP	Green Fluorescent Protein
Glu	Glutanine
HCs	Hyaline cells
H&E	Haematoxylin and Eosin
HHNBV	Hypodermal and haemotopoietic necrosis baculovirus
HM	Haemocyte medium
$HOCl^-$	Hypochlorous acid
H_2O_2	hydrogen peroxide
hpi	hour post inoculation
IIF	Indirect immunofluorescence
kDa	Kilodalton

L15	Leibovitz's medium
2x-L15	Double-stranges Leibovitz's medium
LB	Luria-Bertani broth
LB-A	Luria-Bertani agar
LD_{50}	Lethal dose 50% endpoint
LGBP	lipopolysaccharide and β-1,3-glucan binding protein
LM	Light microscopy
LPS	Lipopolysaccharide
Lys	Lysine
MA	Marine agar
MA	Marine Anticoagulant
MACS	Magnetic activated cell sorting
NaBr	Sodium bromide
NaCl	Sodium chlorua
N:C	Nucleus/cytoplasmic ratio
NO	Nitric oxide
NOS	Nitric oxide synthase
O^-_2	superoxide ions
$^1O_2$	singlet oxygen
OD	Optical density
ODNs	Oligodeoxynucleotides
OH^-	hydroxyl radicals
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen-1	penaeidin 1
Pen-2	penaeidin 2
Pen-3a	penaeidin 3a
PF	Paraformaldehyde
PI	Phagocytic index
PLs	Postlarvae
PMA	Phorbol myristate acetate
PMNOB	*Penaeus monodon* nonoccluded baculovirus
PGBP	Peptidoglycan binding protein

PO	Phenoloxidase
PR	Phagocytic rate
proPO	Prophenoloxidase
ppA	Prophenoloxidase-activating enzyme
PRRs	Pattern recognition receptors
PRPs	Pattern recognition proteins
RV-Pj	Rod-shaped nuclear virus of *Penaeus japonicas*
RPMI	Roswell park memorial institute
Rpm	Rotations per minute
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
SEMBV	Systemic ectodermal and mesodermal baculovirus
SGCs	Semi-granular cells
SID_{50}	Shrimp infectious dose 50% endpoint
shPBS	Shrimp phosphate buffered saline
SOD	Superoxide dismutase
SPF	Specific pathogen free
SPSS	Statistical package for the social sciences
S-S	Disulfide bridges
SSC-A	Side scatter area
Sub 1	Subpopulation 1
Sub 2	Subpopulation 2
Sub 3 + 4	Subpopulation 3 + 4
Sub 5	Subpopulation 5
TLR	Toll-like receptors
TGase	Trans-glutaminase
TSV	Taura syndrome virus
WSBV	White spot baculovirus
WSSV	White spot syndrome virus
WAP	whey acidic protein
YHV	Yellow head disease
UV	Ultraviolet

Chapter 1

Introduction

1.1 Aquaculture production

Aquaculture, known as the farming of aquatic organisms, plays an important role in the global economic growth and is considered as an agro-industrial activity with the highest growth rate worldwide in the last five decades (Figure 1). Aquaculture began to be significant at the global level in the late 1960s when total fisheries production was about 60 million tonnes, of which about 1 million tonnes were farmed. From that point on, aquaculture production grew progressively more rapidly than the capture fisheries, and by the late 1980s production was in excess of 10 million tonnes per year. According to the latest available statistics reported by the FAO (2014), world aquaculture production attained another all-time height of 90.4 million tonnes in 2012 and accounted for about 144.4 billion US$, with an average annual growth rate of 6.2% in the period 2000-2012.

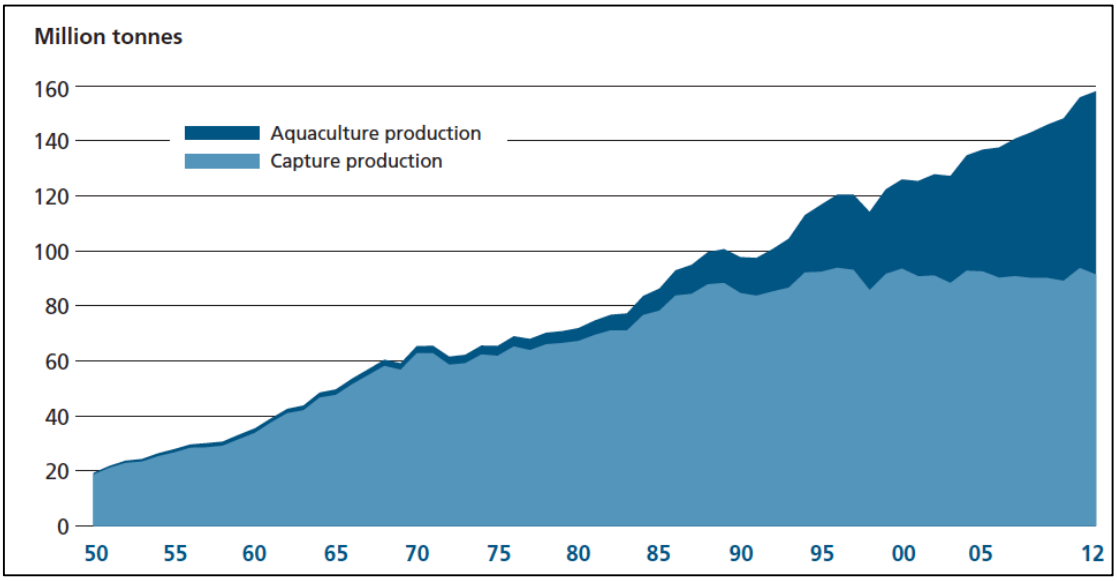

Figure 1. World capture fisheries and aquaculture production (FAO, 2014)

Aquaculture production is vulnerable to adverse impacts of disease and environmental conditions. Disease outbreaks have been increasingly recognized as a significant constraint to the aquaculture sector, affecting the economic and social-economic development of the industry in many countries.

Crustacean production represents one of the most economically important global aquaculture activities, accounting for about US$31 billion annually (FAO, 2015). Culture of crustaceans, especially penaeid shrimp, is an important aquaculture activity in South East Asia and Latin America. Among various species cultured, white leg

shrimp, *Penaeus vannamei*, is considered as a major species contributing to global shrimp aquaculture production. According to the FAO (2012), the total farmed production of *P. vannamei* increased steadily in the period 1980-1998. In 1999, the production of *P. vannamei* rapidly decreased and significantly declined in 2000 due to white spot syndrome virus. It was estimated that around 40% of production, representing a value of over 3 billion US dollar, was lost due to diseases. The production of *P. vannamei* increased again in the next decade due to the rapid expansion of this species in Asian countries (Figure 2).

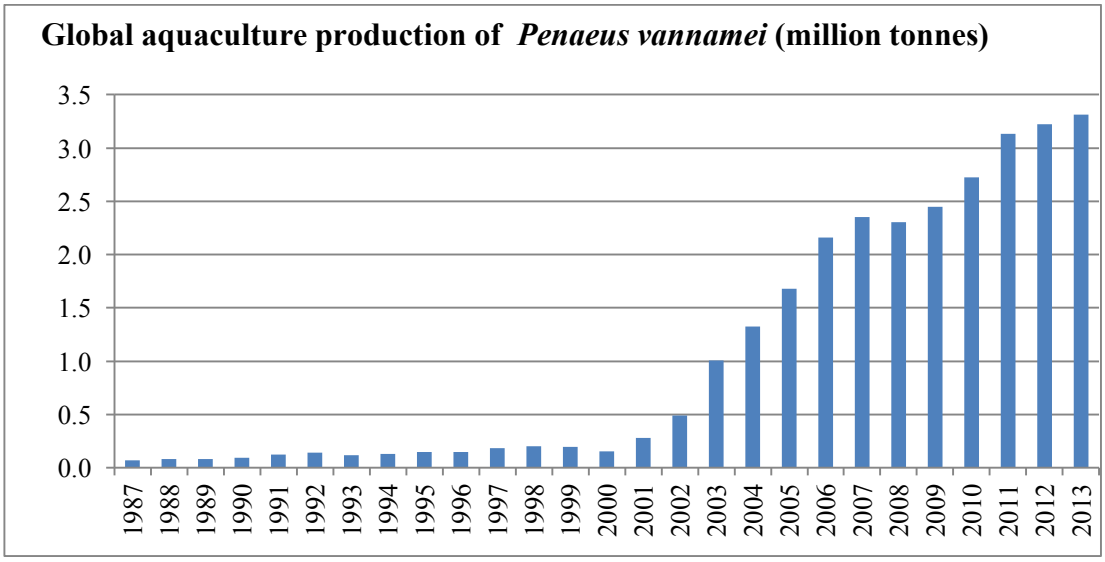

Figure 2. Global production of *Penaeus vannamei* from 1987 till 2013
(Source: http://www.fao.org/fishery/culturedspecies/*Litopenaeus_vannamei*/en)

1.2 Penaeid shrimp biology

1.2.1 Taxonomy

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda, characterized by jointed appendages and an exoskeleton or cuticle that is periodically moulted. There are thousands of terrestrial species in this phylum, and a large, predominately aquatic subphylum, the Crustacea. The subphylum Crustacea contains about 42,000 species belonging to 10 classes. The class Malacostraca contains about three-fourths of the known species and includes crayfish, lobsters, shrimps and crabs (Bailey-Brock, 1992).

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Superfamily: Penaeoidea

Family: Penaeidae

Genus: Penaeus

Species: *Penaeus vannamei*

1.2.2 Morphology

The external morphology of *P. vannamei* (Figure 3) is composed of three parts: pereon, pleon and telson. The pereon where the head and thorax are fused into the cephalothorax has 13 segments (5 in the head and 8 in the thorax). Each segment of the cephalothorax bears a pair of bi- or triramous appendages, which perform sensory or feeding functions (two antennae, a set of mandibles and 5 pairs of maxillae). The last 5 limbs of the cephalothorax are the pereiopods (legs), of which the first 3 are equipped with chelae used for grabbing food and the last 2 used for walking. The exoskeleton of the cephalothorax (carapace) covers the gills with a protective gill chamber (branchiostegite) and forms a dorsal keel-shaped rostrum between the eyes. The pleon (abdomen) has six segments, mainly composed of muscle. The first 5 with paired pleopods (legs) are used for swimming and the sixth is the uropod. The telson is composed of 2 pairs of uropods and used for escaping in case of danger (Ruppert, 1994).

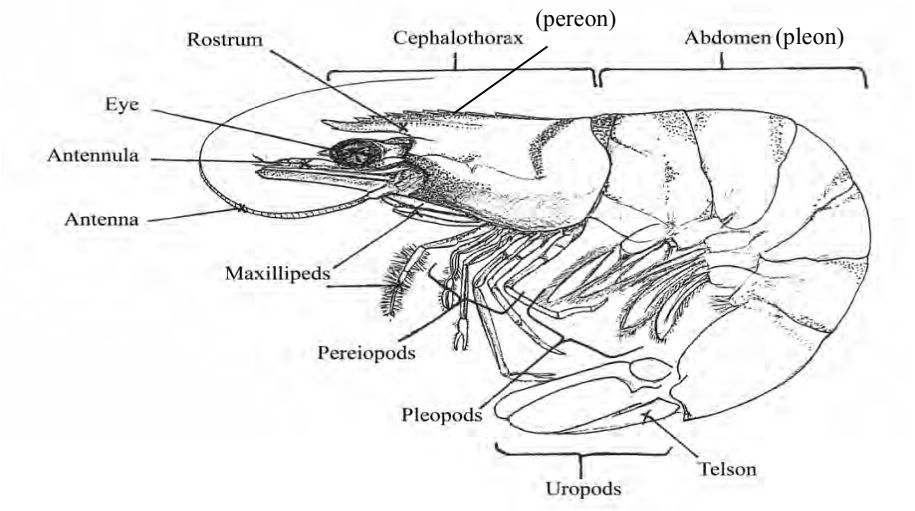

Figure 3. External morphology of penaeid shrimp (Corteel, 2013)

The internal morphology of penaeid shrimp is illustrated in Figure 4. Penaeid shrimp have an open circulatory system. They have a muscular heart that is dorsally located in the cephalothorax. It functions by pumping out hemolymph through anterior, posterior and ventral arteries. Hematopoietic tissue lies dorsally on the stomach and in the onset of the maxillipeds, and has a main function in haemocyte production (van de Braak et al., 2002a). Lymphoid organs lie as a pair of lobes at the end of the subgastric arteries, ventrally of the stomach and just anterior of where the stomach enters the hepatopancreas. The function of the lymphoid organ is primarily involved in elimination of bacteria from the haemolymph (Martin et al., 1996; van de Braak et al., 2002b). The digestive system consists of mouth, esophagus, foregut, midgut and hindgut. The foregut is located dorsally in the cephalothorax. The midgut starts at the end of the stomach. The hindgut is located in the posterior half of the 6th abdominal segment. The digestive tract is responsible for ingestion, mechanical digestion, chemical and biochemical hydrolysis and cellular absorption (Ceccaldi, 1989).

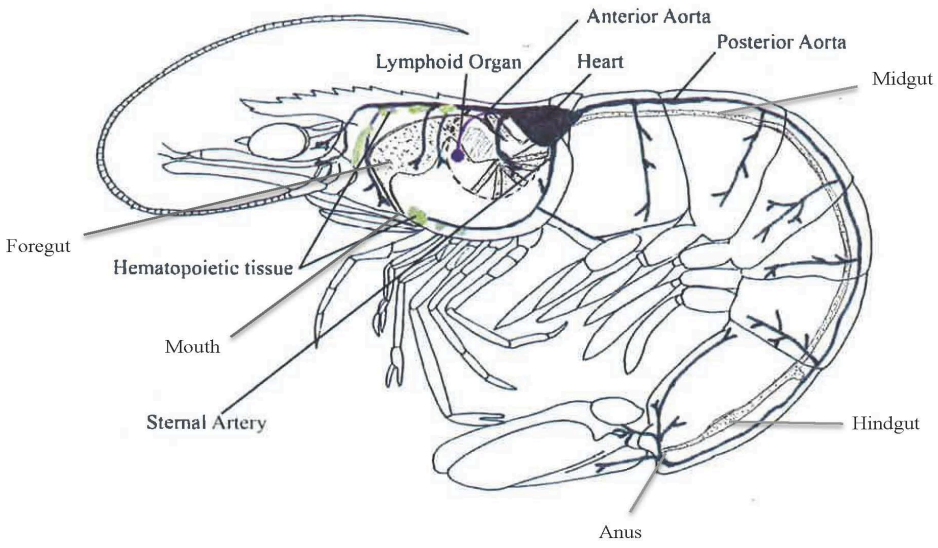

Figure 4. Internal morphology of penaeid shrimp (Corteel, 2013)

1.2.3 Life cycle of penaeid shrimp

The life cycle of penaeid shrimp consists of several distinct stages such as eggs, nauplius, zoea, mysis, and postlarvae that are found in a variety of habitats (Figure 5). Juveniles often prefer brackish water and coastal estuaries, while adults are usually

found offshore at higher salinities and greater depth. Every female of penaeid shrimp produces between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The eggs hatch into the first larval stage, which is the nauplius. The nauplii do not feed, but use their yolk reserves for development. After five moults (instars), the nauplii metamorphose into zoea stage. The zoeas feed on microalgae and metamorphose into mysis. The mysis feed on algae and zooplankton. The mysis then will go through a final metamorphosis and develop into postlarvae (PLs).

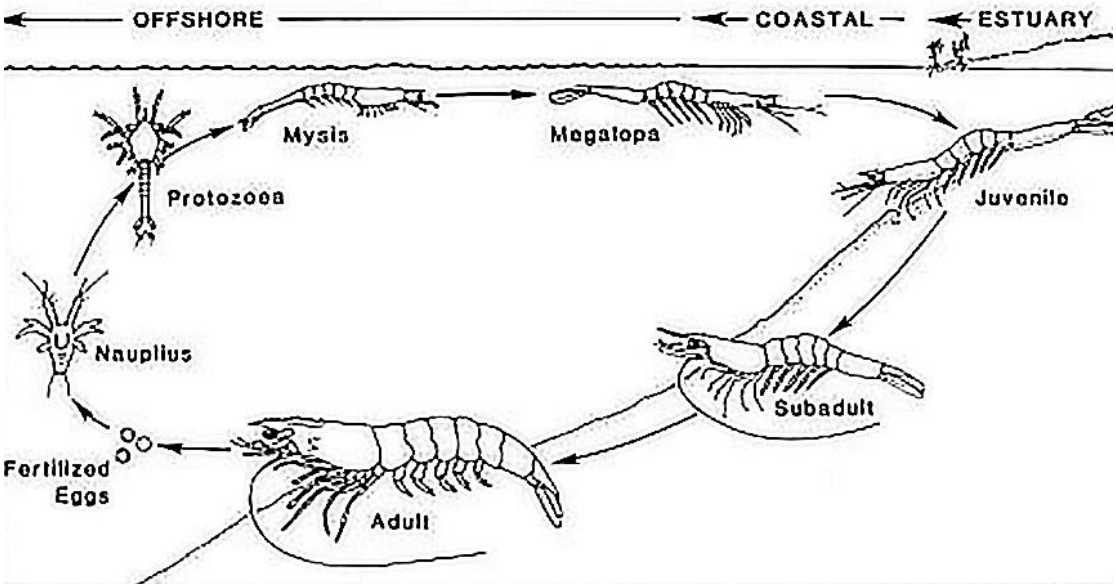

Figure 5. Life cycle of penaeid shrimp (Bailey-Brock, 1992)

1.3 Penaeid shrimp diseases with emphasis on vibriosis and white spot syndrome virus

Penaeid shrimp culture has boomed rapidly all over the world, especially in Asia and South America. With the rapid expansion, however, more disease problems occurred (Lightner, 2011). Among the diseases of penaeid shrimp, those caused by bacteria and viruses have gained more attention.

1.3.1 Vibriosis

Vibriosis is one of the most important diseases for shrimp culture because of its close association with low survival in the hatchery and the grow-out pond (Lavilla-Pitogo et al., 1998; Chen et al., 2000). Vibriosis is caused by Gram-negative, rod shapes, facultative anaerobes, motile bacteria in the family of *Vibrionaceae*. Various species of

Vibrio such as *Vibrio harveyi* (Prayitno & Latchford, 1995), *V. campbellii*-like (Hameed, 1995), *V. parahaemolyticus* (Tran et al., 2013; Lee et al., 2015), *V. alginolyticus* (Lipton, 2003; Liu et al., 2004), which belong to the harveyi clade, and *V. anguillarium* (Lightner, 1996), have been described as pathogenic species that affect penaeid shrimp. These bacteria are also known to take advantage of ecological changes in the culture system and to cause periodic diseases in shrimp. The effect and severity of disease in shrimp are mainly related to the type of *Vibrio* in combination with stress factors, like water quality (pH, salinity, temperature, ammonia…), as well as feed and shrimp quality at the time of stocking (Saulnier et al., 2000). Mortality of shrimp caused by *Vibrio* has been reported in the Philippines (Lavilla-Pitogo et al., 1990), Australia (Hirst, 1995), South America (Alvarez et al., 1998), Mexico (Vandenberghe et al., 1999), India (Jayasree et al., 2006) and South East Asia (Vietnam, Malaysia and Thailand) (Zorriehzahra & Banaederakhshan, 2015).

Many virulence factors have been identified in the harveyi clade, including haemolysins, proteases, phospholipases and chitinase (Ruwandeepika et al., 2012). Haemolysin is an exotoxin that attacks blood cell membranes and causes cell rupture. Haemolysins are produced by many different species of bacteria including *Escherichia coli*, *Pseudomonas aeruginosa* and harveyi clade vibrio (Zhang and Austin, 2000). Proteases represent an important group of lytic enzymes and have been reported in *V. alginolyticus, V. harveyi* and *V. parahaemolyticus*. Proteases have been found to digest a range of host proteins, including gelatin, fibronectin and collagen. Lipases may also cause damage to host tissues. However, little is known about their involvement in the pathogenesis of vibrios belonging to the *harveyi* clade (Ruwandeepika et al., 2012). Chitinases are enzymes that are produced by many marine organisms. They play a potential role in adhesion and penetration of pathogen into host tissues.

Clinical signs of vibriosis in crustaceans include lethargy, slow growth, slow metamorphosis, tissue and appendage necrosis, body malformation, bioluminescence, muscle opacity and melanisation (Aguirre-Guzmán *et al.* 2004).

1.3.2 White spot syndrome virus

White spot syndrome virus (WSSV), a major pathogen of penaeid shrimp, is a pathogen of major economic importance in shrimp aquaculture throughout the world. WSSV was first reported in farmed *Penaeus japonicus* from Japan in 1992/1993, but was thought

to have been imported with live infected postlarvae from mainland China. At roughly the same time, it was discovered in cultured *Penaeus monodon*, *Penaeus japonicus* and *Penaeus penicillatus* in Taiwan. WSSV then spread rapidly throughout most of the shrimp growing regions of Asia such as Thailand, India, Indonesia, Malaysia, Vietnam, the Philippines, and Iran (Durand, 1996; Lo et al., 1996a; Karunasagar et al., 1997; Kasornchandra et al., 1998; Magbanua et al., 2000; Rajan et al., 2000; Bondad-Reantaso, 2001; Dieu et al., 2004). In 1995, WSSV was detected for the first time in farmed *Penaeus setiferus* in Texas and South Carolina in the U.S (Lightner, 1996; Wang et al., 1999). Later on, outbreaks of white spot syndrome were also found in other shrimp farming areas including South, North and Central America, Europe and the Middle East (Walker & Mohan, 2009; Sanchez-Paz, 2010; Lightner et al., 2012).

1.3.2.1 Morphology and classification

White spot syndrome virus (WSSV) is an enveloped, non-occluded and rod-shaped DNA virus with a bacilliform to ovoid or ellipsoid shape. The viral envelope, having a thickness of 6-7 nm, is a lipidic, trilaminar membranous structure with two electron transparent layers divided by an electron opaque layer (Wonteerasupaya et al., 1995; Durand et al., 1997; Nadala et al., 1998). The nucleocapsid is located inside the envelope and has a striated appearance and a size of 420 ± 18 nm in length and 68 ± 5 nm in width (Wonteerasupaya et al., 1995; Hameed et al., 1998). The genome size of WSSV is about 300 kbp and is considered as the largest that has been found until now (van Hulten et al., 2001; Yang et al., 2001).

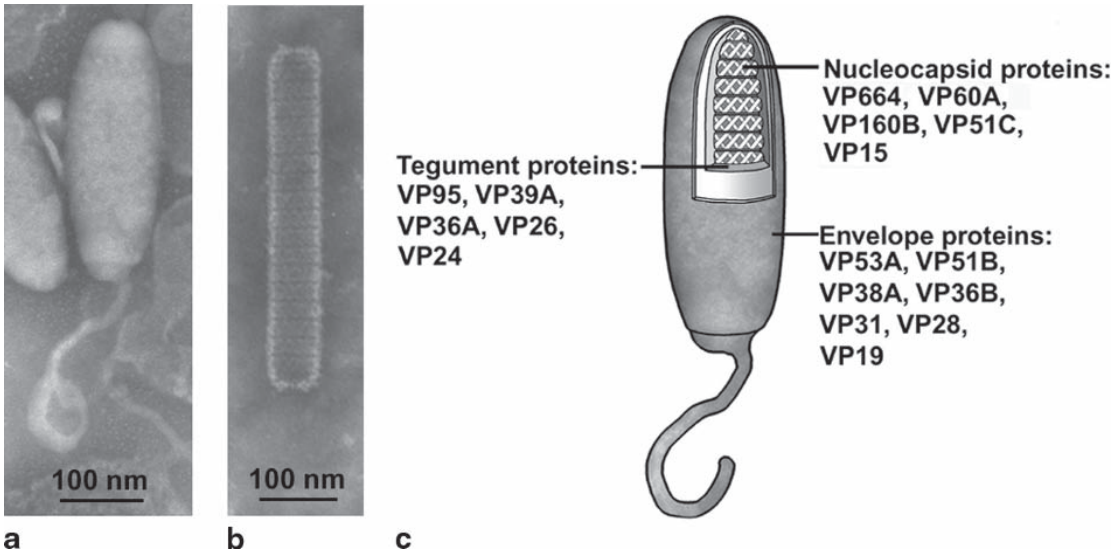

Figure 6. Morphology of WSSV virion. Negative contrast electron micrographs of (**a**) an intact WSSV virion with tail-like extension and (**b**) nucleocapsid. (**c**) layered structures of a WSSV virion (Leu et al., 2009).

WSSV was originally classified as an unassigned member of the *Baculoviridae* family, but later was re-classified as a new virus family, the *Nimaviridae* (genus *Whispovirus)* (van Hulten et al., 2001). Initially, the disease was thought to be caused by different viral agents and a variety of names for the viral agents were given in every region where the disease appeared such as hypodermal and haemotopoietic necrosis baculovirus (HHNBV), rod-shaped nuclear virus of *P. japonicus* (RV-Pj), systemic ectodermal and mesodermal baculovirus (SEMBV), white spot baculovirus (WSBV), and *P. monodon* non-occluded baculovirus (PMNOB) (Durand et al., 1997; Karunasagar et al., 1997; Chou et al., 1998; Hameed et al., 1998). Later, it was recognized that a single viral agent was responsible for these reports and finally an informal consensus was reached and the viral agent was given the name "White Spot Syndrome Virus" (WSSV).

1.3.2.2 Structural proteins

More than 40 WSSV structural proteins with a size from 60 to 6,077 amino acids have been characterized. Of these, 25 have been found in the envelope (VP12, VP19, VP22, VP24, VP28, VP31, VP36B, VP38A, VP39, VP41, VP41A, VP41B, VP51B, VP52A, VP52B, VP53, VP53A, VP68, VP110, VP124, VP150, VP187, VP281, VP292, VP466), six in the nucleocapsid (VP15, VP35, VP51C, VP60B, VP388, VP664) and four in the tegument (a putative structure located between the envelope and

nucleocapsid) (VP26, VP36A, VP39A, VP95) (Escobedo‐Bonilla et al., 2008; Leu et al., 2009).

The interaction between WSSV structural proteins and host proteins plays an important role in viral infection. This virus-host interaction may trigger host immune responses against the invader as well as some modifications of host gene expression to facilitate virus replication (Liang et al., 2015). Several WSSV structural proteins and cellular proteins of shrimp have been reported in relation to the process of viral attachment and entry into the host cells (table 1).

Table 1. WSSV-host proteins interactions (Bas et al., 2016)

Viral protein	**Host protein**	**Species**
VP24, VP32, VP39B, VP41A, VP51B, VP53A, VP53B, VP60A, VP110, VP124, VP337	Chitin-binding protein (PmCBP)	*Penaeus monodon*
VP53A	Glut1	*Penaeus monodon*
VP95, VP28, VP26, VP24, VP19	C-type lectin (LvCTL1)	*Penaeus vannamei*
VP28	C-type lectin (FcLec3)	*Fennerropenaeus chinesis*
VP26, VP28	C-type lectins (MjLecA, MjLecB, MjLecC)	*Marsupenaeus japonicus*
VP28	C-type lectins (MjsvCL)	*M. japonicus*
VP187	β-Integrin	*M. japonicus, P. clarkii*
VP26, VP31, VP37, VP90, VP136	β-Integrin	*Penaeus vannamei*
VP15, VP28	Calreticulin (PlCRT)	*P. leniusculus*
VP466	Rab (PjRab)	*P. japonicus*
VP28	Rab7 (PmRab7)	*P. monodon*
ORF514	PCNA (lvPCNA)	*P. vannamei*
WSSV PK1	Ferritin (lvFerritin)	*P. vannamei*
WSV083	FAK (MjFAK)	*M. japonicus*
AAP1 (WSSV449)	Caspase (PmCaspase)	*P. monodon*
WSSV134, WSSV332	Caspase (PmCasp)	*P. monodon*
WSSV249	Ubc (PvUbc)	*P. vannamei*
ICP11	Histones	*P. monodon*
VP9	RACK1 (PmRACK1)	*P. monodon*
VP15	FKBP46 (PmFKBP46)	*P. monodon*
VP15	CRT (PlgCRT)	*P. leniusculus*
WSSV-miRNA	Dorsha, Dicer, Ago1	-
VP14	Arginine kinase (LvAK)	*P. vannamei*
ORF427	PPs	*P. vannamei*
WSSV IE1, WSSV056	Retinoblastoma protein (Lv-RBL)	*P. vannamei*

The functions of most of these proteins have not been fully elucidated. VP15 appears to be a DNA binding protein (Witteveldt et al., 2005). Neutralization assays suggested that envelope proteins VP24, VP28, VP31, VP36B, VP68, VP76, VP281 and VP466 are involved in early stages of WSSV replication (van Hulten et al., 2001b; Huang et al., 2005; Li et al., 2005; Wu et al., 2005; Hongyan Li et al., 2006; Li Li et al., 2006; Xie & Yang, 2006). VP28 is one of the envelope proteins and is considered to play an important role in the initial steps of systemic WSSV infection in shrimp (van Hulten et al., 2001b; Wu et al., 2005). Yi et al. (2004) reported that VP28 was observed as early as 3 hours post inoculation. VP28 might also be involved in attachment, binding the virus to shrimp cells, and helping it to enter into cell cytoplasm (Yi et al., 2004), because there is a strong hydrophobic region present at the N-terminus of VP28, including a putative trans-membrane region (van Hulten et al., 2001b). This biological structure of VP28 suggests that it might play a role as attachment protein. VP664, a major capsid protein, consists of a remarkable long polypeptide of 6,077 amino acids and is encoded by a giant genome sequence of 18,234 nucleotides (Leu et al., 2005). This protein has a mass of 664 kDa and is considered as the largest viral structure protein ever found (Leu et al., 2005). Based on research done by Li et al. (2015), a hypothesis of WSSV replication and assembly inside susceptible cells is given. First, viral particles will attach to the membrane and then enter the cell via endocytosis. In the early endosome, the envelope and nucleocapsid of WSSV start to separate. The envelope probably fuses with the endosome membrane and the nucleocapsid is released into the cytoplasm. The nucleocapsid migrates close to the cell nuclei and injects its genome into the nuclus via a nuclear pore. Inside the nucleus, the transcription starts and the mRNAs of immediate-early gene are produced. This gene subsequently migrates back to the cytoplasm, where it is translated into proteins by free ribosomes. Afterwards, the capsid protein VP664 is expressed and later also envelope protein VP28. The viral genome multiplies inside the cell nuclei and the viral capsids become assembled around the viral genome. As a result, the nucleocapsids are formed. The nucleocapsids then bud at the inner nuclear membrane with the envelope protein VP28. Finally, the new WSSV particles are released via cell lysis and start a new cycle in other susceptible cells.

1.3.2.3 WSSV infection

Host range

WSSV has a remarkable broad host range among crustacea. This virus can infect marine, brackish and freshwater decapods, such as penaeid shrimp, crayfish, crabs, spiny lobsters and hermit crabs (Lo et al., 1996; Flegel, 1997; Wang et al., 1998; Rajendran et al., 1999; Flegel, 2006). Over 90 species of arthropods have been reported as hosts or carriers of WSSV (Sánchez-Paz, A., 2010).

Clinical signs

The clinical signs of white spot syndrome in infected penaeid shrimp are composed of white spots (0.5-3 mm in diameter) which are the result of calcified deposits embedded within the exoskeleton and epidermis, reduction in food consumption, reddish discoloration of body and appendages due to the expansion of chromatophores, reduced preening and response to stimulus, loose cuticle, swelling of gill covers (branchiostegites) due to accumulation of fluid (Balakrishnan et al., 2012).
WSSV infected shrimp normally concentrate near the pond edge in the field and display clinical signs within 1 or 2 days before the first mortality occurs. Cumulative mortality may reach 100% within 3 to 10 days after the onset of diseases (Lightner, 1996). Juvenile shrimp of all ages and sizes are susceptible to white spot syndrome but massive mortality mainly occurs 1 or 2 months after post-larvae stocking.

Pathogenesis

WSSV infects a wide range of target tissues of ectodermal and mesodermal origins such as epidermis, gills, foregut, hind gut, antennal gland, lymphoid organ, heart, eye-stalk, gonads and hematopoietic cells. In early stages of viral infection, the nuclei of infected cells become hypertrophied with marginalized chromatin, and contain inclusion bodies that stain intensively eosinophilic. In the later stages of infection, the inclusion expands to fill the whole nucleus and stain basophilic (Bas et al., 2016).
How WSSV enters shrimp remains unclear. A study of Chang et al. (1996) on early juvenile of *Penaeus monodon* reported that the primary sites of WSSV infection are the subcuticular epithelial cells of stomach and cells in gills, integument and connective tissue of the hepatopancreas. Another study with *M. japonicus* showed that epithelial

cells in the midgut trunk may be a transient site of WSSV replication (Di Leonardo et al., 2005). An oral route challenge *P. vannamei* using a standardized inoculation technique indicated that WSSV most probably enters via gills and epithelial cells in the foregut (Escobedo-Bonilla *et al*., 2007). The mechanism of viral spread from the primary sites to other target organs has been controversial. Some studies have shown that WSSV infects haemocytes of *Penaeus merguiensis*, *Marsupenaeus japonicus* and *Palaemon sp.* and travels throughout the body in these cells to reach target organs (Wang et al., 2002; Di Leonardo et al., 2005). Other studies have indicated that circulating haemocytes of *Penaeus monodon*, *Procambarus clarkii* and *Penaeus vannamei* are refractory to WSSV infection (van de Braak et al., 2002; Shi et al., 2005, Escobedo-Bonilla et al., 2007). It means that WSSV might reach other target tissues through haemolymph circulation in a cell-free form.

1.4 Crustacean immunity

Crustaceans have a well-developed innate immunity that responds against antigens on the surface of potential pathogens. This innate immunity is activated when pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, such as antimicrobial, clotting and pattern recognition proteins, which, in turn, activate humoral or cellular effector mechanisms to destroy invading pathogens. The innate immune system of crustaceans is composed of humoral and cellular defense mechanisms. Humoral defense includes the production of antimicrobial peptides, reactive oxygen intermediates and the complex enzymatic cascades that regulate coagulation or melanization of haemolymph. In contrast, cellular defense refers to the direct action of haemocytes like phagocytosis, nodulation and encapsulation (Söderhäll, 1999; Jiravanichpaisal et al., 2006; Smith, 2010). However, there is an overlap between humoral and cellular defense. Haemocytes are considered as an important source of many humoral molecules, while many humoral factors also affect haemocyte function (Jiravanichpaisal et al., 2006). The first step of internal defense is the recognition of invading microorganisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores & Yepiz-Plascencia, 2000). Haemocytes can recognize invading pathogens either directly by interaction of surface receptors on haemocytes with molecules on the pathogen, or indirectly by recognition of humoral receptors that bind to the surface of the invader. An overview of crustacean immune responses is illustrated

in Figure 7.

