High quality diet enhances immune response during viral infection in an insect herbivore.

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Abstract

Insect immune response plays a crucial role in how external threats influence overall fitness through life history traits. An understudied question is how the use of different host plants might affect the ability of herbivorous insects to resist viral pathogens. The Melissa blue butterfly (Lycaeides melissa) has colonized the exotic legume Medicago sativa as a larval host within the past 200 years. Here we investigate how novel host plant use affects the immune response of L. melissa when infected with the lepidopteran virus, Junonia coenia densovirus (JcDV). We measured immune strength in response to 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. Viral infection caused total phenoloxidase (total PO) to increase. We detected an interaction between viral infection and host plant for total PO: for control larvae, host plant use had no effect on total PO, whereas for infected larvae, total PO was significantly higher for larvae consuming the native host. Within the exotic host plant treatment, few genes were differentially regulated due to viral infection. Approximately two times more genes were differentially regulated in response to infection for larvae eating the native or exotic host, with differential expression of few putative immune genes. These results demonstrate that consumption of a novel host plant can alter both physiological and transcriptional responses to infection, emphasizing the importance of understanding diet when studying the molecular basis of immune function.

Informative title: High quality diet enhances immune response during viral infection in an insect herbivore.

Running title: Diet and viral infection impact insect immunity.

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Abstract.

Insect immune response plays a crucial role in how external threats influence overall fitness through life history traits. An understudied question is how the use of different host plants might affect the ability of herbivorous insects to resist viral pathogens. The Melissa blue butterfly (*Lycaeides melissa*) has colonized the exotic legume *Medicago sativa* as a larval host within the past 200 years. Here we investigate how novel host plant use affects the immune response of *L. melissa* when infected with the lepidopteran virus, *Junonia coenia* densovirus (JcDV). We measured immune strength in response to 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. Viral infection caused total phenoloxidase (total PO) to increase. We detected an interaction between viral infection and host plant for total PO: for control larvae, host plant use had no effect on total PO, whereas for infected larvae, total PO was significantly higher for larvae consuming the native host. Within the exotic host plant treatment, few genes were differentially regulated due to viral infection. Approximately two times more genes were differentially regulated in response to infection for larvae eating the native or exotic host, with differential expression of few putative immune genes. These results demonstrate that consumption of a novel host plant can alter both physiological and transcriptional responses to infection, emphasizing the importance of understanding diet when studying the molecular basis of immune function.

Key-words: *Lycaeides*, *Medicago*, immune response, lepidopteran virus, phenoloxidase, melanization

Introduction

Organisms in the wild face diverse threats, from pathogens to parasites and predators and even parasitoids. In the face of these numerous life-threatening enemies, the immune response can influence fitness by affecting life history traits, despite the many costs associated with mounting a response (Schulenburg et al., 2009; Catalan 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). The lepidoptera in particular represent an especially interesting test case for ecological immunology given many species within this group are known to have recently colonized novel, introduced host plants, which can provide a tractable model to identify variation in immune response as novel host plant use can influence life history traits which in turn is affected by immunity (Graves and Shapiro, 2003).

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 (the other remaining studies did not detect an effect of host plant use). Thus, further research is needed to disentangle the relationship between host plant quality and the lepidopteran immune

response, as different host plants represent complex combinations of nutritional, phytochemical, and microbial traits (Yoon et al. 2019; Mason, 2020).

We explore these relationships using the butterfly *Lycaeides melissa* (Lycaenidae), a specialist herbivore on legumes including members of *Astragalus* and *Lupinus*, as well the exotic legume *Medicago sativa* (Fabaceae), which it has colonized at least twice and probably many times within the past 200 years (Forister et al., 2009, 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). 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 yet to learn about physiological and genetic processes underlying host plant-specific effects on either development or the immune response, which is the issue that we address in the present study.

Junonia coenia densovirus (JcDV) is a lepidopteran pathogen, first discovered in the buckeye butterfly, *Junonia coenia* (Rivers and Longworth, 1972; Bruemmer et al., 2005), but which 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). For example, in the noctuid moth *Spodoptera frugiperda*, JcDV can infect 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 (Tan et al., 2019; Vogel et al., 2001; Gandhe et al., 2006; Wang et al., 2016), but pathogens and host plant use have rarely been investigated in the same study (but see Tan et al. 2019). One goal of our study is 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 gypsy 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) Viral 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.

