

## Effects of Water Temperature on the White Spot Syndrome Virus Infection in Postlarvae *Litopenaeus vannamei*

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### Abstract

This study evaluated the effects of high water temperature ( $32 \pm 1$  °C) on the white spot syndrome virus (WSSV) infection in *Litopenaeus vannamei* postlarvae (PL<sub>15</sub>). WSSV challenge was done by immersion. One group of PL<sub>15</sub> was continuously maintained at  $32 \pm 1$  °C until the end of the experiment after challenge and a control group of PL<sub>15</sub> was constantly maintained at  $28 \pm 1$  °C until the end of the experiment after challenge. Other groups were kept at  $32 \pm 1$  °C until temperature was altered from  $32 \pm 1$  °C to  $28 \pm 1$  °C at 0, 1, 3, 5 and 7 days after infection. 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 were kept at  $32 \pm 1$  °C for 0, 1, 3 and 5 days before the temperature was reduced to  $28 \pm 1$  °C revealing that maintaining the temperature at  $32 \pm 1$  °C for a longer period could delay clinical signs and onset of mortalities. Nevertheless, 100 % mortalities occurred in all groups and the control group within 7 days. All moribund PL<sub>15</sub> were WSSV-positive by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods. In contrast, PL<sub>15</sub> constantly maintained at  $32 \pm 1$  °C until the end of the experiment, and for 7 days after challenge before switching to  $28 \pm 1$  °C did not show clinical signs and mortality. Surviving PL<sub>15</sub> from both groups were WSSV-negative by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods. This study clearly indicated that postlarvae maintained constantly at  $32 \pm 1$  °C for 7 days were able to eliminate/clear WSSV infection.

**Keywords:** White spot syndrome virus, temperature, infection, *Litopenaeus vannamei*, postlarvae

### Introduction

White spot disease (WSD), commonly known as the white spot syndrome virus (WSSV), is an important viral pathogen and is responsible for huge economic losses in the shrimp culture industry worldwide [1,2]. This pathogen is an enveloped, double stranded DNA virus of the family *Nimaviridae*, genus *Whispovirus* [3,4]. In Thailand, WSSV was first reported in black tiger shrimp (*Penaeus monodon*) from the eastern and southern provinces along the coastal cultured areas

of the Gulf of Thailand and the Andaman Sea in late 1994 [5]. Mortality of WSSV-infected shrimp can reach 100 % within 2 - 10 days after the onset of the disease [1]. Recently, prevention measures have been employed using biosecurity systems including disinfecting ponds, water treatment before postlarvae (PL) stocking, fencing to prevent crabs that may carry infectious agents into the ponds and stocking WSSV-free PL by using a polymerase chain reaction (PCR) assay [6,7]. The

combination of these practices result in decreased risk of WSSV outbreak. However, during low temperature season from November to February water temperature in central and eastern cultured areas is lower than the optimal levels of 28 - 30 °C, which is also the case in the southern provinces during the monsoon season from October to December [8]. Shrimp farmers still faced WSSV infection despite PL were determined to be free from WSSV by using PCR assay and culturing in a biosecure system. This indicates that some PL were probably contaminated with WSSV without clinical signs of the disease and mortalities due to high water temperature ( $30 \pm 1$  °C) in hatcheries or PL samples for PCR detection do not entirely represent PL. In fact, after PL were stocked into a grow-out pond during continuously low water temperature for several days disease outbreak did occur. Therefore, water temperature is considered to be one of the most important environmental factors for outbreak of WSSV [9-10]. Previous studies reported *Litopenaeus vannamei* infected with WSSV and kept continuously at 32 - 33 °C showed no signs of disease or mortality [11-14]. Recently, Wongmaneeprateep *et al* [15] reported that experimentally WSSV-infected juveniles *L. vannamei* being kept constantly at  $32 \pm 1$  °C for 7 days were able to eliminate/clear WSSV infection. In contrast, shrimp constantly maintained at  $28 \pm 1$  °C after infection with WSSV by both immersion and oral procedures showed 100 % mortalities within 7 and 5 days, respectively. This indicated that high water temperature at  $32 \pm 1$  °C has an effect on the clearance of WSSV in shrimp. Moreover, if shrimp are raised constantly at  $32 \pm 1$  °C for at least 7 days before being transferred into grow-out ponds, the WSSV outbreak can be minimized. Although it is impossible to maintain the temperature of water in the pond at  $32 \pm 1$  °C all the time, temperature control during larval rearing can be done in order to reduce the WSSV infection in postlarvae. This method may be the best way to minimize WSSV outbreak in farm-reared *L. vannamei*.

