

Effects of High Water Temperature on the Elimination of White Spot Syndrome Virus in Juveniles of *Litopenaeus vannamei*

Sutee Wongmaneeprateep¹, Niti Chuchird¹, Puttharat Baoprasertkul²,
Piyanuch Prompamorn¹, Kanittada Thongkao³ and Chalor Limsuwan¹

ABSTRACT

This study evaluated the effects of high water temperature ($32\pm 1^\circ\text{C}$) on white spot syndrome virus (WSSV) infection in *Litopenaeus vannamei* juveniles (5-6 g). WSSV challenge was done by immersion and oral routes. One group of shrimp was constantly maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment after challenge while a control group of shrimp was constantly maintained at $28\pm 1^\circ\text{C}$ until the end of the experiment after challenge. Other groups were kept at $32\pm 1^\circ\text{C}$ until the temperature was switched to $28\pm 1^\circ\text{C}$ at 0, 1, 3, 5 and 7 days after challenge. Gross signs and mortality were monitored every 12 h until the end of the experiment. WSSV infections were confirmed by nested-PCR, histopathology, immunohistochemistry and bioassay methods.

Challenged shrimp maintained at $32\pm 1^\circ\text{C}$ for 0, 1, 3 and 5 days before switching to $28\pm 1^\circ\text{C}$ revealed that maintaining for a longer period at $32\pm 1^\circ\text{C}$ could delay clinical signs and onset of mortalities. Nevertheless, 100% mortalities occurred in all groups and control group within 7 days. All moribund shrimp were WSSV-positive by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods. The histopathology of infected shrimp showed hypertrophied nuclei with eosinophilic (Cowdry A-type inclusion) to basophilic inclusion bodies in the cells of the cuticular epidermis, stomach cuticular epithelium, connective tissue, gills, antennal gland, heart and haematopoietic tissue. In contrast, the two groups of shrimp, i.e. those constantly maintained at $32\pm 1^\circ\text{C}$ during the experiment, and another at the same temperature for 7 days after challenge before switching to $28\pm 1^\circ\text{C}$, did not show any clinical signs and mortality. Surviving shrimp from both groups were WSSV-negative by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods. This study clearly indicated that shrimp maintained constantly at $32\pm 1^\circ\text{C}$ for 7 days were able to eliminate/clear WSSV infection.

Key words: White spot syndrome virus, *Litopenaeus vannamei*, Temperature

¹Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.

²Aquatic Animal Health Research Institute, Department of Fisheries, Bangkok 10900, Thailand.

³Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand

INTRODUCTION

White spot syndrome virus (WSSV) infestation poses the greatest threat in shrimp aquaculture. This virus has caused enormous economic losses in the shrimp culture industry worldwide (Lightner, 1996; Flegel, 1997). This pathogen is a bacilliform, non-occluded enveloped virus (van Hulst *et al.*, 2001) of the family *Nimaviridae*, genus *Whispovirus* (Mayo, 2002). In Thailand, WSSV was first reported in black tiger shrimp (*Penaeus monodon*) from the eastern and southern provinces along the coastal areas of the Gulf of Thailand and the Andaman Sea in late 1994 (Limsuwan, 2000). Mortality of WSSV-infected shrimp could reach 100% within 3-10 days after the onset of disease. The cause of death of WSSV infected shrimp could be due to dysfunction of target tissues including gills, stomach epithelium, cuticular epithelium, antennal gland and hematopoietic tissue as suggested by Lightner (1996). Recently, prevention has been employed through a biosecurity system which includes disinfecting of ponds, water treatment before stocking postlarvae (PL), fencing to prevent crabs that may carry infectious agents getting into the ponds, and stocking PL which are free from WSSV ensured by polymerase chain reaction (PCR) assay (Limsuwan, 1997; Withyachumnarnkul, 1999). However, during the winter season from November to February, the water temperature in central and eastern regions is lower than the optimal level of 28-30°C, a similar situation in the southern provinces during the monsoon season from October to December (Limsuwan, 1991; 2003). Thus there is still a threat of WSSV infection. This indicates that some PL were probably

contaminated with WSSV without clinical signs of disease and mortalities due to high water temperature (30±1°C) in hatcheries, or PL samples for PCR detection are not representative of the PL stock population. In fact, after stocking the PL in grow-out ponds during continuously low water temperature for several days, disease outbreak would occur. Therefore, water temperature is considered to be one of the most important environmental factors for WSSV outbreak (Fegan and Clifford, 2001; Vidal *et al.*, 2001; Granja *et al.*, 2003; 2006; Rodríguez *et al.*, 2003; Rahman *et al.*, 2007a). Previous studies reported that *Litopenaeus vannamei* infected with WSSV but were kept continuously at 32-33°C did not show any sign of disease or mortality (Vidal *et al.*, 2001; Rahman *et al.*, 2006; 2007b). However, information on the use of high water temperature to clear or eliminate WSSV in juveniles of *L. vannamei* is not available.

