

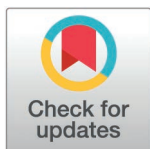
RESEARCH ARTICLE

Transcriptomic study of WSSV infection in *Litopenaeus vannamei* lymphoid organ via single nuclei RNA sequencing

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Abstract

Background

Aquaculture is the fastest growing farmed food sector and is a key part of global food security. Crustacean aquaculture, while being one of the most profitable sectors, is threatened by pathogenic diseases such as white spot disease (WSD), which results in severe stock losses and threatens animal health and welfare. In this study, we used novel techniques to study the impact of white spot syndrome virus (WSSV), the causative agent of WSD, on Pacific whiteleg shrimp (*Litopenaeus vannamei*), and uncover additional information that may be used in creating WSSV-resistant shrimp stocks.

Results

We successfully developed a novel nuclei isolation protocol optimized for shrimp tissues to prepare our samples for single nuclei RNA-sequencing following a pathogen challenge comprising 32 adult whiteleg shrimp infected with WSSV either through their feed or by injection. We constructed the first penaeid shrimp lymphoid organ cell atlas to improve our understanding of the characteristics of this immune organ, constructed UMAPs and identified marker genes that define distinct cell clusters to reveal the biological functions of different cell types within this organ.

Conclusion

By comparing gene expression between the control and WSSV-infected samples we uncovered multiple genes of interest that have the potential to be used as targets for CRISPR gene editing for WSSV-resistance in Pacific whiteleg shrimp. These genes have the potential to be valuable assets for future gene editing studies in commercially important penaeid shrimp species.

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Abbreviations: Cas9, CRISPR-Associated Protein 9; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DEG, Differentially Expressed Genes; DNA, Deoxyribonucleic Acid; EST, Expressed Sequence Tag; GO, Gene Ontology; GSEA, Gene set enrichment analysis; HCMV, Human Cytomegalovirus; HCV, Hepatitis C virus; HIV, Human Immunodeficiency Virus; HTLV,

Introduction

Aquaculture contributes significantly to global food production, and its importance is projected to rise. By 2032, aquaculture is estimated to account for 54 percent of the total production of aquatic animals [1]. Penaeid shrimp, such as Pacific whiteleg shrimp (*Litopenaeus vannamei*), Chinese white shrimp (*Fenneropenaeus chinensis*), kuruma shrimp (*Marsupenaeus japonicus*) and black tiger shrimp (*Penaeus monodon*), accounted for 62.2% of all farmed crustacean species, and remain the second most valuable farmed group after salmonids, accounting for 17% of total global aquaculture value [1]. Whiteleg shrimp was the top produced aquaculture species globally, at 6.8 million tonnes.

Despite their global productivity, shrimp farms frequently face disease outbreaks which severely hamper their production [2]. White spot syndrome virus (WSSV) is recognised as one of the most significant threats to crustacean aquaculture by the World Organization of Animal Health [3], and effective disease control methods are urgently needed.

Invertebrate immune responses are less well characterised than those of mammals, primarily due to their evolutionary distance from humans and mice, where the majority of functional biology studies have been focused [4]. Unlike mammals, penaeid shrimp lack developed antibody-mediated adaptive immunity, relying solely on effective cellular and humoral innate immunity, which in turn limits the potential for vaccine development [5,6]. In addition, crustacean immunity and the interactions between WSSV and host shrimp cells are not well characterised, therefore limiting the development of effective control measures.

It is known that the hepatopancreas, lymphoid organ and haemocytes play crucial roles in controlling the innate immunity responses in penaeid shrimp, making it essential to study their response to pathogens to facilitate disease prevention and resource development for future research [7]. The lymphoid organ, also known as the Oka organ, plays a major immunological role alongside haemolymph cells and hepatopancreas. It is a paired, lobed, immune organ found in penaeid shrimp, and other crustacean species such as *Macrobrachium rosenbergii* [8] and *Procambarus clarkii* [9]. It functions in pathogen defence by facilitating the recognition and elimination of viral and bacterial invaders, as evidenced by its significant role during infections by pathogens such as *Vibrio parahaemolyticus* and WSSV. Mechanisms by which the lymphoid organ fights against invading pathogens are believed to include phagocytosis, filtering of foreign materials from the haemolymph, bacteriostasis and viral degradation via the lymphoid organ spheroid (LOS) cells [7,10,11].

Transcriptomic analyses have shown differential gene expression in the lymphoid organ in response to various pathogens, indicating its adaptive immune functions. For instance, *Vibrio* spp. infections trigger broad immune responses, including the activation of pattern recognition receptors and phagocytosis-related genes, whereas WSSV infections tend to suppress immune responses, highlighting the organ's dynamic role in managing shrimp immunity against diverse threats [11].

Given the potential immunological roles of multiple cell types, distinguishing between them is critical to understanding their individual and collective contributions

Human T-Lymphotropic Virus; IAV, Influenza A Viruses; IFN, Interferon; JNK, c-Jun N-terminal kinases; KEGG, Kyoto Encyclopedia of Genes and Genomes; LIM (in LIM/homeobox), Lin-11, Islet-1, and Mec-3 - (the three original members of the LIM protein family); LSU rRNA, Large Ribosomal RNA Subunit; MAPK, Mitogen-Activated Protein Kinases; MERS-CoV, Middle East Respiratory Syndrome Coronavirus; NK, Natural Killer Cells; QPCR, Quantitative Polymerase Chain Reaction; RNA, Ribonucleic Acid; RNA-seq, RNA Sequencing; SAPK, Stress-Activated Protein Kinase; SARS-CoV-2, Severe-Acute-Respiratory-Syndrome-Related Coronavirus 2; snRNA-seq, Single Nuclei RNA Sequencing; SPF, Specific Pathogen-Free; SSU rRNA, Small Ribosomal RNA Subunit; TNF, Tumor Necrosis Factor; UMI, Unique Molecular Identifier; WSSV, White Spot Syndrome Virus; WSD, White Spot Disease; ZIKV, Zika Virus.

to a host's immune response. For instance, it is estimated that humans have almost 200 different cell types, many of which are well documented [12]. In contrast, few cell types have been documented in crustaceans and they generally lack thoroughly characterised marker genes, or cluster of differentiation (CD) marker genes [6]. Consistent cell type characterizations have been elusive due to variations in cell classification, study methodologies, and the absence of genetic and annotation tools for shrimp cell populations. Studying crustacean immunity at the cellular level would provide insights into the evolution of immune mechanisms and identify fundamental immune processes that are conserved across species. This approach also provides the opportunity to uncover novel immune strategies, improve disease models, and advance biotechnological applications and drug discovery.

Advances in next-generation sequencing technologies, particularly single-cell (scRNA-seq) and single nuclei RNA sequencing (snRNA-seq), have enabled comprehensive analysis of mRNA at the individual cell level, facilitating the study of gene expression differences among single cells [13]. To date, sc/snRNA-seq has been applied to a variety of aquatic animal species including Atlantic salmon, rainbow trout, turbot, grouper, orange spotted grouper, Nile tilapia, Asian sea bass, Kuruma shrimp, white shrimp, black tiger shrimp and Hong Kong Oyster, revealing cellular diversity and gene expression patterns (summarized in [14]). Beyond identifying and transcriptionally characterizing cell types, snRNA-seq has also proven valuable in revealing the specific cell types responding to pathogens as well as those cell types targeted by pathogens to modulate their host's immune response (e.g., [15]).

Capitalizing on the strengths of this technology, snRNA-seq studies on penaeid shrimp species have started to emerge across the literature, focusing on haemocyte and hepatopancreas tissues, studying cell types and differentiation [16,17], and differentially expressed genes (DEGs) during events such as ammonia nitrogen stress [18], cold stress [19], and artificial infections via injection with WSSV inoculum [6,13] (Table 1).

The application of single-nucleus RNA sequencing (snRNA-seq) in this study aims to provide insights into the shrimp lymphoid organ that cannot be obtained using conventional bulk RNA-seq methods. While bulk RNA-seq analyses measure average gene expression across whole tissues, it masks cellular heterogeneity and obstructs the contributions of individual cell populations. In contrast, snRNA-seq enables transcriptional profiling at the level of individual nuclei, allowing distinct cell populations within the lymphoid organ to be identified and their gene expression profiles characterized. This approach facilitated the construction of a cellular atlas of the organ and enabled us to study the infection-associated transcriptional responses of individual cell clusters, allowing us to infer which cells are most actively involved in antiviral responses and immune regulation during WSSV infection.

Materials and methods

Experimental animal care

Adult *L. vannamei* weighing 6–12 g were used for this study and held in the Recirculating Aquaculture System (RAS) shrimp facility at the Roslin Institute (Midlothian,

Table 1. Summary of single cell and single nuclei RNA-sequencing studies in penaeid shrimp of aquaculture importance.

Species	Sample	Approach	Summary	Citation
Kuruma shrimp	HM	scRNA-seq (Drop-seq)	HM cell atlas (6 clusters with 6 haemocyte subtypes). Identified cluster gene markers and evidence for factors driving haemocyte differentiation	[20]
	HM	scRNA-seq (Drop-seq)	HM cell atlas (8 clusters with 6 haemocyte subtypes). Estimated new antimicrobial peptides from functionally unknown genes following WSSV infection	[6]
Chinese white shrimp	HM	scRNA-seq (10x Genomics)	HM cell atlas (8 clusters with 3 cell types). Identified novel macrophage-like cells and their gene markers.	[17]
Black tiger shrimp	HM, HP	scRNA-seq (10x Genomics)	HM & HP cell atlas (7 clusters each with unknown specify cell types). DEG markers associated with ammonia nitrogen stress	[18]
Pacific whiteleg shrimp	HP	scRNA-seq (droplet-based)	HP cell atlas (10 clusters with 4 cell types). DEG markers associated with cold tolerance	[19]
	HM	scRNA-seq (10x Genomics)	HM cell atlas (4 clusters with 3 cell types). Identified HM functional properties, potential differentiation trajectory and correspondence with morphological subpopulations	[16]
	HM	scRNA-seq (10x Genomics)	HM cell atlas (16 clusters with 3 cell types). Identified HM functional subtypes, differentiation characteristic, and their differential immune response to WSSV infection	[13]

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UK) (Fig 1). The shrimp were kept at 28 ± 0.5 °C, housed individually in 3 L plastic tanks containing aerated artificial sea-water with a salinity of 25‰ (ppt) and pH of 8.0. The shrimp were fed a commercial shrimp pellet diet twice a day (~5% body weight/day). Solid waste siphoning, and water tests were performed daily. The aquaria connected to the RAS system had an average water turnover of 3L/h which was filtered using mechanical and biological filters before being passed through a protein skimmer and returned to the system.

