Latin American Journal of Aquatic Research, 50(4): 562-572, 2022
DOI: 10.3856/vol50-issue4-fulltext-2816

*Research Article*

# Gene expression in primary hemocyte culture of the Pacific white shrimp *Penaeus vannamei* infected with different white spot syndrome virus (WSSV) strains

**Delia Patricia Parrilla-Taylor[1], Regina Elizondo-González[2]**
**Jesús Neftalí Gutierrez-Rivera[1], Alejandra García-Gasca[3], Norberto Vibanco-Pérez[4]**
**María de Jesús Durán Avelar[4] & Ricardo Vázquez-Juárez[1]**
[1]Centro de Investigaciones Biológicas del Noroeste, La Paz, BCS, México
[2]CONACyT - Centro de Investigaciones Biológicas del Noroeste, La Paz, BCS, México
[3]Centro de Investigación en Alimentación y Desarrollo, Sinaloa, México
[4]Unidad Académica de Ciencias Químico-Biológicas y Farmacéuticas
Universidad Autónoma de Nayarit, Tepic, Nayarit, México
Corresponding author: Ricardo Vázquez-Juárez (rvazquez04@cibnor.mx)

**ABSTRACT.** Five previously analyzed white spot syndrome virus (WSSV) strains from northwest Mexico, differing in their genome architecture as well as in virulence, were selected (high virulence JP and LG strains; moderate virulence GVE and DIV strains; and low virulence LC10 strain) to evaluate pathogenesis response *in vitro*. Expression of phagocytosis-activating protein PAP, manganese superoxide dismutase MnSOD and peroxiredoxin PRX, and two genes of immediate-early expression (IE1 and WSSV304) were measured by qPCR in a primary hemocyte cell culture from *Penaeus vannamei* at 1, 3, 6, 12, and 24 h post-infection (hpi). PAP expression was significantly higher at 1 and 3 hpi, and JP and LC10 strains induced the highest expression. The response of MnSOD was high at 1 hpi, and a significant increase in PRX expression was detected at 3 hpi, probably due to the occurrence of an oxidative burst; expression levels of MnSOD and PRX were significantly higher at 1 and 3 hpi, respectively, induced by the LG strain (high virulence), suggesting an acute response. In general, expression of most immune-related - genes decreased after the initial hours of infection. Expression levels of IE1 and WSSV304 were exceptionally high at 1 hpi in almost all five WSSV analyzed strains, confirming their efficient mechanism for replication and viral fitness. The results of this study do not show an accurate link between the genome size and WSSV virulence of the strains, albeit the strain with the smallest genome showed the highest virulence. All strains induced an early immune response in heterogeneous ways.

**Keywords:** *Penaeus vannamei*; virulence; gene expression; viral fitness; viral pathogenesis; immune response

## INTRODUCTION

White spot syndrome virus (WSSV) is a large dsDNA virus of approximately 300 kb encoding about 184 open reading frames (ORFs) (Van Hulten et al. 2001). It is the most contagious viral infection of decapod crustaceans and causes up to 100% mortality of shrimp cultures within 3-6 days at any age (Lightner 1996). The control of this disease has been a global priority over the past two decades since it has been causing mass mortalities in shrimp culture worldwide as a consequence of the rapid growth and development of the industry, costing billions of dollars (Lightner 1996, Lightner et al. 2012, Patil et al. 2021).

The host range of WSSV includes many decapod and non-decapod species, including freshwater crayfish

Corresponding editor: Mauricio Laterça

and prawns, crabs, and marine crayfish (Stentiford et al. 2009), which contribute to transmission and thus global dispersion of the disease. Several WSSV genomes have been entirely or partly sequenced, showing differences in genome size and sequence, especially for multiple-repeat regions (Wongteerasupaya et al. 2003, Marks et al. 2004, Durán-Avelar et al. 2015, Oakey & Smith 2018). The consensus is that during the spread of WSSV throughout Asia, the DNA genome has been progressively shrinking (Dieu et al. 2004, Marks et al. 2005, Pradeep et al. 2008). A study focused on the genetic variation present among WSSV isolates found a higher virulence of one Thai isolate of relatively smaller genome size compared to another isolate with a larger one, suggesting a replication advantage (Marks et al. 2005). It has also been proposed that genome shrinkage stabilizes over time and that genome size and fitness are related (Zwart et al. 2010). In addition to differences in genomic architecture, multiple experimental studies have shown differences in virulence between WSSV isolates where infectivity of virions, median lethal time, or both have been used as measures of virulence; besides that, the results of variations and changes in virulence are conflicting (Pradeep et al. 2009, Zwart et al. 2010, Hoa et al. 2012, Li et al. 2017).

Knowledge of the pathogenesis of the major viral diseases is essential; research on crustacean immunity has received high priority during the past decades, mainly induced by the need to control disease outbreaks in shrimp farms. Invertebrates do not possess acquired immunity and rely on innate, non-adaptive immune mechanisms; the first immune process is mediated by hemocytes and plasmatic proteins of the circulatory system (Bachère 2000). Crustacean hemocytes play a central role in the host immune response, including recognizing invading microorganisms and performing functions such as phagocytosis, melanization, encapsulation, nodule formation, mediation of cytotoxicity, and cell-cell communication for detecting and eliminating foreign and potentially harmful microorganisms and parasites (Bachère 2000, Johansson et al. 2000).

The first immune response in arthropods is an immediate and inductive stage corresponding to recognizing non-self-factors and initiating immune reactivity, a response we intended to deal with in this study.

In a previous study, our group reported the draft genomes of five WSSV strains with high (JP and LG), moderate (GVE and DIV), and low virulence (LC10), sampled in the states of Sinaloa and Nayarit, Mexico, showing differences in genome length and multiple mutations, and including significant deletions (Parrilla-Taylor et al. 2018).

Despite the significance of cell culture in shrimp immunology studies, the lack of shrimp immortal cell lines has only allowed the use of primary cell cultures. Due to this limitation, the primary culture of *Penaeus vannamei* hemocytes has been evaluated only by a few researchers (Ellender et al. 1992, Toullec et al. 1996, George & Dhar 2010, George et al. 2011, Dantas-Lima et al. 2012, Vieira-Girao et al. 2017, Thedcharoen et al. 2020). The use of cell cultures has facilitated the study under controlled conditions of different viruses, allowing us to identify the molecular mechanisms involved during the replication of viruses and their interactions with the cell host.

