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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Host plant-dependent effects of microbes and phytochemistry on the insect immune response

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Abstract

Herbivorous insects can defend themselves against pathogens via an immune response, which is influenced by the nutritional quality and phytochemistry of the host plant. However, it is unclear how these aspects of diet interact to influence the insect immune response and what role is played by ingested foliar microbes. We examined dietary protein, phytochemistry, and the caterpillar microbiome to understand variation in immune response of the Melissa blue butterfly, *Lycaeides melissa.* We also asked if these factors have host plant-specific effects by measuring *L. melissa* immune response when reared on a recently colonized exotic host plant (*Medicago sativa*) as compared to the immune response on an ancestral, native host (*Astragalus canadensis*). *L. melissa* did not experience immunological benefits directly related to consumption of the novel plant *M. sativa*. However, we did find negative, direct effects of phytochemical diversity and negative, direct effects of diet-derived microbial diversity on constitutive immune response for caterpillars fed *M. sativa*, as measured by phenoloxidase activity. Foliar protein did not directly influence the immune response, but did do so indirectly by increasing weight gain. Our results highlight the important effects of host diet on caterpillar physiology and raise the possibility that foliar microbiota, despite being rapidly passed through the gut, can affect the caterpillar immune response.

Keywords Lepidoptera · Microbiome · Novel host · Phenoloxidase · Secondary metabolites

Introduction

Immune defense is a major component of organismal fitness because wild organisms face diverse threats from pathogens and parasites (Schmid-Hempel 2005). A primary goal of ecological immunology is to understand the ecological and evolutionary sources of variation that underlie the immune response (Schulenburg et al. 2009). As introduced species sweep through the world's ecosystems, native taxa are increasingly facing novel immune threats in the form of introduced pathogens and microbes (Litchman 2010; Bennett 2013). Moreover, native insect taxa that utilize introduced plant species must cope with traits associated with these new dietary resources, including anti-herbivore plant metabolites, which may substantially differ from those of ancestral hosts (Cappuccino and Arnason 2006), as well as a potentially unfamiliar microbial assemblage (Vandenkoornhuyse et al. 2015). Understanding how native species cope with this kind of novelty is essential for predicting the immunological and ecological impacts of introduced species (Ponton et al. 2013).

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Upon infection with a pathogen or parasitoid, insects have evolved adaptive behaviors that increase their likelihood of survival through preferential consumption of certain secondary metabolites and macronutrients (Lee et al. 2006; Smilanich et al. 2011; Shikano and Cory 2016). For example, some herbivorous insects modulate their consumption of nutrients in response to parasitism, with a few using self-medication with specific secondary metabolites (Keating et al. 1990; Singer et al. 2009; Singer et al. 2014). Conversely, secondary metabolites can negatively influence the immune response; for example, *Junonia coenia* caterpillars that sequester high levels of iridoid glycosides express lower levels of melanization (Smilanich et al. 2009a). While plant chemistry clearly plays a role in mediating herbivore immunity, what is less clear is how different components of plant chemistry, such as dietary protein and secondary metabolites, interact with each other to influence the insect immune response (Shikano 2017).

A further complexity for ecological immunology comes from the potential role of the gut microbiome in mediating immune defense and natural enemy resistance, which has been intensively studied for only a few insect taxa, namely pea aphids (Scarborough et al. 2005), fruit flies (Broderick 2016), and honey bees (Kwong et al. 2017), but is beginning to be appreciated in additional systems (Shikano et al. 2017; Smilanich et al. 2017). For some insects, beneficial microbes can play a role in nutrient uptake, detoxification of chemicals, and resistance against parasitoids (Oliver et al. 2005; Engel and Moran 2013; Hansen and Moran 2013), suggesting that interactions between beneficial microbes and the immune system may be common in insects. The Lepidoptera represent a rich testing ground for examining such interactions due to their diversity, well-studied life histories, and the wide range of secondary metabolites that they consume.

Moreover, host breadth expansion onto non-native plants has been documented for many lepidopterans, many of which are now reliant on these novel resources (Graves and Shapiro 2003). Given that different plant species have divergent foliar microbial assemblages (e.g., Kembel et al. 2014), successful dietary expansion by lepidopterans necessitates surmounting the challenges imposed by exposure to novel microbiota. Recently, it has been proposed that caterpillars do not possess resident gut microbiomes, but instead host a variety of transient microbes (Hammer et al. 2017). However, the likely transience of microbes in the guts of caterpillars does not necessarily mean that these microbes have no impact on their hosts, especially for immune function which has been little studied in this context.

The specialist butterfly *Lycaeides melissa* has colonized the exotic legume *Medicago sativa* (Fabaceae) within the past 200 years (Forister et al. 2009). *M. sativa* supports populations of *L. melissa* throughout the western United States, despite reducing larval performance and adult fecundity compared to a preferred native host *Astragalus canadensis* (Fabaceae; Forister et al. 2009). Given the widespread use of the apparently inferior host plant by *L. melissa*, it is possible that a diet of *M. sativa* confers a yet to be quantified advantage that could include a benefit to the immune system. This possibility is supported by several studies showing lepidopteran herbivores can experience immunological benefits through the utilization of alternative host plants (Yang et al. 2008; Muller et al. 2014). However, other studies have shown no immunological benefit of alternative host plant use (Diamond and Kingsolver 2011), or even negative immunological effects imposed by feeding on otherwise high-quality, alternative host plants (Klemola et al. 2007). These conflicting reports highlight the ecological and physiological complexities associated with dietary expansion and the need for studies that take a comprehensive approach towards characterizing the interplay between aspects of host plant variation, particularly in defenses and nutrition, and any resulting influence on insect fitness and performance.