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 genetic regions and cellular processes might be brought to bear in fighting a pathogen. Understanding these underlying molecular mechanisms will be 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). Plants were collected weekly from the same site where the maternal butterflies were collected. 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 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 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 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 2018). 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. 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 1 □g 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, 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. Trimmed reads were then used to build a de novo transcriptome assembly using Trinity version2.12.0 (Grabher et al. 2011). We built the Trinity transcriptome assembly by specifying a minimum contig length of 150 bp. We then aligned the filtered, quality-checked, and trimmed paired-end reads to the Trinity build denovo transcriptome using TopHat version 2.1.0 (Trapnell et al. 2009). TopHat alignment rate ranged between 80-92% for all sample libraries. We converted TopHat alignments to gene count data for each sample using Cufflinks version 2.1.1 (Trapnell et al. 2012). We used custom R code to create the final count matrix for downstream analyses. 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 gene ontology terms (GO) and interproscan IDs (IPR) for the transcripts using this genome annotation. The scripts are archived one GitHub (https://github.com/chaturvedi-lab/lyc_rnaseq_transcript_annotations).

Differential expression analyses

We then 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 for which

one or fewer samples had nonzero read counts and (ii) genes with low coverage denoted with baseMean (count average across all samples) <1.

We then performed the DESeq2 analyses 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), (ii) native host plant comparison (*A. canadensis* infected vs. *A. canadensis* control), (iii) exotic host plant comparison (*M. sativa* infected vs. *M. sativa* control), and (iv) infected comparison (infected *M. sativa* vs. infected *A. canadensis*). 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 gene ontology (GO) categories of biological process, cellular component, and molecular function. We then performed randomization tests to ask if the number of immune genes differentially expressed for a given pairwise comparison are more than expected under random chance (1000 randomizations were performed to generate null expectations).

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 exotic 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.

Differential gene expression due to host plant use and viral infection

One striking result was that within the native host plant treatment (control caterpillars feeding on the native host plant vs. infected caterpillars feeding on the native host plant), 680 genes were differentially expressed, while only 162 genes were differentially expressed within the exotic host plant treatment (control caterpillars feeding on the exotic host plant vs. infected caterpillars feeding on the exotic host plant), (Fig. 2). When comparing control caterpillars feeding on the native host plant to control caterpillars feeding on the exotic host plant, 333 genes were differentially expressed. In the other host plant comparison involving infected caterpillars across the two hosts, 227 genes were differentially expressed.

Immune genes of interest

Within the control group comparison (*M. sativa* uninfected vs. *A. canadensis* uninfected), there were five immune-associated genes that appear to have a high degree of differential expression certainty according to our model: CUFF_1767.1, CUFF_6977.1, CUFF_23529.1, CUFF_23530.1, and CUFF_25652.1 (Table 1; Fig 3a). The first four IDs are associated with an Immunoglobulin-like domain superfamily and the last one is associated with an Immunoglobulin E-set. The first four genes associated with Immunoglobulin-like domain were upregulated in response to novel host plant use. The last gene associated with Immunoglobulin E-set was also upregulated. Within the native host plant comparison (*A. canadensis* infected vs. *A. canadensis* control), we found eleven immune related genes differentially expressed after viral infection: CUFF_3723.1, CUFF_3726.1, CUFF_6959.1, CUFF_7938.1, CUFF_9596.1, CUFF_12074.1, CUFF_12088.1, CUFF_13421.1, CUFF_19797.1, CUFF_21070.1, and CUFF_22431.1 (Table 2; Fig 3b)Of these, four genes were associated with Immunoglobulin E-set and upregulated in response to infection, while the rest were associated with Immunoglobulin-like domain and also upregulated in response to infection. Within the exotic host plant, *M. sativa*, we found five different immune-associated

