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Host plant associated enhancement of immunity and survival in virus infected caterpillars

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ABSTRACT

Understanding the interaction between host plant chemistry, the immune response, and insect pathogens can shed light on host plant use by insect herbivores. In this study, we focused on how interactions between the insect immune response and plant secondary metabolites affect the response to a viral pathogen. Based upon prior research, we asked whether the buckeye caterpillar, *Junonia coenia* (Nymphalidae), which specializes on plants containing iridoid glycosides (IGs), is less able to resist the pathogenic effects of a densovirus infection when feeding on plants with high concentrations of IGs. In a fully factorial design, individuals were randomly assigned to three treatments, each of which had two levels: (1) exposed to the densovirus versus control, (2) placed on a plant species with high concentrations of IGs (*Plantago lanceolata*, Plantaginaceae) versus low concentrations of IGs (*P. major*), and (3) control versus surface sterilized to exclude surface microbes that may contribute to viral resistance. We measured phenoloxidase (PO) activity, hemocyte counts, and gut bacterial diversity (16S ribosomal RNA) during the fourth larval instar, as well as development time, pupal weight, and survival to adult. Individuals infected with the virus were immune-suppressed (as measured by PO response and hemocyte count) and developed significantly faster than virus-free individuals. Contrary to our predictions, mortality was significantly less for virus challenged individuals reared on the high IG plant compared to the low IG plant. This suggests that plant secondary metabolites can influence survival from viral infection and may be associated with activation of PO. Removing egg microbes did not affect the immune response or survival of the larvae. In summary, these results suggest that plant secondary metabolites are important for survival against a viral pathogen. Even though the PO response was better on the high IG plant, the extent to which this result contributes to survival against the virus needs further investigation.

1. Introduction

Studies in ecoimmunology investigate how variation in the immune response shapes species interactions and contributes to our understanding of life history traits (Zuk and Stoehr, 2002; Martin et al., 2011). The immune response is a physiological process that protects the organism from natural enemies, and the strength of this response can be affected by secondary metabolites from the host plant consumed by the herbivore (Smilanich et al., 2009a; Richards et al., 2012; Lampert and Bowers, 2015; Gowler et al., 2015; Hansen et al., 2017; Trowbridge et al., 2016). Given that host plant characteristics can affect immune strength, which in turn can determine the likelihood of escape from natural enemies (Smilanich et al., 2009b; Hansen et al., 2017; Barthel et al. 2016), host plant associated enhancement of immunity may be a key predictor of host plant use. For example, if feeding on certain host plants enhances the immune response, then the herbivore is better protected from infection by pathogens and more likely to survive, and the continued use of the immune-enhancing host plant would be favored by natural selection (Muller et al., 2015; Barthel et al., 2016). Alternatively, certain host plants may be detrimental to the immune response, and thus could deter herbivores from using them (Castro et al., 2008; Blackiston et al., 2008; Carlsson et al., 2013; Anderson et al., 2013; Engsontia et al., 2014).

There is a rich literature showing host plant associated protection from pathogens (Felton and Duffey, 1990; Cory and Hoover, 2006; Shikano et al., 2010; Shikano and Cory, 2014; Shikano et al., 2017; Shikano, 2017 and references therein), and from predators and parasitoids (Campbell and Duffey, 1979; Dyer, 1995; Gentry and Dyer 2002; Murphy, 2004; Lill et al. 2002; Singer et al., 2009; Smilanich et al., 2011; Hansen et al., 2017). In each of these studies, nutritional

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chemistry or secondary metabolites have been hypothesized to play a significant role in protecting the herbivores from succumbing to natural enemies. In some cases, whether plant chemistry directly interferes with natural enemy physiological functioning, or has beneficial effects through indirect means such as boosting the immune response is unclear (but see Campbell and Duffey, 1979; Felton and Duffey, 1990). The handful of studies that have investigated the effects of plant chemistry on the insect herbivore immune response have found the effects range from positive to negative with no general pattern emerging (Lampert and Bowers, 2015; Gowler et al., 2015). Indeed, it may not be surprising that the relationship between secondary metabolites and a physiological process like the immune response is difficult to predict given the extremely high diversity of secondary metabolites produced by plants, their many different modes of action, and the diverse ability of herbivores to detoxify or sequester them (Smilanich et al., 2009a, 2016).

In this study, we use the buckeye caterpillar, *Junonia coenia* (Nymphalidae: Lepidoptera), which specializes on host plants containing iridoid glycosides (IG) to investigate the interaction between plant chemistry, immune response, and pathogen infection. There is a wealth of background information on the chemical ecology of the buckeyes and their host plants, and we build upon this body of work (Bowers, 1984; Bowers and Puttick, 1988; Bowers, 1992; Bowers and Collinge 1992; Bowers and Stamp, 1993; Dyer and Bowers, 1996; Jarzomski et al., 2000; Richards et al., 2012). Relevant to the study here, we found that buckeyes suffered a lower encapsulation response when sequestering high concentrations of IGs from their host plant (Smilanich et al., 2009a). Interestingly, buckeyes caterpillars will sequester IGs from their diet in proportion to the amount that is found in the plant, such that the more that is present in the host plant, the more they will sequester (Bowers and Collinge, 1992). Additionally, adult buckeyes prefer plants with high concentrations of IGs for oviposition compared to plants with low concentrations of IGs (Pereyra and Bowers, 1988; Klockars et al., 1993; Prudic et al., 2005). If high concentrations of dietary IGs are detrimental to the immune response, then one question that emerges is whether there is a benefit to sequestering and preferring IG producing host plants given that they depress the immune response. Here, we explore this question by exposing buckeye larvae to a pathogenic virus (*Junonia coenia* densovirus, JcDNV) and measuring their immune response, survival, and development on two host plants that vary in IG content.

In addition to understanding the effects of plant chemistry on the immune response, there is increased interest in the effects of microbial communities on chemically mediated insect-plant interactions (Shikano, 2017). Recent investigations of the insect microbiome have provided evidence for positive effects on insects by aiding in digestion (Anand et al., 2010), ameliorating negative effects of secondary metabolites (Mason et al., 2014), and possibly positive interactions with the immune response (Broderick et al., 2010; Freitak et al., 2014). Here, we were specifically interested in examining whether maternally provisioned microbes that were added to the egg surface during oviposition may be beneficial to the offspring by enhancing immunity. This type of transmission of microbes is best known from the plataspid stinkbug (*Megacopta punctatissima*) and has been associated with increased development time and growth rate (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006). Maternal transfer of beneficial microbes has been found in at least four orders of insects (reviewed in Funkhouser and Bordenstein, 2013; Engel and Moran, 2013), but whether they play a role in immune enhancement is not clear. We investigated whether maternal microbes on the egg surface benefit individuals infected with the virus by protecting them through immune-enhancing avenues, or through indirect routes such as developmental changes.

To test the effects of host plant identity and egg microbes on the immune response and its role in survival against the JcDN virus, inoculated *Junonia coenia* larvae were reared on either *Plantago major* or *Plantago lanceolata*. The former has low concentrations of one iridoid glycoside (aucubin), and the latter has high concentrations of two iridoid glycosides (aucubin and catalpol) (Theodoratus and Bowers, 1999). To test the effects of egg microbes on the immune response, eggs were surface sterilized to remove any microbes. Given our prior data showing that sequestration of high concentration of IGs lead to lower melanization in buckeyes (Smilanich et al. 2009a), we predicted that individuals consuming high amounts of iridoid glycosides would be immunosuppressed and more susceptible to the virus. We also predicted that individuals without egg microbes would be more susceptible to the virus and would have a weaker immune response as measured by phenoloxidase activity and hemocyte counts.

