Vol. 126: 125–134, 2017
https://doi.org/10.3354/dao03165

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 18

Experimental inoculation of oriental river prawn *Macrobrachium nipponense* with white spot syndrome virus (WSSV)

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ABSTRACT: The oriental river prawn *Macrobrachium nipponense* is an economically important species that is widely farmed in China. White spot syndrome virus (WSSV) is one of the most devastating pathogens of the cultured shrimp *Litopenaeus vannamei*, responsible for massive loss of its commercial products worldwide. We investigated the infectivity and pathogenicity of WSSV in adult *M. nipponense* using standardized conditions for *L. vannamei*. The median lethal dose of WSSV in adult *M. nipponense* was $10^{3.84\pm0.06}$ copies g^{-1}, which was about 1000-fold higher than in *L. vannamei* ($10^{0.59\pm0.22}$ copies g^{-1}). WSSV was detected by 2-step PCR in the gills, hepatopancreas, muscle, stomach, heart, gut, nerve, integument, pereopod, eyestalk, testis, and ovary of experimentally infected dead *M. nipponense*. Lesions were observed histologically following WSSV injection, showing basophilic intranuclear inclusion bodies in the hepatopancreas and subsequently in the gills. The clearance of WSSV was observed in hepatopancreas and gills at 48 and 96 h post-inoculation, respectively. No histological lesions were detected in muscle from 0–96 h post-injection. The results show that the oriental river prawn *M. nipponense* can be infected by WSSV and the infections are self limiting over time; therefore, *M. nipponense* may serve as a useful model for studying resistance to WSSV.

KEY WORDS: *Nimaviridae* · *Whispovirus* · Crustacean · LD_{50} · Histopathology

INTRODUCTION

White spot syndrome virus (WSSV) is a virus in the genus *Whispovirus*, family *Nimaviridae* (Mayo 2002). WSSV infects a wide spectrum of crustaceans and is highly pathogenic to the farmed shrimp *Litopenaeus vannamei*, where it is responsible for major economic losses (Walker & Mohan 2009, Corteel et al. 2012, Shi et al. 2012, Yuan et al. 2016). WSSV was first recorded in Taiwan in 1992 and subsequently observed worldwide (Chou et al. 1995, Pradeep et al. 2012). Losses due to WSSV are reported every year, and have been estimated at more US$8 billion since 2000 (Bondad-Reantaso et al. 2001, Rosenberry 2001, APHIS-USDA 2005, Dieu et al. 2004, Marks 2005, OIE 2011, Tendencia & Verreth 2011, Tang et al. 2012, World Bank 2013). WSSV outbreaks occur continuously in China and exist widely in the wild (Jang et al. 2009, Ding et al. 2015, Li et al. 2016). The oriental river prawn *Macrobrachium nipponense* can be infected by WSSV via oral administration and intramuscular injection (Yun et al. 2014). In freshwater ponds, *M. nipponense* usually cohabitates with the Chinese mitten crab *Eriocheir sinensis*, and serious outbreaks of WSSV have caused catastrophic losses in harvests of *E. sinensis* (Ding et al. 2015). Red swamp crayfish *Procambarus clarkii* is usually used as a model organism for WSSV infection for research

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on the invertebrate innate immune system (Du et al. 2016). The Chinese mitten crab *E. sinensis* and the red swamp crayfish *P. clarkii* are both important WSSV carriers, and there is expanding literature on infection research for these species (Du et al. 2008, Zeng & Lu 2009, Du et al. 2010, Bateman et al. 2012, Ding et al. 2017a,b). In the wild, *E. sinensis* and *P. clarkii* were more likely to carry WSSV than *M. nipponense*, and the natural prevalence level of *M. nipponense* was about 8.3% (Yin et al. 2017). The virus is transmitted horizontally to *E. sinensis* and *P. clarkia* (Yan et al. 2007) and the infection dynamics in these hosts is well known (Zeng & Lu 2009, Zeng et al. 2011, Huang et al. 2015, Ding et al. 2017b). WSSV infectivity studies have been conducted on *M. nipponense* (Yun et al. 2014, Yin et al. 2017) but little is known about the infection dynamics in this host.

M. nipponense is an important economic species that is farmed widely in China (Ma et al. 2011, Fu et al. 2012, Yuan et al. 2015), with annual yields exceeding 265 061 metric tonnes (Bureau of Fishery, Ministry of Agriculture 2016). Compared with penaeid shrimps, *M. nipponense* is generally considered to be less prone to disease in culture. Although there has been no outbreak caused by WSSV in farmed *M. nipponense* to date, it may serve as a reservoir for WSS and thus poses a potential threat to cultured *L. vannamei*.

