

TESTING HYPOTHESIZED LATITUDINAL
PATTERNS IN PLANT-INSECT INTERACTIONS
FROM TROPICAL TO NORTH-TEMPERATE REGIONS

By

Carina Alexis Baskett

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Biology—Doctor of Philosophy
Ecology, Evolutionary Biology and Behavior—Dual Major

2018

ABSTRACT

TESTING HYPOTHESIZED LATITUDINAL PATTERNS IN PLANT-INSECT INTERACTIONS FROM TROPICAL TO NORTH-TEMPERATE REGIONS

By

Carina Alexis Baskett

Biotic interactions vary geographically, with potential consequences for evolutionary processes such as local adaptation and speciation. The “biotic interactions hypothesis” posits that biotic interactions are stronger at lower latitudes, and that coevolution has contributed to the well-known, poorly understood pattern of greater biodiversity toward the equator. The hypothesis plausibly connects known environmental variation to speciation rates, but strong empirical support is lacking for latitudinal patterns of some interactions, particularly herbivory and pollination. Current data may be insufficient, and methods of quantifying interaction strength lack standardization and rigor. I tested the hypothesis that plant-insect interactions are stronger at lower latitudes by quantifying contemporary herbivory and pollination rates, and patterns of trait evolution that may show signatures of past selection. Herbivory and plant defense were measured along a 15° latitudinal gradient in *Phytolacca americana* in the eastern USA, and in *P. rivinoides*, its tropical congener, in Costa Rica. Both are perennial early-successional herbs studied in their native ranges. Patterns of herbivory rates in the field and plant defense (quantified using a bioassay in a common garden) were consistent with the biotic interactions hypothesis for most metrics, particularly for young leaves, which are rarely measured in comparable studies. Variation in leaf chemistry, which is a strong predictor of palatability for a generalist herbivore, depends strongly on geography and leaf age. In addition, multiple metrics of pollination (e.g., self-pollination ability) were evaluated for *P. americana* at the northern and southern range edges, and for *P. rivinoides* in Costa Rica. Patterns were consistent with a greater

reliance on insect pollination at lower latitudes. For both herbivory and pollination, latitudinal patterns were rarely clinal, instead demonstrating thresholds or scale-dependency. For example, comparisons of *P. rivinoides* and north-temperate *P. americana* were consistent with the biotic interactions hypothesis, but comparisons of either region with subtropical *P. americana* showed a variety of patterns. These three chapters strongly support a long-standing hypothesis, and generate new hypotheses for ecological mechanisms driving geographic variation in biotic interactions. The fourth chapter reviews the state of evidence for evolution and genetics of mutualisms, focusing on the question of how adaptation and speciation may be unique when driven by mutualistic interactions. Together, these studies further our understanding of the ecological causes and evolutionary consequences of variation in plant-insect interactions.

This dissertation is dedicated
to my mother Jenelle O'Sullivan,
my grandmothers Josefina O'Sullivan and Dawn Baskett,
and my great-grandmother Eileen McPhee.
Your stories led me here.

ACKNOWLEDGEMENTS

I am so thankful to my tag-team advisors, Doug Schemske and Marge Weber, for helping me to grow as a researcher more than I thought possible. I feel ready for a lifetime of science, thanks to you. I thank my committee members Jen Lau, Gary Mittelbach, and Jeff Conner, for engaging deeply with me on ideas and practicalities, and putting up with endless downsizing of my ambitions. Chapters 1 and 4 were published with the help of Doug Schemske as a co-author. Marjorie Weber contributed greatly to chapters 2-3. Chapter 2 includes data collected for Lucy Schroeder's senior thesis project. Anthony Schillmiller was instrumental in the chemical analyses in Chapter 3. My research assistants were a joy to work with: Alyssa Tretheway, Abby Sulesky, Carolyn Graham, Emma Clark, Glenda Mesen, Andie Veach, Dani Blumstein, Erin Leestma, and Keegan Mackin. Numerous lab technicians also helped with essential practical advice and data collection: Nick Batora, Jon Spoelhof, Mark Hammond, Rachel Atchison, Jessie Eilers, Inam Jameel, Susan Gordan, and Dan Hughes. There are an astonishing number of people who allowed me to use their property as a field site, helped me find plants, and extended wonderful hospitality during my travels. Many have already been thanked in these chapters, but I particularly wish to highlight the Mesen family, the McPhee family, the Yule family, Rick Toomey, Vince Morris, Reinaldo Aguilar, Albert Meier, Nick Batora, and Betsey Boughton. My parents, Fufi, and Kaily have been very patient, and I appreciate their support of strange hobbies like science. I thank the many other friends and family who supported me, especially my hexen Emily Dittmar, Kristen Nolting, and Heather Stadden. Finally, I am deeply grateful for my partner Georg Rieckh, who is remarkably good at changing my mind, and thereby convinced me that all of this would work out.

TABLE OF CONTENTS

LIST OF TABLES	ix
LIST OF FIGURES	xii
KEY TO SYMBOLS AND ABBREVIATIONS	xvii
INTRODUCTION	1
REFERENCES	7
CHAPTER 1: LATITUDINAL PATTERNS OF HERBIVORE PRESSURE IN A TEMPERATE HERB SUPPORT THE BIOTIC INTERACTIONS HYPOTHESIS	11
Introduction	12
Methods	14
<i>Study system</i>	14
<i>Does herbivory vary with latitude and/or leaf age?</i>	15
<i>What is the shape of the herbivory-latitude relationship?</i>	18
<i>Does herbivore abundance predict herbivory?</i>	19
<i>Does plant defense vary latitudinally and/or with leaf age?</i>	20
Results	23
<i>Does herbivory vary with latitude and/or leaf age?</i>	23
<i>What is the shape of the herbivory-latitude relationship?</i>	23
<i>Does herbivore abundance predict herbivory?</i>	27
<i>Does plant defense vary latitudinally and/or with leaf age?</i>	28
Discussion	30
Acknowledgements	35
REFERENCES	37
CHAPTER 2: MULTIPLE METRICS OF LATITUDINAL PATTERNS IN INSECT POLLINATION AND HERBIVORY FOR A TROPICAL-TEMPERATE CONGENER PAIR	44
Introduction	45
Methods	48
<i>Study system and sites</i>	48
<i>Floral visitation rate in situ: tropical, temperate</i>	52
<i>Self-pollination in a common garden: tropical, subtropical, temperate</i>	55
<i>Floral display and reward in a common garden: tropical, subtropical, temperate</i>	55
<i>Pollinator attraction in a common garden: subtropical, temperate</i>	59
<i>Herbivory rate and herbivore abundance in situ: tropical, temperate</i>	61
<i>Leaf palatability in a common garden: tropical, subtropical, temperate</i>	63
Results	64
<i>Floral visitation rate in situ: tropical, temperate</i>	64

<i>Self-pollination in a common garden: tropical, subtropical, temperate</i>	66
<i>Floral display and reward in a common garden: tropical, subtropical, temperate</i>	66
<i>Pollinator attraction in a common garden: subtropical, temperate</i>	68
<i>Herbivory rate and herbivore abundance in situ: tropical, temperate</i>	69
<i>Leaf palatability in a common garden: tropical, subtropical, temperate</i>	73
Discussion	74
Acknowledgements	79
APPENDIX	80
REFERENCES	83
 CHAPTER 3: LEAF AGE DETERMINES TRAITS UNDERLYING STRONGER HERBIVORE DEFENSE AT LOWER LATITUDES IN A PAIR OF CONGENERS	 89
Introduction	90
Methods	93
<i>Study species</i>	93
<i>Common gardens</i>	93
<i>Leaf trait measurements</i>	94
<i>Palatability</i>	97
<i>Statistical analyses</i>	98
<i>Latitude and leaf age effects on traits and palatability</i>	98
<i>Traits predicting palatability</i>	98
<i>Identification of chemicals predicting palatability</i>	99
Results	100
<i>Latitude and leaf age effects on traits and palatability</i>	100
<i>Traits predicting palatability</i>	106
<i>Identification of chemicals predicting palatability</i>	107
Discussion	111
Acknowledgements	116
APPENDIX	117
REFERENCES	121
 CHAPTER 4: EVOLUTION AND GENETICS OF MUTUALISM	 128
Introduction	129
Natural selection in mutualisms	130
The genetics of mutualism evolution	132
<i>Advanced stages</i>	133
<i>Early stages</i>	135
Emerging systems and tools	140
<i>Moving beyond pollination</i>	140
<i>Genomic approaches</i>	142
<i>Experimental evolution</i>	142
Future directions	143
<i>Comparison to antagonisms</i>	144
<i>Speciation</i>	144
<i>The latitudinal diversity gradient</i>	145

<i>Anthropogenic environmental change</i>	145
<i>Conclusions</i>	146
Acknowledgements	147
REFERENCES	148

LIST OF TABLES

Table 1. Location and sampling details for sites shown in Fig. 1. Site ID refers to Table 3. Dates are shown for when leaves were marked and measured, with the intervening interval in days. More leaves and plants were marked than measured due to disease and mortality. Young and mature refer to leaves marked for each leaf age.	17
Table 2. Results and model comparison for mixed-effects ANCOVA models analyzing per-plant mean herbivory on young <i>P. americana</i> leaves at ten sites in 2015. Models are shown in order of increasing AIC. d.f. = degrees of freedom (Satterthwaite approximation), AIC = Akaike information criterion value, dAIC = difference in AIC from best-fitting model, weight = Akaike weights, Lep. = lepidopteran (abundance was log _e -transformed). Population as a random blocking factor is included in all models. <i>F</i> - and <i>p</i> -values are from restricted maximum likelihood models, while AIC values are from maximum likelihood models. Significant effects (<i>p</i> < 0.05) are bolded.	25
Table 3. Lepidopteran herbivore species collected in 2015 from 3,000 <i>Phytolacca americana</i> leaves from ~30 individual plants per site. Sites are shown sequentially from south to north (see Table 1 for locations). The sum of two collections (late July and early September) is shown. “Recorded Range” refers to the collection sites where each species is expected to occur based on its recorded geographic range. Larval morphospecies A may have been <i>A. velutinana</i> , <i>A. gordialis</i> , or <i>P. obscuralis</i> ; counts for successfully reared adults are shown in italics for each. Morphospecies BG and BQ were never reared to adulthood. Sites with asterisks were only surveyed in late July because there were too few leaves to survey in early September; thus, for all sites only the July survey was used in analyses throughout the manuscript.	26
Table 4. Results for mixed-effects ANCOVAs of biomass of <i>S. exigua</i> larvae per replicate cup (see Fig. 4). Larvae were reared on a diet of young vs. mature <i>P. americana</i> leaves from 13 populations grown in the greenhouse from seeds collected along a latitudinal gradient. Top: effects of latitude and leaf age on larval biomass. Bottom: effects of leaf area consumed and leaf age on larval biomass. Both biomass and leaf area are log _e -transform and standardized by duration of experiment and starting number of larvae. Plant maternal line nested in population is included as a random blocking factor. Significant <i>p</i> -values (<0.05) from Type III SS are bolded.	29
Table 5. Hypotheses and study design for each metric of pollination and herbivory. <i>P. riv.</i> = <i>Phytolacca rivinoides</i> , native to the Neotropics; <i>P. am.</i> = <i>P. americana</i> , native to the eastern USA. Cohort is indicated by a combination of place (<i>in situ</i> vs. common garden) and year.	51
Table 6. Populations of <i>Phytolacca rivinoides</i> (<i>P. riv.</i>) and <i>P. americana</i> (<i>P. am.</i>) used for <i>in situ</i> data collection or seed collection for common gardens at Michigan State University. Site ID corresponds to map in Fig. 5 and studies in Table 5. The	

Finca Bellavista population was spread along a dirt and gravel road of a few kilometers, so the coordinates are less precise; other populations were patches or spread along shorter dirt roads. 52

Table 7. Loadings, proportion of variance explained, and Eigenvalues from principal components analysis of four floral display traits: inflorescence length, open and fresh flowers per inflorescence, and flower diameter. Traits were measured on three populations each of temperate and subtropical *Phytolacca americana* and tropical *P. rivinoides*. For each population, we measured three individuals from three maternal lines and analyzed mean values from multiple inflorescences per individual in a common garden. Temperate plants were measured in the field, subtropical plants were measured in the field and greenhouse (treated as separate measurements), and tropical plants were measured in the greenhouse, due to differences in flowering time. 58

Table 8. Loadings, proportion of variance explained, and Eigenvalues from principal components analysis of five floral display traits: inflorescence length, open and fresh flowers per inflorescence, flower diameter, and inflorescence count. Traits were measured on three temperate and three subtropical populations of potted *Phytolacca americana* in a field common garden in Michigan, USA, and the first PC is used for analysis of traits correlated with floral visitation. For each population, we measured three individuals from three maternal lines and analyzed mean values from multiple inflorescences per individual. 61

Table 9. Effects of region and leaf age (young or mature) on herbivory and palatability. Herbivory (percent leaf area consumed over ~42 days) was measured in three populations in each region over three intervals in tropical *Phytolacca rivinoides* and two in temperate *P. americana*. Palatability (biomass of *Spodoptera exigua* reared on a diet of *Phytolacca* leaves) was measured for three populations in each of three regions: tropical (*P. rivinoides*), subtropical, and temperate (*P. americana*). 70

Table 10. Effects of season and leaf age (young or mature) on herbivory (percent leaf area consumed over ~42 days). Three populations in each region were surveyed over three seasons in the tropics for *Phytolacca rivinoides* (dry, dry-wet transition, and wet; Mar 4-Jul 23, 2016) and two in the temperate zone for *P. americana* (mid- and late summer; Jul 5-Sep 29, 2017). Plant means of 5 marked leaves of each age were analyzed using a mixed model with random effects of individual nested in population. See Fig. 14. 71

Table 11. Daily herbivory rates (percent leaf area consumed per day) for each population in each season. See Table 6 for population key. 81

Table 12. Mean percent leaf area consumed per day by leaf age in dry and wet seasons in Central America. “Gap spp.” = means of 20 gap specialists, not including *P. rivinoides*, measured in Panama by Coley in 1979, from Table 5 in Coley (1983). “*P. riv.*” = *Phytolacca rivinoides* in three populations in Costa Rica in 2016, from this study. 82

Table 13. Interactions between biogeography and leaf age for *Phytolacca* leaf traits hypothesized in the literature to influence palatability. Models for *P. americana* include 13 populations along a gradient and treat latitude as a continuous predictor. The *P. rivinoides* comparison consists of three regions, each containing three populations: tropical *P. rivinoides*, subtropical *P. americana*, and north-temperate *P. americana*. All models contain a random effect of population. 101

Table 14. Effects of leaf age and latitude or region on palatability of *Phytolacca* species (*Spodoptera exigua* larval biomass per leaf area consumed; Fig. 1). Latitude is treated as a continuous variable for analysis of *P. americana* along a gradient of 13 populations in eastern North America. We compared the tropical *P. rivinoides* to subtropical and north-temperate *P. americana* (three populations per region). All models included random effects of maternal line nested in population. Significant effects ($p < 0.05$) are in bold. 106

Table 15. Chemical peaks determined to be predictive of palatability by machine learning analysis using the Random Forest algorithm (RF), shown in rank order of RF importance scores (Gini index). The sign of the importance score is the sign of each peak's correlation with palatability. 109

LIST OF FIGURES

- Figure 1.** *Phytolacca americana* populations in the mid-western and eastern USA used for herbivory surveys and palatability assays. The first year of field herbivory surveys (2014) included five populations (black dots). In 2015, these populations were re-surveyed, and five more were added (black and grey dots). Palatability assays utilized seeds from all 13 populations shown (black, grey, and white dots). See Table 1 for GPS and other details. Plotted with *ggmap* in R (Kahle & Wickham 2013). 16
- Figure 2.** Herbivory in the field (percent leaf area consumed) for young and mature leaves of *P. americana* in 2014. Lines of best fit (solid = mature, dashed = young) and population means \pm S.E. are shown. The mature-leaf line is shown in gray because the 95% confidence interval for the slope barely overlapped zero. 23
- Figure 3.** Herbivory in the field (percent leaf area consumed) for young leaves of *Phytolacca americana* in 2015 plotted against latitude (left) and lepidopteran abundance (right). Each point is a population mean \pm S.E. Each plot shows the results of two models: one where the predictor is continuous, and another with a threshold in the predictor (thicker line). Constant herbivory at the mean of the five populations is shown when the 95% confidence interval of the slope overlapped zero. Letters correspond to models in Table 2; AIC values increase from A to E. 24
- Figure 4.** Larval biomass of *Spodoptera exigua* reared on mature vs. young leaf diets (solid vs. dashed line) of *Phytolacca americana* from seeds collected along a latitudinal gradient. Each point is a population mean \pm S.E. (based on number of plant maternal lines). Biomass and area were measured per replicate cup and standardized by the number of starting caterpillars and duration after a $\log_e + 1$ transformation (mature: 5 caterpillars, 8 days; young: 3 caterpillars, 9 days). The left-hand plot shows results of a model with a continuous predictor, and a model with a threshold in the predictor (thicker line). For the threshold models, constant herbivory at the population means are shown because the 95% confidence interval of the slopes overlapped zero. See Table 4 for model results. 29
- Figure 5.** Populations used for *in situ* studies of pollination and herbivory rate and/or sources of seeds for common gardens planted at Michigan State University. Populations are grouped throughout the paper into three consistently color-coded “regions:” temperate and subtropical for *P. americana*, and tropical *P. rivinoides*. Letters correspond to Table 5 and Table 6; coordinates are in Table 6. Labels for A and B would overlap, so they are labeled “A-B” at coordinates for population A. 50
- Figure 6.** Inflorescences of *Phytolacca americana* (A, B) and *P. rivinoides* (C, D). A representative screenshot of video recordings used to estimate floral visitation rate is shown for each species (B-C). 54

Figure 7. Principal components of floral display traits of *Phytolacca rivinoides* (tropical) and subtropical and temperate *P. americana* (populations A-C, D-F, and G-I in Fig. 5, respectively). Subtropical plants were measured in both the field and greenhouse and show an effect of environment and/or phenology; temperate plants were only measured in the field, and tropical plants only in the greenhouse. Traits were floral diameter (“diam”), inflorescence length (“length”), open flowers per inflorescence (“open”) and fresh flowers per inflorescence (“fresh”). The vectors for open and fresh flowers overlap. 57

Figure 8. Insect visitation rates per flower per hour *in situ* for three populations each of tropical *Phytolacca rivinoides* (pink) versus temperate *P. americana* (blue; Table 5). Bars are raw means \pm SE, although the response was \log_e+1 transformed for analysis; asterisk indicates significant difference in means ($p = 0.002$). 65

Figure 9. Autogamy rate (filled/possible fruits) for *Phytolacca* in a greenhouse nearly absent of pollinators. *Phytolacca rivinoides* is pink, and *P. americana* is dark and light blue; each region was represented by three populations (Table 5). Bars are least-square means \pm SE, and significant differences between regions are indicated with different letters. 66

Figure 10. Comparison of PC1 (A) and PC2 (B) between region of origin and place of measurement. See Fig. 7 and Table 7 for PC loadings. –PC1 is shown because PC1 was negatively correlated with flower size. All plants experienced the same environments at the same times, but due to differences in phenology, measurements were made at different times and places during the common garden experiment. Tropical *Phytolacca rivinoides* was only measured in the greenhouse, temperate *P. americana* was only measured in a field common garden, and subtropical *P. americana* was measured in both locations. Bars are least-square means \pm SE, and asterisks indicate significant differences for comparisons in each measurement location ($p < 0.05$). 67

Figure 11. Regional differences in nectar production and floral visitation in a common garden, and the correlation between nectar and visitation. (A) Floral nectar volume of *Phytolacca rivinoides* (pink) and *P. americana* (dark and light blue). Nectar was quantified in the greenhouse, in the absence of pollinators, for three populations per region (Table 5). (B) Floral visitation rate of subtropical and temperate populations of *P. americana* in a common garden in Michigan, USA. (C) Nectar volume is a significant predictor of floral visitation rate in the common garden. Bars are least-square means \pm SE. Bars with different letters are significantly different (A), and the asterisk indicates a significant difference between regions in (B). Points in (C) are individual means. 68

Figure 12. Display size in the field common garden (PC1 axis of inflorescence count, inflorescence length, total open flowers, fresh flowers, and flower diameter) is not a significant predictor of floral visitation rate. 69

Figure 13. Percent leaf area consumed of young and mature leaves in three populations each of tropical *Phytolacca rivinoides* (pink) and temperate *P. americana* (blue; see Table 5). Herbivory was surveyed by marking and re-measuring leaves ~42 days later

season) and two in the temperate zone (3 months; mid- and late summer). We marked 3,625 leaves (five of each age per plant at each interval) and analyzed individual means of each leaf age in each interval ($N = 674$ after mortality). Bars are least-square means \pm SE, and those that share the same letter are not significantly different according to Tukey's HSD test.

70

Figure 14. Percent leaf area consumed of young and mature leaves in three populations of *Phytolacca rivinoides* in Costa Rica over three seasons. Herbivory was surveyed by marking and re-measuring leaves ~42 days later in three intervals (Mar 4-Jul 23; dry season, dry-wet transition, and wet season). We marked 1,825 leaves (five of each age per plant at each interval) and analyzed individual means ($N = 324$ plants after mortality). Bars are least-square means \pm SE, and those that share the same letter are not significantly different according to Tukey's HSD test.

71

Figure 15. Abundance of lepidopteran larvae in three populations each of *Phytolacca rivinoides* (pink) and *P. americana* (blue). Four censuses of 3000 leaves were conducted in each population. Bars are raw census means \pm SE, although the response was $\log_e + 1$ transformed for analysis; asterisk indicates significant difference in means ($p = 0.02$).

72

Figure 16. Biomass of larval *Spodoptera exigua* reared on a no-choice diet of young or mature leaves of greenhouse-grown *Phytolacca rivinoides* (pink) and *P. americana* (dark and light blue). Three populations per region were assayed (Table 5). Bars are least-square means \pm SE. Biomass was measured per cup (non-survivors included as zero mass) and standardized by initial larvae number and experiment duration after natural log transformation (see text). Bars that share the same letter are not significantly different, according to Tukey's HSD.

73

Figure 17. Chemical composition for young leaves (open circles) and mature leaves (closed circles) of *Phytolacca americana* (light blue, grey, dark blue) and *P. rivinoides* (pink), based on 110 peaks from LC/MS analysis. The grey points are from seven *P. americana* populations that are not included in regional analyses, but lie between the subtropical and temperate regions. A) Plot of the first two NMDS axes. B) Plot of the third and fourth NMDS axes.

