Faculty of Veterinary Medicine

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Development and use of standardized white spot syndrome virus (WSSV) inoculation procedures for studies on pathogenesis and control

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List of acronyms

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AMPs	Antimicrobial peptides
βGBP	Beta glucan-binding protein
bp	Base pairs
CO_2	Carbon dioxide
DAB	3,3-diaminobenzidine
DABCO	1,4-diazobicyclo-2,2,2,-octane
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FITC	Fluorescein isothiocyanate
FREP	fibrinogen-related protein
ha	Hectare (10 000 m^2)
HHNBV	Hypodermal and hematopoietic necrosis baculovirus
hpi	Hours post inoculation
IE	Immediate early
IIF	Indirect immunofluorescence
IHHNV	Infectious hypodermal and hematopoietic necrosis virus
IgG	Immunoglobulin G
IHC	Immunohistochemistry
im	Intramuscular
Imd	Immunodeficiency
ISH	*In situ* hybridization
Kbp	Kilobasepairs
KDa	Kilodalton
*Kpn*I	*Klebsiella pneumoniae* endonuclease I
L-DOPA	L-3,4-dihydroxyphenilalanine
LPS	Lipopolysaccharide
LBP	Lipopolysaccharide-binding protein
LT_{50}	Median lethal time
Mab	Monoclonal antibody

mg	Milligram
ml	Milliliter
mm^{-2}	Square millimeter
mM	Millimolar
mRNA	Messenger RNA
MT	Metric ton (1000 kg)
µl	Microliter
µm	Micrometer
µM	Micromolar
NF-κB	Nuclear Factor kappa B
nm	Nanometer (10^{-9} m)
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain-reaction
PG	Peptidoglycan
PGBP	Peptidoglycan-binding protein
pH	A measure of the activity of hydrogen ions (H+) in a solution
PmNOB III	Third *Penaeus monodon* non-occluded baculovirus
PO	Phenoloxidase
PRDV	Penaeid rod-shaped DNA virus
PRP	Pattern recognition peptides
proPO	Prophenoloxidase
PRPs	Pattern recognition peptides
RFLP	Restriction fragment length polymorphism
RIP	Receptor-interacting protein of TNF
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RV-PJ	Rod-shaped nuclear virus of *Marsupenaeus japonicus*
SEMBV	Systemic ectodermal and mesodermal baculovirus
SID_{50}	Shrimp infectious dose with 50% endpoint
SiRNA	Silencing RNA interference
SPF	Specific pathogen-free

TAE	Tris acetate EDTA buffer pH 7.2
TIS	Transcription initiation site
TNF	Tumor necrosis factor
TSV	Taura syndrome virus
VP	Viral protein
WB	Western blot
WSBV	White spot baculovirus
WSSV	White spot syndrome virus

CHAPTER 1

INTRODUCTION

“The roots of Science are bitter, but the fruits are sweet” - Aristotle (384-322 BC)

INTRODUCTION

1.1 Shrimp aquaculture

Shrimp culture probably originated thousands of years ago when people living in coastal areas incidentally harvested shrimp growing in estuaries and tidal ponds (Fast 1992; Martínez-Córdoba & Peña-Messina 2005). In Asia, shrimp larvae entered milkfish ponds during tidal exchange or were intentionally collected from the wild and directly stocked in ponds. Here, the production was dependent on the seasonal abundance of wild larvae which has yearly variations (Kungvankij 1985). This practice later evolved into the extensive culture systems performed nowadays. Coastal lagoons of several hectares (ha) in size are stocked at a low density (≤ 10 animals m^{-2}) with wild larvae. These larvae develop into juvenile or subadult stages with little human intervention (Fast 1992). In this system, production can reach up to 1 metric ton (MT) ha^{-1} $year^{-1}$ and the culture may last for several months (Fast 1992, Wickins & Lee 2002).

Modern shrimp farming started in Japan in the 1930s with the successful spawning and larviculture of *Marsupenaeus japonicus* under laboratory conditions (Rosenberry 2001). Later, technological advances of artificial fertilization, mass seed production and feed formulation for penaeid shrimp (Lotz 1997; Hsu et al. 2000) contributed to the appearance of the semi-intensive and intensive culture systems. In the semi-intensive system, wild or hatchery-raised postlarvae are stocked at densities of 20 - 60 shrimp m^{-2}, the size of ponds is smaller (2 - 12 ha) and the productivity per hectare may reach 12 - 15 MT ha^{-1} $year^{-1}$. This system requires supply of artificial food, fertilization of the water, additional aeration and water exchange. The intensive system is performed in small ponds (≤ 1 ha), stocked at high density (> 60 shrimp m^{-2}) with postlarvae from hatcheries. It gives a high production (up to 50 MT ha^{-1} $year^{-1}$). This system requires a high water exchange rate and it relies exclusively on artificial feed. Such a system may yield more than two harvests per year (Fast 1992; Wickins & Lee 2002).

The extensive and semi-intensive shrimp farming systems are currently practiced in more than 55 countries. In 2004, the major penaeid shrimp farming countries were: China (935 944 MT), Thailand (390 000 MT), Vietnam (275 569 MT), Indonesia (238 341 MT), India (133 020 MT), Brazil (75 904 MT), Mexico (62 361 MT), Bangladesh (58 044 MT), Ecuador (56 300 MT), Philippines (37 947 MT) and Malaysia (30 838

MT). That year, world farmed penaeid shrimp production reached 2 475 508 MT worth 9.74 billion USD. The main cultured species were *Litopenaeus vannamei* (56% of the world total farmed penaeid shrimp) and *Penaeus monodon* (29%) (FAO 2006).

Shrimp aquaculture has developed and expanded rapidly and is currently recognized as a potential long-term sustainable industry for many tropical countries (Flegel 1997). However, the intensification has also produced a number of problems affecting the industry (Flegel 1997; Alabi et al. 2000). These include environmental (fluctuations in water quality and toxicants from industrial and agricultural origin) and physiological stress factors (overcrowding, overfeeding and lack of essential nutrients) that are often related to disease and mortality (Hsu et al. 2000; Yusoff et al. 2001). These elements have been related to an increased susceptibility to infectious diseases (Lightner & Redman 1998; Hsu et al. 2000).

1.2 The shrimp *Litopenaeus vannamei*

The white-legged shrimp *Litopenaeus vannamei* has been cultured in America since the beginning of the industry in 1969. Although species such as *L. stylirostris* are also attractive for aquaculture, a number of characteristics have made the white-legged shrimp the preferred species for aquaculture not only in America but also in many Asian countries: (1) its remarkable ability to acclimatize to broad environmental variations and (2) its good performance in conditions of limited food diversity. This characteristic is related to its omnivorous feeding habits that include organic matter and detritus from plants and animals. Also, *L. vannamei* has a relatively low requirement of dietary protein and good feed conversion efficiency. These features are very attractive in aquaculture (Moss & Pruder 1995; Martínez-Córdoba & Peña-Messina 2005).

1.2.1 Biology

The white-legged shrimp *L. vannamei* belongs to the decapod crustaceans which is probably one of the most diverse and complex arthropod groups. The taxonomic position of this species is described below (Pérez-Farfante & Kensley 1997; Martin & Davis 2001):

Domain Eukarya
Kingdom Animalia, Linnaeus 1758
Phylum Arthropoda, Latreille 1829
Subphylum Crustacea, Brünnich 1772
Class Malacostraca, Latreille 1802
Subclass Eumalacostraca, Gröbben 1892
Superorder Eucarida, Calman 1904
Order Decapoda, Latreille 1802
Suborder Dendrobranchiata, Bate 1888
Superfamily Penaeoidea, Rafinesque 1815
Family Penaeidae, Rafinesque 1815
Genus *Litopenaeus*, Pérez Farfante 1969
Species *Litopenaeus vannamei* Boone 1931

The shrimp *L. vannamei* is an American species distributed in the Pacific coast, from the Gulf of California, Mexico (28° N, 112°W) to Peru (5° S, 83°W). This species inhabits muddy bottoms from the shoreline (3-5 m) down to 72 m. Adult shrimp are found in warm coastal waters up to 32 km off-shore while larvae and juveniles are found near protected coastal areas such as estuaries and coastal lagoons which serve as nursing grounds rich in food sources (Bailey-Brock & Moss 1992; Primavera, 1998, Sánchez 1997; Eggleston et al. 1999; Corona et al. 2000). The main parameters of water quality in natural environments and culture conditions include temperature, salinity, dissolved oxygen, turbidity, pH and presence of toxic metabolites such as ammonia, nitrites and hydrogen sulfide (Bray & Lawrence 1992; Brock & Main 1994). In estuaries, temperature and salinity show drastic variations according to the season and the interaction between these parameters often determines shrimp distribution and abundance (Lester & Pante 1992; Wyban et al. 1995).

The white-legged shrimp mates and spawns in off-shore waters where fertilized eggs are released as zooplankton in the ocean. Embryological development begins within minutes after fertilization. Fourteen hours later, the first larval stage or nauplius hatches. This stage has six instars (the interval of time between molts) which are completed 1.5 days after hatching. A nauplius has a pear-shaped form with only three pairs of

appendages: antennae 1, 2 and mandibles. A primitive carapace and a furcal tail develop later. Nauplii are zooplanktonic and migrate from oceanic waters to coastal nursing areas driven by tides and waves. The antennae are used for swimming which is very limited. The second larval stage or protozoea has three instars which are completed five days after nauplius. Here the carapace and rostrum develop; compound eyes and rudiments of maxillipeds, pereopods, uropods and telson appear. The third larval stage or mysis also has three instars lasting five days. Here, major metamorphoses occur: the carapace fuses to the pereon; maxillipeds, pereopods and uropods become fully functional and the pleopods first appear. The animal increases in size and the sixth pleonite is larger than all the other pleonites. The last stage of larval development or postlarva lasts several days. A postlarva is morphologically similar to the adult (Bray & Lawrence 1992). At this stage, shrimp enter into nursing coastal areas swimming with pleopods and change habits from zooplanktonic to epibenthic (Sánchez 1997; Corona et al. 2000). Shrimp reach full morphology and function within twenty days after reaching the postlarval stage (pl-20). Postlarvae only grow in size to become juvenile and leave nursing areas (Sánchez 1997). Juvenile still do not develop sexual characters. The ratio of the length of the sixth pleonite to carapace is higher (0.68) than adults (0.52). This stage lasts three to four months. Subadults first show sexual dimorphism (development of petasma and *appendix masculina* in male or thelycum in female) and usually perform the first copulation. This stage last up to four months after the juvenile stage. Adults appear four to seven months after subadult, are sexually mature and show complete dimorphism where females are somewhat larger than males (Bray & Lawrence 1992). The lifespan of shrimp is about 1.5 to 2 years, they can spawn several times and the females can produce in a single spawn between 100 to 500 thousand eggs (Bray & Lawrence 1992).

Feeding habits of shrimp change according to the developmental stage. Larval stages feed upon phyto- or zoo-plankton. When postlarvae change from planktonic to epibenthic they feed upon detritus of animal and/or vegetal origin, epibenthic microalgae, plants, nematodes, copepods, tanaids and larvae from molluscs and other crustaceans. The size of prey increases as the shrimp grow, so the diet of juvenile shrimp include mysid and caridean shrimp, amphipods, polychaetes, molluscs and even fish (Bailey-Brock & Moss 1992). In culture conditions, the abundance of prey is

reduced; therefore shrimp has to rely heavily on the fraction of particulate organic matter and/or detritus generated by the dynamics of culture systems in addition to artificial diets (Moss & Pruder 1995; Martínez-Córdoba & Peña-Messina 2005).

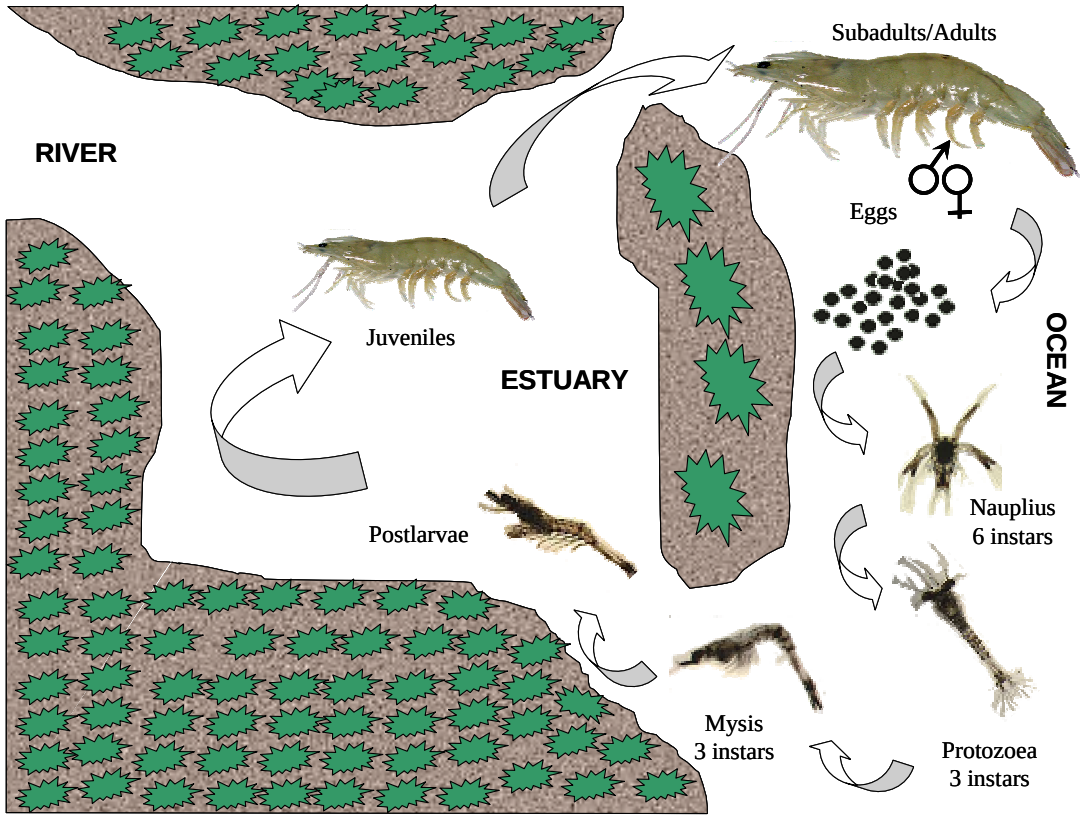


Figure 1. Life cycle of *L. vannamei* (size of the different stages is not in scale)

1.2.2 Morphology

The white-legged shrimp is well adapted to the aquatic environment. It has a laterally-compressed, cylindrical body with bilateral symmetry. Three distinctive external regions are recognized: pereon, pleon and telson (Brusca & Brusca 1990) (Figure 2).

The pereon is formed by the fusion of 13 body segments: five from the head (acron) and eight from the thorax. Each segment of the head bears a pair of limbs which perform sensory or feeding functions (two antennae, one mandible and two maxillae), while the limbs from the thorax perform preening and crawling functions (three pairs of maxillipeds and five pairs of pereopods) (Budd 2002). The pereon is covered by a carapace with a dorsal keel-shaped rostrum.

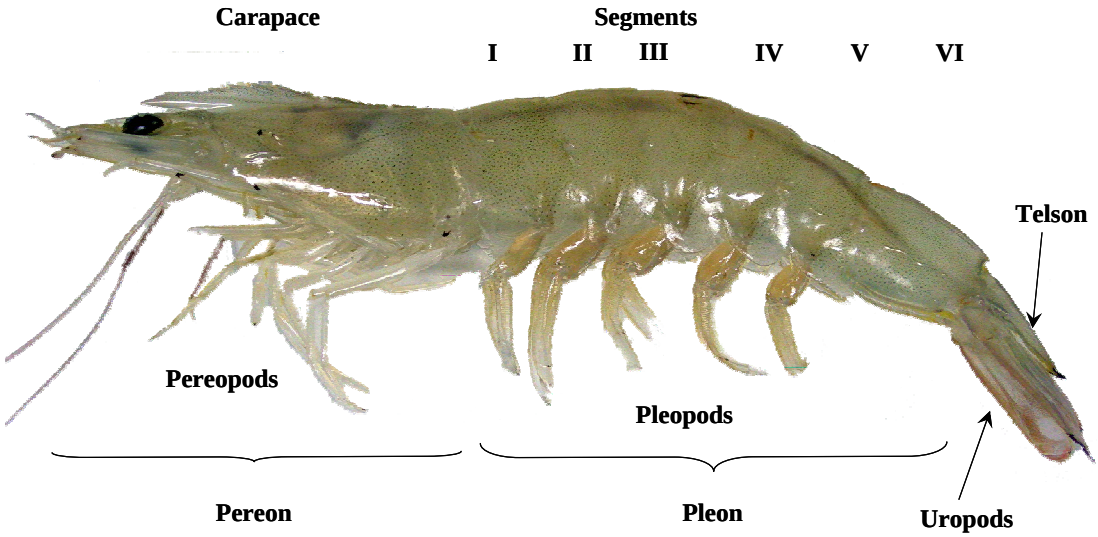


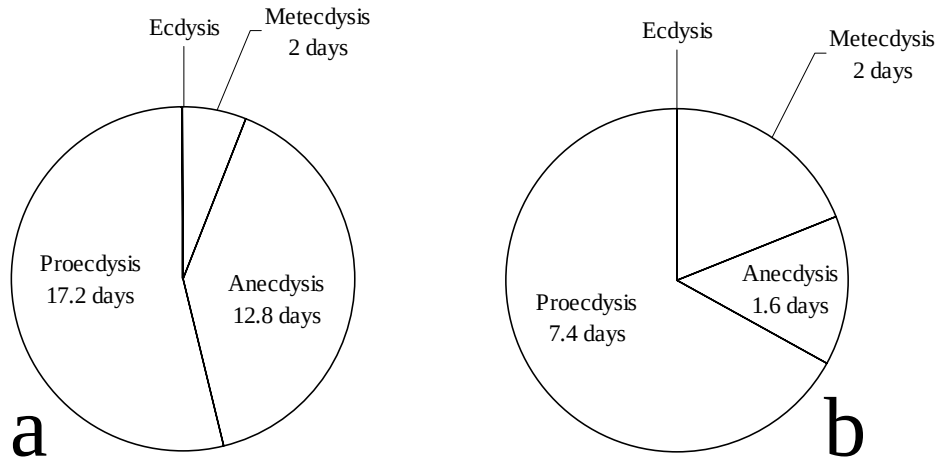
Figure 2. External morphology of *L. vannamei*

The pleon has six segments mainly composed by muscle. Other structures present in this region are gonads, the posterior artery and digestive tract (midgut trunk and hindgut). This region is controlled by the ventral nerve cord and segmental ganglia. Each of the first five segments bears one pair of appendages (pleopods) used for swimming, burrowing and ventilation. The last segment has one pair of uropods. The telson is a flattened structure surrounded by the uropods, which together form the tail fan and is used in escape swimming (Ruppert & Barnes 1994).

Integument - this organ covers and protects the body of shrimp and is a primary defense barrier against pathogen penetration into the body (Armstrong & Quigley 1999; Tincu & Taylor 2004). The integument consists of a bio-mineralized cuticle produced by a monolayer of epithelial cells, a basal membrane and subjacent connective tissues (Felgenhauer 1992a; Wilt et al. 2003; Compére et al. 2004). Chitin (a linear homopolymer of β-1,4-N-acetyl-D-glucosamine with a molecular weight of 10^3 KDa) is the major component of the cuticle (50-75% of decalcified dry weight) together with proteins which constitute 30-40%.

Molting, also known as ecdysis, is a complex process for growth by which shrimp sheds the old exoskeleton and produces a new one. The molting cycle occurs within an instar and its duration increases as shrimp grow older. Molting influences other functions such

as development (from nauplius to larvae, juvenile and adult), growth, regeneration, hematopoiesis and defense response (Skinner 1985; Le Moullac et al. 1997; Chang et al. 2001; Liu et al. 2004). Molting has four main phases: metecdysis (postmolt), anecdysis (intermolt), proecdysis (premolt) and ecdysis (molting) (Figure 3) (Kaestner 1970; Skinner 1985; Chan et al. 1988). Molting is controlled by neurohormones but it is also influenced by environmental factors such as temperature, light and salinity (Kaestner 1970; Skinner 1985) which in turn influence metabolism and neurohormone production.



Duration of one instar = 32 days at 22°C **Duration of one instar = 11 days at 28°C**

Figure 3. Duration of molting stages in *L. vannamei*: (a) 22 °C and 30 g l^{-1} salinity (Chan et al. 1988); (b) 27°C and 35 g l^{-1} salinity (M. Corteel, unpublished results)

Digestive system - the digestive tract of shrimp is divided in three regions: foregut, midgut and hindgut (Figure 4). The foregut and hindgut derive from the ectoderm and the epithelium in these regions is lined with cuticle that is continuous with the exoskeleton. The midgut derives from the endoderm and the epithelial tissues in this region are not covered by cuticle (Brusca & Brusca 1990; Icely & Nott 1992).

The mouth is located sub-ventrally in the pereon underneath the labrum and anterior to the oral appendages. The labrum is a fleshy structure which may prevent reflux of ingested food. The mandibles are grinding structures composed of a molar, an incisor process and a flattened mandibular palp. This palp helps the labrum to push food items into the mouth (Pérez-Farfante & Kenlsey 1997; Garm 2004). The maxilla 1 assists in holding and pushing food items between the mandibles. The maxilla 2 is involved in preening food items and it has an exopod (scaphognatite) which serves as gill bailor

(Garm et al. 2003). The maxillipeds (three pairs) are appendages which assist in gathering and processing food and grooming the gills (Bell & Lightner 1988; Ceccaldi 1997; Bauer 1999).

The foregut is divided into the esophagus and stomach. The epithelium of the foregut is lined by cuticle (140 µm thick) which lacks the epicuticle layer (Icely & Nott 1992; Ceccaldi 1998). The esophagus is a short slender tube made of simple columnar epithelial cells surrounded by muscle acting as a sphincter. It contains numerous tegumental glands which secrete mucus that may lubricate the passage of food (Fingerman 1992; Ceccaldi 1998).

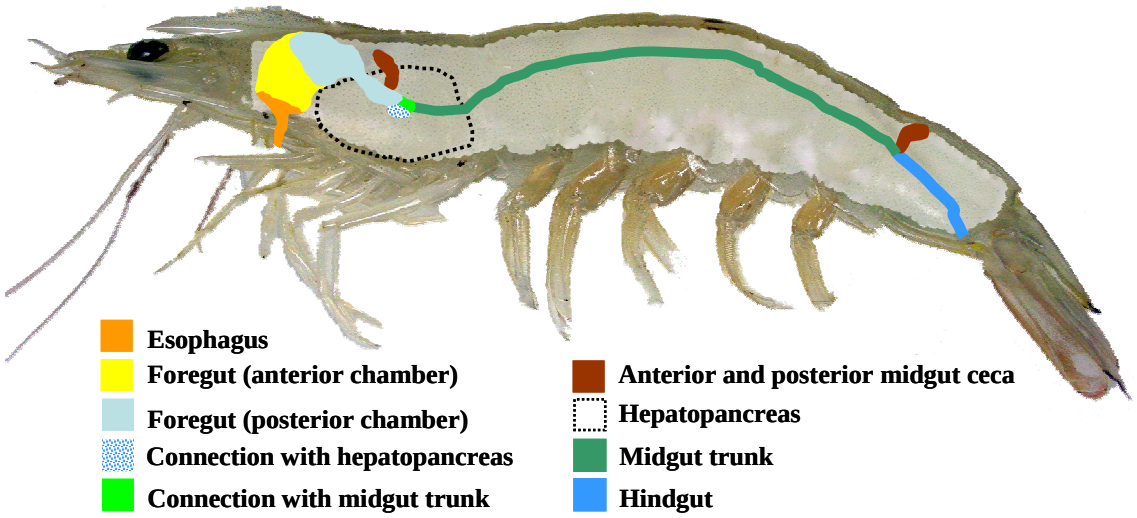


Figure 4. Structure of the digestive system of *L. vannamei*

The stomach performs: (1) physical breakdown of ingested material by mastication, (2) the chemical digestion of food, (3) separation of fine particles and fluids from coarse materials by filtration and (4) transport of fine particles and fluids to the hepatopancreas and transport of undigested materials to the intestine for excretion (Icely & Nott 1992). The stomach is divided into an anterior and posterior chamber: The anterior chamber is muscular and has cuticular tooth-like projections used in food grinding and for this reason it is called gastric mill. The posterior chamber is divided into a dorsal and a ventral sub-chamber. The ventral sub-chamber has a gastric sieve formed by spiny cuticular projections which filter tiny food particles and fluids into the hepatopancreas. Coarse food particles are sent back to the gastric mill for further grinding or towards the intestine for removal (Ceccaldi 1997). The dorsal chamber is beneath the anterior

midgut cecum. This chamber squeezes the residual bolus to extract fluids and fine particles which go to the filter gland, while the compact residues are sent to the intestine (Icely & Nott 1992; Ruppert & Barnes 1994).

The midgut extends from the foregut in the pereon to the hindgut in the sixth segment of the pleon. It is composed by the anterior and posterior midgut ceca, the hepatopancreas or midgut gland and the midgut trunk or intestine (Figure 4) (Icely & Nott 1992; Martin & Chiu 2003). The anterior and posterior midgut ceca and midgut trunk are lined with simple columnar epithelial cells with a basal lamina which becomes thin (0.8 µm) in the intestine. These cells have many apical microvilli with electrondense tips. The anterior midgut cecum is a blind sack with large epithelial folds projecting into the lumen. Bacteria are usually found here and may help to perform biodigestion of food and synthesis of vitamins. The intestine is composed by the following layers from the lumen inside: peritrophic membrane, epithelium, basal lamina, hemocyte layer (exclusively composed by granulocytes), connective tissue layer (which contains one layer of circular muscles and another of longitudinal muscles) and the outer intima (Martin & Chiu 2003). The peritrophic membrane is produced by delamination of epithelial cells in the midgut and its function is to protect the midgut epithelium from mechanical damage, pathogens, toxins and other damaging chemicals (Lehane 1997).

The hepatopancreas is the master digestive gland. It performs functions such as synthesis and secretion of digestive enzymes, absoption of nutrients, storage of mineral reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during intermolt stage, calcium absorption, etc. (Icely & Nott 1992; Ceccaldi 1997, 1998). In the shrimp, the hepatopancreas is bilobed and surrounds the stomach. A duct connects the filter gland of the stomach with the hepatopancreas. The hepatopancreas is composed by a series of blind-ended, finger-like tubules. The tubule walls are composed by simple columnar epithelial cells. The hepatopancreas of *L. vannamei* has four cell types: embryonic (E) undifferentiated cells that undergo active cell division (Caceci et al. 1988); fibrilar (F) cells produce enzymes and store iron; secretory or blister (B) cells contain one or two very large vacuoles filled with a flocculent material and perform absorption of nutrients and storage of glycogen, fats and calcium (Ruppert & Barnes 1994) and reservoir (R) cells that store lipids and glycogen (Icely & Nott 1992; Ceccaldi 1997).

The hindgut is the terminal part of the digestive tract. It is a simple epithelial tube lined with non-calcified cuticle that links the lumen at the posterior end of the intestine with the anus that opens onto the surface of the exoskeleton below the telson. The hindgut begins after the posterior midgut cecum. The junction between midgut and hindgut is composed of longitudinal folds in the hindgut wall. Tegumental glands are present in the hindgut. Some bacterial growth can be seen in hindgut due to secretions of the tegumental glands (Ceccaldi 1997). The anus is the posterior extremity of the gastrointestinal tract and is lined with a thick and calcified cuticle (Bell & Lightner 1988). The epithelial cells of the digestive system are also involved in water and ion balance (Ahearn et al. 1999; Wheatley 1999).

Respiratory system - the gills are the respiratory organs in shrimp. The genus *Litopenaeus* has 19 pairs of gills enclosed in a branchial chamber (Figure 5) (Foster & Howse 1976, 1978; Taylor & Taylor 1992; Pérez-Farfante & Kensely 1997). The gills are attached to the pereon by a tubular structure. The dendrobranchiate gills of shrimp have a tree-like shape, which consist of an axis that bears a series of paired branches at right angles along its length. Each branch gives rise to numerous perpendicularly-oriented filaments that bifurcate at least twice. A longitudinal septum divides the lumen of each tubular structure, axis, branch and filament into afferent (towards the gill) and efferent (away from the gill) branchial channels, which are lined by a basal lamina. A third, smaller channel is located between a layer of nephrocytes that faces the efferent hemolymph and the septum that separates the afferent and efferent channels in the axis and branches (Taylor & Taylor 1992). The interstitial spaces of the branchial tissues are often filled with hemolymph carried by this auxiliary channel (Foster & Howse 1978).

The gills are lined with uncalcified cuticle, which thickness varies from 16 µm in the axis to 2.5 µm in branches and less than 1.0 µm in the filaments. Pore canals (0.14 µm in diameter) enter the cuticle but they do not reach the epicuticle (0.16 µm thick in filaments) (Taylor & Taylor 1992). The gill epithelium is composed by a layer of cuboidal cells in the axis (8 - 10 µm thick in intermolt). In branches and filaments the epithelium is a thin sheet (1.1 - 0.1 µm) with a series of pillar processes (2 - 6 µm wide) that form lacunae beneath the cuticle (Foster & Howse 1978).

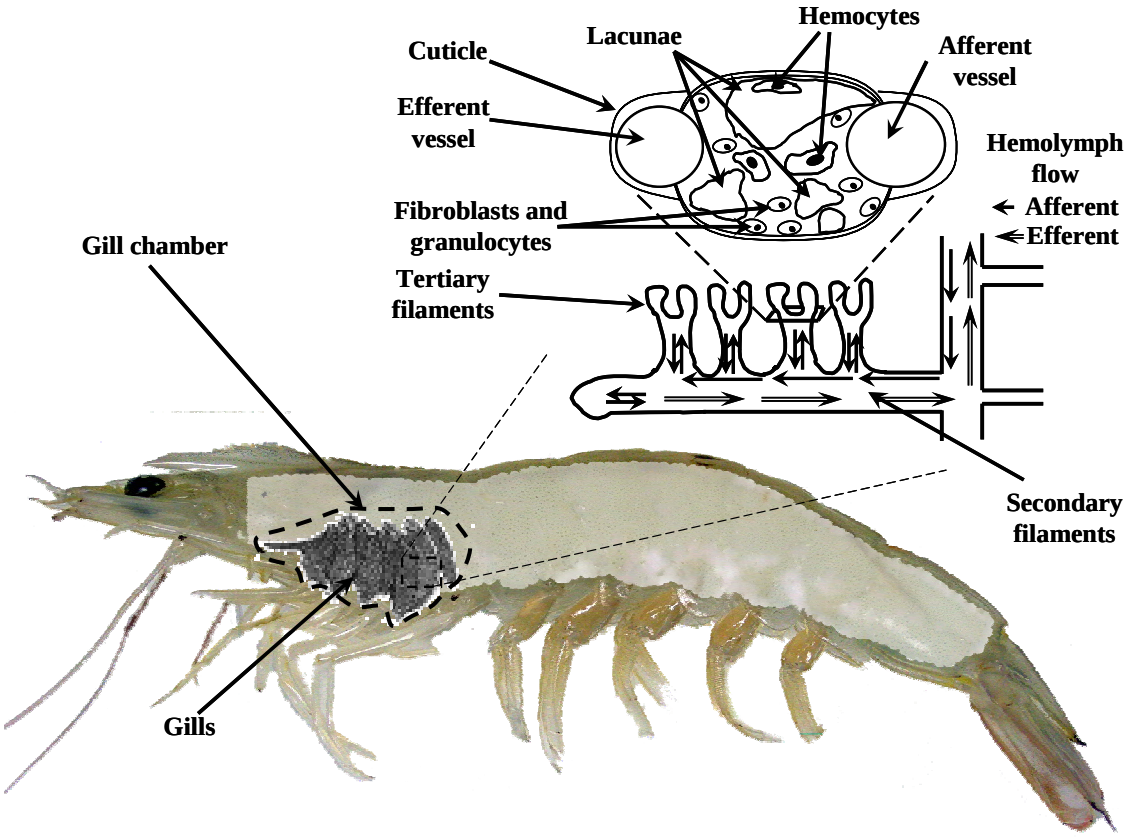


Figure 5. Location and general structure of the dendrobranchiate gills. This figure shows the secondary filaments and a cross-section detail of the tertiary filaments

Gills have specialized epithelial cells: (1) flange cells are the main cell type and may form lacunae (Foster & Howse 1978; Taylor & Taylor 1992); (2) pillar cells are support structures that separate lamellar septa and often define hemolymph flow routes and prevent distortion of filament shape by hemolymph pressure (Bell & Lightner 1988; Taylor & Taylor 1992); (3) nephrocytes are large (20 - 50 µm), vacuolated cells similar to glomerular podocytes of vertebrates and cells of the crustacean antennal gland. Nephrocytes are mainly located in the axial efferent vessels and occasionally in the filaments. These cells do not form a continous epithelial layer but instead they form clusters up to 30 cells (Taylor & Taylor 1992). Fibroblasts, reserve cells and granulocytes are associated to connective tissues supporting the gills and branchiosteguites. Granulocytes are thought to play a role in cuticle formation. Tegumental glands are also present in gills and may play a role in epicuticle formation, tanning and defense against pathogens (Taylor & Taylor 1992).

The gills are protected from mechanical injury in the gill chamber. Here, water is pumped rapidly by the scaphognatite over the gill filaments facilitating gas exchange (Bauer 1999) which occurs as follows: hemolymph moves through the tissues and goes to gills via a pair of infrabranchial sinuses. There, hemolymph flows into the afferent branchial vein, which supplies to individual lamellae. In the lamellae, hemolymph flows along the marginal canal formed by pillar cells and here occurs gas exchange. Once hemolymph passed through the lamellae, it drains into the efferent branchial vein and continues into the branchio-cardiac vein, which carries arterial blood back to the heart (Taylor & Taylor 1992; McGaw & Reiber 2002). Regulation of hemolymph flow through the gills is thought to occur via the efferent valve, which acts as a dike in the efferent channels (Bell & Lightner 1988).

Other functions performed by gill epithelium are: transport and excretion of carbon dioxide (CO_2), salt and water balance, acid-base regulation of hemolymph, ammonia excretion and calcium uptake (Taylor & Taylor 1992; Ahearn et al. 1999; Bauer 1999). The gills also function in capture and elimination of foreign particles in hemolymph especially bacteria (Martin et al. 1993, 1996, 2000).

Excretory system - the paired antennal glands are the main excretory organs located in the anterior part of the pereon. The excretory pore exits on the coxa of the antenna. In shrimp, the antennal gland has three regions: the coelomosac (end sac), labyrinth and bladder (Figure 6) (Felgenhauer 1992b; Fingerman 1992).

The coelomosac derives from the mesoderm and its wall is composed by a single layer of podocytes lined by a basal lamina (Fingerman 1992). Podocytes perform ultra-filtration in a similar way as the glomerular nephron of vertebrates.

The labyrinth is a network of coiled simple columnar epithelial cells. It has two regions: labyrinth I with tall columnar cells with basal nuclei and apical membrane blebs that reach the lumen. The cells in labyrinth II are shorter, with central nuclei, brush border and larger vacuoles (Fingerman 1992). The labyrinth is bathed by hemolymph in a sinus as well as by capillaries that reach the base of the epithelial cells. The epithelium in the labyrinth changes according to its physiological state. Non-secretory cells (inactive secretory state) are cuboidal to low columnar with central or apical nuclei. Secretory cells (active secretory state) are tall columnar epithelial cells with basal nuclei and

vacuolated apical cytoplasm without microvilli (Fingerman 1992). In shrimp, the labyrinth is found scattered throughout the anterior part of the pereon.

The bladder is a large multi-lobed reservoir for urine storage and may play a role in final urine modification (Felgenhauer 1992b). It has epithelial cells similar to the labyrinth (Fingerman 1992).

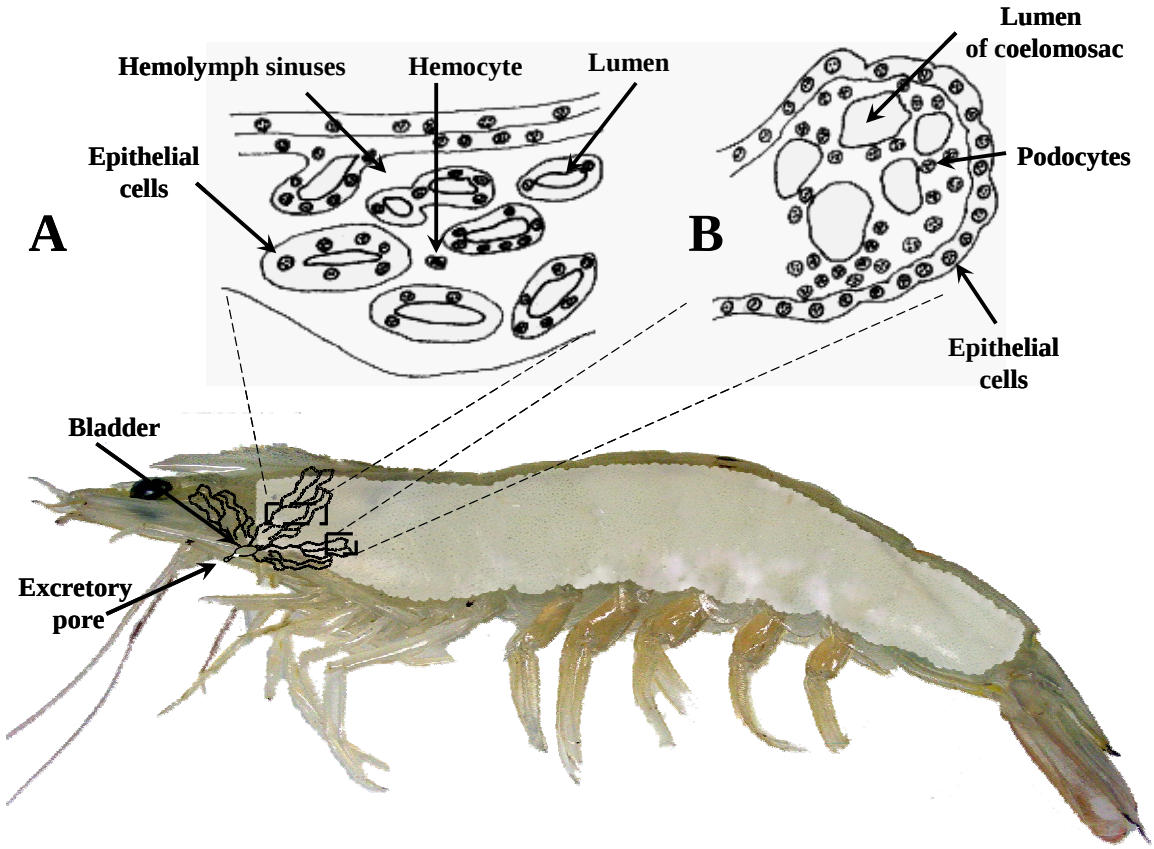


Figure 6. Structure of the antennal gland in *L. vannamei* (After Bell & Lightner 1988) showing details of (A) labyrinth and (B) coelomosac

Hemolymph bathes the antennal gland and flows into the hemocoel through a series of channels in the gland. The excretion products are ammonia and residual salts (Bell & Lightner 1988). The urine of *L. vannamei* remains isosmotic with hemolymph (Lin et al. 2000; Cheng et al. 2002).