WSSV can be diagnosed by PCR and histopathology (Sahul Hameed et al. 2003, Mijangos-Alquisires et al. 2006). Histological examination of WSSV-challenged *M. nipponense* may show visual changes in the target tissues. The gills play a vital role in transporting respiratory gases and controlling the osmotic and ionic balances in aquatic organisms, and WSSV causes histopathologically detectable changes in the gills of *L. vannamei*, *Macrobrachium rosenbergii*, *Panulirus homarus*, *Penaeus indicus*, *Penaeus monodon*, *Panulirus orantus*, and *Scylla serrata* (Rajendran et al. 1999, Yoganandhan et al. 2003, Syed Musthaq et al. 2006, Naresh et al. 2017). In addition, hepatopancreas is the main immune organ in shrimp (Jiang et al. 2014) and the center for storage, metabolism, and detoxification (Bhavan & Geraldine 2009). Many viruses cause histopathologically detectable changes in the epithelium of hepatopancreas of the cultured crustaceans, including *L. vannamei*, *Fenneropenaeus chinensis*, *Marsupenaeus japonicus*, *P. monodon*, and *Macrobrachium rosenbergii* (Bateman & Stentiford 2017, Zhang et al. 2017). However, information on histological changes following infection of WSSV in *M. nipponense* is lacking.

Before studying histological changes following infection of WSSV in *M. nipponense*, the pathogenicity of WSSV to *M. nipponense* should be studied first. One means to examine pathogenicity is to establish the median lethal dose (LD_{50}) of a pathogen or contaminant. The LD_{50} has been adopted extensively to evaluate the toxicity of WSSV in various marine and freshwater crustaceans (Escobedo-Bonilla et al. 2005, Du et al. 2006, Liu et al. 2011, Corteel et al. 2012, Zhu & Quan 2012, Pace et al. 2016). We determined the LD_{50} of WSSV and examined infection levels in tissues of experimentally infected *M. nipponense* by 2-step PCR. We examined the interactions between *M. nipponense* and WSSV by isolating and quantifying viable WSSV copies from infected *L. vannamei*. We also examined the histopathological changes in different tissues at different times following infection. The results of the present study will expound the pathogensis of WSSV in *M. nipponense* and facilitate the further prevention and control of WSSV in crustaceans.

MATERIALS AND METHODS

Maintenance of experimental animals

Litopenaeus vannamei free of WSSV were taken from culture ponds in Wuxi, China. *Macrobrachium nipponense* were purchased from the Lake Tai region of China. *L. vannamei* (weight 5.65±1.93 g) and *M. nipponense* (weight 4.76±1.54 g) were maintained in a recirculating-water aquarium system filled with aerated freshwater (25±1°C) and fed with paludina (freshwater snails with an operculum) twice a day. Animals were acclimated for 7 d prior to experimental treatments.

Viral inoculum preparation and quantification

WSSV-infected *L. vannamei* were obtained from the School of Life Science, Sun Yat-sen University, in 2015. These animals had been diagnosed as positive by 2-step PCR using primers from the World Organisation for Animal Health (OIE 2012). The infected samples were stored at −80°C. DNA was extracted from samples using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit from Takara Biotechnology. The primers used for 2-step PCR are shown in Table 1. A 1 µl aliquot of DNA template solution was add to a PCR tube containing 100 µl of reaction mixture (10 mM Tris–HCl, pH 8.8, 50 mM KCl,

Table 1. Primer sequences used for WSSV 2-step PCR and quantitative real-time PCR

Name	Sequence
146F1	5′-ACT ACT AAC TTC AGC CTA TCTAG-3′
146R1	5′-TAA TGC GGG TGT AAT GTT CTT ACG A-3′
146F2	5′-GTA ACT GCCCCT TCC ATC TCC A-3′
146R2	5′-TAC GGC AGC TGC TGC ACC TTG T-3′
Wq F	5′-CTC TTG TGG TTC ATC AGG GGC-3′
Wq R	5′-CTG GAT TTT CTC TCA GGG TCT TTA-3′

1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTP, 100 pmol of each primer, 2 U of heat-stable DNA polymerase). PCR was carried out as follows: one cycle of 94°C for 4 min, 55°C for 1 min, and 72°C for 2 min, followed by 39 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final 5 min extension at 72°C. The WSSV-specific amplicon from this reaction was 1447 base pairs. To visualize the results of PCR, 10 µl PCR products were electrophoresed on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}.

