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Increased susceptibility of white spot syndrome virus-infected *Litopenaeus vannamei* to *Vibrio campbellii*

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Summary

The concept of polymicrobial disease is well accepted in human and veterinary medicine but has received very little attention in the field of aquaculture. This study was conducted to investigate the synergistic effect of white spot syndrome virus (*WSSV*) and *Vibrio campbellii* on development of disease in specific pathogen-free (SPF) shrimp *Litopenaeus vannamei*. The juvenile shrimp were first injected with *WSSV* at a dose of 30 SID_{50} $shrimp^{-1}$ (SID_{50} = shrimp infectious dose with 50% endpoint) and 24 h later with 10^6 colony-forming units (cfu) of *V. campbellii* $shrimp^{-1}$. Controls receiving just one of the pathogens or negative inocula were included. In the treatment with *WSSV* only, shrimp started to die at 48–108 h post injection (hpi) and cumulative mortality reached 100% at 268–336 hpi. In the treatment with only *V. campbellii* injection (10^6 cfu $shrimp^{-1}$), cumulative mortality reached 16.7%. Shrimp in the dual treatment died very quickly after *V. campbellii* injection and 100% cumulative mortality was obtained at 72–96 hpi. When *WSSV*-injected shrimp were given sonicated *V. campbellii* instead of live *V. campbellii*, no synergistic effect was observed. Density of *V. campbellii* in the haemolymph of co-infected moribund shrimp collected 10 h after *V. campbellii* injection was significantly higher than in shrimp injected with *V. campbellii* only ($P < 0.01$). However, there was no difference in *WSSV* replication between shrimp inoculated with *WSSV* only compared with dually inoculated ones. This study revealed that prior infection with *WSSV* enhances the multiplication and disease inducing capacity of *V. campbellii* in shrimp.

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Introduction

Infectious diseases are a major constraint to shrimp aquaculture production in many countries. The rapid increase in cultured areas since the 1980s facilitated spread and outbreaks of a high number of pathogens, viruses in particular. Since its emergence in 1992 (Chou *et al*., 1995), white spot syndrome virus (*WSSV*) has been one of the major disease problems in shrimp culture around the world (Lightner, 2003; Rosenberry, 2004; Sanchez-Martinez *et al*., 2007; Escobedo-Bonilla *et al*., 2008). In cultured penaeid shrimp, *WSSV* infections can cause a cumulative mortality up to 100% within 3–10 days (Lightner, 1996). Infected shrimp show lethargic behaviour, loss of appetite, reddish discoloration and white spots in the exoskeleton composed of calcified deposits (Chou *et al*., 1995). *WSSV* not only infects all shrimp species, but also a wide range of other decapod crustaceans (Lightner *et al*., 1998). Reports have described both acute and chronic *WSSV* infections which caused different rates of mortality in shrimp ponds (Sudha *et al*., 1998) and under experimental conditions (Wang *et al*., 1999; Rahman *et al*., 2008).

Species of *Vibrio* are well known in penaeid shrimp culture as causative agents of vibriosis. This important disease is mainly caused by *Vibrio anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. penaeicida*, *V. campbellii*, both in hatcheries and in grow-out cultures (Lightner, 1988; Lavilla-Pitogo *et al*., 1990). Infections with luminescent *V. harveyi* strains have been reported to cause major losses in shrimp larviculture in Australia (Pizzutto and Hirst, 1995), South America (Álvarez *et al*., 1998; Robertson *et al*., 1998) and Mexico (Vandenberghe *et al*., 1999). Vibriosis usually occurs during the first month of culture and can cause more than 50% mortality. So far, it is not clear whether *Vibrio* spp. are opportunistic or primary pathogens. According to Saulnier and colleagues

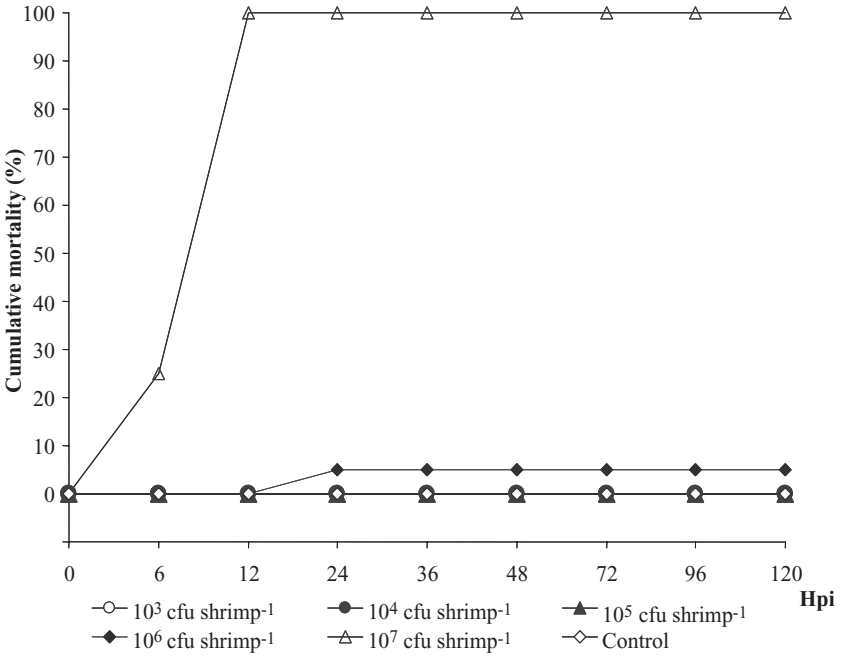

Fig. 1. Cumulative mortality caused by different doses of *V. campbellii* injected in *L. vannamei* (experiment 1)

(2000a), *Vibrio* spp. may act as opportunistic agents in secondary infections or be true pathogens. As low number of *V. penaeicida* bacterial cells were shown to produce the disease in *Penaeus japonicus* and *P. stylirostris*, Saulnier and colleagues (2000b) reported that *V. penaeicida* could act as a primary pathogen. However, pathogenic vibrios were also isolated from apparently healthy penaeid shrimp (Gómez-Gil *et al*., 1998, Vandenberghe *et al*., 1998). These observations lead researchers to consider *Vibrio* spp. are opportunistic pathogens. Horowitz and Horowitz (2001) postulated that if shrimp are not suffering from primary infections, physical damage or stress, their resistance against vibrios is adequate to prevent disease. This idea was further supported by Alday-Sanz and colleagues (2002) who showed that shrimp, when exposed to ammonia prior to immersion challenge with *Vibrio*, suffered more frequent and earlier pathological changes than shrimp exposed to the bacteria alone.

