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A comparative synthesis of transcriptomic analyses reveals major differences between WSSV-susceptible *Litopenaeus vannamei* and WSSV-refractory *Macrobrachium rosenbergii*

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ABSTRACT

Since the 1990s White Spot Syndrome Virus (WSSV) has severely affected shrimp aquaculture worldwide causing a global pandemic of White Spot Disease (WSD) in penaeid culture. However, not all decapod species that can be infected by WSSV show the same susceptibility to the virus, thus raising interesting questions regarding the potential genetic traits that might confer resistance to WSSV. In order to shed light into the genetic markers of WSSV resistance, we employed a dual approach: *i)* we initially analysed the transcriptomes derived from the hepatopancreas of two species, the susceptible white shrimp *Litopenaeus vannamei* and the refractory fresh water prawn *Macrobrachium rosenbergii*, both infected with WSSV. We found a large number of differentially expressed genes (DEGs) belonging to the immune system (mostly anti-microbial peptides and haemolymph clotting components) that were generally up-regulated in *M. rosenbergii* and down-regulated in *L. vannamei*. Further, in both species we identified many up-regulated DEGs that were related to metabolism (suggesting a metabolic shift during the infection) and, interestingly, in *L. vannamei* only, we found several DEGs that were related to moult and suggested an inhibition of the moult cycle in this species following WSSV infection. *ii)* we then identified a limited number of genetic markers putatively linked with WSD tolerance by employing an ecological genomics approach in which we compared published reports with our own RNA-seq datasets for different decapod species infected with WSSV. Using this second comparative approach, we found nine candidate genes which are consistently down-regulated in susceptible species and up-regulated in refractory species and which have a role in immune response. Together our data offer novel insights into gene expression differences that can be found in susceptible and refractory decapod species infected with WSSV and provide a valuable resource towards our understanding of the potential genetic basis of tolerance to WSSV.

1. Introduction

White Spot Syndrome Virus (WSSV) is the causative agent of White Spot Disease (WSD), a viral disease which appeared in the early 90s in China and Taipei (Stentiford et al., 2009) and has, since then, spread across the world causing substantial economic losses for shrimp farmers globally (Hauton et al., 2015). WSSV is a dsDNA virus with a particularly wide range of host species; in fact, thus far 47 different crustacean species have been reported to be infected with WSSV and numerous aquatic organisms (e.g. birds) can act as mechanical vectors (Stentiford et al., 2009). Whilst the majority of decapod species infected with WSSV show 100% mortality within 10 days following the onset of

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[1] 17.02.53–02.05.19.
[2] This work is dedicated to the memory of our colleague and friend, Dr Valerie Smith, who dedicated her career to comparative immunology and, in particular, creating an improved mechanistic understanding of the crustacean immune system.
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disease (Escobedo-Bonilla et al., 2008), the freshwater prawn *Macrobrachium rosenbergii* is reported to present a refractory phenotype (Hossain et al., 2001; Pais et al., 2007). In fact, as reported by Sarathi et al. (2008), Sahul Hameed et al. (2000) and Rajendran et al. (1999), *M. rosenbergii* infected with WSSV (via intramuscular inoculation or oral feeding) showed mortality ≤20% in comparison to mortalities ≥50% observed in other decapod species. Despite the increasing effort in trying to characterize and understand WSD, the molecular/physiological traits that confer resistance to WSD have not been fully elucidated yet and studies comparing the molecular response to WSSV in refractory and susceptible species have still to be conducted.

The era of next-generation sequencing has allowed researches to investigate how tissue gene expression (GE) changes in animals exposed to stressors or infections and thus, in recent years, we have seen increasing efforts to understand GE changes in shrimp infected with WSSV (Cao et al., 2017; Chen et al., 2013; Du et al., 2016; Du, 2016; Du and Jin, 2017; Peruzza et al., 2019; Rao et al., 2016; Shi et al., 2018; Zhong et al., 2017). In this context, the comparison of transcriptomes between refractive and susceptible species would help identify elements that confer refractivity to WSD. However, a large number of differentially expressed genes (DEGs) are classically found in RNA-seq experiments and thus far it has proved difficult to select a few candidate genes for WSD resistance from such lists of DEGs. To overcome this problem Landry and Aubin-Horth (2007) and Pavey et al. (2012) have utilised an ecological genomics approach, whereby gene functions are linked to observed phenotypes (e.g. resistance/vulnerability to virus) from a database that comprises multiple species exposed to the same biotic condition (i.e. WSSV infection). In fact, as stated by Pavey et al. (2012), "the systematic association of one gene across multiple experiments with a particular condition" (e.g. WSSV refractivity) "can help narrow down (from the large number of DEGs of a classic RNA-seq experiment) candidate genes for the trait of interest". Hence, it could be argued that, if a gene contributed to WSSV refractivity, it might be predicted to be activated/up-regulated in databases in which the tolerant species was subjected to WSSV, whereas the same gene would be inactive/down-regulated in susceptible species.

At physiological level some differences exists between refractive and susceptible species when infected with WSSV: for example, following WSSV infection in *M. rosenbergii* the haemolymph is still able to clot (Sarathi et al., 2008) and components of the clotting cascade are activated/up-regulated (Cao et al., 2017; Rao et al., 2016), whilst in the susceptible species *P. indicus*, *P. chinensis* and *L. vannamei*, haemolymph fails to clot (Yoganandhan et al., 2003) and many components of the clotting cascade are down-regulated following WSSV infection (Goncalves et al., 2014; Shi et al., 2018). Similarly, some authors report that prophenoloxidase (proPO (Sarathi et al., 2008)), antioxidant enzymes (e.g. glutathione peroxidase, catalase (Rao et al., 2016)) and antimicrobial peptides (e.g. lectin, galectin, anti-lipopolysaccharide factors (Cao et al., 2017; Rao et al., 2016)) are activated/up-regulated in *M. rosenbergii* and suppressed/down-regulated in susceptible species (Ji et al., 2011; Kulkarni et al., 2014; Mathew et al., 2007; Mohankumar and Ramasamy, 2006; Zhang et al., 2018). Together, these observations suggest the presence of important molecular/physiological differences which confer refractivity to WSD in *M. rosenbergii* (compared to susceptible species). However, a candidate gene (or a handful set of candidates) responsible for WSD refractivity has yet to be identified and key "new" candidate genes might await discovery and characterisation.

In this study, in order to compare GE changes following WSSV infection in susceptible and refractive species, we injected the refractive *M. rosenbergii* (hereafter called: Mr) and the susceptible *L. vannamei* (hereafter called: Lv) with WSSV and we analysed the transcriptomes derived from the hepatopancreas of the two species. We found that a large number of DEGs represented immune system components (mostly anti-microbial peptides and components of the coagulation cascade) and were generally up-regulated in Mr and down-regulated in Lv, many up-regulated DEGs in both species were related to metabolism (suggesting a metabolic boost) and, interestingly, we found several DEGs in Lv only that were related to moult and suggested an inhibition of the moult cycle in this species following WSSV infection. Thereafter, to identify few genetic markers putatively linked with WSD refractivity, we employed an ecological genomics approach in which we analysed published and in-house RNA-seq datasets involving different species infected with WSSV to identify markers that were systematically associated with susceptibility or refractivity across datasets. We report nine candidate genes which have a proven role in the immune response. Overall, our data provide a valuable resource towards our understanding of the genetic basis of WSD resistance.

