High quality diet enhances immune response and affects gene expression during viral infection in an insect herbivore.

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Abstract

Herbivorous insects tolerate chemical and metabolic variation in their host plant diet by modulating physiological traits. Insect immune response is one such trait that plays a crucial role in maintaining fitness but can be heavily influenced by variation in host plant quality. An important question is how the use of different host plants affects the ability of herbivorous insects to resist viral pathogens. Furthermore, the transcriptional changes associated with this interaction of diet and viral pathogens remain understudied. The Melissa blue butterfly (*Lycaeides melissa*) has colonized the exotic legume *Medicago sativa* as a larval host within the past 200 years. We used this system to study the interplay between the effects of host plant variation and viral infection on physiological responses and global gene expression. We measured immune strength in response to infection by the Junonia coenia densovirus (JcDV) in two ways: 1) direct measurement of phenoloxidase activity and melanization, and 2) transcriptional sequencing of individuals exposed to different viral and host plant treatments. Our results demonstrate that viral infection caused total phenoloxidase (total PO) to increase and viral infection and host plant interactively affected total PO such that for infected larvae, total PO was significantly higher for larvae consuming the native host plant. Additionally, *L. melissa* larvae differentially expressed several hundred genes in response to host plant treatment, but with minimal changes in gene expression in response to viral infection. Not only immune genes, but several detoxification, transporter, and oxidase genes were differentially expressed in response to host plant treatments. These results demonstrate that in herbivorous insects, consumption of a novel host plant can alter both physiological and transcriptional responses relevant to viral infection, emphasizing the importance of considering immune and detoxification mechanisms into models of evolution of host range in herbivorous insects.

Key-words: *Lycaeides, Medicago, immune response, lepidopteran virus, phenoloxidase, melanization, gene expression, gene regulation, plant-insect interactions*

Introduction

Herbivorous insects and their host plants represent an intimate interaction where both species exert selective pressures on each other that solicit behavioral, physiological, life-history, and molecular responses (Schoonhoven, Van Loon, and Dicke 2005; Birnbaum and Abbot 2020; Groen and Whiteman 2022). Past work on plant-insect interactions has demonstrated host plants effects on individual fitness and adaptation of insects (Gloss, Groen, and Whiteman 2016; Vertacnik and Linnen 2017). While molecular studies on herbivorous insects have mostly focused on the genomic basis of adaptation to host plants, we are only recently starting to understand the gene regulatory mechanisms underlying tolerance and resistance of chemical challenges presented by host plants (Nallu et al. 2018; Näsvall et al. 2021; Okamura et al. 2019; Tan et al. 2019; Ragland et al. 2015). Furthermore, specialist and generalist herbivores differ in their adaptive mechanisms in response to different host plant species such that while generalists invest in a broad range of chemical detoxification strategies, specialist herbivores have evolved adjust responses to specific plant defenses (Marquis and Koptur 2022; Vogel, Musser, and Paz Celorio-Mancera 2014; War et al. 2012; Lankau 2007) . Moreover, when herbivores are faced with novel host plant challenges, they potentially employ different strategies to detoxify new secondary metabolites, which can be associated with drastic changes in gene regulation. Indeed, studies have shown that different insect species regulate different families of genes in response to variation in chemical content from the different species of plants they utilize as their hosts (Tan et al. 2019; Näsvall et al. 2021).

While herbivorous insects have evolved several strategies to deal with host plant related challenges, pathogens or parasites can present an added layer of selective pressure that can influence fitness and survival in the wild. In this case, the insect immune response can influence fitness by affecting life history traits, despite the many costs associated with mounting a response (Schulenburg et al. 2009; Catalán et al. 2012). The field of ecological immunology seeks to understand how variation in biotic and abiotic factors contributes to immunological variation in the wild, and how immune function evolves and is involved in the evolution of other organisms (Schmid-Hempel 2005). Plant-feeding insects represent a rich testing ground for examining ecological immunology concepts due to their diversity and abundance in natural ecosystems (Janz et al., 2006). Along these lines, the Lepidoptera represent an especially interesting test case for ecological immunology given that many species within this group are known to be specialists on various species of host plants of the same genus, which can provide a tractable model to identify variation in the immune response as variation in host plant diet can influence life history traits which in turn can affect immunity (Graves and Shapiro, 2003). Moreover, plant metabolites can indirectly affect parasites by modulating the insect immune response creating a complex cascade of gene regulatory pathways. Regulation of immune genes is an important adaptive strategy in response to host plant variation or parasite infection. For instance, some species of butterflies, such as *Junonia coenia*, *Militea cinxia*, and *Euphydryas phaeton*, regulate their immune response based on the iridoid glycoside concentrations in their host plants (Smilanich, Dyer, Chambers, & Bowers, 2009; Smilanich et al., 2017; Laurentz et al.,2012; Muchoney et al. 2022). Monarch butterflies reared on different milkweed species exhibit down regulation of immune genes on the species which affects endogenous immune response (Tan et al. 2019).

With respect to larval development and performance, previous meta-analyses have shown that novel host plants generally represent inferior hosts relative to native hosts for larval lepidopterans despite the many butterflies and moths that are known to persist on exotic hosts in the wild (Yoon and Read, 2016). Further, a previous literature survey comparing immune strength on different host plants found that in 5 out of 10 published studies, lepidopteran larvae have higher cellular immune response when reared on high quality host plants, with quality in this instance determined by fitness correlates such as larval weight (Lampert, 2012). In the remaining studies, only one showed that consumption of a comparatively lower quality host plant led to a higher cellular immune response and a variation in regulation of canonical immune genes (the remaining studies did not detect an effect of host plant use) (Yoon et al. 2019; Mason, 2020). Furthermore, transcriptomic variation in response to diet breadth in herbivores is not just dominated by immune genes. In fact, canonical detoxification, chemosensory, cuticle, and transporter genes - all interact to aid herbivores in tolerating low quality hosts and can eventually lead to adaptation and specialization to hosts in their geographic ranges (Birnbaum & Abbot, 2019).

We explore these relationships using the butterfly *Lycaeides melissa* (Lycaenidae), a specialist herbivore on legumes including members of *Astragalus* and *Lupinus* (native hosts), as well as the exotic legume *Medicago sativa* (Fabaceae) (exotic host), which it has colonized at least twice and probably many times within the past 200 years (Forister et al., 2009, Chaturvedi et al. 2018). Across their geographic range in Northern America, these butterflies are locally adapted to their native or exotic host plants (Chaturvedi et al. 2018). *Medicago sativa* supports populations of *L. melissa* heterogeneously throughout the western United States (Forister et al. 2020), despite reducing larval performance and adult fecundity compared to a preferred native host *Astragalus canadensis* (Forister et al. 2009, Harrison et al. 2016). Past work in this system has revealed that *L. melissa* immune strength can be affected by nutritional, phytochemical, and microbial variation, and that these effects are host plant specific (Yoon et al., 2019). Genomic studies on this system have revealed that novel host plant adaptation is a polygenic trait with additive effects associated with larval development and survival on different host plants (Gompert et al. 2015; Chaturvedi et al. 2018; Gompert et al. 2022). However, what is still unclear is how variation in host plant use will affect the ability of *L. melissa* larvae to respond to a live, experimentally introduced pathogenic threat. Moreover, we have much to learn about physiological and genetic processes underlying host plant-specific effects on development, detoxification and the immune response, which is the issue that we address in the present study.

