University of Nevada, Reno

The Causes and Consequences of Variation in the Insect Immune Response

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in Biology

by

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ABSTRACT

Plants produce an array of secondary metabolites that play an important ecological role in defense against herbivores in addition to having varied effects on herbivore natural enemies. Here, I investigate the complex interactions between diet, the immune response, and microbiota. In my first experiment, I asked whether plant chemistry and the presence of egg microbes affect the immune response of a specialist herbivore and its resistance to viral attack. Common buckeye larvae (*Junonia coenia*) were inoculated with a densovirus (JcDNV) and reared on two host plant species (*Plantago lanceolata* and *Plantago major*) that differ in their composition and concentration of iridoid glycosides. In addition, a subset of eggs were surface sterilized to investigate whether microbes on the egg's surface contribute to viral resistance. Survivorship, development time, pupal weight, hemocyte counts, and a colormetric assay of phenoloxidase (PO) enzyme activity were measured to identify what role plant chemistry and microbes play on the outcome of infection by a pathogen, *Junonia coenia* densovirus. I found that individuals exposed to the virus did not have significantly lower PO activity compared to the unexposed individuals. Survival was significantly higher in individuals feeding on high iridoid diets. Individuals reared from surface sterilized eggs suffered higher mortality from the virus than individuals that were not sterilized, indicating beneficial egg microbes may interfere with viral inoculation success. Individuals feeding on *P. lanceolata* had significantly larger pupal mass and increased development time as did those larvae that were exposed to the virus. In summary, these results suggest that plant chemistry and egg microbes play an important role in defense against viral enemies and herbivore performance.

Insect herbivores represent a model system for investigating complex tri-trophic interactions between their parasitoid counterparts, the insect immune response, and plant defense. The selective pressures from natural enemies and secondary plant metabolites play a key role in driving narrow herbivore diet breadth. In parallel, plants utilize a variety of chemical defense strategies to deter and reduce the fitness of natural enemies via direct and indirect mechanisms. In my second study, I investigated how intraspecific variation of amides in *P. cenocladum* affect the immune response of associated specialist neotropical caterpillars, *Eois apyraria* and *Eois nympha* (Lepidoptera: Geometridae). This plant is defended by colonies of *Phiedole bicornis* ants that live in the stems and petioles. When the ants are not present *P. cenocladum* produces high concentrations of three amides: piplartine, cenocladamide, and $4'$-desmethylpiplartine. I hypothesized that intraspecific variation of plant chemistry would affect tri-trophic interactions. Specifically, I predicted there would be a positive affecting parasitoid success through the negative effects on the caterpillar immune response. Phenoloxidase activity was measured from 65 *Eois* caterpillars that were collected from 18 sites across the La Selva Biological station in Costa Rica. Abiotic and biotic factors such as light availability and ant-plant mutualisms that may influence chemotype-mediated effects on the caterpillar immune response were also explored. I concluded that intraspecific chemical variation is influenced by light availability and the presence/absence of ant mutualists. Overall, the immune response of *Eois* caterpillars was not affected by feeding on host plants that varied in their concentration of *Piper* amides.

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CHAPTER 1

The Effects of Plant Chemistry, Egg Microbes, and a Densovirus on the Immune Response of a Specialist Caterpillar

INTRODUCTION

Specialization in resource use is hypothesized to be one important driver of biodiversity (Janzen 1970, Forister et al. 2012). In particular, there is accumulating evidence that insect herbivore diversification is attributed to host plant specialization and host plant shifts (Dyer et al. 2007, Hardy and Otto 2014). Investigating the driving forces that cause host shifts and specialization has been a prominent area of study in ecology and evolution. Studies have found the primary drivers of specialization to be two-fold, namely plant secondary metabolites and natural enemies (Brower 1958, Fraenkel 1959, Ehrlich and Raven 1964, Bernays and Graham 1988). Herbivores can be diet constrained due to plant secondary metabolites acting as feeding stimulants, deterrents, and oviposition stimulants (Bowers 1984, Pereyra1 and Bowers 1988), and in some cases protecting the herbivore through sequestration (Duffey 1980, Dyer and Bowers 1996, Nishida 2002). In the latter case, natural enemies also play a role in narrowing herbivore diet breadth (Smilanich et al. 2009a), where evidence suggests that specialist herbivores may be chemically protected from certain natural enemies, but are at greater risk of attack by parasitoids (Dyer 1995, Gentry and Dyer 2002). Variation on this intriguing intersection between plant secondary chemistry and predators is most likely the rule rather than the exception. Here, I focus on the insect immune response as one possible mediator between plant chemistry and natural enemies that drives herbivore host plant use.

Using the immune response as a mechanism for understanding life history traits and species interactions is one aim for studies centered in ecoimmunology (Zuk and Stoehr 2002, Martin et al. 2011). Since the immune response is a physiological process

that protects the organism from possible death by natural enemies, it is relevant on both ecological and evolutionary timescales, and thus may provide information on why herbivores specialize on particular host plants. For example, if the immune response is enhanced on certain host plants, then the herbivore is better protected from natural enemies and more likely to survive. In this hypothetical scenario, the immune-enhancing host plant is optimal for the herbivore and would be favored by natural selection. Alternatively, certain host plants may be detrimental to the immune response, and thus could deter herbivores from using them. In either case, the effect that the plant has on the insect immune response potentially influences whether or not it is incorporated into, or excluded from, the diet breadth of the herbivore. Furthermore, if the secondary chemistry of the host plant plays a role in enhancing or compromising the immune response, then plant chemistry would be expected to dramatically influence interactions between herbivores and their natural enemies.

The handful of studies that have investigated the effects of plant chemistry on the herbivore immune response have found the effects range from positive to negative with no clear pattern emerging (Lampert and Bowers 2014, 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 enormous variety of compounds produced by plants, their many different modes of action, and the diverse ability of herbivores to detoxify and/or sequester plant secondary metabolites (Smilanich et al. 2009a, Smilanich et al. 2016). For example, in a prior study Smilanich et al (2009) found that specialist buckeye caterpillars (*Junonia coenia,* Nymphalidae: Lepidoptera) suffered a depressed immune response when feeding on diets with a high concentration

of iridoid glycosides. Interestingly, buckeyes will sequester iridoid glycosides from their diet in proportion to the amount that it is found in the plant, such that the more that is present, the more they will sequester (Bowers and Collinge 1992). An obvious question that emerges is why these caterpillars would specialize on a plant and sequester the secondary metabolites if the metabolites are harmful to their immune response.

In addition to understanding the effects of plant chemistry on the immune response and survival against viral infection, I was also interested in the effects of the maternal microbial community on these responses. Recent investigations on the insect gut microbiome have provided evidence for positive effects on insects by aiding in digestion, ameliorating negative effects of secondary metabolites (Mason et al. 2014) and possibly positive interactions with the immune response (Broderick et al. 2004, Freitak et al. 2014). For example, Freitak et al. (2014) found that the progeny of female *Galleria mellonella* (Pyralidae: Lepidoptera) that had been exposed to non-pathogenic bacteria showed an increase in several measures of their immune response, including differential expression of immunity-related genes. In this case, the effects on the immune response were manifested through the bacteria priming the progeny for a possible attack.

Here, I explore whether maternal microbes on the egg surface benefit progeny by protecting them against the *Junonia coenia* densovirus (JcDNV), a nonenveloped, single stranded DNA virus. Insect densoviruses are highly virulent, spreading horizontally through populations via oral inoculation (Rivers and Longworth 1972). When infected with JcDNV, caterpillars experience oxidative stress and molting failure (Mutuel et al. 2010).

