

## Effect of temperature and salinity on the infectivity pattern of white spot syndrome virus (WSSV) in giant tiger shrimp *Penaeus monodon* (Fabricius, 1837)

STALIN RAJ\*, K. K. VIJAYAN, S. V. ALAVANDI, C. P. BALASUBRAMANIAN AND T. C. SANTIAGO

Central Institute of Brackishwater Aquaculture, 75 Santhome High Road, R. A. Puram, Chennai - 600 028, Tamil Nadu, India

\*Department of Infectious and Parasitic Diseases, 43b, University of Liege, B - 4000 Liege, Belgium

e-mail: vijayankk@gmail.com

### ABSTRACT

White spot disease (WSD) caused by the lethal white spot syndrome virus (WSSV) continues to be the major cause of mortality among farmed tiger shrimp in India and elsewhere, resulting in an annual loss of about 4-6 billion US\$. Among the environmental variables, temperature and salinity of the rearing water are considered to be major triggering factors for white spot disease outbreak. In order to characterise the effect of salinity and temperature on the pathogenicity of WSSV infection in giant tiger shrimp *Penaeus monodon*, a laboratory challenge study was conducted at different levels of temperature (16, 25, 27, 28, 30, 32 and 36 °C) and salinity (0.5, 5, 10, 15, 30 and 45 g l<sup>-1</sup>) with virulent white spot syndrome virus. Significant influence of temperature ( $p < 0.05$ ) on the percentage mortality and time until death of shrimp affected by the virus was observed, whereas salinity did not show any effect. Significantly higher survival rate was recorded in animals exposed at 32 °C (37%) and 36 °C (14%), 21 days post-challenge (dpc). All the shrimp challenged at other temperature levels, however, died after 21 dpc. These results demonstrated preference of WSSV for lower temperatures and higher survival in temperature ranges of 32 °C to 36 °C. The present observation may help to develop a management option to control the WSSV inflicted mortalities by selecting favorable hyperthermic rearing conditions for the shrimp.

Keywords: *Penaeus monodon*, Salinity and temperature regimes, White spot syndrome virus

### Introduction

White spot syndrome virus (WSSV) is among the most serious pathogen affecting shrimp aquaculture industry, and it has caused severe disruption to the industry since the early 1990s (Lightner, 2005; Flegel, 2006). The social and economic impacts caused by WSSV pandemic have been profound and the estimated economic loss is about US\$6-8 billion (Lightner, 2005). Several studies have addressed various aspects of WSSV during the past one decade (Flegel, 2006). However, WSSV epizootic, has remained the most serious challenge to the shrimp farming industry, yet to be resolved. Since there are no therapeutic treatments currently available for WSSV, the best management strategy is to prevent WSSV from entering a shrimp farming facility (Lotz, 1997) or use of optimal shrimp culture conditions (Browdy *et al.*, 1993).

It is well established that the disease is the end result of complex interactions between shrimp, its environment and the pathogen itself (Lightner and Redman, 1998). There

is considerable evidence to support the hypothesis that environmental changes induce modification of immune system leading to enhanced susceptibility to infectious disease agents (Liu *et al.*, 2006). It has also been reported that manipulating environmental factors enabled shrimp infected with WSSV to survive whole culture period (Vidal *et al.*, 2001; Liu *et al.* 2006). Therefore, studies on environmental factors on WSSV outbreaks are of major importance to shrimp mariculture.

Temperature and salinity are perhaps the most important environmental variables that directly affect physiology and ecology of farmed penaeid shrimp population. Temperature is both a limiting factor and determinant of growth rate through its impacts on molecular activity (Lester and Pante, 1992). An extreme thermal tolerance (10- 39 °C) has been attributed to all age groups of *P. monodon* (Moto, 1981). Laboratory studies of early juveniles of *Fenneropenaeus indicus* showed a higher growth rate at 31°C (Vijayan and Diwan, 1995). Several extensive reviews have been devoted to the ecological and