The objective of this study was to evaluate the effects of water temperature specifically at 32±1°C on WSSV infection in *Litopenaeus vannamei* juvenile shrimp (5-6 g) by immersion and oral routes.

MATERIALS AND METHODS

Preparation of viral inoculum

WSSV-infected shrimp, *L. vannamei*, with prominent white spots on the exoskeleton and pink to reddish discoloration were collected from a shrimp farm located in Chantaburi Province, Thailand. The virus was maintained through re-infection of specific pathogen-free (SPF) *L. vannamei*. The inoculum was prepared from WSSV-infected tissues (soft

tissue from the cephalothorax including gills and muscle) which were homogenized in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4) at 0.1 g/ml and centrifuged at 3,000g for 20 min at 4°C. The supernatant fluid was re-centrifuged at 8,000g for 30 min at 4°C and the final supernatant fluid was filtered through a 0.45- μ m membrane filter. Aliquots were transferred to 50-ml plastic centrifuge tubes and then stored at -80°C. Before storage, the presence of WSSV in the tissue samples and the final supernatant fluid was determined by a nested-PCR assay (IQ2000™ WSSV Detection and prevention system, Farming IntelliGene Tech. Corp.) and the viral load was quantified using real-time PCR techniques (LightCycler® 480 SYBR green I Master, Roche). The WSSV content of the innoculum prepared from WSSV-infected tissues and WSSV-infected shrimp was quantified by real-time PCR. The innoculum contained 10^8 WSSV copies/ml and 10^7 WSSV copies/ μ g.

Experimental animals

SPF juvenile *L. vannamei* (5-6 g) were obtained from grow-out ponds located in Chantaburi Province, Thailand, and transported to the laboratory of the Aquaculture Business Research Center, Kasetsart University, Bangkok. Shrimp were acclimatized in 3000-L fiberglass tanks with aeration. Water temperature was maintained at $32\pm 1^\circ\text{C}$ with an aquarium heater, with salinity at 25 ppt. Shrimp were fed twice daily with a commercial pelleted feed at 5% body weight/day for 4 days. Prior to the experiments, shrimp were randomly sampled and verified to be WSSV-free by nested-PCR assay. In the immersion challenge, a total of 180 shrimp were transferred into 21 90-L

aquaria (10 shrimp/aquarium) equipped with aeration and heater, then 90 ml (1:1000 dilutions) of the WSSV inoculums was added in the aquarium water as described by Chen *et al.* (2000). One group of shrimp was constantly maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment after challenge and a control group of shrimp was constantly maintained at $28\pm 1^\circ\text{C}$ until the end of the experiment after challenge. Other groups were kept at $32\pm 1^\circ\text{C}$ then temperature was reduced to $28\pm 1^\circ\text{C}$ at 0, 1, 3, 5 and 7 days post-challenge. Water temperature was maintained at $28\pm 1^\circ\text{C}$ until the end of the experiments. Each group had three replicates.

For the oral route, a total of 180 shrimp were transferred into 21 90-L aquaria (10 shrimp/aquarium) equipped with aeration and heater. Shrimp were fed once (only one time on day 0) with WSSV-infected shrimp (10% body weight) and later fed twice a day with commercial pelleted feed at a rate of 5% body weight/day. Shrimp were constantly maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment after challenge and a control group of shrimp was constantly maintained at $28\pm 1^\circ\text{C}$ until the end of the experiment after challenge. Other groups were kept at $32\pm 1^\circ\text{C}$ then temperature was reduced to $28\pm 1^\circ\text{C}$ at 0, 1, 3, 5 and 7 days post-challenge. Water temperature was maintained at $28\pm 1^\circ\text{C}$ until the end of the experiment. Each group had three replicates.

Nitrite and ammonia levels were monitored throughout the experiment to ensure that the concentrations did not exceed 0.1 and 0.25 mg/l, respectively. Gross signs and mortality of experimental shrimp were observed and recorded every 12 h until the end of the experiment. Moribund or surviving

shrimp were confirmed by nested-PCR, histopathology, immunohistochemistry and bioassay methods.

No negative control group was used since previous experiment showed that uninfected juvenile *L. vannamei* maintained at different temperatures had low levels of mortality (Wongmaneeprateep *et al.*, 2009).