To test the effects of iridoid glycosides on the immune response and survival against the JcDNV, inoculated individuals 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 maternally-deposited egg microbes on the immune response, eggs were surface sterilized to remove any microbes. Given our prior knowledge of the interaction between iridoid glycosides and the immune response in buckeyes, I predicted that individuals consuming high amounts of iridoid glycosides would be immunosuppressed and more susceptible to the virus. I also predicted that individuals that lost their egg microbes would be more susceptible to the virus and would have a weaker immune response. I investigate this question by inoculating buckeye larvae with the *Junonia coenia* densovirus (JcDNV) and measuring their immune response, survival, and development on two different host plants that vary in concentrations of iridoid glycosides.

METHODS

Study System

The common buckeye (*J. coenia*) caterpillar specializes on various iridoid glycoside, a class of monoterpene derived secondary metabolites, producing plants within six families (Bowers, 1984). In this study we focused on two common host plants of *J. coenia* that differ in their iridoid glycoside (IG) composition, *Plantago major* and *Plantago lanceolata*. *Plantago major* (Plantaginaceae) contains low concentrations of IG aucubin (no catalpol) and *P. lanceolata* contains higher concentrations of aucubin and

low concentrations of another IG catalpol (Theodoratus and Bowers 1999). *J. coenia* sequester high levels of these IGs in the hemolymph where they are metabolized prior to pupation (Bowers & Collinge 1992).

     Junonia ceonia 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 maintained in the laboratory for two generations prior to this study and reared on an artificial diet with dried *P. lanceolata* leaves added (Smilanich et al. 2009). For this experiment, *Junonia 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 this experiment were reared on diets of *P. major* and *P. lanceolata* leaves that were collected from areas around the University of Nevada, Reno.

Overview of experiments

     I set out to measure the effects that host plant, gut microbial status, and viral infection have on the insect immune response of the *J. coenia* caterpillar. Buckeye larvae were reared on two host plant species, *P. major* (low IG) and *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 when infected with the *Junonia coenia* densovirus. A colony of 150 individuals was split into larval cohorts of 30 caterpillars which were fed a 10 mm leaf disk of *P. major* containing a 1ul drop of isolated *Junonia coenia* densovirus (stock dilution 10^{13}) in dilutions of 10^3, 10^5, 10^7, 10^9, 10^{11} particles per microliter concentration. Larvae were permitted to consume the entire leaf disk for 24

hours 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 per microliter concentration.

After determining the LD_{50}, for our experiment, individuals were randomly assigned to diets containing different levels of IGs (*P. major* and *P. lanceolata*; N= 160 per host plant). A subset of eggs were surface sterilized to remove the presence of maternal microbes prior to viral inoculation of the JcDNV virus at the 4th larval instar. As shown in Figure 1, each host plant group was separated into four treatments (N=40 per treatment): Virus (+)/Microbe (+), Virus (+)/Microbe (-), Virus (-)/Microbe (+), Virus (-)/Microbe (-). A follow up experiment was conducted replicating treatment groups from our initial experiment. A quantitative analysis of viral replication post-inoculation was measured using qPCR.

Experimental Design

To remove any maternally transmitted microbes, I surfaced sterilized eggs using a combination of ethanol, bleach, and deionized water, as outlined in Hail et al. (2012). Eggs that had been oviposited on either *P. major* or *P. lanceolata* were washed three times in 70% ethanol, followed by a wash in 10% bleach, and were then rinsed three times in deionized water. Eggs were left to air dry in incubators until hatching.

Upon hatching, *Junonia coenia* caterpillars were systematically assigned to either *P. lanceolata* or *P. major* (Experiment 1, N=160; Experiment 2, N=240). Caterpillars fed on *P. major*, containing low concentrations of aucubin (0.2 -1% dry weight) (Barton and Bowers 2006) or *P. lanceolata*, primarily containing high concentrations aucubin and

catalpol (5-12% dry weight) (Bowers and Stamp 1992). At the 4th instar, half of the larvae from each treatment group (*P. lanceolata* or *P. major*; wash or unwashed) were inoculated with JcDNV. Caterpillars feeding on each plant group were provided a 10 mm leaf disk containing a 1ul drop of isolated JcDNV (10^{11} viral particles per microliter concentration). Larvae were permitted to consume the entire leaf disk for 24 hours 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. Twenty individuals from each treatment group were used to collect hemolymph samples 4 days post inoculation. A colormetric assay of hemolymph phenoloxidase (PO) activity was measured spectrophotometrically.

Development time, pupal weight, and survival were used as fitness measurements. Development time was measured from date of egg hatching to date of pupation. All pupae were weighed individually and any deformities were noted. Survival was dependent on successful pupation and eclosion into an adult. Larvae that became leaky in nature (indicative of lethal infection) or that experienced partial pupation were determined to have not survived the JcDNV virus.

Immune Assays

Melanization (synthesis and deposition of melanin) is an effective defense response against invaders such as parasatoids, parasites, and various pathogens (Smilanich et al. 2009a) . The phenoloxidase enzyme (PO) is an important indicator of immune status in invertebrates (Gonzalez-Santoyo and Cordoba-Aguilar 2012) and is responsible for the activation of the melanization cascade. For these experiments, a 10 µl

hemolymph sample was taken from each individual by piercing the cuticle of the A1 abdominal segment with a fine needle. The hemolymph was added to 500 µl of ice-cold phosphate-buffered saline in a plastic Eppendorf tube and vortexed. One sample of 100 µl of PBS-bound hemolymph (Standing PO) and one sample of 100 µl PBS-bound hemolymph plus 10 µl chymotrypsin were incubated for 20 minutes on ice. During experiment 1, 200 µl of L-DOPA (0.118g L-DOPA, 30ml deionized water) was used as our substrate and added to each PBS-bound hemolymph sample. Standing and total PO activity was measured using an iMark Microplate Absorbance Reader (Bio-Rad). PO activity was measured every 30s and expressed as the slope of the line over 45 minutes at 490 nm.

In addition to measuring phenoloxidase activity, hemocyte counts can be used as an alternative measure of immune status. Hemolymph samples that contained 4 µl of PBS-bound hemolymph, 12 µl anticoagulant (0.684g EDTA, 0.346g citric acid, 180 ml PBS), and 16 µl glycerol were stored at ~18°C. To perform the cell counts, the cell suspension was diluted with 0.5 µl of Trypan Blue dye. 10 µl of each sample was observed under a light microscope using a Neubauer hemocytometer. I took the average of two cell counts per sample.

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. Total DNA extraction was performed using 50 mg of frozen caterpillar-midsection using Qiagen DNeasy Blood and Tissue Kit (Qiagen #69506) with Supplementary Protocol: Purification of Total DNA

from Insects using the DNeasy Blood and Tissue Kit. All of the DNA samples were normalized to 10 ng/ul and qPCR was performed using (0.2uM) JcDNV-specific primers (Wang et al. 2013) for VP4, and (0.2uM) CO1 primers HCO/LCO were used as a housekeeping gene (Folmer, et. al. 1994), and iTaq Universal SYBR Green Supermix on a BioRad CFX96 Optics Module with C1000 Thermal Cycler. This was run at 95°C for 5 minutes followed by 45 cycles under the following conditions: 95°C for 10 seconds, 60°C for 15 seconds, 72°C for 15 seconds. A melt curve was ran following amplification, and the viral titer (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 (Ct) protocol as outlined in Schmittgen and Livak (2008). Replicates per sample were averaged and Ct values represent the point at which the reporter dye in PCR reaction crosses an arbitrary threshold during the exponential phase, thus, the lower the Ct value, the more virus that is present.

Analyses

All analyses were performed in SAS 9.4 (SAS Institute) using the GLM procedure for ANOVA analyses and the CALIS procedure for path analysis. The immune response variables from the PO assay (rate, maximum rate, and integral) were analyzed for normality of variance. The residuals from rate and maximum rate were not normally distributed and transformations were unable to normalize the data. Thus, I used the integral variable (i.e. the area under the curve of the reaction line) for all analyses since these data were normally distributed after log transformation. Integral data were analyzed using a three-way ANOVA to test for significant interactions between the three

independent variables (host, virus, and wash). None of the interactions were significant (at $P < 0.05$), thus they were removed from the model. The results presented here show the main effects only.