2. Materials and methods

2.1. Study system

2.1.1. Caterpillars and host plants

The common buckeye (*J. coenia*) caterpillar specializes on various IG producing plants within six families (Bowers, 1984). In this study we focused on two common host plants of *J. coenia* that differ in their IG composition, *Plantago major* and *Plantago lanceolata* (Plantaginaceae). Both species were introduced from Europe approximately 200 years ago, and have been incorporated into the diet of buckeye caterpillars. *Plantago major* contains low concentrations of the IG aucubin (0.2–1% dry weight) (Barton and Bowers, 2006) and no catalpol. While *P. lanceolata* contains higher concentrations of aucubin and low concentrations of catalpol (5–12% dry weight total IG) (Bowers and Stamp, 1992; Theodoratus and Bowers, 1999). Buckeye caterpillars sequester high levels of these IGs in the hemolymph where it is metabolized prior to pupation (Bowers and Collinge, 1992).

Junonia coenia larvae used for all experiments were originally collected as adults from natural populations found in the long-leaf pine habitat of Florida and from the Central Valley of California. Populations were allowed to interbreed and maintained as a colony in the laboratory for two generations prior to the first experiment, and for six generations prior to the second experiment. Colony larvae were reared on a wheat germ based artificial diet with dried *P. lanceolata* leaves added (10% of dry weight ingredients) to stimulate feeding (Camara 1997). For all experiments, *J. coenia* caterpillars were reared in growth chambers with a photoperiod of 16:8 L:D at temperatures of 25 °C daytime temperature and 20 °C nighttime temperature. Buckeye larvae in the experiment were reared on diets of *P. major* or *P. lanceolata* leaves that were collected from various public parks around Reno, Nevada.

2.1.2. Virus

The *Junonia coenia* densovirus (JcDNV), is a nonenveloped, single stranded DNA virus belonging to the Parvoviridae family. The virus is named after the buckeye (*Junonia coenia*) since it was first isolated from this lepidopteran species (Rivers and Longworth, 1972). The occurrence of this virus in populations that we study is unknown, but is currently being investigated. Densoviruses infect arthropods with most of the studies on ecological effects focused on crustaceans and insects, and the recent paper by (Francois et al., 2016) greatly expanded the number of known arthropod species carrying the virus. Insect densoviruses are highly virulent, spreading horizontally through populations via oral inoculation (Rivers and Longworth, 1972; Simpson et al., 1998), and they have been detected in the phloem of host plants providing a route for infection to herbivores (van Munster et al., 2005). Vertical transmission of densoviruses have also been found in mosquitos and aphids (van Munster et al., 2003. The JcDNV infects species in a number of different lepidopteran families including Noctuidae, Erebidae (Rivers and Longworth, 1972; Mutuel et al., 2010), and Lycaeides (Yoon et al., unpublished data). Once ingested, the virus infects the midgut epithelium and targets cells in the trachea, visceral muscles, and hemocytes (Wang et al., 2013). Insect host death occurs through asphyxiation and molting failure (Mutuel et al., 2010).

2.2. Overview of experiments

We measured the effects that host plant, viral challenge, and egg microbial status have on the insect immune response, survival, and development of the *J. coenia* caterpillar. Larvae were reared on one of two host plant species, *P. major* (low IG) or *P. lanceolata* (high IG). A preliminary experiment was conducted to determine the lethal dose of JcDNV required to kill 50% (LD_{50}) of a buckeye larvae test group. A group of 150 individuals was split into larval cohorts of 30 individuals which were fed a 10 mm leaf disk of *P. major* containing a 1 µl drop of isolated *Junonia coenia* densovirus (stock solution 10^{13} viral particles per µl) in doses of 10^{11}, 10^{9}, 10^{7}, 10^{5}, 10^{3} viral particles at the beginning of fourth instar. The virus was isolated and purified in the M. Ogliastro Lab (Mutuel et al., 2010) and sent to our lab for experiments. Larvae were permitted to consume the entire leaf disk for 24 h to ensure inoculation. Following inoculation, larvae were maintained on an artificial diet until death. The LD_{50} of JcDNV was determined to be 10^{11} viral particles, 95% CI [0.3130, 0.6870].

After determining the LD50, buckeye larvae were randomly assigned to either *P. major* or *P. lanceolata* (N = 80 per host plant) and reared individually in 4oz. soufflé cups. The immune response was assayed by measuring the activity of the phenoloxidase enzyme, which produces melanin, and by hemocyte counts. Melanization (synthesis and deposition of melanin) is an effective defense response against invaders such as parasitoids, parasites, and various pathogens (Cerenius et al., 2008; Smilanich et al., 2009b). The phenoloxidase enzyme (PO) is an important indicator of immune status in invertebrates (Gonzalez-Santoyo and Cordoba-Aguilar, 2012; Hansen et al., 2017) and is responsible for the activation of the melanization cascade. The PO enzyme is recognized as an effective defense against viral infection (Ourth and Renis, 1993; Shelby and Popham, 2006; Cerenius et al., 2008), and in Lepidoptera is contained within the oenocytoid hemocytes (Gonzalez-Santoyo and Cordoba-Aguilar, 2012).

To quantify viral replication and measure the gut bacterial diversity, we conducted a second experiment using identical treatments as our initial experiment. Quantitative analysis of viral replication was measured using qPCR and gut bacterial diversity was estimated by sequencing a conserved region of the bacterial 16S ribosomal RNA gene (see below).

2.3. Experimental design

To eliminate egg-surface microbes, eggs that had been oviposited on either *P. lanceolata* or *P. major* were washed three times in 70% ethanol, followed by a wash in 10% bleach, and were then rinsed three times in deionized water (as per Hail et al., 2012). Eggs were left to air dry in incubators until hatching. Upon hatching, *J. coenia* caterpillars were systematically assigned to either *P. lanceolata* or *P. major* (Experiment 1, N = 160; Experiment 2, N = 240). At the beginning of the 4^{th} instar, half of the larvae from each treatment group (*P. lanceolata* and *P. major*; washed and unwashed) were inoculated with JcDNV. Caterpillars were provided a 10 mm leaf disk to which was applied a dose of 10^{11} particles of JcDNV in 1 µl of water. Larvae were permitted to consume the leaf disk for 24 h to ensure inoculation. Individuals that did not consume the leaf disk were re-inoculated using a fresh leaf disk. Post-inoculation, larvae were maintained on a leaf diet of their respective host plant treatment groups. In the first experiment, twenty individuals from each treatment group were used to collect hemolymph samples 4 days post inoculation or 4 days after entering 4^{th} instar. Development time, pupal weight, and survival were used as performance measurements. Development time was measured from the date of egg hatching to the date of pupation. All pupae were weighed individually and any deformities were noted. Survival was defined as successful pupation and eclosion into an adult. Larvae that experienced hemolymph leakage and became flaccid, or that experienced partial pupation were determined to have not survived the JcDNV infection (Mutuel et al., 2010).

2.4. Immune assays

A colorimetric assay of hemolymph phenoloxidase (PO) activity was measured using a spectrophotometer to assess insect host immune response (Adamo, 2004a). For these experiments, a 10 µl hemolymph sample was taken using a micropipette (20 µl) from each individual by piercing the cuticle of the A1 abdominal segment with a hand-pulled Pasteur pipette needle (Smilanich et al., 2009a). The hemolymph was added to 500 µl of ice-cold phosphate-buffered saline (PBS) in a 1.5 mL Eppendorf tube and vortexed. From each individual, 100 µl of PBS-bound hemolymph mixture was added to 200 µl of l-DOPA (0.118 g l-DOPA mixed with 30 ml deionized water) in a 96 well plate, and PO activity was measured using an iMark Microplate Absorbance Reader (Bio-Rad). The absorbance from each well was measured every 30 s and expressed as the slope of the line over a total of 45 m at 490 nm. For analyses, we used the linear portion of the curve which was during the first 10 m of the assay.