To address this issue, we quantified phenotypic and transcriptomic responses of caterpillars infected with a lepidopteran virus and reared on a native and a novel host plant. Junonia coenia densovirus (JcDV) was first discovered in the buckeye butterfly, *Junonia coenia* (Rivers and Longworth, 1972; Bruemmer et al., 2005), and has been shown to infect other lepidopteran species and families (Mutuel et al. 2010, Smilanich et al. 2018; Muchoney et al. 2022, 2023; McKeegan et al. 2024). This viral pathogen was chosen because it is common in the environment, is frequently found at *L. melissa* populations in the wild throughout its range and can affect larval survival in the lab (McKeegan et al., 2024, Yoon et al. unpublished data). In the noctuid moth *Spodoptera frugiperda*, JcDV infects larvae through oral ingestion of viral particles, resulting in the virus crossing the midgut, and then finally replicating in visceral tracheae and hemocyte cells, leading to death by hypoxia (Mutuel et

al., 2010). Transcriptome analyses have been successful in elucidating lepidopteran immunological responses to both pathogens and differential host plant use across a wide range of taxa (Vogel et al., 2001; Gandhe et al., 2006; Wang et al., 2016; Tan et al., 2019), but pathogens and host plant use have rarely been investigated in the same study (but see Tan et al. 2019). Therefore, our goal was to investigate whether functional genetic data can complement physiological assays, which have relied in large part on the phenoloxidase pathway. The phenoloxidase pathway is one of the major immunological pathways in insects, and is a generalized pathway that protects against viruses, bacteria, fungi, and parasitoids (González-Santoyo & Córdoba-Aguilar, 2012). Previous experimental work with the tobacco budworm *Heliothis virescens* and the spongey moth *Lymantria dispar* have indicated that the phenoloxidase enzyme has anti-viral properties in response to infection (Shelby and Popham, 2006; McNeil et al., 2009). While these studies suggest that the phenoloxidase pathway and the melanization response may be important components of the lepidopteran antiviral response, other studies have found no notable role for the phenoloxidase enzyme in antiviral immune response (Saejeng et al., 2010; Scholefield et al. 2019). Given uncertainty associated with the phenoloxidase response, we have opted to pair our immune assays of standing and total phenoloxidase and melanization with a transcriptome analysis of global gene expression.

Ecological immunology theory predicts that immune responses are costly (Sheldon and Verhulst, 1996) and that as organisms have access to higher quality nutritional resources, they should have enhanced immune function due to increased resource availability (Ponton et al., 2011). As such, we predict that 1) infection with JcDV will result in physiological changes, including increased phenoloxidase activity and melanization, as well as differential upregulation of immune related genes as measured by transcriptome analysis; 2) *L. melissa* larvae fed the native, nutritionally superior host plant *A. canadensis* will have a heightened immune response compared to larvae fed the novel host plant *M. sativa*, which should be reflected in both immune assays and differential expression of immune-relevant genetic regions; 3) canonical detoxification genes will be upregulated in larvae reared on the novel host plant *M. sativa*. By pairing physiological assays with a survey of gene expression, we create an opportunity for learning about the molecular mechanisms underlying insect immune response and how these mechanisms interact with nutrition. For example, we do not know if, under conditions of poor nutrition, a caterpillar will simply have lower expression levels of immune-related genes, or if different cellular, metabolic, or molecular processes might be brought to bear in fighting a pathogen. Understanding these underlying molecular mechanisms is essential for predicting the trajectory of adaptation to novel host plants in plant-feeding insects and other parasitic organisms.

Materials and methods

Overview of experiments

We conducted two separate viral infection experiments. The first experiment allowed us to ask if viral infection of *L. melissa* larvae would affect the amount of standing and total PO or melanization, and whether these effects would be mediated by host plant use. Next, we asked whether viral infection or different host plant use would affect the global gene expression of *L. melissa* larvae using transcriptomics.

For the first experiment, gravid *L. melissa* females were collected from a population associated with *M. sativa* at Verdi NV, USA (hereafter: VUH) during June 2016. Eggs acquired from these females were randomly assigned to a host plant treatment (*A. canadensis* or *M. sativa*) and larvae were reared individually in petri dishes at ambient temperature and ten hours of light per day, as previously described (Forister et al., 2009). *Medicago sativa* plants were collected weekly from the same site where the maternal butterflies were collected, and *A. canadensis* plants for rearing were collected from a nearby location that similarly supports a population of *L. melissa*. We reared 125 larvae to the fourth (final) instar to be used in immune experiments; 46 on *M. sativa* and 79 on *A. canadensis*. When larvae reached their fourth instar, every other individual from each treatment group was selected to be given 1 µl of Junonia coenia densovirus. Larvae were fed a 10mm diameter leaf disk with 1 µl of 1 x 10^{11} virus particles/µl pipetted onto the leaf surface (purified virus stock courtesy of M. Ogliastro, University of Montpellier, France). This concentration was used as it is considered a "high" dose, which would potentially allow us to detect transcripts that are only expressed during times of high viral load. This concentration has been shown to constitute an LD50 in another lepidopteran species (Smilanich et al., 2018). They were allowed to eat the leaf disk for 16 hours to ensure inoculation. After the inoculation period, larvae were returned to their petri dishes and fed for 48 hours before immune assays.

For the second experiment, approximately 80 eggs from VUH were distributed evenly across the two host plant treatments. From the original 80 larvae reared, approximately 60 survived to fourth instar. Larvae were reared until fourth instar, weighed, and then orally infected in the same manner as described above, with the same concentration of virus. Infection and incubation of larvae was performed in a separate building from the initial rearing process, and infected larvae were kept in a separate growth chamber after viral exposure to prevent cross contamination. Larvae that served as controls were never exposed to the lab/growth chamber housing infected larvae. After 48 hours, all larvae were weighed again and then extracted for RNA. From these 60 extracted larvae, 12 larvae were chosen randomly for sequencing.

Immune assays

Larval immune response was measured using three assays: standing and total phenoloxidase (PO) activity and melanization via nylon filament injections. Standing PO is a measurement of the naturally activated enzyme after the hemolymph is taken from the caterpillars (Gonzalez-Santoyo and Cordoba-Aguilar, 2012). This assay measures the formation of dopachrome, which is assumed to be largely driven by active phenoloxidase. Total PO is a measure of standing PO plus any inactive PO remaining within hemocytes. Filament injections serve as a proxy for a parasitism event and are a useful measure of immune response in caterpillars. Both of these metrics accurately reflect the strength of the immune response (Smilanich et al. 2009b).

Standing and total phenoloxidase were measured by taking 5 µl of hemolymph using a sterile sewing needle from the abdominal cavity. Hemolymph was added to 100 µl of ice-cold phosphate buffered saline (PBS) in an Eppendorf tube and was chilled on ice while a dopamine solution (25.7 mg dopamine in 20 mL water) was prepared. Powdered dopamine (Sigma-Aldrich; St. Louis, Missouri, USA) (0.0257 g) was added to 20 mL of distilled water.

The hemolymph bound PBS solution was split evenly between two well plates to run standing and total PO activity; 10 µl of cetylpridinium chloride solution (1g in 20 mL of distilled water) was added to all wells measuring total PO, then 200 µl of the dopamine solution was added to every well in the plate. Samples were incubated for 20 minutes at room temperature and the reaction then proceeded in a microplate reader (Bio-Rad iMark) for 45 minutes (data recorded every 30 seconds at 490 nm); data were analyzed using Microplate Manager (MPM) software (Bio-Rad v.6.3). We extracted the kinetic rate for the linear phase of the reaction (0–45 minutes). In addition, blanks which consisted of distilled water and dopamine were included as negative controls for each run. We did not run a positive control with each run, however, samples from all treatment groups (both host treatments) were run together to avoid confounding treatment with instrument variation.

After hemolymph extraction, larvae were individually injected with clear nylon filament approximately 2 mm in length. Filaments were injected at the same wound site where hemolymph was previously drawn for PO assay (posterior abdominal segment). Larvae were returned to their respective petri dishes and given access to plant tissue for 24 hours, then frozen and dissected for filaments. Dissected filaments were photographed using a dissecting microscope connected to a digital camera (Carl Ziess Discovery V.8, AXIOCAM Software, Oberkochen, Baden-Wurttenburg, Germany). For each individual, each filament was photographed at 80X magnification, and their melanization value was recorded in ImageJ. For additional details on melanization assay methods, see Smilanich et al., 2009a.

Statistical estimation of Immune Function and Larval Performance

All analyses were conducted in R (R Core Team 2019). Total PO, standing PO, melanization, and larval weight were analyzed using linear models with host plant and treatment as fixed effects, as well as the interaction between host plant and infection status. Assumptions of linear models including normality and homoscedasticity of residuals were inspected. Host plant and larval weight were not included as covariates in models together as variance inflation factors were very high (>7) for these two covariates when they were included simultaneously in linear models.