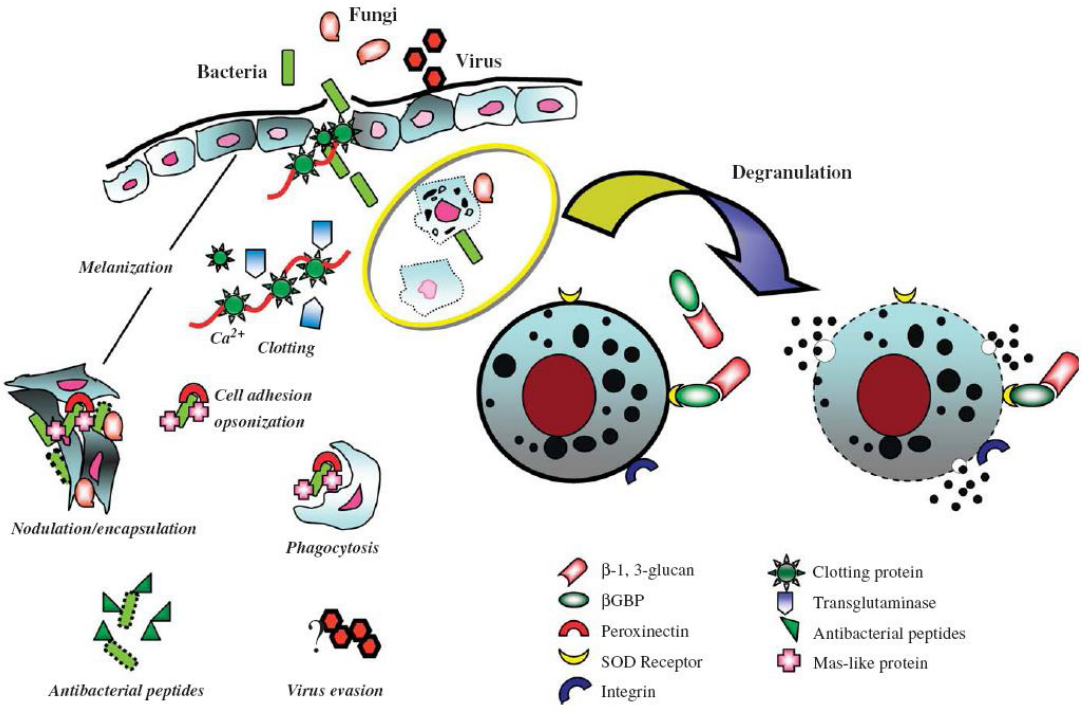

Figure 7. Innate immune responses of crustaceans (Jiravanichpaisal et al., 2006)

It is generally accepted that invertebrates lack a complex adaptive immune response due to the fact that there is no evidence of antigen-specific humoral compound production similar to antibodies in vertebrates. There are no T cells, B cells or major histocompatibility complex molecules (MHC) in invertebrate immune systems (Little et al., 2005). However, the recent experimental data from shrimp and other arthropods demonstrated that invertebrates possess some form of memory immune response. The memory-like phenomenon are termed "immune priming". For instance, vaccinated shrimp with DNA plasmids carrying a white spot syndrome virus (WSSV) envelope protein gene (vp28 or vp281) could protect shrimp against WSSV infection (Rout et al., 2007). This study indicated that the immune priming effect in shrimp is present for 3 to 7 weeks. In some cases, the immune priming effect might extend for almost lifetime of the animal and may even be transmitted to the next generation. For example, in the copepod *Daphnia magna*, offspring from mothers primed with the pathogenic bacteria *Pasteuria ramose* suffered less from a reduction in fitness when subsequently infected with this bacteria (Little et al., 2003). Recently, evidence of a mechanism for a more specific immune response capacity has been discovered in invertebrates. Down syndrome cell adhesion molecule (Dscam) (reviewed by Hauton et al., 2015), a member

of the immunoglobulin super family (IgSF), plays an essential role in the alternative adaptive immune system of invertebrates. Dscam was identified and characterized in crustaceans and insects such as penaeid shrimp (*Penaeus vannamei*, *P. monodon)*, crayfish (*Pacifastacus leniusculus*), Chinese mitten crab (*Eriocheir sinensis*), honey bee (*Apis mellifera*) and mosquito (*Anopheles gambiae*) (Chou et al., 2009; Chou et al., 2011; Watthanasurorot et al., 2011; Wang et al., 2013; Schwarz and Evans, 2013; Dong et al., 2012). The Dscam molecule has a hyper-variable extracellular region that can facilitate phagocytosis of the specific invading pathogens through alternative splicing by recognizing pathogens including bacteria *Escherichia coli*, *Streptococcus aureus, Vibrio alginolyticus* and WSSV (Watthanasurorot et al. 2011, Lin et al. 2013, Wang et al. 2013).

1.4.1 Recognition of non-self molecules

The innate immune system is evolutionarily conserved and is involved in the recognition of invading microorganism, which is mediated by the haemocytes and plasma proteins (Vargas-Albores et al., 1996). It recognizes microorganisms by their characteristic pathogen-associated molecular patterns (PAMPs), such as peptidoglycan, lipopolysaccharide from bacteria, and β-1,3-glucans from fungal cell walls. Upon recognition, these receptors activate distinct signaling cascades that regulate specific immune-related proteins aimed at eliminating pathogenic microorganisms. Several pattern recognition proteins (PRPs) like lipopolysaccharide-binding protein, β-1,3-glucan binding protein, peptidoglycan-binding protein, lipopolysaccharide and β-1,3-glucan binding protein recognize and respond to microbial intruders (Sritunyalucksana & Söderhäll, 2000). Besides, lectins and toll-like receptors also belong to PRPs. Lectins can work directly as agglutinins or opsonins (Marques & Barracco, 2000). Compared to other arthropod groups, such as insects and crabs, the involvement of lectins in shrimp non-self-recognition is still much less well established. However, lectins have the ability to bind carbohydrates and promote the agglutination of different cells like bacteria and other invading pathogens. It was assumed that these molecules maybe regarded as having a potential role in invertebrate non-self-recognition reactions (Marques & Barracco, 2000). This binding directly activates the haemocytes resulting in their degranulation and subsequent activation of prophenoloxidase (proPO) system (Söderhäll et al., 1990; Söderhäll & Cerenius, 1992).

1.4.2 Cellular immunity

1.4.2.1 Phagocytosis

Phagocytosis is one of the major defense mechanisms when foreign particles or microorganisms intrude their host. It is a primitive defense mechanism, conserved in both vertebrates and invertebrates. The phagocytic process is thought to occur in two steps, with the first step involving physio-chemical adherence of the foreign particles to the lining of the haemocoel and the second step involving attachment of haemocytes to the sites of bacterial adherence (Martin et al., 1996), leading to the engulfment of particles into the cell and subsequent formation of phagosomes to stimulate microbial digestion. Target recognition can either occur through direct cell-target interaction or can be mediated by cell adhesion molecules, such as peroxinectin (Johansson et al., 1999). Phagocytosis is believed to be one of the major cellular defense mechanisms in crustaceans. Certain types of haemocytes can phagocytose either biotic targets such as bacteria, yeast, and apoptotic cells or abiotic targets like synthetic beads or India ink particles (Hernández et al., 1999; Jiravanichpaisal et al., 2006; Smith, 2010). The haemocyte types that are responsible for phagocytic reaction have been reported to differ among invertebrates and even within crustacean species.

Smith & Söderhäll (1983) reported that phagocytic activity of the crayfish, *Astacus astacus* and *Pacifastacus leniusculus*, was evident only for hyaline cells and semi-granular cells. In red swamp crayfish, *Procambarus clarkii*, hyalinocytes are considered as phagocytes (Söderhäll et al., 1986); semi-granulocytes, which have limited phagocytic capacities, would be specialized in particle encapsulation and granulocytes would participate in the pro-phenoloxidase (proPO) system (Söderhäll & Smith, 1983). Another study conducted by Söderhäll et al. (1986) also reported hyaline cells of the shore crab, *Carcinus maenas*, as the primary phagocytic cells, which were capable of engulfing both Gram-negative and Gram-positive bacteria. In penaeid shrimp, *Penaeus indicus*, semi-granular and granular cells were responsible for phagocytosis (Jayasree, 2009). In ridgeback prawn, *Sicyonia ingentis*, phagocytosis of the Gram-negative marine bacterium (*Cytophaga* sp.) was accomplished primarily by small granule haemocytes, rarely by large granule haemocytes, and never by hyaline cells (Hose et al., 1990). In freshwater prawn, *Macrobrachium rosenbergii*, phagocytosis was observed to be primarily carried out by granular cells and semi-

granular cells (Gargioni, 1998; Sung et al., 2000).

1.4.2.2 Nodulation and encapsulation

Nodulation/encapsulation is a process that responds to infection and is a part of cellular defense reaction of crustacean against invading microorganisms. Nodulation refers to multiple haemocytes binding to aggregates of bacteria, while encapsulation refers to the binding of haemocytes to larger targets like parasites. Nodulation and encapsulation are actually the same process, albeit against different targets. When the body cavity is invaded by a large number of foreign particles, like bacteria, fungi or parasites, which cannot be removed by phagocytosis, nodulation/encapsulation is formed. During a nodulation/encapsulation response, there is a co-operation of different types of haemocytes that can recognize and attach to the foreign target and one another, eventually forming a smooth capsule comprising overlapping cellular layers. Kobayashi et al. (1990) and Liu et al. (2005) proved that peroxinectin, a cell adhesion molecule, plays an important role in nodulation/encapsulation enhancement. The biological activity of peroxinectin is related to the activation of the proPO system (Johansson et al., 1995). Under normal conditions, this protein is synthesized and stored in granules of semi-granular and granular cells in an inactive form. In response to a stimulus, peroxinectin is released from the haemocytes by degranulation and activated outside the cells to mediate haemocytes attachment (Johansson & Söderhäll, 1988). This process kills pathogens or, at least, restricts their movement and growth in the haemocoel cavity. The nodulated/encapsulated organism seems to be killed by asphyxia, toxic action of quinones or semi-quinones via the proPO activation cascade (precursors of melanin), free radicals reactive oxygen intermediates and antimicrobial peptides (Nappi et al., 1995; Gillespie et al., 1997; Nappi et al., 2000).

1.4.2.3 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are evolutionarily conserved in crustaceans. ROS are involved in processes of cellular immunity in which foreign particles or microorganisms are internalized into the phagocytic cells, followed by the release of enzymes and generation of reactive oxygen species. ROS also play a positive role in the phagocytic activity of crustacean haemocytes. Upon stimulation, phagocytic cells increase O_2 consumption and produce several reactive oxygen intermediates such as

superoxide ions (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$). ROS were first discovered in the shore crab, *Carcinus maenas* (Bell & Smith, 1993). They showed that superoxide production was generated by hyaline cells using phorbol myristate acetate (PMA) as elicitor. Then, several approaches have been used to evaluate the superoxide production with different elicitors during phagocytic processes in shrimp (Muñoz et al., 2000; Jayasree, 2009). Although, ROS play an important role in host defense, high concentrations of ROS produce oxidative stress and increase the risk of cell damage (Lambeth, 2004; Guertler et al., 2010).

1.4.3 Humoral immunity

1.4.3.1 Clotting process

Since crustacean have an open circulatory system, wounds must be sealed immediately to prevent blood loss and also hinder pathogenic microorganisms from entering and spreading throughout the haemocoel. The activity that contributes to stopping loss of body fluids is called clotting. Haemolymph clotting is an important part of the innate immune system, which overlaps with the humoral/cellular boundary and involves a combination of soluble and cell-derived factors (Johansson et al., 1999; Theopold et al., 2002; Jiravanichpaisal et al., 2006). The clotting system has been shown to depend on the activity of the calcium-dependent enzyme trans-glutaminase (TGase), which has cross-linking activity. Clotting occurs through polymerization of a clotting protein in plasma and is catalyzed by a calcium ion dependent TGase (Yeh et al., 1998; Hall et al., 1999; Wang et al., 2001). Haemolymph clotting is induced when TGase is released from haemocytes or tissues, and starts cross-linking plasma derived clotting protein in the presence of Ca^{2+}.

Montaño-Pérez et al. (1999) purified the clotting protein of white leg shrimp, *Penaeus vannamei,* by using affinity chromatography. The protein was found to be a lipoglycoprotein and consisted of two 210-kDa subunits covalently bound by disulfide bridges. Every 210 kDa subunit has lysine and glutamine side chains, which are covalently cross-linked to each other by TGase. TGase was also purified and characterized. It is a homodimeric cytosolic protein with 84.2 kDa subunits, abundant in haemocytes and hepatopancrease (Yeh et al., 2006).

In insects, the clotting system consists of four steps: (i) primary or soft clotting step - in this step, degranulation of haemocytes leads to the establishment of extracellular

aggregates which serve to seal the wound; (ii) hard clotting step - activation of the proPO cascade/tranglutaminase subsequently leads to crosslinking of the clot; (iii) scab formation step - plasmatocytes are attracted, spread across the clot and seal it off from the haemocoel; (iv) replaced scab step - regeneration of the epidermis and growing across the wound site (Theopold et al., 2004).

1.4.3.2 Prophenoloxidase (proPO) system and melanisation

ProPO system is considered to be one of the main non-self recognition and defense systems in invertebrates (Söderhäll & Cerenius, 1998). The proPO system can be activated by minute amounts of microbial components like lipopolysaccharide, peptidoglycan from bacteria and β-1, 3-glucan from fungi (Söderhäll & Cerenius, 1998). This activation causes degranulation in granular and semi-granular cells and release of prophenoloxidase-activating enzyme (ppA) (Aspán et al., 1990; Barracco et al., 1991). Via limited proteolysis in the presence of calcium (Ca^{2+}), ppA becomes active and cleaves proPO into active phenoloxidase (PO). The PO, an active form of proPO, is responsible for the melanisation process in arthropods where melanin synthesis is involved in the process of sclerotisation and wound healing of the cuticle as well as in defense reactions (nodule formation and encapsulation) against invading microorganisms entering the haemocoel. This active PO will catalyse both *o*-hydroxylation of monophenols and the oxidation of phenols to quinones. Finally, quinones are converted to melanin, a brown pigment (Figure 8) (Ratcliffe et al., 1985; Cerenius & Söderhäll, 2004).

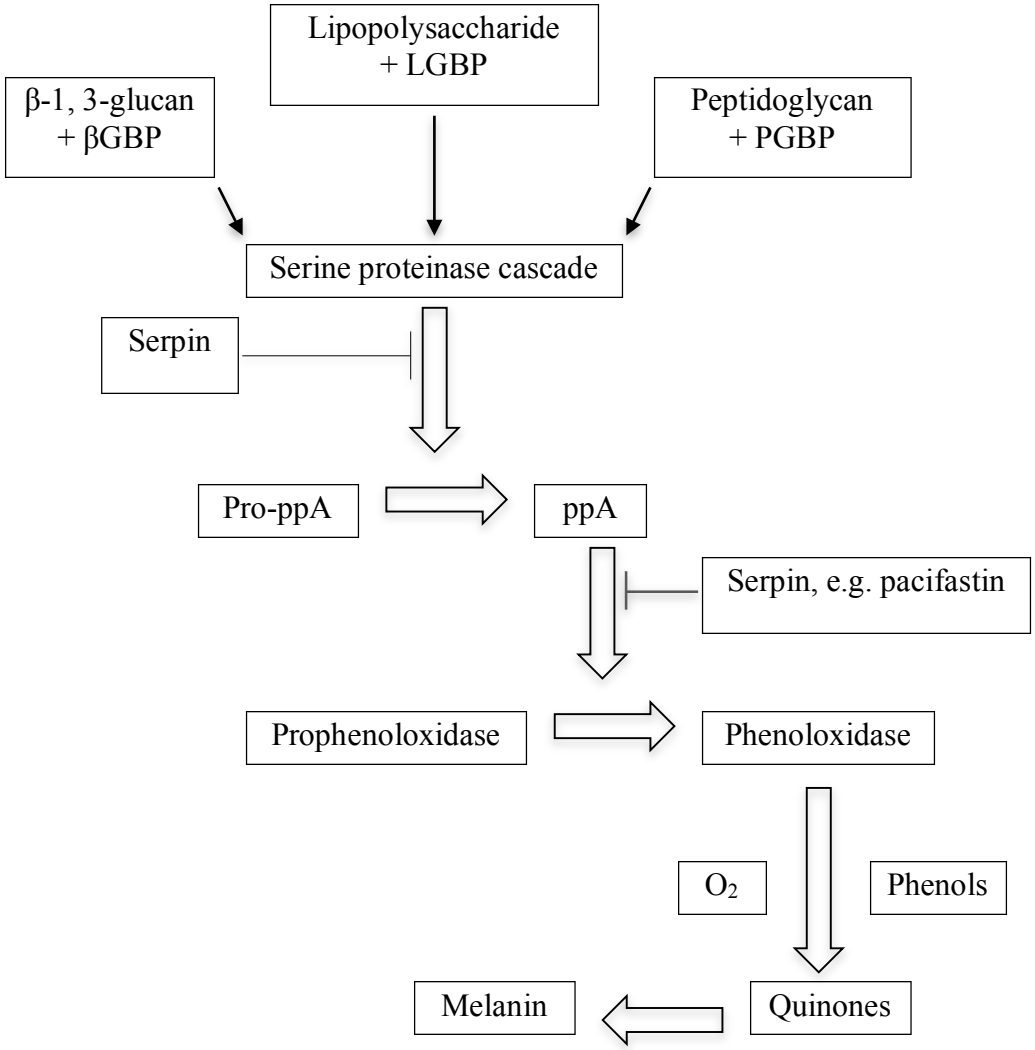

Figure 8. Overview of the arthropod prophenoloxidase (proPO)-activating system (Cerenius & Söderhäll, 2004) (βGBP: β-glucan binding protein; LGBP: lipopolysaccharide and β-1,3-glucan binding protein; PGBP: peptidoglucan binding protein; ppA: prophenoloxidase-activating enzyme).

1.4.3.3 Antimicrobial peptides

Antimicrobial peptides (AMPs), ubiquitously found in all living kingdoms from bacteria to mammals including fungi and plants, are small molecules with a mass less than 10 kDa. AMPs are a major component of the innate immunity in invertebrates and play an important role in the innate immune defense with an ability to neutralize and/or kill invading microorganisms (Brown & Hancock, 2006). They are primarily known as natural antibiotics because of their rapid and efficient antimicrobial effects against a broad range of microorganisms, including Gram-positive and Gram-negative bacteria, yeast, filamentous fungi and, to a lesser extent, protozoans and enveloped viruses (Bulet

et al., 2004; Yount et al., 2006; Guaní-Guerra et al., 2010; Rosa & Barracco, 2010). They are synthesized and stored in granule haemocytes (Destoumieux et al., 2000; Rosa & Barracco, 2010). The major classes of antimicrobial peptides include (i) α-helices, (ii) β-sheet and small proteins, (iii) peptides with thio-ether rings, (iv) peptides with an over-representation of one or two amino acids, (v) lipopeptides, and (vi) macrocyclic cystine knot peptides (Epand & Vogel, 1999). The majority of antimicrobial peptides are amphiphilic, displaying both hydrophilic and hydrophobic surfaces. These peptides generally act by forming pores in microbial membranes or otherwise disrupting membrane integrity of the cell target (Tam et al., 2000), which is facilitated by their amphiphilic structure. The cationic portion of the peptide is first attracted to the negatively charged bacterial and fungal cell walls and/or membranes, and following this first electrostatic interaction, the peptide inserts into and permeabilizes the microbial cell membranes through its hydrophobic portion. The microorganisms are then destroyed via membrane destabilization and/or pore formation (Brogden, 2005; Yount et al., 2006; Rosa & Barracco, 2010). Beyond this direct interaction with microbial membranes, AMPs may have additional mechanisms to inactivate pathogens. They can be translocated into the cytoplasm of the microorganism where they act on specific intracellular targets. Once inside, the peptides interfere with several essential metabolic functions, such as protein, nucleic acid and cell wall synthesis, leading to bacterial cell death (Kamysz et al., 2002; Brogden, 2005; Yount et al., 2006; Hale & Hancock, 2007; Nicolas, 2009).

The first AMP isolated from plasma and haemocytes of penaeid shrimp, *Litopenaeus vannamei*, was penaeidins (Destoumieux et al., 1997). So far, the penaeidins have been discovered from at least eight shrimp species (Song and Li, 2014). The masses of these penaeidins are around 5.5-6.5 kDa and fully characterized at amino acid levels (Pen-1, Pen-2, and Pen-3a) (Destoumieux et al., 2000). They are composed of a N-terminal proline domain and a cyclic C-terminal domain containing three intra-molecular disulfide bridges. Penaeidins have both antibacterial and antifungal properties. The antibacterial properties are mainly direct against Gram-positive bacteria with either bactericidal or bacteriostatic effects. These peptides have no effect on the activity of Gram-negative pathogenic *Vibrio* spp. However, these peptides can inhibit the growth of a large range of filamentous fungi like *Fusarium oxysporum* (Destoumieux et al., 1997; Destoumieux et al., 2000).

Beside penaeidins, other AMPs like crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes (Bartlett et al., 2002; Hikima et al., 2003; Sotelo-Mundo et al., 2003; Supungul et al., 2004; Amparyup et al., 2008a; Amparyup et al., 2008b) have also been discovered in crustacean species.

Crustins are defined as multi-domain cationic antibacterial polypeptides (7-14 kDa) that contain one whey acidic protein (WAP) domain at the C-terminus (Smith et al., 2008) and have a mass of 11.5 kDa protein. Crustins were first reported in the granular haemocytes of the shore crab, *Carcinus maenas* that exhibits specific activity towards Gram-positive bacteria (Relf et al., 1999), and recently have been reported in *Litopenaeus vannamei* and *L. setiferus* (Bartlett et al., 2002). Three main types of crustins were identified as type I, II and III based on their structural features (Smith et al., 2008). Type I crustins, mainly present in crabs (Relf et al., 1999; Imjongjirak et al., 2009; Sperstad et al., 2009a; Mu et al., 2010; Yue et al., 2010), lobsters (Stoss et al., 2004; Hauton et al., 2006; Christie et al., 2007; Pisuttharachai et al., 2009), crayfish (Jiravanichpaisal et al., 2007; Shi et al., 2009), freshwater prawn (Dai et al., 2009), and shrimp (Sun et al., 2010), comprise the members most related to carcinin and possess a cysteine-rich region of variable length between the leader sequence and the WAP domain. On the other hand, type II crustins are characterized by the presence of a hydrophobic region containing an overrepresentation of glycine residues upstream of the cysteine-rich and WAP domains found in type I. This type of crustins is reported in penaeid shrimp (Bartlett et al., 2002; Rattanachai et al., 2004; Supungul et al., 2004; de Lorgeril et al., 2005; Rosa et al., 2007; Zhang et al., 2007; Antony et al., 2011) and crayfish (Jiravanichpaisal et al., 2007). Conversely, type III crustins, found in shrimp and crayfish species (Jiménez-Vega et al., 2004; Jia et al., 2008; Amparyup et al., 2008a; Du et al., 2010), possess a short PRP-rich region between the leader sequence and the single WAP domain, but do not contain the characteristic cysteine-rich domain present in both type I and II crustins nor the glycine region motif.

Anti-lipopolysaccharide factors (ALFs), initially purified and characterized from haemocytes of the horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka et al., 1982; Ohashi et al., 1984), are small basic proteins that inhibit the lipopolysaccharide (LPS)-mediated coagulation cascade. Recently, several ALFs have been isolated and characterized from various prawns, crabs, lobsters and crayfish (Supungul et al., 2004; Liu et al., 2006; Nagoshi et al., 2006; Imjongjirak et al., 2007). ALFs have strong antibacterial activity against, in particular, Gram-negative bacteria

but also display activity against Gram-positive bacteria and fungi (De la Vega et al., 2008; Jiang et al., 2015).

Lysozyme, one of the first discovered antibacterial proteins, is widely distributed in invertebrate animals (Söderhäll, 1999; Zhao et al., 2007) and is considered as a molecule involved in non-specific innate immunity. Lysozyme is thought as one of the main enzymes that exist in the lysosome (Misra et al., 2004). The biological function of this enzyme is believed to be self-defense from bacterial infection, because it induces bacterial cell lysis by hydrolyzing β-1,4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan layer in the bacterial cell wall (Bachali et al., 2002; Yao et al., 2008). Six types of lysozymes are classified (Hikima *et al.,* 2003): (i) chicken-type lysozyme (c-type), (ii) goose-type lysozyme (g-type), (iii) plant-type lysozyme, (iv) T4-phage lysozyme (phage-type), (v) bacterial-type lysozyme and (vi) invertebrate-type lysozyme (i-type). In shrimp, lysozyme was found to display antimicrobial activity against both Gram-negative and Gram-positive bacteria including *Vibrio* species that are pathogenic to shrimp (Hikima et al., 2003; Tyagi et al., 2007; Xing et al., 2009; Supungul et al., 2010).

1.4.4 Apoptosis

Apoptosis, also termed programmed cell death, is a critically important cellular process for the survival of multicellular organisms by getting rid of damaged or infected cells that may interfere with normal function (Vicencio et al., 2008). The apoptotic cells are characterized by morphological changes that include condensation of nuclear chromatin, formation of apoptotic bodies, cytoplasmic vacuolization, and blebbing of the cell membrane. The major players of apoptosis are a group of caspases, a family of structurally related cysteine proteases. Caspases play a vital role at various stages of the apoptotic process, which involves an intricate cascade of events including interactions among several protein families (Jin & El-Deiry, 2005). During viral infection, apoptosis plays a key role in reducing viral replication by containing the virus within the cell and limiting the spread of viral particles (Liu et al., 2009; Menze et al., 2010; Flegel & Sritunyalucksana, 2011; Xu et al., 2014).

1.4.5 RNA interference

RNA interference (RNAi) is a biological process that mediates gene silencing in a sequence specific manner and plays a crucial role in controlling virus replication. The mechanism and the function of the biochemical molecules that are fundamental to a functional RNAi pathway have been extensively studied during the last decade. The administration of dsRNA or small interference RNA (siRNA) specific to particular viral genes could protect shrimp against WSSV infection. Robalino et al. (2004, 2005) demonstrated that the treatment of penaeid shrimp, *Penaeus vannamei*, with sequence-specific RNA leads to increased resistance to viral infection. Another study on *Penaeus monodon* reported that shrimp infected with WSSV following treatment with dsRNA showed a much lower mortality (Westenberg et al., 2005). However, the basic research and application of RNAi in shrimp aquaculture are still in early developmental stages.

1.4.6 Crustacean haemocytes and their functions

Crustacean haemocytes play an important role in the host immune response including recognition, phagocytosis, melanization, cytotoxicity and cell-cell communication. Classification of the haemocytes in crustaceans is based mainly on the presence of cytoplasmic granules and the size of cells. Basically, three types of circulating haemocytes: (i) hyaline cells, (ii) semigranular cells, and (iii) granular cells, are involved in the cellular immune reactions (Söderhäll & Smith, 1983; van de Braak et al., 1996; Vargas‐Albores et al., 2005; Li & Shields, 2007; Smith, 2010; Hong et al., 2013). Recently, five types of haemocytes were identified in spider crab, *Hyas araneus* (L.) (Roulston & Smith, 2011) and penaeid shrimp, *Litopenaeus vannamei* (Dantas-Lima et al., 2013).

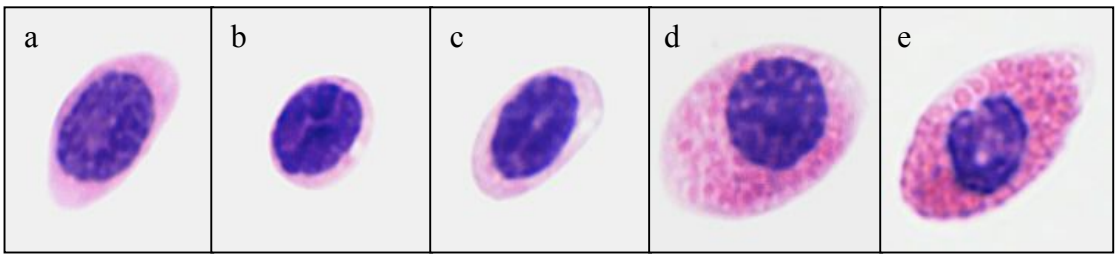

Figure 9. Morphology of haemocytes from penaeid shrimp, *Litopenaeus vannamei*, stained with haematoxilin & eosin, a: hyaline cells (sub 1), b-c: prohaemocyte-like cells (sub 2 and sub 3), d: semi-granular cells (sub 4), e: granular cells (sub 5) (Dantas-Lima et al., 2013).

Hyaline cells have a spindle or ovoid shape with few small basophilic and eosinophilic granules. Semi-granular cells have an ovoid shape and contain several eosinophilic granules. Granular cells have a spherical shape and consist of many large eosinophilic granules. Prohaemocyte-like cells have a small and spherical or slightly oval shape and possess only a thin ring of basophilic cytoplasm around a central nucleus.

An *in vitro* experiment showed that prohaemocyte-like cells display only very weak adherence to glass with no evidence of cytoplasmic spreading, whereas hyalinocytes and semi-granulocytes are known to present strong adherence to the substrate and display acute spreading behaviour. Adherence appears to be more limited in the granular cells (Vargas‐Albores et al., 2005; Roulston & Smith, 2011; Dantas‐Lima et al., 2013). Each cell type is active in defense reactions and carries out different functions in immunity. In crayfish, *Astacus astacus*, *Pacifastacus leniusculus*, *Procambarus clarkii*, the hyaline cells are mainly involved in phagocytosis, the semi-granular cells, which have limited phagocytic capacities, would be specialized in particle encapsulation, while the granular cells participate in storage and release of the prophenoloxidase (proPO) (Smith & Söderhäll, 1983). In penaeid shrimp, *Penaeus indicus*, semi-granular and granular cells are responsible for phagocytosis (Jayasree, 2009) while hyaline and semi-granular cells of *Penaeus vannamei* mainly function in the phagocytosis of pathogenic and non-pathogenic bacteria (Tuan et al., 2015). In ridgeback prawn, *Sicyonia ingentis*, phagocytosis of the Gram-negative marine bacterium (*Cytophaga* sp.) is accomplished primarily by small granule haemocytes, rarely by large granule haemocytes, and never by hyaline cells (Hose et al., 1990).

Both semi-granular and granular cells can be induced to degranulate by foreign molecules, such as lipopolysaccharides (LPS) or β-1,3-glucans. The semi-granular cells are the first haemocyte type to react to foreign particles *in vivo* and they respond by degranulation (Johansson and Söderhäll, 1985), releasing the proPO system including the cell adhesion/degranulating factor peroxinectin from their granules into the plasma (Johansson et al., 1995).

1.5 Separation of biological particles

Several techniques such as density gradient centrifugation, fluorescent activated cell sorting, magnetic activated cell sorting and monoclonal antibodies have been developed for the purification of specific types of biological particles. Selection of the technique,

however, depends on the characteristics of the biological particles and the objectives of the study. Density gradient centrifugation is probably preferable. Percoll (colloidal silica), Ficoll (high molecular weight organics), iodixanol (iodinated organic compounds), sucrose (small hydrophilic organic molecules), caesium chloride (CsCl; salt of alkali metal) and sodium bromide (NaBr; inorganic salt) are the media mostly selected (Lawrence & Steward, 2010). In general, iodixanol and Percoll have more advantages than other media. These media have a low viscosity, are non-toxic to cells and have the capacity to form self-generating gradients.

The viscosity influences not only the speed of the separation but also the structure of the particles. The non-toxicity of these products excludes the need for washing steps (Graham, 2001). The capacity to form self-generating gradients depends more on the centrifugation time. Percoll tends to form non-linear S-shaped gradients in short centrifugation time and nearly linear gradients in longer centrifugation time. The density curve of Percoll gradient presents two steep regions on the top and at the bottom of the gradient. Between those regions, there is a wide and shallow region with a shorter density range. Therefore, these gradients tend to excessively concentrate cells in the steep areas on the top and at the bottom of the gradient and to disperse them in the shallow area in-between (Graham, 2001). In contrast, iodixanol can form a linear, continuous gradient more easily by diffusion of preformed discontinuous gradients.

Iodixanol, a non-ionic iodinated compound with a molecular mass of 1550, was developed in the early 1990s as an X-ray contrast medium and has been subjected to rigorous clinical testing. It is considered as non-toxic to cells and can be made iso-osmotic at all useful densities. It also has low viscosity and osmolality. Besides, iodixanol is capable of forming self-generating gradients in 1 to 3 h. Its systematic chemical name is 5,5′-[(2-hydroxy-1,3-propanediyl)-(acetylamino)] bis-[N,N-bis(2,3-dihydroxypreopyl)-2,4,6-triiodo-1,3-benzenecarboxamide.

1.6 Methods for crustacean haemocyte separation

In order to study crustacean haemocytes in an optimal way, it is preferable to work with isolated populations of different cell types. Density gradient centrifugation is the first technique used for separating crustacean haemocytes into subpopulations. This technique was developed by Söderhäll and Smith (1983) for several marine crab species such as *Carcinus maenus*, *Cancer pagurus*, *Macropipus depurator*, *Eupagurus*

bernhardus and *Nephrops noriegicus*. The success of the isolation of haemocytes depends on the efficiency of the anti-coagulant used. Haemolymph of marine crab was collected in a low pH citrate-EDTA anticoagulant buffer and separated by centrifugation on a continuous density gradient of Percoll. After the separation in this type of density gradient, hyaline and granular cells were separated. In this technique, citric acid serves to delay cellular breakdown, while EDTA inhibits prophenoloxidase (proPO) activation and prevents the clotting reaction, which is dependent on Ca^{2+} and transglutaminase (Hall et al., 1999). Low pH in this buffer, in combination with citrate, glucose and NaCl, provides an optimal medium for maintenance of cell integrity without significant loss of cell viability. With some modification, this methodology was applied to separate haemocytes of the crayfish *Astacus astacus* (Smith & Söderhäll, 1983), the mussel *Mytilus edulis* (Pipe et al., 1997), the swimming crab *Liocarcinus depurator* (Hammond & Smith, 2002), the penaeid shrimp (Liu et al., 2005; Vargas‐Albores et al., 2005), the Caribbean spiny lobster, *Panulirus argus* (Li & Shields, 2007), and the spider crab *Hyas araneus* (Roulston & Smith, 2011).

Fluorescence activated cell sorting (FACS) is another option for separation of crustacean haemocytes. It is a simple, reproducible, and sensitive method. However, there are no data on the *in vitro* culture of separated cells after sorting. Furthermore, there are no fully characterized monoclonal antibodies available to sort certain subpopulations. Several attempts were made to produce monoclonal antibody markers specific for certain haemocyte subpopulations of crustacea (van de Braak et al., 2000; Sung & Sun, 2002; Wu et al., 2008). Unfortunately, none of these specific markers are commercially available.

Recently, another method using different concentrations of iodixanol was successful not only for separation of haemocyte subpopulations of penaeid shrimp, *Litopenaeus vannamei*, but also for *in vitro* culture of separated haemocytes (Dantas-Lima et al., 2013).

