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# Development of Techniques to Culture Shrimp Haemocytes and Purify White Spot Syndrome Virus (WSSV) in Order to Study WSSV-Haemocyte Interactions

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Thesis for obtaining the degree of Doctor in Veterinary Sciences (PhD)

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# Table of Contents

## List of abbreviations

| | |
|---|---|
| βGBP | β-glucan-binding protein |
| ALPF | Anti-lipopolysaccharide factors |
| AMPs | Antimicrobial peptides |
| ARC | Artemia Reference Center |
| BCA | Bicinchoninic acid |
| BD | Billion dollars |
| BSA | Bovine serum albumin |
| CFU | Colony forming units |
| CS | Chen's salts |
| DW | Distilled water |
| EMA | Ethidium monoazide bromide |
| FACS | Fluorescence activated cell sorting |
| FAO | Food and Agriculture Organization of The United Nations |
| FASW | Filtered and autoclaved seawater |
| FCS | Foetal calf serum |
| FSC-H | Forward scatter height |
| GCs | Granular cells |
| GFP | Green Fluorescent Protein |
| GSH | L-glutathione |
| HCs | Hyaline cells |
| H&E | Haematoxylin and Eosin |
| HM | Haemocyte medium |
| HOCl | Hypochlorous acid |
| ICTV | International Committee on Taxonomy of Viruses |
| IIF | Indirect immunofluorescence |
| IHHNV | Infectious hypodermal and haematopoietic necrosis virus |
| IMNV | Infectious myonecrosis virus |
| kDa | Kilodalton |
| $LD_{50}$ | Lethal dose 50% endpoint |
| LGBP | Lipopolysaccharide and glucan-binding protein |
| LPS | Lipopolysaccharide |
| MA | Marine Anticoagulant |
| MACS | Magnetic activated cell sorting |
| MT | Million tones |
| NADPH | Nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| OD | Optical density |
| OIE | World Animal Health Organization |
| PAMPs | Pathogen-associated molecular patterns |
| PBS | Phosphate buffered saline |
| PF | Paraformaldehyde |
| PGBP | Peptidoglycan binding protein |
| PGN | Peptidoglycan |
| PO | Phenoloxidase |
| PNA | Peanut agglutinin |
| proPO | Prophenoloxidase |
| ppA | Prophenoloxidase-activating enzyme |
| PRRs | Pattern recognition receptors |
| ROIS | Reactive oxygen intermediates |
| ROS | Reactive oxygen species |
| SGCs | Semi-granular cells |
| $SID_{50}$ | Shrimp Infectious Dose 50% endpoint |
| shPBS | Shrimp phosphate buffered saline |
| SOD | Superoxide dismutase |
| SPF | Specific pathogen free |
| SSC-A | Side scatter area |
| TEM | Transmission electron microscopy |
| TLR | Toll-like receptors |
| TSV | Taura syndrome virus |
| WSD | White spot disease |
| WSSV | White spot syndrome virus |
| YHV | Yellow head disease |

# Chapter 1

## Literature review

## 1.1 Aquaculture production

According to the latest statistics released by the Food and Agriculture Organization of the United Nations (FAO) in 2011, the world seafood production (fisheries and aquaculture combined) reached 154 million tonnes (MT). From these, 131 MT were used directly for human consumption. This represented an average seafood consumption of 18.8 kg per capita (Figure 1). Globally, seafood provides 2.9 billion people with almost 20% of their needs in animal protein. The seafood sector has a high economic and social importance since it provides 45 million direct jobs (FAO, 2012a; c).

Although the seafood supply in 2011 was still dominated by products coming from fisheries (90.4 MT), the aquaculture (63.6 MT) sector presented an extreme growth during the last decades. It increased from 4% in 1970 to 41% of the total seafood production in 2011. In the last decade, its average annual growth rate was 6.3%. In 2010, the top-five producers of farmed aquatic animals were China (36.7 MT), India (4.6 MT), Vietnam (2.7 MT), Indonesia (2.3 MT) and Thailand (1.4 MT). In terms of regional aquaculture production, Asia contributed 90% to the world production while China alone contributed 60%. The value of the total production was estimated at 119.4 USD billion dollars (BD) in 2010. Based on this growth rate, aquaculture is currently classified as the growing animal-based food production sector (FAO, 2012a; b).

It is predicted that world fisheries and aquaculture production will reach around 172 MT in 2021. It is expected that aquaculture will be the main contributor for this growth, which is projected to reach a production of about 79 MT. This is a rising of 33% against the 3% growth expected for capture fisheries. Although it is expected that the annual growth of aquaculture will diminish to less than half, this sector will remain one of the fastest-growing animal food production sectors (FAO, 2012c).

### 1.1.1 Crustacean aquaculture

In 2010, FAO reported 62 crustacean species being used in aquaculture production worldwide. The total production was 5.7 MT, representing an estimated value of 26.9 BD. That production volume represented 70.6% from marine species and 29.4% from freshwater species. The production of marine species was dominated by white leg

shrimp (*Penaeus vannamei*), which accounted for 2.7 MT (11.3 BD). From these, 77.9% were produced in Asia and the rest in the Americas. Another important species was the giant tiger shrimp (*Penaeus monodon*) (0.78 MT). The most important freshwater crustacean species were red swamp crayfish (*Procambarus clarkii*; 0.62 MT), Chinese mitten crab (*Eriocheir sinensis*; 0.59 MT), oriental river prawn (*Macrobrachium nipponense*; 0.23 MT) and giant river prawn (*Macrobrachium rosenbergii*; 0.20 MT). Concerning the prawns, despite *M. nipponense* presenting a slightly higher production volume, *M. rosenbergii* generated more economical value (1.2 BD). Moreover, while *M. nipponense* only has a significant production in China, *M. rosenbergii* is being produced in over 31 countries (FAO, 2012c; b).

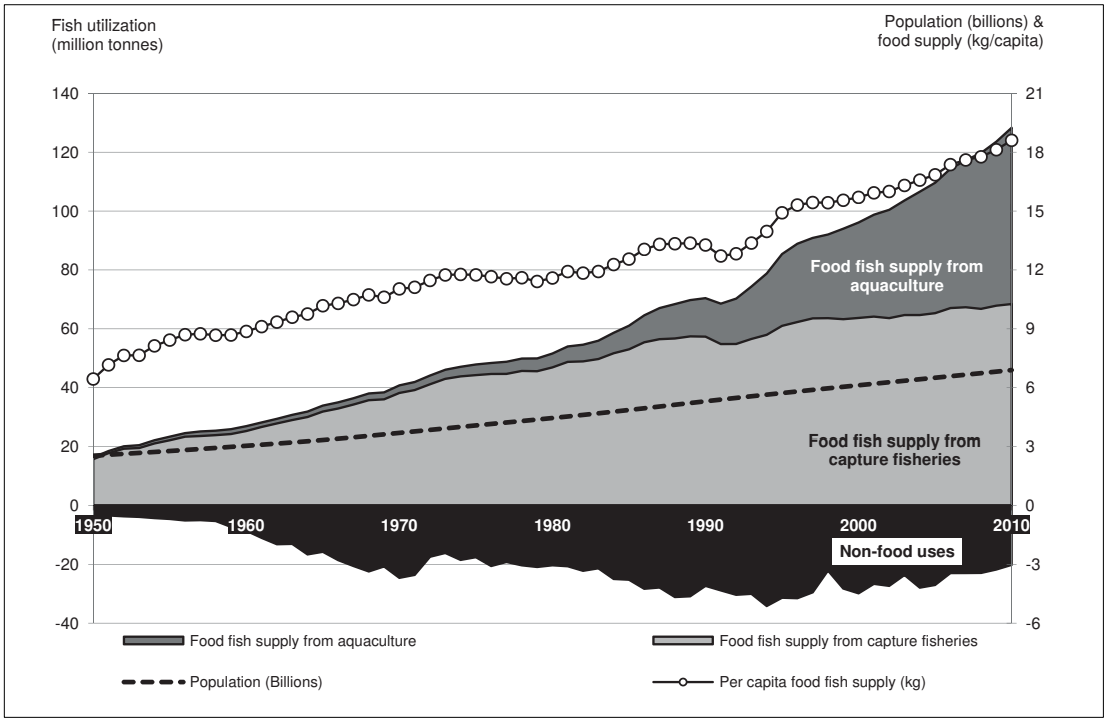


**Figure 1.** World seafood utilization and supply (FAO, 2012b).

### 1.1.2 *Penaeus vannamei*

*Penaeus vannamei* (Figure 2) is native to the western pacific coast of the Americas, from Mexico to Peru, in areas where water temperatures are normally over 20ºC throughout the year. The first spawning in captivity was achieved in Florida in 1973. The commercial culture began a few years later in 1976 in South and Central America, expanding subsequently to Hawaii, mainland United States of America, and much of Central and South America. This expansion was mainly driven by the

development of intensive breeding and rearing techniques in the early 1980s. The commercial production showed a rapid increase in Latin America, with peak cycles every 3-4 years, which were coinciding with the cycles of the climate phenomena 'el niño' and 'la niña'. In 1996, specific pathogen free (SPF) *P. vannamei* raised in Hawaii were introduced in Taiwan and in 1998 in Mainland China. This introduction was a big success and since then, Asia has seen a phenomenal increase in the production. In 2004, *P. vannamei* production had overtaken the production of *P. monodon* in China, Taiwan and Thailand (FAO, 2006; Chamberlain, 2010).

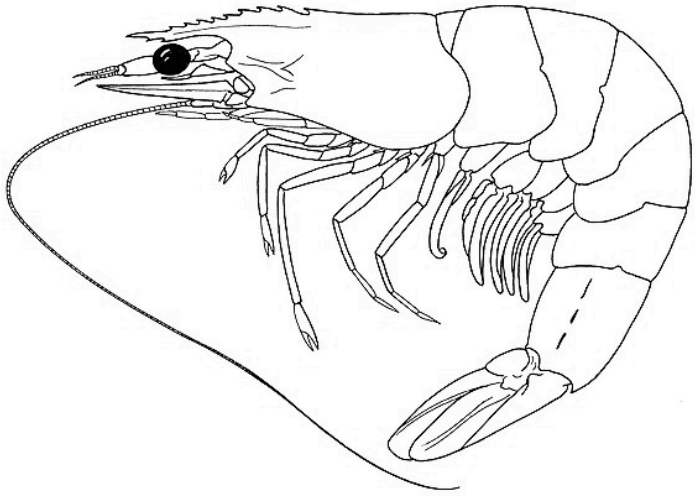

**Figure 2.** *Penaeus vannamei* drawing (FAO, 2006).

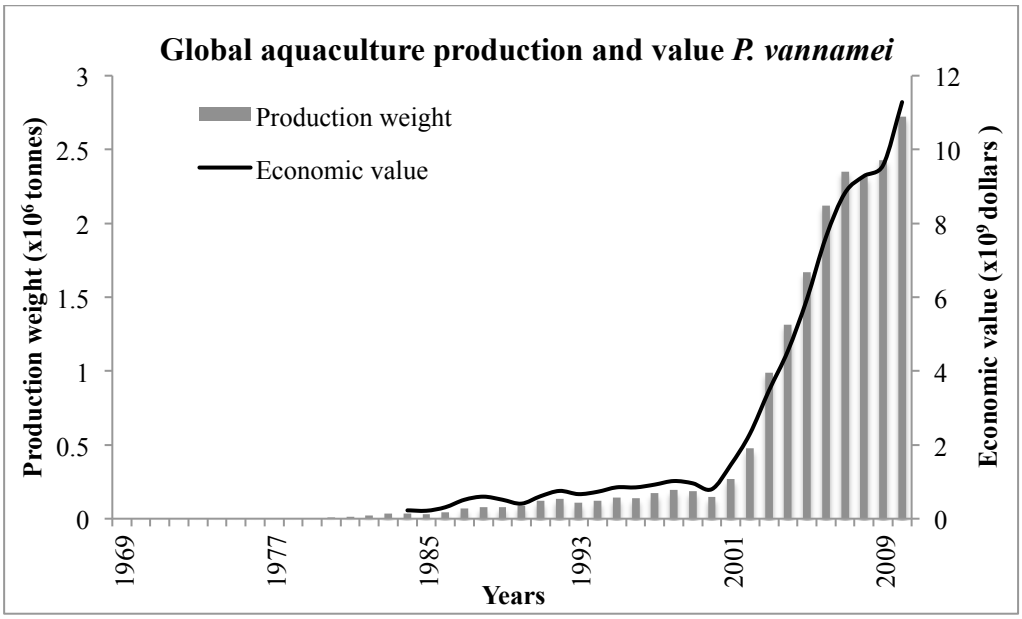


**Figure 3.** Global production of *P. vannamei* and respective economic value between the years 1969 and 2010 (Source: FAO - Fisheries and Aquaculture Information and Statistics Service).

Globally, the commercial production started in the 1970's with 2,000 tonnes reported in 1976 and increased to 194,000 tonnes in 1998. In 2000, it declined to 146,000 tonnes due to disease problems (white spot syndrome virus, WSSV) in Latin America. Afterwards, with the rapid spread of this species through Asia, it experienced a spectacular growth and by 2010 it was 2.7 MT (Figure 3). The main producer countries in 2010 were China (1.2 MT), Thailand (0.56 MT), Ecuador (0.22 MT), Indonesia (0.21 MT) and Vietnam (0.14 MT) (Chamberlain, 2010; FAO, 2012b).

### 1.1.3 *Macrobrachium rosenbergii*

*Macrobrachium rosenbergii* (Figure 4) is native to the Indo-Pacific region, northern Australia and Southeast Asia. Modern aquaculture of *M. rosenbergii* started in 1961, when the reproduction cycle was closed in captivity. The key discovery was the requirement for brackish water in the early life cycle. Afterwards, the culture remained at an experimental level for almost one decade. In 1972, when mass-rearing techniques for commercial-scale hatchery production appeared, the industry started to develop. The first commercial farms were established in Hawaii and spread to Thailand and Taiwan, which became pioneers in modern giant freshwater prawn culture. The introduction of broodstock, initially from Hawaii and Thailand, into non-indigenous areas (North, Central and South America and Africa) began in the 1970s. Another milestone in the development of the industry was a project that FAO implemented in 1978, which intended to expand the culture in Thailand. From then on, *M. rosenbergii* culture has developed in every continent, particularly in Asia and the Americas (FAO, 2004; New, 2010).

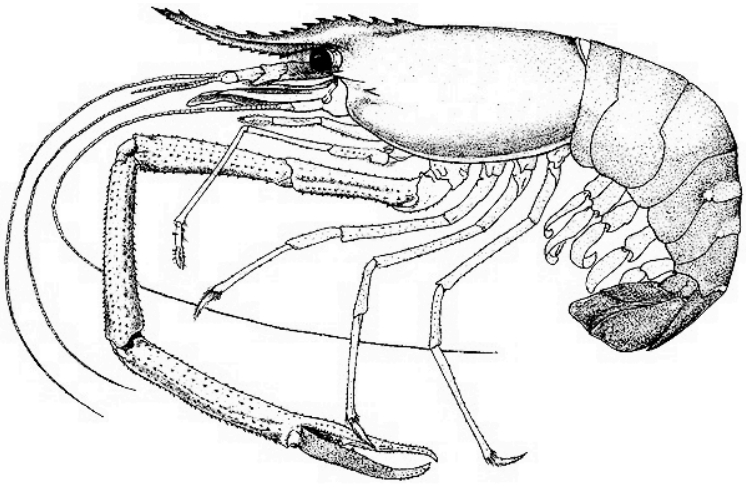

**Figure 4.** *Macrobrachium rosenbergii* drawing (FAO, 2004).

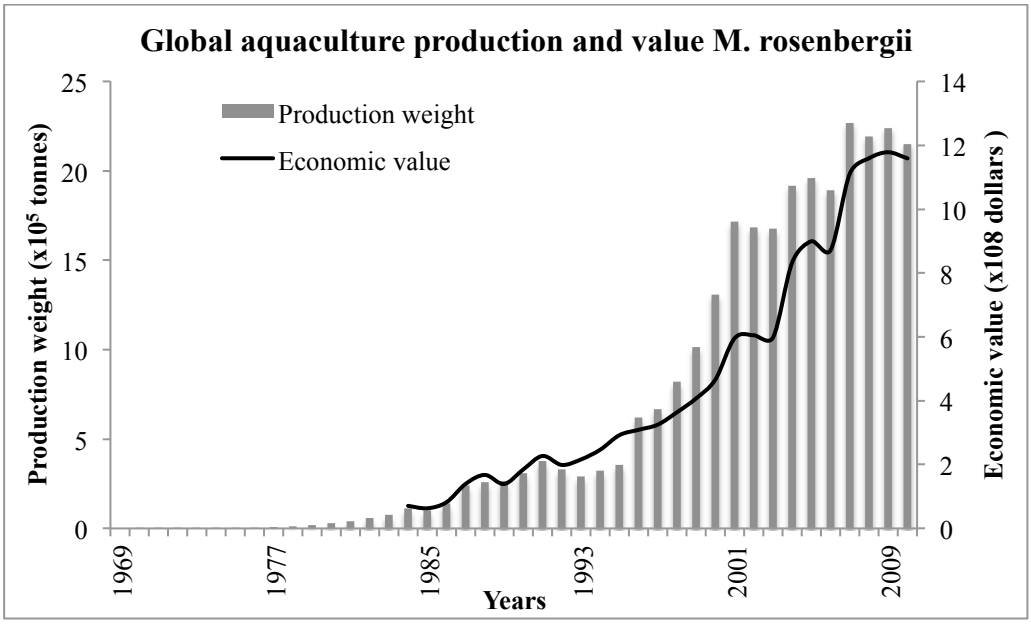


**Figure 5.** Global production of *M. rosenbergii* and respective economic value between the years 1969 and 2010 (Source: FAO - Fisheries and Aquaculture Information and Statistics Service).

For the first time in 1970, FAO estimated a global production of 10 tonnes for this species (Figure 5). Afterwards, it grew considerably until it reached a peak of 38,000 tonnes in 1991. A major expansion in the industry was noticed in the mid 1990s, when the Chinese production of *M. rosenbergii* registered levels significant enough to be presented in separated reports. Later, it registered an overall growth, with peaks in 2001 (172,000 tonnes), 2005 (196,000 tonnes) and the highest production ever registered was in 2007 (227,000 tonnes). In 2010, the production was slightly diminished to 215,000 tonnes. The main producing countries were China (130,000 tonnes), Bangladesh (31,000 tonnes), Thailand (26,000) and India (14,000) (New, 2010; FAO, 2012b)

## 1.2 Crustacean immunity

The innate immune response is an ancient and highly conserved mechanism within animal taxa. It is well known that the defence of crustaceans against infection relies on strong and effective innate immune reactions. In contrast to vertebrates, there is no substantial evidence for the existence of an adaptive immune defence in crustaceans (Iwanaga *et al.*, 1998; Hauton and Smith, 2007; Smith, 2010). Their innate immunity is composed of two main branches, the cellular and humoral immunity. The cellular

immunity is defined by the direct action of haemocytes (invertebrate's immune cells) in the defence against invading microorganisms. The humoral immunity is composed of a diversity of defence immune molecules that are mainly produced, stored and released by haemocytes (Jiravanichpaisal *et al.*, 2006; Vazquez *et al.*, 2009; Smith, 2010). Since haemocytes are involved, directly or indirectly, in all known immune reactions, cellular and humoral immunity tend to overlap.

The immune response starts with the recognition of a non-self target by granular haemocytes, which subsequently triggers their degranulation and the release of immune effectors into the haemolymph (invertebrate's blood) (Johansson and Söderhäll, 1985). These immune effectors are the constituents of the humoral branch of innate immunity and eventually triggers and/or promoters of other cellular reactions (Jiravanichpaisal *et al.*, 2006; Cerenius *et al.*, 2008). After degranulation, a diverse range of immune processes takes place. The most immediate is the clotting (Martin *et al.*, 1991; Theopold *et al.*, 2004). Other components of humoral immunity, which have antimicrobial activity are the prophenoloxidase-activating system (proPO system), antimicrobial peptides, lectins, reactive oxygen species (ROS), lysosomal enzymes and agglutinins (Smith and Chisholm, 1992; Bachère *et al.*, 1995; Bachère *et al.*, 2000; Muñoz *et al.*, 2000; Sritunyalucksana and Söderhäll, 2000; Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2010). Some of these components also trigger and promote cellular immunity processes such as phagocytosis and nodulation/encapsulation of pathogens. An overview of the main happenings during the crustacean immune response is presented in the diagram of Figure 6.

### 1.2.1 Recognition

Resembling the situation in vertebrates, the immune response of crustaceans starts with the recognition of microorganism cell wall compounds and consequently the triggering of immune processes, e.g. degranulation, specific gene activation and phagocytosis. This recognition is mediated by pattern recognition receptors (PRRs) located in the plasmatic membrane of haemocytes or dissolved in the body fluids. The main PRRs in crustaceans are β-glucan-binding protein (βGBP), lipopolysaccharide and glucan-binding protein (LGBP) and peptidoglycan binding protein (PGBP). Other examples of PPRs are lectins and toll-like receptors (TLR). The TLR are highly conserved trans-membrane protein structures with extra and intracellular domains.

Toll-like genes have been identified in several penaeid shrimp and they are expressed in haemocytes and other cells throughout the body (Smith *et al.*, 2010). Lectins are described in more detail in point 2.2.3. These receptors recognize and bind to the pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and peptidoglycan (PGN) from bacteria and β-1,3-glucan from fungi. They are conserved structures within microorganisms but absent in the host. In crustaceans, the recognition happens in the presence of small amounts of these microbial components (Medzhitov and Janeway, 2000; Roux *et al.*, 2002; Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2008; Cerenius *et al.*, 2010; Smith, 2010). The recognition and the binding of PAMPs to PRRs triggers signalling pathways that upregulate immune genes for production of compounds such as anti-microbial peptides (AMPs) and cytokines (Smith *et al.*, 2010). In *Drosophila*, the mechanical damage of tissues and probably enzymes released by invading microorganisms, were also reported to work as stimulus for the activation of immune reactions such as the proPO system (Galko and Krasnow, 2004).

### 1.2.2 Humoral immunity

#### 1.2.2.1 ProPO system

The proPO enzymatic cascade (Figure 7) is by far the most studied immune process in shrimp. Phenoloxidase (PO), which is a copper-containing tyrosinase, is the active form of proPO. The components of this enzymatic cascade are synthesized in haemocytes and stored in their cytoplasmic granules (Johansson and Söderhäll, 1985). After recognition of non-self, the degranulation (regulated exocytosis) of haemocytes is induced and the components of the proPO system are released into the blood stream (Johansson and Söderhäll, 1985). Hence, the enzymatic cascade starts with the activation of the prophenoloxidase-activating enzyme by a serine protease cascade (Figure 7). This prophenoloxidase-activating enzyme catalyses the conversion of proPO into active phenoloxidase by a process of limited proteolysis. The ultimate function of phenoloxidase is the production of melanin and toxic (antimicrobial) intermediate compounds such as quinones. Melanin is a black insoluble pigment that forms the melanotic capsules that are typical after wounding and microbial infection.

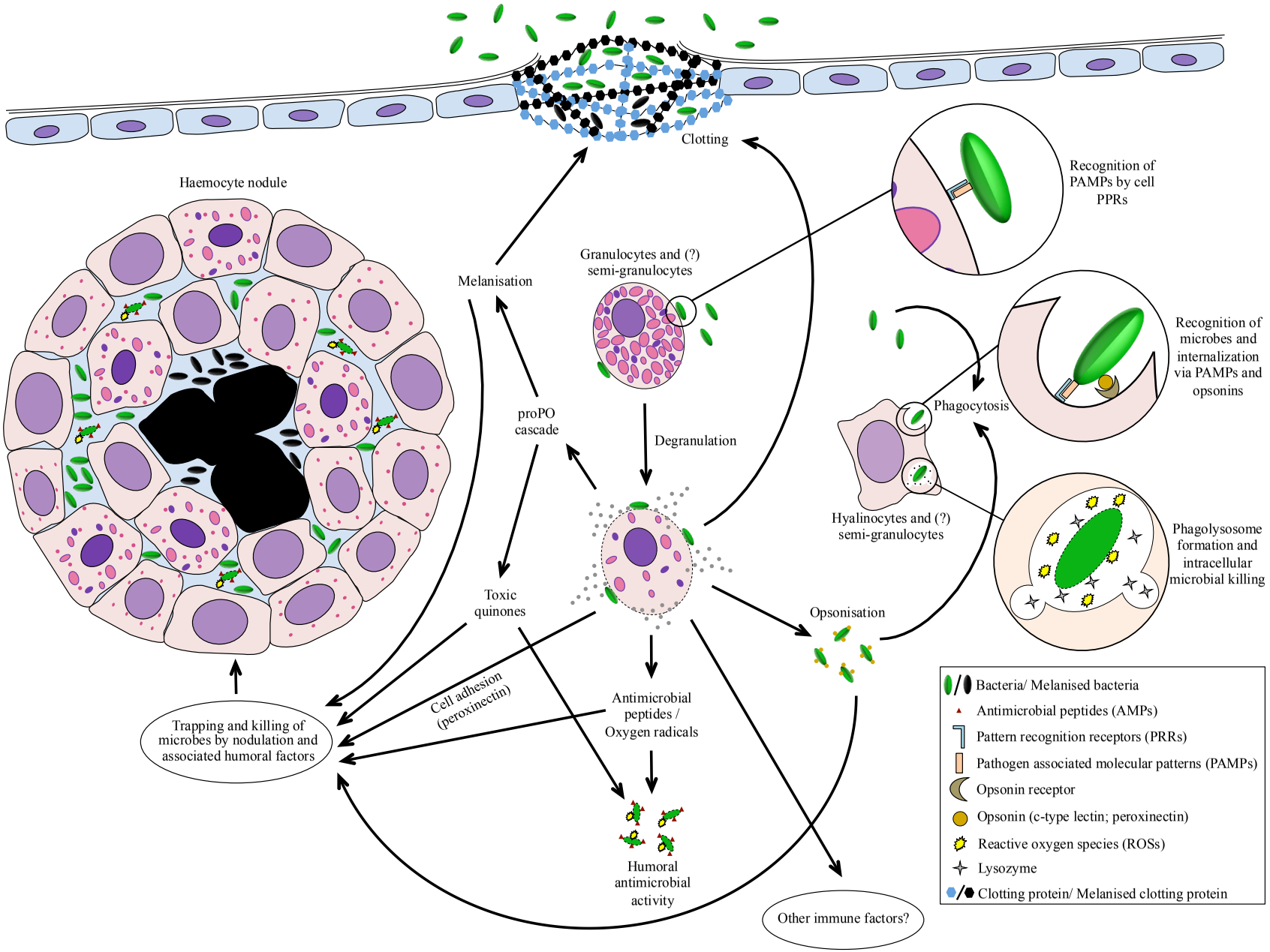


**Figure 6**. Overview of the main events towards infection in crustacean immunity upon exposure to a pathogen (adapted from Jiravanichpaisal *et al.* 2006 and Smith *et al.* 2010).

These melanotic capsules physically control the spreading of microorganisms. The active phenoloxidase catalyses the hydroxylation of monophenols into *o*-diphenols and their oxidation into toxic quinones. Quinones are converted into melanin by a non-enzymatic process. Nevertheless, this process needs to be strictly regulated in order to prevent excessive activation and consequent damage to the host. This regulation is mediated by serine proteinase inhibitors present in the haemolymph plasma, which regulate the activity of the prophenoloxidase-activating enzyme, the key enzyme in the prophenoloxidase cascade. These proteinase inhibitors are members of the pacifastin family (Aspan *et al.*, 1990; Söderhäll and Cerenius, 1992; Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2008; Smith *et al.*, 2010).

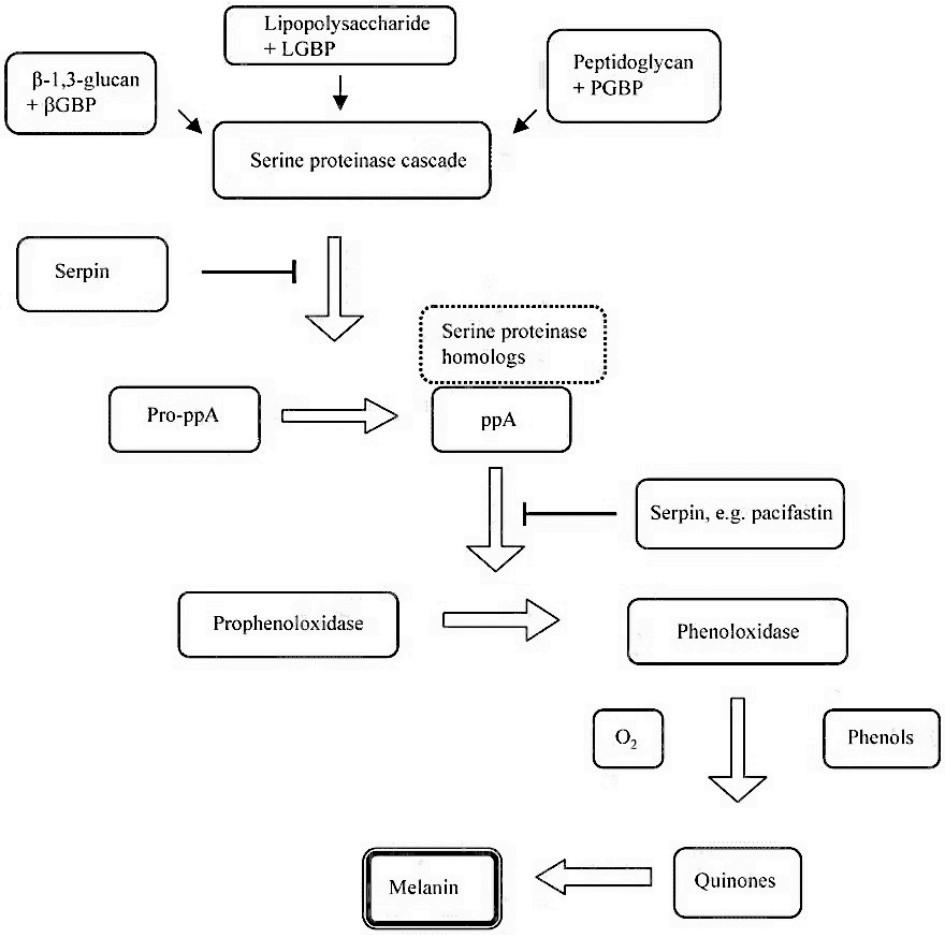


**Figure 7.** Overview of the main actors and reactions of the enzymatic cascade of the arthropod prophenoloxidase activating system. Microbial compounds (e.g. β-1,3-glucan) are recognized by haemocytes and a degranulation reaction is triggered. Degranulation releases the components of the prophenoloxidase cascade into the animal's haemocoel. The inactive prophenoloxidase-activating enzyme (pro-ppA) is converted into prophenoloxidase activating enzyme (ppA), which in its turn converts prophenoloxidase into active phenoloxidase. In the presence of oxygen, phenoloxidase catalyses the conversion of phenols into quinones and later, quinones are non-enzymatically converted into melanin. This enzymatic cascade is regulated by protease inhibitors (serpins) (Cerenius and Söderhäll, 2004).

#### 1.2.2.2 Clotting

The crustacean clotting process is an extremely rapid and efficient reaction that simultaneously prevents the excessive loss of haemolymph, by sealing the wound with a "rubbery plug", and the spreading of infection. In some cases, this process happens within seconds after wounding. The formation of the clot happens by polymerization of clotting proteins present in the haemolymph by transglutaminases released by degranulating haemocytes (Martin *et al.*, 1991; Söderhäll and Cerenius, 1992; Theopold *et al.*, 2004). In *Drosophila melanogaster*, the stability of the clot might be enhanced by phenoloxidase since its activity creates further protein-protein crosslinking (Theopold *et al.*, 2004).

#### 1.2.2.3 Antimicrobial peptides and proteins

Antimicrobial peptides (AMPs) are one of the first lines of defence of crustacean immunity towards infection. Other important antimicrobial factors are the protein lysozyme and anti-lipopolysaccharide factors (ALPF). In this context, there is another class of proteins that act more as promoters of certain antibacterial processes instead of direct antibacterial activity. These are lectins (Smith *et al.*, 2010).