Other roles of the antennal gland include water and calcium balance, maintenance of hemolymph volume, ionic and osmotic regulation, gastric acidification and heavy metal detoxification (Ahearn et al. 1999; Wheatly 1999; Lin et al. 2000).

Circulatory system - this system is composed by hemolymph, heart, associated conduits and haemal sinuses, hematopoietic tissues and lymphoid organ.

Hemolymph is the bodily fluid that transports nutrients, salts, water and oxygen to all tissues. It also carries metabolic waste, excess salts and/or water for excretion. The hemolymph is composed by proteins, lipoproteins, glycoproteins, free aminoacids, carbohydrates, fatty acids, electrolytes and metals (Shimizu et al. 2001). Hemocyanin (a glycosylated copper-containing protein) is dissolved in hemolymph of crustaceans and molluscs and has an oxygen-carrier function. Its oxygen-binding capacity might change as required under hypoxic or stress conditions (McMahon 2001). In *L. vannamei*, hemocyanin accounts for 87% of total hemolymph proteins (Figueroa-Soto et al. 1997; Cheng et al. 2002) and it has other functions such as an energy-storage protein (Sánchez et al. 2001; Pascual et al. 2003) and humoral defense (Destomieux-Garzón et al. 2001).

The circulatory system consists of a single-chambered dorsal heart suspended in a pericardial chamber (Figure 7). The pericardial sinus is composed of spongy connective tissue. The heart wall is composed of an outer adventitia (or epicardium) and an inner muscular layer (Ruppert & Barnes 1994; Brusca & Brusca 1990). Cardiac muscle is transversally striated, separated from the hemolymph by a basal lamina. Cardiac cells are fed directly by hemolymph.

The heart pumps out hemolymph through an anterior (ophthalmic), posterior (dorsal) and ventral (sternal) arteries (Figure 7). The diameter of these arteries decreases as they separate from the heart (Martin & Hose 1992). Large arteries such as the ophthalmic, contains a thick basal lamina lining the lumen of the vessel, an endothelial layer, loose connective tissue and an outer basal lamina. The dorsal artery also contains a single layer of striated muscle adjacent to the inner basal lamina (Martin et al. 1989). A pair of hematopoietic arteries branches from the ophthalmic artery shortly after leaving the heart. These pass in front of the dorsolateral surface of the foregut where they branch repeatedly to form the lymphoid organ. Here, the inner basal lamina is very thin and it entirely surrounds the endothelial cells and the rest of the vessel is composed of hemocyte-like cells (Martin et al. 1987; Martin & Hose 1992; van de Braak et al. 2002a).

Small vessels lose first the layer of connective tissue and as it decreases its size, the endothelium is also reduced. Here, less than four endothelial cells and often only one

cell encircle the vessel. Eventually, only the basal lamina or possibly the fusion between the internal and external basal lamina remains and lines the sinuses. Small vessels are thought to conduct hemolymph to sinuses which bathe the tissues and organs. The sinuses are interstitial spaces into which the hemolymph flows after leaving the arteries and vessels (Martin & Hose 1992). The hemolymph in tissues and sinuses is channeled into the infrabranchial sinus to be oxygenated in gills and transported to the heart (Bell & Lightner 1988; McGaw and Reiber 2002).

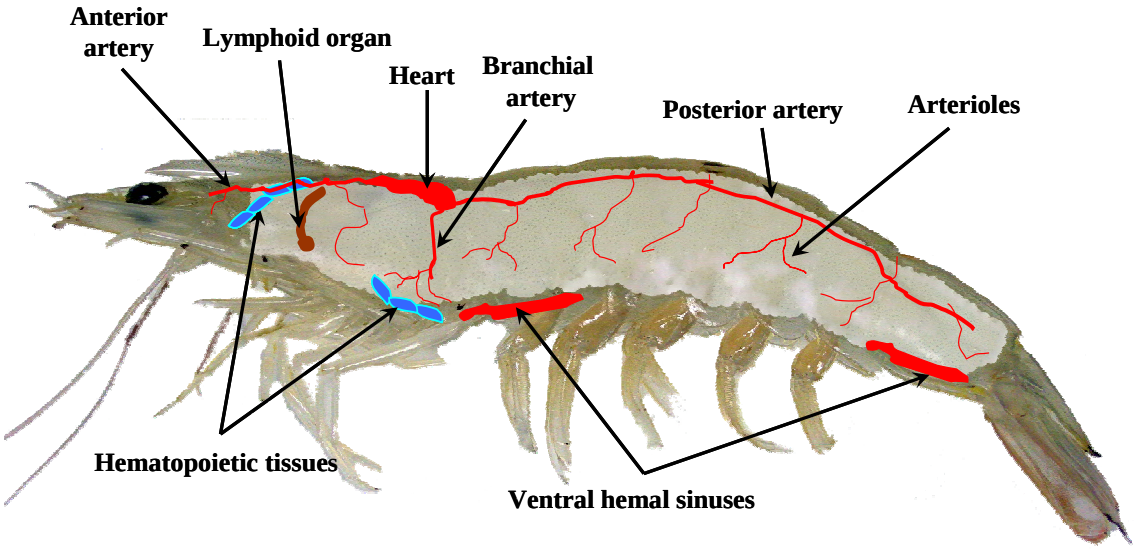


Figure 7. Circulatory system and associated organs in *L. vannamei*

Hematopoietic tissues are located dorsal to the anterior chamber of the stomach (thickness 20 - 600 µm) and it often continues towards the antennal gland. They are also found at the base of the maxillipeds (20 - 80 µm thick) (Figure 7) (Bell & Lightner 1988; Martin & Hose 1992). These tissues are arranged as spherical and elongated lobules which are surrounded by fibrous connective tissues and are partly embedded in muscle or spongy connective tissue (Martin & Hose 1992; van de Braak et al. 2002a).

Hematopoiesis is thought to occur in two main cell lines: the hyaline cell line and the granular cell line (Martin & Graves 1985; Hose et al. 1987; Omori et al. 1989; Hose & Martin 1989; Hose et al. 1990; Martin & Hose 1992). Mitosis has only been observed in stem cells, hyaline hemocytes and small granular hemocytes within hematopoietic tissues (Martin & Hose 1992). Four main cellular types were described in hematopoietic tissues of *Penaeus monodon* (van de Braak et al. 2002a): type 1 cells are considered to be undifferentiated giving rise to the other hemocyte types. Type 2 cells probably

differentiate into small granular hemocytes (Hose et al. 1987). Type 3 cells may differentiate into large granular hemocytes (Hose et al. 1987). The cell types 2 and 3 probably represent the starting point of maturation of the hyaline and granular hemocytes respectively. The cell type 4 was considered to be an interstitial cell (van de Braak et al. 2002a).

The lymphoid organ is a paired nodular structure located at the anterodorsal surface of the hepatopancreas (Martin et al. 1987; Bell & Lightner 1988). Each nodule (1 X 1.5 mm to 1.5 X 4.0 mm) consists of a series of folded tubules with a central haemal lumen (van de Braak et al. 2002b). Each tubule is a modified artery that branches from each of the ophthalmic arteries just anterior to the heart. As the vessel approaches the cardiac stomach, it divides repeatedly to form a network of smaller tubules lying dorsolateral to the foregut (Figure 7). The tubules are circular and surround a central haemal space. Adjacent tubules can be contigous or separated by loose connective tissue. The wall of each small tubule has the same structure of a larger artery (Martin et al. 1987). A layer of flattened endothelial cells lines the inner wall of each small tubule and surrounds the haemal space. Cells are connected by desmosomal junctions and interdigitations of their lateral plasma membranes. The endothelial layer is covered by a thin *intima* layer that hemocytes have to cross to enter hemolymph circulation. The tubules of lymphoid organ are composed of mature hemocytes. These are embedded in stromal cells similar to fibroblasts which are arranged perpendicular to the long axis of the tubule. The tubule is partially surrounded by capsular cells. Hemocytes can also go into circulation through this outer layer (Martin & Hose 1992).

The lymphoid organ is primarily involved in elimination of bacteria from the hemolymph (Martin et al. 1996; van de Braak et al. 2002b). A special change in the structure of this organ is observed upon viral infections. Spheroids or hyperplastic tubules lacking a central lumen and composed of cells with hypertrophied nuclei and prominent cytoplasmic inclusions are found in shrimp with viral infections. It is suggested that the lymphoid organ acts as a hemolymph-filtering organ that primarily removes bacteria and virus-infected cells. These are engulfed, destroyed or encapsulated by hemocyte-like cells in the spheroids. These structures detach from the lymphoid organ as ectopic metastatic bodies that migrate towards different epidermal surfaces (integument, gills, etc.) and may be eliminated from the body during molting

(Anggraeni & Owens 2000).

Central nervous system - in Crustacea, this compartment originates as embryonic pairs of ganglia joined in the midline by commisures and longitudinally by connectives (Sandeman 1982). The brain is formed by three ganglia: proto-, deutero-, and trito-cerebrum. These ganglia probably derive from pre-oral structures (Sandeman 1982; Govind 1992; Budd 2002). The protocerebrum innervates optic ganglia in eyestalks. Deuterocerebrum controls motor and sensory neurons such as mechanoreceptors, chemoreceptors and statocysts on eyestalk and antennae 1. Tritocerebrum controls motor neurons, proprioreceptors and mechanoreceptive hairs in antennae 2 and labrum and almost all sensory neurons in pereon (Sandeman 1982).

The brain is located dorsally in the pereon and is connected to the ventral cord by two connectives passing around the esophagus (periesophageal ring). The paired thoracic and abdominal ganglia in shrimp are medially fused but separated from ganglia of adjacent segments by paired connectives (Figure 8) (Sandeman 1982; Govind 1992).

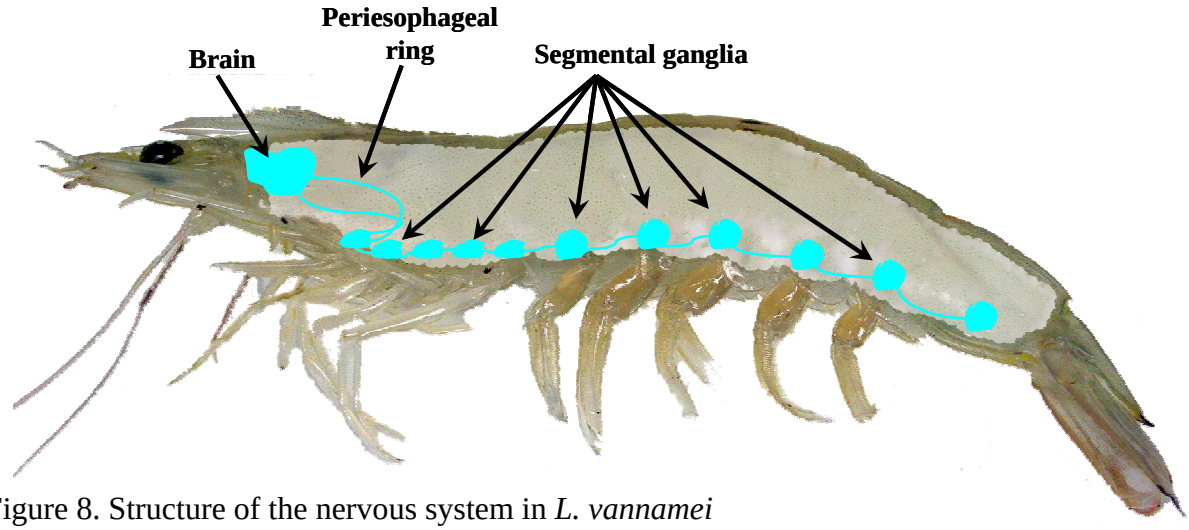


Figure 8. Structure of the nervous system in *L. vannamei*

The central nervous system is regulated through neurohormones secreted by (1) neuroendocrine glands such as X-organ/sinus gland (SG) complex, postcommisural and pericardial organs, and (2) epithelial endocrine glands such as Y-organ, mandibular organ and androgenic glands (Cooke & Sullivan 1982; Skinner 1985; Fingerman 1992; Subramoniam 2000; Diwan 2005).

Reproductive system - shrimp is a dioecious animal. Males and females are only recognized at late life stages when gonads mature and sexual characters develop.

The male reproductive tract is composed by paired testes, a *vas deferens* or genital duct and an ejaculatory duct or terminal ampoule which exits at paired gonopores in the coxae of the 5th pereopods (Krol et al. 1992).

Testes are multilobed and each lobe has one convoluted seminiferous tubule surrounded by haemal sinuses. Germinal cells in testes differentiate into spermatogonia or nurse cells. Spermatogonia produce spermatids that mature sequencially in different regions of the seminiferous tubule. Mature spermatids leave testes through the *vas deferens*. This conduct protects and transports sperm cells to the terminal ampoule where the spermatophore is produced. The spermatophore encloses sperm cells and contains attachment structures and adhesive materials. In *L. vannamei*, the spermatophore is placed externally on a hardened intermolt female. Shrimp sperm cells are nail-shaped.

In males, the first two pairs of pleopods are modified into copulatory organs. The endopod of the first pair of pleopods is modified into a petasma, a slender, bag-like membrane interlocked by minute hooks which is used to collect and transfer the spermatophore to the female. The endopod of the second pair of pleopods bears an *appendix masculina* which is an oval structure with numerous marginal spine-like projections that divides the petasma into two halves to help in spermatophore transfer (Bailey-Brock & Moss 1992; Kroll et al. 1992).

In females, the reproductive tract has paired ovaries and oviducts leading to gonopores opening at the base of the third pereopod. The thelycum is an external reproductive structure located between the 5th pair of pereopods. It is not enclosed by plates (open) and is formed by modifications of the sternites XII-XIII (Krol et al. 1992; Pérez-Farfante & Kensley 1997). The spermatophore is placed on the thelycum of a female in intermolt (Bailey-Brock & Moss 1992).

Ovaries are dorsolateral to the hepatopancreas, multilobed and may extend until the pleon. Ovaries contain germinal epithelium, oogonia and follicle cells. Follicle cells produce vitellogenin which is taken up and stored by oocytes as yolk. Follicle cells renew continouosly after ovulation and before each gametogenesis. Ovulation occurs by the separation of follicle cells from the oocytes. Ova go from the ovaries to the gonopores through the oviducts (Krol et al. 1992). Spawning takes place after ovulation.

Eggs are fertilized upon release into water (Brusca & Brusca 1990; Ruppert & Barnes 1994; Bailey-Brock & Moss 1992). In *L. vannamei*, mating occurs mainly in the evening. In this process, the male places the spermatophore into the open thelycum of the female, which retains it for a few hours before spawning. Multiple spawns may occur within one molt cycle (Bray & Lawrence 1992).

1.2.3 Defense - the defense system of crustaceans, like that of most organisms, is innate. This type of system appeared millions of years ago and as consequence, it is often regarded as 'primitive'. Nonetheless, it is very complex and almost perfect in its function (Beutler 2004). The innate system is the most universal, rapidly acting and in some respects, the most important defense system. The innate system performs a series of defense functions such as (1) recognizing non-self molecules, (2) killing different types of pathogens and (3) limiting the host's tissue damage (self-tolerance) (Beutler 2004).

In shrimp, the defense system is composed of a cellular and a humoral arm (Beutler 2004). The defense cells are called hemocytes and they are classified into hyaline or granular according to the presence and relative size of cytoplasmic granules observed by light microscopy (Martin & Graves 1985). The humoral arm consists of several molecules present in hemolymph which perform different functions (Cerenius & Söderhäll 1998).

In the cellular arm, the hyaline hemocytes do not contain cytoplasmic granules. Instead, they have abundant cytoplasmic glycoprotein deposits with staining properties similar to plasma coagulogen (Omori et al. 1989). A fraction of small granule hemocytes also contain these cytoplasmic deposits. About 54% of all hemocytes contain these deposits and trigger hemolymph coagulation within 30s upon body damage and contact with seawater (Omori et al. 1989). These cytoplasmic deposits form long filamentous strands which rapidly expand and form a fine filamentous matrix of extracellular clot. Lysed cells form the core of a sphere of clotted material. Several of these foci coalesce and form an extensive gel matrix. Two minutes after lysis, hemolymph becomes a soft gel and after 5 minutes, the coagulated hemolymph became a solid gel. This coagulation pattern is known as type C which occurs in spiny lobsters and shrimp (Omori et al. 1989; Hose et al. 1990; Yeh et al. 1998). Coagulation is an essential defense mechanism

of crustaceans. It prevents hemolymph loss and pathogen entry (Yeh et al. 1998; Wang et al. 2001; Lee & Söderhäll 2002).

The other cellular type is the granular hemocytes. These are divided into small or large granule subpopulations. These two subtypes appear to be different maturation stages (Hose et al. 1990). Both subpopulations synthesize α_2-macroglobulins (Gollas-Galván et al. 2003) and upon microbial infection they release cytoplasmic granules containing peroxinectin (Sritunyalucksana et al. 2001), agglutinins such as peptidoglycan binding protein [PGBP], lipopolysaccharide binding protein [LBP] (Lee & Söderhäll 2002), β-glucan binding protein (βGBP) (Vargas-Albores et al. 1996; Romo-Figueroa et al. 2004), prophenoloxidase (proPO) (Hernández-López et al. 1996; Johansson et al. 2000; Lai et al. 2005), penaeidins (Muñoz et al. 2002) and other antimicrobial peptides, oxygen reactive species (ROS), acid phosphatase, lysozyme, β-glucuronidase (Hose et al. 1987, 1990; Sotelo-Mundo et al. 2003), non-specific esterase and serine proteinases (Jiménez-Vega et al. 2005).

The small granule hemocytes perform phagocytosis and contain hydrolytic enzymes and lysosomes used to degrade foreign particles within phagosomes. These cells are also involved in encapsulation and nodule formation upon fungal infection. Large granule hemocytes mainly contain large refractile granules in their cytoplasm. These granules contain more proPO and less acid phosphatase than the small granule hemocytes. These hemocytes are primarily involved in fungal encapsulation and nodule formation (Hose & Martin 1989). Upon infection, degranulation of these cells activates the proPO cascade for cell adhesion, phagocytosis and cytotoxicity (Johansson et al. 2000).

The humoral arm of shrimp is comprised of the prophenoloxidase (proPO) system, agglutinins and opsonizing lectins, bactericidins and antimicrobial peptides, lysozymes, reactive oxygen species (ROS), serine proteinases and proteinase inhibitors (Bachère et al. 1995; Söderhäll & Cerenius 1998; Gollas-Galván et al. 1999; Roch 1999; Sritunyalucksana & Söderhäll 2000; Cerenius & Söderhäll 2004; Lai et al. 2005).

Hemolymph-soluble pattern recognition peptides (PRP) with cell attachment motifs (RGD, KGD) recognize foreign molecules and bind an integrin-like protein in the surface of shrimp hemocytes inducing hemocyte spreading and degranulation (Holmblad & Söderhäll 1999; Sritunyalucksana & Söderhäll 2000; Lee & Söderhäll

2002). A serine proteinase cascade activates the proPO activating enzyme (ppA) that cleaves proPO into the active phenoloxidase (PO) (Cerenius & Söderhäll 2004).

The PO molecule is a copper oxidase that catalyzes the o-hydroxylation of tyrosine or monophenols such as L-3,4-dihydroxyphenilalanine (L-DOPA) to *o*-diphenol (monophenoloxidase activity) and oxidates *o*-diphenol to *o*-quinones (diphenoloxidase activity) to produce melanin (Sritunyalucksana & Söderhäll 2000; Lai et al. 2005). Melanin biosynthesis is important for cuticular sclerotization, wound healing and encapsulation of foreign materials (Lee & Söderhäll 2002; Cerenius & Söderhäll 2004). Several quinone intermediates are highly toxic and prevent or delay the replication of pathogens (fungistatic activity) (Sritunyalucksana & Söderhäll 2000). PO triggers other defense mechanisms such as cell adhesion (Holmblad & Söderhäll 1999), opsonization (Söderhäll & Cerenius 1998), phagocytosis (Roch 1999), encapsulation (Lee & Söderhäll 2002), melanization (Sritunyalucksana & Söderhäll 2000), antibacterial activity and bacterial clearance (Bachère et al. 1995; Vargas-Albores et al. 1996; Yeh et al. 1998; Yepiz-Plascencia et al. 1998; Marques & Barracco 2000; Gollas-Galván et al. 2003; Pascual et al. 2003; Alpuche et al. 2005; Jimenez-Vega et al. 2005; Lai et al. 2005).

The innate system in shrimp has been studied for some years and much of it is still unknown. The presence of other defense mechanisms such as an antiviral defense response has just recently been explored. Two studies have shown the activation of an unspecific antiviral mechanism by injecting double-stranded RNA sequences of viral and eukaryotic origin (Robalino et al. 2004; Westenberg et al. 2005).

The function of the innate system of invertebrates has been better studied in the fruit-fly *Drosophila*. In this species, the innate defense response is complex and not well understood. Two different systems are involved in defense against microbial infections. One is the Toll system and it detects and generates a defense response against fungi and gram-positive bacteria (see Beutler 2004). This pathway induces seven distinct antimicrobial peptides which have high specific activities against fungi and gram-positive bacteria (Hoffman 2003; Beutler 2004). In bacterial infections, the activation of the Toll pathway begins with the recognition of specific structural motifs from the bacterial cell wall by a peptidoglycan binding protein (PGBP). This event triggers a proteolytic cascade inducing the cleavage of a protein called Spaetzle, which in turn

activates the Toll system (Hoffman 2003). This pathway may also be activated by two other proteins found in hemolymph called *Semmelweis* and *Osiris* (Hoffman 2003). These proteins are a PGBP and a gram-negative binding protein, respectively. The function of these two proteins is not completely known but they may induce the cleavage of Spaetzle by recognizing and binding to the cell wall of gram-positive bacteria (Hoffman 2003). In the case of fungal infections, cleavage of Spaetzle is mediated by a trypsin-like protease called *Persephone* (Hoffman 2003).

A second system involved in the defense response of *Drosophila* is the immunodeficiency (Imd) pathway. This system induces the expression of several antimicrobial peptides such as diptericin, drosocin, cecropins and attacins which are active against gram-negative bacteria. The activation of this pathway also requires a putative transmembrane protein that belongs to the PGBP family. Such a protein may recognize and bind to the cell wall of gram-negative bacteria. Then, a eukaryotic transcription factor (NF-kB)-protein called Relish, is activated through a signal-induced endoproteolytic cleavage. The subsequent steps that lead to phosphorylation and cleavage of Relish as well as the molecular mechanism of Imd activation are not known (Hoffman 2003).

The existence of the Toll and Imd pathways indicates the high specificity of the innate defense response in invertebrates (Kurtz 2004). Furthermore, the regulatory mechanisms shown in the proPO system, the Toll and the Imd pathways suggest a rudimentary form of short-term memory (Kurtz 2005). A more complex type of memory may be present in invertebrates. A group of lectin-like peptides known as fibrinogen-related proteins (FREPs) contain one or two immunoglobulin superfamily (IgSF) domains and a fibrinogen domain (Kurtz 2005). These molecules have a high rate of genome polymorphism. Analysis of a snail's FREP3 showed the existence of a high number of individual (up to 45 different) sequences which are generated by a small set of source sequences (2 to 10 alleles). This finding suggests that such diversity may occur by somatic recombination following a different way from that of vertebrates (Kurtz 2005).

1.3 White spot syndrome virus

In 1992, a new virus appeared in shrimp farms in northern Taiwan causing disease and massive mortality (Chou et al. 1995). In late 1993, the viral agent was first isolated from an outbreak in Japan (Inouye et al. 1994). In a few years, this new pathogenic agent spread to several shrimp farming countries (Flegel 1997). At first, it was thought that different viral agents simultaneously appeared in different regions and were given a specific name: hypodermal and hematopoietic necrosis baculovirus (HHNBV) (see Durand et al. 1996), third *Penaeus monodon* non-occluded baculovirus (PmNOB III) (see Wang et al. 1995; see Karunasagar & Karunasagar 1997), rod-shaped nuclear virus of *Marsupenaeus japonicus* (RV-PJ) (Inouye et al. 1994, 1996), penaeid rod-shaped DNA virus (PRDV) (see Venegas et al. 2000), systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya et al. 1995; Sahul-Hameed et al. 1998) or white spot baculovirus (WSBV) (Chou et al. 1995; Lightner 1996). Later, it was recognized that a single viral agent was responsible for these outbreaks. Eventually an informal consensus was reached to call it white spot syndrome virus (WSSV).

WSSV has become the major disease threat to shrimp aquaculture worldwide. In 1993, losses of 80% of farmed shrimp production in China were attributed to WSSV (Zhan et al. 1998) (Figure 9a). During the same interval, WSSV outbreaks were recorded in Japan (Inouye et al. 1994) and Korea (Park et al. 1998) and since then, it spread to several Asian countries such as Thailand (1994) (Lo et al. 1996a), India (1994) (Karunasagar & Karunasagar 1997), Indonesia (1996) (see Durand et al. 1996), Malaysia (1996) (Kasornchandra et al. 1998; Rajan et al. 2000), Vietnam (1997) (Bondad-Reantaso et al. 2001), the Philippines (1999) (Magbanua et al. 2000) and Iran (2002) (see Dieu et al. 2004).

In 2000, crabs and crayfish were found WSSV-positive in Australia but later these results were proven to be false positive (Claydon et al. 2004). This country remains at risk of a WSSV outbreak given its proximity to south-east Asia where the pathogen is endemic (Chang et al. 1999, 2003). It is possible that some Asian crustaceans carrying WSSV may reach Australia and spread it to shrimp farming areas (Claydon et al. 2004). Furthermore, the introduction of WSSV-infected organisms through ballast water from cargo ships (Flegel & Fegan 2002) or even frozen commodities (Durand et al. 2000) may be possible.

In the American continent, WSSV was first recorded in 1995 from hatchery facilities in Texas and South Carolina in the U. S (Lightner 1996; Wang et al. 1999a). It was later reported in Peru (1998) (Rosenberry 2001), Ecuador (1999) (see Durand et al. 2000), Colombia (1999), Panama (1999), Honduras (1999), Nicaragua (1999), Guatemala (1999), Belize (1999), Mexico (1999) (Bondad-Reantaso et al. 2001; Houssain et al. 2001; Wu et al. 2001) and most recently, Brazil (2005) (APHIS 2005). The impact of WSSV on shrimp production in Ecuador has been especially disastrous (Figure 9b). In 2002, WSSV was found in wild crustaceans off the Mediterranean French coast (see Marks 2005).

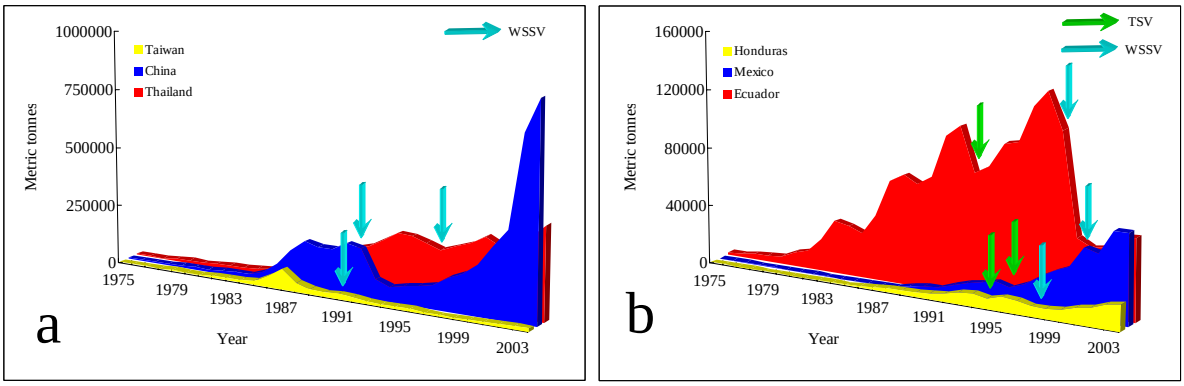


Figure 9. Impact on farmed shrimp production of viral outbreaks of white spot syndrome virus (WSSV) in Asia (a) and outbreaks of WSSV and Taura syndrome virus (TSV) in America (b) (FAO 2006)

1.3.1 Morphology - WSSV is a bacilliform, non-occluded enveloped virus (Chou et al. 1995; Wang et al. 1995; Wongteerasupaya et al. 1995). Intact enveloped virions range between 210 and 380 nm in length and 70 to 167 nm of maximum width (Chang et al. 1996; Flegel & Alday-Sanz 1998; Park et al. 1998; Rajendran et al. 1999). A characteristic feature is the presence of a tail-like appendage at one end (Wongteerasupaya et al. 1995; Durand et al. 1996) (Figure 10).

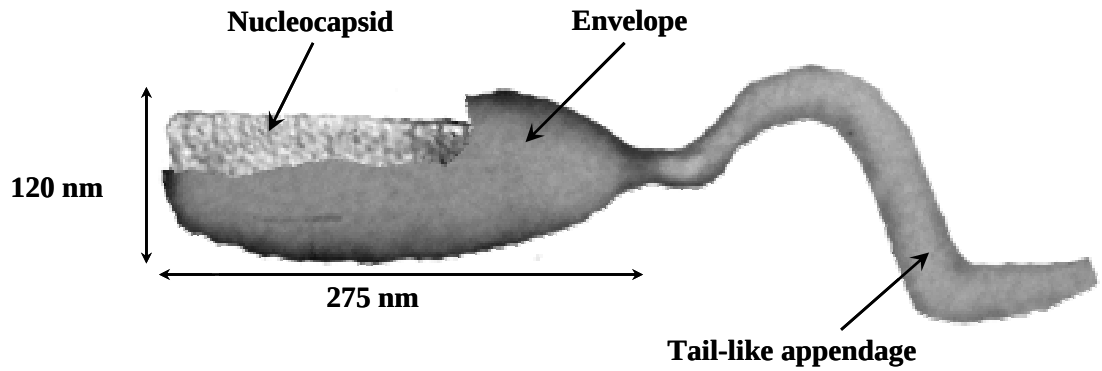


Figure 10. Morphology of the WSSV virion

The envelope is 6-7 nm thick and is a lipidic, trilaminar membranous structure with two electron-transparent layers divided by an electron-opaque layer (Wongteerasupaya et al. 1995; Durand et al. 1997; Nadala et al. 1998).

The nucleocapsid is located inside the envelope and it is a stacked-ring structure composed of globular protein subunits of 10 nm in diameter arranged in 14-15 vertical striations located every 22 nm along the long axis giving it a cross-hatched appearance (Durand et al. 1997; Nadala & Loh 1998). When released from the envelope, the nucleocapsid increases in length indicating that within the virion it is tightly packed. The size of the nucleocapsid varies from isolate to isolate and ranges between 180 and 420 nm long and 54 to 85 nm width and with a 6 nm-thick external wall (Kasornchandra et al. 1998; Sahul-Hameed et al. 1998; Rajendran et al. 1999).

A highly electrodense core comprised of the DNA binding protein VP15 and the viral DNA is found inside the nucleocapsid (Durand et al. 1997; Wang et al. 1999b; van Hulten et al. 2001a).

1.3.2 Structural proteins - about 41 WSSV proteins with a putative structural function have been characterized (Table 1, see references below). Of these, 14 have been located in the envelope, four in the tegument (a putative structure located between the envelope and nucleocapsid) and six in the nucleocapsid.

A cell attachment motif that suggests a role in viral entry has been found in the envelope proteins VP31 and VP281 (Huang et al. 2002a; Tsai et al. 2004; Li et al. 2005a), the tegument protein VP36A and the nucleocapsid VP664 (Tsai et al. 2004; Leu et al. 2005) as well as in the uncharacterized proteins VP110 and VP136A (Tsai et al. 2004). Other proteins such as VP28, VP68, VP124, VP281, VP292, VP466 (van Hulten et al. 2001a, 2001b, 2002; Huang et al. 2002a, 2002b; Zhang et al. 2004; Zhu et al. 2005; Wu et al. 2005), and a collagen-like protein (Li et al. 2004) have been located in the envelope; whereas the proteins VP35 (Chen et al. 2002b), VP15 (van Hulten et al. 2002), VP664 (Leu et al. 2005) and others have been located in the nucleocapsid and may have different putative functions (van Hulten et al. 2001a, Yang et al. 2001).

In vivo neutralization assays using antibodies against different envelope proteins showed a significant delay of shrimp mortality, indicating that proteins such as VP28 (van Hulten et al. 2001b; Yi et al. 2004) and the proteins VP68, VP281 and VP466 (Wu et al. 2005), might have an important role in virus penetration.

1.3.3 Genome and classification - the WSSV genome is a circular, double-stranded DNA molecule with an A + T content of 59% homogeneously distributed. The genome size varies according to the viral isolate (Thailand 293 kilobase pairs (kbp), China 305 kbp, Taiwan 307 kbp) (van Hulten et al. 2001a; Yang et al. 2001; Chen et al. 2002b).

This viral genome is one of the largest sequenced so far, only behind a huge virus (mimivirus) infecting an amoeba (1’181 404 bp), a canarypox (359 853 bp), a virus from the brown algae *Ectocarpus siliculosus* (335 593 bp) and a virus from *Paramecium bursaria* (PBCV-1) (330 743 bp) (van Hulten et al. 2001b; Raoult et al. 2004; http://www.giantvirus.org).

Table 1 - List of WSSV proteins so far characterized.

Protein name	Size (Aminoacid residues)	Apparent size (KDa)	Putative function	Location in WSSV virion *
VP11	433	11	Unknown	Not determined [8]
VP12A (VP95)	95	11	Structural	Tegument [8, 9]
VP12B (VP68)	68	7	Structural	Envelope [8, 12, 13]
VP13A	100	13	Energy metabolism	Not determined [8]
VP13B	117	13	Unknown	Not determined [8]
VP15	80	15	DNA binding protein	Nucleocapsid/core [8, 11]
VP19	121	19	Structural	Envelope [8, 9, 11]
VP22 (VP184)	891	100	Unknown	Not determined [8]
VP24 (VP208)	208	24	Structural	Nucleocapsid [8, 10, 13]
VP26	204	26	Structural	Tegument [8, 10]
VP28	204	28	Structural	Envelope [8, 9, 10]
VP31	261	31	Cell attachment	Envelope [6, 8, 9]
VP32	278	32	Unknown	Not determined [8]
VP35	228	26	Structural	Nucleocapsid [1]
VP36A	297	36	Cell attachment	Tegument [8, 9]
VP36B (VP281)	281	32	Cell attachment	Envelope [2, 8, 12]
VP38A	309	38	Structural	Envelope [8, 9]
VP38B	321	38	Endonuclease	Not determined [8]
VP39A	419	39	Structural	Tegument [8, 9]
VP39B	283	39	Unknown	Not determined [8]
VP41A (VP292)	292	33	Structural	Envelope [2, 8, 13]
VP41B (VP300)	300	34	Unknown	Not determined [8]
VP51A	486	51	Eggshell protein	Envelope [8]
VP51B (VP384)	384	50	Structural	Envelope [8, 9]
VP51C (VP466)	466	50	Structural	Nucleocapsid [3, 8, 12]
VP53A	1301	53	Structural	Envelope [8, 9]
VP53B	968	53	Signal transduction pathway	Not determined [8]
VP53C	489	53	Unknown	Not determined [8]
VP55 (VP448)	448	55	Unknown	Not determined [8]
VP60A	465	60	Unknown	Not determined [8]
VP60B (VP544)	544	60	Adenovirus fiber-like protein	Nucleocapsid [8, 9, 13]
VP75	786	75	Unknown	Not determined [8]
VP76 (VP73)	675	76	Class 1 cytokine receptor	Envelope [4, 8]
VP110	972	110	Cell attachment	Not determined [8]
VP124	1194	124	Structural	Envelope [8, 14]
VP136A	1219	136	Cell attachment	Not determined [8]
VP136B	1243	136	Unknown	Not determined [8]
VP180 (VP1684)	1684	169	Collagen-like protein	Envelope [8]
VP187	1606	187	Structural	Envelope [7]
VP664	6077	664	Cell attachment	Nucleocapsid [5, 8, 9]
VP800	800	90	Unknown	Not determined [8]

*References: [1]Chen et al. 2002b, [2]Huang et al. 2002a, [3]Huang et al. 2002b, [4]Huang et al. 2005, [5]Leu et al. 2005, [6]Li et al. 2005a, [7]Li et al. 2006, [8]Tsai et al. 2004, [9]Tsai et al. 2006, [10]van Hulten et al. 2000b, [11]van Hulten et al. 2002, [12]Wu et al. 2005, [13]Zhang et al. 2004, [14]Zhu et al. 2005.

Sequence analysis shows that the WSSV genome contains between 531 and 684 open reading frames (ORFs) with an ATG initiation codon. Of these, 181 to 184 ORFs are likely to encode functional proteins with sizes between 51 and 6077 aminoacids, which represent 92% of the genetic information contained in the genome (van Hulten et al.

2001b; Yang et al. 2001). About 21 to 29% of such ORFs have been shown to encode WSSV proteins or share identity with other known proteins. These proteins include enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase (Chen et al. 2002a), a non-specific nuclease (Witteveldt et al. 2001; Li et al. 2005b), a small and a large subunits of ribonucleotide reductase (van Hulten et al. 2000a; Tsai et al. 2000a), thymidine kinase, thymidylate kinase, a chimeric thymidine-thymidylate kinase (Tsai et al. 2000b), a thymidylate synthase (Li et al. 2004), a dUTPase (Liu & Yang 2005) and two protein kinases (van Hulten et al. 2001b; van Hulten & Vlak 2001; Yang et al. 2001). Other proteins with a putative function include a collagen-like protein (Li et al. 2004), flagellin, a chitinase, a pupal cuticle-like protein, a cell surface flocculin, a kunitz-like proteinase inhibitor, a class 1 cytokine receptor, a *sno*-like peptide and a chimeric anti-apoptotic protein (van Hulten et al. 2001b; Yang et al. 2001; Marks 2005). Three ORFs (151, 366 and 427 of the Thailand isolate) may encode putative proteins involved in WSSV latency (Khadijah et al. 2003).

Recently, it was found that WSSV also has three immediate early (IE) genes (ORFs 126, 242 and 418 of the Taiwan isolate). These genes are transcribed independently of any viral protein synthesized *de novo* by the host cell machinery and are directly expressed *in vitro*. These IE genes may be important to determine host range and also can function as regulatory trans-acting factors during infection (Liu et al. 2005).

Transcriptional analysis of genes coding for proteins required in DNA replication and nucleotide metabolism are synthesized early during virus replication. Early transcribed WSSV genes in general have a TATA box 20-30 nucleotides upstream of the transcription initiation site (TIS) (A/C)TCANT (Chen et al. 2002a; Liu et al. 2005; Marks 2005). Structural proteins are synthesized later during infection and generally have a degenerate TIS motif (A/TNAC/G) located 25 nucleotides downstream of an A/T rich region; which is similar to the TIS motif found in arthropods (Tsai et al. 2004; Marks 2005).

Sequence analysis of the DNA polymerase and the organization of several ORFs known to encode WSSV structural proteins were different from those of known baculoviruses, demonstrating that WSSV is not closely related to this virus group (Nadala et al. 1998; van Hulten et al. 2000b, 2001b, 2002; Chen et al. 2002a, 2002b; Huang et al. 2002a,

2002b, 2005; Tsai et al. 2004; Zhan et al. 2004; Zhang et al. 2004; Leu et al. 2005; Marks 2005; Xie & Yang 2005; Zhu et al. 2005). As a result that WSSV is a distinct new virus, it has been assigned to its own virus family *Nimaviridae* (van Hulten & Vlak 2001; Vlak et al. 2002).