Histopathology

Moribund or surviving shrimp (10 shrimp in each group) were preserved in Davison's fixative for 24 h and then transferred to 70% ethanol until they were processed according to the method described by Bell and Lightner (1988). All samples was sectioned and stained with hematoxylin and eosin (H&E). Infections were considered positive when sample showed typical WSSV histopathological features including hypertrophied nuclei with basophilic inclusions in cuticular epithelium, connective tissue, gills, antennal gland, and/or haematopoietic tissue (Lightner, 1996).

Immunohistochemistry

Paraffin sections from moribund or surviving shrimp were deparaffinized, rehydrated and processed for indirect immunoperoxidase antibody staining. Monoclonal antibodies specific to VP28 of white spot syndrome virus (WSSV) (W29; Chaivisuthangkura *et al.*, 2004) were used as the prime antibodies. Goat anti-mouse IgG H&L horseradish peroxidase conjugate (BioRad) was used as the second antibody. Peroxidase activity was revealed by incubation with 0.03% diaminobenzidine, 0.006% hydrogen peroxide in PBS, then

counterstained with eosin, and processed for permanent slides. WSSV-positive cells showed a brown precipitate.

Bioassay

Soft tissue from cephalothorax including gills and muscle collected from WSSV-infected shrimp were fed twice daily to juvenile SPF *L. vannamei*. Moribund shrimp were collected and detected for WSSV by using nested-PCR assay and histopathological technique. After challenge for 7 days, if there were no moribund or dead shrimp and all test results were negative, then it was safe to conclude that the bioassay results were negative.

RESULTS

Clinical signs were observed at 48, 72, 120, 168 and 48 hpc on juvenile shrimp challenged with WSSV-immersion at $32\pm 1^\circ\text{C}$ for 0, 1, 3 and 5 days before switching to $28\pm 1^\circ\text{C}$ and control group, respectively. Mortalities were first observed at 60, 84, 144, 192 and 60 hpc and reached 100% at 168, 192, 252, 300 and 168 hpc, respectively (Figure 1). All moribund shrimp were WSSV-positive by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods (Table 1). In contrast, shrimp constantly maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment and 7 days after challenge before switching to $28\pm 1^\circ\text{C}$ did not show clinical signs or mortality. Surviving shrimp were WSSV-negative by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods (Table 1).

Table 1. The results of nested-PCR, histopathology, immunohistochemistry and bioassay methods of *Litopenaeus vannamei* juveniles after challenge with WSSV

Water temperature ^a	Detection method			
	Nested-PCR ^b	Histopathology ^b	Immunohistochemistry ^b	Bioassay ^c
<u>Juvenile shrimp challenged with WSSV by immersion</u>				
32±1°C-32±1°C	0/10	0/10	0/10	NM
28±1°C-28±1°C	10/10	10/10	10/10	M
32±1°C-28±1°C/ 0 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 1 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 3 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 5 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 7 d	0/10	0/10	0/10	NM
<u>Juvenile shrimp challenged with WSSV by oral route</u>				
32±1°C-32±1°C	0/10	0/10	0/10	NM
28±1°C-28±1°C	10/10	10/10	10/10	M
32±1°C-28±1°C/ 0 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 1 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 3 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 5 d	10/10	10/10	10/10	M
32±1°C-28±1°C/ 7 d	0/10	0/10	0/10	NM

^aWater temperature before challenge-water temperature after challenge/days post-challenge

^bValues represent the number of shrimp positive in PCR or histopathology or immunohistochemistry /number of shrimp tested.

^cM, mortality; NM, no mortality.