physiological significance of salinity on crustaceans (Anger, 2003). Osmo and ionic regulation require energy expenditure which could have supported growth. Thus, it was suggested that the maximum growth of an organism occurs in iso-osmotic medium (Le Molluc and Heffner, 2000). Low salinity was associated with faster growth in *Penaeus aztecus* (Venkatramaiah *et al.*, 1974), and high salinity appears to retard growth. A correlation between salinity and pond production of *P. monodon* has also been reported in extensive shrimp farms in India (Chakraborti *et al.*, 1985). Researchers have been focusing mainly on the effect of salinity and temperature on growth and survival of shrimp. In contrast, the works on relationship between environmental conditions and susceptibility to viral infection is limited though increasing in recent years (de la Vega *et al.*, 2007). A relationship between salinity and infectious hypodermal and hematopoietic necrosis virus has been observed in *Litopenaeus vannamei* (Bray *et al.*, 1994). The effect of water temperature on the infectivity of WSSV are addressed by few workers in *L. vannamei* (Vidal *et al.*, 2001, Granja *et al.*, 2003, 2006; Rahman, 2006, 2007a, b), in *Marsupenaeus japonicus* (Guan *et al.*, 2003) and in *Procambarus clarkii* (Du *et al.*, 2006). Though *P. monodon* is the prime species in shrimp aquaculture in India and other Asian countries, the effect of temperature and salinity on infectivity of WSSV has not been previously tested. Therefore, we performed a study to determine the effect of salinity and temperature changes on WSSV outbreak in *P. monodon*. The information generated would be a useful guide to the industry in developing proper health management strategies to tackle the problem of WSSV.

## Materials and methods

### Experimental animals

White spot syndrome virus (WSSV) free *P. monodon* juveniles (500 nos. having (mean body weight:  $5 \pm 1$  g) were obtained from a commercial shrimp farm in Chennai, India. The animals were brought to the laboratory of the Central Institute of Brackishwater Aquaculture (CIBA) and maintained in 500 l fiber glass tanks for a period of one week. Rearing conditions: water temperature. 27- 31 °C, salinity 28-30 g l<sup>-1</sup>. Shrimps were fed with a commercial dry diet (35% protein) twice daily at 5% body weight. The WSSV status was verified by PCR prior to the initiation of the experiments.

### Source of WSSV

Moribund symptomatic shrimps were collected from a WSSV infected shrimp farm in Andhra Pradesh, India. Gills from 25 animals were preserved in Davidson's AFA for confirmatory histology (Bell and Lightner, 1988), and pleopods were preserved in 95% ethanol for nested PCR

diagnosis. The shrimps were placed in ice and transferred immediately to the laboratory and maintained at -70 °C until used. The presence of WSSV was confirmed by PCR analysis, and gill tissues of these animals were used for oral challenges.

### Effect of temperature on WSSV infectivity

To test the effect of temperature on WSSV infectivity, six temperature treatments of 16°C, 20°C, 28°C, 30 °C, 32°C and 36°C were used in triplicate, giving a total of 18 experimental treatments. In addition, positive and negative control groups in triplicate were also set. The experiment was carried out in a refrigerated water bath (Gambaks, India). The salinity of the water used in all the groups was  $21 \pm 0.5$  g l<sup>-1</sup>. A total of 240 shrimps from the stock were distributed into 24 rearing units giving a total of 10 shrimps in each tank. The animals were gradually acclimatised to the test temperature by increasing or decreasing the temperature at the rate of 1 °C per h. All the animals were subjected to starvation 3 days prior to the exposure to WSSV. All the test groups and positive control groups were then fed with gills of WSSV infected *P. monodon* (~5% body weight) twice daily for 2 days whereas negative control group was fed with gill tissues from WSSV negative *P. monodon*. After 2 days of exposure, all the groups were fed with commercial formulated feed. The tanks were continuously aerated and 10% of water was exchanged daily. The day of post-exposure mortality was recorded for shrimps died during the 21 days experimental period. Dead animals were preserved at -70 °C for molecular detection of WSSV, and gill tissues from the moribund animals were fixed in Davidson's AFA for histopathological analysis. After 24-72 h fixation in the Davidson's AFA, tissues were transferred to 70% ethanol and stored until further analysis.

