

Transcriptomic analysis of genes related to neuronal functions in *Apis mellifera*: expression dynamics during the progression of DWV-A infection

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Article

Keywords: *Apis mellifera*, DWV-A, antenna, head, transcriptome

Posted Date: March 18th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9087825/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on June 20th, 2026. See the published version at <https://doi.org/10.1038/s41598-026-57691-z>.

Abstract

Deformed Wing Virus type A (DWV-A) is one of the primary threats to honeybees (*Apis mellifera*), significantly impacting their nervous system physiology and behavior. While its neurotropic nature is well-recognized, the temporal dynamics of the neuronal transcriptomic response following oral infection, the natural transmission route, remains poorly understood. In this study, we analyzed gene expression in the heads of worker bees orally inoculated with DWV-A over a 16-day time course (1, 4, 7, 10, 13, and 16 days post-inoculation). RNA-seq analysis at day 10 identified 147 differentially expressed genes, primarily associated with mitochondrial function, energy metabolism, and neuronal processes. RT-qPCR validation revealed a persistent downregulation of key genes related to glutamatergic system (*eaat-2*, *neto*, and *kainate*) and sensory perception related-genes in the antennae. Notably, the simultaneous co-expression of nurse-associated and forager-associated marker genes suggests that DWV-A infection induces an asynchrony in behavioral maturation. Our findings demonstrate that DWV-A disrupts neuronal homeostasis and peripheral sensory perception in a tissue-specific and time-dependent manner, providing a molecular framework to understand the behavioral impairment and the loss of coordination at the colony level.

INTRODUCTION

Pollination is a key ecosystem service that sustains biodiversity and agricultural productivity. Among pollinating insects, the honeybee (*Apis mellifera* L.) plays a central role, being responsible for the pollination of many crop species worldwide, particularly in extensive monoculture systems^{1, 2, 3}. This ecological and economic relevance is closely linked to its polylectic foraging behavior and its ability to detect, discriminate, and memorize a wide diversity of floral odors⁴⁻⁶. Such behavioral complexity relies on the integration of olfactory and visual information through learning and memory processes supported by a highly specialized nervous system⁶⁻⁸.

The honeybee olfactory system is organized as an extensive neural network connecting peripheral sensory structures in the antennae with central processing centers in the brain. Odorant signals are transduced by olfactory receptor neurons and transmitted to the antennal lobes, where information is encoded within glomerular circuits before being relayed to higher brain regions^{6, 9, 10}. This architecture enables the precise processing of complex odor landscapes underlying orientation, learning, and foraging decisions¹¹.

Olfactory performance and neural function in *A. mellifera*, as in other insects such as *Drosophila melanogaster*, are regulated by dynamic cellular and molecular interactions, including glial neuronal coupling, neuromodulatory signaling, and gene regulatory programs. Glial cells contribute to the maintenance and modulation of olfactory networks^{12, 13}. These regulatory mechanisms include pathways associated with neurotransmitter dynamics, such as glutamatergic signaling, supporting the functional plasticity required for adaptive behavior¹⁴.

The integrity of neural system is compromised by the Deformed Wing Virus (DWV), one of the most prevalent and damaging pathogens of honeybees worldwide^{15, 16}. Mainly transmitted by the ectoparasitic mite *Varroa destructor*, DWV comprises several epidemiologically relevant variants, among which DWV-A is the most prevalent in Chile and is frequently associated with colony losses^{17, 18}.

DWV is neurotropic and can replicate in both central and peripheral nervous tissues, including the brain and antennae, where it disrupts neuronal ultrastructure, synaptic gene expression, and olfactory sensitivity^{19–21}. High viral loads are associated with transcriptomic alterations affecting synaptic function and neurotransmission, impairments in memory, and reduced behavioral performance^{14, 22}. Furthermore, DWV-A alters the expression of odorant-binding proteins and pre- and postsynaptic genes in the antennae, directly compromising peripheral olfactory processing^{22, 23}.

Despite recent advances, how DWV-A induced molecular alterations affect information flow, and their functional consequences on odor-guided behaviors remain poorly understood. Here, we combined transcriptomic and targeted expression analyses to test whether DWV-A infection induces coordinated metabolic, proteostatic, and glutamatergic alterations in honeybee heads and antennae, potentially underlying previously reported olfactory and cognitive impairments.

MATERIALS AND METHODS

Preparation of the viral inoculum

The viral inoculum used for all bioassays was prepared as described by Gusachenko et al.²⁴ and Silva et al.²². The viral inoculum was obtained from field honey bee colonies maintained in an experimental apiary. Colonies were previously screened by quantitative PCR (qPCR) to determine the presence and viral load of Deformed Wing Virus variant A (DWV-A). In parallel, the presence of other pathogens, *Nosema* spp., *Lotmaria* spp., DWV-B, Acute Bee Paralysis Virus (ABPV), and Chronic Bee Paralysis Virus (CBPV), were also assessed. The selection of the colony used for viral inoculum preparation was based on viral quantification criteria, including a Ct value > 15 for DWV-A and Ct values < 32 for the other pathogens described above. A group of 20 symptomatic bees was homogenized in phosphate-buffered saline (1X PBS) for 90 seconds at high-speed using a Stomacher 80 laboratory blender (Seward, London, UK). The homogenate was then centrifuged twice: first at 1500× g for 10 minutes, followed by 10,000× g for 10 minutes, both at 4°C. The supernatant was purified by filtration through a 0.22 µm filter (PES, Merck Millipore, Darmstadt, Germany). To determine the exact concentration of viral particles present in the inoculum, a 200 µL aliquot of the supernatant was collected for RNA extraction, cDNA synthesis, and subsequent quantification of the viral load via qPCR, following the methodology detailed in the RNA extraction section. The remaining extracted inoculum was stored at – 80°C until further use.

Viral inoculation

A. mellifera workers used in all experimental procedures were obtained from the apiary of the experimental station "El Nogal" (36°35'58.25" S, 72°04'51.77" W), University of Concepción, Chillán, Chile. To ensure uniformity in age, brood frames were extracted directly from healthy hives and maintained under controlled conditions ($30 \pm 1^\circ\text{C}$, $60 \pm 3\%$ RH) until adult emergence. Donor colonies were pre-selected based on pathogen screening, prioritizing those free from *Nosema ceranae* (Ct < 32), *Lotmaria passim* (Ct < 32), and exhibiting either undetectable or minimal viral loads of acute bee paralysis virus (ABPV, Ct < 32), chronic bee paralysis virus (CBPV, < 32), and DWV (< 32).

Newly emerged bees were randomly collected and transferred to cylindrical plastic cages (base diameter: 8 cm; top diameter: 10 cm; height: 15 cm). Viral infection procedures followed previously established protocols²³, with modifications. Briefly, bees in the inoculated group (Treatment i-DWV) were orally inoculated with 5 μL of a viral suspension containing 1.0×10^9 genome copies per bee, prepared in 60% (w/v) sucrose solution. Individuals that did not ingest the full inoculum were excluded. The control group (Treatment n-DWV) received only sucrose solution (60% w/v), without viral particles.

A total of 600 worker bees were used, distributed into 8 independent plastic cages (replicates), each containing 75 bees. Four cages were assigned to the inoculated group (n = 300) and four to the control group (n = 300). All bees received *ad libitum* access to a 60% sucrose syrup and 3 g of a standardized pollen substitute composed of soybean flour, brewer's yeast, cornstarch, wheat flour, corbicular pollen, and canola oil, following Arismendi et al.²⁵.

Sampling was performed every 3 days, starting 24 hours after viral inoculation. At each time point, a pool of five bees was collected per replicate cage. Sampling days included 1, 4, 7, 10, 13, and 16 days post-inoculation (dpi). Additionally, a specific sample was taken at 10 dpi for subsequent RNA-seq analysis. All collected bees were immediately frozen at -80°C until use.

RNA extraction and quality assessment

Total RNA was extracted from bee heads with antennae collected at each bioassay time point, following the protocols described by Kim et al.²⁰, Mondet et al.²⁶, and Silva et al.²², with minor modifications. Briefly, pools of dissected heads including antenna from each replicate and sampling day were homogenized in 500 μL of TRIzol™ Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) using a Retsch homogenizer at maximum speed. Following homogenization, 5 μL of RNA Carrier (Invitrogen) was added according to the manufacturer's instructions. The homogenate was incubated on ice for 5 minutes, followed by the addition of 200 μL of chloroform. Samples were then vortexed briefly, incubated on ice for an additional 3 minutes, and centrifuged at $10,000 \times g$ for 15 minutes at 4°C . The aqueous phase was carefully transferred to a new tube for RNA purification. For most samples, RNA was extracted using the E.Z.N.A.® Total RNA I Kit (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's protocol. In the case of samples collected at 10 dpi, used for transcriptomic analysis, total RNA was isolated using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, CA, USA), according to the manufacturer's instructions.

RNA concentration and purity were determined spectrophotometrically using an Infinite® 200 PRO NanoQuant microplate reader (Tecan Group, Männedorf, Switzerland). RNA integrity was evaluated by electrophoresis on 1% agarose gels. All RNA extracts were stored at – 80°C until downstream applications.

Sequencing

Library construction and high-throughput sequencing services were outsourced to Austral-omics (Valdivia, Chile). A total of six cDNA libraries were generated (three per experimental condition). For mRNA library preparation, 300 ng of total RNA per sample was used as input, and the RNA Directional Library Prep Set (MGI Tech) was employed, following the manufacturer's instructions. Insert size and final library integrity were verified using capillary electrophoresis on a Fragment Analyzer system (Agilent Technologies) with the DNF-474 High Sensitivity NGS Fragment Analysis Kit. Library quantification was performed using the Qubit™ DNA High Sensitivity Assay Kit (Thermo Fisher Scientific), and the volume required for a final concentration of 1 pM was calculated accordingly.

cDNA strand separation and circularization were carried out, followed by DNA nanoball (DNB) synthesis, loading of the flow cell, and sequencing. Short-read sequencing was performed using the DNBSeg technology (MGI Tech) on the DNBSeg-G400 platform, generating paired-end reads of 150 nucleotides.

