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Effect of white spot syndrome virus on the activity of immune-related enzymes in the red claw crayfish (*Cherax quadricarinatus*)

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Abstract

In this study, we explored the pathogenic effects of white spot syndrome virus (WSSV) and effects of yeast cell wall in the red claw crayfish, *Cherax quadricarinatus*, by investigating the activity of enzymes related to innate immune function following artificial infection of immunized and non-immunized crayfish. Our results reveal that the activity of four enzymes, phenoloxidase (PO), peroxidase (POD), superoxide dismutase (SOD) and lysozyme (LSZ), increased in the hepatopancreas and antennal gland of *C. quadricarinatus* 6 – 24 hours after exposure to WSSV. Activity levels of these enzymes decreased rapidly, so that 72 h post-exposure the activity of the enzymes in WSSV-infected crayfish were significantly lower than those of the control individuals (except for LSZ). Interestingly, we found that immunization with yeast cell wall before challenge with WSSV conferred an immune protection rate of 51.86% at 7 days post-infection, demonstrating that yeast cell wall could improve immune-related enzyme activity in the crayfish and enhance the anti-viral defenses of this species. Morphological examination by transmission electron microscopy revealed significant damage in the hepatopancreas and antennal glands of infected crayfish. WSSV infection caused damage to the epithelium of the hepatopancreas and antennas and reduced the number and size of microvilli. In addition, mitochondria morphology changed following infection, with parts of the cristae diminishing leaving large vacuoles. Moreover, the dictyosome morphology changed, the lysosome membranes ruptured, and heterochromatinized nuclei could be seen in cells with ruptured nuclear membranes although no WSSV particles appeared in the hepatopancreas or in the antennal gland.

Keywords: *Cherax quadricarinatus*, WSSV, Hepatopancreas and antennal gland, Immune enzymes activity, Ultrastructure

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Introduction

The Australian red claw crayfish, *Cherax quadricarinatus*, is one of the most important farmed freshwater crayfish in the world as it is easily cultured and exhibits numerous commercially desirable traits (large size, fast growth, polyphagia, high protein content and low cholesterol content; Mu *et al.,* 2007). In recent years, the constantly expanding scale of *C. quadricarinatus* aquaculture operations and the development of increasingly intensive culture techniques has resulted in the outbreak of infectious diseases in this species. White spot syndrome virus (WSSV) is a highly pathogenic virus with a wide distribution and broad host coverage including marine and freshwater crustaceans such as crabs and crayfish. When they are wounded, sick or weak, they are readily infected which leads to severe disease and death. WSSV is one of the most devastating crustacean viruses in cultured and wild species (Chen *et al.,* 2000; Song *et al.,* 2006; Huang *et al.,* 2007). Natural WSSV infections in wild populations of red claw crayfish are present in many regions of China potentially compromising commercial breeding attempts (Chen *et al.,* 2000). To date, numerous studies have investigated the epidemiology and prevention of WSSV in crustaceans (Shi *et al.,* 2000; Bowater *et al.,* 2002; Claydon *et al.,* 2004; Unzueta-Bustamante *et al.,* 2004; Shen *et al.,* 2007; Hao *et al.,* 2009; Wang *et al.,* 2012 a,b; Liu *et al.,* 2013; Duan *et al.,* 2014; Gao *et al.,* 2014). However, a thorough examination of the pathological effects of WSSV on immune function of breeding red claw crayfish is lacking.

Improving immune function and enhancing disease resistance is one of the fundamental measures for preventing and controlling disease in crustaceans. The enzymes, PO, POD, SOD and LSZ, play an important role in measuring immune function and health status in crustaceans (Rameshthangam and Ramasamy, 2006; Wang *et al.,* 2012a,b). In crustaceans, the hepatopancreas stores nutrients, detoxifies the tissues and removes foreign organisms, while the antennal gland remove wastes and maintains body water balance (Chen *et al.,* 2007). Invading viruses can damage the hepatopancreas and the antennal gland and impair their physiological functioning (Xue *et al.,* 1992).

In this study we present the investigation of the pathogenic effects of WSSV on immune-related enzyme activity in the hepatopancreas and antennal gland tissue of red claw crayfish. In particular, we investigate (a) changes in the activity of key enzymes involved in oxidative stress, (b) the ultrastructure of normal and WSSV-infected cells of the hepatopancreas and antennal gland tissue, and (c) monitor changes in immune function after infection with WSSV under different experimental conditions.