### Effect of salinity on infectivity of WSSV

The experiment was carried out using six different salinity treatments, 0.5, 5, 10, 15, 30 and 45 g l<sup>-1</sup> in five replicates (three test groups and two controls for each salinity). Salinity was adjusted from initial 32 g l<sup>-1</sup> at the rate of 1 g l<sup>-1</sup> per 3 h until treatment salinities (higher or lower) were reached. Ten animals were held in each group, and all groups were maintained in 50 l fiber glass tanks. To prepare the test salinity below 30 g l<sup>-1</sup>, seawater was diluted with freshwater whereas to prepare test salinity above 30 g l<sup>-1</sup>, artificial sea salt (Southern India Aquaculture, Chennai, India) was added. The animals were acclimatised in each test salinity for 48 h, and following acclimatisation, the test groups were fed (~ 5% biomass) with WSSV infected gills of *P. monodon* twice daily for 2 days. The

control groups were fed with gill tissues from WSSV negative *P. monodon* for 2 days. Water temperature in all the treatments was maintained at 27 °C. After 2 days, all the groups were fed with commercial diet. Dead shrimps noticed were collected and frozen at -70 °C. The experiment was terminated at 10 d post-exposure, and gill tissues from moribund animals were fixed in Davidson's AFA for histopathological analysis. After 24-72 h fixation in the Davidson's AFA, tissues were transferred to 70% ethanol and stored until further analysis

#### Diagnosis of WSSV infection

The diagnosis of WSSV was confirmed by 2 step PCR (nested) assays as described by Kimura *et al.* (1996). The DNA template was prepared from each sample using a modified alkaline lysis method (Vijayan *et al.*, 1998). Briefly, 40 to 50 mg tissue sample was homogenised in sterile disposable tissue homogeniser with 500 µl lysis buffer (25 mM Tris HCl, 10 mM EDTA, 50 mM glucose, 0.2 N NaOH and 1% SDS at pH 8), and the suspension was boiled for 10 min, cooled and centrifuged at 12 000 g for 10 min. Two microliters of the clear supernatant was used as the template in the PCR reaction. The 2<sup>nd</sup> step PCR procedure comprised an external and internal primer. The amplification was performed in an Eppendorf Master Cycler. The first and second step PCR amplifications were expected to amplify a WSSV DNA fragment of 982 and 570 bp respectively. The PCR products were analysed on 1.5% agarose gel and bands were visualised by ethidium bromide staining using UV transilluminator.

#### Statistical analysis

The difference between mortality levels between treatments were compared using students' t test. Statistical analysis was completed with the use of Microsoft® Excel for Windows®.

## Results

#### Effect of water temperature on the infectivity of WSSV

A constant high level mortality was observed in the treatment groups set below 32 °C. Mortality started on 3<sup>rd</sup> day (3.5%), and cumulative mortality reached 100% within 8-13 d in all these treatments. In contrast, shrimp exposed to 32 °C and 36 °C showed a lower rate of mortality, and 37% and 14% of animals survived respectively at the end of the experiment (Fig. 1). No significant difference was observed in the mortality rate among the treatment groups exposed to lower water temperatures of 16 - 25 °C), whereas a constant higher mortality was observed in the group exposed to 28 °C (Fig. 1). Further, there was a statistically significant difference in the mean lethal time (LT<sub>50</sub>) between treatment groups held

at 32 °C and all other treatment groups ( $p > 0.05$ ) (Fig. 2). All the dead shrimps were WSSV positive by first step PCR assay and histopathological analysis; whereas all shrimps that survived at higher temperatures (32 °C and 37 °C) were found to be second step positive for WSSV, indicating that these animals harbored the virus (Fig. 3 and 4).

#### Effect of salinity on infectivity of WSSV

Shrimps exposed to WSSV in all salinity treatment groups showed a constant high level of mortality. Mortality started on 3<sup>rd</sup> day (6.7-10%) and cumulative mortality reached 100% on 10<sup>th</sup> day in all the treatment groups (Fig. 5). No mortality was observed in the control groups at all salinity levels. All dead animals were WSSV positive (first step) by PCR assay (Fig. 3 and 4).

## Discussion

The effect of temperature and salinity on the pathogenicity of *P. monodon* was investigated for the first time. Further, feeding with infected tissue made it possible to carry out reproducible studies of experimental induction of WSSV infection. A constant high level mortality was observed in all the experimental salinity treatments (Fig. 5). The lack of significant difference in mortalities between different salinity treatments in the present study suggests that mortalities were not influenced by exposure to different salinity levels. Yu and Guan (2003), however, reported that salinity changes have been associated with increased susceptibility to WSSV. High incidence of WSSV outbreaks have also been reported in shrimp farms along the south-east coast of India during wet season in association with drop in salinity (Vijayan, unpublished data). The reason for this may be that an already infected shrimp relapse to acute stage and die upon stress associated with lower salinity and low water temperature. In contrast, the present experiment was conducted at constant temperature and animals were acclimatised to the various salinities to avoid the stress associated with sudden fall in salinity.

