Transcriptome of *Nosema ceranae* and Upregulated Microsporidia Genes during Its Infection of Western Honey Bee (*Apis mellifera*)

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**Abstract:** *Nosema ceranae* is one of the fungal parasites of *Apis mellifera*. It causes physical and behavioral effects in honey bees. However, only a few studies have reported on gene expression profiling during *A. mellifera* infection. In this study, the transcriptome profile of mature spores at each time point of infection (5, 10, and 20 days post-infection, d.p.i.) were investigated. Based on the transcriptome and expression profile analysis, a total of 878, 952, and 981 differentially expressed genes (DEGs) (fold change \( \geq 2 \) or \( \leq -2 \)) were identified in *N. ceranae* spores (NcSp) at 5 d.p.i., 10 d.p.i., and 20 d.p.i., respectively. Moreover, 70 upregulated genes and 340 downregulated genes among common DEGs (so-called common DEGs) and 166 stage-specific genes at each stage of infection were identified. The Gene Ontology (GO) analysis indicated that the DEGs and corresponding common DEGs are involved in the functions of cytosol (GO:0005829), cytoplasm (GO:0005737), and ATP binding (GO:0005524). Furthermore, the pathway analysis found that the DEGs and common DEGs are involved in metabolism, environmental information processing, and organismal systems. Four upregulated common DEGs with higher fold-change values, highly associated with spore proteins and transcription factors, were selected for validation. In addition, the stage-specific genes are highly involved in the mechanism of pre-mRNA splicing according to GO enrichment analysis; thus, three of them showed high expression at each d.p.i. and were also subjected to validation. The relative gene expression levels showed a similar tendency as the transcriptome predictions at different d.p.i., of nosemosis at the genetic level in apiculture.

**Keywords:** common DEGs; stage-specific genes; transcriptome; *Nosema ceranae*; *Apis mellifera*

1. **Introduction**

Microsporidia are considered primitive eukaryotic and obligate intracellular parasites; they have 70S ribosomes of small subunit (SSU) and large subunit (LSU) rRNA, which is similar to eukaryotes, but they lack mitochondria, peroxisomes, and centrioles. Nonetheless,
molecular phylogeny shows that microsporidia are regarded as highly specialized parasitic fungi [1–6]. Nosemosis is a serious and common microsporidial disease of adult honey bees that is mainly caused by two species of microsporidians, Nosema apis and Nosema ceranae [7,8]. N. ceranae and N. apis are described as midgut pathogens found in adult honey bees that reproduce only in midgut epithelial cells [9]. There are no clear outward symptoms of nosemosis; however, whitish and swollen midguts are observed in heavily infected honey bees, honey bees become weak, and colonies can even collapse.

In general, Nosema spp. have two distinct phases in their life cycle, including the proliferative phase (merogony) and sporogonic phase (sporogony). The morphology and life cycle of N. apis and N. ceranae are quite similar, and previous studies have also proven that N. apis and N. ceranae can infect Apis mellifera [9–11]. However, some different features are found between N. apis and N. ceranae, including genome size, temperature resilience, survival, and impacts on infected honey bees [9,12–14]. Compared with N. apis, N. ceranae has a higher virulence and appears to successfully compete with N. apis in many A. mellifera populations globally. Therefore, N. ceranae manifests as a long-term chronic infection in warmer areas and then causes colony loss [15–17].

As mentioned above, N. ceranae mainly infects the midgut tissues of honey bees, and many studies have demonstrated the impacts of an N. ceranae infection on A. mellifera. Once honey bees are infected with N. ceranae, immunity and physiological conditions decrease, eventually leading to colony weakness and losses [18,19]. Because of the pressure of the lack of nutrition and energy, a higher hunger level is observed in infected honey bees, and more sugar is consumed [19–21]. Moreover, N. ceranae replicates with considerable energy taken from the host, causing malnutrition and increased mortality in honey bees [22]. The expression profile of the genes associated with the carbohydrate metabolism of A. mellifera are changed during the process of an N. ceranae infection. Besides, severe physiological effects caused by an infection with N. ceranae, including the expression of age-inappropriate behaviors, a reduced flying capability, and a shortened lifespan, were also reported [23]. It was reported that an infection with N. ceranae does not cause large compositional changes to the honey bee midgut microbiota, but the relative abundances of Proteobacteria and Firmicutes were significantly changed under a severe N. ceranae infection, indicating the dysbiosis state of the bacteriome in the honey bee gut [24]. In addition, it has also been reported that co-exposure to pesticides, such as fipronil or thiamethoxam, and N. ceranae decreased the survival rate of honey bees [25]. In terms of microbiota composition, co-exposure to pesticides and N. ceranae also negatively influenced the abundance of Alphaproteobacteria, but increased the Gammaproteobacteria abundance, indicating that the pesticides and N. ceranae are associated with the pathobiome status of honey bees [25]. Even though some bacterial species exhibited inhibitory activity on N. ceranae, it has been reported that the enhancement of Bifidobacterium spp. in the hindgut after N. ceranae infection may not inhibit nosemosis [25–27].

Previous studies have examined the gene expression profile and corresponding differentially expressed genes of N. ceranae-infected A. mellifera [28–30]. However, few studies have emphasized either the comprehensive gene expression of N. ceranae during its infection of A. mellifera or the common DEGs, which are important to N. ceranae for completing its life cycle [31]. Moreover, the genome of N. ceranae has been studied and reported for a better understanding of the architecture, regulation, and evolution of this pathogen [31,32]. From our previous study, the identification of N. ceranae-specific genes during its infection of A. mellifera by suppressive subtractive hybridization (SSH) has been performed [33], but comprehensive data on the gene expression profile during the infection process are still unavailable. In this study, we attempted to reveal the transcriptomic profiling of N. ceranae during its infection of A. mellifera to complete the information on the infection cycle of this parasite. To this end, a transcriptomic approach at different N. ceranae infection stages in A. mellifera was followed, and common DEGs and stage-specific genes among different infectious periods were identified and validated. This study provides information to better understand the mechanism of N. ceranae and guide basic disease-control studies.
2. Materials and Methods

- **Honey bees**
  
  Worker honey bees were collected from *A. mellifera* colonies in apiaries at the National Chung Hsing University (NCHU, Taiching, Taiwan). Each honey bee colony had six to eight frames and the presence of a normal spawning queen. Brood cells of honeycombs from the six colonies, which were capped at more than 70–80%, were selected and incubated at 34 °C until honey bees emerged. The newly emerged adult honey bees were collected in a rearing cage and supplied with 50% syrup for the following experiments.

- **Artificial infection of Nosema ceranae**
  
  Mature *N. ceranae* spores were collected from the midgut tissues of honey bees from apiaries at the NCHU (National Chung Hsing University, Taichung, Taiwan) and purified by the protocol proposed by Huang et al., 2007 [34]. Briefly, the midgut tissues of honey bees were collected in 500 µL 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) (Bioman, New Taipei, Taiwan) and homogenized with a pestle. The homogenized tissues were then filtered with 100 mesh stainless steel wire cloth. The filtrate was centrifuged at 1000 × g for 10 min, and the supernatant and tissue debris were removed. Then, the pellet was resuspended in 500 µL 1 × TE buffer. The wash step was repeated to remove any remaining tissue debris. The spore suspension was centrifuged at 16,000 × g for 3 min at 4 °C, and the collected spores were counted with a haemocytometer (Paul Marienfeld GmbH & Co. KG, Baden-Württemberg, Germany). The concentration of the mature spore suspension was adjusted to 1 × 10^5 spore/2 µL for the infection of honey bees [35].

