Differential expression of proteins in the gills of *Litopenaeus vannamei* infected with white spot syndrome virus

Pedro A. Valentim-Neto · Ana P. M. Fraga · Maria R. F. Marques

Abstract Determination of differentially expressed protein profile is necessary to understand the host response to viral infection. Proteomics can be applied as a tool to examine white shrimp *Litopenaeus vannamei* molecular responses against white spot syndrome virus (WSSV) infection, thus enabling development of effective strategies to reduce their impact on farms. In the present study, specific pathogen-free shrimp was tested against WSSV infection under several time intervals. Shrimps were submitted to a viral load of with 5.5×10^6 viral copies in 100 μL/shrimp. The monitoring of infection was performed in intervals of 6, 12, 24, 48 and 72 h after infection. The analysis was realized using 2-DE, and differentially expressed proteins were identified by MALDI-TOF mass spectrometry (MS) peptide mass fingerprint (PMF). Between the differentially expressed proteins found in the infected animals, the most important were identified as caspase-2, ubiquitin and F1-ATP synthase. They are interesting candidates for biomarkers because could be related to the beginning of apoptosis process. The differentially expressed protein profile creates a new paradigm in the analysis of *L. vannamei* shrimp molecular response to WSSV infection and in virus–host relationship. Furthermore, it proposes potential biomarkers that allow strategies both selecting less susceptible individuals and reducing the impact of viruses on farms.

Keywords WSSV · Shrimp · Infection · Biomarkers

Introduction

Shrimp farming has grown significantly in recent years, and diseases have had a major impact particularly in the Americas and Asia, where most of the world's farmers are

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located. Only in the Americas, the shrimp industry is responsible for approximately 20 % of the farmed shrimp produced worldwide. The main producers are Ecuador, Brazil, Honduras and Mexico. Shrimp *Litopenaeus vannamei* is the most cultivated accounting for more than 95 % of the total. The second most cultivated is the *Litopenaeus stylirostris* species (Lightner 2011).

Viruses are the most common pathogens found in the sea. Up to 10 billion viruses can be found in one liter of seawater, and they can infect nearly all organisms such as phytoplankton, bacteria and others (Fuhrman 1999). Viral infections are common in crustaceans such as penaeid shrimps, which can be infected by more than twenty different viruses (Loh et al. 1997). Crustacean viruses belong or are related to various viral families such as Baculoviridae, Bunyaviridae, Herpesviridae, Picornaviridae, Parvoviridae, Reoviridae, Rhabdoviridae, Togaviridae and, Iridoviridae, or to a new family called Nimaviridae (Jiravanichpaisal 2005).

In order to have an improved disease control, more understanding concerning the immune system of the shrimp is necessary. The crustacean defense mechanisms depend completely on the innate immune system that is activated by the recognition of molecular patterns. Pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, such as lectins, antimicrobial, clotting and pattern recognition proteins, which, in turn, activate cellular or humoral effectors mechanisms to destroy invading pathogens (Kawabata and Iwanaga 1999; Marques and Barracco 2000; Vazquez et al. 2009).

In this context, the study of crustacean immune system is emerging as a promising strategy, since it allows better understanding about the susceptibility and resistance of animals to pathogens and parasites and provides valuable insights into the establishment of health indicators and immunomarkers for genetic selection of animals resistant to infection (Barracco et al. 2007; Robalino et al. 2009).

The advance of analytical techniques, additional biochemical approaches and molecular biology enables proteomic analysis to become a new improved tool in the study of protein expression patterns in organs, cells or subcellular compartments. Therefore, opportunities are opening up for major effectors of the crustacean immune response, including the prophenoloxidase system and antimicrobial peptides that can be quickly clarified with a reasonable level of confidence. In several circumstances, such studies may provide a better understanding of the cellular response to various external factors (Bachère et al. 2004; Cerenius and Soderhall 2004; Somboonwiwat et al. 2010). The aim of this study was to characterize the profile of differentially expressed proteins in *L. vannamei* in response to white spot syndrome viral infection, by using specific pathogen-free (SPF) shrimp gills experimentally infected with the virus. Shrimps selection was realized by assessment of pleopods that are abdominal appendages of crustaceans. The gills were chosen because they are a target tissue in WSSV infection. Furthermore, potential protein targets were proposed, such as molecular biomarkers for the development of new diagnostic methods. The results are discussed in light of new tools for preventive monitoring of cultures, moreover for the selection of animals less susceptible to WSSV, and for conducting studies to better understand the etiology of this infection in cultured shrimp.

Materials and methods

Biological material

Juvenile SPF shrimps of about 0.3 g individual body mass provided by Quality farm were stocked into 50-L plastic tanks and divided into control and treatment groups. Each group

consisted of 15 shrimps subdivided into 3 groups with 5 replicates to sample gills and pleopods at 6, 12, 24, 48 and 72 h after viral inoculation. The shrimps were kept in constant aeration under the following conditions, 4.0 mg/mL dissolved oxygen, 0.5–1.5 mg/L (NH_3), pH 8.5, temperature 23 °C and 25 ‰ salinity. The animals were conditioned for a period of 3 days. After this period, the experimental infection was performed with an inoculum prepared from positive animals, farmed in the region of Santa Catarina, Brazil. Some of this with historic of white spot and the presence of the virus were confirmed by nested PCR. The inoculum was 5.5 $\times$ 10^6 viral copies in 100 μL/shrimp quantified by real-time PCR (qPCR). The viral load quantification of the animals collected was performed using RT-qPCR (Table 1). Pleopods and gills were collected using sterile material and kept in liquid nitrogen until they were stored at −80 °C for subsequent procedures.

Detection of WSSV

Shrimp pleopods were collected, and total genomic DNA was extracted for detection of WSSV by nested PCR according to OIE (2003) following procedures described in Lo et al. (1996). The following primer pairs were used: WS146 F1 (5′-ACT ACT AAC TTC AGC CTA TCT AG-3')/WS146 R1 (5′-TAA TGC GGG TGT AAT GTT CTT ACG-3′) and WS146 F2 (5′-GTA ACT GCC CCT TCC ATC TCC A-3')/WS146 R2 (5′-TAC GGC AGC TGC TGC ACC TTG T-3′), generating a 941 bp length fragment. Amplification results were viewed in 2 % agarose gel stained with ethidium bromide.

