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White spot syndrome virus strains of different virulence induce distinct immune response in *Cherax quadricarinatus*

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ABSTRACT

In this study, we identified three white spot syndrome virus (WSSV) strains (WSSV-CN01, WSSV-CN02 and WSSV-CN03) with significant differences in virulence. Among them, WSSV-CN01 caused significant higher and earlier mortality in redclaw crayfish *Cherax quadricarinatus*, thus was determined as high-virulent, while WSSV-CN02 and WSSV-CN03 were moderate-virulent and low-virulent. By investigating the total number of the circulating haemocytes and the activity of immune relative enzymes, we demonstrated that the different virulent WSSV strains induced distinct immune response in the host. Notably, a dramatic reduction of circulating haemocytes was observed in the crayfish infected with WSSV-CN01 and WSSV-CN02 but not WSSV-CN03. Further analysis revealed that cell death induced by WSSV-CN01 and WSSV-CN02 might be responsible for the decrease of circulating haemocytes.

1. Introduction

White spot syndrome virus (WSSV) is one of the devastating shrimp pathogens which can cause up to 100% mortality in commercial shrimp farms [1]. Previous studies have shown that WSSV has a broad host range among marine and freshwater crustaceans including shrimp, crayfish and crab [2–4], and virulence differences among WSSV strains have been observed [5–8]. However, previous studies regarding the relationship between WSSV genetic variations and virulence were limited and conflicting. Marks et al. reported that WSSV-TH, a WSSV strain with a smaller genome, was more virulent than the TH-96-II strain that contains a larger genome [5]. They suggested that smaller genome size might give WSSV a replication advantage. On the contrary, Lan et al. showed that a 4.8 kb deletion of the WSSV genome led to a dramatic decrease in virulence [7]. In addition, studies focus on the variable number tandem repeats associate with WSSV ORF94 suggested that WSSV isolates with fewer repeat units were more virulent [6].

The outcome of an infectious disease is not only determined by the pathogen, but also by the immune response of the host. Research of mammalian viruses shows that virus strains of different virulence may induce distinct response in the hosts and lead to diverse disease outcomes [9,10]. Crustaceans rely on the innate immune system to defense against invading pathogenic organisms, while haemolymph is where most immune responses take place [11,12]. The circulating haemocytes protect the animal by participating in recognition, phagocytosis, melanization and cytotoxicity [13]. Immune relative enzymes in the haemolymph like phenoloxidase (PO) and peroxidase (POD) are believed to function in pathogen clearance and are often used to evaluate the immune status of crustaceans [14,15]. WSSV infection has been found to induce enzymatic change in the haemolymph of the hosts [16,17], and stimulate apoptosis of circulating haemocytes [18–21]. However, the relationship between host immune response and WSSV virulence remains unexplored.

In this study, we identified three WSSV strains differs in virulence and compared the immune responses they induced in *Cherax quadricarinatus*. The findings will extend our knowledge of WSSV virulence and pathogenesis of white spot syndrome.

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2. Materials and methods

2.1. Animals

Healthy redclaw crayfish *C. quadricarinatus* were purchased from Xiamen Xinrongteng Aquatic Technology Development Company and *Procambius clarkii* were purchased from a local market of Xiamen. They were determined to be WSSV free before experiments, using WSSV-PCR detection kit (Xiamen Lulong Biotech Company, China).

2.2. WSSV stocks and purification of WSSV virions

The virus strain WSSV-CN01 was collected from infected *Marsupenaeus japonicus* from Xiamen, China in 1994. WSSV-CN02 was collected from crayfish *P. clarkii* from Xiamen, China in 2010. WSSV-CN03 was collected from infected *Litopenaeus vannamei* from Zhangpu, China in 2010. The infected animals were stored at −80 °C until experiment. To amplify the virus, 0.1 g tissue from each infected animal was homogenized in 10 ml normal saline (0.9% NaCl) and then centrifuged at 5000× *g* for 5 min at 4 °C. The supernatant was filtrated through a 0.2-μm membrane filter, and then used to inoculate healthy crayfish *P. clarkii* at a dose of 100 μl/each. WSSV virions were purified from moribund *P. clarkii* and their concentration was determined as described before [22,23].