Antimicrobial peptides are highly conserved components of the innate immune defence that are found in all kingdoms, from bacteria to mammals, including fungi and plants. In crustaceans, more than 60 AMPs have been described. They comprise many different molecular groups, but the predominant ones are penaeidins and crustins. These are small molecules (normally less than 10kDa), which are synthesized and stored in granular haemocytes (Destoumieux *et al*., 2000; Munoz *et al*., 2002). They are active cationic agents that have the ability of killing microbial pathogens by destabilization and disruption of their membranes. This killing mechanism starts with their attachment to the cell membrane of the target organism by electrostatic affinity and its subsequent permeation by creating holes in the structure. This eventually provokes the destruction of the invader by cell membrane disruption. A very interesting feature of antimicrobial peptides is their apparent lack of activity against host tissues. However, the current information shows a broad activity of these compounds against microbes, information which also shows disagreement in between reports (Destoumieux *et al.*, 1997; Bachère *et al.*, 2000; Destoumieux *et al.*, 2000; Rosa and Barracco, 2010; Smith *et al.*, 2010; Tassanakajon

*et al.*, 2010; Hauton, 2012).

Crustaceans possess c-type lysozymes, which are stored in haemocytes and probably released during degranulation together with antimicrobial peptides and other substances. The killing of pathogenic *Vibrio* species by lysozyme has been reported (Callewaert and Michiels, 2010; Smith *et al.*, 2010). Lysozyme is a muramidase enzyme with strong antibacterial activity that cleaves *β*-(1,4)-glycosidic bonds in the structure of peptidoglycan, the polymer that composes the cell wall of Gram-positive bacteria. When this enzyme acts on the cell wall of bacteria, they become sensitive to hypotonic environments and eventually undergo lysis. However, the peptidoglycan wall of Gram-negative bacteria is not directly accessible for lysozymes, due to the phospholipidic lipopolysaccharide-containing outer membrane. Thus, other more conventional antimicrobial peptides act synergistically with lysozyme by creating holes in the outer membrane and making the peptidoglycan accessible. In the case of Gram-positive bacteria, the synergism happens in the opposite way. Lysozyme compromises the structure of the peptidoglycan wall making the phospholipidic membrane accessible to antimicrobial peptides.

Anti-lipopolysaccharide factors are small basic proteins that bind to lipopolysaccharides present in the surface of bacteria. They induce a strong antibacterial activity, in particular towards gram-negative bacteria, but also against gram-positive bacteria and fungi. These immune effectors were initially identified in *Limulus polyphemus* and more recently in several commercially important penaeid shrimp, crayfish and crabs (Fredrick and Ravichandran, 2012; Hauton, 2012). The mechanism of action of these proteins it is not yet clear but is very likely associated to binding and agglutination of bacteria, with or without direct killing (Smith *et al.*, 2010).

Lectins are proteins or glycoproteins, normally without catalytic activity, that can specifically recognize and non-covalently bind to sugars. They are regarded as an important element of innate immunity in non-self recognition and elimination of invading microorganisms by promoting phagocytosis through opsonisation and agglutination. They are considered to be the functional evolutionary precursors of antibodies. In crustaceans, they can be found in haemocyte cytoplasmic membrane and granules or dissolved in the haemolymph. A long list of lectins have been identified in different species of penaeid shrimp and more recently, special attention has been directed to calcium dependent lectins (c-type lectins) (Marques and

Barracco, 2000; Cerenius *et al.*, 2010; Smith *et al.*, 2010; Fredrick and Ravichandran, 2012).

### 1.2.3 Cellular immunity

#### 1.2.3.1 Phagocytosis and oxygen radicals

Phagocytosis is an ancient mechanism that is present among a wide range of animal taxa. While protozoans use it to acquire nutrients, in higher organisms it is performed by specialized cells (macrophages and neutrophils) for defence against infection and maintenance purposes. The process starts with the attachment of the target particle to the phagocytic cell via direct binding of PAMPs to the cell PRRs or/and by opsonin-mediated attachment. The following step is the engulfment of the particle into the phagosome through cytoskeleton modification driven by polymerization of F-actin. The intracellular transport is done inside phagosomes (using actin and tubulin networks), which is followed by fusion with lysosomes and formation of the phagolysosomes, where the engulfed target is destroyed. The process of particle engulfment, phagosome transport and fusion with the lysosome is mediated by rabGTPases (Bayne, 1990; Aderem and Underhill, 1999; Smith *et al.*, 2010). It is believed that phagocytosis is the major cellular defence reaction towards infection in crustaceans. Certain types of haemocytes are specialised phagocytes that can engulf biotic targets such as bacteria, yeast and apoptotic cells or abiotic targets like synthetic beads (Jiravanichpaisal *et al.*, 2006; Smith *et al.*, 2010). The cell types involved in this process are variable within crustaceans (described in more detail under 1.2.5).

The production of oxygen radicals, which are potent microbicidal metabolites, is intimately associated with phagocytosis. During the process of uptake, phagocytic cells increase the oxygen consumption and produce several reactive oxygen intermediates (ROIs). This process is characterized by the activation of a NADPH oxidase (membrane-bound enzyme complex), which is usually induced by PAMPs. The first metabolite produced in the extracellular or intraphagosomal spaces, is the superoxide anion ($O^{2-}$). This is readily converted into hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD). This oxygen radical is further converted into several toxic oxygen intermediates, such as hypochlorous acid (HOCl) by action of a myeloperoxidase or nitric oxide (NO) by action of nitric oxide synthase (NOS). These

compounds form an efficient defence system against invading bacteria, parasites and other dangerous intruders. Nevertheless, if not properly controlled by endogenous antioxidant enzymes such as SOD, catalase and glutathione peroxidase, they can also damage the host (Bell and Smith, 1993; Lambeth, 2004; Guertler *et al.*, 2010; Smith *et al.*, 2010).

#### 1.2.3.2 Nodulation and encapsulation

If the number of the foreign entities invading the haemocoel is too high or their size is too large to be eliminated by phagocytosis, invertebrates may apply another cellular immune response known as nodulation (bacteria, fungus) or encapsulation (parasites). These two processes seem to be variations of the same immune reaction. This immune process is characterised by the trapping and isolation of foreign entities by attachment of concentric layers of adherent haemocytes around them. The process starts with the recognition of non-self. Next, haemocytes become activated, pass to an adherent state and release the components of an adhesive extracellular matrix that will compose the binding material for the cell aggregate. The subsequent step is the killing of the invaders within the nodule/capsule by asphyxiation and action of several toxic compounds released by haemocytes and enzymatic cascades (proPO). These compounds are quinones or semiquinones, reactive oxygen species (ROS) and antibacterial peptides (Gillespie *et al*., 1997; Nappi *et al*., 1995, 2000). The blackening of the capsule (melanisation) due to phenoloxidase activity is the final stage of this process (Söderhäll and Cerenius, 1992; Nappi *et al.*, 1995; Lavine and Strand, 2002). Although the composition of the binding matrix is still largely unknown, one probable candidate protein in crustaceans is peroxinectin (Johansson *et al.*, 1995; Sritunyalucksana *et al.*, 2001; Liu *et al.*, 2005). In other invertebrates the proteins that were found to support haemocyte aggregation were: lacunin and the lectin peanut agglutinin (PNA) in *Manduca sexta* (Nardi *et al.*, 2005) and calreticulin in *Galleria melonella* (Choi *et al.*, 2002).

### 1.2.4 Apoptosis

Apoptosis is a complexly regulated cellular suicide mechanism. Morphologically, it is characterised by nuclear condensation, cell shrinkage, membrane blebbing, and DNA fragmentation. Biochemically, caspases (a family of cysteine proteases) play a major

role as regulators of this process. During the biochemical cascade that induces apoptosis, a series of dramatic perturbations to the cellular architecture contribute not only to cell death, but also prepare cells for removal by phagocytes. This process plays an important role not only in eliminating potentially harmful cells, but it is also vital for normal cell turnover and proper functioning of the immune system. Apoptosis is an important strategy for elimination of infected cells without spilling of their contents and, thus, limiting the spread of infection (Elmore, 2007; Taylor *et al.*, 2008; Smith *et al.*, 2010). More information on apoptosis in shrimp can be found in Sahtout *et al*. (2001), Granja *et al.* (2003), Wongprasert *et al.* (2003), Wang *et al*. (2008) and Zhi *et al*. (2011).

### 1.2.5 Crustacean haemocyte subpopulations and their functions

Haemocytes are the immune cells of invertebrate animals. Unlike the variety of cells in the blood of vertebrates, haemocytes are the only cells in the haemolymph. Besides their key role in immunity, they participate in diverse biological functions such as hardening (Vacca and Fingerman, 1983; Hose *et al.*, 1992) and repair of the cuticle and muscle (Halcrow and Smith, 1986; Uhrík *et al.*, 1989) in crustaceans. Despite some disagreement in the terminology for classifying the different haemocyte subpopulations, there is now an almost consensual classification into three subpopulations: hyalinocytes or hyaline cells (HCs), semi-granulocytes or semi-granular cells (SGCs) and granulocytes or granular cells (GCs) (Söderhäll and Smith, 1983; Martin and Graves, 1985; Hose *et al.*, 1990; Giulianini *et al.*, 2007). Nevertheless, variations of these names can be found in literature (Hose *et al.*, 1990; Johansson *et al.*, 2000). On the other hand, this simplistic classification system solely based on the presence and size of cytoplasmatic granules, is very likely the origin of confusion and disagreement concerning haemocyte subtyping. While the visual differentiation of hyalinocytes and granulocytes is relatively easy and straightforward, the classification of semi-granulocytes becomes very subjective. This may be the cause of discrepancies in differential counts of shrimp haemocyte subpopulations, made by different research groups (Tsing *et al.*, 1989; Le Moullac *et al.*, 1997; Liu *et al.*, 2005; Vargas-Albores *et al.*, 2005).

The possible existence of more than three functionally distinct subpopulations also reinforces the need for a revision of this classification system. This revision should be

complemented with extensive and objective functional/biochemical studies, as previously done for vertebrate white blood cells. This was attempted in decapod crustaceans (Hose *et al.*, 1990). It is generally accepted that in crustaceans, granulocytes and semi-granulocytes are cell types producing and storing immune-related molecules in cytoplasmatic granules, performing nodulation/encapsulation of pathogens and cytotoxicity (Johansson and Söderhäll, 1985; Söderhäll *et al.*, 1985; Persson *et al.*, 1987; Kobayashi *et al.*, 1990; Roulston and Smith, 2011). Confusing findings were reported on the cell type that phagocytise. Hyalinocytes were found to be the major phagocytic cell type in crayfish, crabs and prawns (Söderhäll and Smith, 1983; Thornqvist *et al.*, 1994; Sung *et al.*, 2000; Roulston and Smith, 2011). In contrast, granular cells were found to be the major phagocytic cell type in shrimp and again in prawn by other research groups, (Hose and Martin, 1989; Vázquez *et al.*, 1997; Gargioni and Barracco, 1998; Jayasree, 2009). These differences might be due to species differences and/or defective isolation/identification techniques. This subject clearly needs further investigation.

### 1.2.6 Haemocyte culture

It is generally accepted that L-15 (Leibovitz, 1963) is the best medium to keep crustacean haemocyte cultures. It is mostly used at double concentration for marine crustaceans, while for freshwater crustaceans it is used at single concentration in order to meet the lower osmolality requirements. The supplements that are considered to be essential for maintaining *in vitro* cultures of haemocytes are mammalian serum (often bovine), balanced salt mixtures for levelling the specific osmolality requirements and antibiotics for bacterial contamination control. The most common pH values are 7.0-7.4 and temperature ranges between 25-28°C (Toullec, 1999).

The first attempts to culture crustacean haemocytes under defined *in vitro* conditions started in the early nineties (Ellender *et al.*, 1992; Chen and Wang, 1999; Itami *et al.*, 1999; Walton and Smith, 1999; Jiang *et al.*, 2006; Li and Shields, 2007; George and Dhar, 2010; Jose *et al.*, 2010; Roulston and Smith, 2011). These reports used whole haemocyte populations and described diverse survival times, ranging from 2-4 days (Chen and Wang, 1999) to 3-4 weeks (Ellender *et al.*, 1992). However, the survival evaluation was made using basic techniques such as trypan blue exclusion or visual evaluation. Evaluation of survival by MTT (tetrazolium dye) assay was also used

later, but the results were not clear (Jose *et al.*, 2010). The poor description of the methodologies and interpretation of results (mostly culture dynamics and behaviour) lead to difficulties in reproduction and standardization. Other reports described the culture of separated haemocyte subpopulations and obtained viabilities of 80% for separated hyalinocytes after 14 to 18 days of culture (Walton and Smith, 1999; Li and Shields, 2007).

Other studies presented a different approach, where undifferentiated haemocytes were isolated and cultured. Roulston (2011) isolated a population of circulating immature haemocytes (prohaemocytes) using the traditional separation procedure in Percoll density gradient centrifugation. These cells showed proliferation *in vitro*. Other used stem cells (precursors of circulating haemocytes), which were isolated by enzymatic digestion of haematopoietic tissue or by *in vitro* cell migration directly from the organ explants (West *et al.*, 1999; Mulford *et al.*, 2000; Söderhäll *et al.*, 2003; Söderhäll *et al.*, 2005). Here, the work of Söderhäll *et al.* (2005) should be highlighted due to the innovative incorporation of a crayfish haematopoietic cytokine named astakine. This supplementation induced the *in vitro* differentiation and growth of haematopoietic cells.

Besides the promising results on *in vitro* proliferation of immature haemocytes, there is still a long way to go in order to achieve a standardized and reproducible culture technology, especially when using mature circulating haemocytes. While the culture of haemotopoietic cells represents a good model for studying the haematopoiesis process in crustaceans, the culture of mature haemocytes is particularly useful for studying cell-mediated immune reactions.

## 1.3 Diseases in crustacean aquaculture

Presently, the terrestrial livestock production and fisheries supply the biggest percentage of protein for human consumption. However, aquaculture gains every year a significant percentage of that quote. This development unavoidably requires growth and intensification of aquaculture production systems. Unfortunately, this growth is not always followed by a proportional development of the culture technologies and consequently diseases start to strike. This is one of the biggest threats to the further development of shrimp aquaculture. The most threatening diseases are the ones provoked by viruses, such as white spot syndrome virus (WSSV) and infectious

hypodermal and haematopoietic necrosis virus (IHHNV). Other sources of problematic diseases are Gram-negative bacteria (e.g. *Vibrio* harveyi, *Vibrio penaecidia*) and protozoa (e.g. *Hematodinium* spp). Non-infectious diseases caused by environmental extremes, nutritional imbalances, pollutants and genetic factors, may also have negative impacts on the shrimp industry (Lightner and Redman, 1998; Lightner, 2011; Hauton, 2012; Stentiford *et al.*, 2012).

### 1.3.1 Viral diseases in shrimp aquaculture

It was estimated that viruses provoke 60% of the disease-associated losses in shrimp aquaculture. The World Animal Health Organization (OIE, 2012) listed white spot syndrome virus (WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV) and white tail disease (WTD provoked by *Macrobrachium rosenbergii* nodavirus - *Mr*NV- and extra small virus - XSV) as the viruses with major impact on the worldwide shrimp aquaculture production. At present, WSSV alone accounts for 1 billion dollar loss, which represents 10% of the total production value. The other major viruses together cause 500 million dollar loss. Up to date, WSSV is still considered as the biggest threat to sustainable development of the crustacean aquaculture industry (Lightner *et al.*, 2012; Stentiford *et al.*, 2012).

### 1.3.2 WSSV

WSSV is the etiological agent of the white spot disease (WSD) in shrimp. It was first detected in Taiwan in 1992 and the first major outbreaks were recorded in Japan and China in 1993 in cultures of *Marsupenaeus japonicus* and *Fenneropenaeus chinensis*. In the following 10 years, it spread to the main shrimp producing countries through Asia and the Americas. This virus can infect all cultured penaeid species and potentially a wider range of other decapod crustaceans. It is a very aggressive virus that leads to mortality rates up to 100% from 3 to 10 days after the onset of the symptoms. At present, this pathogen causes one of the most prevalent and widespread diseases in the history of modern shrimp aquaculture industry (Escobedo-Bonilla *et al.*, 2008).

After extensive molecular and phylogenetic analysis and considering its morphological particularities, the International Committee on Taxonomy of Viruses

(ICTV) approved in 2002 to include WSSV in a new virus family named *Nimaviridae*. This name refers to the thread-like extension present in one of the extremities of the virus envelope ("nima" is Latin for "thread"). This new virus family consists of a single genus Whispovirus where WSSV is the sole member so far (Nunan and Lightner, 1997; Sánchez-Paz, 2010; Lo C.F. *et al.*, 2012).

It is a large non-occluded, rod- to elliptical-shaped (80–120 nm x 250–380 nm) DNA virus (Figure 8). The mature virions enclose a rod-shaped tight-fitting nucleocapsid inside an envelope composed by a trilaminar cell membrane-like layer, which contains as major proteins VP28 and VP19. The nucleocapsid is formed by a stack of 14 rings, each one composed of two parallel rows of spaced globular subunits. The virion has a unique and peculiar tail-like extension at one of the extremities, of which the function is unknown. It has a circular dsDNA genome of approximately 300 kbp. (Nakano *et al.*, 1994; Chou *et al.*, 1995; Lo *et al.*, 1996; Durand *et al.*, 1997; Zhan *et al.*, 1998; van Hulten *et al.*, 2000; Escobedo-Bonilla *et al.*, 2008; Leu *et al.*, 2009; Sánchez-Paz, 2010). Recent advances in the understanding of the virion's structure illustrate the distribution and interactions of several structural proteins. It was suggested that the tegument protein VP24 acts as a core protein that directly associates with other tegument (VP26, WSV010 and VP38A) and envelope (VP28 and VP51A) proteins and form a membrane-associated protein complex. VP19 and VP37 are also associated with this complex. The anchorage of this structure to the globular subunits of the nucleocapsid (VP664) happens through the VP51C-VP26 interaction (Chang *et al.*, 2010; Li *et al.*, 2011)

WSSV replicates and assembles in the nucleus of susceptible cells and its life cycle is completed within approximately 24 hours (Leu *et al.*, 2009). After entering the cell, WSSV probably loses its envelope and the naked nucleocapsid releases the genome into the nucleus. Here the replication process starts. During the process, there is a visible chromatin marginalization. After assembling the nucleocapsids, they become tightly wrapped in envelopes, which activate compression and the decrease of its original length. In the cell cytoplasm, organelles become disintegrated, the cellular and nuclear membranes are disrupted and the virions are released for starting a new cycle in other susceptible cells. WSSV targets cells in organs of ectodermal and mesodermal origin, e.g. epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eyestalk, heart, gonads, haematopoietic cells and cells associated with the nervous system (Escobedo-Bonilla *et al.*, 2008).

To date, more than 93 species of arthropods have been reported as hosts or carriers of WSSV (Sánchez-Paz, 2010). However, the major route of transmission is still not clear. It was suggested that the infection of shrimp can happen horizontally via wounds depending on the animal's moult stage (Corteel *et al.*, 2009), by ingestion of infected shrimp tissues (Soto *et al.*, 2001), by passive carriers such as polychaete worms (Vijayan *et al.*, 2005) and rotifers (Yan *et al.*, 2007). Another alternative is the transmission by water-borne route (Arts *et al.*, 2007; Esparza-Leal *et al.*, 2009). Recently, a breed line of *P. vannamei* was reported to be partially resistant to WSSV (Cuéllar-Anjel *et al.*, 2012).

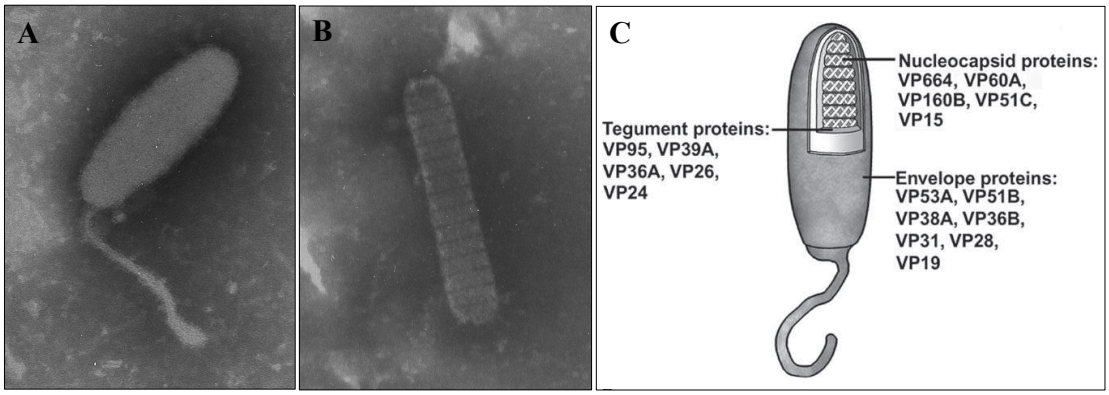


**Figure 8.** (A) Intact WSSV viral particle. (B) Naked WSSV nucleocapsid. (C) Diagram representing the main structural parts as respective constituent proteins (Leu *et al.*, 2009).

### 1.3.3 Interference with viral infections in crustaceans

Despite the dramatic impact of viral diseases on the shrimp aquaculture industry, effective preventive/curative treatments are not available yet. One of the main reasons is the lack of knowledge on antiviral immunity of shrimp. The need for control has stimulated research in this field. As a result, several compounds, processes and genes were found to be involved in crustacean antiviral defence, but the molecular mechanisms of their activity are only starting to be addressed (Liu *et al.*, 2009). From those, the administration of dsRNA seems to be the most promising. Administration of dsRNA for knocking down the expression of WSSV genes, enabled a high degree of protection against WSSV infection in shrimp (Robalino *et al.*, 2005; Kim *et al.*, 2007; Sarathi *et al.*, 2008; Sarathi *et al.*, 2010; Sanjuktha *et al.*, 2012). However, in order to be applied commercially, this method still has to go through an extensive validation process with different doses of different WSSV strains and under representative field conditions. Furthermore, an easy and efficient dsRNA delivery

route has to be found. In insects, oral delivery is extensively used with reasonable success (Yu *et al.*, 2012).

It was previously demonstrated that high water temperature (33°C) could completely protect *P. vannamei* from WSSV infection, when applied at the right time and regime (Rahman *et al.*, 2007a; Rahman *et al.*, 2007b). However, the applicability of this system is limited mainly due to the impossibility of controlling the temperature in shrimp grow-out culture ponds. An alternative to overcome this limitation is to unveil the mechanisms involved in the antiviral effect that is expressed when shrimp are exposed to these environmental conditions. A better insight in this matter was found when the knocking down of aldehyde dehydrogenase and Hsp70 genes provoked a severe infection of shrimp exposed to high temperature (Lin *et al.*, 2011).

## 1.4 Purification of biological particles

There are several techniques to separate specific types of biological particles from heterogeneous suspensions. If the sedimentation rate (see definition in point 2.4.1) of the particles to separate is very different from the rest of the particles in suspension, (e.g. cells and subcellular structures) they can be separated by differential centrifugation. This technique is based on the application of increasing centrifugation forces to pellet particles with decreasing sedimentation rates. Nevertheless, this technique does not achieve good particle purity. Other alternatives based only on particle size are filtration and dialysis. Using these techniques, one must keep in mind that all the contaminants within the size range of the particles to separate will not be excluded. If the intention is to separate particles with similar sizes (e.g. suspension of cell subpopulations or virus with subcellular debris), a good alternative is the separation according to their density. This is done by separation in density gradients. If the particles of interest have distinctive morphological characteristics, demonstrate autofluorescence or contain specific antigens (need for specific markers), the separation can be done using fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS), immune panning or affinity chromatography (Freshney, 2005).

The selection of the method or combination of methods is dependent on the characteristics of the particles and objective of the work. If the preservation of the biological activity is important, as normally required for cells and viruses, less

invasive and aggressive techniques such as density gradient centrifugation and flow cytometry (without antibody binding) are preferable.

### 1.4.1 Purification of particles by density gradient centrifugation

The centrifugation of particle suspensions through density gradients, achieves at the same time the concentration and purification of the particles of interest. For deciding which centrifugation procedure should be used, the physical behaviour of sinking particles must be considered. The formula that describes the velocity (m $s^{-1}$) of a particle under centrifugation through a fluid column is:

$$v = [d^2(\rho_p - \rho_m)\, \omega^2 r]/18\mu$$

where d is the diameter of the particle, $\rho_p$ is the density of the particle (kg $m^{-3}$), $\rho_m$ is the density of the liquid medium (kg $m^{-3}$), $\omega$ is the angular velocity (rad $s^{-1}$), and $\mu$ is the viscosity (Pa s). Having the velocity of a particle under specific centrifugation conditions, the sedimentation coefficient expressed in Svedbergs (S), can be calculated using the following formula:

$$s = v/\omega^2 r$$

where v is the velocity previously calculated, $\omega$ is the angular velocity and r the distance between the particle and the axis of rotation. This value expresses the particle's sedimentation rate per unit of centrifugal force and, as such, allows comparing the behaviour of different particles under certain centrifugation conditions. There are two types of separation procedures that can be performed using density gradients: rate-zonal and isopycnic. Rate-zonal is based mainly on the size (to a smaller extent on the density and shape of the particle) and associated speed (sedimentation rate) of a particle under centrifugation through a certain density gradient. This procedure requires the use of continuous gradients in which the maximum density is always lower than the density of the particles of interest, otherwise the sedimentation will terminate when the density of the medium is equal to the density of the particle. The centrifugation is stopped before the densest particle reaches the bottom of the gradient. As a result, the particles of interest will be grouped (concentrated) in an area of the tube, depending on the centrifugation time and/or centrifugal force used. An isopycnic centrifugation takes into account the particle's

buoyant density. This technique requires the composition of an adequate density gradient (continuous or discontinuous) in which the highest density is always higher than the density of the particles of interest. The centrifugation is ran until all the particles reach their buoyant density (density of the particle=density of the medium). The particles are grouped (concentrated) in a certain area according to their density.
For separating different cell subpopulations (e.g. white blood cells), the best strategy is to apply isopycnic centrifugation. Cells are relatively big particles with a very high sedimentation coefficient, which is also similar among cell types, and, as such, they move fast and at similar speeds. This does not allow an efficient separation by rate-zonal centrifugation. In the case of viruses, both techniques can be applied. In this case, the decision will depend on the physical characteristics of the contaminating particles (Graham, 2001; Lawrence and Steward, 2010).

### 1.4.2 Density gradient media

The density gradient media most widely used for separation of biological particles are: Percoll (colloidal silica), Ficoll (high mass hydrophilic polysaccharide), iodixanol (iodinated alcohol), sucrose (small hydrophilic organic sugar), caesium chloride (CsCl; salt of alkali metal) and sodium bromide (NaBr; inorganic salt). These media are used routinely for separation of cells, subcellular organelles and other subcellular structures, viruses and proteins (Lawrence and Steward, 2010).
Iodixanol and Percoll have advantages over all the other media because of their low viscosity, non-toxicity for cells and capacity to form self-generating gradients. The viscosity is a property that strongly influences the speed of the separation (the higher the viscosity, the slower the separation will be) and the damages to the structure of the particles (the higher the viscosity, the more damages). The non-toxicity of these products excludes the need for washing steps before using the purified particles in biological evaluations (Ford *et al.*, 1994; Graham, 2001). The creation of self-forming gradients is different in Percoll and iodixanol. While in Percoll they are formed at a relatively low speed and centrifugation times, this process in iodixanol needs much higher speed and more time. However, when using short centrifugation times (20 minutes), Percoll tends to form non-linear S-shaped gradients, which are characterized by steep density profiles at the top and bottom areas and a shallow area in between. When using more extended centrifugation times, these gradients become nearly linear

but display a very steep density profile (Figure 9). Iodixanol on the other hand, has the property of forming linear continuous gradients from preformed discontinuous gradients, by diffusion of iodixanol in between the gradient fractions (Figure 9) (Pertoft *et al.*, 1978; Graham, 2001). These properties can be both useful in different situations. For separation of big particles such as cells, both gradients media are suitable. However, in the particular case of separation of immune cell subpopulations (e.g. mammalian macrophages) it is advisable to wash out Percoll as it might be taken up by these cells (Wakefield *et al.*, 1982). For separation of smaller particles such as viruses, there is the need to apply high centrifugation speeds in order to displace the particles inside the gradient. Therefore, due to the easy sedimentation and pelleting of Percoll, this media is not the best choice for purification of viruses (Lawrence and Steward, 2010).

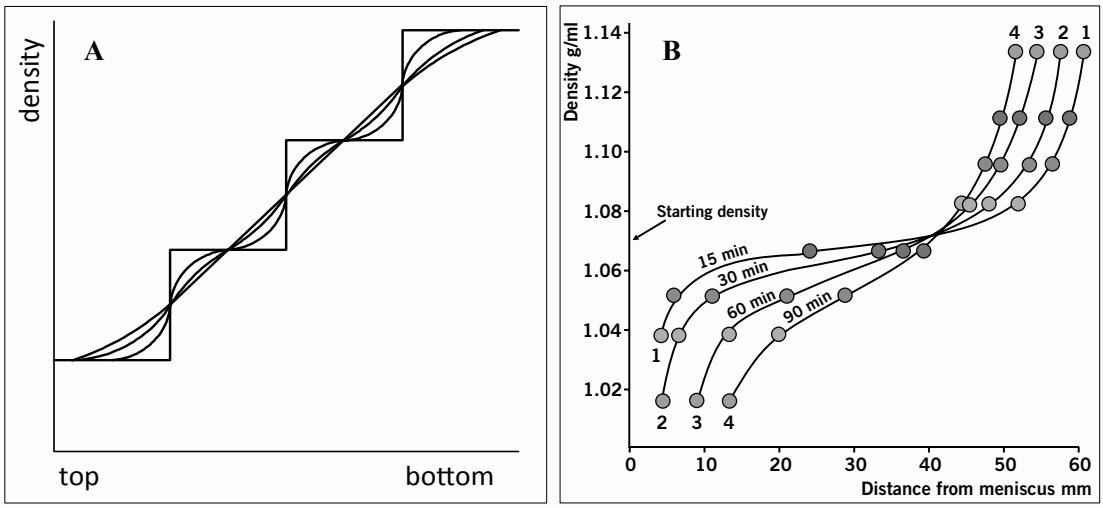


**Figure 9.** Iodixanol pre-formed discontinuous gradient profile becoming continuous by solute diffusion over time (A) (Graham, 2001). Percoll self-forming gradient profile evolution after different centrifugation periods (B). The circles on the density curves represent the position of density marker beads in the gradient (Amersham Biosciences, 2001).

### 1.4.3 Iodixanol

Iodixanol is an iodinated alcohol (Figure 10). It was developed in the early 1990's for use as X-ray contrast agent. Due to its inherent characteristics (inertness and low half-life period), it is readily excreted in an unchanged form when injected into the organism. This allows its utilization as contrast agent for coronary angiography in humans. Its systematic chemical name is 5,5-[(2-hydroxy-1,3-propanediyl)-bis(acetylimino)]bis-[N,Nbis(2,3dihydroxypropyl)-2,4,6-triiodo-

benzenedicarboxamide] whose molecular structure is given in Figure 10. Its molecular mass is 1550. In aqueous solutions, iodixanol is iso-osmotic for terrestrial animals (aproximately 300mOsmol/kg) up to a density of 1.32 g/ml. This product is capable of forming self-generating gradients in 1 to 3 h. It has a very low toxicity towards biological material and enzyme assays can be carried out in its presence (Axis-Shield, 2012).