Materials and methods

Crayfish sampling and virus preparation

A total of 150 healthy and uninfected *C. quadricarinatus* (75.26 ± 6.13 g body

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weight; 14.65 ± 1.16 cm in length) were collected from the aquafarm located at Jingshan, Shanghai, China, in 2010. They were transported to the laboratory and maintained in freshwater at 25±1° C in an aerated system with autoclaved pellet feed. These crayfish were confirmed not to be WSSV-infected by PCR (data not shown) before each experiment. WSSV virus was obtained from an infected *C. quadricarinatus* kept at the Key Laboratory of Fish Immunology and Health of the Chinese Academy of Fishery Sciences, Zhejiang Institute of Freshwater Fisheries, Huzhou, Zhejiang, China. Samples of the hemolymph and gills were obtained by using the following procedure from a crayfish confirmed to be infected with WSSV by PCR (Shen *et al.*, 2007, EF078890.1, China strains, data not shown). These tissues were homogenized at a 1:10 W:V ratio in saline solution and centrifuged at 2358 g for 5 min and 3929 g for 10 min at 4°C. The supernatant was then passed through a 220-nm membrane filter. Filtrates were stored at -80°C. To amplify the virus, uninfected red claw crayfish were artificially infected with the prepared supernatant (0.14 – 0.20 mL per crayfish). Three to five days after infection, the hemolymph of the infected crayfish was collected and treated with repeat freezing and thawing, followed by centrifugation at 1571 g for 5 min. The supernatant was then collected to once again inoculate uninfected crayfish (0.14 mL per crayfish， Shen *et al.*, 2007). These infected individuals were then fed and maintained at 25°C.

Amylose immunization

Amylose extracted from yeast cell wall was purchased from Hubei Angel Yeast Limited Company. The product contained β-glucan ≥29.0%, α-mannan peptide ≥20.0%, peptide and protein ≥30.0%, chitin ≥2.0% (Xu and Wei, 2005). The 0.5%, 1.0% and 2.0% immune polysaccharide (yeast cell wall) solution was prepared with normal saline and sterilized.

Experimental challenge with WSSV

After acclimatization for seven days with no obvious signs of infection in any individual, nor any mortality, the experimental animals were randomly assigned into four groups (with six crayfish per aquarium and three replicates per group). Individuals in the control group were inoculated with 0.10 mL PBS buffer. Individuals in the immunized non-infected group (Group I) were inoculated with 0.10 mL of 1.0% immune yeast cell wall solution. Individuals in the non-immunized infected group (Group II) were inoculated with 0.10 mL PBS buffer and then 48 h later injected with 0.14 mL of the virus solution. Finally, individuals in the immunized infected group (Group III) were inoculated with 0.10 mL of 1.0% immune yeast cell wall and then 48 h later inoculated with 0.14 mL of the virus solution. All inoculations were administered by intramuscular injection between the first and second abdominal sections.

Randomly selected crayfish from the control group and the experimentally infected groups were euthanized at 0, 6, 12, 24, 48 and 72 h after injection, at

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which point 0.5 g of the hepatopancreas and the antennal gland tissues were removed by dissection. These tissues were then homogenized by the addition of saline solution (10% W:V; Precellys24, Bertin Technologies, Montigny-le-Bretonneux, France). The samples were then centrifuged at 1454 g for 10 min at 4°C to obtain both the hepatopancreas and antennal gland tissue extracts and the precipitate that would be used for immune enzyme analysis. In addition, tissue samples from the experimentally infected groups (Group II and Group III) were obtained prior to their infection (i.e. at 0 h) and used as internal 'controls'. The supernatant fractions (tissue extracts) and hepatopancreas and antennal gland tissues were stored at -80°C prior to analyses.

Determination of enzyme activities

Phenoloxidase (PO) activity was quantified following the method of Ashida (1971), with some modifications (Wang *et al.,* 2015) Briefly, 100 µL of each tissue extract were placed in a microtiter tube with 100 µL of 3,4-dihydroxyphenylalanine (L-DOPA, Sigma, St. Louis, MO). The mixture was incubated for 40 min at 28°C, followed by the addition of 3 mL of pre-chilled 0.l mol L^{-1} potassium phosphate buffer (0.1 M $K_2HPO_4 \bullet 3H_2O$, 0.1 M KH_2PO_4, adjusted pH to 6.0). The reaction was read at 490 nm.

The activity of peroxidase (POD), superoxide dismutase (SOD) and lysozyme (LSZ) in the gills of crayfish were determined with kits (Jiancheng, Ltd, Nan-jing, China) and read on a spectrophotometer (Model Spectrumlab 22 pc, Lengquang Tech, Shanghai, China). The protein content of in the crude tissue extracts were determined using the Coomassie brilliant blue protein assay kit (Jiancheng, Ltd, Nan-jing, China)

Electron microscopy sample preparation and observation

Crayfish tissues were processed according to published procedures (Rachel *et al.,* 2002; Shen *et al.,* 2007). The hepatopancreas and antennal gland tissues of the uninfected 0 h group and the group infected with WSSV at 72 h were fixed in 2.5% glutaraldehyde ($C_5H_8O_2$) for 24 h, post-fixed in 1% osmium tetraoxide (OsO_4) for 1 h and then stored at 4°C. Sections were embedded in epoxy resin (Epon812) and cut in an RMC PowerTome XL microtome (Boeckeler Instruments, Tuscon, AZ). They were then stained with uranyl acetate and lead citrate and examined under a Hitachi H-7650 transmission electron microscope at 80 kV.

Determination of immune protection rate

In a separate experiment, 60 uninfected crayfish were divided into two groups (10 crayfish per aquarium, with three replicates per group). Individuals in the infected group (control group) crayfish were then injected with PBS solution and then inoculated 48 h later with 0.10 mL of the virus solution. Individuals in the yeast cell wall-injected infected group were injected with 0.10 mL of the 1.0% yeast cell wall solution and

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then inoculated 48 h later with 0.10 mL of the virus solution. All inoculations were administered by intramuscular injection between the first and second abdominal sections. The cumulative mortality rate of individuals in each group was recorded for seven days. We then calculated the immune protection rate according to the following formula: (control group mortality − yeast cell wall injected infected group mortality)/control group mortality.