The objective of this study was to evaluate the effects of water temperature at  $32 \pm 1$  °C on WSSV infection in *Litopenaeus vannamei* postlarvae (PL<sub>15</sub>) by immersion challenge.

## 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 by 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) was homogenized in a TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4) at 0.1 g/ml and centrifuged at 3,000 g for 20 min at 4 °C. The supernatant was removed to a new tube and centrifuged at 8,000 g for 30 min at 4 °C and the final supernatant was filtered through a 0.45-µm 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 final supernatant was determined by a nested-PCR assay (IQ2000™ WSSV Detection and Prevention System, Farming IntelliGene Tech. Corp.) and the viral load was quantified by using real-time PCR techniques according to the method of Sritunyalucksana *et al* [16]. The WSSV content of the inoculum prepared from WSSV-infected tissues was quantified by real-time PCR. The inoculum contained  $1 \times 10^8$  WSSV copies/ml.

### Experimental Animals

Specific pathogen free (SPF) postlarvae *L. vannamei* (PL<sub>10</sub>) were obtained from a hatchery in Chachoengsao Province, Thailand, and then transported to the Aquaculture Business Research Center (ABRC) laboratory, Kasetsart University. Shrimps were acclimatized in a 500-L fiberglass tank with aeration at 25 ppt salinity. Water temperature was maintained at  $32 \pm 1$  °C using an aquarium heater. Shrimps were fed 3 times daily with WSSV-free *Artemia* nauplii for 4 days before being transferred for WSSV-immersion challenge. Shrimp were randomly sampled and verified to be WSSV-free by nested-PCR assay. A total of 2,100 shrimps were transferred into twenty one 90-L aquaria (100 PL/aquarium) equipped with aeration and heater. The shrimp were challenged by inoculation of 90 ml (1:1000 dilutions) of the WSSV inoculum in the aquarium water [17,18]. One group of shrimps was constantly maintained at  $32 \pm 1$  °C until the end of the experiment after

challenge and a control group was constantly maintained at  $28 \pm 1$  °C until the end of the experiment after challenge. Other groups were kept at  $32 \pm 1$  °C until the temperature was reduced to  $28 \pm 1$  °C at 0, 1, 3, 5 and 7 days post-challenge. Water temperature was then maintained at  $28 \pm 1$  °C until the end of the experiments. Each group was conducted in 3 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 experiments. Moribund or surviving shrimp were confirmed by nested-PCR, histopathology, immunohistochemistry and bioassay methods.

### Histopathology

Moribund or surviving shrimp (30 shrimps in each group) were preserved in Davison's fixative for 24 h and then transferred to 70 % ethanol until processing as previously described [19]. All samples were sectioned and stained with hematoxylin and eosin (H & E). Infections were considered positive when samples showed typical WSSV histopathological features including hypertrophied nuclei with basophilic inclusions in subcuticular epithelium, gills and stomach cuticular epithelium [1].

