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# 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 variation in their host plant diet by modulating several traits. Insect immune response is one 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. Here we study the interplay between the effects of host plant variation and viral infection on larval immune responses and global gene expression. We measured immune strength in response to infection by the Junonia coenia densovirus (JcDV) and performed transcriptional sequencing of L. melissa larvae 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, larvae differentially expressed hundredgenes in response to host plant treatment, but with minimal changes in gene expression in response to viral infection. These results demonstrate that in herbivorous insects, diet 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 insects.

# 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*

48 ## Introduction
49 Herbivorous insects and their host plants represent an intimate interaction where both spe-
50 cies exert selective pressures on each other that solicit behavioral, physiological, life-history,
51 and molecular responses (Schoonhoven, Van Loon, and Dicke 2005; Birnbaum and Abbot
52 2020; Groen and Whiteman 2022). Past work on plant-insect interactions has demonstrated
53 host plants effects on individual fitness and adaptation of insects (Gloss, Groen, and
54 Whiteman 2016; Vertacnik and Linnen 2017). While molecular studies on herbivorous in-
55 sects have mostly focused on the genomic basis of adaptation to host plants, we are only re-
56 cently starting to understand the gene regulatory mechanisms underlying tolerance and re-
57 sistance of chemical challenges presented by host plants (Nallu et al. 2018; Näsvall et al.
58 2021; Okamura et al. 2019; Tan et al. 2019; Ragland et al. 2015). Furthermore, specialist and
59 generalist herbivores differ in their adaptive mechanisms in response to different host plant
60 species such that while generalists invest in a broad range of chemical detoxification strate-
61 gies, specialist herbivores have evolved adjust responses to specific plant defenses (Marquis
62 and Koptur 2022; Vogel, Musser, and Paz Celorio-Mancera 2014; War et al. 2012; Lankau
63 2007) . Moreover, when herbivores are faced with novel host plant challenges, they poten-
64 tially employ different strategies to detoxify new secondary metabolites, which can be asso-
65 ciated with drastic changes in gene regulation. Indeed, studies have shown that different in-
66 sect species regulate different families of genes in response to variation in chemical content
67 from the different species of plants they utilize as their hosts (Tan et al. 2019; Näsvall et al.
68 2021).
69
70 While herbivorous insects have evolved several strategies to deal with host plant related
71 challenges, pathogens or parasites can present an added layer of selective pressure that can
72 influence fitness and survival in the wild. In this case, the insect immune response can influ-
73 ence fitness by affecting life history traits, despite the many costs associated with mounting
74 a response (Schulenburg et al. 2009; Catalán et al. 2012). The field of ecological immunology
75 seeks to understand how variation in biotic and abiotic factors contributes to immunological
76 variation in the wild, and how immune function evolves and is involved in the evolution of
77 other organisms (Schmid-Hempel 2005). Plant-feeding insects represent a rich testing
78 ground for examining ecological immunology concepts due to their diversity and abundance
79 in natural ecosystems (Janz et al., 2006). Along these lines, the Lepidoptera represent an es-
80 pecially interesting test case for ecological immunology given that many species within this
81 group are known to be specialists on various species of host plants of the same genus, which
82 can provide a tractable model to identify variation in the immune response as variation in
83 host plant diet can influence life history traits which in turn can affect immunity (Graves
84 and Shapiro, 2003). Moreover, plant metabolites can indirectly affect parasites by modulat-
85 ing the insect immune response creating a complex cascade of gene regulatory pathways.
86 Regulation of immune genes is an important adaptive strategy in response to host plant var-
87 iation or parasite infection. For instance, some species of butterflies, such as *Junonia coenia*,
88 *Militea cinxia*, and *Euphydryas phaeton*, regulate their immune response based on the iri-
89 doid glycoside concentrations in their host plants (Smilanich, Dyer, Chambers, & Bowers,
90 2009; Smilanich et al., 2017; Laurentz et al.,2012; Muchoney et al. 2022). Monarch butter-
91 flies reared on different milkweed species exhibit down regulation of immune genes on the
92 species which affects endogenous immune response (Tan et al. 2019).
93

94 With respect to larval development and performance, previous meta-analyses have shown
95 that novel host plants generally represent inferior hosts relative to native hosts for larval
96 lepidopterans despite the many butterflies and moths that are known to persist on exotic
97 hosts in the wild (Yoon and Read, 2016). Further, a previous literature survey comparing im-
98 mune strength on different host plants found that in 5 out of 10 published studies, lepidop-
99 teran larvae have higher cellular immune response when reared on high quality host plants,
100 with quality in this instance determined by fitness correlates such as larval weight (Lampert,
101 2012). In the remaining studies, only one showed that consumption of a comparatively
102 lower quality host plant led to a higher cellular immune response and a variation in regula-
103 tion of canonical immune genes (the remaining studies did not detect an effect of host plant
104 use) (Yoon et al. 2019; Mason, 2020). Furthermore, transcriptomic variation in response to
105 diet breadth in herbivores is not just dominated by immune genes. In fact, canonical detoxi-
106 fication, chemosensory, cuticle, and transporter genes - all interact to aid herbivores in tol-
107 erating low quality hosts and can eventually lead to adaptation and specialization to hosts in
108 their geographic ranges (Birnbaum & Abbot, 2019).
109
110 We explore these relationships using the butterfly *Lycaeides melissa* (Lycaenidae), a special-
111 ist herbivore on legumes including members of *Astragalus* and *Lupinus* (native hosts), as
112 well as the exotic legume *Medicago sativa* (Fabaceae) (exotic host), which it has colonized at
113 least twice and probably many times within the past 200 years (Forister et al., 2009, Chatur-
114 vedi et al. 2018). Across their geographic range in Northern America, these butterflies are
115 locally adapted to their native or exotic host plants (Chaturvedi et al. 2018). *Medicago sativa*
116 supports populations of *L. melissa* heterogeneously throughout the western United States
117 (Forister et al. 2020), despite reducing larval performance and adult fecundity compared to
118 a preferred native host *Astragalus canadensis* (Forister et al. 2009, Harrison et al. 2016).
119 Past work in this system has revealed that *L. melissa* immune strength can be affected by
120 nutritional, phytochemical, and microbial variation, and that these effects are host plant
121 specific (Yoon et al., 2019). Genomic studies on this system have revealed that novel host
122 plant adaptation is a polygenic trait with additive effects associated with larval development
123 and survival on different host plants (Gompert et al. 2015; Chaturvedi et al. 2018; Gompert
124 et al. 2022). However, what is still unclear is how variation in host plant use will affect the
125 ability of *L. melissa* larvae to respond to a live, experimentally introduced pathogenic threat.
126 Moreover, we have much to learn about physiological and genetic processes underlying
127 host plant-specific effects on development, detoxification and the immune response, which
128 is the issue that we address in the present study.
129
130 To address this issue, we quantified phenotypic and transcriptomic responses of caterpillars
131 infected with a lepidopteran virus and reared on a native and a novel host plant. Junonia
132 coenia densovirus (JcDV) was first discovered in the buckeye butterfly, *Junonia coenia* (Riv-
133 ers and Longworth, 1972; Bruemmer et al., 2005), and has been shown to infect other lepi-
134 dopteran species and families (Mutuel et al. 2010, Smilanich et al. 2018; Muchoney et al.
135 2022, 2023; McKeegan et al. 2024). This viral pathogen was chosen because it is common in
136 the environment, is frequently found at *L. melissa* populations in the wild throughout its
137 range and can affect larval survival in the lab (McKeegan et al., 2024, Yoon et al. un-
138 published data). In the noctuid moth *Spodoptera frugiperda*, JcDV infects larvae through
139 oral ingestion of viral particles, resulting in the virus crossing the midgut, and then finally
140 replicating in visceral tracheae and hemocyte cells, leading to death by hypoxia (Mutuel et

