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The white spot syndrome virus hijacks the expression of the *Penaeus vannamei* Toll signaling pathway to evade host immunity and facilitate its replication

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ABSTRACT

The white spot syndrome virus (WSSV), the most lethal pathogen of shrimp, is a dsDNA virus with approximately a 300,000 base pairs and contains approximately 180–500 predicted open reading frames (ORFs), of which only 6% show homology to any known protein from other viruses or organisms. Although most of its ORFs encode enzymes for nucleotide metabolism, DNA replication, and protein modification, the WSSV uses some of its encoded proteins successfully to take control of the metabolism of the host and avoid immune responses. The contribution of the shrimp innate immune response to prevent viral invasions is recognized but yet not fully understood. Thus, the role of several components of Toll pathway of the shrimp *Penaeus vannamei* against WSSV has been previously described, and the consequential effects occurring through the cascade remain unknown. In the current study the effects of WSSV over various components of the shrimp Toll pathway were studied. The gene expression of Spätzle, Toll, Tube, Cactus and Dorsal was altered after 6–12 h post inoculation. The expression of *LvToll3*, *LvCactus*, *LvDorsal*, decreased ~4.4-, ~3.7- and ~7.3-fold at 48, 24 and 48 hpi, respectively. Furthermore, a remarkable reduction (~18-fold) in the expression of the gene encoding *LvCactus* in WSSV infected specimens was observed at 6 hpi. This may be a sophisticated strategy exploited by WSSV to evade the Toll-mediated immune action, and to promote its replication, thereby contributing to viral fitness.

1. Introduction

All living forms are exposed continuously to biotic stressors that include, among others, challenges to the immune system. Thus, from the simplest prokaryotes to the most complex vertebrates, all species must defend themselves from invading pathogens [1], and the response triggered relies on both the immune defenses they have fixed through their evolutionary history and the nature of the pathogen [2,3].

Invertebrates represent up to 97% of animal diversity and inhabit nearly every environment on Earth, which implies that they should adapt to a plethora of challenges in order to survive and thrive [4]. Viral pathogens are among the most serious threats for the survival of invertebrates, and the immune response is the major physiological mechanism ensuring the survival and fitness of these organisms [5]. In the never-ending battle between a virus and its host, the innate immune system plays an essential role in preventing the harmful effects of pathogens [6,7], and it consists of two cooperative branches: the humoral and cell-mediated immune responses, which interact tightly [8–10].

Shrimp diseases are highly relevant due to the adverse effect they pose over one of the most successful fishing industries in the world [11]. It has been reported that viral diseases represent the most serious risk to the shrimp farming industry, as nearly 60% of disease losses in shrimp aquaculture could be attributed to viral pathogens [12–15]. Among all the different viruses infecting shrimp, the white spot syndrome virus (WSSV) has been reported as the most prevalent, highly lethal, contagious, and widespread [16]. This virus can cause

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cumulative mortalities of up to 100% within three days after the onset of the clinical signs [17,18], resulting in enormous economic losses amounting to billions of US dollars for the global shrimp farming industry [19], and currently, there are no effective treatments against this disease [16,20]. Furthermore, a wide range of crustaceans is susceptible to WSSV infection [16,21], which may pose a serious threat to biodiversity. It is well known that the WSSV has evolved several mechanisms to evade, or annex on its own benefit, the host defenses [22–31], but, on the other way, shrimp have also evolved a number of immune strategies to provide maximum protection against the harmful effects of WSSV [32], a sort of competitive, cyclical Red Queen evolutionary dynamics [33].