RNA Extraction and Sequencing

Larval samples were homogenized in trizol (Life Technologies), and stored at -80C prior to homogenization with a motorized pellet pestle. Larvae were extracted at the 4th instar stage 48 hours after viral infection. Total RNA was extracted using the Purelink RNA mini kit with DNAse treatment per manufacturer's protocol (Ambion). Ethanol precipitated pellets were resuspended in sterile water and quantified by Nanodrop. Barcoded mRNA libraries were prepared with 1g of total RNA using the TruSeq Stranded mRNA kit (Illumina) and sequenced using on the HiSeq4000 platform at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley.

Quality filtering, sequence alignment, and generating count matrix

We checked the quality of raw reads using FastQC before proceeding to downstream processing of reads. We then used RCorrector (Song and Florea 2015) to detect unfixable k-mers in the RNA sequences and corrected these k-mer based read errors. RCorrector compares k-mer based error correction tools and identifies whether the read has been corrected

or has been detected as containing an uncorrectable error. We then used a custom python script to discard unfixable reads identified by RCorrector. Reads were then trimmed using Trim Galore (version 0.3.3) (https://github.com/FelixKrueger/TrimGalore) to remove Illumina adapter sequences. Filtered and quality-checked paired-end reads were aligned to an existing, annotated genome of *L. melissa* (Chaturvedi et al. 2018, 2020) using STAR (version 1.5.2) (Dobin et al. 2013). STAR alignment rate ranged between 70-85% for all sample libraries. We converted STAR alignments to gene count data for each sample using featureCounts (version v2.0.0) (Liao, Smyth, and Shi 2014). Finally, we assigned gene annotations to transcripts using the genome annotation for the *L. melissa* genome (for details of the genome assembly and annotation see Chaturvedi et al. 2020). We used custom python scripts to identify the interproscan IDs (IPR), PANTHER and Pfam IDs for the transcripts using this genome annotation. The scripts are archived on GitHub (https://github.com/chaturvedi-lab/lyc_rnaseq_transcript_annotations). Whenever we discuss gene functions in the text, we refer to their IPR domain and superfamily classification. These gene functions were also validated using PANTHER and Pfam modules.

Differential expression analyses

We used the final raw gene counts file from above as an input to perform standardized differential gene expression analyses using DESeq2 version 3.18 (Love et al., 2014). This analysis was implemented in R version 4.1.0 (R Core Team, 2013). We filtered the dataset by removing genes if they met any of the following criteria: (i) genes with non-zero counts in at least two samples and (ii) genes with low coverage denoted with baseMean (count average across all samples) <1. The DESeq2 analyses were performed using the default settings where we normalized counts per gene by library size (the number of reads in a specific library) and used the Wald test to carry out significance testing for individual genes (Love et al., 2014). We used the Benjamini and Hochberg (1995) method to produce adjusted significance levels (p_{adj}) for each gene based on the false discovery rate (FDR) and thereby account for multiple testing. We investigated the effect of host plant and viral infection on caterpillar gene expression by using the following pairwise comparisons: (i) control group comparison (*M. sativa* uninfected vs. *A. canadensis* uninfected) (Treatment 1), (ii) infected comparison (infected *M. sativa* vs. infected *A. canadensis*) (Treatment 2), (iii) native host plant comparison (*A. canadensis* infected vs. *A. canadensis* control) (Treatment 3), and (iv) exotic host plant comparison (*M. sativa* infected vs. *M. sativa* control) (Treatment 4). We identified genes as exhibiting statistically significant differential expression for given pairwise comparison if p_{adj} was < 0.05. We then used the gene annotations (as described in previous section) to identify gene functions of differentially expressed gene sets for each comparison based on InterProScan terms and the Pfam and PANTHER modules.

Results

Viral infection effects on phenoloxidase, melanization, and larval weight

A series of linear models were run to examine the effects of host plant use and viral treatment on total PO, standing PO, melanization, and fourth instar larval weight. For total PO, we found a direct effect of viral treatment ($F_{(1, 67)}$ = 13.128, p = 0.0005, Std. coefficient = 1.11 [0.66, 1.56]), along with a two-way interaction between host plant use and viral infection (Figure 1a; $F_{(1, 67)}$ = 5.693, p = 0.0198, Std. coefficient = -0.58 [-1.29, 0.13]). Infected larvae had higher total PO than control larvae. For control larvae, host plant use had no detectable effect on

total PO, whereas for infected larvae, total PO was higher for larvae consuming the native host (Fig 1a).

For standing PO, we did not detect an effect of viral treatment (Figure 1b, $F_{(1, 67)} = 0.207$, $p = 0.65$), or an interaction between infection and host plant ($F_{(1, 67)} = 0.267$, $p=0.60$). However, host use did have a direct effect on standing PO, with larvae consuming the exotic host having higher levels of standing PO ($F_{(1, 67)} = 4.999$, $p = 0.0287$, Std. coefficient = -0.10 [-0.62, 0.41]). For percent melanization, we did not detect an interaction between host plant use and treatment ($F_{(1, 67)} = 1.199$, $p=0.277$), however we found evidence for direct effects of both host ($F_{(1, 67)} = 10.274$, $p=0.001$, Std. coefficient = -0.56 [-1.03, -0.10]) and treatment ($F_{(1, 67)} = 8.754$, $p=0.003$, Std. coefficient = 0.66 [0.18, 1.14]), with larvae having higher melanization with viral infection and lower melanization on the exotic host (Figure 1c).

For fourth instar larval weight, we found direct effects of both host use ($F_{(1, 73)} = 414.09$, $p<0.0001$, Std. coefficient = -1.82 [-2.08, -1.57]), and viral treatment ($F_{(1, 73)} = 7.264$, $p=0.008$, Std. coefficient = 0.17 [-0.08, 0.42]), however, we did not find an interaction between host and treatment (Fig 1d). Fourth instar larval weight was higher on the native host plant and in infected individuals.

Alignment, gene count estimation, and differential gene expression

We obtained ~43 million reads after aligning our samples to the *L. melissa* reference genome. The number of reads per sample varied from 3.4 to 5.9 million. After quality filtering we ended up with a global gene expression dataset of 11, 214 genes. The DESeq2 filtering of gene counts revealed that the median gene counts of the 12 samples were similar, and the normalized gene expression values ranged from 6.20 to 9.49 (meaning the amount of mRNA detected in each sample) (Figure 2A). We then visualized variation in gene expression between individual larvae using principal component analysis using the plotPCA function in DESeq2 (PCA). The normalized gene expression values were transformed using the DESeq2 getVariationStabilizedData function prior to performing the PCA. Our PCA results revealed that host plant is the main determinant of variation in gene expression in our dataset with the first two principal components explaining most of the variation (PC1=50%, PC2=19%, Figure 2B). Larvae reared on the same host plant clustered together irrespective of their infection status. This result was mirrored by our heatmap and hierarchical clustering analysis which showed that individual larvae are more clustered by plant diet irrespective of viral infection with some subtle but variable clustering based on viral infection (Figure 3). Overall, larvae showed gene expression similarity based on host plant treatment with high variation between plant treatments but minimal variation within plant treatments irrespective of viral infection.

Effect of experimental treatments on gene expression

We next quantified differences in the number of differentially expressed genes between treatment groups. We first compared gene expression between all uninfected larvae to examine the host plant specific effects on gene expression (control group comparison: No virus ASCA versus No virus MESA). We found that 140 genes showed significant differential expression for this comparison. Interestingly, the majority (123) of the genes were upregulated in larvae fed with *M. sativa,* with 17 genes being downregulated in the same larvae (Figure 2C, Figure 4A, Supp. Table 1). We then compared gene expression between infected larvae reared on the two host plant species separately (infected comparison: Virus ASCA versus virus

MESA). For this comparison, we found that a total of 31 genes showed significant differential expression for this comparison where 17 genes were upregulated in larvae reared on *M. sativa* and 14 genes downregulated (Figure 2C, Figure 4B, Supp. Table 2).

We then compared gene expression between infected and uninfected larvae reared on the same host plant to identify the effect of viral infection while controlling host plant treatment (native host plant comparison: No virus ASCA versus virus ASCA, and exotic host plant comparison: No virus MESA versus virus MESA). Surprisingly, we found one or zero significantly differentially expressed genes for these treatments. We found only one gene was significantly upregulated for the native host plant comparison where uninfected larvae and infected larvae were reared on *A. canadensis,* and none were differentially expressed for the exotic host plant comparison where uninfected larvae and infected larvae were reared on *M. sativa* (Figures 2C and Figure 4C and 4D). Overall, these results were indicative of a strong effect of host plant and a weak to negligible effect of viral infection on larval gene expression response.

