



Proteomic profiling of the white shrimp *Litopenaeus vannamei* (Boone, 1931) hemocytes infected with white spot syndrome virus reveals the induction of allergy-related proteins

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ABSTRACT

To elucidate the proteomic responses of shrimp hemocytes to white spot syndrome virus (WSSV) infection at the proteome level, a quantitative shotgun proteomic analysis was performed to detect differentially synthesized proteins in infected hemocytes of white shrimp (*Litopenaeus vannamei*). We identified 1528 proteins associated to 203 gene ontology (GO) categories. The most representative GO categories were regulation of cellular processes, organic substance metabolic processes and nitrogen compound metabolic processes. Most of the 83 detected up-regulated proteins are involved in DNA regulation and organization and cell signaling. In contrast, most of the 40 down-regulated proteins were related to immune defense processes, protein folding, and development. Differentially induced proteins were further analyzed at the transcript level by RT-qPCR to validate the results. This work provides new insights into the alterations of *L. vannamei* hemocytes at the protein level at 12 h post-infection with WSSV. Interestingly, several of the up-regulated proteins are allergy-related proteins in humans. Based on our results, we suggest a deeper analysis of the effects of this interaction on the regulation of allergy related-proteins as their up-regulation during WSSV could represent a threat to human health.

1. Introduction

The white spot syndrome virus (WSSV) is the sole member of the Nimaviridae family. It is a large enveloped, ellipsoid, dsDNA virus, with a genome size of about 300 kb (Nakano et al., 1994; Escobedo-Bonilla et al., 2008). This is the most notorious aquatic virus infecting shrimp, with more than 93 species of arthropods reported as hosts and/or

carriers (Sánchez-Paz, 2010). WSSV is the causal agent of a disease that can provoke severe mortalities of cultured shrimps (of up to 100% within 3–10 days), producing enormous economic losses in the shrimp farming industry worldwide (Lightner, 2003; Tan and Shi, 2008).

The white shrimp *Litopenaeus vannamei* has become one of the most cultivated in the world with profitable outcomes (FAO, 2016). Like other crustaceans, *L. vannamei* completely depends on its innate

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immune system to defend itself against pathogens, and this immune system is directed by the hemocytes, the mature cells for immunological defense (Söderhäll and Cerenius, 1992). Hemocytes play a crucial role as effectors of cellular and humoral immunological response and participate in the recognition of pathogens, phagocytosis, encapsulation, and apoptosis, to name a few. Likewise, circulating hemocytes rely on the prophenoloxidase (proPO) activating system to achieve pathogen entrapment and wound healing (Cerenius and Söderhäll, 2018). This system is activated by lipopolysaccharides (LPS), β -glucans, or peptidoglycans, causing exocytosis of granular cells and formation of phenoloxidase (PO), an enzyme that initiates melanization (Chen et al., 2014). However, it has been shown that a viral protein (WSSV164) suppresses the activation of PO, suggesting that the infection route of WSSV occurs by interfering with the activation of the melanization cascade in shrimps (Sangsuriya et al., 2018). By performing this action WSSV might replicate in the hemocytes of *L. vannamei* (Wu et al., 2015; Hernández-Pérez et al., 2017a; Zhang et al., 2018). However, the study of the protein profile of these effector cells at different stages of infection remains unexplored.

In this sense, quantitative proteomics can help to show the link between the proteome and its functions under specific conditions. This platform provides comprehensive information about the biochemical status of biological systems during the presentation of diseases, which is especially important during the study of veterinary pathogens and their pathogenesis (Bilic et al., 2018; Fricker, 2018). Therefore, to eradicate the WSSV disease, we need detailed information about the interactions of the binomial WSSV-shrimp hemocytes to understand the relation between the pathogen and the immune system of the host.

Despite the great importance of WSSV, data on the proteomic alterations of *L. vannamei*'s hemocytes when exposed to the virus are not available. This is because most of the efforts have been focused on the global understanding of the proteome adjustment in response to the WSSV infection directed to other organs of *L. vannamei* or they have been studied either in other crustaceans. For example, different proteomics approaches were used to study the WSSV infection response of *Fenneropenaeus chinensis* hepatopancreas at 6, 12, and 24 h post-infection (hpi) (Chai et al., 2010), as well as its complete cephalothoraxes during latent and acute infection (Li et al., 2016, 2013). Other analyses included the proteomic profiling of hematopoietic tissue (hpt) cells from *Cherax quadricarinatus* at 1 and 12 hpi (Jeswin et al., 2016). Also, previous proteomic studies have described the interaction between *L. vannamei* and WSSV in stomachs at 48 hpi, and in the hepatopancreatic cells at 72 hpi (Sun et al., 2018; Wang et al., 2007). However, in the shrimp's hemocytes, the proteomics of the WSSV infection has been evaluated only in *F. chinensis* at 24 hpi by two-dimensional gel electrophoresis (2-DE) (Li et al., 2014). Although 2-DE is extensively used, this method falls short in its reproducibility. It has an inability to detect low abundant and hydrophobic proteins, and low sensitivity in identifying proteins with extreme pH and molecular masses (Baltimore, 2001; Godovac-Zimmermann and Brown, 2001; Alaoui-Jamali and Xu, 2006; Chandramouli and Qian, 2009). In contrast, liquid chromatography coupled to tandem mass spectrometry can overcome several limitations of 2-DE proteomics. Further, tandem mass tags (TMT) and synchronous precursor selection (SPS)-MS³ have displayed a much better approach in comparative proteomic studies (Chick et al., 2015; McAlister et al., 2014).

In the present study, an isobaric labeling-based quantitative shotgun proteomics approach was used to identify and quantify the changes in the proteome of *L. vannamei* hemocytes infected with WSSV at 12 hpi. A revision of the principal functions of differentially expressed proteins families was included. Data provided herein can provide new clues on WSSV control by identifying proteins that are important for the immune defense of *L. vannamei*, but also highlight that allergy-related proteins are induced during infection and this could have implications for human health, a concern not considered previously.

2. Material and methods

2.1. Inoculum preparation and viral quantification

The viral inoculum was prepared from frozen shrimp infected with WSSV. Gills, muscle, and pleopods were used to prepare the viral inoculum using the procedure described by Gracia-Valenzuela et al. (2009) with minor modifications. Briefly, 9 g of the tissues were homogenized in 50 mL of sterile water and centrifuged 3000 $\times$ g for 20 min and 4 °C. The supernatant was recovered and filtered by passing both through 0.45 and 0.22 μ m ultrafiltration membranes (Corning®). The flow-through was concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck-Millipore®) of 100 kDa cut-off at 1000 $\times$ g for 30 min and 4 °C. The retained material was used as inoculum.

To quantify the WSSV viral particles, primers for qPCR were designed with Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) to amplify a 126 bp fragment of the WSSV immediate early (IE) gene (Genbank Accession number: AF440570) previously described (Hernández-Pérez et al., 2017b). Resulting primers, IE-126Fw (TGAAACGGTGTGCTGTTAGC) and IE-126Rv (AAGTTCCTCCATCGTCATCG), were used to quantify the viral load. The 126 pb fragment was amplified from the inoculum and cloned into the CloneJET PCR Cloning Kit (Thermo Scientific™). The recombinant plasmid was sequenced, quantified, and used as a template to perform serial dilutions, ranging from 2×10^6 to 20 copies- μ L⁻¹. qPCRs were performed using the 2 $\times$ QuantiNova SYBR Green PCR Master Mix (QIAGEN®) in a 15 μ L total reaction volume containing 2 μ L of each dilution or 50 ng of gDNA template, 0.5 μ L of each primer (5 μ M), and 5.5 μ L of nuclease-free water. qPCRs were performed in a Rotor-Gene Q (Qiagen®), as follows: initial denaturation step of 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, and a final extension step of 72 °C during 5 min. Amplification specificity was assessed by melting curve analysis.

The presence of virions in the inoculum was confirmed by transmission electron microscopy (TEM). In this case, 1 mL of the inoculum was put on 10 mL of 30% sucrose and centrifuged for 2 h at 20,000 $\times$ g and 4 °C. The pellet was resuspended in 50 μ L of water, and virions were negatively stained with 2% phosphotungstic acid (PTA) and visualized in a transmission electron microscope JEOL model Jem-1400.