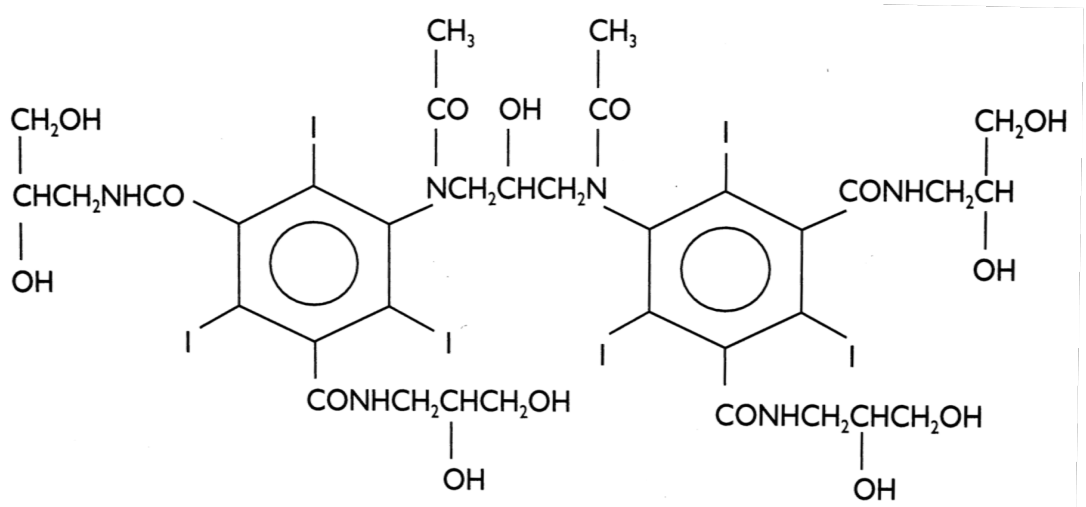


**Figure 10.** Molecular structure of iodixanol (Graham, 2001).

1.5 Separation of crustacean haemocyte subpopulations.

The first separation of crustacean haemocytes was described by Söderhäll and Smith (1983) for several crab species. This technique described the use of a self-forming Percoll gradient for separating cell subpopulations based on their buoyant density. The preparation of the gradient was done by centrifuging a solution of 60% Percoll in 3.2% NaCl at 25,000 g for 20 min. Haemolymph was collected in a citrate/EDTA anticoagulant, topped on the gradient and centrifuged for 10 min at 2,900 g. They were able to separate biologically active hyaline and granular cells. Furthermore, this methodology was adapted to other invertebrate species (Smith and Söderhäll, 1983; 1991; Pipe *et al.*, 1997; Sritunyalucksana *et al.*, 2001; Hammond and Smith, 2002; Liu *et al.*, 2005; Vargas-Albores *et al.*, 2005; Li and Shields, 2007; Sperstad *et al.*, 2010; Falwell *et al.*, 2011; Roulston and Smith, 2011). Since the development of this technique, no other new techniques and significant improvements to separate living crustacean haemocytes were described in literature.

Flow cytometry and fluorescence activated cell sorting (FACS) were successfully used to analyse and sort crustacean haemocyte subpopulations (Sequeira *et al.*, 1995;

Owens and O'Neill, 1997; Lee *et al.*, 2001; Yip and Wong, 2002; Cardenas *et al.*, 2004; Taylor *et al.*, 2009; Sun *et al.*, 2010; Roulston and Smith, 2011). Unfortunately, no convincing data on the *in vitro* cultivation of sorted subpopulations could be found. Crustacean haemocytes are extremely reactive cells which promptly react in response to any external stimulus starting a clustering chain reaction when cultured in suspension (Dantas-Lima *et al.*, 2012). This reaction can be delayed using a citrate/EDTA buffer but not completely stopped. This makes FACS-based sorting of living haemocyte subpopulations difficult (Dantas-Lima *et al.*, unpublished results). Therefore, to successfully accomplish the separation of these cells, a more efficient anticoagulant buffer is needed. While FACS is used to separate cells marked with fluorescent dyes, magnetic activated cell sorting (MACS) separate cells coupled to magnetic beads. In both cases, subpopulation specific markers are necessary. Several attempts were made to produce markers specific for crustacean haemocyte subpopulations (Rodriguez *et al.*, 1995; Sung *et al.*, 1999; van de Braak *et al.*, 2000; Zhan *et al.*, 2001; Wu *et al.*, 2008). Unfortunately, none of these makers are commercially available. This limitation together with the difficulty of handling haemocytes at an individual level makes these techniques at this stage unsuitable to separate biological active crustacean haemocyte subpopulations.

### 1.6 WSSV purification

Up to date, WSSV purification was performed by density centrifugation and filtration techniques. Centrifugation was done in sucrose, NaBr or CsCl density gradients (Wang *et al.*, 1995; Huang *et al.*, 2001; van Hulten and Vlak, 2001; Tsai *et al.*, 2004; Escobedo-Bonilla *et al.*, 2006; Du *et al.*, 2007; Chen *et al.*, 2010). Because of the known deleterious effects of these media on virus structure and toxic effects for cells, better alternatives are needed. Others used simple differential centrifugation or filtration techniques (Durand *et al.*, 1996; Xie *et al.*, 2005; Gracia-Valenzuela *et al.*, 2009). These methodologies, besides easy and fast to perform, often result in low purity. In a few reports, the infectivity of the purified stocks was tested by bio-assays (Xie *et al.*, 2005; Du *et al.*, 2007; Gracia-Valenzuela *et al.*, 2009), however none evaluated the exact quantity of infectious virus and infectivity recovery during the purification procedures. These types of evaluations are particularly important when a high reproducibility of experiments is required. In general, the existing methodologies

for purifying WSSV were presented in such a resumed way that is difficult to reproduce them.

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# Chapter 2

## Aims of this thesis

Aquaculture is one of the main sources of quality animal protein for human consumption, together with terrestrial livestock and fisheries. In 2011, aquaculture produced almost half of the world seafood commodities (64 out of 154 BD). The value of its production was estimated to be 199 BD in 2010. This output is expected to increase in the coming years. After four decades of continuous expansion, crustacean aquaculture reached the production mark of 5.7 million tonnes, which was equivalent to 27 BD. The white leg shrimp (*Penaeus vannamei*) was by far the most cultivated crustacean species. Its production was 2.7 MT with an estimated value of 11 billion dollars in 2011.

The study of the physiology of farmed crustaceans is still a young scientific field compared to that of terrestrial livestock. The same can be said about the viral diseases that appeared following the expansion and intensification of shrimp farming. Shrimp viruses are associated with high morbidity and mortality, which provoke huge economic losses every year. Among these, the most important pathogen is WSSV. To be able to control WSSV-associated problems, it is necessary to acquire an extensive knowledge on shrimp physiology and particularly on shrimp immunity. Two of the main obstacles in this process are the lack of continuous cell lines and standardized *in vitro* models for evaluating host-pathogen interactions.

The general aim of this thesis was to develop and improve *in vitro* techniques for supporting the study of shrimp immunity.

The particular aims of this thesis were:

(1) To develop standardized shrimp haemocyte culture techniques for use in *in vitro* immunity studies.

(2) To develop an improved haemocyte subpopulation separation procedure that may serve as a basis for haemocyte subtyping and functional characterization of subpopulations.

(3) To develop an improved virus purification protocol for producing well-characterized WSSV stocks for use in experiments on the interaction of virus particles with the immune system of shrimp.

# Chapter 3

## Haemocyte culture systems

# 3.1 Development of two haemocyte culture systems (in attachment and in suspension) for shrimp immunity studies

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*Aquaculture 366-367(0): 17-26*

**Abstract**

For studying shrimp immunity, in vitro haemocyte cultures are essential. Despite various reported attempts, well-described and reproducible culture techniques are lacking. The current work aimed to establish two *in vitro* haemocyte culture systems for *Penaeus* (*Litopenaeus*) *vannamei*. Haemocyte suspensions were either seeded in conventional Nunc® Nunclon™Δ Surface 24-well cell culture plates with glass cover slips (haemocytes in attachment) or in Nunc® Hydrocell Surface 24-well cell culture plates (haemocytes in suspension). The culture medium was based on L-15 (Leibovitz), and was further supplemented with L-glutathione and protease inhibitors in an attempt to improve haemocyte survival. Parameters such as number of living adherent and single cells, number and average diameter of clusters and survival of cells inside clusters were evaluated. Additionally, live-cell imaging videos were recorded. It was found that haemocytes cultured for 1 hour on glass coverslips in Nunc® Nunclon™Δ Surface plates could be separated in two cell fractions: adherent or non-adherent. Shrimp haemocytes cultured in Nunc® Hydrocell Surface plates remained in suspension and over time formed cell clusters which melanised. L-glutathione supplementation clearly improved haemocyte survival up to 48 h and delayed clustering and melanisation; addition of protease inhibitors did not. To validate the system, the phagocytic and antibacterial activities of adherent haemocytes towards *V. campbellii* were evaluated. After one hour of co-culture, 11.5±0.14% of haemocytes showed phagocytosis with an average of 2.4±0.1 bacteria internalised per haemocyte. Furthermore, haemocytes clearly demonstrated an antibacterial activity. It was concluded that these systems were reproducible and could keep haemocytes functionally active during the time required for the study of innate immune processes. Consequently, these techniques represent powerful tools for studying a variety of cell-mediated and humoral immune responses of shrimp *in vitro*.

## Introduction

Research on crustacean immunity has received high priority during the past two decades, mainly triggered by the need of controlling disease outbreaks in shrimp farms. Invertebrates do not possess an acquired immunity. Instead they rely on innate, non-adaptive immune mechanisms. Despite this restriction, shrimp defence has the ability to effectively control most microbial challenges within hours after they occur (Jiravanichpaisal *et al.*, 2006; Liu *et al.*, 2009). The innate immune system consists of a humoral and a cellular branch, with the haemocytes occupying a central position in both. These immune effector cells are known to be involved in all major immune processes such as the prophenoloxidase (proPO) system, clotting, antimicrobial action, opsonisation, phagocytosis, cell agglutination and nodulation/encapsulation of foreign material (Johansson *et al.*, 2000; Jiravanichpaisal *et al.*, 2006). Of these processes, the proPO activation cascade and the clotting system are the most extensively studied in decapod crustaceans and this knowledge even serves as a research model (reviewed by Jiravanichpaisal *et al.*, 2006; Cerenius *et al.*, 2008). There is, however, still a long way to go in unveiling the mechanisms of other important immune reactions and the role of haemocytes in these reactions.

Decapod crustacean haemocytes are traditionally divided into three morphologically distinct subpopulations: hyalinocytes or hyaline cells, semigranulocytes or small granule cells and granulocytes or large granule cells (Söderhäll and Smith, 1983; Martin and Graves, 1985; Hose *et al.*, 1990; Giulianini *et al.*, 2007). In order to initiate any immune response, it is necessary for haemocytes to identify a target as non-self. In homology with the innate immunity of vertebrates, they use pattern-recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs) specific for microorganisms like bacteria and fungi (Fearon, 1997). The invertebrate immune system can also very efficiently recognise abiotic targets such as plastic and glass as non-self (Nardi *et al.*, 2006). This feature demonstrates its broad immune reactivity. The innate immune response in vertebrates is initiated with the phagocytosis of pathogens by macrophages. This process is characterised by the uptake of large particles by an actin-dependent mechanism (pathogens or other non-self structures and cell debris) by cells for their further degradation (Aderem and Underhill, 1999). In crustaceans, the cells and processes by which phagocytosis is carried out are still poorly understood and subject of debate in literature. In previous publications, besides

the phagocytic cell type differed or was not mentioned, the percentage of cells performing phagocytosis was greatly variable (Smith and Ratcliffe, 1978; Hose *et al.*, 1990; Itami *et al.*, 1998; Deachamag *et al.*, 2006; Li *et al.*, 2008). Interestingly, invertebrates exhibit another peculiar cell-mediated immune reaction described as nodulation or encapsulation. This function appears to be a very important response against invading microorganisms (Ratcliffe and Rowley, 1979; Söderhäll *et al.*, 1984; Auffret and Oubella, 1997; Johansson, 1999; Lavine and Strand, 2002; Nardi *et al.*, 2005). The phenomenon consists of haemocytes identifying a target as being non-self, followed by adhesion of newly attracted haemocytes, forming overlapping layers of cells around the foreign entity. While nodulation immobilises large amounts of biotic targets with the size of bacteria or fungal spores, encapsulation is directed to bigger targets such as parasites (Ratcliffe and Gagen, 1976; 1977) or even abiotic targets such as plastic beads (Pech and Strand, 1996). Nodulation and encapsulation appear to be the same process but directed against different targets (Lavine and Strand, 2002). The process normally ends with the melanisation of the nodule/capsule and the killing of the invader by asphyxiation and action of toxic products (Nappi *et al.*, 1995). Although all factors involved in the capsule formation in arthropods are not completely understood, it is known that after the identification of the foreign target, haemocytes have to pass from a non-adherent to a strongly adherent state, at which point they activate their immune functions (Schmidt *et al.*, 2001; Nardi *et al.*, 2006). Some of the most crucial immune effectors are the proPO system, antimicrobial peptides and reactive oxygen species (ROS). Especially the latter pathway, if not properly balanced by antagonists (enzyme inhibitors, antioxidants), can comprise a serious threat to the host (Johansson and Soderhall, 1989). The major antioxidant molecule present in animal cells is glutathione (GSH), which is a very effective actor in preventing the damage to important cellular components by ROS. Moreover, GSH is also involved in preventing the toxic effects of quinones, which are produced in the proPO cascade (Monks and Lau, 1992; Uhlig and Wendel, 1992; Söderhäll *et al.*, 1994).

Throughout the advancement of crustacean immunity research, methodologies for culturing mature haemocytes and *in vitro* differentiated haematopoietic stem cell have been reported (Smith and Söderhäll, 1983; Söderhäll and Smith, 1983; Johansson and Söderhäll, 1988; Hose and Martin, 1989; Chisholm and Smith, 1992; Rodriguez *et al.*, 1995; Gollas-Galván *et al.*, 1997; Vázquez *et al.*, 1997; Gargioni and Barracco, 1998; Muñoz *et al.*, 2000; van de Braak *et al.*, 2000; Lee *et al.*, 2001; Söderhäll *et al.*, 2003;

Cardenas *et al.*, 2004; Söderhäll *et al.*, 2005; Vargas-Albores *et al.*, 2005; Jiang *et al.*, 2006; Jiang *et al.*, 2007; Vidya *et al.*, 2007; Jose *et al.*, 2010; Sun *et al.*, 2010). Although some were properly designed, most of these studies did not take into account or mention important parameters such as the animal's moult stage and age and did not control cell survival and clustering activity over time. Due to poor methodological descriptions, most experimental protocols are difficult to reproduce.

The current work aimed to establish and compare two *in vitro* haemocyte culture systems, in attachment and in suspension, for a representative penaeid shrimp species, the marine *Penaeus* (*Litopenaeus*) *vannamei*. The ultimate goal was to provide reproducible and well-characterised haemocyte culture systems for use in shrimp immunological research and at the same time to provide a solid base for its adaptation to other shrimp species.

## Materials and methods

### *Experimental animals*

Specific pathogen-free (SPF) *Penaeus* (*Litopenaeus*) *vannamei* were imported from Piti Syaqua Farm, Syaqua Siam Co. Ltd., Thailand. The shrimp were certified to be SPF for the major shrimp viruses by the Phuket Coastal Fisheries Research and Development Centre of the Thai Department of Fisheries. Batches of postlarvae were reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium (water temperature at 27±1°C, pH at 7.8-8.1 and salinity at 35±1g $l^{-1}$). Adult shrimp of 12 months old with a mean body weight of 40±5g were used in this study. All were selected when they were in inter-moult (C) stage (Corteel *et al.*, 2012).

### *Haemocyte medium*

Osmolality and pH of *P. vannamei* haemolymph were determined (903±13 mOsmol $kg^{-1}$,pH 7.5±0.2). Haemocyte medium (HM) and anticoagulant were adapted to these parameters. L-15 medium (Leibovitz; Sigma-Aldrich) was prepared at double strength (2xL-15) and used as basal medium. HM was composed of 2xL-15, 10.5% (v/v) Chen's salts (CS) (Chen and Wang, 1999b), 10% (v/v) foetal calf serum (FCS) and penicillin/streptomycin (P/S: 100units $ml^{-1}$/100µg $ml^{-1}$) (pH 7.5; 900 mOsmol $kg^{-1}$). In survival improvement experiments, L-glutathione (GSH) (Sigma Aldrich) and EDTA-

free protease inhibitors cocktail (Roche) were added individually to HM at a final concentration of 0.1% (w/v) and 1x, respectively. The media were filtered through a 0.20µm filter before use.

*Haemolymph collection and haemocyte isolation*

Marine Anticoagulant (MA) (Söderhäll and Smith, 1983) with the pH adjusted to 5.4 was used as anticoagulant solution. Haemolymph was taken from the ventral sinus in the second abdominal segment using a 2 ml syringe with a 20G hypodermic needle filled with MA (1:2 with haemolymph). Haemolymph was poured into Eppendorfs and centrifuged at 250 g during 5 minutes at 4ºC. Supernatant was discarded and the pellet was immediately resuspended in HM. Cell concentration was evaluated using the Bürker-Türk counting chamber. All the materials and solutions were pre-chilled and kept on ice during the whole procedure to prevent clotting and haemocyte activation.

*Haemocyte culture systems and experimental setup*

Haemocyte culture in attachment: Freshly collected haemocyte suspensions were seeded into Nunc® Nunclon™Δ Surface 24-well cell culture plates. Heat-sterilised (180°C for 2h) round glass cover slips were brought in each well. Cells were seeded at $3x10^5$ cells $well^{-1}$ in a total volume of 400 µl. Plates were incubated for 1h at 27ºC in order to allow cell attachment to the cover slips. Subsequently, non-adherent/weakly adherent haemocytes were resuspended by pipetting gently up and down six times throughout the well. This washing procedure was repeated twice and the wells were finally filled with 400µl of medium. The cells that remained attached to the glass cover slip were designated as "adherent cell fraction". The supernatant from each well was recovered in a new well also supplied with a glass cover slip. The cells in this supernatant were designated as "non-adherent cell fraction". The number of living individual haemocytes in time was determined for both adherent and non-adherent cell fractions. Additionally, the number of living cells from the adherent cell fraction that detached from the glass cover slip in time was evaluated. Samples were collected at 1, 3, 24, 48, 72, 96 and 120 hours post seeding (hps). The cells were kept at 27ºC during the entire experimental period. Haemocyte culture in suspension: Different plastics and coating materials were tested for their suitability to prevent haemocyte adherence. We tested conventional Eppendorf tubes, siliconised glass bottles, Sarstedt® Flat Bottom

Suspension Cells 24-well plates and Nunc® Hydrocell™ surface 24-well plates. In Nunc® Hydrocell™ surface 24-well plates, the attachment of haemocytes was completely prevented and therefore these plates were chosen for use in haemocyte suspension experiments. Cells were seeded and cultured in the same way as for culture in attachment except that the sampling started immediately after seeding.

*Survival staining*

Haemocyte culture in attachment: Adherent cells were transferred to new, pre-chilled wells and immediately stained with ethidium monoazide bromide (EMA, Sigma) for survival evaluation (Costers *et al.*, 2006). Cells were incubated with EMA solution diluted in HM (0.02 mg $ml^{-1}$) in the dark and on ice for 30 minutes. Afterwards, Hoechst dye (Invitrogen, Life Technologies) diluted in HM (0.01 mg $ml^{-1}$) was added. The plates were exposed to incandescent light for 10 minutes and then washed 1x with HM. After staining, cells were fixed for 10 minutes with paraformaldehyde (PF) 2% and washed with PBS and distilled water (DW). Finally, cover slips with attached cells were mounted on glass slides with 2µl of anti-fading mounting medium (Glycerine-DABCO). Detached and non-adherent cells were transferred to new, pre-chilled wells and immediately stained with EMA and Hoechst as described above. After, the washing steps were performed in Eppendorf tubes by centrifugation at 250 g for 10 minutes. At the end of the procedure, the cell pellet was resuspended in a volume of 10µl, mounted on a glass slide with 2µl of glycerine-DABCO and covered with a glass cover slip (24x24mm). Haemocyte culture in suspension: Cell suspension was pipetted gently up and down six times throughout the well and transferred to pre-chilled Hydrocell wells. This procedure was repeated twice and cells were immediately stained with EMA and Hoechst as described above. The washing and mounting procedures were the same as described above for detached and non-adherent cells.

*Live-cell imaging*

Live-cell imaging was performed with Olympus IX81®. Plates were seeded with $3x10^5$ cells per well and micrographs of 5 predetermined positions in the wells were taken every 2 minutes for 2 hours post seeding (hps). During the experiment, the temperature was kept at 27ºC. The micrographs from each location were put together and saved as a video file (15 seconds; 4 frames $second^{-1}$).

*Evaluation of the number and survival of haemocytes*

Haemocyte culture in attachment: The total number of cells (Hoechst staining) and the number of dead cells (EMA staining) were evaluated. For adherent cells (round cover slips), counting was done in 9 standardly positioned microscopic fields (Leica DM IRBE, magnification 400x) making up a total area of 2.1 $mm^2$. The number of cells in this area was extrapolated to the area of a well and the total number of living cells calculated. For detached and non-adherent cells (square cover slip), the evaluation was done as described for individual cells in the culture in suspension. Haemocyte culture in suspension: The total number of cells, the number of dead individual cells and the average size of cell clusters were determined. Individual cells were counted in 20 microscopic fields (magnification 200x) making up a total area of 18.3 $mm^2$. This number was extrapolated to the area of a square glass cover slip for determination of the total number of cells. The average diameter of haemocyte clusters was assessed by taking micrographs of at least 20 random haemocyte clusters per sample and measuring their diameter using the software ImageJ (Abramoff, 2004). The number of living cells was determined by subtraction of dead cells from the total number of cells. The experiments were repeated 3 times and the mean values and respective standard deviations calculated.

*Validation of the culture systems*

In order to demonstrate that the set of culture systems was suitable to be used in immunological studies, phagocytosis and inactivation of bacteria were evaluated in the adherent cell fraction.

Production of GFP-labelled bacterial stocks

Rifampicin and kanamycin-resistant *Vibrio campbellii* (LMG 21363) (Phuoc *et al.*, 2009), were transfected with a plasmid containing Green Fluorescent Protein (GFP). *Escherichia coli* DH5α was used as plasmid carrier. Briefly, the two bacteria species were co-cultured in order to transfect *V. campbellii* with the plasmid. After transfection, colonies of GFP-labelled *V. campbellii* were isolated based on their antibiotic resistance. GFP-labelled *V. campbellii* colonies were subsequently grown in marine broth for 12h at 27°C, washed with filtered and autoclaved seawater (FASW)

and stored at -80°C in 20% glycerol.

Bacteria inactivation by haemocytes

GFP-labelled *V. campbellii* were sub-cultured twice in HM containing selective antibiotics (20µl of bacterial suspension in 20ml of HM for 12h and 14h at 27°C). Suspensions were washed as described above. The concentration of bacteria in the suspension was determined by measuring the optical density at 600nm (OD600). The concentration in colony forming units (CFU) was calculated using the formula CFU/ml=(10xOD600-1)x$10^8$. During the challenge period, antibiotics (P/S) were not included in HM in order to prevent unwanted bacterial inactivation.

Haemocytes cultured in attachment were inoculated with 0.1 CFU haemocyte$^{-1}$. Wells with medium and bacteria only were used as control. Samples of the supernatants were taken as previously described, at 0 and 1 hour post inoculation (hpi). These samples were serially 10-fold diluted and 3 dilutions of interest plated (100µl) in duplicate on marine agar plates containing selective antibiotics. Plates were incubated for 24h at 27°C and bacterial colonies counted.

The experiments were repeated 3 times and the mean values and respective standard deviations calculated.

Percentage of phagocytic cells and phagocytic index

A sample of *V. campbellii* frozen stock was diluted and plated in marine agar plates containing selective antibiotics. After 24h of incubation at 27°C, a single fluorescent colony was inoculated in 20ml of HM containing selective antibiotics and incubated for 12h at 27°C. Bacteria suspensions were washed twice in HM and the concentration in CFU determined as previously described. Cultures of adherent haemocytes (177,000 cells well$^{-1}$) were inoculated with 100 CFU haemocyte$^{-1}$ at 1 hps and samples were taken at 0 and 1 hpi. Before sampling, wells were washed 2x with HM. The sampling procedure was as previously described for adherent cells. A survival control stained with EMA was included in each trial. At the moment of sampling, cells were fixed with 4% PF for 10 minutes, permeabilised with 0.1% Triton X-100 for 5 minutes and stained with Texas Red-labelled phalloidin (Invitrogen, Life Technologies$^{TM}$) (1:50 in PBS) for 1h at 37°C. Ten minutes before the end of this staining, Hoechst (1:100 dilution) was added. The mounting procedure was the same as described above. The

percentage of phagocytic haemocytes (haemocytes uptaking bacteria) and the phagocytic index (number of bacteria internalised per phagocytic haemocyte) were determined using confocal microscopy. Sequential confocal pictures in three different wavelength emission channels (Hoechst: 461nm; Texas Red: 615nm; GFP: 509nm) were taken from the cell base to its apex. These pictures were taken in 9 random confocal visual fields, where at least 150 cells were analysed.
The experiments were repeated 3 times and the mean values and respective standard deviations calculated.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 5.0d for Mac (Graphpad software, San Diego California USA). Survival curves of cells cultured in HM and GSH-supplemented HM were analysed for significant differences using a Kaplan-Meier estimator. Differences between the number of cells, number of clusters and average cluster diameters in both media were analysed using the Wilcoxon matched-pairs signed rank test at a confidence interval of 95%. The bacteria inactivation level was analysed using an independent sample *t*-test.

**Results**

*Haemocyte culture in attachment*

The live-cell imaging videos showed that part of the haemocytes strongly attached to the substrate (glass cover slip) and spread and moved with continuous amoeboid motion (showing pseudopodia) over short distances (Video 1). The round, weakly attached cells had variable movement patterns, i.e. either they were completely static, had erratic movement or moved in a way similar to the adherent cells, by means of small pseudopodia. At no point cells were observed clustering to each other.
Part of the cells attached to the culture substrate (adherent cell fraction) and part remained non-attached (non-adherent cell fraction). The cell attachment efficiency was 59±14% and 49±6% for cells cultured in HM and in GSH supplemented HM (HM+GSH), respectively. The non-adherent cell fraction was washed away from the original wells and plated into new wells (Figure 1). The number of living cells in adherent and non-adherent cell fractions, cultured in HM and in HM+GSH are presented in Figure 3.

In the adherent cell fraction, the total number of living cells at 1 hps was nearly identical in both HM (93,401±9,878 cells well$^{-1}$) and GSH supplemented HM (97,690±9,903 cells well$^{-1}$). This scenario was maintained until 3 hps, but at 24 hps the difference in cell number was very pronounced between the two treatments. In HM, the total number of living cells was nearly half of the initial (46,441±13,219 cells well$^{-1}$)

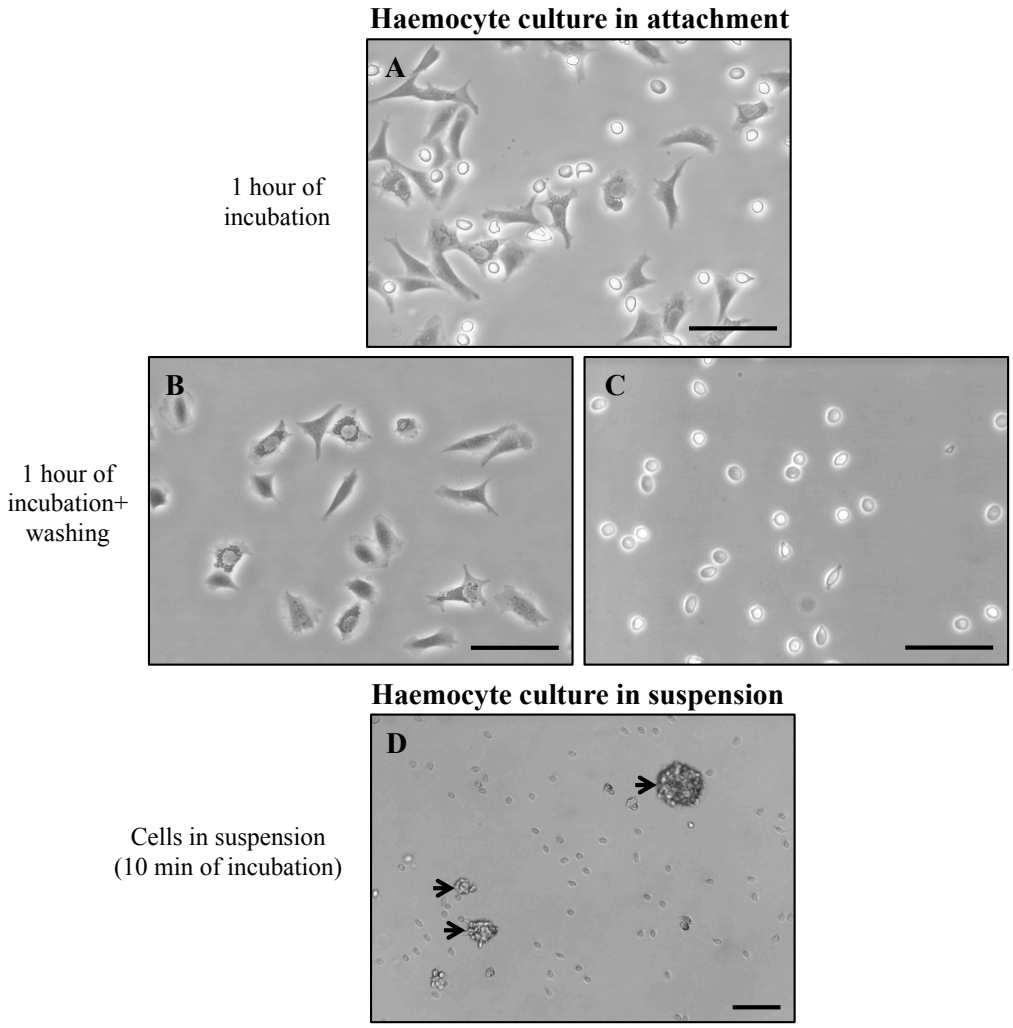



**Figure 1.** Representative pictures of cultures in attachment (conventional plates with glass cover slips) and in suspension (Hydrocell plates) of *P. vannamei* haemocytes. After one hour of incubation, the culture in attachment (A) could be divided into 2 cell fractions, an adherent cell fraction (B) and a non-adherent cell fraction (C). In the culture in suspension (D), cells either appeared individually or clustered together (arrows). Scale bars = 50µm.

while in HM+GSH the decrease was smaller (85,210±17,661 cells well$^{-1}$). At this time, a significant part of the cells detached from the substrate, in both treatments. The number of living detached cells was 10,962±3,120 cells well$^{-1}$ for HM and 19,026±4,256 for HM+GSH. The living cell number (adherent+detached cells) diminished in both treatments until the end of the experiment (120 hps), with a higher

performance of cell cultures in HM supplemented with GSH.

In the non-adherent cell fraction, the number of single living cells in HM+GSH was always higher. At 1 hps the living cell number was 91,764±24,919 cells well$^{-1}$ and 125,363±625 cells well$^{-1}$ for HM and HM+GSH, respectively. These numbers remained constant in the first 3 hours of culture, resembling the adherent cell fraction. At 24 hps the number of single living cells in HM diminished to 39,407±15,538 cells well$^{-1}$ while in HM+GSH it was 78,900±7,021 cells well$^{-1}$. This scenario remained almost unchanged at 48 hps. In the subsequent samples, the number of cells gradually diminished, nevertheless the performance was better in GSH.

The statistical comparison between HM and GSH supplemented HM showed significant differences ($p$<0.05) in the survival curves and number of living cells, both for adherent and non-adherent cell fractions. Nevertheless, no statistical differences ($p$>0.05) were found when cell fractions were compared. The inclusion of protease inhibitors did not improve the survival of the cultures (data not shown).

*Haemocyte culture in suspension*

Of all tested culture vessels, only Nunc® 24-well Hydrocell™ plates completely prevented adherence of *P. vannamei* haemocytes to the culture surface. This was checked by microscopic observations and was further verified by the complete recovery of cells after washing the well. Although haemocytes did not adhere to the culture substrate, they did adhere to each other, forming cell clusters. All the other culture vessels were ineffective in completely preventing cell attachment.