Only a few cases of polymicrobial disease have been described in shrimp aquaculture. In 2001–2, a retardation of the *Penaeus monodon* growth rate was noted in shrimp ponds in Thailand. This problem was named Monodon Slow Growth Syndrome. Samples of affected shrimp were screened by histopathology, polymerase chain reaction (PCR) and transmission electron microscopy for a wide range of pathogens. It was discovered that several causative agents were involved. Many shrimp specimens had dual or multiple infections with monodon baculovirus (MBV), heptopancreatic parvovirus (HPV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Chayaburakul *et al*., 2004). After screening shrimp samples from 18 ponds in India, Umesha and colleagues (2006) found that the animals in seven ponds showed dual infections with *WSSV* and MBV and in 10 ponds triple infections with HPV, *WSSV* and MBV could be found. Selvin and Lipton (2003) demonstrated the presence of a virulent strain of *V. alginolyticus* in shrimp from a pond hit by a *WSSV* outbreak. Although both pathogens could not be isolated from all sampled shrimp, it was stated that shrimp weakened by *WSSV* would succumb to a secondary infection by *V. alginolyticus.*

Vibrio is known to be one of the dominant species of bacteria living in shrimp ponds (Hisbi *et al*., 2000). As a known facultative pathogenic bacterium, it is probable that *Vibrio* co-infects shrimp with *WSSV* regularly in the field.

The objective of this study was to reproduce a co-infection of shrimp with *WSSV* and *Vibrio* under laboratory conditions using standardized challenge protocols and to investigate the existence of any synergistic effect. More specifically, the question was raised whether a *WSSV* infection already present in specific pathogen-free (SPF) *Litopenaeus vannamei* shrimp would allow *Vibrio* to cause faster and higher mortality rates than the virus or bacteria administered separately.

Results

Experiment 1: dose effect of V. campbellii *on mortality of* L. vannamei

In treatments with lower than 10^6 colony-forming units (cfu) of *V. campbellii* shrimp^{-1}, no mortality was observed (Fig. 1). When 10^6 cfu was administered, 5% cumulative mortality was recorded during the 5-day experimental

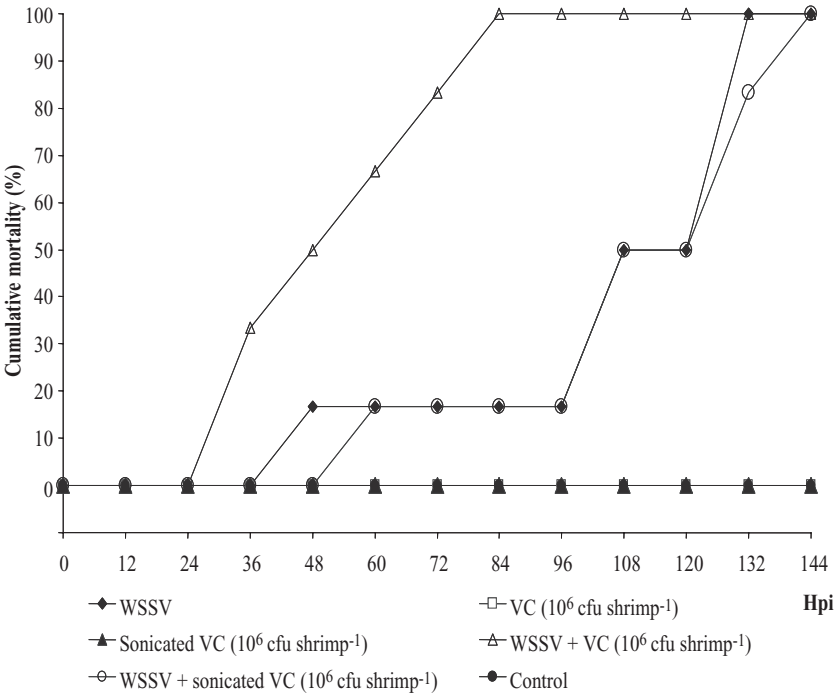

Fig. 2. Cumulative shrimp mortality after challenge with *WSSV* and sonicated *V. campbellii* (VC) (experiment 3).

period. On the other hand, shrimp died very quickly after injection with 10^7 cfu of *V. campbellii* and 100% cumulative mortality was found at 12 h post injection (hpi). From these results it was concluded that up to a dose of 10^6 cfu shrimp^{-1}, *V. campbellii* on its own was not capable of causing any significant mortality. Therefore, this sublethal dose was chosen for the following co-infection experiments of *WSSV* and *Vibrio*.

Experiment 2: effect of sonicated V. campbellii *and supernatant from* V. campbellii *cultures on mortality of* L. vannamei

No mortality was observed when shrimp were injected with supernatant, sonicated *V. campbellii*, Marine Broth (MB) or filtered autoclaved seawater (FASW). It was therefore concluded that no toxic products from *V. campbellii* which could influence shrimp mortality were present in the inocula.

Experiment 3: effect of sonicated V. campbellii *on mortality of* WSSV*-infected* L. vannamei

Mortalities in the treatments with *WSSV* only and in the dual treatment with *WSSV* and sonicated *V. campbellii* evolved in a similar, slow manner (Fig. 2). Cumulative mortality reached 100% at 132 hpi and 144 hpi respectively. At 84 hpi, when cumulative mortality was only 16.7% in both groups, all shrimp had already died in the dual treatment of *WSSV* and live *V. campbellii*. These results clearly demonstrated that injection of sonicated *V. campbellii* in combination with *WSSV* did not result in an increase of shrimp mortality.

Experiment 4: co-infection of L. vannamei *with* WSSV *and* V. campbellii *(first run)*

Shrimp injected with *WSSV* only started to die at 72 hpi and cumulative mortality reached 100% at 252 hpi (Fig. 3). In the treatment with 10^6 cfu of *Vibrio* only, one shrimp died within 12 h post *Vibrio campbellii* injection (hpvi) and cumulative mortality reached 16.7% at the end of the experiment. As in experiment 1, shrimp died quickly after injection with 10^7 cfu of *V. campbellii*. In the treatment with co-infection of *WSSV* and *V. campbellii*, shrimp started to die at 36 hpi and 66.7% mortality occurred within 48 hpi. These data indicated that the accelerated mortality caused by co-infection as observed in experiment 3 could be reproduced.

