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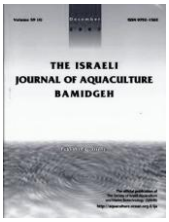

Temperature Fluctuation, Low Salinity, Water Microflora: Risk Factors for WSSV Outbreaks in *Penaeus monodon*

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Abstract

White spot syndrome virus (WSSV) has been devastating the shrimp industry for almost a decade. This study compares water parameters, alkalinity, and microflora in three ponds on a farm on Negros Island (Philippines) during two production cycles where WSSV infection resulted in an outbreak in 2006 but not in 2005. The total bacterial count of the pond water in 2005 was about twice as high as in 2006. However, luminous bacterial counts were twice as high in 2006 than in 2005 and total presumptive *Vibrio*, as counted on *Vibrio* selective thiosulfate citrate bilesalt sucrose (TCBS) agar, was over ten times higher, with a greater percentage of green colonies. More green colonies might indicate a higher concentration of harmful *Vibrio* bacteria. Total alkalinity for both production cycles was within the normal range while temperature, salinity, pH, and dissolved oxygen varied and sometimes fell below or exceeded the acceptable range. In 2006, there were more instances during which the temperature fluctuated 3-4°C within the period of 07:00-17:00, and salinity more often dropped below 15 ppt. Our survey suggests that WSSV outbreak are triggered by water temperature fluctuations of 3-4°C, coupled with low salinity and a high presumptive *Vibrio* count.

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Introduction

White spot syndrome virus has been devastating the shrimp industry for almost a decade. It was probably introduced in Asia by careless importation (Flegel, 2009). Exposure of shrimp to stressors increases the risk of WSSV, since stressors compromise the shrimp immune system (Takahashi et al., 1995). Consequently, under stressful conditions, WSSV can proliferate rapidly and cause mortality (Lo and Kou, 1998; Doan et al., 2009). WSSV outbreaks may be preceded by or coincide with high pH and un-ionized ammonia in shrimp pond water (Corsin et al., 2001). Clinical white spots associated with high water pH disappear after molting (Sahoo et al., 2005). Ammonia-N at 5 mg/l appears to reduce the immunocompetence of *Penaeus japonicus* but may also decrease the virulence of WSSV (Jiang et al., 2004). Temperature is associated with mortality in Ecuadorian farms (Rodriguez et al., 2003). Abrupt fluctuations in temperature and salinity due to heavy rains contribute to increased viral loads in shrimp in Mexico, resulting in 80% mortality (Peinado-Guevara and Lopez-Meyer, 2006).

A WSSV outbreak occurred on the west coast of India when the monsoon peaked and salinity approached 0 ppt (Karunasagar et al., 1997). Fluctuations in salinity and temperature can weaken the shrimp's immune system and affect viral replication. Low salinity and low hardness of pond water are stress factors that increase the susceptibility of shrimp to *Vibrio* and, subsequently, white spot disease (Hettiarachchi et al., 1999). Acute salinity changes of greater than 4 ppt within one hour can lead to rapid WSSV replication and decreased disease-resistance in *Fenneropenaeus chinensis* (Liu et al., 2006). Continual small salinity adjustments may also result in increased WSSV replication and the loss of self-adaptive ability after a long period of salinity stress (Liu et al., 2006).

Water temperature affects WSSV infection and the outbreak of clinical disease. Survival of WSSV-infected *M. japonicus* was low at 23°C and 28°C and high at 15°C and 33°C (Guan et al., 2003) while shrimp maintained at or transferred from a lower temperature to 33°C after WSSV challenge did not show signs of disease (Rahman et al., 2006). Mortality was 100%, however, in WSSV-infected shrimp transferred from 32°C to 25.8±0.7°C (Vidal et al., 2001).

Most of the above reports are tank based. The current study compares and correlates water parameters measured daily, and alkalinity and microflora observed every 2-3 days, in three ponds in a *Penaeus monodon* farm in the Philippines where a disease outbreak associated with WSSV infection was observed in 2006 but not in 2005.