2.2. Virus challenge experiment

Eighteen healthy adult shrimps (*L. vannamei*) (24.53 ± 4.3 g weight and 14.07 ± 1.3 cm height), were used in the experimental infections. The organisms were acclimatized for 10 days in tanks with 150 L of 35 PSU water, at 26 ± 2 °C and constant aeration. Shrimps were fed twice a day with commercial food of 35% protein. Only intermolt animals were used for the experiments.

The shrimps of the WSSV-infected group (n = 9) were injected with $\sim 2.16 \times 10^6$ viral copies in 10 μ L, while in the control group 9 shrimps were injected with sterile water. Infected and control groups consist of three groups of three organisms each one. 100 μ L of hemolymph were collected from each shrimp in 200 μ L of anticoagulant solution (NaCl 450 mM, KCl 10 mM, HEPES 10 mM, EDTA 10 mM, pH 7.0) (1:2) according to Vargas-Albores et al. (1993). The hemolymph from infected and uninfected organisms were centrifuged for 5 min at 2500 $\times$ g and 4 °C. The cell pellet from each organism was washed twice with 200 μ L of anticoagulant without EDTA in the same conditions.

2.3. Viral quantification in hemocytes

To confirm the integrity of isolated hemocytes, they were visualized with scanning electron microscopy (adapted from Manachini et al., 2011). For this purpose, the pellet was fixed with fixation solution (3% glutaraldehyde and 0.1 M sodium cacodylate), washed twice with saline phosphate buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄,

1.47 mM KH_2PO_4 , pH 7.4), washed with distilled water, and dehydrated in ethanol series (30%, 50%, 70%, 90%, and 100%) of 15 min each. The sample was dropped on a 13 mm Thermanox Plastic® coverslip (Nunc) pretreated with 0.2% gelatin and finally dried using a critical point drier (Quorum®, model K850) and coated with gold-palladium using an ion sputter coater (Quorum, Q150R ES). The hemocytes pellets were observed under the scanning electron microscope (FESEM Jeol 7600F) with 25 kV at 2500 $\times$.

Hemocytes sub-samples were taken out from each pellet to isolate gDNA, using the Quick-gDNA™ MiniPrep kit (Zymo Research®) and used as template to quantify the viral charge by qPCR.

2.4. Extraction of proteins from hemocytes

Three groups of hemocytes (each group consisting of hemocytes of three organisms each) of infected animals and three equivalent groups from uninfected animals, were immediately homogenized with 500 μL of TRIzol® and incubated for 5 min at room temperature (RT). Then, 100 μL of chloroform was added and mixed vigorously for 30 s. The samples were incubated for 3 min at RT and centrifuged at 12,000 $\times$ g for 15 min at 4 °C. The phenolic phase was recovered, and 300 μL of absolute ethanol was added and incubated for 5 min at RT and centrifuged at 2000 $\times$ g for 5 min at 4 °C. The aqueous phase was recovered for RNA isolation and this fraction was used for gene expression analysis (see section 2.10).

The resulting supernatant was carefully transferred and mixed with 3.5 vol of protein precipitation solution (100 mM sodium acetate in methanol) and kept at –70 °C overnight. Samples were centrifuged at 12,000 $\times$ g for 10 min at 4 °C. Pellets were washed twice with 1 mL of protein precipitation solution. Subsequently, they were washed twice with 1 mL of 80% acetone and twice with 1 mL of 70% ethanol. Protein pellets were stored at –70 °C till use. For proteomics analysis, proteins were solubilized in resuspension buffer (1% SDS in phosphate buffer, 100 mM, pH 7.5) and quantified using a micro BCA assay (Thermo Scientific®).

2.5. Protein digestion

A total of 100 μg of protein were reduced with 10 mM Tris (2-carboxyethyl) phosphine (TCEP) for 45 min at 60 °C, alkylated with 30 mM iodoacetamide (IA) for 1 h at RT and darkness, and the remaining IA was quenched with 30 mM DTT for 10 min. Proteins were then precipitated with cold acetone overnight at –20 °C. The mix was centrifuged at 10,000 $\times$ g for 15 min at 4 °C, and the pellets dried at RT for 15 min. Then, proteins were suspended with 100 μL of digestion buffer containing 50 mM triethylammonium bicarbonate (TEAM) and 0.1% SDS and digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison WI) at a 1:30 w/w trypsin protein ratio overnight at 37 °C. Thereafter, a 1:60 w/w freshly made trypsin was added during four h at 37 °C. The reaction was stopped by denaturing the trypsin at 80 °C.

2.6. Isobaric tag protein labeling, simple fractionation, and desalting

TMT six-plex reagents (Thermo Scientific®) with varying molecular weights were applied as isobaric labels for the quantification of differential protein between the digested samples, 126, 127 and 128 for control group and 129, 130 and 131 for infected group, according to the manufacturer's protocol. The labeled peptide was combined at equal ratios and fractionated by using a strong cation exchange with HyperSep SCX cartridges (Thermo Scientific®) loaded in 5 mM KH_2PO_4 /25%ACN pH 3. Then, the fractions were eluted using three salt steps: 75 mM KCl, 250 mM KCl, and 500 mM KCl, all together in 10 mM KH_2PO_4 /25% ACN pH 3. In order to desalt the fractions, a C18 solid phase extraction with HyperSep™ C18 Cartridges (Thermo Scientific®) was used. The eluted samples were dried using a CentriVap (Labconco Kansas, Missouri).

2.7. Nano-LC-MS/MS analysis

Samples were analyzed by nano-LC-MS/MS analysis using an Orbitrap Fusion TMTribid TM (Thermo-Fisher Scientific®) mass spectrometer equipped with an “EASY Spray” nano ion source (Thermo-Fisher Scientific). The Orbitrap Fusion TMTribid TM was interfaced with an UltiMate 3000 RSLC system (Dionex®). Each reconstituted sample (5 μL) was injected into a nanoviper C18 trap column (3 μm , 75 $\mu\text{m} \times 2$ cm, Dionex®) at 3 $\mu\text{L min}^{-1}$ flow rate, and then separated on an EASY spray C18 RSLC column (2 μm , 75 $\mu\text{m} \times 25$ cm), using a 100 min gradient with a flow rate of 300 nL min^{-1} , and using 0.1% formic acid in LC-MS grade water (solvent A) and 0.1% formic acid in 90% acetonitrile (solvent B). The gradient was as follows: 10 min solvent A, 7–20% solvent B within 25 min, 20% solvent B for 15 min, 20–25% solvent B for 15 min, 25–95% solvent B for 20 min, and 8 min for solvent A. The mass spectrometer was operated in positive ion mode with nanospray voltage set at 3.5 kV and source temperature at 280 °C. External calibrant included caffeine, Met-Arg-Phe-Ala (MRFA), and Ultramark 1621.

2.8. Synchronous precursor selection (SPS)-MS³ for TMT analysis

Full MS scans were run in the Orbitrap analyzer with 120,000 (FWHM) resolution, scan range 350–1500 m/z, AGC of 2.0e5, the maximum injection time of 50 ms, intensity threshold of 5.0e3, dynamic exclusion one at 70s, and 10 ppm mass tolerance. For MS2 analysis, the 20 most-abundant MS1s were isolated with charge states set to 2–7. Fragmentation parameters included, CID with 35% of collision energy and activation Q of 0.25, AGC of 1.0e4 in maximum injection time of 50 ms, precursor selection mass range of 400–1200 m/z, precursor ion exclusion width low 18 m/z and high 5 m/z, isobaric tag loss TMT, and detection run in the ion trap. Afterward, MS³ spectra were acquired as previously described (McAlister et al., 2014; Ting et al., 2011) using synchronous precursor selection (SPS) of 10 isolation notches. MS³ precursors were fragmented by HCD with 65% of collision energy and analyzed using the Orbitrap with 60,000 resolution power at 120–500 m/z scan range, two m/z isolation window, 1.0e5 AGC, and maximum injection time of 120 ms with one microscan.

2.9. Bioinformatic analysis

Raw data were processed with Proteome Discoverer 2.1 (PD, Thermo Fisher Scientific Inc.). The subsequent searches were carried out using Mascot search engine (version 2.4.1, Matrix Science, Boston, MA), SEQUEST HT (Eng et al., 1994), and AMANDA (Dorfer et al., 2014). We considered a 25 score for protein identification. The search with both engines was conducted against a custom database. For this, we built a specific database from non-redundant transcript sequences of *L. vannamei* deposited in the NCBI (RefSeq), and ORFs (> 100 aa) were predicted with TransDecoder (<https://github.com/TransDecoder/TransDecoder/wiki>). These predicted sequences were added to the sequences downloaded from UniProt (Bateman et al., 2017) for Arthropoda (UniRef) and were filtered with CD-HIT (with -c 0.98 and -n 5 as parameters) to reduce redundancies (Li and Godzik, 2006).