Ethics approval for the use of *Litopenaeus vannamei* prawns in this study. This study involved the use of Pacific whiteleg shrimp (*Litopenaeus vannamei*) which are not currently regulated under A(SP)A 1986 but have recently become recognized under the Animal Welfare (Sentience) Act 2022. This study has been reviewed and approved by the Roslin Institute Animal Welfare and Ethical Review Body (AWERB).

WSSV quantification

A WSSV-standard curve was used to quantify all WSSV samples. The qPCRs were done with Brilliant III SYBR Master Mix (Agilent), with published primer and probe sequences [21] (Table 2). Briefly, each 20 µL reaction contained 10 µL of 2× SYBR Master Mix, 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 0.2 µL of probe, 0.2 µL of ROX dye, 1 µL of template DNA, and 7.6 µL of nuclease-free water.

All reactions were prepared on ice and briefly centrifuged prior to the run. The thermal cycling was performed with an initial denaturation and polymerase activation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 10 s with fluorescence reading. A melt curve from 65 °C to 95 °C was performed at the end of the run to verify amplification specificity.

WSSV stock expansion

Purified viral stock and infected shrimp tissue were sourced from CEFAS – *Centre of Environment Fisheries and Aquaculture Science* (Lowestoft, England). We expanded our viral stock by bath challenge and feed challenges; each performed on adult *L. vannamei* shrimp. For the bath challenge we decanted a 2 mL vial of purified WSSV stock into a 20 L aquarium with three adult shrimp. Separately, we prepared infected shrimp material and mixed it with the standard food pellets which was fed to six adult shrimp. The challenges were performed in two isolated aquaria with filtration and heating.



Fig 1. Shrimp holding facility. The picture shows the RAS system setup used for the viral challenges in adult *L. vannamei* shrimp. Each tank is fitted with an individual aeration system. A small-scale RAS system is used to keep the water parameters constant and water quality within safety levels. The feeding was done manually.

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Table 2. Primers and probe used for WSSV 69 bp amplicon amplification in qPCR.

Forward primer (WSS1011F)	5'-TGGTCCCGTCCTCATCTCAG-3'
Reverse primer (WSS1079R)	5'-GCTGCCTTGCCGAAATT-3'
WSSV probe	FAM-AGCCATGAATGCCGTCTATCACACA-BHQ1

<https://doi.org/10.1371/journal.pone.0348006.t002>

Both groups were maintained for 7 days before shrimps were sacrificed on ice. The tail muscle of the sacrificed shrimps was extracted and macerated using a tissue homogenizer. All the tissue samples were tested for viral load using qPCR. Viral tissue stocks that exceeded 1.5×10^6 WSSV copies/ μ L were kept frozen at -80°C until their use in the main WSSV challenge.

WSSV infection and viral load quantification *in-vivo*. For our experimental viral challenge, we used 32 adult shrimp, split into two main groups of 16 shrimp: control and infected. Each of the groups were further split in two eight-shrimp sub-groups by infection method: feed and injection. The WSSV-challenged shrimp were maintained in isolated aquaria which were closed off from the recirculation system for the duration of the challenge in order to prevent cross-contamination of the control group. The aquaria on this tray had daily manual water exchanges, with the displaced water going straight into the waste tank for decontamination. To ensure identical conditions in both groups, the control shrimp were held in equivalent static systems with manual water changes.

Injection challenges were performed with 50 μ L of saline solution (Tris-HCl 20mM & NaCl 400mM in dH_2O , pH 7.4, 0.45 μ m syringe filtered) delivered intra-muscularly in the dorsal side between the 3rd and 4th abdominal segment using a 26G needle. Animals in the WSSV-challenged group were injected with a mix of base saline solution and 5 μ m-filtered WSSV

stock, prepared from the previous challenge (approx. 1.5×10^6 WSSV copies/ μL) at a 50:1 ratio. The control group was injected using 100% saline solution.

For the feed challenge group, we prepared balls of food made out of macerated shrimp tail muscle tissue and shrimp food pellets at a 3:1 ratio. The balls were approximately 0.5 cm^3 and weighed 0.5–0.55 g, corresponding to 5–6% of the average body weight of the shrimp used in the challenge. For the infected group, the muscle tissue came from the WSSV-infected shrimp (approx. 3×10^5 WSSV copies/ μL), while for the control group we used WSSV-free SPF shrimp (CEFAS, Lowestoft, England)

Post-infection, all shrimps were fed a normal diet twice a day. The WSSV-infected shrimp were monitored several times during the day, between early morning and later afternoon, for mortality and infection symptoms. Due to the infection timeline being slightly different for each group, the shrimp were sampled separately between 48 and 72h post-infection for the injection group (rapid infection, following an artificial infection method) and between 5- and 7-days post-infection for the feed group (slow infection, following a natural mechanism of infection) to ensure they were all in the same mid to late infection stage (Fig 2). Mortalities were discarded to avoid capturing cell death processes in transcriptomic analysis. Data on weight and moult stage was collected upon sacrificing the shrimp. Only inter-moult stage shrimp (C-D1) were used for the downstream analysis to ensure differential gene expression was not biased due to differences in moult stage.

Sampling and nuclei processing

Tissue sampling. Following the WSSV trial, all the shrimp with suitable moult stages were sacrificed on ice. The individuals were dissected; the lymphoid organs were extracted (Fig 3) and then immediately frozen on dry ice. Tissues were stored at -80°C until processing.

A muscle sample from each individual was subsequently tested for the presence or absence of WSSV (viral load). The 17 lymphoid organ pairs carried forward for the snRNA-seq experiment were chosen for their intermediate moult stage, absence of WSSV in control groups and high viral load in WSSV-positive samples

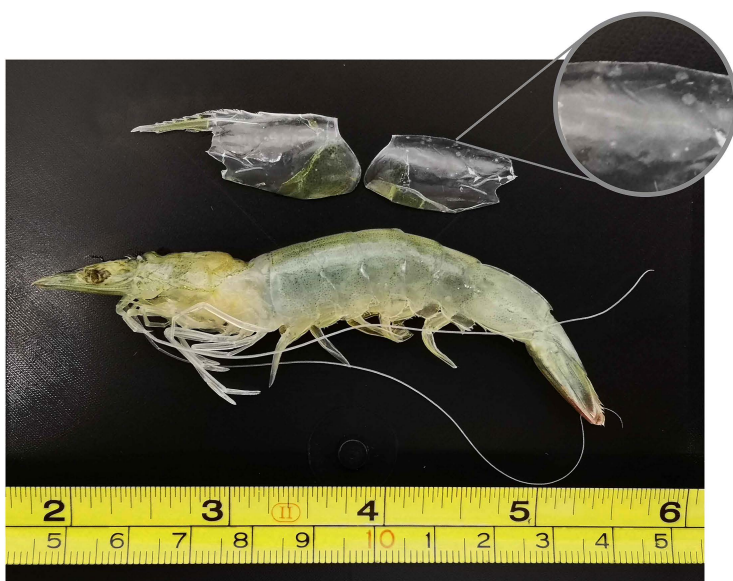


Fig 2. White spot disease symptoms in WSSV-challenged shrimp from the 'feed' group. The top-right close-up image shows white-spots on the carapace which are characteristic of white spot disease.

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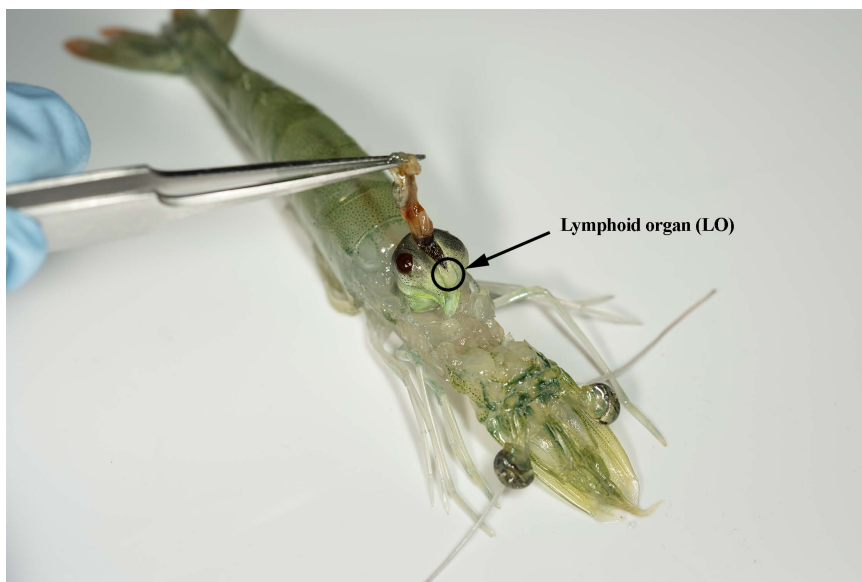


Fig 3. Pacific whiteleg shrimp dissection. The arrow and circle show lymphoid organ harvested for the transcriptomic analysis.

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Nuclei isolation and fixation. The nuclei were extracted using a modified version of the TST-based method [22]. Briefly, we prepared the 2X stock of salt-Tris solution (ST buffer), 1X ST-buffer solution working solution (TST) as below:

- **2X ST buffer** (for 2 samples):

Reagent	Volume	Final concentration (mM)
NaCl	292 μ L	146
Tris-HCl pH 7.5	100 μ L	10
CaCl ₂	10 μ L	1
MgCl ₂	210 μ L	21
dH ₂ O	9.388 mL	N/A

- **1X ST buffer:** Dilute 2x in nuclease-free water (1:1) and add 5.2 μ L of RNase Inhibitor (final conc. 40 U/mL).
- **TST solution:** Mix 2 mL of 2X ST buffer, 120 μ L of Tween-20 1%, 20 μ L BSA 2% and 1.86 mL of dH₂O.

For each sample, a 6-well tissue culture plate was placed on ice and 1 mL of TST buffer was added to a well. The frozen tissue sample (~10–20 mg) was placed in the buffer straight from dry ice. Note that due to the lymphoid organs being very small, we could only get sufficient material to process with the Parse kit by pooling samples together. Lymphoid organ pairs from 2–3 individuals were pooled together for each sample depending on their size. The sample was then macerated as finely as possible with spring scissors for 4 minutes. The sample was then sequentially pipetted for 30 seconds with a P1000 and 30 seconds with a P200. Each sample lysate was then filtered through a 40 μ m Falcon cell-strainer into a clean well of the plate, and then again through a 20 μ m cell strainer. 1 mL of TST was added to wash the initial maceration well and material passed through the filters again to collect the remaining nuclei. The volume was brought up to 5 mL with the addition of 3 mL of 1X ST buffer which also inactivates the Tween-20. The lysate was transferred to a 15 mL Falcon tube and centrifuged at 4 °C for 5 minutes at 500 g. The pellet was resuspended in 200 μ L 1X ST buffer.