Experiment 5: co-infection of L. vannamei *with* WSSV *and* V. campbellii *(second run)*

Shrimp injected with *WSSV* only started to die at 108 hpi and cumulative mortality reached 100% at 336 hpi (Fig. 4). Shrimp injected with *Vibrio* only showed exactly the same mortality rate as in the previous experiment (16.6%). Animals co-infected by *WSSV* and *V. campbellii* died quickly after the challenge with *V. campbellii*, with mortality reaching 66.7% by 48 hpi and 100% by 96 hpi. Again, the observations showed that the results of the first run of experiment 4 could be reproduced.

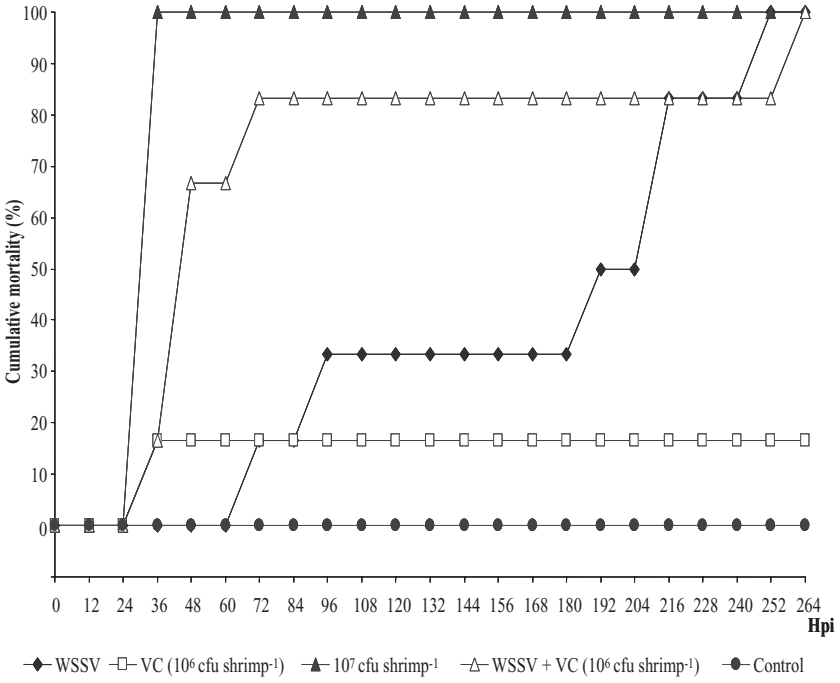

Fig. 3. Cumulative shrimp mortality after challenge with *WSSV* and *V. campbellii* (first run, experiment 4).

Experiment 5 and 6: quantification of WSSV *and* V. campbellii *in co-infected* L. vannamei

In experiment 5, shrimp injected with *WSSV* only died between 119 and 334 hpi. Dually infected animals started to die much faster, between 29 and 96 hpi. These moribund shrimp were collected for quantification of *WSSV*. Gills, stomach and cuticular epithelium and haematopoietic tissue were screened for *WSSV*-infected cells. In the treatment with *WSSV* only, all shrimp were found to be infected in all organs (Table 1). Overall, fewer *WSSV*-infected cells were counted in shrimp of the dual treatment and not in all organs.

In experiment 6, shrimp were collected at 10 hpvi for quantification of *WSSV* and *V. campbellii* (Table 2). Dual infections did not appear to cause any change in the

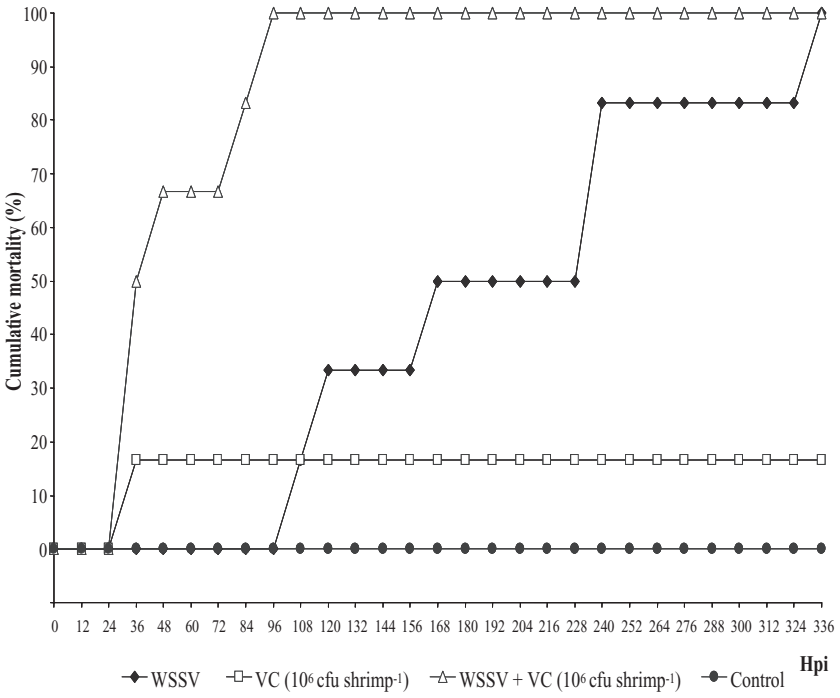

Fig. 4. Cumulative shrimp mortality after challenge with *WSSV* and *V. campbellii* (second run, experiment 5).

Table 1. Quantification of *WSSV*-infected cells in gills (G), stomach and cuticular epithelium (SE and CE) and haematopoietic tissue (HP) of shrimp collected at time of death (experiment 5).