The Toll pathway, which was identified for the first time in the early 1980s in the fruit fly *Drosophila melanogaster* as a critical factor for dorsal-ventral patterning during embryonic development [34], is also intimately involved in the immune response. The shrimp defense system involves the synthesis of a variety of potent antimicrobial peptides (AMPs), as penaeidins [35,36], crustins [37–40], antilipopolysaccharide factors [41,42], and stylicins [43,44], among others. The induction of the expression of such AMPs in shrimp is regulated by the different components of the Toll signaling pathway [22,45–49]. However, to the best of our knowledge, the information on the expression of the different components of the Toll pathway, as Spätzle, Toll, Tube, Cactus, and Dorsal, of the shrimp *Penaeus vannamei* during a viral infection remains largely fragmented, and there are no comprehensive studies dealing with the expression of the majority of the genes encoding the components of the Toll pathway throughout the course of a viral disease. Therefore, the present study aimed to explore the changes in the gene expression patterns of the main components of the Toll pathway cascade of the shrimp *Penaeus vannamei* induced by the experimental infection with WSSV. Understanding the molecular basis of disease processes and the immune response of the shrimp may contribute significantly to the development of a treatment against this viral pathogen.

2. Materials and methods

2.1. Shrimp samples

Three hundred specimens of the Pacific whiteleg shrimp *Penaeus vannamei* (average weight of 12.5 g) were donated by Quinta San Fabián Acuacultores, S.P.R. de R.L. de C.V. Organisms were acclimatized for five days in two 1000 L-plastic tanks containing 500 L of purified, aerated and UV-treated seawater (34 ppt) at 28 °C. Shrimp were fed twice daily ad libitum with a commercial feed (Camaronina 35, Agribrands Purina®). Prior to use, specimens were PCR-tested for detection of WSSV, and only WSSV-free individuals were used for experiments. One-hundred μL of hemolymph were aseptically withdrawn from the ventral sinus of each organism using a sterile 1 mL tuberculin syringe (with a 26 gauge needle) filled with 300 μL of shrimp anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM Na_2-EDTA, 10 mM HEPES, pH 7.3) [50]. Hemolymph samples were centrifuged at 600 rpm for 10 min at 4 °C to separate hemocytes from the plasma. DNA was isolated from hemocytes using the DNAzol reagent (Life Technologies) according to the manufacturer's instructions. Samples were screened for WSSV and PstDV-1 following the diagnostic procedure previously described by Refs. [51,52].

Healthy shrimp were randomly divided into two groups of 42 organisms in tanks with 500 L of seawater. Shrimp were maintained under the same acclimation conditions until the end of the experiment.

2.2. WSSV inoculum preparation and experimental infection

A WSSV inoculum was prepared according to a modified protocol described previously [53]. A WSSV-infected shrimp (*P. vannamei*) was sacrificed by decapitation, and the abdominal muscle was weighed and transferred to a 250 mL glass blender containing six volumes of a 15.4 mM precooled saline solution. The muscle was homogenized at maximum speed using 10 pulses of 10 s with 30 s pauses for cooling in crushed ice. The mixture was poured into 1.7-mL tubes and centrifuged at 3000 × *g* for 20 min at 4 °C. The supernatant was transferred into new tubes, and centrifugation was repeated; all supernatant was collected and filtered through a 0.22 μm syringe-filter membrane (Acrodisc, Pall Gelman), and the inoculum obtained was stored at −80 °C until used. Viral load was quantified by real-time PCR using the method described by Ref. [51].

Healthy shrimp were then distributed in two treatments: the control group (n = 42), which was inoculated with 100 μL of a 15.4 mM saline solution, and the infected group (n = 42), injected with 100 μL of viral inoculum (5.71×10^4 total copies). Organisms were fed ad libitum 2 h before sampling and food particles were removed 1 h prior sampling. Six shrimp were sampled from each treatment at 0, 3, 6, 12, 24, 48, and 72 h post inoculation (hpi). Pleopods and gills were carefully dissected and fixed in 1 mL of RNAlater™ (Thermo Fisher Scientific) and stored at −80 °C until use.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the gills (50 mg) using TRIzol reagent (Invitrogen) following the manufacturer instructions. The RNA concentration and purity were determined through a NanoDrop Lite spectrophotometer (Thermo Scientific, USA) at 260/280 nm wavelengths. Then, the RNA integrity was analyzed in 1% agarose gels following the methodology described by Ref. [54]. Genomic DNA (gDNA) was removed from RNA samples with DNase I recombinant RNase-free (Roche) according to manufacturer's instructions. The complementary DNA (cDNA) synthesis was prepared from 3.2 μg of total RNA using the commercial kit SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions.