Here, we used the colonization of a novel host plant as a framework to investigate the relative effects of body condition, nutrition, plant chemistry, and microbes on the insect immune response. In doing so, we sought to understand how immunological factors may facilitate the colonization of a novel resource. Specifically, we investigated the direct and indirect effects of host plant-associated variation in phytochemistry, dietary protein, and microbes on immune response in *L. melissa* reared on either its native host plant *A. canadensis,* or the introduced host plant, *M. sativa*. We asked the following questions: (i) does host plant species affect immune response? (ii) what is the relative importance of foliar dietary protein, the caterpillar gut microbiome, and caterpillar body condition (weight) for predicting the insect immune response and are these relationships the same across and within host plants? (iii) how does phytochemistry affect immune response for larvae feeding on the novel host plant *M. sativa*? Answers to these questions will reveal the complexity of immune regulation in a wild insect and will suggest future avenues of research with both applied and basic importance for organismal biology.

Materials and methods

Overview of experiments

Gravid *L. melissa* females were collected from a population associated with the native host plant *A. canadensis* at Silver Lake NV, USA (hereafter referred to as SLA) and from a population utilizing *M. sativa* at Verdi NV, USA (hereafter: VUH) during late May and June 2015 (30 females from each location; see Supplemental Figure S1 for an illustration of our experimental design). Eggs acquired from these females

were randomly assigned to a host plant treatment (approximately $n = 94$ eggs per plant), and larvae were reared individually in petri dishes at ambient temperature and 10 h of light per day, as previously described (Forister et al. 2009). Plant samples were collected weekly from the same localities where the maternal butterflies were collected. Plant stems in rearing dishes were replaced every 2–3 days, with the exception of a focused feeding trial, in which stems were changed every day (see below). We reared 79 larvae to the fourth (final) instar to be killed in immune experiments; 32 on *M. sativa* and 47 on *A. canadensis*.

We also conducted a focused feeding trial to understand the relationship between mass of plant tissue consumed and larval weight gain. For this trial, weight gain and plant tissue consumed were measured daily from the beginning of the second instar until the molt into third instar. Fresh plant tissue was provided to larvae daily. Weight gain during the feeding trial was calculated by subtracting final weight from starting weight. In addition, tissue from each leaflet consumed was collected during this trial for protein analysis (both host plants) and secondary metabolite extraction (only *M. sativa*).

Phytochemistry was investigated only from the novel host plant, *M. sativa*, partly for reasons of logistics and expense, but also because we were most interested to understand performance on the novel host plant (where growth and survival has been previously observed to be both poor and highly variable; Harrison et al. 2016a, b). At the beginning of the last instar, final weight was noted and immune assays were conducted (as described below) on all surviving larvae. Finally, the microbiome of whole larvae was characterized and associations between individual microbial taxa and the immune response explored.

Immune assays

Larval immune response was measured using two assays: baseline phenoloxidase (PO) concentration and melanization via bead injections. Baseline 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. Bead injections serve as a proxy for a parasitism event and are a useful measure of immune response in caterpillars (Lavine and Beckage 1996). Both of these metrics accurately reflect the strength of the immune response (Smilanich et al. 2009b; Hansen et al. 2017).

PO activity was measured by taking an aliquot of hemolymph (5 µL) from the posterior abdominal segment of individual larvae using a sterilized sewing pin. Pins and handling instruments were sterilized with 100% ethanol before each extraction. Individuals were observed after extraction to ensure no larvae bled excessively, which could have confounded our measures of immune response. Hemolymph was added to 50 µL of ice-cold, distilled water in an Eppendorf tube and chilled on ice while dopamine solution was prepared. Powdered dopamine (Sigma-Aldrich; St. Louis, Missouri, USA) (0.0257 g) was added to 20 mL of distilled water. Samples were incubated for 20 min at room temperature, then 50 µL of the hemolymph/distilled water solution was added to a 96-well plate with 200 µL of dopamine. The reaction then proceeded in a microplate reader (Bio-Rad iMark) for 45 min (data recorded every 30 s 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 min). 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 approximately 5 µL Ringer's solution containing 10–12 DEAE Sephadex-A25 chromatography beads (Sigma-Aldrich; St. Louis, Missouri, USA). Sephadex beads measured 40–120 µm in diameter and were dyed with 0.1% [w/v] solution Congo Red. Injections were performed using hand-drawn glass syringes fashioned from Pasteur pipettes. Pipettes were flame sterilized prior to injections. Beads were injected at the same wound site where hemolymph was previously drawn for PO assay (posterior abdominal segment). Prior to injection, 5 µL of ringer solution was pipetted using a micropipette, then the beads were added to this pool of ringer solution. Larvae were returned to their respective petri dishes and given access to plant tissue for 24 h, then frozen and dissected for beads. Dissected beads were photographed in 70% [v/v] ethanol solution using a dissecting microscope connected to a digital camera (Carl Ziess Discovery V.8, AXIOCAM Software, Oberkochen, Baden-Wurttemberg, Germany). For each individual, 10 beads were photographed at 80× magnification, and their red value was recorded in Adobe Photoshop (v6.0; Adobe System Inc., San Jose, California, USA). Red value (r-value) is calculated on a scale of 0–250, with 250 being pure red, and 0 being pure gray. Average red values were converted into average percent melanization for each individual using the following equation: [1 − (r-value/maximum r-value] (for additional details on melanization assay methods, see Smilanich et al. 2009a).