Treatments	Shrimp	hpi	G (cells mm^{-2})	SE (%)	CE (%)	HP (cells mm^{-2})
WSSV	1	119	68	34	19	50
	2	119	132	21	17	190
	3	170	52	16	19	80
	4	232	9	7	8	13
	5	237	32	13	24	168
	6	334	39	15	19	118
WSSV + VC	1	29	0	0	0	0
	2	35	10	0.8	0.5	2.5
	3	37	0	0	0	0
	4	41	1	0	0	0
	5	83	199	12.4	14.9	115
	6	96	85	23.6	18.9	30

number of *WSSV*-infected cells. Positive cells were found in all organs, but no significant difference was found between groups which were administered both pathogens or *WSSV* alone ($P < 0.01$). The number of *WSSV*-infected cells in haematopoietic tissue (15–568 cells mm^{-2}) was higher than that in gills (55–241 cells mm^{-2}) and lymphoid organs (3–165 cells mm^{-2}) (Table 2). In the stomach epithelium, 2–29% of cells were infected. The number of *V. campbellii* isolated from bacteria-only-injected shrimp was lower than 100 cfu ml^{-1}. In contrast, a very high density of *V. campbellii* (1.8×10^6 cfu ml^{-1}) was observed in the haemolymph of shrimp in the dual treatment with *WSSV* and *Vibrio*.

Discussion

The *WSSV* Viet strain used in the current study caused a rather low mortality rate compared with other strains reported in literature (Lightner, 1996; Sanchez-Martinez *et al*., 2007; Escobedo-Bonilla *et al*., 2008). Shrimp injected with only *WSSV* started to die at 48–108 hpi and cumulative mortality reached 100% at 132–336 hpi. This outcome is not completely in concurrence with previous observations but still within the reported range. Working with the same *WSSV* strain, injected dose and the same shrimp species but different batch, Rahman and colleagues (2008) documented an onset of mortality at 36–60 hpi and 100% of cumulative mortality at 204–348 hpi. It is interesting to note that the *WSSV*-induced mortality rate is hardly dependent on the injected dose. Using the same virus batch, Rahman and colleagues (2007a) injected 10 000 SID_{50} (SID_{50} = shrimp infectious dose with 50% endpoint), obtaining 15–25% cumulative mortality after 36–48 hpi and 100% mortality after 168–196 hpi. All moribund shrimp injected with *WSSV* only in experiment 5 were positive for *WSSV* on immunohistochemistry (IHC) staining, confirming that all shrimp died due to the *WSSV* infection.

The current study supported the hypothesis that *V. campbellii* used in this study does not cause primary disease in healthy juvenile shrimp, unless high doses were administered. Shrimp injected with *V. campbellii* alone in doses of 10^5 cfu or lower did not present any signs of mortality. However, the surviving shrimp showed a black spot at the site of *V. campbellii* injection. This observation has also been described by Sarathi and colleagues

Table 2. Quantification of *WSSV*-infected cells (mean ± SD) and *V. campbellii* (cfu ml^{-1} of haemolymph) in gills (G), stomach epithelium (SE), lymphoid organ (LO) and haematopoietic tissue (HP) of shrimp collected 10 h after *V. campbellii* injection (experiment 6; shrimp in dual treatment were moribund).

	WSSV-infected cells in organs				
Treatments	G (cells mm^{-2})	SE (%)	LO (cells mm^{-2})	HP (cells mm^{-2})	VC (cfu ml^{-1})
WSSV	167 ± 58^a (55–221)	17 ± 10^a (2–29)	83 ± 56^a (12–138)	315 ± 194^a (70–568)	–
VC	–	–	–	–	43 ± 61^a (0–157)
WSSV + VC	184 ± 40^a (130–241)	14 ± 5^a (4–19)	90 ± 67^a (3–165)	208 ± 168^a (15–503)	183 721 ± 73 177^b (113 000–314 600)

Numbers between brackets are minimum and maximum values of six shrimp. Numbers of infected cells in the same tissue or cfu ml^{-1} with different superscripts were significantly different between the two treatments ($P < 0.01$).

(2007), who saw haemocytic infiltration and melanization at the injection site upon injections with *V. alginolyticus* in *Fenneropenaeus indicus* shrimp. In the present study, cumulative mortality reached 16.7% when shrimp were injected with 10^6 cfu of *V. campbellii* (Figs 2 and 3). Because shrimp died within hours after injection with 10^7 cfu of *V. campbellii*, it was first considered that shrimp might die as a consequence of toxins produced by the bacteria. However, as no mortality was observed when shrimp were challenged with the supernatant of *V. campbellii* cultures or with sonicated *V. campbellii*, this possibility was ruled out and it was concluded that live bacteria were required to reproduce the disease in shrimp.

In dual treatments of *WSSV* and *V. campbellii*, a clear acceleration of the mortality of *WSSV*-infected shrimp was noticed shortly after inoculation with bacteria. Only 1 out of 18 co-infected shrimp survived longer than 96 hpi. A similar observation has been published by Pakingking and colleagues (2003). They reported that the mortality rate of flounder fish (*Paralichthys olivaceus*) increased by secondary infection with *Streptococcus iniae* or *Edwardsiella tarda* at 1 week post marine birnavirus (MABV) infection. In the same fish species, Oh and colleagues (2006) also found an increase in mortality of fish co-infected with MABV and *V. harveyi* or *E. tarda.*

To find out the underlying cause for the rapid mass mortality of the shrimp in the dual treatment, quantification of *WSSV* in different organs and *V. campbellii* in the shrimp's haemolymph was performed. It has been established that gills, stomach and cuticular epithelium, haematopoietic tissue and lymphoid organ are major target organs of *WSSV* replication (Chang *et al*., 1996). Escobedo-Bonilla and colleagues (2007) and Rahman and colleagues (2008) also selected these organs for enumerating *WSSV*-infected cells. In the present study, all observed organs of moribund shrimp collected at 10 hpvi (34 hpi) were positive with *WSSV*. However, the obtained counts were different from the ones obtained by Rahman and colleagues (2008) who reported lower numbers of *WSSV*-infected cells. This difference might be contributed to the use of shrimp from a different batch and different size. The shrimp in the present study were 4.5 times smaller than the ones used by Rahman and colleagues (2008). Additionally, moribund shrimp were collected in this study instead of euthanized shrimp as mentioned in their report. The most important finding, however, was that the quantification did not reveal any significant difference in the number of *WSSV*-infected cells between the single and dual treatments. This observation showed that injection of *V. campbellii* did not result in any increase of *WSSV* replication, thus making it very unlikely that *WSSV* was responsible for the accelerated mortality. As antibody staining methods for *V. campbellii* are not readily available, re-isolation was chosen as means to quantify *V. campbellii* in the shrimp's haemolymph. This procedure was facilitated by the use of a rifampycin-resistant strain of *V. campbellii*. Plating of haemolymph samples is one of the few ways to estimate the amount of bacteria replicating inside the body of shrimp and has been used with success in previous studies (Mermound *et al*., 1998; Van de Braak *et al*., 2002). In contrast to the viral load, the *V. campbellii* load showed a highly significant difference between single and dual treatments. At 10 hpvi, the amount of *V. campbellii* in the haemolymph of moribund shrimp inoculated with both *WSSV* and *V. campbellii* was more than 10^3 times higher than that in shrimp that received bacteria only. Apparently, low densities of bacteria could be eliminated quickly by shrimp that were not coping with a *WSSV* infection. Van de Braak and colleagues (2002) also recorded that the concentration of live bacteria in haemolymph of shrimp decreased by 97% within 2 h after injection. This was attributed to the host's defence system, more specifically, the clearing mechanism of the shrimp's haemocytes.

