Scale Drop Disease Virus (SDDV) and *Lates calcarifer* Herpes Virus (LCHV) Coinfection Downregulate Immune-Relevant Pathways and Cause Splenic and Kidney Necrosis in Barramundi Under Commercial Farming Conditions

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Marine farming of barramundi (*Lates calcarifer*) in Southeast Asia is currently severely affected by viral diseases. To better understand the biological implications and gene expression response of barramundi in commercial farming conditions during a disease outbreak, the presence of pathogens, comparative RNAseq, and histopathology targeting multiple organs of clinically “sick” and “healthy” juveniles were investigated. Coinfection of scale drop disease virus (SDDV) and *L. calcarifer* herpes virus (LCHV) were detected in all sampled fish, with higher SDDV viral loads in sick than in healthy fish. Histopathology showed that livers in sick fish often had moderate to severe abnormal fat accumulation (hepatic lipidosis), whereas the predominant pathology in the kidneys shows moderate to severe inflammation and glomerular necrosis. The spleen was the most severely affected organ, with sick fish presenting severe multifocal and coalescing necrosis. Principal component analysis (PC1 and PC2) explained 70.3% of the observed variance and strongly associated the above histopathological findings with SDDV loads and with the sick phenotypes, supporting a primary diagnosis of the fish being impacted by scale drop disease (SDD). Extracted RNA from kidney and spleen of the sick fish were also severely degraded likely due to severe inflammation and tissue necrosis, indicating failure of these organs in advanced stages of SDD. RNAseq of sick vs. healthy barramundi identified 2,810 and 556 differentially expressed genes (DEGs) in the liver and muscle, respectively. Eleven significantly enriched pathways (e.g., phagosome, cytokine-cytokine-receptor interaction, ECM-receptor interaction, neuroactive ligand-receptor interaction, calcium signaling, MAPK, CAMs, etc.) and gene families (e.g., tool-like receptor, TNF, lectin, complement, interleukin, chemokine, MHC, B and T cells,
INTRODUCTION

Barramundi (Lates calcarifer), also known as Asian seabass, is an important tropical aquaculture food fish and a species that is receiving increased global attention due to its good attributes for aquaculture and consumer attractiveness (Jerry, 2013). As the barramundi industry grows and production intensifies, commercial farmers have seen the occurrence of several emerging viral and bacterial diseases that affects the survival of farmed stocks (Gibson-Kueh et al., 2012; de Groof et al., 2015; Dong et al., 2017a,b; Chang et al., 2018; Chen et al., 2019; Girisha et al., 2020). Emerging diseases are a particular threat to intensive barramundi aquaculture, where fish are directly exposed to the natural environment. In these open systems, it is impractical, due to the unavailability of efficacious commercial vaccines.

Scale drop disease virus (SDDV), a novel Megalocytivirus of the Iridoviridae family, has had devastating consequences on the production of barramundi in Southeast Asia, particularly during early juvenile phases (~50–500 g) reared in sea cages. SDDV is often associated with mortalities ranging from 40 to 50% of cultured stock (Gibson-Kueh et al., 2012; de Groof et al., 2015; Senapin et al., 2019). While initial reports of the disease were from farms in Singapore (Gibson-Kueh et al., 2012; de Groof et al., 2015), SDDV has been reported in other Southeast Asian producing countries, such as Malaysia (Nuriliana et al., 2020), Thailand (Senapin et al., 2019), and Indonesia (de Groof et al., 2015). SDDV was also recently reported in barramundi farmed in freshwater ponds in Thailand, suggesting this disease is not confined to marine aquaculture (Kerddee et al., 2020). Clinically affected fish show darkened bodies, scale loss, fin and tail erosion, and occasionally, cloudy eyes and red bellies (Gibson-Kueh et al., 2012; de Groof et al., 2015; Senapin et al., 2019; Nuriliana et al., 2020). Many of these gross abnormalities observed are similar to that caused by opportunistic bacterial infections, such that SDDV was initially misdiagnosed as tenacibaculosis (Gibson-Kueh et al., 2012). AFFECTED fish show systemic vasculitis and resulting tissue necrosis in all major organs, particularly in the spleen and kidney (Gibson-Kueh et al., 2012; Senapin et al., 2019).

Other emerging pathogens in cultured barramundi include L. calcarifer herpes virus (LCHV) (Chang et al., 2018; Meemetta et al., 2020), infectious spleen and kidney necrosis virus (ISKNV) (Dong et al., 2017a), Vibrio harveyi causing scale drop and muscle necrosis (Vh-SDMND) (Dong et al., 2017b), L. calcarifer birnavirus (LCBV) (Chen et al., 2019), and red sea bream iridovirus (RSIV) (Girisha et al., 2020). SDDV and LCHV infections in barramundi reportedly caused similar gross signs of scale loss (Chang et al., 2018), reiterating the need to use more specific diagnostic tests. PCR-based diagnostic methods have been established for these pathogens (Gias et al., 2011; de Groof et al., 2015; Meemetta et al., 2020; Srisan et al., 2020). However, epidemiological studies characterizing the prevalence of these pathogens in farming sites have not yet been carried out. In fact, co-infections, rather than a single pathogen are commonly responsible for mortalities and production losses (Dong et al., 2015; Bastos Gomes et al., 2019; Nguyen et al., 2019; Kerddee et al., 2020). In some cases, such interactions might change how the host responds to a secondary infection in a counterintuitive manner, as for instance persistently nervous necrosis virus (NNV)-infected barramundi were shown to exhibit resistance to RSIV coinfection (Wu et al., 2013).

Recent genomic studies in barramundi have resulted in the development of linkage maps (Wang et al., 2015a, 2015b), molecular markers (Zhu et al., 2006a; Wang et al., 2015b), transcriptomes (Xia et al., 2011, 2013; Thevasagayam et al., 2015), and whole genome assemblies (Domingos et al., 2015; Vij et al., 2016). These studies have contributed to a better understanding of the biology of wild barramundi (Zhu et al., 2006b; Yue et al., 2009; Loughnan et al., 2019) and in aquaculture production (Domingos et al., 2013, 2014a,b, 2018, 2021; Loughnan et al., 2013, 2016; Ravi et al., 2014; Ngoh et al., 2015; Wang et al., 2015b). A number of studies have employed “omics” approaches targeted to better understand molecular pathways and genes involved in L. calcarifer’s adaptive stress response (Newton et al., 2013; Hook et al., 2017; Ma et al., 2020; Vij et al., 2020). Other studies investigated immune functions (Xia and Yue, 2010; Xia et al., 2011, 2013; Jiang et al., 2014; Liu et al., 2016), QTLs (Wang et al., 2015b) and disease resistance genes (Fu et al., 2013, 2014; Sun et al., 2020). Laboratory challenge trials have been carried out to understand the genetic basis of immune response and survival in barramundi associated with V. harveyi (Fu et al., 2013; Xia et al., 2011, 2013), Photobacterium damselae (Fu et al., 2013), Streptococcus iniae (Jiang et al., 2014), iridovirus (Wang et al., 2017b; Sun et al., 2020), and NNV (Liu et al., 2016). Transcriptome analyses based on RNAseq have proven to be a powerful tool to understand pathogenicity and fish immunity (Sudhagar et al., 2018).

To better understand recurring mortalities experienced during the early phases of sea cage culture of barramundi, this study investigated the transcriptional changes and associated histopathology in fish sampled during a major disease outbreak event. Tissues of clinically healthy and sick fish were collected, and histological analyses, qPCR, and PCR for significant
pathogens and RNAseq methodologies were carried out. Our results showed significant changes in pathogen loads of SDDV but not LCHV, pathology, and differential gene expression between clinically healthy and sick barramundi.