Tissue samples from infected *L. vannamei*, except for the hepatopancreas, can be used to prepare a WSSV inoculum (Rajendran et al. 2005). We prepared purified WSSV by homogenizing gills from infected shrimp in phosphate-buffered saline, followed by centrifugation at 8000 × *g* for 30 min at 4°C, and then filtering the supernatant fluid through a 0.4 µm filter using a vacuum filter apparatus (Sahul Hameed at al. 2000). The filtrate was stored at –20°C for subsequent studies.

We determined the WSSV copy number in triplicate using quantitative real-time PCR (qPCR) with the primers Wq-F and Wq-R (Table 1). A 1.5 µl DNA sample was added to 15 µl reaction mixture containing 1 µl each primer, and qPCR was performed as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 10 s and 60°C for 10 s. Melting curve analysis was performed at the end of the qPCR reaction at 65–95°C (in 0.5°C increments) for 10 s. The WSSV-specific amplicon using Wq-F and Wq-R was cloned into the vector pMD 19-T Simple (Takara) and sequenced to confirm the sequences. The obtained recombinant plasmid was then transformed into competent *Escherichia coli* (DH5α, TransGen Biotech) and cultured for 12 h. The recombinant plasmid was extracted from the *E. coli* to detect its concentration using an Eppendorf 2000 spectrophotometer. The WSSV DNA copy number was calculated as described by Sun et al. (2013). A standard curve for quantifying WSSV DNA copy numbers was made using serially diluted solutions of the recombinant plasmid.

Dilutions, inoculation, and WSSV infectivity

After quantification, the concentration of WSSV inoculum was $10^{6.2}$ copies g^{-1}. *L. vannamei* and *M. nipponense* were stocked in 36 l aquaria (10 animals per aquarium). For each trial, 10 animals were injected intramuscularly with WSSV inoculum (20 µl) at 10-fold serial dilutions (10^0–10^{-6}). Animals serving as negative controls were injected with the same volume of phosphate-buffered saline. Each treatment was performed in triplicate. The inoculum was injected at the junction between the 3rd and 4th abdominal segments. Dead and moribund animals in each treatment group were recorded at 24 h intervals and examined by 2-step PCR to ensure the accuracy of the LD_{50}. The WSSV LD_{50} was calculated using the Behrens-Kärber method (Kärber 1931).

Distribution of WSSV in *Macrobrachium nipponense*

Dead *M. nipponense* exposed to WSSV were collected and heart, gill, stomach, gut, hepatopancreas, nerve, integument, muscle, pereopod, eyestalk, testis, and ovary tissue samples were sampled for PCR processing. Each tissue was examined in triplicate. DNA was extracted from the samples using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit and prepared for 2-step PCR to detect the presence of WSSV.

Histopathology

Hepatopancreas, gill, and muscle tissues were collected for histopathology studies at 0, 24, 48, 72, and 96 h post-inoculation (hpi) with WSSV. At each time point, 3 individuals were selected randomly. The concentration of WSSV injected was 40% of the LD_{50} for *M. nipponense*. Gill, hepatopancreas, and muscle were removed from *M. nipponense* and immersed immediately in Davidson's fixative for 48 h, and then transferred to 70% ethanol for subsequent paraffin histology. Tissues were sectioned at 5–6 µm and stained with hematoxylin and eosin. WSSV copy number in the hepatopancreas, gill, and muscle tissues of *M. nipponense* were quantified by qPCR as above at each time point.

RESULTS

LD_{50} of WSSV in *Litopenaeus vannamei* and *Macrobrachium nipponense*

For each trial, 10 animals were injected with WSSV inoculum at 10^0–10^{-6} dilutions. *Litopenaeus vannamei* injected with 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions began to show disease signs from 24 hpi, whereas those injected with 10^{-6} dilutions began to show disease signs from 48 hpi. The clinical signs of WSSV infection in *L. vannamei* included lethargy, reduced appetite, and reddish coloration of body and appendages. White spots were seen on several individuals. Changes in behavior patterns included reduced swimming activity and disorientation with swimming to one side. Deaths occurred from 24 to 144 hpi, and dead animals were subjected to bioassays. The LD_{50} determined from 3 replicate treatments of *L. vannamei* ($10^{0.51}$, $10^{0.42}$, and $10^{0.83}$ LD_{50} copies g^{-1}) was $10^{0.59\pm0.22}$ copies g^{-1} (Fig. 1).