102

Figure 18. Latitudinal patterns in various leaf traits (A-H) for *Phytolacca americana* for young and mature leaves (open circles and dashed line vs. closed circles and solid line, respectively). All traits significantly differed by leaf age, and we plotted best-fit lines for latitude if there was a significant main effect of latitude or a latitude-age interaction (Table 13). Points are population means (\pm SE), and points for young leaves are jittered 0.3 degrees south to minimize point overlap. NMDS scores are from Fig. 17.

103

Figure 19. Geographic and ontogenetic differences in various leaf traits (A-H) for three populations per region of *Phytolacca rivinoides* (tropical) and *P. americana* (subtropical and temperate). Young leaves are open bars, and mature leaves are filled bars. Bars are means (\pm SE). Bars that share letters are not significantly different according to Tukey's HSD; panels D and E only had significant main effects of region, while all others had

significant main effects of the interaction of region and leaf age (Table 13). NMDS scores are from Fig. 17. 104

Figure 20. Palatability of *Phytolacca americana* and *P. rivinoides*, quantified by *Spodoptera exigua* larval biomass per leaf area consumed, as a function of latitude and leaf age (mature = filled circles and solid line, young = open circles and dashed line). Each point is a population mean (\pm SE) of per-cup palatability. Lines of best fit are shown for analysis of *P. americana* populations along a latitudinal gradient. Different letters show significant differences between regions and leaf ages for comparison of tropical *P. rivinoides* (pink) to north-temperate (light blue) and subtropical (dark blue) *P. americana*, according to Tukey's HSD. Grey populations are not included in regional analyses. See Table 14 for results. 105

Figure 21. Palatability (*Spodoptera exigua* biomass per leaf area consumed) as a function of various leaf traits for young (A-B) and mature (C-D) *Phytolacca* leaves (open vs. closed circles, respectively). Leaf ages were modeled separately, and slopes were obtained from mixed models including both traits and a random effect of population. 107

Figure 22. Model comparison using R^2 from machine learning analyses of the chemical peaks predicting palatability, using the Random Forest algorithm (RF). Chemical models began with 110 peaks; shown are results from the best model for each leaf age, with 27 peaks for young leaves and 3 peaks for mature leaves. Each bar is the mean (\pm SE) of 100 RF iterations. Model selection procedures are detailed in Supplemental Information. 108

Figure 23. Chemical composition, colored by region, for young leaves (A, open circles) and mature leaves (B, closed circles), based on peaks from LC/MS determined to be important predictors of palatability for each leaf age by Random Forest analysis (27 for young leaves, 3 for mature). *Phytolacca americana* is light blue, grey, and dark blue; *P. rivinoides* is pink. The grey points are from seven *P. americana* populations that are not included in regional analyses, but lie between the subtropical and temperate regions. 110

Figure 24. Scree plots of stress values for up to 11 NMDS dimensions using chemical abundances of 110 LC/MS peaks from young and mature leaves. Analyses are based on Bray-Curtis distances. We used four dimensions for further analyses, because stress was 0.058 at this threshold. 118

Figure 25. Model selection steps for predicting young-leaf palatability with chemical peak areas. A) Feature importance scores (Gini index) for initial model with 110 chemical peaks. B) Model fit based on number of features in the model. C) Actual and predicted values of palatability for the best-fitting model (highest R^2 in B, which includes 27 chemical peaks). 118

Figure 26. Model selection steps for predicting mature-leaf palatability with chemical peak areas. A) Feature importance scores (Gini index) for initial model with 110 chemical peaks. B) Model fit based on number of features in the model. C) Actual and

predicted values of palatability for the best-fitting model (highest R^2 in B, which includes only three chemical peaks).

119

Figure 27. Scree plots of stress values based on number of NMDS dimensions using chemical abundances of LC/MS peaks that were found to be important for predicting palatability in young (left) and mature (right) leaves. Twenty-seven peaks were used for young leaves, and three for mature leaves. Analyses are based on Bray-Curtis distances. We used two dimensions for further analyses for each leaf age, because stress was an average of 0.075 at this threshold.

119

Figure 28. Leaf trait correlations for young (A) and mature (B) leaves. Circle color and size correspond to correlation magnitude according to key at right. Blank boxes are not significantly correlated ($\alpha = 0.05$).

120

KEY TO SYMBOLS AND ABBREVIATIONS

°	Degree
±	Plus or minus
%	Percent
ANCOVA	Analysis of Co-Variance
ANOVA	Analysis of Variance
BIH	Biotic Interactions Hypothesis
C	Celsius
Ch.	Chapter
d	days
d.f.	degrees of freedom
e.g.	for example
et al.	and others
<i>F</i>	F-statistic
Fig.	figure
h	hours
i.e.	that is
HSD	Honestly Significant Difference
L.	Linnaeus
LC/MS	Liquid Chromatography-Mass Spectrometry
min	minutes
ML	Machine Learning
mL	milliliter

N	sample size
NMDS	Nonmetric Multidimensional Scaling
p	probability value
RF	Random Forest
v	volume
USA	United States of America

INTRODUCTION

Biotic interactions are predicted to play a major role in shaping adaptive evolution, community assembly, and biodiversity. Unlike interactions with abiotic factors, biotic interactions can coevolve, leading to a variety of evolutionary feedbacks. For example, the Red Queen hypothesis predicts that coevolution contributes to the maintenance of sexual reproduction (Jaenike 1978), and “Janzen-Connell effects” predict that specialized enemies promote coexistence in diverse communities (Janzen 1970, Connell 1971). Furthermore, spatial variation in biotic interactions has been hypothesized to contribute to the latitudinal diversity gradient (Wallace 1878, Dobzhansky 1950, Fischer 1960, Schemske 2009), the striking and poorly understood biogeographic pattern of greater biodiversity at lower latitudes (Mittelbach et al. 2007). This “biotic interactions hypothesis” has two testable predictions: 1) at lower latitudes, biotic interactions are more important selective agents, because the abiotic environment is less stressful. That is, in the absence of strong selection from cold temperatures, populations must adapt chiefly to challenges arising from other organisms. 2) This geographic variation in the importance of biotic interactions leads to faster speciation rates at lower latitudes because populations may diverge from each other faster if their main environmental challenges are biotic, and thus continuously evolving.

The biotic interactions hypothesis plausibly connects known environmental gradients to differential speciation rates, but it is controversial (Moles and Ollerton 2016), and there are many competing explanations for the latitudinal diversity gradient. For example, Palmer (1994) lists 120 hypotheses. Despite the preponderance of ideas, testing them is difficult at best due to the massive temporal and spatial scales involved. Thus, we still lack a generally accepted explanation for the latitudinal diversity gradient, reflecting a fundamental missing piece in our understanding of ecology and evolution (Mittelbach et al. 2007).

The biotic interactions hypothesis is considered an “evolutionary” hypothesis (Mittelbach et al. 2007) because it posits differential diversification rates between tropical and temperate regions. There is evidence for greater diversification rates in the tropics from phylogenetic comparative analyses (Condamine et al. 2012, Pyron and Wiens 2013, Rolland et al. 2014), and patterns of fossils and endemism show higher speciation rates at lower latitudes (e.g., Jablonski et al. 2013, Hanly et al. 2017). However, the bulk of research on the latitudinal diversity gradient has focused on “ecological” hypotheses (e.g., high tropical productivity supports large populations, less likely to go extinct (Currie et al. 2004)) and “historical” hypotheses (e.g., tropics are historically larger and less disturbed (Fine and Ree 2006)), without achieving a widely-accepted explanation (Mittelbach et al. 2007). Evolutionary explanations, then, may be necessary to fill the gap, and may act in conjunction with other hypotheses (Mittelbach et al. 2007, Schemske 2009).

There are three intellectual strengths of the biotic interactions hypothesis: 1) It is based on temperature, known to correlate tightly with species diversity. 2) It explicitly provides a mechanism by which temperature and biotic interactions contribute to speciation rates. 3) It is generalizable beyond latitude, potentially applying to other gradients in abiotic stressors that covary with species richness: altitude, ocean depth, precipitation, etc. (Schemske et al. 2009). Nevertheless, testing the hypothesis is still in early stages, and there is disagreement about how best to test it and how to interpret existing evidence (Schemske et al. 2009, Moles 2013, Anstett et al. 2016, Moles and Ollerton 2016).

One major open question is, are biotic interactions stronger at lower latitudes? Schemske et al. (2009) reviewed 39 types of biotic interactions, and determined that 30 were more prevalent or stronger at lower latitudes, with the remainder showing no pattern. Some examples

related to plants include a higher tropical prevalence of animal pollination (Regal 1982), animal seed dispersal (Moles et al. 2007), endophyte infection (Arnold and Lutzoni 2007), and extrafloral nectaries, which are both mutualistic and defensive (Coley and Aide 1991). Herbivory and plant defense have been shown to be more important in the tropics in some reviews (e.g., Coley and Aide 1991, Dyer and Coley 2002; reviewed in Schemske, Mittelbach et al. 2009), but not others (Moles et al. 2011). Anstett et al. (2016) argue that current data are insufficient and methods have been too heterogeneous to draw conclusions on latitudinal patterns in herbivory and plant defense. They suggested improvements for future work, many of which have been applied in my research.

Chapter 1 tests the hypothesis that herbivore pressure increases toward the equator. I measured herbivory and defense along a latitudinal gradient from 27°-42°N in the USA in *Phytolacca americana*, a native herbaceous pioneer species. I found higher herbivory rates at lower latitudes, and palatability experiments revealed that low-latitude plants are better defended, consistent with the hypothesis that the impact of herbivory on defense evolution varies latitudinally. Furthermore, I found evidence that past studies may have unintentionally overlooked latitudinal patterns by not measuring tender young leaves that are often the target of herbivory. This study strongly supports a long-standing hypothesis, and was published in *Ecology Letters* (Baskett and Schemske 2018).

Chapter 2 is an in-depth cross-species investigation comparing both herbivory and pollination in *P. americana* to its ecologically similar congener, *P. rivinoides*, in Costa Rica. The biotic interactions hypothesis was proposed at a global scale, to explain differences between tropical and temperate regions (Dobzhansky 1950). However, most latitudinal comparisons of biotic interactions within species or genera are conducted within temperate regions, and evidence

is mixed that the hypothesis is predictive at this scale (Anstett et al. 2016). Additionally, although there is evidence that mutualisms are more prevalent in tropical communities (Schemske et al. 2009), to our knowledge there are no studies quantifying latitudinal patterns in strength of a plant-insect mutualism. I quantified eight metrics of biotic interaction strength for herbivory and pollination. Rates of both interactions are higher for *P. rivinoides* than *P. americana*, and trait evolution is consistent with greater importance of these interactions for driving adaptation in the tropical species.

Chapter 3 investigates geographic patterns in the evolution of leaf traits important for plant defense in *P. americana* and *P. rivinoides*. I quantify latitudinal patterns in traits in young and mature leaves that are often used as proxies of palatability (leaf toughness and nitrogen content) and in leaf chemistry (using liquid-chromatography mass spectrometry). All traits differ with geography and leaf age, though not always in directions predicted by the biotic interactions hypothesis. However, geographic analyses are paired with functional bioassays to ask which traits are predictive of palatability, and I find that only leaf chemistry is an important defense against a generalist lepidopteran. Thus, patterns of trait evolution support the biotic interactions hypothesis when defensive trait function is known.

Another open question regarding the biotic interactions hypothesis is, does population divergence and speciation occur more rapidly when selective agents coevolve? There are several cases where traits associated with species interactions are implicated in driving higher diversification rates in sister taxa comparisons: phytophagy in insects (Mitter et al. 1988), plant latex canals (Farrell et al. 1991), animal pollination in plants (Dodd et al. 1999), nectar spurs (Hodges and Arnold 1995), and extrafloral nectaries (Weber and Agrawal 2014). In addition, host-pathogen coevolution is considered an important ultimate cause of selection for sexual

reproduction in hosts (Jokela et al. 2009). Despite these anecdotes, and despite much active research on coevolution, to my knowledge there is little explicit discussion or synthesis of *how*, *why*, or even *if* we expect evolutionary outcomes to differ when the selective agent is biotic or abiotic (but see Schemske 2009).

Chapter 4 is a review of our current understanding of the evolution and genetics of mutualisms (Baskett and Schemske 2015). A key contribution, related to the biotic interactions hypothesis, was to ask how evolution in a mutualism differs from evolution driven by antagonisms or the abiotic environment. For example, how might the magnitude and direction of natural selection differ for a mutualistic versus abiotic selective agent; are adaptive peaks more likely to be “moving” when selective agents are co-evolving? One of the main conclusions was that we lack explicit conceptual frameworks and empirical evidence to address how evolution in mutualisms is unique.

The latitudinal diversity gradient is a long-standing biological puzzle, with many proposed hypotheses but few firm answers. Research is lacking for evolutionary hypotheses that explain why diversification rates may be faster at lower latitudes. The biotic interactions hypothesis connects the dots to explain why observed variation in the abiotic environment would lead to higher speciation rates at lower latitudes. My work on geographic patterns in plant-insect interactions is a rigorous test of the prediction that biotic interactions are stronger at lower latitudes, with data on interactions and traits, studies of both an antagonism and a mutualism, and a wide latitudinal range. Even more broadly than the biotic interactions hypothesis, my dissertation addresses fundamental questions about the causes and consequences of variation in the importance of biotic interactions for adaptive evolution.

REFERENCES

REFERENCES

- Anstett, D. N., K. A. Nunes, C. A. Baskett and P. M. Kotanen (2016). "Sources of controversy surrounding latitudinal patterns in herbivory and defense." Trends in Ecology & Evolution **31**(10): 789-802.
- Arnold, A. E. and F. Lutzoni (2007). "Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots?" Ecology **88**(3): 541-549.
- Baskett, C. A. and D. W. Schemske (2015). Evolution and genetics of mutualism. Mutualism. J. L. Bronstein. Oxford, Oxford University Press: 77-92.
- Baskett, C. A. and D. W. Schemske (2018). "Latitudinal patterns of herbivore pressure in a temperate herb support the biotic interactions hypothesis." Ecology Letters **21**(4): 578-587.
- Coley, P. D. and T. M. Aide (1991). Comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. P. W. Price, T. M. Lewinsohn, G. W. Fernandes and W. W. Benson. New York, USA, John Wiley and Sons: 25-49.
- Condamine, F. L., F. A. H. Sperling, N. Wahlberg, J. Y. Rasplus and G. J. Kergoat (2012). "What causes latitudinal gradients in species diversity? Evolutionary processes and ecological constraints on swallowtail biodiversity." Ecology Letters **15**(3): 267-277.
- Connell, J. H. (1971). On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees. Dynamics of Populations. P. J. Boer and G. Gradwell. The Netherlands, PUDOC: 298-310.
- Currie, D. J., G. G. Mittelbach, H. V. Cornell, R. Field, J. F. Guegan, B. A. Hawkins, D. M. Kaufman, J. T. Kerr, T. Oberdorff, E. O'Brien and J. R. G. Turner (2004). "Predictions and tests of climate-based hypotheses of broad-scale variation in taxonomic richness." Ecology Letters **7**(12): 1121-1134.
- Dobzhansky, T. (1950). "Evolution in the tropics." American Scientist **38**: 209-221.
- Dodd, M. E., J. Silvertown and M. W. Chase (1999). "Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families." Evolution **53**(3): 732-744.
- Farrell, B. D., D. E. Dussourd and C. Mitter (1991). "Escalation of plant defense: Do latex and resin canals spur plant diversification?" American Naturalist **138**(4): 881-900.
- Fine, P. V. A. and R. H. Ree (2006). "Evidence for a time-integrated species-area effect on the latitudinal gradient in tree diversity." American Naturalist **168**(6): 796-804.
- Fischer, A. G. (1960). "Latitudinal variation in organic diversity." Evolution **14**: 64-81.

- Hanly, P. J., G. G. Mittelbach and D. W. Schemske (2017). "Speciation and the Latitudinal Diversity Gradient: Insights from the Global Distribution of Endemic Fish." The American Naturalist **189**(6): 604-615.
- Hodges, S. A. and M. L. Arnold (1995). "Spurring plant diversification: Are floral nectar spurs a key innovation?" Proceedings of the Royal Society B-Biological Sciences **262**(1365): 343-348.
- Jablonski, D., C. L. Belanger, S. K. Berke, S. Huang, A. Z. Krug, K. Roy, A. Tomasovych and J. W. Valentine (2013). "Out of the tropics, but how? Fossils, bridge species, and thermal ranges in the dynamics of the marine latitudinal diversity gradient." Proceedings of the National Academy of Sciences of the United States of America **110**(26): 10487-10494.
- Jaenike, J. (1978). "An hypothesis to account for the maintenance of sex within populations." Evol. theory **3**: 191-194.
- Janzen, D. H. (1970). "Herbivores and the number of tree species in tropical forests." American Naturalist **104**(940): 501-528.
- Jokela, J., Mark F. Dybdahl and Curtis M. Lively (2009). "The Maintenance of Sex, Clonal Dynamics, and Host-Parasite Coevolution in a Mixed Population of Sexual and Asexual Snails." The American Naturalist **174**(S1): S43-S53.
- Mittelbach, G. G., D. W. Schemske, H. V. Cornell, A. P. Allen, J. M. Brown, M. B. Bush, S. P. Harrison, A. H. Hurlbert, N. Knowlton, H. A. Lessios, C. M. McCain, A. R. McCune, L. A. McDade, M. A. McPeck, T. J. Near, T. D. Price, R. E. Ricklefs, K. Roy, D. F. Sax, D. Schluter, J. M. Sobel and M. Turelli (2007). "Evolution and the latitudinal diversity gradient: speciation, extinction and biogeography." Ecology Letters **10**(4): 315-331.
- Mitter, C., B. Farrell and B. Wiegmann (1988). "The phylogenetic study of adaptive zones: Has phytophagy promoted insect diversification?" American Naturalist **132**(1): 107-128.
- Moles, A. T. (2013). "Dogmatic is problematic: Interpreting evidence for latitudinal gradients in herbivory and defense." Ideas in Ecology and Evolution **6**: 1-4.
- Moles, A. T., D. D. Ackerly, J. C. Tweddle, J. B. Dickie, R. Smith, M. R. Leishman, M. M. Mayfield, A. Pitman, J. T. Wood and M. Westoby (2007). "Global patterns in seed size." Global Ecology and Biogeography **16**(1): 109-116.
- Moles, A. T., S. P. Bonser, A. G. B. Poore, I. R. Wallis and W. J. Foley (2011). "Assessing the evidence for latitudinal gradients in plant defence and herbivory." Functional Ecology **25**(2): 380-388.
- Moles, A. T. and J. Ollerton (2016). "Is the notion that species interactions are stronger and more specialized in the tropics a zombie idea?" Biotropica **48**(2): 141-145.
- Orr, H. A. (2005). "Theories of adaptation: what they do and don't say." Genetica **123**(1-2): 3-13.

- Palmer, M. W. (1994). "Variation in species richness: towards a unification of hypotheses." Folia Geobotanica & Phytotaxonomica **29**(4): 511-530.
- Pyron, R. A. and J. J. Wiens (2013). "Large-scale phylogenetic analyses reveal the causes of high tropical amphibian diversity." Proceedings of the Royal Society B: Biological Sciences **280**(1770).
- Regal, P. J. (1982). "Pollination by wind and animals--ecology of geographic patterns." Annual Review of Ecology and Systematics **13**: 497-524.
- Rolland, J., F. L. Condamine, F. Jiguet and H. Morlon (2014). "Faster speciation and reduced extinction in the tropics contribute to the mammalian latitudinal diversity gradient." Plos Biology **12**(1): e1001775.
- Schemske, D. W. (2009). Biotic interactions and speciation in the tropics. Speciation and Patterns of Diversity. R. K. Butlin, J. R. Bridle and D. Schluter. Cambridge, UK, Cambridge University Press: 219-239.
- Schemske, D. W., G. G. Mittelbach, H. V. Cornell, J. M. Sobel and K. Roy (2009). "Is there a latitudinal gradient in the importance of biotic interactions?" Annual Review of Ecology Evolution and Systematics **40**: 245-269.
- Wallace, A. R. (1878). Tropical Nature, and Other Essays. London, UK, MacMillan and Co.
- Weber, M. G. and A. A. Agrawal (2014). "Defense mutualisms enhance plant diversification." Proceedings of the National Academy of Sciences **111**(46): 16442-16447.

CHAPTER 1: LATITUDINAL PATTERNS OF HERBIVORE PRESSURE IN A TEMPERATE
HERB SUPPORT THE BIOTIC INTERACTIONS HYPOTHESIS

Introduction

It has long been predicted that the strength of biotic interactions increases closer to the equator. This pattern is hypothesized to influence latitudinal variation in species coexistence (LaManna *et al.* 2017), ecosystem function (Violle *et al.* 2014), trophic cascades (Marczak *et al.* 2011), and speciation rates (Schemske 2009). For example, greater importance of biotic interactions at lower latitudes is hypothesized to contribute to the latitudinal diversity gradient, the poorly understood pattern of greater biodiversity at lower latitudes (the "biotic interactions hypothesis;" Wallace 1878; Dobzhansky 1950; Fischer 1960; Mittelbach *et al.* 2007; Schemske 2009). However, while evidence for stronger or more prevalent biotic interactions in the tropics has been found for many mutualisms and some antagonisms (Schemske *et al.* 2009), evidence for herbivory, one of the most ecologically significant biotic interactions, has been mixed (Moles *et al.* 2011).

Herbivory is particularly important because it involves a large proportion of biodiversity and energy flow in terrestrial ecosystems (Price 2002), and it has been hypothesized to play a major role in the origin and maintenance of biodiversity (Ehrlich & Raven 1964; Janzen 1970; Connell 1971). The existence of latitudinal patterns in herbivore pressure is controversial; perspectives have shifted over the decades (Ehrlich & Raven 1964; Coley & Aide 1991; Coley & Barone 1996; Moles *et al.* 2011). While Coley and Barone (1996) and Schemske *et al.* (2009) found support with literature syntheses for higher herbivory rates and stronger defense in the tropics, Moles *et al.* (2011) found little evidence for latitudinal patterns in herbivore pressure with a meta-analysis. The discrepancy led Anstett *et al.* (2016) to conclude that available studies are too few and heterogeneous in approach and quality to justify drawing conclusions.