Statistical analysis

The results were subjected to one-way ANOVA followed by a least significant difference test with significance level set to p=0.05. Differences among groups were assessed by a Duncan's multiple comparison test. All data were analyzed using SPSS software, version 14.0 (Chicago, IL, USA).

Results

Effect on WSSV infection on the activity of immune enzymes

Our results reveal that the activity levels of PO in the hepatopancreas of both the non-immunized infected group (Group II) and the immunized infected group (Group III) were significantly lower than that of the control group (p<0.01) with the exception of individuals in Group III at 24 h (Fig. 1). In addition, PO activity in the hepatopancreas rapidly declined over the infection period. In contrast, the activity of PO in antennal gland tissue in Group II and Group III dramatically increased to 4-6 times that of the PO levels observed in the control group within 24 h of virus exposure (p<0.01; Fig. 1). PO activity peaked at 12 h in Group II and at 24 h in Group III and also showed a quick downward trend (Fig. 1). Meanwhile, the activity of PO in the uninfected group (Group I) increased post-infection but only significantly so in the antennal gland tissues (p<0.01; Fig. 1). Our results suggest that the immunization of individuals in Group III caused the higher activity level of PO observed in that group compared to Group II which was not immunized (p<0.01). Overall we observed that the activity of PO in the hepatopancreas was greater than the activity of PO in the antennal gland (Fig. 1).

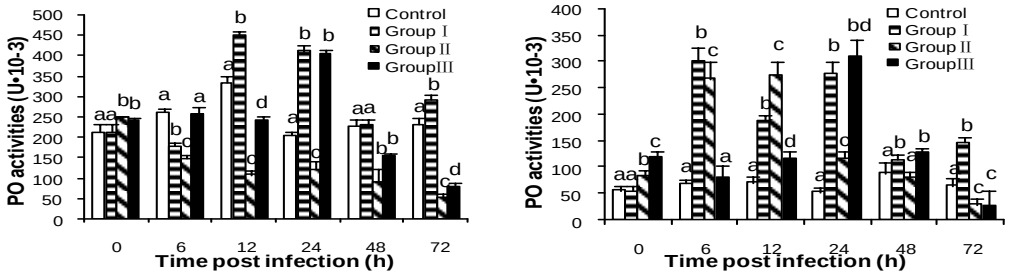


Figure 1: The effect of immunization and WSSV on the activity of PO in the hepatopancreas (left) and the antennal gland (right) of red claw crayfish (*Cherax quadricarinatus*). Results shown are the means (± S.D.) of three replicates for each of four groups: Control group, Group I, Group II, and Group III. Different letters show significant differences among the groups at each exposure time (p<0.05).

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The activity of POD in both the hepatopancreas and antennal gland tissue of red claw crayfish in groups II and III was variable over the course of the experiment (Fig. 2). We observed that the activity of POD in the antennal gland of individuals in Group I was significantly higher than that of individuals in the control group but only at 24 h (p<0.01; Fig. 2). Similarly, the activity of POD in the hepatopancreas of individuals in Group I was only significantly higher than that of individuals in the control group at 72 h post-infection (p<0.01; Fig. 2). The activity of POD activity in individuals in Group III did not differ significantly to that of individuals in Group II. Overall, we observed that POD activity was greater in the hepatopancreas than in the antennal gland tissue.

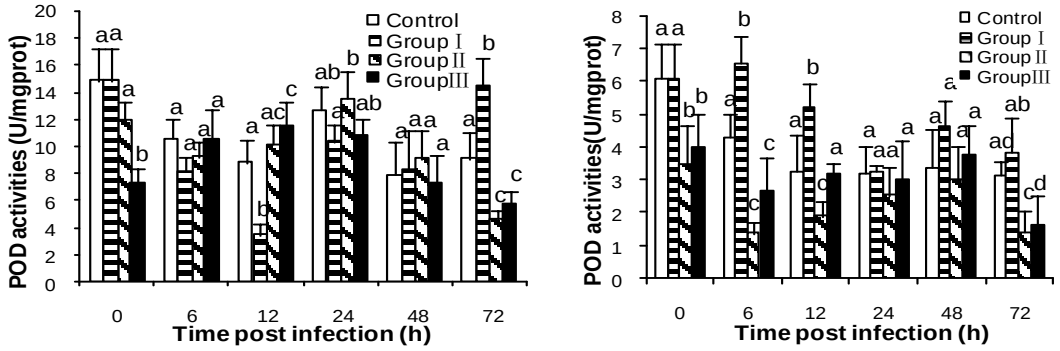


Figure 2: The effect of immunization and WSSV on the activity of POD in the hepatopancreas (left) and the antennal gland (right) of red claw crayfish (*Cherax quadricarinatus*). Results shown are the means (± S.D.) of three replicates for each of four groups: control group, Group I, Group II, and Group III. Different letters show significant differences among the groups at each exposure time (p<0.05).