Survivorship data were analyzed using log-linear models with host (*P. major* or *P. lanceolata*), virus (yes or no), and wash (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 analysis. Both pupal weight and development time were normally distributed. Pupal weight was measured one to two days after pupation. Pupal weight was analyzed using a three-way ANOVA with host, wash, and virus as the independent variables. Development time (1/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 of virus was set at beginning of fourth instar to pupation data for all individuals including those not receiving the virus. Total development time and development time post inoculation were analyzed using a three-way ANOVA with host, wash, and virus as the independent variables.

Quantitative PCR residuals were normally distributed. Quantitative PCR data were analyzed using a two-way ANOVA with host plant and wash as the independent variables.

Three *a priori* causal hypothesis models were tested using the immune data and fitness data as response variables, and infection status as the independent variable. In

SAS, I used the Calis (Covariance Analysis of Linear Structural Equations) Procedure to test goodness of fit of specific *a priori* causal hypotheses, examining direct and indirect relationships between infection status, immune response, and fitness variables. Data for path analyses were from experiment 1. All models were specified based on our predictions; specification, estimation, and tests of model fit followed recommendations of Ullman (1996) and Shipley (2000, and references therein). Maximum likelihood (ML) methods were used for estimation, and parameter vectors were estimated iteratively by a nonlinear optimization algorithm to optimize a goodness of fit function. Chi-squares were calculated for the ML goodness of fit to assess the fit of the models to the data; P-values of greater than 0.05 are considered to indicate a good fit (Ullman 1996). When appropriate, models were statistically compared to one another by subtracting the chi-squares and degrees of freedom for the two models being compared (P < 0.05 indicates a significantly better fit for the model with lower chi-square value; Ullman 1996).

RESULTS

Immune response

Host plant identity had no significant effect on the phenoloxidase activity ($F_{[1,293]}= 1.29$, P = 0.256, N = 294; Figure 2). Likewise, infection status (virus or no virus) and microbe status (wash or no wash) had no significant effect on PO activity (Infection: $F_{[1,293]} = 0.04$, P = 0.8440, N = 294; Microbe: ($F_{[1,293]} = 0.30$, P = 0.5817, N = 294). However, for hemocyte counts, individuals that were inoculated with the virus had a significantly reduced total hemocyte count ($F_{[1,143]} = 6.91$, P = 0.0095, N = 147, Figure

3), while host plant identity and microbe status had no significant effect (Host plant: $F_{[1,143]} = 0.99$, $P = 0.3219$, $N = 147$; Microbe: $F_{[1,143]} = 0.00$, $P = 0.950$, $N = 147$).

Survival

Using the parsimonious model, host plant, virus, and wash were all significant predictors of survival, and based upon the standardized parameter estimates (Table 1), virus was the best predictor of survival, followed by wash, and then host. The optimal conditions for survival were no virus, no wash, and feeding on *P. lanceolata* (Table 1). Analysis of inoculated individuals showed that those feeding on *P. lanceolata* had significantly higher survivorship compared to individuals feeding on *P. major* (53% *P. lanceolata* vs. 30% *P. major*, $\chi 2 = 4.5$, $P = 0.033$, Figure 4).

Fitness

Pupal weight was significantly higher on *P. lanceolata* compared to *P. major* ($F_{[1,115]} = 5.57$, $P = 0.045$, $N = 116$). Wash and virus did not have significant effects on pupal weight (wash: $F_{[1,112]} = 0.10$, $P = 0.7567$, $N = 116$; virus: $F_{[1,115]} = 2.10$, $P = 0.1506$, $N = 116$). Overall, the development time from egg emergence to pupal date was significantly faster when individuals were reared on *P. lanceolata* ($F_{[1,115]} = 6.32$, $P = 0.0134$, $N = 116$), when they were not washed as eggs $F_{[1,115]} = 15.39$, $P = 0.0002$, $N = 116$). None of the interactions were significant and are not reported here. The analysis of developmental time post inoculation (or starting at fourth instar for controls) showed that individuals feeding on *P. lanceolata* still had a significantly faster development time compared to individuals on *P. major* ($F_{[1,114]} = 53.11$, $P < 0.0001$, $N = 115$; Figure 5). Having the virus significantly increased developmental time post inoculation ($F_{[1,114]} =$

53.56, P < 0.0001, N = 115), and there was a significant interaction between host plant

and viral status with infected individuals on *P. lanceolata* developing fastest ($F_{[1,114]}$ =

11.06, P = 0.0012, N = 115).

Viral quantification

      There was no effect of host plant ($F_{[1,59]}$ = 0.61, P = 0.439, N = 60) or egg wash

$F_{[1,59]}$ = 0.23, P = 0.632, N = 60) on the quantity of JcDNV in larvae.

Path Analysis

      The path analysis model that best fit the data (χ^2 = 1.03, df = 2, P = 0.595, Figure

6) included the pathways showing that viral infection had a significant positive effect on

larval development time post inoculation (standardized parameter estimate = 0.55, P <

0.0001), a significant negative effect on pupal weight (standardized parameter estimate =

-0.15, P = 0.0221), and a weak negative effect on the immune response (standardized

parameter estimate = -0.07, P = 0.2755). These data included caterpillars reared on both

Plantago major and *Plantago lanceolata*. This model also showed that development time

post inoculation had a significant positive effect on the immune response (standardized

parameter estimate = -0.05, P < 0.0001).

DISCUSSION

      Here, we provide evidence that the immune response influences insect diet

breadth. We tested the effects of iridoid glycosides on the immune response and survival

against a pathogen and found that buckeye larvae infected with the *Junonia coenia*

densovirus have differential survivorship that is dependent upon host plant species. Infected larvae were more likely to survive when feeding 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 functionality for their own benefit (Smilanich et al. 2016). Indeed, many herbivores like the buckeye sequester specific compounds from their host plants thereby becoming defended against predators (Dyer and Bowers 1996, Nishida 2002). Prior studies also 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 iridoid glycosides interferes with viral activity. Defensive molecules and pathogen resistance in herbivores have been demonstrated in other insect herbivore systems. For example, recent studies with the monarch (*Danaus plexippus*) found that parasitized individuals had a longer adult lifespan when they consumed high concentrations of cardenolides from their host plant as larvae (Gowler et al. 2015). In another study with bumblebees (*Bombus impatiens*), plant secondary metabolites in nectar significantly reduced parasite load leading to higher survival (Richardson et al. 2015). 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.

Immune response

To take a deeper look at how host plant identity might be enhancing survival against pathogens, I quantified the strength of the immune response in the buckeye caterpillar by measuring phenoloxidase activity and hemocyte abundance when infected and uninfected individuals were feeding on *P. lanceolata* and *P. major*. Prior results with buckeyes suggest that sequestering high concentrations of iridoid glycosides detrimentally affects the herbivore immune response through reducing the melanization of a foreign body (Smilanich et al. 2009a). However, in this study I did not find a difference in the immune response as measured by phenoloxidase activity. One main difference between the current study and Smilanich et al (2009) was the method by which the melanization response was measured. In the prior study, melanization was evaluated by measuring the response to beads injected into the caterpillar's hemocoel, and in this study, the activity of the phenoloxidase enzyme and hemocyte count was used to assess immunity. Bead melanization and PO activity are two measures that occur at different points on the melanization cascade; phenoloxidase at the beginning, catalyzing the cascade, and melanin at the end of the cascade as the product (Gonzalez-Santoyo and Cordoba-Aguilar 2012). This result reinforces the need to measure multiple parameters of the immune response instead of focusing on one measure as they can give different results (Adamo 2004). Altogether, a puzzling pattern emerges showing that even though the immune response as measured by bead melanization is depressed when buckeyes sequester and feed on plants with high concentrations of iridoid glycosides (Smilanich et al. 2009), here I found that there is no difference in the immune response as measure by PO activity between individuals fed high and low iridoid glycoside plants. In addition, survival

against the virus is highest when individuals fed on the high iridoid glycoside plant. Nonetheless, I found that the abundance of hemocyte cells was significantly reduced in individuals infected with the virus. While research suggests that the release of the phenoloxidase enzymes is dependent on cell lysis (Cornelis, L. and Soderhall 2004), a reduction in hemocyte cells in our system may be better explained as a direct result of the virus. For example, Wan et al. (2015) found that viral infection induced hemocyte apoptosis (programmed cell death) in *Spodoptera exigua* larvae. These results lead us to infer that the activity of the phenoloxidase enzyme may not be playing a large role in the defense of the buckeye caterpillar against the JcDNV.