Hemocyte counts were used as an additional measure of immune status (Triggs and Knell, 2012). Hemolymph samples containing 4 µl of PBS-bound hemolymph, 12 µl anticoagulant (0.684 g EDTA, 0.346 g citric acid in 180 ml PBS), and 16 µl glycerol were stored at 4 °C. To perform the cell counts, the cell suspension was diluted with 0.5 µl of Trypan Blue dye and 10 µl of each sample was examined under a light microscope using a Neubauer Bright-Line hemocytometer. The entire gridded area was counted, and the average of two cell counts per sample was used for all analyses.

2.5. qPCR

To test for host plant and egg microbe effects on the replication of JcDNV, the amount of virus present was calculated using quantitative PCR (qPCR). For each treatment group the number of total individuals used was as follows: $L_{anceolata}V_{irus} = 11$; $L_{anceolata}V_{irus}W_{ash} = 17$; $M_{ajor}V_{irus} = 16$; $M_{ajor}V_{irus}W_{ash} = 17$. Larvae used for the qPCR assay were frozen 4 days post-inoculation. Total DNA extraction was performed by homogenizing 50 mg of individual frozen caterpillar-midsection that included midgut, tracheal tissue, and hemolymph, using a Qiagen DNeasy Blood and Tissue Kit (Purification of Total DNA from Insects using the DNeasy Blood and Tissue Kit, Qiagen #69506), since the virus is known to replicate in all three of these tissue types (Wang et al., 2013). All of the samples were normalized to 10 ng/µl of DNA and qPCR was performed using 0.2 µM each of JcDNV specific forward and reverse primers for the VP4 gene (Wang et al., 2013), and 0.2 µM each of the CO1 primers HCO/LCO for use as a housekeeping gene (Folmer et al., 1994) and 5 µl iTaq Universal SYBR Green Supermix in a total volume of 9 µl. Reactions were run using a BioRad CFX96 Thermal Cycler with an initial denaturing step at 95 °C for 5 min followed by 45 cycles under the following parameters: 95 °C for 10 s, 60 °C for 15 s, 72 °C for 15 s. A melt curve was run following amplification, the JcDN virus stock solution (provided by M. Ogliastro) was serially diluted and used to make a standard curve. Viral load was calculated as absolute quantification of gene copy number using the threshold cycle (C_t) protocol as outlined in Schmittgen and Livak (2008). Samples were run in triplicate and averaged. C_t values were standardized against an internal control gene (COI). Normalized C_t values are reported as $2^{\sim\Delta Ct}$. Small C_t values indicate the presence of higher quantities of the targeted DNA, and in this case higher numbers of virus.

2.6. Microbial analysis

Larvae used for sequenced-based characterization of the gut bacterial community were frozen 4 days after entering 4^{th} instar, then stored at −4 °C for 2 months until dissection. The midgut of ten larvae from each treatment group (8 treatment groups × 10 = 80 individuals) was dissected and removed in a hood under sterile conditions. Tissues from ten individuals per treatment were homogenized and the tissue sent to the

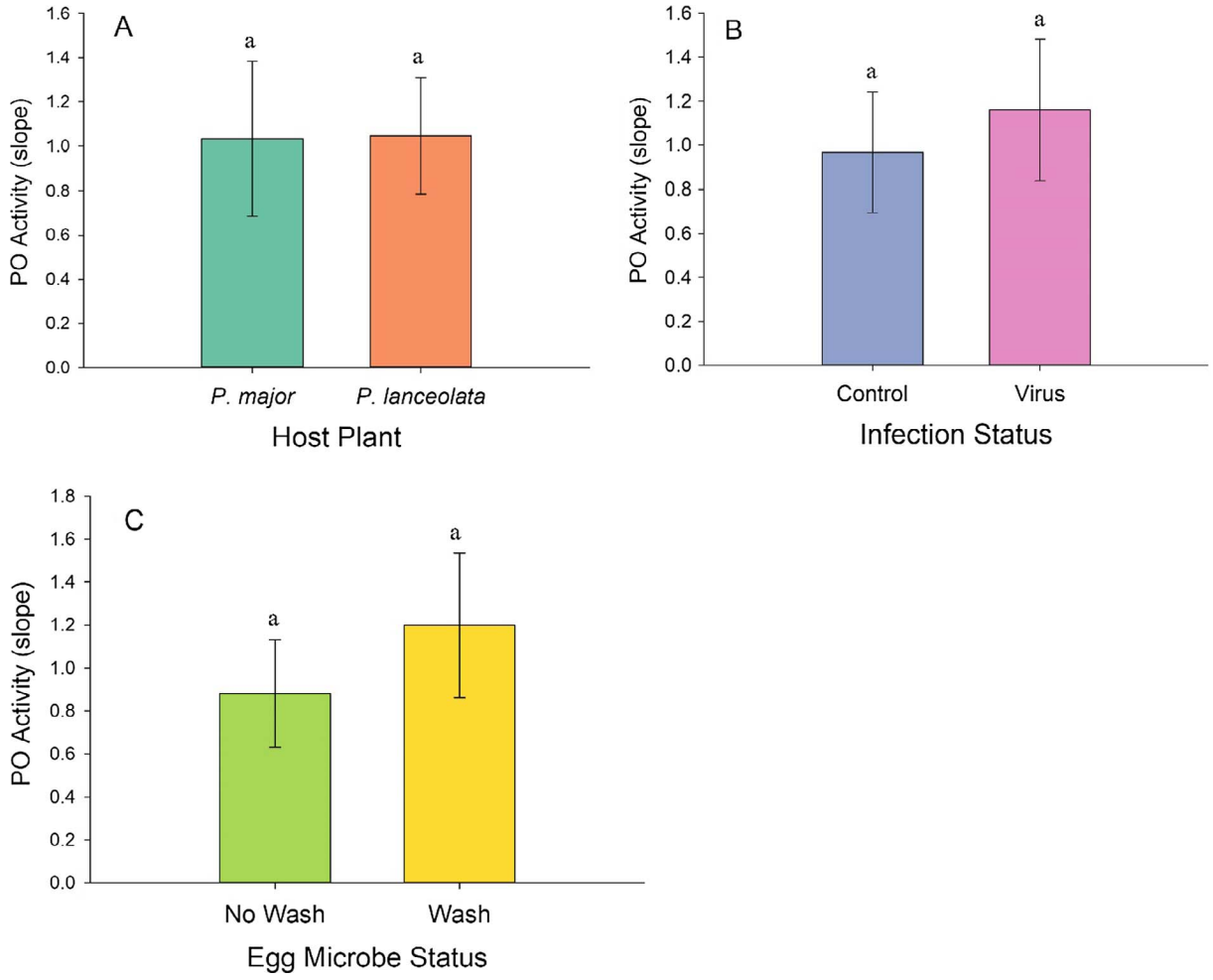

Fig. 1. Phenoloxidase activity (mean ± SE) as measured by the slope of the kinetic enzyme assay of buckeye caterpillars associated with host plant, virus challenge, and egg microbe status. **A**. No significant difference was detected in caterpillar PO activity between the two host plants (P = .9776, N = 85). **B**. No significant difference was found in PO activity between virus challenged and unchallenged caterpillars (P = .7204, N = 85). **C.** No significant difference was found in PO activity between individuals washed as eggs and those with intact egg microbes (P = .4531, N = 85).

