On the Role of the Polychaete *Dendronereis* spp. in the Transmission of White Spot Syndrome Virus in Shrimp Ponds

Desrina

Propositions

1. White Spot Syndrome Virus is widely distributed in *Dendronereis* spp. (This thesis)

2. Polychaetes are vectors of white spot syndrome virus in shrimp ponds. (This thesis)

3. Pathogens exist in nature in balance with host populations and it is up to us humans to determine which direction the balance will tilt.

4. The internet built-world prompts individuals to have an amazing virtual social life, while they are less sociable to their immediate surroundings.

5. Animal rights should be based on ecological balance instead of on human perception of animal welfare.

6. 'What's in a name' is culturally determined.

7. The h-factor is in fact an age-factor.

Propositions belonging to the thesis entitled:

On the Role of the Polychaete *Dendronereis* spp. in the Transmission of White Spot Syndrome Virus in Shrimp Ponds

Desrina
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Desrina

Thesis

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ABSTRACT

Desrina. (2014). On the role of the polychaete *Dendronereis* spp. in the transmission of white spot syndrome virus in shrimp ponds. Ph.D thesis, Wageningen University, the Netherland.

White spot syndrome virus (WSSV) is by far the most devastating shrimp virus. Control measures have lowered the WSSV incidence to various degrees, but the pathogen remains plaguing shrimp culture worldwide. Continuous exposure may cause WSSV to adapt and infect non-crustacean benthic fauna in ponds such as polychaetes, hence, extending WSSV host range to maintain virus persistence in ponds. *Dendronereis* spp. (Pieters 1854) are ubiquitous Nereid polychaetes in shrimp ponds in Indonesia and part of the shrimp's natural diet. This thesis aimed to investigate the possible role of *Dendronereis* spp. in the transmission of WSSV in shrimp ponds. The significance of the findings may provide new insight on the persistence of WSSV in the pond environment and novel strategies for disease management. The investigation started with a survey to determine the occurrence of WSSV in *Dendronereis* spp. in Indonesia, followed by subsequent laboratory observations to determine the role of *Dendronereis* spp. in white spot syndrome disease development. Field surveys in selected ponds in two research locations in Indonesia, the Mahakam delta (East Kalimantan) and the vicinity of Semarang (Central Java), showed that WSSV infection in *Dendronereis* spp. is quite common. Point prevalence of WSSV infected *Dendronereis* spp. was 44 ± 27% (± SD). The average prevalence in Mahakam delta was 73 ± 22% and in Java 26 ± 38%. This result implied that WSSV-infected *Dendronereis* spp. are widely distributed. WSSV replicated in the gut of naturally-infected *Dendronereis* spp. as detected in cell nuclei via immunohistochemistry (IHC) using monoclonal antibodies and via RT-PCR to detect the viral mRNA. These experiments showed that *Dendronereis* spp. are natural and susceptible hosts of WSSV. WSSV was transmitted from naturally infected *Dendronereis* spp. to *Litopenaeus vannamei* (Boone 1931) through the oral route and further to new naïve shrimp showing natural transmission of WSSV from polychaetes to shrimp. This indicates that the transmission of WSSV from polycheates to shrimp is possible. An experiment using *Hediste diversicolor* (O.F. Müller 1776) as a more amenable alternative model animal to study WSSV infection in polychaetes showed that this polychaete

was not susceptible to WSSV infection using methods commonly used to induce infection in shrimp. In ponds, WSSV infection incidence in *Dendronereis* spp. correlated positively with *Dendronereis* spp. density and with the proportion of WSSV infection in shrimp. Findings of the present study underscore that *Dendronereis* spp., as ubiquitous and resident animals in the shrimp ponds can be reservoir hosts of WSSV and responsible for disease transmission. However, further studies are needed to obtain a better understanding of the importance of *Dendronereis* spp in WSSV epidemiology in and beyond shrimp ponds.

ABSTRAK

Desrina. (2014). On the role of the polychaete *Dendronereis* spp. in the transmission of white spot syndrome virus in shrimp ponds. Ph.D thesis, Wageningen University, the Netherland.

White spot syndrome virus (WSSV) adalah virus udang yang paling merugikan sampai saat ini. Walau metoda pengendalian yang digunakan telah mampu menurunkan insidensi serangan patgogen ini sampai tahap tertentu, namun WSSV tetap menjadi ancaman bagi budidaya udang di dunia. Paparan yang terus menerus terhadap WSSV bisa mengakibatkan virus teradaptasi dan menginfeksi fauna bentik selain udang yang hidup di tambak seperti cacing polychaeta. Hal ini akan dapat memperlebar rentang inang WSSV dan membantu mempertahankan keberadaan virus di tambak sehingga sukar untuk kendalikan. *Dendronereis* spp. (Pieters 1854) adalah polychaeta dari family Nereidae yang banyak hidup di dasar tambak di Indonesia dan merupakan pakan alami udang. Thesis ini bertujuan untuk meneliti tentang peran *Dendronereis* spp. dalam penyebaran WSSV di tambak. Hasil penelitian akan menyumbangkan pengetahuan baru tentang rentang inang yang dapat menjadi faktor dalam persistensi WSSV di tambak dan pengendaliannya. Penelitian diawali dengan survey di lapangan untuk menentukan keberadaan WSSV di *Dendronereis* spp. di Indonesia, diikuti dengan serangkaian penelitian laboratorium. Survei lapangan dilakukan di tambak udang yang terletak di delta Mahakam (Kalimantan Timur) dan di sekitar kota Semarang (Jawa Tengah). Hasil survey menunjukkan bahwa infeksi WSSV di *Dendronereis* spp. umum dijumpai di dua lokasi ini. Prevalensi sesaat infeksi WSSV di *Dendronereis* spp. adalah 44 ± 27% (± SD). Prevalensi sesaat di lokasi di delta Mahakam adalah 73 ± 22% dan Semarang 26 ± 38%. Hasil survei juga menunjukkan bahwa distribusi *Dendronereis* spp. yang terinfeksi WSSV di Indonesia cukup luas. WSSV bereplikasi di dalam saluran pencernaan *Dendronereis* spp. yang terinfeksi secara alami seperti yang ditunjukkan hasil uji immunohistochemistry (IHC) dengan menggunakan monoclonal antibodi dan adanya virus mRNA berdasarkan hasil uji RT-PCR. Hal ini menguatkan bahwa *Dendronereis* spp. secara alami rentan terifeksi dan inang dari WSSV. WSSV ditularkan dari *Dendronereis* spp. yang terinfeksi secara alami ke udang vanname *Litopenaeus vannamei* (Boone 1931), dan selanjutnya ke udang baru yang sehat

melalui oral. Percobaan untuk menguji kelayakan *Hediste diversicolor* (O.F. Müller 1776) sebagai hewan model untuk mempelajari infeksi WSSV di polychaete belum memberikan hasil yang diharapkan. *H. diversicolor* tidak rentan terinfeksi WSSV menggunakan metoda yang biasa digunakan untuk menimbulkan infeksi pada udang. WSSV infeksi di *Dendronereis* spp. di tambak berkorelasi positif dengan densitas cacing ini dan infeksi WSSV di udang yang ada di tambak. Hasil penelitian dalam thesis ini menekankan bahwa *Dendronereis* spp. berpotensi menjadi sumber penularan WSSV di tambak. Diperlukan penelitian lebih lanjut untuk menentukan seberapa penting peran cacing *Dendronereis* spp. dalam transmisi WSSV dibandingkan inang dan vektor krustase lain di tambak.

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CHAPTER 1.
GENERAL INTRODUCTION

BACKGROUND

Shrimp culture.

Shrimp culture has evolved in less than four decades from a small sector producing for local markets (Bardach et al. 1972) into a major industry producing for the global market (FAO 2012). Total shrimp culture production in 2011 was close to 5 million metric tons (FAO 2013), in which the Pacific white leg shrimp (*Litopenaeus vannamei* Boone 1931) and the giant tiger shrimp (*Penaeus monodon* Fabricius 1798) are the two major traded species. However, in the last decade *P. monodon* has been replaced by *L. vannamei* as the main cultured shrimp for a variety of reasons: the availability of hatchery- produced specific pathogen-free (SPF) larvae (Cock et al. 2009), lower dietary protein requirements, greater tolerance to high density and a wider range of salinity and presumed increased pathogen tolerance. The giant tiger prawn was originally the major cultured species, but between 2000 and 2011 its production stagnated around 600,000 metric tons per year. Over the same period, the production of Pacific white leg shrimp grew explosively from 146,000 metric tons in 2000 to close to 3 million metric tons today (FAO 2013), primarily due to its introduction and production in Asia. Although brackish water aquaculture by weight represents only 8 % of the total world aquaculture production, it represents 13% by value, because of the high price of the cultured shrimp (FAO 2012).

Sustainability of shrimp culture has been questioned in relation to the boom and bust pattern associated with transformation of coastal areas into shrimp ponds, destruction of mangrove forests, farming intensification, environmental deterioration and farming collapse due to diseases (Cock et al. 2009; Kautsky et al. 2000). Improvements and innovations to reduce disease incidence included breeding for disease resistance (Cock et al. 2009), use of specific pathogen free (SPF) post larvae (PL) and broodstock (Baliao 2000; Corsin et al. 2003; Lotz 1997; Menasveta 2002; Withyachumnarnkul 1999; Withyachumnarnkul et al. 2003), better designs for shrimp grow out systems (Menasveta 2002) and pond management strategies (Baliao 2000; Subasinghe 2005), and implementation of an ecosystem based approach for disease control (Soto et al. 2008). This has resulted in a promising outlook for sustainable shrimp culture in the future.

WSSV, an important pathogen of shrimp culture.

Since its intensification shrimp aquaculture has been plagued by diseases. This had a huge economic impact on smallholder shrimp farmers in developing countries, and almost brought down national economies, such as the one in Ecuador in 1999. The rapid development in shrimp culture worldwide has been followed by the emergence of a plethora of viral and bacterial diseases. To date, the emergence of eleven viral pathogens of shrimp in the last two decades had a major impact on shrimp culture and has caused huge economic losses amounting to an estimated 2 billion US dollars annually (Walker and Winton 2010). The emergence viruses associated with farmed shrimp diseases are listed (Table 1) and many of these are notifiable to the World Organization for Animal Health (OIE) in Paris, France. Although once restricted to the region of origin, many diseases quickly spread around the world through false assumptions of biosecurity in trading live animal. A typical disease is such as the one caused by the Taura syndrome virus

which was initially only occurred in South America. However, it was introduced into Asia along with *L. vannamei* when that species was thought to be resistant against white spot syndrome virus (WSSV).

WSSV causes white spot disease (WSD) and is by far the most important viral pathogen to shrimp culture industry worldwide (Flegel 2012), with associated economic losses reaching USD 1 billion/year (Stentiford et al. 2012). The disease is called white spot disease because of the prominent external clinical signs in the form of white spots on the carapace of *P. japonicus* and *P. monodon*. The pathogenesis and the clinical signs in penaeid shrimp and crabs have been described in much detail (Escobedo-Bonilla et al. 2008; Flegel 2006; Sahoo et al. 2005; Wang et al. 1999a; Yoganandhan et al. 2003). In infected shrimp these signs include body discoloration, loss of appetite and slower movement. These properties often make WSSV-infected shrimp a target for predation and cannibalism by healthy crustaceans causing quick dissemination of the disease.

The first report of WSSV epizootic was the outbreak in the kuruma shrimp (*Marsupenaeus japonicus* Spence Bate 1988) cultured in Taiwan in 1992 (Chou et al. 1995) and in Japan in 1993 (Nakano et al. 1994). Soon thereafter, the pathogen spread in Asia (Flegel 1997; Kasornchandra et al. 1998; Rajan et al. 2000; Shin et al. 2001) and the Americas in 1999 (Lightner et al. 2012). More recently, the virus was reported from Europe (Stentiford and Lightner 2011), Mozambique, Madagascar and Saudi Arabia (Tang et al. 2013), which were areas that were not affected by the WSSV pandemic until shrimp culture was introduced.

Table 1. Viral diseases of cultured shrimp

Virus	Abbreviation	Genome	Viral Family	Host	Notifiable to OIE	Reference
DNA Virus						
Monodon baculovirus	MBV	Ds DNA	Baculoviridae	*Penaeus monodon, Macrobrachium rossenbergii, Metapenaeus monoceros, M.elegans*	No	(Gangnonngiw et al. (2010); Lightner and Redman (1998); Manivannan et al. (2004))
Baculoviral midgut gland necrosis virus	BMNV	dsDNA	Baculoviridae	*P.monodon*	No	(Walker and Winton 2010)
White spot syndrome virus	WSSV	dsDNA	Nimaviridae	Penaeids shrimp, crabs, lobster,	Yes	Stentiford et al. (2009) (de la Vega et al. (2008); Walker and Winton (2010))
Infectious hypodermal and haematopoietic necrosis virus	IHHNV	ssDNA	Parvoviridae	Cultured penaeid shrimp, *Macrobrachium rossenbergii*	Yes	(Vega-Heredia et al. 2012)
Hepatopancreatic parvovirus	HPV	ssDNA	Parvoiridae	Penaeid shrimp	No	Safeena et al. (2012)

Virus	Abbreviation	Genome	Viral Family	Host	Notifiable to OIE	Reference
RNA virus						
Yellow head virus	YHV	(+)ss RNA	Roniviridae	Penaeid shrimp	Yes	Munro and Owens (2007)
Taura Syndrome Virus	TSV	(+)ss RNA	Dicistroviridae	Penaeid shrimp	Yes	(Brock (1997); Flegel (2012))
Laem-Singh virus	LSNV	(+) ds RNA	Unclassified	Penaeid shrimp	No	(Kumar et al. (2011); Walker and Winton (2010))
Infectious Myonecrosis virus	IMNV	ds RNA	Totiviridae	*L.vannamei*	Yes	(Lightner 2011)

This showed that the virus follows the expansion of the shrimp culture, presumably through transportation of infected live or frozen shrimps or crabs. A concomitant effect has been that the virus appeared to have increased its virulence over time and space (Zwart et al. 2010) .

WSSV is one of the giant DNA viruses (giruses) (Claverie et al. 2006; Van Etten 2009) known today from the living kingdom. It is an enveloped, non-occluded, double-stranded DNA virus with a genome size of about 305 kilobase pairs (kbp). WSSV is a single virus species within the genus Whispovirus, family Nimaviridae (Lo et al. 2012) and has, in suspension, a rod- to ovoid-shaped virion with a tail-like structure (nima = thread in Greek) at one end. The inner core of the virion, the nucleocapsid of 220 x 70 nm in size, is wrapped in a proteinaceous envelope (Chou et al. 1995; Van Hulten and Vlak 2001). WSSV infect tissues of ectodermal and mesodermal origins such as gills, epithelial lining of the anterior and posterior gut, the hepatopancreas and the cuticular epidermis. Inside the animal the virus causes a lytic infection and infected shrimp shed the virions into the hemolymph. The virus replicates in the nucleus of infected cells and causes hypertrophy, necrosis and organ dysfunction, which ultimately leads to death of the shrimp (Chang et al. 1996; Durand et al. 1997).

WSSV has a broad range of hosts and vectors that mostly consist of decapod crustacean shrimp and crabs (Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010; Stentiford et al. 2009), and the spread over long distance by birds cannot be excluded. Among crustaceans, cultured penaeid shrimps are highly susceptible for the WSSV infection (Chou et al. 1995; Chou et al. 1998; Wang et al. 1995; Wang et al. 1998). The susceptibility of crabs and crayfish to WSSV varies among species (Bateman et al. 2012b; Hameed et al. 2003; Kanchanaphum et al. 1998; Waikhom et al. 2006). Being a generalist virus infecting a plethora of hosts, continuous exposure may cause the virus to adapt and infect less susceptible and

non-natural host such as non-crustacean benthic fauna, hence, extending its host range. Benthic fauna found in shrimp ponds consists primarily of Annelida (Polychaetes and Oligochaetes), Molusca (bivalves, gastropods), Nematoda and Rotifera (Boyd 1995). WSSV has been reported from molluscs (Chang et al. 2011; Vazquez-Boucard et al. 2010), annelids (Vijayan et al. 2005) and rotifers. It is equally possible that non-crustacean hosts only 'carry' the virus and only passively transmit the virus. The current strategies to control WSSV have not given satisfying results, may be because among others the impact of potential hosts for WSSV in the pond system other than shrimp has been underestimated.

Experimental transmission studies showed that WSSV is easily transmitted from infected shrimp to other susceptible and healthy shrimp *per os* (scavenging infected shrimp), via water/cohabitation and via feces (Rajan et al. 2000) and from decapods from the wild to cultured shrimp and vice versa (Esparza-Leal et al. 2009; Kanchanaphum et al. 1998). WSSV transmission can occur via hosts in which the virus is amplified (active hosts) or through hosts in which the viral DNA just accumulates (carrier or passive host). Ingestion of tissue from an infected host is the most important mode of transmission during the outbreak (Soto and Lotz 2001). Numerous hosts and carriers of the virus that are present in the pond environment and their relative abundance may contribute to the dynamics of WSSV in pond systems finally resulting in the occurrence of disease in the pond. Also, the genetic structure of the virus population and the shrimp culture system (intensive, extensive) may contribute to the outcome of an infection (Hoa et al. 2012a). Conversely, pond management may influence the number and type of host/vectors that are present and may affect the WSSV genotypes presence in the pond (Hoa et al. 2012a; Walker et al. 2011a). Improved-extensive shrimp culture system, being more open and less managed than semi-intensive culture systems, had a larger variety of WSSV genotypes (Hoa et al. 2011a).

Chapter 1

Polychaetes as potential hosts of WSSV in pond systems

Polychaetes contribute significantly to the benthic fauna of soft bottom estuaries (De Oliveira et al. 2012; García-Arberas and Rallo 2002). These organisms comprised 13% of the benthic community in a zero-water-exchange extensive pond (Balasubramanian et al. 2004). Through bioturbation, polychaetes reduce anaerobic zones in the sediment and stimulate oxidation of the organic matter (Kristensen et al. 2008). They are preferred natural food of shrimp (Fujioka et al. 2007b; Nunes et al. 1997). In addition, they induce gonad maturation (Hoa et al. 2009) by providing progesterone and 17α-hydroxy-progesterone, which enhances giant tiger shrimp oocyte maturation (Meunpol et al. 2010). Polychaetes survive and even thrive in low oxygen environments in tubes and burrows (Meksumpun and Meksumpun 1999; Sarkar et al. 2005). In shrimp ponds the polychaete community is affected by the amount of nutrients reaching the sediment and by shrimp predation (Martinez-Cordova et al. 1998). In extensive pond systems, they contribute significantly to shrimp production. Most polychaetes found in shrimp ponds are burrowers that live in the sediment up to 20 cm deep. Polychaetes are exposed to WSSV when ingesting infected sediment, i.e. detritus, shrimp feces and carcasses. Taken together, these conditions make polychaetes potential natural vectors, either as replicative or carrier hosts and important players in WSSV transmission in ponds.

THESIS RATIONALE

This study aimed to investigate the role of polychaetes as vector or carrier in WSSV transmission in shrimp ponds. Such role has been inferred from the previously known host range of WSSV, the relative abundance of polychaetes in shrimp ponds and from the observation that WSSV has been found in polychaetes

(Vijayan et al. 2005). These observations may imply that polychaetes are potential replicative vectors or carriers of WSSV.

On the basis of the existing knowledge on polychaetes and WSSV transmission, the following research hypotheses were formulated:

1. Polychaetes are natural replicative hosts of WSSV and are involved in the transmission of WSSV in pond systems.
2. Sediment conditions in pond systems influence the relative contribution of polychaetes in the transmission of WSSV.

The research focused on *Dendronereis* spp., cosmopolitan Nereid polychaete in the soft sediment estuary, where most shrimp ponds in shrimp-producing areas are located. This polychaete is a natural prey of shrimps in the pond. The research was carried out in East Kalimantan and Central Java (Indonesia), as representative areas for extensive and intensive shrimp culture.

The objective of this thesis was approached by investigating the following research questions:

1. Does WSSV occur in *Dendronereis* spp. in shrimp ponds in Indonesia and are WSSV-infected *Dendronereis* animals commonly found?
2. Does WSSV replicate in *Dendronereis* spp.?
3. Can *Hediste diversicolor* be used as a model animal to study WSSV infection in polychaetes?
4. Can WSSV be transmitted from polychaetes to shrimp?
5. Are there pond factors related to the presence of WSSV in polychaetes in shrimp ponds and is this related to shrimp culture practices?

OUTLINE OF THE THESIS

This thesis investigates the role of *Dendronereis* spp. in the ecology and transmission of WSSV in shrimp ponds. This investigation is part of a more

general issue and that is 'what are the factors that determine the development of WSSV into a world-wide epidemic in such a relatively short period of time'. A major element is the observation that WSSV is a 'generalist' virus, i.e. it infects a plethora of crustaceans and possibly other aquatic invertebrate animals. This wide host range may be a major reason not only for the development of the epidemic, but also for the frequent outbreaks in ponds, even when methods for the disease control are implemented such as the use of SPF shrimp and modern pond management systems. Many benthic invertebrates species can serve as a reservoir for WSSV or provide a shelter for the virus when its natural hosts (shrimp) are not readily available.

In **Chapter 2** a survey of the literature has been made on the host range of WSSV in crustaceans and other aquatic invertebrate organisms, with particular emphasis on (i) the techniques used to detect the virus, (ii) to show that the virus can replicate in that host and (iii) that the virus can be transmitted.

A major, outstanding question has been whether WSSV can replicate in a non-crustacean host. The virus was demonstrated to be present in polychaetes (Vijayan et al., 2005), but whether the virus could replicate in this organism and whether that organism can serve as a propagative host/vector or not was not elucidated. This was the objective of **Chapter 3**, e.g., to investigate whether or not the virus replicates in *Dendronereis* spp..

In **Chapter 4** it was investigated whether there is an alternative system to study WSSV replication and transmission, since *Dendronereis* cannot (yet) be cultivated in the laboratory. Therefore a polychaete that could be cultivated in the laboratory, *Hediste diversicolor*, was chosen. This polychaete occurs in the more temperate areas and may be susceptible to WSSV, just as crustaceans from temperate regions are, such as *Pacifastacus* spp..

It is important to show that the virus present in polychaetes can be transmitted to shrimp. Therefore in **Chapter 5** a bioassay was set up in which WSSV-containing polychaetes were fed to naive shrimp and the shrimp were checked for symptoms and for the virus. The potentially infected shrimp were fed to another batch of naive shrimp in order to confirm the transmission of the virus.

Finally an analysis was carried out on a number of shrimp ponds in different geographic regions of Indonesia, with different shrimp culture regimens (intensive versus extensive) to see whether there is a correlation between the abiotic environment, pond management regimes and the incidence of WSSV-infected polychaetes (**Chapter 6**).

In **Chapter 7** the results of the various chapters are discussed in the light of the importance of polychaetes in the transmission of WSSV in shrimp pond systems. A model will be presented on the role of polychaetes in WSSV transmission in shrimp ponds and/or ecosystems. Finally, a forward view is presented on the impact of this study on pond management strategies and the control of WSSV in pond systems.

CHAPTER 2.

ON THE TRANSMISSION OF THE WHITE SPOT SYNDROME VIRUS: A REVIEW

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ABSTRACT

White pot syndrome virus (WSSV) is a major pathogen of the shrimp farming industry. After its emergence in the early-1990s, the virus quickly spread around the globe mainly due to human actions including the transfer of life animal, poor pond management, and the availability of an ever increasing number of naive shrimp. Containment of the virus is difficult. WSSV persistence in the pond environment is aggravated by the observation that WSSV has a very wide host range among crustaceans, which could serve as a reservoir for the virus and a source of new infections of shrimp. Little is known about the presence of WSSV in non-crustaceans and their role in the transmission of WSSV. This review made a critical evaluation of existing literature on the presence of WSSV in benthic organisms with special attention for the potential role these organisms might play in the transmission of WSSV and the factors which influence WSSV outbreaks in ponds.

Keywords: WSSV, transmission, shrimp, benthic organisms.

Chapter 2

I. INTRODUCTION

White spot syndrome virus (WSSV), the etiological agent of white spot disease (WSD), has plagued the global shrimp culture industry for the last two decades causing massive production losses (Chou et al. 1998; Momoyana et al. 1994; Nakano et al. 1994; Stentiford et al. 2012). The spread of WSSV at a global level is influenced by transport of (i) WSSV-infected live animals (Nakano et al. 1994; Zwart et al. 2010) for farming purposes, (ii) WSSV-contaminated frozen shrimp products for human consumption (Bateman et al. 2012a) and (iii) crustacean broodstock for shrimp rearing or fishing purposes (Hasson et al. 2006). WSSV was first reported in *Marsupeneus japonicus* 1992 in Taiwan (Chou et al. 1998) and Japan (Momoyana et al. 1994; Nakano et al. 1994). Soon thereafter, WSD spread to other shrimp producing countries in Asia (Karunasagar et al. 1998; Shin et al. 2001; Sunarto et al. 2004) and developed into a pandemic, causing recurrent losses in shrimp production (Flegel 2006; Stentiford et al. 2012; Walker and Winton 2010). Since the mid-1990s WSD also occurs in the Americas where it almost pushed a country like Ecuador into bankruptcy (Lightner 2011). More recently, some EU countries (Stentiford and Lightner 2011), and Mozambique and Madagascar in Africa and Saudi Arabia (Tang et al. 2013; Tang et al. 2012) were affected by WSD. Transmission at the regional level can be partially controlled by implementing regulation to prevent trans-boundary disease spreading (Lightner 2012).

At the farm or estuary scale, WSSV spreads and maintains itself in the aquatic environment by transmission via intricate interactions of pathogens, numerous hosts or carriers and environmental factors (Flegel et al. 2004; Tendencia et al. 2010a; 2011; Tendencia et al. 2010b; Walker et al. 2011c). WSSV transmission in nature is complex, as numerous organisms present in ponds can act

as vector or host, while not all of these species are equally susceptible to or propagate the virus (Hameed et al. 2003; Hameed et al. 2001; Kanchanaphum et al. 1998; Somboonna et al. 2010; Waikhom et al. 2006). WSSV has a broad range of hosts and vectors, belonging mainly to 34 families of crustaceans (Sánchez-Paz 2010; Stentiford et al. 2009). In addition, nine non-crustacean species (this review) were reported as hosts of WSSV, showing the generalist nature of this virus.

As shrimp are cannibalistic and predaceous, oral transmission is the most important route followed by the water (immersion) route (Soto et al. 2008; Soto and Lotz 2001). Active carriers in which WSSV can replicate, such as shrimp, crabs and crayfish, can also be asymptomatic, hence being a potential reservoir for the virus. Non-crustacean invertebrates such as molluscs, polychaetes and plankton were, until recently, mainly regarded as passive or mechanical carriers of WSSV (Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010) meaning that the virus may simply 'passages' through the gut and is excreted with the feces. However, more in-depth studies showed that the polychaete *Dendronereis* spp. can be WSSV natural host (Desrina et al. 2013), constituting a potential WSSV reservoir in ponds (Haryadi et al. 2014). Plankton, as passive carrier, can play a role in WSSV transmission in ponds when taken up as food (Liu et al. 2007; Zhang et al. 2007; Zhang et al. 2006).

The ability of WSSV to infect a broad range of invertebrate species may relate to the adaptability of the virus to different hosts. As such, WSSV can become genetically diverse (Caipang et al. 2012; Hoa et al. 2012b; Waikhom et al. 2006) and modify existing proteins for entering and overcoming the shrimp's immune system (Chang et al. 2008; Hossain et al. 2004; Huang et al. 2013; Li et al. 2006; Sangsuriya et al. 2011; Song et al. 2010). Pond management regimens may create conditions enabling contact between WSSV and different resident host species, many of which are ubiquitous in ponds (Dsikowitzky et al. 2011; Fujioka et al.

2007a; Ngqulana et al. 2010). Furthermore, pond management regimens (Tendencia et al. 2011; Walker et al. 2011a; Walker et al. 2011c) and water condition such as salinity (Joseph and Philip 2007; Liu et al. 2006) and temperature (Rahman et al. 2007a; Rahman et al. 2006; You et al. 2010) increase the risk of disease occurrence. In this literature review, all currently known potential carriers of WSSV are documented, with special emphasis on the status in crustacean and non-crustacean hosts. This information is important to evaluate the risk of WSSV transmission in shrimp pond settings and to develop or implement effective disease control strategies. Finally, in this review, factors contributing to WSSV transmission in ponds are also reviewed and knowledge gaps identified.

II. CHARACTERISTICS OF WSSV

WSSV is a large double stranded DNA virus belonging to the genus *Whispovirus* of the *Nimaviridae* family (Lo et al. 2012) and one of the few giant viruses or giruses (Claverie et al. 2006). Currently, complete genome sequences of WSSV isolates from Thailand, China, Taiwan and Korea are listed in Gene-Bank. The genome size of WSSV varies between 290 kilobase pairs (kbp) (Thailand isolate; (van Hulten et al. 2001)) and 307 kbp (China isolate; (Yang et al. 2001)). The most recently published sequence from Korea has a size of 295 kbp (Chai et al. 2013).

The virion or virus particle is rod-to-ovoid in shape, with a length of 330 – 350 nm and a diameter of 65 – 80 nm. The nucleocapsid size is 220 x 70 nm, with a characteristic tail-like structure at one end (Chou et al. 1995; van Hulten et al. 2001; Wang et al. 1995). This extension (nima = thread) formed the rationale for the family name of the virus (Vlak et al. 2005). The proteinaceous envelope is 6-7 nm thick (Durand et al. 1997) and consists predominantly of a single protein, VP28, which is an important target for WSSV diagnostics. The virus was originally

classified as a non-occluded Baculovirus (NoB) (Wang et al. 1995), until it was re-assigned to its present name and classification (Lo et al. 2012).

III. DETECTION OF WSSV

The World Organization for Animal Health (OIE) as the authority to monitor and control animal diseases at the global level, prescribes microscopic, immunological and molecular techniques to detect WSSV (Lo 2014). Diagnosis of WSSV usually starts with visible, behavioral or clinical signs, which are then confirmed by microscopy or histological (staining) examination, or molecular techniques. Confirmation is important, especially at shrimp stocking and when testing a new potential host for WSSV. The Polymerase Chain Reaction (PCR) is a widely used and robust nucleic-acid-based method to detect WSSV. If not detected in a single step PRC, a nested PCR can be employed to further enhance the detection level. Its sensitivity and specificity to detect minute amounts of WSSV DNA is useful to investigate suspected carrier species or lightly infected animals because (i) clinical signs might be barely visible or not present at all (asymptomatic carriers) and (ii) white spots on the carapace may also be due to bacterial infection (Wang et al. 2000).

Historically, most reports of WSSV infection are based on PCR. However, to determine whether a species is a natural (replicative) host for the virus or just a mechanical vector, further testing either singular or a combination of the following assays is necessary: (i) immunohistochemistry to detect infected tissue using virus-specific antibodies; (ii) electron microscopy to view virions in nuclei of infected tissue cells; or (iii) Reverse Transcriptase PCR (RT-PCR) to detect viral mRNA. The mere presence of viral DNA with one-step or two-step (nested) PCR does not necessarily implies that the virion is ‘alive’ and is able to replicate in a particular host. Immunohistochemistry uses specific monoclonal or polyclonal antibodies,

e.g. against VP28, to detect viral protein in infected tissue. To be positive with immunohistochemisty, a cell must contain a high amount of viral protein, which is very likely the result of replicated virus. Immunohistochemisty is less sensitive than PCR and more time consuming, requiring skilled technicians able to recognize specific histopathological patterns. Electron microscope yields images of virions in specific tissues, but is not easily available. PCR is the most robust and most widely used technique, although with a slight risk of false-positives, and required high capital investment in equipment.

IV. WSSV HOST AND VECTORS

The principal phyla of macro-benthic organisms found in brackish water ponds are arthropods (*Arthropoda*), molluscs (*Mollusca*), annelids (*Annelida*) and aschhelminths (*Aschelminthes*), the latter including the nematods (*Nematoda*) (Abu Hena et al. 2011; Fujioka et al. 2007b). By far, arthropods are the most frequently reported hosts of WSSV (Table 1). Moreover, realizing the nature of WSSV as a generalist virus, there is a growing number of studies of potential non-crustacean hosts and vectors of WSSV such as mollusc and annelids in shrimp pond environments (Table 2).

WSSV is highly contagious to numerous shrimp and decapod species present in brackish water ponds (Bateman et al. 2012b; Cheng et al. 2013; Chou et al. 1995; Gitterle et al. 2005; Kasornchandra et al. 1998; Lo and Kou 1998; Nakano et al. 1994; Wang et al. 1998). Of these, the 22 reported penaeid (shrimp) species (Table 1) are considered the most susceptible to WSSV. In addition to the 4 phyla mentioned above, plankton (Esparza-Leal et al. 2009), phytoplankton (Jiang 2012; Liu et al. 2007) and the rotifers *Brachionus urceu*s (Linneaus 1758) (Jiang 2012) and *B. plicatilis* (Müller 1787) (Corre Jr et al. 2012) are also reported to be susceptible to WSSV (Table 2). Such a broad host and vector range is rather

unusual for viral pathogens, even in aquaculture, and the list of species is continuously expanding. The number of reported hosts or vectors increased from 31 in 1997 (Flegel 1997) to 46 in 2006 (Flegel 2006), then to 94 in 2008 (Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010), reaching 119 today (Tables 1 and 2).