A 9 μ L sub-sample of the nuclei was taken and stained with 1 μ L of Acridine Orange/Propidium Iodide. The LUNA-FX7™ Automated Cell Counter was used to count the nuclei as well as to determine the nuclei quality, degradation level, shape, size, and non-singlet percentage.

Following nuclei isolation and removal of a sub-sample for quality testing, nuclei were fixed immediately using the Evercode™ Nuclei Fixation v2 kit from Parse Biosciences according to manufacturer's instructions. At the end, the nuclei were re-counted as above using the LUNA-FX7™ Automated Cell Counter. Finally, the fixed nuclei samples were chilled using a Mr. Frosty™ freezing container and stored at -80°C until all other samples had been processed.

RNA library generation and sequencing

Once all the nuclei had been processed, the samples were prepared for sequencing using the Evercode™ WT Mega v2 from Parse Biosciences according to manufacturer's instructions. Six total libraries, each containing 5000 nuclei (number limited by the maximum capacity of the kit and the number of allocated sample slots on the library plate), were prepared using the 17 lymphoid organ pairs as follows:

- Library 1 (Sample name in repository: S3): Feed control
- Library 2 (S4): Injection control
- Libraries 3 (S9) and 4 (S10): Feed WSSV infection
- Libraries 5 (S11) and 6 (S12): Injection WSSV infection.

Following library preparation, samples were sequenced by Novogene using Illumina NovaSeq X Plus, 150 bp paired end sequencing (PE150) on the 25B flow cell. Libraries were spiked with 2% PhiX. Approximately 1.5 billion reads across six libraries were obtained, for an estimated 50,000 reads per cell.

The full sequencing dataset has been uploaded to the SRA database, under BioProject accession PRJNA1307069. The eight Parse sub-libraries can be found under BioSample accessions: SAMN50653283, SAMN50653284, SAMN50653285, SAMN50653286, SAMN50653287, SAMN50653288, SAMN50653289, SAMN50653290.

Data analysis

The data analysis was performed using a homemade pipeline (available: https://github.com/Roslin-Aquaculture/Parse-sn-RNAseq_LV-WSSV.git) that combined Parse Biosciences pipeline for Linux and the options from Seurat single cell analysis package (v4.4.0, Satija Lab, [23]) for R (v4.2.0, [24]). Below is an overview of the analysis steps undertaken. The full details, including individual sample thresholds for filtering can be found inside the GitHub repository.

Data de-multiplexing. A genome file made from *Litopenaeus vannamei* (Genome assembly ASM378908v1, NCBI RefSeq assembly GCF_003789085.1) concatenated with the *White spot syndrome virus* (Genome assembly ASM397252v1, NCBI RefSeq assembly GCF_003972525.1) annotation was used in Parse split-pipe v1.2.1 (use_case “normal”; mode “comb”; chemistry “v2”; kit “WT_mega 96 wells”) to demultiplex sequence data into biological samples, align the reads against the genome, generate gene annotations, and estimate gene expression per cell, resulting in a unique molecular identifier (UMI) count matrix per sample.

Cluster and differential gene expression analysis using Seurat R package. The sample preprocessing, including quality controls (QC), was performed in Seurat v4.4.0 (SeuratObject v 4.1.4) as Seurat v4.3.0 or higher is required to open the ‘count_matrix.mtx’ file generated by Parse's split-pipe. This included removing the cells with low UMI or feature numbers (detected genes), as well as those cells which contained more than 5% mitochondrial DNA genes (features). The UMI and feature thresholds vary with each sample and are specified inside the GitHub repository. This was followed by SCTransformation and cell cycle (st.flavor = “v2”; method = “glmGamPoi”; assay = “RNA”) data normalisation steps.

The highly variable genes were identified (feature selection), the data was scaled accordingly, and a linear dimensional reduction was performed. Finally, the ‘dimensionality’ of the dataset was determined using Elbowplots (dims = 1:15), and the cells were clustered, generating the initial UMAP (Uniform Manifold Approximation and Projection) (resolution = 0.4).

After the initial UMAP, the DoubletFinder package was used to remove the doublets from the dataset. The dataset was integrated using integration anchors before downstream analysis. Individual UMAPs were generated per group (control or infected) and treatment (feed or injection). The cell distribution throughout the clusters was assessed based on group (control or infected) and treatment (feed or injection). From this point on Seurat versions v4.1.0 (Seurat Object 4.0.4) was used for all other analysis steps to accommodate for an already existing pipeline we developed for 10x Genomics data (available: https://github.com/Roslin-Aquaculture/10x-snRNAseq_LV-HP-atlas.git). Differential gene expression analysis (DEG) was performed in order to determine the gene markers for each cluster. At this point, a list containing the top 20 marker genes (by positive expression level) for each cluster was generated (assay = “SCT”; slot = “data”; only.pos = TRUE; min.pct = 0.25; return.thresh = 0.01; pseudocount.use = 0.001; logfc.threshold = 0.25; test.use = “LR”; latent.vars = “sample”). The complete list of these cluster markers can be found in the supplementary file “S1 File”. Each gene was analysed manually with a combination of NCBI (www.ncbi.nlm.nih.gov/gene/; [25]), UniProt (www.uniprot.org/; [26]) and The Human Protein Atlas (www.proteinatlas.org/; [27]). Following this, the UMAP clusters were annotated, and a series of plots (Heatmaps, Dotplots, Feature plots, Violin plots) were generated characterising each cluster and its relevant markers using Seurat.

Finally, expression differences between control and WSSV-infection groups by treatment were analysed using Seurat DEG analysis (Wilcoxon Rank Sum test using SCT assay; min.pct = 0.1; return.thresh = 0.01; pseudocount.use = 0.001; logfc.threshold = 0.25). The function of all the differentially expressed genes was assigned manually with a combination of NCBI (www.ncbi.nlm.nih.gov/gene/; [25]), UniProt (www.uniprot.org/; [26]) and The Human Protein Atlas (www.proteinatlas.org/; [27]). A Dotplot of the top 10 differentially expressed genes (by logFC value for both negative and positive values) by treatment was created per group and plotted DEGs were studied regardless of their inclusion in the Dotplots.

GSEA analysis using GO and KEGG. Gene enrichment analysis was done via “SRplot: A free online platform for data visualization and graphing” (<http://www.bioinformatics.com.cn/en>), using the “GO, pathway Enrichment Analysis” module (http://www.bioinformatics.com.cn/basic_local_go_pathway_enrichment_analysis_122_en). Only genes with homology to human genes and a known well-established gene symbol were analysed due to the restrictions imposed by the method.

Results

WSSV infections and viral quantification

For the ‘WSSV injection’ group, the CT values were between 20.97 and 24.10, which indicates a viral load of between 3×10^6 and 3×10^7 WSSV particles per μL . For the ‘WSSV feed’ group, the CT values were between 22.41 and 29.32, indicating a viral load between 3×10^5 and 1.5×10^7 WSSV particles per μL (Fig 4).

Nuclei isolation

The lymphoid organ nuclei had minimal debris inside the final samples and had >95% singlet rate as counted using the LUNA-FX7™ Automated Cell Counter (Fig 5). The number of nuclei varied slightly between samples. The nuclei had minimal amount of degradation and were generally high quality,

Cell atlas construction and histopathological data integration

All six snRNA-seq libraries passed filtration, with over 1.5 billion reads across all samples (0.89 sequencing saturation). The final number of cells obtained after the QC and filtering was 18,541 (Fig 6A). The mean number of UMIs and genes was 1,173 UMIs and 654 features per cell (Fig 6D and 6E).

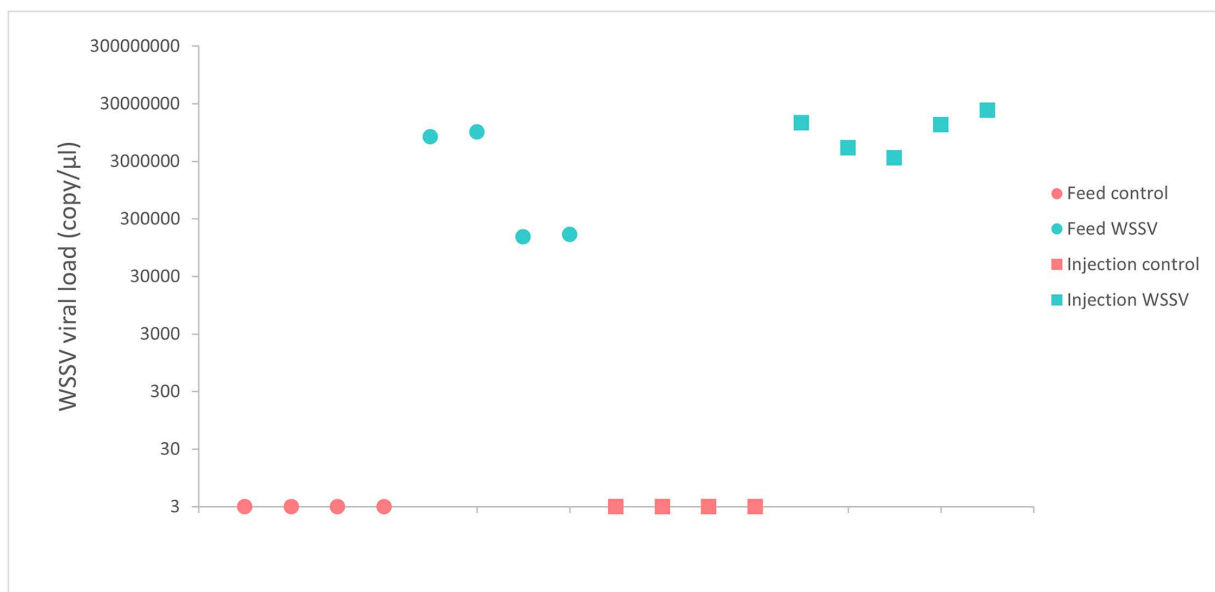


Fig 4. Average viral load of shrimp selected for snRNA sequencing analysis. The viral load was quantified using 50 mg of muscle tissue from the last abdominal segment. The viral load was inferred from the WSSV standard curve derived by qPCR ($Ct = -3.7913 \times \log(\text{Concentration}) + 48.887$).

