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Differences in virulence between white spot syndrome virus (WSSV) isolates and testing of some control strategies in WSSV infected shrimp

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List of abbreviations

%	Percentage
°C	Celsius temperature scale
µm	micrometer
ABV	Angelfish birnavirus
ALF	Antipolysaccharide factor
AMP	Antimicrobial peptide
ARC	Laboratory of Aquaculture and *Artemia* Reference Center
BMNV	Baculovirus midgut gland necrosis virus
BP	Baculovirus penaei
BTC	Belgian Technical Co-operation
DAB	3,3 diaminobenzidine
DABCO	1, 4-diaza-bicyclo[2.2.2]octan
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
dpi	days post inoculation
dsRNA	double stranded RNA
e.g.	exempli gratia (for example)
ELISA	Enzyme linked immunosorbent assay
et. al.	et alii (and others)
FAO	Food and Agriculture Organization
fg	Femtogram
FITC	Fluorescein isothiocyanate
FV3	Frog virus 3
GAV	Gill associated virus
h	hour
HD	High dose = 10000 SID_{50}
HIV-1	Human immunodeficiency virus type 1
hpi	Hours post inoculation
HPMPC	(s)-1-(3-hydroxy-2-phosphonylmethoxy propyl)cytosine

HPMPCpp	(s)-1-(3-hydroxy-2-phosphonylmethoxy propyl)cytosine diphosphate
HPV	Hepatopancreatic parvovirus
HSP	Heat shock protein
HSV-1	Herpes simplex virus type 1
Ig	Immunoglobulin
IHC	Immunohistochemistry
IHHNV	Infectious hypodermal and hematopoietic necrosis virus
IIF	Indirect immunoflourescence
IMNV	Infectious myonecrosis virus
IPNV	Infectious pancreatic necrosis virus
KHV	Koi herpesvirus
L	Litre
LAMP	Loop mediated isothermal amplification
LD	Low dose = 30 SID_{50}
LDV	Lymphocystis virus
LMBV	Largemouth bass virus
LOV	Lymphoid organ virus
LOVV	Lymphoid organ vacuolization virus
LPS	Lipopolysaccharide
LPV	Lymphoidal parvo-like virus
LT_{100}	Time to reach 100% mortality
LT_{50}	Time to reach 50% mortality
MAb	Monoclonal antibody
MBV	Monodon baculovirus
MBW	Mean body weight
MI	Mock inoculated
min	minute
MOV	Mourilyan virus
MSGS	Monodon slow growth syndrome
MT	Metric tons
NHP	Necrotizing hepatopancreatitis

NPV	Nucleopolyhedrovirus
PAb	Polyclonal antibody
PBS	Phosphate-buffered solution
PCR	Polymerase chain reaction
PL	Post larvae
ppm	parts per million
REO	Reo-like virus
RIA-2	Research Institute for Aquaculture N^{O} 2 in Vietnam
RNA	Riboxynucleic acid
RPS	Rhabdovirus of penaeid shrimp
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SGIV	Singapore grouper iridovirus
SID_{50}	Shrimp infectious dose with 50% endpoint
siRNA	Short interfering RNA
SMV	Spawner-isolated mortality virus
SPF	Specific pathogen free
SPR	Specific pathogen resistant
TEM	Transmission electron microscopy
ThRV	Threadfin reovirus
TLR	Toll like receptor
TPS	Trehalose-phosphate synthase
ts	Temperature-sensitive
TSV	Taura syndrome virus
USA	United States of America
UV	Ultra-violet
VP	Viral protein
WSS	White spot syndrome
WSSV	White spot syndrome virus
YHV	Yellow head virus

CHAPTER 1

General introduction

1.1 **Shrimp aquaculture**

In 2005, global marine shrimp production reached about 4 million metric tons. Shrimp aquaculture contributed to two third of this production (Figure 1). It is one of the fastest growing agricultural industries, with more than 10% average growth in the last decade (FAO, 2005).

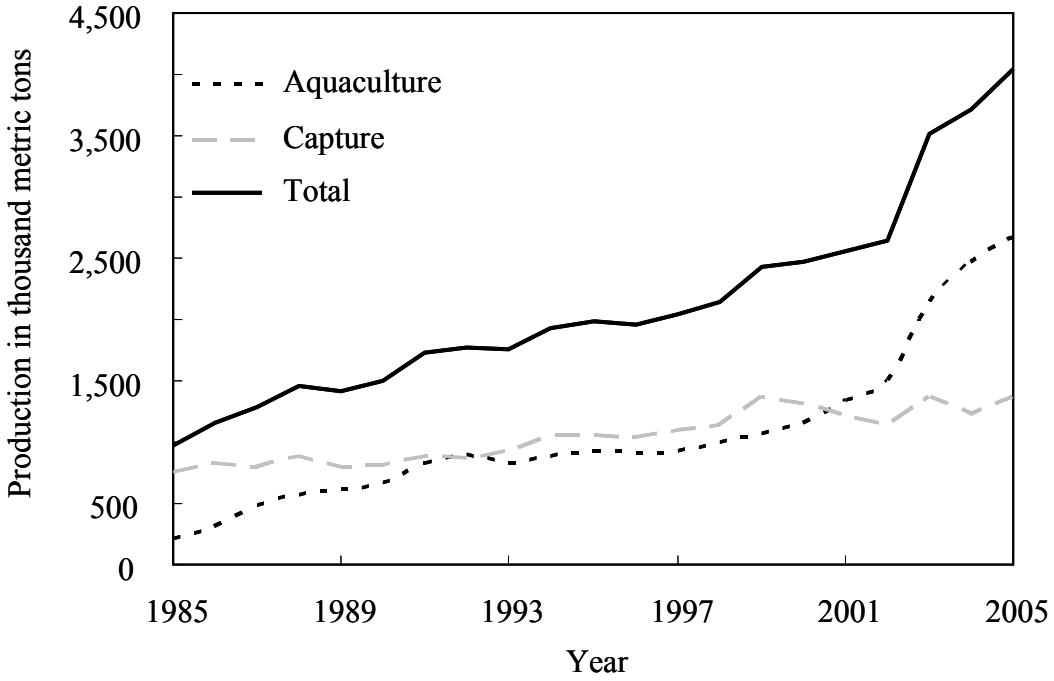

Figure 1. Trends in penaeid shrimp aquaculture production of the world compared to capture (FAO, 2005).

This rapid increase in cultured shrimp production was achieved by geographical expansion and technological advancements in reproduction in captivity, larval rearing, artificial diet and intensification in growout systems. Shrimp growout systems around the world are very diverse. They are generally divided into extensive, semi-intensive, intensive and ultra-intensive depending on characteristics including production per unit, stocking density, type and amount of feed used, percentage of water exchange, aeration, pond size, pond shape and water depth (Fast, 1992). Culture intensity, production rate and application of advanced technologies in growout, greatly vary in between shrimp farming countries. More than fifty countries, mainly from Asia, North, Central and South America, were involved in shrimp farming in 2005. China, Thailand, Vietnam and Indonesia produced 75% of the

aquaculture production (FAO, 2005). The major shrimp producing countries of the world are listed in Table 1.

Table 1. Major penaeid shrimp producing countries of Asia and American continents in 2005

Asia	Production in metric tons (MT)	Americas	Production in MT
China	1024049	Mexico	72279
Thailand	375320	Brazil	63134
Vietnam	327200	Ecuador	56300
Indonesia	279375	Colombia	18040
India	143170	Venezuela	16500
Bangladesh	63052	Belize	10433
Myanmar	48640	Peru	9809
Philippines	39909	Nicaragua	9633
Malaysia	33364	Panama	7098
Taiwan	14760	Costarica	5714

Source: FAO (2005)

Penaeus vannamei is the most important shrimp species in terms of aquaculture production (Figure 2). The other important species are *P. monodon*, *P. chinensis*, *P. merguiensis*, *P. japonicus* and *P. indicus*. *P. vannamei* is naturally present along the pacific coast of Central and South America (Holthuis, 1980). It was originally cultured in North, Central and South American countries. At the end of the 1970s, this species was introduced in Asia (Briggs et al., 2004). In 2005, 83% of global farm raised *P. vannamei* was produced in Asia (FAO, 2005).

P. vannamei has several advantages compared to other cultured species. These include the availability of specific pathogen free (SPF) and specific pathogen resistant (SPR) strains, a higher growth rate, suitability to higher stocking density, tolerance to a wider range of temperature and salinity, a lower protein requirement in the diet, easier to breed and higher

survival in larval rearing (Briggs et al., 2004). These aspects could explain the increasing preference to culture this species.

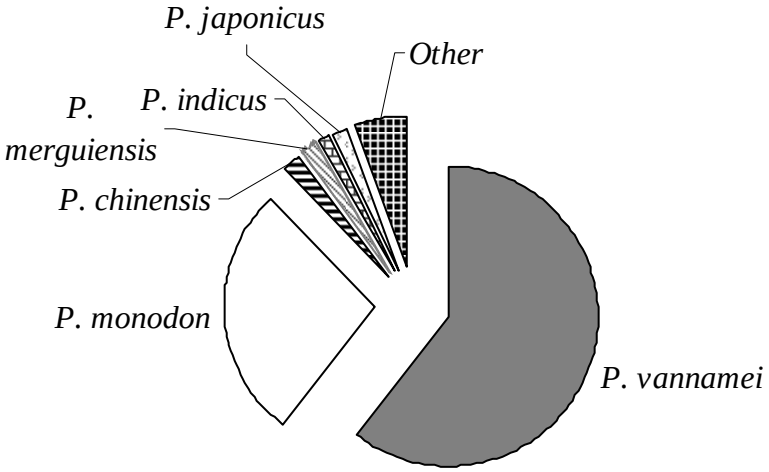

Figure 2. Proportion of species in total penaeid aquaculture production (FAO, 2005)

1.2 **Infectious shrimp diseases**

Diseases are one of the major constraints for the sustainable increase of shrimp production. Shrimp diseases can be divided into non infectious and infectious in origin (Lightner and Redman, 1998). Infectious diseases are caused by viruses, bacteria, fungi and parasites. Biological factors such as microbial flora present in the pond play a role on the susceptibility of shrimp to pathogens. Proper management of the microbial flora is done by biosecurity measures, aeration, reduction or elimination of pathogens and their carriers, application of probiotics, sludge management, waste treatment, reduction of the amount of water exchange and treatment of the incoming water. These are all important issues in prevention of shrimp disease (Horowitz and Horowitz, 2001).

Viral diseases - Viruses are considered to be the most important pathogens in shrimp. Different life stages of shrimp may be susceptible to certain viral infections causing mortality, slow growth and deformations. More than 20 viruses have been reported as pathogenic to shrimp. Table 2 gives an overview of the most important viral diseases.

Table 2. Viral pathogens of penaeid shrimp

Family	Virus
DNA virus	
Parvoviridae	Infectious hypodermal and hematopoeitic necrosis virus (IHHNV)[1]
	Hepatopancreatic parvovirus (HPV)[1]
	Spawner-isolated mortality virus (SMV)[2]
	Lymphoidal parvo-like virus (LPV)[1]
Baculoviridae	Baculovirus penaei (BP)[1]
	Monodon baculovirus (MBV)[1]
	Baculovirus midgut gland necrosis virus (BMNV)[1]
	Type C baculovirus of *Penaeus monodon*[3]
	Hemocyte infecting non-occluded baculo-like virus[4]
Iridovidae	Shrimp iridovirus (IRIDO)[5,6]
Nimaviridae	White spot syndrome virus (WSSV)[7]
RNA Virus	
Picornaviridae	Taura syndrome virus (TSV)[1]
Roniviridae	Yellow head virus (YHV)[8]
	Gill associated virus (GAV)[7]
	Lymphoid organ virus (LOV)9
Reoviridae	Reo-like virus (REO) type II and IV[1]
Rhabdoviridae	Rhabdovirus of penaeid shrimp (RPS)[1]
Togaviridae	Lymphoid organ vacuolization virus (LOVV)[1]
Totiviridae	Infectious myonecrosis virus (IMNV)[10]
Bunyaviridae	Mourilyan virus (MOV)[11]
unclassified	Monodon slow growth syndrome (MSGS)[12]

[1]Lightner, 1996; [2]Owens et al., 1998; [3]Chang et al., 1993, [4]Owens, 1993; [5]Lightner and Redman, 1993; [6]Tang et al., 2007; [7]Mayo, 2002; [8]Soowannayan et al., 2003; [9]Spann et al., 1995, [10]Tang et al., 2005; [11]Cowley et al., 2005; [12]Sritunyalucksana et al., 2006b

Bacterial diseases - Bacteria involved in shrimp disease can be pathogenic or opportunistic. Under unfavorable environmental circumstances for shrimp, opportunistic bacteria may cause disease. Bacterial infections in shrimp may cause mortality, cuticular lesions, necrosis, opacity of muscle, discoloration of gills, slow growth, loose cuticle, white gut, lethargia and reduced feed uptake. Major bacterial diseases in shrimp are vibriosis, filamentous bacterial disease, necrotizing hepatopancreatitis, mycobacteriosis, chitinolytic bacterial shell disease and rickettsial infection, (Lightner, 1996, Horowitz and Horowitz, 2001; Nunan et al., 2005; Goarant et al., 2006; Jayasree et al., 2006). Vibriosis is caused by *Vibrio alginolyticus*, *V. anguillarum*, *V. campbelli*, *V. damsela*, *V. harveyi*, *V. parahaemolyticus*, *V. penaeicida*, *V. vulnificus*, *V. nereis*, *V. tubiashi*, *V. fluvialis, V. splendidus, V. nigripulchritudo*. The causative agents of filamentous bacterial disease are *Leucothrix mucor*, *Thiothrix* sp., *Flexibacter* sp., *Cytophaga.* The pathogen responsible for necrotizing hepatopancreatitis is an alpha proteobacterium. Mycobacteriosis is due to *Mycobacterium marinum*, *Mycobacterium fortuitum* and other *Mycobacterium* sp. The chitinolytic bacterial shell diseases are caused by *Benekea, Pseudomonas, Aeromonas*, *Spirillum* and rickettsial infection by *Rickettsia* like organisms. Other gram positive bacteria such as *Aerococcus*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Staphylococcus* and gram negative bacteria *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, *Chromobacterium, Spiroplasma penaei* sp. have also been described as the cause of disease in shrimp.

Fungal diseases - Fungal infections may also cause disease mainly in larval stages of shrimp in hatcheries. The major pathogenic species are *Lagenidium callinectes*, *L. marina*, *Sirolpidium* spp., *Phythium* spp., *Leptolegnia marina*, *Haliphthoros milfordensis*, *Fusarium solani*, *F. moniliformae* and *F. incarnatum*. (Lightner, 1996; Alday and Felgel, 1999; Khoa et al., 2004).

Parasitic diseases - The diseases called cotton shrimp, gregarine disease and black gill/ brown gill disease are caused by infections with (i) protozoa - *Zoothamnium*, *Epistylis*, *Vorticella*, *Anophrys*, *Acineta* sp., *Lagenophrys*, *Ephelota,* (ii) ciliates - *Paranophrys* spp., *Parauronema* sp., (iii) flagellates - *Leptomonas* sp., (iv) annelid worms - *Nematopsis* spp., *N. litopenaeus*, *Paraphioidina scolecoide*, *Cephalobolus litopenaeus*, *C. petiti*,

Cephaloidophoridae stenai, (v) microsporidian - *Ameson* sp., *Agmasoma* sp., *Pleistophora* sp., *Microspridium* sp., and (vi) haplosporidian (Lightner, 1996; Alday and Flegel, 1999; Gopalakrishnan and Parida, 2005).

Overall, pathogens are divided into three categories depending on the potential impact. The viruses WSSV, YHV, GAV, LOV, TSV, IHHNV, SMV are listed in the category 1 (C-1); the viruses BP, MBV, BMN, HPV, the bacteria - α-proteobacterium and the protozoa microsporidians and haplosporidians in category 2 (C-2) and parasites causing gregarines in category 3 (C-3). C-1 pathogens may cause catastrophic losses of more than one species of shrimp. C-2 pathogens are less but still serious, while C-3 pathogens have minimal effects (Lightner, 2005). Other bacteria such as *Vibrio* sp. were not included in the list because of their availability in normal microflora of shrimp and unavailability of a diagnostic kit to distinguish pathogenic isolates from harmless isolates (D.V. Lightner, personal communication).

Shrimp diseases have caused significant losses of production and jobs, reduced earning, export restrictions, failure and closing of business and decreased confidence of consumers (Bondad-Reantaso et al., 2005). WSSV is considered as a serious pathogen. It affected shrimp producing countries of Asia -Bangladesh, Cambodia, China, India, Indonesia, Iran, Japan, Korea, Malaysia, Myanmar, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam- and North, Central and South America -Belize, Brazil, Columbia, Ecuador, Guatemala, Hondurus, Mexico, Nicaragua, Panama, Peru and United states- (Bondad-Reantaso et al., 2005; Lightner, 1996; Rosenberry, 2002; WB/NACA/WWF/FAO, 2001; FAO/NACA, 2003). WSS outbreaks in China in 1992 (Feigon, 2000), Thailand in 1995 to 1997 (Flegel, 2006) and Ecuador in 1999 (Rodríguez et al., 2003) caused losses in production with thousands of metric tons, jobs (thousands) and export with millions of dollars.

1.3 **White spot syndrome virus (WSSV) - a review**

1.3.1 **Virus**

WSSV is an enveloped, double stranded DNA virus, ovoid to bacilliform in shape with a tail like extension at one end (van Hulten et al., 2001a; Yang et al., 2001). A schematic diagram of the WSS virion is shown in Figure 3. The virus is the only member of the family *Nimaviridae*, genus *Whispovirus* (Mayo et al., 2002). WSSV is pathogenic to at least 78 species, mainly to decapod crustaceans including marine and freshwater shrimp, crab, crayfish and lobsters (Lightner, 1996; Flegel, 2006). The first outbreak due to WSSV was reported in shrimp farms in Taiwan in 1992 (Chou et al., 1995) followed by other shrimp farming countries of South East Asia, Middle East, North, Central and South America (Lightner, 1996; Rossenberry, 2002; Rodríguez et al., 2003; Flegel, 2006).

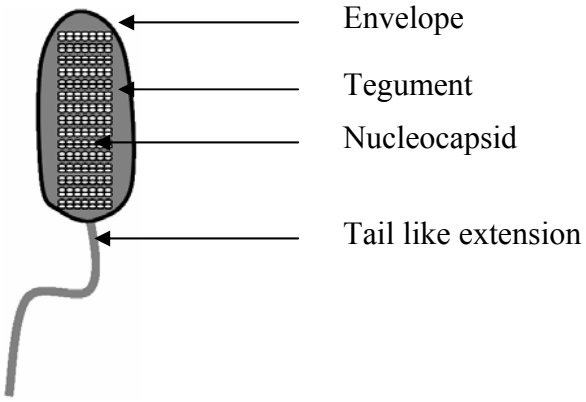

Figure 3. Schematic diagram of WSSV

The genome of different WSSV isolates ranges from 292 kbp to 307 kbp in size (van Hulten et al., 2001a; Yang et al., 2001; Chen et al., 2002). The virions are 70-138 nm x 240-340 nm in diameter. It contains a rod shaped nucleocapsid of 70-90 x 200-350 nm (Kasornchandra et al., 1998; Wang, Q. et al., 2000a). Genomic deletions were observed among isolates from different geographical areas (1 to 13 kb) (Marks et al., 2004) and from different host species of the same area (Lan et al., 2002). High variations in 54 bp DNA repeats were found in the samples from different regions in Thailand (Wongteerasupaya et

al., 2003) and the intensity of hybridization signals varied between isolates by dot blot hybridization and some samples even failed to hybridize with some of the probes. This indicated the existence of WSSV mutants (Lo et al., 1999).

WSSV is inactivated by various physical and chemical treatments including heat treatment at 55°C for 90 min, 70°C for 10 min, desiccation in filter paper within 3 h at 26°C, very acidic pH 1 for 10 min, pH 3 for 1 h, very alkaline pH 12 for 10 min, UV irradiation at 9 x 10^5 µW s/cm^2 for 60 min, different concentrations of disinfectants - ozone 0.5 and 0.8 µg/ml, formalin 200 parts per million (ppm), 25% sodium chloride within 24 h, chloroform within 15 min (Chang et al., 1998; Nakano et al., 1998; Balasubramanian et al., 2006). The effective concentrations of sodium hypochlorite, povidone iodine, benzalkonium chloride were between 75 - 200 ppm (Chang et al., 1998; Balasubramanian et al., 2006).

More than 50 structural proteins and one non structural protein VP9 (Liu, Y et al., 2006) have been detected in WSSV up till now. They were named according to the estimated molecular weights of the protein bands in SDS-PAGE or the number of amino acids. Proteins located in the envelope are: VP12, VP19, VP22, VP24, VP28, VP31, VP36B, VP38A, VP39, VP41, VP41A, VP41B, VP51B, VP52A, VP52B, VP53, VP53A, VP68, VP110, VP124, VP150, VP187, VP281, VP292, VP466 (Van Hulten et al., 2000; van Hulten et al., 2001b; Huang et al., 2002a; Huang et al., 2002b; van Hulten et al., 2002; Zhang et al., 2002; Tsai et al., 2004; Yi et al., 2004; Wu et al., 2005; Zhu et al., 2005; Li, H et al., 2006; Li, L.J. et al., 2006; Li, L et al., 2006, Tsai et al., 2006; Xie and Yang, 2006; Xie et al., 2006; Zhu et al., 2006), in the nuclocapsid: VP15, VP35, VP51C, VP60B, VP388, VP664 (van Hulten et al., 2002; Tsai et al., 2004; Leu J.H. et al., 2005; Witteveldt et al., 2005; Tsai et al., 2006; Xiao et al., 2006) and in the tegument: VP36A, VP39A, VP95 (Tsai et al., 2006). Locations of other proteins are not known. The functions of most of these proteins have not been fully elucidated. VP15 appears to be a DNA binding protein (Witteveldt et al., 2005). Neutralization assays suggested that envelope proteins VP24, VP28, VP31, VP36B, VP68, VP76, VP281 and VP466 are involved in early stages of WSSV replication (van Hulten et al., 2001b; Huang et al., 2005; Li, L et al., 2005; Wu et al., 2005; Li, H. et al., 2006; Li, L.J et al., 2006; Xie and Yang, 2006). VP28 is involved in attachment and penetration into cells (Yi et al., 2004) and systemic infection (van Hulten et al., 2001b; Wu et al., 2005). Primers

designed against VP28 gene or antibodies produced against VP28 (Poulos et al., 2001) were found to be suitable to detect different isolates (Musthaq et al., 2006).

1.3.2 **Pathogenesis and virulence**

Pathogenicity describes the ability of a pathogen to cause disease; and virulence is the degree of pathogenicity within a group or species (Shapiro-Ilan et al., 2005). Virulence of a pathogen can be measured by the time of onset of disease (clinical signs), onset of mortality, time to reach cumulative mortality 100%, median lethal time (LT_{50}) and severity of infection in tissues. Under experimental conditions, intramuscular or oral inoculation of the virus, immersion in viral suspension, feeding of infected tissue or cohabitation with infected animals cause infection in shrimp at post larval stage onwards (Chou et al., 1998; Kanchanaphum et al., 1998; Prior et al., 2003; Yoganandhan et al., 2003a; Leonardo et al., 2005; Escobedo-Bonilla et al., 2006). WSSV infection could not be induced in the early larval stages of *P. monodon* (nauplii, zoea, mysis) by immersion and oral challenge (Yoganandhan et al., 2003a). Apparently, shrimp become susceptible to infection from PL 6 (Venegas et al., 1999), PL 10 (Flegel, 2007) or PL 30 onwards (Pérez et al., 2005). The different findings in these studies could be due to shrimp species, inoculation procedure, infectious titer used, WSSV isolates or detection limit. Susceptibility differences for WSSV between life stages of a host and between host species were indicated in earlier studies (Momoyama et al., 1999; Wang, Q. et al., 1999).

WSSV replication occurs in the nucleus of the cells (Wongteerasupaya et al., 1995; Durand et al., 1997; Wang, Y. et al., 1999). A recent study showed that the cell surface molecule integrin is involved in WSSV infection, which might serve as a cellular receptor (Li, D-F. et al., 2007). WSSV infections have been detected in hemolymph, gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ, antennal glands, connective tissues, muscle tissues, hepatopancreas, heart, midgut, hindgut, nervous tissues, compound eyes, eye stalks, pleopods, pereiopods, testes and ovaries of naturally and experimentally infected shrimp (Wongteerasupaya et al., 1995; Chang et al., 1996; Lo et al., 1997; Sahul Hameed et al., 1998; Rajan et al., 2000; Yoganandhan et al., 2003b; Escobedo-

Bonilla et al., 2007). Quantitative pathogenic analyses suggest that the major target tissues for replication are gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ and antennal glands (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007). Major target tissues for WSSV replication in shrimp are shown in Figure 4. Hepatopancreas and heart were infected only in the connective tissues (Chang et al., 1996; Lo et al., 1997). Infected cells could not be found in the midgut cecum (Sahul Hameed et al., 1998; Escobedo-Bonilla et al., 2007). At a terminal stage of infection, the epithelial cells, hematopoietic tissues, tubules of antennal gland become degenerative and lysed (Chang et al., 1996; Lo et al., 1997). The route of WSSV entry and spreading mechanism among the tissues has recently been shown by Escobedo-Bonilla et al., (2007). Gills and cuticular epithelium of foregut in *P. vannamei* are portals of entry after oral inoculation of WSSV. After primary replication in these tissues, the virus crosses the basal membrane and reaches the associated hemal sinuses. Through hemolymph circulation, the virus is spread to internal organs where it causes a new wave of infections.

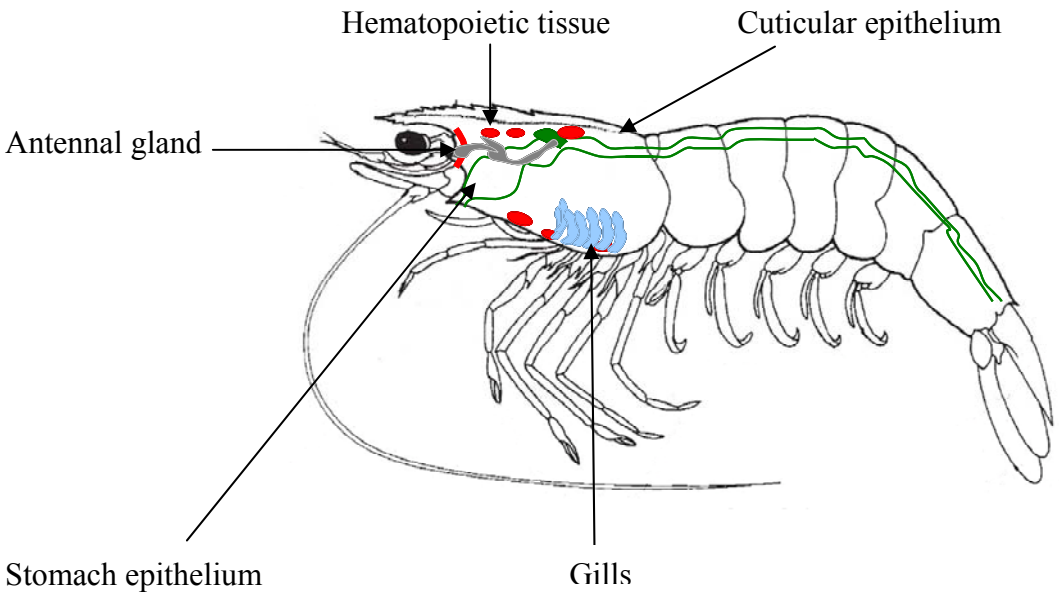

Figure 4. Major target tissues of WSSV replication in shrimp

Natural outbreaks of WSSV are categorized into peracute, acute to subacute and chronic forms, where mortality occurs within 2-3 days, 7-10 days and 15-28 days, respectively (Sudha et al., 1998). Differences in virulence between WSSV isolates have been

examined on the basis of time to reach a cumulative mortality of 50% (LT_{50}) and 100% (LT_{100}) (Wang, Q. et al., 1999; Lan et al., 2002; Marks et al., 2005). In those studies, an unknown amount of infectious virus was used for inoculation. Different methods (feeding infected tissue or intramuscular inoculation) were used, different species were inoculated (*P. vannamei*, *P. monodon*, *Procambarus clarkii*). Furthermore, the experiments were not repeated. Two studies have reported opposite findings over the relationship of genome size with virulence of the strains. Lan et al., (2002) found that in cray fish *P. clarkii* WSSV containing a 305 kb genome gave 100% mortality earlier than WSSV with a 4.8 kb deletion. Marks et al., (2005) found that *P. monodon* infected with WSSV containing the smallest genome (293 kb) reached 100% mortality at 8 days post inoculation (dpi) (LT_{50} = 3.5 dpi) compared to 17 dpi (LT_{50} = 14 dpi) with WSSV containing the largest genome (312 kb).