Data preprocessing and identification of differentially expressed genes

Raw read quality and the presence of adapter sequences were assessed using FastQC (available at www.bioinformatics.babraham.ac.uk/projects/fastqc). To remove adapter sequences and to trim low-quality bases from the raw reads, we used Trimmomatic (v0.39) in paired-end mode with the following parameters: TRAILING:10, LEADING:5, SLIDINGWINDOW:4:20, ILLUMINACLIP:DNBSEQ_adapter.fa:2:30:10. Reads shorter than 50 bp were filtered out, using MINLEN:50 parameter in the same step.

The filtered reads obtained in the previous step were aligned to the most recent *A. mellifera* genome assembly, Amel_HAV3.1 along with its corresponding *gtf* file (NCBI accession number: GCF_003254395)²⁷, using STAR software (v2.7.6a)²⁸ with the option `--outFilterMultimapNmax` set at 1.

To improve statistical power, a filtering step to remove low-expressed genes was performed using the HTSFilter package²⁹ with a modeling length of $s.len = 100$. Differential expression analysis was subsequently conducted on the filtered genes using the DESeq2 package v1.42.0³⁰ through the DESeqfunction, employing a Wald test with parametric dispersion fitting. To control the False Discovery Rate (FDR), Storey's q-values³¹ were calculated from the raw p-values using the qvalue package. Throughout this study, these significance values are referred to as 's-values' to maintain consistency with the bioinformatics pipeline outputs. Genes with an s-value (q-value) < 0.05 were considered statistically significant.

Subsequently, the sequences corresponding to all RNA-seq analysis and all DEGs, were analyzed for functional enrichment in terms of Gene Ontology (GO) categories. To assess the functional enrichment of GO terms, a Gene Score Resampling (GSR) analysis was performed using ErmineJ (V.3.1.2 desktop version for macOS). The analysis utilized the s-values as input, with the 'big is better' option enabled to prioritize higher values. The 'Mean' class scoring method was applied, and statistical significance was determined through 200,000 iterations to ensure robust empirical p-value estimation. Gene set size constraints were set to a minimum of 10 and a maximum of 200 genes. GO terms with a corrected p-value < 0.05 were considered statistically significant.

Pfam protein domains associated with each DEG were identified using InterProScan (v 5.73–104.0), considering the longest transcript as the representative for each gene. Significantly overrepresented domains were identified through a hypergeometric test ($p < 0.05$). The background set consisted of all domains identified in the longest transcripts of the total gene set encoded in the genome

cDNA synthesis and qPCR

For viral load quantification, a standard curve was generated using purified PCR products (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA), quantified by spectrophotometry (Epoch™ Microplate Spectrophotometer, BioTek, Winooski, VT, USA). Copy numbers were calculated following Wu et al.³², and standard curves were generated using serial dilutions (1.0×10^1 to 1.0×10^9). Ct values were plotted against log₁₀ copy numbers, and viral loads were expressed as DWV-A copies per bee after normalization and dilution correction³³.

cDNA derived from head and antenna samples collected at 10 dpi, the same material used for RNA-seq, was included. In parallel, independent samples collected at 4, 7, 13 and 16 dpi were dissected into heads and antennae, and processed for cDNA synthesis and qPCR.

For cDNA synthesis, the High-Capacity Reverse Transcription Kit (Applied Biosystems; Invitrogen, Life Technologies, Carlsbad, CA, USA) was used according to the manufacturer's instructions. Viral quantification and host gene expression analyses were performed using cDNA corresponding to samples collected at each point, with *β-actin* and *18 rps* used as normalization genes (Table 1). qPCR reactions were performed using Applied Biosystems MasterMix (Thermo Fisher Scientific, Wilmington, MA, USA) in a final volume of 10 μL containing 10 ng of cDNA and 530 μM of each primer. Thermal cycling conditions were optimized for each primer pair. Reactions were run on a StepOne Real Time PCR System de Applied Biosystems (Waltham, MA 02451, USA), and data were analyzed using StepOne software.

Table 1
Primer sequences designed for quantitative real-time PCR (qPCR) analysis of gene expression, including DWV, housekeeping genes, and genes of interest (Neto, Kainate, and Eaat-2).

Gene	F/R	Sequence	Target
DWV-A	F	TATCTTCATTAAAGCCACCTGGAA	Virus
	R	TTTCCTCATTAAGTGTGTCGTTGAT	
<i>Arp1</i>	F	ATGCCAACACTGTCCTTTCTGG	housekeeping 1
	R	GACCCACCAATCCATACGGA	
<i>18rps</i>	F	GATTCCCGATTGGTTTTTGAATAG	housekeeping 2
	R	AACCCCAATAATGACGCAAACC	
<i>Neto</i>	F	ATCAGAGGCGACCTTTCAGC	Neto (LOC411019)
	R	TGACAGTTTGACGGGGATGG	
<i>Kainate</i>	F	TGCTTCCTCCCGACAATGAC	Kainate (LOC102653800)
	R	AGCAATCCCGTAACCCTTGG	
<i>Eaat-2</i>	F	TTATCGCGGCGCTCATTCTT	Eaat-2 (LOC406113)
	R	TCCTCTTACGTGCAACACAGG	

Data Analysis

qPCR data were analyzed to evaluate the effects of viral inoculation and time post infection on gene expression and viral load. For each gene, relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Genes were analyzed independently, and at each dpi, inoculated (i-DWV) and non-inoculated (n-DWV) groups were compared.

Data normality was assessed using the Shapiro–Wilk test and Q–Q plots. As the data did not consistently meet parametric assumptions, pairwise comparisons were performed using the Wilcoxon rank-sum test. p-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate correction. Viral load data are presented as mean $\pm$ standard error (SE). All analyses and graphical visualizations were performed in R (RStudio 2025.09.1). Statistical significance was set at adjusted $p < 0.05$.

RESULTS

Sequencing and mapping metrics

The RNA-seq analysis yielded over 145 million paired-end reads in total, with an average of 24.29 millions per experimental replicate. After preprocessing, more than 91.5% of the raw reads were

retained. These high-quality reads were subsequently aligned to the *A. mellifera* reference genome, resulting in an average of 20 million mapped reads per replicate, corresponding to 90.23% of the processed reads (see Supplementary Table S1).

Identification of Differentially Expressed Genes (DEGs)

Differential expression analysis was conducted on 9,113 genes, representing 74% of the total genes encoded in the genome. A total of 147 genes (1.61% of the analyzed genes) were identified as differentially expressed (DEGs) between the i-DWV and n-DWV treatments ($s\text{-value} < 0.05$) (Fig. 1, Supplementary Table S2). Among these DEGs, 109 genes were up-regulated and 38 were down-regulated in the i-DWV treatment compared to the n-DWV condition (Fig. 2, Supplementary Table S2).

Notably, several immune and metabolic genes were among the most highly up-regulated transcripts, including the antimicrobial peptides *hymenoptaecin*, *abaecin*, and *apidaecin*, as well as *L-lactate dehydrogenase* (LDH; LOC411188), each exhibiting a $\log_2\text{FoldChange} > 1.5$. Additionally, our analysis identified the up-regulation of three key genes representing complementary regulatory layers of the glutamatergic synapse: *Neto* (LOC411019), involved in synaptic scaffolding; *Eaat-2* (LOC406113), responsible for extracellular glutamate clearance; and the *Kainate receptor* (LOC102653800), central to synaptic signal reception. We also detected the induction of transcription factors associated with behavioral maturation, specifically *cwo* (LOC726204) and *nau* (LOC724636).

Conversely, critical components of the cellular proteostasis network were significantly down-regulated ($\log_2\text{FoldChange} < -1$). These included *Stip1* (*Stress-induced phosphoprotein 1*, LOC102656934) and *Hsp70* (*Heat shock protein 70*, Gene ID: 409587). (see Supplementary Table S2 for details)

Enrichment analysis

To provide a functional context, a GO enrichment analysis was first performed using the entire set of 9,113 genes as a background. This identified 176 significantly enriched terms ($p < 0.05$), primarily associated with essential constitutive processes such as protein synthesis, tRNA processing, and peptide metabolism (Fig. 3). This baseline profile reflects the high metabolic and translational activity required for cellular maintenance under the studied conditions.

Subsequently, a targeted GO enrichment analysis was conducted specifically on the 147 DEGs to identify biological functions specifically modulated by the i-DWV treatment. This analysis revealed 23 significantly enriched terms ($p < 0.05$) (Fig. 4). The most represented categories included cellular anatomical structure (GO:0110165; 68 genes), cellular process (GO:0009987; 56 genes), and catalytic activity (GO:0003824; 39 genes), spanning the three main GO domains.

Focusing on the cellular process category, the increased viral load in the heads of i-DWV bees specifically modulated genes associated with cell communication and transmembrane transport. Within this group, several genes related to neurotransmitter signaling were up-regulated, suggesting an alteration in synaptic excitability and amino acid homeostasis. For instance, we identified the up-

regulation of *Glutamate receptor ionotropic, kainate* (LOC102653800) and *Excitatory amino acid transporter 2* (LOC406113), both key players in glutamatergic neurotransmission. Additionally, *Neuropilin and tolloid-like* (LOC411019), involved in axon guidance, and the *Sodium-dependent nutrient amino acid transporter 1* (LOC725346) showed increased expression.