genes significantly upregulated in response to infection: CUFF_2310.1, CUFF_4747.1, CUFF_10473.1, CUFF_12984.1, and CUFF_13730.1 (Table 3; Fig 3c). Of these five, four genes are associated with Immunoglobulin-like domain superfamily and the other gene is associated with Immunoglobulin E-set. All five were upregulated. When we compared across both infected groups (infected *M. sativa* vs. infected *A. canadensis*) we found nine immune-associated genes that were significantly differentially expressed: CUFF_2309.1, CUFF_2310.1, CUFF_4747.1, CUFF_7531.1, CUFF_9718.1, CUFF_11292.1, CUFF_13336.1, CUFF_13421.1, and CUFF_22433.1 (Table 4; Fig. 3d). Of these nine, six were associated with Immunoglobulin-like domain and the other three were associated with Immunoglobulin E-set. All genes associated with Immunoglobulin-like domain were upregulated while the genes associated with E-set were mixed in their response (two downregulated and one upregulated). Our randomization results indicated a significant enrichment of immune genes only for the comparison of both infected groups (infected *M. sativa* vs. infected *A. canadensis*) where the number of immune genes which were differentially expressed were two times more than it would be expected under a null model (expectation value = 2.04; p-value = 0.032). We did not see a significant enrichment of immune genes in the differentially expressed dataset for any other comparisons.

Discussion

In this study, we examined the effects of viral infection and of consuming a nutritionally inferior host plant on multiple physiological parameters with known immunological roles, specifically standing PO, total PO, and melanization. Previous studies have shown that host plant associated nutritional and/or 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 important consequences for

total PO and melanization, with higher activity on the native host. This result is consistent with previous studies that have compared performance on native host plants to introduced host plants and found heightened cellular immune response on the native host plants (Diamond and Kingsolver 2011, Lampert 2012, Muchoney et al., 2022). Interestingly, this result was not found by a previous experiment measuring similar immune parameters in *L. melissa* (Yoon et al., 2019), however, our current study has a comparatively larger sample size and greater statistical power. Our results are also consistent with predictions from ecological immunology theory which posits that greater access to high quality nutritional resources will lead to a strengthened immune response, due to the costly trade-offs involved in maintaining an effective and robust immune system (Ponton et al., 2011). With respect to the experimental manipulation of a virus, our results demonstrate that 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 also other insects (Rodriguez-Andres et al., 2012), that show that PO can have anti-viral properties in the hemolymph.

When comparing across non-infected larvae consuming different host plants, we found some evidence for differential regulation of immune genes in the absence of a pathogen. We found that while several hundred genes were differentially expressed (333 genes in total) across the two control host plant treatments, only a small number were related to immunity (5 immune-associated genes). We observed that the majority of differentially expressed genes across host treatments in the absence of a pathogen were related to cellular processes such as transcriptional regulation, DNA replication, or cellular metabolic processes. This is consistent with previous transcriptome work involving Monarch butterflies that showed most differences in expression were due to host plant use and not parasite infection (Tan et al., 2019).

We found evidence for the differential expression of a small number of immune-related genes in response to viral infection and found 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,* upregulated genes associated with Immunoglobulin E-set and Immunoglobulin-like domain superfamily. These genes have been implicated in other studies with PO activity, which is thus consistent with our experimental result of elevated total PO associated with infection. Previous studies in this system have identified genomic regions associated with these functional annotations. For example, the Immunoglobulin E-set/oxidoreductase activity genes are associated with genomic loci which act as barrier loci in *Lycaeides* butterflies hybrid zones where parental and hybrid populations utilize different host plants (Chaturvedi et al., 2020). This gene is also identified as a possible functional annotation for genomic loci associated with larval performance across host plants in *L. melissa* (Gompert et al., 2015). Thus, variation in genes associated with this functional annotation is implicated with larval performance across host plants, in the absence of a pathogen.

The identification of specific immune-relevant genes can hopefully provide targets for future studies on the molecular basis of immune function in insects, but our study was also designed 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 as opposed to the native plant (as can be seen 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.

Conclusions

Our study demonstrates that consumption of a nutritionally inferior host plant can alter both physiological and transcriptional responses to infection, and we identified a handful of immune genes that are differentially expressed both in response to a novel host and 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 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 immune response in lepidopterans (Lampert, 2012). Interesting caveats to this trend include species such as *J. coenia* that derive benefits from sequestering secondary metabolites such as iridoid glycosides from their novel host plants, which appear to have anti-viral benefits. 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. SY made the figures and SY and SC prepared 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. Variation in total PO, standing PO, melanization, and fourth instar larval weight by host plant use and viral treatment.