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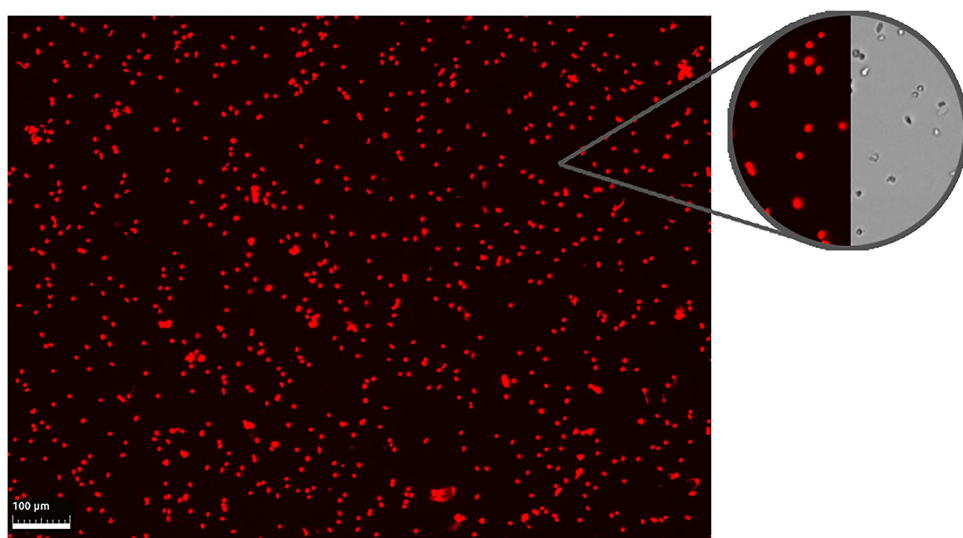


Fig 5. Example of isolated and fixated nuclei from lymphoid organ. The images are taken using the LUNA-FX7™ Automated Cell Counter. For the red field imaging, the nuclei were stained with Acridine Orange/Propidium Iodide dye. The top-right circles show a closer look at the nuclei in both red-field and bright-field.

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The nuclei number and distribution by sample group or treatment was also assessed. Overall, our experimental data contained 12,117 nuclei from WSSV-infected group and 6,424 from control (Fig 6B), 11,284 nuclei from injection treatment and 7,257 from feed (Fig 6C).

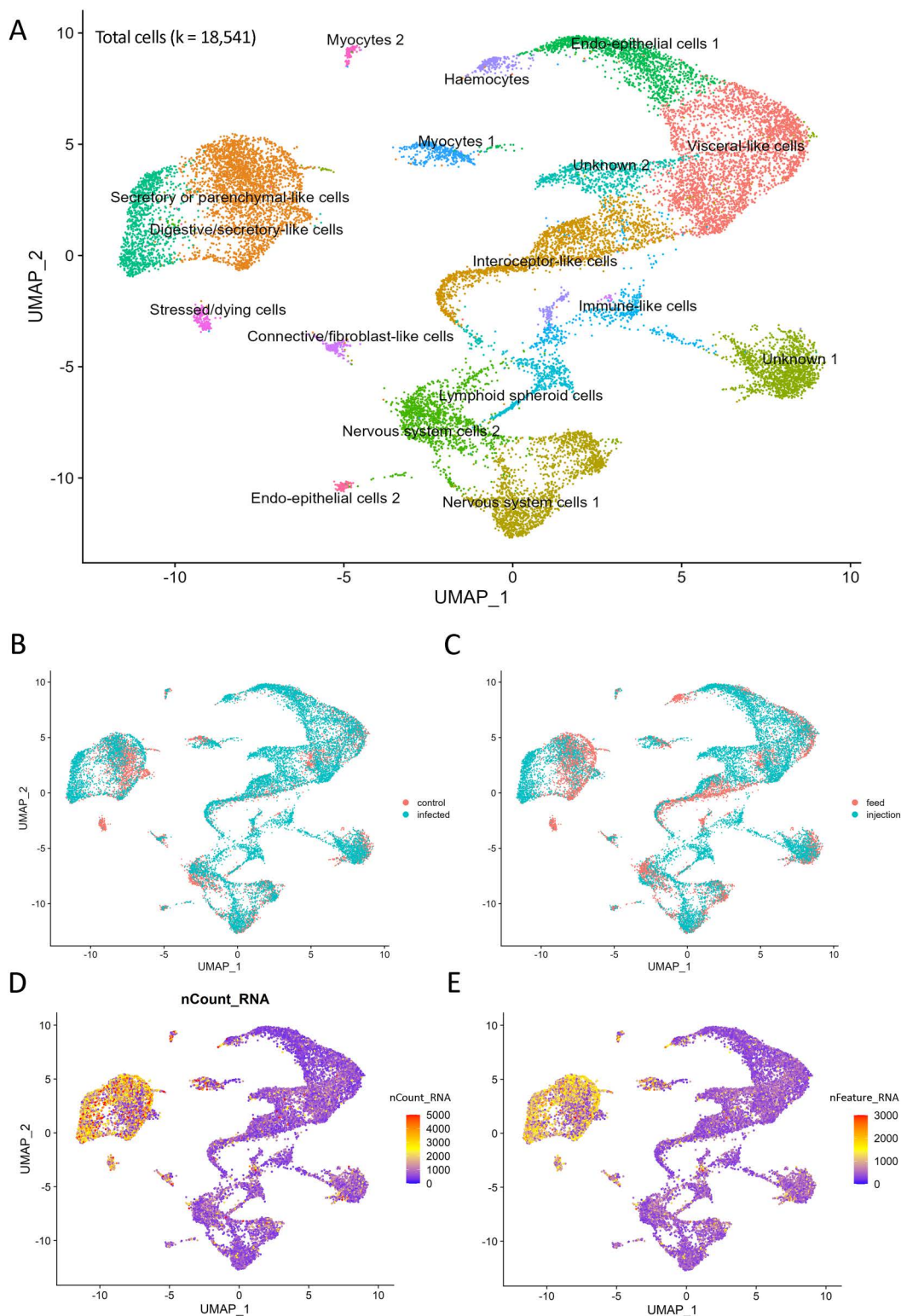


Fig 6. UMAPs. **A)** UMAP plot of cell clusters from shrimp lymphoid organ tissue. **B)** UMAP sample spread by treatment group (control and WSSV-infected). **C)** UMAP by infection method (feed vs injection). **D)** Total number of molecules (UMIs) detected within cells (nCount_RNA). **E)** Total number of genes detected in each cell (nFeature_RNA).

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A total of 17 clusters were observed (Fig 6A). The majority of clusters had distinct and recognisable transcriptomic profiles, with two clusters remaining undetermined due to the high number of uncharacterised marker genes and lack of cell type specificity of the annotated marker genes. The lymphoid organ cluster identification was partly based on the histopathological data from Rusaini and Owens [7].

Lymphoid organ cell clusters. Distribution of the number of UMIs and features, as well as the percentage of UMIs that were mtDNA genes for each cluster can be seen in Fig 7. Marker genes differentiating clusters within the lymphoid organ can be seen in Fig 8. Here we aim to assign identity to each of the cell clusters based on expression of key and unique genes. As a reminder, the clusters were assigned based on the localisation and function of the top expressed genes (human or mouse homologues in most part) according to data from NCBI, UniProt and The Human Protein Atlas. Unless otherwise specified.

Non-immune cell clusters: The clusters we identified include many of the cell types expected to be present within lymphoid tissues based on histopathological data present in the literature. We identified Cluster 0 as “Visceral-like cells” with low confidence due to a higher percentage of genes with low tissue specificity, and the presence of only a handful of genes (*Clca1*, *Ptprt*, *Pdgfrb*) indicative of the identity. It is worth noting that there are several genes in this cluster with additional roles in hydrolysis and pro-inflammation, including *Lgals*, *Casp1*, *LyzPtprt*, *clca1* and *Fbh1*, but their localisation is very broad or non-specific. Nonetheless, it could indicate that this cluster may also be associated with immune-like cells such as macrophages instead. Cluster 1, “Secretory or parenchymal-like cells”, expresses genes involved in secretion, including transporters and solute carriers such as *Tret1–2*, *Slc5a12*, *Slc6a5*, *Naat1*, *Slco5a1*, *Slc5a8*, *Slc13a2*, and two homologues of *Slc22a1* and *Slco3a1*. Cluster 7 has a similar gene profile and was identified as “Digestive/Secretory-like cells” due to the presence of *Slc12a2*, *Clca1*, *Naat1*, *Ces3* and *Ces2a*, often seen expressed in digestive glands due to their roles in transport (ion, calcium, sodium, potassium, amino-acid transporters) and hydrolysis. Cluster 2 expresses a mix of receptor-coding genes (*Gfra4*, *Scarb1*, *Hr96*), generally enriched in internal-organ cells, as well as genes coding for proteins involved in neurogenesis (*Dar1*) and gap-junction structure (*Inx2*). As such, the cells belonging to this cluster are identified as potential interceptors (sensory receptor for internal organs). Cluster 3 and Cluster 5 are both identified as nervous system cell types with high confidence due to the expression of CNS and PNS-enriched genes. Cluster 3 expressed *Gbx2*, *Hth*, two homologues of *Cadn*, and *Ap*, which have functions in DNA binding and/or cell adhesion inside the brain, bone marrow and peripheral neurons and *Glr* which codes for glycine receptors (in ligand-gated chloride channels). Cluster 5 expresses *Slc1a3* (transporter enriched in astrocytes), *Nrxn1* (neuronal cell surface protein with role in cell adhesion), *Grik2* (receptor for the excitatory neurotransmitter L-glutamate) and *Ten-M* (cell adhesion role in neural development) among its top 20 gene markers. Although we cannot assign a definitive identity to Cluster 4, multiple embryonic-enriched genes such as *Rst*, *If*, and two copies of *Trim9*, indicate an undifferentiated cluster of cells. The “Endo-epithelial cells 1” cluster (Cluster 6) has gene markers enriched in both epithelial tissue (*Bm*, *Notch1*, *Mys*) as well as a variety of genes that are typically enriched in internal organs lining or tubes in vertebrates. An additional smaller cluster of “Endo-epithelial cells 2” (Cluster 16) was also identified, although we predict that this cluster might contain undifferentiated cells, possibly similar to pluripotent stem cells. This is due to the presence of *Lhx5*, two homologues of *Lhx1*, two LIM/homeobox protein-coding genes that are enriched in the developing vertebrate brain and kidney, as well as a mix of genes that are generally expressed in endothelial or epithelial-like cells from a multitude of organs. Cluster 8 remains uncharacterised due to half of the top 20 markers belonging to uncharacterised loci, as well as the low tissue expression specificity of the characterised markers. Clusters 11 and 15 are both myocytes. Both clusters express multiple copies of *Ttn*, *Tnni1* and *Myh1*. In addition, Cluster 11 expresses *Mt-Nd3* and Cluster 15 expresses *Tnni1* as a marker. We note that Cluster 11 has an unusually high proportion of mitochondrial DNA comparing to the other clusters (including *Cox1* gene as a marker) (Fig 7C), in addition to three RNA-specific genes coding for large and small RNA subunits (one SSU and two LSU rRNA genes), which could be indicative of a poorer quality cluster. Cluster 13 has gene markers for different cell types and was difficult to assign to a single identity. Nevertheless, there were several genes (*Lama4*, *Angptl2*, *Reln*)