1.3.4 Genetic, antigenic and virulence variability in WSSV isolates - the genome of three WSSV isolates has been fully sequenced: Thailand 293 kilobase pairs (kbp), (van Hulten et al. 2001b), China 305 kbp (Yang et al. 2001), Taiwan 307 kbp (Chen et al. 2002a). The nucleotide identity between these isolates is 99.3% (Marks 2005).

In silico restriction analysis with the enzyme *Kpn*I predicts 27 fragments for the Chinese and Taiwanese isolates and 25 for the Thai isolate (Figure 11). Nine fragments of 0.3, 0.5, 0.7, 4.2, 4.7, 5.3, 5.4, 8.3 and 10.8 kbp are identical in size for all three isolates. Two fragments of approximate sizes of 9 and 20 kbp respectively are missing in the Thai isolate. The remaining 14 to 16 fragments vary in size from 1.2 to 18 kbp between the isolates.

Experimental restriction analysis with *Hin*dIII done in several WSSV isolates also found differences in restriction fragment length polymorphism (RFLP) between a Chinese isolate (*F. chinensis*), two isolates from Indonesia (*P. monodon*) and one from the U.S. (*F. setiferus*). The latter two isolates were more similar to each other (Nadala & Loh 1998).

Other WSSV isolates from China (*F. chinensis*), India (*P. monodo*n), Thailand (*P. monodon* and *L. vannamei*) and U.S. (crayfish *Orconectes punctimanus* from Washington and *L. vannamei* from South Carolina and Texas) were compared by dot blot hybridization using a DNA probe from a Taiwanese isolate. With this method, negative results or a very faint signal were found in some samples from India, Thailand and Texas. This finding suggests important differences between these isolates. Further RFLP analysis of PCR products from 10 different primer sets showed that the Texas isolate was very different from the others (Lo et al. 1999).

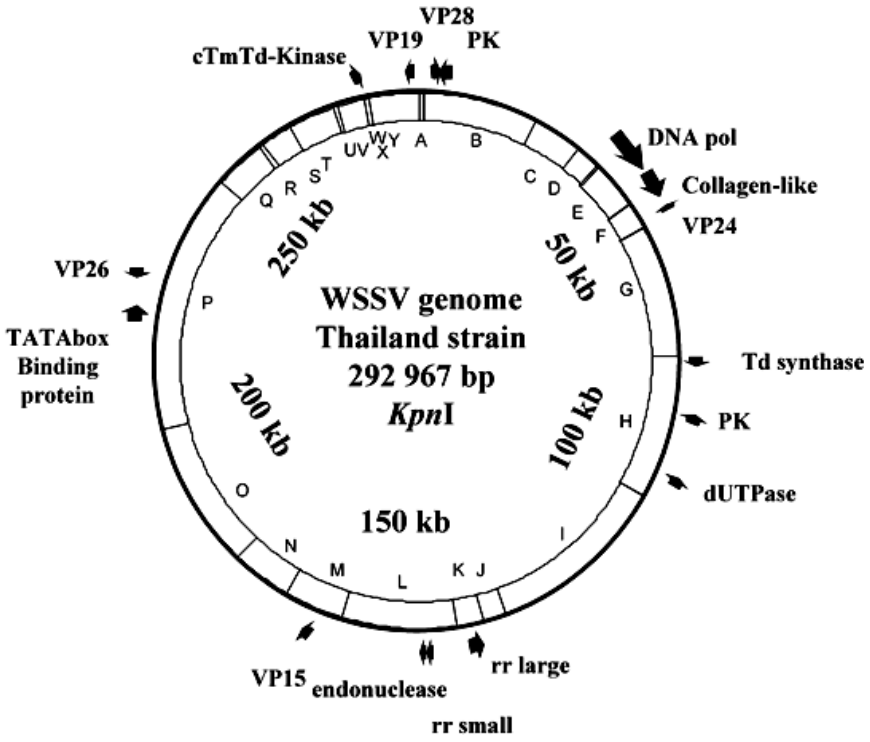


Figure 11. Structure of the WSSV genome (Thailand isolate)

Different regions of the WSSV genome display important sequence variations which can be used to establish the origin of a WSSV outbreak and its spread in a certain area (Dieu et al. 2004; Hoa et al. 2005) and also to differentiate isolates in the field (Wongteerasupaya et al. 2003; Marks 2005). Such variability may also induce false-negative results when using certain PCR primers (Claydon 2004; Kiatpathomchai et al. 2005).

The protein profiles of the six WSSV isolates described in Lo et al. (1999) and isolates from India and Korea were very similar as all of them displayed at least three major structural proteins (VP28, VP24 and VP19). An additional band corresponding to VP15 was found in four isolates. The sequence of the amino-terminal portion of these proteins was identical between isolates (Wang et al. 2000; Rajendran et al. 2004).

Several WSSV isolates (from U.S. [*F. setiferus*, *L. vannamei*], Panama, China [*F. chinensis*, *M. japonicus*], Indonesia [*P. monodon*], Japan [*M. japonicus*], Thailand, Malaysia, Taiwan or different isolates from India) were shown to have low antigenic variability using polyclonal or monoclonal antibodies (from whole WSSV virions or raised against full or truncated recombinants of VP28) in different immunoassays such

as immunodot assays (Nadala & Loh 2000; You et al. 2002), western blot (WB) (Shih et al. 2001; Yoganandhan et al. 2004), indirect immunofluorescence (IIF) (Poulos et al. 2001), immunohistochemistry [IHC]) (Anil et al. 2002) or enzyme-linked immunosorbent assay (ELISA) (Zhang et al. 2001).

Differences in virulence of six WSSV isolates were found in postlarvae of *L. vannamei* and juveniles of *F. duorarum* inoculated *per os*. Virulence was determined as the time required to induce 100% mortality in *L. vannamei*. The Texas isolate was the most virulent while the Washington isolate (from crayfish) was the least virulent. The shrimp *F. duorarum* is known to be more resistant to WSSV infection. In this species, cumulative mortality was 60% with the Texas isolate and 35% with the WSSV isolate from crayfish (Wang et al. 1999a). Another study showed that differences in virulence and competitive fitness may be dependent of the genomic size. A putative ancestral WSSV isolate (WSSV-TH-96-II) with the largest genome size recorded (312 kbp), showed a lower virulence (median lethal time [LT_{50}] = 14 d) and competitive fitness compared to another WSSV isolate (WSSV-TH) with a smaller genome size (292 kbp) (LT_{50} = 3.5 d). This study indicated that WSSV isolates with a smaller genome size may represent an advantage for virus replication (Marks 2005).

1.4 WSSV infection

1.4.1 Host range - WSSV has a broad host range within decapod crustaceans. At least, 18 cultured and/or wild penaeid shrimp (Wongteerasupaya et al. 1996; Durand et al. 1997; Lu et al. 1997; Chou et al. 1998; Lightner et al. 1998; Park et al. 1998), eight caridean species (Sahul-Hameed et al. 2000; Shi et al. 2000; Pramod-Kiran et al. 2002), seven species of lobster (Chang et al. 1998; Rajendran et al. 1999), seven of crayfish (Wang et al. 1998a; Corbel et al. 2001; Jiravanichpaisal et al. 2001, 2004; Edgerton 2004), 38 crab species (Lo et al. 1996a; Kanchanaphum et al. 1998; Kou et al. 1998; Sahul-Hameed et al. 2001, 2003), six non-decapod crustacean species (Otta et al. 1999; Supamattaya et al. 1998; Hossain et al. 2001; Yan et al. 2004), members of the phyla Chaetognata and Rotifera (Ramirez-Douriet et al. 2005), polychaete worms (Vijayan et al. 2005) and some aquatic insect larvae (Lo et al. 1996b; Flegel 1997; Ramirez-Douriet et al. 2005) have been found to be WSSV-positive by a PCR method (Table 2).

Although many of these species have been confirmed to support WSSV replication under experimental conditions, some others may only be mechanical carriers of WSSV in the wild, such as the case of the polychaete worms (T.W. Flegel, pers. comm.).

Table 2 - WSSV host range

Animal	Species	Type of infection		Detection method	Country *
		Natural	Experimental		
Penaeid shrimp	*Farfantepenaeus aztecus*		X	Histopathology	U.S.A. [10]
	F. duorarum		X	Histopathology	U.S.A. [10]
	Fenneropenaeus chinensis	X	X	Histopathology, ISH, PCR	China, Korea, Thailand [13, 25, 26]
	F. indicus	X	X	Histopathology, PCR, TEM	India, Indonesia, Thailand [14, 15]
	F. merguiensis	X	X	Histopathology, PCR, IIF	Malaysia, Thailand [3, 4, 18, 19]
	Litopenaeus setiferus		X	Histopathology	U.S.A. [10]
	L. stylirostris	X	X	Histopathology	U.S.A., Latin America [10, 13]
	L. vannamei	X	X	Histopathology, ISH, TEM	U.S.A., Latin America [10, 13]
	Marsupenaeus japonicus	X	X	Histopathology, PCR, TEM	China, Japan, India [11, 13, 23, 24, 26]
	Metapenaeus dobsonii	X	X	Histopathology, PCR, TEM	India [4, 16]
	M. ensis	X	X	ISH, PCR	Taiwan [1, 11, 23, 24]
	M. monoceros		X	PCR	India [16]
	Penaeus monodon	X	X	Histopathology, ISH, PCR	At least eight Asian countries [1, 11, 14, 15, 23, 24, 26]
	P.penicillatus	X		ISH, PCR	Taiwan [11, 23]
	P. semisulcatus	X	X	ISH, PCR	India, Taiwan [11, 15, 23]
	Parapenaeopsis stylifera	X		PCR	India [4]
	Solenocera indica	X		PCR	India [4]
	Trachypenaeus curvirostris	X	X	ISH, PCR	Taiwan [23, 24]
Caridean shrimp	*Alpheus sp.*		X	PCR	Thailand [11]
	Callianassa sp.		X	PCR	Thailand [11]
	Exopalaemon orientalis		X	ISH, PCR	Taiwan [23, 24]
	Palaemon sp.	X		ISH, PCR	Taiwan [11]
	P. adspersus		X	TEM, ISH, PCR, dot-blot	France [2]
	Macrobrachium idella		X	Histopathology, WB	India [15]
	M. lamerrae		X	Histopathology, WB	India [17]
	M. rosenbergii	X	X	Histopathology, ISH, PCR	India, Taiwan [4, 11, 15, 23, 24]
Lobster	*Panulirus homarus*		X	Histopathology	India [15]
	P. longipes	X	X	ISH, PCR	Taiwan [24]
	P. ornatus	X	X	Histopathology, ISH, PCR	India, Taiwan [15, 23]
	P. penicillatus		X	ISH, PCR	India, Taiwan [1, 23]
	P. polyphagus	X	X	Histopathology	India [15]
	P. versicolor	X	X	ISH, PCR	Taiwan [1, 23]
	Scyllarus arctus		X	TEM, ISH, PCR, dot-blot	France [2]

Table 2 - WSSV host range (continued)

Animal	Species	Type ofinfection		Detection method	Country *
		Natural	Experimental		
Crayfish	*Astacus astacus*		X	PCR	Sweden [7]
	A. leptodactylus		X	TEM, ISH, PCR, dot-blot	France [2]
	Cherax destructor		X	Histopathology, dot-blot	Australia [3]
	C. quadricarinatus		X	Histopathology, ISH, TEM	China [20]
	Pacifastacus leniusculus		X	Histopathology, ISH	Sweden [6]
	Procambarus clarkii		X	ISH, PCR	Taiwan [1, 5, 23]
	Orconectes limosus		X	TEM, ISH, PCR, dot-blot	France [2]
Crab	*Atergatis integerrimus*		X	PCR	India [19]
	Calappa philarigus	X	X	Histopathology, ISH, PCR	India, Taiwan [9, 19]
	Callinectes lophos		X	ISH, PCR	Taiwan [23]
	Cancer pagurus		X	TEM, ISH, PCR, dot-blot	France [2]
	Carcinus maenas		X	TEM, ISH, PCR, dot-blot	France [2]
	Charybdis annulata	X	X	Histopathology, PCR	India [4, 19]
	C. cruciata		X	PCR	India [4]
	C.feriatus	X	X	Histopathology, ISH, PCR	India, Taiwan [9, 11, 23]
	C. granulata		X	ISH	Taiwan [1, 23]
	C. lucifera	X	X	Histopathology, PCR	India [12, 19]
	C. natatus	X	X	Histopathology, ISH PCR	India, Taiwan, Thailand [9, 19]
	Demania splendida		X	PCR	India [19]
	Doclea hybrida		X	Histopathology, PCR	India [19]
	Gelasimus marionis nitidus	X		PCR	India [4]
	Grapsus albolineatus		X	Histopathology, PCR	India [19]
	Halimede ochtodes		X	Histopathology, PCR	India [19]
	Helice tridens	X		PCR	Taiwan, Thailand [9, 11]
	Liagore rubronaculata		X	Histopathology, PCR	India [19]
	Liocarcinus depurator		X	TEM, ISH, PCR, dot-blot	France,India [8, 15]
	L. puber		X	TEM, ISH, PCR, dot-blot	France, India [8, 15]
	Lithodes maja		X	Histopathology, PCR	India [19]

Table 2 - WSSV host range (continued)

Animal	Species	Type of infection		Detection method	Country *
		Natural	Experimental		
Crab	*Macrophthalmus sulcatus*	X		PCR	India [4]
	Matuta miersi		X	Histopathology, PCR	India [19]
	M. planipes	X		PCR	India [12]
	Menippe rumphii		X	PCR	India [19]
	Metapograpsus sp.		X	Histopathology	India, Taiwan [15]
	Metapograpsus messor	X		PCR	India [4]
	Paradorippe granulata		X	Histopathology, PCR	India [19]
	Paratelphusa hydrodomous		X	Histopathology, PCR,	India [18]
	P. pulvinata		X	Histopathology, PCR,	India [18]
	Parthenope prensor		X	Histopathology, PCR	India [19]
	Phylira syndactyla		X	Histopathology, PCR	India [19]
	Podophthalmus vigil		X	Histopathology, PCR	India [19]
	Portunus pelagicus	X	X	Histopathology, ISH, TEM	Taiwan, Thailand [9, 11, 21]
	P. sanguinolentus	X	X	Histopathology, ISH, PCR	India, Taiwan [1, 9, 11, 19, 24]
	Sesarma sp.		X	Histopathology, ISH, PCR	India, Thailand [8, 15]
	S. oceanica	X		PCR	India [12]
	Scylla serrata	X	X	Histopathology, ISH, PCR	India, Taiwan, Thailand [8, 9, 11, 15, 19, 21]
	S. tranquebaricca		X	Histopathology	India [15]
	Thalamite danae		X	Histopathology, PCR	India [19]
	Uca pugilator		X	Histopathology, ISH	Thailand [8]
Other	Sergestoidea *Acetes sp.*	X	X	Histopathology, ISH, PCR	Thailand [21]
	Cirripedia *Balanus sp.*	X	X	PCR	Mexico, Thailand [11, 16]
	Branchiopoda Cladocera	X		PCR	Mexico [16]
	Branchiopoda (*Artemia sp.*)	X		PCR	India [12]
	Stomatopoda *Squilla mantis*	X		PCR	India [4]
	Copepoda	X		PCR	Mexico, Thailand [11, 16]
	Chaetognata	X		PCR	Mexico [16]
	Rotifera	X		PCR	China [25]
	Polychaeta *Marphysa sp.*	X		PCR	India [22]
	Coleoptera (Ephydridae)	X		PCR	Taiwan [11]

*References: [1]Chang et al. 1998, [2]Corbel et al. 2001, [3]Edgerton 2004, [4]Hossain et al. 2001, [5]Huang et al. 2001, [6]Jiravanichpaisal et al. 2001, [7]Jiravanichpaisal et al. 2004, [8]Kanchanaphum et al. 1998, [9]Kou et al. 1998, [10]Lightner et al. 1998, [11]Lo et al. 1996b, [12]Lo et al. 1999, [13]Lu et al. 1997, [14]Rajan et al. 2000, [15]Rajendran et al. 1999, [16]Ramírez-Douriet et al. 2005, [17]Sahul-Hameed et al. 2000, [18]Sahul-Hameed et al. 2001, [19]Sahul-Hameed et al. 2003, [20]Shi et al. 2000, [21]Supamattaya et al. 1998, [22]Vijayan et al. 2005, [23]Wang et al. 1998a, [24]Wang et al. 1998b, [25]Yan et al. 2004, [26]Zhan et al. 1998.

1.4.2 Pathogenesis - experimental methods of WSSV inoculation that simulate natural routes of virus entry have been developed. These inoculation methods are: (1) waterborne, by immersing animals in water containing WSSV cell-free suspensions (Chou et al. 1998; Supamattaya et al. 1998) and (2) feeding WSSV-infected tissues to the animals for a single time or once daily for up to seven days (Chang et al. 1998; Wang et al. 1998b; Lightner et al. 1998; Rajan et al. 2000). The latter route is considered the most important in natural and culturing conditions (Chou et al. 1998; Wu et al. 2001; Lotz & Soto 2002; Pramod-Kiran et al. 2002).

The portals of WSSV entry into the shrimp have not yet been clearly identified. According to experimental data, putative sites of WSSV entry may be the foregut (Chang et al. 1996) or midgut (Di Leonardo et al. 2005), gills and integument (Chang et al. 1996, 1998). The mechanism of viral spread from the primary replication sites to other target organs has been controversial. Some studies have indicated that WSSV infects hemocytes in crayfish and travels throughout the body in these cells to reach distant target organs (Wang et al. 2002; Di Leonardo et al. 2005). Other studies have shown by ISH and IHC that circulating hemocytes in freshwater prawns and shrimp are refractory to WSSV infection (van de Braak et al. 2002c; Shi et al. 2005) thus indicating that WSSV might reach other target organs through hemolymph circulation in a cell-free form. It is possible that these mechanisms of spread may be species-dependent.

WSSV targets cells of organs of ectodermal and mesodermal origin, including the epidermis, gills, foregut, hindgut (Wongteerasupaya et al. 1995; Chang et al. 1996), antennal gland, lymphoid organ (Chang et al. 1998; Durand et al. 1996), muscle, eye-stalk, heart (Kou et al. 1998), gonads (Lo et al. 1997), haematopoietic cells and cells associated to the nervous system (Wang et al. 1999b; Rajendran et al. 1999). Epithelial cells of organs of endodermal origin such as hepatopancreas, anterior and posterior midgut ceca and midgut trunk are refractory to WSSV infection (Sahul-Hameed et al. 1998). In the late stages of infection, the epithelia of the stomach, gills and integument may become severely damaged (Chang et al. 1996; Wang et al. 1999b). This may cause multiple organ dysfunction and probably lead to death.

1.4.3 Clinical signs and pathology - under culture conditions, many Asian and American penaeid species infected with WSSV display obvious white spots or patches of 0.5 to 3.0 mm in diameter embedded in the exoskeleton (Lo et al. 1996b; Kasornchandra et al. 1998; Wu et al. 2001; T.W. Flegel, pers. comm.). These patches are probably formed by the accumulation of calcium salts that results from the vacuolization of the cuticular epithelium (Wang et al. 1999b). Other signs of disease include a reddish discoloration in body and appendages due to the expansion of chromatophores (Lightner et al. 1998; Nadala et al. 1998); a reduction in feed uptake (Chou et al. 1995; Durand et al. 1996; Flegel 1997) preening and response to stimulus (Wongteerasupaya et al. 1995; Durand et al. 1997), loose cuticle (Lo et al. 1996b), swelling of branchiostegites due to accumulation of fluid (Otta et al. 1999), enlargement and yellowish discoloration of the hepatopancreas (Sahul-Hameed et al. 1998), thinning and delayed clotting of hemolymph (Wang et al. 2000; Kiatpathomchai et al. 2001).

In the field, WSSV-infected shrimp gather near the pond edge and display clinical signs one or two days before the first mortalities occur (Kou et al. 1998). Cumulative mortality may reach up to 100% within 10 days after the onset of disease (Karunasagar et al. 1997; Lotz & Soto 2002). In grow-out ponds, juvenile shrimp of all ages and sizes are susceptible to the disease but massive mortality usually occurs one or two months after stocking (Kasornchandra et al. 1998).

By histopathology, WSSV infection is characterized by cells with hypertrophied nuclei showing amphophilic intranuclear inclusions and marginated chromatin (Durand et al. 1997; Wang et al. 2000). These intranuclear inclusions are markedly distinct and bigger than the cowdry A-type inclusions characteristic of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Wongteerasupaya et al. 1995). Infected nuclei become progressively more basophilic and enlarged (Chang et al. 1996; Lo et al. 1996b; Durand et al. 1996, 1997; Flegel 1997; Wang et al. 1998a; Otta et al. 1999; Takahashi et al. 2000). In the late stages of infection, karyorrhexis and cellular disintegration may occur, leading to the formation of necrotic areas characterized by vacuolization (Karunasagar et al. 1997; Kasornchandra et al. 1998; Wang et al. 1999b).

1.4.4 Diagnosis - detection methods for WSSV are used for two main purposes: (1) confirmation of WSSV infections in ponds (Lightner 1996; Anonymous 2003) and (2) certification of health status in broodstock and postlarvae used for larviculture and/or grow-out ponds (Anonymous 2003). Diagnostic techniques can be divided in:

(1) presumptive, which includes: (i) on site pond observations of clinical signs such as white spots, reduction in feed uptake, locomotion and response to stimulus in cultured animals (Lightner 1996; Anonymous 2003), (ii) the recent history of disease outbreaks at the shrimp farm, (iii) the origin of stocked postlarvae and (iv) the presence of hypertrophied or vacuolated nuclei in rapid stainings (fresh mounted squashes or tissue smears) from gills or stomach from shrimp displaying signs of disease (Lightner 1996; Anonymous 2003).

(2) Confirmatory, which includes: (i) histopathological analysis of target organs (Bell & Lightner 1988; Lightner 1996; Anonymous 2003); (ii) immunoassays, done with polyclonal or monoclonal antibodies raised against whole WSSV virions or recombinant WSSV envelope proteins. These include western blot (WB) (Nadala et al. 1997; Sahul-Hameed et al. 1998), immuno-dot blot (Nadala & Loh 2000), enzyme-linked immunosorbent assay (ELISA) (Zhang et al. 2001), indirect immunofluorescence (IIF) (Escobedo-Bonilla et al. 2005) and immunohistochemistry (IHC) (Poulos et al. 2001). Recently, an immunochromatographic detection kit has been developed for rapid and specific diagnosis of WSSV in the field (Powell et al. 2006). (iii) Nucleic acid technology includes various polymerase chain reaction (PCR) protocols which may be qualitative (Lo et al. 1996a, 1996b; Tapay et al. 1999; Kiatpathomchai et al. 2001) or quantitative (Tang & Lightner 2000; Tan et al. 2001; Dhar et al. 2001; Durand & Lightner 2002). PCR is commonly used to screen potential WSSV-carriers and particularly to determine the sanitary status of postlarvae before stocking into grow-out ponds (Otta et al. 1999; Houssain et al. 2001). Other DNA based-methods include dot blot and *in situ* hybridization (ISH) (Chang et al. 1996; Durand et al. 1996; Wongteerasupaya et al. 1996; Nunan & Lightner 1997; Wang et al. 1998). These DNA-based techniques have been used for the early detection of WSSV infection (Chang et al. 1996; Lo et al. 1997; Lightner & Redman 1998; van de Braak et al. 2002c).

1.4.5. Measures for WSSV control - to date, only preventive measures can be applied to reduce the risk of a WSSV outbreak in shrimp farming facilities (Lotz 1997; Lightner & Pantoja 2001).

In farms, pathogen exclusion is done through the use of domesticated specific pathogen-free shrimp stocks and by using screens at the inlet and outlet of the ponds. Fences around ponds prevent the entry of potential carriers of WSSV. In some Asian and American countries, a series of chemicals such as disinfectants (chlorine), pesticides (carbaryl, malathion) or other biocidal substances (tea seed cake), are commonly used to eliminate pathogens, their vectors and predators of shrimp (Weston 2000).

The use of domesticated shrimp stocks may be a critical step for the sustainability of shrimp aquaculture in the future (Flegel & Alday-Sanz 1998). Stocking ponds with domesticated shrimp free of specific pathogens (SPF) has been practiced in recent years (Lotz 1997). By doing this, farmers ensure the absence of viral pathogens at the beginning of the culture; they improve the chance of a good production and significantly reduce the risk of an outbreak (Lotz 1997).

Recently, several products aimed to reduce the negative impact of WSSV infection in shrimp aquaculture have been experimentally tested. These include (1) immunostimulants added to the diet to boost the proPO system (Chang et al. 2003; Chotigeat et al. 2004), (2) ‘vaccines’ with whole inactivated virus or with recombinant envelope proteins to stimulate an antiviral response in shrimp (Namikoshi et al. 2004; Witteveldt et al. 2004), (3) antimicrobial peptides or drugs with antiviral activity *in vivo* (Dupuy et al. 2004; Rahman et al. 2006), (4) non-specific RNA interference (Robalino et al. 2004; Westenberg et al. 2005) and (5) the manipulation of water temperature to ≥ 32°C (Vidal et al. 2001, Granja et al. 2003).

The experimental challenge tests currently used have a number of drawbacks that compromise the reproducibility of results. This situation has motivated the search for the standardization of experimental inoculation procedures.

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CHAPTER 2

AIMS OF THE THESIS

Aims of the thesis

White spot syndrome virus (WSSV) is the agent that causes disease and high mortality to cultured shrimp. This virus is also infectious to many decapod crustaceans. The devastating economic impact of WSSV to several Asian and American shrimp farming countries threatens the further development of shrimp aquaculture in these regions. Therefore, the search for effective control measures against WSSV is urgently needed.

In the past, experimental WSSV challenge tests were developed to evaluate the efficacy of different products or methods to control the disease. Such challenge models used undefined viral doses and different routes of inoculation, which made it difficult to interpret the results and to compare different studies. Further, no effective control measures are available in the field. Therefore, novel and effective methods to control WSSV are needed.

A better knowledge on the early events of WSSV pathogenesis may give useful information for the development of new methods for the prevention and/or treatment of the disease caused by this pathogen.

The aims of this thesis were:

(1) To develop standardized WSSV inoculation procedures using intramuscular and oral routes.

(2) To study the pathogenesis of a WSSV infection with emphasis on the portal(s) of WSSV entry and the spread to other target organs in SPF *Litopenaeus vannamei*.

(3) To use the standardized inoculation procedures developed in (1) for the evaluation and comparison of antiviral products and the manipulation of water temperature to control WSSV infection.

CHAPTER 3

DEVELOPMENT OF STANDARDIZED WSSV INOCULATION PROCEDURES IN SPECIFIC PATHOGEN-FREE (SPF) *Litopenaeus vannamei*

“We learn from failure much more than from success; we often discover what we will do by finding out what we will not do; and probably he who never made a mistake, never made a discovery.” - Samuel Smiles

3.1 *In vivo* titration of white spot syndrome virus (WSSV) in SPF *Litopenaeus vannamei* by intramuscular and oral routes

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C.M. Escobedo-Bonilla, M. Wille, V. Alday Sanz, P. Sorgeloos, M.B. Pensaert, H.J. Nauwynck.

ABSTRACT

White spot syndrome virus (WSSV) is a devastating pathogen in shrimp aquaculture. Standardized challenge procedures using a known amount of infectious virus would assist in evaluating strategies to reduce its impact. In this study, the shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) of a Thai isolate of WSSV was determined by intramuscular inoculation (IM) in 60 days-old and 135 days-old SPF *Litopenaeus vannamei* using indirect immunofluorescence (IIF) and one-step polymerase-chain reaction (PCR). Also the lethal dose 50% endpoint (LD_{50} ml^{-1}) was determined from the proportion of dead shrimp. The median virus titers of infection in 60 days-old and 135 days-old juveniles were $10^{6.8}$ and $10^{6.5}$ SID_{50} ml^{-1}, respectively. These titers were not significantly different ($P \geq 0.05$). The titration of the WSSV stock by oral intubation in 80 days-old (PL 80) juveniles resulted in approximately ten - fold reduction in virus titer compared to IM route. This lower titer is probably the result of physical and chemical barriers in the digestive tract of shrimp that hinders WSSV infectivity. The titers determined by infection were identical to the titers determined by mortality in all experiments using IM and oral routes at 120 hours post inoculation (hpi) indicating that every infected shrimp died. The determination of WSSV titers by IM and oral routes constitutes the first step towards the standardization of challenge procedures to evaluate strategies to reduce WSSV infection.

INTRODUCTION

White spot syndrome virus (WSSV) is a devastating pathogen causing disease and mortality in shrimp aquaculture. First recorded in 1992 in Taiwan (Chou et al. 1995), it has spread to several shrimp-farming countries in Asia and Latin America (Wang et al. 2000, Hill 2002).

In culture ponds, mortalities up to 100% may occur within 10 days after the onset of disease (Kasornchandra et al. 1998). In many Asian shrimp species the acute phase of disease is characterized by the presence of white spots on the inner surface of the exoskeleton (Lo et al. 1996) from which the disease name is derived. Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Otta et al. 1999).

WSSV is an enveloped, non-occluded bacilliform-shaped virus containing a double-stranded DNA genome with a size between 293 and 308 kbp (van Hulten et al. 2001, Yang et al. 2001). This pathogen was first grouped with the non-occluded enveloped baculoviruses (Durand et al. 1996), but gene sequencing and characterization of its major structural proteins have shown low homology with this virus family. These molecular differences supported the formation of a new virus family for WSSV: the *Nimaviridae* (Vlak et al. 2002). Several decapod crustaceans (Chang et al. 1998, Sahul-Hameed et al. 2003) and shrimp species (Wongteerasupaya et al. 1996, Chou et al. 1998, Wang et al. 1999) are susceptible to WSSV infection.

Several experiments have been carried out with WSSV to determine its pathogenicity in crustacean hosts using intramuscular (IM) inoculation (Jiravanichpaisal et al. 2001), the *per os* route by feeding WSSV-infected tissues to experimental animals (Rajendran et al. 1999, Wang et al. 1999) and by immersion (Chou et al. 1998, Rajan et al. 2000). In these studies, the dose of infectious virus given to each animal was not known. A standardized inoculation procedure requires two major components: (1) the use of animals with low genetic variability and high susceptibility to the virus, preferably free of specific pathogens (SPF) and (2) a WSSV stock with a known titer of infection. Such a standardized procedure is essential (*i*) to compare the susceptibility of different host species and life stages to WSSV, (*ii*) to determine the virulence of different WSSV strains, and (*iii*) to test the efficacy of strategies aimed to control the disease.

To date, no shrimp cell cultures are available for *in vitro* titration of WSSV therefore *in vivo* titration is the only alternative. Only one previous study has determined the LD_{50} of a WSSV tissue suspension. A South Carolina isolate was inoculated by IM route in 1 g SPF *Litopenaeus vannamei* and the virus titer was determined using probit analysis (Prior et al. 2003).

The aims of the present study were: (1) to determine the shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) and the lethal dose 50% endpoint (LD_{50} ml^{-1}) of a Thai isolate of WSSV in two sizes of juvenile SPF *Litopenaeus vannamei* by IM route, (2) to determine these virus titers by oral inoculation and (3) to establish the relationship between WSSV infection and shrimp mortality for the two routes of inoculation.

MATERIAL AND METHODS

Experimental shrimp and rearing conditions. Specific pathogen-free (SPF) *Litopenaeus vannamei* of the Kona strain was used (Wyban et al. 1992). Batches of shrimps arrived as postlarvae (PL 8 - 12; mean body weight [MBW] = 0.0013 g) and were acclimatized and reared for a period of 36 to 50 days after arrival. Postlarvae were fed with *Artemia* nauplii once daily. Juveniles were fed with a commercial pelleted feed (A2 monodon high performance shrimp feed/shrimp complete grower, INVE aquaculture NV) at a rate of 2.5% MBW twice daily. Water temperature was kept at 27°C ± 1°C, salinity between 30 and 35 g l^{-1}, total ammonia-N less than 0.5 mg l^{-1} and nitrite-N between 0.05 and 0.15 mg l^{-1}.

Experimental conditions. Shrimp were acclimatized to a salinity of 15 g l^{-1} over four days prior to experimental infectivity trials. Shrimp were kept individually in 10 l plastic aquaria provided with aeration and covered with acrylic plates. Shrimp were fed a restricted diet of six pellets divided into two rations a day to maintain water quality. Water temperature was between 25 and 28°C, total ammonia levels were between 0 and 5 mg l^{-1} and nitrite was between 0 and 0.15 mg l^{-1} as monitored daily. Artificial seawater at a salinity of 15 g l^{-1} was prepared with instant ocean (Marine systems, France) dissolved in distilled water.

Virus and production of the WSSV stock. A Thai isolate of WSSV from naturally-infected *Penaeus monodon* was used. This virus isolate was passaged once in crayfish

Pacifastacus leniusculus (Jiravanichpaisal et al. 2001). A gill suspension from crayfish (10^{-2} in L-15 medium) was kindly donated by P. Jiravanichpaisal and K. Söderhäll, (Uppsala University, Sweden). It was diluted 10^{-1} in phosphate-buffered saline (PBS) pH 7.4 and 50 µl were injected intramuscularly (IM) into SPF *Litopenaeus vannamei* to amplify the virus. The inoculated shrimp were collected at 48 hours post inoculation (hpi) and were frozen at -70°C. Tissues from these shrimps were analyzed by indirect immunofluorescence (IIF) to confirm WSSV infection. Thawed carcasses without hepatopancreas, gut and exoskeleton were minced. A 10^{-1} suspension was made in PBS and centrifuged (3000 x g at 4°C for 20 min). The supernatant was centrifuged (13000 x g at 4°C for 20 min), filtered (0.45 µm) and aliquoted for storage at -70°C. The total volume was 250 ml. Samples from tissues used to produce the viral stock were sent to Dr. James Brock (Moana Technologies LLC, Hawaii) for detection of the major viral pathogens of shrimp by polymerase-chain reaction (PCR). PCR analysis confirmed the sole presence of WSSV DNA in the tissues.

***In vivo* titration by intramuscular inoculation.** *Virus titer of infection in 135 days-old juveniles and its relationship to the titer of mortality* - Five experiments were performed using shrimp of this age (MBW = 13.34 ± 4.08 g, n = 172). In three experiments shrimp were inoculated by IM route with 200 µl of a tenfold serial dilution of WSSV. Four shrimp were used per dilution. Moribund and dead shrimp were recorded, removed from the aquaria and processed for detection of WSSV infection. Surviving shrimp were sacrificed at the end of the experiments and analyzed for WSSV infection. These experiments were terminated at 72, 96 and 168 hpi. In the other two experiments shrimp were inoculated by IM route with 50 µl of a tenfold serial dilution of WSSV. Twelve shrimp were used per dilution. Moribund, dead and surviving shrimp were analyzed for WSSV infection. These experiments were terminated at 120 and 168 hpi (Table1).

Virus titers in 60 days - old juveniles - Three experiments were performed with shrimp (MBW = 3.00 ± 1.18 g; n = 75) inoculated by IM route with 50 µl of a tenfold serial dilution of WSSV. Five shrimp were used per dilution. Moribund and dead shrimp were processed for detection of WSSV infection. These experiments were terminated at 120 hpi (Table 2).

The statistical comparison of the virus titers in these two sizes of juvenile shrimp was undertaken using the non-parametric test of Mann-Whitney (Zar, 1996).

***In vivo* titration by oral inoculation** *Virus titers in 80 days-old juvenile shrimp* - The minimum size of shrimp that could be inoculated by oral route was 6 - 7 g. The virus titer of the WSSV stock by oral inoculation was evaluated using the overall median virus titer found by IM route as the basis to define five doses (10^3, 10^2, 10^1, 10^0 and 10^{-1} SID_{50} [IM] in 50 µl). This procedure allowed a direct comparison of the infectivity of the virus stock between the IM and the oral routes. Two experiments were performed in 80 days-old shrimp (MBW = 7.75 ± 2.83 g, n = 50). Five shrimp per dose were intubated using a sterile flexible pipette tip (Biozym, The Netherlands) and a magnifying glass (2.5 X) to locate the mouth. Shrimp were placed with the ventral side up, the pipette tip was introduced into the oral cavity and the viral inoculum was delivered into the lumen of the oral tract. Moribund and dead shrimp were analyzed for WSSV infection.

Determination of the virus titers. The virus titers of infection (SID_{50} ml^{-1}) and mortality (LD_{50} ml^{-1}) were calculated using the method of Reed & Muench (1938). Briefly, data of infected and uninfected shrimp at each dilution were ordered. Infected shrimp were summed up from the lowest to the highest concentration as well as the uninfected ones but in the inverse direction to obtain the percentage of infected shrimp for each dilution. The two dilutions with the nearest percentage above (a) and below (b) 50% were used to calculate a proportional distance (50% - b / a - b) which was added to the $\log_{10}$ of the dilution next below to 50% in order to determine the 50% endpoint of infection (SID_{50} ml^{-1}) according to the volume inoculated.

Evaluation of WSSV infection. Moribund, dead and surviving shrimp at the end of the experiments were processed to detect WSSV infection using indirect immunofluorescence (IIF) and one-step PCR.

Indirect immunofluorescence analysis (IIF) - Tissues from the pereon were embedded in methylcellulose (Fluka, Germany) and frozen at -20°C. Cryosections (5 - 6 µm) were made and fixed in absolute methanol at -20°C, washed with PBS, incubated for 1 h at 37°C with 2 mg ml^{-1} of the monoclonal antibody 8B7 specific for VP28 (Poulos et al. 2001), washed and incubated for 1 h at 37°C with 0.02 mg ml^{-1} of fluorescein isothiocyanate (FITC) -labeled goat anti-mouse antibody (F-2761, Molecular Probes,

The Netherlands) in PBS, washed with PBS, rinsed in deionised water, dried and mounted. Slides were analyzed by fluorescence microscopy (Leica DM RBE, Germany). Tissues of moribund shrimp infected with WSSV and uninfected shrimp were stained and used as positive and negative controls respectively.

One-step PCR - Total DNA was extracted from shrimp muscle with lysis buffer (Intelligene Corp. Taiwan). Primers F002 and R002 were used to amplify WSSV DNA and primers F and R3 which amplify β-actin from shrimp were used as control. The amplicon for WSSV was 306 bp, while that for β-actin was 339 bp (Dhar et al. 2001).

Extracted DNA (2 µl) was added to a PCR tube containing 48 µl of a PCR master mix [1X PCR buffer (Eurogentec, Belgium), 1.5 mM $MgCl_2$, 0.3 mM of each of the respective forward and reverse primers for WSSV or β-actin, 1.6 mM dNTPs (Eurogentec, Belgium), 1 U hot goldstar *Taq* polymerase (Eurogentec, Belgium) total reaction volume 50 µl]. One-step PCR was carried out as follows: a preheating step at 95°C for 10 minutes followed by 35 cycles each with the following steps: denaturation (94°C for 45 s), annealing (55°C for 45 s), (72°C for 75 s) and a final extension at 72°C for 5 minutes. PCR products were stored at 4°C. PCR products (12 µl), negative (ultrapure water) and positive (DNA from a 10^{-2} dilution of WSSV stock) controls, as well as DNA markers (smart ladder, Eurogentec, Belgium) were resolved on a 1.2% agarose gel in tris-acetate-EDTA (TAE) buffer. The gel was stained with ethidium bromide (0.02 mg ml^{-1}) and visualized by UV transillumination.