Therefore, this study aimed to evaluate three genes of the shrimp's immune response against viral infection (phagocytosis-activating protein PAP, manganese superoxide dismutase MnSOD and peroxiredoxin PRX) and two viral genes of immediate-early expression (IE1 and WSSV304) in a primary hemocyte cell culture from *P. vannamei* challenged with five different WSSV strains of high (JP and LG), moderate (GVE and DVI), and low (LC10) virulence. The intention was to determine the possible link between the genome size and virulence of WSSV strains, contributing to the knowledge of virulence and pathogenesis of WSSV in a highly productive area with recurrent disease events.

## MATERIALS AND METHODS

### Animals

Pathogen-free *Penaeus vannamei* (SPF) juvenile shrimp (12 ± 1 g) were supplied by Pichilingue, Aquaculture Laboratory, Universidad Autónoma de Baja California Sur (UABCS), La Paz, Mexico and transported to a wet laboratory at Centro de Investigaciones Biológicas del Noroeste (CIBNOR). Shrimp were acclimatized for 20 days in a recirculating water tank system with constant aeration (DO > 4 mg $L^{-1}$), with a 12:12 h light:dark photoperiod at 28 ± 0.5°C and salinity at 35. Tanks were cleaned daily, during which 20% of the total volume of water was changed; shrimp were fed twice a day (9:00 and 15:00 h) with a commercial food (35% protein, Purina).

### WSSV strains, inoculum preparation, and viral load quantification

One inoculum for each of the five WSSV strains (Parrilla-Taylor et al. 2018) was prepared from the gills

**Table 1.** Description of the white spot syndrome virus (WSSV) strains isolated from *Penaeus vannamei* used in this study. *Data reported by farms where the strain was isolated, not experimental data.

| WSSV strain | GenBank accession | Virulence* | Deletion in 130 and 275 kb position | Genome length (bp) |
|---|---|---|---|---|
| JP | MG432479 | High | 4 kb, 2.2 kb | 283,858 |
| LC10 | MG432480 | Low | 2.7 kb, 9.9 kb | 270,274 |
| LG | MG432482 | High | 2.7 kb, 9.9 kb | 257,675 |
| GVE | MG432478 | Moderate | 8.8 kb, 5.7 kb | 272,607 |
| DVI | MG432477 | Moderate | 2.7 kb, 9.9 kb | 290,879 |

and muscles of *P. vannamei* infected with WSSV strains (under laboratory conditions) according to the method described by Escobedo-Bonilla et al. (2005). WSSV strains are described in Table 1 (JP, LG, GVE, DVI and LC10); previously identified metadata are publicly available as NCBI Bioproject ID PRJNA413204 (Parrilla-Taylor et al. 2018). Tissues (1.3 g) were disrupted with a tissue homogenizer in 6 mL of PBS solution and were clarified by centrifugation at 5000 *g* at 4°C for 30 min. The supernatants were aseptically filtered through a 0.45 µM pore-size membrane, recovered in sterile centrifuge tubes, and stored at -80°C until further use. An aliquot of every inoculum was used to quantify the viral load of WSSV by real-time PCR (qPCR) at the CIBNOR-Hermosillo reference laboratory (Mendoza-Cano & Sánchez-Paz 2013).

**Development of primary hemocyte culture**

Every shrimp was dipped in 70% ethanol, and the cephalothorax was rinsed and disinfected with 96% ethanol under a laminar flood hood. Hemolymph was withdrawn from the ventral sinus at the base of the second abdominal pleonite using a 1 mL insulin syringe filled with 200 µL of pre-cooled SIC-EDTA marine anticoagulant in a proportion of 1:1 with hemolymph volume; it was mixed immediately and maintained under the ice. Cell count and viability were determined by microscopy using a Neubauer counting chamber and trypan blue exclusion; the mixture was diluted in a modified 2×L-15 medium (L-15, Leibovitz; Sigma-Aldrich) composed of 10.5% (v/v) Chen's salts (CS) (Chen & Wang 1999), 10% (v/v) fetal calf serum (FCS), and penicillin/streptomycin (P/S: 100 units $mL^{-1}$ 100 µg $mL^{-1}$) (pH 7.5).

**Titration of WSSV inoculum**

In aseptic conditions, 96-well plates were prepared and seeded with $1\times10^5$ cells/well in a total volume of 200 µL. Plates were incubated at 28°C for 20 h to allow cell adherence; the medium was discarded, and 100 µL of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$ viral dilution (eight replicates) of WSSV inoculum in 2×L-15 medium was added. The plates were incubated at 28°C for 1 h; after that, media were discarded, and 200 µL of 2×L-15 was added. Cytopathic effects (CPE) observations were made in an inverted microscope after incubation at 28°C for 20 h. Dilution endpoints were calculated by the method of Reed & Muench (1938), and the viral titer was expressed as the 50% tissue culture infective dose ($TCID_{50}$) for each of the five strains.

The titer of the five strains of WSSV was $10^{2.66}$ $TCID_{50}$ $mL^{-1}$ for the DVI strain (moderate virulence, genome length 290,879 bp) and $10^{3.5}$ $TCID_{50}$ $mL^{-1}$ for the rest of the strains (JP and LG strains, characterized as high virulence, genome length 283,858 and 257,675 bp, respectively; GVE05 strain with moderate virulence, 272,607 bp in length; and LC10 strain with low virulence and 270,274 bp in length).

**Haemocyte culture and challenge with WSSV**

Freshly collected hemocyte suspensions were prepared by seeding each of the 24 well cell culture plates with 600 µL of hemolymph suspension containing $1\times10^6$ cells. After 20 h of incubation at 28°C, plates were observed for adherence, and the medium was removed under aseptic conditions. The attached cells were exposed to 100 µL of inoculum dilution containing $4.99\times10^{3.5}$ $\mu L^{-1}$ viral copies for JP inoculum; $4.23\times10^{3.5}$ $\mu L^{-1}$ for LC10 inoculum; $1.03\times10^{2.5}$ $\mu L^{-1}$ for LG inoculum; $1.04\times10^{2.5}$ $\mu L^{-1}$ for GVE inoculum; and $6.48\times10^{3.3}$ $\mu L^{-1}$ viral copies for DVI inoculum and incubated at 28°C; after 1 h the inoculum was removed, and 600 µL of modified 2×L-15 medium was added. Control wells with hemocytes were treated with the modified 2×L-15 medium instead of WSSV. The plate cells were incubated and collected at 1, 3, 6, 12, and 24 h post-infection with four replicates in every case.