**MATERIALS AND METHODS**

**Sample Collection**

Juvenile barramundi were collected in a sea cage with a history of recent mortalities from a commercial farm in Singapore, in June 2019. Eight apparently diseased fish (hereafter termed “sick” fish—length, 26.5 ± 3.0 cm; weight, 231.3 ± 67.4 g) displaying lethargy (slow swimming at the surface), fin and body rot, and scale loss (or which scales were easily removed) were sampled for tissue collection, along with eight clinically healthy fish (hereafter termed “healthy”—length, 28.0 ± 2.5 cm; weight, 290.1 ± 71.6 g) exhibiting active swimming patterns, smooth body and skin, and clear eyes. Fish were euthanized in buckets containing 15 L of seawater and 15 ml of 10% clove oil (100 ppm), and immediately dissected after decapitation to collect the kidney, muscle, spleen, and liver (~0.5 cm³). Tissues were subsampled and preserved either in RNAlater (Ambion, Austin, TX, United States) for RNA sequencing and pathogen screening by PCR and/or qPCR or 10% phosphate-buffered formalin for histology. Tissue samples in formalin later were kept on ice and transferred to a −20°C freezer on the same day for storage until processed for analyses. Formalin-fixed tissues were processed into 5 µm hematoxylin and eosiin (H&E)-stained tissue sections at the Institute of Molecular and Cell Biology (IMCB) histology laboratory in Singapore. H&E-stained tissue sections were viewed under bright field microscopy, and images were captured using the Olympus Research Microscope BX53, Digital Camera DP74, and CellSens™ Standard Imaging System (Olympus Corporation, Tokyo, Japan).

**Screening of Five Putative Fish Pathogens by PCR Methods**

The presence and load of pathogens in fish was identified via PCR/qPCR methods (Table 1). Firstly, genomic DNA was extracted from kidney and liver tissues of healthy (n = 8) and sick (n = 8) barramundi using a conventional phenol/chloroform and ethanol precipitation method. From this extract, 200 ng of DNA template was then used in each PCR reaction. Along with all tests for the target pathogen, amplification of the cytomegocoxidase gene (COI) was included (Ivanova et al., 2007) to ensure quality of the DNA template. PCR diagnosis were conducted for three viral pathogens namely SDDV, LCHV, *Megalocytivirus*, and two bacterial pathogens *Tenacibaculum maritimum* and *Vibrio harveyi* causing scale drop and muscle necrosis (Vh-SDMND), as per Table 1.

Copy numbers of SDDV and LCHV were calculated from respective standard graphs generated by qPCR amplifications of serially diluted plasmid containing corresponding viral insert target as previously described (Meemetta et al., 2020; Sriisan et al., 2020).

**Table 1** Summary of molecular tests employed to detect and/or quantify pathogens present in kidney and liver of barramundi.

| Pathogen | Method | Target | Reaction | Cycling conditions | Positive controls | References |
|----------|--------|--------|----------|-------------------|------------------|------------|
| Scale drop disease virus (SDDV) | SYBR qPCR | SDDV ATPase gene | A 20-µl qPCR reaction contained the DNA template, 150 nM of each primer and 2 x KAPA SYBR FAST master mix (Kapa Biosystems, Inc., Wilmington, WA, United States) | 95°C for 3 min and 40 cycles of 95°C for 3 s and 63°C for 30 s followed by melt curve analysis | DNA from SDDV-infected barramundi | Sriisan et al., 2020 |
| Late caecal enteritis virus (LCHV) | SYBR qPCR | LCHV major envelop protein gene | A 20-µl qPCR reaction contained the DNA template, 200 nM of each primer and 1x Taq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) | 95°C for 10 min and 40 cycles of 95°C for 10 s and 63°C for 30 s followed by melt curve analysis | DNA from LCHV-infected barramundi | Meemetta et al., 2020 |
| Megalocytivirus | Single PCR | Megalocytivirus major capsid protein gene | A 25-µl PCR reaction contained the DNA template, 200 nM of each primer, 200 µM dNTP, 1.25 units of Taq DNA polymerase enzyme (RBC Bioscience, New Taipei City, Taiwan) and 1 x supplied buffer | 94°C for 5 min and 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min | DNA from Megalocytivirus-infected Asian sea bass (Dong et al., 2017a) | Glass et al., 2011 |
| *Vibrio harveyi* causing scale drop and muscle necrosis (Vh-SDMND) | Duplex PCR | Vh-SDMND hypothetical protein gene and SDDV ATPase gene | A 25-µl PCR reaction contained the DNA template, 200 nM of each primer, and 1 x AccuStart II GelTrack PCR SuperMix (Quantabio, Beverly, MA, United States) | 94°C for 3 min and 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and an extension step at 72°C for 5 min | Plasmid harboring dual targets for both Vh-SDMND and SDDV | Taengphu et al., unpublished |
| *Tenacibaculum maritimum* | Single PCR | 16S rDNA gene | A 20-µl PCR reaction contained the DNA template, 200 nM of each primer, 2 units of Taq polymerase (PCR Biosystems), and 1 x supplied buffer | 94°C for 1 min and 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and an extension step at 72°C for 5 min | Plasmid containing the T. maritimum insert target | Toyama et al., 1996 |
Statistical Analyses
Assessment of statistical differences between sick and healthy fish viral loads in kidney and liver was performed with a Mann-Whitney U-test at a significance level of 0.05. The association between the viral loads and the observed histopathological scores (hepatic reserves, lipodrosis, glomerulonephritis, and splenitis) was explored using PCA with Spearman rank metrics in Xlstat® software (Addinsoft, Paris, France). The original set of eight variables of interest was reduced into two components of eigenvalues of 4.72 and 1.61 and represented as a two-dimensional plot.

RNA Extraction, Library Preparation, and Sequencing
Total RNA was extracted from three immune competent tissues (liver, spleen, and kidney) and muscle from all 16 fish using an RNeasy® Mini kit (Qiagen 74104, Qiagen, Frankfurt, Germany). All RNA samples were treated with RNase free DNase-I (M610A, Promega, Madison, WI, United States) to remove genomic DNA contamination. The quality and quantity of the total RNA was determined with an Agilent 2100 Bioanalyzer (RNA 6000 Nano Chip Assay, Agilent, Böblingen, Germany) and a Qubit 3.0 (Quant-It dsRNA BR Assay, ThermoFisher Scientific, Waltham, MA, United States).

For the cDNA library preparations, 1 μg RNA was used as an input material for each sample. VAHTS mRNA-seq V3 Library Prep Kit for Illumina (NR611, Vazyme; San Diego, CA, United States) was used to generate sequencing libraries. In brief, mRNA with poly(A) was enriched by mRNA Capture Beads and fragmented by heating. Short mRNA was reverse-transcribed with random hexamer primers to generate the first cDNA, and then the second cDNA was synthesized. The cDNA fragments then went through an end repair process, the addition of a single “A” base to the 3′ end and then ligation of the adapters. The products were then purified and size selected (350 bp range). At the end, fragments were enriched by PCR and purified using VAHTSTM DNA Clean Beads. The quality and quantity of PCR product was determined by the Agilent Bioanalyzer 2100 and Qubit 2.0 (ThermoFisher). Finally, sequencing was undertaken on an Illumina Novaseq 6000 platform generating 150 bp paired-end reads.

RNA-Seq Data Mapping, Gene Differential Expression, and Enrichment Analysis
To obtain high-quality clean data for downstream analyses, raw reads of FASTQ format were firstly processed through in-house perl scripts. In this step, the low-quality reads and reads containing adaptors or poly-N were removed. At the same time, Q20, Q30, GC content, and sequence duplication level of the clean data were calculated.

Reference genome and gene model annotation files of *L. calcarifer* were downloaded from the NCBI1 genome website directly. Index of the reference genome was built using Bowtie2 (Langmead and Salzberg, 2012), and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 (Kim et al., 2013). The process of genetic quantification of gene expression level was carried out by HTSeq v0.6.1 by counting the read numbers mapped to each gene (Anders et al., 2015), and then the expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Differential expression statistical analysis between organs from sick and healthy fish was performed using the DESeq R package (1.18.0) (Wang et al., 2010). The resulting P-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false-discovery rate. Genes with an adjusted P-value < 0.05 found by DESeq were assigned as the threshold for indicating significantly differential expression. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected (Young et al., 2010). GO terms with corrected P-values < 0.05 were considered significantly enriched by differentially expressed genes. KOBAS software (Mao et al., 2005) was utilized to test the statistical enrichment of those differential expression genes in KEGG2 pathways.