Sizeable individual variations in numbers of *WSSV*-infected cells and *V. campbellii* in the haemolymph at certain time points were observed in dual and single treatments. Individual variations in the load of *WSSV* in shrimp were reported earlier (Tan *et al*., 2001; Durand and Lightner, 2002) and can be explained by differences in viral replication, defence response of the host and/or susceptibility among individuals of the same species. Differences in bacterial density between individual shrimp can also reflect the variable health status of shrimp. It appears that stronger shrimp can eliminate bacteria very fast, while weaker ones do it slowly or cannot resist to the bacteria and finally die.

To explain the findings of the current study, it is postulated that the bacterial clearing capacity of shrimp can be severely undermined by a *WSSV* infection, even in the early stages of the viral disease. Consequently, *V. campbellii* is allowed to multiply unchecked in the shrimp's body, rapidly leading to death. Previous research has documented negative effects, both of *WSSV* and of *V. campbellii*, on vital physiological processes. Jiravanichpaisal and colleagues (2006) reported that granular haemocytes of *WSSV*-infected crayfish had lost their capacity to induce melanization. *WSSV* inhibits the proPO system upstream of phenoloxidase or simply consumes the native substrate for the enzyme so that no activity can occur. Scholnick and colleagues (2006) found that *L. vannamei* injected with *V. campbellii* decreased oxygen uptake by 27% after 4 h. This phenomenon persisted 24 h after *Vibrio* injection. The inhibition of melanization due to *WSSV* might be directly linked with improved dissemination of *V. campbellii* inside shrimp. The combination with a reduced oxygen level caused by the *Vibrio* can result in an acute threat to the survival of shrimp.

In conclusion, an injection with live *V. campbellii* 24 h after a *WSSV* injection clearly accelerated mortality in juvenile SPF *L. vannamei* shrimp. Such accelerated mortality was not observed when shrimp were injected with very high dose of *WSSV* (Rahman *et al*., 2007a). The presence of *V. campbellii* did not result in any increase of *WSSV* replication, but the density of *V. campbellii* in haemolymph increased spectacularly, resulting in much faster mortality of dually inoculated shrimp. The combination of these findings strongly argues for a synergistic effect in which a low non-lethal *WSSV* load allows for a rapid *Vibrio* multiplication. All research performed on *Vibrio* infection of shrimp can no longer deny this phenomenon, as viral load at the start of the experiment has a dramatic effect on the outcome. The use of SPF shrimp is paramount, or the quantification of the viral load should be taken into account.

With their wide distribution, it is probable that co-infections of *WSSV* and *Vibrio* can occur regularly in the field. However, detailed research is required to elucidate the importance and the exact mechanism of polymicrobial infections in shrimp ponds.

Experimental procedures

Viral and bacterial stocks

Viral stock. A Vietnamese *WSSV* isolate was used in this study. This isolate was studied before and was shown to be significantly less virulent than two other isolates from Thailand (Rahman *et al*., 2007a,b). The original *WSSV* isolate from naturally infected *P. monodon* was passaged once into crayfish (*Cherax quadricarinatus*). Crayfish gill suspension containing *WSSV* was received from Research Institute for Aquaculture No2, Vietnam. The isolate was amplified in SPF *L. vannamei* juveniles. The virus stock was titrated *in vivo* by intramuscular route and the virus titre was $10^{5,8}$ SID_{50} ml^{-1} as determined by indirect immunofluorescence (IIF) and one-step PCR (Escobedo-Bonilla *et al*., 2005). A dose of 30 SID_{50} was prepared in a volume of 50 µl by diluting the stock with phosphate-buffered saline (PBS). As a control inoculum (mock), PBS alone was used.

Bacterial stock. Vibrio campbellii (LMG21363) was obtained from the Laboratory of Microbiology (Ghent University, Belgium). The strain, previously stored in 20% glycerol at −80°C, was aseptically inoculated in Marine Agar (MA). The plates were incubated for 24 h. Single colonies were subsequently transferred and grown in MB 2216 (Difco Laboratories, USA) by incubation overnight (28°C, 150 r.p.m.). The culture was then transferred to centrifugation tubes and centrifuged at 2200 *g* for 15 min. The supernatant was discarded and pellets were washed twice and finally re-suspended in FASW. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm assuming that an optical density of 1.0 corresponds to 1.2×10^9 cells ml^{-1}, the McFarland standard (Bio Merieux, France). A bacterial dose of 10^6 cfu 100 μl^{-1} was prepared. As a control, FASW was used.

Experimental animals and conditions

Specific pathogen-free *L. vannamei* of the Kona-Hawaii (USA) strain (Wyban *et al*., 1992) were used in this study. Shrimp were imported from Molokai Sea Farms, Hawaii (USA). Animals were certified to be free of *WSSV*, IHHNV, MBV, HPV, Taura syndrome virus (TSV), Yellow head virus (YHV) and Gill-associated virus (GAV), as well as other pathogens (fungi, protozoa) as verified by PCR and histopathology. Batches of shrimp arrived at the Laboratory of Aquaculture and Artemia Reference Center (ARC), Ghent University, as post-larvae (PL_{8-12}). They were kept in a recirculation system at a water temperature of 28–29°C, 34 mg l^{-1} salinity and pH of 7.8–8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After 1 week their diet was shifted to A2 monodon high-performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) day^{-1}. In this study, shrimp were taken randomly from the population and gradually acclimatized to a salinity of 15 g l^{-1} over 4 days. Acclimatized shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the infection experiments were performed under biosafety conditions. One day before and during the entire period after the inoculation, shrimp were housed individually in covered 10 l aquaria, filled with artificial seawater prepared with distilled water at a salinity of 15 g l^{-1}, provided with constant aeration and maintained at 27 ± 1°C by air heaters. Feeding was skipped for 24 h prior to the injection, and resumed 6 hpi. During the experiments, shrimp were fed three pellets of feed every 12 h.