Regarding the protein domain analysis, a total of 77 Pfam domains were significantly enriched within the DEG set. The 13 most significantly overrepresented domains (those with the lowest p-values, $p < 0.05$) are shown in Fig. 5, while the complete list is provided in Supplementary Table S3.

Notably, the Chitin binding Peritrophin-A domain (PF01607) and the Polysaccharide deacetylase domain (PF01522) were among the enriched categories; these domains are present in the genes *cda5* (LOC552276) and *serp* (LOC408365), respectively, which are known to modulate structural architecture. Additionally, we found significant enrichment of the Helix-loop-helix DNA-binding domain (PF00010), a feature of transcription factors such as *Cwo* (LOC726204) and *Nau* (LOC724636), previously described as regulators of the nurse-to-forager transition.

The analysis also highlighted the enrichment of the CUB domain (PF00431) and the Receptor family ligand binding region domain (PF01094). These domains are structural components of the synaptic gene *Neuropilin and tolloid-like* (LOC411019) and the *Glutamate receptor ionotropic, kainate* (LOC102653800), respectively. These findings reinforce the relevance of the glutamate-related regulatory genes identified in the DEG analysis, all of which were up-regulated in i-DWV bees.

Conversely, the Tetratricopeptide repeat domain (PF00515) and the Hsp70 protein domain (PF00012) were also significantly enriched. These domains are characteristic of *Stress-induced phosphoprotein 1* (LOC102656934) and *Heat shock protein 70* (Gene ID: 409587), both of which were down-regulated in i-DWV bee heads.

Temporal dynamics of viral load and gene expression validation

To validate the transcriptional profile observed at 10 dpi and explore the temporal dynamics of the infection, we focused on the glutamatergic triad: *Neto*, *Eaat-2*, and *Kainate*. In head samples, viral loads were significantly higher in the i-DWV group compared to the control (n-DWV) across all time points ($p < 0.0001$; Fig. 6). Notably, the qPCR results at 10 dpi confirmed the up-regulation of all three genes observed in the RNA-seq analysis, reinforcing the reliability of the transcriptomic snapshot (Fig. 7).

The temporal analysis in heads revealed a biphasic response: *Neto* showed early up-regulation at 7 and 10 dpi ($p < 0.01$), followed by a significant reduction at 13 and 16 dpi ($p < 0.01$). Similarly, *Eaat-2* and *Kainate* peaked at 10 dpi ($p < 0.001$ and $p < 0.05$, respectively) before declining significantly by 16 dpi ($p < 0.0001$) (Fig. 7).

The antennal dynamics showed a progressive down-regulation of the triad, which became statistically significant at the later stages of infection. Specifically, *Neto* expression decreased at 13 and 16 dpi ($p < 0.0001$), while *Eaat-2* and *Kainate* reached significant down-regulation at 16 dpi ($p < 0.001$) (Fig. 9). This

suggests that while the initial viral impact triggers a compensatory response in the brain (heads), the sensory tissues (antennae) exhibit a steady decline in the expression of these synaptic integrity genes. For the load virus and expression analysis in antennae, the 10 dpi time point was integrated into the head analysis, as the head capsule constitutes most of the tissue mass and RNA yield at this stage. In antennal samples, viral loads were also consistently higher in inoculated bees ($p < 0.001$; Fig. 8).

DISCUSSION

Numerous transcriptomic studies in *A. mellifera* have addressed the consequences of infections by different pathogens, generally analyzing body segments such as the thorax, abdomen, or whole individuals^{34–38}. In contrast, only a few studies have focused on specific tissues such as the brain and/or other specialized tissues^{21, 39, 40}. In this study, although we included a complete head segment (comprising the brain, antennae, and other sensory organs), our DEG analysis aimed to identify global changes that could encompass both sensory receptor cells and those responsible for processing stimuli in higher-order organs.

In our analysis, the increase in viral loads in bee heads was primarily associated with an overexpression of most DEGs. We observed a consistent induction of three antimicrobial peptides (AMPs), *hymenoptaecin*, *abaecin*, and *apidaecin*, under conditions of high viral load. Although AMPs are typically induced by bacterial infections⁴¹, they have also been frequently associated with viral challenges³⁶. This observation is further supported by a meta-analysis of transcriptomes from DWV-infected heads, which identified *apidaecin* as a gene consistently overexpressed in correlation with viral increase⁴². These consistencies across studies highlight that the response to viral infection involves the activation of immune pathways; however, it is important to note that the AMP expression detected in our head samples might not be restricted to cephalic structures but could also reflect the immune activity of circulating hemocytes present in the cephalic hemolymph.

Our findings regarding gene up-regulation contrast with the results reported by Pizzorno et al.²¹ in bee brains, where a general repression of gene expression predominated. This divergence suggests that DWV exerts a dramatic and distinct effect on the physiology of the head compared to isolated brain tissue. Furthermore, the route of infection appears to be a decisive factor in these differences. While abdominal injection of DWV has been widely used to mimic transmission by *V. destructor*, generating pathogenic infections in the brain^{19, 21, 43}, our oral inoculation method represents a non-invasive and less stressful approach. Oral infection has been shown to trigger specific behavioral and physiological repercussions⁴⁴, as well as changes in gene expression related to memory¹⁴. Therefore, despite the overlaps in immune markers like AMPs with the study by Pizzorno et al.²¹, the overall transcriptional landscape seems to be strongly shaped by the mode of viral entry and the specific tissues analyzed.

The GO term analysis of our DEGs revealed a pattern centered on metabolic processes, protein synthesis, macromolecule modification, transcriptional regulation, and nucleotide metabolism. Taken

together, these results suggest that DWV reprograms the cellular machinery to favor its replication, enhancing translation and adjusting key metabolic pathways without markedly activating stress responses or apoptosis. This is consistent with the idea that the virus manipulates the host cell rather than inducing immediate damage detectable solely at the transcriptional level. In this context, recent studies have highlighted key metabolic genes such as *kynurenine/α-aminoadipate aminotransferase* (LOC724239) and *l-lactate dehydrogenase, LDH* (LOC411188), which emerge as central DEGs and 'hub genes' in responses to viral infections, including DWV, along with Toll pathway genes and AMPs⁴⁵.

In agreement with these reports, our analysis identified the overexpression of AMPs and *LDH* (LOC411188), whose function of catalyzing the reversible conversion of pyruvate to lactate is directly linked to aerobic glycolysis (AG). This process, described in cancer cells as the 'Warburg effect'⁴⁶, enables the rapid generation of ATP and NAD⁺ from glucose, even in the presence of oxygen. Such metabolic reprogramming has been proposed as a mechanism that benefits both viral replication and the availability of cellular metabolites⁴⁷. The increase in *LDH* and AG in DWV-infected bee heads could therefore have a dual impact: metabolic, by facilitating favorable conditions for viral replication, and behavioral/physiological, by modulating processes associated with the nurse to forager transition. Supporting this, AG in bee heads has been reported to be associated with increased aggressiveness⁴⁸, cellular proliferation⁴⁶, and higher glutamate production⁴⁹. Collectively, these factors could stimulate an accelerated maturation of infected bees.

Our temporal expression data further support this neurometabolic disruption, suggesting that *Eaat-2* acts as a metabolic sensor within this pathological process. As *Eaat-2* is the primary transporter responsible for removing glutamate from the synaptic cleft through an active, ATP-dependent mechanism^{50, 51}, its regulation is intrinsically tied to the cell's energetic status. While increased AG may lead to higher glutamate production, the observed downregulation of *Eaat-2*, particularly at late stages of infection, points to a critical failure in glutamate clearance. In the context of a 'Warburg-like' metabolic shift, the high energetic cost of maintaining *Eaat-2* activity likely becomes unsustainable, creating a 'double hit' effect: the convergence of increased neurotransmitter production and impaired removal. This imbalance leads to synaptic glutamate accumulation and potential excitotoxicity, providing a mechanistic link between viral-induced metabolic reprogramming and the subsequent cognitive and behavioral decline observed in infected workers. This breakdown coincides with the attainment of peak viral loads (~ 10⁹ copies/bee), suggesting that the high viral replication pressure eventually exhausts the energetic and homeostatic capacity of the neuron.

However, contrary to the effects proposed because of a possible increase in AG, Chen et al.¹⁴ determined that the increase in DWV viral loads reduced glutamate concentration due to decreased expression of genes involved in glycolysis, while still observing symptoms of both short- and long-term memory loss. In summary, our results and previous studies converge on the idea that DWV, in addition to activating immune responses (AMPs), induces profound metabolic reprogramming centered on aerobic

glycolysis. This shift could not only favor the virus but also accelerate maturation processes and alter cognitive and behavioral functions in bees.

Traniello et al.⁴² proposed the hypothesis of genetic markers explaining this accelerated maturation, identifying the increased expression of the transcription factor *cwo*, a known regulator of the nurse-to-forager transition, as a consistent feature across multiple datasets^{52, 53}. The relevance of *cwo* lies in its role as a key coordinator of circadian rhythms⁵⁴. In healthy honey bees, circadian regulation intensifies as they progress toward the foraging state, transitioning from the arrhythmic behavior of newly emerged bees to the robust circadian gene expression required for foraging⁵⁵. This rhythmicity is essential for the spatiotemporal memory needed to anticipate floral resource availability⁵⁶, which explains why *cwo* expression is naturally higher in foragers⁵².