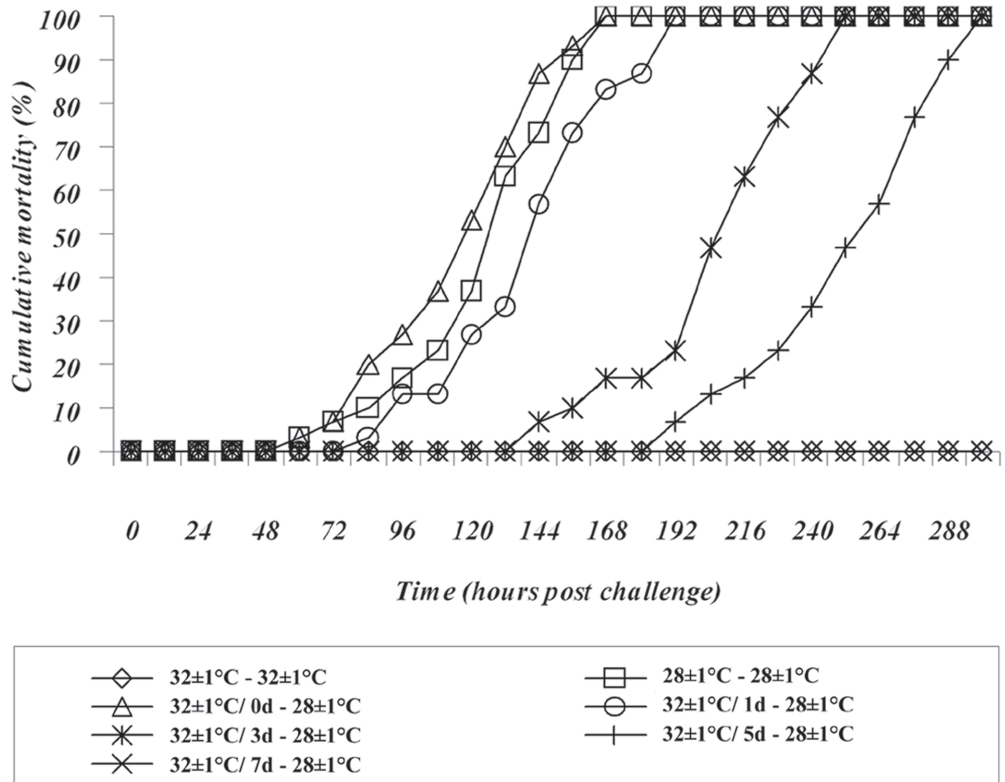


Figure 1. Cumulative mortalities of *L. vannamei* juveniles, challenged with WSSV by immersion. Shrimp were constantly maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment ($32\pm 1^\circ\text{C}$ - $32\pm 1^\circ\text{C}$) after challenge and a control group of shrimp were constantly maintained at $28\pm 1^\circ\text{C}$ until the end of the experiment ($28\pm 1^\circ\text{C}$ - $28\pm 1^\circ\text{C}$) after challenge. Other groups were kept at $32\pm 1^\circ\text{C}$ and temperature was switched to $28\pm 1^\circ\text{C}$ at 0 day ($32\pm 1^\circ\text{C}/0\text{d}-28\pm 1^\circ\text{C}$), 1 day ($32\pm 1^\circ\text{C}/1\text{d}-28\pm 1^\circ\text{C}$), 3 days ($32\pm 1^\circ\text{C}/3\text{d}-28\pm 1^\circ\text{C}$), 5 days ($32\pm 1^\circ\text{C}/5\text{d}-28\pm 1^\circ\text{C}$) and 7 days ($32\pm 1^\circ\text{C}/7\text{d}-28\pm 1^\circ\text{C}$) post-challenge

For oral infection, juvenile shrimp were fed with WSSV-infected shrimp and kept at $32\pm 1^\circ\text{C}$ for 0, 1, 3 and 5 days before the temperature was switched to $28\pm 1^\circ\text{C}$ and control group. Shrimp first showed clinical signs at 24, 60, 132, 216 and 24 hpc, respectively, and later all shrimp stopped eating. Mortalities started at 36, 72, 144, 228 and 36 hpc and cumulative mortalities reached 100% at 120, 156, 252, 372 and

120 hpc, respectively. In contrast, shrimp which were constantly maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment and 7 days after challenge before switching to $28\pm 1^\circ\text{C}$ showed no clinical signs or mortality (Figure 2). Confirmation for WSSV infection by nested-PCR assay, histopathology, immunohistochemistry and bioassay methods were similar to the immersion challenge studies (Table 1).