2.4. DNA isolation

DNA was isolated from pleopods samples using DNAzol® reagent (Invitrogen) following the manufacturer's instructions for viral load estimation for monitoring the WSSV replication kinetics. The DNA concentration and purity were determined through spectrophotometric analysis at 260/280 nm.

2.5. Primer design and PCR amplification

Primers for amplification of the genes encoding the components of the Toll signaling pathway, Spätzle, Toll, Cactus, and Dorsal of *P. vannamei*, and WSSV-VP28 used in this study were previously described by others (see Table 1). Primers for amplification of the gene encoding Tube were designed according to the sequence of *P. vannamei* (GenBank accession number: KC346865), using the online software Primer 3 v.4.1.0 (http://bioinfo.ut.ee/primer3/) [55], and the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [56] was used to determine the sequence identity of each primer. The sequences of primers and the length of the PCR products are listed in Table 1.

Endpoint PCR was performed to amplify a fragment of each gene in a 15 μL final volume reaction mix containing 7.5 μL of iQ SYBR® Green Supermix (Bio-Rad), 0.5 μL of each primer (10 pmol), 1 μL of DNA template (10 ng/μL), and 5.5 μL of nuclease-free water. Cycling conditions for the amplification were: 1 cycle of 98 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and a final cycle at 72 °C for 10 min. PCR products were loaded and electrophoresed in a 2% agarose gel stained with GelRed® Nucleic Acid Gel dye (Biotium), and visualized under UV light and documented by using a Gel Doc™ EZ imaging system (Bio-Rad). Purification of the PCR products was achieved using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), and the concentration of the obtained

Table 1
List of primers used for gene expression analysis and amplicon size.

Gen	Oligo Name	Sequence (5′-3′)	Amplicon (bp)	Reference
Spätzle	LvSpz3-F	ACACCCTGGAGGAGGAAACT	216	[65]
	LvSpz3-R	TCTGCAGACACTTGGACTCG		
Toll	LvToll3-F	TCGTACAACCAGCTGACGAG	195	[65]
	LvToll3-R	ATACTTCAGGTGGGCCACAG		
Tube	LvTube F	CAGTTAGCGCACATCTTGGA	102	GenBank accesión number: KC346865
	LvTube R	AAGACCCTCTGGGATTGGTT		
Dorsal	LvDorsal-F	TGGGGAAGGAAGGATGC	130	[94]
	LvDorsal-R	CGTAACTTGAGGGCATCTTC		
Cactus	LvCactus-F	GGAGGCGTGCCAGTGACTATG	75	[83]
	LvCactus-R	GAAGTAACGATCTGCATTGAAGGG		
VP28	vp28 140-F	AGGTGTGGAACAACACATCAAG	140	[51]
	vp28 140-R	TGCCAACTTCATCCTCATCA		

products was quantified using a NanoDrop™ Lite spectrophotometer (Thermo Scientific).

2.6. Quantitative real-time PCR (qPCR)

For this study, a comparative analysis of the expression changes of the genes encoding Spätzle, Toll, Cactus, and Dorsal of *P. vannamei*, was evaluated through the absolute quantification of mRNA transcripts by using real-time PCR. Accordingly, standard curves of each gene were constructed from 10-fold dilution series of the purified amplicons obtained previously by endpoint PCR. All standard-curve dilution points were tested by triplicate. The number of copies of each gene was calculated online through the following formula:

$$Number\ of\ copies = (mass \times 6.022 \times 10^{23}) \div (length \times 10^{9} \times 650)$$

Where the mass is the amount of DNA in nanograms, the length is the size of the amplicon in base pairs, and the average weight of a base pair is assumed to be 650 Da.

Quantification cycle (Cq) [57] values in each dilution were determined in triplicate using the Roche LightCycler 480 software v1.5 default threshold settings. The Cq values were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated by a linear regression of the plotted points. From the slope of each standard curve, PCR amplification efficiencies (E) were calculated according to the equation described by Ref. [58]:

$$E = 10^{-1 \div slope} - 1$$

PCR efficiencies were determined for each run and were considered as acceptable when slopes were in the range of −3.2 and −3.5, and R^2 values over 0.98 [59], and the values are given in Table 2.