2.3. Analysis of crayfish mortality rate

C. quadricarinatus were divided into four groups (21 individuals in each group) and intramuscularly injected with WSSV-CN01, -CN02, -CN03 at a dose of 1×10^4 virions per individual, or normal saline as a negative control. Crayfish mortality was recorded twice a day.

2.4. Virus quantification

Four groups of *C. quadricarinatus* were injected with WSSV-CN01, -CN02, -CN03 at a dose of 1×10^4 virions per individual, or normal saline as a negative control. Five individuals from each challenged group and five individuals from the control group were randomly selected for sampling at certain time interval until all animals in the challenge groups were dead. For each sampling, 10 μl of haemolymph was withdrawn from the base of the fifth walking leg of each crayfish and analyzed separately. The viral load in the samples was measured using quantitative real-time PCR (qPCR) according to the instruction of WSSV-qPCR detection kit (Xiamen Lulong Biotech Co., Ltd., Xiamen, Fujian, China).

2.5. Analysis of C. quadricarinatus immune response

2.5.1. Sample preparation

C. quadricarinatus were divided into four groups (three individuals in each group) and challenge was performed at a dose of 1×10^5 virions per individual as described in Section 2.3. At 24 h and 72 h post-infection (p.i.), 0.5 ml of haemolymph was withdrawn from each crayfish using a 1 ml syringe preloaded with 0.5 ml of anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM citric acid, 30 mM trisodium citrate, 10 mM EDTA, pH5.8). Fifty microliters of the mixture were used directly for haemocyte count. Five hundred microliters of the mixture were centrifuge at 300× *g* for 5 min, then the pellet was used for PO activity analysis and the supernatant (plasma) was used to determine the haemocyanin concentration and POD activity.

2.5.2. Total haemocyte count

The haemolymph samples were immediately fixed with the same volume of 20% formalin in 0.15 M NaCl [24] and the haemocytes were counted using cell counting board. The total haemocyte count (THC) in the haemolymph was presented as: number of cells/ml of haemolymph.

2.5.3. Haemocyanin level

The haemocyanin concentration in the haemolymph was determined by the absorbance at 335 nm using a SpectraMax microplate reader (Molecular Devices, USA) as described previously [25]. Twenty microliters of the plasma were diluted with 180 μl ultrapure water and loaded into 96-well plates. The concentration of haemocyanin was calculated as the following: haemocyanin concentration (mM) = 17.26 × OD value × 10 × 2.

2.5.4. Determination of PO activities

The total PO activity in the haemocytes was analyzed in our experiments. All the intracellular prophenoloxidase (proPO) were completely catalyzed to form PO before measurement of the enzyme activity. Haemocyte lysate supernatant (HLS) for PO activity analysis was prepared as previously described [26]. Haemocytes collected from 0.25 ml of haemolymph were washed with 1 ml PBS and resuspended in 0.3 ml PBS. The cell suspension was homogenized with a homogenizer (IKA T10 basic) at 5000 rpm for 15 s on ice and then centrifuged at 43,000× *g* for 20 min at 4 °C to remove cell debris. Determination of PO activity was performed according to Perazzolo and Barracco [27]. Briefly, the resulting HLS (50 μl) was pre-incubated with an equal volume of trypsin (1 mg/ml) at 20 °C for 10 min to activated proPO, and then 50 μl L-DOPA was added to the mixture. The reaction was carried on at 37 °C for 30 min and the yield of dopachrome was measured by the absorbance at 490 nm.

2.5.5. Determination of POD activities

The activity of POD in the plasma was determined with the peroxidase detection kit (Jiancheng, Ltd, Nanjing, China). The plasma was diluted with ultrapure water (1:4) and 5 μl diluted plasma was added to the reaction mixture (from detection kit). The optical density at 470 nm was measured using the SpectraMax microplate reader.

