

# Horizontal transmission dynamics of White spot syndrome virus by cohabitation trials in juvenile *Penaeus monodon* and *P. vannamei*



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## ABSTRACT

White spot syndrome virus (WSSV), a rod-shaped double-stranded DNA virus, is an infectious agent causing fatal disease in shrimp farming around the globe. Within shrimp populations WSSV is transmitted very fast, however, the modes and dynamics of transmission of this virus are not well understood. In the current study the dynamics of disease transmission of WSSV were investigated in small, closed populations of *Penaeus monodon* and *Penaeus vannamei*. Pair cohabitation experiments using PCR as a readout for virus infection were used to estimate transmission parameters for WSSV in these two species. The mortality rate of contact-infected shrimp in *P. monodon* was higher than the rate in *P. vannamei*. The transmission rate parameters for WSSV were not different between the two species. The relative contribution of direct and indirect transmission rates of WSSV differed between the two species. For *P. vannamei* the direct contact transmission rate of WSSV was significantly lower than the indirect environmental transmission rate, but for *P. monodon*, the opposite was found. The reproduction ratio  $R_0$  for WSSV for these two species of shrimp was estimated to be above one: 2.07 (95%CI 1.53, 2.79) for *P. monodon* and 1.51 (95%CI 1.12, 2.03) for *P. vannamei*. The difference in  $R_0$  between the two species is due to a lower host mortality and hence a longer infectious period of WSSV in *P. monodon*.

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## 1. Introduction

White spot syndrome virus (WSSV) is a serious pathogen of penaeid shrimp. Outbreaks of WSSV were first reported in 1992 for cultured *Penaeus japonicus* shrimp in

Zhangpu in Fujian Province, China (Lo et al., 2005). Within two years, Taiwan reported that WSSV caused disease in three cultured shrimp species *Penaeus monodon*, *P. japonicus*, and *P. penicillatus* (Chou et al., 1995). Within a decade from these first observations WSSV had spread very fast and caused losses in fourteen shrimp producing countries of Asia (NACA, 2002). In 1995, WSSV was found in shrimp farms along the coastal area in the Gulf of Mexico (Lightner et al., 1997). Subsequently WSSV was reported in nine countries in the Americas (OIE, 2003), most notably in Brazil (Cavalli et al., 2008) and Argentina (Martorelli et al., 2010). WSSV is now considered a global epidemic, having

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also recently been found in Saudi Arabia and off the African coast in Madagascar (Flegel, 2012). Transmission of WSSV between countries is thought to occur mainly through the transport of both live and frozen uncooked shrimp (Nunan et al., 1998; Durand et al., 2000), as well as imports of brood stock with latent WSSV (Stentiford et al., 2012).

A typical outbreak in cultured shrimp starts one or two days after the introduction of virus or virus-infected shrimp and is usually followed by a mass mortality of up to 70–100% of the shrimp population in the next five to seven days (Chou et al., 1995). *P. monodon*, infected by WSSV, often show reduced movement and food consumption, discoloration of the body from pinkish to reddish, occurrence of white spots of 0.5–2.0 mm in diameter under the surface of the exoskeleton, and rapid mortality (Lightner, 1996). WSSV is a non-occluded virus (Wang et al., 1995) with a large double-stranded super-coiled DNA genome that varies in size from 292.96 to 305.1 kilobase pairs in size (van Hulst et al., 2001; Yang et al., 2001). It was classified to belong to the genus *Whispovirus* of a newly established virus family *Nimaviridae* (Lo et al., 2011).

As reviewed by Sánchez-Paz (2010), WSSV has a wide range of arthropods, particularly of decapods, as hosts and carriers which have been identified in both challenge experiments and in the wild. All cultured marine shrimps are susceptible to WSSV (Walker and Mohan, 2009). Other aquatic and benthic organisms can be virus carriers or reservoirs and these include polychaete worms (Vijayan et al., 2005; Desrina et al., 2013), microalgae (Liu et al., 2007) and rotifer eggs (Yan et al., 2004).