RESULTS
Pathogen Screening Using PCR and qPCR
While histopathology strongly suggested a primary infection with scale drop virus in both sick and clinically healthy fish, qualitative molecular tests (PCR) were carried out to rule out other possible concurrent infections that could cause the scale loss observed (Vh-SDMDN, *T. maritimum*), or other viral diseases that could cause the occasional inclusion bodies observed in renal glomeruli or connective tissues within skeletal muscles in sick fish (*Megalocyttivirus*). Based on PCR, liver and kidney samples returned negative for the presence of *Megalocyttivirus*, Vh-SDMDN, and *T. maritimum* in both sick and healthy fish, while all fish sampled were positive for SDDV and LCHV, with exception of two sick fish liver samples (out of seven) (Supplementary Table 1 and Supplementary Figure 1). SDDV and LCHV loads in the healthy fish ranged from 1 to 1,853 copies and 17 to 475 copies/qPCR reaction, respectively. SDDV and LCHV loads were higher in the sick fish, from 131 to 22,549 and 0 to 4,045 copies/qPCR reaction, respectively (Supplementary Table 2 and Figure 1A). SDDV overall loads were seven times higher than those for LCHV. SDDV loads in kidney and liver of sick fish were 8.4 and 3.6 times higher than those of healthy fish (Figure 1A). However, differences in loads between the two groups were not statistically significant (P > 0.05). The kidneys of sick fish had 3.6 times higher LCHV loads than those of healthy fish. Comparatively, LCHV loads in the liver were one magnitude lower than those in the kidney and two orders of magnitude lower than that of SDDV (Figure 1A).

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1https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Lates_calcarifer/100/

2http://www.genome.jp/kegg/
Domingos et al. Barramundi SDDV and LCHV Co-infection

FIGURE 1 | (A) Viral copy number (mean + SD) determined by qPCR assays for SDDV and LCHV of samples derived from kidney and liver (n = 8, except for liver of the sick fish, where n = 7). (B) Distribution of samples according to histopathology scores of liver (hepatic reserves and lipidosis), spleen (splenitis), and kidney (glomerulonephritis) of apparently healthy (H, n = 8) and sick (S, n = 8) barramundi.

right). Amplicons from selected positive test samples (marked with an asterisk in Supplementary Table 1) were subjected to DNA sequence analysis and found to show one (99.3%) to zero nucleotide change (100% identity) among the SDDV product sequences. In contrast, two (97.85%) to no nucleotide differences (100% identity) were observed among the LCHV product sequences (Supplementary Figure 2).

Histopathology

Raw data on individual viral loads and histopathology scores are presented in Supplementary Table 2. Overall, clinically sick fish showed a greater degree of abnormalities, often related to inflammatory processes (Figure 1B). The wispy cytoplasmic appearance of H&E-stained livers with good hepatocellular reserves in healthy fish, differs from the more homogenous, basophilic cytoplasm in sick fish, with depleted hepatic reserves (Figures 2A,B). Moderate (score 2) to good (score 3) hepatocellular lipid and glycogen reserves were observed in most of the livers of clinically healthy barramundi (Figures 1B, 2A), in contrast to low hepatic reserves observed in all sick fish (Figures 1B, 2B). Moderate to severe, accumulation of large, round, lipid vacuoles or macrovesicles in liver cells (lipidosis), was observed in most sick barramundi (Figures 1B, 2C). Lipidosis is either not observed or mild in healthy fish, with the exception of one individual which had the highest SDDV loads within this group (Figure 1A and Supplementary Table 2).

Moderate to severe splenic inflammation (splenitis) were observed in both healthy and sick fish (Figure 1B). Moderate splenitis is characterized by multifocal necrosis (Figure 2D), while severe splenitis has multifocal to coalescing necrosis, affecting extensive areas of the spleen. Spleens with multifocal infarcts, or tissue deaths (necrosis), had occluded blood vessels, due to marked inflammatory response in the endothelium (obliterative endarteritis) (Figure 2D). While there was splenitis in a significant number of both clinically healthy and sick fish, moderate to severe kidney inflammation (glomerulonephritis) was observed only in all sick fish. The kidneys of diseased barramundi showed glomerular necrosis, protein effusion, and mixed infiltration of macrophages and lymphocytes into the Bowman’s space (protein loosing, necrotizing glomerulonephritis) (Figures 2E,F).

Mild multifocal interstitial myositis was observed in both healthy (H02, H04, and H05) and sick fish (S07), and occasionally in association with the presence of inclusion bodies (Figure 2H).
Moderate, diffuse dermatitis was observed where skin was intact in tissue sections examined, in both healthy (H01, H02, H04, and H06) and sick fish (S02 and S04). In addition, severe thinning of epidermis was observed in the sick fish S04, lymphocytic-monocytic infiltration surrounding dermal blood vessels (perivasculitis) and occluded dermal blood vessels with obliterator endarteritis was observed in sick fish S06 (Figure 2I).

Two principal components derived from PCA explained 70.3% of the variance among SDDV and LCHV viral loads and histopathological findings (Figure 3). A positive association was observed between SDDV loads (both in the liver and in the kidney) and the major negative histopathological findings (glomerulonephritis, splenitis, and lipidosis) (component F1 > 2), which were inversely related to lipid score (component F1 < –2). In contrast, the PCA revealed no direct association of LCHV loads with any of the histopathological findings (and neither with SDDV loads). Although all healthy individuals were positive for both viruses (albeit with lower loads than sick individuals) and did present some of the histopathological alterations (most notably on the spleen, Figure 1B), the PCA revealed a marked difference in grouping of sick (F1 > 0) and healthy (F1 < 0) fish, with exception of S08 and H05 individuals, which respectively had the lowest and the highest SDDV loads within their groups (Supplementary Table 2).

Severe RNA Degradation Observed in Spleen and Kidney of Sick Fish
Major differences were observed in the quality of RNA extracted from spleen and kidney of healthy vs. sick fish and between different organs in sick fish, as assessed with an Agilent 2100 Bioanalyzer (Figure 4). RNA integrity number (RIN, mean ± S.D.) for extracts from spleen and kidney of sick fish were 2.50 ± 0.55 and 3.39 ± 1.15, respectively. This contrasts with RIN of 9.93 ± 0.09 for liver, and 8.86 ± 1.03 for muscle RNA extracts for sick fish, and 9.37 ± 0.11 for RNA derived from all organs of healthy fish. As RNA in spleen and kidney samples of sick fish were degraded and did not pass quality
control for sequencing, only liver and muscle tissues were used for transcriptomic comparisons between the two groups.

Liver and Muscle RNA Sequencing, Mapping, and Analysis of Differentially Expressed Genes

RNA sequencing performed on eight sick and eight healthy fish generated a total of 1,426.10 and 1,831.68 million clean reads, from the liver and muscle tissues, respectively. An average of 101.80 million clean reads (ranging from 82.48 to 167.31 million) was obtained from each sample. The Q30 (i.e., probability of an incorrect base call of 1 in 1,000) and GC percentages of the reads were 92.84 and 50.08%, respectively. All the filtered clean reads were mapped individually against the annotated genome of *L. calcarifer*. In total, 2,504.23 million reads were successfully mapped, with approximately 2,306.56 million reads (92.1%) uniquely mapped (i.e., mapped to a single locus in the genome). Detailed sequencing and mapping results are summarized in Supplementary Table 3. RNAseq data (32 transcriptomes) have been deposited in NCBI under the accession number PRJNA713978.

