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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)

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Abstract

This study evaluated the effect of daily fluctuations between optimum (27 °C) and high water temperature (33 °C) on the clinical and virological outcome of a WSSV infection in *Penaeus vannamei* juveniles. Shrimp were inoculated intramuscularly with a dose of 10,000 SID_{50} of either virulent WSSV Thai-1 or less virulent WSSV Viet or mock inoculated. Temperature was kept either continuously at 27 °C or switched from 27 °C to 33 °C at 12 h post inoculation (hpi) and maintained at that temperature for 6 h (6 h 33 °C), 12 h (12 h 33 °C), 18 h (18 h 33 °C) or 24 h (24 h 33 °C) per day. Temperature was then lowered and maintained at 27 °C for the remaining hours of the day. The experiments ran for 132 hpi with WSSV Thai-1 and 168–192 hpi with WSSV Viet. WSSV infections were demonstrated by indirect immunofluorescence (IIF).

Shrimp kept continuously at 27 °C, started to show clinical signs at 24 hpi and mortalities started at 36 hpi with both strains. Cumulative mortalities reached 100% at 60 hpi with WSSV Thai-1 and at 168–192 hpi with WSSV Viet in two experiments. At constant 33 °C (24 h 33 °C), mortalities were 0% with WSSV Thai-1 and 5% or 10% with WSSV Viet. With exposure to 33 °C for 6 or 12 h per day (6 h 33 °C or 12 h 33 °C), cumulative mortalities reached 90–100% at 72–96 hpi with WSSV Thai-1. With WSSV Viet cumulative mortalities reached 100% at 84–96 hpi or 50–95% at 96–108 hpi with 33 °C for 6 h or 12 h per day. Mortalities were clearly reduced with WSSV Thai-1 (0–40%) and WSSV Viet (5–15%) at 33 °C for 18 h per day. At constant 27 °C and at 33 °C for 6 h or 12 h per day, dead shrimp were WSSV positive and euthanized shrimp were WSSV negative. At 33 °C for 18 h or 24 h per day, the dead and euthanized shrimp were WSSV negative except the dead shrimp at 18 h 33 °C with Thai-1 were WSSV positive.

The present study showed that daily temperature fluctuations have negative (6 h 33 °C with WSSV Viet) or positive effects (6 h 33 °C with WSSV Thai-1 and 12 h or 18 h 33 °C with both strains) on disease and mortality of shrimp infected with WSSV.

Keywords: WSSV; Temperature fluctuations; *Penaeus vannamei*

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1. Introduction

Water temperature is an important environmental factor for shrimp. It has a direct influence on metabolic rate (Allan et al., 2006), growth and survival of shrimp (Wyban et al., 1995). The optimum temperature for growth and survival of *Penaeus vannamei* juveniles of more than 5 g is 27 °C (Wyban et al., 1995). Water temperature in shrimp farms fluctuates diurnally and seasonally as it depends on air temperature, water depth, pond design and water management. Water temperature can easily reach 33 °C in shrimp ponds at least for several hours per day in many tropical countries such as Bangladesh (Wahab et al., 2003), China (Wang et al., 2005), Thailand (Thongrak et al., 1997), Vietnam (Alongi et al., 1999) and Mexico (Ruiz-Fernández and Páez-Osuna, 2004).

White spot syndrome virus (WSSV) is an enveloped, double stranded DNA virus (Van Hulten et al., 2001) of the family Nimaviridae, genus *Whispovirus* (Mayo, 2002). This pathogen causes disease and mortality in many shrimp producing countries. Cumulative mortality of WSSV infected shrimp may reach 100% within 3–10 days (Lightner, 1996). At optimum temperature (26 °C–27 °C), differences in virulence between WSSV strains have been reported (Wang et al., 1999, Rahman et al., 2006b). High water temperature (32–33 °C) reduces mortality in WSSV inoculated *P. vannamei* postlarvae and juveniles (Vidal et al., 2001; Rahman et al., 2006a). At 33 °C, an inhibition of WSSV replication (Rahman et al., 2006a) and a reduction of viral load (Granja et al., 2006) have been shown as possible explanations for this reduced mortality.