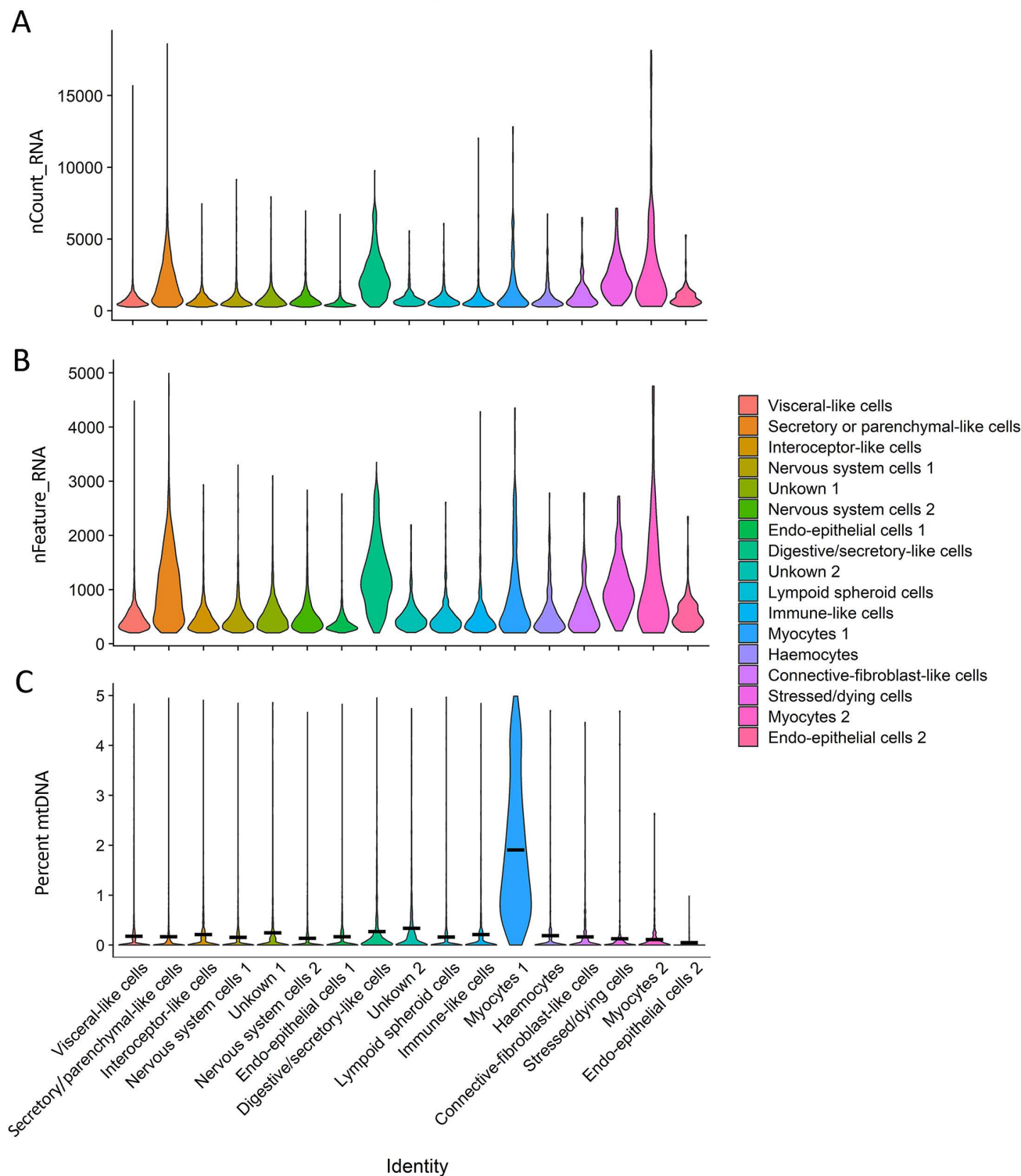


Fig 7. Data quality-violin plots by cluster. **A)** Average number of molecules (UMIs) detected within cells by cluster (nCount_RNA). **B)** Average number of genes detected in each cell by cluster (nFeature_RNA). **C)** Mitochondrial DNA percentage by cluster. The black bar represents average mtDNA percentage per cluster.

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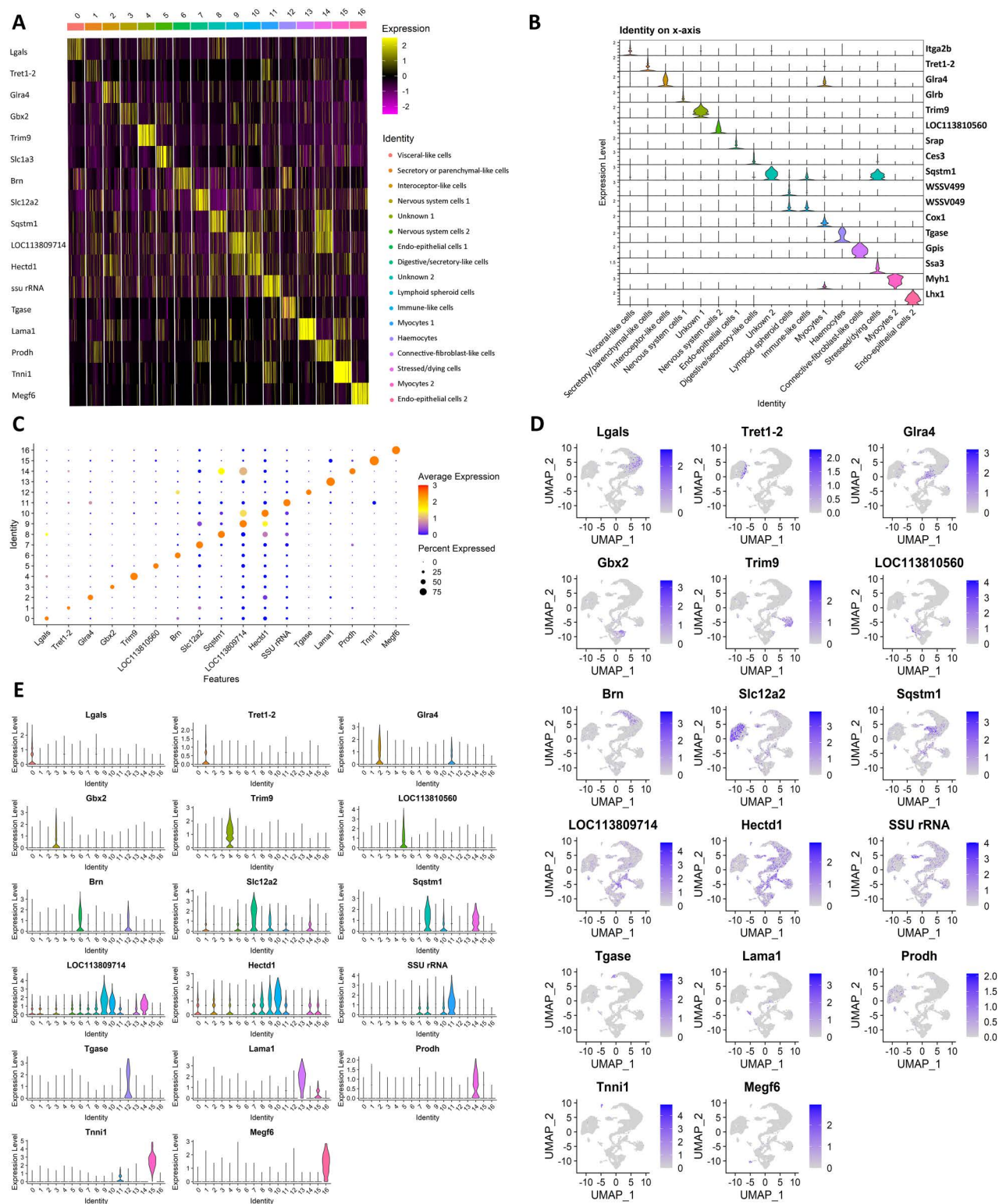


Fig 8. Cluster analysis. **A)** Heatmap of the top 8 marker genes in lymphoid organ. **B)** Violin plots of the significantly marker genes per cluster. **C)** Dot plot of significantly marker genes per cluster. Cluster numerical labels on the Y axis are as follows: 0=Visceral-like cells, 1=Secretory or parenchymal-like cells, 2=Interoceptor-like cells, 3=Nervous system cells 1, 4=Unknown 1, 5=Nervous system cells 2, 6=Endo-epithelial cells 1, 7=Digestive/secretory-like cells, 8=Unknown 2, 9=Lymphoid spheroid cells, 10=Immune-like cells, 11=Myocytes 1, 12=Haemocytes, 13=Connective-fibroblast-like cells, 14=Stressed/dying cells, 15=Myocytes 2, 16=Endo-epithelial cells 2.

7=Digestive/secretory-like cells, 8=Unknown 2, 9=Lymphoid spheroid cells, 10=Immune-like cells, 11=Myocytes 1, 12=Haemocytes, 13=Connective-fibroblast-like cells, 14=Stressed/dying cells, 15=Myocytes 2, 16=Endo-epithelial cells 2. **D)** UMAPs showing the expression level and location of the top cluster markers. **E)** Violin plots showing expression of top cluster markers by cluster.