RESULTS

***In vivo* titration by intramuscular inoculation.** *Virus titer of infection in 135 days-old juveniles and its relationship to the titer of mortality* - The number of shrimp that died before termination of the experiments at 72, 96, 120 and 168 hpi and the number of dead or euthanized shrimp found positive by IIF (Figure 1) and one-step PCR are presented in Table 1. The virus titers of infection in the experiments terminated at 72 and 96 hpi were $10^{6.0}$ and $10^{6.4}$ SID_{50} ml^{-1} respectively, and the titer of infection in the experiment terminated at 168 hpi was $10^{6.5}$ SID_{50} ml^{-1}. The experiments performed with 12 shrimps per dilution had virus titers of infection of $10^{6.4}$ SID_{50} ml^{-1} at 120 hpi and

$10^{6.6}$ SID_{50} ml^{-1} at 168 hpi. The same proportion of infected shrimp was detected by IIF and one-step PCR in each of these experiments.

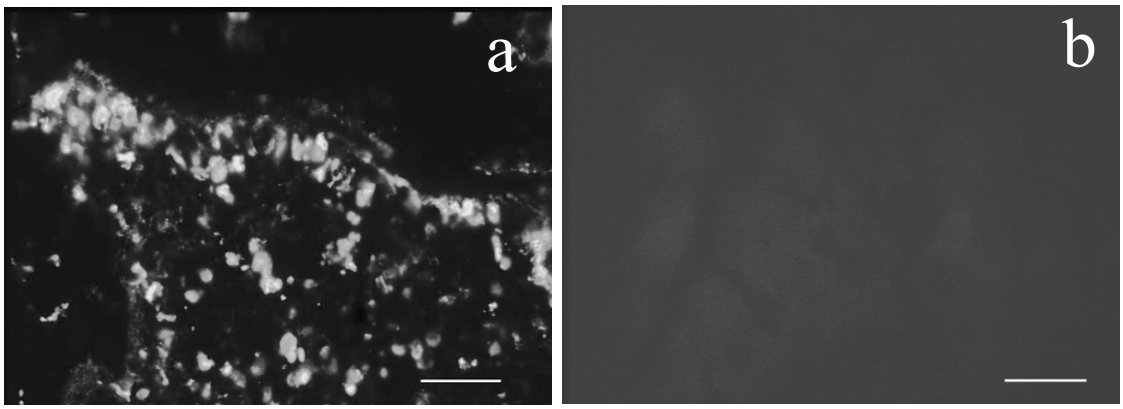

Figure 1 - Presence (a) or absence (b) of WSSV-infected cells in tissues of (a) WSSV-infected or (b) uninfected shrimp as determined by indirect immunofluorescence. Bar = 100 μm

Virus titers in 60 days - old juveniles - The virus titers of infection in each of the three experiments performed in this size of juveniles were $10^{6.8}$, $10^{6.9}$ and $10^{6.5}$ SID_{50} ml^{-1} (Table 2). The same proportion of WSSV-infected shrimp was determined by IIF and one-step PCR (Figure 2). Likewise, the titers of mortality were the same as the titers of infection for each experiment.

The titers of mortality were lower than those of infection when the experiments were terminated at 72 or 96 hpi ($10^{5.7}$ and $10^{5.4}$ LD_{50} ml^{-1}, respectively), whereas the titer of mortality fully matched that of infection in the experiment terminated at 120 hpi ($10^{6.4}$ LD_{50} ml^{-1}) as well as in the two experiments terminated at 168 hpi ($10^{6.5}$ and $10^{6.6}$ LD_{50} ml^{-1}).

The statistical comparison of the three virus titers determined at 120 and 168 hpi in 135 days-old shrimp and the three performed in 60 days-old juveniles inoculated by IM route showed no significant differences ($P \geq 0.05$). Therefore these six virus titers were used to establish the overall median virus titer of infection of the WSSV stock at $10^{6.6}$ SID_{50} ml^{-1} and the overall median virus titer of mortality was $10^{6.6}$ LD_{50} ml^{-1}.

Table 1.- Titers by mortality (LD_{50} ml^{-1}) and infection (SID_{50} ml^{-1}) of the WSSV stock by IM inoculation in 135 days-old *Litopenaeus vannamei*.

Experiment	Termination (hpi)	Dilution	Number of shrimps	Mortality	Infection determined by IIF	Infection determined by PCR
1	72	10^{-5}	4	2/4	2/4	2/4
		10^{-6}	4	0/4	1/4	1/4
		10^{-7}	4	0/4	0/4	0/4
		10^{-8}	4	0/4	0/4	0/4
			Titer (IM route)	$10^{5.7}$ LD_{50} ml^{-1}	$10^{6.0}$ SID_{50} ml^{-1}	$10^{6.0}$ SID_{50} ml^{-1}
2	96	10^{-2}	4	4/4	4/4	4/4
		10^{-3}	4	4/4	4/4	4/4
		10^{-4}	4	4/4	4/4	4/4
		10^{-5}	4	1/4	4/4	4/4
		10^{-6}	4	0/4	1/4	1/4
			Titer (IM route)	$10^{5.4}$ LD_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}
3	120	10^{-4}	12	11/12	11/12	11/12
		10^{-5}	12	7/12	7/12	7/12
		10^{-6}	12	1/12	1/12	1/12
		10^{-7}	12	0/12	0/12	0/12
		10^{-8}	12	0/12	0/12	0/12
			Titer (IM route)	$10^{6.4}$ LD_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}	$10^{6.4}$ SID_{50} ml^{-1}
4	168	10^{-5}	4	4/4	4/4	4/4
		10^{-6}	4	1/4	1/4	1/4
		10^{-7}	4	1/4	1/4	1/4
		10^{-8}	4	0/4	0/4	0/4
			Titer (IM route)	$10^{6.5}$ LD_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}
5	168	10^{-4}	12	12/12	12/12	12/12
		10^{-5}	12	7/12	7/12	7/12
		10^{-6}	12	3/12	3/12	3/12
		10^{-7}	12	0/12	0/12	0/12
		10^{-8}	12	0/12	0/12	0/12
			Titer (IM route)	$10^{6.6}$ LD_{50} ml^{-1}	$10^{6.6}$ SID_{50} ml^{-1}	$10^{6.6}$ SID_{50} ml^{-1}

Table 2.- Titers by mortality (LD_{50} ml^{-1}) and infection (SID_{50} ml^{-1}) of the WSSV stock by IM inoculation in 60 days-old *Litopenaeus vannamei*.

Experiment	Termination (hpi)	Dilution	Number of shrimps	Mortality	Infection determined by IIF	Infection determined by PCR
1	120	10^{-4}	5	5/5	5/5	5/5
		10^{-5}	5	5/5	5/5	5/5
		10^{-6}	5	0/5	0/5	0/5
		10^{-7}	5	0/5	0/5	0/5
		10^{-8}	5	0/5	0/5	0/5
			Titer (IM route)	$10^{6.8}$ LD_{50} ml^{-1}	$10^{6.8}$ SID_{50} ml^{-1}	$10^{6.8}$ SID_{50} ml^{-1}
2	120	10^{-4}	5	5/5	5/5	5/5
		10^{-5}	5	5/5	5/5	5/5
		10^{-6}	5	1/5	1/5	1/5
		10^{-7}	5	0/5	0/5	0/5
		10^{-8}	5	0/5	0/5	0/5
			Titer (IM route)	$10^{6.9}$ LD_{50} ml^{-1}	$10^{6.9}$ SID_{50} ml^{-1}	$10^{6.9}$ SID_{50} ml^{-1}
3	120	10^{-4}	5	5/5	5/5	5/5
		10^{-5}	5	3/5	3/5	3/5
		10^{-6}	5	0/5	0/5	0/5
		10^{-7}	5	0/5	0/5	0/5
		10^{-8}	5	0/5	0/5	0/5
			Titer (IM route)	$10^{6.5}$ LD_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}	$10^{6.5}$ SID_{50} ml^{-1}

***In vivo* titration by oral inoculation** *Virus titers in 80 days-old juvenile shrimp* - The 50% endpoint of the virus titers by oral inoculation was 1.2 log_{10} and 0.7 log_{10} times higher than by IM inoculation (Table 3). Thus the titers of the WSSV stock determined using the oral route of inoculation were $10^{5.6}$ SID_{50} ml^{-1} and $10^{5.6}$ LD_{50} ml^{-1}.

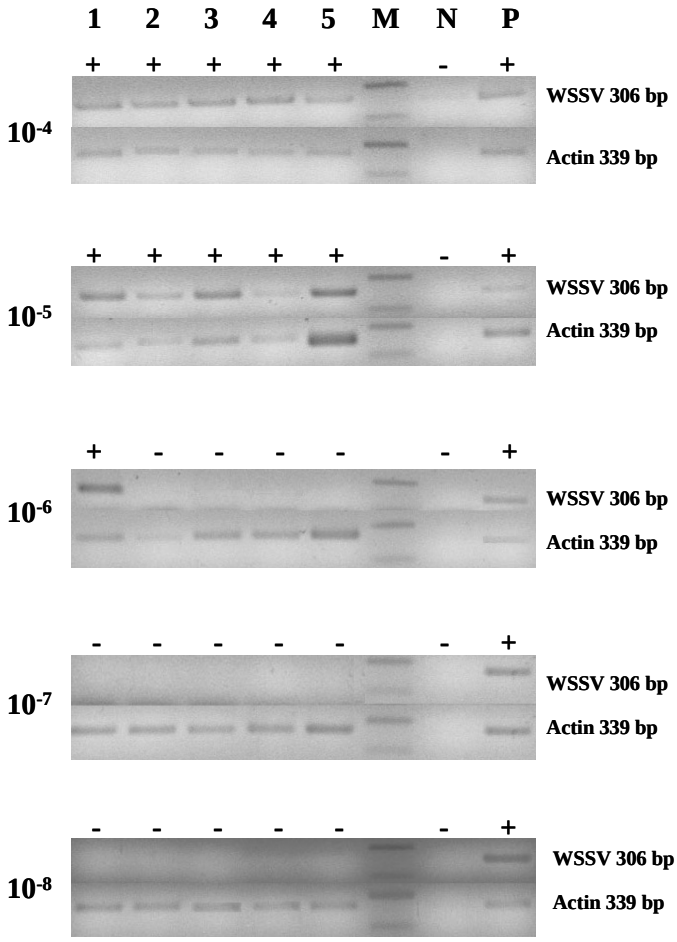

Figure 2 - *In vivo* titration of WSSV by one-step PCR. WSSV-positive tissues of 60 days-old *Litopenaeus vannamei*. Lanes 1 to 5, shrimp number. M, DNA weight marker (upper = 400 bp, lower = 200 bp). N, negative control. P, positive control

Table 3.- Doses of WSSV (based on the median virus titer by IM route) orally inoculated in 80 days-old *Litopenaeus vannamei* and virus titers of mortality (LD_{50} ml^{-1}) and infection (SID_{50} ml^{-1}) of the WSSV stock obtained by oral route.

Experiment	Termination (hpi)	Dose (SID_{50} IM)	Number of shrimps	Mortality	Infection determined by IIF	Infection determined by PCR
1	120	10^{3}	5	5/5	5/5	5/5
		10^{2}	5	5/5	5/5	5/5
		10^{1}	5	2/5	2/5	2/5
		10^{0}	5	0/5	0/5	0/5
		10^{-1}	5	0/5	0/5	0/5
				$10^{1.2}$*	$10^{1.2}$*	$10^{1.2}$*
		Titer (oral route)		$10^{5.4}$ LD_{50} ml^{-1}	$10^{5.4}$ SID_{50} ml^{-1}	$10^{5.4}$ SID_{50} ml^{-1}
2	120	10^{3}	5	5/5	5/5	5/5
		10^{2}	5	5/5	5/5	5/5
		10^{1}	5	3/5	3/5	3/5
		10^{0}	5	1/5	1/5	1/5
		10^{-1}	5	0/5	0/5	0/5
				$10^{0.7}$*	$10^{0.7}$*	$10^{0.7}$*
		Titer (oral route)		$10^{5.9}$ LD_{50} ml^{-1}	$10^{5.9}$ SID_{50} ml^{-1}	$10^{5.9}$ SID_{50} ml^{-1}

*Times the 50% endpoint by oral route higher than by IM route

DISCUSSION

The *in vivo* titration of viral stocks using the 50% endpoint dilution assay is commonly used when virus titers cannot be calculated *in vitro* (Flint et al. 2000). In the present study the virus titers of infection and mortality of a WSSV stock inoculated by IM and oral routes were determined using this method. To our knowledge, this is the first study to describe the relationship between routes of exposure (IM vs. oral) and virus infectivity of a WSSV stock in *Litopenaeus vannamei.* The virus titers of infection and mortality in 60 and 135 days-old juveniles were not significantly different, indicating that these two stages of juvenile shrimp had the same susceptibility to WSSV when the virus is inoculated by IM route.

In vivo titrations are important to evaluate differences in susceptibility between life stages within a host species or between related species (Plumb & Zilberg 1999). In shrimp, there are indications that susceptibility to WSSV may differ between life stages (Pramod-Kiran et al. 2002, Yoganandhan et al. 2003), shrimp species (Lightner et al. 1998, Wang et al. 1999) and different decapods (Wang et al. 1998, Sahul-Hameed et al. 2003). However, the use of a known dose of infectious virus is critical to demonstrate these differences.

The virulence of a pathogen or its power to produce disease in a host is a measurable feature (Shapiro-Ilan et al. 2005). In order to compare the virulence of different virus strains their infectivity (SID_{50} ml^{-1}) should be known. Every shrimp should be inoculated with the same amount of infectious virus. The inoculation techniques described in the present study may be used to determine the infectivity of different WSSV strains and to test their virulence.

The virus titers of infection determined both by IIF and one-step PCR were identical in each of the experiments performed either by intramuscular or oral inoculations. Every shrimp detected positive by PCR was equally detected by IIF. All surviving shrimp at 120 hpi or longer were not infected with WSSV as determined by these two methods. Although one-step PCR is probably more sensitive than IIF (Sizun et al. 1998, Walker et al. 1998) under these experimental conditions IIF was able to detect WSSV-infected cells in all shrimp detected positive by PCR. In addition it was very convenient: cheap, easy to perform, yielding rapid results (within 4 hours) and with good sensitivity. Thus these results indicate that IIF may be considered as a suitable diagnostic tool in areas where PCR is not available.

The relationship between the virus titers of infection and mortality with the Thai isolate of WSSV by IM or oral route was 1:1 only in experiments terminated at 120 hpi or later. Thus, every shrimp that became infected with this strain of WSSV by any of these routes of inoculation died within 120 hpi. Although previous studies have determined WSSV infection and mortality of *Litopenaeus vannamei*, none has indicated a full match between these two parameters. All surviving shrimp analyzed by histopathology were WSSV-negative, but not all the dead shrimp (6%) were WSSV-positive (Soto & Lotz 2003), or the analysis of WSSV infection was not performed in all the survivors (Prior et al. 2003). Therefore, the present study is the first to show the identity between

infection (SID_{50} ml^{-1}) and mortality (LD_{50} ml^{-1}) in shrimp inoculated by IM or oral routes. Moreover, the present study extends the findings of Prior et al. in the following points: (i) the virus titers were determined by IM injection and oral intubation in contrast to the evaluation of the LD_{50} only by IM inoculation but not by immersion, (ii) the WSSV strain used is different (a Thai strain vs. an American strain), (iii) the infectivity of the WSSV stock was determined by IM route in two sizes of juvenile shrimp, in contrast to only one shrimp size and (iv) the use of IIF to detect WSSV replication in infected cells represents a more appropriate virological technique than histopathology to determine WSSV infection.

The oral route represents the natural way of WSSV entry into shrimp through ingestion (Wu et al. 2001, Lotz & Soto 2002). Experimental infections using the oral route have been performed by feeding shrimp and other crustaceans with WSSV-infected tissues. However, this procedure cannot guarantee that every animal receives the same amount of infectious virus. The results presented here demonstrate that oral intubation makes it possible to deliver a fixed quantity of virus to all inoculated shrimp and therefore represents a solution to this problem.

Once WSSV enters the oral cavity it has to overcome a series of physical and chemical barriers in the digestive tract of the shrimp in order to reach the susceptible epithelial cells. The cuticle sheath coating the epithelium of the foregut forms an important physical barrier (Ceccaldi, 1997, Martin & Chiu 2003). Digestive enzymes such as trypsin, amylases and lipases (Gamboa-Delgado et al. 2003) form a chemical barrier. These barriers may account for the reduction of WSSV infection by approximately 1 log_{10} compared with the IM route of inoculation. It is possible that through these barriers shrimp may be able to prevent WSSV to reach the epithelial cells in the stomach when it is orally inoculated.

In conclusion, the virus titers of the WSSV stock determined *in vivo* by intramuscular and oral routes constitute the first step towards standardization of infectivity models. These models can be used for the evaluation of different strategies (immunostimulants, antivirals and vaccines) aimed to reduce the impact of WSSV disease and for comparing the virulence of WSSV strains.

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3.2 Standardized white spot syndrome virus (WSSV) inoculation procedures for intramuscular or oral routes

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ABSTRACT

In the past, strategies to control white spot syndrome virus (WSSV) were mostly tested by infectivity trials *in vivo* using immersion or *per os* inoculation of undefined WSSV infectious doses, complicating the comparison between experiments. In this study, the reproducibility of three defined doses (10, 30 and 90 shrimp infectious doses 50% endpoint [SID_{50}]) of WSSV was determined in three experiments using the intramuscular (im) or oral inoculation in specific pathogen-free (SPF) *Litopenaeus vannamei*. Reproducibility was determined by the time of onset of disease, cumulative mortality and median lethal time (LT_{50}). By im route, the three doses induced disease between 24 and 36 hours post inoculation (hpi). Cumulative mortality was 100% at 84 hpi with doses 30 and 90 SID_{50} and 108 hpi with dose 10 SID_{50}. The LT_{50} of doses 10, 30 and 90 SID_{50} was 52, 51 and 49 hpi respectively and not significantly different ($p > 0.05$). Shrimp orally inoculated with 10, 30 or 90 SID_{50} developed disease between 24 and 36 hpi. Cumulative mortality was 100% at 108 hpi with doses 30 and 90 SID_{50} and 120 hpi with dose 10 SID_{50}. The LT_{50} of 10, 30 and 90 SID_{50} was 65, 57 and 50 hpi respectively and significantly different from each other ($p < 0.05$). A dose of 30 SID_{50} was selected as the standard for further WSSV challenges by i.m. or oral routes. These standardized inoculation procedures may be applied to other Crustacea and WSSV strains to achieve comparable results between experiments.

INTRODUCTION

White spot syndrome virus (WSSV) is one of the most lethal pathogens in shrimp aquaculture. First reported in Taiwan in 1992 (Chou et al. 1995) it has spread to several shrimp farming countries. Within a decade, it has become a serious threat to the shrimp culture industry throughout Asia and Latin America (Hill 2002). WSSV also infects many other crustacean species from several regions in the world (Lo et al. 1996, Chang et al. 1998, Kanchanaphum et al. 1998, Kasornchandra et al. 1998, Wang et al. 1998, Rajendran et al. 1999, Corbel et al. 2001, Hossain et al. 2001, Sahul-Hameed et al. 2003).

The WSSV virion is bacilliform, non-occluded and enveloped. It contains a circular, double-stranded DNA genome with size between 293 and 307 kilobasepairs (kbp) (van Hulten et al. 2001, Yang et al. 2001, Chen et al. 2002). Several WSSV strains have been identified by differences in their genomic size (Wang et al. 2000), restriction enzyme profile (Nadala & Loh, 1998), deletion variants (Lan et al. 2002) or pathogenicity (Wang et al. 1999a).

The disease caused by this virus is characterized by the presence of white spots in the inner surface of the exoskeleton of *Penaeus monodon* and other Asian shrimp species during the acute phase. Other clinical signs include a reduced feed uptake, locomotion and reddish discoloration of the body (Otta et al. 1999). Mass mortalities (up to 100%) have been reported within 10 days after the onset of disease (Wang et al. 1999b).

Several approaches to reduce mortality due to WSSV have been tested using experimental challenges. Some of these include (i) feeding shrimp with immunostimulants to enhance the defense response (Chang et al. 1999, 2003, Huang & Song, 1999, Newman, 1999, Takahashi et al. 2000, Yusoff et al. 2001, Chotigeat et al. 2004), (ii) ‘vaccinating’ shrimp with formalin-fixed virus or recombinant WSSV-envelope proteins (Namikoshi et al. 2004, Witteveldt et al. 2004a, 2004b), (iii) administering antimicrobial peptides (mytilin) (Dupuy et al. 2004) or double-stranded RNA (dsRNA) (Robalino et al. 2004) and (iv) manipulating water temperature (Vidal et al. 2001, Granja et al. 2003, Guan et al. 2003, Jiravanichpaisal et al. 2004).

Other strategies with potential to combat WSSV infections include the induction of antiviral genes present in shrimp (Luo et al. 2003), the application of synthetic antiviral

peptides (Yi et al. 2003) and the induction of a 'WSSV neutralizing factor' in shrimp using sublethal concentrations of WSSV (Venegas et al. 2000, Wu et al. 2002).

So far, most of the WSSV challenge tests developed to control WSS disease have used different inoculation routes and undefined amounts of infectious virus. The routes of inoculation used are immersion, *per os* feeding of infected tissues and intramuscular (i.m.) injection. The amount of infectious virus taken up by each animal using immersion or feeding may be quite different, making it very difficult to compare results from different studies. The development of standardized WSSV inoculation procedures that yield reproducible results in terms of onset and severity of disease would be significant to solve this problem.

One of the main requirements for a reproducible model of infection is to use a virus stock with known infectivity titer. The shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) of the Thai WSSV stock used in this study was determined by *in vivo* titration in specific pathogen-free (SPF) *Litopenaeus vannamei* by i.m. and oral routes (Escobedo-Bonilla et al. 2005). Determination of the infectivity titer allows the establishment of a reproducible dose-response curve for experimental WSSV infections in shrimp.

The objectives of this study were (1) to develop standardized WSSV inoculation procedures by IM and oral routes and (2) to characterize the mortality pattern (time of onset of disease, median lethal time [LT_{50}] and cumulative mortality) of three doses of a Thai WSSV stock. Thus, a reproducible dose-response relationship was established to determine an appropriate WSSV dose to be used as a standard in further experimental challenges.

MATERIAL AND METHODS

Shrimp and rearing conditions Specific pathogen-free (SPF) *Litopenaeus vannamei* of the Kona strain (Wyban et al. 1992) were used. Batches of shrimp arrived at the Laboratory of Aquaculture & *Artemia* Reference Center (ARC), Ghent University, as postlarvae (PL 8 - 12; mean body weight [MBW] = 0.0013 g). Shrimp at this stage were fed *Artemia* nauplii once daily for one week. Afterwards, they were fed with a crumbled commercial pelleted feed (A2 monodon high performance shrimp feed/shrimp complete grower, INVE aquaculture NV) at a rate of 2.5% MBW twice daily. Older juvenile

shrimp were fed a pelleted feed at the same rate twice daily. Water temperature was 27°C ± 1°C, salinity ranged between 30 and 35 g l^{-1}, total ammonia was less than 0.5 mg l^{-1} and nitrites ranged between 0.05 and 0.15 mg $^{-1}$.

WSSV stock and *in vivo* infectivity titers The WSSV stock used in this study was prepared and titrated by i.m. or oral inoculations as described previously (Escobedo-Bonilla et al. 2005). The median virus titer of infection was $10^{6.6}$ shrimp infectious dose 50% endpoint (SID_{50} ml^{-1}) by IM route and $10^{5.6}$ SID_{50} ml^{-1} by oral route.

Doses Three doses of the WSSV stock were prepared in phosphate-buffered saline pH 7.4 (PBS) for i.m. or oral inoculation: 10, 30 and 90 SID_{50} in a volume of 50 µl.

Experimental conditions Shrimp were acclimatized to a salinity of 15 g l^{-1} over four days at the ARC and then transported to the Laboratory of Virology, Faculty of Veterinary Medicine Ghent University, where the experiments were carried out under biosafety conditions. Shrimp were accustomed to the experimental conditions 24 h before challenge and during this time they were not fed. After inoculation, shrimp were fed daily with only six pellets in order to maintain the water quality.

Groups of 10 shrimp each were placed in 50 l glass aquaria with glass covers and a plastic sheath to prevent transmission by aerosol. Artificial seawater was prepared at 15 g l^{-1} with Instant Ocean® (Marine systems, France) in distilled water. Each aquarium was fitted with a mechanical filter (Eheim classic 2213, Germany), a water heater (Visitherm, aquarium systems, USA) and aeration. Water temperature was 27 ± 1°C, total ammonia was between 0 and 5 mg l^{-1} and nitrites were between 0 and 0.15 mg l^{-1} as monitored daily.

Intramuscular inoculation procedure Three experiments were performed using the i.m. route. In each experiment, three groups of 10 shrimp (MBW = 9.40 ± 4.92 g, n = 120) were inoculated with 10, 30 or 90 SID_{50}. In addition, a group of 10 shrimp was mock-inoculated with 50 µl of PBS and used as controls. Shrimp were injected between the third and fourth segments of the pleon. Before and after injection, this surface was wiped with 70% ethanol and finally shrimp were re-placed into their respective aquarium. These experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 120 hours post inoculation (hpi).

Oral inoculation procedure Three experiments were performed using the oral route. In every experiment, three groups of 10 shrimp (MBW = 9.72 ± 2.24 g, n = 120) were

intubated with one of three doses (10, 30 and 90 SID_{50}). Ten shrimp were mock-inoculated with 50 µl of PBS and used as control. Oral inoculation was performed as follows: Shrimp were placed in a tray with the ventral side up. A flexible and slender pipette tip (no. 790004 Biozym, The Netherlands) was introduced into the oral cavity, the inoculum was delivered into the lumen of the foregut and shrimp were re-placed into their respective aquarium. These experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 120 hpi.

Evaluation of WSSV infection Inoculated shrimp were monitored every 12 h throughout the experiment. Moribund and dead shrimp were removed and processed for indirect immunofluorescence (IIF) analysis as well as control shrimp.

Clinical signs - Litopenaeus vannamei rarely display white spots during WSSV infection (Nadala et al. 1998, Rodriguez et al. 2003). Empty guts and reduced response to mechanical stimulation are the first clinical signs to appear in WSSV-diseased shrimp and are good indicators of infection and mortality. These clinical signs were used to monitor the onset of disease in shrimp inoculated by IM or oral routes.

Indirect immunofluorescence analysis (IIF) - Shrimp were processed for detecting WSSV-infected cells as follows: pereons were excised and embedded in methylcellulose (Fluka, Germany) and frozen at -20°C. Cryosections (5 - 6 µm) were made and tissues fixed in absolute methanol at -20°C, washed with PBS and incubated for 1 h at 37°C with 2 mg ml^{-1} of the monoclonal antibody 8B7 against VP28 (Poulos et al. 2001). Tissues were washed and incubated for 1 h at 37°C with 0.02 mg ml^{-1} of fluorescein isothiocyanate (FITC) -labeled goat-anti mouse antibody (F-2761 Molecular Probes, The Netherlands) in PBS, washed with PBS, rinsed in deionised water and mounted with a solution containing glycerin and 1,4-diazobicyclo-2,2,2,-octane (DABCO). Tissue sections were analyzed by fluorescence microscopy (Leica DM RBE, Germany).

Statistical analysis The cumulative mortality and the standard deviation of the three experiments performed by IM or oral routes were calculated for each dose. The mean cumulative mortality was analyzed by probit, which is a generalized linear model with a probit link function (Agresti, 1996). After checking that no significant interactions exist between dose and time, the probit model has the form:

$$\text{Probit}(x) = \alpha + \beta(\text{time}) + \gamma(\text{dose}) \qquad (1)$$

Where:

α is the intercept

β is the rate of probability change per unit change of time (for a constant dose)

γ is the rate of probability difference for each dose (for a constant time)

The statistical software Minitab (Minitab v. 14, Minitab Inc. USA) was used to calculate the parameters of the regression and to determine the median lethal time (LT_{50}) or the time at which 50% of the tested organisms die (Yi et al. 2003) for each dose. Differences in the LT_{50} of the doses were evaluated by the significance of dose in the probit model (1) (significance level = 0.05) using the same statistical software.

RESULTS

Intramuscular inoculation *Clinical signs and onset of disease* - Shrimp inoculated with the three doses of WSSV by IM route first displayed empty guts and reduced response to mechanical stimulus between 24 and 36 hours post inoculation (hpi). The proportion of shrimp from each of the three doses that displayed these clinical signs is presented in Tables 1 & 2. Shrimp used as controls did not display any of these clinical signs, remained healthy and survived throughout the experiments.

Table 1.- Proportion of shrimp with empty guts after IM inoculation with three doses of WSSV.

Group	Number of shrimp*	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	6/30	12/30	9/22	18/20	4/6	2/2	2/2	1/1	
30 SID_{50}	30	0/30	0/30	5/30	16/29	13/22	11/15	5/5	2/2			
90 SID_{50}	30	0/30	0/30	6/30	18/30	14/26	13/17	3/4	1/1			

* Total of the three experiments

Table 2.- Proportion of shrimp with reduced response to mechanical stimulus after IM inoculation with three doses of WSSV.

Group	Number of shrimp*	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	0/30	0/30	5/22	13/20	4/6	2/2	2/2	1/1	
30 SID_{50}	30	0/30	0/30	2/30	11/29	8/22	9/15	3/5	2/2			
90 SID_{50}	30	0/30	0/30	1/30	7/30	12/26	11/17	3/4	1/1			

* Total of the three experiments

Mortality - Each of the three doses of WSSV inoculated by IM route induced 100% mortality. The first mortalities were recorded at 36 hpi with each of the three doses tested. The cumulative mortality reached 100% at 84 hpi in shrimp inoculated with doses 30 and 90 SID_{50}, while shrimp inoculated with 10 SID_{50} were all dead at 108 hpi (Fig. 1a).

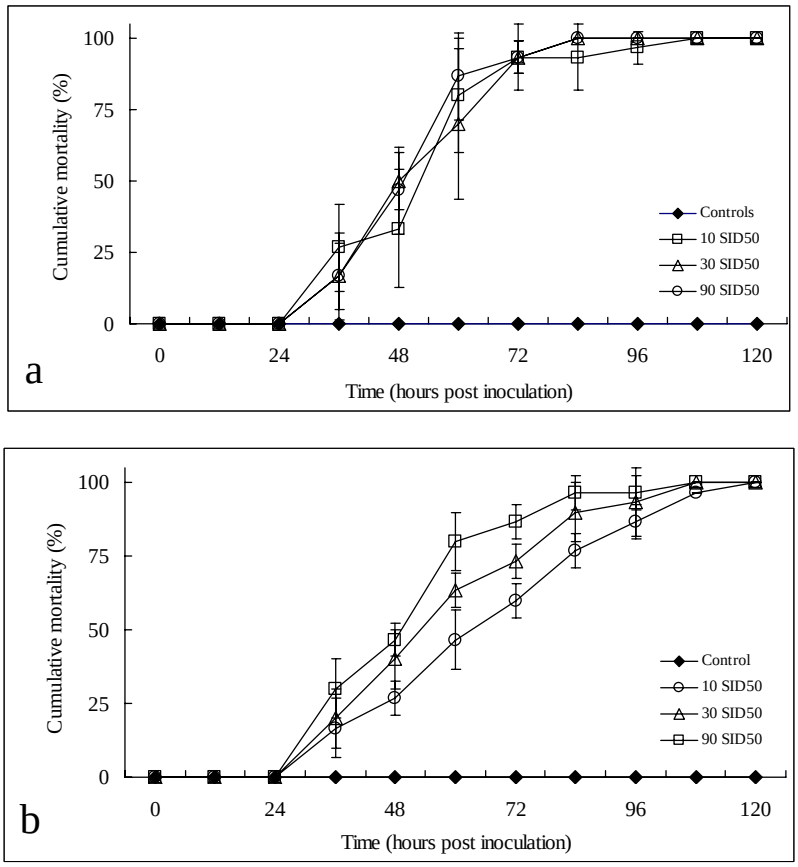

Figure 1. Cumulative mortality (mean of three experiments and standard deviation) of shrimp inoculated with three doses of WSSV by intramuscular (a) or oral routes (b)

Oral inoculation *Clinical signs and onset of disease* - Shrimp inoculated with any of the three doses of WSSV by oral route first displayed empy guts and reduced response to mechanical stimulus between 24 and 36 hpi. The proportion of shrimp from each of the three doses that displayed these clinical signs is presented in Tables 3 & 4. Control shrimp did not display any of these clinical signs, remained healthy and survived throughout the experiments.

Table 3.- Proportion of shrimp with empty guts after oral inoculation with three doses of WSSV.

Group	Number of shrimp*	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	7/30	11/30	13/25	15/22	10/16	8/12	6/7	4/4	1/1
30 SID_{50}	30	0/30	0/30	6/30	13/30	14/24	13/18	7/11	7/8	3/3	2/2	
90 SID_{50}	30	0/30	0/30	7/30	24/30	16/24	14/16	4/6	3/4	1/1	1/1	

* Total of the three experiments

Table 4.- Proportion of shrimp with reduced response to mechanical stimulus after oral inoculation with three doses of WSSV.

Group	Number of shrimp*	Proportion of shrimp showing clinical signs at each time point (hpi)										
		0	12	24	36	48	60	72	84	96	108	120
Control	30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
10 SID_{50}	30	0/30	0/30	2/30	4/30	8/25	12/22	9/16	6/12	6/7	4/4	1/1
30 SID_{50}	30	0/30	0/30	0/30	12/30	11/24	13/18	7/11	7/8	3/3	2/2	
90 SID_{50}	30	0/30	0/30	1/30	13/30	8/24	11/16	3/6	2/4	1/1	1/1	

* Total of the three experiments

Mortality - Each of the three doses of WSSV inoculated by oral route induced 100% mortality. By oral inoculation, the first mortalities due to WSSV were recorded at 36 hpi for each dose. Cumulative mortality was 100% at 108 hpi in shrimp inoculated with doses 30 and 90 SID_{50}, while the cumulative mortality of shrimp inoculated with 10 SID_{50} was 100% at 120 hpi (Fig. 1b). Probit analysis (Fig. 2b) showed significant differences ($P < 0.05$) in the LT_{50} of each of the three doses inoculated by oral route (Table 5). The LT_{50} of doses 10, 30 and 90 SID_{50} was 65, 57 and 50 hpi respectively.

IIF analysis confirmed infection in all the shrimp inoculated with WSSV. Control shrimp were WSSV-negative.

The cumulative mortality of the three doses was analyzed with the probit model (Fig. 2a) and the LT_{50} of the three doses were compared. The LT_{50} with doses 10, 30 and 90 SID_{50} had values of 52, 50 and 49 hpi respectively, which was not significantly different (Table 5). IIF analysis confirmed that all WSSV-inoculated shrimp became infected. Control shrimp were WSSV-negative.

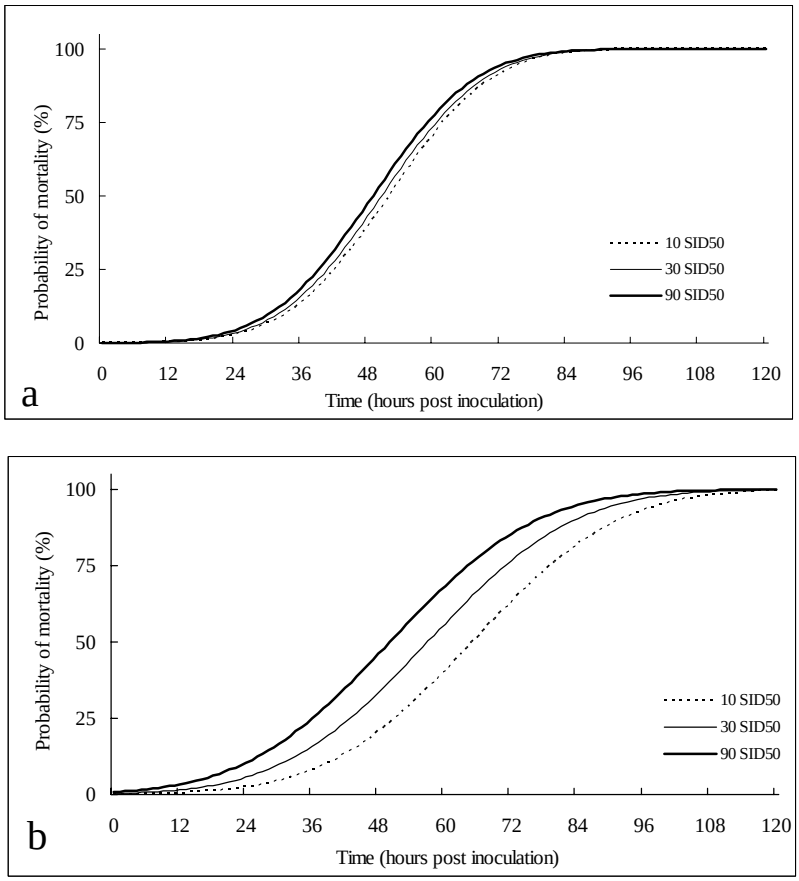

Figure 2. Probability of mortality (probit) of the three doses of WSSV inoculated into shrimp by intramuscular (a) or oral routes (b)

Table 5.- Parameters of the probit regression model of the three doses inoculated by i.m. or oral routes.

Inoculation route	Dose (SID_{50})	Time of 100% mortality (hpi)	α	β	γ (dose)	LT_{50}	LT_{50} similarity (Z, p = 0.05)
IM	10^a	108	-3.5616	0.06866	0	51.87	c ≤ b ≤ a
	30^b	84	-3.5616	0.06866	0.09214	50.53	
	90^c	84	-3.5616	0.06866	0.19966	48.96	
Oral	10^a	120	-3.1279	0.04809	0	65.04	c* < b* < a*
	30^b	108	-3.1279	0.04809	0.393898	56.85	
	90^c	108	-3.1279	0.04809	0.721668	50.03	

* Significant differences

DISCUSSION

In the past, experimental challenge tests have been used to determine the pathogenicity of WSSV, the susceptibility of different species to the virus and to test products and strategies to control the disease (Lu et al. 1997, Lightner et al. 1998, Chang et al. 2003). In all these experiments, different viral strains, shrimp species, ages and routes of inoculation were used, which makes it difficult to compare the results from different studies. Moreover, the infectivity of the virus stock is mostly undefined.

This study is the first that used defined infectious doses of WSSV to standardize experimental challenge protocols by i.m. and oral routes using SPF shrimp of similar age.

Each of three doses of WSSV inoculated by either i.m. or oral route induced infection and mortality in all shrimp and their mortality patterns were reproducible according to the criteria used. The clinical signs used in these experiments were useful to indicate the time of onset of disease caused by WSSV infection in every dose. Clinical signs appeared at least 12 h before the first mortalities and were displayed by similar

proportions of shrimp whether inoculated by i.m. or oral routes. The onset of disease and the first mortalities occurred at the same time whether shrimp were inoculated i.m. or by the oral route. However, shrimp inoculated orally died between 12 and 24 hpi later than shrimp inoculated i.m. with the equivalent doses. Accordingly, the LT_{50} is earlier for the doses delivered by i.m. route compared with the LT_{50} of the equivalent doses inoculated orally.