Crabs comprised the largest group of reported WSSV vectors and are considered to be the most important source of WSSV infection in nature because of their ubiquitous occurrence and mobility in aquatic and terrestrial environments, and the absence of clinical signs. Susceptibility to WSSV differs between crab species. Some species show asymptomatic WSSV infection as the infection persisted up to 45 dpi without visible signs of disease and showing no mortality (Hameed et al. 2003; Kanchanaphum et al. 1998). For example, the mud crab *Scylla serrata* (Forsskål, 1775) (Somboonna et al. 2010), the fiddler crab *Uca pugilator* (Bosc 1802) (Kanchanaphum et al. 1998), *Atergatis integerrimus*, *Demania splendida*,*Charybdis natator*, *Menippe rumphii* (Hameed et al. 2003), the terrestrial crab *Sesarma* spp. (Say 1817), and the blue swimmer crab *Portunus pelagicus* (Linnaeus 1758) (Waikhom et al. 2006) showed some degree of resistance to WSD, while other crabs, such as *S. olivaceae* (Somboonna et al. 2010), *Paratelphusa hydrodomous* and *P. pulvinata* (Hameed et al. 2001), *Charybdis annulata*, *Grapsus albolineatus* (Hameed et al. 2003) are highly susceptible. Being phylogenetically close to shrimp, crabs exhibit similar tissue tropism to WSSV infection as shrimp (Chen et al. 2000). Although the number of hypertrophied nuclei was high, less tissue damage was observed in severely infected non-penaeid shrimp and crabs than in penaeid shrimp (Flegel 1997) explaining the general absence of disease symptoms. The ability to carry WSSV

Table 1. Crustacean hosts and vectors of WSSV

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Brackish water and marine shrimp										
Malacostraca	Decapoda	Penaeidae	*Metapenaeus brevicornis*	N	PCR	NR	NR	NR	Potential vector	(Hossain et al. 2001a)
Malacostraca	Decapoda	Penaeidae	*M. dobsoni*	N,F, IN	Histo	Y	Y	F	Host	(Hossain et al. 2001b; Rajan et al. 2000)
Malacostraca	Decapoda	Penaeidae	*M.ensis*	N, F	PCR, DNA Probe	NR	NR	NR	Potential vector	(Chang et al. 1998)
Malacostraca	Decapoda	Penaeidae	*M. monoceros*	N, IM, F	Nested -PCR, Probe, Histo	Y	Y	F	Host	(Hossain et al. 2001a; Rajendran et al. 1999)
Malacostraca	Decapoda	Penaeidae	*Parapeneopsis stylifera*	N	Nested PCR, Probe	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Decapoda	Penaeidae	*Farfantepenaeus aztecus*	N	PCR, Histo	N	Y	IN	Vector	(Chapman et al. 2004)
Malacostraca	Decapoda	Penaeidae	*F. duorarum*	F	Histo	Y	NR	NR	Host	(Wang et al. 1999a)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Penaeidae	*F. paulensis*	N	PCR, Histo	NA	NR	NR	Host	(Cavalli et al. 2010)
Malacostraca	Decapoda	Penaeidae	*F. brasiliensis*	N	PCR, Histo	NA	NR	NR	Host	(Cavalli et al. 2010)
Malacostraca	Decapoda	Penaeidae	*Penaeus merguiensis*	N	Histo, DNA probe	Y	Y	NR	Host	(Flegel 1997)
Malacostraca	Decapoda	Penaeidae	*P. penicillatus*	N	Nested- PCR	NR	NR	NR	Potential vector	(Lo et al. 1996)
Malacostraca	Decapoda	Penaeidae	*P. schmitti*	IN	Histo, ISH	Y	NR	NR	Host	(Unzueta-Bustamante et al. 2004)
Malacostraca	Decapoda	Penaeidae	*L. setiferus*	N	PCR, Histo	Y	Y	IN	Host	(Chapman et al. 2004)
Malacostraca	Decapoda	Penaeidae	*P. stylirostris*	N,	TEM, PCR, Histo	Y	NR	NR	Host	(Galavíz-Silva et al. 2004)
Malacostraca	Decapoda	Penaeidae	*L. vannamei*	N, F	TEM, PCR, Histo	Y	NR	NR		(Galavíz-Silva et al. 2004; Wang et al. 1999a)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Penaeidae	P. japonicus	N, IM,F	TEM, Histo	Y	Y	IM	Host	(Chou et al. 1995; Takahashi et al. 2007)
Malacostraca	Decapoda	Penaeidae	P.monodon	N, IM	TEM, histo	Y	Y	IM	Host	(Chou et al. 1995; Wang et al. 1999a)
Malacostraca	Decapoda	Penaeidae	P.indicus	N, IN, F	TEM, Histo	Y	Y	F	Host	(Rajan et al. 2000; Rajendran et al. 1999)
Malacostraca	Decapoda	Penaeidae	P.chinensis	N, IN	EM, Histo	Y			Host	(Zhan et al. 1998)
Malacostraca	Decapoda	Penaeidae	P.semisulcatus	N, F, IN	Nested PCR, Histo	Y	Y	F	Host	(Rajendran et al. 1999; Wang et al. 1998)
Malacostraca	Decapoda	Penaeidae	Trachypenaeus curvirostris	N, F	DNA Probe	Y	NR	NR	Potential vector	(Chang et al. 1998; Wang et al. 1999a)
Malacostraca	Decapoda	Sergestidae	Acetes sp	IN,F,IM	EM, IH, Histo	Y	NR	NR	Host	(Supamattaya et al. 1998)
Malacostraca	Decapoda	Alpheidae	Alpheus brevicristatus	N	Nested PCR	NR	NR	NR	Potential vector	(Takahashi et al. 2007)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Alpheidae	A. lobidens	N	Nested PCR	NR	NR	NR	Potential vector	(Takahashi et al. 2007)
Malacostraca	Decapoda	Solenoceridae	Solenocera indica	N	Nested PCR, Probe	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Stomato	Squillidae	Squilla mantis	N	Nested PCR, Probe	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
					Crabs					
Malacostraca	Decapoda	Xanthidae	Atergatis integerrimus,	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Xanthidae	Demania splendida	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Xanthidae	Halimede ochtodes	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Xanthidae	*Liagore rubronaculata*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Calappidae	*Calappa philargius*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Calappidae	*C. lophos*	F	PCR	NR	NR	NR	Potential vector	(Wang et al. 1999a)
Malacostraca	Decapoda	Majidae	*Doclea hybrida*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Grapsidae	*Grapsus albolineatus*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Grapsidae	*Metopograpsus messor*	N	Nested PCR	NA	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Decapoda	Grapsidae	*Pseudograpsus intermedius*	N	Nested PCR	NR	NR	NR	Potential vector	(Hossain et al. 2001a)
Malacostraca	Decapoda	Lithodidae	*Lithodes maja*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Matutidae	*Matuta miersi*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Matutidae	*M. Planipes*	N	Nested PCR	NR	NR	NR	Potential vector	(Otta et al. 1999)
Malacostraca	Decapoda	Eriphiidae	*Menippe rumphii*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Dorippidae	*Paradorippe granulata*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Partheno pidae	*Parthenope prensor*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Leucosiidae	*Philyra syndactyla*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Portunidae	*Podophthal mus vigil*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Portunidae	*Portunus sanguinolen tus*	IN, F	PCR, Histo	Y	NR	NR	Host	(Chang et al. 1998; Hameed et al. 2003)
Malacostraca	Decapoda	Portunidae	*P. pelagicus*	IN,F	DNA Probe, Histo, EM	Y	NR	NR	Host	(Supamattaya et al. 1998)
Malacostraca	Decapoda	Portunidae	*Thalamita danae*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Portunidae	*Calinectes arcuatus*	N	PCR	NR	NR	NR	Potential vector	(Galavíz-Silva et al. 2004)
Malacostraca	Decapoda	Portunidae	*C. sapidus*	N	PCR, ISH	NR	NR	NR	Potential vector	(Chang et al. 2001)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Portunidae	*Carcinus maenas*	IN, F	TEM, Dot Blots, ISH 1step PCR, Histo	Y	Y	Y	Host	(Bateman et al. 2012b; Corbel et al. 2001)
Malacostraca	Decapoda	Portunidae	*Portunus trituberculatus*	N, IN,F	Histo, Real Time PCR	Y	NR	NR	Host	(Meng et al. 2009; Momoyama et al. 1999)
Malacostraca	Decapoda	Portunidae	*Charybdis granulata*	F	DNA Probe	NR	NR	NR	Potential vector	(Chang et al. 1998)
Malacostraca	Decapoda	Portunidae	*Ch. annulata*	N, IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003; Hossain et al. 2001b)
Malacostraca	Decapoda	Portunidae	*Ch. lucifera*	N,IN, F	Nested PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Portunidae	*Ch. natator*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2003)
Malacostraca	Decapoda	Portunidae	*Ch. cruciata*	N	Nested PCR,	NR	NR	NR	Potential vector	(Hossain et al. 2001b)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Portunidae	*Ch. Feriata*	F	Nested- PCR	NR	NR	NR	Potential vector	(Wang et al. 1999a)
Malacostraca	Decapoda	Portunidae	*Ch.japonica*	N	Nested PCR	NR	NR	NR	Potential vector	(Takahashi et al. 2007)
Malacostraca	Decapoda	Portunidae	*Ch. callinassa*	N	Nested PCR,	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Decapoda	Portunidae	*Scylla serrata*	IN, F	DNA Probe, PCR, Histo	Y	Y	F, C	Host	(Kanchanaphum et al. 1998; Rajendran et al. 1999)
Malacostraca	Decapoda	Portunidae	*S. tranquebarica*	N, IN, F	PCR, SEM, Histo	Y	Y	F, IM	Host	(Gopalakrishnan et al. 2011; Kanchanaphum et al. 1998; Rajendran et al. 1999)
Malacostraca	Decapoda	Portunidae	*S. olivacea*	IN	Q PCR Histo, IHC		NR	NR	Host	(Somboonna et al. 2010)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Portunidae	*S. paramamosain*	IN	Q PCR, Histo, IHC		NR	NR	Host	(Somboonna et al. 2010)
Malacostraca	Decapoda	Portunidae	*Liocarcinus depurator*	IN, F	TEM, Dot Blots, ISH, 1step PCR	Y	NR	NR	Host	(Corbel et al. 2001)
Malacostraca	Decapoda	Portunidae	*L. puber*	IN, F	TEM, Dot Blots, ISH, 1step PCR	Y	NR	NR	Host	(Corbel et al. 2001)
Malacostraca	Decapoda	Sesarmidae	*Sesarma* sp.	IN, F	DNA Probe, PCR, Histo	Y	Y	C	Host	(Kanchanaphum et al. 1998; Rajendran et al. 1999)
Malacostraca	Decapoda	Sesarmidae	*Sesarma oceanica*	N	Nested PCR, Probe	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Decapoda	Ocypodidae	*Uca pugilator*	IN	DNA Probe, PCR, Histo	Y	Y	C	Host	(Kanchanaphum et al. 1998)
Malacostraca	Decapoda	Ocypodidae	*Gelasimus marionis nitidu*	N	Nested PCR,	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Decapoda	Ocypodidae	*Macropthalmus sulcatus*	N	Nested PCR,	NR	NR	NR	Potential vector	(Hossain et al. 2001b)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Grapsidae	*Metapograpsus* sp.	F	Histo	Y	Y	F	Host	(Rajendran et al. 1999)
Malacostraca	Decapoda	Grapsidae	*Metapograpsus messor*	N	Nested PCR,	NR	NR	NR	Potential vector	(Hossain et al. 2001b)
Malacostraca	Decapoda	Cancridae	*Cancer pagurus*	IN, F	TEM, ISH, Histo, 1step PCR	Y	Y	IN	Host	(Bateman et al. 2012b; Corbel et al. 2001)
Malacostraca	Decapoda	Varunidae	*Helice tridens*	N	Nested- PCR	NR	NR	NR	Potential vector	(Lo et al. 1996)
Malacostraca	Decapoda	Varunidae	*Chasmagnathus granulata*	N	nested PCR	NR	NR	NR	Potential vector	(Marques et al. 2011)
Malacostraca	Decapoda	Varunidae	*Eriocheir sinensis*	IN	Nested-PCR, TEM, Histo	Y	Y	IN	Host	(Bateman et al. 2012b)
Malacostraca	Decapoda	Paguridae	*Pagurus minutus*	IN	Nested-PCR, TEM	Y	NR	NR	Host	(Chang et al. 2012)
Malacostraca	Decapoda	Paguridae	*P. angustus*	IN	Nested-PCR	NR	NR	NR	Potential vector	(Chang et al. 2012)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Diogenidae	*Diogenes aff. nitidimanus*	IN	Nested-PCR	NR	NR	NR	Potential vector	(Chang et al. 2012)
Malacostraca	Decapoda	Parathelphusidae	*Paratelphusa hydrodomus*	IN, F	PCR, Histo, RT PCR, ELISA	Y	Y	IN	Host	(Hameed et al. 2001; Sundar Raj et al. 2012)
Malacostraca	Decapoda	Parathelphusidae	*P. pulvinata*	IN, F	PCR, Histo	Y	NR	NR	Host	(Hameed et al. 2001)
					Lobster					
Malacostraca	Decapoda	Scyllaridae	*Scyllarus arctus*	IN, F	TEM, Dot Blots, ISH, 1step PCR	Y	NR	NR	Host	(Chang et al. 1998; Wang et al. 1999a)
Malacostraca	Decapoda	Palinuridae	*Panulirus homarus*	F	Histo	Y	Y	F	Host	(Rajendran et al. 1999)
Malacostraca	Decapoda	Palinuridae	*P. ornatus*	F	Nested PCR, Histo	Y	Y	F	Host	(Rajendran et al. 1999; Wang et al. 1999a)
Malacostraca	Decapoda	Palinuridae	*P. polyphagus*	F	Histo	Y	Y	F	Host	(Rajendran et al. 1999)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Palinuridae	*P. versicolor*	F	Nested PCR, DNA Probe	NR	NR	NR	Potential vector	(Chang et al. 1998; Wang et al. 1999a)
Malacostraca	Decapoda	Palinuridae	*P. penicillatus*	F	Nested PCR, DNA Probe	NR	NR	NR	Potential vector	(Chang et al. 1998; Wang et al. 1999a)
Malacostraca	Decapoda	Palinuridae	*P. longipes*	F	Nested PCR	NR	NR	NR	Potential vector	(Wang et al. 1999a)
Malacostraca	Decapoda	Nephropidae	*Nephrops norvegicus*	F	Nested-PCR, TEM, Histo	Y	Y	IN	Host	(Bateman et al. 2012b)
Malacostraca	Decapoda	Nephropidae	*Homarus gammarus*	F	Nested-PCR, TEM, Histo	Y	Y	IN	Host	(Bateman et al. 2012b)
Malacostraca	Decapoda	Nephropidae	*Homarus americanus*	IN	RT-qPCR, histo, TEM	Y	NR	NR	Host	(Clark et al. 2013)
Freshwater shrimp and crayfish										
Malacostraca	Decapoda	Palaemonidae	*Exopalaemon orientalis*	N, F	Nested PCR, DNA Probe	NR	NR	NR	Potential vector	(Chang et al. 1998; Wang et al. 1998)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Palaemonidae	*Palaemon adspersus*	IN, F	TEM, Dot Blots, ISH, 1step PCR	Y	NR	NR	Host	(Corbel et al. 2001)
Malacostraca	Decapoda	Palaemonidae	*Macrobrachium* sp.	F	DNA Probe	Y	NR	NR	Potential vector	(Chang et al. 1998)
Malacostraca	Decapoda	Palaemonidae	*M. rosenbergii*	N,IM, F	Nested PCR, Southern blot, Histo	Y	Y	F	Host	(Hossain et al. 2001a; Peng et al. 1998; Rajendran et al. 1999)
Malacostraca	Decapoda	Palaemonidae	*M. idella*	F, IM, IN	Western blot, Histo	Y	NR	NR	Host	(Hameed et al. 2000)
Malacostraca	Decapoda	Palaemonidae	*M.lamarrae*	F, IM, IN	Western blot, Histo	Y	NR	NR	Host	(Hameed et al. 2000)
Malacostraca	Decapoda	Cambaridae	*Procambarus clarkii*	F, IN	Histo, Nested PCR, TEM	Y	NR	NR	Host	(Baumgartner et al. 2009; Huang et al. 2001; Wang et al. 1999a)
Malacostraca	Decapoda	Cambaridae	*Orconectes limosus*	IN, F	TEM, Dot Blots, ISH, 1step PCR	Y	NR	NR	Host	(Corbel et al. 2001)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Malacostraca	Decapoda	Cambaridae	*O. punctimanus*	N	DNA probe	NR	NR	NR	Potential vector	(Lo et al. 1996)
Malacostraca	Decapoda	Astacidae	*Astacus leptodactylus*	IN, F	TEM, Dot Blots, Insitu Hybridizatio, 1step PCR	Y	NR	NR	Host	(Corbel et al. 2001)
Malacostraca	Decapoda	Astacidae	*A.astacus*	IN, F	PCR	NR	NR	NR	Potential vector	(Jiravanichpaisal et al. 2004)
Malacostraca	Decapoda	Astacidae	*Pacifastacus leniusculus*	IN, F	Nested-PCR, TEM, Histo	Y	Y	Y	Host	(Bateman et al. 2012b; Jiravanichpaisal et al. 2004)
Malacostraca	Decapoda	Parastacidae	*Cherax destructor albidus*	F, IN	DNA Probe, Histo	Y	NR	NR	Host	(Edgerton 2004)
Malacostraca	Decapoda	Parastacidae	*C. quadricarinatus*	IN, F, C	TEM, ISH, IHC, Nested PCR	Y	Y	C	Host	(Shi et al. 2000; Soowannayan and Phanthura 2011)
Malacostraca	Decapoda	Astacidae	*Austropotamobius pallipes*	F	Nested-PCR, TEM, Histo	Y	Y	IN	Host	(Bateman et al. 2012b)
Brine shrimp and copepods										
Branchiopoda	Anostraca	Artemiidae	*Artemia franciscana*	IM	Nested PCR	N	N	F		(Hameed et al. 2002)

Class	Order	Family	Species	Infection method	Detection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Branchiopoda	Anostraca	Artemiidae	*Artemia*	F	Nested PCR	NR	Y	F	Vector	(Zhang et al. 2010)
Maxillopoda	Harpacticoida	Amei ridae	*Nitocra* sp	F	Nested PCR	NR	Y	F, IN	Vector	(Zhang et al. 2008)

N = Natural infection; F = Experimental infection/transmission through feeding; IM = Experimental infection/transmission by immersion; IN = Experimental infection/transmission by injection; C= Experimental infection/transmission by cohabitation. NR = Not reported in the reference; NA= Not applicable; Histo = Histopathology; TEM = Transmission Electron Microscope; ISH= In situ hybridization; Y= Yes; N= No.
Host = WSSV replication in this animal has been shown based on a detection method besidesPCR or DNA Probe.
Vector = WSSV was detected with PCR or DNA probe and the virus was transmitted to shrimp or crabs.
Potential vector = WSSV detected in this animal based on PCR or DNA probe and no transmission to shrimp or crab was reported.

Table 2. Reported non-crustacean host and vectors of WSSV

Phylum	Class	Order	Family	Species	Detection method	Infection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
					Plankton						
Chlorophyta	Trebuxiophyceae	Chlorellales	Chlorellaceae	*Chlorella sp*	Nested PCR	C	NR	N	Y	Vector	(Liu et al. 2007)
Dinophyta	Dinophyceae	Gonyaulacales	Goniomataceae	*Alexandrium tamarense*	Probe	IM	NR	Yes	F	Vector	(Jiang 2012)
Dinophyta	Dinophyceae	Gonyaulacales	Goniomataceae	*Alexandrium minutum*	Probe	IM	NR	Yes	F	Vector	(Jiang 2012)
Rotifera	Eurotatoria	Ploima	Brachionidae	*Brachionus urceus*	Nested PCR, Dot Blot hybridization	F	NR	Yes	F	Vector	(Jiang 2012; Zhang et al. 2006)
Rotifera	Eurotatoria	Ploima	Brachionidae	*Brachionus plicatilis*	Nested-PCR	IM	NR	Yes	F, C	Vector	(Corre Jr et al. 2012)
					Polychaete						
Annelida	Polychaeta	Eunicida	Eunicidae	*Marphysa gravelyi*	Nested and 1-step PCR	N, F	NR	Y	F	Vector	(Vijayan et al. 2005)

Phylum	Class	Order	Family	Species	Detection method	Infection method	Replication	Transmission to Shrimp	Transmission route	Status	Reference
Annelida	Polychaeta	Phyllodocida	Nereididae	*Dendronereis* spp	Nested PCR, IHC, RT-PCR	N	Y	Y	F	Host	(Desrina et al. 2013; Haryadi et al. 2014)
Oyster and Clam											
Mollusca	Bivalvia	Ostreoidea	Ostreidae	*Crassostreagigas*	Nested PCR	N	NR	NR	NR	Potential vector	(Vazquez-Boucard et al. 2010)
Mollusca	Bivalvia	Veneroida	Veneridae	*Meretrix lusoria*	Nested-PCR, qPCR, RT-PCR	IM	N	Y	F	Vector	(Chang et al. 2011)
Insects											
Arthropoda	Hexapoda	Diptera	Ephydridae		Nested- PCR	N	NR	NR	NR	Potential vector	(Lo and Kou 1998)

N = Natural infection; F = Experimental infection/transmission through feeding; IM = Experimental infection/transmission by immersion; IN = Experimental infection/transmission by injection; C= Experimental infection/transmission by cohabitation. NR = Not reported in the reference; NA= Not applicable; Histo = Histopathology; TEM = Transmission Electron Microscope; ISH= In situ hybridization; Y= Yes; N= No.
Host = WSSV replication in this animal has been shown based on a detection method besides PCR or DNA Probe.
Vector = WSSV was detected with PCR or DNA probe and the virus was transmitted to shrimp or crabs.
Potential vector = WSSV detected in this animal based on PCR or DNA probe and no transmission to shrimp or crab was reported.

infection without being affected make crabs important WSSV reservoir hosts besides infected cultured shrimp species.

The Pacific oyster *Crassostrea gigas* (Thunberg 1793) (Vazquez-Boucard et al. 2010), the common orient clam *Meretrix lusoria* (Roeding 1798) (Chang et al. 2011) and the annelid *Marphysa* spp. (Quatrefages 1865) (Vijayan et al. 2005) were regarded as passive vectors of WSSV. Macrobenthic invertebrates live in the pond sediment and can acquire WSSV, which make these animals potential vectors for WSSV due to the niche they occupy and their eating habits. For instance, as filter feeders and with their restricted mobility, molluscs can accumulate the virus in their bodies. WSSV was detected in the gills and gut of Pacific oyster *C. gigas* (Vazquez-Boucard et al. 2010) and *M. lusoria* (Chang et al. 2011) and transmitted the virus to shrimp (Chang et al. 2011). However, no replication has been reported so far in molluscs. The ability of WSSV to infect numerous species, belonging to different phyla is unique for an aquatic virus, and demonstrates the generalist nature of this virus, which in turn could enhance its fitness by infecting multiple hosts (Elena et al. 2009).

Polychaetes are dominant in soft bottom estuaries (De Oliveira et al. 2012; García-Arberas and Rallo 2002), representing 13% of the benthic animal biomass in extensive shrimp ponds with zero water exchange (Balasubramanian et al. 2004). Polychaetes are preferred, highly nutritious prey for shrimp (Abu Hena et al. 2011; Nunes et al. 1997). They are often included in maturation diets of shrimp broodstock (Vijayan et al. 2005). Their burrowing behaviour, mobility, scavenging attitude and detritofeeding make polychaetes, more relevant potential vectors for WSSV than sedentary molluscs. However, WSSV transmission in ponds is still poorly understood. While Vijayan et al. (2005) considered the polychaete *Marphyssa*s spp. a passive WSSV vector, recently Desrina et al. (2013) reported that WSSV replicates in the naturally infected *Dendronereis* spp.. *Dendronereis* spp. live in burrows in mangroves and tropical estuaries (Gowda et al. 2009;

Ngqulana et al. 2010; Watts et al. 2013). Its foregut has a proboscis equipped with a pair of sclerotized jaws indicating this animal is both a carnivore and a scavenger (Tzetlin and Purschke 2005). Their mobility and feeding habits certainly brings *Dendronereis* spp. in close contact with WSSV in 'infected' ponds. Recently, it was found that WSSV from infected *Dendronereis* spp. could be transmitted to naïve shrimp (Haryadi et al. 2014) and that the prevalence of WSSV in *Dendronereis* spp. was positively correlated with the occurrence of WSSV in ponds (Desrina et al; submitted). This strongly suggests that *Dendronereis* spp., being a permanent pond resident, may play an important role in the transmission of WSSV in pond systems.

V. EPIDEMIOLOGY

The various animal species present in ponds may simultaneously affect WSSV presence and abundance. A mix of living or dead infected animals continuously releases viruses, maintaining the presence of the virus in the system. WSSV persisted for 1 year after a WSD outbreak in Vietnamese ponds (Quang et al. 2009) and for 10 months in pond soil stored at room temperature (Natividad et al. 2008). Moreover, WSSV remained viable for nearly 40 days in 30 ºC sea water in absence of a host species (Momoyama et al. 1998), for 19 days in sun-dried sediment and for 35 days in waterlogged sediment (Satheesh Kumar et al. 2013). So, even in the absence of carriers the virus can survive for a reasonable period of time in pond systems.

WSSV prevalence in non-cultured animals is generally low. When infected under culture conditions, WSSV prevalence in *L. vannamei* ranged from 40 to 71% (Cheng et al. 2013) and was 100% during WSD outbreak (Cheng et al. 2013). Withyachumnarnkul et al. (2003) and De la Peña et al. (2007) estimated the prevalence of WSSV infection in broodstock collected at sea to vary between

seasons. In the Philippines WSSV prevalence was higher during the dry season (10%) than during the wet season (0.3%) (De La Peña et al. 2007), while it was the opposite in Thailand (Withyachumnarnkul et al. 2003). This could imply that the transmission in wild shrimp is higher at certain times of the year. WSSV prevalence in the mud crab *Scylla serrata* ranged from 18% in India (Sethi et al. 2011), 34.82% in China (Liu et al. 2011b), to 60% in Taiwan (Chen et al. 2000; Lo and Kou 1998). Hence, WSSV prevalence in general is highly variable and site-dependent.

High genetic variation might be advantageous to a generalist virus to enhance its survival by infecting a broad range of host species (Woolhouse et al. 2001). The genetic variation of WSSV is mostly assessed by quantifying the number of repeat units (RU) in the open reading frames (ORF) 94, ORF75 and ORF125 that have 54 bp, 69 bp and compound 45 bp and 102 bp RU respectively (Dieu et al. 2004; Hoa et al. 2012b; Marks et al. 2005a; van Hulten et al. 2001). The number of RU of the same ORF differed between WSSV from Thailand (Wongteerasupaya et al. 2003), Vietnam (Hoa et al. 2011b) and India (Pradeep et al. 2008). ORF94 is generally considered the most sensitive for detecting genetic variation in ponds (Hoa et al. 2011b; Pradeep et al. 2008) and was used to identify genotypes in epidemiology (Dieu et al. 2010; Pradeep et al. 2008; Wongteerasupaya et al. 2003). Examples included mixed genotype infection of shrimps in ponds (Hoa et al. 2011b), virus passage through different host species (Waikhom et al. 2006) and virus passage through different hosts and vectors during a WSD outbreak (Walker et al. 2011b). Mixed genotype infections of WSSV are common with pond-reared shrimp (Hoa et al. 2011b; Pradeep et al. 2008; Walker et al. 2011c; Wongteerasupaya et al. 2003). Combining variation of ORFs75, 94 and 125 to elucidate the spatio-temporal WSSV transmission in ponds revealed that low genetic variation is linked to a WSD outbreak, suggesting that some WSSV

genotypes are more prevalent during WSD outbreak than others (Hoa et al. 2011a; Pradeep et al. 2008). In general, genotypes associated with WSD have a lower number of RUs for the ORFs studied than those in non-outbreak ponds (Hoa et al. 2012a). In improved extensive culture systems, more WSSV genotypes tend to be present than in semi-intensive systems, most likely due to a higher frequency of within-pond transmission (Hoa et al. 2011a; Hoa et al. 2011b; Pradeep et al. 2008). Genotypes with less than 8 RUs for ORF94 are more common in ponds with WSD, while more than 9 RUs are more common in WSD-free ponds. Variation in WSSV genotype related to location (geographical area) and infected wild shrimp (*Acetes spp.*, H. Milne-Edwards 1830) and mud crab showed the same RU pattern for ORF125 as cultured shrimp (Pradeep et al. 2008).

'Extensive' ponds provide more opportunities for cross-species transmission because (1) macrobenthic invertebrate species enter and leave easily, either settling or moving between ponds, (2) stocked shrimp are mostly not WSSV specific pathogen free (SPF) (3) continuous partial cropping and stocking is practiced, allowing transmission between successive cohorts, and (4) the ponds are rarely drained, dried or fully disinfected. Based on studies on human pathogens, cross-host exposure is an important factor in cross-species virus transmission and is affected by the ecological and geographical distribution of potential hosts or vectors (Parrish et al. 2008). During a WSD outbreak, high numbers of virions are released into the water and sediment. The presence of different macrobenthic invertebrate species in ponds provides favorable conditions for WSSV host jumping. A virus mostly infects host species that are phylogenetically close. Nevertheless, the intensity of contact between virus and potential host is equally important (Parrish et al. 2008).

WSSV transmission between shrimp and other crustacean and non-crustacean host species may bounce back and forth in an environment showing a

high degree of spatio-temporal variation and supporting genetic variation. In Vietnam, the so-called improved extensive system tends to harbor more WSSV genotypes than semi-intensive farms (Hoa et al. 2011a; Walker et al. 2011a), maybe because of the frequency of in-pond transmission is higher (Hoa et al. 2011a). Walker et al. (2011a) detected in total 25 WSSV genotypes in infected wild crabs and crustaceans, plankton and cultured shrimp during a WSD outbreak in a traditional pond in India. The plankton contained the highest number of genotypes, with less genotypes found in farmed shrimps than in wild crustaceans and planktonic species. Moreover, the dominant genotypes in farmed shrimps differed from the dominant genotypes present in non-cultured species. Repeated virus transfer between closely related hosts could also lead to host switching (Parrish et al. 2008). Hence, a high genetic variability might be the result of cross-species transmission in a cascading way. Nevertheless, maintaining a high genetic variability could also be a survival strategy of WSSV. Both high genetic variation and repeated cross-species transmission foster the survival of WSSV over time and space.

VI. VIRUS-HOST INTERACTION

Once WSSV entered a host, survival depends on its successfully binding on a cell membrane receptor, and its ability to overcome or avoid the host's cellular and humoral defenses. WSSV replicates primarily in the gills and foregut of shrimp and reaches a detectable concentration with 1-step PCR around 18 hours post infection (hpi) (Escobedo-Bonilla et al. 2007). Gill tissues had the highest viral load after oral transmission (Tan et al. 2001).

WSS virus proteins (VP) which might act as attachment proteins are VP28 (Chang et al. 2011; Van Hulten et al. 2000a; Yi et al. 2004), VP 26 (Liu et al. 2011a), and VP281 (VP37) (Huang et al. 2002; Liang et al. 2005). For instance,

WSSV VP37 was shown to bind to membranes of shrimp gill and haemocytes (Liu et al. 2009). Other proteins were suggested to have a role in infection without specifying their function, such as VP76, which is involved in cellular infection (Huang et al. 2005).

Several receptor-binding proteins found on cell surfaces of shrimp tissues have been published. Huang et al. (2012) identified glucose transporter 1 (Glut 1) as WSSV receptor, which is ubiquitously present in tissues of *P. monodon* and *L. vannamei*. A cell membrane protein found in the giant tiger prawn called P. monodon-chitin-binding-protein (PmCBP) interacted with several WSSV envelope proteins, including VP24, VP32, VP39B, VP41A, VP53A, VP53B, VP51B, VP60A, VP110, VP124 and VP337 (Sánchez-Paz 2010). Liang et al. (2010) identified BP53, a F1-ATP synthase beta subunit that exists on the surface of the gills and haemocytes of *L. vannamei*. PmRab7 (*P. monodon* Rab7) is likely a receptor protein of VP28 (Somboonna et al. 2010), but also the heat-shock cognate protein 70 (Hsc70) in the haemocyte cytoplasm (Xu et al. 2009) bound to VP28. A WSSV binding protein (WBP) from the shrimp bound to WSSV VP26 protein (Youtong et al. 2011). Chen et al. (2007) showed the *P. monodon* chitin-binding-protein (PmCBP) bound to the WSSV structural protein WSSV067C/VP53A. Hence, numerous binding sites are available to WSSV in different tissues of shrimp and potentially other hosts and the future will tell which of these proteins the genuine (co) receptor of WSSV is.

VII. QUALITATIVE AND QUANTITATIVE ASPECTS OF TRANSMISSION

WSSV can be transmitted experimentally by injection, feeding, immersion or cohabitation. Injection is not a natural way for the virus to enter the animal. The

reaction may not reflect the natural susceptibility to WSSV. Possibly the immune system is overwhelmed by the high number of viruses injected. Therefore, focusing on information on transmission through feeding, immersion or cohabitation might yield better insight in natural infection routes. A major problem in comparing WSSV transmission studies is that there is no standard viral concentration norm, also for transmission studies through feeding. Cross species transmission of WSSV further complicates the picture (Esparza-Leal et al. 2009) in ponds and the environment in general. WSSV transmission through rearing water (Chou et al. 1998) was less effective than transmission through ingestion of (parts of) infected animals (Chou et al. 1995; Soto and Lotz 2001), while dead infected shrimp are the most important route for WSSV transmission once a WSD outbreak occurred (Lotz and Soto 2002). Under experimental exposure with ingestion and immersion methods, WSSV transmission occurred and the virus was detectable within 24 hpi.

Immersion in infected water gives insight of natural route of infection among hosts and vectors in pond. WSSV transmission through immersion route has been reported for mud crab (Chen et al. 2000). In addition, WSSV was transmitted to healthy shrimp by immersion in brackish water-containing WSSV filtrate (Chou et al. 1998) or through cohabitation with infected animals, including Australian red clawcrayfish, *Cherax quadricarinatus* (von Martens 1868) (Soowannayan and Phanthura 2011), and different crab species (Kanchanaphum et al. 1998). In contrast, cohabitation with infected *P.monodon* did not induce infection in Australian red clawcrayfish (Soowannayan and Phanthura 2011). Care must be taken in interpreting these results. In cohabitation experiments in which the infection source and victim are housed in the same aquarium, the possibility that transmission resulted from eating infected tissue cannot be excluded completely (Soowannayan and Phanthura 2011).

Vijayan et al. (2005) with the polychaete *Marphysa gravelyi*., Zhang et al. (2010) with brine shrimp *Artemia salina* (Linneaus 1758) and Hameed et al. (2003) with crabs showed the importance of the oral route in WSSV transmission. WSSV binds in vitro to the Artemia cell membrane which might have a WSSV receptor similar to receptors described for shrimp gill cells (Feng et al. 2013). However, a full-blown infection of Artemia has not yet been reported.

The role of plankton as WSSV mechanical vector has not gained much attention until today. WSSV was present in brine shrimp obtained from shrimp ponds (Otta et al. 1999). WSSV was transmitted from infected phytoplankton to brine shrimp (Zhang et al. 2010), to the rotifer *B. urceu* (Jiang 2012) and to the copepod *Apocyclops royi* (Lindberg 1940) (Chang et al. 2011) before being consumed by the shrimp. Dinoflagellates of the genus *Alexandrium* (Halim 1960) became WSSV positive after 24 h co-culture with WSSV infected shrimp. The WSSV attachment to phytoplankton is however temporal (Liu et al. 2007). Walker et al (2011a) showed that high WSSV prevalence in plankton preceded WSD outbreak in extensive farms. Taken together, the phytoplankton-zooplankton route for WSSV transmission in ponds might play a more important role than currently assumed and requires further research.

Crabs may be the most important biological vectors of WSSV in nature. Fifty-one crab species were shown to carry the infection by oral and cohabitation transmission with or without clinical signs but overall showed more resistance to infection than penaeid shrimp. Mortality is relatively low and occurs over a long period (> 1 week-1 month) and some time the infection is repressible (Hameed et al. 2003). WSSV was transmitted to mud crab *S. serrata* and blue swimmer crab *P. pelagicus* by consuming WSSV infected shrimp without causing mortality or clinical signs (Supamattaya et al. 1998).

In quantitative terms transmission rate is an important parameter in the dynamics of WSSV transmission and the emergence of an epidemic. The transmission rate of the virus (e.g. WSSV) depends on the reproduction ratio (R_0) of the virus, host density and environmental factors such as temperature, salinity, dissolved oxygen, etc. Very little information is available in the literature on the dynamics of WSSV transmission. Soto and Lotz (2001) used cohabitation experiments and found a low level of transmission. Very recently Tuyen et al., (2014) also using pair cohabitation conditions found that the reproduction ratio (R_0) of WSSV was 3.19 for *P. monodon* and 1.97 for *L. vannamei*. These values are well in a range that an outbreak would quickly occur once infected shrimp is present in a pond system.

VIII. WSSV TRANSMISSION AT POND LEVEL AND INTERRELATEDNESS BETWEEN VECTORS AND CULTURE CONDITIONS

Emergence of new pathogens is often associated with the development of farming systems which hold culture animals in a setting different from their natural environment (Johnson 2013; Woolhouse 2002). In shrimp pond ecosystems, tidal currents and wave turbulence, which are typical for estuaries and maintain the sediment well-oxygenated, are absent. Hence ponds provide a suboptimal living environment for shrimp. In addition, the shrimp are reared at densities much higher than found in nature and feeds produced outside the pond are applied. This aggravates the flux of organic matter to the sediment (Cock et al. 2009; Kautsky et al. 2000), creating an unfavorable low-oxygen benthic environment, causing increased disease incidence.

Farming practices were linked to WSSV transmission within and between ponds. Transmission in extensive farms originated mainly from shrimp in

neighboring ponds and channels, followed by shrimp and crab in the same ponds (Hoa et al. 2011b). In the semi intensive ponds the main source of infection was shrimp from neighboring ponds. Based on analysis of ORF94, WSSV infection in the wild shrimp (*Acetes spp.*), wild crabs (*Scylla serrata*) and plankton were identified as WSSV infection sources in ponds (Pradeep et al. 2008; Walker et al. 2011a). Sharing the same water source between culture units, feeding of live molluscs are major risk factors for the occurrence of WSSV while a high pond:mangrove ratio had the opposite effect (Tendencia et al. 2010a). The latter reduced WSSV infection presumably by absorbing excess nutrients such as NO_2 (Tendencia et al. 2012).

Environmental conditions affect the physiology and shrimp response to the invading pathogen, and hence the transmission. Stocking WSSV infected larvae increased the risk of a WSD outbreak (Withyachumnarnkul 1999), however, under good culture conditions, shrimp tolerated light WSSV infection without developing WSD (Tsai et al. 1999). Poor water quality influenced shrimp health and aggravated WSSV infection in shrimp. A sudden change in salinity and water temperature due to heavy rain triggered WSSV incidence in ponds (Peinado-Guevara and López-Meyer 2006). Acute salinity changes increased susceptibility to WSSV infection (Carbajal-Sánchez et al. 2008), were linked to reduced haemolymph osmotic pressure during infection (Ramos-Carreño et al. 2014), reduced haemocytes count and lowered phenoloxidase activity (Joseph and Philip 2007), and increased WSSV load in infected shrimp (Liu et al. 2006). Temperature yielded different WSSV-infection outcomes in different hosts. Prolonged exposures of *L. vannamei* at high water temperature (33 ^{o}C) delayed mortality due to WSSV infection (Rahman et al. 2006) while daily temperature fluctuations of 5 ^{o}C above the optimum shrimp culture temperature had either a positive or negative effect (Rahman et al. 2007b). Warm water culture conditions (29±0.5 °C) increased

WSSV load in *L. vannamei* (Moser et al. 2012). Cold water culture conditions (4-12 ^{o}C) reduced WSSV pathogenicity in the temperate crustacean species *P. leniusculus* and *A. astacus* because of lower WSSV replication (Jiravanichpaisal et al. 2004). On the other hand, Lavilla-Pitogo et al. (2007) reported that low temperature increased WSSV load in the mud crab *S. serrata*. Although all these reports were based on laboratory experiments and focused only on shrimp, the findings give insight on how salinity and temperature may affect WSD outbreaks in ponds.

IX. CONCLUSION AND WAY FORWARD

This review has brought to light that WSSV is present in a plethora of crustacean and non-crustacean invertebrates in aquatic and benthic environments (Tables 1 and 2). These organisms thus may form in principle an important WSSV reservoir from which the virus can initiate infection in susceptible hosts. This is particularly the case, when naïve shrimp are brought into a pond system and the virus finds virgin territory for infection and reproduction. These virus reservoirs cannot only initiate an outbreak in pond systems, but can also cause a WSD epidemic in an area or region.