The present study indicated that mortality in WSSV infected *P. monodon* significantly reduced at higher temperature (Fig. 1). The effect of temperature in reducing mortality has previously been reported in experimentally infected decapod crustaceans other than *P. monodon* viz., *L. vannamei* (Vidal *et al.*, 2001; Granaja *et al.*, 2003; Rahman *et al.*, 2006, 2007); in *M. japonicus* (Guan *et al.*, 2003); in freshwater crayfish *Procambarus clarkii* (Du *et al.*, 2001). Vidal *et al.* (2001) reported >80% survival rate in WSSV infected *L. vannamei* reared at higher temperature ( $32.8 \pm 0.8$  °C) in contrast to the control shrimp kept at low water temperature ( $25.9 \pm 0.7$  °C).

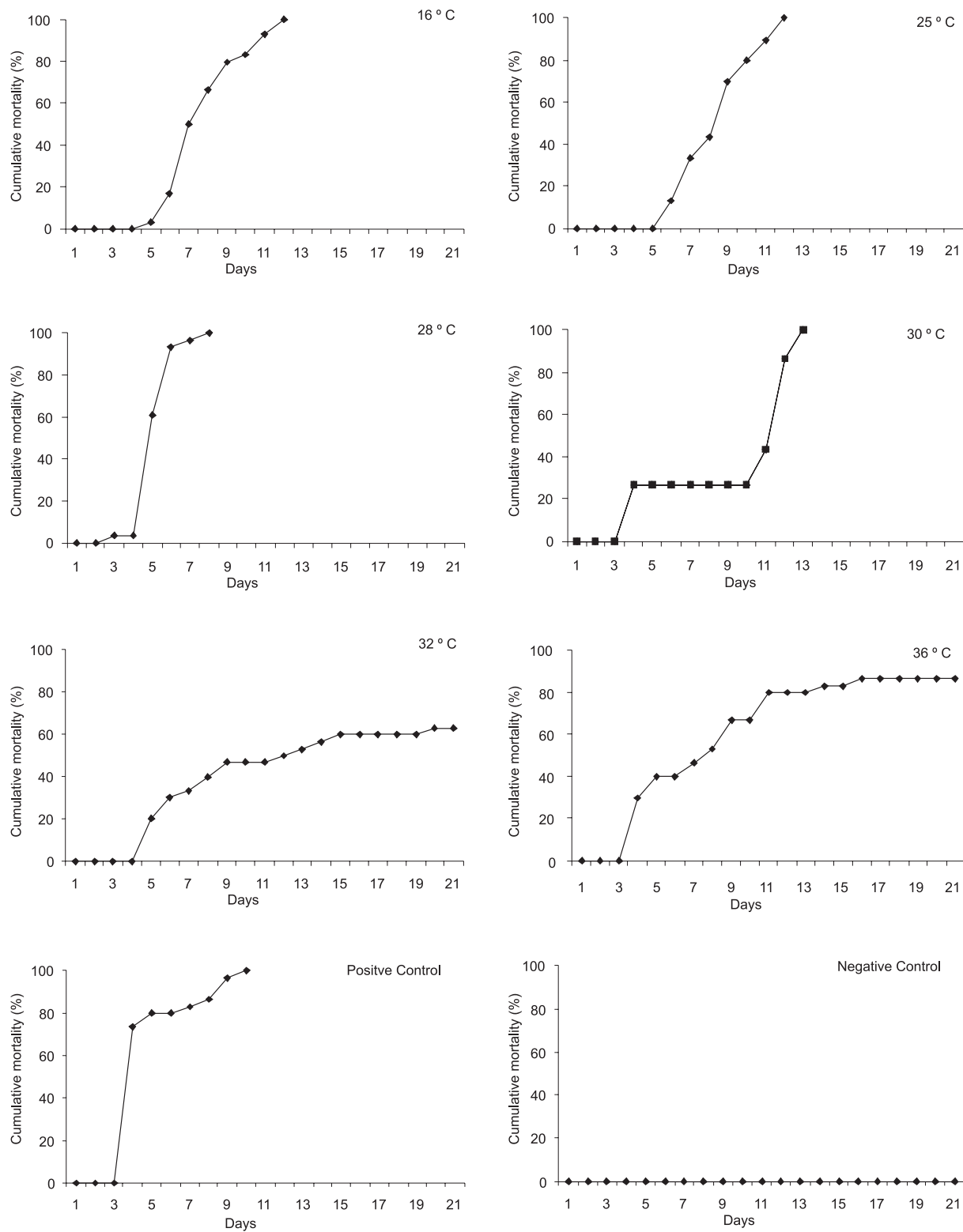


Fig. 1. Cumulative mortality in *Penaeus monodon* after oral exposure of WSSV, held at different temperature levels