141 al., 2010). Transcriptome analyses have been successful in elucidating lepidopteran immu-
142 nological responses to both pathogens and differential host plant use across a wide range of
143 taxa (Vogel et al., 2001; Gandhe et al., 2006; Wang et al., 2016; Tan et al., 2019), but patho-
144 gens and host plant use have rarely been investigated in the same study (but see Tan et al.
145 2019). Therefore, our goal was to investigate whether functional genetic data can comple-
146 ment physiological assays, which have relied in large part on the phenoloxidase pathway.
147 The phenoloxidase pathway is one of the major immunological pathways in insects, and is a
148 generalized pathway that protects against viruses, bacteria, fungi, and parasitoids (Gonzá-
149 lez-Santoyo & Córdoba-Aguilar, 2012). Previous experimental work with the tobacco bud-
150 worm *Heliothis virescens* and the spongey moth *Lymantria dispar* have indicated that the
151 phenoloxidase enzyme has anti-viral properties in response to infection (Shelby and Pop-
152 ham, 2006; McNeil et al., 2009). While these studies suggest that the phenoloxidase path-
153 way and the melanization response may be important components of the lepidopteran anti-
154 viral response, other studies have found no notable role for the phenoloxidase enzyme in
155 antiviral immune response (Saejeng et al., 2010; Scholefield et al. 2019). Given uncertainty
156 associated with the phenoloxidase response, we have opted to pair our immune assays of
157 standing and total phenoloxidase and melanization with a transcriptome analysis of global
158 gene expression.
159
160 Ecological immunology theory predicts that immune responses are costly (Sheldon and Ver-
161 hulst, 1996) and that as organisms have access to higher quality nutritional resources, they
162 should have enhanced immune function due to increased resource availability (Ponton et
163 al., 2011). As such, we predict that 1) infection with JcDV will result in physiological changes,
164 including increased phenoloxidase activity and melanization, as well as differential upregula-
165 tion of immune related genes as measured by transcriptome analysis; 2) *L. melissa* larvae
166 fed the native, nutritionally superior host plant *A. canadensis* will have a heightened im-
167 mune response compared to larvae fed the novel host plant *M. sativa*, which should be re-
168 flected in both immune assays and differential expression of immune-relevant genetic re-
169 gions; 3) canonical detoxification genes will be upregulated in larvae reared on the novel
170 host plant *M. sativa*. By pairing physiological assays with a survey of gene expression, we
171 create an opportunity for learning about the molecular mechanisms underlying insect im-
172 mune response and how these mechanisms interact with nutrition. For example, we do not
173 know if, under conditions of poor nutrition, a caterpillar will simply have lower expression
174 levels of immune-related genes, or if different cellular, metabolic, or molecular processes
175 might be brought to bear in fighting a pathogen. Understanding these underlying molecular
176 mechanisms is essential for predicting the trajectory of adaptation to novel host plants in
177 plant-feeding insects and other parasitic organisms.
178

## 179 Materials and methods

### 180 Overview of experiments

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

186 For the first experiment, gravid *L. melissa* females were collected from a population associ-
187 ated with *M. sativa* at Verdi NV, USA (hereafter: VUH) during June 2016. Eggs acquired from
188 these females were randomly assigned to a host plant treatment (*A. canadensis* or *M. sa-*
189 *tiva*) and larvae were reared individually in petri dishes at ambient temperature and ten
190 hours of light per day, as previously described (Forister et al., 2009). *Medicago sativa* plants
191 were collected weekly from the same site where the maternal butterflies were collected,
192 and *A. canadensis* plants for rearing were collected from a nearby location that similarly
193 supports a population of *L. melissa*. We reared 125 larvae to the fourth (final) instar to be
194 used in immune experiments; 46 on *M. sativa* and 79 on *A. canadensis*. When larvae
195 reached their fourth instar, every other individual from each treatment group was selected
196 to be given 1 µl of Junonia coenia densovirus. Larvae were fed a 10mm diameter leaf disk
197 with 1 µl of 1 x $10^{11}$ virus particles/µl pipetted onto the leaf surface (purified virus stock
198 courtesy of M. Ogliastro, University of Montpellier, France). This concentration was used as
199 it is considered a "high" dose, which would potentially allow us to detect transcripts that are
200 only expressed during times of high viral load. This concentration has been shown to consti-
201 tute an LD50 in another lepidopteran species (Smilanich et al., 2018). They were allowed to
202 eat the leaf disk for 16 hours to ensure inoculation. After the inoculation period, larvae were
203 returned to their petri dishes and fed for 48 hours before immune assays.
204
205 For the second experiment, approximately 80 eggs from VUH were distributed evenly across
206 the two host plant treatments. From the original 80 larvae reared, approximately 60 sur-
207 vived to fourth instar. Larvae were reared until fourth instar, weighed, and then orally in-
208 fected in the same manner as described above, with the same concentration of virus. Infec-
209 tion and incubation of larvae was performed in a separate building from the initial rearing
210 process, and infected larvae were kept in a separate growth chamber after viral exposure to
211 prevent cross contamination. Larvae that served as controls were never exposed to the
212 lab/growth chamber housing infected larvae. After 48 hours, all larvae were weighed again
213 and then extracted for RNA. From these 60 extracted larvae, 12 larvae were chosen ran-
214 domly for sequencing.
215

## 216 Immune assays

217 Larval immune response was measured using three assays: standing and total phenoloxi-
218 dase (PO) activity and melanization via nylon filament injections. Standing PO is a measure-
219 ment of the naturally activated enzyme after the hemolymph is taken from the caterpillars
220 (Gonzalez-Santoyo and Cordoba-Aguilar, 2012). This assay measures the formation of do-
221 pachrome, which is assumed to be largely driven by active phenoloxidase. Total PO is a
222 measure of standing PO plus any inactive PO remaining within hemocytes. Filament injec-
223 tions serve as a proxy for a parasitism event and are a useful measure of immune response
224 in caterpillars. Both of these metrics accurately reflect the strength of the immune response
225 (Smilanich et al. 2009b).
226
227 Standing and total phenoloxidase were measured by taking 5 µl of hemolymph using a ster-
228 ile sewing needle from the abdominal cavity. Hemolymph was added to 100 µl of ice-cold
229 phosphate buffered saline (PBS) in an Eppendorf tube and was chilled on ice while a dopa-
230 mine solution (25.7 mg dopamine in 20 mL water) was prepared. Powdered dopamine
231 (Sigma-Aldrich; St. Louis, Missouri, USA) (0.0257 g) was added to 20 mL of distilled water.