This present study evaluated the effect of daily fluctuations of optimum (27 °C) and high water temperature (33 °C) on the clinical and virological outcome of specific pathogen free (SPF) shrimp *P. vannamei* juveniles inoculated with either of two WSSV strains with difference in virulence (Rahman et al., 2006b).

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 and WSSV Viet was kindly provided by K. Söderhäll (Uppsala University, Sweden) and Research Institute for Aquaculture-2 (RIA-2), Vietnam, respectively. 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 infectivity titers of stocks were determined according to the procedure described by Escobedo-Bonilla et al. (2005a). The median infectious titres of stocks determined by intramuscular inoculation in SPF *P. vannamei* were $10^{5.9}$ and $10^{5.8}$ SID_{50} per ml for WSSV Thai-1 and WSSV Viet, respectively.

2.2. Shrimp

A total of 517 shrimp were used in the four experiments of the present study. Mean body weights (MBW) of shrimp were 4.9±1.3 g, 15.7±2.6 g and 6.5±1.7 g, 8.6±2.1 g in

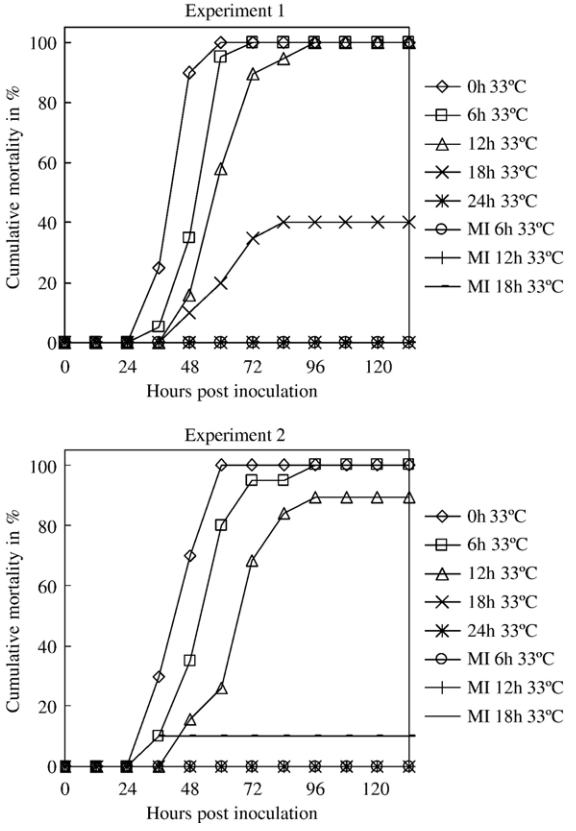

Fig. 1. Cumulative mortalities of SPF *Penaeus vannamei* juveniles intramuscularly inoculated either with a dose of 10000 SID_{50} of WSSV Thai-1 or mock inoculated (MI). Shrimp were either kept at constant 27 °C (0 h 33 °C) or from 12 hpi exposed to 33 °C for 6 h (6 h 33 °C), 12 h (12 h 33 °C), 18 h (18 h 33 °C) and 24 h (24 h 33 °C) per day. Afterwards, temperature was lowered and maintained at 27 °C for the rest of the day. These temperature regimes were repeated every 24 h up to 132 hpi.

the first and second experiment with WSSV Thai-1 and WSSV Viet, respectively.

2.3. Experimental conditions

SPF *P. vannamei* from Molakai farm, Hawaii (USA) were imported at early postlarval (PL) stage and reared in a recirculation system at the Laboratory of Aquaculture & *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 for WSSV challenge studies. Nine to eleven shrimp were housed per 50 litre aquarium, equipped with aeration, mechanical filtration (Eheim, Germany), water pump (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 maintained at 27 °C before inoculation and also during the first twelve hours post inoculation (hpi). From 12 hpi, temperature was maintained either at 27 °C or switched to 33 °C depending on temperature fluctuation regimes described below. Approximately 0.2 g of a commercial shrimp diet was provided for each shrimp per day. About 80% brackish water was renewed at 120 hpi in experiments with WSSV Viet. Water quality was checked by measuring ionized ammonia (NH_4^+) using test kits (Aquamerck, Germany).