Research and Testing Laboratory (RTL) (Lubbock, TX, USA) where DNA was extracted and the 16S ribosomal RNA region amplified to identify bacteria inhabiting the midgut tissues (primer sequence: Gray28F (GAGTTTGATCNTGGCTCAG)/Gray519R (GTNTTACNGCGGCKGCTG)). Sequencing was conducted using the Roche 454 platform, and potential sequencing errors were corrected by RTL using a proprietary denoising procedure. Corrected reads were quality filtered again to < 1% errors per read using USEARCH v8.1 (Edgar, 2010). Reads were prefix dereplicated to determine unique sequences. Using this technique, shorter sequences that match the initial portion of longer sequences are deemed identical. Consensus sequences output from the dereplication step were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm with a 97% sequence similarity threshold (Edgar, 2013). This process also removes chimeric sequences. Taxonomy of OTUs was assigned using UTAX in USEARCH and the Ribosomal Database Project training database (Cole et al., 2014; accessed November 2016). Unfiltered reads were queried against bacterial OTUs using a 97% similarity threshold. Some sequences that did not pass initial stringent filtering were thus assigned to bacterial OTUs, thereby providing a more accurate estimate of sequence count for a particular OTU. Read counts were normalized using the relative log expression method in the R package edgeR v3.12.1 (Anders and Huber, 2010). Normalization resulted in read count estimates for each OTU normalized to counts per million reads. This method is statistically preferable to rarifying reads (McMurdie and Holmes, 2014). Microbial diversity in each sample (alpha diversity) was determined using the species equivalents of Shannon-Wiener and Simpson's diversity indices (Jost, 2006), as well as the normal entropy measurements.

2.7. Statistical analyses

All statistical analyses were performed in SAS 9.4 (SAS Institute) using the GLM (general linear model) procedure for ANOVA analyses and CATMOD for logit models. The residuals for the slope data from the PO assay were not normally distributed, so this variable was log transformed to normalize the residuals. Slope data were analyzed using a three-way ANOVA to test for significant interactions between the three independent variables (host, virus, and egg wash). None of the interactions were significant (at $P < .05$), thus they were removed from the model. The results presented here show the main effects only. For the PO assay, we found that the slope of the line was 0 (flat) or slightly negative in many cases. Therefore, we transformed the data to categorical variables ("yes response" or "no response"), and added a second method where the data were analyzed as part of a logit model with plant host, virus challenge, and egg microbe status predicting survival.

Patterns of survivorship for individual caterpillars were analyzed using logit models with host (*P. major* or *P. lanceolata*), virus challenge (yes or no), and egg wash (yes or no), and immune response (yes or no)

as predictor variables. The saturated model with all interactions was run first to identify important interactions. Non-significant interactions were dropped from the model, and the results from the most parsimonious model that fit the data were reported using chi-square goodness of fit.

Residuals for models including pupal mass or development time were normally distributed. Pupal mass was measured once the cuticle of the pupa was hardened, one to two days after pupation. Pupal mass data were analyzed using a three-way ANOVA with host (*P. lanceolata* or *P. major*), virus challenge (yes or no), and egg wash (yes or no) as the independent variables. Development time was analyzed as total development time (egg hatch to pupal date), and as development time post inoculation of virus. Since the individuals in the virus treatment group were inoculated at the beginning of the 4th instar, development time post inoculation was standardized as the beginning of the fourth instar to pupation date for all individuals, including those not receiving the virus (controls). Total development time and development time post inoculation were analyzed using a three-way ANOVA with host, virus challenge, and egg wash status as the independent variables.

Quantitative PCR data were log transformed to meet the assumptions of normality, and analyzed using a two-way ANOVA with host plant and egg wash status as the independent variables. ANOVA was used to test for an effect of host plant, virus, and egg microbe removal on the diversity of microbes. None of the two-way interactions were significant and were removed from the model. Given the low sample size after sample homogenization (host N = 4, virus N = 4, and egg wash N = 4), we were unable to run the three-way interaction.

3. Results

3.1. Immune response

Host plant identity did not have a significant effect on phenoloxidase activity (F[1,84] = 0.00, P = .9776, N = 85, Fig. 1A) as measured by the slope in the kinetic assay. Likewise, virus challenge (virus or no virus) and microbe status (wash or no wash) did not significantly affect the rate of the reaction (Infection: F[1,84] = 0.13, P = .7204, N = 85, Fig. 1B) (Microbe: F[1,84] = 0.57, P = .4531, N = 85, Fig. 1C). However, when phenoloxidase activity was changed to a binary response (activity or no activity), we found that infected individuals feeding on *P. lanceolata* were more likely to have active PO (non-zero absorbance) compared to infected individuals feeding on *P. major* (standardized parameter estimate = 3.09, χ^2 = 9.58, P = .002, Fig. 2A). In addition, the PO enzyme was less likely to be active (zero absorbance) in infected individuals compared to non-infected individuals (standardized parameter estimate = 2.43, χ^2 = 5.92, P = .0149, Fig. 2B).

For hemocyte counts, host plant identity was not significant (Host plant: F[1137] = 0.13, P = .7194, N = 138, Fig. 3A). However, individuals that were challenged with the virus had a significantly reduced total hemocyte count compared to the controls (F[1137] = 5.76, P = .0177, N = 138, Fig. 3B), while egg microbe status had no significant effect (Microbe: F[1137] = 0.04, P = .9227, N = 138, Fig. 3C).

3.2. Survival

Within the inoculated individuals, the best model data showed that survival was higher on *P. lanceolata* compared to *P. major* (standardized parameter estimate = 2.12, χ^2 = 4.53, P = .0334, 30% survival on *P. lanceolata* vs. 15% survival on *P. major*, Fig. 4A). When all individuals were included in the model, survival was best predicted by virus challenge, with those individuals that were not challenged having higher survival (standardized parameter estimate = 4.97, χ^2 = 24.76, P < .0001, 67% survival on control vs. 32% survival with virus, Fig. 4B). The other predictor variables (egg microbes: no wash = 57%, wash = 43% survival; host plant: *P. lanceolata* = 57%, *P. major* = 43% survival) were not significant and were dropped from the model along with all non-significant interaction terms.

In the second experiment, mortality was very high in the JcDNV infected group (81%), even though the JcDNV concentration was the same as the first experiment. High mortality may have been due to the many generations (6th) of the colony being reared in a lab setting, or possible inbreeding effects. In this case, the best predictor of survival was not receiving the virus (standardized parameter estimate = 4.74, χ^2 = 22.54, P < .0001, virus = 19%, control = 81% survival), while microbe status (standardized parameter estimate = 0.772, χ^2 = 0.60, P < .4401, wash = 63%, no wash = 38% survival) and host plant (standardized parameter estimate = 1.12, χ^2 = 1.27, P < .2596, *P. lanceolata* = 47%, *P. major* = 53% survival) were not significant predictors of survival.

3.3. Fitness

Pupal weight was significantly higher in the *P. lanceolata* reared group compared to *P. major* (F[1115] = 5.57, P = .045, N = 116, Fig. 5A). Viral infection and egg washing did not have significant effects on pupal weight (virus: F[1115] = 2.10, P = .1506, N = 116, Fig. 5B; egg wash: F[1112] = 0.10, P = .7567, N = 116, Fig. 5C).