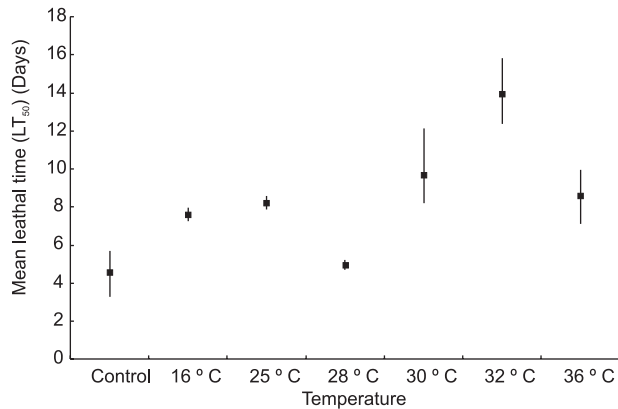


Fig. 2. Mean lethal time (LT<sub>50</sub>) of WSSV exposed *P. monodon* under different temperature regime

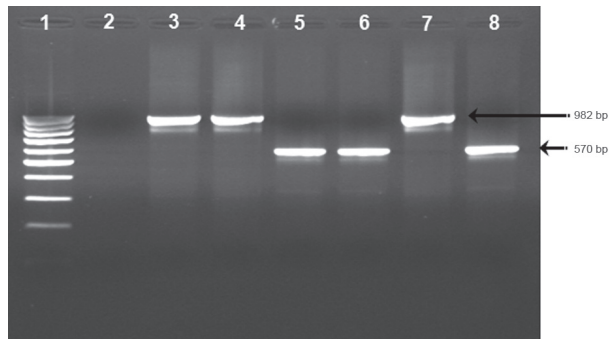


Fig. 3. Polymerase Chain Reaction (PCR) analysis for the presence of WSSV in *P. monodon* in temperature and salinity experiments. Lanes: 1: DNA marker (100 bp); 2: negative control; 3, 4 and 7: 1<sup>st</sup> step PCR positive; 5, 6 and 8: 2<sup>nd</sup> step PCR positive

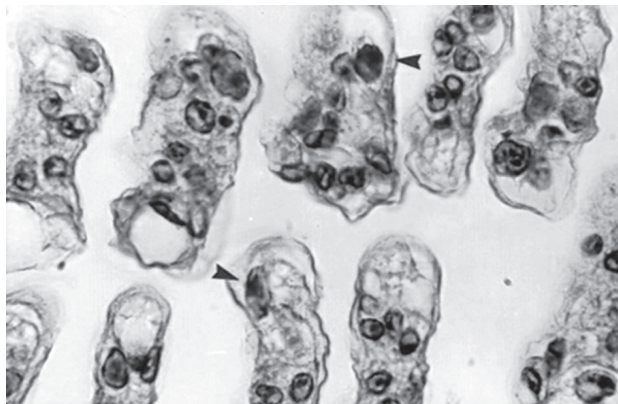


Fig. 4. Histopathology of H&E stained gill tissue from moribund *P. monodon* infected with WSSV in temperature and salinity experiments. Hypertrophied nuclear cells identifies infected cells (arrows), X400

Rahman *et al.* (2006) also reported that raising temperature to 33 °C in *L. vannamei* is effective to prevent disease. Nevertheless, they further reported that increasing temperature to 33 °C could not reduce mortality in shrimps already infected for 24 h, as 24 h viral infection had already become systemic causing irreversible damages. Few studies, however, report low mortality in WSSV infected animals at low water temperature (Jirvanichapaisal *et al.*, 2004). Pathogenicity of WSSV in temperate waters needs further investigations on the basis of comparative immunity of shrimp from temperate and tropical environments.

