LATITUDINAL VARIATION IN HERBIVORE INTERACTIONS AND THE EVOLUTION OF DEFENSIVE METABOLITES IN *PASSIFLORA INCARNATA*

by

NICHOLAS LEE BATORA

(Under the Direction of Arthur S. Edison & Rodney Mauricio)

ABSTRACT

Plants produce an outstanding diversity of metabolites and many of these metabolites are hypothesized to be involved in coevolutionary interactions. Plants in the genus *Passiflora* and butterflies in the tribe heliconiine are model systems in the field of coevolutionary biology and a class of defensive metabolite, cyanogenic glycoside (CNglc), is hypothesized to mediate coevolutionary interactions within this system. CNglc are abundant and diverse within the genus *Passiflora*, however, little is still known regarding the microevolutionary forces that dictate the evolution of CNglc within *Passiflora*. Here, I describe research aimed at assessing the contribution of specialist heliconiine herbivores in the evolution of CNglc in *Passiflora incarnata* (purple passionflower). Specifically, I investigated predictions of the geographic mosaic theory of coevolution and investigate the relationship between CNglc, herbivory, and plant fitness. To achieve this, I sampled *P. incarnata* populations across a large latitudinal transect in the southeastern United States across which the presence and abundance of heliconiine herbivores vary. I then took a combination of experimental approaches to

assess variation in defensive metabolites among populations. First, I utilized an untargeted NMR metabolomics methodology to identify metabolites associated with lepidopteran interactions and their corresponding latitudinal distributions. I found 43 metabolites or unknown NMR features that are associated with lepidopteran interactions, however, only 15 had a clinal distribution across latitude. Additionally, I identified multiple cyclopentenoids, which are precursors involved in CNglc biosynthesis in *P. incarnata*. In my next experiment, I investigated if CNglc varied over latitude and the influence of this defense on herbivory. I found a positive latitudinal distribution for CNglc with greater CNglc concentrations at high-latitude. Furthermore, CNglc was positively correlated with herbivory from a primarily specialist heliconiine herbivore community. Finally, I investigated if CNglc is under natural selection from herbivores. I measured natural selection in the presence and absence of herbivores to determine the effect they have on fitness and how this relates to CNglc. I found that this defense is not under natural selection, but the presence of herbivores influences the relationship of this trait to fitness. From this body of work, I show that cyanogenic glycosides mediate coevolutionary interactions with heliconiine specialists.

INDEX WORDS: Coevolution, *Passiflora*, heliconiine, cyanogenic glycoside, NMR metabolomics, Latitudinal Herbivory-Defense Hypothesis

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DEDICATION

To Jessie, Dan, Ashleh, Alyx, Abby, Mom, and Dad. Your love and support has made it possible for me to achieve this. I love you all.

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CHAPTER 1

INTRODUCTION

Plants produce an extraordinary array of secondary metabolites, which are utilized by humans as medicines, fragrances, dyes, and spices (Osbourn and Lanzotti 2009). A large goal in the fields of ecology and evolutionary biology is to understand the forces that have contributed to the generation of secondary metabolite variation both within and among plant species (Wink 2003, Rosenthal and Berenbaum 2012, Dyer et al. 2018). Early researchers recognized that plant secondary metabolites are likely not metabolic waste products, but rather serve as deterrents against insect herbivores (Fraenkel 1959). Following this pivotal observation, Ehrlich and Raven (1964) postulated that the diversity of these metabolites arises as a consequence of reciprocal adaptations between plants and their natural enemies. This evolutionary arms race between interacting species is formally known as coevolution (Ehrlich and Raven 1964).

Measuring coevolution is particularly challenging, as it is difficult to measure evolutionary changes in interacting taxa over evolutionary relevant time scales (Gomulkiewicz et al. 2007). As a substitute, biologists often measure traits that potentially mediate these interactions across geographic space where there is variation in coevolutionary strength (Berenbaum and Zangerl 1998, Burdon and Thrall 2000, Fornoni et al. 2004, Brodie et al. 2005). The notion that interactions between species vary in their form and strength across vast geographic space is referred to as the geographic mosaic theory of coevolution (Thompson 2005), which hypothesizes that traits that mediate

interactions among coevolving species are variable across geographic space as a result of spatial variation in reciprocal selection and trait remixing (Thompson 2005). Although geographic variation in traits that mediate interactions is compelling evidence for coevolution, geographic variation alone is not sufficient evidence to claim coevolution is the causal source. It is also necessary to demonstrate that these geographic patterns in traits arose from natural selection varying among populations (Laine 2009). In order to determine the source of natural selection on a trait, it is necessary to look at the dynamics of selection in the presence and absence of a putative selective agent (Endler 1986). Thus, to demonstrate that the evolution of plant secondary metabolites was driven by coevolution between plants and herbivores, one must: 1) assess plant secondary metabolite variation over geographic space where coevolutionary interactions are predicted to vary, and 2) determine if plant secondary metabolites are indeed under selection from herbivores, which can be achieved by measuring selection on plant secondary metabolites in the presence and absence of herbivores (Mauricio and Rausher 1997).

One way to investigate coevolutionary forces acting on plant secondary metabolites is through the use of metabolomics techniques. The two primary analytical tools utilized in metabolomics studies are nuclear magnetic resonance (NMR) and mass spectroscopy (MS) (Emwas 2015). These two analytical techniques provide different information on the metabolites within a complex mixture (Emwas 2015). MS ionizes metabolites and sorts the ions based upon their mass-to-charge ratio and is very sensitive, detecting metabolites at picomolar levels compared to micromolar levels observed in NMR experiments (Emwas 2015). However, NMR provides many advantages over MS

when conducting metabolomics experiments. This includes high reproducibility and the acquisition of more accurate structural information (Dunn et al. 2011, Robinette et al. 2011, Athersuch 2016, Dyer et al. 2018). Due to these features of NMR metabolomics, this technique is ideal for untargeted studies, such as investigations into unknown metabolites that mediate coevolutionary interactions. Therefore, this introduction will focus on using NMR for untargeted metabolomics studies.

NMR measures nuclei with spins of 1/2 by applying an external magnetic field that causes these nuclei to precess at a measurable resonance frequency (Figure 1.1). Proton nuclear magnetic resonance (^{1}H-NMR) is most commonly used in metabolomics experiments, as protons have a high natural abundance (99%). For 1-dimensional ^{1}H-NMR experiments, the local environment of protons in a single metabolite will affect where the peak is seen in the resulting spectrum (Figure 1.1A). Each metabolite therefore generates a unique spectrum. However, in complex mixtures, a spectrum will be composed of many metabolites, resulting in signal overlaps (Figure 1.2) and creating challenges metabolite annotation and data analysis (Kim et al. 2011). However, due to the non-destructive nature of NMR metabolomics, a single sample can be used in multiple NMR experiments that can assist in annotating spectra. To address these challenges from overlap in 1D ^{1}H-NMR experiments, 2-dimensional NMR experiments can be utilized, which provide additional information on metabolite structures (Figure 1.1B). These 2D-experiments include ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), 1H-1H total correlation spectroscopy (TOCSY), and a combination of the two, 1H-13C heteronuclear single quantum correlation total correlation spectroscopy (HSQC-TOCSY) (Figure 1.1B). By utilizing both 1D- and 2D-NMR experimentation, NMR

metabolomics experiments can result in the identification of 30 – 150 metabolites as well as many unknown analytes that may be important in biological processes (Kim et al. 2011). NMR metabolomics has been used widely in the plant metabolomics (Kim et al. 2011) and to specifically study plant defensive metabolites (Leiss et al. 2011, Dyer et al. 2018).

In this dissertation, I will investigate the role of plant-herbivore interactions in shaping geographic variation secondary metabolites within the purple passionflower, *Passiflora incarnata,* and the role coevolutionary interactions with heliconiine butterflies play in the evolution of cyanogenic glycosides. Interactions between individuals in the genus *Passiflora* and butterflies in the tribe heliconiine are classic examples of coevolution between plants and the insects that feed on them (Ehrlich and Raven 1964). Multiple adaptations to *Passiflora* defenses have been observed in specialists heliconiine (Benson et al. 1975, Turner 1981). This includes adaptations to cyanogenic glycosides, the major class of defensive metabolites in *Passiflora* plants (de Castro et al. 2017). This specific class of defensive metabolites is thought to mediate coevolution between *Passiflora* and heliconiine (Spencer 1988, Engler et al. 2000, Engler-Chaouat and Gilbert 2007). We therefore test for evidence that cyanogenic glycosides mediate coevolutionary dynamics within *P. incarnata* and explore other metabolites that may be involved in these interactions using an NMR metabolomics. In chapter 2 we investigate geographic variation in putative metabolites involved in herbivore interactions including cyanogenic glycoside biosynthesis. In chapter 3 we investigate geographic variation in cyanogenic glycosides and its ability to prevent herbivory, and in chapter 4 we investigate natural selection on cyanogenic glycosides from herbivores.

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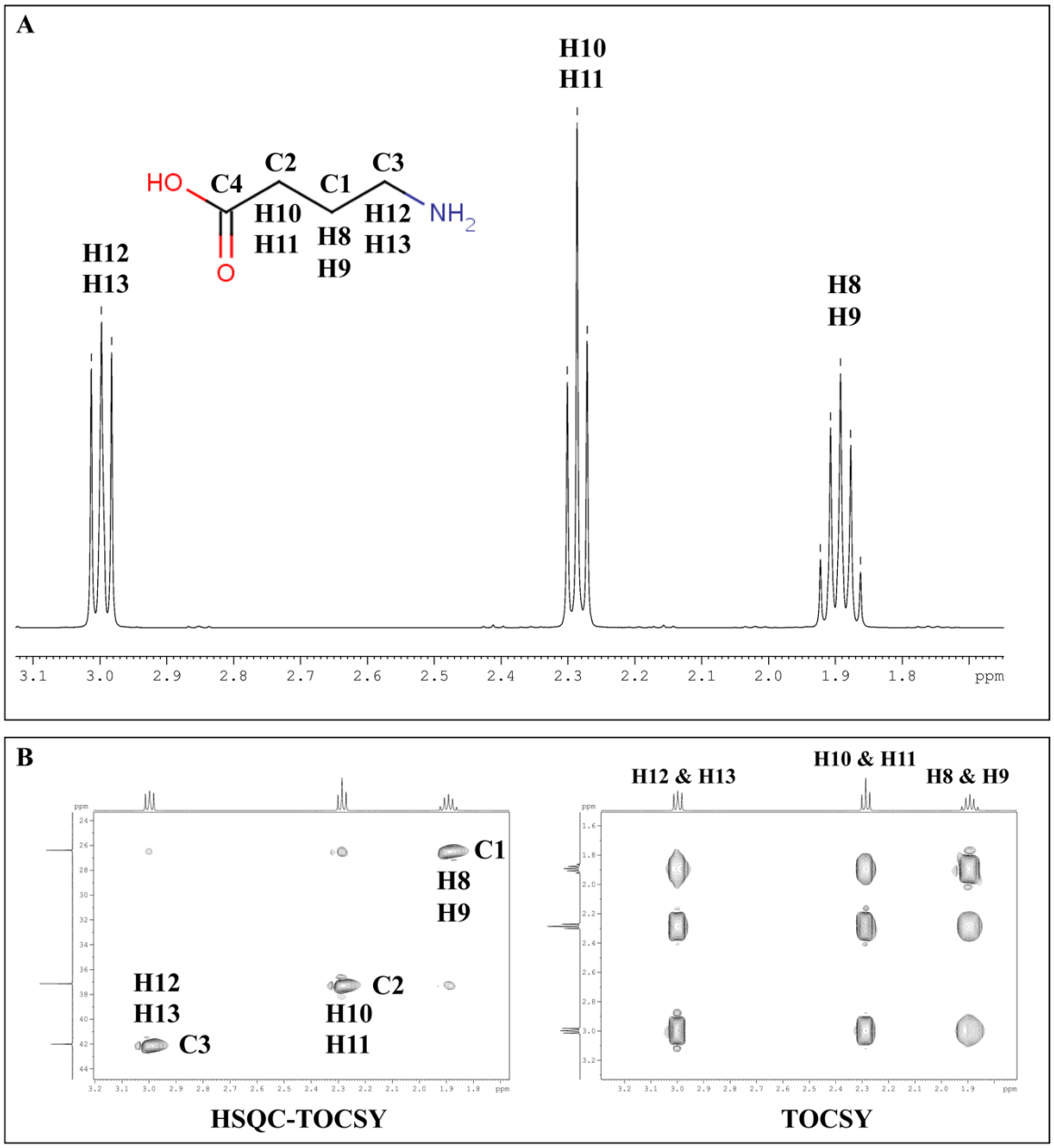

Figure 1.1)

Example ^{1}H-NMR spectrum of gamma-Aminobutyric acid, a common plant metabolite. Both carbon (13C) and proton (1H) numbers are indicated on the molecule with their corresponding chemical shifts indicated in each NMR spectra. **A)** ^{1}H-NMR spectrum with corresponding proton chemical shifts. **B)** 2D NMR experiments such as HSQC-TOCSY (left) with corresponding carbon and proton chemical shifts and TOCSY (right) with corresponding proton chemical shifts. Spectrum were obtained from the Biological Magnetic Resonance Bank.

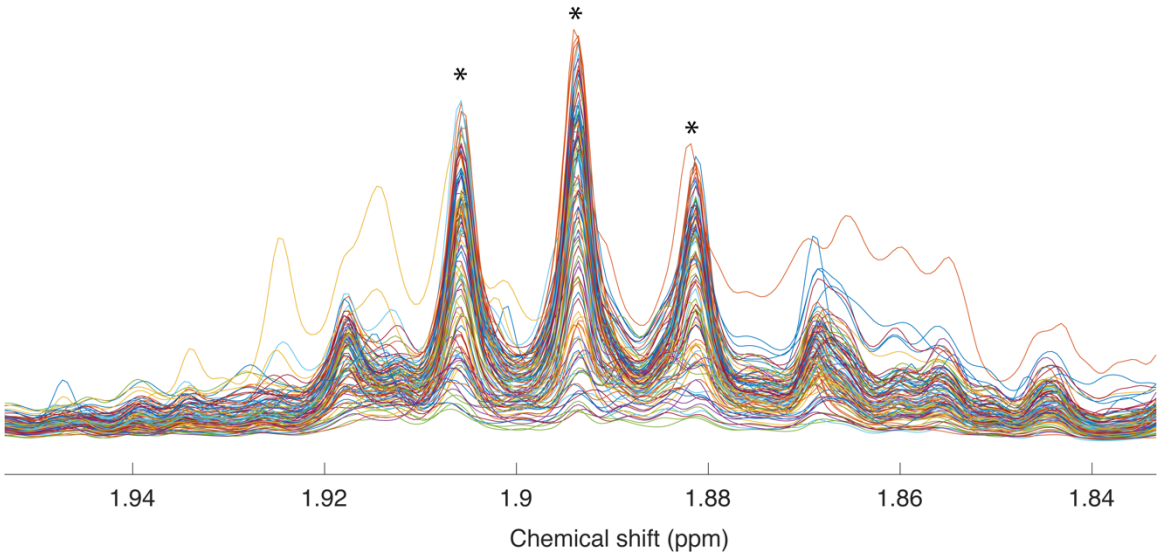

Figure 1.2)

Example of 1H-NMR spectrum of a complex mixture. Here, there is a triplet peak corresponding to gamma-Aminobutyric acid (denoted with *). Adjacent to it are peaks corresponding to unknown metabolites. Each colored line represents a different NMR spectrum obtained from a different sample.

CHAPTER 2

GEOGRAPHIC VARIATION IN METABOLITES ASSOCIATED WITH LEPIDOPTERAN INTERACTIONS IN *PASSIFLORA INCARNATA* (PURPLE PASSIONFLOWER)

Introduction

Plants produce an extraordinary array of metabolites, which have been utilized by humans for centuries for both medicinal and cultural purposes (Osbourn and Lanzotti 2009). These metabolites are important mediators of interactions between plants and other biotic organisms, and have been shown to attract mutualists, inhibit competing plants, and defend against natural enemies (Lovett et al. 1989, Dicke 1994, Baldwin 2010, Rosenthal and Berenbaum 2012). Due to the ability of plant metabolites to mediate a myriad of biotic interactions, they are a major focus for the study of the geographic mosaic of coevolutionary interactions (Ehrlich and Raven 1964, Cornell and Hawkins 2003, Dyer et al. 2018).

The geographic mosaic of coevolution theory predicts that traits that mediate interactions between interacting species will vary across geographic space in their form and strength (Thompson 2005). For plant-herbivore interactions, this occurs when plant populations are spread out over a vast heterogeneous landscape interspersed with varying herbivore communities. Within herbivore communities, there are animals that feed on a diversity of host plants (i.e., generalists) and others feed exclusively on a subset of

closely related plant species (i.e., specialists). Across plant populations the relative contribution of these two forms of herbivore can vary (Castillo et al. 2014). This variation in herbivore communities can result in variable selective pressures among plant populations. Consequently, plant traits that mediate interactions with herbivores, such as defensive metabolites, can also vary among populations (Berenbaum and Zangerl 1998, Zangerl and Berenbaum 2003).

The concentrations of some plant defensive metabolites change after herbivore attack, while others do not; these differences are classified as constitutive and induced defenses (Karban 2011). Constitutive defenses are continuously expressed and are not altered in response to herbivore attack, while induced defenses are those which are specifically altered in response to herbivory (Karban and Myers 1989). Further, the induced responses driven by herbivory in the expression of gene transcripts and metabolites can differ between generalist and specialist herbivores (Mewis et al. 2006, Zong and Wang 2007), but this is not always the case (seeAli and Agrawal 2012). Therefore, when investigating geographic variation in plant metabolites it is important to compare both constitutive and induced defenses while controlling the type of herbivore that is eliciting the induced response (Rasmann and Agrawal 2011, Anstett et al. 2016a, Dyer et al. 2018).

Plant-herbivore interactions and the traits that mediate them are specifically predicted to vary across latitude (Schemske 2002), as selection from biotic interactions is predicted to be greater at low-latitudes due to longer growing seasons, higher temperatures, and an increase in the diversity of species (Dobzhansky 1950). At high-latitudes species are predicted to experience stronger selection from abiotic factors

(Dobzhansky 1950). As such, plants are hypothesized to experience stronger selection from herbivores at lower latitudes and therefore invest more in defenses (Levin 1976, Coley 1991, Coley and Barone 1996, Pennings et al. 2009, Schemske et al. 2009). This hypothesis is referred to as the Latitudinal Herbivory-Defense Hypothesis (Johnson and Rasmann 2011). Some investigations of this hypothesis with single plant species have found greater concentration of chemical defenses at low-latitudes (Pearse and Hipp 2012, Anstett et al. 2015), while others studies on chemical defenses found either no pattern or greater defenses at high latitudes (Moles et al. 2011, Abdala-Roberts et al. 2016). This observed discrepancy has led to a recent review of methodologies, which called for more studies that account for induction of plant defenses (Anstett et al. 2016b). This is further complicated by the fact that induction of defense traits is also predicted to vary across latitude, with increased constitutive defenses predicted to be favored at low-latitudes and increased induced responses at high-latitudes as a strategy to mitigate the costs of defenses (Figure 2.1) (Karban and Myers 1989, Karban 2011, Anstett et al. 2016a). But as mentioned previously, plants from low latitudes are predicted to invest more in herbivory defense in general (Figure 2.1).

Here, we assess geographic variation in constitutive and induced plant metabolites from populations sampled across a latitudinal transect. To achieve this, we utilized the plant species *Passiflora incarnata* (purple passionflower). For this analysis, we sampled from 8 populations spanning a 10.67º latitudinal transect across the southeastern United States (Figure 2.2A). Plants in the genus *Passiflora* and butterflies in the tribe heliconiine are classic examples of coevolution (Ehrlich and Raven 1964). Multiple adaptations to *Passiflora* defenses have been observed and quantified in specialist

heliconiine butterflies (Benson et al. 1975, Turner 1981). This includes adaptations to cyanogenic glycosides, the major class of defensive metabolites in *Passiflora* plants (de Castro et al. 2017). Most of the cyanogenic glycosides in *Passiflora* are derived from forms of the non-protein forming amino acids, cyclopentenoids (Figure 2.3A) (Forslund et al. 2004, de Castro et al. 2017). Heliconiine butterflies are capable of sequestering or tolerating cyclopentenoids, and thus plant-herbivore interactions involving these metabolites have been hypothesized to mediate coevolution between *Passiflora* and heliconiine (Spencer 1988, Engler et al. 2000, Engler-Chaouat and Gilbert 2007). The distribution and abundance of specialist heliconiine butterflies vary considerably across the latitudinal transect we covered in this study (Figure 2.2B; Chapter 3). Thus, *P. incarnata* populations that span this gradient are an ideal resource for testing predictions generated from the Latitudinal Herbivory-Defense Hypothesis. In this study, we assess variation in leaf metabolites through the use of Nuclear Magnetic Resonance (NMR) spectroscopy, which is an ideal technology for this study because it is untargeted, quantitative, and highly reproducible (Dunn et al. 2011, Robinette et al. 2011, Athersuch 2016, Dyer et al. 2018). Specifically, we asked the following questions: 1) What NMR features are associated with biotic interactions from two different lepidopteran herbivores (one generalist and one specialist)? 2) How are they distributed over latitude? 3) Does induction of plant NMR features vary over latitude? 4) What is the distribution of cyclopentenoids over latitude and are they inducible?

Methods

Study System

The purple passionflower, *Passiflora incarnata* Linn. (Passifloraceae), is an herbaceous, perennial vine native to the southeastern United States with a distribution extending from central Florida to southern Pennsylvania (McGuire 1999). It can be found primarily growing in disturbed locations and is thought of as an early successional species (Radford et al. 2010). The plant reproduces either vegetatively through root and rhizome fragments or through seed production *via* an andromonoecious mating system (Wehtje et al. 1985).

Passiflora incarnata interacts with several different species of lepidopteran herbivores across the southeastern United States, all from the family Nymphalidae (Opler 1998). These include specialists *Heliconius charithonia* (Zebra Heliconian; Passiflora specialist), *Dryas iulia* (Julia Heliconian; Passiflora specialist), *Agraulis vanillae* (Gulf Fritillary; Passifloraceae specialist), and *Euptoieta claudia* (Variegated Fritillary; generalist). Published field guides indicate that there may be spatial variation in the distribution of these herbivores across the range of *P. incaranta* (Opler 1998), and unpublished citizen science and museum specimen data corroborate these claims (Figure 2.2B; Chapter 3).

Plant Samples

To investigate latitudinal variation in plant metabolites, we collected *P. incarnata* from 8 populations that span a latitudinal range of 27.6205°N – 38.0299°N (Figure 2.2A, Table S2.1, Figure S2.1A). We sampled plants in the field that were at least 10 meters away from each other in order to decrease the likelihood of sampling the same individual,

as rhizomes grow, on average, 6 meters (McGuire 1999). Thus, each plant rhizome sampled in the field was treated as a unique genotype and the number of genotypes per population varied depending on local densities (Table S2.1). After field collection, we transplanted rhizomes into 1 gallon pots in a 1:1 ratio of potting soil mix and vermiculite. Plants were watered and provided fertilizer sporadically (Osmocote Plus fertilizer (15 – 9 – 12 NPK)), beginning in 2014 and allowed to grow under ambient greenhouse conditions in Athens, Georgia for two years prior to experiments reported in this study.

Damage study (DAM Experiment)

To investigate how plant metabolic responses were associated with interactions from lepidopteran herbivores, we first crossed plants originally collected from the field (Figure S2.1). First, plants from all Florida and Virginia populations were randomly paired together to generate an F1 population resulting in 20 different F1 combinations from a total of 8 different populations. We then crossed F1s together in a partial diallelic breeding design to generate an F2 population (Figure S2.1B, Chapter 4). From this F2 population, we randomly selected 15 full-sib families for our damage experiment. Because we eventually exposed this experimental population to damage from herbivores, we refer to this populations of plants as "DAM".

To assess metabolic responses involved in lepidopteran herbivore interactions, we grew plants from seed for 15 F2 full-sib families that were randomly selected for this study. To germinate seeds, we first soaked seeds in de-ionized water for 48 hours at 4ºC and then placed them on filter paper in an incubator set to 37ºC. Once seeds began to germinate, we transplanted seedlings into 1:1 potting soil mixture and vermiculite and placed them under lights in the greenhouse to continue to grow. Once seedlings grew

their first true leaf, we transplanted them into 10-inch pots filled with the same soil mixture of pine bark, vermiculite, superphosphate, calcium nitrate, potassium nitrate, micronutrients, gypsum, and dolomite limestone. We arranged plants in a randomized design in the greenhouse under ambient conditions in early October 2017. We watered plants regularly, provided each with a single tablespoon of fertilizer (Osmocote Plus fertilizer (15 – 9 – 12 NPK)), and allowed vines to grow on a 1.2-meter bamboo stick.

To investigate the response of *P. incarnata* to different types of damage, we harvested leaves that were undamaged (control), mechanically damaged, damage from *C. includens* (generalist), and damage from *A. vanillae* (specialist) from each full-sib family. Each full-sibling family was composed of 12 individual plants, and we randomly assigned one of 4 treatments to each plant (undamaged, mechanically damaged, generalist herbivory, or specialist herbivory). Thus, our damage experiment constituted a total of 180 individual plants (3 reps/family * 15 plants families * 4 treatments). For each treatment, we covered the third, fully expanded adult leaf on the primary shoot with a 5" x 7" white mesh bag for 72 hours prior to harvest. After this time period, we harvested by clipping leaves off at the base where the petiole is connected and immediately flash freezing leaves in liquid nitrogen. Undamaged leaves had no damage at the time of harvest while damaged leaves had different damage treatments 72 hours prior to harvest. For mechanically damaged leaves, we used a hole punch where we removed three holes adjacent to the midrib on each leaf. For generalist damage, we used laboratory reared *Chrysodeixis includens* (Soybean Looper moth) that were at the 2^{nd}-3^{rd} instar developmental stage. All *C. includens* utilized in this study were kept in standard conditions and reared on artificial diets prior to our experiment (Strand 1990). For

specialist damage, we used *Agraulis vanillae* (Gulf Fritillary) that were in the 1^{st}-3^{rd} instar developmental stage. All *A. vanillae* utilized in this study were collected from the wild in Athens, Georgia and were reared on a combination of *Passiflora suberosa* and *Passiflora edulis* in the greenhouse prior to our study. Prior to beginning our herbivore damage treatments, all caterpillars were weighed after a 12-hour fasting period. After herbivores were allowed to feed on leaves for 72 hours, we determined the amount of herbivory by each caterpillar. To achieve this, we placed a clear plastic 1x1 cm grid on each leaf surface and counted the number of squares missing due to caterpillar feeding. We then measured leaf length from the base of the leaf to the tip. Previous analyses indicated that leaf length was an excellent predictor for total leaf area by utilizing the equation: leaf area = $0.3795(\text{leaf length})^{2.1272}$ ($N=97$; $R^2=0.928$). Thus, leaf herbivory was determined using the equation: [leaf area missing cm^2] / [total leaf area cm^2].

From our DAM experiment, we first sought to determine what NMR features showed differences between control (undamaged) and each form of damage (mechanical, generalist, and specialist). To achieve this, we separately compared each damage treatment (mechanical, generalist, and specialist) to control (undamaged) leaves and constructed analysis of variance models where treatment was a fixed effect and full-sib family was a random effect. The resulting p-values were then corrected for multiple comparisons using the Benjamini and Hochberg (B-H) FDR correction with a significance threshold of 0.1 (Hochberg and Benjamini 1990).