The influence of the route of inoculation on the speed of mortality produced by WSSV infection has been determined previously in *Penaeus monodon* and *Fenneropenaeus indicus.* Shrimp infected *per os* displayed 100% mortality two to four days later than those inoculated by IM route (Sahul-Hameed et al. 1998, Rajendran et al. 1999, Rajan et al. 2000). In the sergestoid shrimp *Acetes,* animals inoculated by IM route had 100% mortality by the third day post inoculation (Supamattaya et al. 1998). In contrast, the mortality due to WSSV infection was reduced five-fold when shrimp were inoculated with infected tissues *per os* and shrimp mortality occurred over a period of nine days post feeding.

By i.m. inoculation, infectious viral particles are placed directly into the shrimp's body, avoiding any natural barrier in the shrimp to prevent pathogen entry. With this inoculation technique most of the injected infectious viral particles have a high probability of reaching susceptible cells and to initiate the infection process. In contrast, the oral inoculation places the virus in the lumen of the foregut, which represents a hostile environment. The cuticle layer lining the epithelial cells in the foregut (Icely & Nott 1992, Ceccaldi 1997, Martin & Chiu 2003) constitutes an important physical barrier that may hinder infectious WSSV particles to reach the epithelial cells. The pH and enzymes present in the digestive tract of the shrimp (Lovett & Felder, 1990, Talbot & Demers 1993, Lemos et al. 1999, Ribeiro & Jones, 2000, Gamboa-Delgado et al. 2003) may damage infectious viral particles leading to their inactivation. It is likely that only a small proportion of infectious virus inoculated orally actually infects cells and is the reason why it is necessary to use ten times more virus to infect shrimp by the oral route compared with the i.m. inoculation (Escobedo-Bonilla et al. 2005).

Even when the doses inoculated were increased ten times for oral intubation, there was still a difference in the time required to produce 100% mortality between i.m. and oral inoculation of WSSV suggesting the existence of barriers other than those alluded to,

for example, the basal lamina (Mellon 1992) underlying the epithelial cells of the foregut. Once epithelial cells are infected with WSSV, the newly-produced infectious virus has to break through the basal lamina to reach the underlying connective tissues in order to spread to other organs. It is possible that a critical number of epithelial cells has to be infected before that infectious virus can cross the basal lamina, thus explaining the dose-dependent pattern. Once infectious WSSV particles reach the connective tissues that may be in contact with hemolymph sinuses and lacunae bathing these tissues, the infectious WSSV particles can be carried by the hemolymph circulation and spread to other target organs. Mortality of WSSV-infected shrimp probably occurs when the level of infection in target organs causes necrosis and loss of function.

Based on the cumulative mortality patterns of the three doses used in these experiments, a dose of 30 SID_{50} was selected as the standard for further WSSV inoculation procedures by i.m. and oral routes. Such a dose ensures infection in every inoculated shrimp, but is not so excessive as to cause acute mortality. This is a desirable feature, especially when these inoculation protocols will be applied to test the efficacy of WSSV control strategies. The oral inoculation procedure may be more relevant for testing these strategies because it mimics the natural mode of WSSV infection. Further, it allows testing products that may have a synergistic effect with the natural physico-chemical barriers to viral entry.

The standardized inoculation procedures described in the present study may be applied for other crustacean species and different WSSV strains. Parameters such as onset and severity of disease and LT_{50} are specific for the viral strain and experimental conditions used. Therefore it is necessary to determine these parameters under specific experimental conditions when other WSSV strains, shrimp species and laboratory conditions are used.

These standardized inoculation procedures may also be used for (i) comparing the susceptibility of different shrimp species to WSSV; (ii) determining the virulence of different WSSV strains and (iii) evaluating the effect of different strategies with potential to control WSSV.

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CHAPTER 4

PATHOGENETIC FEATURES OF WHITE SPOT SYNDROME VIRUS (WSSV) IN JUVENILE SPF *Litopenaeus vannamei*

“Science sometimes requires at the very least the courage to question the conventional wisdom”. - Carl Sagan (Broca's Brain: Reflections on the Romance of Science, 1979)

4.1 Pathogenesis of white spot syndrome virus (WSSV) in juvenile specific pathogen-free *Litopenaeus vannamei*

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ABSTRACT

White spot syndrome virus (WSSV) causes disease and mortality in cultured and wild shrimp. A standardized WSSV oral inoculation procedure was used in specific pathogen-free (SPF) *Litopenaeus vannamei* (also called *Penaeus vannamei*) to determine the primary sites of replication (portal of entry), to analyze the viral spread and to propose the cause of death. Shrimp were inoculated orally with a low ($10^{1.5}$ shrimp infectious doses 50% endpoint [SID_{50}]) or a high (10^4 SID_{50}) dose. Per dose, six shrimp were collected at 0, 6, 12, 18, 24, 36, 48 and 60 hours post inoculation (hpi). WSSV-infected cells were located in tissues by immunohistochemistry and in hemolymph by indirect immunofluorescence. Cell-free hemolymph was examined for WSSV DNA using one-step PCR. Tissues and cell-free hemolymph were first positive at 18 hpi (low dose) or at 12 hpi (high dose). With the two doses, primary replication was found in cells of the foregut and gills. The antennal gland was an additional primary replication site at the high dose. WSSV-infected cells were found in hemolymph starting from 36 hpi. At 60 hpi, the number of WSSV-infected cells found in the epithelial cells of foregut (36%), gills (98 cells mm^{-2}), antennal gland (26 cells mm^{-2}), hematopoietic tissue (78 cells mm^{-2}), lymphoid organ (49 cells mm^{-2}) and epithelial cells of integument (27%). Areas of necrosis were observed in infected tissues starting from 48 hpi (low dose) or 36 hpi (high dose). Since the foregut, gills, antennal gland and integument are essential for the maintenance of shrimp homeostasis, it is likely that WSSV infection leads to death due to their dysfunction.

INTRODUCTION

White spot syndrome virus (WSSV) has caused serious economic losses to the shrimp farming industry in many countries in Asia, Latin America and the U.S. (Lu et al. 1997, Chou et al. 1998, Wang et al. 1999, Hill 2002, Chapman et al. 2004). This has prompted the search for control measures and their evaluation through experimental inoculation tests. The development of standardized inoculation tests has been described in previous publications (Escobedo-Bonilla et al. 2005, 2006) where we showed that such inoculation methods yield reproducible results.

In order to help formulating new control methods against disease it is important to have a better understanding of WSSV pathogenesis. At present, aspects of WSSV pathogenesis are known mainly from studies of naturally infected Asian shrimp species. Controversial results have stirred the debate about the sites of WSSV entry, primary replication and the mode of spread to distant target organs. Early juvenile (0.45 g) *Penaeus monodon* inoculated *per os* first showed WSSV-infected cells at 16 hours post feeding in cells of the foregut, gills, integument and connective tissue of the hepatopancreas as determined by *in situ* hybridization (ISH) (Chang et al. 1996). However, in another study done with *Marsupenaeus japonicus,* (also called *Penaeus japonicus*) epithelial cells in the midgut were suggested as the portal of WSSV entry *per os* (Di Leonardo et al. 2005). Likewise, the debate on the role of circulating hemocytes in the systemic spread of WSSV is rejected by some results obtained by ISH (van de Braak et al. 2002), while it is supported by others using immunofluorescence and transmission electron microscopy (Wang et al. 2002).

The main target organs of WSSV found in marine shrimp and many other crustaceans inoculated *per os* include the foregut, hindgut, gills, antennal gland, integument, gonads, muscle, nervous tissues, lymphoid organ, haematopoietic tissues, heart and hemocytes. All these organs are of ectodermal or mesodermal origin (Wongteerasuypaya et al. 1995, Durand et al. 1996, Lo et al. 1997, Sahul-Hameed et al. 1998, Mohan et al. 1998). At present, no information is available on the pathogenesis of WSSV infection in the American shrimp *Litopenaeus vannamei* (also called *Penaeus vannamei*).

In the present study, two different doses of a Thai WSSV stock were orally inoculated to juvenile specific pathogen-free (SPF) *L. vannamei* using a standardized oral inoculation procedure previously described (Escobedo-Bonilla et al., 2006). A low inoculation dose resulted in a slower rate of disease progression than a high dose. The objectives were (1) to determine the sites of virus replication with emphasis on the portal of entry, (2) to analyze how WSSV spreads from the primary replication sites to other distant target organs and (3) to search for the cause of death.

MATERIAL AND METHODS

Shrimp and experimental conditions - Specific pathogen-free (SPF) *Litopenaeus vannamei* Kona-strain (Wyban et al. 1992) were used. Shrimp (14.6 ± 3.3 g mean body weight [MBW], n = 102) were acclimatized to a salinity of 15 g l^{-1} and 27 °C over four days at the facilities of the Laboratory of Aquaculture and Artemia Reference Center (ARC), Ghent University. Afterwards, they were transported to facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where experiments were performed under biosafety conditions. Two experiments were done. In a first experiment, shrimp (n = 54) were inoculated with a low dose and in a second experiment, shrimp (n = 48) were inoculated with a high dose. In each experiment, groups of six shrimp were kept in 50 l glass aquaria equipped with water heaters (Visitherm, aquarium systems, USA), mechanical filters (Eheim classic 2213, Germany) and continuous aeration. Aquaria were covered with glass and a plastic sheet to prevent virus dispersion by aerosol.

Virus - A Thai WSSV stock was used. WSSV from naturally infected *P. monodon* was passaged once into crayfish and grown to high titers in SPF *L. vannamei*. The virus stock was titrated *in vivo* by intramuscular (im) and oral routes. The virus titer by oral route was $10^{5.6}$ shrimp infectious doses 50% endpoint (SID_{50} ml^{-1}) as determined by indirect immunofluorescence (IIF) and one-step polymerase chain-reaction (PCR) (Escobedo-Bonilla et al. 2005). A low ($10^{1.5}$ SID_{50}) or a high ($10^{4.0}$ SID_{50}) dose was made in phosphate-buffered saline (PBS) pH 7.4 in a volume of 50 µl.

Oral inoculation procedure - The inoculation was performed by placing the shrimp with the ventral side up. A magnifying glass was used to locate the mouth. A long and

flexible pipette tip (Biozym no. 790 004, The Netherlands) was inserted beneath the labrum and anterior to the mandibles. The WSSV inoculum was delivered into the lumen of the foregut.

Time course - For each experiment, six shrimp were collected at 0, 6, 12, 18, 24, 36, 48 and 60 hours post inoculation (hpi). In addition, six control shrimp were inoculated only with PBS and collected at 72 hpi.

Sampling

Tissue processing - At each time point, the pereons of three shrimp were sectioned longitudinally, fixed with Davidson's for 24 to 48 h and changed to 50% ethanol for at least 24 h before paraffin embedding (Bell & Lightner 1988, Lightner 1996). The pereons of the remaining shrimp were cross-sectioned at three different levels and processed for paraffin embedding:

The first cross-section was made at the anterior part of the pereon. In this section, organs of the digestive system (foregut), excretory system (antennal gland), integument and internal organs (hematopoietic tissue) were located. The second cross-section comprised the central part of the pereon. In this part, organs of the digestive (foregut, anterior midgut cecum and hepatopancreas), respiratory (gills and branchial chamber), excretory (antennal gland) and nervous (ganglia and ventral cord) systems, integument and internal organs (lymphoid organ, gonads) were present. The third cross-section included the posterior part of the pereon. Here, organs of the digestive (posterior part of the hepatopancreas and its junction with the midgut trunk) and respiratory (gills, branchial chamber) systems, integument and internal organs (heart and gonads) were found. Organs of the digestive tract located in tail were not analyzed.

Hemolymph collection - Hemolymph from every shrimp was collected at the different time points as follows: shrimp were anaesthetized by placing them on ice. Then, shrimp were injected with 200 µl of ice-cold Alsever's (AS) buffer pH 7.0 (Rodríguez et al. 1995) in the anterior part of the pereon using a 24 gauge needle (Terumo Europe NV, Belgium) and 400 µl of hemolymph mixed with AS buffer was obtained. An aliquot (100 µl) was diluted 1:10 in PBS and 70 µl of the diluted hemolymph was placed into a cytospin (Cytospin 3, Shandon USA) fixed to a glass slide. The slides were centrifuged at 300 g for 4 min and immediately fixed at -20 °C in 100% methanol for 15 min. The slides were air-dried and stored at -20 °C until indirect immunofluorescence (IIF)

analysis was performed. Another aliquot (100 µl) was spun down at 300 g for 5 min at 4 °C to obtain cell-free hemolymph. This fraction was used to perform one-step PCR for the early detection of WSSV DNA.

Analysis of WSSV infection - *Immunohistochemistry (IHC)* - Paraffin-embedded tissue sections were cut at 4 µm and placed onto Silane-coated slides (A3648, Sigma-Aldrich, USA). Sections were deparaffinized and rehydrated. The endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in tris buffer pH 7.4. Sections were incubated for 1 hr at 37 °C with 2 mg ml^{-1} of monoclonal antibody 8B7 raised against WSSV envelope protein VP28 (Poulos et al. 2001). Sections were washed in Tris buffer (pH 7.6) and incubated for 1 hr at 37 °C with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001 Amersham Biosciences, UK). Afterwards they were washed, incubated for 30 min at room temperature (RT) with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences, UK) and washed again. Color development was made with 0.01% of 3, 3'-diaminobenzidine (DAB) (D8001 Sigma-Aldrich, Germany). Sections were counter-stained with Gill's hemaluin and washed in water, dehydrated and mounted. WSSV-infected cells were counted using light microscopy (Leica DM RBE, Germany) at a magnification of 400 X. Two different methods were used to quantify WSSV-infected cells in shrimp tissues: (1) WSSV-infected epithelial cells from the foregut and integument were counted in five fields randomly selected and expressed as percentage of the total number of cells. (2) WSSV-infected cells located in tissues / organs such as antennal gland, hematopoietic tissue, lymphoid organ, heart, gonads, and connective tissues were counted in five fields selected at random and expressed as the number of WSSV-infected cells per square millimeter (cells mm^{-2}). The quantitative method used depended on the tissues evaluated. For example, epithelial tissues are linearly arranged, so the proportion of WSSV-infected cells was determined as a percentage. In contrast, most of the internal organs are solid and composed of different cell types, so the proportion of WSSV-positive cells was determined as the number of infected cells per square millimeter.

Indirect immunofluorescence (IIF) - Single- or double-stainings were made on cytospins with circulating hemocytes. Single stainings were done to detect WSSV-infected cells.

Here, the cytospins were washed for five min in PBS and incubated for 1 h at 37 °C with 2 mg ml^{-1} of monoclonal antibody 8B7 against VP28 (Poulos et al. 2001), washed twice for five min each in PBS and incubated for 1 h at 37 °C with 0.2 mg ml^{-1} of fluorescein isothiocyanate (FITC) -labeled goat anti mouse IgG antibodies (F-2761 Molecular probes, The Netherlands) and finally washed twice for 5 min each in PBS. Nuclear counter-stain of hemocytes was performed by incubating the slides for 10 min at room temperature with 0.01 mg ml^{-1} solution of bisbenzimide H 33342 (H1399 Molecular probes, The Netherlands). After two washings with PBS, cytospins were mounted. Double stainings were performed to characterize infected hemocytes. Here, a first incubation was made for 1 h at 37 °C (1:70 dilution in PBS or 0.02 mg ml^{-1} solution) with hemocyte markers from *P. monodon* (Winotaphan et al. 2005) or with wheat germ agglutinin (WGA) labeled with FITC (L4895 Sigma) (Martin et al. 2003). Cytospins were washed and incubated with 0.02 mg ml^{-1} of goat anti-mouse IgG-FITC for 1 h at 37 °C. After washing, a second staining was done by incubating the slides with a 1:100 dilution of a polyclonal antiserum raised in rabbit against recombinant VP28 for 1 h at 37 °C. Slides were washed and incubated for 1 h at 37 °C with 0.04 mg ml^{-1} of goat anti-rabbit IgG-Texas Red (TR) (T6391, Molecular probes, The Netherlands). Afterwards, slides were washed and counter-stained with bisbenzimide H 33342 before they were washed and mounted. The analysis was done using fluorescence microscopy (Leica DM RBE, Germany). Per shrimp, 500 hemocytes or hemocytes from five fields (400 X) randomly selected were counted and the percentage of WSSV-infected cells was determined. Slides without infected cells in this restricted number of cells counted were screened entirely.

One-step PCR - Cell-free hemolymph supernatant (1 µl) was directly used to perform a one-step PCR analysis as described elsewhere (Dhar et al. 2001). Each sample was added to a PCR tube containing 48 µl of a PCR master mix [1X PCR buffer (Eurogentec, Belgium), 1.5 mM $MgCl_2$, 0.3 mM of each of the respective forward and reverse primers for WSSV or β-actin, 1.6 mM dNTPs (Eurogentec, Belgium), 1 U hot goldstar *Taq* polymerase (Eurogentec, Belgium) in a total reaction volume of 50 µl]. The primers F002 and R002 were used to amplify WSSV DNA, as well as the primers F and R3 which amplified β-actin from shrimp used as control. The expected amplicon for WSSV was 306 bp, while that for β-actin was 339 bp. A preheating step at 95 °C for 10

min was followed by 35 cycles of the following steps: denaturation (94 °C for 45 s), annealing (55 °C for 45 s) and extension (72 °C for 75 s). A final extension step (72 °C for 5 min) was made. PCR products were stored at 4°C. PCR products (12 µl), negative (ultrapure water) and positive (DNA from a 10^{-2} dilution of WSSV stock) controls, as well as DNA markers (smart ladder, Eurogentec, Belgium) were resolved on a 1.2% agarose gel in tris-acetate-EDTA (TAE) buffer. The gel was stained with ethidium bromide (0.02 mg mL^{-1}) and DNA bands were visualized by UV transillumination.

Histopathology - Deparaffinized tissue sections were stained with hematoxilin-eosin-phloxine (Lightner 1996) and analyzed by light microscopy at a magnification of 400X. Cellular changes and tissue damage were determined.

RESULTS

WSSV pathogenesis with the low dose ($10^{1.5}$ SID_{50}) - *Virus replication* - *Tissues* - The first WSSV-infected cells detected by IHC in tissues occurred at 18 hpi in one out of six shrimp. The primary sites of WSSV replication were epithelial cells in foregut (0.08%) (digestive system) and cells in gills (0.26 cells mm^{-2}) (respiratory system) (Figures 1a & b, 2 and 4). At 24 hpi, five out of six shrimp were WSSV-positive by IHC. In the digestive system, WSSV infection was observed in the epithelium (2.7%) and connective tissues (3.0 cells mm^{-2}) of foregut and connective tissues of organs in midgut (anterior midgut cecum 2.8 cells mm^{-2} and hepatopancreas <0.1 cells mm^{-2}). Epithelial cells in midgut were refractory to WSSV (Figure 1a). Other organs infected with WSSV were gills (6.1 cells mm^{-2}) and integument of the gill chamber (0.4%), antennal gland (2.0 cells mm^{-2}) (excretory system) and internal organs associated to hemolymph circulation such as lymphoid organ (0.4 cells mm^{-2}) and hematopoietic tissue (0.3 cells mm^{-2}) (Figures 2, 3 and 4). In heart, muscle and nervous system, only a few cells of the connective tissue were WSSV-positive. From 36 hpi until the end of the experiment (60) hpi, 100% of the shrimp collected were WSSV-positive by IHC. At 60 hpi, the number of infected cells was 81.9 cells mm^{-2} in gills, 62.8 cells mm^{-2} in hematopoietic tissue, 34.1% in epithelial cells and connective tissue (28.8 cells mm^{-2}) of foregut, lymphoid organ (28.7 cells mm^{-2}), epithelial cells (27.7%) and connective tissue (14.1 cells mm^{-2}) of integument and antennal gland (15 cells mm^{-2}) (Figures 1a, & b, 2, 3 and

4). Organs such as heart, gonads, muscle, neuronal ganglia and nerve cord had only a few WSSV-positive cells located in connective tissues. In gonads, no reproductive cells (eggs or sperm) were detected WSSV-positive by IHC.

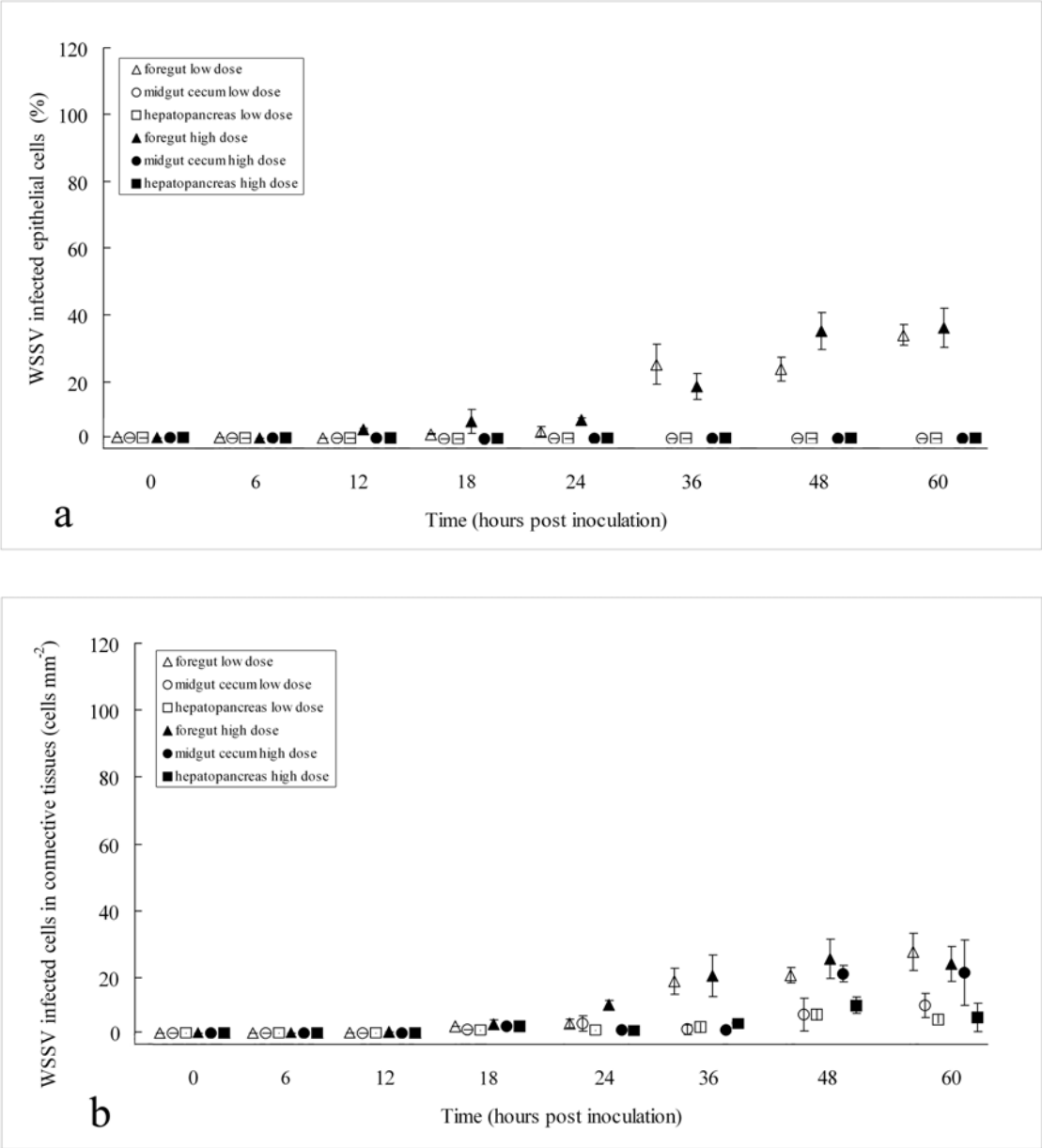

Figure 1 - Proportion of WSSV-infected cells in a) epithelium and b) connective tissues of organs of the digestive system of *Litopenaeus vannamei* inoculated with a low or a high dose. Six shrimp were used per time and the error bars represent the standard error

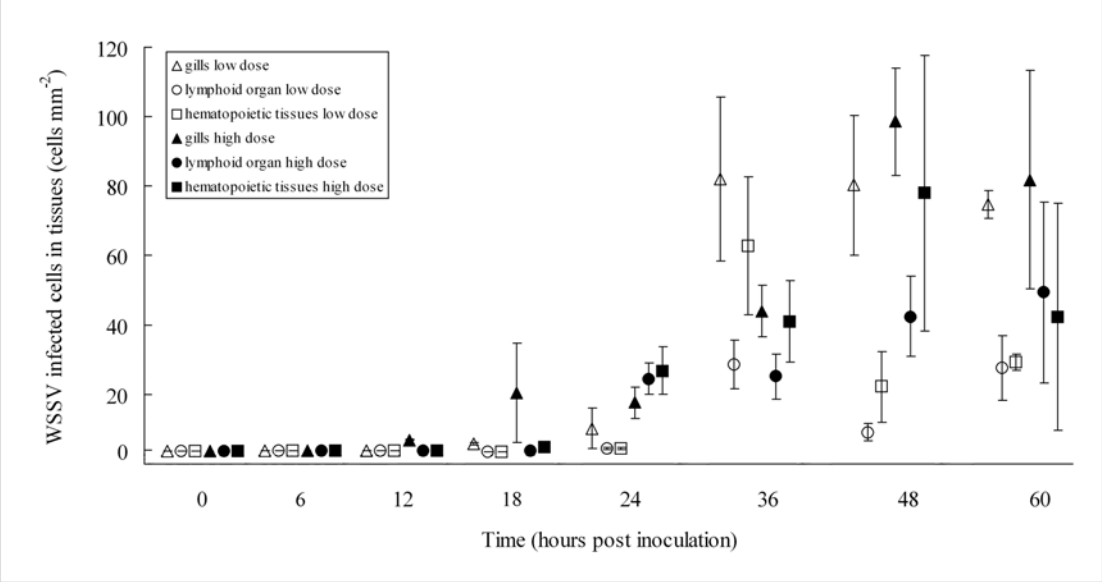

Figure 2 - Proportion of WSSV-infected cells in gills and organs associated to the circulatory system (hematopoietic tissues and lymphoid organ) of shrimp inoculated with a low or a high dose. Six shrimp were used per time and the error bars represent the standard error

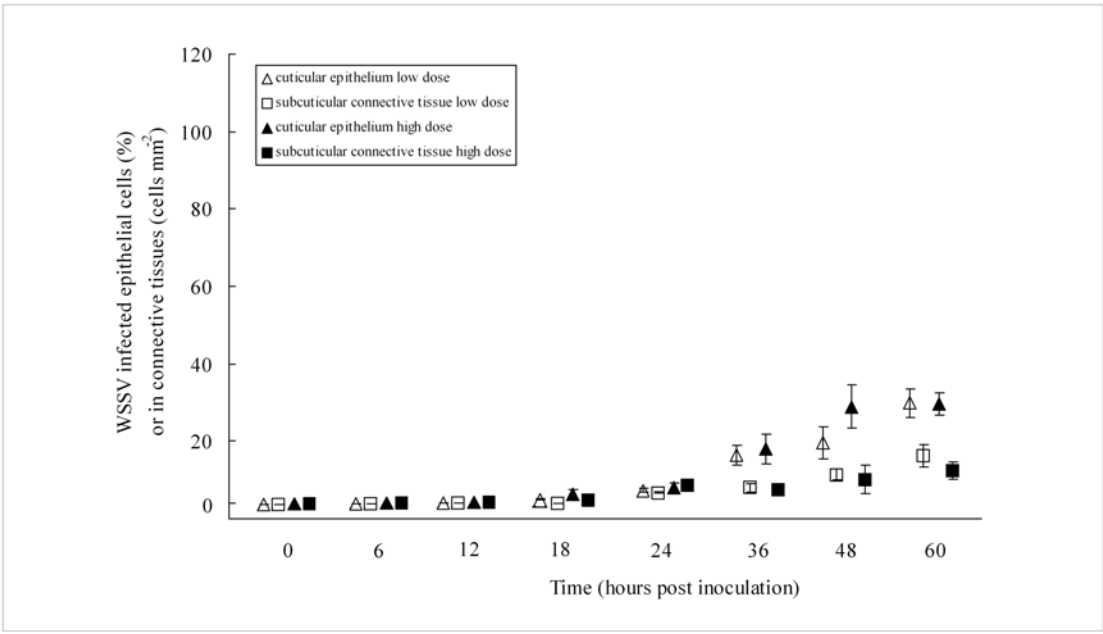

Figure 3 - Proportion of WSSV-infected cells in cuticular epithelium and subcuticular connective tissues of shrimp inoculated with a low or a high dose. Six shrimp were used per time and the error bars represent the standard error

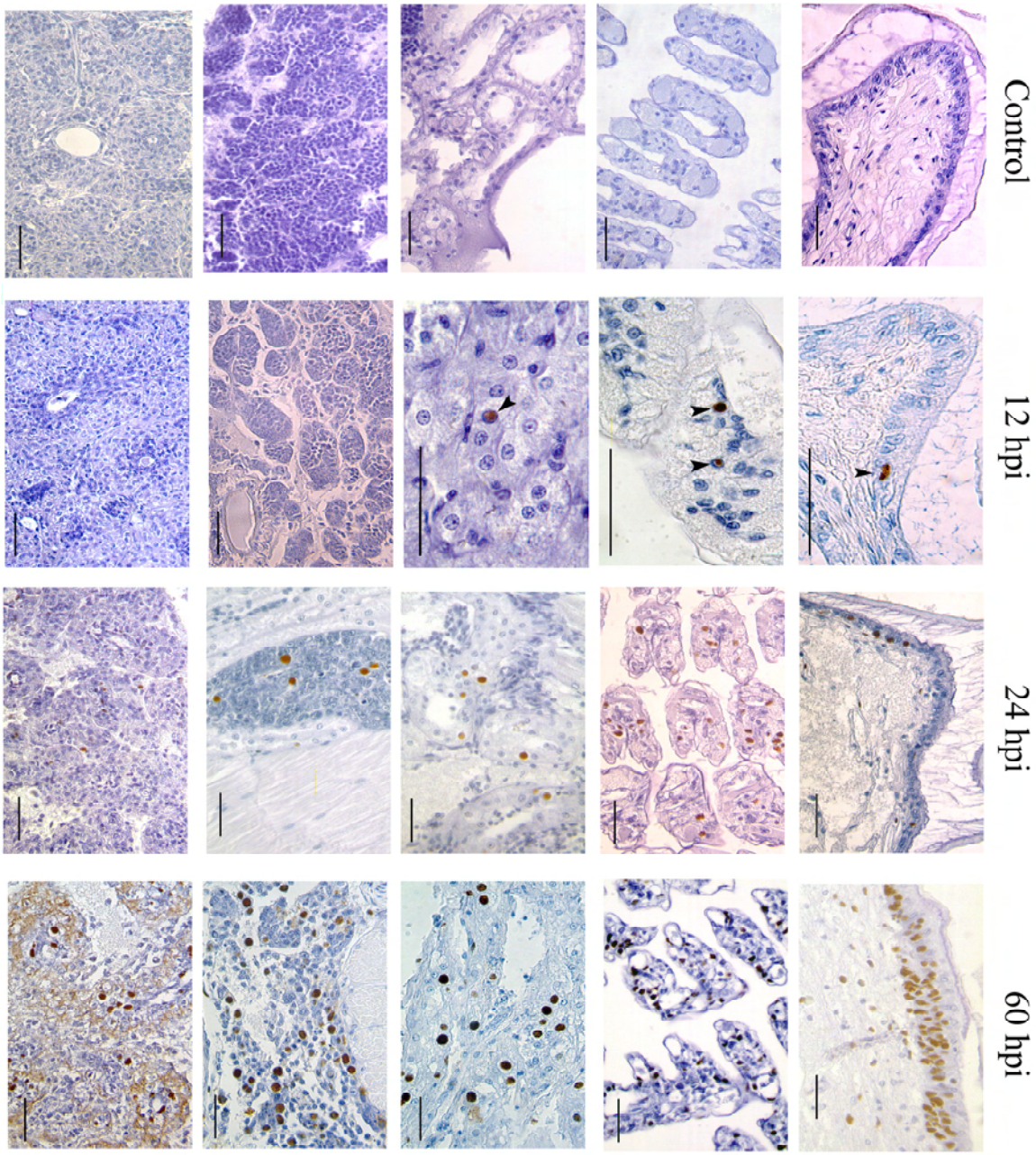

Figure 4 - Progression of WSSV infection in tissues of the foregut, gills, antennal gland, hematopoietic tissues and lymphoid organ. Arrowheads indicate infected cells at 12 hpi. Bar = 50 µm

Hemolymph - At 18 hpi, WSSV DNA was first detected in cell-free hemolymph in four out of five shrimp (Figure 5) and at 24 hpi in five out of six shrimp. From 36 hpi until the end of the experiment (60 hpi), hemolymph from all shrimp collected was WSSV DNA positive (not shown). In contrast, the first WSSV-positive cells in hemolymph were detected at 36 hpi in three out of six shrimp; at 48 hpi in one out of six shrimp and at 60 hpi in two out of six shrimp, respectively (Figure 6).

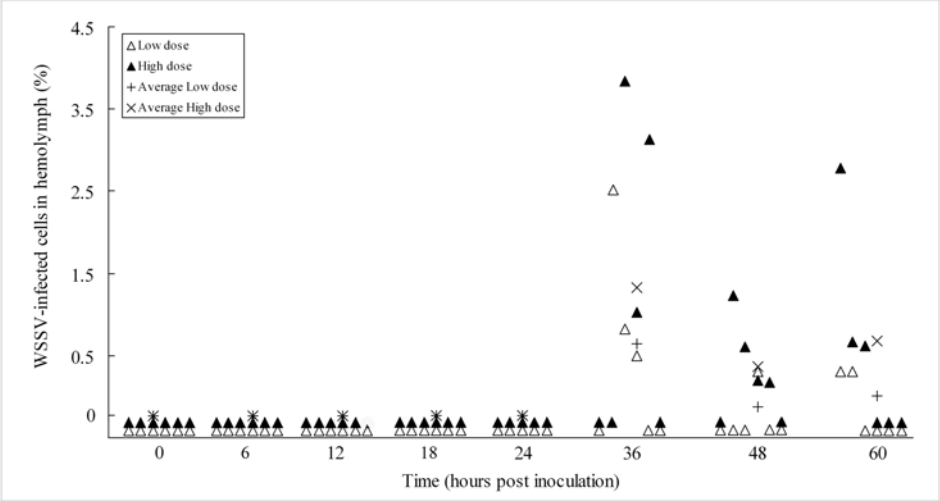

Figure 5 - Percentage of WSSV-infected cells in hemolymph of shrimp inoculated with a low or a high dose

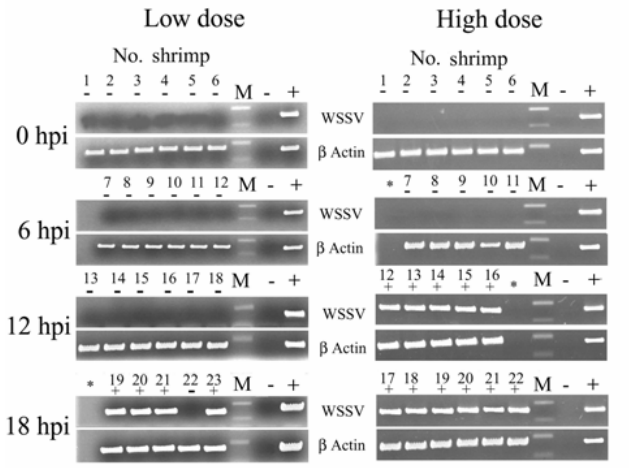

Figure 6 - WSSV DNA detection by one-step PCR in cell-free hemolymph of shrimp inoculated with a low or a high dose. (*) DNA samples not available, (M) DNA weight marker, (-) negative control, (+) Positive control

Histopathology - WSSV-infected cells with hypertrophied nuclei and amphophilic inclusions were first observed at 36 hpi. Affected tissues were located in the digestive (foregut, anterior midgut cecum and hepatopancreas), respiratory (gills) and excretory (antennal gland) systems, integument and internal organs (hematopoietic tissue and lymphoid organ). Although hydropic degeneration was occasionally observed in cells of foregut, antennal gland, hematopoietic tissue and lymphoid organ, the structure of these organs remained intact. At 48 hpi, a higher proportion of hypertrophied cells showed hydropic degeneration and some areas of focal necrosis were observed in foregut, gills, antennal gland, hematopoietic tissue and lymphoid organ. Occasionally, zones of erosion with hemocytic infiltration were observed in foregut and anterior midgut cecum at 60 hpi. The structure of organs such as heart, gonads, muscle, neuronal ganglia and nerve cord was intact throughout the duration of the experiment.

WSSV pathogenesis with the high dose ($10^{4.0}$ SID_{50}) - *Virus replication* - *Tissues* - The first WSSV-infected cells were detected at 12 hpi by IHC in four out of five shrimp. The primary sites of WSSV replication were located in epithelial cells of the foregut (0.3%) (digestive system), gills (0.4 cells mm^{-2}) (respiratory system) and antennal gland (0.4 cells mm^{-2}) (excretory system) (Figures 1a & b, 2 and 4). At 18 hpi, five out of five shrimp were WSSV-positive. In the digestive system, WSSV-positive cells were observed in epithelium (6.6%) and connective tissue (2.5 cells mm^{-2}) of foregut. In the anterior midgut cecum and hepatopancreas, WSSV-infected cells were only found in the connective tissues (0.2 cells mm^{-2}) (Figures 1a & b, 4). At this time point, cells in gills (18 cells mm^{-2}), integument of the branchial chamber (1.6%), lymphoid organ (0.5 cells mm^{-2}) and hematopoietic tissues (1.3 cells mm^{-2}) were also WSSV-positive (Figures 2, 3 and 4). Organs such as heart (0.2 cells mm^{-2}), gonads (0.2 cells mm^{-2}) and neuronal ganglia (0.9 cells mm^{-2}) showed WSSV-infected cells only in connective tissues. Cardiac, neuronal and reproductive (eggs or sperm) cells were WSSV-negative. From 24 hpi until the end of the experiment (60 hpi) the mean number of WSSV-infected cells increased in tissues of the foregut, gills, antennal gland, integument and internal organs such as hematopoietic tissues and lymphoid organ (Figures 1a & b, 2, 3 and 4). At 60 hpi, the number of WSSV-infected cells in epithelial cells of the foregut was 36.2% and 23.1 cells mm^{-2} in the connective tissue, 27.5% in epithelial cells and connective tissue (10.1 cells mm^{-2}) of integument and 26 cells mm^{-2}

in antennal gland. The most affected organs were associated with hemolymph such as gills (98.5 cells mm^{-2}), hematopoietic tissue (78 cells mm^{-2}) and lymphoid organ (49.4 cells mm^{-2}).

Hemolymph - At 12 hpi, WSSV DNA was found by one-step PCR in all of the shrimp in cell-free hemolymph (Figure 5). From this time point and until the end of the experiment, all collected shrimp were WSSV DNA-positive (not shown). In contrast, the first WSSV-infected cells in hemolymph were detected in three out of six shrimp at 36 hpi by IIF and at 48 hpi in four out of six shrimp. At the end of the experiment (60 hpi), three out of six shrimp showed WSSV-positive cells in hemolymph (Figure 6).