We then checked if the same genes show significant differential expression across treatments. We found that two genes were downregulated on *M. sativa*, and seven genes were upregulated on *M. sativa* between uninfected and infected comparisons. This was indicative of minimal levels of convergence in gene regulation in response to viral and diet stress. No genes showed opposite directionality in regulation between the two treatments. We refer to these genes as "common" genes from here on.

Genomic distribution and functional properties of DEGs

We evaluated the distribution of genes showing significant differential expression in our treatment comparisons to identify the underlying genetic architecture of infection and diet related traits in *L. melissa*. For the uninfected larvae comparison (No virus – ASCA versus No virus – MESA), differentially expressed genes were present on all 23 chromosomes of the *L. melissa* genome (Figure 5A). Nineteen out of the total 140 genes were present on the Z chromosome (18 genes upregulated and 1 gene downregulated on MESA). For the infected larvae comparison (Virus – ASCA versus Virus – MESA), relevant genes were present on 10 chromosomes, none on the Z chromosome (Figure 5B). The one significantly differentially expressed gene for native host plant comparison (No virus ASCA versus Virus ASCA) was present on chromosome 11.

We then evaluated the functional properties of the DEGs for each treatment. We saw significant up-regulation or down-regulation of several insect immune genes and detoxification genes (Table 1 and 2). The immune genes were involved in different immune processes such as response, signaling, and resistance (Table 1). Similarly, the detoxification genes underlie different proximate mechanisms such as signaling and chemosensory behavior (Table 2). Besides these categories of genes, for the uninfected larvae comparison (No virus ASCA versus No virus MESA) the top genes which were significantly up-regulated in those feeding on *M. sativa* were associated with the tubulin protein, FAD/NAD(P)-binding, and the Zinc finger protein (Supp. table 1). Interestingly, the top five down-regulated genes for this treatment were associated with immune responses such as Serine/Protease function and the immunoglobulin E family (Table 2). For the infected larvae comparison, the top genes significantly upregulated in caterpillars feeding on *M. sativa* were associated with Zinc finger proteins, protein kinase, neurotransmitter genes, cytochrome C oxidase genes, and olfactory receptor genes (Table 2,

Supp. Table 2). Here too, the top down-regulated genes for this treatment were also associated with immune response such as immunoglobulin genes and hemocyanin genes with some detoxification genes showing differential expression such as hemolymph protein genes, and zinc finger genes. For the native host plant comparison, which included infected and uninfected larvae fed with *A. canadensis*, only one gene was significantly upregulated in the infected group when compared to the uninfected group: a chitin binding domain gene. Overall, our results indicate that detoxification genes are upregulated, and immune genes are significantly downregulated in response to exotic host plant diet and viral infection. For the "common" genes, one of the down regulated genes was associated with immune response and three of the upregulated genes were associated with detoxification response such as Zing finger binding and proteinase kinase activity.

Discussion

Our study investigated the effects of viral infection and consumption of a nutritionally inferior host plant on the phenotypic and transcriptional responses of *Lycaeides melissa* caterpillars, which use both native and exotic host plants. We quantified the impact of viral infection and host plants on larval performance and gene expression variation. Our experimental treatments affected immunological genes and expression of genes which could be associated with immune, detoxification, and chemosensory functions. As such, we identify the following answers to our predictions: 1) Viral infection with JcDV results in physiological changes, including increased phenoloxidase activity and melanization, with no evidence of gene regulation changes as measured by transcriptome analysis; 2) *L. melissa* larvae fed the native, nutritionally superior host plant *A. canadensis* had a heightened immune response compared to larvae fed the novel host plant *M. sativa*, which was reflected in both immune assays and differential expression of canonical immune genes; 3) several canonical detoxification genes were upregulated in larvae fed with the novel host plant *M. sativa* as well as in infected larvae fed with native and exotic host plants. Specifically, we found that host plants caused significant differential gene expression responses in larvae, while viral infection had a minimal effect on these responses. Given the nutrient composition differences between the two host plant species and the genomic regions affecting *L. melissa* larval performance on these plants, these phenotypic and transcriptional responses result from the combined effects of several genes involved in tolerance and detoxification of plant compounds. In line with this hypothesis, the differentially expressed genes are widespread across the genome, with several genes being upregulated and downregulated in response to host plant treatments. We discuss these results in detail below.

Viral Infection Effects on Larval Performance

We examined the effects of viral infection and consumption of a nutritionally inferior host plant on multiple physiological parameters with known immunological roles, specifically standing phenoloxidase (PO), total PO, and melanization. Previous studies have shown that host plant-associated nutritional and phytochemical variation can have immunological consequences for lepidopteran larvae (Ponton et al. 2023; Muchoney et al. 2022; Resnik and Smilanich 2020). We found that for infected larvae, host use had significant consequences for total PO and melanization, with higher activity on the native host. This result is consistent with previous studies comparing performance on native host plants to introduced host plants, showing heightened cellular immune response on native host plants (Diamond and Kingsolver

2011; Lampert 2012; Muchoney et al., 2022, but see Mo and Smilanich 2023). Interestingly, a previous experiment measuring similar immune parameters in *L. melissa* did not find this result (Yoon et al., 2019). However, the current study has a pathogen challenge which was missing in the previous study which could be driving this disparity in the results between the two studies. Our results align with ecological immunology theory, which posits that access to high-quality nutritional resources strengthens the immune response due to the costly trade-offs involved in maintaining an effective immune system (Ponton et al., 2011). Concerning the experimental manipulation of a virus, our results demonstrate that Junonia coenia densovirus (JcDV) infection is associated with a heightened physiological immune response, specifically for total PO and melanization. These results are consistent with previous studies of lepidopteran larvae (Shelby and Popham, 2006; Li et al., 2021) and other insects (Rodriguez-Andres et al., 2012), showing that PO can have antiviral properties in the hemolymph.

Effect of Host Plant Diet on Differential Expression

Among our four comparisons, only two showed a substantial number of differentially expressed genes, revealing a strong effect of plant diet on gene regulation in *L. melissa* larvae. *L. melissa* has recently colonized the novel host plant, *Medicago sativa*, across their geographic range (Chaturvedi et al. 2018). Despite several generations of selection on the novel host, M. sativa, *L. melissa* populations still show lower survival and weight measures when reared on these plants compared to their native hosts. Herbivores overcome host plant phytochemical defenses by employing various behavioral, physical, and physiological mechanisms to prevent toxin ingestion and penetration through cuticle surfaces, gut surfaces and membranes (Groen and Whiteman 2022). Additionally, herbivores show a strong immune response to different host plant diets (Schmid-Hempel 2005; Tan et al. 2019). Interestingly, the differentially expressed genes for the uninfected larvae comparison (No Virus ASCA vs. No Virus MESA) and the infected larvae comparison (Virus ASCA vs. Virus MESA) represent several mechanistic gene regulation categories, indicating that in *L. melissa*, gene regulation in response to host plant diet is complex (Keehnen et al. 2018). For example, several canonical immune genes were upregulated and downregulated in uninfected larvae feeding on the novel host *M. sativa* (Supplementary Table 1). These include immune genes which are involved in recognition of pathogens, modulation of immune response, effector genes (Table 1). These genes have also been shown to play a role in gene regulation in response to host plant diet in other Lepidopterans (Keehnen et al. 2018; Tan et al. 2019). In addition to immune genes, several detoxification genes were upregulated in uninfected larvae feeding on *M. sativa* in (No Virus ASCA vs. No Virus MESA) such as Cytochrome C oxidase and Insect cuticle protein. Several other genes associated with GTPase proteins (hydrolase activity), FAD activity (oxidoreductase activity), and ATP binding (transporter activity) were also differentially expressed. These functions could broadly represent groups of genes associated with detoxification and excretion of toxic compounds (Groen and Whiteman 2022; Jeckel et al. 2022). Interestingly, the same larvae downregulated genes associated with immune response, primarily the Immunoglobulin E-set and Immunoglobulin-like domain superfamily. Previous studies on *L. melissa* have identified genomic regions associated with similar functional annotations. For example, the Immunoglobulin E-set/oxidoreductase activity genes are associated with genomic loci that act as barrier loci in *Lycaeides* butterfly hybrid zones where parental and hybrid populations utilize different host plants (Chaturvedi et al., 2020). Genes in this superfamily are also identified as a possible functional annotation for genomic loci associated with larval performance across host plants in *L. melissa* (Gompert et al., 2015). More broadly, these

genes have been implicated in other studies with PO activity, consistent with our experimental result of elevated total PO associated with infection. Thus, variation in genes associated with this functional annotation is implicated in larval performance across host plants in the absence of a pathogen.