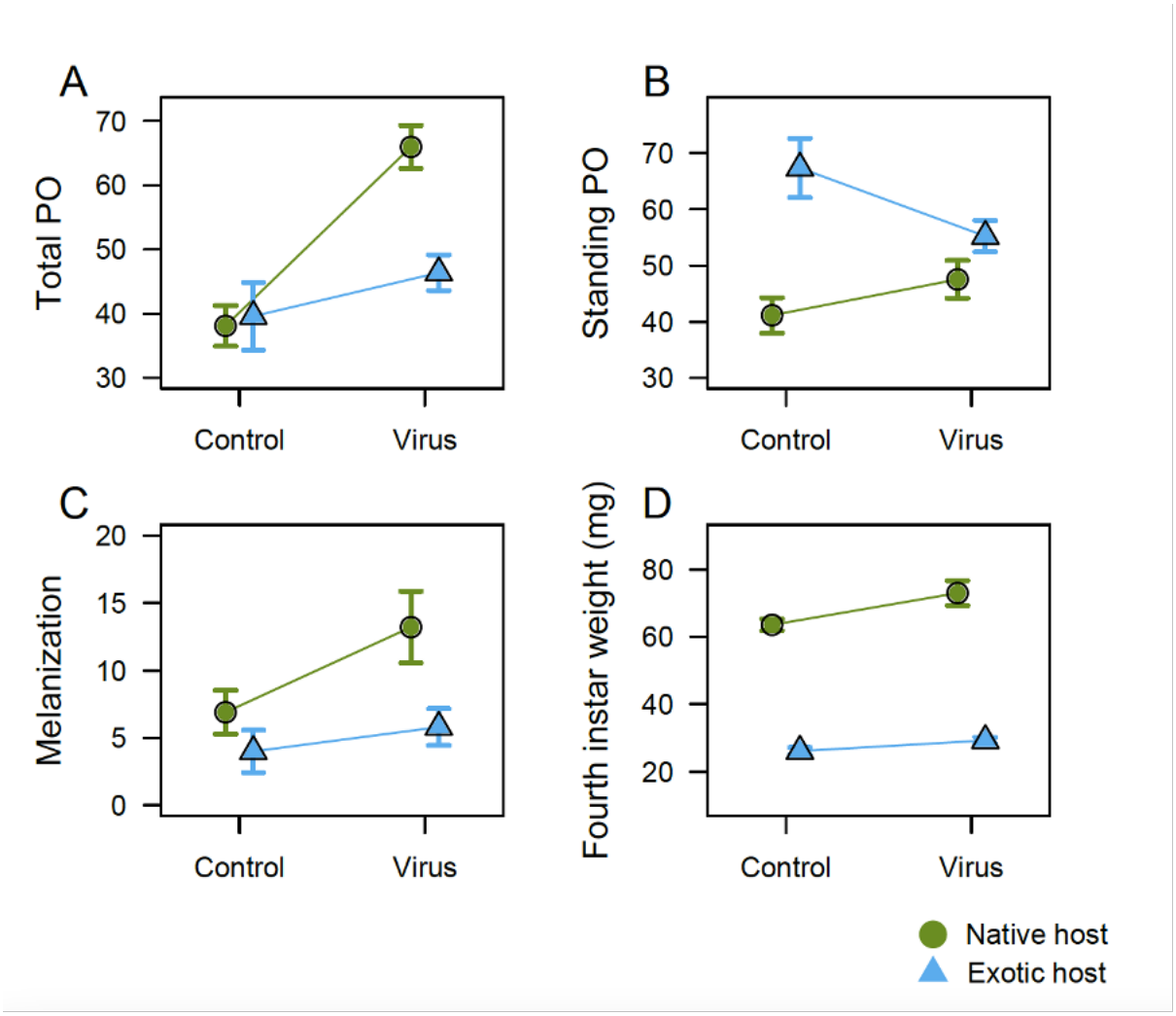

Figure 2. Number of genes differentially expressed across treatment comparisons.