Extraction and quantification of proteins from gills

The gills was macerated in mortar and pestle and kept cooled in liquid nitrogen to prevent degradation. Immediately, PMSF in PBS buffer was added, and the mixture was centrifuged at 15,000×g for 1 h at 4 °C, the supernatant was transferred into a new microtube. Four volumes of acetone containing 12.5 % TCA and 0.125 % DTT (final concentration 10 and 0.1 %, respectively) was added to the extracted proteins. The mixture was maintained on ice for 1 h to precipitate. Another centrifugation was made at 20,000×g for 20 min at 4 °C, the supernatant was discarded. The precipitate, containing the total protein, was washed with 1 mL cold methanol. It was then suspended in cold methanol, stirred for homogenization and submitted to new centrifugation at 20,000×g for 5 min at 4 °C, the supernatant was discarded. Washing was performed twice more. After washing with methanol, the precipitate was washed twice with cold acetone to remove any traces of methanol and TCA. The precipitate containing the total protein was resuspended in acetone containing 0.1 % DTT and once again centrifuged at 10,000×g for 30 min at 4 °C, and the supernatant was discarded. The final precipitate was air dried and dissolved in 1 mL rehydration solution (7 M urea, 2 M thiourea, 2 % CHAPS) with the addition of 20 μL of PMSF (4 mg/mL). A final

Table 1 Viral load quantification at different intervals after infection with WSSV in *L. vannamei* shrimp

Real-time PCR	Viral charge
Hours after infection	Viral copies/μL
6	$3,00 \times 10^1$
12	$7,37 \times 10^2$
24	$8,38 \times 10^1$
48	$2,57 \times 10^6$
72	$7,79 \times 10^5$

10,000×*g* centrifugation at 4 °C was performed for 30 min, and the supernatant containing soluble protein fraction was collected. A 100 μL aliquot was used for quantification of total protein content by Bradford (1976). BSA was used as the standard for quantification. The remainder of the sample was stored at −80 °C for subsequent purification procedures.

2-DE

2-DE was performed by Ettan IPGphor 3, SE 600 Ruby system (GE healthcare®, Uppsala, Sweden) using protocol in accordance with manufacturer. Total proteins (250 μg) were mixed in 250 μL rehydration buffer (7 M urea, 2 M thiourea, 2 % w/v CHAPS, 0.28 % w/v DTT, 1 % v/v IPG buffer and 0.002 % bromophenol blue) and isoelectric focusing (IEF) using 13-cm IPG strips immobilized with 3–10 linear gradient pH (GE Healthcare®). IEF was performed until a total of 20 kWh has been accumulated. Proteins were subsequently reduced with DTT and alkylated with iodoacetamide before the second dimension (SDS-PAGE). Strips were equilibrated with solution containing 6 M urea, 30 % glycerol, 2 % SDS, 75 mM Tris–HCl, pH 8.8, 0.002 % bromophenol blue and 1 % w/v DTT for 15 min. Then, proteins were treated with the same solution containing 2.5 % w/v iodoacetamide instead of DTT for 15 more min. The equilibrated gel strip was then placed on top of SDS-PAGE gel 12.5 % and sealed with 0.5 % agarose containing bromophenol blue. SDS-PAGE gel run was performed at 15 mA per gel during 25 min, then 30 mA per gel until the frontline of the run reached the end of the gel. Subsequently, the gels were fixed in a solution containing 50 % (v/v) methanol, 10 % (v/v) acetic acid, and Coomassie Brilliant Blue stain Neuhoff et al. (1985), containing 10 % (w/v) ammonium sulfate, 10 % (v/v) phosphoric acid, 20 % (v/v) methanol and 0.12 % (w/v) CBB-G250 (Merck, Germany). Samples were produced in triplicates for the infected and control group, and each sample gel was composed of gill tissue proteins of three animals.

Imaging analysis

Gel images were acquired in an Image Scanner III (GE healthcare®) and examined using the Image Master 2D Platinum 7.0 software (GE healthcare®). Individual spots on each gel were detected by their limit, and the volume (abundance) of the spot was automatically calculated. The volume percentage (% vol), as well as the normalized value of volume intensity for each spot and the volume intensity of all the spots detected in the gel were statistically analyzed by ANOVA using a 0.05 level of significance. Spots were considered differentially expressed based on their ratio between infected and control groups (relative expression) and divided into three categories: greater or exclusive expression in the infected group; deleted or higher expression in the control group; and weakly but not significantly different "constants" (present in both groups).

In gel protein digestion and protein identification

The differentially expressed spots of protein were excised from gel, and individually transferred into sterile tubes. According to the protocol of Chai et al. (2010), each spot was washed thrice with distilled water, and 200 μL of acetonitrile 40 % (ACN) in 200 mM of ammonium bicarbonate (pH 8.5) was added at 37 °C for 30 min. Each spot was then dehydrated with 100 μL ACN for 5 min at room temperature, dried for 15 min and rehydrated with 5 μL of trypsin solution containing 20 mg/mL of trypsin (Proteomics

Grade, Sigma, EUA), 40 mM of ammonium bicarbonate and 9 % ACN in ice for 45 min. Trypsin solution surplus was removed, and 5 μL of ammonium bicarbonate 40 mM in 9 % ACM was added to each tube at 37 °C for 16 h. After incubation, the liquid was transferred into a new container. A total of 5 μL trifluoroacetic acid 0.1 % (TFA) in ACN solution 50 % was added to each and incubated for 30 min at 37 °C. Finally, the solution was mixed with 40 mM ammonium bicarbonate liquid in 9 % ACN. The mixture was dried under vacuum, dissolved in 0.1 % TFA and then subjected to MALDI-TOF. Mass spectrometry (MS) data were obtained from Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics). The mass spectrometer was set to peptide mass fingerprint (PMF), with full automatic mode using FlexControl^{TM}. In this study, 20 kV acceleration and 50 Hz laser frequency were used. The spectrum was analyzed by FlexAnalysis 3.0 (Bruker Daltonics, Bremen, Germany). Proteins were determined by PMF, and the list of masses derived from peptides was searched in NCBI non-redundant database (NCBInr) using the MASCOT program (http://www.matrixscience.com/cgi/protein-view). Research in the database was performed using the following parameters: database, NCBInr, taxonomy; metazoa, enzyme; trypsin. Peptide mass error limit was set at ±100 ppm, or MS ion mass tolerance was set at 0.6 Da. Carbamidomethylation of cysteine and methionine oxidation was selected as fixed and variable modifications, respectively. Significant hits (as defined by Mascot probability analysis) were regarded as positive identification.