Overall, the development time from larval emergence to pupal date was significantly shorter when individuals were reared on *P. lanceolata*

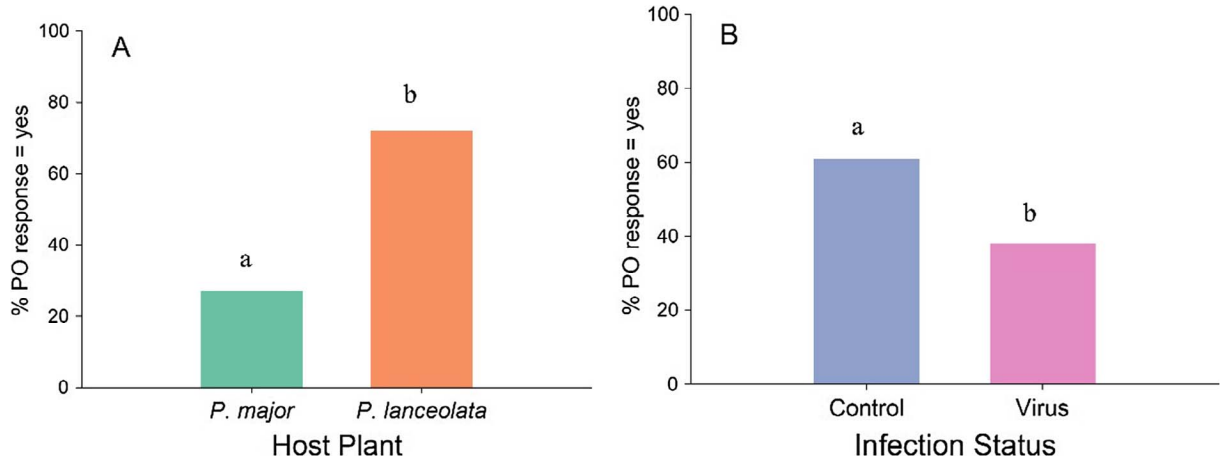

Fig. 2. The frequency (displayed as percentage) of buckeye individuals with a positive phenoloxidase response (i.e. non-zero) associated with host plant and virus challenge. **A**. The frequency of the PO enzyme with non-zero activity was significantly higher when caterpillars ate *P. lanceolata* (χ^2 = 9.58, P = .002). **B**. The frequency of the PO enzyme with non-zero activity was significantly higher in unchallenged caterpillars (χ^2 = 5.92, P = .0149).

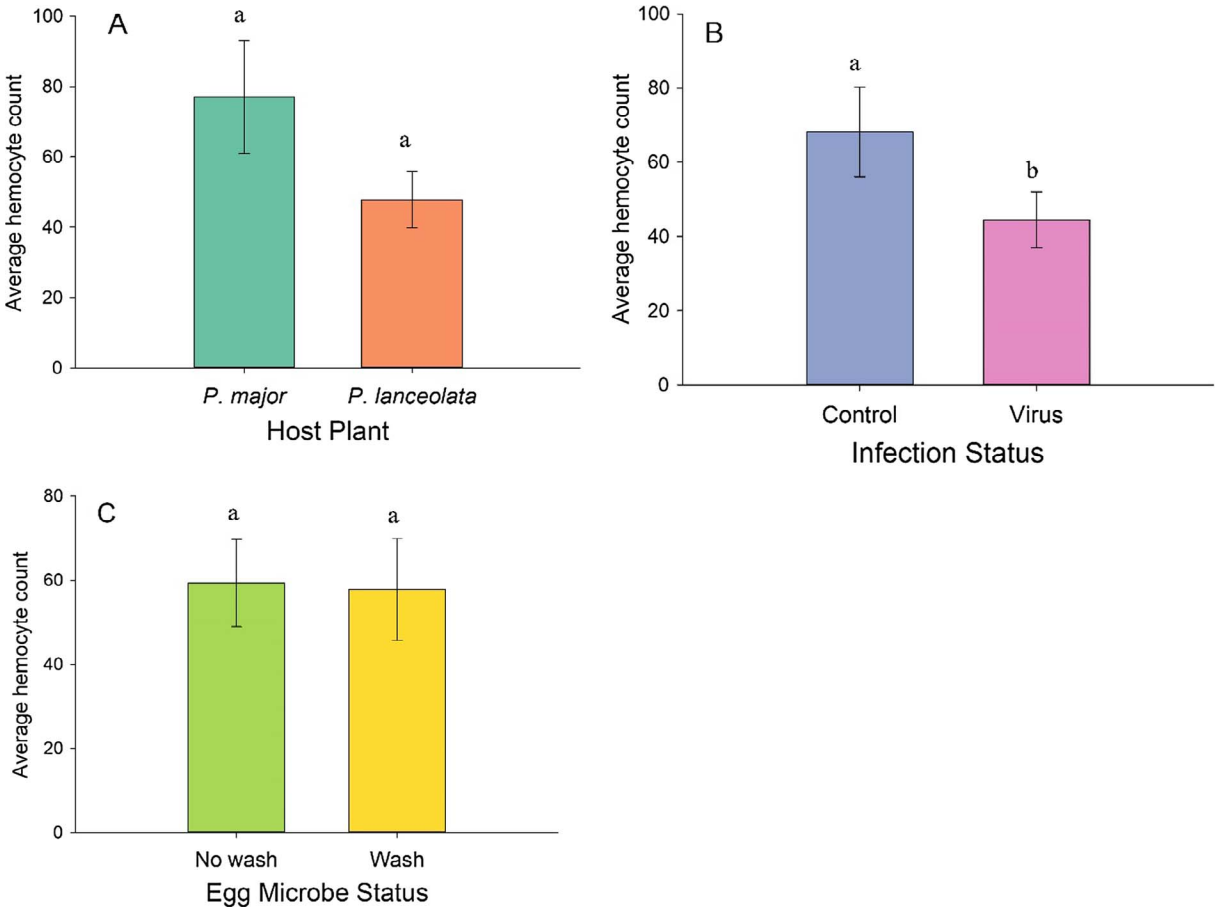

Fig. 3. Hemocyte count (mean ± SE) associated with host plant, virus challenge, and egg microbe status. **A.** There was no significant difference in hemocyte count totals between host plants (P = .7194, N = 138). **B.** Hemocyte count was significantly lower in the virus challenged individuals (P = .0177, N = 138). **C.** Egg washing had no significant effect on hemocyte count (P = .9227, N = 138).

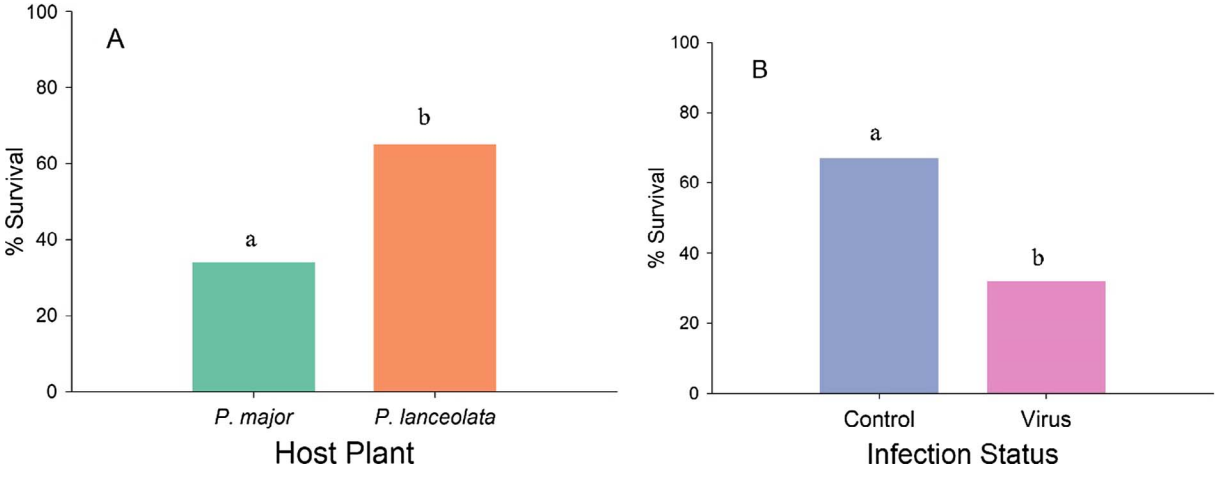

Fig. 4. Survival frequency (displayed as percentage) of infected buckeye caterpillars associated with host plants and virus challenge. **A**. For the challenged individuals, the likelihood of survival was significantly higher when buckeye caterpillars ate *P. lanceolata* (χ^2 = 4.53, P = .0334). **B.** For all individuals, the likelihood of survival was higher when individuals were not challenged with the virus (χ^2 = 24.76, P < .0001).