The activity of SOD in the hepatopancreas and the antennal gland in the immunized groups was significantly higher than that observed in individuals in the control group (p<0.05; Fig. 3). In particular, SOD activity in both tissues was higher in the immunized infected crayfish (Group III) than in the non-immunized infected crayfish (Group II, p<0.01; Fig. 3). However, the activity of SOD in individuals in Group II was variable over the course of the experiment, increasing at 12 h, but declining by 24 h post-infection (Fig. 3). In addition, the activity of SOD in the antennal gland of individuals in Group II (non-immunized) was significantly lower than that of individuals in the control group (Fig. 3). The total SOD activity levels did not differ between the two tissues types.

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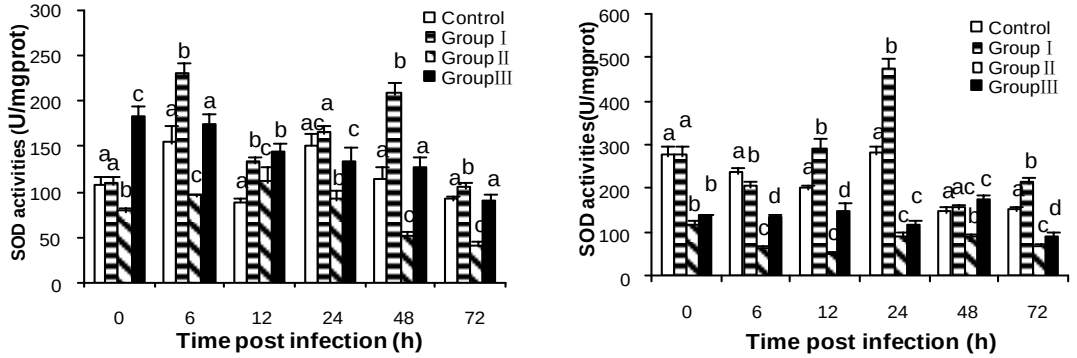


Figure 3: The effect of immunization and WSSV on the activity of SOD in the hepatopancreas (left) and the antennal gland (right) of red claw crayfish (*Cherax quadricarinatus*). Results shown are the means (± S.D.) of three replicates for each of four groups: control group, Group I, Group II, and Group III. Different letters show significant differences among the groups at each exposure time (p<0.05).

LSZ activity in individuals from the immunized infected group (Group III) was significantly higher in both tissue types than for individuals in the control group (Fig. 4). Meanwhile, LSZ activity in individuals in Group II was variable, increasing between 6 and 24 h post-infection, but then declining at 48 h post-infection. In this group LSZ activity was both significantly higher and significantly lower than the control group at particular times during the experiment (Fig. 4). The LSZ activity of the hepatopancreas and antennal gland in the immunized groups increased over the course of the experiment (Fig. 4). Overall we observed that LSZ activity was greater in the antennal gland tissue than in the hepatopancreas.

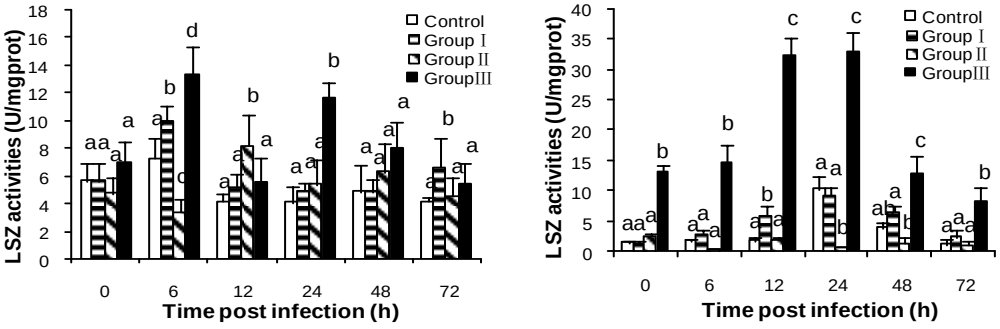


Figure 4: The effect of immunization and WSSV on the activity of LSZ in the hepatopancreas (left) and the antennal gland (right) of red claw crayfish (*Cherax quadricarinatus*). Results shown are the means (± S.D.) of three replicates for each of four groups: control group, Group I, Group II, and Group III. Different letters show significant differences among the groups at each exposure time (p<0.05).

Protective efficacy of yeast cell wall in C. quadricarinatus

After seven days exposure to WSSV, the mortality rate of the control infected group was as high as 90%, while that of the immunized infected group was

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significantly lower at 43.33% (p<0.05; Fig. 5). The overall rate of immune protection was 51.86% after 7 days of exposure.

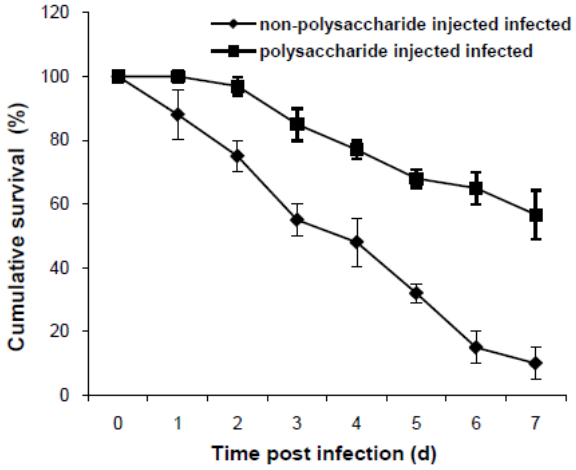


Figure 5: Cumulative survival of red claw crayfish *Cherax quadricarinatus* infected with WSSV as a function of time post-infection.