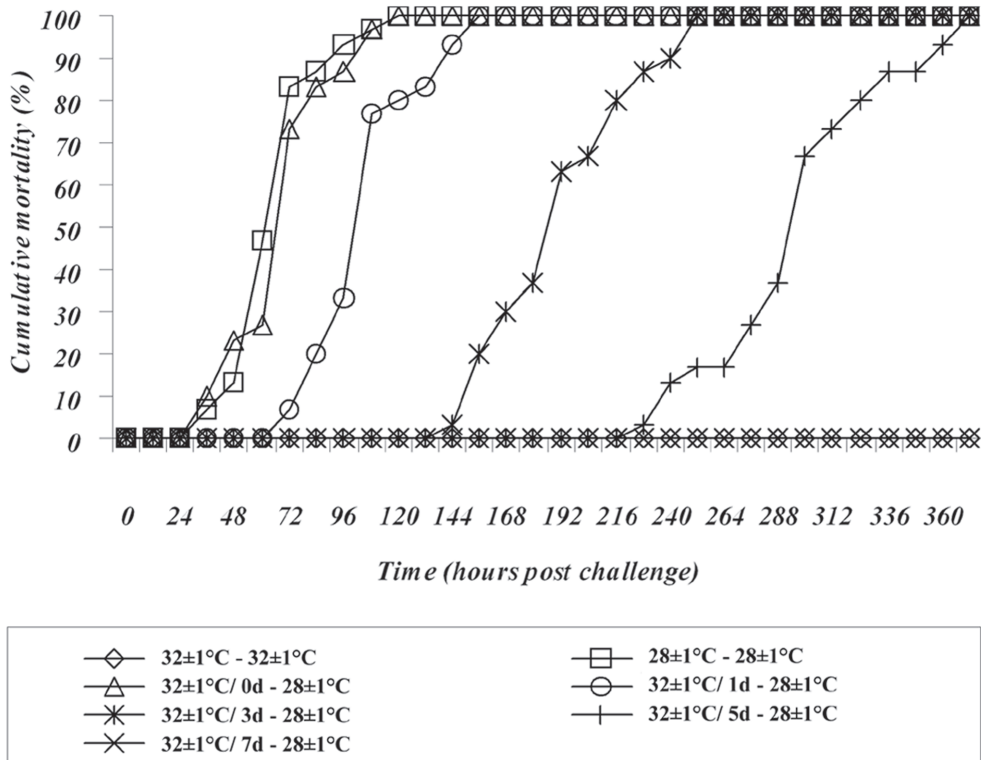


Figure 2. Cumulative mortalities of *L. vannamei* juveniles, challenged with WSSV by oral route. Shrimp were constantly maintained at 32±1°C until the end of the experiment (32±1°C-32±1°C) after challenge and a control group of shrimp were constantly maintained at 28±1°C until the end of the experiment (28±1°C-28±1°C) after challenge. Other groups were kept at 32±1°C and temperature was switched to 28±1°C at 0 day (32±1°C/0d-28±1°C), 1 day (32±1°C/1d-28±1°C), 3 days (32±1°C/3d-28±1°C), 5 days (32±1°C/5d-28±1°C) and 7 days (32±1°C/7d-28±1°C) post-challenge

All moribund shrimp from groups kept at 32±1°C for 0, 1, 3 and 5 days after infection with WSSV before temperature was switched to 28±1°C and control group with both immersion and oral routes developed clinical signs of WSSV with white spots or patches in the carapace and on various parts of the body (Figure 3). Histological sections of moribund shrimp revealed hypertrophied nuclei with eosinophilic (Cowdry A-type

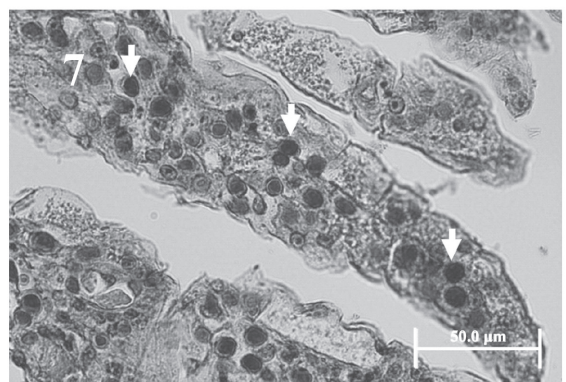
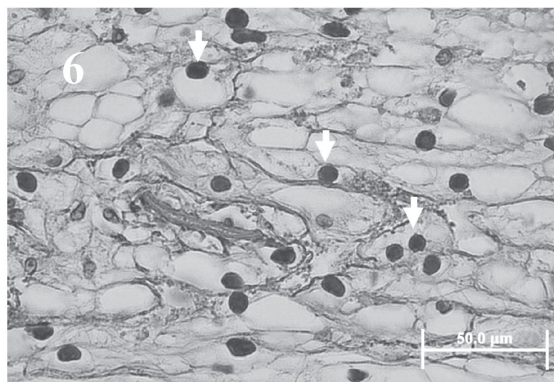
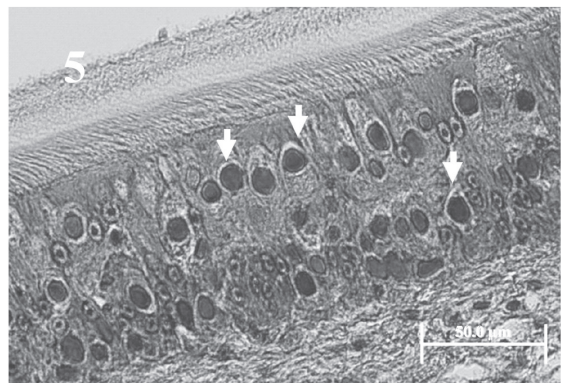
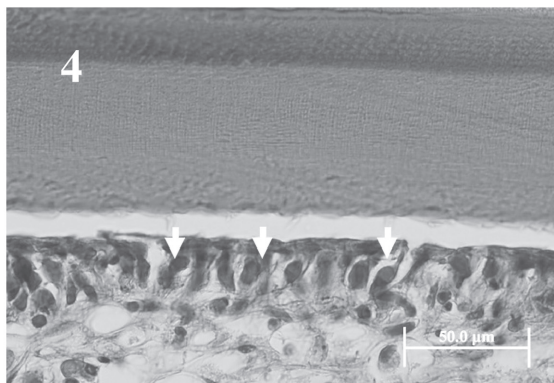
inclusion) to basophilic inclusion bodies in subcuticular epidermis, stomach cuticular epithelium, connective tissue, gills, antennal gland, heart and haematopoietic tissue (Figures 4-9). For immunohistochemistry method, WSSV infection was observed in the moribund shrimp from groups kept at 32±1°C for 0, 1, 3 and 5 days after infection and control group in various organs including subcuticular epidermis, stomach cuticular