2. Materials and methods

2.1. Collection and maintenance of shrimp and freshwater prawn

Adult White leg shrimp, *Litopenaeus vannamei* (12–15 g body weight) were collected from grow-out ponds located at Minjur, near Chennai, Tamilnadu, India (N 13°14.307′ E 080°15.302′). In these grow-out ponds, the post-larvae were negative for WSSV, IHHNV and EHP, at stocking and the shrimp were monitored regularly for these pathogens thereafter. The collected shrimp were transported to the laboratory in live condition with continuous aeration. The WSSV-free status of the animals was further confirmed by nested PCR as described by Lo et al. (1996) using viral DNA extracted from the gill tissue of shrimp. In the laboratory, the shrimp were maintained in 1000-l fiberglass tanks with airlift biological filters at an ambient temperature of 27–30 °C with salinity between 20 and 25. Natural seawater pumped from the Bay of Bengal, India was used in all the experiments. Suspended particles including sand were removed by allowing the suspended particulates to settle. Then, it was treated with sodium hypochlorite at the concentration of 25 ppm and dechlorinated by vigorous aeration. Finally, it was passed through a sand filter and used for the experiments. The animals were fed with artificial pellet feed (CP feed, Thailand). Temperature and pH were recorded, salinity was measured with a salinometer (Aquafauna, Japan) and dissolved oxygen was estimated by the Winkler method. Before conducting experiments, the shrimp were acclimated to the experimental tanks for 5 days. After acclimation, shrimp were randomly selected and tested for the presence of WSSV by polymerase chain reaction (PCR) using virus specific primers (Sivakumar et al., 2018). Only healthy WSSV-free shrimp free were used for the experiments.

Healthy adult prawns *M. rosenbergii* (30–50 g body weight) were collected from grow-out ponds located at Atmakur village near Nellore, India (N 14°35.009′ E 079°30.277′) and transported in live condition with continuous aeration. In the laboratory, the prawns were maintained in 1000 l fiberglass tanks with continuous aeration at room temperature (27–30 °C) in freshwater. The animals were fed with commercial pellet feed (CP shrimp feed, Thailand).

2.2. Preparation of WSSV inoculum

The WSSV inoculum was prepared from WSSV-infected shrimp, *L. vannamei* with clinical signs of reddish coloration, swelling of branchiostegites, whitish abdominal muscle and minute spots on the carapace collected from shrimp farms located near Nellore, India (N 14°48.417′ E 080°02.521′). Head soft tissues from cephalothorax region including gills were dissected and homogenized. A 10% suspension was made in NTE buffer (0.2 M NaCl, 0.02 M Tris-HCl and 0.02 M EDTA, pH 7.4). This suspension was frozen and thawed three times, and centrifuged at 3000 g for 20 min at 4 °C. The collected supernatant was re-centrifuged at 8000 × *g* for 20 min at 4 °C and the final supernatant fluid was filtered through a 0.4 μm membrane filter. The filtrate was then stored at −80 °C and used for experimental infection after confirming the presence of WSSV by PCR using virus specific primer (Sivakumar et al., 2018).

2.3. Experimental infection in shrimp and prawn

Shrimp were injected intramuscularly with WSSV inoculum prepared as described above with viral copy number of 10^5 per individual. Given the fact that a WSSV dose up to 8-times higher (i.e. 8×10^5 viral copies) fails to produce mortality in Mr (Sahul Hameed et al., 2000), we decided to use the same dose for the two species. Viral copy number was estimated by StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using TaqMan Assay. A forward primer 5′ CCC ACA CAG ACA ATA TCG AGA C 3′ and reverse primer 5′ TCG CTG TCA AAG GAC ACA TC 3′ were used to amplify a 109-bp fragment from the VP28 gene of WSSV, with a TaqMan hydrolysis probe, RT-WSSV-TP109 (5′ FAM-TTC CTG TGA CTG CTG AGG TTG GAT-TAMRA 3'). The sample of diluted DNA (20–100 ng) total DNA was added to a TaqMan Universal Master Mix (ABI, USA) containing 0.25 μM of each primer and 0.125 μM of TaqMan probe in a final reaction volume of 20 μl. The amplification programme consisted of 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. A standard curve was obtained using serial dilutions of plasmid pVP28 (full-length ORF of VP28 gene of WSSV was cloned into pRSETB vector) that were used to quantify the WSSV copy number. Each assay was carried out in triplicate (Jang et al., 2009).

Control animals were inoculated with tissue suspension prepared from uninfected shrimp. The experimental shrimp were examined twice a day for gross signs of disease, and hepatopancreas sample was collected at different time points and used for transcriptomic analysis after confirming the presence of WSSV by PCR as described above.

Freshwater prawns were injected intramuscularly in the second abdominal segment with WSSV inoculum (10^5 WSSV copy number per prawn) as described above. Control animals were injected with the filtrate prepared from healthy shrimp. The experimental prawns were examined twice per day for gross signs of disease, and the hepatopancreas was collected at different time points and stored −80 °C for transcriptomic analysis after confirming the presence of WSSV by PCR (Sivakumar et al., 2018).

2.4. RNA extraction and library preparation

Hepatopancreatic tissue (30 mg) from Lv and Mr was used to extract total RNA using HiPurA™ total RNA miniprep purification kit (Himedia, India) as per the manufacturer's instructions. The RNA quality was checked using Agilent Bioanalyzer RNA 6000 nano kit (Agilent Technologies). RNA quantity was estimated with Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific). Samples with > 7 RNA Integrity Number (RIN) were taken further for library preparation.

mRNA sequencing libraries were prepared using NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® following the manufacturer's instructions. Briefly, 800 ng of total RNA was used as input for poly(A) mRNA enrichment using NEBNext® Poly(A) mRNA Magnetic Isolation Module followed by fragmentation and reverse transcription to generate cDNA. Hairpin adapters were ligated to fragmented double strand cDNA and USER enzyme was used to cleave the hairpin structure. Ampure beads were used to purify adapter ligated fragments and the purified product was amplified using Illumina Multiplex Adapter primers (NEBNext Multiplex Oligos for Illumina Cat#E7500S) to generate sequencing libraries with barcodes for each sample. Library quality was checked using Agilent Bioanalyzer DNA 1000 assay kit and quantity was estimated Qubit HS DNA assay kit. The QC passed libraries were sequenced on Illumina Hiseq at Clevergene Biocorp Ltd. (Bangalore, India).

2.5. Transcriptome assembly, annotation and analysis

Raw reads from all libraries from Lv and Mr inoculations were quality checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and low quality reads were removed using AfterQC (Chen et al., 2017) with default parameters. Each species was processed separately, and all subsequent steps were repeated for both species. For each species, all libraries were used for *de novo* transcriptome assembly using Trinity/v2.5.1 (Haas et al., 2013) with "Kmer = 25, Min contig length 300" and default parameters. The quality of the assembly was assessed using BUSCO/v3.0.2 (Simao et al., 2015) using the arthropoda_odb9 database. Reads were mapped to the transcriptome using hisat2 (Kim et al., 2015) and featureCounts (Liao et al., 2014) with default parameters.

For each species, all contigs with at least 1 CPM in ≥2 infected replicate samples were submitted to the KEGG (Kanehisa and Goto, 2000) Automatic Annotation Server (KAAS, http://www.genome.jp/tools/kaas/) to retrieve KEGG pathway maps for each contig using GHOSTZ with the single-directional best hit (SBH) method.

Pairwise differential expression analysis was performed using DESeq (Anders and Huber, 2010). Contigs were considered as significantly differentially expressed if the absolute log2FoldChange was greater than 2 with p-value ≤ 0.05.

Each transcriptome was annotated against UniProt (The UniProt Consortium, 2017), NCBI nr and UniRef 100 databases with BlastX (Altschul et al., 1990) (e-value 1E-04 and default options) and InterPro Scan/v5.29–68.0 (Jones et al., 2014) (default options). Hits were imported in Blast2GO/v5.2.5 (Conesa et al., 2005) and GO numbers were retrieved using "mapping" and "annotation" functions with default options. Blast2GO function "Fisher enrichment analysis" was used to perform GO enrichment analysis using default options.