WSSV can transmit between individuals of the same host species (Chou et al., 1998) or between different host species (Waikhom et al., 2006). Horizontal transmission of WSSV within a host population is influenced by particular biological factors, including aggressive behavior and predation (Wu et al., 2001; Soto et al., 2001), different ages of shrimp (Lightner et al., 1998; Venegas et al., 2000), difference in the virulence of WSSV strains (Wang et al., 1999; Marks et al., 2005; Zwart et al., 2010), virus passing through different hosts (Lightner et al., 1998; Rajendran et al., 1999; Waikhom et al., 2006), high densities of hosts (Wu et al., 2001) and meteorological conditions (Tendencia et al., 2011). Physiological stressors such as water quality parameters (temperature, dissolved oxygen, salinity, etc.), outside the optimal ranges for shrimp growth can also influence transmission (Rahman et al., 2006; Esparza-Leal et al., 2010; Gunalan et al., 2010). Transmission through different infection routes (Soto and Lotz, 2001) can affect the rate of horizontal transmission of WSSV, and there is also evidence that WSSV can be vertically transmitted to the next generation from brooders (Lo et al., 1997; Hsu et al., 1999). Observationally epidemiological studies on the risk factors of WSSV under culture pond conditions identified the following risk factors: stocking outside of right time of the year to stock, slow growth of shrimp (Corsin et al., 2001), sharing water with other farms and high stocking density (Tendencia et al., 2011).

Shrimp aquaculture offers a suitable system to improve our currently limited understanding of the dynamics of aquatic animal diseases transmission. However, one experiment has been conducted on the transmission by

cohabitation of infected and susceptible shrimp (Soto and Lotz, 2001). In that paper four experimental groups were used starting with one infected shrimp and 12 susceptible in-contact shrimp. The results showed that contact infection was only observed in one. It can be concluded that they only observed a minor outbreak with a reproduction ratio  $R_0$  (the average number of new cases caused by one typical infected shrimp in a susceptible population) estimated less than one (Diekmann et al., 1990; de Jong, 1995). However, the  $R_0$  should be larger than one. Their experiments still leave the possibility for  $R_0 > 1$ , as their results the estimated  $R_0 = 0.22$  with 95% confidence interval (0.0065; 2.15), calculated as described in van der Goot et al. (2005). However, to obtain an estimate for  $R_0$  with narrow confidence intervals pairwise cohabitation experiments are better suited (Velthuis et al., 2002).

The purpose of this paper was therefore to use pairwise cohabitation experiments, (i.e. one infected shrimp cohabitated with one healthy shrimp), to estimate the transmission rate and mortality rate parameters for WSSV in the two shrimp species that are most frequently used in the production of *Penaeus vannamei* and *P. monodon*. In addition we wanted to know which routes of transmission are responsible for the transmission of WSSV in these species and whether or not they differ between the two species. To that end we compared transmission between pairs of shrimp where we removed dead shrimp as quickly as possible ('remove') and pairs where the dead shrimps were left ('keep'). Also we estimated transmission rate parameters both for the transmission between in-contact shrimp (direct contact transmission) and spatially separated shrimp in the same tank (indirect environment transmission).

## 2. Materials and methods

### 2.1. Virus and inoculum preparation

The WSSV strain (VN-T) used to prepare the inoculum was derived from diseased *P. monodon* collected from a farm in the Central region of Vietnam (Dieu et al., 2004). Virus propagated once through *Orconectes limosus* crayfish (Jiravanichpaisal et al., 2001) was used as an inoculum for *P. vannamei* and virus propagated once through juvenile *P. monodon* was used as an inoculum for *P. monodon*. WSSV was purified from ~10 g gill tissue homogenized in 500 ml TNE buffer (50 mM Tris-HCl, 400 mM NaCl, 5 mM EDTA; pH 8.5) as described by Xie et al. (2005). After the addition of the protease inhibitors phenylmethylsulfonyl fluoride, benzamidine, and  $\text{Na}_2\text{S}_2\text{O}_5$  each to a 1 mM final concentration, the homogenate was centrifuged at  $3500 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant was filtered through a 400 mesh nylon net and centrifuged at  $30,000 \times g$  for 30 min at  $4^\circ\text{C}$ . The pellet was suspended in 10 ml TM buffer (50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ ; pH 7.5), the suspension centrifuged at  $3500 \times g$  for 5 min at  $4^\circ\text{C}$  and WSSV particles were pelleted by ultracentrifugation at  $30,000 \times g$  for 20 min and  $4^\circ\text{C}$ . The white pellet was suspended in 1 ml TM buffer containing 0.1%  $\text{NaN}_3$  and stored at  $-80^\circ\text{C}$  as WSSV stock. The lethal-dose 50% end-point ( $\text{LD}_{50}$  per ml) was determined by muscle injection at the middle of the

lateral part of the 2nd segment with 40  $\mu\text{l}$  of a 10-fold dilution series of the WSSV stock suspension as described previously (Escobedo-Bonilla et al., 2005, 2006) using a Novopen-3 syringe with a gauge 29 needle (Microfine B&D).