In laboratory challenge tests, WSSV as sole pathogen may cause disease and mortality in SPF *P. vannamei* and other shrimp and crayfish species. In case of natural infection, several biotic and abiotic factors may influence the course of a WSS outbreak. Co-infections of different viruses including HPV, MBV, IHHNV together with WSSV have been reported (Manivannan et al., 2002; Flegel et al., 2004; Umesha et al., 2006).

1.3.3 **Clinical signs**

WSSV infected shrimp display clinical signs such as anorexia, lethargia, swollen branchiostegites due to fluid accumulation, white spots in the cuticle, separated/ loose cuticle from underlying epidermis, yellowish-white and enlarged hepatopancreas, hemolymph which fails to coagulate and reddish discoloration of the moribund shrimp (Lightner, 1996; Sahul-Hameed et al., 1998; Wang, Y. et al., 1999, this thesis). Shrimp with clinical signs are shown in Figure 5. Clinical signs do not allow a diagnosis of WSS (Flegel, 2006) because anorexia is observed in uninfected shrimp before and after molting (Jory et al., 2001), white spots in the carapace can also be caused by bacterial infection (Wang, Y. et al., 2000) and other clinical signs are unspecific and common to other diseases.

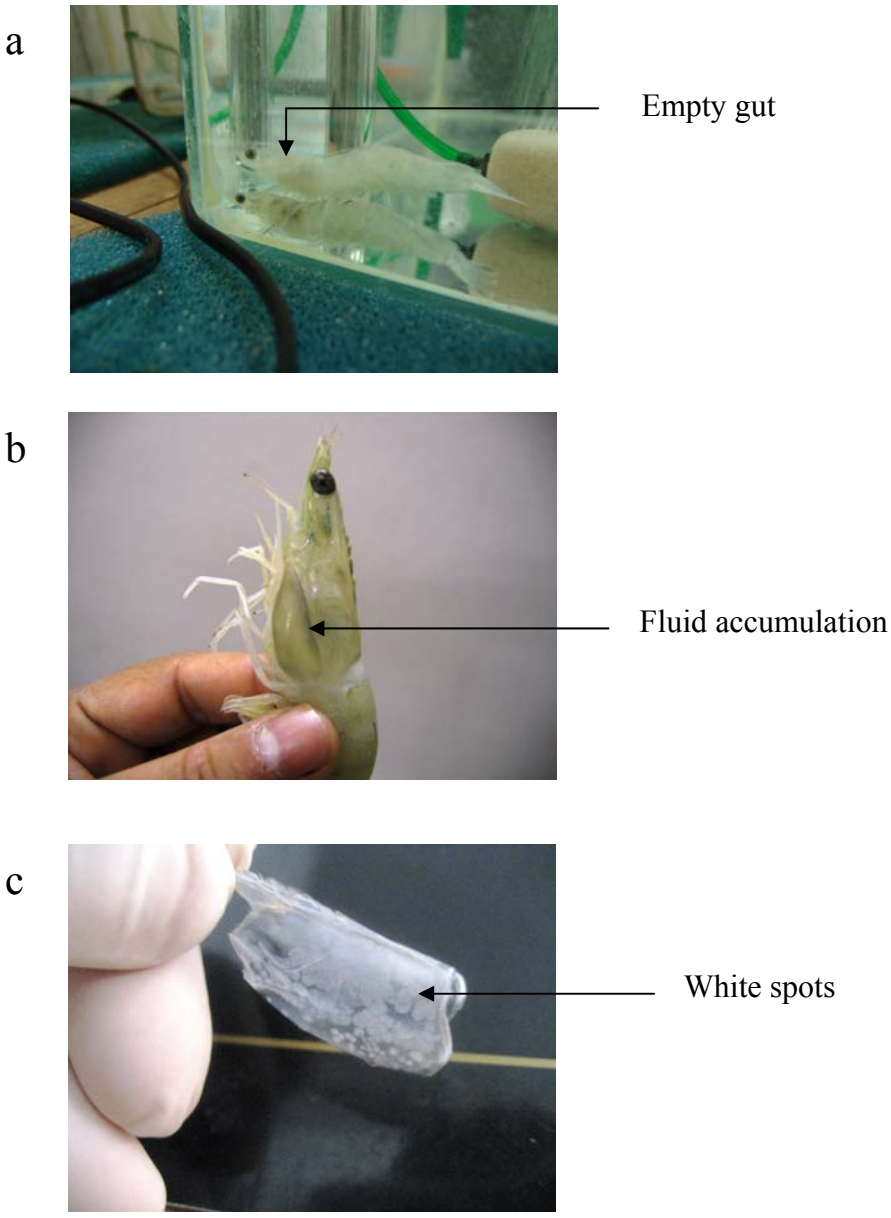

Figure 5. Clinical signs in white spot syndrome virus infected *Penaeus vannamei* juveniles, (a) anorexia and lethargia leading to empty gut (b) swollen branchiostegites, (c) white spots in the carapace.

1.3.4 **Antiviral immunity**

In crustaceans, only innate immunity without immunological memory exists (Lee and Söderhäll, 2002). Innate immunity includes physical barriers, humoral and cellular responses. The rigid and wax covered cuticle in crustaceans serves as a mechanical barrier against pathogen invasion (Brey et al., 1993; Lee and Söderhäll, 2002). Humoral factors originate mainly from hemocytes and are released during immune response. The substances released during humoral responses include lectins, defensive enzymes (phenol oxidase), lipoproteins, antimicrobial peptides, β-1,3 glucan binding protein, lipopolysaccharide (LPS) binding protein, peptidoglycan binding protein and reactive oxygen intermediates. Hemocytes are involved in clotting, recognition of foreign particles, phagocytosis, melanization, encapsulation, cytotoxicity and cell-cell communication (Newman and Bullis, 2001; Lee and Söderhäll, 2002). Most of the cellular and humoral responses were studied against bacterial, fungal and parasitic infections.

Recent studies showed evidences of an antiviral immunity in shrimp including the existence of toll like receptors (TLR), RNA interference, antiviral substances in tissues and immune genes. TLR in mammals function as activators of immune cells, intracellular signaling against infection, recognition of LPS, peptidoglycan, lipoprotein and are involved in antiviral immunity (Schröder and Bowie, 2005; Barton, 2007). TLR have been discovered in *L. vannamei* (Yang et al., 2007) and *P. monodon* (Arts et al., 2007). The roles of TLR in shrimp against viral infection still have to be examined. Genes encoding for RNA interference were found in *L. vannamei* (Robalino et al., 2007). Injection of double stranded RNAs (dsRNAs) induced antiviral immunity in *L. vannamei* (Robalino et al., 2004) and in *P. chinensis* (Kim et al., 2007). Short interfering RNAs (siRNAs) corresponding to viral proteins VP15 or VP28 were effective to reduce mortality in *P. monodon* (Westenberg et al., 2005) and *P. japonicus* (Xu et al., 2007). Virus replication was inhibited by multiple injection of VP28-si RNA (Xu et al., 2007). An antiviral gene *PmAV* was cloned from WSSV infected *P. monodon*. *PmAV* was highly expressed in the hepatopancreas from the second day post infection with a high viral load. This gene showed efficacy *in vitro* against the cytopathic effect of singapore grouper iridovirus (SGIV) (Luo et al., 2003; 2007). Another gene named

antipolysaccharide factor was upregulated in *Pacifastacus leniusculus* when treated with UV inactivated WSSV. The antipolysaccharide factor upregulated crayfish had lower virus replication and slower increase of cumulative mortality than those inoculated with virus only (Liu, H. et al. 2006). Twenty two antiviral genes which encode proteins including interferon-like protein and oligo synthetase like protein were found in the hemocytes of *P. japonicus* surviving from natural WSSV outbreaks (He et al. 2005). Two proteins were isolated from hemocyanin of WSSV infected *P. monodon*. These proteins inhibited replication *in vitro* of SGIV, frog virus 3 (FV3), lymphocystis virus (LDV), threadfin reovirus (ThRV), angelfish birnavirus (ABV) and infectious pancreatic necrosis virus (IPNV) (Zhang et al., 2004). Antiviral substances were found in tissue extracts of shrimp *P. setiferus*, crab *Callinectes sapidus*, crayfish *Procambarus clarkii*. These substances were effective against vaccinia virus, poliovirus, mengovirus, sindbis virus, banzi virus, vesicular stomatitis virus *in vitro* (Pan et al., 2000). Immune genes in hemocytes and hepatopancreas of WSSV infected *L. vannamei* and *L. setiferus* were reported (Gross et al., 2001). Heat shock protein (HSP 70, HSP 90), trehalose-phosphate synthase (TPS) and ubiquitin C were upregulated in WSSV infected *P. chinensis* (Wang et al., 2006). Levels of other immune factors such as phenoloxidase, lysozyme, hemolysis and hemaglutinin were higher in survivors from natural outbreaks than in normal shrimp (He et al., 2005). A quasi immune response was reported in *P. japonicus* surviving from natural WSSV outbreaks. This study also indicated the presence of a WSSV neutralizing molecule in the plasma of survivor shrimp (Venegas et al., 2000). Larvae derived from β 1,3-1,6 glucan injected spawner (*P. monodon*) had a better survival upon WSSV challenge than those from saline injected spawner. The authors suggested transmission of immunity from spawner to larvae (Huang and Song, 1999). Lipopolysaccharide and β 1,3 glucan binding protein gene was upregulated in WSSV infected *P. stylirostris* (Roux et al., 2002). This indicates a common defense response of crustaceans to different types of pathogens (virus, bacteria or fungus).

Interaction between virus infection and shrimp defense was described in the 'viral accommodation' concept. Disease outbreaks in shrimp caused by viruses such as IHHNV, YHV, TSV and WSSV were characterized by initial catastrophic losses followed by persistent infection in survivors after some time. This phenomenon of persistent infection was

termed as viral accommodation. The mechanism of persistent infection in shrimp is not known. Defective viral particles or an effect on viral triggered apoptosis could play a role in this process (Flegel, 2007). Apoptotic cells were detected both in naturally and experimentally WSSV infected shrimp (Sahtout et al., 2001; Wongprasert et al., 2003; Sahul Hameed et al., 2006). Apoptotic cells increased during a time course study of WSSV infection (Wongprasert et al., 2003). However, it is not clear whether apoptosis is a process that is linked to death or protection.

1.3.5 **Environmental factors and WSSV infection**

Environmental factors may play a key role on the severity of disease outbreaks of aquatic animals (Snieszko, 1974). Water temperature, salinity, dissolved oxygen, ammonia, pH and toxins derived from pesticides might be associated with mass mortalities due to WSSV (Fegan and Clifford, 2001). A significant effect of water temperatures on the disease and mortality of WSSV infected animal was reported in several studies (Vidal et al., 2001; Guan et al., 2003; Jiravanichpaisal et al., 2004). Higher incidences of WSSV outbreaks in Ecudaor (Rodriguez et al., 2003) and higher prevalence of infection in broodstock, nauplii and postlarvae in Thailand (Withyachumnarnkul et al., 2003) were observed during colder periods of the year. These results could be due to the effect on susceptibility for infection and activity of the host immune system. The effect of temperature on viral disease and mortality has also been reported in several other ectothermic animals, including *Cyprinus carpio koi* fish infected with koi herpesvirus (Gilad et al., 2003), *Micropterus salmoides* fish infected with largemouth bass virus (Grant et al., 2003), *Bombyx mori* insect larvae infected with nucleopolyhedrovirus (Kobayashi et al., 1981). A sudden drop in salinity might increase mortality rate of WSSV infected shrimp (Liu, B. et al., 2006; Peinado-Guevara and López-Meyer, 2006). A higher concentration of ammonia (5 mg/ml) in rearing water is thought to reduce the infectivity of WSSV (Jiang et al., 2004). Extreme conditions of environmental factors suppress the innate immunity including reduction of total hemocyte count, proPO activation, phagocytic index and release of oxygen radicals (Le Moullac and Hafner, 2000).

1.3.6 **WSSV diagnosis**

Several methods including microscopic observation under light, dark field, phase contrast microscope, bioassay, transmission electron microscopy, immunological, molecular and histopathological methods were developed to detect WSSV infection (Lightner and Redman, 1998). The selection of a method is dependent on the purpose. For instance, for screening brood stock and nauplii in hatcheries different detection methods will be used than for research on WSSV pathogenesis. The most frequently used histopathological, immunological and molecular methods are described below.

Histopathology: In the early stage of WSSV infection in *P. vannamei*, Cowdry A type inclusions are present, which are characterized by marginated chromatin separated from nucleoplasm. At more advanced stages of infection, basophilic inclusion bodies and at a chronic stage of infection, pyknosis and karyorrhexis was observed (Lightner, 1996; V. Alday, personal communication). This method is less sensitive and specific than molecular and immunological methods and requires highly trained personnel.

Immunological methods: These methods are based on monoclonal or polyclonal antibodies produced against viral antigens or recombinant viral antigens. Monoclonal and polyclonal antibodies produced against VP28 or rVP28 were used to develop several methods including immunofluorescence, immunohistochemistry (Poulos et al., 2001; Anil et al., 2002; Escobedo-Bonilla et al., 2005; Escobedo-Bonilla et al., 2007), immunoblot assays (Anil et al., 2002; You et al., 2002; Makesh et al., 2006), immunochromatographic test strips (Sithigorngul et al., 2006), enzyme linked immunosorbent assay (ELISA) (Liu, W. et al., 2002), and western blotting (Nadala et al., 1997; Yoganandhan et al., 2004). These methods are confirmatory to detect infection and demonstrate infected cells or quantitate viral antigens. Immunological methods are dependent on the expression of viral antigens and specificity of the antibody against viral antigens. Shrimp endogenous peroxidase might cause false positive results in immuno peroxidase assays (Zhan et al., 2003).

Molecular methods : These include several protocols of polymerase chain reaction (PCR), *in situ* hybridization and dot blot hybridization.

PCR - These methods are based on primers designed against a specific part of the genome sequence of WSSV. PCR methods include one step PCR (Lightner 1996; Lo et al., 1996), semi nested PCR (Kiatpathomchai et al., 2001), two step PCR (Hsu et al., 1999; Tapay et al., 1999; Hossain et al., 2004), quantitative competitive PCR (Tang and Lightner, 2000) and real time PCR (Durand and Lightner 2002). One step PCR detects WSSV in shrimp containing a substantial concentration of viral DNA which is usually the case in animals displaying gross signs of disease (Otta et al., 1999; Jian et al., 2005). Two step PCR can detect light infections in brood stock, nauplii, postlarvae and juveniles (Lo et al., 1996, 1997; Hsu et al., 1999) and quantitative PCR can be used for the quantification of the viral load. The disadvantages are misdiagnosis (false positive) (Claydon et al., 2004; Sritunyalucksana et al., 2006a), the inability to confirm whether the detected DNA is infectious or not, the fact that the sensitivity depends on the primer used (Hossain et al., 2004), the lack of localization of the infection in tissues and possible presence of inhibitory factors in some tissues (false negative) (Shekhar et al., 2006). The protocols of multiplex PCR to simultaneously detect WSSV and other viruses such as IHHNV (Quéré et al., 2002; Yang et al., 2006), TSV (Tsai et al., 2002), MBV (Natividad et al., 2006) or IHHNV and TSV have also been developed (Xie et al., 2007). A new protocol called *in situ* PCR can detect light infections in tissues at early stage of infection (Jian et al., 2005). Another method named loop mediated isothermal amplification (LAMP) is claimed to be more sensitivity than other PCR protocols. It can detect up to 1 femtogram (fg) of virus (Kono et al., 2004).

In situ hybridization - This method detects viral DNA in the host tissues through hybridization with a DNA probe (Chang et al., 1996; Wongteerasupaya et al., 1996). It is useful to detect infected cells in tissues, but it is less sensitive than PCR and requires histopathology facilities.

Dot blot hybridization - This procedure detects a fragment of viral DNA by hybridization with a DNA probe (Dupuy et al., 2004; Shekhar et al., 2006). This technique is also less sensitive than PCR.

WSSV infections in target tissues were detected at 12 hpi by one step PCR, 36 hpi by histopathology (Yoganandhan et al., 2003b), 12 hpi by indirect immunofluorescence and immunohistochemistry (Escobedo Bonilla et al., 2007), 16 hpi by in situ hybridization (Chang et al., 1996) and 12 to 24 hpi by western blot (Yoganadhan et al., 2004).

1.3.7 **Strategies to control disease and mortality due to WSSV**

Panzootics caused by WSSV resulted in serious production losses to Asian and American shrimp farmers. In response to this devastation, researchers have tested several preventive and curative measures. These are briefly described below.

Biosecurity - The term biosecurity was defined as practices to prevent introduction, contact, carry and spread of pathogens to shrimp (Menasveta, 2002; Lightner, 2005). It includes the development of SPF shrimp stock, exclusion of pathogens from broodstock in hatcheries and farms, 'zero' water exchange and water treatment before filling in growout, hygiene of workers and the use of quality feed (Lightner, 2005). At present, biosecurity measures are widely used in shrimp farms, however under field conditions, biosecurity is not absolute (Schuur, 2003). For example, success in preventing the introduction of diseased shrimp in a farm does not depend only on the testing of PL before stocking. Sampling errors or misdiagnosis may occur (Fegan and Clifford, 2001). In case diseased shrimp are introduced, quarantine might be useful to reduce the risk of transmission. Removal of dead shrimp from the farm, a quality diet and better feed management can also reduce the risk.

Antiviral - An antiviral is a compound that suppresses the ability of a virus to replicate at any step of the replication cycle (attachment, entry, uncoating, transcription, translation, and assembly).

The antiviral effect of sulfated polysaccharides extracted from marine algae against mammalian viruses *in vitro* is well known (Witvrouw and De Clercq, 1997). Both *Spirulina platensis* (Hayashi and Hayashi, 1996) and *S. maxima* (Hernández-Corona et al., 2002) have antiviral activity. Sulfated fucans (fucoidan) can be isolated from 43 species of brown algae. Fucoidans from brown algae are extremely complex and heterogenous in structure (Berteau and Mulloy, 2003). In a study, fucoidan extracted from marine algae *Sargassum polycystum*

was incorporated in a diet. The diet was fed to *P. monodon* juveniles for four days before and after challenge with WSSV. Shrimp receiving the highest amount of fucoidan in the diet (100, 200 and 400 mg/ kg body weight of shrimp) showed increased survival rate (Chotigeat et al., 2004).

Seed extract of *Pongamia pinnata* plant has antiviral activity against herpes simplex virus (Elanchezhiyan et al., 1993). An ethanolic extract bis(2-methylheptyl)phthalate from *P. pinnata* leaves was incorporated in a diet and tested for antiviral activity against WSSV infection in *P. monodon*. *P. monodon* were fed for 4 days before and 15 days after WSSV challenge (200 and 300 µg/g body weight of shrimp/ day). Increased survival (40 to 80%) was found with the diet containing the highest concentration of the extract (Ramesthangam and Ramasamy, 2007). Increased survival of *P. monodon* was also observed after administration of a mixture of WSSV and extracts of *Aegle marmelos*, *Cynodon dactylon*, *Lantana camara*, *Mimosa charanita*, *Phyllanthus amarus* plants (Balasubramanian et al., 2007). The mechanisms of the antivial activity of these plant extracts against WSSV are not known.

A phage display peptide was claimed to be effective against WSSV *in vivo* in crayfish and *in vitro* in primary culture of lymphoid organ of shrimp (Yi et al., 2003). Injection of double stranded RNA (dsRNAs) induced protection in *L. vannamei* depending on the WSSV inoculation dose (Robalino et al., 2004). The degree of protection differed between dsRNAs targeted genes (Robalino et al., 2005). DsRNAs corresponding to viral proteins VP28, VP281, protein kinase gene of WSSV and green fluorescence protein (GFP) gene gave protection to *P. chinensis* (Kim et al., 2007). Injection of the short interfering RNAs (siRNA) VP15-siRNA or VP28-siRNA were found to be effective in *P. monodon* (Westenberg et al., 2005). Multiple injections of VP28-siRNA delayed and reduced mortality of WSSV infected *P. japonicus* due to the inhibition of the virus replication (Xu et al., 2007).

'Vaccination' or increased resistance/ tolerance - The term vaccination may not be appropriate in shrimp, as they have only an innate immunity (Smith et al., 2003). Though, it has been used in different papers. 'Resistance' includes evidence of defense, clearance activities and ultimately elimination of the virus. 'Tolerance' implies a persistent infection, which is transmissible with or without detectable signs of viral pathology (Flegel, 2001).

Several studies have claimed success in different shrimp species. Administration of viral proteins or recombinant viral proteins VP19, VP26, VP28, VP31, VP292 provided protection in shrimp or crayfish (Namikoshi et al., 2004; Witteveldt et al., 2004a, 2004b; Du et al., 2006a; Vaseeharan et al., 2006; Witteveldt et al., 2006; Jha et al., 2006, 2007; Rout et al., 2007). The durations of protection (maximum seven weeks) and efficacies varied with viral proteins and between studies. Vaccination with nucleocapsid protein VP15, VP35 had no effect (Rout et al., 2007). Dietary rVP28 was effective to reduce mortality in *P. monodon* and *Procambarus clarkii* (Witteveldt et al., 2004b; Jha et al., 2007) but comparatively less effective in *P. vannamei* (Witteveldt et al., 2006). The method used for the production of rVP28 might have played a role on its efficacy. rVP28 expressed in BmN cells was more effective in protecting crayfish than *E. coli* expressed rVP28 (Du et al., 2006a). Monoclonal antibody (MAbs) against rVP28 (Musthaq et al., 2006; Natividad et al., 2007) or polyclonal antibodies (PAbs) against rVP(19+28) (Li, H-X. et al., 2005) can neutralize the infectivity of WSSV. The neutralization efficacy of MAb against VP28 was dependent on the inoculation dose and was less effective *in vitro* (Natividad et al., 2007). On the contrary, according to Robalino et al., (2006), MAbs and PAbs raised against VP28 could not inhibit WSSV infectivity and the observed protection in Witteveldt et al. (2004b) was possibly due to components other than antibodies in the rabbit antiserum. Administration of formalin inactivated WSSV induced a protection to subsequent challenge in *P. japonicus*, *P. indicus* and *P. vannamei* (Namikoshi et al., 2004; Bright Singh et al., 2005; Melena et al., 2006) whereas heat inactivated WSSV had no effect (Namikoshi et al., 2004).

Interference with other virus - IHHNV infected *P. vannamei* and *P. stylirostris* postlarvae and juveniles had a reduced mortality upon challenge with WSSV (Tang et al., 2003; Bonnichon et al., 2006; Melena et al., 2006). Survivors of *P. vannamei* during a natural WSSV outbreak were found to be positive for runt deformity syndrome. This protection could be either due to an induced immunity or blocked/ depleted host components such as cellular receptors required for WSSV replication (Tang et al., 2003).

Immunostimulants - Diets containing microbial components such as β-1,3 -glucan from *Schizophyllum commune* (Chang et al., 1999, 2003), lipopolysaccharide from *Pantoea agglomerans* (Takahashi et al., 2000) or extracts of plants (*Cyanodon dactylon*, *Aegle*

marmelos, *Tinospora cordifolia*, *Picrorhiza kurooa*, *Eclipta alba*) (Citarasu et al., 2006) have been shown to improve the immunity and reduce the mortality of WSSV infected shrimp. The mechanism was attributed to an activation of innate immunity. The best results were obtained when immunostimulants were applied before exposure to the pathogen. Excess or indiscriminate use of immunostimulants may not be useful or even cause negative effects (Horowitz and Horowitz, 2001).

Antimicrobial peptide (AMP) - A few reports described the activity of AMPs against different viruses. AMPs are part of the innate immunity of shrimp. In two studies, WSSV was incubated *in vitro* with a synthetic AMP 'mytilin' before inoculation in shrimp *Palaemon serratus*. Mortality of *P. serratus* was lowered by the pretreatment with AMPs (Dupay et al., 2004; Roch et al., 2007 in press).

Water temperature - Mortality of WSSV infected shrimp or crayfish was reduced or even totally stopped at a water temperature higher (32-33°C) or lower (<15°C) than optimum (Vidal et al., 2001; Guan et al., 2003; Jiravanichpaisal et al., 2004; Du et al., 2006b). The suggested mechanisms are including reduced replication (Du et al., 2006b), reduction of viral load (Granja et al., 2006), apoptosis (Granja et al., 2003) and altered gene expression of WSSV (Reyes et al., 2007). WSSV replication was inhibited at 4°C and at 32°C in *in vitro* primary culture of hematopoietic tissue of crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2006). Heat shock protein expression might play a role to prevent mortality of infected shrimp at 32°C (Vidal et al., 2001).

It is important to note that the WSSV challenge procedures were different in between the above mentioned studies. The inoculation was done by intramuscular injection, immersion in viral suspension or feeding of infected tissue. Further, the amount of infectious virus given to each animal was not defined. This variation makes comparison of the results impossible. More standardization is required.

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CHAPTER 2

Aims of the thesis

White spot syndrome virus (WSSV) is the causative agent of white spot syndrome (WSS) in shrimp. This disease has caused huge production losses to most of the shrimp producing countries of the world. Earlier studies were indicative for differences in virulence of WSSV geographical isolates. Researchers have tested several strategies including antivirals, immunostimulants and high (32-33°C) or low (< 15°C) water temperature to control WSS. In those studies, the virulence of the isolates and titer of the virus stock were unknown and the inoculation method did not ensure the exposure of a certain amount of infectious titer to each animal. Further, experiments were rarely repeated.

The general aims of this thesis are (i) to examine differences in virulence between WSSV isolates and (ii) to study the effect of antivirals, and high water temperature using a fully standardized challenge procedure and several repeats.

The specific aims of this thesis are as follows:

- To evaluate the differences in virulence between three WSSV isolates (WSSV Thai-1, WSSV Thai-2 and WSSV Viet) through intramuscular inoculation with a fixed infectious titer (30 SID_{50}) in specific pathogen free *Penaeus vannamei* juveniles.
- To test the efficacy of cidofovir - an antiviral, a diet supplemented with *Spirulina platensis* and high water temperature (33°C) to control disease and mortality in WSSV infected shrimp.
- To determine the minimal duration of exposure to high water temperature (33°C) and the latest time point in infection of exposure to 33°C to be effective in inducing protection. This will allow to determine the usefulness of high water temperature to control WSS in the field.

CHAPTER 3

Virulence of white spot syndrome virus (WSSV) isolates may be determined by the degree of replication in gills of *Penaeus vannamei* juveniles

Diseases of Aquatic organisms, in press

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Abstract

The virulence of three white spot syndrome virus isolates (WSSV Thai-1, WSSV Thai-2 and WSSV Viet) was assessed by a standardized inoculation model and the degree of virus replication in tissues was compared between the most virulent WSSV Thai-1 and the least virulent isolate WSSV Viet to find a basis for differences in virulence. *Penaeus vannamei* juveniles were inoculated intramuscularly with 30 SID_{50} (SID_{50} = shrimp infectious dose with 50% endpoint) of each isolate. WSSV infections in dead shrimp were demonstrated by indirect immunofluorescence. Virus replication was analysed in the naturally dead shrimp and in the shrimp euthanized at 0, 12, 24, 48, 72, 120 and 168 hpi by quantifying the number of infected cells using immunohistochemistry.

Mortalities started at 36 hpi with both Thai isolates and 36-60 hpi with the Viet isolate. In three experiments, 100% mortality was reached 96 to 240 h later in shrimp inoculated with the Viet isolate compared to those inoculated with the Thai isolates.

WSSV positive cells were detected in every investigated tissue of naturally dead and euthanized shrimp from 24 hpi onwards when inoculated with WSSV Thai-1 and from 36 hpi onwards when inoculated with WSSV Viet. Higher numbers of infected cells were found in tissues, especially in gills (6 times), of naturally deceased shrimp inoculated with WSSV Thai-1 than Viet isolate, except in hematopoietic tissues.

This study indicates that the difference of virus replication in tissues, particularly in gills may determine the virulence of WSSV isolates.

Keywords: WSSV, virulence, quantitative analysis, *Penaeus vannamei*

1. **Introduction**

White spot syndrome virus (WSSV) is an enveloped, double stranded DNA virus (Van Hulten et al. 2001a), which is pathogenic mainly to decapod crustaceans including penaeid shrimp, caridean shrimp, crayfish, crabs and lobsters (Flegel, 2006). The first WSSV epidemic was reported in shrimp farms of South East Asia in 1992 (Chou et al., 1995). The virus then spread to other shrimp farming countries in Asia, North, Central and South America and Middle East (Lightner 1996; Flegel 2006; Rosenberry 2002). Different forms of WSSV outbreaks (acute, per-acute and chronic) in shrimp farms have been reported (Sudha et al., 1998).

Virulence has been defined as the power of a pathogen to produce disease within a group or species (Shapiro-Ilan et al., 2005). Differences in virulence between WSSV strains have been suggested through challenge studies in shrimp and crayfish (Wang et al., 1999; Lan et al., 2002; Marks et al., 2005). However, those studies were performed without using a standardized challenge procedure to deliver a certain amount of infectious virus. Genomic and antigenic variations between WSSV isolates have been reported (Wang et al., 2000; Marks et al., 2004). Previous studies have reported conflicting findings concerning the effect of genomic deletions on WSSV strain virulence (Lan et al., 2002; Marks et al. 2005).