232 The hemolymph bound PBS solution was split evenly between two well plates to run stand-
233 ing and total PO activity; 10 µl of cetylpridinium chloride solution (1g in 20 mL of distilled
234 water) was added to all wells measuring total PO, then 200 µl of the dopamine solution was
235 added to every well in the plate. Samples were incubated for 20 minutes at room tempera-
236 ture and the reaction then proceeded in a microplate reader (Bio-Rad iMark) for 45 minutes
237 (data recorded every 30 seconds at 490 nm); data were analyzed using Microplate Manager
238 (MPM) software (Bio-Rad v.6.3). We extracted the kinetic rate for the linear phase of the re-
239 action (0–45 minutes). In addition, blanks which consisted of distilled water and dopamine
240 were included as negative controls for each run. We did not run a positive control with each
241 run, however, samples from all treatment groups (both host treatments) were run together
242 to avoid confounding treatment with instrument variation.
243
244 After hemolymph extraction, larvae were individually injected with clear nylon filament ap-
245 proximately 2 mm in length. Filaments were injected at the same wound site where hemo-
246 lymph was previously drawn for PO assay (posterior abdominal segment). Larvae were re-
247 turned to their respective petri dishes and given access to plant tissue for 24 hours, then
248 frozen and dissected for filaments. Dissected filaments were photographed using a dissect-
249 ing microscope connected to a digital camera (Carl Ziess Discovery V.8, AXIOCAM Software,
250 Oberkochen, Baden-Wurttenburg, Germany). For each individual, each filament was photo-
251 graphed at 80X magnification, and their melanization value was recorded in ImageJ. For ad-
252 ditional details on melanization assay methods, see Smilanich et al., 2009a.
253

254 ## Statistical estimation of Immune Function and Larval Performance

255 All analyses were conducted in R (R Core Team 2019). Total PO, standing PO, melanization,
256 and larval weight were analyzed using linear models with host plant and treatment as fixed
257 effects, as well as the interaction between host plant and infection status. Assumptions of
258 linear models including normality and homoscedasticity of residuals were inspected. Host
259 plant and larval weight were not included as covariates in models together as variance infla-
260 tion factors were very high (>7) for these two covariates when they were included simulta-
261 neously in linear models.
262

263 ## RNA Extraction and Sequencing

264 Larval samples were homogenized in trizol (Life Technologies), and stored at -80C prior to
265 homogenization with a motorized pellet pestle. Larvae were extracted at the 4$^{th}$ instar stage
266 48 hours after viral infection. Total RNA was extracted using the Purelink RNA mini kit with
267 DNAse treatment per manufacturer's protocol (Ambion). Ethanol precipitated pellets were
268 resuspended in sterile water and quantified by Nanodrop. Barcoded mRNA libraries were
269 prepared with 1g of total RNA using the TruSeq Stranded mRNA kit (Illumina) and se-
270 quenced using on the HiSeq4000 platform at the Vincent J. Coates Genomics Sequencing La-
271 boratory at UC Berkeley.
272

273 ## Quality filtering, sequence alignment, and generating count matrix

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

278 or has been detected as containing an uncorrectable error. We then used a custom python
279 script to discard unfixable reads identified by RCorrector. Reads were then trimmed using
280 Trim Galore (version 0.3.3) (https://github.com/FelixKrueger/TrimGalore) to remove Illu-
281 mina adapter sequences. Filtered and quality-checked paired-end reads were aligned to an
282 existing, annotated genome of *L. melissa* (Chaturvedi et al. 2018, 2020) using STAR (version
283 1.5.2) (Dobin et al. 2013). STAR alignment rate ranged between 70-85% for all sample librar-
284 ies. We converted STAR alignments to gene count data for each sample using featureCounts
285 (version v2.0.0) (Liao, Smyth, and Shi 2014). Finally, we assigned gene annotations to tran-
286 scripts using the genome annotation for the *L. melissa* genome (for details of the genome
287 assembly and annotation see Chaturvedi et al. 2020). We used custom python scripts to
288 identify the interproscan IDs (IPR), PANTHER and Pfam IDs for the transcripts using this ge-
289 nome annotation. The scripts are archived on GitHub (https://github.com/chaturvedi-
290 lab/lyc_rnaseq_transcript_annotations). Whenever we discuss gene functions in the text,
291 we refer to their IPR domain and superfamily classification. These gene functions were also
292 validated using PANTHER and Pfam modules.
293

## 294 Differential expression analyses

295 We used the final raw gene counts file from above as an input to perform standardized dif-
296 ferential gene expression analyses using DESeq2 version 3.18 (Love et al., 2014). This analy-
297 sis was implemented in R version 4.1.0 (R Core Team, 2013). We filtered the dataset by re-
298 moving genes if they met any of the following criteria: (i) genes with non-zero counts in at
299 least two samples and (ii) genes with low coverage denoted with baseMean (count average
300 across all samples) <1. The DESeq2 analyses were performed using the default settings
301 where we normalized counts per gene by library size (the number of reads in a specific li-
302 brary) and used the Wald test to carry out significance testing for individual genes (Love et
303 al., 2014). We used the Benjamini and Hochberg (1995) method to produce adjusted signifi-
304 cance levels ($p_{adj}$) for each gene based on the false discovery rate (FDR) and thereby account
305 for multiple testing. We investigated the effect of host plant and viral infection on caterpillar
306 gene expression by using the following pairwise comparisons: (i) control group comparison
307 (*M. sativa* uninfected vs. *A. canadensis* uninfected) (Treatment 1), (ii) infected comparison
308 (infected *M. sativa* vs. infected *A. canadensis*) (Treatment 2), (iii) native host plant compari-
309 son (*A. canadensis* infected vs. *A. canadensis* control) (Treatment 3), and (iv) exotic host
310 plant comparison (*M. sativa* infected vs. *M. sativa* control) (Treatment 4). We identified
311 genes as exhibiting statistically significant differential expression for given pairwise compari-
312 son if $p_{adj}$ was < 0.05. We then used the gene annotations (as described in previous section)
313 to identify gene functions of differentially expressed gene sets for each comparison based
314 on InterProScan terms and the Pfam and PANTHER modules.
315

# 316 Results

## 317 Viral infection effects on phenoloxidase, melanization, and larval weight

318 A series of linear models were run to examine the effects of host plant use and viral treatment
319 on total PO, standing PO, melanization, and fourth instar larval weight. For total PO, we found
320 a direct effect of viral treatment ($F_{(1, 67)}$ = 13.128, p = 0.0005, Std. coefficient = 1.11 [0.66,
321 1.56]), along with a two-way interaction between host plant use and viral infection (Figure
322 1a; $F_{(1, 67)}$ = 5.693, p = 0.0198, Std. coefficient = -0.58 [-1.29, 0.13]). Infected larvae had higher
323 total PO than control larvae. For control larvae, host plant use had no detectable effect on

324 total PO, whereas for infected larvae, total PO was higher for larvae consuming the native
325 host (Fig 1a).
326
327 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,
329 host use did have a direct effect on standing PO, with larvae consuming the exotic host having
330 higher levels of standing PO ($F_{(1, 67)} = 4.999$, $p = 0.0287$, Std. coefficient = -0.10 [-0.62, 0.41]).
331 For percent melanization, we did not detect an interaction between host plant use and treat-
332 ment ($F_{(1, 67)} = 1.199$, $p=0.277$), 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 ($F_{(1, 67)} = 8.754$,
334 $p=0.003$, Std. coefficient = 0.66 [0.18, 1.14]), with larvae having higher melanization with viral
335 infection and lower melanization on the exotic host (Figure 1c).
336
337 For fourth instar larval weight, we found direct effects of both host use ($F_{(1, 73)} = 414.09$,
338 $p<0.0001$, Std. coefficient = -1.82 [-2.08, -1.57]), and viral treatment ($F_{(1, 73)} = 7.264$, $p=0.008$,
339 Std. coefficient = 0.17 [-0.08, 0.42]), however, we did not find an interaction between host
340 and treatment (Fig 1d). Fourth instar larval weight was higher on the native host plant and in
341 infected individuals.
342

## 343 Alignment, gene count estimation, and differential gene expression

344 We obtained ~43 million reads after aligning our samples to the *L. melissa* reference genome.
345 The number of reads per sample varied from 3.4 to 5.9 million. After quality filtering we ended
346 up with a global gene expression dataset of 11, 214 genes. The DESeq2 filtering of gene counts
347 revealed that the median gene counts of the 12 samples were similar, and the normalized
348 gene expression values ranged from 6.20 to 9.49 (meaning the amount of mRNA detected in
349 each sample) (Figure 2A). We then visualized variation in gene expression between individual
350 larvae using principal component analysis using the plotPCA function in DESeq2 (PCA). The
351 normalized gene expression values were transformed using the DESeq2 getVariationStabi-
352 lizedData function prior to performing the PCA. Our PCA results revealed that host plant is
353 the main determinant of variation in gene expression in our dataset with the first two princi-
354 pal components explaining most of the variation (PC1=50%, PC2=19%, Figure 2B). Larvae
355 reared on the same host plant clustered together irrespective of their infection status. This
356 result was mirrored by our heatmap and hierarchical clustering analysis which showed that
357 individual larvae are more clustered by plant diet irrespective of viral infection with some
358 subtle but variable clustering based on viral infection (Figure 3). Overall, larvae showed gene
359 expression similarity based on host plant treatment with high variation between plant treat-
360 ments but minimal variation within plant treatments irrespective of viral infection.
361