Parameters in the search using AMANDA, MASCOT, and SEQUEST engines included: full-tryptic protease specificity, two missed cleavages allowed. In addition, static modifications covered carbamidomethylation of cysteine (+57.021 Da) and TMT 6-plex N-terminal/lysine residues (+229.163 Da). Furthermore, dynamic modifications included methionine oxidation (+15.995 Da) and deamidation in asparagine/glutamine (+0.984 Da). For the SPS-MS³ method, in which identification was performed at lower resolution in the linear ion trap, tolerances of ± 10 ppm and ± 0.6 Da were applied. Resulting peptide hits were filtered for maximum 1% FDR using the Percolator algorithm (Käll et al., 2007).

The TMT 6-plex quantification method within PD software was used to calculate the reported ratios with mass tolerance ± 10 ppm, with the

most confident centroid and a precursor co-isolation filter of 45% applied. For the SPS-MS³ method, the quantification was run at the MS³ level. Only peptide spectra containing all reporter ions were designated as “quantifiable spectra”. For protein-level reporting, protein grouping was enabled, and values were calculated from the median of all quantifiable PSMs for each group (master proteins). TMT values were then reported as a ratio to the sum of reporters in each spectrum. The determination of differential proteins was performed using the Bioconductor package “edgeR” v3.16.5 (Robinson et al., 2010), generating a normalized matrix for each protein, the fold of change of WSSV-infected samples with respect to non-infected samples (logFC), and P y P-adjusted values (FDR, correction of Bonferroni-Holms, Holm, 1979).

The annotation of differential proteins was performed with Blast2GO (Conesa et al., 2005). This was complemented with information from InterProScan (Zdobnov and Apweiler, 2001).

2.10. Proteomic data validation with real-time quantitative PCR

Total RNA was isolated from hemocytes using the TRIzol[®] protocol (see section 2.4). Real-time qPCR was performed in a Rotor Gene-Q (QIAGEN) using the Quantinova system (QIAGEN) and 7 differentially regulated proteins were randomly chosen to validate the inferred proteomic profile at transcript level. For these, 14 sets of primers (forward and reverse) were designed using the program Primer3 (Table 1 supplementary).

The expression level of mRNA was normalized to the L8 ribosomal protein transcript (Valenzuela-Castillo et al., 2017). All data were presented as relative gene expression levels using the ddCt method (Livak and Schmittgen, 2001).

3. Results and discussion

This study denotes the first analysis of the proteome profile of hemocytes exposed to WSSV infection in *L. vannamei* and contributes to a better understanding of this interaction as information on this topic is

scarce. In the present study, shotgun quantitative proteomics was used to investigate the proteomic adjustment of hemocytes to WSSV infection in *L. vannamei* performed on hemocytes at 12 hpi. This approach revealed 123 proteins being regulated under the viral infection, including many proteins associated with allergenic reactions in humans.

3.1. Viral quantification of inoculum and hemocytes samples

Viral particles were monitored by electron microscopy and qPCR. The electron micrographs obtained showed naked and complete WSSV virions ranging in size from 270 to 310 nm length and 40–58 nm weight (Fig. 1a), ensuring the presence of viral particles in the inoculum. Additionally, hemocytes were micrographed to evaluate the ability of the isolation protocol to maintain their cell integrity under the isolation conditions (Fig. 1b). The average quantification of the virus from hemocytes isolated at 12 hpi was $57,866 \pm 32,117$ viral copies μL^{-1} , while the control group remained negative.

3.2. Protein identification and GO annotation

Through our pipeline, we detected isobaric tag signals associated with 6854 identified peptides and 5691 unique peptides, corresponding to 1528 different proteins.

The use of different proteomics pipelines included various extraction protocols and mass spectrometers making it challenging to conduct comparative analyses across similar studies reported in the literature. It is worth noting that in this study we used the synchronous precursor selection (SPS)-MS³, which provides accurate comparative analysis by reducing the co-isolation of tagged interferences during the mass spectrum acquisition.

The top 20 GO annotations for molecular functions, cellular component, and biological process are shown in Fig. 2. The resulting proteome was distributed into 22 molecular function categories, where the most notorious are hydrolase activity, protein binding, transferase activity, oxidoreductase activity, and catalytic activity acting on a protein. At cellular components at GO level, 89 categories were

Table 1

Functional classification of total proteins identified in different tissues performed during WSSV pathogenesis studies in crustaceans. WSSV: White spot disease virus; hpi: hours post-infection.

Reference	Species	Organ	WSSV hpi	Number of desregulated proteins	Analysis technique	Principal functional classification of total proteins reported by different studies
Present study	<i>Litopenaeus vannamei</i>	Hemocytes	12	123	iTRAQ LC-MS/MS	Regulation of cellular process Organic substance metabolic process Nitrogen compound metabolic process Cellular metabolic process
Sun et al., 2018	<i>Litopenaeus vannamei</i>	Hepatopancreas	72	161	iTRAQ LC-MS/MS	Metabolic processes Response to stimulus Binding Catalytic activity
Jeswin et al., 2016	<i>Cherax quadricarinatus</i>	Hematopoietic tissue cells	1 and 12	17 → 1 hpi 30 → 12 hpi	iTRAQ TOF-MS/MS	Cellular processes Metabolic processes Single-organism processes
Li et al., 2016	<i>Fenneropenaeus chinensis</i>	Cephalothorax	Not specified	240	iTRAQ LC-ESI-MS/MS	Molecular functions Biological processes Cellular components
Li et al., 2014	<i>Fenneropenaeus chinensis</i>	Hemocytes	24	45	2-DE MALDI-TOF-MS/ MS	Cytoskeleton Immune related Stimulus response DNA of binding
Chai et al., 2010	<i>Fenneropenaeus chinensis</i>	Hepatopancreas	6, 12 and 24	60	2-DE MALDI-TOF-MS MS/MS	Immune related
Wang et al., 2007	<i>Litopenaeus vannamei</i>	Stomach	48	26	2-DE LC-nano ESI-MS/ MS	Biological processes
Wu et al., 2007	<i>Penaeus monodon</i>	Subcuticular epithelium	72	12	2D-LC MALDI-TOF/TOF- MS/MS	Not specified

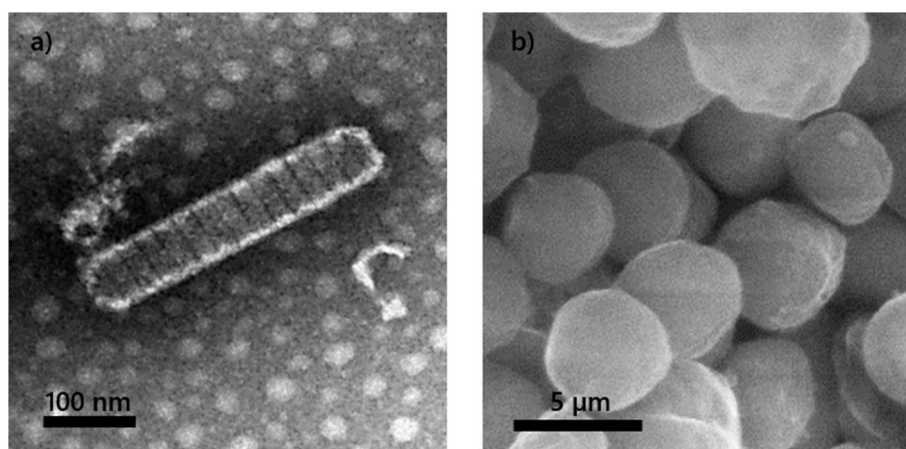


Fig. 1. Electron micrographs of purified WSSV viral particles stained with 2% PTA and hemocytes pellet coated with gold-palladium. a) WSSV virion showed a size of 310 nm in length and 58 nm in width (Scale bar = 100 nm). b) Purified hemocytes' pellet showed cell integrity (Scale bar = 5 μm).