Temperatures above 16 °C and below 32 °C allow WSSV replication in susceptible hosts such as shrimps, crabs and crayfish (Corbel *et al.*, 2001; Guan *et al.*, 2003; Jirvanichapaisal *et al.*, 2004; Rahman *et al.*, 2006). Although the above discussed reports clearly show that temperature can affect WSSV infection, the mechanism of beneficial effect of hypothermia on the immune response remains largely unknown. Rahman *et al.* (2006) reported that high water temperature completely inhibits the expression of the protein VP28 *in vivo* and blocks WSSV replication at an early stage.

The outcome of pathogen challenge is determined by many interacting factors including host species, biology, age and health (Edgerton, 2004). However, based on the present results and published data from trials conducted with other penaeids, it appears that tropical penaeids have a lower level of susceptibility to WSSV at hyperthermic conditions. The extreme variation in development of WSSV disease observed experimentally and seasonally in commercial shrimp farms could be attributed to the influence of environmental temperature (Vidal *et al.*, 2001). Further, the beneficial effect of hyperthermia in reducing the mortality of *P. monodon* reported here, opens the possibility to apply high water temperature to manage WSSV infection. Management practices that increase pond temperature offer probable strategy to manage devastating WSSV pandemic. Reducing water depth in ponds and reducing water exchange may contribute to increase pond temperature and hence reduce mortality due to WSSV infection. Indian shrimp farmers have already adopted farming strategies to raise one shrimp crop during the summer months, to take advantage of the higher water temperature, and shifting to a finfish crop during the winter months to avoid increased WSSV occurrence due to cooler water temperatures. Further, techniques such as growing shrimp under green house condition may provide a management option in tackling the lethal WSSV epizootic.