WSSV infected cells have been detected in tissues of ectodermal and mesodermal origin in both naturally and experimentally infected shrimp. Qualitative (Chang et al., 1996; Lo et al., 1997) and quantitative pathogenic analyses (Tan et al., 2001; Durand and Lightner 2002; Escobedo-Bonilla et al., 2007) have shown that the major target tissues for WSSV replication are gills, stomach cuticular epithelium, cuticular epithelium of the body wall, hematopoietic tissues, antennal glands and lymphoid organ. To our knowledge, quantitative analysis of virus replication of WSSV isolates with different virulence has not been done before.

The objectives of this study were (i) to compare the virulence of three WSSV isolates (WSSV Thai-1, WSSV Thai-2 and WSSV Viet) by intramuscular inoculation with the same amount of infectious virus (30 SID_{50}) in specific pathogen free (SPF) *Penaeus vannamei*

juveniles of the same size/age and (ii) to compare the virus replication in specific tissues of shrimp inoculated with the most and least virulent isolate.

2. **Materials and methods**

2.1 Virus

Three WSSV isolates were used in the present study. All three isolates were collected from naturally infected *Penaeus monodon*, two from Thailand in 1996 and one from Vietnam in 2003. WSSV Thai-1 was passaged in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001), WSSV Thai-2 in *Procambarus clarkii* (Van Hulten et al., 2001b) and WSSV Viet in *Cherax quadricarinatus.* Crayfish gill suspension of WSSV Thai-1 was provided by K Söderhäll (Uppsala University, Sweden), purified WSSV Thai-2 was given by JM Vlak (Wageningen University, The Netherlands) and gill suspension of WSSV Viet was received from the Research Institute for Aquaculture n^{o} 2, Vietnam. Each isolate was amplified in specific pathogen free (SPF) *Penaeus vannamei* juveniles and infectivity titres of the stocks were determined according to the procedure described by Escobedo-Bonilla et al., (2005). The median infectious titres of stocks as determined by intramuscular inoculation in SPF *P. vannamei* were $10^{5.8}$, $10^{5.9}$ and $10^{5.8}$ SID_{50} (SID_{50} = shrimp infectious dose with 50% end point) per ml for WSSV Thai-1, WSSV Thai-2 and WSSV Viet, respectively.

2.3 Experimental conditions and shrimp

SPF *P. vannamei* were imported from Molakai farm, Hawaii (USA) at an early postlarval stage and reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Faculty of Bioscience Engineering, Gent University, Belgium. Rearing conditions were: water temperature 27-28 °C, salinity 34-36 g/l. Before each experiment, shrimp were gradually acclimatized to a salinity of 15 g/l at the ARC over four days. Acclimatized shrimp were transported to the facilities of Laboratory of Virology, Faculty of Veterinary Medicine, Gent University for WSSV challenge studies. Nine to eleven shrimp were housed per 50 litre (L) aquarium, equipped with aeration, mechanical filtration (Eheim, Germany), water pump (Eheim, Germany), aquarium heater (Model VTX 300,

Aquarium systems, France). Two 50 L tanks were used per isolate in each experiment for the comparison of clinical outcome. In the comparison of virus replication in target tissues experiment, four and six 50 L tanks were used with Thai-1 and Viet isolate, respectively. Brackish water with a salinity of 15 g/l was prepared using artificial sea salt (Instant Ocean, Aquarium systems, France) and de-ionized water. Water temperature was maintained at 27 °C. Approximately 0.2 g of a commercial shrimp diet was provided for each shrimp per day. Water quality was checked everyday by measuring ionized ammonia (NH_4^+) using test kits (Aquamerck, Germany). Ninety percent water was renewed at 120 hpi in the experiment with WSSV Viet. In total, 280 shrimp were used in the present study. In the three bioassay experiments, 181 shrimp with a mean body weight (MBW) of 8.8 ± 2.6 g were used. For the quantitative analysis of infection in target tissues, 99 shrimp with a MBW of 16.6 ± 2.8 g were used.

2.4 WSSV inoculation procedure

Shrimp were inoculated intramuscularly with 50 µl of inoculum containing 30 SID_{50} of each isolate in the junction between the third and fourth abdominal segments. A very precise syringe (P/N: 81001/00, 1710 LT, 100 µl, Hamilton Bonaduz AG, Switzerland) and 25 G needle (Terumo, Belgium) was used for intramuscular inoculation.

2.5 Experimental design

2.5.1 Comparison of clinical outcome

The three WSSV isolates (WSSV Thai-1, WSSV Thai-2 and WSSV Viet) were used in this study. Nineteen to twenty one shrimp were inoculated with each isolate per experiment. Shrimp were observed for clinical signs including anorexia and lethargia was recorded every 12 h till the end of the experiment. Dead shrimp were collected 4 times per day. The experiments ran until cumulative mortalities reached 100% for each isolate and were repeated three times. Dead shrimp were collected and WSSV infections were demonstrated by indirect immunofluorescence (IIF).

2.5.2 *Comparison of virus replication in target tissues*

Two WSSV isolates were used in this experiment. Thirty nine shrimp were inoculated with the most virulent WSSV Thai-1 and sixty shrimp were inoculated with the least virulent WSSV Viet. Shrimp inoculated with each isolate were euthanized at 0, 12, 24, 36, 48 and 72 hpi. In addition, WSSV Viet inoculated shrimp were euthanized at 120 and 168 hpi. Five shrimp were euthanized at each time point with each isolate except for WSSV Thai-1 at 72 hpi when only two survivors were euthanized. During this period, naturally dead shrimp inoculated with each isolate were also collected. WSSV infected cells were quantified in gills, stomach cuticular epithelium, cuticular epithelium of body wall, hematopoietic tissues and antennal glands of both euthanized and naturally deceased shrimp by immunohistochemistry (IHC). The experiment was conducted once.

2.6 Demonstration of WSSV infection by IIF

The procedure to detect WSSV infection by IIF was described before (Escobedo-Bonilla et al., 2005). In brief, the cephalothoraxes of dead shrimp were dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at -20 °C. Cryosections (5 µm) were made and immediately fixed in 100% methanol at -20 ºC for 20 min. Sections were washed three times for 5 min each in phosphate buffered saline (PBS) and incubated with 2 µg ml^{-1} of the monoclonal antibody 8B7 (Diagxotics Inc. USA) directed against viral protein VP28 (Poulos et al. 2001) for 1 h at 37 ºC. 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 (Leica DM RBE).

2.7 Quantification of infected cells by IHC

2.7.1 Fixation and processing of tissues

The cephalothoraxes of dead and euthanized shrimp were cut into two halves and then fixed in davidson's for 48-72 h (Bell and Lightner, 1988). Fixed tissues were transferred and

kept in 50% ethanol till paraffin embedding. The two sides of the cephalothoraxes embedded in paraffin in separate cassettes. Sections of 5 μm were made; one side of the cephalothorax was used for sections of the gills and the other side for sections of the internal organs including stomach cuticular epithelium, cuticular epithelium of body wall, hematopoietic tissues and antennal glands. Sections were placed on silane-coated slides (A3648, Sigma-Aldrich) and stained for IHC according to the procedure described in Escobedo-Bonilla et al., (2007).

2.7.2 Staining procedure for IHC

Tissue sections were deparaffinized by heating at 55-60°C for 30 min and rehydrated by immersed in xylene. Then, tissues were rehydrated by immersion in gradual decrease of several concentrations of ethanol from 100% up to 50%. The endogenous peroxidase was blocked by incubation for 30 min at room temperature in sodium azide (1%) and hydrogen peroxidase (0.02%) in Tris buffer pH 7.4. Sections were incubated with monoclonal antibody 8B7 (Diagxotics Inc. USA) of 2 μg ml^{-1} against WSSV envelope protein VP28 (Poulos et al., 2001) for 1 h at 37°C. Then, sections were washed in Tris buffer (pH 7.6) and incubated for 1 h at 37°C with 1:200 dilution of biotinylated sheep antimouse IgG antibodies (RPN 1001, Amersham Biosciences) followed by washing and incubation in streptavidine-biotinylated horseradish peroxidase complex (RPN 1051, Amersham Biosciences) for 30 min at room temperature. Afterwards, tissues were incubated for 30 min in 0.01% of 3,3′ diaminobenzidine (D8001, Sigma Aldrich) for the color development and counterstained with Gill's hemaluin, washed, dehydrated and mounted (DPX mountant for histology, Fluka, Biochemika, 44581, United Kingdom).

2.7.3 Quantification of WSSV infected cells

WSSV infected cells were counted with a light microscope (Leica DMRBE) using a 400x magnification. WSSV infected cells in 5 fields in gills selected at random and 2-3 fields in hematopoietic tissue were counted and converted to the number of WSSV infected cells/mm^2. Both WSSV infected and uninfected cells in stomach cuticular epithelium,

cuticular epithelium of body wall and antennal glands were counted in 5 fields selected at random and the average percentage (%) of infected cells was calculated.

2.8 Statistical analysis

The cumulative mortalities of shrimp inoculated with three WSSV isolates in bioassay experiments was analysed by probit (Agresti, 1996). When no significant interactions exist between time and isolate, the probit model has the form:

Probit (x) = $\alpha + \beta$ time + γ isolate

Where

α is the intercept,

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

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

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

3. **Results**

3.1 Comparison of clinical outcome

Shrimp inoculated with each isolate started to show clinical signs consisting of anorexia and lethargia from 24-36 hpi. Mortalities started from 36 hpi with both Thai isolates and 36-60 hpi with WSSV Viet in the three experiments (Figure 1a). Cumulative mortalities reached 100% at 72-84 hpi, 108 hpi and 204-348 hpi of shrimp inoculated with WSSV Thai-1, WSSV Thai-2 and WSSV Viet, respectively. All dead shrimp were WSSV-positive. Median lethal times (LT_{50}) were 47 and 61 hpi with the Thai isolates 1 and 2 and 120 hpi with WSSV Viet, which were all significantly different from each other (Table 1 and Figure 1b) ($p<0.05$).

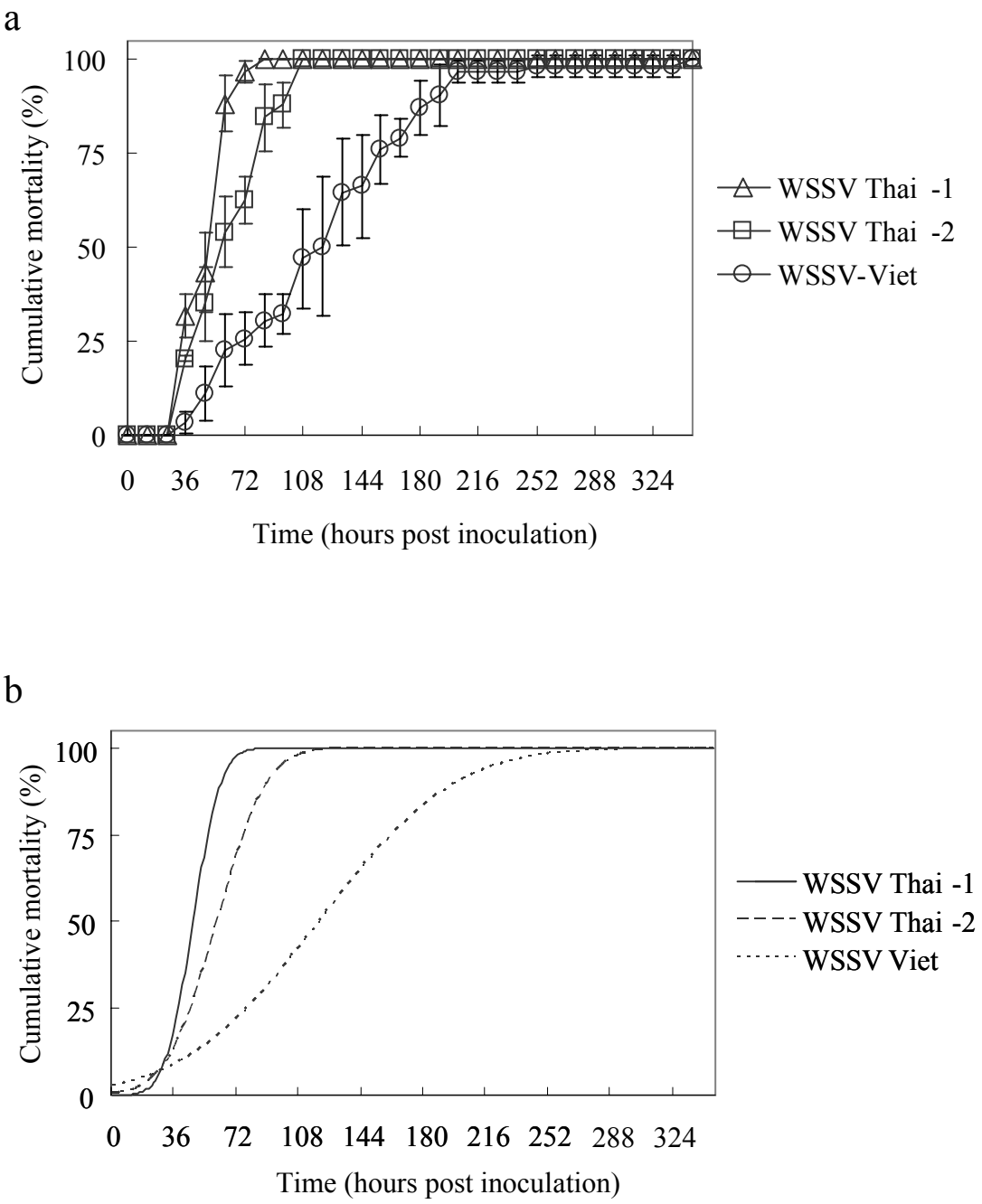

Figure 1. (a) Cumulative mortalities (mean ± standard deviation [SD]) in SPF *Penaeus vannamei* juveniles inoculated intramuscularly with 30 SID_{50} of WSSV Thai-1, WSSV Thai-2 or WSSV Viet. The experiments were repeated three times. (b) Probability of mortality (probit) of shrimp inoculated intramuscularly with 30 SID_{50} of each of the three isolates.

Table 1. Median lethal times (LT_{50}) of *Penaeus vannamei* juveniles inoculated intramuscularly with 30 SID_{50} of WSSV Thai-1, WSSV Thai-2 or WSSV Viet.

WSSV isolates	Inoculation dose	LT_{50}	α	β	γ	δ	LT_{50}[a] comparison
WSSV Thai-1	30 SID_{50}	46.9[a]	3.713	-0.0792	-	-	
WSSV Thai-2	30 SID_{50}	60.8[b]	3.713	-0.0792	-1.035	0.0352	a<b<c
WSSV Viet	30 SID_{50}	119.7[c]	3.713	-0.0792	-1.776	0.063	

[a]Differences in LT_{50} are significant ($p<0.05$)

3.2 *Comparison of virus replication in target tissues*

The evolution of number of infected cells in gills, stomach cuticular epithelium, cuticular epithelium of body wall, hematopoietic tissues and antennal gland in the course of infection with WSSV Thai-1 and WSSV Viet is given in Figure 2. Differences in number of infected cells in gills, stomach cuticular epithelium, cuticular epithelium of body wall, hematopoietic tissues and antennal gland at 48 hpi between shrimp inoculated with WSSV Thai-1 and shrimp inoculated with WSSV Viet are illustrated in Figure 3.

Gills - WSSV infected cells in gills were first found at 24 hpi in euthanized shrimp inoculated with WSSV Thai-1. The mean numbers of infected cells were: 2 ± 1.4, 31 ± 53, 276 ± 318 and 342 ± 479 cells/mm^2 in euthanized shrimp at 24, 36, 48 and 72 hpi, respectively. The mean number of infected cells in dead shrimp was 523 ± 321 cells/mm^2. WSSV infected cells in gills were also first found at 24 hpi in euthanized shrimp inoculated with WSSV Viet. The mean numbers of infected cells were: 3 ± 4, 9 ± 16, 114 ± 52, 101 ± 26, 58 ± 10 and 17 ± 6 cells/mm^2 in euthanized shrimp at 24, 36, 48, 72, 120 and 168 hpi, respectively. The mean number of infected cells in dead shrimp was 86 ± 63 cells/mm^2.

Stomach cuticular epithelium - WSSV infected cells in stomach epithelium were first found at 24 hpi in euthanized shrimp inoculated with WSSV Thai-1. The mean percentages of infected cells were: 1 ± 1, 4 ± 7, 18 ± 18, 18 ± 26 % in shrimp euthanized at 24, 36, 48 and 72 hpi, respectively. The mean percentage of infected cells in dead shrimp was 38 ± 7 %. WSSV infected cells in stomach epithelium were also first found at 24 hpi in euthanized shrimp inoculated with WSSV Viet. The mean percentages of infected cells were: 0.1 ± 0.1, 6

± 11, 24 ± 13, 24 ± 3, 27 ± 6 and 14 ± 6 % in shrimp euthanized at 24, 36, 48, 72, 120 and 168 hpi, respectively. The mean percentage of infected cells in dead shrimp was 23 ± 8 %.

Cuticular epithelium of body wall - WSSV infected cells in cuticular epithelium were first found at 24 hpi in euthanized shrimp inoculated with WSSV Thai-1. The mean percentages of infected cells were: 0.1 ± 0.2, 5 ± 6, 13 ± 14, 17 ± 24 % in shrimp euthanized at 24, 36, 48 and 72 hpi, respectively. The mean percentage of infected cells in dead shrimp was 28 ± 8 %. WSSV infected cells in cuticular epithelium were first found at 36 hpi in shrimp inoculated with WSSV Viet. The mean percentages of infected cells were: 3 ± 5, 21 ± 8, 18 ± 6, 18 ± 15, 13 ± 9 % in shrimp euthanized at 36, 48, 72, 120 and 168 hpi, respectively. The mean percentage of infected cells in dead shrimp was 18 ± 8 %.

Hematopoietic tissues - WSSV infected cells in hematopoietic tissue were first found at 24 hpi in euthanized shrimp inoculated with WSSV Thai-1. The mean numbers of infected cells were: 2 ± 4, 42 ± 57, 257 ± 348 and 58 ± 83 cells/mm^2 in shrimp euthanized at 24, 36, 48 and 72 hpi, respectively. The mean number of infected cells in dead shrimp was 259 ± 155 cells/mm^2. WSSV infected cells in hematopoietic tissue were first found at 36 hpi in euthanized shrimp inoculated with WSSV Viet. The mean numbers of infected cells were 109 ± 232, 308 ± 195, 56 ± 41, 106 ± 111 and 140 ± 95 cells/mm^2 in shrimp euthanized at 36, 48, 72, 120 and 168 hpi, respectively. The mean number of infected cells in dead shrimp was 160 ± 103 cells/mm^2.

Antennal glands - WSSV infected cells were first found at 24 hpi in euthanized shrimp inoculated with WSSV Thai-1. Mean percentages of infected cells were: 0.3 ± 0.7, 2 ± 4, 10 ± 14 and 5 ± 8 % in shrimp euthanized at 24, 36, 48 and 72 hpi, respectively. The mean percentage of infected cells in dead shrimp was 11 ± 6 %. WSSV infected cells were first found at 36 hpi in euthanized shrimp inoculated with WSSV Viet. Mean percentages of infected cells were: 0.5 ± 0.5, 3 ± 2, 5 ± 2, 2 ± 1 and 2 ± 2 % in shrimp euthanized at 36, 48, 72, 120 and 168 hpi, respectively. The mean percentage of infected cells in dead shrimp was 5 ± 3 %.

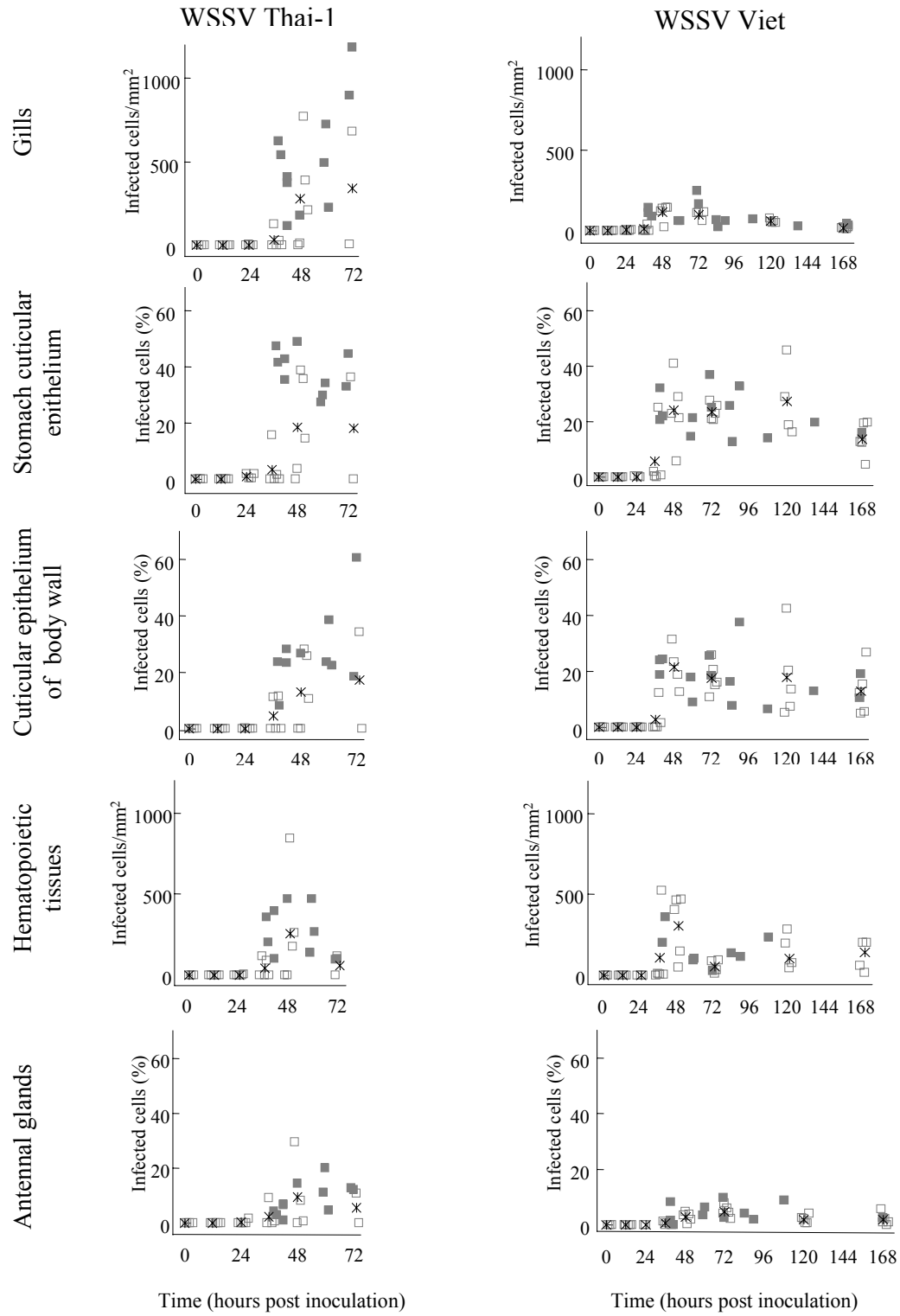

Figure 2. Number of WSSV infected cells per mm^2 in gills and hematopoietic tissues and percentages of infected cells in stomach cuticular epithelium, cuticular epithelium of body wall and antennal glands of dead and euthanized shrimp. *Penaeus vannamei* juveniles were inoculated intramuscularly with 30 SID_{50} of WSSV Thai-1 or WSSV Viet. (■ Dead shrimp,□ euthanized shrimp, ⁎ mean of the euthanized shrimp).

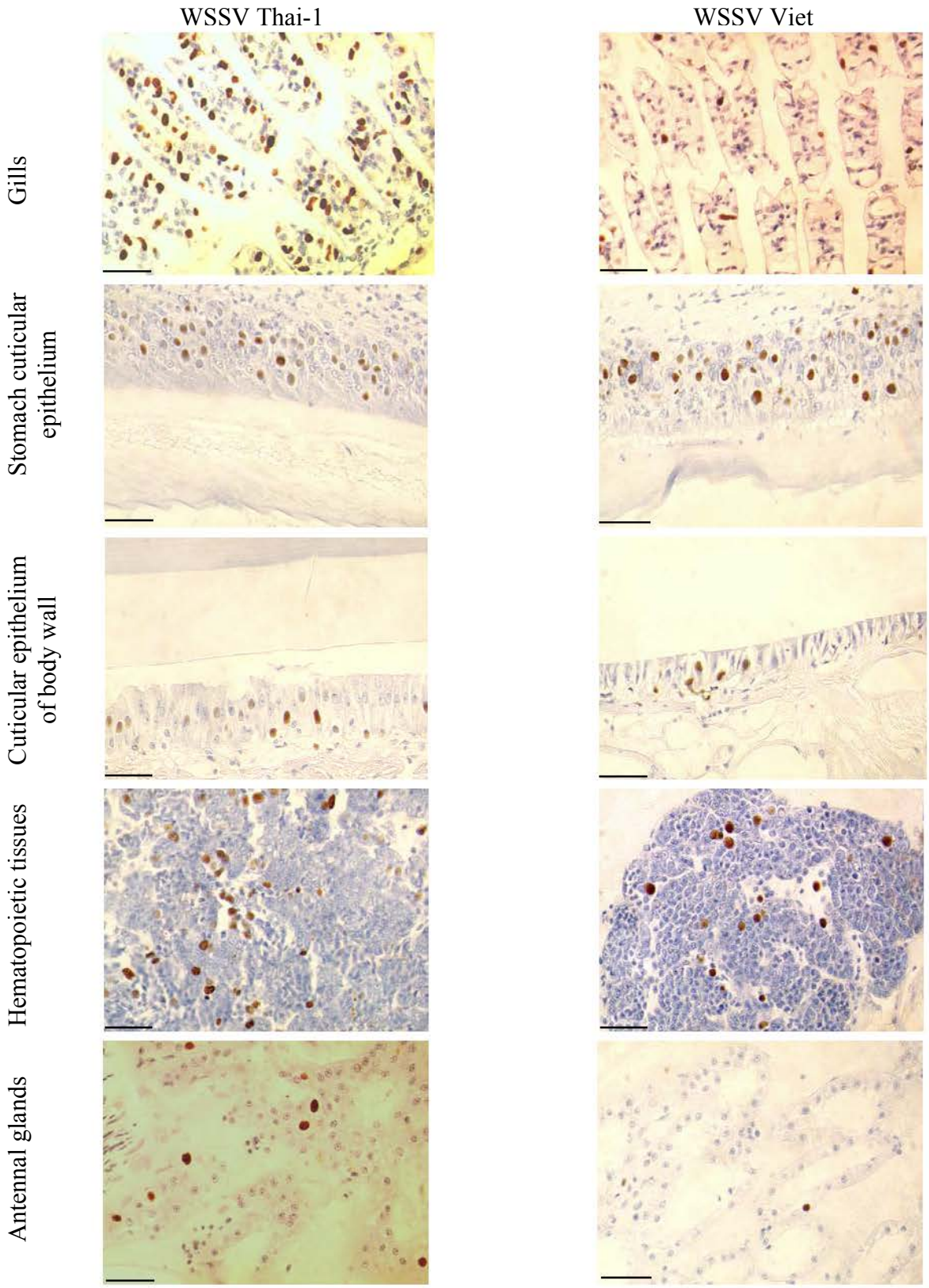

Figure 3. WSSV infected cells (VP28 positive by immunohistochemistry) in gills, stomach cuticular epithelium, cuticular epithelium of body wall, hematopoietic tissues and antennal gland of shrimp. *Penaeus vannamei* juveniles were inoculated intramuscularly with 30 SID_{50} of WSSV Thai-1 or WSSV Viet and euthanized at 48 h post inoculation. Scale bars = 50 μm.

4. **Discussion**

This study demonstrated the existence of clear differences in virulence between WSSV isolates. Further, it was shown that deceased shrimp inoculated with the highly virulent isolate WSSV Thai-1 had a more extensive replication (higher number of WSSV infected cells) in gills, stomach cuticular epithelium, cuticular epithelium of the body wall, hematopoietic tissues and antennal gland than deceased shrimp inoculated with the least virulent isolate WSSV Viet.

The difference in virulence between the three isolates Thai-1, Thai-2 and Viet was characterized by differences in onset of mortality (36, 36 and 36-60 hpi, respectively), time to reach a cumulative mortality 100% (72-84, 108 and 204-348 hpi, respectively) and median lethal time (47, 61 and 120 hpi, respectively). The reproducible results in the experiments showed the usefulness of the challenge procedure through intramuscular inoculation of a well defined amount of WSSV infectious titre (30 SID_{50}) in SPF *Penaeus vannamei* juveniles of the same age. This method can now be used to evaluate the virulence of many other geographical isolates. The observed differences in virulence between WSSV geographical isolates is a confirmation of indications reported earlier (Wang et al. 1999, Lan et al. 2002, Marks et al. 2005).