Developmental data

 I used real-time PCR to understand whether host plant chemistry or egg microbes would have a significant effect on viral replication of JcDNV in buckeye larvae. I found no difference of viral quantification between individuals feeding on *P. lanceolata* and *P. major*, or between individuals washed as eggs and those with intact egg microbes. While I was not able to measure survival on individuals used for viral quantification, our results from the developmental data suggest that higher survival in buckeye larvae is facilitated through faster development.

 To elaborate, I found that post-infection, individuals in the viral treatment group developed faster than individuals in the control group that did not receive the virus (post-inoculation time was standardized for both groups). Moreover, there was a significant interaction between development time and host plant, showing that feeding on *P. lanceolata* increased development time compared to those feeding on *P. major.* These

results pose two questions: (1) what is driving faster development when larvae are infected? and (2) what is driving faster development time on *P. lanceolata*? One scenario is that by decreasing 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 individuals can develop quickly, then they may avoid succumbing to the virus if viral replication is life cycle dependent and can only successfully infect larval stage individuals. Faster development on *P. lanceolata* has been found before with *J. coenia* larvae when compared to *P. major* (Smilanich et al. 2009). These data clearly show that being infected with the virus induces physiological changes that lead to faster development time, and since post-inoculation development time was measured up to pupal date, these data only included individuals that survived to pupation. Thus, there is a strong correlation between faster development and surviving the virus. Of the forty individuals included in the post-inoculation development data (i.e. those reaching pupation), only seven succumbed to the virus and did not survive to adulthood. Consequently, it appears that developing faster when infected is advantageous to the diseased individual and not necessarily advantageous for the virus, suggesting that this physiological change evolved in response to viral infection. In contrast, other studies have demonstrated developmental retardation when insects are infected with entomopathic viruses, thus it appears that the results here are unique for investigations of insect-viral interactions.

Microbial Data

Insects are inhabited by a diverse array of microorganisms which play key roles in the digestion of nutrients and secondary metabolites, defending against harmful pathogens, and aiding the limitation of parasite development (Engel and Moran 2013). However, the mechanisms of microbial acquisition vary widely from maternal bacterial transfer to transient bacteria obtained environmentally through host plant diet. I found that individuals that retained their egg microbes had a higher survival when infected with a virus. While the retention of the egg microbes showed no effect on the immune response in buckeyes, it is possible that they are functioning to defend against the virus through a more direct method such as interference with viral replication or a similar method of viral suppression. Similar studies have shown that symbiotic bacteria play an important role in the interference of vector borne diseases (Engel and Moran 2013). For example, antimicrobial phenolics produced by bacteria in the gut play a role in helping to protect the desert locust (*Schistocerca gregaria*) from pathogenic bacteria (Dillon and Charnley 2002). This has also been seen in the endosymbiont *Wolbachia*, which can directly interfere with pathogenic transmission and inhibit viral replication of dengue in the mosquito, *Aedes aegypti* (Moreira et al. 2009).

Conclusion

I set out to investigate herbivore specialization and the role of the immune response as potential mediators of plant chemistry and defense against natural enemies. Selection for a specific host plant and sequestration of secondary metabolites may elicit life-history trade offs when an insect herbivore is faced with harmful pathogens. While

no direct correlation was found between host plant use and the immune response, host plant did influence survival when infected with a virus. Our results imply that egg microbes are also key players for development and survival, where changes in development time is both dependent on host plant and infection status. I gather that the most favorable conditions for survival against a viral pathogen are retention of egg microbes and host plant, with *P. lanceolata* being the optimal diet. Future studies examining the role of diet breadth on the insect immune response should consider nutritional differences between host plants in addition to alternative hosts that do not produce iridoid glycosides, such as *Mimulus guttatus*. Furthermore, a comprehensive scan of all immunity vectors may help better determine the role microbial communities play on pathogens.

Chapter 2:

Intraspecific variation of piper amides and the insect immune response: Exploring the effects on tri-trophic interactions

INTRODUCTION

Plants utilize a broad array of defense strategies to both deter and reduce the fitness of herbivores, and in response insect herbivores have counter adapted and coevolved with their host plants (Ehrlich and Raven 1964) Many of these compounds facilitate avoidance of predation and parasitism (Dyer and Bowers 1996, Nishida 2002). These range from constitutive defenses, which are always present, to induced defenses that are initiated in response to herbivory (Strauss et al. 2002). Plant defenses can also mediate herbivore interactions with predators via indirect mechanisms that vary from nutritional quality to the production of toxic compounds (Kessler and Baldwin 2002, Strauss et al. 2002). Examples of indirect defense mechanisms against herbivores include the attraction of natural enemies via volatile emissions (Turlings et al. 1990, Dicke and van Loon 2000) or modifying existing plant structures as shelter for predators (Janzen 1966, Rehr et al. 1973). Kessler (2001) demonstrated that volatile plant emissions of *Nicotiana attenuata* as a response to herbivory caused an increase in the rate of predation on lepidopteran eggs. Plants also provide food bodies and shelter in the form of hollow thorns and stems for the enemies of herbivores such as ants (Agrawal and Rutter 1998). Examples of direct defenses include structural adaptations such as thickened cuticles that aid in herbivore deterrence and decrease feeding efficiency or the production of secondary metabolites (phenolics, terpenoids, alkaloids) which cause specialist herbivores to invest in detoxification efforts (Kessler and Baldwin 2002). Another example of an adaptive strategy plants utilize against herbivores is the production of multiple secondary compounds that allow the plant to produce greater toxicity at a decreased cost (Nelson and Kursar 1999). The effects of these plant toxins can be

antagonistic resulting in reduced toxicity or can provide higher activity by acting synergistically in lowering the total concentration of individual compounds (McKey 1979, Berenbaum et al. 1991, Hay et al. 1994, Nelson and Kursar 1999).

A more complex evolutionary response to herbivory is found in plants that possess multiple defenses as in the tropical shrub *Piper cenocladum* (Piperaceae). This plant relies on two distinct nitrogen-based defenses, specifically a class of compounds called *Piper* amides, in addition to food bodies that attract *Pheidole bicornis* (Formicidae: Myrmicinae*)* ants (Dyer et al. 2001). The ants take shelter in the petiole and hollowed stems, and in return protect the plant from invading herbivores. Plant -insect mutualisms can also play a role in intraspecific variation of plant chemistry, where significantly higher levels of amides have been found in *P. cenocladum* that do not house ants in the hollow petiole and stem (Dodson et al. 2000, Dyer et al. 2001). Similar studies have shown that the presence of ants can inhibit plants from producing any toxic secondary metabolites. Rehr et al. (1973) found that *Acacia* spp. that did not house mutualistic ants (*Pseudomyrmex*) produced leaves with cyanogenic glycosides, while species that hosted ant mutualists did not. In addition to defense via ant-plant mutualisms, plants also benefit from reduced herbivory by encouraging specialists to switch between individual plants that vary in their concentration of secondary metabolites (Moore et al. 2014). In response to this induced defense, herbivores that switch plants are in turn better able to dilute toxic secondary metabolites. Herbivore performance has been linked to intraspecific variation in *Combretum fragrans* plants, where *Chrysopsyche imparilis* caterpillars that switched regularly between individual plants that varied in their secondary chemistry, had increased growth and fecundity (Mody et al. 2007).