2.4. WSSV inoculation procedure

Shrimp were inoculated intramuscularly with 50 µl of inoculum containing 10000 SID_{50} of either WSSV

Table 1
Median lethal times (LT_{50}) of shrimp inoculated with a high dose of either of WSSV Thai-1 or WSSV Viet exposed to 33 °C for different periods (0 h, 6 h, 12 h, 18 h and 24 h) per day

Ex. no.	WSSV	h/day at 33 °C	LT_{50}	α	β	γ	δ	LT_{50} [a] comparison
1	Thai-1	0 h	40.2[a]	6.802	−0.169			$a=b<c<d$
		6 h	49.6[b]	6.802	−0.169	0.166	0.029	
		12 h	59.4[c]	6.802	−0.169	−1.638	0.082	
		18 h	122.7[d]	6.802	−0.169	−4.705	0.152	
		24 h	–	6.802	−0.169	−2.48	0.169	
		NC 6 h	–	6.802	−0.169	−2.48	0.169	
		NC 12 h	–	6.802	−0.169	−2.48	0.169	
		NC 18 h	–	6.802	−0.169	−2.48	0.169	
2	Thai-1	0 h	42.0[e]	5.098	−0.121			$e=f<g<h$
		6 h	52.6[f]	5.098	−0.121	−1.123	0.046	
		12 h	73.2[g]	5.098	−0.121	−2.377	0.084	
		18 h	–	5.098	−0.121	−0.776	0.121	
		24 h	–	5.098	−0.121	−0.776	0.121	
		NC 6 h	–	5.098	−0.121	−0.776	0.121	
		NC 12 h	–	5.098	−0.121	−0.776	0.121	
		NC 18 h	308.2[h]	5.098	−0.121	−3.208	0.115	
1	Viet	0 h	95.3[i]	2.022	−0.021			$j<k<i<l=m$
		6 h	59.8[j]	2.022	−0.021	3.22	−0.066	
		12 h	69.5[k]	2.022	−0.021	0.047	−0.009	
		18 h	263.4[l]	2.022	−0.021	0.044	0.013	
		24 h	350.4[m]	2.022	−0.021	0.168	0.015	
		NC 6 h	–	2.022	−0.021	2.068	0.021	
		NC 12 h	–	2.022	−0.021	2.068	0.021	
		NC 18 h	–	2.022	−0.021	2.068	0.021	
2	Viet	0 h	125.4[n]	2.121	−0.017			$o<n<p<r=q$
		6 h	73.5[o]	2.121	−0.017	3.651	−0.062	
		12 h	148.3[p]	2.121	−0.017	−0.433	0.006	
		18 h	615.2[q]	2.121	−0.017	0.022	0.013	
		24 h	506.5[r]	2.121	−0.017	−0.346	0.013	
		NC 6 h	–	2.121	−0.017	1.969	0.017	
		NC 12 h	–	2.121	−0.017	1.969	0.017	
		NC 18 h	–	2.121	−0.017	1.969	0.017	

[a] Differences in LT_{50} are significant ($p<0.05$).

Thai-1 or WSSV Viet in the junction between the third and fourth abdominal segments. Mock inoculated (MI) shrimp were inoculated with 50 μl of phosphate buffered saline (PBS) only. Inoculated shrimp were observed for clinical signs including anorexia and lethargia and mortality was recorded every 12 h till the end of experiment.

2.5. Temperature fluctuations

Two temperatures, 27 °C and 33 °C, were used for daily temperature fluctuation regimes. WSSV inoculated and mock inoculated shrimp were kept at 27 °C till 12 h post inoculation (hpi). Starting from 12 hpi, different regimes of temperature fluctuations were used. WSSV inoculated shrimp were kept either continuously at 27 °C (0 h 33 °C) or temperature was increased to 33 °C at 12 hpi and maintained for 6 h (6 h 33 °C), 12 h (12 h 33 °C), 18 h (18 h 33 °C), 24 h (24 h 33 °C) per day. Nineteen to twenty one WSSV inoculated shrimp were used per temperature regime. After exposure to 33 °C (6 h, 12 h and 18 h), temperature was lowered and maintained at 27 °C for the rest of the day. This temperature regime was repeated every 24 h up to 132 hpi with WSSV Thai-1 and 168–192 hpi with WSSV Viet inoculated shrimp. Mock inoculated (MI) shrimp were also exposed to the temperature fluctuation regimes. Ten mock inoculated shrimp were used per temperature regime. At the end of the experiment surviving shrimp were euthanized.