In hemolymph, the marker HC55 showed 53.5% of the circulating hemocytes to be semigranular and granular. The marker HC47d detected 34% of hemocytes as semigranular and hyaline whereas 28.1% of the hemocytes were recognized as hyaline and semigranular with the monoclonal antibody HC201d. The lectin wheat-germ agglutinin (WGA) showed that 20.9% of the hemocytes were hyaline and semigranular while the marker HC200 found 11.7% of the circulating hemocytes to be hyaline and semigranular. A proportion of 8.5% of the hemocytes were semigranular and granular according to the marker HC249d and 5.3% of the circulating hemocytes were granular as they reacted with the monoclonal antibody HC114. None of these circulating hemocytes were found WSSV-positive by IIF using double staining.

Histopathology - WSSV-infected cells with hypertrophied nuclei and amphophilic inclusions were first observed at 24 hpi. Affected organs were foregut, gills, antennal gland, integument and internal organs such as heart, hematopoietic tissue and lymphoid organ. In the digestive system, the anterior midgut cecum and hepatopancreas only showed these intranuclear inclusions in connective tissues. Cells from muscle, gonads and neuronal ganglia did not show such cellular lesions. At 36 and 48 hpi, the number of damaged cells increased in foregut, anterior midgut cecum, hepatopancreas, gills, antennal gland, integument and internal organs (hematopoietic tissue and lymphoid organ) and hydropic degeneration occurred in some cells. Small areas of erosion and focal necrosis appeared in foregut epithelium (digestive system) and hemocytic infiltration occurred in the subjacent connective tissues. Areas of the integument, antennal gland and hematopoietic tissue occasionally showed some loss of structure, whereas the gills and internal organs such as heart, lymphoid organ, gonads, neuronal

ganglia and nerve cord were still intact. At 60 hpi, cytoplasmic detachment and focal necrosis was observed in foregut epithelium. Some loss of structure was observed in gills, antennal gland, integument and internal organs (hematopoietic tissue and lymphoid organ). The structure of heart, gonads, muscle and tissues of the nervous system appeared undamaged.

DISCUSSION

This study shows that upon oral inoculation of WSSV, the portals of virus entry in *L. vannamei* are epithelial cells of the foregut, cells in the gills and only with a high dose, cells in the antennal gland as well. Foregut epithelium and cells in gills were also described as primary sites of WSSV replication in early juvenile *P. monodon* fed infected tissues *per os* (Chang et al. 1996). In contrast, another study done in *M. japonicus* inoculated *per os*, described the epithelial cells of the midgut trunk as the primary WSSV replication site (Di Leonardo et al. 2005). The size/age of shrimp and the method of inoculation used may explain these differences.

After primary replication, WSSV spread to other target organs where the number of WSSV-infected cells increased causing cellular and tissue damage. A dose-dependent effect was observed in time at which primary WSSV replication was detected and proportion of shrimp found positive by PCR and IHC at early time points after inoculation. However, at the end of the experiments, the number of WSSV-infected cells in target organs was similar in shrimp inoculated with both doses. The comparison of numbers of WSSV-positive cells between the various tissues and organs should be made with caution because of the two different methods used for its quantification.

Although foregut and gills are armed with a layer of cuticle, this could not prevent infection. The reason for this can be sought in the lack of an epicuticle layer, the absence of calcification and the presence of numerous pore canals (Compére et al. 2004, Taylor & Taylor 1992, Icely & Nott 1992, Pratoomchat et al. 2002). Further, it cannot be excluded that during the oral intubation, fissures were made in the cuticle of the foregut resulting in a free access of the virus to epithelial cells. The fact that cells in gills and/or antennal gland were also primary replication sites suggests that WSSV might have reached these organs by regurgitated or spilled inoculum. After primary

replication (12 or 18 hpi, depending on the dose), newly produced WSSV would have been released from epithelial cells and in one way or another, crossed the basal membrane to reach the underlying connective tissues and associated haemal sinuses. By hemolymph circulation the virus would have reached other organs so that WSSV-infected cells could be observed in various organs throughout the body by 18 or 24 hpi (depending on the dose).

It appeared that early in infection, circulating hemocytes were refractory to WSSV infection and that WSSV spread in a cell-free form via hemolymph circulation. The absence of infected circulating hemocytes early in infection was also noticed in other WSSV pathogenesis studies in *P. monodon* inoculated *per os* (van de Braak et al. 2002) or crayfish inoculated intramuscularly (Shi et al. 2000, 2005). We found a small proportion of shrimp with a few WSSV-infected cells in their hemolymph late during infection, but none of these cells were recognized by the hemocyte markers we used. Wang et al. (2002) also found WSSV-positive cells in the hemolymph of diseased *Fenneropenaeus merguiensis* by IIF and proposed that they were exclusively granular hemocytes. Since none of the hemocyte markers tested could bind to the infected cells we saw in *L. vannamei*, they may have belonged to an unrecognized hemocyte type or alternatively, may not have been hemocytes at all. Because of the late time post inoculation at which these WSSV-positive cells were first recorded, it is possible that they were cells detached from infected tissues or disrupted during hemolymph extraction. Overall, our results suggest that hemocytes do not play an important role in the systemic spread of WSSV, at least in *L. vannamei*.

Under culturing conditions, many Asian and American shrimp species display white spots in the cuticle (T.W. Flegel, pers. comm.) but the exact mechanism of white spots formation is largely unknown. A WSSV-infection may induce dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle (Wang et al. 1999). In the present study it was shown that the integument is one of the most affected organs in *L. vannamei* although no white spots were observed. It is possible that under our experimental conditions the infection spreads and kills shrimp within 5 d post inoculation (dpi) (Escobedo-Bonilla et al. 2006), which is much faster than in culture conditions. Experiments done with the penaeid species *Trachypenaeus curvirostris* and *Metapenaeus ensis* fed with WSSV-infected tissues, showed appearance of white spots

as the disease progressed slowly (100% mortality at 18 dpi) (Chang et al. 1998; Wang et al. 1998). In the field, the progression of disease before the acute stage may also be slow and this may explain the appearance of white spots in the cuticle.

Previous studies done in shrimp brooders (Lo et al. 1997) or in animals of undetermined age (Chang et al. 1998) showed by ISH analysis that connective tissues and muscle sheath around ovary or testes/spermatophore were susceptible to WSSV infection. In ovary of brooders, WSSV was detected in follicle cells and oogonia. A few developing oocytes were WSSV-positive. In testes, no reproductive cells were found infected with WSSV (Lo et al. 1997). In the present study, immature gonads of female or male juveniles showed WSSV-infected cells only in connective tissues. In a few males, epithelial cells of the *vas deferens* were also infected, but the reproductive cells from the two sexes were always WSSV-negative. These results suggest that the stage of gonad maturation may influence WSSV susceptibility of reproductive cells in the ovary. WSSV infection in gonads has been associated with poor spawning performance and low quality offspring (Lo et al. 1997) and it may also play a role in the vertical transmission of WSSV.

Gills, foregut, integument and antennal gland were among the main WSSV target organs in *L. vannamei*. The epithelial cells of these organs perform important functions such as gas exchange, transport and excretion of CO_2 and ammonia, the salt regulation and the control of the acid-base balance. These functions are critical to maintain shrimp homeostasis and all are involved in molting and growth rate (Ahearn et al. 1999, Wheatley 1999). Epithelial cells in these organs were increasingly damaged as WSSV infection progressed, which most probably led to dysfunction of these organs and death. Conversely, small numbers of WSSV-infected cells were found in the heart, neuronal ganglia, nerve cord and muscle and the structure of these organs remained intact throughout the experiments.

In conclusion, a standardized oral inoculation procedure consistently showed foregut and gills as primary sites of WSSV replication. Systemic spread of WSSV occurred mainly in cell-free form. Although hemocytic infiltration was observed, this reaction did not control virus replication in affected cells/tissues. The gills, foregut, integument and antennal gland were main WSSV target organs. Because they perform critical functions

for the maintenance of shrimp homeostasis, WSSV infection may lead to dysfunction of these organs and to the death of infected shrimp.

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CHAPTER 5

USE OF STANDARDIZED INOCULATION PROCEDURES TO EVALUATE STRATEGIES FOR THE CONTROL OF WSSV

“Science is magic that works”. -- Kurt Vonnegut (Cat's Cradle, 1963)

5.1 Clinical effect of cidofovir and a diet supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles

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ABSTRACT

The antiviral product cidofovir and a diet supplemented with *Spirulina platensis* were tested for their efficacy to prevent or delay/reduce mortality due to white spot syndrome virus (WSSV) infection in specific pathogen free (SPF) *Litopenaeus vannamei*. Cidofovir was injected intramuscularly at 200 mg/kg shrimp mean body weight (MBW) at the moment of WSSV challenge. *Spirulina* was supplemented in the shrimp diet at 25% w/w and shrimp were fed for 4 days at 5% of the MBW per day before WSSV challenge. Shrimp were orally inoculated with a WSSV dose of 30 shrimp infectious doses 50% endpoint (SID_{50}). Clinical signs and mortality were monitored for 120 hours post inoculation (hpi). WSSV infection was determined by indirect immunoflourescence (IIF) in dead and survivor shrimp at the end of the trial. In two experiments, mortality was delayed approximately for 24 hours by cidofovir treatment. Mortality of 100% was reached at 96-108 hpi in mock treated shrimp, whereas in shrimp treated with cidofovir, mortality of 80-90% was recorded at the end of experiment (120 hpi). Significant differences ($p<0.05$) in the median lethal time (LT_{50}) of cidofovir-treated shrimp and mock-treated shrimp were found by probit analysis. A *Spirulina* supplemented diet delayed the onset of clinical signs for 12 hours but had no effect on the cumulative mortality at the end of the experiment. This study opens perspectives for the evaluation of antiviral drugs to treat shrimp infected with WSSV.

INTRODUCTION

White spot syndrome (WSS) is one of the most damaging diseases in penaeid shrimp characterized by 100% mortality within 3-10 days (Lightner 1996). Since its first report in Taiwan in 1992, it has spread over both the eastern and western hemisphere, causing losses of billions of dollars every year to the shrimp farming industry. The viral agent, (WSSV) is a bacilliform, enveloped, double-stranded DNA virus measuring 270 x 120 nm in size and with a tail-like extension at one end. Based on sequence information of the genome, structural proteins and phylogenetic analysis of viral DNA polymerase, WSSV was assigned to the new virus family *Nimaviridae*, genus Whispovirus (Van Hulten et al. 2001).

Different approaches to control WSSV have been successful under experimental conditions. Some of these include (i) increasing or decreasing the optimal water temperature of shrimp (Vidal et al. 2001, Guan et al. 2003, Jiravanichpaisal et al. 2004), (ii) administering feed supplemented with immunostimulants such as peptidoglycans, lipopolysaccharides and β-1,3 glucans (Itami et al. 1998, Takahashi et al. 2000, Chang et al. 2003), (iii) 'vaccinating' shrimp by giving feed coated with formalin-inactivated WSSV or recombinant WSSV envelope proteins VP19 and VP28 (Namikoshi et al. 2004, Witteveldt et al. 2004) and (iv) feeding shrimp with a diet containing fucoidan, a sulfated polysaccharide with antiviral activity *in vitro* (Chotigeat et al. 2004).

Cidofovir [(s)-1-(3-hydroxy-2-phosphonylmethoxy propyl) cytosine] (HPMPC) is an acyclic nucleoside phosphonate. It is an antiviral drug that enters the cell by fluid phase endocytosis. After cellular uptake, two phosphorylation steps done by cellular kinases are necessary to reach its active metabolite stage (HPMPCpp). Phosphorylation occurs in both virus infected and uninfected cells. It does not require prior activation by virus encoded kinases. The antiviral activity of cidofovir is by blocking DNA synthesis through incorporation of HPMPCpp molecules in the viral DNA. Cidofovir has proven effective against a number of human DNA viruses such as polyomavirus, papillomavirus, adenovirus, herpesvirus and poxvirus (De Clercq 2003).

Spirulina platensis is a marine blue-green alga. It contains calcium spirulan, a sulfated polysaccharide soluble in water. This polysaccharide consists of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid, galacturonic acid,

sulfate, and calcium. It inhibits replication of enveloped viruses such as herpes simplex virus type 1 (HSV-1), human cytomegalovirus, measles virus, mumps virus, influenza A virus and human immunodeficiency virus type 1 (HIV-1) *in vitro* (Hayashi et al. 1996). The concentration of calcium spirulan that reduces viral replication by 50% was between 11.4 to 2600 µg ml^{-1} added to the medium immediately after virus inoculation. *S. platensis* was also found to improve the immune system by suppressing cancer development and viral infection in man (Hirahashi et al. 2002).

In the present study, the effects of an intramuscular (im) injection of cidofovir or a diet supplemented with *S. platensis* were compared on the outcome of WSSV infection in shrimp.

MATERIAL AND METHODS

Virus - A Thai WSSV isolate from naturally-infected *Penaeus monodon* was passaged once into crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al. 2001). Gills from infected crayfish were collected and suspended (10^{-2}) in L-15 medium. The gill suspension was diluted (10^{-1}) in phosphate-buffered saline (PBS) pH 7.4 and 50 µl were injected intramuscularly (im) into specific pathogen-free (SPF) *Litopenaeus vannamei* to amplify the virus. Moribund and dead shrimp were collected 48 hours post inoculation (hpi). Carcasses without hepatopancreas, gut and exoskeleton were minced and diluted (10^{-1}) in PBS. The suspension was centrifuged at 3000 x g and 13000 x g at 4ºC for 20 min, respectively. The supernatant was collected and filtered (0.45 µm), aliquoted and stored at -70ºC. The total volume was 250 ml. Samples from tissues used to produce the viral stock were sent to Dr. James Brock (Moana Technologies LLC, Hawaii) for PCR screening of all known viral pathogens of shrimp. The results confirmed the only presence of WSSV DNA. The infectivity titer of the stock was determined *in vivo* through an oral titration using the formula of Reed & Muench (1938). The infectivity titer by oral route was $10^{5.6}$ shrimp infectious doses 50% endpoint (SID_{50} ml^{-1}) (Escobedo-Bonilla et al. 2005).

Shrimp - Specific pathogen-free (SPF) *L. vannamei* Kona-strain were reared and acclimatized at the facilities of the Laboratory of Aquaculture and *Artemia* Reference Center (ARC), Ghent University. Shrimp used for testing cidofovir toxicity and its

antiviral properties, and those used for testing the effect of a diet supplemented with *Spirulina* had a mean body weight (MBW) of 2.7 ± 0.6g (n=36); 5.9 ± 1.6 g (n=78) and 9.5 ± 1.9 g (n=20), respectively.

Experimental conditions - Shrimp reared at the ARC were acclimatized to a salinity of 15 g l^{-1} over a period of four days, and then transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University, where the experiments were carried out under biosafety conditions. Depending on the experiment, six or ten animals were housed in 50 l aquaria equipped with aeration, heating (Visitherm, Aquarium systems, France) and mechanical filtration (Eheim, Germany). Salinity was maintained at 15 g l^{-1} using artificial seawater (Instant Ocean, Aquarium systems, France) prepared with distilled water. Shrimp were fed with six pellets daily equally divided in the morning and afternoon. Water quality (NH_4^+ and NO_2^-) was monitored daily using test kits (Aquamerck, Germany). The maximum value was 3 mg l^{-1} for NH_4^+ and 0.05 mg l^{-1} for NO_2^-.

Cidofovir - *Toxicity* - Toxicity of cidofovir was tested first. Six cidofovir concentrations (0, 12.5, 25, 50, 100 and 200 mg kg^{-1} of shrimp body weight) were prepared with PBS in a volume of 50 µl. Each concentration of cidofovir was injected im to six shrimp between the 3rd and 4th pleonite. All animals were followed clinically for 120 hours.

Efficacy - The highest concentration that showed no apparent signs of toxicity was used as treatment in the efficacy experiments. The effect of cidofovir on the outcome of WSSV infection was evaluated in two experiments. In the first experiment, twenty shrimp were treated with cidofovir and eighteen shrimp were mock treated with PBS. In the second experiment, twenty shrimp were used in each treatment.

Spirulina - Shrimp were fed with a diet supplemented with *Spirulina* (n = 10) or a normal diet (n=10) at 5% MBW per day for four days before the WSSV challenge. BESTMIX computer software was used to formulate the diets, where nutrient settings were fixed and ingredient values were allowed to shift between determined ranges. Both diets had equal settings for protein, fat, starch, fiber, calcium, phosphorous, cholesterol, phospholipid, essential fatty acids and carotenoids. The formulation program selected fish meal and soymeal as the major ingredients to be replaced with *S. platensis* meal, due to their similar gross composition. The compositions of the diets are given in Table

1.

Table 1.- Composition of a normal diet and a diet supplemented with *S. platensis*.

Composition (% w/w)		
Ingredient	Normal diet	Diet supplemented with *Spirulina platensis*
S. platensis meal		25.0
Fish meal	40.0	22.0
Wheat fluor	15.0	12.9
Krill	13.0	12.3
Fish oil	9.5	10.5
Soy meal	5.2	0.0
Squid meal	5.0	5.0
Mineral mix	5.0	5.0
Liquid binder	4.9	4.9
Vitamin mix	2.4	2.4
Total	100.0	100.0

WSSV oral inoculation procedure - Shrimp were inoculated orally with a WSSV dose of 30 SID_{50} in 50 µl. After inoculation, shrimp were monitored for clinical signs twice daily for 5 days. Clinical signs included empty gut and reduced response to stimulus. Mortality was recorded twice daily.

Evaluation of WSSV infection by indirect immunofluorescence (IIF) - The pereon of dead, moribund and surviving shrimp were dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at -20°C. Tissues (5 µm) were cryo-sectioned longitudinally and immediately fixed in 100% methanol at -20ºC for 20 min. Sections were washed three times for 5 min. each in PBS and incubated with 2 mg ml^{-1} of the monoclonal antibody 8B7 (DiagXotics Inc. USA) directed against the WSSV envelope protein VP28 (Poulos et al. 2001) for 1 h at 37ºC. Then, sections were washed three times for 5 min. each in PBS and incubated with fluorescein isothiocyanate (FITC) - labeled goat anti-mouse antibody (F-2761, Molecular Probes, The Netherlands) for 1 h

at 37ºC. Sections were finally washed in PBS, rinsed in deionised water, dried and mounted with a solution of glycerin and 1, 4-diaza-bicyclo[2,2,2]-octan (DABCO) (ACROS organics, USA). Slides were analyzed by fluorescence microscopy.

Statistical analysis - The cumulative mortality of the two cidofovir experiments was analysed by probit, which is a generalized linear model with a probit link function (Agresti 1996). When significant interactions exist between treatment and time, the probit model has the form:

$$\text{Probit}(x) = \alpha + \beta\ \text{time} + \gamma\ \text{treatment} + \delta\ \text{time*}\ \text{treatment}$$

Where:

α is the intercept

β is the rate of probability change per unit change of time (for a constant dose)

γ is the rate of probability difference for each treatment (for a constant time)

δ is the change in rate of probability per unit change of time depending on the treatment

When no significant interactions are found, the probit model becomes:

$$\text{Probit}(x) = \alpha + \beta\ \text{time} + \gamma\ \text{treatment}$$

The interactions between treatment and time, as well as each of the parameters were determined using the statistical software s-plus version 6.1 (Lucent technologies Inc., USA). Differences between treatment and control were determined by t-tests using the same statistical software.

RESULTS

Cidofovir - *Toxicity* - none of the cidofovir concentrations tested caused disease (evaluated by clinical signs) in shrimp. Hence, the maximum dose of 200 mg kg^{-1} body weight was used to further assess its antiviral effect during a WSSV infection.

Efficacy - Mock-treated and cidofovir-treated shrimp started to show clinical signs (empty gut and reduced response to stimulus) at 24-36 hpi. Cidofovir treatment delayed

mortality with approximately 24 hours when compared to mock-treated shrimp. In the two experiments, 56% mortality was observed in the controls at 60 hpi, compared to only 15-30% mortality in cidofovir treated animals (Fig 1a & 1b). The 60% mortality was reached in cidofovir treated shrimp at 84 hpi. 100% mortality of mock treated shrimp was observed at 96-108 hpi, while cidofovir treated shrimp had 80-90% mortality at the end of the experiment (120 hpi). All dead shrimp in the two experiments were infected with WSSV as determined by IIF. Surviving shrimp in both cidofovir experiments were also infected and found positive by IIF except for one in the second cidofovir experiment. Probit analysis showed significant differences ($p < 0.05$) in the median lethal times (LT_{50}) (Table 2) between shrimp treated with cidofovir and the mock-treated (Fig 2a & 2b).

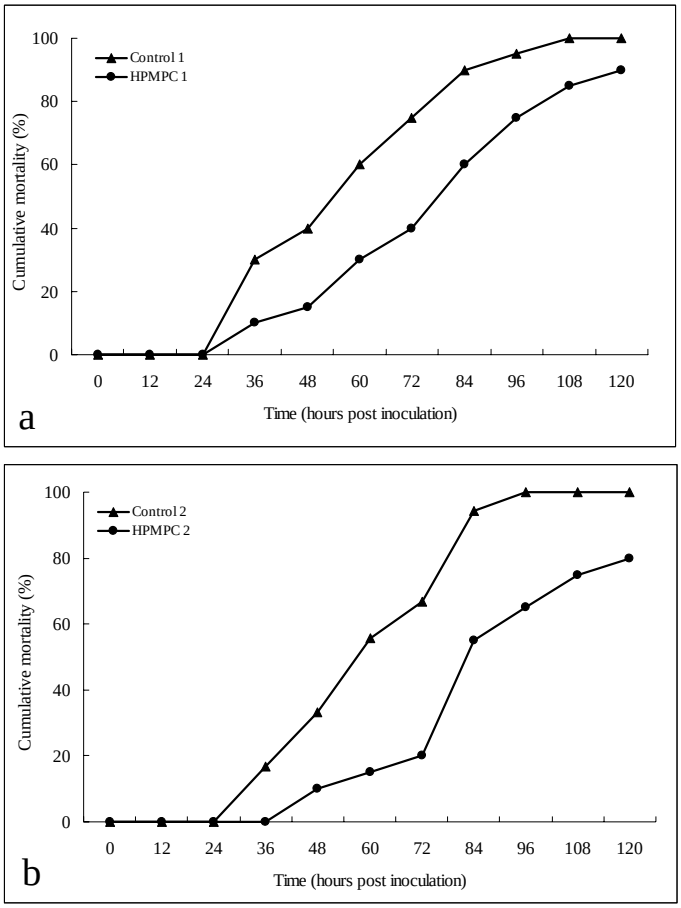

Figure 1. Cumulative mortality of *L. vannamei* challenged with 30 SID_{50} of WSSV and treated with cidofovir (200 mg kg^{-1} shrimp body weight in 50 µl) or mock-treated (50 µl of PBS) in (a) experiment 1 and (b) experiment 2

Table 2.- Median lethal times (LT_{50}) and its comparison between cidofovir-treated and mock-treated shrimp.

Experiment	Group	α	β	γ	δ	LT_{50}	comparison (p = 0.05)
1	Control	-2.2352	0.04034	0	----	55.41	Significant
	Cidofovir	-2.2352	0.04034	-0.9020	----	77.78	difference
2	Control	-3.2492	0.05606	0	0	57.97	Significant
	Cidofovir	-3.2492	0.05606	0.0070	-0.01963	89.02	difference

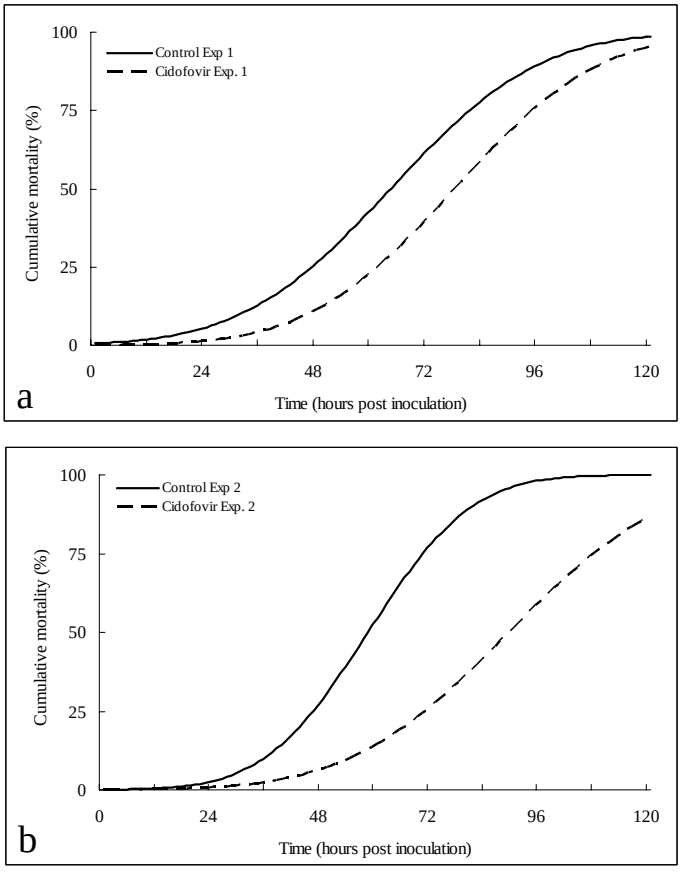

Figure 2. Probability of mortality of cidofovir-treated and mock-treated shrimp in experiment 1 (a) and experiment 2 (b)

Spirulina - Shrimp fed the normal diet showed clinical signs starting at 24-36 hpi. Shrimp fed the *S. platensis* supplemented diet started to show disease at 36-48 hpi. Both groups reached 100% mortality at 84 hpi (Fig 3) and all dead shrimp were WSSV-positive by IIF.

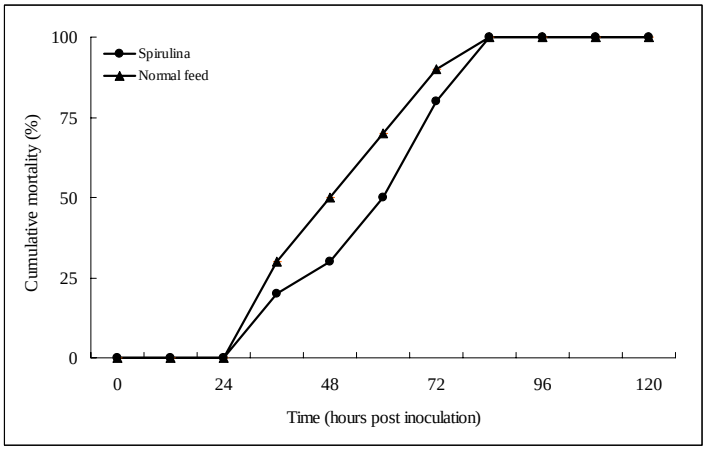

Figure 3. Cumulative mortality of shrimp challenged with 30 SID_{50} of WSSV and fed a diet supplemented with *Spirulina*

DISCUSSION

In this study, a standardized WSSV challenge procedure was used to test two candidate antiviral products, cidofovir and a diet supplemented with *Spirulina platensis*. Only WSSV-infected shrimp treated with cidofovir showed a considerable delay in mortality (24 h).

In the past, inoculation methods that mimic natural routes of infection such as feeding WSSV-infected tissues or immersing shrimp in water containing viral suspensions were used. However, these methods do not ensure that each individual takes up the same amount of infectious virus particles, causing a high variability between animals and between experiments. Therefore, a more reproducible WSSV challenge procedure is needed (Prior et al. 2003). The new challenge procedure used in this study may give an answer to this problem.

In the control groups of the different experiments, the clinical outcome always was very similar, showing the reproducibility of the procedure. The fact that 100% mortality was

reached at 84-108 hpi indicates that the challenge was quite severe. This may be due to the high virulence of the WSSV strain and/or the high susceptibility of the SPF shrimp. Previous studies have indicated possible differences in virulence of WSSV strains and different susceptibility between shrimp species (Lightner et al. 1998, Wang et al. 1999). In this study, the infectious doses of virus inoculated to shrimp could not be lowered because this would have resulted in an increased chance to get uninfected animals.

Cidofovir is very effective in controlling infections in different mammalian DNA viruses (De Clercq 2003). At least three different explanations can be proposed to explain why cidofovir did not show a sufficient protection against a fatal WSSV infection in shrimp. First, the cellular uptake of cidofovir by shrimp cells may be lower than that of mammalian cells, making the compound less effective even at a very high dose (200 mg/kg body weight). This problem could be solved by using an esterified form of cidofovir, which undergoes a better cellular uptake. Second, it is possible that intracellular cidofovir is not sufficiently metabolized into its active form (HPMPCpp). Since the antiviral effect of cidofovir depends on the intracellular concentration of this metabolite (De Clercq 2003), a low metabolite concentration will not be able to stop viral DNA synthesis. Third, the lower effectiveness of cidofovir against WSSV infection compared to other DNA viruses may also be attributed to differences in affinity of HPMPCpp to the viral DNA polymerase.

Cidofovir induced a significant delay in mortality in WSSV infected shrimp. However, most of the surviving shrimp were infected and would probably have died at a later time, thus minimizing the potential use of cidofovir as therapeutic agent in aquaculture.

In the present study, dietary supplementation of *S. platensis* had no clear effect on the mortality of infected shrimp, which was different from the results obtained by Chotigeat et al. (2004) using another sulfated polysaccharide, fucoidan. However, due to differences in challenge procedures and WSSV strains used in each study, it is difficult to make firm conclusions on the comparison of the efficacy of both products. In the future, other products will be tested with this challenge procedure in order to end up with some promising products that could be used in the field.

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5.2 Effect of high water temperature on the virological and clinical outcome of the intramuscular or oral inoculation of white spot syndrome virus (WSSV) in *Litopenaeus vannamei*

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ABSTRACT

White spot syndrome virus (WSSV) is the most lethal pathogen of cultured shrimp. Previous studies done with undefined WSSV titers showed that high water temperature (32-33°C) reduced / delayed mortality of WSSV-infected shrimp. This study evaluated the effect of high water temperature on the clinical and virological outcome of a WSSV infection under standardized conditions. Groups of specific pathogen-free *Litopenaeus vannamei* were challenged either by intramuscular or oral routes with a low (30 SID_{50}) or a high (10000 SID_{50}) virus titer. Shrimp were kept (i) continuously at 27°C, (ii) 30°C or (iii) 33°C; (iv) maintained at 33°C before challenge and 27ºC afterwards, or (v) kept at 27°C before challenge and 33°C afterwards. Shrimp were maintained at the respective temperatures for 120 h before challenge and 120-144 h post challenge (hpc). Gross signs and mortality were monitored every 12 h until the end of the experiment. Dead and surviving shrimp were screened for WSSV infection (VP28-positive cells) by indirect immunofluorescence (IIF). Shrimp kept continuously at 27°C or 30°C, or switched to 27°C post challenge developed gross signs within 24 hpc, first mortalities at 36-60 hpc and 100% cumulative mortality between 60 and 144 hpc depending on the virus titer. All dead shrimp were WSSV-positive. In contrast, shrimp kept at 33°C continuously or after WSSV challenge showed no signs of disease and low mortalities (0-30%) regardless of the virus titer. Dead and surviving shrimp were WSSV-negative. Further, early virus replication was studied in two groups of shrimp: one maintained at 27°C before and after challenge and one switched from 33°C to 27°C after challenge with 10000 SID_{50}. Immunohistochemistry (IHC) analysis showed that WSSV-positive cells were first displayed at 12 hpc in shrimp kept at 27°C and by 24 hpc the infection became systemic. In contrast, shrimp kept at 33°C did not display WSSV-positive cells at 12 or 24 hpc. This work confirms previous reports that high water temperature prevents the onset of disease and significantly reduces mortality of WSSV-inoculated shrimp regardless of the route of inoculation or virus titer used. This strategy may have practical applications to control WSSV in tropical shrimp farming countries.

INTRODUCTION

White spot syndrome virus (WSSV) has caused disease and mortality resulting in huge production losses in shrimp aquaculture for more than a decade. This pathogen is a bacilliform, enveloped, double stranded (ds) DNA virus of the family *Nimaviridae* (Mayo, 2002). In WSSV-infected shrimp 100% mortality occurs within 3-10 days (Lightner, 1996). Signs of disease include white spots in the carapace, reddish discoloration, anorexia, lethargy and swelling of branchiostegites in infected *Penaeus monodon* (Lightner, 1996, Sahul-Hameed et al., 1998). Experimental infections in *Litopenaeus vannamei* showed as signs of disease a reduction in feeding and in response to stimulus (Escobedo-Bonilla et al., 2006).

Disease is the end result of complex interactions between host, pathogen and environment (Lightner and Redman, 1998). In this context, water temperature is considered to be one of the most important environmental factors for shrimp since it influences metabolism, oxygen consumption, feeding rate, growth, molting, survival and tolerance to toxic metabolites (Wyban et al., 1995, Ponce-Palafox et al., 1997, Jackson and Wang, 1998, Hewitt and Duncan, 2001, Coman et al., 2002, Spanopoulos-Hernández et al., 2005). Optimum temperature for growth and survival of shrimp varies according to the life stage and the species. For small *L. vannamei* (<5g), optimum temperature is higher than 30°C and for large shrimp (16 g) optimum temperature is around 27°C (Wyban et al., 1995). Highest survival of juvenile *L. vannamei* is obtained between 20°C and 30ºC (Ponce-Palafox et al., 1997). The upper lethal temperature limit for juvenile penaeid shrimp is 34°C - 36°C (Dall et al., 1990).

The effect of temperature on the outcome of WSSV infections is already documented. In tropical countries such as Ecuador and Thailand, the prevalence of WSSV in grow-out ponds and hatcheries is reduced in the warm season (Rodríguez et al., 2003, Withyachumnarnkul et al., 2003). Further, experimentally WSSV-infected shrimp kept at high (>32ºC) (*L. vannamei* or *Marsupenaeus japonicus*) or at low (12°C - 15ºC) (*M. japonicus* or crayfish *Astacus astacus* and/or *Pacifastacus leniusculus*) water temperatures showed reduced / delayed mortality (Vidal et al., 2001, Guan et al., 2003, Jiravanichpaisal et al., 2004). Temperature also influences the outcome of viral infections in other ectothermic animals such as fish and insects. Examples are infections

caused by a koi herpesvirus (KHV) (Gilad et al., 2003, Iida and Sano, 2005), a largemouth bass virus (LMBV) (Grant et al., 2003) and a nucleopolyhedrovirus (NPV) of the silkworm *Bombyx mori* (Kobayashi et al., 1981, Shikata et al., 1998).

The mechanism by which high water temperature induces a reduction in mortality of WSSV-infected *L. vannamei* is not known. It has been suggested that hyperthermia may trigger a host defense response (e.g. apoptosis). Alternatively, it may affect WSSV replication (Vidal et al., 2001, Granja et al., 2003). Granja et al., (2006) showed a reduction of the WSSV DNA load in WSSV-infected shrimp at 32°C. *In vitro* studies demonstrated a progressive reduction of WSSV replication and amount of WSSV DNA in haematopoietic stem cells of *P. leniusculus* at 16°C and 4°C (Jiravanichpaisal et al., 2006).

The objective of this study was to evaluate the effect of high water temperature (33°C ± 0.5) before and/or after challenge on the clinical and virological outcome of WSSV infection in specific pathogen-free *L. vannamei* using standardized WSSV inoculation procedures (intramuscularly and orally; high and low virus titers).

MATERIAL AND METHODS

Virus and infectivity titers - a Thai isolate of WSSV was kindly provided by P. Jiravanichpaisal and K. Söderhäll (Upsala University, Sweeden). This isolate was passaged into *L. vannamei* to produce high virus titers. The determination of the infectivity titers *in vivo* was done as follows: tenfold serial dilutions of the WSSV stock were made in phosphate buffered saline (PBS) pH 7.4. Per dilution, five shrimp were injected intramuscularly (50 µl). The proportion of infected shrimp at each dilution was determined by indirect immunofluorescence (IIF) and one-step PCR. The infectivity titer (shrimp infectious dose 50% endpoint [SID_{50} ml^{-1}]) was calculated with the method of Reed and Muench. By intramuscular route the median infectivity titer was $10^{6.6}$ SID_{50} ml^{-1} and by oral route it was $10^{5.6}$ SID_{50} ml^{-1} (Escobedo-Bonilla et al., 2005a).

Shrimp - specific pathogen-free (SPF) shrimp *L. vannamei* Kona strain from (i) Molakai Sea Farms or (ii) Ceatech, both farms from Hawaii, were imported as postlarvae (PL) and reared at the facilities of the Laboratory of Aquaculture and Artemia Reference Center (ARC), Ghent University. Water temperature was 27°C -

28°C and salinity 35 - 37 g l^{-1}. A total of 240 shrimp with a mean body weight (MBW) of 14.3 ± 3.4 g were used for intramuscular inoculation, 73 shrimp with a MBW of 6.6 ± 1.5 g for oral inoculation and 24 shrimp with a MBW of 14.3 ± 3.4 g were used for the time course study.

Experimental conditions - shrimp were first acclimatized to water temperatures of 27°C, 30°C or 33°C (see Table 1) at the facilities of the Laboratory of Aquaculture and Artemia Reference Center (ARC) and maintained at that temperature for 96 h. Water temperature was controlled with an aquarium heater (VTX 300 aquarium systems, France). Simultaneously, shrimp were acclimatized to a salinity of 15 g l^{-1}.

After acclimatization at the ARC, shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University to perform the experimental WSSV challenges. Immediately after arrival, shrimp were weighed and housed in 50 l aquaria with artificial seawater (salinity of 15 g l^{-1}) and equipped with aeration, mechanical filtration and water heaters. Shrimp were kept at the same temperature as at the ARC for another 24 h. Immediately after WSSV challenge, the different groups were kept at the same temperature or switched to another water temperature as described in Table 1.

In these experiments no control groups were used since we know from unpublished experiments that uninfected juvenile *L. vannamei* maintained at the different temperatures had low levels of mortality (data not shown).

Experimental design

Clinical and virological outcome - for the intramuscular route, groups of 9 to 11 shrimp were continuously kept at (i) 27°C or (ii) 33°C, or switched (iii) from 27°C before WSSV injection to 33°C afterwards, or (iv) from 33°C before injection to 27°C afterwards. For each temperature regime one group was injected with a low virus titer (30 SID_{50}) (SID_{50} = shrimp infectious dose with 50% endpoint) and a second with a high virus titer (10000 SID_{50}). Shrimp were monitored twice daily for signs of disease and mortality. Dead and surviving shrimp were processed for IIF analysis. These experiments were run for 120 to 144 hpc and were repeated three times.

For the oral route, groups of 13 to 28 shrimp were continuously maintained at (i) 27°C; (ii) 30°C or (iii) 33°C. A group of 16 shrimp was kept at 27°C before challenge and switched to 33°C after challenge. All these shrimp were orally challenged with a low

virus titer (30 SID_{50}). Shrimp were monitored twice daily for signs of disease and mortality. Dead and surviving shrimp were processed for detection of WSSV-infected cells by indirect immunofluorescence (IIF) analysis. These experiments lasted 120 hours post challenge (hpc) and were done only once.

Time course of viral replication - four groups of six shrimp were kept at 27°C before inoculation for 120 h. These shrimp were orally inoculated with 10000 SID_{50} in a volume of 50 µl. After WSSV challenge, the water temperature of two groups was raised to 33°C while the other two groups were maintained at 27°C. Per temperature regime, one group was collected at 12 and the other at 24 hpc. Tissues from the pereon were fixed in Davidson's for 48 h and changed to 50% ethanol for paraffin embedding. Tissue sections (4 µm) were stained by the immunohistochemistry (IHC) technique described by Escobedo-Bonilla et al., (2005b). Briefly, tissue sections were deparaffinized, rehydrated, washed in tris buffer pH 7.4 and the endogenous peroxidase was blocked. Sections were incubated with the monoclonal antibody 8B7 (DiagXotics Inc. USA) directed against the WSSV envelope protein VP28 (Poulos et al., 2001). Afterwards, sections were incubated with biotinylated sheep anti-mouse IgG and streptavidine-biotinylated horseradish peroxidase complex. Development was done with 3,3 diaminobenzidine (DAB) and counter-stained with Gill's hemaluin. Sections were washed, dehydrated, mounted and analyzed by light microscopy. WSSV-positive cells showed a brown precipitate.