(F[1115] = 6.32, P = .0134, N = 116, Fig. 6A), and when they were not washed as eggs (F[1115] = 15.39, P = .0002, N = 116, Fig. 6B). The analysis of development time post inoculation (or starting at fourth instar for controls) showed that individuals challenged with the virus had a significantly shorter development time post inoculation compared to control individuals (F[1114] = 53.56, P < .0001, N = 115, Fig. 7A), and there was a significant interaction between host plant treatment and virus challenge with challenged individuals reared on *P. lanceolata* (mean = 17.83) developing faster than individuals reared on *P. major* (mean = 22.25) (F[1114] = 11.06, P = .0012, N = 115, Fig. 7B). There was no difference in development time from egg hatch to 4th instar between individuals in the viral group and individuals in the control group indicating that the change in development time was stimulated after the virus was administered (F[1114] = 0.00, P = .9654, N = 115).

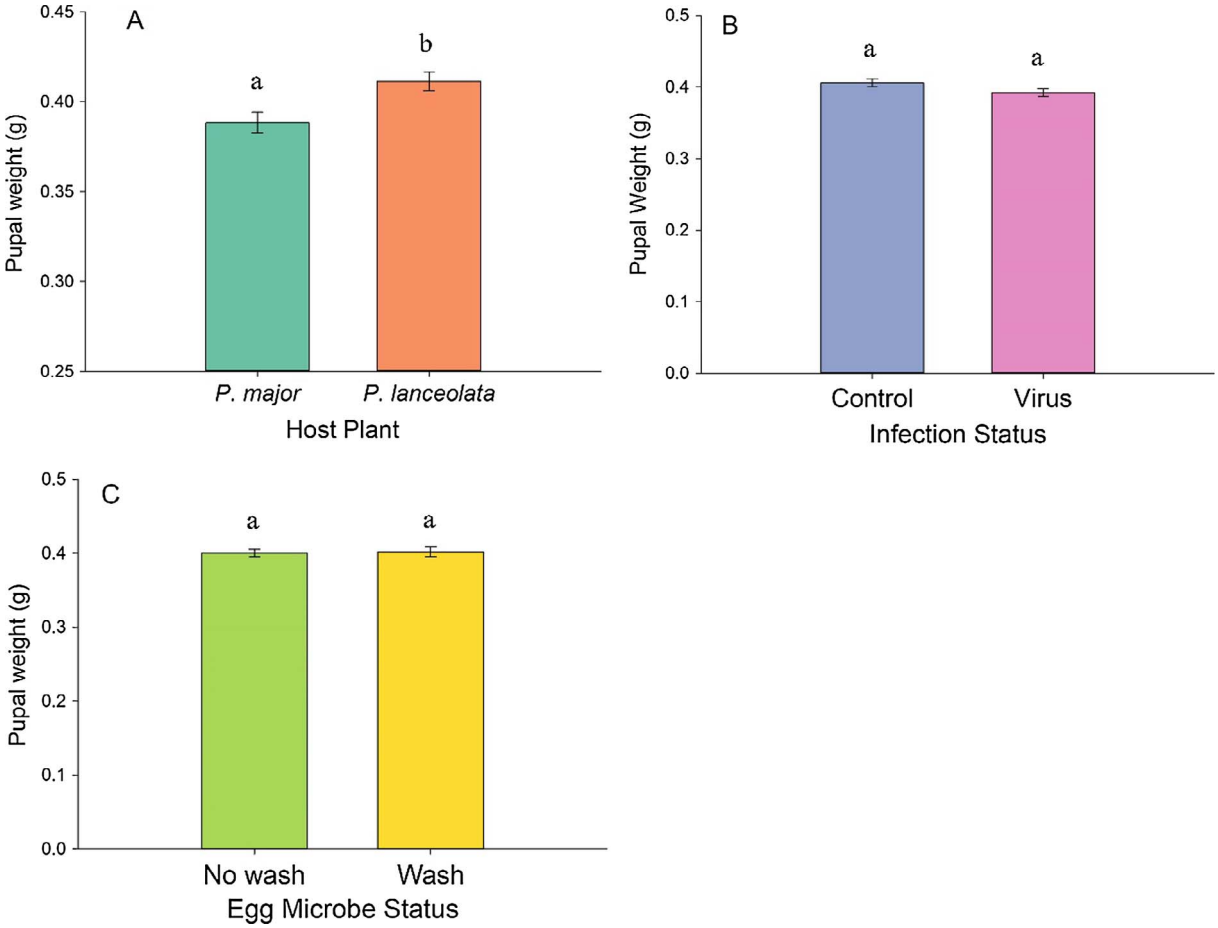

Fig. 5. Average pupal mass (grams) (mean ± SE) associated with host plant, virus challenge, and egg microbe status. **A.** Pupal mass was significantly higher when caterpillars were reared on *P. lanceolata* (P = .045, N = 116). **B.** There was no significant difference in pupal mass between challenge and unchallenged individuals (P = .1506, N = 116). **C.** There was no significant difference in pupal mass between individuals with intact egg microbes and those washed as eggs (P = .7567, N = 116).

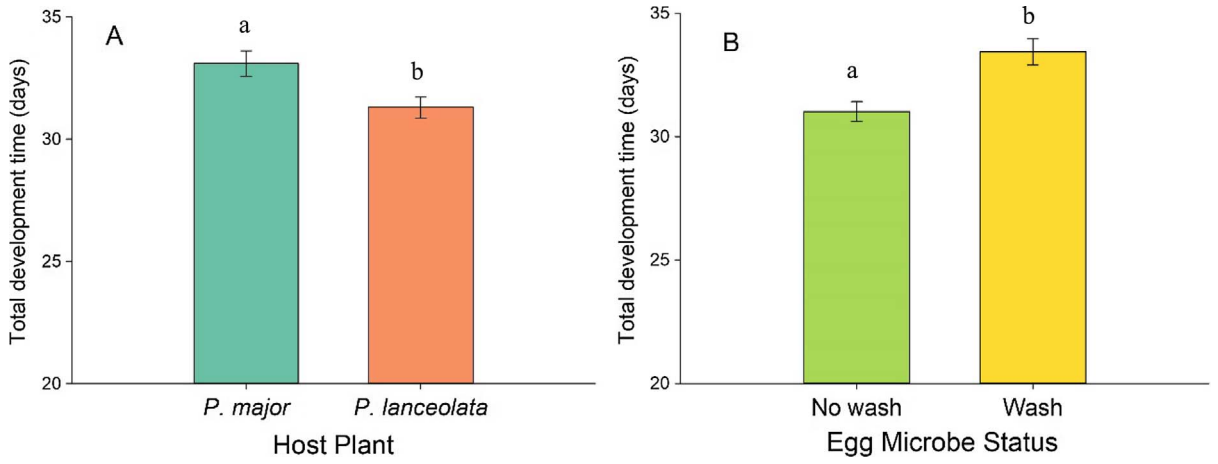

Fig. 6. Development time (days) from egg hatch to pupal date (mean ± SE) of buckeye caterpillars associated with host plant treatment, virus challenge, and egg microbe status. **A**. Total development time (egg to pupa) was significantly shorter on *P. lanceolata* (P = .0134, N = 116). **B**. Total development time was significantly longer when individuals had their egg microbes removed (P = .0002, N = 116).