Contributing to the controversy is that quantifying geographic variation in biotic interaction strength is not straightforward (Schemske *et al.* 2009), leading Anstett *et al.* (2016) to

suggest several methodological improvements. For one, herbivore pressure is quantified by herbivory rates and/or defensive traits (Coley & Barone 1996), which are interpreted differently, and thus best assessed in tandem. Greater herbivore damage indicates stronger current-day pressure, while more effective plant defense is assumed to be a signature of stronger selection by herbivores (Coley & Barone 1996; Hahn & Maron 2016). Second, herbivory is estimated as standing damage on mature leaves in the majority of studies (Anstett *et al.* 2016), despite evidence that young leaves receive more damage and complete consumption may not be apparent on older branches (Lowman 1984; Filip *et al.* 1995). Thus, restricting estimates to mature leaves may underestimate herbivory if young leaves are often completely consumed, obscuring geographic patterns. Third, comparisons between sites that differ in growing season and leaf lifespan are facilitated by marking and re-measuring leaves over a standard exposure time, but this approach is rare in latitudinal studies (Anstett *et al.* 2016). Fourth, latitudinal comparisons of plant defense suffer from a lack of standardized methods; e.g., many quantify secondary metabolites without evidence that they are defensive (Anstett *et al.* 2016). Bioassays to quantify plant defense when important traits are unknown can integrate leaf chemistry, physical defense, and nutrition (Miller & Hanson 1989; Pennings *et al.* 2001; Kim 2014). Finally, it is unclear whether the relationship between herbivore pressure and latitude is linear or non-linear. For example, a step function may be more appropriate if a latitudinal gradient spans a climatic or biological threshold (Anstett *et al.* 2016). More data on the shape of the latitude-herbivory relationship would potentially support or rule out some of the many proposed mechanisms for latitudinal gradients in herbivory, such as greater lower-latitude abundance, diversity, or specialization of herbivores (e.g., Pennings *et al.* 2001; Gaston *et al.* 2004; Pennings & Silliman 2005; Salazar & Marquis 2012; Lim *et al.* 2015).

Here, we ask whether plant populations at lower latitudes in the temperate zone experience greater herbivore pressure within a species, addressing major gaps in previous studies related to leaf age, defense and herbivory quantification, and non-linearity. We integrated field and laboratory approaches to quantify insect herbivory and plant defense on both young and mature leaves in the perennial herb *Phytolacca americana* L. (pokeweed; Phytolaccaceae) along a continental latitudinal gradient between Michigan and Florida (27°N to 42°N). Herbivory was measured in the field to test the prediction that plants at lower latitudes experience greater consumption. We also quantified *P. americana* herbivore abundance, to test for positive correlations with herbivory. We performed palatability experiments with a generalist lepidopteran using greenhouse-grown plants to test the prediction that lower-latitude populations are better defended. For both field and lab studies, we predicted that young leaves are consumed at higher rates than mature leaves.

Methods

Study system

Phytolacca americana is a herbaceous short-lived perennial, native to the eastern US. It is a bird-dispersed gap specialist with a long-lived seed bank (Hardin 1964), common in disturbed areas. Its mating system is unknown, but it is both visited by insects and capable of high autogamy rates (pers. obs.). The gradient in this study covers most of its native latitudinal range (Sauer 1952).

Does herbivory vary with latitude and/or leaf age?

We measured herbivory rates in 2014 in five populations of *P. americana* between central Florida and southwest Michigan (27°N to 42°N; mean interval between sites = 3.8°, range = 3.2°-5.2°; Fig. 1, Table 1). Populations ranged from partially shaded to full sun and were either abutting or surrounded by natural vegetation. To estimate herbivory over a standardized time period, we marked young and mature leaves on 15 plants per population in late July and returned ~34 days later to measure changes in damage. Plants were haphazardly selected, with a range of sizes and locations in the patch, but each plant had at least five growing branches. Five young and five mature leaves per plant were haphazardly chosen and marked with colored bands and permanent marker. For young leaves, buds were just beginning to burst. Mature leaves were fully expanded and toughened up (Coley 1983) and less than 50% damaged. Initial herbivory of mature leaves and final herbivory for both ages (consumed area/total leaf area) was estimated with a plastic 6.35 x 6.35 mm grid. A leaf was considered 100% consumed if only the midvein or petiole stub remained. Sample sizes at high-mortality sites, particularly in Florida, were smaller than the number of marked leaves (Table 1) because some leaves could not be found for later sampling. Herbivory can be converted to daily rates for a standard comparison to other studies (Coley & Barone 1996; Anstett *et al.* 2016), but since intervals were so similar among sites, analyzing rates does not change results (data not shown). Thus, we present percentage for ease of interpretation.

Lepidopteran larvae are the primary herbivores (pers. obs.). *P. americana* experiences occasional deer herbivory (less than 5% of branches at our sites), which is easily differentiated from insect herbivory because deer strip leaves. Variation in deer herbivory likely is explained more by idiosyncratic features of the immediate patch surroundings than climatic factors

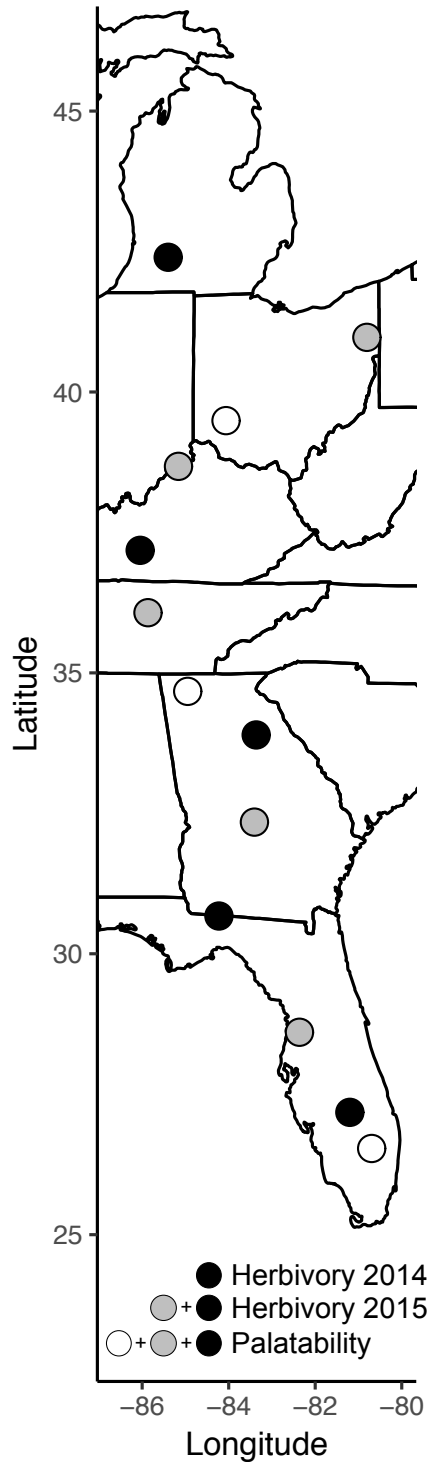


Figure 1. *Phytolacca americana* populations in the mid-western and eastern USA used for herbivory surveys and palatability assays. The first year of field herbivory surveys (2014) included five populations (black dots). In 2015, these populations were re-surveyed, and five more were added (black and grey dots). Palatability assays utilized seeds from all 13 populations shown (black, grey, and white dots). See Table 1 for GPS and other details. Plotted with *ggmap* in R (Kahle & Wickham 2013).

Table 1. Location and sampling details for sites shown in Fig. 1. Site ID refers to Table 3. Dates are shown for when leaves were marked and measured, with the intervening interval in days. More leaves and plants were marked than measured due to disease and mortality. Young and mature refer to leaves marked for each leaf age.

ID	Site name	Latitude	Longitude	2014 dates			Sample sizes measured			2015 dates			Sample sizes	
				Mark	Meas.	Days	Plants	Young	Mature	Mark	Meas.	Days	Plants	Young
	Highway 27	26.53707	-80.69487											
	MacArthur													
	Agroecology	27.17758	-81.20727											
	Ranch													
A	MacArthur-2015	27.17833	-81.19387	07/18	08/21	34	13*	34	7	07/24	09/05	43	37	154
B	Withlacoochee State	28.60387	-82.36100							07/26	09/06	42	4	18
	Forest													
C	Tall Timbers	30.67389	-84.22306	07/20	08/23	34	15	69	52	07/28	09/07	41	17	53
	Research Station													
D	Ocmulgee WMA	32.34554	-83.40459							07/29	09/09	42	26	123
E	Whitehall Forest	33.89577	-83.36165	07/21	08/24	34	15	71	70	07/30	09/11	43	26	90
	Dalton	34.67088	-84.94345											
F	Center Hill	36.06935	-85.86862							08/01	09/12	42	20	59
G	Hamilton Valley	37.18180	-86.04253	07/23	08/26	34	12**	51	52	08/03	09/14	42	24	96
H	General Butler State	38.67552	-85.16030							08/04	09/16	43	27	83
	Resort Park													
	Caesar Creek State	39.48870	-84.05828											
	Park													
I	McPhee Farm	40.97133	-80.80262							08/05	09/18	44	28	131
	Kellogg Biological	42.39993	-85.39643	07/25	08/29	35	15	72	74					
	Station													
J	Kellogg Biological	42.48810	-85.45091							08/06	09/21	46	30	149
	Station-2015													

*Only 4 plants were measured for mature leaf herbivory.

**Only 11 plants were measured for mature leaf herbivory.

correlated with latitude (Prendeville *et al.* 2015), so it was not quantified for the purpose of testing our hypotheses.

Mean herbivory per plant was analyzed with mixed-effects ANCOVA using the *lme* function in the *nlme* package in R (R Core Team 2014; Pinheiro *et al.* 2017). Fixed effects were latitude, leaf age, and their interaction, and population was a random blocking factor. To compare our results to studies that only measure mature-leaf herbivory, we also ran the model on mature leaves only.

Our analyses are presented in their respective sections, but have details in common. Mixed ANCOVA models met assumptions of normally distributed residuals, and we used the *lmer* function in the *lme4* package (Bates *et al.* 2015) if variance was homogeneous; if not, we used the *lme* function in the *nlme* package and estimated variance separately for each block. Results were obtained with restricted maximum likelihood estimates, while model comparisons using Akaike information criterion (AIC) were based on maximum likelihood estimates.

What is the shape of the herbivory-latitude relationship?

To provide greater spatial resolution for estimating the shape of the herbivory-latitude relationship, we repeated the methods described above and added five intermediate sites in 2015 (Fig. 1, Table 1; mean interval between sites = 1.7°, range = 1.1°-2.3°). Locations of two sites changed slightly between years (Table 1). Sampling was restricted to young leaves because they received the majority of herbivory in 2014. Five leaves per plant were marked in late July 2015 for 30-40 individuals per population (Table 1). Populations were re-visited in September (~43 days after marking; Table 1), when young leaves were expected to have fully expanded.

Using mixed-effects ANCOVA, we analyzed herbivory per plant with latitude as a fixed effect and population as a random blocking factor in *lmer*. Using AIC, we compared this model fit to a piecewise linear regression which fit different slopes around a threshold at 35°N, between the five southern and five northern sites, following the example of Wagner *et al.* (2012). This threshold was obtained by comparing AIC of nine models with thresholds at midpoints of adjacent populations. Finally, to test whether there were strong effects of sampling year, we tested a fixed effect of the interaction of year and latitude for young leaves in the five populations common to 2014-2015. We used mixed-effects ANCOVA in *lmer* with population as a random blocking factor. Since intervals between marking and measuring leaves differed between years, we also analyzed herbivory as a daily rate, which gave qualitatively similar results (data not shown).

Does herbivore abundance predict herbivory?

To test whether *P. americana* herbivore abundance varies latitudinally, insects were collected during herbivory surveys in 2015, with the exception of two sites in September because too few leaves were available due to herbivory and disease (Table 1). Thus, all September collections were used only for identification and not included in analyses. For each collection, the top and bottom surfaces of 3,000 leaves per population were checked between 7 AM and 1 PM. Approximately 100 leaves of all sizes were examined on each of 30 plants.

Lepidopteran larvae were tallied as morphospecies. Up to ten individuals of each morphospecies were collected per site and reared on a *P. americana* diet. Six to 18 specimens were successfully reared to adulthood for each of the three common morphospecies. Singletons were dropped from the dataset due to uncertainty about their status as *Phytolacca* consumers.

Lepidopteran abundance per population was \log_e -transformed for analysis because it varied by nearly two orders of magnitude.

We tested whether lepidopteran abundance was correlated with latitude using Pearson's product-moment correlation. We analyzed whether latitude or herbivore abundance best predicted herbivory in 2015. AIC was used to compare mixed-effects ANCOVA models in *lmer* with fixed effects of latitude, herbivore abundance, or both; all included population as a random blocking effect. To determine whether including both factors significantly reduced residual sum of squares, we performed Chi-squared tests. In addition, we compared fits of models with continuous predictors to the piecewise latitudinal model (see previous section) and a piecewise herbivore abundance model obtained in the same manner.

Does plant defense vary latitudinally and/or with leaf age?

To compare the effectiveness of plant defense between populations and leaf ages, we performed no-choice palatability experiments using the generalist lepidopteran *Spodoptera exigua* (Benzon Research Inc., Carlisle, USA). *S. exigua* has been used for similar purposes in other studies (e.g., Gomez & Stuefer 2006; Hendriks *et al.* 2009), and it tolerates a *P. americana* diet better than other commercially available species (pers. obs.).

Larvae ate a diet of young or mature leaves of greenhouse-grown *P. americana* from 4-5 maternal lines per population for 13 populations (Fig. 1; 64 total lines). Leaf material was from greenhouse-grown plants from field-collected seeds. Fruits were collected in 2014-2015 from each population surveyed for herbivory, plus three additional populations (Table 1, Fig. 1). In summer 2016, seeds were soaked in concentrated sulfuric acid for five minutes (Farmer & Hall 1970), rinsed in DI water, and sown on Petri plates with moistened filter paper in an incubator at

31°C for 16 hour days and 27°C nights. Seedlings were transplanted to a peat soil mix (Suremix Perlite, Michigan Growers Products, Inc., Galesburg, USA) in plug trays in the Michigan State University greenhouse with natural lighting. Seedlings were transplanted to 1.3 L pots when needed and finally to 5 L pots in September 2016. For the last transplant, each plant received 12 mL of Miracle Gro All-Purpose or Fruit and Vegetable Mix slow release fertilizer (Scotts Miracle-Gro Company, Marysville, USA). Virtually all plants had flowers and/or fruits by the time of leaf harvest. Plants were randomly arranged in the greenhouse until the palatability experiment. During the experiment, individuals were blocked within randomly ordered maternal lines.

Starting from neonates, larvae were reared at 22°C for 16 hour days with 35% relative humidity in 59-mL plastic cups on a diet of fresh, entire leaves. Cups contained 7 mL of 7% plain Phytoblend agar (Caisson Labs, North Logan, USA) to provide moisture. For mature leaves, the four bottom-most leaves on haphazardly selected branches were harvested, while avoiding the three newest fully expanded leaves. The mature leaf treatment had four replicate cups per maternal line with five larvae per cup (1,280 larvae). Leaves ~2.5-5 cm long that had not fully expanded and toughened were considered young and were harvested from multiple haphazardly chosen branches. Due to limited young leaf material, the young leaf treatment had three replicate cups per maternal line with three larvae per cup (576 larvae). For both treatments, leaves were pooled from two to three individual plants per maternal line, and we provided more leaf material than larvae could consume. Large leaves were rolled to fit in cups; ripping was minimized. In August-September, one mature and two young leaves per individual were sampled for another study, but leaf removal typically does not induce defenses (Agrawal 1998). Thus, the plants likely began the experiment in October expressing only constitutive defenses.

We monitored leaf consumption and measured final larval mass after 8-9 days. Daily for eight days in the mature leaf treatment, leaves were refreshed, and consumed leaf area was estimated with a plastic 3.175 x 3.175 mm grid. The same measurements were performed on alternate days for nine days in the young leaf treatment. Survivors on the last day were placed in individual 1.5 mL microcentrifuge tubes and frozen at -20°C after one day to allow defecation. Wet weight was obtained with a microbalance, as drying would damage the smallest individuals. Total biomass and total leaf area consumed were analyzed per cup, with a $\log_e + 1$ transformation, because growth and consumption are exponential. After transformation, responses were standardized by initial caterpillar count per day in order to compare the young and mature leaf treatments (three larvae over nine days and five larvae over eight days, respectively).

Treatment effects on larval biomass were analyzed with mixed-effect ANCOVA in *lme*; for each analysis, maternal line nested within population was a random blocking effect. To analyze latitudinal patterns, fixed effects were latitude, leaf age, and their interaction. We compared a model with latitude as a continuous predictor to a piecewise regression (see field herbivory analyses). Piecewise regressions were run separately on young and mature leaves, so they were compared to linear regression models on separate leaf ages. We also asked whether larval growth per leaf area consumed depended on leaf age by analyzing larval biomass with fixed effects of area consumed, leaf age, and their interaction.

Results

Does herbivory vary with latitude and/or leaf age?

Herbivory was negatively correlated with latitude in 2014, but slopes depended on leaf age ($F_{1,123} = 26.13$, $p < 0.0001$ for latitude*age). The slope was steeper for young leaves, which were eaten more at low-latitude sites (Fig. 2). In fact, the 95% confidence interval of the slope for mature leaves barely overlapped zero (-5.9 to 0.7). When we subset data to mature leaves only, the relationship between herbivory and latitude was only marginally significant ($F_{1,3} = 7.73$, $p = 0.0690$).

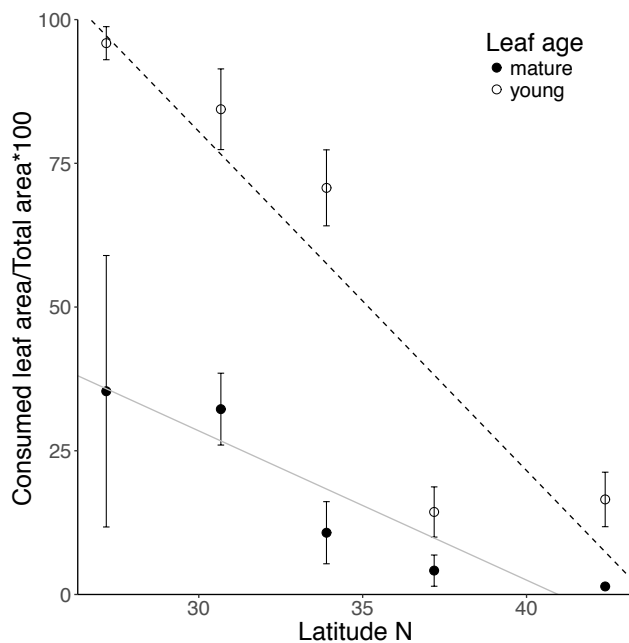


Figure 2. Herbivory in the field (percent leaf area consumed) for young and mature leaves of *P. americana* in 2014. Lines of best fit (solid = mature, dashed = young) and population means \pm S.E. are shown. The mature-leaf line is shown in gray because the 95% confidence interval for the slope barely overlapped zero.

What is the shape of the herbivory-latitude relationship?

We found greater herbivory on young leaves at lower latitudes in 2015 as well (Fig. 3, Table 2).

With higher resolution than in 2014, we were able to fit a piecewise regression model with a

breakpoint of 35°N, which was better supported than a linear gradient (Table 2, Models A vs. E). The threshold of 35°N received 99% of AIC weight compared to the eight other possible thresholds between sampling sites. On both sides of the threshold, 95% confidence intervals of the slopes overlapped zero (Fig. 3). Mean herbivory in populations south of the threshold was 79%, and mean herbivory in populations north of the threshold was 15%, a 5.3-fold difference. For comparison, mean herbivory on young leaves in 2014 was 84% and 15% (a 5.6-fold difference) for populations south vs. north of 35°N, respectively. The slope of herbivory vs. latitude was 26% greater in 2014 than in 2015 for young leaves at the five sites common in both years (latitude*year: $F_{1,197} = 5.30$, $p = 0.0224$). However, this effect was driven by the southernmost site, and disappeared if that site was removed.

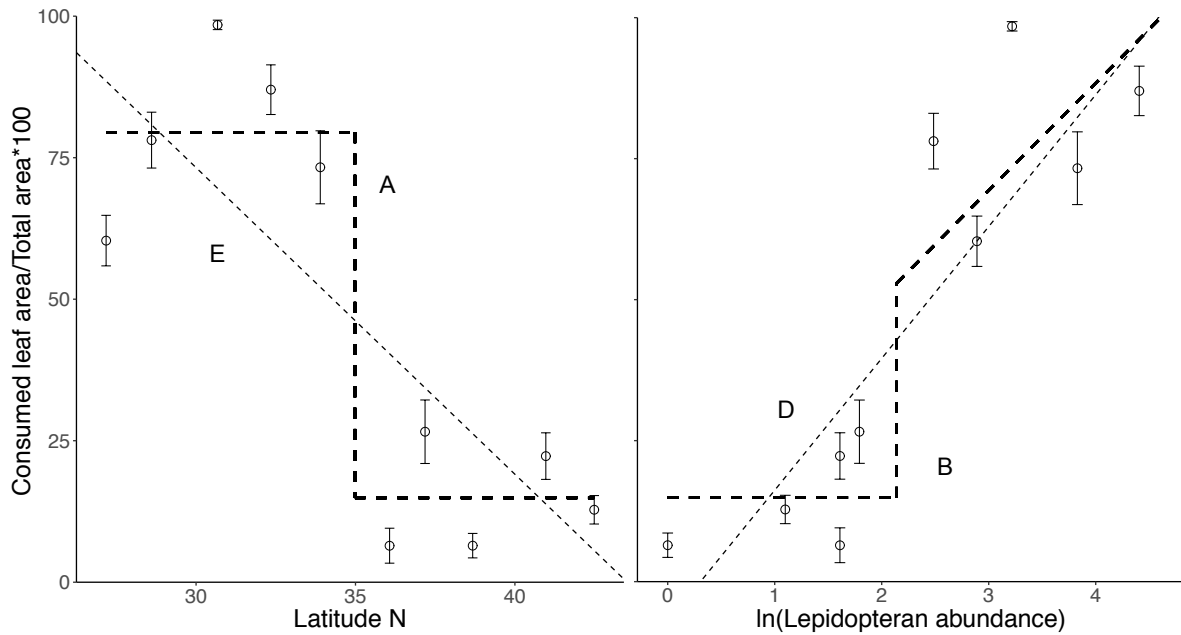


Figure 3. Herbivory in the field (percent leaf area consumed) for young leaves of *Phytolacca americana* in 2015 plotted against latitude (left) and lepidopteran abundance (right). Each point is a population mean \pm S.E. Each plot shows the results of two models: one where the predictor is continuous, and another with a threshold in the predictor (thicker line). Constant herbivory at the mean of the five populations is shown when the 95% confidence interval of the slope overlapped zero. Letters correspond to models in Table 2; AIC values increase from A to E.