All samples were examined in triplicate by quantitative RT-PCR (qRT-PCR) on 96-well plates (LightCycler® 480 Multiwell Plate 96, Roche), using the LightCycler® 480 Real-Time PCR platform (Roche). The reaction mixture of 10 μL contained 5 μL SsoAdvanced TM Universal SYBR™ Green Supermix (Bio-Rad), 0.25 μL of each primer (10 pmol), 1 μL of cDNA template (produced from 160 ng/μL of total RNA) and 3.5 μL of nuclease-free water. Cycling conditions for the amplification were as follows: 1 cycle of 98 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 10 s.

Table 2
Copy numbers of the serial dilutions used for to quantitative real-time PCR using a Real-Time PCR System and PCR efficiencies determined for each standard curve.

Gen	Interval of standard curve (copies/μL)	Efficiency
Spätzle	1.67×10^{9} to 1.67×10^{5}	92.3%
Toll	1.55×10^{11} to 1.55×10^{5}	98%
Tube	3.11×10^{9} to 3.11×10^{5}	92.5%
Dorsal	3.11×10^{11} to 3.11×10^{4}	91.2%
Cactus	4.26×10^{11} to 4.26×10^{4}	92.7%
VP28	1.57×10^{10} to 1.57×10^{2}	95.2%

2.7. Statistical analysis

The statistical analysis was performed using the software SigmaPlot 11.0. Data sets were first tested for normality using the Shapiro-Wilk test. For parametric data (Toll and Dorsal), the statistical significance was evaluated using one-way ANOVA analysis followed by Tukey's post hoc multiple comparisons test. Data sets with varying degrees of non-normality (Spätzle, Tube, and Cactus) were analyzed for significance using the Kruskal-Wallis test followed by Tukey's post hoc multiple comparisons test. In all experiments, significant differences were accepted at $p \leq 0.05$.

3. Results

3.1. WSSV replication kinetics

The WSSV replication kinetics was monitored by using quantitative real-time PCR in pleopods of infected specimens over a 72 h period (Fig. 1). An estimation of the WSSV replication kinetics indicated an early (0–24 hpi) low number of viral copies and subsequently (24–72 hpi) the viral progeny accumulate from 48 hpi and reach maximal levels at 72 hpi. This seems to be consistent with an initial antiviral immune response of the organism against this viral pathogen, a race between the immune system and the replication of the WSSV until the virus finally overcomes the immune system.

3.2. Expression profiles of Spätzle, Toll, Tube, Cactus, and Dorsal during WSSV-infection

The expression of the gene encoding Spätzle of the shrimp *Penaeus vannamei* (*LvSpz2*) remained relatively stable in response to a WSSV

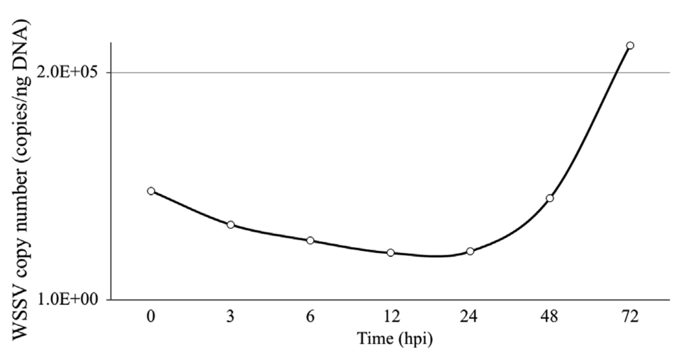

Fig. 1. The kinetics of WSSV replication. Shrimp were infected 5.71×10^{4} total copies of a WSSV freshly prepared inoculum. The number of copies of viral DNA were measured by quantitative real-time polymerase chain reaction [51].