For the infected larvae comparison (Virus ASCA vs. Virus MESA), where infected larvae were reared on both host plant species, we found a broader set of genes that showed significant differential expression (Supplementary Table 2). Along with the oxidoreductase and transporter genes, we also identified genes associated with cytochrome C oxidase activity, olfaction, transportation, coloration, and hemolymph activity upregulated in infected larvae reared on *M. sativa*. Given the complex nature of gene regulation in response to infection and host plant diet, these were interesting findings. Cytochrome P450s are known to play a role in detoxification of plant secondary metabolites and insecticides, consistent with a general pattern of increased plasticity of detoxification genes in herbivorous insects (Rêgo et al. 2020; Groen and Whiteman 2022). Insect olfaction is a crucial chemosensory response associated with larval response to variation in host plant diet in European corn borer and Adzuki bean borer species (Orsucci et al. 2018). Studies on novel host plant adaptation in spider mites have found variation in the expression of major facilitator transporter and lipocalin genes, indicating that these genes can affect novel host plant use across herbivorous insects (Dermauw et al. 2013; Wybouw et al. 2015). Interestingly, the differential expression of a single gene associated with the invertebrate coloration gene could suggest mechanisms associated with melanization (Li et al. 2021). Lastly, the haemolymph insect juvenile hormone-binding gene was downregulated, which regulates insect metamorphosis and reproduction. For the same treatment, several immune genes were downregulated in larvae reared on *M. sativa*, including Immunoglobulin E set genes and Serine/Protease genes. These genes have been implicated as canonical immune genes which show differential expression in other Lepidopterans (Keehnen et al. 2018; Tan et al. 2019).

For the native host plant comparison (No Virus ASCA vs. Virus ASCA), only one gene was upregulated, associated with the chitin-binding protein superfamily. These proteins are constituents of the peritrophic membrane or matrix, which lines the midgut of caterpillars and can act as a physical barrier to prevent toxin absorption. Studies have shown that insect herbivores show increased expression of this gene and other cuticle genes to activate jasmonic acid-mediated defensive signaling and production of reactive oxygen in response to host plant diets (Groen et al., 2016; Mittapalli et al., 2007; Whiteman et al., 2011).

We found evidence for the differential expression of a small number of canonical immune-related genes in response to viral infection and a significant enrichment of immune genes for this comparison. Both larvae feeding on the novel host plant, *M. sativa*, and larvae feeding on the native host, *A. canadensis*, regulated genes associated with immune response. The identification of specific immune-relevant genes can hopefully provide targets for future studies on the molecular basis of immune function in insects. Our study also aimed to advance understanding of the molecular mechanisms underlying host use and response to infection in butterflies. For example, it is interesting to note that the overall number of genes differentially expressed in response to viral infection was considerably lower for caterpillars raised on the exotic plant compared to the native plant (as shown in Fig. 2). This raises the possibility that larvae on a nutritionally superior host also mount a more extensive genetic response to

infection. However, whether similar effects occur in complex, natural environments and whether the stronger response results in stronger selection on immune function remains unknown.

Effect of Viral Infection on Differential Expression

Despite the possibility of interactive effects discussed above (such that the expression of immune genes is contingent on diet), when we tested for the effect of viral infection by controlling for host plant diet, we observed almost no transcriptional response to viral infection regardless of host plant treatment. Our results align with previous findings in lepidopteran systems showing a lack of transcriptional response to parasitic/viral infection. There are two possible explanations for our results. First, while JcDV can cause mortality at high concentrations, the load and prevalence that occur in natural populations are low (McKeegan et al. 2024, Muchoney et al. 2022). Thus, it is possible and even likely that this virus-host interaction represents a stable interaction and thus a strong physiological response is not needed. Second, the virus could suppress or escape the host immune system, as is evident in several other insect parasites (Gurung and Kanneganti 2015; Mahanta et al. 2023).

Distribution of differentially expressed genes across the genome

Theoretical models investigating the genetic basis of adaptation to host plant diets have traditionally assumed a simplistic or monogenic architecture of resistance and tolerance to plant toxins in herbivorous insects (Hardy et al. 2020; Hardy and Forister 2023). However, recent studies have identified an oligogenic and even polygenic architecture of host plant adaptation in insects (Chaturvedi et al. 2018; Rêgo et al. 2020; Sezer and Butlin 1998; Simon et al. 2015). Our previous work supports a polygenic model, with several loci across the genome potentially underlying adaptation to the novel host plant, *Medicago sativa*, in *L. melissa* butterflies (Gompert et al., 2015; Chaturvedi et al., 2018). Furthermore, polygenic and mostly additive genetic architectures affect growth and development in butterfly species on different plant genotypes (Gompert et al. 2022).

Our current results further support a polygenic model for host plant use, as we found that differentially expressed genes are distributed across the genome without specific regional enrichment, although there is some concentration on the Z chromosome. This distribution supports the idea that gene regulation for complex life history traits in *L. melissa* is likely polygenic, involving several genes that could form modules to regulate detoxification and immune responses (Fagny et al., 2021). While this model has been tested in studies of gene expression underlying development and wing pattern formation in butterflies (Wu et al., 2022), few studies have extended this model to identify transcriptomic variation underlying host plant diet adaptation in Lepidoptera and other insects, making our findings novel.

Conclusions

Our study demonstrates that consumption of a nutritionally inferior host plant can alter both physiological and transcriptional responses to infection, and we identified canonical detoxification and immune genes that are differentially expressed both in response to a novel host but not in response to a viral pathogen. These genes have the potential to undergo natural selection in the wild as immunological genes tend to evolve faster than average (Obbard et al., 2006; Jiggins and Kim, 2007). As anthropogenic change and effects on natural systems

continue to accelerate, it is reasonable to expect that native lepidopterans will continue to be exposed to novel and introduced host plants, and colonization of these host plants will occur, especially as native host plants become displaced (Tallamy et al., 2020). Thus, as we accumulate more examples of novel host use affecting the lepidopteran immune and detoxification response, incorporating immunity into our models of host range evolution should be a priority. This study, combined with previous literature reviews, demonstrates that there is growing evidence that consumption of novel host plants, especially nutritionally inferior ones, often results in a suppressed cellular and metabolic response in lepidopterans (Lampert, 2012). Interesting caveats to this trend include species such as the Baltimore checkerspot (*Euphydryas phaeton*) that derive benefits from sequestering secondary metabolites such as iridoid glycosides from their novel host plants, which appear to have anti-viral benefits (Muchoney et al. 2022, Christensen et al. 2024). Future meta-analyses are needed to assess the effect size of the relative benefits and disadvantages of novel host plant use on the lepidopteran immune response, while accounting for differences in sequestration strategy.

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Authors' Contributions

SY, JH, AS, and MF conceptualized the project. SY and KU performed fieldwork and experiments. KS conducted the RNA extractions, library prep, and prepared samples for sequencing. JH, VS, and SC ran bioinformatics and statistical analyses. SC and SY made the figures. SY and SC prepared and revised the manuscript. All authors reviewed and provided comments on the manuscript.

Data Accessibility

All sequence data and metadata will be available on dryad.org upon acceptance of the manuscript. Custom scripts for transcriptome analysis will be uploaded to GitHub as well upon acceptance of the manuscript.

Competing Interests

The authors have no competing interests.

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Figure 1. Line plots show A) variation in total PO, B) variation in standing PO, C) variation in melanization, and D) variation in fourth instar larval weight by host plant use and viral treatment. In each case, "native host" is A. canadensis and "exotic host" is M. sativa.