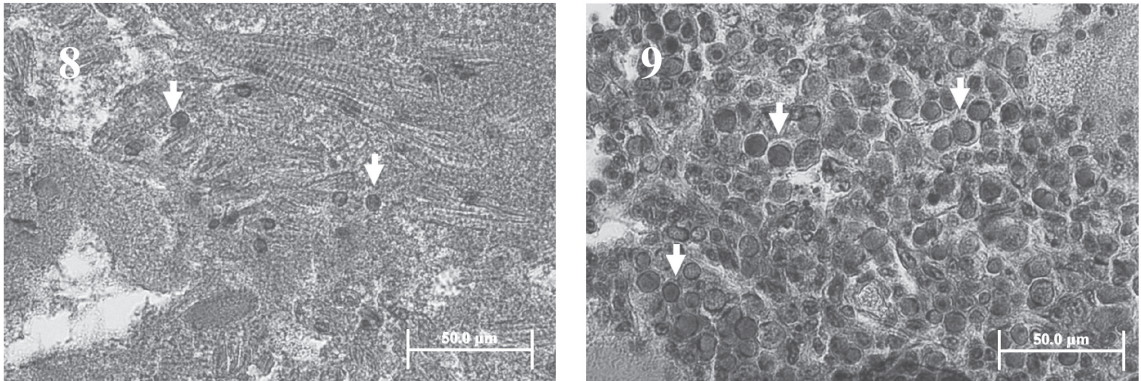
epithelium, connective tissue, gills, antennal gland, heart and haematopoietic tissue (Figures 10-13). In contrast, shrimp maintained at $32\pm 1^\circ\text{C}$ until the end of the

experiment and 7 days after infection before switching to $28\pm 1^\circ\text{C}$ did not display WSSV-positive cells at all sampling times. (Figure 14-17).