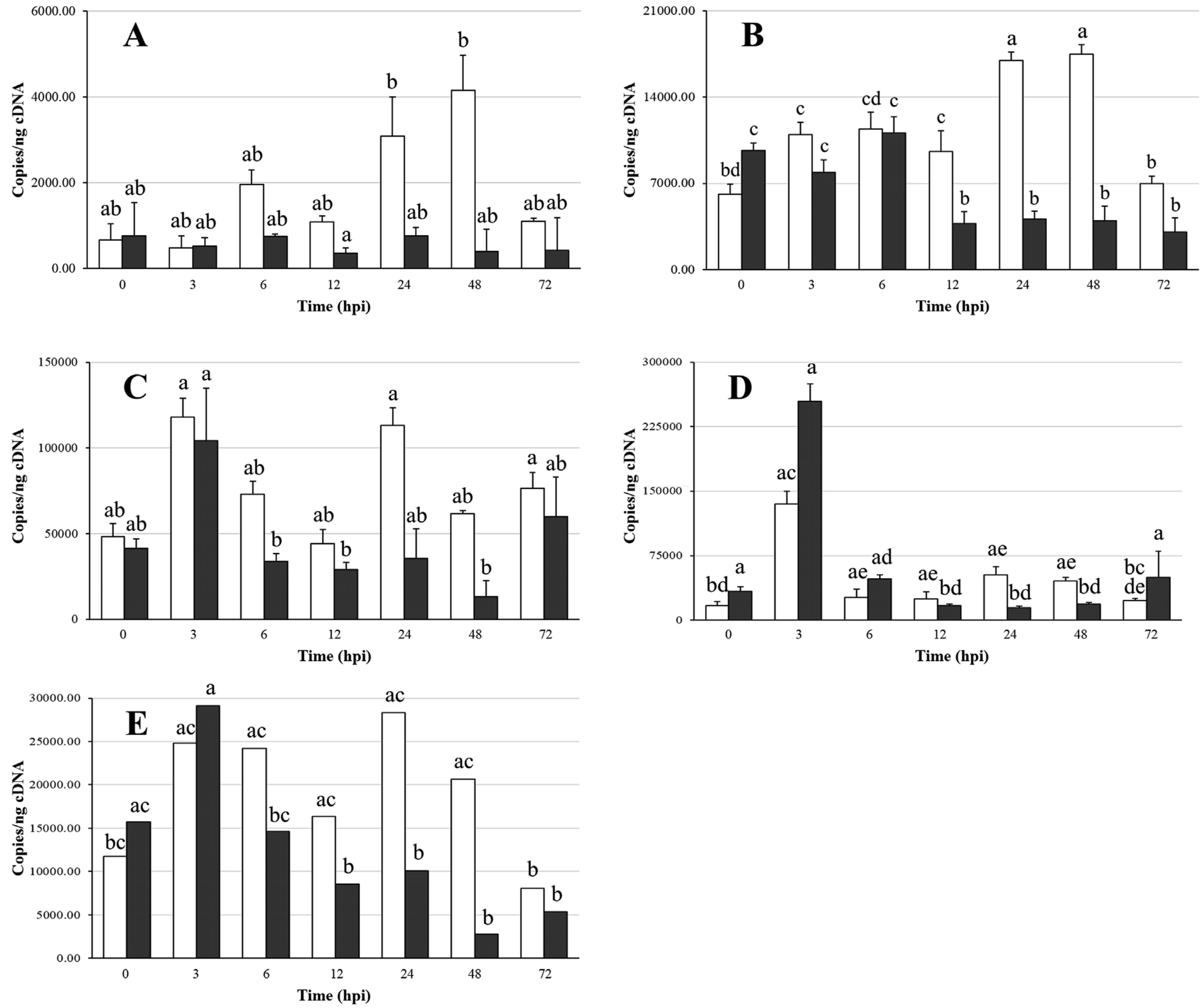

Fig. 2. Temporal expressions of the genes encoding for (A) Spätzle, (B) Toll, (C) Tube, (D) Cactus, and (E) Dorsal, in gills of the whiteleg shrimp *Penaeus vannamei* after an experimental challenge with WSSV. In all panels white bars represent the mock control group, and the black bars represent the WSSV infected group. Results are presented as the mean ± standard deviation. Letters represent statistical differences among data. In all experiments, significant differences were accepted at $p \leq 0.05$.