The live-cell imaging videos revealed that small haemocyte clusters were formed in the beginning of the experiment and further increased in size by recruitment of new cells (Video 2). This recruitment was done by the projection of pseudopod-like structures, pulling inside passing cells. The fusion of small clusters was observed at later time points.

Figure 4 presents the number of living individual (A) cells and clusters per well (B), the average diameter of clusters and the percentage of living cells inside clusters, both for HM and GSH supplemented HM. The number of living individual cells and clusters decreased over time while the average diameter of the clusters increased. This demonstrated the fusion of clusters over time. The number of living individual cells at 0hps was 46,691±7,731 and 50,721±4,749 cell well$^{-1}$ for HM and HM+GSH,

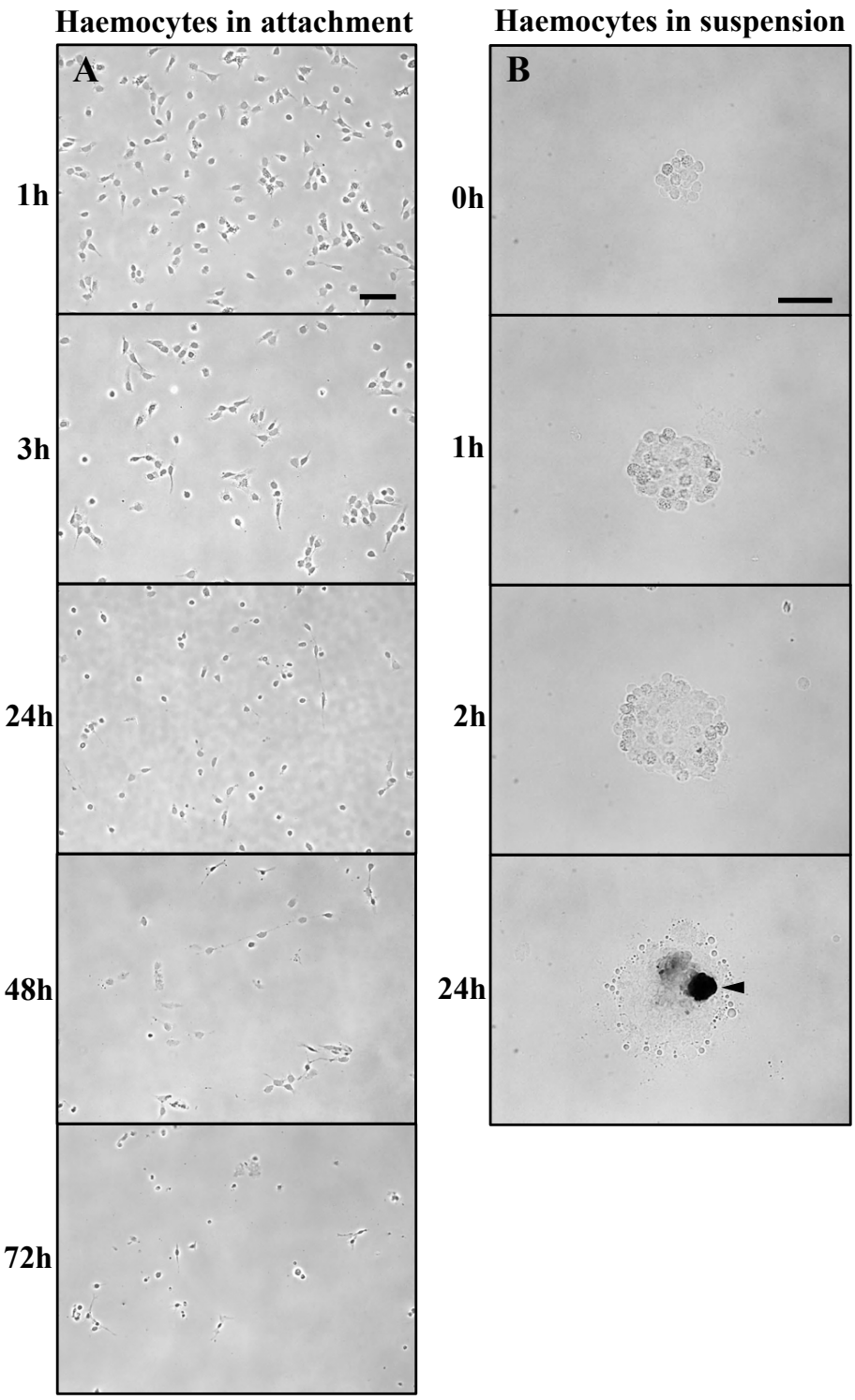



**Figure 2.** *P. vannamei* haemocyte cultures in attachment (A) and in suspension (B) at different time points after seeding. The cultures in attachment (conventional plates with glass cover slips) displayed attachment and cell spreading/stretching from 1hps, followed by detachment and finally cellular degeneration at later time points. The cultures in suspension (Hydrocell plates) showed haemocyte clusters with increasing diameter in time. By 24 hours, the intercellular separations of clustered cells have disappeared (indicating cell fusion) and melanisation of the cell mass could be observed (arrowhead). Scale bars = 50μm.

respectively. This number decreased similarly until 24hps for both treatments. At 48hps, the difference between treatments was more accentuated. In HM the number of living individual cells was 2,979±1,729 cells well$^{-1}$ while HM+GSH was 12,267±1,610 cells well$^{-1}$. The number and diameter of clusters was nearly the same in almost all the sampling time points for both treatments. As from 24hps, the survival of cells inside clusters was higher in GSH medium than in the HM. Experiments were terminated when no individual cells remained and/or clusters showed heavy melanisation (Figure 2 B). The termination time was at 48hps for the HM and 72hps for GSH medium. The comparison between the HM and GSH supplemented medium only showed statistical differences ($p$<0.05) in the survival curves of cells inside the clusters. The cluster melanisation process culminated at 48 hps in the HM and at 72 hps in GSH supplemented medium. Moreover, at 24 hps the clusters in GSH showed a less tight structure. In general, the life span of cultures of haemocytes in suspension was increased 24 hours by the addition of GSH to the culture medium. The inclusion of protease inhibitors in the medium did not improve any of the evaluated parameters.

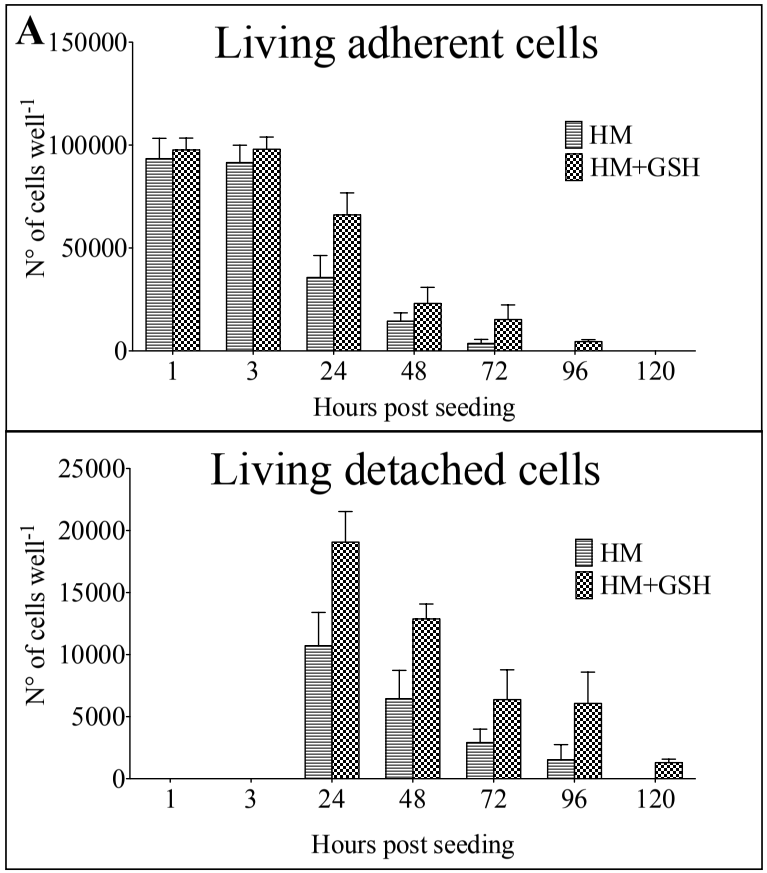
*Validation of the culture systems*

Bacteria inactivation by haemocytes

Starting with a similar concentration of bacteria (control: 23,613±3,184 CFU ml$^{-1}$; treatment: 25,729±4,879 CFU ml$^{-1}$), at 1hpi the number of bacteria in wells containing haemocytes (50,867±22,762 CFU ml$^{-1}$) was significantly lower ($p$<0.05) than in the ones were no haemocytes were present (113,867±23,832 CFU ml$^{-1}$) (Figure 5).

Percentage of phagocytic haemocytes and phagocytic index

The visualisation of the confocal picture sequence revealed whether the bacteria were localised inside or outside the cell, depending on its position relatively to the three-dimensional cell structure. The percentage of phagocytic haemocytes at 1 hpi was 11.5±0.14% and the phagocytic index was 2.4±0.1 bacteria per haemocyte. Confocal microscopy was effective for discriminating between internalized and non-internalised bacteria (Figure 6).



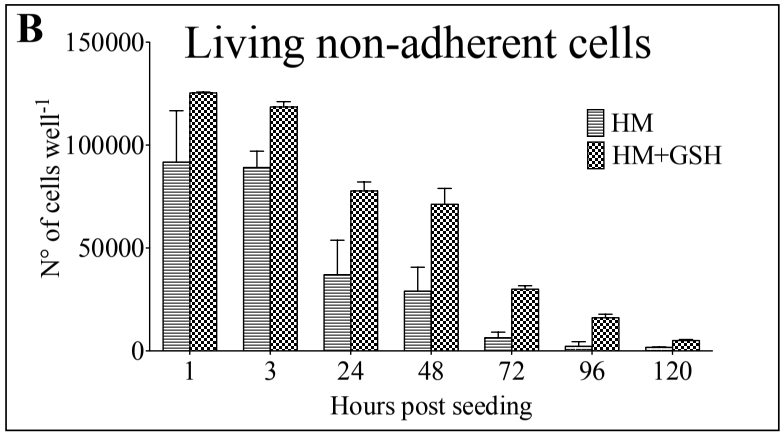




**Figure 3.** *P. vannamei* haemocytes cultured in attachment. The number of living cells cultured in haemocyte medium (HM) or L-glutathione supplemented HM (HM+GSH) is presented for the whole culture period. The values given are means ± standard deviations, *n*=3. Haemocytes were categorised in two cell fractions: adherent (A) and non-adherent (B). The adherent cell fraction was further divided in cells that remained attached to the glass cover slip and cells that detached over time.

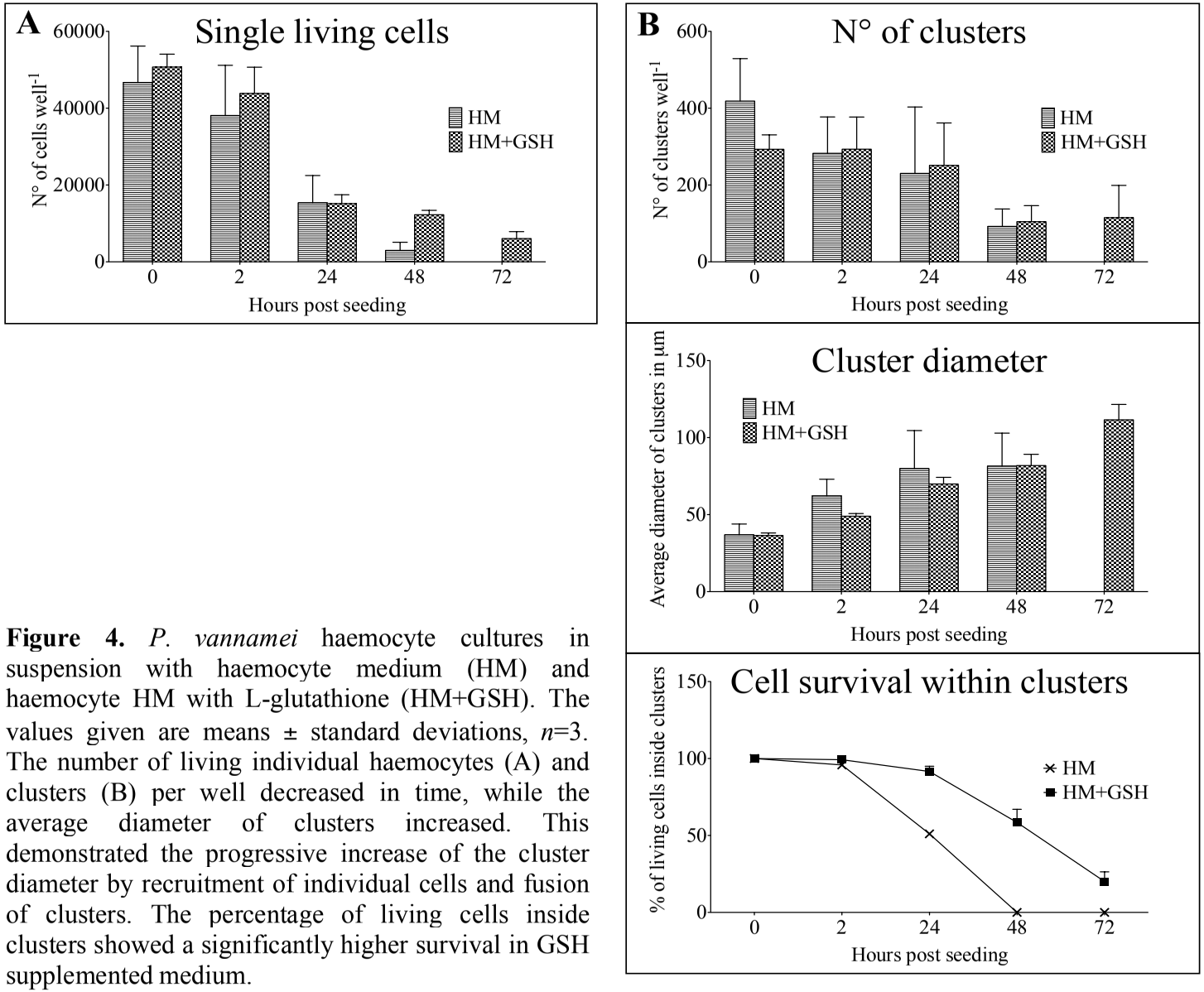



**Figure 4.** *P. vannamei* haemocyte cultures in suspension with haemocyte medium (HM) and haemocyte HM with L-glutathione (HM+GSH). The values given are means ± standard deviations, $n$=3. The number of living individual haemocytes (A) and clusters (B) per well decreased in time, while the average diameter of clusters increased. This demonstrated the progressive increase of the cluster diameter by recruitment of individual cells and fusion of clusters. The percentage of living cells inside clusters showed a significantly higher survival in GSH supplemented medium.

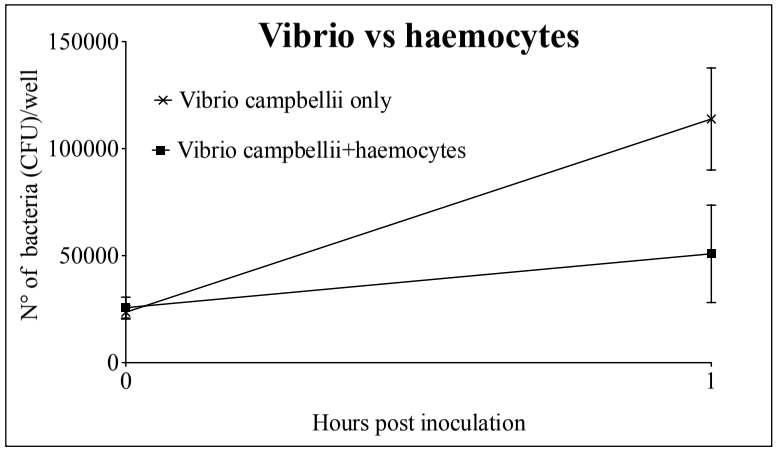



**Figure 5.** Inactivation of *V. campbelii* by *P. vannamei* haemocytes cultured in attachment. The values given are means ± standard deviations, *n*=3.

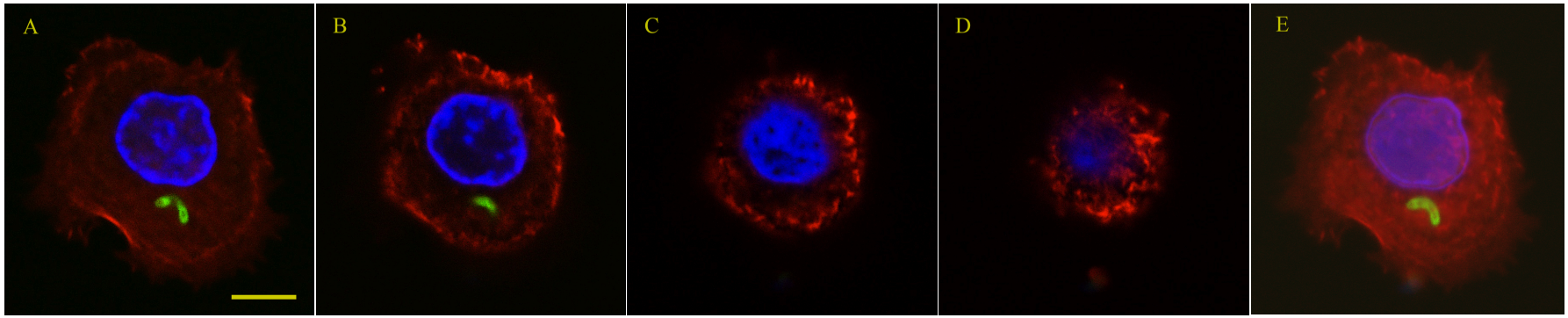
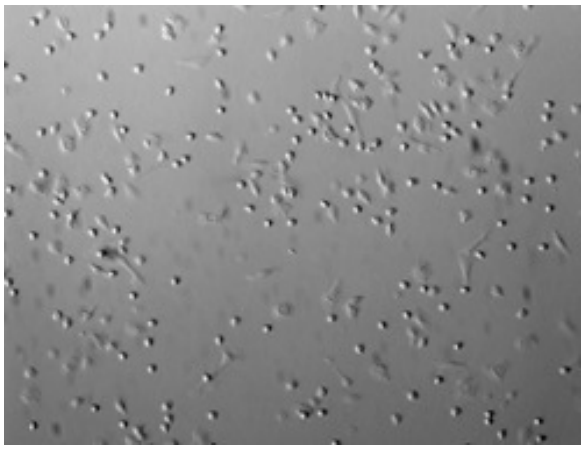
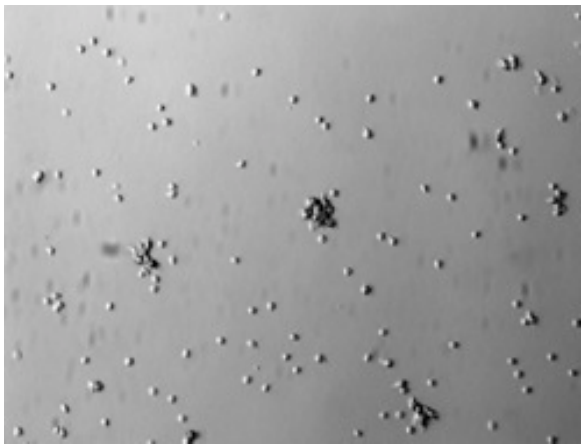



**Figure 6.** Phagocytosis of *V. campbelli* by an adherent *P. vannamei* haemocyte. The images are a sequence of confocal microscopic pictures taken from the cell base (A) to its apex (D). F-actin fibres are stained with phalloidin-Texas Red (red), the nucleus is stained with Hoechst (blue) and GFP-labelled *V. campbellii* exhibit green fluorescence. (E) overlay of the confocal microscopic pictures. Scale bar = 5µm.



**Video 1.** Live-cell imaging of haemocytes in attachment. For visualization consult the online paper (http://www.sciencedirect.com/science/article/pii/S0044848612005133)



**Video 2**. Live-cell imaging of haemocytes in suspension. For visualization consult the online paper (http://www.sciencedirect.com/science/article/pii/S0044848612005133)

**Discussion**

Under normal circumstances, haemocytes are transported as single cells through the shrimp's circulation system. In case of infection, part of them adheres to the walls of the haemal spaces and infiltrate in tissues. Here, they accomplish immune reactions such as phagocytosis, clotting and encapsulation/nodulation of pathogens (Martin *et al.*, 1993; Supamattaya *et al.*, 2003; Cerenius and Söderhäll, 2004). In order to investigate these cell-mediated immune responses *in vitro*, representative culture systems for the *in vivo* situation are needed. The main objective of our work was precisely to develop these systems in a reproducible way. Our system of cells in attachment can be considered as a representation of the morphological differentiation

of circulating haemocytes into adhering and infiltrating haemocytes. This resembles well the *in vitro* differentiation of mammal peripheral blood leucocytes leaving the circulation and infiltrating the surrounding tissues. The system in suspension is a system to represent haemocytes in circulation. To the best of our knowledge, this is the first report where a system to culture shrimp haemocytes in suspension was developed and analysed. Here, the attachment of cells to the culture vessel was totally prevented. This makes the system useful for studying cell-to-cell and cell-to-pathogen interactions in suspension, process of capsule/nodule formation in shrimp and trapping in nodules and inactivation of pathogens by "circulating haemocytes".

Although it was previously reported that penaeid haemocyte cultures in attachment could only be kept alive for 4 days (Chen and Wang, 1999a), other reports describe culture periods of 3-4 weeks (Ellender *et al.*, 1992; Jiang *et al.*, 2006). Unfortunately, the two latter works did not include any specific survival evaluation. Other works with non-penaeid marine decapods evaluated the survival of isolated haemocyte subpopulations. In these trials, separated cultures of hyalinocytes (Walton and Smith, 1999) and hyalinocytes and semigranulocytes were viable for approximately two weeks. Cultures degraded rapidly in the presence of granulocytes (Li and Shields, 2007). Given this, the short life span found in our and other previous works might be related with interactions between different cell types translated into induced or self-induced immune related killing mechanisms. In insects, the cooperation between different haemocyte subpopulations in the accomplishment of immune processes was previously reported (Anggraeni and Ratcliffe, 1991; Pech and Strand, 1996; Tojo *et al.*, 2000; Lavine and Strand, 2001). However, these interactions are not restricted only to positive cooperation but also apply to deleterious actions as for example the induction of apoptosis in other immune cells (Pech and Strand, 2000). This might explain why certain haemocyte subpopulations can survive longer when cultured separately. Because we used a minimal medium, it could be argued that the cell mortality was induced by nutritional deficiencies. However, we considered that improbable because after 24 hours of culture we already observed some haemocytic degeneration and that was probably too fast to be induced by nutritional stress (Bruno *et al.*, 2007). Paradoxically, the relatively fast degradation of our haemocyte cultures might demonstrate the good physiological state of the haemocytes since they reacted as expected to the strange environment they were exposed to. This reinforces the usefulness of our two new systems for studies of shrimp immunity *in vitro*. The

addition of GSH to the culture medium clearly improved the performance of the cells *in vitro*. This could be observed by the prolonged cell survival and the delay of the clustering processes and melanisation of clusters. It is known that extensive cell damage can be caused by ROS produced by immune cells (respiratory burst), when not properly regulated by enzymes and antioxidant systems (Smith *et al.*, 2010). Since GSH is a powerful antioxidant which is known to offset the effect of ROS (reviewed by Meister and Anderson, 1983), its addition to the culture medium might explain the significant increase in survival we observed. Also, the delay of the clustering activity and melanisation demonstrated that GSH could counteract the proPO cascade and consequently minimize its toxic effects on the cells. Furthermore, the less tight appearance of the GSH-treated clusters, suggested that GSH could interfere with cell-adhesion activity.

Knowledge on shrimp haemocyte clustering activity is lacking. Since invertebrates share an innate immune system where haemocytes are the major players, we used literature on insects (and other invertebrates) to discuss our findings. For capsule formation, haemocytes have to switch to an adherent state. They have to recognise and adhere to pathogens, abiotic foreign targets or other activated haemocytes at the surface of the capsule. In the case of abiotic targets, this phenomenon is dependent on the charge and wettability of the target surface and presence of certain surface functional groups as well (Lackie, 1983; Lackie, 1986; Lavine and Strand, 2001; Vargas-Albores *et al.*, 2005; Nardi *et al.*, 2006). Although in our research all procedures were done under aseptic conditions, where no pathogens or inert materials (beads, fibres, etc.) were presented to the haemocytes, there were diverse abiotic surfaces with which haemocytes made contact during the procedure (needles, syringes, Eppendorfs, pipette tips, culture plates, etc.). It is then logical to assume that our haemocytes became activated by those materials and consequently started to attach to each other and formed nodule-like structures. In a work with oyster, Auffret and Oubella (1997) also reported the spontaneous aggregation of haemocytes *in vitro* without the addition of any foreign material and this was used as an index for immune competence. Lackie (1983) characterised the *in vitro* attachment of insect haemocytes to polystyrene culture plates (depending on the surface charge and wettability) and used it as reference for the power of haemocytes to encapsulate polystyrene beads *in vivo*. The conclusion was that for the same substratum, the *in vitro* haemocyte attachment capacity was closely related to the thickness of haemocyte capsules formed

around the beads *in vivo*. This strongly suggests that haemocytes can express encapsulation behaviour *in vitro*. Given this, we hypothesised that the clustering reaction found in our study is similar to a typical *in vivo* encapsulation response. In agreement with previous data on haemocyte capsule formation (Persson *et al.*, 1987; Nappi *et al.*, 1995; Schmidt *et al.*, 2001), our cell clusters also melanised and died. Further, it was reported that the process of nodule formation in *Penaeus monodon* takes between 24 and 48 hours *in vivo* (Supamattaya *et al.*, 2003). Recently, a report on crayfish haematopoiesis described the *in vitro* clustering of one type of haematopoietic stem cell (Noonin *et al.*, 2012). These cells formed densely packed clusters after 3-5 days of culture and the process was prolonged until 12-14 days. Although this system seems promising for studying the process of haemocyte clustering, unfortunately the comparison with the present work was difficult since we have used mixed subpopulations of mature haemocytes. In order to verify if haemocytes did get activated during the culture procedure, we measured the phenoloxidase activity in the supernatant of the cultures. Although the activity measured was low, it was still detectable and increased over the culture period (data not shown). This proved some degree of haemocytic activation. Also, to consider other activation factors (not only activation by abiotic targets), we analysed the level of bacterial endotoxins present in the culture medium. As expected, the medium was tested positive for endotoxins, although it was within the acceptable levels for cell culture ($0.125<X<1.250$ EU $ml^{-1}$). From this data, we can hypothesize that low level of bacterial endotoxins might have contributed to the activation of haemocytes and consequently had an influence on the survival. In an attempt to prevent the activation and adhesion of haemocytes, we performed a trial where an EDTA-free cocktail of protease inhibitors (including serine proteases) was included in the culture medium. It is known from literature that the proPO cascade initiates the activation of many immune processes and that serine proteases are involved in its activation. It has been demonstrated in previous works that activation of the proPO activating system and cell adhesion could be inhibited by endogenous/exogenous protease inhibitors, (Johansson and Söderhäll, 1988; Aspan *et al.*, 1990; Söderhäll and Cerenius, 1998). Nevertheless, in our experiment this supplementation did not influence cell survival, clustering activity and cluster melanisation. In this situation, either the activation of haemocytes occurring during the collection and isolation could not be stopped, or the inhibitors were somehow not available for blocking the process. This was probably one of the factors that limited the

survival of haemocytes. Therefore, work regarding inhibition of cell activation is necessary. This should include the testing of chemical inhibitors and different types of materials and coatings used for haemocyte isolation and culture.

Another interesting finding in this work was the difference in attachment behaviour of haemocytes. This difference provides a simple but interesting method for isolation of morphological/functionally different haemocyte subpopulations. In mammals, a similar attachment procedure makes it possible to isolate monocytes from the peripheral blood mononuclear cells (Nauwynck and Pensaert, 1994). Besides the diminished attaching capability of our non-adherent cells, they had a very limited clustering capability and a more extended life span when supplied with GSH. We also found remarkable differences in their morphology when compared with adherent haemocytes. Its morphology and adherence behaviour suggested that they are prohaemocytes or immature haemocytes (Roulston and Smith, 2011; Noonin *et al.*, 2012). However, these cells represented around 50% of the haemocytes, which is in disagreement with 10% previously described by Roulston and Smith (2011). Moreover, it is unlikely that healthy animals would have such an amount of immature cells in their blood.

The validation experiments clearly showed that our system to culture haemocytes in attachment can support functionally active haemocytes. This was proven by the phagocytic and the significant antibacterial activities of attached haemocytes. The phagocytic activity was comparable to previously reported data (Mori and Stewart, 2006; Pope *et al.*, 2011). This validation can be extrapolated to the culture in suspension since the origin of the cells, medium and physical conditions were the same as for the attachment system. These systems are, thus, potentially useful for screening and studying immune molecules produced by haemocytes, cell-to-cell communication and many other immune-related mechanisms. Practically, these systems can be very useful for screening immunostimulants by measuring phagocytic parameters, pathogen inactivation and clustering activity of haemocytes. We believe that the methodologies described in the present work have advantages over the existing ones, mainly due to its controlled execution, detailed description and extensive analysis of the results. This originated a reproducible evaluation of different parameters. Moreover, these cultures bring up for the first time the possibility of using haemocytes of the same animal under two different *in vitro* conditions, in suspension and in attachment.

**Acknowledgements**

The first author was financially supported by a scholarship from the Foundation for Science and Technology (FCT, Portugal), and the second author by a scholarship from the Institute for the Promotion of the Innovation through Science and Technology in Flanders (IWT-Vlaanderen, Belgium).

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# 3.2 Haemocyte culture systems for the freshwater prawn *Macrobrachium rosenbergii*

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*Submitted to Aquaculture Research*

**Abstract**

As for other crustacean aquaculture species, *M. rosenbergii* production faces disease-related problems. In order to control them, it is necessary to obtain a better knowledge on its immunity. *In vitro* haemocyte culture techniques are essential tools for achieving this objective.

The present work established two *M. rosenbergii* haemocyte culture systems, one with cells cultured in attachment and another with cells cultured in suspension. In the cultures in attachment, cells were seeded into conventional Nunc® Nunclon™Δ Surface cell culture plates with glass cover slips. In the cultures in suspension, the cells were seeded into Nunc® Hydrocell Surface cell culture plates. The culture medium was based on L-15 (Leibovitz) and supplemented with Chen's salts (5%), foetal calf serum (10%) and 100units $ml^{-1}$ penicillin/100µg $ml^{-1}$ streptomycin (pH 7.5, 490mOsmol $kg^{-1}$). Parameters such as number of living attached and suspended single cells, number and average diameter of cell clusters and survival of cells inside clusters, were evaluated. In the cultures in attachment ($3x10^5$ cells seeded per well), 41% of the cells attached to the glass after one hour of incubation at 27°C. The total number of living cells at 3, 24, 48 and 72 hours after seeding was 58,987±8,647, 32,490±4,687, 12,364±2,426 and 9,232±1,029, respectively. In the cultures in suspension ($3x10^5$ cells seeded per well), the number of individual living cells at 2, 24 and 48 hours after seeding was 88,636±15,760, 32,753±17,521 and 6,478±3,110, respectively. The total number of haemocyte clusters at 2, 24 and 48 hours after seeding was 382±132 (45±2 µm cluster diameter; 100% survival), 419±153 (68±13 µm diameter; 87±8% survival) and 209±145 (101±3 µm diameter; 35±12% survival), respectively.

It was concluded that these systems could keep haemocytes alive up to 48 hours after seeding and that the evaluation of culture parameters was reproducible. Therefore, these techniques can be potentially used to study *M. rosenbergii* immunity *in vitro*.