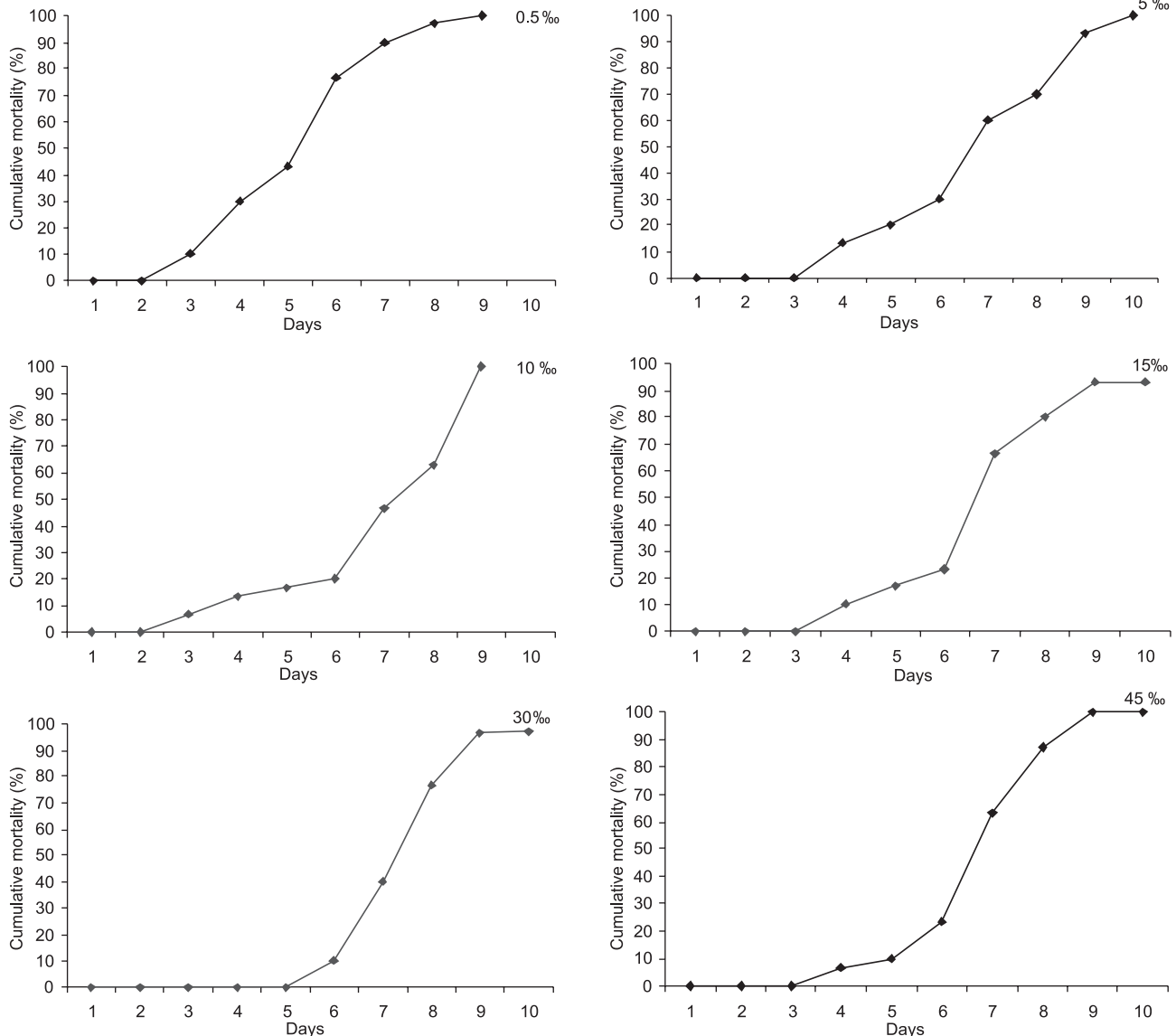


Fig. 5. Cumulative mortality of *Penaeus monodon* after oral exposure of WSSV at different salinity levels

**Acknowledgements**

The work was supported by the World Bank aided National Agricultural Technology project (NATP), ICAR. We are grateful to the Director, Central Institute of Brackishwater Aquaculture, Chennai for the encouragement. We thank Miss. Sanjukta, M. for her assistance in the preparation of the manuscript.

**References**

Angrer, K. 2003. Salinity as a key parameter in the larval biology of decapod crustaceans. *Invertebr. Repr. Dev.*, 43: 29-45

Bell, T. A. and Lightner, D. V. 1988. *A handbook of normal penaeid histology*. Special publication of the World Aquaculture Society. Baton Rouge, Louisiana, USA., 114 pp.

Berlim, M. T. and Abeche, A. M. 2001. Evolutionary approach to medicine. *Southern Med. J.*, 94: 26-32.

Bray, W. A., Lawrence, A. L. and Leung-Trujillo 1994. The effect of salinity on growth and survival of *Penaeus vannamei*, with observation on the interaction of IHNV virus and salinity. *Aquaculture*, 122: 133-146

Browdy, C. L., Holloway, J. D., King, C. O., Stokes, A. D., Hopkins, J. S. and Sandifer, P. A. 1993. IHNV virus and intensive culture of *Penaeus vannamei*: Effect of stocking density and water exchange rates. *J. Crustacean Biol.*, 13(1): 87-94.

Chakraborti, R. K., Holder, D. D., Das, N. K., Mandal, S. K. and Bhowmik, M. L. 1986. Growth of *Penaeus monodon*, Fabricius, under different environmental conditions. *Aquaculture*, 51: 189- 194.