2.6. Cell death analysis

C. quadricarinatus were grouped and infected following the methods described in Section 2.5.1. Haemocytes collected from 0.2 ml of haemolymph at 12 h p.i. and 24 h p.i. were resuspended in Leibovitz's L-15 medium (Gibco) and seeded into a glass bottom cell culture dish. After incubation at 27 °C for 30 min to allow cell attachment, the death of haemocytes was analyzed with Annexin-V-FLOUS staining kit (Roche). In brief, the haemocyte monolayer was incubated in Annexin-V-FLOUS and PI labeling solution for 10 min at room temperature and observed by confocal microscopy immediately.

2.7. Statistical analysis

The SPSS 16.0 software was used for statistical analysis. The data were presented as means ± SD. The comparison of multiple groups was performed with one-way ANOVA test, using Dunnett's T3 method for haemocyanin concentration and cell death, or the LSD method for the rests.

3. Results

3.1. Significant virulence differences between WSSV strains

The relative virulence of three WSSV strains (WSSV-CN01, -CN02 and -CN03) isolated from infected animals in 1994 and 2010 were compared through time-mortality assays in redclaw crayfish *C. quadricarinatus*. As shown in Fig. 1, crayfish mortality occurred as early as 3 days p.i. (d p.i.) of WSSV-CN01, which was earlier than those infected with WSSV-CN02 (5 d p.i.) and WSSV-CN03 (6 d p.i.). All three WSSV strains caused 100% mortality in crayfish finally, but the 100% mortality occurred at an earlier time for WSSV-CN01 (5 d p.i.), than for WSSV-CN02 (12 d p.i.) and WSSV-CN03 (15 d p.i.). The median lethal times (LT50s) of crayfish infected with WSSV-CN01, -CN02 and -CN03 were 3.9 days, 6.5 days and 8.6 days respectively. Therefore, we assume that WSSV-CN01, -CN02 and -CN03 are high-virulent, moderate-virulent and low-virulent, respectively.

To compare the replication kinetics of the three WSSV strains, viral copies in the haemolymph of the infected and un-infected crayfish were measured using real-time PCR at certain time interval. Five crayfish per group were sampled at each time point until all the challenged animals were dead. The data show that the replication efficiency of WSSV-CN01 was higher than WSSV-CN02 and WSSV-CN03 in the first 2 days. The WSSV-CN01 DNA copies in infected crayfish reached the peak level (4.3×10^6 virion/μl haemolymph) at 3 d p.i., and did not increase anymore. The viral load in WSSV-CN03 infected crayfish became similar to WSSV-CN01 infected ones at 3 d p.i., and continuously increased to 9.1×10^7 virion/μl haemolymph at 11 d p.i., which was ~21-fold of WSSV-CN01 peak level. Among the three strains, WSSV-CN02 replicated at the lowest speed, and the viral copies steadily increased to 4.4×10^6 virion/μl haemolymph at 9 d p.i.. Therefore, WSSV-CN01 did have a replication efficiency over -CN02 and -CN03 in the early stage of infection, but it could caused a much rapid death of infected animals with a relative low viral load when compared to WSSV-CN03.

3.2. Host immune response against WSSV strains

To explore how the immune system of crayfish responded to different strains of WSSV, haemolymph was collected from challenged crayfish at 24 h p.i. and 72 h p.i.. The THC, haemocyanin concentration, PO activity in the haemocytes, and POD activity in the plasma were further analyzed. As shown in Fig. 3, there were no significant differences in the THC between three infected groups and the control group at 24 h p.i.. However, at 72 h p.i., a dramatic decline of the haemocyte number was observed in WSSV-CN01 ($P < 0.01$) and -CN02 ($P < 0.001$) infected groups compared with the control, while the THC of WSSV-CN03 challenged crayfish was similar to the control. Besides, when we compared the data from 24 h p.i. and 72 h p.i., THC decrease was observed in both the WSSV-infected groups and the negative control, which suggested that the haemolymph withdraw at 24 h p.i. might lead to some physical damages to the animals.