2.6. Ecological genomics analysis

A comparative analysis was performed using our RNA-seq datasets comprising of Lv gill tissue infected with WSSV from Peruzza et al. (2019) (Accession: PRJNA524934) and Lv and Mr hepatopancreas (from this study) and datasets available from NCBI (Accessions: PRJNA437347, PRJNA252894, PRJNA267515, PRJNA428228) to identify important genes implicated in the refractivity/susceptibility to WSSV. All available RNA-Seq projects concerning WSSV infection experiments on Crustaceans were downloaded from NCBI-SRA archive (see details in Fig. 4A). From each dataset reads were mapped to their transcriptome with kallisto/v0.43.1 (Bray et al., 2016) and DEGs were detected with edgeR/v3.22.3 (Robinson et al., 2010) (FDR cut-off 0.0001 and fold-change ≥ |2|) and retained for comparative analysis. The "*L. vannamei* gill" dataset (from Peruzza et al. (2019)) was used as a Reference Set. DEGs from all other datasets were BLASTed/v2.6.0 (Altschul et al., 1990) against the Reference Set (e-value cut-off 1E −05). To identify DE contigs in common between experiments, BLAST hits from all datasets were merged together and their frequency in the resulting dataset was determined. Only blast hits with a frequency ≥3 were retained and the direction of change in GE (i.e. up- or down-regulation) was determined for all hits in their respective dataset. We did not consider the ratio of change in GE, as this would not be comparable among different datasets given the fact that each dataset was independently mapped to its own transcriptome and DEGs were independently calculated for each dataset. BLAST hits were then subjected to a conservative validation criterion: *viz.* to be concordant with the direction of GE change in reference set and 3 or more other databases (i.e. be downregulated in reference and > 3 other datasets), allowing for the opposite direction in GE change in datasets with refractive species. Only BLAST hits passing the frequency threshold and the validation criterion were deemed as suitable candidates in determining susceptibility to WSSV in susceptible species, such as Lv.

2.7. Validation of bioinformatic analyses by means of real-time PCR (qPCR)

The expression of some DEGs was further confirmed by means of quantitative PCR analysis. For each biological sample (n = 3

independent biological samples per treatment) the mRNA levels of interesting genes was determined using SYBR green quantitative real-time PCR (StepOnePlus, Applied Biosystems, USA). The amplification was performed in a 96-well plate in a 10 μL reaction volume containing 5 μL of 2 X SYBR Green Master Mix (ABI, USA), 1 μL of diluted cDNA, 0.5 μL (each) of each forward and reverse primers and 3.0 μL of DEPC-treated water. DEPC-water for the replacement of cDNA template was used as the negative control. The thermal profile for the SYBR green real-time PCR was 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s. The expression of immune-related genes was normalised to the expression of ß-actin gene for each sample. Data analysis was executed using mathematical model for relative quantification in real-time PCR described by Pfaffl (2001). ΔCT represented the difference between CT for target gene and the internal control, and ΔΔCT was obtained by subtracting the ΔCT for the control (PBS) from the ΔCT for each sample. The sequence of each primer used can be found in Supplementary Table 1. For each gene in each species, an unpaired *t*-test was used to assess for statistical differences between control and WSSV-infected animals and p-values were deemed significant at $p \leq 0.05$.

3. Results

3.1. Transcriptome assembly

Illumina RNA-sequencing was conducted on hepatopancreas tissue samples from WSSV-infected and control animals (n = 3 different individuals for each treatment) sampled at 1 day-post-inoculation (dpi) for Lv and sampled at 5 dpi for Mr; the difference in sampling time for the two species resides in the different timing required for the development of the infection in the two species: while for Lv mortalities > 60% are found within 72 hpi (Han-Ching Wang et al., 2010; Sarathi et al., 2008), infection in Mr develops slowly, with very low mortality rates (i.e. ~0%) and with WSSV being detected up to 50dpi in several tissues (Sarathi et al., 2008). Given this substantial difference in duration of (and development of) WSSV infection, we decided to sample the two species at different time points in order to compare "early stage" of infection in both species. For each species one transcriptome was generated by combining clean reads from WSSV-infected and control samples. Assembly generated 102,290 transcripts (with an N50 value of 1529) for Mr and 84,229 transcripts (with an N50 value of 1769) for Lv (Supplementary Table 2). The completeness and integrity of the assemblies were evaluated by using BUSCO, which revealed that 81.1% and 90.6% of the benchmarking orthologous genes were present in Mr and Lv assemblies, respectively. Raw sequence data associated with this project have been deposited at NCBI with BioProject accession number PRJNA554075 and SRA accession numbers from SRR9670544 to SRR9670555.

3.2. Transcriptome comparison

To provide a descriptive overview of the composition (in terms of % of genes) in relation to the function (in terms of KEGG categories) of all protein coding genes (≥50 residues) in the transcriptomes of the two species, we retained contigs from WSSV-infected libraries with CPM ≥2 in at least two replicates, annotated them with KEGG Orthology (KO) numbers and retrieved KEGG distribution across different biological pathways (Fig. 1A, left panel). Overall the two transcriptomes presented a similar composition of genes across KEGG categories, with only small differences: in example "signal transduction", "cancer: overview" and "immune system" were more abundant in Lv, while "carbohydrate metabolism" and "amino acid metabolism" were more abundant in Mr (Fig. 1A, middle panel). Pathways composing the most different categories were further investigated (Fig. 1B and Supplementary Fig. 1): inside "Carbohydrate metabolism", more transcripts mapping to "Glycolysis/Gluconeogenesis", "Citrate cycle" and "Galactose metabolism" (essential sugar for the production of Galectins) were found in Mr (Supplementary Fig. 1) while inside "immune system" differences were mainly linked with the coagulation cascade (e.g. "platelet activation and complement", "coagulation cascade", Supplementary Fig. 1).

3.3. Differentially expressed genes (DEGs)

Differential expression analysis was performed for each species separately. In total we found 199 DEGs in Mr and 3106 DEGs in Lv (Fig. 2A). DEGs from both species were mapped to KEGG categories (Fig. 2B) and revealed an important difference between up-regulated and down-regulated DEGs, with the vast majority of down-regulated DEGs in Lv belonging to metabolism and therefore suggesting an important shift in metabolic processes.

Fisher's enrichment analysis on up-regulated DEGs from Mr revealed that several immune mechanisms were enriched (e.g. "positive regulation of immune system processes", "defence response to other organism", "immune response", "detection of other organism", Fig. 3A). Among the up-regulated DEGs in Mr (Supplementary Table 3) we found multiple contigs that BLAST matched to the metabolic enzyme *phosphoenolpyruvate carboxykinase* and several contigs with immune functions, such as antimicrobial peptides (e.g. *C-type lectins* or *Ficolin*), the *phenoloxidase-activating enzyme*, integrins and haemolymph clotting components (e.g. *coagulation-factor IX* or *Carboxypeptidase B* which downregulates fibrinolysis, hence facilitating haemolymph clotting).

Fisher's enrichment analysis on up-regulated DEGs from Lv revealed that immune pathways and metabolic processes were enriched in WSSV-infected samples (Fig. 3B): in example we found an enrichment of "positive regulation of Wnt signalling pathway", "phosphoenolpyruvate carboxykinase activity", "glycolytic process" and "gluconeogenesis" in up-regulated DEGs (Fig. 3B). Among the up-regulated DEGs in Lv (Supplementary Table 4) we found contigs blasting against immune genes (e.g. *Lectins*, a *single VWC domain protein* and a *beta-arrestin 2*), stress genes (e.g. *Heat shock proteins 21 and 90*), several metabolic enzymes involved in gluconeogenesis, glycolysis and anaerobic metabolism, two important crustacean hormones, the crustacean hyperglycaemic hormone (cHH) and the moult inhibiting hormone (MIH), the endocytosis protein *flotillin-2* and the cytoskeletal protein *actin*.