Results

Protein identification

The gills of *L. vannamei* from both infected and control groups were collected, homogenized and subjected to 2-DE analysis. After staining with CBB, about 1,500 spots on each gel were detected using ImageMaster Platinum 7.0, software, but only those with $p < 0.05$ were considered significantly different by analysis of variance (ANOVA). Gel analysis (see Fig. 1) showed the protein expression profile in gills of WSSV-infected and control shrimp. A total of 47 proteins were submitted to PMF, and subsequently to MASCOT for identification. The searches were performed in the NCBI nr for protein homology identification. Groups were divided as follows: 6 h: 10 proteins (1 up-regulated, 4 down-regulated and 5 with decreased expression in the infected group); 12 h: 07 proteins (5 up-regulated and 2 down-regulated in the infected group); 24 h: 06 proteins (4 up-regulated and 2 with increased expression in the infected group); 48 h: 11 proteins (3 down-regulated, 7 with decreased expression in the infected group and 1 constant for both groups) and 72 h: 13 proteins (8 up-regulated, 1 down-regulated and 4 with increased expression in the infected group). In the total of proteins, 28 were differentially expressed, and these were of both positive and negative animals for WSSV. With exception of the constant protein, all the other mentioned above showed either increased or decreased significant difference ($p < 0.05$) between groups. The identified proteins and related information are listed in Table 2.

Discussion

Differential protein profile of gills was assessed by the spot analysis comparing infected and uninfected animal group gels. Gills were chosen because they are a target tissue in
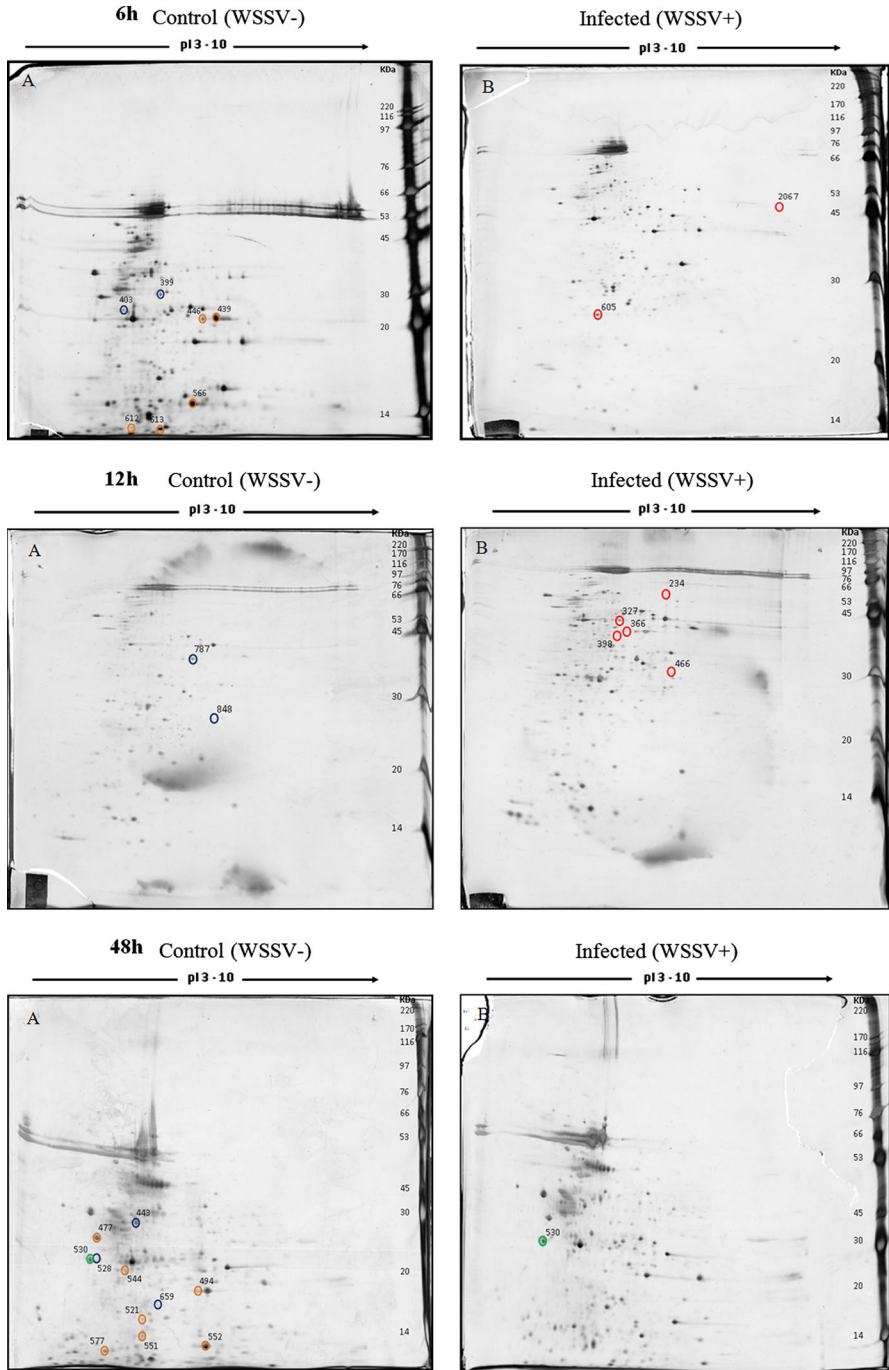
Fig. 1 2-DE protein profile of gills from *Litopenaeus vannamei,* 6, 12, 48 h after WSSV infection. (**a**) IPG 3-10, PBS control, (**b**) IPG 3-10, WSSV-infected. Eligible protein spots that showed consistent change or were constant during WSSV infection are *circled. Numbers* correspond to the entries in Table 1

Table 2 Altered proteins after WSSV infection in gills of *L. vannamei* identified by peptide mass fingerprint (PMF)