Analysis of haemocyanin concentration in the haemolymph of infected crayfish showed that there was no great difference in the haemocyanin titers between WSSV challenged groups and the un-infected control at 24 h p.i. and 72 h p.i. (Fig. 4). However, it is notable that the haemocyanin concentration in WSSV-CN01 infected crayfish was slightly higher than those of WSSV-CN02 and -CN03 infected crayfish at 24 h p.i. ($P < 0.05$) and 72 h p.i. ($P = 0.001$).

In addition, two immune-related enzymes, PO and POD were also used as markers to evaluate the immune response associated with WSSV infection. Here we measured the PO activity in the haemocytes collected from 42 μl of haemolymph. As shown in Fig. 5, the PO activity in the haemocytes remained almost unchanged at 24 h p.i. with all three WSSV strains, while the PO activity increased with the progress of WSSV-CN03 infection to a significant higher level at 72 h p.i. ($P < 0.001$), as compared to WSSV-CN01 and -CN02 challenged groups and the negative control. On the other hand, the POD activity in the plasma remained at the similar level as the control after challenged with WSSV-CN01, -CN02 or -CN03 at 24 h p.i. (Fig. 6). However, infection of WSSV-CN01 led to a significantly rise of POD activity at 72 h p.i. ($P \leq 0.001$) compared with the other groups.

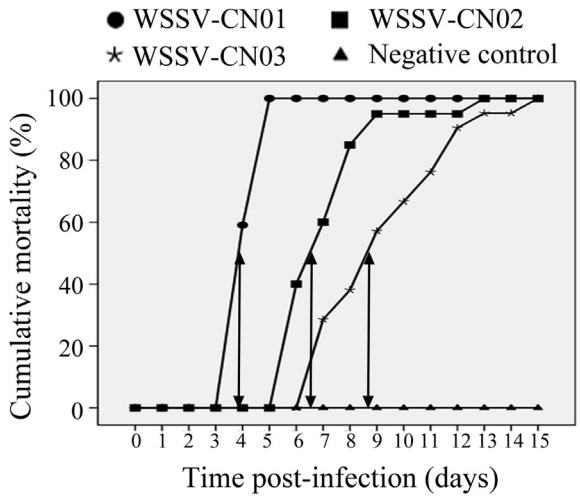


Fig. 1. Cumulative mortality of *C. quadricarinatus* challenged with WSSV strains. Cumulative mortality rates of crayfish infected with different WSSV strains are plotted against d p.i.. The vertical arrows indicate the LT50s associated with three WSSV strains.

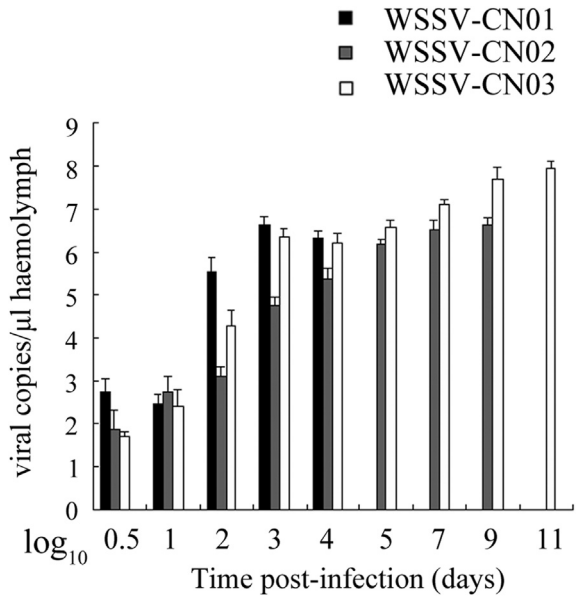
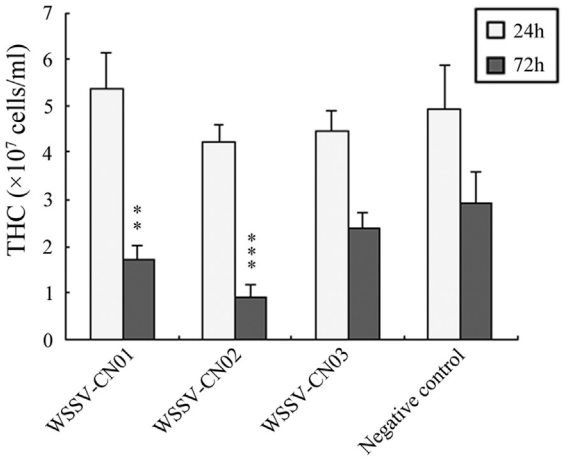