To identify gene expression changes between liver (or between muscle) of sick and healthy fish, the FPKM method was used to calculate the expression levels of genes (Figure 5). For liver tissues, the average Pearson correlation coefficient ($R^2$ values) for gene expression values was 0.92 (0.89–0.97) in healthy fish and 0.95 (0.93–0.98) in sick fish, indicating the high repeatability of biological replicates. For muscle tissues, the average $R^2$ values was 0.82 (0.71–0.94) in healthy fish and 0.80 (0.51–0.95) in sick fish, which suggested less similarity in the gene expression patterns of muscle relative to liver between sick and healthy fish. Given the high number of biological samples randomly sampled within each group (n = 8) for a transcriptomic study, but still limited in relation to the farmed population subjected to the disease outbreak, all samples were utilized in the analysis. Comparison between liver of two groups (sick vs. healthy fish) revealed 2,810 significantly differentially expressed genes (DEGs). Among them, 1,083 were upregulated and 1,727 were downregulated in the liver of sick fish (Figure 5 and Supplementary Table 4). In the muscle, a total of 556 significantly DEGs were discovered, with 144 genes upregulated and the rest downregulated in sick fish (Figure 5 and Supplementary Table 4). To illustrate the DEGs detected in sick and healthy fish, heatmaps were generated for both liver and muscle separately (Supplementary Figures 3A,B). The transcriptomic profile in the livers of healthy fish was obviously different from that of the sick fish, with all eight samples from each group contained within two main clusters (Supplementary Figure 3A). Whereas in muscle, the expression pattern of the DEGs showed a less distinct clustering between
FIGURE 4 | Box plots of RNA integrity number (RIN) of total RNA extracted from liver (L), kidney (K), spleen (S), and muscle (M) tissues of eight “sick” and eight “healthy” juvenile barramundi from a sea cage farm, as determined with an Agilent 2100 Bioanalyzer RNA 6000. Nanochip assay with representative gel images (top) and electropherogram (bottom) of total RNA extracts of sick and healthy fish, indicated severe degradation in RNA extracted from kidneys and spleens of sick fish.

the two groups compared with that of liver, with some sample overlap between muscle of sick and healthy animals between the two main clusters (Supplementary Figure 3B), probably because muscle is not an immune-related organ like liver and is thus less affected by the disease. About 32% of the DEGs found in the muscle (n = 176) were also differentially expressed in the liver, whereby a 97% in concordance between up- or downregulation was observed. In terms of gene ontology, DEGs in both tissues were primarily classified within “cellular component,” followed by “biological processes” and then “molecular function” (Supplementary Figure 4).

The top 20 significantly up- or downregulated DEGs identified in liver and muscle are listed in Tables 2, 3, respectively.

A number of these genes were related to inflammatory and immune responses of juvenile barramundi to viral infections. Upregulated gene expression in liver of sick fish included interleukin-1 receptor type 2-like (il-1r2l), cytokine-like protein 1 (cytl1), toll-like receptor 13 (tlr13), hepatitis A virus cellular receptor 1 homolog (havcr-1), and leucine-rich repeat-containing protein 52-like (lrrc52), whereas the complement C1q-like protein 4 (c1ql4), complement C1q tumor necrosis factor-related protein 1-like (c1r-p1), phospholipase A2-like (pla2s), and retinol-binding protein 2 (rbp2) were downregulated (Table 2).

In muscle of sick fish, vital components of innate immunity, including toll-like receptor 13 (tlr13) and toll-like receptor 5 (tlr5), Fc receptor-like protein 5 (fcrl5), and interleukin-1
FIGURE 5 | Volcano plots of differently expressed genes (DEGs; numbers up- or downregulated in brackets) in liver and muscle of sick vs. healthy barramundi. x-axis represents \( \log_2 \)-transformed fold change and the y-axis indicates \( -\log_{10} \)-transformed adjusted significance. Each dot indicates an individual gene that was significantly upregulated (red), downregulated (green), and non-differentially regulated (blue). The horizontal line represents adjusted \( P \)-value (\( P < 0.05 \) cutoff).

receptor type 2-like (il-1r2) were significantly upregulated. In contrast, interferon-induced protein 44-like (ifi44l), E3 ubiquitin-protein ligase TRIM21-like (trim21), complement C1q tumor necrosis factor-related protein 1-like (ctrp1), golgi-associated plant pathogenesis-related protein 1-like (gapr-1), and G protein-coupled receptor 17 (gpr17), associated to immune response were downregulated in the muscle of sick fish (Table 3).

Immune-Relevant Pathways Identified in Liver and Muscle of Farmed Barramundi

Based on the DEG findings in sick vs. healthy fish, we performed KEGG pathway classification and functional enrichment analysis. Liver DEGs were classified into a total of 141 pathways, whereby 42 pathways showed statistical significance (corrected \( P \)-value < 0.05) (Supplementary Table 6). Muscle DEGs were classified into 69 pathways, whereby four pathways were statistically significant (corrected \( P \)-value < 0.05) (Supplementary Table 7). The top 20 enriched KEGG pathways in the liver and muscle in relation to their rich factor (i.e., the ratio of DEG numbers to all gene numbers annotated in a pathway) are shown in Supplementary Figures 5, 6, respectively.

Furthermore, a total of 27 pathways relevant to innate and adaptive immunity function were identified based on 525 DEGs in the liver, whereby 10 pathways were significantly enriched. Out of those 27 immune-related pathways, 22 were also shared by 127 DEGs in the muscle, whereby four of those pathways were significantly enriched (Table 4). Based on the significance value, the phagosome was the most enriched pathway in the liver, whereas the shared ECM-receptor interaction was the most enriched pathway in the muscle. Most of these immune relevant pathways (16) were classified under environmental information processing, including cytokine-cytokine receptor interaction, calcium signaling pathway, ECM-receptor interaction, cell adhesion molecules (CAMs), neuroactive ligand-receptor interaction, and MAPK signaling pathways. According to the KEGG organismal immune system, five pathways were identified: intestinal immune network for IgA production, Toll-like receptor, cytosolic DNA-sensing, NOD-like receptor, and RIG-I-like receptor signaling pathways. In addition, DEGs were also classified under the broader pathways of endocrine and metabolic disease, infectious diseases (viral and bacterial), cell growth and death, and xenobiotics biodegradation and metabolism (Table 4).

Key DEGs Related to Immune Responses Are Mostly Downregulated in Liver and Muscle of Sick Barramundi

DEGs associated to immune-relevant pathways are listed in Supplementary Table 8 (liver) and Supplementary Table 7 (muscle), whereas DEGs, further classified by immune relevant gene families, are presented in Table 5. About 65 and 89% of these DEGs were downregulated in the liver and in the muscle, respectively. Most genes in Table 5 are related to innate immunity: pattern recognition receptors (PRRs) including
TABLE 2 | The top 20 significantly up- or downregulated genes in liver of sick barramundi.