WSSV has been demonstrated in crustacean and non-crustacean invertebrates mostly by (nested) PCR, but only in a limited number of cases it became clear whether this reflected reproductive infection or the mere presence of the virus in a carrier host. It is highly relevant when testing for WSSV presence to also verify if the virus replicates. This can be done by using, preferentially in combination, immunological and molecular (RT-PCR) techniques.

The non-crustacean organisms in pond systems received less attention, but they are probably equally important in the initial transmission of WSSV from within the pond. The finding that *Dendronereis* spp., important benthic polychaete

in pond systems in Indonesia, are reproductive carrier of WSSV supports this view. It is recommended to continue screening other resident organisms in pond systems for the presence of WSSV and to check if the virus replicates in these organisms. The relative importance of active versus passive vectors in the transmission of WSSV is enigmatic, but the high reproduction rate of WSSV in shrimp explains in part the speed WSD outbreaks in ponds. In the transmission of WSSV, a high virus load in passive carrier hosts can be offset by a low virus load in a replicative carrier host, but the latter may have more impact in the long term because the virus may become persistent in this host. A confounding factor in all of this is the presence of active carriers which do not show symptoms as has been documented for crabs. Considering shrimp are cultured primarily in ponds, which are semi-open ecosystems with an abundance of natural species present, field studies targeting to elucidate the ecology of WSSV in relation to non-shrimp host and vectors in ponds, are needed.

In summary, the number of reported crustacean and non-crustacean WSSV hosts is ever-increasing, with many species remaining present in pond systems even after shrimp farming stopped. Some of these hosts are present in high numbers and hence important reservoirs for WSSV. It would be highly relevant to determine the relative importance of each of these host species in the development of WSSV in shrimps in pond systems.

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CHAPTER 3.

REPLICATION OF WHITE SPOT SYNDROME VIRUS (WSSV) IN THE POLYCHAETE *Dendronereis* spp.

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ABSTRACT

This study investigated whether WSSV replicates in naturally infected *Dendronereis* spp., common polychaete (Nereididae*)* species in shrimp ponds in Indonesia. To detect WSSV replication, (i) immunohistochemistry (IHC) using a monoclonal antibody against WSSV VP28 protein and (ii) nested RT-PCR using specific primers set for the *vp28* gene to detect WSSV-specific mRNA were applied. WSSV immunoreactive-nuclei were detected in the gut epithelium of the polychaete and WSSV mRNA was detected with nested RT-PCR. This, together with the IHC results, confirmed that WSSV could replicate in *Dendronereis* spp.. This is the first report showing that WSSV replicated in a naturally infected non-crustacean host.

Keywords: *Dendronereis* spp., WSSV, replication, immunohistochemistry, RT-PCR

Chapter 3

INTRODUCTION

White spot syndrome virus (WSSV), the causative agent of white spot disease (WSD) in penaeid shrimp, belongs to the genus *Whispovirus*, of the Nimaviridae family (Lo et al. 2012). There are two factors that may contribute to the persistence of the virus. Firstly, the virus can persist for a long period in the environment during which susceptible host(s) can be infected. WSSV remained infectious in the seawater up to 40 days (Momoyama et al. 1998). The viral DNA could still be detected in water 20 months after a disease outbreak (Quang et al. 2009) and in pond soil after 10 months of storage at room temperature (Natividad et al. 2008). Secondly, WSSV is unique among shrimp viruses because of its broad host range among crustaceans. The range of reported host species increased from 46 to 94 between 2005 and 2010 (Flegel 2006; Sánchez-Paz 2010). In many instances WSSV virulence tended to be lower, sometimes without causing mortality, so the host can survive longer (Chang et al. 2011; Esparza-Leal et al. 2009; Waikhom et al. 2006) and form a WSSV reservoir. These factors may promote horizontal transmission of WSSV in ponds and contribute to its persistence in pond environments.

Polychaetes (Phylum *Annelida*) are common macroinfauna in mangroves (Fujioka et al. 2007b) and are an important prey for shrimp (Shishehchian et al. 2001). They are often found in and around shrimp pond sites. WSSV was taken in by immersion and accumulated in the gut of the polychaete *Marphysa* spp. (Vijayan et al. 2005). However, whether the virus replicated in the polychaete or was passively carried is a matter of debate. Here, we investigated whether the polychaete *Dendronereis* spp. are susceptible host of WSSV by showing the presence of WSSV-infected cells in tissue using immunohistochemistry (IHC) and

by verifying the presence of WSSV messenger RNA (mRNA) for the major late virion protein, VP28 .

MATERIAL AND METHODS

Dendronereis spp. (9 to 11 cm in length) were randomly collected from a shrimp pond (2.5 ha) in the Semarang district, Central Java, Indonesia. *Penaeus monodon* was cultured traditionally in this pond and the farmer suffered persistent reoccurrence of WSSV infection. The specimens for immunohistochemical (IHC) analysis were collected in January 2010 at which time the farmer was forced to harvest early because of WSSV infection. Seven animals were fixed in Davidson's solution for 48 h and subsequently transferred to 50% ethanol, processed and embedded in paraffin (Lightner 1996) for immunohistochemical analysis. Animals used for Reverse Transcriptase-PCR (RT-PCR) analysis (n=10), were collected in February 2013 at which time the pond contained juvenile *P. monodon* that had been stocked 1 month earlier without signs of WSD.

Immunohistochemistry (IHC) to detect WSSV infected cells

Nested-PCR was done on the *Dendronereis* spp. paraffin-embedded specimens prior to IHC analysis to verify the presence of WSSV using previously described primer sets for *vp28* (amplicon size 529 bp) (Marks et al. 2003) and a purposely designed *vp28* nested primer set (VP28nest F1: 5'CAT TCC TGT GAC TGC TGA GG 3'; VP28nest R1: CCA CAC ACA AAG GTG CCA AC 3') (amplicon size 364 bp). The DNA template was prepared using DNeasy Blood and Tissue (QIAGEN) kit following the protocol from the manufacturer. Artificially infected *P. monodon* (positive control) and *Nereis virens* (negative control) were tested alongside WSSV infection tests with *Dendronereis* spp.

The PCR reaction was carried out in a 0.2 ml PCR tube (final reaction volume 25 µl) containing 40-50 ng/µl of DNA, 10 pmol of each forward and

reverse primer, 0.5 µl of dNTP (10 mM), 5 µl of 5X PCR buffer (Promega), 1.5 µl of $MgCl_2$ (25 mM) and 2.5 µl of GoTaq Flexy DNA Polymerase (Promega) using Gene Amp PCR System 9600 (Applied Biosystems, Foster City, USA). The 1-step PCR conditions were: initial denaturation (94 ^{o}C, 3 min); denaturation (94 ^{o}C, 50 sec); annealing (50 ^{o}C, 50 sec) and elongation (72 ^{o}C, 1 min) for 30 cycles and a final extension at 72 ^{o}C (7 min). One µl of the product of the 1-step PCR was used in the nested-PCR with the same conditions as the 1-step (25 cycles) PCR. The WSSV positive specimens along with uninfected specimens as negative control were further analyzed by IHC.

Paraffin-embedded *Dendronereis* spp. were cut in 5 µm thick sections, mounted on silane-coated slides, deparaffinized, and then rehydrated in a series of ethanol. Endogenous hydrogen peroxidase was blocked by immersion in methanol +0.3 % hydrogen peroxide. Tissue was pre-incubated in 5 % normal goat serum (30 min), subsequently incubated for 1 h in a 1:100 diluted mouse monoclonal antibody (mAb) solution, specifically reacting with clone C5 expressing VP28 (Anil et al. 2002). The sections were washed twice in phosphate-buffered saline triton (PBS-t), incubated in Goat Anti Mouse-Alkaline Phosphatase (GAM-AP, Dako; 1:200) for 1 h and washed twice in PBS-t. Tissue was incubated in an alkaline phosphatase-buffer (pH 9, 0) (10 min) followed by incubation in the alkaline phosphatase substrate BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate - nitro blue tetrazolium) until color developed. The reaction was stopped by washing the slides in distilled water.

RT-PCR to detect WSSV mRNA

Live *Dendronereis* spp. were brought to the laboratory, the head was individually stored in Trizol (n=10) at -80 °C for RT-PCR analysis and the rest of the body was checked for WSSV infection by nested PCR. Three WSSV positive individuals were used in the RT-PCR analysis along with one WSSV-negative individual.Total RNA was extracted from 50 mg *Dendronereis* spp. tissue including the head, the first 20 proximal segments and part of the gut using Trizol (Invitrogen). Residual DNA was removed with a DNA-*free* kit (Invitrogen), both following the protocol recommended by the manufacturer, and diluted 50x before proceeding to the next step. First cDNA strand was synthesized using the Superscript III Reverse Transcriptase enzyme (Invitrogen) and an oligo (dT) anchor primer. One µl of the cDNA was used in 1-step RT-PCR reaction and 1µl of product was used in nested-RT PCR, both using gene specific primer for *vp28* and PCR condition as described in 2.1.The annealing temperature was raised to 55 °C to increase the specificity. WSSV genomic DNA from the infected shrimp was used as positive control and sterile miliQ water was used as no template control for the PCR.18s rRNA of the host (internal control) was detected with primer pair (NVF1: GTTGATCCTGCCAGTAGTCATATGC; NVR1: TTTCTCATGCTCCCTCTCCGG, amplicon size = 406 bp based on the published sequence of 18s rRNA of *Nereis virens*) (GenBank: Z83754.1). PCR condition for host 18s rRNA was as for *vp28* primer pairs with annealing temperature set at 57 °C for 30 sec. The products of RT-PCR were confirmed by sequencing.

RESULTS

WSSV was detected in paraffin-embedded *Dendronereis* spp. with 1-step PCR in 2 out of 7 individuals and with nested- PCR in 5 out of 7 individuals.

WSSV immunoreactive cells were detected only in the stomach and intestinal tissue of *Dendronereis* spp. (Figure 1) that were positive with 1-step PCR. The nuclei of those cells were enlarged and contained dense and prominent nucleoli and showed strong affinity to the antibody against VP28 as indicated by staining. Infected cells were clearly different from non-infected cells. The latter were homogenous in size and well-bordered cells with relatively similar sized and regularly spaced nuclei. These nuclei were not stained with anti-VP28 serum.

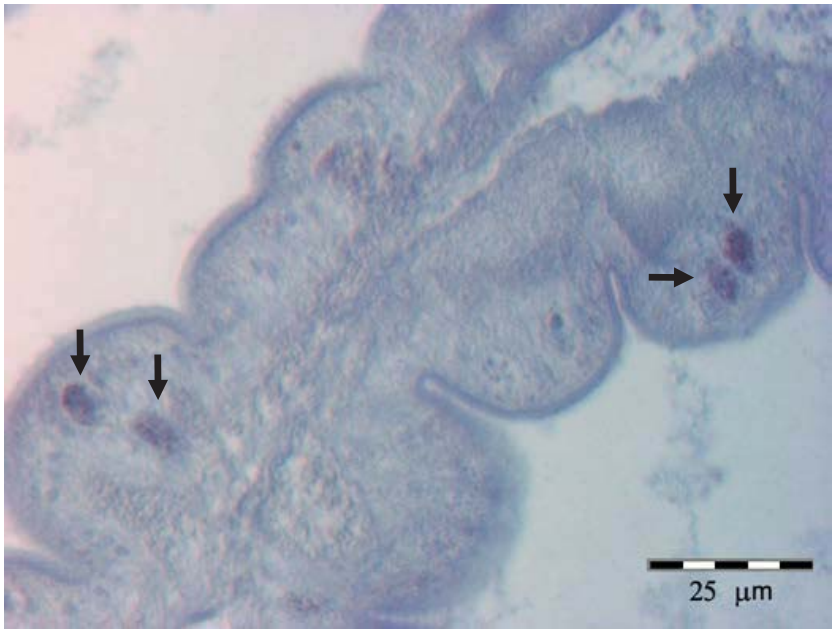

Figure 1. WSSV-immunoreactive nuclei in the front gut of *Dendronereis* spp. (arrow) and adjacent uninfected cells

Taking advantage of the fact that the *vp28* transcript is polyadenylated (Marks et al. 2003), RT-PCR was used to detect the presence of *vp28* mRNA. Since the expression of VP28 occurs late after infection and is dependent on viral DNA replication, the presence of *vp28* transcripts would signal viral DNA replication. WSSV mRNA encoding the virion envelope protein VP28 was detected in samples of *Dendronereis* spp. (Fig. 2A) as a nested RT-PCR product at the expected size of about 364 bp in 1 out of 3 WSSV-infected *Dendronereis* spp..

No PCR product was seen with mRNA template only (without addition of Reverse Transcriptase enzyme) and with no template control (without addition of RNA). The latter served as negative control for the RT reaction and confirmed that the cDNA was amplified only when viral RNA was present. The nested RT-PCR product of *vp28* gene obtained from *Dendronereis* spp. was sequenced and aligned with BLAST (http://blast.ncbi.nlm.nih.gov) against known sequence in the GenBank. It had 99% identity with the WSSV *vp28* gene of an Indonesian isolate (GenBank accession number AY24944) and WSSV genome segment of Thai isolate (AF369029.2, nucleotide 159-491). The host's 18s rRNA was detected in all specimens (Fig 2B) and showed, upon sequencing of the PCR product, 80 % identity with 18s RNA of *Nereis virens* and other Nereididae.

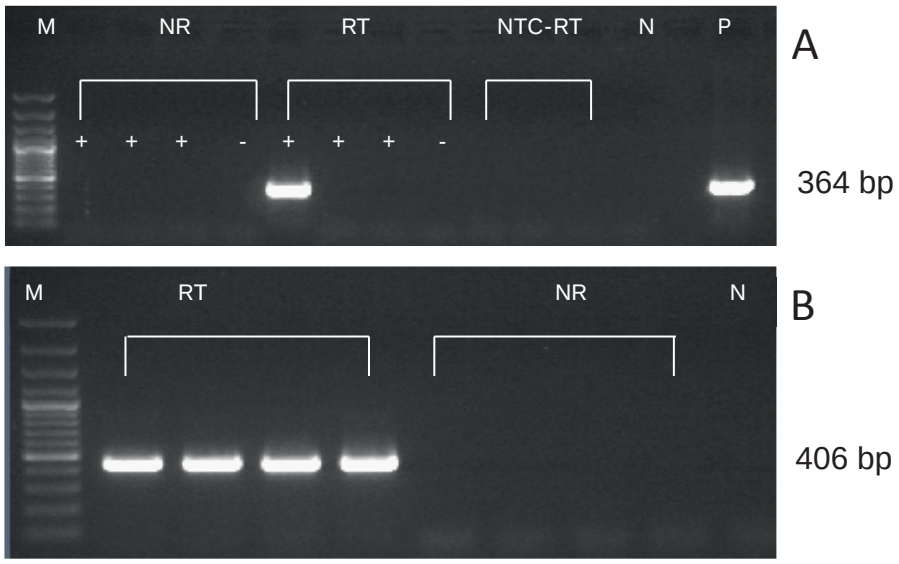

Figure 2. Nested RT-PCR of the WSSV mRNA of the vp28 gene (A). One out of 3 individuals of WSSV-positive *Dendronereis* spp. showed positive signal. M= Marker (100 bp DNA ladder), NR = No addition of RT-enzymes; RT= with addition of RT enzyme; NTC-RT= No template control for RT step (RNA was replaced with MQ water); + = positive for WSSV with nested PCR; - = negative for WSSV with nested PCR. N= Negative control of PCR; P= Positive control of PCR (DNA of infected shrimp). RT-PCR of messenger of 18s rRNA of *Dendronereis* spp.(B). For explanation see part A.

Chapter 3

DISCUSSION

Our observations suggest that *Dendronereis* spp. are propagative host of WSSV. The identification of immunoreactive nuclei in the gut tissue as indicated by IHC and the presence of WSSV-specific mRNA as indicated by the RT-PCR, both support the view that WSSV replicates, at least in some cells, in this polychaete. Despite the difference in sensitivity of the two methods, the results of IHC and PCR converge in their interpretation. WSSV-immunoreactive nuclei were detected only in specimens that were positive with 1-step PCR, indicative of the extent of the WSSV infection in the *Dendronereis* spp. Newly-made WSSV virions accumulate in the nuclei of infected cells (Lo et al. 2012) and VP28 is a major constituent of these virions. Generally, 1- step PCR is positive with heavily infected individuals. So, it can be concluded from the results of the PCR on paraffin-embedded specimens that these individuals contained relatively high concentration of viral DNA, as viral DNA in the paraffin material must have been partially degraded by the chemicals used.

The result of RT-PCR supports the IHC findings. The *vp28* gene transcript was detected in one of three animal tested with nested-RT-PCR indicating the expression level was low. The animals had light infection as shown by nested-PCR. Since mRNA synthesis is an intermediate step in the synthesis of VP28, the presence of mRNA signals late RNA transcription, which can only occur after DNA replication. The fact that complementing results were obtained with naturally infected specimens collected in the same pond three years apart strengthens evidence that WSSV replicated in *Dendronereis* spp.

WSSV morphogenesis occurs in the nucleus of foregut and stomach epithelium of shrimp and crab (Durand et al. 1997). Our results showed that WSSV also infected and replicated in the foregut epithelium of *Dendronereis spp*., despite the fact that *Dendronereis* spp. belong to a different phylum in the animal kingdom

(Annelida). Hence, WSSV has a wider host range than Crustaceans alone and must be considered an even more generalist virus than previously thought.

Dendronereis spp. are widely distributed in the mangrove areas in Southeast Asia (Kumar 2003; Pillai 1965; Sarkar et al. 2005) and Africa (Ngqulana et al. 2010) and are natural prey of shrimp in traditional ponds in Indonesia. Even though WSSV replication in penaeid shrimp and decapods has been documented, until today, no replication in planktonic crustaceans and polychaetes has been reported. These organisms were considered to be passive vectors (Escobedo-Bonilla et al. 2008; Stentiford et al. 2009). Our findings showed that *Dendronereis* spp. can also be a propagative carrier. Although the result of (Vijayan et al. 2005) suggested that transmission is likely, transmission experiments are still needed to confirm if infected *Dendronereis* spp. also transmit WSSV disease to shrimp when cannibalizing these polychaetes.

ACKNOWLEDGEMENT

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CHAPTER 4.

Hediste diversicolor (O.F. MÜLLER 1776) AS A POSSIBLE MODEL TO STUDY WHITE SPOT SYNDROME VIRUS INFECTION IN POLYCHAETES

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ABSTRACT.

The white spot syndrome virus (WSSV) is a highly contagious shrimp pathogen world-wide for which polychaetes are among the many biological vectors. In a previous study, WSSV infection was detected in the naturally infected *Dendronereis* spp. (Nereidae). To further study WSSV infection in polychaetes, a model polychaete that is easy to handle and propagate, and is free of and susceptible to WSSV infection is needed. In the present study the suitability of *Hediste diversicolor* (Nereidae) was tested as a model animal. WSSV-free *H. diversicolor* was infected by injection, feeding and immersion, and the infection was followed for 12 days post infection (dpi). In addition, polychaete survival was determined 40 dpi. *Hediste diversicolor* was able to clear the virus within 4 dpi without showing clinical signs and WSSV-associated mortality. Although a first attempt, it was concluded that *H. diversicolor* may not be an immediately suitable model animal for WSSV studies in polychaetes.

Chapter 4

INTRODUCTION

White Spot Syndrome Virus (WSSV) is the most damaging viral pathogen to the shrimp culture industry worldwide (Stentiford et al. 2012). WSSV is a large double-stranded DNA virus and the only member of the genus *Whispovirus* within the family *Nimaviridae* (Lo et al. 2012). The disease was first reported in Taiwan in 1992 and was named 'white spot syndrome' after the pathognomonic clinical signs in the form of small white spots that occur on the carapace of infected animals (Chou et al. 1995). The primary sites of WSSV replication in shrimp are tissues of mesodermal and ectodermal origin especially the epithelium of the foregut, the gills and the antennal gland of large decapod crustaceans (Escobedo-Bonilla et al. 2007). WSSV is infectious to shrimp *per os* and horizontal transmission via water and cannibalism has been documented (Chou et al. 1998).

The virus is not only highly infectious to penaeid shrimp, but also to many other crustaceans including crabs and fresh and salt water crayfish. Hence WSSV has a very broad host range among aquatic crustaceans (Bateman et al. 2012b; Escobedo-Bonilla et al. 2008; Flegel 2006; Liu et al. 2011b; Marques et al. 2011; Sánchez-Paz 2010; Soowannayan and Phanthura 2011; Witteveldt 2006). The only reported non-crustacean host for WSSV is the polychaete *Dendronereis* spp. (Desrina et al. 2013). Such a broad host range and tissue tropism makes WSSV a generalist virus.

Nereid polychaetes (Family *Nereidae*) are abundantly present in soft sediments typically present in shrimp ponds. The presence of these errant polychaetes in the sediment is beneficial for improving sediment quality by bioturbation (Carvalho et al. 2007). Hence, these animals promote microbial activity, influence chemical nutrient fluxes (Brown et al. 2011; Kristensen et al. 2011), and reduce the anaerobic area thereby increasing oxidation of organic matter and pollutants. In addition, polychaetes are highly nutritious and preferred prey for

shrimp (Nunes et al. 1997; Reymond and Lagardère 1990). This is why they are used as supplemental food to induce gonad maturation in broodstock (Chung et al. 2011; Nguyen et al. 2012; Poltana et al. 2007).

WSSV can be transmitted via polychaetes as evidenced from feeding experimentally infected *Marphysa* spp. (Vijayan et al. 2005) and naturally infected *Dendronereis* spp. (Haryadi et al. 2014) to naive shrimp. In contrast to what others have assumed (Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010; Vijayan et al. 2005), polychaetes may potentially act as a propagative vector for WSSV. WSSV replicates in *Dendronereis* spp. (Desrina et al. 2013) and may serve as a reservoir for this virus. As such, polychaetes may play a role in the epidemiology of WSSV in pond systems.

To study WSSV infection in polychaetes in more detail, WSSV-free polychaetes should be available. Because *Dendronereis* spp. collected in pond systems in Indonesia were often found positive for WSSV infection (Desrina, personal communication), another nereid polychaete *Hediste diversicolor* (Müller 1776) (the common ragworm) was selected as model organism. This species is abundant in the Northern hemisphere where WSSV infections have not been reported. It is a euryhaline and eurythermal species and has a wide distribution in the estuaries and intertidal zone of the north Atlantic region throughout Europe (Scaps 2002). It does not have planktonic larval stages, lives in U-shaped burrows (Durou et al. 2008), and is a non-selective deposit feeder (Esselink and Zwarts 1989).

Due to the ease of handling under experimental conditions, *H. diversicolor* has been used as a model animal to study heavy metal contamination in marine sediments (Caçador et al. 2012; Durou and Mouneyrac 2007; Durou et al. 2008; Kalman et al. 2009) and to test diets and culture conditions for commercial production (Nesto et al. 2012). We assumed that *H. diversicolor*, being a close

relative to *Dendronereis* spp., could potentially be used as a more convenient model animal to study WSSV infection in polychaetes, provided WSSV replicates in this species.

The objectives of this study were to determine (i) the infectivity of WSSV in *H. diversicolor* and (ii) the time span to develop infection or WSSV persistence in this animal.

MATERIALS AND METHODS

Hediste diversicolor

Adult *H. diversicolor* was obtained from a commercial ragworm producer (Fish-bait BV, Yerseke, the Netherlands). The worm can grow in captivity using sand or mud substrate (Fidalgo e Costa 1999). Upon arrival, the worms were kept in 96-L aerated sea water aquaria with an 8 cm sand layer at the bottom. *H. diversicolor* naturally lives in 5 – 15 ºC water. The animals arrived in 15 ppt water at 10 ºC and were over a 2-months period acclimatized to 27 ºC and 27 ppt, the conducive conditions for WSSV to develop disease in shrimp (Chou et al. 1998; Moser et al. 2012; Rahman et al. 2006). The animals were fed a commercial shrimp feed twice daily at 2% of the total biomass.

A total of 360 *H. diversicolor* (average individual weight 2.1-2.3 g) were used. The animals were randomly distributed in groups of 15, over 24 20-L aquaria with an 8-cm sand layer and 4 L artificial sea water. Each aquarium was aerated with an air-stone laying on the bottom in the center of each aquarium. During the experiment, water temperature was maintained at 25-27 ºC and salinity at 27 ppt. The animals were fed shrimp feed at 2% of body weight per day, given in two meals per day to prevent cannibalism. Water quality was maintained by replacing 1 L per day with sterile artificial sea water. During acclimatization, dead animals were removed and replaced so that each tank contained 15 animals at the start of

the experiment. From the common stock population, 5 animals were tested for the presence or absence of WSSV with nested PCR prior to stocking. Since this analysis invariably gave negative results, it can be assumed that this polychaete species was free of infection at the beginning of trial.

Inoculum preparation

Inoculum was prepared from the hemolymph of artificially WSSV- infected whiteleg shrimp *Litopenaeus vannamei* (Boone 1931). Thirty µl of purified WSSV (originated from infected *Penaeus monodon* (Fabricius 1798) obtained from Vietnam) was diluted 100x, and injected into 10 healthy specific pathogen free (SPF) *L. vannamei* (average weight 6 g/animal). The shrimp were observed during 1 week for clinical signs. Two days post infection (dpi) shrimp became lethargic and stopped feeding. Moribund shrimp were removed, washed in sterile-cold sterile artificial sea water, dried with paper towel and stored at -20 ºC. All shrimp were tested for the presence of WSSV with 1-step PCR and all were found positive. Four shrimps were used for inoculum preparation and the rest was used in oral route infection experiments (see the 'Infectivity of WSSV in *H. diversicolor*' section below). To prepare the inoculum, shrimps were thawed, cut into head and body portion and placed in a 50 ml- sterile- conical tube containing anticoagulant Alsever's solution and filled with cut yellow tips arranged compactly at the base of the tube with the fine tip end positioned toward the bottom which function both as channel and suction of the hemolymph. The shrimp was placed with severed parts towards the base of the tube. The tube was centrifuged at 1500 g for 3 min at 4 ºC. Then the shrimp were removed, TNE buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 0.1 mM EDTA) was added and the tubes were centrifuged at 3000 x g for 8 min at 4 ºC. The clear fluid was passed through a 0.45 µm sterile membrane filter and the collected filtrate was used as inoculum and stored at -80 ºC until use.

Infection of WSSV in *H. diversicolor*

White spot syndrome virus was introduced into *H. diversicolor* by intra-coelomic injection (injection route), feeding (oral route) and immersion (water-borne route). Eight aquaria were randomly assigned for each route. Per route six aquaria were used in time series sampling to determine WSSV persistence in the polychaete over time. The other two aquaria were observed until 40 dpi to determine polychaete survival. Per administration route, half of the aquaria were exposed to WSSV, the other half served as the negative control.

For the injection route, individual worms were placed in cold sterile phosphate buffered saline (PBS, pH 7.8) until they relaxed, put onto sterile paper towel moistened with cooled PBS, injected with WSSV inoculum (diluted 100x in 330 mM NaCl) in between the antero-lateral segments (segments 10-15) with 5 µL inoculum$\cdot g^{-1}$ body weight while gently holding the body during 1 min to prevent contraction, which would cause loss of inoculum. The polychaetes from 'negative control' aquaria were injected with 5 µL sterile 330 mM NaCl$\cdot g^{-1}$ body weight. After injection, *H. diversicolor* was added to a 1-L glass beaker filled with 500 mL water from its own aquarium. Once all ragworms from one aquarium were injected, they were returned into their aquarium. The ragworms were closely observed for burrowing behaviour during 60 min. To confirm the infectivity and pathogenicity of the WSSV inoculum, five 7-g *P. vannamei* were each injected with 35 µL inoculum.

For the waterborne route, the ragworms from each tank were separately immersed for 2 h in either 0.1% inoculum solution or in sterile sea water (negative control). For the oral route, the ragworms were fed during four consecutive days minced WSSV-infected shrimp at 1% of total body weight$\cdot day^{-1}$ and subsequently returned to normal shrimp feed. Animals in control aquaria were fed the same ration of WSSV-free shrimp (stock shrimp and proven WSSV negative with nested

PCR one day before) with the same dose. Total ammonia, pH, nitrate, nitrite and salinity were measured daily.

H. diversicolor behaviour and physical observations

The number of worm on the sand surface and the body condition was recorded twice daily at 7 am (after being in the dark for 12 h) and at 7 pm (after being in the light for 12 h). Afterwards, a predetermined amount of feed was placed on the sand surface of each tank and feeding appetite was observed for 1 h. The number of ragworms that grabbed the food was recorded. During each time series sampling, *H. diversicolor* in each tank was observed for burrowing as follows: six worms from each tank were randomly taken and placed on a plastic tray (20 x 10 x 5 cm) that has been assigned for the particular tank. Color and degree of body damage were recorded at sampling. Ragworms were left in the tray for 10 min and observed whether or not the ragworms were huddled. Next, two ragworms were taken as sample (see next section: time series sampling) and the rest were placed back in the tank. After being put back in the tank, the ragworms' digging activity was observed for 10 min.

Time series sampling

To determine persistence of WSSV in the polychaete over time, two ragworms from each tank were taken at 2, 4, 6, 8, and 10 and 12 dpi, and pooled: 2 ragworms were preserved in 70 % alcohol for PCR testing, 2 ragworms in Davidson's solution for histopathology and 2 ragworms were stored at -80 ºC for RT-PCR to detect WSSV replication. However, we choose not to carry out RT-PCR and histology analysis, when the PCR result on the viral genomic DNA was negative soon after infection and throughout the observation period.

Survival observation

Mortality, feeding activity, changes in burrowing behavior, response to touching and changes in coloration were recorded daily until 40 dpi. At 40 dpi all ragworms were harvested and survival calculated as S (%) = ([number at T_{40} - number at T_0] / number at T_0) x 100 where T_0 and T_{40} are 0 and 40 dpi.

WSSV detection

WSSV was detected in experimental animals with 1-step and nested-PCR using primer pairs for *vp26* (Marks et al. 2005b) for 1-step PCR and *vp28* and *vp28*-nested according to Desrina et al. (2013). 18s rRNA of *H. diversicolor* was used as internal control of successful DNA extraction and detected according to Desrina et al. (2013). Selected PCR products of WSSV DNA from *H. diversicolor*, *L. vannamei* along with 18s rRNA of *H. diversicolor* were sequenced (Macrogen Europe). The results were aligned to known sequences present in GenBank based on the BLAST program.

RESULTS

Behaviour and physical observation

The ragworms crawled on the surface, dispersed and started to burrow within 30 min post infection and returned to a normal feeding pattern at 2 dpi. During the experiment, the ragworms showed normal burrowing behaviour (e.g. within 15 min ragworms were burrowing), flocked together when put together in a tray, and fed normally (the ragworm head was out of the burrow, grabbed the food and dragged it into the burrow). There was no obvious difference in body coloration, movement, feeding, and burrowing activity between the WSSV-infected *H. diversicolor* and the negative control ragworms (no virus) during 11

days of observation. Shrimp injected with WSSV inoculum became lethargic, showed reduced feeding and all died within 6 dpi.

Time series analysis

WSSV was only detected up to 4 dpi in *H. diversicolor* individuals subjected to infection by injection (Fig.1). Other infection methods (oral, immersion) gave negative results with nested-PCR. 18s rRNA of *H. diversicolor* was detected in all samples tested (Fig. 2). No WSSV was detected in the negative control animals. All whiteleg shrimps injected with the inoculum were WSSV positive with 1-step PCR (Fig.3). Alignment of sequencing results showed 100% identity with the *vp28* gene of known WSSV isolates in GenBank (Accession Numbers AF 369029, AF440570, AF 332093). The 18s rRNA of *H. diversicolor* used in this study showed 97% identity with 18s rRNA of members of genus *Nereis* (Accession number U36270, EF117897.1, AY210447.1).

Survival observation

Out of 5 *P. vannamei* used as positive control for the injection route, 3 (60%) shrimp died at 3 dpi; the other 2 died at 4 and 6 dpi, respectively. Survival of the *H. diversicolor* at 40 dpi was comparable among treatments and between each treatment and its control. The summary of survival of each treatment is presented in Table 1. Ragworms that did not survive had no particular symptoms or abnormal pathology. Apparently, no WSSV-related mortality occurred during the experiment. The experiment was considered a pilot and carried out once.

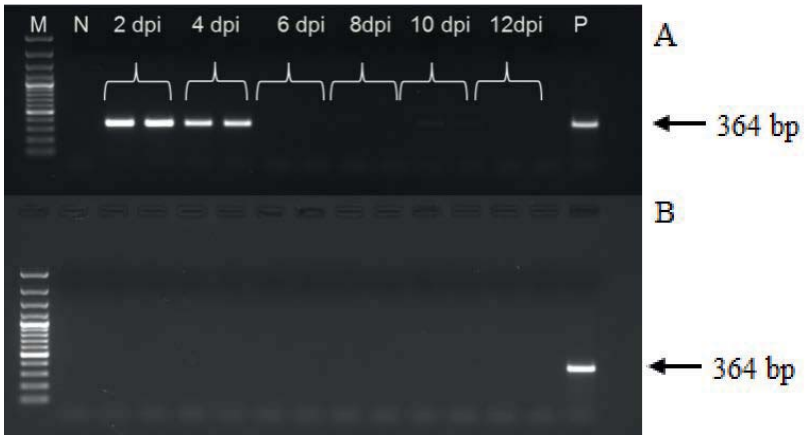

Figure 1. WSSV was detected with nested-PCR using *vp28*-nested primer pair (amplicon 364 bp) in the *H. diversicolor* upon injection up to 4 dpi (T2) (Panel A) and was not detected in negative control specimens sampled at the same time (Panel B). M = Marker (100 bp DNA ladder). N= Non-template control (NTC); P = Positive control of PCR (experimentally infected *L. vannamei*).

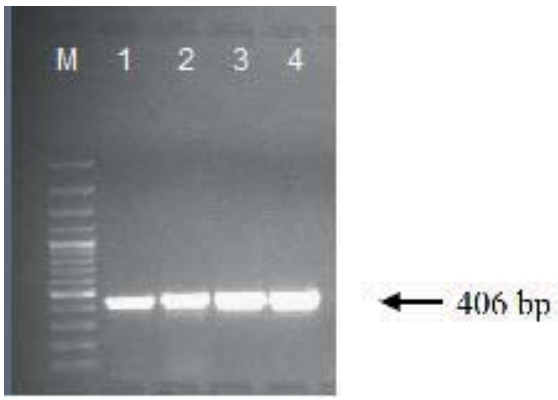

Figure 2. 18s rRNA gene of selected *H. diversicolor* (lane 1-4) used in this experiment served as internal control for adequate DNA extraction. M= Marker (100 bp DNA ladder).

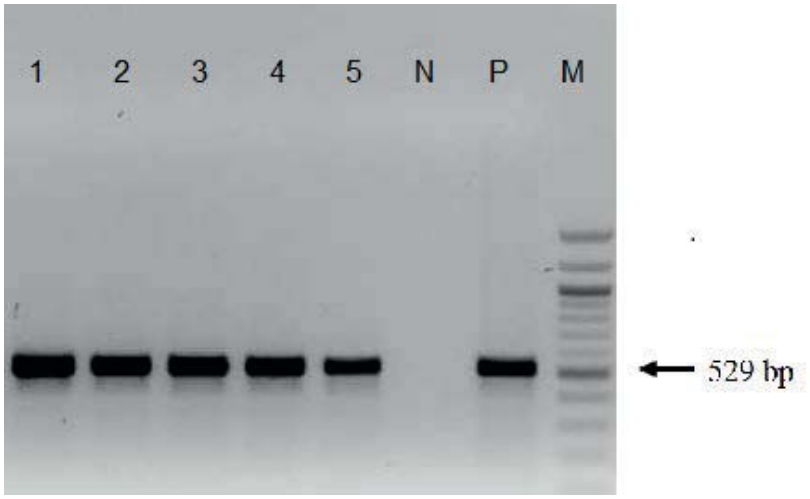

Figure 3. WSSV DNA was detected with 1-step PCR in the *L. vannamei* injected with WSSV inoculum (positive control of infection by injection route). Lane 1= WSSV DNA obtained from *L. vannamei* at 1 dpi; Lane 2-4= WSSV DNA obtained from *L. vannamei* at 2 dpi; lane 5 =WSSV DNA obtained from *L. vannamei* at 4 dpi; N = No template control of PCR; P = positive control of PCR; M = Marker (100 bp DNA ladder).

Table 1. Survival of H. diversicolor infected by injection, oral and immersion routes at 40 dpi

	Infection route	Survival (%)
Injection	Injection with WSSV inoculum	53
	Injection with sterile 330 mM NaCl	53
Waterborne	Immersed in 0.1% WSSV inoculum	53
	Immersed in sterile sea water	60
Oral	Fed with minced WSSV-infected shrimp	53
	Fed with minced WSSV free shrimp	66

Chapter 4

DISCUSSION

The goal of this is study was to evaluate *H. diversicolor* as a potential model animal to further investigate WSSV infection in polychaetes. This polychaete did not show basic indicators of WSSV infection in penaeid shrimp, such as clinical signs, body coloration, and/or behavioral changes. The animals fed and burrowed shortly after infection and during the experiment appeared to be healthy, hence, the presence of the virus did not affect this worm. Burrowing activity of *H. diversicolor* is an indicator of the worm's well-being (Esselink and Zwarts 1989) and has been used to study effects of heavy metal contamination in this polychaete (Bonnard et al. 2009; Kalman et al. 2010; Kalman et al. 2009; Mouneyrac et al. 2010). We adapted the parameters to our study because generally, reduced feeding and abnormal movement were the first behavioral change attributed to disease or adverse environmental conditions in fish or shrimp. Therefore, burrowing activity is an applicable proxy to assess *H. diversicolor*'s health condition. Furthermore, there was no difference in body coloration between infected and mock-infected worms, even at 40 dpi.