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that indicated it may be formed of connective tissue or fibroblast-like cells. Lastly, Cluster 14 is the only cluster to be made up of cells entirely from the control group samples. This cluster expresses a variety of heat-shock or heat-shock-binding protein-encoding genes: *Hsc70*, *Hsc70-4*, *Dnajb4*, *Ssa3*, *Hsp20* and *Hspbp1*. These types of proteins maintain cellular proteostasis and protect cells from stresses [28]. As such, we concluded that this cluster is likely stressed or dying cells. **Immune cell clusters:** Clusters 9 and 10 were made almost entirely out of cells from the WSSV infection samples, potentially indicating an influx of these particular cell types into the lymphoid organ at the time of infection. Additionally, these clusters were the only two to contain WSSV genes as markers. Cluster 9 is likely to represent lymphoid organ-specific spheroid cells. Nine out of the top 20 markers were WSSV genes. Alongside them, multiple *L. vannamei* genes were expressed, including *Abcc1*, a membrane pump which mediates the export of foreign material from the cytoplasm, *Cpne8*, which regulate molecular events at the interface of the cell membrane and cytoplasm, as well as two cancer and tumour related genes: *Brat* and *Unc5c*. Similarly, Cluster 10 had two WSSV genes as markers. In addition to that, it expressed genes generally associated with immune cells (plasma and NK cells) such as *Npc1* and *Lpin3*, which suggest the cluster is of “Immune-like cells”. Furthermore, we also observed the presence of several genes involved in early cell development and embryogenesis, meaning that this cluster could potentially be young spheroid or young immune (haemocyte) cells. Finally, Cluster 12 has been identified as haemocytes due to the expression of the hemocytin-like gene, *proclotting enzyme-like* gene and two *Tgase* homologues.

Differential gene expression (DEGs) analysis

The infection routes (injection vs. feed) and sampling times (48–72 h vs. 5–7 days) were not intended to be directly compared; rather, our goal was to identify differentially expressed genes associated with WSSV infection regardless of route or precise infection stage. Nonetheless, to acknowledge potential transcriptional differences between the two groups, DEGs are presented separately (Figs 9 and 10).

Across the whole dataset, we found 85 upregulated and 70 downregulated genes in the WSSV-infected group. From these, 13 upregulated genes and 19 downregulated genes had an average log fold change of over 5.0, and there were three genes with values over 9.0. Several differentially expressed genes are likely to play a role in viral immunity or apoptosis. *Slc2a1*, a viral receptor in humans was upregulated in the “Myocytes 2” cluster (avg_log2FC=5.71) but also downregulated in “Endo-epithelial cells 1” cluster (avg_log2FC=-4.39). Two different copies of *Abcc5* gene were downregulated in the “Myocytes 2” cluster (avg_log2FC=-2.87 and avg_log2FC -4.50). *Ivns1abp*, an antiviral-related gene, was downregulated in the “Secretory/parenchymal-like cells” cluster (avg_log2FC=-4.71). Two genes involved in host-virus interactions were also found among the differentially expressed genes. *Xpo1* (avg_log2FC=3.71) was upregulated in the “Connective tissue-fibroblast-like” cluster while *Cebpa* (avg_log2FC = -2.65) was downregulated in the “Endo-epithelial cells 1” cluster. Apoptosis-related genes such as *Slk* (avg_log2FC=4.82) and *Plxna3* (avg_log2FC=4.15) were upregulated in the “Connective -fibroblast-like” and “Visceral-like cells” clusters respectively, while others such as *Ptpn4* (avg_log2FC=-2.05), *Ntrk2* (avg_log2FC=-6.72) and *Comp* (avg_log2FC=-6.88) were downregulated in the “Nervous system cells 2”, “Lymphoid spheroid cells” and “Endo-epithelial cells 1” cluster respectively. *Hr3*, a putative receptor, was the only differentially expressed gene present in both tissues. This gene was downregulated in the “Nervous system cells 2” cluster (avg_log2FC=-3.92).

Discussion

In this study, we conducted a white spot syndrome virus challenge on Pacific whiteleg shrimp in order to understand the molecular mechanisms underlying both the host response to WSSV and how the virus modulates gene expression in host

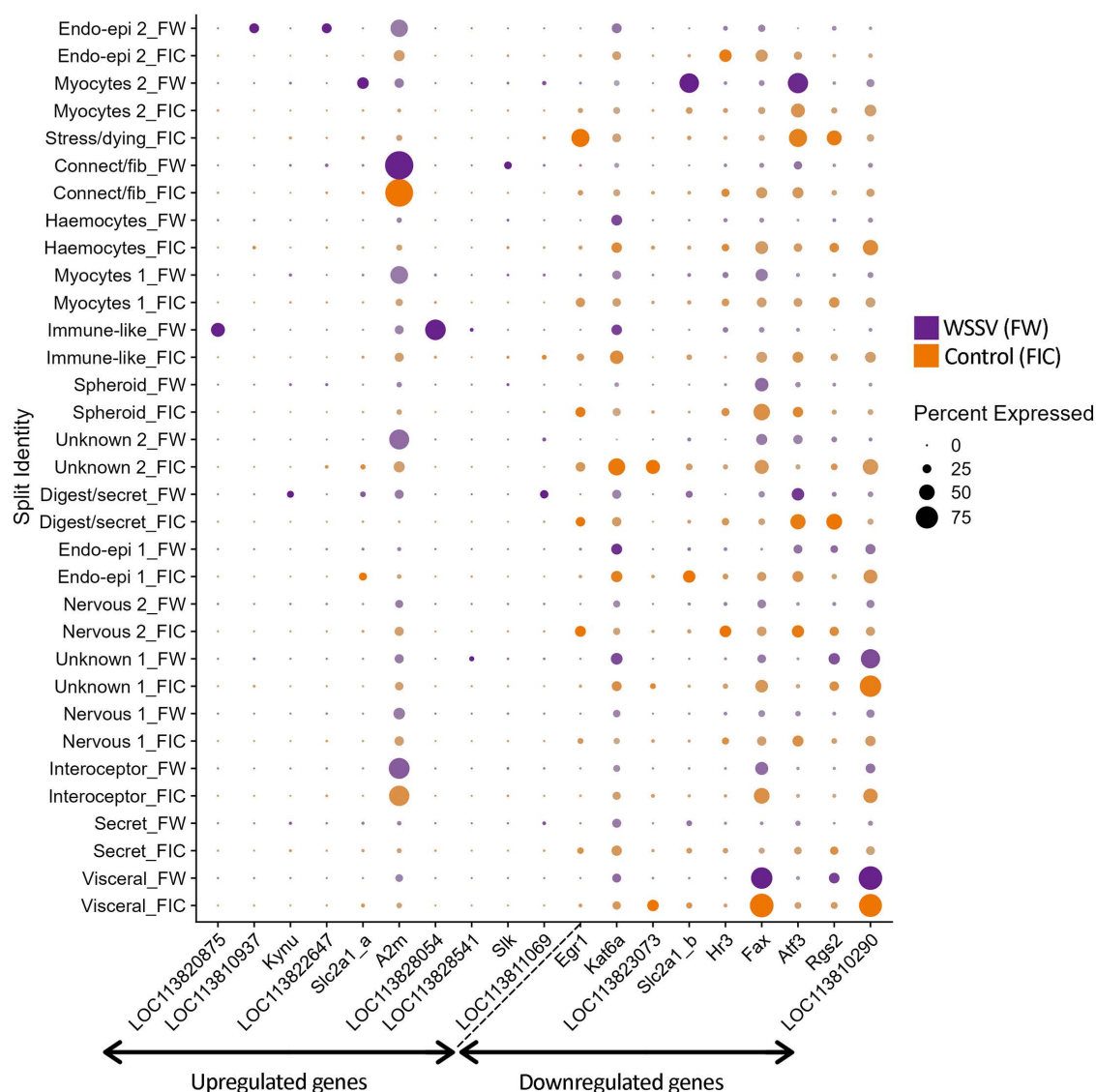


Fig 9. Differential gene expression dotplot in the 'Feed' group. The plots show the differentially expressed genes between the control and WSSV-infection samples in the 'Feed' infection group. The top 10 upregulated and top 10 downregulated genes per infection method were chosen for these plots. The size of the dots coincides with the gene expression percentage while the different colours represent the control or infected groups. The colour intensity coincides with the difference in expression levels, with the most intense colour representing up to 9.89 times expression level difference.

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cells. All WSSV-challenged animals exhibited clinical symptoms associated with white spot disease. In the case of acute infections ("Injection" group), the shrimp only exhibited lethargy, reduction in feeding and slight red discoloration on the body. The shrimp belonging to the "Feed" group also developed white spots on the exoskeleton, the "hallmark" of WSD, due to the more chronic nature of this infection method, as seen in other penaeid shrimp such as *P. indicus* [29]. These behavioural and morphological changes were assessed alongside quantitative PCR to confirm that all shrimp selected for RNA sequencing were genuinely infected with WSSV, rather than exhibiting similar symptoms due to unrelated causes. It should be noted that the viral load was assessed using tail muscle rather than lymphoid organ due to the limited tissue available for both nuclei isolation and DNA extraction, a consequence of the small size of the lymphoid organ in Pacific