| Gene ID/name | Log2 fold change | P (adj) | Up/downregulation | Gene description |
|--------------|------------------|---------|-------------------|------------------|
| LOC108880246 | 9.21             | 1.44E-09| Up                | Excitatory amino acid transporter 5-like |
| LOC108884404 | 7.94             | 1.26E-06| Up                | Fibroblast growth factor 21 |
| slc7a11      | 7.94             | 1.03E-06| Up                | Solute carrier family 7 member 11 |
| LOC108897018 | 7.74             | 3.82E-20| Up                | Interleukin-1 receptor type 2-like |
| LOC108893249 | 7.20             | 0.00550 | Up                | Cytokine-like protein 1 |
| LOC108874878 | 7.17             | 6.74E-09| Up                | Solute carrier family 1 member 8b |
| LOC108885470 | 6.95             | 3.63E-07| Up                | Hepatitis A virus cellular receptor 1 homolog |
| LOC108879612 | 6.74             | 1.95E-05| Up                | Insulin-like growth factor binding protein 1a |
| LOC108879014 | 6.55             | 0.00090 | Up                | Leucine-rich repeat-containing protein 52-like |
| LOC108897016 | 5.91             | 6.64E-05| Up                | Interleukin-1 receptor type 2-like |
| LOC108887824 | 5.80             | 0.02700 | Up                | Hepcidin-like |
| LOC108885100 | 5.80             | 5.26E-24| Up                | Toll-like receptor 13 |
| LOC108902039 | 5.74             | 0.00060 | Up                | Polycystic kidney disease 1b |
| LOC108890793 | 5.74             | 0.00560 | Up                | IgGFe-binding protein-like |
| LOC108874726 | 5.72             | 1.68E-17| Up                | Neoverrucotoxin subunit alpha-like |
| LOC108895006 | 5.60             | 6.07E-09| Up                | Cytosolic sulfotransferase 3-like |
| nim1k         | 5.57             | 3.70E-07| Up                | NIM1 serine/threonine protein kinase |
| LOC108886837 | 5.54             | 1.27E-09| Up                | Protein NDRG1-like |
| Tub           | 5.53             | 0.00389 | Up                | TUB bipartite transcription factor |
| LOC108902348 | 5.50             | 3.15E-10| Up                | Ladderlectin-like |
| LOC108891007 | –6.21            | 9.61E-09| Down              | Complement C1q-like protein 4 |
| LOC108890805 | –6.25            | 0.01456 | Down              | Protein jagged-1-a-like |
| LOC108881856 | –6.28            | 3.94E-10| Down              | P17/29C-like protein DDB_G0287399 |
| abcg4         | –6.34            | 0.00404 | Down              | ATP-binding cassette, subfamily G (WHITE), member 4a |
| pacr5         | –6.41            | 0.03062 | Down              | Progestin and adipQ receptor family member Vb |
| LOC108891005 | –6.45            | 4.60E-09| Down              | Complement C1q-like protein 4 |
| LOC108878005 | –6.49            | 0.04627 | Down              | Neuropeptide Y receptor Ybb |
| LOC108887476 | –6.54            | 1.73E-10| Down              | Cytochrome P450 2K1-like |
| LOC108879156 | –6.70            | 1.65E-15| Down              | Granzyme E-like |
| LOC108893889 | –6.84            | 8.61E-11| Down              | High choriolytic enzyme 1-like |
| LOC108890633 | –6.85            | 3.83E-14| Down              | Solute carrier family 12 member 3-like |
| LOC10882788  | –6.97            | 3.75E-05| Down              | Glutamate receptor, ionotropic, AMPA 1a |
| LOC108899398 | –6.98            | 1.96E-07| Down              | Fatty acid binding protein 10a, liver basic |
| LOC108881836 | –7.07            | 0.00086 | Down              | Semaphorin 5A |
| LOC108878838 | –7.63            | 2.81E-08| Down              | Chymotrypsin-like elastase family member 3B |
| LOC108880291 | –7.88            | 7.82E-09| Down              | Phospholipase A2-like |
| LOC108890739 | –7.91            | 4.23E-17| Down              | Complement C1q tumor necrosis factor-related protein 1-like |
| LOC108876188 | –8.35            | 0.01616 | Down              | Mdm1-interacting protein 1-B-like |
| LOC108899606 | –9.42            | 0.01790 | Down              | L-Rhamnose-binding lectin SML-like |
| rbp2          | –9.53            | 0.00918 | Down              | Retinol-binding protein 2 |

Given in descending order of log2 fold change.

Toll-like receptors (tlr5, tlr13) and C-type lectin receptors (cl-11l, colec12, and selp); inflammatory cytokines and receptors including interleukins (il-1β, ilr4, and illa1a) and interleukin receptors (ill13r1, ill17r, and ill1r1l), TNF including (tnfsf11a, tnfap3, tnsf10l, and tnsf12); chemokines (ccl4, ccl25b, and c-x-c12a) and chemokines receptors (ccr7, ccr3l, crc6b, and ackr3b); complement factors (C3l and Clq); collagen (col4a5, col6a6, col6a3, col1a1a, col1a1b, and col2a1b); and myD88 as innate immune signal transduction adaptor CD molecules. In addition, gene families related to the adaptive immune response, identified only in the liver, such as B cell receptor CD22-like (down), and T cell tcrig1 (up) and nfatc1 (down), and major histocompatibility complex (MHC) such as h2-aa, h2-eb1, and hla-dap1 were significantly downregulated (except for tcrig1) in sick barramundi.

**DISCUSSION**

Barramundi farming in Southeast Asia has been severely affected by disease outbreaks. In Singapore, where this particular study has taken place, farmers have reported mass mortalities of...
TABLE 3 | The top 20 significantly up- or downregulated genes in muscle of sick barramundi.

| Gene ID/name | Log₂ fold change | P (adj) | Up/downregulation | Gene description |
|--------------|------------------|--------|-------------------|-----------------|
| LOC108885100| 9.14             | 9.88E-10| Up                | Toll-like receptor 13 |
| acd1         | 8.21             | 0.01243| Up                | Aconitate decarboxylase 1 |
| LOC108874726| 7.77             | 1.16E-06| Up                | Neovaccinocucin subunit alpha-like |
| donson       | 7.55             | 0.00698| Up                | DNA replication fork stabilization factor DONSON |
| LOC108873250| 7.52             | 0.00011| Up                | Granulocyte colony-stimulating factor-like |
| LOC108889912| 7.50             | 0.00171| Up                | Toll-like receptor 5 |
| chrng        | 7.40             | 0.02103| Up                | Cholinergic receptor, nicotinic, gamma |
| LOC108880557| 7.35             | 0.00275| Up                | Fc receptor-like protein 5 |
| LOC108893722| 7.15             | 0.00854| Up                | Mucin-SAC-like |
| LOC108897018| 7.04             | 0.00344| Up                | Interleukin-1 receptor type 2-like |
| LOC108884001| 7.01             | 0.01199| Up                | Protein OSCP1-like |
| LOC108890609| 6.55             | 0.00172| Up                | Hydroxycarboxylic acid receptor 2-like |
| LOC108880702| 6.54             | 0.00167| Up                | Carcinoembryonic antigen-related cell adhesion molecule 20-like |
| LOC108894065| 6.29             | 0.02952| Up                | Matrix metallopeptidase 13a |
| gadi1        | 6.09             | 1.56E-06| Up                | Glutamate decarboxylase like 1 |
| LOC10884332| 5.74             | 0.00011| Up                | Acyl-coenzyme A thioesterase 2, mitochondrial-like |
| irriq1       | 5.72             | 0.01210| Up                | Leucine-rich repeats and IQ motif containing 1 |
| LOC108886122| 5.54             | 1.40E-06| Up                | Arginase 1 |
| LOC108883738| 5.433            | 0.00090| Up                | Carboxypeptidase N subunit 2 |
| alpl         | 5.335            | 5.08E-05| Up                | Alkaline phosphatase, biomineralization associated |
| lh8          | –6.40            | 0.04081| Down              | LIM homeobox 8 |
| fosex1       | –6.42            | 0.00211| Down              | Forkhead box E1 |
| cd248        | –6.50            | 0.00630| Down              | CD248 molecule |
| dbx6         | –6.52            | 0.03813| Down              | Distal-less homeobox 6a |
| LOC108900437| –6.55            | 0.00378| Down              | Tissue alpha-L-fucosidase-like |
| LOC108990438| –6.61            | 0.00038| Down              | Nuclear factor 7, ovary-like |
| folh1b       | –6.67            | 0.00124| Down              | Folate hydrolase 1B |
| LOC108877493| –6.87            | 0.00073| Down              | EF-hand and coiled-coil domain-containing protein 1-like |
| LOC108892256| –6.95            | 0.00378| Down              | Dickkopf-related protein 2-like |
| LOC108890633| –6.97            | 0.02433| Down              | Solute carrier family 12 member 3-like |
| LOC108893465| –7.23            | 0.00627| Down              | von Willebrand factor A domain-containing protein 7-like |
| LOC108874532| –7.23            | 0.00811| Down              | Interferon-induced protein 44-like |
| gop17        | –7.35            | 0.00062| Down              | G protein-coupled receptor 17 |
| LOC108896888| –7.68            | 0.00657| Down              | Growth arrest-specific 2b |
| LOC108900725| –7.71            | 0.00162| Down              | E3 ubiquitin-protein ligase TRIM21-like |
| mei1         | –8.39            | 0.03185| Down              | Meiotic double-stranded break formation protein 1 |
| LOC108896482| –8.64            | 0.02613| Down              | Secretory calcium-binding phosphoprotein 7 |
| LOC108878074| –8.92            | 0.04369| Down              | Golgi-associated plant pathogenesis-related protein 1-like |
| LOC108897956| –9.83            | 0.01199| Down              | Calpain-2 catalytic subunit-like |
| LOC108890739| –10.12           | 0.038363| Down              | Complement C1q tumor necrosis factor-related protein 1-like |