## 2.2. DNA extraction and WSSV PCR

Total DNA was extracted from  $\sim 50 \mu\text{g}$  of gill tissue. The tissue was homogenized in 200  $\mu\text{l}$  of 5% (w/v) Chelex X-100 resin (Bio-Rad, US) solution, 16  $\mu\text{l}$  of 20  $\text{mg ml}^{-1}$  Proteinase K (Promega, US) in 50 mM Tris-HCl pH 8.0 was then added and the mixture incubated at 56 °C for at least 6 h to overnight, followed by 95 °C for 10 min. The mixture was micro-centrifuged at 13,000 rpm for 5 min and the supernatant containing DNA was stored at  $-20 \text{ }^\circ\text{C}$ .

The one-step PCR method described previously (Dieu et al., 2004) employing primers VP26F and VP26R were used to amplify a 304 bp region of the WSSV VP26 gene. Each PCR (50  $\mu\text{l}$ ) contained 1  $\mu\text{l}$  of gill DNA solution and was thermal cycled at 94 °C for 3 min, 40 cycles at 94 °C for 30 s, at 52 °C for 30 s, at 72 °C for 50 s and then at 72 °C for 7 min.

## 2.3. Shrimp and aquarium systems

Specific pathogen free (SPF) stocks of *P. vannamei* and *P. monodon* were used in transmission trials. SPF *P. vannamei* postlarvae originating from Oceanboy Farms Inc., FL, USA, were cultured in indoor systems at Happy Shrimp Farm B.V., Rotterdam, the Netherlands. Transmission trials using *P. vannamei* were conducted at Wageningen University, the Netherlands. SPF *P. monodon* were obtained from The National Breeding Center for Southern Marine Aquaculture of Research Institute for Aquaculture No. 2, Vietnam. Transmission trials using *P. monodon* were carried out in the laboratory of Research Institute for Aquaculture No. 2 in HoChiMinh City – Vietnam.

Two sets of experiments were conducted, one used SPF *P. vannamei* and another used SPF *P. monodon*. Each set of experiments was replicated twice and involved a total of 120 pairs divided into 60 pairs of “keep” and 60 pairs of “remove” shrimp. These subgroups were further divided into three tanks containing 20 pairs. Shrimp ( $8 \pm 2 \text{ g}$  body weight) were acclimated for at least 3 days before each trial was initiated. Shrimp were maintained in an air-conditioned room in three 250 l glass aquaria. Each aquarium contained 120 l salt water. Two plastic frames each divided into 10 cubicles 22 cm  $\times$  12 cm  $\times$  20 cm in size were installed in each aquarium (Fig. 1). Each cubicle had 10 cm diameter holes at both ends which were covered with 1 mm mesh to allow flow-through of water and small particulate matter. Water in the aquarium was aerated continuously and recycled through a bio-mechanic filter (Eheim, Germany). Water was maintained at  $28 \pm 0.5 \text{ }^\circ\text{C}$  using a water heater, at pH  $8 \pm 0.2$ , at 25 ppt salinity, and  $>4 \text{ mg l}^{-1}$  dissolved oxygen. The total ammonia-N and  $\text{NO}_2$  were maintained at  $<0.5 \text{ mg l}^{-1}$  and  $<1 \text{ mg l}^{-1}$ , respectively (Escobedo-Bonilla et al., 2006; FAO, 2007).

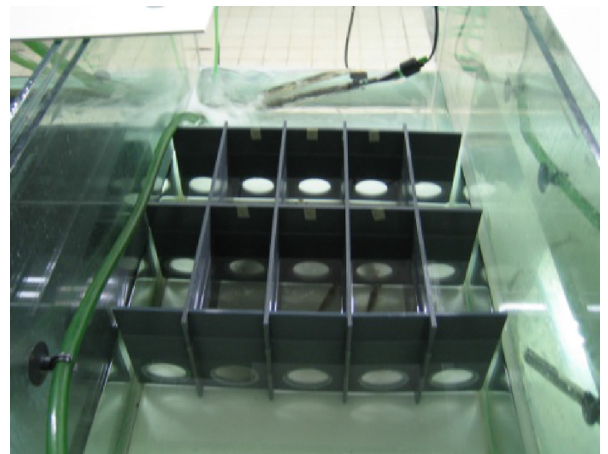


Fig. 1. Glass aquarium with a plastic frame forming 10 cubicles.