challenge, except for a decrease after 12 hpi. The only significant variation in *LvSpz2* in WSSV-infected specimens, relative to the control group was observed between infected organisms at 12 hpi and control specimens at 24 and 48 hpi ($p < 0.05$). No significant variations were observed between infected specimens or the control group. Interestingly, the expression levels of *LvSpz2* in WSSV-infected shrimp were lower than those observed in healthy specimens (Fig. 2a).

The steady-state transcript abundance of Toll (*LvToll3*) showed significant differences between infected shrimp and the control group at 12, 24 and 48 hpi ($p < 0.001$), when a sharp decrease on its expression was observed in infected specimens (Fig. 2b). In addition, striking differences were observed in healthy shrimp at 24 and 48 hpi ($p < 0.001$), when compared against other times of the control group and the infected group.

There were no significant differences ($p < 0.05$) in expression levels of the Tube encoding gene (*LvTube*) between the groups at each corresponding time (Fig. 2c). The expression of *LvTube* in infected specimens was about 2.5-fold higher at 3 hpi than its initial value (0 hpi), and then there was a sharp reduction on the amount of *LvTube* transcripts from 6 to 48 hpi, where the lowest levels of this gene were observed (a ~8-fold decrease). Finally, a subsequent increase in the expression of *LvTube* was observed in WSSV-infected specimens. However, statistical differences were only observed between the control group at 3 and 24 hpi, and the infected organisms at 6, 12, and 48 hpi.

The temporal expression of Cactus transcripts (*LvCactus*) in WSSV-infected shrimp was low at early times (0 hpi), but a dramatic increase of ~7.5-fold was observed in the abundance of these transcripts at 3 hpi. Subsequently, a marked downregulation of the *LvCactus* expression levels was observed from 6 to 24 hpi, where the lowest *LvCactus* mRNA abundance was detected (~18-fold decrease). Finally, the amount of *LvCactus* transcripts showed a slight increase from 48 to 72 hpi. However, despite this tendency, significant differences were found between treatments on the expression of the gene encoding Cactus from 12 to 72 hpi (Fig. 2d).

Finally, The expression of the gene encoding the transcription factor Dorsal (*LvDorsal*) showed a significant increase in WSSV-infected shrimp reaching a peak at 3 hpi ($p < 0.05$), and subsequently its expression dropped abruptly, reaching its lowest levels at 48 hpi (~10-

fold reduction) (Fig. 2e). Significant differences between groups were only detected from 12 to 48 hpi ($p < 0.001$).

4. Discussion

Due to the improved formulation and feeding management among, other things, the global shrimp production capacity has exceeded four million tons per year [60]. However, and despite the increase in production, the shrimp aquaculture industry is negatively impacted, in a frequent fashion, by an increasing number of diseases, among which those of viral origin represent the most serious threat to shrimp farming. Unfortunately, due to the lack of a comprehensive understanding of the shrimp innate immune response against viral pathogens, shrimp diseases are currently difficult to prevent and control.

It is well known that the Toll pathway, an essential component of the innate immune system of shrimp, regulates the expression of several genes, including those encoding for antimicrobial peptides (AMPs) [61]. In shrimp, several constituents of the Toll pathway, as Spätzle, Toll receptors, MyD88, Tollip, Tube, Pelle, TRAF6, Dorsal, and Cactus, have been identified [62].

Spätzle, an endogenous extracellular ligand of the Toll receptor, plays a critical role in innate immunity and controlling dorsal-ventral axis formation in *Drosophila* [63]. It has been well documented that during the immune response, Spätzle is processed from its inactive proform by the serine protease Spätzle-processing enzyme (SPE) [64], and the pro-domain prevents Spätzle to bind the Toll receptor [65]. Cleavage of the pro-domain causes a conformational modification that uncovers critical determinants for binding the Toll receptor [63], leading to the subsequent immune signal transduction.