Rifampycin-resistant V. campbellii

Rifampycin-resistant (RR) *V. campbellii* was produced as follows: a colony picked from MA plates was cultured for 24 h in 25 ml of MB in 100 ml erlenmeyer. After incubation, 50 µl of the culture was taken by micropipette and transferred to 25 ml of fresh MB containing 0.5 mg l^{-1} rifampycin. The culture was incubated for 24 h at 28°C. The growth of bacteria was indicated by turbidity of the culture. In the following days, further subcultures were made in MB, increasing the rifampycin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100 mg l^{-1}. When bacteria cultures were growing well in the final concentration of rifampycin (100 mg l^{-1}), they were inoculated on MA plates containing 100 mg l^{-1} rifampycin for obtaining single colonies. The stock was stored in 20% glycerol at −80°C for long-term storage.

IHC and quantification of WSSV*-infected cells*

Shrimp samples were collected and fixed in Davidson's fixative for 36 h and kept in 50% ethanol afterwards. Samples were processed as described by Bell and Lightner (1988). Paraffin-embedded tissue sections were cut at 5 mm and placed onto Silane-coated slides (A3648, Sigma-Aldrich). Sections were deparaffinized and rehydrated. The endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in Tris buffer pH 7.4.

Sections were incubated for 1 h at 37°C with 2 μg ml^{-1} of monoclonal antibody 8B7 raised against *WSSV* envelope protein VP28 (Poulos *et al*., 2001). Sections were washed in Tris buffer (pH 7.6) and incubated for 1 h at 37°C with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001, Amersham Biosciences). Afterwards they were washed, incubated for 30 min at room temperature with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences) and washed again. Colour was developed with 0.01% of 3, 3′-diaminobenzidine (D8001 Sigma-Aldrich). Sections were counterstained with Gill's haemaluin and washed in water, dehydrated and mounted. *WSSV*-infected cells were counted using light microscopy (Leica DM RBE) at a 400× magnification in five fields in gills and lymphoid organs and in two to three fields in haematopoietic tissue. These counts were converted to the number of *WSSV*-infected cell mm^{-2}. Both *WSSV*-infected and uninfected cells in stomach epithelium and cuticular epithelium were counted in five fields and the average percentage (%) of infected cells was calculated.

Enumeration of V. campbellii

Rifampycin-resistant *V. campbellii* were enumerated on MA + 100 mg l^{-1} rifampycin (MAR). Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment, using a 25-gauge needle and a 1 ml syringe rinsed with cold modified Alsever's solution [AS; 19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA (ethylene diaminetetra-acetic acid); pH 7.2] as an anticoagulant (Rodriguez *et al*., 1995). Directly after sampling, haemolymph was serially diluted in FASW and plated on MAR. The plates were incubated at 28°C for 24 h. As RR *V. campbellii* are luminous bacteria, all colonies were checked for luminescence before counting to ensure that no contamination had occurred during the sampling procedure.

Destruction of bacterial cells by sonicator

A bath sonicator and glass beads were used for destruction of the bacterial cells. For this purpose, *V. campbellii* were up-scaled for 12 h in an erlenmeyer containing 25 ml of MB. Harvesting of bacteria was performed by centrifugation for 15 min at 2200 *g*. The cells were washed twice and finally re-suspended in FASW. Six millilitres of bacterial suspension with a density of 10^7 cfu ml^{-1} was transferred to a 50 ml falcon tube containing glass beads and was vortexed for 3 min. Then, it was kept in −80°C for 20 min. Subsequently, it was thawed in sonicator for 3 min, vortexed for 3 min, sonicated for 6 min and was placed back in −80°C for 20 min. The freeze and thaw cycle was repeated for six times. After each cycle, samples were collected and plated on MA to check whether the bacteria had been killed by the procedure. The supernatant was prepared by culturing of *V. campbellii* in MB for 24 h. The cultures were then transferred to the falcon tube and centrifuged for 15 min at 2200 *g*. The supernatant was collected and filtered through 0.2 μm filter paper.

Experimental design

Experiment 1: dose effect of V. campbellii *on mortality of* L. vannamei. The aim of this experiment was to find the highest possible dose of *V. campbellii* which does not cause significant mortalities in *L. vannamei*. This sublethal dose was used for the co-infection experiments. Juvenile shrimp (MBW = 12.47 ± 0.89 g) were injected (100 μl) with different doses of *V. campbellii* containing 10^3, 10^4, 10^5, 10^6 or 10^7 cfu per shrimp respectively. As control inoculum, shrimp were injected with the same volume of FASW. Each treatment was performed with two replicates of 10 shrimp and the experiment lasted 5 days. Every 6 h, shrimp were monitored for disease symptoms and moribund/dead shrimp were collected.

Experiment 2: effect of sonicated V. campbellii *and supernatant from* V. campbellii *cultures on mortality of* L. vannamei. This experiment was conducted to test whether toxic products produced by *V. campbellii*, both extra- or intracellular, could be responsible for shrimp death. The experiment was set up with four different treatments. Shrimp (MBW = 1.1 ± 0.17 g) were injected either with supernatant or sonicated *V. campbellii* or with MB or FASW as a control. Each treatment was performed with three replicates of 10 shrimp. Every 6 h, they were monitored for disease symptoms and moribund/dead shrimp were removed. The experiment was terminated after 5 days.

Experiment 3: effect of sonicated V. campbellii *on mortality of* WSSV*-infected* L. vannamei. This experiment tested the effect of sonicated *V. campbellii* on the mortality of *WSSV*-infected shrimp. The experiment was set up with six treatments. At 0 hpi, groups of six shrimp (MBW = 5.17 ± 0.9 g) were either injected with *WSSV* at a dose of 30 SID_{50} shrimp^{-1}(treatment 1, 4 and 5) or mock inoculated (treatment 2, 3 and 6). At 24 hpi, shrimp were either injected with 10^6 cfu of *V. campbellii* (treatment 2 and 4), negative inocula (treatment 1 and 6) or sonicated *V. campbellii* (treatment 3 and 5). After each injection procedure, shrimp were placed in their individual 10 l aquarium. Every 12 h, they were monitored for disease symptoms and moribund/dead shrimp were collected. Every 5 days, 75% of water was replaced with new seawater (15 g l^{-1} salinity) to minimize ammonia build-up. The experiment was terminated when mortality reached 100% in the *WSSV* only group.