### 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) were used as the prime antibodies [20]. Goat anti-mouse IgG H & L horseradish peroxidase conjugate (BioRad)

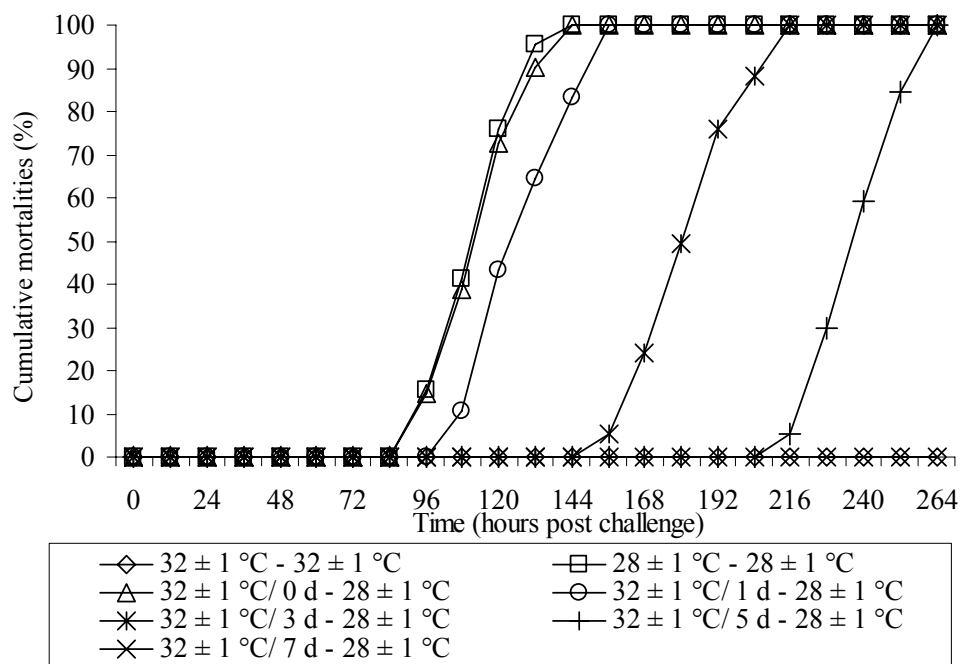
was used as the second antibody. Peroxidase activity was revealed by incubation with 0.03 % diaminobenzidine, 0.006 % hydrogen peroxide in PBS, then counterstained with eosin, and processed for permanent slides. WSSV-positive cells showed a brown precipitate.

### Bioassay

Moribund or surviving shrimps were feed twice daily for SPF postlarvae *L. vannamei* (PL<sub>15</sub>). Moribund shrimp were collected and detected for WSSV by using nested-PCR assay and histopathological techniques. Seven days after challenge, if there were no moribund or dead shrimps and all test results were negative, then it was safe to conclude that the bioassay results were negative.

### Results

The cumulative mortalities of *L. vannamei* PL<sub>15</sub> challenged with WSSV-immersion are shown in **Figure 1**. For PL<sub>15</sub> exposed to WSSV at  $32 \pm 1$  °C for 0, 1, 3 and 5 days before reducing the temperature to  $28 \pm 1$  °C and control group, mortalities were first observed at 96, 108, 156, 216 and 96 h post-challenge (hpc) and cumulative mortalities reached 100 % at 144, 156, 216, 264 and 144 hpc, respectively. All moribund shrimp were WSSV-positive by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods (**Table 1**). In contrast, shrimp continuously maintained at  $32 \pm 1$  °C until the end of the experiment and 7 days after challenge before switching to  $28 \pm 1$  °C did not show mortality and surviving shrimp were WSSV-negative by nested-PCR assay as well as histopathology, immunohistochemistry and bioassay methods (**Table 1**).



**Figure 1** Cumulative mortalities of *L. vannamei* PL<sub>15</sub>, challenged with WSSV by immersion. PL<sub>15</sub> 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 PL<sub>15</sub> 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 days (32 ± 1 °C/ 0 d - 28 ± 1 °C), 1 day (32 ± 1 °C/ 1 d - 28 ± 1 °C), 3 days (32 ± 1 °C/ 3 d - 28 ± 1 °C), 5 days (32 ± 1 °C/ 5 d - 28 ± 1 °C) and 7 days (32 ± 1 °C/ 7 d - 28 ± 1 °C) post-challenge.

