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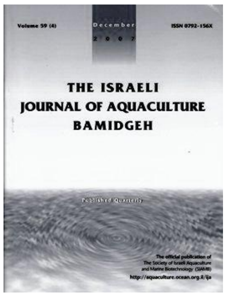

Identification of Stressors that Affect White Spot Syndrome Virus (WSSV) Infection and Outbreak in Pond Cultured *Penaeus monodon*

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Abstract

White spot syndrome virus (WSSV) has been a big problem to the worldwide shrimp industry. Exposure to stressors related to physicochemical water parameters affect WSSV infection but not all WSSV infections result in outbreaks. This paper describes a detailed monitoring of important physicochemical water parameters on a farm with 11 ponds that had WSSV infection. The virus was detected in shrimp exposed to two or more simultaneous stress factors (temperature, pH, water level) or multiple stressors for a number of days. Exposure to more than three stressors resulted in an outbreak of the disease within 3-6 days. Outbreaks were experienced in ponds with a temperature of 26-27°C, a pH lower than 8.0, pH fluctuation of 1.0, and a water depth of less than 1 m. Shrimp stocked in eight of the ponds were successfully harvested after 128-173 days of culture.

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Introduction

White spot syndrome virus (WSSV) is an important shrimp disease that infects arthropods and other organisms as a host or a carrier. WSSV affects the most commercially important species of penaeid shrimp and crabs (Otta et al., 1999; Momoyama, 2003). Clinical signs of WSSV infection are usually visible 24-96 h after exposure to stressors, depending on the degree of infection or the viral titer (Vidal et al., 2001; Rahman et al., 2007).

Stress increases the susceptibility of shrimp to WSSV infection. In tank-based experiments, physicochemical water parameters can act as stressors that trigger WSSV infection by compromising the defense system of shrimp (Takahashi et al., 1995). WSSV propagates rapidly under stressful conditions, resulting in shrimp mortality (Lo and Kou, 1998).

Studying details that affect WSSV infection, such as the level, number, and type of stressors and the interaction between them, is difficult in large datasets; case studies are more appropriate for generating such information. The objective of this study was to determine the number and types of stressors that affect WSSV infections and outbreaks in pond-cultured *Penaeus monodon*. Our strategy was to count the number of stressors to which the shrimp were exposed in 11 WSSV-infected *P. monodon* ponds of which only three experienced an outbreak of the disease.

Materials and Methods

Farm site. The farm has a total area of 47 ha of which 42.7 ha is covered with water. The farm consists of 52 ponds varying in size from 0.22 to 1.0 ha. During the experiment, four ponds served as reservoirs and the rest were used to culture *P. monodon*, tilapia, and milkfish. The main water sources were a river and several deep or shallow wells. Salinity ranged from 10 ppt during the rainy season to 38 ppt during the peak of summer.

Pond management. In all 11 shrimp ponds, the top soil was scraped and plowed to a depth of 6-10 inches. After plowing, the pond bottom was dried and flushed by filling with water to a depth of 30 cm. The water remained in the pond 2-3 days, then was drained. This sequence was repeated four times before compaction of the pond bottom and application of agricultural lime. Hydrated lime was applied after the final flushing for disinfection. After preparation, the pond was filled with water to a depth of 30 cm. Probiotics (*Bacillus* and other presumed beneficial microorganisms) and molasses were applied to condition the pond bottom. The water level was adjusted to 1.0 m after 24 h. Probiotics and molasses (5 kg/ha) were again added to the water four days before stocking.

The ponds were stocked with WSSV-free *P. monodon* $PL_{18\text{-}20}$ at 20-25/m^2; shrimp were previously analyzed with nested polymerase chain reaction to ascertain they were not infected by WSSV. The fry were acclimatized following a standard protocol: unopened plastic bags containing fry were allowed to float in the pond for about one hour. The bags were then opened and pond water allowed to enter the bags slowly until the salinity, temperature, and pH of the water inside the transport bags were equal to that of the pond water. The fry were then allowed to swim out of the bags.

Paddlewheels were installed in the ponds to provide 24-h aeration. Shrimp were fed a commercial diet in which commercial probiotics and vitamin C (Charoen Pokphand Foods) were incorporated at 10 g and 2-5 g/kg feed, respectively. Molasses were added to the water at 5 kg/ha.