Our findings reinforce this link between viral infection and disrupted maturation. The enrichment analysis of Pfam domains in our DEGs highlighted the Helix-loop-helix DNA-binding domain (PF00010), associated with both *cwo* (LOC726204) and *nau* (LOC724636). While these transcription factors typically act as markers for different life stages, *cwo* for foragers and *nau* for nurses^{52, 53}, our results show that both were simultaneously up-regulated in the heads of bees with high viral loads. This suggests that DWV-A does not simply push the bee into a forager state but rather triggers a lack of coordination in the developmental process. The simultaneous increase of forager-like (*cwo*) and nurse-like (*nau*) signals could lead to a physiological noise that explains the behavioral deficiencies and the breakdown of normal colony transitions observed in DWV-infected bees.

Although some effects of DWV-A are not lethal at the individual level, they can still threaten colony fitness by disrupting organizational cohesion^{42,44}. A striking finding in our study was the significant down-regulation of genes containing the Tetratricopeptide repeat (PF00515) and *Hsp70* (PF00012) domains, such as *Stress-induced phosphoprotein 1* and *Heat shock protein 70*. In *A. mellifera*, the expression of HSPs typically serves as a stress indicator; for instance, their up-regulation is commonly correlated with high infestation rates of pathogens like *V. destructor*⁶⁶. Similarly, increases in *Hsp90*, which in our study also showed a downward trend (log2FoldChange 0.9, s-value 0.07), have been associated with neurotoxic damage in bees exposed to glyphosate⁵⁷.

These precedents reinforce the idea that HSP up-regulation is a standard defense mechanism under stress. However, our results show a contrary pattern, with DWV-A inducing a down-regulation of these chaperones, opposing the behavior observed for AMPs. A possible explanation for this relates to the essential role of these proteins in proteostasis, where they act as critical regulators of proper protein folding and functional homeostasis⁵⁸. We hypothesize that the viral-induced suppression of these folding machineries, combined with high viral replication in the head, could be a key driver of the cognitive impairment reported in infected bees. This link between viral load and the breakdown of cellular homeostasis may ultimately manifest as the memory deficits and learning alterations observed in previous studies^{14, 59, 60}.

It has been validated that the ortholog of *Hsp70* in *Drosophila melanogaster* can act as a neuroprotective agent, as it has been demonstrated that in mutant insects expressing $A\beta 42$, *Hsp70* reduces protein aggregation, thereby preventing the loss of cognitive abilities and maintaining neuronal functionality^{61, 62}. Furthermore, studies in *D. melanogaster* have shown that the regulation of *Hsp70* expression under normal conditions is maintained at a constantly low level, rising exponentially under different stress conditions (thermal, pathogens, etc.)⁶³. It has also been determined that memory consolidation requires structural remodeling and protein formation, in which the role of HSPs is key for memory stabilization⁶⁴. Their involvement has also been observed in memory associated with alert responses: when activated under stress, HSPs stimulate memory formation in response to stressful events, playing a fundamental role in behavioral responses and environmental interactions of the insect⁶⁵. However, downregulation of the gene encoding *Hsp70* in *D. melanogaster* impairs long-term memory formation, with consequences for social behavioral responses⁶⁴.

This viral-induced suppression of the folding machinery could be a contributing factor to the aberrant behavioral phenotypes associated with DWV-A. In models such as *D. melanogaster*, *Hsp70* has been shown to play a neuroprotective role by preventing protein aggregation and supporting neuronal functionality. While HSPs are highly dynamic and respond to a wide array of environmental stressors⁶⁶, their down-regulation in our study, alongside the significant enrichment of the Tetratricopeptide repeat domain (PF00515), is noteworthy. This domain is a core feature of *Stress-induced phosphoprotein 1* (LOC102656934; also known as *Stip1* or *Hop*), a co-chaperone that coordinates the interaction between *Hsp70* and *Hsp90* to maintain efficient proteostasis^{67, 68}.

Although *Stip1* remains little studied in honey bees, the neuroprotective function of its homolog, *Hop*, in *D. melanogaster* is well-documented; it facilitates the removal of exogenous misfolded proteins⁶⁹ and regulates cell communication via exosome release⁷⁰. Furthermore, the human homolog *Stip1* is a recognized regulator in preventing neurodegenerative pathologies associated with proteostatic loss, such as Alzheimer's, Parkinson's, Huntington's, and amyotrophic lateral sclerosis^{71, 72}. Therefore, the simultaneous decrease of *Hsp70* and *Stip1/Hop* in bee heads might reflect a virus-driven compromise of the cellular stress network, potentially increasing neuronal vulnerability and facilitating the cognitive and behavioral deficits previously described in infected individuals^{14, 59, 60}.

Our Pfam domain analysis revealed a significant enrichment of the CUB domain, associated with the synaptic gene *Neuropilin and tolloid-like (Neto)*, and the *ligand-binding domain of ionotropic glutamate receptors of the kainate type (Kainate)*, together with the overexpression of the glutamate transporter *Excitatory amino acid transporter 2 (Eaat-2)*. These findings suggest that the alteration in the expression of genes essential for synaptic plasticity is not an isolated event, but rather the convergent outcome of the three main pathogenic axes of DWV-A that we have identified. First, the deregulation of the transcription factors *cwo* and *nau* indicates a state of premature and uncoordinated maturation, producing a physiologically immature and vulnerable brain. Second, the metabolic shift toward aerobic glycolysis (Warburg effect) alters the neuronal metabolic landscape, potentially increasing glutamate

precursors and modifying the bioenergetic environment of the synapse. Finally, the collapse of proteostasis mechanisms, evidenced by the suppression of *Hsp7* and *Stip1/Hope*, deprives neurons of their essential quality-control and neuroprotective system. It is within this scenario of physiological immaturity, metabolic stress, and disrupted proteostasis that the DWV-A–infected brain appears to attempt compensating for physiological imbalance. This compensation is visibly manifested in the transient upregulation of the 'glutamatergic triad' (*Neto*, *Eaat-2*, and *Kainate*) at 10 dpi. However, this effort is short-lived; the subsequent collapse of these targets at 16 dpi in head and antennae identifies the glutamatergic synapse as the primary site of functional failure, marking the transition from subclinical stress to terminal cognitive impairment.

The dynamic changes in the expression in the head of *Neto*, *Eaat-2* and *Kainate* observed during infection suggest a biphasic process. The early overexpression detected at 10 dpi, is consistent with a homeostatic response aimed at maintaining synaptic functionality under viral stress. However, the downregulation of these same genes at 16 dpi in bees with high viral loads suggests that this compensatory mechanism cannot be sustained over time, giving rise to a state of dysregulation. This breakdown in glutamatergic homeostasis could promote the disorganization of postsynaptic densities, compromise synaptic plasticity, and contribute to the deterioration of higher-order functions such as associative learning and olfactory memory, which have been previously reported in bees with high viral loads.

In particular, the loss of synaptic plasticity may be strongly influenced by the changed expression of the *Neto* gene. Although poorly studied in *A. mellifera*, its ortholog in *D. melanogaster* has been shown to act as an essential co-regulator of ionotropic glutamate receptors (iGluRs) at the neuromuscular junction (NMJ), enabling iGluR clustering in postsynaptic densities and ensuring stable and functional synaptic transmission^{73, 74}.

Neto is indispensable for invertebrate development: null mutations result in paralyzed embryos that never hatch, whereas hypomorphic alleles generate locomotor defects⁷⁵. It localizes mainly in type I NMJs, together with iGluRs at postsynaptic densities⁷⁵. Interestingly, in the absence of *Neto*, synapses form normally during the prepatterning stage, but iGluRs fail to cluster at postsynaptic regions, disrupting synapse maintenance and producing electrophysiological defects⁷⁵. Moreover, in iGluR mutants, *Neto* aggregates do not form, highlighting a codependent relationship between the two⁷⁵. Conversely, *Neto* overexpression, as identified in our study, causes iGluRs in adult *D. melanogaster* to accumulate at extrajunctional sites, leading to synaptic defects likely due to the trapping of iGluRs outside the synapse⁷⁶.

The iGluRs comprise three major subtypes of glutamatergic receptors: AMPARs, NMDARs, and kainate receptors⁷⁷. Regulatory changes in these receptors, particularly kainate-type receptors, have been directly linked to synaptic plasticity and memory capacity, processes that have also been associated with neurodegenerative diseases such as Alzheimer's disease in mammalian models⁷⁸.

Additionally, changes in *Eaat-2* expression may also be involved, together with *Neto* and *kainate* receptors, in behavioral alterations, synaptic imbalance, and the loss of synaptic plasticity. The taurine/aspartate transporter *Eaat-2* is the ortholog of vertebrate glutamate transporters⁵⁰, and in *D. melanogaster* it has been described in glial cells of the central nervous system during larval stages⁵¹. More recently, its functional relevance in peripheral sensory processing and behavior has been reported: adults with *Eaat-2* silenced in perineurial olfactory glial cells exhibited behavioral alterations, showing increased olfactory sensitivity when exposed to ethyl acetate (EA). This led to the proposal that *Eaat-2* plays a key role in modulating OSN activity by regulating neuronal signal intensity and preventing excessive responses¹³.

Altogether, our results support the idea that DWV-A infection profoundly disrupts glutamatergic homeostasis in honey bees through coordinated and dynamic alterations in *Neto*, *Eaat-2* and ionotropic glutamate receptors, *Kainate*. The early induction and subsequent collapse of this regulatory network suggest a transient compensatory attempt that ultimately fails as viral loads increase, giving rise to a state of synaptic dysregulation. This imbalance is likely to compromise the structural and functional integrity of synapses, reduce neuronal plasticity, and contribute to the sensory and cognitive impairments reported in DWV-infected bees.