  In the *N. ceranae*-infected group, the newly emerged honey bees were fed 2 µL of 50% sugar syrup with *N. ceranae* spores (1 × 10^5 spores per honey bee), while the honey bees in the control group were fed 2 µL of 50% sugar syrup. Each group contained fifty honey bees in one rearing cage, which were supplied with 50% syrup and incubated at 34 °C until sampling. Samples for both RNA-seq and the detection of *N. ceranae* infections were selected in triplicate, and qPCR validation for gene expression was repeated in four replicates. The survival rates of the infected and control groups were observed and recorded daily. A Kaplan–Meier survival curve analysis was performed using SPSS (Norman H. Nie, C. Hadlai (Tex) Hull and Dale H. Bent, IBM SPSS Statistics version 20, Palo Alto, CA, USA).

- **Confirmation of Nosema ceranae infection**
  
  Before the experiment, multiplex PCR [36] was performed to confirm the absence of *Nosema* spp. infection in newly emerged adult honey bees (Supplementary Figure S1). To confirm the *N. ceranae* infection, three midgut tissues of *N. ceranae*-infected bees and noninfected honey bees were collected in 1 × TE buffer. The midgut tissues were homogenized with a pestle, and the tissue suspension was subjected to observation, spore counting, and DNA extraction. Mature spores were observed under 400× light microscopy (WHITED, Taiwan) and counted by a haemocytometer (Paul Marienfeld GmbH & Co. KG, Baden-Württemberg, Germany). A total of 100 µL of homogenized tissue suspension was subjected to DNA extraction by the EDNA HISPEX (Easy DNA High-Speed Extraction) tissue kit (Fisher Biotec, Stirling, Australia) according to the user manual. The DNA sample was then subjected to PCR detection with the microsporidia universal primer set 18f/1537r, and the Hb18f/Hb18r primer set was used as an internal control (Supplementary Table S1). For the PCR, each 20 µL PCR contained 10 µL 2× SuperRed PCR Master mix (BIOTOOLs, New Taipei, Taiwan), 1 µL 100 mM of each primer, and 1 µL template DNA. PCR amplification was performed by a MiniAmp™ Thermal Cycler (Thermo Scientific, Waltham, MA, USA) with the following program: the thermal cycle was preheated to 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min 30 s, and then a final extension was performed of 72 °C for 10 min and storage at 20 °C. The PCR product was analyzed by electrophoresis on a 1% agarose gel in 1 × Tris-acetate-EDTA (TAE) buffer.
Preparation of RNA samples for next-generation sequencing

Based on the data of the survival curve and mature spore counts, midgut tissues from three honey bees were collected at 0 (3 h), 5, 10, and 20 days post-infection (d.p.i.) and purified *N. ceranae* mature spores (\(1 \times 10^8\)) were homogenized by a Tissue Lyser II (QIAGEN, Solingen, Germany) at \(f = 30/s\) for 1 min. This procedure was repeated at three time points. The total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Waltham, MA, USA) following the user’s manual. The extracted RNA sample was then purified using a Locking Gel (BIOTOOLS, New Taipei, Taiwan). The RNA purity and integrity were checked by a Nanodrop 1000 Spectrophotometer V3.5 (Thermo Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Invitrogen Life Technologies, Waltham, MA, USA), respectively. Total RNA samples of three biological repeats were pooled and used for library construction. The size distribution of each RNA sample was determined by Fragment Analyzer Systems (Agilent, Santa Clara, CA, USA). The RNA sample was then used to construct a library for transcriptome sequencing.

RNA-Seq library construction and sequencing

The mRNA from each sample was further purified for sequencing library construction using an Ultra RNA Library Prep Kit (NEB, Essex, MA, USA) according to the manufacturer’s protocol. Briefly, poly-A-containing mRNA was purified by oligo d (T) magnetic beads. During the final step of mRNA purification, RNA was fragmented and primed with NEBNext random primers for cDNA synthesis. After mRNA was reverse-transcribed, end-repair and adaptor ligation (NEBNext Adaptor) were performed. Finally, DNA fragments were selectively enriched by PCR and then qualified and quantified by Fragment Analysis Systems (Agilent, Santa Clara, CA, USA) and qPCR, respectively, for the DNA library quality control analysis. The libraries were then pooled and sequenced by the Illumina MiSeq sequencer with a paired-end (PE) technology of 301 bp in length to generate high-throughput transcriptome sequencing data. Sequencing adaptors were trimmed using Trimmomatic [37] from raw PE reads, and then a read quality check was performed by PRINseq [38] from trimmed PE reads. To confirm the pathogen load in the sequencing data of the newly emerged adult honey bees, the trimmed PE reads were mapped to the genomes of *Nosema apis* and 6 other viruses.

Differentially expressed gene (DEG) analysis

The raw PE reads were trimmed by Trimmomatic [37], and then the clean reads were obtained. The quality reads were mapped to the *N. ceranae* genome index (ASM98816v1) using HISAT2 with default parameters, and the corresponding gene annotation information (GCF_000988165.1_ASM98816v1_genomic.gff) from the National Biotechnology Information Center (NCBI) (https://www.ncbi.nlm.nih.gov/ (accessed on 19 January 2021)) was used to obtain the *N. ceranae*-related PE reads for further analysis. Differentially expressed genes (DEGs) among experimental groups were analyzed via Cuffdiff, and the gene expression heatmap was generated by the R package [39] (Supplementary Figure S2). For the DEG analysis, genes with a zero FPKM value from each group of the analysis were filtered out. To facilitate logarithmic transformation, a value of 1 was added to the FPKM value of the remaining genes. Raw signals (FPKM t 1) were then transformed using log2-based transformation. A statistical test of fold changes was performed, and a significant difference in the expression of these two groups was defined as a log2 ratio \(\geq 1\) or \(\leq -1\) (fold change \(\geq 2\) or \(\leq -2\)). The DEGs from each comparison (NcSp/5 d.p.i., NcSp/10 d.p.i., and NcSp/20 d.p.i.) were further compared by Venny 2.1.0 (Juan Carlos Oliveros, Madrid, Spain) [40] to obtain the common DEGs of *N. ceranae* in different infection stages. The DEGs and common DEGs were further used in the GO enrichment analysis and submitted to the KEGG Automatic Annotation Server (KAAS, http://www.genome.jp/tools/kaas/ (accessed on 23 April 2021)) for gene orthologue assignment and pathway mapping [41]. The selected *N. ceranae* common DEGs were further analyzed by EMB BLAST (European Molecular Biology Network—Swiss node) for a functional homology search. The results with an E-value \(< 10^{-4}\) were recorded for further discussion of functional proteins.
qRT–PCR validation

For validation, three midgut tissues of infected honey bees were collected into 300 μL of TE buffer at 0.5 (12 h), 1, 3, 5, 10, 15, and 20 d.p.i., with four replicates, and were homogenized for RNA extraction. Four replicates of 1 × 10⁸ purified mature *N. ceranae* spores were also subjected to RNA extraction. The total RNA for qRT–PCR validation was extracted from 200 μL of homogenized midgut tissue and the *N. ceranae* mature spore sample using the GENEzol™ TriRNA Pure Kit (Geneaid, New Taipei, Taiwan) as described in the user manual. The concentration and quantity of the total RNA were measured using a Nanodrop™ spectrophotometer (Thermo Scientific, Waltham, USA). Each RNA sample was adjusted to 1 μg/mL for cDNA synthesis by BioDiamond RT–PCR mix (Origin Pure BioSci & Tech.Co., Yilan, Taiwan) as described in the user manual. Four highly upregulated common DEGs, AAJ76_1600052943, AAJ76_2000149161, AAJ76_2000141845, and AAJ76_500091146, and three highly expressed stage-specific genes, AAJ76_500026485, AAJ76_2400020190, and AAJ76_2300035467, were selected for RT–qPCR validation. β-Tubulin from *N. ceranae* served as the internal control. Real-time PCR was performed by iQ™ SYBR® Green Supermix (Bio–Rad, Hercules, CA, USA) in a CFX Connect Real-Time PCR Detection System (Bio–Rad, Hercules, CA, USA). qRT–PCR was performed as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 45 s. The relative gene expression levels for common DEGs were calculated by the 2–△△Ct method, and stage-specific genes were calculated by the 2–△Ct method [42].