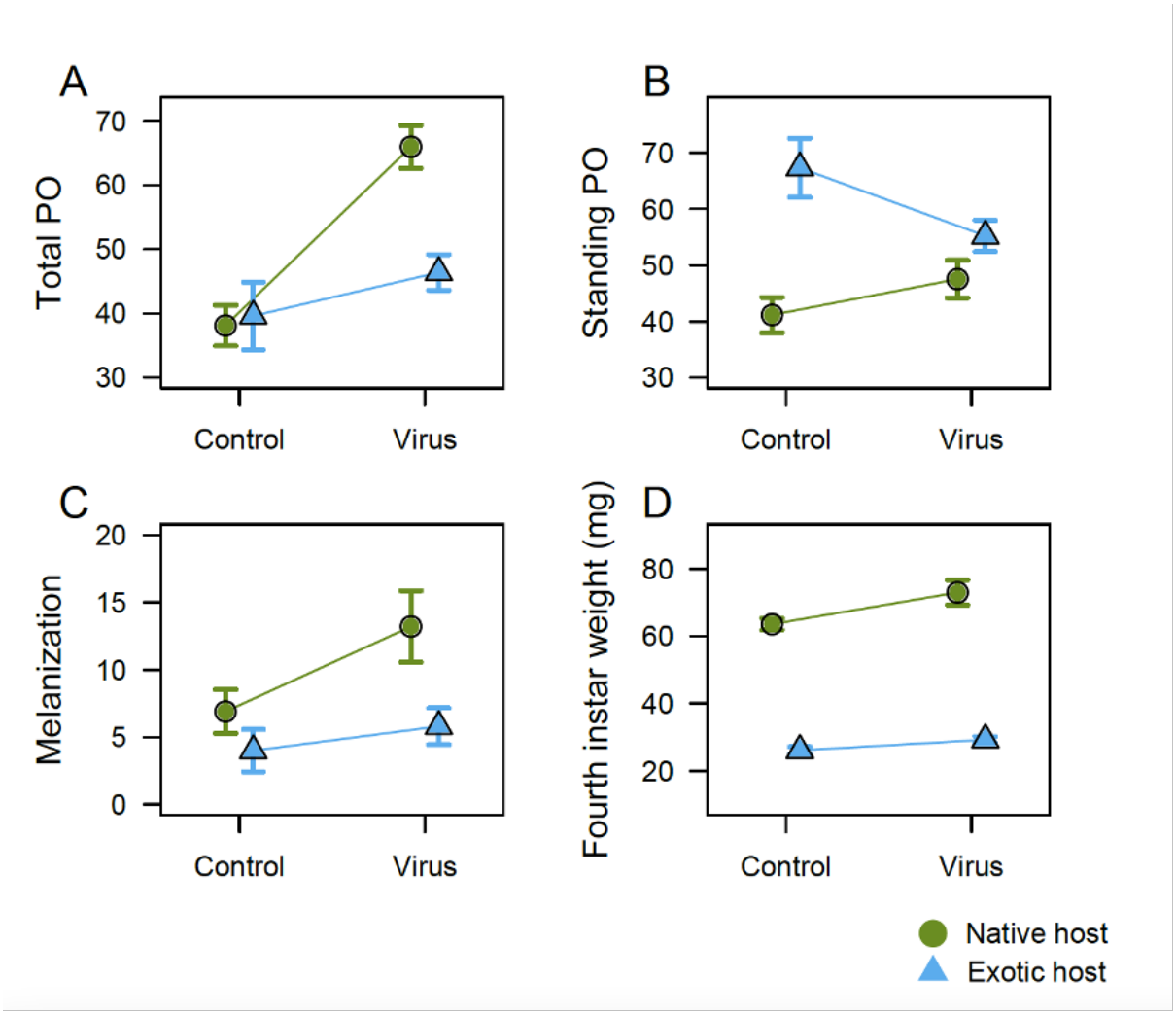

Figure 2. Variation in gene expression across samples. A) Violin plots showing horizontal distribution of normalized gene expression in all samples included in this study. The values from top to bottom represent the maximum, the upper quartile, the median, the lower quartile and the minimum in turn. The width of each violin represents the number of genes under the same expression. B) Principal Component Analyses (PCA) based on normalized gene expression of all genes (N=11,214) included in this dataset. C) Barplot showing the number of genes upregulated versus downregulated for the four comparisons included in the study. "No virus - ASCA" represents uninfected larvae fed with A. canadensis; "Virus ASCA" represents infected larvae fed with A. canadensis; "No virus - MESA" represents uninfected larvae fed with M. sativa;"Virus - MESA" represents infected larvae fed with M. sativa. This labelling is followed in all figures and tables below.

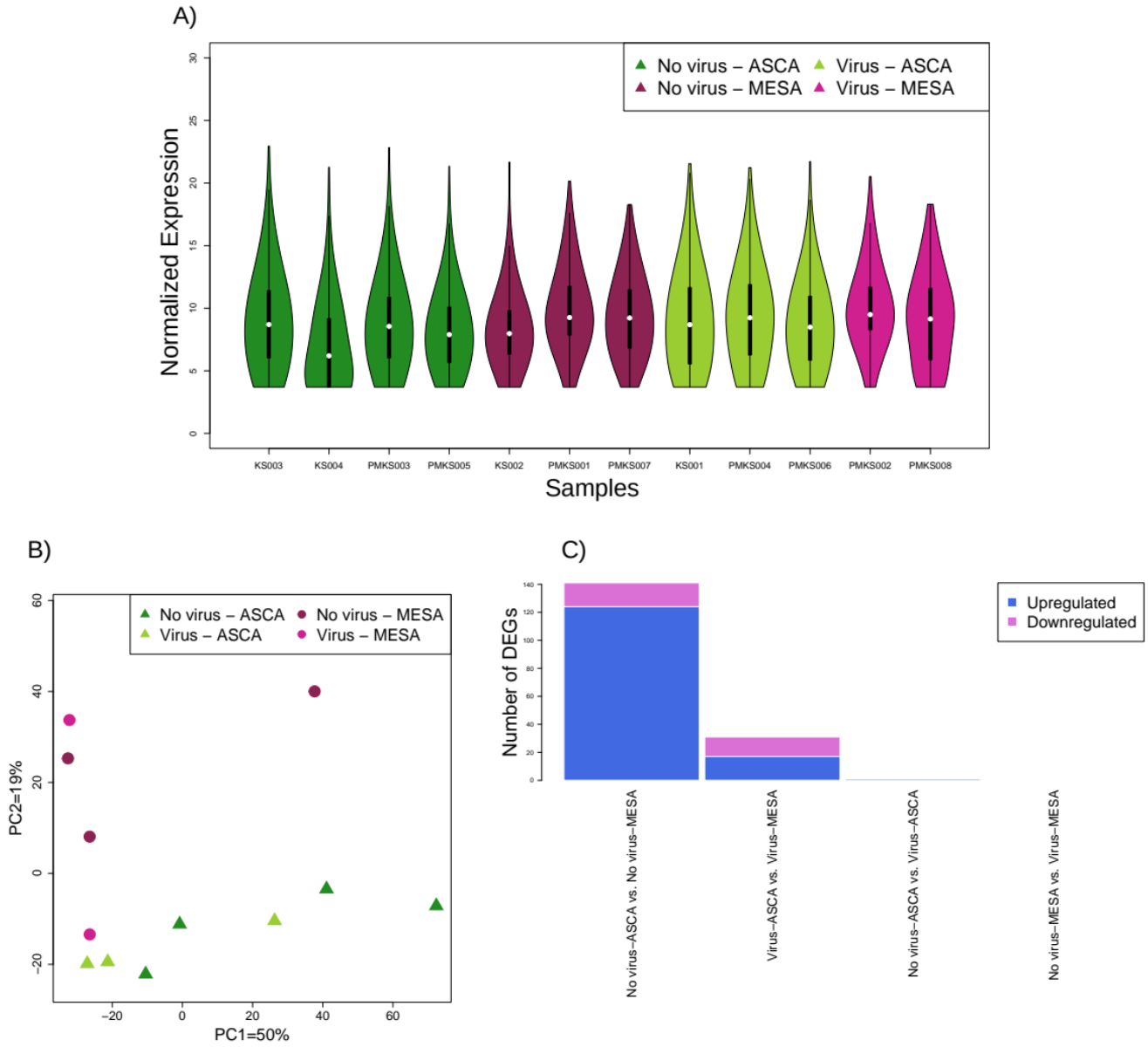

Figure 3: Heatmap and hierarchical clustering of the top 150 differentially expressed genes between samples included in this study show that host plant treatment affects clustering of differentially expressed genes.

