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The effect of raising water temperature to 33 °C in *Penaeus vannamei* juveniles at different stages of infection with white spot syndrome virus (WSSV)

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Abstract

This study investigated the effect of high water temperature (33 °C) at different stages of infection with a highly virulent and low virulent white spot syndrome virus strain (WSSV Thai-1 and WSSV Viet) in *Penaeus vannamei* juveniles. Shrimp were inoculated intramuscularly with either a high dose (HD) or low dose (LD). Water temperature was kept either at continuously 27 °C or switched from 27 °C to 33 °C at 0, 12 or 24 h post inoculation (hpi) for both strains and in addition at 48 or 96 hpi for WSSV Viet. The increased temperature 33 °C was maintained till the end of the experiments (120–144 hpi with WSSV Thai-1 and 240 hpi with WSSV Viet). To determine the infection status at the moment of temperature increase, five shrimp that were kept continuously at 27 °C were euthanized at 0, 12, 24, 48 and 96 hpi with each dose of two strains. WSSV infections (viral antigen VP28) in dead and euthanized shrimp were demonstrated by indirect immunofluorescence.

Shrimp inoculated with HD or LD of WSSV Thai-1 and kept continuously at 27 °C till euthanasia were 100% viral antigen positive from 12 (HD) or 24 hpi (LD). Shrimp inoculated with WSSV Viet were 100% positive from 24 (HD) and 48 hpi (LD). Shrimp kept at 27 °C, showed clinical signs from 24 (HD) or 24–36 hpi (LD) with both strains. Cumulative mortalities reached 100% with WSSV Thai-1 at 60 (HD) or 84–144 hpi (LD) and with WSSV Viet 100% at 216 hpi (HD) or 90% at 240 hpi (LD). Switch of temperature to 33 °C from 0, 12 or 24 hpi was effective in reducing mortality of shrimp inoculated with the LD of both strains and with the HD of WSSV Viet. The switch to 33 °C from 24 hpi with the Thai strain (HD) and from 48 and 96 hpi with the Viet strain (LD or HD) had no effect or even accelerated the mortality rate (80–100%). All shrimp were viral antigen positive at death and euthanasia (one shrimp LD WSSV Viet) when kept continuously at 27 °C. All dead and euthanized shrimp kept at 33 °C from 0 or 12 hpi were viral antigen negative. With 33 °C from 24, 48 or 96 hpi, all dead shrimp were viral antigen positive and euthanized shrimp were negative.

This study showed that 33 °C is effective to prevent disease, reduce mortality and block WSSV replication, but only in the early stages of infection.

Keywords: Stages of WSSV infection; *Peaneus vannamei*; Water temperature

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1. Introduction

White spot syndrome virus (WSSV) has caused severe mortalities in penaeid shrimp farms for more than one decade (Chou et al., 1995; Lightner, 1996; Rodríguez et al., 2003; Flegel, 2006). A WSSV epidemic was first reported in east Asia (Chou et al., 1995) and afterwards spread to almost all shrimp producing countries of Asia, North, Central and South America (Lightner, 1996; Rodríguez et al., 2003; Flegel, 2006). Under standardized experimental conditions with a water temperature of 27 °C, differences between strains have been found in *Penaeus vannamei* juveniles in the onset of clinical signs, onset of mortality and time to reach a cumulative mortality of 100% (Rahman et al., 2006c). The cause of death of WSSV infected shrimp has been suggested to be due to dysfunction of target tissues including gills, stomach epithelium, cuticular epithelium, antennal gland and hematopoietic tissue. WSSV replication can be demonstrated in target tissues from 12 to 24 h post inoculation (hpi) (Chang et al., 1996; Yoganandhan et al., 2003; Escobedo-Bonilla et al., 2007) depending on the titer of inoculation (Escobedo-Bonilla et al., 2007) and virulence of the WSSV strain used (Rahman et al., 2007b). Natural outbreaks of WSSV are suggested to be associated with stress caused by environmental factors such as water temperature (Fegan and Clifford, 2001; Rodríguez et al., 2003).