Among *Macrobrachium nipponense* injected with serial dilutions of WSSV, animals injected with 10^0 or 10^{-1} dilutions began to show disease signs after 48 hpi, and those injected with 10^{-2} began to show disease signs after 72 hpi. The clinical signs in *M. nipponense* included lethargy, reduced appetite, and reddish coloration of body and appendages, but no white spots in the epidermal tissues were observed. Changes in behavior included reduced swimming activity and disorientation with swimming to one side. Deaths occurred from 48–144 hpi and dead animals were bioassayed as above. The LD_{50} determined from the 3 replicate treatments of *M. nipponense* ($10^{3.91}$, $10^{3.82}$, and $10^{3.79}$ LD_{50} copies g^{-1}) was $10^{3.84\pm0.06}$ copies g^{-1} (Fig. 2).

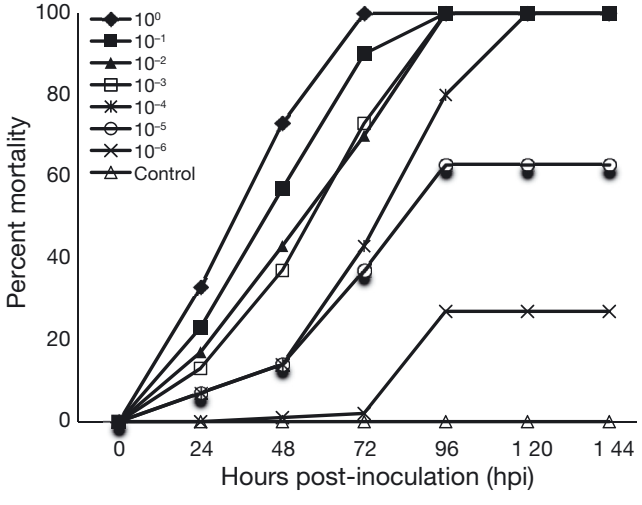

Fig. 1. Cumulative mortality for *Litopenaeus vannamei* injected with white spot syndrome virus and held over time. The viral dilution regime ranged from 10^0 to 10^{-6}. Data were from 3 replicates of 10 *L. vannamei* individuals

Distribution of WSSV in *M. nipponense*

WSSV was detected in the gills, hepatopancreas, muscle, stomach, heart, gut, nerve, epidermis, pereopod, eyestalk, testis, and ovary by simple PCR (Fig. 3). WSSV was detected as intranuclear inclusions in the epidermal cells of hepatopancreas and the hemocytes of gills by histological observation (Figs. 4B & 5D). The WSSV copy number was significantly higher in the gills, hepatopancreas, and muscle compared with the other tissues, as assayed by simple PCR. The results of 3 replicates were the same.

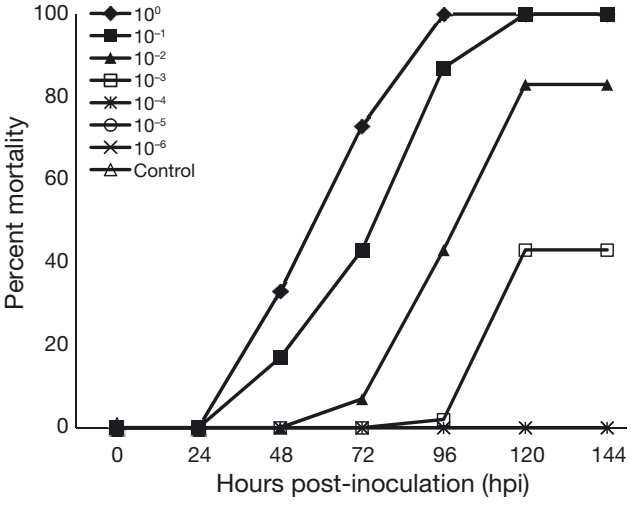

Fig. 2. Cumulative mortality for *Macrobrachium nipponense* injected with white spot syndrome virus and held over time. The viral dilution regime ranged from 10^0 to 10^{-6}. Data were from 3 replicates of 10 *M. nipponense* individuals