As recently reported by Ferreira et al.⁷⁹, DWV-A infection triggers a cascade of behavioral changes in honey bees, including precocious foraging and impaired resource collection, that directly impact pollination services and agronomic stability. Our results suggest that the neurophysiological alterations identified in this work provide a plausible mechanistic explanation for such phenomena, acting as the bridge between viral infection and impaired colony performance. Within this context, *Neto*, *Eaat-2* and *Kainate* emerge not only as molecular targets of DWV-A infection, but also as potential nodes linking viral replication to synaptic dysfunction and, ultimately, to the progressive behavioural and ecological deterioration observed at the colony level.

CONCLUSION

In conclusion, oral DWV-A infection triggers a sequential transcriptomic reprogramming in *Apis mellifera*, characterized by a biphasic CNS response, involving Warburg-like metabolic activation at 10 dpi followed by homeostatic collapse and terminal sensory impairment in the antennae at 16 dpi. The desynchronization of maturation markers (*nau* and *cwo*) demonstrates that the virus dismantles the behavioral genetic program even under moderate viral loads. These findings reveal that subclinical neurobiological damage, rather than overt physical symptoms, is a key driver of colony functional decline, providing a novel molecular framework to understand hive collapse during persistent, asymptomatic infections.

Declarations

Acknowledgments: D.S. thanks the grant from the National Agency for Research and Development (ANID) through the National Ph.D. Scholarship ID 21240845. The authors would like to express their gratitude the Doctoral Program in Agronomic Sciences of the University of Concepción (Chile). Authors would like to acknowledge the technical support provided by Jose Luis to the Servicios Científico-Técnicos from the Universidad de Oviedo (Spain). Finally the authors would like to acknowledge to the PhD. Giovanni Galizia from Department of Neuroscience, University of Konstanz, Konstanz, Germany for his insightful comments and the stimulating discussions that helped refine the ideas presented in this manuscript.

Author Contributions: Conceptualization: M.V., C.G-D., Sh.O., D.S.; Methodology: M.V., C.G-D., Sh.O., D.S.; Formal analysis: L.T., D.S.; Investigation: F.B., Y.F., G.D., L.T., D.S.; Writing original draft: D.S.; Writing review & editing: M.V., C.G-D., Sh.O., L.T., D.S.; Supervision: V.M., G-D.C., O. Sh, S.D.; Project administration: M.V., C.G-D.; Funding acquisition: M.V., C.G-D., D.S.; Resources: M.V., C.G-D., Sh.O. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the FONDECYT N° 1241994 grant from the National Research and Development Agency (ANID), Chile and by the Spanish Agency for Research (AEI, PID2023-149482NB-100).

Competing interests: The authors declare no competing interests.

Data Availability Statement: All new transcriptomic data have been deposited under the Bioproject number PRJNA1419292 in the NCBI Genbank repository.

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Figures

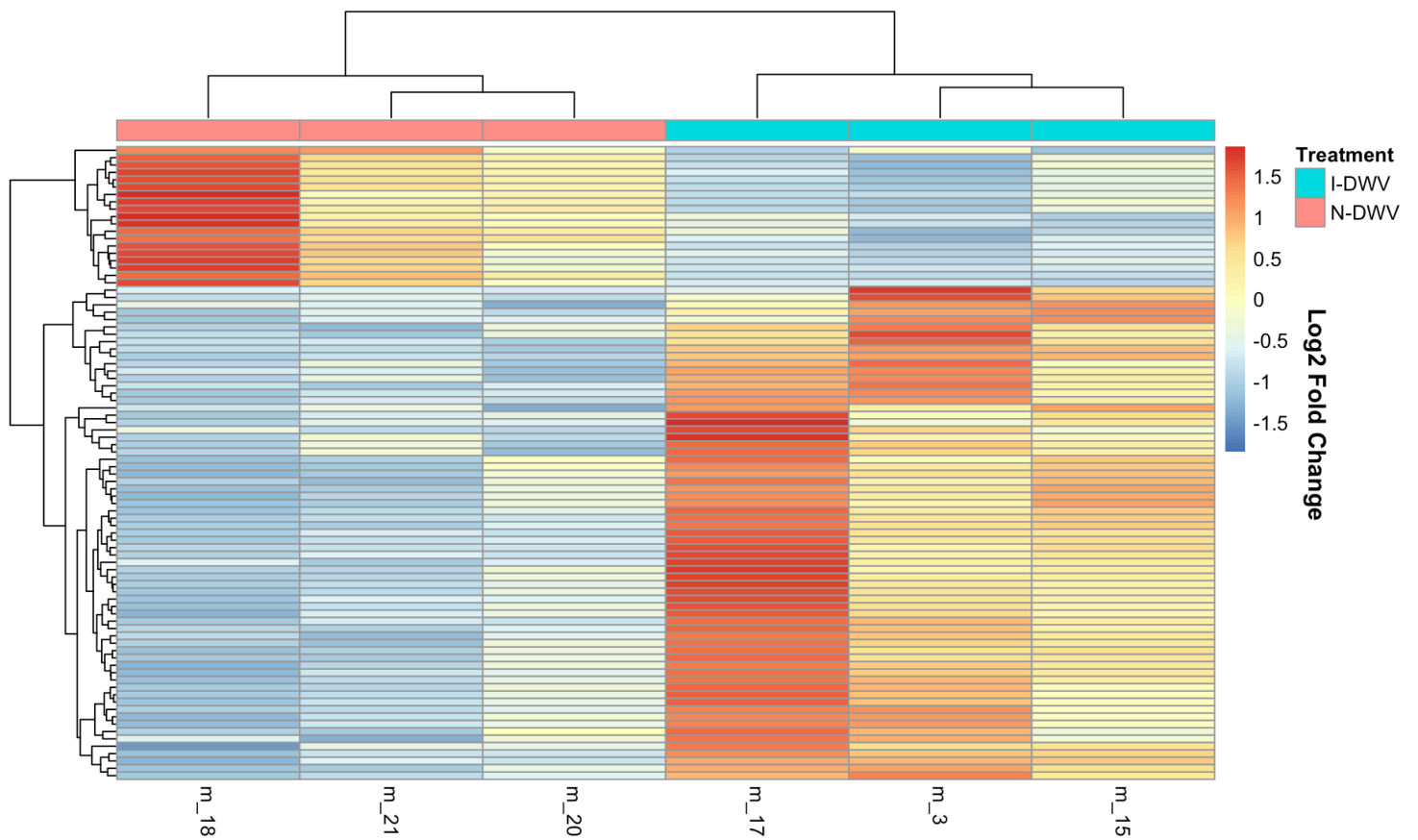


Figure 1

Heatmap of differentially expressed genes (DEGs) in bees inoculated with Deformed Wing Virus variant A (DWV-A) at 10 dpi. Inoculated (m_3, m_15, m_17) and non-inoculated (m_18, m_20, m_21) samples are shown. Columns correspond to biological replicates and rows to individual DEGs. The dendrogram above the heatmap depicts hierarchical clustering of samples. Red denotes upregulated genes and blue denotes downregulated genes.