Phytochemical variation between plant species is common and chemical diversity between species appears to have effects on higher trophic levels (Richards et al. 2015). Specifically, diet specialization in herbivores has been demonstrated to increase parasitism rates (Dyer and Gentry 1999). For example, defensive secondary chemicals (*Piper* amides) produced in the plant family Piperaceae have been found to have negative effects on both generalist and specialist herbivores (Dyer et al. 2003). At high concentrations, these amides have toxic effects, decreasing development time (Dyer et al. 2003). Amides are suspected to cause interruptions in the immune response of *Eois* caterpillars, therefore increasing parasitoid success (Dyer et al. 2003, Richards et al. 2010, 2012). There is also an indirect positive effect of plant chemistry on parasitoids through plant chemical interference of the caterpillar immune response (Smilanich et al. 2009). Parallel to this work, I am interested in the effects of intraspecific chemical variation of *Piper* on upper trophic levels and how this may be mediated by ant mutualisms.

I tested how intraspecific variation in *Piper* amide concentration and composition (chemotype) affect the *Eois* immune response. I explored whether ant-plant mutualisms play a role in intraspecific variation of plant chemistry and the resultant effects on herbivore function. I hypothesize that plants with higher concentrations of amides will positively affect parasitoid success through the negative effects on the caterpillar immune response, thus having tri-trophic repercussions.

METHODS

Study System

The genus *Piper* (Piperaceae) is a dominant understory shrub common in lowland wet forests of Costa Rica (Burger 1971). The diversity of *Piper* makes it a model system for chemical ecologists to study plant-insect interactions. In this study I focused on two specialist lepidopterans, *Eois nympha* and *Eois apyraria* (Lepidoptera: Geometridae) that are commonly found on *Piper cenocladum.* This plant contains three amides in high concentrations: piplartine (0.58% dry mass), cenocladamide (0.33% dry mass), and 4'-desmethylpiplartine (0.45% dry mass) (Dodson et al. 2000). In addition to being chemically defended, this shrub is defended against herbivores by a mutualistic ant, *Pheidole bicornis* (Formicidae). When these ants are present lepidopteran populations decrease (Letourneau 1983).

Sample Collection and Rearing

Eois nympha and *Eois apyraria* caterpillars were collected from 65 individuals of *Piper cenocladum* from 18 sites at La Selva Biological Research Station in Costa Rica during June 2014. Caterpillars used in our experiment were collected in instars 1-4 and were housed at ambient conditions (mean monthly temperatures at La Selva are 24.7-27.1°C) in 2 oz. plastic cups containing the leaves of their host plant, *P. cenocladum*. Leaves (one from individual plant) were collected and air dried to measure the concentration of the three key amides. Canopy cover was measured for each individual plant using a convex spherical densitometer. The immune response of each individual was assessed at the 5th instar by piercing the cuticle of the A1 abdominal segment with a

fine needle to take a hemolymph sample. Hemolymph phenoloxidase activity was assayed spectrohotometrically (Thermos, Spectronic Helios UV Vis) with L-DOPA as the substrate. Hemolymph (4 µL) was added to 25 µl of ice-cold PBS in a plastic Eppendorf tube and vortexed. The PBS-bound hemolymph was combined with 7µl chymotrypsin (0.032g/25ml deionized water) and the mixture was incubated for 20 minutes. 0.9 µl of L-DOPA (0.2958g /100ml deionized water) was added to each PBS-bound hemolymph sample. PO activity was expressed as the slope of the line over 40 minutes at 490 nm (modified from Adamo 2004). Parasitoid success (presence/absence) was measured by rearing caterpillars on their host plants until they eclosed as adults or until a parasitoid emerged.

Quantitation of amides

Air dried leaves (0.3-1.0g) were ground/milled using liquid nitrogen and were extracted in 5ml 1:1 $MeOH/H_2O$ and 5ml $CHCl_3$, vortexed, sonicated for 30 minutes, and centrifuged for 5 minutes. The aqueous layer was partitioned from the organic methanol/chloroform layer. The aqueous partition was returned to the pellet of plant tissue, $CHCl_3$ (5 mL) was added and the extraction was repeated two times. The chloroform was removed from the combined organic layers using a stream of nitrogen at room temperature and a Visiprep SPE Vacuum Manifold (Supelco).

GC FID Protocol

The crude extract was redissolved in 2ml CH_2Cl_2, filtered, and 0.9ml was analyzed separately by GC/FID using commercially available piperine as an internal standard (809 ppm per sample). A series of pipleroxide, alkene, piplartine,

cenocladamide and 4'-desmethylpiplartine standard solutions in methylene chloride were prepared via 2-fold serial dilutions to yield stocks containing 11.9-760 ppm standards and 809 ppm piperene internal standard. All samples and standards were analyzed using Agilent 7980 A gas chromatograph with flame ionizaton detection, fitted with a 320 µm x 30.0 m HP-5 5% Phenyl Methyl Siloxane column (Agilent). The gas chromatography parameters were as follows: A 1 µL injection was split 30:1 with 3 pre-injection and 6 post-injection washes with methylene chloride and the inlet temperature was set to 280 °C with He gas flow of 50 mL/min at 7.54 psi. Carrier gas flow was constant at 1.5 mL/min with the column temperature starting at 50 °C for 1 min, then raising 25 °C/min to 325 °C over 5 min, and holding at 325 °C for the remaining 11 minutes of the run. The detector temperature was 300 °C with 35 mL/min H_2, 300 mL/min Air and 25 mL/min makeup gas (N_2). Data were collected and batch analyzed using ChemStation (Agilent B.04.02) by auto-integration of the resulting chromatograms and comparison of peak areas to that of calibration curves for pipleroxide (11.7 min), *Piper imperiale* alkene (11.3 min), piplartine (12.5 min), cenocladamide (13.3 min) and 4'-desmethylpiplartine (12.8 min) weighted against piperene (12.7 min) internal standard area ($r^2 > 0.99$).

Statistical Analysis

All analyses were performed using SAS software v.9.4 (SAS Institute, Cary, North Carolina). The residuals were normally distributed for all variables, thus no transformations were performed.

Amide Diversity

Amide diversity was calculated using Simpson's diversity index. Since all individuals contained the same three amides, I were interested in how the abundance (i.e. concentration) of each amide changed between individuals and whether the evenness (in relative abundance) of the amides affected immunity and parasitism. To test for an effect of the presence of ants on amide diversity, an ANOVA was used with ant status (present or absent) as the predictor variable and amide diversity as the response variable. I used linear regression to test for a relationship between amide diversity and the immune response as measured by the slope of the line during the first five minutes of the PO kinetic assay. Linear regression was also used to test for a relationship between amide diversity and light. ANOVA was used to test for a difference in the PO activity between the two caterpillar species. Logistic regression was used to test for an association between amide diversity and parasitism status (yes/no). Finally, I tested for an interaction between amide diversity, ant presence, light, and parasitism on the immune response using ANOVA.

Amide Concentration

ANOVA was used to test for an effect of the presence or absence of ants on the concentration of each amide individually. Linear regression was used to test for a relationship between amide concentrations and PO activity, and for a relationship between amide concentration and light, that was used as a covariate.

RESULTS

There was a significant difference in the diversity of amides between plants housing ants versus plants with no ants ($F_{[1,83]} = 5.89$, P = 0.0174, N = 84, Figure 1). On plants where ants were not present, a higher diversity of compounds was detected. There was no significant relationship between diversity of amides in *P. cenocladum* and the *Eois* immune response ($F_{[1,65]} = 2.31$, $r^2 = 0.03$, P = 0.1335, N = 66). No significant difference was found between the immune function of *Eois apyraria* and *Eois nympha* caterpillars ($F_{[1,71]}=0.33$, N=72, P=0.5681). In addition, I did not detect a significant effect of ant presence on the immune function of *Eois* caterpillars ($F_{[1,77]} = 0.01$, N = 78, P = 0.9303). The diversity of amides showed no significant effect on parasitoid success in *Eois* caterpillars ($\chi^2 = 0.3887$, df =1, p-value = 0.5330;17 parasitized, 96 not parasitized). There was no interactive effect of ants, light, amide diversity, and parasitism status on the immune response ($F_{[39, 59]} = 1.47$, N = 60, P = 0.1806).