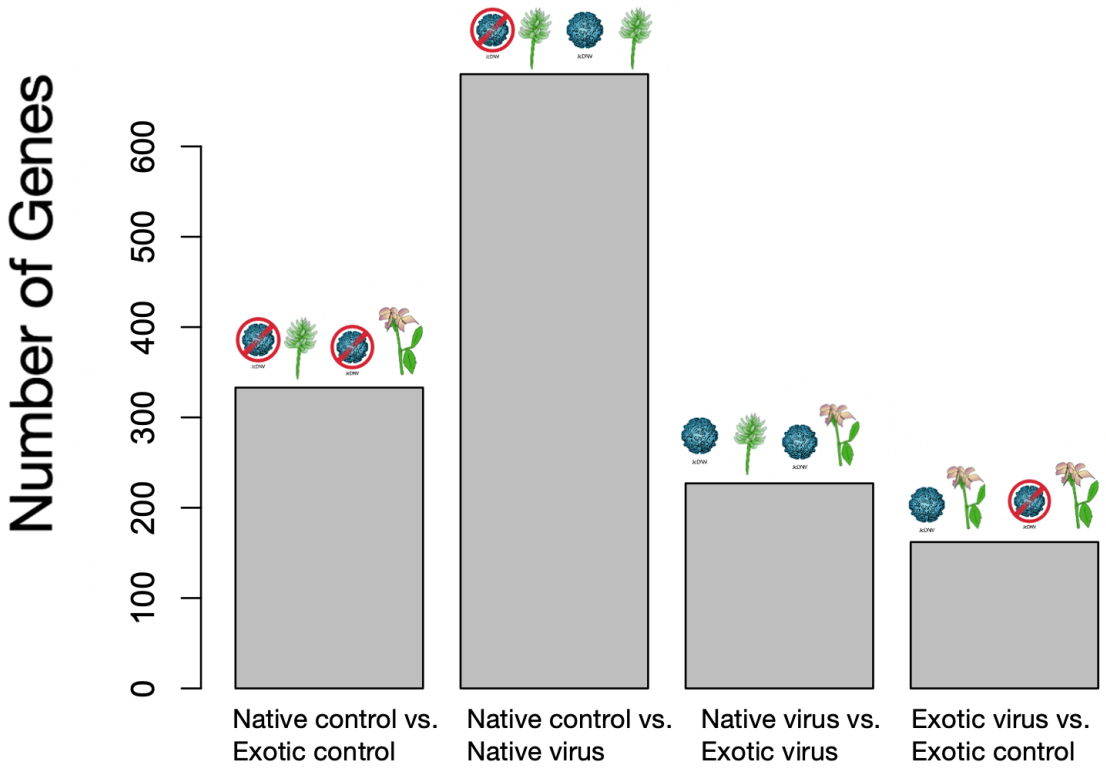

Figure 3. Volcano plots displaying mean of normalized counts versus log fold change.