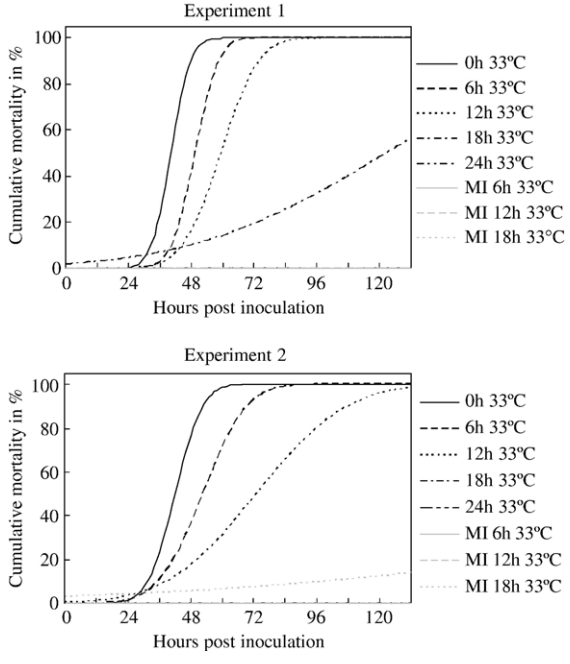

Fig. 2. Probability of mortality (probit) of shrimp inoculated either with a dose of 10000 SID_{50} of WSSV Thai-1 or mock inoculated (MI) and exposed to different regimes of temperature fluctuations.

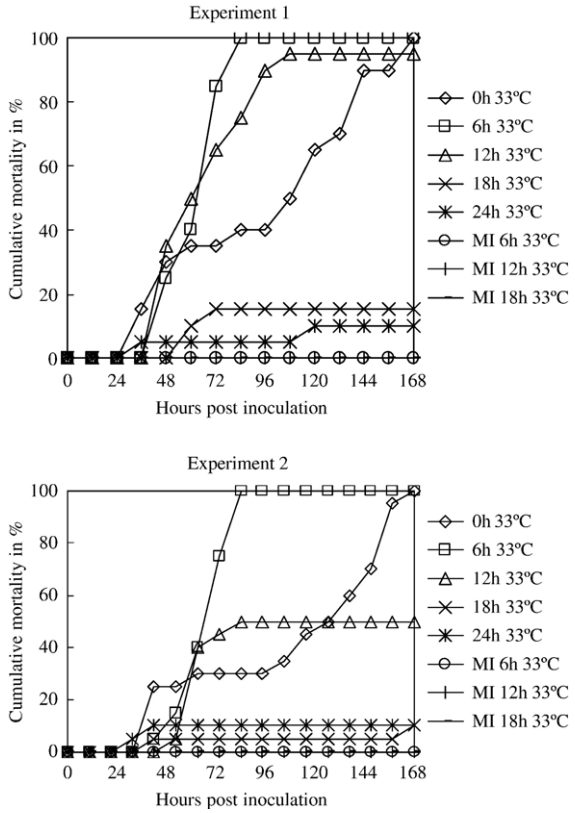

Fig. 3. Cumulative mortalities of SPF *Penaeus vannamei* juveniles intramuscularly inoculated either with a dose of 10000 SID_{50} of WSSV Viet or mock inoculated (MI). Shrimp were either kept at constant 27 °C (0 h 33 °C) or from 12 hpi exposed to 33 °C for 6 h (6 h 33 °C), 12 h (12 h 33 °C), 18 h (18 h 33 °C) and 24 h (24 h 33 °C) per day. Afterwards, temperature was lowered and maintained at 27 °C for the rest of the day. These temperature regimes were repeated every 24 h up to 168–192 hpi.

2.6. Evaluation of WSSV infection

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 directed against VP28 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 deionized water, dried and mounted with a solution of glycerin and 1, 4-diaza-

bicyclo[2,2,2]-octan (DABCO) (ACROS organics, USA). Slides were analyzed by fluorescence microscopy.