Effect of WSSV infection on the ultrastructure of the hepatopancreas and the antennal gland in C. quadricarinatus

The hepatopancreas in *C. quadricarinatus* is composed of numerous hepatopancreas tubules whose walls consist of four different morphological cell structures (Li. 2008). In this study, we observed that many hepatopancreas cells contained abundant mitochondria (Fig. 6A, B), and that the hepatopancreas tubules contained neat rows of microvilli (Fig. 6B). In addition, we report that the structure of the rough endoplasmic reticulum, mitochondria, Golgi and lysosomes within the hepatopancreas cells was clear and complete (Fig. 6B, C, H, I), and the nuclear chromatin was evenly distributed (Fig. 6G).

We observed that infection with WSSV caused significant changes in the ultrastructure of the hepatopancreas. For example, the rough endoplasmic reticulum was unordered, the Golgi contraction boundary was not clear (Fig. 6F); the mitochondria were damaged, showing partly missing cristae and thin matrix, and some mitochondria remained as microbubbles (Fig. 6D). In addition, the lysosome membranes had ruptured (Fig. 6K), the cell nuclei showed high hetero-chromatinization, with deformation of the nuclear membrane, and there were microbubbles in the cytoplasm (Fig. 6J). Finally, the nuclei of hepatopancreas cells showed rupturing of the nuclear membrane and outflow of nucleoplasm (Fig. 6L), while some of the hepatopancreas tubules' parietal

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microvilli were damaged and detached (Fig.6E).