Statistical analysis

Statistical analysis and plotting were performed with R 4.0.3 [43] and the heatmap, ggplot2, and ggpubr [39,44,45] packages. The statistical analysis of the Kaplan–Meier survival curves was performed using the R packages survminer [46] and survival [47]. The statistical analysis of the spore count and qRT–PCR data was performed using one-way ANOVA with SPSS® [48].

3. Results

3.1. *N. Ceranae* Infection

The Kaplan–Meier survival curve results showed that survival fitness was significantly reduced (*p* < 0.01) in the *N. ceranae*-infected *A. mellifera* (Figure 1A). Mature spores were observed from 5 to 20 d.p.i. in the homogenized midgut tissues of the *N. ceranae*-infected honey bees under microscopy; while only a few mature spores were observed at 5 d.p.i., the spore counts significantly increased from 10 to 20 d.p.i (Figure 1B,C). Molecular detection was performed to detect the infection process of *N. ceranae* on *A. mellifera*. Although the earliest time that mature spores were observed was at 5 d.p.i., the results showed that *N. ceranae* infection was first detected at 3 d.p.i. by PCR (Figure 1D).
3.2. Sequencing Data Summary

The sequencing data are summarized in Supplementary Table S2. A total of 1.68 million (M), 6.85 M, 1.58 M, and 2.91 M raw PE reads were obtained from 0 (3 h), 5, 10, and 20 d.p.i. and mature \textit{N. ceranae} spores. The number of clean reads was 1.44 M, 2.35 M, 1.57 M, 1.38 M, and 1.58 M for 0 (3 h), 5, 10, and 20 d.p.i. and mature \textit{N. ceranae} spores, respectively. The mappability of each sample to the \textit{N. ceranae} genome was 0%, 4%, 3%, 3%, and 12% for 0 (3 h), 5, 10, and 20 d.p.i. and mature \textit{N. ceranae} spores, respectively. In addition, the newly emerged adult honey bees were confirmed to be free of \textit{N. apis} and common honey bee viral infections (Supplementary Table S3). These mapped reads were subjected to further analysis of the expression of different genes.

3.3. DEG Analysis

For the DEG analysis, a total of 878, 952, and 981 genes were identified to have significant fold changes (fold change $\geq 2$ or $\leq -2$) in NcSp/5 d.p.i., NcSp/10 d.p.i., and NcSp/20 d.p.i., respectively. Among these DEGs, 429, 117, and 113 genes were upregulated and 449, 835, and 868 genes were downregulated in NcSp/5 d.p.i., NcSp/10 d.p.i., and NcSp/20 d.p.i., respectively (Figure 2A; Tables 1, 2, and S4–S9). The common DEGs were selected from all three groups of common DEGs by set intersection, and stage-specific genes were defined as the genes that were only expressed at 5, 10, and 20 d.p.i., respectively (Tables 3 and S10). There was a total of 70 upregulated and 340 downregulated common DEGs (Figure 2B; Tables 1, 2, and S4–S9), and the fold-change distribution at each infection stage is shown in Figure 2B. The results also showed that a greater upregulation of \textit{N. ceranae} common DEGs and stage-specific genes was found in the initial infectious stage,
whereas more genes of *N. ceranae* were suppressed in the late stage of infection (Figure 2). For the stage-specific genes, a total of 25, 71, and 70 genes were identified at each time post-infection (Supplementary Table S10), and the top 10 expression values (FPKM) of the stage-specific genes are summarized in Table 3.

![Figure 2. DEG and common gene identification. (A) Scatter plot of DEGs (red dots = upregulation and blue dots = downregulation); (B) Venn plot and fold-change number for the distribution of common DEGs.](image-url)
Table 1. List of upregulated common DEGs with FPKM values > 1000 at different times post-infection.

| No. | Gene ID    | FPKM (Mature Spore/5 d.p.i./10 d.p.i./20 d.p.i.) | Fold Change | Gene Type | Protein ID | Length (aa) | Protein Name | E-Value | KO Annotation |
|-----|------------|-----------------------------------------------|-------------|-----------|------------|-------------|--------------|---------|---------------|
| 1   | AAJ76_16_00052943 | 15,626.3/115,903/34,433.6/47165.2 | 7.417124574/2.030556935/3.018307934 | Protein coding | XP_024331363.1 | 265 | Hypothetical protein | Spore wall protein 25 (SWP25_NOSB) | 1.50 x 10^{-13} | - |
| 2   | AAJ76_20_00149161 | 12,535.3/91,333/34,160.1/43545.1 | 7.286065679/2.725120453/3.473788695 | Protein coding | XP_024332331.1 | 263 | Hypothetical protein | Spore wall protein 30 (SWP32_NOSB) | - | - |
| 3   | AAJ76_20_00141845 | 11,759.8/65,562.7/29,751.5/34741.6 | 5.575147599/2.529916904/2.95426655 | Protein coding | XP_024332256.1 | 341 | Hypothetical protein | Spore wall protein 25 (SWP25_NOSB) | 6.00 x 10^{-13} | - |
| 4   | AAJ76_16_00051435 | 189.533/21,504.1/1488.81/3201.29 | 113.4584304/7.855156397/16.89038139 | Protein coding | XP_024331362.1 | 286 | Hypothetical protein | Spore wall protein 25 (SWP25_NOSB) | 1.50 x 10^{-6} | - |
| 5   | AAJ76_23_00022418 | 6875.45/16,903.7/17,987.8/19,037.2 | 2.458553927/2.616261210/2.03897491 | Protein coding | XP_024331106.1 | 198 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | 3.30 x 10^{-3} | - |
| 6   | AAJ76_80_00022418 | 836.132/12,300.3/3753.56/3436.68 | 9.714636403/2.279662102/2.03897491 | Protein coding | XP_024331997.1 | 187 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | - | - |
| 7   | AAJ76_50_00022418 | 783.185/14,628.1/1618.08/1750.26 | 18.67776596/4.110203999/2.03897491 | Protein coding | XP_024331997.1 | 187 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | - | - |
| 8   | AAJ76_50_00022418 | 292.943/6718.83/2035.24/2486.37 | 22.93565176/6.947577391/8.487560776 | Protein coding | XP_024331997.1 | 187 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | - | - |
| 9   | AAJ76_70_00022418 | 142.197/5518/1477.09/1142.36 | 38.80552048/10.387668689/8.038167865 | Protein coding | XP_024331997.1 | 187 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | - | - |
| 10  | AAJ76_15_00022418 | 121.197/5518/1477.09/1142.36 | 38.80552048/10.387668689/8.038167865 | Protein coding | XP_024331997.1 | 187 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | - | - |
| 11  | AAJ76_10_00022418 | 794.305/5221.61/2185.48/1886.35 | 6.573811096/2.735122262/3.274839476 | Protein coding | XP_024331404.1 | 233 | Hypothetical protein | WD repeat-containing protein 48 homolog (WDR48_DICDI) | - | - |
| 12  | AAJ76_50_00022418 | 612.16/4981.59/1332.9/1251.91 | 8.137750225/2.177375154/2.03897491 | Protein coding | XP_024331065.1 | 26 | Proteasome subunit alpha type-2 (PSA2_ORYSJ) | 3.40 x 10^{-30} | K02726 |
| 13  | AAJ76_79_00022418 | 530.27/3753.56/2918.4/1222.32 | 7.04469616/5.505540416/3.20569010 | Protein coding | XP_024330110.1 | 199 | Hypothetical protein | Ubiquitin-conjugating enzyme E2 e2 Forkhead transcription factor (SLP1_DROME) | - | - |
| 14  | AAJ76_50_00022418 | 487.495/2612.36/2107.93/1320.58 | 5.35862956/4.24004124/2.667114941 | Protein coding | XP_024330474.1 | 149 | Hypothetical protein | Ubiquitin-conjugating enzyme E2 e2 Forkhead transcription factor (SLP1_DROME) | - | - |
| 15  | AAJ76_50_00022418 | 417.662/1822.04/1577.38/2178.81 | 4.36248448/3.776683799/5.21667299 | Protein coding | XP_024330780.1 | 205 | Hypothetical protein | Ubiquitin-conjugating enzyme E2 e2 Forkhead transcription factor (SLP1_DROME) | - | - |