1.7 Crustacean haemocyte culture

Crustacean haemocyte cultures have been studied for the last two decades and the survival time of these haemocytes has been reported (Ellender et al., 1992; Chen & Wang, 1999; Itami et al., 1999; Jiang et al., 2006; Li & Shields, 2007; Jose et al., 2010; Roulston & Smith, 2011; Dantas-Lima et al., 2012; Dantas-Lima et al., 2013), ranging

from 2-4 days (Chen & Wang, 1999) to 3-4 weeks (Ellender et al., 1992).

Culture medium is the most important element having an impact on the survival of animal cells (Mothersill & Austin, 2000). Many attempts have focused on the selection and optimization of culture medium in order to prolong the survival of crustacean cells and haemocytes. Generally, a selected culture medium must supply all essential nutrients for cellular growth, such as amino acid, salts, and vitamins. Out of 17 publications, Leibovitz's medium has been a popular choice for crustacean haemocyte culture due to its strong buffering capacity.

Regardless to the choice of basic culture medium, the culture medium must be adjusted to the physico-chemical requirements of crustacean cells, depending on whether the species are freshwater or marine water. For instance, a double concentration of Leibovitz's medium (2x-L15) is used to culture of marine crustacean haemocytes, while a single concentration (L15) is applied to freshwater ones. Three main factors must be considered. The osmolality should be adjusted to 400-500 mOsmol/kg for freshwater species and to 700-1000 mOsmol/kg for marine species. The pH of the culture medium should be relatively alkaline (7.0-7.4) (Toullec, 1999). Last but not least, the temperature for penaeid shrimp haemocyte culture should be between 25-28^{o}C (Toullec, 1999; Jose et al., 2011; Dantas-Lima et al., 2012). Besides, other supplements like mammalian serum and antibiotics are also considered to be essential for maintaining *in vitro* cultures of crustacean haemocytes.

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Chapter 2

Aims of thesis

Knowledge on crustacean immunology is mainly based on research with crayfish. Mainly processes mediated by circulating haemocytes such as phagocytosis, encapsulation, coagulation and cytotoxic reaction have been elucidated. However, the knowledge on shrimp's immune system, *Penaeus* (*Litopenaeus*) *vannamei*, is still limited. Currently, the research on the immunity of penaeid shrimp is expanding because of the economic importance of shrimp aquaculture throughout the world and the significant impact of infectious diseases. Diseases could be prevented by regularly monitoring the immune state of the shrimp to detect as early as possible any abnormal condition. In order to develop effective strategies for disease control in shrimp culture, it is necessary to acquire thorough knowledge on shrimp immune system, especially the role of each type of haemocytes in shrimp defense. Therefore, the general objective of this thesis was to obtain a better understanding of the functions of haemocyte subpopulations in the defense system of *Penaeus (Litopenaeus) vannamei*.

The specific objectives of this thesis were:

(1) To develop a technique for separating haemocyte subpopulations and to characterize these subpopulations.

(2) To study the differences in uptake and killing of pathogenic and non-pathogenic bacteria by different haemocyte subpopulations.

(3) To investigate the uptake and disassembly of white spot syndrome virus by haemocyte subpopulations and the induction of apoptosis.

Chapter 3

Separation of *Penaeus vannamei* haemocyte subpopulations by iodixanol density gradient centrifugation

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** Equally contributed*

Abstract

Methodologies for separation of immune cell subpopulations are essential tools in immunology studies. Up to date, only one methodology for separating crustacean haemocyte subpopulations using Percoll density gradient centrifugation has been described.

In the present work, a new methodology to separate *Penaeus vannamei* haemocyte subpopulations was developed, using a two-step iodixanol density gradient centrifugation. *P. vannamei* haemolymph was collected with anticoagulant and centrifuged through a first gradient (densities from 1.063 to 1.109 g/ml) for 10 min at 2000 *g*. Three bands were formed: two bands with lower density close together, and a third band with higher density. The first two were collected together, whilst the third band was collected separately. The volume fraction in-between these bands contained dispersed cells and was also collected. The suspension containing the mixture of the first two bands was centrifuged through a second gradient (densities from 1.047 to 1.087 g/ml) for 15 min at 2000 *g*. Two bands were formed and collected individually. All the cell suspensions were used for *in vitro* culture (cell survival evaluation) and for evaluation of cell morphology by flow cytometry and light microscopy. Each of the three bands contained a major cell type with distinct morphology and behaviour. The dispersed cell fraction contained a mixture of two different cell types, which were distinct from the cell types in the bands. By order of appearance from the top of the gradient, the cell types were named: subpopulations (Sub) 1 (band 1), Sub 2 (band 2), Sub 3 + 4 (dispersed cells) and Sub 5 (band 3). The purity level (percentage of the major cell type) of Sub 1, 2 and 5 was 95.0 ± 1.0%, 97.7 ± 1.2% and 99.4 ± 0.8%, respectively. Cells of Sub 2 showed the best survival time *in vitro* (up to 96 h) followed by cells from Sub 1, Sub 3 + 4 and Sub 5. Phagocytic activity was detected in Sub 1 and 4.

This methodology allowed the separation and characterization of five morphologically distinct and physiologically active *P. vannamei* haemocyte subpopulations, from which three were isolated with a very high degree of purity. Therefore, we consider this methodology a valuable alternative for the traditional crustacean haemocyte separation procedure in Percoll.

1. Introduction

Haemolymph (blood of invertebrates) is composed of a liquid and a cellular fraction. The liquid fraction is named plasma. The cellular fraction is solely composed of haemocytes, the shrimp immune cells. Haemocytes are key players in invertebrate immunity since they mediate, directly or indirectly, all known invertebrate immune reactions. Crustacean haemocytes are traditionally divided into subcategories or subpopulations according to their morphological characteristics and/or functionality. Three morphologically distinct subpopulations have been described: (i) hyalinocytes or hyaline cells, (ii) semi-granulocytes or semi-granular cells, (iii) granulocytes or granular cells (Li, Shields, 2007; Söderhäll, Smith, 1983; van de Braak et al., 1996; Vargas-Albores et al., 2005). When stained with histological dyes, hyalinocytes display a spindle/ovoid shape and few small basophilic and eosinophilic granules, semi-granulocytes an ovoid shape and several medium-large eosinophilic granules and granulocytes a spherical shape and many large eosinophilic granules (Roulston, Smith, 2011; Smith, 2010). When exposed to foreign environments or substances, hyalinocytes and semi-granulocytes present strong adherence to the substrate and acute spreading behaviour. Adherence is more limited in granular cells (Roulston, Smith, 2011; Vargas-Albores et al., 2005). However, there is still some inconsistency in the description of the morphology, functionality and proportion of each of these cell types. This may be due to species-specific differences, but also the subjective classification of semi-granulocytes.

A protocol to separate crustacean haemocytes using a percoll density gradient was developed for the first time by Söderhäll and Smith (1983). This protocol was afterwards adapted to other invertebrate species (Falwell et al., 2011; Hammond, Smith, 2002; Li, Shields, 2007; Liu et al., 2005; Pipe et al., 1997; Roulston, Smith, 2011; Smith, Söderhäll, 1983; 1991; Sperstad et al., 2010; Sritunyalucksana et al., 2001; Vargas-Albores et al., 2005). This methodology allowed the separation of haemocyte subpopulations with no apparent deleterious effects since the cell functionality was preserved in most of the cases. Nevertheless, due to the density that is intrinsic to each cell type, only granulocytes were isolated efficiently.

Iodixanol is an alternative separation medium to percoll. Both media share suitable characteristics for an efficient separation of cells, cell organelles, and other subcellular structures. Nevertheless, iodixanol possesses some advantages over percoll. While

percoll has a very low osmolality (Pertoft et al., 1978) and as such often requires sucrose for the preparation of stock solutions, iodixanol has an osmolality of 290 mOsmol/kg (Solomon, 2005), what makes the preparation of isosmotic solutions easier. Percoll is light scattering at all wavelengths (Jenkins et al., 1979) and thus needs to be removed prior to most flow cytometry and spectrophotometry analysis. Iodixanol on the other hand, only exhibits absorbance at the UV range (Jacobsen, 2000). Another advantage of iodixanol is the formation of linear continuous gradients by passive diffusion of the preformed discontinuous gradient. This excludes the need of ultracentrifugation as for percoll self-forming gradients. The shape of iodixanol continuous gradients can be easily customized by manipulating the concentration and volume of the initial gradient fractions and diffusion time (Axis-Shield, 2012). On the other hand, self-forming percoll gradients present non-linear S-shaped gradients with two steep density profiles on top and bottom of the gradient and a shallow zone in between (Amersham Biosciences, 2001). These gradients have limited manipulation possibilities. The damage of cells centrifuged in percoll was previously reported (Juan et al., 2012; Oliveira et al., 2011). Conversely, no literature reporting cell damage by iodixanol centrifugation was found.

To the best of our knowledge, the present work described for the first time a procedure to efficiently separate highly pure *P. vannamei* haemocyte subpopulations using iodixanol density gradient centrifugations. The high purity of the subpopulations was obtained by centrifuging the haemocytes through a sequence of two differently shaped iodixanol density gradients. Additionally, cell subpopulations were subjected to morphological and viability analyses.

2. Materials and methods

2.1 Shrimp and experimental conditions

Penaeus (*Litopenaeus*) *vannamei* post-larvae which were certified to be specific pathogen free (SPF) for WSSV, TSV, YHV and IHHNV were imported from Piti Syaqua Farm, Syaqua Siam Co. Ltd., Thailand. Upon arrival, they were reared in a recirculation system at the Laboratory of Aquaculture and Artemia Reference Center (ARC), Ghent University, Belgium until they reached an adult size. Water temperature was kept at 27 ± 1°C, pH at 7.8-8.1 and salinity at 35 ± 1ppt. A biological filter and

regular water changes kept the total ammonia below 0.5 mg/L and nitrite below 0.15 mg/L. The room was illuminated 12 hours a day by dimmed TL-light.
For this study, adult shrimp with a mean body weight of 40 ± 10 g in C moult stage (inter-moult stage) (Corteel et al., 2012) were selected. They were transported to the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University and acclimatized at 27 °C for 1 hour before extracting haemolymph.

2.2 Iodixanol density gradient preparation

A discontinuous density gradient composed of 2.5 ml iodixanol (Optiprep, Axis-Shield) fractions of different concentrations was prepared by fraction under-layering (Figure 1). The fractions were loaded in 15 mL non-pyrogenic polypropylene centrifuge tubes (SARSTEDT) using 2 ml syringes and 20G (0.9x70mm) needles. The gradient concentration profile (from the top to the bottom of the tube) was: 10%, 15% and 20%. The iodixanol solutions were prepared by diluting the stock solution (60% iodixanol) in shrimp PBS (shPBS; PBS adjusted to 900 mOsmol/kg with NaCl; pH 7.4). This preparation was kept for 18 h at 4°C to allow the formation of a continuous gradient. Afterwards, the gradient was either used for haemocyte separation or entirely collected in 0.5 ml fractions for determination of its density profile. The density was determined by measuring the absorbance at 244 nm (Nanodrop 2000 spectrophotometer), following the procedure specified by the Optiprep manufacturer (Axis-Shield, UK). Briefly, a set of solutions with known iodixanol concentrations (5, 10, 15, 20, 25 and 30%) were prepared in distilled water (DW) and further diluted 1:1000 (v/v). The absorbance was measured and the iodixanol concentration converted into density (g ml^{-1}) using the formula indicated by the manufacturer. A standard curve expressing absorbance vs. density was made and a correlation formula was calculated. The absorbance of the gradient samples was measured following the same procedure and its density calculated using the correlation formula.

2.3 Haemolymph extraction and haemocyte separation

Haemolymph was extracted with cold Marine Anticoagulant as described by Dantas-Lima et al. (2012). Briefly, haemolymph was collected with syringes filled with anticoagulant at a proportion of 1:1 with the required volume of haemolymph. The collection was done from the ventral sinus located at the second tail segment and immediately layered carefully on top of the iodixanol gradient using a pipette (Figure 1). This preparation was centrifuged in a swinging bucket centrifuge (Rotina 380R, Hettich Lab Technology) at 2000 *g* for 10 minutes at 4°C. The haemocyte bands that were formed in the gradient were collected and the haemocytes were either used for *in vitro* culture and survival evaluation or fixed for morphological (H&E staining) and flow cytometry analysis. The separated haemocytes were fixed in an equal volume of Marine Fixative at double concentration (2% glutaraldehyde with 2% saccharose in seawater) (Cima, 2010) for 30 minutes at 4°C.

2.4 Morphological characterization

2.4.1 Light microscopy and live cell imaging of haemocyte cultures

Haemocyte cultures were observed under a light microscope (Olympus IX50) 1 h after seeding. Morphological characteristics of haemocytes and purity of the cultures (percentage of the major cell type) were evaluated for each cell band and fraction. Additionally, live cell imaging videos were made using ImageJ software and the behaviour of the cells was registered (Abramoff, 2004).

2.4.2 Flow cytometry

Fixed haemocytes were analysed on a FACS Aria III (Beckton Dickinson). Analysis was performed using the 488 nm laser. Statistics were obtained by the FACS Diva software (Version 6.1.3, Beckton Dickinson). The relative size and granularity (subcellular complexity) were assessed by forward scatter height (FSC-H) and side scatter area (SSC-A), respectively. For each sample, at least 20,000 events were counted. Results were expressed as contour plot graphs indicating the relative size (FSC) and granularity (SSC) of the cells of each band.

2.4.3 Light microscopy of fixed haemocytes

Fixed haemocytes were cytospinned (Cytospin 3, Shandon) at 700 rpm for 5 minutes onto glass slides. After drying, cells were stained with Haematoxylin and Eosin (H&E) in an automatic staining machine (Linear Stainer II SAKURA). Slides were dipped in each staining bath for 105 seconds. The bath sequence was: 1x distilled water (DW), 2x haematoxylin, 2x DW, 3x eosin, 2x DW, a dehydration series of 50%, 70%, 80%, 94%, 100% ethanol and finally 2x in xylene. After drying, slides were mounted with DPX mounting medium. Cells were observed under light microscope (Olympus BX61) and pictures were taken. The size and morphological characteristics of cells were evaluated.

2.5 Haemocyte in vitro culture and survival evaluation

Haemocyte survival evaluation was done as previously described by Dantas-Lima et al. (2012). Cell bands were collected from the gradient and immediately diluted with Haemocyte Medium (HM; 2xL-15 medium, 10.5% Chen's salts, 10% FBS, 1% penicilline/streptomycine; pH 7.5; 900 mOsmol/kg). Cells were seeded in 24-well plates (Nunc® Nunclon™ Δ Surface) in which round glass coverslips were previously brought in each well. A volume of 400 µl of cell suspension (150,000 cells well^{-1}) was seeded in each well. Samples were taken at 0, 2 and 24 h and after every 24 h until the end of each experiment. Cells were stained with EMA (ethidium monoazide bromide) dye for 30 minutes and with Hoechst dye for 10 minutes. At the end of the procedure, haemocytes were fixed and mounted on glass slides. Survival was evaluated under fluorescence microscope and expressed as total number of living cells per well over time. Each experiment was repeated 3 times and the average values were calculated.

2.6 Validation of the functionality of separated haemocyte subpopulations

2.6.1 Production of inactivated GFP-labelled bacterial stocks

GFP-labelled *Vibrio campbellii* (LMG 21363) was obtained as previously described by

(Dantas-Lima et al., 2012). Briefly, *V. campbellii* was transformed with a plasmid containing Green Fluorescent Protein (GFP), which was carried by *Escherichia coli* DH5α. After transfection colonies of GFP-labelled *V. campbellii* were isolated based on their antibiotic resistance and subsequently grown in marine broth, washed and stored at -80 °C in 20% glycerol.

2.6.2 Detection of phagocytic activity

GFP-labelled *V. campbellii* were sub-cultured twice in HM containing selective antibiotics (20 µl of bacterial suspension in 20 ml of HM for 12 h and 14 h at 27°C). Suspensions were washed as described above. The concentration of bacteria in the suspension was determined by optical density at 600 nm (OD600) and by the conversion formula CFU/ml = (10 x OD_{600} - 1) x 10^8.

Cultures of separated haemocyte subpopulations (150,000 cells $well^{-1}$) were inoculated with 100 bacteria per haemocyte at 1 h after seeding. Samples were taken at 0 and 1 h after inoculation. Before sampling, wells were washed 2 times with HM. At the moment of sampling, cells were fixed with 4% PF for 10 min, permeabilized with 0.1% Triton X-100 for 5 min and stained with Texas Red-labelled phalloidin (Invitrogen, Life Technologies™) diluted in PBS (4 units ml^{-1}) for 1 h at 37°C. Ten minutes before the end of this staining, Hoechst (0.01 mg ml^{-1}) was added. After, cells were washed and mounted on glass slides. The detection of phagocytic activity (haemocytes uptaking bacteria) was made using confocal microscopy. Sequential confocal pictures in three different wavelength emission channels (Hoechst: 461 nm; Texas Red: 615 nm; and GFP: 509 nm) were taken from the cell base to its apex. This was made in 10 cells that presented signs of phagocytosis.

3. Results

3.1 Separation of haemocytes in iodixanol density gradients

After 18h of incubation at 4°C, the iodixanol gradient became nearly linear. The density profile from the top to the bottom of the tube was: 1.063, 1.063, 1.064, 1.067, 1.070, 1.075, 1.080, 1.084, 1.089, 1.093, 1.095, 1.100, 1.105, 1.110, 1.109 g/ml (Figure 2A). After centrifugation, three cell bands were clearly formed (Figure 1). They were

collected and starting from the top of the gradient, they were named Band 1, 2 and 3. The volume in between bands 2 and 3 was also collected. Although this volume contained a substantial amount of cells, they did not form a sharp band. Therefore, this fraction was described as “dispersed cells”. The tube’s volumetric graduation was used as a marker for the location of the bands in the gradient. Bands 1 and 2 were located in between the marks of 4.3-5 ml and band 3 in between the marks of 2.5-3 ml (Figure 1). Bands 1 and 2 were physically too close to each other to allow their individual collection without cross-contamination. Thus, they were collected together in a volume of 1 ml, diluted in 2 ml of shPBS and loaded in another gradient. This gradient was designed to promote the physical separation of the bands. From then on, the first gradient and the new gradient were named Gradient 1 and Gradient 2, respectively. The procedure for preparing Gradient 2 was similar as for Gradient 1. It was composed of 2.5 ml iodixanol fractions with concentrations of 7%, 10%, 13% and 16%. Its density profile after 18h at 4°C was: 1.047, 1.046, 1.048, 1.050, 1.052, 1.054, 1.058, 1.060, 1.062, 1.064, 1.069, 1.071, 1.076, 1.077, 1.078, 1.082, 1.084, 1.087 g/ml (Figure 2B). The suspension containing Band 1 and 2 was loaded on Gradient 2 and centrifuged at 2000 *g* for 15 minutes at 4°C. The formed bands were more diffuse than in Gradient 1 (more difficult to visualize), but also more physically separated. This allowed their individual collection with a low cross-contamination. Band 1 was located in between the marks of 4-4.5 ml and Band 2 in between the marks of 3-3.5 ml (Figure 1). The approximate average buoyant density in iodixanol of the cells from Band 1 was 1.075, Band 2 was 1.078 and Band 3 was 1.095 g/ml (Figure 2).

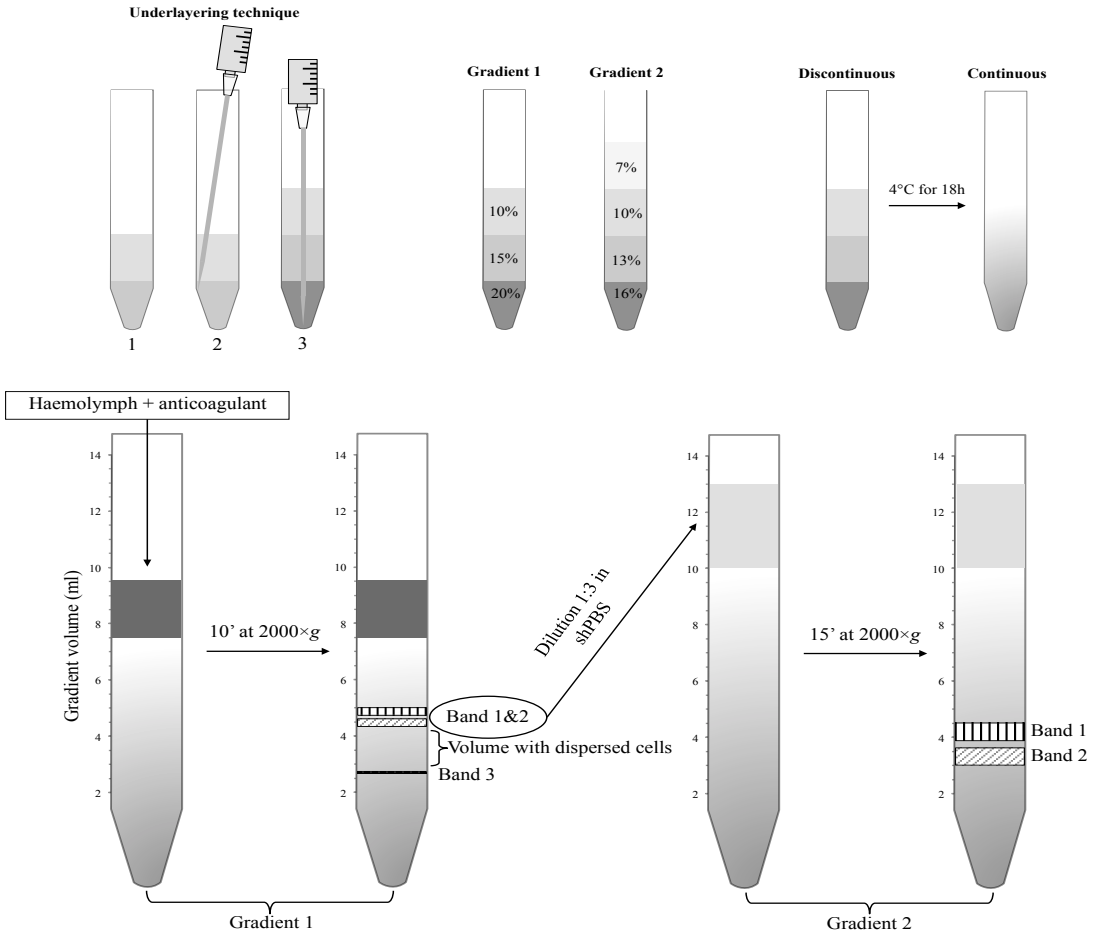

Figure 1. Diagram illustrating the preparation of continuous iodixanol density gradients and centrifugation procedure for separation of *P. vannamei* haemocyte subpopulations. The gradients were prepared by under-layering of 2.5 ml iodixanol fractions with different concentrations. An incubation step (18h at 4°C) allowed the diffusion of the fractions and the formation of linear continuous gradients. The haemocyte separation procedure included 2 centrifugation steps with two different gradients. This procedure was necessary because bands 1 and 2 obtained in gradient 1 were to close to each other to be collected individually without cell type cross-contamination. The centrifugation of these two bands in gradient 2, which had narower density profile (see figure 2A), promoted the physical separation of bands 1 and 2, allowing their individual collection without significant cross-contamination (high purity).

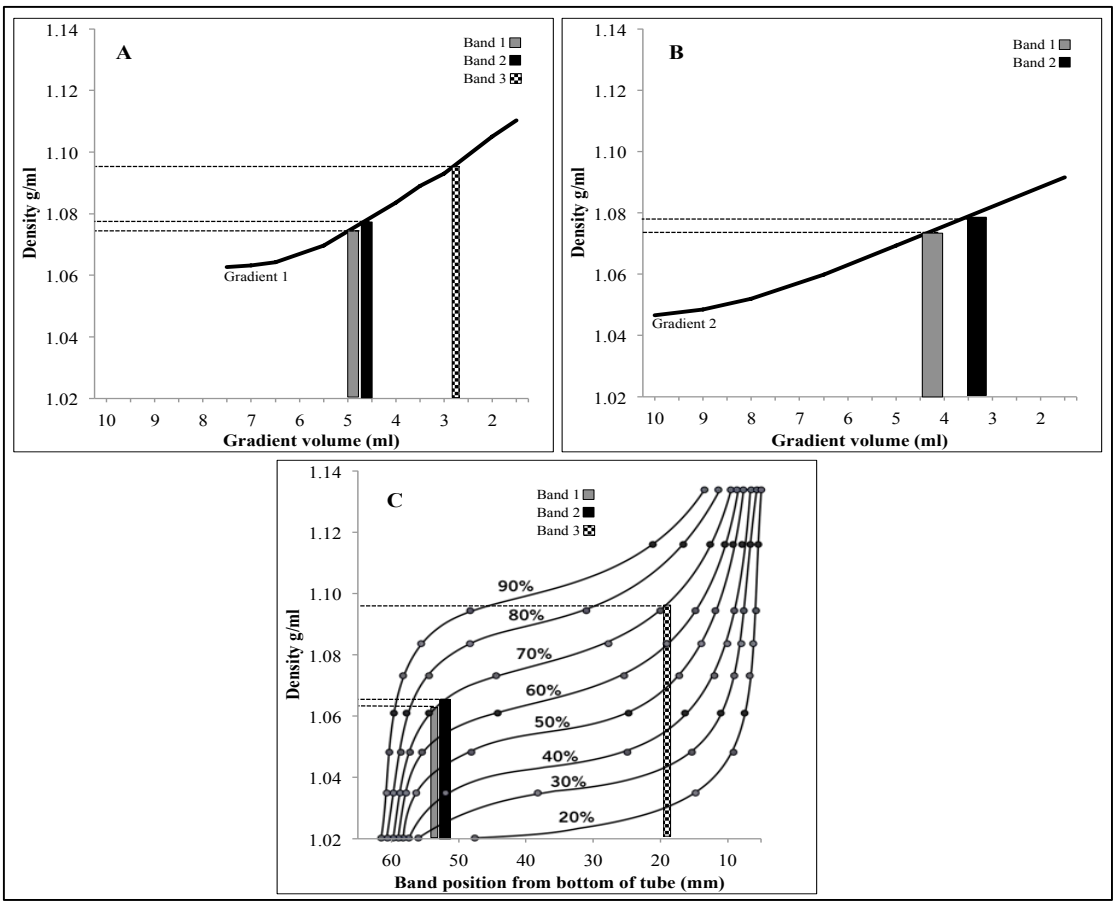

Figure 2. Iodixanol density gradients used in the present work (A and B) and self-forming percoll gradients using several initial concentrations (C) (Adapted from Amersham Biosciences, 2001). The iodixanol gradients display a continuous and nearly linear density profile. The vertical bars represent the haemocyte bands indicating their position in the gradients. They also indicate the approximate buoyant density of each cell type. Percoll gradient density curves represent self-forming gradients with starting concentrations of stock isotonic percoll from 20% to 90% in 0.15 M NaCl. Running conditions were: 23º angle-head rotor 30000 *g* for 15 minutes. The vertical bars represent the approximate positions of the bands in the gradient according to the work of Liu et al. (2005) on *P. vannamei* haemocyte separation. The 70% gradient profile was created using similar conditions to the ones this author used.

3.2 Morphological characterization

3.2.1 Light microscopy and live cell imaging of haemocyte cultures

Figure 3 and live cell imaging videos provided visual details of the morphology and behaviour of cells in culture. Cells from Band 1 adhered very strongly to the glass by means of pseudopod-like projections, which was translated in a high degree of cell spreading. Cells from Band 2 on the other hand, presented a very limited spreading activity and adherence to the glass. These cells were easily resuspended by gentle

pipetting. Cells from Band 3 adhered strongly to the glass with moderate spreading. The “dispersed cells” fraction enclosed 2 cell types; small cells and big cells with a similar morphology to the cells from Band 2 and 3, respectively. From then on, the different haemocytes were classified as subpopulations (Sub): Sub 1 (Band 1), Sub 2 (Band 2), Sub 3 (small cells of dispersed cells), Sub 4 (big cells from dispersed cells) and Sub 5 (Band 3). The purity (percentage of the major cell type) of Sub 1, 2 and 5 was 95.0 ± 1.0%, 97.7 ± 1.2% and 99.4 ± 0.8%, respectively. Since Sub 3 and 4 were mixed in the “dispersed cells” fraction, the purity level could not be evaluated. Starting from 24 h of culture, it was common to observe cellular breakdown due to cellular over-spreading in Sub 1. In Sub 2 and 3, some cell lysis was observed after 1 h of culture and some clustering activity after 24 h of culture. Cells of Sub 4 and 5 started to show signs of apoptosis (nuclear condensation and fragmentation) and degranulation after 2 h of culture. Massive apoptosis was observed at 24 h.

The live cell-imaging videos revealed differences in cellular motility and morphology and confirmed population’s purity level. Sub 1 cells adhered and spread strongly over the glass but did not display an intense movement. In contrast, Sub 2 and 3 cells remained mostly rounded and exhibited very limited pseudopod-like projections and adherence. The movement displayed by these cells was mainly caused by brownian motion. Cells of Sub 4 and 5 demonstrated more intense activity, both by diapedesis and cytoplasmic granules displacement.

3.2.2 Flow cytometry

For each subpopulation, the values of forward and side scatter were related with the cell diameter and granularity, respectively. The standard deviations were supplied for each average value. Cells of Sub 1 had a small average diameter (126.4 ± 4.6) and presented the lowest granularity (2.3 ± 0.1) (Figure 3 and Table 1). Sub 2 contained the smallest cells (107.2 ± 4.5), which were more granular (3.7 ± 0.5) than cells of Sub 1. The cells of Sub 3 were slightly bigger (118.2 ± 9.4) and less granular (3.4 ± 0.6) than Sub 2 cells. Sub 4 cells were bigger (171.5 ± 16.3) but less granular (6.0 ± 2.9) than cells of Sub 5. The latter were the biggest cells (166.2 ± 6.0) with the highest granularity (10.6 ± 2.9).

3.2.3 Light microscopy of fixed haemocytes

The H&E staining of fixed cell cytospins provided morphological details of separated haemocytes (Figure 3). The average cell diameter was the smallest in Sub 2 (7.5 ± 1.3μm) followed by Sub 3 (7.8 ± 0.9μm), Sub 1 (8.6 ± 0.8 μm), Sub 5 (9.9 ± 1.0 μm) and finally Sub 4 (10.5 ± 1.5μm). The nucleus/cytoplasm ratio was high in Sub1 and 3 and very high in Sub 2. In Sub 4 and 5 this ratio was low. The cytoplasm was eosinophilic in all the cells but with a more intense staining in Sub 4 and 5. The granularity content increased from the cells on top of the gradient (Sub 1) to the ones at the bottom (Sub 5). These granules were always basophilic (when present) in Sub 1, 2 and 3. Sub 4 presented a high number of granules that were predominately eosinophilic with sporadic appearance of basophilic ones. This situation was the same in Sub 5, although the number of granules and their staining intensity was higher. The nuclei of cells in Sub 1 and 4 were in general large with dispersed chromatin (euchromatin). The nuclei of cells in Sub 2 and 3 were small, folded and with very condensed chromatin (heterochromatin). Sub 5 cells had in general small and condensed nuclei. Table 1 presents a resume of the parameters described above.

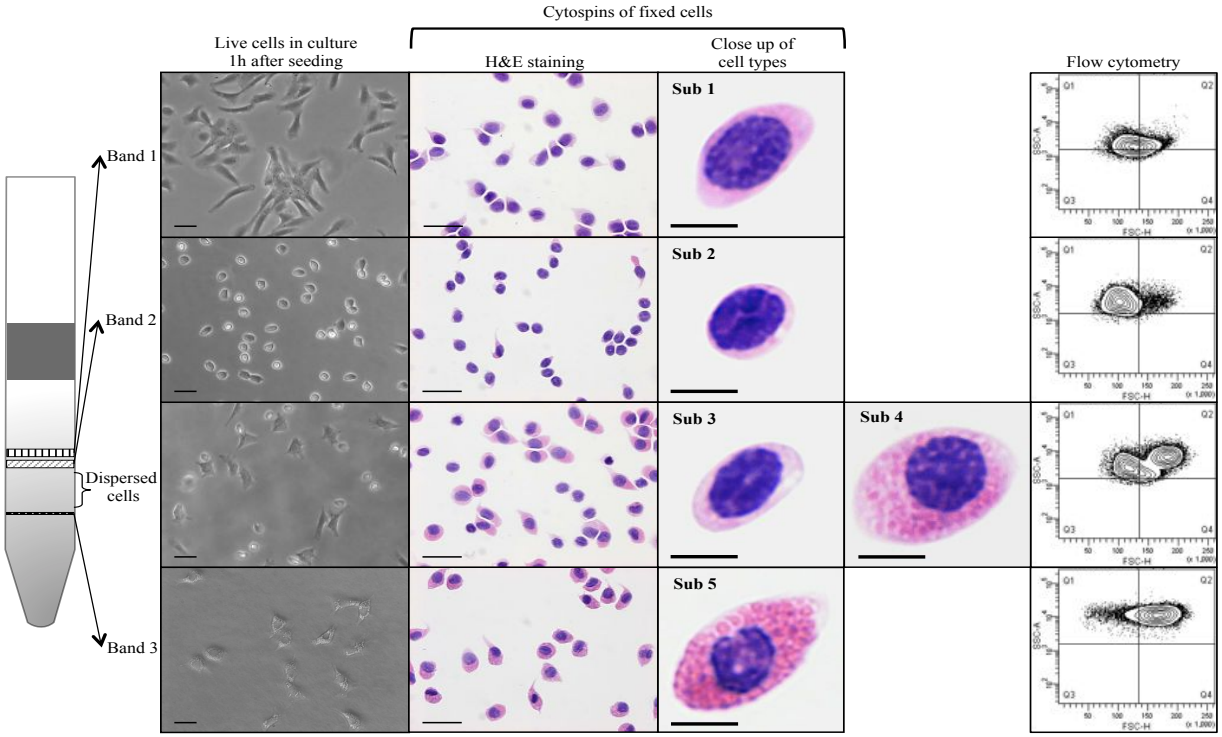

Figure 3. Analysis of individual *P. vannamei* haemocyte bands by *in vitro* culture, H&E staining and flow cytometry. Subpopulation 1 (Sub 1) showed cells that attached strongly to glass, had an average diameter size, contained little or no basophilic granules in the cytoplasm and a high nucleus/cytoplasmic ratio (N:C). Subpopulation 2 (Sub 2) enclosed cells that did not attach or attached very weakly to glass, had a small diameter size, contained few small basophilic cytoplasmatic granules and had a very high N:C ratio. The dispersed cells fraction enclosed two cell types, cells of Subpopulation 3 (Sub 3) and cells of Subpopulation 4 (Sub 4). Subpopulation 3 cells behaved similarly to Subpopulation 2 cells but had a higher N:C ratio and diameter. Subpopulation 4 cells behaved similarly to Subpopulation 5 (Sub 5) cells but the granularity was lower and the diameter was the biggest. Subpopulation 5 attached moderately to glass, their diameter was big, contained a high amount of large eosinophilic/basophilic granules and low N:C ratio. Scale bars in living cells and H&E staining = 20 µm and in individual cells = 5 µm.