Fig. 2. Replication kinetics of WSSV strains in *C. quadricarinatus*. Haemolymph of five crayfish were collected from WSSV-CN01, -CN02 and -CN03 infected groups and the control group injected with normal saline at different time points. Viral load in the haemolymph of each crayfish was measured using real-time PCR and converted to log base 10. The standard deviations were calculated from five repeats. The data of the negative control group were zero, thus they cannot be seen in the graft.



Groups		24h ×10⁷cells/ml	72h ×10⁷cells/ml
WSSV-CN01	Mean value	5.353	1.710
	± SD	0.782	0.325
WSSV-CN02	Mean value	4.247	0.913
	± SD	0.341	0.253
WSSV-CN03	Mean value	4.453	2.380
	± SD	0.456	0.362
Negative control	Mean value	4.933	2.907
	± SD	0.952	0.677

Fig. 3. Total haemocyte count (THC) in *C. quadricarinatus* infected with WSSV strains. Haemolymph of crayfish infected with WSSV isolates differ in virulence were collected at 24 h p.i. and 72 h p.i.. The haemolymph was fixed with formalin and the haemocytes were counted. Asterisks represent statistical significant ($n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$) compared with the control group using one-way ANOVA test, LSD method.

3.3. A rapid haemocyte death induced by high- and moderate-virulent WSSV strains

WSSV-CN01 and -CN02 infection resulted in significant reduction of haemocytes in haemolymph, suggesting that the two strains might induce apoptosis or necrosis of haemocytes. To explore the possibility, we analyzed the effects of the three WSSV strains on crayfish haemocytes apoptosis by examining phosphatidylserine exposure and membrane permeability. Haemocytes were taken from different challenged groups as well as the negative control at 12 h p.i., and 24 h p.i. and examined by Annexin V/PI double staining. The fluorescent images of the haemocytes stained with Annexin V/PI at 24 h p.i. were shown in Supplementary Fig. 1. For statistical analysis, the Annexin V single stained cells were consider as early apoptotic cells, while the Annexin V/PI double stained cells or PI single stained cells were considered as late apoptotic/necrotic cells. As shown in Fig. 7, early apoptosis cells was undetectable in the WSSV challenged groups or the negative control at 12 h p.i., while basal level of late apoptosis/necrosis was observed. At 24 h p.i. the percentage of late apoptotic/necrotic haemocytes in WSSV-CN01 and -CN02 challenged crayfish rapidly raised to 19.7% and 36.0% respectively, while no late apoptotic/necrotic haemocytes were detected in WSSV-CN03 challenged group or the negative control. At this moment, early apoptotic haemocytes could be found in all the groups in our experiment, but at a relatively low level (<5%).