* = selected gene for validation; FPKM = fragment per kilobase per million; d.p.i. = days post-infection; aa = amino acid; NCBI = National Center for Biotechnology Information; EMBL-EBI = the European Molecular Biology Laboratory European Bioinformatics Institute.
| No. | Gene ID     | FPKM (Mature) Spore/5 d.p.i/10 d.p.i/20 d.p.i | Fold Change | Gene Type               | Protein ID | Length (aa) | Protein Name                                                                 | E-Value    | KO Annotation |
|-----|-------------|---------------------------------------------|-------------|-------------------------|------------|------------|--------------------------------------------------------------------------------|------------|---------------|
| 1   | AAJ76_63   | 398,502/32947/61,754/70,676/1                | -12.0951772 | NeSp5/10 d.p.i.         | Protein coding | 107        | Hypothetical protein                                                        | -          | K09140        |
| 2   | AAJ76_20   | 522,948/111,302/18,531/16,522/1              | -4.69843405 | NeSp10 d.p.i.             | Protein coding | 183        | Hypothetical protein                                                        | 2.00 \times 10^{-7} |               |
| 3   | AAJ76_16   | 978,848/95,231/2/24,913/3/19,641/3          | -10.27865792 | NeSp20 d.p.i.            | Protein coding | 267        | Hypothetical protein                                                        | 2.40 \times 10^{-20} |               |
| 4   | AAJ76_19   | 321,105/84,821/6/14,069/3/17,252/2          | -3.785647288 | Protein coding           | 275        | Protein coding | Polar tube protein 2                                                         | 2.60 \times 10^{-6} |               |
| 5   | AAJ76_21   | 282,631/79,179/8/8975/3/7515/33             | -3.56949504 | Prolin-rich protein 2 (PRP2_MOUSE) | 446        | Protein coding | Elongation factor 1-alpha (EFIA_ENCCU)                                      | 3.10 \times 10^{-22} | K10844        |
| 6   | AAJ76_12   | 104,357/36,502/7/9664/2/6697/23             | -2.85889688 | Protein coding           | 464        | Protein coding | Hypothetical protein                                                        | -          |               |
| 7   | AAJ76_60   | 135,745/34,449/1/7043/6/6780/66             | -3.940448921 | Protein coding           | 236        | Protein coding | Hypothetical protein                                                        | -          |               |
| 8   | AAJ76_45   | 80,571/7/30,587/7/9618/9/9332/46             | -2.634118045 | Protein coding           | 239        | Protein coding | Hypothetical protein                                                        | -          |               |
| 9   | AAJ76_21   | 59,830/8/26,523/1/6066/5/3556/9             | -2.25501308 | Protein coding           | 678        | Protein coding | Heat shock protein 70                                                      | -          |               |
| 10  | AAJ76_13   | 105,634/24,439/2415/33/1928/25             | -4.322332282 | Protein coding           | 204        | Protein coding | Hypothetical protein                                                        | -          |               |
| 11  | AAJ76_31   | 54,826/6/22,173/9/2287/8/2631/64            | -2.4725666  | Protein coding           | 338        | Protein coding | Uncharacterized protein                                                   | 1.60 \times 10^{-10} | K03857        |
| 12  | AAJ76_42   | 91,596/5/21,857/3/5018/44/4703/25          | -4.19067331 | Protein coding           | 221        | Protein coding | Hypothetical protein                                                        | 6.70 \times 10^{-25} | K01689        |
| 13  | AAJ76_27   | 39,025/5/19,267/6/3853/6/3059/65            | -2.02544653 | Protein coding           | 156        | Protein coding | Spore wall protein 7                                                        | -          |               |
| 14  | AAJ76_12   | 84,245/8/18,063/1/3850/84/6236/68           | -4.663974821 | Protein coding           | 222        | Protein coding | ABC-type multidrug transporter Atpase and permease component                | -          |               |
| 15  | AAJ76_47   | 67,259/6/16,069/1/3500/31/3049/83           | -1.85645126 | Protein coding           | 284        | Protein coding | Spore wall protein 7 (SWP7_NOSB1)                                          | 2.80 \times 10^{-10} |               |
| 16  | AAJ76_66   | 34,656/4/16,003/4/2672/38/2821/32          | -2.16559782 | Protein coding           | 317        | Protein coding | Transcription initiation factor TFIIID subunit 3 (TAF3_CICICK)              | 3.10 \times 10^{-6} |               |
| 17  | AAJ76_14   | 95,516/2/14,664/3/5701/6/3838/4            | -6.513531706 | Protein coding           | 226        | Protein coding | Histone-lysine N-methyltransferase, HD lysine-79 specific                  | -          |               |
| 18  | AAJ76_24   | 38,208/39,133/2/2471/53/3059/12           | -2.852862075 | Protein coding           | 131        | Protein coding | Nuclear protein 58                                                          | 1.10 \times 10^{-6} | K07178        |
Table 2. Cont.