Water temperature has a direct effect on metabolic rate (Allan et al., 2006), growth and survival (Wyban et al., 1995; Ponce-Palafox et al., 1997), molting rate (Vijayan and Diwan, 1995), requirement of dissolved oxygen (Tian et al., 2004), lethal dissolved oxygen level (Zhang et al., 2006), tolerance to ammonia-N (Barajas et al., 2006) and immune response of shrimp (Le Moullac and Haffner, 2000; Cheng et al., 2005). The optimum temperature for growth of *P. vannamei* juveniles of above 5 g has been shown to be 27 °C (Wyban et al., 1995).

Previous studies reported prevention of clinical signs, reduced mortality and absence of infection in target tissues of shrimp kept continuously at 32–33 °C before and after WSSV inoculation or immediately after inoculation (Vidal et al., 2001; Rahman et al., 2006b). However, the effect of increasing water temperature from 27 °C to 33 °C during the course of infection is unknown.

The aim of this study was to evaluate the effect of increasing water temperature from 27 °C to 33 °C on the clinical and virological outcome in *P. vannamei* juveniles at different time points after inoculation with a highly (Thai-1) and low (Viet) virulent WSSV strain (Rahman et al., 2006c).

2. Materials and methods

2.1. Virus

Two WSSV strains were used in this study. WSSV Thai-1 was collected from naturally infected *Penaeus monodon* in Thailand and passaged in crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001). WSSV Viet was collected from naturally infected *P. monodon* in Vietnam and passaged in crayfish *Cherax quadricarinatus*. Crayfish gill suspension of WSSV Thai-1 was kindly provided by K. Söderhäll (Uppsala University, Sweden) and WSSV Viet was received from the Research Institute for Aquaculture no. 2, Vietnam. Both strains were amplified in specific pathogen free (SPF) *P. vannamei* juveniles in the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University, Belgium and the infectivity titers of the stocks were determined according to the procedure described by Escobedo-Bonilla et al. (2005). The median infectious titers of the stocks as determined by intramuscular inoculation in SPF *P. vannamei* were $10^{5.9}$ and $10^{5.8}$ SID_{50} (shrimp infectious dose with 50% endpoint) per ml for WSSV Thai-1 and WSSV Viet, respectively.

2.2. Shrimp

In total, 437 shrimp were used in the present study. In the experiment to determine WSSV infection status at different hours post inoculation (hpi) mean body weights (MBW) of shrimp were 16.5±2.7 g. To evaluate the effect of a switched temperature from 27 °C to 33 °C on the virological and clinical outcome MBW of shrimp were 10.7±2.2 g, 13.8±2.7 g, 16.5±2.7 g in the three experiments with WSSV Thai-1 and 16.6±2.8 g in the experiment with WSSV Viet.

2.3. Experimental conditions

SPF *P. vannamei* from Molakai farm, Hawaii were imported at early post larval stage and reared in a recirculation system at the Laboratory of Aquaculture and Artemia Reference Center (ARC), Faculty of Bioscience Engineering, Gent University, Belgium. Rearing conditions were: water temperature 27–28 °C, salinity 34–36 g/l. Before each experiment shrimp were gradually acclimatized to the salinity of 15 g/l at the ARC over four days. Acclimatized shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Gent University. Nine to eleven shrimp were housed per 50 litre aquarium, equipped with aeration, mechanical filtration (Eheim, Germany) and aquarium heater (Model VTX 300, Aquarium systems, France). Brackish water with a salinity of 15 g/l was prepared using artificial sea salt (Instant Ocean, Aquarium systems, France) and de-ionized water. Water temperature was kept at 27 °C before WSSV inoculation and temperature was switched to 33 °C at different hpi as described below. Approximately 0.2 g of a commercial shrimp diet was provided per shrimp per day. Water quality was checked by measuring ionized ammonia (NH_4^+) using test kits (Aquamerck, Germany) and

90% of water was renewed at 120 hpi in the experiment with WSSV Viet.

2.4. WSSV inoculation procedure

Shrimp were inoculated intramuscularly in the junction between the third and fourth abdominal segments with 50 μl of inoculum containing a low dose (LD) (30 SID_{50}) or a high dose (HD) (10,000 SID_{50}) of either of WSSV Thai-1 or WSSV Viet. Shrimp were observed for clinical signs including anorexia and lethargia and mortality was recorded every 12 h.