Plant protein, secondary metabolite, and microbial processing

For full details on protein assays, secondary metabolite extraction, DNA extractions, and sequence processing, see the Supplemental Methods. Briefly, a bicinchoninic acid

assay (BCA; Pierce Biotechnology) was used to quantify extracted protein content using bovine serum albumin as a standard. Protein concentration was standardized by the mass of the ground plant material.

Phytochemical variation in *M. sativa* foliar tissue fed to caterpillars was assayed using high-performance liquid chromatography and mass spectrometry (LC–MS). A DNeasy blood and tissue kit (Qiagen) was used to extract DNA from whole caterpillars. DNA was sent to the Genome Sequencing and Analysis Facility (GSAF) at the University of Texas, Austin for 16S library preparation and sequencing on the Illumina MiSeq platform.

DNA sequence processing

Sequencing data were processed using USEARCH v8.1.1831 (Edgar 2010, 2013). The template sequence may bind to the primer imperfectly, thus this region is prone to sequencing errors and was removed. Trimmed forward and reverse reads were merged and then quality filtered using USEARCH. Reads with more than a single expected error were removed. Resulting high-quality sequences were clustered into OTUs using the UPARSE algorithm set at a 97% similarity threshold. This step also removes potentially chimeric sequences. Taxonomic status was assigned to each OTU using the UTAX algorithm as implemented in USEARCH. UTAX was trained using the 16S dataset from the Ribosomal Database Project (Wang et al. 2007; Training Set 15; accessed January 22, 2016). Those OTUs designated as bacterial in origin were queried using original merged, but unfiltered reads, at a 97% match using the "usearch_global" function in USEARCH. This step allows information to be recovered from reads that did not pass our initial stringent quality criteria, thus facilitating a more accurate estimate of total reads for a particular OTU. OTU tables were normalized using the relative log expression method of the edgeR package in R (Robinson et al. 2010). This approach normalizes read counts with respect to variation in sequencing depth across samples and is preferable to rarefying (McMurdie and Holmes 2014).

Statistical analyses

All statistical analyses were run in R (v3.4.1) (Team RD 2009). We used one-way analyses of variance (ANOVA) to examine the direct effects of host plant on weight gain, protein content, and immune response. For PO and protein, data were log transformed before analysis. For more complex hypotheses addressing direct and indirect effects, we utilized structural equation models (SEMs). In initial models, population of origin (site) was included as a predictor variable; however, we did not detect an effect of site for any variables of interest, and site was removed from later models. For all SEMs, PO activity was log transformed before model comparison. In addition, microbial diversity (represented using Shannon's entropy) was exponentiated prior to analyses to reflect effective species number (Jost 2006). To represent beta-diversity within a treatment group while accounting for variation in alpha diversity, "turnover" (see Equation 25 from Jost 2007) was calculated using the R package vegetarian (Charney and Record 2012).

For calculating phytochemical diversity for each individual, peak areas for each chemical feature were divided by total peak area across features to give relative peak area. These relative peak areas were then used to calculate exponentiated Shannon's entropy. Phytochemical diversity was used as a predictive variable for immune response because we were interested in quantifying the effect of the host plant-specific phytochemical mixtures encountered by larvae. Phytochemical diversity is a useful index specifically because our experiment was designed to encompass a broad picture of caterpillar immunity and physiology but was not designed to detect the effects of individual chemical compounds on caterpillars.

We used structural equation modeling to examine direct and indirect effects of host plant species on immune response and performance. Weight gain was used as the performance response variable in all SEMs because preliminary analyses found that models containing final weight as a measure of performance ad variance inflation factors > 5 and our ability to test multiple variables was limited. All path analyses were run in the R package *lavaan* using the SEM function (Rosseel 2012). Model goodness of fit was determined using χ^2, with $p < 0.05$ indicating a poor fit to the data. Combining all of the data on both host plants, two models were specified with PO activity and melanization as response variables; host plant use was hypothesized to have both direct effects on immune response, as well as indirect effects mediated through weight gain, microbial diversity, and protein content. When appropriate, we further investigated host plant-specific interactions through pairwise linear regression models. We were especially interested in how microbes may differentially affect immune response across host plant treatments.

Additional hypotheses concerning the effects of phytochemical variation on the immune response were tested using data from caterpillars fed *M. sativa* because phytochemical data was only collected for this host. A total of six models were tested with *M. sativa*: three using either phytochemical diversity, protein, or microbial diversity as independent variables and weight gain and PO activity as dependent variables, and three using the same independent variables with weight gain and melanization as dependent variables. A total of four models were tested with *A. canadensis*: two using either protein or microbial diversity as independent variables and weight gain and PO activity as

dependent variables, and two using the same independent variables and weight gain and melanization as dependent variables. Models that fit the data, as determined by χ^2, are presented here.