### Isolation of RNA from cells and RT-PCR

After incubation, the medium was removed, and 300 µL of TRIzol reagent (Invitrogen, USA) was added; cells were removed by repeated pipetting. The suspension was collected in micro-centrifuge tubes, and RNA extraction was accomplished according to the following protocol: 70 µL of chloroform was added and shaken vigorously for 15 s; the mixture was centrifuged at 11000 *g* for 15 min at 4°C. The upper phase was transferred, and 150 µL of isopropanol was added; the mixture was kept for 20 min at -4°C and hen centrifuged at 11000 *g* at 4°C for 20 min. The supernatant was discarded, and 200 µL of 70% ethanol was added to wash the pellet. The pellet RNA was air-dried and resuspended in 15 µL of DEPC water. The RNA was treated with 2 U of RNase-free DNAse I (Promega, Madison, WI, USA) at 37°C for 30 min to remove residual DNA. The enzyme was inactivated at 65°C for 10 min. The isolated nucleic acids were quantified and quality verified at 260/280 nm using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

RNA was reverse-transcribed into cDNA using ImProm II reverse transcriptase (Promega, Madison, WI, USA) and oligo (dT) primer with 20 µL of reaction mix following the manufacturer's instructions. RNA integrity was evaluated by PCR using shrimp β-actin (forward primer 5′-GAGACCTTCAACACCCAGC-3′ and reverse primer 5′-TAGGTGGTCTCGTGATGCC-3′) with the following conditions: 94°C for 3 min; 30 cycles of 94°C, 60°C for 30 s, and 72°C for 1 min; and elongation at 72°C for 8 min. The expression of the genes PAP, MnSOD and PRX were evaluated as a response to viral infection; moreover, two immediate-early expression genes of WSSV, IE1, and WSSV304, crucial for WSSV pathogenesis, were analyzed.

### Quantitative real-time PCR analysis of gene expression

Gene expression was analyzed by quantitative real-time PCR (qPCR) in 96-well optical plates with a Bio-Rad CFX384 system (Bio-Rad Laboratories, Richmond, CA) using SensiFAST Sybr NO-rOX Mix (Bioline, England) in 12 µL reactions. The qPCR conditions were 98°C for 2 min, 39 cycles of 95°C for 5 s, and 60°C for 15 s, including a dissociation protocol of 65°C for 5 s, 95°C for 5 s. All samples were run with three replicates, and no-template controls (NTC) were included in every run to monitor DNA contamination. The threshold cycle (Ct) values, which represented the PCR cycle when a fluorescent signal reached the threshold, were measured according to the setting of an auto-calculated baseline threshold in Bio-Rad CFX Manager software (Bio-Rad, USA). As a reference, the F0 subunit of the ATP synthase gene was used as the internal control (Alvarez-Lee et al. 2020), and the expression of immune-related and viral genes was determined relative to the expression of the internal control. Relative gene expression was calculated as $2^{-ddct}$ (Livak & Schmittgen 2001). The primers used are listed in Table 2.

### Statistical analysis

The results given are average values of replicates ± standard deviation. Data were evaluated for normal distribution and homogeneity of variance. Multiple comparisons were performed using the non-parametric Kruskal-Wallis test, and differences were considered significant at $P < 0.05$. Statistical analyses were conducted using Statistica version 7 software (StatSoft; Tulsa, OK, USA).

## RESULTS

### Expression of immune-related genes

The expression profiles of the three immune-related genes in a hemocyte culture after WSSV infection are shown in Figure 1. The maximum expression of PAP in the five strains was detected after 3 hpi (Fig. 1a). Comparing among hpi, PAP expression was significantly higher at 3 hpi compared to 6 hpi for LC10, 12 hpi for JP, and 24 hpi for LG. PAP expression levels in hemocytes remained at similar levels from 6 to 24 hpi, for all strains ($P > 0.05$) and among hpi. PAP expression in JP was significantly higher than in GVE at 1 hpi ($P < 0.05$). No significant differences in PAP expression were observed at 3 hpi, despite the same trend being shown at 1 hpi, which might have been due to the high standard error in this condition.

Significant differences were observed in the expression of MnSOD (Fig. 1b), which showed the earliest response with the highest values at 1 hpi, decreasing afterward and remaining stable for the rest of the infection. The LG strain showed significantly higher MnSOD expression at 1 hpi than at 24 hpi ($P < 0.05$). At 1 hpi, LG had significantly higher MnSOD expression than LC10.

Differences in expression levels of PRX in hemocytes after inoculation with WSSV are shown (Fig. 1c). PRX expression was significantly higher at 3 hpi than at 24 hpi in JP, LC10, LG, and GVE strains ($P < 0.05$). PRX expression was significantly higher in JP than in GVE at 1 hpi, in LC10 than in DVI at 3 hpi, and in DVI than in JP at 24 hpi ($P < 0.05$).

**Table 2.** The Primer sequences used in this study for real-time PCR quantification of gene expression in *Penaeus vannamei*.

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) | Accession # |
|---|---|---|---|
| Endogene - F0 | CGTCGTTAGGTTAAGTGCGTT | TTTCAGCGCATTAAGACGTGTT | AY368151.1 |
| Immune-related | | | |
| - PAP | CTAGCCCCCTATCAAAGGAACTGC | GGATGCCAACATAGACTGATGCAC | EF523242.1 |
| - MnSOD | CGTTGGAGTGAAAGGCTCTG | GATTTGCAAGGGATCCTGGT | DQ029053.1 |
| - PRX | TGGACCTCGCGGGAGAT | GACCGATAGCCACCATGCTT | AF188840.1 |
| Immediate-early of WSSV | | | |
| - IE1 | TGGCACAACAACAGACCCTA | TGCATTATCTTCGAGCCAGA | AAL88937.1 |
| - WSSV304 | CCGGACGGAGACGTGATAAG | AGCCCTGTACAATGCCTTCC | AAL88937.1 |

**Expression of WSSV-related genes**

Viral gene expression of IE1 and WSSV304 was selected and quantified to investigate *in vitro* expression of early WSSV genes. The expression of IE1 was induced in the first hpi (Fig. 2a). Comparing among hpi, LC10 was the only strain to show a significant difference in IE1 expression among hpi, being significantly higher at 3 hpi than at 6 hpi. Differences in the expression levels between strains were observed only at 24 hpi, with IE1 expression significantly lower in LC10 than in GVE and DVI.

As shown in Figure 2b, expression of the immediate-early gene WSSV304 was detected from the first hour. The expression of WSSV304 at 1 hpi in the LG strain was significantly higher than in GVE ($P < 0.05$). The DVI strain showed an increase in WSSV304 expression at 12 hpi, significantly higher than its expression at 3 hpi. Statistically significant differences were found when comparing the genetic expression of WSSV304 in the strains; expression in LG was significantly downregulated compared with the expression of DVI at 12 hpi; likewise, the expression of WSSV304 in JP was downregulated when compared to the DVI at 24 hpi.