Fig. 3. Detection of white spot syndrome virus in different tissues of experimentally infected *Macrobrachium nipponense* by 1-step PCR. Lane 1: gill; lane 2: hepatopancreas; lane 3: muscle; lane 4: stomach; lane 5: heart; lane 6: gut; lane 7: nerve; lane 8: integument; lane 9: pereopod; lane 10: eyestalk; lane 11: testis; lane 12: ovary; lane 13: negative control; lane 14: positive control; lane M: marker

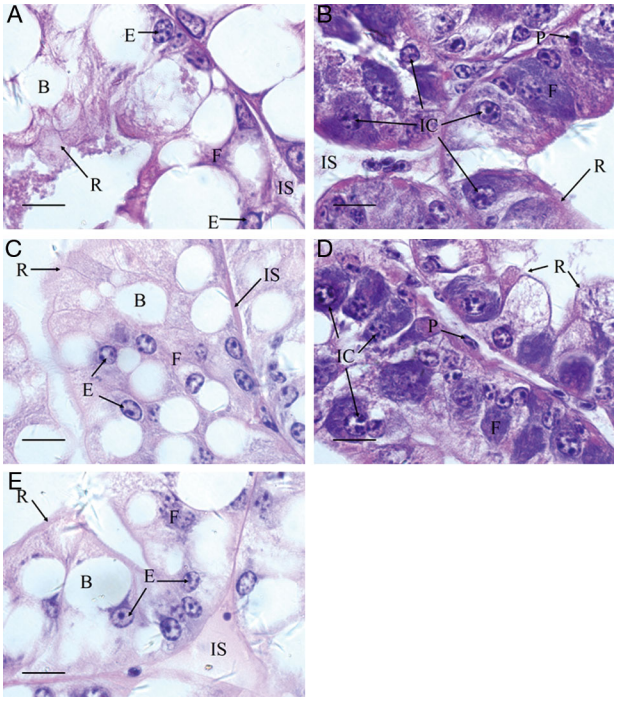

Fig. 4. (A) Hepatopancreas from *Macrobrachium nipponense* experimentally infected with white spot syndrome virus (WSSV) at 0 h post-inoculation (hpi). Typical organization of the hepatopancreas. E: embryonic cells. B: blasenzellen; R: restzellen; F: fibrillenzellen; IS: interstitial sinus. (B) Hepatopancreas from experimentally infected *M. nipponense* at 24 hpi. Presence of more R cells in the hepatopancreas tubules of infected prawns, basophilic intranuclear inclusion bodies (IC), and pyknosis (P). (C) Hepatopancreas from experimentally infected *M. nipponense* at 48 hpi. Hepatopancreas tubules showed clearance of WSSV and turn to typical organization. (D) Hepatopancreas from experimentally infected *M. nipponense* at 72 hpi showing basophilic IC. (E) Hepatopancreas from experimentally infected *M. nipponense* at 96 hpi. Presence of more Band R cells in the hepatopancreas tubules, decreased basophilic IC. All scale bars = 2 µm