| No. | Gene ID | FPKM (Mature Spore/5 d.p.i./10 d.p.i./20 d.p.i.) | Fold Change | Gene Type | Protein ID | Length (aa) | Protein Name | E-Value | KO Anotation |
|-----|---------|-----------------------------------------------|-------------|-----------|-----------|-------------|-------------|---------|--------------|
| 19  | AAJ76_53 00022630 | 39,320.1/12,093.2/3489.63/3466.58 | −3.251442701 | −11.267691 | −11.34260414 | Protein coding | XP_024330400.1 | 295 | - | 1.60 × 10<sup>−37</sup> |
| 20  | AAJ76_10 00065899 | 96,438.3/11,363/4552.52/4869.86 | −8.487013111 | −21.18351365 | −19.80303982 | Protein coding | XP_024331635.1 | 216 | - | - |
| 21  | AAJ76_40 00112883 | 117,599/9836.2/2044.46/1175.49 | −11.95572738 | −57.52072681 | −100.0425551 | Protein coding | XP_024332099.1 | 327 | - | 2.10 × 10<sup>−120</sup> |
| 22  | AAJ76_50 00109749 | 21,356.7/9085.52/2351.82/2527.34 | −2.350634122 | −9.080929654 | −8.450285003 | Protein coding | XP_024332015.1 | 229 | - | 2.20 × 10<sup>−76</sup> |
| 23  | AAJ76_23 00036398 | 21,838.9/8533.66/3482.57/2535.92 | −2.559141644 | −6.270932541 | −8.611771259 | Protein coding | XP_024331119.1 | 380 | 37.0 × 10<sup>−41</sup> |
| 24  | AAJ76_27 00031779 | 26,552.2/7739.67/1271.69/1831.42 | −3.430669148 | −20.87942389 | −14.498181 | Protein coding | XP_024330086.1 | 167 | - | K011838 |
| 25  | AAJ76_66 00019728 | 28,729.1/5107.81/2222.85/3656.61 | −5.624520074 | −12.9244412 | −7.856735542 | Protein coding | XP_024330233.1 | 154 | - | - |
| 26  | AAJ76_42 00041343 | 16,934/5107.54/1904.29/1739.95 | −3.707071651 | −9.942853842 | −10.88191078 | Protein coding | XP_024330582.1 | 219 | - | - |
| 27  | AAJ76_20 0059488 | 19,359.4/4708.45/1884.78/1879.72 | −4.111628734 | −10.47167266 | −10.29905455 | Protein coding | XP_024332275.1 | 250 | - | 3.70 × 10<sup>−15</sup> |
| 28  | AAJ76_16 00068502 | 14,848.7/4309.26/2652.73/2834.43 | −3.445778582 | −5.597352862 | −5.238704187 | Protein coding | XP_024331374.1 | 122 | - | 1.20 × 10<sup>−24</sup> |
| 29  | AAJ76_11 00066671 | 659,735/4001.88/4268.18/8495.99 | −164.85668473 | −154.570373 | −77.65247484 | Protein coding | XP_024331582.1 | 132 | 3.70 × 10<sup>−41</sup> |
| 30  | AAJ76_10 00142585 | 18,723.8/3995.19/1946.34/2392.26 | −4.686945887 | −6.920020711 | −7.826480255 | Protein coding | XP_024332442.1 | 268 | - | K01151 |
| 31  | AAJ76_28 0006733 | 32,568.2/3941.14/1832.71/1589.86 | −8.253064983 | −17.70593999 | −20.48491498 | Protein coding | XP_024330038.1 | 201 | - | - |
| 32  | AAJ76_17 00062503 | 19,005.2/2941.7/7507.76/2855.59 | −6.44711209 | −2.53149707 | −6.655421322 | Protein coding | XP_024331841.1 | 190 | - | - |
| 33  | AAJ76_71 0008572 | 22,045.4/2941.14/1179.25/1071.71 | −7.495527438 | 16.69447434 | −14.6010111 | Protein coding | XP_024331959.1 | 209 | - | K00162 |
| 34  | AAJ76_10 0008081 | 73,487.3/2295.2/1218.66/2315.53 | −32.01774949 | −60.30141705 | −31.73669519 | Protein coding | XP_024330233.1 | 347 | - | - |
| 35  | AAJ76_10 00162053 | 8466.55/1704.59/1290.67/1686.56 | −4.864191406 | −6.599719681 | −5.02009172 | Protein coding | XP_024332456.1 | 8.40 × 10<sup>−110</sup> |
| 36  | AAJ76_10 00016205 | 97,815.6/1570.42/2985.17/2356.31 | −62.28660354 | −32.76718881 | −41.51209968 | Protein coding | XP_024331606.1 | 186 | - | - |

**FPKM** = fragment per kilobase per million; d.p.i. = days post-infection; aa = amino acid; NCBI = National Center for Biotechnology Information; EMBL-EBI = the European Molecular Biology Laboratory European Bioinformatics Institute.
| Stage | No. | Gene ID          | FPKM   | Protein ID         | Length (aa) | Protein Name                                      | NCBI | E-Value                | KO Annotation   |
|-------|-----|------------------|--------|-------------------|-------------|---------------------------------------------------|------|------------------------|----------------|
|       | 1 * | AAJ76_5000026485 | 2388.86| XP_024330451.1    | 116         | g10 protein                                       |      | 7.40 × 10^-22          | -              |
| 5 d.p.i| 2   | AAJ76_500072312  | 1067.07| XP_024331999.1    | 149         | Hypothetical protein                              |      | -                      | -              |
| 5 d.p.i| 3   | AAJ76_4508025263 | 987.901| XP_024330536.1    | 176         | Hypothetical protein                              |      | -                      | -              |
| 5 d.p.i| 4   | AAJ76_500090033  | 754.257| XP_024332000.1    | 164         | Nuclear essential protein 1                       |      | 8.40 × 10^-11          | K14568         |
| 5 d.p.i| 5   | AAJ76_4800030660 | 679.592| XP_024330467.1    | 132         | Hypothetical protein                              |      | -                      | -              |
| 5 d.p.i| 6   | AAJ76_2700033485 | 540.13 | XP_024330988.1    | 174         | Hypothetical protein                              |      | -                      | -              |
| 5 d.p.i| 7   | AAJ76_2200018137 | 404.002| XP_024331140.1    | 145         | Deoxyctydylate deaminase                          |      | 8.60 × 10^-22          | K15441         |
| 5 d.p.i| 8   | AAJ76_900062461  | 401.31 | XP_024331679.1    | 148         | Bis(5'-adenosyl)-triphosphatase-like protein      |      | 1.00 × 10^-25          | K01522         |
| 5 d.p.i| 9   | AAJ76_1000019447 | 326.831| XP_024331608.1    | 187         | Hypothetical protein                              |      | -                      | -              |
| 5 d.p.i| 10  | AAJ76_1400060351 | 208.221| XP_024331456.1    | 199         | Hypothetical protein                              |      | 1.60 × 10^-5           | -              |
| 10 d.p.i| 1   | AAJ76_2850002256 | 5462.22| XP_024329453.1    | 41          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 2   | AAJ76_2900015300 | 3735.49| XP_024330918.1    | 41          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 3   | AAJ76_1100010265 | 326.98 | XP_024331553.1    | 43          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 4 *  | AAJ76_2400020190 | 2691.71| XP_024331062.1    | 53          | Haca ribonucleoprotein complex subunit 3          |      | 8.80 × 10^-11          | -              |
| 10 d.p.i| 5   | AAJ76_900027941  | 1915.36| XP_024331657.1    | 63          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 6   | AAJ76_1100054191 | 1914.44| XP_024331571.1    | 56          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 7   | AAJ76_21600003   | 1472.66| XP_024329567.1    | 62          | Nol1 nol2 sun tRNA tRNA cytosine-c5-methylase     |      | 8.00 × 10^-12          | K14835         |
| 10 d.p.i| 8   | AAJ76_1120001085 | 1441.18| XP_024329876.1    | 52          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 9   | AAJ76_600074061  | 1344.19| XP_024330748.1    | 63          | Hypothetical protein                              |      | -                      | -              |
| 10 d.p.i| 10  | AAJ76_1000166313 | 1281.3 | XP_024332457.1    | 64          | Hypothetical protein                              |      | -                      | -              |
|       | 1   | AAJ76_3000132534 | 5797.14| XP_024332206.1    | 46          | Hypothetical protein                              |      | -                      | -              |
|       | 2 * | AAJ76_2300035467 | 5519.29| XP_024331118.1    | 67          | Hypothetical protein                              |      | 8.70 × 10^-27          | -              |
| 20 d.p.i| 3   | AAJ76_6100016064 | 2662.52| XP_024330287.1    | 53          | Hypothetical protein                              |      | -                      | -              |
| 20 d.p.i| 4   | AAJ76_600074061  | 1345.98| XP_024331899.1    | 87          | Zinc finger-domain-containing protein             |      | 7.20 × 10^-10          | K13127         |
| 20 d.p.i| 5   | AAJ76_3470001388 | 1394.2 | XP_024329388.1    | 62          | Hypothetical protein                              |      | 3.60 × 10^-9           | -              |
| 20 d.p.i| 6   | AAJ76_1720003146 | 1256.07| XP_024329669.1    | 61          | Calcium-binding protein of the recoverin subfamily |      | 5.40 × 10^-7           | -              |
| 20 d.p.i| 7   | AAJ76_400028944  | 1057.47| XP_024332054.1    | 86          | Ubiquitin-related modifier 1                     |      | -                      | -              |
| 20 d.p.i| 8   | AAJ76_230009864  | 789.777| XP_024331097.1    | 82          | Hypothetical protein                              |      | -                      | -              |
| 20 d.p.i| 9   | AAJ76_500080905  | 769.682| XP_024330308.1    | 84          | Hypothetical protein                              |      | -                      | -              |
| 20 d.p.i| 10  | AAJ76_610001     | 724.872| XP_024330279.1    | 69          | Hypothetical protein                              |      | -                      | -              |