## DISCUSSION

The innate immune system of shrimp relies on a variety of mechanisms, among which are cellular (as phagocytosis, encapsulation, among others, and mediated by hemocytes) and humoral components (including agglutinins, protease inhibitors, prophenoloxidase-activating systems) (Tyagi et al. 2007, Banerjee et al. 2015, Li et al. 2019, Kulkarni et al. 2021). The innate immune system recognizes and rapidly and efficiently eliminates foreign and toxic materials that can cause diseases (Casadevall & Pirofsky 1999, Aguirre-Guzmán et al. 2009). A disease is defined as the result of a complex interaction between the host, pathogen, and the environment, but the outcome of infectious diseases is mainly determined by the pathogen's virulence and the host's immune defense against invaders (Casadevall & Pirofsky 1999). The primary culture of hemocytes is a simple method of studying the mechanisms associated with host-virus interactions (Dantas-Lima et al. 2012). However, only Jose et al. (2010) have used a primary hemocyte culture from *P. monodon* as an *in vitro* model to study viral and immune-related gene expression of WSSV, suggesting that primary hemocyte cultures could be used to describe viral mechanisms of infection.

The pathogenesis of WSSV disease is complicated because of the genetic variation between strains (Marks et al. 2005, Gao et al. 2014, Parrilla-Taylor et al. 2018). Therefore, this study explores whether five WSSV strains can differentially modulate the expression of effector molecules of the innate immune system in shrimp with different genome lengths (257-290 kb) in hemocyte culture *in vitro*. They were previously shown to differ in virulence (JP, LG, GVE, DVI and LC10) (previously described by Parrilla-Taylor et al. 2018). The role of apoptosis (mainly the role played by initiator and effector caspases) in controlling the infection outcome has been studied, and the expression level of the (PAP) gene has been used as an indicator of activation of the immune response (Deachamag et al. 2006, Khimmakthong et al. 2013, Romo-Quiñonez et al. 2020). In this study, the expression of PAP was significantly higher at 1 and 3 hpi in JP compared with the rest of the strains. These results differ from a previous study in which Deachamag et al. (2006) challenged shrimp with inactivated WSSV, IVH, and fucoidan, detecting activation of the PAP gene at 6 h post-immunostimulation in the hemolymph. The eleva-

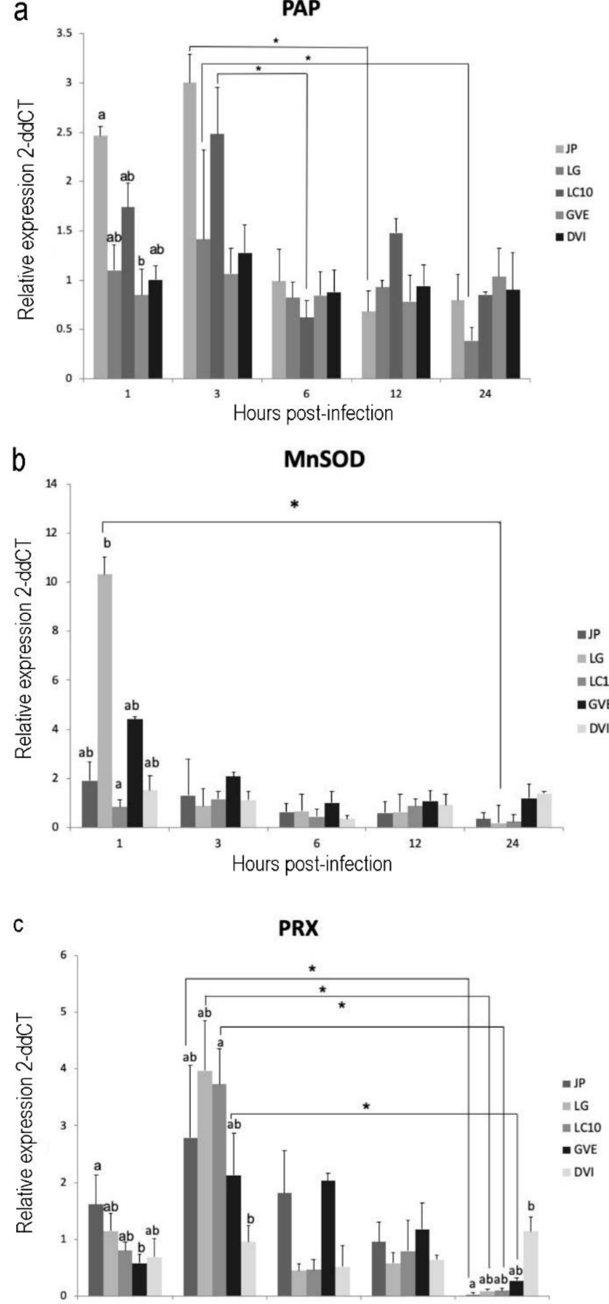
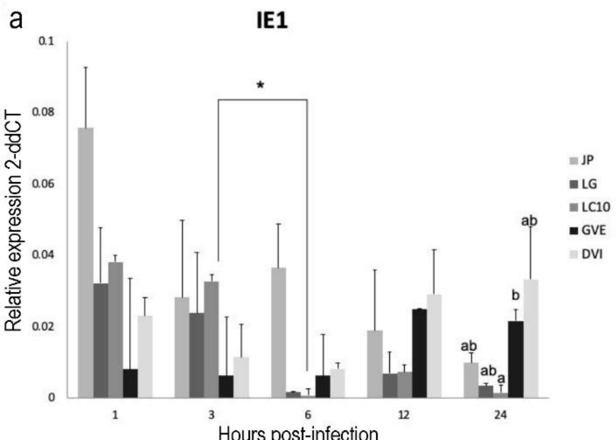



**Figure 1.** a) Relative expression levels of a) phagocytosis-activating protein (PAP), b) manganese superoxide dismutase (MnSOD), and c) peroxiredoxin (PRX) immune-related genes in an *in vitro* culture of hemocytes from *Penaeus vannamei* infected with JP, LC10, LG, GVE, and DVI WSSV strains. Bars represent mean values with standard errors. Statistically significant differences among hours post-infection are indicated with an asterisk, and differences among strains are indicated with superscript letters.