Table 2. Results and model comparison for mixed-effects ANCOVA models analyzing per-plant mean herbivory on young *P. americana* leaves at ten sites in 2015. Models are shown in order of increasing AIC. d.f. = degrees of freedom (Satterthwaite approximation), AIC = Akaike information criterion value, dAIC = difference in AIC from best-fitting model, weight = Akaike weights, Lep. = lepidopteran (abundance was log_e-transformed). Population as a random blocking factor is included in all models. *F*- and *p*-values are from restricted maximum likelihood models, while AIC values are from maximum likelihood models. Significant effects (*p* < 0.05) are bolded.

Model	Fixed Effects	<i>F</i>	<i>p</i>	d.f.	AIC	dAIC	Weight
A	Latitude below threshold	1.79	0.2248	1,6.8			
	Latitude above threshold	0.03	0.8658	1,6.8			
	Overall model			5	2177.7	0	0.725
D	Lep. abundance below threshold	0.16	0.6993	1,6.1			
	Lep. abundance above threshold	24.86	0.0026	1,5.9			
	Overall model			5	2180.2	2.6	0.202
C	Latitude	2.83	0.1357	1,7.1			
	Lep. abundance	10.66	0.0147	1,6.7			
	Overall model			5	2183.1	5.4	0.048
D	Lep. abundance	28.70	0.0010	1,7.0			
	Overall model			4	2184.5	6.8	0.024
E	Latitude	12.11	0.0079	1,8.2			
	Overall model			4	2190.3	12.6	0.001

Table 3. Lepidopteran herbivore species collected in 2015 from 3,000 *Phytolacca americana* leaves from ~30 individual plants per site. Sites are shown sequentially from south to north (see Table 1 for locations). The sum of two collections (late July and early September) is shown. “Recorded Range” refers to the collection sites where each species is expected to occur based on its recorded geographic range. Larval morphospecies A may have been *A. velutinana*, *A. gordialis*, or *P. obscuralis*; counts for successfully reared adults are shown in italics for each. Morphospecies BG and BQ were never reared to adulthood. Sites with asterisks were only surveyed in late July because there were too few leaves to survey in early September; thus, for all sites only the July survey was used in analyses throughout the manuscript.

Species	Collections/Site (south to north; see Table 1)										Total	Recorded Range
	A	B*	C*	D	E	F	G	H	I	J		
Larval "species A"	21	1	14	40	60	8	9	1	4	7	165	
<i>Argyrotaenia velutinana</i>							1	1		2		D-J ¹
<i>Asciodes gordialis</i>	3											A-C ²
<i>Psara obscuralis</i>	1		3	2	3	2						A-H ³
<i>Disclisioprocta stellata</i>	1	10	11	120	13	2	1	1	1		160	A-H ³
<i>Spodoptera eridania</i>	11	1		3							15	A-H ³
Larval "species BG"									2		2	
Larval "species BQ"	2										2	
Total	35	12	25	163	73	10	10	2	7	7	344	

¹Freeman, T. (1958). The Archipinae of North America (Lepidoptera: Tortricidae). *Mem Entomol Soc Can*, 90, 5-89.

²Allyson, S. (1984). Description of last-instar larvae of 22 species of North American Spilomelini (Lepidoptera, Pyralidae, Pyraustinae) with a key to species. *Can Entomol*, 116, 1301-1334.

³Moth Photographers Group, Mississippi Entomological Museum. (2012). *Distribution Maps*. Available at: <http://mothphotographersgroup.msstate.edu/>. Last accessed 14 SEPTEMBER 2017.

Does herbivore abundance predict herbivory?

Log_e-abundance (hereafter referred to as “abundance”) of lepidopterans collected from *P. americana* plants was negatively correlated with latitude (Pearson’s $r = -0.6547$, $t_8 = -2.4497$, $p = 0.0400$, $R^2 = 0.429$). Lepidopteran abundance was a better predictor of herbivory on young leaves in 2015 than latitude, as shown by improved model fit (Table 2 Models D vs. E, Fig. 3). A model that included both herbivore abundance and latitude had a lower AIC value than that with either factor alone; herbivore abundance was a significant predictor, but not latitude. The fit of this model was significantly better than a model with latitude alone ($X^2 = 9.15$, $p = 0.0025$ for Table 2 Models C vs. E), but not for lepidopterans alone ($X^2 = 3.40$, $p = 0.0654$ for Table 2 Models C vs. D). Similar to the results for latitude, we found that a piecewise regression with a threshold at ln(abundance) of 2.1 (~8 larvae per 3000 leaves, which occurred at 35°N) improved model fit compared to treating abundance as a continuous fixed effect (Table 2 Models B vs. D, Fig. 3). This threshold received 53% of AIC weight compared to all other possibilities. At low herbivore abundance, the 95% confidence interval of the slope overlapped zero, but at high abundance, there was a positive correlation with herbivory (Fig. 3). Despite support for lepidopteran abundance as a stronger predictor of herbivory than latitude, the latitudinal threshold model was the best fit of all compared models (Table 2 Model A). Nevertheless, considering the fairly similar AIC values across models, we lack power to confidently disentangle the relative effects on herbivory of herbivore abundance and unmeasured factors associated with latitude. A structural equation modeling approach would be useful (e.g., Kim 2014), but the number of populations sampled is insufficient.

Little was known about *P. americana* herbivores prior to our study, so we present natural history details here. Two lepidopteran groups were common consumers, each representing

~47% of 344 total collections (Table 3): *Disclisioprocta stellata* (Geometridae; found at 90% of sites) and three species whose indistinguishable larvae were found sheltering in a web in young leaves at all sites: *Psara obscuralis* (Crambidae), *Asciodes gordialis* (Crambidae), and *Argyrotaenia velutinana* (Tortricidae). Leaf sheltering is hypothesized to be an anti-phototoxin defense (Sandberg & Berenbaum 1989), and phototoxic metabolites are reported for tropical Phytolaccaceae (Downum *et al.* 1991). Three additional taxa were collected: *Spodoptera eridania* (Noctuidae; 4% of collections), and two morphospecies that were not successfully reared (combined, 1% of collections). Localized outbreaks of *S. eridania* have been observed decimating *P. americana* in Florida (Scriber 1986), and it may be a more important herbivore at high-consumption sites than our collections would suggest because it feeds nocturnally, and its life cycle is short enough that outbreaks could have swept through sites between surveys. *A. velutinana* and *S. eridania* are highly polyphagous (Summerland & Hamilton 1955; Robinson *et al.* 2010). Information about host breadth for the three other species is sparse, but each has host records in the Caryophyllales (Cuenoud *et al.* 2002): either Nyctaginaceae (sister family to Phytolaccaceae) or Amaranthaceae (Allyson 1984; Robinson *et al.* 2010).

Does plant defense vary latitudinally and/or with leaf age?

In palatability assays, there was a positive correlation between larval biomass and plant latitude of origin for both leaf ages (Fig. 4, Table 4). In addition, larvae grew 1.5 times larger in the mature-leaf treatment overall. Mean leaf area consumed was similar for mature and young leaves (0.165 vs. 0.178 $\ln(\text{mm}^2)$), but larvae gained less biomass per leaf area consumed when they ate young leaves (Fig. 4, Table 4).

Table 4. Results for mixed-effects ANCOVAs of biomass of *S. exigua* larvae per replicate cup (see Fig. 4). Larvae were reared on a diet of young vs. mature *P. americana* leaves from 13 populations grown in the greenhouse from seeds collected along a latitudinal gradient. Top: effects of latitude and leaf age on larval biomass. Bottom: effects of leaf area consumed and leaf age on larval biomass. Both biomass and leaf area are \log_e -transform and standardized by duration of experiment and starting number of larvae. Plant maternal line nested in population is included as a random blocking factor. Significant p -values (<0.05) from Type III SS are bolded.

Response	Fixed Effect	d.f.	F	p
Total biomass	Latitude	1, 11	17.45	0.0015
	Leaf age	1, 373	9.09	0.0027
	Latitude*Age	1, 373	0.04	0.8489
Total biomass	Leaf area eaten	1, 372	661.91	<0.0001
	Leaf age	1, 372	280.22	<0.0001
	Area*Age	1, 372	452.13	<0.0001

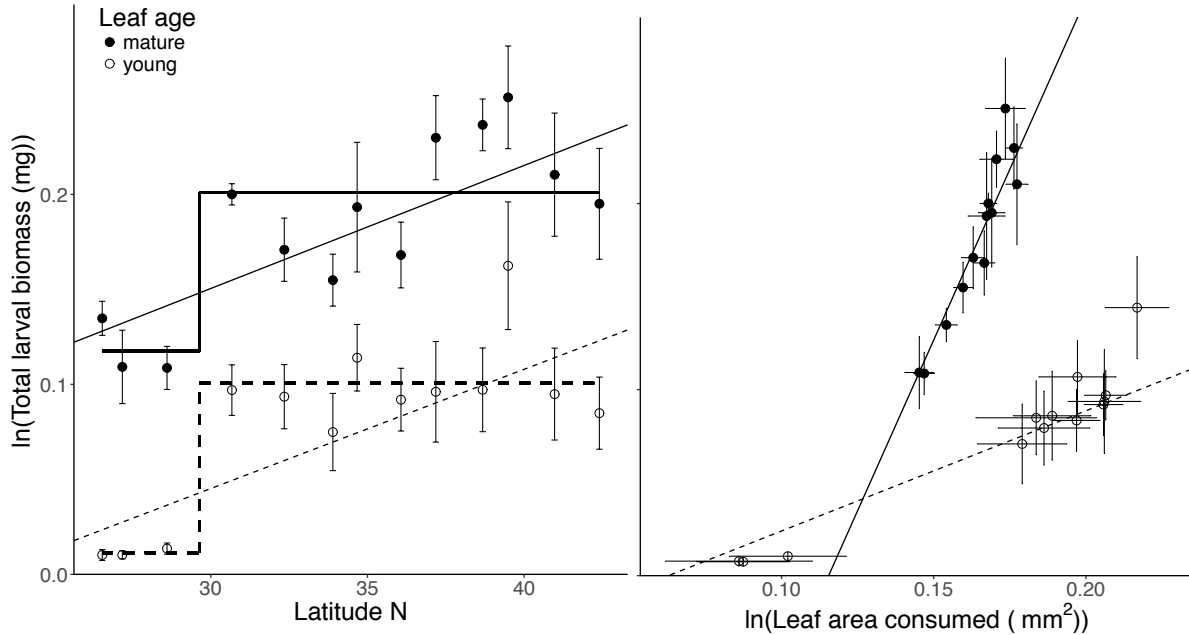


Figure 4. Larval biomass of *Spodoptera exigua* reared on mature vs. young leaf diets (solid vs. dashed line) of *Phytolacca americana* from seeds collected along a latitudinal gradient. Each point is a population mean \pm S.E. (based on number of plant maternal lines). Biomass and area were measured per replicate cup and standardized by the number of starting caterpillars and duration after a \log_e+1 transformation (mature: 5 caterpillars, 8 days; young: 3 caterpillars, 9 days). The left-hand plot shows results of a model with a continuous predictor, and a model with a threshold in the predictor (thicker line). For the threshold models, constant herbivory at the population means are shown because the 95% confidence interval of the slopes overlapped zero. See Table 4 for model results.

Similar to herbivory in the field, a piecewise regression fit better than a linear regression, but the optimal latitudinal threshold was about 5° further south in the defense analysis compared

to the herbivory analysis (Fig. 4 vs. Fig. 3). For young leaves, a piecewise regression model with a breakpoint at 29.6°N was a better fit than a linear regression model (AIC = -630.2 vs. -606.8). A piecewise regression was also a better fit for mature leaves, although the difference in AIC values was small (AIC = -556.8 vs. -553.7). For populations north vs. south of the threshold, \log_e -biomass per cup was 7.9 times larger in the young-leaf treatment and 0.7 times higher in the mature-leaf treatment. In piecewise regressions for both leaf ages, latitude was not correlated with biomass on either side of the threshold ($p > 0.15$).

Discussion

The idea that plants experience greater herbivore pressure at lower latitudes has interested ecologists since Dobzhansky first posited the biotic interactions hypothesis (1950). However, previous work has not consistently supported this prediction (reviewed in Moles *et al.* 2011; Andrew *et al.* 2012; Kozlov *et al.* 2015; Anstett *et al.* 2016), possibly due to a relative lack of studies capturing biologically meaningful measures of defense and consumption of young leaves, which can be substantial. Here, we examined relationships between latitude, herbivory, and defense in *P. americana* across a large temperate latitudinal gradient. We found evidence supporting several long-standing hypotheses: (1) Plants at lower latitudes experienced greater herbivory in nature, with young leaves demonstrating a particularly strong pattern compared to mature leaves. Herbivory sharply decreased around 35°N, and was predicted by herbivore abundance, suggesting that biotic or abiotic thresholds may drive latitudinal patterns. (2) Lower-latitude populations had more effective leaf defense, shown by positive correlation of *S. exigua* growth with latitude in palatability experiments. Palatability also showed non-linear patterns, but

with a different threshold than we found for herbivory. Larvae grew less on young than mature leaves, suggesting that young leaves are better defended.

Our evidence for higher herbivory rates at lower latitudes agrees with studies of salt marsh plants (Pennings & Silliman 2005; Pennings *et al.* 2009), and some terrestrial plants (Garibaldi *et al.* 2011; Kim 2014; Lehdal & Agren 2015b; Lehdal & Agren 2015a; Moreira *et al.* 2015). However, to our knowledge these are the first results finding a latitudinal threshold in herbivory. It is possible that short transects cannot capture thresholds; 16 single-species gradient studies have had transects that were shorter than ours (15°), and 15 have been the same or longer (Anstett *et al.* 2016). Many other studies compare high and low latitude regions rather than sampling a continuous gradient (e.g., Pennings *et al.* 2009; Feller *et al.* 2013; Lehdal & Agren 2015a). Thus, it is unclear whether thresholds are rare, or only rarely documented or noticed.

Mechanisms proposed to explain latitudinal gradients in herbivore damage within plant species include greater herbivore abundance, per capita consumption, or specialization at lower latitudes. Several studies have found similar results to ours: a negative correlation between herbivore abundance and latitude (Pennings *et al.* 2009; Salazar & Marquis 2012; Kim 2014). However, Pennings and Silliman (2005) found that *Spartina alterniflora* herbivore abundance did not differ with latitude, but that high-latitude herbivore species consumed less per capita. Herbivores are more specialized at lower latitudes at the global scale (Forister *et al.* 2015), but more generalist consumers were found at lower latitudes in two *Piper* species (Salazar & Marquis 2012). Information on host ranges for *P. americana* herbivores is sparse, but there are no obvious patterns of latitudinal differences in specialization.

The threshold in herbivory that we observed generates hypotheses about mechanisms driving latitudinal patterns in herbivore abundance and herbivory. First, temperature thresholds

could limit herbivore species' ranges or reduce abundance at higher latitudes; e.g., *S. eridania* cannot survive extended freezing and re-colonizes northward each year from subtropical areas (Mitchell & Tumlinson 1994). Second, fewer life cycles can be completed in shorter growing seasons (Roff 1980; Scriber 2002); *Papilio glaucus*, for example, transitions from trivoltine to bivoltine at our latitudinal threshold in herbivory (Scriber *et al.* 2014). The discrete nature of life cycles could lead to a threshold pattern in abundance within species. Third, the driver may not be herbivore population sizes, but latitudinal shifts in host preference or availability of alternative hosts (Logarzo *et al.* 2011), which can interact with time available for development (Scriber 2002). That is, *P. americana* could be preferred in lower-latitude communities and not at higher latitudes, despite our evidence of greater palatability at higher latitudes, akin to an associational effect (Barbosa *et al.* 2009). For example, Descombes *et al.* (2017) found that high-elevation plant communities were more palatable than low-elevation communities.

Quantifying latitudinal variation in plant defense is a powerful approach to testing the biotic interactions hypothesis because trait variation in a common garden demonstrates genetically based differences that could be due to natural selection (Woods *et al.* 2012; Anstett *et al.* 2015; Lehdal & Agren 2015b). Using this approach, we found evidence that *P. americana* populations are better defended at lower latitudes, measured by palatability (herbivore growth). Other studies that have examined latitudinal patterns of insect herbivore defense by using functional assays (e.g., palatability, herbivore preference, resistance) have found stronger defenses in lower-latitude populations (Miller & Hanson 1989; Pennings *et al.* 2001; Salgado & Pennings 2005; Pennings *et al.* 2007; Anstett *et al.* 2015; Lehdal & Agren 2015b), but see (Kim 2014). In contrast to mixed results seen for putative physical and chemical defenses, insect

herbivores reliably preferred higher latitude leaves in a meta-analysis (Moles *et al.* 2011).

Bioassays of plant defense thus may give more consistent results than traits as defense proxies.

We found that young leaves are consumed at higher rates than mature leaves in the field, which agrees with other data on consumption over leaf lifetime (Lowman 1984; Filip *et al.* 1995). We speculate that low herbivory rates on mature leaves may explain some of the mixed results across latitudinal studies of herbivory, which almost exclusively measure mature leaves (Moles *et al.* 2011; Moles 2013; Anstett *et al.* 2016; Moles & Ollerton 2016). In fact, we found only a marginally significant relationship between latitude and herbivory for mature leaves in the field. Despite young leaves being eaten more in nature, we unexpectedly found them less palatable in bioassays. Coley and Barone (1996) hypothesize that young leaves do not have structural defenses and are defended by chemicals, while mature leaves rely on toughness for defense. Young *P. americana* leaves may be more nutritious and less tough than mature leaves, explaining greater herbivory in the field by *P. americana* consumers, but also contain chemical defenses that are unpalatable to the naïve generalist in our experiments. Further study using specialist herbivores is needed to test the defensive functions of leaf traits in *P. americana*.

We found support for greater herbivore pressure at lower latitudes for both herbivory and defense, but the relationship between the two indices is unclear. Stronger defense in southern populations seem to maintain mature-leaf herbivory at similar levels to northern populations, but potent young-leaf defense is ineffective against herbivores in peninsular Florida. Low-latitude *Lythrum salicaria* populations in Sweden were also more attacked in the field but showed greater resistance in a common garden, and damage in the field and greenhouse were negatively correlated (Lehndal & Agren 2015a; Lehndal & Agren 2015b). In contrast, a post-hoc test of whether palatability and field herbivory were correlated in our study found no relationship

because of their distinct thresholds (Pearson's product-moment correlation on population means from 2015 herbivory survey: $t_8 = -0.9312$, $p = 0.3790$); the use of a naïve herbivore for palatability may contribute to this inconsistency. Herbivore resistance in *Solanum carolinense* and *Solidago altissima* was also unrelated to field herbivory (Kim 2014), and Marczak *et al.* found that latitudinal differences in plant quality in *Spartina alterniflora* (2013) and *Iva frutescens* (2011) were relatively unimportant predictors of herbivory compared to food web structure. In other systems with data on both herbivory and defense, latitudinal patterns are only sometimes consistent between indices (Salgado & Pennings 2005; Pennings *et al.* 2009; Woods *et al.* 2012; Anstett *et al.* 2014; Wieski & Pennings 2014; Anstett *et al.* 2015). Thus, it is clear that measuring both is crucial to understand geographic patterns in herbivore pressure, but relationships between herbivory and defense are inconsistent and not straightforward (Hahn & Maron 2016).

The lack of a relationship between herbivory and defense may be complicated by latitudinal differences not only in ecological context such as food webs and plant community composition, but also in evolutionary processes such as coevolutionary dynamics, gene flow, and fitness impacts of herbivores, although latitudinal patterns in these mechanisms have been poorly explored. Herbivory on young leaves at some of our sites exceeded 90%, more than enough to impact fitness (Agrawal 2005; Lehndal & Agren 2015b), but further work is needed to show that herbivores exert stronger selection on defense at lower latitudes, as variation in tolerance could mediate fitness impacts (Woods *et al.* 2012). In addition, selection on defense could be influenced by phenology and leaf turnover (e.g. herbivory during growth vs. reproduction), so more information on spatiotemporal variation in herbivory is needed to understand its fitness impacts (Anstett *et al.* 2016).

In conclusion, we find strong support for the prediction that herbivore pressure is greater at lower latitudes in *P. americana*. Leaf age affected herbivory and defense, and should be considered in future studies, as latitudinal patterns may be weaker for mature leaves. The nonlinear patterns that we observed for both herbivory and defense suggest avenues for future research on how community context and the abiotic environment interact to affect biotic interactions. Further research is also needed to understand how single-species results scale up to affect food webs, community assembly, and biodiversity.

Acknowledgements

Assistance with lepidopteran identification was kindly provided by C.V. Covell, Jr. (University of Florida, Gainesville), James Hayden (Florida Department of Agriculture and Consumer Services), and Gary Parsons (Michigan State University). For hospitality and help finding plants, we thank Nick Batora, Betsey Boughton, Anna Groves, Mike Hunter, the Canfield McPhees, Vince Morris, Bill Palmer, Eric Staller, Rick Toomey, Randy Wood, Jim and Susanne Yule, and many others who found plants that we did not use. Emma Clark, Carolyn Graham, Jon Spoelhof, Abby Sulesky, and Alyssa Trethewey assisted substantially with data collection. Anurag Agrawal, Emily Dittmar, Marc Johnson, Christopher Oakley, Mark Scriber, Nash Turley, Marjorie Weber, Will Wetzal, and Chad Zirbel provided valuable feedback on ideas, writing, and/or data analysis. Comments from three anonymous reviewers greatly improved the manuscript. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant Nos. DGE0802267 and DGE1424871. Funding also provided by the Social Sciences Research Council-Mellon Mays Graduate Initiatives Program and Michigan State University (University Distinguished Fellowship, Dr. Marvin Hensley

Endowed Fellowship in Science, and travel grants from Department of Plant Biology and Ecology, Evolutionary Biology, and Behavior Program).