Table 1. Characteristics of the haemocyte bands and diffused cells fraction by Flow Cytometry (FC) and Light Microscopy (LM). The values of haemocyte average size and granularity were obtained from FC forward scatter (FSC) and side scatter (SSC), respectively. The FC calibration and settings were kept between measurements. The percentage of each subpopulation in the whole haemocyte population was determined by cell counts before seeding into the culture plates. The purity % represents the percentage of the major cell type in each subpopulation counted by LM.

	Average size		Granularity/Granule staining		Attachment to glass	% of the total	% of Purity	Average density
Subpopulation	FC (FSC)	LM (µm)	FC (SSC)	LM	LM	LM	LM	(g/ml)
1	126.4±4.6	8.6±0.8	2.3±0.1	Very low/Basophilic	Yes/Very strong	41.7±3.3	95.0±1.0	1.075
2	107.2±4.5	7.5±1.3	3.7±0.5	Low/Basophilic	No/Very weak	48.8±3.3	97.7±1.2	1.078
3	118.2±9.4	7.8±0.9	3.4±0.6	Low/Basophilic	No/Very weak	-	-	-
4	171.5±16.3	10.5±1.5	6.0±2.9	Moderate/ Eosinophilic and basophilic	Yes/Strong	-	-	-
5	166.2±6.0	9.9±1.0	10.6±2.9	High/ Eosinophilic and basophilic	Yes/Strong	5.3±0.7	99.4±0.8	1.095

3.3 Survival evaluation

The survival evaluation for each haemocyte subpopulation is presented in Figure 4. The cells of Sub 2 showed the best survival performance, followed by the cells of Sub1, Sub 3+4 and finally Sub 5. Living cells were detected up to 96, 48, 24 and 24 hours in Sub 2, Sub 1, Sub 3+4 and Sub 5, respectively.

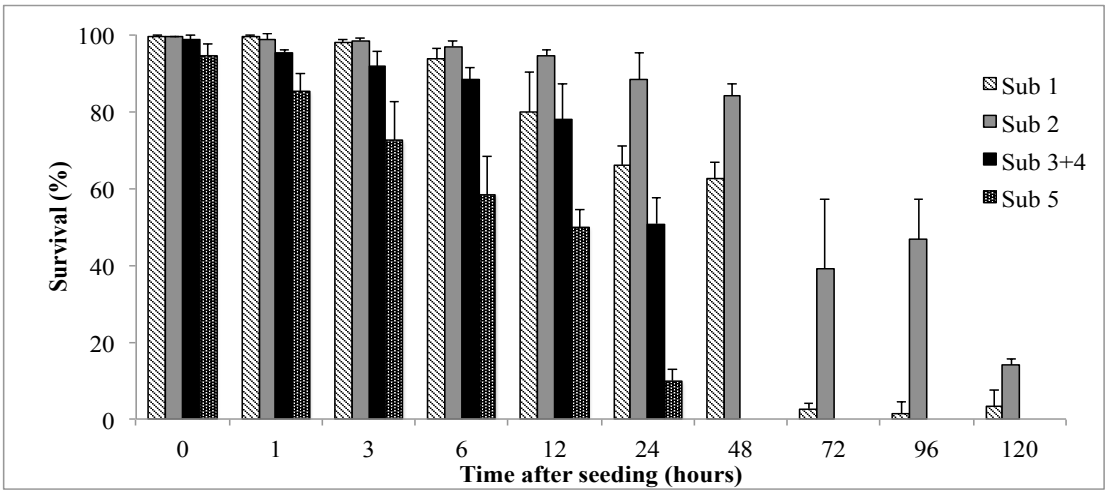

Figure 4. *In vitro* survival of separated *P. vannamei* haemocyte subpopulations.

3.4 Detection of phagocytic activity

After 1 h of co-culture with GFP-labelled *V. campbellii*, phagocytosis was only detected in Sub 1 and Sub 4. The remaining subpopulations did not show any uptake (internalization) of bacteria (Figure 5).

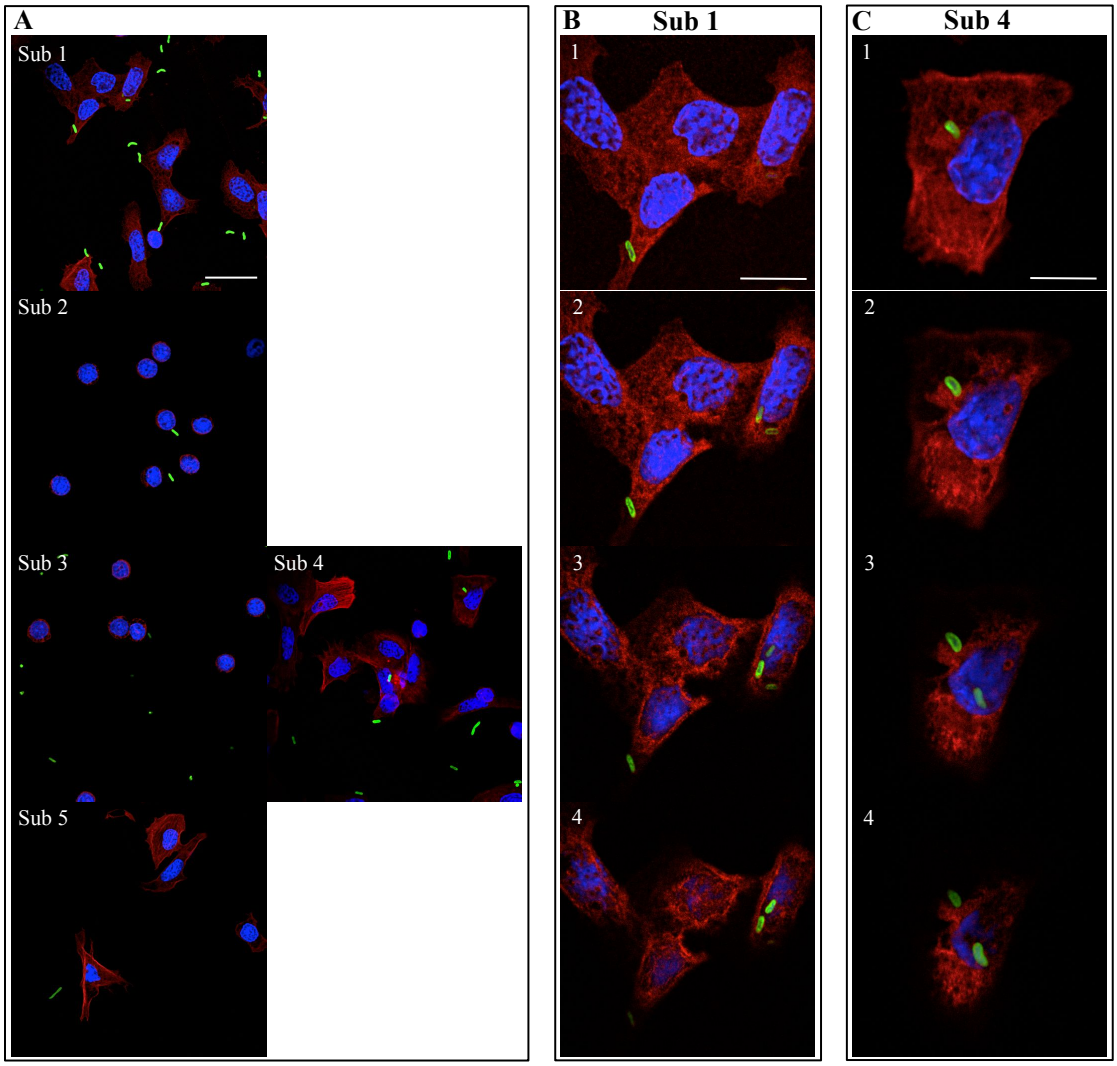

Figure 5. Phagocytosis of *V. campbellii* by separated *P. vannamei* haemocyte subpopulations at 1 hour post inoculation (hpi). The images in A provide a general view of the cultures at 1 hpi. The image sequences presented for Sub 1 (B) and Sub 4 (C) are a magnification of the images presented in A. These images are a sequence of confocal microscopy pictures taken from the cell base (1) to its apex (4). This ilustrates the process of bacteria uptake and therefore proves that cells from Sub 1 and Sub 4 express phagocytic activity. F-actin fibres are stained with phalloidin-Texas Red (red), the nucleus is stained with Hoechst (blue) and GFP-labelled *V. campbellii* exhibit green fluorescence. Scale bars: A= 15 µm; B= 8 µm; C= 5 µm.

4. Discussion

The centrifugation of *P. vannamei* haemocytes through iodixanol density gradients allowed the isolation and collection of three visibly distinct haemocyte bands. Additionally, the cells collected from the interphase between those bands (dispersed cells) also presented morphological differences with the cells from the neighbouring bands.

Since Bands 1 and 2 were too close to each other to be collected without cross-contamination (Figure 1), we composed a new gradient (Gradient 2) especially designed to promote the physical separation of these two bands. The principle was to compose a gradient with a narrower density range (Gradient 1: 10-20% iodixanol; Gradient 2: 7-16% iodixanol) in a higher total volume (Gradient 1: 7.5 ml; Gradient 2: 10 ml). This created a gradient density profile curve with a smaller slope (narrower density range per unit of volume), when compared with Gradient 1 (Figure 2). Although the average density of cells of Sub 1 and 2 was similar (1.075 and 1.078, respectively) this strategy promoted their physical separation and consequently their collection with a high purity level was possible.

The analysis of the haemocyte bands revealed the existence of 5 haemocyte morphotypes or subpopulations. Two of them were easily identified based on existing literature (reviewed by Jiravanichpaisal et al., 2006). Sub 1 exhibited all the characteristics typically attributed to hyalinocytes and Sub 5 were clearly granulocytes. The classification of Sub 2, 3 and 4 was not that straightforward. Sub 4 resembled typical semi-granulocytes. The classification of the haemocytes of Sub 2 and 3 was very difficult. These cells were smaller but more granular than hyalinocytes and interestingly did not adhere to glass and presented folds in the nucleus. These characteristics suggested that Sub 2 could be classified as small hyaline cells (Rodriguez et al., 1995), small granule haemocytes or lymphocyte-like hyalinocytes (Hose et al., 1987; Vargas-Albores et al., 2005) and prohaemocytes or immature haemocytes (Roulston and Smith, 2011).

The objective of the present work was to develop a system to efficiently separate biologically-active haemocyte subpopulations. The efficiency was proven by the identification of 2 currently undescribed *P. vannamei* haemocyte subpopulations, the high degree of purity of the separated subpopulations and the reproducibility of the procedures. The experiments on the *in vitro* cell viability and phagocytic activity

proved that the isolated cells were biologically-active. However, in order to make a clear classification of these cell types, the performance of detailed histochemical and functional studies will be necessary.

The density profiles of the iodixanol gradients used in this study and several Percoll gradients are presented in Figure 2. The Percoll self-forming gradients traditionally used to separate crustacean haemocyte subpopulations are prepared with initial concentrations of 60-70% Percoll (Liu et al., 2005; Roulston and Smith, 2011; Söderhäll and Smith, 1983). The density curve of these gradients presents two steep regions on the top and at the bottom of the gradient. Between those regions, there is a high range of density values per unit of volume. In-between those regions, there is a wide and shallow region with a shorter density range. Therefore, these gradients tend to excessively concentrate cells in the steep areas on the top and at the bottom of the gradient and to disperse them in the shallow area in between (Graham, 2001). Our iodixanol density gradients were nearly linear, displaying a constant density increment over the entire gradient. This potentiated the formation and visualization of individual cell bands. Therefore, we considered this property an advantage of our procedure over the traditional one using Percoll.

To the best of our knowledge, this is the first report describing the separation of crustacean haemocytes using iodixanol density gradients. This efficient and reproducible separation procedure, allowed the identification of five *P. vannamei* haemocyte subpopulations. From those, three were separated with a high degree of purity. It was proven that these cells were alive over different time periods and functionally active, and as such suitable to be used in further functionality studies. This procedure appears to be a valuable alternative for the traditional separation in Percoll gradients.

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Chapter 4

Differences in uptake and killing of pathogenic and non-pathogenic bacteria by haemocyte subpopulations of the penaeid shrimp, *Litopenaeus vannamei,* (Boone)

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Abstract

Phagocytosis is an important function of both invertebrate and vertebrate blood cells. In this study, the phagocytic activity of haemocyte subpopulations of penaeid shrimp, *Litopenaeus vannamei*, (Boone), against pathogenic and non-pathogenic particles was investigated *in vitro*. The haemocytes of penaeid shrimp were firstly separated by centrifugation on a continuous density gradient of iodixanol into 4 fractions with 5 subpopulations (sub), of which sub 1 (hyalinocytes) and sub 4 (semi-granulocytes) mainly function in phagocytosis of both pathogenic and non-pathogenic bacteria as well as fluorescent polystyrene beads. It was found that these haemocyte subpopulations engulfed virulent *Vibrio campbellii* and *Vibrio harveyi* at a higher rate than non-virulent *Escherichia coli* and polystyrene beads. When these bacteria were mixed with shrimp haemocyte subpopulations and incubated for 180 min, the percentage of culturable intracellular *V. campbellii* (25.5 ± 6.0%) recovered was significant higher than the percentage recovered from *V. harveyi* (13.5 ± 1.1%). No culturable intracellular *E. coli* was observed in this study. In contrast with *V. harveyi* and *E. coli*, *V. campbellii* containing endosomes did not acidify in time. Incubation of haemocyte subpopulations with the most virulent *V. campbellii* strain resulted in a significant drop in haemocyte viability (41.4 ± 6.3% in sub 1 and 30.2 ± 15.1% in sub 4) after 180 min post inoculation in comparison with the less virulent *V. harveyi* (84.1 ± 5.6% in sub 1 and 83.4 ± 4.1% in sub 4) and non-virulent *E. coli* (92.7 ± 2.8% in sub 1 and 92.3 ± 5.6% in sub 4) and polystyrene beads (91.9 ± 1.6% in sub 1 and 84.4 ± 3.4% in sub 4). These findings may be a valuable tool for monitoring shrimp health and immunological studies.

1. Introduction

Penaeus (*Litopenaeus*) *vannamei* is one of the most commonly cultured species of penaeid shrimp in the Western hemisphere (Menz & Blake, 1980) and accounts for more than 95% of the total production (Lightner, 2011). With the rapid expansion, more disease problems have occurred (Lightner, 2011). One approach to control diseases in shrimp is to increase their internal defense against pathogens.

Immunity in crustacea is defined as a non-specific internal defense response that includes both humoral and cellular components, which cooperate to eliminate microorganisms. The blood cells (haemocytes) of crustacea play an important role in the defense reactions against pathogenic and non-pathogenic microorganisms, parasites and other foreign targets that might enter into the haemocoel (Bachere et al., 2004; Jose et al., 2010; Matozzo & Marin, 2010). The ability of blood cells to recognize non-self particles has been studied in a variety of species and recognition has been found to result in a number of cell-associated responses such as coagulation, phagocytosis, encapsulation and nodule formation (Hose et al., 1987; Johansson & Söderhäll, 1989; Hose et al., 1990).

Crustacean haemocytes are traditionally divided on the basis of morphology into three distinct subpopulations: (i) hyaline cells which possess an ovoid shape and have few small basophilic and eosinophilic granules, (ii) semi-granular cells which have an ovoid shape and contain a variable number of small eosinophilic granules, and (iii) granular cells which have a spherical shape and contain many large eosinophilic granules (Söderhäll & Smith, 1983; Van de Braak et al., 1996; Li & Shields, 2007; Smith, 2010; Hong et al., 2013). Recently, haemocytes of penaeid shrimp (*Litopenaeus vannamei*) were separated into 5 subpopulations (Dantas-Lima et al., 2013).

Phagocytosis is one of the major defense mechanisms when foreign particles or micro-organisms intrude their host. The phagocytic process is thought to occur in two steps, with the first step involving physio-chemical adherence of the foreign particles to the lining of the haemocoel and the second step involving attachment of haemocytes to the sites of bacterial adherence (Martin et al., 1996), leading to the engulfment of particles into the cell and subsequent formation of phagosomes to stimulate the microbial digestion.

Although the contributions of both hyalinocytes and granulocytes in cellular immune responses in haemolymph of crustacea are broadly studied (Smith & Söderhäll, 1983;

Goldenberg et al., 1984; Söderhäll et al., 1986), the haemocyte types involved in the phagocytic reaction have been reported to differ among crustacean species. Smith & Söderhäll (1983) reported that phagocytic activity of the crayfish, *Astacus astacus* and *Pacifastacus leniusculus*, was evident only for hyaline cells and semi-granular cells. In red swamp crayfish, *Procambarus clarkii*, hyalinocytes are considered as phagocytes (Söderhäll et al., 1986); semi-granulocytes, which have limited phagocytic capacities, would be specialized in particle encapsulation and granulocytes would participate in the pro-phenoloxidase (proPO) system (Söderhäll & Smith, 1983). Another study conducted by Söderhäll et al (1986) also reported phagocytic activity for hyaline cells of the crab, *Carcinus maenas*. In penaeid shrimp, *Penaeus indicus*, semi-granular and granular cells were responsible for phagocytosis (Jayasree, 2009). In ridgeback prawn, *Sicyonia ingentis*, phagocytosis of the Gram-negative marine bacterium (*Cytophaga* sp.) was accomplished primarily by small granule haemocytes, rarely by large granule haemocytes, and never by hyaline cells (Hose et al., 1990). In a previous study, the haemocyte subpopulations of penaeid shrimp were separated into 5 separated populations by centrifugation in a two-step continuous density gradient of iodixanol (Dantas-Lima et al., 2013).

The aims of the work described here were to investigate the phagocytic activity of *Litopenaeus vannamei* haemocyte subpopulations towards pathogenic and non-pathogenic bacteria as well as fluorescent polystyrene beads and to analyse the fate of both bacteria and cell upon ingestion.

2. Materials and methods

2.1 Experimental animals

Specific pathogen-free (SPF) penaeid shrimp, *Litopenaeus vannamei*, with a mean body weight of 25 ± 5 g in inter-molt (C) stage (Corteel et al., 2012) were used. The shrimp were imported from Holland and were reared in a recirculation system at the Laboratory of Artemia & Reference Center (ARC), Faculty of Bioscience Engineering, Ghent University, Belgium. They were fed twice daily at a total rate of 5% of their mean body weight. Water temperature was kept at 27 ± 1^{o} C, pH 7.5-8.0, and salinity at 35 ± 1 g l^{-1}. Regular water changes and bio-filters kept total ammonia-N below 0.5 mg l^{-1} and nitrite-N below 0.15 mg l^{-1}.

2.2 Bacteria and culture conditions

The bacteria *Vibrio campbellii* (LMG 21363) and *Vibrio harveyi* (BB 120), that are pathogenic to penaeid shrimp, and *Escherichia coli* DH5α, that is non-pathogenic to penaeid shrimp, were obtained from the Laboratory of Artemia and Reference Center, Ghent University, Belgium. These bacteria were labeled with Green Fluorescent Protein (GFP) and Fluorescent Isothiocyanate (FITC) as follows:

GFP-labeled *V. campbellii* and *V. harveyi* were obtained as previously described by Dantas-Lima et al (2012). Firstly, these bacteria were transfected with a plasmid containing Green Fluorescent Protein (GFP), which was carried by *E. coli* DH5α. After transfection, colonies of GFP-labelled bacteria were isolated based on their antibiotic resistance (Phuoc et al., 2009) and grown in marine broth, washed and stored at -80° C in 20% glycerol.

From the stock, 20 µl of bacterial suspension were sub-cultured in 20 ml haemocyte medium (2x Leibovitz's (L-15) basal medium (Sigma-Aldrich), 10.5% Chen's salts, 10% foetal calf serum, pH 7.55) containing 100 mg l^{-1} of rifampicin and kanamycin for 12 h at 27^{o} C in a shaker at 90 rotations per minute (rpm). Then, the bacteria were sub-cultured again at the same conditions for 14 h. The suspension was washed and centrifuged three times at 2000 *g* for 10 minutes. The concentration of bacteria in the suspension was determined through spectrophotometry at an absorbance of 600 nm (OD_{600}) and through the conversion formula from the standard curve, CFU/ml = $(10xOD_{600}-1)x10^{8}$ for *V. campbellii* and CFU/ml = $(40xOD_{600}-2)x10^{7}$ for *V. harveyi*.

FITC-labeled *E. coli* were labeled by a modification of the procedure of Weingart et al (1999). Briefly, bacteria were cultured in Luria-Bertani broth (LB) for 24 h at 37^{o} C in a shaker at 90 rotations per minute (rpm). The suspension was washed three times in 100 mM $NaHCO_3$, pH 9 at 2000 *g* for 10 min. Optical density readings at 600 nm were used to calculate microbial cell densities. An OD value of 1.0 corresponds to $1.2x10^{9}$ cells ml^{-1} (McFarland standard). The pellet was re-suspended in 100 mM $NaHCO_3$, pH 9 containing 0.5 mg ml^{-1} fluorescein isothiocyanate (FITC, Sigma-Aldrich; F7250) and incubated in the dark for 1 h at room temperature. After incubation, bacteria were pelleted and washed in the same manner until a clear supernatant was observed. The final pellet containing the fluorescent-labeled bacteria was re-suspended in 1 ml haemocyte medium and used for in *vitro* challenge.

2.3 Haemolymph collection and haemocyte separation

Haemolymph collection

Haemolymph was extracted from the ventral sinus located at the base of the second abdominal pleonite, using a pre-cooled 2 ml syringe with 20 gauge needle (0.9x25mm) filled with pre-cooled marine anti-coagulant (450 mM NaCl, 100 mM glucose, 30 mM tri-sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4) (Söderhäll & Smith, 1983) in a proportion of 1:1 with the required volume of haemolymph. Care was taken to disinfect the area with 70% ethanol before haemolymph withdrawal to prevent the entry of opportunistic micro-organisms into the haemocoel.

Haemocyte separation

The different haemocyte subpopulations of *Litopenaeus vannamei* were separated by a two-step continuous density gradient of iodixanol (Optiprep 60%, Axis-Shield, UK) as described by Dantas-Lima et al (2013). The two gradients were prepared by under-layering of 2.5 ml of each iodixanol concentration (10%, 15%, 20% iodixanol for the first gradient and 7%, 10%, 13%, 16% iodixanol for the second gradient) into 15 ml non-pyrogenic Sarstedt tube, and incubated at 4^{o} C for 18 h to allow the formation of a continuous gradient.

Haemolymph was extracted, poured into pre-cooled 15 ml non-pyrogenic polypropylene centrifuge tube (Sarstedt, Germany) and immediately layered onto the first gradient (10%, 15%, 20% iodixanol; 2.5 ml per fraction). This gradient was centrifuged at 2000 *g* for 10 min at 4^{o} C with a Beckman CPR centrifuge (Rotina 380R, Hettich Lab Technology, Germany). Three sharp cell bands (band 1, band 2 and band 3 determined as subpopulation 1 (sub 1 - hyalinocytes), 2 (sub 2 - prohaemocytes) and 5 (sub 5 - granulocytes)) (Dantas-Lima et al., 2013) and one dispersed cell band in between band 2 and band 3 containing a mixture of subpopulation 3 and 4 (sub 3+4 - prohaemocytes + semigranulocytes) were formed. The first two bands (band 1 and band 2) from the top were very close to each other, and impossible to harvest without cross-contamination. To improve the purity, these two bands were collected together in 1 ml and put into a pre-cooled 15 ml tube containing 2 ml sPBS (shrimp phosphate buffered

saline, 18.1g NaCl in 1l of PBS: 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4), and layered onto the second gradient (7%, 10%, 13%, 16% iodixanol; 2.5 ml per fraction). The second gradient was then centrifuged at 2000 *g* for 15 min at 4° C with Beckman CPR centrifuge. Afterwards, band 1 (sub 1) and band 2 (sub 2) were physically separated.

To separate haemocytes of sub 3 and 4 of the dispersed cell band, we took advantage of the differential adhesion characteristics of cells from sub 3 and sub 4 to glass insert. Haemocytes were seeded in Nunc® 24-well cell culture plates with glass inserts for 1 h. Haemocytes of sub 4 strongly adhered to the insert surface whereas haemocytes of sub 3 did not.

2.4 Phagocytic assays

Haemocyte subpopulations from each band on the first and the second gradient were seeded into Nunc® 24-well cell culture plates supplied with glass inserts at a concentration of $3x10^5$ cells $well^{-1}$. Plates were incubated at 27° C for 1 h allowing haemocytes of subpopulation 1 (sub 1), 4 (sub 4) and 5 (sub 5) to attach on the substratum. Non-adherent haemocytes of subpopulation 2 (sub 2) and 3 (sub 3) were suspended by pipetting gently up and down six times and put into the new Nunc® 24-well cell culture plates. Afterwards, the bacterium suspension (GFP-labeled *V. campbellii*, *V. harveyi*, and FITC-labeled *E. coli*) and fluorescent polystyrene beads (1 µm in size) at a ratio of 1 bacteria or bead per haemocyte were added. The glass inserts containing haemocytes of sub 1, sub 4, sub 5 and the supernatant containing haemocytes of sub 2, sub 3 were collected at different time points of 0, 30, 60, 120, 180 min post inoculation. For evaluation of the uptake kinetics of foreign particles by haemocytes of sub 1, sub 4 and sub 5, the glass inserts with haemocytes were transferred to a new Nunc® 24-well cell culture plates, washed three times with cold HM and immediately fixed with 500 µl of paraformaldehyde 4% for 10 min. Samples were then washed once with PBS (phosphate buffered saline), permeabilized with Triton X-100 0.1% for 5 min, and washed two times with PBS. After that, samples were stained with 200 µl phalloidin Texas Red® diluted in PBS (4 units ml^{-1}) and incubated at 37° C for 1 h, rinsed three times with PBS (5 min each), and stained with 200 µl Hoechst (1:100 dilution of Hoechst stock in PBS, stock solution 1 mg ml^{-1}) for 15 min at room temperature. Finally, the samples were washed and mounted upside down on a drop of

glycerine. For the evaluation of the uptake kinetics of foreign particles by non-adherent haemocytes of sub 2 sub 3, the supernatant with haemocytes were collected and transferred to eppendorf tubes, fixed with paraformaldehyde 4% for 10 min and permeabilized with Triton X-100 0.1% for 5 min. Washing steps were performed in eppendorf tubes by centrifugation at 500 *g* for 5 min at 4° C. Then fixed haemocytes of sub 2 and sub 3 were cytospinned (Shandon Cytospin 3, Thermo Scientific, USA) at 700 rpm for 5 min onto glass slides. Afterwards, samples were stained with phalloidin Texas Red® and Hoechst as described above.

The percentage of phagocytic haemocytes and the phagocytic index was evaluated in 9 visual fields by confocal microscopy. In every field, the total number of cells and number of phagocytic cells were counted.

PR (phagocytic rate) = (Number of cells showing phagocytosis/total number of cells) x 100

PI (phagocytic index) = Number of engulfed bacteria/number of phagocytic cells

2.5 Intracellular killing of bacteria by separated haemocytes

To determine the fate of intracellular bacteria post-phagocytosis, haemocyte subpopulations were infected with bacteria (*V. campbellii, V. harveyi*, and *E. coli*) for 2 h, washed twice with HM and treated with gentamycin (100 µg ml^{-1} for *Vibrio* species and 50 µg ml^{-1} for *E. coli*) for 1 h to kill extracellular bacteria. Gentamycin was discarded and the cells were washed three times with HM and directly lysed in 400 µl cold PBS containing 0.1% Triton X-100 for 10 min. After lysis, serial dilutions were plated out in marine agar containing selective antibiotics (*V. campbellii, V. harveyi*) and Luria-Bertani agar (*E. coli*) to quantify the number of culturable intracellular bacteria as colony forming units (CFU) per well. Then the percentage of culturable intracellular bacteria was calculated as follow:

Percentage of culturable intracellular bacteria (%) = (Number of culturable intracellular bacteria/number of phagocytosed bacteria) x 100

Each experiment was repeated three times and the average values were calculated.

2.6 Detection of phagolysosome acidification with pHrodo labeling

The pHrodo™ green dye, a novel fluorogenic dye that dramatically increases the fluorescence as the pH of its surroundings becomes more acidic, was used to label bacteria with a concentration of 0.1 mg ml^{-1} according to the manufacturer's manual. The pHrodo-based system could measure the acidification of particles upon infection. Haemocyte subpopulations were inoculated with the pHrodo-labeled *V. campbellii*, *V. harveyi* and *E. coli* (1 bacteria/haemocyte) for 3 h. Then, samples were collected, fixed with 4% PF for 10 min, permeabilized with 0.1% Triton X-100 for 5 min and stained with Texas Red-labeled phalloidin (Invitrogen, Life Technologies™) diluted in PBS (4 units ml^{-1}) for 1 h at 37° C. Ten minutes before the end of this staining, Hoechst (0.01 mg ml^{-1}) was added. Finally, they were analyzed by confocal microscopy.

2.7 Effect of bacteria on the viability of haemocytes within subpopulations

The cytotoxic effect of bacteria on the survival of haemocyte subpopulations from penaeid shrimp was assessed *in vitro* using ethidium bromide monoazide (EMA, Sigma-Aldrich) and Hoechst (Invitrogen, Life Technologies) staining as described by Dantas-Lima et al. (2012). EMA binds to nucleic acid in cells with damaged membranes and was used for the detection of non-viable cells.
Haemocyte subpopulations were seeded in 24-well cell culture plate ($3x10^5$ cells $well^{-1}$). Each well was supplied with a glass insert. After one hour of incubation at 27^o C, allowing the cells to attach onto the substratum, the bacterium suspension (GFP-labeled *V. campbellii*, *V. harveyi*, and FITC-labeled *E. coli*) and fluorescent polystyrene beads (1 μm in size) with the ratio of one bacteria or bead per haemocyte were added. As a control, haemocytes were incubated in HM. The haemocytes were sampled at 0, 30, 60, 120 and 180 min post inoculation and immediately stained with 200 μl of EMA (ethidium bromide monoazide, 1:50 dilution of EMA stock in HM). Samples were then incubated for 30 min on ice and in the dark, followed by exposure to incandescent light for 10 min, washed once with cold HM and fixed with 500 μl paraformaldehyde 4% for 10 min. Cells were then washed once with PBS (phosphate buffered saline), stained with 200 μl of Hoechst (0.01mg ml^{-1}) (Invitrogen, Life Technologies) for 10 min, washed again and mounted on glass slides with 2 μl of anti-fading mounting medium

(glycerine-DABCO). The survival rate was evaluated in 9 visual fields of the inverted fluorescence microscope (Leica DM IRBE). In every field, the total number of cells and number of dead cells were counted. The total number of cells was determined by counting the cell nuclei stained by Hoechst counter staining (blue); the number of dead cells was assessed by counting the cells stained by EMA (red).
Survival rate (%) = (1 - (number of dead cells/total number of cells)) x 100
Each experiment was repeated three times and the average values were calculated.

2.8 Statistical analysis

All treatments were performed in three different experiments. The effect of treatments was statistically analyzed by analysis of variance (ANOVA). Differences were considered significant at $p<0.05$. All statistical analyses were conducted by SPSS software.

3. Results

3.1 Kinetic uptake of GFP-labeled V. campbellii and V. harveyi, FITC-labeled E. coli, and fluorescent polystyrene beads

Haemocytes from two subpopulations (sub 1 and sub 4) of penaeid shrimp were able to phagocytose a variety of biological particles (*V. campbellii*, *V. harveyi*, and *E. coli*) as well as fluorescent polystyrene beads (Fig. 1−3). The percentage of sub 1 and sub 4 that contained ingested bacteria and beads varied according to the type of particles. The percentage of sub 1 and sub 4, which ingested the non-virulent *E. coli* and beads, was significantly lower ($p<0.05$) than the percentage that contained virulent *V. campbellii* and *V. harveyi*. Haemocytes of sub 2, sub 3 and sub 5 did not internalize bacteria/beads, indicating that these hemocyte subpopulations do not have phagocytic capacities (Fig. 4).

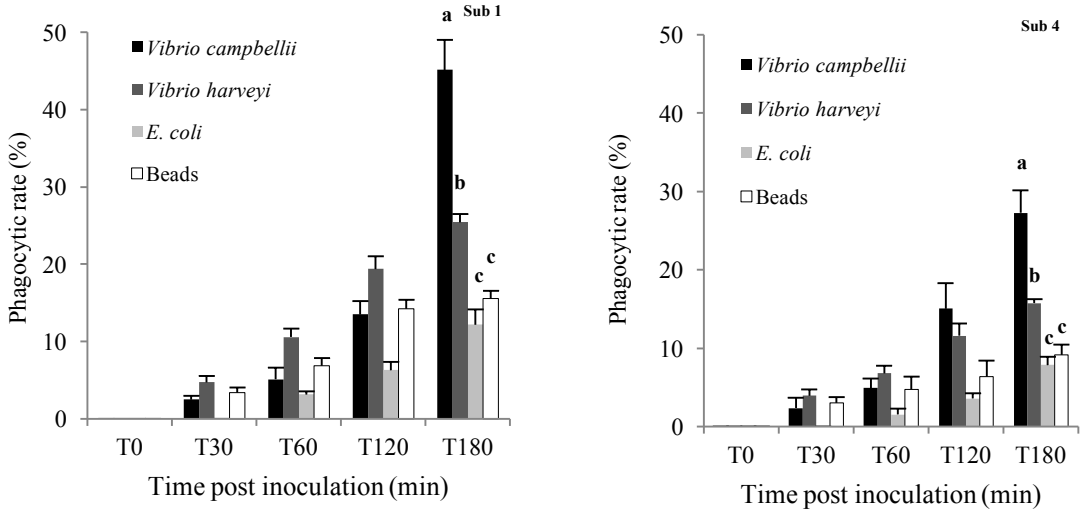

Figure 1. Kinetic uptake of GFP-labeled *V. campbellii* and *V. harveyi*, FITC-labeled *E. coli*, and fluorescent beads by haemocytes of sub 1 and sub 4. Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$).

After 60 min of incubation, the percentage of haemocytes of sub 1 taking up *V. campbellii*, *V. harveyi*, *E. coli* and polystyrene beads was 5.1 ± 1.5, 10.6 ± 1.1, 3.1 ± 0.4, 6.8 ± 1.0, respectively; while the percentage of bacteria (*V. campbellii*, *V. harveyi*, *E. coli*) and polystyrene beads engulfed by haemocytes of sub 4 was 4.9 ± 1.2, 6.8 ± 0.9, 1.6 ± 0.7, 4.7 ± 1.6, respectively. This percentage increased to 45.2 ± 3.9, 25.4 ± 1.1, 12.2 ± 1.9, 15.5 ± 1 in sub 1 and 27.3 ± 2.9, 15.7 ± 0.6, 7.9 ± 1.1, and 9.1 ± 1.3 in sub 4 after 180 min of post incubation.