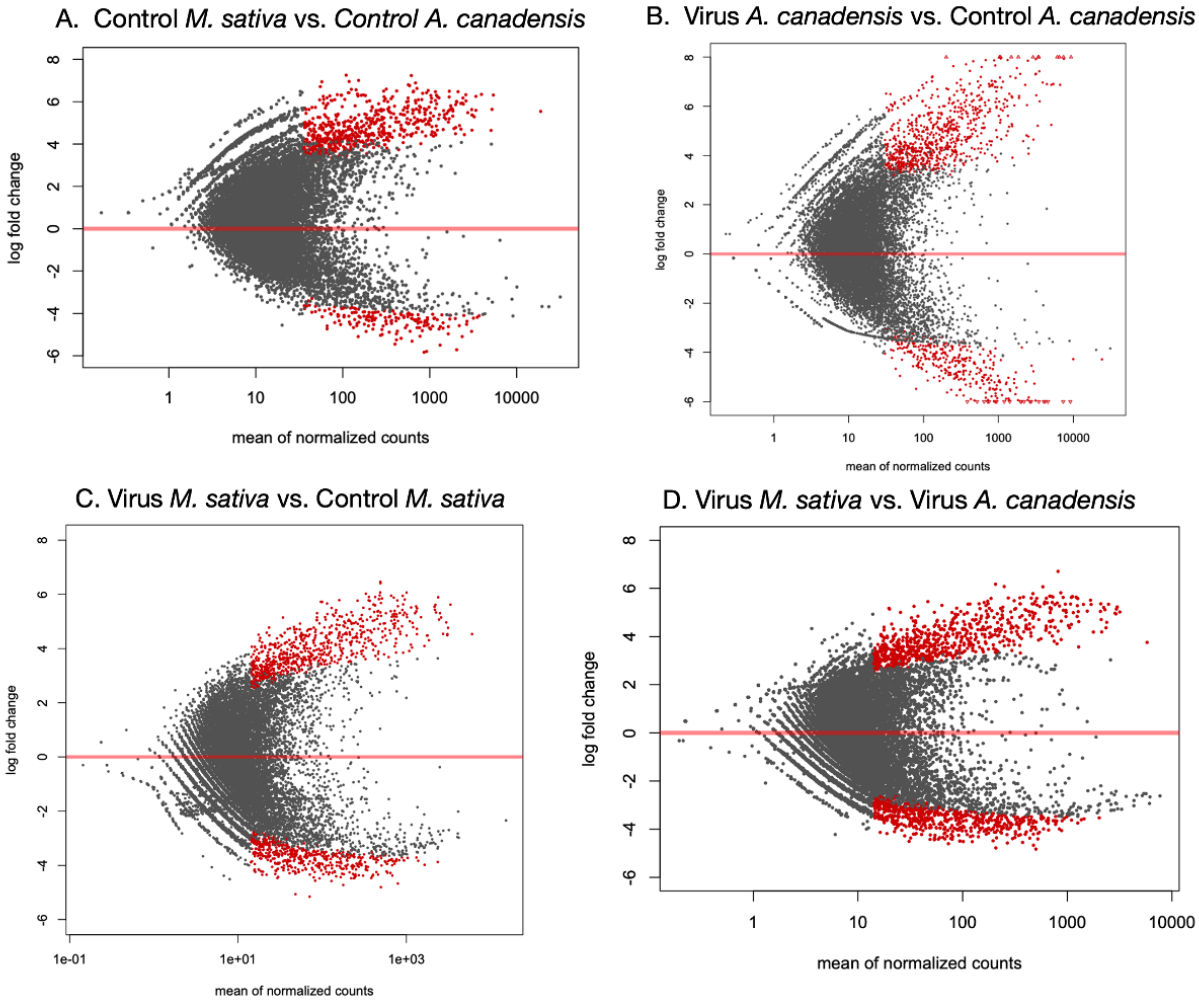

Table 1. Immune genes of interest: *M. sativa* (control) versus *A. canadensis* (control) comparison

CUFF ID	P-value (adjusted)	Log_2 fold change	Description
1767.1	0.0447	4.345	Domain-C-type lectin-like; Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Fibronectin type III; Domain-Immunoglobulin-like domain; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-C-type lectin-like/link domain superfamily; Homologous_superfamily-C-type lectin fold; Homologous_superfamily-Fibronectin type III superfamily; Homologous_superfamily-Immunoglobulin-like domain superfamily
6977.1	0.0312	5.612	Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Fibronectin type III; Domain-Immunoglobulin-like domain; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Fibronectin type III superfamily; Homologous_superfamily-Immunoglobulin-like domain superfamily
23529.1	0.0224	6.953	Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Immunoglobulin-like domain; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily
23530.1	0.0398	4.948	Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Immunoglobulin-like domain; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily
25652.1	0.0224	6.112	Family-Arrestin; Domain-Arrestin-like, N-terminal; Homologous_superfamily-Arrestin, N-terminal; Homologous_superfamily-Immunoglobulin E-set; Conserved_site-Arrestin, conserved site~

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Table 2. Immune genes of interest: *A. canadensis* (infected) versus *A. canadensis* (control) comparison