d.p.i. = days post-infection; * = selected gene for validation; FPKM = fragment per kilobase per million; aa = amino acid; NCBI = National Center for Biotechnology Information; E-Value = European Molecular Biology Laboratory European Bioinformatics Institute.
3.4. Gene Ontology (GO) Enrichment Analysis

The results of the GO annotation are shown in Figure 3. At NcSp/5 d.p.i., some genes belonging to GO pathways were significantly upregulated, including cytosol (GO:0005829), cytoplasm (GO:0005737), ATP binding (GO:0005524), protein binding (GO:0005515), and translation (GO:0006412), which were commonly found in NcSp/5 d.p.i., NcSp/10 d.p.i., and NcSp/20 d.p.i.; meanwhile, the genes involved in the aforementioned GO terms were reduced in number and downregulated in the NcSp/10 d.p.i. and NcSp/20 d.p.i. groups (Figure 3 and Supplementary Tables S11–S16). For the GO annotation of common DEGs, cytosol (GO:0005829) and cytoplasm (GO:0005737) were also found in the upregulated common DEGs group; however, metal ion binding (GO:0046872), GTP binding (GO:0005525), and the ubiquitin-dependent protein catabolic process (GO:0006511) showed high gene counts as observed in the downregulated common DEGs (Figure 4A and Supplementary Tables S11–S16). On the other hand, many stage-specific genes of *N. ceranae* were involved in pre-mRNA-associated pathways, including “RNA binding” (GO:0003723) and “mRNA splicing, via spliceosome” (GO:0000398) (Figure 4B).

Figure 3. Dot plot of *N. ceranae* Gene Ontology (GO) term enrichment analysis, showing the enriched pathways with *p* < 0.05. (A) Upregulated DEGs; (B) downregulated DEGs. Each comparison is indicated at the bottom. The total number of regulated genes within the GO pathways selected on the dot plot are indicated in brackets. Colors indicate the *p* values from Fisher’s exact test, and the dot size is proportional to the number of DEGs and common DEGs in the pathway.

Figure 4. Dot plot of *N. ceranae* Gene Ontology (GO) term enrichment analysis, showing the enriched pathways with *p* < 0.05. (A) Core genes; (B) stage-specific genes. Each trial is indicated at the bottom; the total number of regulated genes within the GO pathways selected on the dot plot are indicated in brackets. Colors indicate the *p* values from Fisher’s exact test, and the dot size is proportional to the number of DEGs and common DEGs in the pathway.
3.5. KEGG Analysis

The results of the KEGG analysis show the pathway annotation of the DEGs and common DEGs at different stages and indicate the mechanisms of *N. ceranae* infection (Figure 5 and Supplementary Tables S17–S22). In the comparison of NcSp at 5 d.p.i., there were 211 pathways that included upregulated genes and 200 pathways that included downregulated genes (Figure 5A). Among these pathways, highly abundant *N. ceranae* DEGs were involved in the metabolism (KO:09100), environmental information processing (KO:09130), and organismal systems (KO:09150) categories, including the carbohydrate metabolism (KO:09101), signal transduction (KO:09132), and immune system (KO:09151) pathways (Figure 5A and Supplementary Tables S17 and S18). In the NcSp at 10 d.p.i. group, 112 pathways included upregulated genes and 122 pathways included downregulated genes. Among these pathways, three categories, including the metabolism pathway (KO:09100), human diseases (KO:09160), and environmental information processing (KO:09130) were the most mapped, and they contained the pathways of carbohydrate metabolism (KO:09101) and signal transduction (KO:09132) (Figure 5B; Supplementary Tables S19 and S20). For the NcSp/20 d.p.i. group, there were 129 pathways that included upregulated genes and 267 pathways that included downregulated genes, and the pathway categories that highly regulated genes belonged to, including metabolism pathways (KO:09100), environmental information processing (KO:09130), human diseases (KO:09160), and organismal systems (KO:09150), were identified. These pathways also included carbohydrate metabolism (KO:09101) and signal transduction (KO:09132) (Figure 5C; Supplementary Tables S21 and S22).

![Figure 5](image)

**Figure 5.** KEGG annotation of (A–C) DEGs and (D) common DEGs. The bar plots show the number of DEGs identified in the relative pathway based on the KEGG pathway database.

The KEGG analysis results of the common DEGs were similar to those of the DEGs (Figure 5D; Supplementary Tables S23 and S24). A total of 80 pathways included upregulated genes and 199 pathways included downregulated genes during *N. ceranae* infection (Figure 5D). A high number of downregulated common DEGs were involved in the pathways of signal transduction (KO:09132); protein families: genetic information processing (KO:09182) and carbohydrate metabolism (KO:09101) within the environmental information process (KO:09130); brite hierarchies (KO:09180); and metabolism (KO:09100) categories.
3.6. Validation

Four highly upregulated common DEGs (AAJ76_1600052943, AAJ76_2000149161, AAJ76_2000141845, and AAJ76_500091146) and three highly expressed stage-specific genes (AAJ76_5000026485, AAJ76_2400020190, and AAJ76_2300035467) at each time post-infection were selected to validate the RNA-seq results during infection by *N. ceranae*. The results showed that all of the upregulated common DEGs increased at 3 d.p.i. and reached a high peak at 15 to 20 d.p.i., while no or a low abundance of gene expression was detected at 0.5 to 1 d.p.i. (Figure 6). The validation result matched the detection of *N. ceranae* infection, which was detectable at 3 d.p.i. (Figure 1D), suggesting that *N. ceranae* begins the infection process and initiates gene expression at 3 d.p.i. In addition, the upregulated common DEGs, reaching a high peak at 15–20 d.p.i., also showed coordination with the increasing spore count of *N. ceranae* (Figure 1C).