Quantification of WSSV infected cells (VP28 positive) in naturally deceased shrimp and euthanized shrimp at different hpi in five tissues was performed using IHC with the hope of explaining the differences between highly virulent WSSV Thai-1 and less virulent Viet isolate. For this purpose, tissues with an important role in shrimp physiology were selected. Gills serve for respiration, osmoregulation, excretion and acid base homeostasis (Taylor & Taylor 1992); stomach takes care of the digestion (Icely & Nott 1992); cuticular epithelium produces cuticle which forms a physical barrier against pathogen invasion (Brey et al. 1993); hematopoietic tissue produces hemocytes which are important for the defence and the antennal gland is the excretory organ of shrimp (Felgenhauer 1992). The quicker mortality in shrimp inoculated with WSSV Thai-1 than in those inoculated with WSSV Viet may be explained by a more pronounced dysfunction due to the more extensive replication in the important tissues, especially the gills (average of six times in dead shrimp). The higher

numbers of infected cells in tissues of deceased shrimp inoculated with WSSV Thai-1 compared to those inoculated with WSSV Viet may be the result of one or a combination of the following factors. First, the replication cycle of WSSV Thai-1 may be shorter than that of WSSV Viet. This can only be examined when shrimp cell lines become available. Second, the higher number of infected cells in gills of shrimp inoculated with WSSV Thai-1 may be due to a higher number of susceptible cell types that can be infected. Six cell types have been differentiated within gills: chief cells, pillar cells, striated cells, glycocytes, nephrocytes and granular cells (Felgenhauer 1992). Genomic differences between WSSV Thai-1 and WSSV Viet have been demonstrated (JM Vlak, personal communication) and these differences may result in infecting different cell types in the host. With other viruses, it has already been shown that genomic variation in viruses may result in a shift of receptor usage and as a consequence of target cell (Baranowski et al. 2001). The lack of cell markers for these cell types hinders further research to confirm the hypothesis on cell specificity. Third, the host defense response during WSSV Thai-1 infection may be less effective from that during a WSSV Viet infection. Recent studies showed the existence of antiviral defense in shrimp including RNA interference (Robalino et al. 2007), the production of an interferon-like protein (He et al. 2005) and an antipolysaccharide factor (Liu et al. 2006) and upregulation of antiviral gene *PmAV* expression (Luo et al. 2007) in WSSV infected animals.

The early mortalities (36 hpi) of a few shrimp inoculated with the less virulent WSSV Viet, showing a low number of infected cells in the gills indicate the involvement of other factors in causing death than the degree of replication. One of the other factors might be hemocyte infiltration in infected tissues that may induce tissue damage leading to dysfunction.

Individual variations in numbers of infected cells were observed in shrimp inoculated with both WSSV isolates. These individual variations in viral load of shrimp were reported earlier (Tan et al. 2001, Durand & Lightner 2002) and can be explained by differences in degree of virus replication and defense response of the host.

In conclusion, this study showed that an intramuscular inoculation with 30 SID_{50} in *P. vannamei* juveniles can be used as a tool to compare virulence of WSSV isolates. There were

clear indications that the degree of replication in certain tissues, especially gills, may determine the virulence of isolates.

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CHAPTER 4

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) shrimp *Litopenaeus vannamei* juveniles. 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 inoculated orally with WSSV at a dose of 30 SID_{50} (SID_{50} = shrimp infectious dose with 50% endpoint) and clinical signs and mortality were followed for 120 h post inoculation (hpi). WSSV infection status 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 h by cidofovir treatment. The 100% mortality level was reached at 96-108 hpi in mock treated shrimp, whereas in cidofovir treated shrimp, 80-90% mortality was reached 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 h but had no effect on the cumulative mortality at the end of the experiment. This study opens perspectives for antiviral drugs to treat shrimp infected with WSSV.

Keywords: *Litopenaeus vannamei,* SPF, WSSV, antiviral, cidofovir, *Spirulina platensis*

1. **Introduction**

White spot syndrome (WSS) is one of the most damage causing viral 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, yearly causing billions of dollars losses to the shrimp farming industry worldwide. WSS virus (WSSV) is an enveloped double stranded DNA virus with a bacilliform morphology measuring 270x120 nm in size and a tail-like extension at one end. Based on the genomic information, and the phylogenetic analysis of the viral DNA polymerase, WSSV is assigned to a new virus family, the *Nimaviridae*, genus *Whispovirus* (Van Hulten et al., 2001).

Different approaches knew already some success to control WSSV, including (i) higher or lower than normal water temperatures (Vidal et al., 2001, Guan et al., 2003 and Jiravanichpaisal et al., 2004), (ii) treatment with the immunostimulants peptidoglycan, lipopolysaccharide and ß-1,3 glucan (Itami et al., 1998, Takahashi et al., 2000 and Chang et al., 2003), (iii) vaccination with formalin inactivated WSSV and WSSV envelope proteins VP19 and VP28 (Namikoshi et al., 2004, Witteveldt et al, 2004) and (iv) feeding antiviral fucoidan, a sulfated polysaccharide extracted from *Sargassum polycystum* supplemented diet (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 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 mode of action of cidofovir is blocking DNA synthesis through incorporation of the HPMPCpp molecule in the viral DNA. Cidofovir has already been proven to be effective against the following human DNA viruses: polyomavirus, papillomavirus, adenovirus, herpes virus and poxvirus (De Clercq, 2003).

Spirulina platensis is a marine blue green alga. It contains calcium spirulan, a sulfated polysaccharide that can be extracted using hot water. This polysaccharide consists of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid,

galacturonic acid, sulfate, and calcium. It inhibits *in vitro* 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) (Hayashi and Hayashi, 1996). The concentration of calcium spirulan that reduces viral replication by 50% was between 11.4 to 2600 µg/ml added to the medium immediately after viral infection. *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, it was examined if intramuscular injection of cidofovir and feeding shrimp a diet supplemented with *S. platensis* have an effect on the outcome of a WSSV infection in shrimp.

2. Materials and methods

2.1 Virus

A Thai isolate of WSSV from naturally-infected *Penaeus monodon* was passaged once into crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001). Gills from infected crayfish were collected and suspended in L-15 medium (1%). A 1:10 dilution of gill suspension was prepared in phosphate-buffered solution (PBS) pH 7.4, and injected intramuscularly (50 µl) into SPF *L. vannamei* to amplify the virus. Moribund and dead shrimp were collected at 48 h post inoculation (hpi). Carcasses without hepatopancreas, gut and exoskeleton were minced. A 1:10 dilution of the suspension was made and centrifuged subsequently at 3000 g and 13000 g at 4ºC for 20 min. The supernatant was filtered (0.45µm) and aliquoted for storage 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 presence of only WSSV DNA. The viral titre of the stock was calculated by an *in vivo* oral inoculation procedure based on Reed and Muench (1938). The viral titre was $10^{5.6}$ SID_{50} per ml (oral) (Escobedo-Bonilla et al., 2005).

2.2 Shrimp

SPF *L. vannamei,* Kona-strain from Ceatech farm, Hawaii were acclimatized and reared at the facilities of the Laboratory of Aquaculture and *Artemia* Reference Center (ARC), Ghent University. Shrimp used for testing cidofovir toxicity, testing the antiviral properties of cidofovir and the effect of feeding a *Spirulina* supplemented diet had MBW of 2.7g (n=36), 5.6 g (n=78) and 9.4 g (n=20) respectively.

2.3 Experimental conditions

Shrimp reared at the ARC were acclimatized to a salinity of 15 g/l over a period of four days, and then transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the challenge procedure was carried out under biosafety conditions. Six to ten animals were housed in 50 l aquaria equipped with aeration, mechanical filtration (Eheim, Germany) and heating (aquarium heater set at 27ºC, Model VTX 300, Aquarium systems, France). The salinity was maintained at 15 g/l using artificial seawater (Instant Ocean, Aquarium systems, France) prepared with distilled water. Six pellets of a normal shrimp diet were fed per shrimp per day equally divided in the morning and afternoon. Water quality (NH_4^+ and NO_2^-) were monitored daily using test kits (Aquamerck, Germany). The maxium value was 3 mg/l for NH_4^+ and 0.05 mg/l for NO_2^-.

2.4 Cidofovir

2.4.1 Toxicity

The toxicity of cidofovir was tested first. Fifty microliter of six cidofovir concentrations, (0, 12.5, 25, 50, 100 and 200 mg/kg shrimp body weight) were prepared in PBS. The concentrations of cidofovir were injected intramuscularly between the 3rd and 4th abdominal segment of the shrimp. Six shrimp were used for each concentration. All animals were followed clinically for 120 h.

2.4.2 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.

2.5 Feeding shrimp with a diet supplemented with Spirulina

Shrimp were fed a *Spirulina* supplemented diet (n=10) or a normal diet (n=10) at 5% MBW per day for four days before challenge with WSSV. 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, fibre, 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 normal diet and diet supplemented with *S. platensis*

	Composition (% w/w)	
Ingredient	normal diet	diet supplemented with *S. platensis*
S. platensis meal	-	25.0
Fish meal	40.0	22.0
Wheat flour	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
	100.0	100.0

2.6 WSSV challenge procedure

Shrimp were inoculated orally with WSSV at a dose of 30 SID_{50} [oral] in 50µl. After inoculation, shrimp were examined clinically, twice a day for 5 days. Clinical signs included empty gut and lethargy. Mortality was recorded twice daily.

2.7 Evaluation of the infection status by indirect immunoflourescence (IIF)

The cephalothoraxes of dead, moribund and euthanised shrimp were dissected longitudinally and embedded in 2% methylcellulose and quickly frozen at -20°C. Tissues were cryosectioned longitudinally with 5 µm thickness, fixed in 100% methanol at -20ºC for 20 minutes. Sections were washed three times for five minutes in PBS and incubated with monoclonal antibody 8B7 (DiagXotics Inc., USA) directed against VP28 of WSSV (Poulos et al., 2001) for one hour at 37ºC. Then, sections were washed three times for five minutes in PBS and incubated with goat anti-mouse Ig-FITC (Molecular Probes, Eugene, Oregon) for another hour at 37ºC. Sections were finally washed in PBS and deionised water, dried and mounted in glycerin with 1, 4-diaza-bicyclo[2.2.2]octan (DABCO) (ACROS organics, USA). Slides were analyzed by using a fluorescence microscope.

2.8 Statistical analyses

The cumulative mortality of the two experiments of cidofovir 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:

Probit (x) = $\alpha + \beta$ time + γ treatment + δ time* 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:

Probit (x) = $\alpha + \beta$ time + γ 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.

3. Results

3.1 Cidofovir

Toxicity - none of the concentrations tested caused disease (evaluated by observation of empty gut and lethargic movement) in shrimp. Hence, it was decided to use the maximum dose of 200 mg/kg body weight to further assess its antiviral effect during a WSSV infection.

Efficacy - Mock treated and cidofovir treated shrimp started to show clinical signs including empty gut and lethargic movement at 24-36 hpi. Cidofovir treatment delayed mortality with approximately 24 h 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).

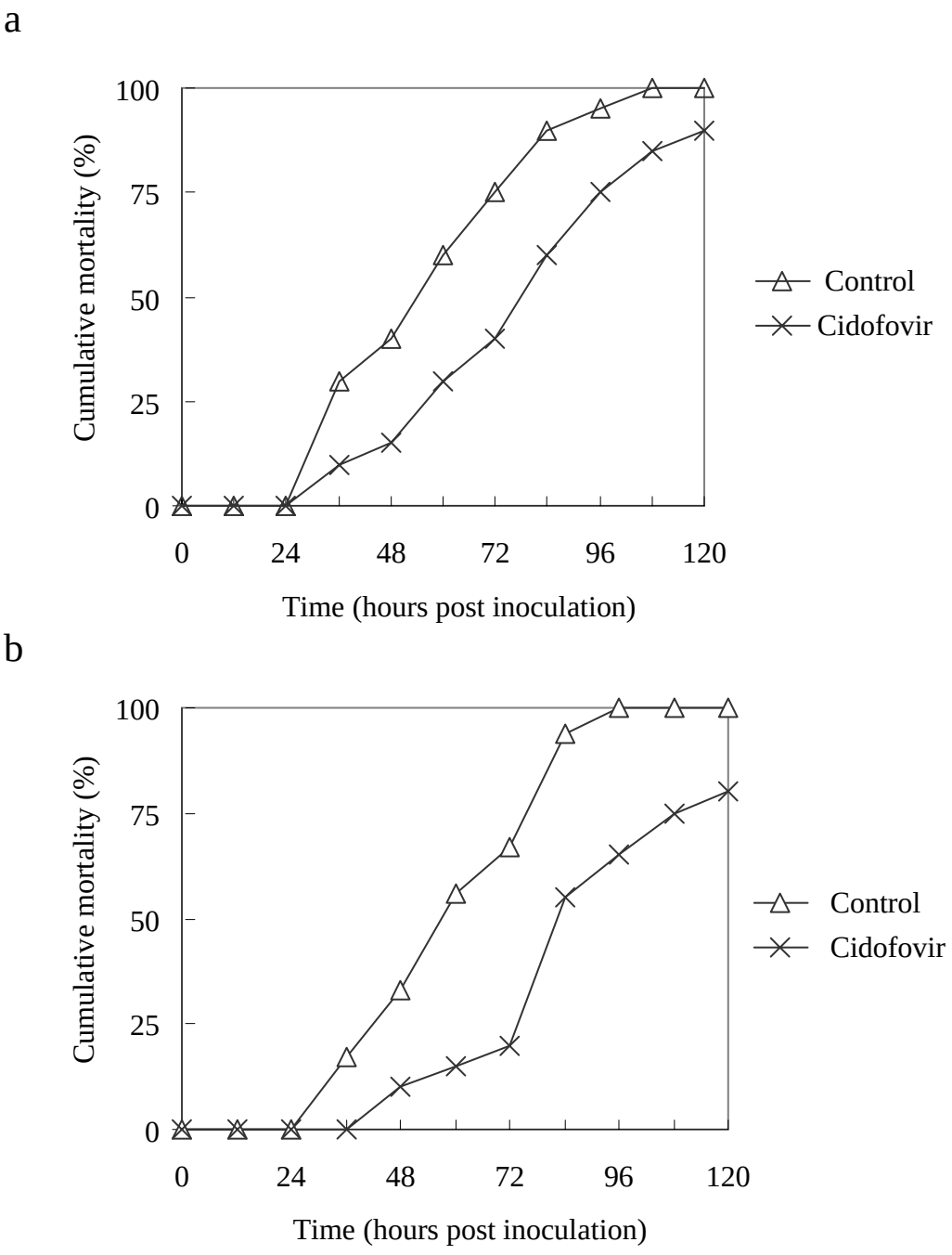

Figure 1. Cumulative mortality (%) of SPF *Litopenaeus vannamei* juveniles upon WSSV challenge. Shrimp were injected intramuscularly with 50µl of cidofovir (200 mg/kg shrimp body weight) or with 50µl of PBS (mock). All shrimp were challenged with 30 SID_{50} [oral] of WSSV, a) experiment 1 and b) experiment 2.

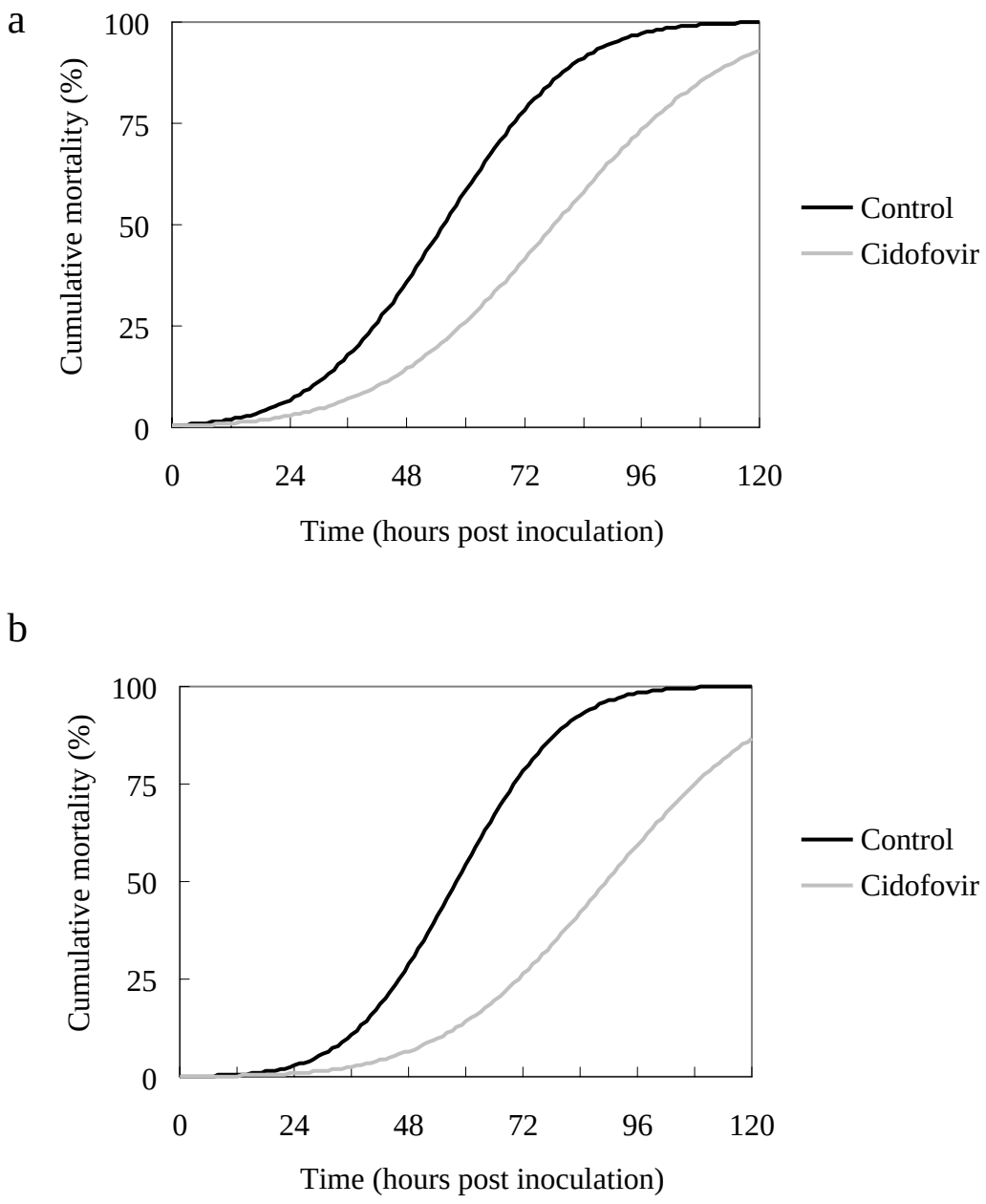

Figure 2. Probit regression of the cumulative mortality of shrimp treated with cidofovir and mock-treated, a) experiment 1 and b) experiment 2.

Table 2. Probit analysis in the median lethal times (LT_{50}) of shrimp treated with cidofovir and mock treated.

Experiment	Group	α	β	γ	δ	LT_{50}	Comparison (p = 0.05)
1	Control	-2.23518	0.040335	0	-	55.41	Significant difference
	Cidofovir	-2.23518	0.040335	-0.90197	-	77.78	
2	Control	-3.24922	0.056055	0	0	57.97	Significant difference
	Cidofovir	-3.24922	0.056055	0.00690	-0.01963	89.02	

3.2. S. platensis

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 were found WSSV positive by IIF.

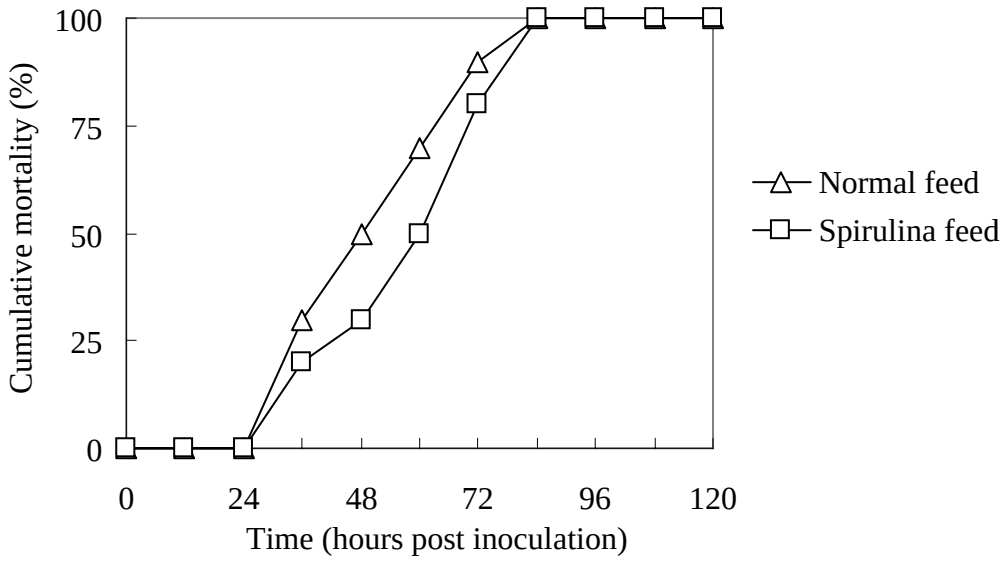

Figure 3. Effect of *Spirulina* extract on cumulative mortality (%) of SPF *Litopenaeus vannamei* juveniles upon WSSV challenge. Shrimp were fed normal feed or a diet supplemented with *Spirulina* during 4 days before challenge at 5% body weight per day. All shrimp were challenged with 30 SID_{50} [oral] of WSSV.

4. Discussion

In this study, a standardized WSSV challenge procedure was used to test two candidate antiviral products, cidofovir and *Spirulina platensis* extract. Only shrimp treated with cidofovir considerably delayed the mortality in WSSV infected shrimp (with 24 h).

In the past, inoculation methods that mimic natural routes of infection such as feeding of infected tissues or submersion of 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, there is a need for a more reproducible WSSV challenge procedure (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 was quite 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. Other authors already found indications for the existence of differences in virulence of WSSV strains and in susceptibility of shrimp (Wang et al, 1999). In this study, the infectious doses of virus inoculated per 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 with different DNA viruses in mammals (De Clercq, 2003). At least three different explanations can be proposed why it did not protect sufficiently shrimp against a fatal outcome of a WSSV infection. Firstly, the cellular uptake of cidofovir by shrimp cells may be lower than that in 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 esterified cidofovir, which undergoes a better cellular uptake. Secondly, 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 its metabolites (De Clercq, 2003), a low concentration of the latter will not be able to stop viral DNA synthesis. Thirdly, the lower effectiveness of

cidofovir to WSSV 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 with the results obtained by Chotigeat et al., (2004) with 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.

5. Acknowledgements

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CHAPTER 5

Testing of the potential of high water temperature (33°C) to control WSSV infection and disease

CHAPTER 5.1

Effect of high water temperature (33°C) on the clinical and virological outcome of experimental infections with white spot syndrome virus (WSSV) in specific pathogen-free (SPF) *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 inoculated 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 inoculation and 27ºC afterwards, or (v) kept at 27°C before inoculation and 33°C afterwards. Shrimp were maintained at the respective temperatures for 120 h before inoculation and 120-144 h post inoculation (hpi). 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 after inoculation developed gross signs within 24 hpi, first mortalities at 36-60 hpi and 100% cumulative mortality between 60 and 144 hpi depending on the virus titer. All dead shrimp were WSSV-positive. In contrast, shrimp kept at 33°C continuously or after WSSV inoculation 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 inoculation and one switched from 27°C to 33°C after inoculation with 10000 SID_{50}. Immunohistochemistry (IHC) analysis showed that WSSV-positive cells were first displayed at 12 hpi in shrimp kept at 27°C and by 24 hpi the infection became systemic. In contrast, shrimp kept at 33°C did not display WSSV-positive cells at 12 or 24 hpi. 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.

Key words: WSSV replication, VP28, water temperature, *Litopenaeus vannamei*, SPF

1. **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 inoculation 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).

2. **Materials and methods**

2.1 Virus and infectivity titers

A Thai isolate of WSSV was kindly provided by P. Jiravanichpaisal and K. Söderhäll (Uppsala University, Sweden). 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).

2.2 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.

2.3 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 inoculation, 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).

2.4 Experimental design

2.4.1 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 inoculation to 33°C afterwards,

or (iv) from 33°C before inoculation 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 hpi 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 inoculation and switched to 33°C after inoculation. All these shrimp were orally inoculated 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 inoculation (hpi) and were done only once.

2.4.2 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 inoculation, 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 hpi. Tissues from the cephalothorax 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.

2.5 Evaluation of WSSV infection by indirect immunofluorescence (IIF)

The cephalothoraxes 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 µg 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.

2.6 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.

3. **Results**

3.1 Intramuscular inoculation

3.1.1 Low virus titer

Shrimp maintained continuously at 27°C or switched from 33°C to 27°C after WSSV inoculation started to show signs of disease at 24 hpi. Mortality was first recorded between 36 - 60 hpi (27°C/27°C) and 36 - 48 hpi (33°C/27°C) and cumulative mortality reached 100% at 84 - 144 hpi (27°C/27°C) and 72-96 hpi (33°C/27°C). All dead shrimp were WSSV-positive. 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 inoculation did not develop signs of disease and showed cumulative mortality between 0% and 30%. In these groups, all shrimp were WSSV-negative. Dead shrimp were found to have molted recently. Body parts such as eyestalks, pleopods, uropods and cephalothoraxes were eaten by the surviving shrimp.

3.1.2 High virus titer

Shrimp kept continuously at 27°C or switched from 33°C to 27°C after inoculation first showed signs of disease between 12 and 24 hpi and almost all shrimp stopped eating at 24 hpi. Mortality started at 36 hpi and 100% cumulative mortality was reached at 60 hpi in both groups. All dead shrimp were WSSV-positive. 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 inoculation did not show signs of disease and had cumulative mortality between 0% and 20%. All dead and surviving shrimp were WSSV-negative and the dead shrimp found in these groups had molted recently.

3.2 Oral inoculation

Shrimp kept continuously at 27°C and 30°C first showed signs of disease at 24 hpi. First mortality occurred at 36 hpi and cumulative mortality in these groups reached 100% at 96 and 108 hpi, respectively. All dead shrimp were positive by IIF. Probit analysis showed significant differences ($p<0.05$) in the median lethal times (LT_{50}) of these two temperatures

(Table 1 and Fig. 3a, b). 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 (Fig. 4a, b).