Fisher's enrichment analysis on down-regulated DEGs from Lv revealed that immune, metabolic and moult related processes were enriched in WSSV-infected samples (Fig. 3C): in example we found "complement activation, lectin pathway", "haemolymph coagulation", "fatty acid beta-oxidation" and "ecdysone binding" in down-regulated DEGs (Fig. 3C). Among the down-regulated DEGs in Lv in response to WSSV (Supplementary Table 5) we found multiple immune genes, including several AMPs (e.g. penaeidins, lectins, hemocyanin and macroglobulin), the melanisation protein and the prophenoloxidase activating enzyme, and several haemolymph clotting peptides. Further, several metabolic enzymes belonging to TCA cycle and oxidative phosphorylation were down-regulated and the *ecdysteroid receptors A* and *B* (responsible for binding ecdysone and triggering moult) were down-regulated. Finally, we found down-regulation of apoptotic proteins (e.g. *caspase* and *caspase-3*).

Viral sequences were identified in transcriptomes from both species (191 and 69 WSSV contigs in Lv and Mr, respectively, Fig. 4A). Interestingly, no WSSV contig was found as DEG in Mr, while 39 WSSV DEGs were identified in Lv. These 39 contigs were exclusively expressed in infected Lv (Fig. 4B) and were mostly comprised of genes with no known function, followed by genes encoding for Envelope/Structural proteins and collagen-like proteins (Fig. 4C).

3.4. Ecological genomics analysis

A comparative analysis was performed using our RNA-seq datasets and datasets available from NCBI to identify important genes implicated with tolerance to WSSV. The relevant features of all datasets

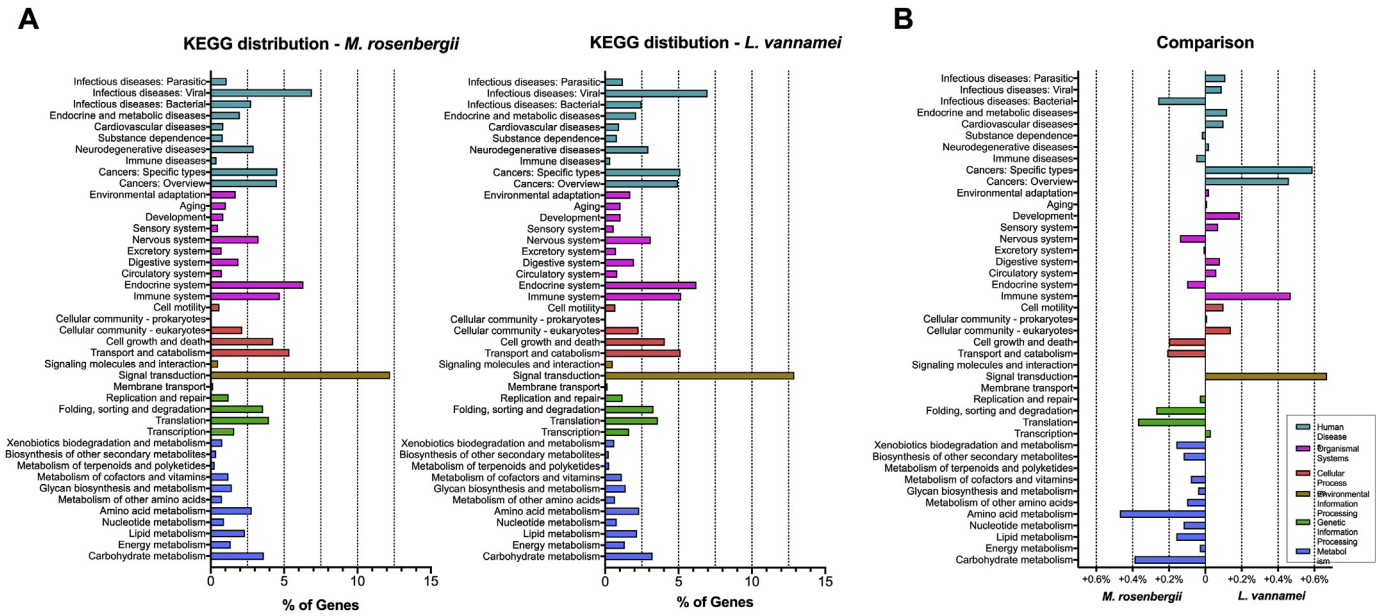

Fig. 1. A) Transcriptome comparison in WSSV infected Mr (left panel) and Lv (middle panel). This figure shows the composition (in terms of % of genes, X-axis) in relation to the function (in terms of KEGG categories, Y-axis) of protein coding genes (≥50 residues) in the transcriptomes of the two species. **B)** Comparison of each KEGG category from **A** obtained by subtracting for each category the % of genes of Mr from Lv to appreciate the subtle differences between the two transcriptomes.

A

Species	# DE Contigs	# Up-regulated	# Down-regulated
M. rosenbergii	199	189	10
L. vannamei	3106	1043	2063

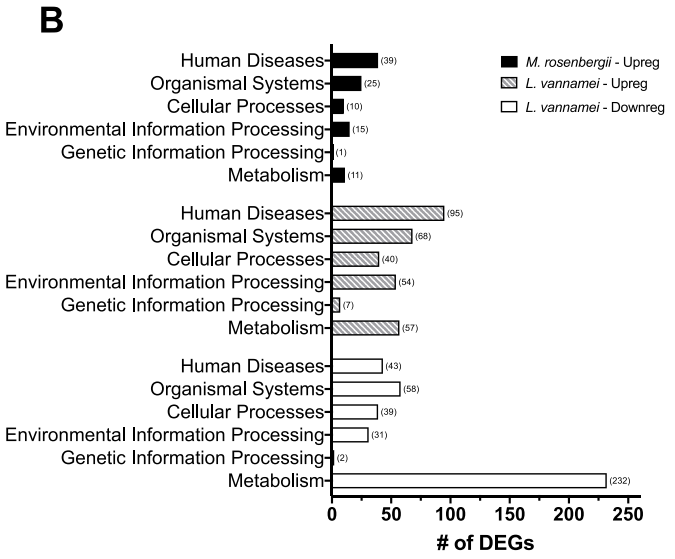

Fig. 2. A) Summary table reporting the number of differentially expressed genes (DEGs) found in WSSV infected Mr and Lv, in comparison to control animals. **B)** DEGs distribution across KEGG categories in the two species.

used in the comparative analysis are reported in Fig. 5A. From all DEGs considered in the analysis, in total only 22 genes from the Reference Set appeared in 3 or more other datasets (Fig. 5B) and were further subjected to the additional validation criterion. In total, 9 genes passed this criterion (Fig. 6). These 9 candidate genes were, in major part, downregulated in susceptible species while they were up-regulated in refractive species. These included the transporters *Sialin* and *Trehalose transporter 1*, two *C-type lectins*, *Phenoloxidase 2*, two dehydrogenases *Formyltetrahydrofolate dehydrogenase* and *Sorbitol dehydrogenase*, and *Sulfotransferase* and *Acetylcholinesterase*.

3.5. Validation of bioinformatic analyses by means of real-time PCR (qPCR)

Quantitative PCR analysis on DEGs confirmed both bioinformatic analyses (i.e. the ecological genomics and transcriptomic analysis, Fig. 7 and Fig. 8 respectively). The 9 candidate genes, found in the ecological genomics analysis, were investigated by means of qPCR in control ("Ctrl") and WSSV-infected ("WSSV") animals from the refractive Mr and susceptible Lv species (Fig. 7). Genes that were classified as up-regulated in WSSV-infected Mr and down-regulated in WSSV-infected Lv at the bioinformatic analysis were DE in a similar fashion following qPCR analysis (compare Figs. 6 and 7). Note that genes *Sulfotransferase* and *Trehalose transporter 1* were not investigated by qPCR in Mr (Fig. 7) as they were not classified as DE in the refractory species' database (see Fig. 6). Additional DEGs from Lv (mapping to haemolymph clotting processes and immune functions) identified as down-regulated from the transcriptomics analysis, were found as down-regulated in WSSV-infected Lv animals (Fig. 8) by means of qPCR.