Spot no.	Protein	Specie	Accession number	Mascot score/SC (%)	Fold change	Observed pI/MW (kDa)
Protein spots 6 h.a.i.						
399	PREDICTED: kelch domain-containing protein 8A-like	[*Oreochromis niloticus*]	gi|348518042	79/7	D	9.27/39
403	Hypothetical protein SINV_05700	[*Solenopsis invicta*]	gi|322796548	78/5	D	5.20/19
439	Hypothetical protein CBG21133	[*Caenorhabditis briggsae*]	gi|268575544	75/6	−1.43	5.45/15
446	Protein kinase anchor (AKAP)	[*Homo sapiens*]	gi|119610330	67/10	−2.46	8.48/76
566	Calcitonin	–	gi|356170	61/3	−2.50	8.04/3.4
605	CBR-LIG-4 protein	[*Caenorhabditis briggsae*]	gi|351700892	60/6	D	6.94/82
612	AGAP003687-PA	[*Anopheles gambiae* str. PEST]	gi|158291944	70/8	−2.32	6.07/102
613	IP20448p	[*Drosophila melanogaster*]	gi|189181950	61/5	−13.33	6.75/34
2067	Unnamed protein	[*Homo sapiens*]	gi|189054178	80/9	U	7.62/66
605	CBR-LIG-4 protein	[*Caenorhabditis briggsae*]	gi|351700892	60/6	D	6.94/82
Protein spots 12 h.a.i.						
234	PREDICTED: uncharacterized protein KIAA1462-like	[*Anolis carolinensis*]	gi|327274528	58/4	U	8.48/19
327	Hypothetical protein CLF_015359, partial	[*Clonorchis sinensis*]	gi|350033705	78/9	U	10.01/38
366	Unnamed protein product	[*Tetraodon nigroviridis*]	gi|47212035	65/8	U	6.61/59
398	PREDICTED: nudix-type motif 9-like	[*Saccoglossus kowalevskii*]	gi|291234261	58/6	U	7.15/35
466	Metionin sintase	[*Danio rerio*]	gi|37620202	63/9	U	5.26/140
787	Arginin quinase	[*Litopenaeus vannamei*]	gi|307334072	132/15	D	6.84/36
848	Unnamed protein product	[*Tetraodon nigroviridis*]	gi|47224481	61/5	D	9.45/20
Protein spots 24 h.a.i.						
162	Putative ribosomal protein L11	[*Trypanosoma vivax*]	gi|340052452	81/7	U	9.75/39
258	Beta actin	[*Litopenaeus vannamei*]	gi|10304437	100/9	U	5.30/42
275	Protein phosphatase-1 regulatory subunit 7 beta 1	[*Homo sapiens*]	gi|4633068	77/5	U	4.66/32
285	Ubiquitin	[*Glossina morsitans morsitans*]	gi|289741017	40/5	0.21	5.23/26
343	Mef2 myocyte enhancer factor 2	[*Podocoryna carnea*]	gi|18676385	68/7	0.08	9.17/48

Table 2 continued

Spot no.	Protein	Specie	Accession number	Mascot score/SC (%)	Fold change	Observed pI/MW (kDa)
426	Caspase-2	[Heliothis virescens]	gi|339830904	64/5	U	5.92/32
Protein spots 48 h.a.i.						
443	Vacuolar ATP sintase	[Carcinus maenas]	gi|6425061	101/13	D	5.31/54
477	F1-ATP sintase beta subunit	[Litopenaeus vannamei]	gi|170779040	192/24	−1.44	5.03/55
494	Arginine quinase	[Litopenaeus vannamei]	gi|307334072	136/13	−1.36	6.84/36
521	Amylase precursor	[Mus musculus]	gi|553866	67/5	−2.92	9.43/20
528	Laminine receptor	[Litopenaeus vannamei]	gi|111572543	75/8	D	4.79/34
530	Hypothetical protein SINV_06746	[Solenopsis invicta]	gi|322782939	77/6	C	6.32/27
544	PREDICTED: hypothetical protein LOC480064 isoform7	[Canis lupus familiaris]	gi|345803227	79/10	−1.76	5.29/93
551	60S ribosomal	[Salmo salar]	gi|209735198	78/6	−2.32	10.42/11
552	Triosephosphate isomerase	[Procambarus clarkii]	gi|328900101	79/8	−2.03	5.68/27
577	Hypothetical protein BRAFLDRAFT_68898	[Branchiostoma floridae]	gi|260795164	86/17	−1.90	6.49/190
659	Proteasome subunit β-3	[Sus scrofa]	gi|42600873	86/5	D	9.62/4.4
Protein spots 72 h.a.i.						
158	DNA topoisomerase 3-beta-1	[Rattus norvegicus]	gi|157786738	58/11	U	8.37/98
162	Hypothetical protein EAG_13577	[Camponotus floridanus]	gi|307175491	60/3	U	8.96/4.9
266	PREDICTED: apolipoprotein A-I-like	[Oreochromis niloticus]	gi|348523730	67/9	6.34	5.60/29
275	GE14732	[Drosophila yakuba]	gi|195497248	53/4	U	8.80/15
300	MCG147328	[Mus musculus]	gi|148677657	53/4	0.57	8.69/11
350	28S ribosomal	[Schistosoma mansoni]	gi|256083308	48/5	U	9.35/28
356	Cholesterol ester transfer	[Oryctolagus cuniculus]	gi|291190970	61/5	U	6.04/54
357	GK15763	[Drosophila willistoni]	gi|195431373	51/4	3.24	5.93/12
360	GG16269	[Drosophila erecta]	gi|194876300	54/4	U	8.52/31
368	Glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase	[Schistosoma mansoni]	gi|256068995	65/5	0.71	9.64/6.9
399	Ubiquitin	[Glossina morsitans morsitans]	gi|289741017	53/5	U	5.23/26

Table 2 continued

Spot no.	Protein	Specie	Accession number	Mascot score/SC (%)	Fold change	Observed pI/MW (kDa)
404	Hypothetical protein CRE_01080	[Caenorhabditis remanei]	gi|308483758	49/4	U	9.41/18
457	PREDICTED: similar to aldehyde dehydrogenase 9 family, member A1a	[Ciona intestinalis]	gi|198413878	52/5	D	5.86/57

− indicates the decrease in the spot intensity at a given time and point after WSSV infection, compared with that of normal shrimp

+ indicates the increase in the spot intensity at a given time and point after WSSV infection, compared with that of normal shrimp

U indicates up-regulated protein spot detected only in extracted proteins from infected animals

D indicates down-regulated protein detected only in extracted proteins from normal animals

C indicates constant proteins detected in both protein extracts with the same expression intensity

Mascot scores greater than 40 indicate proteins with statistical significance ($p < 0.05$)

SC: amino acid sequence covering to the proteins identified

WSSV infection (Escobedo-Bonilla et al. 2007). Forty-seven spots were selected in groups 6, 12, 24, 48 and 72 h after infection. In 24 h, the viral load decreases what is showed in Table 1. It can be possible due to an effective response of shrimp to viral infection, which was identified by up-regulated expression of caspase-2 that marks the onset of apoptosis in infected cells. The 28 differentially expressed proteins are probably enzymes involved in the amino acid metabolism, as well as proteins that are active in other cell functions in accordance with results found by Rattanarojpong et al. (2007), Wang et al. (2007) and Chongsatja et al. (2007).