2.5. WSSV infection status at different hpi

Eighty shrimp were kept at continuously 27 °C. Thirty shrimp were inoculated with WSSV Thai-1 and fifty shrimp with WSSV Viet, equally divided in groups for inoculation with a LD and HD. Five shrimp inoculated with each dose of each strain were euthanized at 0, 12 and 24 hpi. In addition, five shrimp inoculated with each dose of WSSV Viet were euthanized at 48 and 96 hpi.

2.6. Effect of switched temperature to 33 °C on virological and clinical outcome

Shrimp were kept either at continuously 27 °C (27 °C–27 °C) or temperature was switched from 27 °C to 33 °C at 0 (27 °C–33 °C/0 hpi), 12 (27 °C–33 °C/12 hpi)) or 24 hpi (27 °C–33 °C/24 hpi). In addition, temperature of WSSV Viet inoculated shrimp was switched to 33 °C at 48 (27 °C–33 °C/48 hpi) and 96 hpi (27 °C–33 °C/96 hpi). Afterwards, increased temperature (33 °C) was maintained till the end of the experiments. The experiments ran for 120–144 hpi with WSSV Thai-1 and 240 hpi with WSSV Viet. Nine to eleven shrimp were used for each temperature treatment with LD or HD of a strain. With WSSV Thai-1, experiments were repeated three times and with WSSV Viet one experiment was conducted.

2.7. Evaluation of WSSV infection by indirect immunofluorescence (IIF)

The cephalothoraxes of dead and euthanized shrimp were dissected longitudinally, embedded in 2% methylcellulose and quickly frozen at −20 °C. Cryosections (5 μm) were made and immediately fixed in 100% methanol at −20 °C for 20 min. Sections were washed (three times for 5 min) in PBS and incubated with 2 μg ml^{-1} of the monoclonal antibody 8B7 (Diagxotics Inc., USA) directed against viral antigen VP28 (Poulos et al., 2001) of WSSV for 1 h at 37 °C. Then, sections were washed in PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (F-2761, Molecular Probes, The Netherlands) for 1 h at 37 °C. Sections were finally washed in PBS, rinsed in de-ionized water, dried and mounted with a solution of glycerin and 1, 4-diaza-bicyclo [2,2,2]-octan (DABCO) (ACROS organics, USA). Sections were analyzed by fluorescence microscopy.

3. Results

3.1. WSSV infection status at different hpi by IIF

3.1.1. WSSV Thai-1

Viral antigen (VP28) positive cells were found in all euthanized shrimp starting from 24 hpi when inoculated with LD and from 12 hpi when inoculated with a HD.

3.1.2. WSSV Viet

Viral antigen positive cells were found in 60% (LD) and 100% (HD) of the euthanized shrimp at 24 hpi. All euthanized shrimp were viral antigen positive at 48 and 96 hpi with LD and HD.

3.2. Effect of temperature switch to 33 °C on virological and clinical outcome

3.2.1. WSSV Thai-1

3.2.1.1. Low dose. At continuously 27 °C (27 °C–27 °C), shrimp started to show clinical signs at 24–36 hpi, mortalities started at 36–60 hpi and cumulative mortalities reached 100% at 84–144 hpi in the three experiments (Fig. 1a). When water temperature increased to 33 °C from 0 (27 °C–33 °C/0 hpi) or 12 hpi (27 °C–33 °C/12 hpi), shrimp did not show clinical signs and mean cumulative mortalities were 10±17.3% and 10±10%, respectively. With 33 °C from 24 hpi (27 °C–33 °C/24 hpi), clinical signs were observed from 24–36 hpi and the mean cumulative mortality was 24±5.3% at the end of the experiment. With 33 °C from 0 or 12 hpi, all dead and euthanized shrimp were viral antigen negative. At constant 27 °C or when temperature was switched to 33 °C from 24 hpi, the shrimp that died were viral antigen positive and the euthanized shrimp were negative.