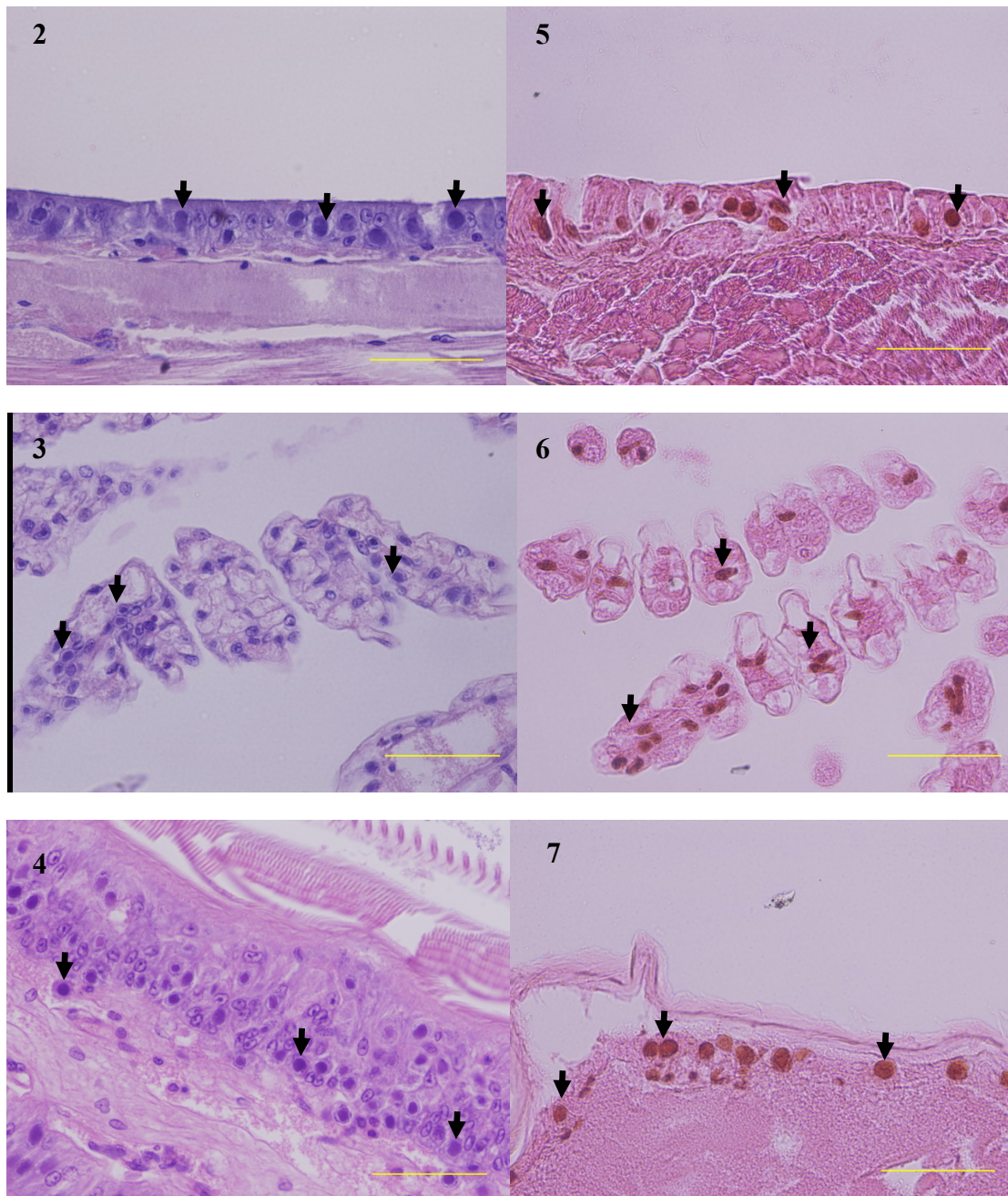
**Table 1** The results of nested-PCR, histopathology, immunohistochemistry and bioassay methods of *L. vannamei* PL<sub>15</sub> challenged with WSSV by immersion and maintained at different temperatures.