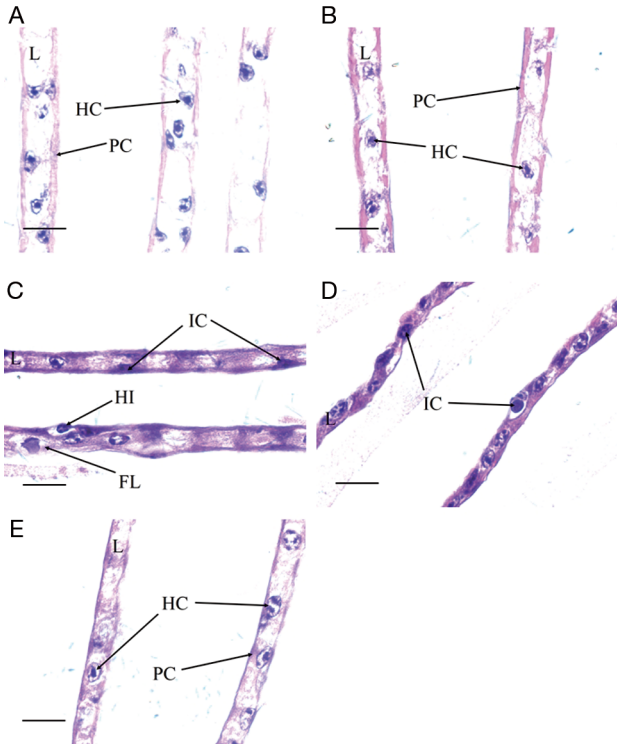

Fig. 5. (A) Gills from *Macrobrachium nipponense* experimentally infected with white spot syndrome virus (WSSV) at 0 h post-inoculation (hpi). Normal organization of the gills. L: lamellae; PC: pillar cells; HC: Hemocytes. (B) Gills from experimentally infected *M. nipponense* at 24 hpi. Normal organization of the gills. (C) Gills from experimentally infected *M. nipponense* at 48 hpi. Hemocytic infiltration (HI), fusion of gill lamellae (FL), and basophilic IC in infected prawns. (D) Gills from experimentally infected *M. nipponense* at 72 hpi. FL and basophilic IC in infected prawns. (E) Gills from experimentally infected *M. nipponense* at 96 hpi showing clearance of WSSV and turn to normal gill organization. All scale bars = 2 µm

Histopathology

Hepatopancreas from *M. nipponense* at 0 hpi showed a normal, well-organized glandular structure. The tubules were closed distally on one side and open proximally into ducts, which united to form longer ducts, ultimately connected to the digestive tract. Embryonic (E) cells were found at the narrow distal end of the tubule, some showing mitotic figures. Restzellen (R) cells, and non-vacuolated and deeply stained fibrillenzellen (F) cells were found a short distance away from the distal region. Vacuolated blasenzellen (B) cells were found in the middle and proximal regions of the tubules. The interstitial sinuses between the tubules were normal (Fig. 4A). The hepatopancreas of *M. nipponense* at 24 hpi exhibited increased numbers of F and R cells compared with 0 h hpi, as well as enlarged nuclei with marginalized chromatin, and often contained a distinct basophilic inclusion body. Pyknotic nuclei were more abundant than at 0 hpi and the size of the B cells was decreased (Fig. 4B). At 48 hpi, the hepatopancreas of *M. nipponense* exhibited a well-organized glandular structure again showing the clearance of WSSV and normal numbers of B, F, and R cells; the basophilic intranuclear inclusion bodies had decreased or even disappeared, and pyknotic nuclei were decreased (Fig. 4C). The hepatopancreas of *M. nipponense* at 72 hpi exhibited abundant basophilic intranuclear inclusion bodies again, and similar numbers of F and R cells to the hepatopancreas at 24 hpi. By 96 hpi, the hepatopancreas showed the

clearance again (Fig. 4D), with decreased basophilic intranuclear inclusion bodies and increasing numbers of B and R cells (Fig. 4E). The WSSV concentration of hepatopancreas at 0, 24, 48, 72, and 96 hpi were 0, $10^{3.9}$, $10^{2.8}$, $10^{3.5}$, and $10^{2.6}$ copies g^{-1}. The clearance of WSSV in hepatopancreas came out together with the decrease in both WSSV concentration and number of basophilic inclusion bodies at 48 and 96 hpi, respectively.

The gills of *M. nipponense* at 0 hpi showed normal lamellae (L) and hemocoelic sinuses, with hemocytes (HC) in the circulation and no structural abnormalities or abnormal gill lesions. Specialized epithelial cells, pillar cells (PC), stretched into the lamellar sinus at intervals and adjoined similar cells stretching from the opposite surface (Fig. 5A). At 24 hpi, the gills showed normal lamellae, similar to 0 hpi (Fig. 5B). However, by 48 hpi, the gills of *M. nipponense* showed hemocytic infiltration (HI) in the hemocoelic sinuses, fusion of lamellae (FL), and basophilic inclusion bodies (Fig. 5C). The number of basophilic inclusion bodies had increased by 72 hpi, showing severe pathology induced by WSSV (Fig. 5D). The gills cleared WSSV and turned to normal organization at 96 hpi together with the decreased WSSV concentration, showing a decreased number of basophilic inclusion bodies (Fig. 5E). The WSSV concentration of gills at 0, 24, 48, 72, and 96 hpi were 0, 0, $10^{3.0}$, $10^{3.5}$, and $10^{2.7}$ copies g^{-1}.

There were no discernible pathological changes in muscle cells, with apparently normal skeletal muscle and fiber cells from 0 to 96 hpi and the WSSV concentration at each time point was also 0 copies g^{-1}.