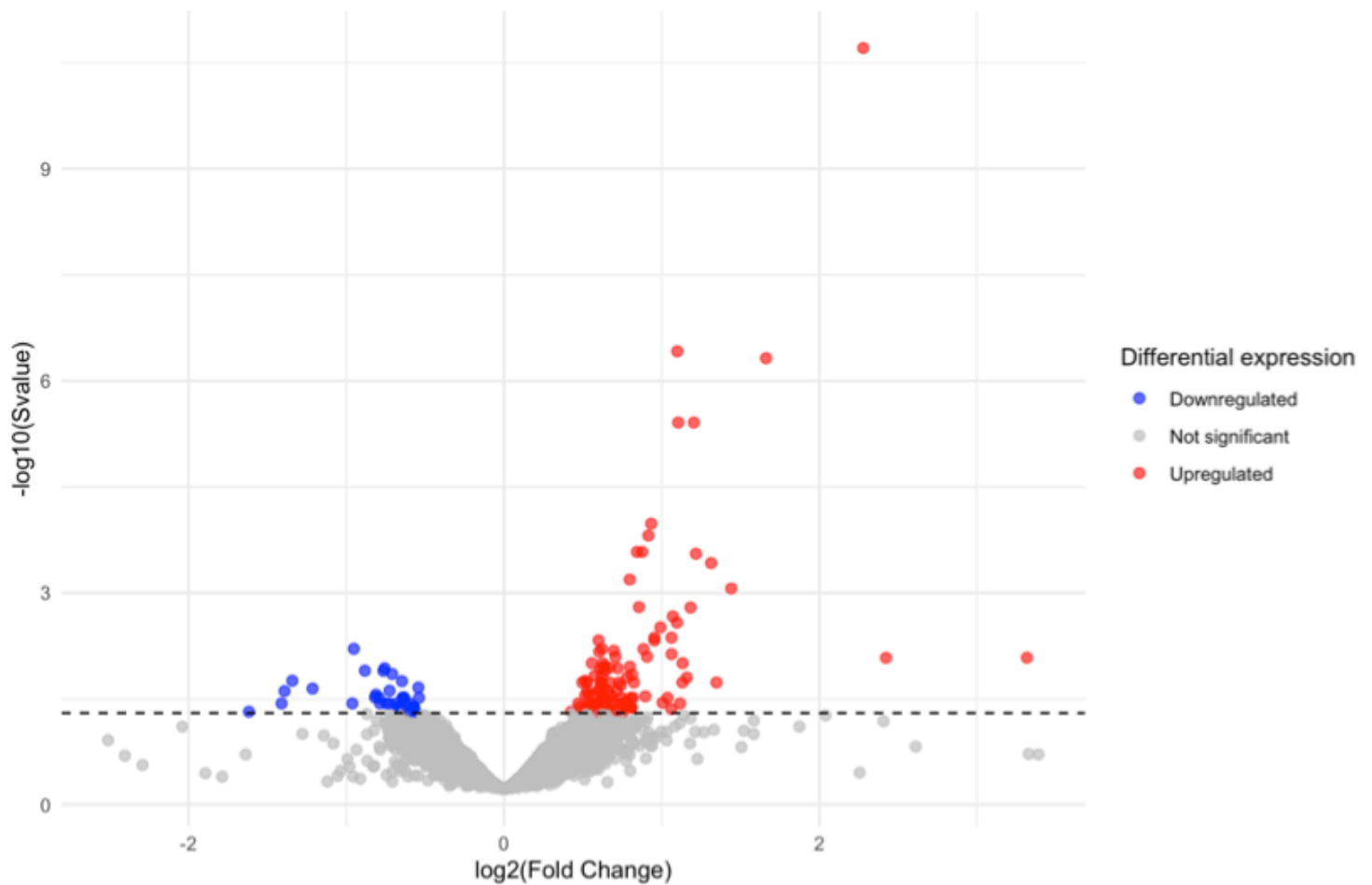


Figure 2

Volcano plot summarizing the differentially expressed sequences in i-DWV vs. n-DWV. Transcript abundance is represented as Log10 (TPM + 1). The x-axis and y-axis represent Log2 fold-change (Log2FC) differences between the contrasted groups and statistical significance as the negative Log10 P-value (s-value), respectively. A total of 9,113 genes were included in the analysis, yielding 109 and 38 transcripts, up- and downregulated.

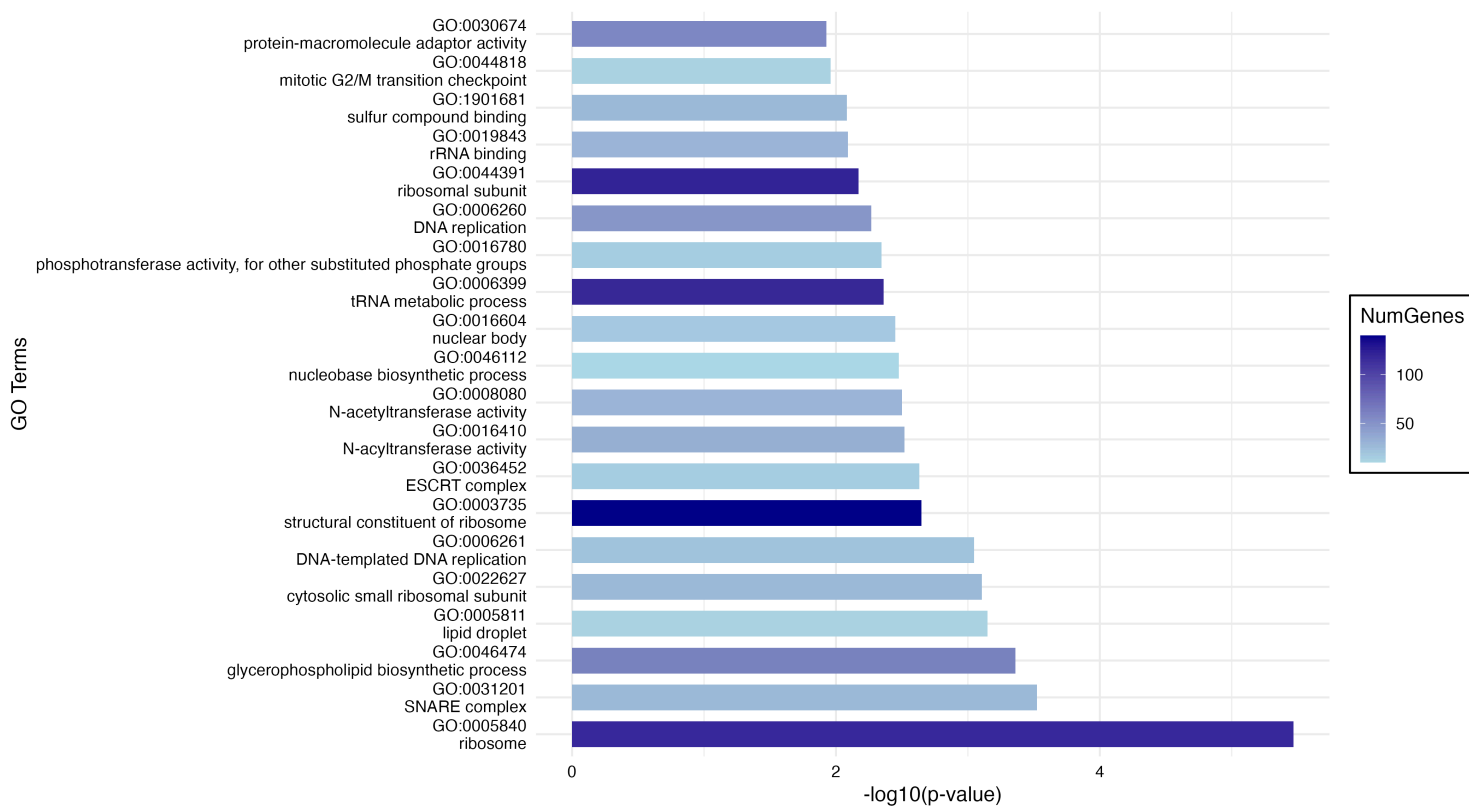


Figure 3

Gene Ontology (GO) enrichment analysis. The results display the top 20 most significant GO terms enriched in inoculated bees (i-DWV) compared to non-inoculated controls (n-DWV). The Y-axis lists the enriched GO terms and their access codes, while the X-axis represents the statistical significance expressed as $-\log_{10}(\text{p-value})$. The color intensity of the bars indicates the number of genes associated with each specific GO term (count). A total of 9,113 genes were used as the background set for the analysis (corrected $p < 0.05$).

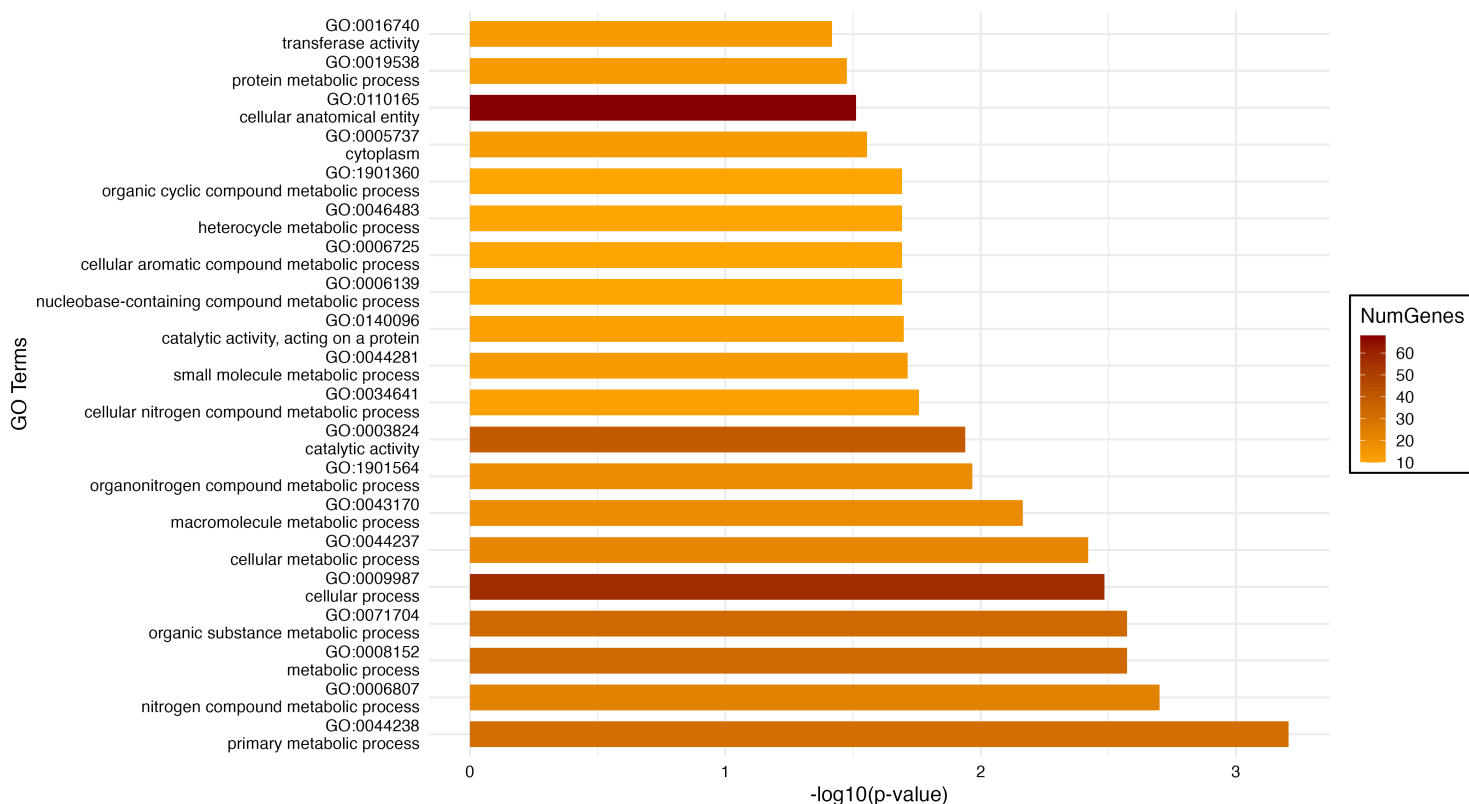


Figure 4

Gene Ontology (GO) enrichment analysis. The results display the top 20 most significant GO terms enriched in inoculated bees (i-DWV) compared to non-inoculated controls (n-DWV). The Y-axis lists the enriched GO terms and their access codes, while the X-axis represents the statistical significance expressed as $-\log_{10}(\text{p-value})$. The color intensity of the bars indicates the number of genes associated with each specific GO term (count). A total of 147 genes (DEGs) were used as the background set for the analysis (corrected $p < 0.05$).