Water temperature <sup>a</sup>	Detection method			
	Nested-PCR <sup>b</sup>	Histopathology <sup>b</sup>	Immunohistochemistry <sup>b</sup>	Bioassay <sup>c</sup>
32 ± 1 °C - 32 ± 1 °C	0/30	0/30	0/30	NM
28 ± 1 °C - 28 ± 1 °C	30/30	30/30	30/30	M
32 ± 1 °C/ 0 d - 28 ± 1 °C	30/30	30/30	30/30	M
32 ± 1 °C/ 1 d - 28 ± 1 °C	30/30	30/30	30/30	M
32 ± 1 °C/ 3 d - 28 ± 1 °C	30/30	30/30	30/30	M
32 ± 1 °C/ 5 d - 28 ± 1 °C	30/30	30/30	30/30	M
32 ± 1 °C/ 7 d - 28 ± 1 °C	0/30	0/30	0/30	NM

<sup>a</sup> Water temperature before challenge/days-water temperature after challenge post challenge.

<sup>b</sup> Values represent the number of shrimp positive in PCR or histopathology or immunohistochemistry /number of shrimp tested.

<sup>c</sup> M, mortality; NM, no mortality.



**Figures 2 - 7** Hypertrophied nuclei with basophilic inclusions (arrows): (2) subcuticular epidermis; (3) gills and (4) stomach cuticular epithelium (H & E, bar = 50  $\mu$ m) and WSSV-positive cells (arrows) in (5) subcuticular epidermis, (6) gills and (7) stomach cuticular epithelium as determined by immunohistochemistry (bar = 50  $\mu$ m) of *L. vannamei* challenged with WSSV and maintained at  $32 \pm 1$   $^{\circ}$ C for 0, 1, 3 and 5 days before the temperature was reduced to  $28 \pm 1$   $^{\circ}$ C and control group.

Histological section of all moribund shrimp from groups kept at  $32 \pm 1$  °C for 0, 1, 3 and 5 days after infection with WSSV before the temperature was altered to  $28 \pm 1$  °C and control group revealed hypertrophied nuclei with eosinophilic (Cowdry A-type inclusion) to basophilic inclusion bodies in subcuticular epidermis, gills and stomach cuticular epithelium (**Figure 2 - 4**). For immunohistochemistry method, WSSV infection were observed in the moribund shrimp from groups kept at  $32 \pm 1$  °C for 0, 1, 3 and 5 days after infection and control group in subcuticular epidermis, gills and stomach cuticular epithelium (**Figure 5 - 7**). In contrast, shrimp maintained at  $32 \pm 1$  °C until the end of the experiment and 7 days after infection before switching to  $28 \pm 1$  °C did not display WSSV-positive cells at all sampling times.

### Discussion

In this study, PL<sub>15</sub> continuously maintained at  $32 \pm 1$  °C after WSSV-infection by immersion showed no clinical signs or mortalities. This result agreed with previous reports that high water temperature (32 - 33 °C) reduced/delayed mortality in WSSV inoculated *L. vannamei* PL and juveniles [12,13,21]. Moreover, other studies done *in vivo* with WSSV-infected kuruma shrimp (*Marsupenaeus japonicus*) or crayfish (*Procambarus leniusculus*) revealed that maintaining these species at water temperatures below 16 °C was also effective in reducing mortality [22,23]. Maintaining shrimp at  $32 \pm 1$  °C for 0, 1, 3 and 5 days after infection with WSSV by immersion before reducing the temperature to  $28 \pm 1$  °C revealed that maintaining the temperature at  $32 \pm 1$  °C for a longer period could delay clinical signs and the onset of mortalities. However, 100 % mortalities occurred in all groups within 7 days, the same as the control group. Only the group constantly maintained at  $32 \pm 1$  °C for 7 days before reducing to  $28 \pm 1$  °C did not show clinical signs or mortalities the same as the group continuously kept at  $32 \pm 1$  °C throughout the experiment.