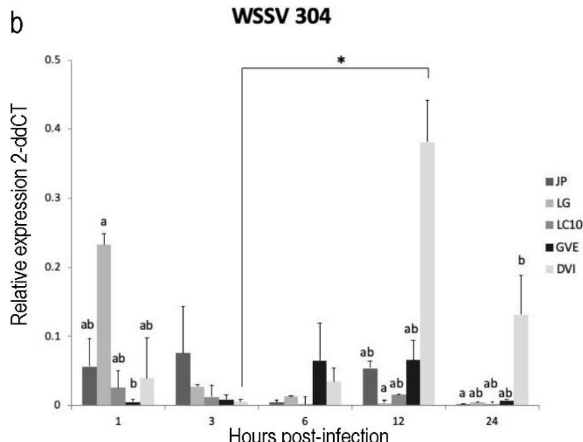




**Figure 2.** a) Relative expression levels of a) IE1 and b) WSSV304 immediate-early white spot syndrome virus (WSSV) genes in an *in vitro* culture of hemocytes from *Penaeus vannamei* infected with JP, LC10, LG, GVE, and DVI WSSV strains. Bars represent mean values with standard errors. Statistically significant differences among hours post-infection are indicated with an asterisk, and differences among strains are indicated with superscript letters.

ted expression of immune-related genes at 1 and 3 hpi observed in this study may be due to the rapid cellular response since the WSSV was introduced directly into the cells, resulting in a rapid infection. JP and LC10 were reported as high and low virulence strains, respectively, which induced the highest expression levels of PAP at the beginning of infection without an apparent relationship with genome length or virulence.

Reactive oxygen species (ROS) are a two-edged sword that can kill pathogens and damage the host (Yu 1994, Tripathy & Mohanty 2017, Maldonado et al. 2020). WSSV infection has been reported to induce oxidative stress in the host by releasing ROS (Mohankumar & Ramasamy 2006), with their level

increasing on hemocytes shrimp during the initial 30-120 min of the infection (Chen et al. 2016). SOD is the first line of defence against ROS to eliminate possible oxidative harm and is essential for reducing oxidative stress. High MnSOD expression levels can indicate a better opportunity to cope with oxidative stress. Our data in Figure 1b indicate a rapid response of MnSOD detected at 1 hpi, decreasing afterward. In a previous study and consistent with these results, the level of MnSOD activity in the hemolymph of *Penaeus vannamei* infected with WSSV was higher at 1 hpi than at 24 hpi (Parrilla-Taylor et al. 2013).

Peroxiredoxins are a family of antioxidant enzymes that modulate biological functions such as apoptosis, lipid metabolism, and immune response, among others (Abbas et al. 2019). They remove hydrogen peroxide to reduce oxidative stress and protect against the inactivation of multiple cellular enzymes closely related to the generation and elimination of ROS during phagocytosis (Le Moullac et al. 1998, Rhee et al. 2005, Zheng et al. 2015); MnSOD primarily produces hydrogen peroxide and, as a consequence, both of them may function as an efficient microbicidal attack system against invading microorganisms (Sritunyalucksana et al. 2001). The results were consistent with those for PAP expression, which showed the highest levels at 3 hpi, allowing the occurrence of an oxidative burst. The significant increase of PRX expression at 3 hpi may have been due to the ROS increase produced during the infection that had to be scavenged by extra PRX translated from more PRX transcripts. In general, the expression of most immune-related genes decreased after the first hours of infection.

As previously suggested, the genome evolves through shrinking, and a WSSV strain with a small genome tends to be more virulent and fit (Pradeep et al. 2009). In this study, the WSSV strain with the smallest genome (LG, 257 kb genome length) induced a significantly higher level of host mortality, suggesting a high viral fitness. According to mortality data from the reactivation bioassay of the WSSV strains, JP (283 kb genome length) and LG (257 kb genome length) were highly virulent, showing 100% mortality at 48 hpi as opposed to the DVI (290 kb genome length), which displayed lower mortality and has a larger genome (Parrilla-Taylor et al. 2018). In the LG strain, expression levels of MnSOD and PRX were significantly higher at 1 and 3 hpi, respectively, suggesting an acute response to avoid cellular damage and oxidative stress due to infection.

WSSV infection caused a substantial decrease in the expression of genes of antioxidants such as MnSOD and PRX. These results might be accompanied by an increase in lipid peroxidation and carbonylated proteins, indicating that increases in ROS after lethal challenges with WSSV cause cellular damage and death (Rameshthangam & Ramasamy 2006, Parrilla-Taylor et al. 2013, Zheng et al. 2015).

IE WSSV genes encode regulatory proteins critical for viral infection and the switch from latency to lytic cycle, viral replication, and transcription (Honess & Roizman 1974). Several IE genes have been identified; among them, the IE1 gene has been particularly studied. IE1 is a highly expressed transcription factor with DNA-binding and transac-tivation activity that plays a fundamental role in promoting viral replication and proliferation for WSSV infection (Liu et al. 2008). IE1 also interacts with host proteins, such as JNK, and establishes a positive feedback loop to promote viral replication (Wang et al. 2020). A less studied IE gene is WSSV304, which contains RING domains and functions as a ubiquitin E3 ligase, which is important for pathogenesis in shrimps (Huang et al. 2014). IE1 and WSSV304 are regulated by Kruppel-like factors (KLFs), and their transcrip-tional regulation is critical to WSSV replication (Huang et al. 2014).

In this study, the expression of IE1 was especially high at 1 hpi in almost all the analyzed strains; the high expression at the beginning of the infection indicated the WSSV strains' efficient mechanism for replication, given that viral IE genes did not require *de novo* synthesis to be activated (Huang et al. 2014). Early genes, such as IE1, *tk-tmk*, and *rr1*, are expressed as early as 2 hpi in infected hemocyte culture (Jose et al. 2010).

The DVI strain, with the largest genome (290 kbp), induced a moderate level of mortality which could have been correlated with lower levels of PAP expression and delayed expression of IE1 and WSSV304. It has been shown that WSSV strains of different virulence can affect the immune system of crayfish *Cherax quadricarinatus* and shrimp *P. vannamei* in various ways (Gao et al. 2014, Sun et al. 2016, 2018). Isolates with greater virulence may encode virulence-associated factors that modulate the host's physical or immune status and are absent or have lost their function in less virulent isolates (Gao et al. 2014).