![Figure 6](image-url)

**Figure 6.** Validation of *N. ceranae* gene expression in upregulated common DEGs during *N. ceranae* infection. The bar plots show the relative gene expression levels of different common DEGs at different d.p.i. Different letters indicate significant differences (*p* < 0.05).

For the stage-specific gene validations, the stage-specific genes were only expressed at specific times post-infection, while the gene expression of AAJ76_5000026485, AAJ76_2400020190, and AAJ76_2300035467 was detected at all times post-infection, including in mature spores (Figure 7). However, the results of three stage-specific genes showed higher expression levels at 5, 10, and 20 d.p.i., revealing a similar expression trend as the transcriptomic prediction (Figure 7).

![Figure 7](image-url)

**Figure 7.** Validation of *N. ceranae* gene expression in upregulated stage-specific genes during *N. ceranae* infection. The bar plots show the relative gene expression levels of different stage-specific genes at different d.p.i. The black arrowheads indicate the time point at which the highest expression level of the gene was predicted based on RNA-seq data. Different letters indicate significant differences (*p* < 0.05).
4. Discussion

The pattern of *N. ceranae* infection in this study was similar to a previous report that *N. ceranae* was detectable in an early infection stage at 3 d.p.i., while mature spores were only observed at 5 d.p.i. [18,49]. Negative impacts on the survival fitness of *N. ceranae*-infected honey bees have been reported, and they explain the changes to immune suppression, nutritional deficiencies, and hormone regulation [18,50–52]. These data indicate that infected *N. ceranae* might begin to affect and have consistent negative effects on honey bees at earlier infectious stages. Altogether, these data reveal that *N. ceranae* infections can be detected as early as 3 d.p.i. and the infection level dramatically increases from 10 to 20 d.p.i.

The gene expression profiling of *N. ceranae* was described at the transcriptomic level in this study. The results indicated that a greater upregulation of *N. ceranae*'s common and stage-specific genes was found in the initial infectious stage, whereas more genes of *N. ceranae* were suppressed in the late stage of infection. This tendency was similar to the study of *N. ceranae* gene expression in *Apis cerana*, suggesting that the *N. ceranae* genes were expressed at an early infection stage, followed by the gene expression profile of *N. ceranae* switching from the merogony to sporogony stages [53]. Microsporidia lack mitochondria, and the loss of most metabolic pathways might involve an alteration of gene expression during the course of infection; therefore, host-generated ATP would be imported to support their metabolism [54,55]. Several highly expressed stage-specific genes (AAJ76_5000026485, AAJ76_2400020190, AAJ76_2300035467, and AAJ76_600074061) were identified as pre-mRNA splicing factors, indicating that post-transcriptional regulation was continually activated during *N. ceranae* infection.

For the GO annotation of common DEGs, cytosol (GO:0005829) and cytoplasm (GO:0005737) were also found in the upregulated common DEG group. The cytoplasm mainly comprises microsporidia, containing many ribosomes in a regular array [56]. During the replication of sporonts, division by binary fission produces two sporoblasts and infects the hosts through karyokinesis and cytokinesis [2]. Therefore, the upregulation of genes belonging to the cytoplasm with corresponding GO terms might contribute to the early propagation of *N. ceranae*. At the initiation of microsporidial infection, sporoplasm was injected into the cytoplasm of the host cell by the germination of the polar tube [2]. Because of the lack of genes for many metabolic pathways in the microsporidia, the microsporidia acquire nutrients from the host. Microsporidia in contact with the host cytoplasm could provide more opportunities to export cytotoxic compounds across the plasma membrane as an alternative to compensate for development [57]. In addition, upregulated genes belonging to the ATP-binding (GO:0005524) pathway were found in the early stage of *N. ceranae* infection (NcSp/5 d.p.i.). In the ATP-binding (GO:0005524) pathway, the transmembrane protein superfamily of microsporidia, which includes ATP-binding cassette (ABC) transporters, plays an important role in substrate transportation and pathogen development, indicating that energy transport is abundant in this stage [57].

It was noted that metal ion binding (GO:0046872), GTP binding (GO:0005525), and the ubiquitin-dependent protein catabolic process (GO:0006511) showed high gene counts as observed in the downregulated common DEGs. Metal ions are vital elements for proper folding and the ribozyme catalysis of nucleic acids [58–60]. GTP-binding proteins regulate cellular processes for multiple protein biosynthesis and intracellular membrane traffic [61]. The role of the ubiquitin–proteasome pathway has been reported to be the selective degradation of soluble cellular proteins under most conditions [62]. The downregulation of the genes belonging to the GO term indicate the requirement of nucleotide and protein bases from host cells in *N. ceranae* infections. In contrast, many stage-specific genes of *N. ceranae* were involved in pre-mRNA-associated pathways, including “RNA binding” (GO:0003723) and “mRNA splicing, via spliceosome” (GO:0000398), suggesting the impacts on the transcriptional level for *N. ceranae* infection.

Based on the result of KEGG pathway analysis on DEGs, the categories of metabolism and environmental information processing included a high number of downregulated genes. Moreover, in the pathways of metabolism, the “carbohydrate metabolism” included
a high number of downregulated genes at each infection stage. It has been reported that *Nosema* acquires carbohydrates from the epithelial cells of the honey bee gut lining, leading to a series of metabolic changes in the host [3,20]; therefore, this point was also proven by the downregulation of genes belonging to “carbohydrate metabolism”.

Instead of mitochondria, microsporidia contain a genome-less organelle called a mitosome, which encodes only 20 mitosomal proteins in microsporidia, indicating the loss of function of the mitosome organ compared to that of normal mitochondria in the yeast *Saccharomyces cerevisiae* [32,63]; therefore, the spore cores still maintain basic metabolism by their own system. In the beginning of infection, genes belonging to the “folding, sorting, and degradation (K09123)” and “replication and repair (K09124)” pathways in the genetic information processing (K09120) categories, the cellular processes pathway (KO:09140) of “cell growth and death” (K09143), and the environmental information processing pathway (KO:09130) of “signal transduction” (K09132) were highly upregulated in the NcSp/5 d.p.i. group. Meanwhile, the genes involved in these pathways were downregulated at a late stage of *N. ceranae* infection (NcSp/10 d.p.i. and NcSp/20 d.p.i. groups), suggesting that the merogony stage needs to initiate more functions of genetic and information processes than mature spores to facilitate the propagation of *N. ceranae* and maintain the activities of infection. Lastly, from our data, the downregulated genes involved in the pathway of carbohydrate metabolism suggest that *N. ceranae* takes energy from honey bees during infection [8].