Injection, immersion and feeding are three commonly used methods to test infectivity of WSSV in shrimps and crabs (Bateman et al. 2012b; Chen et al. 2000; Liu et al. 2011b). We used all three types of exposure to overcome the limitation of each method. Published studies on WSSV infectivity in polychaetes gave variable results. *Marphysa* spp. developed light infection within 7 days post exposure to WSSV by immersion in sediment-contaminated WSSV, as evidenced by nested PCR (Vijayan et al. 2005). Laoaroon et al. (2005) were able to induce light infection in *Perinereis nuntia* (Savigny in Lamarck, 1818) fed with WSSV infected *P. monodon* and by immersion methods as evidenced by nested PCR. In the latter report, 40 - 90% of the tested polychaetes were found infected within 2 weeks pi. However, these results should be regarded with caution because the experiment did

not start with WSSV-free animals. In contrast, we very likely failed to induce infection in *H. diversicolor* by both feeding and immersion.

It is possible that in our case the dose used was too low to initiate infection in this polychaete, although the same dose was readily able to induce infection in the control positive shrimp. There may be a huge difference in susceptibility between nereids and penaeids, and among nereids. Another explanation may be that the virus was diluted quickly in the coelomic cavity. The WSSV inoculum mixed with 1 µl of patent Blue V color was spread and diluted in the coelomic cavity within 30 min (data not shown) indicating that this may have been the case. In addition, the polychaete species and type of sediment used in our experiment as compared to the literature (Laoaroon et al. 2005; Vijayan et al. 2005), may have attributed to the different outcome. The behaviors and lack of external symptoms in WSSV infected *H. diversicolor* were in accordance with the PCR result, showing that *H. diversicolor* was able to clear the virus within 4 days.

WSSV occurrence in wild *Marphysa* spp. (Vijayan et al. 2005) and *P. nuntia* (Laoaroon et al. 2005) was reported. The authors hypothesized that WSSV was naturally acquired along with ingested sediment and that the virus accumulated in the polychaete to make it only a passive vector. Later, Desrina et al. (2013) showed that WSSV replicated in *Dendronereis* spp., which makes these polychaete active WSSV carrier. Likewise, *H. diversicolor* can be a potential carrier of WSSV once the virus establishes itself in the environment where this polychaete lives. On the basis of this first attempt though, it seems that *H. diversicolor* is not an immediately suitable proxy model animal for WSSV studies in polychaetes. No clinical or typical signs were noted and no WSSV-associated mortality occurred, not even in animals infected by injection. It is possible that (i) WSSV in polychaetes is attenuated because of the phylogenetic distance between polychaete and penaeid shrimp as natural host of WSSV, including the associated immune

response or (ii) there is specific coadaptation between WSSV and polychaete that enable them to adapt to each other.

CONCLUSIONS

Studies on WSSV infection in polychaetes and roles played by polychaetes in transmission of WSSV in shrimp ponds are still at early stage. To further study WSSV infection in polychaetes, a model animal is needed. In the present study, WSSV infection could not be induced in *H. diversicolor* through oral, injection and immersion routes. Therefore, other routes of infection such as indirect transmission through virions attached to sediment particles or benthic algae should be explored. If the latter would be not successful, then the use of other nereids polychaetes as model species for WSSV infection studies in polychaetes should be explored.

ACKNOWLEDGEMENT

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CHAPTER 5.

TRANSMISSION OF WHITE SPOT SYNDROME VIRUS (WSSV) FROM *Dendronereis* spp. (PETERS) (NEREIDIDAE) TO PENAEID SHRIMP

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ABSTRACT

Dendronereis spp. (Peters) (Nereididae) are common polychaete in shrimp ponds built on intertidal land and are natural food for shrimp in traditionally-managed ponds in Indonesia. White spot syndrome virus (WSSV), an important viral pathogen of the shrimp, can replicate in this polychaete (Desrina et al. 2013), therefore, they are potential propagative vectors for virus transmission. The major aim of present study was to determine whether WSSV can be transmitted from naturally infected *Dendronereis* spp. to specific pathogen free (SPF) pacific white shrimp *Litopenaeus vannamei* (Boone) through feeding. WSSV was detected in naturally infected *Dendronereis* spp. and *Penaeus monodon* Fabricius from a traditional shrimp pond and the positive animals were used in the current experiment. WSSV infected *Dendronereis* spp. and *P. monodon* in a pond had a point prevalence of 90% and 80%, respectively, as measured by PCR. WSSV was detected in the head, gills, blood and mid-body of *Dendronereis* spp.. WSSV from naturally infected *Dendronereis* spp. was transmitted to SPF *L. vannamei* and subsequently from this shrimp to new naïve-SPF *L. vannamei* to cause transient infection. Our findings support the contention that *Dendronereis* spp., upon feeding, can be a source of WSSV infection of shrimp in ponds.

Keywords: WSSV, *Dendronereis* spp., shrimp, oral transmission, infection

Chapter 5

INTRODUCTION

White spot syndrome virus (WSSV), the etiological agent of white spot disease (WSD), is a significant viral shrimp pathogen worldwide. The virus was first reported in Taiwan in 1992 (Chou et al. 1995) and quickly spread around the world (Muroga 2001; Rajan et al. 2000; Wang et al. 1999b). WSSV is one of the most damaging shrimp pathogens to date, inflicting loss of production of approximately 300,000 tons of shrimp or about 1 billion US$ annually (Stentiford et al. 2012). The first report of WSSV in Indonesia was in 1993 (Sunarto et al. 2004), today WSSV is endemic in Indonesia as well as in the rest of Southeast Asia.

WSSV is a double stranded DNA virus belonging to the genus *Whispovirus*, family *Nimaviridae* (Lo et al. 2012). It is unique among shrimp viruses, because it has a large genome size of approximately 290 kbp (Van Hulten et al. 2000b) and is a generalist virus of crustaceans. In addition to those listed by Flegel (2006), Escobedo-Bonilla et al. (2008) and (Sánchez-Paz 2010) recently reported hosts of WSSV including wild crab *Casmagnathus granulata* (Dana) (Marques et al. 2011), red claw crayfish *Cherax quadricarinatus* (von Martens) (Soowannayan and Phanthura 2011), mud crab *Scylla serrata* (Forskål) (Liu et al. 2011b), edible crab *Cancer pagurus* (Linnaeus), lobster *Homarus gammarus* (Linnaeus), Norway lobster *Nephros norvegicus* (Linnaeus), shore crab *Carcinus maenas* (Linnaeus), Chinese mitten crab *Eriocheir sinensis* (H. Milne Edwards), white claw crayfish *Austropotamobius pallipes* (Lereboullet) and signal crayfish *Pacifastacus leniusculus* (Dana) (Bateman et al. 2012b). Moreover, organisms other than decapods have been regarded as mechanical vectors of WSSV without evidence of virus replication, except for the copepod *Apocyclops royi* (Lindberg) (Chang et al. 2011) and the polychaete *Dendronereis* spp. (Peters) (Desrina et al. 2013).

WSSV is highly pathogenic and very infectious to decapods, however, variation in pathogenicity among isolates from different geographical regions (Laramore et al. 2009; Zwart et al. 2010) and cross-species passaging (Waikhom et al. 2006) have been reported. The virus initially replicates in the nucleus of epithelium cells lining the stomach wall (Durand et al. 1997), gills (Rahman et al. 2008) and later spreads via the hemolymph causing WSSV pathology in tissues that are of ectodermal and mesodermal origin (Chang et al. 1996). Transmission of WSSV from one shrimp species to another occurs by feeding on infected animals, direct contact with WSSV-contaminated water or cohabitation with infected animals (Chou et al. 1998; Kanchanaphum et al. 1998; Supamattaya et al. 1998; Wang et al. 1998). Hundred percent mortality was reported within one week of infection (Rajan et al. 2000). However, under favourable culture conditions shrimp can harbour WSSV for a long period without causing mortality or showing clinical signs (Tsai et al. 1999; Withyachumnarnkul 1999).

Polychaetes are common inhabitants of soft bottom coastal ecosystems and a few have been used as food in shrimp hatcheries to enhance brood stock maturation (Poltana et al. 2007; Vijayan et al. 2005). Despite its close proximity to shrimp, very limited research has been done on the role of indigenous polychaetes on the epidemiology of WSSV in shrimp ponds. A single report on *Marphysa* spp. (Quatrefages) showed that this animal acquired WSSV infection through feeding on 'infected' detritus, accumulated the virus in the gut and transmitted it to shrimp, thus, acted as a passive vector (Vijayan et al. 2005). *Dendronereis* spp. (Family *Nereididae*) are ubiquitous polychaete in shrimp ponds in Indonesia (Pillai 1965). In a previous study we reported evidence that WSSV replicates in this polychaete (Desrina et al. 2013). Hence, *Dendronereis* spp. may be a natural propagative host and vector of WSSV in earthen shrimp ponds. The aims of this study were to determine (i) the prevalence of WSSV both in polychaetes and shrimp in a pond,

(ii) whether WSSV in polychaetes and shrimp is the same by genetic analysis, (iii) whether transmission of WSSV could occur from naturally infected *Dendronereis* spp. to pacific white shrimp *Litopenaeus vannamei*, and (iv) whether the transmitted virus can be further transmitted to naïve-SPF *L. vannamei* shrimp. To our knowledge this is the first report on the possible transmission of WSSV from naturally-infected *Dendronereis* spp. to penaeid shrimp.

MATERIALS AND METHODS

Dendronereis spp. and *Penaeus monodon*

Dendronereis spp. (average wet weight 0,8-0.9 g/individual) and *Penaeus monodon* (average wet weight 6-8 g/individual) used throughout this study were obtained from a grow-out pond (0.8 ha, about 50 years old) located on the north coast of Java close to the city of Semarang. The pond was used to culture juvenile *P. monodon* (average weight when stocking 4-6 g shrimp, density 2 shrimp/m^2, 3 crops/year) and was managed by traditional methods (no aeration, tidal water exchange and no feeding), without biosecurity measurements employed and relying solely on natural food (among others *Dendronereis* spp.). The pond was never completely dried during the last 15 years. However, the sludge was removed and placed on the dike every year. When the farmers observe shrimp with white spots on the carapace, this results in an early harvest. *P. monodon* and *Dendronereis* spp. were collected one month after stocking. Sediment containing *Dendronereis* spp. was removed using a PVC pipe (diameter 10 cm, height 40 cm with the lid at one end) and gently passed through a series of sieves (mesh sizes: 2.0, 0.6 and 0.3 mm) to collect the animals. The animals were cleaned with pond water and a fine brush. The *P. monodon* were also obtained from the same pond at the same time as *Dendronereis* spp.. Two out of 20 of the shrimp showed white spots on the carapace, which was the specific clinical sign in *P. monodon* for WSSV infection

(Chou et al. 1998). None of the polychaetes showed these clinical signs. Live shrimp and polychaetes were brought to the Centre of Medical Research (CEBIOR) laboratory, Diponegoro University, rinsed thoroughly in sterile sea water and sterile, cooled phosphate-buffer saline (PBS, pH 7.4), dried with paper towel and immediately preserved at -80 ºC to be used later for transmission experiments.

Point prevalence of WSSV infection in *Dendronereis* spp. and *P. monodon*

Polymerase Chain Reaction tests to detect WSSV infection

Dendronereis spp. (n=20) and *P. monodon* (n=20) from the pond were individually tested for presence of WSSV. Twenty-five mg worm tissue from the head and 25 mg of the gills of *P. monodon* were extensively washed to remove adhering WSSV or DNA and grinded in a sterilized pestle. Total DNA was extracted using DNeasy Hemolymph and Tissue Kit (QIAGEN, Germany) following the protocol outlined by the manufacturer. PCR to detect the WSSV infection in *Dendronereis* spp. and other animals used in this study was conducted according to Desrina et al. (2013). The DNA was amplified using Gene Amp PCR System 9600 (Applied Biosystems, Foster City, USA) using *vp28* as a target gene (Desrina et al. 2013). WSSV-containing DNA extracted from experimentally-infected *L. vannamei* (gills) was used as positive control throughout the experiment (sequence-checked). The result was visualized using a UV illuminator Gel Doc XR System (Biorad). Point prevalence of WSSV infection in *Dendronereis* spp. and *P. monodon* was calculated according to Cameron (2002).

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PCR analysis to determine WSSV infection in parts of *Dendronereis* spp.

Five live *Dendronereis* spp. were used for this analysis. *Dendronereis* spp. were placed on a paper towel to absorb the fluid on the body surface and gills before being transferred to a sterile aluminium sheet (15 x 15 cm). Gills, hemolymph, head (up to 20^{th} segment) and body (segment 70-100) were dissected out employing the principle of clinical examination of fish necropsy. The respective body parts (5 each) were pooled in microfuge tubes placed on ice. The *Dendronereis* spp. gills were cut using ophthalmic scissors, the head and body parts were cut using scalpels. Hemolymph was carefully drawn from the dorsal blood vessel using a tuberculin syringe filled with 50 µl of anticoagulant Alsever buffer (Rodriguez et al. 1995) and placed in a sterile micro-centrifuge tube. Cross contamination between worm body parts was prevented by working aseptically using sterilized equipment and solution and a disinfected work area. Equipment was changed after taking a body part, preventing spreading of body fluid by blotting with sterile paper towel and gently wiping the worm surface with sterile cotton dipped in cool sterile PBS before proceeding to the next body part. DNA extraction was done immediately using QIAGEN DNeasy kit (Qiagen, Germany). Hemolymph (total 600 µl) was centrifuged (4500xg, 4 ^{0}C, 20 min). Both supernatant and pellet were used for PCR assay. For other parts (gills, head and body) each was homogenized in cool sterile PBS (1: 10 w/v), and 100 µl suspension was used for DNA extraction with DNeasy kit (QIAGEN, Germany). The DNA suspension was subjected to PCR analysis.

Bioassay I to determine transmission of WSSV from naturally infected *Dendronereis* spp. to juvenile *L. vannamei* through feeding

The transmission experiment consisted of two bioassays that were conducted in a stepwise fashion (Figure 1). Healthy, specific SPF for WSSV (as well as for TSV, IMNV and IHHNV), juvenile *L. vannamei* animals (average weight 3 g) were obtained from a research hatchery in Lampung, Indonesia. The animals were placed in aquaria (volume 30 L, 11 shrimp/aquarium) filled with disinfected sea water (calcium hypochlorite 30 mgL^{-1} for overnight, neutralized with 30 mgL^{-1} sodium thiosulfate with strong aeration and kept aerated for 1 week before use) and equipped with air stones. Water temperature was kept at 28±1 ^{o}C and salinity at 30 gL^{-1}. The shrimps were fed commercial shrimp food at 1% body weight per day, which is below satiation to increase appetite and reduce waste. Bottom water was siphoned out (about 20 % of aquarium volume) daily and replaced with disinfected seawater. One day before the infection, one shrimp of each aquarium was randomly chosen and checked for WSSV again with a nested-PCR and all were negative for WSSV.

Shrimp were divided into three treatment groups (4 aquaria/treatment, 10 shrimp/aquarium). The first group was fed with commercial shrimp pellets (type 8003 VAN, CP Group Indonesia, contained 30% protein) (treatment P) at 2% body weight as negative control; the second group was fed with WSSV naturally infected *Dendronereis* spp. (treatment D) obtained from WSSV infected shrimp pond as tested group and the third group was fed WSSV naturally infected *P. monodon* (treatment M) as positive control. Shrimp of treatments D and M were fed at 6% (w/w) body weight per day with either *Dendronereis* spp. or *P. monodon* for five consecutive days and afterward were fed with commercial shrimp pellet at 2% body weight as for treatment P. Two aquaria per treatment were assigned for

time series sampling and the other two aquaria were used for infection development and mortality observation.

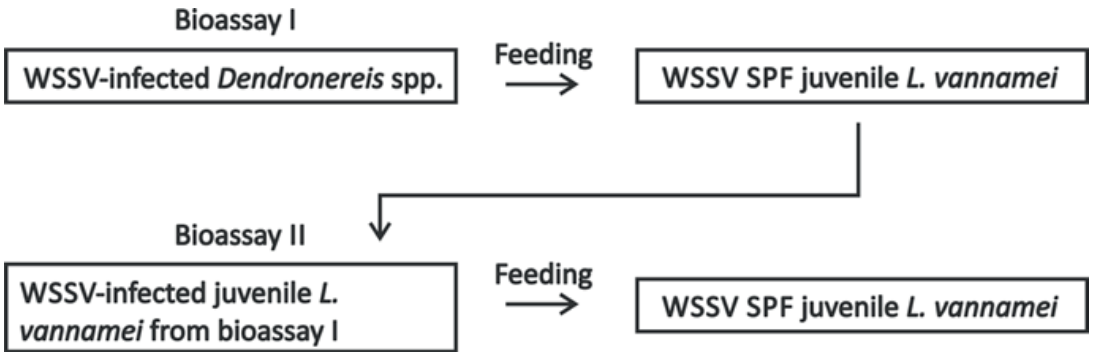

Figure 1. Schematic diagram of bioassays in this paper

Time series sampling

The observation started at 1 day post inoculation (dpi) (= day 2 of the experiment). Two shrimps from each aquarium allocated for time series sampling were taken (in total 4 shrimps per treatment) at 3, 5, 7, 9 and 11 dpi and stored at -80 ^{o}C to be used later in the transmission experiment to conspecifics (Bioassay II). Gills of all four specimens were individually tested for WSSV with nested-PCR. Point prevalence for each sampling time was calculated according to Cameron (2002) as the percentage of animals that tested positive for WSSV with PCR. Cumulative prevalence was calculated as the total number of shrimp infected during 11 days of observation.

Survival measurement

Shrimp in 2 aquaria of each group were maintained until 18 dpi, and observed daily for survival (%) and clinical signs. At 18 dpi, all shrimps were harvested and tested for WSSV infection.

WSSV detection post transmission

WSSV infection in the shrimp used in both bioassays was determined with nested-PCR as described above. Effect of difference of source of WSSV on the occurrence of infection post transmission was analyzed with Pearson's Chi Square Analysis using R program R 2.15.3 (http://cran.r-project.org/bin/windows/base/)

Bioassay II to determine transmission of WSSV from infected-*Dendronereis*-fed *L. vannamei* to naïve *L. vannamei*.

A total of nineteen juvenile *L. vannamei* from the same batch as used in bioassay I was randomly divided over 4 aquaria. Shrimp (n=5) which were infected by WSSV (based on result of nested-PCR) were minced and fed to healthy juvenile *L. vannamei* in 2 aquaria (total n=10) for 5 consecutive days at 6% body weight/day and afterwards fed commercial pellets at 3% body weight/day. Another batch of shrimp (total n= 9) were fed a commercial shrimp food pellet as negative control. The bioassay was run for 18 days, at which time all shrimps were harvested and tested for the presence of WSSV.

Sequencing of the *vp28* gene of WSSV from *Dendronereis* spp., *P. monodon* and post-infected *L. vannamei*.

Nested-PCR products of WSSV DNA from naturally infected *Dendronereis* spp., *P. monodon* and *L. vannamei* after feeding with infected *Dendronereis* spp. were sent to 1st BASE Laboratory (Selangor, Malaysia) for sequencing. Results were aligned for homology among sequences of WSSV *vp28* obtained in this study and with known sequence contained in GenBank using the BLAST program (www.ncbi.nlm.nih.gov).

Chapter 5

RESULTS

Point prevalence of WSSV in *Dendronereis* spp. and *P. monodon* used in the transmission experiment

In order to determine the point prevalence of WSSV in both *Dendronereis* spp. and *P. monodon* from a pond with traditional management, twenty animals of each were used and the viral DNA detected by 1-step and 2-step PCR. Fifty-five % of either *P. monodon* or *Dendronereis* spp. tested were positive for WSSV in a 1-step PCR indicating infection of a large proportion of the sample. The samples negative in the 1-step PCR were exposed to a 2-step PCR, which enhanced the proportion of PCR-positive animals. *Dendronereis* spp. and *P. monodon* were infected with high point prevalence: 80% for *P. monodon* and 90% for *Dendronereis* spp., indicating that WSSV infection occurs in both hosts, shrimp and polychaete (Table 1). The observation that not 100% of the animals were infected can be explained by the fact that the animals were not infected or resistant, or that the infection is extremely low, below the detection level of the nested-PCR. The infectious load of shrimp and *Dendronereis* spp., that were positive with 1-step PCR, was different, because the PCR bands of DNA extracted from *Dendronereis* spp. and shrimp were highly variable in intensity. This was confirmed by qPCR on selected samples (data not shown).

Table 1. Summary of result of 1-step and nested-PCR of *P. monodon* and *Dendronereis* spp. used in the transmission study.

Specimen		Positive for WSSV infection		Negative for WSSV	Point Prevalence of infection (%)
	Number tested	1-step PCR	Nested-PCR		
P. monodon	20	11	5	4	80
Dendronereis spp.	20	11	7	2	90

WSSV infection of parts of *Dendronereis* spp.

Pooled specimens of parts of *Dendronereis* spp. obtained from five animals were tested for the presence of WSSV. WSSV DNA was detected by 1-step PCR in the head region, gills, blood, and mid-body of *Dendronereis* spp. (Figure 2) indicating that the virus was present in these tissues. This would imply that WSSV was available for infection via *Dendronereis* spp. when ingested by the shrimp through predation. The absence of a signal in lane 5 while present in lane 3 could be explained by the presence of competing factors in the whole blood (Hedman and Rådström, 2013) which are less or not at all present in the polychaete tissues.

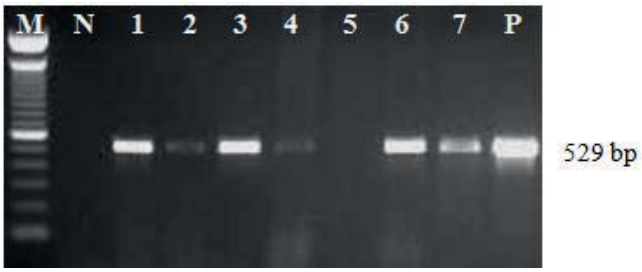

Figure 2. WSSV DNA detected with 1-step PCR on different parts of *Dendronereis* spp. M = Marker (100 bp ladder); N = Non-template control (NTC); P = Positive control of PCR. Lanes 1-6 parts of *Dendronereis* spp. tested. 1 = Head; 2 = Gills; 3 Blood plasma; 4 = Blood cells pellet; 5 = Whole blood; 6 = Mid-body part; 7 = WSSV inoculum from a macerated WSSV-infected *Dendronereis* spp.

Transmission of WSSV from infected *Dendronereis* spp to *L. vannamei* through feeding

WSSV transmission from naturally infected *Dendronereis* spp. to SPF *L. vannamei* was determined in a series of bioassays. WSSV was transmitted through feeding from naturally infected *Dendronereis* spp. to juvenile *L. vannamei*. The shrimp started showing mild clinical signs, such as lethargy, swimming on its side and light pink body coloration on the second day of feeding which remained visible until 6 dpi. The symptoms receded at 7 dpi and the animals appeared to have

recovered. This is in accordance with the results of the PCR test. The WSSV DNA in the gills of tested shrimp was detected with nested-PCR at 3 up to 11 dpi with a final prevalence 70% (Table 2). However, the number of WSSV-positive shrimp declined after 5 dpi. In contrast, juveniles fed with naturally infected *P. monodon* obtained from the same pond at the same time caused severe clinical signs that appeared at 1 dpi and all the shrimp in time series sampling aquaria were dead within 5 dpi and were positive for WSSV infection with 1-step PCR indicating heavy infection. None of juveniles fed commercial pellets got infected (Figure 3).

Table 2. Result of time series sampling of bioassay I. WSSV was detected in juvenile *L. vannamei* after feeding with WSSV-naturally-infected *Dendronereis* spp. and *P. monodon*. Two shrimps from two aquaria per treatment (total 4 shrimp) were tested at each sampling time. WSSV DNA was detected with 1-step and nested-PCR in the shrimp gills.

Treatment	Number shrimp tested	Number of juvenile *L. vannamei* infected byWSSV/number of tested at sampling time					Cumulative prevalence (%) at 11 dpi of WSSV infection
		3 dpi	5 dpi	7 dpi	9 dpi	11 dpi	
D	20	4/4	4/4	2/4	2/4	2/4	70*
M	20	18/18	2/2	-	-	-	100**
P	20	0	0	0	0	0	0*

D = *L. vannamei* fed with naturally WSSV-infected *Dendronereis* spp. for 5 consecutive days; M = *L. vannamei* fed with naturally WSSV-infected *P. monodon* for 5 consecutive days (positive control); P = *L. vannamei* fed with commercial shrimp pellet (negative control).

* WSSV DNA was detected with nested-PCR

** WSSV DNA was detected with 1-step PCR

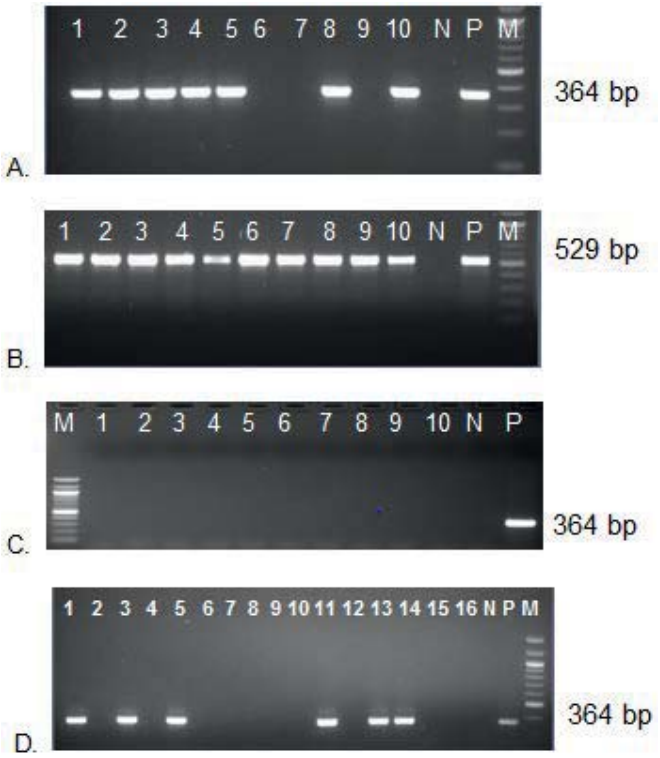

Figure 3. Results of Bioassay I. Panel A-C WSSV DNA detection in juvenile *L. vannamei* after oral transmission at time series sampling up to 11 dpi. (A) WSSV detected in the gills of shrimp fed with naturally infected *Dendronereis* spp. (Treatment D), (B) Shrimp fed with naturally infected *P. monodon* (positive control) (Treatment M), and (C) Shrimp fed with commercial shrimp feed (Treatment P, negative control). WSSV DNA was detected in the gills by 1-step PCR in juveniles fed with infected *P. monodon* (Panel B), and nested-PCR in those fed with *Dendronereis* spp. (Panel A,C and D). Panel D shrimp fed with naturally infected *Dendronereis* spp. (Treatment D) at 18 dpi. No WSSV DNA was detected in juvenile fed with commercial shrimp feed (C). N = Non-template control (NTC); P = Positive control of PCR; M (Size marker) = 100 bp DNA ladder. For panels A, B and C : lane 1&2 = 2 dpi, lane 3&4 = 5 dpi, lane 5&6 = 7 dpi, lane 7&8 = 9 dpi, lane 9&10= 11 dpi. The numbers above the lanes represent individual shrimp.

Table 3. Survival of juvenile *L. vannamei* of bioassay I after feeding with WSSV naturally- infected *Dendronereis* spp. and *P. monodon* at 18 dpi.

Treatment	Number of animal at T0	Number of animal at 18 dpi	Survival (%)	WSSV infection prevalence (%)
D	20	20	100	37.5*
M	20	0	0	100**
P	10	9	95	0*

D = *L. vannamei* fed with naturally WSSV-infected *Dendronereis* spp. for 5 consecutive days; M = *L. vannamei* fed with naturally WSSV-infected *P. monodon* for 5 consecutive days (positive control); P = *L. vannamei* fed with commercial shrimp pellet (negative control).
* WSSV DNA was detected with nested-PCR
** WSSV DNA was detected with 1-step PCR

Survival of juvenile shrimp fed infected *Dendronereis* spp. was 100%, and 95 % for the negative control, hence no mortality associated with WSSV occurred in either situation. One shrimp died in the negative control because it jumped out the tank. The prevalence of WSSV infection in juveniles fed infected *Dendronereis* spp. at 18 dpi declined to 37% (Table 3). In contrast, cumulative mortality of juvenile fed infected *P. monodon* was 100% within 1 week and prevalence of WSSV infection was 100%. None of the negative control shrimp was infected with WSSV. The Pearson's chi-square test showed that there was a significant association between the source WSSV to be transmitted and whether or not it will result in infection in the tested shrimp. At 11 dpi, the infection caused by WSSV originating from *P. monodon* was significantly higher than that of *Dendronereis* spp. ($X^2(1) = 18.25$, $p<.001$). Subsequent feeding on infected *Dendronereis* spp. resulted in infection that was significantly higher than when feeding on pellets ($X^2(1) = 8.58$, $p<.001$).

WSSV transmission from infected-*Dendronereis*-fed juvenile *L. vannamei* to naïve *L. vannamei*

Shrimp that had been infected with WSSV by feeding on naturally infected *Dendronereis* spp. was offered as food to healthy SPF juveniles by ingestion. This resulted in a prevalence of infection of 70%, as measured by nested-PCR (Figure 4) in which 20% of the tested animals was WSSV-positive in the gills. Infected shrimps were slightly lethargic, less active as compared to uninfected shrimp. No mortality occurred during the observation period and none of the negative control animals was infected.

Figure 4. Result of bioassay II. WSSV DNA was detected in the gills in new naïve juvenile *L. vannamei* after feeding with WSSV-infected-*Dendronereis L. vannamei* juveniles. M = Marker (100 bp DNA ladder); N = Non-template control (NTC); P = Positive control of PCR. Lane 1-10 WSSV DNA extracted from juvenile *L. vannamei*

Sequencing of the WSSV *vp28* gene

Result of sequencing of WSSV*vp28* obtained from naturally infected *Dendronereis* spp. and *P. monodon*, and from *L. vannamei* post transmission showed 99%, 100% and 99% nucleotide sequence identity with the *vp28* gene of WSSV isolate Indonesia 97 (Accession code AY249441.1), respectively. This indicates not only that the PCR product genuinely is WSSV DNA, but also that the WSSV in the polychaete and in the shrimp from the same pond were nearly identical.

Chapter 5

DISCUSSION

In the present study we provide evidence that WSSV from *Dendronereis* spp., ubiquitous Nereid in shrimp ponds in Indonesia was transmitted to WSSV-SPF *L. vannamei*, and may play a role in WSSV transmission in nature. *Dendronereis* spp. are reservoir host and susceptible to WSSV infection (Desrina et al. 2013). WSSV coexisted in both *Dendronereis* spp. and *P. monodon* and the point prevalence of infection in *Dendronereis* spp. and *P. monodon* from the same pond used in this study was comparable. Considering that we took samples of both species randomly from the same pond, the point prevalence may reflect the pervasiveness of WSSV in both hosts in this particular pond. Since the samples were obtained from only one pond, any broader interpretation requires a more extensive study involving multiple ponds and locations.

It is not yet clear how the infection occurs in *Dendronereis* spp.. Both the shrimp and polychaete were confined in the same pond for a long period of time, facilitating a close contact between infected shrimp and *Dendronereis* spp., and hence promoting the transmission of the virus. It is possible that the infection bounced back and forth over time between these cohabiting animal species. Considering that the number of WSSV hosts other than penaeid shrimp is large (Desrina *et al.*, submitted) and the traditional pond is a semi-closed water system, it will be interesting to study to what extent pathogen spill-over occurs in the traditional pond environment and what the epidemiological relevance of the *Dendronereis* spp. and shrimp interaction is with regard to occurrence of white spot disease. The similarity between the WSSV *vp28* gene sequences suggest that the viruses are similar, but further conclusions should not be drawn, as this would either require the comparative analysis of a less conserved gene or deep sequencing and population structure determination.

In recent study we detected WSSV-infected cells by immunohistochemistry in the intestine and stomach of naturally infected *Dendronereis* spp. (Desrina et al. 2013). WSSV was now also detected in gills, hemolymph, head and body containing digestive tract suggesting that WSSV travelled beyond the site of entry. The presence of WSSV in the hemolymph suggests that the virus circulated in the body cavity of *Dendronereis* spp., which is an essential step in the systemic infection of this polychaete. Our findings thus support the hypothesis that *Dendronereis* spp. are replicative host of WSSV and not merely a passive vector as reported in *Marphyssa* sp by Vijayan et al. (2005), and can be a vector of WSSV.

Oral transmission is the natural way of WSSV and the most important route of transmission in ponds (Lotz and Soto 2002). In our study, WSSV was transmitted from naturally infected *Dendronereis* spp. to healthy SPF juvenile *L. vannamei* through feeding, causing light infection without mortality, but the infection persisted until the end of observation (18 dpi). Since *L. vannamei* is susceptible to WSSV (Escobedo-Bonilla et al. 2007; Pérez et al. 2005), this finding suggests that WSSV in *Dendronereis* spp. was viable and retained its infectivity but did not cause a full-blown infection. The virus might have been attenuated while replicating in the *Dendronereis* spp., its pathogenicity may have altered as a consequence of replication in an alternate host or the shrimp defence allowed accommodation of WSSV (Flegel 2007). As a result the shrimp was able to overcome the WSSV isolate originated from *Dendronereis* spp., so that the number of shrimp with WSSV detected was reduced by about half within a week.

Pathogenicity variation of WSSV isolates obtained across host species (Waikhom et al. 2006), and even from shrimp from the same country (John et al. 2010) and across–countries (Laramore et al. 2009; Rahman et al. 2008; Zwart et al. 2010) was reported. However, attempts to reveal the WSSV pathogenicity alteration gave divergent results. Susceptibility of shrimp and crabs to WSSV

infection varies and is species specific (Wang et al. 1998), which may have contributed to the variation in the outcome of pathogenicity studies. Passage through different shrimp and crabs species altered the pathogenicity of WSSV to various degrees and induced genomic variation in ORF 94 (Waikhom et al. 2006). Difference in virulence of WSSV may also be related with certain proteins produced by the shrimp, so that it may 'accommodate' the virus without causing the disease (Stalinraj et al. 2009). Surprisingly, the conspecific transmission experiment showed that the virulence of WSSV from *Dendronereis* spp. did not restore even after the WSSV passed once through the shrimp. It is possible that more than one passage is needed to revive the pathogenicity and the virulence of WSSV from *Dendronereis* spp. Further study at the WSSV genome level is needed to test this hypothesis.

The present study was conducted to provide support for the role of *Dendronereis* spp. in the transmission of WSSV to shrimp. We used naturally infected *Dendronereis* spp. to mimic nature. The degree of the infection may vary among individual polychaetes. The point prevalence for WSSV in Dendronereis was 90% (55% in the 1-step PCR positive and another 35 % in the nested-PCR). We are inclined to think that the 30% of acceptor shrimp not showing infection of WSSV by 2-step PCR in time series sampling may have been feeding on not or lightly infected animals. Another possibility is that these shrimps consumed less food because there is variation in food intake among animals.

Other experiments on WSSV transmission from polychaetes to shrimp by oral route gave conflicting results. Vijayan et al. (2005) reported that this virus was transmitted from *Marphysa* spp. to *P. monodon*. In contrast, Laoaroon et al. (2005) claimed that WSSV transmission from *Perinereis nuntia* (Savigny in Lamarck) failed to induce infection in *P. monodon*. It is possible that the ability of WSSV to retain its infectivity when passaging through polychaetes is species specific.

Dendronereis spp. lives in burrows up to 30 cm down in the mud (data not shown). In contrast to shrimp, the animal does not show gross signs of the disease, such as body coloration, sluggishness or white spots. It is not yet clear where and when WSSV entered the *Dendronereis* spp., but it is safe to assume that the *Dendronereis* spp. acquired the infection in the shrimp pond.

The current study provides insight in the possible role of the polychaete *Dendronereis* spp. in WSSV epidemiology in traditional shrimp ponds in Indonesia. *Dendronereis* spp. are reservoir host of WSSV in earthen shrimp pond, in which it retained its infectivity. The virus can be transmitted to shrimp through feeding. However, how important this animal is as the source of infection compared to other decapods in ponds and whether environmental conditions influence the transmission to shrimp, need further studies. It is very important to study the behaviour of WSSV in vectors and carriers especially on those that have a wide geographic distribution and can cause direct transmission to shrimp in the pond, such as *Dendronereis* spp. This knowledge will help us to have a better understanding of the epidemiology of the virus and find an eco-friendly method to control the disease.

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CHAPTER 6.