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]	2.948	-0.058	----	----	
30°C / 30°C	Oral	Low	48.93[b]	2.948	-0.058	-0.357	0.00538	
27°C / 33°C	Oral	Low	----	2.948	-0.058	1.1414	0.00583	b = a < d
33°C / 33°C	Oral	Low	394.9[d]	2.948	-0.058	-0.599	0.05239	
27°C / 27°C	IM	Low	73.29[e]	2.858	-0.039	----	----	
33°C / 27°C	IM	Low	57.05[f]	2.858	-0.039	1.4556	-0.0366	
27°C / 33°C	IM	Low	257.8[g]	2.858	-0.039	-0.453	0.02966	f < e < g = h
33°C / 33°C	IM	Low	374.6[h]	2.858	-0.039	-0.168	0.0318	
27°C / 27°C	IM	High	45.2[i]	5.188	-0.115	----	----	
33°C / 27°C	IM	High	42.0[j]	5.188	-0.115	0.1690	-0.0128	
27°C / 33°C	IM	High	260.1[k]	5.188	-0.115	-2.766	0.1054	j = i < l = k
33°C / 33°C	IM	High	188.7[l]	5.188	-0.115	-1.58	0.0956	

‡temperature before inoculation/temperature after inoculation

*differences in LT_{50} are significant ($P < 0.05$)

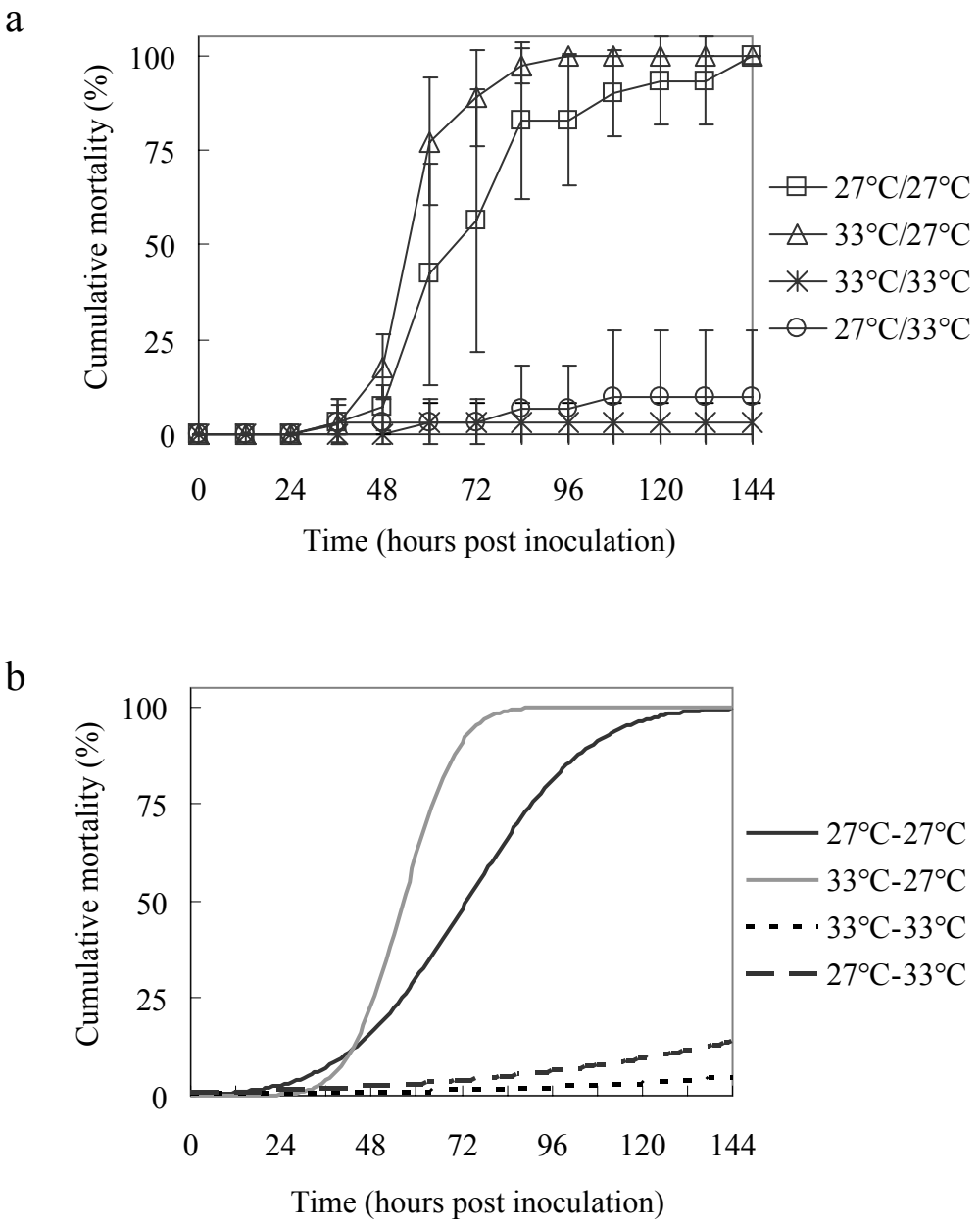


Figure 1. (a) Cumulative mortality (mean ± standard deviation [SD]) of SPF *Litopenaeus vannamei* intramuscularly inoculated with 30 SID_{50}. Shrimp were continuously kept at 27°C or 33°C, or switched from 33°C before inoculation to 27°C afterwards, or changed from 27°C before inoculation to 33°C afterwards.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

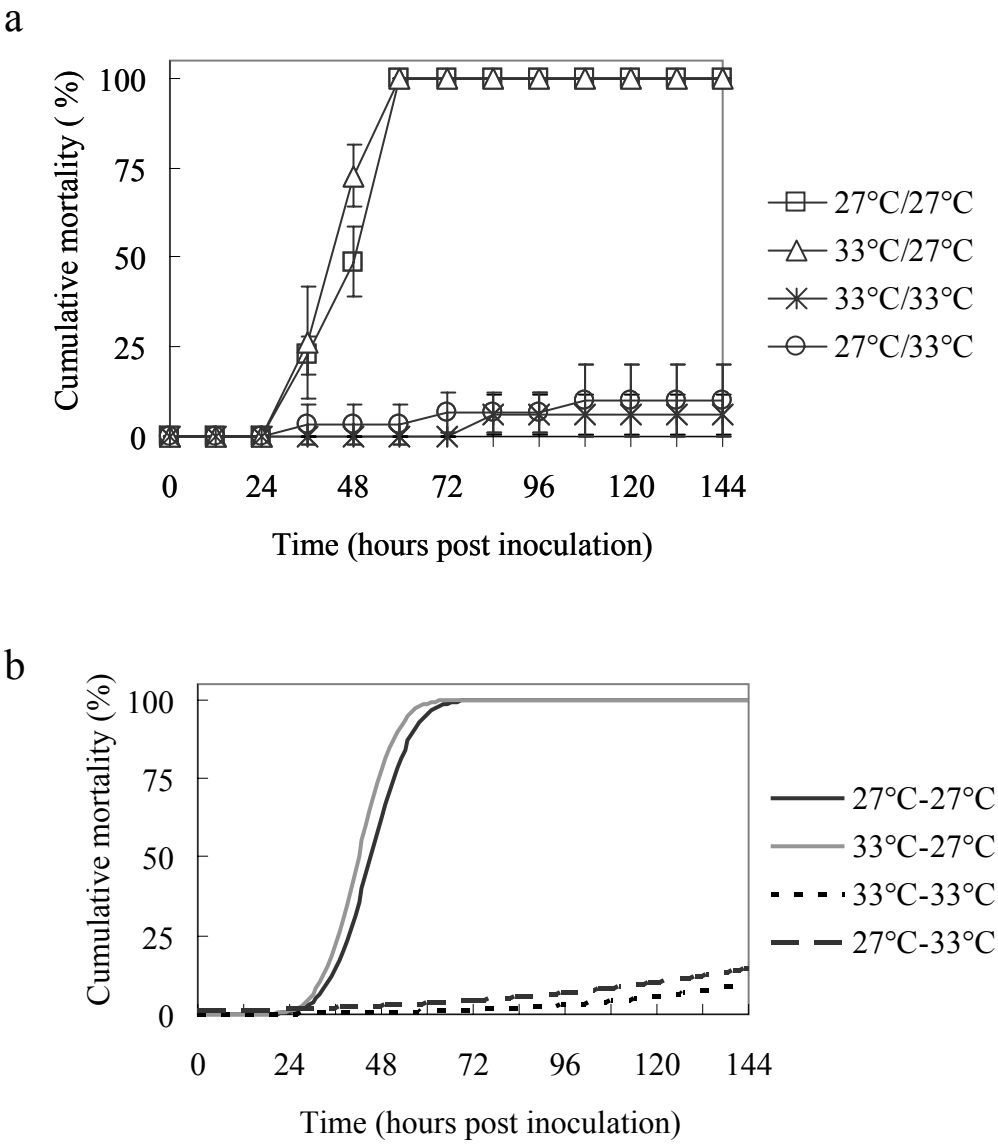
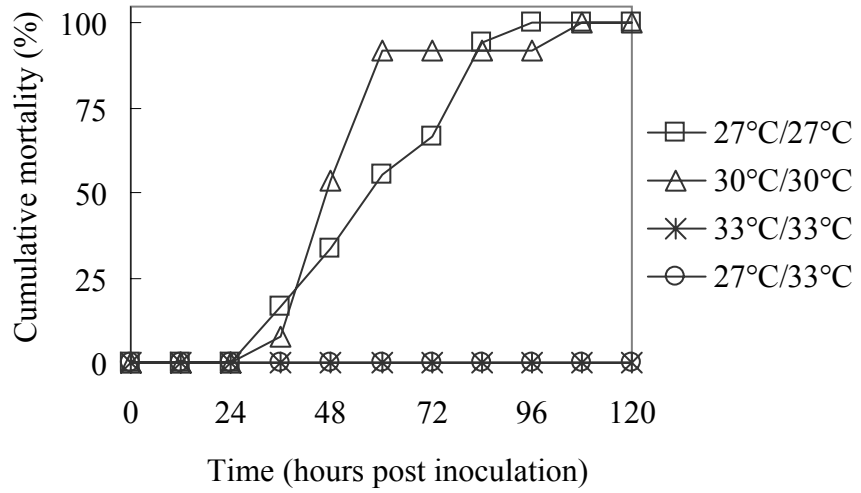


Figure 2. (a) Cumulative mortality (mean ± SD) of SPF *Litopenaeus vannamei* intramuscularly inoculated with 10000 SID_{50} of WSSV. Shrimp were continuously kept at 27°C or 33°C, or kept at 33°C before inoculation and switched to 27°C after inoculation, or kept at 27°C before inoculation and switched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

a



b

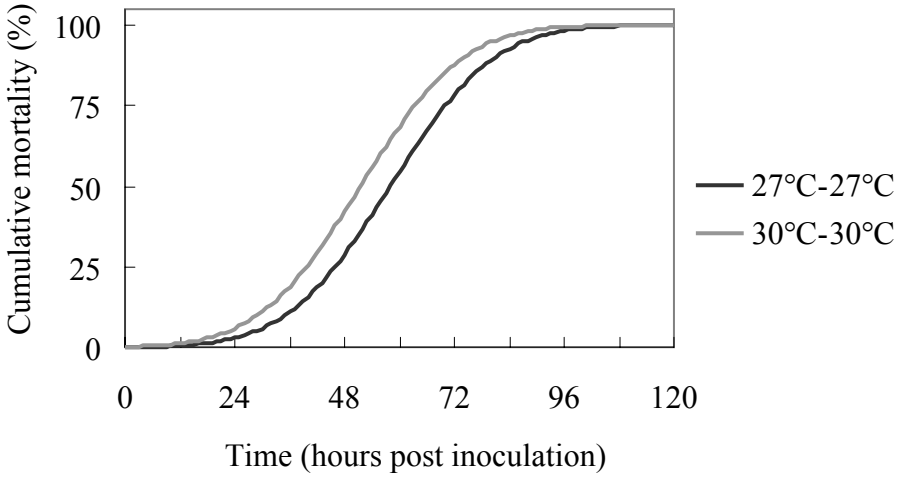


Figure 3. (a) Cumulative mortality of SPF *Litopenaeus vannamei* continuously kept at 27°C, 30°C, 33°C or maintained at 27°C before inoculation and switched to 33°C after WSSV oral inoculation. Shrimp were inoculated with 30 SID_{50}. (b) Probability of mortality (probit) for the different temperature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

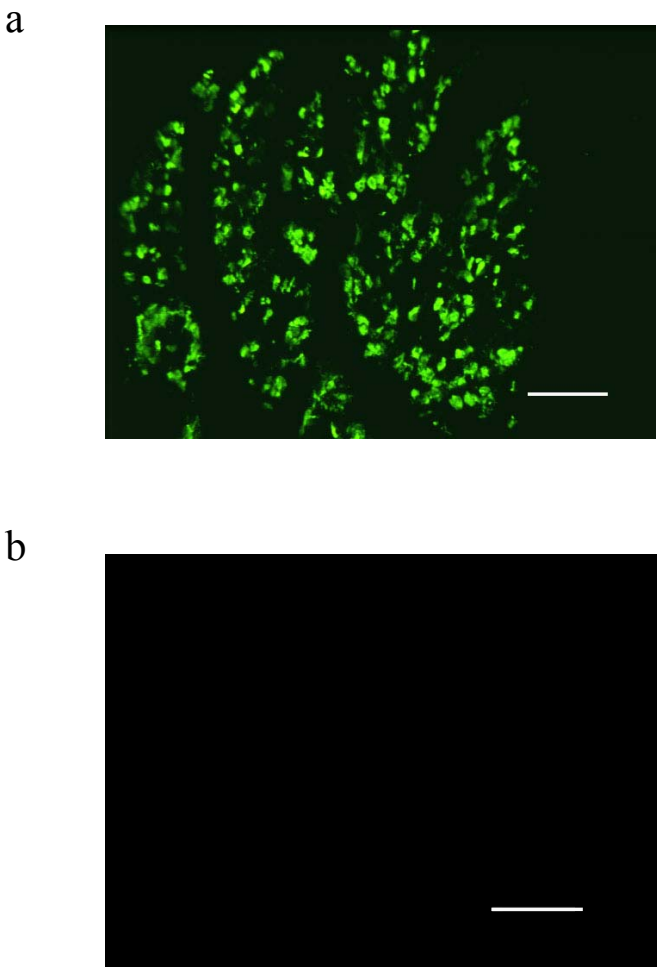
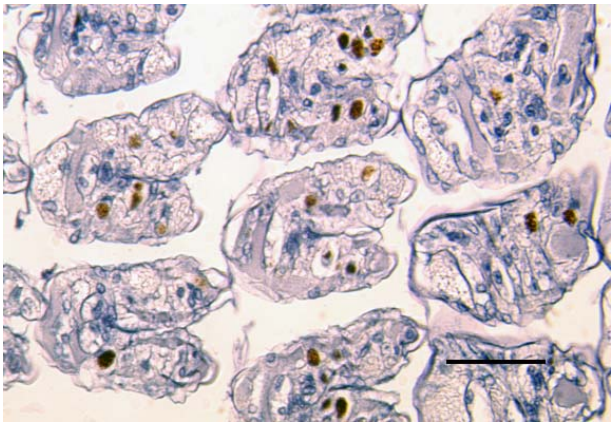


Figure 4. (a) WSSV-positive and (b) WSSV-negative cells in gills of SPF *Litopenaeus vannamei* as determined by indirect immunofluorescence (IIF). Magnification 200X, bar = 100 μm.

3.3 Time course

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

a



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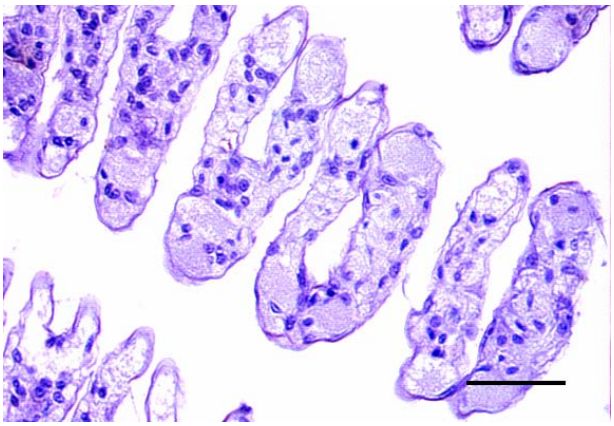

Figure 5. (a) WSSV-positive and (b) WSSV-negative cells in gills of SPF *Litopenaeus vannamei* as determined by immunohistochemistry (IHC). Magnification 400X, bar = 50 µm

4. **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. Taken together, these studies indicate that high temperature may affect enzyme activity during different phases of early viral replication in ds DNA viruses. In WSSV, it is not known which enzymes are impaired by high water temperature. It may be proposed that in WSSV infection, the activity of the DNA polymerase (van Hulten et al., 2001, Yang et al., 2001, Chen et al., 2002) or the protein kinase (van Hulten and Vlak, 2001) is impaired by high water temperature. This hypothesis should be confirmed 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 inoculation was sufficient to block viral replication at an early stage, resulting in the inhibition of expression of the structural protein VP28. The hypothesis that the activity of one or more molecules involved in DNA synthesis and expression of late proteins may be affected by high water temperature should be further confirmed by biochemical assays. High water temperature may be applied in nursery and grow-out facilities in tropical shrimp farming countries to control WSSV.

5. **Acknowledgements**

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CHAPTER 5.2

Impact of daily fluctuations of optimum (27°C) and high water temperature (33°C) on *Penaeus vannamei* juveniles infected with white spot syndrome virus (WSSV)

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Abstract

This study evaluated the effect of daily fluctuations between optimum (27°C) and high water temperature (33°C) on the clinical and virological outcome of a WSSV infection in *Penaeus vannamei* juveniles. Shrimp were inoculated intramuscularly with a dose of 10000 SID_{50} of either virulent WSSV Thai-1 or less virulent WSSV Viet or mock inoculated. Temperature was kept either continuously at 27°C or switched from 27°C to 33°C at 12 hours post inoculation (hpi) and maintained at that temperature for 6 h (6 h 33°C), 12 h (12 h 33°C), 18 h (18 h 33°C) or 24 h (24 h 33°C) per day. Temperature was then lowered and maintained at 27°C for the remaining hours of the day. The experiments ran for 132 hpi with WSSV Thai-1 and 168-192 hpi with WSSV Viet. WSSV infections were demonstrated by indirect immunofluorescence (IIF).

Shrimp kept continuously at 27°C, started to show clinical signs at 24 hpi and mortalities started at 36 hpi with both strains. Cumulative mortalities reached 100% at 60 hpi with WSSV Thai-1 and at 168-192 hpi with WSSV Viet in two experiments. At constant 33°C (24 h 33°C), mortalities were 0% with WSSV Thai-1 and 5% or 10% with WSSV Viet. With exposure to 33°C for 6 or 12 h per day (6 h 33°C or 12 h 33°C), cumulative mortalities reached 90-100% at 72-96 hpi with WSSV Thai-1. With WSSV Viet cumulative mortalities reached 100% at 84-96 hpi or 50-95% at 96-108 hpi with 33°C for 6 h or 12 h per day. Mortalities were clearly reduced with WSSV Thai-1 (0-40%) and WSSV Viet (5-15%) at 33°C for 18 h per day. At constant 27°C and at 33°C for 6 h or 12 h per day, dead shrimp were WSSV positive and euthanized shrimp were WSSV negative. At 33°C for 18 h or 24 h per day, the dead and euthanized shrimp were WSSV negative except the dead shrimp at 18 h 33°C with Thai-1 were WSSV positive.

The present study showed that daily temperature fluctuations have negative (6 h 33°C with WSSV Viet) or positive effects (6h 33°C with WSSV Thai-1 and 12 h or 18 h 33°C with both strains) on disease and mortality of shrimp infected with WSSV.

Key words: WSSV, temperature fluctuations, *Penaeus vannamei*

1. Introduction

Water temperature is an important environmental factor for shrimp. It has a direct influence on metabolic rate (Allan et al., 2006), growth and survival of shrimp (Wyban et al., 1995). The optimum temperature for growth and survival of *Penaeus vannamei* juveniles of more than 5 g is 27°C (Wyban et al., 1995). Water temperature in shrimp farms fluctuates diurnally and seasonally as it depends on air temperature, water depth, pond design and water management. Water temperature can easily reach 33°C in shrimp ponds at least for several hours per day in many tropical countries such as Bangladesh (Wahab et al., 2003), China (Wang et al., 2005), Thailand (Thongrak et al., 1997), Vietnam (Alongi et al., 1999) and Mexico (Ruiz-Fernández and Páez-Osuna, 2004).

White spot syndrome virus (WSSV) is an enveloped, double stranded DNA virus (van Hulten et al., 2001) of the family *Nimaviridae*, genus *Whispovirus* (Mayo, 2002). This pathogen causes disease and mortality in many shrimp producing countries. Cumulative mortality of WSSV infected shrimp may reach 100% within 3-10 days (Lightner, 1996). At optimum temperature (26°C-27°C), differences in virulence between WSSV strains have been reported (Wang et al., 1999, Rahman et al., 2006b). High water temperature (32-33°C) reduces mortality in WSSV inoculated *P. vannamei* postlarvae and juveniles (Vidal et al., 2001, Rahman et al., 2006a). At 33°C, an inhibition of WSSV replication (Rahman et al., 2006a) and a reduction of viral load (Granja et al., 2006) have been shown as possible explanations for this reduced mortality.

This present study evaluated the effect of daily fluctuations of optimum (27°C) and high water temperature (33°C) on the clinical and virological outcome of specific pathogen free (SPF) shrimp *Penaeus vannamei* juveniles inoculated with either of two WSSV strains with difference in virulence (Rahman et al., 2006b).

2. Materials and methods

2.1 *Virus*

Two WSSV strains were used in this study. WSSV Thai-1 was collected from naturally infected *Penaeus monodon* in Thailand and passaged in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001). WSSV Viet was collected from naturally infected *Penaeus monodon* in Vietnam and passaged in crayfish *Cherax quadricarinatus*. Crayfish gill suspension of WSSV Thai-1 and WSSV Viet was kindly provided by K. Söderhäll (Uppsala University, Sweden) and Research Institute for Aquaculture - 2 (RIA-2), Vietnam, respectively. Both strains were amplified in specific pathogen free (SPF) *Penaeus vannamei* juveniles in the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University, Belgium and infectivity titers of stocks were determined according to the procedure described by Escobedo-Bonilla et al., (2005). The median infectious titres of stocks determined by intramuscular inoculation in SPF *P. vannamei* were $10^{5.9}$ and $10^{5.8}$ SID_{50} per ml for WSSV Thai-1 and WSSV Viet, respectively.

2.2 *Shrimp*

A total of 517 shrimp were used in the four experiments of the present study. Mean body weights (MBW) of shrimp were 4.9±1.3 g, 15.7±2.6 g and 6.5±1.7 g, 8.6±2.1 g in the first and second experiment with WSSV Thai-1 and WSSV Viet, respectively.

2.3 *Experimental conditions*

SPF *P. vannamei* from Molakai farm, Hawaii (USA) were imported at early postlarval (PL) stage and reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Faculty of Bioscience Engineering, Gent University, Belgium. Rearing conditions were: water temperature 27-28°C, salinity 34-36 g/l. Before each experiment, shrimp were gradually acclimatized to the salinity of 15 g/l at the ARC over four days. Acclimatized shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University for WSSV challenge studies. Nine to eleven shrimp were housed per 50 litre aquarium, equipped with aeration, mechanical filtration

(Eheim, Germany), water pump (Eheim, Germany) and aquarium heater (Model VTX 300, Aquarium systems, France). Brackish water with a salinity of 15 g/l was prepared using artificial sea salt (Instant Ocean, Aquarium systems, France) and de-ionized water. Water temperature was maintained at 27°C before inoculation and also during the first twelve hours post inoculation (hpi). From 12 hpi, temperature was maintained either at 27°C or switched to 33°C depending on temperature fluctuation regimes described below. Approximately 0.2 g of a commercial shrimp diet was provided for each shrimp per day. About 80% brackish water were renewed at 120 hpi in experiments with WSSV Viet. Water quality was checked by measuring ionized ammonia (NH_4^+) using test kits (Aquamerck, Germany).

2.4 *WSSV inoculation procedure*

Shrimp were inoculated intramuscularly with 50 µl of inoculum containing 10000 SID_{50} of either WSSV Thai-1 or WSSV Viet in the junction between the third and fourth abdominal segments. Mock inoculated (MI) shrimp were inoculated with 50 µl of phosphate buffered saline (PBS) only. Inoculated shrimp were observed for clinical signs including anorexia and lethargia and mortality was recorded every 12 h till the end of experiment.

2.5 *Temperature fluctuations*

Two temperatures, 27°C and 33°C, were used for daily temperature fluctuation regimes. WSSV inoculated and mock inoculated shrimp were kept at 27°C till 12 hours post inoculation (hpi). Starting from 12 hpi, different regimes of temperature fluctuations were used. WSSV inoculated shrimp were kept either continuously at 27°C (0 h 33°C) or temperature was increased to 33°C at 12 hpi and maintained for 6 h (6 h 33°C), 12 h (12 h 33°C), 18 h (18 h 33°C), 24 h (24 h 33°C) per day. Nineteen to twenty one WSSV inoculated shrimp were used per temperature regime. After exposure to 33°C (6 h, 12 h and 18 h), temperature was lowered and maintained at 27°C for the rest of the day. This temperature regime was repeated every 24 h up to 132 hpi with WSSV Thai-1 and 168-192 hpi with WSSV Viet inoculated shrimp. MI shrimp were also exposed to the temperature fluctuation regimes. Ten MI shrimp were used per temperature regime. At the end of the experiment surviving shrimp were euthanized.

2.6 Evaluation of WSSV infection

The cephalothoraxes of dead and euthanized shrimp were 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) in PBS and incubated with 2 µg ml^{-1} of the monoclonal antibody 8B7 directed against VP28 for 1 h at 37ºC. Then, sections were washed 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.

2.6 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: Probit (x) = $\alpha + \beta$ time + γ temperature + δ time* 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-test using the same statistical software.

3. **Results**

3.1 *WSSV Thai-1*

Shrimp kept continuously at 27°C (0 h 33°C), showed clinical signs within 24 hpi. Mortalities started at 36 hpi (25-30%) and cumulative mortalities reached 100% at 60 hpi in the both experiments (Figure 1). At constant 33°C (24 h 33°C), no clinical signs were observed and no mortality occurred. Shrimp exposed to 33°C for 6 h (6 h 33°C), 12 h (12 h 33°C) or 18 h (18 h 33°C) per day, started to show clinical signs at 24-36 hpi. Mortalities started at 36 hpi in 6 h 33°C shrimp (5% or 10%), at 48 hpi in 12 h 33°C shrimp (15.8%) and in 18 h 33°C shrimp (10% or 0%) in the first or second experiment. Cumulative mortalities reached 100% at 72 hpi or at 96 hpi in 6 h 33°C shrimp, 100% or 90% at 96 hpi in 12 h 33°C shrimp and 40% at 84 hpi or 0% in 18 h 33°C shrimp. Out of two experiments, only one mock inoculated shrimp died at 33°C for 18 h per day (MI 18 h 33°C). At continuously 27°C, or at 33°C for 6 h, 12 h and 18 h per day, dead WSSV inoculated shrimp were WSSV positive while euthanized WSSV inoculated and dead and euthanized mock inoculated shrimp were all WSSV negative. At constant 33°C, dead and euthanized shrimp were WSSV negative. Significant differences ($p<0.05$) in the median lethal times (LT_{50}) were found between the groups of temperature fluctuation regimes (Table 1 and Figure 2).

3.2 WSSV Viet

Shrimp kept continuously at 27°C (0 h 33°C), started to show clinical signs at 24 hpi. Mortalities started at 36 hpi (15%) or at 48 hpi (25%) and cumulative mortalities reached 100% at 168 hpi or at 192 hpi, in the first or second experiment (Figure 3). At constant 33°C (24 h 33°C), shrimp did not show clinical signs and cumulative mortalities were 5% or 10%. Shrimp exposed to 33°C for 6 h (6 h 33°C) and 12 h (12 h 33°C) per day, started to show clinical signs at 24-36 hpi. Mortalities started at 48 hpi (25% or 5%) in 6 h 33°C shrimp and at 48 hpi (35%) or at 60 hpi (5%) in 12 h 33°C shrimp in two experiments. Cumulative mortalities reached 100% at 84 hpi or at 96 hpi in 6 h 33°C shrimp, 95% at 108 hpi or 50% at 96 hpi in 12 h 33°C shrimp. At 33°C for 18 h per day (18 h 33°C), shrimp did not show clinical signs and cumulative mortalities were 15% or 5%. None of the mock inoculated shrimp died in both experiments. At continuously 27°C or at 33°C for 6 h and 12 h per day, dead shrimp were WSSV positive and euthanized shrimp were WSSV negative. All of the euthanized WSSV inoculated shrimp kept continuously or at least 18 h per day at 33°C and mock inoculated shrimp were WSSV negative. Significant differences ($p<0.05$) in the median lethal times (LT_{50}) were found between the groups of temperature fluctuation regimes (Table 1 and Figure 4).