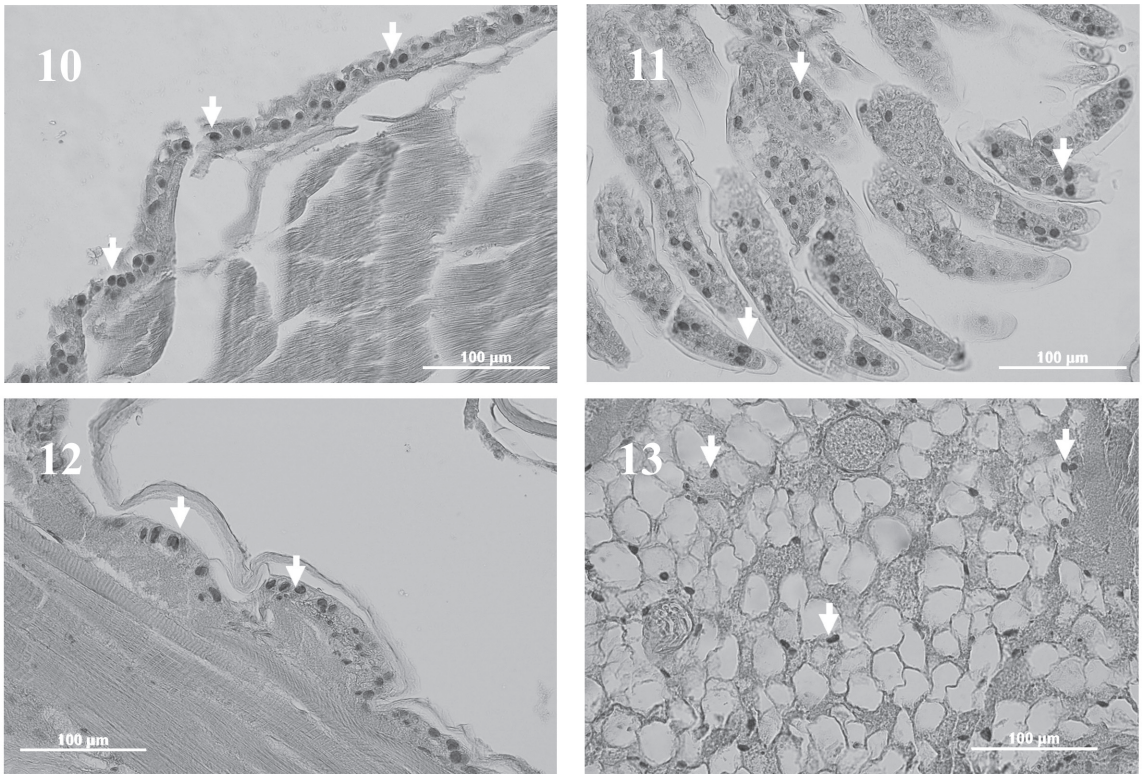


Figure 3. External appearance of moribund *L. vannamei* experimentally infected with WSSV, showing white spots (arrows) in the carapace and on various parts of the body

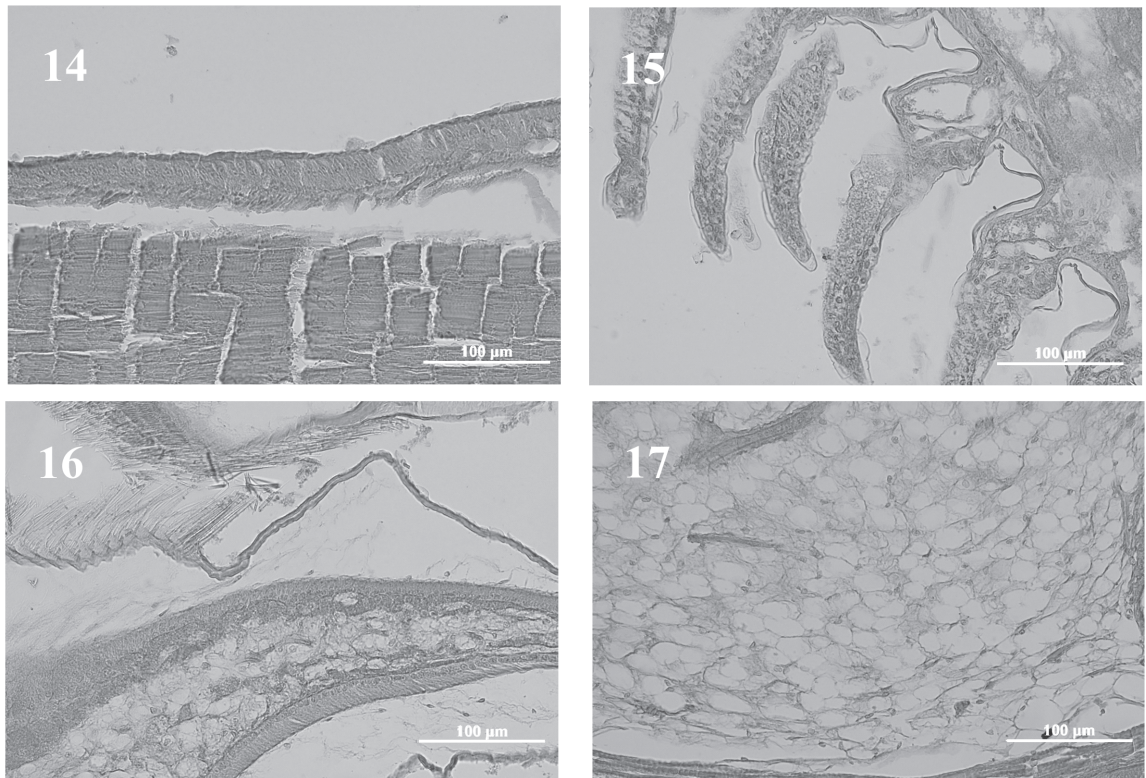




Figures 4 - 9. Photomicrographs of tissues from moribund *L. vannamei* after experimental infection with WSSV and maintained at $32\pm 1^\circ\text{C}$ for 0, 1, 3 and 5 days before the temperature was switched to $28\pm 1^\circ\text{C}$ and control group showing hypertrophied nuclei with basophilic inclusions (arrows): (4) subcuticular epidermis; (5) stomach cuticular epithelium; (6) connective tissue; (7) gills; (8) heart; and (9) haematopoietic tissue (H&E, bar = 50 μm)



Figures 10-13. WSSV-positive cells (arrows) in (10) subcuticular epidermis, (11) gills, (12) stomach cuticular epithelium and (13) connective tissue of *L. vannamei* challenged with WSSV and maintained at $32\pm 1^\circ\text{C}$ for 0, 1, 3 and 5 days before the temperature was switched to $28\pm 1^\circ\text{C}$ and control group as determined by immunohistochemistry (bar = 100 μm)