3.2.1.2. High dose. At continuously 27 °C (27 °C–27 °C), shrimp showed clinical signs within 24 hpi, mortalities started at 36 hpi and cumulative mortalities reached 100% at 60 hpi in the three experiments (Fig. 1b). After a temperature switch to 33 °C at 0 (27 °C–33 °C/0 hpi) or 12 hpi (27 °C–33 °C/12 hpi), shrimp did not show clinical signs and mean cumulative mortalities were 10±10% and 6.6±5.8%, respectively. With 33 °C from 24 hpi (27 °C–33 °C/24 hpi), shrimp showed clinical signs within 24 hpi, mortalities started at 36 hpi and cumulative mortalities reached 90±10% at 60 hpi. With 33 °C from 0 or 12 hpi, all dead and euthanized shrimp were viral antigen negative. At continuously 27 °C or when temperature was switched to 33 °C from 24 hpi, all dead shrimp were viral antigen positive and the euthanized shrimp were negative.

3.2.2. WSSV Viet

3.2.2.1. Low dose. At continuously 27 °C (27 °C–27 °C), shrimp started to show clinical signs at 24–36 hpi, mortality started at 72 hpi, cumulative mortality reached 90% and one

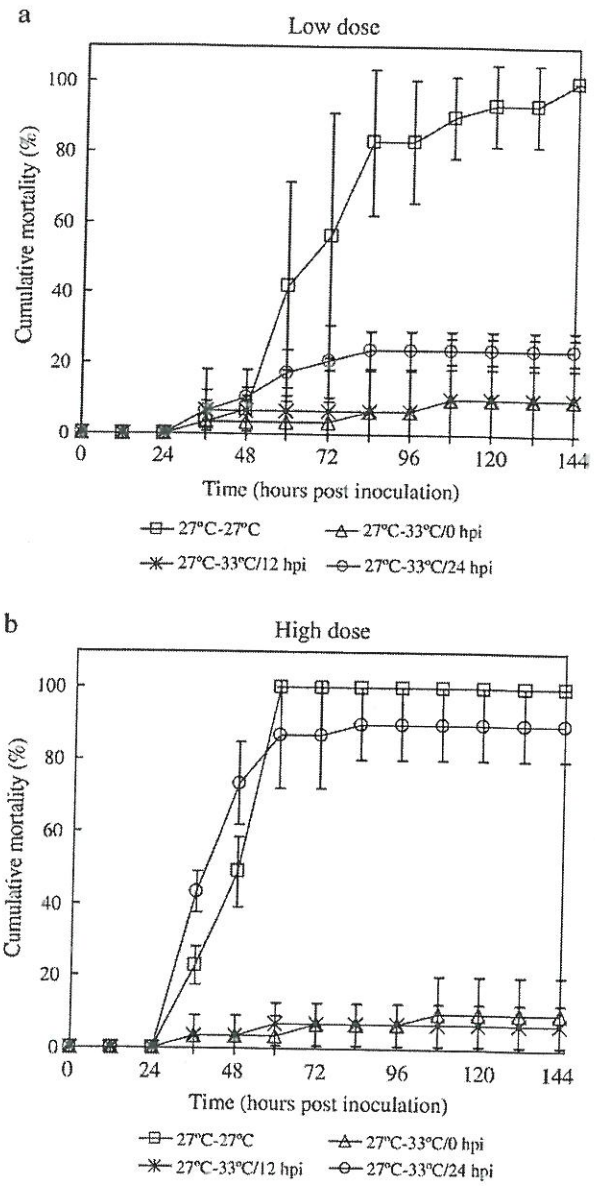

Fig. 1. Cumulative mortalities (mean±standard deviation [SD]) of SPF *Penaeus vannamei* juveniles, intramuscularly inoculated with (a) 30 SID_{50} or (b) 10000 SID_{50} of WSSV Thai-1. Water temperature was either kept continuously at 27 °C (27 °C–27 °C) or switched to 33 °C at 0 h post inoculation (hpi) (27 °C–33 °C/0 hpi), 12 hpi (27 °C–33 °C/12 hpi) or 24 hpi (27 °C–33 °C/24 hpi) and then maintained till 120–144 hpi.