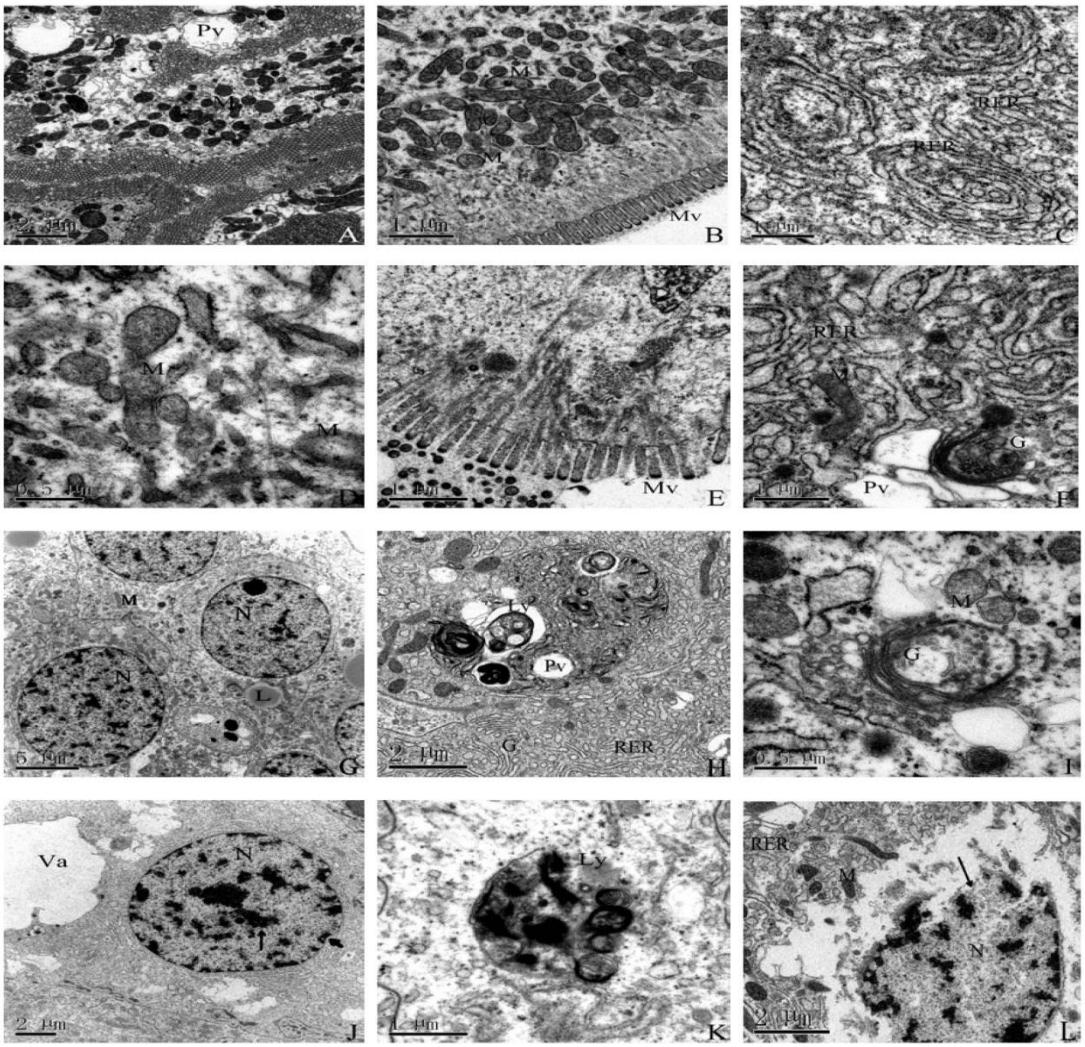


Figure 6: Ultrastructure of the hepatopancreas of red claw crayfish infected with WSSV. (A) Control group, mitochondria (×10000); (B) control group, microvilli and mitochondria (×25000); (C) control group, rough endoplasmic reticulum (×25000); (D) infected group, damaged mitochondria (×50000); (E) infected group, deciduous and damaged microvilli (×30000); (F) infected group, disordered rough endoplasmic reticulum, contracting and incomplete Golgi apparatus in border (×30000); (G) control group, chromatin of uniform distribution in nucleus (×5000); (H) control group, lysosome, rough endoplasmic reticulum, and golgi apparatus (×15000); (I) control group, golgi apparatus (×50000); (J) infected group, nucleus hetero-chromatinization (thin arrow), nuclear membrane transubstantiation (thick arrow), vacuole in the cytoplasm (×8000); (K) infected group, lysosome membrane ruptured (×30000); (L) infected group, nuclear membrane ruptured (thin arrow) and nucleoplasm outflow into the hepatopancreas cell (×15000).

In the antennal gland tissue of C. *quadricarinatus* of the control group, the basement membrane was complete and the epithelial cell bodies were ordered (Fig. 7A, B). In addition, cells were rich in mitochondria (Fig. 7C). In

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the WSSV-infected group, however, we observed significant changes in the antennal gland ultrastructure. For example, part of the basement membrane and some of the epithelial cells were damaged and detached (Fig. 7D), the nuclear membrane was damaged (Fig. 7E), while some mitochondria were deformed and their boundary was not clear (Fig. 7F).