We used principal coordinates analysis (PCoA) and PERMANOVA (using the adonis function of the vegan R package; Oksanen et al. 2007) to compare microbial assemblage similarity across host treatments and population of origin. Data were represented as Euclidean distances of Hellinger standardized data. To determine how specific microbial taxa or compounds affected the caterpillar immune response, we used linear regression where PO was the response variable and the relative abundance of a microbial taxon or a particular phytochemical was the sole predictor variable. Models were created for every microbial taxon, saponin, and phenolic with a Benjamini–Hochberg multiple comparison correction. For a microbial taxon or phytochemical to be considered it must have occurred in at least 15 caterpillars. Predictor variables were converted to z scores prior to regression. Models with significant predictors were examined for undue influence of outliers and to ensure model assumptions were met. Analyses were repeated independently for caterpillars fed *M. sativa* or *A. canadensis.*

Results

The influence of host plant species on larval immune response and performance

To determine if host plant affected the immune response of *L. melissa*, we examined the direct effects of host taxon on larval melanization and PO activity, as well as larval performance and foliar protein content. As hypothesized, we found that larval performance differed between host plants (Fig. 1a, $F(1,76) = 123.8$, $p = 0.000$) with individuals fed *A. canadensis* gaining dramatically more weight compared to individuals fed *M. sativa*. Protein content did not differ between host plants (Fig. 1b, $F(1,75) = 1.5738$, $p = 0.2135$). Also, neither mean PO activity (Fig. 1c, $F(1,65) = 2.6345$, $p = 0.1094$), nor average melanization differed by host plant (Fig. 1d, $F(1,75) = 0.1124$, $p = 0.7384$) in simple models not controlling for other factors.

For both the native and the novel host, we used SEM to quantify the relative importance of foliar dietary protein, the caterpillar gut microbiome and body condition (weight gain) on mean PO activity and melanization. We also asked if host plant use indirectly affected these immune responses. Both the PO model (Fig. 2a, $\chi^2 = 3.001$, $df = 3$, $p = 0.391$), and the model of melanization were good fits to the data (Fig. 2b, $\chi^2 = 6.854$, $df = 3$, $p = 0.077$). Host plant significantly affected both weight gain and microbial diversity (Fig. 2a, b), with the native host supporting a higher microbial diversity and larger caterpillars. However, we found no

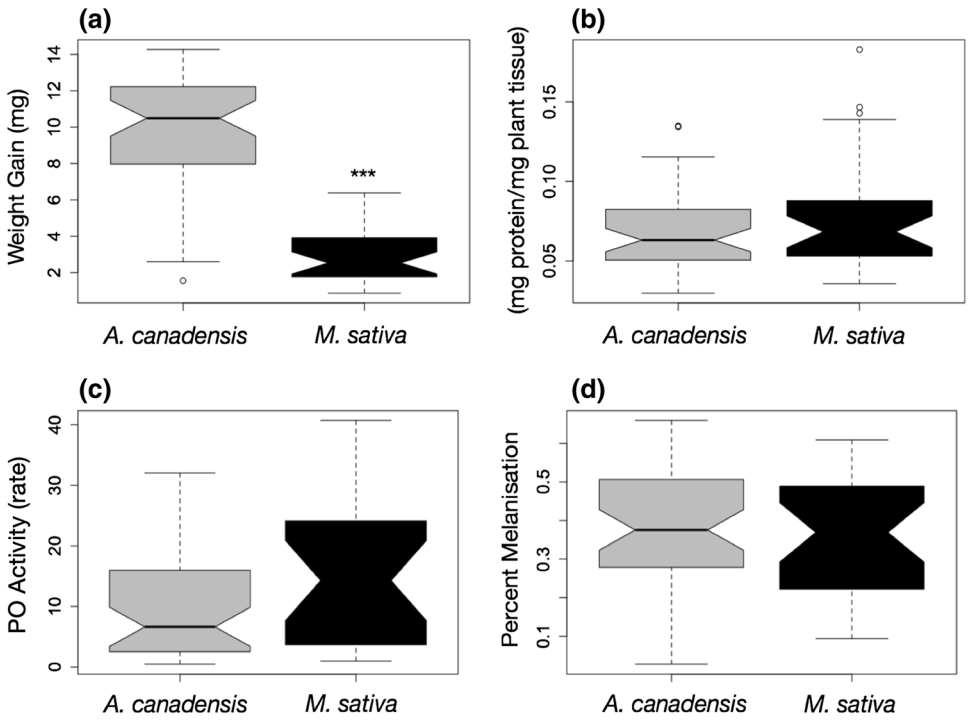

Fig. 1 Notched boxplots showing differences in **a** weight gain (mg), **b** average protein content by host plant (mg protein/mg plant tissue), **c** PO activity, and **d** percent melanization. Asterisks indicate significant levels at $\alpha = 0.05$ (ANOVA). Notched areas indicate 95% confidence intervals around the median

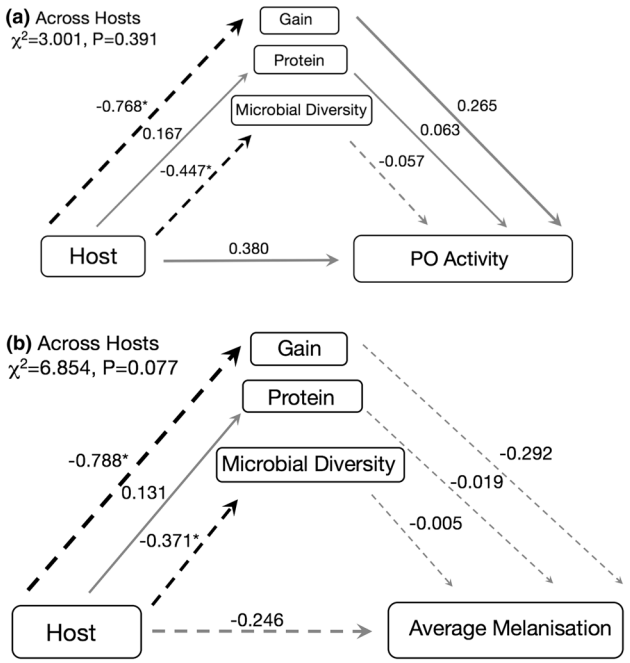

Fig. 2 Path models testing a priori hypotheses regarding the effects of host, weight gain, protein, and microbial diversity on **a** PO activity and **b** melanization response. Black arrows and asterisks indicate significant, standardized path coefficients, while gray arrows indicate non-significant relationships. Negative relationships are shown as dashed lines, and positive relationships are shown as solid lines. Strength of the relationship is proportional to line thickness, with weaker relationships indicated by thinner lines