4. Discussion

In the present study we investigated the transcriptomic responses of two decapod species (the susceptible Lv and the refractive Mr) infected with WSSV. In the refractive Mr we mainly found up-regulation of immune genes, whereas within the susceptible Lv we found that many DEGs belonged to the immune system (with the majority of them being down-regulated), to metabolic pathways and to the moult process (which was inhibited in WSSV-infected animals). Finally, to identify a small set of candidate genes involved in conferring WSD refractivity we employed a comparative approach by comparing our RNA-Seq datasets (from this study and Peruzza et al. (2019)) and datasets available on NCBI-SRA archive and we identified nine candidate genes which regularly appear down-regulated in susceptible species but up-regulated in

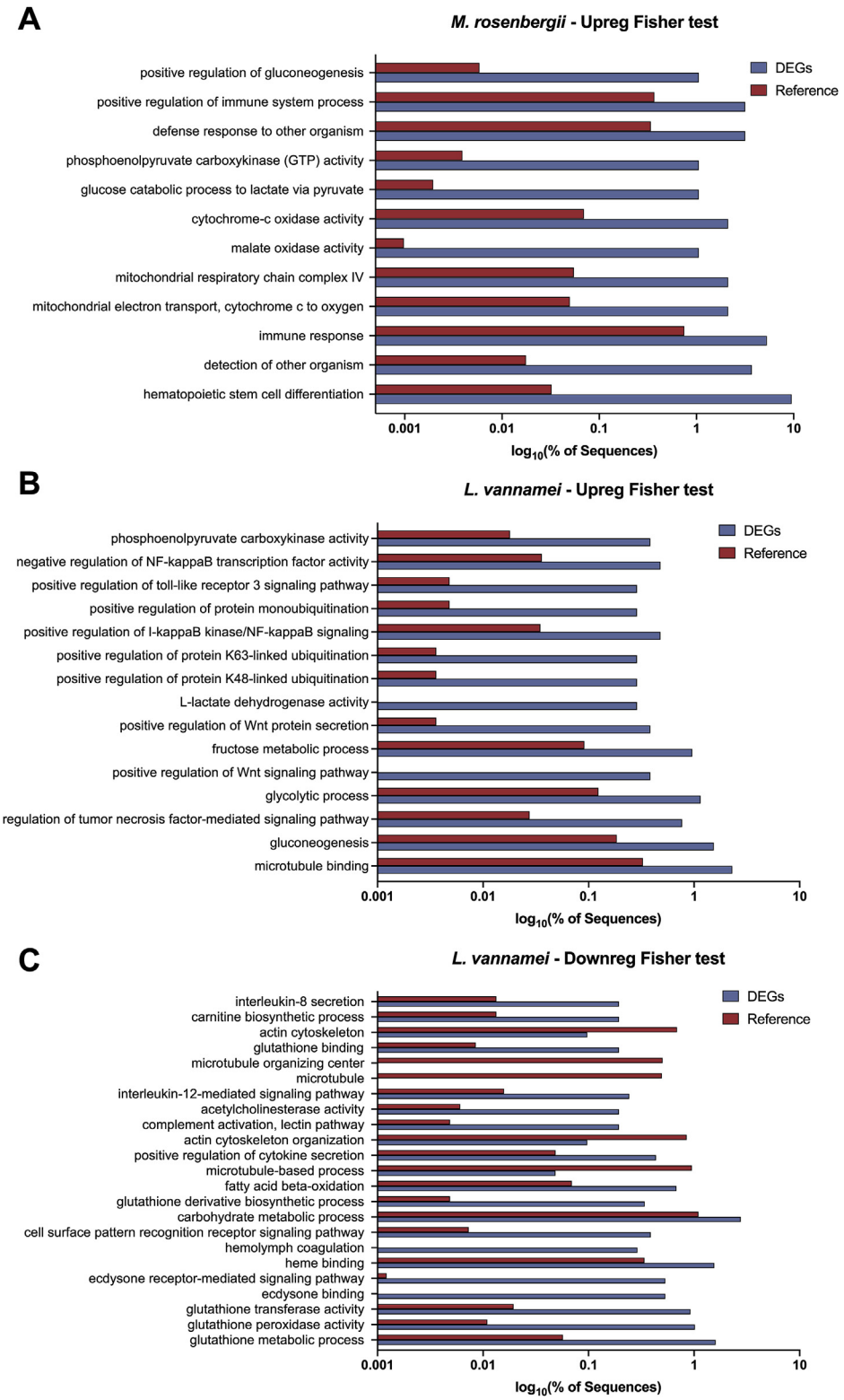

Fig. 3. Enrichment analysis on DEGs in comparison to the remaining transcriptome (Reference): **A)** DEGs up-regulated in Mr; **B)** DEGs up-regulated in Lv; **C)** DEGs down-regulated in Lv.

refractive species. Overall, our comparative approach and our results provide an important resource for understanding the genetic differences which confer WSD resistance in some species.

4.1. Differentially expressed genes

To date many transcriptomics studies have been performed to describe GE changes in different decapod species infected with WSSV (Cao et al., 2017; Chen et al., 2013; Du et al., 2016; Du, 2016; Du and Jin, 2017; Peruzza et al., 2019; Rao et al., 2016; Shi et al., 2018; Zhong

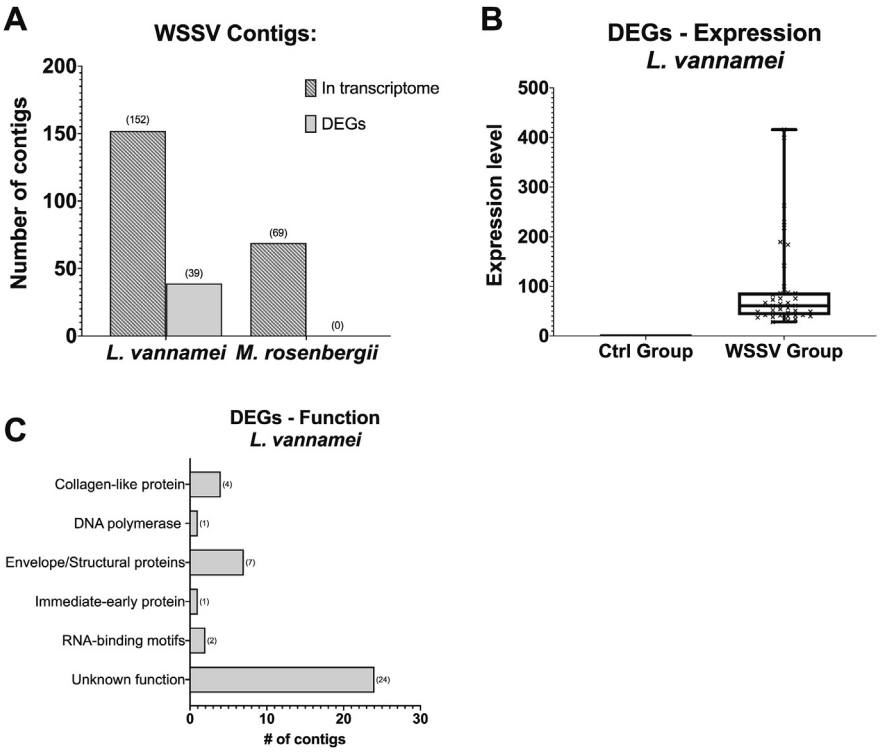

Fig. 4. Viral contigs. **A)** Number of viral contigs found in each transcriptome and number of DEGs viral contigs in each transcriptome. **B)** Box-and-whiskers plot showing the expression levels of DE viral genes in Lv injected with PBS (i.e. "Control animals") and WSSV-injected animals (i.e. "WSSV Group"). Each "X" represents the average (across three biological samples) expression level of one gene. **C)** Function of DE viral genes according to NCBI's nr database.