We also sought to determine what NMR features were shared exclusively between plants exposed to herbivory from *C. includens* and those exposed to herbivory by *A. vanillae*. To achieve this, we first standardized the amount of leaf area consumed

by each caterpillar by dividing leaf area consumed by the caterpillar's initial mass prior to the beginning of the experiment. This was necessary due to differences in size between each lepidopteran species and therefore allowed us to compare the relationship of NMR features to leaf area consumed among the two treatments. We then investigated if NMR features showed correlations to herbivory that were significantly different from zero using the corr function in MATLAB (The MathWorks Inc., 2017). From this analysis, we corrected for multiple comparisons using a B-H FDR procedure with a significance cutoff of 0.1 (Hochberg and Benjamini 1990). We then visualized these results using statistical total correlation spectroscopy (STOCSY) (Cloarec et al. 2005).

All statistical analyses were carried out using MATLAB v.2017b. Any NMR features discovered in our DAM experiment that were considered to be involved exclusively in lepidopteran interactions were (1) uniquely identified in the one of the two herbivore treatments as different from control or (2) identified as being involved with leaf herbivory from either herbivore species. These were then considered candidates to pursue in the POP experiment.

Population Experimental Design (POP Experiment)

In our next experiment, we attempted to test predictions of the Latitudinal Herbivory-Defense Hypothesis with NMR features involved in lepidopteran interactions. To assess latitudinal variation in plant metabolites associated with lepidopteran herbivore interactions, we first generated individual plants from 8 different populations by clonally propagating them in mid-June of 2016 (Figure S2.1A). We clonally propagated plants by cutting off portions of the vine, dipping one end of the vine in rooting hormone (Hormodin 3; active ingredient Indole-3-butyric Acid 00.8%), placing them in soil (1:1

ratio of potting soil mixture and vermiculite), and leaving them on a mist bench for several weeks until plants developed sufficient roots for transplanting. We refer to this population sample set as "POP".

In mid-July 2016, we transplanted clonal propagules (2 per genotype) of similar size into 8-inch pots filled with a pine bark soil mixture similar to the DAM experiment. After transplanting, we placed pots in a randomized design with plants positioned 6 inches apart from each other under ambient greenhouse conditions. We watered plants regularly, provided each with a single tablespoon of fertilizer (Osmocote Plus fertilizer (15 – 9 – 12 NPK)), and allowed vines to grow on a 1.2-meter bamboo stick.

To investigate plant metabolite production and induction over latitude, we randomly assigned a damage treatment to each pair of clones propagated from the same plant, where one plant was assigned the undamaged treatment and the other was assigned the herbivore damage treatment. Thus, our population experiment constituted a total of 96 individual plants (48 genotypes * 2 treatments/genotype). For all leaves collected, we covered the third, fully expanded adult leaf on the primary shoot with a 5" x 7" white mesh bag for 72 hours prior to harvest. To minimize any confounding effects of altered plant defenses due to plant volatiles released during herbivore attack (Kessler and Baldwin 2001), we first harvested our undamaged treated leaves prior to beginning of damage treatment. After undamaged leaf harvest, we began our damage treatment by placing a single, neonate *Agraulis vanillae* caterpillar (Passifloraceae specialist) purchased from Shady Oak Butterfly Farm (Brooker, FL) and left caterpillars on leaves for 72 hours. When leaves were harvested, we removed the leaf by clipping it at the base

where the petiole is connected and immediately flash freezing the leaf in liquid nitrogen. All leaves were stored at -80 ºC until processing for NMR spectroscopy.

Information on induced plant metabolome natural variation is poorly known (Li et al. 2015), therefore we sought to assess this variation in our POP dataset. To assess within and among population variation in plant metabolites, we utilized both a principal component analysis as well as calculated Euclidean distances among each sample. To perform the principal component analysis, we scaled our dataset using Pareto (Eriksson 1999). For Euclidean distances (ED), we calculated inter-individual distances and plotted that against geographic distance based upon latitude. This ED analysis has been utilized in other studies that investigate natural variation in plant metabolomes across geographic space (Li et al. 2015).

In our previously described DAM experiment, we determined which features were associated specifically with lepidopteran interactions. By comparing data from the both experiments (DAM vs POP), we were able to identify NMR features in the DAM experiment that were also present in the POP experimental dataset. The Latitudinal Herbivory-Defense Hypothesis only makes predictions on plant traits associated with defense, thus we aimed to look at only NMR features associated with lepidopteran interactions. To do this, we used statistical total correlation spectroscopy (STOCSY) (Cloarec et al. 2005) to determine if features shared similar splitting patterns and correlations with other features in both datasets. From this, we then integrated features in the POP experimental dataset with bounds of each feature determined using SRV (Navratil et al. 2013).

With our POP dataset, we tested the hypothesis that these features would vary in their quantity and inducibility over latitude. To determine if features involved in lepidopteran interactions showed latitudinal clines, we evaluated the linear relationships between feature quantity and latitude of plant population origin (Table S2.1). To do this, we constructed an analysis of variance model where the fixed effects were latitude, treatment, and the interaction of latitude*treatment with a response variable of area under the curve (AUC) for each NMR feature. In all of our models, the interaction term was not significant therefore we removed this effect from the analyses reported in this study. To determine if induction varied across latitude, we pooled samples from high and low-latitudinal regions (Table S2.1). We then performed a paired t-test (paired by genotype) for high and low-latitudinal regions for each metabolite involved in biotic interactions. All statistical analyses were carried out using MATLAB v.2017b.

NMR Metabolomics

To identify and quantify plant leaf metabolites, we utilized NMR spectroscopy. We freeze dried leaves for 72 hours, weighed out 50 mg (+/- 2 mg) dried leaf tissue, added three 3.5mm glass beads and one 1.6mL scoop of 2.0mm zirconia beads, and extracted metabolites by homogenizing (MP tissue homogenizer) tissue in a 1:4 mixture of water and methanol kept on dry ice. Samples were then sonicated for five minutes in ice water and then spun at 10,000 rpm at 4 ºC for 10 minutes. Supernatant was then extracted and this process was repeated again, starting at the homogenization step. Both extracts for each sample were pooled together and then dried down in a speed vacuum for 19 hours. Samples were reconstituted in 750 µL of a 1:1 mixture of deuterated water and methanol with 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) such that the final

concentration of DSS was 333 μM. This was followed by vortexing samples and then spinning in a centrifuge for 15 minutes at 14,000 rpm. Then, 550 μL from each sample were added into 5 mm NMR tubes (Bruker Biospin, Billerica, MA, USA). Additionally, 38 μL from each sample were pooled together for an internal standard.

NMR data collection

NMR data were obtained under automation using ICONNMR and Topspin (v. 3.5pl5) using a 600 MHz Bruker AVIII-HD NMR spectrometer equipped with a Bruker SampleJet cooled to 5.6 ºC. One-dimensional (1D) experimental data were obtained using the NOESYPR pulse sequence for residual water presaturation with 128 scans, spectral width of 16.0221, and free induction decay (FID) size of 32768.

We also acquired two-dimensional (2D) data using an internal pooled sample for metabolite identification. To do this, we performed the following experiments: ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), HSQC-TOCSY (total correlation spectroscopy), TOCSY, and ^{1}H-^{13}C HMBC (heteronuclear multiple bond correlation). HSQC pulse sequence was hsqcetgpprsisp2.2 with 32 scans, spectral width of 16.3102 in the second dimension and 165 in the first dimension, and FID size of 2048 in the second dimension and 1024 in the first dimension. HSQC-TOCSY pulse sequence was hsqcdietgpsisp.2, with 32 scans, spectral width of 16.3102 in the second dimension and 165 in the first dimension, and FID size of 2048 in the second dimension and 1024 in the first dimension. TOCSY pulse sequence was dipshi2esgpph, with 32 scans, spectral width of 16.3102 in both dimensions, and FID size of 2048 in the second dimension and 512 in the first dimension. Finally, HMBC pulse sequence was hmbcetgpl3nd with 32

scans, spectral width of 16.3102 in the second dimension and 220 in the first, and FID size of 4096 in the second dimension and 1024 in the first dimension.

NMR data processing: All NMR spectra were first processed using NMRPipe (Delaglio et al. 1995). Line broadening of 0.5 was applied and then the NMR spectra were zero filled, Fourier transformed, phased, and the baseline was corrected using an automatic polynomial baseline correction.

Next, spectra were imported into MATLAB (The MathWorks Inc., 2017) for further data processing and analysis. Spectra were referenced to 0.0 ppm using DSS, methanol and water solvent peaks removed, aligned using a modified version for increased efficiency of correlation optimized warping (Nielsen et al. 1998, Tomasi et al. 2004), and normalized using probabilistic quotient normalization (PQN) (Dieterle et al. 2006).

We sought to apply univariate statistics to our metabolomics data set, however, due to the complexity of NMR data where many data points are from single NMR features and multiple NMR features correspond to single metabolites (Blaise et al. 2009), we sought to further reduce the redundancy of these data. First, we utilized Statistical Recoupling of Variables (SRV) (Navratil et al. 2013) by setting resolution size to 0.01, correlation threshold of 0.9, and removing peaks under the size of 0.1ppm peak width. Next, we used recoupled-STOCSY (R-STOCSY) (Blaise et al. 2011) to cluster multiple NMR features together from the same molecule by setting our correlation threshold to 0.95 as this has been demonstrated as a good means of detecting multiple NMR features coming from the same metabolite (Couto Alves et al. 2009). All raw and processed data are available on the Metabolomics workbench

(http://www.metabolomicsworkbench.org/), along with detailed experimental NMR and analysis methods.

Resonance Assignments: 1D and 2D NMR resonances were compared to databases such as Bruker AssumreNMR software (Bruker Biospin, USA) and COLMARm (Bingol et al. 2016). Briefly, we first used COLMARm, which uses a combination of HSQC, TOCSY, and HSQC-TOCSY data to generate putative assignments to different resonances. Next, we further matched our HSQC data using AssureNMR. We then compared these matches to 1D spectra in both Biological Magnetic Resonance Bank (Ulrich et al. 2007) and Human Metabolome Database (Wishart et al. 2017) to assign ^{1}H-NMR peaks to annotated metabolites. Metabolites were assigned a confidence level ranging from 1 to 5, with 5 being the highest confidence. The scale is defined as: (1) putatively characterized compound classes or annotated compounds, (2) matched to literature, (3) matched to HSQC (AssureNMR), (4) matched to HSQC and validated by HSQC-TOCSY (COLMARm), and (5) validated by spiking the authentic compound (Walejko et al. 2018).

NMR Quality Assurance: In addition to our experimental samples, internal and external controls, solvent blanks consisting of sample buffer, and extraction blanks were randomized prior to sample preparation for quality assurance purposes. For internal controls, 38 µL from each sample were pooled together for an internal standard. For external controls, we used *P. incarnata* leaves that were not a part of either the reported experiments. Following data acquisition and processing, we found that internal and external controls were tightly clustered using PCA for both experimental datasets (Figure

S2.2). Based upon these results, we were confident our data were not biased due to sample preparation, data acquisition, and data processing (Dunn et al. 2012).

Results

Throughout the course of this study, we utilized two distinct data sets of *P. incarnata* samples in two separate but interconnected NMR experiments. First, a damage study (DAM; Figure S2.3) was performed to identify NMR features associated with lepidopteran interactions. Next, population study (POP; Figure S2.4) was performed to determine the geographical distribution of NMR features identified from the DAM assay. For the DAM samples, we compared changes in NMR spectra in leaves with mechanical damage, damage from a generalist herbivore, and damage from a specialist herbivore to control leaves that experienced no damage (Figure S2.3). For the POP samples, we compared NMR spectra over latitude in both undamaged and damaged leaves (Figure S2.4). We were particularly interested in NMR features that significantly changed in both studies.

By visual inspection of the two datasets, we found NMR features around 5.73 ppm that appeared to be important in both datasets (Figure 2.3). Upon recognition of the 5.73 ppm feature, we also noticed another interesting feature adjacent to it at 5.66 ppm with similar multiplet splitting patterns (Figure 2.3). When we attempted to annotate these features, we found that neither of these two features matched with any of our database searches. Thus, we then began a literature search of NMR data on plants in the genus *Passiflora* (Clausen et al. 2002). From this, we found that the multiplet at 5.73 ppm matched with published data for (S)-2-(3'-cyclopentenyl)glycine, which we referred to as

isocyclopentenylglycine (Figure 2.3B). The multiplet at 5.66 ppm matched with published data for (2S,1'R)-2-(2'-cyclopentenyl)glycine, which we referred to as cyclopentenylglycine (Figure 2.3B). Collectively, these metabolites are known as cyclopentenoids and are precursors to the majority of cyanogenic glycoside biosynthesized in the genus *Passiflora*. Both of these cyclopentenoids displayed interesting patterns in both the DAM and POP datasets (Figure S2.5). We overview results from analyses of both of our datasets with a focus on these cyclopentenoids.

DAM Experiment: Induced responses in leaf metabolomes is greater for a generalist herbivore relative to a specialist herbivore

We initially set out to determine which metabolic changes were specific to lepidopteran herbivore interactions in our DAM experimental population. Many features demonstrated significant alterations due to damage and they were distributed across the NMR spectrum in the aliphatic, sugar, and aromatic regions (Figure 2.4A). Among different damage treatments, we found that the majority of features changed in response to all forms of damage (Figure 2.4B). Of the 414 total NMR features we identified from the deconvolution analyses of SRV (Blaise et al. 2009) and RSTOCSY (Blaise et al. 2011), 73 significantly changed in response to all three of our damage treatments (Figure 2.4B). Consistent with this, we additionally found that all three treatments overlapped in our principal component analysis (Figure S2.2A).

From the features that responded to damage, we were interested in determining which were involved in lepidopteran interactions. From this, we found that a total of 120 features responded specifically to only herbivore treatments (Figure 2.4B). Of the 65 features that significantly changed due to both of the herbivore treatments, we found that

33 decreased relative to undamaged control leaves, while 32 increased. Interestingly, the direction of change in the abundance of these metabolites was the same for both the specialist and generalist herbivore treatments. One specific metabolite that changed in response to both herbivores is isocyclopentenylglycine, which was decreased in both specialist and generalist herbivore treatments relative to undamaged control and mechanically damaged leaves (Figure 2.3C; Figure S2.5C).

We also observed a large difference in features that changed uniquely among the two herbivore treatments, with 49 features changing solely due to the generalist herbivore treatment and 6 features changing solely due to the specialist herbivore treatment. Of the 49 generalist-specific features, we found 25 features decreased relative to undamaged control while 32 increased. For the 6 unique specialist herbivore treatment features, we found 3 decreased relative to control while 3 increased.

DAM Experiment: Leaf metabolites, including both cyclopentenoids, have greater impact on C. includens herbivory but not A. vanillae herbivory

In addition to quantitative differences in leaf metabolomes, we also strove to identify other NMR features associated with lepidopteran interactions by associating NMR features with the amount of leaf area eaten from each herbivore species. We found large differences in the amount of standardized leaf area eaten when plants were exposed to different herbivores (t-Ratio = -6.515; $P < 0.0001$). The generalist *C. includens* ate on average 1.76 cm^2 (standard error = 0.099) of leaf area in proportion to their starting size, while the specialist *A. vanillae* ate on average 0.965 cm^2 (SE = 0.071) of leaf area in proportion to their starting size.

In order to visualize the relationship between NMR feature intensity and the amount of standardized leaf area eaten, we performed a STOCSY analysis where the driver peak was the standardized leaf area eaten for each separate herbivore treatment (Figure 2.5). From this, we found that many NMR features had strong correlations for the *C. includens* treatment (Figure 2.5A), while there were no strong correlations in the *A. vanillae* treatment (Figure 2.5B). Notably, the cyclopentenoids had similar patterns to each herbivore species. Specifically, cyclopentenylglycine and isocyclopentenylglycine had negative relationships to *C. includens* herbivory (Figure 2.5A), while neither had strong correlations with *A. vanillae* herbivory (Figure 2.5B). Finally, we found that a total of 48 features were significantly correlated with herbivory from *C. includens* after multiple comparisons corrections while none were significantly correlated with *A. vanillae*.

POP Experiment: Passiflora incarnata populations have tremendous amounts of metabolic variation both within and among populations

Our knowledge of how plant metabolic profiles are distributed across geographic space is limited (Li et al. 2015). Thus, given the unique opportunity to investigate this, we sought to quantify these patterns in our POP dataset. To assess the variability of leaf metabolic profiles within and among populations of *P. incarnata*, we utilized 8 populations from across the southeastern United States that span a total of 10.41 degrees latitude (Figure 2.1A; Table S2.1). Using a Principal Component Analysis, we found a large amount of variation both within and among populations of *P. incarnata* with no clear separation of populations in multivariate space (Figure S2.2B). We further investigated variation in metabolic profiles through the use of pairwise Euclidean distances (ED) calculations as a means of estimating "metabolic distance" (Li et al.

2015). Using deconvoluted spectra, we found a substantial amount of variation both within and among populations in both undamaged and damaged leaves (Figure S2.6; Figure S2.7). Within populations, we found ED measurements ranging from 0.733 – 7.998 for undamaged leaves (Figure S2.6) and 0.7154 – 8.457 for damaged leaves (Figure S2.7). To assess if metabolic distance changes the further populations are from one other, we calculated absolute geographic distances among populations using latitude and compared that to inter-sample ED. We found that there is no pattern of metabolic distance as the distance among plant populations increased for both undamaged (Pearson correlation coefficient = 0.0013; P = 0.9653) and damaged leaves (Pearson correlation coefficient = -0.0207; P = 0.4876).

POP Experiment: NMR features associated with lepidopteran interactions show sporadic distributions over latitude

We were interested in testing predictions of the Latitudinal Herbivory-Defense Hypothesis using features that are involved in lepidopteran interactions. Specifically, we determined which NMR features showed differences when exposed *C. includens* (generalist herbivore; Figure 2.4B green portion of Venn diagram), *A. vanillae* (specialist herbivore; Figure 2.3B orange portion of Venn diagram), both *C. includens* and *A. vanillae* (Figure 2.4B purple portion of Venn diagram), or were strongly correlated to herbivory from *C. includens* (Figure 2.5A). We detected a total of 120 features that were significantly different between lepidopteran herbivores treatments and undamaged control and a total of 48 features that significantly correlated with herbivory from *C. includens*. We rigorously screened these features against our POP experimental dataset to determine what features appeared in both datasets and were not overlapped with other

features. This screening resulted in us identifying a total of 43 features that were shared between both the DAM and POP data sets. We were able to identify a total of 18 metabolites, while the remaining 25 were unknown features (Table 2.1). Identified metabolites included protein forming amino acids, organic acids, a nucleic acid, sugars, an alkaloid, two cyclopentenoids, and many unknown features (Table 2.1). Of these 43 metabolites and unknown features, 37 were derived from comparing herbivore treatments to control (Figure 2.4C) and 6 were derived from correlations from the *C. includens* herbivory analysis (Figure 2.5A). Only 1 was in common to both of those analyses, which was isocyclopentenylglycine (Table 2.1).

We investigated linear trends of all 43 metabolites and unknown features over latitude and found that 15 had significant linear trends over latitude. However, a total of 28 did not show such a relationship, including the cyanogenic glycoside precursor cyclopentenylglycine. Of the 15 metabolites and unknown features with latitudinal clines, 7 had a negative trend with increasing latitude (Figure 2.6A), including multiple unknown features and the amino acid proline. A total of 8 metabolites and unknown features showed a positive trend with increasing latitude including shikimic acid, aspartic acid, myo-Inositol, and the cyclopentenoid isocyclopentenylglycine (Figure 2.6B).

Metabolites and unknown features associated with differences when exposed to *C. includens* (generalist herbivore; Figure 2.4B green) or differences when exposed to both *C. includens* and *A. vanillae* (Figure 2.4B purple) had mixed patterns over latitude. However, the unknown features associated with herbivory from the generalist species *C. includens* (Figure 2.5A) that had significant latitudinal trends all increased with increasing latitude. Included in this group is isocyclopentenylglycine. Finally, we found

that proline, the single metabolite associated with differences when exposed to *A. vanillae* (specialist herbivore; Figure 2.4B orange), had a negative trend with increasing latitude.

POP Experiment: Induction of NMR features is greater at high-latitudes

To assess if induced responses vary over latitude, we evaluated differences in signal intensity between undamaged and damaged leaves from high and low latitudinal regions (Figure 2.7; Table S2.1). Of the 43 metabolites and unknown features identified as being involved in lepidopteran interactions, we found a total of 13 showed significantly induced changes in plants from high-latitude populations. Among the metabolites that showed significant induced changes for the high-latitude populations were branch chain amino acids such as valine, isoleucine, leucine, the organic acid fumarate, the nucleic acid uracil, and the cyclopentenoid isocyclopentenylglycine. Only 5 metabolites and unknown features showed significantly induced changes in plants from low-latitude populations, including myo-Inositol and uracil. Interestingly, only uracil showed significantly induced changes between undamaged and damaged leaves in both high and low-latitudinal populations.

Discussion

Here, we evaluated the geographic distribution of leaf metabolites in *Passiflora incarnata* (purple passionflower) that are involved in lepidopteran interactions. We first evaluated which metabolites and unknown features showed significant differences between two different herbivore species treatments and undamaged control. We additionally evaluated which metabolites and unknown features were correlated with leaf

damage from these two species. We then determined if these metabolites and unknown features displayed latitudinal clines, as well as clines in induction, across *P. incarnata* populations that span 10.04 degrees in latitude across the southeastern United States. Using this untargeted metabolomics experimental approach, we were able to test specific predictions of the Latitudinal Herbivory-Defense Hypothesis as well as identify many unknown and known metabolites that show interesting ecological interactions. Notably, we identified two precursors to cyanogenic glycoside biosynthesis in *Passifora*, the cyclopentenoids isocyclopentenylglycine and cyclopentenylglycine.

Variation in the distribution of metabolites over latitude

The Latitudinal Herbivory-Defense Hypothesis predicts that due to increased selection from biotic sources at low-latitudes, plant defenses will be greater at low-latitudes relative to high-latitudes. In this study, we identified metabolites and unknown features involved in lepidopteran interactions using two different forms of criteria. 1) We identified metabolites and unknown features that were different between herbivore damaged leaves and undamaged leaves and 2) by correlating NMR features to amount of herbivory from each herbivore species. Only a single metabolite, isocyclopentenylglycine, matched both of these criteria. From this, we found mixed results consistent with predictions of the Latitudinal Herbivory-Defense Hypothesis, however, the roles in defense these metabolites provide may explain, in part, the patterns we observed.

Of the 43 metabolites and unknown features that were involved in lepidopteran interactions, we found mixed patterns for their distributions over latitude: some increased in abundance, others decreased over latitude, and many did not show any patterns over

latitude. Indeed, we found that of the 43 metabolites and unknown features identified, only 15 showed any latitudinal pattern. Of these 15, we identified 6 unknown features that were more abundance at low-latitudes, while 4 unknown features were more abundant at high-latitudes. All of these unknown features are ideal candidates to pursue by natural product chemists interested in describing biologically relevant metabolites. We also annotated some metabolites with interesting patterns over latitude. These metabolites include shikimic acid, aspartic acid, isocyclopentenylglycine and myo-inositol, which increased in abundance with increasing latitude. Only a single metabolite, proline, had a negative cline over latitude, which is consistent with predictions of the Latitudinal Herbivory-Defense Hypothesis.

Of the metabolites that did show trends over latitude, some have known functions in plant defense and/or stress response. The metabolite myo-Inositol is involved in many cellular processes such as biogenesis of cell wall and membrane structures, phosphate storage, cell signaling, and stress responses such as plant tolerance to salt, cold, and UV stress (Loewus and Murthy 2000, Valluru and Van den Ende 2011, Lytvyn et al. 2016). *Arabidopsis atips1* mutants that were unable to accumulate as much myo-Inositol relative to wild-type showed changes in the constitutive activity of biotic stress response genes, indicating that myo-Inositol could play broad roles in plant stress responses (Meng et al. 2009, Bruggeman et al. 2014). Another metabolite with a latitudinal cline that we identified is shikimic acid. Shikimic acid is a precursor to many different types of secondary metabolites such as phenolics and indolic alkaloids (Bennett and Wallsgrove 1994). Furthermore, it is estimated that as much as 20% of the total fixed carbon flows through the shikimate pathway, and an increase in this flow can occur in response to

general stress (Tohge et al. 2013). Shikimic acid therefore plays an important role in stress responses, including those from natural enemies (Bennett and Wallsgrove 1994). The observation that both of these metabolites are involved in general stress responses suggests that the positive latitudinal clines we observed may be due to selection from increased abiotic stress at higher latitudes, although further studies would be needed to determine this.

Aspartic acid is a major amino acid involved in a myriad of plant cellular functions (Kirma et al. 2012). Included in this is the role aspartic acid has in plant defense priming against natural enemies (Zeier 2013). Additionally, aspartic acid is a product of a detoxification pathway plants utilize when free HCN is released at the end of cyanogenesis (Gleadow and Møller 2014). The turnover rate of HCN to aspartic acid can be quite rapid (Jenrich et al. 2007, Gleadow and Møller 2014), therefore, one possibility is that the increased aspartic acid observed at high-latitudes is related to high cyanogenic glycoside capacity observed in other studies (Chapter 3). We did not measure cyanogenic glycoside capacity in the same individuals used for these metabolomics studies, therefore we cannot experimentally determine this. Thus, future studies should focus on connecting aspartic acid to HCN detoxification in *P. incarnata*.

Finally, we also observed that proline abundance varied over latitude with higher amounts at low-latitudes. Proline plays are its major roles in stress responses (Hayat et al. 2012). Proline has been identified as a major contributor in plant mechanisms to cope with environmental stresses and has been identified as an antioxidative defense molecule (Smirnoff and Cumbes 1989) as well as a major signaling molecule (Hayat et al. 2012). Thus, the latitudinal pattern we observed here, greater proline abundance at low-latitude,

may be due to some other ecological role. Ultimately, all metabolites identified in this study will need to be explored further to unraveling the evolutionary and ecological forces that are contributing to geographic variation in their abundance.

A caveat to the first criteria is that metabolites and unknown features that showed a significant difference in response to herbivores are not necessarily directly involved in plant defense. However, they may be involved in priming a defensive response (Karban 2011). Indeed, little is known regarding the ecological forces that shape variation in plant defense priming and therefore it is still informative to investigate the metabolites and unknown features identified through this approach over latitude (Martinez-Medina et al. 2016).

Variation in induced responses across high & low latitudinal regions

One prediction of the Latitudinal Herbivory-Defense Hypothesis is that plants at lower latitudes will evolve greater constitutive resistance if they indeed experience consistently high herbivory. In contrast, plants at higher latitudes are predicted to evolve greater inducibility if herbivory is less frequent and or predictable, and thus inducibile defenses will evolve as a means of mitigating the costs of defenses. We tested this prediction by comparing inducibility of plants from high vs low-latitudinal regions for all metabolites identified in this study that are involved in lepidopteran interactions. From this, we found evidence in support of these predictions, as plants from high-latitude populations had a total of 13 metabolites and unknown features that showed induced changes while plants from low-latitude populations had a total of only 5 metabolites and unknown features that showed induced changes. Interestingly, many of the induced changes for the high-latitude plants were for metabolites and unknown features identified

as being involved in exclusively generalist herbivore interactions (a total of 6). Given that we expect generalist herbivores to make up a relative greater proportion of the herbivore community at high-latitudes (Chapter 3), then this result suggests that plant populations may evolve the ability to respond appropriately to the type of herbivores they encounter more frequently. Finally, only one of the induced changes were shared among the high and low-latitudinal region regions. This suggest there may be variation in the genetic control of induced changes in metabolites that are segregating among populations in nature. Future studies should look into this possibility.