WHITE SPOT SYNDROME VIRUS INFECTION IN THE POLYCHAETE *Dendronereis* spp. IN SHRIMP PONDS

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ABSTRACT

Extensively managed shrimp ponds in East Kalimantan and Central Java in Indonesia were sampled for the presence of white spot syndrome virus (WSSV) infection in nereid polychaete, *Dendronereis* spp.. In total, 5 ponds in Kalimantan and 8 in Java were sampled. All ponds experienced white spot disease between 1 month and 1 year before sampling. Each pond was sampled at 9 or 12 uniformly spaced locations; per sampling location the polychaetes were checked for WSSV with nested PCR. In addition, pH, total N, P and organic carbon concentrations, bulk density and the percentages of clay, sand and silt in the mineral fraction of the sediment were determined. On average, ponds with WSSV infected *Dendronereis* spp. showed a point prevalence of 44 ± 27% (± SD). The average prevalence in East Kalimantan was 73 ± 22% and in Java 26 ± 38%, with point prevalence in Central Java varying between 0 and 100%, explaining the large standard deviation. *Dendronereis* spp. density and C:N ratio were associated with WSSV infection in *Dendronereis* spp.. The other soil parameters had no effect on polychaete density or prevalence of WSSV in *Dendronereis* spp.. Prevalence of WSSV in shrimp was positively correlated with WSSV prevalence in the polychaete. We conclude that WSSV infection in *Dendronereis* spp. in shrimp ponds is common and that this polychaete can be a WSSV reservoir, which needs to be considered when designing disease prevention strategies.

Key words: WSSV, *Dendronereis* spp., infection, sediment, shrimp

Chapter 6

INTRODUCTION

White spot syndrome virus (WSSV) is an important shrimp pathogen inflicting severe production losses worldwide (Stentiford et al. 2012). WSSV was reported in numerous invertebrate organisms belonging to different taxonomic groups (Sánchez-Paz 2010; Stentiford et al. 2009). Mostly crustacean species were reported (Chen et al. 2000; Kanchanaphum et al. 1998; Marques et al. 2011; Supamattaya et al. 1998), although recently other benthic invertebrates, including molluscs (Chang et al. 2011) and polychaetes (Desrina et al. 2013) are getting attention.

In Asia losses due to white spot disease (WSD) declined somewhat after 2004 when WSSV specific pathogen free (SPF) larvae of *Litopenaeus vannamei* became commonly available as part of on-farm biosecurity measures (Cock et al. 2009; Flegel 2012). Unfortunately, in spite of stocking SPF larvae, disease outbreaks are still common because WSSV maintains itself transmitting between wild shrimp, crabs, benthos and plankton in ponds and adjacent surface waters (Walker et al. 2011a; Walker et al. 2011c). Stocking SPF larvae for WSSV only reduces one of many potential infection sources, but other routes of infection remain open.

In perennial ponds, sedimentation of suspended solids continuously adds new layers of sediment, with deeper and older layers being more compact than the newly formed flocculent layers at the water-sediment interface (Avnimelech and Ritvo 2003). In intensive ponds, C, N and P in the sediment originate mainly from feed and feces (Xia et al. 2004), while in extensive non-fed or additionally fed ponds they originate mainly from suspended organic matter in the incoming water or in situ primary production (Nhan et al. 2006). Shrimp scavenge for food at the bottom (Pontes et al. 2006), preferring oxygen rich and clean sediments (Avnimelech and Ritvo 2003). For instance, a low soil pH of 6-7 caused by a high

loading with organic matter reduced the osmotic pressure in hemolymph, and hence influenced shrimp wellbeing (Lemonnier et al. 2004). In fact, shrimp were shown to avoid anaerobic sediments rich in organic matter (Delgado et al. 2003). In contrast, burrowing polychaetes tolerate low oxygen conditions in organic sediments and re-oxidize reduced sediments while enhancing solute fluxes at the water-sediment interphase. The more persistent the oxygen depletion of the sediment the stronger the re-oxidizing feedback from burrowing will be, as long as a minimum oxygen influx prevents the system from collapse (Bartoli et al. 2009). As such, polychaetes are not only a natural food for shrimp (Nunes and Parsons 2000) but also contribute to nutrient cycling and the maintenance of a favorable pond culture environment.

Before Vijayan *et al*. (2005) reported polychaetes as a potential vector for WSSV, these animals were not considered to be involved in WSSV infection because they are taxonomically very distant from shrimp. They were considered to be mechanical vectors of the virus, but not natural hosts (Escobedo-Bonilla et al. 2008; Stentiford et al. 2009). However, Desrina et al. (2013) reported WSSV replication in *Dendronereis* spp. adding these polychaete species to the list of WSSV natural hosts. Furthermore, there was comparable prevalence of WSSV infection in *Dendronereis* spp. and shrimp *Penaeus monodon* in a traditional shrimp pond (Haryadi et al. 2014), suggesting a link of infection between these two classes of animals in ponds. In the present study, *Dendronereis* spp. were collected from extensively managed shrimp ponds which experienced production losses due to WSD between 1 year and 1 month before sampling. The goal of the current study was to (1) determine the extent of WSSV infection in the *Dendronereis* spp. in shrimp ponds in Indonesia; (2) verify whether there is a correlation between polychaete density and infection rate with pond parameters such as shrimp density and sediment condition. To this end, the prevalence of WSSV in *Dendronereis* spp.

and shrimp in these ponds was determined. Soil samples were also taken to check if the sediment conditions influenced the presence of *Dendronereis* spp. in the ponds and presence of WSSV in the polychaete.

MATERIAL AND METHODS

Study Sites

Polychaetes were collected from 13 ponds: 5 traditional ponds in the Mahakam delta, East Kalimantan (will be referrred as Kalimantan) and 8 in the vicinity of Semarang, Central Java (will be referred as Java). Information on pond management was obtained by interviewing farmers and included: pond age, pond size, pond preparation procedures, post larvae (PL) stocking density and stocking frequency, source and size of PLs, feed, pesticide use, water exchange and the number of production cycles per year. All farmers knew WSD clinical signs, especially white spots, reddish coloration and lethargy, but none had had WSSV infection confirmed through PCR.

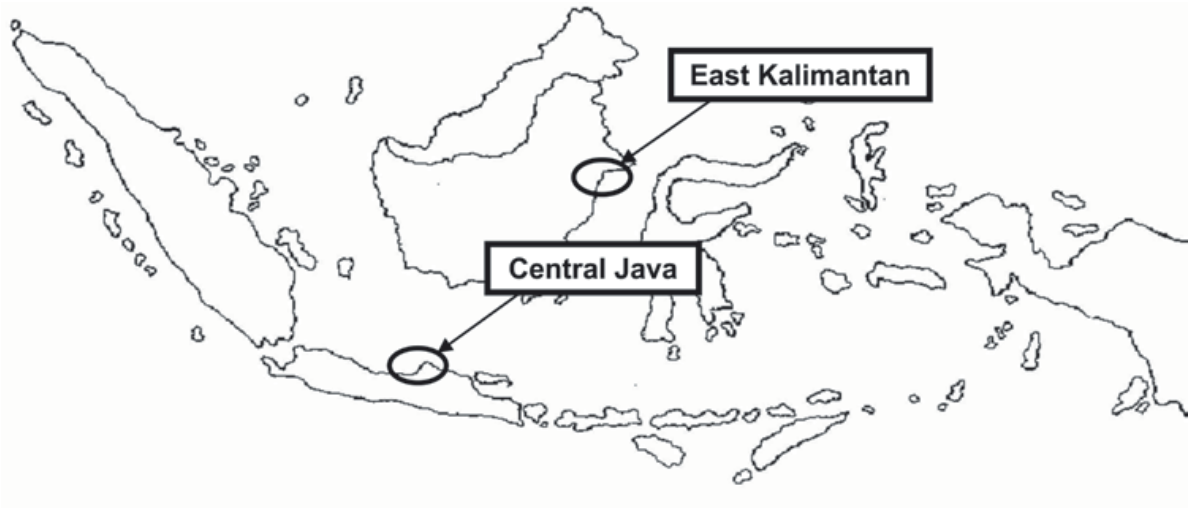

Figure 1. Study sites in Indonesia.

Polychaete collection

In Kalimantan, ponds were sampled at 12 locations along the peripheral ditch. In Java, ponds were sampled at 9 locations. The sample locations were equally spaced covering the whole peripheral channel in Kalimantan and the whole pond area in Java. At each sampling location, 3 sediment cores were collected with a 40-cm long PVC pipe with an inner diameter of 15.2 cm. The pipe was pushed into the mud, closed on the top with a PVC cap and then slowly removed from the mud. A depth of 40 cm was used, as most burrowing polychaetes remain within the top 20-30 cm of the sediment (Gowda et al. 2009). The 3 cores per sample location were pooled in a large plastic bucket. By adding pond water, the mud cores were weakened and then successively washed through a 2, 0.6 and 0.3 mm mesh sieve to collect all *Dendronereis* spp. individuals. This polychaete species belongs to the Nereidae family and individuals were easily recognizable by their red colored external gills. *Dendronereis* spp. individuals retained on the surface of each sieve were gently removed with a fine brush, counted, cleaned and stored. In all ponds, *Dendronereis* spp. were the most abundant polychaete species present. Per sample location, most of animals were stored in 96% ethanol for PCR, and the remaining animals in 70% ethanol. Damaged individuals were only counted when the head section was intact. The number of polychaetes collected per sample location was expressed as individuals per m^2.

Soil analysis

Sediment samples were taken at the same locations and time as the polychaetes and within a few cm from each core taken for polychaete collection. Each sediment sample was taken with a PVC pipe with an inner diameter of 5.1 cm, which was driven minimum 20 cm into the sediment. The top 5-6 cm was collected from one core and stored in a plastic bag, sealed and placed on ice in an

ice box, and later used to measure the bulk density. The two other cores were pooled, sealed in a plastic bag and transported to the laboratory on ice. Soil was dried in a convection oven at 40 ^{o}C until constant weight was achieved and then crumbled. Soil parameter measures included pH, total organic matter, total nitrogen and total phosphorous. The percentages clay, silt and sand were also determined. Soil pH was measured in a 1:1 mixture of dried soil and distilled water. Soil parameters were determined following Boyd (1979), unless stated otherwise. Soil organic matter was measured using the Walkley and Black method. Soil organic carbon was calculated assuming the carbon represents 50% of the organic matter. Soil total nitrogen was measured using the Kjeldahl method. Soil total phosphorous was determined by the ascorbic acid reduction method (Sletten and Bach 1961). The particle size distribution was determined according to Gee & Bauder (1986).

PCR analysis to detect WSSV in *Dendronereis* spp. and shrimp

Genomic DNA was extracted from tissue of the head area, defined as body segments 1 to 20, applying a modified protocol according to Dixit (1998). Briefly, 30 mg of minced *Dendronereis* spp. tissue was mixed with 300 µl lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM ß-mercapthoethanol, 20% SDS, Proteinase K 0.5 mg/ml) and incubated at 55 °C for 2 h. The DNA was precipitated by adding 300 µl 5M potassium acetate, purified with a mixture of phenol:chloroform:isoamyl alcohol (1:1:1) and washed in cold isopropanol followed by 70% ethanol to remove residual phenol and salt. The DNA pellet was dissolved in sterile TE buffer and stored at -20 °C until used. The presence of WSSV DNA was detected by 2-step nested-PCR using a WSSV Detection Kit (PT Nugen Bioscience Indonesia, Jakarta, Indonesia) following the manufacturer's protocol. For first-step PCR, 2 µl of tested DNA, along with negative (DNA replaced with sterile nuclease free water) and positive (DNA from WSSV infected

shrimp) control was used and amplified (15 cycles) with the Gene Amp R PCR System 2720 (Applied Biosystem). Subsequently, 2 µl of product of first step PCR was used in second step PCR (40 cycles). The manufacturer's guidelines were followed: 5 µl of second step product was loaded in to 1.5% agarose gel and visualized under UV light. Presence of a band at 250 bp indicated positive for WSSV. WSSV detection in shrimp followed protocol according to Haryadi et al. (2014). Point Prevalence of WSSV infection in *Dendronereis* spp. was calculated according to Cameron (2002).

Statistical analysis

Soil properties were compared between extensive ponds in the Kalimantan and Java, using principal component analysis (PCA) based on Euclidian distances and analysis of similarity (ANOSIM). The parameters explaining the highest percentage of variation in the data set were identified. Association between soil parameters and *Dendronereis* spp. density with WSSV presence in polychaete was analyzed with binary logistic regression. Pearson correlation was used to evaluate the relation between WSSV infection in *Dendronereis* and shrimp. Depending on the analysis, the statistical analysis packages Primer-Permanova or SPSS were used.

RESULTS

All farmers indicated they suffered losses due to WSD during the last year before sampling. Farmers stocked shrimp post-larvae irregularly, depending on cash availability and expectations of possible losses due to disease. All farmers practiced some degree of pest control before stocking PLs. Each farmer seemed to follow his own protocol. The most commonly applied pesticides before stocking were Brestan-60 (tri-phenyl tin acetate) to reduce snail numbers and saponin (by-

product of *Camillia* sp. oil extraction) to eradicate predatory fishes. Information on sampled ponds is summarized in Table 1, with additional information given below.

Traditional ponds in the Mahakam Delta, Kalimantan

In the early 1990's the mangrove - nipah palm area of the Mahakam Delta was converted into aquaculture ponds. At the moment of sampling, ponds were 11 to 15 years old. Soil to raise pond dikes was obtained by digging a 10-20 m wide ditch along the pond perimeter. The sediment in the pond center remained undisturbed. Some farmers removed the vegetation, others left it untouched. Each pond got one sluice gate serving as water inlet and outlet. Through a network of channels all ponds in the area were connected. A depth of 0.2 – 0.5 m was maintained in the pond center; in the peripheral ditch the depth fluctuated between 0.6 – 1.0 m.

The difference between high and low tide fluctuated with the lunar cycle between 1.0 and 1.5 m and was used to exchange water. Wild shrimp, crab and fish can enter the pond with incoming water, and nets were used to trap animals from the outgoing water. Besides relying on natural recruitment, some farmers stocked *P. monodon* PLs once or twice annually, at densities of 0.11 – 1.33 individuals m^{-2}. Farmers bought healthy looking post-larvae, but not SPF animals. When shrimp cultures failed due to WSD, farmers often opted to postpone stocking for months switching for the time being to collecting shrimp, crab and fish that entered with tidal water and subsequently grew these animals in the pond. They were mainly milkfish, tilapia and mullets which then contributed to production.

Traditional ponds in Semarang area, Java

This pond area was developed in the 1930's. After 1985, in most locations, intensive or semi-intensive shrimp farming was practiced at one moment (Hariati et al. 1995), but due to disease problems, most farms were converted to extensive operations with the aim to minimize capital costs and financial risks. The original ponds were 4-15 ha large, but smaller units of 0.4 – 1.0 ha were developed for intensive farming. Many of these smaller ponds are still in use today. When stocking, farmers preferred to stock *P. monodon* at a density of 0.5 – 5.0 individuals m^{-2}, considering its high market price. Weekly collection of 25-30 g individuals started two months after stocking until no shrimp were left. Nevertheless, recently, some farmers stocked 16-20 *L. vannamei* PLs m^{-2}, aiming to compensate lower market prices with higher production volumes. Ponds were slightly deeper than in Kalimantan with an average depth of 0.8 – 1.1 m during culture. Some farmers practiced a form of crop rotation, switching between shrimp and finfish in monoculture or polyculture.

Sediment properties

There was a high variation in individual soil parameters between ponds (Figure 1). The soil properties in the Kalimantan and Java locations were different (ANOSIM, $R = 0.679$, $P < 0.001$), with soils in Kalimantan having a higher fraction of silt and sand, being richer in total phosphorous and having a lower pH and bulk density than ponds in Java. Total nitrogen, C:N ratio and organic matter content were not different between Kalimantan and Java, but were highly variable, especially for ponds in Java (Figure 1).

Table 1. Farming information of sampled ponds.

Pond #	Stocking density (#/m^{-2})	Size (ha)	Sediment removal	Shrimp harvest (kg ha^{-1} crop^{-1})	Months since WSD outbreak	Farming information
Kalimantan						
1	0.36	3	Irregularly, last time 5 years ago	33	3	Pond not restocked since last WSD outbreak. Harvesting natural recruits.
2	0.25	8	Never	?	6	Stocked 2 times per year. Continuous harvesting, including natural recruits of shrimp and milkfish. No supplemental feeding.
3	1.33	6	Never	16-20	2	Previous crop was harvested after 2 months due to WSD. Immediately stocked new PLs. No supplemental feeding.
4	0.16	4	Never	?	1	Lost previous crop within one month after stocking due to WSD. Pond restocked 2 days before sampling.
5	-none-	6	Never	16	1	Not stocked for last 4 years., Wild shrimp, crab and milkfish harvest.
Java						
6	1.0	0.8	Annually, put on dike	150	2	Regularly observed WSD, never mass mortality. Stocks PLs regularly, weekly partial harvest.
7	5.0	0.4	Annually, put on dike	350	6	Originally intensive *P. monodon*, later semi-intensive *L. vannamei*, present extensive *P. monodon*.
8	5.0	1.0	Annually, put on dike	50-80	6	Extensive polyculture with milkfish. Some diseased animals present, but does not consider it a problem.
9	4.0	0.4	Annually, put on dike	100-120	4	Extensive polyculture with milkfish. Some diseased animals present,, but does not consider it a problem.
10*	20.0	0.5	Annually, put on dike	500	6	Stocked *L. vannamei* in monoculture. Practiced crop rotation and polyculture with milkfish or tilapia. Before, the pond belonged to an intensive shrimp farm.
11	1.5	1.0	Annually, put on dike	80	6	Stocked also 0.5 milkfish m^{-2} besides shrimp.

12	3.0	0.8	Annually, put on dike	40-100	12	Stocked 0.8 milkfish m^{-2}, which should yield 300 kg ha^{-1}. Fish irregularly fed. Prefers polyculture, because shrimp monoculture often fails. WSD is present, but with no major impact on production.
13	16	0.7	Annually, put on dike	500	12	Stocked *L. vannamei* and 1 tilapia m^{-1}, with the latter aiming for 1500 kg ha^{-1}. Fish irregularly fed. Previous crop failed due to WSD.

* Farmers stocked *Litopenaeus vannamei*. All other farms stocked *Penaeus monodon*

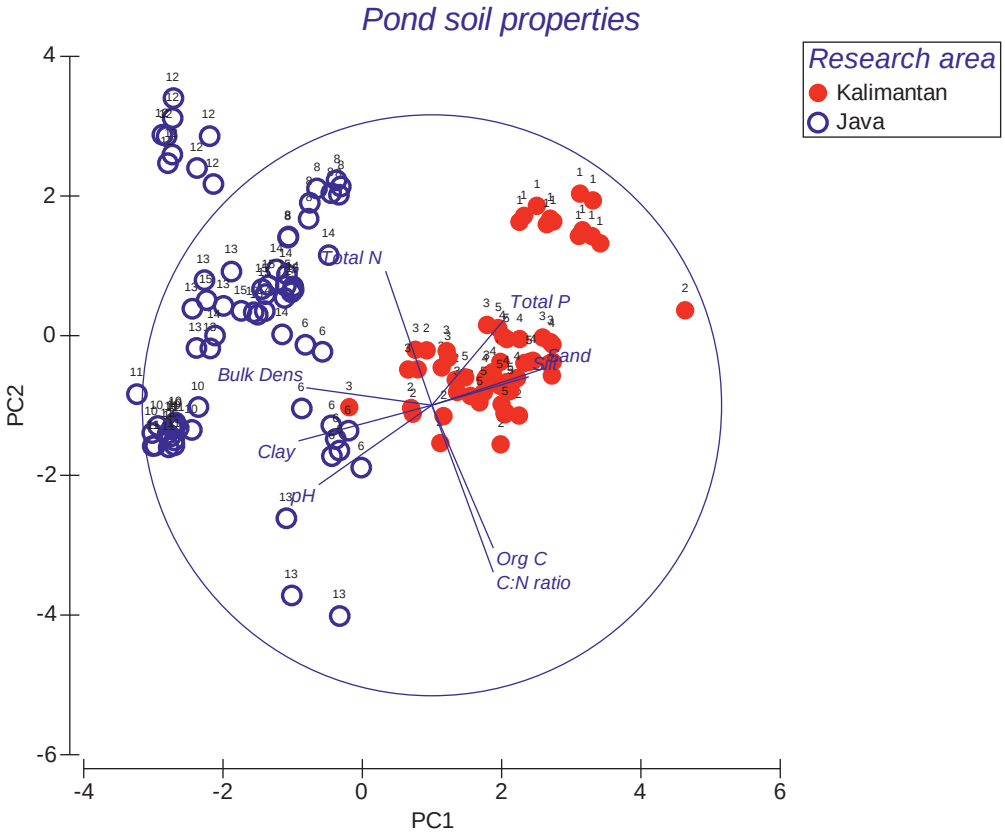

Figure 2. Principal component diagram of soil parameters in research areas in Kalimantan and Java. Numbers in figure relate to pond number (12 sampling sites per pond in Kalimantan, 9 sites per pond in Java). Vectors showing pH, Total N, Total P, Org(anic) C, bulk dens(ity), and sand, silt and clay fractions. Principal Coordinate (PC) axis 1 explained 47.3 % of variation; PC2 explained 21.7 % of variation.

WSSV prevalence in *Dendronereis* spp. in ponds

Dendronereis spp. were present in all ponds and WSSV infected individuals were present in all ponds in Kalimantan and in 4 of the 8 ponds in Java. Figure 3 is a typical example of a pond analysis indicating the presence (+) or absence (-) of WSSV in polychaete or shrimp samples. The 4 ponds with uninfected *Dendronereis* spp. experienced the last WSSV infection of shrimp more than a half year before sampling, after which no PLs were restocked. On average, ponds with WSSV infected *Dendronereis* showed point prevalence 44 ± 27% (± SD). The average prevalence in Kalimantan was 73 ± 22% and in Java 26 ± 38%, with point prevalence's per pond in Java varying between 0 and 100%, rationalising the large standard deviation.

Association between WSSV infection in *Dendronereis* spp., polychaete density and sediment condition.

Dendronereis spp. density in shrimp ponds ranged from 13 ± 12 to 2517 ± 908 individuals m^{-2} (± SD). Soil parameters were not significantly associated (Pearson correlation, $P > 0.05$) with *Dendronereis* spp. density, except for a weak positive correlation to C:N ratio (r^2 0.20, $P = 0.014$, N=150). However, WSSV prevalence in *Dendronereis* spp. was positively correlated with polychaete density (Pearson correlation 0.51, $P = 0.000$, N=150) and confirmed by binary logistic regression (P=0.001, odd ratio =1.006). Prevalence of WSSV in shrimp and in polychaetes were positively correlated at pond level (Pearson correlation 0.78, $P = 0.003$, $N = 12$), but this was not the case at individual sample sites level (logistic regression, $P > 0.05$).

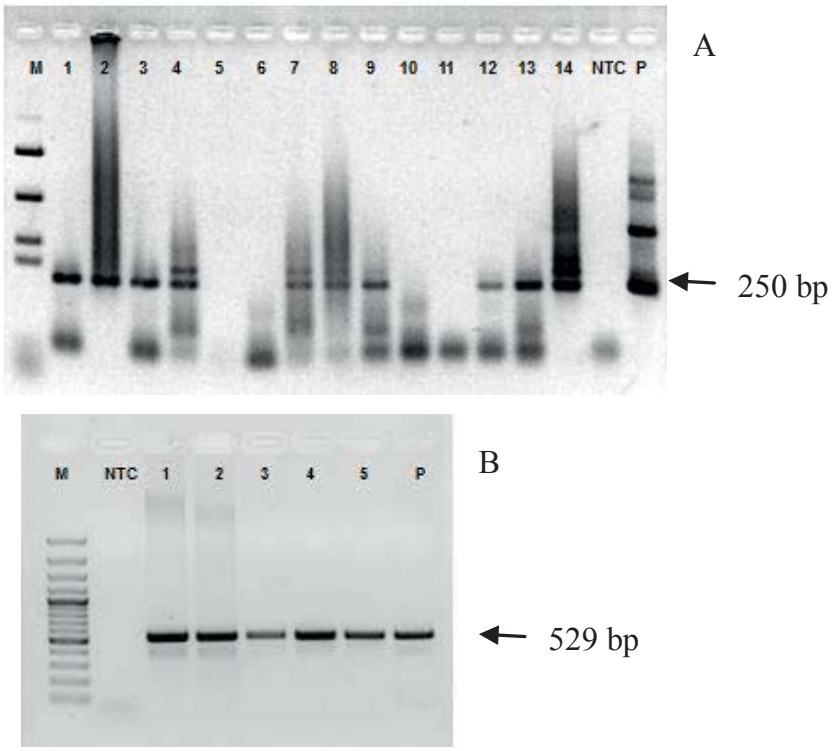

Figure 3.(A) Result of nested PCR on *Dendronereis* spp. and (B) result of 1-step PCR on the shrimp P. *monodon* from selected study ponds. M= marker (DNA ladder); NTC= No template control (negative control), P= Positive controlof PCR. Lane 1- 14 (panel A) and lane 1-5 (panel B) samples of *Dendronereis* spp and *P. monodon* specimens, respectively.

DISCUSSION

WSSV-infected *Dendronereis* spp. were found in all ponds where WSD had occurred less than six months prior to sampling. The sampling locations in Java and Kalimantan are more than 1000 km apart and separated by the Java Sea. Although the number of ponds sampled was small, the high point prevalences in both areas indicate that the occurrence of WSSV in *Dendronereis* spp. is common. The fact that none of the extensive ponds was drained completely since WSD occurred might have contributed to the local survival of the virus (Satheesh Kumar et al. 2013) which possibly circulated between carriers present in the pond (Hoa et al. 2011b).

In four ponds in Java with a WSD history there was no WSSV-infected *Dendronereis* spp. present. (Tendencia et al. 2013) found improved water quality, a higher redox potential in the sediment and lower luminous bacteria counts in greenwater rearing systems, correlated with reduced occurrence of WSD in shrimp. In greenwater systems the shrimp culture unit receives water from a fish pond or contains fish cages. In our case, these four ponds were alternately used for shrimp and monoculture milkfish production in the months before sampling. Few shrimp might have been present, while excessive plankton blooms, which could also be potential source of WSSV infection (Esparza-Leal et al., 2009; Walker et al., 2011b),. were controlled through fish grazing. Using logistic regression to identify risk factors for shrimp disease, (Leung et al. 2000) concluded that polyculture reduced the risk of disease occurrence in extensive and semi-intensive ponds. In all the system mentioned above, shrimp which are the main infection source were present at low density, while in addition co-cultured fish graze or prey on potential carriers such as plankton and polychaetes or filter away circulating viruses. As a result, the virus concentration in the pond is reduced.

Dendronereis spp. are ubiquitous macroinfauna of mangrove and other coastal soft sediment environments in the tropics. For instance, *D. arborifera* (Peters 1854) was reported in ponds in Africa (Ngqulana et al. 2010) and India (Gowda et al. 2009; Watts et al. 2013). Since most shrimp ponds are built in the intertidal zone, it is not surprising that these animals are abundant in these ponds where they are natural food for shrimp. In our study, *Dendronereis* spp. were the most abundant polychaete species, and often the only polychaete species present, especially in Java. The ubiquitous distribution might be related to tolerance to a wide salinity range (Roy and Nandi 2012), high organic content (Gowda et al. 2009) and pollution (Watts et al. 2013). Extensively managed ponds receive large quantities of suspended solids with tidal water exchange and act as nutrient traps

(Nhan et al. 2008). High loading with organic matter creates a low oxygen environment to which *Dendronereis* spp. are among a few species that can adapt (Bartoli et al. 2009).

The observed prevalence of WSSV infection in *Dendronereis* spp. in some ponds in this study was higher than previously reported by Vijayan et al. (2005) on *Marphysa* spp. in India or by Laoaroon et al. (2005) on *Perinereis nuntia* (Savigny in Lamarck, 1818) in Thailand. The fact that in this study *Dendronereis* spp. were collected from previously infected ponds, might have contributed to the higher WSSV prevalence. However, the prevalence of WSSV in *Dendronereis* spp. was within the range reported on naturally infected wild crabs in Taiwan (Lo and Kou 1998; Wang et al. 1998), Thailand (Chen et al. 2000), China (Liu et al. 2011b) and Brazil (Marques et al. 2011). This indicates that polychaetes, either as carrier or replicative host for WSSV in ponds, might be as important as vectors as crabs.

WSSV infection rate in *Dendronereis* spp. was positively correlated to polychaete density, and to a lesser extent to C:N ratio in the sediment. Shrimp density is an important factor in the occurrence of WSSV in ponds (Owens 2011; Soto and Lotz 2001; Tendencia et al. 2011) because shrimp-to-shrimp transmission dramatically increases the virus load. If this is also the case with polychaete-to-polychaete transmission still needs to be elucidated, as it is not certain the same mechanisms are involved. A high C:N ratio was associated with increased bacterial biomass and enriched benthic communities in sediments (Asaduzzaman et al. 2008). (Doan et al. (2014)) reported that organic matter load affects the total virus count in the soil by enhancing virus adsorption to soil particles. Hence, a higher C:N ratio might have influenced WSSV occurrence in *Dendronereis* spp. by enhancing WSSV presence in the sediments in which the polychaete lives.

We found a positive correlation between WSSV prevalence in *Dendronereis* spp. and prevalence in the shrimp at pond level, but could not confirm this when

comparing individual sampling sites. Despite the relatively small sample size it is clear that the individual sampling sites showed a high degree of variation in this analysis. None of the ponds had been dried in the year before sampling. In water-logged soils, WSSV remained viable for 35 days (Satheesh Kumar et al. 2013) or remain detectable up to 10 months in sediment at room temperature (Natividad et al. 2008) and up to 20 months in ponds after disease outbreaks (Quang et al. 2009), providing an ample time window to *Dendronereis* spp. to acquire the virus through ingestion (Vijayan et al. 2005) and propagate it (Desrina et al. 2013). In turn, the virus might re-infect shrimps predating or scavenging on polychaetes (Haryadi et al. 2014). However, more research is necessary as the time needed for WSSV to proliferate upon infection to detectable levels in *Dendronereis* spp. remains unknown. *Dendronereis* spp. are unique among WSSV carriers in the pond. They make the entire sediment up to a depth of 20-40 cm a possible infection source of WSSV, where the virus can remain present for a long time in the polychaete.

In conclusion, *Dendronereis* spp. commonly present in shrimp ponds in Kalimantan and Java and its prevalence was not dependent on soil conditions, except slightly for the C:N ratio. Polychaete density was positively-linked to WSSV prevalence in *Dendronereis.* At the pond level, there was a positive correlation between WSSV prevalence in shrimp and polychaete, and a broader study, involving more ponds and sampling over time will be needed to get a better understanding of factors influencing WSSV infection in this polychaete.

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CHAPTER 7.
GENERAL DISCUSSION

THESIS FINDINGS

White spot syndrome virus (WSSV) was unknown prior to its emergence in the early 1990s when it started to cause heavy losses in the penaeid shrimp industry. WSSV is a 'multihost'-virus (Chou et al. 1998; Flegel 2006; Sánchez-Paz 2010) infecting a range of invertebrates living in ponds and estuarine environments. About 50% of reported WSSV hosts are crabs (Chapter 2), which are considered a non-penaeid crustacean reservoir for the virus. Improving pond management and increasing biosecurity reduced white spot disease (WSD) incidence, but did not eradicate it. WSSV continues plaguing the major shrimp culture producing countries in Asia (NACA-FAO 2014) and WSD outbreaks re-occur even after fallowing and strict implementation of sanitation measures. The question remains whether the stocked shrimp receives the pathogen from an outside source or from within the pond environment. In the latter case the question is how WSSV maintains its persistence in ponds. Experience with human pathogens showed that multihost-pathogens are difficult to control considering complexities and dynamics of the underlying host-to-host interactions and the virus genetic plasticity (Woolhouse et al. 2001). Getting better insight in the host-to-host interactions of WSSV in the pond environment becomes a priority to effectively contain WSD. Identifying all possible reservoir host species for WSSV is an important aspect of this effort.

Recognizing the persistence of WSSV, this study started with the assumption that some WSSV reservoir host-species are always present, even in fallowed or

derelict ponds. This comes from the observation that even when ponds are stocked with SPF animals, WSD frequently occur. These pond species live in close contact with shrimps and are able to escape the various, often chemical, decontamination protocols. This thesis focused on *Dendronereis* spp., Nereid polychaete commonly and ubiquitously present in shrimp ponds in Indonesia. The objective was to study the potential role of *Dendronereis* spp. in the ecology and transmission of WSSV in shrimp ponds, focusing on the following research questions:

1. Does WSSV occur in *Dendronereis* spp. in shrimp ponds in Indonesia, and are WSSV-infected *Dendronereis* spp. indeed commonly present?
2. Does WSSV replicate in *Dendronereis* spp.?
3. Can *Hediste diversicolor* be used as a model animal to study WSSV infection in polychaetes?
4. Can WSSV be transmitted from *Dendronereis* spp. to shrimp?
5. Are there pond factors related to the presence of WSSV in *Dendronereis* spp.?

Thesis findings showed that WSSV is a generalist virus and that *Dendronereis* spp. can be non-crustacean reservoir host of WSSV in shrimp ponds. Although the attempt to use another nereid species as model polychaete for WSSV infection did not yield the expected outcome, the findings suggested that the route of infection in a non-crustacean host might be different from that in crustaceans. Considering this is a first attempt to study the role of polychaetes in WSSV ecology in shrimp ponds, the implications of the current findings are discussed, knowledge gaps are identified and suggestions to move forward are given.

WSSV infects the Nereid polychaete *Dendronereis* spp. in shrimp ponds

The thesis results showed that WSSV infection in *Dendronereis* spp. is widespread in the shrimp ponds in Kalimantan and Central Java. It is proposed that WSSV infection in this polychaete is linked to its detritivorous and herbivorous feeding habits (Watts et al. 2013) . WSSV enters *Dendronereis* spp. by ingesting 'infectious' sediment. Sediment was visible in a dissection microscope in the digestive tract of freshly caught *Dendronereis* spp. put in sterile PBS or cleaned in sea water. Although WSSV presence in the soil and water was not checked in this study, it is known that WSSV DNA can be present for a long time in pond soil (Natividad et al. 2008) and water (Quang et al. 2009), and could remain infectious to shrimp up to 35 days in pond sediments (Satheesh Kumar et al. 2013). Considering the history of WSSV infection in the study ponds, we assumed WSSV was present in the sediment. Because *Dendronereis* spp. has a broad geographical distribution (Gowda et al. 2009; Ngqulana et al. 2010; Pillai 1965) overlapping with the current study areas in Kalimantan and Java, findings in this thesis are of general interest for polychaete ecology and shrimp farming alike.

Host-pathogen encounter is the most important factor for cross host-infection of multihost-pathogens (Parrish et al. 2008; Rigaud et al. 2010; Woolhouse 2002). Mangrove and estuarine areas are rich in macrobenthic invertebrate species (Nordhaus et al. 2009). Accordingly, in locations where WSSV is endemic, benthic invertebrates such as gastropods, bivalves, amphipods, decapods and benthic phyto-periphyton mats associated organisms (locally named 'klekap' or 'lab lab') are potential WSSV hosts or vectors because they are continuously exposed to WSSV contaminated pond water and sediment. Bivalves and polychaetes are more exposed to WSSV because (1) they are confined in the pond where they are exposed continuously to WSSV containing sediment, and (2) their filter and detritus feeding might also include WSSV-infected particulate material.

Considering, WSSV can be attached to phytoplankton (Jiang 2012; Liu et al. 2007), benthic algae could also be vectors transferring WSSV infection to *Dendronereis* spp.

Opportunities for host-virus encounters in semi-intensive and intensive ponds are different from extensive ponds. Under more intensive farming conditions, the biodiversity and abundance of benthic invertebrates can remain low due to constant cleaning of the pond bottom. However, a high shrimp density provides ideal conditions for WSSV to thrive (Cock et al. 2009), resulting in a high viral load which expose biotic as well as abiotic elements in the pond ecosystem to the virus. In contrast, in extensive ponds, the benthic community can be more diverse, harboring more potential host and vector species that can contribute to WSD outbreaks. Repeated exposures of WSSV among different host species through complex interactions may induce genetic adaptations in the virus or alterations of the genetic population structure of the WSSV community, both of which potentially might result in successful transmission to a new host species. An example was the repeated transmission of a Simian retrovirus from non-human primates to humans which ultimately caused the emergence of HIV (Tebit and Arts 2011).

WSSV replication in *Dendronereis* spp.

WSSV replicated in naturally infected *Dendronereis* spp. (Desrina et al. 2013), making the existing view that polychaetes are only a mechanical vector no longer valid and changing *Dendronereis* spp. into the first reported non-crustacean WSSV host species (chapter 2). Overall, result of IHC, PCR and RT-PCR indicated that WSSV caused a persistent low infection in *Dendronereis* spp. In a multihost-pathogen setting, each host species can have a different susceptibility to infection and might apply different mechanisms to evade virus infection and transmission.

Similar to herpes virus infection in humans (Modrow et al. 2013), the low level of infection observed in *Dendronereis* spp. could reflect WSSV persistence in the aquatic environment. The fact that *Dendronereis* spp. individuals used in WSSV replication (chapter 3) and transmission studies (chapter 5) were collected at the same extensive shrimp farm with a two year interval in between, indicated continuous presence of WSSV–infected *Dendronereis* spp. This supports the assumption that WSSV replicated and maintained itself in the polychaete in the pond over a longer period of time. Checking *Dendronereis* spp. over time with IHC and RT-PCR, however remains necessary to confirm this assumption.