CUFF ID	P-value (adjusted)	Log_2 fold change	Description
3723.1	0.00796	5.943	~Domain-Sema domain; Repeat-Plexin repeat; Domain-IPT domain; Homologous_superfamily-Rho GTPase activation protein; Domain-Plexin, cytoplasmic RasGAP domain; Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Immunoglobulin E-set;Homologous_superfamily-WD40/YVTN repeat-like-containing domain superfamily;Domain-PSI domain;Family-Plexin family;Homologous_superfamily-Sema domain superfamily
3726.1	0.0118	5.653	Domain-Sema domain; Repeat-Plexin repeat; Domain-IPT domain; Homologous_superfamily-Rho GTPase activation protein; Domain-Plexin, cytoplasmic RasGAP domain; Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Immunoglobulin E-set;Homologous_superfamily-WD40/YVTN repeat-like-containing domain superfamily;Domain-PSI domain;Family-Plexin family;Homologous_superfamily-Sema domain superfamily
6959.1	0.0160	5.157	Domain-Immunoglobulin subtype 2;Domain-Immunoglobulin subtype;Domain-Fibronectin type III;Domain-Immunoglobulin-like domain;Domain-Immunoglobulin I-set;Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Fibronectin type III superfamily;Homologous_superfamily-Immunoglobulin-like domain superfamily~Domain-Immunoglobulin subtype 2;Domain-Immunoglobulin subtype;Domain-Immunoglobulin-like domain;Domain-Immunoglobulin I-set;Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Immunoglobulin-like domain superfamily
7938.1	0.0338	4.554	Domain-Ovarian carcinoma immunoreactive antigen domain; Family-OCIA domain-containing protein 1/2
9596.1	0.0088	5.960	Domain-MD-2-related lipid-recognition domain; Homologous_superfamily-Immunoglobulin E-set; Family-Sterol transport protein NPC2-like
12074.1	0.0316	4.775	Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Fibronectin type III; Domain-Immunoglobulin-like domain;Domain-Immunoglobulin I-set;Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Fibronectin type III superfamily;Homologous_superfamily-Immunoglobulin-like domain superfamily
12088.1	0.0411	-4.513	Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Immunoglobulin-like domain; Domain-Immunoglobulin I-set;Homologous_superfamily-

			Immunoglobulin-like fold;Homologous_superfamily-Immunoglobulin-like domain superfamily
13421.1 **GO:0016491**	0.0179	5.123	Domain-Hemocyanin/hexamerin middle domain; Domain-Tyrosinase copper-binding domain; Domain-Hemocyanin, C-terminal; Homologous_superfamily-Di-copper centre-containing domain superfamily; Family-Hemocyanin/hexamerin; Homologous_superfamily-Immunoglobulin E-set; Homologous_superfamily-Hemocyanin, C-terminal domain superfamily
19797.1	0.0259	4.921	Family-NF-kappa-B/Dorsal; Homologous_superfamily-p53-like transcription factor, DNA-binding domain superfamily; Domain-Rel homology domain, DNA-binding domain; Homologous_superfamily-Immunoglobulin E-set; Conserved_site-Rel homology domain, conserved site; Homologous_superfamily-Rel homology domain (RHD), DNA-binding domain superfamily
21070.1	0.0184	5.206	Domain-Association with the SNF1 complex (ASC) domain; Homologous_superfamily-Immunoglobulin-like fold; Domain-AMP-activated protein kinase, glycogen-binding domain; Homologous_superfamily-ASC domain superfamily
22431.1	0.0185	5.453	Domain-Sec63 domain; Homologous_superfamily-Immunoglobulin E-set; Homologous_superfamily-P-loop containing nucleoside triphosphate hydrolase; Homologous_superfamily-C2 domain superfamily

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Table 3. Immune genes of interest: *M. sativa* (infected) versus *M. sativa* (control) comparison

CUFF ID	P-value (adjusted)	Log_2 fold change	Description
2310.1	0.0458	5.157	Domain-Immunoglobulin subtype; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily
4747.1	0.0454	5.313	Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin E-set; Family-Suppressor of hairless-like
10473.1	0.0454	5.220	Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Immunoglobulin-like domain; Domain-WAP-type 'four-disulfide core' domain; Domain-PLAC; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily; Homologous_superfamily-Elafin-like superfamily
12984.1	0.0454	4.450	Homologous_superfamily-Potassium channel, inwardly rectifying, Kir, cytoplasmic; Homologous_superfamily-Immunoglobulin E-set; Family-Potassium channel, inwardly rectifying, Kir; Domain-Potassium channel, inwardly rectifying, transmembrane domain~~Domain-3-oxo-5-alpha-steroid 4-dehydrogenase, C-terminal; Family-Probable O-methyltransferase UstE-like
13730.1	0.0474	4.153	Domain-SH3 domain; Domain-Fibronectin type III; Homologous_superfamily-Immunoglobulin-like fold; Domain-RIMS-binding protein, second SH3 domain; Domain-RIMS-binding protein, third SH3 domain; Homologous_superfamily-SH3-like domain superfamily; Homologous_superfamily-Fibronectin type III superfamily