This chapter is modified and reprinted with permission from John Wiley and Sons. The published article is: Baskett, C. A. and D. W. Schemske (2018). "Latitudinal patterns of herbivore pressure in a temperate herb support the biotic interactions hypothesis." Ecology Letters **21**(4): 578-587.

REFERENCES

REFERENCES

- Agrawal, A. A. (1998). "Induced responses to herbivory and increased plant performance." Science **279**(5354): 1201-1202.
- Agrawal, A. A. (2005). "Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores." Evolutionary Ecology Research **7**(5): 651-667.
- Allyson, S. (1984). "Description of last-instar larvae of 22 species of North American Spilomelini (Lepidoptera, Pyralidae, Pyraustinae) with a key to species." Canadian Entomologist **116**(10): 1301-1334.
- Andrew, N. R., I. R. Roberts and S. J. Hill (2012). "Insect herbivory along environmental gradients." Open Journal of Ecology **2**(4): 202-213.
- Anstett, D. N., J. R. Ahern, J. Glinos, N. Nawar, J.-P. Salminen and M. T. J. Johnson (2015). "Can genetically based clines in plant defence explain greater herbivory at higher latitudes?" Ecology Letters **18**(12): 1376-1386.
- Anstett, D. N., I. Naujokaitis-Lewis and M. T. J. Johnson (2014). "Latitudinal gradients in herbivory on *Oenothera biennis* vary according to herbivore guild and specialization." Ecology **95**(10): 2915-2923.
- Anstett, D. N., K. A. Nunes, C. A. Baskett and P. M. Kotanen (2016). "Sources of controversy surrounding latitudinal patterns in herbivory and defense." Trends in Ecology & Evolution **31**(10): 789-802.
- Barbosa, P., J. Hines, I. Kaplan, H. Martinson, A. Szczepaniec and Z. Szendrei (2009). Associational resistance and associational susceptibility: Having right or wrong neighbors. Annual Review of Ecology Evolution and Systematics. **40**: 1-20.
- Bates, D., M. Maechler, B. Bolker and S. Walker (2015). "Fitting linear mixed-effects models using lme4." Journal of Statistical Software **67**(1): 1-48.
- Coley, P. D. (1983). "Herbivory and defensive characteristics of tree species in a lowland tropical forest." Ecological Monographs **53**(2): 209-233.
- Coley, P. D. and T. M. Aide (1991). Comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. P. W. Price, T. M. Lewinsohn, G. W. Fernandes and W. W. Benson. New York, USA, John Wiley and Sons: 25-49.
- Coley, P. D. and J. A. Barone (1996). "Herbivory and plant defenses in tropical forests." Annual Review of Ecology and Systematics **27**: 305-335.

- Connell, J. H. (1971). On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees. Dynamics of Populations. P. J. Boer and G. Gradwell. The Netherlands, PUDOC: 298-310.
- Cuenoud, P., V. Savolainen, L. W. Chatrou, M. Powell, R. J. Grayer and M. W. Chase (2002). "Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid rbcL, atpB, and matK DNA sequences." American Journal of Botany **89**(1): 132-144.
- Descombes, P., J. Marchon, J. N. Pradervand, J. Bilat, A. Guisan, S. Rasmann and L. Pellissier (2017). "Community-level plant palatability increases with elevation as insect herbivore abundance declines." Journal of Ecology **105**(1): 142-151.
- Dobzhansky, T. (1950). "Evolution in the tropics." American Scientist **38**: 209-221.
- Downum, K. R., L. A. Swain and L. J. Faleiro (1991). "Influence of light on plant allelochemicals: A synergistic defense in higher plants." Archives of Insect Biochemistry and Physiology **17**(4): 201-211.
- Ehrlich, P. R. and P. H. Raven (1964). "Butterflies and plants - A study in coevolution." Evolution **18**(4): 586-608.
- Farmer, R. E. and G. C. Hall (1970). "Pokeweed seed germination: effects of environment, stratification, and chemical growth regulators." Ecology **51**(5): 894-&.
- Feller, I. C., A. H. Chamberlain, C. Piou, S. Chapman and C. E. Lovelock (2013). "Latitudinal patterns of herbivory in mangrove forests: Consequences of nutrient over-enrichment." Ecosystems **16**(7): 1203-1215.
- Filip, V., R. Dirzo, J. M. Maass and J. Sarukhan (1995). "Within-year and among-year variation in the levels of herbivory on the foliage of trees from a Mexican tropical deciduous forest." Biotropica **27**(1): 78-86.
- Fischer, A. G. (1960). "Latitudinal variation in organic diversity." Evolution **14**: 64-81.
- Forister, M. L., V. Novotny, A. K. Panorska, L. Baje, Y. Basset, P. T. Butterill, L. Cizek, P. D. Coley, F. Dem, I. R. Diniz, P. Drozd, M. Fox, A. E. Glassmire, R. Hazen, J. Hreck, J. P. Jahner, O. Kaman, T. J. Kozubowski, T. A. Kursar, O. T. Lewis, J. Lill, R. J. Marquis, S. E. Miller, H. C. Morais, M. Murakami, H. Nickel, N. A. Pardikes, R. E. Ricklefs, M. S. Singer, A. M. Smilanich, J. O. Stireman, S. Villamarin-Cortez, S. Vodka, M. Volf, D. L. Wagner, T. Walla, G. D. Weiblen and L. A. Dyer (2015). "The global distribution of diet breadth in insect herbivores." Proceedings of the National Academy of Sciences of the United States of America **112**(2): 442-447.
- Garibaldi, L. A., T. Kitzberger and A. Ruggiero (2011). "Latitudinal decrease in folivory within *Nothofagus pumilio* forests: Dual effect of climate on insect density and leaf traits?" Global Ecology and Biogeography **20**(4): 609-619.

- Gaston, K. J., D. R. Genney, M. Thurlow and S. E. Hartley (2004). "The geographical range structure of the holly leaf-miner. IV. Effects of variation in host-plant quality." Journal of Animal Ecology **73**(5): 911-924.
- Gomez, S. and J. F. Stuefer (2006). "Members only: Induced systemic resistance to herbivory in a clonal plant network." Oecologia **147**(3): 461-468.
- Hahn, P. G. and J. L. Maron (2016). "A framework for predicting intraspecific variation in plant defense." Trends in Ecology & Evolution **31**(8): 646-656.
- Hardin, J. W. (1964). "A comparison of *Phytolacca americana* and *P. rigida*." Castanea **29**(3): 155-164.
- Hendriks, R. J. J., L. Luijten and J. M. van Groenendael (2009). "Context-dependent defence in terrestrial plants: The effects of light and nutrient availability on plant resistance against herbivory." Entomologia Experimentalis Et Applicata **131**(3): 233-242.
- Janzen, D. H. (1970). "Herbivores and the number of tree species in tropical forests." American Naturalist **104**(940): 501-528.
- Kahle, D. and H. Wickham (2013). "ggmap: Spatial Visualization with ggplot2." The R Journal **5**(1): 144-161.
- Kim, T. N. (2014). "Plant damage and herbivore performance change with latitude for two old-field plant species, but rarely as predicted." Oikos **123**(7): 886-896.
- Kozlov, M. V., V. Lanta, V. Zverev and E. L. Zvereva (2015). "Global patterns in background losses of woody plant foliage to insects." Global Ecology and Biogeography **24**(10): 1126-1135.
- LaManna, J. A., S. A. Mangan, A. Alonso, N. A. Bourg, W. Y. Brockelman, S. Bunyavejchewin, L.-W. Chang, J.-M. Chiang, G. B. Chuyong, K. Clay, R. Condit, S. Cordell, S. J. Davies, T. J. Furniss, C. P. Giardina, I. A. U. N. Gunatilleke, C. V. S. Gunatilleke, F. He, R. W. Howe, S. P. Hubbell, C.-F. Hsieh, F. M. Inman-Narahari, D. Janik, D. J. Johnson, D. Kenfack, L. Korte, K. Král, A. J. Larson, J. A. Lutz, S. M. McMahon, W. J. McShea, H. R. Memiaghe, A. Nathalang, V. Novotny, P. S. Ong, D. A. Orwig, R. Ostertag, G. G. Parker, R. P. Phillips, L. Sack, I.-F. Sun, J. S. Tello, D. W. Thomas, B. L. Turner, D. M. Vela Díaz, T. Vrška, G. D. Weiblen, A. Wolf, S. Yap and J. A. Myers (2017). "Plant diversity increases with the strength of negative density dependence at the global scale." Science **356**(6345): 1389-1392.
- Lehndal, L. and J. Agren (2015). "Herbivory differentially affects plant fitness in three populations of the perennial herb *Lythrum salicaria* along a latitudinal gradient." Plos One **10**(9).
- Lehndal, L. and J. Agren (2015). "Latitudinal variation in resistance and tolerance to herbivory in the perennial herb *Lythrum salicaria* is related to intensity of herbivory and plant phenology." Journal of Evolutionary Biology **28**(3): 576-589.

- Lim, J. Y., P. V. A. Fine and G. G. Mittelbach (2015). "Assessing the latitudinal gradient in herbivory." Global Ecology and Biogeography: 1-7.
- Logarzo, G. A., M. A. Casalinuovo, R. V. Piccinali, K. Braun and E. Hasson (2011). "Geographic host use variability and host range evolutionary dynamics in the phytophagous insect *Apagomerella versicolor* (Cerambycidae)." Oecologia **165**(2): 387-402.
- Lowman, M. D. (1984). "An assessment of techniques for measuring herbivory: Is rainforest defoliation more intense than we thought?" Biotropica **16**(4): 264-268.
- Marczak, L. B., C. K. Ho, K. Wieski, H. Vu, R. F. Denno and S. C. Pennings (2011). "Latitudinal variation in top-down and bottom-up control of a salt marsh food web." Ecology **92**(2): 276-281.
- Marczak, L. B., K. Wieski, R. F. Denno and S. C. Pennings (2013). "Importance of local vs. geographic variation in salt marsh plant quality for arthropod herbivore communities." Journal of Ecology **101**(5): 1169-1182.
- Miller, J. C. and P. E. Hanson (1989). "Laboratory feeding tests on the development of gypsy moth larvae with reference to plant taxa and allelochemicals." Bulletin of the Agricultural Experimental Station of Oregon State University **674**: 1-63.
- Mitchell, E. R. and J. H. Tumlinson (1994). "Response of *Spodoptera exigua* and *S. eridania* (Lepidoptera, Noctuidae) males to synthetic pheromone and *S. exigua* females." Florida Entomologist **77**(2): 237-247.
- Mittelbach, G. G., D. W. Schemske, H. V. Cornell, A. P. Allen, J. M. Brown, M. B. Bush, S. P. Harrison, A. H. Hurlbert, N. Knowlton, H. A. Lessios, C. M. McCain, A. R. McCune, L. A. McDade, M. A. McPeck, T. J. Near, T. D. Price, R. E. Ricklefs, K. Roy, D. F. Sax, D. Schluter, J. M. Sobel and M. Turelli (2007). "Evolution and the latitudinal diversity gradient: speciation, extinction and biogeography." Ecology Letters **10**(4): 315-331.
- Moles, A. T. (2013). "Dogmatic is problematic: Interpreting evidence for latitudinal gradients in herbivory and defense." Ideas in Ecology and Evolution **6**: 1-4.
- Moles, A. T., S. P. Bonser, A. G. B. Poore, I. R. Wallis and W. J. Foley (2011). "Assessing the evidence for latitudinal gradients in plant defence and herbivory." Functional Ecology **25**(2): 380-388.
- Moles, A. T. and J. Ollerton (2016). "Is the notion that species interactions are stronger and more specialized in the tropics a zombie idea?" Biotropica **48**(2): 141-145.
- Moreira, X., L. Abdala-Roberts, V. Parra-Tabla and K. A. Mooney (2015). "Latitudinal variation in herbivory: Influences of climatic drivers, herbivore identity and natural enemies." Oikos **124**: 1444-1452.

- Pennings, S. C., C.-K. Ho, C. S. Salgado, K. Wieski, N. Dave, A. E. Kunza and E. L. Wason (2009). "Latitudinal variation in herbivore pressure in Atlantic Coast salt marshes." *Ecology* **90**(1): 183-195.
- Pennings, S. C. and B. R. Silliman (2005). "Linking biogeography and community ecology: Latitudinal variation in plant-herbivore interaction strength." *Ecology* **86**(9): 2310-2319.
- Pennings, S. C., E. L. Siska and M. D. Bertness (2001). "Latitudinal differences in plant palatability in Atlantic coast salt marshes." *Ecology* **82**(5): 1344-1359.
- Pennings, S. C., M. Zimmer, N. Dias, M. Sprung, N. Dave, C. K. Ho, A. Kunza, C. McFarlin, M. Mews, A. Pfaunder and C. Salgado (2007). "Latitudinal variation in plant-herbivore interactions in European salt marshes." *Oikos* **116**(4): 543-549.
- Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar and R. C. Team (2017). nlme: Linear and nonlinear mixed effects models. R package version 3.1-131, <https://CRAN.R-project.org/package=nlme>.
- Prendeville, H. R., J. C. Steven and L. F. Galloway (2015). "Spatiotemporal variation in deer browse and tolerance in a woodland herb." *Ecology* **96**(2): 471-478.
- Price, P. W. (2002). "Resource-driven terrestrial interaction webs." *Ecological Research* **17**(2): 241-247.
- R Core Team (2014). *R: A language and environment for statistical computing*. Vienna, Austria, R Foundation for Statistical Computing.
- Robinson, G. S., P. R. Ackery, I. J. Kitching, G. W. Beccaloni and L. M. Hernandez (2010). HOSTS - A Database of the World's Lepidopteran Hostplants. London, Natural History Museum.
- Roff, D. (1980). "Optimizing development time in a seasonal environment: The 'ups and downs' of clinal variation." *Oecologia* **45**(2): 202-208.
- Salazar, D. and R. J. Marquis (2012). "Herbivore pressure increases toward the equator." *Proceedings of the National Academy of Sciences of the United States of America* **109**(31): 12616-12620.
- Salgado, C. S. and S. C. Pennings (2005). "Latitudinal variation in palatability of salt-marsh plants: Are differences constitutive?" *Ecology* **86**(6): 1571-1579.
- Sandberg, S. L. and M. R. Berenbaum (1989). "Leaf-tying by Tortricid larvae as an adaptation for feeding on phototoxic *Hypericum perforatum*." *Journal of Chemical Ecology* **15**(3): 875-885.
- Sauer, J. D. (1952). "A geography of pokeweed." *Ann Missouri Bot Gard* **39**((2)): 113-125.

- Schemske, D. W. (2009). Biotic interactions and speciation in the tropics. Speciation and Patterns of Diversity. R. K. Butlin, J. R. Bridle and D. Schluter. Cambridge, UK, Cambridge University Press: 219-239.
- Schemske, D. W., G. G. Mittelbach, H. V. Cornell, J. M. Sobel and K. Roy (2009). "Is there a latitudinal gradient in the importance of biotic interactions?" Annual Review of Ecology Evolution and Systematics **40**: 245-269.
- Scriber, J. M. (1986). "Local food plant specialization in natural field populations of the southern armyworm, *Spodoptera eridania* (Lepidoptera, Noctuidae)." Entomological News **97**(4): 183-185.
- Scriber, J. M. (2002). "Latitudinal and local geographic mosaics in host plant preferences as shaped by thermal units and voltinism in *Papilio* spp. (Lepidoptera)." European Journal of Entomology **99**(2): 225-239.
- Scriber, J. M., B. Elliott, E. Maher, M. McGuire and M. Niblack (2014). "Adaptations to "thermal time" constraints in *Papilio*: Latitudinal and local size clines differ in response to regional climate change." Insects **5**: 199-226.
- Summerland, S. A. and D. W. Hamilton (1955). "Biology of the red-banded leaf roller in southern Indiana." Journal of Economic Entomology **48**(1): 51-53.
- Violle, C., P. B. Reich, S. W. Pacala, B. J. Enquist and J. Kattge (2014). "The emergence and promise of functional biogeography." Proceedings of the National Academy of Sciences of the United States of America **111**(38): 13690-13696.
- Wagner, C. E., L. J. Harmon and O. Seehausen (2012). "Ecological opportunity and sexual selection together predict adaptive radiation." Nature **487**(7407): 366-369.
- Wallace, A. R. (1878). Tropical Nature, and Other Essays. London, UK, MacMillan and Co.
- Wieski, K. and S. Pennings (2014). "Latitudinal variation in resistance and tolerance to herbivory of a salt marsh shrub." Ecography **37**(8): 763-769.
- Woods, E. C., A. P. Hastings, N. E. Turley, S. B. Heard and A. A. Agrawal (2012). "Adaptive geographical clines in the growth and defense of a native plant." Ecological Monographs **82**(2): 149-168.

CHAPTER 2: MULTIPLE METRICS OF LATITUDINAL
PATTERNS IN INSECT POLLINATION AND HERBIVORY FOR
A TROPICAL-TEMPERATE CONGENER PAIR

Introduction

A pattern of stronger and more prevalent biotic interactions at lower latitudes has been hypothesized to contribute to high tropical biodiversity (the “biotic interactions hypothesis,” (Dobzhansky 1950, Fischer 1960, Pianka 1966, Schemske 2009, Schemske et al. 2009)). Many plant mutualisms have been shown to be more prevalent in tropical communities, such as animal pollination (Regal 1982, Rech et al. 2016), animal seed dispersal (Moles et al. 2007), ant-plant interactions involving extrafloral nectaries (Coley and Aide 1991), ant domatia (Davidson and McKey 1993), and endophytic fungi (Arnold and Lutzoni 2007). However, quantifying the strength of ubiquitous interactions, rather than their presence or absence in a community, is more challenging and controversial (Schemske et al. 2009, Anstett et al. 2016). In particular, latitudinal patterns in herbivore pressure, plant defense, and pollination specialization have been contested, with no clear resolution (Moles et al. 2011, Ollerton 2012, Moles 2013, Moles and Ollerton 2016). Here, we focus on herbivory and pollination, important plant-animal interactions that are still poorly understood with respect to latitudinal patterns.

Comparisons of latitudinal trends in biotic interaction strength are generally conducted at one of two scales, each with advantages and disadvantages: 1) within a wide-ranging species or genus, or 2) across communities surveyed at different latitudes. Focusing on a wide-ranging species allows for quantifying detailed metrics of interaction strength. This approach is commonly used to study plant-herbivore interactions (e.g., Pennings and Silliman 2005, Salgado and Pennings 2005, Anstett et al. 2014, Anstett et al. 2015, Baskett and Schemske 2018), but has found mixed support for the biotic interactions hypothesis (Moles et al. 2011). One of its major limitations is that species’ ranges are only so large, and studies have largely focused on the temperate zone, typically spanning 15° (Anstett et al. 2016). This is problematic because the

biotic interactions hypothesis is a comparison of temperate and tropical communities (Dobzhansky 1950), and it remains unclear whether interaction strength should scale linearly with latitude, or change abruptly at climatic thresholds that may only be captured by large-scale studies (Moles et al. 2007, Kozlov et al. 2015, Anstett et al. 2016, Baskett and Schemske 2018). Additionally, attributes associated with large ranges could impact biotic interactions; for example, self-pollination was associated with larger ranges in a comparison of largely animal-pollinated sister species (Grossenbacher et al. 2015). Studies of wide-ranging genera can address these issues by spanning a wider geographic range than any one species. Traits such as secondary metabolites with known defensive function can be compared among species with a shared evolutionary history (Rasman and Agrawal 2011, Pearse and Hipp 2012). However, there is a trade-off with detail, as measuring interactions in the field across many species is infeasible, and species mean trait values may obscure signals of latitudinal patterns within species. For example, greater herbivore resistance observed in northern populations of *Asclepias syriaca* (Woods et al. 2012) contrasts with trends across *Asclepias* of more toxic, diverse, and inducible cardenolides at low latitudes (Rasman and Agrawal 2011).

In contrast to single-system studies, community surveys can span wide geographic scales and encompass a diversity of abiotic environments and taxonomic groups (Adams et al. 2009, Moles et al. 2011, Kozlov et al. 2015, Lim et al. 2015, Moeller et al. 2017), so biases of focusing on temperate biomes and/or wide-ranging taxa are avoided. However, community sampling has drawbacks; for example, we lack “common currencies” of traits to compare plant defenses between unrelated species (Agrawal and Weber 2015), and communities sampled at large geographic scales will have mostly separate evolutionary histories (e.g., one median species per plant family in Moles et al. 2011). Additionally, the most diverse terrestrial communities, wet

lowland tropical forests, are often poorly represented in community-level studies (e.g., Olesen and Jordano 2002, Moles et al. 2011, Kozlov et al. 2015). Similar to genus-level studies, collecting detailed interaction data is infeasible at large geographic and taxonomic scales. For example, estimating herbivory from leaf litter is a high-throughput approach to randomly sample forest communities (Adams et al. 2009), but cannot capture complete leaf consumption, which can be a substantial fraction of herbivory (Lowman 1984, Baskett and Schemske 2018).

Here, we bridge the gap between the detailed single-species studies limited to smaller latitudinal ranges, and community-level surveys that can cover large latitudinal ranges but suffer from poor phylogenetic control. We combine species- and genus-level sampling by quantifying species interactions in depth in tropical and temperate regions, using a pair of closely related, ecologically similar species. Specifically, we compare several ecological and evolutionary metrics of the importance of herbivory and pollination in the Neotropical *Phytolacca rivinoides* (Phytolaccaceae) in Costa Rica to its congener *P. americana* at its southern and northern range edges in the USA, spanning 32° latitude. For both herbivory and pollination, we pair *in situ* field studies evaluating ecological interactions with common garden phenotyping to quantify evolution in interaction-related traits. We hypothesize that pollination and herbivory rates are greater at lower latitudes, and that trait evolution shows a signature of stronger selection by herbivores and pollinators. Specifically, we predict that (1) *P. rivinoides* is more reliant than *P. americana* on insects for pollination, has evolved a reduced ability to self-pollinate, and has more attractive and rewarding flowers, and (2) that *P. rivinoides* experiences greater herbivore pressure (higher herbivory rate and greater herbivore abundance) and thus has evolved to be better defended than *P. americana*. We predict that these various measures of interaction strength are intermediate for *P. americana* at its subtropical southern range edge compared to its

northern range edge and to *P. rivinoides*, assuming that interaction strength decreases linearly with latitude. To our knowledge, this is the first study to examine latitudinal patterns for multiple biotic interactions in a single system, allowing us to compare results for a mutualistic and an antagonistic interaction. Furthermore, while latitudinal patterns in herbivory and plant defense have been studied using approaches at multiple scales (Anstett et al. 2016), to our knowledge, all latitudinal comparisons of plant-pollinator interactions thus far have been at the community level (e.g., Rech et al. 2016, Moeller et al. 2017), making this the first study of latitudinal trends in pollination within a wide-ranging genus.