## 362 Effect of experimental treatments on gene expression

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

371 MESA). For this comparison, we found that a total of 31 genes showed significant differential
372 expression for this comparison where 17 genes were upregulated in larvae reared on *M. sa-*
373 *tiva* and 14 genes downregulated (Figure 2C, Figure 4B, Supp. Table 2).
374
375 We then compared gene expression between infected and uninfected larvae reared on the
376 same host plant to identify the effect of viral infection while controlling host plant treatment
377 (native host plant comparison: No virus ASCA versus virus ASCA, and exotic host plant com-
378 parison: No virus MESA versus virus MESA). Surprisingly, we found one or zero significantly
379 differentially expressed genes for these treatments. We found only one gene was significantly
380 upregulated for the native host plant comparison where uninfected larvae and infected larvae
381 were reared on *A. canadensis,* and none were differentially expressed for the exotic host plant
382 comparison where uninfected larvae and infected larvae were reared on *M. sativa* (Figures
383 2C and Figure 4C and 4D). Overall, these results were indicative of a strong effect of host plant
384 and a weak to negligible effect of viral infection on larval gene expression response.
385
386 We then checked if the same genes show significant differential expression across treat-
387 ments. We found that two genes were downregulated on *M. sativa*, and seven genes were
388 upregulated on *M. sativa* between uninfected and infected comparisons. This was indicative
389 of minimal levels of convergence in gene regulation in response to viral and diet stress. No
390 genes showed opposite directionality in regulation between the two treatments. We refer to
391 these genes as "common" genes from here on.
392

## 393 Genomic distribution and functional properties of DEGs

394 We evaluated the distribution of genes showing significant differential expression in our treat-
395 ment comparisons to identify the underlying genetic architecture of infection and diet related
396 traits in *L. melissa*. For the uninfected larvae comparison (No virus – ASCA versus No virus –
397 MESA), differentially expressed genes were present on all 23 chromosomes of the *L. melissa*
398 genome (Figure 5A). Nineteen out of the total 140 genes were present on the Z chromosome
399 (18 genes upregulated and 1 gene downregulated on MESA). For the infected larvae compar-
400 ison (Virus – ASCA versus Virus – MESA), relevant genes were present on 10 chromosomes,
401 none on the Z chromosome (Figure 5B). The one significantly differentially expressed gene for
402 native host plant comparison (No virus ASCA versus Virus ASCA) was present on chromosome
403 11.
404
405 We then evaluated the functional properties of the DEGs for each treatment. We saw signifi-
406 cant up-regulation or down-regulation of several insect immune genes and detoxification
407 genes (Table 1 and 2). The immune genes were involved in different immune processes such
408 as response, signaling, and resistance (Table 1). Similarly, the detoxification genes underlie
409 different proximate mechanisms such as signaling and chemosensory behavior (Table 2). Be-
410 sides these categories of genes, for the uninfected larvae comparison (No virus ASCA versus
411 No virus MESA) the top genes which were significantly up-regulated in those feeding on *M.*
412 *sativa* were associated with the tubulin protein, FAD/NAD(P)-binding, and the Zinc finger pro-
413 tein (Supp. table 1). Interestingly, the top five down-regulated genes for this treatment were
414 associated with immune responses such as Serine/Protease function and the immunoglobulin
415 E family (Table 2). For the infected larvae comparison, the top genes significantly upregulated
416 in caterpillars feeding on *M. sativa* were associated with Zinc finger proteins, protein kinase,
417 neurotransmitter genes, cytochrome C oxidase genes, and olfactory receptor genes (Table 2,

418 Supp. Table 2). Here too, the top down-regulated genes for this treatment were also associ-
419 ated with immune response such as immunoglobulin genes and hemocyanin genes with some
420 detoxification genes showing differential expression such as hemolymph protein genes, and
421 zinc finger genes. For the native host plant comparison, which included infected and unin-
422 fected larvae fed with *A. canadensis*, only one gene was significantly upregulated in the in-
423 fected group when compared to the uninfected group: a chitin binding domain gene. Overall,
424 our results indicate that detoxification genes are upregulated, and immune genes are signifi-
425 cantly downregulated in response to exotic host plant diet and viral infection. For the "com-
426 mon" genes, one of the down regulated genes was associated with immune response and
427 three of the upregulated genes were associated with detoxification response such as Zing
428 finger binding and proteinase kinase activity.
429

## 430 Discussion

431 Our study investigated the effects of viral infection and consumption of a nutritionally inferior
432 host plant on the phenotypic and transcriptional responses of *Lycaeides melissa* caterpillars,
433 which use both native and exotic host plants. We quantified the impact of viral infection and
434 host plants on larval performance and gene expression variation. Our experimental treat-
435 ments affected immunological genes and expression of genes which could be associated with
436 immune, detoxification, and chemosensory functions. As such, we identify the following an-
437 swers to our predictions: 1) Viral infection with JcDV results in physiological changes, including
438 increased phenoloxidase activity and melanization, with no evidence of gene regulation
439 changes as measured by transcriptome analysis; 2) *L. melissa* larvae fed the native, nutrition-
440 ally superior host plant *A. canadensis* had a heightened immune response compared to larvae
441 fed the novel host plant *M. sativa*, which was reflected in both immune assays and differential
442 expression of canonical immune genes; 3) several canonical detoxification genes were upreg-
443 ulated in larvae fed with the novel host plant *M. sativa* as well as in infected larvae fed with
444 native and exotic host plants. Specifically, we found that host plants caused significant differ-
445 ential gene expression responses in larvae, while viral infection had a minimal effect on these
446 responses. Given the nutrient composition differences between the two host plant species
447 and the genomic regions affecting *L. melissa* larval performance on these plants, these phe-
448 notypic and transcriptional responses result from the combined effects of several genes in-
449 volved in tolerance and detoxification of plant compounds. In line with this hypothesis, the
450 differentially expressed genes are widespread across the genome, with several genes being
451 upregulated and downregulated in response to host plant treatments. We discuss these re-
452 sults in detail below.
453

### 454 Viral Infection Effects on Larval Performance

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

464 2011; Lampert 2012; Muchoney et al., 2022, but see Mo and Smilanich 2023). Interestingly, a
465 previous experiment measuring similar immune parameters in *L. melissa* did not find this re-
466 sult (Yoon et al., 2019). However, the current study has a pathogen challenge which was miss-
467 ing in the previous study which could be driving this disparity in the results between the two
468 studies. Our results align with ecological immunology theory, which posits that access to high-
469 quality nutritional resources strengthens the immune response due to the costly trade-offs
470 involved in maintaining an effective immune system (Ponton et al., 2011). Concerning the
471 experimental manipulation of a virus, our results demonstrate that Junonia coenia densovirus
472 (JcDV) infection is associated with a heightened physiological immune response, specifically
473 for total PO and melanization. These results are consistent with previous studies of lepidop-
474 teran larvae (Shelby and Popham, 2006; Li et al., 2021) and other insects (Rodriguez-Andres
475 et al., 2012), showing that PO can have antiviral properties in the hemolymph.
476