DISCUSSION

WSSV infects a broad range of crustaceans, including the shrimps *Fenneropenaeus indicus* (Bright Singh et al. 2005), *Marsupenaeus japonicus* (Satoh et al. 2008), and *Penaeus monodon* (Das et al. 2010), fresh water prawns *Macrobrachium idella*, *Macrobrachium lamerrae* (Sahul Hameed et al. 2000), and *Macrobrachium rosenbergii* (Rao et al. 2016), the crayfish *Astacus*, *Pacifastacus leniusculus* (Jiravanichpaisal et al. 2004), and *Cherax quadricarinatus* (Mrugała et al. 2015), and the crabs *Calappa philargius*, *Paradorippe granulata*, *Scylla serrata*, and *Thalamita danae* (Chen et al. 2000, Sahul Hameed et al. 2003). The susceptibility of a host to WSSV can be reflected in the copy numbers found during active infections (Sahul Hameed et al. 2003, Mijangos-Alquisires et al. 2006, Bateman et al. 2012), and the LD_{50} is a common mean to examine its pathogenicity. The LD_{50} value of *Litopenaeus vannamei* to a Thai isolate of WSSV was $10^{6.6}$ copies ml^{-1} (Escobedo-Bonilla et al. 2005), that of *M. rosenbergii* to WSSV-Thai-1 and WSSV-Viet were $10^{5.4\pm0.4}$ and $10^{2.3\pm0.3}$ copies ml^{-1}, respectively (Corteel et al. 2012), that of *Procambarus clarkii* to the native WSSV was $10^{1.3}$–$10^{3.3}$ copies ml^{-1} (Pace et al. 2016), and of *S. serrata* was 10^5 copies ml^{-1} (Liu et al. 2011). In the present study, *M. nipponense* readily supported WSSV replication, leading to disease and mortality. The LD_{50} values of *L. vannamei* and *M. nipponense* to WSSV were $10^{0.59\pm0.22}$ and $10^{3.84\pm0.06}$ copies g^{-1}, respectively, indicating an approximately 1000-fold higher LD_{50} for *M. nipponense* compared with *L. vannamei*. Compared with *L. vannamei*, *M. nipponense* appeared to be more capable of resisting WSSV infection and disease, similar to the situation for WSSV-Thai-1 and WSSV-Viet in *M. rosenbergii* (Corteel et al. 2012). These data indicate that higher doses of WSSV are required to establish infection in the oriental river prawn *M. nipponense*.

The success of a viral infection (successful replication) depends mainly on the interactivity between the viral attachment proteins and the host's specific cellular receptors (Sánchez-Paz 2010). WSSV's ability to infect a broad range of crustaceans suggests that the virus interacts with a general cell surface receptor common to most crustaceans (Liang et al. 2005). WSSV can replicate in all the vital organs of infected penaeid shrimps (Syed Musthaq et al. 2006). However, WSSV cannot infect all types of cells indiscriminately. The main target tissues for WSSV infection are the epidermis, foregut, gills, antennal gland, hindgut, gonads, lymphoid organ, hematopoietic cells, cells associated with the nervous system, and connective tissue (Jiravanichpaisal et al. 2006, Reddy et al. 2010, Han et al. 2013, Söderhäll 2013, Li et al. 2014, Yan et al. 2016), whereas the other tissues, such as the hepatopancreas and gut, are refractory to WSSV infection (Sahul Hameed et al. 1998, Wang et al. 1997). WSSV was detected as intranuclear inclusions in eyestalk, gills, head soft tissue, connective tissue, appendages, and Y-organ by histopathological observations (Vijayan et al. 2003, Yoganandhan et al. 2003). However, WSSV was detected in the hepatopancreas in *P. monodon* and *M. rosenbergii* and in the gut of *M. japonicus* by *in situ* hybridization (Chang et al. 1996, Di Leonardo et al. 2005, Corteel et al. 2012). The hepatopancreas is involved in filtering out stuff from the hemolymph and that would include products of tissue damage; hence, the WSSV presented in these types of cells could be transited from

other organs (Di Leonardo et al. 2005). Pleopods, gills, hemolymph, stomach, and abdominal muscle are the 5 most prevalent infected organs in shrimps (Lo et al. 1997, Jeswin et al. 2013), while the gills, heart, stomach, gut, hepatopancreas, nerve, epidermis, muscle, pereopod, eyestalk, testis, and ovary were shown to be WSSV-positive by 2-step PCR in the current study. These results were consistent with others for *L. vannamei* (Ashikaga et al. 2009).