Experiment 4: co-infection of L. vannamei *with* WSSV *and* V. campbellii *(first run).* The experiment was set up with five treatments to investigate the synergistic effect of *WSSV* and *V. campbellii* on the mortality of *L. vannamei*. At 0 hpi, groups of six shrimp (MBW = 16.8 ± 2.1 g) were either injected with *WSSV* at a dose of 30 SID_{50} shrimp^{-1} (treatment 1 and 3) or mock inoculated (treatment 2, 4 and 5). After 24 h, all shrimp were either injected with 10^6 cfu (treatment 2 and 3) or 10^7 cfu of *V. campbellii* (treatment 5) or mock inoculated (treatment 1 and 4). After each injection procedure, shrimp were placed in their individual 10 l aquarium. Every 12 h, they were monitored for disease symptoms and moribund/dead shrimp were removed. Temperature and ammonium were checked daily. Every 5 days, 75% of water was replaced with new seawater (15 g l^{-1} salinity) to minimize ammonia build-up.

Experiment 5: co-infection of L. vannamei *with* WSSV *and* V. campbellii *(second run) and quantification of* WSSV*.* In this experiment, we aimed at repeating experiment 4, and at the same time collect samples that would allow for the quantification of *WSSV*. The experiment was identical to experiment 4, except that the positive control group was omitted. Moribund shrimp (MBW = 21.5 ± 2.4 g) were collected at different time points for quantification of *WSSV*-infected cells by IHC.

Experiment 6: quantification of WSSV *and* V. campbellii *in co-infected* L. vannamei*.* This experiment was set up to collect moribund shrimp for quantification of *WSSV* and *V. campbellii.* The experimental design was identical to experiment 5 but shrimp were injected with 10^5 cfu of *V. campbellii* instead of 10^6 cfu as mentioned in previous experiments. This dose was chosen as it did not cause any mortality when injected alone but could lead to a very clear acceleration of mortality in co-infections with *WSSV*. In this experiment, RR *V. campbellii* were used to avoid contamination during re-isolation. A test confirmed that the selection process had not altered the virulence of this strain. Each treatment started with 12 shrimp (MBW = 4.35 ± 0.72 g). Ten hours post *V. campbellii* injection, six shrimp were collected from each treatment for quantification of the two pathogens.

Statistical analysis

Differences between treatments were evaluated by performing *t*-test analysis using statistical analysis software SPSS (version 13.0 for Windows). Values in percentages (*WSSV*-infected cells in stomach epithelium) were ArcSin-transformed to satisfy normal distribution.

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References

Alday-Sanz, V., Roque, A., and Turnbull, J.F. (2002) Clearing mechanisms of *Vibrio vulnificus* biotype I in the black tiger shrimp *Penaeus monodon*. *Dis Aquat Organ* **48:** 91–99.

Álvarez, J.D., Austin, B., Álvarez, A.M., and Reyes, H. (1998) *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J Fish Dis* **21:** 313–316.

Bell, T.A., and Lightner, D.V. (1988) *A Handbook of Normal Penaeid Shrimp Histology.* Baton Rough, LA, USA: The World Aquaculture Society.

Chang, P.S., Lo, C.F., Wang, Y.C., and Kou, G.H. (1996) Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. *Dis Aquat Organ* **27:** 131–139.

Chayaburakul, K., Nash, G., Pratanpipat, P., Sriurairatana, S., and Withyachumnarnkul, B. (2004) Multiple pathogens found in growth-retarded black tiger shrimp *Penaeus monodon* cultivated in Thailand. *Dis Aquat Organ* **60:** 89–96.

Chou, H.Y., Huang, C.Y., Wang, C.H., Chiang, H.C., and Lo, C.F. (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis Aquat Organ* **23:** 165–173.

Durand, S.V., and Lightner, D.V. (2002) Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. *J Fish Dis* **25:** 381–389.

Escobedo-Bonilla, C.M., Wille, M., Alday-Sanz, V., Sorgeloos, P., Pensaert, M.B., and Nauwynck, H.J. (2005) *In vivo* titration of white spot syndrome virus (*WSSV*) in SPF *Litopenaeus vannamei* by intramuscular and oral routes. *Dis Aquat Organ* **66:** 163–170.

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

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

Gómez-Gil, B., Tron-Mayén, L., Roque, A., Turnbull, J.F., Inglis, V., and Guerra-Flores, A.L. (1998) Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. *Aquaculture* **163:** 1–9.

Hisbi, D., Vandenberghe, J., Robles, R., Verdonck, L., Swings, J., and Sorgeloos, P. (2000) Characterisation of *Vibrio and* related bacteria associated with shrimp *Penaeus monodon* larvae in Indonesia. *Asian Fish Sci* **13:** 57–64.

Horowitz, A., and Horowitz, S. (2001) Disease control in shrimp aquaculture from a microbial ecology perspective. In *The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001.* Browdy, C.L., and Jory, D.E. (eds). Baton Rouge, LA, USA: The World Aquaculture Society, pp. 199–218.

Jiravanichpaisal, P., Sricharoen, S., Söderhäll, I., and Söderhäll, K. (2006) White spot syndrome virus (*WSSV*) interaction with crayfish haemocytes. *Fish Shellfish Immunol* **20:** 718–727.

Lavilla-Pitogo, C.R.M., Baticados, C.L., Cruz Lacierda, E.R., and De La Peña, L.D. (1990) Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture* **91:** 1–13.

Lightner, D.V. (1988) *Vibrio* disease in penaeid shrimp. In *Disease Diagnosis and Control in North American Marine Aquaculture*, 2nd edn. Sinderman, C.J., and Lightner, D.V. (eds). Amsterdam: Elsevier, pp. 42–47.

Lightner, D.V. (1996) *A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp.* Baton Rouge, LA, USA: World Aquaculture Society.