477 ## Effect of Host Plant Diet on Differential Expression

478 Among our four comparisons, only two showed a substantial number of differentially ex-
479 pressed genes, revealing a strong effect of plant diet on gene regulation in *L. melissa* larvae.
480 *L. melissa* has recently colonized the novel host plant, *Medicago sativa*, across their geo-
481 graphic range (Chaturvedi et al. 2018). Despite several generations of selection on the novel
482 host, M. sativa, *L. melissa* populations still show lower survival and weight measures when
483 reared on these plants compared to their native hosts. Herbivores overcome host plant phy-
484 tochemical defenses by employing various behavioral, physical, and physiological mecha-
485 nisms to prevent toxin ingestion and penetration through cuticle surfaces, gut surfaces and
486 membranes (Groen and Whiteman 2022). Additionally, herbivores show a strong immune re-
487 sponse to different host plant diets (Schmid-Hempel 2005; Tan et al. 2019). Interestingly, the
488 differentially expressed genes for the uninfected larvae comparison (No Virus ASCA vs. No
489 Virus MESA) and the infected larvae comparison (Virus ASCA vs. Virus MESA) represent sev-
490 eral mechanistic gene regulation categories, indicating that in *L. melissa*, gene regulation in
491 response to host plant diet is complex (Keehnen et al. 2018). For example, several canonical
492 immune genes were upregulated and downregulated in uninfected larvae feeding on the
493 novel host *M. sativa* (Supplementary Table 1). These include immune genes which are in-
494 volved in recognition of pathogens, modulation of immune response, effector genes (Table
495 1). These genes have also been shown to play a role in gene regulation in response to host
496 plant diet in other Lepidopterans (Keehnen et al. 2018; Tan et al. 2019). In addition to immune
497 genes, several detoxification genes were upregulated in uninfected larvae feeding on *M. sa-*
498 *tiva* in (No Virus ASCA vs. No Virus MESA) such as Cytochrome C oxidase and Insect cuticle
499 protein. Several other genes associated with GTPase proteins (hydrolase activity), FAD activity
500 (oxidoreductase activity), and ATP binding (transporter activity) were also differentially ex-
501 pressed. These functions could broadly represent groups of genes associated with detoxifica-
502 tion and excretion of toxic compounds (Groen and Whiteman 2022; Jeckel et al. 2022). Inter-
503 estingly, the same larvae downregulated genes associated with immune response, primarily
504 the Immunoglobulin E-set and Immunoglobulin-like domain superfamily. Previous studies on
505 *L. melissa* have identified genomic regions associated with similar functional annotations. For
506 example, the Immunoglobulin E-set/oxidoreductase activity genes are associated with ge-
507 nomic loci that act as barrier loci in *Lycaeides* butterfly hybrid zones where parental and hy-
508 brid populations utilize different host plants (Chaturvedi et al., 2020). Genes in this super-
509 family are also identified as a possible functional annotation for genomic loci associated with
510 larval performance across host plants in *L. melissa* (Gompert et al., 2015). More broadly, these

511 genes have been implicated in other studies with PO activity, consistent with our experi-
512 mental result of elevated total PO associated with infection. Thus, variation in genes associ-
513 ated with this functional annotation is implicated in larval performance across host plants in
514 the absence of a pathogen.
515
516 For the infected larvae comparison (Virus ASCA vs. Virus MESA), where infected larvae were
517 reared on both host plant species, we found a broader set of genes that showed significant
518 differential expression (Supplementary Table 2). Along with the oxidoreductase and trans-
519 porter genes, we also identified genes associated with cytochrome C oxidase activity, olfac-
520 tion, transportation, coloration, and hemolymph activity upregulated in infected larvae
521 reared on *M. sativa*. Given the complex nature of gene regulation in response to infection and
522 host plant diet, these were interesting findings. Cytochrome P450s are known to play a role
523 in detoxification of plant secondary metabolites and insecticides, consistent with a general
524 pattern of increased plasticity of detoxification genes in herbivorous insects (Rêgo et al. 2020;
525 Groen and Whiteman 2022). Insect olfaction is a crucial chemosensory response associated
526 with larval response to variation in host plant diet in European corn borer and Adzuki bean
527 borer species (Orsucci et al. 2018). Studies on novel host plant adaptation in spider mites have
528 found variation in the expression of major facilitator transporter and lipocalin genes, indicat-
529 ing that these genes can affect novel host plant use across herbivorous insects (Dermauw et
530 al. 2013; Wybouw et al. 2015). Interestingly, the differential expression of a single gene asso-
531 ciated with the invertebrate coloration gene could suggest mechanisms associated with
532 melanization (Li et al. 2021). Lastly, the haemolymph insect juvenile hormone-binding gene
533 was downregulated, which regulates insect metamorphosis and reproduction. For the same
534 treatment, several immune genes were downregulated in larvae reared on *M. sativa*, includ-
535 ing Immunoglobulin E set genes and Serine/Protease genes. These genes have been impli-
536 cated as canonical immune genes which show differential expression in other Lepidopterans
537 (Keehnen et al. 2018; Tan et al. 2019).
538
539 For the native host plant comparison (No Virus ASCA vs. Virus ASCA), only one gene was up-
540 regulated, associated with the chitin-binding protein superfamily. These proteins are constit-
541 uents of the peritrophic membrane or matrix, which lines the midgut of caterpillars and can
542 act as a physical barrier to prevent toxin absorption. Studies have shown that insect herbi-
543 vores show increased expression of this gene and other cuticle genes to activate jasmonic
544 acid-mediated defensive signaling and production of reactive oxygen in response to host plant
545 diets (Groen et al., 2016; Mittapalli et al., 2007; Whiteman et al., 2011).
546
547 We found evidence for the differential expression of a small number of canonical immune-
548 related genes in response to viral infection and a significant enrichment of immune genes for
549 this comparison. Both larvae feeding on the novel host plant, *M. sativa*, and larvae feeding on
550 the native host, *A. canadensis*, regulated genes associated with immune response. The iden-
551 tification of specific immune-relevant genes can hopefully provide targets for future studies
552 on the molecular basis of immune function in insects. Our study also aimed to advance un-
553 derstanding of the molecular mechanisms underlying host use and response to infection in
554 butterflies. For example, it is interesting to note that the overall number of genes differen-
555 tially expressed in response to viral infection was considerably lower for caterpillars raised on
556 the exotic plant compared to the native plant (as shown in Fig. 2). This raises the possibility
557 that larvae on a nutritionally superior host also mount a more extensive genetic response to

558 infection. However, whether similar effects occur in complex, natural environments and
559 whether the stronger response results in stronger selection on immune function remains un-
560 known.
561

### 562 Effect of Viral Infection on Differential Expression

563 Despite the possibility of interactive effects discussed above (such that the expression of im-
564 mune genes is contingent on diet), when we tested for the effect of viral infection by control-
565 ling for host plant diet, we observed almost no transcriptional response to viral infection re-
566 gardless of host plant treatment. Our results align with previous findings in lepidopteran sys-
567 tems showing a lack of transcriptional response to parasitic/viral infection. There are two pos-
568 sible explanations for our results. First, while JcDV can cause mortality at high concentrations,
569 the load and prevalence that occur in natural populations are low (McKeegan et al. 2024,
570 Muchoney et al. 2022). Thus, it is possible and even likely that this virus-host interaction rep-
571 resents a stable interaction and thus a strong physiological response is not needed. Second,
572 the virus could suppress or escape the host immune system, as is evident in several other
573 insect parasites (Gurung and Kanneganti 2015; Mahanta et al. 2023).
574