The present findings clearly demonstrate that PL<sub>15</sub> infected with WSSV and kept continuously at  $32 \pm 1$  °C for 7 days showed no clinical signs or mortalities. 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* [24] which indicated that high water temperature may affect enzyme activity during the early stages of WSSV replication. Recent studies done with temperature-sensitive mutant baculoviruses showed that mutations in protein kinase-1 [25] or in a putative RNA polymerase [26] 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 - 25 °C and in the afternoon from 26 - 28 °C, while in the southern provinces during the monsoon season between October to December water temperature does not fluctuate greatly but is still at a low level of 25 - 27 °C all day for several days due to continuous rain most of the time. 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 in years that experience a long winter. Solving the problems related to water temperature in the grow-out ponds is impossible and most farms cannot do anything. On the other hand, during the larval rearing in hatcheries, temperatures of  $32 \pm 1$  °C can easily be maintained by equipping with adequate heaters. So far not many hatcheries pay attention to elevating water temperature to  $32 \pm 1$  °C; instead they normally keep it continuously at 28 - 30 °C or even lower during the winter period in some hatcheries that do not have heaters. In order to successfully prevent WSSV outbreaks we should adapt or modify larval rearing practices by elevating water temperature to  $32 \pm 1$  °C for the last 7 days before PL are transported and stocked into the grow-out ponds.

### Conclusions

This study clearly indicated that PL maintained constantly at  $32 \pm 1$  °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 for preventing WSSV outbreak in pond-reared *L. vannamei* by rearing PL at  $32 \pm 1$  °C for at least 7 days before stocking the PL in the culture ponds.