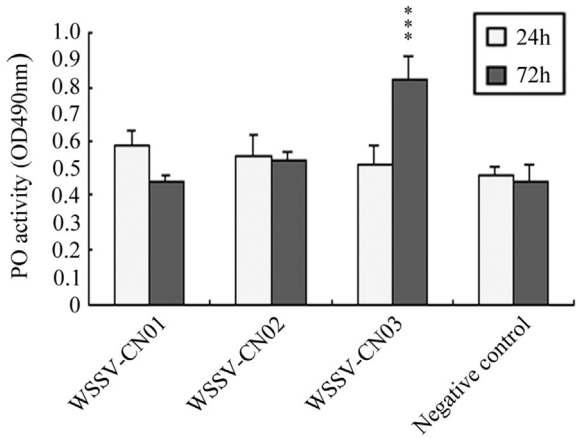


Fig. 5. PO activity in the haemocytes of *C. quadricarinatus* infected with WSSV strains. PO activity in the haemocytes collected from 42 μl haemolymph was measured spectrophotometrically by recording the formation of dopachrome produced from L-DOPA. The data display in the figure are the absorbance at 490 nm. Asterisks represent statistical significant ($n = 3$, $^{***}P < 0.001$) compared with the control group using one-way ANOVA test, LSD method.

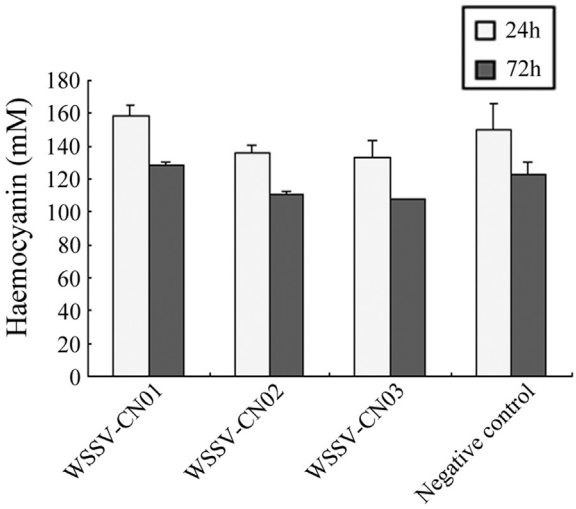


Fig. 4. Haemocyanin concentration in *C. quadricarinatus* infected with WSSV strains. Haemolymph was collected from crayfish infected with different WSSV strains at 24 h p.i. and 72 h p.i. and centrifuged at 300× *g* for 5 min. The supernatant was used for measurement of haemocyanin concentration $n = 3$.

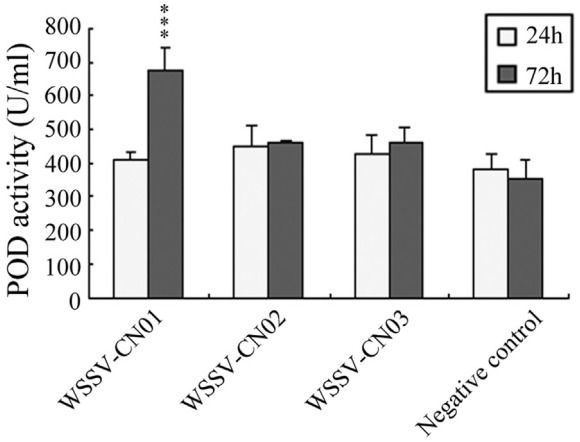
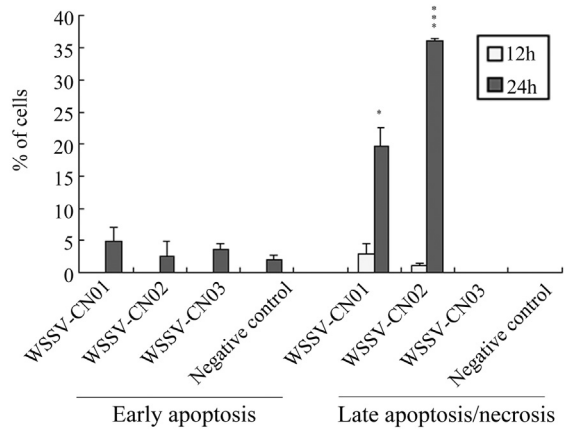


Fig. 6. POD activity in haemolymph plasma of *C. quadricarinatus* infected with WSSV strains. POD activity in the plasma of crayfish was analyzed at 24 h p.i. and 72 h p.i.. Asterisks represent statistical significant ($n = 3$, $^{***}P \leq 0.001$) compared with the control group using one-way ANOVA test, LSD method.