Materials and Methods

Farm site. The farm is located on Negros Island, Philippines. It has a total area of 32 ha with 29 ponds ranging 5996-11000 m^2 and a reservoir of 10.5 ha. Water is supplied mainly from the sea and irrigation systems.

Shrimp. Penaeus monodon were purchased for both production runs (2005 and 2006) from the same hatchery, located in a different province. Postlarvae

were acclimatized in unopened plastic bags allowed to float in the pond in which the shrimp were to be stocked and stocked as shown in Table 1.

Table 1. Stocking of *Penaeus monodon* in three ponds on a farm in 2005, when there was no WSSV outbreak, and in 2006, when there was a WSSV outbreak.

	Pond					
	2005			*2006*		
	A	*B*	*C*	*A*	*B*	*C*
Stocking density (ind/m^2)	12	13	15	20	13	15
Age at stocking (postlarvae stage)	19	20	18	19	20	18
Stocked in:	Jun	Jul	Jun	Jul	Jun	Aug

Farm inputs. The three ponds were managed identically during both production cycles. The shrimp were fed a commercial pellet diet to which probiotics, vitamin C, immune enhancers, and molasses were added. Bio-remediation products were applied to the water weekly. Water was not changed for the first 30 days, but as required between days 31 and 90, and every 2-5 days after day 90 until harvest.

Monitoring. Monitoring of physico-chemical parameters and microflora in the water was identical during 2005 and 2006. Temperature, salinity, and pH were measured twice daily, at 8:00 and 18:00. Dissolved oxygen (DO) was measured before dawn at 5:00 and at 18:00. Alkalinity was measured every 2-3 days (APHA, 1995). Total bacteria, luminous bacteria, and presumptive *Vibrio* were counted on *Vibrio* selective thiosulfate bilesalt citrate sucrose agar (TCBS) every 2-3 days using the plate count method. Water samples (1 l) were collected in sterile bottles and serially diluted using autoclaved seawater. Representative dilutions of (0.1 ml) were plated onto nutrient agar (NA) and TCBS agar plates, in duplicate. Inoculated plates were incubated at room temperature (approx. 30°C) for 24 h. After incubation, total bacteria counts were determined on NA plates, presumptive *Vibrio* on TCBS plates, and luminous bacteria on NA plates in a dark room.

Results

Total alkalinity was within the normal range during both cycles but temperature, salinity, pH, and dissolved oxygen varied and sometimes fell out of the acceptable range (Table 2). Generally, temperature, DO, and pH were lower in the morning than in the afternoon. The water temperature fluctuated more than 3-4°C between 07:00 and 17:00 more often during 2006, when there was an outbreak of disease due to WSSV infection, than in 2005. Likewise, salinity dropped below 15 ppt more often in 2006. The total bacterial count in the pond water was twice as high in 2005 than in 2006, but the luminous bacteria count was twice as high in 2006 as in 2005 and the total presumptive *Vibrio* count was over ten times higher in 2006 than in 2005, with a greater percentage of green colonies.

Table 2. Physico-chemical parameters and microflora of culture water in ponds without (2005) and with (2006) WSSV outbreaks.

Parameter	*Acceptable range*	*Time*	*Without WSSV (n = 3)*		*With WSSV (n = 3)*	
			Range	*Mean±SD*	*Range*	*Mean±SD*
Temperature (°C)	28-32	8:00	23-31	28.56±1.25	26-31	28.4±1.1
		15:00	26-33	29.72±1.45	26.2-34	30.2±1.6
Salinity (ppt)	15-25	8:00	7-25	16.11±4.45	9-22	15.9±3.4
		15:00	7-25	15.89±4.48	10-22	15.8±3.4
Dissolved oxygen (ppm)	>4	5:00	3.2-12.6	4.71±0.63	3.6-5.8	4.4±0.5
		15:00	4.6-6.2	7.6±1.4	5.4-12.1	7.7±0.9
pH	7.5-8.5	8:00	7.4-8.9	8.11±0.25	7.6-8.4	8.1±0.2
		15:00	7.5-8.9	8.34±0.28	7.4-8.6	8.3±0.2
Alkalinity (ppm)	>80	-	108-222	155.8±29.87	110-180	147.4±19.6
Total bacterial count (cfu/ml)	10^3-10^4	-	1.1^2-2.7^4	3.4^3±3.4^3	2.5^3-2.1^2	8.48^2±4.01^2
Luminous bacterial count (cfu/ml)	10^2	-	<1-2.5^3	3.14^2±4.27^2	<1-9.4^4	6.87^2±1.2^3
Presumptive *Vibrio* count (cfu/ml)	<10^2	-	<1-1.8^3	3.14^2±3.15^2	6-8.8^3	5.39^3±1.99^4
Green *Vibrio* colonies (%)	-	-	0-100	25.77±25.76	0-96.55	50.00±30.00
Days of culture	-	-	143		95	
Culture days wherein temperature fluctuated 3-4°C (%)			0.005		11.5	
Culture days wherein salinity was <15 ppt (%)			34.8		45.1	