3.4. Viral quantification and bacterial diversity

Even though the mean JcDNV load was higher when individuals were feeding on *P. lanceolata* and when eggs were washed, the differences were not significant (host plant: F[1,60] = 1.62, P = .2083, N = 61) (egg wash: F[1,60] = 2.69, P = .1062, N = 61, Table 1).

A total of 183,751 reads were generated from sequencing, 151,969 of which were assigned to 138 bacterial OTUs. Actinobacteria and Proteobacteria were particularly well represented with 49 OTUs each. Bacterial diversity was higher in individuals that were not infected with JcDNV, and significance varied depending upon the diversity index used (Table 2). Across all indices, individuals feeding on *P. lanceolata* had significantly higher bacterial diversity (Table 2). Finally, egg washing tended to reduce bacterial diversity, although only Shannon's diversity index was significant (Table 2).

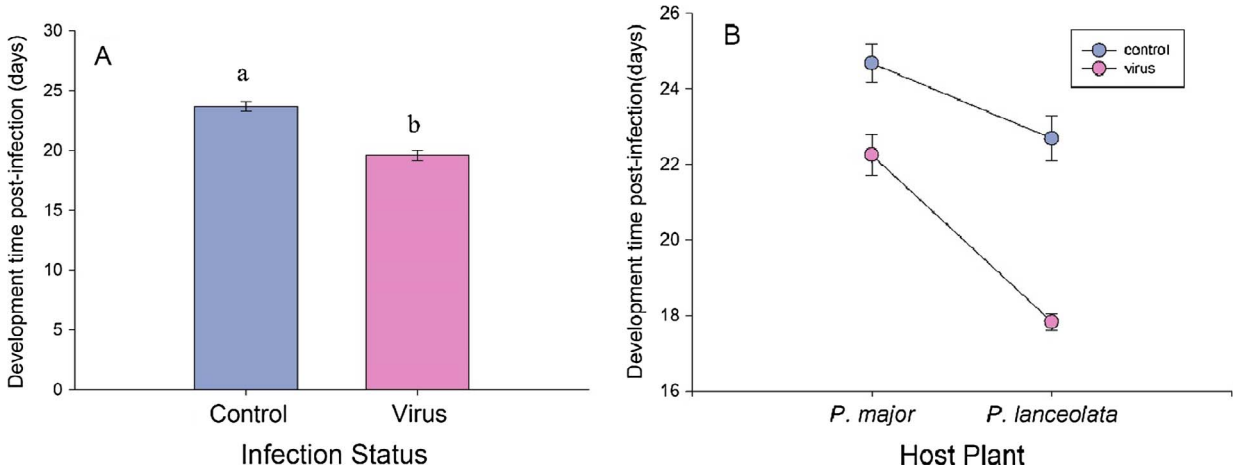

Fig. 7. Development time (days) (mean ± SE) post-infection (starting at 4 th instar for controls). **A.** Development time post-infection was significantly shorter in virus challenged individuals compared with controls (P < .0001, N = 115). **B.** There was a significant interaction between host plant and infection with faster development when virus challenged individuals were feeding on *P. lanceolata* (P = .0012, N = 115).

Table 1
Mean, standard deviation, and sample size for qPCR data from each treatment group exposed to the virus. Mean data were not log-transformed.

Treatment	X	S.D.	N
Egg wash	32995.90	152160.91	34
Unwashed control	513.39	2467.91	27
P. lanceolata	4421.86	19986.58	28
P. major	30663.94	153995.45	33

Table 2
Mean diversity value according to each diversity index for the eight treatment groups followed by main effects of ANOVAs for each diversity index. F-statistics and P-values in bold indicate a significant result.

Treatment	Shannon's	Shannon's species equivalent	Simpson's	Simpson's species equivalent
L	2.39	10.90	0.18	5.55
LV	2.30	9.94	0.15	6.77
LW	2.26	9.62	0.18	5.42
LVW	0.85	2.34	0.55	1.82
M	2.02	7.52	0.36	2.80
MV	0.98	2.66	0.64	1.57
MW	1.18	3.27	0.53	1.88
MVW	0.46	1.59	0.83	1.21
Host plant	**$F_{1,7}$ = 10.49, P = .031**	**$F_{1,7}$ = 10.93, P = .030**	**$F_{1,7}$ = 17.68, P = .013**	**$F_{1,7}$ = 9.49, P = .037**
Virus infection	**$F_{1,7}$ = 11.21, P = .029**	$F_{1,7}$ = 7.56, P = .051	**$F_{1,7}$ = 8.75, P = .041**	$F_{1,7}$ = 1.19, P = .336
Egg wash	**$F_{1,7}$ = 8.98, P = .040**	$F_{1,7}$ = 6.99, P = .057	$F_{1,7}$ = 6.30, P = .066	$F_{1,7}$ = 5.06, P = .181

[i]Treatment abbreviations are as follows: L = *P. lanceolata*; LV = *P. lanceolata* + virus; LW = *P. lanceolata* + egg wash; LVW = *P. lanceolata* + virus + egg wash; M = *P. major*; MV = *P. major* + virus; MW = *P. major* + egg wash: MVW = *P. major* + virus + egg wash.

4. Discussion

4.1. Survival

We provide evidence that host plant diet and densovirus infection can influence insect herbivore immune response and survival. Specifically, larvae were more likely to survive JcDNV infection when reared on *P. lanceolata* compared to *P. major*. As previously mentioned, these two host plants differ in their iridoid glycoside composition, with *P. lanceolata* containing high concentrations of both aucubin and catalpol (Bowers and Stamp, 1992) and *P. major* containing low concentrations of aucubin only (Barton and Bowers, 2006). Many plant secondary metabolites function as a defense against herbivores and pathogens (Fraenkel, 1959; Ehrlich and Raven, 1964; de la Fuente et al., 1994), and it is possible herbivores have co-opted this biological activity for their own benefit (Smilanich et al., 2016). Buckeyes, like many other herbivores, sequester specific compounds from their host plants, which can provide a measure of defense against some predators (Dyer and Bowers, 1996; Nishida, 2002). Prior studies found that buckeyes will sequester IGs proportional to the concentration found in the leaves (Theodoratus and Bowers, 1999), thus it is possible that sequestering high concentrations of IGs interferes with viral replication. However, if this were the case, we would have expected to see a difference in virus abundance between individuals feeding on the two host plants, which we did not. Nonetheless, Richardson et al. (2015) found that bumblebees (*Bombus impatiens*) had significantly reduced parasite loads of *Crithidia bombi* when consuming nectar containing the iridoid glycoside, catalpol. Catalpol is found in *P. lanceolata*, but not in *P. major*, thus, our results support that this compound may protect insects during viral infection, though the mechanism remains unknown. In addition, aucubin and other iridoid glycosides show strong anti-inflammatory activity in mice (Recio et al., 1994), thus consuming a diet with high aucubin concentration, as found in *P. lanceolata*, could mitigate immunopathology, allowing higher survival. The results found here support a growing body of evidence showing that host plant chemistry is an important factor influencing survival when insects are attacked by pathogens (Singer et al., 2009; Smilanich et al., 2011; Gowler et al., 2015).