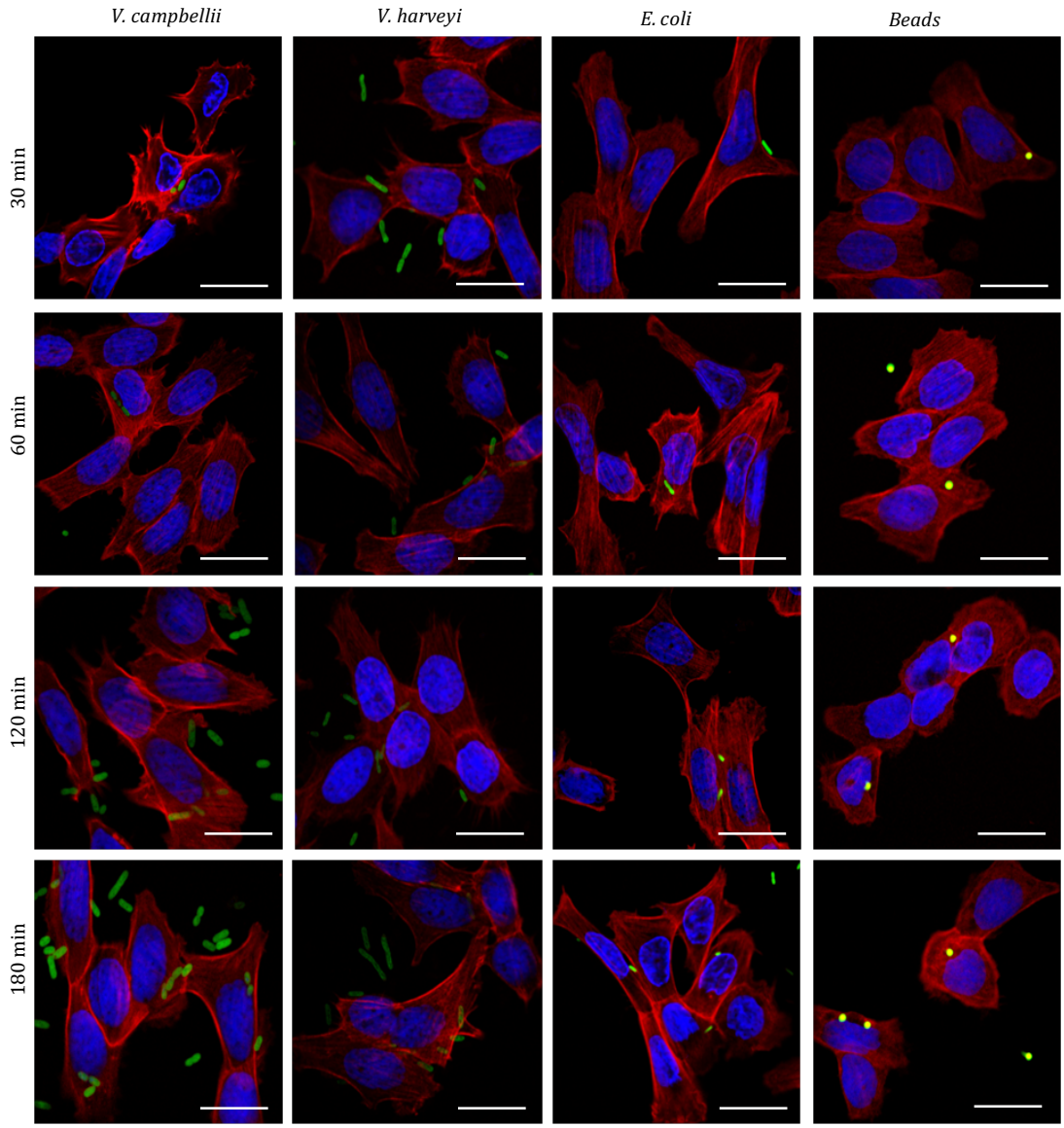

Figure 2. Kinetic uptake of *V. campbellii* and *V. harveyi*, *E. coli* and beads by haemocytes of sub 1. Scale bar: 15 µm.

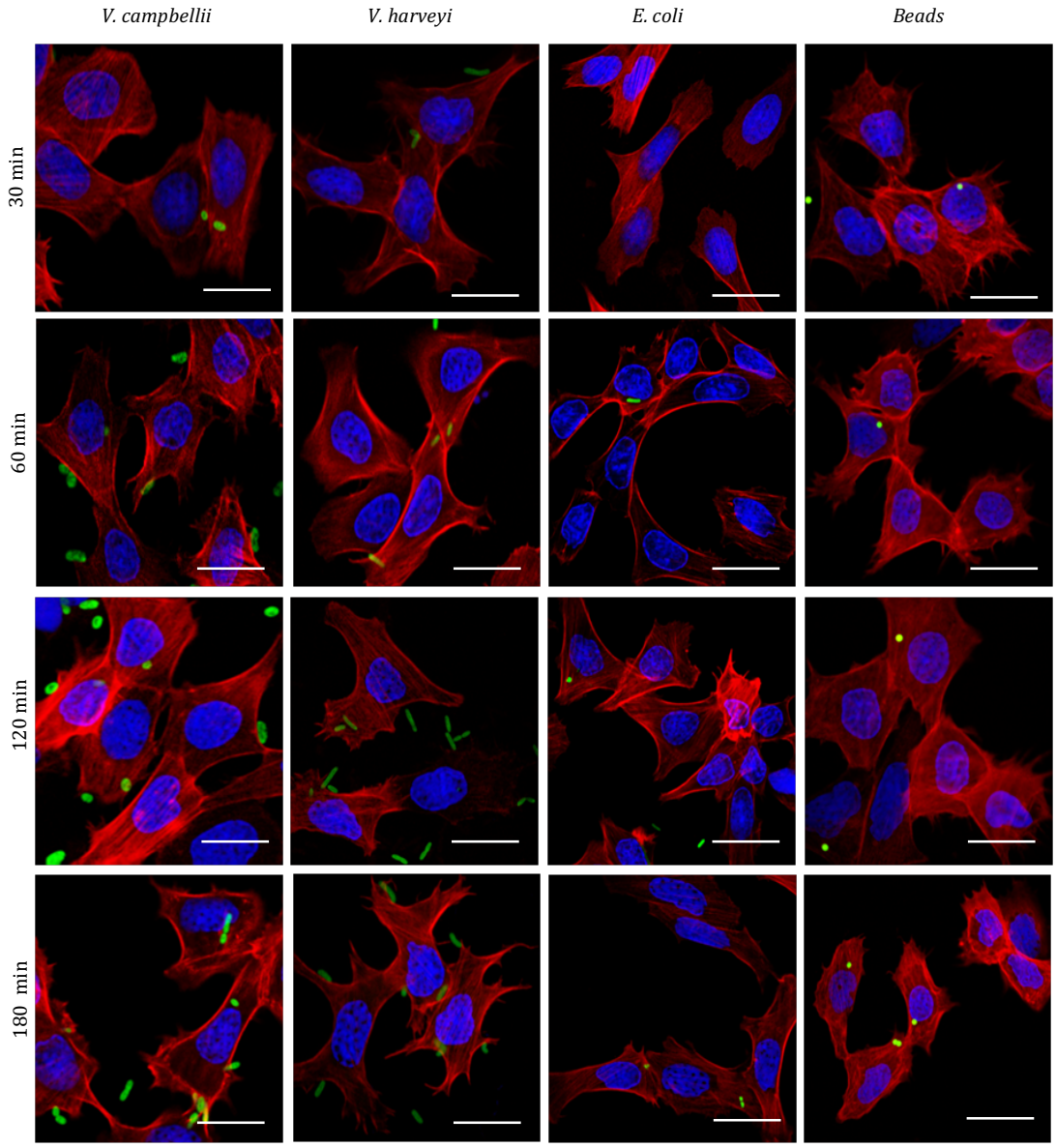

Figure 3. Kinetic uptake of *V. campbellii* and *V. harveyi*, *E. coli* and beads by haemocytes of sub 4. Scale bar: 15 µm.

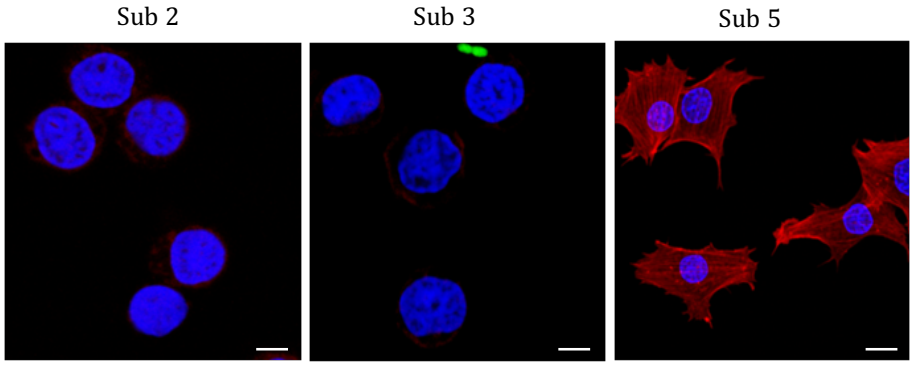

Figure 4. Absence of bacteria and bead uptake in sub 2, 3 and 5. Scale bar: 15 µm.

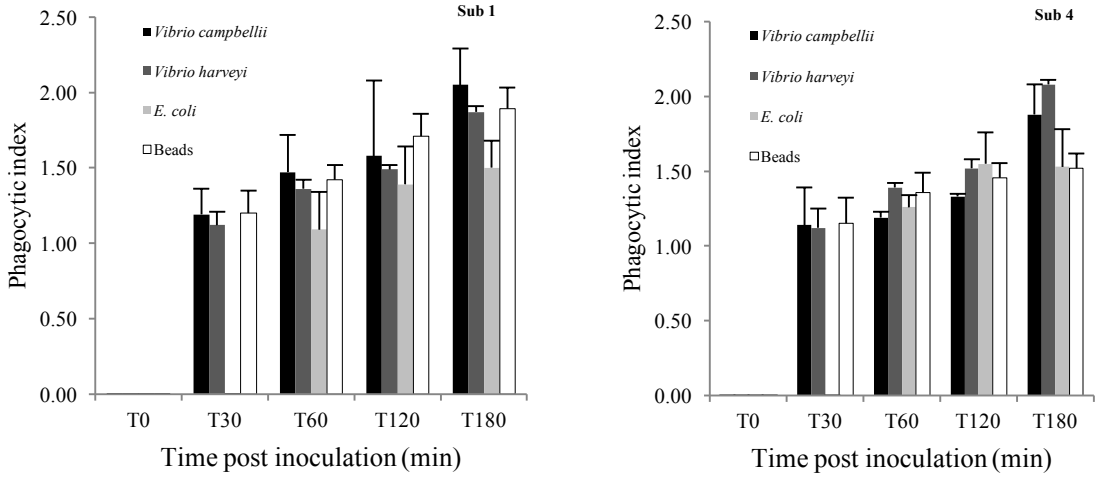

Figure 5. Number of bacteria/beads taken up by phagocytic haemocytes of sub 1 and sub 4. Error bars are standard error of mean (n = 3).

To determine the phagocytic index (PI), at least two hundred haemocytes in each group were analysed. The PI after 60 min of incubation in sub 1 and sub 4 were 1.5 ± 0.3, 1.2 ± 0.1 for *V. campbellii*; 1.4 ± 0.1, 1.4 ± 0.1 for *V. harveyi*, 1.1 ± 0.3, 1.3 ± 0.1 for *E. coli*, and 1.4 ± 0.1, 1.3 ± 0.1 for polystyrene beads, respectively (Fig. 5). After 180 min post incubation, the PI of sub 1 and sub 4 were 2.1 ± 0.2, 1.9 ± 0.2 for *V. campbellii*; 1.9 ± 0.1, 2.1 ± 0.1 for *V. harveyi*, 1.5 ± 0.2, 1.5 ± 0.3 for *E. coli*, and 1.8 ± 0.1, 1.5 ± 0.1 for polystyrene beads, respectively.

3.2 Intracellular killing of bacteria by haemocyte subpopulations

To determine the fate of intracellular bacteria post-phagocytosis, haemocytes of sub 1 and sub 4 were inoculated with pathogenic *V. campbellii, V. harveyi* and non-pathogenic *E. coli* bacteria for 2 h, washed and treated with gentamycin. The percentage of culturable intracellular bacteria in phagocytic cells was assessed by CFU counts obtained by inoculating lysates of infected cells at specific time points post-phagocytosis on marine agar for *Vibrio* species and LB-agar for *E. coli*. The percentage of internalized culturable *V. campbellii*, *V. harveyi*, and *E. coli* in sub 1, just after inoculation, was 23.9 ± 5.9%, 30.2 ± 6.1%, and 3.4 ± 0.2%, respectively. After 180 min post inoculation, there was a rapid drop in the percentage of culturable *V. harveyi* (13.5 ± 1.1%); while the percentage of culturable *V. campbellii* slightly increased (25.5 ± 6.0%).

The same case was also observed in sub 4. Just after inoculation, the percentage of internalized culturable *V. campbellii*, *V. harveyi*, and *E. coli* was 20.3 ± 6.3%, 7.9 ± 5.9%, and 6.2 ± 0.3%, respectively. After 180 min post inoculation, there was a slightly drop in the percentage of culturable *V. harveyi* (6.5 ± 1.8%); while the percentage of culturable *V. campbellii* slightly increased (24.1 ± 6.4%). No culturable *E. coli* was observed at 180 min post inoculation in sub 1 and sub 4. For the control group, bacteria were not detected, showing the effectiveness of a 100 µg ml^{-1} dose of gentamycin for killing extracellular bacteria (Fig. 6).

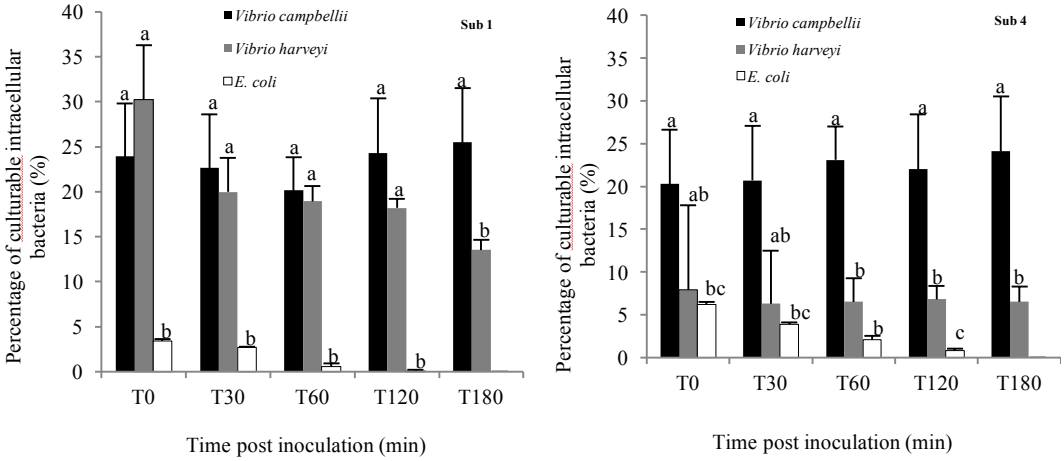

Figure 6. Intracellular culturability of bacteria after exposure to phagocytic haemocytes of sub 1 and sub 4. Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$).

3.3 Acidification of pHrodo-labeled bacteria upon internalization

In phagocytic cells, pathogens are internalized into phagosomes, which undergo a gradual maturation by fusion with lysosomes to become the phagolysosome, an efficient microbicidal compartment. A pH-sensitive dye, non-fluorescent at neutral pH typically found in the extracellular environment, can be used to label bacteria and monitor their acidification upon engulfment in a mature phagosome simply by examining whether these pHrodo-labeled bacteria fluoresce.

To investigate the acidification of internalized pathogenic and non-pathogenic bacteria, these bacteria were labeled with pHrodo and examined by confocal microscopy. The results indicated that non-pathogenic bacteria (*E. coli*) as well as less pathogenic bacteria (*V. harveyi*) were engulfed by sub 1 and 4 of *Litopenaeus vannamei* into an

acidified mature phagosome at 3 h after inoculation, whereas the pathogenic bacteria (*V. campbellii*) did not end up in a phagosome that undergoes acidification (Fig. 7−8).

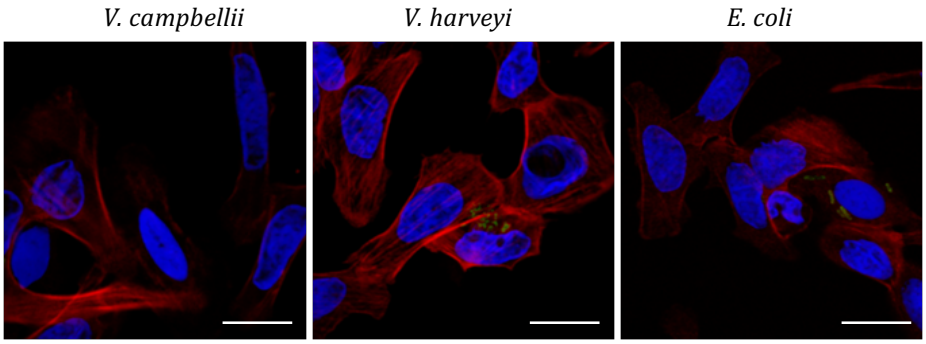

Figure 7. Phagocytosis of pHrodo-labeled bacteria (*V. campbellii*, *V. harveyi*, and *E. coli*) by haemocytes of sub 1 under confocal microscopy. Bacteria became green during acidification. Scale bar: 15 µm.

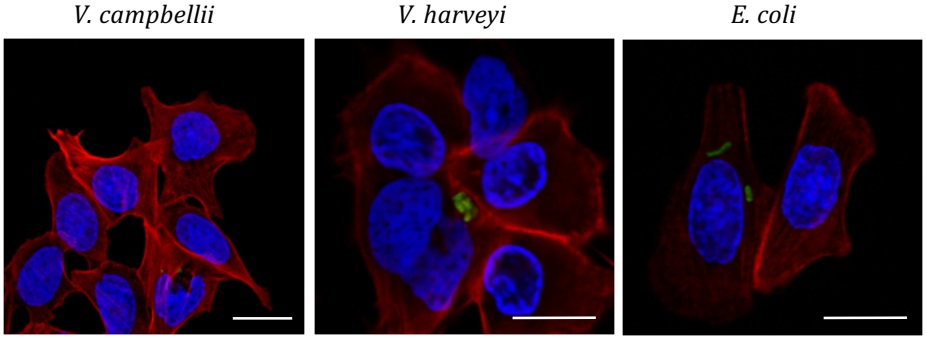

Figure 8. Phagocytosis of pHrodo-labeled bacteria (*V. campbellii*, *V. harveyi*, and *E. coli*) by haemocytes of sub 4 under confocal microscopy. Bacteria became green during acidification. Scale bar: 15 µm.

3.4 Viability of different haemocyte subpopulations from Litopenaeus vannamei

The exposure of haemocyte subpopulations to abiotic and biotic particles induced significant changes in cell viability, and the results are reported in Fig. 9. Incubation of haemocyte monolayers for 30 min with both pathogenic and non-pathogenic particles did not significantly affect haemocyte survival. However, after 180 min post inoculation, *V. campbellii* induced a dramatic decrease of haemocyte survival in comparison with *V. harveyi*, *E. coli*, polystyrene beads and control group. The haemocyte viability in sub 1 and sub 4 was 41.4 ± 6.3% and 30.2 ± 15.1% for *V. campbellii*; 84.1 ± 5.6% and 83.4 ± 4.1% for *V. harveyi*; 92.7 ± 2.8% and 92.3 ± 5.6%

for *E. coli*; 83.8 ± 7.9% and 83.4 ± 4.0% for polystyrene beads, and 91.9 ± 1.6% and 84.4 ± 3.4% without bacteria, respectively.

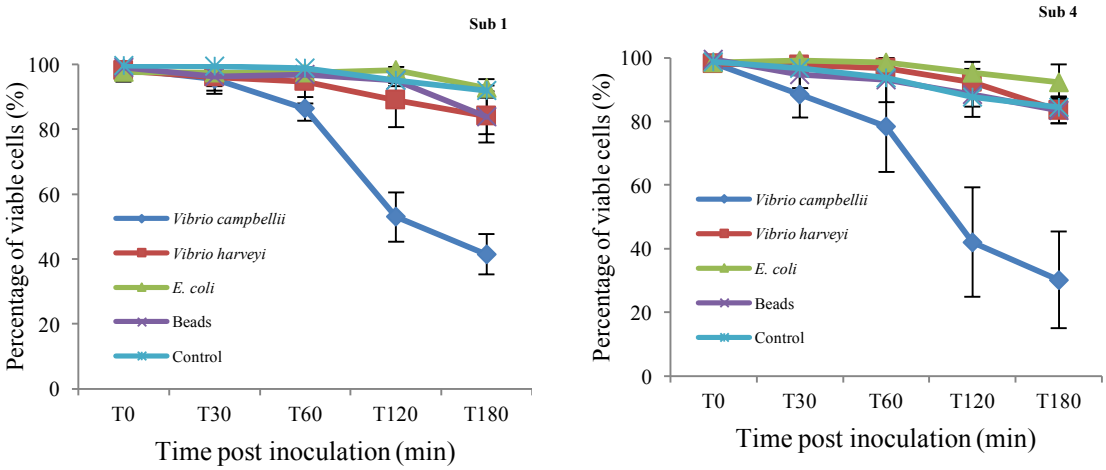

Figure 9. Effect of beads and bacterial species on survival of haemocytes of sub 1 and sub 4 as determined by ethidium monoazide bromide (EMA). Error bars are standard error of mean (n = 3).

4. Discussion

The results obtained in this study showed different *in vitro* responses of penaeid shrimp haemocytes to pathogenic and non-pathogenic bacteria as well as polystyrene beads in terms of phagocytosis, bactericidal capacity and survival of haemocytes.

Penaeid shrimp hemocytes are able to phagocytose both biotic particles, such as bacteria, yeasts and apoptotic cells, as well as abiotic targets, such as fluorescent polystyrene beads. A previous study has shown that sub 1 (hyalinocytes) and sub 4 (semi-granulocytes) efficiently phagocytosed the pathogenic bacteria *V. campbellii in vitro* (Dantas-Lima et al., 2013). In the current study, we demonstrated that these subpopulations were the only ones involved in phagocytosis of both pathogenic and non-pathogenic bacteria as well as abiotic particles like fluorescent polystyrene beads. The pathogenic bacteria (*V. campbellii*) was engulfed by a much larger percentage of cells compared to *V. harveyi*, *E. coli* and beads. The role of the non-phagocytic haemocytes of sub 2 and sub 3 in defense is unknown. They most probably perform immunological functions different from phagocytosis.

To the best of our knowledge, this is the first report where the phagocytic activity of *Litopenaeus vannamei* haemocyte subpopulations was studied *in vitro*. The maximum level of phagocytosis of bacteria recorded in haemocytes of sub 1 (hyalinocytes) and sub 4 (semi-granulocytes) in the present study was 45% and 27%, respectively. This is different from the level reported by Jayasree *et al.* (2009) on *Penaeus indicus*. They found a higher percentage of phagocytosis of *Vibrio alginolyticus* in semi-granular (91%), no phagocytosis in hyalinocytes, but some in granular cells (33%). However, the authors did not mention the ratio between bacteria and haemocytes. Hose and Martin (1989) also achieved high phagocytic rates of gram-negative bacterium *Cytophaga* sp. in semi-granulocytes (31-90%) of ridgeback prawn *Sicyonia ingentis*. Kondo et al. (1992) reported that all three types of kuruma prawn haemocytes are involved in phagocytosis of sheep red blood cells. However, they concluded that the phagocytic activities of semi-granular and granular cells were much higher than that of hyaline cells. All these results seem to be different to our experiments, in which hyaline cells (sub 1) and semi-granular cells (sub 4) are the main cell types involved in phagocytosis. The lower value and the difference of cell types participating in phagocytosis in our research may have been caused by (1) species differences, (2) culture medium, (3) type of particles and (4) opsonization of particles before inoculation (more details: see general discussion).

Both pathogenic and non-pathogenic bacteria were phagocytosed by penaeid shrimp haemocytes, however, only non-pathogenic bacteria were destroyed upon phagocytosis. When these bacteria were mixed with shrimp haemocyte subpopulations and incubated for 180 min, the percentage of culturable intracellular *V. campbellii* recovered was significantly higher than the percentage recovered from *V. harveyi* group treated in the same manner ($p<0.05$). No culturable intracellular *E. coli* was observed under the same condition. The present data suggest that haemocytes of sub 1 and 4 are responsible for most of the bacterial killing of non-pathogenic bacteria, but failed to kill pathogenic ones. The absence of acidification of the endosome carrying the bacteria *V. campbellii* was demonstrated in the present study by the use of pHrodo-labeled bacteria indicating that these pathogenic bacteria most probably inhibited phagosome maturation and phagosome-lysosome fusion. An interesting observation was observed during the uptake experiment. The majority of *V. campbellii* cells were rod-shaped immediately after ingestion but converted into a coccal form after 2 h of incubation (Fig. 2 and 3). It was speculated that the conversion from rod-shaped to a coccal form

may represent a survival strategy of the bacteria against the intracellular killing by phagocytic cells. Similarly in mammalian phagocytes, both pathogenic and non-pathogenic *Streptococcus suis* are engulfed, but only non-pathogenic bacteria are killed whereas the pathogenic strain survives and even grows intracellularly (Williams, 1990). Also in insect, most of the *E. coli* strains are killed by phagocytic plasmatocytes (swelling of intracellular bacteria, nuclear and cytoplasmic disruption) after 120 min post inoculation (Rowley & Ratcliffe, 1976). Another study in mussel also reported that phagocytic haemocytes are able to engulf pathogenic vibrio species (*Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32) *in vitro* (Balbi et al., 2013) and that just like for *Vibrio harveyi* in our study they are able to kill about 50% of bacteria within 90 min.

V. campbellii caused the greatest drop of haemocyte viability. The other two strains of bacteria (*V. harveyi* and *E. coli*) as well as polystyrene beads did not have an effect on haemocyte viability when compared with the control group. Experiments with extracellular products of these bacteria indicated that they were not toxic to shrimp haemocytes (data not shown). Therefore, it seems that *V. campbellii* possesses a cytotoxic effect upon ingestion. Our results are in agreement with the findings of Nottage & Birkbeck, 1990. They reported that certain vibrio strains were toxic to *Mytilus edulis* haemocytes when present in large numbers. Another study conducted by Lambert et al., 2001 also showed that *Vibrio pectinicida* can cause a decrease in haemocyte viability of the scallop *Pecten maximus* upon contact with live bacteria. In the future, more work will be done to better understand the mechanisms of the haemocyte killing by *V. campbellii* upon uptake.

Acknowledgements

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Chapter 5

Kinetic analysis of internalization of white spot syndrome virus (WSSV) by haemocyte subpopulations of the penaeid shrimp, *Litopenaeus vannamei* (Boone), and the outcome for virus and cell

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Abstract

Little is known about the innate antiviral defense of shrimp haemocytes. In this context, the haemocytes of penaeid shrimp *Litopenaeus vannamei* (Boone) were separated by iodixanol density gradient centrifugation into 5 subpopulations (sub): sub 1 (hyalinocytes), sub 2 & 3 (pro-hyalinocytes), sub 4 (semi-granulocytes) and sub 5 (granulocytes) and exposed to beads, white spot syndrome virus (WSSV) and UV-killed WSSV. In a first experiment, the uptake of beads, white spot syndrome virus (WSSV) and UV-killed WSSV by these different haemocyte subpopulations was investigated using confocal microscopy. Only haemocytes of sub 1, 4 and 5 were internalizing beads, WSSV and UV-killed WSSV. Beads were engulfed by a much larger percentage of cells (91.2 % in sub 1; 84.1% in sub 4 and 58.1% in sub 5) compared to WSSV (9.6% in sub 1; 10.5% in sub 4 and 7.9% in sub 5) and UV-killed WSSV (12.9% in sub 1; 13.3% in sub 4 and 11.8% in sub 5). In a second experiment, it was shown that upon internalization, WSS virions lost their envelope most probably by fusion with the cellular membrane of the endosome (starting between 30 and 60 min post inoculation) and that afterwards the capsid started to become disintegrated (from 360 min post inoculation). Expression of new viral proteins was not observed. Incubation of haemocyte subpopulations with WSSV but not with UV-killed WSSV and polystyrene beads resulted in a significant drop in haemocyte viability. In order to find the underlying mechanism, a third experiment was performed in which haemocyte subpopulations were exposed to a short WSSV DNA fragment (VP19) and CpG ODNs. These small DNA fragments induced cell death. In conclusion, WSSV is efficiently internalized by hyalinocytes, semi-granulocytes and granulocytes, after which the virus loses its envelope; as soon as the capsids start to disintegrate, cell death is activated, which in part may be explained by the exposure of viral DNA to cellular sensing molecules.

1. Introduction

Crustacean immunity is defined as a non-specific internal defense response that includes both humoral and cellular components, in which the blood cells (haemocytes) play an important role in the defense reactions against microorganisms, parasites and other foreign materials that might enter into the haemocoel (Bachere et al., 2004; Jose et al., 2010; Matozzo & Marin, 2010). The haemocytes are directly involved in recognition, coagulation, phagocytosis, encapsulation, nodule formation, and cytotoxic reaction (Hose et al., 1987; Johansson & Söderhäll, 1989; Hose et al., 1990). Haemocytes are traditionally classified into three distinct subpopulations: hyaline cells, semi-granular cells and granular cells, according to the number and size of granules (Söderhäll & Smith, 1983; van de Braak et al., 1996; Li & Shields, 2007; Smith, 2010; Hong et al., 2013). Recently, haemocytes of penaeid shrimp (*Litopenaeus vannamei*) were separated into 5 subpopulations (Dantas-Lima et al., 2013). The haemocytes of two additional subpopulations resemble the so-called pro-hyalinocytes. These subpopulations need further characterization.

Phagocytosis is one of the major defense mechanisms when foreign particles intrude their host. The phagocytic process is thought to occur in two steps: first, the attachment of the particles to the cell surface and second, the internalization into the cytoplasm. This process has extensively been studied by the authors for bacteria (Tuan et al., 2015), however, information on the uptake of viruses is scarce.

White spot syndrome virus (WSSV), the most serious pathogen in penaeid shrimp (Lo et al., 1996a) was reported since 1992-1993 throughout the world (Durand et al., 1996; Lo et al., 1996b; Karunasagar et al., 1997; Kasornchandra et al., 1998; Magbanua et al., 2000; Rajan et al., 2000; Bondad-Reantaso et al., 2001; Dieu et al., 2004). It was originally classified as an unassigned member of the *Baculoviridae* family, but has been later re-classified as a new virus family, the *Nimaviridae* (genus *Whispovirus)* (van Hulten et al., 2001). WSSV is an enveloped, non-occluded and rod-shaped DNA virus with a bacilliform to ovoid or ellipsoid shape. The viral envelope, having a thickness of 6-7 nm, is a lipidic, trilaminar membranous structure with two electron transparent layers divided by an electron opaque layer (Wonteerasupaya et al., 1995; Durand et al., 1997; Nadala et al., 1998). The nucleocapsid is located inside the envelope and has a striated appearance and a size of 420 ± 18 nm in length and 68 ± 5 nm in width (Wonteerasupaya et al., 1995; Hameed et al., 1998). WSSV has a remarkable broad

host range among crustaceans. It can infect a wide range of aquatic crustaceans living in marine, brackish and fresh water (Lo et al., 1996b; Flegel, 1997; Peng et al., 1998; Wang et al., 1998; Rajendran et al., 1999; Flegel, 2006) and can cause extensive mortality in shrimp pond within a period of 3 to 7 days and massive production losses to aquaculture industry (Lightner, 1996). Curing and preventing WSSV disease is still impossible, because the basic mechanisms of WSSV infection and replication are poorly understood.

In literature, there are conflicting data regarding the susceptibility of crustacean haemocytes to WSSV. Some researchers have claimed that haemocytes of crustacean are susceptible to WSSV (Wang et al., 2002; Jiang et al., 2006; Jiravanichpaisal et al., 2006a). Others mentioned that crustacean haemocytes could not support WSSV replication (Itami et al., 1999; Shi et al., 2005; Escobedo-Bonilla et al., 2007; Wu et al., 2015). In addition, WSSV has been found to induce apoptosis in host haemocytes during an experimental WSSV infection in shrimp (Hameed et al., 2006; Jiravanichpaisal et al., 2006a). The aims of the present work were to (i) investigate the kinetics of the uptake of both intact and UV-inactivated WSSV in the different haemocyte subpopulations of *Litopenaeus vannamei* as well as fluorescent polystyrene beads, (ii) to analyze the fate of virus and cell upon ingestion and (iii) to examine underlying mechanisms.

2. Materials and methods

2.1 Experimental animals

Specific pathogen-free (SPF) penaeid shrimp, *Litopenaeus vannamei*, with a mean body weight of 25 ± 5 g in inter-molt (C) stage (Corteel et al., 2012) were used. The shrimp were imported from Holland and were reared in a recirculation system at the Laboratory of Artemia & Reference Center (ARC), Faculty of Bioscience Engineering, Ghent University, Belgium. They were fed twice daily at a total rate of 5% of their mean body weight. Water temperature was kept at 27 ± 1^{o} C, pH 7.5-8.0, and salinity at 35 ± 1 g l^{-1}. Regular water changes and bio-filters kept the total ammonia-N below 0.5 mg l^{-1} and nitrite-N below 0.15 mg l^{-1}.

2.2 Preparation of viral inoculum

To prepare purified virus, shrimp (15 ± 5g) were intramuscular injected with 50 µl of a ten times diluted white spot syndrome virus stock. This WSSV stock was isolated from naturally infected *Penaeus monodon* in 1996 in Thailand and passaged once in crayfish. Shrimp were monitored every 6 h over 72 h. When shrimp became moribund, haemolymph was withdrawn directly from the ventral sinus located at the base of the second abdominal pleonite, using a pre-cooled 2 ml syringe with 20-gauge needle (0.9x25mm). Haemolymph was centrifuged at 500 *g* for 10 min at 4°C and the supernatant was collected, pooled together, and centrifuged again in the same way. The supernatant was filtered through a 0.4µm Millipore filter membrane. WSSV was purified by a discontinuous iodixanol-gradient ultracentrifugation (Dantas-Lima et al., 2013).

2.3 Treatment with UV-irradiated virus

WSSV was ultraviolet (UV) irradiated at 1000 mJ/cm^2 for 10 min to inactivate its infectivity. The UV-irradiated virus was injected into penaeid shrimp. The loss of infectivity was confirmed as previously described by Escobedo-Bonilla et al. (2005).