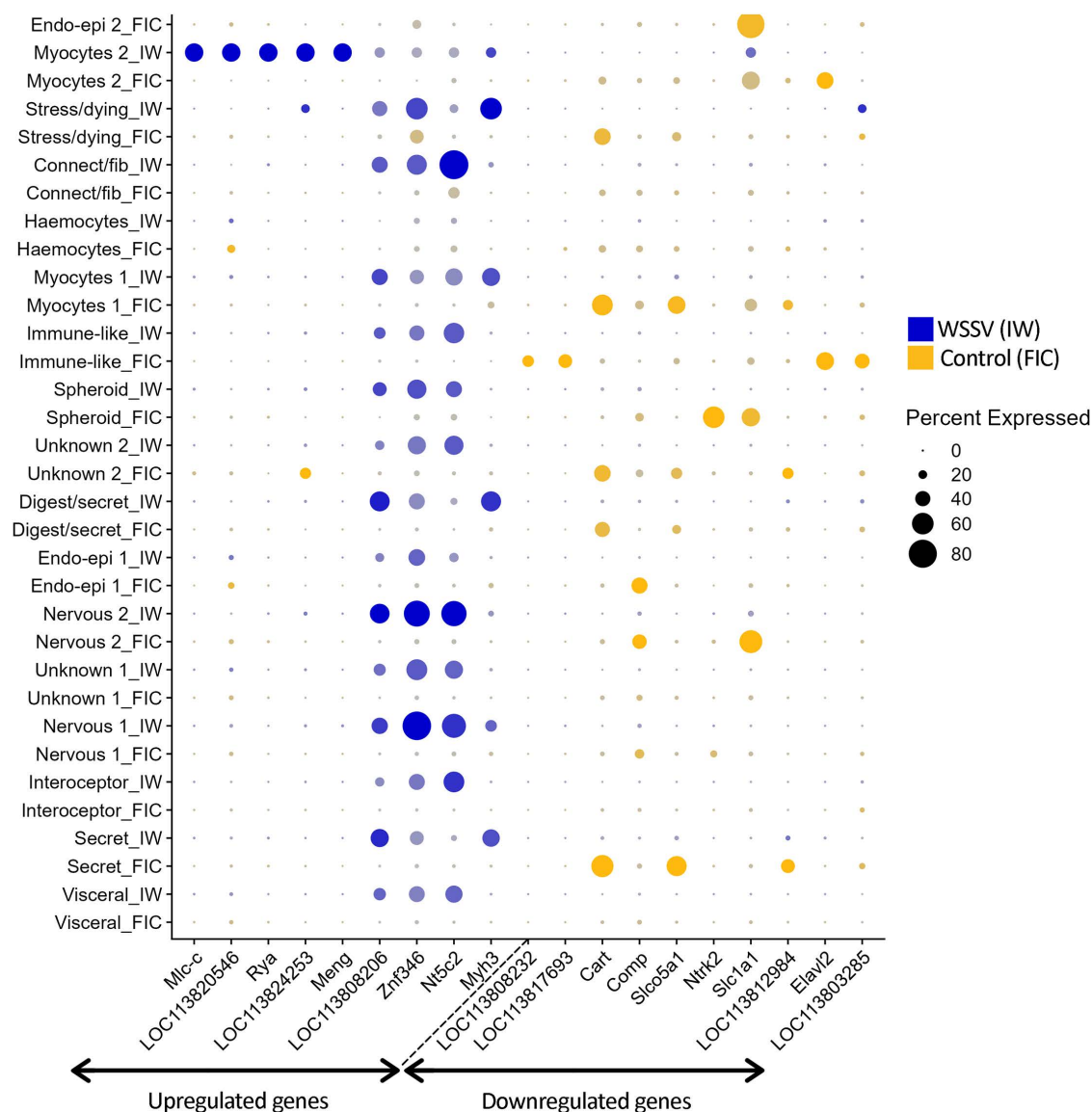


Fig 10. Differential gene expression dotplot in the 'Injection' group. The plots show the differentially expressed genes between the control and WSSV-infection samples in the 'Injection' infection group. The top 10 upregulated and top 10 downregulated genes per infection method were chosen for these plots. The size of the dots coincides with the gene expression percentage while the different colours represent the control or infected groups. The colour intensity coincides with the difference in expression levels, with the most intense colour representing up to 9.89 times expression level difference.

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whiteleg shrimp. While this approach was sufficient to confirm WSSV infection, we acknowledge that the viral burden in muscle may not fully reflect infection levels in the lymphoid organ.

As necessary, the lymphoid organs from 2–3 shrimp within the same treatment group were pooled to generate a single sample. This approach was required because the lymphoid organs of individual shrimp are too small to yield sufficient nuclei for the Parse Biosciences Evercode™ Nuclei Fixation v2 kit, which requires a substantial number of nuclei going into the fixation procedure as a minimum. While pooling allowed us to meet these technical requirements, we acknowledge that it limits the resolution of differential gene expression analyses at the individual-animal level, as natural biological

variation can be partially masked and subtle expression changes may be flattened. To mitigate these effects, our analyses focused on genes exhibiting robust expression differences (average log₂ fold-change ≥ 2 or ≤ -2), which are less likely to be affected by pooling. Furthermore, sample similarity was assessed via UMAP overlays and multivariate analyses (data not shown), confirming that the pooled biological replicates were largely consistent. Notably, pooling does not impact the generation of the single-cell atlas, as all cells are analysed individually, preserving cell-level resolution.

Cell types found in the lymphoid organ

The lymphoid organ is a paired, lobed, immune organ and a crucial component of the immune system, exhibiting significant differential expression of genes involved in viral receptor activity, immune modulation, cytokine production, pathogen-host interactions, cellular stress responses and apoptosis (Rusaini and Owens, 2010); [11]. It is involved in cellular and humoral immune responses and acts as the primary site of viral degradation via its spheroid cells [10,11]. Located ventro-anterior to the hepatopancreas, it is structurally characterized by lymphoid tubules and hemal sinuses, where it functions primarily as a haemolymph filter. The two lobes are surrounded by connective tissue capsules and muscle, and are connected to the heart by the subgastric artery. The tubules are surrounded by connective tissue fibre, have a central haemal lumen, stromal matrix cells and interstitial sinuses. The lumen is lined with flattened endothelial cells and often blocked with haemocytes [7].

We have successfully developed a new protocol for nuclei isolation from this important organ which will crucially inform future snRNA-seq studies on the immunological role of this organ. We have also generated a detailed cell atlas of the lymphoid organ, identifying multiple cell types with distinct roles in both function and viral immunity. Our samples contained various clusters with structural supportive or motility role, including epithelial cells (Clusters 6 and 16), muscle cells (Clusters 11 and 15) and connective tissue cells (Cluster 13). Three distinct clusters with roles in secretion were also identified (Cluster 0, 1 and 7). In vertebrates, lymphoid secretory cells are known to play important roles in immune function by facilitating communication, pathogen defences, and tissue organization [30]. While lymphoid tissues (in vertebrates) are not primarily sensory organs, they do contain nervous elements (such as autonomic nerve fibres and sensory nerve endings) that can influence immune function and that provide a major pathway through which the brain can alter immune reactivity [31]. We identified three different clusters that potentially fulfil these roles (Clusters 2,3 and 5).

Lymphoid organ plays a key role in pathogen defence by facilitating the recognition and elimination of viral and bacterial invaders and has a significant role during pathogenic infections [7,11]. The spheroid cells (Cluster 9) are formed in the haemal sinuses and appear to have a more basophilic cytoplasm and lack of a central lumen as compared to normal lymphoid tubules [7]. The spheroid cells phagocytize foreign material, particularly viruses [10]. These cells form during infection, encapsulate the pathogen and then degenerate. They are known to exhibit anaplasia (characteristic of malignant tumours) and to have an increased cytoplasm to nuclear ratio [7].

Infection- and immunity-related DEGs in lymphoid organ (Table 3)

Functional annotation in this study relied primarily on vertebrate homology-based gene annotations due to the limited availability of well-curated genomic and functional data for Pacific whiteleg shrimp and other non-model crustaceans. Where possible, we supplemented this approach with annotations from more closely related invertebrate species, including silkworm (*Bombyx mori*), horseshoe crab (Limulidae family), and fruit fly (*Drosophila spp*), to provide additional biological context. We acknowledge that the evolutionary distance between vertebrates and crustaceans imposes limitations on the precision of functional inference, and interpretations should be considered in light of these constraints.

The GSEA analysis (S1–S3 Figs) indicated that the main molecular mechanisms enriched following WSSV-infection were associated with transporter activities, including those for xenobiotics, organic and inorganic anions, chloride, active ions, dicarboxylic acid and sulphur. Other functions included calmodulin binding, actin binding, adenylate cyclase activity and sodium:chloride symporter activity.

Table 3. Immune- and infection-related genes differentially expressed in lymphoid organ.

Gene role	Gene symbol	avg_log2FC	Expression cluster	Associated protein function	Reference
Cellular Stress	<i>Slk</i>	4.82	CT	Regulates apoptosis and cytoskeletal dynamics. It plays a role in immune signalling pathways and cellular stress responses, influencing apoptosis.	[32]
	<i>Myl2</i>	8.96	MYO2	Involved in muscle contraction and cellular stress responses.	[33]
	<i>Sacs</i>	4.38, 3.70, 3.61, 3.48	NS1, NS2, VIS, MYO1	Associated with neurodegenerative disorders, role in cellular stress responses.	[34]
IS Modulators/Cellular Stress	<i>Znfx1</i>	3.11, 2.55	NS1, NS2	Antiviral defence by enhancing the recognition of viral RNA.	[35]
	<i>Ptpn4</i>	-2.06	NS2	Involved in signalling pathways that can modulate apoptosis and signal transduction pathways. Important role in innate immunity during MERS-CoV infection, Rabies virus infections	[36, 37]
	<i>Cebpa</i>	-2.65	EE1	Regulates expression of genes related to immune response, including cytokines. Negatively regulates IFN- γ Expression in T cells.	[38]
	<i>Egr1</i>	-9.89, -8.22	LOS, NS2	Multifunctional TF. Can regulate immune response genes, including those involved in cytokine production and response to viral infections by both DNA and RNA viruses	[39]
	<i>Atf3</i>	-1.94, -3.83	NS1, NS2	Stress-induced transcriptional effector. Modulates genes involved in many cellular processes, including inflammation and antiviral responses (e.g., Zika virus) to restore cellular homeostasis.	[40]
IS Modulators/Pathogen-Host	<i>Ifi30</i>	3.11	CT	Important role in MHC class II-restricted antigen processing.	[41]
	<i>A2m</i>	5.03	DS	"Double-edged sword. Traps and inactivates the host proteases that disrupt the viral infectivity but can also obstruct viruses (swine influenza A virus)	[42]
Pathogen-Host/Cellular Stress	<i>Xpo1</i>	3.71	CT	Nuclear export. XPO1-mediated protein/RNA export is necessary for viral replication. XPO1 contributes to the immunopathology by activating inflammatory signalling. Oncogenic driver in humans.	[43, 44]
	<i>Ivns1abp</i>	-4.71	DS	Specifically interacts with the NS1 protein of the influenza virus, impacting host-virus interactions and the host's ability to counteract viral replication.	[45]
SP/Cellular Stress	<i>Nphs1</i>	2.11	UN1	Can participate in immune response signalling pathways.	[46]
	<i>Piezo2</i>	3.03	MYO2	Involved in mechanotransduction; indirectly related through cellular stress mechanisms.	[47]
SP/Pathogen-Host	<i>Lrp1b</i>	-4.64	INTR	Receptor-mediated endocytosis, potentially influencing viral entry mechanisms.	[48]
SP/VR/Pathogen-Host	<i>Slc2a1</i> (<i>Glut1</i>)	5.71, -4.39	MYO2, EE2	Major glucose transporter found on cell membrane and cell surface. Receptor for HTLV-1 and HTLV-2.	[49]

TF = transcription factor, VR = Viral receptor, IS = Immune system, INTR = Interoceptor-like cells, CT = connective fibroblasts, MYO = Myocytes, DS = Digestive/secretory-like cells, LOS = Lymphoid spheroid cells, VIS = Visceral-like cells, UN = Unknown, EE = Endo-epithelial cells, NS = Nervous system cells. The gene relative expression level between control and WSSV-infected samples is shown as avg_log2FC, where positive numbers represent upregulated genes in infected samples, and negative numbers represent downregulated genes. All the functions stated have been primarily studied in mammals, unless stated otherwise. All underlined genes have also been studied in invertebrates. The gene underlined with a double line was differentially expressed in both tissues.

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Among the enriched pathways, two contained genes linked to viral (T-cell leukaemia virus 1) and bacterial (*Vibrio cholerae*) infections in other species, while several others were linked to secretion (salivary, pancreatic, gastric acid, insulin, parathyroid hormone, cortisol, thyroid hormone) and signalling pathways (Adrenergic signalling in cardiomyocytes, apelin, oxytocin, GnRH, cGMP-PKG and chemokine signalling).