A

ID	NCBI Accession	Species	Tissue	Experimental design	# DE Contigs
Lv_1.5hpi	PRJNA524934	*L. vannamei*	Gill	1.5hpi Infected vs Control	5093
Lv_18hpi	PRJNA524934	*L. vannamei*	Gill	18 hpi Infected vs Control	5096
Lv_56hpi	PRJNA524934	*L. vannamei*	Gill	56 hpi Infected vs Control	5092
Mn_mor	PRJNA437347	*M. nipponense*	Hepatopancreas	Moribund vs Control	167
Mn_surv	PRJNA437347	*M. nipponense*	Hepatopancreas	Survived vs Control	13
Mr_inf1	PRJNA252894	*M. rosenbergii*	Hepatopancreas	Infected vs Control	215
Pm_inf	PRJNA267515	*P. monodon*	Hepatopancreas	Infected vs Control	819
Lv_hep	PRJNA428228	*L. vannamei*	Hepatopancreas	Ill vs Healty	22
Lv_mus	PRJNA428228	*L. vannamei*	Muscle	Ill vs Healty	8
Mr_inf2	This study	*M. rosenbergii*	Hepatopancreas	Infected vs Control	199
Lv_inf2	This study	*L. vannamei*	Hepatopancreas	Infected vs Control	3106

B

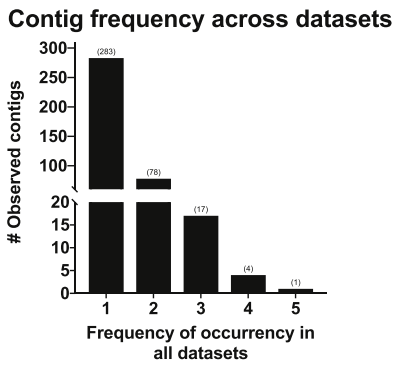

Fig. 5. Ecological genomics analysis. **A)** Summary table of all dataset included in the comparative analysis together with the relevant features of the downloaded datasets. From each dataset DEGs were obtained and retained for further analysis. "*L. vannamei* gill" (bold, raw 1–3 from the table) was used as a Reference Set against which, DEGs from other datasets, were blasted. **B)** Frequency of blast-hits across all datasets. This figure shows the number of times each contig from the Reference Set was found in the other datasets.

et al., 2017), but there is still limited knowledge in relation to the genetic traits that confer WSD resistance in some species. By comparing GE changes across multiple susceptible and refractive species we were able to identify major cellular and physiological processes altered upon WSSV infection and start unravelling the genetic basis of refractivity to WSD.

In total we identified 199 DEGs in Mr and 3106 DEGs in Lv. Not surprisingly a good proportion of DEGs in both species were involved with the immune system and some important differences were observed. In fact haemolymph coagulation, one of the major arms of the humoral immune response in crustaceans (Perdomo-Morales et al., 2018), was triggered in the refractive Mr (as suggested by the up-regulation of facilitators of haemolymph clotting such as *coagulation-factor IX* and *Carboxypeptidase B* (Broze and Higuchi, 1996)), while it was inhibited in the susceptible Lv (Figs. 3 and 8 and Supplementary Tables 1 and 3). The ability to form haemolymph clots provides a physical barrier that prevents microbial entrance/diffusion in the hemocoel (Perdomo-Morales et al., 2018) and several authors have reported that Mr haemolymph is still able to clot after WSSV infection (Cao et al., 2017; Sarathi et al., 2008; Zhong et al., 2017) while Lv haemolymph is not (Yoganandhan et al., 2003). Given the fact that many pathogens possess some fibrinolytic protease systems to prevent clotting (Perdomo-Morales et al., 2018), it could be hypothesised that WSSV possesses one which is effective against the susceptible Lv but not against Mr.

In Lv, we observed an activation of the RNAi pathway, an important anti-viral immune defence mechanism (Li et al., 2018) and an activation of the Wnt signalling pathway, involved in innate immunity (Du, 2016) and essential for WSSV phagocytosis, as demonstrated by Zhu and Zhang (2013) in *D. melanogaster*. However, while WSSV infection triggered an immune response which resulted in the up-regulation of many AMPs (e.g. C-type lectin, Ficolin) and the proPO in Mr, in Lv AMPs and the proPO were down-regulated, in agreement with Ji et al. (2011) (Figs. 7 and 8 and Supplementary Table 4). In particular, among

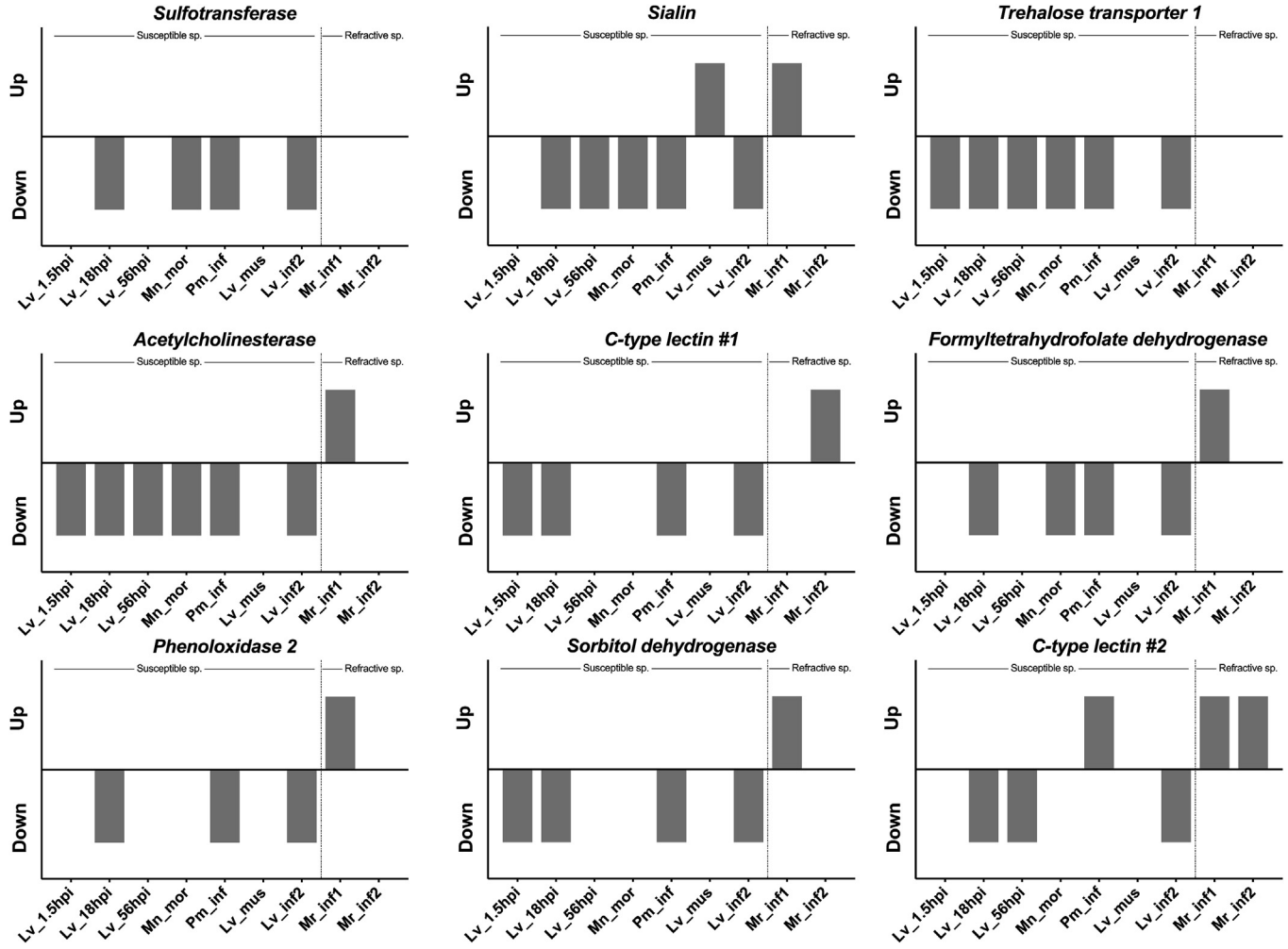

Fig. 6. Schematic of all genes that were observed in 3 datasets or more. For each gene, the columns represent each dataset (to simplify the visualisation "Mn_surv" and "Lv_hep" were removed as they had no positive match with any of the selected genes) the bars indicate the direction of change (i.e. up-regulation or down-regulation) of that gene in the original dataset (measured as: $CPM_{infected} - CPM_{control}$). Lack of the bar indicates absence of the gene from the particular dataset. All genes passed the validation criterion: be concordant with the direction of GE change in reference set and 3 or more other databases (i.e. be downregulated in reference and > 3 other datasets), allowing opposite direction in GE change in datasets with refractive species.