Monitoring. Temperature, salinity, pH, transparency, and water depth were monitored twice daily at 08:00 and 15:00. Every two weeks and whenever disease signs (white spots on the shrimp carapace) were observed, five shrimp were sampled for WSSV analysis using a SVBBU kit developed by the Shrimp Biotechnology Business Unit (SBBU) of Mahidol University, Thailand (Withyachumnarnkul, 1999). The kit uses a lateral flow dipstick (LFD) for molecular diagnosis of WSSV. Total DNA from gills of juvenile and adult shrimp was extracted using the DNA Purification Kit (SBBU) according to the manufacturer's protocol and amplified using the kit's reagents. Amplified DNA was applied to the LFD and placed in an assay buffer to allow bands to develop. Bands were interpreted based on the kit manual.

Definition of terms. Stressors were defined as physicochemical water parameters outside the suitable range for shrimp culture (Table 1). The stressors to which shrimp were exposed four days prior and six days after infection were determined. WSSV infection was determined using the SBBU kit. WSSV was considered an outbreak when mortality exceeded 90% on a single day.

Table 1. Physicochemical water parameters beyond the suitable range for *Penaeus monodon* culture, thus considered stressors.

Water parameter	*Value*	*Reference*
Low temperature	≤28°C	Guan et al. 2003
High temperature	≥33°C	Guan et al. 2003
Low pH	<8.0	Pan et al. 2005
High pH	>8.5	Pan et al. 2005
pH fluctuation	≥0.5	Pan et al. 2005
Water transparency	<30 cm	Baliao & Tookwinas 2002
Water depth	<100 cm	Baliao & Tookwinas 2002

Statistical analysis. The Mann-Whitney Test was used to test differences between means. The presence or absence of an outbreak was the dependent variable while the average number and quantified kinds of stressors were the independent variables. The same set was used as dependent and independent variables for Spearman's rho correlation analysis to determine the linear relationship between factors. All analyses were done using the statistical program for the social sciences (SPSS v. 16).

Results

WSSV infection and outbreak. All 11 ponds were infected by WSSV. Four were infected after 68-88 days of culture, seven after 103-127 days of culture (Table 2). WSSV outbreak was experienced in three ponds, 3-6 days after WSSV was detected, in shrimp younger than 90 days, exposed to an average of five stressors per day and a combination of at least four kinds of stressors. Exposure to more than four stressors that included low temperature and low pH four days prior and six days after infection resulted in an outbreak. There were no outbreaks in ponds exposed to less than four stressors, even when those stressors included low temperature and low pH. Low pH, high pH fluctuation, and low transparency were observed in almost all ponds four days prior to infection (Table 3). Low pH and high pH fluctuations were the most common stressors in ponds that did not result in outbreaks. In spite of the infection, shrimp from eight ponds were successfully harvested after 128-173 days of culture.

There were significant differences in temperature, pH, pH fluctuation, water level, average number of stressors per day, and types of stressors between ponds that resulted in outbreaks and those that did not (Table 4). Outbreaks correlated positively with age of

Table 2. Day of culture at which white spot syndrome virus (WSSV) infection and outbreak occurred, numbers and kinds of stressors per day before and after infection.

	Day of culture		*Avg no. stressors/day*	*Kinds of stressor 4 days before infection*		*Kinds of stressor 6 days after infection*	
Pond	*at infection*	*at outbreak*	*before infection*	*No.*	*Kind*	*No.*	*Kind*
1	68	71	5.53	6	D, pH, S, T, Tr, W	5	D, pH, Sf, Tf, Tr
2	84	87	5.4	5	D, pH, T, Tr, W	5	D, pH, T, Tr, W
3	87	93	5.16	4	pH, T, Tr, W	5	pH, pHf, T, Tr, W
4	103	None	2.81	3	pH, pHf, Tr	3	pH, T, Tr
5	88	None	4.82	3	pH, T, Tr	4	T, pH, Tr, D
6	126	None	3.07	3	pH, pHf, Tr	3	pH, pHf, Tr
7	126	None	2.51	2	pH, Tr	3	pH, pHf, Tr
8	119	None	3.18	3	pH, pHf, Tr	3	pH, pHf, Tr
9	119	None	3.43	3	pH, pHf, Tr	3	pH, pHf, Tr
10	127	None	3.11	4	pH, pHf, S, Tr	4	pH, pHf, S, Tr
11	127	None	3.42	4	D, pH, pHf, Tr	3	pH, pHf, Tr

D = water depth (<100 cm), pHf = pH fluctuation (>0.5), S = salinity (≤10 ppt), Sf = salinity fluctuation (≥4 ppt in 12 h), T = temperature (≤27°C), Tf = temperature fluctuation (≥4°C within a 7-17 h period), Tr = water transparency (<30 cm), W = weather (rain)

Table 3. Significant water parameters four days prior to WSSV infection.