Of the metabolites that showed induced changes, several are known to be altered due to biotic stress in other plant systems. For example, we observed induced changes in amino acid quantities in this study. Amino acids are nitrogen rich metabolites and therefore are important nutritional sources of nitrogen in herbivore diets (Zhou et al. 2015). Thus, plants may alter the abundance of amino acids when damaged in order to deprive herbivores of these limited nutrients (Schwachtje et al. 2018). Future studies should look into this more deeply.

Interactions with a specialist and a generalist herbivore species

We found that the metabolomics response of *Passiflora incarnata* differed when the individual interacted with generalist herbivores compared to those that interacted with specialist herbivores. Specifically, we found both positive and negative correlations of various metabolites and the amount of leaf area eaten from the generalist herbivore, *Chrysodeixis includens*. However, we did not find any such correlations for leaf area eaten from the specialist herbivore, *Agraulis vanillae*. Furthermore, we found a larger proportion of metabolites were altered when plants were fed upon by *C. includens*

relative to when they were fed upon by *A. vanillae* (49 and 6, respectively). Asymmetry in induced plant chemical responses to generalist and specialist herbivores and how these types of herbivores respond to these metabolites are common (Ali and Agrawal 2012). Indeed, there are many examples where plant metabolites negatively impact generalist herbivores, yet have negligible effects on specialist herbivores (Whittaker and Feeny 1971). Moreover, one prediction of plants interactions with generalist vs specialist herbivores is that plants should respond as strongly as possible to most generalists is consistent with results reported here (Ali and Agrawal 2012). Thus, our results in this study comparing a generalist and a specialist species are not atypical.

It is important to note that in this study we only included one generalist herbivore and one specialist herbivore. Thus, we do not know if the alteration in metabolites and their relationship to leaf herbivory is broadly associated with these different life history strategies in herbivores or if they are specific to these two taxa (Ali and Agrawal 2012). Another interesting result of this study is the observed variation in induced changes of metabolites identified between both of the experiments. One potential explanation for this discrepancy is that plants used in both studies differed in the way they were generated. For the population study, we collected rhizomes from the field and clonally propagated them to use in the greenhouse study (Table S2.1). It is possible that maternal effects from plants in the field could have still had effects on metabolic phenotypes were quantified in the population study. In contrast, plants utilized in the damage experiment were generated after two generations of crosses in the greenhouse. Thus, maternal effects would have been drastically reduced given similar maternal environments in which plants were grown. Furthermore, any type of genetic control of metabolic phenotypes through

dominance or epistasis would have been reduced in the F2 population utilized in the damage study while this type of variation that could be fixed among populations in the population study would have an impact on metabolic phenotypes.

Metabolites involved in cyanogenic glycoside biosynthesis

Although cyanogenic glycosides are produced in plant species that span many different plant families (Gleadow and Møller 2014), the large diversity of structures of these chemicals in plants from the Passifloraceae is noteworthy (Spencer 1988). Furthermore, butterflies in the tribe heliconiine have multiple adaptations that counter the negative effects of cyanogenic glycosides. These adaptations include their ability to tolerate and sequester these metabolites (Engler et al. 2000, Engler-Chaouat and Gilbert 2007), and in certain species they even have evolved the ability to de novo synthesize their own (Engler-Chaouat and Gilbert 2007). These observations have led some to hypothesize that these metabolites may mediate coevolutionary interactions between these two lineages (Spencer 1988). The structural diversity of cyanogenic glycosides in the Passifloraceae is in part due to the variety of amino acid precursors from which these molecules are biosynthesized from (Gleadow and Møller 2014). Many cyanogenic glycosides are derived from common amino acids such phenylalanine, tyrosine, valine, isoleucine, and leucine. However, many cyanogenic glycosides in the Passifloraceae are derived from the less common, non-protein forming amino acid group called cyclopentenoids (Forslund et al. 2004). In this study, we identified two different amino acid precursors that are a part of the cyclopentenoids group (Figure 2.3B). These amino acids are similar in structure, but differ in the position of the amino group that is attached to the cyclopentenyl 5-carbon ring (Clausen et al. 2002). Based upon the well resolved

process of biosynthesis of cyanogenic glycosides from amino acids (Gleadow and Møller 2014), we predict that *P. incarnata* is actually producing multiple forms of cyanogenic glycosides based upon the variation in the precursors. Traditionally, *P. incarnata* is thought to only produce a single cyanogenic glycoside, gynocardin (Spencer 1988). Future work should aim at testing this prediction.

Finally, we also observed a marked decrease in isocyclopentenylglycine production when lepidopteran herbivores. This change in response to herbivore attack seems to vary somewhat across latitude where high-latitude populations show this general pattern for both precursors, while low-latitude plants do not. The alteration in the production and the overall differences in isocyclopentenylglycine quantity across latitude in this study could be indicative of adaptive evolution. Due to the known adaptations of specialist lepidopteran species of *Passiflora* to cyanogenic glycosides, a decrease in production of these metabolites may be a response to the stimuli from these herbivores. It is therefore possible that these plants alter their production of these chemicals as a means to mitigate costs of these metabolites. Costs may manifest in the form of allocation costs (e.g., the use of energy to produce something that provides no fitness advantage in return) and/or in the form of ecological costs (Strauss et al. 2002). An ecological cost that may be associated with the cyclopentenoid metabolites in our study may be that they are oviposition cues. Indeed, in other plant taxa that are acyanogenic, but still produce these precursors, butterflies still are able to seek them out and oviposit on them (Clausen et al. 2002). These same butterflies also oviposit on plants in the Passifloraceae, therefore, these authors speculate that the cyclopentenoids are actually the cue these herbivores use to identify host plants (Clausen et al. 2002). Thus, a decrease in the production of these

metabolites may be favorable under certain ecological conditions, especially since specialist herbivores seem to be unaffected by this defensive metabolite. These reports are tantalizing and future studies will need to unravel the role cyclopentenoids play in host recognition.

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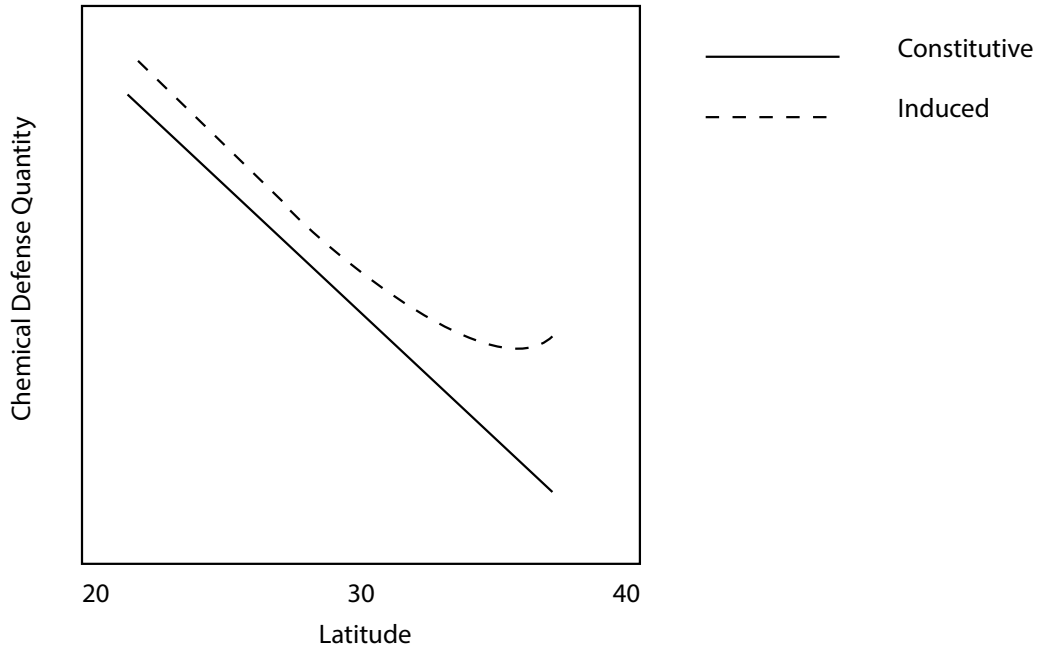

Figure 2.1)

Predictions of the Latitudinal Herbivory-Defense Hypothesis on the concentration of plant chemical defenses across latitude with modified predictions of a trade-off between induced and constitutive defenses based upon (Anstett et al. 2016a).

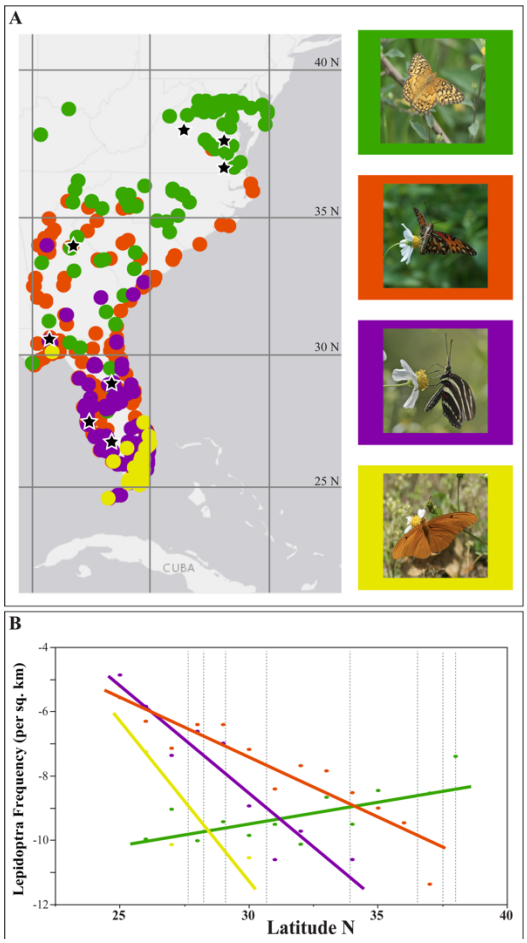

Figure 2.2)

Populations of *Passiflora incarnata* sampled across the southeastern United States and their corresponding lepidoptera herbivore communities. **A)** Populations of *P. incarnata* sampled across the southeastern United States. Populations are distinguished with black stars with populations at the extremes of the distribution (Florida and Virginia) used to generate the F2 full-sib families in the study on damage response. Colored dots indicate observation data from the Global Biodiversity Information Database (gbif.org) for lepidoptera that utilize *P. incarnata* as a host plant. Colored dots are representative of reported sightings of *Euptoieta claudia* (green), *Agraulis vanillae* (orange), *Heliconius charithonia* (purple), and *Dryas iulia* (yellow). **B)** Quantitative representation of lepidoptera that feed on *Passiflora incarnata* across latitude. Specialist lepidoptera frequency was standardized by land area (sq. km) over latitudinal degrees. Vertical dotted lines match the latitudinal degree for populations of *P. incarnata* used in this study.

Gulf Fritillary: http://doi.org/10.15468/dl.ji9ffs
Zebra Heliconia: http://doi.org/10.15468/dl.pgsbdx
Julia Heliconia: http://doi.org/10.15468/dl.mudnty
Variegated Fritillary: http://doi.org/10.15468/dl.xjecor

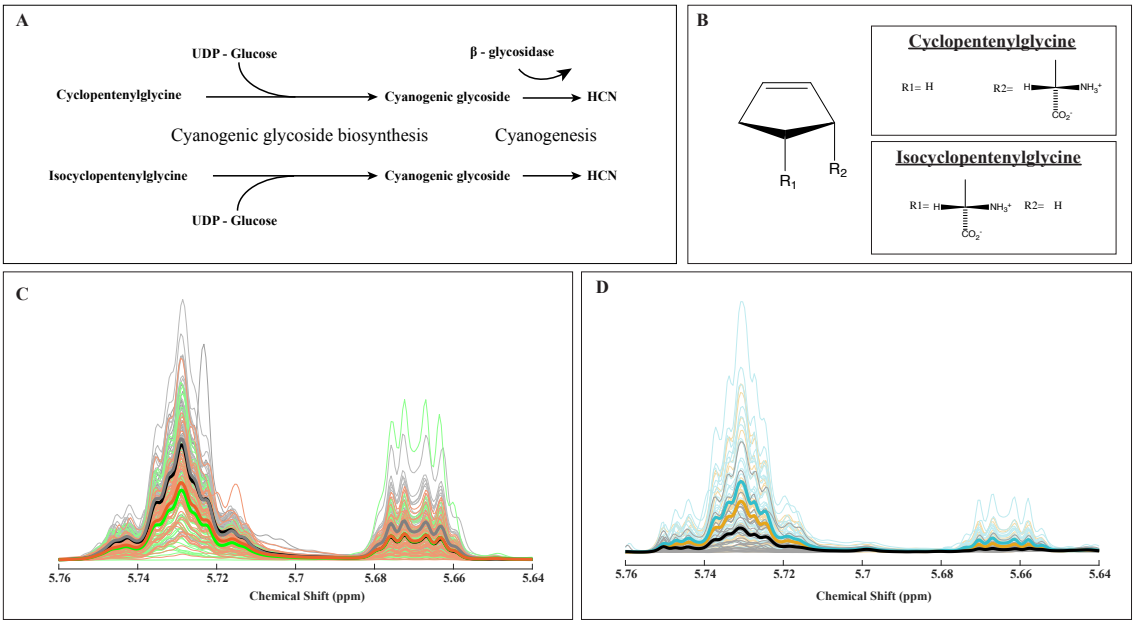

Figure 2.3)

Cyclopentenoids identified in *Passiflora incarnata* and their role in cyanogenic glycoside biosynthesis and bioactivation. **A)** Model of biosynthesis and bioactivation of cyanogenic glycosides in *P. incarnata*. Cyanogenic glycosides are synthesized from cyclopentenoid precursors where the final step is the addition of UDP-glucose. Cyanogenesis occurs upon the reaction between β-glycosidase and cyanogenic glycoside. The ultimate product of this reaction results in the release of hydrogen cyanide (HCN). **B)** Structures of the two forms of cyclopentenoids identified in *P. incarnata*. **C & D)** NMR spectra of cyclopentenoids in the damage (DAM) and population (POP) experimental datasets. Distinctive chemical shifts are 5.66 ppm for cyclopentenylglycine and 5.73 ppm for isocyclopentenylglycine. Bold lines denote treatment means and thin lines represent real sample spectra. **C)** NMR spectra from DAM. Colors correspond to undamaged control (black), mechanical damage (grey), generalist herbivore damage of *C. includens* (green), and specialist herbivore damage of *A. vanillae* (orange). **D)** NMR spectra from POP. Colors correspond to high (gold), intermediate (teal), and low (black) latitudinal regions (Table S2.1).

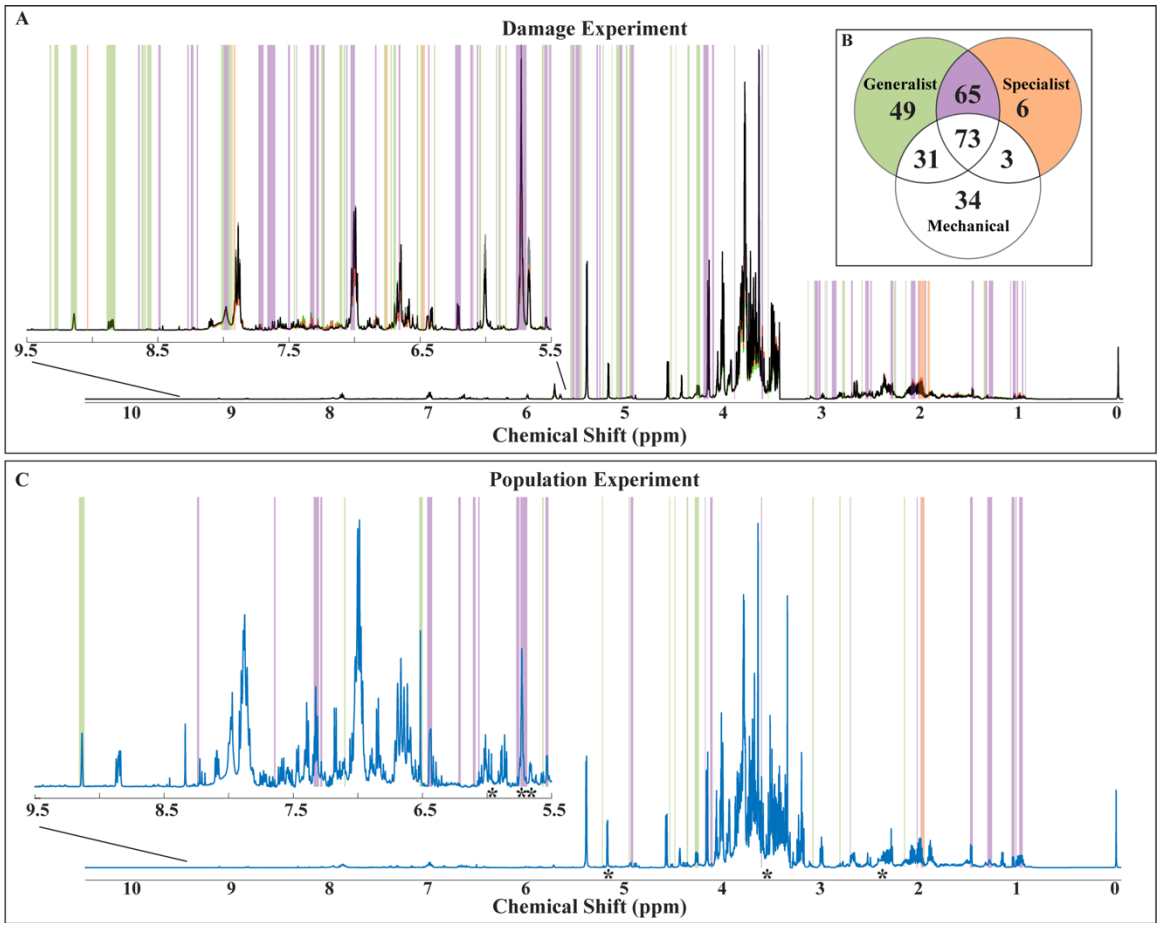

Figure 2.4)

^{1}H-NMR features that are associated with biotic interactions in *Passiflora incarnata* leaves. **A)** Average ^{1}H-NMR profile of *P. incarnata* leaves from undamaged (black), mechanically damaged (grey), damaged by a generalist lepidoptera (green), and damaged by a specialist lepidoptera (orange) treatments. Colored bars over spectra represent metabolites that significantly differ for herbivore treatments only [both specialist and generalist (purple); generalist only (green); specialist only (orange)] or are associated with general damage (black). **B)** Venn diagram depicting number of metabolites that significantly change between control and all three damage treatments. Numbers indicate the number of features which are significantly different to control after Benjamini-Hochberg FDR correction. **C)** Average ^{1}H-NMR spectrum from *P. incarnata* leaf samples from different populations. Bars over spectrum indicate features that were present in both studies that are associated with biotic interactions.

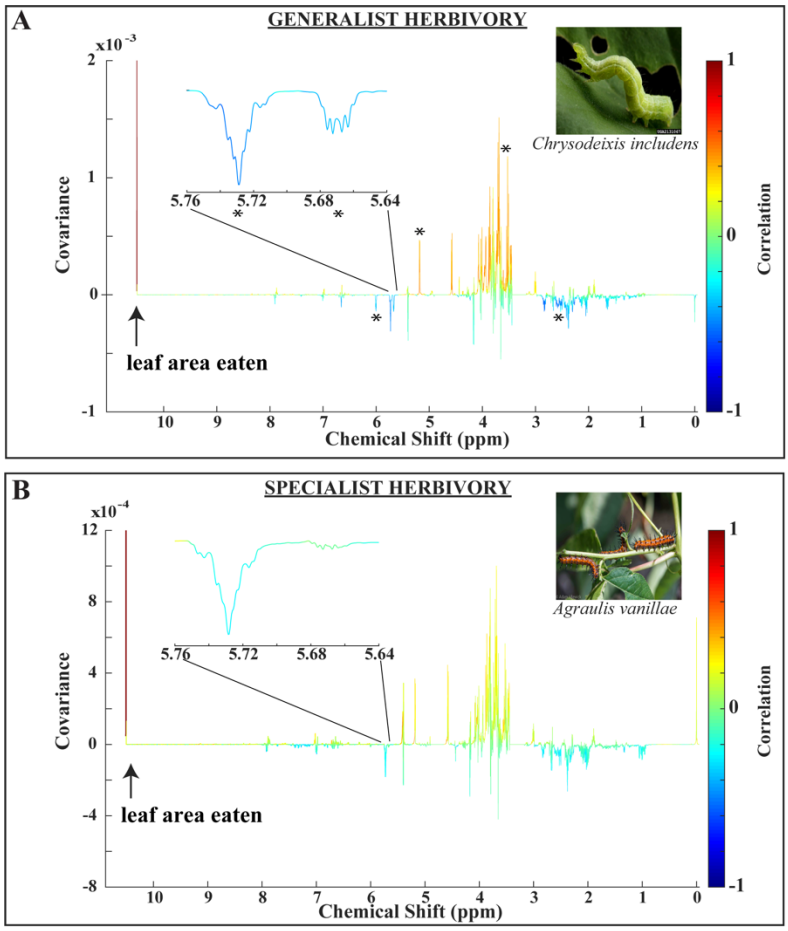

Figure 2.5)

Statistical Total Correlation Spectroscopy (STOCSY) of herbivore damage indicating ^{1}H-NMR features that correlate with leaf area eaten standardized by initial herbivore mass. **(A)** Generalist herbivore damage from *Chysodeixis includens.* * indicate features that were significantly associated with damage that were utilized for the POP dataset. **(B)** Specialist herbivore damage from *Agraulis vanillae*. Zoomed in insets focus in both A & B show features associated with cyclopentenylglycine and isocyclopentenylglycine (5.66ppm & 5.73ppm, respectively).

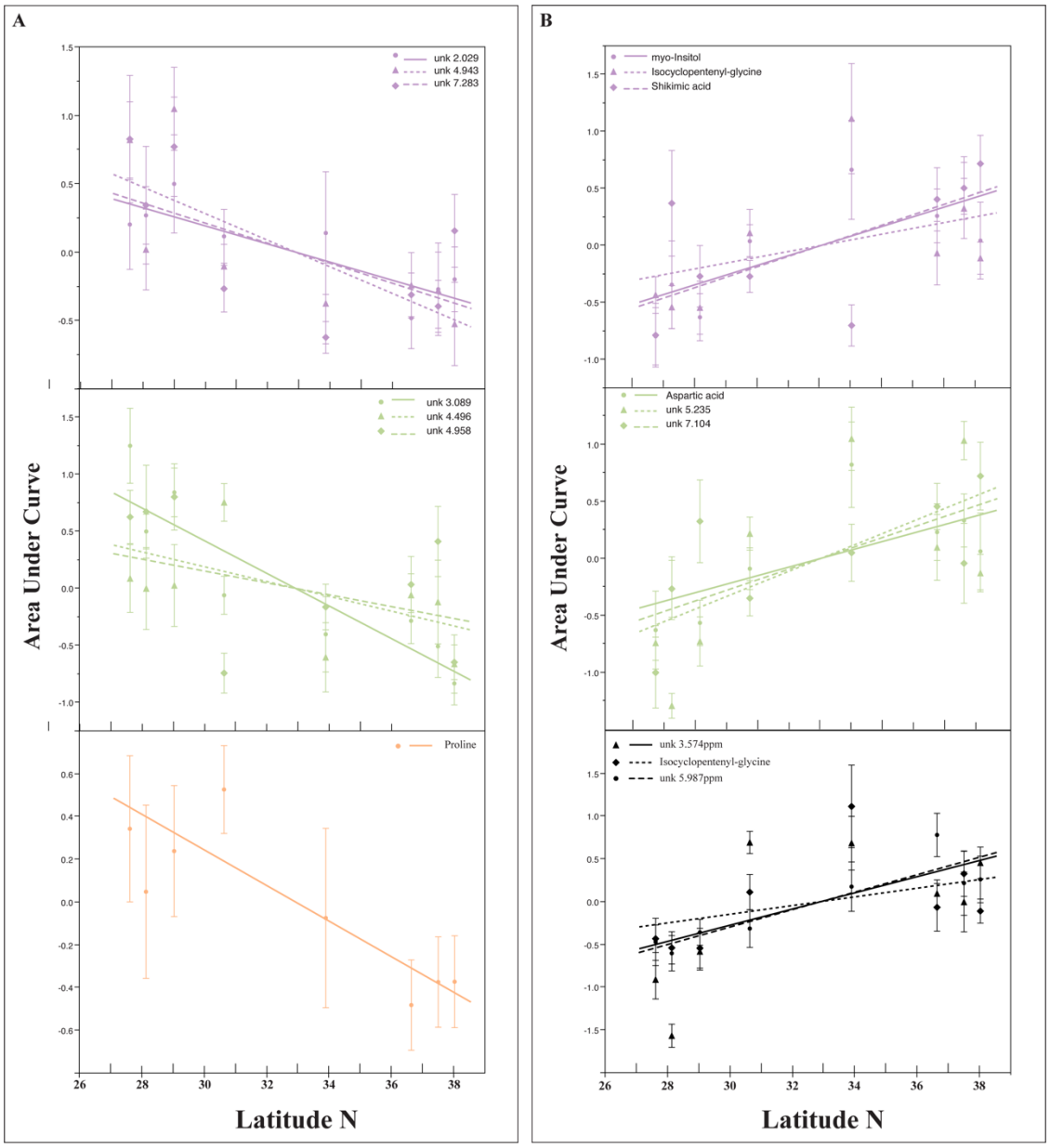

Figure 2.6)

Metabolites and unknown features related to lepidopteran interactions that had significant trends over latitude. Colors correspond to the type of lepidopteran interaction that each metabolite and unknown feature was identified through (purple = both generalist and specialist; green = generalist only; orange = specialist only; and black = generalist damage) **A)** Metabolites and unknown features that had a significant negative linear trend over latitude. **B)** Metabolites and unknown features that had a significant positive linear trend over latitude. Error bars represent one standard error.