## Introduction

*Macrobrachium rosenbergii* is an economically important freshwater prawn species (New and Nair, 2012). As for other aquaculture species, *M. rosenbergii* production faces disease threats. Some diseases are difficult to control what represents an impediment to the sustainability of its industry (Devika Pillai *et al.*, 2009; Hameed, 2009; New, 2009). In order to control these pathologies, a better knowledge of crustacean immunity and especially that of *M. rosenbergii* is necessary. The successful accomplishment of this task unavoidably implies the need of an *in vitro* culture technique for haemocytes. Reports on *M. rosenbergii* haemocyte culture and characterization have already been published (Vázquez *et al.*, 1997; Gargioni and Barracco, 1998; Sung *et al.*, 2000; Sierra *et al.*, 2001; Hsu *et al.*, 2005; Raman *et al.*, 2008; Du *et al.*, 2012). In general, they kept haemocytes *in vitro* for a very short time and the methodologies were mostly not described in a detailed way. Therefore, they are difficult to reproduce.

Recently, well-characterised haemocyte culture systems were developed for *Penaeus vannamei* (Dantas-Lima *et al.*, 2012). This report enclosed two haemocyte culture systems: one for culturing haemocytes in attachment and another for culturing them in suspension. The methodology was extensively described and the results were easy to reproduce. The current work aimed to adapt this technology to haemocytes of *M. rosenbergii.* In a long term, the established haemocyte *in vitro* culture systems will allow to get better insights in the immunity of *M. rosenbergii* against pathogens.

## Materials and methods

### *Experimental animals*

Adult *M. rosenbergii* were selected at the inter-molt stage (C stage) (Corteel *et al.*, 2012). Prawns were obtained from a long lasting in-house domestication program performed at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. After hatching, larvae were fed with *Artemia* nauplii twice daily for 3 weeks and then weaned onto a commercial pelleted feed, which was fed twice daily at a total rate of 5% of their mean body weight. The water temperature was maintained at 27±1ºC, pH 7.5, carbonate hardness 25º and total hardness 17º. A recirculation system and regular water changes kept the total ammonia at less than 0.5mg $l^{-1}$ and nitrite at less than 0.15mg $l^{-1}$. The room was illuminated 12 hours a day by dimmed light. The mean body weight of

shrimp used in this study was 50±10g.

*Haemocyte medium and anticoagulant*

The osmolality of the haemolymph and the body cavity pH of 10 *M. rosenbergii* were determined (489±14mOsmol $kg^{-1}$, pH 7.5±0.1). L-15 medium (Leibovitz; Sigma-Aldrich, USA) was prepared at single strength (1×L-15) and used as basal medium. The haemocyte medium (HM) was composed of L-15, Chen's salts (CS) (Chen and Wang, 1999), foetal calf serum (FCS) and penicillin/streptomycin (P/S). The formulation was: 1×L-15, 5% CS, 10% FCS and 100units $ml^{-1}$ penicillin/100µg $ml^{-1}$ streptomycin (pH 7.5, 490mOsmol $kg^{-1}$). The medium was filtered through a 0.20µm membrane before use.
An anticoagulant buffer previously developed for marine crabs (Söderhäll and Smith, 1983) was adapted to the parameters required by *M. rosenbergii* and named Freshwater Anticoagulant. The formulation was the following: sodium chloride (187mM), glucose (100mM), tri-sodium citrate (30mM), citric acid (26mM) and EDTA (10mM), (pH 5.4, 630mOsmol $kg^{-1}$).

*Haemolymph collection and haemocyte isolation*

Haemolymph was taken from the anterior part of the ventral sinus (second abdominal segment) using a 2ml syringe with a 20G hypodermic needle. Syringes were pre-filled with ice-cold anticoagulant solution (1:1 with haemolymph). After collection, haemolymph was centrifuged at 250 *g* during 5 minutes at 4ºC. Supernatant was discarded and the pellet was immediately resuspended in ice cold HM. Cell concentration was evaluated using a Bürker-Türk counting chamber.

*Haemocyte culture systems and experimental setup*

Culture in attachment: Freshly collected and quantified haemocyte suspensions were seeded into conventional Nunc® 24-well cell culture plates. The wells were supplied with round glass coverslips. Each well was seeded with $3\times10^5$ cells in a total volume of 400 µl. Plates were incubated for 1 h at 27ºC to allow the cell to attach to the substratum. Subsequently, the culture medium was discarded and wells were washed once with HM at 27ºC. The wells were finally filled with 400µl of HM. These plates were used for the determination of attachment rate, number and survival of haemocytes over time. For survival evaluation, samples were collected at 1, 3, 24, 48, 72 hours after seeding. The

plates were kept at 27ºC during the whole experimental period. Samples were stained with ethidium bromide monoazide (EMA) and Hoechst for survival evaluation (Costers *et al.*, 2006). The total number (Hoechst positive) and the number of dead cells (EMA positive) were evaluated. The survival was expressed as number of living cells per well over time. Culture in suspension: Freshly collected and quantified haemocyte suspensions were seeded into Nunc® 24-well Hydrocell™ cell culture plates. The number of cells seeded per well was $3\times10^5$ in 400 µl of HM. Plates were kept at 27ºC during the whole experimental period. Samples were collected at 0, 2, 24 and 48 hour after seeding and transferred to new pre-chilled Hydrocell plates. Cells were immediately stained with EMA and Hoechst for survival evaluation. The total number (Hoechst positive) and the number of dead individual cells (EMA positive) over time were determined. Additionally, the number of haemocyte clusters, their diameter and viability of the cells were also quantified.

*Survival staining*

Haemocyte culture in attachment: Glass cover slips with adherent cells were transferred to new, pre-chilled wells and immediately stained for survival evaluation with ethidium bromide monoazide at a concentration of 0.02 mg $ml^{-1}$ (Sigma-Aldrich, USA) and incubated in the dark and on ice for 30 minutes. Afterwards, cells were stained with Hoechst at a concentration of 0.01 mg $ml^{-1}$ (Invitrogen, Life Technologies, USA) and exposed to incandescent light for 10 minutes on ice. In between the staining steps, cells were washed with ice cold HM. After staining, cells were fixed for 10 minutes at room temperature with paraformaldehyde (PF) 2%, washed with phosphate buffer saline and finally mounted on glass slides with anti-fading mounting medium (Glycerine-DABCO). Haemocyte culture in suspension: For sampling, cell suspensions were pipetted gently up and down six times throughout the well and transferred to pre-chilled Hydrocell wells. This procedure was repeated twice and cells were immediately stained and fixed as described above. The washing steps were done by centrifugation at 250 *g* for 10 minutes and after pipette up and down in the appropriated solution. After staining, cells were resuspended in a volume of 10µl, mounted on a glass slide with 2µl of glycerine-DABCO and covered with a square glass cover slip (24x24mm).

*Live-cell imaging*

Live-cell imaging videos of cultures in attachment and in suspension were recorded with an automatic live cell imaging system (Olympus IX81, USA). Micrographs of 5 predetermined positions in the wells were taken every 2 min for 2 h at 27°C.

**Results**

*Culture in attachment*

The live-cell imaging video 1 shows differences in the attachment capability and movement of cells. There were cells that attached strongly to the substrate with a high degree of stretching. These cells had a continuous motion by projection of pseudopodia. There were also weakly attached fusiform cells that expressed different movement patterns, mainly by means of Brownian motion.

The observation of the cultures by stereo light microscope confirmed previous observations made by live-cell imaging. One hour after seeding, although there were cells that visibly spread and strongly attached, some were weakly attached. These cells presented spindle or fusiform shape. A majority of these cells was lost after washing the wells (Figure 1 A). At this time point, the cell attachment rate was 40,6±8,3%. Figure 2 A presents an overview of the culture's morphology at every sampling point. Figure 3 A shows the evolution in terms of attached living cells present in a well over time. At one hour after seeding, the number of cells was 57,839±8,353 and it was nearly the same (58,987±8,647) at 3 hours. At 24, 48 and 72 hours, the number decreased to 32,490±4,687, 12,364±2,426 and 9,232±1,029, respectively.

*Culture in suspension*

Video 2 illustrates the behaviour of *M. rosenbergii* haemocytes cultured in suspension. Small clusters were formed during the first 2 hours of the experiment but most of the cells remained at an individual state.

Figure 3 B represents the evolution of the number of individual living cells, number of clusters, average diameter of clusters and survival of cells within the clusters over time. At the time of seeding, the cells were mainly at an individual state (90,401±16,359) but a part was already immobilized in small clusters (272±151 clusters with average diameter of 36±2 μm). After 2 hours, the number of individual cells slightly decreased

(88,636±15,760) while the number of clusters (382±132) and its average diameter (45±2 µm) increased. At 24 hours, the number of individual cells was reduced to nearly 1/3 (32,753±17,521) and consequently the number (419±153) and diameter (68±13 µm) of clusters increased. At the end of the experiment (48 hours) the number of individual cells was less than 10% of the initial number (6,478±3,110). By this time, the number of clusters also decreased (209±145) but its diameter increased (101±3 µm). The survival of cells within clusters was kept near to 100% until 2 hours after seeding. At 24 and 48 hours the survival was 87±8% and 35±12%, respectively. Clusters displayed melanisation at 48 hours. The average diameter of individual haemocytes was 9.7±2µm.

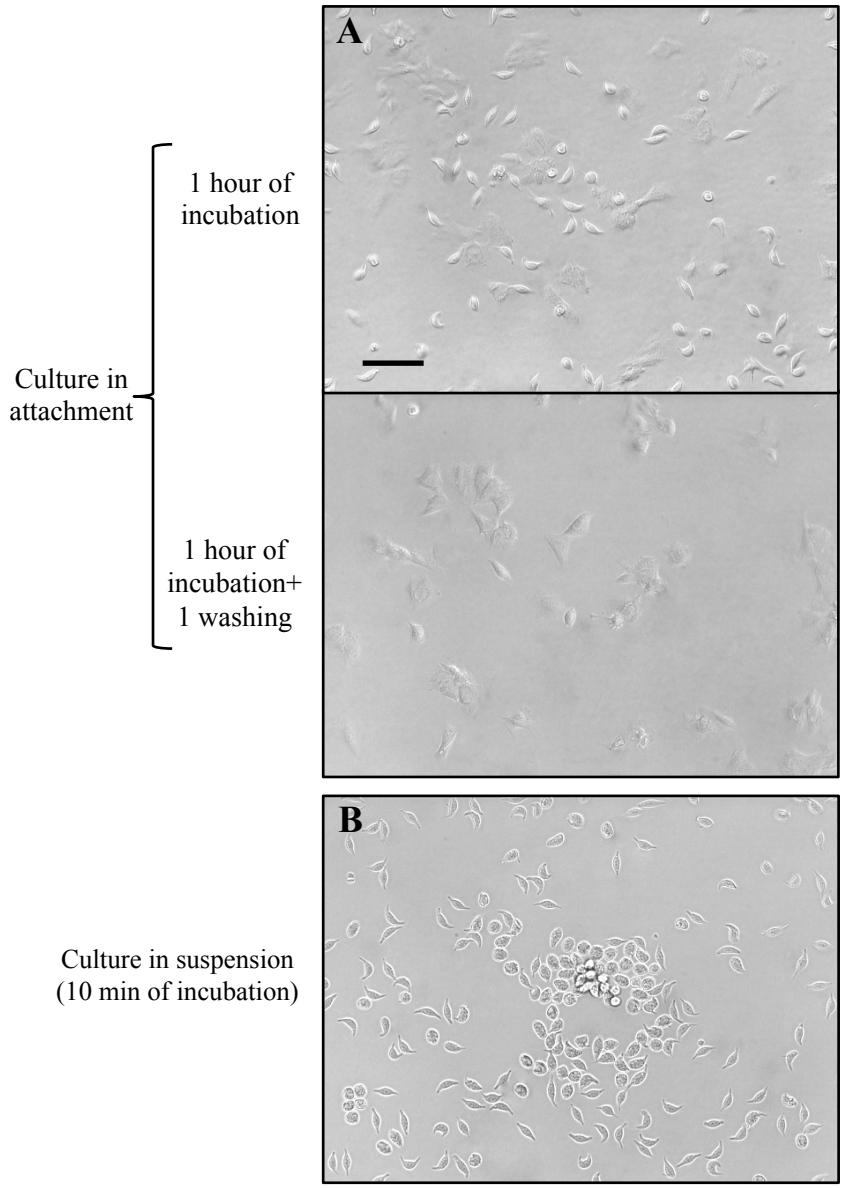



**Figure 1.** *M. rosenbergii* haemocyte cultures in attachment (A) and in suspension (B). The culture in attachment shows attached and non-attached cells before washing and only attached cells after washing. The culture in suspension shows the start of the formation of a haemocyte cluster. Scale bar=50µm.

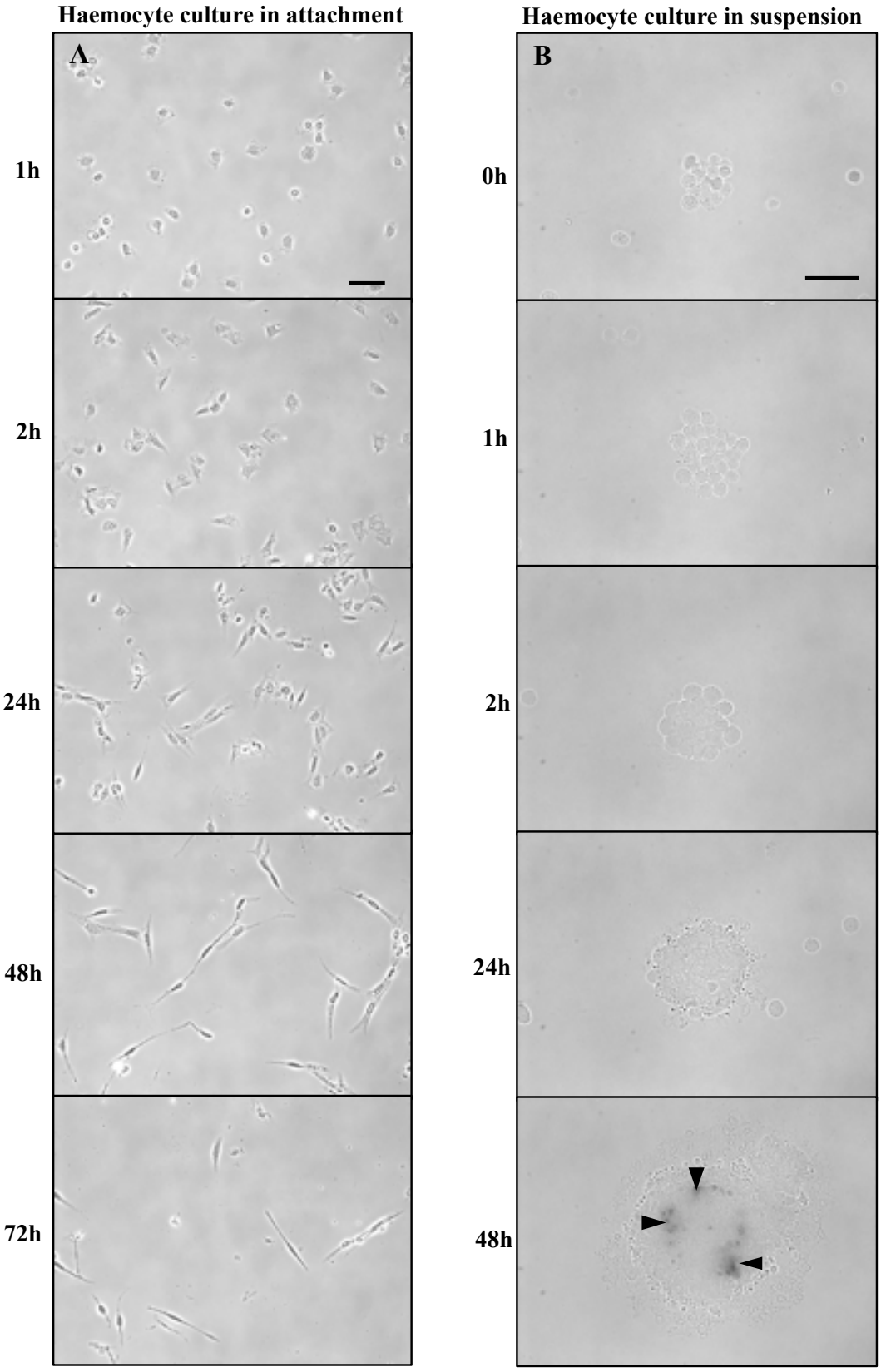



**Figure 2.** Overview of the morphological changes of haemocytes in attachment and in suspension. The culture in attachment shows the progression of cell stretching. The culture in suspension shows the formation of clusters and an increase of the cluster size by recruitment of new cells. At 24 h the cells fused into a cell mass. At 48 h, the cluster presented melanisation (arrowheads). Scale=50µm.

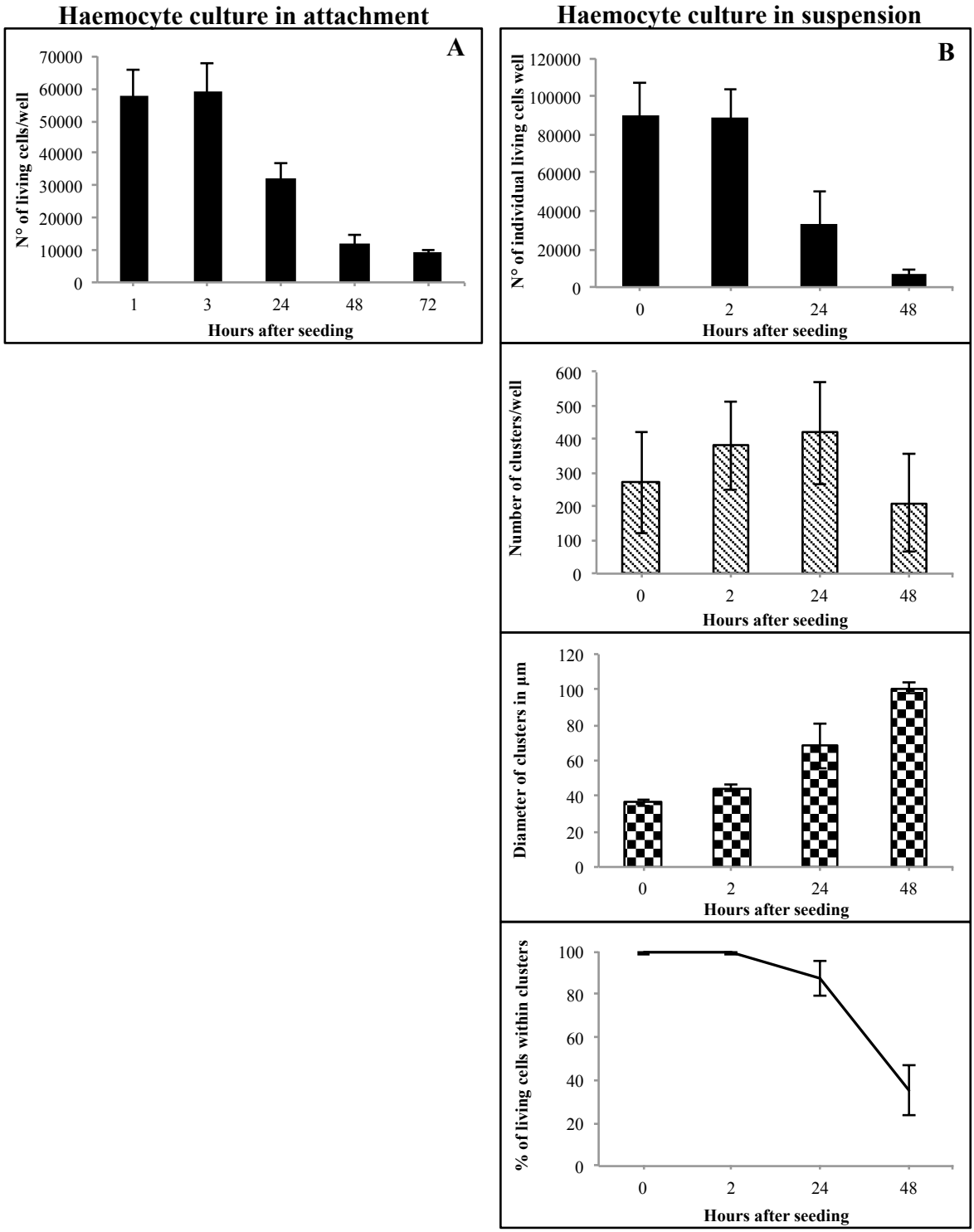
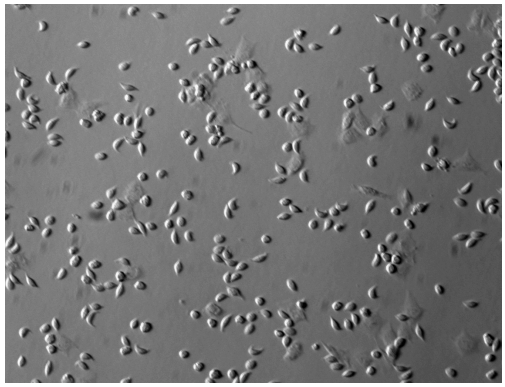



**Figure 3.** Overview of the parameters evaluated for haemocyte cultures in attachment and in suspension over time.



**Video 1.** Live-cell imaging of *M. rosenbergii* haemocytes in attachment. For visualisation consult the electronic version of the article.

**Video 2.** Live-cell imaging of *M. rosenbergii* haemocytes in suspension. For visualisation consult the electronic version of the article.

**Discussion**

*M. rosenbergii* is a freshwater prawn species with high economical importance in several countries worldwide. As a consequence of the on-going industrialization of prawn production, disease-related problems are striking, causing economical losses (Devika Pillai *et al.*, 2009). Together with obvious farm management measures, a better knowledge on *M. rosenbergii* immunity is a prerequisite for controlling and overcoming these problems.

Recently, Dantas-Lima *et al*. (2012) developed systems for culturing *P. vannamei* haemocytes *in vitro* and measure their immune reactions. Since we have followed similar protocols for culturing *M. rosenbergii* haemocytes, some degree of comparison between these species was possible. In the cultures in attachment, the number of attached cells after 1 hour of culture in *M. rosenbergii* was lower than in *P. vannamei*, even though the initial number of seeded cells was the same for both species. This was correlated with the

haemocyte attachment rate. While the attachment rate was 41% in *M. rosenbergii*, it was 59% in *P. vannamei*. In cultures in suspension, *M. rosenbergii* haemocytes expressed a slower formation of clusters and consequently a higher number of individual cells was found over the whole culture period. Moreover, the melanisation of haemocyte clusters was only visible after 48 hours of culture, contrasting with the 24 hours observed for *P. vannamei*. *M. rosenbergii* haemocytes had longer survival times, both in culture in attachment and in suspension. The survival of cells within clusters was also higher in *M. rosenbergii.* Although we do not know the exact nature of these differences, they are most likely species-dependent and probably related with a lower sensitivity/reactivity of *M. rosenbergii* haemocytes to some component(s) of our culture system. These findings require further investigation.

Du *et al.* (2012) claimed that they have developed the first primary haemocyte culture system for *M. rosenbergii*. This work described that cultures could be kept up to 12 days with significant cell proliferation. Curiously, the paper did not enclose any cell proliferation assay, survival evaluation or cell count over time. Other reports that used *M. rosenbergii* haemocytes used very short-term cultures without testing the viability.

To the best of our knowledge, our work was the first where a primary culture of *M. rosenbergii* haemocytes was performed in a controlled and reproducible way, with an extensive analysis of culture parameters over time. The primary objective of our work, which was to create tools for use in *M. rosenbergii* immunity research, was successfully achieved.

**Acknowledgements**

The first author was financially supported by a scholarship from the Foundation for Science and Technology (FCT, Portugal).

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# Chapter 4

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

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*Submitted to Aquaculture*

**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 minutes at 2,000 *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 while 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 to1.087 g/ml) for 15 minutes at 2,000 *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 characterisation 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.

## Introduction

Haemolymph (blood of invertebrates) is composed of a liquid fraction called plasma and a cellular fraction 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 (Söderhäll and Smith, 1983; van de Braak *et al.*, 1996; Vargas-Albores *et al.*, 2005; Li and Shields, 2007). When stained with histological dyes, hyalinocytes display a spindle/ovoid shape and few small basophilic and eosinophilic granules. Semi-granulocytes have an ovoid shape and contain several eosinophilic granules. Granulocytes have a spherical shape and have many large eosinophilic granules (Smith, 2010; Roulston and Smith, 2011). When exposed to foreign environments or substances, hyalinocytes and semi-granulocytes are known to present strong adherence to the substrate and acute spreading behaviour. Adherence appears to be more limited in granular cells (Vargas-Albores *et al.*, 2005; Roulston and Smith, 2011). 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 differences between different species, differences in analytical methodologies and the somewhat subjective classification of semi-granulocytes.

A protocol to separate crustacean haemocytes using a Percoll density gradient was developed by Söderhäll and Smith (1983), and was later adapted to other invertebrate species (Smith and Söderhäll, 1983; 1991; Pipe *et al.*, 1997; Sritunyalucksana *et al.*, 2001; Hammond and Smith, 2002; Liu *et al.*, 2005; Vargas-Albores *et al.*, 2005; Li and Shields, 2007; Sperstad *et al.*, 2010; Falwell *et al.*, 2011; Roulston and Smith, 2011). 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, only granulocytes were isolated efficiently probably due to the density that is intrinsic to each cell type.

With the aim of improving the separation of the haemocyte populations, we turned our attention to 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 possesses some advantages over Percoll. 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), making 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 cytometrical and spectrophotometrical analysis. Iodixanol on the other hand, only exhibits absorbance in the UV range (Jacobsen, 2000). Another advantage of iodixanol is the formation of linear continuous gradients by allowing passive diffusion of pre-formed discontinuous gradients. This excludes the need of ultracentrifugation as is the case of Percoll self-forming gradients. The shape of iodixanol continuous gradients can be customized by manipulating the concentration and volume of the initial gradient fractions and diffusion times (Axis-Shield, 2012). On the other hand, self-forming Percoll gradients present non-linear, S-shaped gradients with two steep density profiles on the top and on the bottom of the gradient and a shallow zone in between (Amersham Biosciences, 2001). These gradients have limited manipulation possibilities. Furthermore, it has also been reported that cells can become damaged during centrifugation in Percoll (Oliveira *et al.*, 2011; Juan *et al.*, 2012), while no such reports on iodixanol were 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.

## Materials and methods

### *Shrimp and experimental conditions*

*Penaeus* (*Litopenaeus*) *vannamei* post-larvae which were certified to be specific pathogen-free for the viruses 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 & Artemia Reference Center,

Ghent University, Belgium until they reached an adult size (±40g). 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 at <0.5 mg/L and nitrite at <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.

*Iodixanol density gradients preparation*

Two discontinuous density gradients composed of 2.5 ml iodixanol (Optiprep, Axis-Shield, UK) fractions of different concentrations were prepared by fraction underlayering (Figure 1). The fractions were loaded in 15 mL non-pyrogenic polypropylene centrifuge tubes (Sarstedt, Germany) using 2 ml syringes and 20G (0.9x70mm) needles. The gradient concentration profiles (from the top to the bottom of the tube) were: (1) 10%, 15% and 20% (2) 7%, 10%, 13% and 16%. The iodixanol solutions were prepared by diluting the stock solution (60% iodixanol) in shrimp phosphate buffered saline (shPBS = PBS adjusted to 900 mOsmol/kg with NaCl; pH 7.4). These preparations were kept for 18 h at 4°C to allow the formation of a continuous gradient. Afterwards, the gradients were 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 244nm (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 1:1,000 (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.

*Haemolymph extraction and haemocyte separation*

Haemolymph was extracted with ice-cold marine anticoagulant as described by Dantas-Lima *et al.* (2012). Briefly, the collection was made 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 abdominal 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, Germany) at 2,000 *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 fixation was done in an equal volume of marine fixative (Cima, 2010) at double concentration (2% glutaraldehyde with 2% saccharose in seawater) for 30 minutes at 4°C.

*Morphological characterization*

Light microscopy and live-cell imaging of haemocyte cultures

Haemocyte cultures were observed by inverted microscope (Olympus IX50, USA) 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, the behaviour of the cells was registered by live-cell imaging videos using ImageJ software (Abramoff, 2004).

Flow cytometry

Fixed haemocytes were analysed with a FACSAria III (Beckton Dickinson, USA) using the 488 nm laser. Statistics were obtained by the FACSDiva software (Version 6.1.3, Beckton Dickinson, USA). 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 cells were counted. Results were expressed as contour plot graphs indicating the relative size (FSC) and granularity (SSC) of the cells of each band.

Light microscopy of fixed haemocytes

Fixed haemocytes were cytospined (Shandon Cytospin 3, Thermo Scientific, USA) 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 (Sakura Linear Stainer II, The Netherlands). 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, USA) and pictures were taken. The size and morphological characteristics of the cells were evaluated.

*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% FCS, 1% Peninciline/Streptomycine; pH 7.5; 900 mOsmol/kg). Cells were seeded in 24-well plates (Nunc® Nunclon™ Δ Surface, Thermo Scientific, USA) in which round glass coverslips were previously brought in each well. A volume of 400 µl of cell suspension was seeded in each well. Samples were taken at 0, 1, 3, 6, 12 and 24 h and every 24 h after until the end of each experiment. Cells were stained with ethidium monoazide bromide (EMA) 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 by fluorescence microscopy and expressed as total number of living cells per well over time. Each experiment was repeated 3 times and the average values were calculated.

*Validation of the functionality of separated haemocyte subpopulations*

Production of inactivated GFP-labelled bacterial stocks

GFP-labelled *V. campbellii* (LMG 21363) was obtained as previously described by (Dantas-Lima *et al.*, 2012). Briefly, *V. campbellii* was transfected 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.

Detection of phagocytic activity

GFP-labelled *V. campbellii* were sub-cultured twice in HM containing selective antibiotics (20µl of bacterial suspension in 20ml of HM for 12h and 14h at 27°C). Suspensions were washed as described above. The concentration of bacteria in the suspension was determined by optical density at 600nm (OD600) and by the conversion formula CFU/ml=(10xOD600-1)x$10^8$.

Cultures of separated haemocyte subpopulations (150,000 cells $well^{-1}$) were inoculated with 100 bacteria $haemocyte^{-1}$ at 1 hour after seeding. Samples were taken at 0 and 1 hour after inoculation. Before sampling, wells were washed 2x with HM. At the moment of sampling, cells were fixed with 4% PF for 10 minutes, permeabilised with 0.1% Triton X-100 for 5 minutes and stained with Texas Red-labelled phalloidin (Invitrogen, Life Technologies™) diluted in PBS (4 units $ml^{-1}$) for 1h 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: 461nm; Texas Red: 615nm; GFP: 509nm) were taken from the cell base to its apex. This was made in 10 cells that presented signs of phagocytosis.

**Results**

*Separation of haemocytes in iodixanol density gradients*

After 18h of incubation at 4°C, the iodixanol gradients became nearly linear. The density profile Gradient 1, 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 5A). 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 Gradient 2. This gradient was designed to promote the physical separation of the bands. 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 5B). The preparation containing Gradient 2 loaded with the suspension of Band 1 and 2 was centrifuged at 2,000 *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 degree of cross-contamination. Band 1 was located in-between the marks of 4-4.5 ml and Band 2 in between the marks of 3-3.5ml (Figure 1). The 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 5).