Methods

Study system and sites

Phytolacca rivinoides Kunth & C.D. Bouché and *P. americana* L. are well-suited for a tropical-temperate test of the biotic interactions hypothesis. *Phytolacca rivinoides* is native throughout the Neotropics, from 16° S to 20° N (Murray 1988), and *P. americana* is native from subtropical to north-temperate eastern USA (27-44° N) (Sauer 1952). Both species are bird-dispersed, short-lived perennial herbs that rely on long-lived seed banks to colonize disturbed areas, where they are abundant and easily found (Sauer 1952, Murray 1988, Veldman et al. 2007). One ecological difference between the species that could potentially mediate fitness effects of pollination and herbivory is that *P. rivinoides* is semelparous, with a lifespan of about two years (Murray 1988), while *P. americana* is an iteroparous perennial, re-emerging each spring from a large taproot. We found no reports of a mean or maximum lifespan, but it can live at least four years (Sauer 1952). The genus *Phytolacca* is monophyletic and contains 20 species, mostly in the New World tropics, but relationships within the genus are poorly resolved (Ali et al. 2015).

Pollination in *Phytolacca* has not been studied to our knowledge. Both species have racemes with many white flowers (6-mm diameter), whose nectaries are tucked at the base of a green pistil. Anthers are dehiscent when flowers open, and tend to fall off the second day of flowering, but the period of stigma receptivity is unknown. *Phytolacca americana* has 5 tepals and around 10 stamens and carpels (Hardin 1964). *P. rivinoides* has 5 tepals and around 16 stamens and carpels (Fassett and Sauer 1950).

Baskett and Schemske (2018) determined that lepidopterans are the primary herbivores of *P. americana*, and identified five consumers along a gradient from Michigan to Florida: *Disclisioprocta stellata* Guenée (Geometridae), *Spodoptera eridania* Stoll (Noctuidae), and three species that shelter in and consume young leaves: *Psara obscuralis* Lederer (Crambidae), *Asciodes gordialis* Guenée (Crambidae), and *Argyrotaenia velutinana* Walker (Tortricidae). *Spodoptera eridania* and *A. velutinana* are highly polyphagous, while the other species have more restricted diets in host families closely related to Phytolaccaceae.

We evaluated species interaction strength using multiple field and common garden studies in three regions, which we refer to as “tropical, subtropical, and temperate” (Fig. 5, Tables 5 and 6): *P. rivinoides* in Costa Rica (8.7°-10.4° N), *P. americana* in Florida (26.5°-28.6° N), and *P. americana* in Michigan and Ohio (39.5°-42.7° N). All measurements of interaction strength were quantified in three populations in each of two or three regions (Table 5). Some of the temperate populations used in our studies differed between *in situ* field studies and common gardens (Table 5). All populations were abutting or surrounded by natural vegetation in rural landscapes, and had varying disturbance histories (Table 6).

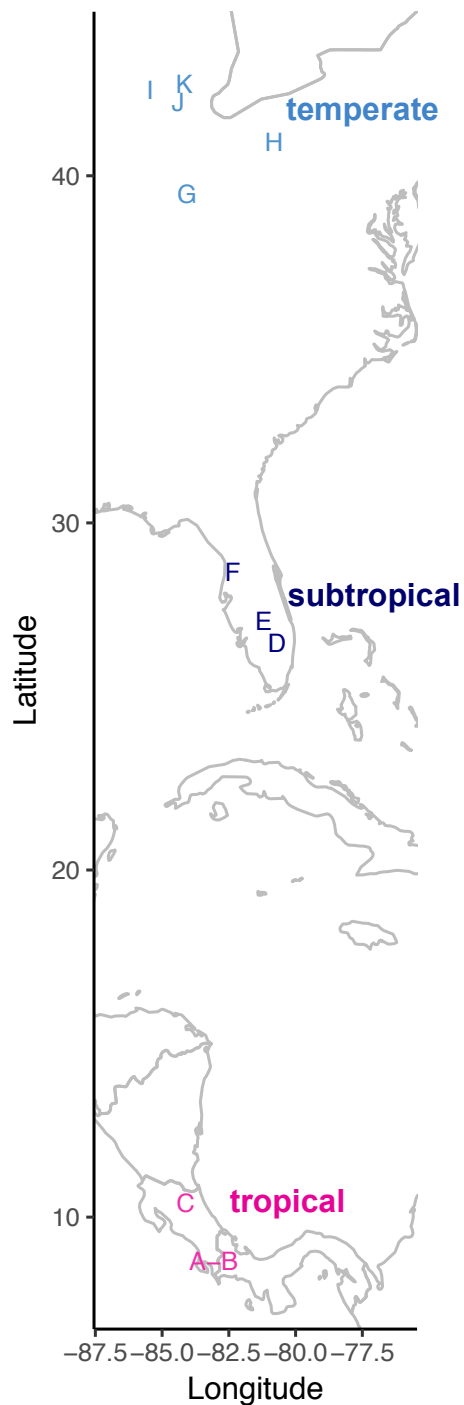


Figure 5. Populations used for *in situ* studies of pollination and herbivory rate and/or sources of seeds for common gardens planted at Michigan State University. Populations are grouped throughout the paper into three consistently color-coded “regions:” temperate and subtropical for *P. americana*, and tropical *P. rivinoides*. Letters correspond to Table 5 and Table 6; coordinates are in Table 6. Labels for A and B would overlap, so they are labeled “A-B” at coordinates for population A.

Table 5. Hypotheses and study design for each metric of pollination and herbivory. *P. riv.* = *Phytolacca rivinoides*, native to the Neotropics; *P. am.* = *P. americana*, native to the eastern USA. Cohort is indicated by a combination of place (*in situ* vs. common garden) and year.

Biotic interaction metric	Hypothesis: low-latitude plants...	Populations compared*	Place and cohort measured	Results
Visitation rate <i>in situ</i>	higher visitation rate	tropical <i>P. riv.</i> (A-C), temperate <i>P. am.</i> (I-K)**	<i>in situ</i> 2016, 2017	Fig. 8
Self-pollination (autogamy)	lower autogamy rate		common garden 2016 (greenhouse)	Fig. 9
Floral display size	larger floral display	tropical <i>P. riv.</i> (A-C), subtropical <i>P. am.</i> (D-F), temperate <i>P. am.</i> (G-I)	common garden 2017 (field + greenhouse)***	Fig. 10
Floral reward	more nectar		common garden 2017 (greenhouse)	Fig. 11
Pollinator attraction	attract more pollinators	subtropical <i>P. am.</i> (D-F), temperate <i>P. am.</i> (G-I)	common garden 2017 (field)	Fig. 11
Herbivory rates <i>in situ</i>	higher herbivory rate	tropical <i>P. riv.</i> (A-C), temperate <i>P. am.</i> (I-K)**	<i>in situ</i> 2016, 2017	Fig. 13, Table 9
Herbivore abundance	higher herbivore abundance	tropical <i>P. riv.</i> (A-C), temperate <i>P. am.</i> (I-K)**	<i>in situ</i> 2016, 2017	Fig. 15
Plant defense (palatability)	less palatable leaves	tropical <i>P. riv.</i> (A-C), subtropical <i>P. am.</i> (D-F), temperate <i>P. am.</i> (G-I)	common garden 2016 (greenhouse)	Fig. 16, Table 9

*Letters correspond to Fig. 5 and Table 6.

**Note that two of these populations differ from those used in common gardens to represent temperate *P. americana*.

***This cohort of plants was moved from field to greenhouse; see main text for details.

Table 6. Populations of *Phytolacca rivinoides* (*P. riv.*) and *P. americana* (*P. am.*) used for *in situ* data collection or seed collection for common gardens at Michigan State University. Site ID corresponds to map in Fig. 5 and studies in Table 5. The Finca Bellavista population was spread along a dirt and gravel road of a few kilometers, so the coordinates are less precise; other populations were patches or spread along shorter dirt roads.

ID	Site name	Latitude	Longitude	Region	Species	Description
A	Finca Gavilan	8.73126	-83.0665	tropical	<i>P. riv.</i>	Landslide
B	Finca Bellavista Tirimina Biological	8.79	-83.2	tropical	<i>P. riv.</i>	Dirt road edge
C	Reserve	10.41549	-84.1215	tropical	<i>P. riv.</i>	River flood Farm field edge
D	Highway 27 MacArthur Agroecology	26.53707	-80.69487	subtropical	<i>P. am.</i>	Farm field edge
E	Ranch Withlacoochee	27.17833	-81.1939	subtropical	<i>P. am.</i>	Gaps in thin forest
F	State Forest Caesar Creek	28.60387	-82.361	subtropical	<i>P. am.</i>	Mowed area edge
G	State Park	39.4887	-84.05828	temperate	<i>P. am.</i>	Dirt road edge
H	McPhee Farm Kellogg	40.971333	-80.802617	temperate	<i>P. am.</i>	Gaps in thin forest
I	Biological Station MacCready	42.4881	-85.4509	temperate	<i>P. am.</i>	Gaps in thin forest
J	Reserve	42.13145	-84.40182	temperate	<i>P. am.</i>	Farm and dirt road edge
K	Grieb Farm	42.65821	-84.17213	temperate	<i>P. am.</i>	

Floral visitation rate in situ: tropical, temperate

To quantify pollination rates across populations we used videos of insect visitation to flowers over multiple seasons in native habitats. We quantified field visitation at three tropical populations of *P. rivinoides* and three north temperate populations of *P. americana* (Table 5, Fig. 5), as we were logistically constrained to conduct repeated monitoring at only two sites. Visits were recorded with GoPro cameras, positioned 30-50 cm away from inflorescences. In the tropics, videos were recorded during three observation blocks, spaced six weeks apart: dry season, dry-wet transition, and wet season (Apr 11-Jul 24, 2016). In the temperate region, we

recorded in three observation blocks, spaced 3-4 weeks apart during the flowering season (Jul 5-Aug 24, 2017). During each observation block, 2-4 cameras were deployed for 1-3 days in each population. Cameras were rotated among flowering individuals each day, and in each observation block the median number of videos per plant was one, although some were observed up to four times (never the same inflorescence on the same day). Video duration ranged from 20 to 160 min (median = 62), and we recorded between 7:30 AM and 4:30 PM.

For each inflorescence on a particular day, we counted total number of open flowers (with tepals, without swollen pistil). From each video, we recorded the number of flowers visited (contact with anthers or stigma) by all insects except ants and lepidopterans (Fig. 6). Total visits were divided by the number of flowers and video duration to obtain visits per flower per hour as the response variable for analysis. Rainy or windy videos were excluded. Number of floral visitors per flower per hour of observation was \log_e+1 transformed to meet assumptions of normality, and analyzed using a mixed model with fixed effect of region to test whether plants in tropical or temperate regions received more floral visits in the field. The model included the random effect of individual nested within population, and variance was estimated separately for each population to reduce effects of heteroscedasticity. To test whether per-flower visitation increases non-linearly with display size, we analyzed visits per flower as a function of flower number, with random effect of individual nested in each population. We also asked whether there is seasonal variation in floral visitation rates. The seasons are not comparable between regions, so we analyzed tropical and temperate regions separately. Floral visitation was analyzed as a function of season with random effects of individual nested in population and separate estimates of variance for each population.

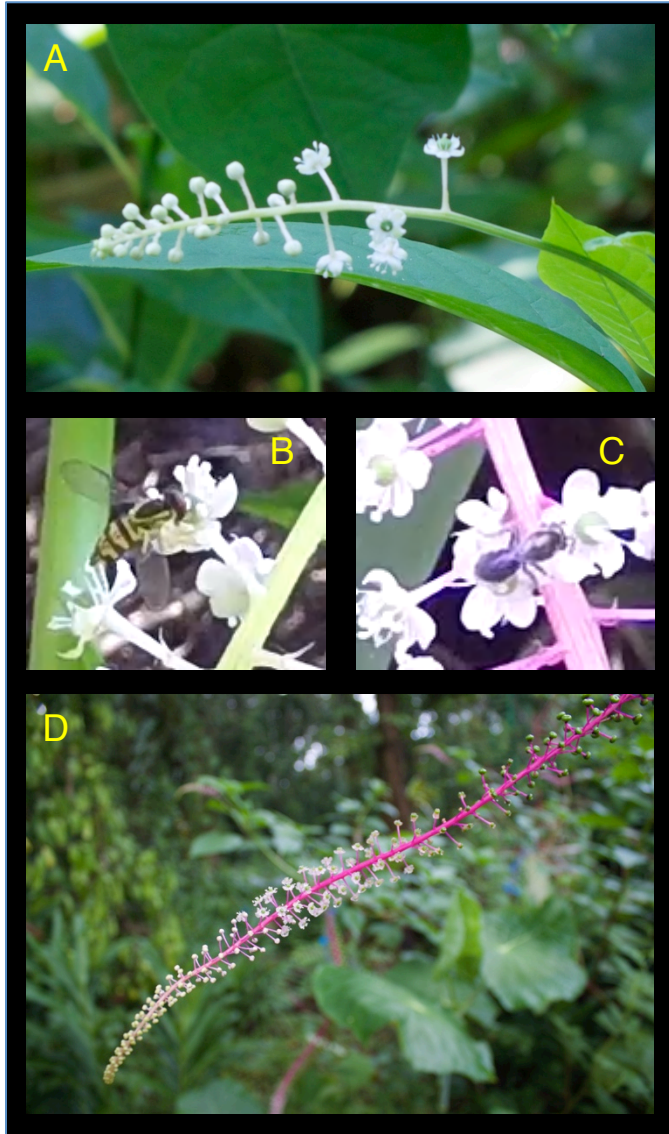


Figure 6. Inflorescences of *Phytolacca americana* (A,B) and *P. rivinoides* (C,D). A representative screenshot of video recordings used to estimate floral visitation rate is shown for each species (B-C).

The following details of analysis are common to all mixed effects models in the present study unless specified. We used the *lme* function in the *nlme* package in R version 3.3.3 (R Core Team 2014, Pinheiro et al. 2017) and obtained results using restricted maximum likelihood estimates. Variance was rarely homogeneous between populations, so we often estimated variance separately for each population (specified in each analysis). Models fit assumptions of

normally distributed residuals. Where there was a significant fixed effect of region for analyses with three regions, we used Tukey's HSD test to compare regional means ($\alpha = 0.05$).

Self-pollination in a common garden: tropical, subtropical, temperate

We evaluated self-pollination ability in the absence of pollinators (autogamy rate) for tropical, subtropical, and temperate plants grown together in a greenhouse common garden at Michigan State University (Table 5). *Phytolacca rivinoides* seeds were collected between Feb and Apr 2016. For *Phytolacca americana*, seeds were collected 2014-2015. Tropical populations are the same as those used for *in situ* pollinator video recordings, but two of the temperate populations are not (see Table 5).

Plants were germinated in summer 2016 and autogamy was estimated from Jan to Aug. For each population, we attempted to grow three plants from each of three "maternal lines" (seeds collected from one individual in the field), resulting in a total of 74 plants (22-27 per region). See Baskett and Schemske (2018) for husbandry details for this cohort of plants. We sampled up to three mature infructescences per individual and counted the number of total flowers and filled fruits (>50% swollen). Autogamy rate was calculated as the ratio of filled fruits versus flowers. Autogamy rate (individual means) was analyzed with a mixed effects model as a function of region, with maternal line nested in population as a random effect. Variance was estimated separately for each population.

Floral display and reward in a common garden: tropical, subtropical, temperate

To compare floral traits and their effects on pollinators both within and between species, in 2017 we planted a cohort of 81 plants in a greenhouse at Michigan State University. The experiment

consisted of three populations from tropical, subtropical, and temperate regions (Table 5), with three maternal lines per population. Most maternal lines (26 of 27) were the same as those used in the autogamy study. Potted plants were germinated in the greenhouse, moved temporarily outdoors to assess pollinator preference (see “Pollination in a common garden” below), and then returned to the greenhouse to measure nectar in the absence of pollinators and to avoid exposure to freezing temperatures.

Plant husbandry details follow Baskett and Schemske (2018), with the following exceptions: seeds were sown after acid treatment between Feb and May on Petri plates with 7% plain Phytoblend agar (Caisson Labs, Smithfield, UT, USA) and incubated at 28 °C 16 h days and 25 °C nights. Osmocote Plus was used according to the label when transplanting to 5 L pots, and a 3% 20-20-20 fertilizer solution (Peter’s) was applied during watering while plants were in the Michigan State University greenhouse. On June 28, plants were sprayed with fertilizer (On Gard, Verdanta OFE) and pesticide (Aria, Mainspring) to target a thrip outbreak. One maternal line was low on field-collected seeds, so we used seeds from a greenhouse-grown, self-pollinated plant; traits did not differ significantly from the two lines in that population grown directly from field-collected seeds (data not shown).

Several measurements of floral display were used to evaluate investment in pollinator attraction. For up to three flowering inflorescences per individual, we measured length (excluding bottom portion of peduncle before flowers) and diameter of three flowers. Flowers per inflorescence were counted on up to three inflorescences throughout the season, including the number of open flowers and the number of fresh flowers (defined as opening that day, with anthers intact and stigma lobes starting to spread). Individual means of all traits were used for analysis, because the number of measurements per individual varied.

Because our study accommodated asynchronous flowering times and a field and greenhouse component, we took measurement location (field or greenhouse) into account in analyses. Display size traits were measured during the field phase of the common garden for subtropical and temperate populations (Jul 11-Aug 1). Subtropical plants had just started flowering in the field, while temperate plants were at peak flowering. From Nov 11 to Jan 5, display size traits were measured in the greenhouse on subtropical and tropical populations at peak flowering, but we did not re-measure temperate plants in the greenhouse, as they had already finished flowering. There was evidence of genotype-by-environment effects on display size for subtropical plants, which were measured in both locations, so we analyzed regional effects on traits separately for each environment.

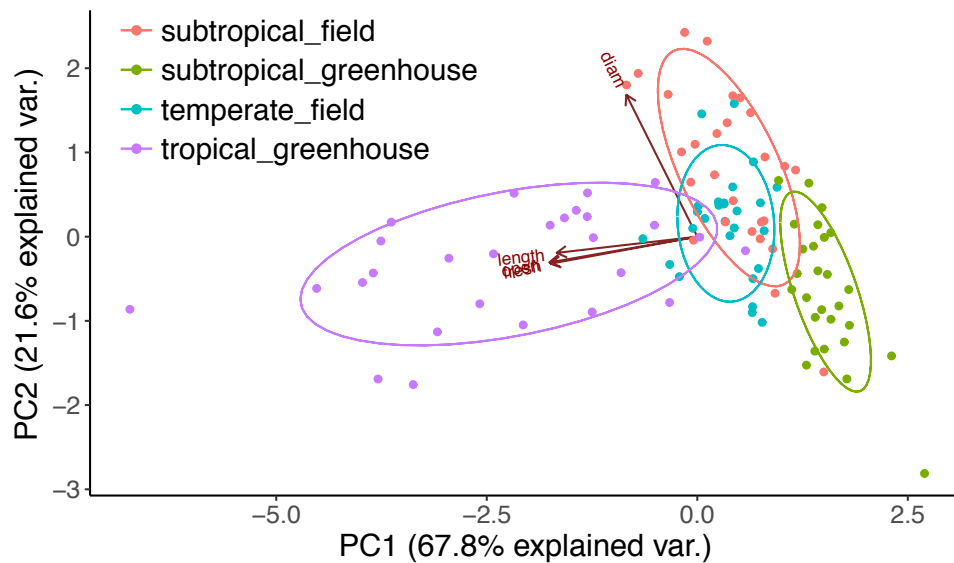


Figure 7. Principal components of floral display traits of *Phytolacca rivinoides* (tropical) and subtropical and temperate *P. americana* (populations A-C, D-F, and G-I in Fig. 5, respectively). Subtropical plants were measured in both the field and greenhouse and show an effect of environment and/or phenology; temperate plants were only measured in the field, and tropical plants only in the greenhouse. Traits were floral diameter (“diam”), inflorescence length (“length”), open flowers per inflorescence (“open”) and fresh flowers per inflorescence (“fresh”). The vectors for open and fresh flowers overlap.

Table 7. Loadings, proportion of variance explained, and Eigenvalues from principal components analysis of four floral display traits: inflorescence length, open and fresh flowers per inflorescence, and flower diameter. Traits were measured on three populations each of temperate and subtropical *Phytolacca americana* and tropical *P. rivinoides*. For each population, we measured three individuals from three maternal lines and analyzed mean values from multiple inflorescences per individual in a common garden. Temperate plants were measured in the field, subtropical plants were measured in the field and greenhouse (treated as separate measurements), and tropical plants were measured in the greenhouse, due to differences in flowering time.

	PC1	PC2	PC3	PC4
inflor. length	-0.5384	-0.1101	-0.8333	0.0602
open flowers	-0.5666	-0.1746	0.3363	-0.7317
fresh flowers	-0.5615	-0.1832	0.4361	0.6789
flower diameter	-0.2716	0.9612	0.0487	0.0034
Proportion of variance	0.6776	0.2163	0.0733	0.0329
Eigenvalue	2.7100	0.8652	0.2930	0.1315

All display size traits were significantly correlated with each other (mean Pearson's $r = 0.53$, range = 0.27—0.87), so we used Principal Components Analysis to reduce dimensionality. We used the *prcomp* function in the *stats* package on scaled and centered variables. The first PC was negatively correlated with inflorescence length and open and fresh flowers (68% variance explained, Eigenvalue = 2.7; Fig. 7, Table 7), and the second PC was highly positively correlated with flower diameter (22% variance explained, Eigenvalue = 0.9). These two axes were retained for further analysis.

We tested whether PC1 and PC2 for floral display size differed by region in each measurement location, using a mixed model with random effects of maternal line nested in population. Thus, for each PC, we compared tropical and subtropical plants measured in the greenhouse separately from subtropical and temperate plants measured in the field. For PC1 in the greenhouse, we estimated separate variance for each population.