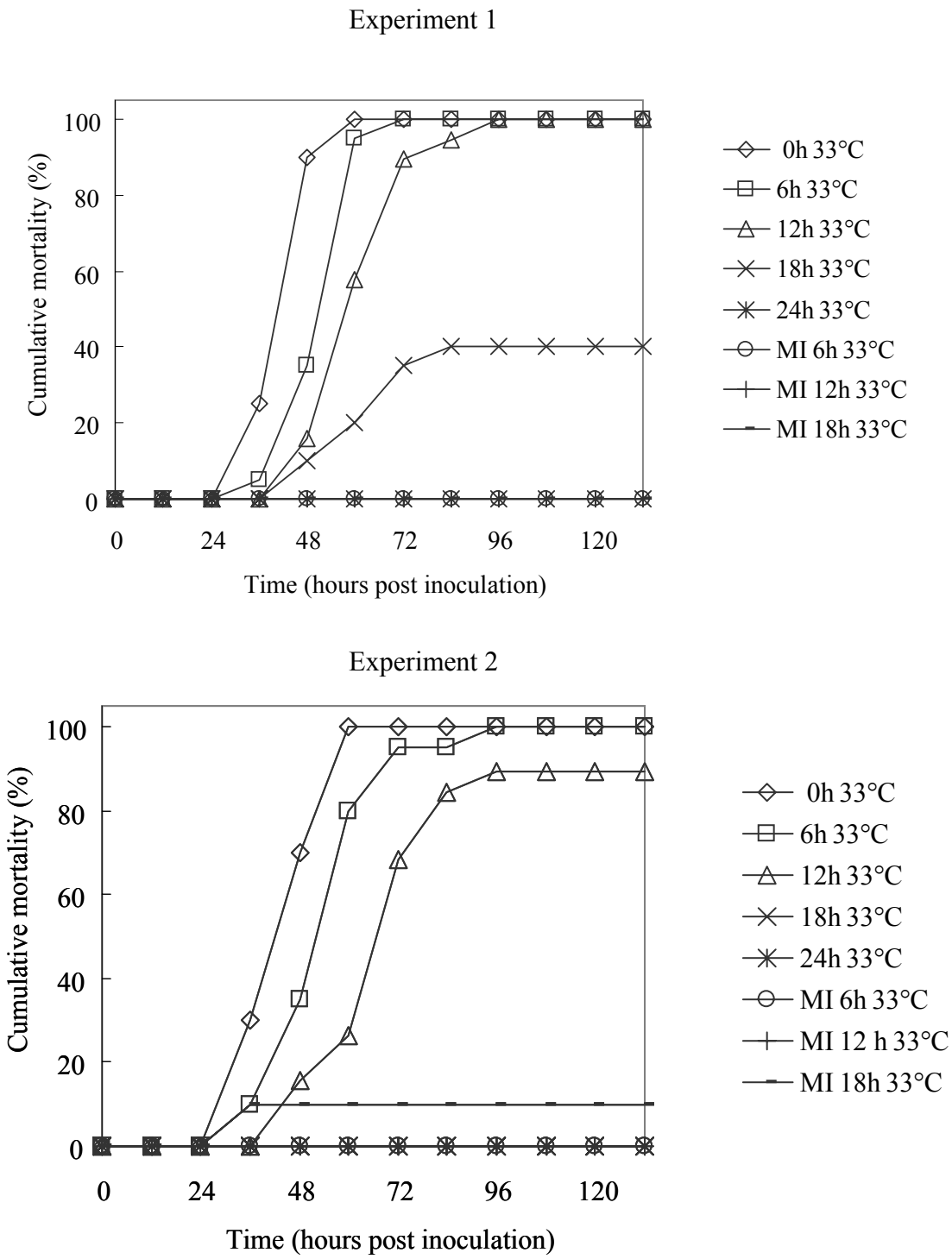
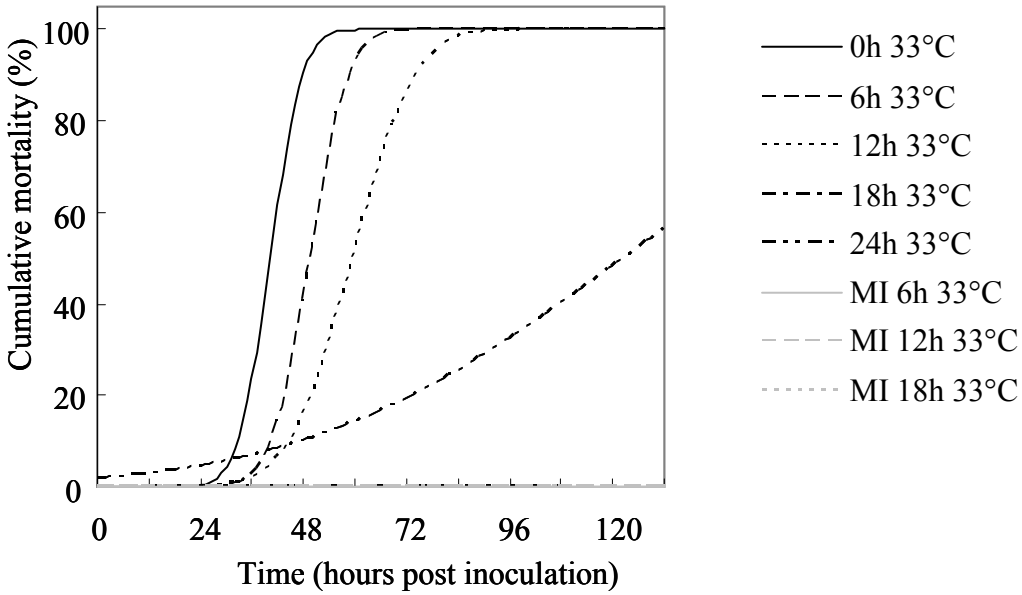


Figure 1. Cumulative mortalities of SPF *Penaeus vannamei* juveniles intramuscularly inoculated either with a dose of 10000 SID_{50} of WSSV Thai-1 or mock inoculated (MI). Shrimp were either kept at constant 27°C (0 h 33°C) or from 12 hpi exposed to 33°C for 6 h (6 h 33°C), 12 h (12 h 33°C), 18 h (18 h 33°C) and 24 h (24h 33°C) per day. Afterwards, temperature was lowered and maintained at 27°C for the rest of the day. These temperature regimes were repeated every 24 h upto 132 hpi.

Experiment 1



Experiment 2

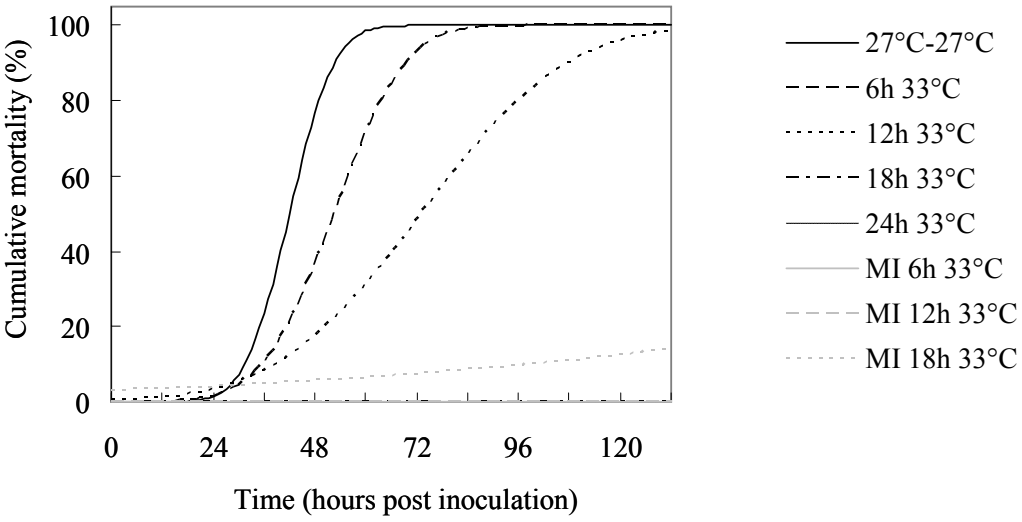


Figure 2. Probability of mortality (probit) of shrimp inoculated either with a dose of 10000 SID_{50} of WSSV Thai-1 or mock inoculated (MI) and exposed to different regimes of temperature fluctuations.

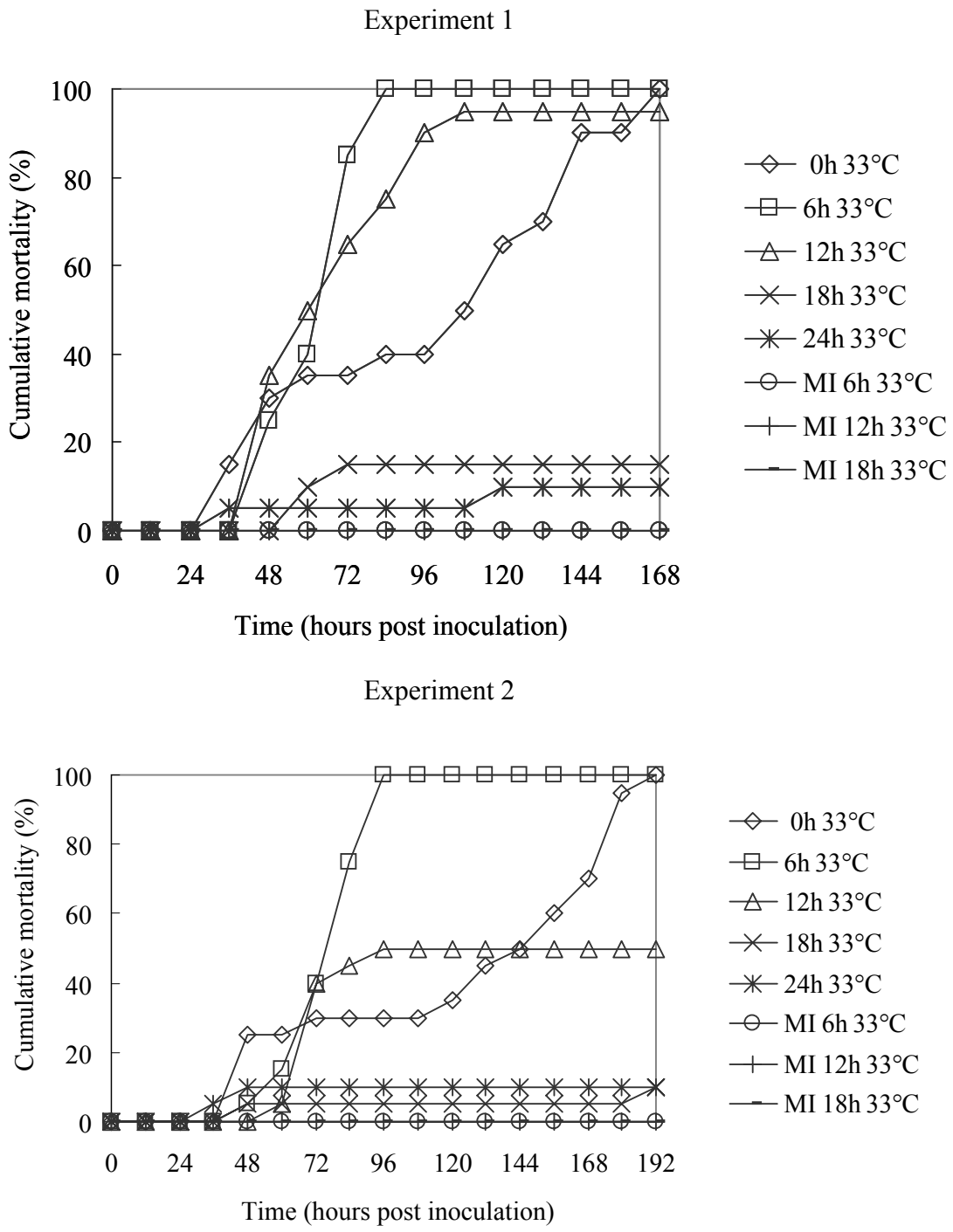


Figure 3. Cumulative mortalities of SPF *Penaeus vannamei* juveniles intramuscularly inoculated either with a dose of 10000 SID_{50} of WSSV Viet or mock inoculated (MI). Shrimp were either kept at constant 27°C (0 h 33°C) or from 12 hpi exposed to 33°C for 6 h (6 h 33°C), 12 h (12 h 33°C), 18 h (18 h 33°C) and 24 h (24h 33°C) per day. Afterwards, temperature was lowered and maintained at 27°C for the rest of the day. These temperature regimes were repeated every 24 h upto 168-192 hpi.

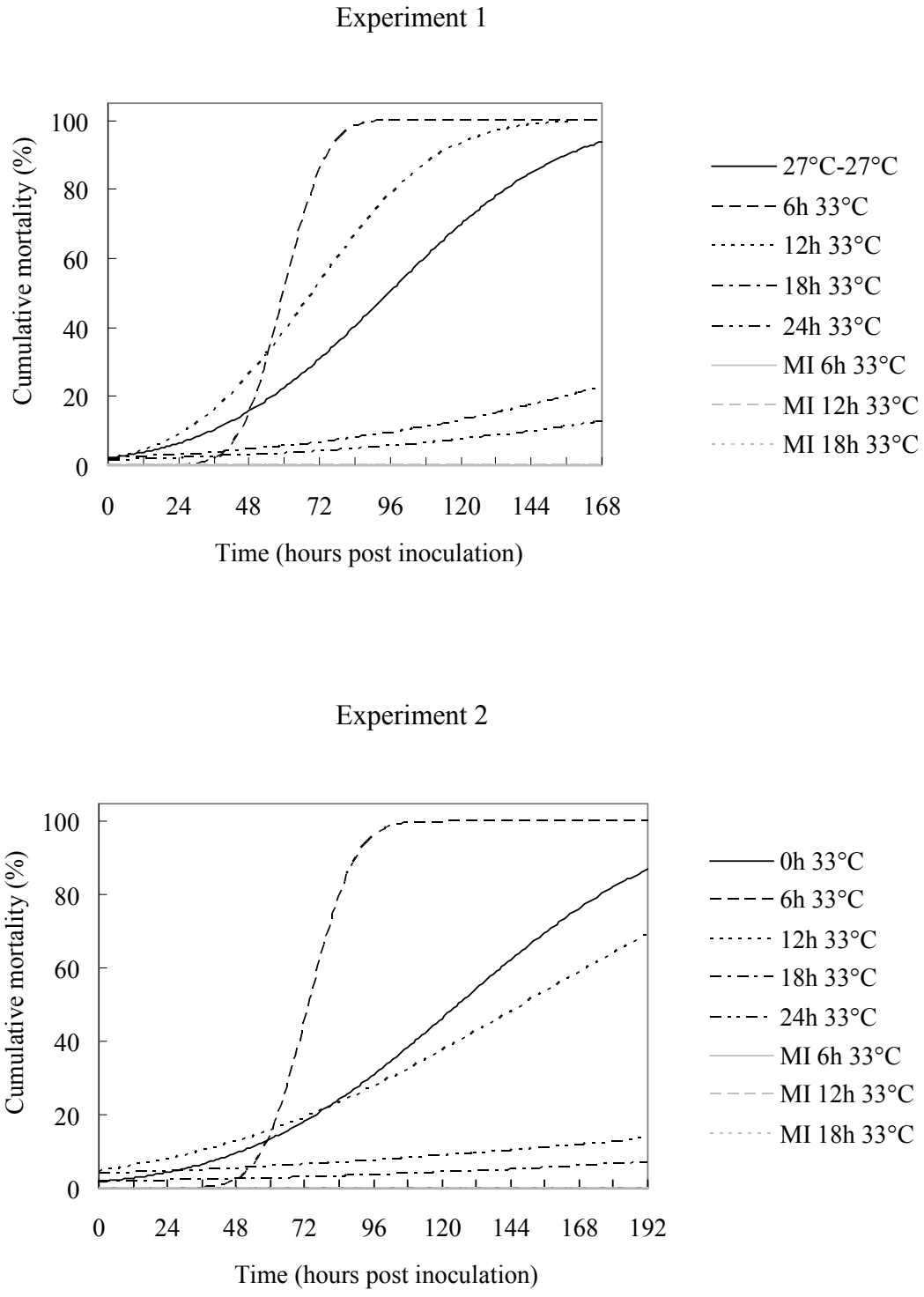


Figure 4. Probability of mortality (probit) of shrimp inoculated either with a dose of 10000 SID_{50} of WSSV Viet or mock inoculated (MI) and exposed to different regimes of temperature fluctuations.

Table 1. Median lethal times (LT_{50}) of shrimp inoculated with a high dose of either of WSSV Thai-1 or WSSV Viet exposed to 33 °C for different periods (0 h, 6 h, 12 h, 18 h and 24 h) per day.

Ex. No.	WSSV	h/day at 33°C	LT_{50}	α	β	γ	δ	LT_{50}* comparison
1	Thai-1	0 h	40.2[a]	6.802	-0.169			
		6 h	49.6[b]	6.802	-0.169	0.166	0.029	a=b<c<d
		12 h	59.4[c]	6.802	-0.169	-1.638	0.082	
		18 h	122.7[d]	6.802	-0.169	-4.705	0.152	
		24 h	-	6.802	-0.169	-2.48	0.169	
		NC 6 h	-	6.802	-0.169	-2.48	0.169	
		NC 12 h	-	6.802	-0.169	-2.48	0.169	
		NC 18 h	-	6.802	-0.169	-2.48	0.169	
2	Thai-1	0 h	42.0[e]	5.098	-0.121			e=f<g<h
		6 h	52.6[f]	5.098	-0.121	-1.123	0.046	
		12 h	73.2[g]	5.098	-0.121	-2.377	0.084	
		18 h	-	5.098	-0.121	-0.776	0.121	
		24 h	-	5.098	-0.121	-0.776	0.121	
		NC 6 h	-	5.098	-0.121	-0.776	0.121	
		NC 12 h	-	5.098	-0.121	-0.776	0.121	
		NC 18 h	308.2[h]	5.098	-0.121	-3.208	0.115	
1	Viet	0 h	95.3[i]	2.022	-0.021			j<k<i<l=m
		6 h	59.8[j]	2.022	-0.021	3.22	-0.066	
		12 h	69.5[k]	2.022	-0.021	0.047	-0.009	
		18 h	263.4[l]	2.022	-0.021	0.044	0.013	
		24 h	350.4[m]	2.022	-0.021	0.168	0.015	
		NC 6 h	-	2.022	-0.021	2.068	0.021	
		NC 12 h	-	2.022	-0.021	2.068	0.021	
		NC 18 h	-	2.022	-0.021	2.068	0.021	
2	Viet	0 h	125.4[n]	2.121	-0.017			o<n<p<r=q
		6 h	73.5[o]	2.121	-0.017	3.651	-0.062	
		12 h	148.3[p]	2.121	-0.017	-0.433	0.006	
		18 h	615.2[q]	2.121	-0.017	0.022	0.013	
		24 h	506.5[r]	2.121	-0.017	-0.346	0.013	
		NC 6 h	-	2.121	-0.017	1.969	0.017	
		NC 12 h	-	2.121	-0.017	1.969	0.017	
		NC 18 h	-	2.121	-0.017	1.969	0.017	

* Differences in LT_{50} are significant ($p<0.05$)

4. Discussion

This study showed that daily fluctuations of two temperatures (27°C and 33°C) with durations of 6 h, 12 h or 18 h can influence the outcome of disease, mortality and infection status of shrimp inoculated with WSSV. The results obtained with 33°C for 12 h and 18 h per day in delaying and reducing mortality could be useful to diminish disease and mortality of WSSV infected shrimp in the field. These findings might also help to understand some of the dynamics of disease outbreaks in shrimp farms due to WSSV. Under shrimp farming conditions, durations and range of temperature fluctuations might vary with geographical locations, season of the year and types of management in shrimp culture operations.

At constant 27°C, the differences in onset of mortality (36 hpi and 36-48 hpi) and time to reach a cumulative mortality of 100% (60 hpi and 168-192 hpi) between the two WSSV strains agree with previous work on virulence difference (Rahman et al., 2006b). The reproducible results of the experiments showed the usefulness of the standardized inoculation procedure (Escobedo-Bonilla et al., 2006) with a well defined amount of infectious virus.

At constant 33°C (24 h 33°C), absence of clinical signs and reduced mortalities (0-10%) of WSSV infected shrimp is in accordance with other studies (Vidal et al., 2001, Rahman et al., 2006a). The results showed similar effects of constant 33°C with the two WSSV strains used.

The shorter periods (6 h, 12 h or 18 h) of 33°C per day were effective in delaying the development of clinical signs and onset of mortality with both strains. However, cumulative mortalities reached always 100%, when shrimp infected with either strain were exposed to 33°C for 6 h per day only. This suggests that the progression of infection/replication at 27°C for 18 h per day was sufficient to cause disease and mortality. Surprisingly, cumulative mortalities of WSSV Viet inoculated shrimp kept at 6 h 33°C reached 100% even earlier than shrimp kept continuously at 27°C (84-96 hpi and 168-192 hpi). This indicates that combining temperature fluctuations with progressed infection (3-4 days) might cause a sharp rise in mortality. The underlying mechanisms will be investigated in future experiments. At 12 h 33°C, delayed/ reduced mortality with both strains could be due to the limited time (12 h per day) for replication (Rahman et al., 2006a). An exposure to 33°C for 18 h per day

significantly reduced mortality of shrimp inoculated with both strains (0-40% with WSSV Thai-1 and 5-15% with WSSV Viet). The reasons why these two temperature regimes (12 h and 18h 33°C) were more effective in bigger sized shrimp (second experiment) and with the less virulent strain are unknown. The findings with 33°C for 18 h per day might be applied in shrimp farms with consideration of proper season, aeration, stocking density and temperature tolerance of the species. Maintaining shrimp at 33°C for 18 h compared to constant 33°C is cheaper and might be easier to maintain in many tropical shrimp farming countries. Furthermore, the disadvantages of high water temperature on shrimp such as increased requirement of dissolved oxygen (DO) (Zhang et al., 2006) and increased rate of ammonia-N excretion (Jiang et al., 2000) could be minimized by shortening the exposure to 33°C by 6 h.

In summary, this study illustrates that daily fluctuations of optimum (27°C) and high water temperature (33°C) may have positive or negative effects on disease, mortality and infection status of WSSV inoculated shrimp depending on temperature regime and strain used.

5. Acknowledgements

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CHAPTER 5.3

The effect of raising water temperature to 33°C in *Penaeus vannamei* juveniles at different stages of infection with white spot syndrome virus (WSSV)

Aquaculture 272, 240-245

M.M. Rahman, M. Corteel, M.Wille, V. Alday-Sanz, M.B. Pensaert, P. Sorgeloos and H.J. Nauwynck

Abstract

This study investigated the effect of high water temperature (33°C) at different stages of infection with a highly virulent and low virulent white spot syndrome virus strain (WSSV Thai-1 and WSSV Viet) in *Penaeus vannamei* juveniles. Shrimp were inoculated intramuscularly with either a high dose (HD) or low dose (LD). Water temperature was kept either at continuously 27°C or switched from 27°C to 33°C at 0, 12 or 24 hours post inoculation (hpi) for both strains and in addition at 48 or 96 hpi for WSSV Viet. The increased temperature 33°C was maintained till the end of the experiments (120-144 hpi with WSSV Thai-1 and 240 hpi with WSSV Viet). To determine the infection status at the moment of temperature increase, five shrimp that were kept continuously at 27°C were euthanized at 0, 12, 24, 48 and 96 hpi with each dose of two strains. WSSV infections (viral antigen VP28) in dead and euthanized shrimp were demonstrated by indirect immunofluorescence.

Shrimp inoculated with HD or LD of WSSV Thai-1 and kept continuously at 27°C till euthanasia were 100% viral antigen positive from 12 (HD) or 24 hpi (LD). Shrimp inoculated with WSSV Viet were 100% positive from 24 (HD) and 48 hpi (LD). Shrimp kept at 27°C, showed clinical signs from 24 (HD) or 24-36 hpi (LD) with both strains. Cumulative mortalities reached 100% with WSSV Thai-1 at 60 (HD) or 84-144 hpi (LD) and with WSSV Viet 100% at 216 hpi (HD) or 90% at 240 hpi (LD). Switch of temperature to 33°C from 0, 12 or 24 hpi was effective in reducing mortality of shrimp inoculated with the LD of both strains and with the HD of WSSV Viet. The switch to 33°C from 24 hpi with the Thai strain (HD) and from 48 and 96 hpi with the Viet strain (LD or HD) had no effect or even accelerated the mortality rate (80-100%). All shrimp were viral antigen positive at death and euthanasia (one shrimp LD WSSV Viet) when kept continuously at 27°C. All dead and euthanized shrimp kept at 33°C from 0 or 12 hpi were viral antigen negative. With 33°C from 24, 48 or 96 hpi, all dead shrimp were viral antigen positive and euthanized shrimp were negative. This study showed that 33°C is effective to prevent disease, reduce mortality and block WSSV replication, but only in the early stages of infection.

Key words: Stages of WSSV infection, *Penaeus vannamei*, Water temperature

1. Introduction

White spot syndrome virus (WSSV) has caused severe mortalities in penaeid shrimp farms for more than one decade (Chou et al., 1995, Lightner, 1996, Rodríguez et al., 2003, Flegel, 2006). A WSSV epidemic was first reported in east Asia (Chou et al., 1995) and afterwards spread to almost all shrimp producing countries of Asia, North, Central and South America (Lightner, 1996, Rodríguez et al., 2003, Flegel, 2006). Under standardized experimental conditions with a water temperature of 27°C, differences between strains have been found in *Penaeus vannamei* juveniles in the onset of clinical signs, onset of mortality and time to reach a cumulative mortality of 100% (Rahman et al., 2006c). The cause of death of WSSV infected shrimp has been suggested to be due to dysfunction of target tissues including gills, stomach epithelium, cuticular epithelium, antennal gland and hematopoietic tissue. WSSV replication can be demonstrated in target tissues from 12 to 24 hours post inoculation (hpi) (Chang et al., 1996, Yoganandhan et al., 2003, Escobedo-Bonilla et al., 2007) depending on the titer of inoculation (Escobedo-Bonilla et al., 2007) and virulence of the WSSV strain used (Rahman et al., 2007b). Natural outbreaks of WSSV are suggested to be associated with stress caused by environmental factors such as water temperature (Fegan and Clifford, 2001, Rodríguez et al., 2003).

Water temperature has a direct effect on metabolic rate (Allan et al., 2006), growth and survival (Wyban et al., 1995, Ponce-Palafox et al., 1997), molting rate (Vijayan and Diwan, 1995), requirement of dissolved oxygen (Tian et al., 2004), lethal dissolved oxygen level (Zhang et al., 2006), tolerance to ammonia-N (Barajas et al., 2006) and immune response of shrimp (Le Moullac and Haffner, 2000, Cheng et al., 2005). The optimum temperature for growth of *P. vannamei* juveniles of above 5 g has been shown to be 27°C (Wyban et. al., 1995).

Previous studies reported prevention of clinical signs, reduced mortality and absence of infection in target tissues of shrimp kept continuously at 32-33°C before and after WSSV inoculation or immediately after inoculation (Vidal et al., 2001, Rahman et al., 2006b). However, the effect of increasing water temperature from 27°C to 33°C during the course of infection is unknown.

The aim of this study was to evaluate the effect of increasing water temperature from 27°C to 33°C on the clinical and virological outcome in *P. vannamei* juveniles at different time points after inoculation with a highly (Thai-1) and low (Viet) virulent WSSV strain (Rahman et al., 2006c).

2. Materials and methods

2.1 Virus

Two WSSV strains were used in this study. WSSV Thai-1 was collected from naturally infected *Penaeus monodon* in Thailand and passaged in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001). WSSV Viet was collected from naturally infected *Penaeus monodon* in Vietnam and passaged in crayfish *Cherax quadricarinatus*. Crayfish gill suspension of WSSV Thai-1 was kindly provided by K. Söderhäll (Uppsala University, Sweden) and WSSV Viet was received from the Research Institute for Aquaculture nº 2, Vietnam. Both strains were amplified in specific pathogen free (SPF) *Penaeus vannamei* juveniles in the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University, Belgium and the infectivity titers of the stocks were determined according to the procedure described by Escobedo-Bonilla et al. (2005). The median infectious titers of the stocks as determined by intramuscular inoculation in SPF *P. vannamei* were $10^{5.9}$ and $10^{5.8}$ SID_{50} (shrimp infectious dose with 50% endpoint) per ml for WSSV Thai-1 and WSSV Viet, respectively.

2.2 Shrimp

In total, 437 shrimp were used in the present study. In the experiment to determine WSSV infection status at different hours post inoculation (hpi) mean body weights (MBW) of shrimp were 16.5 ± 2.7 g. To evaluate the effect of a switched temperature from 27°C to 33°C on the virological and clinical outcome MBW of shrimp were 10.7 ± 2.2 g, 13.8 ± 2.7 g, 16.5 ± 2.7 g in the three experiments with WSSV Thai-1 and 16.6 ± 2.8 g in the experiment with WSSV Viet.

2.3 Experimental conditions

SPF *P. vannamei* from Molakai farm, Hawaii were imported at early postlarval stage and reared in a recirculation system at the Laboratory of Aquaculture and Artemia Reference Center (ARC), Faculty of Bioscience Engineering, Gent University, Belgium. Rearing conditions were: water temperature 27-28°C, salinity 34-36 g/l. Before each experiment shrimp were gradually acclimatized to the salinity of 15 g/l at the ARC over four days. Acclimatized shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University. Nine to eleven shrimp were housed per 50 litre aquarium, equipped with aeration, mechanical filtration (Eheim, Germany) and aquarium heater (Model VTX 300, Aquarium systems, France). Brackish water with a salinity of 15 g/l was prepared using artificial sea salt (Instant Ocean, Aquarium systems, France) and de-ionized water. Water temperature was kept at 27°C before WSSV inoculation and temperature was switched to 33°C at different hpi as described below. Approximately 0.2 g of a commercial shrimp diet was provided per shrimp per day. Water quality was checked by measuring ionized ammonia (NH_4^+) using test kits (Aquamerck, Germany) and 90% of water was renewed at 120 hpi in the experiment with WSSV Viet.

2.4 WSSV inoculation procedure

Shrimp were inoculated intramuscularly in the junction between the third and fourth abdominal segments with 50 µl of inoculum containing a low dose (LD) (30 SID_{50}) or a high dose (HD) (10,000 SID_{50}) of either of WSSV Thai-1 or WSSV Viet. Shrimp were observed for clinical signs including anorexia and lethargia and mortality was recorded every 12 h.

2.5. WSSV infection status at different hpi

Eighty shrimp were kept at continuously 27°C. Thirty shrimp were inoculated with WSSV Thai-1 and fifty shrimp with WSSV Viet, equally divided in groups for inoculation with a LD and HD. Five shrimp inoculated with each dose of each strain were euthanized at 0, 12 and 24 hpi. In addition, five shrimp inoculated with each dose of WSSV Viet were euthanized at 48 and 96 hpi.

2.6 Effect of switched temperature to 33°C on virological and clinical outcome

Shrimp were kept either at continuously 27°C (27°C-27°C) or temperature was switched from 27°C to 33°C at 0 (27°C-33°C/0 hpi), 12 (27°C-33°C/12 hpi)) or 24 hpi (27°C–33°C/24 hpi). In addition, temperature of WSSV Viet inoculated shrimp was switched to 33°C at 48 (27°C-33°C/48 hpi) and 96 hpi (27°C-33°C/96 hpi). Afterwards, increased temperature (33°C) was maintained till the end of the experiments. The experiments ran for 120-144 hpi with WSSV Thai-1 and 240 hpi with WSSV Viet. Nine to eleven shrimp were used for each temperature treatment with LD or HD of a strain. With WSSV Thai-1, experiments were repeated three times and with WSSV Viet one experiment was conducted.