Groups		Early apoptosis of haemocyte (%)		Late apoptosis/necrosis of haemocyte (%)	
		12h	24h	12h	24h
WSSV-CN01	Mean value	0	4.7	3.4	19.7
	± SD	0	2.3	1.5	2.9
WSSV-CN02	Mean value	0	2.5	1.5	36.0
	± SD	0	2.2	0.05	0.4
WSSV-CN03	Mean value	0	3.6	0	0
	± SD	0	0.8	0	0
Negative control	Mean value	0	2.4	0	0
	± SD	0	0.8	0	0

Fig. 7. Cell death induced by WSSV strains. Haemocytes were collected and seeded in glass bottom cell culture dish. The haemocyte monolayer was stained with Annexin V-FLUOS staining kit (Roche). The Annexin V single stained cells were considered as early apoptosis, and the Annexin V/PI double stained cells or PI single stained cells were considered as late apoptosis/necrosis. The experiments were repeated three times and ~300 cells were counted each time. The standard deviations (SD) were indicated. Asterisks represent statistical significant ($n = 3$, $^{*}P < 0.05$, $^{***}P < 0.001$) compared with the control group using one-way ANOVA test, Dunnett's T3 method.

4. Discussions

Although WSSV was discovered more than 20 years ago, the pathogenesis of the disease it caused remains to be explored. The outcome of infectious diseases is determined by both the virulence of the pathogen and the host immune defense against the invaders [28]. Difference in virulence has been observed in WSSV since the 1990s, but no efforts have been made to understand the relationship between virus virulence and host anti-viral defense. In this study, we identified three WSSV strains differed in virulence, the high-virulent strain WSSV-CN01, moderate-virulent strain WSSV-CN02, and low-virulent strain WSSV-CN03. Compared with WSSV-CN03, WSSV-CN01 caused a rapider and earlier mortality in crayfish when the viral load was relatively low (Figs. 1 and 2). Therefore, the higher virulence in WSSV-CN01 may not due to a higher viral load in the host. Instead, the high-virulent strain may encode virulence-associate proteins that modulate host physical or immune status and thus lead to more severe symptoms.

To extend our observation, we analyzed the immune responses induced by the three WSSV strains through investigation of four immune markers in the haemolymph including: THC, haemocyanin concentration, and the activity of immune-related enzymes PO and POD. In crustacean, haemolymph is where many immune responses take place [11,12], and haemocytes are key players that protect the animal from invading microorganisms [13,29]. WSSV infection has been found to induce a reduction of circulating haemocytes in the hosts [16,19,30,31]. In the current study, we found that WSSV-CN01, -CN02 and -CN03 differed in their ability to affect the host circulating haemocytes. WSSV-CN01 and -CN02 induced a significant reduction in the total number of circulating haemocytes at 72 h p.i., while WSSV-CN03 infection had little effect on the number of circulating haemocytes (Fig. 3). Further investigation revealed that infection of WSSV-CN01 and -CN02 but not -CN03 led to an obvious increase of haemocyte mortality in *C. quadricarinatus* (Fig. 7). Notably, the haemocyte mortality rate was higher in WSSV-CN02 infected crayfish than in WSSV-CN01 infected crayfish, which was coincident with what we observed in the THC. Thus the decrease of haemocyte number in the haemolymph may due to the increase of cell death induced by WSSV infection. Together with the fact that WSSV-CN01 and -CN02 caused a much earlier and rapider death in *C. quadricarinatus* than WSSV-CN03, we propose that depletion of circulating haemocytes is an important cause of the death of infected animals. However, when we compared the crayfish infected with WSSV-CN01 and -CN02, we noticed that although WSSV-CN02 induced a more dramatic reduction of THC than WSSV-CN01, it did cause a less severe mortality. Therefore depletion of circulating haemocytes may not be the only cause of the death of the host.