## 2.4. Transmission trials

To evaluate WSSV transmission dynamics, shrimp inoculated with WSSV were cohoused with naïve shrimp. One inoculated shrimp and one naïve shrimp were placed into each aquarium cubicle (Fig. 1). To distinguish contact shrimp from inoculated shrimp, a red thread was tied around an eyestalk of the contact shrimp. Inoculated shrimp were infected with WSSV by injection laterally into the muscle of the 2nd tail segment with a dose of 40  $\mu\text{l}$  inoculum diluted to contain 10 times the calculated  $\text{LD}_{50}$  per ml (Escobedo-Bonilla et al., 2006) as described above.

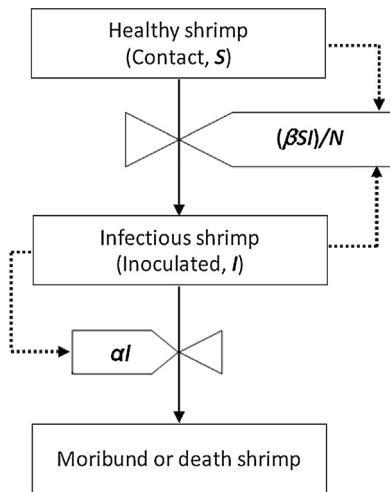
Two replicate trials (1 and 2) were conducted. Each trial employed 60 pairs of shrimp in a ‘remove group’, in which moribund and dead shrimp were removed immediately upon WSSV detection, and 60 pairs of shrimp in a ‘keep group’, in which small pieces of gill tissues were sampled from each moribund or dead shrimp, which was then returned to the cubicle.

In each trial 10 control pairs of shrimp, comprising one shrimp injected with inoculum buffer only and one naïve shrimp, were kept in cubicles of a separate aquarium. Control shrimps were sampled at the end of the experiment. Starting at 12 h post-injection (pi) in each trial and at 8 h intervals thereafter, 2 portions of gill were sampled destructively from 5 shrimp pairs per group. One gill portion was placed into Davidson’s fixative ( $\sim 1/10 \text{ w/v}$ ) for histology and the other portion was preserved in 70% ethanol for WSSV PCR analysis.

## 2.5. WSSV transmission dynamics

The cohabitation trials employed the stochastic Susceptible-Infected-Recovered (SIR) model to evaluate WSSV transmission dynamics (Kermack and McKendrick, 1927). For this, WSSV infection progress in shrimp was grouped into three states, (i) healthy, (ii) infectious, and (iii) moribund/dead, as defined in Fig. 2.

Two transmission rate parameters were estimated, the first between the infected and naïve shrimp in each cubicle (direct contact) and the second between shrimp spatially separated but within the same aquarium (indirect contact



**Fig. 2.** Diagram of changes in health stages of WSSV-infected shrimp in a population. ( $N$ ,  $S$ , and  $I$  are the total population, healthy shrimp, and infected shrimp, respectively;  $\beta$  and  $\alpha$  are the transmission rate and mortality rate parameter).

via water). This is matched in the model of WSSV-infected shrimp population by dividing the total population  $N$  in three compartments; the susceptible compartment  $S$ , the infected compartment  $I$  (either  $I_{pair}$  or  $I_{tank}$  for  $I$  of the pair or of the environment see below) and the mortality compartment  $R$ . It is assumed that the dynamics of disease transmission of WSSV in this population proceeds according to true mass theory (de Jong, 1995), which stipulates:

- (1) The number of  $S$  individuals move to  $I$  compartment with an infection rate  $\beta \cdot S \cdot I / N$ ;
- (2) The number of  $I$  individuals move to  $R$  compartment with an infection rate  $\alpha \cdot I$ ;

with  $\beta$  and  $\alpha$  are respectively the transmission rate and mortality rate parameter.

All shrimp injected with WSSV will progress from  $S$  to  $I$  and over time from  $I$  to  $R$ . Progress of naïve shrimp, cohabitated with WSSV-infected shrimp through these stages, will occur according to chance as given by the model described above. The reproduction ratio  $R_0$ , which defines the average number of secondary infections that will arise in a large population of susceptible individuals due to exposure to a single infected shrimp during its infectious period, can be estimated by the equation  $R_0 = \beta / \alpha$  or  $\beta \cdot T$ , where the average duration of the infectious period  $T = 1 / \alpha$ .