The presence of ants had no effect on the concentration of piplartine ($F_{[1,85]} =1.99$, P = 0.1623 , N = 86). However the ant mutualists did have a significant effect on the concentration of both 4'-desmethylpiplartine ($F_{[1,85]} =12.03$, P = 0.0008, N = 86) and cenocladamide ($F_{[1,84]} = 11.90$, P = 0.0009, N = 85). When ants are absent the concentration of 4'-desmethylpiplartine increases and concentrations of cenocladamide decrease (Figure 2).

I did not detect a relationship between the diversity of amides and light availability ($F_{[1,79]} = 0.65$, $r^2 =0.0082$, df = 80, p-value = 0.4238, Figure 3). However, light availability did play a role in the concentrations of the individual amides piplartine, cenocladamide, and 4'-desmethylpiplartine. As light increases the

concentration decreases in both piplartine ($F_{[1,80]}$ =7.47, r^2= 0.0864, P =0.0077, N =81 , Figure 4) and cenocladamide ($F_{[1,79]}$ =6.78, r^2= 0.0799, P =0.0111 , N =80 , Figure 4), while light has no significant effect on the concentration of 4'-desmethylpiplartine ($F_{[1,80]}$ =3.27 , r^2=0.0397, P =0.0744 , N =81 , Figure 4)

DISCUSSION

Our goals were to understand how the abundance and composition of amides within *Piper cenocladum* might influence the immune response of two Eois species. We tested the hypothesis that intraspecific variation of individual plant chemotypes will affect tri-trophic interactions by positively affecting parasitoid success through the negative effects on the caterpillar immune response. In this study, biotic and abiotic factors such as light availability and ant-plant mutualisms that may influence chemotype-mediated effects on the caterpillar immune response were also explored. I found that the diversity of three amides (piplartine, cenocladamide, and 4'-desmethylpiplartine) found in *Piper cenocladum* varied greatly between plants housing ant mutualists as compared to those that did not. Similar to previous studies, *Piper* amides were found in greater concentration when ants were not present (Dodson et al. 2000, Dyer et al. 2001). Interestingly, when these compounds were analyzed individually it was found that concentrations of 4'-desmethylpiplartine increase and cenocladamide decreases. As posited by Dyer et al. (2003), it is possible that *Piper* is utilizing the toxic qualities of amides in addition to synergy as a defensive adaptation against a broad range of herbivores, specifically generalists (Dyer et al. 2004).

I considered the possible role light availability plays on variation of plant chemotypes and found that light does not have a significant effect on the chemical diversity of our three focal amides. In particular, the evenness (i.e. concentration of each compound per individual) of the three compounds was not affected. However, we found a decrease in the concentration of piplartine and cenocladamide with light. The results of this study bring into question how light availability could change how these shade tolerant plants allocate resources. One hypothesis that warrants examination is that greater canopy openings may cause these shade tolerant plants to reserve more energy for biomass and growth versus defensive chemistry and the production of amides. Alternatively, photoprotection in plants may be a mechanism for resource allocation, where excessive light availability can alter photosynthetic efficiency in plants (Niyogi 1999). This may suggest that variations in plant secondary chemistry are protecting the plant from photodamage and not from herbivory (Close and McArthur 2002).

Interestingly, the concentration of amides played no role in the immune response of *Eois* caterpillars and had no significant effect on parasitoid success in our study, possibly suggesting that these specialist herbivores have adapted to feed on these plants. Past studies on the synergistic and additive effects of *Piper* amides on *Eois* caterpillars have shown that specialist caterpillars experience increased parasitism rates when amides are working synergistically (Dyer et al. 2003, Richards et al. 2010). When these compounds are ingested alone, development time, larval mass, and pupal mass are affected by the consumption of cenocladamide but not piplartine (Dyer et al. 2003). However, my results were not consistent with the inference that plant chemistry indirectly effects parasitoid success via suppression of the immune response (Richards et

al. 2010). In contrast, an alternative explanation to Richards et al. (2010), is that parasitoid wasps are protected from the immune response of caterpillars by means of an immunosuppressive agents such as a polydnaviruses (PDVs). These obligate mutualist symbionts (PDVs) found in some parasitoids, suppress the immune response of their lepidopteran hosts (Stoltz and Guzo 1986) by preventing encapsulation (Lovallo et al. 2002). Thus, when the host immune system is compromised, parasitoid wasps would be more likely to successfully reproduce. In my study, this could imply that high levels of parasitism at this site have left the immune response of *Eois* caterpillars functioning at maximum efficiency. Therefore, when measuring the immune response, no variation was seen.

There has been little evidence linking parasitoid success and infection of PDVs to plant secondary metabolites. However, based on these patterns of changes, it should not be ruled out that intraspecific variation of *Piper* amides isn't having a tri-trophic effect on these specialist herbivores. It should be considered that only one immune parameter, phenoloxidase activity, was measured. Additionally, *Eois* caterpillars were collected in varying instars which could have contributed to both parasitoid success, where parasitoid wasps may have preference for smaller caterpillars in earlier instars (van Driesche 1988).

In conclusion, my work shows that intraspecific chemical variation does occur in a narrow geographic span (Glassmire et al. 2016) and can be mediated by ant mutualisms and light availability. However, changes in secondary chemistry were not shown to affect the third trophic level. There was a lack of evidence to support that intraspecific chemical variation of *Piper* amides positively affect parasitoid success through the negative effects on the caterpillar immune response. Further research to more definitively explain the

interaction between *Piper* amides, the immune response of *Eois* caterpillars, and

parasitoid wasps might focus on the impact pathogens such as viruses have on higher

trophic levels.

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TABLES: Chapter 1

Table 1. Using the parsimonious log-linear model. The optimal conditions for survival were no virus, no wash, and feeding on *P. lanceolata.* All were significant predictors.

Parameter	χ^2	Parameter Estimate	P-value
P. lanceolata (high IG)	4.59	0.314	0.032
No Virus	54.2	1.18	<0.0001
Maternal Microbes	10.07	0.474	0.0015

FIGURES: Chapter 1

Figure 1. Summary of experimental design where *Junonia coenia* larvae were reared on one of two host plants (*P. major* and *P. lanceolata*), inoculated with JcDNV, and either retained maternal microbes or had them removed.

Figure 2. Average rate of phenoloxidase (PO) activity of buckeye larvae when feeding on *P. lanceolata* and *P. major*. Host plant identity had no significant effect on the phenoloxidase activity ($F_{[1,293]}$= 1.29, P = 0.256, N = 294; means ± SE).

Figure 3. Hemocyte cell count of buckeye larvae when feeding on *P. lanceolata* (L) or P. major (M), when infected with a virus (V), or when maternal microbes are washed (W)) Individuals that were inoculated with the virus had a significantly reduced total hemocyte count ($F_{[1,143]}$ = 6.91, P = 0.0095, N = 147; means ± SE).

Figure 4. Survival frequency of buckeye larvae when feeding on *P. lanceolata* (L) or *P. major* (M), when infected with a virus (V), or when maternal microbes are washed (W). Analysis of inoculated individuals showed that those feeding on *P. lanceolata* had significantly higher survivorship compared to individuals feeding on *P. major* (53% *P. lanceolata* vs. 30% *P. major*, χ2 = 4.5, P = 0.033).