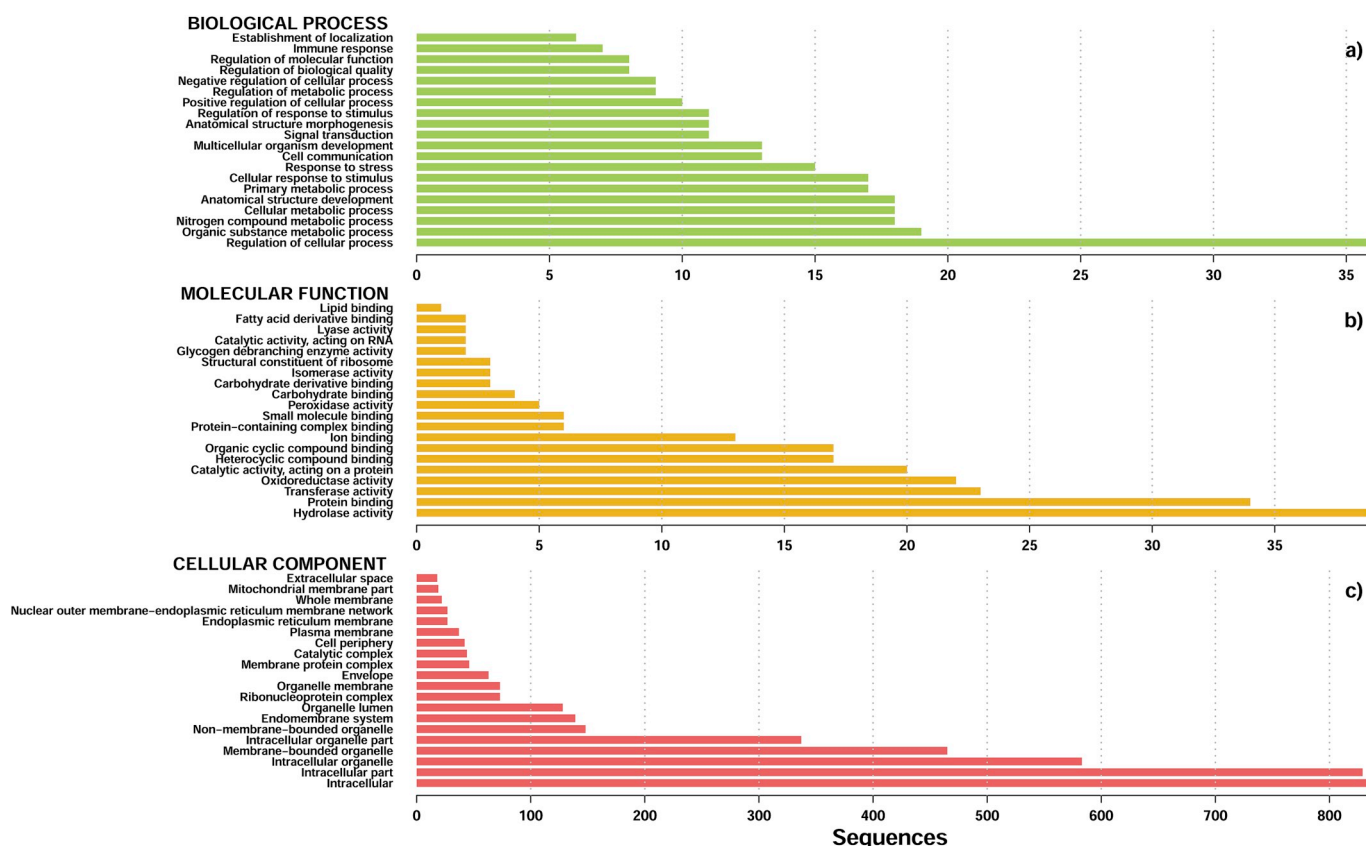


Fig. 2. Top 20 GO distribution of total proteins sequences involved in a) biological processes, b) molecular functions and c) cellular components identified in *L. vannamei* hemocytes infected with WSSV.

distinguished, and the most important are intracellular, intracellular part, intracellular organelle, membrane-bounded organelle, and intracellular organelle part. Finally, the biological processes related to the proteins detected showed 90 categories. The most enriched are regulation of cellular process, organic substance metabolic process, nitrogen compound metabolic process, cellular metabolic process, and anatomical structure development.

Zhu et al. (2018), established the profile of hemocyte subpopulations of *F. chinensis* reporting that in normal conditions these cells are involved mainly in five biological processes: binding proteins, proteins involved in the catalytic activity, enzyme regulator activity, structural molecule activity, and translation regulator activity. The 90 biological

processes identified in this work extend the possibility of elucidating important pathways and physiological processes performed by hemocytes.

In bacterial infections, proteomic studies have shown changes of traditionally recognized immune-related proteins during phagocytosis associated with the prophenoloxidase (proPO) system, showing that most of the proteins affected act at the cytoskeletal level (Tassanakajon et al., 2013), whereas viral infections appear to affect other processes. For example, studies focused on WSSV pathogenesis and its interaction with hemocytes at late stages of WSSV infection (24 hpi) revealed that the biological processes were represented by nine categories, with the most important being cytoskeleton proteins, immune-related proteins,

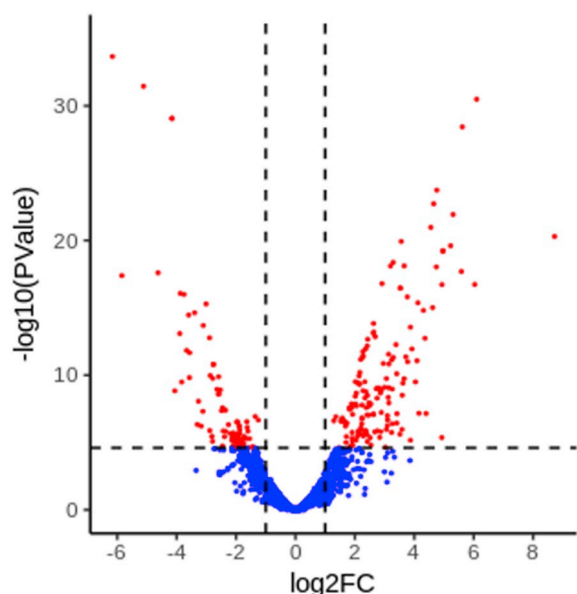


Fig. 3. Volcano plot of differentially expressed proteins from WSSV infected shrimps and uninfected shrimps performed in triplicate with a pool of three organisms each one. Red if $\log_2FC > 2$ and $P \text{ value} < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DNA and protein binding proteins, stimulus-response proteins, and proteins involved in glucose metabolic process (Li et al., 2014). Furthermore, Jeswin et al. (2016) reported that in cultivated hpt cells of

crayfish, the biological processes were registered into 23 categories, and the most well represented were related to cellular and metabolic processes. A comparison of the functional classification of total proteins identified in different tissues performed during WSSV pathogenesis studies in crustaceans is summarized in Table 1.

3.3. Analysis of differentially expressed proteins

Only proteins with a fold change < 2 or > 2 and a $p\text{-value} < 0.01$ were considered as differentially accumulated. The results showed a total of 123 proteins being regulated, 40 were down-regulated, and 83 up-regulated (Fig. 3). From the up-regulated group, 10 proteins were uncharacterized. The 123 proteins affected by the WSSV are shown in Table 2 as well as the $\log_2$ fold change and $p\text{-values}$ of each one.

Fig. 4 resumes and compares the biological processes in which both groups are involved. In the upregulated group, the GO enrichment analyses showed over-synthesized proteins that are involved in processes of biological regulation, protein binding, regulation of biological process, and response to stimulus. In the down-regulated group, the GO enrichment analyses showed the least-synthesized proteins are involved with biological regulation, response to stimulus, regulation of biological process, multicellular organismal process, and regulation of cellular process.

3.4. Classification of regulated proteins

A protein family is defined as a group of proteins that share a common evolutionary origin reflected by their related functions, similarities in sequence, or similar primary, secondary, or tertiary structure (EMBL-EBI, 2016). The protein families that present changes during pathogenesis are crucial to identifying cellular proteins and pathways

Table 2

Description of the 123 differentially expressed proteins from 12 hpi WSSV-infected hemocytes from *L. vannamei*.