With  $S$ ,  $I$ ,  $R$ , and  $N$  being the numbers of contact shrimp, WSSV-injected shrimp, moribund/dead shrimp, and total shrimp population size, respectively. At the beginning of the trials, for the direct transmission both  $S$  and  $I = 1$  and  $R = 0$ . Assuming that  $S$  and  $I$  are homogeneous groups of individuals, the infection rate is constant for the entire infectious period. Based on this assumption,  $\text{Pr}[(S_{t+\Delta t}, I_{t+\Delta t}) = (S_t - 1, I_t + 1)] = \beta \cdot (S_t \cdot I_t / N_t) \cdot \Delta t$ , and the probability of any individual becoming infected in a time interval  $\Delta t$  is  $p = 1 - e^{-(\beta \cdot I_t / N_t) \cdot \Delta t}$ .

The rate at which  $I$  shrimp become  $R$  shrimp is proportional to number of  $I$  shrimp and mortality parameter  $\alpha$ .

The transition state from  $(S_t, I_t)$  to  $(S_t, I_t - 1)$  is  $\text{Pr}[(S_{t+\Delta t}, I_{t+\Delta t}) = (S_t, I_t - 1)] = \alpha \cdot I_t \cdot \Delta t$  and the probability of any infected shrimp becoming removed in a time interval  $\Delta t$  is  $p = 1 - e^{-(\alpha \cdot \Delta t)}$ .

The unknown transmission rate parameter  $\beta$  was estimated using the Generalized Linear Model (GLM) method, with the complimentary loglog link, the error term being binomial and the binomial total being  $S$ . The above expression for the transmission rate resulted in an offset equal to  $\text{LN}(I_{total} \cdot \Delta t / N)$ . The total infectivity  $I_{total}$  consisted of the contribution of the pair partner  $I_{pair} / 2$ , where  $I_{pair}$  is either 0 or 1, and the contribution from the whole tank  $I_{tank} / N$ , where  $N$  is the total number of shrimp in the tank (i.e., 40 shrimp). The direct and indirect transmission rates were estimated by using the cofactor  $F$  as explanatory variable:

$$F = \frac{I_{pair} / 2}{(I_{pair} / 2) + (I_{tank} / N)}$$

which defines the contribution of the infectivity of the pair partner to the total infectivity. Note that  $F = 1$  implies infectivity is only from within the pair (direct) and  $F = 0$  implies that infectivity is only from the rest of the tank (indirect).

The GLM equation, i.e. the expected value of the dependent variable using the link function, shows the linear relationship to the explanatory variable  $F$  and the offset:

$$\text{cloglog} = \left( \in \frac{C_t}{S_t} \right) = \text{intercept} + C \cdot F + \text{Offset}$$

where intercept and  $C$  are estimated using:

$$\text{Offset} = \text{LN} \left[ \Delta \text{time} \left( \left( \frac{I_{pair}}{2} \right) + \left( \frac{I_{tank}}{N} \right) \right) \right]$$

$$F = \frac{I_{pair} / 2}{(I_{pair} / 2) + (I_{tank} / N)}$$

Thus  $\beta_{direct} = e^{\text{intercept}}$  and  $\beta_{indirect} = e^{\text{intercept} + C}$  and the total  $\beta$  is the sum of both.

The upper and lower bound of reproduction ratio  $R_0$  both for transmission in the pair (direct) and in the tank (indirect) was based on the upper and lower bound of  $\text{LN}(R_0) = \text{LN}(\beta) - \text{LN}(\alpha)$ . Thus we have  $\text{Var}(\text{LN}(R_0)) = \text{Var}(\text{LN}(\beta)) + \text{Var}(\text{LN}(\alpha))$  assuming that covariance is zero. For the overall rate of transmission we use the beta estimated for the model without  $F$ . Note that as in that case direct and indirect transmission are still counted separately because of the offset where each infected is counted twice. Thus the overall beta is still the sum of the contribution of direct and indirect transmission albeit with them now being equal.

The statistical test used for comparisons of percentages in Tables 1–4 was Excel Chisq.dist function. The statistical test used for comparisons of parameter values in Table 5 was Log-rank test for equality of survivor functions in STATA.

Calculations were performed using IBM SPSS Statistics Package 15 for Microsoft Windows and Stata 11.1 (Stata-corp, USA).