Given in descending order of log₂ fold-change.

juveniles during the transition between nursery phases (∼50 to 500 g) and the final grow out period as a recurring phenomenon over the last few years. Although several novel viral pathogens (SDDV, LCHV, LCBV) affecting barramundi have been first reported and identified in Singaporean farms (Gibson-Kueh et al., 2012; de Groof et al., 2015; Chang et al., 2018; Chen et al., 2019), their occurrence is now known to extend throughout Southeast Asia (Senapin et al., 2019; Nurliyana et al., 2020; Meemetta et al., 2020), affecting the industry as a whole. To better understand what is happening to the biology of barramundi in commercial farming conditions during one such disease outbreak, the presence of five putative pathogens was investigated using a comparative RNAseq and histological approach targeting multiple organs of affected and non-affected juveniles. This study for the first time identified a concurrent infection of SDDV and LCHV in all barramundi samples (including all those apparently healthy), while ruling out infection of Megalocytivirus ISKNV/RSIV and two bacteria, T. maritimum and Vh-SDMN, which were associated with scale drop and muscle necrosis disease events in Vietnam (Dong et al., 2017b). Although lower LCHV detection in all samples were indicative of an underlying herpes viral coinfection, LCHV presence, or loading
TABLE 4 | Distribution of the differentially expressed genes in immune-relevant pathways in sick (vs. healthy) barramundi.

| KEGG pathway                                      | Liver                  | Muscle                | Pathway ID  | Pathway subclass                        |
|---------------------------------------------------|------------------------|-----------------------|-------------|-----------------------------------------|
|                                                   | DEG Corr. P-value      | DEG Corr. P-value     |             |                                         |
| Phagosome                                         | 41 5.39E-08            | 4 *                   | Ko04145     | Transport and catabolism                |
| Cytokine-cytokine receptor interaction            | 35 3.06E-05            | 6 *                   | Ko04060     | Signaling molecules and interaction     |
| Calcium signaling pathway                         | 46 3.92E-05            | 10 *                  | Ko04020     | Signal transduction                     |
| ECM-receptor interaction                          | 21 0.00141             | 13 1.41E-07           | Ko04512     | Signaling molecules and interaction     |
| Cell adhesion molecules (CAMs)                    | 29 0.00141             | 7 *                   | Ko04514     | Signaling molecules and interaction     |
| Focal adhesion                                    | 38 0.001475            | 17 2.81E-06           | Ko04510     | Cellular community                      |
| Neuroactive ligand-receptor interaction            | 53 0.003021            | 20 0.000240           | Ko04080     | Signaling molecules and interaction     |
| AGE-RAGE signaling pathway in diabetic complications | 22 0.005143         | 4 *                   | Ko04933     | Endocrine and metabolic disease         |
| Intestinal immune network for IgA production      | 11 0.005172            | 2 *                   | Ko04672     | Immune system                          |
| Insulin signaling pathway                         | 25 0.019897            | 1 *                   | Ko04910     | Endocrine system                        |
| MAPK signaling pathway                            | 35 *                   | 13 0.033187           | Ko04010     | Signal transduction                     |
| Wnt signaling pathway                             | 18 *                   | 7 *                   | Ko04310     | Signal transduction                     |
| Herpes simplex infection                          | 21 *                   | 1 *                   | Ko05168     | Infectious diseases: Viral              |
| mTOR signaling pathway                            | 21 *                   | 4 *                   | Ko04150     | Signal transduction                     |
| Apoptosis                                         | 15 *                   | 3 *                   | Ko04210     | Cell growth and death                   |
| Lysosome                                          | 14 *                   | 3 *                   | Ko04142     | Transport and catabolism                |
| PPAR signaling pathway                            | 13 *                   | 1 *                   | Ko03320     | Endocrine system                        |
| TGF-beta signaling pathway                        | 11 *                   | 2 *                   | Ko04210     | Signal transduction                     |
| Ubiquitin mediated proteolysis                    | 10 *                   | 1 *                   | Ko04120     | Folding, sorting and degradation        |
| Toll-like receptor signaling pathway              | 10 *                   | 1 *                   | Ko04620     | Immune system                          |
| Salmonella infection                              | 10 *                   | 1 *                   | Ko05132     | Infectious disease: bacterial           |
| p35 signaling pathway                             | 6 *                    | 3 *                   | Ko04115     | Cell growth and death                   |
| Drug metabolism—other enzymes                     | 6 *                    | 0 *                   | Ko00983     | Xenobiotics biodegradation and metabolism|
| Drug metabolism—cytochrome P450                    | 6 *                    | 0 *                   | Ko00982     | Xenobiotics biodegradation and metabolism|
| Cytosolic DNA-sensing pathway                     | 2 *                    | 0 *                   | Ko04623     | Immune system                          |
| NOD-like receptor signaling pathway               | 4 *                    | 0 *                   | Ko04621     | Immune system                          |
| RIG-I-like receptor signaling pathway             | 2 *                    | 0 *                   | Ko04622     | Immune system                          |

* Corr. P-value > 0.05.

was not associated to any of the histopathological findings, or to clinical disease expression (sick/healthy groups) in the PCA. In contrast, kidney and liver SDDV loads were strongly associated with the severity of histopathological alterations observed in several organs, and sick individuals. Clinical disease, severity of pathology observed in the kidney and spleen, and viral loads support the diagnosis of primary scale drop disease, whereby apparently healthy fish were in subclinical stages while sick fish were in advanced disease stages of SDD. Furthermore, this study unveiled 2,810 and 556 differentially expressed genes in the liver and muscle respectively of sick and healthy fish, and importantly, identified immune-related pathways and genes which were predominantly downregulated in sick juveniles, thus contributing to broaden our understanding of the effects of SDD in barramundi farmed under commercial culture conditions.

The severity of tissue inflammation and necrosis in spleen and kidney of sick fish may explain why the RNA extracted from these organs were too degraded for further transcriptomic analyses. Severe and extensive tissue necrosis during the later clinical phase of SDD progression is expected to cause disruption of cellular, tissue, and organ functions. RNA is highly susceptible to degradation by reactive oxygen species (ROS), and oxidative RNA damage has been recently found to be involved in the pathogenesis of several chronic degenerative diseases (Fimognari, 2015). Barramundi spleen was the most affected organ by SDD (and where RNA was most degraded in sick fish), followed by the kidney, liver, and muscle. In addition, histological observations from apparently healthy, but subclinically infected fish indicated that spleens are likely the first organ to be compromised by SDDV. The spleen stores erythrocytes (red pulp) and lymphocytes (white pulp) (Noga, 2006). The importance of spleen in modulating barramundi immune response and the severe damage observed in this organ may somewhat explain the inability of barramundi to fight against SDD and high mortality rates observed in farmed animals. Vaccination trials in barramundi against *S. iniae* revealed that spleen (but not the kidney) responded transcriptionally at 25–29 h postchallenge to activate NFκ-B, chemokine, and toll-like receptor genes, whereby vaccinated fish had increased survival and reduced pathogen shedding (Jiang et al., 2014). Occlusion of splenic arteries from chronic obliteratorive endarteritis and subsequent multifocal splenic necrosis from infarcts are further evidence of strong non-specific innate inflammatory response and failure to control the SDDV infection. Pathology observed suggests
## Table 5: Summary of immune-relevant genes characterized from sick (vs. healthy) barramundi based on KEGG functional analysis.