The validation of four highly upregulated common DEGs and three highly expressed stage-specific genes at each time post-infection showed similar tendencies to those of the DEG predictions. The AAJ76_1600052943 and AAJ76_2000141845 showed a functional homology to the spore wall proteins SWP25 and SWP30, respectively. In *Nosema bombycis*, the two spore wall proteins have been characterized as an endosporal protein (SWP30) and an exosporal protein (SWP32) [64,65]. These two spore wall proteins bind to the chitin component of *N. bombycis*, implying that these proteins are the structural support proteins in the spore wall. SWP25 was first found in *N. bombycis*; the localization of SWP25 is the endospore, and this protein possesses a signal peptide and a heparin-binding motif. In addition, SWP25 is critical for host-to-host transmission and persistence in the environment [66]. Furthermore, SWP25 has been reported to be related to spore wall integrity and germination, and recent research has shown that the induction of SWP25 expression after binding with haematopoietin may change the integrity of pathogens and affect the clearance of immune cells or the transmission of pathogens in haemolymph [65,67]. Therefore, the upregulation of AAJ76_1600052943 and AAJ76_2000141845 might also play roles similar to those mentioned above to facilitate spore germination and mature spore development. The highly expressed SPW was consistent with our previous study on *N. ceranae* gene expression in *A. mellifera* [33] and the research on the *N. ceranae* gene expression in *A. cerana*, suggesting a significant role for SPW during *N. ceranae* infection [53].

AAJ76_2000149161 was mapped to a DNA-dependent protein kinase catalytic subunit (DNA-PKcs) by SWISS-MODEL [68]. DNA-PKcs is a key member of the phosphatidylinositol-3 kinase-like (PIKK) family of protein kinases and plays an essential role in the repair of DNA double-strand breaks by nonhomologous end joining (NHEJ) [69]. The upregulation of this gene might contribute to the relative function of *N. ceranae* in using host DNA fragments for its replication. AAJ76_500091146 is described as a forkhead hnf3 transcription factor based on protein functional prediction. HNF-3/FKH genes are a large family of transcriptional activators, and all forkhead proteins contain a highly conserved DNA binding domain and play numerous regulatory roles in eukaryotes. At least four genes in yeast are involved in a wide range of biological functions, such as apoptosis, cell cycle, immunity, development, growth, stress resistance, metabolism, and reproduction [70,71]. Based on the above functions, the upregulated AAJ76_500091146 might be involved in multiple regulatory roles in the development and growth of *N. ceranae*.

Interestingly, the functional prediction of all three stage-specific genes indicated post-transcriptional modification. AAJ76_5000026485 is a functional homologue of the pre-
mRNA-splicing factor cwf14, which is a type of spliceosome that catalyzes the excision of introns from pre-mRNA [72]. A study from yeast showed that cwf14 functions in the regulation of cell growth and mRNA splicing and is also a key factor regulating cell wall biogenesis [73]. The function of AAJ76_2400020190 was found to match that of the putative H/ACA ribonucleoprotein complex subunit 3, which is known as a small nuclear RNP (snRNP). snRNP predominantly guides the site-directed pseudouridylation of target RNAs, processes ribosomal RNA, and stabilizes telomerase RNA [74]. Moreover, the function of H/ACA ribonucleoproteins is essential for ribosome biogenesis, pre-mRNA splicing, and telomere maintenance [74]. AAJ76_2300035467 is functionally similar to U6 snRNA-associated Sm-like protein LSm6. The Sm proteins are RNA–protein complex proteins, and LSm6 is an Sm-like protein (LSM) in S. cerevisiae. A study of S. cerevisiae suggested that LSm6 is associated with U6 snRNA and is required for the maintenance of normal U6 snRNA levels and for pre-mRNA splicing [75]. In summary, the primary transcription products (pre-mRNAs) undergo a variety of post-transcriptional modifications to create functional mRNAs [76]. These three stage-specific genes were identified at different infection stages, and all associations with pre-mRNA splicing suggest that they are highly involved in the activation of post-transcriptional modifications of N. ceranae at each infectious stage.

5. Conclusions

In this study, the transcriptomic analysis results indicated alterations in the N. ceranae gene expression status during A. mellifera infection. The identification of the common and stage-specific genes expressed during the infection process revealed the interaction between N. ceranae and A. mellifera. Downregulated genes during the infection, especially at a late period of infection, were found. Regarding RT–qPCR validation, the common DEGs showed higher expression levels from 5 d.p.i. to 20 d.p.i., indicating the importance of common DEGs. In our study, we attempted to identify the DEGs resulting from gene expression profiling based on transcriptomic analysis and proposed the common and stage-specific genes of N. ceranae during its infection of A. mellifera. Our data also describe the relatively comprehensive gene expression of the N. ceranae infection stage and analyze the details during infection, thus providing basic information on the N. ceranae mechanism during infection and furthering the application, including the early detection of nosemosis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13080716/s1, Figures S1 and S2; Tables S1–S24. Figure S1. The detection of Nosema spp. of newly emerged adult honey bees by multiplex PCR before experiment. The information of primer sets were listed in Supplementary Tables S1; Figure S2. The pipeline of transcriptome analysis. The commend lines and analysis purposes are noted pairwise the item. DEG = differential expressed gene; FPKM = fragment per kilobase per million; GO = gene ontology; KEGG = Kyoto Encyclopedia of Genes and Genomes; Table S1. Primer sets list; Table S2. RNA sequencing summary; Table S3. Transcriptomic data for honey bee infected with Nosema ceranae were screened by mapping to the genomes of Nosema apis and 6 honey bee infecting viruses; Table S4. Up-regulated DEG list of NcSp/5 d.p.i. comparison group; Table S5. Down-regulated DEG list of NcSp/5 d.p.i. comparison group; Table S6. Up-regulated DEG list of NcSp/10 d.p.i. comparison group; Table S7. Down-regulated DEG list of NcSp/10 d.p.i. comparison group; Table S8. Up-regulated DEG list of NcSp/20 d.p.i. comparison group; Table S9. Down-regulated DEG list of NcSp/20 d.p.i. comparison group; Table S10. Non-core genes list at each time post infection; Table S11. Up-regulated GO list of NcSp/5 d.p.i. comparison group; Table S12. Down-regulated GO list of NcSp/5 d.p.i. comparison group; Table S13. Up-regulated GO list of NcSp/10 d.p.i. comparison group; Table S14. Down-regulated GO list of NcSp/10 d.p.i. comparison group; Table S15. Up-regulated GO list of NcSp/20 d.p.i. comparison group; Table S16. Down-regulated GO list of NcSp/20 d.p.i. comparison group; Table S17. Up-regulated KEGG list of NcSp/5 d.p.i. comparison group; Table S18. N.c spore vs. 5 d.p.i. Down regulated KEGG list; Table S19. N.c spore vs. 10 d.p.i. Up regulated KEGG list; Table S20. N.c spore vs. 20 d.p.i. Up regulated KEGG list; Table S21. N.c spore vs. 20 d.p.i. Down regulated KEGG list; Table S22. N.c spore vs. 20 d.p.i. Down regulated KEGG list; Table S23. Up-regulated KEGG list of core genes; Table S24. Down-regulated KEGG list of core genes.
Author Contributions: Conceptualization, Y.-H.L. and Y.-S.N.; methodology, Y.-H.L., Z.-T.C., T.-H.C. and J.-C.C.; software, Y.-H.L., Y.-F.H. and M.-R.Y.; validation, Y.-H.L.; formal analysis, Y.-H.L. and M.-R.Y.; investigation, Y.-H.L.; resources, M.-C.W. and Y.-L.Y.; data curation, Y.-H.L.; writing—original draft preparation, Y.-H.L.; writing—review and editing, Y.-F.H., M.-C.W., Y.-C.W. and Y.-S.N.; supervision, Y.-S.N.; project administration, Y.-S.N.; funding acquisition, Y.-S.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by 109-2313-B-005-048-MY3 from the Ministry of Science and Technology (MOST).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The RNA-Seq data were deposited in NCBI with BioProject ID: PRJNA814859.

Conflicts of Interest: The authors declare no conflict of interest.

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