Down-regulated proteins			
blast hit	Description	$\log_2FC$	P-value
gi 1275508098 ATU31747.1	Calcium-activated chloride channel regulator 1	6.16	4.07E-10
gi 313747943 ADR74382.1	Phenoloxidase-activating factor 3 heavy chain	5.84	3.02E-09
gi 1067069117 XP_018028022.1	Cartilage oligomeric matrix protein	5.12	7.85E-11
gi 33318289 AAQ05018.1	beta-actin	4.63	3.46E-07
gi 1067064068 XP_018015971.1	Ubiquitin-conjugating enzyme E2 N	4.16	1.09E-11
gi 1022766446 KZS11497.1	ATP synthase subunit d, mitochondrial	4.13	2.02E-08
gi 225718928 ACO15310.1	serine protease K12H4.7	4.06	8.48E-09
gi 146217394 ABQ10739.1	Cathepsin L	3.89	3.77E-06
gi 17223040 AAL36897.1	putative antimicrobial peptide	3.87	9.75E-07
gi 1022761450 KZS07405.1	Alpha-2-macroglobulin receptor	3.83	2.02E-05
gi 1067064614 XP_018016259.1	Poly(U)-specific endoribonuclease	3.66	5.22E-09
gi 238683637 ACR54097.1	Glyceraldehyde-3-phosphate dehydrogenase	3.59	1.56E-07
gi 1067069907 XP_018018296.1	2,4-dienoyl-CoA reductase, mitochondrial	3.57	1.26E-05
gi 409711855 AFV39710.1	Beta-thymosin 4	3.56	6.20E-11
gi 1067096129 XP_018010006.1	venom serine protease Bi-VSP-like	3.39	1.10E-09
gi 1067087235 XP_018027601.1	FK506-binding protein 2-like	3.30	9.66E-09
gi 328796228 AEB40303.1	ATP synthase subunit delta, mitochondrial	3.26	5.07E-06
gi 988045886 AME17864.1	Beta-arrestin-1	3.17	3.02E-09
gi 1067095441 XP_018009638.1	Peptidyl-prolyl cis-trans isomerase FKBP2; Full = Immunophilin FKBP13	3.12	5.66E-08
gi 1067093389 XP_018008548.1	Wnt inhibitory factor 1	3.10	2.11E-09
gi 1293743678 AUB13319.1	C-type lectin containing low-density lipoprotein receptor domain	3.01	1.75E-10
gi 328899962 AEB54653.1	Ubiquitin-40S ribosomal protein S27a	2.89	1.01E-09
gi 868477782 AKO90283.1	cathepsin L-like protein 2	2.88	1.52E-05
gi 1067071924 XP_018019383.1	Cold shock domain-containing protein CG9705	2.87	7.99E-08
gi 1067075523 XP_018021310.1	Glucosidase 2 subunit beta	2.79	4.93E-07
gi 1325273903 XP_023321602.0	basement membrane-specific heparan sulfate proteoglycan core protein-like isoform X2	2.78	1.07E-09
gi 946526317 ALM09354.1	ubiquitin carboxyl-terminal esterase L3	2.75	2.36E-09
gi 1102610569 APC62653.1	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	2.76	2.35E-09

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Table 2 (continued)

Down-regulated proteins			
blast hit	Description	log2FC	P-value
gi 1067090541 XP_018007023.1	Sulfotransferase 1E1	2.64	1.04E-07
gi 1067095441 XP_018009638.1	Peptidyl-prolyl cis-trans isomerase, Immunophilin FKBP13	2.59	4.67E-09
gi 1022755884 KZS02794.1	Serine/arginine-rich splicing factor 1	2.59	6.53E-09
gi 1067096129 XP_018010006.1	venom serine protease Bi-VSP-like	2.49	3.21E-08
gi 395484805 AFN66647.1	chitin binding-like protein	2.45	5.36E-07
gi 946526317 ALM09354.1	Ubiquitin carboxyl-terminal hydrolase isozyme	2.45	1.18E-07
gi 293652146 ADE60733.1	Myosin-2 essential light chain	2.43	1.37E-11
gi 1067075455 XP_018021276.1	Isochorismatase domain-containing protein 2	2.39	6.33E-06
gi 944542876 YP_009176794.1	Cytochrome c oxidase polypeptide II	2.35	8.91E-08
gi 326579691 ADZ96217.1	JHE-like carboxylesterase 1	2.34	4.53E-08
Up-regulated proteins			
KZS21418.1	Tubulin alpha-1 chain	8.72	1.53E-09
XP_018026249.1	60S ribosomal protein L11-like	6.10	5.75E-14
AAR82846.1	actin E	6.04	5.40E-08
EFX73805.1	cytoplasmic actin	5.72	7.74E-17
XP_018013473.1	AP-2 complex subunit alpha-like	5.62	4.47E-13
XP_018027502.1	splicing factor U2AF 50 kDa subunit-like isoform X2	5.58	2.06E-08
XP_018025728.1	tropomyosin-1, isoforms 9A/A/B isoform X30	5.22	3.05E-09
XP_018009404.1	failed axon connections-like isoform X1	4.96	4.37E-09
XP_023327939.1	solute carrier family 12 member 9-like isoform X2	4.93	5.47E-08
ASC55673.1	putative IST1 protein	4.76	4.91E-11
KZS21756.1	Histone H2B	4.74	1.47E-08
XP_018006479.1	putative pre-mRNA-splicing factor ATP-dependent RNA helicase PRP1	4.65	1.35E-10
ABD92928.1	hemocyte transglutaminase	4.62	3.01E-07
AEC12820.1	lactate dehydrogenase	4.56	7.79E-10
EFX76617.1	hypothetical protein DAPPUDRAFT_248788	4.40	7.85E-04
AKI81624.1	hemocyanin V4	4.36	2.94E-06
XP_021921765.1	actin-related protein 2/3 complex subunit 3-like	4.31	3.72E-07
XP_018014697.1	serine/threonine-protein kinase PAK 3-like	4.16	7.72E-04
PSN55383.1	Eukaryotic translation initiation factor 3 subunit M	4.12	2.11E-07
XP_018027233.1	S-adenosylmethionine synthase-like isoform X1	4.09	1.56E-05
XP_018015790.1	heterogeneous nuclear ribonucleoprotein U-like protein 1	4.04	7.50E-05
ABW77320.1	clottable protein 2C-type lectin	3.92	6.49E-06
ALF45199.1	C-type lectin	3.87	1.29E-06
XP_018007503.1	dnaJ homolog subfamily C member 25 homolog	3.87	4.3E-03
XP_023348951.1	putative all-trans-retinol 13,14-reductase isoform X1	3.77	1.36E-07
KZS15317.1	Histone H2A	3.72	1.17E-05
XP_018027836.1	histone H4	3.66	1.36E-08
XP_018006579.1	40S ribosomal protein S17-like	3.66	5.94E-05
XP_023718244.1	serine/threonine-protein phosphatase 4 regulatory subunit 3 isoform X2	3.62	8.18E-05
KZS07264.1	Ubiquilin-2	3.59	2.02E-04
XP_015839050.1	hemocytin	3.59	1.60E-04
ANZ78205.1	clottable protein	3.56	2.21E-09
AEC22816.1	tsunagi/Y14	3.59	7.13E-08
ACY66444.1	actin-depolymerizing factor	3.41	1.10E-04
XP_018011640.1	purine nucleoside phosphorylase-like	3.41	4.10E-05
KZS08489.1	Beta-arrestin-1	3.38	4.71E-06
JP432686.1	uncharacterized protein	3.37	6.54E-06
KJH43687.1	uncharacterized protein	3.29	1.22E-05
XP_018027035.1	synaptogyrin-1-like	3.29	1.05E-08
AIT98358.1	histone 3 subunit a	3.26	1.28E-04
AHY28871.1	threonine/serine protein kinase	3.25	1.26E-03
XM_018161641.1	uncharacterized protein	3.26	2.60E-03
XP_018023505.1	probable serine-tRNA ligase, cytoplasmic	3.20	1.37E-08
XP_018025281.1	chymotrypsin-like protease	3.14	3.56E-04
CAG29666.1	translation initiation factor 2 gamma subunit	3.13	9.60E-06
XP_023335573.1	cytochrome b-c1 complex subunit 2, mitochondrial-like	3.13	1.16E-04
XP_018027527.1	ras-related protein Rap-2c-like	3.12	2.37E-04
KZS21198.1	Germinal-center associated nuclear-like protein	3.10	9.46E-04
XP_018008511.1	uncharacterized protein	3.10	1.35E-03

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Table 2 (continued)