Viral infection starts with virus-host encounter, followed by attachment of viral attachment protein to receptor protein on the surface of host cells, penetration, uncoating and transfer to the nucleus. Once inside the nucleus, morphogenesis depends on success of transcription of viral RNA and the subsequent assemblage of virions (Modrow et al. 2013). Multihost-viruses often have properties facilitating cross-host infection including a high genetic variability such as is the case with the influenza virus (Baigent and McCauley 2003). High genetic variation of WSSV (Hoa et al. 2011a; Walker et al. 2011a; Wongteerasupaya et al. 2003) may enable WSSV to infect an array of host species, including polychaetes. Moreover, multi-genotype WSSV infection has been reported in shrimp collected from culture ponds (Hoa et al. 2011b). Whether the WSSV genome varies and multi-genotype infection is possible in *Dendronereis* spp. requires further research. For this, extensive deep-sequencing would be required to see if the WSSV is the same or that the population structure is different. This is a challenging task as the level of WSSV in an individual polychaete is quite low. The combination of repeated passage through a multihost-community and multi-genotype infection in one specific host could facilitate expansion of the host range (Rigaud et al. 2010). In addition, the connectivity between ponds, channels and estuaries in coastal areas

and the intensive trading of live and frozen shrimps, enlarges possibilities for the emergence of new WSSV host species.

Despite the phylogenetic distance between decapods and polychaetes, WSSV replicated in the nucleus of stomach and foregut cells of *Dendronereis* spp. (Chapter 3), shrimps (Durand et al. 1997) and crabs (Chen et al. 2000; Supamattaya et al. 1998). Similar to shrimps and crabs, the gut tissue in polychaetes is also of ectodermal and mesodermal origin (Tzetlin and Purschke 2005). WSSV may have *per os* infectivity factors (PIFs) enabling the virus to bind to the protein in the gut tissue in both *Dendronereis* spp. and shrimp upon entry. PIFs have been described in other large enveloped DNA viruses of invertebrates, such as baculoviruses (Peng et al. 2011), hytrosaviruses (Abd-Alla et al. 2008) and nudiviruses (Wang and Jehle (2009)). Although baculoviruses, hytrosaviruses and nimaviruses (WSSV) are evolutionarily very distinct, this suggests that oral infectivity using PIFs is an evolutionarily conserved mechanism of virus entry operating in a wide range of terrestrial and aquatic invertebrates. Furthermore, the WSSV surface proteins were recognized by receptors on the *Artemia* cell membrane and shrimp gill tissue (Feng et al. 2013). Another possibility is that several WSSV binding proteins exist with one of them being specific for polychaetes gut tissue. Whether similar binding proteins are involved in WSSV infections in crustaceans and polychaetes needs further investigation.

In search of a model species to study WSSV infection in polychaetes.

The commonly used methods to infect shrimp were not effective with the Nereid polychaete *H.diversicolor*. The possibility that other routes of infection with this polychaete are more effective cannot be excluded, hence alternative routes should be explored. For example, direct contact with WSSV through immersion did not induce infection in *Artemia* (Hameed et al. 2002), but exposure to a virus-

phytoplankton-rotifer complex did (Jiang 2012). In retrospect, it might require virus-sediment attachment to induce WSSV infection in polychaetes (Vijayan et al. 2005).

Other possible explanations are that *H. diversicolor* is not susceptible to WSSV, that the specimens used were not of a size vulnerable to infection, or that the exposure time was too short. In ponds, *Dendronereis* spp. is continuously exposed to WSSV during all life stages. Studying WSSV in non-crustacean species requires researchers to broaden the 'shrimp centris' concept, considering that a multihost pathogen uses host specific strategies, especially when phylogenetically distant species are involved (Rigaud et al. 2010). More research on possible infection routes with a broad range of species is needed.

For the work on this thesis a reliable model species to study WSSV infection in polychaetes would have been highly desirable. Due to lack of a polychaete model, naturally infected *Dendronereis* spp. was used, limiting the option to include a non-infected-polychaete control treatment in experiments (chapter 5). Considering the preliminary experiment with *H.diversicolor* did not give satisfying result, additional routes of infection such as via sediment, should be tested before abandoning it as model species. Concurrently, endemic polychaete species such as *Perinereis nuntia,* which can be cultured in captivity (Poltana et al. 2007), can also be evaluated as model species. This would increase flexibility to further study WSSV infection in polychaetes.

WSSV transmission from *Dendronereis* spp. to penaeid shrimp

WSSV can be transmitted from naturally infected *Dendronereis* spp. to healthy shrimp and further to naive shrimp, causing light infection (chapter 5) (Haryadi et al. 2014).Combined with the fact that WSSV replicated in *Dendronereis* spp. (chapter 3), it indicates this polychaete can be a productive

reservoir host species (active carrier) in ponds. Crabs are another group of reservoir and active carrier species without a high degree of mortality. However, whereas *Dendronereis* spp. showed a low level of infection (chapter 3, 5 and 6), infection in crabs can be very heavy (Chen et al. 2000; Supamattaya et al. 1998). In addition, crabs are free-roaming and even semi-terrestrial (Le Vay et al. 2007; Qureshi and Saher 2012) while polychaetes are benthic, more confined inside the pond. As a consequence, crabs are more important in spreading WSSV between ponds while polychaetes are mainly important for transmission within the pond. *Dendronereis* spp. will remain present in the pond, even after shrimp culture was stopped, and will survive adverse conditions by retreating in burrows in the mud. In addition, the polychaetes facilitate spreading of WSSV to other benthic animals by shedding the virus through feces in the sediment. As such, polychaetes may maintain a WSSV reservoir in the pond, even long after abandoning shrimp culture. This could be tested by analysis of polychaetes shrimp ponds restocked with fish.

The arbitrariness by which WSSV infection doses are assigned to experiments reported in literature make it necessary to interpret results cautiously. Many transmission studies applied a heavy infection dose which tested positive with 1-step PCR. Under such conditions, mortality and observed infections in the potential host might be due primarily to the high dose, telling us less about susceptibility, let alone replication. In consequence, using naturally infected animals with a lower infection level is recommended, as it mimics better the conditions under which WSD affects shrimp culture ponds. A better marker for showing a productive infection is the presence of messenger RNA for the major late envelope protein VP28 in polychaete guts (chapter 3).

Multihost pathogens were commonly described in terrestrial animals, of which 60 % were zoonoses (Woolhouse et al. 2001). Several viruses of terrestrial animals such flaviviruses (Gale and Johnson 2014) and parvoviruses (Allison and

Parrish 2014) are known to infect cross-phylum hosts. However, it is quite rare for a single virus species to infect such a wide range of aquatic invertebrate hosts species as is the case for WSSV. Many studies dealt with bi-directional transmission between shrimp and the suspected vector like in chapter 5 in this thesis, using a simple experimental design. However, in ponds WSSV spreads through a broad array (figure 1) of host species interacting at numerous levels (Walker et al. 2011a; Walker et al. 2011b; Walker et al. 2011c), and is also influenced by environmental factors (Tendencia et al. 2011). Transmission of WSSV through the pond food web may affect shrimps and other decapod hosts which occupy overlapping feeding niches alike. A macrocosm study of WSSV prevalence involving species with (partially) overlapping feeding niches might provide insight in the population dynamics of WSSV in cultured ponds. This may link to differences in WSSV virulence between host species, as interspecies transmission is the most important factor in the evolution of virulence of multihost pathogens (Rigaud et al. 2010).

A study of variation in WSSV virulence and pathogenicity across different host species revealed differences in pathogenicity already after 1 passage (Waikhom et al. 2006). In the current study such variation was not observed, which could be attributed to the phylogenetic distance between the virus origin (crustacean) and new host (annelid) (chapter 5). Waikhom et al. (2006) showed genotype variation in ORF94 after one passage through different species, suggesting high adaptability of WSSV to different crustacean host species. Virus virulence may increase or decrease when passing through different hosts as a result to adaptation to the host (Rigaud et al. 2010; Woolhouse et al. 2001). Repeated passage through a susceptible host increased the virulence of the arbovirus Venezuelan Equine Encephalitis Virus (VEEV) (Coffey et al. 2008). Passage through an unnatural host species may induce genotype and virulence changes

(Elena et al. 2009). Further research on genetic changes and variation in pathogenicity of WSSV after alternating passage through *Dendronereis* spp. and shrimp is recommended to gain better insight in the role of this polychaete as WSSV reservoir species in ponds. For this, deep sequencing approaches are recommended (see above).

Genome analysis suggested that WSSV shares evolutionary links with the other giruses (giant viruses): nudiviruses, baculoviruses and hytrosaviruses. This led to the hypothesis that these viruses specialized to become arthropod specific viruses (Wang and Jehle 2009). In the current study, it was suspected that cross-phylum WSSV infection and pathogen spillover in *Dendronereis* spp. may occur in ponds. With more WSSV host species involved, highly complex transmission modes may evolve. (Wang and Jehle (2009)) described such strategy for nudiviruses for successful infection of taxonomically diverse invertebrate host species occupying different ecological niches.

Pond conditions and WSSV occurrence in polychaete *Dendronereis* spp.

Because of its economic value, pond management aims at providing optimal rearing conditions that support maximum shrimp growth and production (Boyd 2003; Saraswathy et al. 2013; Yuvanatemiya et al. 2011). Although shrimps are important members of the benthic community, little is known about interactions within and between member species of this community, especially in relation to WSSV dynamics. This thesis investigated possible correlations between soil parameters, polychaete density and WSSV infection in *Dendronereis* spp.

The extensive ponds monitored in this thesis exchanged water regularly through tidal exchange, and soils conditions differed between locations. This had no effect on WSSV prevalence in *Dendronereis* spp. However, WSSV prevalence in *Dendronereis* spp. was positively correlated with polychaete density (chapter 6).

From a disease management perspective, the polychaetes extend the WSSV reservoir in the ponds. Polychaetes thrive in soft bottom estuaries (Ngqulana et al. 2010; Nordhaus et al. 2009) and the species composition often changes over time (Hutchings 1998; Sarkar et al. 2005). Conditions in the sediment affect the composition of the benthic community, which in turn may affect WSSV prevalence and susceptibility in vectors and host species, the WSSV adsorption to soil particles (Hurst et al. 1980) and WSSV attachment to benthic algae. Which role *Dendronereis* spp. plays in the dynamics of WSSV in the pond ecosystem and WSD occurrence needs furthers research.

Infection occurs when there is overlap between the ecological niches of host and vector species (Hurst et al. 1980). WSSV infection in *Dendronereis* spp. was apparent (chapters 3, 5 and 6), but no correlation was found between soil parameters and *Dendronereis* spp. density (Chapter 6). The number of ponds sampled was small, and no time series were taken. A broader study, including time series samples to elucidate the population dynamics and life cycle analysis of *Dendronereis* spp. in shrimp ponds is thus needed. By also monitoring WSSV prevalence during such a study, the understanding of the role polychaetes play in WSD occurrence in shrimp farming can be increased. This is a first step in analyzing how sediment conditions influence the benthic community and WSSV persistence in ponds.

A proposed model on the role of polychaete in WSSV transmission

WSSV can infect a broad range of benthic invertebrates that as a whole cover a wide salinity and temperature range. Figure 1 shows possibly directions of WSSV transmission in aquatic environments, including ponds, illustrating the complexities of the WSSV dynamics in ponds when all possible interactions would

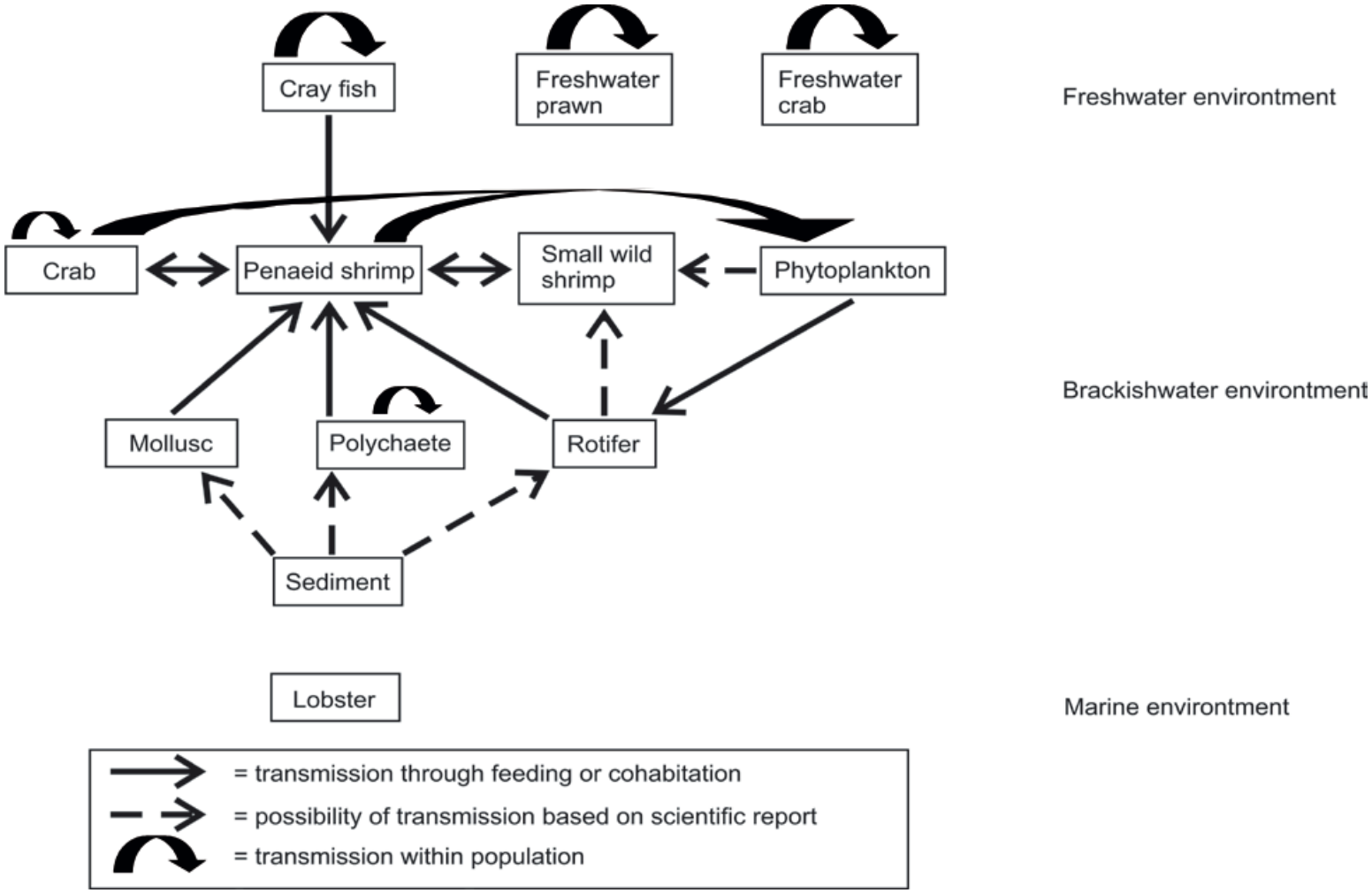
Cray fish
Freshwater prawn
Freshwater crab
Freshwater environtment
Crab
Penaeid shrimp
Small wild shrimp
Phytoplankton
Brackishwater environtment
Mollusc
Polychaete
Rotifer
Sediment
Lobster
Marine environtment
= transmission through feeding or cohabitation
= possibility of transmission based on scientific report
= transmission within population

Figure 1. Routes of WSSV transmission among reported host groups in ponds and the proposed role of polychaete as WSSV reservoir host species. Penaeid shrimp are the most dominant and susceptible host in this model. In an extensively managed pond, crabs, polychaetes and non-WSSV-SPF larvae are WSSV reservoirs. WSSV is transmitted within and among host species cohabiting in the pond. Stocking with non-WSSV-SPF shrimp larvae raises the viral load and increases contacts with other host and vectors species in the pond. In return, these organisms expose the shrimp to WSSV through various transmission routes. Burrowing polychaetes such as *Dendronereis* spp. maintain WSSV presence in the pond. In semi-intensive and intensive ponds stocked with WSSV-SPF larvae, polychaetes constitute an important WSSV reservoir. The infection may start when shrimp prey on WSSV infected polychaetes. The high shrimp density will facilitate WSSV transmission among shrimps and will quickly raise the overall viral load in the pond. Whether other invertebrate species play a similar role in freshwater and marine environments need further investigation.

be considered. The latter remain largely unexplored, underscoring the need and importance of further research.

PRACTICAL IMPLICATIONS OF THE STUDY

The overall findings suggested *Dendronereis* spp. are both natural food source and WSSV reservoir in ponds. Measures to control WSD should also consider control of WSSV infected polychaete stocks. Especially in extensive and semi-intensive culture systems, pond management is often aiming to increase the presence of natural food species to support shrimp production. Accordingly, polychaetes as natural food of shrimp should not be eliminated because of its susceptibility to WSSV infection, but be tested for the presence of WSSV prior to use. Instead, the pond management should focus on regulating the density of the principal reservoir and most susceptible host species in shrimp culture ponds, of which the cultured penaeid shrimp is one of various species. An "end of the pipe" disease control method (Kautsky et al. 2000) which aims to fully eradicate the WSSV from the culture environment may not be the best approach. An ecological approach which includes a broader range of WSSV host and vector species holds more promise for successful control of WSD. Such an approach would also allow for a more in-depth evaluation of the effectiveness of BMPs, polyculture and crop rotation schemes in controlling WSD.

THE WAY FORWARD

Recognizing the complexities of WSSV transmission in ponds, much more research is needed to elucidate the importance of different reservoir host species in relation to WSD. The observations on WSSV infection in polychaetes in this thesis research were only a first step. The availability of WSSV-free polychaete as a model species for WSSV infection and proliferation studies will certainly facilitate

further research. More insight is also needed into the ecology and biology of *Dendronereis* spp. in ponds and estuarine environments and parameters influencing WSSV infection and WSSV replication in this polychaete. This approach should be widened to other benthic invertebrate species possibly involved in WSSV proliferation in ponds. More info will also be needed on the genotypic variation of WSSV present in reservoir species present in ponds. Finally, deepening insights in the types and roles of binding proteins in WSSV infection and existing mechanism by which WSSV avoids polychaete anti-viral defense measures will allow better interpretation of the results of ecological and epidemiological studies.

REFERENCES

Abd-Alla, A.M.M., F. Cousserans, A.G. Parker, J.A. Jehle, N.J. Parker, J.M. Vlak, A.S. Robinson and M. Bergoin. 2008. Genome analysis of a *Glossina pallidipes* salivary gland hypertrophy virus reveals a novel, large, double-stranded circular DNA virus. Journal of Virology 82:4595-4611.

Abu Hena, M.K., O. Hishamuddin and K. Misri. 2011. Benthic meiofaunal predation and composition in the tiger shrimp *Penaeus monodon* culture ponds, Malaysia. Advances in Environmental Biology 5:605-611.

Allison, A.B. and C.R. Parrish. 2014. Chapter 3 - Parvoviruses of carnivores: Their transmission and the variation of viral host range. In: Johnson, N. (Ed.), The Role of Animals in Emerging Viral Diseases. Academic Press, Boston, pp. 39-61.

Anil, T.M., K.M. Shankar and C.V. Mohan. 2002. Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. Diseases of Aquatic Organisms 51:67-75.

Asaduzzaman, M., M.A. Wahab, M.C.J. Verdegem, S. Huque, M.A. Salam and M.E. Azim. 2008. C/N ratio control and substrate addition for periphyton development jointly enhance freshwater prawn *Macrobrachium rosenbergii* production in ponds. Aquaculture 280:117-123.

Avnimelech, Y. and G. Ritvo. 2003. Shrimp and fish pond soils: Processes and management. Aquaculture 220:549-567.

Baigent, S.J. and J.W. McCauley. 2003. Influenza type A in humans, mammals and birds: Determinants of virus virulence, host-range and interspecies transmission. Bioessays 25:657-671.

Balasubramanian, C., S. Pillai and P. Ravichandran. 2004. Zero-water exchange shrimp farming systems (extensive) in the periphery of Chilka lagoon, Orissa, India. Aquaculture International 12:555-572.

Baliao, D.D. 2000. Environment-friendly schemes in intensive shrimp farming. Aquaculture Department, Southeast Asian Fisheries Development Center.

References

Bardach, J.E., J.H. Ryther and W.O. MacLarney. 1972. Aquaculture : the farming and husbandry of freshwater and marine organisms. [s.n.], New York, 686 pp.

Bartoli, M., D. Longhi, D. Nizzoli, S. Como, P. Magni and P. Viaroli. 2009. Short term effects of hypoxia and bioturbation on solute fluxes, denitrification and buffering capacity in a shallow dystrophic pond. Journal of Experimental Marine Biology and Ecology 381:105-113.

Bateman, K.S., J. Munro, B. Uglow, H.J. Small and G.D. Stentiford. 2012a. Susceptibility of juvenile European lobster *Homarus gammarus* to shrimp products infected with high and low doses of white spot syndrome virus. Disease of Aquatic Organisms 100:169-184.

Bateman, K.S., I. Tew, C. French, R.J. Hicks, P. Martin, J. Munro and G.D. Stentiford. 2012b. Susceptibility to infection and pathogenicity of White Spot Disease (WSD) in non-model crustacean host taxa from temperate regions. Journal of Invertebrate Pathology 110:340-351.

Baumgartner, W.A., J.P. Hawke, K. Bowles, P.W. Varner and K.W. Hasson. 2009. Primary diagnosis and surveillance of white spot syndrome virus in wild and farmed crawfish (*Procambarus clarkii*, *P. zonangulus*) in Louisiana, USA. Diseases of Aquatic Organisms 85:15-22.

Bonnard, M., M. Roméo and C. Amiard-Triquet. 2009. Effects of copper on the burrowing behavior of estuarine and coastal invertebrates, the polychaete *Nereis diversicolor* and the bivalve *Scrobicularia plana*. Human and Ecological Risk Assessment 15:11-26.

Boyd, C.E. 1979. Water quality in warmwater fish ponds. Auburn University, Auburn, USA, pp. 359.

Boyd, C.E. 1995. Bottom soils, sediment and pond aquaculture. Chapman & Hall, New York [etc.], 348 pp.

Boyd, C.E. 2003. Bottom soil and water quality management in shrimp ponds. Journal of Applied Aquaculture 13:11-33.

Brock, J.A. 1997. Taura syndrome, a disease important to shrimp farms in the Americas. World Journal of Microbiology and Biotechnology 13:415-418.

Brown, N., S. Eddy and S. Plaud. 2011. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*. Aquaculture 322-323:177-183.

Caçador, I., J.L. Costa, B. Duarte, G. Silva, J.P. Medeiros, C. Azeda, N. Castro, J. Freitas, S. Pedro, P.R. Almeida, H. Cabral and M.J. Costa. 2012. Macroinvertebrates and fishes as biomonitors of heavy metal concentration in the Seixal Bay (Tagus estuary): Which species perform better? Ecological Indicators 19:184-190.

Caipang, C.M.A., M.F.J. Sibonga, J.S. Geduspan and M.J.A. Amar. 2012. An optimized loop-mediated isothermal amplification (LAMP) assay for the detection of white spot syndrome virus (WSSV) among cultured shrimps in the Philippines. Journal of Animal and Plant Sciences 22:927-932.

Cameron, A. 2002. Survey Toolbox for Aquatic Animal Diseases. A Practical Manual and Software Package, ACIAR Monograph 94, Canberra, Australia, pp. 375.

Carbajal-Sánchez, I.S., R. Castro-Longoria and J.M. Grijalva-Chon. 2008. Experimental white spot syndrome virus challenge of juvenile *Litopenaeus vannamei* (Boone) at different salinities. Aquaculture Research 39:1588-1596.

Carvalho, S., M. Barata, M.B. Gaspar, P. Pousão-Ferreira and L. Cancela da Fonseca. 2007. Enrichment of aquaculture earthen ponds with *Hediste diversicolor*: Consequences for benthic dynamics and natural productivity. Aquaculture 262:227-236.

Cavalli, L.S., B.F.S. Nornberg, S.A. Netto, L. Poersch, L.A. Romano, L.F. Marins and P.C. Abreu. 2010. White spot syndrome virus in wild penaeid shrimp caught in coastal and offshore waters in the southern Atlantic Ocean. Journal of Fish Diseases 33:533-536.

Chai, C.Y., J. Yoon, Y.S. Lee, Y.B. Kim and T.J. Choi. 2013. Analysis of the complete nucleotide sequence of a white spot syndrome virus isolated from pacific white shrimp. Journal of Microbiology 51:695-699.

Chang, P.-S., C.F. Lo, Y.C. Wang and G.H. Kou. 1996. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp

Penaeus monodon by in situ hybridization. Diseases of Aquatic Organisms 27:131-139.

Chang, P.S., H.C. Chen and Y.C. Wang. 1998. Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by in situ hybridization. Aquaculture 164:233-242.

Chang, Y.S., T.C. Chen, W.J. Liu, J.S. Hwang, G.H. Kou and C.F. Lo. 2011. Assessment of the roles of copepod *Apocyclops royi* and bivalve mollusk *Meretrix lusoria* in white spot syndrome virus transmission. Marine Biotechnology 13:909-917.

Chang, Y.S., W.J. Liu, T.L. Chou, Y.T. Lee, T.L. Lee, W.T. Huang, G.H. Kou and C.F. Lo. 2008. Characterization of white spot syndrome virus envelope protein VP51A and its interaction with viral tegument protein VP26. Journal of Virology 82:12555-12564.

Chang, Y.S., S.E. Peng, H.C. Wang, H.C. Hsu, C.H. Ho, C.H. Wang, S.Y. Wang, C.F. Lo and G.H. Kou. 2001. Sequencing and amplified restriction fragment length polymorphism analysis of ribonucleotide reductase large subunit gene of the white spot syndrome virus in blue crab (*Callinectes sapidus*) from American coastal waters. Marine Biotechnology 3:163-171.

Chang, Y.S., W.J. Liu, T.C. Chen, T.Y. Chan, K.F. Liu, J.C. Chuang, G.H. Kou, C.F. Lo and H.C. Wang. 2012. Feeding hermit crabs to shrimp broodstock increases their risk of WSSV infection. Diseases of Aquatic Organisms 98:193-199.

Chapman, R.W., C.L. Browdy, S. Savin, S. Prior and E. Wenner. 2004. Sampling and evaluation of white spot syndrome virus in commercially important Atlantic penaeid shrimp stocks. Diseases of Aquatic Organisms 59:179-185.

Chen, L.L., C.F. Lo, Y.L. Chiu, C.F. Chang and G.H. Kou. 2000. Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab *Scylla setrata*. Diseases of Aquatic Organisms 40:157-161.

Chen, L.L., L.C. Lu, W.J. Wu, C.F. Lo and W.P. Huang. 2007. White spot syndrome virus envelope protein VP53A interacts with *Penaeus monodon*

chitin-binding protein (PmCBP). Diseases of Aquatic Organisms 74:171-178.

Cheng, L., W.H. Lin, P.C. Wang, M.A. Tsai, J.P. Hsu and S.C. Chen. 2013. White spot syndrome virus epizootic in cultured pacific white shrimp *Litopenaeus vannamei* (Boone) in Taiwan. Journal of Fish Diseases 36:977-985.

Chou, H.-Y., C.-Y. Huang, C.-H. Wang, H.-C. Chiang and C.-F. Lo. 1995. Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Diseases of Aquatic Organisms 23:165-173.

Chou, H., C. Huang, C. Lo and G. Kou. 1998. Studies on transmission of white spot syndrome associated baculovirus (WSBV) in *Penaeus monodon* and *P. japonicus* via waterborne contact and oral ingestion. Aquaculture 164:263-276.

Chung, M.Y., C.H. Liu, Y.N. Chen and W. Cheng. 2011. Enhancing the reproductive performance of tiger shrimp, *Penaeus monodon*, by incorporating sodium alginate in the broodstock and larval diets. Aquaculture 312:180-184.

Clark, K.F., S.J. Greenwood, A.R. Acorn and P.J. Byrne. 2013. Molecular immune response of the American lobster (*Homarus americanus*) to the white spot syndrome virus. Journal of Invertebrate Pathology 114:298-308.

Claverie, J.M., H. Ogata, S. Audic, C. Abergel, K. Suhre and P.E. Fournier. 2006. Mimivirus and the emerging concept of "giant" virus. Virus Research 117:133-144.

Cock, J., T. Gitterle, M. Salazar and M. Rye. 2009. Breeding for disease resistance of Penaeid shrimps. Aquaculture 286:1-11.

Coffey, L.L., N. Vasilakis, A.C. Brault, A.M. Powers, F.R. Tripet and S.C. Weaver. 2008. Arbovirus evolution in vivo is constrained by host alternation. Proceedings of the National Academy of Sciences of the United States of America 105:6970-6975.

References

Corbel, V., Z. Zuprizal, C. Shi, Huang, Sumartono, J.M. Arcier and J.R. Bonami. 2001. Experimental infection of European crustaceans with white spot syndrome virus (WSSV). Journal of Fish Diseases 24:377-382.

Corre Jr, V., J. Faisan Jr, R.J. Carton-Kawagoshi, B.J. Elle, R.F. Traifalgar and C.M. Caipang. 2012. Evidence of WSSV transmission from the rotifer (*Brachionus plicatilis*) to the black tiger shrimp (*Penaeus monodon*) postlarvae and means to control rotifer resting eggs using industrial disinfectants. Aquaculture, Aquarium, Conservation & Legislation 5:64-68.

Corsin, F., P.C. Thakur, P.A. Padiyar, M. Madhusudhan, J.F. Turnbull, C.V. Mohan, N.V. Hao and K.L. Morgan. 2003. Relationship between white spot syndrome virus and indicators of quality in *Penaeus monodon* postlarvae in Karnataka, India. Diseases of Aquatic Organisms 54:97-104.

De La Peña, L.D., C.R. Lavilla-Pitogo, C.B.R. Villar, M.G. Paner, C.D. Sombito and G.C. Capulos. 2007. Prevalence of white spot syndrome virus (WSSV) in wild shrimp *Penaeus monodon* in the Philippines. Diseases of Aquatic Organisms 77:175-179.

de la Vega, E., N.A. O'Leary, J.E. Shockey, J. Robalino, C. Payne, C.L. Browdy, G.W. Warr and P.S. Gross. 2008. Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): A broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Molecular Immunology 45:1916-1925.

De Oliveira, A.B., A.E. Rizzo, E. Da Conceicao and G. Couto. 2012. Benthic macrofauna associated with decomposition of leaves in a mangrove forest in Ilhéus, State of Bahia, Brazil. Journal of the Marine Biological Association of the United Kingdom 92:1479-1487.

Delgado, P.C., Y. Avnimelech, R. McNeil, D. Bratvold, C.L. Browdy and P. Sandifer. 2003. Physical, chemical and biological characteristics of distinctive regions in paddlewheel aerated shrimp ponds. Aquaculture 217:235-248.

Desrina, J.A.J. Verreth, S.B. Prayitno, J.H.W.M. Rombout, J.M. Vlak and M.C.J. Verdegem. 2013. Replication of white spot syndrome virus (WSSV) in the polychaete *Dendronereis* spp. Journal of Invertebrate Pathology 114:7-10.

Dieu, B.T.M., H. Marks, M.P. Zwart and J.M. Vlak. 2010. Evaluation of white spot syndrome virus variable DNA loci as molecular markers of virus spread at intermediate spatiotemporal scales. Journal of General Virology 91:1164-1172.

Dieu, B.T.M., H. Marks, J.J. Siebenga, R.W. Goldbach, D. Zuidema, T.P. Duong and J.M. Vlak. 2004. Molecular epidemiology of white spot syndrome virus within Vietnam. Journal of General Virology 85:3607-3618.

Dixit, A. 1998. A simple and rapid procedure for isolation of total DNA suitable for fingerprint analysis from *Amaranthus*. Plant Molecular Biology Reporter 16:91-91.

Doan, T.T., C. Bouvier, Y. Bettarel, T. Bouvier, T. Henry-des-Tureaux, J.L. Janeau, P. Lamballe, B.V. Nguyen and P. Jouquet. 2014. Influence of buffalo manure, compost, vermicompost and biochar amendments on bacterial and viral communities in soil and adjacent aquatic systems. Applied Soil Ecology 73:78-86.

Dsikowitzky, L., I. Nordhaus, T.C. Jennerjahn, P. Khrycheva, Y. Sivatharshan, E. Yuwono and J. Schwarzbauer. 2011. Anthropogenic organic contaminants in water, sediments and benthic organisms of the mangrove-fringed Segara Anakan Lagoon, Java, Indonesia. Marine Pollution Bulletin 62:851-862.

Durand, S., D.V. Lightner, R.M. Redman and J.R. Bonami. 1997. Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). Disease of Aquatic Organisms 29:205-211.

Durou, C. and C. Mouneyrac. 2007. Linking steroid hormone levels to sexual maturity index and energy reserves in *Nereis diversicolor* from clean and polluted estuaries. General and Comparative Endocrinology 150:106-113.

Durou, C., C. Mouneyrac and C. Amiard-Triquet. 2008. Environmental quality assessment in estuarine ecosystems: Use of biometric measurements and fecundity of the ragworm *Nereis diversicolor* (Polychaeta, Nereididae). Water Research 42:2157-2165.

Edgerton, B.F. 2004. Susceptibility of the Australian freshwater crayfish *Cherax destructor albidus* to white spot syndrome virus (WSSV). Disease of Aquatic Organisms 59:187-193.

References

Elena, S.F., P. Agudelo-Romero and J. Lalić. 2009. The evolution of viruses in multi-host fitness landscapes. The Open Virology Journal 3:1-6.

Escobedo-Bonilla, C.M., M. Wille, V. Alday Sanz, P. Sorgeloos, M.B. Pensaert and H.J. Nauwynck. 2007. Pathogenesis of a Thai strain of white spot syndrome virus (WSSV) in juvenile, specific pathogen-free *Litopenaeus vannamei*. Diseases of Aquatic Organisms 74:85-94.

Escobedo-Bonilla, C.M., V. Alday-Sanz, M. Wille, P. Sorgeloos, M.B. Pensaert and H.J. Nauwynck. 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. Journal of Fish Diseases 31:1-18.

Esparza-Leal, H.M., C.M. Escobedo-Bonilla, R. Casillas-Hernández, P. Álvarez-Ruíz, G. Portillo-Clark, R.C. Valerio-García, J. Hernández-López, J. Méndez-Lozano, N. Vibanco-Pérez and F.J. Magallón-Barajas. 2009. Detection of white spot syndrome virus in filtered shrimp-farm water fractions and experimental evaluation of its infectivity in *Penaeus (Litopenaeus) vannamei*. Aquaculture 292:16-22.

Esselink, P. and L. Zwarts. 1989. Seasonal trend in burrow depth and tidal variation in feeding activity of *Nereis diversicolor*. Marine Ecology Progress Series 56:243-254.

FAO. 2012. The state of world fisheries and aquaculture 2012 FAO Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations. Rome. Pages 209.

FAO. 2013. FishStatJ, a tool for fishery statistics analysis. Fishery and Aquaculture Department, Policy and Economics Division, Statistics and Information Service [downloaded 25 June 2013] (www.fao.org/fishery/statistics/software/fishstatj/en):release 2.0.0.

Feng, S., G. Li, W. Feng and J. Huang. 2013. Binding of white spot syndrome virus to *Artemia* sp. cell membranes. Journal of Virological Methods 193:108-111.

Fidalgo e Costa, P. 1999. Reproduction and growth in captivity of the polychaete *Nereis diversicolor* O.F. Muller, 1776, using two different kinds of

sediment: preliminary assays. Boletin-Instituto Espanol de Oceanografia 15:351-356.

Flegel, T.W. 1997. Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World Journal of Microbiology and Biotechnology 13:433-442.

Flegel, T.W. 2006. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture 258:1-33.

Flegel, T.W. 2007. Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. Developmental and Comparative Immunology 31:217-231.

Flegel, T.W. 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. Journal of Invertebrate Pathology 110:166-173.

Flegel, T.W., L. Nielsen, V. Thamavit, S. Kongtim and T. Pasharawipas. 2004. Presence of multiple viruses in non-diseased, cultivated shrimp at harvest. Aquaculture 240:55-68.

Fujioka, Y., T. Shimoda and C. Srithong. 2007a. Diversity and community structure of macrobenthic fauna in shrimp aquaculture ponds of the gulf of Thailand. Japan Agricultural Research Quarterly 41:163.

Fujioka, Y., T. Shimoda and C. Srithong. 2007b. Diversity and community structure of macrobenthic fauna in shrimp aquaculture ponds of the Gulf of Thailand. Japan Agricultural Research Quarterly 41:163-172.

Galavíz-Silva, L., Z.J. Molina-Garza, J.M. Alcocer-González, J.L. Rosales-Encinas and C. Ibarra-Gámez. 2004. White spot syndrome virus genetic variants detected in Mexico by a new multiplex PCR method. Aquaculture 242:53-68.

Gale, P. and N. Johnson. 2014. Chapter 7 - The role of birds in the spread of west Nile virus. In: Johnson, N. (Ed.), The role of animals in emerging viral diseases. Academic Press, Boston, pp. 143-167.