| Gene family       | Gene name | Liver | Muscle |
|-------------------|-----------|-------|--------|
|                   | Gene name | Log2 fold change | Adj P | Up/downregulated | Log2 fold change | Adj P | Up/downregulated |
| Tolllike receptor | tlr5      | 2.83  | 0.012439 | Up | 7.50 | 0.00171 | Up |
|                   | tlr13     | 5.80  | 5.26E-24  | Up | 9.14 | 9.88E-10 | Up |
| Lectin            | cl-11l    | -1.33 | 0.004857  | Down | -a  | -a  | -a  |
|                   | colec12   | 2.60  | 0.00014   | Up | -a  | -a  | -a  |
| Complement        | C1q4      | -6.21 | 9.61E-09  | Down | -a  | -a  | -a  |
| CTRP              | ctrp1     | -7.91 | 4.23E-17  | Down | -10.12 | 0.038363 | Down |
| Chemokine         | ccl4      | -3.15 | 0.000577  | Down | -a  | -a  | -a  |
|                   | cct25b    | 0.92  | 0.04234   | Up | -a  | -a  | -a  |
|                   | c-x-cl12a | 0.99  | 0.031141  | Up | -a  | -a  | -a  |
| Interleukin       | il-1β     | 3.91  | 5.09E-06  | Up | -a  | -a  | -a  |
|                   | irak4     | 1.24  | 0.007064  | Up | -a  | -a  | -a  |
|                   | il11a     | 3.08  | 0.000064  | Up | -a  | -a  | -a  |
|                   | il13ra1   | 1.75  | 0.000044  | Up | -a  | -a  | -a  |
|                   | il1r1l    | 1.02  | 0.031425  | Up | -a  | -a  | -a  |
|                   | il17ra    | -1.33 | 0.031206  | Down | -a  | -a  | -a  |
|                   | il7r-αl   | -1.37 | 0.002399  | Down | -a  | -a  | -a  |
| Tumor necrosis    | tnfsf11a  | 1.89  | 0.013310  | Up | -a  | -a  | -a  |
| factor (TNF)      |          |       |         |     |     |     |     |
|                   | tnfap3    | 1.78  | 0.000031  | Up | -a  | -a  | -a  |
|                   | tnfsf10l  | -2.05 | 0.003045  | Down | -a  | -a  | -a  |
|                   | tnfsf12   | -2.54 | 0.000000  | Down | -3.86 | 0.002753 | Down |

(Continued)
| Gene family | Gene name | Liver | Muscle |
|-------------|-----------|-------|--------|
| Collagen    | col4a5    | Log2 fold change | 1.87E-08 | Log2 fold change |
|             |           | P (adj) | Down   | P (adj) | Down   |
|             |           | Up/ downregulated | -3.78 | Up/ downregulated | -a |
|             |           |           | -a     |           | -a     |
|             | col6a6    | -1.78   | 0.046855 | -5.04 | 0.003999 |
|             |           | Down     | Down | Down     |
|             | col6a6    | -3.93   | 0.000086 | -4.43 | 0.001080 |
|             |           | Down     | Down | Down     |
|             | col6a3    | -1.52   | 0.027935 | -a   | -a   |
|             |           | Down     | Down | Down     |
|             | col1a1b   | -3.32   | 0.000073 | -3.63 | 0.004069 |
|             |           | Down     | Down | Down     |
|             | col1a2    | -3.80   | 0.000046 | -3.69 | 0.003305 |
|             |           | Down     | Down | Down     |
|             | col1a1    | -1.75   | 0.000495 | -3.79 | 0.001714 |
|             |           | Down     | Down | Down     |
|             | col1a2    | -2.18   | 1.56E-06 | -2.81 | 0.046610 |
|             |           | Down     | Down | Down     |
|             | col2a1b   | -a      | -a      | -2.96 | 0.036736 |
|             |           | -a      | -a      | Down     |
|             | col1a1a   | -3.94   | 0.000201 | -4.67 | 0.007110 |
|             |           | Down     | Down | Down     |
|             | col6a6    | -3.82   | 0.005404 | -a   | -a   |
|             |           | Down     | Down | Down     |
| Glutathione S-transferase (gst) | gstm3l | -1.22 | 0.004676 | -a | -a |
|             |           | Down     | Down | Down     |
|             | gsta.1    | -1.96   | 0.038464 | -a | -a |
|             |           | Down     | Down | Down     |
|             | mgst3a    | -1.28   | 0.002452 | -a | -a |
|             |           | Down     | Down | Down     |
| CD molecules | myd88 | 1.15 | 0.010149 | Up | -a |
|             |           | -a | -a | -a |
|             | cd276    | 1.27   | 0.008465 | -a | -a |
|             |           | Up | -a | -a |
|             | cd74     | -1.64  | 0.000159 | -a | -a |
|             |           | Down | -a | -a |
|             | cd276    | -1.05  | 0.038810 | -a | -a |
|             |           | Down | -a | -a |
|             | cd34     | -a     | -a     | -3.89 | 0.018911 |
|             |           | -a     | -a     | Down | -a |
|             | cd166    | -a     | -a     | -2.95 | 0.021215 |
|             |           | -a     | -a     | Down | -a |
| B cell      | cd248    | -a     | -a     | -6.50 | 0.00630 |
|             |           | -a     | -a     | Down | -a |
|             | cd22     | -1.15  | 0.017344 | -a | -a |
|             |           | Down | -a | -a |
|             | tcrg1    | 1.25   | 0.006142 | -a | -a |
|             |           | Up | -a | -a |
|             | nfatc1   | -1.97  | 0.010264 | -a | -a |
|             |           | Down | -a | -a |
| T cell      | h2-aa    | -2.37  | 0.027046 | -a | -a |
| Major histocompatibility complex (MHC) |           | Down | -a | -a |
|             | h2-eb1   | -1.60  | 0.016974 | -a | -a |
|             |           | Down | -a | -a |
|             | h2-eb1   | -1.97  | 0.008253 | -a | -a |
|             |           | Down | -a | -a |
|             | h2-eb1   | -2.62  | 6.93E-06 | -a | -a |
|             |           | Down | -a | -a |
|             | h2-eb1   | -3.02  | 0.000078 | -a | -a |
|             |           | Down | -a | -a |
|             | hla-dap1 | -1.67  | 0.000442 | -a | -a |

No differential expression of the gene in liver or muscle.
that SDD is a chronic viral disease that develops over time (potentially weeks), before presenting as clinically diseased fish. The severe necrotizing glomerulonephritis in sick fish may be directly as a result of prolonged inflammation because of release of chemokines, interleukins, and tumor necrosis factors (TNF).

In this study, the KEGG metabolic pathway was the most enriched and significant pathway with 208 DEGs. Insulin signaling and AGE-RAGE signaling pathway in diabetic complications (among 27 immune-relevant pathways) were identified based on DEGs in sick vs. healthy fish, suggesting a disease-induced endocrine and metabolic disorder. In barramundi subjected to various stressors (LPS, \textit{V. harveyi}, high salinity and fasting), DEGs associated with metabolic pathways were also notably downregulated (Xia et al., 2013). It is expected that genes associated with cellular processes and/or homeostasis will be affected by severe tissue damage during the advance stages of disease. It is likely that SDDV and underlying LCHV infections contributed to depletion of energy stores in liver from disease, when fish are stressed and stop feeding. Cortisol is known to cause insulin resistance which disrupts glucose metabolism (Kamba et al., 2016), and stress-related cortisol spikes are well described in fish (Sadoul and Geffroy, 2019). Starvation stage can cause abnormal fat accumulation in liver, from a disorder of glucose metabolism and energy for processing fat (Rui, 2014). There was consistently abnormal accumulation of fat macrovesicles in the liver (lipidosis) in all sick fish, in which functional hepatic genes (e.g., insulin-like growth factor binding protein 1a; progesterin and adipoQ receptor family member Vb; glutamate receptor, ionotropic, AMPA 1a; fatty acid-binding protein 10a; phospholipase A2-like; etc.) were differentially expressed. Dietary fat is processed in the livers of fish very similarly to that in mammals, via lipoprotein conjugation and subsequent storage in adipose tissues throughout the body (Yan et al., 2015). In fact, \textit{ctrp1}, a C1q/TNF-related adipokine strongly implicated in pathogenesis of non-alcoholic fatty liver disease (NAFLD), a human chronic liver disease associated with several metabolic-related disorders including insulin resistance (diabetes) and inflammation (Shabani et al., 2017), was one of the most significantly downregulated genes both in the liver and in the muscle of sick animals.