Down-regulated proteins			
blast hit	Description	log2FC	P-value
XP_018020355.1	vitamin K epoxide reductase complex subunit 1-like	3.06	1.61E-03
XP_018012347.1	transcription factor BTF3 homolog 4-like isoform X1	3.06	5.85E-05
AEL23015.1	protein spaetzle	2.96	1.92E-05
ABI95361.1	hemolymph clottable protein	2.92	5.09E-08
XP_023328726.1	cAMP-dependent protein kinase catalytic subunit isoform X6	2.89	7.25E-04
ALP82438.1	coatamer subunit epsilon	2.88	1.39E-04
AJS14565.1	cysteine-rich protein	2.87	1.33E-04
XP_018027717.1	protein Mo25-like	2.86	1.71E-04
XP_018008264.1	pleiotropic regulator 1-like	2.84	3.72E-03
KZS15994.1	Flavin reductase (NADPH)	2.80	1.79E-04
KOB75909.1	putative Broad-complex core-protein isoform 6	2.76	1.23E-04
XP_018012773.1	exportin-1-like isoform X1	2.75	1.80E-03
AAG16253.1	beta-actin	2.75	2.51E-04
XP_022326973.1	Adhesion G-protein coupled receptor G6	2.72	1.24E-04
XP_018026409.1	coatamer subunit delta-like isoform X2	2.68	2.62E-06
XP_018017808.1	uncharacterized protein	2.67	2.56E-03
KZS21346.1	Histone H4 type G-like protein	2.64	1.85E-06
HAAW01014979.1	uncharacterized protein	2.63	9.81E-07
XP_018021477.1	histone H2A.V	2.63	8.55E-04
XP_018025485.1	ribonucleoside-diphosphate reductase large subunit-like	2.54	5.90E-03
KZS06960.1	Trifunctional enzyme subunit beta, mitochondrial	2.53	3.81E-04
KZS08360.1	titin-like	2.52	3.77E-03
XM_013920344.2	60S ribosomal protein L6-like, transcript variant X1	2.48	4.49E-04
XP_018015072.1	dipeptidyl peptidase 3-like isoform X1	2.47	3.45E-04
ACU31810.1	alpha2 macroglobulin isoform 2	2.47	8.81E-03
ADB44900.1	DEAD(Asp-Glu-Ala-Asp) box polypeptide 5	2.43	3.18E-06
AEK99382.1	lipoprotein receptor	2.42	5.09E-03
XP_015912217.1	septin-9 isoform X3	2.42	5.20E-06
XP_023329260.1	regulator of G-protein signaling loco-like	2.41	3.02E-04
XP_018021677.1	eukaryotic translation initiation factor 3 subunit H-like	2.41	8.73E-04
AST11587.1	ficolin 2	2.38	5.06E-04
XP_018019403.1	beta-hexosaminidase subunit beta-like isoform X1	2.37	1.10E-04
KZS11752.1	Calcium/calmodulin-dependent protein kinase type 1	2.33	7.45E-05
BAP28203.1	copper/zinc superoxide dismutase isoform 4	2.32	3.08E-03

perturbed by viral infection as well as to identify important diagnostic and therapeutic targets (Bilic et al., 2018). The families of differentially accumulated proteins identified were obtained through an InterProScan analysis and are shown in Fig. 5. The functions of the three most representative families of proteins altered during infection with WSSV are described.

3.4.1. Down-regulated proteins families

Papain-like cysteine peptidase superfamily. The papain-like cysteine peptidase superfamily acts as proteinases inhibitors and has an important role in defending against pathogens infection. Cysteine proteinases perform crucial functions in different levels of biological processes, such as intracellular protein degradation, antigen processing, and inflammatory responses (Tassanakajon et al., 2013). Our results showed that four proteins from this family were down-regulated: ubiquitin carboxyl-terminal esterase L3, ubiquitin carboxyl-terminal hydrolase isozyme, cathepsin L-like protein 2, and cathepsin L. Previously, two crustacean cysteine cathepsins had been characterized and evaluated at the mRNA level during infection, cathepsin B from *P. clarkii* (Dai et al., 2016) and cathepsin C from *F. chinensis* (Wang et al., 2012). Cathepsin B transcripts in *P. clarkii* were up-regulated after lipopolysaccharide (LPS) injection (Dai et al., 2016). Cathepsin C from *F.*

chinensis was reported to occur in hemocytes, and its transcripts up-regulated in hemocytes after 24 hpi WSSV infection. These results backed up the idea about the need to perform a specific analysis for different crustacean species (Söderhäll, 2016), even if the viral agent is the same.

Peptidyl-prolyl cis-trans isomerase, FKBP-type. The FK Binding Proteins (FKPBs) participate in the protein quality control process. The main enzymatic function of FKPBs is the peptidyl-prolyl cis/trans isomerase (PPIase) activity, which is essential for the final structure and correct folding of a protein (Galat, 1993). They act as chaperones guaranteeing correct protein folding and participate in cellular processes during growth and development (Breiman and Camus, 2002). In *Artemia* species, this protein family is also involved in homeostasis and provides fitness and adaptation during environmental changes (Maniatsi et al., 2015). Our results showed three proteins from this family as down-regulated: FK506-binding protein 2-like, peptidyl-prolyl cis-trans isomerase-immunophilin FKBP13, and peptidyl-prolyl cis-trans isomerase FKBP2. Sangsuriya et al. (2011) reported an FKBP in *P. monodon* (PmFKBP46) and observed an interaction between PmFKBP46 and the WSSV viral protein VP15, suggesting the virus recruits PmFKBP46 during virion assembly. However, to unveil a more precise interaction between FKPBs and viral pathogenesis, further studies are needed.

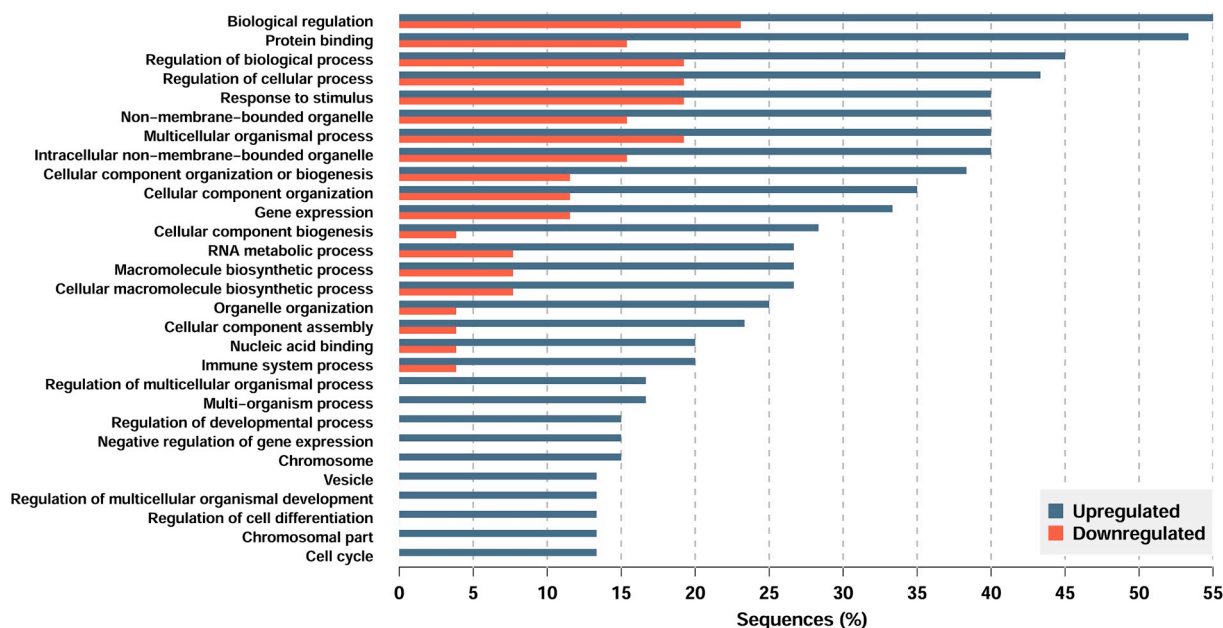


Fig. 4. GO analysis of differential proteins involved in biological processes identified in *L. vannamei* hemocytes infected with WSSV.