Lightner, D.V. (2003) *The Penaeid Shrimp Viral Pandemics due to IHHNV, WSSV, TSV and YHV: Current Status in the Americas*, Vol. 1. World Aquaculture Society Meeting,

Salvador, Brazil, Book of Abstracts, Baton Rouge, LA, USA: World Aquaculture Society, p. 418.

Lightner, D.V., Hasson, K.W., White, B.L., and Redman, R.M. (1998) Experimental infection of western hemisphere Penaeid shrimp with Asian white spot syndrome virus and Asian yellow head virus. *J Aquat Anim Health* **10:** 271–281.

Mermound, I., Costa, R., Ferré, O., Goarant, C., and Haffner, P. (1998) 'Syndrome 93' in New Caledonian outdoor rearing ponds of *Penaeus stylirostris*: history and description of three major outbreaks. *Aquaculture* **164:** 323–335.

Oh, M.J., Kim, W.S., Kitamura, S.I., Lee, H.K., Son, B.W., Jung, T.S., and Jung, S.J. (2006) Change of pathogenicity in Olive flounder *Paralichthys olivaceus* by co-infection of *Vibrio harveyi*, *Edwardsiella tarda* and marine birnavirus. *Aquaculture* **257:** 156–160.

Pakingking, J.R., Takano, R., Nishizawa, T., Mori, K., Lida, Y., Arimoto, M., and Muroga, K. (2003) Experimental coinfection with aquabirnavirus and viral hemorrhagic septicemia virus (VHSV), *Edwardsiella tarda* or *Streptococcus iniae* in Japanese Flounder *Paralichthys olivaceus*. *Fish Pathol* **38:** 15–21.

Pizzutto, M., and Hirst, R.G. (1995) Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting. *Dis Aquat Organ* **21:** 61–68.

Poulos, B.T., Pantoja, C.R., Bradley-Dunlop, D., Aguila, J., and Lightner, D.V. (2001) Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *Dis Aquat Organ* **47:** 13–23.

Rahman, M.M., Corteel, M., Dantas-Lima, J.J., Wille, M., Alday-Sanz, V., Pensaert, M.B., *et al.* (2007a) 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.

Rahman, M.M., Corteel, M., Wille, M., Alday-Sanz, V., Pensaert, M.B., Sorgeloos, P., and Nauwynck, H.J. (2007b) 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., Corteel, M., Escobedo-Bonilla, C.M., Wille, M., Alday-Sanz, V., Pensaert, M.B., *et al.* (2008) Virulence of white spot syndrome virus (*WSSV*) isolates may be correlated with the degree of replication in gills of *Penaeus vannamei* juveniles. *Dis Aquat Organ* **79** (3)**:** 191–198.

Robertson, P.A.W., Calderon, J., Carrera, L., Stark, J.R., Zherdmant, M., and Austin, B. (1998) Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. *Dis Aquat Organ* **32:** 151–155.

Rodriguez, J., Boulo, V., Mialhe, E., and Bachère, E. (1995) Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. *J Cell Sci* **108:** 1043–1050.

Rosenberry, B. (2004) World shrimp farming 2004. In *Shrimp News International*. Rosenberry, B. (ed.). San Diego, CA, USA.

Sanchez-Martinez, J.G., Aguirre-Guzman, G., and Mejia-Ruiz, H. (2007) White spot syndrome virus in cultured shrimp: a review. *Aquacult Res* **38:** 1339–1354.

Sarathi, M., Ishaq Ahmed, V.P., Venkatesan, C., Balasubramanian, G., Prabavathy, J., and Sahul Hameed, A.S. (2007) Comparative study on immune response of *Fenneropenaeus indicus* to *Vibrio alginolyticus* and white spot syndrome virus. *Aquaculture* **271:** 8–20.

Saulnier, D., Haffner, P., Goarant, C., Levy, P., and Ansquer, D. (2000a) Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture* **191:** 133–144.

Saulnier, D., Avarre, J.C., Le Moullac, G., Ansquer, D., Levy, P., and Vonau, V. (2000b) Evidence that *Vibrio penaeicida* is a putative etiological agent of syndrome'93 in New Caledonia and development of a rapid and sensitive PCR assay for its detection in shrimp and sea water. *Dis Aquat Org* **40:** 109–115.

Scholnick, D.A., Burnett, K.G., and Burnett, L.E. (2006) Impact of exposure to bacteria on metabolism in the Penaeid shrimp *Litopenaeus vannamei*. *Biol Bull* **211:** 44–49.

Selvin, J.L., and Lipton, A.P. (2003) Vibrio alginolyticus associated with white spot disease of Penaeus monodon. *Dis Aquat Organ* **57:** 147–150.

Sudha, P.M., Mohan, C.V., Shankar, K.M., and Hegde, A. (1998) Relationship between White Spot Syndrome Virus infection and clinical manifestation in Indian cultured penaeid shrimp. *Aquaculture* **167:** 95–101.

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

Umesha, K.R., Dass, B.K.M., Naik, B.M., Venugopal, M.N., Karunasagar, I., and Karunasagar, I. (2006) High prevalence of dual and triple viral infections in black tiger shrimp ponds in India. *Aquaculture* **258:** 91–96.

Van de Braak, C.B.T., Botterblom, M.H.A., Taverne, N., van Muiswinkel, W.B., Rombout, J.H.W.M., and van der Knaap, W.P.W. (2002) The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. *Fish Shellfish Immunol* **13:** 293–309.

Vandenberghe, J., Li, Y., Verdonck, L., Li, J., Sorgeloos, P., Xu, H.S., and Swings, J. (1998) *Vibrio* associated with *Penaeus chinensis* (Crustacea: Decapoda) larvae in Chinese shrimp hatcheries. *Aquaculture* **169:** 121–132.

Vandenberghe, J., Verdonck, L., Robles Arozarena, R., Rivera, G., Bolland, A., Balladares, M., *et al.* (1999) Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. *Appl Environ Microbiol* **65:** 2592–2597.

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

Wyban, J.A., Swingle, J.S., Sweeney, J.N., and Pruder, G.D. (1992) Development and commercial performance of high health shrimp using specific pathogen free (SPF) broodstock *Penaeus vannamei*. In *Proceedings of the Special Session on Shrimp Farming.* Wyban, J.A. (ed.). Baton Rouge, LA, USA: World Aquaculture Society, pp. 254–266.