significant direct or indirect effects of predictor variables on PO activity or melanization.

Interestingly, bacterial diversity had a host plant-specific effect on PO activity, as determined through pairwise linear regression. For caterpillars fed *M. sativa,* microbial diversity had a negative effect on PO activity (Fig. 3, $\beta = -0.634$, $p = 0.001$), but this effect was not observed in caterpillars fed *A. canadensis* (Fig. 3, $\beta = 0.124$, $p = 0.425$).

Effects of intraspecific variation in hosts on the larval immune response

We also asked whether variation in foliar protein, larval gut microbiota, or body condition affected immune response *within* host treatments, and whether these effects differed from the patterns obtained in across-host models. Using SEM, within the native host plant *A. canadensis,* we tested whether the aforementioned predictor variables directly affected mean PO activity (Fig. 4b, $\chi^2 = 0.038$, $df = 3$, $p = 0.846$) or melanization (Fig. 5b, $\chi^2 = 0.134$, $df = 3$, $p = 0.714$). For the novel host plant *M. sativa,* we augmented model structure to include phytochemical variation, which was only characterized for this host. The model structure we used to test associations between predictors and PO (Fig. 4a,

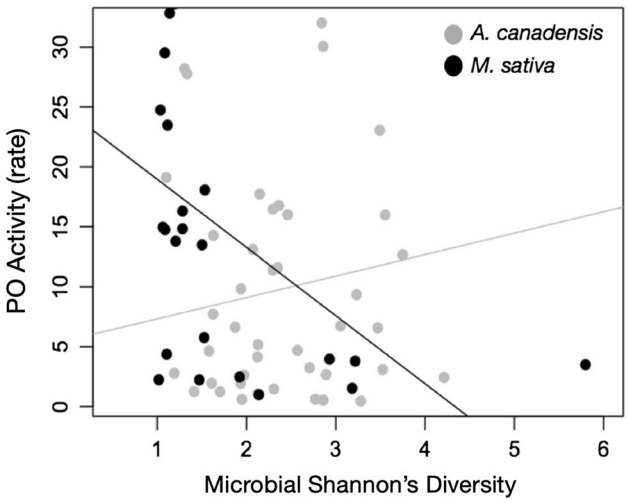

Fig. 3 Interaction between microbial diversity and host plant in predicting PO activity

$\chi^2 = 0.854$, $df = 1$, $p = 0.355$) and melanization (Fig. 5a, $\chi^2 = 1.072$, $df = 1$, $p = 0.300$) was a good fit to the data.

Among larvae fed the native host, *A. canadensis*, we did not detect any significant direct effects of protein, weight

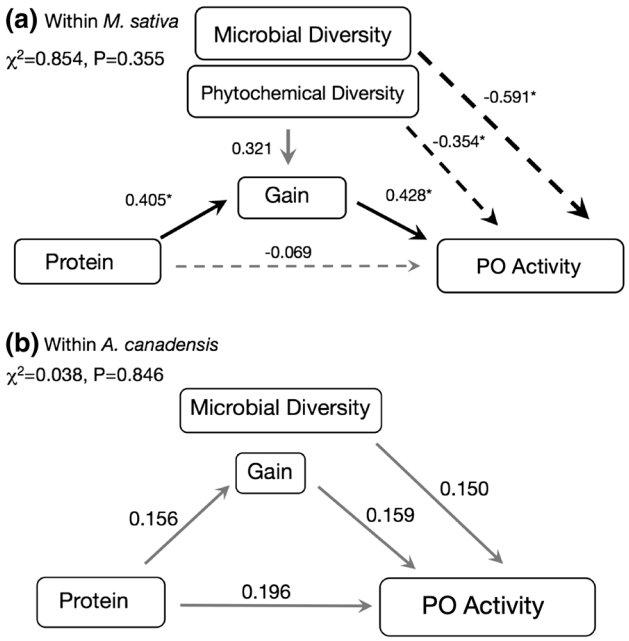

Fig. 4 Path models testing a priori hypotheses about factors mediating PO activity within **a** the novel host, *M. sativa* and **b** the native host *A. canadensis*. Black arrows and asterisks indicate significant standardized path coefficients, and gray arrows indicate non-significant relationships. Negative relationships are shown as dashed lines, while positive relationships are shown as solid lines. Strength of the relationship is proportional to line thickness, with weaker relationships indicated by thinner lines