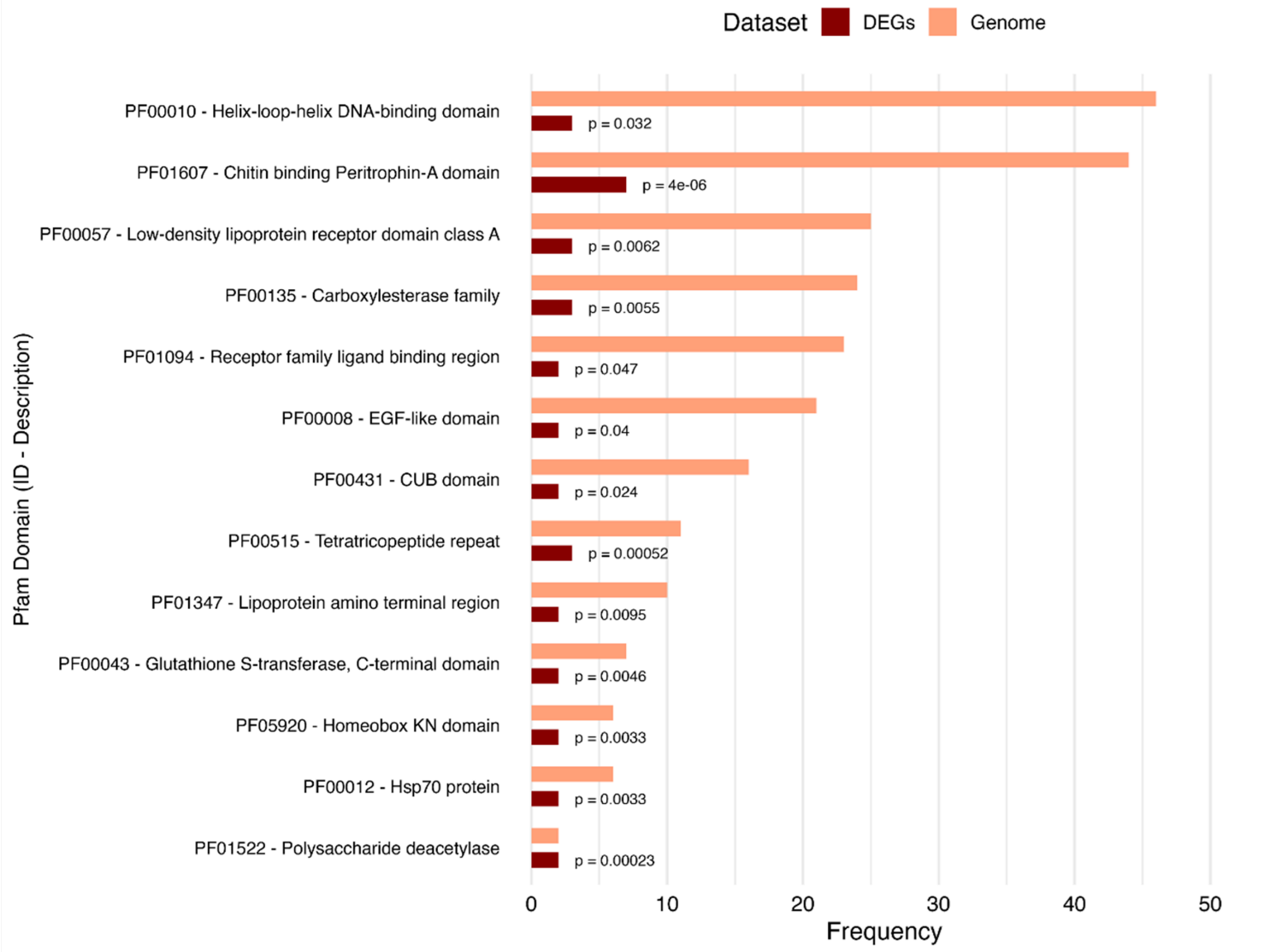


Figure 5

Hypergeometric test results for enrichment analysis of DEGs. Horizontal bars indicate total count gene in the genome of *A. mellifera* (Amel_HAv3.1) and in DEGs dataset (i-DWV vs n-DWV). P-values for each Pfam domain are indicated.

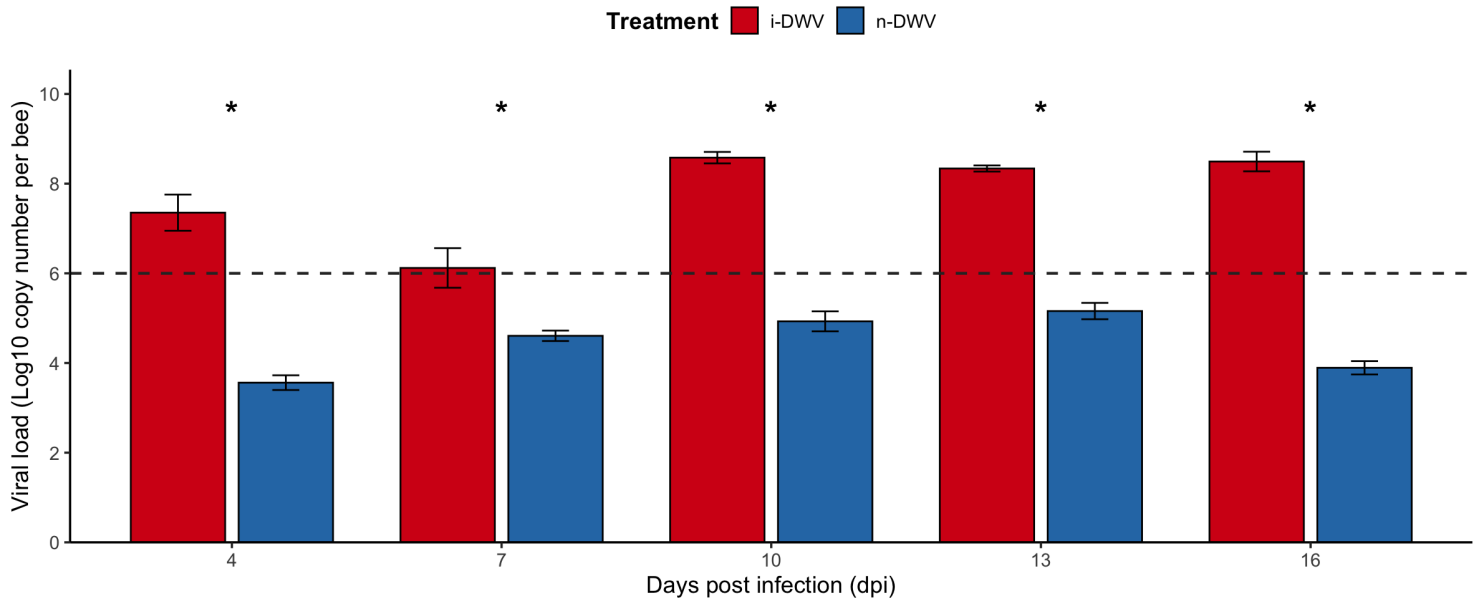


Figure 6

Temporal dynamics of DWV-A viral loads in honey bee heads. Viral titers in inoculated bees (i-DWV) ranged from to copies/bee, significantly exceeding non-inoculated controls (n-DWV) at all time points (4–16 dpi). Values at 10 dpi represent the pooled material used for RNA-seq. The horizontal dashed line indicates the basal viral load detected in field-sampled bees, representing the natural background levels of the pathogen in the apiaries Asterisks (*) above the means indicate significant differences between treatments at the same dpi, according to the Wilcoxon test ($p < 0.05$).