Sarathi et al. (2007) and Zwart et al. (2010) hypothesized that genome shrinkage of WSSV is an adaptive process that might give the virus a replication advantage and contribute to increasing viral fitness and virulence. Our results do not show an accurate link between WSSV genome size and virulence (Table 1);

however, there is a trend supporting this observation since the strain with the smallest genome showed high virulence while the strains with the largest genomes showed moderate to low virulence. The possibility exists that, beyond the genome size effect, proteins with minor alterations in their amino acid sequence and genomic variations in non-coding regions may also contribute to differences in virulence as reported for other viruses such as African swine fever virus (Chapman et al. 2008), frog virus 3 (Morrison et al. 2014), human adenovirus (Hage et al. 2014) and influenza A (Yamayoshi et al. 2014). Li et al. (2017) suggested the presence of factors related to virulence on WSSV isolates that might be absent on low virulent isolates. The pathogenesis of individual WSSV strains is not yet completely understood, and further research is required to identify a virulence-determining mechanism. Despite the link between genome size and virus fitness in WSSV, we could not demonstrate virulence determinants by analyzing the response of shrimp to infection with virus strains differing in genome size, raising new questions about the host-pathogen interactions.

## ACKNOWLEDGMENTS

The first author was funded by Consejo Nacional de Ciencia y Tecnología (CONACYT) (Grant Number 267040) in Mexico. The authors thank Zulia F. Nieves-López (UAN) and Sandra de La Paz for technical support; and Diana Fischer for editorial services.

## REFERENCES

Abbas, M.N., Kausar, S. & Cui, H. 2019. The biological role of peroxiredoxins in innate immune responses of aquatic invertebrates. Fish and Shellfish Immunology, 89: 91-97. doi: 10.1016/j.fsi.2019.03.062

Aguirre-Guzmán, G., Sánchez-Martínez, J.G., Campa-Córdova, A.I., Luna-González, A. & Ascencio, F. 2009. Penaeid shrimp immune system. Thai Journal of Veterinary Medicine, 39: 205-215.

Alvarez-Lee, L., García-Gasca, A., Martínez-Díaz, S. & Gutiérrez-Rivera, J.N. 2020. Selection of reference genes for the study of relative gene expression during development of *Litopenaeus vannamei* larvae. Aquaculture Research, 5: 2997-3006. doi: 10.1111/are.14638

Bachère, E. 2000. Shrimp immunity and disease control. Aquaculture, 191: 3-11. doi: 10.1016/S0044-8486(00)00413-0

Banerjee, D., Maiti, B., Girisha, S.K., Venugopal, M.N. & Karunasagar, I. 2015. Broad spectrum anti-bacterial activity of a recombinant phosphatase-like protein (rPLP), isolated from the shrimp *Penaeus monodon*. Israeli Journal of Aquaculture-Bamidgeh, 67: 1-7.

Casadevall, A. & Pirofski, L.A. 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infection and Immunity, 67: 3703-3713.

Chapman, D.A., Tcherepanov, V., Upton, C. & Dixon, L.K. 2008. Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. Journal of General Virology, 89: 397-408. doi: 10.1099/vir.0.83343-0

Chen, S.N. & Wang, C.S. 1999. Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses. Methods in Cell Science, 21: 199-206. doi: 10.1023/a:1009885929335

Chen, I., Lee, D.Y., Huang, Y.T., Kou, G.H., Wang, H.C., Chang, G.D. & Lo, C.F. 2016. Six hours after infection, the metabolic changes induced by WSSV neutralize the host's oxidative stress defenses. Scientific Reports, 6: 1-14. doi: 10.1038/srep27732

Dantas-Lima, J.J., Corteel, M., Oanh, D.T.H., Bossier, P., Sorgeloos, P. & Nauwynck, H.J. 2012. Development of two haemocyte culture systems (in attachment and in suspension) for shrimp immunity studies. Aquaculture, 366-367: 17-26. doi: 10.1016/j.aquaculture.2012.08.032

Deachamag, P., Intaraphad, U., Phongdara, A. & Chotigeat, W. 2006. Expression of a phagocytosis activating protein (PAP) gene in immunized black tiger shrimp. Aquaculture, 255: 165-172. doi: 10.1016/j.aquaculture.2006.01.010

Dieu, B.T.M., Marks, H., Siebenga, J.J., Goldbach, R.W., Zuidema, D., Duong, T.P. & Vlak, J.M. 2004. Molecular epidemiology of white spot syndrome virus within Vietnam. Journal of General Virology, 85: 3607-3618. doi: 10.1099/vir.0.80344-0

Durán-Avelar, M.J., Pérez-Enríquez, R., Zambrano-Zaragoza, J.F., Montoya-Rodríguez, L., Vázquez-Juárez, R. & Vibanco-Pérez, N. 2015. Genotyping WSSV isolates from northwestern Mexican shrimp farms affected by white spot disease outbreaks in 2010-2012. Diseases of Aquatic Organisms, 114: 11-20. doi: 10.3354/dao02844

Ellender, R.D., Najafabadi, A.K. & Middlebrooks, B.L. 1992. Observations on the primary culture of hemo-

cytes of *Penaeus*. Journal of Crustacean Biology, 12: 178-185. doi: 10.2307/1549072

Escobedo-Bonilla, C.M., Wille, M., Alday-Sanz, V., Sorgeloos, P., Pensaert, M.B. & Nauwynck, H.J. 2005. *In vivo* titration of white spot syndrome virus (WSSV) in specific pathogen-free *Litopenaeus vannamei* by intramuscular and oral routes. Diseases of Aquatic Organisms, 66: 163-170. doi: 10.3354/dao066163

Gao, M., Li, F., Xu, L. & Zhu, X. 2014. White spot syndrome virus strains of different virulence induce distinct immune response in *Cherax quadricarinatus*. Fish and Shellfish Immunology, 39: 17-23. doi: 10.1016/j.fsi.2014.04.011

George, S.K. & Dhar, A.K. 2010. An improved method of cell culture system from eyestalk, hepatopancreas, muscle, ovary, and hemocytes of *Penaeus vannamei*. In Vitro Cellular & Developmental Biology - Animal, 46: 801-810. doi: 10.1007/s11626-010-9343-x

George, S.K., Kaizer, K.N., Betz, Y.M. & Dhar, A.K. 2011. Multiplication of Taura syndrome virus in primary hemocyte culture of shrimp (*Penaeus vannamei*). Journal of Virological Methods, 172: 54-59. doi: 10.1016/j.jviromet.2010.12.020

Hage, E., Huzly, D., Ganzenmueller, T., Beck, R., Schulz, T.F. & Heim, A. 2014. A human adenovirus species B subtype 21a associated with severe pneumonia. Journal of Infection, 69: 490-499. doi: 10.1016/j.jinf. 2014.06.015

Hoa, T.T.T., Zwart, M.P., Phuong, N.T., Oanh, D.T.H., de Jong, M.C.M. & Vlak, J.M. 2012. Indel-II region deletion sizes in the white spot syndrome virus genome correlate with shrimp disease outbreaks in southern Vietnam. Diseases of Aquatic Organisms, 99: 153-162. doi: 10.3354/dao02463

Honess, R.W. & Roizman, B. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. Journal of Virology, 14: 8-19.