Discussion

Total alkalinity during both production cycles was above 80 ppm and suitable for *P. monodon* culture (Chanratchakool, 1995; Tookwinas, 2000). During the 2005 cycle, the mean total bacterial count of the shrimp ponds was within the prescribed range of 10^3-10^4 cfu/ml (Tookwinas, 2000), but it was lower during the WSSV-associated disease outbreak in 2006. Total bacterial count includes all kinds of beneficial as well as harmful bacteria. The beneficial bacteria might have conditioned the pond water, making environmental conditions less stressful to the shrimp and rendering them less susceptible to WSSV infection. Further, probiotics encourage the proliferation of bacteria that inhibit the colonization of pathogens (Das et al., 2006; Ganguly et al., 2010). The presumptive *Vibrio* count was over 10 times higher in the 2006 cycle than the maximum 10^2 cfu/ml recommended for shrimp culture (Baliao, 2000). Further, more green colonies, believed to be pathogenic, were recovered in 2006 than in 2005, which may have stressed the shrimp, making them more susceptible to WSSV infection. *Vibrio* infection due to poor environmental conditions makes shrimp susceptible to WSSV (Hettiarachchi et al., 1999).

The poor conditions may have been attributable to sub-optimal temperature and salinity. Temperature and salinity affect the immune response of crustaceans (Vargas-Albores et al., 1998; Le Moullac and Haffner, 2000). Temperature changes are one of the factors that trigger WSSV infection in shrimp culture (Kautsky et al., 2000). Temperature shifts can induce an outbreak of WSSV disease in lightly infected shrimp (Hsu et al., 2000). There were a greater number of 3-4°C temperature fluctuations during the WSSV outbreak in 2006 than during 2005. The temperature fluctuations could have stressed the shrimp, lowering their immune response and making them susceptible to WSSV infection. Temperature effects survival of WSSV infected shrimp (Rodríguez and Sonnenholzher, 2001; Guan et al., 2003).

Salinity affects the immune response of *Marsupennaeus japonicus*; the further from the original salinity the shrimp are maintained, the weaker their immune response (Yu et al., 2003). The ideal salinity for shrimp culture is 15-25 ppt (Baliao, 2000). Salinities dropped below 15 ppt in both production cycles but more instances were observed during 2006. Acute salinity stress is more significant at low salinity than at high, affecting the immunocompetence of *P. monodon* and resulting in increased susceptibility to WSSV infection (Joseph and Philip, 2007). Although their immune defense and metabolic response overwhelmed the pathogen during the early stages of infection, delaying the onset and pace of mortality, shrimps maintained at 15 ppt did not completely eliminate virus particles from circulation and thwart infection (Joseph and Philip, 2007).

Results indicate that temperature fluctuations of 3-4°C between 07:00 and 17:00, salinity below 15 ppt, high presumptive *Vibrio* counts, and presence of 50% green *Vibrio* colonies are important risk factors in WSSV outbreaks. Our work suggests that appropriate management measures such as the use of reservoirs should be adopted in *P. monodon* culture ponds to minimize acute salinity stress or salinity below 15 ppt, temperature fluctuations, and dominance of green *Vibrio* colonies. Whether induced dominance of yellow *Vibrio* colonies would limit WSSV outbreaks might be a subject of study.

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