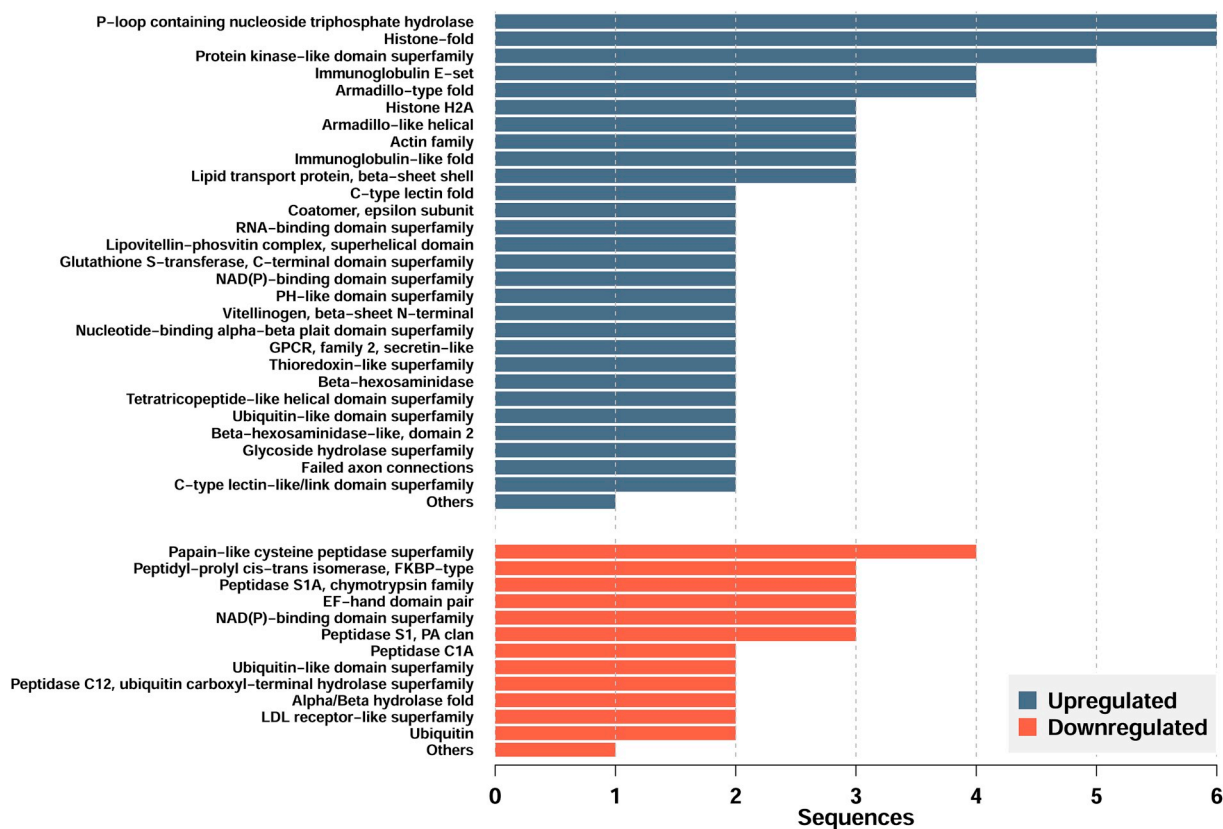


Fig. 5. InterProScan analysis of protein families corresponding to differential proteins identified in *L. vannamei* hemocytes infected with WSSV.

Peptidase S1A, chymotrypsin family. S1 family of serine proteases (SPs) mediates embryonic development and immune defenses (Jiang and Kanost, 2000). Our results showed three proteins from this family as down-regulated: two venom serine proteases Bi-VSP-like, and the phenoloxidase-activating factor 3 heavy chain. Two serine proteinase homologs (SPH) have been studied in shrimps, SPH with

prophenoloxidase activating activity of *P. monodon* (PmPPAF) (Ma et al., 2014), and a masquerade SPH from *F. chinensis* (FcMas) (Ren et al., 2011). Both PmPPAF and FcMas are mainly expressed in hemocytes (Amparyup et al., 2007; Ren et al., 2016) and a decrease in the expression level of this enzyme during WSSV infection was also reported (Ma et al., 2014; Ren et al., 2011).

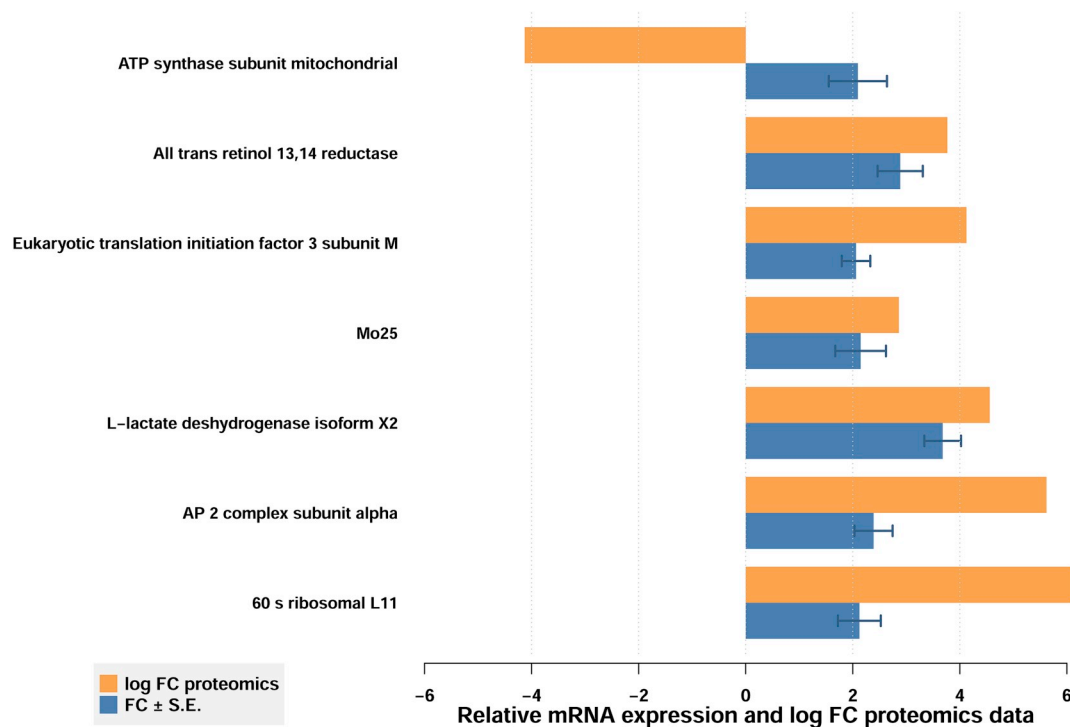


Fig. 6. Validation of proteomic data using RT-qPCR. Relative mRNA levels (Fold of change FC mean $\pm$ S.E. in blue) and log FC proteomics (in orange) data of WSSV infected hemocytes. Genes validated were: ATP synthase subunit mitochondrial, all-trans retinol 13,14 reductase, eukaryotic translation initiation factor 3 subunit M, Mo25, L-lactate dehydrogenase isoform $\times$ 2, AP 2 complex subunit alpha and 60 s ribosomal L11. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4.2. Up-regulated proteins families

P-loop containing nucleoside triphosphate hydrolase superfamily. P-loop_NTPase is the most common domain of the distinct nucleotide-binding protein folds. The energy from NTP hydrolysis is used to induce conformational changes in molecules. Our results showed six proteins from this superfamily as up-regulated: two DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX), protein Spätzle, septin-9 isoform $\times$ 3, Ras-related protein Rap-2c-like, and protein Mo25-like.

Soponpong et al. (2018) characterized *P. monodon* DDX (PmDDX41), a cytosolic DNA sensor in shrimp that participates during the antiviral response. Also, the authors reported up-regulation of PmDDX41 transcripts during WSSV infection, suggesting that PmDDX41 plays a role in the immune response against viruses via a DNA-sensing pathway.

Spätzle protein is a signaling ligand in innate immune response that signals pathogenic infection via Toll receptor and Toll pathway into the cells for the synthesis of antimicrobial proteins. This protein activates the Toll pathway, making the shrimp less susceptible to infections. Spätzle is expressed in several tissues, including the hemocytes of *P. monodon* (Boonrawd et al., 2017). During Toll pathway, Spätzle also activates the synthesis of four antimicrobial proteins (crustinPm1, crustinPm7, ALFPm3, penaeidin3) in late stages of infection (Boonrawd et al., 2017), which were not detected in this study.

Septins act as nucleic acid-binding proteins and are also utilized for cytokinesis and autophagy at the cytoplasmic level (Barve et al., 2018). Transcripts from a neuronal-specific septin 3 were up-regulated after WSSV and *Vibrio* challenge in the lymphoid organ (Wang et al., 2008a,b); however, the specific role of septin 9 during WSSV infection remains unclear.