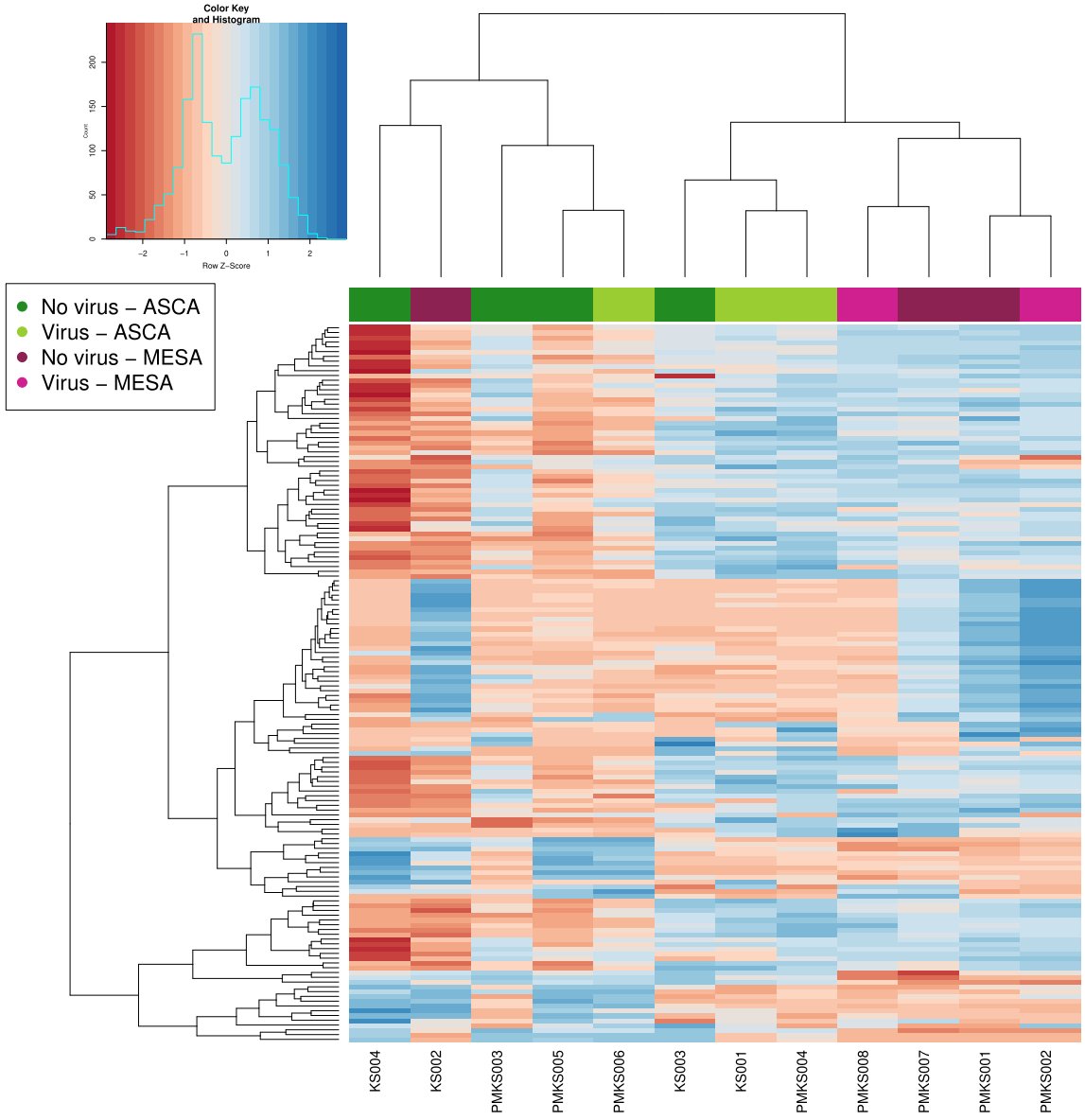

Figure 4. Volcano plots show differentially expressed genes for each of the four comparisons. In each figure panel, solid red lines delimit gene expression above a $\log^2$ fold change of 1 (upregulated on MESA, downregulated on ASCA) or below a $\log^2$ fold change of -1 (downregulated on MESA, upregulated on ASCA). Black dots indicate genes which show significant log2 fold change values.

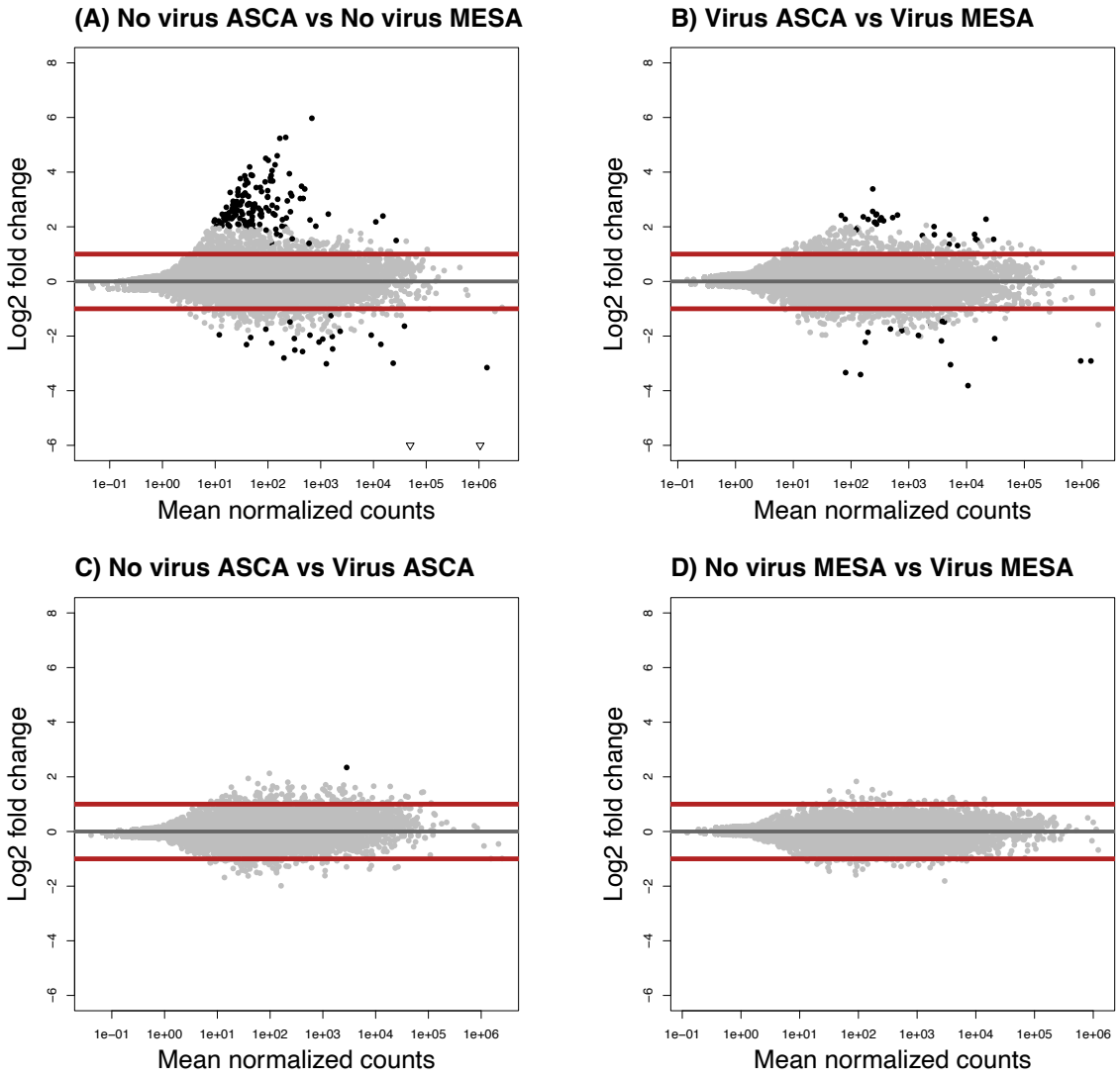

Figure 5: Boxplots show distribution of log^2 fold change values of differentially expressed genes for the following two comparisons A) Treatment 1: No virus - ASCA versus No virus - MESA (Total 140 DEGs), and B) Treatment 2: Virus - ASCA versus Virus - MESA (Total 31 DEGs). The boxplots are plotted for 23 chromosomes in the *Lycaeides melissa* genome to show how differential gene expression occurs across the genome for each comparisons. Chromosome 23 is the Z chromosome in the *L. melissa* genome.