The histological changes induced by WSSV have been widely investigated in shrimps. Histological studies of WSSV-infected shrimps showed degenerated cells, characterized by basophilic intranuclear inclusion bodies in hypertrophied nuclei of ectodermal and mesodermal origins (Sahul Hameed et al. 2002, Durand et al. 2003, Rodríguez et al. 2003, Tang et al. 2013).The present study revealed discernible cellular degeneration, nuclei hypertrophy with basophilic intranuclear bodies, and chromatin margination in the hepatopancreas and gill cells at 24 and 48 hpi, respectively. The hepatopancreas consists of branched tubules made up of 4 different types of epithelial cells (B, F, R, and E cells). The hepatopancreas functions of storage, metabolism, and detoxification are mediated by R cells (Li et al. 2007). WSSV infection of *M. nipponense* resulted in increased numbers of F and R cells in the hepatopancreas, accompanied by basophilic intranuclear Cowdry type-A inclusion bodies and decreased size of B cells. The WSSV concentration had the same fluctuation with the changes in histopathology following different infection times in the hepatopancreas of *M. nipponense*, suggesting that hepatopancreas at 48 and 96 hpi had an efficient resistance to WSSV. The increasing number of R cells in the hepatopancreas in WSSV-infected *M. nipponense* may be a stress response to the virus, be a defensive reaction to the virus or simply be a metabolic response to the disease in terms of mobilizing resources.

Histopathological changes in the gills of WSSV-infected *M. nipponense* were similar to those reported in *L. vannamei* (Rajendran et al. 2005, Afsharnasab et al. 2009, Pazir et al. 2011). The gills of *M. nipponense* showed hemocytic infiltration in the hemocoelic sinuses, fusion of lamellae, and basophilic inclusion bodies at 48 hpi. The basophilic inclusion bodies induced by WSSV observed in gills were the same as those reported by Yun et al. (2014). The reduction of viral load and mitigation histopathology of hepatopancreas and gills were indicated by the decreased number of basophilic inclusion bodies and WSSV concentration, and the increased number of B cells in hepatopancreas. The WSSV concentration had the same fluctuation as the changes in histopathology following different infection time in the gills of *M. nipponense*. Yoganandhan et al. (2003) recorded histopathological evidence of WSSV in gills of *P. indicus* at 36 hpi, whereas the hepatopancreas remained negative, even when shrimp were in a moribund state. Afsharnasab et al. (2009) found intranuclear Cowdry type-A inclusion bodies in all tissues except the hepatopancreas in *L. vannamei*. However, the hepatopancreas and gill cells in the current study showed signs of clearance of WSSV at 48 and 96 hpi, respectively. To the best of our knowledge, this is the first evidence for this phenomenon in crustaceans. The results of our study differed from those of Yoganandhan et al. (2003) and Afsharnasab et al. (2009) in that the hepatopancreas cells in *M. nipponense* were more likely to be damaged by WSSV than *L. vannamei*. Furthermore, the clearance of WSSV in the hepatopancreas and gills also suggested that *M. nipponense* was more able to resist WSSV infection than *L. vannamei*. The earlier histopathological signs of infection in the hepatopancreas suggests that the hepatopancreas is more likely to be affected by WSSV than the gills in *M. nipponense*.

The immune response of crustaceans to invading pathogens includes phagocytosis, encapsulation, and melanization. Encapsulated pathogens are often collected in the gills or hepatopancreas, where they are broken down and subsequently cleared (Hauton 2012). High tolerance and clearing mechanisms for WSSV have been reported in the giant freshwater prawn *M. rosenbergii* (Pais et al. 2007, Sarathi et al. 2008). WSSV-infected shrimp such as *F. indicus* show an efficient immune response to WSSV, but the compensation mechanism appears unable to maintain resistance against infection, suggesting that this shrimp cannot protect itself from WSSV infection (Sarathi et al. 2007). However, the clearance of WSSV in the hepatopancreas and gills of *M. nipponense* implies that it has an effective immune response with efficient healing compensation against WSSV.

Acknowledgements. This work was supported by the special items of basic research projects of Chinese Academy of Fishery Sciences (2016HY-ZD0402), the National Natural Science Foundation of China (Grant No. 31572617), the Science and Technology Supporting Program of Jiangsu Province (BE2016308), the Fund of Independent Innovation of Agricultural Sciences of Jiangsu province (CX (15)1012-4), the key project of provincial key research and development plan (Modern Agriculture) (BE2016308), the Three Aquatic Projects of Jiangsu Province (D2015-16), and the Science and Technology Development Fund of Wuxi (CLE02N1514).

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Editorial responsibility: Jeffrey Shields, Gloucester Point, Virginia, USA

Submitted: March 9, 2017; Accepted: August 4, 2017
Proofs received from author(s): October 2, 2017