### 575 Distribution of differentially expressed genes across the genome

576 Theoretical models investigating the genetic basis of adaptation to host plant diets have tra-
577 ditionally assumed a simplistic or monogenic architecture of resistance and tolerance to plant
578 toxins in herbivorous insects (Hardy et al. 2020; Hardy and Forister 2023). However, recent
579 studies have identified an oligogenic and even polygenic architecture of host plant adaptation
580 in insects (Chaturvedi et al. 2018; Rêgo et al. 2020; Sezer and Butlin 1998; Simon et al. 2015).
581 Our previous work supports a polygenic model, with several loci across the genome poten-
582 tially underlying adaptation to the novel host plant, *Medicago sativa*, in *L. melissa* butterflies
583 (Gompert et al., 2015; Chaturvedi et al., 2018). Furthermore, polygenic and mostly additive
584 genetic architectures affect growth and development in butterfly species on different plant
585 genotypes (Gompert et al. 2022).
586
587 Our current results further support a polygenic model for host plant use, as we found that
588 differentially expressed genes are distributed across the genome without specific regional
589 enrichment, although there is some concentration on the Z chromosome. This distribution
590 supports the idea that gene regulation for complex life history traits in *L. melissa* is likely pol-
591 ygenic, involving several genes that could form modules to regulate detoxification and im-
592 mune responses (Fagny et al., 2021). While this model has been tested in studies of gene
593 expression underlying development and wing pattern formation in butterflies (Wu et al.,
594 2022), few studies have extended this model to identify transcriptomic variation underlying
595 host plant diet adaptation in Lepidoptera and other insects, making our findings novel.
596

## 597 Conclusions

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

604 continue to accelerate, it is reasonable to expect that native lepidopterans will continue to be
605 exposed to novel and introduced host plants, and colonization of these host plants will occur,
606 especially as native host plants become displaced (Tallamy et al., 2020). Thus, as we accumu-
607 late more examples of novel host use affecting the lepidopteran immune and detoxification
608 response, incorporating immunity into our models of host range evolution should be a prior-
609 ity. This study, combined with previous literature reviews, demonstrates that there is growing
610 evidence that consumption of novel host plants, especially nutritionally inferior ones, often
611 results in a suppressed cellular and metabolic response in lepidopterans (Lampert, 2012). In-
612 teresting caveats to this trend include species such as the Baltimore checkerspot (*Euphydryas*
613 *phaeton*) that derive benefits from sequestering secondary metabolites such as iridoid glyco-
614 sides from their novel host plants, which appear to have anti-viral benefits (Muchoney et al.
615 2022, Christensen et al. 2024). Future meta-analyses are needed to assess the effect size of
616 the relative benefits and disadvantages of novel host plant use on the lepidopteran immune
617 response, while accounting for differences in sequestration strategy.
618

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623

## 624 Authors' Contributions

625 SY, JH, AS, and MF conceptualized the project. SY and KU performed fieldwork and experi-
626 ments. KS conducted the RNA extractions, library prep, and prepared samples for sequenc-
627 ing. JH, VS, and SC ran bioinformatics and statistical analyses. SC and SY made the figures. SY
628 and SC prepared and revised the manuscript. All authors reviewed and provided comments
629 on the manuscript.
630

## 631 Data Accessibility

632 All sequence data and metadata will be available on dryad.org upon acceptance of the man-
633 uscript. Custom scripts for transcriptome analysis will be uploaded to GitHub as well upon
634 acceptance of the manuscript.
635

## 636 Competing Interests

637 The authors have no competing interests.
638

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909 **Figure 1.** Line plots show A) variation in total PO, B) variation in standing PO, C) variation in
910 melanization, and D) variation in fourth instar larval weight by host plant use and viral treat-
911 ment. In each case, “native host” is A. canadensis and “exotic host” is M. sativa.
912