(Spot 258) Beta actin, a ubiquitous protein involved in the formation of filaments which are important components of the cytoskeleton. The interaction with myosin provides the basis of muscle contraction and many aspects of cell motility. Each actin protomer binds an ATP molecule and calcium ions or magnesium. Actin exists as a monomer in low salt concentrations, but filaments form rapidly as salt concentration rises, with the consequent hydrolysis of ATP. Polymerization is regulated by so-called capping proteins. Actin is a member of a superfamily of proteins, which includes hsp70. The ATPase domain of actin shares similarities with ATPase domains of hsp70 protein (Van Den Ent et al. 2001). In this study, increased expression was observed 24 h.a.i., probably due to increased muscle fiber recruitment during contraction, cell motility and possible involvement in the process of phagocytosis (May and Machesky 2001). (Spot 544) Important mitochondrial transcription factor in mitochondrial biogenesis during mitochondrial DNA development and differentiation (Larsson et al. 1998) and its regulation seem to be linked to the FAK protein (Tornatore et al. 2011).

(Spots 494, 787) Arginine kinase (AK) cell buffer of ATP level, which reversibly, catalyzes transphosphorylation between phosphoarginine and ADP. AK had increased expression in *Jasus edwardsii* and *P. stylirostris* both infected with WSSV (Speed et al. 2001; Astrofsky et al. 2002). Similar increased expression of AK was also observed in *S. serrata* infected with that virus, indicating that AK regulates physiological response during viral infection, and that it is associated to the mechanism of innate immunity (Robalino et al. 2009), this could explain low metabolism observed in some species infected with WSSV. It is interesting that among the identified proteins, two AKs have different pIs and molecular weight, suggesting that there are different AKs in the hemolymph of *S. serrata* and they should play different roles in cellular response to viral infection (Liu et al. 2011). In our study, we found a decrease in the production of AK 12 and 48 h after viral infection, but results found in the field showed that AK levels remained constant for both groups (control and infected), probably due to persistent viral infection and possible accommodation process in animals infected in the field, in response to white spot virus, since it was the same protein (Flegel and Pasharawipas 1998; Flegel 2007; Flegel and Sritunialucksana 2011).

(Spot 426) Caspases are central effectors in apoptosis with a key role in the antiviral process of many organisms, including shrimps, by eliminating cells infected with viruses (O'brien 1998; Granja et al. 2006; Wang et al. 2008; Zhi et al. 2011). It has been shown that 24 h.a.i. by WSSV, the nucleus of the cell is filled with enveloped and non-enveloped virions (Wongprasert et al. 2003). At this stage, the mechanism cannot be as effective in preventing animal death. In our work, caspase-2 had its expression enhanced 24 h.a.i., which indicates animal response to WSSV, as to hinder viral infection by means of the apoptosis. However, at this stage, such mechanism will favor the virus, since it will be ready to infect other cells (Wongprasert et al. 2003; Barracco et al. 2007). (Spot 528) Laminin receptor is required for the mounting and/or stability of the 40S ribosomal subunit. Important for the processing of 20S ribosomal subunit, precursor to mature 18S

rRNA, in a final stage of 40S ribosomal subunits maturation, and vital to *L. vannamei* shrimp in protein synthesis (Phiwsaiya et al. 2010). This could explain why this protein has had its expression suppressed in the infected group 48 h, probably a viral mechanism of action to prevent the induction of apoptosis in infected host cells. Thus, favoring a higher synthesis activity in the process of viral infection. (Spot 551) Recent studies on 60S ribosomal protein have shown that when bound to 40S subunit by QM protein, it is in charge of inducing cellular apoptosis. Researches have shown that depletion of units of 60S ribosomal protein is associated with inhibition of proliferation and apoptosis, where p53 plays a central role in this process as in *Drosophila melanogaster* (Yan et al. 2010). Apparently, QM protein participates in different processes in antiviral defense, and this indicates its role in the defense against pathogens (Xu et al. 2008). According to Gonçalves-Soares et al. (2012), infected shrimp have increased transcription to the gene of QM, probably as defense against WSSV. In this study, we have observed a decrease of 60S ribosomal protein in the infected animals after 48 h, which, according to the authors mentioned above, could induce apoptosis, probably in response to this virus infection.

(Spot 566) Calcitonin is involved in the metabolism of calcium during the molt cycle of crustaceans (Arlot-Bonnemains et al. 1986; Luquet and Marin 2004). It had its expression reduced in animals with virus, 6 h.a.i., possibly because the animals were in the intermolt period. (Spot 466) Methionine synthase is an enzyme involved in the regeneration of methionine an important amino acid in the metabolism of shrimp needed for protein synthesis (Richard et al. 2011) had its expression increased in the group infected after 12 h, perhaps because of greater synthesis activity. (Spot 399) Kelch-containing domain (KD) is a protein linked to regulation of p53, which plays a central role in cell integrity in response to stress factors. Fine regulation of p53 is essential for maintaining genome integrity and normal cell proliferation. Importantly, KD inhibits p53 transcription and reduction in its level stabilizes activity of p53, promotes cellular apoptosis and maintains the cell in the G1 phase. This finding indicates that KD is a critical negative regulator of p53 and represents a way of maintaining it stable in cells that are not stressed (Sun et al. 2009). Results found show that this protein had its expression level reduced in infected animals after 6 h, which may already indicate the start of animal response to the virus in the early hours of infection.

(Spot 446) A-kinase anchor protein (AKAP) responsible for a variety of physiological responses and component of the tyrosine kinases. AKAP is associated to cytoskeleton cells and calcium anchor (Mochly-Rosen 1995). (Spot 566) calcitonin, in insects, is a family of peptides with sequence homology to vertebrate calcitonin and it has been implicated in the control of diuresis, a process which comprises hemolymph mixture. It was also described in crustaceans, such as *Homarus americanus* lobster and *Palaemon serratus* shrimp, during the molt cycle showing a decrease in the circulating calcium in hemolymph with the increase in calcitonin (Lamharzi et al. 1992; Christie et al. 2010).