- Corbel, V., Zuprizal, S. Z., Huang, Z., Arcier, J. M. and Bonami, J. R. 2001. Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *J. Fish. Dis.*, 24: 377-382.
- de la Vega, E., Hall, M. R., Wilson, K. J., Reverter, A., Woods, R. G., and Degnan, B. M. 2007. Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*: *Physiol. Genomics*, 31: 126-138.
- Du, H. H., Li, W. F., Xu, Z. R. and Kil, Z. S. 2006. Effect of hyperthermia on the replication of white spot syndrome virus (WSSV) in *Procambarus clarkii*. *Dis. Aquat. Org.*, 71: 175-178.
- Edgerton, B. F. 2004. Susceptibility of the Australian freshwater crayfish *Cherax destructor albidus* to white spot syndrome virus. *Dis. Aquat. Org.*, 59: 187-193.
- Flegel, T. W. 2006. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. *Aquaculture*, 258: 1-33.
- Granja, C. B., Aranguren, L. F., Vidal, O. M., Aragón, L. and Salazar, M. 2003. Does hyperthermia increase apoptosis in white spot syndrome virus (WSSV) -infected *Litopenaeus vannamei*? *Dis. Aquat. Org.*, 54: 73-78.
- Granja, C. B., Vidal, O. M., Parra, G. and Salazar, M. 2006. Hyperthermia reduces viral load of white spot syndrome virus in *Penaeus vannamei*. *Dis. Aquat. Org.*, 68: 175-180.
- Guan, Y., Yu, Z. and Li, C. 2003. The effects of temperature on white spot syndrome infections in *Marsupenaeus japonicus*. *J. Invertebr. Pathol.*, 83: 257-260.
- Jiravanichpaisal, P., Soderhall, K. and Soderhall, I. 2004. Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. *Fish Shellfish Immunol.*, 17: 265-275.
- Kimura, T., Yamano, K., Nakano, H., Monoyama, K., Hiraoka, M. and Frousp, K. 1996. Detection of penaeid rod shaped DNA (PRVD) by PCR (In Japanese). *Fish Pathol.*, 31: 93-98.
- Le Moullac, G. and Heffner, P. 2000. Environmental factors affecting immune response in crustacea. *Aquaculture*, 191: 121-131.
- Lester, L. J. and Pante, M. J. R. 1992. Penaeid temperature and salinity responses. In: Fast, A. W. and Lester, L. J. (Eds.), *Marine shrimp culture: Principles and practices*. Elsevier, Amsterdam, p. 515-534.
- Lightner, D. V. 2005. Biosecurity in shrimp farming: Pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquacult. Soc.*, 36: 229-248.
- Lightner, D. V. and Redman, R. M. 1998. Shrimp disease and current diagnostic methods. *Aquaculture*, 164: 201-220.
- Lindquist, S. and Craig, E. A. 1988. The heat shock proteins. *Ann. Rev. Genetics*, 22: 631- 677.
- Liu, B., Yu, Z., Song, X., Guan, Y., Jian, X. and He, J. 2006. The effect of acute salinity change on white spot syndrome (WSS) outbreaks in *Fenneropenaeus chinensis*. *Aquaculture*, 205: 163-170.
- Lotz, J. M. 1997. Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. *World J. Microbiol. Biotechnol.*, 13: 405-413.
- Moto, H. 1981. *Studies on the fishery biology of the giant tiger prawn, Penaeus monodon in the Philippines*. Technical Report No. 7, Aquaculture Department, SEAFDEC, Philippines, 128 pp.
- Rahman, M. M., Escobedo-Bonilla, C. M., Corteel, M., Dantas-Lima, J. J., Wille, M., Alday Sanz, V., Pensaert, M. B., Sorgeloos, P. and Nauwynck, H. J. 2006. Effect of high water temperature (33 °C) on the clinical and virological outcome of experimental infections with white spot syndrome virus (WSSV) in specific pathogen-free (SPF) *Litopenaeus vannamei*. *Aquaculture*, 261: 842-849.
- Rahman, M. M., Corteel, M., Dantas-Lima, J. J., Wille, M., Alday-Sanz, V., Pensaert, M. B., Sorgeloos, P. and Nauwynck, H. J. 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.
- Venkataramaiah, A., Lakshmi, G. J. and Gunter, G. 1974. *Studies on the effect of salinity and temperature on the commercial shrimp, Penaeus aztecus, Ives, with special regard to survival limits, growth, oxygen consumption and ionic regulation*. Contract report H-74-2. Gulf Coast Research Laboratory, Ocean springs, MS, USA, 147 pp.
- Vidal, M. O., Granja, B. C. and Aranguren, F. 2001. A profound effect of hyperthermia on survival of *Litopenaeus vannamei* juveniles infected with white spot syndrome virus. *J. World Aquacult. Soc.*, 32: 364-372.
- Vijayan, K. K., and Diwan, A. D. 1995. Influence of temperature, salinity, pH and light on moulting and growth in the Indian white prawn *Penaeus indicus* under laboratory conditions. *Asian Fish. Sci.*, 8: 63 - 72.
- Vijayan, K. K., Rajendran, K. K. and Santiago, T. C. 1998. A simple and rapid molecular diagnostic technique for white spot syndrome virus (WSSV) infection. In: Balachandran, K. K. (Ed.), *Advances and priorities in fisheries technology*, Society of Fisheries Technologists (India), Cochin, India, p. 398-401.
- Yu, Z. and Guan, Y. 2003. Effect of salinity on the immune response and outbreak of white spot syndrome in the shrimp *Marsupenaeus japonicus*. *Ophelia*, 57: 99-106.

Date of Receipt : 18.02.2012

Date of Acceptance : 25.07.2012