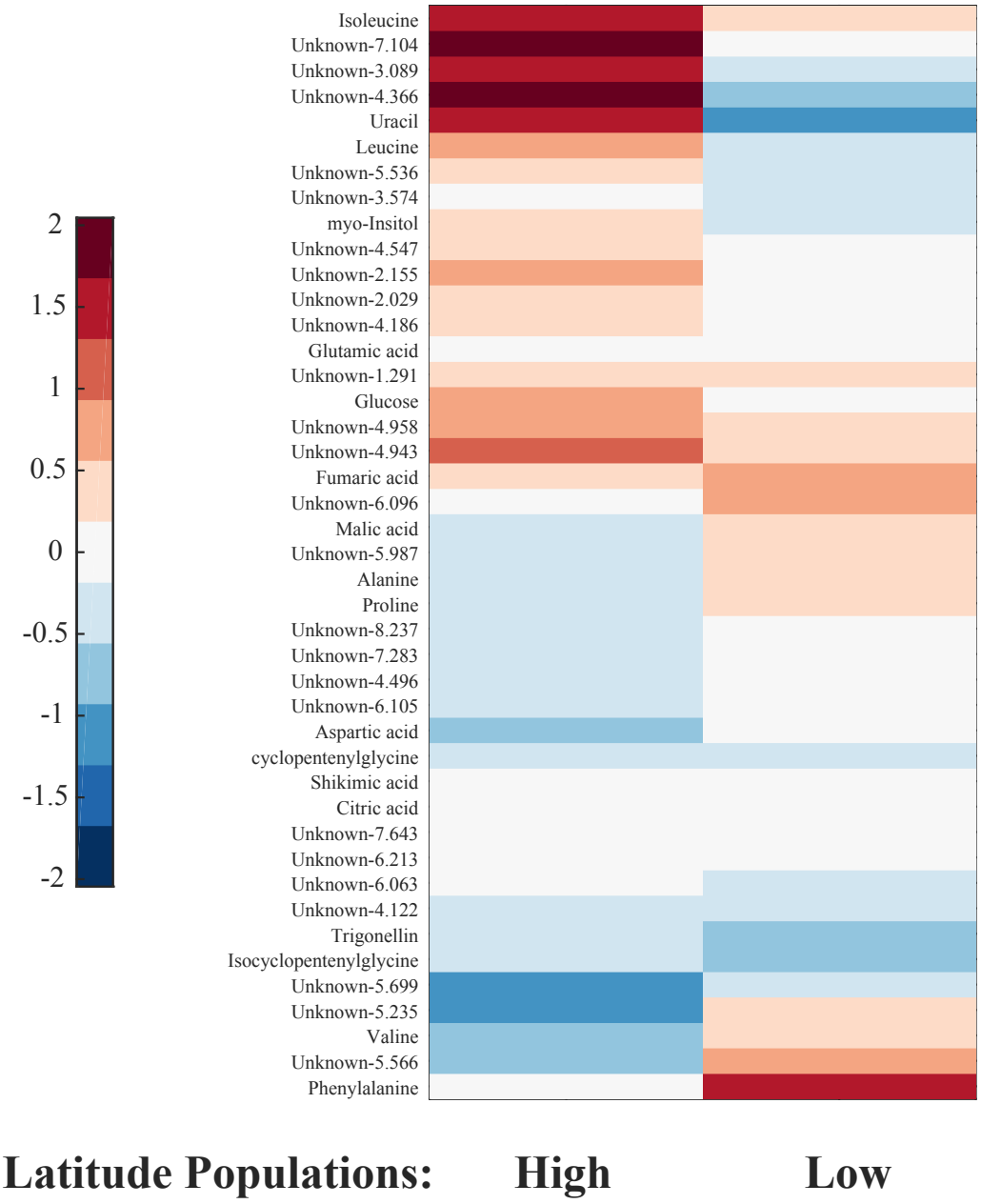

Figure 2.7)

Induced changes for high and low-latitudinal regions in metabolites and unknown features that are involved in lepidopteran herbivore interactions. Colors are based upon Z-transformed values from (damage – undamaged) leaves.

Table 2.1)

Metabolites and unknown features associated with various damage treatments with corresponding ppm values and ppm range used to determine bounds for integration of various metabolites. This range value was determined through the use of SRV. Biotic interaction category represents the damage treatment(s) in which a metabolite significantly changed relative to the undamaged control. Mean fold change is the log2 changed in treatment/control for each metabolite reported with corresponding FDR corrected p-values. Latitudinal trend reports p-values associated with metabolite patterns over latitude. Fold change p-value is associated with induction changes within high and low-latitudinal regions. Confidence in metabolite annotation is indicated by the confidence score. Bold numbers indicate statistical significance.

				Mechanical Damage		Genearlist Herbivore		Specialist Herbivore			Fold Change *p-value*		
Metabolite Name	**Biotic Interaction Category**	**Chemical shift (ppm)**	**ppm range**	**Fold Change**	***FDR***	**Fold Change**	***FDR***	**Fold Change**	***FDR***	**Latitudinal Trend *p-value***	**High-latitude**	**Low-latitude**	**Confidence Score**
Unknown-4.943	Generalist & Specialist	4.943	4.92-4.946	0.089	0.590053	0.391	**0.000536**	0.259	**0.020772**	**0.000104**	**0.010146**	0.541302	-
Shikimic acid	Generalist & Specialist	6.441	6.425-6.462	-0.059	0.771267	-0.603	**0.000014**	-0.358	**0.007142**	**0.000280**	0.185568	0.623554	4
myo-Insitol	Generalist & Specialist	3.612	3.609-3.617	-0.023	0.731958	0.182	**0.000015**	0.200	**0.000230**	**0.000670**	0.272488	**0.025815**	4
Unknown-7.283	Generalist & Specialist	7.283	7.275-7.289	-0.278	0.193465	0.615	**0.000250**	0.322	**0.087651**	**0.004383**	0.373194	0.173254	-
Unknown-2.029	Generalist & Specialist	2.029	2.023-2.031	0.089	0.247845	0.148	**0.042545**	0.182	**0.004189**	**0.009896**	**0.046432**	0.283662	-
Isocyclopentenylglycine	Generalist & Specialist / Generalist Herbivory	5.731	5.703-5.741	0.068	0.673030	-0.721	**0.000037**	-0.533	**0.000976**	**0.041043**	**0.044598**	0.051246	2
Unknown-7.643	Generalist & Specialist	7.643	7.638-7.648	-0.233	0.220002	-0.411	**0.017678**	-0.312	**0.058430**	0.052254	0.321517	0.169602	-
Citric acid	Generalist & Specialist	2.706	2.704-2.71	0.223	0.110313	0.309	**0.016307**	0.312	**0.029090**	0.053153	0.599627	0.731157	4
Unknown-4.186	Generalist & Specialist	4.186	4.183-4.189	-0.061	0.463324	0.167	**0.015804**	0.166	**0.020772**	0.081928	0.355286	0.762716	-
Unknown-6.063	Generalist & Specialist	6.063	6.058-6.067	-0.049	0.767537	-0.270	**0.017140**	-0.180	**0.060247**	0.159071	0.436073	**0.023932**	-
Unknown-4.122	Generalist & Specialist	4.122	4.111-4.135	-0.068	0.570244	-0.223	**0.003167**	-0.140	**0.080020**	0.173824	0.823101	0.945931	-
Unknown-5.536	Generalist & Specialist	5.536	5.525-5.546	-0.035	0.627369	-0.099	**0.048575**	-0.096	**0.088667**	0.224508	0.086995	**0.042405**	-
Phenylalanine	Generalist & Specialist	7.325	7.301-7.341	0.516	0.114155	1.367	**0.000001**	1.130	**0.000004**	0.273803	0.081314	0.060282	4
Alanine	Generalist & Specialist	1.477	1.461-1.487	0.057	0.679950	0.203	**0.016408**	0.176	**0.086286**	0.287098	0.057421	0.529121	4
Leucine	Generalist & Specialist	0.9692	0.953-0.986	0.315	0.309043	0.638	**0.005615**	0.626	**0.003322**	0.326742	**0.035737**	0.061373	4
Uracil	Generalist & Specialist	5.757	5.749-5.772	0.172	0.147505	-0.452	**0.003438**	-0.345	**0.011019**	0.333526	**0.029974**	**0.008909**	4
Isoleucine	Generalist & Specialist	1.022	1.017-1.025	0.351	0.110929	0.704	**0.000327**	0.610	**0.001733**	0.416224	**0.042854**	0.082809	4
Unknown-1.291	Generalist & Specialist	1.291	1.265-1.311	0.360	0.198273	0.654	**0.000046**	0.502	**0.049689**	0.502660	0.280534	0.742833	-
Valine	Generalist & Specialist	1.049	1.035-1.064	0.286	0.178721	0.764	**0.000048**	0.710	**0.000128**	0.535495	**0.028896**	0.114884	4
Unknown-6.096	Generalist & Specialist	6.096	6.089-6.102	-0.116	0.497890	-0.477	**0.000010**	-0.364	**0.003162**	0.591562	0.533238	0.691723	-
Unknown-5.699	Generalist & Specialist	5.699	5.689-5.703	-0.064	0.728337	-0.711	**0.000002**	-0.401	**0.014068**	0.645184	**0.046152**	0.050740	-
Unknown-6.213	Generalist & Specialist	6.213	6.203-6.222	-0.195	0.489106	-1.509	0.000000	-1.193	**0.000044**	0.697245	0.636983	0.770255	-
Unknown-8.237	Generalist & Specialist	8.237	8.23-8.244	-0.112	0.489106	-0.290	**0.020663**	-0.295	**0.001765**	0.779833	0.744529	0.836908	-
Unknown-6.105	Generalist & Specialist	6.105	6.102-6.108	-0.145	0.612362	-0.592	**0.000549**	-0.351	**0.040003**	0.994589	0.556741	0.506801	-
Unknown-3.089	Generalist Only	3.089	3.083-3.093	0.063	0.792911	0.304	**0.052523**	0.162	0.426285	**0.000000**	0.110808	0.384334	-
Unknown-5.235	Generalist Only	5.235	5.23-5.238	0.133	0.360860	0.205	**0.069011**	0.154	0.291552	**0.000011**	0.125625	0.823241	-
Unknown-7.104	Generalist Only	7.104	7.097-7.105	0.119	0.220002	0.175	**0.032893**	0.037	0.738826	**0.000152**	**0.003262**	0.901696	-
Aspartic acid	Generalist Only	2.814	2.809-2.818	-0.078	0.312994	-0.135	**0.024105**	0.004	0.968423	**0.003695**	0.923606	0.790487	4
Unknown-4.496	Generalist Only	4.496	4.492-4.499	0.030	0.751865	0.138	**0.042545**	0.088	0.179619	**0.012249**	0.960414	0.550615	-
Unknown-4.958	Generalist Only	4.958	4.955-4.964	-0.092	0.342911	0.213	**0.031032**	0.146	0.122393	**0.041645**	**0.014670**	0.233472	-
Malic acid	Generalist Only	4.274	4.252-4.292	-0.033	0.905841	-0.241	**0.046772**	0.003	0.931812	0.090180	0.434173	0.961896	4
Unknown-5.566	Generalist Only	5.566	5.563-5.571	0.095	0.489106	0.172	**0.091358**	0.114	0.393382	0.210758	0.155290	**0.020598**	-
Unknown-4.547	Generalist Only	4.547	4.543-4.55	0.106	0.239115	0.222	**0.010761**	0.089	0.319725	0.282714	0.690913	0.987165	-
Trigonellin	Generalist Only	9.137	9.116-9.158	-0.186	0.128080	-0.317	**0.000965**	-0.146	0.163714	0.365131	0.051678	0.097411	4
Fumaric acid	Generalist Only	6.517	6.498-6.525	0.114	0.698718	-0.352	**0.048401**	-0.088	0.813201	0.365313	**0.004729**	0.082020	3
Unknown-2.155	Generalist Only	2.155	2.152-2.159	0.096	0.253326	0.132	**0.092709**	0.076	0.349784	0.400540	0.349958	0.362042	-
Unknown-4.366	Generalist Only	4.366	4.362-4.375	0.096	0.607343	0.390	**0.000633**	0.220	0.199097	0.506852	**0.008821**	0.309166	-
Unknown-5.987	Generalist Herbivory	5.987	5.982-5.99	0.091	0.230201	-0.007	0.976741	0.095	0.291552	**0.000029**	**0.040846**	0.320316	-
Unknown-3.574	Generalist Herbivory	3.574	3.568-3.578	-0.114	**0.046774**	-0.161	**0.001750**	-0.120	**0.019843**	**0.000198**	0.897628	0.086501	-
Glucose	Generalist Herbivory	5.183	5.169-5.195	-0.514	**0.000987**	-0.552	**0.000088**	-0.384	**0.005620**	0.068532	0.294921	0.499773	4
cyclopentenylglycine	Generalist Herbivory	5.664	5.649-5.677	0.575	**0.000987**	0.003	0.827335	0.051	0.812690	0.114048	0.093560	0.591926	2
Glutamic acid	Generalist Herbivory	2.374	2.361-2.394	0.072	0.464676	-0.080	0.410668	0.013	0.931812	0.938386	0.185257	0.457215	5
Proline	Specialist Only	1.979	1.953-1.992	-0.046	0.852601	0.233	0.116812	0.256	**0.060430**	**0.001149**	0.365908	0.647955	4

CHAPTER 3

LATITUDINAL TRENDS IN TRICHOME DENSITY, LEAF TOUGHNESS, AND CYANOGENIC GLYCOSIDE CAPACITY IN *PASSIFLORA INCARNATA* IS ASSOCIATED WITH SPECIALIST LEPIDOPTERAN DISTRIBUTION

Introduction

Host plants and the insect herbivores that feed on them are often thought to be in a coevolutionary dynamic (Rausher 2001). Indeed, coevolution is thought to have generated a tremendous degree of phenotypic diversity in both interacting partners (Marquis 1992, Mauricio and Rausher 1997, Agrawal 2011), including plant defensive traits such as trichomes, spines, latex, and secondary chemicals (Levin 1973, Mauricio 1998, Rausher 2001, Rosenthal and Berenbaum 2012, Speed et al. 2015). Thus, when interactions between coevolving plant and insect herbivore species vary over geographic space, the defensive traits that mediate these interactions can also vary over this space (Thompson 2005).

Moreover, plant-herbivore interactions are specifically predicted to vary substantially across latitudinal gradients (Coley and Barone 1996). Natural selection from biotic interactions is predicted to be greater at lower latitudes, which is in part due to longer growing seasons, higher temperatures, and an increase in the diversity of species, (Dobzhansky 1950, Schemske et al. 2009). At higher latitudes, species are predicted to experience stronger natural selection from abiotic factors such as low temperature (Dobzhansky 1950, Schemske et al. 2009). To test these predictions,

biologists have often investigated plant defensive traits across latitude (Coley 1991, Moles et al. 2011a). If plants at lower latitudes experience higher rates of herbivory relative than plants at higher latitudes, then plants at lower latitudes should have increased defenses to herbivory (i.e., greater concentration of defensive chemicals, higher density of trichomes, greater tolerance, etc.) relative to plants at higher latitudes (Levin 1976, Coley 1991, Coley and Barone 1996). This specific hypothesis is referred to as the Latitudinal Herbivory-Defense Hypothesis (Johnson and Rasmann 2011) and has been supported in multiple empirical studies (Siska et al. 2002, Schemske et al. 2009, Rasmann and Agrawal 2011, Pearse and Hipp 2012, Salazar and Marquis 2012, Anstett et al. 2015, Lim et al. 2015, Wang et al. 2016, Baskett and Schemske 2018). However, the universality of this pattern has been recently challenged (Adams et al. 2009, Moles et al. 2011b).

One contributing factor to this controversy is that there are several confounding factors that can occur when comparing plant defenses from populations that span a large geographic space (Schemske et al. 2009). Recently, Anstett *et al.* (2016) proposed several methodological improvements when testing the Latitudinal Herbivory-Defense Hypothesis. First, for studies on a single species, there can be improved comparisons among populations by reducing environmental effects on phenotypic variation through the use of common garden experiments (Anstett et al. 2015) and controlling for induced responses in plants (Moreira et al. 2014). Induced plant defense responses from herbivore damage can be specific to the feeding guild of the herbivore (i.e., phloem feedings vs chewing damage) (Walling 2000), as well as the species that is inflicting damage (Van Zandt and Agrawal 2004, Ali and Agrawal 2012). Thus, plant defenses

that do have induced responses can bias estimates of trait values and therefore confound the comparison of defenses among populations (Anstett et al. 2016a). Second, studies that account for variability in herbivore communities among populations and assess the functional capacity of defense traits to minimize herbivore attack from different herbivore species provide much needed insight into how these traits perform in nature and against what type of herbivore (Anstett et al. 2016b). This is particularly important when plants defend against attack from generalist or specialist herbivores. The paradigm for the impact of plant defenses on specialist and generalist herbivores is that specialist herbivores frequently have adaptations allowing them to circumvent (and in some instances, utilize) host plant defenses, while generalists are often susceptible to these defenses (Krieger et al. 1971, Cornell and Hawkins 2003, Hanley et al. 2007, Ali and Agrawal 2012). However, there are examples where this assumption is violated (Van Zandt and Agrawal 2004, Zarate et al. 2007). Therefore, in order to formulate predictions for latitudinal patterns in plant populations, it is necessary to evaluate how traits perform as defenses against different types of herbivores. Further, the proportion of specialists and generalists in herbivore communities might also vary over latitude with a greater density of specialists occurring at lower latitudes (MacArthur 1969, Schemske et al. 2009, Forister et al. 2015). Therefore, if the composition of specialists and generalists in herbivore communities do vary over latitude and if the ability of defense traits to thwart attack is variable among specialist and generalist, then it can impact predictions of the Latitudinal Herbivory-Defense Hypothesis.

Here, we examine *Passiflora incarnata* (Purple Passionflower) across a latitudinal transect in the southeastern United States. Plants in the genus *Passiflora* and butterflies

in the tribe heliconiine represent a classic model of coevolution between plants and insect herbivores (Ehrlich and Raven 1964). Specialists heliconiine butterflies possess multiple adaptations that allow them to overcome herbivore defenses in *Passiflora* (Benson et al. 1975, Turner 1981), including the ability to sequester and/or tolerate cyanogenic glycoside, which is the major chemical defense utilized by these plants (Engler et al. 2000). Perhaps in response to this, *Passiflora* plants have evolved alternative defense mechanisms against these herbivores, including physical defenses such as trichomes (Gilbert 1971) and tough leaves (Kerpel et al. 2006). Finally, published field guides suggest that there may be variability in the presence and abundance of heliconiine across latitude, which spans the native range of *P. incarnata* (Opler 1998). Thus, this system provides a rich natural experiment for testing predictions of the Latitudinal-Herbivory-Defense Hypothesis for both chemical (cyanogenic glycoside) and physical (trichomes and leaf toughness) defenses and the potential contribution of specialist herbivores.

In this study, we evaluate the Latitudinal Herbivory-Defense Hypothesis in *Passiflora incarnata*. We utilized a common garden experiment in Athens, Georgia with a total of 208 individual's representative of populations that span over 10° latitude. We then contextualize our results by assessing the distribution of lepidopteran herbivores across our latitudinal transect and by quantifying the functional importance of defense traits to mitigate herbivory in the field. We specifically test the following three questions: (1) Is there a latitudinal pattern in cyanogenic glycoside capacity, trichome density, and leaf toughness in *P. incarnata*? (2) Are any of these defenses inducible? and (3) Are these defenses functionally important in mitigating herbivory?

Methods

Study Systems

The purple passionflower, *Passiflora incarnata* Linn. (Passifloraceae), is an herbaceous, perennial vine native to the southeastern United States with a distribution extending from central Florida to southern Pennsylvania (McGuire 1999). It can be found primarily growing in disturbed locations and is thought of as an early successional species (Radford et al. 2010). The plant reproduces either vegetatively through root and rhizome fragments or through seed production *via* an andromonoecious mating system (Wehtje et al. 1985). *Passiflora incarnata* interacts with several different species of lepidopteran herbivores across its range, all from the family Nymphalidae (Opler 1998). These include specialists *Heliconius charithonia* (Zebra Heliconian; Passiflora specialist), *Dryas iulia* (Julia Heliconian; Passiflora specialist), *Agraulis vanillae* (Gulf Fritillary; Passifloraceae specialist), and *Euptoieta claudia* (Variegated Fritillary; generalist).

Plant Sampling

To investigate regional variation in defense traits, we collected *P. incarnata* from 8 populations that span a latitudinal range of 27.6205°N – 38.0299°N (Figure 3.1A and Table S3.1). We sampled plants in the field that were at least 10 meters away from each other in order to decrease the likelihood of sampling the same individual, as rhizomes grow, on average, 6 meters (McGuire 1999). Each plant rhizome sampled in the field was treated as a unique genotype. The number of rhizomes per population varied depending on local densities (Table S3.1).

Regional Variation in Lepidopteran Herbivores

Published field guides indicate that there may be variability in the distribution of these herbivores across the range of *P. incaranta* (Opler 1998). However, we sought to determine if there is significant variation in the lepidopteran herbivore community across latitude. We acquired data from the Global Biodiversity Information Facility Data Portal (http://www.gbif.org/, 22 November 2016) and calculated the abundance of *Passiflora* lepidopteran herbivores along the eastern coast of the United States (W 85-degree longitudinal cutoff), where *P. incarnata* is natively found. Our estimates of lepidopteran sightings spanned from 25 – 39°N and we binned observations by each latitudinal degree. We then standardized each latitudinal degree by the amount of land area (square kilometers) using the Google Earth tracing program (version 7.1.8.3036) to estimate land area coverage.

To quantify variability of lepidopteran herbivore community across latitude, we performed analysis of variance for each species with latitudinal degree as the predictor variable and number of sightings standardized for square kilometer land area as the response variable. Unless otherwise noted, all statistical analyses were carried out using SAS JMP v. 12 (SAS Institute Inc., Cary, NC, USA).

Pesticide Pilot Experiment

The main common garden experiment involved the application of pesticide to a specific portion of the plants in order to prevent herbivory in the field. In order to confirm that the application of pesticide itself did not effect biomass production or cyanogenic glycoside capacity, we performed two separate greenhouse control experiments. For each experiment, plants were arranged in a split-plot randomized block design where half of the plants were sprayed with pesticide (0.36% wt/volume

Acephate® 97UP, O,S-Dimethyl acetylphosphoramidothioate) and the other half with water. Plants were sprayed every two weeks for a similar duration as the field experiment.

To determine if the application of pesticide impacted biomass production, we used an experimental population composed of 16 genotypes with 6 clonal replicate individuals per genotype. Each individual was generated in the exact same way and at the exact same time as plants used in the field experiment described above. Thus, plants used in the biomass control experiment were representative of populations from across the range of *P. incarnata*. Plants were transplanted into 20-inch pots filled with Faford soil, placed in the greenhouse under ambient conditions, and watered regularly. Plants were arranged such that each subplot was 1-meter away from each other in order to minimize the chance of cross-contamination among treatments. After the duration of the pesticide spraying regimen, we harvested all above ground biomass from each individual. We then measured total dry biomass after oven-drying at 60°C for 72 hours.

To determine if the application of pesticide impacted cyanogenic glycoside capacity, we used an experimental population of 12 full-sib families with 4 replicate individuals per family. Full-sib families were F2 hybrids generated from 8 populations of *P. incarnata*. Briefly, plants from all Florida and Virginia populations were randomly paired together to generate an F1 population resulting in 20 different F1 combinations from a total of 8 different populations. To germinate seeds, we soaked them in DI water for 48 hours at 4°C and then placed them on filter paper in an incubator set to 37°C. Once seeds began to germinate, we transplanted seedlings into 1:1 Fafard potting mix and vermiculite soil and placed them under lights in the greenhouse to continue to grow.

Once seedlings grew their first true leaf, we transplanted them into 10-inch pots filled with a pine bark soil mixture soil mixture of pine bark, vermiculite, superphosphate, calcium nitrate, potassium nitrate, micronutrients, gypsum, and dolomite limestone. Plants were arranged in the greenhouse such that each subplot was separated by 1-meter in order to minimize cross-contamination among treatments. Plants were under ambient greenhouse conditions and watered regularly. After the duration of the spraying regimen, we harvested one fully-expanded adult leaf from each individual, placed them on ice, and then stored them at -80° C until cyanogenic glycoside capacity quantification. To determine if the application of pesticide impacted biomass production, we constructed a mixed model with biomass as a response variable and the fixed effects of treatment (pesticide vs water sprayed plants) and random effects of block, population, and plant genotype nested within population. To determine if cyanogenic glycosides capacity changes due to exposure to pesticide, we performed a t-test with cyanogenic glycoside capacity as a response variable and treatment (pesticide vs water sprayed plants) as a predictor variable. In all of our analyses, we logged transformed dry end-of-season biomass in order to improve normality.

Common Garden Experiment: Experimental Design

To test for regional differences in defense traits, we conducted a common garden experiment in a split-plot completely randomized block design in Athens, GA (33.9519°N). In mid-May of 2014, we transplanted clonal propagules of similar size into the field. Individuals were positioned approximately 1 meter apart and blocks containing each genotype were positioned approximately 6 meters apart. After transplanting, we applied 20-10-20 NPK fertilizer at 15 ml per 3.78 L of water to minimize variation in

nutrient availability across microhabitats, and allowed each vine to grow onto a 1.2-meter-tall bamboo stick. Due to high initial mortality after transplantation, each plant genotypes were represented by 3-6 clonal replicates, which resulted in our experimental population size of 208 individuals.

To investigate if defense traits influence end-of-season biomass through interactions with herbivores, we generated an experimental design similar to those that investigate natural selection from herbivores on plant defense traits (Mauricio and Rausher 1997). Specifically, we experimentally removed herbivores to determine if this changes the relationship of defense traits to end-of-season biomass. To achieve this, we protected half of the plants with pesticide, while the other half were exposed to herbivores at natural densities. For the protected plants, we sprayed them with a broad spectrum pesticide (0.36% wt/volume Acephate® 97UP, O,S-Dimethyl acetylphosphoramidothioate) in two-week intervals throughout the field season. For the control plants, we performed a similar treatment application during this time, but with only water. As results will show, the application of the pesticide itself had no effect on either biomass production or cyanogenic glycoside capacity.

Using this common garden design, we first determined if specific plant defenses varied over latitude. We measured two structural plant defenses (trichome density and leaf toughness) and one chemical defense (cyanogenic glycosides). Three months after the start of the field experiment, we randomly selected four adult leaves from each individual (two each for trichome density measurements and cyanogenic glycoside measurements; N = 208 individuals). We quantified cyanogenic glycoside capacity using the technique detailed in (Brinker and Seigler 1989) with modifications by (Alonso-

Amelot and Oliveros 2000) where toluene is used as a strong solvent capable of disrupting cell walls and membranes and therefore bringing into contact the enzyme β-glycosidase and cyanogenic glycoside. Hence, this measurement of cyanogenic glycoside capacity is expressed as 1 HCN = 1 cyanogenic glycoside per gram leaf (wet weight), as the assay measures the product of the catalysis of the mixture of cyanogenic glycoside and endogenous β-glycosidase. We estimated leaf trichome density as total number of trichomes within a 2.3 mm^2 area of the upper central area on the adaxial side of the leaf (Mauricio and Rausher 1997). We estimated leaf toughness on 10 October 2014 in the field. For each individual, we randomly selected two adult leaves and determined the amount of pressure required to punch through the upper central area on the adaxial side for each leaf using a penetrometer (Chatillon DFM-2 Cadet CD1 Series; FL, USA). Towards the end of the field season, before plants started to senesce, we harvested all above ground biomass for each individual. We then measured total dry biomass after oven-drying at 60°C for 72 hours. We then constructed mixed models where latitude, treatment (pesticide or no pesticide), and the interaction between latitude and treatment were treated as fixed effects while plant genotype and block were treated as random effects in order to determine if these factors had a significant effect on the magnitude of these plant defenses.

To determine the functional capacity of plant defense traits to prevent herbivory, we investigated the relationship between defense traits and end-of-season biomass production in the presence and absence of herbivores. First, we sought to relate end-of-season biomass to herbivory. For each individual, we randomly selected two adult leaves with no damage and marked each leaf with a colored string for continued monitoring.