**Table 1**

Two-sided tests for comparisons of virus infection of inoculated shrimp compared between two treatments, two species, and two replications.

Comparison of infection (%) in inoculated shrimp between	Two treatments		$\chi^2$	P-values (2-sided)
	Keep	Remove		
Overall two species	73.7	70.4	0.11	0.74
In <i>P. vannamei</i> only	79.2	72.5	0.20	0.65
In <i>P. monodon</i> only	68.3	68.3	0.00	1.00
Comparison of infection (%) in inoculated shrimp between	Two species		$\chi^2$	P-values (2-sided)
	<i>P. vannamei</i>	<i>P. monodon</i>		
Overall two species	75.8	68.3	0.54	0.46
Comparison of infection (%) in inoculated shrimp between	Two replications		$\chi^2$	P-values (2-sided)
	Replication 1	Replication 2		
In <i>P. vannamei</i> only	74.2	77.5	0.05	0.82
In <i>P. monodon</i> only	67.5	69.2	0.01	0.90

### 3. Results

In order to evaluate WSSV transmission dynamics shrimp inoculated with WSSV were cohabitated with naive shrimp and the transmission rates were determined. The dead shrimp of the inoculated group were either kept ('keep' group) or removed ('remove' group) to see if there is a difference in transmission rates. Infection was determined by PCR. The percentages of WSSV infection of inoculated shrimp ranged between 67.5% and 79.2%, but were found to be not significantly different between 'keep' and 'remove' treatments, between the two species *P. vannamei* and *P. monodon*, and between two replications (Table 1).

The number of deaths of inoculated shrimp was determined, as these can influence the transmission rates of

the virus. Percentages of mortality of inoculated shrimp ranged between 38.3% and 66.5%, but were found not significantly different between the 'keep' and 'remove' treatments, between the two species *P. vannamei* and *P. monodon*, and between two replications (Table 2).

The observed percentages of WSSV infection of contact-exposed shrimp in the 'keep' treatments were higher than those percentages in the 'remove' treatments for the both species, particular in *P. vannamei* (Table 3), but none of these percentages were significantly different.

Percentages of mortality of contact-infected shrimp observed for *P. monodon* were higher than in *P. vannamei* ( $p < 0.05$ ) (Table 4). Percentages of mortality of contact shrimp were found not to differ significantly between the 'keep' treatment and the 'remove' treatment. Percentages of mortality of contact shrimp were also not significantly

**Table 2**

Two-sided tests for comparisons of the mortality of inoculated shrimp: comparisons between treatments, species, and replications.

Comparison of mortality (%) of inoculated shrimp between	Two treatments		$\chi^2$	P-values (2-sided)
	Keep	Remove		
Overall two species	50.5	45.0	0.27	0.60
In <i>P. vannamei</i> only	49.2	51.7	0.34	0.56
In <i>P. monodon</i> only	51.7	38.3	1.43	0.23
Comparison of mortality (%) of inoculated shrimp between	Two species		$\chi^2$	P-values (2-sided)
	<i>P. vannamei</i>	<i>P. monodon</i>		
Overall two species	50.5	45.0	0.27	0.60
Comparison of mortality (%) of inoculated shrimp between	Two replications		$\chi^2$	P-values (2-sided)
	Replication 1	Replication 2		
In <i>P. vannamei</i> only	45.8	55.0	0.34	0.56
In <i>P. monodon</i> only	43.3	46.6	0.04	0.84

**Table 3**

Two-sided tests for comparisons of WSSV infection of contact shrimp: comparison between treatments, species, and replications.

Comparison of WSSV infection (%) in contact shrimp between			$\chi^2$	P-values (2-sided)
Two treatments				
	Keep	Remove		
Overall two species	32.1	27.9	0.53	0.46
In <i>P. vannamei</i> only	31.7	28.3	0.17	0.68
In <i>P. monodon</i> only	32.5	27.5	0.39	0.53

Comparison of WSSV infection (%) in contact shrimp between			$\chi^2$	P-values (2-sided)
Two species				
	<i>P. vannamei</i>	<i>P. monodon</i>		
Overall two species	30.0	30.0	0.00	1.00

Comparison of WSSV infection (%) in contact shrimp between			$\chi^2$	P-values (2-sided)
Two replications				
	Replication 1	Replication 2		
In <i>P. vannamei</i> only	30.0	30.0	0.00	1.00
In <i>P. monodon</i> only	30.8	29.2	0.01	0.90

**Table 4**

Two-sided tests for comparisons of mortality of contact-shrimp: comparison between treatments, species, and replications.