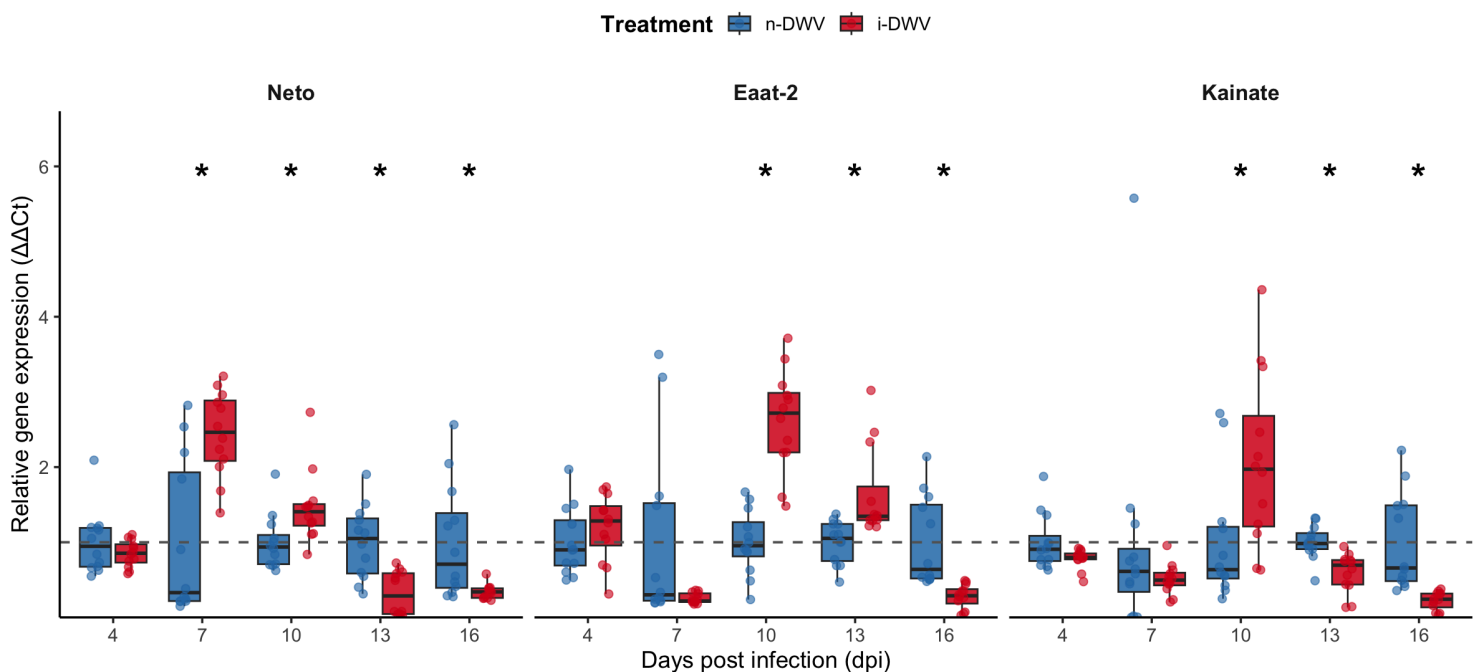


Figure 7

Temporal gene expression patterns of the nervous system genes *Neto*, *Eaat-2*, and *Kainate* in head samples of honeybees inoculated (i-DWV) and non-inoculated (n-DWV) with a viral suspension. Values at 10 dpi represent the pooled material (head with antennae) used for RNA-seq. Asterisks (*) above the means indicate significant differences between treatments at the same dpi, according to the Wilcoxon test ($p < 0.05$).

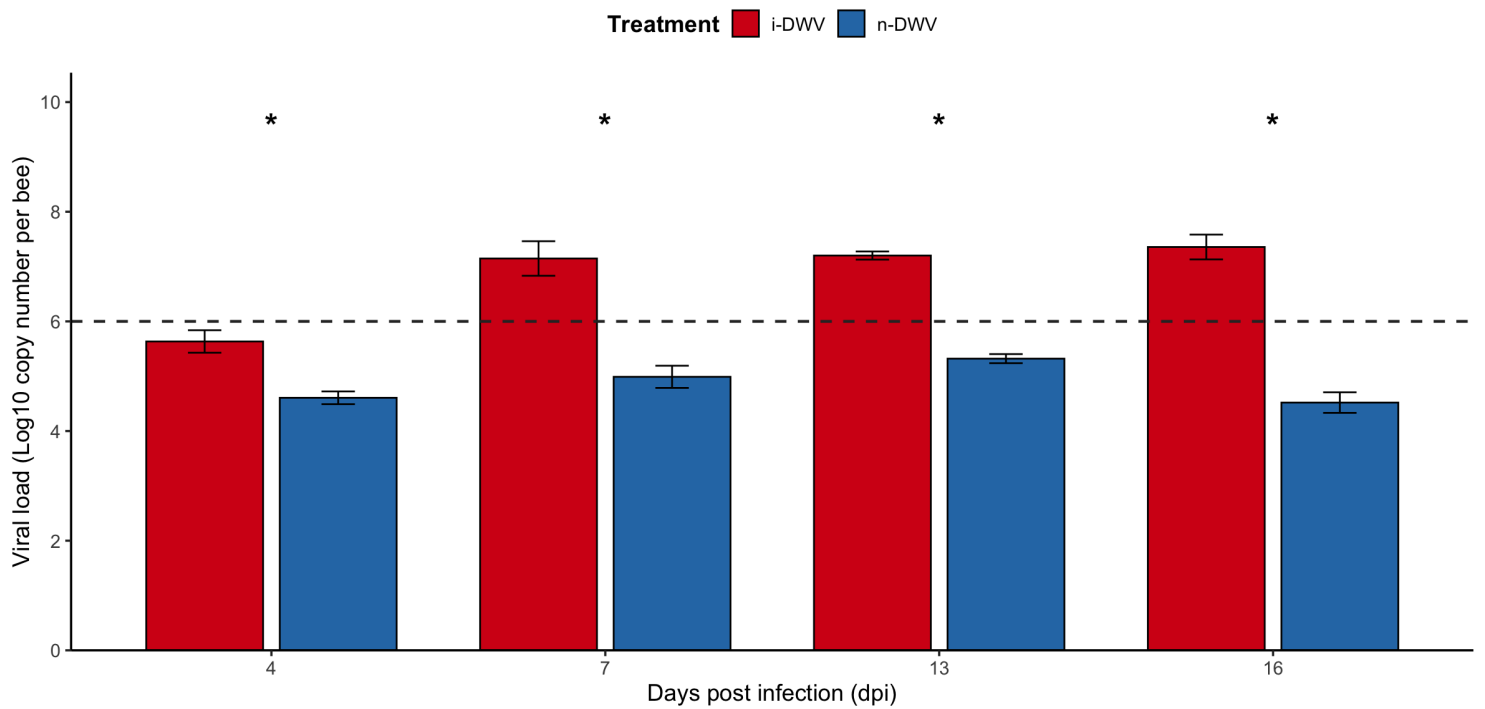


Figure 8

Temporal dynamics of DWV-A viral loads in honey bee antennae. Viral titers in inoculated bees (i-DWV) ranged from to copies/bee, significantly exceeding non-inoculated controls (n-DWV) at all time points (4–16 dpi). The horizontal dashed line indicates the basal viral load detected in field-sampled bees, representing the natural background levels of the pathogen in the apiaries Asterisks (*) above the means indicate significant differences between treatments at the same dpi, according to the Wilcoxon test ($p < 0.05$).

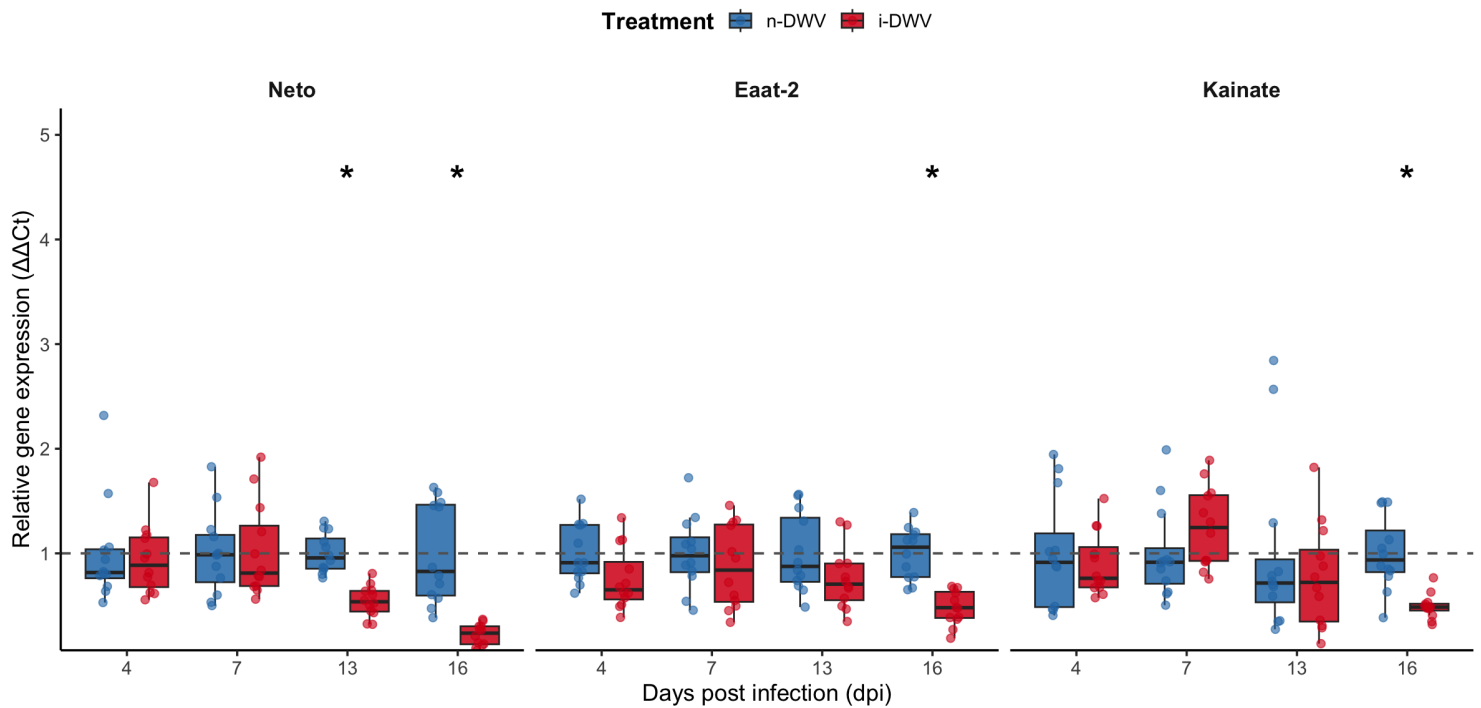


Figure 9

Temporal gene expression patterns of the nervous system genes *Neto*, *Eaat-2*, and *Kainate* in antennae samples of honeybees inoculated (i-DWV) and non-inoculated (n-DWV) with a viral suspension. Asterisks (*) above the means indicate significant differences between treatments at the same dpi, according to the Wilcoxon test ($p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarytableSilvaetal2026.xlsx](#)