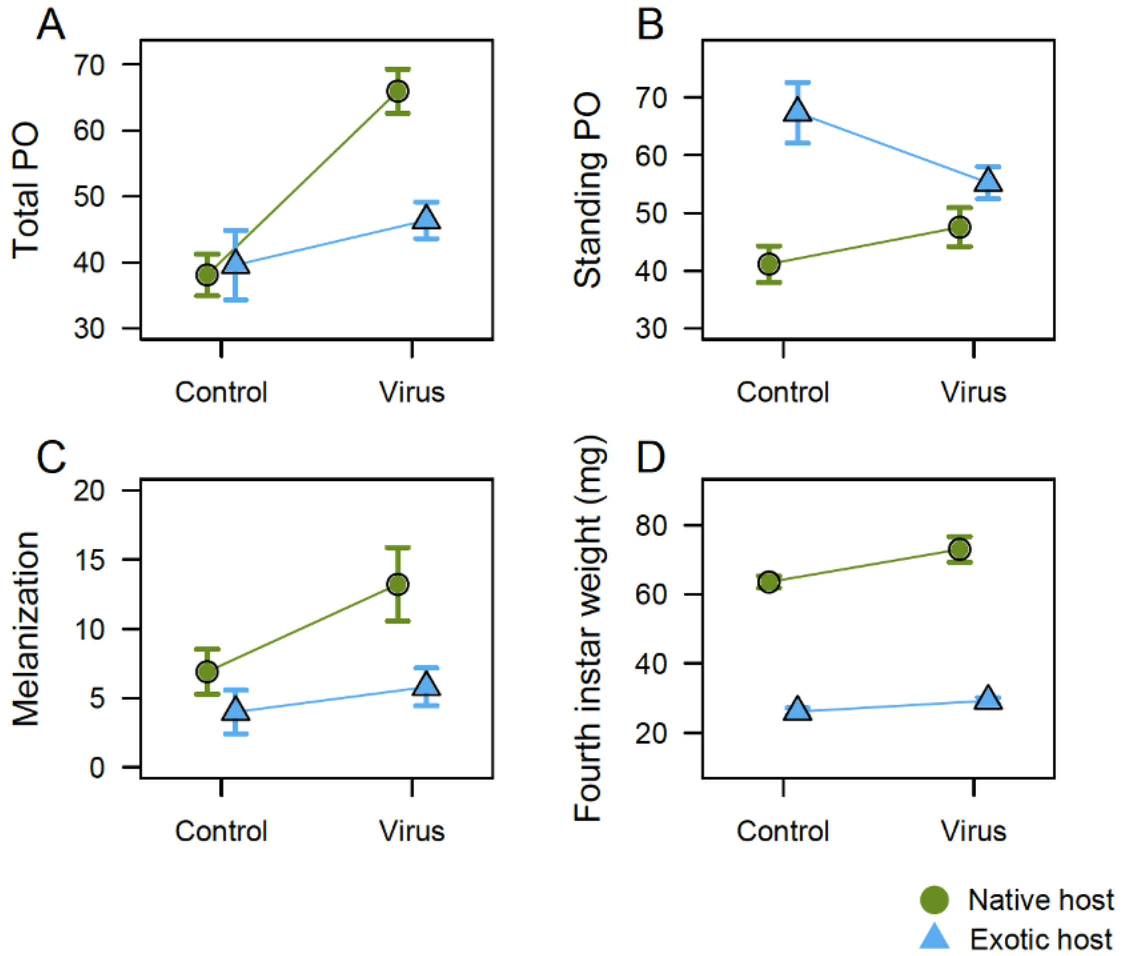


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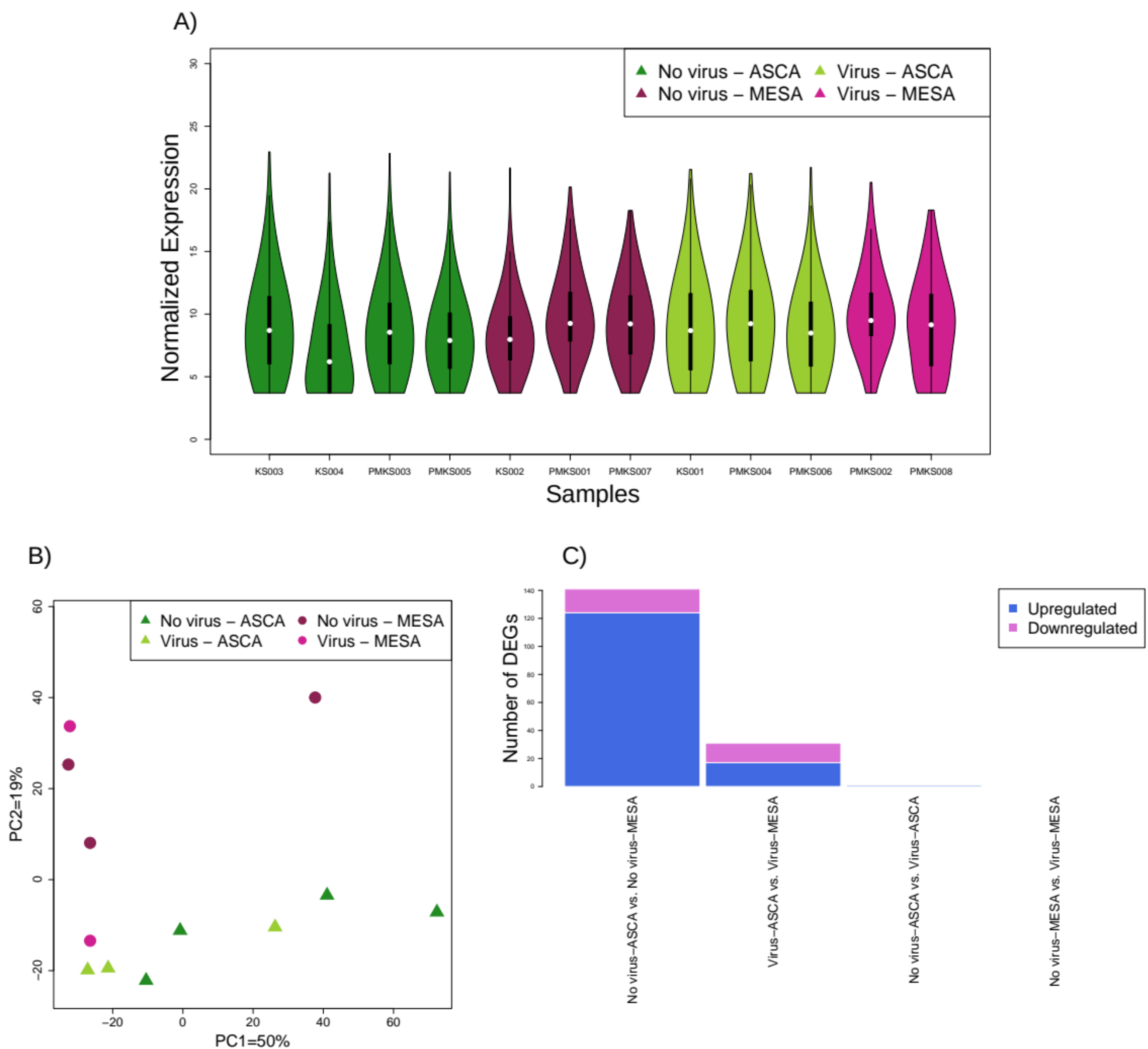
**Figure 2.** Variation in gene expression across samples. A) Violin plots showing horizontal distribution of normalized gene expression in all samples included in this study. The values from top to bottom represent the maximum, the upper quartile, the median, the lower quartile and the minimum in turn. 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.



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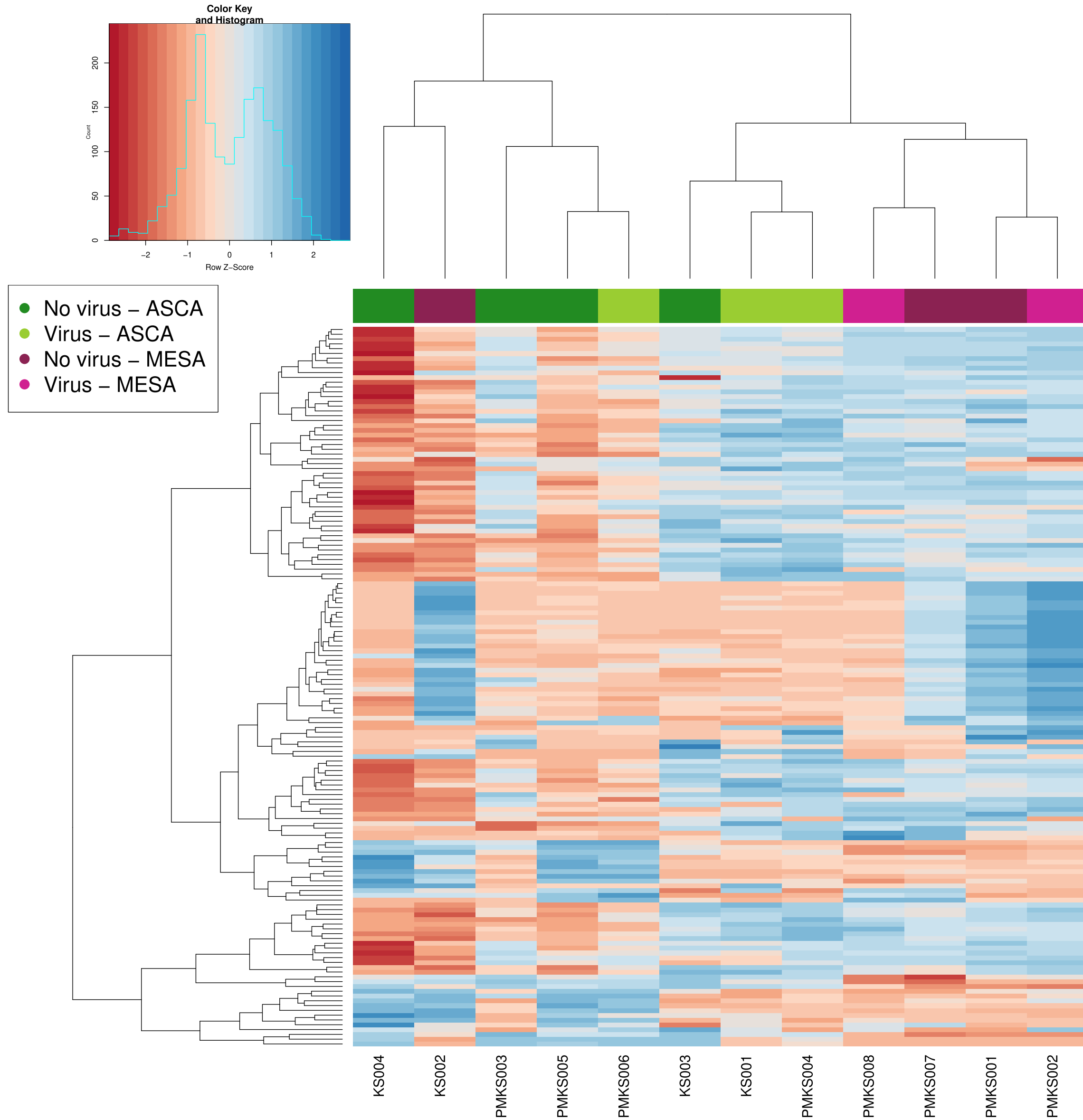


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**Figure 4.** Volcano plots show differentially expressed genes for each of the four comparisons. In each figure panel, solid red lines delimit gene expression above a $\log^2$ fold change of 1 (upregulated on MESA, downregulated on ASCA) or below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