To quantify nectar in the absence of visiting insects, we estimated nectar volume in the greenhouse (Table 5). Nectar was measured on three flowers per plant using 0.5 and 1 μL microcapillary tubes between 8:00 and 10:30 AM on up to three separate mornings per plant between Sep 7 and Dec 13 (median Sep 21). To access nectaries, the pistil was removed with forceps. We were able to measure nectar for 77 of the 81 plants (four temperate plants had finished flowering and were not measured). For analysis, we took the mean nectar volume of three flowers per measurement averaged across up to three replicate measurements per individual. Nectar volume was analyzed with a mixed-model ANOVA with a fixed effect of region and random effects of maternal line nested in population.

Pollinator attraction in a common garden: subtropical, temperate

To determine whether floral visitation depended on plant region of origin and floral traits, plants were observed in the field in a common garden in rural southwest Michigan (42.53621° N, -84.40528° W), using the same cohort as “Floral display in a common garden.” Only *P. americana* could be observed for floral visitors because *P. rivinoides* flowered later in the year. Floral visitors were observed between 9 AM and 5 PM from July 14 to July 27. The number of flowers visited by each insect was counted over a period of 15 minutes for blocks of 3-5 individual plants. A visit was counted if the insect contacted the stigma or anthers, and visitors were identified to morphospecies on the wing. Observer was constant throughout the experiment. Each plant was observed between 8 and 12 times; temperate plants overall were observed 262 times, and subtropical plants 273 times, for a total of 134 observer-hours.

To determine whether floral visitation depended on putative attraction and reward traits, we used data on field-measured display traits and greenhouse-measured nectar volume (see

“Floral display” and “Floral reward in a common garden”). For each individual, every 1-3 days we estimated the total number of flowers by multiplying the total number of inflorescences on that plant times mean flower number of three inflorescences. For analysis of visitation rates, we calculated mean visits per hour for each individual, and divided by the mean number of open flowers per individual to obtain an estimate of visits per flower per hour.

To test whether floral visitation rate depended on plant region of origin, we analyzed visits/flower/hour as a function of region (subtropical vs. temperate) using a mixed model with random effects of maternal line nested in population. Variance was estimated separately for each population to account for heteroscedasticity. To test whether floral traits are predictive of insect visitation, we modeled visits/flower/hour as a function of display size and nectar. We performed a Principal Components Analysis using individual plant means of display size traits to reduce dimensionality of display size, since traits were all significantly correlated with each other (mean Pearson’s $r = 0.65$, range = 0.52—0.78). Display size traits consisted of inflorescence length, flower diameter, inflorescence count, open flowers per inflorescence, and fresh flowers per inflorescence. The first PC was positively correlated with all traits, explained 72% of variation, and had an Eigenvalue of 3.5 (Table 8). The second PC had an Eigenvalue of 0.53, so only the first PC was retained for analysis of floral visitors. The principal component representing display size was significantly negatively correlated with nectar (Pearson’s $r = -0.564$, $p < 0.0001$), so models with both predictors had problems with multicollinearity. Thus, we used nectar and display size as predictors in separate models. Total visits were modeled as a function of display size PC1 or nectar volume with random effects of maternal line nested in population. Variance was estimated separately for each population to account for heteroscedasticity.

Table 8. Loadings, proportion of variance explained, and Eigenvalues from principal components analysis of five floral display traits: inflorescence length, open and fresh flowers per inflorescence, flower diameter, and inflorescence count. Traits were measured on three temperate and three subtropical populations of potted *Phytolacca americana* in a field common garden in Michigan, USA, and the first PC is used for analysis of traits correlated with floral visitation. For each population, we measured three individuals from three maternal lines and analyzed mean values from multiple inflorescences per individual.

	PC1	PC2	PC3	PC4	PC5
inflor. length	0.4372	0.5482	-0.5017	0.3156	0.3963
open flowers	0.4818	0.2678	-0.0537	-0.2046	-0.8071
fresh flowers	0.4559	0.1756	0.6201	-0.4605	0.4060
flower diameter	0.4181	-0.6725	-0.4759	-0.3531	0.1476
inflor. count	0.4405	-0.3804	0.3667	0.7223	-0.0708
Proportion of variance	0.7199	0.1083	0.0710	0.0670	0.0339
Eigenvalue	3.5990	0.5416	0.3548	0.3347	0.1696

Herbivory rate and herbivore abundance in situ: tropical, temperate

To compare pressure from chewing herbivores between tropical and temperate *Phytolacca*, we surveyed herbivory rates in tropical and temperate regions (Table 5; same populations as “Pollination rate *in situ*”). Young and mature leaves were marked and re-measured in order to capture complete consumption and standardize over time to compare to other studies (Coley 1983, Coley and Barone 1996, Anstett et al. 2016, Baskett and Schemske 2018). Herbivory rate was measured over three intervals in the tropics between Mar 4 and Jul 23, 2016; each interval was 38-47 days (median = 43), and the three intervals covered the end of the dry season, the dry-wet transition, and the beginning of the wet season. Herbivory rate was measured over two intervals in the temperate zone, due to its shorter growing season, between Jul 5 and Sep 29, 2017. Each interval was 40-43 days (median = 41), and the two intervals covered mid- and late summer, after the rapid early summer growth.

Herbivory rate (chewing damage) was measured following methods of Baskett and Schemske (2018), a separate study of herbivory rates along a latitudinal gradient in *P. americana*. In short, at the start of each interval, five mature and five young leaves were marked per plant for 20-30 plants per population, using colored bird bands and permanent marker. Initial percent damage was measured on mature leaves ($\text{consumed area}/\text{total leaf area} \times 100$), using a transparent plastic grid with 40.3 mm^2 squares. Young leaves were marked when buds were bursting, and had initial herbivory of zero. An average of 203 and 300 leaves per population per season were marked in tropical and temperate regions, respectively, for a total of 3,625 marked leaves. Final percent consumption was measured approximately 6 weeks later, after most young leaves had expanded. Due to mortality, at the end of each interval we were able to measure on average 18 plants in tropical and 29 plants in temperate regions per population per season.

To test whether herbivore pressure varies with region, we analyzed mean per-plant herbivory using a mixed model with fixed effects of region, leaf age, and their interaction, and random effects of individual nested in population, due to repeated measures over multiple seasons. Variance was estimated separately for each leaf age in each population due to heteroscedasticity. For clarity, we present results for final minus initial consumption, expressed as a percentage, because the interval over which we measured was similar for all populations. Analyzing herbivory as a daily rate does not qualitatively change results. We also asked whether there is seasonal variation in herbivory rates, following similar methods to analysis of floral visitation rates. Herbivory was analyzed as a function of season, leaf age, and their interaction; individual nested in population was a random effect.

Concurrent with herbivory surveys, we collected lepidopteran larvae in each population to estimate herbivore abundance. There were four censuses in each region: at the first leaf-

marking and last leaf-measurement, and two in between (3-6 week intervals). At each census, we searched the top and bottom surfaces of 3,000 leaves of all sizes per population, divided among the individuals surveyed for herbivory. Larvae were counted as morphospecies, and we attempted to rear representative specimens to adulthood for identification on a *Phytolacca* diet.

Herbivore abundance was \log_e+1 transformed for analysis to meet assumptions of normally distributed residuals. We removed singletons (morphospecies that were only collected once out of 36,000 leaves checked in each region), assuming they are not important consumers, which did not qualitatively change results. Total abundance from 3,000 leaves in each population at each census was analyzed using a mixed-effects model with population as a random effect to account for repeated measures of populations.

Leaf palatability in a common garden: tropical, subtropical, temperate

To compare latitudinal patterns in evolution of plant defense, we performed a bioassay using performance of *Spodoptera exigua* larvae (Lepidoptera, Noctuidae) as a metric of strength of plant defense. We reanalyzed palatability data from Baskett and Schemske (2018) for subtropical and temperate *P. americana* and included new data on three tropical populations of *P. rivinoides* (Table 5). Detailed methods for the palatability experiment are described in Baskett and Schemske (2018). In brief, neonate larvae were fed a no-choice diet of young or mature leaves of *P. americana* or *P. rivinoides* from a greenhouse common garden. Larvae were reared in cups (the experimental unit); each cup received leaves from multiple plants in one maternal line. For the mature-leaf treatment, cups began with 5 individuals, the experiment ran for 8 days, and there were 4 replicate cups per maternal line. Young leaf material was more limited, so cups began with 3 individuals and ran for 9 days, with 3 replicate cups per maternal line. We measured total

larval biomass of survivors per cup at the end, counting non-survivors as zero biomass to integrate survival and growth in one response. We report survivorship and growth separately in Supplemental Information.

For analysis, larval biomass per cup was \log_e+1 transformed because growth is exponential. After transformation, biomass was standardized by the number of starting caterpillars and the duration to compare leaf age treatments (5 individuals X 8 days for mature, 3 individuals X 9 days for young leaves). We analyzed larval biomass as a function of region, leaf age, and their interaction (tropical, subtropical, temperate), with random effects of maternal line nested in population. Variance was estimated separately for each leaf age in each population.

Results

Floral visitation rate in situ: tropical, temperate

Overall, we recorded 177 usable hours of video from 97 plants, resulting in 104 data points in tropical and 62 in temperate populations. During the last temperate observation block, one population was no longer flowering and another had only one inflorescence, but there was no seasonal variation in visitation in either region (Supplemental Information). Tropical *P. rivinoides* flowers were visited at a 13-fold higher rate than temperate *P. americana* flowers ($F_{1,4} = 51.65, p = 0.0020$; Fig. 8). There was no significant effect of floral display size (number of open flowers) on visits per flower ($F_{1,68} = 2.17, p = 0.1454$).

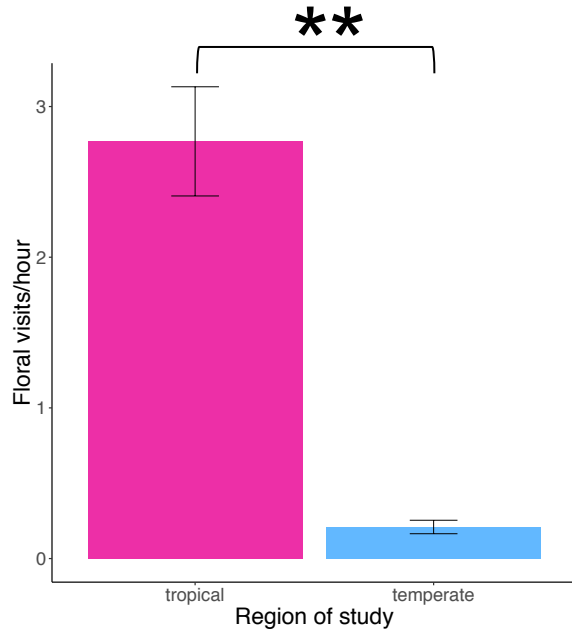


Figure 8. Insect visitation rates per flower per hour *in situ* for three populations each of tropical *Phytolacca rivinoides* (pink) versus temperate *P. americana* (blue; Table 5). Bars are raw means \pm SE, although the response was \log_e+1 transformed for analysis; asterisk indicates significant difference in means ($p = 0.002$).

Visitors to *P. americana* and *P. rivinoides* included small bees (especially *Lasioglossum*, but also *Augochlora*, *Augochlorella*, *Ceratina*, and *Trigona*), Vespidae (*Parancistrocerus*, *Stenodynerus*), Syrphidae, other Diptera (Tephritidae), and Coleoptera (Curculionidae, Chrysomelidae). Twenty-four *P. americana* visitors were collected in the common garden and at other sites in southeast Michigan for morphospecies identification purposes. Pollen loads were examined by T. Wood and compared to a reference library. Bees were the only visitors with *P. americana* pollen on their bodies (5 of 10 bees). Thirty-three *P. rivinoides* visitors were collected for identification purposes, and again bees were the only visitors with *P. rivinoides* pollen on their bodies (13 of 27 bees). There was no significant seasonal variation in floral visitation rates in either the tropics ($F_{2,59} = 0.88$, $p = 0.4212$) or temperate zone ($F_{2,6} = 4.51$, $p = 0.0638$).

Self-pollination in a common garden: tropical, subtropical, temperate

Phytolacca americana populations from both subtropical and temperate regions had a high autogamy rate, with 91% of possible fruit set. This was 2.6-fold higher than the 36% autogamy rate of *P. rivinoides* ($F_{2,6} = 96.76$, $p < 0.0001$; Fig. 9). In April, we observed a small number of wasps (unidentified Vespidae) in the greenhouse despite freezing temperatures outside, suggesting that pollinator exclusion was not completely effective. Thus, for both species, rates may be slightly overestimated because of incomplete pollinator exclusion; autogamy was 7% higher in this study than in preliminary studies of *P. americana* with complete exclusion (data not shown).

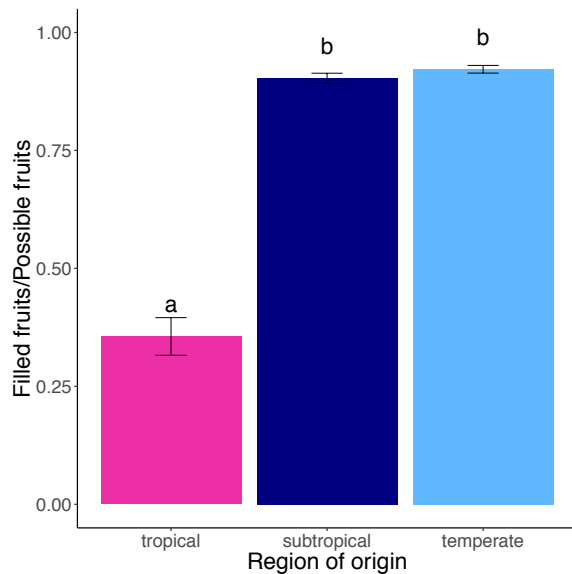


Figure 9. Autogamy rate (filled/possible fruits) for *Phytolacca* in a greenhouse nearly absent of pollinators. *Phytolacca rivinoides* is pink, and *P. americana* is dark and light blue; each region was represented by three populations (Table 5). Bars are least-square means \pm SE, and significant differences between regions are indicated with different letters.

Floral display and reward in a common garden: tropical, subtropical, temperate

Region of origin had strong effects on floral display size in each experimental location for PC1 (negatively correlated with inflorescence length and flowers per inflorescence; Fig. 7). Measured in the greenhouse, tropical *P. rivinoides* had 804% larger floral displays than subtropical *P.*

americana ($F_{1,4} = 86.23, p = 0.0007$). Measured in the field, temperate *P. americana* had 306% larger floral displays (PC1) than subtropical *P. americana* ($F_{1,4} = 35.78, p = 0.0039$). For PC2 (positively correlated with flower diameter; Fig. 7), there was no difference between tropical and subtropical populations ($F_{1,4} = 4.48, p = 0.1017$; Fig. 10). Temperate *P. americana* had 226% wider flowers than subtropical populations ($F_{1,4} = 9.24, p = 0.0384$; Fig. 10). Due to GxE effects (e.g., subtropical *P. americana* populations measured in the greenhouse had 77% larger displays than when the same plants were measured earlier in the field), and flowering phenology mismatches that precluded measuring all populations in the same environment, we could not confidently compare temperate and tropical populations.

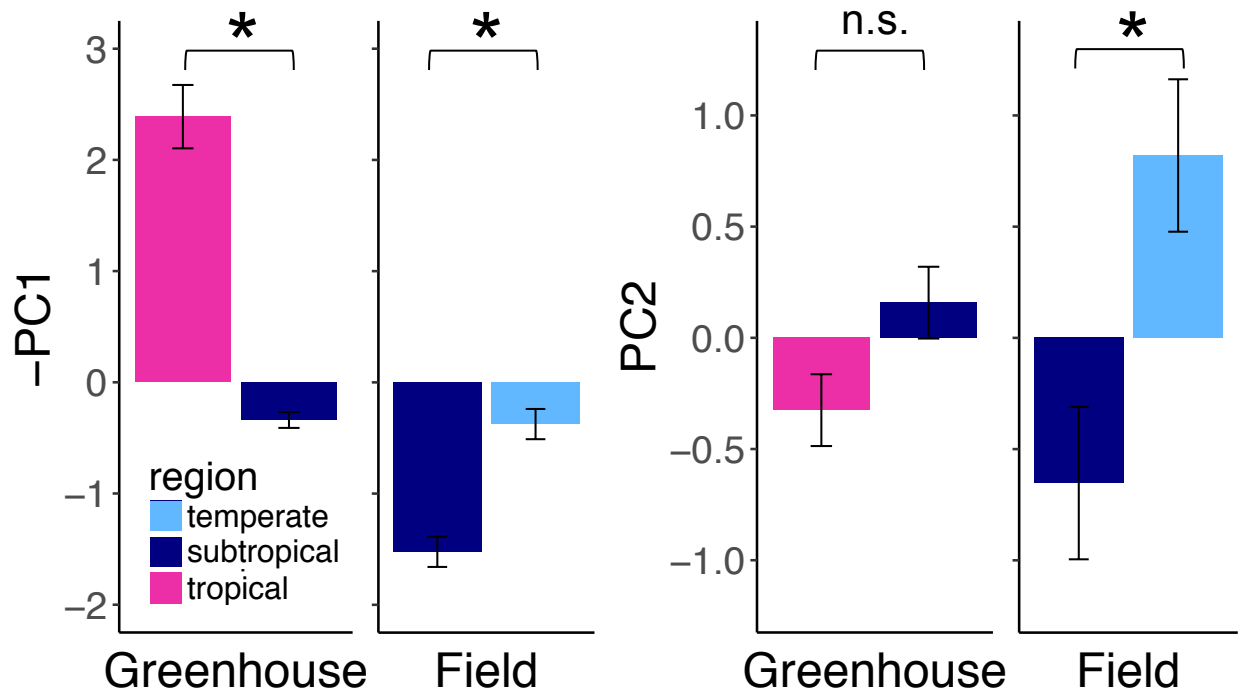


Figure 10. Comparison of PC1 (A) and PC2 (B) between region of origin and place of measurement. See Fig. 7 and Table 7 for PC loadings. -PC1 is shown because PC1 was negatively correlated with flower size. All plants experienced the same environments at the same times, but due to differences in phenology, measurements were made at different times and places during the common garden experiment. Tropical *Phytolacca rivinoides* was only measured in the greenhouse, temperate *P. americana* was only measured in a field common garden, and subtropical *P. americana* was measured in both locations. Bars are least-square means \pm SE, and asterisks indicate significant differences for comparisons in each measurement location ($p < 0.05$).

The tropical and subtropical plants produced twice as much nectar per flower than temperate plants ($F_{2,6} = 19.63$, $p = 0.0023$; Fig. 11A).

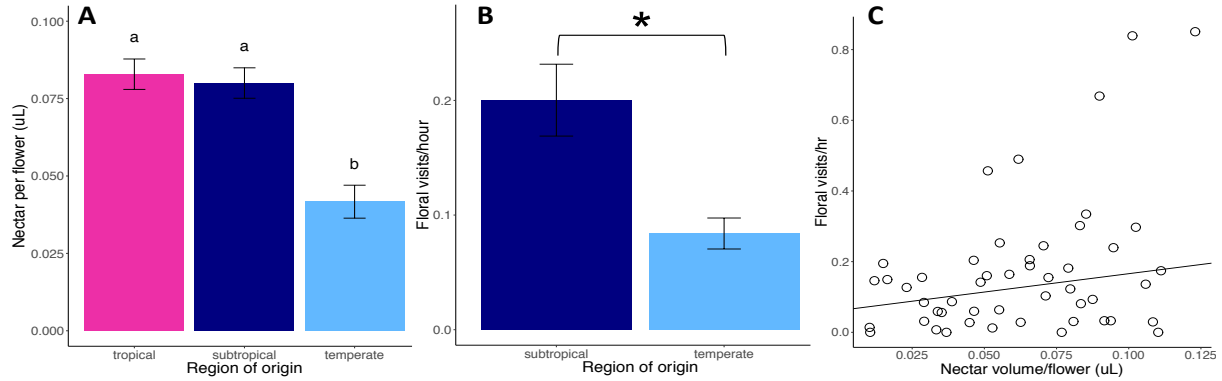


Figure 11. Regional differences in nectar production and floral visitation in a common garden, and the correlation between nectar and visitation. (A) Floral nectar volume of *Phytolacca rivinoides* (pink) and *P. americana* (dark and light blue). Nectar was quantified in the greenhouse, in the absence of pollinators, for three populations per region (Table 5). (B) Floral visitation rate of subtropical and temperate populations of *P. americana* in a common garden in Michigan, USA. (C) Nectar volume is a significant predictor of floral visitation rate in the common garden. Bars are least-square means \pm SE. Bars with different letters are significantly different (A), and the asterisk indicates a significant difference between regions in (B). Points in (C) are individual means.

Pollinator attraction in a common garden: subtropical, temperate

Although the subtropical populations had significantly smaller floral displays than temperate populations in the field common garden, subtropical plants received 2.4 times more visits per flower per hour ($F_{1,4} = 11.61$, $p = 0.0271$; Fig. 11B). Visitation to subtropical flowers (mean \pm SE: 0.20 ± 0.03) was similar to the rates we observed in videos of three natural populations of temperate *P. americana* (0.21 ± 0.04). Nectar volume was positively correlated with floral visitation rate ($F_{1,31} = 4.27$, $p = 0.0473$; Fig. 11C), but nectar is also confounded with region, so an unmeasured trait could be driving this pattern. PC1 axis of floral display traits was not a significant predictor of visitation ($F_{1,35} = 2.55$, $p = 0.1195$; Fig. 12).

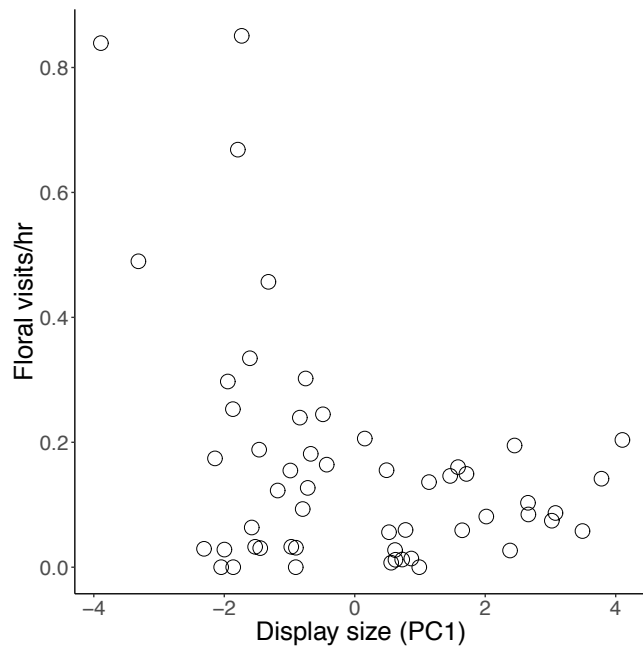


Figure 12. Display size in the field common garden (PC1 axis of inflorescence count, inflorescence length, total open flowers, fresh flowers, and flower diameter) is not a significant predictor of floral visitation rate.