Huang, P-H., Lu, S-C., Yang, S-H., Cai, P-S., Lo, C-F. & Chang, L-K. 2014. Regulation of the immediate-early genes of white spot syndrome virus by *Litopenaeus vannamei* Kruppel-like factor (LvKLF). Developmental and Comparative Immunology, 46: 364-367. doi: 10.1016/j.dci.2014.05.012

Johansson, M.W., Keyser, P., Sritunyalucksana, K. & Soderhall, K. 2000. Crustacean haemocytes and haematopoiesis. Aquaculture, 191: 45-52. doi: 10.1016/ S0044-8486(00)00418-X

Jose, S., Mohandas, A., Philip, R. & Bright, S.I. 2010. Primary hemocyte culture of *Penaeus monodon* as an *in vitro* model for white spot syndrome virus titration, viral and immune-related gene expression and cytotoxicity assays. Journal of Invertebrate Pathology, 105: 312-321. doi: 10.1016/j.jip. 2010.08.006

Khimmakthong, U., Kongmee, P., Deachamag, P., Leggat, U. & Chotigeat, W. 2013. Activation of an immune response in *Litopenaeus vannamei* by oral immunization with phagocytosis activating protein (PAP) DNA. Fish and Shellfish Immunology, 34: 929-938. doi: 1016/j.fsi.2013.01.004

Kulkarni, A., Krishnan, S., Deepika, A., Kokkattunivarthil, D., Uthaman, S., Kumar-Otta, S., et al. 2021. Immune responses and immunoprotection in crustaceans with special reference to shrimp. Reviews in Aquaculture, 13: 431-459. doi: 10.1111/raq.12482

Le Moullac, G., Soyez, C., Saulnier, D., Ansquer, D., Christophe, J. & Levy, P. 1998. Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*. Fish and Shellfish Immunology, 8: 621-629. doi: 10.1006/fsim.1998. 0166

Li, C., Weng, S. & He, J. 2019. WSSV-host interaction: host response and immune evasion. Fish and Shellfish Immunology, 84: 558-571. doi: 10.1016/j.fsi.2018. 10.043

Li, F., Gao, M., Xu, L. & Yang, F. 2017. Comparative genomic analysis of three white spot syndrome virus isolates of different virulence. Virus Genes, 53: 249-258. doi: 10.1007/s11262-016-1421-z

Lightner, D.V. 1996. A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge.

Lightner, D.V., Redman, R.M., Pantoja, C.R., Tang, K.F.J., Noble, B.L., Schofield, P., et al. 2012. Historic emergence, impact and current status of shrimp pathogens in the Americas. Journal of Invertebrate Pathology, 110: 174-183. doi: 10.1016/j.jip.2012.03. 004

Liu, W.J., Chang, Y.S., Wang, H.C., Leu, J.H., Kou, G.H. & Lo, C.F. 2008. Transactivation, dimerization, and DNA-binding activity of white spot syndrome virus immediate-early protein IE1. Journal of Virology, 82: 11362-11373. doi: 10.1128/JVI.01244-08

Livak, K.J. & Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods, 25: 402-408. doi: 10.1006/meth.2001.1262

Maldonado, E., Rojas, D.A., Morales, S., Miralles, V. & Solari, A. 2020. Dual and opposite roles of reactive oxygen species (ROS) in Chagas disease: beneficial on

the pathogen and harmful on the host. Oxidative Medicine and Cellular Longevity, 2020: 1-17. doi: 10.1155/2020/8867701

Marks, H., Goldbach, R.W., Vlak, J.M. & Van Hulten, M.C.W. 2004. Genetic variation among isolates of white spot syndrome virus. Archives of Virology, 149: 673-697. doi: 10.1007/s00705-003-0248-9

Marks, H., Van Duijse, J.J., Zuidema, D., Van Hulten, M.C.W. & Vlak, J.M. 2005. Fitness and virulence of an ancestral white spot syndrome virus isolate from shrimp. Virus Research, 110: 9-20. doi: 10.1016/j.virusres.2005.01.002

Mendoza-Cano, F. & Sánchez-Paz, A. 2013. Development and validation of a quantitative real-time polymerase chain assay for universal detection of the white spot syndrome virus in marine crustaceans. Virology Journal, 10: 186. doi: 10.1186/1743-422X-10-186

Mohankumar, K. & Ramasamy, P. 2006. White spot syndrome virus infection decreases the activity of antioxidant enzymes in *Fenneropenaeus indicus*. Virus Research, 115: 69-75. doi: 10.1016/j.virusres.2005.07.006

Morrison, E.A., Garner, S., Echaubard, P., Lesbarrères, D., Kyle, C.J. & Brunetti, C.R. 2014. Complete genome analysis of a frog virus 3 (FV3) isolate and sequence comparison with isolates of differing levels of virulence. Virology Journal, 11: 1-13. doi: 10.1186/1743-422X-11-46

Oakey, H.J. & Smith, C.S. 2018. Complete genome sequence of a white spot syndrome virus associated with a disease incursion in Australia. Aquaculture, 484: 152-159. doi: 10.1016/j.aquaculture.2017. 11.009

Parrilla-Taylor, D.P., Zenteno-Savin, T. & Magallon-Barajas, F. 2013. Antioxidant enzyme activity in Pacific whiteleg shrimp (*Litopenaeus vannamei*) in response to infection with white spot syndrome virus. Aquaculture, 380-383: 41-46. doi: 10.1016/j.aquaculture.2012.11.031

Parrilla-Taylor, D.P., Vibanco-Pérez, N., Durán-Avelar, M.J., Gómez-Gil, B., Llera-Herrera, R. & Vázquez-Juárez, R. 2018. Molecular variability and genetic structure of white spot syndrome virus strain from northwest Mexico based on the analysis of genomes. FEMS Microbiology Letters, 365: fny216. doi: 10.1093/femsle/fny216

Patil, P.K., Geetha, R., Ravisankar, T., Avunje, S., Solanki, H.G., Abraham, T.J., et al. 2021. Economic loss due to diseases in Indian shrimp farming with special reference to *Enterocytozoon hepatopenaei* (EHP) and white spot syndrome virus (WSSV). Aquaculture, 533: 736231. doi: 10.1016/j.aquaculture.2020.736231

Pradeep, B., Karunasagar, I. & Karunasagar, I. 2009. Fitness and virulence of different strains of white spot syndrome virus. Journal of Fish Diseases, 32: 801-805. doi: 10.1111/j.1365-2761.2009.01053.x

Pradeep, B., Shekar, M., Karunasagar, I. & Karunasagar, I. 2008. Characterization of variable genomic regions of Indian white spot syndrome virus. Virology, 376: 24-30. doi: 10.1016/j.virol.2008.02.037

Rameshthangam, P. & Ramasamy, P. 2006. Antioxidant and membrane-bound enzymes activity in WSSV-infected *Penaeus monodon* Fabricius. Aquaculture, 254: 32-39. doi: 10.1016/j.aquaculture.2005.10.011

Reed, L.J. & Muench, H. 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene, 27: 493-497.