In *P. vannamei*, three different genes encoding Spätzle-like proteins (*LvSpz1-3*) have been identified and characterized [66]. In the current study, the expression levels of the gene encoding the *LvSpz2* isoform in gills of the white shrimp *Penaeus vannamei* showed no significant changes in response to a WSSV challenge, except for a minor reduction on the transcripts abundance after 12 hpi. This observation is in agreement with previous reports, where only a slight downregulation was observed on the expression of *LvSpz2* in gills after 12 h of the experimental infection trial of shrimp specimens with WSSV [66]. Since it has been reported that the expression of *LvSpz1* and *LvSpz3* was upregulated after a WSSV challenge [66], it is conceivable that different pathogens may activate the expression of the diverse Spätzle isoforms in *P. vannamei*.

The *Toll* gene encodes a protein that controls the dorsal-ventral patterning during the embryonic development of the fruit fly [34]. Further studies in *Drosophila* identified that the Toll protein is also involved in a potent innate immune response against fungal infections [67]. Recent studies have revealed the presence of three genes encoding different isoforms of Toll receptors in the whiteleg shrimp *P. vannamei* (*LvToll1-3*) [66,68]. While some evidence suggests that *LvToll1* may not play a role in dsRNA-induced antiviral immunity [69], *LvToll3* may play a critical role in shrimp antiviral immunity when viral molecular patterns, such as dsRNA, are detected, mounting an antiviral response by activating the expression of genes encoding the interferon regulatory factor (IRF) and Vago 4/5, a peptide that restricts virus infection [70]. In our study, the transcript abundance of *LvToll3* showed significant differences between infected shrimp and the control group at 12, 24 and 48 hpi, when a sharp decrease on its expression was observed in infected specimens. These results contradict previous studies where the expression of *LvToll3* in gills of *P. vannamei* was upregulated at 3 and 12 hpi when specimens were challenged with WSSV [66]. From our perspective, the kinetics of viral replication may explain this apparent discrepancy. In the current study, the expression levels of *LvToll3* remained relatively unaltered during the first 6 hpi. Previous studies have demonstrated an increase of the total hemocytes count (THC) and on the expression of proPO during the first 5 h after a WSSV challenge, as an antiviral response. Hemocytes are a vital part of the immune shrimp response because they are the major store of the prophenoloxidase (proPO) system, which plays an essential role in defense against pathogens. Subsequently, however, both THC and the expression of proPO decreased significantly after 24 hpi [71]. This coincides with the kinetics of the WSSV replication observed in the current study. Thus, the immune system of shrimp, including *LvToll3*, combats the replication and spread of the WSSV during the first 24 hpi, but subsequently, the virus hijacks the cellular machinery, and the viral replication reaches its maximum levels, inhibiting the expression *LvToll3*. The inhibition of immune defenses is a common strategy used by pathogens to infect hosts successfully [72].

It is well known that the signal transduction from Toll to the Dorsal/Cactus complex requires the Tube and Pelle proteins [73]. The adaptor protein Tube contains two motifs that recruit MyD88, Pelle (at the N-terminal region), and Dorsal (at the C-terminal end) [74,75]. The interaction of Pelle, a serine/threonine kinase, and Tube leads to the phosphorylation, and subsequent degradation, of Cactus, and once Cactus is degraded, Dorsal is translocated into the nucleus inducing the expression of AMPs [76]. In the current study, no significant differences were found in the expression levels of *LvTube* between healthy and WSSV-infected specimens at each corresponding time. Similarly, a previous study reported that the expression levels of the gene encoding Tube showed moderated variations, including a conspicuous increase at 48 hpi, in response to WSSV infection relative to the control group [77]. However, in our study, the *LvTube* mRNA abundance showed a striking reduction from 3 to 48 hpi, where it reached its lowest levels (~70% less than those observed at 0 hpi). Thus, it seems plausible to suggest that the virus modulates the expression of *LvTube* to enhance its replication by inhibiting the synthesis of the protein, which may decrease the interaction with Pelle to hamper the phosphorylation of Cactus and the subsequent translocation of Dorsal into the nucleus, a key event in controlling the expression of AMPs.