Evaluation of WSSV infection by indirect immunofluorescence (IIF) - the pereon of dead and surviving shrimp was dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at -20°C. Cryosections (5 µm) were made and immediately fixed in 100% methanol at -20ºC for 20 min. Sections were washed three times for 5 min. each in PBS and incubated with 2 mg ml^{-1} of the monoclonal antibody 8B7 directed against VP28 for 1 h at 37ºC. Then, sections were washed three times for 5 min. each in PBS and incubated with fluorescein isothiocyanate (FITC) -labeled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1 h at 37ºC. Sections were finally washed in PBS, rinsed in deionised water, dried and mounted with a solution of glycerin and 1, 4-diaza-bicyclo[2,2,2]-octan (DABCO) (ACROS organics, USA). Slides were analyzed by fluorescence microscopy.

Statistical analysis - the cumulative mortality of all groups was submitted to probit analyses (Agresti 1996). When significant interactions exist between temperature and time, the probit model has the form:

$$\text{Probit}(x) = \alpha + \beta \text{ time} + \gamma \text{ temperature} + \delta \text{ time*} \text{ temperature}$$

Where:

α is the intercept

β is the rate of probability change per unit change of time (for a constant temperature)

γ is the rate of probability difference for each temperature (for a constant time)

δ is the change in rate of probability per unit change of time depending on the temperature

The parameters of this model were determined using the statistical software S-plus version 6.1 (Lucent technologies Inc., USA). Differences between treatment and control were determined by t-tests using the same statistical software.

RESULTS

Intramuscular inoculation - *Low virus titer* - shrimp maintained continuously at 27°C or switched from 33°C to 27°C after WSSV challenge started to show signs of disease at 24 hpc. Mortality was first recorded between 36 - 60 hpc (27°C/27°C) and 36 - 48 hpc (33°C/27°C) and cumulative mortality reached 100% at 84 - 144 hpc (27°C/27°C) and 72-96 hpc (33°C/27°C). All dead shrimp were WSSV-positive by IIF assay. Significant differences ($p<0.05$) in the median lethal times (LT_{50}) between these groups were found (Table 1 and Fig. 1a, b). In contrast, shrimp continuously kept at 33°C or maintained at 33°C only after challenge did not develop signs of disease and showed cumulative mortality between 0% and 30%. All dead shrimp were WSSV negative by IIF assay. Dead shrimp were found to have molted recently. Body parts such as eyestalks, pleopods, uropods and pereon were eaten by the surviving shrimp.

High virus titer - shrimp kept continuously at 27°C or switched from 33°C to 27°C after

challenge first showed signs of disease between 12 and 24 hpc and almost all shrimp stopped eating at 24 hpc. Mortality started at 36 hpc and 100% cumulative mortality was reached at 60 hpc in both groups. All dead shrimp were WSSV-positive by IIF assay. Significant differences ($p<0.05$) in the LT_{50} were found between these groups (Table 1 and Fig. 2a, b). In contrast, shrimp continuously exposed to 33°C or switched to 33°C after challenge did not show signs of disease and had cumulative mortality between 0% and 20%. All dead and surviving shrimp were WSSV-negative by IIF assay and the dead shrimp found in these groups had molted recently.

Table 1. Median lethal times (LT_{50}) of WSSV-inoculated shrimp under different temperature regimes. Oral and intramuscular (IM) inoculations were used as well as a low (30 SID_{50}) and a high (10000 SID_{50}) virus titer.

Temperature regime‡	Inoculation route	Virus titer	LT_{50}	α	β	γ	δ	LT_{50} comparison*
27°C / 27°C	Oral	Low	50.53[a]	2.9483	-0.0583	----	----	
30°C / 30°C	Oral	Low	48.93[b]	2.9483	-0.0583	-0.3570	0.00538	
27°C / 33°C	Oral	Low	----	2.9483	-0.0583	1.1414	0.00583	b = a < d
33°C / 33°C	Oral	Low	394.9[d]	2.9483	-0.0583	-0.5994	0.05239	
27°C / 27°C	IM	Low	73.29[e]	2.8579	-0.0389	----	----	
33°C / 27°C	IM	Low	57.05[f]	2.8579	-0.0389	1.4556	-0.0366	
27°C / 33°C	IM	Low	257.8[g]	2.8579	-0.0389	-0.4526	0.02966	f < e < g = h
33°C / 33°C	IM	Low	374.6[h]	2.8579	-0.0389	-0.1683	0.0318	
27°C / 27°C	IM	High	45.2[i]	5.1882	-0.1148	----	----	
33°C / 27°C	IM	High	42.0[j]	5.1882	-0.1148	0.1690	-0.0128	
27°C / 33°C	IM	High	260.1[k]	5.1882	-0.1148	-2.766	0.1054	j = i < l = k
33°C / 33°C	IM	High	188.7[l]	5.1882	-0.1148	-1.5808	0.0956	

‡temperature before inoculation/temperature after inoculation

*Differences in LT_{50} are significant (P = 0.05)

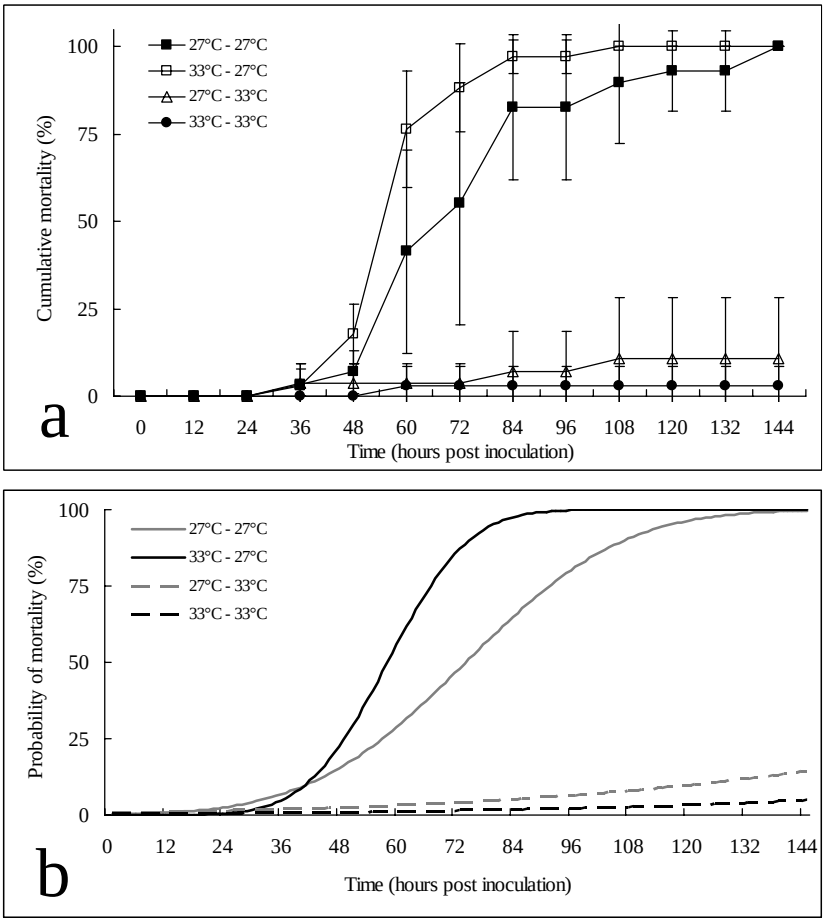

Figure 1. (a) Cumulative mortality (mean ± standard deviation [SD]) of SPF *L. vannamei* intramuscularly inoculated with 30 SID_{50}. Shrimp were continuously kept at 27°C or 33°C, or switched from 33°C before inoculation to 27°C afterwards, or changed from 27°C before inoculation to 33°C afterwards. (b) Probability of mortality (probit) for the different temperature treatments

Oral inoculation - Shrimp kept continuously at 27°C and 30°C first showed signs of disease at 24 hpc. First mortality occurred at 36 hpc and cumulative mortality in these groups reached 100% at 96 and 108 hpc, respectively (Fig. 3a, b). All dead shrimp were positive by IIF (Fig. 4a, b). Probit analysis showed significant differences ($p<0.05$) in the median lethal times (LT_{50}) of these two temperatures (Table 1). Shrimp continuously maintained at 33°C did not show signs of disease; mortality in this group was 0% - 10% and all surviving shrimp were WSSV-negative.

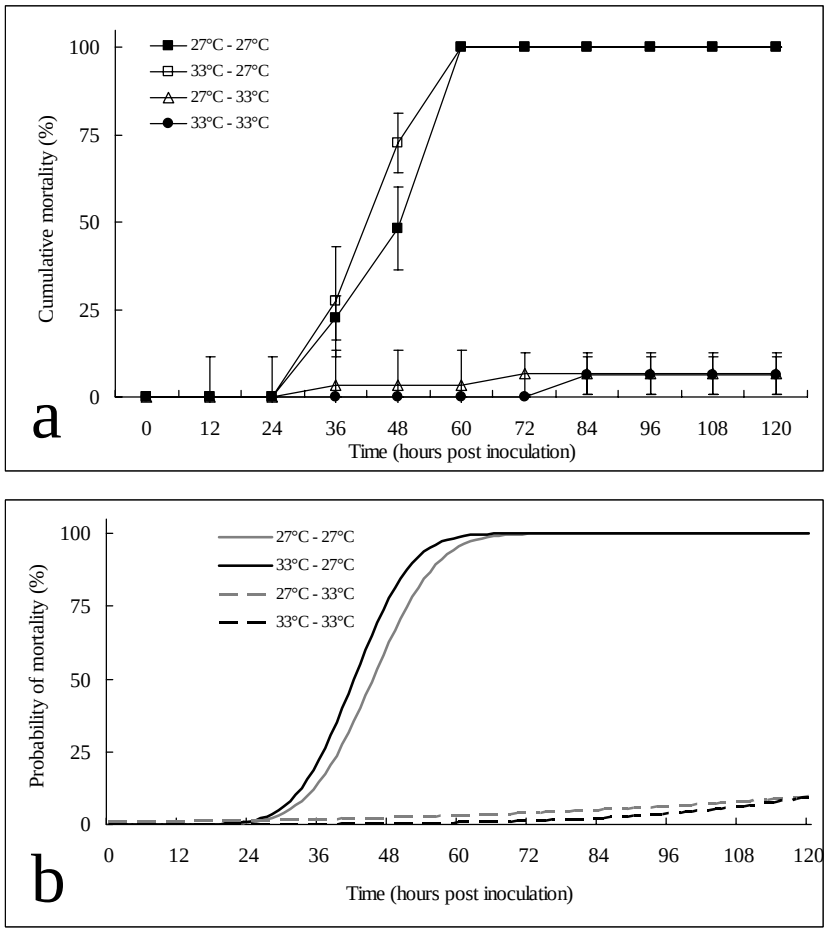

Figure 2. (a) Cumulative mortality (mean ± SD) of SPF *L. vannamei* intramuscularly inoculated with 10000 SID_{50} of WSSV. Shrimp were continuously kept at 27°C or 33°C, or kept at 33°C before inoculation and switched to 27°C after inoculation, or kept at 27°C before inoculation and switched to 33°C after inoculation. (b) Probability of mortality (probit) for the different temperature treatments

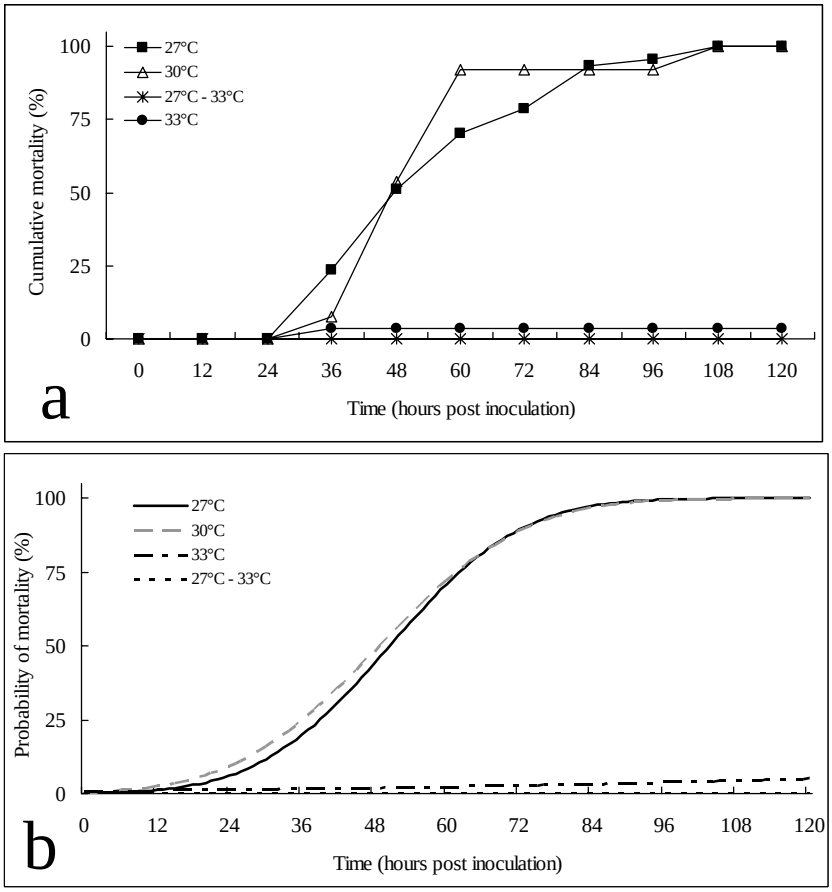

Figure 3. (a) Cumulative mortality of SPF *L. vannamei* continuously kept at 27°C, 30°C, 33°C or maintained at 27°C before challenge and switched to 33°C after WSSV oral inoculation. Shrimp were challenged with 30 SID_{50}. (b) Probability of mortality (probit) for the different temperature treatments

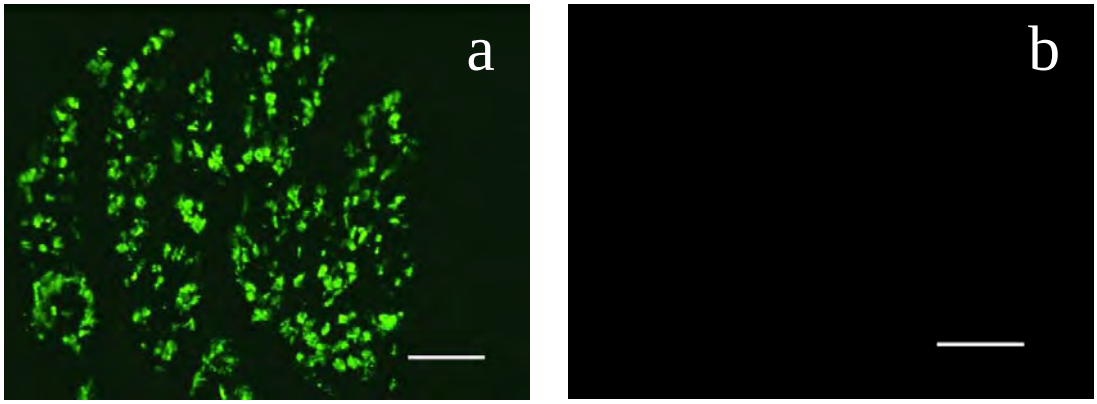

Figure 4. (a) WSSV-positive and (b) WSSV-negative cells in gills of SPF *L. vannamei* as determined by indirect immunofluorescence (IIF). Magnification 200X, bar = 100 µm.

Time course - shrimp maintained at 27°C first displayed WSSV-positive cells at 12 hpc in epithelial cells of foregut, cells in gills and antennal gland. At 24 hpc, WSSV-positive cells were also found in integument, hematopoietic tissue and lymphoid organ. In contrast, shrimp maintained at 33°C after challenge did not display WSSV-positive cells at 12 or 24 hpc (Fig. 5a, b).

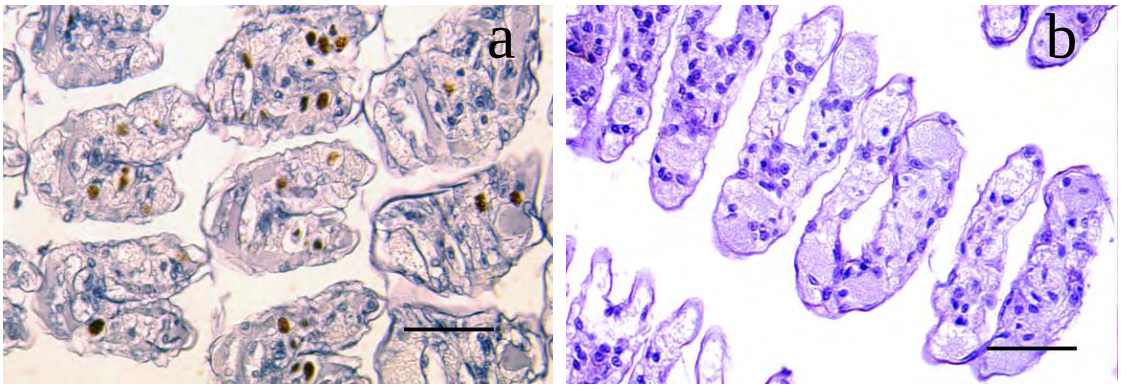

Figure 5. (a) WSSV-positive and (b) WSSV-negative cells in gills of SPF *L. vannamei* as determined by immunohistochemistry (IHC). Magnification 400X, bar = 50 µm

DISCUSSION

The present findings agree with previous work where mortality was reduced in WSSV-infected *L. vannamei* maintained at 32°C (Vidal et al., 2001, Granja et al., 2003, 2006). Other studies done *in vivo* with WSSV-infected shrimp *M. japonicus* or crayfish *P. leniusculus* showed that maintaining these species at water temperature below 16°C was also effective in reducing mortality (Guan et al., 2003, Jiravanichpaisal et al., 2004). Temperatures above 16°C and below 32°C allow WSSV replication in susceptible hosts such as shrimp, crabs and crayfish (Corbel et al., 2001, Guan et al., 2003, Jiravanichpaisal et al., 2004, 2006 and this study). This agrees with the normal temperature range in tropical areas where penaeid shrimp naturally occur and are cultured (Glude, 1978, Dall et al., 1990). In some tropical zones, water temperature may reach 32°C or more for several months (Wahab et al., 2003, Burford et al., 2004). This opens the possibility to apply high water temperature (≥ 32°C) to control mortality due to WSSV infection in several shrimp farming countries. Since outbreaks of WSSV have

been reported to occur in general one or two months after pond stocking (Otta et al., 1999), it may be proposed to start a culture cycle about one month before the season of high water temperature begins to minimize the risk of a WSSV outbreak. Another advantage of culturing shrimp at high temperature may be an increase in the growth rate and as a consequence a shorter time to complete the culture cycle. A disadvantage of high temperature is the negative influence on other environmental variables such as levels of dissolved oxygen, evaporation rate, salinity and concentration of toxic metabolites such as ammonia or nitrites, which are all very critical for the normal shrimp metabolism (Brock and Main, 1994, LeMoullac et al., 1998, van Wyk and Scarpa, 1999, Lemaire et al., 2002).

This study clearly demonstrated that high water temperature completely inhibited the expression of the envelope protein VP28 *in vivo*. This result suggests a block of WSSV replication at an early stage and this finding may help to unravel the protective mechanism of high water temperature against WSSV. Previous studies done with temperature-sensitive (ts) mutant baculoviruses showed that mutations in the protein kinase-1 (Fan et al., 2006) or in a putative RNA polymerase (Shikata et al., 1998) resulted in the lack of expression of late viral proteins such as envelope proteins at high water temperature. These studies indicate that high temperature may affect enzyme activity during different phases of early viral replication of ds DNA viruses. With WSSV, it is not known if enzymes are impaired by high water temperature. This should be studied by biochemical assays.

In summary, the drastic reduction of infected cells and mortality in WSSV-challenged shrimp when maintained at high water temperature was confirmed by using standardized WSSV inoculation models. This effect was always consistent, regardless of the route of inoculation or viral titers used. Keeping shrimp at 33°C after WSSV challenge was sufficient to block viral replication at an early stage, resulting in the inhibition of expression of the structural protein VP28. High water temperature may be applied in nursery and grow-out facilities in tropical shrimp farming countries to control WSSV.

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CHAPTER 6

GENERAL DISCUSSION

“Writing is not necessarily something to be ashamed of, but do it in private and wash your hands afterwards”. - Robert A. Heinlein

In the present thesis, infectivity titers of a WSSV stock were determined *in vivo* in specific pathogen-free (SPF) *Litopenaeus vannamei* by intramuscular and oral routes. These titers were the basis to define the infectious doses that induced infection and mortality to the complete population of inoculated shrimp. Doses were subsequently used in standardized inoculation models to evaluate the efficacy of products with a potential to control WSSV infection. The oral inoculation procedure resembled a natural way of WSSV infection and was therefore used to study aspects of WSSV pathogenesis, particularly the portals of entry in shrimp and the spread to other target organs.

Continuous cell lines derived from vertebrates or insects are routinely used to determine virus titers *in vitro* (Plumb & Zilberg 1999; Grasela et al. 2000). The lack of a continuous shrimp cell line makes it difficult to establish such a titer for shrimp viral pathogens. The only way to adequately determine virus titers was to perform *in vivo* titrations with SPF shrimp. The present thesis is the first study that determined the infectivity titer (shrimp infectious dose 50% endpoint [SID_{50} ml^{-1}]) of a WSSV stock and the relationship between infectivity and mortality upon intramuscular or oral inoculations of WSSV. By any of these routes, the relationship between infection and mortality was 1:1 at 120 hours post inoculation (hpi), indicating that every shrimp that became infected actually died.

We showed that the titers of infection and mortality were statistically similar in shrimp at the stages of early juvenile (≤ 60 days-old) to subadult (≥ 135 days-old). Previous studies have indicated differences in susceptibility between developmental stages. In general, juvenile, subadult and adult stages are more susceptible to WSSV than the larval or postlarval ones (Lightner et al. 1998; Yoganandhan et al. 2003; Pérez et al. 2005). However, some species showed a different pattern in susceptibility. Postlarvae (PL19) of the shrimp *Farfantepenaeus aztecus* were very susceptible to WSSV when fed with infected tissues (cumulative mortality ≥ 75% at 9 days post inoculation [dpi]), whereas the juvenile stage showed a lower mortality (27% after 10 dpi).

The fact that shrimp were inoculated by feeding with WSSV-infected tissues does not ensure that all the animals took up an equal dose of infectious virus, especially when the amount of infectious tissues consumed by the shrimp varies even within a group of shrimp of the same age. In order to objectively establish differences in susceptibility to WSSV, it is necessary to challenge shrimp at different developmental stages or different

shrimp species with a standardized inoculation protocol. Because it is impossible to intubate postlarvae for the administration of a certain virus titer due to their minute size, the oral inoculation protocol cannot be used to make a comparison in earlier developmental stages. The intramuscular model is more suitable but the disadvantage with this technique is that protection by the different barriers at the primary replication site cannot be tested.

The oral inoculation model described in this thesis was developed to mimic a natural pathway of WSSV infection *in vivo* under natural and culture conditions. The oral inoculation differed from the intramuscular model in two important aspects: (1) it required about 1 $\log_{10}$ more infectious virus than the intramuscular route to reach the 50% endpoint of infection (SID_{50} ml^{-1}) and (2) it delayed the 100% mortality with one day compared to the intramuscular route. These characteristics indicated that the oral route was less aggressive than the intramuscular route, probably due to the fact that the virus was delivered in the lumen of the digestive tract and that it had to overcome some physical and chemical hindrances. In the foregut of shrimp, the epithelial cells are lined with cuticle (Icely & Nott 1992). This component of the digestive tract probably acts as a barrier and effectively hinders virus entry. The presence of digestive enzymes in the lumen of the foregut (Icely & Nott 1992) may inactivate viral particles.

With the oral inoculation a slower mortality rate (LT_{50}) was observed compared to the intramuscular route. This result is similar to that of previous studies in which Asian shrimp species fed with WSSV-infected tissues died two or three days later than shrimp challenged by injection (Sahul-Hameed et al. 1998; Rajendran et al. 1999; Rajan et al. 2000). Other decapod species such as *Acetes sp*. and *Palaemon sp*. fed with WSSV-infected tissues showed a significant reduction (75% to 100%) in mortality when fed WSSV-infected tissues compared to a challenge by injection (Suppamattaya et al. 1998; Di Leonardo et al. 2005).

The oral inoculation model described in this thesis resembles a natural way of WSSV infection. As a consequence, we used the oral route to study different aspects of WSSV pathogenesis and to evaluate the effect of antiviral products to control WSSV infection.

After oral inoculation, WSSV was able to reach the epithelial cells in the foregut but also simultaneously infected cells in the gills. White spot syndrome virus may have reached the gills by one of the following ways: (1) through inoculum spilled into the gill

chamber or (2) by crossing the epithelial layer of the foregut shortly after inoculation. The first hypothesis was supported by the fact that during inoculation, some of the inoculum was spilled over the shrimp which may have reached the branchial chamber. The second possibility is based on the assumption that the oral inoculation technique may have caused fissures to the cuticle and/or epithelium of the foregut facilitating WSSV entry into the shrimp. To rule out the second hypothesis, an alternative standardized inoculation model that resembles a natural infection but that will not damage the cuticle may be used. Such an alternative model is immersion. This standardized inoculation procedure could be very useful to determine the susceptibility of shrimp at different stages of development to WSSV infection. It also can be used to confirm the primary sites of replication found by oral inoculation.

For the pathogenesis study, a standardized oral inoculation model was chosen because it allowed us to determine the sites of primary WSSV replication when the virus was delivered only through this route. With the immersion route it is possible that WSSV may enter through any of the body surfaces including the integument or gills, making it difficult to determine the main portal of entry.

Foregut and gills were previously also found as primary replication sites for WSSV in early juvenile *Penaeus monodon* fed with infected tissues (Chang et al. 1996) and in other naturally infected decapods (Lo et al. 1996; Chang et al. 1998; Chou et al. 1998). However, the latter findings together with our observations are in contrast with results of another study where upon ingestion of WSSV-infected tissues, the epithelial cells in foregut of juvenile *Marsupenaeus japonicus* were not infected. Instead, a transient replication of WSSV was observed in epithelial cells of the intestine (Di Leonardo et al. 2005).

In the present thesis, WSSV DNA was found in hemolymph at the same time when WSSV-positive cells were found in the primary replication sites (12 or 18 hpi depending on the dose used) whereas WSSV-positive hemocytes in hemolymph were found only at a later time during infection (36 hpi onwards). These results indicate that WSSV reached other target organs through hemolymph circulation in cell-free form and agree with previous studies done in shrimp and crayfish experimentally infected with WSSV (Shi et al. 2000, 2005, van de Braak et al. 2002). The results of a study done with naturally-infected *Fenneropenaeus merguiensis* suggested that circulating

hemocytes may be involved in the systemic infection of shrimp by WSSV (Wang et al. 2002). However, this work demonstrated the presence of WSSV-infected hemocytes in naturally-infected shrimp which already displayed signs of disease. Because we showed in this thesis that clinical signs appear from 24-36 hpi onwards, it is very likely that the infection was already at an advanced stage. It is not clear why in the present thesis it took 36 hpi before infected cells were detected in the hemolymph and more importantly, what the origin of these cells was. It is possible that they are cells originating from different internal organs in which the replication started from 12 or 18 hpi (depending on the dose used) after which infected cells got loose and as a result, entered the hemolymph circulation. Another possibility is the infection of hematopoietic tissues; the organ where all hemocytes are produced. Infection in this organ started at 18 to 24 hpi (depending on the dose used) and may have released WSSV-positive hemocytes in the general hemolymph circulation starting from 36 hpi onwards.

According to our results, we currently view the WSSV pathogenesis in *L. vannamei* as follows: shortly after primary replication at the sites of virus entry (epithelial cells in foregut and gills), WSSV crosses the basal lamina and reaches the underlying connective tissues and/or its associated haemal sinuses. Here, WSSV is transported in a cell-free form by hemolymph circulation to other target organs. The most susceptible target organs are either in close contact with hemolymph circulation or in direct contact with the exterior such as gills, foregut, integument and antennal gland.

Although no shrimp died at the time when the pathogenesis study was stopped, it was proposed that death of WSSV-infected shrimp probably occurs by the multiple dysfunction of organs such as gills, foregut, integument and antennal gland. These organs were highly affected by WSSV infection in *L. vannamei.* Such target organs are critical for the maintenance of the homeostasis of the animal since they perform gas exchange, transport and excretion of CO_2 and ammonia, the salt regulation and the control of the acid-base balance (Ahearn et al. 1999; Wheatley 1999).

Since WSSV is a very important pathogen and has profound economical consequences for aquaculture in tropical regions, we also assessed possible treatment options. In this thesis, we showed that the antiviral drug cidofovir significantly delayed mortality for a duration of 24 h compared to mock-treated shrimp, but it did not fully control infection. Cidofovir is successfully used to control infections caused by other double-stranded

DNA viruses such as herpesviruses, poxviruses, adenoviruses, polyomaviruses and papillomaviruses in both humans and other mammals (De Clerq 2002). It is believed that an antiviral product does not completely inhibit a viral infection in mammals, but only helps to control the infection until the specific immunity consisting of a humoral and a cellular component is activated.

In shrimp, the following possibilities can be formulated to explain the failure of cidofovir to control WSSV infection: (1) despite the high dose of cidofovir administered to the shrimp just after inoculation, only a small amount was taken up and/or stored by the shrimp cells. This could be improved by administering the drug well in advance to WSSV challenge or alternatively, by using the esterified form. (2) Shrimp cells may not efficiently convert cidofovir into its antiviral form. (3) The lack of a specific immunity (Söderhäll & Cerenius 1998) does not allow the shrimp to finally control viral replication. A repeated administration of the drug would probably only prolong the survival as long as the product is present. It is possible that once WSSV starts replicating in shrimp cells, no shrimp defense response is able to delay or stop the infection. These results minimize the potential use of cidofovir as a therapeutic agent against WSSV.

The use of drugs to treat infections in aquaculture systems in general is under debate. It may pose environmental risks such as pollution and toxic effects to other aquatic species and it also may accelerate the appearance of drug-resistant pathogens. It is necessary to search for new antiviral products which can be used to completely block viral infections in shrimp and which are not harming the ecosystem. Furthermore, these products should be suitable for oral delivery for a potential use in the field.

Previous studies have used bacterial or algal cell wall components (β-1,3 glucans, lipopolysaccharides [LPS], peptidoglycans [PG] or Fucoidan) to stimulate the defense response in shrimp and even to show an antiviral effect against WSSV (Itami et al. 1998; Takahashi et al. 2000; Chang et al. 2003; Chotigeat et al. 2004). In the present thesis we performed trials to determine whether the alga *Spirulina platensis* would be a suitable antiviral agent. The antiviral activity displayed *in vitro* by the blue green alga *S. platensis* (Lee et al. 2003) had no effect against a WSSV infection *in vivo*. It is possible that the oral inoculation procedure described in this thesis delivered a much higher virus titer to the challenged animals compared to the experimental challenge tests used from

previous studies. In these, the infectivity titers of the virus stock used to challenge shrimp were undefined. Furthermore, such challenge procedures often used different routes of inoculation such as feeding shrimp with infected tissues or immersing shrimp in water containing a certain volume of virus stock. These non-standardized conditions did not allow the estimation of how much infectious virus was taken up per individual animal. Influences of food uptake by the shrimp in one way or another may have important effects on infection and mortality.

Another variable which influenced the pathogenicity of WSSV was water temperature. This thesis confirmed that high water temperature (33°C) was effective to reduce mortality (70 to 100%) of shrimp inoculated with low or high infectious doses by intramuscular or oral routes. Furthermore, we showed that the protective effect not only occurred in shrimp maintained continuously at 33°C but also in those switched from 27° to 33°C immediately after WSSV inoculation. These results agree with previous studies where high water temperature effectively reduced the severity of clinical signs and mortality in WSSV-infected shrimp (Vidal et al. 2001, Granja et al. 2003).

Although the action of high water temperature was suggested to be either by an increase of a host defense response (apoptosis) or alternatively, as a result of a direct effect on viral replication, the exact mechanism is still unknown. In this thesis, some shrimp were also submitted to 33°C before WSSV inoculation. This treatment was done in order to evaluate whether the protective effect of high water temperature was related to a possible heat-shock protein (HSP) response.

The HSP are molecules found in many different organisms and are classified into well studied families (hsp40, hsp60, hsp70, hsp90 hsp110 and grp94) (Lee et al 1996; Dong et al. 2006). These proteins are involved in a number of functions including protein folding, translocation across membranes and assembly of oligomeric complexes. They are always expressed at low levels in normal cells thus indicating housekeeping activities. The over-expression of these molecules is triggered by several ‘stressful’ stimuli such as heat-shock, UV radiation, glucose deprivation and viral infection (Dong et al. 2006; Pérez-Vargas et al. 2006). The families hsp60, hsp70 and hsp90 are especially involved in innate immunity and immune response (Dong et al. 2006).

In mammalian cells, induction of hsp70 occurs by incubation at 41°C and is consistently maintained for up to 48 h. The induction of this protein in Vero cells inhibits the

production of a Japanese Sendai virus. Upon heat-shock, all the structural viral proteins are expressed, transported and assembled at the host cell membrane. Then, the over-expressed hsp70 interacts with the viral hemaglutinin-neuraminidase (HN) and prevents its integration to the plasma membrane. This results in a great reduction in infectivity and virus production (Hirayama et al. 2006 in press). In another study using mammalian cells, a soluble form of a cognate of hsp70 (hsc70) interacts with a rotavirus infecting human enteric cells and reduced its infectivity *in vitro* probably by inducing conformational changes (Pérez-Vargas et al. 2006).

Also in lower vertebrates, stress proteins have important effects on viral infection. In fish, infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that causes an acute disease in wild and hatchery-raised animals. Upon viral infection in a fish cell line, a novel 90 kDa stress protein was induced at a late stage of IHNV infection. This protein was found attached to surface proteins of IHNV particles in fish surviving a natural IHNV infection. It was proposed that such a protein is involved in the fish immune response to neutralize IHNV (Lee et al. 1996; Cho et al. 1997).

Recently, heat shock proteins (hsp86 and hsp70) were induced respectively in crayfish and shrimp by a short heat shock treatment (from 24°C to 32°C and maintained at high temperature for 2 h or from 29°C to 35°C and maintained in hyperthermia for 24 h) (Cimino et al. 2003; de la Vega et al. 2006). Shrimp chronically infected with a gill-associated virus (GAV) showed no differences in mortality compared to controls or shrimp submitted to osmotic or hypoxic stress. However, the heat shock induced a significantly higher level of hsp70 and produced significantly less amounts of GAV RNA copies (de la Vega et al. 2006).

In this thesis, all shrimp kept at 33°C during five days before WSSV challenge and switched to 27°C after inoculation, became infected and died at earlier times than control shrimp upon WSSV inoculation. This result suggests that a HSP response was not triggered by this high water temperature treatment or that it had no effect on the viral replication. The death of shrimp at a faster rate was probably due to the combination of stress by the inoculation of WSSV and the stress caused by the changes in water temperature. The interaction between environmental stress and microbial infection to induce mortality in aquatic organisms is well documented (Le Moullac et al. 1998; Lightner & Redman 1998; Le Moullac & Haffner 2000).

The present thesis clearly showed that high water temperature inhibited the expression of VP28 in cells of shrimp inoculated with WSSV. This result indicates that high water temperature negatively affects viral replication. It is most likely that a temperature-dependent enzymatic process which is critical for WSSV replication is impaired at 33°C. Inhibition of viral replication by high temperature has already been demonstrated for other viruses such as baculoviruses (Kobayashi et al. 1981), herpesviruses (Schildgen et al. 2005) and poxviruses (Luttge & Moyer 2005). The mechanism is mostly based on temperature-sensitive viral enzymes which are essential in the virus replication cycle. The application of high water temperature may be useful to gain insight in aspects of WSSV replication and its pathogenesis. Molecular studies aimed at the differential expression of WSSV genes in infected shrimp maintained at 33°C may give information on the mechanism of action of high water temperature on viral replication. Biochemical assays that evaluate the optimal temperatures for a number of enzymes involved in WSSV replication may identify the affected molecule(s).

Shrimp maintained at high water temperature just after WSSV inoculation were also protected from infection and mortality. This result indicates that shrimp already infected with WSSV can also survive when switched to a high water temperature. Further experiments are being performed to determine the efficacy of high water temperature as a therapeutic method in shrimp at different stages of WSSV infection. This is an effective, cheap and easy method to control WSSV infection compared to the lower efficacy and the difficulty of administering antiviral drugs to aquatic animals.

Raising water temperature to ≥ 32°C may have practical applications in many tropical shrimp farming countries where water temperature can reach 32°C or more for several months. Culturing shrimp during the season of high water temperature may reduce significantly the risk of a WSSV outbreak during the grow-out phase. However, such a practice would require a much closer monitoring of water quality parameters and probably also an increase in water exchange as high water temperature negatively influences other variables of water quality such as dissolved oxygen, pH and toxicity of ammonia and other metabolites.

This thesis has contributed to improve the reproducibility of experimental WSSV inoculation techniques. The way of working can now be used by other researchers not only for WSSV but also for other viruses and not only in shrimp but also other

crustaceans, defense against viruses or look for differences in virulence of virus isolates or to find genetic determinants for susceptibility of hosts. The protocols are very useful to study aspects of viral pathogenesis and to develop, test and compare methods for control.

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CHAPTER 7

SUMMARY/SAMENVATTING/RESUMEN

Summary

White spot syndrome virus (WSSV) has become the most important pathogenic agent in shrimp aquaculture. Under natural and culturing conditions, WSSV infection is thought to occur mainly via ingestion of WSSV-infected tissues. Signs of disease include reduced feeding and locomotion. Diseased shrimp gather around the pond shores. Mortalities up to 100% have been recorded within 10 days after the onset of disease. The severe impact of WSSV has prompted the search for effective control strategies. Before the start of the thesis, different control measures have been experimentally tested using non-standardized challenge procedures. This made it difficult to reproduce and to compare results between experiments.

In Chapter 1, an overview is given on the current knowledge of the shrimp *Litopenaeus vannamei* and WSSV.

Chapter 2 described the aims of this thesis. The main goals were: (1) to develop standardized WSSV inoculation procedures by intramuscular and oral routes; (2) to study the WSSV pathogenesis with emphasis on the portals of WSSV entry and the spread to other target organs and (3) to apply the standardized WSSV challenge models for the evaluation and comparison of control strategies against WSSV.

In Chapter 3, standardized WSSV inoculation models by intramuscular and oral routes were developed and used to evaluate strategies to control WSSV.