Figure 5. Average development time of buckeye larvae when feeding on *P. lanceolata* and *P. major*. The analysis of developmental time post inoculation (or starting at fourth instar for controls) showed that individuals feeding on *P. lanceolata* had a significantly faster development time compared to individuals on *P. major* ($F_{[1,114]}$ = 53.11, P < 0.0001, N = 115; means ± SE).
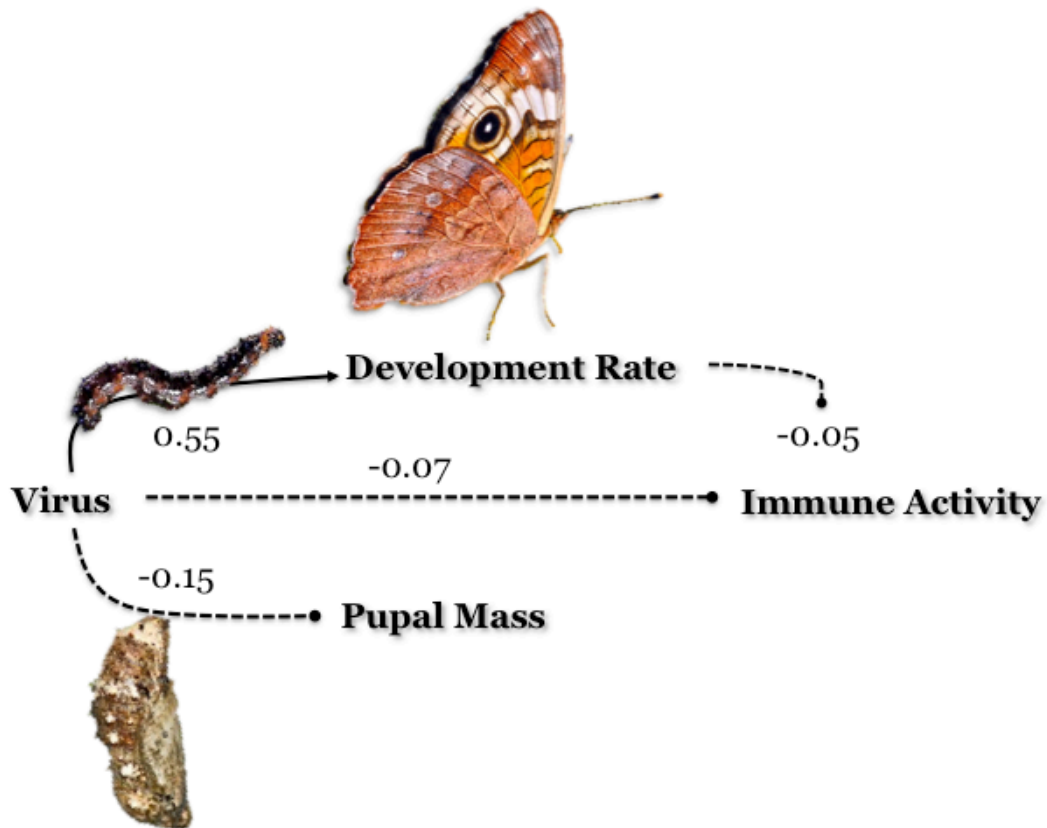
Figure 6. Path diagram showing the relationship between viral infection, development time, immune activity, and pupal mass when larvae were reared on both hosts. Numbers above pathways are standardized path coefficients. Negative numbers and bulleted lines indicate a negative effect of one variable on another. The model is a significant fit to the data ($\chi^2 = 1.03$, df = 2, P = 0.595)
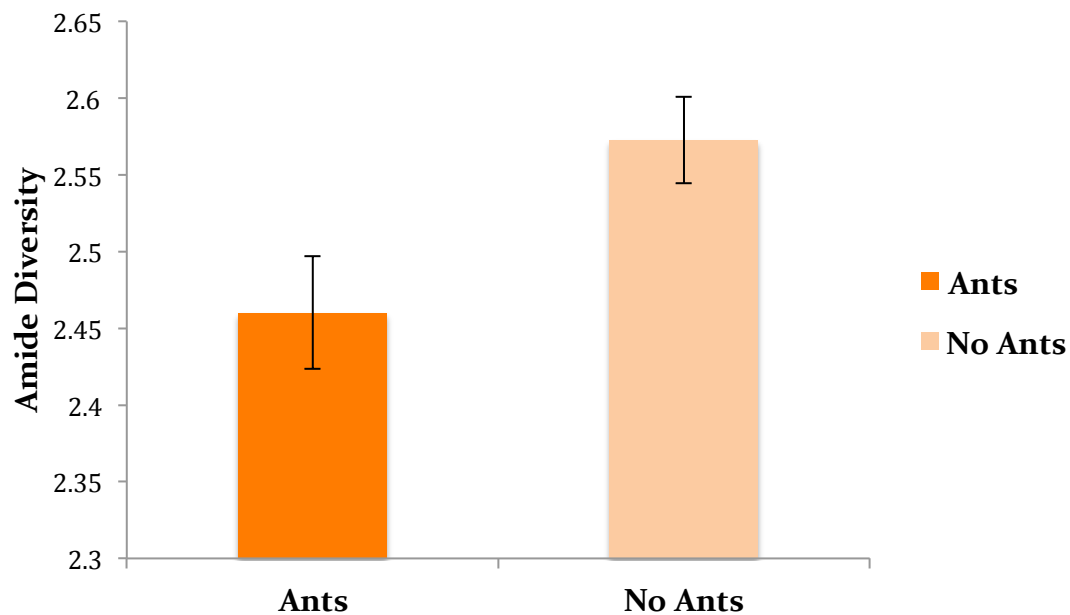
FIGURES: Chapter 2