2.4 Titration of WSSV suspension prepared from purified virus

In vivo WSSV titration: the infectivity titer in the gradient samples was assessed as previously described by Escobedo-Bonilla et al. (2005). Briefly, samples were diluted in steps, with dilutions ranging from 10^{-4} to 10^{-8}. Fifty microlitres of each dilution was intramuscularly injected in the junction between the third and fourth abdominal segment of shrimp. Five shrimp (2-5g/shrimp) were used per dilution. Shrimps were housed individually in 10 l aquarium tank supplied with aeration. The temperature was maintained at 27 ± 1°C. Over the course of 5 days, dead and surviving shrimp were collected and the cephalothorax was dissected longitudinally, embedded in 2% methylcellulose (Fluka) and quickly frozen at -20°C. Cryosections of 5 µm were made and immediately fixed in absolute methanol at -20°C for 20 min. Sections were washed three times for 5 min each in phosphate buffered saline (PBS) and incubated for 1 h at 37°C with W29 monoclonal antibody (kindly provided by Parin Chaivisuthangkura,

Srinakharinwirot University, Thailand) against VP28 (Poulos *et al.*, 2001). Then they were washed three times for 5 min each in PBS and incubated for 1 h at 37°C with 0.02 μg ml^{-1} of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG antibodies (F-2761 Molecular Probes, The Netherlands). Normal goat serum was added at 10% (v:v) in both antibody suspensions. Finally, the stained sections were analyzed by fluorescence microscopy (Leica DM IRBE) to confirm the presence or absence of WSSV infection in dead and surviving shrimp. The infectivity titer was calculated from these infection data using the Reed and Muench formula (Reed & Muench, 1938) and expressed as shrimp infectious dose 50% endpoint (SID_{50}).

The titer of purified virus in the gradient sample was $10^{6.1} SID_{50}$ ml^{-1}.

Quantification of viral particles by confocal microscopy: the number of viral particles in the samples was estimated using the method described by Dantas-Lima et al. (2013). Briefly, the samples collected after purification were tenfold diluted in PBS. Red fluorescent 0.2 μm polystyrene beads (Fluospheres, Invitrogen) at a concentration of 10^8 ml^{-1} and collagen type I (Sigma) at a ratio of 1:15 (v/v) were added to each sample. Three microlitres of these suspensions were placed on glass slides coated with 3-aminopropyl- triethoxysilane (Sigma), allowed to dry for 20 min at 37°C and immediately fixed with 4% paraformaldehyde for 10 min at room temperature. These smears were stained by indirect immunofluorescence as described above for staining of shrimp tissues. The number of beads and viral particles was counted using confocal microscopy. The concentration of viral particles was determined by calculating its proportion to the number of fluorescent beads that were added to each sample at a known concentration.
It was shown that 1 ml of purified viral particle suspension contained 7.2 x 10^8 WSSV particles.

2.5 Haemolymph extraction and haemocyte separation

Haemolymph was extracted from the ventral sinus located at the base of the second abdominal pleonite, using a pre-cooled 2 ml syringe with 20 gauge needle (0.9x25mm) filled with pre-cooled marine anti-coagulant (450 mM NaCl, 100 mM glucose, 30 mM tri-sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4) (Söderhäll & Smith, 1983)

in a proportion of 1:1 with the required volume of haemolymph. Care was taken to disinfect the area with 70% ethanol before haemolymph withdrawal to prevent the entry of opportunistic microorganisms into the haemocoel.

The different haemocyte subpopulations of *Litopenaeus vannamei* were separated by a two-step continuous density gradient of iodixanol (Optiprep 60%, Axis-Shield, UK) as described by Dantas-Lima et al. (2013). The two gradients were prepared by under-layering 2.5 ml of each iodixanol concentration (10%, 15%, 20% iodixanol for the first gradient and 7%, 10%, 13%, 16% iodixanol for the second gradient) into a 15 ml non-pyrogenic Sarstedt tube, and incubated at 4^{o}C for 18 h to allow the formation of a continuous gradient.

Haemolymph was extracted, poured into pre-cooled 15 ml non-pyrogenic polypropylene centrifuge tube (Sarstedt, Germany) and immediately layered onto the first gradient (10%, 15%, 20% iodixanol; 2.5 ml per fraction). This gradient was centrifuged at 2000 *g* for 10 min at 4^{o}C with a Beckman CPR centrifuge (Rotina 380R, Hettich Lab Technology, Germany). Three sharp cell bands (band 1, band 2 and band 3 determined as subpopulation 1 (sub 1), 2 (sub 2) and 5 (sub 5)) (Dantas-Lima et al., 2013) and one dispersed cell band in between band 2 and band 3 containing a mixture of subpopulation 3 and 4 (sub 3+4) were formed. The first two bands (band 1 and band 2) from the top were located very close to each other, and impossible to harvest without cross-contamination. To improve the purity, these two bands were collected together in 1 ml and put into a pre-cooled 15 ml tube containing 2 ml sPBS (shrimp phosphate buffered saline, 18.1g NaCl in 1 l of PBS: 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4), and layered onto the second gradient (7%, 10%, 13%, 16% iodixanol; 2.5 ml per fraction). The second gradient was then centrifuged at 2000 *g* for 15 min at 4^{o}C with Beckman CPR centrifuge. Afterwards, band 1 (sub 1) and band 2 (sub 2) were physically separated.

To separate haemocytes of sub 3 and 4 of the dispersed cell band, we took advantage of the differential adhesion characteristics of cells from sub 3 and sub 4 to glass inserts. Haemocytes were seeded in Nunc® 24-well cell culture plates with glass inserts for 1 h. Haemocytes of sub 4 strongly adhered to the insert surface, whereas haemocytes of sub 3 did not.

2.6 Internalization of WSSV by haemocyte subpopulations of penaeid shrimp

Haemocyte subpopulations from each band of the first and the second gradient were seeded into Nunc® 24-well cell culture plates supplied with glass inserts at a concentration of $1x10^5$ cells $well^{-1}$. Plates were incubated at 27°C for 1 h allowing haemocytes of subpopulation 1 (sub 1), 4 (sub 4) and 5 (sub 5) to attach on the substratum. Non-adherent haemocytes of subpopulation 2 (sub 2) and 3 (sub 3) were suspended by pipetting gently up and down six times and were put into Nunc® 24-well cell culture plates. Afterwards, the viral suspension and fluorescent polystyrene beads (0.2 µm in size) at a ratio of 100 viral particles or beads per haemocyte were added. The glass inserts containing haemocytes of sub 1, sub 4, sub 5 and the supernatant containing non-adherent haemocytes of sub 2, sub 3 were collected at different time points of 30, 60, 120, 180, 360 and 720 min post inoculation.

For evaluation of the internalization kinetics of foreign particles by adherent haemocytes of sub 1, sub 4 and sub 5, the glass inserts with haemocytes were transferred to new Nunc® 24-well cell culture plates, washed three times with cold HM and immediately fixed with 500 µl of paraformaldehyde 4% for 10 min. Samples were then washed once with PBS (phosphate buffered saline), permeabilized with Triton X-100 0.1% for 5 min, washed two times with PBS and incubated for 1 h at 37°C with 200 µl of monoclonal antibody W29 (1:50 in PBS) against VP28 envelop protein. Then, they were washed three times for 5 min each in PBS and incubated for 1 h at 37°C with 0.02 µg ml^{-1} of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibodies (F-2761 Molecular Probes). Normal goat serum was added at 10% (v:v) in both antibody suspensions. Afterwards, they were washed again and stained with 200 µl phalloidin Texas Red® diluted in PBS (4 units ml^{-1}) for 1 h at 37°C, rinsed three times with PBS (5 min each), and stained with 200 µl Hoechst (1:100 dilution of Hoechst stock in PBS, stock solution 1 mg ml^{-1}) for 10 min at room temperature. Finally, the samples were washed and mounted upside down on a drop of glycerine.

For evaluation of the uptake kinetics of foreign particles by non-adherent haemocytes of sub 2 and sub 3, the supernatant with non-adherent haemocytes were collected and transferred to eppendorf tubes, fixed with paraformaldehyde 4% for 10 min and permeabilized with Triton X-100 0.1% for 5 min. Washing steps were performed in eppendorf tubes by centrifugation at 500 *g* for 5 min at 4°C. Then, fixed haemocytes of

sub 2 and sub 3 were cytospinned (Shandon Cytospin 3, Thermo Scientific, USA) at 700 rpm (50 *g*) for 5 min onto glass slides. Afterwards, samples were stained with primary antibody, secondary antibody, phalloidin Texas Red® and Hoechst as described above.

The percentage of endocytosis in haemocytes and the endocytosis index was evaluated in 9 visual fields by confocal microscopy, using the following calculation:

ER (Endocytosis rate) = (Number of cells showing endocytosis/total number of cells) x 100

EI (Endocytosis index) = Number of engulfed particles/number of endocytosis cells

Sequential confocal pictures were taken from the cell base to its apex to determine internalized particles.

2.7 Kinetics of WSSV disassembly in cytoplasmic endosome of shrimp haemocytes within subpopulation 1, 4 and 5

$1x10^5$ cells $well^{-1}$ were seeded into Nunc® 24-well cell culture plates supplied with glass insert. After 1 h of incubation at 27°C allowing cells to attach onto the glass insert, the virus suspension (100 viral particles/haemocyte) was added. The glass inserts were sampled at 30, 60, 120, 180, 360 and 720 mpi and washed 3 times with cold HM. Then they were fixed with 500 µl paraformaldehyde (4%) for 10 minutes, permeabilized with triton X-100 0.1% for 5 minutes, and washed two times with PBS. 200 µl of primary antibody solution W29 against VP28 envelope protein (1:50 in PBS together with 10% normal goat serum) was added and incubated at 37°C for 1h. Then, they were washed three times for 5 min each in PBS and incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibodies (F-2761 Molecular Probes) (GαM-FITC; 1:200 in PBS together with 10% normal goat serum). The samples were stained with 200 µl of rabbit polyclonal antibody WSSV419 (1:200 in PBS together with 10% normal goat serum), which is directed against WSSV nucleocapsid protein VP664 (Leu et al., 2005) and goat anti-rabbit IgG Alexa Fluor 647 (1:300 in PBS together with 10% normal goat serum). Each antibody was incubated for 1 h at 37°C. Samples were washed after each incubation and stained afterwards with

200 µl Hoechst (1:100 dilution of Hoechst stock in PBS, stock solution 1 mg ml^{-1}) for 10 min at room temperature. Finally, the samples were washed and mounted upside down on a drop of glycerine.

2.8 Effect of WSSV on the viability of haemocytes within subpopulation 1, 4 and 5

The effect of virus on the survival of haemocyte subpopulations of penaeid shrimp was assessed *in vitro* using ethidium bromide monoazide (EMA, Sigma-Aldrich) and Hoechst (Invitrogen, Life Technologies) staining as described by Dantas-Lima et al. (2012). EMA binds to nucleic acid in cells with damaged membranes and is used for the detection of non-viable cells.

Haemocyte subpopulations were seeded in 24-well cell culture plates ($1x10^5$ cells $well^{-1}$). Each well was supplied with a glass insert. After one hour of incubation at 27°C, allowing the cells to attach onto the substratum, the viral inoculum (live and UV-killed WSSV) and fluorescent polystyrene beads (0.2 µm in size) with the ratio of 100 viral particles or beads per haemocyte were added. The glass inserts were sampled at 0, 30, 60, 120 and 180, 360 and 720 min post inoculation and immediately stained with 200 µl of EMA (ethidium bromide monoazide, 1:50 dilution of EMA stock in HM). Samples were then incubated for 30 min on ice and in the dark, followed by exposure to incandescent light for 10 min, washed twice with cold HM and fixed with 500 µl paraformaldehyde 4% for 10 min. Cells were then washed once with PBS (phosphate buffered saline), stained with 200 µl of Hoechst (0.01 mg ml^{-1}) (Invitrogen, Life Technologies) for 10 min, washed again and mounted on glass slides with 2 µl of anti-fading mounting medium (glycerine-DABCO). The survival rate was evaluated in 9 visual fields of the inverted fluorescence microscope (Leica DM IRBE). In every field, the total number of cells and number of dead cells were counted. The total number of cells was determined by counting the cell nuclei stained by Hoechst counter staining (blue) while the number of dead cells was assessed by counting the cells stained by EMA (red).

Survival rate (%) = (1 - (number of dead cells/total number of cells)) x 100

Each experiment was repeated three times and the average values were calculated.

2.9 Effect of a small DNA WSSV fragment (111 bp) and CpG oligodeoxynucleotides on the viability of haemocytes within subpopulation 1, 4 and 5

DNA extraction: DNA was extracted from WSSV-Thai 1 stock using the QIAamp DNA mini kit (Qiagen, California, USA) and amplified by polymerase chain reaction. Primers were designed in a conserved region of the VP19 coding sequence using the Primer3Plus website (Li et al., 2015). The VP19 DNA fragment was amplified in a 50 µl of polymerase chain reaction mixture using the primers designed previously in our lab (forward primer 5'-ATTGGTATCCTCGTCCTGGC-3' and reverse primer 5'-GTTATCGTTGGCAGTGTCGTC-3'). Fragment length (111 bp) with a sequence ATTGGTATCCTCGTCCTGGCCGTCATGAATGTATGGATGGGACCAAAGAA GGACAGCGATTCTGACACTGATAAGGACACCGATGATGATGACGACACTG CCAACGATAAC was cut from the band of agarose gel electrophoresis. This band was then purified using the Nucleospin® Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany). The amount of DNA fragment was determined using the Nanodrop 2000 system.

Exposure of haemocytes to small DNA WSSV fragment and CpG: to determine the effect of a small fragment of WSSV DNA and CpG ODNs on the viability of haemocytes, separated haemocytes of penaeid shrimp were seeded on a glass insert in 24-well cell culture plate ($1x10^5$ cells $well^{-1}$). After one hour of incubation at 27^oC, allowing the cells to attach onto the substratum, the small WSSV DNA fragment and CpG oligodeoxynucleotides (CpG ODNs 2006, 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3', purchased from Invivogen-USA) at a dose of 1 µg/ml were added. The haemocytes on glass inserts were sampled at 0, 180, 360 and 720 min post inoculation and stained with EMA and Hoechst as described above.

2.10 Statistical analysis

All exposures were performed in three different experiments. The effect of exposures was statistically analyzed by analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. All statistical analyses were conducted by SPSS software.

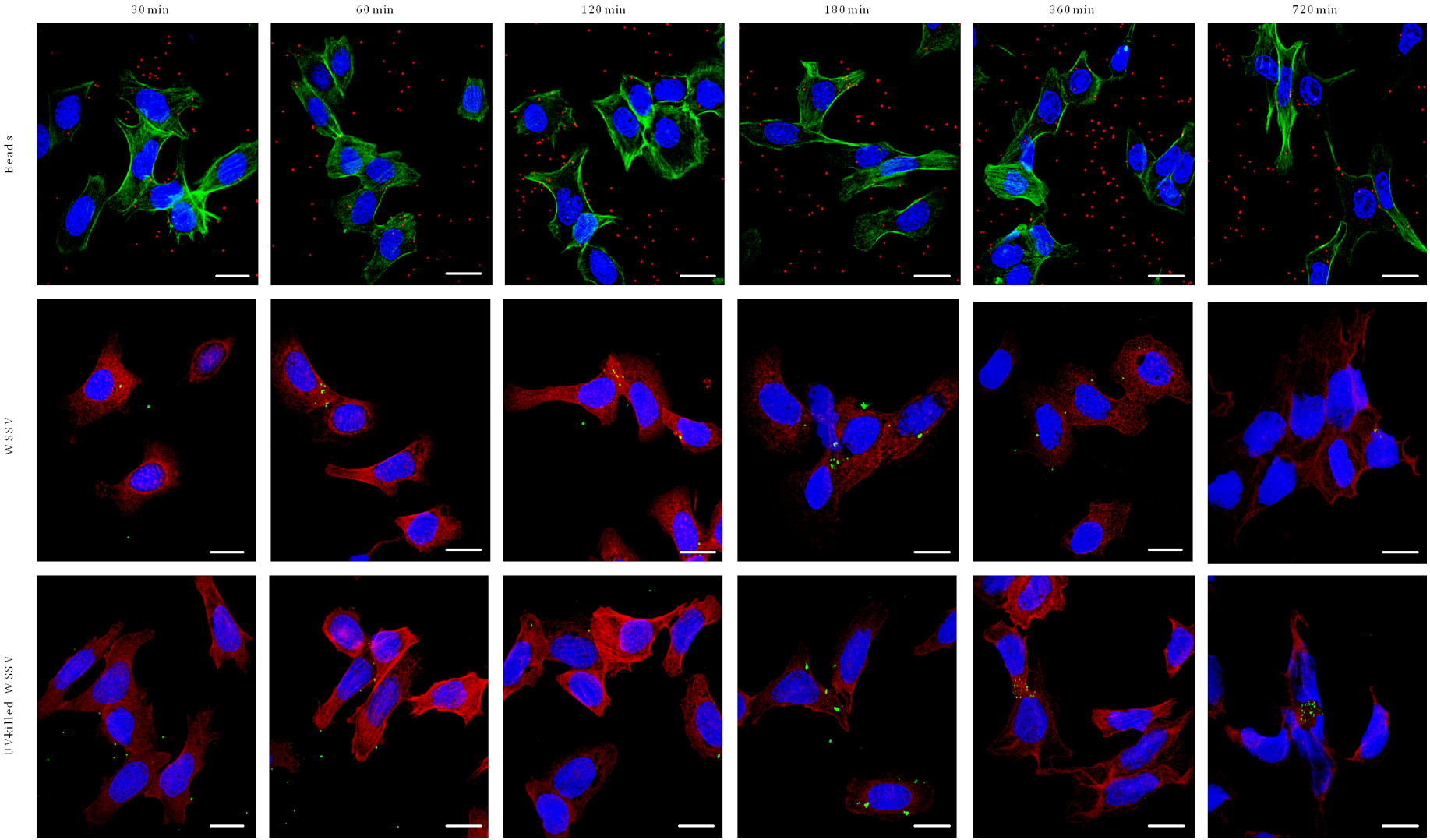
3. Results

3.1 Kinetic analysis of internalization of WSSV by haemocyte subpopulations of penaeid shrimp

To analyze the internalization kinetics of WSSV (both intact and UV-inactivated WSSV) and fluorescent polystyrene beads by shrimp haemocyte subpopulations, the cells were seeded in 24-well culture plates with glass inserts and incubated with beads or virus at a MOI of 100. Beads and viral internalization were analyzed by using confocal microscopy at different time points.

Haemocytes from sub 1, sub 4 and sub 5 of penaeid shrimp were able to endocytose WSSV and UV-inactivated WSSV as well as fluorescent polystyrene beads. The percentage of haemocytes of sub 1, sub 4 and sub 5 that contained endocytosed WSSV and beads varied according to the type of particles. After 30 min of incubation, the percentage of haemocytes that taking up WSSV, UV-inactivated WSSV and polystyrene beads was 5.7 ± 0.4%, 7.7 ± 0.8%, 40.5 ± 6.0% in sub 1; 4.0 ± 1.6%, 5.0 ± 2.5%, 35.4 ± 7.2% in sub 4 and 3.9 ± 1.6%, 8.4 ± 0.6%, 14.6 ± 1.7% in sub 5, respectively. The number of internalized WSSV, UV-inactivated WSSV and polystyrene beads per internalizing cell was 1.7 ± 0.5, 1.4 ± 0.1, 2.0 ± 0.1 in sub 1; 1.6 ± 0.1, 1.4 ± 0.1, 2.0 ± 0.4 in sub 4 and 1.4 ± 0.1, 1.4 ± 0.1, 1.5 ± 0.1 in sub 5, respectively.

The percentage and the number of WSSV, UV-inactivated WSSV and polystyrene beads that internalized by haemocytes of sub 1, 4 and 5 was significantly different at 720 mpi ($p < 0.05$). At this time point, the percentage of haemocytes that phagocytosed WSSV, UV-inactivated WSSV and polystyrene beads was 9.6 ± 2.4%, 12.9 ± 0.4%, 91.2 ± 8.5% in sub 1; 10.5 ± 3.0%, 13.3 ± 0.7%, 84.1 ± 6.6% in sub 4 and 7.9 ± 0.5%, 11.8 ± 0.6%, 58.1 ± 5.9% in sub 5, respectively. The number of internalized WSSV, UV-inactivated WSSV and polystyrene beads per internalizing cell was 1.4 ± 0.1, 2.0 ± 0.1, 4.8 ± 0.3 in sub 1; 1.7 ± 0.1, 1.9 ± 0.3, 4.2 ± 0.2 in sub 4 and 1.4 ± 0.1, 1.6 ± 0.3, 2.7 ± 0.3 in sub 5, respectively.


30 min
60 min
120 min
180 min
360 min
720 min
Beads
WSSV
UV-killed WSSV

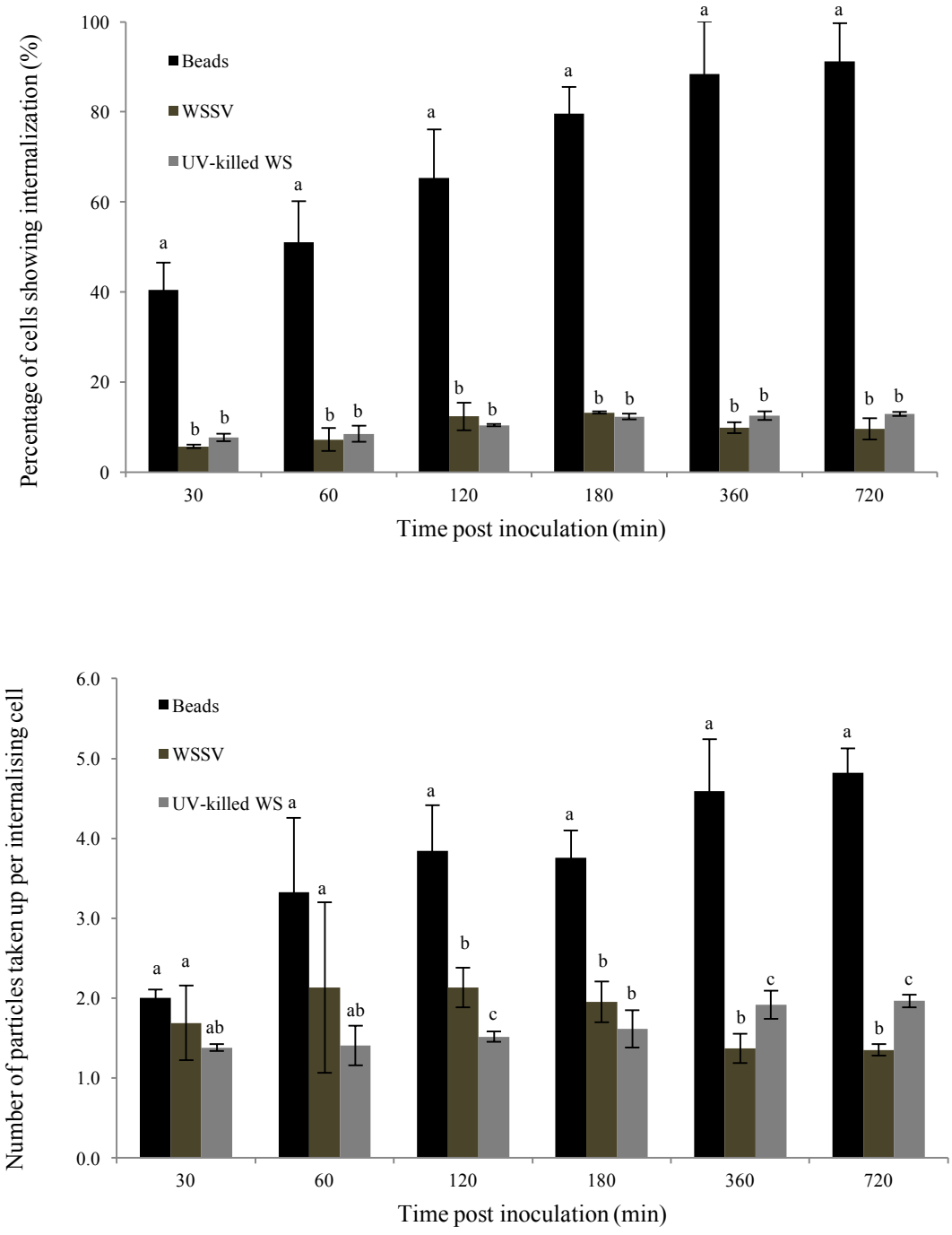
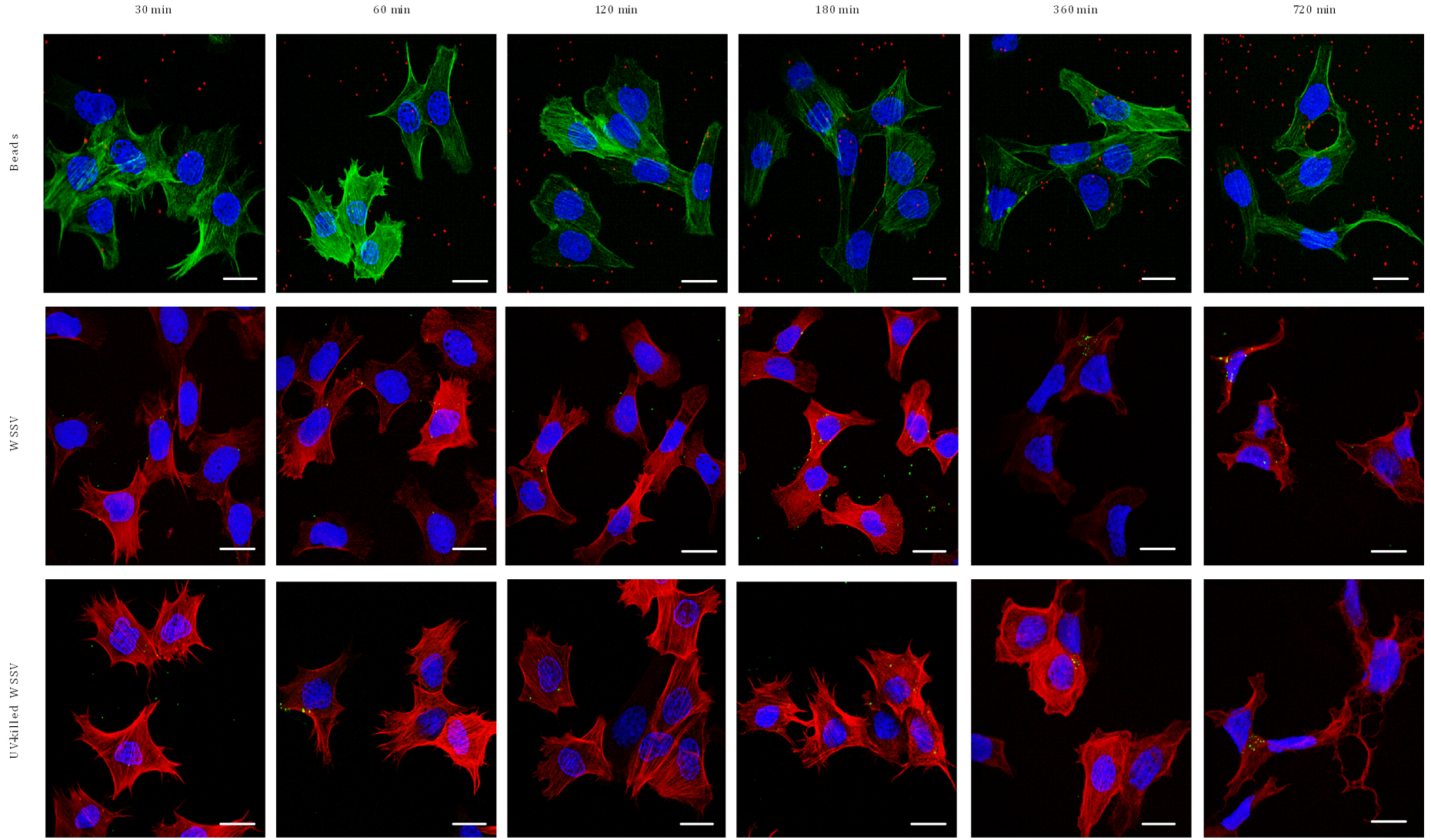


Figure 1. Uptake process of polystyrene beads, WSSV and UV-killed WSSV in haemocytes of sub 1. Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$). Scale bars: 15 µm.


30 min
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Beads
WSSV
UV-killed WSSV

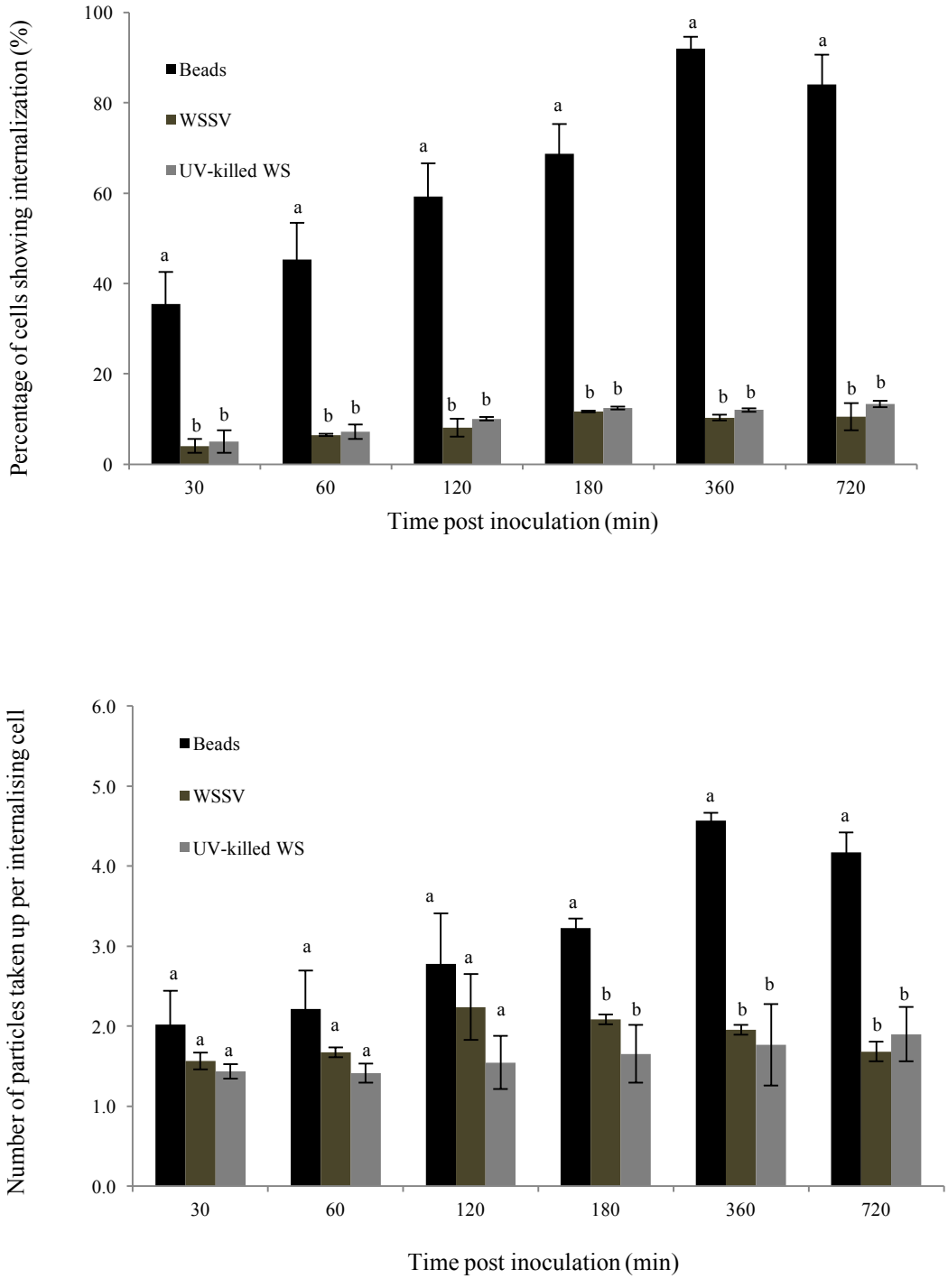
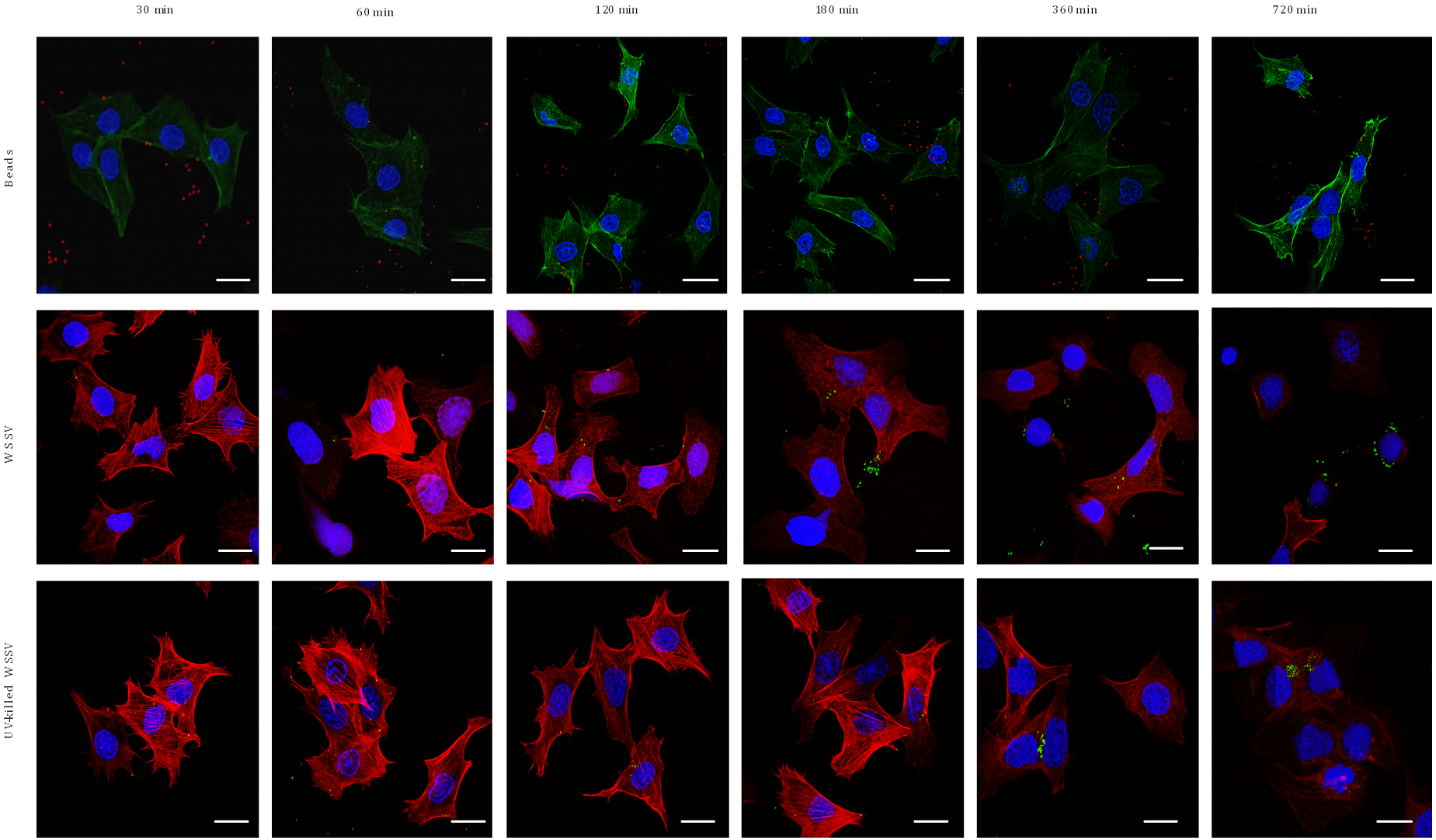


Figure 2. Uptake process of polystyrene beads, WSSV and UV-killed WSSV in haemocytes of sub 4. Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$). Scale bars: 15 μm.