In part 3.1, *in vivo* titrations of a Thai WSSV stock were done by intramuscular route into shrimp of 60 to 135 days-old. The infectivity (shrimp infectious dose 50% endpoint [SID_{50} ml^{-1}]) and lethal (LD_{50} ml^{-1}) titers were recorded at 72, 96, 120 and 168 hours post inoculation (hpi). The SID_{50} ml^{-1} of the WSSV stock as determined by indirect immunofluorescence (IIF) and one-step PCR was between 0.2 to 1.0 log_{10} higher than the LD_{50} ml^{-1} at 72 or 96 hpi, respectively. The infectivity and lethal titers reached the same values from 120 hpi onwards. At 120 hpi the median infectivity and lethal titers of the WSSV stock by intramuscular route were $10^{6.6}$ SID_{50} ml^{-1} and $10^{6.6}$ LD_{50} ml^{-1} respectively.

Based on these infectivity titers, doses of WSSV (10^{-1}, 10^{0}, 10^{1}, 10^{2} and 10^{3} SID_{50} in 50 µl) were orally inoculated into five 80 days-old shrimp. The infectivity titers of the same WSSV stock when orally inoculated required 1 log_{10} more infectious virus to

reach the 50% endpoint. Therefore, the median infectivity titer by oral route was $10^{5.6}$ SID_{50} ml^{-1}. This result indicates the presence of barriers in the digestive tract of shrimp that hinder virus entry. The infectivity titers of the Thai WSSV strain obtained by intramuscular and oral routes were reproducible. The determination of the infectivity titers of the WSSV stock constitutes the first step towards standardization of experimental WSSV challenge tests.

In part 3.2, a minimal infectious dose of WSSV (10, 30 or 90 SID_{50}) that induced infection and mortality in all inoculated shrimp was determined. The evaluation was made by time of appearance of clinical signs, cumulative mortality and the median lethal time (LT_{50}). By intramuscular route, all shrimp inoculated with 30 and 90 SID_{50} died within 84 hpi while all shrimp inoculated with 10 SID_{50} died within 108 hpi. The LT_{50} of doses 10, 30 and 90 SID_{50} was 52, 50 and 49 hpi, respectively and were not significantly different ($P > 0.05$). All shrimp orally inoculated with doses 30 and 90 SID_{50} died within 108 hpi, while shrimp inoculated with 10 SID_{50} died within 120 hpi. The LT_{50} of doses 10, 30 and 90 SID_{50} was 65, 56 and 50 hpi respectively and all were statistically different, indicating a dose-dependent effect. These results indicate the presence of barriers in the digestive tract that may hinder virus entry and delayed mortality up to 24 h compared to intramuscular route. Such a route of inoculation may also mimic a more natural way of infection / transmission. This makes the oral route a preferable model for testing control strategies against WSSV. The dose of 10 SID_{50} showed more variability in cumulative mortality than the doses of 30 and 90 SID_{50}, suggesting that such a dose may be close to the 50% endpoint. Therefore, a dose of 30 SID_{50} was chosen as the standard dose for testing the efficacy of control strategies against WSSV.

In Chapter 4, the primary replication sites and the spread of WSSV to other target organs were determined. Shrimp orally inoculated with a low (30 SID_{50}) or a high (10000 SID_{50}) virus titer were collected at 0, 6, 12, 18, 24, 36, 48 and 60 hpi. In cell-free hemolymph, WSSV DNA was detected by one-step PCR and WSSV-infected hemocytes by IIF. In tissues, WSSV infection was analyzed by immunohistochemistry and histopathology. Epithelial cells in foregut and cells in gills were the primary replication sites at 18 hpi (low dose) or at 12 hpi (high dose). The antennal gland was a primary replication site only with a high dose. At 60 hpi, the most affected organs were

gills, hematopoietic tissues, foregut, lymphoid organ, integument and antennal gland. In these organs, histopathological lesions caused by WSSV were first detected at 36 hpi (low dose) or 24 hpi (high dose) and the proportion of cells with lesions increased with time. Heart, gonads, muscle, neuronal ganglia and nerve cord were little affected by WSSV infection and the epithelial cells of midgut were refractory. In hemolymph, WSSV DNA was first detected at 18 hpi (low dose) or 12 hpi (high dose). With the two doses, a few WSSV-positive cells were detected in circulating hemolymph starting from 36 hpi. These results suggest that WSSV spreads in *L. vannamei* to other target organs in cell-free form.

The oral inoculation induced primary WSSV replication in epithelial cells of foregut, cells in gills and only with a high dose, cells of the antennal gland. At the same time, WSSV DNA was detected in cell-free hemolymph. Critical organs for the maintenance of shrimp homeostasis such as gills, foregut, antennal gland and integument might lose structure and function due to WSSV infection inducing death.

In Chapter 5, the standardized inoculation models were used to evaluate the efficacy of antiviral products and the manipulation of water temperature against WSSV infection.

In part 5.1, cidofovir and a diet supplemented with *Spirulina platensis* were tested. The efficacy was evaluated by the proportion of WSSV-infected shrimp (IIF), cumulative mortality and LT_{50}.

The highest concentration of cidofovir (200 mg kg^{-1}) that showed no toxicity was used in two experiments using 20 shrimp orally inoculated with 30 SID_{50} of WSSV and immediately injected with cidofovir. A control group was mock-treated with PBS. Shrimp treated with cidofovir first showed signs of disease at 24-36 hpi but showed a delay in mortality (24 h) compared to the mock-treated shrimp. At the end of the experiments (120 hpi), cumulative mortality in the cidofovir-treated groups was 80-90% in contrast to 100% mortality in the mock-treated groups. The LT_{50} was statistically different ($P< 0.05$) between the cidofovir-treated and the mock-treated groups. All shrimp were WSSV-positive except for one survivor.

A group of 10 shrimp was fed with a diet supplemented with *S. platensis* during four days before the WSSV challenge. Another group of 10 shrimp was fed with a normal diet and used as control. Shrimp fed with *Spirulina* only showed a delay in onset of

signs of disease. Cumulative mortality was 100% at 84 hpi in the *Spirulina*-treated and the control group. All dead shrimp were WSSV-positive.

This study showed that cidofovir was more effective than a diet supplemented with *Spirulina* to delay/reduce shrimp mortality due to WSSV infection. However, none of these products may be successfully applied in the field.

In part 5.2 the standardized intramuscular and oral inoculation models were used to evaluate the effect of high water temperature (33°C) on WSSV infection. A low (30 SID_{50}) or a high (10000 SID_{50}) infectious dose was inoculated to groups of shrimp continuously maintained at 27°C, 30°C or 33°C, or switched from 33°C to 27°C or from 27°C to 33°C immediately after inoculation. Shrimp continuously maintained at 27°C or 30°C and those switched from 33°C to 27°C after WSSV inoculation first showed signs of disease at 24 hpi and first mortalities at 36 hpi. In these groups, cumulative mortality reached 100% and all dead shrimp were WSSV-positive by IIF. The LT_{50} showed that shrimp switched from 33°C to 27°C and those continuously maintained at 30°C died faster than shrimp kept continuously at 27°C.

In contrast, shrimp continuously maintained at 33°C or those kept at 33°C immediately after inoculation did not develop signs of disease, had a reduced cumulative mortality (0-30%) and all were WSSV-negative by IIF. The dead shrimp probably died due to cannibalism during molting. These results showed that the protective effect of high water temperature was independent of the route of inoculation and virus titer used.

In a time course, groups of shrimp maintained at 27°C were orally inoculated with a high dose of WSSV and afterwards kept at 27°C or switched to 33°C. One group maintained at 27°C and another kept at 33°C were simultaneously collected at 12 and 24 hpi. Immunohistochemistry (IHC) analysis revealed that shrimp maintained at 27°C displayed WSSV-positive cells at 12 hpi in foregut, gills and antennal gland. By 24 hpi, WSSV-positive cells were also found in integument, hematopoietic tissue and lymphoid organ. In contrast, all shrimp switched to 33°C after inoculation were WSSV-negative at 12 or 24 hpi.

This study showed that high water temperature effectively prevented onset of disease and significantly reduced mortality in WSSV-inoculated shrimp. The fact that high water temperature inhibited the expression of a structural protein such as VP28 suggests

that a critical enzymatic process may be impaired at an early stage of viral replication. These results suggest that high water temperature may not induce a heat-shock protein response by the host.

In this thesis, WSSV inoculation procedures by intramuscular and oral routes were developed and used to study the pathogenesis and to evaluate different strategies aimed to control WSSV. The quantitative comparison of the efficacy of experimental measures to control WSSV was done with the median lethal time (LT_{50}). Virological detection methods such as IIF/IHC were used to determine the infectivity titers of a WSSV stock and the relationship between infection and mortality.

The oral inoculation model was developed to mimic a common route of WSSV entry under culturing and natural conditions. With this method it was possible to successfully reproduce the primary replication sites described for *P. monodon* fed WSSV-infected tissues.

The antiviral cifodovir significantly delayed mortality in WSSV-infected shrimp and was more effective than a diet supplemented with *Spirulina*. High water temperature was very effective to prevent the onset disease and to reduce mortality of WSSV-infected shrimp regardless of the dose and/or route of inoculation used. This strategy is very attractive for field application in many tropical countries.

Samenvatting

White spot syndrome virus (WSSV) is uitgegroeid tot de belangrijkste ziekteverwekker bij gekweekte garnalen. Aangenomen wordt dat WSSV infecties onder natuurlijke en kweekomstandigheden vooral optreden door het opnemen van geïnfecteerde weefsels. De symptomen zijn onder andere verminderde eetlust en activiteit. Stervende garnalen worden opgemerkt aan de randen van de kweekvijvers. Binnen 10 dagen na de start van een uitbraak kan de mortaliteit reeds tot 100% oplopen. De ernstige impact van WSSV infecties heeft tot gevolg gehad dat een zoektocht naar effectieve controlestrategieën op gang is gekomen. Vóór deze thesis werd aangevat, werden bestrijdingsmethoden experimenteel getest met behulp van ongestandaardiseerde blootstellingstesten. Hierdoor waren de resultaten van experimenten moeilijk te reproduceren en te vergelijken.

In Hoofdstuk 1 wordt een overzicht gegeven van wat er momenteel bekend is over de garnaal *Litopenaeus vannamei* en het WSSV.

In Hoofdstuk 2 worden de doelstellingen van deze thesis beschreven: (1) de ontwikkeling van gestandaardiseerde WSSV inoculatieprocedures via intramusculaire en orale routes; (2) de bestudering van de pathogenese van WSSV, met nadruk op de toegangspoorten van het virus, de spreiding naar andere doelwitorganen en de uiteindelijke doodsoorzaak; (3) de toepassing van de gestandaardiseerde WSSV testmodellen om bestrijdingsstrategieën tegen WSSV te evalueren en te vergelijken.

In Hoofdstuk 3 werden gestandaardiseerde WSSV inoculatiemodellen via intramusculaire en orale routes ontwikkeld en gebruikt om strategieën ter controle van WSSV infecties te evalueren. In deel 3.1 werden *in vivo* titraties uitgevoerd met een Thais WSSV isolaat via de intramusculaire route bij garnalen van 60 tot 135 dagen oud. De infectiviteits- ("shrimp infectious dose 50% endpoint" [SID_{50} ml^{-1}]) en lethale (LD_{50} ml^{-1}) titers werden berekend op 72, 96, 120 en 168 uur na inoculatie (uni). Met behulp van indirecte immunofluorescentie (IIF) en 1-staps PCR werd de SID_{50} ml^{-1} van de WSSV stock bepaald. Deze bleek 0.2 en 1.0 $\log_{10}$ hoger te liggen dan de LD_{50} ml^{-1} op respectievelijk 72 en 96 uni. Op 120 uni bereikten de infectiviteits- en lethale titers gelijke waarden waarbij de mediane infectiviteitstiter van de WSSV stock via de intramusculaire route $10^{6.6}$ SID_{50} ml^{-1} was en de lethale titer $10^{6.6}$ LD_{50} ml^{-1}. Gebaseerd

op deze infectiviteitstiters werden dosissen van WSSV (10^{-1}, 10^{0}, 10^{1}, 10^{2} en 10^{3} SID_{50} in 50 µl) oraal geïnoculeerd bij telkens vijf garnalen van 80 dagen oud. Om het 50% eindpunt te bereiken na orale inoculatie van dezelfde WSSV stock was 1 log_{10} meer infectieus virus nodig, waardoor de gemiddelde infectiviteitstiter via de orale route $10^{5.6}$ SID_{50} ml^{-1} was. Dit resultaat duidt op het bestaan van barrières in het spijsverteringsstelsel van garnalen die het binnentreden van het virus kunnen hinderen. De bekomen infectiviteitstiters van het Thaise WSSV isolaat waren reproduceerbaar, zowel via de intramusculaire als de orale route. De bepaling van de infectiviteitstiters van de WSSV stock was de eerste stap in de standaardisatie van experimentele WSSV blootstellingstesten.

In deel 3.2 werd een minimale infectieuze dosis van WSSV (10, 30 en 90 SID_{50}) bepaald die infectie en sterfte veroorzaakte bij alle geïnoculeerde garnalen. De evaluatie gebeurde aan de hand van het tijdstip waarop de symptomen verschenen, de cumulatieve mortaliteit en de bepaling van de "median lethal time" (LT_{50}). Via de intramusculaire route stierven alle garnalen geïnoculeerd met 30 en 90 SID_{50} binnen 84 uni, terwijl alle garnalen geïnoculeerd met 10 SID_{50} stierven binnen 108 uni. De LT_{50} van de dosissen 10, 30 en 90 SID_{50} waren respektievelijk 52, 50 en 49 uni en niet significant verschillend van elkaar (P = 0.05). Alle garnalen, die oraal geïnoculeerd waren met dosissen van 30 en 90 SID_{50}, stierven binnen 108 uni, terwijl garnalen, oraal geïnoculeerd met 10 SID_{50}, binnen 120 uni stierven. De LT_{50} van de dosissen 10, 30 en 90 SID_{50} was respektievelijk 65, 56 en 50 uni en statistisch verschillend van elkaar, wat duidt op een dosis-afhankelijk effect. Deze resultaten wijzen op de aanwezigheid van barrières in het spijsverteringsstelsel die het intreden van het virus kunnen belemmeren zodat sterfte tot 24 uur later optreedt, vergeleken met de intramusculaire route. De orale inoculatie bootst een meer natuurlijke infectie- en overdrachtsweg na. Hierdoor is de orale route te verkiezen als model om bestrijdingsstrategieën tegen WSSV te testen. De cumulatieve mortaliteit vertoonde meer variatie bij een dosis van 10 SID_{50} dan bij de dosissen van 30 en 90 SID_{50}, wat suggereert dat deze dosis dicht bij het 50% eindpunt ligt. Daarom werd een dosis van 30 SID_{50} gekozen als de standaarddosis voor blootstellingstesten om de doeltreffendheid van bestrijdingsstrategieën tegen WSSV na te gaan.

In Hoofdstuk 4, werden de primaire vermeerderingsplaatsen en de spreiding van WSSV naar andere doelwitorganen bepaald. Garnalen werden oraal geïnoculeerd met een lage (30 SID_{50}) of een hoge (10000 SID_{50}) virustiter en gecollecteerd op 0, 6, 12, 18, 24, 36, 48 en 60 uni. Met behulp van een één-staps PCR werd WSSV DNA gedetecteerd in celvrije hemolymfe en werden WSSV-geïnfecteerde cellen in de hemolymfe aangetoond met IIF. In weefsels werd de WSSV infectie geanalyseerd aan de hand van immunohistochemie en histopathologie. Epitheelcellen in de voormaag en cellen in de kieuwen waren plaatsen van primaire vermeerdering op 18 uni (lage dosis) en op 12 uni (hoge dosis). De antenneklier was enkel een primaire vermeerderingsplaats bij een hoge dosis. Op 60 uni waren de meest getroffen organen: de kieuwen, het hematopoïetisch weefsel, de voormaag, het lymfoïde orgaan, het integument en de antenneklier. In deze organen werden de eerste histopathologische letsels waargenomen op 36 uni (lage dosis) of op 24 uni (hoge dosis) en de laesies namen toe in de tijd. Het hart, de gonaden, de spieren, de neuronale ganglia en de zenuwbanen waren amper aangetast door de WSSV infectie en de epitheelcellen van de middendarm waren refractair. In de hemolymfe werd voor het eerst WSSV DNA gedetecteerd op 18 uni (lage dosis) en op 12 uni (hoge dosis). Voor beide dossisen werd een klein aantal WSSV-positieve cellen in de circulerende hemolymfe gedetecteerd vanaf 36 uni. Deze resultaten suggereerden dat WSSV zich in *L. vannamei* naar andere doelwitorganen verspreidde in celvrije vorm.

De orale inoculatie induceerde primaire WSSV vermeerdering in de epitheliale cellen van de voormaag en in de cellen van de kieuwen, en enkel bij een hoge dosis ook in de antenneklier. Op datzelfde moment werd WSSV DNA gedetecteerd in de celvrije hemolymfe. Vervolgens verloren organen van vitaal belang voor de homeostasis in de garnaal, zoals de kieuwen, de voormaag, de antenneklier en het integument, tengevolge van de WSSV infectie aan structuur en functie waardoor het dier uiteindelijk zou sterven.

In Hoofdstuk 5, werden de gestandaardiseerde inoculatiemodellen toegepast om de doeltreffendheid van antivirale producten en een aangepaste watertemperatuur tegen WSSV te testen.

In deel 5.1 werden cidofovir en een dieet gesupplementeerd met *Spirulina platensis* onderzocht. De doeltreffendheid van de behandeling werd geëvalueerd aan de hand van

het aantal WSSV-geïnfecteerde garnalen (IIF), de cumulatieve mortaliteit en de LT_{50}.

De hoogste concentratie cidofovir (200 mg kg^{-1} MBW) die geen duidelijke tekens van toxiciteit vertoonde, werd gebruikt in twee experimenten waarbij 20 garnalen intramusculair ingespoten werden met cidofovir onmiddellijk nadat ze oraal geïnoculeerd waren met 30 SID_{50} WSSV. Een controlegroep werd geïnoculeerd met PBS. De garnalen behandeld met cidofovir vertoonden net zoals de onbehandelde controledieren de eerste ziektetekens op 24-36 uni. De eerste dode dieren in de met cidofovir behandelde groep werden echter 24 uur later aangetroffen dan in de controlegroep. Op het einde van de experimenten (120 uni) was de cumulatieve mortaliteit 80-90% in de cidofovir-behandelde groep, tegen 100 % in de controle groepen. De LT_{50} waarde van de cidofovir-behandelde groep was statistisch verschillend ($P< 0.05$) van die van de controlegroep. Alle dode en overlevende garnalen waren WSSV-positief behalve één overlevende.

Een groep van 10 garnalen werd gedurende vier dagen gevoederd met een dieet gesupplementeerd met *S. platensis*, voordat ze aan WSSV werden blootgesteld. Een controlegroep van 10 garnalen werd gevoederd met een normaal dieet. Garnalen die het dieet met *Spirulina* kregen, vertoonden een vertraging in het verschijnen van de symptomen. Cumulatieve mortaliteiten bereikten 100% op 84 uni zowel in de *Spirulina*-behandelde groep als de controle groep. Alle dode garnalen waren positief voor WSSV. Deze studie toonde aan dat cidofovir effectiever was dan een dieet gesupplementeerd met *Spirulina* om de mortaliteit bij garnalen ten gevolge van WSSV te vertragen/verminderen. Geen van beide producten zal echter met succes kunnen worden toegepast in het veld.

In deel 5.2 werden de gestandaardiseerde intramusculaire en orale inoculatie procedures toegepast om het effect van een hoge watertemperatuur (33°C) op WSSV infecties te evalueren. Een lage (30 SID_{50}) of een hoge (10000 SID_{50}) infectieuze dosis werd geïnoculeerd in groepen garnalen die continu bij 27°C, 30°C of 33°C werden gehouden, of waarbij de temperatuur onmiddellijk na de inoculatie werd gewijzigd van 33°C naar 27°C of van 27°C naar 33°C. Garnalen die continu bij 27°C of 33°C werden gehouden, of waarbij de temperatuur werd gewijzigd van 33°C naar 27°C na de WSSV inoculatie, vertoonden de eerste ziektetekens op 24 uni en de eerste mortaliteit op 36 uni. In deze groepen bereikte de cumulatieve mortaliteit 100% en alle dode garnalen waren positief

voor WSSV op IIF. De LT_{50} toonde aan dat garnalen die van 33°C naar 27°C werden gebracht of continu bij 30°C werden gehouden sneller stierven dan garnalen die continu bij 27°C werden gehouden. Hier staat tegenover dat garnalen die continu bij 33°C werden gehouden of naar 33°C werden gebracht onmiddellijk na inoculatie, geen symptomen ontwikkelden, een verminderde cumulatieve mortaliteit vertoonden (0-30%) en allen WSSV-negatief waren op IIF. De dode exemplaren stierven vermoedelijk tengevolge van kannibalisme tijdens het vervellen. Deze resultaten toonden aan dat het beschermend effect van een hoge watertemperatuur onafhankelijk was van de gebruikte inoculatieroute en virustiter.

In een opvolging in de tijd werden garnalen bij 27°C gehouden en oraal geïnoculeerd met een hoge dosis. Daarna werden zij voort bij 27°C gehouden of opgewarmd tot 33°C. Eén groep gehouden bij 27°C en een tweede bij 33°C werden tesamen verzameld op 12 uni en 24 uni. Met behulp van immunohistochemie (IHC) werden in de garnalen die bij 27°C werden gehouden de eerste positieve cellen aangetoond in de voormaag, de kieuwen en de antenneklier op 12 uni. Op 24 uni werden WSSV-positieve cellen ook gezien in andere doelwitorganen zoals het integument, het hematopoïetisch weefsel en het lymfoïde orgaan. Garnalen gehouden bij 33°C na de WSSV inoculatie vertoonden daarentegen geen enkele WSSV-positieve cel 12 of 24 uni.

Deze studie toonde aan dat een hoge watertemperatuur het optreden van de ziekte kon voorkomen en de sterfte bij WSSV-geïnoculeerde dieren significant kon verminderen. Het feit dat een hoge watertemperatuur de expressie van een structureel proteïne zoals VP28 inhibeert, suggereert dat een cruciaal proces belemmerd wordt in een vroeg stadium van de virusreplicatie. Deze resultaten suggereren dat een hoge watertemperatuur geen “heat-shock protein” respons opwekt in de gastheer.

In deze thesis werden gestandaardiseerde inoculatieprocedures voor WSSV via intramusculaire en orale routes ontwikkeld. De kwantitatieve vergelijking van enkele experimentele maatregelen tegen WSSV werd uitgevoerd aan de hand van de median lethal time (LT_{50}). Virologische detectietechnieken zoals IIF en IHC werden gebruikt om de infectiviteitstiters van een WSSV stock te berekenen. Verder werd het verband tussen de infectie en de mortaliteit bepaald. Met de ontwikkeling van een oraal inoculatiemodel kon de toegangsroute van WSSV die het meest voorkomt onder

natuurlijke en kweekcondities nagebootst worden. Gebruik makend van deze inoculatietechniek werden dezelfde primaire vermeerderingsplaatsen geïdentificeerd zoals reeds beschreven bij *P. monodon* die gevoederd waren met WSSV-geïnfecteerde weefsels: de kieuwen en de voormaag. Het antivirale effect van cidofovir vertraagde significant de sterfte bij met WSSV-geïnfecteerde garnalen en bleek effectiever te zijn dan een dieet gesupplementeerd met *Spirulina*. Een hoge watertemperatuur was erg doeltreffend om het onstaan van symptomen te voorkomen en deed in grote mate de sterfte afnemen bij garnalen geïnoculeerd met WSSV, ongeacht de dosis en/of de inoculatieroute. Deze strategie is erg aantrekkelijk voor toepassing in het veld in vele tropische landen.

Resumen

El virus de la mancha blanca (WSSV) es el agente viral más dañino para el cultivo de camarón. En condiciones naturales y de cultivo, la infección de WSSV ocurre principalmente por medio de la ingestión de tejidos infectados con el virus. Los signos de la enfermedad no son específicos e incluyen la reducción en las actividades alimenticia y locomotora. Los animales enfermos se aglomeran a las orillas de los estanques y mortalidad de hasta 100% ha sido reportada 10 dias después de la aparición de los signos clínicos.

El impacto negativo de WSSV ha propiciado la búsqueda de nuevos y efectivos métodos para controlar la enfermedad. Varias de estas medidas han sido evaluadas experimentalmente usando diferentes rutas de inoculación, especies de camarón y estadios de desarrollo. En estudios previos se hacían inoculaciones experimentales con dosis indefinidas de WSSV. Todos estos factores han hecho dificil la reproducibilidad y comparación de resultados entre experimentos.

En el Capítulo 1 se hizo una revisión actualizada del conocimiento sobre el camarón *Litopenaeus vannamei* y de WSSV.

En el Capítulo 2 se describieron los objetivos de esta tesis. Los objetivos principales fueron: (1) desarrollar procedimientos de inoculación estandarizados para WSSV por vía intramuscular y oral; (2) estudiar la patogénesis de WSSV con énfasis en los portales de entrada de WSSV, su dispersión a otros órganos blanco y (3) aplicar los modelos de inoculación descritos en (1) para la evaluación y comparación de la eficacia de métodos de control contra WSSV.

En el Capítulo 3, se desarrollaron los modelos de inoculación por vía intramuscular y oral. En la parte 3.1, titulaciones *in vivo* de un inóculo de WSSV fueron hechas por ruta intramuscular en camarones de 60 a 135 días de edad. El título de infectividad (dosis infecciosa en camarón al 50% [SID_{50} ml^{-1}]) y la dosis letal al 50% (LD_{50} ml^{-1}) fueron evaluadas a las 72, 96, 120 y 168 horas post inoculación (hpi). El título de infectividad determinado por inmunofluorescencia indirecta (IIF) y reacción en cadena de la polymerase (one-step PCR) fue entre 0.2 y 1.0 log_{10} mayor que el LD_{50} ml^{-1} a las 72 ó 96 hpi respectivamente. Los títulos de infectividad y letal alcanzaron el mismo valor a partir de las 120 hpi. A este tiempo, los valores medios de los títulos de infectividad y

letal del inóculo de WSSV fueron $10^{6.6}$ SID_{50} ml^{-1} y $10^{6.6}$ LD_{50} ml^{-1} respectivamente. Basados en el título de infectividad, distintas dosis de WSSV (10^{-1}, 10^{0}, 10^{1}, 10^{2} y 10^{3} SID_{50} en 50 µl) fueron inoculados oralmente en camarones de 80 días de edad. El mismo inóculo de WSSV requirió 1 log_{10} más cantidad del virus infectioso en comparación con la ruta intramuscular para alcanzar la dosis infecciosa al 50%. Por lo tanto, el título medio de infectividad por ruta oral fue $10^{5.6}$ SID_{50} ml^{-1}. Este resultado indica la presencia de barreras en el tracto digestivo del camarón que interfieren la entrada del virus. Los títulos de infectividad de WSSV fueron reproducibles tanto por la vía intramuscular como la oral. La determinación de dichos títulos de infectividad del inóculo de WSSV constituye el primer paso hacia la estandarización de modelos experimentales de infección.

En la parte 3.2, se determinó la mínima dosis infeccciosa de WSSV (10, 30 and 90 SID_{50} en 50 µl) que produjo infección y mortalidad en todos los camarones inoculados. La evaluación se hizo considerando el tiempo de aparición de signos clínicos, la mortalidad cumulativa y el tiempo letal medio (LT_{50}). Por ruta intramuscular, todos los camarones inoculados con las dosis 30 y 90 SID_{50} murieron hacia las 84 hpi, mientras que todos los camarones inoculados con la dosis de 10 SID_{50} murieron a las 108 hpi. El valor del LT_{50} para las dosis de 10, 30 y 90 SID_{50} fueron respectivamente de 52, 50 y 49 hpi y dichos valores no fueron significativamente differentes ($P > 0.05$).

Todos los camarones inoculados por vía oral con las dosis de 30 y 90 SID_{50} murieron hacia las 108 hpi, mientras que los camarones inoculados con la dosis de 10 SID_{50} murieron dentro de las 120 hpi. Los valores de LT_{50} de las dosis de 10, 30 and 90 SID_{50} fueron respectivamente 65, 56 and 50 hpi y cada uno de estos valores fueron estadísticamente distintos, lo cual indica un efecto dosis-dependiente. Estos resultados indican la presencia de barreras en el tracto digestivo que impiden la entrada del virus hacia el hospedero y retrasó la mortalidad en hasta 24 h en comparación con la ruta intramuscular. La inoculación por vía oral representó una forma más natural de infección / transmisión del virus. Esto hace de la inoculación oral un modelo más favorable para evaluar estrategias de control contra WSSV.

La dosis de 10 SID_{50} dió resultados más variables en mortalidad cumulativa que las dosis de 30 ó 90 SID_{50}, lo cual sugiere que tal dosis puede estar muy cerca del valor de infectividad al 50%. Por lo tanto, la dosis de 30 SID_{50} fue elegida como la dosis

estándar para los dos modelos de inoculación y para evaluar la eficacia de medidas de control contra WSSV.

En el Capítulo 4 se determinaron los sitios de replicación primaria de WSSV y el modo de dispersión viral a otros órganos blanco. Camarones inoculados oralmente con una dosis baja (30 SID_{50}) ó alta (10000 SID_{50}) fueron colectados a las 0, 6, 12, 18, 24, 36, 48 y 60 hpi. El DNA de WSSV fue detectado en la fracción de hemolinfa libre de células por PCR y en hemocitos circulantes, WSSV fue detectado por IIF. En tejidos de camarón, la infección de WSSV fue detectada por inmunohistoquímica (IHC) e histopatología.

Células epiteliales en la región anterior del tracto digestivo y células en branquias fueron los sitios de replicación primaria a las 18 hpi (dosis baja) ó a las 12 hpi (dosis alta). La glándula antenal fue un sitio de replicación primaria solamente con la dosis alta. A las 60 hpi, los órganos más afectados fueron branquias, tejidos hematopoiéticos, parte anterior del tracto digestivo, órgano linfoide, integumento y glándula antenal. En éstos, lesiones histopatológicas causadas por WSSV fueron detectadas por primera vez a las 36 hpi (dosis baja) ó 24 hpi (dosis alta) y la proporción de células con lesiones aumentó con el tiempo. Organos tales como el corazón, gónadas, músculo, ganglios y cordón nerviosos fueron muy poco afectados por WSSV y las células epiteliales de la región media del tracto digestivo fueron refractarias a la infección por WSSV.

En hemolinfa, el DNA de WSSV fue detectado por primera vez a las 18 hpi (dosis baja) ó a las 12 hpi (dosis alta). Con ambas dosis, solamente algunas células positivas para WSSV fueron detectadas en hemolinfa a partir de las 36 hpi. Estos resultados sugieren que WSSV se dispersó a otros órganos blanco en *L. vannamei* de una manera no asociada a células.

La técnica de inoculación oral indujo replicación primaria de WSSV en células epiteliales de la región anterior del tracto digestivo, branquias y solamente con una dosis alta, en células de la glándula antenal. Al mismo tiempo, el DNA viral fue detectado en hemolinfa en la fracción libre de células. Los órganos que resultan críticos para mantener el equilibrio fisiológico en el camarón son branquias, la región anterior del tracto digestivo, glándula antenal e integumento. Estos órganos podrían perder estructura y función debido a la infección por WSSV lo cual podría causar la muerte.

En el Capítulo 5 se evaluó la eficacia de productos antivirales y la manipulación de la temperatura del agua en el control de la infección de WSSV. En la parte 5.1, el efecto antiviral de cidofovir y de una dieta supplementada con *Spirulina platensis* fue determinada con el modelo de inoculación oral. La eficacia fue evaluada por la proporción de animales infectados con WSSV por medio de la técnica de IIF, la mortalidad cumulativa y el LT_{50}.

La concentración más alta de cidofovir (200 mg kg^{-1}) que no produjo signos de toxicidad fue usada en dos experimentos con 20 camarones inoculados oralmente con 30 SID_{50} de WSSV e inmediatamente después fueron inyectados intramuscularmente con cidofovir. Al mismo tiempo, un grupo control fue tratado con buffer salino de fosfatos (PBS) como placebo. Los camarones tratados con cidofovir mostraron los primeros signos de la enfermedad a las 24-36 hpi pero empezaron a morir solo 24 h más tarde que aquellos que recibieron el placebo. Al final de los experimentos (120 hpi), la mortalidad cumulativa fue de 80-90% en el grupo tratado con cidofovir, en contraste con los camarones del grupo placebo que alcanzaron el 100% de mortalidad. Los valores de LT_{50} fueron significativamente distintos ($P< 0.05$) entre los dos tratamientos. Tanto todos los camarones muertos como los sobrevivientes resultaron infectados con WSSV excepto por un sobreviviente tratado con cidofovir en el segundo experimento.

Un grupo de 10 camarones fue alimentado con una dieta suplementada con *S. platensis* durante cuatro días antes de la infección experimental con WSSV. Otro grupo de 10 camarones fue alimentado con una dieta comercial normal y sirvieron como control. Los camarones alimentados con la dieta de *Spirulina* solamente mostraron un retraso de 12 h en la aparición de signos clínicos. La mortalidad cumulativa alcanzó 100% a las 84 hpi tanto en el grupo tratado con *Spirulina* como en el control y todos los camarones muertos resultaron infectados con WSSV.

Estos estudios mostraron que cidofovir fue más efectivo que la dieta con *Spirulina* para retrasar o reducir la mortalidad del camarón debido a la infección de WSSV. No obstante, ninguno de estos productos parecen tener valor práctico en el campo.

En la parte 5.2, se usaron los modelos de inoculación intramuscular y oral para evaluar el efecto de la alta temperatura del agua (33°C ± 0.5°C) sobre una infección de WSSV. Una dosis infecciosa baja (30 SID_{50}) y una alta (10000 SID_{50}) fueron inoculadas a grupos de camarones mantenidos continuamente a 27°C, 30°C ó 33°C, ó en grupos de

camarón cambiados de 33°C a 27°C ó de 27°C a 33°C inmediatamente después de la inoculación con WSSV. Los camarones mantenidos continuamente a 27°C ó 30°C y aquellos cambiados de 33°C a 27°C inmediatamente después de la inoculación mostraron los primeros signos clínicos a las 24 hpi y las primeras mortalidades a las 36 hpi. En estos grupos, la mortalidad cumulativa alcanzó 100% y todos los camarones muertos estuvieron infectados con WSSV de acuerdo al análisis de IIF. El valor de LT_{50} mostró que los camarones que fueron cambiados de 33°C a 27°C y aquellos mantenidos continuamente a 30°C murieron más rápido que los camarones mantenidos continuamente a 27°C y sirvieron como controles.

En contraste, los camarones mantenidos continuamente a 33°C ó aquellos cambiados de 27°C a 33°C inmediatamente después de la inoculación con WSSV no desarrollaron signos de enfermedad, tuvieron una reducida mortalidad cumulativa (0-30%) y todos fueron negativos para WSSV usando IIF. Los camarones que murieron fueron probablemente devorados durante su muda. Estos resultados mostraron que el efecto protector de agua a alta temperatura fue independiente de la ruta de inoculación y la dosis viral infecciosa usadas.

En un curso de tiempo, grupos de camarón mantenidos a 27°C fueron inoculados oralmente con una dosis alta de WSSV y después fueron mantenidos a 27°C ó cambiados a 33°C. Un grupo de camarones mantenido a 27°C y otro cambiado a 33°C fueron colectados simultáneamente a las 12 y a las 24 hpi. El análisis por IHC reveló que los camarones a 27°C presentaron células positivas para WSSV a las 12 hpi en la parte anterior del tracto digestivo, branquias y glándula antenal. A las 24 hpi, células infectadas con WSSV se encontraron además en integumento, tejidos hematopoiéticos y órgano linfoide. En contraste, todos los camarones cambiados a 33°C después de la inoculación fueron negativos para WSSV en los dos tiempos de colecta.

Este estudio mostró que el agua a alta temperatura efectivamente previno el inicio de la enfermedad y redujo significativamente la mortalidad de camarones inoculados con WSSV. El hecho de que el agua a alta temperatura inhibiera la expresión de la proteína viral VP28 sugiere que un proceso enzimático crítico para la replicación viral es afectado. Estos resultados también sugieren que el tratamiento con agua a alta temperatura no induce una respuesta de proteínas de estrés (heat-shock) en el camarón.

En esta tesis, los procedimientos estandarizados de inoculación para WSSV por rutas intramuscular y oral fueron desarrollados y utilizados para estudiar la patogénesis y para evaluar diferentes productos y métodos dirigidos a controlar la infección de WSSV. La comparación cuantitativa de la eficacia de estos métodos experimentales fue hecha por medio del valor letal medio (LT_{50}). El uso de técnicas virológicas como IIF e IHC fueron útiles para la determinación de los títulos de infectividad de un inóculo de WSSV y también establecer la relación entre infección y mortalidad.

El modelo de inoculación oral fue desarrollado para simular una ruta común y natural de entrada de WSSV en el camarón bajo condiciones naturales y de cultivo. Con este método fue posible reproducir con éxito los sitios de replicación primaria descritos para *Penaeus monodon* alimentado con tejidos infectados con WSSV.

El producto antiviral cidofovir retrasó significativamente la mortalidad en camarones infectados con WSSV y fue mucho más efectivo que una dieta suplementada con *Spirulina*. El agua a alta temperatura fue muy efectiva para prevenir el inicio de enfermedad y para reducir la mortalidad de camarones infectados con WSSV de manera independiente a la ruta de inoculación o las dosis infecciosas utilizadas. El manejo de agua a alta temperatura puede ser un método muy atractivo y efectivo para el control de WSSV en granjas e instalaciones de acuacultura de camarón en varios países tropicales.

Curriculum vitae

Cesar Marcial Escobedo Bonilla was born in Mexico City on May 6th, 1968. In 1990, he obtained the Bachelor of Science in Biology at Universidad Nacional Autónoma de México Campus Iztacala.

From 1991 to 1993, he collaborated at the Departament of Ecological Studies at the Mexican Institute of Oil describing the benthic communities of crustacean peracarids in the continental shelf of the Gulf of Mexico and the Campeche Sound.

From 1993 to 1994, he worked at Charoen Pokphand Aquaculture Mexico, performing technology transfer and consultant in semi-intensive shrimp culture to shrimp farms in Sinaloa and Nayarit, Mexico.

From 1995 to 1996, he worked as teacher of general chemistry and biology (University level) at Unidad Profesional Interdisciplinaria y Biotecnologia (UPIBI) in Mexico City.

From 1996 to 1999, he enrolled in a MSc program in aquaculture with major in shrimp pathology. His MSc thesis was entitled: "Susceptibility of wild and laboratory batches of shrimp *Litopenaeus vannamei* (BOONE 1931) and *Litopenaeus stylirostris* (STIMPSON 1874) to Taura syndrome virus (TSV) and its evaluation by histopathology and *in situ* hybridization".

From 2000 to 2001, he collaborated at the Laboratory of Virology, Department of Plant Sciences, Wageningen University, The Netherlands, working on the cloning of the white spot syndrome virus (WSSV) genes encoding structural proteins and their expression in bacterial cells.

In 2002, he started a joint PhD program between the Laboratory of Virology, Faculty of Veterinary Medicine and the Laboratory of Aquaculture and Artemia Reference Center (ARC), Faculty of Bioscience Engineering, Gent University, on shrimp viral diseases which resulted in the present thesis.

Mr. Escobedo-Bonilla is author or co-author of five articles published in international aquaculture journals and is member of scientific societies related to crustacean ecology and aquaculture. He has participated in several international meetings and symposia concerning crustacean ecology and shrimp viral diseases in aquaculture.

PUBLICATIONS

Publications in international peer-reviewed journals:

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