2.7. Statistical analysis

The cumulative mortality of all groups was submitted to probit analyses (Agresti, 1996). When significant interactions exist between temperature and time, the probit model has the form: Probit $(x)=\alpha+\beta$ time $+\gamma$ temperature $+$ δ time * temperature

Where:

- α is the intercept
- β is the rate of probability change per unit change of time (for a constant temperature)
- γ is the rate of probability difference for each temperature (for a constant time)
- δ is the change in rate of probability per unit change of time depending on the temperature

The parameters of this model were determined using the statistical software S-plus version 6.1 (Lucent technologies Inc., USA). Differences between treatment and control were determined by *t*-test using the same statistical software.

3. Results

3.1. WSSV Thai-1

Shrimp kept continuously at 27 °C (0 h 33 °C), showed clinical signs within 24 hpi. Mortalities started at 36 hpi (25–30%) and cumulative mortalities reached 100% at 60 hpi in the both experiments (Fig. 1). At constant 33 °C (24 h 33 °C), no clinical signs were observed and no mortality occurred. Shrimp exposed to 33 °C for 6 h (6 h 33 °C), 12 h (12 h 33 °C) or 18 h (18 h 33 °C) per day, started to show clinical signs at 24–36 hpi. Mortalities started at 36 hpi in 6 h 33 °C shrimp (5% or 10%), at 48 hpi in 12 h 33 °C shrimp (15.8%) and in 18 h 33 °C shrimp (10% or 0%) in the first or second experiment. Cumulative mortalities reached 100% at 72 hpi or at 96 hpi in 6 h 33 °C shrimp, 100% or 90% at 96 hpi in 12 h 33 °C shrimp and 40% at 84 hpi or 0% in 18 h 33 °C shrimp. Out of two experiments, only one mock inoculated shrimp died at 33 °C for 18 h per day (MI 18 h 33 °C). At continuously 27 °C, or at 33 °C for 6 h, 12 h and 18 h per day, dead WSSV inoculated shrimp were WSSV positive while euthanized WSSV inoculated and dead and euthanized mock inoculated shrimp were all WSSV negative. At constant 33 °C, dead and euthanized shrimp were WSSV negative. Significant differences ($p<0.05$) in the median lethal times (LT_{50}) were found between the groups of temperature fluctuation regimes (Table 1 and Fig. 2).

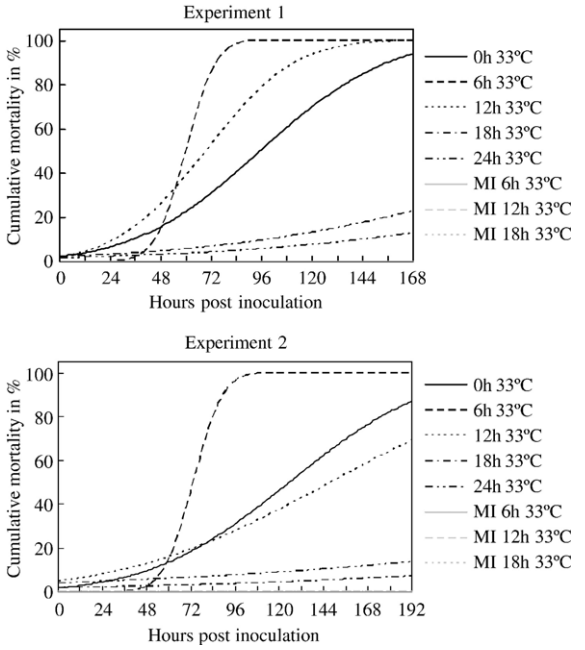

Fig. 4. Probability of mortality (probit) of shrimp inoculated either with a dose of 10,000 SID_{50} of WSSV Viet or mock inoculated (MI) and exposed to different regimes of temperature fluctuations.