shrimp was still alive at the end of the experiment (240 hpi) (Fig. 2a). When temperature was switched to 33 °C from 0 (27 °C–33 °C/0 hpi), 12 (27 °C–33 °C/12 hpi), or 24 hpi (27 °C–33 °C/24 hpi), shrimp did not show clinical signs and no mortality occurred. With 33 °C from 48 (27 °C–33 °C/48 hpi) or 96 hpi (27 °C–33 °C/96 hpi), shrimp started to show clinical signs at 24–36 hpi, the onset of mortality was at 48 or 72 hpi and cumulative mortality reached 80% at 84 hpi and 90% at 120 hpi, respectively. With 33 °C from 0, 12 or 24 hpi, all euthanized shrimp were viral antigen negative. At continuously 27 °C, all dead and survivor shrimp were viral antigen positive. When temperature was switched to 33 °C from 48 and 96 hpi, dead shrimp were viral antigen positive and euthanized shrimp were negative.

3.2.2.2. High dose. At continuously 27 °C (27 °C–27 °C), shrimp started to show clinical signs at 24 hpi, mortality started at 60 hpi and cumulative mortality reached 100% at 216 hpi (Fig. 2b). With temperature switched to 33 °C from 0 (27 °C–33 °C/0 hpi) or 12 hpi (27 °C–33 °C/12 hpi), shrimp did not show clinical signs and cumulative mortality reached 20%. With 33 °C from 24 hpi (27 °C–33 °C/24 hpi), shrimp started

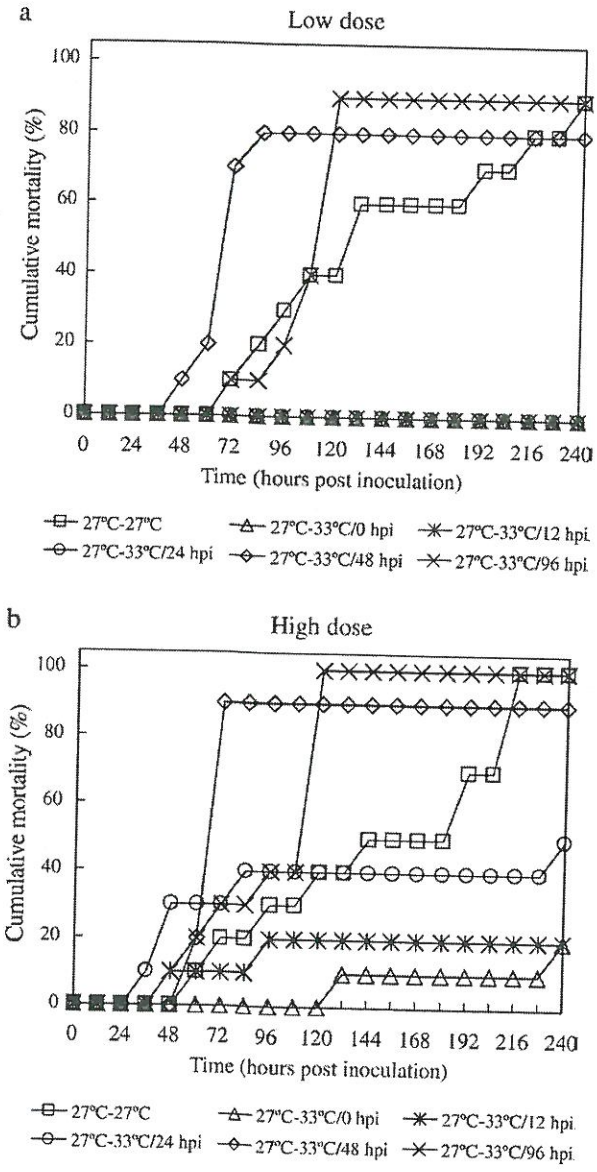

Fig. 2. Cumulative mortalities of SPF *Peaneus vannamei* juveniles, intramuscularly inoculated with (a) 30 SID_{50} or (b) 10000 SID_{50} of WSSV Viet. Water temperature was either kept continuously at 27 °C (27 °C–27 °C) or switched to 33 °C at 0 h post inoculation (hpi) (27 °C–33 °C/0 hpi), 12 hpi (27 °C–33 °C/12 hpi), 24 hpi (27 °C–33 °C/24 hpi), 48 hpi (27 °C–33 °C/48 hpi) or 96 hpi (27 °C–33 °C/96 hpi) and then maintained till 240 hpi.