Comparison of contact shrimp mortality (%) between			$\chi^2$	P-values (2-sided)
Two treatments				
	Keep	Remove		
Overall two species	9.2	4.2	2.48	0.11
In <i>P. vannamei</i> only	5.8	0.8	3.53	0.06
In <i>P. monodon</i> only	12.5	7.5	0.50	0.48

Comparison of contact shrimp mortality (%) between			$\chi^2$	P-values (2-sided)
Two species				
	<i>P. vannamei</i>	<i>P. monodon</i>		
Overall two species	3.3	10.0	6.6	0.01*

Comparison of contact shrimp mortality (%) between			$\chi^2$	P-values (2-sided)
Two replications				
	Replication 1	Replication 2		
In <i>P. vannamei</i> only	3.3	3.3	0.00	1.00
In <i>P. monodon</i> only	11.7	8.3	0.35	0.56

\*  $p < 0.05$ .

**Table 5**

Model parameters estimated for the two species overall and for each species separately.

	$\beta$		$\alpha$		$R_0$	
	$\beta$ ( $h^{-1}$ )	95% CI	$\alpha$ ( $h^{-1}$ )	95% CI	$R_0$	95% CI
<b>Both species</b>						
Direct transmission	0.0081	(0.0068, 0.0095)	0.0091	(0.0080, 0.010)	0.89	(0.72, 1.10)
Indirect transmission	0.0081	(0.0068, 0.0095)			0.89	(0.72, 1.10)
Overall	0.016	(0.013, 0.019)			1.77	(1.44, 2.19)
<b><i>P. monodon</i></b>						
Direct transmission	<sup>a</sup> 0.022*	(0.0024, 0.20)	<sup>d</sup> 0.0077	(0.0064, 0.0093)	2.85	(0.31, 25.82)
Indirect transmission	<sup>a</sup> 0.0026	(0.00092, 0.0077)			0.35	(0.12, 1.02)
Overall	<sup>c</sup> 0.016	(0.013, 0.020)			2.07	(1.53, 2.79)
<b><i>P. vannamei</i></b>						
Direct transmission	<sup>b</sup> 0.0038	(0.00084, 0.017)	<sup>d</sup> 0.011**	(0.0091, 0.013)	0.35	(0.08, 1.61)
Indirect transmission	<sup>b</sup> 0.018*	(0.0088, 0.035)			1.62	(0.80, 3.32)
Overall	<sup>c</sup> 0.016 <sup>o</sup>	(0.013, 0.021)			1.51	(1.12, 2.03)

Statistical tests are done between pairs indicated by the same letter a, b, c or d at: <sup>o</sup>not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ .

different between the two replications for both species (Table 4).

For *P. vannamei* the direct transmission rate parameter  $\beta$  of WSSV was significantly lower ( $p < 0.05$ ) when compared to the indirect transmission rate parameter (Table 5). For *P. monodon* the direct transmission rate parameter  $\beta$  was significantly higher ( $p < 0.05$ ) when compared to indirect transmission (Table 5). For both species combined, the  $\beta$  for direct and indirect transmission was not significantly different (Table 5). The  $\beta$  value of the overall transmission rate observed in *P. monodon* ( $0.00164 \text{ h}^{-1}$ ) was higher compared to the  $\beta$  value in *P. vannamei* ( $0.00159 \text{ h}^{-1}$ ), but not significantly different.

There was a significant difference between the mortality rates ( $\alpha$ ) found in *P. vannamei* and in *P. monodon* ( $p < 0.01$ ) (Table 5). The value of the reproduction ratio  $R_0$  for the two species overall was 1.77 (Table 5). The value of the reproduction ratio  $R_0$  for *P. monodon* (2.07 with 95% CI 1.53, 2.07) was mainly the result of direct transmission, while for *P. vannamei* it was mainly the result of indirect transmission (1.51 with 95% CI 1.12, 2.03). The model with intercept and offset had a lower AIC than the model with intercept without offset (*P. monodon* 115.7 vs 174.2 and *P. vannamei* 122.1 vs 184.0). Thus the probability of infection did depend on the number of infected individuals in the same tank.

All control shrimps were negative for WSSV, which shows that there was no cross contamination of WSSV between tanks during the experiments. No shrimp died in the control group, which indicates the culture system for shrimp did work well throughout the experiments.