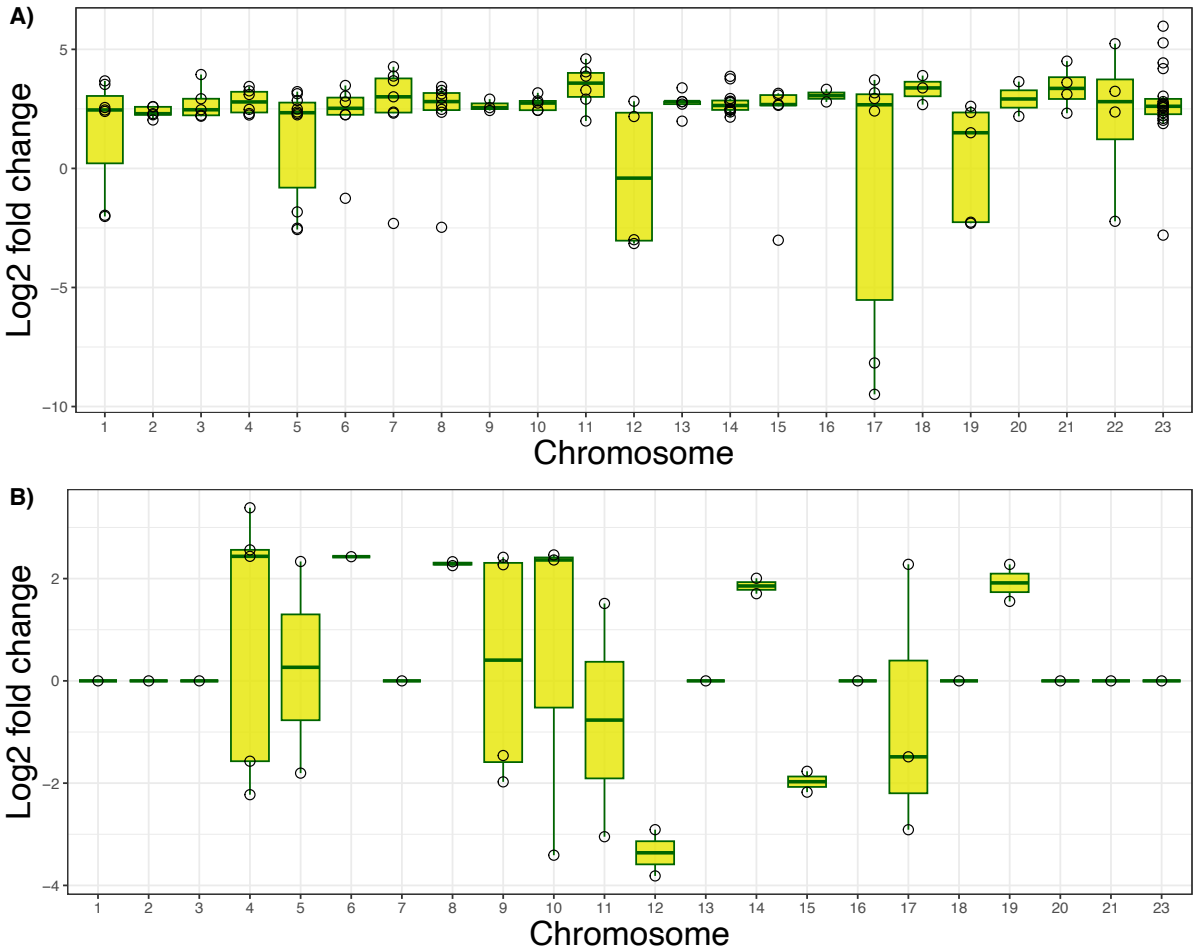

Table 1: Table shows details of the samples include in this study.

Sample name	Viral treatment	Plant treatment
KS001	Virus	ASCA
KS002	No virus	MESA
KS003	No virus	ASCA
KS004	No virus	ASCA
PMKS001	No virus	MESA
PMKS002	Virus	MESA
PMKS003	No virus	ASCA
PMKS004	Virus	ASCA
PMKS005	No virus	ASCA
PMKS006	Virus	ASCA
PMKS007	No virus	MESA
PMKS008	Virus	MESA

944

Table 2: Table shows list of canonical immune genes differentially expressed in the treatment 1 contrast (No virus – ASCA versus No virus – MESA) and treatment 2 contrast (Virus – ASCA versus Virus MESA). In each case, "upregulated" means genes which show upregulation in larvae feeding on *M. sativa*, and "downregulated" means genes which show down regulation in larvae feeding on *M. sativa*. Immune genes and their functions are assigned based on InteProScan super family domain classifications and Pfam and PANTHER modules.

Gene ID	Log2 Fold Change	Regulation	Chromosome	IPR Superfamily	Immune gene function
Contrast 1: No virus ACA versus No virus MESA					
melissa_00003657-RA	3.435	upregulated	8	Galectin	Regulation
melissa_00005721-RA	2.249	upregulated	6	Gloverin	Effector
melissa_00000412-RA	2.455	upregulated	1	Immunoglobulin-like fold	Recognition
melissa_00001048-RA	2.608	upregulated	23	Lymphocyte expansion molecule	Recognition
melissa_00000753-RA	3.675	upregulated	1	Pacifastin	Regulation
melissa_00000571-RA	2.393	upregulated	1	Serine proteases	Modulation
melissa_00001943-RA	-2.566	downregulated	5	Immunoglobulin subtype 2	Modulation
melissa_00001736-RA	-1.826	downregulated	5	Serine proteases	Modulation
Contrast 2: Virus ACA versus Virus MESA					
melissa_00003956-RA	3.384	upregulated	4	Lipocalin	Modulation
melissa_00001612-RA	2.334	upregulated	5	Serine/threonine-protein kinase	Modulation
melissa_00008868-RA	2.279	upregulated	17	Serine/threonine-protein kinase	Modulation
melissa_00009066-RA	-2.911	downregulated	17	Immunoglobulin E-set	Recognition
melissa_00007165-RA	-2.909	downregulated	12	Immunoglobulin E-set	Recognition

Table 3: Table shows list of canonical detoxification genes differentially expressed in the treatment 1 contrast (No virus – ASCA versus No virus – MESA) and treatment 2 contrast (Virus – ASCA versus Virus MESA). In each case, "upregulated" means genes which show upregulation in larvae feeding on *M. sativa*, and "downregulated" means genes which show down regulation in larvae feeding on *M. sativa*. Immune genes and their functions are assigned based on InteProScan super family domain classifications and Pfam and PANTHER modules.

Gene ID	Log2 Fold Change	Regulation	Chromosome	IPR Superfamily	Function
Comparison 1: No virus ASCA versus No virus MESA					
melissa_00005476-RA	1.991	upregulated	11	ABC transporter type 1	Detoxfication
melissa_00010472-RA	2.318	upregulated	21	Cytochrome c oxidase	Detoxification
melissa_00006291-RA	2.322	upregulated	7	Insect cuticle protein	Resistance
Comparison 2: Virus ASCA versus Virus MESA					
melissa_00004891-RA	2.462	upregulated	10	Olfactory receptor	Chemosensory
melissa_00006663-RA	-1.975	downregulated	9	Haemolymph juvenile hormone binding	Signaling

947

Supplementary Table 1: Table shows list of significantly differentially expressed genes for treatment 1 contrast (No virus – ASCA versus No virus – MESA) including uninfected larvae fed with *A. canadensis* versus infected larvae fed with *M. sativa*. The list includes the top genes significantly up-regulated in larvae fed with *M. sativa* and the top genes significantly down-regulated larvae fed with *M. sativa*.

Supplementary Table 2: Table shows list of significantly differentially expressed genes for treatment 2 contrast (Virus – ASCA versus Virus – MESA) including infected larvae fed with *A. canadensis* versus infected larvae fed with *M. sativa*. The list includes the top 15 genes significantly up-regulated when fed with *M. sativa* and the top 15 genes significantly up-regulated when fed with *M. sativa*.