30 min
60 min
120 min
180 min
360 min
720 min
Beads
WSSV
UV-killed WSSV

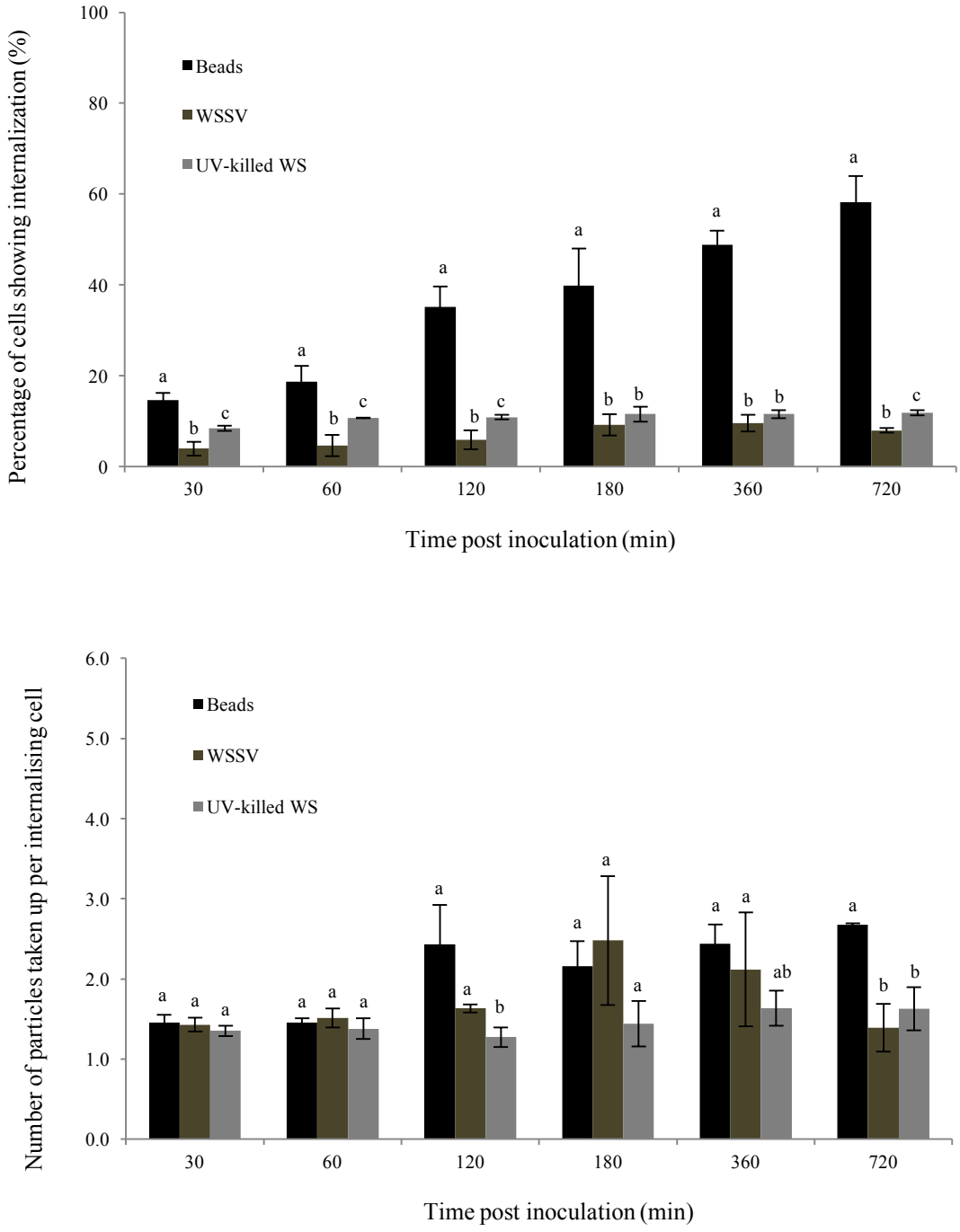


Figure 3. Uptake process of polystyrene beads, WSSV and UV-killed WSSV in haemocytes of sub 5. Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$). Scale bars: 15 µm.

Haemocytes of sub 2 and sub 3 did not internalize WSSV/beads, indicating that these haemocyte subpopulations do not have phagocytic capacities for small particles.

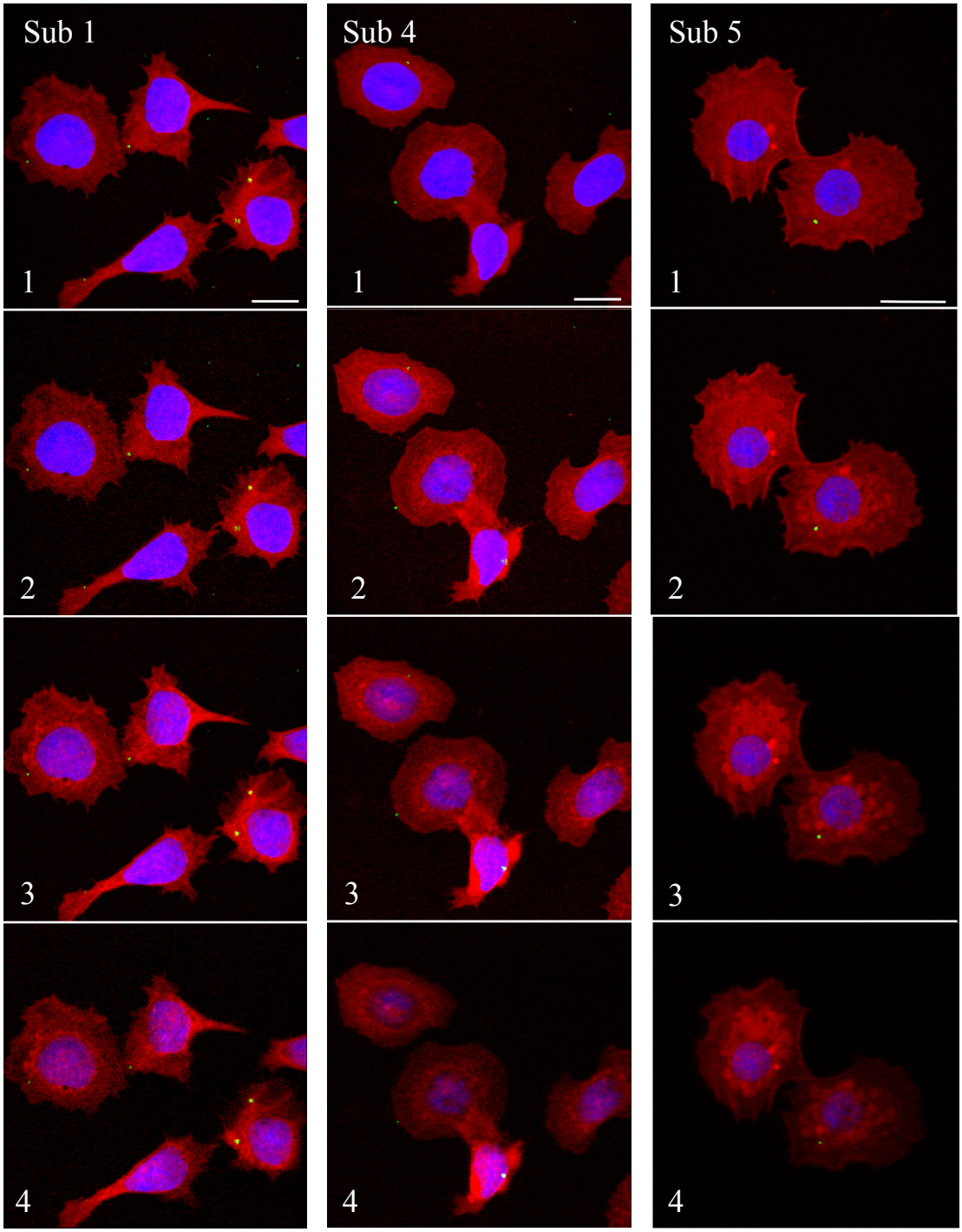


Figure 4. The images are a sequence of confocal microscopy pictures taken from the cell base (1) to its apex (4). This illustrates the process of virus internalization and therefore proves that cells from sub 1, sub 4 and sub 5 express endocytic activity.

3.2 Kinetics of WSSV disassembly in cytoplasmic endosome of shrimp haemocytes within subpopulation 1, 4 and 5

Intact WSSV virions (colocalization of nucleocapsid and envelope) were observed in the cytoplasm of penaeid shrimp haemocytes shortly after incubation. At 30 mpi, the

envelope and capsid of WSSV started to separate as demonstrated by staining with W29 (mouse monoclonal antibodies directed against WSSV envelope VP28) and WSSV419 (rabbit polyclonal antibodies against WSSV capsid protein VP664). The number of complete virions (co-localization of VP28 and VP664), single VP28 and single VP664 was 1.76 ± 0.21, 1.19 ± 0.13 and 1.55 ± 0.17 particles per positive cell in sub 1; 1.46 ± 0.06, 1.12 ± 0.08 and 1.50 ± 0.21 particles per positive cell in sub 4; 1.61 ± 0.13, 1.22 ± 0.20 and 1.33 ± 0.16 particles per positive cell in sub 5. At 180 mpi, the number of single VP28 and VP664 positive particles reached a maximum level (1.94 ± 0.04 and 2.07 ± 0.01 particles per positive cell in sub 1; 2.15 ± 0.21 and 2.36 ± 0.10 particles per positive in sub 4; 1.94 ± 0.01 and 2.11 ± 0.02 particles per positive cell in sub 5), while the complete virions dropped to a minimum (0.04 ± 0.0 particles per positive cell in sub 1; 0.08 ± 0.01 particles per positive cell in sub 4; 0.25 ± 0.02 particles per positive cell in sub 5). Later on, the capsids started to disintegrate (reduction from 2.07 ± 0.01 particles per positive cell at 180 min pi to 1.44 ± 0.05 particles per positive cell at 720 min pi in sub 1; from 2.36 ± 0.1 particles per positive cell at 180 min pi to 1.81 ± 0.01 particles per positive cell at 720 min pi in sub 4; from 2.11 ± 0.02 particles per positive cell at 180 min pi to 1.49 ± 0.37 particles per positive cell at 720 min pi in sub 5).

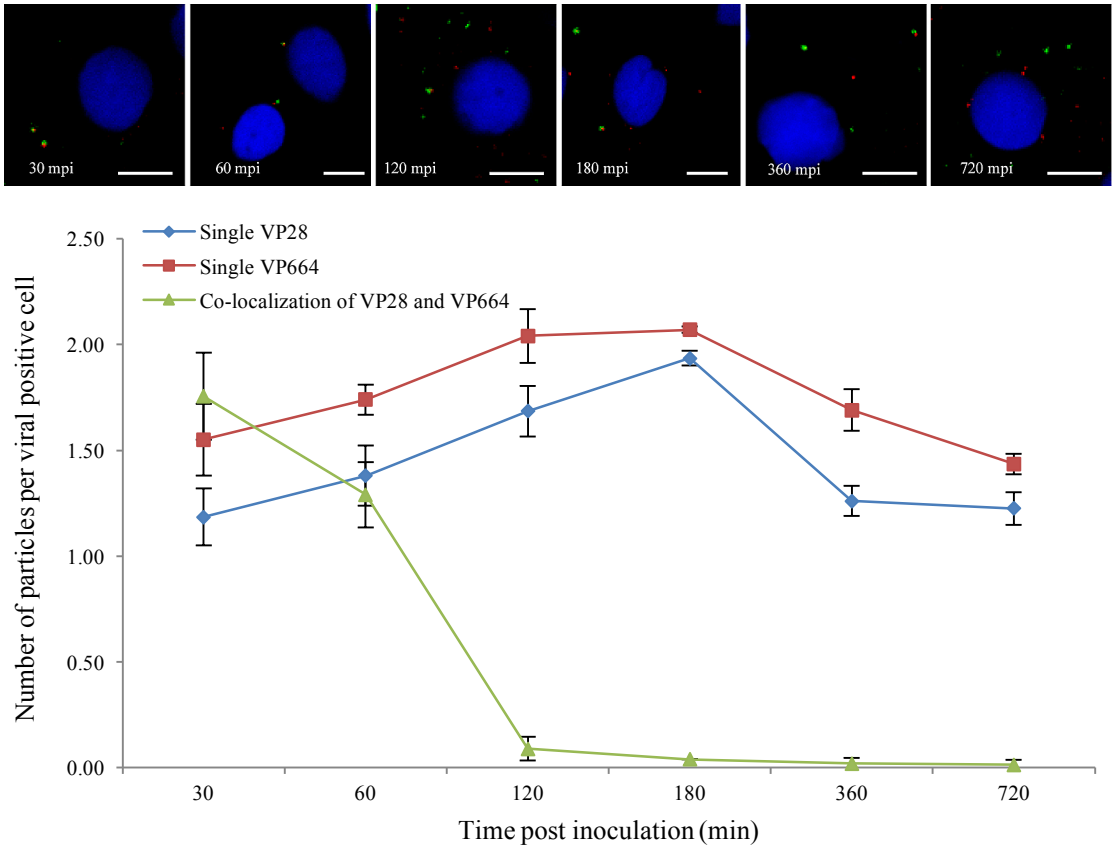


Figure 5. Kinetics of WSSV disassembly in cytoplasmic endosome of penaeid shrimp haemocytes within sub 1. Error bars are standard error of mean (n = 3). Scale bar: 5 µm.

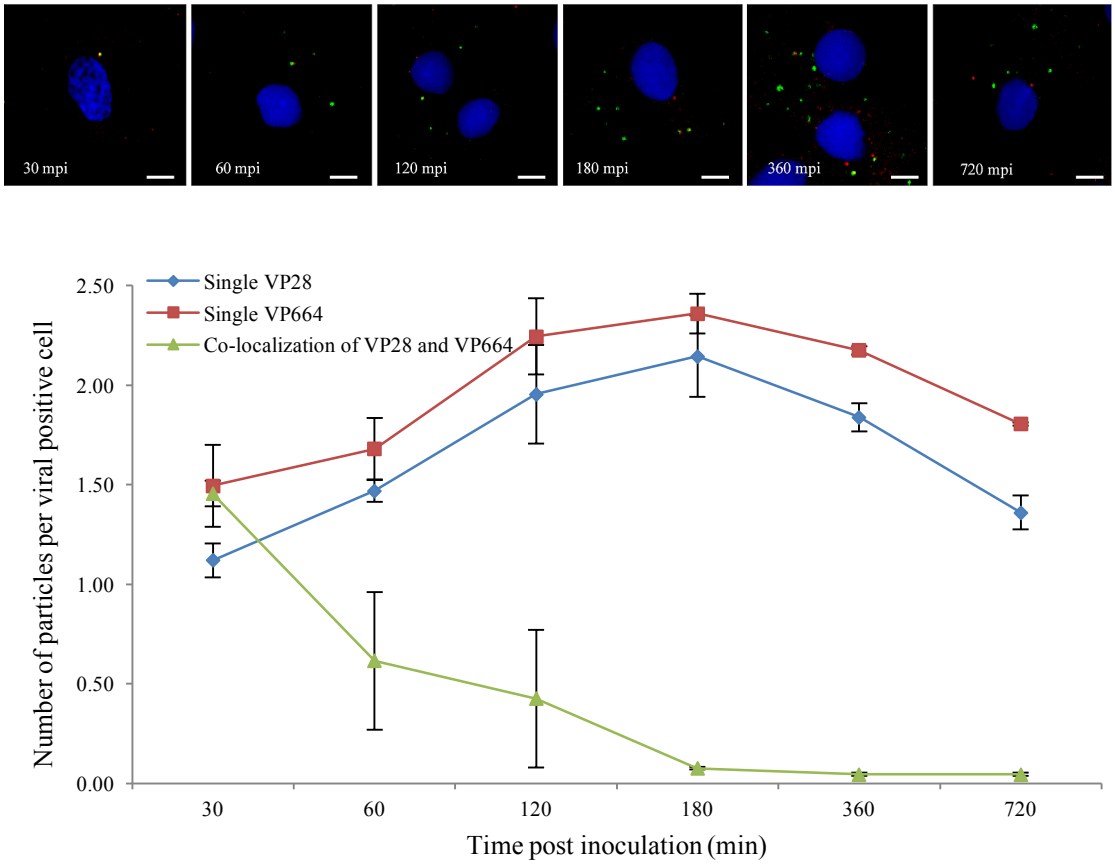


Figure 6. Kinetics of WSSV disassembly in cytoplasmic endosome of penaeid shrimp haemocytes within sub 4. Error bars are standard error of mean (n = 3). Scale bar: 5 µm.

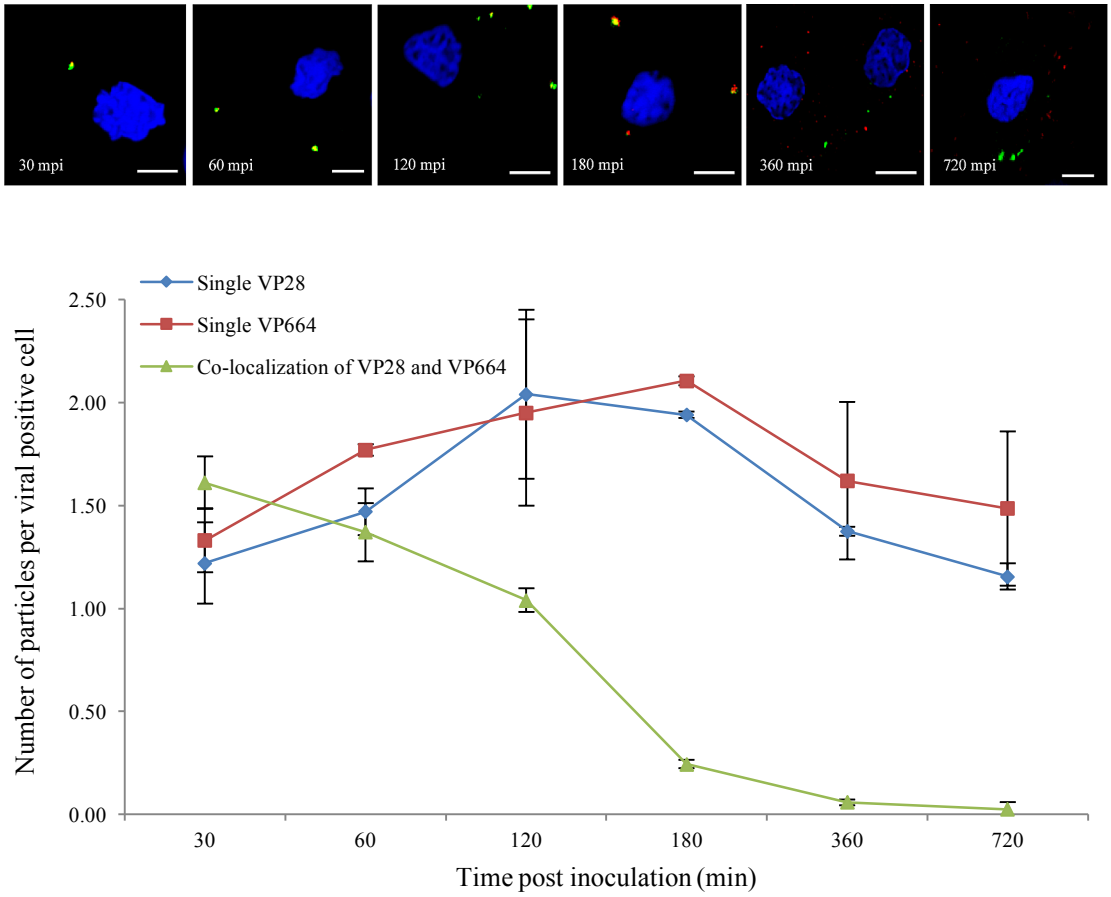


Figure 7. Kinetics of WSSV disassembly in cytoplasmic endosome of penaeid shrimp haemocytes within sub 5. Error bars are standard error of mean (n = 3). Scale bar: 5 µm.

3.3 Effect of WSSV on the viability of haemocytes within subpopulations

The exposure of haemocyte subpopulations to abiotic and biotic particles induced significant changes in cell viability. Incubation of haemocyte monolayers for 30 min with both abiotic and biotic particles did not significantly affect haemocyte survival. However, after 720 min post inoculation, WSSV induced a dramatic decrease of haemocyte survival in comparison with UV-inactivated WSSV and polystyrene beads. The haemocyte viability in sub 1, 4 and 5 was 49.6 ± 5.2%, 38.5 ± 5.7%, and 12.2 ± 1.1% for WSSV; 83.8 ± 2.3%, 81.1 ± 5.8%, and 46.9 ± 4.2% for UV-inactivated WSSV; 82.9 ± 8.2%, 70.7 ± 16.7% and 39.4 ± 7.5% for polystyrene beads; 82.8 ± 5.4%, 70.8 ± 1.8% and 49.7 ± 6.1% for mock exposure, respectively.

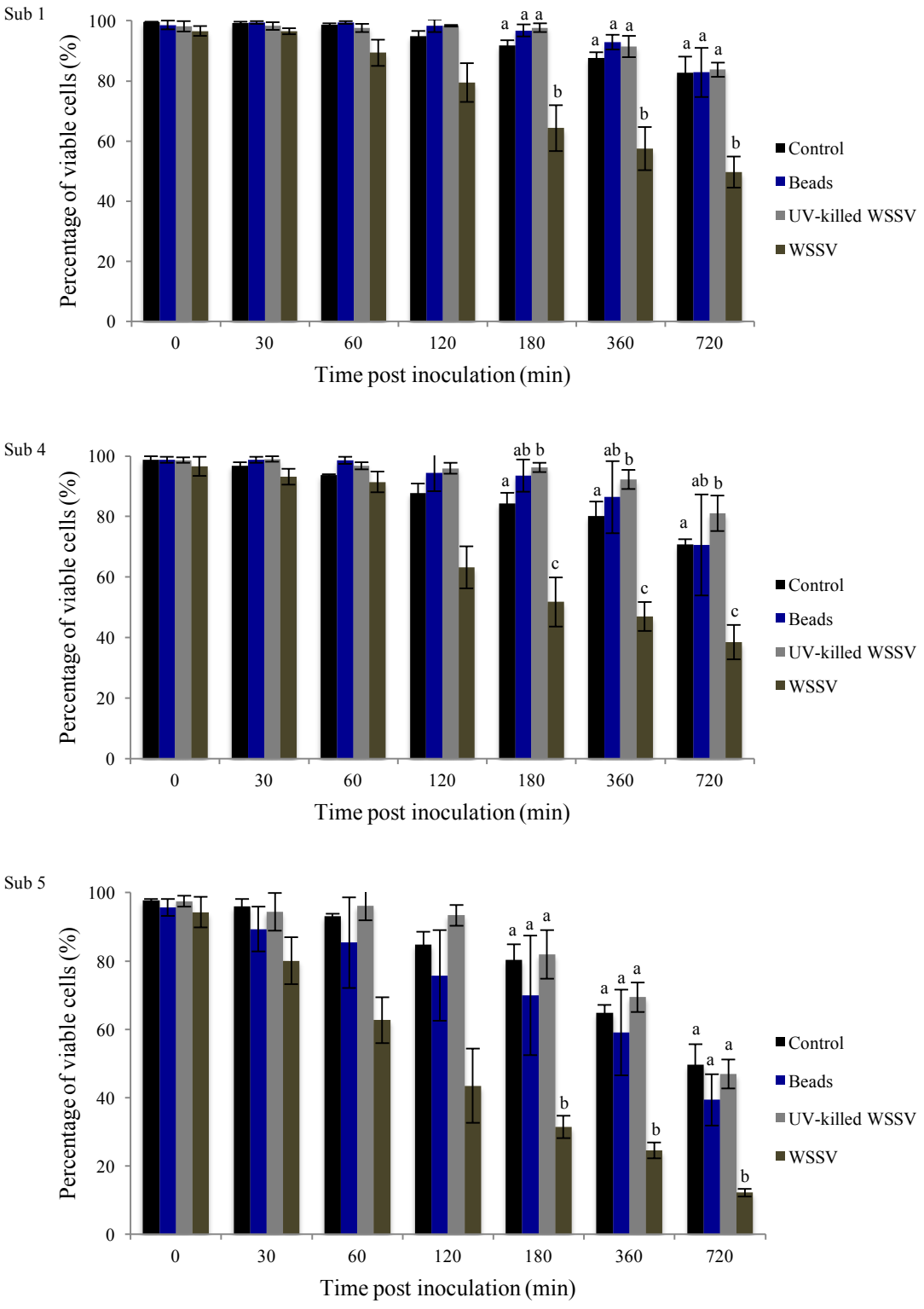


Figure 8. Effect of beads, WSSV and UV-killed WSSV on survival of haemocytes of sub 1, 4 and 5 as determined by ethidium monoazide bromide (EMA). Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$).

3.4 Effect of small WSSV DNA fragment (VP19) and CpG oligodeoxynucleotides on the viability of haemocytes within subpopulations

Small WSSV DNA fragment (VP19) and CpG ODNs were added to shrimp haemocyte subpopulations as described above to determine if there was any effect of DNA genetic material on the viability of shrimp haemocytes within the different subpopulations. The percentage of viable haemocytes within sub 1, 4 and 5 upon mock exposure decreased slightly during the observation period of 180 mpi (99.1 ± 0.2% in sub 1; 97.5 ± 2.0% in sub 4; 91.0 ± 7.5% in sub 5). Both WSSV DNA fragment and CpG ODNs potently induced cell death and caused a statistically significant difference of cell viability in culture at 720 mpi (79.5 ± 12.7% and 84.9 ± 1.8% in sub 1; 73.4 ± 13.9% and 77.8 ± 2.2% in sub 4; 50.8 ± 8.4% and 58.0 ± 3.3% in sub 5). UV treatment of the WSSV DNA fragment did not affect the viability.

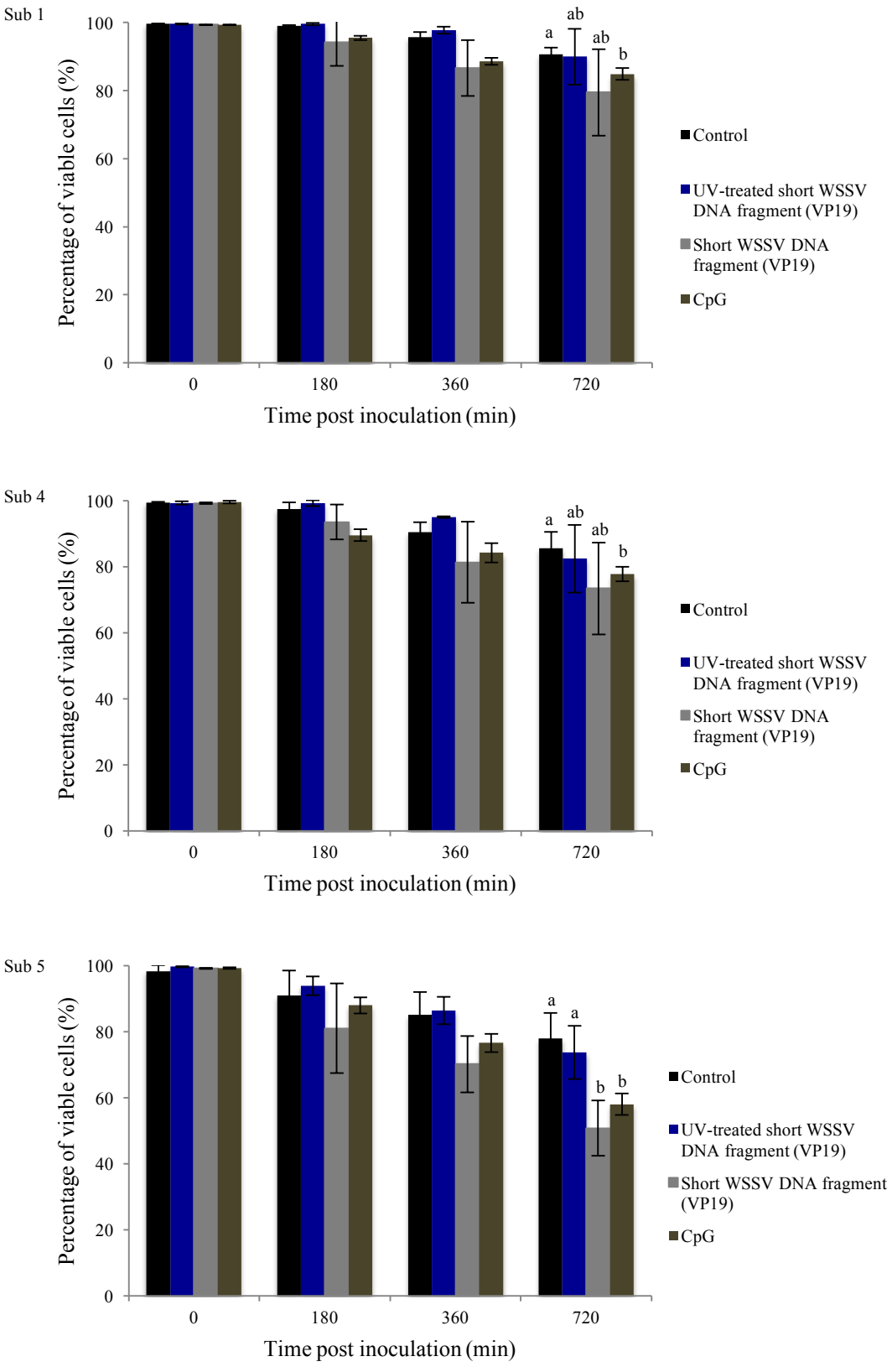


Figure 9. Effect of short DNA fragment of WSSV (111 bp) and CpG on survival of haemocytes of sub 1, 4 and 5 as determined by ethidium monoazide bromide (EMA). Data (mean ± SE; n = 3) with different letters were significantly different ($p < 0.05$).

4. Discussion

The haemocyte subpopulations of penaeid shrimp can be separated into five different subpopulations by centrifugation in a two-step continuous density gradient of iodixanol (Dantas-Lima et al., 2013). Each subpopulation has different functions and is involved in different defense reactions. A previous study has shown that haemocytes of sub 1 (hyalinocytes) and sub 4 (semi-granulocytes) efficiently phagocytose large abiotic particles (fluorescent polystyrene beads with a diameter of 1 μm) and biotic particles (pathogenic and non-pathogenic bacteria) *in vitro* (Dantas-Lima et al., 2013; Tuan et al., 2015). In the current study, we demonstrated that the same subpopulations, together with haemocytes of sub 5 (granulocytes) were the ones involved in endocytosis of small abiotic (fluorescent polystyrene beads with a diameter of 0.2 μm) and biotic particles (WSSV and UV-killed WSSV). The role of the non-phagocytic haemocytes of sub 2 and sub 3 in defense is totally not clear and more research is necessary to elucidate their immunological function.

To the best of our knowledge, this is the first report where the endocytic activity of the five different haemocyte subpopulations of *P. vannamei* was studied *in vitro*. WSS virions appear to bind directly to cell surface molecules that subsequently mediate internalization in hyalinocytes, semi-granulocytes and granulocytes. This binding is most probably mediated by viral attachment proteins on the surface of the virus particle (viral ligands) and certain virus receptors on the target cells. VP28 of WSSV has been considered as attachment and penetration protein (Yi et al., 2004). This protein has a strong hydrophobic region and can bind to the shrimp cells, and then help the virus to enter the cytoplasm. By confocal microscopy, it is interesting to note that some virus particles were present as single particles while others grouped together on certain areas of the cell surface. These findings suggest that WSSV accumulates at areas with high endocytic activity on the plasma membrane. Besides, we also observed that a lot of virus particles were sticking to the cell membrane, but did not internalize. This suggests that WSSV has the ability to escape immune recognition by host haemocytes. Another possibility is that the host cells recognized WSSV and initiate an efficient immune response to block the endocytosis. This explains also the five to 10 fold lower percentages of cells that take up WSSV in comparison with abiotic beads.

After entry, the virus envelope and nucleocapsid started to separate from each other. The nucleocapsid seemed to be transported to the region nearby the nucleus. This

process resembles well the early stage of a WSSV infection of secondary cells of the lymphoid organ as earlier demonstrated by Li et al. (2015). However, the uptake of WSSV by haemocytes of penaeid shrimp did not result in efficient expression of new viral proteins. It looks like the viral replication cycle is blocked at the viral genome expression in the nucleus. How the replication was blocked is not clear. It is possible that there is a cellular mechanism that inhibits viral replication in endocytic haemocytes. This antiviral mechanism could restrict infection at the transcriptional/translational level. Another explanation could be that virus may enter latency. A similar phenomenon was reported in human monocytes by Noriega et al. (2014). These authors demonstrated that human cytomegalovirus utilizes cellular miRNAs to repress expression of viral transcripts. Another reason could be the activation of cell death before viral protein expression. By committing suicide, cells become recognized and are targets for adjacent non-infected haemocytes. Upon uptake, these cells become eliminated. This system fully blocks viral spread.

Viruses can be distinguished from other organisms because they are obligatory intracellular pathogens. They absolutely require living host cells in order to replicate. In the current experiment, WSSV, the most serious pathogen to penaeid shrimp, induced cell death, which was found to be time-dependent as the percentage of apoptotic haemocytes increased with the time course of the infection. More than 80% of haemocytes of sub 5 and 50% of haemocytes of sub 1 and 4 were dead at 720 mpi. Our findings are in agreement with the finding of Wongprasert et al. (2003) who reported that 60% of haemocytes of *Penaeus indicus* were dead during an *in vivo* WSSV infection. By administering CpG oligodeoxynucleotides and a short WSSV DNA fragment (VP19) to shrimp haemocytes *in vitro*, cells also died, showing that DNA on itself may activate cell death. The molecular mechanism involved in CpG ODNs and viral DNA mediated cell death is unknown. It is very well possible that CpG ODNs and viral DNA are sensed by Toll like receptors and then activate Toll and JAK/STAT signaling pathway, resulting in the death of cells as earlier has been shown for mammalian cells (Liang et al., 2010; Sun et al., 2013). By committing suicide, the virus can no longer replicate and the dead haemocytes, that contain fragmented viruses, are destroyed by neighboring haemocytes. This is a beautiful example of the strong antiviral innate immunity in shrimp.

In conclusion, our study showed that WSSV is efficiently internalized by hyalinocytes, semi-granulocytes and granulocytes of penaeid shrimp. Upon internalization, the virus

is losing its envelope and the capsid becomes disintegrated, however new viral proteins are not expressed. WSSV internalization leads to cell death and it was shown that released DNA may be responsible for this. In the future, more research will be performed to better understand the mechanism of the haemocyte killing by WSSV upon endocytosis.