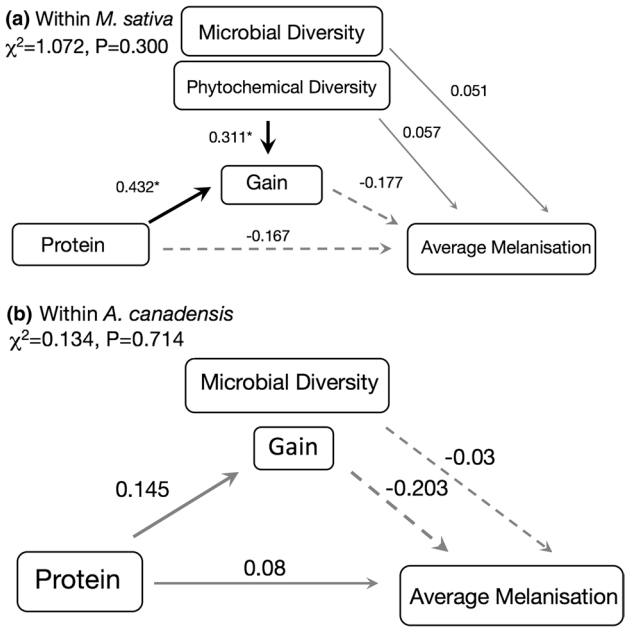

Fig. 5 Path models testing a priori hypotheses about factors mediating melanization response within **a** the novel host, *M. sativa* and **b** the native host *A. canadensis*. Black arrows and asterisks indicate significant standardized path coefficients, and gray arrows indicate non-significant relationships. Negative relationships are shown as dashed lines, while positive relationships are shown as solid lines

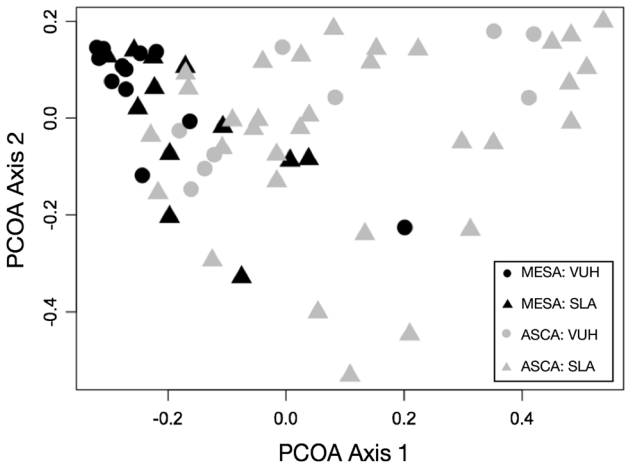

Fig. 6 Principal coordinates analysis (PCoA) plot showing larval microbial assemblages across host plants and sites. Points are individual larvae. See legend for hosts and populations of origin

gain, or microbial diversity on PO activity, nor did we find evidence for indirect effects of protein as mediated by weight gain. In contrast, among larvae fed *M. sativa,* we found a negative relationship between immune response and microbial diversity and a positive relationship between immune response and weight gain. Protein concentration did not directly influence the immune response, but did do so indirectly via increasing weight gain. Lastly, phytochemical diversity had a direct negative effect on the immune response.

Phytochemical analysis revealed that, on average, samples contained 33.7 effective compounds (exponentiated Shannon's diversity), with individual plants ranging from 25.87 to 40.19 effective compounds. Phytochemical Shannon's diversity equaled 3.510 on average, with values ranging from 3.253 to 3.694. Turnover was very low across samples (0.0022), indicating plant samples contained similar phytochemical mixtures. No individual saponin or phenolic compound was significantly associated with PO activity. Principal Component Analysis of phytochemical data revealed four main axes which explained 87.5% of variation within the data; however, none of these components significantly predicted PO activity as determined through linear regression of principal components on PO activity.

Shifts in the caterpillar gut microbiome with diet and taxon-specific effects on immune response

PCoA revealed that the bacterial assemblages within larvae were influenced by diet and host population (Fig. 6, PERMANOVA $p = 0.000$ and 0.0168, respectively). Diversity of the gut microbiome was higher for larvae fed the native host plant, *A. canadensis*, compared to those fed *M. sativa* ($t = 3.289$, $df = 75.778$, $p = 0.0001$). Turnover in microbial assemblages, as calculated by the numbers equivalent of Shannon beta-diversity, was low across all individuals (0.0119). Turnover was higher among larvae fed *A. canadensis* (0.0255), compared to larvae fed *M. sativa* (0.00953). The most prevalent bacterial phyla observed included Actinobacteria, Proteobacteria, and Firmicutes (see Fig. S1). We also observed Acidobacteria, Armatimonadetes, Bacteroidetes, Cyanobacteria, Deinococcus–Thermus, Euryarchaeota, Fusobacteria, and Verrucomicrobia.

Multiple regression revealed four microbial taxa that were significantly associated with PO activity on caterpillars fed *M. sativa.* Of all bacteria sequenced, the most reads were obtained for these four taxa. BLAST searches of the National Center for Biotechnology Information nucleotide collection (Johnson et al. 2008) revealed one of these OTUs matched members of *Enterobacter* and *Klebsiella* (100% match). This OTU was positively associated with PO activity based on multiple regression analysis. Two additional OTUs matched members of *Acinetobacter* and the fourth OTU was assigned to *Wolbachia*. These three OTUs all were negatively associated with PO activity. No microbial taxa significantly predicted PO activity for caterpillars fed *A. canadensis*, though taxa found in fifteen or more caterpillars fed this host were negatively correlated with larval PO activity (nine out of twelve taxa).