Ras-related protein GTPase (Rap) has been implicated in cell adhesion, cell proliferation, and cell junction formation (Lundquist, 2006). Ren et al. (2012) identified a shrimp Rap from *F. chinensis* (FcRap). FcRap is present in hemocytes and at the transcriptional level could be detected as upregulated from 2 h post WSSV challenge, with a

peak at 6 h before declining at 12 h. Post-transcriptional studies should be assessed to clarify its role during viral infections.

Mo25 has been identified as a protein involved in asymmetric cell divisions in *Drosophila* and change during its expression leads to mutations (Yamamoto et al., 2008). Studies in vertebrates have shown Mo25 is involved in the regulation of ion homeostasis and blood pressure, as well as in development control and morphogenesis (Filippi et al., 2011). To our knowledge, this is the first report of Mo25 up-regulation during WSSV infection.

Histone-fold superfamily. Histones are proteins that play an important role during the regulation of genes at the nuclear level. A multi-omic analysis in *Machrobrachium rosenbergii* reported histone as one of the differentially expressed proteins in antennal gland involved with intermolt, premolt, and postmolt processes (Bose et al., 2017). Histone has also shown antibacterial activity in oysters (Dorrington et al., 2011). Our results showed six proteins from this family as up-regulated: histone H2A, one uncharacterized protein, histone H2B, histone H4 type G-like protein, histone 3 subunit a, and histone H2A.V. Wang et al. (2008a,b) discovered that ICP11, a WSSV protein, bound with histone proteins in the cytosol of WSSV-infected hemocytes and HeLa cells, preventing them to participate in nucleosome assembly, inducing incidental apoptosis. Li et al. (2014) showed histone H2B as a down-regulated protein during WSSV in hemocytes measured with 2-DE analysis.

Protein kinase-like domain superfamily. The serine/threonine kinase proteins are associated with multiple cellular functions including proliferation, survival, differentiation, apoptosis, tumorigenesis, immune regulation, and inflammation in vertebrates (Wang et al., 2001). Our results showed five proteins from this family as up-regulated: cAMP-dependent protein kinase catalytic subunit isoform $\times$ 6, serine/threonine-protein kinase PAK 3-like, calcium/calmodulin-dependent protein kinase type 1, titin-like, and threonine/serine protein kinase. Recently, a serine/threonine gene from *L. vannamei* (LvPim1) which shares a close evolutionary relationship with those from vertebrates,

was discovered (Aweya et al., 2018). Junkunlo et al. (2017) demonstrate how a tyrosine kinase (TK) receptor whose ligand is platelet-derived growth factor (PDGF) is crucial in the transduction of extracellular signals into cells and mediates numerous processes, such as cell proliferation, differentiation, survival, and migration. They also discovered these molecules are highly expressed in hemocytes and the hpt. Aweya et al. (2018) reported LvPim1 transcript level was induced in hemocytes upon challenge with WSSV, followed by an increase in hemocyte apoptosis after its knockdown, suggesting its probable involvement in shrimp immune response.

3.4.3. Allergy-related proteins

Consumption of shrimps sometimes causes allergic reactions in humans (Faber et al., 2017), and although WSSV infection is not considered zoonotic to humans, up to date, there is lack of information about the potential risk to human health of the enhanced synthesis of allergens during infection with WSSV or another virus in shrimps. This is a remarkable topic to study since most of the shrimp's diseases are globally distributed, and sanitary regulations are not always strictly followed. Thus, the link of allergy to the ingestion of fresh shrimp infected with WSSV or other viruses is a risk that has not been addressed previously. Herein we discuss the main allergy-related proteins found.

Ubiquitin. Protein degradation is the most well-studied aspect of the ubiquitin-proteasome system; protein ubiquitination is also responsible for regulating cell signaling by controlling the endocytosis of plasma membrane receptors (Yi and Ehlers, 2007). In this study, five ubiquitin proteins were detected as down-regulated: polyubiquitin, ubiquitin-conjugating enzyme E2 N, ubiquitin-40S ribosomal protein S27a, ubiquitin carboxyl-terminal esterase L3, and ubiquitin carboxyl-terminal hydrolase isozyme. Levels of ubiquitin vary depending on the time post-infection with WSSV. In a previous study, the concentration of this protein during WSSV infection was recorded with a sharp decrease at 12 hpi and an induction at 18 hpi, suggesting the virus sequesters the protein and uses it for viral replication (Vidya et al., 2013). Ubiquitin is considered an allergy related protein (Faber et al., 2017) and it is abundant in the abdominal muscle of shrimps (Schey et al., 2016). Further studies should be conducted to establish the kinetics of these proteins.

Tropomyosin. This protein regulates actin filaments related to muscle contraction and cell functions such as cytoskeleton structure. There are more than 40 tropomyosin isoforms involved in the actin filament system of these cell functions (Gunning et al., 2005). Our results showed the upregulation of tropomyosin-1, isoforms 9A/A/B isoform $\times 30$. The upregulation of tropomyosin during WSSV infection was reported previously (Jeswin et al., 2016; Li et al., 2016). Tropomyosin is considered to be the major cross-reactive allergen among crustaceans and mollusks.

Hemocyanin. This protein is involved in oxygen transport within the hemolymph of invertebrates and has been linked to key aspects of antiviral innate immunity (Coates and Nairn, 2014). Hemocyanin V4 was detected as up-regulated in this study. This protein is heat stable and has cross-reactivity with its homolog in the house dust mite (Giuffrida et al., 2014).

Others. Some other proteins detected in the present study as up-regulated have shown allergen activity evidence (Faber et al., 2017), including titin-like, actin E, cytoplasmic actin, actin-related protein 2/3 complex subunit 3-like, actin-depolymerizing factor, and beta-actin. However, a deeper characterization is still required.

3.5. Validation of proteomic data

A group of seven differentially expressed proteins were selected to validate the proteomic data using relative quantification by real-time PCR. Protein expression levels and their mRNA levels did not match perfectly (Fig. 6), however, this could be attributed to post-transcriptional mechanisms involved during the translation process (Greenbaum et al., 2003).

4. Conclusions

Herein we provide the first record of the perturbed proteome profile of the hemocytes of *L. vannamei* at 12 hpi. Relevant information from this study shows that the immune system is strongly compromised at early stages of WSSV infection. Unknown proteins must be characterized to have a complete knowledge of the critical processes involved during the host immune response. The generation of more detailed proteomic data in mature hemocytes with new technologies will contribute to the knowledge of crustacean immune mechanisms, necessary for a sustainable aquaculture industry. Finally, we strongly recommend studying allergy-related proteins during viral infections and further studies on this topic when a host-parasite interaction occurs. Moreover, important differences exist among crustacean species' response to WSSV infection and it is mandatory to conduct specific studies to achieve accurate host-specific immune response against WSSV.

Author contribution statement

HPA, ZBJA, and RCR conceived the study. HPA and ZBJA designed the research and conducted experiments and molecular analysis. HPA and PGS carried out the TEM analysis. HPA carried out the SEM analysis. HPA, ZBJA, RME, PSA, ECJM carried out the proteomic and biostatistics analysis. TIE helped with the edition of the graphics. HPA, ZBJA, ECJM, HLJ, GCMG and RCR analyzed data and drafted the manuscript. GCMG provided the organisms for the experiments. RCR and RME provided financial support. All authors read and approved the manuscript.

Declaration of interest

None.

Selection of editor

Kenneth Söderhäll.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.dci.2018.10.002>.

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Table 1 supplementary. Primers sequences designed for proteomic data confirmation with quantitative real-time PCR.

Gene	Sequence (5' - 3')	Accession number
60S ribosomal L11	CAAGATCTCCTCAGCCTTGG	GETD01034607.1
	TCAACATCTGCGTTGGAGAG	
AP-2 complex subunit alpha	TCATCTGTGCCGTCTGAGTC	GETD01037536.1
	TCTTCCATCTTAGCGGTGCT	
L-lactate dehydrogenase isoform X2	GACGAGCACCAGCAGTAACA	GETD01035385.1
	AAGCTGCGTGGAGAGATGAT	
Mo25	GACGAGCAGTTCAATGACGA	GETZ01047375.1
	ATGAGCCTCTCAGTGCGAAT	
eukaryotic translation initiation factor	GTGGACCCTTGAGGTTACGA	GETD01038532.1
	ACGGAAATGACCTTCGACAC	
all-trans-retinol 13,14-reductase	ACTGGGTATTTGCGAACTGG	GETD01030029.1
	TCGACCAAATCACAGATGGA	
ATP synthase subunit mitochondrial	GCAGGTTGATGTCCCTTCAT	GCVY01000156.1
	GACAATGCCAGAGCTCACAA	