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#### References

- [1] DV Lightner. *A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp*, Vol. 80. World Aquaculture Society, Baton Rouge, LA, 1996, p. 3021-9.
- [2] TW Flegel. Major viral disease of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J. Microbiol. Biotechnol.* 1997; **13**, 433-42.
- [3] MCW van Hulten, J Wittevelde, S Peters, N Kloosterboer, R Tarchini, M Fiers, H Sandbrink, RK Lankhorst and JM Vlak. The white spot syndrome virus DNA genome sequence. *Virology* 2001; **286**, 7-22.
- [4] MA Mayo. A summary of taxonomic changes recently approved by ICTV. *Arch. Virol.* 2002; **147**, 1655-63.
- [5] C Limsuwan. *Thai Shrimp 2000 (in Thai)*, Charoenrat Publishing Company, Bangkok, 2000, p. 102-6.
- [6] C Limsuwan. Reducing the effects of white-spot baculovirus using PCR screening and stressors. *AAHRI Newsletter* 1997; **6**, 1-2.
- [7] B Withyachumnarnkul. Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). *Dis. Aquat. Org.* 1999; **39**, 21-7.
- [8] C Limsuwan. Diseases of Pacific white shrimp (*Litopenaeus vannamei*) in Thailand. *AAHRI Newsletter* 2003; **12**, 1-4.
- [9] DF Fegan and HC Clifford III. *Health Management for Viral Diseases in Shrimp Farms*. In: Browdy CL and Jory DE (eds.). The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001. The World Aquaculture Society, Baton Rouge, 2001, p. 168-92.
- [10] J Rodríguez, B Bayot, Y Amano, F Panchana, I de Blas, V Alday and J Calderón. White spot syndrome virus infection in cultured *Penaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and ultrastructure. *J. Fish Dis.* 2003; **26**, 439-50.
- [11] OM Vidal, CB Granja, LF Aranguren, JA Brock and M Salazar. A profound effect of hyperthermia on survival of *Litopenaeus vannamei* juveniles infected with white spot syndrome virus. *J. World Aquac. Soc.* 2001; **32**, 364-72.
- [12] CB Granja, LF Aranguren, OM Vidal, L Aragon and M Salazar. Does hyperthermia increase apoptosis in white spot syndrome virus (WSSV)-infected *Litopenaeus vannamei*? *Dis. Aquat. Org.* 2003; **54**, 73-8.
- [13] CB Granja, OM Vidal, G Parra and M Salazar. Hyperthermia reduces viral load of white spot syndrome virus in *Penaeus vannamei*. *Dis. Aquat. Org.* 2006; **68**, 175-80.
- [14] MM Rahman, M Corteel, JJ Dantas-Lima, M Wille, V Alday-Sanz, MB Pensaert, P Sorgeloos and HJ Nauwynck. 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* 2007; **269**, 107-13.
- [15] S Wongmaneeprateep, N Chuchird and C Limsuwan. Effect of high water temperature on clearance of white spot syndrome virus (WSSV) in *Litopenaeus vannamei*. Sustainable aquaculture and quality seafood for all. In: Asian Pacific Aquaculture 2009, Kuala Lumpur, Malaysia. 2009, p. 568.
- [16] K Sritunyalucksana, J Srisala, K McColl, L Nielsen and TW Flegel. Comparison of PCR testing methods for white spot syndrome virus (WSSV) infections in penaeid shrimp. *Aquaculture* 2006; **255**, 95-104.
- [17] CA Venegas, L Nonaka, K Mushiake, K Shimizu, T Nishizawa and K Muroga. Pathogenicity of penaeid rod-shaped DNA virus PRDV to Kuruma prawn at different development stages. *Fish Pathol.* 1999; **170**, 179-94.

- [18] L Chen, CF Lo, YL Chiu, FC Chen and GH Kou. Experimental infection of white spot syndrome virus WSSV in benthic larvae of mud crab *Scylla serrata*. *Dis. Aquat. Org.* 2000; **40**, 157-61.
- [19] TA Bell and DV Lightner. *A Handbook of Normal Penaeid Shrimp Histology*. World Aquaculture Society, Baton Rouge, LA. 1988.
- [20] P Chaivisuthangkura, J Tangkhabuanbutra, S Longyant, W Sithigorngul, S Rukpratanporn, P Menasveta and P Sithigorngul. Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. *Sci. Asia* 2004; **30**, 359-63.
- [21] MM Rahman, M Corteel, M Wille, V Alday-Sanz, MB Pensaert, P Sorgeloos and HJ Nauwynck. 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* 2007; **272**, 240-5.
- [22] Y Guan, Z Yu and C Lia. The effects of temperature on white spot syndrome infections in *Marsupenaeus japonicus*. *J. Invertebr. Pathol.* 2003; **83**, 257-60.
- [23] P Jiravanichpaisal, K Soderhall and I Soderhall. Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. *Fish Shellfish Immunol.* 2004; **17**, 265-75.
- [24] MM Rahman, CM Escobedo-Bonilla, M Corteel, JJ Dantas-Lima, M Wille, V Alday-Sanz, MB Pensaert, P Sorgeloos and HJ Nauwynck. 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* 2006; **261**, 842-9.
- [25] X Fan, K Thirunavukkarasu and RF Weaver. Temperature sensitive mutations in the protein kinase-1 (pk-1) gene of the *Autographa californica* nuclear polyhedrosis virus that block very late gene expression. *Virology* 1996; **224**, 1-9.
- [26] M Shikata, Y Sano, Y Hashimoto and T Matsumoto. Isolation and characterization of a temperature-sensitive mutant of *Bombyx mori* nucleopolyhedrovirus for a putative RNA polymerase gene. *J. Gen. Virol.* 1998; **79**, 2071-8.