*Morphological characterization*

Light microscopy and live-cell imaging of haemocyte cultures

Figure 2 and live-cell imaging videos provided details of the morphology and behaviour of the haemocytes in culture. Cells from Band 1 adhered very strongly to the glass by means of pseudopod-like projections, which resulted in a high degree of cell spreading. Cells from Band 2 on the other hand, presented a very limited spreading and adherence to the glass. These cells were easily re-suspended by gentle pipetting. Cells from Band 3 adhered strongly to the glass with moderate spreading. The fraction of dispersed cells contained 2 cell types: small and big cells with morphological characteristics similar 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 from the fraction of dispersed cells), Sub 4 (big cells from the fraction of 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,

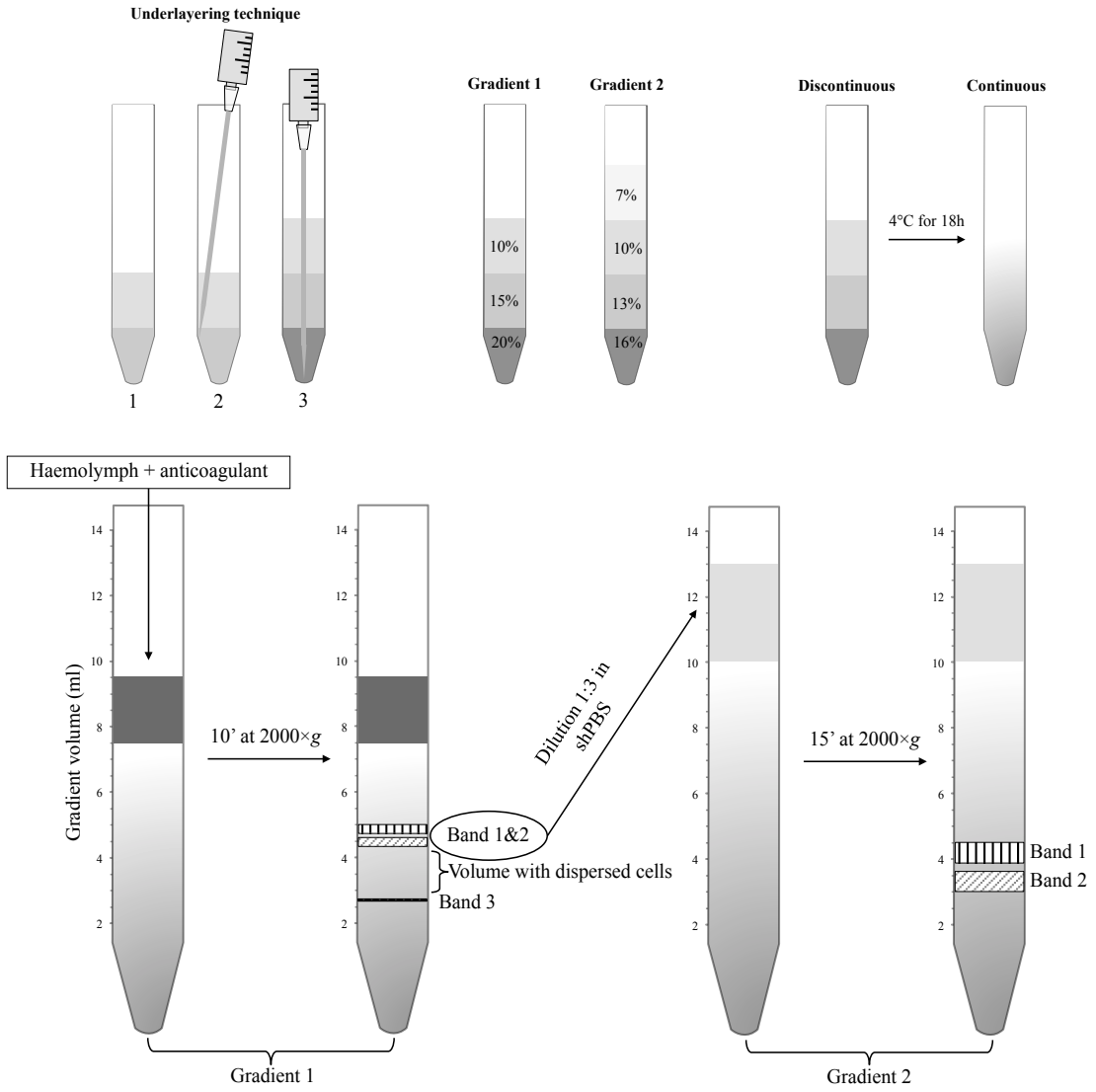


**Figure 1.** Diagram illustrating the preparation of continuous iodixanol density gradients and centrifugation procedure for separation of *P. vannamei* haemocyte subpopulations. The gradients were prepared by underlayering of 2.5 ml iodixanol fractions with different concentrations. An incubation step (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 a narrower density profile (see figure 5A), promoted the physical separation of bands 1 and 2, allowing their individual collection without significant cross-contamination (high purity).

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, cell lysis was observed after 1 h of culture and clustering activity after 24 h of culture. Cells of Sub 4 and 5 started to show signs of degranulation and deterioration (cell fragmentation) after 2 h of culture, which continuously increased up to 24 h.
The live-cell imaging videos revealed differences in cellular motility and morphology and confirmed the purity levels of the subpopulations. Sub 1 cells adhered and spread strongly over the glass and displayed active 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 than the other subpopulations, both by projection of pseudopodia and cytoplasmatic granules displacement.

Flow cytometry

For each subpopulation, the values of forward and side scatter were related with the cell diameter and granularity, respectively. Cells of Sub 1 had a small average diameter (126.4±4.6) and presented the lowest granularity (2.3±0.1) (Figure 2 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. These latter were big cells (166.2±6.0) with the highest granularity (10.6±2.9).

Light microscopy of fixed haemocytes

The H&E staining of fixed cell cytospins provided morphological details of separated haemocytes (Figure 2). The average cell diameter was the smallest in Sub 2 (7.5±1.3 μm) followed by Sub 3 (7.8±0.9 μm), Sub 1 (8.6±0.8 μm), Sub 5 (9.9±1.0 μm) and finally Sub 4 (10.5±1.5 μm). The nucleus: cytoplasm ratio was high in Sub 1 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 summary of the parameters described above.

*Survival evaluation*

The survival evaluation for each haemocyte subpopulation is presented in Figure 3. The cells of Sub 2 showed the best survival performance, followed by the cells of Sub 1, Sub 3+4 and finally Sub 5. Living cells were detected up to 120 hours in Sub 1 and Sub 2, and up to 24 hours in Sub 3+4 and Sub 5. In the first 12 hours of culture, all the subpopulations presented viability over 50%.

*Detection of phagocytic activity*

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

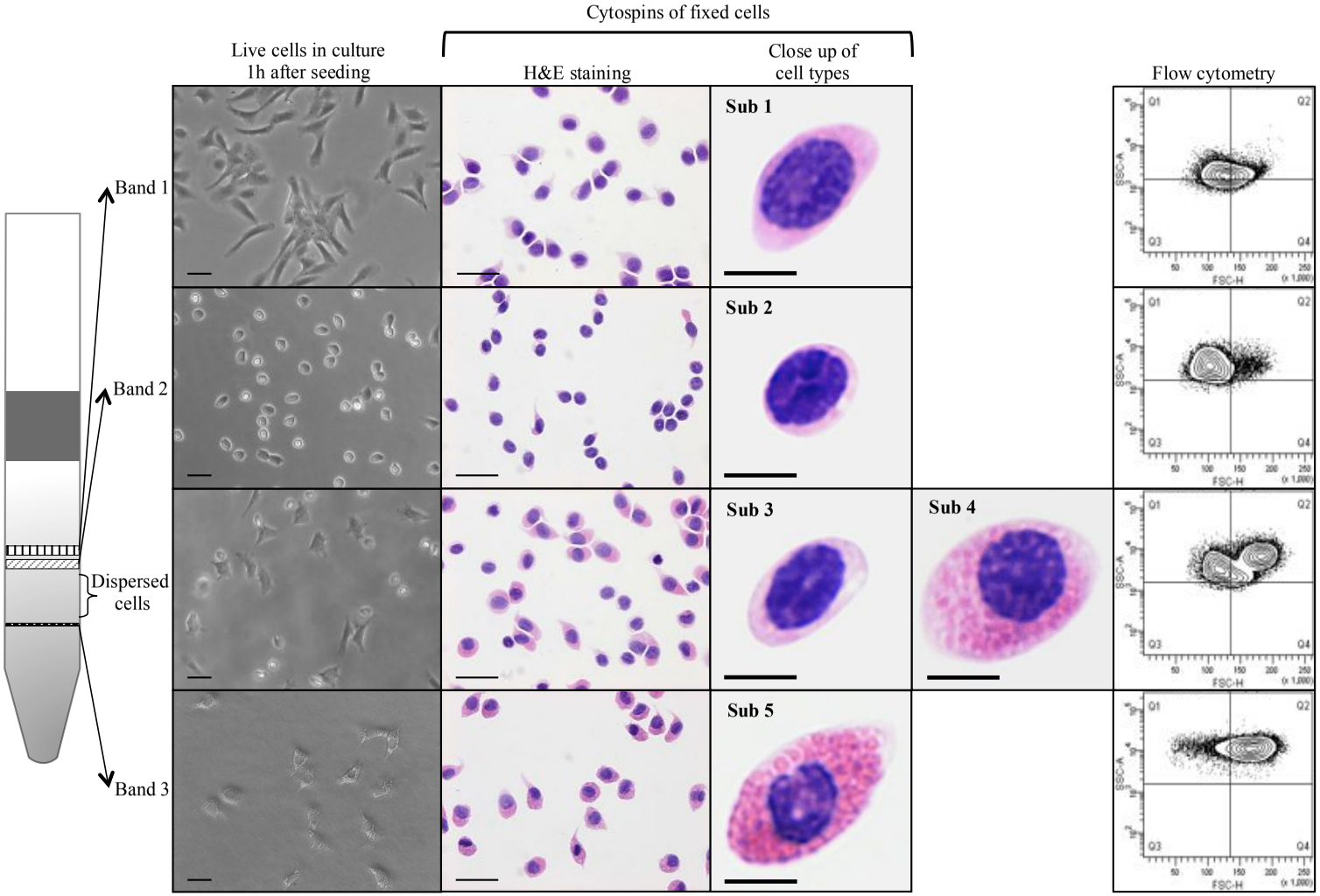


**Figure 2.** Analysis of individual *P. vannamei* haemocyte bands by *in vitro* culture, H&E staining and flow cytometry. Subpopulation 1 (Sub 1) showed cells that attached strongly to glass, had an average diameter size, contained little or no basophilic granules in the cytoplasm and a high nucleos:cytoplasm 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 cells attached moderately to glass, their diameter was big, contained a high amount of large eosinophilic/basophilic granules and had a low N:C ratio. Scale bars in living cells and H&E staining=20µm and in individual cells=5µm.

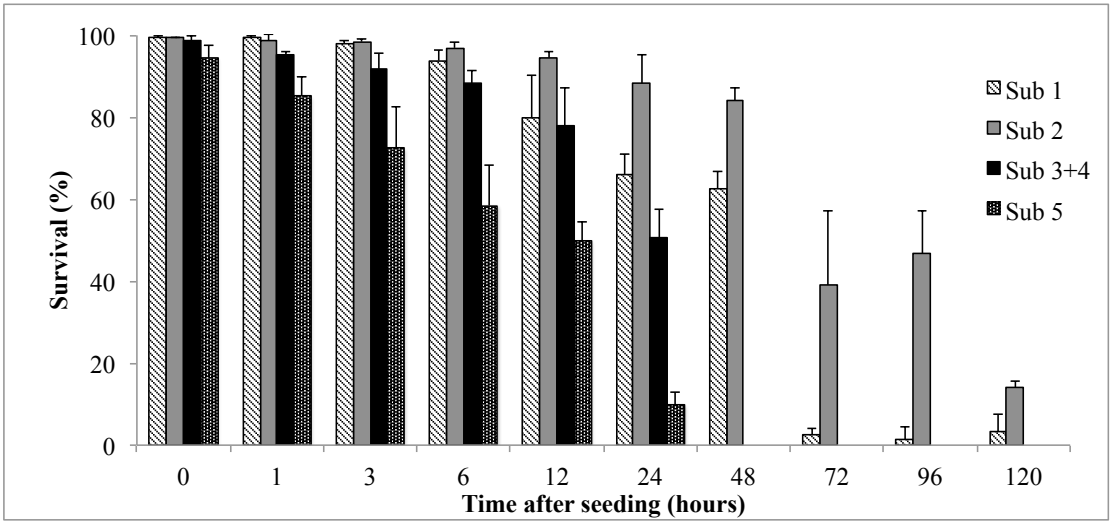


**Figure 3.** *In vitro* survival of separated *P. vannamei* haemocyte subpopulations as determined by ethidium monoazide bromide staining.

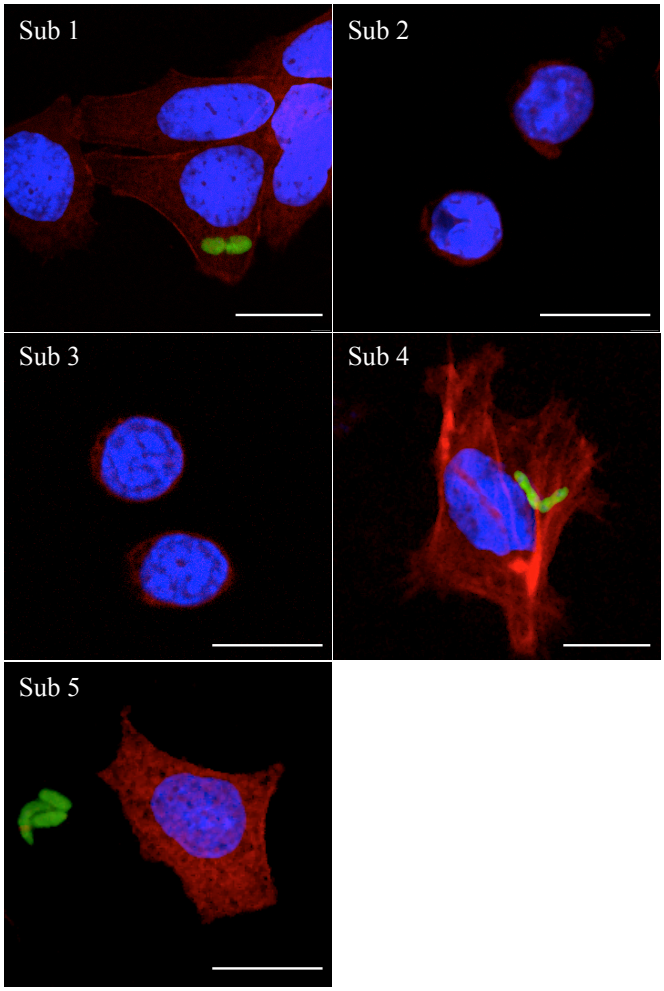


**Figure 4.** Phagocytosis of *V. campbelli* by separated *P. vannamei* haemocyte subpopulations at 1 hour after inoculation. The images are an overlay of confocal microscopic pictures taken from the cell base to its apex. F-actin fibres are stained with phalloidin-Texas Red (red), the nucleus is stained with Hoechst (blue) and GFP-labelled *V. campbellii* exhibit green fluorescence. Only Sub 1 and 4 showed phagocytic activity. Scale bar = 10µm.

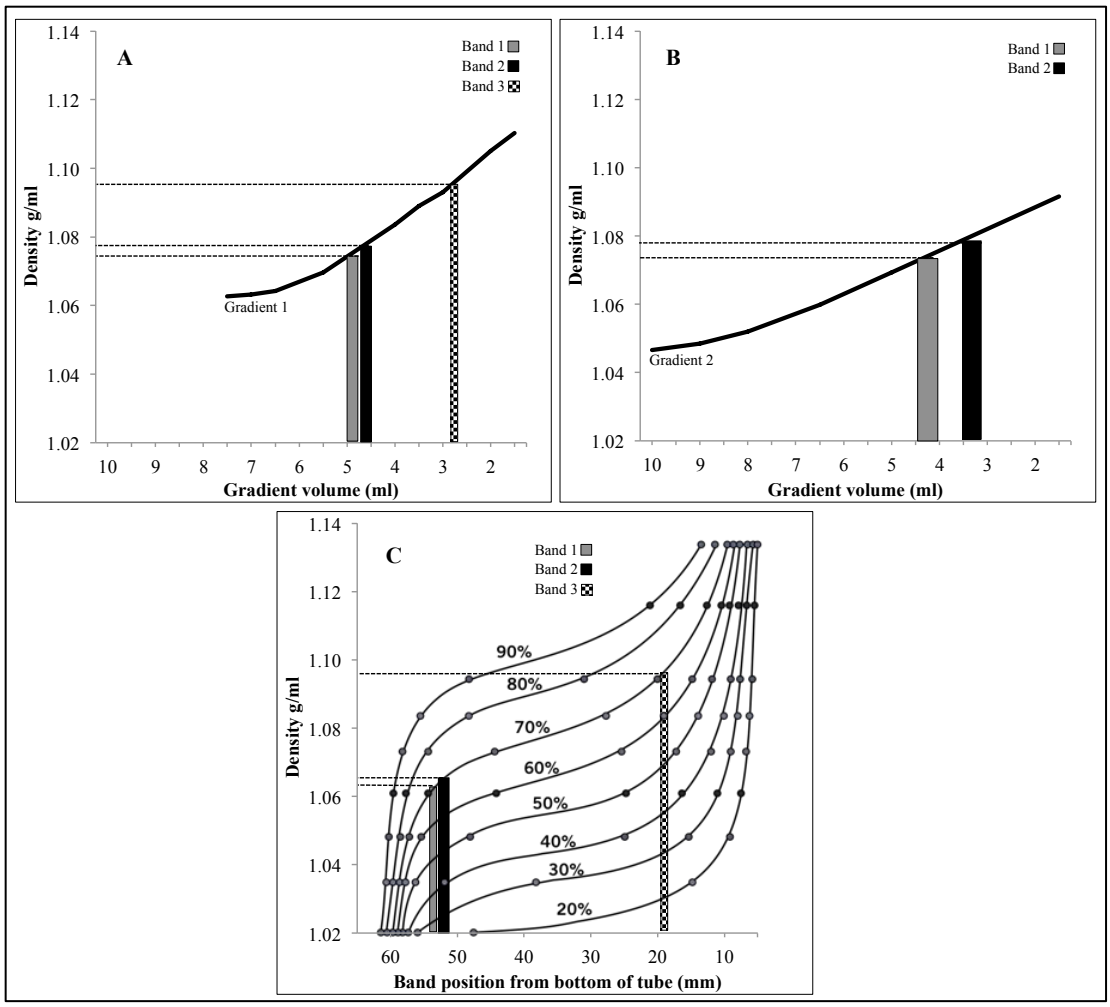


**Figure 5.** Iodixanol density gradients used in the present work (A and B) and self-forming Percoll gradients using several initial concentrations (C) (Adapted from Amersham Biosciences, 2001). The iodixanol gradients display a continuous and nearly linear density profile. The vertical bars represent 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 30,000 *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.

**Table 1.** Characteristics of the haemocyte bands and dispersed cells fraction by flow cytometry (FC) and light microscopy (LM). The values of average haemocyte size and granularity were obtained from FC forward scatter (FSC) and side scatter (SSC), respectively. The FC calibration and settings were standardized in-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 | Phagocytic activity | % 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 | Yes | 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 | No | 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 | No | - | - | - |
| 4 | 171.5±16.3 | 10.5±1.5 | 6.0±2.9 | moderate/ eosinophilic and basophilic | yes/strong | Yes | - | - | - |
| 5 | 166.2±6.0 | 9.9±1.0 | 10.6±2.9 | high/ eosinophilic and basophilic | yes/strong | No | 5.3±0.7 | 99.4±0.8 | 1.095 |

**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 in-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 5). 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 (Figure 1 and 4) 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 5. The Percoll self-forming gradients traditionally used to separate crustacean haemocyte subpopulations are prepared with initial concentrations of 60-70% Percoll (Söderhäll and Smith, 1983; Liu *et al.*, 2005; Roulston and Smith, 2011). The density curve of these gradients presents two steep regions on the top and at the bottom of the gradient. In these 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 5

## Purification of white spot syndrome virus by iodixanol density gradient centrifugation

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*Journal of fish diseases "in press"*

**Abstract**

White spot disease (WSD) is currently recognized as the most aggressive and devastating viral disease of shrimp aquaculture worldwide. The etiological agent is white spot syndrome virus (WSSV). At present, there are still gaps in the knowledge of WSD pathogenesis, and virus-host interactions. In order to fill these gaps, it is essential to perform *in vitro* experiments, and in this context, purified virus is required. Since WSSV first appeared in 1992, only a few brief purification procedures have been described. These protocols were mainly based on density gradient centrifugation using traditional media such as sucrose, NaBr and CsCl.

The present work describes for the first time the purification of WSSV using iodixanol density gradients. The virus was isolated from infected tissues and haemolymph of initially specific pathogen-free *Penaeus vannamei* (Boone, 1931) shrimp. The purification from tissues included an initial concentration of the viral suspensions by centrifugation (2.5 h at 60,000x*g*) onto a 50% iodixanol cushion. The virus purification was done by centrifugation (3 h at 80,000x*g*) through a discontinuous iodixanol density gradient (PBS, 5, 10, 15 and 20%). The purification from infected cell-free haemolymph enclosed a first dialysis step with a membrane of 1000 kDa cut off during 18 h. The purification step was done by centrifugation through the earlier mentioned iodixanol gradient. The gradients were collected in fractions and extensively analysed. Parameters such as number of particles, infectivity titre (*in vivo* titration), total protein and viral protein content were evaluated. These data were used to determine the efficiency of the procedures. The purification of WSSV from infected tissues resulted in viral suspensions with a very high infectivity and an acceptable purity, while virus purified from haemolymph had a high infectivity and a very high purity. Additionally, it was observed that WSSV has an unusually low buoyant density and that it is very sensitive to high external pressures.

These novel methodologies allowed an efficient purification of WSSV infectious viral particles. The high reproducibility of the procedures and clear characterization of these inocula makes them valuable tools for WSSV research where high quality of the results is required.

## Introduction

White spot disease (WSD) is a devastating viral disease that is extremely difficult to control in shrimp aquaculture. It causes huge economical losses worldwide and threatens the sustainability of the shrimp industry. The causative agent is white spot syndrome virus (WSSV), a virus with many exceptional characteristics (Escobedo-Bonilla *et al.*, 2008). It is the sole member of the family *Nimaviridae*, showing no direct relation with other virus families. It has a remarkable virion size (~250x120nm) and large genome (~300 kbp). By transmission electron microscopy (TEM), an unusual tail-like appendage is often observed at one pole of the virus. The nucleocapsid also shows an interesting pattern of compressed stacked rings, which are composed of the largest known viral protein (664 kDa).

In order to develop control strategies against WSD, a better understanding of the interactions of WSSV with its host's immune system and its target cell(s) is crucial. These aspects of the pathogenesis should be evaluated with well-characterized inocula, produced with controlled and reproducible concentration and purification procedures.

So far, published methods for purification of WSSV have been by centrifugation in sucrose, NaBr or CsCl density gradients (Wang *et al.*, 1995; Huang *et al.*, 2001; van Hulten and Vlak, 2001; Tsai *et al.*, 2004; Du *et al.*, 2007; Chen *et al.*, 2010) or simple differential centrifugation or filtration techniques (Durand *et al.*, 1996; Xie *et al.*, 2005; Gracia-Valenzuela *et al.*, 2009). In a few reports, the infectivity of the purified stocks was tested by bio-assays (Xie *et al.*, 2005; Du *et al.*, 2007; Gracia-Valenzuela *et al.*, 2009), however none evaluated the exact number of infectious virus and infectivity recovery during the purification procedures. These types of evaluations are particularly important when a high reproducibility of experiments is required. In general, the existing methodologies for purifying WSSV were presented in such a resumed way that is difficult to reproduce them.

The aim of the present study is to produce concentrated, highly purified WSSV inocula with known number of viral particles and infectivity by using well-described and reproducible methodologies and different virus sources (infected tissues or cell-free haemolymph) from SPF shrimp. The present work describes for the first time the purification technique for of WSSV using iodixanol density gradients. This separation medium has several advantages over those traditionally used, such as sucrose, CsCl or

NaBr, i.e. iodixanol is non-toxic for cells and it has limited interference in further analysis (no need for dialysis of the inocula). Moreover, it has practically no deleterious effects on the infectivity nor the physical structure of viruses treated with iodixanol (Ford *et al.*, 1994; Møller-Larsen and Christensen, 1998; Hermens *et al.*, 1999).

**Materials and methods**

*WSSV multiplication*

Specific pathogen free (SPF) *Penaeus (Litopenaeus) vannamei* (Boone, 1931) juveniles (Syaqua Siam Co., Ltd, Thailand) with an average weight of 10 grams were used for virus multiplication. A previously profusely studied WSSV strain (Jiravanichpaisal *et al.*, 2001; Escobedo-Bonilla *et al.*, 2005; Escobedo-Bonilla *et al.*, 2006; Rahman *et al.*, 2006a; Rahman *et al.*, 2006b; Escobedo-Bonilla *et al.*, 2007; Rahman *et al.*, 2007a; Rahman *et al.*, 2007b; Rahman *et al.*, 2008; Corteel *et al.*, 2009) was used in the purification experiments. This strain was isolated from naturally-infected *P. monodon* in 1996 in Thailand and passaged once in crayfish. Gill homogenates from crayfish were diluted $10^{-1}$ in phosphate-buffered saline (PBS, GIBCO) pH 7.4 and 50µl were intra-muscularly injected in each shrimp, between the 3$^{rd}$ and 4$^{th}$ tail segments. Shrimp were monitored every 6 h over 72 h. Moribund shrimp were collected and either stored at -70°C (purification from tissues) or used to extract haemolymph by hypodermal needle and syringe without anticoagulant, which was also stored at -70°C (purification from haemolymph). In both cases, WSSV infection in the moribund shrimp was confirmed by indirect immunofluorescence (IIF) as described below for *in vivo* titrations.

*Purification of WSSV from infected tissues*

WSSV suspension

Infected *P. vannamei* carcasses (at least 10) were thawed on ice and rinsed with cold PBS. Hepatopancreas, gut and exoskeleton were discarded. Infected tissues were weighed, cut into small pieces with a scalpel blade and finally ground in sterile PBS

(1:10 w/v) using an electric grinder (T25 digital Ultra-Turrax) at 5,500 rotations per minute (rpm). The whole procedure was executed on ice with pre-cooled (4ºC) solutions and materials. The suspension was clarified by centrifugation (Sorvall RC 5C plus; fixed angle rotor SLA-600 TC) at 3000 *g* for 20 minutes at 4ºC. Supernatants were collected and filtered through a 0.45 µm membrane.

WSSV suspension concentration

A 50% iodixanol solution (w/v; 5 vol. of OptiPrep diluted with 1 vol. of PBS) (Optiprep, Axis-Shield, UK) was prepared and 10ml were layered under 60 ml of WSSV tissue suspension with a 10 ml syringe and a 20G (0.9x70mm) needle in 70ml tubes. This preparation was centrifuged at 60,000 *g* during 2.5 hours at 4ºC with a Beckman Optima LE-80K ultra-centrifuge with a Type 35 rotor. After ultracentrifugation, 7.5 ml of the iodixanol cushion were first collected from the bottom of the tube with the same needle-syringe preparation and discarded afterwards. The following 5 ml were collected and kept for further purification. This suspension contained the band of concentrated WSSV suspension in 25% iodixanol (2.5 ml 50% iodixanol + 2.5 ml PBS containing the band which formed on top of the cushion) (Figure 1A).

WSSV suspension purification

This purification step was performed using a Beckman Optima LE-80K ultra-centrifuge with a SW41TI rotor. Prior to centrifugation, an iodixanol discontinuous gradient of PBS, 5, 10, 15 and 20% iodixanol in PBS (w/v) was composed with 2 ml fractions. This gradient was prepared by underlayering the fractions of increasing concentration in the bottom of the tube, using 2 ml syringes and 20G needles. The concentrated virus suspension (25% iodixanol) was layered under this gradient and the tubes were centrifuged at 80,000 *g* during 3 hours at 4ºC (Figure 1A). The gradient was collected in 1 ml fractions (13 fractions) using a 1 ml pipette, except for the first and the last fractions, which were collected in 0.5 ml. From these, only the ones which contained visible bands (always located on the interface between two gradient fractions) were kept and frozen at -70°C for further analysis. This experiment was repeated three times, starting from the preparation of the tissue suspensions.

*Purification of WSSV from infected cell-free haemolymph*

WSSV suspension

Haemolymph was collected from at least 20 WSSV-infected shrimp with 2 ml syringes with 20G needles and immediately centrifuged at 500 *g* for 10 min at 4°C. All the materials were pre-cooled on ice. The supernatants were individually frozen at -70°C. At the end of the experiment and after WSSV infection was confirmed, the samples were thawed on ice, pooled together, filtered through a 0.45 μm membrane and immediately frozen at -70°C until further use.

WSSV suspension dialysis

The WSSV suspension was dialysed using a membrane with size cut-off of 1000 kDa (Float-A-Lyzer G2 CE, Spectrum Laboratories, the Netherlands) during 18 h at 4°C (Figure 1B). PBS was used as dialysis buffer and totally replaced after 2, 6 and 12 h. Samples of the suspension were taken at 0, 6 and 18 h. The total protein content in the samples was determined and used as indicator for the success of the dialysis. The dialysed suspension was mixed with 60% iodixanol at a final concentration of 25% and immediately loaded under the purification gradient (Figure 1B).

WSSV dialysed suspension purification

This step was performed as described above for the purification of WSSV isolated from infected tissues (Figure 1B).

*Gradient analysis*

Determination of the number of viral particles by confocal microscopy

The number of viral particles in the samples was estimated using a modification of the method described by Pizzato, Marlow, Blair & Takeuchi (1999). We determined the number of virus particles by calculating its proportion to the number of fluorescent beads that were added to each sample at a known concentration. Briefly, the samples

collected after purification were diluted tenfold in PBS. Red fluorescent 0.2µm polystyrene beads (Fluospheres, Invitrogen, Belgium) at a concentration of $10^8$ $ml^{-1}$ and collagen Type I (Sigma, Belgium) at a proportion of 1:15 (v/v) were added to each sample. Drops of these suspensions (3 µl) were placed on glass slides coated with (3-Aminopropyl) triethoxysilane (Sigma), allowed to dry for 20 min at 37ºC and immediately fixed with 4% paraformaldehyde for 10 min at room temperature. These smears were double stained by IIF as described below for staining of shrimp tissues. Confocal microscopic pictures of 4 random locations in the smear were taken with two fluorescence emission wavelengths (green: 521 nm; red: 605 nm) (Figure 3C). The number of beads and viral particles in each picture was counted using ImageJ software (Abramoff, 2004). The concentration of viral particles was calculated using the proportion beads/viral particles.

*In vivo* WSSV titration

The infectivity titre in the gradient samples was evaluated as previously described by Escobedo-Bonilla *et al.* (2005). Briefly, samples were diluted in tenfold steps and dilutions from $10^{-5}$ to $10^{-9}$, in the case of purification from tissues and from $10^{-4}$ to $10^{-7}$, in the case of purification from haemolyph. Each of these dilutions was intramuscularly injected (50µl) in five *P. vannamei*. Animals were housed individually in covered 10 L aquaria supplied with aeration. The temperature was maintained at 27±1ºC. Over the course of 5 days, dead and surviving shrimp were collected and the cephalothorax was frozen in 2% methylcellulose (Fluka, Belgium). These blocks were cryosectioned and stained by (IIF) with 8B7 mouse monoclonal antibody against VP28 envelope protein (Poulos *et al.*, 2001) (10 µg $ml^{-1}$ in PBS) and goat-α-mouse IgG FITC-conjugate (Invitrogen; 4 µg/ml in PBS). Each antibody was incubated during 1 h at 37°C. Normal goat serum was added at 10% (v:v) in both antibody suspensions. The stained sections were analysed by fluorescence microscopy (Leica DM IRBE) to confirm the presence or absence of WSSV infection in dead and surviving shrimp. The infectivity titre was calculated from these infection data using the Reed & Muench formula (Reed and Muench, 1938) and expressed as Shrimp Infectious Dose 50% endpoint ($SID_{50}$). This methodology was also used to determine the efficiency of the purification steps (infectivity recovery).

Gradient density profile determination

The density of the gradient fractions was determined by measuring the absorbance at 244nm (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%; w/v) 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.

Total protein determination

The amount of protein present in each gradient fraction was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Belgium). Briefly, a bovine serum albumin (BSA) concentration series standard was prepared according to the manufacturer's manual and its absorbance measured at 550nm using an ELISA. A standard curve expressing absorbance vs. BSA concentration was made and a correlation formula calculated. The gradient samples were serially diluted and the absorbance of 3 of those dilutions determined. The protein concentration was calculated using the correlation formula.