Figure 1. Amide diversity (piplartine, cenocladamide, and 4'-desmethylpiplartine) of *P. cenocladum* with and without ant mutualists (*Pheidole bicornis)*. There was a significant difference in the diversity of amides between plants housing ants versus plants with no ants ($F_{[1,83]} = 5.89$, $P = 0.0174$, N = 84; means ± SE)
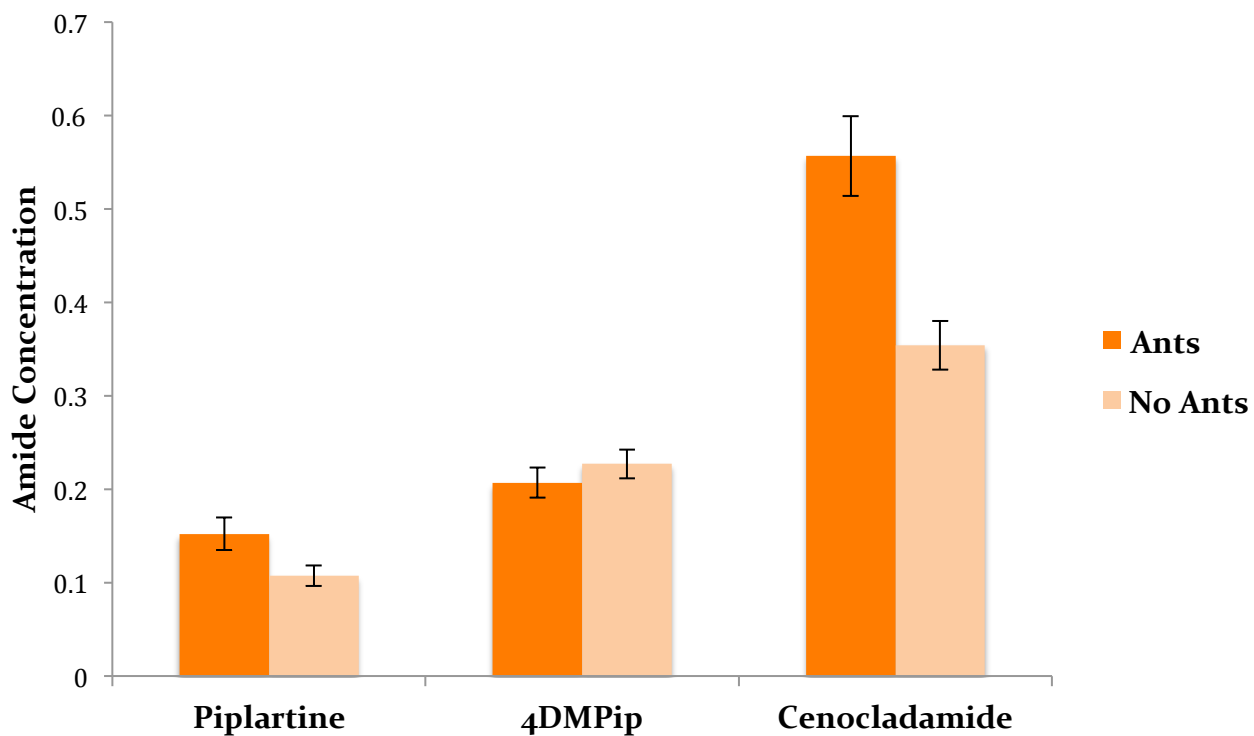
Figure 2. Amide concentration of piplartine, cenocladamide, and 4'-desmethylpiplartine (4DMPip) in *P. cenocladum* with and without ant mutualists. The presence of ants had no effect on the concentration of piplartine ($F_{[1,85]}$ =1.99, P = 0.1623, N = 86; means ± SE). There was a significant effect on the concentration of both 4'-desmethylpiplartine ($F_{[1,85]}$ =12.03 , P = 0.0008, N = 86; means ± SE) and cenocladamide ($F_{[1,84]}$ = 11.90, P = 0.0009, N = 85; means ± SE).
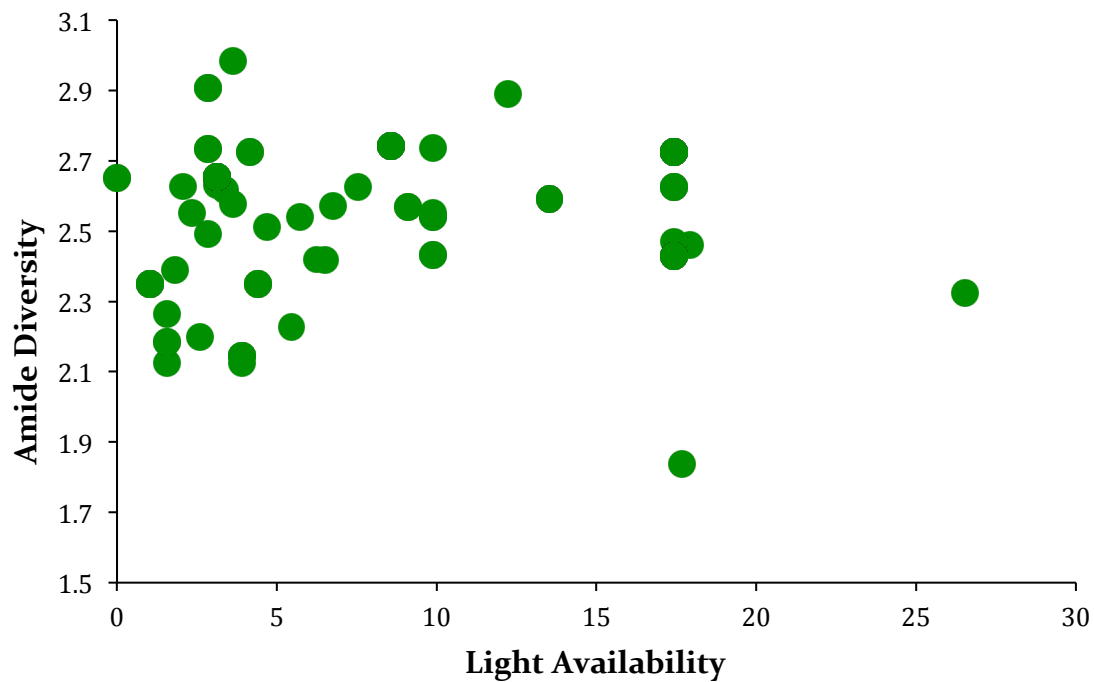
Figure 3. The effect of light availability in the canopy of a tropical forest on the amide diversity (piplartine, cenocladamide, and 4'-desmethylpiplartine) of *P. cenocladum*. There was no relationship between the diversity of amides and light availability ($F_{[1,79]}$= 0.65, r^2 =0.0082, df = 80, p-value = 0.4238).
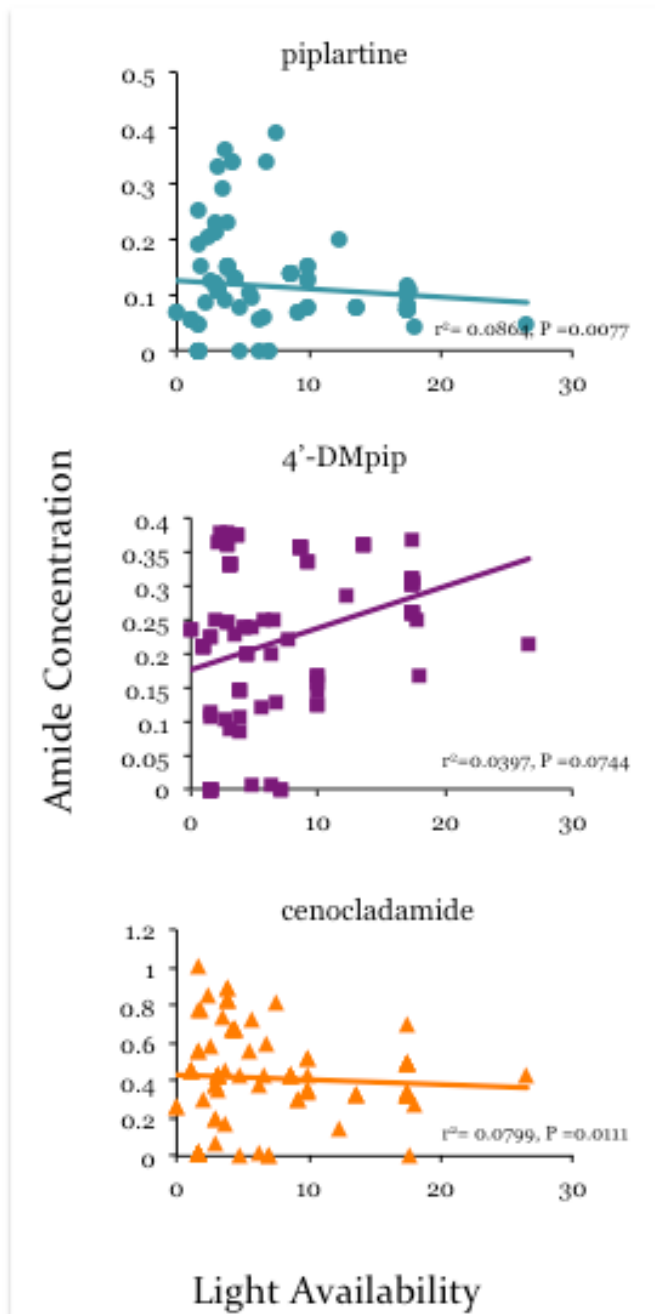
Figure 4. The effect of light availability in the canopy of a tropical forest on the amide concentration of a) piplartine, b) 4'-desmethylpiplartine (4DMPip), and c) cenocladamide, in *P. cenocladum*. As light increased the concentration decreased in both piplartine ($F_{[1,80]}$ =7.47, r^2= 0.0864, P =0.0077, N =81) and cenocladamide ($F_{[1,79]}$ =6.78, r^2= 0.0799, P =0.0111 , N =80 , Figure 4). Light had no significant effect on the concentration of 4'-desmethylpiplartine ($F_{[1,80]}$ =3.27 , r^2=0.0397, P =0.0744 , N =81)