(Spot 343) Myocyte enhancer factor (MEF) is a calcium/calmodulin-dependent protein, and it is involved in the process of skeletal muscle cellular differentiation (McKinsey et al. 2000), it had its expression enhanced in the group infected 24 h, which suggests greater control in the calcium/calmodulin mechanism. Some researches speculate that it mediates the process of apoptosis (Yu et al. 2002). (Spot 443) Vacuolar ATP synthase is molecular motor linked to the membrane and converts stored free energy in ATP molecule and electrochemical gradient of cations, H^+ or Na^+, through the membrane (Lolkema and Boekema 2003; Nakano et al. 2006). (Spot 477) The mitochondrial F_0F_1 ATP synthase, described as a splendid molecular machine (Boyer 1997), accomplishes a dual role since it is able to synthesize and hydrolyze ATP in eukaryotes and bacteria. The enzyme consists of two nanomotors, the F_1 (driven by ATP hydrolysis) and the F_0 (embedded in the

mitochondrial membrane and driven by a proton gradient) (Leslie and Walker 2000). F_1, the catalytic portion of the enzyme, is composed of five major subunits α3, β3, γ, δ and ε and includes the three catalytic sites formed by three alternating pairs of subunits α/β forming a sphere that functions as an ATPase by hydrolyzing ATP to ADP + Pi, and as an ATP-producer by synthesizing ATP from ADP + Pi (Futai et al. 1989; Lai-Zhang and Mueller 2000). The mitochondrial ATPase activity of shrimp is promoted during environmental stress conditions then, a specific mechanism may exist to regulate the activity of the enzyme (Martinez-Cruz et al. 2012). F_1-ATP synthase beta similar to BP53 in *L. vannamei* is associated to the process of WSS viral infection. It plays a central role in the synthesis of ATP in several organisms, which was originally described from the inner mitochondrial membrane. BP53 was found in both cell surface of gills and hemocytes, confirming that F_1-ATP synthase beta exist in shrimp, working as a receptor for invertebrates. It is believed that F_1-ATP synthase beta may be related to the WSSV host infection process (Liang et al. 2010), which could explain the reduction of such protein in animals infected after 48 h, possibly a defense mechanism in response to the virus. (Spot 552) Triosephosphate isomerase (TIM), this enzyme plays an important role in glycolysis and is essential for an efficient energy production and enzymatic activity in all tissues (Schneider 2000; Olah et al. 2002). Its expression was reduced in the group corresponding to the 48 h.a.i. with WSSV, possibly due to decreased feeding and metabolism, causing lethargy, against the advance of viral infection.

(Spot 350) 28S ribosomal protein was also found in the hemolymph of the crab *Scylla serrata* (Liu et al. 2011) and had a greater induction after infection with WSSV, as shown in this work.

(Spot 266) Apolipoprotein functions in the transport and increase in cholesterol and lipids to different receptors (Wilson et al. 1991). It is known that this mechanism works in humans but nothing has been described for crustacea yet. (Spot 356) Cholesterol ester transfer protein binds and neutralizes lipopolysaccharide in gram-negative bacterial outer membrane, function similar to that of crustacean LGBP described by Lee et al. (2000). They are standard recognition proteins that bind to lipopolysaccharide in gram-negative bacteria, in fungi and are present in the hemocytes, leading to the activation of the proPO system. In WSSV-infected shrimp, the LGBP was increased in the acute phase of infection, showing also a response to the virus (Roux et al. 2002), the same was found in this work 72 h.a.i., probably as a response mechanism to viral action. (Spot 285, 399) Ubiquitin plays an important role in the process of proteolysis and is an evolutionarily conserved protein. It functions in various processes, protein degradation in the cell, cell progression, organelle biogenesis, transcriptional regulation, antigen processing, and is involved in the anti-apoptosis process (Chen et al. 2008; Shen et al. 2009). Following WSSV viral infection in shrimp, it functions as a suppressor protein. Thus, it provides the virus greater chance of viral replication and dissemination (Flegel 2007; He et al. 2009; Sudhakaran et al. 2011). According to Wang et al. (2005), an increased expression of ubiquitin conjugating enzyme in *P. vannamei* (PvUbc) was detected in a time course study by real-time PCR as well as RT PCR and reported that RING H2 protein WSSV 249 from WSSV may function as E3 ligase by sequestering PvUbc for viral pathogenesis in shrimp. Their study also revealed a weak expression of PvUbc in uninfected shrimp, suggesting that PvUbc expression is induced in WSSV infection till 48 h.a.i. Keezhedath et al. (2013), the ubiquitin increased expression in *P. monodon* (PmUbc) post WSSV infection. In our study, the groups 24 and 72 h.a.i., ubiquitin protein had greater expression, agreeing with the findings of the authors cited above. Probably, the virus is using this mechanism to be able to propagate during the infectious process, blocking and degrading pro-apoptotic proteins.

Shrimps may express or suppress proteins in response to the viral infection. In this context, a better understanding of the host response to WSSV will help elucidate its mechanism of infection and pathogenicity. Some of these proteins play an important biological role in the mechanism of apoptosis and can be used as biomarkers. In this work, we had suggested three proteins, caspase-2, ubiquitin and F1-ATP synthase, as potential biomarkers. It is possible because they presented alteration in its expression between control and infected groups. Since these proteins were detected in the first 72 h and they are directly related to the infection process, they can be used as a detection tool. Therefore, if one of these proteins presents altered expression, it can indicate a WS viral infection, since it happens in the beginning of the process (until 72 h). Thus, knowing the probable infection, biosafety measures can be adopted early and applied for future development of antiviral defenses, because the animal needs a fast response in the beginning of infection to combat the virus with efficacy and minimize the impacts of infection in shrimp farming. In recent years, studies on shrimp immunity at the molecular level has increased rapidly for both cellular and humoral responses, in order to elucidate host immune response mechanisms to viral infection (Bourchookarn et al. 2008; Robalino et al. 2009; Chai et al. 2010; Zhang et al. 2010; Somboonwiwat et al. 2010).