Genes associated with the adaptive immune system, in particular those involved in immune effector process, such as T cells, B cells, and the major histocompatibility complex (MHC) were also predominantly downregulated in SDD barramundi. SDDV differs from other systemic iridoviral disease, where infected cells are filled with large numbers of virions in crystalline array (Gibson-Kueh et al., 2003). In contrast, few mature virions are observed in fish with SDD using transmission electron microscopy (TEM), suggesting that SDDV infection results in the continual release of mature viral particles (Gibson-Kueh et al., 2012). It is also likely that continual viral shedding results in a prolonged inflammatory host immune response that causes severe tissue damage but fails to overcome SDDV infections. This is consistent with the observed involvement of a number of cytokines (e.g., chemokines, interleukins, and TNFs) and toll-like receptors, predominantly upregulated in SDD fish, which suggest an ongoing strong response by the innate immune system of fish in advance stages of SDD. Cytokines such as interleukins stimulate T cells, and TNF activates macrophages (Uribe et al., 2011). Toll-like receptors recognize viral infections and trigger the release of cytokines and chemokines (Xagorari and Chichilia, 2008), a general mechanism which has also been previously observed in transcriptomic studies of barramundi infected by other pathogens such as \textit{V. harveyi} (Xia et al., 2013), \textit{S. iniae} (Jiang et al., 2014), and NNV (Liu et al., 2016). In the giant grouper (Epinephelus lanceolatus) infected with \textit{Vibrio alginolyticus}, upregulation of the \textit{tlr}5 gene leading to cytokine regulation has been suggested to induce proinflammatory and/or chemotactic effects (Wang et al., 2016). Here, \textit{tlr}5 and \textit{tlr}13 were significantly upregulated in both liver (8- and 33-fold) and muscle (56- and 83-fold) of sick fish, respectively. Similarly to what was observed in our study, \textit{tlr}5 was upregulated by over 150-fold in spleen of barramundi challenged with \textit{S. iniae} but not in vaccinated challenged fish (Jiang et al., 2014). Moreover, TLR signaling pathway has been shown ubiquitously upregulated in the intestines of barramundi after exposure to LPS, \textit{V. harveyi} challenge, high salinity, and fasting (Xia et al., 2013). Taken together, these studies suggest that there is a coordinated response among several organs upregulating the TLR signaling pathway in response to pathogens, whereby our study suggests that \textit{tlr}5 and \textit{tlr}13 are key markers in SDD barramundi.

Lectins such as ladderlectin, t-ramnose-binding lectin, and collectins were among the most differentially expressed genes in the livers of SDD barramundi. Lectins were identified as part of the barramundi immune response to foreign antigens (LPS) as early as 35 days posthatch (Xia and Yue, 2010). Lectins are assumed to mediate pathogen recognition, cell adhesion, the activation of complement pathway, and facilitate pathogen clearance by phagocytosis, thus playing an important role in innate immunity and disease resistance in fish (Elumalai et al., 2019). While the phagosome was the most enriched immune-related pathway in the livers of SDD barramundi, there was an overall downregulation of C3 and C1q, key complement proinflammatory genes in sick fish. Differential regulation of complement genes was observed in barramundi intestine, whereby LPS challenge upregulated C3 and downregulated C1q; the latter also downregulated at 40 h post-V. harveyi challenge (Xia et al., 2013). Complement genes coordinate the communication between the innate and the adaptive immune system (Bergman, 2011). Their activity is tightly regulated to avoid immune dysregulation and tissue damage as a consequence of excessive expression and inflammation (Wasiak et al., 2017). In giant grouper, \textit{V. alginolyticus} challenge had time-dependent effects on several genes related to the complement pathway, with expression levels of most genes (including C3 and C1q) peaking between 4 and 8 h postinfection and returning to basal (preinfection) levels at 48 h postinfection (Wang et al., 2014). It is hypothesized here that the observed downregulation of C1q and C3 genes in SDD barramundi might be due to the fact that organs had already reached advanced inflammatory stages thus precluding further activation, which is not the case of apparently healthy, but subclinically SDDV- and LCHV-coinfected fish. However, the observed upregulation of C5 and C6 genes in sick fish might indicate that complement genes may be differentially activated in different phases in the animal’s immune response and disease progression.
Like in humans and other animals, collagen plays an important role in strengthening skin and its elasticity in the fish. If downregulation of collagen gene clusters in both liver and muscle of sick fish would also occur in skin tissue (not assessed in this study), it might possibly explain the observed damage in the epidermis and reduction of scale adhesion, which results in obvious scale detachment in clinically sick fish (for which SDD is known for). Systemic iridoviruses of fish target fibroblasts in connective tissues surrounding blood vessels (Gibson-Kueh et al., 2003). The inflammation in skeletal muscles involved mainly the connective tissues and not muscles, with the occasional presence of viral inclusion bodies. This is consistent with the lower number of DEGs, lower Pearson $R^2$ values, and less evident heatmap clustering of muscle samples when compared with liver tissues between clinically sick and healthy *L. calcarifer*. This is also consistent with tissue predilection of systemic iridoviruses, targeting fibroblasts of mesothelial origin in all organs (Gibson-Kueh et al., 2003). The inflammation in dermis of skin is centered on the fibroblastic connective tissues of tunica adventitia of blood vessels. The marked occlusion of blood vessels would further explain infarct of epidermis of skin and scale loss characteristic of SDD, and the multifocal infarcts in spleen (Gibson-Kueh et al., 2012).

Finally, it is important to note that the transcriptomic comparison (and DEGs) between the healthy and sick fish groups evaluated in this field outbreak would likely be different if the comparison were made between the sick group and an “uninfected control group.” Such comparison would likely reveal more immune genes be identified as differentially regulated between sick and uninfected groups. Further studies investigating transcriptome of uninfected fish under laboratory conditions may be required to clarify this uncertainty. However, the data provided in this study might be an indication that SDDV (and LCHV) is currently endemic in sea-caged farmed barramundi juveniles within this site, which should also be confirmed by follow-up epidemiological surveys. This is not unlikely because in recent years, all batches farmed in the area get ubiquitously affected with SDD mortalities during the juvenile stages, whereby some animals get clinically sick and succumb to the disease, whereas others with mild infection and which look “apparently healthy” survive. Therefore, also of importance, future studies should evaluate when animals become infected after stocking, with time-series sampling plan over the course of multiple outbreaks (e.g., prior, during, and after) to better understand transcriptomic responses over the progression of disease.

In conclusion, SDDV infection (and to a lesser but unknown extent of LCHV coinfection) resulted in upregulation of genes associated with innate immunity, downregulation of genes associated with adaptive immunity and homeostatic regulation of cellular and tissue function, and severe inflammatory response that resulted in destruction of spleen followed by the kidney. Research to understand why SDD viral infection is not brought under control by the host immunity may be key to developing effective vaccines and/or immunostimulants to alleviate the effects of SDD in farmed barramundi.

### DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA (Accession: PRJNA713978).

### ETHICS STATEMENT

The animal study was reviewed and approved by the James Cook University Singapore, Animal Ethics Committee (Approval No. 2019-A07).

### AUTHOR CONTRIBUTIONS

JD and DJ contributed to conception and design of the study. CT and MT collected specimens and organized the database. SS and HD performed the molecular diagnostics. XS and JD performed the RNAseq statistical analysis. SG-K performed the histopathology analysis. JD wrote the first draft of the manuscript. All authors contributed to the manuscript writing, revision, read, and approved the submitted version.

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### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.666897/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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