Discussion

We found that host plant-specific variation in nutrition, phytochemistry, and the foliar microbiome affected the immune response of *L. melissa*. Specifically, we found that phytochemical and microbial diversity had direct, negative effects on constitutive immune response for caterpillars fed *M. sativa*, as measured by phenoloxidase activity. Further, larval body condition had a direct, positive effect on constitutive immune response. Protein concentration did not directly influence the immune response but did do so indirectly via increasing weight gain. In contrast, variation in microbial diversity, protein, and weight gain had no effect on the immune response of larvae feeding on the native, ancestral host plant *A. canadensis*. These results demonstrate the value of measuring multiple ecological determinants of immune response in natural systems, as well as the importance of quantifying both direct and indirect effects of such determinants.

Variation in microbial assemblages and the microbial modulation of the insect immune response

Microbial assemblages in our study were influenced by larval diet (Fig. 1), which is consistent with previous work investigating the effects of diet on microbiome composition in other systems (i.e., flies, gypsy moths, and cotton bollworms); for instance, flies taken from different populations and raised on the same diet developed highly similar microbiomes (Ponton et al. 2013). Furthermore, work with *Heliconius* butterflies has shown that microbial assemblages shift during metamorphosis, presumably, at least partially due to the shift in diet from plant tissue during the larval stage to pollen and nectar in the adult stage (Hammer et al. 2014). Harrison et al. (2016b) also report a shift in the fungal assemblage of *L. melissa* over ontogeny, and fungi recovered from all life history stages were a subset of those associated with host plant tissue, which suggests acquisition of the microbiome from diet either via ingestion or simply direct contact. Additionally, in a survey across Lycaenidae, Whitaker et al. (2016) report highly variable larval microbial assemblages across taxa. Recent work on lepidopteran larvae representing 124 species from 15 families has also shown that most larval gut microbes are transient, i.e., caterpillars lack large populations of resident microbes (Hammer et al. 2017). When taken together, these studies and our results suggest that much of the lepidopteran microbiome is highly plastic and diet-derived. Interestingly, our results contrast with a recent microbial survey of *L. melissa* larvae (Chaturvedi et al. 2017), which found that larval microbial assemblages were only very weakly affected by host plant or source population. That study involved the same novel host plant as our study (*M. sativa*) but a different native host plant, *Lupinus argenteus*, thus it is likely that the host-specific clustering of microbial assemblages that we report is dependent on the identity of the plant species involved.

Microbiome plasticity associated with diet is interesting in light of our observation that microbial diversity had a direct negative effect on PO activity for individuals reared on the novel host plant, *M. sativa*. The direct effect of microbial diversity was 40% greater than phytochemical diversity and 28% greater than weight gain, suggesting that microbes have a comparatively greater influence on constitutive immune response than phytochemistry or body condition. This result is similar to that reported by Freitak et al. (2007), who fed non-pathogenic bacteria to *Trichoplusia ni* (Noctuidae) larvae and assessed immunological and performance consequences. These authors observed that *T. ni* larvae fed bacteria-rich diets had lower PO activity and increased antibacterial activity. In our study, we focused on microbial diversity. Given that diversity is a function of both richness and evenness of taxa, the immune response of *L. melissa* may be responding to either increased richness of bacteria (more species present) or increased evenness of bacteria (relative abundance of species present). Post hoc analyses showed that variation in diversity was best explained by evenness, not richness (in a linear model, evenness explained 15.5% of the variation in Shannon's diversity, while species richness only explained 0.57% of the variation). It is possible that as the immune system has to contend with a greater range of bacterial taxa of similar abundance, less investment is put into the constitutive immune response. This strategy may seem counter-intuitive, but could be beneficial in the long run, as continual upregulation of the constitutive response to non-lethal threats may lead to self-harm (Sadd and Siva-Jothy 2006). Alternatively, the PO cascade involves both activators and inhibitors. It is also possible that the relationship between higher microbial diversity and lower PO is a function of increased activity of the inhibitors, instead of lowered investment in immune response. Further work is needed to disentangle these relationships. Another important caveat is that most larvae reared on *M. sativa* harbored low microbial diversity (Fig. 4), so the negative relationship we observed between PO activity and microbial diversity is driven by a few high diversity individuals.

We found four bacterial OTUs which were significantly associated with PO activity in larvae reared on *M. sativa*; of these, only one bacterial OTU was positively associated with PO, belonging to the genus *Klebsiella*. Previous work with a pathogenic strain of *Klebsiella* in the Noctuid moth *Galleria mellonella* found that infection with *Klebsiella pneumoniae* resulted in an increase in PO activity, but

only for more virulent strains (Wand et al. 2013). The other three OTUs were negatively associated with PO activity and included two members of *Acinetobacter* and one member of *Wolbachia*. Wolbachia are known to have complex effects on lepidopteran physiology and fitness, including manipulation of host reproduction, nutritional supplementation, and possible protection against wing deformity (Duplouy and Hornett 2018). Interestingly, Wolbachia have been shown to negatively affect immune related traits in *Drosophila simulans* and the parasitoid wasp *Leptopilina heterotoma* (Fytrou et al. 2005).