Gangnonngiw, W., K. Laisutisan, S. Sriurairatana, S. Senapin, N. Chuchird, C. Limsuwan, P. Chaivisuthangkura and T.W. Flegel. 2010. Monodon

baculovirus (MBV) infects the freshwater prawn *Macrobrachium rosenbergii* cultivated in Thailand. Virus Research 148:24-30.

García-Arberas, L. and A. Rallo. 2002. The intertidal soft-bottom infaunal macrobenthos in three Basque estuaries (Gulf of Biscay): a feeding guild approach. Hydrobiologia 475/476:457-468.

Gee, G.W. and J.W. Bauder. 1986. Particle-Size Analysis. In: Klute, A., G.S. Campbell (Eds.), Methods of soil analysis: Physical and mineralogical methods, Agronomy no. 9. American Society of Agronomy, Madison, pp. 383-411.

Gitterle, T., R. Salte, B. Gjerde, J. Cock, H. Johansen, M. Salazar, C. Lozano and M. Rye. 2005. Genetic (co) variation in resistance to White Spot Syndrome Virus (WSSV) and harvest weight in *Penaeus (Litopenaeus) vannamei*. Aquaculture 246:139-149.

Gopalakrishnan, A., M. Rajkumar, J. Sun, M. Wang and K. Senthil Kumar. 2011. Mud crab, *Scylla tranquebarica* (Decapoda: Portunidae), a new host for the white spot syndrome virus. Aquaculture Research 42:308-312.

Gowda, G., K.M. Rajesh and R.M. Mridula. 2009. Vertical distribution of polychaetes in brackishwater pond of Nethravathi estuary, India. Journal of Environmental Biology 30:1025-1029.

Hameed, A.S., G. Balasubramanian, S.S. Musthaq and K. Yoganandhan. 2003. Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). Diseases of Aquatic Organisms 57:157-161.

Hameed, A.S., K. Yoganandhan, S. Sathish, M. Rasheed, V. Murugan and K. Jayaraman. 2001. White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphusa hydrodomous* and *P. pulvinata*). Aquaculture 201:179-186.

Hameed, A.S.S., M.X. Charles and M. Anilkumar. 2000. Tolerance of *Macrobrachium rosenbergii* to white spot syndrome virus. Aquaculture 183:207-213.

Hameed, A.S.S., B.L.M. Murthi, M. Rasheed, S. Sathish, K. Yoganandhan, V. Murugan and K. Jayaraman. 2002. An investigation of Artemia as a

possible vector for white spot syndrome virus (WSSV) transmission to Penaeus indicus. Aquaculture 204:1-10.

Hariati, A.M., A. D. G. R. Wiadnyaprajitno, M. Sukkel, J.H. Boon and M.C.J. Verdegem. 1995. Recent developments of shrimp, *Penaeus monodon* (Fabricius) and *Penaeus merguinensis* (de Man), culture in East Java. Aquaculture Research 26:819-829.

Haryadi, D., J.A.J. Verreth, M.C.J. Verdegem and J.M. Vlak. 2014. Transmission of white spot syndrome virus (WSSV) from *Dendronereis* spp. (peters) (Nereididae) to penaeid shrimp. Journal of Fish Diseases:early view (doi 10.1111/jfd.12247).

Hasson, K.W., Y. Fan, T. Reisinger, J. Venuti and P.W. Varner. 2006. White-spot syndrome virus (WSSV) introduction into the Gulf of Mexico and Texas freshwater systems through imported, frozen bait-shrimp. Diseases of Aquatic Organisms 71:91-100.

Hoa, N.D., R. Wouters, M. Wille, V. Thanh, T.K. Dong, N. Van Hao and P. Sorgeloos. 2009. A fresh-food maturation diet with an adequate HUFA composition for broodstock nutrition studies in black tiger shrimp *Penaeus monodon* (Fabricius, 1798). Aquaculture 297:116-121.

Hoa, T.T.T., M.P. Zwart, N.T. Phuong, J.M. Vlak and M. de Jong. 2011a. Transmission of white spot syndrome virus in improved-extensive and semi-intensive shrimp production systems: A molecular epidemiology study. Aquaculture 313:7-14.

Hoa, T.T.T., M.P. Zwart, N.T. Phuong, M.C.M. De Jong and J.M. Vlak. 2012a. Low numbers of repeat units in variable number of tandem repeats (VNTR) regions of white spot syndrome virus are correlated with disease outbreaks. Journal of Fish Diseases 35:817-826.

Hoa, T.T.T., M.P. Zwart, N.T. Phuong, D.T.H. Oanh, M.C.M. De Jong and J.M. Vlak. 2011b. Mixed-genotype white spot syndrome virus infections of shrimp are inversely correlated with disease outbreaks in ponds. Journal of General Virology 92:675-680.

Hoa, T.T.T., M.P. Zwart, N.T. Phuong, D.T.H. Oanh, M.C.M. De Jong and J.M. Vlak. 2012b. Indel-II region deletion sizes in the white spot syndrome

virus genome correlate with shrimp disease outbreaks in southern Vietnam. Diseases of Aquatic Organisms 99:153-162.

Hossain, M.S., S. Khadijah and J. Kwang. 2004. Characterization of ORF89 - A latency-related gene of white spot syndrome virus. Virology 325:106-115.

Hossain, M.S., S.K. Otta, I. Karunasagar and I. Karunasagar. 2001a. Detection of white spot syndrome virus (WSSV) in wild captured shrimp and in non-cultured crustaceans from shrimp ponds in Bangladesh by polymerase chain reaction. Fish Pathology 36:93-95.

Hossain, M.S., A. Chakraborty, B. Joseph, S.K. Otta, I. Karunasagar and I. Karunasagar. 2001b. Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction. Aquaculture 198:1-11.

Huang, C., X. Zhang, Q. Lin, X. Xu and C.L. Hew. 2002. Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. Journal of General Virology 83:2385-2392.

Huang, C.H., L.R. Zhang, J.H. Zhang, L.C. Xiao, Q.J. Wu, D.H. Chen and J.K.K. Li. 2001. Purification and characterization of White Spot Syndrome Virus (WSSV) produced in an alternate host: Crayfish, *Cambarus clarkii*. Virus Research 76:115-125.

Huang, H.H., X.L. Liu, J.H. Xiang and P. Wang. 2013. Immune response of *Litopenaeus vannamei* after infection with *Vibrio harveyi*. Aquaculture 406-407:115-120.

Huang, H.T., J.H. Leu, P.Y. Huang and L.L. Chen. 2012. A putative cell surface receptor for white spot syndrome virus is a member of a transporter superfamily. PLoS ONE 7.

Huang, R., Y. Xie, J. Zhang and Z. Shi. 2005. A novel envelope protein involved in white spot syndrome virus infection. Journal of General Virology 86:1357-1361.

Hurst, C.J., C.P. Gerba and I. Cech. 1980. Effects of environmental variables and soil characteristics on virus survival in soil. Applied and Environmental Microbiology 40:1067-1079.

Hutchings, P. 1998. Biodiversity and functioning of polychaetes in benthic sediments. Biodiversity & Conservation 7:1133-1145.

Jiang, G. 2012. Can white spot syndrome virus be transmitted through the phytoplankton →rotifer →artemia →shrimp pathway? African Journal of Biotechnology 11:1277-1282.

Jiravanichpaisal, P., K. Söderhäll and I. Söderhäll. 2004. Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. Fish and Shellfish Immunology 17:265-275.

John, K.R., M.R. George, T. Iyappan, A.J. Thangarani and M.J.P. Jeyaseelan. 2010. Indian isolates of white spot syndrome virus exhibit variations in their pathogenicity and genomic tandem repeats. Journal of Fish Diseases 33:749-758.

Johnson, N. 2013. A short introduction to disease emergence. In: Johnson, N. (Ed.), The role of animals in emerging viral diseases. Elsevier. pp 344., Amsterdam, pp. 1-19.

Joseph, A. and R. Philip. 2007. Acute salinity stress alters the haemolymph metabolic profile of *Penaeus monodon* and reduces immunocompetence to white spot syndrome virus infection. Aquaculture 272:87-97.

Kalman, J., B.D. Smith, I. Riba, J. Blasco and P.S. Rainbow. 2010. Biodynamic modelling of the accumulation of Ag, Cd and Zn by the deposit-feeding polychaete *Nereis diversicolor*: Inter-population variability and a generalised predictive model. Marine Environmental Research 69:363-373.

Kalman, J., F. Palais, J.C. Amiard, C. Mouneyrac, A. Muntz, J. Blasco, I. Riba and C. Amiard-Triquet. 2009. Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation. Marine Ecology Progress Series 393:55-67.

Kanchanaphum, P., C. Wongteerasupaya, N. Sitidilokratana, V. Boonsaeng, S. Panyim, A. Tassanakajon, B. Withyachumnarnkul and T.W. Flegel. 1998. Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon*. Diseases of Aquatic Organisms 34:1-7.

References

Karunasagar, I., S.K. Otta and I. Karunasagar. 1998. Disease problems affecting cultured penaeid shrimp in India. Fish Pathology 33:413-419.

Kasornchandra, J., S. Boonyaratpalin and T. Itami. 1998. Detection of white-spot syndrome in cultured penaeid shrimp in Asia: Microscopic observation and polymerase chain reaction. Aquaculture 164:243-251.

Kautsky, N., P. Rönnbäck, M. Tedengren and M. Troell. 2000. Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture 191:145-161.

Kristensen, E., S. Bouillon, T. Dittmar and C. Marchand. 2008. Organic carbon dynamics in mangrove ecosystems: a review. Aquatic Botany 89:201-219.

Kristensen, E., T. Hansen, M. Delefosse, G.T. Banta and C.O. Quintana. 2011. Contrasting effects of the polychaetes *Marenzelleria viridis* and *Nereis diversicolor* on benthic metabolism and solute transport in sandy coastal sediment. Marine Ecology Progress Series 425:125-139.

Kumar, R.S. 2003. A checklist of polychaete species some mangroves of Asia. Zoos' Print Journal 18:1017-1020.

Kumar, T.S., P. Krishnan, M. Makesh, A. Chaudhari, C.S. Purushothaman and K.V. Rajendran. 2011. Natural host-range and experimental transmission of Laem-Singh virus (LSNV). Diseases of Aquatic Organisms 96:21-27.

Laoaroon, S., A. Boonnat, P. Poltana, P. Kanchanaphum, W. Gangnonngiw, G. Nash and B. Withyachumnarnkul. 2005. Infectivity of white spot syndrome virus (WSSV) to the polychaete *Pereneis nuntia* and a possibility of WSSV transmission from the polychaete to the black tiger shrimp *Penaeus monodon*. In: Walker, P., R. Lester, M.G. Bondad-Reantaso (Eds.), Diseases in Asian Aquaculture V, Fish Health Section, Asian Fisheries Society, Manila, pp. 353-361.

Laramore, S.E., J. Scarpa, C.R. Laramore and J. Lin. 2009. Virulence variation of white spot syndrome virus in Pacific white shrimp *Litopenaeus vannamei*. Journal of Aquatic Animal Health 21:82-90.

Lavilla-Pitogo, C.R., L.D. De La Peña and D.D. Catedral. 2007. Enhancement of white spot syndrome virus load in hatchery-reared mud crab *Scylla serrata*

(Forsskål, 1775) juveniles at a low temperature. Aquaculture Research 38:1600-1603.

Le Vay, L., V.N. Ut and M. Walton. 2007. Population ecology of the mud crab *Scylla paramamosain* (Estampador) in an estuarine mangrove system; a mark-recapture study. Marine Biology 151:1127-1135.

Lemonnier, H., E. Bernard, E. Boglio, C. Goarant and J.C. Cochard. 2004. Influence of sediment characteristics on shrimp physiology: pH as principal effect. Aquaculture 240:297-312.

Leung, P., L.T. Tran and A.W. Fast. 2000. A logistic regression of risk factors for disease occurrence on Asian shrimp farms. Diseases of Aquatic Organisms 41:65-76.

Li, L., S. Lin and F. Yang. 2006. Characterization of an envelope protein (VP110) of White spot syndrome virus. Journal of General Virology 87:1909-1915.

Liang, Y., J.J. Cheng, B. Yang and J. Huang. 2010. The role of F1 ATP synthase beta subunit in WSSV infection in the shrimp, *Litopenaeus vannamei*. Virology Journal 7:144.

Liang, Y., J. Huang, X.L. Song, P.J. Zhang and H.S. Xu. 2005. Four viral proteins of white spot syndrome virus (WSSV) that attach to shrimp cell membranes. Diseases of Aquatic Organisms 66:81-85.

Lightner, D. 1996. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp, World Aquaculture Society, Baton Rouge, LA, USA.

Lightner, D.V. 2011. Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review. Journal of Invertebrate Pathology 106:110-130.

Lightner, D.V. 2012. Global transboundry disease politics: The OIE perspective. Journal of Invertebrate Pathology 110:184-187.

Lightner, D.V. and R.M. Redman. 1998. Shrimp diseases and current diagnostic methods. Aquaculture 164:201-220.

Lightner, D.V., R.M. Redman, C.R. Pantoja, K.F.J. Tang, B.L. Noble, P. Schofield, L.L. Mohney, L.M. Nunan and S.A. Navarro. 2012. Historic emergence,

impact and current status of shrimp pathogens in the Americas. Journal of Invertebrate Pathology 110:174-183.

Liu, B., X. Tang and W. Zhan. 2011a. Interaction between white spot syndrome virus VP26 and hemocyte membrane of shrimp, *Fenneropenaeus chinensis*. Aquaculture 314:13-17.

Liu, B., Z. Yu, X. Song and Y. Guan. 2007. Studies on the transmission of WSSV (white spot syndrome virus) in juvenile *Marsupenaeus japonicus* via marine microalgae. Journal of Invertebrate Pathology 95:87-92.

Liu, B., Z. Yu, X. Song, Y. Guan, X. Jian and J. He. 2006. The effect of acute salinity change on white spot syndrome (WSS) outbreaks in *Fenneropenaeus chinensis*. Aquaculture 253:163-170.

Liu, Q.H., X.L. Zhang, C.Y. Ma, Y. Liang and J. Huang. 2009. VP37 of white spot syndrome virus interact with shrimp cells. Letters of Applied Microbiology 48:44-50.

Liu, W., D. Qian and X.J. Yan. 2011b. Studies on pathogenicity and prevalence of white spot syndrome virus in mud crab, *Scylla serrata* (Forskal), in Zhejiang Province, China. Journal of Fish Diseases 34:131-138.

Lo, C.F. 2014. White spot disease, Manual of Diagnostic Tests for Aquatic Animals 2013. OIE World Organization of Animal Health, pp. 177 - 190.

Lo, C.F. and G.H. Kou. 1998. Virus-associated white spot syndrome of shrimp in Taiwan: A review. Fish Pathology 33:365-371.

Lo, C.F., J.H. Leu, C.H. Ho, C.H. Chen, S.E. Peng, Y.T. Chen, C.M. Chou, P.Y. Yeh, C.J. Huang, H.Y. Chou, C.H. Wang and G.H. Kou. 1996. Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Diseases of Aquatic Organisms 25:133-141.

Lo, C.F., T. Aoki, J.R. Bonami, T. Flegel, J.H. Leu, D.V. Lightner, G. Stentiford, K. Söderhäll, P.J. Walker, H.C. Wang, X. Xun, F. Yang and J.M. Vlak. 2012. Family Nimaviridae. In: King, A.M.Q., M.J. Adams, E.B. Carstens, E.J. Lefkowitz (Eds.), Virus Taxonomy: Classification and Nomenclature

of Viruses, Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier–Academic Press, Amsterdam, pp. 229-234.

Lotz, J.M. 1997. Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. World Journal of Microbiology and Biotechnology 13:405-413.

Lotz, J.M. and M.A. Soto. 2002. Model of white spot syndrome virus (WSSV) epidemics in *Litopenaeus vannamei*. Diseases of Aquatic Organisms 50:199-209.

Manivannan, S., B. Kennedy, I. Karunasagar and I. Karunasagar. 2004. Prevalence of monodon baculovirus in wild *Metapenaeus* species along the southwest coast of India. Aquaculture 232:63-67.

Marks, H., M. Mennens, J.M. Vlak and M.C. van Hulten. 2003. Transcriptional analysis of the white spot syndrome virus major virion protein genes. Journal of General Virology 84:1517-1523.

Marks, H., J.J.A. Van Duijse, D. Zuidema, M.C.W. Van Hulten and J.M. Vlak. 2005a. Fitness and virulence of an ancestral White Spot Syndrome Virus isolate from shrimp. Virus Research 110:9-20.

Marks, H., O. Vorst, A.M.M.L. van Houwelingen, M.C.W. van Hulten and J.M. Vlak. 2005b. Gene-expression profiling of white spot syndrome virus in vivo. Journal of General Virology 86:2081-2100.

Marques, J.S., I.C. Müller, J.R. Moser, T.C. Sincero and M.R.F. Marques. 2011. Wild captured crab, *Chasmagnathus granulata* (Dana, 1851), a new host for white spot syndrome virus (WSSV). Aquaculture 318:20-24.

Martinez-Cordova, L.R., M.A. Porchas-Cornejo, H. Villarreal-Colemnares, J.A. Calderon-Perez and J. Naranjo-Paramo. 1998. Evaluation of three feeding strategies on the culture of white shrimp *Penaeus vannamei* Boone 1931 in low water exchange ponds. Aquacultural Engineering 17:21-28.

Meksumpun, C. and S. Meksumpun. 1999. Polychaete–sediment relations in Rayong, Thailand. Environmental Pollution 105:447-456.

References

Menasveta, P. 2002. Improved shrimp growout systems for disease prevention and environmental sustainability in Asia. Reviews in Fisheries Science 10:391-402.

Meng, X.H., I.K. Jang, H.C. Seo and Y.R. Cho. 2009. White spot syndrome virus quantification in blue crab *Portunus trituberculatus* hatchery-produced larvae and wild populations by TaqMan real-time PCR, with an emphasis on the relationship between viral infection and crab health. Aquaculture 291:18-22.

Meunpol, O., E. Duangjai, R. Yoonpun and S. Piyatiratitivorakul. 2010. Detection of prostaglandin E2 in polychaete *Perinereis* sp. and its effect on *Penaeus monodon* oocyte development in vitro. Fisheries Science 76:281-286.

Modrow, S., D. Falke, U. Truyen and H. Schätzl. 2013. Molecular Virology. Springer, Berlin, Heidelberg.

Momoyama, K., M. Hiraoka and C.A. Venegas. 1999. Pathogenicity of Penaeid Rod-shaped DNA Virus (PRDV) to juveniles of six crustacean species. Fish Pathology 34:183-188.

Momoyama, K., M. Hiraoka, H. Nakano and M. Sameshima. 1998. Cryopreservation of penaeid rod-shaped DNA virus (PRDV) and its survival in sea water at different temperatures. Fish Pathology 33:95-96.

Momoyana, K., M. Hiraoka, H. Nakano, H. Koube, K. Inouye and N. Oseko. 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: histopathological study. Fish Pathology 29:141-148.

Moser, J.R., D.A.G. Álvarez, F.M. Cano, T.E. Garcia, D.E.C. Molina, G.P. Clark, M.R.F. Marques, F.J.M. Barajas and J.H. López. 2012. Water temperature influences viral load and detection of White Spot Syndrome Virus (WSSV) in *Litopenaeus vannamei* and wild crustaceans. Aquaculture 326-329:9-14.

Mouneyrac, C., H. Perrein-Ettajani and C. Amiard-Triquet. 2010. Influence of anthropogenic stress on fitness and behaviour of a key-species of estuarine ecosystems, the ragworm *Nereis diversicolor*. Environmental Pollution 158:121-128.

Munro, J. and L. Owens. 2007. Yellow head-like viruses affecting the penaeid aquaculture industry. Aquaculture Research 38:893-908.

Muroga, K. 2001. Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. Aquaculture 202:23-44.

NACA-FAO. 2014. Quarterly Aquatic Animal Disease Report (Asia and Pacific Region), 2013/3, July - August 2013. NACA: Bangkok, Thailand.

Nakano, H., H. Koube, S. Umezawa, K. Momoyama, M. Hiraoka, K. Inouye and N. Oseko. 1994. Mass mortalities of cultures kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: epizootiological survey and infection trials. Fish Pathology 29:135-139.

Natividad, K.D.T., N. Nomura and M. Matsumura. 2008. Detection of white spot syndrome virus DNA in pond soil using a 2-step nested PCR. Journal of Virological Methods 149:28-34.

Nesto, N., R. Simonini, D. Prevedelli and L. Da Ros. 2012. Effects of diet and density on growth, survival and gametogenesis of *Hediste diversicolor* (O.F. Müller, 1776) (Nereididae, Polychaeta). Aquaculture 362-363:1-9.

Ngqulana, S., R. Owen, L. Vivier and D. Cyrus. 2010. Benthic faunal distribution and abundance in the Mfolozi–Msunduzi estuarine system, KwaZulu-Natal, South Africa. African Journal of Aquatic Science 35:123-133.

Nguyen, B.T., S. Koshio, K. Sakiyama, M. Ishikawa, S. Yokoyama and M.A. Kader. 2012. Effects of polychaete extracts on reproductive performance of kuruma shrimp, *Marsupenaeus japonicus* Bate. - Part II. Ovarian maturation and tissue lipid compositions. Aquaculture 334-337:65-72.

Nhan, D.K., A. Milstein, M.C.J. Verdegem and J.A.V. Verreth. 2006. Food inputs, water quality and nutrient accumulation in integrated pond systems: A multivariate approach. Aquaculture 261:160-173.

Nhan, D.K., M.C.J. Verdegem, A. Milstein and J.A.V. Verreth. 2008. Water and nutrient budgets of ponds in integrated agriculture-aquaculture systems in the Mekong Delta, Vietnam. Aquaculture Research 39:1216-1228.

Nordhaus, I., F.A. Hadipudjana, R. Janssen and J. Pamungkas. 2009. Spatio-temporal variation of macrobenthic communities in the mangrove-fringed

Segara Anakan lagoon, Indonesia, affected by anthropogenic activities. Regional Environmental Change 9:291-313.

Nunes, A., T. Gesteira and S. Goddard. 1997. Food ingestion and assimilation by the southern brown shrimp *Penaeus subtilis* under semi-intensive culture in NE Brazil. Aquaculture 149:121-136.

Nunes, A.J. and G.J. Parsons. 2000. Effects of the Southern brown shrimp, *Penaeus subtilis*, predation and artificial feeding on the population dynamics of benthic polychaetes in tropical pond enclosures. Aquaculture 183:125-147.

Otta, S.K., G. Shubha, B. Joseph, A. Chakraborty, I. Karunasagar and I. Karunasagar. 1999. Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. Diseases of Aquatic Organisms 38:67-70.

Owens, L. 2011. The viral ecology of aquatic crustaceans. In: Hurst, C.J. (Ed.), Studies in Viral Ecology: Animal Host Systems. Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 177-189.

Parrish, C.R., E.C. Holmes, D.M. Morens, E.C. Park, D.S. Burke, C.H. Calisher, C.A. Laughlin, L.J. Saif and P. Daszak. 2008. Cross-species virus transmission and the emergence of new epidemic diseases. Microbiology and Molecular Biology Reviews 72:457-470.

Peinado-Guevara, L.I. and M. López-Meyer. 2006. Detailed monitoring of white spot syndrome virus (WSSV) in shrimp commercial ponds in Sinaloa, Mexico by nested PCR. Aquaculture 251:33-45.

Peng, K., J.W.M. van Lent, J.M. Vlak, Z. Hu and M.M. van Oers. 2011. In situ cleavage of baculovirus occlusion-derived virus receptor binding protein p74 in the peroral infectivity complex. Journal of Virology 85:10710-10718.

Peng, S.E., C.F. Lo, K.F. Liu and G.H. Kou. 1998. The transition from pre-patent to patent infection of white spot syndrome virus (WSSV) in *Penaeus monodon* triggered by pereiopod excision. Fish Pathology 33:395-400.

Pérez, F., F.A.M. Volckaert and J. Calderón. 2005. Pathogenicity of white spot syndrome virus on postlarvae and juveniles of *Penaeus (Litopenaeus) vannamei*. Aquaculture 250:586-591.

Pillai, T.G. 1965. Annelida polychaeta from the Philippines and Indonesia. Ceylon Journal of Science, Biological Sciences 5:110–177.

Poltana, P., T. Lerkitkul, P. Pongtippatee-Taweepreda, S. Asuvapongpattana, K. Wongprasert, S. Sriurairatana, J. Chavadej, P. Sobhon, P.J.W. Olive and B. Withyachumnarnkul. 2007. Culture and development of the polychaete *Perinereis* cf. *nuntia*. Invertebrate Reproduction and Development 50:13-20.

Pontes, C.S., M.D.F. Arruda, A.A. De Lara Menezes and P.P. De Lima. 2006. Daily activity pattern of the marine shrimp *Litopenaeus vannamei* (Boone 1931) juveniles under laboratory conditions. Aquaculture Research 37:1001-1006.

Pradeep, B., M. Shekar, N. Gudkovs, I. Karunasagar and I. Karunasagar. 2008. Genotyping of white spot syndrome virus prevalent in shrimp farms of India. Diseases of Aquatic Organisms 78:189-198.

Quang, N.D., P.T.P. Hoa, T.T. Da and P.H. Anh. 2009. Persistence of white spot syndrome virus in shrimp ponds and surrounding areas after an outbreak. Environmental Monitoring and Assessment 156:69-72.

Qureshi, N.A. and N.U. Saher. 2012. Burrow morphology of three species of fiddler crab (*Uca*) along the coast of Pakistan. Belgian Journal of Zoology 142:114-126.

Rahman, M.M., M. Corteel, M. Wille, V. Alday-Sanz, M.B. Pensaert, P. Sorgeloos and H.J. Nauwynck. 2007a. 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.

Rahman, M.M., M. Corteel, J.J. Dantas-Lima, M. Wille, V. Alday-Sanz, M.B. Pensaert, P. Sorgeloos and H.J. Nauwynck. 2007b. 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). Aquaculture 269:107-113.

References

Rahman, M.M., M. Corteel, C.M. Escobedo-Bonilla, M. Wille, V. Alday-Sanz, M.B. Pensaert, P. Sorgeloos and H.J. Nauwynck. 2008. Virulence of white spot syndrome virus (WSSV) isolates may be correlated with the degree of replication in gills of *Penaeus vannamei* juveniles. Diseases of Aquatic Organisms 79:191-198.

Rahman, M.M., C.M. Escobedo-Bonilla, M. Corteel, J.J. Dantas-Lima, M. Wille, V.A. Sanz, M.B. Pensaert, P. Sorgeloos and H.J. Nauwynck. 2006. 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*. Aquaculture 261:842-849.

Rajan, P.R., P. Ramasamy, V. Purushothaman and G.P. Brennan. 2000. White spot baculovirus syndrome in the Indian shrimp *Penaeus monodon* and *P. indicus*. Aquaculture 184:31-44.

Rajendran, K.V., K.K. Vijayan, T.C. Santiago and R.M. Krol. 1999. Experimental host range and histopathology of white spot syndrome virus (WSSV) infection in shrimp, prawns, crabs and lobsters from India. Journal of Fish Diseases 22:183-191.

Ramos-Carreño, S., R. Valencia-Yáñez, F. Correa-Sandoval, N. Ruíz-García, F. Díaz-Herrera and I. Giffard-Mena. 2014. White spot syndrome virus (WSSV) infection in shrimp (*Litopenaeus vannamei*) exposed to low and high salinity. Archives of virology:DOI 10.1007/s00705-00014-02052-00700.

Reymond, H. and J.P. Lagardère. 1990. Feeding rhythms and food of *Penaeus japonicus* bate (Crustacea, Penaeidae) in salt marsh ponds; role of halophilic entomofauna. Aquaculture 84:125-143.

Rigaud, T., M.J. Perrot-Minnot and M.J.F. Brown. 2010. Parasite and host assemblages: Embracing the reality will improve our knowledge of parasite transmission and virulence. Proceedings of the Royal Society B: Biological Sciences 277:3693-3702.

Rodriguez, J., V. Boulo, E. Mialhe and E. Bachere. 1995. Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. Journal of Cell Science 108:1043-1050.

Roy, M. and N. Nandi. 2012. Distribution pattern of macrozoobenthos in relation to salinity of Hugli-Matla estuaries in India. Wetlands 32:1001-1009.

Safeena, M.P., P. Rai and I. Karunasagar. 2012. Molecular biology and epidemiology of hepatopancreatic parvovirus of penaeid shrimp. Indian Journal of Virology 23:191-202.

Sahoo, A., P. Patil and K. Shankar. 2005. White spots? A loaded question for shrimp farmers. Current Science 88:1914-1917.

Sánchez-Paz, A. 2010. White spot syndrome virus: An overview on an emergent concern. Veterinary Research 41:43.

Sangsuriya, P., S. Senapin, W.P. Huang, C.F. Lo and T.W. Flegel. 2011. Co-interactive DNA-binding between a novel, immunophilin-like shrimp protein and VP15 nucleocapsid protein of white spot syndrome virus. PLoS ONE 6.

Saraswathy, R., M. Muralidhar, M. Kailasam, P. Ravichandran, B.P. Gupta, K.K. Krishnani, A.G. Ponniah, J.K. Sundaray, A. Panigrahi and A. Nagavel. 2013. Effect of stocking density on soil, water quality and nitrogen budget in *Penaeus monodon* (Fabricius, 1798) culture under zero water exchange system. Aquaculture Research 44:1578-1588.

Sarkar, S.K., A. Bhattacharya, S. Giri, B. Bhattacharya, D. Sarkar, D.C. Nayak and A.K. Chattopadhaya. 2005. Spatiotemporal variation in benthic polychaetes (Annelida) and relationships with environmental variables in a tropical estuary. Wetlands Ecology and Management 13:55-67.

Satheesh Kumar, S., R. Ananda Bharathi, J.J.S. Rajan, S.V. Alavandi, M. Poornima, C.P. Balasubramanian and A.G. Ponniah. 2013. Viability of white spot syndrome virus (WSSV) in sediment during sun-drying (drainable pond) and under non-drainable pond conditions indicated by infectivity to shrimp. Aquaculture 402-403:119-126.

Scaps, P. 2002. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O.F. Müller)(Annelida: Polychaeta). Hydrobiologia 470:203-218.

References

Sethi, S.N., V. Mahendran, K. Nivas, P. Krishnan, S. Damroy, N. Ram and S. Sethi. 2011. Detection of white spot syndrome virus (WSSV) in brood stock of tiger shrimp, *Penaeus monodon* and other crustaceans of Andaman waters. Indian Journal of Marine Sciences 40:403-406.

Shi, Z., C. Huang, J. Zhang, D. Chen and J.R. Bonami. 2000. White spot syndrome virus (WSSV) experimental infection of the freshwater crayfish, *Cherax quadricarinatus*. Journal of Fish Diseases 23:285-288.

Shin, E., J.H. Park and Y. Lee. 2001. White spot syndrome virus in penaeid shrimp cultured in Korea. Journal of Microbiology and Biotechnology 11:394-398.

Shishehchian, F., F.M. Yusoff and M. Shariff. 2001. The effects of commercial bacterial products on macrobenthos community in shrimp culture ponds. Aquaculture International 9:429-436.

Sletten, O. and C.M. Bach. 1961. Modified stannous chloride reagent for orthophosphate determination. Journal of American Water Works Association 53:1031 - 1033.

Somboonna, N., S. Mangkalanan, A. Udompetcharaporn, C. Krittanai, K. Sritunyalucksana and T.W. Flegel. 2010. Mud crab susceptibility to disease from white spot syndrome virus is species-dependent. BMC Research Notes 3:Article Number 315.

Song, K.K., D.F. Li, M.C. Zhang, H.J. Yang, L.W. Ruan and X. Xu. 2010. Cloning and characterization of three novel WSSV recognizing lectins from shrimp *Marsupenaeus japonicus*. Fish and Shellfish Immunology 28:596-603.

Soowannayan, C. and M. Phanthura. 2011. Horizontal transmission of white spot syndrome virus (WSSV) between red claw crayfish (*Cherax quadricarinatus*) and the giant tiger shrimp (*Penaeus monodon*). Aquaculture 319:5-10.

Soto, D., J. Aguilar-Manjarrez, C. Brugère, D. Angel, C. Bailey, K. Black, P. Edwards, B. Costa-Pierce, T. Chopin, S. Deudero, S. Freeman, J. Hambrey, N. Hishamunda, D. Knowler, W. Silvert, N. Marba, S. Mathe, R. Norambuena, F. Simard, P. Tett, M. Troell and A. Wainberg. 2008. Applying an ecosystem-based approach to aquaculture: principles, scales and some management measures. In: Soto, D., J. Aguilar-Manjarrez, N.

Hishamunda (Eds.), Building an ecosystem approach to aquaculture. FAO/Universitat de les Illes Balears Expert Workshop. 7-11 May 2007, Palma de Mallorca, Spain. FAO Fisheries and Aquaculture Proceedings, Rome, FAO, pp. 15-35.

Soto, M.A. and J.M. Lotz. 2001. Epidemiological parameters of white spot syndrome virus infections in *Litopenaeus vannamei* and *L. setiferus*. Journal of Invertebrate Pathology 78:9-15.

Stalinraj, V., K.K. Vijayan, M. Sanjuktha, C.P. Balasubramanian, S.V. Alavandi and T.C. Santiago. 2009. Virulence status, viral accommodation and structural protein profiles of white spot syndrome virus isolates in farmed *Penaeus monodon* from the southeast coast of India. Aquaculture Research 40:129-138.

Stentiford, G.D. and D.V. Lightner. 2011. Cases of white spot disease (WSD) in European shrimp farms. Aquaculture 319:302-306.

Stentiford, G.D., J.R. Bonami and V. Alday-Sanz. 2009. A critical review of susceptibility of crustaceans to Taura syndrome, yellowhead disease and white spot disease and implications of inclusion of these diseases in European legislation. Aquaculture 291:1-17.

Stentiford, G.D., D.M. Neil, E.J. Peeler, J.D. Shields, H.J. Small, T.W. Flegel, J.M. Vlak, B. Jones, F. Morado, S. Moss, J. Lotz, L. Bartholomay, D.C. Behringer, C. Hauton and D.V. Lightner. 2012. Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. Journal of Invertebrate Pathology 110:141-157.

Subasinghe, R.P. 2005. Epidemiological approach to aquatic animal health management: Opportunities and challenges for developing countries to increase aquatic production through aquaculture. Preventive Veterinary Medicine 67:117-124.

Sunarto, A., I. Koesharyani, H. Supriyadi, L. Gardenia, B. Sugianti and D. Rukmono. 2004. Current status of transboundary fish diseases in Indonesia: Occurrence, surveillance, research and training, Transboundary Fish Diseases in Southeast Asia: Occurence, Surveillance, Research and Training. Proceedings of the Meeting on Current Status of Transboundary Fish Diseases in Southeast Asia: Occurence, Surveillance, Research and

Training, Manila, Philippines, 23-24 June 2004. SEAFDEC Aquaculture Department, pp. 91-121.

Sundar Raj, N., K.S. Nathiga Nambi, S. Abdul Majeed, G. Taju, S. Vimal, M.A. Farook and A.S. Sahul Hameed. 2012. High efficacy of white spot syndrome virus replication in tissues of freshwater rice-field crab, *Paratelphusa hydrodomous* (Herbst). Journal of Fish Diseases 35:917-925.

Supamattaya, K., R.W. Hoffmann, S. Boonyaratpalin and P. Kanchanaphum. 1998. Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus pelagicus*, mud crab *Scylla setrata* and krill *Acetes* sp. Diseases of Aquatic Organisms 32:79-85.

Takahashi, E., Q. Yu, G. Eaglesham, D.W. Connell, J. McBroom, S. Costanzo and G.R. Shaw. 2007. Occurrence and seasonal variations of algal toxins in water, phytoplankton and shellfish from North Stradbroke Island, Queensland, Australia. Marine Experimental Research 64:429-442.

Tan, L.T., S. Soon, K.L. Lee, M. Shariff, M.D. Hassan and A.R. Omar. 2001. Quantitative analysis of an experimental white spot syndrome virus (WSSV) infection in *Penaeus monodon* Fabricius using competitive polymerase chain reaction. Journal of Fish Diseases 24:315-323.

Tang, K., M. Le Groumellec and D.V. Lightner. 2013. Novel, closely related, white spot syndrome virus (WSSV) genotypes from Madagascar, Mozambique and the Kingdom of Saudi Arabia. Diseases of Aquatic Organisms 106:1-6.

Tang, K.F.J., S.A. Navarro, C.R. Pantoja, F.L. Aranguren and D.V. Lightner. 2012. New genotypes of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) from the Kingdom of Saudi Arabia. Diseases of Aquatic Organisms 99:179-185.

Tebit, D.M. and E.J. Arts. 2011. Tracking a century of global expansion and evolution of HIV to drive understanding and to combat disease. The Lancet Infectious Diseases 11:45-56.

Tendencia, E.A., R.H. Bosma and J.A.J. Verreth. 2010a. WSSV risk factors related to water physico-chemical properties and microflora in semi-intensive

Penaeus monodon culture ponds in the Philippines. Aquaculture 302:164-168.

Tendencia, E.A., R.H. Bosma and J.A.J. Verreth. 2011. White spot syndrome virus (WSSV) risk factors associated with shrimp farming practices in polyculture and monoculture farms in the Philippines. Aquaculture 311:87-93.