the downregulated AMPs in Lv, we identified multiple contigs blasting hemocyanin. This gene has been recently implicated in the production of anti-viral peptides which are able to inhibit the transcription of WSSV genes wsv069 and wsv421, resulting in a significant reduction in WSSV copy numbers in the initial stage of infection (i.e. 2hpi) (Zhan et al., 2018). Further, we found that in Lv *beta-arrestin 2* was up-regulated. This gene, as demonstrated by Sun et al. (2016), is able to negatively regulate the Toll-like receptor pathway which is one of the pathogen recognition receptor (PRR) pathways involved in innate immunity (Du and Jin, 2017). Interestingly, Cao et al. (2017) found that this pathway was up-regulated in Lv 3-h after WSSV inoculation, suggesting that, while in Lv in the initial stages of infection (i.e. < 3 hpi) an immune response can be triggered (i.e. by up-regulating hemocyanin or the toll-like receptor pathway), in the later stages of the infection WSSV may be able to actively regulate AMPs production, arguably acting on *beta-arrestin 2*.

In Lv many DEGs belonged to major glucose metabolic pathways, suggesting an important metabolic shift in animals following WSSV inoculation, in agreement with Peruzza et al. (2019), Li et al. (2018) and Chen et al. (2016). In fact, the up-regulation of *lactate dehydrogenase* (involved in anaerobic metabolism), *fructose 1,6-biphosphate-aldolase A* (involved in glycolysis) and *phosphoenolpyruvate carboxykinase* (PEPCK, involved in gluconeogenesis) coupled with the downregulation of enzymes from Krebs cycle and oxidative phosphorylation suggests a shift towards glycolysis (Chen et al., 2016; Li et al., 2018), known as the Warburg effect. In agreement with the model proposed by Escobedo-Bonilla et al. (2008), this metabolic boost, which was observed in Lv at 1dpi, is found in the early stages of infection to support the energetic demands of viral replication. Interestingly a metabolic boost was also observed in Mr, with the up-regulation of PEPCK, *triosephosphate isomerase* (involved in glycolysis) and *ATP-synthase*. However, in the light of Mr's resistance to WSD and given the fact that no viral gene was found as DE in this species, we could hypothesize that the metabolic boost was the result of a physiological response to counteract the virus by triggering an immune response (further supported by the up-regulation of genes belonging to the clotting cascade and AMPs genes).

Multiple physiological processes can be altered by WSSV during the infection as demonstrated by Peruzza et al. (2019). Interestingly, we found that WSSV induced an effect on the moult cycle of Lv: we observed the up-regulation of MIH, a hormone belonging to the crustacean hyperglycaemic family (Manfrin et al., 2015, 2016), and the simultaneous down-regulation of ecdysteroid receptors A and B, nuclear receptors activated by 20-hydroxyecdysone and responsible for transcriptional activation of moulting (Song et al., 2017). In accordance with these data, we recently reported that MIH was highly up-regulated

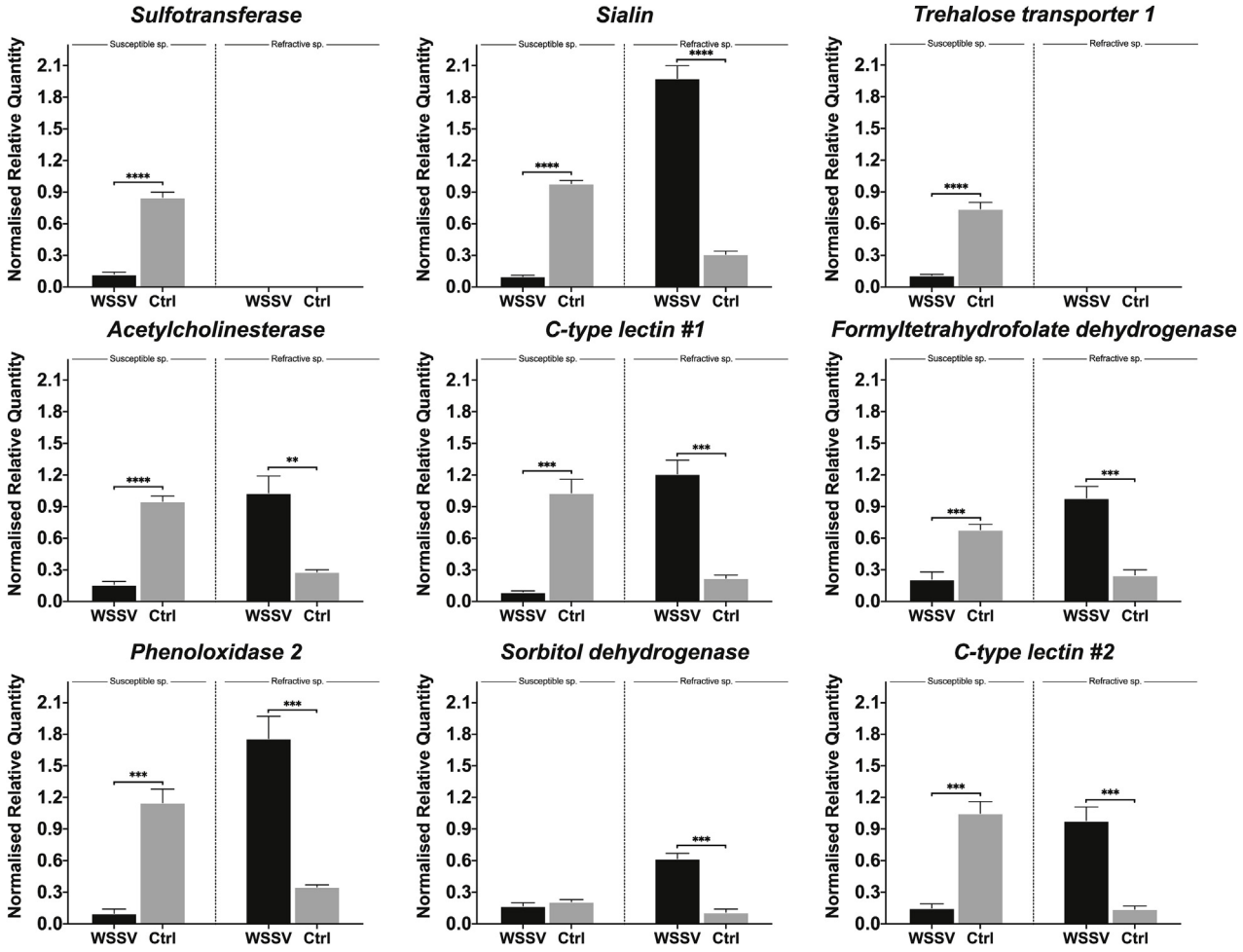

Fig. 7. Confirmatory qPCRs of DE genes from the comparative genomics analysis. Normalised Relative Quantities of selected genes were compared using ß-Actin as housekeeping gene in susceptible species (i.e. Lv, left part of each graph) and refractory species (i.e. Mr, right part of each graph) in WSSV-injected (i.e. "WSSV", black columns) and control (i.e. "Ctrl", grey columns) animals. Data are shown as mean + SD of n = 3 independent biological replicates. Note that genes *Sulfotransferase* and *Trehalose transporter 1* were not investigated by qPCR in Mr as they were not classified as DE in the refractory species' database. **: p-val < 0.01; ***: p-val < 0.001; ****: p-val < 0.0001.