References

Arlot-Bonnemains Y, Van-Wormhoudt A, Favrel P, Fouchereau-Péron M, Milhaud G, Moukhtar MS (1986) Calcitonin-like peptide in the shrimp *Palaemon serratus* (Crustacea, Decapoda) during the intermolting cycle. Cell Mol Life Sci 42:419–420

Astrofsky KM, Roux MM, Klimpel KR, Fox GJ, Dhar AK (2002) Isolation of differentially expressed genes from white spot virus (WSV) infected Pacific blue shrimp (*Penaeus stylirostris*). Arch Virol 147:1799–1812

Bachère E, Gueguen Y, Gonzalez M, De Lorgeril J, Romestand B (2004) Insights into the anti-microbial defense of marine invertebrates, the penaeid shrimps and the oyster *Crassostrea gigas*. Immunol Rev 198:149–168

Barracco MA, Perazzolo LM, Rosa RD (2007) Imunologia de crustáceos, com ênfase em camarões. Florianópolis, SC, p 80. http://www.liaaq.ufsc.br. Accessed in May 2012

Bourchookarn A, Havanapan P, Thongboonkerd V, Krittanai C (2008) Proteomic analysis of altered proteins in lymphoid organ of yellow head virus infected *Penaeus monodon*. Biochim Biophisica Acta 1784:504–511

Boyer PD (1997) The ATP synthase—a splendid molecular machine. Annu Rev Biochem 66:717–749

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cerenius L, Soderhall K (2004) The prophenoloxiase-activating system in invertebrates. Immunol Rev 198:116–126

Chai Y, YU S, Zhao X, Zhu Q, Wang J (2010) Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* to white spot syndrome virus. Fish Shellfish Immunol 29:480–486

Chen YT, Lin CH, Ji WT, Li SK, Liu HJ (2008) Proteasome inhibition reduces avian reovirus replication and apoptosis induction in cultured cells. J Virol Methods 151:95–100

Chongsatja P, Bouchookarn A, Lo CF, Thongboonkerd V, Krittanai C (2007) Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon taura syndrome virus infection. Proteomics 7:3592–3601

Christie AE, Stevens JS, Bowers MR, Chapline MC, Jensen DA, Schegg KM, Goldwaser J, Kwiatkowski MA, Pleasant TK (2010) Identification of a calcitonin-like diuretic hormone that functions as an

intrinsic modulator of the American lobster, *Homarus americanus*, cardiac neuromuscular system. J Exp Biol 213:118–127

Escobedo-Bonilla CM, Wille M, Alday-Sanz V, Sorgeloos O, Pensaert MB (2007) Pathogenesis of a Tai strain of White spot syndrome virus (WSSV) in juvenile, specific pathogen-free *Litopenaeus vannamei*. Dis Aquat Org 74:85–94

Flegel TW (2007) Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. Dev Comp Immunol 31:217–231

Flegel TW, Pasharawipas T (1998) Active viral accommodation, a new concept for crustacean response to viral pathogens. In: Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok, pp 245–250

Flegel TW, Sritunialucksana K (2011) Shrimp molecular responses to viral pathogens. Mar Biotechnol 13(4):587–607

Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548

Futai M, Noumi T, Maeda M (1989) ATP synthase (H+−ATPase): results by combined biochemical and molecular biological approaches. Annu Rev Biochem 58:111–136

Gonçalves-Soares D, Seiffert WQ, Schlindwein AD, Toledo-Silva G, Zanette J, Marques MRF, Bainy ACD (2012) Identification of differentially transcribed genes in shrimp *Litopenaeus vannamei* exposed to osmotic stress and challenged with WSSV virus. Comp Biochem Physiol Part D 7:73–81

Granja CB, Vidal OM, Parra G, Salazar M (2006) Hyperthermia reduces viral load of white spot syndrome virus in *Penaeus vannamei*. Dis Aquat Organ 68:175–180

He F, Syed SM, Hameed ASS, Kwang J (2009) Viral ubiquitin ligase WSSV222 is required for efficient white spot syndrome virus replication in shrimp. J Gen Virol 90:1483–1490

Jiravanichpaisal P (2005) White spot syndrome virus interaction with a freshwater crayfish. Digital comprehensive summaries of Uppsala dissertations. Uppsala University

Kawabata SI, Iwanaga S (1999) Role of lectins in the innate immunity of horseshoe crab. Dev Comp Immunol 23:391–400

Keezhedath J, Kurcheti PP, Pathan MK, Babu GP, Tripathi G, Sudhagar A, Rao SP (2013) Expression profile of *Penaeus monodon* ubiquitin conjugating enzyme (PmUbc) at protein level in white spot syndrome virus challenged shrimp. Indian J Virol 24:48–53

Lai-Zhang J, Mueller DM (2000) Complementation of deletion mutants in the genes encoding the F1-ATPase by expression of the corresponding bovine subunits in yeast *S. cerevisiae*. Eur J Biochem 267:2409–2418

Lamharzi N, Arlot-Bonnemains Y, Milhaud G, Fouchereau-Peron M (1992) Calcitonin and calcitonin gene-related peptide in the shrimp, *Palaemon serratus*, variations during the molt cycle. Comp Biochem Physiol 4:679–682

Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS, Clayton DA (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat Genet 3:231–236

Lee SY, Wang R, Söderhäll K (2000) A lipopolysaccharide- and beta-1, 3-glucanbinding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization, and cDNA cloning. J Biol Chem 275:1337–1343

Leslie AGW, Walker JE (2000) Structural model of F1–ATPase and the implications for rotary catalysis. Philos Trans R Soc Lond B 355:465–472

Liang Y, Cheng J, Yang B, Huang J (2010) The role of F_1 ATP synthase beta subunit in WSSV infection in the shrimp, *Litopenaeus vannamei*. Virol J 7:144–153

Lightner DV (2011) Virus diseases of farmed shrimp in the Western Hemisphere (the Americas), a review. J Invertebr Pathol 106:110–130

Liu W, Qian D, Yan X (2011) Proteomic analysis of differentially expressed proteins in hemolymph of *Scylla serrata* response to white spot syndrome virus infection. Aquaculture 314:53–57

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH, Kou GH (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Organ 25:133–141

Loh PC, Tapay LM, Lu Y, Nadala-Junior EC (1997) Viral pathogens of the penaeid shrimp. Adv Virus Res 48:263–312

Lolkema JS, Boekema EJ (2003) The A-type ATP synthase subunit K of *Methanopyrus kandleri* is deduced from its sequence to form a monomeric rotor comprising 13 hairpin domains. FEBS 543:47–50

Luquet G, Marin F (2004) Biomineralisations in crustaceans, storage strategies. CR Palevol 3:515–534

Marques MRF, Barracco MA (2000) Lectins, as non-self-recognition factors, in crustaceans. Aquaculture 191:23–44

Martinez-Cruz O, Calderon De La Barca AM, Uribe-Carvajal S, Muhlia-Almazan A (2012) The function of mitochondrial F_0F_1 ATP-synthase from the whiteleg shrimp *Litopenaeus vannamei* muscle during hypoxia. Comp Biochem Phys Part B 162:107–112

May RC, Machesky LM (2001) Phagocytosis and the actin cytoskeleton. J Cell Sci 6:1061–1077

McKinsey TA, Zhang CL, Olson EN (2000) Activation of the myocyte enhancer factor-2 transcription factor by calcium calmodulin dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. PNAS 26:14400–14405

Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins, a theme in signal transduction. Science 268:247–251

Nakano T, Ikegami T, Suzuki T, Yoshida M, Akutsu H (2006) A new solution structure of ATP synthase subunit c from *Thermophilic Bacillus* PS3, suggesting a local conformational change for HC-translocation. J Mol Biol 358:132–144

Neuhoff V, Stamm R, Eibl H (1985) Clear background and highly sensitive protein staining with Coomassie blue dyes in polyacrylamide gels, a systematic analysis. Electrophoresis 6:427–448

OIE. World Organization for Animal Health (2003) Diagnostic manual for aquatic animals. http://www.oie.int. Accessed in May 2010

Phiwsaiya K, Saenapi S, Anantasomboon G, Sriphaijit T, Browdy CL, Flegel TW (2010) Knocking down a Taura syndrome virus (TSV) binding protein *Lamr* is lethal for the whiteleg shrimp *Penaeus vannamei*. In: Proceedings of the 7th annual conference of Marine Shrimp Culture of Thailand. Oral presentation, Set. 7–8, 4p

Rattanarojpong T, Wang H, Lo CF, Flegel TW (2007) Analysis of differently expressed proteins and transcripts in gills of *Penaeus vannamei* after yellow head virus infection. Proteomics 7:3809–3814

Richard L, Vachot C, Surger A, Rigolet V, Kaushik SJ, Geurden I (2011) The effect of choline and cystine on the utilisation of methionine for protein accretion, remethylation and trans-sulfuration in juvenile shrimp *Penaeus monodon*. Br J Nutr 106:825–835

Robalino J, Carnegie RB, O'leary N, Ouvry-Patat SA, De La Vega E, Prior S, Gross PS, Browdy CL, Chapman RW, Schey KL, Warr G (2009) Contributions of functional genomics and proteomics to the study of immune responses in the pacific white leg shrimp *Litopenaeus vannamei*. Vet Immunol Immunopathol 128:110–118

Roux MM, Pain A, Klimper KR, Dhar AY (2002) The lipopolysaccharide and b-1,3-glucan binding protein gene is up-regulated in white spot virus-infected shrimp *Penaeus stylirostris*. J Virol 76:7140–7149

Schneider AS (2000) Triosephosphate isomerase deficiency, historical perspectives and molecular aspects. Baillière's Clin Haematol 1:119–140

Shen B, Zhang Z, Wang Y, Wang G, Chen Y, Lin P, Wang S, Zou Z (2009) Differential expression of ubiquitin-conjugating enzyme E2r in the developing ovary and testis of penaeid shrimp *Marsupenaeus japonicas*. Mol Biol Rep 36:1149–1157

Somboonwiwat K, Chaikeeratisak V, Wang H, Lo CF, Tassanakajon A (2010) Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harvey* infection. Proteome Sci 8:1–11

Speed SR, Baldwin J, Wong RJ, Wells RMG (2001) Metabolic characteristics of muscles in the Spiny lobster *Jasus edwardsii*, and response to emersion during simulated live transport. Comp Biochem Phys 128:435–444

Sudhakaran R, Okugawa S, Mekata T, Inada M, Yoshimine M, Nishi J, Ozono C, Kono T, Sakai M, Itami T (2011) Deciphering the DNA repair protein, Rad23 from kuruma shrimp *Marsupenaeus japonicus*, full-length cDNA cloning and characterization. Appl Microbiol 53:63–72

Sun L, Shi L, Li W, YU W, Liang J, Zhang H, Yang X, Wang Y, Li R, Yao X, Yi X, Shang Y (2009) JFK, a Kelch domain-containing F-box protein, links the SCF complex to p53 regulation. PNAS 25:10195–10200

Tornatore TF, Costa APD, Clemente CFMZ, Rocco CJSA, Calegari VC, Cardoso L, Cardoso AC, Júnior AG, Franchin KG (2011) A role for focal adhesion kinase in cardiac mitochondrial biogenesis induced by mechanical stress. Am J Physiol Heart Circ Physiol 300:902–912

Van Den Ent F, Amos LA, Löwe J (2001) Prokaryotic origin of the actin cytoskeleton. Nature 413:39–44

Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A (2009) Immunity mechanisms in crustaceans. Innate Immun 15(3):178–188

Wang Z, Chua HK, Gusti AARA, He F, Fenner B, Manopo I, Wang H, Kwang J (2005) RING-H2 protein WSSV249 from white spot syndrome virus sequesters a ubiquitin-conjugating enzyme, PvUbc, for shrimp viral pathogenesis. J Virol 79:8764–8772

Wang H, Wang H, Leu J, Kou G, Wang A, Lo C (2007) Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. Dev Comp Immunol 31:672–686

Wang L, Zhi B, Wu W, Zhang X (2008) Requirement for shrimp caspase in apoptosis against virus infection. Dev Comp Immunol 32:706–715

Wilson C, WardelL MR, Weisgraber KH, Mahley RW, Agard DA (1991) Three-dimensional structure of the LDL receptor-biding domain of human apolipoprotein E. Science 5014:1817–1822

Wongprasert K, Khanobdee K, Glunukarn SS, Meeratana P, Withyachumnarnkul B (2003) Time-course and levels of apoptosis in various tissues of black tiger shrimp *Penaeus monodon* infected with white-spot syndrome virus. Dis Aquat Organ 55:3–10

Xu J, Wu S, Zhang X (2008) Novel function of QM protein of shrimp (*PenaeusJaponicus*) in regulation of phenol oxidase activity by interaction with hemocyanin. Cell Physiol Biochem 21:473–480

Yan LH, Xia PL, Mian GK (2010) Depletion of ribosomal protein L8 impairs *Drosophila* development and is associated with apoptosis. Sci China 9:1092–1097

Yu W, Niwa T, Miura Y, Horio F, Teradaira S, Ribar TJ, Means AR, Hasegawa Y, Senda T, Niki I (2002) Calmodulin overexpression causes Ca_2-dependent apoptosis of pancreatic cells, which can be prevented by inhibition of nitric oxide synthase. Lab Invest 82:1229–1239

Zhang J, Li F, Jiang H, Yu Y, Liu C, Li S, Wang B, Xiang J (2010) Proteomic analysis of differentially expressed proteins in lymphoid organ of *Feneropenaeus chinensis* response to *Vibrio anguilarum* stimulation. Fish Shellfish Immunol 29:186–194

Zhi B, Tang W, Zhang X (2011) Enhancement of shrimp antiviral immune response through caspase-dependent apoptosis by small molecules. Mar Biotechnol 3:575–583