After 4 weeks, we visually evaluated selected leaves and estimated the amount of missing area in categories of 0%, 25%, 50%, 75%, and 100%, where 0% was no visual damage and 100% was the entirety of the leaf was eaten except for portions of the mid-vein. Due to the nature of this measurement, we only assessed damage by chewing herbivores. However, we believe this is a good measure of herbivory in our field experiment. We completed a 2-week herbivore census at the time we were estimating herbivore damage and found that 98% of observed herbivores were *A. vanillae* and the remaining 2% were the generalist lepidoptera *E. claudia*.

To estimate the functional capacity of defense traits to thwart herbivore damage, we investigated phenotypic selection gradients (Lande and Arnold 1983) on end-of-season biomass production by evaluating both linear and quadratic phenotypic selection gradients in the presence and the absence of herbivores. Hence, we estimated a directional selection gradient though a linear regression between end-of-season biomass and all three defense traits and estimated stabilizing/disruptive selection gradients though a quadratic regression of end-of-season biomass and all three defense traits together. To achieve this, we *z*-transformed each raw defense trait values to a have a mean of 0 and a standard deviation of 1. We then constructed mixed models that included each standardized trait, treatment, and all of their interaction terms as fixed effects, while block and plant genotype were treated as random effects. A significant interaction term of defense trait * treatment is interpreted as that the presence of herbivores is important indicator on how the defense trait influences end-of-season biomass production.

To investigate the connection of herbivory to end-of-season biomass, we investigated the linear relationship of end-of-season biomass production and herbivory

for control plants only (i.e., those not treated with pesticide). We additionally established that herbivory was different among treatments by performing a t-test on herbivory among treatments. As noted above, we again logged transformed dry end-of-season biomass in order to improve normality.

Post-hoc Cyanogenic Glycoside Capacity Induction Assay

In order to confirm that the results we obtained during the common garden experiment were due directly to the effects of herbivory and no other environmental factors, we sought to further explore the inducibility of cyanogenic glycoside capacity in a controlled greenhouse experiment. Additionally, we were interested in determining if cyanogenic glycoside capacity differed among leaves at different developmental stages. To achieve this, we conducted a greenhouse experiment utilizing the plant defense hormone methyl jasmonate. Methyl jasmonate is a plant hormone involved in plant defense responses to chewing herbivores (Walling 2000) and is often used as a means to elicit induced defense responses (Thaler et al. 1996, Agrawal et al. 1999). Individuals used in this experiment were half-sib families generated from openly pollinated *P. incarnata* in Athens, GA after they had been collected from across the range *of P. incarnata* (Figure 3.1A). We germinated 6 seedlings from 12 half-sib families after soaking seeds in water at 4°C for 48 hours followed by placing seeds on moist filter paper left in an incubator set to 30°C. After germination, we transplanted seedlings into 8-inch pots filled with Faford soil. We then arranged plants in a split-plot randomized block design where we sprayed half the plants until runoff with 150 μM methyl jasmonate (Sigma-Aldrich Lot # MKBT7772V) solution while control plants were sprayed with water. Each subplot was positioned ¾ yard away from each other in order to minimize

the chances of treatment cross-contamination. We then harvested leaves from each individual 48 hours following application of treatments. We harvested 4 leaves per individual, targeting 2 young leaves and 2 adult leaves with all leaves harvested being fully expanded. Thus, we collected a total of 284 leaves and all leaves were stored on ice after harvest and then left at -80°C until cyanide extraction similar to previous experiments. One individual died over the course of this experiment and thus data from this plant was excluded. To determine if cyanogenic glycoside capacity could be induced due to exposure to methyl jasmonate, we performed a standardized least squares regression. In this model, we included the response variable of cyanogenic glycoside capacity and the fixed effects of plant family, treatment, block, leaf age, and leaf age*treatment.

Results

Regional Variation in lepidopteran Herbivores

We estimated the range of the observed herbivores of Purple passionflower across latitude using data from the Global Biodiversity Information Facility (GBIF) (http://www.gbif.org/, 22 November 2016). From GBIF, we obtained a total of 722 taxon observations from a combination of museum specimens and citizen science databases that span multiple years (1894 – 2016). We found that the abundance of most of the lepidopteran species that feed on *P. incarnata* significantly varied across latitude in the eastern United States (Figure 3.1B). *Euptoieta claudia* showed a significant positive linear relationship to latitude ($R^2 = 0.416$; $F_{1,12} = 8.53$; $P = 0.0128$), while *Agraulis vanillae* ($F_{1,12} = 24.32$; $P = 0.0003$) and *Heliconius charithonia* ($F_{1,12} = 9.52$; $P = 0.0094$) showed significant negative relationships with latitude ($R^2 = 0.670$, $R^2 = 0.443$,

respectively). *Dryas iulia* did not show a significant relationship to latitude ($R^2 = 0.25$; $F_{1,12} = 4.00$, $P = 0.0686$).

Pesticide Pilot Experiment

To determine if our application of pesticide treatments in our common garden experiment biased our estimates of plant biomass or cyanogenic glycoside capacity, we performed two controlled greenhouse experiments. From this, we found that treatment of pesticide had no impact on above ground biomass production ($F = 0.576$; $P = 0.527$). Therefore, our estimates on the relationship between *P. incarnata* defensive traits and biomass production when herbivores are present or absent do not appear to be biased through our application of pesticides. Further, we determined that the treatment of pesticide did not have any measurable impact on cyanogenic glycoside capacity (t-Ratio = 1.099; $P = 0.2781$). Thus, our measurement of increased cyanogenic glycoside capacity for plants protected from herbivores with pesticides was not an artifact that is due to the application of the pesticide.

Common Garden Experiment: Variation in Plant Defenses over Latitude

We first found a substantial amount of variation in defenses measured (Figure S3.1A) with leaf toughness and cyanogenic glycoside capacity tending to tradeoff (Figure S3.1B). Further, we found that cyanogenic glycoside capacity and leaf toughness differed highly across latitude; however, latitudinal patterns varied by trait (Figure 3.2; Table S3.2). Cyanogenic glycoside capacity showed a significant latitudinal cline and increased with increasing latitude ($R^2 = 0.096$, $P < 0.0001$). Further, we found that cyanogenic glycoside capacity differed between control and treatment plants (Table S3.2). For the two structural defenses measured in this study, we found that leaf

toughness showed a significant latitudinal pattern with a general decrease with increasing latitude ($R^2 = 0.101$, $P < 0.0001$); however, trichome density did not show any pattern over latitude (Figure 3.1; Table S3.2). Finally, both of these defenses did not significantly differ among control and experimental treatments, and no defense trait showed a significant interaction term between latitude and treatment (Table S3.2).

Common Garden Experiment: Defense traits and herbivory impact end-of-season biomass

We next assessed the functional importance of defense traits in *Passiflora incarnata* by evaluating their relationship to end-of-season biomass production in our common garden experiment and the connection of biomass to herbivory. To determine if herbivore damage had a measurable impact on end-of-season biomass, we assessed herbivory in the field over a 4-week period when herbivores were abundant. Overall, we found that plants protected by pesticide had significantly lower herbivore damage relative to control plants (Figure S3.2A). Plants with pesticide had a mean percentage of herbivore damage of 0.45% (standard deviation = 3.74) while those without pesticide had a mean percentage of herbivore damage of 50.80% (standard deviation = 42.03). A t-test showed that this difference in herbivory was significant (t-ratio = 11.574; $P<0.0001$). From a field herbivore census during our common garden assay, we determined that nearly all of the herbivore damage was caused by the specialist, *A. vanillae*. Further, we determined that end-of-season biomass for plants that lacked pesticide were strongly negatively correlated with herbivory in the field ($r = -0.366$; $P = 0.0003$; Figure S3.2B).

We further assessed the functional capacity of defense traits in *P. incarnata* through the use of a multiple regression analysis with end-of-season biomass as our

response variable. We investigated both linear and quadratic relationships of plant defense traits and end-of-season biomass (Table 3.2). We found that the linear term of cyanogenic glycoside capacity significantly predicted end-of-season biomass production in both treatments, while neither of the two physical defenses did (Table 3.2). Further, we constructed models to determine if the linear and quadratic relationship of defense traits to end-of-season biomass changed in the presence of herbivores. From our linear models, we found that the relationship of cyanogenic glycoside capacity and leaf toughness to end-of-season biomass changed between plants that were treated with pesticides and those that were not (Figure 3.3, Table 3.1). However, we did not find any evidence of significant quadratic terms with treatments (Table S3.3) and therefore we did not detect any evidence of intermediate levels of defense traits influencing end-of-season biomass.

Post-hoc Cyanogenic Glycoside Capacity Induction Assay

To determine if the response of decreased cyanogenic glycoside capacity that was observed in our field experiment was due to a response to herbivore attack, we determined if plants treated with the plant defense hormone methyl jasmonate also showed a similar decrease in cyanogenic glycoside capacity. We found that methyl jasmonate treated plants do alter their cyanogenic glycoside capacity (Figure 3.4, Table S3.4). Our model was highly significant ($F_{12,262} = 7.4494$; $P < 0.0001$) between control and methyl-jasmonate treated plants, with methyl-jasmonate treated plants having lower cyanogenic glycoside capacity than control plants and that this pattern was consistent even among leaves at different developmental stages (Figure 3.4).

Discussion

The Latitudinal Herbivory-Defense Hypothesis predicts that due to greater herbivore pressure at lower latitudes, plant populations at low latitudes will have stronger/a wider array of defense mechanisms against biotic selective agent's relative to high-latitude populations. We tested this prediction by comparing both constitutive and induced expression of three defense traits in *Passiflora incarnata* populations collected across a large latitudinal range; we then contextualized our results by assessing lepidopteran herbivore community across latitude and ability of these traits to mitigate herbivore attack. We found that specialist lepidopteran herbivores are highly abundant at lower latitudes, which is consistent with patterns observed in other studies (Dyer et al. 2007, Salazar and Marquis 2012). These results are also consistent with published field guides for all of the species investigated in this study (Opler 1998). Further, our study revealed significant geographic variation in plant defenses across latitude. Leaf toughness and cyanogenic glycoside capacity showed latitudinal patterns, although the direction of these latitudinal patterns differed between these traits. Finally, trichome density did not show any clear latitudinal pattern. Thus, our test of the Latitudinal Herbivory-Defense Hypothesis showed mixed results among the plant defenses investigated in this study. Based on our assessment of the ability of these defense traits to thwart herbivore damage from a specialist lepidopteran herbivore, geographic variation in cyanogenic glycoside capacity and leaf toughness among *P. incarnata* populations may depend, in part, on the distribution of specialist herbivores rather than latitude, *per se*.

Natural selection by specialist and generalist herbivores on plant defense traits can differ and in certain instances, can be opposing (Meijden 1996, Salazar and Marquis

2012). This is due in part to adaptations to circumvent host defenses that are typical of specialists (Krieger et al. 1971, Cornell and Hawkins 2003). Of note are plant chemical defenses, which often have contrasting effects on specialist and generalist herbivores (Roslin and Salminen 2008, Macel 2011), resulting in natural selection from these herbivores to favor decreasing or increasing concentration of chemical defenses, respectively (Lankau 2007). Therefore, variation in the relative composition of specialist and generalist herbivores across latitude can complicate classic predictions of the Latitudinal Herbivory-Defense Hypothesis as it pertains to chemical defenses.

In this study, we quantified a class of defensive chemical known as the cyanogenic glycosides, which are known to mediate interactions with specialist herbivores (Gleadow and Woodrow 2002). In the Passifloraceae, previous work has found that cyanogenic glycoside capacity is not an effective defense against lepidopteran larva in the tribe heliconiine (Spencer 1988, Alonso Amelot et al. 2006). Passifloraceae specialists lepidoptera are capable of tolerating cyanogenic glycosides (de Castro et al. 2017), with some species capable of utilizing these compounds through sequestration or *de novo* synthesis (Engler et al. 2000, Engler-Chaouat and Gilbert 2007). Thus, our finding that cyanogenic glycoside capacity is negatively related to end-of-season biomass is consistent with the expectation that cyanogenic glycoside capacity does not serve as an effective defense against heliconiine specialists. Therefore, greater cyanogenic capacity is potentially costly where heliconiine specialists are abundant. Thus, at low-latitude, where *P. incarnata* populations interact with a greater proportion of lepidopteran specialists, natural selection could favor decreased cyanogenic glycoside capacity. Consistent with this expectation, we found that the relationship between cyanogenic

glycoside capacity and end-of-season biomass production significantly changed in the presence and absence of herbivores. In both cases, cyanogenic glycoside capacity was negatively correlated to end-of-season biomass, however, the strength of this negative relationship increased when exposed to herbivores relative to pesticide treated plants. This suggests that producing greater cyanogenic glycosides when exposed to a primarily specialist herbivore community is costly in some form.

Natural selection could favor increased cyanogenic glycoside capacity in regions where there is a greater proportion of herbivory from generalist herbivores, which are often susceptible to cyanogenic glycosides (Gleadow and Woodrow 2002). Consistent with this, we found that cyanogenic glycoside capacity was greater at high-latitudes where the sole generalist herbivore known to frequently interact with *P. incarnata*, *E. claudia*, is also more abundant. However, this is assuming that herbivory from generalist herbivores at high-latitudes is occurring at great enough frequency for natural selection to favor greater quantities in these locations. Therefore, in order to elucidate the actual mechanism behind this latitudinal pattern in cyanogenic glycoside capacity, future studies should investigate the intensity of herbivory and the abundance of all generalist herbivores that interact with *P. incarnata* populations across latitude.

In addition to plant chemical defenses, structural defenses often are not ubiquitously capable of being effective against both specialist and generalist herbivores (Hanley et al. 2007, Volf et al. 2015). Therefore, it is necessary to functionally characterize these traits against specialist and generalist herbivores when assessing the Latitudinal Herbivory-Defense Hypothesis. For the two structural defenses investigated in this study, leaf toughness and trichome density, we did not find that either of these

traits related to end-of-season biomass production. However, we did find that the relationship of leaf toughness to end-of-season biomass changed in the presence and absence of herbivores. Thus, our results suggest that there may be a role of leaf toughness in defense against herbivory in *P. incarnata*. To reduce herbivory, leaf toughness acts as a mechanical and nutritional barrier to herbivores by prolonging growth, potentially exposing herbivores to greater risk from predators or parasitoids (Feeny 1976, Price et al. 1980, Coley 1983, Lowman and Box 1983). Thus, tough leaves can act as effective defenses against herbivory from specialist herbivores that attack *P. incarnata*. Consistent with this hypothesis, *P. incarnata* populations at low-latitude have tougher leaves where specialists are abundant. In contrast, we did not observe a latitudinal cline in trichome density. Although trichomes have been shown to be an effective defense against both specialist and generalist herbivores (Dimarco et al. 2012), there are instances where they are not (Volf et al. 2015). Trichomes are known to also protect plants against abiotic stresses as well (Levin 1973, Hanley et al. 2007), therefore the lack of a latitudinal pattern in trichome density in our study suggests that this trait is not important for defense against specialists, but rather maybe serving some other ecological function for the plant. This is also true for leaf toughness, where tougher leaves protect against a variety of abiotic stresses such as UV light and heat (Hanley et al. 2007).

Certain plant defenses can be induced following an attack from an herbivore. In this study, we found that cyanogenic glycoside capacity significantly decreased in plants exposed to herbivores. Due to the non-intuitive nature of this result, we sought to confirm it in a more controlled setting. From our controlled greenhouse experiment

where plants were not exposed to any herbivores, but were only sprayed with the plant defense hormone methyl jasmonate, we again observed a decrease in plant cyanogenic glycoside capacity. Thus, these results suggest that indeed *P. incarnata* has a marked decrease in cyanogenic glycoside capacity when exposed to herbivores. Although cyanogenic glycosides are often considered a constitutive defense (Gleadow and Møller 2014), there are examples where HCN quantity changed between plants damaged vs undamaged. In lima beans, wounded plants have a marked increase in HCN quantity, but this was due to an increase in β-glycosidase and not a change in the amount of cyanogenic glycoside metabolites (Ballhorn et al. 2006). Thus, the decrease in cyanogenic glycoside capacity that we observe maybe due to an alteration in enzyme production. If the decrease in cyanogenic glycoside capacity is adaptive is unknown, but given the negative relationship between this trait and end-of-season biomass, it is possible that this is a mechanism to mitigate potential costs associated with this trait.

In their meta-analysis of chemical defenses, Moles et al. (2011) did not find strong evidence in support of the Latitudinal Herbivory-Defense Hypothesis. In fact, broad plant defense chemical classes such as tannins or phenolics, flavonoids, resins or oils, and the macroalgae-specific dimethylsulfoniopropionate, did not show a trend of higher concentrations at lower latitudes. The only chemical defense that did show such a pattern were alkaloids. As noted by Moles et al. (2011), one possibility for these results is that some compounds have mixed effects on herbivores and therefore the presence of such a compound does not necessarily predict herbivore deterrence (Carmona et al. 2011, Moles et al. 2011b). In light of this, we argue that it is essential to assess herbivore community among populations/species under study and to design experiments such that a

clear understanding of how plant defense traits will actually function in herbivore deterrence.

Connecting patterns of herbivory with patterns of defense is particularly important when assessing plant chemical defenses over latitude. Due to the ability of many specialist herbivores to tolerate chemical defenses of their host plant, any latitudinal pattern in chemical defenses can be a result of multiple scenarios that are a combination of variation in herbivore pressure and variation in herbivore identity. For example, if plant chemical defenses are observed to be lower for low-latitude populations relative to high-latitude populations, then hypotheses to explain this observation are: 1) herbivore pressure is driven primarily by generalists and it is greater at high-latitudes relative to low-latitudes; 2) herbivore pressure is primarily from specialists and it is greater at low-latitudes relative to high-latitudes; 3) herbivore pressure is equal across latitude, but composition of generalist and specialist herbivores varies across latitude; 4) some combination of 1 & 2 where herbivore identity and pressure varies across geographic space and among populations. Hence, it is difficult to interpret latitudinal patterns of "defensive traits" without the context of the effectiveness of defenses, against what herbivores, and where are those herbivores located. With all of this information, it will be possible to connect patterns of herbivory with patterns of defense.

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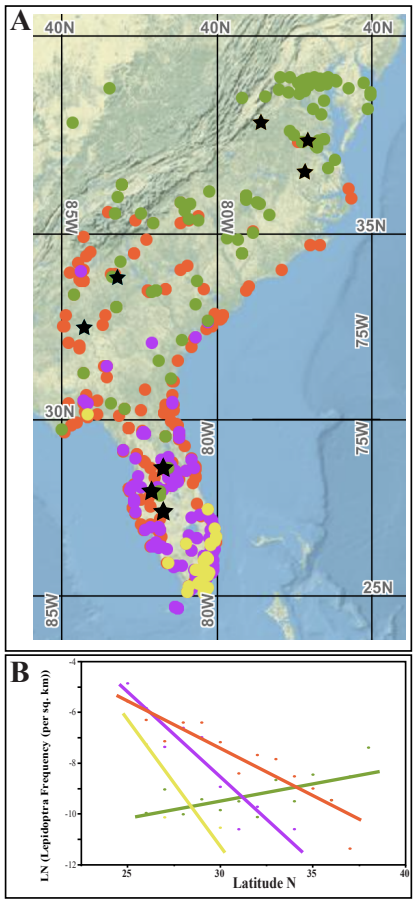

Figure 3.1)
Populations of *Passiflora incarnata* sampled across the southeastern United States and their corresponding lepidoptera herbivore communities. **A)** Populations of *P. incarnata* sampled across the southeastern United States. Populations are distinguished with black stars with populations at the extremes of the distribution (Florida and Virginia) used to generate the F2 full-sib families in the study on damage response. Colored dots indicate observation data from the Global Biodiversity Information Database (gbif.org) for lepidoptera that utilize *P. incarnata* as a host plant. Colored dots are representative of reported sightings of *Euptoieta claudia* (green), *Agraulis vanillae* (orange), *Heliconius charithonia* (purple), and *Dryas iulia* (yellow). **B)** Quantitative representation of variation in the presence and abundance of specialist lepidoptera across latitude. Specialist lepidoptera frequency was standardized by land area (sq. km) over latitudinal degrees.

Gulf Fritillary: http://doi.org/10.15468/dl.ji9ffs
Zebra Heliconia: http://doi.org/10.15468/dl.pgsbdx
Julia Heliconia: http://doi.org/10.15468/dl.mudnty
Variegated Fritillary: http://doi.org/10.15468/dl.xjecor

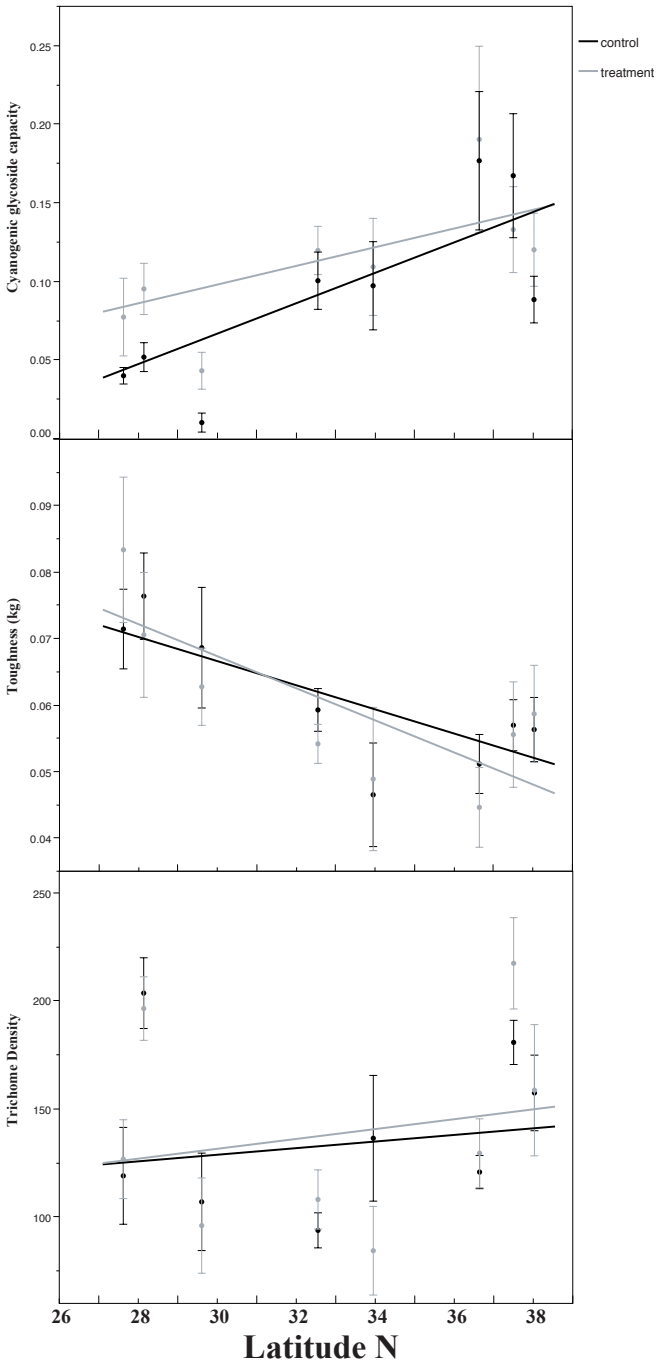

Figure 3.2)
Variation in plant defenses among *Passiflora incarnata* populations across latitude. Grey represents samples that were exposed to herbivores (control) and black represents samples that were protected from herbivores (treatment). Each dot is a population mean for a given trait ± standard error.

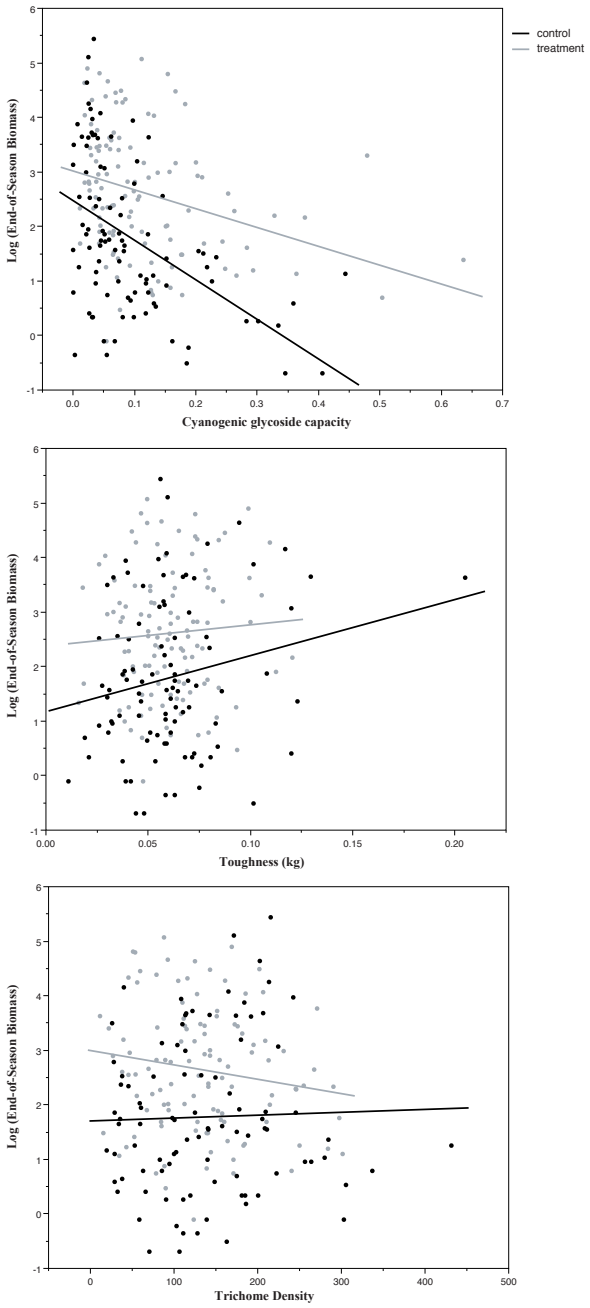

Figure 3.3)

Relationship between end-of-season biomass and plant defense traits. Black represents samples that were exposed to herbivores (control) and grey represents samples that were protected from herbivores (treatment).

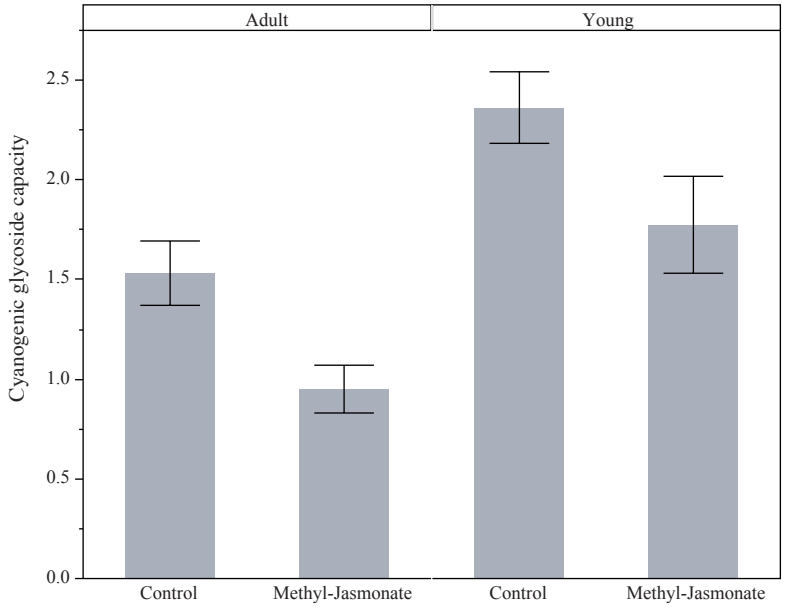

Figure 3.4)
Cyanogenic glycoside capacity as measured by the amount of HCN released from leaf tissue in methyl jasmonate treated and control leaves. Measurements were obtained from both young and adult leaves. Error bars represent one standard error.