Western Blot analysis

Samples from the gradient fractions were mixed 1:1 with loading buffer containing β-mercaptoethanol. This suspension was heated at 90°C for 3 minutes and loaded in a 14% acrylamide gel. After 60 minutes of electrophoresis, proteins were transferred from the gel to a PVDF membrane (Membrane Hybond-P, GE Healthcare, Belgium) via Western Blotting (BioRad Mini Trans Blot, Belgium). Blotted proteins were stained with a primary mouse monoclonal antibody (8B7; stock diluted 1:300) directed against VP28 WSSV envelope protein (Poulos *et al.*, 2001) and a secondary goat anti mouse-HRP monoclonal antibody (DAKO, Belgium; stock diluted 1:2000).

All the antibodies were diluted in a blocking buffer composed of 0.1% tween (v/v) and 5% milk powder (w/v) in PBS.

Transmission Electron Microscopy (TEM)

Due to the possible interference of iodixanol (x-ray opaque compound) with TEM visualization, samples were dialysed using a membrane with a 12kDa cut-off size. Afterwards, formvar-coated and carbon-stabilized 200 mesh copper grids (Jeol, Belgium) were incubated with alcian blue 1% for 10 minutes and afterwards washed 5 times in DW. Grids were then incubated with the sample suspensions for 30 minutes, washed 2 times in DW and finally negatively stained with 2% phosphotungstic acid (Sigma), pH 7.4 for 10 seconds and dried. The grids were analysed by TEM (Jeol JEM-1200EX II) for evaluation of the samples purity degree by visualization of virus particles and identification of subcellular structures. Additionally, pictures were taken and the dimensions (width and length) of 20 viral particles per sample measured using ImageJ software (Abramoff, 2004). The length of the viral particles of each fraction was analysed for statistical differences by one-way ANOVA test at a confidence interval of 95%.

**Results**

*WSSV purification procedures*

The diagrams in Figure 1 describe the steps followed for purifying WSSV either from infected tissues (A) or haemolymph (B).

Purification from tissues: After the concentration step, a dense and thick band of sediment material was found on top of the 50% iodixanol cushion. No material (pellet) was found at the bottom of the tube. The sediment band was collected together with part of the cushion and loaded under the discontinuous iodixanol gradient for further purification. After ultracentrifugation, sharp bands were found in the areas corresponding to the interfaces in- between the gradient fractions (Figure 2A). The density profile of fractions 1, 2, 3, 4, 5 and 6 was 1.02, 1.06, 1.08, 1.10, 1.12 and 1.16 g/ml, respectively.

Purification from haemolymph: After the dialysis step, the protein content of the

suspension was reduced nearly 50%. Its colour changed from intense blue to lighter blue, indicative for a reduction in haemocyanin content. This suspension was mixed with iodixanol at a final concentration of 25%, loaded under the standard discontinuous iodixanol gradient and further centrifuged for virus purification. A sharp and thin band was found in fraction 4 (between 15% and 20% iodixanol) (Figure 3A). As for the purification from tissues, 6 fractions were sampled and analysed. The density profile was 1.03, 1.05, 1.07, 1.09, 1.12 and 1.15 g/ml for the fraction 1, 2, 3, 4, 5 and 6, respectively.

*Gradient analysis*

Figures 2 and 3 present an overview of the results obtained from the analysis of the fractions sampled after the purification procedures.

Number of viral particles counted by confocal microscopy

Purification from tissues: The concentration step increased the concentration of viral particles approximately 11 times without significant losses in its absolute number. The total number of particles originally loaded under the purification gradient was $32.0\pm5.6x10^9$. After centrifugation, fraction 1 held the lowest number ($1.1\pm0.5x10^9$) of viral particles. The number started to increase in fractions 2 ($5.2\pm2.4x10^9$) and 3 ($4.9\pm0.7x10^9$) and even more in fraction 4 ($9.7\pm3.4x10^9$). After a decrease in fraction 5 ($3.3\pm1.3x10^9$), it reached a peak in fraction 6 ($11.6\pm0.7x10^9$). The recovery percentage of viral particles in the analysed fractions was 76.9±26.1%.
Purification from haemolymph: The dialysis step did not influence the number of viral particles. A total amount of $99.8x10^7$ particles was loaded under the purification gradient. After centrifugation, the number of particles peaked in fraction 4 ($35.2x10^7$). Fractions 3, 5, and 6 accounted with $7.6x10^7$, $11.9x10^7$ and $20.8x10^7$ particles, respectively. The recovery after centrifugation was 75.1%.

Infectivity

Purification from tissues: The concentration step did not influence negatively the infectivity. The total infectivity of the concentrated WSSV suspension loaded under

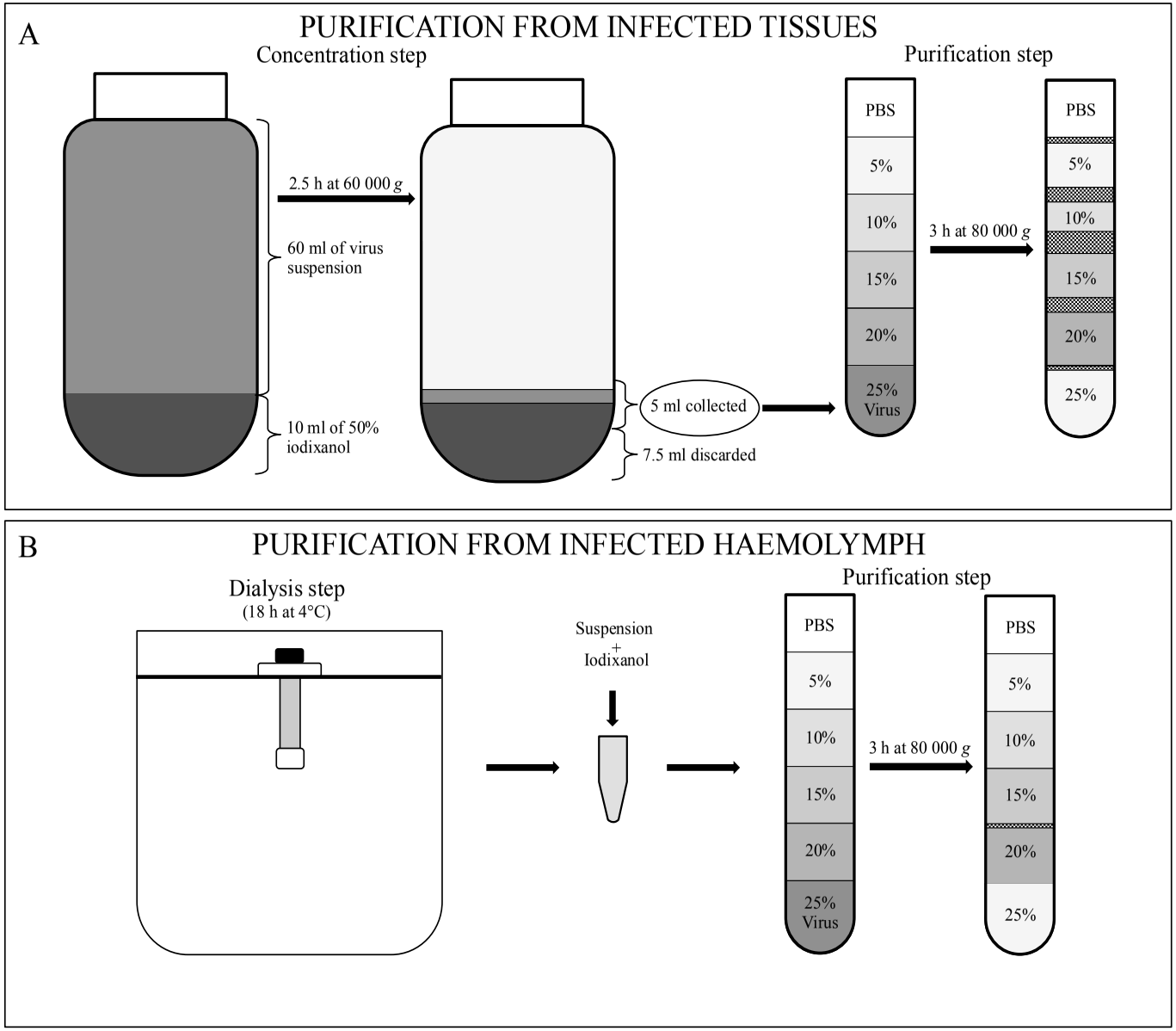


**Figure 1.** Schematic representation of the procedures to purify WSSV from infected shrimp tissues (A) and from haemolymph (B) of *P. vannamei*.

the purification gradient was $10^{8.7}$±0.0 SID50. After ultracentrifugation, the total infectivity recovered from all the gradient fractions was 53.4±7.1%. Fractions 2, 3 and 4 together contained 93% of that infectivity. Fraction 3 alone ($10^{8.2\pm0.2}$ $SID_{50}$) represented 57.4% of the recovered infectivity, while fractions 2 ($10^{7.6\pm0.3}$ $SID_{50}$) and 4 ($10^{7.7\pm0.2}$ $SID_{50}$) represented 15.4% and 20.2%, respectively. Fractions 5 ($10^{7.1\pm0.1}$ $SID_{50}$/ml) and 6 ($10^{6.4\pm0.1}$ $SID_{50}$/ml) together accounted for only 6.9% of the infectivity. The infectious titre in fraction 1 was below the detection limit of the *in vivo* titrations. Since all the shrimp that were confirmed to be infected with WSSV died, the lethal dose 50% endpoint ($LD_{50}$) was identical to the $SID_{50}$ in all the fractions.

Purification from haemolymph: The dialysis step did not have any inactivating effect on the infectivity of the virus (data not show). The total infectivity loaded under the purification gradient was $10^7$ $SID_{50}$. After purification, an infectivity recovery of 65.2% was obtained. A clear infectivity peak appeared in fraction 4 ($10^{6.5}$ $SID_{50}$), which was 69.5% of the total recovered infectivity. Fractions 5 ($10^{5.3}$ $SID_{50}$) and 6 ($10^{6.1}$ $SID_{50}$) together accounted for 30.5% of that infectivity. Fraction 3's infectivity titre was below the detection limit. Here, the $LD_{50}$ was also identical to the $SID_{50}$ in all the fractions.

Protein content

Purification from tissues: Fraction 1 held the lowest amount of protein (0.20±0.01 mg/ml). Fractions 2, 3 and 4, which showed a similar protein content, had 1.18±0.16, 1.16±0.19 and 1.23±0.14 mg, respectively. The majority of proteins was found in fractions 5 (2.73±0.26 mg) and 6 (10.31±2.13 mg). These two fractions together accounted for 77.5% of the total amount of protein found in the gradient.
Purification from haemolymph: Fractions 5 (10.33 mg) and 6 (41.60 mg) enclosed almost all protein found in the gradient (99.3%). Fraction 4 (0.26 mg) only accounted for 0.5% of that protein.

Western blot

Purification from tissues: The amount of VP28 was similar in fractions 2, 3, 4 and 6. This amount was visibly lower in fraction 5 and very low in fraction 1 (Figure 2B).
Purification from haemolymph: VP28 was only detected in fractions 3-6. It increased from fraction 3 to 5 and decreased again in 6 (Figure 3B).

TEM

Purification from tissues: Figure 2 shows representative pictures of the gradient fractions. The analysis revealed differences in the distribution of subcellular debris and amorphous protein matrix through the gradient fractions. In fraction 1, an enrichment in cell membranes and large vesicles (probably early endosomes) was clear. This scenario was similar in fraction 2. In fraction 3, the presence of cellular contaminants was visibly reduced. Membranous material started to appear in fraction 4, although it seemed to be from a different origin than in fractions 1 and 2, possibly endoplasmatic reticulum. Fraction 5 showed an increased content in amorphous protein patches. Fraction 6, displayed among others, small vesicles and granular material.
The dimensions of virus particles (length x width in nm) measured in fractions 2, 3, 4, 5 and 6 was 280±23 x 92±8, 278±18 x 106±8, 270±19 x 87±14, 260±19 x 99±12 and 249±21 x 97±15, respectively. The analysis of the viral particles' length by one-way ANOVA showed significant differences in between the different fractions (*p=0.0136*).
Purification from haemolymph: Representative pictures of each fraction are presented in Figure 3. No subcellular or amorphous protein contaminants were found in fraction 4. On the other hand, enrichment in nucleocapsids and especially a crossed-patterned protein matrix were visualized in fraction 6.

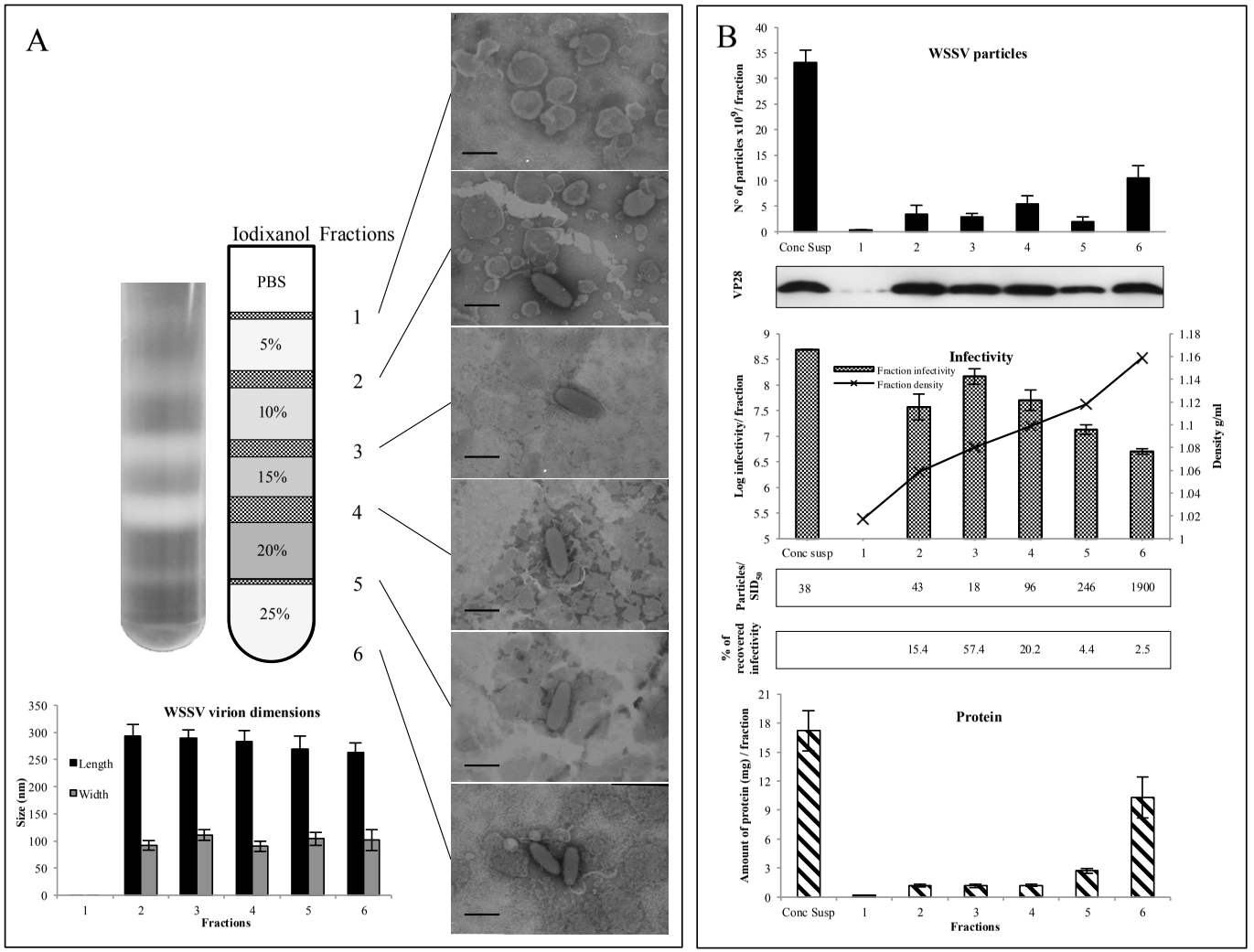


**Figure 2.** Purification of WSSV from shrimp infected tissues. Figure 2A encloses a picture of a centrifuge tube after the purification step (left) and its respective schematic representation (right). Very clear bands were visualised in the areas corresponding to the interfaces between the gradient fractions. The lines projected from the tube scheme show representative TEM pictures of each of the gradient fractions. These pictures showed differences in the distribution of sub-cellular debris and other contaminates over the gradient fractions. Scale bar = 200µm. Figure 2B presents the analysis of the individual gradient fractions. These figures are vertically aligned by gradient fractions (1-6) and including the concentrated suspension (Conc Susp), which was used for WSSV purification.

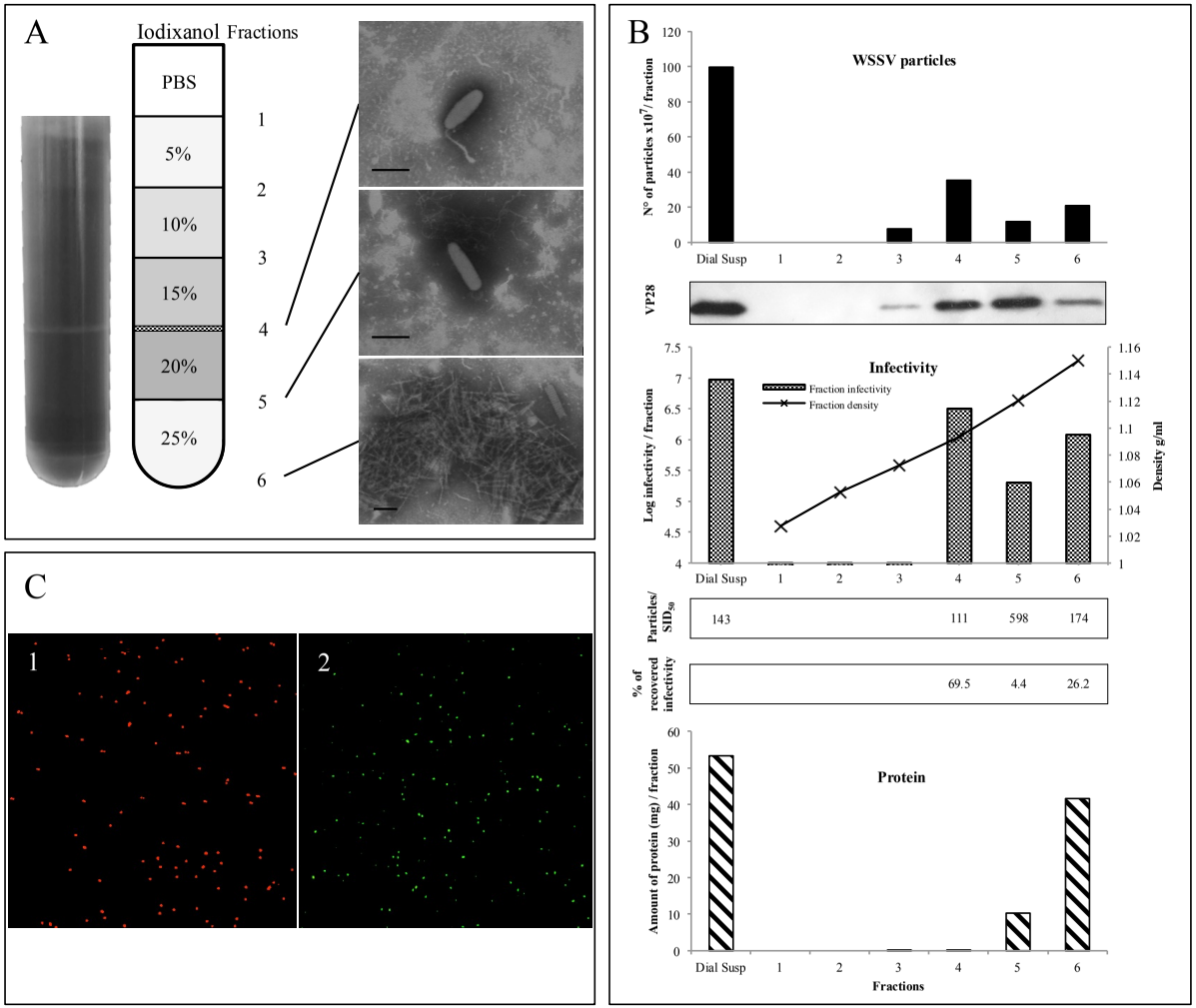


**Figure 3.** Purification of WSSV from infected shrimp haemolymph. Figure 3A encloses a picture of a centrifuge tube after the purification step (left) and its respective schematic representation (right). A thin and sharp band could be visualised in the area corresponding to fraction 4 (interface between 15 and 20% iodixanol). The lines projected from the tube scheme show representative TEM pictures of the fractions 4 and 6. These pictures showed the absence of sub-cellular debris of fraction 4 and enrichment in clotting protein and nucleocapsids in fraction 6. Scale bar = 200µm. Figure 3B presents the analysis of the individual gradient fractions. These figures are vertically aligned by gradient fractions (1-6) and also including the dialysed suspension (Dial Susp), which was used for WSSV purification. Confocal microscope pictures of the gradient fraction smears are presented in Figure 3C. Red fluorescent beads are shown in Picture 1 and FITC-labelled viral particles in Picture 2. These pictures were used to calculate the number of viral particles.

**Discussion**

One of the prerequisites for high quality virological research is to have viral stocks that are well characterized, in terms of infectivity and purity. The present work described in detail the purification of WSSV from infected shrimp carcasses and haemolymph. As expected, the shrimp material used for purification influenced the characteristics of the final stock, mostly in terms of purity. The virus suspensions obtained from infected tissues had a high infectivity titre and a satisfactory purity level. On the other hand, those obtained from infected haemolymph, had much lower infectivity titres but the purity was greatly improved. Thus, according to the aim of the work, it might be convenient to select the purification procedure. If highly pure virus gets priority, then virus from haemolymph should be used. If a high virus titre is needed then virus from infected tissues should be preferred.

Analysis of the results of isolation and purification WSSV from infected tissues showed that fraction 3 is the best source of purified WSSV. Besides enclosing 57.6% of the recovered infectivity, this fraction showed the highest concentration of infectious virus particles (lowest quotient particles/infectious dose; Figure 2B). The amount of protein (total protein analysis) and cellular contaminants (TEM) were greatly reduced when compared with the initial virus suspension and the other gradient fractions. Furthermore, it was clear that fraction 6 enclosed high amounts of inactivated virus (highest quotient particles/infectious dose) and possibly pieces of virus and other subcellular contaminants. Fractions 2 and 4 also contained a considerable amount of inactivated viral particles as demonstrated by the quotient viral particles/infectivity. The results of the purification of WSSV from infected haemolymph were easier to interpret, i.e. fraction 4 was clearly the purest WSSV suspension as it contained 69.5% of the total infectivity, the highest percentage of infectious viral particles and a very low amount of protein and subcellular contaminants. Nevertheless, it must be pointed out that its content in non-infectious viral particles is 6 times higher than fraction 3 from infected tissues. Since that disproportion was already present in the initial suspensions before dialysis, this might be explained by the inactivation of the virus when in direct contact with some antiviral component present in shrimp haemolymph.

The virus isolated from infected tissues appeared in all the fractions, but with a distinctive infectivity peak in fraction 3. This finding indicates that the buoyancy of

WSSV viral particles is highly variable. As reviewed by Escobedo-Bonilla *et al.* (2008), towards the end of the WSSV virion maturation, there is a tightening of the envelope wrapping which provokes a reduction of its length due to the nucleocapsid compression. Since this inoculum was obtained from infected tissues (and, thus, infected cells), the presence of virions in different maturation stages (and, thus, different sizes) was probable. Given this, we hypothesise that the virion's density variation could be related with its size, which is in turn dependent on the maturation stage. To test this hypothesis we measured the dimensions of the viral particles in all the fractions, and indeed we found differences mostly in terms of length (Figure 2A). The length varied inversely with the gradient density supporting our hypothesis. To reinforce this idea, the virions isolated from cell-free haemolymph, which are per definition mature virions, showed a narrower density distribution over the gradient.

In general, the density of mammalian enveloped viruses in iodixanol is situated in between 1.12-1.20 g $ml^{-1}$ (Fowler *et al.*, 1985; Yi *et al.*, 2006; Dormond *et al.*, 2010; Tseng *et al.*, 2010). On the other hand, the density range of marine viruses is higher and varies between 1.18-1.29g/ml (Lawrence and Steward, 2010). According to our findings, WSSV is an atypical virus, which does not fit in any of those density ranges. WSSV buoyancy was much lower, i.e. within 1.06-1.16 g $ml^{-1}$ in virus isolated from tissues and 1.07-1.15 g/ml in virus isolated from haemolymph.

Another interesting finding was the inactivation of part of the virus during the purification procedure. It was previously reported that iodixanol has little adverse effects on the infectivity or integrity of viruses in contradiction to traditional products such as sucrose or CsCl (Dettenhofer and Yu, 1999; Hermens *et al.*, 1999; Zhu and Yuan, 2003). Given this, a damaging impact of iodixanol on WSSV infectivity is less likely. On the other hand, in preliminary purification trials we have found that using a higher centrifugal force of 200,000 *g* for 3 hours inactivated 98% of the virus (data not shown). This strongly suggested that WSSV is extremely sensitive to high pressures. It may be hypothesized that the tight packing of the nucleocapsid (high internal pressure) could provoke a collapse and loss of the envelope when applying a high external pressure.

In conclusion, to the best of our knowledge, this work is the first to purify WSSV using an iodixanol density gradient. By using well-controlled and reproducible procedures we successfully purified WSSV from infected tissues and from cell-free haemolymph. The accurate determination of the number of particles and their

infectivity will facilitate reproducible infection experiments with high accuracy. Additionally, the experiments revealed that WSSV virions have a variable and unusually low buoyancy and are prone to inactivation during purification, possibly by the high pressure during ultracentrifugation.

**Acknowledgements**

The first author was financially supported by a scholarship from the Foundation for Science and Technology (FCT, Portugal), and the second author by a scholarship from the Institute for the Promotion of the Innovation through Science and Technology in Flanders (IWT-Vlaanderen, Belgium).

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# Chapter 6

## General discussion

The development of shrimp aquaculture and its associated obstacles gave an impulse to research on several aspects of shrimp physiology similarly to what happened before with other animal species that are bred as human food source. The research on shrimp immunity was driven by the emergence of diseases in the 1970's as a result of the intensification of the shrimp farming industry (Chamberlain, 2010). The recent development of this branch of research and the specificities of invertebrate physiology explain the relative lack of information on shrimp immunity, when compared to that of terrestrial livestock.

Important in the progress of any biological research field is the development of reliable laboratory techniques. Despite four decades of research on shrimp, there is still a generalized lack of systematic approaches in the development of standardized laboratory techniques. Examples of such techniques are the development of primary and continuous cell lines, models for studying pathogen-host interactions and purification and full characterization of important pathogens. The present work approached this subject by developing new and improving existing tools that are essential for research on the interaction of WSSV with shrimp immunity: (i) cultivation of haemocytes, (ii) haemocyte subpopulation separation and (iii) purification of WSSV. While the haemocyte culture systems are important for evaluating general immune reactions towards pathogens, the separation of haemocytes into subpopulations is preferable for the evaluation of subtype specific reactions. The purification of WSSV is crucial for the performance of experiments on antiviral immunity because contaminants (e.g. cell debris, and proteins), which are normally present in material obtained from infected animals, animal fluids or cell cultures, may interfere. With some adaptation, this tool can be applied to virtually all the important pathogens affecting shrimp culture.

An important obstacle for the development of research on crustacean physiology is the lack of continuous cell lines. This is less limiting in immunity research because immune cells can be easily isolated from haemolymph and kept temporarily *in vitro* for studies on immune responses. In Chapters 3 of this thesis, we were able to keep haemocytes in culture for (at least) 48 hours. During this period, we evaluated survival of haemocytes from two crustacean species (*P. vannamei* and *M. rosenbergii*) using two distinct culture systems (attachment and suspension). The culture in attachment of *P. vannamei* haemocytes allowed the quantification of haemocyte-mediated immune reactions such as phagocytosis and inactivation of bacteria. The

phagocytic haemocytes internalised (as shown by confocal microscopy) in average 2.4 bacteria in one hour. At the same time, the bacteria in the culture supernatant grew much slower than in the control (culture without haemocytes), confirming that haemocytes were alive and functionally active during the culture period. The cultures in suspension allowed the study of general aspects of haemocyte nodulation such as kinetics of nodule formation and maturation. The kinetics study characterised the evolution of the nodules in terms of number and average diameter. These nodules became mature (stable diameter and showing melanisation) after 24 and 48 hours of culture for *P. vannamei* and *M. rosenbergii*, respectively. This demonstrated that haemocytes cultured in suspension were also functionally active. However, the use of this type of primary cell cultures has also its limitations. The high reactivity of haemocytes often leads to short survival times and reproducibility issues. In order to overcome the high reactivity of haemocytes and the consequent clotting of haemolymph during collection, Söderhäll and Smith (1983) developed a citrate/EDTA anticoagulant adapted to the osmolality of marine crustaceans. This was a crucial step for establishing *in vitro* haemocyte culture techniques. As demonstrated in Chapter 3 of this thesis, the *in vitro* survival of haemocytes can be prolonged by the addition of a major antioxidant. Therefore, a possible approach for improving haemocyte survival is to optimise the media with supplements that prevent haemocyte activation and/or control the effect of toxic substances (e.g. quinones, ROS, etc.) produced by haemocytes during activation. This strategy together with the optimization of the nutritional composition of the media may be valuable in the process of developing shrimp haemocyte cell lines. However, if the objective is to study immune reactions, attention must be paid to avoid disabling the immune competence of haemocytes by damaging their reactivity. The experimental reproducibility should be addressed by developing culture procedures that can be performed in a fully standardized way. The work described in Chapter 3 gave an answer to this issue by following well-defined working protocols and by analysing an extensive number of haemocyte culture parameters. This strategy gave reproducible results, which proved that our procedure was one step further in the establishment of standardized laboratory techniques for use in routine evaluations of shrimp immunity. The next step will be to optimize these basic techniques. Special attention must be paid to the control of haemocyte reactivity and optimization of media formulation, optimization of anticoagulant buffers and automation of the evaluation procedures. Application of newly established cell culture

techniques might also be a valuable asset. From these, new culture plate coatings and techniques targeting the control of physical conditions of the *in vitro* environment such as cell density, three-dimensional culture surfaces, movement during incubation, gases and atmospheric pressure, must be prioritized. Finally, we would like to state that the extensive knowledge on immunity of terrestrial livestock and the culture of their immune cells, might be in part negatively influencing the progress of research on crustacean immunity. The tendency that crustacean immunologists have to think in a "mammalian" immunity oriented way might not be the right strategy to follow. The known peculiarities of crustacean immunity, namely the likely inexistence of an adaptive immune system (Hauton and Smith, 2007), the high reactivity of haemocytes (Dantas-Lima *et al.*, 2012), the nature and efficiency of their clotting system (Theopold *et al.*, 2004), the nodulation of pathogens (Söderhäll *et al.*, 1984) and probably other unknown particularities, suggest that an attitude of "thinking outside the box" might be the strategy to follow in order to solve the current issues and to make a faster progress in this field.

The creation of detailed and well-characterized haemocyte culture systems for the marine *Penaeus vannamei* enabled us to adapt it to one of the most commercially important freshwater shrimp species *Macrobrachium rosenbergii*. This also allowed us to make a proper comparison between immune parameters of both species. The anticoagulant and culture media were adapted to the specific requirements of *M. rosenbergii* as measured directly in the experimental animals (osmolality and pH). Afterwards, haemocytes were cultured and the immune parameters were evaluated as for *P. vannamei*. Although haemocytes of both species shared many common characteristics such as total and differential haemocyte counts and adherence behaviour, we have found that haemocytes of *M. rosenbergii* are less reactive (slower formation of haemocyte clusters) and survive longer under the similar *in vitro* conditions. These differences make *M. rosenbergii* haemocytes easier to isolate and to handle under *in vitro* conditions. Consequently, *M. rosenbergii* appears to be a suitable alternative to *P. vannamei* for performing studies on general crustacean immunity. The identification of the differences in survival times may also help in designing strategies to improve the survival of crustacean haemocytes *in vitro*.