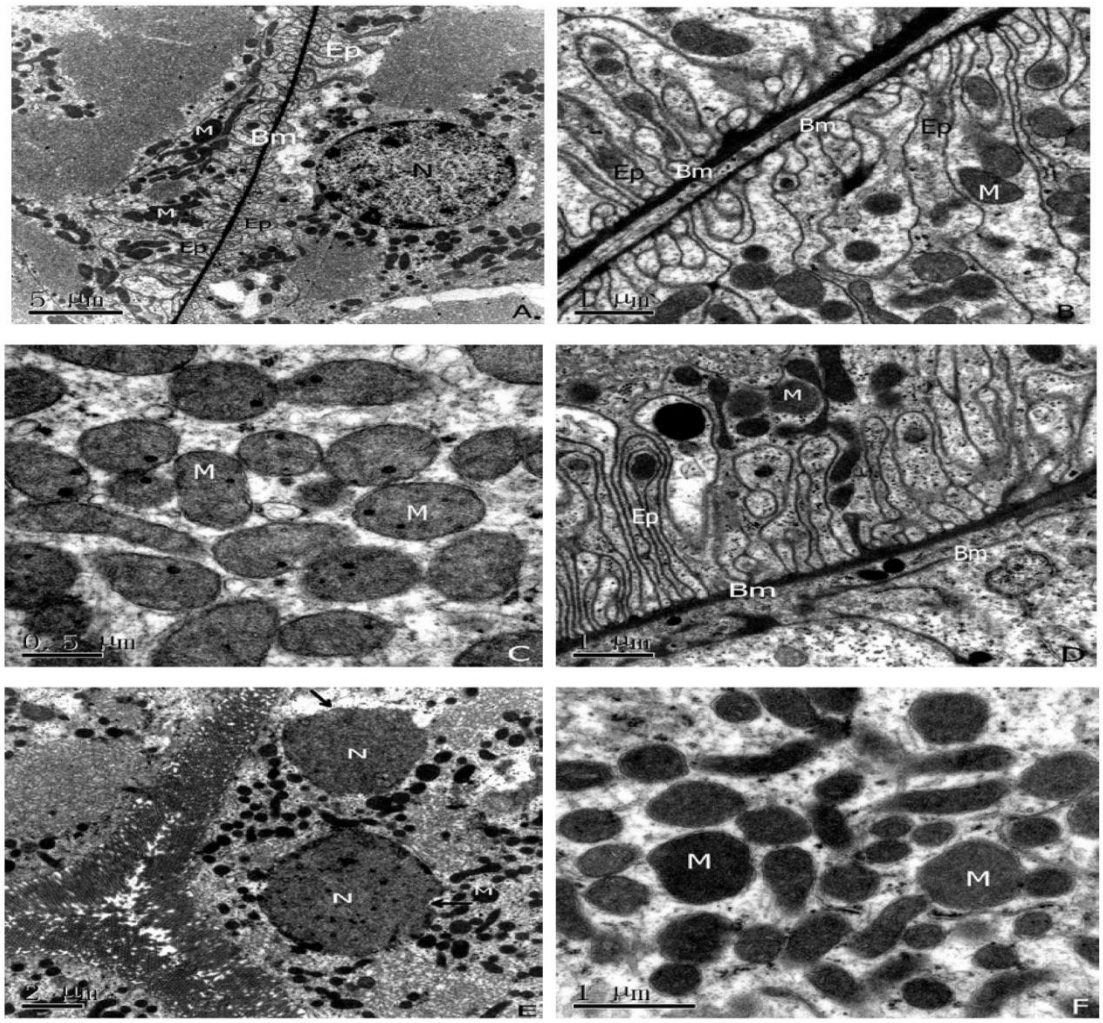


Figure 7: Ultrastructure of the antennal gland of red claw crayfish infected with WSSV. (A) control group, basement membranes, epithelium, nucleus and abundant mitochondria (×5000); (B) control group, basement membranes, epithelium and mitochondria (×20000); (C) control group, mitochondria (×40000); (D) infected group, parts of deciduous and damaged basement membranes and epithelium (×20000); (E) infected group, nuclear membrane ruptured (black arrow, ×8000); (F) infected group, parts of transubstantiation and incomplete mitochondria in border (×30000). Cm=cutin membrane; Bm=basement membranes; Ep=epithelium; Mv=microvilli; Va= vacuole; M= mitochondria; N=nucleus; RER=rough endoplasmic reticulum; G=Golgi apparatus; Ly=lysosome; Pv=pinocytosis vesicle.

Discussion

Impact of WSSV infection on enzyme activity in C. quadricarinatus

As prophenoloxidase (proPO) can be activated by β-1,3 glucans, lipopolysaccharides and peptidoglycans,

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the activity level of PO is, to some extent, a reflection of the strength of resistance of *C. quadricarinatus* to disease. Following its release from granulosa cells into the plasma, proPO can be converted by serine proteases into active PO, which then catalyzes phenolic compounds into melanin. The melanin and its intermediate products can insulate and kill the invading pathogen (Sritunyalucksana *et al.*, 1999; Lei *et al.*, 2001). In this study, we observed that PO activity was significantly elevated in infected *C. quadricarinatus*. This result indicates that the virus triggers the immune system of the crayfish (i.e. the biosynthesis of PO) to limit proliferation of the pathogen. However, our results also showed that by spreading rapidly, the virus overwhelmed the host's defense system, thereby increasing the mortality rate and causing a dramatic decrease in PO activity during the late stage of infection. This finding is consistent with previous reports (Liu *et al.*, 2000; Huang *et al.*, 2007; Mathew *et al.*, 2007; Wang *et al.*, 2012 a,b). The antennal gland is also an important excretion organ in crustaceans, and also maintains ionic balance and adjusts osmotic pressure (Chen *et al.*, 2007). May be to maintain ionic balance, the stress response of crayfish after being infected first appeared in the antennal gland showing that PO activity increased in Group II and Group III， and PO activity in hepatopancreas peaked late in Group II at 24h.

Similarly, POD can protect cells by decomposing harmful metabolites such as H_2O_2 into non-toxic water and oxygen through oxidative reactions. Therefore, activity of POD can be used as an index to evaluate the immune function of crustaceans (Liu *et al.*, 2000). In this study, the activity of POD in infected *C. quadricarinatus* initially increased but then decreased within 12h to 24 h after infection. As discussed above, WSSV infection in *C. quadricarinatus* induced the biosynthesis of POD as part of the host's anti-viral defense mechanism. However, as has been previously reported (Lei *et al.*, 2001), this efficacy of this defense mechanism declined over time, such that the activity of POD was lower than that observed in the control group during late infection.

The enzyme, SOD, plays an important role in clearing superoxide radicals and preventing injury to cells, and exhibits differing levels of activity in different tissues and organs (Chen *et al.*, 2007). SOD activity is induced when crayfish and crabs suffer from slight stress but is inhibited under severe stress, and hence, can serve as an index of immune function in crustaceans. The results of this study demonstrate that while SOD activity in the hepatopancreas of individuals in Group II and GroupⅢ was higher than that of control individuals, SOD activity in the antennal gland was lower than the uninfected control individuals at 12 h. Moreover, in both tissues SOD activity waned over time. We also observed that the activity of SOD in both tissues of individuals in Group II was significant lower than for control individuals at 72 h, which indicates that

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immune function had been damaged. This finding is consistent with results of previous research in tiger prawns, *Penaeus monodon* (Rameshthangam and Ramasamy, 2006) and *C. quadricarinatus* (Wang *et al.,* 2012). SOD activity did not differ between the two tissue types indicating that both are important antioxidant tissues in *C. quadricarinatus.* Indeed, it is known that the hepatopancreas and antennal gland contain many antioxidant enzymes that operate at high activity to avoid injury from free radicals produced by metabolism (Zeng *et al.,* 2008).

Finally, we examined changes in LSZ activity following WSSV infection. LSZ can hydrolyze the cell wall of Gram-positive bacteria and destroy invading foreign organisms. In this study, the activity of LSZ in both tissues in individuals in the non-immunized infected group (Group II) was lower than that in control individuals. Similar observations have been reported for LSZ activity in *Fenneropenaeus chinensis* (Huang *et al.,* 2007) and in *C. quadricarinatus* (Wang *et al.,* 2012 a,b), and suggest that this is a weak defense mechanism against WSSV-infection in *C. quadricarinatus* (Rameshthangam and Ramasamy, 2006). However, the activity of phenoloxidase (PO), peroxidase (POD) and superoxide dismutase (SOD), increased in the hepatopancreas and antennal gland of *C. quadricarinatus* 6 – 24 hours after exposure to WSSV. Based on our results we surmise that the mechanism underlying changes in LSZ activity differed to that regulating changes in PO, POD and SOD, revealing that immune defenses are regulated differently throughout the body. LSZ activity in the context of diverse pathological processes should be further studied.