Table 3.1)
Analysis of variance for end-of-season biomass of *P. incarnata* grown in the field and the effect of linear trait terms. Treatments were plants exposed to herbivores or plants protected from herbivores through the use of a pesticide. Only model fixed effects are reported. Bold numbers indicate significant p-values.

	Log End-of-Season Biomass		
Source of Variation	df	*F*	*P*
Treatment	1	42.165555	**<.0001**
Cyanogenic glycoside capacity	1	7.3248373	**0.0074**
Toughness	1	0.9019346	0.3434
Trichome Density	1	4.7472149	**0.0306**
Cyanogenic glycoside capacity * Treatment	1	4.813547	**0.0295**
Toughness * Treatment	1	8.2982705	**0.0045**
Trichome Density * Treatment	1	3.8407863	0.0516

Table 3.2)
Phenotypic selection gradients of defense traits and end-of-season biomass production by each treatment. Treatment represents plants protected from herbivores with a pesticide and control represents plants exposed to herbivores.

	Treatment		Control	
Resistance Character	β	γ	β	γ
Cyanogenic Glycoside Production	-0.346***	0.055	-0.708****	0.185
	(0.100)	(0.055)	(0.140)	(0.115)
Toughness	0.050	0.057	0.178	0.007
	(0.125)	(0.105)	(0.112)	(0.043)
Trichome Density	-0.165	-0.016	0.046	-0.085
	(0.114)	(0.108)	(0.119)	(0.073)

* P < 0.05; ** P < 0.01, *** P < 0.001, **** P < 0.0001

CHAPTER 4

NATURAL SELECTION BY HERBIVORES ON CYANOGENIC GLYCOSIDE CAPACITY IN *PASSIFLORA INCARNATA*

Introduction

Plants and their associated insect herbivores make up more than half of the macroscopic diversity of life on the planet, with herbivory being the dominant ecological interaction that occurs in terrestrial ecosystems (Strong et al. 1984, Turcotte et al. 2014). In response to herbivory, plants have evolved a suite of defensive traits to protect themselves including trichomes, toughened exterior leaf layers, thorns, and natural chemical products (Ehrlich and Raven 1964, Levin 1973, Mauricio 1998, Rausher 2001, Rosenthal and Berenbaum 2012, Speed et al. 2015). In parallel, insect herbivores have evolved adaptations to mitigate host plant structural and chemical defenses (Zangerl and Berenbaum 1993). Strategies to circumvent plant chemical defenses include dextofication of harmful metabolites (Scott and Wen 2001), avoidance of toxic plant tissues (Zangerl and Berenbaum 1990), and sequestration and or utilization of defensive metabolites to their own benefit (Nishida 2002). Due to the plethora of adaptations in both plants and insects, these systems influenced Ehrlich and Ravens (1964) seminal paper on coevolution and thus highlight the potential of this evolutionary force as a major contributor in the evolution of phenotypic diversity.

Measuring coevolution between biological systems is challenging due to the difficulty in quantifying adaptations in interacting taxa over evolutionary relevant time

scales (Gomulkiewicz et al. 2007). As an alternative, biologists often test for results of coevolutionary interactions by measuring traits that mediate biotic interactions across geographic space where there is variation in coevolutionary strength (Burdon and Thrall 2000, Fornoni et al. 2004). Spatial variation in the magnitude of plant-herbivore interactions is common in nature, and this phenomenon is commonly referred to as the geographic mosaic theory of coevolution (Thompson 2005). However, geographic variation in the degree of interaction between taxa alone is not sufficient evidence to denote coevolution. It is also necessary to demonstrate that the geographic distribution of traits involved in coevolutionary interactions arose from natural selection varying among populations (Laine 2009). In order to determine the source of selection on a trait, it is necessary to look at the dynamics of selection in the presence and absence of putative selective agent (Endler 1986). In the case of plant-herbivore interactions, this can be achieved by measuring natural selection on plant defenses in the presence and absence of herbivores (Mauricio and Rausher 1997).

Numerous studies have demonstrated that insect herbivores are the agents of natural selection on plant defenses, and it has been demonstrated that they influence the distribution of these traits through a combination of directional and stabilizing selection (Fritz and Simms 1992, Mauricio and Rausher 1997, Fornoni et al. 2004, Agrawal 2005, Lankau 2007). These studies therefore generate valuable information on which specific traits are under selection, what mode of selection is currently operating on them, and what species of herbivores act as selective agents. This information, in combination with geographic variation in defensive traits, provides strong evidence that coevolutionary

dynamics contributes to plant defensive trait evolution (Muola et al. 2010, Castillo et al. 2014).

Plants in the genus *Passiflora* and butterflies in the tribe heliconiine are classic examples of coevolution between plants and herbivores (Ehrlich and Raven 1964). Heliconiine butterfly larva feed nearly exclusively on plants in the genus *Passiflora* and closely related plant genera (de Castro et al. 2017). This observation led to studies that elucidated taxonomic correlations, ecological interactions, and numerous adaptations within this system (Gilbert 1971, Benson et al. 1975, Turner 1981, Gilbert 1982). Adaptations include the ability to sequester and/or tolerate cyanogenic glycoside, which is a major chemical defense in *Passiflora* (de Castro et al. 2017). Other adaptation include the ability of some species to *de novo* synthesize of their own cyanogenic glycosides (Nahrstedt and Davis 1985, Engler et al. 2000, Engler-Chaouat and Gilbert 2007). Acquisition of cyanogenic glycosides is ultimately beneficial to heliconiine butterflies through defense against predation and are also used as nuptial gifts (Cardoso and Gilbert 2013). The fact that cyanogenic glycosides are not harmful to heliconiine is a major factor in why they can utilize *Passiflora* as a host (de Castro et al. 2017).

Cyanogenic glycosides are a broadly distributed chemical in the plant kingdom and are considered defensive due to their capacity to release hydrogen cyanide (HCN) and a toxic aglycone after coming into contact with the enzyme beta-glycosidease (Gleadow and Møller 2014). Different forms of cyanogenic glycosides are classified based upon which amino acid precursor they are derived from (e.g., aliphatic, aromatic, and cyclopentenoid) (Gleadow and Møller 2014). Aliphatic and aromatic amino acids are present in >2500 plant species, while cyclopentenoids are relatively rare in the plant

kingdom and have only been discovered in six plant families to date, including Passifloraceae (de Castro et al. 2017). Further, of the 27 reported structures for cyclopentenoids, 15 are found in plants in the genus *Passiflora* (Spencer 1988, Jaroszewski et al. 2002, Dhawan et al. 2004). Thus, due to the adaptations by heliconiines to cyclopentoids and the diversity of these metabolites in *Passiflora*, some have postulated that these metabolites are mediated by coevolution with heliconiines (Spencer 1988). But to date no study has demonstrated that cyanogenic glycosides in *Passiflora* are indeed under natural selection from herbivores.

In Chapter 3, we found that the herbivore community of *P. incarnata* is primarily composed of the specialist heliconiine species *A. vanillae*. Further, we found that in the presence of herbivores cyanogenic glycoside capacity had a negative impact on end-of-season biomass. Thus, we predicted that cyanogenic glycoside capacity would be selected against in the presence of a similar herbivore community. Here, we aim to test the hypothesis that herbivores are the selective agents that impact the evolution of the cyanogenic glycoside capacity in *Passilfora incarnata*. To achieve this, we generated an F2 population from parental lines with substantial variation in cyanogenic glycoside capacity. We then planted this F2 population in a common garden located in Athens, Georgia, where the primary herbivores is *Agraulis vanillae* (Passifloraceae specialist). We examined whether removal of herbivores as putative selective agents impacted the overall pattern of selection acting on cyanogenic glycoside capacity. We also assessed leaf damage and surveyed herbivores throughout the field season to determine what herbivores were attacking *P. incarnata*. Any difference between in gradients of natural

selection would indicate that herbivores exerted selection on cyanogenic glycoside capacity.

Methods

Study System

The purple passionflower, *Passiflora incarnata* Linn. (Passifloraceae) is an herbaceous, perennial vine native to the southeastern United States with a distribution extending from central Florida to southern Pennsylvania (McGuire 1999). It can be found primarily growing in disturbed locations and is thought of as an early successional species (Radford et al. 2010). The plant reproduces either vegetatively through root and rhizome fragments or through seed production *via* an andromonoecious mating system (Wehtje et al. 1985).

Passiflora incarnata interacts with several different species of lepidopteran herbivores across the southeastern United States, all from the family Nymphalidae (Opler 1998). These include specialists *Heliconius charithonia* (Zebra Heliconian; Passiflora specialist), *Dryas iulia* (Julia Heliconian; Passiflora specialist), *Agraulis vanillae* (Gulf Fritillary; Passifloraceae specialist), and *Euptoieta claudia* (Variegated Fritillary; generalist). Published field guides indicate that *H. charithonia*, *A. vanillae*, and *E. claudia* are the most likely herbivores of *P. incaranta* in Athens, Georgia (Opler 1998), and unpublished citizen science and museum specimen data corroborates these claims (N. Batora, Chapter 3).

In order to protect against herbivore attack, *Passiflora incarnata* produces a variety of direct and indirect defenses. These defenses include extrafloral nectaries (McLain 1983) and chemicals such as flavonoids, alkaloids, and cyanogenic glycoside

(Dhawan et al. 2004). In this study, we focused on only a single defensive chemical: cyanogenic glycoside. Heliconiine larva are known to possess adaptation to cyanogenic glycoside, and it is hypothesized that this metabolite mediates coevolutionary interactions between these taxa (Spencer 1988). Notably, in Chapter 2 of this dissertation we showed that cyanogenic glycoside capacity varied considerably across latitude, which led us to want to study if this trait is under natural selection.

Effect of Pesticide application on flower production

Our main common garden experiment required the application of a pesticide to determine if the presence of herbivory affected flower production. We therefore sought to determine if the application of this pesticide biased any of our estimates of fitness. Thus, we performed a controlled greenhouse experiment utilizing these plants. Similar to our subsequent field study, plants were arranged in a split-plot randomized block design where half of the plants were sprayed with pesticide (0.36% wt/volume Acephate® 97UP, O,S-Dimethyl acetylphosphoramidothioate) and the other half with water. Plants were treated with either pesticide or water every two-weeks for a similar duration as the subsequent field experiment. The experiment contained 16 plant genotypes with 6 replicate clonal individuals per genotype. Experimental methods for the generation of the control experiment were the same as those used to test for the impact of pesticide application on end-of-season biomass (Chapter 3). Throughout the duration of the experiment, we counted the number of flowers produced and used this as a proxy for fruit production. We opted to measure total flower production as a proxy for fruit production due to the nature of the mating system in this species. *Passiflora incarnata* is an obligate outcrosser that is primarily pollinated by *Bombus* species (May and Spears Jr 1988) and

thus was not capable of producing fruits in the greenhouse where there are no pollinators. To determine if the application of pesticide impacted fruit production, we constructed a partial least squares regression model with our greenhouse control experimental data where we assessed if the fixed effects of treatment (pesticide vs water sprayed plants), population, and plant genotype nested within population had a significant effect on total flower production.

Common Garden Experiment: Experimental Design

To investigate if herbivores are selecting on cyanogenic glycoside capacity, we first generated an F2 population from *Passiflora incarnata* individuals collected from the extreme ends of its native range. By generating F2s from plants from disparate populations that differed considerably in cyanogenic glycoside capacity (N. Batora, unpublished data), we aimed to increase phenotypic variance in cyanogenic glycoside production in our study population. First, parental plants collected from 8 different populations (4 northern populations and 4 southern populations) were randomly paired together to generate an F1 population. This resulted in 20 different F1 full-sib combinations. In each combination, we randomly assigned which plant would be the pollen donor (sire) and which would be the pollen recipient (dam). F2 seeds were obtained from a replicated partial-diallel cross involving 30 different F1 individuals. There were three diallels where each group was composed of 10 different individual plants. No two plants in a diallel group were from the same F1 family. Each plant was crossed as a female with five of the nine other plants in the same diallel and also crossed as a male with five of the plants in the diallel. However, many crossing pairs failed to ever set fruit or resulted in inviable seeds. Nevertheless, we succeeded to generate a total

of 68 full-sib families from this design, which were distributed among 19 dam half-sib families and 20 sire half-sib families.

To investigate if herbivores are selecting on cyanogenic glycoside capacity, we first germinated seeds by soaking them in deionized water for 48 hours at 4°C and then planted them directly into Jiffy-strips filled with Faford soil. The individuals were then placed in the greenhouse under ambient conditions and the seeds were allowed to germinate. In early July 2017, we transplanted a total of 408 individuals into the field just as they were beginning to germinate. Our field site was located at the University of Georgia Horticultural Farm (Watkinsville, Georgia) where *P. incarnata* grows naturally. The field was not fertilized at the beginning of our experiment; however, we did provide a teaspoon of instant release fertilizer (NPK 10-10-10) after transplanting to each plant in order to decrease microhabitat variation in nutrients. Prior to being used in this study, our field site had cover crops growing in it for two years.

We arranged plants in the field in a split-plot completely randomized block design. This consisted of 3 spatial blocks (separated by 8 meters) where each block consisted of two subplots (separated by 6 meters), one for each treatment. One individual from each full-sib family was randomly positioned in each subplot such that each plant in the plot was separated by 4-meters. We utilized a split-plot design due to the implementation of our treatment, which required the use of spraying a pesticide. As results will show, the application of pesticide alone did not have any effects on flower production in these plants. Thus, half of the plants were exposed to herbivores at natural densities (hereafter referred to as control) and the other half were protected from herbivores through the use of a pesticide (hereafter referred to as treatment). Treatment

plants were sprayed with the broad spectrum pesticide Acephate® 97UP (O,S-Dimethyl acetylphosphoramidothioate) at 0.36% wt/volume every two-weeks throughout the entirety of the field season, while control plants were sprayed with water similar to treatment plants.

Herbivory estimates

We sought to quantify herbivory for multiple reasons. First, it is important to determine if herbivory significantly changed between treatments, which would indicate that our application of pesticide was successful in removing herbivores. Second, we aimed to determine if herbivory had any impact on fitness. Finally, we sought to determine the relationship of cyanogenic glycoside capacity to herbivory. Thus, we measured leaf damaged on each individual plant throughout the course of the season. To assess leaf herbivory, we randomly selected a leaf at the top, middle, and bottom of the plant as a means of broadly assessing herbivory on the entire plant across leaves at different developmental stages. For each leaf, we estimated total leaf area and the amount of leaf area eaten by chewing herbivores. We estimated total leaf area by measuring leaf length from the base of the leaf to the tip. In previous unpublished work, we determined that leaf length was an excellent predictor for total leaf area in an independent study with the equation: leaf area = $0.3795(\text{leaf length})^{2.1272}$ (N=97; R^2=0.928). To calculate the amount of leaf area eaten by a chewing herbivore, we used a clear plastic 1 x 1 cm grid, which we laid on top of the leaf surface and counted the amount of leaf area missing. Thus, our measurement of leaf herbivory was the average proportion of leaf area missing relative to total leaf size (Leaf Area Missing/Total Leaf Area) from all three leaves on each plant. We took these measurements at three different

time points throughout the field season (both control and treatment: August 28, September 11, control only: October 30).

Herbivore survey

We aimed to connect the amount of leaf herbivory to the number of *E. claudia* and *A. vanillae*. Previous observations led us to believe that these two species are the main herbivores of *P. incarnata* in Athens, Georgia (N. Batora, personal observations). To achieve this, we surveyed herbivores throughout the course of the season. To survey herbivores, we visually assessed each control plant in the field thoroughly by walking around them and also looking under leaves. We did not do this for treatment plants because we expected herbivore interactions with treatment plants to be minimal. Any herbivore species that were present on a plant were noted. These surveys took place seven different times during our field season starting on August 28th and ending on October 23rd with one survey each week starting August 28th and ending October 5th followed by one final survey October 23rd.

Cyanogenic glycoside capacity estimate

To calculate cyanogenic glycoside capacity, we harvested a single leaf from each plant on September 21st. Immediately after harvest, we flash froze leaves in the field with liquid nitrogen and then stored them at -80°C until analysis. We quantified cyanogenic glycoside capacity using the technique detailed in (Brinker and Seigler 1989) with modifications by (Alonso-Amelot and Oliveros 2000). This measurement is expressed as 1 HCN = 1 cyanogenic glycoside per gram leaf (dry weight) as the assay measures the product of the catalysis of the mixture of cyanogenic glycoside and endogenous β-glycosidase and is referred to as cyanogenic glycoside capacity.

Fitness estimate

To estimate plant fitness, we counted the number of fruits produced on each plant in mid-October. At this point, plants were no longer producing new flowers and thus fruit count represented total progeny produced during the growing season. In previous work, we found that total fruit number is a good estimate of overall seed set in field collected *P. incarnata*. We estimated the correlation between seed number and fruit count per plant and found these values are highly correlated (N = 107; t Ratio = 30.10; $P<0.0001$; $R^2 = 0.896$). Given that we only counted fruits and that *P. incarnata* is an obligate outcrosser, our estimate of fitness is only representative of female fitness. Finally, not all plants produced fruits in our common garden, therefore we utilized two measures of fitness in our analyses. First, fitness was whether or not a plant produced a fruit. Then, for the plants that did produce a fruit, we used fruit count as a fitness measure. For fruit counts, we calculated relative fitness by dividing by the mean number of fruits produced.

Selection Analyses

In this study, our genetic level of analysis is half-sibs generated by similar sires or dams. This is because our crossing design to generate full-sib families in this study was a partial-diallelic design and therefore different genetic families could share either the same sire, but different dam or vice versa. This type of design is appropriate for an obligate outcrossing species like *P. incarnata* where natural selection acts on additive genetic variance (Conner and Hartl 2004). Thus, any effect of sire on phenotypic variation is indicative of segregating additive genetic variation contributing to phenotypic variation.

In order to determine if natural selection is being exerted on cyanogenic glycoside capacity, we used a genotypic selection analysis (Rausher 1992). In this analysis, the measurement of selection on a quantitative trait is similar to a phenotypic selection analysis, however, the genotypic selection analysis accounts for any biases introduced by the environment that may lead to spurious correlations between fitness and phenotypes of interest (Stinchcombe et al. 2002). Thus, our level of analysis is on genotypic means (i.e., means of full-sib families) rather than on phenotypic means (Mauricio and Mojonniner 1997). Here, partial regression coefficients are utilized in order to estimate selection gradients acting directly on cyanogenic glycoside capacity. Hence, we estimated a directional selection gradient though a linear regression between relative fitness and cyanogenic glycoside capacity and estimated stabilizing/disruptive selection gradients thought a quadratic regression of relative fitness and cyanogenic glycoside capacity.

In order to determine if herbivores are selecting on cyanogenic glycoside capacity, we constructed partial least squares regression models for relative fitness and logistic regression models for fruit production. To test for differences in directional selection among treatments, we included model effects of treatment, sire half-sib families, dam half-sib families, cyanogenic glycoside capacity, and the interaction of cyanogenic glycoside capacity and treatment. To test for differences in stabilizing/disruptive selection, we created additional models, but also with the quadratic term for cyanogenic glycoside capacity. Model terms were the same for both relative fitness and fruit production. By using both the linear and quadratic trait terms, as well as including an interaction between treatment and cyanogenic glysocide capacity, we could

compare if the slope (direction selection gradient) or the curvature (stabilizing/disrupting selection gradient) differed in the presence and absence of herbivores. If we found evidence for a significant effect of interaction term in our models, then we interpret this as indicating that herbivores are selecting on cyanogenic glysocide capacity (Simms and Rausher 1989, Mauricio and Rausher 1997). Finally, we *z*-transformed each raw cyanogenic glycoside capacity values to a have a mean of 0 and a standard deviation of 1 in order to accurately estimates the modes of selection that affect variances of the trait (Lande and Arnold 1983). All statistical analyses were carried out using SAS JMP v. 12 (SAS Institute Inc., Cary, NC, USA).

Results

In this study, we tested the hypothesis that herbivores are the agents of natural selection on cyanogenic glycoside capacity in *P. incarnata*. Based upon previous work, we predicted that greater cyanogenic glycoside capacity would be selected against in an herbivore community primarily composed of the specialist heliconiine *A. vanillae*. To evaluate this, we surveyed herbivore community across the season, determined if plants are incurring damage in the presence of herbivores, and assessed if the presence of herbivores influences natural selection on cyanogenic glycoside capacity.

Effect of Pesticide application on flower production

As a means of controlling for any bias that our pesticide spraying treatment may have introduced to our large common garden study, we performed a controlled greenhouse study where we sprayed half the plants under the same pesticide regime and the remaining half with only water. We then counted the number of flowers produced over the course of a few months to estimate the impact of our treatment on our fitness

component in this study. We found that our treatment of pesticide did not influence flower production under greenhouse conditions ($F_{1,44} = 0.1829$; $P = 0.6710$).

Overall herbivore numbers peaks in early fall driven mainly by Agraulis vanillae

To determine if the primary putative selective agent on *P. incarnata* was the specialist *A. vanillae*, we noted what herbivore species attacked plants in the field throughout the growing season of our common garden experiment. We found that plants were almost exclusively attacked by lepidopteran herbivores *Euptoieta claudia* and *Agraulis vanillae* (Figure 4.1). Furthermore, we found that lepidopteran herbivore abundance increased later in the season (around early October) as the total sum of all herbivores on control plants increased to 250, with the vast majority being *A. vanillae*. At this peak period, we found the average number of *E. claudia* was 0.231 ($\sigma = 0.543$) across all control plants and the average number of *A. vanillae* was 0.971 ($\sigma = 1.27$) per plant. These results are consistent with previous field experiments and observations of natural populations of *P. incarnata* in Athens, Georgia over the previous five years (N. Batora, personal observation).

Herbivore load predicts damage, but damage does not predict relative fitness

The explanation for why herbivores are selective agents on plant defenses is that as plants are eaten by herbivores, their relative fitness decreases (Mauricio 1998). In our study, we were explicitly interested in determining if (1) lepidopteran herbivores were the cause of damage to *P. incarnata*, (2) if our pesticide treatment was effective in preventing damage, and (3) if this leaf damage impacted an individual plant's fitness.

To determine if plants in different treatments were experiencing different levels of damage, we estimated plant damage from herbivory at three time points throughout the

field season. Here, we only report our estimates on September 11th and October 30th. This is because our first estimate on August 28th was not qualitatively different from that of September 11th. From our September 11th estimate, we found there was already a substantial difference in herbivore damage between our control and treatment plots (Table S4.1). Control plants experienced significantly more damage relative to plants who were exposed to the pesticide ($F_{1, 96} = 53.26$; $P < 0.0001$). Control plants had an average of 0.057 cm^2 (standard error = 0.005) of damage, while plants exposed to the pesticide had only an average of 0.003 cm^2 (standard error = 0.005) of damage. Our final estimate of damage took place on October 30th after the peak of lepidopteran herbivore abundance in the field (Figure 4.1). At this point, we only estimated damage on control plants and we further explored if the damage experienced by control plants could be predicted by average herbivore load per plant. We found heritable variation predicted damage in the field (sire effect: $F_{19,29} = 1.99$; $P = 0.045$) and, importantly, we found that average herbivore load predicted damage in the field ($F_{1,29} = 4.45$; $P = 0.044$) (Table 4.1). Finally, we were interested in exploring the effect damage had on plant fitness. Our measure of fitness was total number of fruits; however, many plants did not fruit at all (N = 282 / 408 total individuals). Hence, we had two different fitness components in our genotypic selection analysis. One was whether or not a plant fruited (henceforth, fruit production) and therefore had a binomial distribution. Then, for only the plants that did produce a fruit, we calculated relative fitness for fruit number (henceforth, relative fruit count). Thus, we created models for each fitness components where response variables were one of the fitness components and the predictor variables were damage, sire, and dam (Table 4.2). For relative fruit count, we did not find an effect on damage ($F_{1,8} =$

0.372; P = 0.0493). However, for fruit production, we did find an effect of damage ($X^2_{(1)}$ = 8.79; P = 0.003. Therefore, it damage does have an impact on whether or not a plant produced a fruit, but once fruits were produced, the effect of damage went away.

Natural selection does not act on cyanogenic glycoside capacity

One of the requirements for natural selection is that there is heritable variation contributing to phenotypic variation of a given trait or adaptation (Conner and Hartl 2004). In our F2 population of *P. incarnata*, we found evidence that additive genetic variation contributed to phenotypic variation in cyanogenic glycoside capacity, as there is a significant effect of sire on variation in cyanogenic glycoside capacity (Table S4.2). Furthermore, we found that cyanogenic glycoside capacity also differed between treatments ($F_{1,336}$ = 29.21; P < 0.0001), which is consistent with earlier findings in both field and greenhouse experiments (Chapter 3).

In order to determine if herbivores influenced the general pattern of selection on cyanogenic glycoside capacity, we manipulated the presence of these putative selective agents. In our control treatments, we sprayed plants with only water allowing them to be freely attacked by herbivores, while in our spray treatment, plants were sprayed with pesticide in order to protect them from any herbivore attack. We then quantified both directional and stabilizing selection gradients on these characters in the presence and absence of putative selection agents. We found that the directional selection gradients differed significantly between treatments for cyanogenic glycoside capacity (i.e., significant treatment * cyanogenic glycoside capacity interaction). Indeed, this pattern was found for both fitness components, indicating that herbivores modify the pattern of directional selection on fruit production (Table 4.4A) and relative fruit count (Table

4.3A). Further, we found that stabilizing selection gradients on cyanogenic glycoside capacity differed significantly between treatments when fitness was fruit production (Table 4.4B). However, we did not observe this for relative fruit count (Table 4.3B).

Although herbivores appear to change the nature of selection acting on cyanogenic glycoside capacity, we did not find that natural selection is indeed acting on cyanogenic glycoside capacity. We did not find a relationship between cyanogenic glycoside capacity and fitness in either of our treatments in this study. Estimated directional selection gradients for both of our fitness components were similar (Table 4.5). When herbivores were present, there were general positive trends of increasing cyanogenic glycoside capacity and fitness (Figure 4.2A; Figure 4.2C). However, when herbivores were not present, these trends were reversed where increasing cyanogenic glycoside capacity generally included a decrease in fitness (Figure 4.2B: Figure 4.2D). Further, we did not detect any evidence of a significant quadratic selection gradient, which would indicate stabilizing or disruptive selection (Table 4.5).

In order to gain greater insight into how herbivores alter the general shape of the selective surface on cyanogenic glycoside capacity, we visualized the selective surface for cyanogenic glycosides. This allowed us to see how herbivores modify the selective surface where we plotted relative fitness and cyanogenic glycoside capacity. Patterns were generally similar for each fitness component utilized in this study (Figure 4.2). For control plants exposed to herbivores, the selective surface represents all selective forces acting on cyanogenic glycoside capacity (Figure 4.2A&C). The surface increases generally, although not significantly (Table 4.5). The selective surface for plants that we sprayed with pesticide represent the total amount of selective forces acting on cyanogenic

glycoside capacity excluding herbivory (Figure 4.2B&D). Here, the selective surface has fallen flat and there appears to be no relationship between either fitness component and cyanogenic glycoside capacity. Thus, we did not appear to detect any fitness cost to cyanogenic glycoside capacity in the absence of herbivores.