In *Drosophila*, Cactus is a homolog of the mammalian IκB anchor protein and regulates the nuclear translocation of NF-κB proteins, which control the antimicrobial response [78–80]. As previously mentioned, activation of Pelle leads to the degradation of Cactus, releasing the NF-κB family protein Dorsal into the nucleus for the transcriptional induction of immune-related genes, such as AMPs [22,81–83]. In the current study, the temporal expression of *LvCactus* in gills of the shrimp *P. vannamei* was detected at low levels at early times of infection (0 hpi) with WSSV. However, a dramatic increase (~7.5-fold) in the abundance of these transcripts was subsequently observed (3 hpi), which was followed by a marked decrease on the *LvCactus* expression levels from 6 to 24 hpi. Finally, the amount of *LvCactus* transcripts showed a slight increase from 48 to 72 hpi. These observations are consistent with previous findings showing that the mRNA abundance of *LvCactus* in hemocytes of *P. vannamei*, during a WSSV challenge, decreased continuously after 0 hpi, reached lowest levels at 48 hpi (a ~63% decrease), and then slightly increase at 72 hpi [84]. This suggests that WSSV may obstruct the *LvCactus* expression to inhibit the *LvCactus-LvDorsal* interaction, to inhibit the synthesis of AMPs and facilitate the viral proliferation.

Finally, it is well known that the Dorsal protein, a transcription factor that plays a central role in the dorsoventral axis patterning of the *Drosophila* embryo [85], also participates as a critical regulator of the transcription of several antimicrobial peptide genes [86,87]. In the current study, the expression of the gene encoding the transcription factor *LvDorsal* showed a significant increase in WSSV-infected shrimp at 3 hpi, but subsequently, its expression dropped abruptly, reaching its lowest levels at 48 hpi (~10-fold reduction). To the best of our knowledge, no other study has investigated the expression of *Dorsal* in gills of WSSV-infected shrimp. However, the changes in the temporal expression profile of *LvDorsal* might be attributed to the effect exerted by two WSSV microRNAs (miRNAs). A recent study reported that the WSSV genome encodes 89 distinct miRNAs [88], and a further study found that two miRNAs, WSSV-miR-N13 and WSSV-miR-N23, target the *Dorsal* gene in the shrimp *Marsupenaeus japonicus*, suppressing the Spz-Toll-Dorsal-ALFs signaling pathway in shrimp, which promotes virus replication [89]. It has been previously demonstrated that viral miRNAs can inhibit the shrimp immune response to facilitate WSSV infection [88,90–92]. A similar strategy to regulate the host immune system to increase viral replication has been reported in several other viruses [93–95]. Therefore it seems plausible to suggest that the WSSV may control the expression of *LvDorsal*, and consequently a part of the antiviral immunity of shrimp, through miRNAs, as occurs in *M. japonicus*. However, this mechanism deserves further investigation.

The two most commonly used methods to analyze data from quantitative PCR experiments are relative and absolute quantification. Relative quantification depends on the use of reference genes whose expression is undoubtedly stable even when exposed to stress conditions [96]. This is necessary for suitable normalization to compensate for the intra- and inter-kinetic real-time RT-PCR variations that result from basic difficulties related to the method [97]. Absolute quantification, on the other hand, calculates the copy number of a transcript of interest, by relating the PCR signal to a standard curve [98]. Thus, it is worth mentioning that in the current study, the expression of the genes encoding Spätzle, Toll, Cactus, and Dorsal of *P. vannamei* was evaluated through absolute quantification and normalization was carried out against the same total RNA content in all samples as previously described [99,100].

Collectively, our results show the occurrence of changes in the expression of a number of constituents of the shrimp Toll pathway that may facilitate the replication of WSSV. To date, the fact that viruses have evolved innumerable strategies to evade such immune control mechanisms is well recognized. Thus, it seems that WSSV modulates the expression of key components of the Toll pathway to promote its replication, thereby contributing to viral fitness.

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