Influence of host plant use on immune response in *L. melissa*

We found no evidence that *L. melissa* receives an immunological benefit by utilizing the exotic host plant; levels of constitutive immune response were not significantly different between host plant treatments (Fig. 2). This result contrasts with other lepidopteran systems (e.g., *Manduca sexta*; Diamond and Kingsolver 2011), where utilization of the ancestral, higher-quality host plant has been associated with increased encapsulation and melanization. Our results also partially contrast with previous work using the autumnal moth (*Epirrita autumnata*) where alternative host plant use led to higher phenoloxidase (PO) activity on three out of four alternative host plants, but encapsulation rate did not differ between host plant treatments (Yang et al. 2008). Assays with the European grapevine moth (*Lobesia botrana*) found that alternative host plant use led to higher PO activity and hemocyte count, but no increase in antimicrobial activity (Muller et al. 2014). In this example, alternative host plant use also led to increased performance as measured by growth and survivorship. Given that the exotic host plant, *M. sativa,* is inferior to *A. canadensis* in terms of performance and survivorship (Forister et al. 2009), these studies taken together with our results suggest that low-quality host plants are unlikely to bolster the insect immune response.

The finding that a diet of *M. sativa* does not inhibit immune response suggests that while utilization of this novel host plant does not confer an immunological benefit, it also does not confer an immunological cost. A possible exception to this is the interaction we detected between host plant and microbial diversity, suggesting that the latter is important for immune response but only on the exotic host (further discussion below). Thus, at the population level, our results suggest that *L. melissa* larvae are not at a heightened risk of disease or parasitoid susceptibility when colonizing *M. sativa* due to a weakened immune response. Indeed, previous work with *L. melissa* has shown that parasitism is not higher at sites associated with *M. sativa* (Scholl et al. 2013). Another possibility is that additional measures of immune response are needed to detect the effect of host plant, such as antimicrobial activity, hemocyte count, or lysozyme-like activity. For example, Adamo (2004) demonstrated the importance of multiple measures of immunity within the same system; resistance to bacterial challenge was not predicted by total PO or baseline lysozyme-like activity, rather, it was predicted by total hemolymph protein concentration. Consequently, further work using a wider array of immune assays is needed to confirm the relationship between host plant and immune response for *L. melissa*.

Direct and indirect effects of dietary protein on immunity

We did not detect a difference in foliar protein content between native and exotic hosts. Somewhat surprisingly, dietary protein did not directly affect immune response in any of our SEMs, either within or across host plants (Figs. 4, 5, 6). However, protein was a good predictor of larval mass for larvae consuming the novel host plant, which confirms previous results in this system (Harrison et al. 2016a). Previous studies have found that high dietary protein enhances growth rate, antibacterial activity, and cuticle melanization (Lee et al. 2008), without affecting PO activity. Therefore, it is possible that protein content affected one arm of the immune system (antibacterial activity) without affecting the components of the immune system actually measured in this experiment (PO activity and bead melanization). In addition, previous studies have typically challenged larvae with bacterial pathogens in order to elucidate the effects of dietary protein on the insect immune response (Povey et al. 2009), whereas our study measured standing PO activity in healthy larvae and in response to artificial challenge. Thus, the lack of an actual pathogenic threat may have affected protein allocation and measurable costs of immunity.

Phytochemical variation and immune response

Phytochemicals have long been studied as putative anti-herbivory compounds that reduce insect damage and performance, leading to direct reductions in insect fitness (Fraenkel 1959; Raguso et al. 2015). Previous studies have shown that increasing the concentration of specific secondary compounds consumed by caterpillars can reduce the strength of the immune response (Smilanich et al. 2009a). Our results show that increased phytochemical diversity correlated with decreased spontaneous activity of PO, which suggests that a combination of phytochemicals appears to alter the dynamics of the PO pathway. A study by Slinn et al. (2018) also found that caterpillars reared on plants with high phytochemical diversity had lower PO activity, suggesting that this may be a general outcome of feeding on phytochemically diverse plants. This result may be a consequence of the costs of detoxification, because

as a wider array of allelochemicals are ingested, more resources may be required to process these compounds. Detoxification is an energetically costly process (Cresswell et al. 1992), which may lead to fewer resources to allocate to other mechanisms such as immune defense. In our study, we were not able to determine which compounds were detoxified by *L. melissa* versus which compounds passed through the gut unmodified. Further, while we currently have no evidence that *L. melissa* sequesters any metabolites from its host plants, it is possible that larvae may sequester flavonoids, as reported for several Lycaenid species (Burghardt et al. 1997; Geuder et al. 1997; Mizokami and Yoshitama 2009).

Conclusions

We found substantial variation in the factors mediating immune response within the context of a dietary expansion. To the best of our knowledge, this is the first study that has examined the effects of larval performance, caterpillar microbiota, dietary protein, and phytochemicals simultaneously. Both microbial and phytochemical diversity had direct negative effects on constitutive immune response within the novel host plant, *M. sativa*. Therefore, it is possible that in *L. melissa* populations where the threat from parasitism and pathogens is high, individuals consuming *M. sativa* with relatively low levels of microbial and phytochemical diversity will be more successful than individuals consuming *M. sativa* with high levels of microbial and phytochemical diversity. These results suggest that ecological variation in host plant traits can influence immunological variation in a highly context specific way. Lastly, when larvae consumed the exotic host plant *M. sativa*, the effect of microbial diversity on immunity was strong relative to other factors, suggesting that while microbes living in caterpillars may be transient, they might still play important ecological roles.

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Author contribution statement SY, MF, and AS conceived the ideas and experimental design; SY performed the experiment and field work and was in charge of immune assays and statistical analyses. JH processed and analyzed sequencing data; IW and DJ processed protein samples; CP and CD processed and analyzed phytochemical data. SY and JH prepared the manuscript; all authors reviewed the manuscript.

Data accessibility The datasets supporting this article have been uploaded as part of the Supplementary material.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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