2.7 Evaluation of WSSV infection by indirect immunofluorescence (IIF)

The cephalothoraxes of dead and euthanized shrimp were 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) in PBS and incubated with 2 µg ml^{-1} of the monoclonal antibody 8B7 (Diagxotics Inc., USA) directed against viral antigen VP28 (Poulos et al., 2001) of WSSV for 1 h at 37ºC. Then, sections were washed in PBS and incubated with fluorescein isothiocyanate (FITC) -labeled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1 h at 37 ºC. 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). Sections were analyzed by fluorescence microscopy.

3. Results

3.1 WSSV infection status at different hpi by IIF

3.1.1 WSSV Thai-1

Viral antigen (VP28) positive cells were found in all euthanized shrimp starting from 24 hpi when inoculated with LD and from 12 hpi when inoculated with a HD.

3.1.2 WSSV Viet

Viral antigen positive cells were found in 60% (LD) and 100% (HD) of the euthanized shrimp at 24 hpi. All euthanized shrimp were viral antigen positive at 48 and 96 hpi with LD and HD.

3.2 Effect of temperature switch to 33°C on virological and clinical outcome

3.2.1 WSSV Thai-1

3.2.1.1 Low dose

At continuously 27°C (27°C-27°C), shrimp started to show clinical signs at 24-36 hpi, mortalities started at 36-60 hpi and cumulative mortalities reached 100% at 84-144 hpi in the three experiments (Figure 1a). When water temperature increased to 33°C from 0 (27°C-33°C/0 hpi) or 12 hpi (27°C-33°C/12 hpi), shrimp did not show clinical signs and mean cumulative mortalities were 10 ± 17.3% and 10 ± 10%, respectively. With 33°C from 24 hpi (27°C-33°C/24 hpi), clinical signs were observed from 24-36 hpi and the mean cumulative mortality was 24 ± 5.3% at the end of the experiment. With 33°C from 0 or 12 hpi, all dead and euthanized shrimp were viral antigen negative. At constant 27°C or when temperature was switched to 33°C from 24 hpi, the shrimp that died were viral antigen positive and the euthanized shrimp were negative.

3.2.1.2 High dose

At continuously 27°C (27°C-27°C), shrimp showed clinical signs within 24 hpi, mortalities started at 36 hpi and cumulative mortalities reached 100% at 60 hpi in the three experiments (Figure 1b). After a temperature switch to 33°C at 0 (27°C-33°C/0 hpi) or 12 hpi (27°C-33°C/12 hpi), shrimp did not show clinical signs and mean cumulative mortalities were 10 ± 10% and 6.6 ± 5.8%, respectively. With 33°C from 24 hpi (27°C-33°C/24 hpi), shrimp showed clinical signs within 24 hpi, mortalities started at 36 hpi and cumulative mortalities reached 90 ± 10 % at 60 hpi. With 33°C from 0 or 12 hpi, all dead and euthanized shrimp were viral antigen negative. At continuously 27°C or when temperature was switched to 33°C from 24 hpi, all dead shrimp were viral antigen positive and the euthanized shrimp were negative.

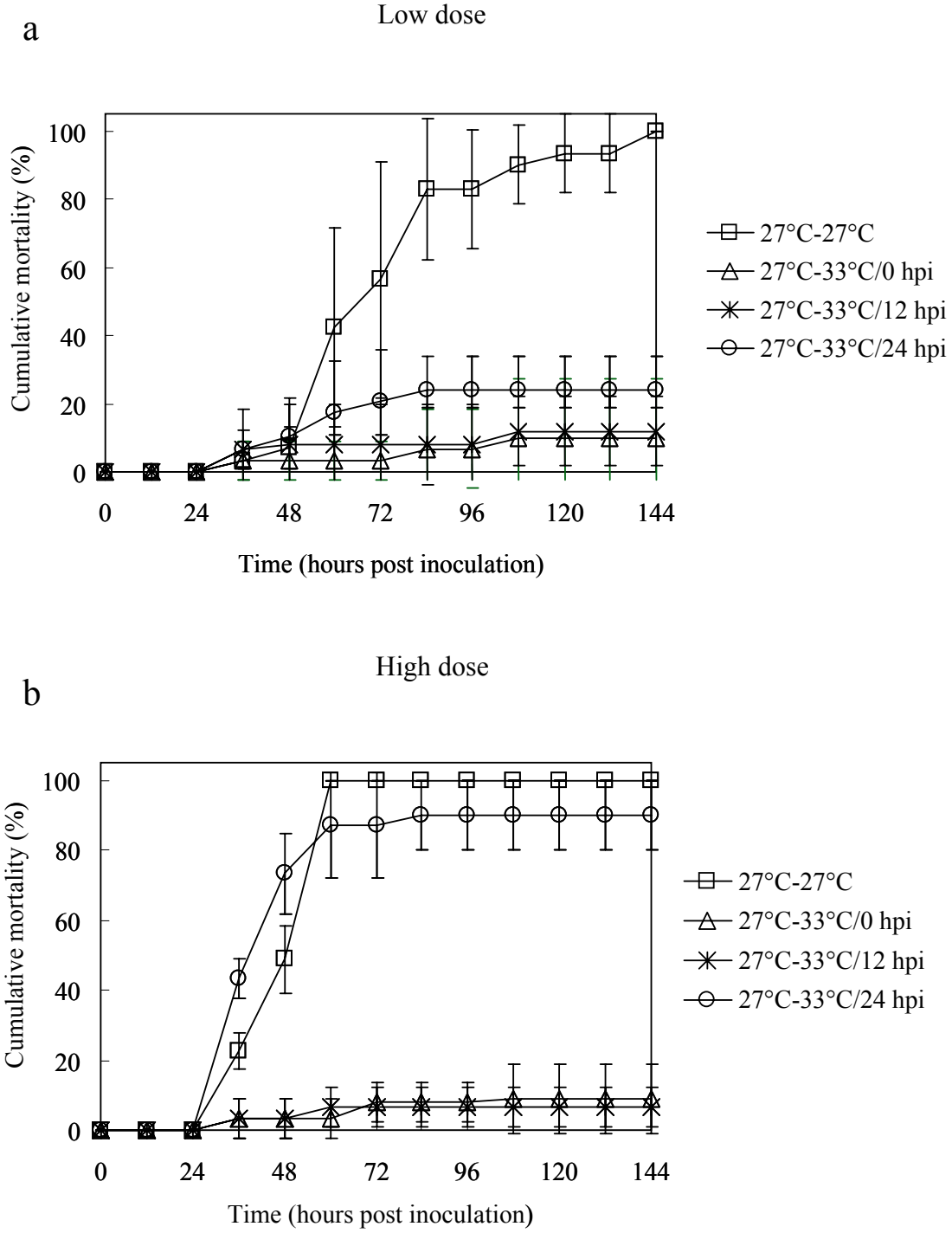

Figure 1. Cumulative mortalities (mean ± standard deviation [SD]) of SPF *Penaeus vannamei* juveniles, intramuscularly inoculated with (a) 30 SID_{50} or (b) 10000 SID_{50} of WSSV Thai-1. Water temperature was either kept continuously at 27°C (27°C-27°C) or switched to 33°C at 0 hours post inoculation (hpi) (27°C-33°C/0 hpi), 12 hpi (27°C-33°C/12 hpi) or 24 hpi (27°C-33°C/24 hpi) and then maintained till 120-144 hpi.

3.2.2 WSSV Viet

3.2.2.1 Low dose

At continuously 27°C (27°C-27°C), shrimp started to show clinical signs at 24-36 hpi, mortality started at 72 hpi, cumulative mortality reached 90% and one shrimp was still alive at the end of the experiment (240 hpi) (Figure 2a). When temperature was switched to 33 °C from 0 (27°C-33°C/0 hpi), 12 (27°C-33°C/12 hpi), or 24 hpi (27°C-33°C/24 hpi), shrimp did not show clinical signs and no mortality occurred. With 33°C from 48 (27°C-33°C/48 hpi) or 96 hpi (27°C-33°C/96 hpi), shrimp started to show clinical signs at 24-36 hpi, the onset of mortality was at 48 or 72 hpi and cumulative mortality reached 80% at 84 hpi and 90% at 120 hpi, respectively. With 33°C from 0, 12 or 24 hpi, all euthanized shrimp were viral antigen negative. At continuously 27°C, all dead and survivor shrimp were viral antigen positive. When temperature was switched to 33 °C from 48 and 96 hpi, dead shrimp were viral antigen positive and euthanized shrimp were negative.

3.2.2.2 High dose

At continuously 27°C (27°C-27°C), shrimp started to show clinical signs at 24 hpi, mortality started at 60 hpi and cumulative mortality reached 100% at 216 hpi (Figure 2b). With temperature switched to 33°C from 0 (27°C-33°C/0 hpi) or 12 hpi (27°C-33°C/12 hpi), shrimp did not show clinical signs and cumulative mortality reached 20%. With 33°C from 24 hpi (27°C-33°C/24 hpi), shrimp started to show clinical signs at 24 hpi, mortality started at 36 hpi and cumulative mortality reached 50% at 240 hpi. With 33°C from 48 (27°C-33°C/48 hpi) or 96 hpi (27°C-33°C/96 hpi), shrimp started to show clinical signs at 24 hpi, mortality started at 48 or 60 hpi and cumulative mortality reached 90% at 72 hpi or 100% at 120 hpi. With 33°C from 0 or 12 hpi, all dead and euthanized shrimp were viral antigen negative. At continuously 27°C, all dead shrimp were viral antigen positive. When temperature was switched to 33°C from 24, 48 and 96 hpi, all dead shrimp were viral antigen positive except one (died at 240 hpi with 33°C from 24 hpi) and euthanized shrimp were negative.

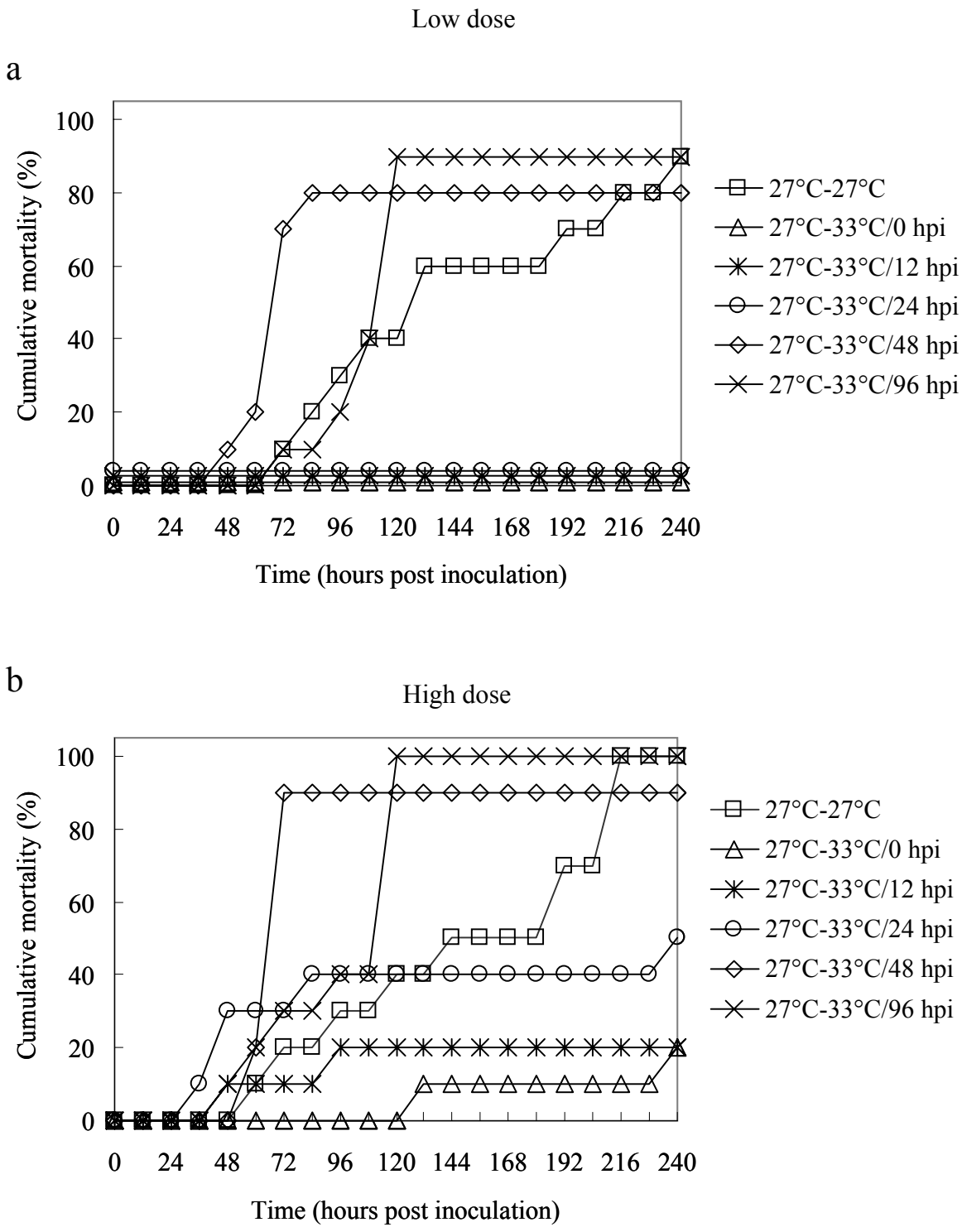

Figure 2. Cumulative mortalities of SPF *Penaeus vannamei* juveniles, intramuscularly inoculated with (a) 30 SID_{50} or (b) 10000 SID_{50} of WSSV Viet. Water temperature was either kept continuously at 27°C (27°C-27°C) or switched to 33°C at 0 hours post inoculation (hpi) (27°C-33°C/0 hpi), 12 hpi (27°C-33°C/12 hpi), 24 hpi (27°C-33°C/24 hpi), 48 hpi (27°C-33°C/48 hpi) or 96 hpi (27°C-33°C/96 hpi) and then maintained till 240 hpi.

4. Discussion

This study showed that increasing water temperature from 27°C to 33°C can have two opposite effects in WSSV infected shrimp depending on the stages of infection. In the acute stage of infection before clinical signs are observed, it shuts off virus replication and disease/mortality. In a more subacute, chronic stage of infection when clinical signs are present, the outcome is detrimental with quicker progression of disease/mortality in WSSV infected shrimp. The positive result with 33°C in acutely infected shrimp opens a scope to apply 33°C as a strategy to control infection, disease and mortality in a shrimp farm with the first signs of white spot syndrome (WSS). This may give the shrimp farmer more time to organize an emergency harvest. Exposure to 33°C might also support the efficacy of other control strategies such as antivirals (Rahman et al., 2006a). However, the benefit from exposure to 33°C of WSSV infected shrimp in the field has its limits because of the short time span during which the temperature has to be increased (between 12 and 24 hpi), negative effects of 33°C on shrimp (Ponce-Palafox et al., 1997, Le Moullac and Haffner, 2000, Cheng et al., 2005, Zhang et al., 2006), restricted availability of logistics to raise temperature, the need for a quick diagnosis of WSS and the cost effectiveness.

Prevention of clinical signs, reduced mortality and absence of viral antigen in shrimp exposed to 33°C at inoculation (27°C-33°C/0 hpi) agrees with previous work (Granja et al., 2006, Rahman et al., 2006b). The results of this study suggest that a similar mechanism is effective, even in shrimp in which WSSV has already replicated for 12 to 24 h.

Increasing temperature to 33°C could not reduce mortality or was at least less effective in shrimp infected for 24 h at 27°C with a HD of WSSV Thai-1 or WSSV Viet. This can be explained by the fact that at 24 hpi the viral infection had already become systemic (Escobedo-Bonilla et al., 2007), causing irreversible tissue damage. The mechanism of accelerated mortalities with exposure to 33°C from 48 hpi or later (27°C-33°C/48 hpi and 27°C-33°C/96 hpi) with each dose of WSSV Viet is not clear. Similar accelerated mortalities were also observed in a study with diurnal fluctuations of temperature (27°C and 33°C) (Rahman et al., 2007a). Several factors may be involved in this phenomenon. An increased requirement for oxygen in shrimp kept at 33°C (Tian et al. 2004) combined with the reduced

amount of dissolved oxygen in the warmer water and a reduced oxygen exchange due to tissue damage in infected gills may be forwarded as the most plausible explanations. This sharp rise of mortality in shrimp with a progressed WSSV infection might explain the quick and high mortality during some of the natural outbreaks in shrimp farms.

With 33°C from 0 or 12 hpi there were still a few shrimp that died. This was not due to infection since viral antigen positive cells were absent. This low mortality was probably due to the negative effects of 33°C on the physiological condition of shrimp. Similar mortalities were also found in both WSSV inoculated and uninoculated shrimp in other studies (Ponce-Palafox et al., 1997, Rahman et al., 2006b).

In conclusion, this study shows that the efficacy of increasing temperature to 33°C to prevent disease, reduce mortality and diminish infection depends on the progression of infection in shrimp.

5. Acknowledgements

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CHAPTER 6

General discussion

In this thesis, a standardized challenge procedure with a fixed amount of infectious virus (Escobedo-Bonilla et al., 2006) was used to inoculate specific pathogen free (SPF) *Penaeus vannamei* juveniles either intramuscularly or orally in order to study the virulence of WSSV isolates and to test a few approaches to control white spot syndrome (WSS) in shrimp.

Differences in virulence between three WSSV isolates were demonstrated. Quantitative analysis showed a higher number of WSSV infected cells in five target tissues of shrimp inoculated with a highly virulent isolate than with a low virulent isolate. Virulence has been defined as the degree of pathogenicity of an agent to cause disease in a host (Shapiro-Ilan et al., 2005). Different forms of outbreaks in the field (peracute, acute and chronic) (Sudha et al., 1998) and earlier laboratory studies were only indicative for the existence of differences in virulence between WSSV strains (Wang et al., 1999; Lan et al., 2002; Marks et al., 2005). The severity of clinical outbreaks in the field is influenced by biotic and abiotic factors together with the pathogenicity of the WSSV isolate. These factors include (i) water temperature, dissolved oxygen, salinity, (ii) infection with other pathogens (bacteria, fungus, parasites), (iii) type of management in culture (stocking density, nutritional status of shrimp). In the laboratory studies, the challenge procedures did not ensure the uptake of a certain amount of infectious virus by each shrimp at the same moment (Wang et al., 1999), different routes of inoculation were used (fed infected tissue or inoculated intramuscularly), the experiments were not repeated and different species (*P. vannamei*, *P. monodon* and *Procambarus clarkii*) were used. In the present thesis, 30 SID_{50} (SID_{50} = shrimp infectious dose with 50% end point) were inoculated intramuscularly to compare the virulence of WSSV isolates in *P. vannamei*. Two isolates were from Thailand (WSSV Thai-1, WSSV Thai-2) and one from Vietnam (WSSV Viet). The experiments were repeated three times to demonstrate the reproducibility of the results. The differences in virulence between WSSV isolates were characterized by the observed differences in the time for onset of mortality, time to reach a cumulative mortality of 100% and median lethal time (LT_{50}). Differences in LT_{50} were significant between the three isolates. WSSV Thai-1 was highly virulent and WSSV Viet was a low virulent isolate. This method is now ready to be used worldwide in order to compare virulence of field isolates from many other geographical areas. The basis of these differences in virulence between isolates was unknown. In two

previous reports, contradictory hypotheses were forwarded on the correlation of genomic differences and differences in virulence. Virulence was correlated with a larger genome by Lan et al. (2002) and with a smaller genome by Marks et al. (2005). In the present thesis, WSSV replication in tissues (number of VP28 positive cell) was compared between shrimp inoculated with WSSV Thai-1 and shrimp inoculated with WSSV Viet to find out the basis for the differences in virulence. Tissues with important roles in shrimp physiology including gills, stomach cuticular epithelium, cuticular epithelium of the body wall, hematopoietic tissues and antennal glands were selected. Gills are important for respiration, osmoregulation, excretion and acid base homeostasis (Taylor and Taylor, 1992); the stomach for digestion (Icely and Nott, 1992); the antennal glands for excretion (Felgenhauer, 1992); cuticular epithelium of the body wall produces cuticle which forms a physical barrier against pathogen invasion (Brey et al., 1993); hematopoietic tissues produce hemocytes which are important for defense. Higher numbers of infected cells in tissues of naturally dead and euthanized shrimp were found with WSSV Thai-1 compared to WSSV Viet. A six times higher number of infected cells was found in gills of naturally dead shrimp inoculated with the WSSV Thai-1 than with the WSSV Viet. The following explanations or combinations can be given for these differences. First, the replication cycle of WSSV Thai-1 may be shorter than WSSV Viet. This permits WSSV Thai-1 to produce more progeny virus in a shorter period of time, which causes infection in more tissues (gills, stomach cuticular epithelium, cuticular epithelium of body wall, hematopoietic tissues and antennal glans) within the same time (at 24 hpi) than with WSSV Viet (gills and stomach cuticular epithelium). Second, a higher number of cell types may be susceptible to infection by WSSV Thai-1. For example, at least six cell types have been differentiated within gills: chief cells, pillar cells, striated cells, glycocytes, nephrocytes and granular cells (Felgenhauer 1992). It is possible that WSSV Thai-1 infects more cell types than WSSV Viet. Due to lack of cell markers, this hypothesis could not be examined. Third, WSSV Thai-1 may be more efficient in escaping from the immune response than WSSV Viet.

WSSV is highly pathogenic to both *P. vannamei* (Escobedo-Bonilla et al., 2007) and *P. monodon* (Chang et al., 1996). Susceptibility differences between shrimp species (Wang et al., 1999), crab species (Sahul Hameed et al., 2003) and *Artemia* (Li et al., 2003) were

extensively documented before. Marine shrimp are more susceptible to WSSV than freshwater species (Sahul Hameed et al., 2000). The less susceptibility of *Macrobrachium rosenbegii* has been attributed to an increased antiviral response (Pais et al., 2007). Early life stages (post larvae onwards) of both marine (Wang et al., 1999) and freshwater shrimp (Kiran et al., 2002), were indicated to be more susceptible than juvenile, subadult or adult.

The efficacy of an antiviral cidofovir and a diet supplemented with *Spirulina platensis* were tested against WSSV infection in shrimp. Only cidofovir had a slightly positive effect in delaying the disease and mortality. In earlier studies, researchers have tested several strategies to control disease and mortality of WSSV infected shrimp, including antivirals (Chotigeat et al., 2004), immunostimulants (Itami et al., 1998; Chang et al., 2003; Citarasu et al., 2006), antibodies raised against viral proteins (Jiang et al., 2007; Natividad et al., 2007) or viral proteins (Witteveldt et al., 2004), RNA interference (Xu et al., 2007) and interference with IHHNV (Bonnichon et al., 2006; Melena et al., 2006). The results of those studies are difficult to compare and reproduce due to the lack of standardization of the challenge procedure, which include different animal species, non titrated virus stocks, different routes/methods of inoculation and virulence of isolates. In most cases, the results obtained in those studies are not feasible or not adequate for practical applications. For example, injecting every shrimp as a delivery method of a product is time consuming, laborious, expensive and difficult to implement in postlarval stage of shrimp.

Cidofovir, a nucleoside phosphonate analogue was proven to be effective against human DNA viruses including polyomavirus, papillomavirus, adenovirus, herpesvirus and poxvirus. The molecule is incorporated into the viral DNA and blocks DNA synthesis (De Clercq, 2003). Generally, the use of antiviral drugs in aquaculture is never considered due to concerns about residuals and food safety. In the present study, cidofovir was tested for an academic purpose. The results showed that cidofovir was clearly less effective in shrimp than against viruses in mammals. Several explanations may be proposed for this less effectiveness. First, shrimp cells may be less efficient for the uptake of cidofovir. Second, intracellular cidofovir might not be metabolized by shrimp cells into its active form. Third, the active form of cidofovir may have differences in its affinity for viral DNA polymerase of WSSV.

S. platensis is a marine alga, which contains calcium spirulan. Calcium spirulan is a sulphated polysaccharide, which was found to be effective *in vitro* against herpes simplex virus type 1 and human immunodeficiency virus type 1 (HIV-1). In this thesis, the diet supplemented with *S. platensis* had no effect on the onset of mortality and time to reach a cumulative mortality of 100% compared to the diet without supplementation. From these experiments, it cannot be predicted what the result would be with a low virulent isolate, lower infectious titer, other route of inoculation and other animal species.

The clinical and virological outcome was studied in WSSV infected shrimp, (i) kept continuously at 27°C, 30°C and 33°C and (ii) exposed to 33°C before or from the moment after inoculation. The results of this study, support previous reports of reduced mortality at continuously 32-33°C (Vidal et al., 2001). Diseases are a complex interaction between host, pathogen and environment, particularly in aquatic animals (Snieszko, 1974). Water temperature is one of the most important environmental factors for shrimp. Earlier field and laboratory studies reported the positive effects of water temperature higher (>32°C) or lower (<15°C) than optimal on mortality of WSSV infected shrimp and crayfish. In the present thesis, several new findings were provided. First, it was shown that 27°C, which is the optimum water temperature for growth and survival of *P. vannamei* (Wyban et al., 1995) and 30°C are permissive for WSSV replication, disease and mortality. Second, exposure to 33°C for four days before inoculation only and lowering water temperature from 33°C to 27°C at the moment of inoculation had no beneficial effect. In contrast, cumulative mortalities reached 100% in a shorter period of time than shrimp kept continuously at 27°C. The faster mortality rate may indicate an increased susceptibility of shrimp to WSSV infection or a hampered defense response of shrimp (total hemocyte count, phenol oxidase acitivity, phagocytic index) due to the exposure at 33°C for four days and a quick drop to 27°C. Third, increased temperature from 27°C to 33°C at the moment of inoculation is very effective to prevent disease and reduce mortalities (mean cumulative mortality was 10% in three experiments). This was due to a full inhibition of replication in WSSV infected shrimp at 33°C. The inhibition of replication at 33°C was effective in shrimp independent of inoculation route and amount of virus that had been administered. Similarly, a reduction of viral load was found in infected *P. vannamei* kept at 32-33°C (Granja et al., 2006) as a

consequence of reduced virus replication. An *in vitro* study also showed inhibition of replication in cells of hematopoietic tissue of crayfish kept at high temperature (32°C) (Jiravanichpaisal et al., 2006). The result with increasing temperature from 27°C to 33°C at the moment of inoculation could be applied as a strategy to prevent WSSV infections. Shrimp farmers should be advised to grow shrimp only during seasons with warmer water temperature (32-33°C) in order to have less problems with WSSV. Culturing shrimp in green houses may allow to control high water temperature during colder periods. The mechanism of the inhibition of virus replication at 32-33°C is not clear. It could be a direct effect due to an inhibition of viral enzymes involved in replication (e.g. DNA polymerase). Altered gene expression (Reyes et al., 2007), apoptosis (Granja et al., 2003) or heat shock protein expression (Vidal et al., 2001) were also proposed as mechanism. The latter factors may influence virus replication as described for other viruses (Clem, 2005, Mayer, 2005).

In this thesis, the effect of 33°C for different periods per day (6h, 12 h, 18 h and 24 h) on the clinical and virological outcome of WSSV infected shrimp was determined. Water temperature in shrimp farms fluctuates diurnally and seasonally as it depends on air temperature, water depth and water management. Maintaining temperature at constant 33°C (24 h per day) may not always be practical due to the unavailability of logistics and the high costs. However, in shrimp ponds water temperature of 33°C may be available at least for several hours per day in many tropical countries (Alongi et al., 1999; Ruiz-Fernández and Páez-Osuna, 2004; Thongrak et al., 1997; Wahab et al., 2003; Wang et al., 2005). A duration of 6 h per day at 33°C could not reduce mortality of shrimp inoculated with either WSSV Thai-1 or WSSV Viet. In contrast, cumulative mortalities reached 100% in shorter periods of time (84-96 hpi) with a sharper rise than shrimp kept continuously at 27°C (168-192 hpi) inoculated with WSSV Viet in two experiments. This suggests that 6 h per day at 33°C is not sufficient to block WSSV replication and accordingly to prevent disease and mortality. By the time of mortality (84-96 hpi), virus may have completed several replication cycles and infection could have spread to all target tissues including gills, hematopoietic tissues, stomach cuticular epithelium, cuticular epithelium of body wall and antennal glands. A possible reason of the sharp rise of mortality may be explained by the effect on shrimp physiology including increased metabolic rate and requirement of oxygen (Allan et al., 2006,

Tian et al., 2004), increased lethal dissolved oxygen level (Zhang et al., 2006) and decreased immune reactivity (total hemocyte count, phenoloxidase activity, phagocytic index) (Cheng et al., 2005). The level of dissolved oxygen in the water might decrease at 33°C than 27°C even at constant aeration. A combination of damaged tissue with an important role in shrimp physiology such as gills due to infection or defense response and negative effects of 33°C on shrimp mentioned above could trigger the sharp rise of mortality. A daily regime of 12 - 18 h per day at 33°C can significantly delay and reduce mortality of shrimp inoculated with WSSV Thai-1 or WSSV Viet. This was most effective in larger sized shrimp and with less virulent WSSV Viet. Better results with larger sized shrimp (juvenile to subadult) could be due to reduced susceptibility or improved immunity. A better effectiveness with the less virulent isolate may be related to the ability of shrimp tissues to keep on performing their physiological role. The lower mortality (≤ 40%) with 33°C for 18 h per day has broadened the application scope of 33°C to control WSSV infection by minimizing the negative effects of constant 33°C on shrimp and lowering the cost involved with warming up water.