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Table 4. Immune genes of interest: *M. sativa* (infected) versus *A. canadensis* (infected) comparison

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CUFF ID	P-value (adjusted)	Log_2 fold change	Description
2309.1	0.0465	4.086	Domain-Immunoglobulin subtype; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily
2310.1	0.0388	5.331	Domain-Immunoglobulin subtype; Domain-Immunoglobulin I-set; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily
4747.1	0.0388	5.393	Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin E-set; Family-Suppressor of hairless-like
7531.1	0.0388	5.365	Domain-Dbl homology (DH) domain;Domain-Protein kinase domain;Domain-Pleckstrin homology domain;Domain-Fibronectin type III;Homologous_superfamily-Protein kinase-like domain superfamily;Homologous_superfamily-PH-like domain superfamily;Homologous_superfamily-Immunoglobulin-like fold;Binding_site-Protein kinase, ATP binding site;Domain-Kalirin/Triple functional domain protein, SH3 domain 1;Homologous_superfamily-Dbl homology (DH) domain superfamily;Homologous_superfamily-Immunoglobulin-like domain superfamily
9718.1	0.0479	4.940	Domain-Immunoglobulin-like domain; Homologous_superfamily-Immunoglobulin-like fold; Homologous_superfamily-Immunoglobulin-like domain superfamily
11292.1	0.0458	4.698	Domain-Cysteine-rich flanking region, C-terminal; Repeat-Leucine-rich repeat; Repeat-Leucine-rich repeat, typical subtype; Domain-Immunoglobulin subtype 2; Domain-Immunoglobulin subtype; Domain-Immunoglobulin-like domain; Domain-Immunoglobulin I-set;Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Leucine-rich repeat domain superfamily;Homologous_superfamily-Immunoglobulin-like domain superfamily
13336.1	0.0465	3.895	Domain-Exoribonuclease, phosphorolytic domain 1;Homologous_superfamily-Ribosomal protein S5 domain 2-type fold;Homologous_superfamily-PNPase/RNase PH domain superfamily;Homologous_superfamily-Exoribonuclease, PH domain 2 superfamily~Family-Maternal protein exuperantia~Domain-TrmO-like, N-terminal domain;Homologous_superfamily-YaeB-like

			superfamily;Homologous_superfamily-YaeB, N-terminal domain superfamily;Family-YaeB-like~Family-Glycoside hydrolase family 31;Domain-Fibronectin type III;Homologous_superfamily-Galactose mutarotase-like domain superfamily;Homologous_superfamily-Glycosyl hydrolase, all-beta;Homologous_superfamily-Immunoglobulin-like fold;Homologous_superfamily-Glycoside hydrolase superfamily;Domain-Domain of unknown function DUF5110~Domain-Proteasome alpha-subunit, N-terminal domain;Family-Proteasome, subunit alpha/beta;Family-Proteasome alpha-type subunit;Homologous_superfamily-Nucleophile aminohydrolases, N-terminal;Family-Proteasome subunit alpha5
13421.1	0.0449	-4.826	Domain-Hemocyanin/hexamerin middle domain; Domain-Tyrosinase copper-binding domain; Domain-Hemocyanin, C-terminal; Homologous_superfamily-Di-copper centre-containing domain superfamily; Family-Hemocyanin/hexamerin; Homologous_superfamily-Immunoglobulin E-set; Homologous_superfamily-Hemocyanin, C-terminal domain superfamily
22433.1	0.0467	-4.368	Domain-Sec63 domain; Homologous_superfamily-Immunoglobulin E-set; Homologous_superfamily-P-loop containing nucleoside triphosphate hydrolase; Homologous_superfamily-C2 domain superfamily