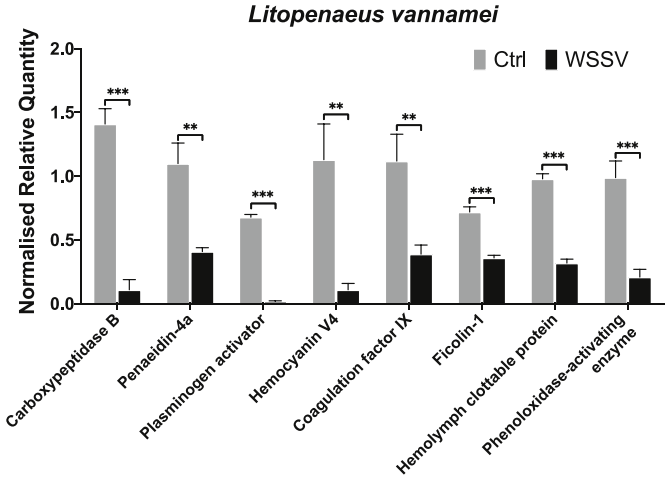

Fig. 8. Confirmatory qPCRs of DE genes from the transcriptomics analysis. Normalised Relative Quantities of selected genes mapping to haemolymph clotting processes and immune functions from *L. vannamei* were compared in WSSV-injected (i.e. "WSSV", black columns) and control (i.e. "Ctrl", grey columns) animals using ß-Actin as housekeeping gene. Data are shown as mean + SD of n = 3 independent biological replicates. **: p-val < 0.01; ***: p-val < 0.001.

in WSSV infected Lv after 18 and 56hpi (Peruzza et al., 2019). Further, Shekhar et al. (2019) reported the up-regulation in WSSV-infected Lv of a miRNA miR-8-5p; this miRNA is associated with the modulation of chitin biosynthesis, which is maximal during inter-moult period (Hornung and Stevenson, 1971), and therefore its up-regulation in infected animals suggests an effect of WSSV on moult cycle. Collectively, it could be hypothesised that the observed interference of WSSV with the moult cycle is arguably a viral mechanism to stop energetically demanding physiological activities in the host, such as moult or reproduction (Peruzza et al., 2018), and focus host energies towards viral replication.

4.2. Ecological genomics analysis

The comparative bioinformatic approach was thereafter adopted to identify genetic markers putatively linked with refractivity to WSD by comparing our in-house and on-line available datasets involving crustaceans infected with WSSV. We believe the major strength of this analysis is that it allowed us identify common trends (in terms of DEGs being repeatedly flagged as DE in different datasets) in a wide range of datasets generated by different research groups and involving different species (i.e. Lv, Mr, *P. monodon* and *Macrobrachium. nipponense*) and different tissues (i.e. gill, hepatopancreas and muscle) at different time points. The fact that some genes were repetitively found as DE in

several datasets despite the overall variability present in the dataset (e.g. different species, tissues, time points and research groups) is a clear indication of the importance of the genes in association with WSSV infection. The analysis highlighted nine candidate genes, which were deemed as putatively involved in WSD resistance as they were down-regulated in susceptible species and up-regulated in refractive host species.

Sialin is the main transporter of sialic acid from the lysosome (Morin et al., 2004). Sialic acids have different functions and, among all, are involved in immune cell maturation and activation (Wasik et al., 2016). Recently Liu et al. (2018) demonstrated that in the crab *Helice tientsinensis Sialin* was upregulated in response to LPS and played a critical role in immune responses and in supporting the defence of the host through activation of the lysosome pathway. A key step for viral replication that is still not understood is the ability of WSSV to escape from the lysosome (known as lysosomal escape; reviewed in Verbruggen et al. (2016)) and the down-regulation of *Sialin* may arguably be key in determining if the virus can get out from the lysosome and replicate or not.

Trehalose transporter 1 (Tret-1) is the transporter of trehalose, the main disaccharide used by immune system cells (especially differentiated immune cells, such as haemocytes (Bajgar et al., 2015)) during infection (Zhang et al., 2019; Zhu et al., 2018). In fact, Zhu et al. (2018) demonstrated that dietary supplementation with trehalose increased activity of immune system in Lv. Further, Bajgar et al. (2015) demonstrated in *Drosophila* sp. that infection induces the up-regulation of *Trehalose transporter* in haemocytes and the transporter is then used to absorb trehalose from haemolymph and subsequently trigger the immune response.

Formyltetrahydrofolate dehydrogenase (FDH) is a member of the "one-carbon (1C) metabolism pathway", a universal metabolic source of 1C units (based on folate) for biosynthetic processes, mainly cell proliferation and division (Ducker and Rabinowitz, 2017). The immune response requires high levels of folate since it is among the most proliferative processes in the body. In fact, as reported by Ducker and Rabinowitz (2017) T-cell activation in mice requires rapid 1C metabolism, involving large GE changes to mobilize existing stored 1C and to produce new 1C compounds.

Phenoloxidase 2 and *C-type lectin* are well known for their involvement in the immune system: both genes have been reported as down-regulated in Lv and in *Fenneropenaeus chinensis* infected with WSSV (Ji et al., 2011; Liu et al., 2007) and up-regulated in Mr (Cao et al., 2017; Zhang et al., 2014). Finally, *Sorbitol dehydrogenase* was reported by Zeng et al. (2013) as down-regulated in Lv following Taura Syndrome Virus infection, *Sulfotransferase* is involved in metabolism of xenobiotics (Ikenaka et al., 2006) and *Acetylcholinesterase* is present on the surface of granulocytes of *Limulus Polyphemus* (Gupta et al., 1991).

Overall, our approach identified nine putative candidates that would require additional in-depth investigation in relation to their role during WSSV infection as they could, arguably, be important genes actively controlled by WSSV. In particular, in the light of their role in immune functions, Sialin, Tret-1 and FDH could arguably be targeted by WSSV to overcome the host defences, hence explaining their down-regulation in susceptible species. On the other hand, their up-regulation in Mr could contribute to explaining the refractivity of Mr toward WSD (Hossain et al., 2001; Pais et al., 2007; Rajendran et al., 1999; Sahul Hameed et al., 2000; Sarathi et al., 2008).

5. Conclusion

In order to improve our knowledge on WSSV infection with particular emphasis on the molecular mechanisms of susceptibility/refractivity of some species, we compared transcriptomes of *Litopenaeus vannamei* and *Macrobrachium rosenbergii* after inoculation with WSSV. Our results indicated an activation of the immune system in Mr which up-regulated many components of the coagulation cascade and several AMPs, while an opposite pattern (i.e. down-regulation) was observed in Lv. In addition, we found several DEGs, exclusively in Lv, that were related to moult and suggested an inhibition of the moult cycle in this species following WSSV infection, suggesting that the virus was deviating host's energy allocation away from normal physiological processes (i.e. moult) to support its replication.

We further identified nine candidate genes which play an important role in the immune response and are consistently down-regulated in the susceptible species and up-regulated in the refractive Mr. We conclude that these genes may be actively regulated by WSSV while infecting susceptible species and, thus, may help overcome the immune defences and allow the progress of the infection. Together these results offer a valuable resource towards our understanding of the genetic basis of WSSV resistance.

Declaration of competing interest

The author(s) declare no competing interests.

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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.2019.103564.

Author contributions

ST and SV carried infection experiments, RNA isolation and library preparation. LP performed the bioinformatic data analysis, the ecological genomics analysis and their interpretation with inputs from CH, VJS, and ASSH. LP drafted the manuscript with inputs from all other authors. CH, LP, VJS, ASSH, KVK, KKV and MSS conceptualised the project. All authors reviewed and agreed the final version of the manuscript.

Data availability

Raw sequence data associated with this project have been deposited at NCBI with BioProject accession number PRJNA554075 and SRA accession numbers from SRR9670544 to SRR9670555.

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