The haemocyte culture systems are ready to be applied in evaluations of immune reactions (cellular and humoral) towards pathogens. They can be used in the evaluation of traditional immune parameters such as phenoloxidase activity and

reactive oxygen species production and cellular reactions such as phagocytosis. Less common immune reactions such as nodulation of pathogens, cell adhesion and spreading index, anti-bacterial/viral activity (cellular and humoral), cell survival and cell image velocimetry, can also be evaluated (Auffret and Oubella, 1997; Liu *et al.*, 2005; Le Foll *et al.*, 2010; Pope *et al.*, 2011). Another application for these technologies is the evaluation of the effect of specific substances on shrimp immunity, e.g. immunostimulants and pollutants (Perez and Fontanetti, 2011). Practically, these haemocyte culture systems may develop into useful tools in the functional validation of commercial nutraceuticals and in the assessment of the pollution levels in ecosystems and freshwater sources. However, for making these applications possible, these techniques still have to be submitted to a validation process that will determine if they are sensitive enough to detect differences between samples.

While cultures of mixed populations of haemocytes are suitable for quantification of general immune reactions, their separation into subtypes or subpopulations is necessary for studying the underlying mechanisms of these processes. Up to date, there is only one procedure based on Percoll density gradient centrifugation being used to separate crustacean haemocyte subpopulations (Söderhäll and Smith, 1983). Although allowing subpopulation separation, these gradients display an S-shaped density curve that may cause separation problems (Figure 9 of chapter 1). The two steep density areas formed on the top and at the bottom of the gradient, display a broad density range in a small gradient volume. Although these areas conveniently promote the concentration of cell subpopulations into sharp bands, they can create problems in separating subpopulations with similar buoyant density. This can result in the concentration of different cell subpopulations in one sole band. In between those areas the situation is the opposite. Here, the shallow density profile (low density range in a big gradient volume) may cause a spatial dispersion of cell subpopulations, resulting in less visible bands. In contrast, the nearly linear density profiles (smooth increment of density all over the gradient) of the iodixanol density gradients, as we described in Chapter 5, promoted the formation and clear visualization of haemocyte bands. The collection of these bands was accomplished with a very high degree of purity, which was never reported before for shrimp haemocyte subpopulations. Given this, we considered that the present separation methodology significantly improved the traditional procedure for separation of crustacean haemocytes in Percoll gradients. This technology allowed us to confirm previous claims about the existence of more

than three haemocytes types (Hose *et al.*, 1987; Giulianini *et al.*, 2007). Five different subpopulations were identified based on their density and morphology. They showed different adherence behaviour and morphological features, confirming their different physiological characteristics. Two of these subpopulations were easily identified based on the current crustacean haemocyte classification system (reviewed by Jiravanichpaisal *et al.*, 2006). Sub 1 displayed the morphological characteristics of hyalinocytes (high nucleus/cytoplasm ratio and absence of cytoplasmatic granules) and Sub 5 were clearly granulocytes (low nucleus/cytoplasmatic ratio and abundant cytoplasmatic granules). Sub 4 resembled typical semi-granulocytes. The experiments for detection of phagocytic activity gave additional indications about the identity of these subpopulations. Phagocytosis was detected only in Sub 1 and 4 as previously described for hyalinocytes and semi-granulocytes. The classification of Sub 2 and 3 was not that straightforward. Interestingly, we have found that besides Sub 2 was very abundant (approximately 50% of the total) in the haemolymph of *P. vannamei*, it did not fit in any morphological category of the traditional crustacean haemocyte classification system. These cells displayed a completely different morphology when compared with the other subpopulations. They were small and had a very high nucleus cytoplasm ratio. The nucleus presented a folded structure and the cells did not attach to the culture surface. Therefore, since they do not attach, they are easily lost during washing steps of the cultures. This characteristic might have been the reason why in past studies this cell type was never considered as a haemocyte subpopulation. However, there are reports describing cells with similar morphological and behavioural characteristics. These cells could be classified either as prohaemocytes or immature haemocytes (Roulston and Smith, 2011), small hyaline cells (Rodriguez *et al.*, 1995), small granule haemocytes or lymphocyte-like hyalinocytes (Hose *et al.*, 1987; Vargas-Albores *et al.*, 2005). In Chapter 3 of this thesis, the existence of similar cells in *M. rosenbergii* was also confirmed. These cells were morphologically different from the ones of *P. vannamei* (spindle shaped instead of round shaped) but the behaviour in culture was comparable. They did not attach and moved erratically over the culture surface. Moreover, the percentage of these cells in the haemolymph of *M. rosenbergii* was also similar to that in haemolymph of *P. vannamei*. Therefore, their existence in such different crustacean species (one marine and another from freshwater) makes their existence also likely in other crustaceans. This generates questions about their functional role in immunity. Our work demonstrated that they

are a separate haemocyte subpopulation with distinctive morphological and behavioural characteristics. Since they do not express adherence/diapedesis behaviour and their cytoplasm is small, they are most likely not phagocytes. The presence of cytoplasmatic granules indicates their possible involvement in the production of immune related compounds. Nevertheless, future research will be needed in order to determine the exact functionality of these and the other haemocyte subpopulations. It could be argued that the haemocyte subpopulations separated in this study could be easily identified and classified using monoclonal antibodies against crustacean haemocytes subpopulations obtained in previous studies (Rodriguez *et al.*, 1995; Sung *et al.*, 1999; van de Braak *et al.*, 2000; Zhan *et al.*, 2001; Winotaphan *et al.*, 2005). However, none of these studies produced antibodies completely specific for the main 3 haemocytes subpopulations, which certainly would limit the use of these tools in our work. Moreover, our study identified 2 undescribed subpopulations against which no antibodies were ever produced. Works on other invertebrate species, as for example the mussel *Mytilus edulis* (Dyrynda *et al.*, 1997), were able to raise subpopulation-specific antibodies. Nevertheless, the physiological differences between bivalves and crustaceans and the variation in the haemocyte classification system, makes the use of these antibodies in *P. vannamei* unlikely of reaching useful results. Given this, a complete and accurate functional classification of our *P. vannamei* haemocyte subpopulations must enclose tests to detect the presence and activity of all known shrimp haemocyte immune-related components, and/or the production of new specific monoclonal antibodies against each of the 5 subpopulations. The latter approach encompasses good chances of success due to the unprecedentedly high purity obtained in the separation of at least 3 of the subpopulations. In order to maximise the chance success of this work, the strategy to follow should be based on a proteomic screening of subpopulation-specific molecules. Practically, this haemocyte separation technique can be useful in the study of immune processes performed (or mediated) by specific haemocyte types. This can also give new insights in the level of specificity of shrimp immunity towards certain pathogens. The clear identification of one or more subpopulations that are involved in antiviral defence and the production of antiviral molecules, can give new information to support the design of strategies to control WSSV outbreaks.

The purification of virus suspensions is a useful tool in the study of virus-pathogen interactions. Impurities such as particulate material (e.g. cell debris and subcellular

structures), proteins and toxic molecules (e.g. quinones and ROS), that are normally present in virus suspensions extracted from animal tissues, fluids and cell cultures, can negatively influence the evaluation of immunity *in vitro*. The density gradient centrifugation is still the most widely used technique for purification of viral suspensions. This methodology offers the best cost-benefit relation. It can isolate (depending on the source and type of virus) pure viral suspensions without excessively compromising the structure and functionally of the virus particles (if applied properly). Moreover, it is still a simple, cheap and a relatively easy procedure to execute. Fundamental characteristics that play a role in the choice of gradient media are toxicity, viscosity, ionic strength and osmolality (Lawrence and Steward, 2010). Among the exiting protocols for purification of WSSV, most used gradients were composed by sucrose (Wang *et al.*, 1995; van Hulten and Vlak, 2001; Tsai *et al*., 2004), CsCl (Chen *et al*., 2010) and NaBr (Huang *et al*., 2001). The use of these particular media has significant disadvantages. Sucrose forms solutions with high viscosity and osmolality. This originates a slow movement of particles in the gradient (long centrifugation times) and inactivation of the virus infectivity (Gias *et al.*, 2008). In CsCl gradients, the main disadvantage is the high ionic strength of the solutions, which disrupt protein-protein and nucleic acid-protein bonds. This will unavoidably originate unwanted inactivation of virus during centrifugation (Lawrence and Steward, 2010). The main disadvantage of NaBr is its toxicity and, thus, needs to be removed prior the inoculation of cell cultures with virus suspensions purified with this compound. Iodixanol on the other hand, does not present any of these disadvantages. Since the purification techniques that we presented in Chapter 6 used iodixanol as gradient media, our purification methodology automatically has advantages over the existing ones. Additionally, we made a more extensive characterization of the resulting viral suspension, mostly in terms of quantification of infectivity and number of particles, confirming the high reproducibility and efficiency of the procedures. We have proven that our methodology for purifying WSSV from infected shrimp haemolymph can produce highly pure virus suspensions with a high concentration of viral particles. The purification from infected tissues on the other hand, resulted in suspensions with an acceptable purity level and a very high concentration in viral particles. These techniques can both be useful depending on the application. As additional information, we have found that WSSV has a wider density distribution over the purification gradient and an unusually low average buoyant density and that

the virus appears more prone to inactivation by high centrifugal force than common viruses of terrestrial animals. This raised more questions about the origin and evolution of this peculiar virus and its strategies for host transmission and infection. The use of these purified inocula in combination with the haemocyte culture techniques for studying the shrimp antiviral defence, must be considered. The purification of WSSV, besides the obvious advantage of removing contaminants from the suspensions, also increased the concentration of infectious viral particles (lower ratio viral particles/infectious dose). This will allow a more accurate quantification of the antiviral activity of haemocyte subpopulations, immune metabolites and antiviral drugs. Moreover, the use of these inocula to infect shrimp primary cell cultures, will increase the chance of cell infection and consequently will facilitate the study of the underlying mechanisms.

In conclusion, we have fulfilled the aims of this thesis by making a substantial contribution to (i) a better understanding of the *in vitro* behaviour of shrimp haemocytes, (ii) how to improve and control haemocyte culture conditions, (iii) how to perform an efficient separation of haemocyte subpopulations and (iv) how to prepare high quality WSSV inocula. The combined application of these tools can help to in the study of shrimp immunity and its interactions with pathogens. This will eventually help unravelling the current disease-related problems and contribute for the sustainable development of the shrimp producing industry.

Finally, we would like to emphasise that this work created a solid ground for continuing existing WSSV/shrimp research lines, with the possibility for the creation of new ones. We considered this one of the biggest achievements of this PhD thesis.

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# Chapter 7

## Summary - Samenvatting

**Summary**

The knowledge on immunity of farmed crustaceans is still at an early developmental stage when compared to that of terrestrial livestock. There are many fundamental questions that still need to be answered and others are currently under academic debate. In contrast, the shrimp farming industry has experienced a huge expansion in the last four decades. The conjugation of these two factors originated the emergence of many infectious diseases that threaten the sustainable development of the shrimp farming industry. Shrimp viruses are the biggest threat. They are associated with high mortalities, which provoke huge economic losses every year. The solution for these problems requires a deeper knowledge on shrimp immunity.

In chapter 1, we provided a general overview of the current status of *Penaeus vannamei* and *Macrobrachium rosenbergii* farming and general characteristics of white spot syndrome virus (WSSV). Furthermore, we made a more detailed overview of the current knowledge on crustacean immune system and techniques for WSSV purification and haemocyte subpopulations separation.

In chapter 2, we introduced the present status of the global shrimp aquaculture industry and associated problems. We linked the relative lack of knowledge on crustacean immunity with the prevalent disease problems affecting shrimp farming. Therefore, the need for a deeper knowledge on the mechanisms of shrimp immunity was highlighted. This existing state of affairs led us to outline the main aim of this PhD thesis. We aimed to develop laboratory tools that would serve as a basis for studies on shrimp immunity and consequently contribute for the control of shrimp farming-associated problems.

In chapter 3 we aimed to establish improved *in vitro* systems for culturing crustacean haemocytes. The main objective was to develop procedures that are reproducible and to present them with enough detail for making its repetition easy. In part 3.1 we have focused on the culture of *P. vannamei* haemocytes. Here, we established techniques to culture haemocytes in suspension and in attachment, and distinguished between fractions of adherent and non-adherent cells. Parameters such as number of living adherent and single cells, number and average diameter of clusters and survival of cells inside clusters were evaluated during the culture period. Additionally, we have tested the influence of a major antioxidant (L-glutathione) on haemocyte survival and haemocyte cluster formation and maturation. In order to prove that the cells cultured

under these systems were functionally active, we inoculated them with *Vibrio campbellii* and evaluated parameters such as bacteria inactivation and phagocytic activity. L-glutathione supplementation clearly improved haemocyte survival up to 48h and delayed clustering and melanisation. After one hour of co-culture of haemocytes and *V. campbellii*, 11.5±0.14% of haemocytes showed phagocytosis with an average of 2.4±0.1 bacteria internalised per haemocyte. Furthermore, haemocytes clearly demonstrated an antibacterial activity. In part 3.2 we have adapted these culture techniques to the freshwater prawn *M. rosenbergii* and evaluated the immune parameters as established for *P. vannamei*. Therefore, it was possible to compare the behaviour of haemocytes of both species under similar *in vitro* culture conditions. Haemocytes of *M. rosenbergii* were less reactive (slower formation of haemocyte clusters) and survive longer. These differences made *M. rosenbergii* haemocytes easier to isolate and to handle under *in vitro* conditions. Consequently, *M. rosenbergii* appeared as a suitable alternative to *P. vannamei* for performing studies on general crustacean immunity.

In chapter 4 the main objective was to establish an improved methodology for separation of *P. vannamei* haemocyte subpopulations. The separation was made using a two-step iodixanol density gradient centrifugation. Three bands were formed in the first gradient: two bands with lower density close together, and a third band with higher density. The first two bands were collected together and centrifuged through a second gradient which was designed for promoting their separation. The separated cells were cultured *in vitro* and their survival quantified. Their morphology was evaluated by flow cytometry and light microscopy. Moreover, the immune functionality of each haemocyte subpopulation was validated by analysis of their phagocytic activity. Each of the three bands contained a major cell type with distinct morphology and behaviour. The gradient fraction between the second and third band was also collected and analysed. It was found that this 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 as follows: Subpopulation 1, 2, 3+4 (dispersed cells) and 5. The purity level (percentage of the major cell type) of Subpopulations 1, 2 and 5 was, 95.0±1.0%, 97.7±1.2% and 99.4±0.8%, respectively. Cells of Subpopulation 2 showed the best survival time *in vitro* (up to 96 h) followed by cells from Subpopulation 1, Subpopulations 3+4 and Subpopulation 5. This separation technique proved to be a valid alternative to the

existing ones.

In chapter 5, we aimed to solve the problem of the lack of techniques for the purifying WSSV in an efficient way. This work described for the first time the purification of WSSV through iodixanol density gradients, using virus isolated from infected tissues and haemolymph of *P. vannamei*. The purification from tissues included a concentration step by centrifugation onto a 50% iodixanol cushion and a purification step by centrifugation through a discontinuous iodixanol gradient. The purification from infected haemolymph enclosed a dialysis step with a membrane of 1000 kDa and a purification step through the earlier iodixanol gradient. The gradients were collected in fractions and analysed in detail. This included the quantification of the number of viral particles, infectivity titre, total protein and viral protein content. The purification from infected tissues generated WSSV suspensions with a very high infectivity and an acceptable purity, while virus purified from haemolymph had a high infectivity and a very high purity. Additionally, it was observed that WSSV has an unusually low buoyant density and that it is very sensitive to high external pressures.

In chapter 6, the main findings of each individual work were discussed in an integrated way and conclusions were drawn. The technical knowledge generated in this thesis was considered to represent a solid ground for continuing the present research lines. The importance of a full control of the *in vitro* haemocyte culture conditions and the production of purified virus suspensions was also highlighted. Furthermore, the combined use of the techniques described in this thesis represents a valuable asset for studying the shrimp immune system mechanisms and its interaction with pathogens such as WSSV. This knowledge can eventually be used for designing strategies aiming to control the disease-related problems affecting shrimp farming. The practical application of these techniques was also analysed and it was concluded that they can be used for evaluating the effect of compounds and environmental factors on shrimp immunity.

**Samenvatting**

De kennis over de immuniteit van gekweekte schaaldieren bevindt zich nog in een vroeg ontwikkelingsstadium in vergelijking met terrestrisch vee. Vele fundamentele vragen zijn nog onbeantwoord en het onderwerp van academische discussie. De industriële kweek van garnalen kende daarentegen een enorme expansie in de laatste vier decennia. De samenloop van deze twee factoren heeft aanleiding gegeven tot de opkomst van vele besmettelijke ziekten die de duurzame ontwikkeling van de garnalenteelt bedreigen. Hierbij presenteren virussen zich als het grootste gevaar. Zij veroorzaken veel sterfte, wat leidt tot grote economische verliezen elk jaar. Om tot een oplossing te komen voor deze problemen is er nood aan een dieper inzicht in de immuniteit van garnalen.

In hoofdstuk 1 introduceerden we de kweek van *Penaeus vannamei* en *Macrobrachium rosenbergii* en bespraken we de algemene kenmerken van het white spot syndrome virus (WSSV). Verder schetsten we een overzicht van wat er geweten is over het afweersysteem van schaaldieren, het opzuiveren van WSSV en het scheiden van haemocyte subpopulaties.

In hoofdstuk 2 gaven we een algemeen overzicht van de stand van zaken van de garnalenaquacultuur in de wereld en de daarmee samenhangende problemen. We legden het verband tussen het relatieve gebrek aan kennis over de immuniteit van schaaldieren en de heersende ziekteproblemen in de garnalenteelt. Hieruit kwam naar voren dat er een nood bestaat om meer kennis te vergaren omtrent de afweer van garnalen. Het is vanuit deze situatie dat het hoofddoel van deze thesis werd afgelijnd: het ontwikkelen van laboratorium tools die konden dienen als basis voor studies op garnalenimmuniteit, om zo bij te dragen tot de controle van garnalenteelt-geassocieerde problemen.

In hoofdstuk 3 was de doelstelling om verbeterde *in vitro* systemen op punt te stellen om haemocyten van schaaldieren in cultuur te houden. Het voornaamste doel was om reproduceerbare procedures te ontwikkelen en deze voldoende gedetailleerd te documenteren zodat deze gemakkelijk te herhalen zouden zijn.

In deel 3.1 lag de focus op het in cultuur houden van *P. vannamei* haemocyten. Hierbij werden technieken ontwikkeld voor de cultuur van cellen in suspensie en van aangehechte cellen, en werd een onderscheid gemaakt tussen hechtende en niet-hechtende celfracties.

Tijdens de kweekperiode werden parameters geëvalueerd zoals het aantal levende, hechtende en individuele cellen, het aantal celclusters en hun gemiddelde diameter en de overleving van cellen binnenin clusters. Daarnaast hebben we de invloed getest van een belangrijke antioxidant (L-glutathione) op haemocyte overleving en haemocyte clustervorming en rijping. Om aan te tonen dat de cellen gekweekt in deze systemen functioneel actief waren, hebben we de celculturen geïnoculeerd met *Vibrio campbellii* en parameters geëvalueerd zoals bacteriële inactivatie en fagocytose. De supplementatie van L-glutathione resulteerde in een duidelijke verbetering van de overleving van haemocyten tot 48u en een vertraging van het aggregeren en melaniseren van cellen. Een uur nadat haemocyten en *V. campbellii* samen in cultuur werden gebracht, vertoonde 11,5±0,14% van de haemocytes fagocytose, met een gemiddelde van 2,4±0,1 opgenomen bacteriën per haemocyte. Tenslotte werd ook een duidelijke antibacteriële activiteit van de haemocyten aangetoond.

In deel 3.2 pasten we de kweektechnieken aan voor de zoetwatergarnaal *M. rosenbergii* en evalueerden we de immuniteitsparameters zoals voor *P. vannamei*. Daardoor werd het mogelijk om het gedrag van haemocyten van beide species te vergelijken onder gelijkaardige kweekomstandigheden. Haemocyten van *M. rosenbergii* bleken minder reactief (vormden trager clusters) en overleefden langer. Deze verschillen toonden aan dat *M. rosenbergii* haemocyten makkelijker te isoleren en hanteren waren onder *in vitro* condities. Bijgevolg ziet het er naar uit dat *M. rosenbergii* een geschikt alternatief kan zijn voor *P. vannamei* voor de uitvoering van onderzoek naar algemene immuniteit bij schaaldieren.

In hoofdstuk 4 was het hoofddoel om een verbeterde methodologie uit te werken voor de scheiding van *P. vannamei* haemocyten in subpopulaties. De scheiding werd bekomen met een twee-staps iodixanol dichtheidsgradiënt centrifugatie. Drie banden vormden zich in de eerste gradiënt: twee met een lage densiteit, dicht bij elkaar, en een derde band met een hogere densiteit. De eerste twee banden werden samen gecollecteerd en gecentrifugeerd in een tweede gradiënt welke aangemaakt was met het oog op een betere scheiding. De gescheide cellen werden in celcultuur gehouden en de overleving werd gekwantificeerd. De morfologie van de cellen werd geëvalueerd met behulp van flowcytometrie en lichtmicroscopie. Daarnaast werd ook de immuunfunctie van iedere haemocyten subpopulatie gevalideerd door middel van analyse van hun fagocytotische activiteit. Elk van de drie banden bevatte hoofdzakelijk één celtype met een bepaalde morfologie en gedrag. De gradiënt tussen

de tweede en de derde band werd ook verzameld en geanalyseerd. Deze fractie bevatte een mengeling van twee celtypes welke verschillend waren van de types in de banden. In de volgorde van verschijning vanaf de top van de gradiënt werden de celtypen als volgt genoemd: Subpopulatie 1, 2, 3+4 (verspreide cellen) en 5. De zuiverheid (percentage van het belangrijkste celtype) van subpopulaties 1, 2 and 5 was respectievelijk: 95,0±1,0%, 97,7±1,2% and 99,4±0,8%. De cellen in Subpopulatie 2 vertoonden de beste overleving *in vitro* (tot 96u), gevolgd door de cellen in Subpopulatie 1, Subpopulaties 3+4 en Subpopulatie 5. Deze scheidingstechniek bleek een goed alternatief voor de bestaande technieken te zijn.

In hoofdstuk 5, was de doelstelling een oplossing te vinden voor het gebrek aan technieken om WSSV op te zuiveren op een efficiënte manier. Dit werk beschreef voor de eerste maal de opzuivering van WSSV met behulp van iodixanol densiteitsgradiënten, gebruikmakend van virus geïsoleerd uit geïnfecteerde weefsels en haemolymfe van *P. vannamei*. De opzuivering vanuit weefsels omvatte een concentratie stap via centrifugatie op een 50% iodixanol kussen en een zuiveringsstap doormiddel van een centrifugatie door een discontinue iodixanol gradiënt. De opzuivering vetrekkende van geïnfecteerde haemolymfe omvatte een dialyse stap met een 1000 kDa membraan en een zuiveringsstap door een discontinue iodixanol gradiënt. De gradiënten werden in fracties verzameld en in detail geanalyseerd. Hierbij kwantificeerden we het aantal virusdeeltjes, de infectieuze titer en de hoeveelheid totaal en viraal eiwit. De opzuivering vetrekkende van geïnfecteerd weefsel leverde een WSSV suspensie op met een zeer hoge infectiviteit en een aanvaardbare puurheid, terwijl virus opgezuiverd vanuit haemolymfe een hoge infectiviteit had en een zeer hoge zuiverheid. Bijkomend werd vastgesteld dat WSSV een ongewoon laag drijvend vermogen bezit en erg gevoelig is voor grote externe druk.

In hoofdstuk 6 werden de belangrijkste bevindingen van elk individueel werk geïntegreerd en besproken, en werden er conclusies getrokken. De technische kennis die in dit proefschrift werd gegenereerd kan als een stevige basis worden beschouwd voor de vederzetting van de huidige onderzoekslijnen. Het belang van een volledige controle over de celcultuur condities voor haemocyten en de productie van zuivere virussuspensies werd onderlijnd. Bovendien is het gecombineerde gebruik van de technieken beschreven in dit proefschrift een waardevolle aanwinst voor het bestuderen van de werking van het immuunsysteem van garnalen en de interactie met

ziekteverwekkers zoals WSSV. Deze kennis kan uiteindelijk worden gebruikt voor het ontwerpen van strategieën die gericht zijn op het controleren van de ziekte-gerelateerde problemen in de garnalenteelt. De praktische toepassing van deze technieken werd ook uitgetest en er werd vastgesteld dat zij gebruikt kunnen worden om het effect van chemische stoffen en omgevingsfactoren op garnalen immuniteit te evalueren.

# Curriculum vitae

João Lima was born on the 5th of April, 1979 in Ponte de Lima, Portugal.

In 2003 he obtained a degree in Zootechnical Engineer at the University of Trás-os-Montes e Alto Douro (UTAD, Vila Real, Portugal). At the end of this degree he made an internship at the department of aquaculture nutrition of the same university and wrote a thesis on "Partial substitution of fishmeal by macroalgae meal (*Gracilaria cornea*, *Ulva lactuca* and *Gracilaria bursa-pastoris*) in diets of seabass juveniles (*Dicentrarchus labrax*)". The same year he started an internship funded by the European Education & Training Programme Leonardo Da Vinci at the Spanish Oceanographic Institute (IEO Vigo, Spain), where he assisted in research on reproduction and grow-out of new fish species for marine aquaculture and improvement of *Artemia salina* growing techniques.

In 2004, he started a Master in Aquaculture and Fisheries at the University of Algarve (Faro, Portugal). In parallel, he worked as a research assistant at the National Research Institute for Fisheries and Sea (IPIMAR Olhão, Portugal), where he assisted in research on the development of hatchery, nursery and grow-out techniques of new fish species for marine aquaculture. During his studies, in 2005, he performed practical work at the Laboratory of Veterinary Virology of Ghent University, which resulted in a thesis on "Experiments on the Effect of Temperature on white spot syndrome virus Infection in *Litopenaeus vannamei* shrimp". This work was funded by the European Education & Training Programme Socrates/Erasmus.

As a continuation of this work, in 2007, he started his doctoral studies under the supervision of Prof. Dr. Hans Nauwynck at the Laboratory of Veterinary Virology. In 2008 he obtained a 4-year scholarship from the Foundation for Science and Technology (FCT, Portugal), after which he was employed for 6 months as research assistant by Gent University.

Since January 2013, he is working as a postdoctoral researcher at the Laboratory of Veterinary Virology.

João Lima is co-author of 7 scientific publications and he presented his work orally at 2 international conferences. He supervised 6 Master students.

**Publications**

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**Dantas-Lima J.J.**, Corteel M., Sorgeloos P., Nauwynck H. Establishment of *in vitro* haemocyte culture models for studying immune responses of penaeid and palaemonid shrimps. World Aquaculture 2011, June 7-10, Natal, Brazil.

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# Acknowledgements

The achievement of this high degree of education and professionalism was a long and difficult task that required a lot of my dedication and effort. This experience was, nevertheless, rewarding and interesting. During this time, my path crossed with many of you who had a role in this process (sometimes bigger than you imagine). You supported me in many different ways, being role models, providing an inspiration, motivation, understanding, friendship, love or even plain physical help and financial support. Some of you provided me with all of this at the same time. All of you became a part of me and made me the man I am today, the man who is now completing this PhD degree.

In particular, I would like to thank:

Prof. Dr. Hans J. Nauwynck (promoter) for having believed in my potential and accepted me as student at the Laboratory of Veterinary Medicine of Ghent University. Thank you for all these years of coordination, scientific support, challenge, motivation, heated debates, pressure (I needed it!), criticism and endless will of going further and achieving more. Thank you for continuing believing in me and in my future endeavours.

My co-promoter, Prof. Dr. Patrick Sorgeloos, for accepting me as his student and for giving me the opportunity of being a part of such an important laboratory in the world´s aquaculture scene. Thank you for the scientific support, criticism, unconditional availability, mediation and diplomacy. Thank you also for believing in my future.

The members of the examination committee, Prof. Dr. Piet Deprez (Chairman), Prof. Dr. Annemie Decostere, Prof. Dr. Peter Bossier (also for all the past years of scientific support, constructive criticism and availability), Prof. Dr. Just Vlak, Prof. Dr. Valerie Smith, Dr. Mathias Corteel, Prof. Dr. Wim Van den Broeck (also for the past microscopy support), Prof. Dr. H. Favoreel (also for the past scientific support) and Prof. Dr. Em. M. Pensaert (also for the past advice). Thank you for contributing to bringing this thesis to a higher level.

Dr. Victoria Alday-Sanz for all the advice and support in various subjects and fields.

Prof. Dr. Maria Cornelissen, for the support with Transmission Electron Microscopy and for the paper review.

Prof. Dr. Ann Van Soom for the all support with lab instruments.

Dr. John Graham for the scientific advice on purification of viruses.

Professor Dr. Chu-Fang Lo for supplying precious WSSV antibodies.

Prof. Dr. Guy Smagghe for the support with insect cell lines and scientific discussions on shrimp cell cultures.

I would like to thank all friends and colleagues from work, Mathias (a book the size of this thesis would not be enough, and actually not advisable, to compile everything we lived together, inside and outside work in the last 6 years! Thanks!), Dries (another book! Thanks!), Uladzimir (….and yet another book! Thanks!), Constantinos (another kind of a book!), Georgios and Petro (not from work but also thanks!). Without you this task would have been Belgian weather style. Past and present members of the shrimp group, Cesar, Meezanur, Wenfeng, Thuong, Tuan, Tu, Lang, Bing, Tien, Edward (Chen's salt!) Aludee and An, thank you for all the challenges, discussions and help. I really learned a lot from you. All the friends and colleagues from the virology laboratory, Gerald, Sjouke, Sarah and Sarah, Annebel, Kalina, Miet, Kathlyn, Korneel, Dominique, Wander, Merijn, Ilias, Lesli, Sabrina, Amy, Nik, Hanne, Hannah, Mieke, and many others , who helped me (physically and mentally!) to go on through this task more easily and pleasantly. To Anamaria, Marlien, Annelies and Lobke, thank you for the help, discussions and good atmosphere you created! The veterinary Portuguese and Spanish crew, thanks!

Margriet and Thomas, thanks for the motivation to finish this step and to start the next one!

Lieve, Carine, Nele, Chantal, Zerger, Bart, Fernand, Karen, Melanie and Tim, the staff from the Laboratory of Virology, thank you for all the help and encouragement. Without you this would not have been possible.

Leen, for all the friendly help with the TEM.

Tom (tubes, pumps, glue & social life!), Mathieu and Jorg (for nannying the shrimp and shrimp group!) and Brigitte, the staff for the Laboratory of Aquaculture, thank you for everything.

The Gent "Family", Ani (The Mother), Jorge, Fernando, Petia, Alexandrina, Aleksandra (the siblings) and Kostas (The !Grand! Father), I can't imagine this adventure without you (another set of books for the encyclopedia!). Many, many thanks!

Thank you to those who I forgot to mention (not on purpose!), but somehow also contributed to the success of this task.

Also to those who never believed in me, thank you for giving me extra motivation!

Finalmente à minha família, Pai Nico (Fernando), Mãe Isabel, Irmão Tico (Fernando

Guilherme), Avós Maria e Amália, Avôs Zé Tordo e Zé Corinheira, (Tias), Mina, Gusta, Mena, Filipa, Lola, (Tios) Filinto, Chico, Zequinha, Miguel, Zé Greu, (Primas) Márcia, Tânia e Jéssica, (Primos) Nando e Zé Miguel, não existem palavras para descrever a minha gratidão e apreço. Obrigados por aquilo que sou hoje e por aquilo que serei no futuro!

Elija (and my daughter to be born), thank you for all the support, patience, motivation and love…. this is also your achievement! Thank you.