Discussion

The prospect that herbivores are selecting on plant defensive traits is fundamental to the theory of coevolution (Ehrlich and Raven 1964). In their seminal paper on coevolution, Ehrlich and Raven (1964) noted the close relationship between heliconiine butterflies and *Passiflora*. Coevolution in Passifloraceae system was then further explored through a combination of field and laboratory experiments in which mutual adaptations in the *Passiflora*-heliconiine system were cataloged (Gilbert 1971, Benson et al. 1975, Turner 1981, Gilbert 1982). This resulted in this system becoming a model in the study of coevolution (de Castro et al. 2017).

In the Passifloraceae, multiple different defensive chemicals have been identified (de Castro et al. 2017). These defensive chemicals partially contribute to the dearth of herbivore species have been observed feeding on *Passiflora* plants (Turner 1981, Smiley 1982, Miller et al. 1997). The majority of herbivores that are observed to feed on *Passiflora* in natural populations are larva from the tribe heliconiine (Turner 1981, Smiley 1982, Miller et al. 1997). Since heliconiine larvae do not feed on plants other than those in the Passifloraceae, it is hypothesized that they may exert strong selective pressure on *Passiflora* populations, including contributing to the evolution of their chemical defense repertoire (Ehrlich and Raven 1964). Specifically, selection from heliconiine is hypothesized to explain the large diversity of a class of chemical defense,

the cyanogenic glycosides (Benson et al. 1975, Spencer 1988). There are many known adaptations within heliconiine that allow them to tolerate (Engler et al. 2000), sequester, and even *de novo* synthesize cyanogenic glycoside (Engler-Chaouat and Gilbert 2007), and thus the hypothesis that cyanogenic glycosides mediate coevolutionary dynamics between these organisms is enticing. However, there has never been a demonstration that cyanogenic glycosides in *Passiflora* are under natural selection from herbivores. Here, we find evidence that in an herbivore community primarily composed of a heliconiine species, *Agraulis vanillae*, and a generalist lepidopteran species from the tribe argynnini, *Euptoieta claudia*, affects the relationship of cyanogenic glycoside capacity to relative fitness. On the other hand, we do not find evidence that natural selection on cyanogenic glycoside capacity is occurring.

In order to demonstrate that herbivores are implementing natural selection on a particular plant defense trait, there are three criteria that must be met (Endler 1986, Mauricio and Rausher 1997): (1) there is genetic variation contributing to phenotypic variation in the trait, (2) individuals with different trait values have differential fitness, and (3) the putative selective agent must to be shown to contribute to evolution of the trait. Below, we elaborate on all three of these requirements.

We first detected additive genetic variation contributing to phenotypic variation in cyanogenic glycoside capacity. Given that *P. incarnata* is an obligate outcrosser, it is not sufficient to quantify only total genetic variation as natural selection only acts on additive genetic variation in outcrossing species (Conner and Hartl 2004). Although *P. incarnata* can reproduce vegetatively through clonal sprouts from underground rhrizomes (McGuire 1999), a spatial genetic structure analysis of *P. incarnata* populations spaced out as far as

10 km found that majority of genetic variation was occurring within a patch and little among patches (Tague and Foré 2005). Thus, populations of *P. incarnata* appear to be established more so through sexual reproduction rather than asexual (Tague and Foré 2005).

We did not find evidence that individuals with different trait values had differential fitness values for either one of the fitness components (fruit production vs. relative fruit count) in this study. One possible explanation for this is that cyanogenic glycoside capacity variation is not shaped by natural selection, but rather other evolutionary forces like genetic drift (Lynch and Hill 1986). Another possibility is that cyanogenic glycoside capacity may also serve other functions for *P. incarnata*, such as a nitrogen storage molecule (Gleadow and Møller 2014). Thus, in our common garden, we provided all individuals with a small amount of fertilizer early in the season, therefore minimizing the need for plants to utilize cyanogenic glycosides. Finally, we may have not observed natural selection on cyanogenic glycoside capacity due to a combination of *P. incarnata* life history and the onset of herbivory during the field season.

Passiflora incarnata is a perennial, living for multiple years and sprouts up each year from an extensive root system that stores resources (McGuire 1999). Given that herbivore numbers peaked later in the growing season, around early October, then any potential opportunity for natural selection imposed by herbivore that acts on cyanogenic glycoside capacity may only manifest in the next growing season. To elaborate, plants that incur greater damage as a consequence of their level of cyanogenic glycoside capacity likely will have a marked reduction in their ability to photosynthesize (Coley et al. 1985). This could then impact the amount of stored resources in their root system,

which may have consequences on their fitness in the next growing season. Indeed, herbivory has been shown to affect plant fitness in the following growing season for perennial plants (Knight 2003). Consistent with this hypothesis is that damage was only a good predictor when we considered only fruit production. If an individual opted to not flower or produce a fruit due to high damage, then it is possible that this could mitigate predicted costs of cyanogenic glycoside capacity in the presence of the heliconiine specialists *A. vanillae*. Indeed, other studies of *P. incarnata* have found that the number of male flowers increased relative to hermaphroditic flowers when plants were exposed to low resources (May and Spears Jr 1988). Thus, *P. incarnata* may elicit other mechanisms to cope with damage from herbivores such as tolerance (Mauricio et al. 1997). Future studies on natural selection in P. incarnata should monitor plants over multiple generations to disentangle these effects.

Finally, in order to show that herbivores are contributing to the nature of selection on cyanogenic glycoside capacity, we manipulated the presence and absence of herbivores through the use of pesticide. From our greenhouse control experiment, we showed that the implementation of pesticides did not bias our estimate of fitness in that it did not cause plants to produce a greater number of flowers. Furthermore, in previous work we showed that this treatment did not influence our estimate of cyanogenic glycoside capacity (Chapter 3). Thus, the forces that could act on cyanogenic glycoside capacity between the treatments were all forces at once (no pesticide) and all forces except herbivores (pesticide present). From this, we found evidence that the presence of herbivores alters the relationship between cyanogenic glycoside capacity and both of our fitness components. Therefore, the presence of herbivores could be important for how

cyanogenic glycoside capacity relates to fitness, but the strength of this interaction was not strong enough to cause natural selection to shape cyanogenic glycoside capacity. of multiple seasons.

The lack of a strong costs of cyanogenic glycoside capacity in the absence of herbivores is interesting. This lack of a relationship may have something to do with the reduction in cyanogenic glycoside capacity in response to herbivores that we observed. This reduction maybe a result of reallocation of cyanogenic glycosides in *P. incarnata* from leaf tissue to the roots. This phenomenon has been observed in other cyanogenic plant species, such as Cassava (*Manihot esculenta*) (Siritunga and Sayre 2004). In this scenario, allocation costs of cyanogenic glycosides may be negligible and the ecological costs are minimized. Future studies should investigate the actual mechanism behind the decrease in cyanogenic glycoside capacity and its role in minimizing costs of cyanogenic glycosides.

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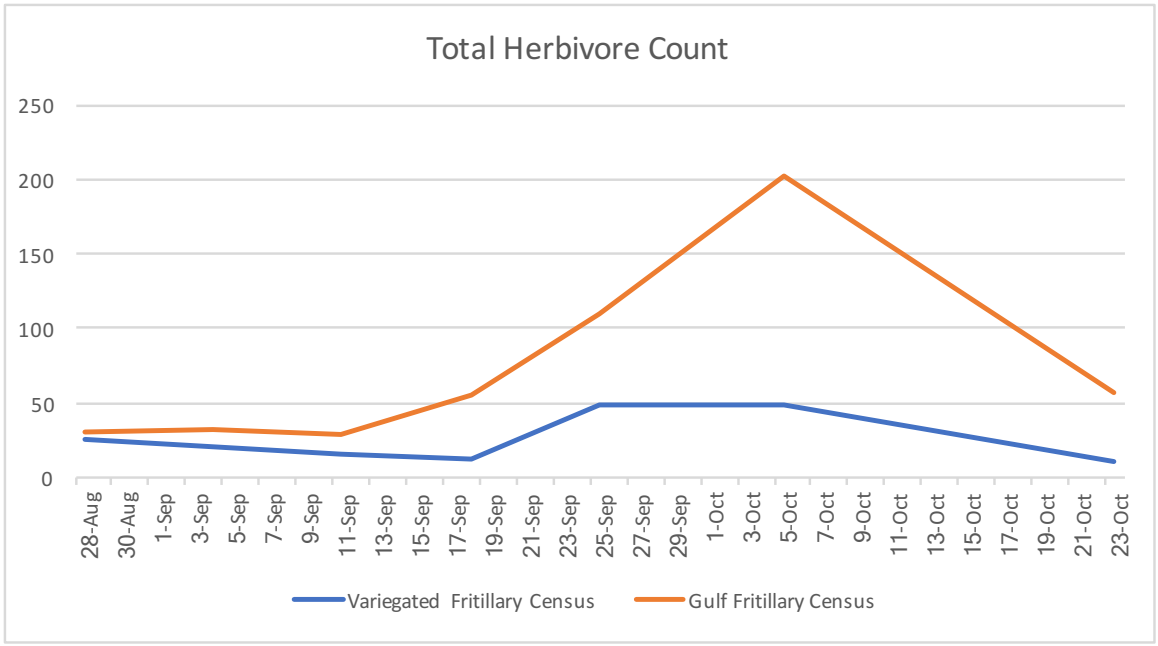

Figure 4.1)

Herbivore survey results across the duration of the experiment.

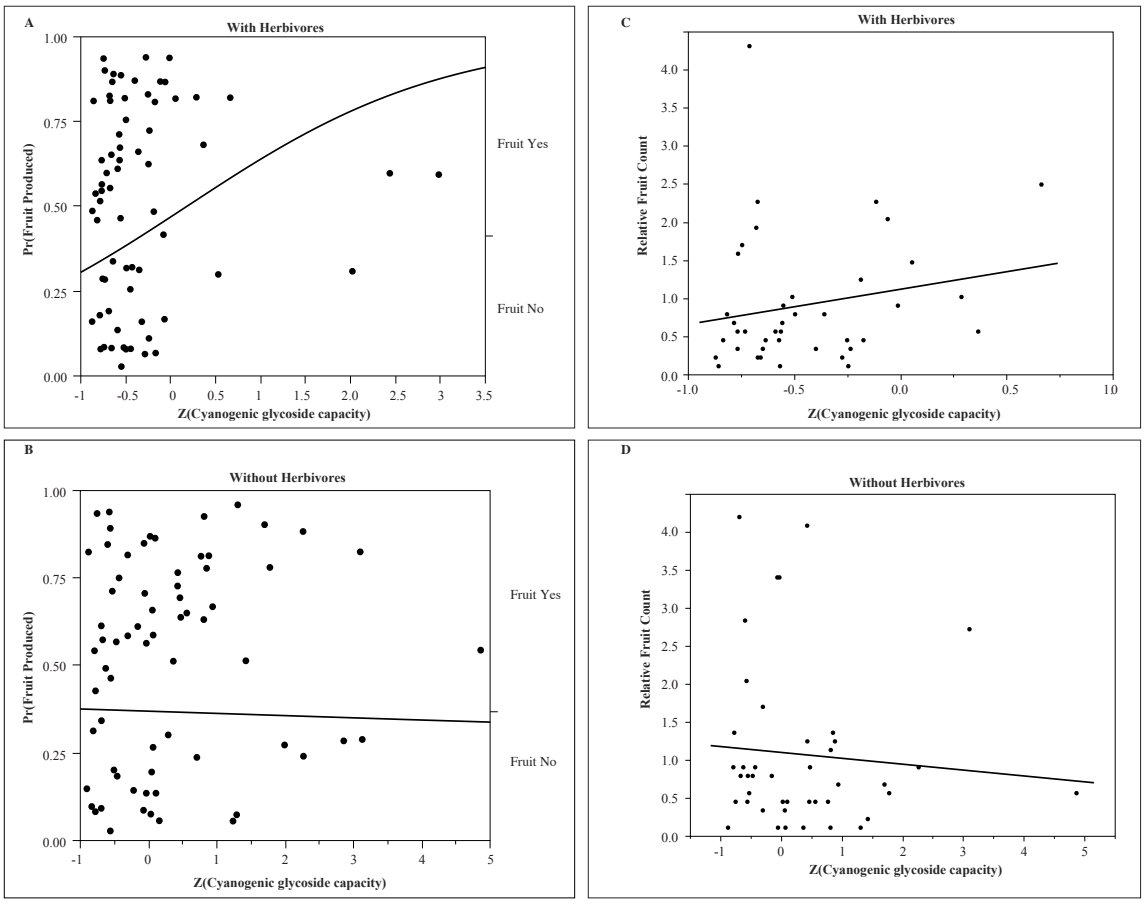

Figure 4.2)

Fitness surfaces for cyanogenic glycoside capacity where cyanogenic glycoside values are z-transformed and relative fitness is from fruit counts. Separate fitness surfaces are depicted among both of the treatments and for each form of fitness. **A&B)** Logistic regression where fitness is production. **A)** Pattern of selection in the presence of herbivores (control plants). **B)** Pattern of selection in the absence of herbivores (treatment plants). **C&D)** Linear regression where relative fitness is based upon fruit counts. **C)** Pattern of selection in the presence of herbivores (control plants). **D)** Pattern of selection in the absence of herbivores (treatment plants).

Table 4.1)

Analysis of variance of damage in the field during the week of October 30, 2017. Bold numbers indicate significant p-values.

Source	df	Sum of Squares	F Ratio	p - value
dam	18	6.4134563	2.4051	**0.0171**
sire	19	5.624957	1.9984	**0.0451**
Herbivore load	1	0.6597538	4.4535	**0.0436**
Error	29	4.29612		

Table 4.2)

Analysis of variance and logistic regression of fitness components and damage in the field during the week of October 30, 2017. Bold numbers indicate significant p-values. **A)** Relative fruit count for only plant full-sib families that did produce any fruits. **B)** Fruit production as a yes or no response variable.

A)

Source	df	Sum of Squares	F Ratio	p - value
dam	15	8.806763	0.8132	0.6529
sire	15	10.732263	0.991	0.531
damage	1	0.372274	0.5156	0.4931
Error	8	5.775768		

B)

Source	df	Chi-Square	p - value
dam	18	47.9437233	**0.0002**
sire	19	29.8150526	0.0542
damage	1	8.79637862	**0.003**

Table 4.3)

Analysis of variance for relative fitness where fitness was fruit count. The treatments were plants exposed to herbivores or protected from them with the use of pesticide. All effects reported are fixed and bold numbers indicate significant p-values. **A)** Analysis of direction selection. **B)** Analysis of stabilizing/disruptive selection. Linear terms in this analysis are biased estimates of directional selection gradients.

A)

Source	df	F Ratio	p - value
dam	17	0.7447	0.7412
sire	18	2.7138	**0.0036**
Treatment	1	5.3333	**0.0257**
Cyanogenic glycoside capacity	1	4.0635	**0.05**
Cyanogenic glycoside capacity * Treatment	1	5.5371	**0.0232**

B)

Source	df	F Ratio	p - value
dam	17	0.7459	0.7393
sire	18	2.5924	**0.0056**
Treatment	1	3.3288	0.0752
Cyanogenic glycoside capacity	1	0.0059	0.9394
Cyanogenic glycoside capacity * Treatment	1	0.4072	0.5269
Cyanogenic glycoside capacity^2	1	0.1637	0.6878
Cyanogenic glycoside capacity^2 * Treatment	1	0.0582	0.8105

Table 4.4)

Logistic regression for fruit production where fitness was whether a plant produced any fruits. The treatments were plants exposed to herbivores or protected from them with the use of pesticide. All effects reported are fixed and bold numbers indicate significant p-values. **A)** Analysis of direction selection. **B)** Analysis of stabilizing/disruptive selection. Linear terms in this analysis are biased estimates of directional selection gradients.

A)

Source	df	Chi-Square	p - value
dam	18	19.00778	0.3913
sire	19	24.94762	0.1623
Treatment	1	4.504429	**0.0338**
Cyanogenic glycoside capacity	1	9.033456	**0.0027**
Cyanogenic glycoside capacity * Treatment	1	4.632075	**0.0314**

B)

Source	df	Chi-Square	p - value
dam	18	23.25084	0.1811
sire	19	31.36329	**0.0368**
Treatment	1	10.42742	**0.0012**
Cyanogenic glycoside capacity	1	2.873632	0.09
Cyanogenic glycoside capacity * Treatment	1	1.859209	0.1727
Cyanogenic glycoside capacity^2	1	8.202433	**0.0042**
Cyanogenic glycoside capacity^2 * Treatment	1	7.460583	**0.0063**

Table 4.5)

Directions ($\boldsymbol{\beta}$) and stabilizing/disruptive ($\boldsymbol{\gamma}$) selection gradients from selection analysis in the presence and absence of herbivores. Standard errors are in parentheses.

		Herbivores present (control)		Herbivores absent (sprayed)	
Fitness trait	Resistance trait	$\boldsymbol{\beta}$	$\boldsymbol{\gamma}$	$\boldsymbol{\beta}$	$\boldsymbol{\gamma}$
Fruit production (y/n)	Cyanogenic glycoside capacity	0.697	0.544	-0.027	0.029
		(0.440)	(0.572)	(0.223)	(0.127)
Fruit count	Cyanogenic glycoside capacity	0.460	0.981	-0.077	0.040
		(0.376)	(0.840)	(0.147)	(0.076)

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

In this dissertation, we sought out to determine if a type of plant secondary metabolite, cyanogenic glycoside, is involved in a coevolutionary dynamic between *Passiflora incarnata* and heliconiine butterflies. Additionally, we took an untargeted NMR metabolomics approach to identify other, potentially biologically important, metabolites. To achieve this, we undertook several experiments to investigate different predictions of coevolutionary theory (Thompson 2005, Laine 2009). In our estimates of cyanogenic glycoside, we measured the cyclopentenoid precursors to cyanogenic glycoside in *Passiflora incarnata*, as well as the ultimate product of cyanogenesis, hydrogen cyanide (HCN) (Gleadow and Møller 2014). We investigated geographic variability in cyclopentenoids and putative metabolites associated with biotic interactions using an NMR metabolomics approach. We further investigated geographic variability in HCN and the influence of this and other physical defense traits on thwarting herbivore attack. Finally, we tested the hypothesis that herbivores are selecting on cyanogenic glycosides in natural populations.

In the second chapter, we performed two, separate metabolomics experiments. First, we explored changes in leaf metabolomes among different forms of damage. This experiment allowed for us to determine what changes in leaf metabolomes were associated with damage in general and damage from an herbivore. In our second experiment, we measured leaf metabolomes in plants collected from a large latitudinal

transect. With both of these experiments combined, we were able to assess how NMR features involved in lepidopteran interactions were distributed across latitude. From these studies, we found many different NMR features that had latitudinal patterns. Most interestingly, we also found evidence of two different cyclopentenoids in *P. incarnata* that we called cyclopentenylglycine and isocyclopentenylglycine. These cyclopentenoids differed in their relative concentrations and in their induced responses to herbivores. Further, we found that both of the cyclopentenoids negatively impacted herbivory from a generalist lepidopteran species, *Chrysodeixis includens*. However, both had no impact on herbivory from a specialist lepidopteran species, *Agraulis vanillae*.

In the third chapter, we conducted a common garden experiment to assess geographic variation in three defense traits and their impact on herbivory. We found that two defense traits, leaf toughness and cyanogenic glycoside capacity, had negative and positive distributions over latitude, respectively. Further, we found that cyanogenic glycoside capacity was negatively related to end-of-season biomass. This relationship was exacerbated by the presence of herbivores. Finally, we also determined that cyanogenic glycoside capacity decreased in response to herbivores.

In the fourth chapter, we conducted a common garden experiment to quantify if cyanogenic glycoside capacity is under natural selection from herbivores. We found significant additive genetic variation contributing to variation in cyanogenic glycoside capacity. Additionally, we found that the relationship of cyanogenic glycoside capacity to fitness changed in the presence and absence of herbivores. However, we did not find evidence that cyanogenic glycoside capacity is under natural selection.

Together, all of these results are consistent with coevolutionary theory that cyanogenic glycoside mediates coevolutionary dynamics with heliconiine butterflies. Further, they open up several new areas of inquiry that will be of value in future studies on the evolutionary ecology of cyanogenic glycoside.

Coevoluionary theory predicts that there will be geographic variation in traits that mediate interactions among different species when the form and strength of these interactions vary of space (Thompson 2005). Here, we found that across latitude, where the abundance of heliconiine butterflies varies, so do cyclopentenoid precursors to cyanogenic glycoside as well as cyanogenic glycoside capacity. In regions where these butterflies are the most abundant, *P. incarnata* populations produce the lowest levels of cyclopentenoids and HCN. This suggest that these traits are costly in the presence of these herbivores. However, the exact mechanism behind these costs is still unknown. Heliconiine adults assess plant suitability after landing on a leaf, followed by tapping their antennae and rubbing their forelegs on the plant surface (Williams and Gilbert 1981). This behavior may stimulate chemoreceptors in the insect, which results in the determination if a host plant is suitable for oviposition based on chemical cues (Williams and Gilbert 1981). It is possible that this chemical cue, whether directly or indirectly, is related to cyanogenic glycosides within the host (Spencer 1988), however, this needs to be further explored. Further, heliconiine larva can sequester cyanogenic glycosides from *Passiflora* as they feed (Engler-Chaouat and Gilbert 2007). This includes *A. vanillae*, the most abundant herbivore in all of the common garden experiments reported here. Thus, cyanogenic glycoside production could be costly in the form of allocation costs in the

presence of heliconiine specialists or it could be costly in its attractiveness to heliconiine specialist. Future studies should investigate these possibilities.

Although we attempted to determine the exact dynamics of cyanogenic glycoside and specialist and generalist herbivores, our experiments were not all encompassing. We did not replicate specialist and generalist herbivore treatments in our experiments, and therefore we can only state that the patterns we observed were in response to these species and not specialism vs generalism, *per se* (Ali and Agrawal 2012). Future studies should include more specialist and generalist herbivores where replication is not confounded by common ancestry (i.e., phylogenetic independent contrast).

Future experiments within *P. incarnata* need to explicitly determine if HCN is indeed defensive against generalist herbivores and ineffective against specialist herbivores. In Chapter 2 we did show that the precursors had different impacts on *A. vanillae* and *C. includens*. We found that the cyclopentenoids were negatively correlated with the amount of leaf area eaten by *C. includens*, while they had no such effect on *A. vanillae*. These results are consistent to predictions that cyanogenic glycosides are effective defenses against generalists (Gleadow and Woodrow 2002) and ineffective against heliconiine specialists (de Castro et al. 2017). This experiment only investigated the precursors, so we do not know if the pattern observed was due to the precursor, the possible correlation of the precursors to cyanogenesis, or some other cryptic source that is highly correlated with the precursors. Future studies need to quantify all components of cyanogenic glycoside biosynthesis and cyanogenesis in *P. incarnata* in order to disentangle the patterns we observed. Specifically, future experiments should measure the precursors, cyanogenic glycosides, β -glycosidase, and HCN to investigate if all have

similar impacts on herbivory. One possible avenue to achieve this can be to experimentally manipulate each component of this pathway through the use of transgenics.

In our study system, we developed a model of how the precursors relate to cyanogenic glycoside production and hydrogen cyanide production. Cyanogenic glycoside biosynthesis has been elucidated in several plant species including flax (*Linum usitatissimum*; (Cutler and Conn 1981)), white clover (*Trifolium repens*; (Hughes 1991)), cassava (*Manihot esculenta*; (Andersen et al. 2000)), almonds (*Prunus amygdalus*; (Sánchez-Pérez et al. 2008)), and sorghum (*Sorghum bicolor;* (MacFarlane et al. 1975)). In each of these taxa, the biosynthesis of cyanogenic glycoside is highly similar (Gleadow and Møller 2014). Using an amino acid precursor, plant cells quickly biosynthesize cyanogenic glycoside into benign molecules that are then separated from the enzyme in which they interact, β-glycosidase (Gleadow and Møller 2014). Upon tissue disruption, these two molecules come together and result in the production of hydrogen cyanide (Gleadow and Woodrow 2002). If we assume parsimony and that cyanogenic glycoside biosynthesis in *P. incarnata* is similar to other plant species in all of these steps, then based upon our discovery of two cyclopentenoid precursors, we hypothesize that *P. incarnata* is synthesizing multiple cyanogenic glycosides. However, it has been previously reported that *P. incarnata* produces only a single cyanogenic glycoside, gynocardin (Jaroszewski et al. 2002). Therefore, future studies need to investigate if there are multiple cyanogenic glycosides produced in *P. incarnata*.
In several of our experiments, we found the pattern of a decrease in cyanogenic glycoside capacity and the precursor, isocyclopentenylglycine, when plants are exposed to

herbivores. For isocyclopentenylglycine, this response is due specifically to herbivore feeding as we did not observe this response in mechanically damaged leaves (Chapter 2). While, for cyanogenic glycoside capacity, our results also suggest that this response is due to interactions from herbivores. Indeed, in each field experiment in Chapter 3, cyanogenic glycoside capacity decreased when plants were exposed to herbivores. Further, in a controlled greenhouse study, plants sprayed with methyl jasmonate also had a marked reduction in cyanogenic glycoside capacity (Chapter 3). Together, these results suggest that the decrease in cyanogenic glycoside capacity maybe due to a decrease in production of one putative form of cyanogenic glycoside over the other. Indeed, we did not observe a reduction in the precursor cyclopentenylglycine like we did with isocyclopentenylglycine (Chapter 2). Further, isocyclopentenylglycine was at a relative ratio of 3:1 with cyclopentenylglycine (Chapter 2). Thus, if the drop we observe in cyanogenic glycoside capacity is due to a drop in one of the two different forms of cyclopentenoids, it would likely be due to isocyclopentenylglycine. However, an alternative hypothesis is that the drop of isocyclopentenylglycine is not related to the drop in cyanogenic glycoside capacity. It is possible that the drop in cyanogenic glycoside capacity is rather due to an alteration of the enzyme β-glycosidease. In other plant species where cyanogenic glycoside capacity changed in response to herbivores, it was found that this alteration was due to changes in the quantities of β -glycosidease (Ballhorn et al. 2006). Future investigations in *P. incarnata* should try to disentangle the dynamics between the precursors and cyanogenic glycoside capacity. One possible experiment would be using labeled carbon or nitrogen to determine the flux between the precursors, cyanogenic glycoside, and ultimately hydrogen cyanide.