Pond	Parameter				
	Temperature (°C)	pH	pH fluctuation	Water depth (cm)	Transparency (cm)
1	26-27	7.3±0.1	0.1-0.2	90-95	25
2	26-27	7.25±0.15	0.1-0.3	102-104	20
3	26-27	7.2±0.1	0-0.2	110-112	20
4	28-32	8.05±0.55	0.5-1.1	110-112	20
5	26-27	7.7±0.2	0.1-0.4	95-100	20
6	28-32	8.05±0.55	0.5-1.05	110-117	25
7	28-32	7.25±0.15	0-0.3	120-124	25
8	28-32	7.8±0.4	0.5-0.8	120	20
9	28-30	7.6±0.4	0.5-0.8	120	20
10	28-32	7.9±0.3	0.4-0.6	107	25
11	28-32	7.9±0.4	0.6-0.8	100	25

shrimp and temperature fluctuations but negatively with the number of stressors per day, which correlated negatively with days of culture (Table 5). Temperature correlated positively with pH, pH fluctuations, and temperature fluctuation, which correlated positively with days of culture and transparency, but negatively with number of stressors per day.

Table 4. Comparison of average levels of stressors in WSSV-infected shrimp ponds with and without an outbreak.

Parameter	Outbreak	
	Yes	No
pH*	7.5	8.0
pH fluctuation*	1.0	0.1
Temperature (°C)*	27.5	29.0
Salinity (ppm)	10.3	11.0
Transparency (cm)	22.9	25.0
Depth (cm)*	98.3	109.5
Average no. of stressors/day*	5	3
Number of types of stressors*	5	3

*significant differences between ponds with and without outbreak

Discussion

WSSV was detected in shrimp after exposure to stressors, confirming that environmental stress increases the risk of WSSV. Stressors can reduce resistance to infection (Le Moullac and Haffner, 2000). Physicochemical changes in sea water can influence the immune system (Chen et al., 1995). WSSV multiplies and spreads quickly in *P. monodon* when triggered by a stressor (Peng et al., 1998).

Outbreaks occurred 3-6 days after WSSV detection. Likewise, mortality was 100% in *Penaeus vannamei* juveniles 60-192 h after inoculation (Rahman et al., 2007) and in *Litopenaeus vannamei* transferred from 32°C to 25.8±0.7°C within 3-4 days where outbreaks were attributed to an actively-proliferated viral infection (Vidal et al., 2001) that became systemic (Escobedo-Bonilla et al., 2007), causing irreversible tissue damage.

Exposure to three or less stressors in shrimp older than 90 days resulted in infection but not an outbreak while exposure to five stressors in shrimps of this age resulted in an outbreak. Temperature, temperature fluctuation, pH, pH fluctuation, water depth, number of stressors per day, and types of stressor were important factors that resulted in outbreaks.

Table 5. Pearson's correlation coefficient for WSSV outbreaks, the number of stressors per day to which the shrimp were exposed, and water parameters.

	Outbreaks	DOC	S/D	T	pH	pHf	Sal	Trans	Depth	tf
Outbreaks	-	-	-	-	-	-	-	-	-	-
DOC	**0.79	-	-	-	-	-	-	-	-	-
S/D	**-0.86	**-0.92	-	-	-	-	-	-	-	-
T	0.511	0.58	-0.56	-	-	-	-	-	-	-
pH	0.49	0.34	-0.49	**0.77	-	-	-	-	-	-
pHf	0.33	0.43	-0.43	**0.89	**0.83	-	-	-	-	-
Sal	0.57	**0.88	*-0.73	*0.61	0.27	0.45	-	-	-	-
Trans	0.55	*0.63	**-0.77	*0.62	*0.62	0.57	0.52	-	-	-
Depth	0.59	**0.79	**-0.80	0.15	-0.08	0.03	*0.64	0.49	-	-
tf	*0.64	*0.69	**-0.77	**0.88	**0.79	**0.85	0.55	**0.81	0.41	-
sf	0.33	0.52	-0.33	-0.08	-0.09	-0.12	0.56	-0.07	0.31	-0.12