Figures 14-17. WSSV-negative cells in (14) subcuticular epidermis, (15) gills, (16) stomach cuticular epithelium and (17) connective tissue of *L. vannamei* challenged with WSSV and maintained at $32\pm 1^\circ\text{C}$ until the end of the experiment and 7 days before the temperature was switched to $28\pm 1^\circ\text{C}$ as determined by immunohistochemistry (bar = 100 μm)

DISCUSSION

In the present study, juvenile shrimp continuously maintained at $32\pm 1^\circ\text{C}$ after WSSV-infection by immersion challenge and by the oral route showed no clinical signs or mortalities. This study agreed with previous reports that high water temperature ($32\text{-}33^\circ\text{C}$) reduced or delayed mortality in WSSV inoculated *L. vannamei* postlarvae and juveniles (Vidal *et al.*, 2001; Rahman *et al.*, 2006; 2007b). Moreover, other studies

done in vivo with WSSV-infected shrimp (*Marsupenaeus japonicus*) or crayfish (*Procambarus leniusculus*) revealed that maintaining these species at water temperature below 16°C was also effective in reducing mortality (Guan *et al.*, 2003; Jiravanichpaisal *et al.*, 2004). Maintaining shrimp at $32\pm 1^\circ\text{C}$ for 0, 1, 3 and 5 days after infection with WSSV both by immersion and oral routes before reducing the temperature to $28\pm 1^\circ\text{C}$ revealed that maintaining for a longer period at $32\pm 1^\circ\text{C}$ could delay clinical signs and

onset of mortalities. However, 100% mortalities occurred in all groups within 7 days, as with that in the control group. Only the group constantly maintained at $32\pm 1^{\circ}\text{C}$ for 7 days before switching to $28\pm 1^{\circ}\text{C}$ did not show clinical signs or mortalities, similar to the group continuously kept at $32\pm 1^{\circ}\text{C}$ throughout the experiment.

The present findings clearly demonstrated that juvenile shrimp infected with WSSV and kept continuously at $32\pm 1^{\circ}\text{C}$ for 7 days showed no clinical signs or mortalities regardless of infection by immersion challenge or oral route. WSSV was completely inhibited at this temperature as confirmed by nested-PCR, histopathology and bioassay methods. In addition, monoclonal antibodies specific to VP28 of WSSV did not give positive immunoreactions with tissues of infected shrimp. This result suggested that high water temperature completely inhibited the expression of the envelope protein VP28 *in vivo* similar to the report by Rahman *et al.* (2006), indicating that high water temperature may affect enzyme activity during the early stage of WSSV replication. Recent studies done with temperature-sensitive mutant baculoviruses showed that mutations in protein kinase-1 (Fan *et al.*, 1996) or in a putative RNA polymerase (Shikata *et al.*, 1998) resulted in the lack of expression of late viral proteins such as envelope proteins at high water temperature. In shrimp farms water temperature fluctuates diurnally and seasonally. In winter from November to the middle of February most cultivated areas in the central and eastern provinces experience low water temperature in the morning ranging from $23\text{--}25^{\circ}\text{C}$ and in the afternoon from $26\text{--}28^{\circ}\text{C}$. Meanwhile, in the southern provinces

during the monsoon season between October to December, water temperature does not highly fluctuate but is still low at $25\text{--}27^{\circ}\text{C}$ all day for several days due to continuous rain.

Despite biosecurity measures being employed in conjunction with zero water exchange during the first 60 days post-stocking, WSSV outbreaks still occur within 30 days and cause a lot of damage, particularly during prolonged winter. Solving the problem related to water temperature in grow-out ponds is impossible and most farms could not do anything. On the other hand, during the larval rearing in hatcheries, temperatures of $32\pm 1^{\circ}\text{C}$ can easily be maintained through heaters. So far not many hatcheries pay attention to elevating water temperature to $32\pm 1^{\circ}\text{C}$; instead they normally keep it continuously at $28\text{--}30^{\circ}\text{C}$ or even lower during the winter period in hatcheries which do not have heaters. In order to successfully prevent WSSV outbreaks hatcheries should adapt or modify larval rearing practices by elevating the water temperature to $32\pm 1^{\circ}\text{C}$ at least 7 days before PL are transported and stocked into grow-out ponds.

CONCLUSIONS

This study clearly indicated that white shrimp maintained constantly at $32\pm 1^{\circ}\text{C}$ for 7 days were able to eliminate/clear WSSV infection, and were WSSV-negative by nested-PCR assay as well as histopathological examination, immunohistochemistry and bioassay methods. Results from the present study can be applied to prevent WSSV outbreak in pond-reared *L. vannamei* by rearing PL at $32\pm 1^{\circ}\text{C}$ for at least 7 days before stocking into grow-out ponds.

ACKNOWLEDGEMENTS

The authors would like to thank the National Research Council of Thailand for the financial support.

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