#### 4. Discussion

The transmission rates, mortality rates, and the basic reproduction ratios are important parameters to quantitatively describe disease transmission. There is a paucity of such data from aquatic systems and therefore we have made an attempt to generate such data, in this case in an invertebrate virus-host system, WSSV and shrimp. The transmission of WSSV was tested by comparing: (i) in-contact shrimp and spatially separated shrimp to determine direct and indirect transmission, respectively, (ii) two shrimp species to determine species-specificity in transmission, and (iii) the 'keep' and 'remove' treatments to estimate the difference in transmission in the absence or presence of cannibalism. The overall transmission rate parameters found for both *P. vannamei* and *P. monodon* was  $0.0016 \text{ h}^{-1}$ . The mortality rate parameter in *P. vannamei* was significantly different from that in *P. monodon*. The basic reproduction ratio of WSSV observed for *P. vannamei* and *P. monodon* was 1.51 and 2.07, respectively.

The values of the transmission rate parameter ( $\beta = 0.016 \text{ h}^{-1}$ ) found in our study for *P. vannamei* was much higher than the rate  $\beta = 0.02/14 \text{ h} = 0.0014 \text{ h}^{-1}$  obtained in the study of Soto and Lotz (2001). However, for comparison it is better to look at the difference in  $R_0$ , as Soto and Lotz (2001) do not give an estimate for the infectious period. The  $R_0 = 1.77$  estimated in our study, is clearly within the confidence limits estimated from the data of Soto and Lotz (2001), i.e. [0.0065; 2.15]. Another way to look at this issue is that these authors

observed four minor outbreaks (counting the groups with no contact infections also as minor outbreaks) and a quick calculation shows that this is not unlikely when  $R_0 = 1.77$ . The probability of a minor outbreak is  $1/R_0 = 0.56$  based on the estimates from our experiments and thus the probability of four minor outbreaks equals 0.102 (i.e.  $(1/R_0)^4$ ), which is not significant at the 5% confidence level. The more minor outbreaks that are observed the more likely it is that the null hypothesis ( $R = 1.77$ ) is not true. Four (the maximum) minor outbreaks were observed and under the null hypothesis the probability of that 4 or more minor outbreaks would be observed is 0.102, which is not significant.

The transmission rate parameter  $\beta$  of WSSV in shrimp was calculated in two ways, the direct and indirect transmission rates. Direct transmission of WSSV, which resulted from direct contact, was the most important component of the transmission of WSSV in *P. monodon*, whereas this type of transmission was of minor importance for *P. vannamei* (Table 5). This suggests that higher shrimp densities are very important for the occurrence of WSSV outbreaks in *P. monodon* (Wu et al., 2001; Ogut et al., 2005), whereas the density parameter may be of lesser importance for the occurrence of WSSV outbreaks in *P. vannamei*. This is in line with the observation that in *P. monodon* farming, WSSV outbreaks are found more often at higher density shrimp culture systems, such as in intensive culture and semi-intensive shrimp culture systems as compared to low density culture systems such as extensive and organic and rice-shrimp farming cultures (Hoa et al., 2012). In practice, this result indicates that systems stocked with *P. vannamei* can tolerate a higher stocking density of shrimp compared to *P. monodon* before running the same risk of a WSSV outbreak.

Cannibalism of shrimp was considered a co-factor of the direct transmission of WSSV (Wu et al., 2001; Soto et al., 2001). In our experiments the cannibalism effect on WSSV transmission was not clear (Table 3). During our experiments dead shrimps were not always found exactly at the time of death, so the occurrence of some degree of cannibalism or scavenging on the dead carcasses cannot be entirely ruled out. The relative role of cannibalism on the disease transmission in cultured ponds is not easy to assess or to mimic in our experimental setting. However, on the basis of the transmission rate values alone (Table 5), it is safe to conclude that transmission of WSSV through direct and indirect (water-borne) contact alone is sufficient to initiate and maintain an epidemic in a pond.

In conclusion, in our experiments the  $R_0$  values for WSSV were found to be larger than one for both *P. monodon* (2.07) and *P. vannamei* (1.51) under pair cohabitation conditions. However, in pond systems the reproduction ratio may be influenced by other factors such as shrimp density and physical parameters (e.g. temperature and salinity). This calls for further studies on the effects of those factors on disease transmission of WSSV in shrimp.

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