to show clinical signs at 24 hpi, mortality started at 36 hpi and cumulative mortality reached 50% at 240 hpi. With 33 °C from 48 (27 °C–33 °C/48 hpi) or 96 hpi (27 °C–33 °C/96 hpi), shrimp started to show clinical signs at 24 hpi, mortality started at 48 or 60 hpi and cumulative mortality reached 90% at 72 hpi or 100% at 120 hpi. With 33 °C from 0 or 12 hpi, all dead and euthanized shrimp were viral antigen negative. At continuously 27 °C, all dead shrimp were viral antigen positive. When temperature was switched to 33 °C from 24, 48 and 96 hpi, all dead shrimp were viral antigen positive except one (died at 240 hpi with 33 °C from 24 hpi) and euthanized shrimp were negative.

4. Discussion

This study showed that increasing water temperature from 27 °C to 33 °C can have two opposite effects in WSSV infected shrimp depending on the stages of infection. In the acute stage of infection before clinical signs are observed, it shuts off virus replication and disease/mortality. In a more subacute, chronic stage of infection when clinical signs are present, the outcome is detrimental with quicker progression of disease/mortality in WSSV infected shrimp. The positive result with 33 °C in acutely infected shrimp opens a scope to apply 33 °C as a strategy to control infection, disease and mortality in a shrimp farm with the first signs of white spot syndrome (WSS). This may give the shrimp farmer more time to organize an emergency harvest. Exposure to 33 °C might also support the efficacy of other control strategies such as antivirals (Rahman et al., 2006a). However, the benefit from exposure to 33 °C of WSSV infected shrimp in the field has its limits because of the short time span during which the temperature has to be increased (between 12 and 24 hpi), negative effects of 33 °C on shrimp (Ponce-Palafox et al., 1997; Le Moullac and Haffner, 2000; Cheng et al., 2005; Zhang et al., 2006), restricted availability of logistics to raise temperature, the need for a quick diagnosis of WSS and the cost effectiveness.

Prevention of clinical signs, reduced mortality and absence of viral antigen in shrimp exposed to 33 °C at inoculation (27 °C–33 °C/0 hpi) agrees with previous work (Granja et al., 2006; Rahman et al., 2006b). The results of this study suggest that a similar mechanism is effective, even in shrimp in which WSSV has already replicated for 12 to 24 h.

Increasing temperature to 33 °C could not reduce mortality or was at least less effective in shrimp infected for 24 h at 27 °C with a HD of WSSV Thai-1 or WSSV Viet. This can be explained by the fact that at 24 hpi the viral infection had already become systemic (Escobedo-Bonilla et al., 2007), causing irreversible tissue damage. The mechanism of accelerated mortalities with exposure to 33 °C from 48 hpi or later (27 °C–33 °C/48 hpi and 27 °C–33 °C/96 hpi) with each dose of WSSV Viet is not clear. Similar accelerated mortalities were also observed in a study with diurnal fluctuations of temperature (27 °C and 33 °C) (Rahman et al., 2007a). Several factors may be involved in this phenomenon. An increased requirement for oxygen in shrimp kept at 33 °C (Tian et al., 2004) combined with the reduced amount of dissolved oxygen in the warmer water and a reduced oxygen exchange due to tissue damage in infected gills may be forwarded as the most plausible explanations. This sharp rise of mortality in shrimp with a progressed WSSV infection might explain the quick and high mortality during some of the natural outbreaks in shrimp farms.

With 33 °C from 0 or 12 hpi there were still a few shrimp that died. This was not due to infection since viral antigen positive cells were absent. This low mortality was probably due to the negative effects of 33 °C on the physiological condition of shrimp. Similar mortalities were also found in both WSSV inoculated and uninoculated shrimp in other studies (Ponce-Palafox et al., 1997; Rahman et al., 2006b).

In conclusion, this study shows that the efficacy of increasing temperature to 33 °C to prevent disease, reduce mortality and diminish infection depends on the progression of infection in shrimp.

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