Rhee, S.G., Chae, H.Z. & Kim, K. 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radical Biology & Medicine, 38: 1543-1552.

Romo-Quiñonez, C.R., Álvarez-Sánchez, A.R., Álvarez-Ruiz, P., Chávez-Sánchez, M.C., Bogdanchikova, N., Pestryakov, A. & Mejía-Ruiz, C.H. 2020. Evaluation of a new argovit as an antiviral agent included in feed to protect the shrimp *Litopenaeus vannamei* against white spot syndrome virus infection. PeerJ, 8: e8446. doi: 10.7717/peerj.8446

Sarathi, M., Ahmed, V., Venkatesan, C., Balasubramanian, G., Prabavathy, J. & Hameed, A. 2007. Comparative study on the immune response of *Fenneropenaeus indicus* to *Vibrio alginolyticus* and white spot syndrome virus. Aquaculture, 271: 8-20. doi: 10.1016/j.aquaculture.2007.07.002

Sritunyalucksana, K., Wongsuebsantati, K., Johansson, M.W. & Soderhall, K. 2001. Peroxinectin, a cell-adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. Developmental and Comparative Immunology, 25: 353-363. doi: 10.1016/s0145-305x(01)00009-x

Stentiford, G.D., Bonami, J.R. & Alday-Sanz, V. 2009. A critical review of the susceptibility of crustaceans to Taura syndrome, yellowhead disease and white spot disease and implications of inclusion of these diseases in European legislation. Aquaculture, 291: 1-17. doi: 10.1016/j.aquaculture .2009.02.042

Sun, X.Y., Liu, Q.H. & Huang, J. 2018. iTRAQ-based quantitative proteomic analysis of differentially expressed proteins in *Litopenaeus vannamei* in response to infection with WSSV strains varying in virulence. Letters in Applied Microbiology, 67: 113-122. doi: 10.1111/lam.13004

Sun, X.Y, Liu, Q.H., Yang, B. & Huang, J. 2016. Differential expression of microRNAs of *Litopenaeus vannamei* in response to different virulence WSSV infection. Fish and Shellfish Immunology, 58: 18-23. doi: 10.1016/j.fsi.2016.08.062

Thedcharoen, P., Pewkliang, Y., Kiem, H.K.T., Nuntakarn, L., Taengchaiyaphum, S., Sritunyalucksana, K., et al. 2020. Effective suppression of yellow head virus replication in *Penaeus monodon* hemocytes using constitutive expression vector for long-hairpin RNA (lhRNA). Journal of Invertebrate Pathology, 175: 107442. doi: 10.1016/j.jip.2020.107442

Toullec, J.Y., Crozat, Y., Patrois, J. & Porcheron, P. 1996. Development of primary cell cultures from the penaeid shrimps *Penaeus vannamei* and *P. indicus*. Journal of Crustacean Biology, 16: 643-649. doi: 10.1163/19 3724096X00711

Tripathy, S. & Mohanty, P.K. 2017. Reactive oxygen species (ROS) are boon or bane. International Journal of Pharmaceutical Sciences and Research, 8: 1. doi: 10.13040/IJPSR.0975-8232.8(1).1-16

Tyagi, A., Khushiramani, R., Karunasagar, I. & Karunasagar, I. 2007. Antivibrio activity of recombinant lysozyme expressed from black tiger shrimp, *Penaeus monodon*. Aquaculture, 272: 246-253. doi: 10.1016/j.aquaculture.2007.08.055

Van Hulten, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., et al. 2001. The white spot syndrome virus DNA genome sequence. Virology, 286: 7-22. doi: 10.1006/viro. 2001.1002

Vieira-Girao, P.R.N., Falcao, C.B., Rocha, I.R.C.B., Lucena, H.M.R., Costa, F.H.F. & Radis-Baptista, G. 2017. Antiviral activity of Ctn [15-34], a cathelicidin-derived eicosapeptide, against infectious myonecrosis virus in litopenaeus vannamei primary hemocyte cultures. Food and Environmental Virology, 9: 277-286. doi: 10.1007/s12560-017-9285-5

*Received: September 8, 2021; Accepted: July 25, 2022*

Wang, S., Li, H., Weng, S., Li, C. & He, J. 2020. White spot syndrome virus establishes a novel IE1/JNK/c-Jun positive feedback loop to drive replication. Iscience, 23: 100752. doi: 10.1016/j.isci.2019.100752

Wongteerasupaya, C., Pungchai, P., Withyachumnarnkul, B., Boonsaeng, V., Panyim, S., Flegel, T.W. & Walker, P.J. 2003. High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. Diseases of Aquatic Organisms, 54: 253-257. doi: 10.3354/dao054253

Yamayoshi, S., Yamada, S., Fukuyama, S., Murakami, S., Zhao, D., Uraki, R., et al. 2014. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses. Journal of Virology, 88: 3127-3134.

Yu, B.P. 1994. Cellular defenses against damage from reactive oxygen species. Physiological Reviews, 74: 139. doi: 10.1152/physrev.1994.74.1.139

Zeng, D., Peng, M., Chen, X., Yang, C., Chen, X., Li, X. & Zhao, Y. 2015. Molecular cloning, characterization, and immune response against white spot syndrome virus and Taura syndrome virus infections of peroxiredoxin in *L. vannamei* and its antioxidant activity. Crustaceana, 88: 1149-1161. doi: 10.1163/15685403-00003476

Zwart, M.P., Dieu, B.T.M., Hemerik, L. & Vlak, J.M. 2010. Evolutionary trajectory of white spot syndrome virus (WSSV) genome shrinkage during spread in Asia. Plos One, 5: e13400. doi: 10.1371/journal.pone.0013400