Looking at the individual gene level, two genes associated with cellular stress response, *Slk* and *Sacs*, were found to be differentially expressed in our data. *Slk* drives protein homodimerization activity and protein serine/threonine kinase activity and plays a role in immune signalling pathways and cellular stress responses [32]. The gene also plays a role in apoptotic signalling pathways, which are crucial for the elimination of infected or damaged cells. The kinase activity of SLK is involved in the activation of stress-activated protein kinase (SAPK) pathways, such as the JNK and p38 MAPK pathways, which are involved in immune responses and inflammation [50]. *Sacs*, though not directly related to viral resistance, may influence cellular stress responses and overall immune function. *Sacs* has chaperon activities, controlling the microtubule balance or cell migration and has been associated with neurodegenerative diseases [34]. SACS contains domains similar to heat shock proteins and J-domain proteins, which are known to participate in stress responses. This function may influence how cells manage protein misfolding during viral infections, which can lead to stress and aggregation of viral proteins.

Znfx1, *Egr1*, *Atf3* and *A2m* code for immune system modulators that also have a role in cellular stress. ZNFX1 is a zinc finger protein involved in antigen processing. It plays multiple roles in immune response modulation. It is an interferon-stimulated double-stranded RNA sensor that recognises viral RNA and restricts the replication of RNA viruses in mice [35], highlighting its potential in enhancing immune recognition against WSSV. EGR1 is involved in cytokine production during viral infections of both DNA and RNA viruses. In mice, several cytokines, including IFN α , IFN β , IFN λ , and TNF α , are capable of increasing *Egr1* transcription [39]. In our study, *Egr1* was highly downregulated in two different cell types, potentially indicating host-cell manipulation by WSSV to create a suitable environment for replication. An additional immune system modulator is ATF3, which is a stress-induced transcriptional effector shown to be induced by viral infections such as the Human cytomegalovirus (HCMV) [51] and Zika virus (ZIKV) in human cells. Additionally, *Atf3* is induced in cells infected with Herpes Simplex Virus (HSV) and helps maintain the virus in a latent (resting) state. It can influence the cellular environment to either promote or inhibit viral replication [52]. In invertebrates, this gene has been characterized in silkworms (*Bombyx mori*) and studied more extensively in *Drosophila*. In the latter species, *Atf3* plays a role in cellular stress responses and immune signalling, safeguarding metabolic and immune system homeostasis [53]. Lastly among immune modulators we highlight A2M which is involved in pathogen-host interactions. A2M, a broad-spectrum protease inhibitor, may play a role in neutralizing WSSV proteases, thereby inhibiting viral spread within the host [54].

XPO1 is crucial for the nuclear export of viral ribonucleoproteins in viruses like HIV [55], Influenza [43], and Ebola [56]. In humans, XPO1 also contributes to immunopathology by activating inflammatory signalling and is an oncogenic driver [44]. In *Drosophila*, *Xpo1* has been studied for its role in nuclear protein and RNA transport which influences developmental and stress response pathways [57]. In crustaceans, modulation of *Xpo1* could potentially disrupt WSSV replication, much like it does during HIV infection, where the inhibition of this gene suppresses the replication of HIV and induces apoptosis in infected cells.

Ivns1abp is involved in host-pathogen interactions, particularly with influenza viruses. In humans, *Ivns1abp* encodes a protein that interacts with the non-structural protein 1 (NS1) of influenza A viruses (IAV) [58]. The NS1 protein of influenza viruses plays crucial roles in evading host immune responses and facilitating viral replication within host cells [45]. In our case, this gene was downregulated in infected samples, potentially as an immunity avoidance mechanism mounted by WSSV.

One final gene that is linked to viral infections is *Slc2a1*, also known as *Glut1*, which codes for a cell membrane glucose transporter implicated in viral entry and replication processes in various viruses such as HIV [59], as well as acting as a cell surface receptor for viral entry in Human T-lymphotropic virus 1 (HTLV-1) and HTLV-2 infections [49]. This gene has also been studied in various invertebrate species, particularly in the context of metabolic and stress responses. It is involved in nutrient transport processes that are crucial for energy balance in invertebrates, such as *D. melanogaster*, and it has been shown to be an important gene in the outcome of Huntington's disease where an increase in gene expression leads to ameliorated disease phenotype [60]. In crustaceans, *Glut1* expression is important for hemocytin adaptation to

hypoxic conditions in the Oriental River prawn (*Macrobrachium nipponense*) [61]. In Pacific whiteleg shrimp, Glut1 was shown to be a conserved protein whose expression changes with dietary carbohydrate level and source, as well as environmental stressors such as salinity, temperature changes and hypoxia [62,63]. Although its direct role in crustacean viral infections remains to be determined, the differential control of the *Slc2a1* among the different lymphoid organ cell clusters suggests its potential involvement in both host defence and WSSV entry and replication, making it an important gene to study in the context of infections and immunity.

WSSV infection mechanism and related genes

Previous transcriptomic studies of shrimp immune responses involving the lymphoid organ have relied on expressed sequence tag (EST) libraries [64] and total RNA-seq analyses [8,11]. These studies have successfully identified numerous immune-related genes and infection-associated transcriptional changes in response to two of the most economically important shrimp pathogens: *Vibrio* spp. [11,64], and WSSV [8,11,64], highlighting the important role of the lymphoid organ in pathogen recognition and immune defence. However, because these approaches measure average gene expression across the entire tissue, they could not resolve the cellular origin of the observed transcriptional changes or capture transcriptional heterogeneity among individual cell populations. By applying single-nucleus transcriptomics to the shrimp lymphoid organ, our current study extended beyond these earlier approaches by providing cell-level resolution of transcriptional activity, enabling a more detailed characterization of the cellular organization of the organ and offering deeper insight into the molecular mechanisms underlying host–virus interactions in shrimp during WSSV infection.

White spot disease (WSD) is one of the most damaging viral diseases in crustacean aquaculture, displaying aggressive pathogenicity that can result in up to 100% mortality 3–10 days post-infection [65]. WSSV's life cycle can be divided into three steps: entry into the host cell, uncoating of the genome followed by replication, and, particle assembly and release [66,67]. The first step of WSSV's cycle is the entry into host cells. This is done by binding to specific receptor molecules on the surface of target cell, which leads to induction of Clathrin-mediated endocytosis. Both LRP1B and SLC2A1 could potentially serve this purpose. Although LRP1B is not studied as a specific receptor, members of the LDL receptor family, including LRP1, are known to be exploited by viruses like Hepatitis C virus (HCV) for cell entry [68,69]. GLUT1 (*slc2a1*) has been identified as a receptor for HTLV-1 and HTLV-2, facilitating the virus's entry into host cells [49].

Once in the cell, host transcription factors, such as *Ie1*, bind the WSSV genome and initiate viral gene expression [70]. WSSV establishes a IE1/JNK/c-Jun positive feedback loop to drive its replication [70]. The SLK kinase is one of the molecules involved in the activation JNK pathway [50]. Other several genes can come into play for the replication step such as *Gsk3b*, *Cebpa*, and *Atf3*. GSK3 β is known to be manipulated by Human cytomegalovirus (HCMV) to create a host cellular environment geared towards accelerated protein translation support viral replication [71]. CEBPA is involved in regulating immune responses and has been shown to interact with viral proteins from Hepatitis B virus (HBV), affecting viral replication via transcriptional regulation of gene expression [72]. The modulation of *Atf3* by the virus could promote or inhibit viral replication by controlling the switch between the latent and active phases of the virus, as in the case of HSV [52]. Lastly, after the viral genome replication, WSSV will use the cell machinery to assemble new viral particles and release them. XPO1 is used by many viruses, including SARS-CoV-2, MERS-CoV, HIV, Influenza, and Ebola, to export viral RNA and proteins from the nucleus to the cytoplasm, which is essential for viral replication and assembly [43]. EGR1 is known to enhance viral gene expression, viral replication, and the release of infectious particles [39].

Conclusion

This study employed single nuclei RNA sequencing transcriptomic analysis of WSSV-infected adult Pacific whiteleg shrimp to uncover genes involved in viral immunity and infection. We generated novel WSSV disease challenge protocols for penaeid shrimp WSSV as well as a novel nuclei isolation protocol for the shrimp lymphoid organ, a critically-important immune organ. The sequencing data we generated was used to create a cell atlas for the lymphoid organ. The differential

expression of genes involved in viral entry, immune modulation, pathogen-host interactions, and cellular stress responses underscores the complexity of the shrimp's immune response to WSSV.

Our research highlighted a selection of WSSV infection-related genes. Future studies should focus on validating these genes through functional assays and exploring their roles in the broader context of crustacean immunity. This could potentially create a future path towards the creation of WSSV-resistant shrimp stocks via CRISPR gene editing and selective breeding, thereby enhancing aquaculture sustainability and productivity.

Supporting information

S1 File. Top 20 cluster marker genes per cell cluster. Marker genes were determined based on differential expression between clusters, ranked by adjusted p-value and average log2 fold change (avg_log2FC). Columns show the raw p-value (p_val), average log2 fold change (avg_log2FC), percentage of nuclei expressing the gene within the cluster (pct.1) and outside the cluster (pct.2), adjusted p-value (p_val_adj), cluster identifier, gene ID, gene symbol, predicted gene name, and the corresponding annotated cluster cell type.

(XLSX)

S1 Fig. Lymphoid organ GO analysis barplot summary. The figure displays the top 10 biological processes (BP), top 10 cellular components (CC) and top 10 molecular functions (MF) enriched in the lymphoid organ during WSSV infection based on GE genes. The length of the bar corresponds to the enrichment, calculated as $-\log_{10}$ of the p-value.

(TIF)

S2 Fig. Lymphoid organ GO analysis visualisation via Dotplots. The figure displays the top 10 biological processes (A), top 10 cellular components (B) and top 10 molecular functions (C) enriched in the lymphoid organ during WSSV infection based on GE genes. The “count” displays the number of DE genes that contributed to the category. The “p-value” shows the significance where $p < 0.05$ is significant. The enrichment score is displayed on the x-axis.

(TIF)

S3 Fig. Lymphoid organ pathway analysis based on KEGG. The figures represent the top 10 KEGG pathways significantly enriched in lymphoid organ tissue during WSSV infection based on DE genes. The “count” displays the number of DE genes that contributed to the category. The “p-value” shows the significance where $p < 0.05$ is significant. The enrichment score is displayed on the x-axis, calculated as $-\log_{10}$ of the p-value. The data is displayed as Barplot (A) and a Dotplot (B).

(TIF)

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