4.2. Immune response

4.2.1. Host plant

To take a deeper look at how consumption of IGs may enhance survival against pathogens, we quantified aspects of the immune response in the buckeye caterpillar by measuring phenoloxidase activity and hemocyte abundance when infected and uninfected individuals were reared on *P. lanceolata* or *P. major*. Prior results with buckeyes suggest that sequestering high concentrations of IGs detrimentally affects the herbivore immune response through reducing the melanization of a foreign object (Smilanich et al., 2009a). Here, we found that PO activity as measured by the slope of the kinetic reaction was not significantly affected by host plant treatment.

When PO responsiveness was measured, individuals reared on *P. lanceolata* had a higher frequency of response (a 2-fold increase in enzyme responsiveness). One possible explanation for this result is that *P.*

major is producing a compound or suite of compounds that inhibit phenoloxidase activity. Other studies have identified inhibitors of insect phenoloxidase. For example, Eleftherianos et al. (2007) found that an antibiotic produced by the insect bacterial pathogen, *Photorhabdus luminescens*, suppressed the host immune response in *Manduca sexta* by inhibiting phenoloxidase activity. Thus, it is possible that compounds associated with *P. major* could inhibit phenoloxidase, resulting in the lack of response from these individuals. Nonetheless, some individuals (27%) still exhibited a response. This could be due to intraspecific variation in concentrations of the putative inhibiting compound from the host plant, or simply genetic variation between buckeye individuals. Within the individuals that did respond, there was no difference between host plants, indicating that once individuals responded, the phenoloxidase activity was similar. In the event of an infection, the consequences of not responding could have dire consequences, leading to lower fitness in infected individuals, or zero fitness if the infection results in death before reproduction. However, it is worth pointing out that the PO assay has suffered from inconsistent results in the literature (Gonzalez-Santoyo and Cordoba-Aguilar, 2012; Phillips and Clark, 2017), thus it is possible that our results do not reflect the enzyme and immune capability of individuals on different plant diets, but problems and errors with the assay itself. The fact that the PO activity as measured by slope and enzyme responsiveness did not predict survival of JcDNV infected individuals provides some support that the assay may have problems with predictability, or that PO is not directly involved in defense against the JcDNV.

4.2.2. Infection

Infection with JcDNV had a negative effect on the activation of the phenoloxidase response. This effect was measured as a dichotomous response with JcDNV infected individuals responding less frequently than control individuals. In addition, we found that the mean hemocyte count was significantly reduced in individuals infected with the virus. While research suggests that the release of the phenoloxidase enzymes is dependent on cell lysis (Cerenius and Soderhall, 2004; Cerenius et al., 2008), the reduction in hemocyte cells found here may be better explained as a direct result of the JcDNV infection alone since PO activity did not increase in the virus infected group. Similarly, Wan et al. (2015) found that viral infection induced hemocyte apoptosis (programmed cell death) in *Spodoptera exigua* larvae, and Yaegashi et al. (1999) showed that the parvovirus (the family to which the JcDNV belongs), B19, is cytotoxic to human red blood cells, inducing apoptosis. Thus, it is possible that the JcDNV is reducing hemocyte cell abundance by the same mechanism.

4.3. Developmental data

Our results from the developmental data suggest that higher survival in buckeye larvae inoculated with the virus is facilitated through faster development. To elaborate, we found that post-inoculation, individuals in the viral treatment group developed faster than individuals in the uninfected control group (post-inoculation development time was standardized for both groups). On average, infected individuals developed four days faster than the uninfected group post-inoculation (infected mean = 19.6 days; uninfected mean = 23.6 days), an effect size of 1.29. For comparison, an effect size of 0.67 (Smilanich et al., 2009a) and 0.49 (current study) was found when comparing development time between buckeyes feeding on two different host plants, *P. lanceolata* and *P. major*. These results prompt the question of whether faster development is advantageous for the infected individual. One possibility is that by quickening development time to pupation, the individual can in a sense “out-run” the effects of the virus. In a prior study with the JcDNV, Mutuel et al. (2010) showed that symptoms of the virus peak at 4 days post-inoculation. If infected individuals can develop quickly, then they may avoid succumbing to the virus if viral replication is life stage dependent and can only successfully infect larval stage individuals.

Considering that the infected individuals had a depressed immune response, as measured by decreased hemocyte cell counts and PO response activation, it is also possible that virus-laden caterpillars allocate resources to faster development rather than investing in an immune response. Since it is resource costly to mount an immune response, individuals may instead invest in faster growth rates to reach sexual maturity and escape the virus. In a natural setting, the effectiveness of faster development would depend upon precisely when the individual contracted the virus. If infection occurred during early instars, then quickening development may not be enough to escape succumbing to the symptoms of the virus. In contrast, other studies have demonstrated developmental retardation when insects are infected with a granulovirus and an entomopoxvirus (Nakai et al., 2002; Nakai et al., 2016), thus it appears that our results are unique for investigations of insect-viral interactions.

4.4. Larval gut microbiome

Independent of the effects of viral infection and host plant, there was a significant slowing of larval development time when egg microbes were reduced via washing. In herbivorous insects, gut endosymbionts can facilitate nutrient provisioning by breaking down tough cellulose tissue (Anand et al., 2010), or by providing limiting resources like nitrogen (Cook and Davidson 2006). In *Drosophila melanogaster*, removal of the commensal bacterium, *Acetobacter pomporum*, slowed development and growth of larvae (Shin et al., 2011). Additionally, removal of maternally provisioned bacteria from stinkbug eggs (*Megacopta punctatissima*) reduces nymphal growth rate and development time (Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006). The concurrence of our results with those from previous studies suggests a plausible beneficial role of egg microbial communities on arthropods. Although used successfully in previous experiments (Hail et al. 2012), the technique for removing eggs microbes by washing with bleach and alcohol may have had detrimental effects on development aside from the loss of microbes. It is also worth noting that we did not test the egg surface for microbes, thus the identity of these microbes is unknown.

A reduction in microbial diversity was associated with JcDNV infection or larvae that were reared *P. major*. The influence of diet on microbial diversity could be due to a variety of factors. The simplest explanation is that the two plants (*P. lanceolata* and *P. major*) have differing microbiomes, and thus the differences that we found in the caterpillar gut microbiome are a direct product of host plant differences. It is also possible that phytochemical differences between host taxa could alter gut chemistry (Mason et al., 2014). Along these same lines, since buckeyes sequester iridoid glycosides at very high concentrations (Bowers and Collinge, 1992), it is possible that having a rich assortment of microbes can ameliorate the negative side effects of high sequestration or in fact facilitate sequestration (Mason et al., 2014).

5. Conclusion

The goal of this study was to investigate the complex relationships between the immune response, plant secondary chemistry, and a pathogen of a specialist caterpillar. We found that even though PO responsiveness was higher on the high IG plant, the kinetic activity of the enzyme did not differ between caterpillars reared on the two host plants. However, development time, and survival were all enhanced on *Plantago lanceolata*. Even though prior studies have shown an immune cost, via encapsulation (Smilanich et al., 2009a) to feeding on this host plant, when larvae are infected by JcDNV, the consumption of *P. lanceolata* provides a performance benefit. Future studies investigating the role of immunity and infection in host plant use should consider pathogen prevalence in field conditions, and subsequent impacts on host plant preference.

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Conflict of interest

The authors state that there is no conflict of interest.

Author contributions

AMS conceived and designed the experiments. TCL, LD, and JH performed the experiments. AMS, LAD, JGH, and MBT analyzed the data. AMS and TCL wrote the manuscript; all other authors provided editorial advice.

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