A number of previous studies reported that WSSV infection could significantly induced haemocyte apoptosis in shrimp [18,19,21]. However, only low level (less than 2%) of apoptotic haemocyte could be detected in crayfish before or after WSSV challenge [32], suggesting that crayfish may response to WSSV infection in a way different from penaeid shrimp. To explored whether crayfish haemocyte undergo apoptosis in our experiment, we employed the Annexin V/PI double staining assay. As shown in Fig. 7, early apoptotic cells (Annexin V single stained) could hardly be observed at 12 h p.i.. A slight increase of the percentage of early apoptotic cells was observed in all the animals at 24 h p.i., but there was no significant difference between the challenged groups and the negative control. On the contrary, there was a sharp increase of the late apoptotic/necrotic cells (Annexin V/PI double stained or PI single stained) at 24 h post-infection of WSSV-CN01 and -CN02. Since the Annexin V/PI assay can not discriminate late apoptotic from necrotic cells [33], more evidences are required to make sure whether haemocytes undergo apoptosis or necrosis during WSSV infection.

In addition, to understand how the immune relative enzymatic system was affected by different WSSV strains, PO and POD activities in the haemolymph were also examined in our experiment. PO is the terminal component of proPO activating cascade. Crustacean proPO activating system is stored in haemocytes and can be activated during pathogen infection. Active PO is released to the plasma and kills invading pathogen by producing toxic product or promoting immune reactions like phagocytosis and coagulation [34]. Here we measured the storage of proPO system inside haemocytes during WSSV infection. Therefore, the intracellular proPO was completely activated before measurement of enzyme activity. The PO activity inside haemocytes from equal volume of haemolymph was especially high in WSSV-CN03 infected crayfish (Fig. 5) when compared with the other groups, implying a more abundant storage of proPO system. Since the THCs were greatly reduced during WSSV-CN01 and -CN02 infection, the lower proPO storage in the animals may due to haemocyte depletion. Although it is still unclear whether the proPO system can function in viral clearance, more abundant proPO storage may represent a more active immune status to fight against invaders.

Moreover, WSSV infection has been reported to induce oxidative stress in the host by releasing reactive oxygen species (ROS) [35,36]. ROS is a two-edge sword that can not only kill the pathogens but also damage the host [37], and PODs are a group of antioxidant enzymes that can detoxify ROS by reducing hydrogen peroxide [38]. The activity of POD usually reduces upon WSSV infection, which is supposed to be caused by increasing oxidative stress [35,36,39].

However, in our experiment the POD activity in the haemolymph plasma was greatly induced by the high-virulent strain WSSV-CN01, but not the moderate- or low-virulent strains at 72 h p.i. Base on the current data, it is hard to explain why the infection of high-virulent WSSV-CN01 can stimulate the activity of the anti-oxidant enzyme POD. Further work is required to understand the role of POD in the pathogenesis of WSSV infection.

Haemocyanin is the most abundant protein in the haemolymph of crustaceans. It has been reported to exhibit PO activity under certain conditions [40–42], and may serve as the precursors of some antimicrobial peptides [43,44]. Besides, haemocyanin has been shown to have anti-viral activity against WSSV [45], and its concentration is especially high in haemolymph of anti-viral shrimp [46]. Considering the possible role of this protein in anti-viral immunity, we examined the concentration of haemocyanin to see how it was affected by the infection of WSSV strains. However, no obvious alternation of haemocyanin concentration was detected in crayfish infected with WSSV-CN01, -CN02 or -CN03.

In conclusion, in this study, we investigated the relationship between WSSV virulence and the immune responses of the host. Our findings demonstrate that WSSV strains differ in virulence can affect the immune system of crayfish in dissimilar ways, and suggest that certain virulence-associate proteins may be responsible for these differences. Genome-wide comparison of the WSSV strains in the future may help to identify the virulence-associate genes and understand the pathogenesis of white spot syndrome.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2014.04.011.

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