The effect of increasing temperature from 27°C to 33°C was investigated at different stages of infection (0, 12, 24, 48 and 96 hpi) in shrimp inoculated with WSSV Thai-1 and WSSV Viet. The results of this study will help us to explore the potential of inducing protection in infected shrimp. Increasing temperature to 33°C is effective at an early stage of infection (≤ 24 hpi) to prevent disease and to reduce mortality in WSSV infected shrimp, independent of virus titer in the inoculum and virulence of the isolates. On the one hand it clearly opens the scope in inducing protection in WSSV infected shrimp by raising temperature to 33°C but on the other hand it shows its limits. Since disease can only be seen from 24 hpi onwards, raising temperature in a pond will not be effective in WSSV diseased animals but may be effective in those animals that are still uninfected or in the early stage of infection without disease. The mortality with increasing temperature from 27°C to 33°C at later time points (48 or 96 hpi) suggest that the beneficial effect of 33°C to prevent disease and mortality is dependent on the severity of infection in tissues with an important role in shrimp physiology. The cause of sharp rise of mortality could be the same as mentioned earlier with 6 h per day at 33°C in shrimp inoculated with the Viet isolate.

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CHAPTER 7

Summary/Samenvatting

White spot syndrome virus (WSSV) has caused serious production losses to almost all shrimp producing countries over the world. Previous field and experimental studies suggested the existence of differences in virulence between geographical isolates. The mechanism of these differences is unknown. Since WSSV appeared, scientists have tested several control strategies to reduce production losses due to WSSV with little success and contradictory results. However, the inoculation procedure in virulence studies did not ensure a certain amount of infectious titer to each animal. Therefore, the results and conclusions from these studies have to be taken with caution. The present thesis aimed to develop an *in vivo* challenge model to compare virulence of WSSV isolates, find out a basis of differences and to obtain better results in control studies by using a standardized inoculation procedure and known virulence of the isolate.

In the introduction in Chapter 1, the present status of shrimp aquaculture and an overview on shrimp diseases were given. The current knowledge on the pathogenesis and virulence differences of WSSV isolates, antiviral immunity in shrimp, roles of environmental factors in WSSV infection and control strategies were reviewed.

In Chapter 2, the aims of the thesis are described.

Chapter 3 presents a study on virulence of WSSV isolates. First, the virulence of three WSSV isolates (WSSV Thai-1, WSSV Thai-2 and WSSV Viet) was compared through intramuscular inoculation with 30 SID_{50} (SID_{50} = shrimp infectious dose with 50% endpoint) in specific pathogen free (SPF) *Penaeus vannamei* juveniles. The difference in virulence was measured by onset of mortality, time to reach a cumulative mortality of 100% and median lethal times (LT_{50}). Shrimp inoculated with each isolate started to show clinical signs from 24-36 h post inoculation (hpi). Mortalities started at 36 hpi of shrimp inoculated with two Thai isolates and at 36-60 hpi with the Viet isolate. Cumulative mortalities reached 100% at 72-84, 108 and 204-348 hpi and median lethal times (LT_{50}) were 47, 61 and 120 hpi with WSSV Thai-1, WSSV Thai-2 and WSSV Viet, respectively. These differences were significant. In the second part, virus replication in tissues including gills, stomach cuticular

epithelium, cuticular epithelium of the body wall, hematopoietic tissues and antennal glands was compared in shrimp inoculated with 30 SID_{50} of a highly virulent WSSV Thai-1 and a low virulent WSSV Viet. Shrimp inoculated with each isolate were euthanized at 0, 12, 24, 36, 48 and 72 hpi. In addition, WSSV Viet inoculated shrimp were euthanized at 120 and 168 hpi. During this period, naturally dead shrimp inoculated with each isolate were also collected. WSSV positive cells in tissues of naturally dead and euthanized shrimp were detected and quantified by using immunohistochemistry. Overall, higher numbers of infected cells were found in each tissue of euthanized shrimp inoculated with WSSV Thai-1 than WSSV Viet. The mean numbers of infected cells in naturally deceased shrimp inoculated with WSSV Thai-1 were 523 ± 321 cells/mm^2, 38 ± 7 %, 28 ± 8 %, 259 ± 155 cells/mm^2 and 11 ± 6 % in gills, stomach cuticular epithelium, cuticular epithelium of the body wall, hematopoietic tissues and antennal glands, respectively. The mean numbers of infected cells of naturally deceased shrimp inoculated with WSSV Viet were 86 ± 63 cells/mm^2, 22.5 ± 7.6 %, 17.7 ± 8.4 %, 160 ± 103 cells/mm^2, 5.2 ± 3.1 % in gills, stomach cuticular epithelium, cuticular epithelium of the body wall, hematopoietic tissues and antennal glands, respectively. This study demonstrated clear differences in virulence between WSSV isolates. It also indicated that the differences in virus replication in tissues especially in gills (factor six in dead shrimp inoculated with WSSV Thai-1) may determine the virulence of WSSV isolates.

Chapter 4 presents a study that tested an antiviral product (cidofovir) and a diet supplemented with *Spirulina platensis* for their efficacy to control disease and mortality of WSSV infected shrimp. Cidofovir, a nucleoside analogue was proven to be effective against DNA viruses in mammals. Cidofovir was injected in shrimp at different concentrations from 12.5 to 200 mg/kg and no toxic effect was found. The highest concentration of cidofovir (200 mg/kg) was injected intramuscularly at the moment of virus inoculation and control shrimp were injected with phosphate buffer saline (PBS). *S. platensis* - a marine alga was supplemented in diet. It contains calcium spirulan, a sulphated polysaccharide that inhibits replication of other viruses *in vitro*. *S. platensis* supplemented diet were fed at 5% of the body weight of shrimp for four days before inoculation and control shrimp were fed a diet without

S. platensis supplementation. Each shrimp was inoculated orally with 30 SID_{50} of highly virulent WSSV Thai-1. Mortalities started 24 h later and cumulative mortalities reached 80-90% of shrimp treated with cidofovir than non treated shrimp. LT_{50} was significantly different between cidofovir treated and non treated shrimp. With or without *S. platensis* supplemented diet, mortality of shrimp started at 36 hpi and cumulative mortality reached 100% at 84 hpi. All dead and euthanized shrimp were WSSV positive except one survivor treated with cidofovir. This study showed that cidofovir is more effective than *S. platensis* supplemented diet to control WSSV infection in shrimp.

In Chapter 5, the effect of water temperature (27°C, 30°C and 33°C) on the clinical and virological outcome of WSSV infected shrimp was investigated. Shrimp were inoculated orally or intramuscularly with a low dose (30 SID_{50}) or a high dose (10000 SID_{50}) of highly virulent WSSV Thai-1. Temperature was kept either (a) continuously at 27°C, 30°C, 33°C before inoculation and after inoculation or (b) switched from 33 °C to 27 °C after inoculation or (c) switched from 27°C to 33°C after inoculation. Respective temperature was maintained for 4 days before inoculation and 5-6 days after inoculation till the end of the experiment. Shrimp kept continuously at 27°C or 30°C or switched from 33°C to 27°C after inoculation showed clinical signs, cumulative mortality reached 100% and all dead shrimp were WSSV positive. The shrimp, kept either continuously at 33°C before and after inoculation or switched from 27°C to 33°C after inoculation did not show clinical signs and mean cumulative mortalities were 10%. All naturally dead and euthanized shrimp were WSSV negative. In a time course study, shrimp were inoculated orally with a high dose (10000 SID_{50}) of highly virulent WSSV Thai-1. Shrimp were kept either continuously at 27°C before and after inoculation or increased from 27°C to 33°C at the moment of inoculation and euthanized at 12 and 24 h post inoculation (hpi). WSSV positive cells were found in the tissues of shrimp kept continuously at 27°C and euthanized at 12 and 24 hpi. On the contrary, WSSV positive cells were not found in the tissues of shrimp exposed to 33°C from the moment of inoculation and euthanized at 12 and 24 hpi. This study showed that high water temperature of 33°C before and after inoculation or only after inoculation was effective to prevent disease and reduce mortality of WSSV infected shrimp. The effect of 33°C was

independent of the route of inoculation and dose used and WSSV replication was inhibited at 33°C. It also suggests that the exposure to 33°C only before inoculation had no beneficial effect.

Further, the impact of daily fluctuations between 27°C and 33°C were evaluated on the clinical and virological outcome of WSSV infection in shrimp. Shrimp were inoculated intramuscularly with 10000 SID_{50} of highly virulent WSSV Thai-1 or low virulent WSSV Viet. Temperature was kept either continuously at 27°C or switched from 27°C to 33°C at 12 hpi and maintained at that temperature for 6, 12, 18 and 24 h per day. Temperature was lowered and maintained at 27°C for the remaining hours of the day. Shrimp kept continuously 27°C showed clinical signs and cumulative mortalities reached 100% with both isolates in two experiments. At constant 33°C, shrimp inoculated with both isolates did not show clinical signs and mortalities were ≤ 10%. With exposure to 33°C for 6 or 12 h per day, cumulative mortalities reached 90-100% of shrimp inoculated with WSSV Thai-1 and 50-95% with WSSV Viet. With 33°C for 18 h per day, cumulative mortalities were 0-40% with WSSV Thai-1 and 5-15% with WSSV Viet. At constant 27°C and at 33°C for 6 h or 12 h per day, dead shrimp were WSSV positive and euthanized shrimp were WSSV negative. At 33°C for 18 h or 24 h per day, the dead and euthanized shrimp were WSSV negative except the dead shrimp at 33°C for 18 h per day with WSSV Thai-1 which were WSSV positive. This study showed that daily fluctuations of temperature between 27°C and 33°C have an effect on WSSV infected shrimp with both highly virulent and low virulent isolate. It also indicates that the exposure to 33°C for 18 h per day is effective to prevent disease and reduce mortality of shrimp independent on the virulence of the isolates.

Finally, the effect of high water temperature (33°C) at different stages of WSSV infection in *Penaeus vannamei* juveniles was investigated. Shrimp were inoculated intramuscularly with either a high dose (HD) (10000 SID_{50}) or a low dose (LD) (30 SID_{50}) of WSSV Thai-1 (highly virulent) or WSSV Viet (low virulent). Water temperature was kept either at continuously 27°C or switched from 27°C to 33°C at 0, 12 or 24 hours post inoculation (hpi) for both isolates and in addition at 48 or 96 hpi for WSSV Viet. The

increased temperature 33°C was maintained till the end of the experiments. To determine the infection status at the moment of temperature increase, five shrimp that were kept continuously at 27°C were euthanized at 0, 12, 24, 48 and 96 hpi with each dose of two isolates. Shrimp inoculated with HD or LD of WSSV Thai-1 and kept continuously at 27°C till euthanasia were 100% WSSV positive from 12 (HD) or 24 hpi (LD). Shrimp inoculated with WSSV Viet were 100% positive from 24 (HD) and 48 hpi (LD). Shrimp kept at 27°C showed clinical signs; cumulative mortalities reached 100% with WSSV Thai-1 at 60 (HD) or 84-144 hpi (LD) and with WSSV Viet 100% at 216 hpi (HD) or 90% at 240 hpi (LD). Switch of temperature to 33°C from 0, 12 or 24 hpi was effective in reducing mortality of shrimp inoculated with the LD of both isolates and with the HD of WSSV Viet. Switch to 33°C from 24 hpi with the Thai isolate (HD) and from 48 and 96 hpi with the Viet isolate (LD or HD) had no effect or even accelerated the mortality rate (80-100%). All shrimp were WSSV positive at death and euthanasia (one shrimp LD WSSV Viet) when kept continuously at 27°C. All dead and euthanized shrimp kept at 33 °C from 0 or 12 hpi were WSSV negative. With 33°C from 24, 48 or 96 hpi, all dead shrimp were WSSV positive and euthanized shrimp were negative. This study showed that 33°C is effective to prevent disease, reduce mortality and block WSSV replication, but only in the early stages of infection.

In conclusion, the findings of this thesis demonstrated clear differences in virulence of WSSV isolates and indicated that the degree of replication in tissues especially in gills may determine the virulence of WSSV isolates. The *in vivo* model can be used to evaluate the virulence of many other geographical isolates as well as the susceptibility of different shrimp species and populations. Among the strategies, high water temperature (33°C) was more effective than antivirals to control disease and mortality of WSSV infected shrimp. The efficacy of 33°C is independent of inoculation route, infectious titer used for inoculation and virulence of the WSSV isolates. A high water temperature is only effective when applied at the early stage of infection. The positive results obtained with 33°C for 18 h per day has demonstrated the potential of its application.

White Spot Syndrome Virus (WSSV) heeft de afgelopen 15 jaar in bijna alle landen waar garnalen worden gekweekt ernstige productieverliezen veroorzaakt. In veld- en experimentele studies werd reeds gesuggereerd dat er verschillen in virulentie bestaan tussen verschillende geografische isolaten, maar het achterliggend mechanisme hiervoor is onbekend. Sinds het verschijnen van WSSV hebben wetenschappers een aantal controle strategieën uitgeprobeerd om de productieverliezen in te perken. Deze testen leverden dusver weinig succes op, maar wel tegenstrijdige resultaten. Dit was voornamelijk te wijten aan het gebruik van willekeurige WSSV isolaten en het toedienen van onbekende dosissen. Het doel van deze doctoraatsthesis was dan ook het ontwikkelen van een *in vivo* model om de virulentie van WSSV isolaten te vergelijken, de achterliggende oorzaak van deze verschillen aan te wijzen en betere resultaten te bekomen in bestrijdingsexperimenten door een gestandaardiseerde inoculatieprocedure te gebruiken met een virusstam met gekende virulentie.

In de inleiding in Hoofdstuk 1 wordt een overzicht gegeven van de kweek en ziekten van garnalen. Verder wordt een bespreking gegeven over de huidige kennis van de pathogenese en verschillen in virulentie bij WSSV isolaten, antivirale immuniteit bij garnalen, rol van omgevingsfactoren op infecties met WSSV en de bestrijding van het virus.

In Hoofdstuk 2 worden de doelstellingen van de thesis beschreven.

Hoofdstuk 3 beschrijft een studie van de virulentie van WSSV isolaten. In een eerste deel werd de virulentie vergeleken tussen 3 isolaten (WSSV Thai-1, WSSV Thai-2 en WSSV Viet) door juveniele specific pathogen free (SPF) *Penaeus vannamei* te injecteren met 30 “shrimp infectious dose with 50 % endpoint” (SID_{50}). De verschillen in virulentie werden opgemeten aan de hand van het optreden van de eerste sterfte, de tijd totdat de cumulatieve sterfte 100 % bereikte en de “median lethal time” (LT_{50}). De garnalen vertoonden symptomen vanaf 24-36 uren na inoculatie (uni) met eender welk isolaat. De eerste mortaliteiten werden genoteerd op 36 uni bij de garnalen die met de Thaise isolaten geïnjecteerd waren, en op 36-60 uni met het Vietnamees isolaat. Respectievelijk voor WSSV Thai-1, WSSV Thai-2 en

WSSV Viet bereikte de cumulatieve mortaliteit 100 % op 72-84, 108 en 204-348 uni en was de LT_{50} 47, 61 en 120 uni. Deze verschillen testten significant.

In een tweede deel werd de virusvermeerdering in de weefsels vergeleken tussen garnalen die geïnoculeerd waren met 30 SID_{50} van de hoog virulente WSSV Thai-1 of de laag virulente WSSV Viet. Voor beide stammen werden dieren geëuthanaseerd op 0, 12, 24, 36, 48 en 72 uni, en voor de WSSV Viet werd nog bijkomend geëuthanaseerd op 120 en 168 uni. Garnalen die een natuurlijke dood stierven gedurende deze periode werden eveneens verzameld. WSSV geïnfecteerde cellen werden gevisualiseerd met immonohistochemie en geteld in de volgende weefsels: kieuwen, maagepitheel, cuticulair epitheel, hematopoietisch weefsel en antennenklier. Globaal beschouwd werd een hoger aantal geïnfecteerde cellen gevonden in de weefsels van garnalen die geïnoculeerd waren met WSSV Thai-1 dan met WSSV Viet. Het gemiddeld aantal positieve cellen in garnalen gestorven ten gevolge van WSSV Thai-1 was 523 ± 321 cellen/mm², 38 ± 7 %, 28 ± 8 %, 259 ± 155 cellen/mm² en 11 ± 6 % in respectievelijk de kieuwen, het maagepitheel, het cuticulair epitheel, het hematopoietisch weefsel en de antennenklier. Het gemiddeld aantal positieve cellen in garnalen gestorven ten gevolge van WSSV Thai-1 was 86 ± 63 cellen/mm², 22,5 ± 7,6 %, 17,7 ± 8,4 %, 160 ± 103 cellen/mm² en 5,2 ± 3,1 % in respectievelijk de kieuwen, het maagepitheel, het cuticulair epitheel, het hematopoietisch weefsel en de antennenklier. Deze studie toonde duidelijke virulentieverschillen aan tussen WSSV isolaten. Vooral de vermeerdering in de kieuwen zou de virulentie van WSSV kunnen bepalen (6 maal meer geïnfecteerde cellen in dode garnalen geïnoculeerd met een hoog virulent isolaat).

In hoofdstuk 4 wordt een studie voorgesteld waarbij een antiviraal product (cidofovir) en een dieet gesupplementeerd met *Spirulina platensis* getest werden op hun efficaciteit om ziekte en sterfte van WSSV geïnfecteerde garnalen te beperken. Cidofovir, een nucleotide-analoog waarvan bewezen is dat het DNA virussen in zoogdieren kan bestrijden, werd geïnjecteerd in garnalen aan concentraties tussen 12,5 en 200 mg/kg zonder enig teken van toxiciteit. De hoogste concentratie (200 mg/kg) werd intramusculair geïnjecteerd op het moment dat garnalen geïnoculeerd werden met virus of fosfaatgebufferde zoutoplossing (controle). Een andere groep dieren kreeg de alg *S. platensis* toegediend in het voedsel. Eén

van de bestanddelen daarvan is calcium spirulan, een gesulfateerde polysaccharide die de vermeerdering van virussen *in vitro* kan inhibiteren. Het dieet met *S. platensis* werd gedurende 4 dagen voor de inoculatie gevoederd aan 5 % van het lichaamsgewicht. Controle dieren kregen een voeder zonder *S. platensis*. Alle garnalen werden per os geïnoculeerd met 30 SID_{50} WSSV Thai-1. Een behandeling met cidofovir stelde de eerste sterfte 24 uur uit in vergelijking met de controle groep en de cumulatieve mortaliteit bereikte 80-90 % na 5 dagen. De LT_{50} was significant verschillend tussen de met cidofovir behandelde groep en de controle. Met of zonder *S. platensis* in het dieet werd de eerste sterfte genoteerd op 36 uni en bereikte de cumulatieve mortaliteit 100 % op 84 uni. Alle dode en geëuthanaseerde garnalen waren positief voor WSSV op IIF behalve 1 met cidofovir behandelde garnaal die overleefde tot 120 uni. Deze studie toonde aan dat cidofovir effectiever was dan *S. platensis* om infecties met WSSV in te perken.

In Hoofdstuk 5 werd het effect onderzocht van de watertemperatuur (27°C, 30°C en 33°C) op de klinische en virologische evolutie van WSSV geïnfecteerde garnalen. Hiervoor werden garnalen per os of intramusculair geïnoculeerd met een lage (30 SID_{50}) of een hoge dosis (10000 SID_{50}) WSSV Thai-1. De temperatuur werd ofwel (a) constant bij 27°C, 30°C of 33°C gehouden voor en na de inoculatie, ofwel (b) van 33°C naar 27°C gebracht na de inoculatie, ofwel (c) van 27°C naar 33°C gebracht na de inoculatie. Deze respectievelijke temperaturen werden behouden gedurende 4 dagen voor en 5-6 dagen na de inoculatie tot het einde van het experiment. Garnalen die continu bij 27°C of 30°C of van 33°C naar 27°C werden gehouden vertoonden symptomen, hadden 100 % mortaliteit en waren allen WSSV positief. De garnalen die ofwel constant bij 33°C werden gehouden of zij die van 27°C naar 33°C werden verwisseld na de inoculatie, vertoonden geen symptomen en de gemiddelde sterfte bedroeg 10 %. Al de gestorven en geëuthanaseerde dieren waren negatief voor WSSV. Voor een opvolging in de tijd werden garnalen oraal geïnoculeerd met 10000 SID_{50} WSSV Thai-1. Deze garnalen werden ofwel constant bij 27°C gehouden voor en na de inoculatie ofwel bij 27°C gehouden vooraf en op het moment van inoculatie bij 33°C geplaatst. Deze dieren werden vervolgens geëuthanaseerd op 12 en 24 uni. Op beide tijdstippen werden WSSV positieve cellen aangetoond in alle dieren die bij 27°C zaten, terwijl bij de groep die

bij 33°C werd gehouden geen positieve cellen werden gevonden. Met deze studie werd aangetoond dat ziekte en sterfte van garnalen tengevolge van WSSV infectie voorkomen kan worden met een hoge watertemperatuur van 33°C, zowel voor en na de inoculatie als enkel na. Een blootstelling aan 33°C enkel vóór de inoculatie had geen bevorderlijk effect. Het effect van 33°C lijkt gebaseerd te zijn op een inhibitie van de replicatie en was onafhankelijk van de inoculatieroute of dosis.

Verder werd de impact van dagelijkse temperatuurschommelingen tussen 27°C en 33°C op de klinische en virologische evolutie van WSSV geïnfecteerde garnalen geëvalueerd. Garnalen werden intramusculair geïnjecteerd met 10000 SID_{50} WSSV Thai-1 of WSSV Viet. De temperatuur werd ofwel constant bij 27°C gehouden, ofwel veranderd van 27°C naar 33°C op 12 uni en dan behouden gedurende 6, 12, 18 en 24 u per dag. De overgebleven tijd van de dag werd de temperatuur teruggebracht naar 27°C. De garnalen die zich bij constante 27°C bevonden vertoonden symptomen en stierven allen in 2 experimenten met beide virusisolaten. In de groepen die bij constant 33°C werd gehouden (na 12 uni) werden voor beide virusisolaten geen symptomen waargenomen en was de mortaliteit ≤ 10 %. Een blootstelling aan 33°C gedurende 6 of 12 u per dag resulteerde in 90-100 % cumulatieve sterfte met WSSV Thai-1 en 50-95 % met WSSV Viet. Met 33°C voor 18 u per dag was de cumulatieve sterfte 0-40 % met WSSV Thai-1 en 5-15 % met WSSV Viet. De dode garnalen gehouden bij constant 27°C of 6 en 12 u per dag aan 33°C waren positief voor WSSV; de geëuthanaseerde waren negatief. Bij 18 of 24 u bij 33°C waren dode en geëuthanaseerde dieren negatief, behalve de gestorven exemplaren die geïnjecteerd waren met WSSV Thai-1 en 18 u per dag bij 33°C werden gehouden. Deze studie toonde aan dat dagelijkse fluctuaties tussen 27°C en 33°C een effect hebben op garnalen geïnfecteerd met hoog of laag virulente WSSV isolaten. Vanaf 18 u per dag kan een blootstelling aan 33°C de ziekte voorkomen en de sterfte beperken, onafhankelijk van de virulentie van het virus.

Tenslotte werd het effect onderzocht van een hoge watertemperatuur in verschillende stadia van WSSV infectie in juveniele *Penaeus vannamei*. Garnalen werden intramusculair geïnoculeerd met een lage (LD: 30 SID_{50}) of een hoge dosis (HD: 10000 SID_{50}) van ofwel WSSV Thai-1 of WSSV Viet. De temperatuur werd ofwel constant bij 27°C gehouden, ofwel veranderd van 27°C naar 33°C op 0, 12 en 24 uni voor beide isolaten en bijkomend op 48 en

96 uni voor WSSV Viet. De verhoogde temperatuur werd dan behouden tot het einde van het experiment. Om de infectiestatus op het moment van de temperatuursverhoging te bepalen werden voor iedere dosis van ieder virusisolaat 5 garnalen die bij constante 27°C werden gehouden geëuthanaseerd op 0, 12, 24, 48 en 96 uni. Deze waren 100 % positief voor WSSV Thai-1 op 12 uni (HD) en 24 uni (LD) en 100 % positief voor WSSV Viet op 24 uni (HD) en 48 uni (LD). De garnalen die bij constant 27°C werden gehouden vertoonden symptomen en een cumulatieve mortaliteit van 100 % ten gevolge van WSSV Thai-1 op 60 uni (HD) of 84-144 uni (LD) of van een HD WSSV Viet op 216 uni of 90 % cumulatieve mortaliteit op 240 uni met een LD WSSV Viet. Een verhoging van de temperatuur naar 33°C op 0, 12 of 24 uni kon de mortaliteit reduceren van garnalen die geïnoculeerd waren met een LD van beide isolaten of een HD van WSSV Viet. Een temperatuursverhoging op 24 uni met een HD WSSV Thai-1 of vanaf 48 of 96 uni met WSSV Viet (LD en HD) had geen effect of versnelde zelfs het tempo waarmee de dieren stierven. Alle garnalen die bij constant 27°C zaten waren WSSV positief op het moment van overlijden of euthanasie (1 exemplaar LD WSSV Viet). Alle dode en geëuthanaseerde garnalen die bij 33°C werden gehouden vanaf 0 of 12 uni waren negatief voor WSSV. Met 33°C vanaf 24, 48 of 96 uni waren alle dode garnalen WSSV positief en alle geëuthanaseerde negatief. Deze studie toonde aan dat een temperatuursverhoging naar 33°C een adequaat middel is om ziekte te voorkomen, sterfte te beperken en vermeerdering van WSSV te blokkeren, maar enkel in de vroege infectiestadia.

Het besluit uit de resultaten van deze thesis is dat er duidelijke virulentieverschillen bestaan tussen WSSV isolaten en dat de graad van vermeerdering in de doelwitorganen (vooral de kieuwen) mogelijk bepalend is voor de virulentie. Het *in vivo* model kan naast het evalueren van de virulentie van andere geografische isolaten, ook gebruikt worden om de gevoeligheid te testen van verschillende garnalenspecies en populaties. Van de geteste bestrijdingsstrategieën was een temperatuursverhoging meer effectief dan het toedienen van antivirale producten om de ziekte en mortaliteit van WSSV geïnfecteerde garnalen te bedwingen. De werkzaamheid van 33°C was onafhankelijk van de inoculatieroute, de geïnoculeerde infectieuze titer en de virulentie van het virusisolaat. Een hoge temperatuur helpt echter enkel in de vroege stadia van de infectie. De positieve resultaten die bekomen

werden door de temperatuur slechts 18 u per dag te verhogen, vergroten de toepasbaarheid van de techniek in het veld.

Curriculum vitae

Muhammad Meezanur Rahman (Meezanur) was born in the district of Laxmipur, Bangladesh, on May 30, 1968. Meezanur graduated in Bachelor of Science in Fisheries (Honours) in 1992 with first class from Bangladesh Agricultural University, Mymensingh, Bangladesh. Then, he worked as a Monitoring and Evaluation Officer and Extension and Training Officer in the Northwest Fisheries Extension Project – Phase II (NFEP II), Parbatipur, Dinajpur, Bangladesh from July 1993 till August 1997. NFEP II was a bilateral project between the Government of Bangladesh and Department for International Development of the United Kingdom (DFID, UK). In 1997, Meezanur was awarded a scholarship from the Belgian Administration for Development Cooperation to follow the courses of Master of Science in Aquaculture at Ghent University. This master is an inter-university programme between Ghent University, Belgium and Wageningen University, The Netherlands. He completed his M.Sc. in Aquaculture in 1999 with great honour. His thesis title was "Heat shock response and induced thermotolerance (ITT) in freshwater prawn *Macrobrachium rosenbergii* larvae (De Man, 1879)". Afterwards, Meezanur returned to Bangladesh and worked as Trial officer and Hatchery Manager (Freshwater prawn *M. rosenbergii*) in NFEP II for another year. In 2001, he joined as an assistant technical specialist (Fisheries) in an Agrobased industries and technology development project, phase II (ATDP II). ATDP II was funded by the United States agency for international development and was implemented by three US companies: The Louis Berger Group Inc., Cargill Inc., and Land O' Lakes Inc. After three months, Meezanur was promoted as Fisheries Technical Specialist and Regional Director of Jessore and later Khulna regional office of the project. Then, he was nominated as Regional Director of shrimp seal of quality programme. In September 2003, Meezanur received a Ph.D. scholarship from the Belgian Technical Cooperation to study at Ghent University. During the last four years, he published six scientific articles in peer reviewed journals and attended international conferences with several oral and poster presentations.

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