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Chapter 6

General discussion

Crustacean aquaculture is one of the fastest growing animal production industries all over the world. The increasing importance of crustaceans in both fisheries and aquaculture has stimulated the research for getting a better understanding of their general physiology. Since crustacean aquaculture is highly affected by infectious diseases (bacteria and viruses), disease control is considered as a priority. However, to control the diseases, it is necessary to understand thoroughly the nature of the host immune system, especially the role of the different types of haemocytes in defense reactions.

Crustacean haemocytes are very reactive and unstable in culture. After removal from the haemocoel, some cell types are rapidly clumping or lysed while others adhere to the surface of the culture vessel and are very mobile. Therefore, research on functional characteristics of these cells is more difficult than with vertebrate blood cells. In previous mixed haemocyte cultures, most of granular and semi-granular cells become disrupted and release their contents into the culture media within 12 hpi. This negatively impacts the survival of the other cell types. Therefore, to study the characteristics of each haemocyte type, it is crucial to separate them and culture them individually (chapter 3).

Development of techniques for separation of haemocyte subpopulations of *Litopenaeus vannamei*

Crustacean haemocytes are traditionally divided on the basis of morphology and number and size of granules into three distinct subpopulations: (i) hyaline cells which possess an ovoid shape and have few small basophilic and eosinophilic granules, (ii) semi-granular cells which have an ovoid shape and contain a variable number of small eosinophilic granules, and (iii) granular cells which have a spherical shape and contain many large eosinophilic granules (Söderhäll & Smith, 1983; van de Braak et al., 1996; Li & Shields, 2007; Hong et al., 2013; Sunjian et al., 2014). Classification of the crustacean haemocytes has been discussed for the last two decades. Up till now, only one procedure based on a Percoll density gradient (Söderhäll & Smith, 1983) was described. This procedure allowed the separation of haemocyte types with no apparent deleterious effects since the cell functionality was preserved in most of the cases. However, only granulocytes were isolated efficiently.

To improve the separation of crustacean haemocytes, especially from *Litopenaeus*

vannamei, we selected iodixanol as an alternative separation medium to Percoll. Although both media share suitable characteristics for an efficient separation of cells, cell organelles, and other subcellular structures, iodixanol has more advantages. The density gradients of iodixanol are nearly linear, while the density gradients of Percoll present two steep regions, one at the top and one at the bottom of the gradient. In these regions, there is a high range of density values per volume unit. The Percoll gradients tend to concentrate cells in sharp bands at the top and bottom of the gradient, and therefore they may have problems in separating haemocytes with rather similar buoyant densities. The nearly linear profile of iodixanol density gradients can promote the formation and visualization of more individual cell bands compared to Percoll gradients. Using iodixanol density gradients, five haemocyte subtypes (subpopulations) of *Litopenaeus vannamei* were identified, from which three were isolated with a very high degree of purity. The purity of these cells was determined based on the percentage of major cell types in each band. Cells from band 3 (sub 5) had the highest purity (99.4%) followed by cells from band 2 (sub 2: 97.7%) and band 1 (sub 1: 95%). Since cells of sub 3 and sub 4 were mixed in the dispersed cell band, we could not evaluate the purity level. These subpopulations could be distinguished by flow cytometry and biological activities as determined by light microscopy. Two of them were easily identified based on existing literature (Jiravanichpaisal et al., 2006). Subpopulation (sub) 1 exhibited all the characteristics of hyalinocytes such as strong adhesion to glass with extensive spreading, high nucleus/cytoplasmic ratio, and absence (or low level) of cytoplasmic granules. The classification of haemocytes of sub 2 and sub 3 (50% of the haemocytes) was much more difficult. The morphology of these cells did not fit in the definition of hyalinocytes, semi-granulocytes or granulocytes. These cells were smaller but more granular than hyalinocytes and had a very high nucleus/cytoplasmic ratio. Interestingly, these haemocytes did not adhere to glass and presented folds in the nucleus. Because these haemocytes did not attach to the substrate, they easily disappeared during washing steps of culture. This could be the reason why these subpopulations were not recorded as haemocyte subpopulations in the past studies. Based on these characteristics, it was speculated that these haemocyte subpopulations could be classified as small hyaline cells (Rodriguez et al., 1995), small granule haemocytes or lymphocyte-like hyalinocytes (Hose et al., 1987; Vargas-Albores et al., 2005) and pro-haemocytes or immature haemocytes (Roulston & Smith, 2011). Haemocytes of sub 4 had the characteristics of semi-granulocytes while haemocytes of

sub 5 were clearly granulocytes: moderate adhesion to glass, small nucleus/cytoplasmic ratio and presence of a lot of big granules in cytoplasm.

A previous experiment showed that a mixture of whole haemocyte subpopulations could not survive longer than 48 hpi *in vitro* (Dantas-Lima et al., 2012), due to disruption and degranulation of the haemocytes. Even some cell types (granulocytes and semi-granulocytes) started to release their contents into the culture media within 6-12 hpi (own observations). As a result, they died in the culture suspension. They could even impact the survival of other cell types. Therefore, to overcome these problems, the haemocyte subpopulations were separated and cultured individually. The results of our study showed that individual cell cultures could prolong their survival. The haemocytes of sub 1 and sub 2 had the best survival performance. These cells could be kept alive up to 120 h (as demonstrated in Figure 3 of chapter 3). The haemocytes of sub 3+4 and sub 5 were less stable. They were maintained in culture until 48 h. All subpopulations demonstrated a viability of over 50% in the first 12 h of culture.

Phagocytic capacity of different haemocyte subpopulations

Haemocytes are excellent biological material to study the interaction between host and pathogen because the internal defense system of crustaceans relies mainly on the activity of these cells. Since individual subtypes/subpopulations of haemocytes are likely involved in different aspects of the cellular immune response, we assessed the functional characteristics of haemocytes after purification by determining the phagocytic capability of the different subpopulations of haemocytes towards abiotic and biotic particles and the fate of biotic particles and cells upon infection. In the present study, the uptake of large particles such as bacteria (chapter 4) and small particles such as viruses (chapter 5) were analyzed.

Phagocytosis is considered as the most primitive immune-defense process. It represents an important defense mechanism in vertebrates and invertebrates. The phagocytic process consists of recognition, adherence, ingestion, destruction and disposal. Recognition of foreign materials is achieved by the direct interaction of surface receptors on haemocytes with molecules on the invading organisms, leading to the engulfment of particles into cell and subsequent formation of phagosomes to initiate the digestion of microbes.

Circulating haemocytes in crustaceans are the main cell type that perform the phagocytosis process and are considered to be crucial to eliminate foreign particles. In the current study, it was demonstrated that hyalinocytes (sub 1) and semi-granulocytes (sub 4) of *Litopenaeus vannamei* were the main cell types involved in phagocytosis of both pathogenic and non-pathogenic bacteria as well as fluorescent polystyrene beads. The role of non-phagocytic pro-hyalinocytes (sub 2 and sub 3) and granulocytes (sub 5) in anti-bacterial defense is unknown. They most probably perform immunological functions different from phagocytosis. Our results are in agreement with the finding of Roulston and Smith (2011). These authors demonstrated that pro-haemocytes or immature haemocytes have no phagocytic activity.

Phagocytosis by penaeid shrimp haemocytes is a strong but apparently non-specific process. Hyalinocytes (sub 1) and semi-granulocytes (sub 4) are extremely active and engulf a wide range of particles from abiotic polystyrene beads to pathogenic and non-pathogenic bacteria. The hyalinocytes (sub 1) of *Litopenaeus vannamei* are the major phagocytic cells with approximately 45% of cells engulfing foreign materials, while the semi-granulocytes (sub 4) have a more limited phagocytic capacity (27% of the cells). In comparison with other studies, the phagocytic activity and the haemocyte types involved in the phagocytic reaction in our study was different. The reasons could be:

(a) Animal species: many authors reported that the cell types participating in phagocytosis are dependent on the animal species. Hyalinocytes and semi-granulocytes are considered as the most important phagocytic cells in freshwater crayfish (Smith & Söderhäll, 1983). In contrast, only hyalinocytes of Chinese mitten crab (*Eriocheir sinensis*) and tiger shrimp (*Penaeus monodon*) are involved in phagocytosis (Sung and Sun, 2002; Sunjian et al., 2014). The present thesis demonstrated that hyalinocytes (sub 1) and semi-granulocytes (sub 4) of white leg shrimp (*Litopenaeus vannamei*) were the main cell types involved in phagocytosis.

(b) Bacterial species: the percentage of cells involved in phagocytosis is dependent on the bacteria used. Indeed, in the current study, different bacteria species such as *Vibrio campbellii*, *Vibrio harveyi* and *E. coli* were used in the phagocytic assay. We found that *Vibrio campbellii* was engulfed by a higher percentage of cells (45%) in comparison with *Vibrio harveyi* (25%) and *E. coli* (13%). Because there are no other reports available in literatures comparing the phagocytosis of different bacteria in the same haemocytes of a certain crustacea species. We could not compare our results with previous publications.

(c) Opsonization of particles: Sahoo et al. (2007) reported that opsinized bacteria *Aeromonas hydrophila* with prawn serum increased phagocytic activity. Opsonized bacteria were cleared from the circulation in *Macrobrachium rosenbergii* faster than non-opsonized bacteria, suggesting that opsonins in serum were one of the most important factors enhancing phagocytosis in prawn. Similarly, in lobster *Homarus americanus*, activated haemocytes showed increased phagocytosis of opsonized sheep red blood cells (Goldenberg et al., 1984). Jayasree (2009) also confirmed that opsonized bacteria *Vibrio alginolyticus* in cell-free haemolymph before addition to the haemocyte cultures increased the phagocytosis rate. The main reason could be due to availability of opsonin binding sites on haemocyte membranes. Lectins or agglutinins (Kondo et al., 1992) and peroxinectin (Johansson et al., 1995) are considered as opsonic factors in crustaceans. In the current study, we did not opsonize the bacteria or beads before inoculation with shrimp haemocytes. May be as a result of that, the percentage of phagocytic cells in our study was lower.

Following phagocytosis, certain bacterial species are killed or at least hampered in their growth by penaeid shrimp haemocytes. *Vibrio campbellii* and *Vibrio harveyi* are considered as pathogenic bacteria in aquaculture. They induce serious disease and elicit strong immune response from the host to fight against invaders, whereas *E. coli* used in the experiment is considered as non-pathogenic bacteria to penaeid shrimp. Our study demonstrated that haemocytes of sub 1 and sub 4 of *Litopenaeus vannamei* could phagocytose and destroy non-pathogenic bacteria after 180 min of incubation, but failed to kill pathogenic ones. It is not fully understood why certain bacteria are more susceptible/resistant to be killed by penaeid shrimp haemocytes. The failure in killing pathogenic bacteria could be due to the capability of these bacteria to inhibit phagosome maturation and phagosome-lysosome fusion. In order to do this, bacteria may respond by a conversion of its morphology from rod-shaped to a coccal form (own observations). This conversion may represent a survival strategy of the bacteria against the intracellular killing by phagocytic cells.

The exposure of haemocyte subpopulations to abiotic and biotic particles also induced significant changes in cell viability. The data presented in chapter 4 showed that the survival of penaeid shrimp haemocytes decreased significantly at 180 mpi after phagocytosis of pathogenic bacteria *Vibrio campbellii*. In contrast, phagocytosis of non-pathogenic bacteria had no such effect on the survival of these cells. These findings suggest that *V. campbellii* could have a cytotoxic effect upon ingestion. The study of

Nottage & Birkbeck (1990) indicated that certain vibrio strains were toxic to *Mytilus edulis* haemocytes when present in large numbers. In addition, Lambert et al. (2001) also confirmed that *Vibrio pectinicida* can cause a decrease in haemocyte viability of the scallop *Pecten maximus* upon contact with live bacteria.

The invasive activity of pathogenic bacteria is usually associated with the potential to inhibit the humoral and cellular defense of the host. In insects, the particular haemocytes reported to be phagocytic varies among insect taxa. For instance, granular cells and plasmatocytes of Lepidoptera (butterflies) are the only haemocyte types reported to be phagocytic, while plasmatocytes of *Drosophila* and wax moth (*Galleria mellonella*) are the main phagocytic haemocytes (Ratcliffe et al., 1984; Elrod-Erickson et al., 2000). These phagocytic cells are capable of phagocytosing bacteria of different pathogenicities. Upon phagocytosis, the virulent strain induced a significant drop in haemocyte viability. These bacteria released enzymatic factors such as phospholipase C and other toxins such as hemolysin that kill phagocytic cells. In mammals, neutrophils and macrophages are considered as professional phagocytic cells. These cells actively engulf microbes and kill them. However, some pathogenic bacteria such as *Streptococcus suis*, *S. aureus, Campylobacter jejuni, Staphylococcus aureus* can survive upon phagocytosis (Kiehlbauch et al., 1985; William, 1990; Kubica et al., 2008). These bacteria appear to be resistant to bactericidal actions inside the phagocytic vacuoles of phagocytic cells. As a results, the bacteria grow intracellular and kill phagocytic cells.

Kinetics of WSSV entry and fate of both WSSV and cells upon infection

Viral diseases have the most devastating impact on aquaculture industry. So far, about 20 types of viruses have been reported in penaeid shrimp, among which white spot syndrome virus (WSSV) has been responsible for major losses in shrimp culture. The control of viral diseases plays an important role in shrimp culture, however little information is available on the basic mechanisms of WSSV infection and replication. To better understand the interaction between virus and host, techniques for culture and maintenance of viruses and their host cells *in vitro* are important. In chapter 5 of this study, haemocytes of penaeid shrimp, *Litopenaeus vannamei*, were separated into 5 subpopulations and used to examine the kinetics of WSSV entry and fate of both WSSV and cells upon infection. Generally, viruses can enter a cell via two pathways: (1) direct

fusion with the plasma membrane or (2) receptor-mediated endocytosis. So far, no evidence existed that WSSV can enter shrimp cells by direct fusion. The interaction between viral proteins and host cell membranes plays a crucial role in helping the virus to enter into host cells. Yi et al. (2004) mentioned that WSSV envelope protein VP28 was responsible for binding to and entering shrimp cells. In the present thesis, the observation of VP28 inside the host cells suggested that the entry of WSSV is not based on fusion of the viral envelope to the host cells, but on a process of endocytosis. Recently, studies of Huang et al. (2013) and Li et al. (2015) confirmed that WSSV uses endocytosis to enter shrimp cells. Besides VP28, other structural proteins of WSSV such as VP37, VP466, VP26 have been reported to interact with host cell components (Xie et al., 2005; Wu et al., 2005; Liu et al., 2009). Our study showed that WSS virions can be bound and internalized by 3 out of 5 different haemocyte subpopulations of penaeid shrimp (sub 1 (hyalinocytes), sub 4 (semi-granulocytes) and sub 5 (granulocytes)). This penetration was probably mediated by a small GTP-binding protein, PmRab7 (Sritunyalucksana et al., 2006). It is interesting to note that haemocytes within sub 5 (granulocytes) have an ability to internalize WSSV, but fail to take up bacteria. The reason is not fully understood. It could be that some virus binding receptors are specifically localized on the surface membranes of granuloctyes. Liang et al. (2015) demonstrated the presence of ATPsyn beta subunit (named as BP53) on the membrane of some circulating haemocytes of *Penaeus vannamei,* which may serve as a receptor for WSSV binding.

After entry, the virus envelope and nucleocapsid started to separate from each other. This process was most probably due to the uncoating of the virus in the endosome complex (Li et al., 2015) with the viral envelope staying in the endosome and the nucleocapsid getting free in the cytosol. The nucleocapsids were then transported to the region close to the cell nucleus. It is possible that viral DNA was entering the nucleus. Because new viral proteins were not expressed later on, one can conclude that genome transcription and translation into new viral proteins did not occur. How the replication was blocked is not clear. It is possible that there is a cellular mechanism that inhibits viral replication in endocytic haemocytes. This antiviral mechanism could restrict infection at the transcriptional/translational level. Another explanation could be that haemocytes commit suicide upon sensing WSSV. Indeed, sensing DNA by toll like receptors may activate an interference phenomenon and/or activate apoptosis. These outcomes have extensively been studied and reported for mammals.

Although viral particles were internalized by shrimp haemocytes, there were still a lot of viral particles sticking to the cell membrane without being internalized. It is possible that the host cells recognize WSSV as a foreign particle and initiate an efficient immune response to prevent the entry of virus into cells. This is indicative for the existence of a WSSV internalization block. Another explanation could be that WSSV has the ability to escape immune recognition by host haemocytes. Further research is necessary to unravel the underlying mechanism of this viral immune evasion system.

The results of chapter 5 showed that the cell death in penaeid shrimp haemocytes occurred at the early stage of virus infection/internalization. It was assumed that WSSV activated a programmed cell death (apoptosis). Apoptosis is considered as an innate cellular response to limit viral replication. The induction of early cell death could severely limit virus production and eliminate spread of progeny virus from host cells. It is very surprising that WSSV did not develop a mechanism to block this antiviral activity. By committing suicide, cells become recognized and are targets for adjacent non-infected haemocytes. Upon uptake, these cells become eliminated. This system fully blocks viral spread.

In literature, some publications mentioned that WSSV can infect penaeid shrimp haemocytes (*Penaeus merguiensis*, *Penaeus chinensis*) (Wang et al., 2002; Jiang et al., 2005). Based on the presence of virus particles in vacuoles and nucleus of semi-granulocytes and granulocytes, these authors concluded that WSSV infects shrimp haemocytes and assembles in the nucleus of these infected cells. However, we do not agree with this conclusion. We think that the presence of virus particles inside shrimp haemocytes could be the result of endocytosis. The results of our study have demonstrated that the uptake of WSSV by haemocytes of penaeid shrimp (*Litopenaeus vannamei*) did not result in efficient expression of new viral proteins. It means that free circulating haemocytes of *Litopenaeus vannamei* are not targets for replication of virus. As far as we know, cells from ectodermal and mesodermal origin, such as epidermis, gills, foregut, hindgut, antennal, lymphoid organ, heart, gonads, haematopoietic tissue are targets for virus replication. Virus infection severely damages these organs and causes organ dysfunctions. This may explain why, upon WSSV infection, the cumulative mortality of cultured shrimp can reach 100% in a short period of time (within 3-10 days).

The results of cell death upon internalization of WSSV in our study may partly explain the previous work of our promoter (Phuoc et al., 2008). In this study, shrimp were first

injected with WSSV and 24 h later with a non lethal dose of bacteria *Vibrio campbellii*. It was shown that WSSV and *Vibrio* co-infection displays a particular dynamic. Shrimp died very quickly after being injected with *V. campbellii*, however that study could not provide mechanistic insights. The results of the present thesis might supply mechanistic insights into the study of Phuoc et al (2008). WSSV interferes with shrimp haemocytes and severely affects them, basically inducing apoptosis in some type of haemocytes. Thus, WSSV infection might considerably reduce the amount of active circulating haemocytes. Our study demonstrated that more than 50% of haemocytes within sub 1 (hyalinocytes) and sub 4 (semi-granulocytes), which are considered as the main phagocytic cells, were dead at 720 mpi after inoculation with WSSV. Based on these results, we could hypothesize that the severe reduction of these types of haemocytes may seriously damage the immune system of shrimp and may explain why, after WSSV infection and subsequent exposure to a non-lethal dose of bacteria, shrimp die very quickly, basically because of a lack of haemocytes to eliminate *Vibrio* by phagocytosis from the haemolymph. Another study of Wongprasert et al. (2003) also indicated that *in vivo* the total haemocyte count of shrimp *Penaeus monodon* significantly drops after WSSV injection. Taken together, WSSV infection causes a dramatically reduction of penaeid shrimp haemocytes, which probably weakens shrimp and makes them more susceptible to bacterial infection.

Conclusions and future perspectives

The research described in this thesis has successfully led to an efficient way for separating penaeid shrimp haemocytes. Five haemocyte subpopulations were classified by using a two-step continuous density gradient of iodixanol: hyalinocytes (sub 1), semi-granulocytes (sub 4), granulocytes (sub 5), and immature hyaline cells or pro-hyalinocytes (sub 2 and sub 3). These haemocyte subpopulations are ideal for studying the interaction between pathogens (bacteria and virus) and the different haemocyte types (uptake, inactivation, cell death, pathogen escape mechanisms). Understanding these mechanisms may allow us to think on strategies to prevent and/or control virus infections in shrimp culture.

The present PhD thesis raises the following questions which could be dealt with in future research:

(1) How can pathogenic bacteria such as *Vibrio campbellii* survive upon phagocytosis?

More work will be done to shed additional light on the survival strategies used by these bacteria. Our experiments provide a useful technique for separating penaeid shrimp haemocytes and comparing the fate of pathogenic and non-pathogenic bacteria upon phagocytosis. This work should be extended with other bacteria species or isolates of known pathogenesis.

(2) How can WSSV escape from immune recognition by penaeid shrimp haemocytes?

(3) What are the functions of haemocytes within sub 2 and sub 3? Although the morphology of these haemocyte subpopulations were characterized in some decapods (Hose et al., 1987; Rodriguez et al., 1995; Vargas-Albores et al., 2005; Roulston and Smith, 2011), the functional characteristics are not fully elucidated. Roulston and Smith (2011) indicated that these cells have no phagocytic capacity, which is in agreement with our findings. These cells constitute more than half of circulating haemocytes in *Litopenaeus vannamei*. They are actively moving in the culture medium and hardly attach to substrates. Based on the morphology of these cells, they look like lymphocytes in mammals.

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Chapter 7

Summary - Samenvatting

Summary

Crustaceans, like other invertebrates, have no adaptive immunity. They fully rely on the innate immune system for their internal defense against foreign material. Innate immunity consists of both humoral and cellular factors mediated primarily by circulating haemocytes. Research on crustacean immunity, especially penaeid shrimp (*Litopenaeus vannamei*), plays a crucial role due to the economic importance of shrimp aquaculture throughout the world and the significant impact of infectious diseases. To obtain a better understanding of the immune system, it is important to separate shrimp haemocyte subpopulations and to determine the role of each type of haemocytes in defense reactions.

In chapter 1, an overview of aquaculture production and associated problems is given, mainly focused on penaeid shrimp, *Litopenaeus vannamei*. Besides, the knowledge on the crustacean immune system is reviewed and the techniques for haemocyte separation are described.

In chapter 2, the aims of the thesis are summarized: (i) development of a technique for separating penaeid shrimp (*Litopenaeus vannamei*) haemocyte subpopulations and (ii) analysis of antibacterial and antiviral activities of these haemocyte subpopulations.

In chapter 3, a new methodology for separation of *Penaeus* (*Litopenaeus*) *vannamei* haemocyte subpopulations was developed, using a two-step continuous density gradient with different concentration of iodixanol. Haemolymph was extracted with anticoagulant, layered on the first gradient and centrifuged at 2000 *g* for 10 min. Three sharp cell bands and one dispersed cell band were formed. The first two bands were collected together and layered on a second gradient. This gradient was centrifuged at 2000 *g* for 15 min. Two bands were physically separated. The separated cells were used for *in vitro* culture to evaluate their survival and phagocytic activity. The morphology of each cell type was determined by flow cytometry and light microscopy. Each of the three bands contained a major cell type with distinct morphology and was designated subpopulation 1 (band 1), subpopulation 2 (band 2) and subpopulation 5 (band 3). The dispersed cell band contained a mixture of subpopulation 3 and 4. The purity level of subpopulation 1, 2 and 5 was 95.0 ± 1.0%, 97.7 ± 1.2% and 99.4 ± 0.8%, respectively. *In vitro* culture of separated cells showed that cells of subpopulation 2 had the best survival (up to 96 h) followed by cells of subpopulation 1, subpopulation 3 + 4 and finally subpopulation 5. Phagocytic activity was only detected in subpopulation 1 and

4.

In chapter 4, haemocyte subpopulations of penaeid shrimp were separated and cultured individually *in vitro* to evaluate the phagocytic activity against pathogenic and non-pathogenic particles. The results showed that haemocytes of subpopulation 1 (hyalinocytes) and 4 (semi-granulocytes) have a main function in phagocytosis of both pathogenic and non-pathogenic bacteria as well as fluorescent polystyrene beads. These haemocyte subpopulations engulfed virulent *Vibrio campbellii* and *Vibrio harveyi* at a higher level than non-virulent *Escherichia coli* and polystyrene beads. The percentage of viable intracellular *V. campbellii* (25.5 ± 6.0%) recovered at 180 mpi was significant higher than the percentage recovered from *V. harveyi* (13.5 ± 1.1%). No viable intracellular *E. coli* was observed in this study. In contrast with *V. harveyi* and *E. coli*, *V. campbellii* containing endosomes did not acidify in time. Virulent *V. campbellii* caused a significant drop in haemocyte viability (41.4 ± 6.3% in sub 1 and 30.2 ± 15.1% in sub 4) after 180 min post inoculation in comparison with the *V. harveyi* (84.1 ± 5.6% in sub 1 and 83.4 ± 4.1% in sub 4) and *E. coli* (92.7 ± 2.8% in sub 1 and 92.3 ± 5.6% in sub 4) and polystyrene beads (91.9 ± 1.6% in sub 1 and 84.4 ± 3.4% in sub 4).

In chapter 5, beads, white spot syndrome virus (WSSV) and UV-inactivated WSSV were used to investigate the uptake kinetics of different haemocyte subpopulations of penaeid shrimp (*Litopenaeus vannamei*) using laser scan confocal microscope. It was shown that haemocytes of subpopulation 1, 4 and 5 engulfed beads at a higher rate than WSSV and UV-inactivated WSSV. No bead/virus uptake was observed in haemocytes of subpopulation 2 and 3. The envelope of WSSV virions was lost after internalization but the capsid did not disintegrate further (remained visible). It is possibly that the envelope was fused with the cellular membrane of the endosome. New viral proteins were not expressed. Incubation of haemocyte subpopulations with WSSV but not with UV-inactivated WSSV and polystyrene beads resulted in a significant drop in haemocyte viability. To fully understand the underlying mechanisms, haemocyte subpopulations of penaeid shrimp were exposed to short WSSV DNA fragment (VP19) and CpG ODNs. The results showed that these dsDNA fragmnets induced cell death. Taken together, WSSV is efficiently internalized by haemocytes within subpopulation 1 (hyalinocytes), 4 (semi-granulocytes) and 5 (granulocytes) and upon internalization, the virus is losing its envelope but the capsid remains intact. This process is activating cell death, which in part may be explained by the internalization of viral DNA with cellular sensing molecules.

In chapter 6, the main findings of this thesis are discussed. The new haemocyte separation method, generated in this thesis, was considered to be of great value for present and future studies on the bacterial and antiviral activities of shrimp haemocytes.

Samenvatting

Schaaldieren, zoals andere ongewervelde dieren, hebben geen adaptieve immuniteit. Voor hun interne verdediging tegen vreemd materiaal zijn ze volledig afhankelijk van het aangeboren immuunsysteem. De aangeboren immuniteit bestaat uit zowel humorale en cellulaire factoren, voornamelijk gemedieerd door circulerende bloedcellen. Onderzoek naar de immuniteit van schaaldieren, voornamelijk van peneïde garnalen (*Litopenaeus vannamei*), is cruciaal wegens het economische belang van de garnaalaquacultuur in de hele wereld en de aanzienlijke impact van infectieuze pathogenen. Om een beter begrip van het immuunsysteem te verkrijgen, is het belangrijk om de verschillende bloedcelsubpopulaties van garnalen te onderscheiden en de rol van elk type bloedcel in de afweerreactie te bepalen.

In hoofdstuk 1 wordt een overzicht, met nadruk op peneïde garnalen (*Litopenaeus vannamei*), gegeven omtrent het productieproces van de aquacultuur en de daarmee geassocieerde problemen. Overigens wordt de huidige kennis van het immuunsysteem van schaaldieren en de scheidingstechnieken van de bloedcelsubpopulaties beschreven.

In hoofdstuk 2 worden de doelstellingen van het proefschrift samengevat: (i) de ontwikkeling van een scheidingstechniek van de verscheidene bloedcelsubpopulaties van peneïde garnalen (*Litopenaeus vannamei*) en (ii) de analyse van de antibacteriële en antivirale activiteit van deze bloedcelsubpopulaties .

In hoofdstuk 3 werd een nieuwe methode voor het scheiden van *Penaeus (Litopenaeus) vannamei* bloedcelsubpopulaties ontwikkeld met behulp van een tweestaps continue dichtheidsgradiënt met verschillende concentraties van iodixanol. Hemolymfe werd geëxtraheerd met anticoagulans, op de eerste gradiënt gebracht en gecentrifugeerd bij 2000 g gedurende 10 min. Drie scherpe zones van cellen en één diffuse celzone werden gevormd. De eerste twee zones werden samen verzameld en op een tweede gradiënt aangebracht. Deze gradiënt werd gedurende 15 min bij 2000 g gecentrifugeerd. Hierbij werden twee celzones fysiek gescheiden. De gescheiden cellen werden gebruikt in een *in vitro* cultuur om hun overleving en fagocytose activiteit te evalueren. De morfologie van elk celtype werd bepaald met behulp van flowcytometrie en lichtmicroscopie. Elk van de drie zones bevatte een belangrijk celtype met een verschillende morfologie en werd benoemd als subpopulatie 1 (groep 1), subpopulatie 2 (groep 2) en subpopulatie 5 (groep 3). De diffuse celzone bevatte beide subpopulaties 3 en 4. De zuiverheid van subpopulatie 1, 2 en 5 was 95,0 ± 1,0%, 97,7 ± 1,2% en 99,4

± 0,8%, respectievelijk. *In vitro* cultivatie van gescheiden cellen toonde aan dat the cellen van subpopulatie 2 het langste *in vitro* overleefden (tot 96 uur), gevolgd door cellen van subpopulatie 1, subpopulatie 3 + 4 en tenslotte subpopulatie 5. Er werd enkel fagocytaire activiteit in subpopulatie 1 en 4 gedetecteerd.

In hoofdstuk 4 werden de bloedcelsubpopulaties van peneïde garnalen gescheiden en afzonderlijk *in vitro* gecultiveerd om de fagocytaire activiteit tegen pathogene en niet-pathogene partikels te evalueren. De resultaten toonden aan dat bloedcellen van subpopulatie 1 (hyalinocytes) en 4 (semi-granulocyten) een belangrijke functie hebben in the fagocytose van zowel pathogene en niet-pathogene bacteriën en fluorescerende polystyreenpartikels. Deze bloedcelsubpopulaties namen de virulente *Vibrio campbellii* en *Vibrio harveyi* meer op dan de avirulente *Escherichia coli* en de polystyreenpartikels. Het percentage levensvatbare opgenomen *V. campbellii* (25,5 ± 6,0%) geanalyseerd bij 180 mpi was significant hoger dan het percentage opgenomen *V. harveyi* (13,5 ± 1,1%). Geen levensvatbare intracellulaire *E. coli* werd waargenomen in deze studie. In tegenstelling met *V. harveyi* en *E. coli*, verzuurden *V. campbellii*-bevattende endosomen niet. Na 180 minuten na incoluatie veroorzaakte de virulente *V. campbellii* een aanzienlijke daling in levensvatbaarheid van de bloedcellen (41,4 ± 6,3% in sub 1 en 30,2 ± 15,1% in sub 4) in vergelijking met *V. harveyi* (84,1 ± 5,6% in sub 1 en 83,4 ± 4,1% in sub 4), *E. coli* (92,7 ± 2,8% in sub 1 en 92,3 ± 5,6% in sub 4) en polystyreenpartikels (91,9 ± 1,6% in sub 1 en 84,4 ± 3,4% in sub 4).

In hoofdstuk 5 werden partikels, wittevlekkenvirus (WSSV) en UV-geïnactiveerd WSSV gebruikt met behulp van een laser scan confocale microscoop om de opname-kinetiek van verschillende bloedcelsubpopulaties van peneïde garnalen (*Litopenaeus vannamei*) te onderzoeken. Er werd aangetoond dat bloedcellen van subpopulatie 1, 4 en 5 de partikels met een hogere snelheid opnamen dan WSSV en UV-geïnactiveerd WSSV. Opname van partikels en virus werd niet waargenomen bij bloedcellen van subpopulatie 2 en 3. De envelop van WSSV-virions werd verloren na internalisatie, maar het kapsied desintegreerde niet verder (bleef zichtbaar). Waarschijnlijk fuseerde het envelop met het celmembraan van het endosoom. Nieuwe virale eiwitten kwamen niet tot expressie. Incubatie van bloedcelsubpopulaties met WSSV, maar niet met UV-geïnactiveerd WSSV en polystyreenpartikels, resulteerde in een aanzienlijke daling van de levensvatbaarheid van de bloedcellen. Om de onderliggende mechanismen volledig te begrijpen werden bloedcelsubpopulaties van peneïde garnalen blootgesteld aan een kort WSSV DNA-fragment (VP19) en CpG ODNs. De resultaten toonden aan dat deze

dsDNA-fragmenten celdood induceerden. Samengevat, WSSV wordt efficiënt geïnternaliseerd door bloedcellen van subpopulatie 1 (hyalinocytes), 4 (semi-granulocyten) en 5 (granulocyten), en bij internalisering verliest het virus zijn envelop, terwijl het kapsied intact blijft. Dit proces induceert celdood, wat gedeeltelijk kan worden verklaard door de internalisatie van viraal DNA gevolgd door een interactie met cellulaire sensormolecules.

In hoofdstuk 6 worden de belangrijkste bevindingen van dit proefschrift besproken. De nieuwe scheidingsmethode van bloedcellen, gegenereerd in dit proefschrift, is van grote waarde voor huidige en toekomstige studies omtrent de bacteriële en anti-virale activiteiten van bloedcellen van garnalen.

Curriculum vitae

Vo Van Tuan was born on 3rd April, 1981 in Tra Vinh city, Vietnam.

In 2005, he obtained the diploma of Aquaculture at Nong Lam University, Hochiminh city, Vietnam (Formerly, University of Agriculture and Forestry). From 2005 to 2007, he worked as a lecturer at the Department of Pathology, Faculty of Fisheries, Nong Lam University. In 2007, he was granted a scholarship by Vlaamse Interuniversitaire Raad (VLIR) in Belgium and enrolled in a two-year international course program "Master of Science in Aquaculture" at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University. From 2009 to 2011, he continued working at Nong Lam University as a lecturer. At the end of 2011, he started a PhD program under the supervision of Prof. Hans Nauwynck at the Laboratory of Virology, Faculty of Veterinary, Ghent University. His PhD study was funded by Vietnamese government scholarship.

Vo Van Tuan is author or co-author of 9 scientific publications. During the PhD study, he has participated in international conferences in Hochiminh City, Vietnam.

Publications

Publications in Peer-reviewed international journals

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<u>International conferences</u>

J.J. Dantas-Lima[#], **V.V. Tuan**[#], M. Corteel, K. Grauwet, N.T.T. An, P. Sorgeloos, H.J. Nauwynck (2013). Separation of Penaeus vannamei haemocyte subpopulations by iodixanol density gradient centrifugation. Conference of World Aquaculture Society in Hochiminh city, Vietnam (14-18th December, 2013).

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