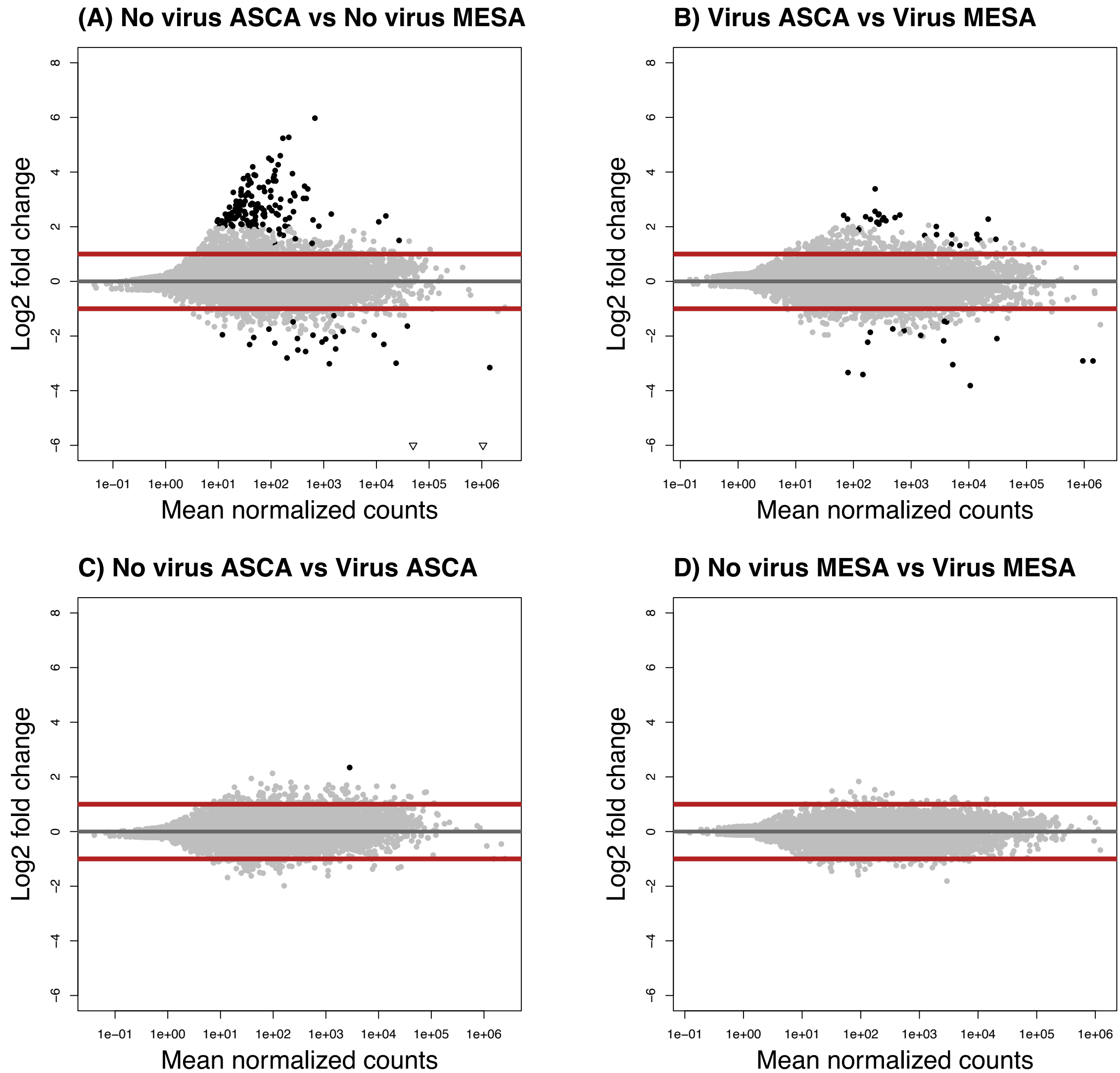

**Figure 5:** Boxplots show distribution of $\log^2$ fold change values of differentially expressed genes for the following two comparisons A) Treatment 1: No virus - ASCA versus No virus - MESA (Total 140 DEGs), and B) Treatment 2: Virus - ASCA versus Virus - MESA (Total 31 DEGs). The boxplots are plotted 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.

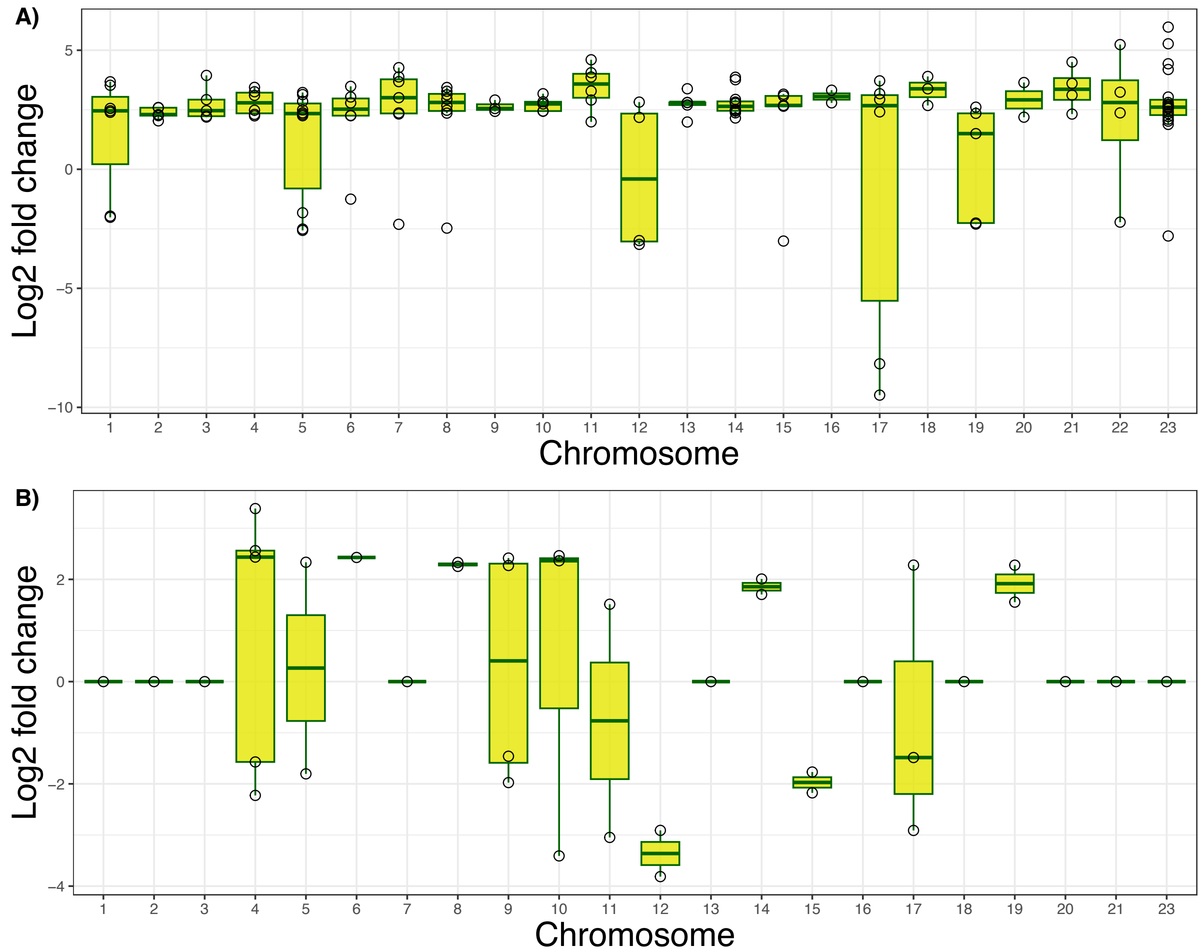

**Table 1: Table shows details of the samples include in this study.**

| 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 |

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**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 |

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**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 |

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**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*.