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SUPPLEMENTARY MATERIAL CHAPTER 2

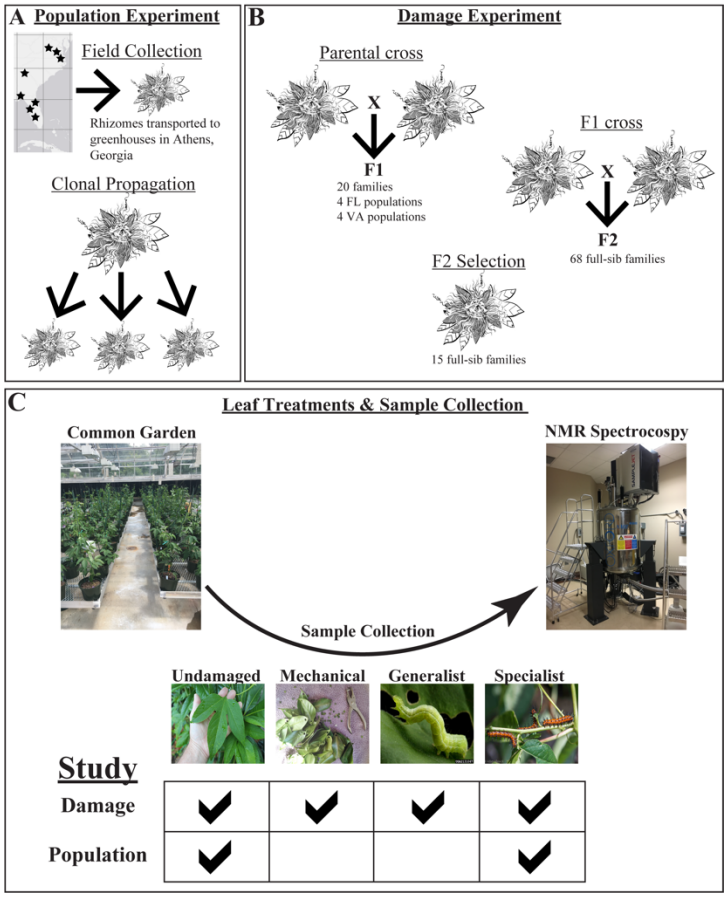

Figure S2.1)

Generation of experimental units (i.e., plants) in our study. **A)** Population experiment. **B)** Damage experiment. **C)** Brief overview of sample generation and differences among the two experiments.

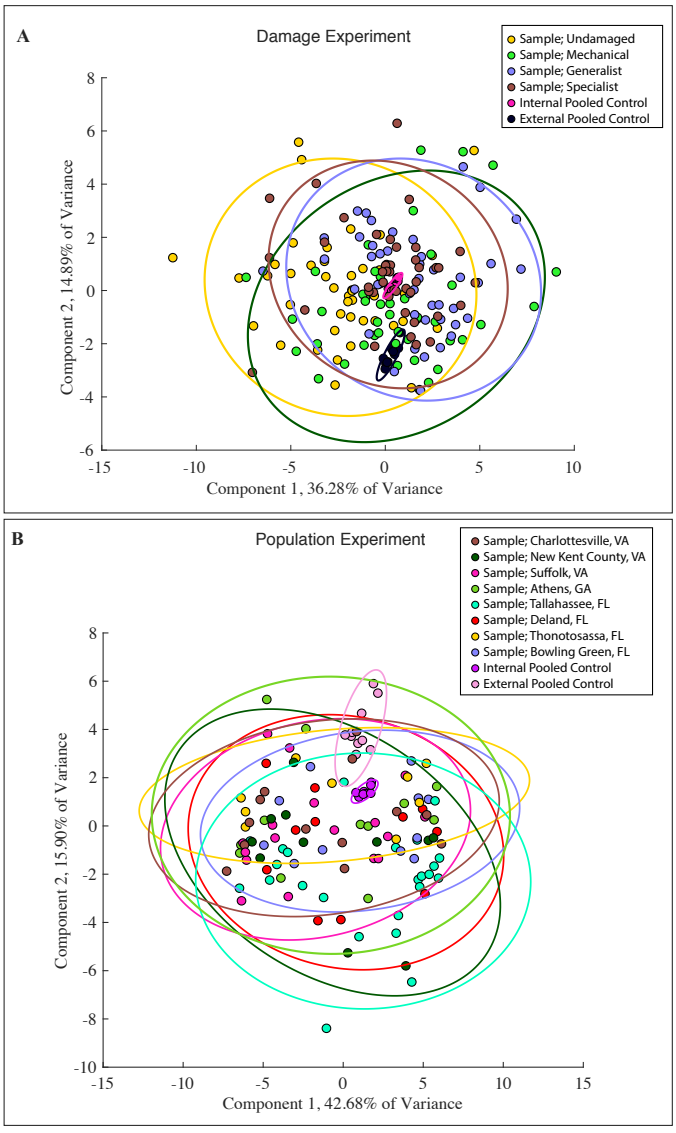

Figure S2.2)

Principal component analysis plots of all experimental samples and internal and external pooled controls used for quality control. Ellipses denote 95% confidence intervals. **A)** PCA of ^{1}H-NMR spectra from samples utilized in the damage experiment. **B)** PCA of ^{1}H-NMR spectra from samples utilized in the population experiment. **A & B)** Pink symbols are from internal pooled controls and black symbols are from external pooled controls.

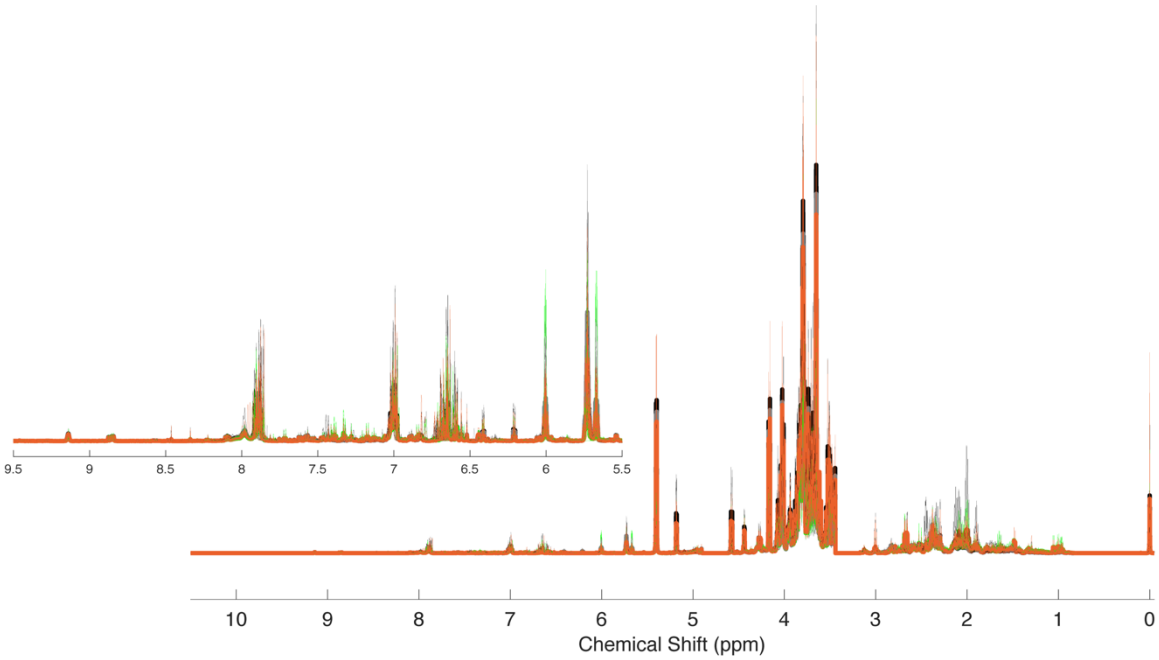

Figure S2.3)

^{1}H-NMR spectra from the damage experiment. Colors of spectra correspond to control (black), mechanical (grey), generalist herbivore (green), and specialist herbivore (orange) forms of damage. Bold lines indicate mean spectra for each latitudinal region, while thin lines correspond to individual samples.

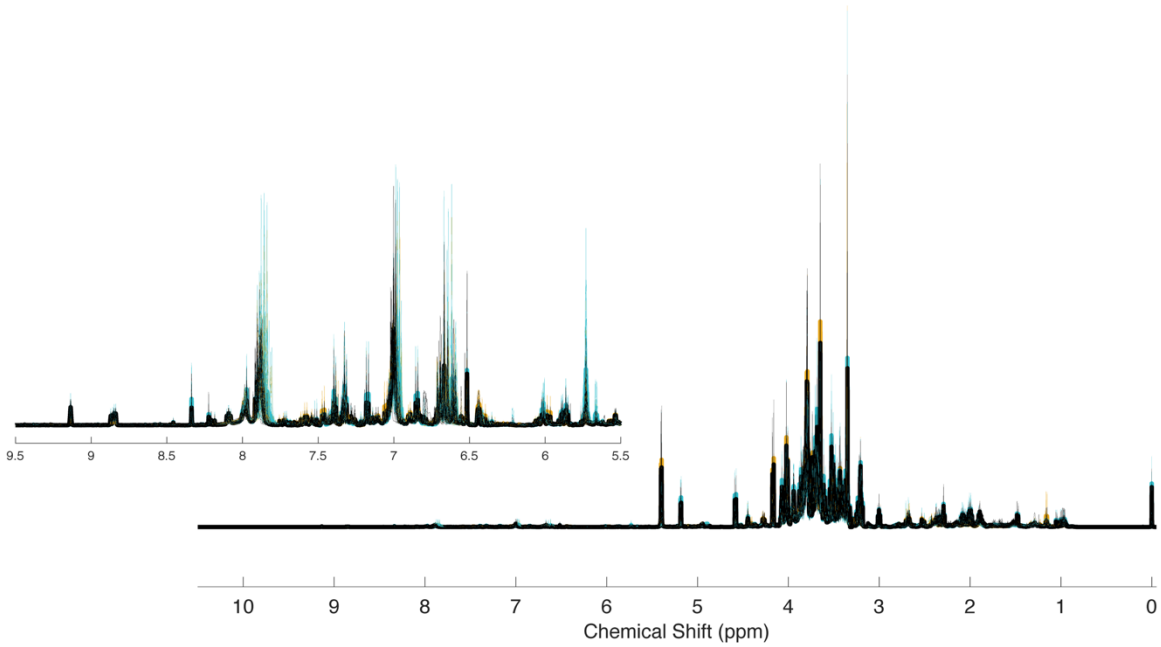

Figure S2.4)

^{1}H-NMR spectra for our population experiment. Colors of spectra correspond to high (gold), intermediate (teal), and low (black) latitudinal regions (Table S1). Bold lines indicate mean spectra for each latitudinal region, while thin lines correspond to individual samples.

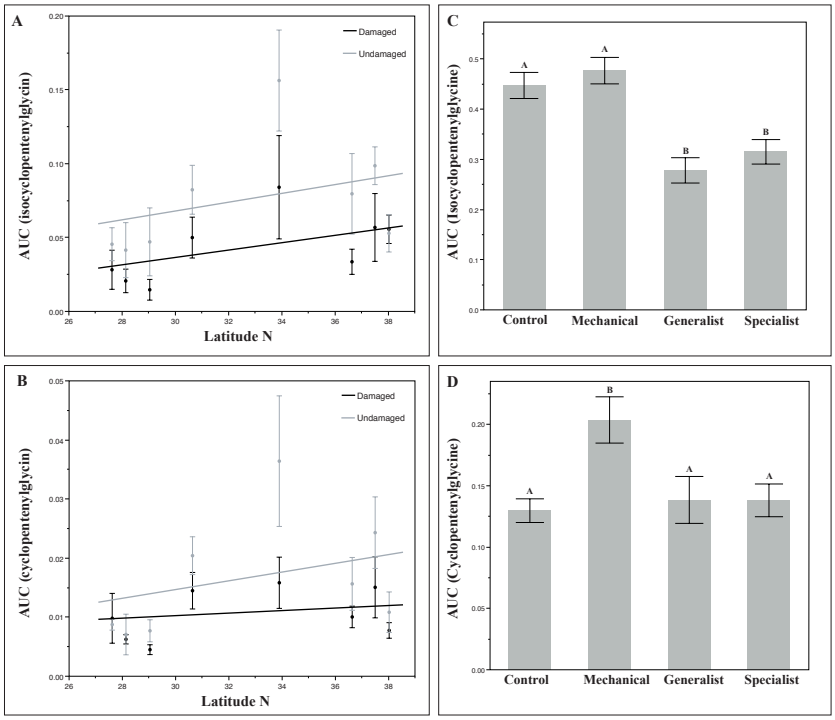

Figure S2.5)

Changes in cyclopentenoid quantity (area under curve; AUC) over latitude and in response to different forms of damage. **A&B)** Latitudinal patterns of cyclopentenoids from the POP dataset. Colors correspond to damaged (black) and undamaged (grey) leaves. Statistical tests results for the effect of latitude and treatment can be found in Table 1. Error bars represent one standard error. **A)** Isocyclopentenylglycine quantity over latitude in both damage and undamaged leaves. **B)** Cyclopentenylglycine quantity over latitude in both damage and undamaged leaves. **C&D)** Response in amounts for cyclopentenoids from the DAM dataset. Letters over figures represent groups corresponding to a *post-hoc* Tukey-HSD test. Error bars represent one standard error. **C)** Isocyclopentenylglycine response to various damage treatments. **D)** Cyclopentenylglycine response to various damage treatments.

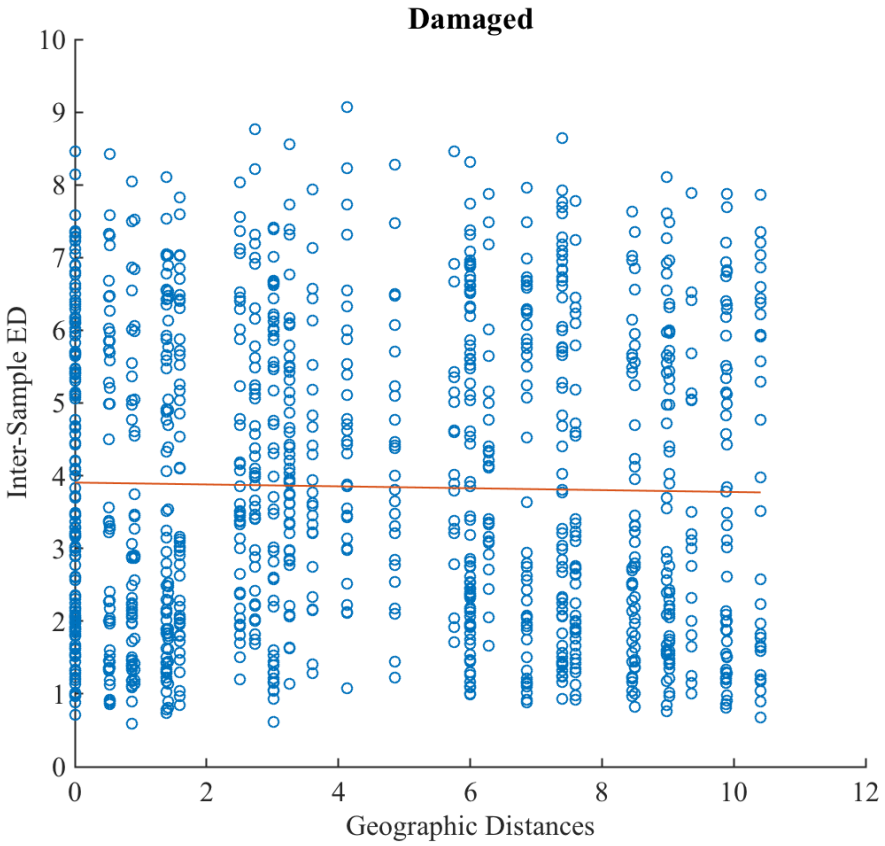

Figure S2.6)

Euclidean distances in metabolic profiles among damaged leaf samples across populations with corresponding trend line (orange). Geographic distance represents latitudinal distance among population of origin for each sample. The trend line is slightly negative; however, it is not significant (r^2 = -.0207; p-value = 0.4876).

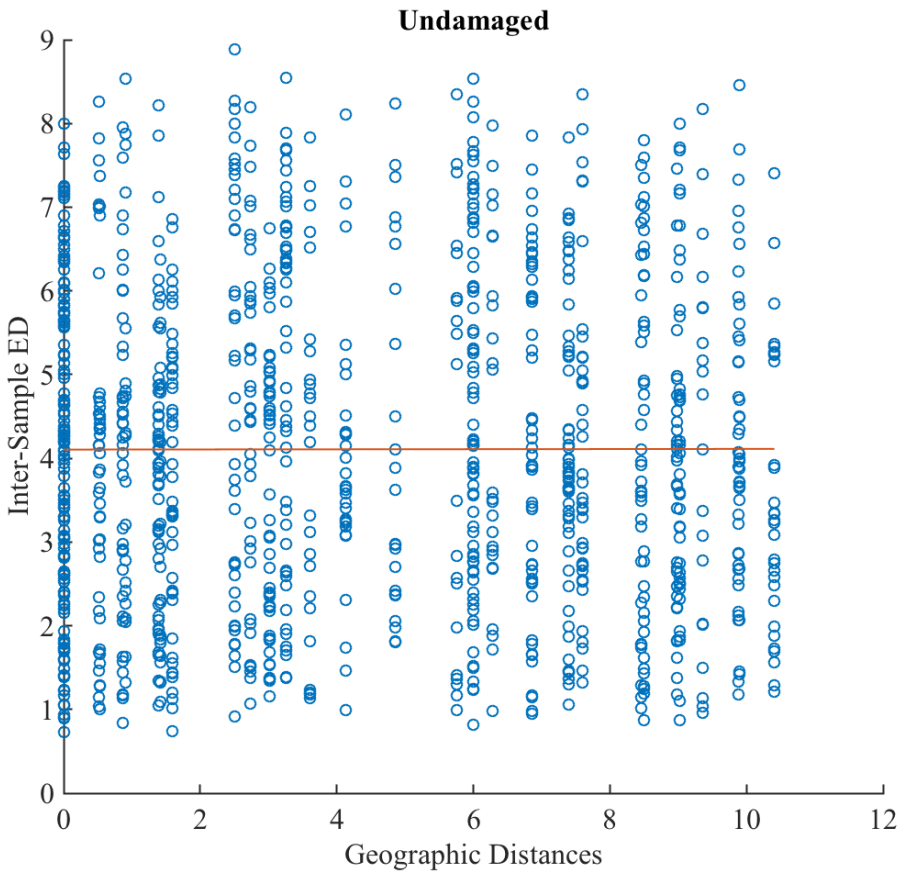

Figure S2.7)

Euclidean distances in metabolic profiles among undamaged leaf samples across populations with corresponding trend line (orange). Geographic distance represents latitudinal distance among population of origin for each sample. The trend line is slightly positive; however, it is not significant ($r^2 = 0.0013$; p-value = 0.9653).

Table S2.1)

Summary of *Passiflora incarnata* populations sampled in this study. Genotype represents a single plant collected from the field.

Latitudinal Region	Population	Latitude	Longitude	Number of Genotypes
High	Charlottesville, Virginia	38.0299°N	78.4790°W	6
High	New Kent County, Virginia	37.5035°N	77.1339°W	5
High	Suffolk, Virginia	36.6408°N	76.8888°W	8
Intermediate	Athens, Georgia	33.9007°N	83.3873°W	5
Intermediate	Tallahassee, Florida	30.6385°N	84.2286°W	10
Low	Deland, Florida	29.0400°N	81.3281°W	5
Low	Thonotosassa, Florida	28.1376°N	82.2311°W	4
Low	Bowling Green, Florida	27.6205°N	81.8139°W	5

SUPPLEMENTARY MATERIAL CHAPTER 3

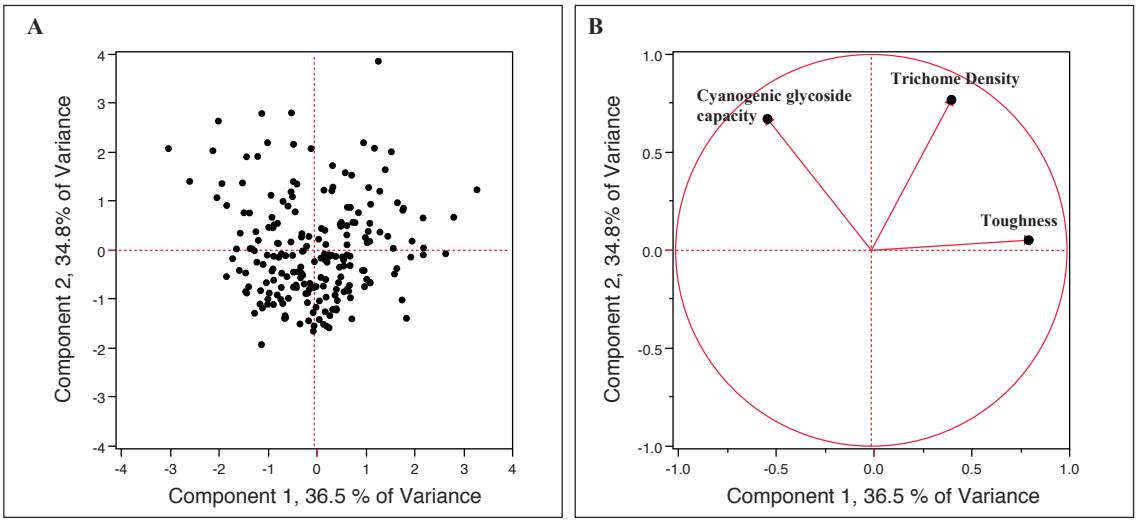

Figure S3.1)
Principle component analysis of all three defense traits measured in the current study. **A)** Score plot for defensive traits in the current study. **B)** Loading plot of defensive traits in the current study.

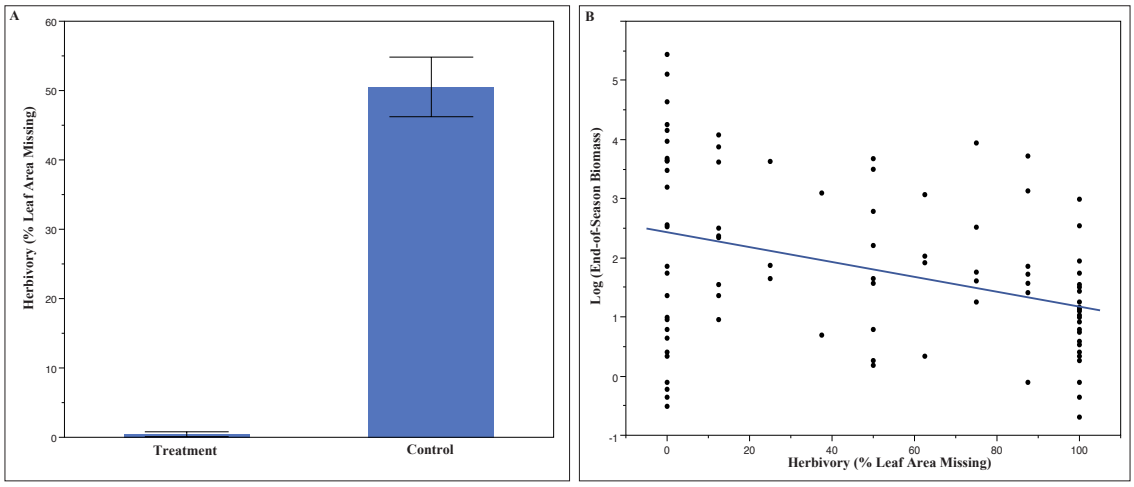

Figure S3.2)
Herbivory among treatments and its influence on end-of-season biomass. **A)** Differences in herbivory among treatments where control represents plants sprayed with water and treatment represents plants sprayed with pesticide. **B)** The linear relationship between end-of-season biomass and herbivory for only control plants (i.e., sprayed with water). Error bars represent one standard error.

Table S3.1)
Summary of *Passiflora incarnata* populations sampled in this study. Genotype represents a single rhizome collected from a population.

Population	Latitude	Longitude	Number of Genotypes
Paynes Creek Historic State Park; Bowling Green, Florida	27.6205°N	81.8139°W	6
Hillsborough River State Park; Thonotosassa, Florida	28.1376°N	82.2311°W	5
Paynes Prairie Preserve State Park; Micanopy, Florida	29.5198°N	82.2950°W	2
Fall Line Sandhills Natural Area; Taylor County; Georgia	32.5500°N	84.2333°W	12
University of Georgia Natural Area; Athens, Georgia	33.9007°N	83.3873°W	2
Great Dismal Swamp National Wildlife Refuge; Suffolk, Virginia	36.6408°N	76.8888°W	4
New Kent County Natural Area; New Kent County, Virigina	37.5035°N	77.1339°W	5
Shadwell Farm; Charlottesville, Virginia	38.0299°N	78.4790°W	7

Table S3.2)
Summary of mixed model fixed effects of treatment, latitude, and latitude*treatment for *Passiflora incarnata* defense traits across populations that span 27.6205°N – 38.0299°N latitude. Bold items are significant p-values.

	Cyanogenic Glycoside Capacity			Trichome Density			Toughness		
Source of Variation	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Treatment	1	4.928	**0.0278**	1	0.7257	0.3955	1	0.0465	0.8296
Latitude	1	12.292	**0.0011**	1	0.8800	0.3535	1	11.81	**0.0013**
Latitude*Treatment	1	1.209	0.2731	1	0.2541	0.6149	1	0.3173	0.574

Table S3.3)
Analysis of variance for end-of-season biomass of P. incarnata grown in the field and the effect of quadratic trait terms. Treatments were plants exposed to herbivores or plants protected from herbivores through the use of a pesticide. Only model fixed effects are reported. Bold numbers indicate significant p-values.

	Log End-of-Season Biomass		
Source of Variation	df	*F*	*P*
Treatment	1	12.30413	**0.0006**
Cyanogenic glycoside production	1	5.169244	**0.0241**
Toughness	1	0.376891	0.54
Trichome Desntiy	1	2.88819	0.091
Cyanogenic glycoside production * Treatment	1	1.380856	0.2416
Toughness * Treatment	1	7.133906	**0.0083**
Trichome Density * Treatment	1	3.844891	0.0516
Cyanogenic glycoside production^2	1	0.743852	0.3896
Toughness^2	1	1.451915	0.2298
Trichome Desntiy^2	1	0.018356	0.8924
Cyanogenic glycoside production^2 * Treatment	1	0.042329	0.8372
Toughness^2 * Treatment	1	0.015896	0.8998
Trichome Desntiy^2 * Treatment	1	0.019027	0.8904

Table S3.4)
Analysis of Variance of cyanogenic glycoside capacity for control experiment. Treatment is plants either treated with methyl jasmonate or sprayed with water. Leaf age is young or adult leaves. All reported model effects are fixed. Bold numbers indicate significant p-values.

	df	Sum of Squares	*F*	*P*
Family	11	52.51	5.615	**<.0001**
Treatment	1	13.57	15.97	**<.0001**
Leaf Age	1	39.84	46.87	**<.0001**
Leaf Age * Treatment	1	0.3181	0.3743	0.5412
Block	3	1.572	0.6165	0.6049
Error	262			

SUPPLEMENTARY MATERIAL CHAPTER 4

Table S4.1)

Explanatory variables for damage in the field during the week of September 11, 2017. Bold numbers indicate significant p-values.

Source	df	Sum of Squares	F Ratio	p - value
dam	18	0.02964251	0.8921	0.5891
sire	19	0.02865758	0.817	0.6821
treatment	1	0.09832609	53.2623	**<.0001**
Error	96	0.17722304		

Table S4.2)

Explanatory variables that contribute to variation in cyanogenic glycoside capacity. Only fixed effects are reported.

Source	df	Sum of Squares	F Ratio	p - value
dam	18	26.017991	2.2783	**0.0024**
sire	19	22.67797	1.8813	**0.0147**
Treatment	1	18.535268	29.2156	**<.0001**
Block	2	1.150036	0.9064	0.405
Error	336	213.17		