Tendencia, E.A., R.H. Bosma, R.C. Usero and J.A.J. Verreth. 2010b. Effect of rainfall and atmospheric temperature on the prevalence of the whitespot syndrome virus in pond-cultured *Penaeus monodon*. Aquaculture Research 41:594-597.

Tendencia, E.A., R.H. Bosma, J.H. Primavera and J.A.J. Verreth. 2012. Effect of different mangrove-to-pond area ratios on influent water quality and WSSV occurrence in *Penaeus monodon* semi-intensive farms using the greenwater culture technique. Aquaculture 362-363:72-79.

Tendencia, E.A., R.H. Bosma, M.C. Verdegem and J.A. Verreth. 2013. The potential effect of greenwater technology on water quality in the pond culture of *Penaeus monodon* Fabricius. Aquaculture Research.

Tsai, M., G. Kou, H. Liu, K. Liu, C. Chang, S. Peng, H. Hsu, C. Wang and C. Lo. 1999. Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. Diseases of Aquatic Organisms 38:107-114.

Tuyen, N.X., J. Verreth, J.M. Vlak and M.C.M. De Jong. 2014. Horizontal transmission dynamics of white spot syndrome virus by cohabitation trials in juvenile *Penaeus monodon* and *L. vannamei*. Preventive Veterinary Medicine in press.

Tzetlin, A. and G. Purschke. 2005. Pharynx and intestine. Hydrobiologia 535:199-225.

Unzueta-Bustamante, M.L., R. Silveira-Cofficny, A.A. Prieto, G. Aguirre-Guzmán and R. Vázquez-Juárez. 2004. Susceptibility of *Litopenaeus schmitti* and *Cherax quadricarinatus* to white spot syndrome virus (WSSV). Ciencias Marinas 30:537-545.

References

Van Etten, J. 2009. Lesser Known Large dsDNA Viruses: Preface. In: Van Etten, J. (Ed.), Current Topics in Microbiology and Immunology. Springer Verlag, Heidelberg, Germany, pp. v-vii.

Van Hulten, M.C., J. Witteveldt, S. Peters, N. Kloosterboer, R. Tarchini, M. Fiers, H. Sandbrink, R.K. Lankhorst and J.M. Vlak. 2001. The white spot syndrome virus DNA genome sequence. Virology 286:7-22.

Van Hulten, M.C.W. and J.M. Vlak. 2001. Identification and phylogeny of a protein kinase gene of white spot syndrome virus. Virus Genes 22:201-207.

Van Hulten, M.C.W., M. Westenberg, S.D. Goodall and J.M. Vlak. 2000a. Identification of two major virion protein genes of White Spot Syndrome virus of shrimp. Virology 266:227-236.

Van Hulten, M.C.W., M.F. Tsai, C.A. Schipper, C.F. Lo, G.H. Kou and J.M. Vlak. 2000b. Analysis of a genomic segment of white spot syndrome virus of shrimp containing ribonucleotide reductase genes and repeat regions. Journal of General Virology 81:307-316.

Vazquez-Boucard, C., P. Alvarez-Ruiz, C. Escobedo-Fregoso, G. Anguiano-Vega, M.d.J. Duran-Avelar, V.S. Pinto and C.M. Escobedo-Bonilla. 2010. Detection of white spot syndrome virus (WSSV) in the Pacific oyster *Crassostrea gigas*. Journal of Invertebrate Pathology 104:245-247.

Vega-Heredia, S., F. Mendoza-Cano and A. Sánchez-Paz. 2012. The infectious hypodermal and haematopoietic necrosis virus: a brief review of what we do and do not know. Transboundary and Emerging Diseases 59:95-105.

Vijayan, K.K., V. Stalin Raj, C.P. Balasubramanian, S.V. Alavandi, V. Thillai Sekhar and T.C. Santiago. 2005. Polychaete worms - A vector for white spot syndrome virus (WSSV). Diseases of Aquatic Organisms 63:107-111.

Vlak, J.M., J.R. Bonami, T.W. Flegel, G.H. Kou, D.V. Lightner, C.F. Lo, P.C. Loh and P.W. Walker. 2005. Nimaviridae. In: Fauquet, C.M., M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (Eds.), VIIIth Report of the International Committee on Taxonomy of Viruses. Princeton University Press, Princeton, NJ, pp. 187-192.

Waikhom, G., K.R. John, M.R. George and M. Jeyaseelan. 2006. Differential host passaging alters pathogenicity and induces genomic variation in white spot syndrome virus. Aquaculture 261:54-63.

Walker, P.J. and J.R. Winton. 2010. Emerging viral diseases of fish and shrimp. Veterinary Research 41:51.

Walker, P.J., N. Gudkovs, C.V. Mohan, V.S. Raj, B. Pradeep, E. Sergeant, A.B.C. Mohan, G. Ravibabu, I. Karunasagar and T.C. Santiago. 2011a. Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) ponds in Andhra Pradesh, India. II. Multiple WSSV genotypes associated with disease outbreaks in ponds seeded with uninfected postlarvae. Aquaculture 319:18-24.

Walker, P.J., N. Gudkovs, B. Pradeep, V. Stalin Raj, E. Sergeant, A.B. Chandra Mohan, G. Ravibabu, N.R. Umesh, I. Karunasagar, T.C. Santiago and C.V. Mohan. 2011b. Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. III. A complex dynamic of WSSV infection and WSSV genotype distribution in farmed shrimp and wild crustaceans. Aquaculture 319:319-327.

Walker, P.J., N. Gudkovs, P.A. Padiyar, V. Stalin Raj, B. Pradeep, E. Sergeant, A.B. Chandra Mohan, G. Ravibabu, K.K. Vijayan, I. Karunasagar, T.C. Santiago and C.V. Mohan. 2011c. Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. I. High prevalence of WSSV infection and low incidence of disease outbreaks in BMP ponds. Aquaculture 318:277-282.

Wang, C.-H., C.-F. Lo, J.-H. Leu, C.-M. Chou, P.-Y. Yeh, H.-Y. Chou, M.-C. Tung, C.-F. Chang, M.-S. Su and G.-H. Kou. 1995. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Diseases of Aquatic Organisms 23:239-242.

Wang, Q., B.L. White, R.M. Redman and D.V. Lightner. 1999a. Per os challenge of *Litopenaeus vannamei* postlarvae and *Farfantepenaeus duorarum* juveniles with six geographic isolates of white spot syndrome virus. Aquaculture 170:179-194.

References

Wang, Y. and J.A. Jehle. 2009. Nudiviruses and other large, double-stranded circular DNA viruses of invertebrates: New insights on an old topic. Journal of Invertebrate Pathology 101:187-193.

Wang, Y.C., C.F. Lo, P.S. Chang and G.H. Kou. 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164:221-231.

Wang, Y.G., M.D. Hassan, M. Shariff, S.M. Zamri and X. Chen. 1999b. Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. Diseases of Aquatic Organisms 39:1-11.

Wang, Y.G., K.L. Lee, M. Najiah, M. Shariff and M.D. Hassan. 2000. A new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon* and its comparison with white spot syndrome (WSS) caused by virus. Disease of Aquatic Organisms 41:9-18.

Watts, M.J., T.S. Barlow, M. Button, S.K. Sarkar, B.D. Bhattacharya, M.A. Alam and A. Gomes. 2013. Arsenic speciation in polychaetes (Annelida) and sediments from the intertidal mudflat of Sundarban mangrove wetland, India. Environmental Geochemistry and Health 35:13-25.

Withyachumnarnkul, B. 1999. Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). Diseases of Aquatic Organisms 39:21-27.

Withyachumnarnkul, B., V. Boonsaeng, R. Chomsoong, T.W. Flegel, S. Muangsin and G.L. Nash. 2003. Seasonal variation in white spot syndrome virus-positive samples in broodstock and post-larvae of *Penaeus monodon* in Thailand. Diseases of Aquatic Organisms 53:167-171.

Witteveldt, J. 2006. On the vaccination of shrimp against white spot syndrome virus. Wageningen University, Wageningen, The Netherlands, pp. 132.

Wongteerasupaya, C., P. Pungchai, B. Withyachumnarnkul, V. Boonsaeng, S. Panyim, T.W. Flegel and P.J. Walker. 2003. High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. Diseases of Aquatic Organisms 54:253-257.

Woolhouse, M.E.J. 2002. Population biology of emerging and re-emerging pathogens. Trends in Microbiology 10:S3-S7.

Woolhouse, M.E.J., L.H. Taylor and D.T. Haydon. 2001. Population biology of multihost pathogens. Science 292:1109-1112.

Xia, L.Z., L.Z. Yang and M.C. Yang. 2004. Nitrogen and phosphorus cycling in shrimp ponds and the measures for sustainable management. Environmental Geochemistry and Health 26:245-251.

Xu, H., F. Yan, X. Deng, J. Wang, T. Zou, X. Ma, X. Zhang and Y. Qi. 2009. The interaction of white spot syndrome virus envelope protein VP28 with shrimp Hsc70 is specific and ATP-dependent. Fish and Shellfish Immunology 26:414-421.

Yang, F., J. He, X. Lin, Q. Li, D. Pan, X. Zhang and X. Xu. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. Journal of Virology 75:11811-11820.

Yi, G., Z. Wang, Y. Qi, L. Yao, J. Qian and L. Hu. 2004. Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. Journal of Biochemisty and Molecular Biology 37:726-734.

Yoganandhan, K., S. Thirupathi and A. Sahul Hameed. 2003. Biochemical, physiological and hematological changes in white spot syndrome virus-infected shrimp, *Penaeus indicus*. Aquaculture 221:1-11.

You, X.X., Y.Q. Su, Y. Mao, M. Liu, J. Wang, M. Zhang and C. Wu. 2010. Effect of high water temperature on mortality, immune response and viral replication of WSSV-infected *Marsupenaeus japonicus* juveniles and adults. Aquaculture 305:133-137.

Youtong, W., P. Deachamag, A. Phongdara and W. Chotigeat. 2011. WSSV: VP26 binding protein and its biological activity. Fish and Shellfish Immunology 30:77-83.

Yuvanatemiya, V., C.E. Boyd and P. Thavipoke. 2011. Pond bottom management at commercial shrimp farms in Chantaburi province, Thailand. Journal of the World Aquaculture Society 42:618-632.

References

Zhan, W.B., Y.H. Wang, J.L. Fryer, K.K. Yu, H. Fukuda and Q.X. Meng. 1998. White spot syndrome virus infection of cultured shrimp in China. Journal of Aquatic Animal Health 10:405-410.

Zhang, J., S. Dong, X. Tian, Y. Dong, X. Liu and D. Yan. 2007. Virus-phytoplankton adhesion: A new WSSV transmission route to zooplankton. Acta Oceanologica Sinica 26:109-115.

Zhang, J.S., S.L. Dong, Y.W. Dong, X.L. Tian and C.Q. Hou. 2008. Bioassay evidence for the transmission of WSSV by the harpacticoid copepod *Nitocra* sp. Journal on Invertebrate Pathology 97:33-39.

Zhang, J.S., S.L. Dong, X.L. Tian, Y.W. Dong, X.Y. Liu and D.C. Yan. 2006. Studies on the rotifer (*Brachionus urceus* Linnaeus, 1758) as a vector in white spot syndrome virus (WSSV) transmission. Aquaculture 261:1181-1185.

Zhang, J.S., S.L. Dong, Y.W. Dong, X.L. Tian, Y.C. Cao, Z.J. Li and D.C. Yan. 2010. Assessment of the role of brine shrimp *Artemia* in white spot syndrome virus (WSSV) transmission. Veterinary Research Communications 34:25-32.

Zwart, M.P., B.T.M. Dieu, L. Hemerik and J.M. Vlak. 2010. Evolutionary trajectory of white spot syndrome virus (WSSV) genome shrinkage during spread in Asia. PLoS ONE 5.

SUMMARY

White spot syndrome virus (WSSV), the etiological agent of white spot disease (WSD) of shrimp is the most devastating virus of farmed shrimp worldwide. It is a large double stranded DNA virus belonging to the genus *Whispovirus* and the family *Nimaviridae*. WSSV is a generalist virus that infects a broad range of benthic invertebrates. Reported WSSV hosts and vectors include members of 34 crustacean families (the majority of which are crabs) and 8 non-crustacean benthic invertebrates (Chapter 2). Considering the close proximity of shrimp and sediment, and the confinement of ponds, it is clear that many non-crustacean invertebrates commonly present in the pond environment may be potential hosts or vectors of WSSV. Non-crustacean invertebrates may acquire WSSV through feeding on WSSV-infected shrimp carcasses, by ingestion of WSSV-contaminated sediment or WSSV attachment to plankton, and later transmit the virus to cultured crustaceans through the food web. *Dendronereis* spp (Pieters 1854) are ubiquitous Nereid polychaete in the pond environment and part of the shrimp's natural diet. Continuous exposure to WSSV may render this polychaete susceptible for WSSV infection. The current study aimed to investigate the role of *Dendronereis* spp. as vector or carrier in WSSV transmission in shrimp ponds. Such a role was inferred from the ecological niche of *Dendronereis* spp. in ponds and from the observation that WSSV persists in the pond environment, even after abandoning shrimps culture.

The study started with a survey to determine the occurrence of WSSV in *Dendronereis* spp. in Indonesia. The sampling was done in 13 traditional shrimp ponds which experienced white spot disease between 1 month and 1 year before sampling. These ponds were located on the Mahakam delta in East Kalimantan (5 ponds) and in the vicinity of Semarang in Central Java (8 ponds). In each pond,

Dendronereis spp. and sediment were obtained from 9 or 12 evenly spaced sampling points. WSSV in *Dendronereis* spp. was detected with nested PCR. In addition, pH, total N, P and organic carbon concentrations, bulk density and the percentages clay, sand and silt in the mineral fraction of the sediment were determined. WSSV-infected *Dendronereis* spp. were detected in 70 % of the surveyed ponds. Average point prevalence of WSSV infection in *Dendronereis* spp. was 44 ± 27% (± SD). The average prevalence in Kalimantan was 73 ± 22% and in Java 26 ± 38%. Overall, the survey results revealed that WSSV infection in *Dendronereis* spp. is common in the traditional ponds surveyed and widely spread in Indonesia (Chapter 6). This finding became the knowledge base to carry out further studies on the role of *Dendronereis* spp. in WSSV transmission in shrimp ponds.

Important questions were whether WSSV replicates in this non-crustacean host and whether *Dendronereis* spp. are vector or a mere carrier (passive vector) of the virus. WSSV replication in naturally infected *Dendronereis* spp. was determined by (i) immunohistochemistry (IHC) using a monoclonal antibody against the WSSV VP28 protein and (ii) nested RT-PCR using a specific primer set for the *vp28* gene to detect WSSV-specific mRNA. The presence of WSSV immunoreactive-nuclei in the gut epithelium of *Dendronereis* spp. and the presence of WSSV mRNA showed that WSSV replicates in *Dendronereis* spp. (Chapter 3). This finding verified that WSSV could replicate in *Dendronereis* spp., and thus changed the view that polychaetes are only a passive or mechanical vector of WSSV. It is also the first report of WSSV replication in a non-crustacean host.

To further study WSSV infection in polychaetes, a model polychaete species which easily adapts and grows under laboratory conditions and is free of, yet susceptible to, WSSV infection was needed. Because the result of the survey indicated that WSSV in *Dendronereis* spp. is widespread, another cultivable nereid

polychaete, *Hediste diversicolor* (Müller 1776) (common ragworm), was selected as candidate for being a model polychaete organism to study WSSV replication. WSSV-free *H. diversicolor* was experimentally infected by injection, feeding or immersion, and the infection was followed for 12 days post infection (dpi). Furthermore, polychaete survival was determined 40 dpi. *H. diversicolor* was able to clear the virus within 4 dpi without showing clinical signs and WSSV-associated mortality. The virulence of the inoculum used was high enough to kill *L. vannamei* shrimp (Chapter 4). It was concluded that *H. diversicolor* at this moment may not be a suitable model animal for WSSV studies in polychaetes. However, other routes of infection such as feeding WSSV-infected plankton or WSSV-containing sediment are worth to try before abandoning *H. diversicolor* as model animal.

WSSV transmission from naturally infected *Dendronereis* spp. to specific pathogen free (SPF) pacific white shrimp *L. vannamei* and further to new naïve shrimp was established through a sequence of bioassays. *Dendronereis* spp. and *Penaeus monodon* (Fabricius) used in the experiments were obtained from a traditional shrimp pond. As measured by PCR, WSSV-infected *Dendronereis* spp. and *P. monodon* in this pond had a point prevalence of 90% and 80%, respectively, indicating that WSSV infection occurred in both species with comparable prevalence. WSSV was detected in the head, gills, blood and mid-body of *Dendronereis* spp. suggesting that WSSV is circulated in the body of *Dendronereis* spp.. WSSV from naturally infected *Dendronereis* spp. was transmitted to *L. vannamei* and subsequently from this shrimp to new naïve-SPF *L. vannamei* to cause transient infection (Chapter 5), suggesting that a similar situation may occur in the pond. This result supports the view that *Dendronereis* spp. can be a source of WSSV infection of shrimp in ponds via feeding. The transient infection observed in *L. vannamei* post transmission might have been caused by the low viral load in *Dendronereis* spp. Another possibility is that the pathogenicity of WSSV

was reduced in *Dendronereis* spp. because of the taxonomic distance to shrimp and the lack of time for adaptation.

Being a benthic animal, sediment conditions influence the physiology of *Dendronereis* spp, hence its susceptibility to viral infection. Among the tested parameters, *Dendronereis* spp. density and, to a lesser extent, C:N ratio were associated with WSSV infection in *Dendronereis* spp. The soil parameters pH, total N, P and organic carbon concentrations, bulk density and the percentages clay, sand and silt had no effect on density and prevalence of WSSV in *Dendronereis* spp. Prevalence of WSSV in shrimp in the sampled ponds was positively correlated with WSSV prevalence in the polychaete (Chapter 6). This may imply that WSSV infection in *Dendronereis* spp. originated from shrimp. In addition, WSSV transmission among many putative hosts and vectors in pond environments is complex as depicted in a model presented in Chapter 7. However, whether pathogen spill-over occurred from the shrimp and the importance and role of this plethora of hosts in WSSV transmission in ponds need further study.

In conclusion, this research showed that *Dendronereis* spp. are reservoir host of WSSV in ponds. WSSV infection in *Dendronereis* spp. was common in the surveyed locations. Using IHC and RT-PCR, we demonstrated that WSSV replicated in *Dendronereis* spp. This confirmed that WSSV infect also cross-phylum hosts. WSSV was transmitted from *Dendronereis* spp. to SPF *L. vannamei* by oral route. Attempts to use *H. diversicolor* as a model animal to study WSSV infection in polychaetes failed, but other nereids may be useful here. WSSV infection in *Dendronereis* spp. in shrimp ponds was influenced by the polychaete density and the prevalence of WSSV infection in shrimp.

SAMENVATTING

White spot syndroom virus (WSSV), de veroorzaker van de witte stip ziekte (WSZ) in garnalen is wereldwijd het meest schadelijke virus in deze teelt. Het is een groot dubbel-strengig DNA-virus, het Whispovirus behorende tot de familie Nimaviridae. WSSV is een generalist-virus dat een breed scala aan ongewervelde bodemorganismen infecteert. Gerapporteerde WSSV-gastheren en -vectoren omvatten 34 families van schaaldieren (waarvan de meerderheid krabben zijn) en 8 niet-schaaldieren (Hoofdstuk 2). Omdat vijvers vrij besloten zijn en garnalen en sediment dus onlosmakelijk met elkaar verbonden zijn, is het niet uitgesloten dat niet-schaaldier ongewervelden, die in vijvers algemeen voorkomen, potentiële gastheren of vectoren van WSSV zijn. Deze niet-schaaldier ongewervelden kunnen besmet worden met WSSV door WSSV-besmette garnaal-karkassen te eten of via inname van WSSV-verontreinigd sediment of plankton, en vervolgens het virus doorgeven aan gekweekte schaaldieren via de voedselketen. *Dendronereis* spp. (Pieters 1854) zijn in garnalenkweekvijvers algemeen voorkomende borstelwormen behorende tot de familie Nereidae, die onderdeel uitmaken van het natuurlijke dieet van garnalen. Voortdurende blootstelling aan WSSV kan deze borstelworm gevoeliger maken voor WSSV-infectie. Deze studie onderzocht de al dan niet mogelijke vector- of dragerrol van *Dendronereis* spp. in de overdracht van WSSV naar garnaal in garnalenvijvers. Dat *Dendronereis* spp. een dergelijke rol zou kunnen vervullen werd deels afgeleid uit de ecologische niche waarin deze worm zich bevindt en uit de voortdurende aanwezigheid van WSSV in vijvers, zelfs na het stopzetten van de teelt.

Het onderzoek begon met het bepalen van de aanwezigheid van WSSV in *Dendronereis* spp. in Indonesië. Voor dit onderzoek werden 13 traditionele garnalen vijvers bemonsterd waarin een uitbraak van WSZ was vastgesteld, 1

maand tot 1 jaar vóór bemonstering. De vijvers waren gelegen in de Mahakam-delta op Oost-Kalimantan (5 vijvers) of in de buurt van Semarang op Midden-Java (8 vijvers). In elke vijver werden *Dendronereis* spp. en sediment bemonsterd in 9 of 12 gelijkmatig over het vijveroppervlak verspreide monsterpunten. WSSV werd vastgesteld in *Dendronereis* spp. met behulp van 'nested PCR', een moleculaire techniek om de aanwezigheid van het virus vast te stellen. In het sediment werden pH, totaal-stikstof (N), -fosfaat (P) en organische koolstof concentraties (OC), bulkdichtheid, en de percentages klei, zand en slib in de minerale fractie, bepaald. Geïnfecteerde *Dendronereis* spp. waren aanwezig in 70 % van de bemonsterde vijvers. De gemiddelde WSSV-puntprevalentie met *Dendronereis* spp. was 44 ± 27 % (± SD). De gemiddelde prevalentie was 73 ± 22% op Kalimantan en 26 ± 38 % op Java. Deze resultaten doen vermoeden dat *Dendronereis* spp. wellicht algemene dragers zijn van WSSV in traditionele vijvers in Indonesië (Hoofdstuk 6). Dit resultaat werd het uitgangspunt voor verder onderzoek naar de mogelijke rol van *Dendronereis* spp. bij WSSV-overdracht in garnaalvijvers.

Belangrijke vragen in dit onderzoek waren of.WSSV zich vermenigvuldigt in deze niet-schaaldier gastheren en of *Dendronereis* spp. vectoren of slechts dragers (passieve vector) zijn van het virus. Dat WSSV zich vermenigvuldigt in natuurlijk geïnfecteerde *Dendronereis* spp. werd vastgesteld aan de hand van 2 onafhankelijke technieken (i) immunohistochemische analyse (IHC) met een monoklonaal antilichaam tegen het WSSV VP28-eiwit en (ii) 'nested RT-PCR' met een primer specifiek voorhet *vp*28-gen om WSSV-mRNA te detecteren. Het aantreffen van WSSV immunoreactieve kernen in het darmepitheel van *Dendronereis* spp. en de detectie van WSSV-mRNA toonden aan dat WSSV zich vermenigvuldigt in *Dendronereis* spp. (Hoofdstuk 3). Op basis van dit resultaat kan de stelling dat borstelwormen slechts een passieve of mechanische vector zijn van

WSSV bijgesteld worden. Dit is tevens ook de eerste keer dat vermenigvuldiging van WSSV in een niet-schaaldier gastheer werd aangetoond.

Om onderzoek naar WSSV-infectie in borstelwormen te bevorderen zou het goed zijn over een model soort te beschikken die WSSV-infectievrij is, maar ook infectiegevoelig, en die zich gemakkelijk aanpast aan en groeit onder laboratoriumomstandigheden. Omdat het veldonderzoek aantoonde dat WSSV-infectie in *Dendronereis* spp. algemeen voorkomt en deze diersoort in gevangenschap niet kan worden gekweekt, werd een andere algemeen voorkomende borstelwormsoort, *Hediste diversicolor* (Müller 1776) (veelkleurige zeeduizendpoot) gekozen als modelorganisme om WSSV-replicatie te bestuderen. Bovendien is *H. diversicolor* gemakkelijk in gevangenschap te houden en voort te planten. WSSV-vrije *H. diversicolor* werd experimenteel geïnfecteerd via injectie, voeding of immersie, en het verloop van de infectie werd gevolgd tot 12 dagen na infectie (dni). Het percentage overlevende dieren werd 40 dni bepaald. *H. diversicolor* was in staat het virus binnen 4 dagen na infectie te elimineren zonder daarbij klinische symptomen te ontwikkelen of WSSV gerelateerde sterfte te vertonen. De virulentie van het gebruikte inoculum was hoog genoeg om witte garnaal *Litopenaeus vannamei* te doden (Hoofdstuk 4). De conclusie was dat *H. diversicolor* niet een geschikt modeldier is om WSSV-infectie in borstelwormen te bestuderen. Andere infectie routes, zoals bijvoorbeeld het voeden met WSSV-geïnfecteerd plankton of WSSV-bevattend sediment, dienen echter onderzocht te worden alvorens voorgoed af te stappen van *H. diversicolor* als model dier.

WSSV-overdracht van natuurlijk-geïnfecteerde *Dendronereis* spp. naar specifiek-pathogeenvrije *L. vannamei* en vervolgens naar gezonde garnaal werd onderzocht in een reeks bio-toetsen. De experimenteel gebruikte *Dendronereis* spp. en *Penaeus monodon* (Fabricius) werden gevangen in een traditionele garnalenvijver, waarin de met PCR gemeten WSSV-punt-prevalentie in

Dendronereis spp. 90% was en 80% in *Penaeus monodon*. Het besmettingsniveau in de bemonsterde vijver was voor beide diersoorten vergelijkbaar. WSSV werd gevonden in hoofd-, kieuw-, bloed- en middenlichaamweefsel van *Dendronereis* spp., hetgeen er op wijst dat WSSV in het garnalenlichaam circuleert. WSSV werd overgedragen van natuurlijk-geïnfecteerde *Dendronereis* spp. naar *L. vannamei* en vervolgens naar specifiek ziektevrije *L. vannamei* bij wie het een infectie van voorbijgaande aard veroorzaakte (Hoofdstuk 5). Hetzelfde gebeurt vermoedelijk ook in kweekvijvers, waar *Dendronereis* spp. een WSSV infectiebron is voor op borstelwormen foeragerende garnalen. Het feit dat de infectie in *L. vannamei* van voorbijgaande aard was werd wellicht mede veroorzaakt door de lage virus concentratie in de toegediende *Dendronereis* spp. Het is ook niet uit te sluiten dat de infectiekracht van WSSV voor garnalen afgezwakt werd in *Dendronereis* spp. omdat borstelwormen en garnalen taxonomisch ver van elkaar staan of omdat het virus onvoldoende tijd had zich aan te passen.

Voor bodembewoners als *Dendronereis* spp. beïnvloeden omgevingsomstandigheden in het sediment de fysiologie, inclusief de vatbaarheid voor virale infecties. Van de geteste parameters, hielden vooral de dichtheid waarin *Dendronereis* spp. voorkomt en, in mindere mate de C:N ratio, verband met het optreden van WSSV-infectie in *Dendronereis* spp. De bodemparameters pH, N-, P- en organische koolstof-concentratie, bulkdichtheid en de percentages aan klei, leem en zand in het sediment, hadden geen effect op de dichtheid en besmettingsgraad van WSSV in *Dendronereis* spp. De besmettingsgraad van WSSV in garnaal was positief gecorreleerd aan de besmettingsgraad in de borstelworm (Hoofdstuk 6). Dit kan er op wijzen dat de WSSV-infectie in *Dendronereis* spp. afkomstig was van garnalen. Bovendien is WSSV-overdracht tussen de vele vermeende gastheren en vectoren in vijvers complex, zoals schematisch getoond werd in Hoofdstuk 7. Echter, of het pathogeen zich daadwerkelijk verspreidt vanuit de garnaal en wat het

belang en rol zijn van de veelheid en verscheidenheid aan mogelijke gastheren in WSSV-overdracht in vijvers, dient verder onderzocht te worden.

Tot slot, dit onderzoek toonde aan dat *Dendronereis* spp. een reservoir-gastheer kan zijn voor WSSV in garnalenvijvers. WSSV-infectie in *Dendronereis* spp. kwam algemeen voor in de onderzochte geografische locaties. Met IHC en RT-PCR werd aangetoond dat WSSV zich vermenigvuldigt in *Dendronereis* spp. en dus gastheren infecteert die behoren tot zeer verschillende hoofdorganismen van het dierenrijk. WSSV werd overgedragen van *Dendronereis* spp. naar specifiek pathogeen-vrije *L. vannamei* via de orale route. Een poging *H. diversicolor* te gebruiken als modeldier om WSSV infectie te bestuderen in borstelwormen mislukte, maar wellicht kunnen andere soorten hiervoor alsnog gebruikt worden. WSSV-infectie in *Dendronereis* spp. was positief gecorreleerd aan de borstelworm dichtheid en prevalentie van WSSV infectie in garnalen.

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WIAS Training and Supervision Plan

The Basic Package (3 ECTS)

WIAS Introduction Course	2007
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Scientific Exposure (11.6 ECTS)

International conferences

Asian Fisheries Aquaculture Forum, Shanghai, China.21-25 April	2011
Performing research, The Indonesian-Netherlands Open Science Meeting, KNAW-LIPI, Amsterdam, 16 November	2009
Valorisation of Knowledge. Boosting technologically oriented cooperation between Indonesia and the Netherlands in the area of Biobased Economy, Denhaag. Open Siciensce Meeting, KNAW-LIPI, 17 Nov 2009.	2009

Seminars and workshops

The 1st RESCOPAR Scientific meeting	2007
RESCOPAR seminar series	2007
Workshop WSSV challenge test, Can Tho University, Can Tho and RIA2 Ho Chi Minh City, Vietnam	2009
The 2nd RESCOPAR Scientific Meeting, Mulawarman University, Samarinda, Indonesia	2009
The 3rd RESCOPAR Scientific meeting, Can Tho University, Vietnam	2010
Ciliary dysfunction in developmental abnormalities and Diseases	2012

Presentations

Oral presentation at the 1st RESCOPAR Scientific meeting Wageningen 30-31 August.	2007
Oral presentation at the Annual seminar on research in Fisheries and Marine Sciences, Gajah Mada University, Jogyakarta.	2008
Oral presentation at the Open Science Meeting, KNAW-LIPI, Amsterdam, 16 November	2009
Oral presentation at the Conference of Indonesian Aquaculture, Indonesian Society of Aquaculture, Jogyakarta.	2009
Poster at the Global Aquaculture Conference, Bangkok, Thailand.	2010

Oral presentation at the Asian Fisheries Aquaculture Forum, Shanghai, China. 21-25 April.	2011
Oral presentation at the Symposium on Coastal Resources Management and Development, Diponegoro University-Indonesian Association of Oceanography, Semarang, Indonesia.	2011

In-Depth Studies (9.7 ECTS)

Disciplinary and interdisciplinary courses

Workshop on Fish Immunology, Wageningen	2007
UT-ITC Master Class Water Research and Management, University of Twente, Twente, The Netherlands. PhD level. 3-day course	2009

Advanced statistics courses

Statistics for animal science	2007

MSc level courses

Aquatic ecology and water quality management	2007

Professional Skills Support Courses (3.5 ECTS)

Ph.D Scientific Writing	2007
Competency for Integrated Agriculture Research	2007
High-Impact writing	2013

Research Skills Training (9.1 ECTS)

Preparing own PhD research proposal	2007
Short Course on *Dendronereis* spp. culture, Jendral Soedirman University, Purwokerto, Indonesia	2008
Laboratory exercise and management workshop at Department and Faculty level, Diponegoro University, Semarang, Indonesia	2010
Laboratory exercise and management workshop at Department and Faculty level, Diponegoro University, Semarang, Indonesia	2010

Didactic Skills Training (15.0 ECTS)

Lecturing

Fish Parasites and Diseases	2007
Fish Parasites and Diseases	2008
Fish Histology	2008
Fish health Management	2009

Supervising theses
Supervising 1 M.Sc. students (major) and 6 B.Sc thesis 2008

Management Skills Training (0.5 ECTS)
Organization of seminars and courses
Assisting 1st RESCOPAR Scientific meeting 2007

Education and Training Total: 52.4 ECTS

LIST OF PUBLICATIONS

Referred Scientific Journal

Desrina, J.A.J. Verreth, S.B. Prayitno, J.H.W.M. Rombout, J.M. Vlak, M.C.J. Verdegem. Replication of White Spot Syndrome Virus (WSSV) In the Polychaete *Dendronereis* spp. Journal of Invertebrate Pathology, 2013, 114: 7–10.

Desrina, J.A.J. Verreth, J.M. Vlak And M.C.J. Verdegem. 2014. *Hediste diversicolor* (O.F. Müller 1776) as a Possible Model to Study White Spot Syndrome Virus Infection in Polychaetes. Journal of the Asian Fisheries Society, 27: 3 (In Press).

D. Haryadi, Johan A.J. Verreth, Marc C.J. Verdegem and Just M. Vlak. Transmission of White Spot Syndrome Virus (WSSV) From *Dendronereis* spp. (Peters) (Nereididae) to Penaeid Shrimp. Journal of Fish Disease (Doi:10.1111/Jfd.12247).

Desrina, Marc C.J. Verdegem, Johan A.J. Verreth, Just M. Vlak. On The Transmission ofthe White Spot Syndrome Virus: A Review. Submitted To Reviews in Aquaculture

Desrina, Just M. Vlak, S. Budi Prayitno, Johan. A. Verreth, Marc C.J. Verdegem. White Spot Syndrome Virus Infection in the Polychaete *Dendronereis* spp. in Shrimp Ponds. Submitted to Aquaculture.

Desrina, Sarjito, A.H. Condro Haditomo D. Chilmawati. 2012. The White Spot Syndrome Virus (WSSV) Load in *Dendronereis* spp. Journal of Coastal Development 15: 270-275.

List of Publications

Desrina, A.Taslihan, Ambariyanto, Budhi Kuncoro Jati. 2011. Effect of dose on the efficacy of vaccine protein outer membrane 74 kDa in Epinephelus fuscogutatus. Indonesian Journal of Marine Sciences 16 (2): 95-102.

Conference Proceedings and Abstracts

Desrina, Slamet Budi Prayitno, Marc Verdegem, J.A J. Verreth, 2009. The occurrence of WSSV in the Polychaete from the shrimp ponds in the Mahakam Delta, Presented on Conference of Indonesian Aquaculture, Indonesian Society of Aquaculture, 27-29 October 2009. Jogyakarta.

Desrina, J. Verreth, Marc Verdegem, Slamet Budi Prayitno. White Spot Syndrome Virus (WSSV) infection in polychaetes from shrimp ponds in Semarang vicinity, Indonesia. Open Science Meeting. Held by KNAW and LIPI.16 November 2009. Amsterdam.

Desrina, Marc Verdegem & Slamet Budi. "Prevalence of WSSV infection in Dendronereis spp from selected ponds in Indonesia", Global Aquaculture 2010, 22-25 Sept. 2010 Phuket, Thailand.

Desrina, Arief Taslihan, Ambariyanto, Ervia Yudiaty, Triyanto, Komari, Andika Tri Kuswara, 2010. Effects of vaccination methods of protein outer membrane Vibrio alginolyticus 74 kDa on specific immunity of grouper Epinephelus fuscoguttatus). Paper presented in Indonesian on the Fifth Annual National Seminar on Research of Fisheries and Marine Sciences, held by Department of Fisheries and Marine Science, Faculty of Agriculture, Gadjah Mada University, Jogjakarta.

Desrina, M.C.J. Verdegem, S.B. Prayitno, J. Rombout, J.A. Vlak, J.A.J. Verreth. Detection of White Spot Syndrome virus (WSSV) in naturally infected Dendronereis spp with immunohistochemistry. Presented in 9 Asian Fisheries Aquaculture Forum, 21-25 April 2011, Shanghai, China.

Desrina, A.H. Condro Haditomo, Sarjito. White Spot Syndrome Virus (WSSV) infection in Dendronereis spp from traditional shrimp pond: viral load and infected tissues. Symposium on Coastal Resources Management and Development. 29-30 November 2011. Semarang.

Desrina, A.H. Condro Haditomo and Sarjito. Polychaete role in WSSV epidemiology in ponds. Presented on Seminar on Integrated Outstanding Research funded by Directorate of Higher Education, Ministry of Culture and Education. 8-9 June 2012. Semarang. (Best Poster Award).

RESCOPAR Project

Rebuilding resilience of coastal populations and aquatic resources: habitats, biodiversity and sustainable use options

On the Role of the Polychaete *Dendronereis* spp. in the Transmission of White Spot Syndrome Virus in Shrimp Ponds

Desrina

2014

Desrina was born in Jakarta 15 December 1965. She graduated from the Faculty of Fisheries Riau University, Pekanbaru, Indonesia in 1989 and employed by this University as junior lecturer. She obtained the M.Sc degree in Aquaculture focusing on desease from Auburn University, Auburn, U.S.A. in 1994 funded by USAID. She started her sandwich Ph.D program at the Aquaculture and Fisheries Departement, Wageningen University in 2007 funded by the Wageningen University through the RESCOPAR project. She is currently working as a lecturer and researcher at the Faculty of Fisheries and Marine Science Diponegoro University, Semarang, Indonesia. Her research included bacterial disease of fish and viral disease of shrimp.