3.2. WSSV Viet

Shrimp kept continuously at 27 °C (0 h 33 °C), started to show clinical signs at 24 hpi. Mortalities started at 36 hpi (15%) or at 48 hpi (25%) and cumulative mortalities reached 100% at 168 hpi or at 192 hpi, in the first or second experiment (Fig. 3). At constant 33 °C (24 h 33 °C), shrimp did not show clinical signs and cumulative mortalities were 5% or 10%. Shrimp exposed to 33 °C for 6 h (6 h 33 °C) and 12 h (12 h 33 °C) per day, started to show clinical signs at 24–36 hpi. Mortalities started at 48 hpi (25% or 5%) in 6 h 33 °C shrimp and at 48 hpi (35%) or at 60 hpi (5%) in 12 h 33 °C shrimp in two experiments. Cumulative mortalities reached 100% at 84 hpi or at 96 hpi in 6 h 33 °C shrimp, 95% at 108 hpi or 50% at 96 hpi in 12 h 33 °C shrimp. At 33 °C for 18 h per day (18 h 33 °C), shrimp did not show clinical signs and cumulative mortalities were 15% or 5%. None of the mock inoculated shrimp died in both experiments. At continuously 27 °C or at 33 °C for 6 h and 12 h per day, dead shrimp were WSSV positive and euthanized shrimp were WSSV negative. All of the euthanized WSSV inoculated shrimp kept continuously or at least 18 h per

day at 33 °C and mock inoculated shrimp were WSSV negative. Significant differences ($p<0.05$) in the median lethal times (LT_{50}) were found between the groups of temperature fluctuation regimes (Table 1 and Fig. 4).

4. Discussion

This study showed that daily fluctuations of two temperatures (27 °C and 33 °C) with durations of 6 h, 12 h or 18 h can influence the outcome of disease, mortality and infection status of shrimp inoculated with WSSV. The results obtained with 33 °C for 12 h and 18 h per day in delaying and reducing mortality could be useful to diminish disease and mortality of WSSV infected shrimp in the field. These findings might also help to understand some of the dynamics of disease outbreaks in shrimp farms due to WSSV. Under shrimp farming conditions, durations and range of temperature fluctuations might vary with geographical locations, season of the year and types of management in shrimp culture operations.

At constant 27 °C, the differences in onset of mortality (36 hpi and 36–48 hpi) and time to reach a cumulative mortality of 100% (60 hpi and 168–192 hpi) between the two WSSV strains agree with previous work on virulence difference (Rahman et al., 2006b). The reproducible results of the experiments showed the usefulness of the standardized inoculation procedure (Escobedo-Bonilla et al., 2005b) with a well defined amount of infectious virus.

At constant 33 °C (24 h 33 °C), absence of clinical signs and reduced mortalities (0–10%) of WSSV infected shrimp is in accordance with other studies (Vidal et al., 2001, Rahman et al., 2006a). The results showed similar effects of constant 33 °C with the two WSSV strains used.

The shorter periods (6 h, 12 h or 18 h) of 33 °C per day were effective in delaying the development of clinical signs and onset of mortality with both strains. However, cumulative mortalities reached always 100%, when shrimp infected with either strain were exposed to 33 °C for 6 h per day only. This suggests that the progression of infection/replication at 27 °C for 18 h per day was sufficient to cause disease and mortality. Surprisingly, cumulative mortalities of WSSV Viet inoculated shrimp kept at 6 h 33 °C reached 100% even earlier than shrimp kept continuously at 27 °C (84–96 hpi and 168–192 hpi). This indicates that combining temperature fluctuations with progressed infection (3–4 days) might cause a sharp rise in mortality. The underlying mechanisms will be investigated in future experiments. At 12 h 33 °C, delayed/reduced mortality with both strains could be due to the limited time (12 h per day) for replication (Rahman et al., 2006a). An exposure to 33 °C for 18 h per day significantly reduced mortality of shrimp inoculated with both strains (0–40% with WSSV Thai-1 and 5–15% with WSSV Viet). The reasons why these two temperature regimes (12 h and 18 h 33 °C) were more effective in bigger sized shrimp (second experiment) and with the less virulent strain are unknown. The findings with 33 °C for 18 h per day might be applied in shrimp farms with consideration of proper season, aeration, stocking density and temperature tolerance of the species. Maintaining shrimp at 33 °C for 18 h compared to constant 33 °C is cheaper and might be easier to maintain in many tropical shrimp farming countries. Furthermore, the disadvantages of high water temperature on shrimp such as increased requirement of dissolved oxygen (DO) (Zhang et al., 2006) and increased rate of ammonia-N excretion (Jiang et al., 2000) could be minimized by shortening the exposure to 33 °C by 6 h.

In summary, this study illustrates that daily fluctuations of optimum (27 °C) and high water temperature (33 °C) may have positive or negative effects on disease, mortality and infection status of WSSV inoculated shrimp depending on temperature regime and strain used.

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