Overall, the activity level of immune-related enzymes reflects the condition of the host's immune system. Our results indicate that the dynamic equilibrium of the micro-environment of uninfected *C. quadricarinatus* became unbalanced after pathogenic infection with WSSV. Although immune responses were stimulated by the virus (as indicated by the elevated activity of the enzymes PO, POD, SOD and LSZ), eventually infection led to high mortality rates for the hosts. The use of the yeast cell wall in this study showed that the immune function of the crayfish could be modulated. It was evident that in crayfish immunized with the yeast cell wall prior to infection (Group III), the activities of the four studied enzymes were generally higher than those in the non-immunized infected group (Group II). This result indicates that the disease resistance in *C. quadricarinatus* is enhanced by greater enzyme activity. Moreover, we found that after seven days exposure to WSSV, individuals exhibited an immune protection rate of 51.86%; a value similar to that reported for *Procambarus clarkii* (Xu and Wei, 2005) and *Penaeus japonicus* (Itami *et al.,* 1998). These observations indicate that the immune- polysaccharides could improve the innate anti-viral ability of crustaceans against WSSV (Itami *et al.,*

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1998; Takahashi *et al.,* 1998; Chen *et al.,* 2004; Huang *et al.,* 2007). In recent years, yeast cell wall was applied in our aquaculture, to crayfish (*C. quadricarinatus*), crabs (*Portunus trituberculatus, Charybdis japonica*), and fishes (*Pampus argenteus, Miichthys miiuy*). We mixed "yeast cell wall" and artificial feed with a certain percentage then fed it to aquatic animals. The "Yeast cell wall" has the potential to improve immunity and reduce mortality of aquatic animals.

Impact of WSSV infection on the ultrastructure of hepatopancreas and the antennal gland in C. quadricarinatus

In the infection environment, the virus was absorbed into the hemolymph via the crayfish's gills and then dispersed throughout the body accumulating mainly in the hepatopancreas (Yang *et al.,* 2005). Infection with WSSV resulted in damage to the physiological structure and function of the hepatopancreas. These included damage to cell absorption processes, secretion and digestive functions, epithelium damage, and damage to mitochondria. In addition, the lysosome membranes ruptured and microvilli were reduced in number and size. The microvilli of hepatopancreas can increase absorption and secretion size of hepatic duct (Li., 1996). A reduction in the number and size of microvilli would lead to poor nutrient absorption and declines in metabolic activity of *C. quadricarinatus.*

The antennal gland is also an important excretion organ in crustaceans, and also maintains ionic balance and adjusts osmotic pressure (Khodabandeh *et al.,* 2005 a,b). The function of secretion and excretion was damaged after *C. quadricarinatus* were WSSV-infected when the basement membrane and epithelial cells were damaged and detached. Mitochondria were partly deformed and the boundary was not clear, and the nuclear membrane was damaged. All of these structural changes will disrupt the physiological function of the antennal gland. As above, damage to epithelial cells and mitochondria weakens the excretory ability of the antennal gland, and impairs the ability of this organ to adjust osmotic pressure.

Within the body toxic substances accumulate in the hepatopancreas. However, in this study no WSSV viral particles were found in either the hepatopancreas or the antennal gland of crayfish. Previous studies have shown that WSSV virions are located primarily in organs of the ectoblast and mesoderm, such as gill, hematopoietic tissue, foregut epithelium, intestinal epithelium, stomach, dermal, etc., *as well as* demonstrating the gills and hemocytes were the main target cells of WSSV (Lo *et al.,* 1997; Zhu *et al.,* 2003). Some studies suggested the distribution of WSSV in hepatopancreas and other organs of diseased shrimp (Chang and Lo,1996; Lo *et al.*,1997), but other experiments showed that there was no distribution in tissues and organs (hepatopancreas, midgut epithelium, etc.) from endoderm system of shrimp (Wang *et al.*,1998; Zhan *et al.*,1999). WSSV was also

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found in the gills and hemocyte of *C. quadricarinatus* (Wang *et al.,* 2012 a,b) and *Homarus gammarus* (Bateman *et al.,* 2012). In this study no WSSV viral particles were found in either the hepatopancreas or the antennal gland of crayfish. Therefore the WSSV distribution is different in crustacean, which may be because of the difference of WSSV isolated strain, or the difference of species, age and sampling time of WSSV-infected shrimp. The factors influencing where WSSV viral particles are distributed requires further research.

In summary, our study demonstrates that infection with WSSV induces the activity of immune-related enzymes (PO, POD, SOD and LSZ) as in the hepatopancreas and antennal gland of *C.quadricarinatus*, but that the effects are limited in duration as their levels declined in the late stage of infection. The overall immune protection rate provided by the yeast cell wall injection after seven days exposure was 51.86%, demonstrating that it could improve immune-related enzyme activity in the crayfish and enhance this species' anti-viral defenses. We found, however, that WSSV infection damaged the ultrastructure and functioning of both the hepatopancreas and the antennal gland, and led to high mortality. Our findings may help to advance future research on strategies for controlling WSSV in red claw crayfish.

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