* significantly correlated at $p<0.05$; ** significantly correlated at $p<0.01$
DOC = days of culture (age), S/D = no. stressors/day, T = temperature, pHf = pH fluctuation, Sal = salinity, Trans = transparency, tf = temperature fluctuation, sf = salinity fluctuation

Low temperatures also caused outbreaks in WSSV-infected *Marsupenaeus japonicus* ponds where survival was low at 23°C and 28°C (Guan et al., 2003) and *L. vannamei* maintained at 27°C after WSSV-challenge showed clinical signs (Rahman et al., 2006). Temperature affects both shrimp and the WSSV virus. Temperature directly affects the metabolism, growth, molting, and immune response of the host organism (Chen et al., 1995; Jiravanichpaisal et al., 2004). At the same time as it weakens the immune response of shrimp, decreased temperature increases the viral load (Rahman et al., 2006; Reyes et al., 2007). High water temperatures reduce or completely inhibit the expression of WSSV genes on shrimp subcuticular epithelial cells, resulting in decreased viral replication (Rahman et al., 2006; Reyes et al., 2007).

Low pH and pH fluctuations four days prior to infection may have immuno-compromised the shrimp, making them susceptible to WSSV. While 6.6-8.5 has been reported as optimum for shrimp culture (Tsai, 1990), later reports indicate that fluctuation of pH should not exceed 0.5 and pH of the environment should be maintained at 8.0-8.5 (Pan et al., 2005). Infectivity of other viruses such as those associated with infectious hypodermal hematopoietic necrosis virus (IHHNV) is affected by low pH (Lu and Loh, 1992). Further, pH affects the shrimp immune system; deviation from the optimal pH induced modification of the hemocyte count and phenoloxidase activity in *Macrobrachium rosenbergii* (Cheng and Chen, 2000) while hemocyte count and antibacterial activity decreased gradually in *L. vannamei* during the first three days of exposure to a variable pH (Lu-Qing et al., 2005).

Salinity was below optimum in all ponds, indicating that the salinity level may not be an important stressor in the triggering of WSSV infection as long as it is constant. Salinity affects shrimp, but has little effect on the infectivity of WSSV (Chang et al., 1998). However, salinity fluctuations of 4 ppt within twelve hours and temperature fluctuations of 4°C twice within 17 hours acted as additional stressors to low pH and low water transparency, resulting in outbreaks in infected ponds. Salinity variation can lower resistance of *P. monodon* to *Photobacterium damselae* (Wang and Chen, 2006). Sudden salinity changes lowered *P. monodon* survival, possibly because acute salinity stress alters the hemolymph metabolic profile of *P. monodon*, reducing immunocompetence to WSSV infection (Joseph and Philip, 2007). Temperature fluctuations of 3-4°C within a 7-17 h period are also WSSV risk factors (Tendencia and Verreth, 2011).

Outbreaks occurred in shrimp infected at younger than 90 days, suggesting that young *P. monodon* are more susceptible to WSSV than old. Likewise, white spot syndrome baculovirus (WSBV) infection was greater in *M. rosenbergii* larvae than in post-larvae and varied between larvae, post-larvae, juveniles, and adults (Peng et al., 1998). In contrast, susceptibility to WSSV disease increased with age in *Penaeus japonicus* (Venegas et al., 1999).

Despite WSSV infection, no outbreak was observed in some of the ponds. Disease outbreaks due to WSSV infection do not occur if the shrimp defense system manages to maintain viral infections at low intensity in low-stress culture conditions. However, outbreaks may occur under stressful conditions (Tsai et al., 1999). In this study, stressors that increased the risk of WSSV infection were low temperature (26-27°C), temperature fluctuations (4°C in 7 h), low pH (<8.0), pH fluctuations (>0.5), and low water depth (<100 cm). Outbreaks occurred in a combination of correlated stressors (e.g., pH and temperature, or pH and pH fluctuation). Thus, WSSV outbreaks could be the synergistic effect of the number and types of stressors and the age of the shrimp, with a greater risk in shrimp younger than 90 days. However, the number and types of stressors to which shrimp are exposed after infection are also important and the possibility that the early WSSV outbreaks were due to the early occurrence of the stressors rather than to the age of the shrimp needs further study.

Exposure of shrimp to these risk factors should be avoided through management strategies. Although water temperature may be difficult to control, abrupt fluctuations can be avoided by increasing the water depth. Lime application usually corrects pH. Other options to reduce exposure to stressors need to be explored.

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