Herbivory rate and herbivore abundance in situ: tropical, temperate

Herbivory in the field depended on both region and leaf age (Table 9, Fig. 13). Young tropical leaves were consumed at 4.8-fold the rate of young temperate leaves. An even larger effect was observed for mature leaves, which were consumed at a 11-fold higher rate in the tropics versus temperate populations, where consumption was essentially zero. In both regions, young leaves were consumed at a higher rate than mature leaves: a 2.3-fold difference in the tropics and a 5.4-fold difference in the temperate region. Tropical populations experienced a 78% increase in herbivory from dry to wet season on young leaves, but no seasonal herbivory changes for mature leaves (Table 10, Fig. 14). There was no change in herbivory from mid- to late summer in the temperate zone (Table 10). Daily herbivory rates for each population at each interval are provided in Appendix (Table 11).

Table 9. Effects of region and leaf age (young or mature) on herbivory and palatability. Herbivory (percent leaf area consumed over ~42 days) was measured in three populations in each region over three intervals in tropical *Phytolacca rivinoides* and two in temperate *P. americana*. Palatability (biomass of *Spodoptera exigua* reared on a diet of *Phytolacca* leaves) was measured for three populations in each of three regions: tropical (*P. rivinoides*), subtropical, and temperate (*P. americana*).

Fixed effect	Herbivory in field			Palatability in lab		
	d.f.	<i>F</i>	<i>p</i>	d.f.	<i>F</i>	<i>p</i>
Region	1,4	11.88	0.0261	2,6	27.00	0.0010
Leaf age	1,507	39.76	< 0.0001	1,239	55.18	< 0.0001
Region X Age	1,507	23.20	< 0.0001	2,239	13.15	< 0.0001

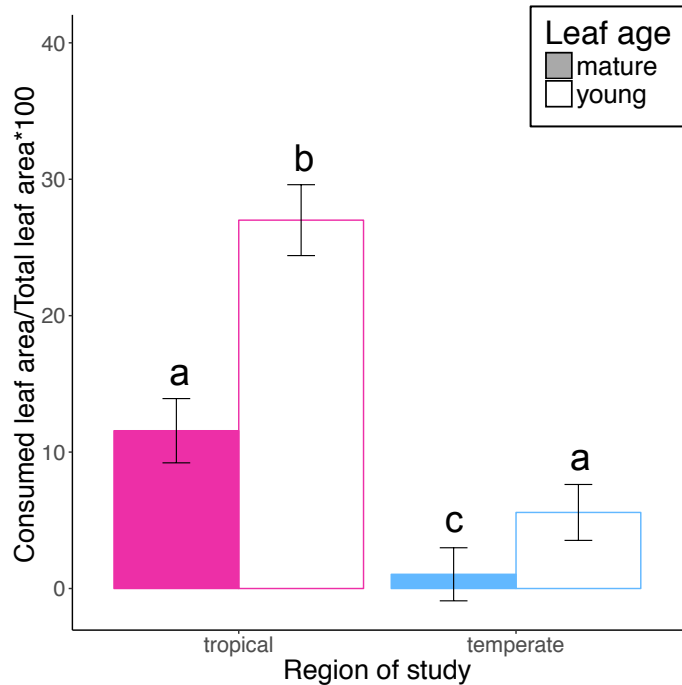


Figure 13. Percent leaf area consumed of young and mature leaves in three populations each of tropical *Phytolacca rivinoides* (pink) and temperate *P. americana* (blue; see Table 5). Herbivory was surveyed by marking and re-measuring leaves ~42 days later over three intervals in the tropics (4.5 months; dry season, dry-wet transition, and wet season) and two in the temperate zone (3 months; mid- and late summer). We marked 3,625 leaves (five of each age per plant at each interval) and analyzed individual means of each leaf age in each interval ($N = 674$ after mortality). Bars are least-square means \pm SE, and those that share the same letter are not significantly different according to Tukey's HSD test.

Table 10. Effects of season and leaf age (young or mature) on herbivory (percent leaf area consumed over ~42 days). Three populations in each region were surveyed over three seasons in the tropics for *Phytolacca rivinoides* (dry, dry-wet transition, and wet; Mar 4-Jul 23, 2016) and two in the temperate zone for *P. americana* (mid- and late summer; Jul 5-Sep 29, 2017). Plant means of 5 marked leaves of each age were analyzed using a mixed model with random effects of individual nested in population. See Fig. 14.

Fixed effect	Tropical			Temperate		
	d.f.	<i>F</i>	<i>p</i>	d.f.	<i>F</i>	<i>p</i>
Season	2,244	0.28	0.7559	1,257	3.54	0.0611
Leaf age	1,244	6.56	0.0110	1,257	17.80	<0.0001
Season X Age	2,244	3.84	0.0229	1,257	0.44	0.5056

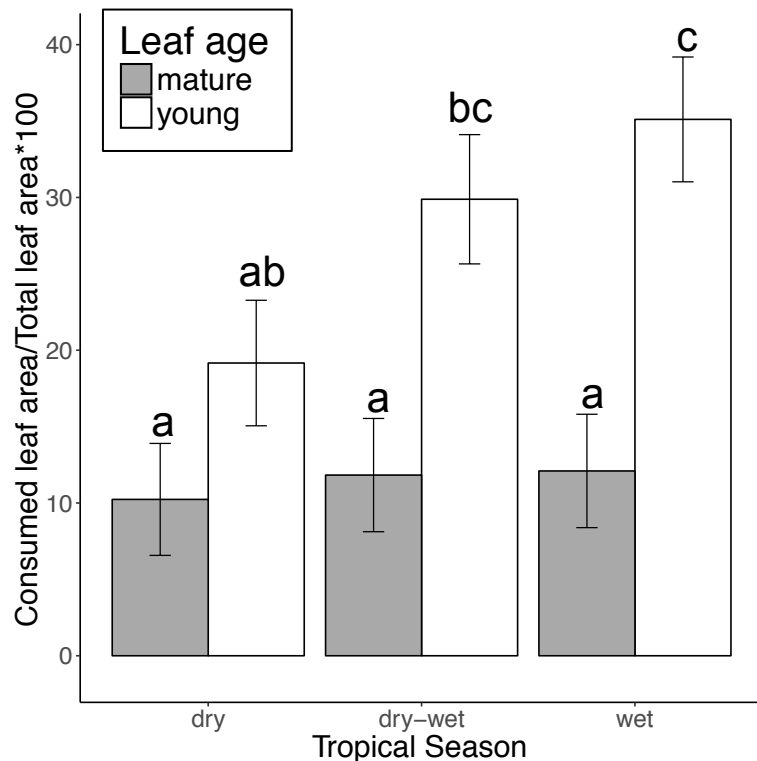


Figure 14. Percent leaf area consumed of young and mature leaves in three populations of *Phytolacca rivinoides* in Costa Rica over three seasons. Herbivory was surveyed by marking and re-measuring leaves ~42 days later in three intervals (Mar 4-Jul 23; dry season, dry-wet transition, and wet season). We marked 1,825 leaves (five of each age per plant at each interval) and analyzed individual means ($N = 324$ plants after mortality). Bars are least-square means \pm SE, and those that share the same letter are not significantly different according to Tukey's HSD test.

Lepidopteran larval abundance (per 3000 leaves) was 42-fold higher in tropical than temperate populations ($F_{1,4} = 12.26$, $p = 0.0249$, Fig. 15). Seven species were observed in the tropics (165 individuals). The most common (61% of observed larvae) was a Crambidae species found consuming and sheltering in young leaves (possibly *Maracayia chlorisalis*), and the second-most common (19%) was *Disclisioprocta stellata* (Geometridae). These are similar (confamilials/conspecifics) to the two common consumers of *P. americana* that we have observed in the eastern USA (Baskett and Schemske 2018). Two species, each at 6-7% abundance, were never reared to adulthood; one is likely either Crambidae or Tortricidae, and the other is unknown. Two Tortricidae moths and a Noctuid (possibly *Tiracola grandirena*) were each found at 1-4% relative abundance. A Noctuid and Tortricid species were also found in low abundance on *P. americana* in a previous study (Baskett and Schemske 2018).

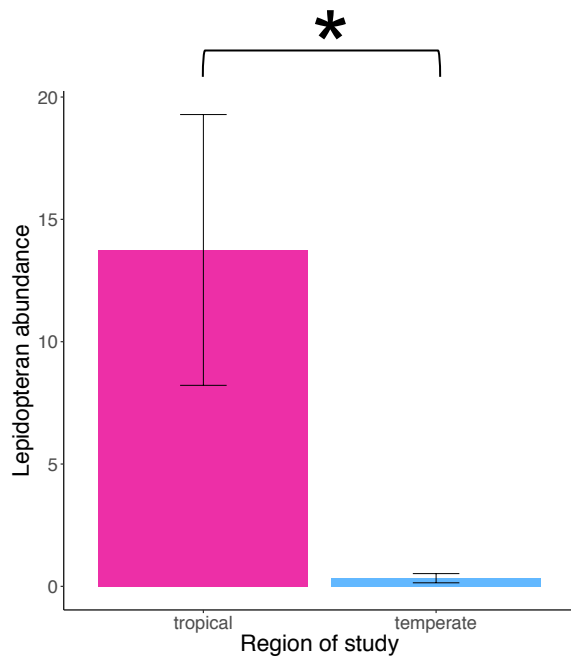


Figure 15. Abundance of lepidopteran larvae in three populations each of *Phytolacca rivinoides* (pink) and *P. americana* (blue). Four censuses of 3000 leaves were conducted in each population. Bars are raw census means \pm SE, although the response was \log_e+1 transformed for analysis; asterisk indicates significant difference in means ($p = 0.02$).

We only found four individuals of two morphospecies after surveying 36,000 total leaves in three populations in the temperate zone. None survived to adulthood. This is a lower abundance than in 2015, when we observed seven individuals of *Argyrotaenia velutinana* upon surveying 6,000 leaves in one of the temperate populations used in the present study (Baskett and Schemske 2018). Even if the 2015 north-temperate abundance is extrapolated, tropical populations still had four-fold the number of larvae.

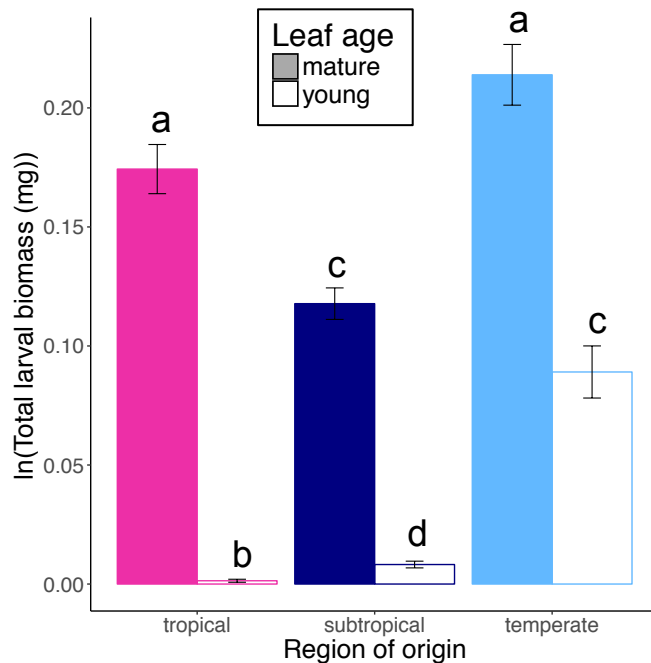


Figure 16. Biomass of larval *Spodoptera exigua* reared on a no-choice diet of young or mature leaves of greenhouse-grown *Phytolacca rivinoides* (pink) and *P. americana* (dark and light blue). Three populations per region were assayed (Table 5). Bars are least-square means \pm SE. Biomass was measured per cup (non-survivors included as zero mass) and standardized by initial larvae number and experiment duration after natural log transformation (see text). Bars that share the same letter are not significantly different, according to Tukey's HSD.

Leaf palatability in a common garden: tropical, subtropical, temperate

In the palatability experiment, caterpillar biomass (*S. exigua*) depended on the interaction of region and leaf age (Table 9, Fig. 16). Larvae grew significantly larger on a diet of mature versus

young leaves within all regions. The tropical young leaf treatment caused high mortality and the poorest larval performance, with 28% of the biomass attained on a diet of subtropical young leaves. In turn, larvae eating subtropical young leaves had only 9% of the biomass of those eating temperate young leaves. Regional patterns for mature leaves differed from young leaves; the subtropical treatment had the lowest biomass, 15% of that on a tropical or temperate diet.

Discussion

A latitudinal trend in the strength of species interactions has been long hypothesized to drive patterns of trait evolution, since Dobzhansky speculated that “the challenges of tropical environments stem chiefly from the intricate mutual relationships among the inhabitants” (1950). However, few studies have provided in-depth comparisons of the strength of herbivory across a temperate-tropical range, and no work has evaluated pollination at this scale. Here, we quantify multiple metrics of interaction strength across a large latitudinal range using the temperate herb *Phytolacca americana* and its tropical congener *P. rivinoides*. Consistent with the biotic interactions hypothesis, nearly all measures of pollination and herbivory show evidence of greater importance of biotic interactions in the tropical *P. rivinoides* than north-temperate *P. americana*: pollination, autogamy, nectar, herbivory rate, herbivore abundance, and palatability. Within *P. americana*, we find evidence that herbivore pressure and defense are stronger at lower latitudes (Baskett and Schemske 2018). Similarly, we found some evidence that subtropical populations of *P. americana* rely more on pollinators than temperate populations (for nectar volume and attraction in a common garden, but not autogamy or display size). Agreement among several metrics of mutualistic and antagonistic interactions bolsters our conclusion that

Phytolacca experiences greater contemporary interaction strength at lower latitudes, and has evolved traits consistent with a history of stronger interactions at lower latitudes.

At the largest scale of our study, comparing tropical *P. rivinoides* (10° N) to temperate *P. americana* (41° N), all but one metric of plant-herbivore and plant-pollinator interaction strength that we have measured support the biotic interactions hypothesis (mature-leaf palatability, equivalent between the two regions, is the exception). The data from subtropical *P. americana* (around 28° N) are less complete (e.g., no pollination in the field), but the available data demonstrate a variety of patterns in relation to temperate and tropical populations. This variation suggests that the shape of latitudinal patterns depends on interactions or traits, perhaps in response to biotic or abiotic thresholds. For example, there is a threshold in herbivory along a latitudinal gradient in *P. americana* at 35° N, and a threshold in palatability at 30° N (Baskett and Schemske 2018). Gene flow within species and a lack of strong selection on some traits could produce patterns such as that seen for autogamy (Fig. 9), whereas interspecific differences in life history could mediate the patterns seen for mature-leaf palatability (Fig. 16). More studies spanning tropical and subtropical regions are needed to clarify their latitudinal patterns and identify putative abiotic mechanisms underlying geographic variation in interactions. We suggest that single-species studies could be useful for focusing on these regional differences, for tighter control of history and ecology than is possible with congeners. We are aware of only one example: a comparison of herbivory in three sites in the mangrove *Rhizophora mangle*; higher folivory was observed in Florida than Belize, and Panama did not significantly differ from either site (Feller et al. 2013).

To our knowledge, all latitudinal comparisons of plant-pollinator interactions thus far have been at the community level. The prevalence of animal pollination increases toward the

tropics (Regal 1982, Rech et al. 2016). For animal-pollinated plants, outcrossing rate increases toward the equator, but this pattern is driven by latitudinal variation in life histories (greater prevalence of herbaceous taxa at higher latitudes (Moeller et al. 2017)). Pollen limitation is greatest in species-rich communities, which is thought to be a signature of greater competition for pollinators (Vamosi et al. 2006). Interaction specialization has also been considered an index of interaction importance, and patterns of specialization in plant-pollinator interactions remains unresolved (Olesen and Jordano 2002, Kay and Schemske 2004, Dalsgaard et al. 2011, Ollerton 2012, Pauw and Stanway 2015). Ours is the first comparison of plant-pollinator interaction strength within a species or genus. Consistent with the biotic interactions hypothesis, we found that the tropical *P. rivinoides* is visited at higher rates by insects in nature, has poorer self-pollination ability, and invests in more nectar than temperate *P. americana*. These pronounced interspecific differences contrast with intraspecific comparisons of *P. americana* populations at the northern and southern edge of the range, which found mixed evidence for regional differentiation in plant-pollinator interactions. Subtropical populations produce more nectar, which is correlated with greater visitation rates to subtropical plants in a common garden. A similar pattern (greater visitation to southern plants, which had more nectar) was also observed in *Gelsemium semperivens* in common gardens at each end of a much smaller latitudinal range in Georgia, USA (32.4° N – 33.9° N) (Adler et al. 2016). However, *P. americana* is capable of very high rates of self-pollination in both regions, consistent with its weedy, gap-specialized ecology (Sauer 1952, Pannell et al. 2015). Future studies should clarify the importance of pollinators to subtropical populations by measuring *in situ* visitation and comparing outcrossing rates between regions.

In contrast to pollination, latitudinal patterns in herbivory and plant defense have been studied using both community- and species-level approaches, and evidence is mixed (Moles et al. 2011), possibly due to inconsistencies and biases in commonly used methods, such as a lack of damage estimates on young leaves (Anstett et al. 2016, Baskett and Schemske 2018). We found evidence of stronger current herbivore pressure (herbivory rate and herbivore abundance) in tropical versus temperate plants, consistent with the biotic interactions hypothesis. For comparison, herbivory on *P. rivinoides* is remarkably similar to that measured across 20 other gap specialist species in 1979 in Panama by Coley (1983) (Appendix: Table 12), and in 2014 we found similar rates of herbivory on young and mature *P. americana* leaves in another Michigan population (Baskett and Schemske 2018). Consistent with previous comparisons of herbivory on different leaf ages, we find that young leaves experience significantly greater herbivory (Coley 1983, Lowman 1984, Filip et al. 1995, Baskett and Schemske 2018). Unlike Baskett and Schemske (2018), we did not find that young leaves showed stronger latitudinal patterns than mature leaves, due to near-zero consumption of mature leaves in Michigan in the present study. In addition, we confirmed previous findings that wet-season herbivory is greater than dry-season herbivory in the tropics (Coley 1983, Aide 1993, Coley and Barone 1996). We lack comparable data of herbivory across the growing season in the subtropics. However, previously we observed approximately three-fold higher herbivory rates on both young and mature leaves of *P. americana* in late summer in the southern US (Florida and Georgia) compared to Costa Rican *P. rivinoides* in the present study (Baskett and Schemske 2018). Further work is needed to understand whether this observation is actually inconsistent with the biotic interactions hypothesis: we lack sufficient data across entire growing seasons to estimate annual herbivory

rates in either region, and we lack data on fitness effects of herbivory in *Phytolacca* (Lehndal and Agren 2015, Anstett et al. 2016).

Plant defense, a metric of how plants have evolved in response to herbivore history, provides evolutionary context for interpreting latitudinal patterns in current-day herbivore pressure (Salgado and Pennings 2005, Woods et al. 2012, Kim 2014, Anstett et al. 2015, Lehndal and Agren 2015). We found that young *P. rivinoides* leaves were highly unpalatable to the generalist *S. exigua*, even more so than unpalatable young subtropical *P. americana* leaves. Despite uncertainty about how annual herbivory rates compare between these two regions, differences in palatability provide evidence that herbivore pressure on young leaves in *Phytolacca* has indeed been very strong in the tropics. We find that mature leaves are more palatable than young leaves in all populations, similar to Alba et al. (2014) in *Verbascum*, likely due to chemical defenses in young leaves that are unpalatable to generalists. Compared to temperate *P. americana*, mature *P. rivinoides* leaves are equally palatable but consumed at higher rates in the field, which is clear evidence of greater herbivore pressure in the tropics. But curiously, compared to subtropical *P. americana*, mature *P. rivinoides* leaves are neither unpalatable nor consumed at high rates in the field during the seasons we have surveyed thus far. It is unclear why mature leaves show different latitudinal patterns than young leaves; performing palatability trials with other species could clarify the generality of plant defense patterns for both leaf ages.

A strength of our study was that it was able to include multiple in-depth metrics of plant-insect interactions, but a drawback of this design is that it is limited in phylogenetic scope. Because two-species comparisons suffer from a lack of statistical power to form strong conclusions about adaptation (Garland and Adolph 1994), a natural follow up would be to

replicate our approach across several other congeneric species pairs to test for generality of the results. Potential New World herbaceous genera for this type of study include *Asclepias*, *Cuphea*, *Desmodium*, *Ipomoea*, *Mirabilis*, *Nicotiana*, *Oxalis*, *Passiflora*, *Physalis*, *Ruellia*, *Sida*, and *Solanum*. Regardless, for a spatial scale larger than the range of any one species, ours is the most in-depth study of biotic interaction strength with the best available phylogenetic control; contrasting with recent claims of little support (Moles et al. 2011, Moles 2013, Moles and Ollerton 2016), we find strong evidence for the biotic interactions hypothesis.

Acknowledgements

Assistance with identification of floral visitors and their pollen loads was kindly provided by Thomas Wood (Michigan State University). We thank Olbert and Susana Mesen, Juan Manuel Ley at Tirimbina Biological Reserve, Erica Andrews and Matt Hogan at Finca Bellavista, the Grieb family, and Doug Schemske allowing data collection on their properties. Reinaldo Aguilar, Emma Clark, Paul Foster of Reserva Ecológica Bijagual, Carolyn Graham, Keegan Mackin, Glenda Mesen, Reyder Mesen, Abby Sulesky, Alyssa Tretheway, and Andie Veach assisted substantially with fieldwork, data collection, or finding plant populations. The Weber and Wetzel labs provided invaluable feedback on analyses and drafts. Research funding was provided by an Organization for Tropical Studies Research Fellowship (Emily Foster Fellowship) and support from the Michigan State University Distinguished Fellowship.

APPENDIX

APPENDIX

Table 11. Daily herbivory rates (percent leaf area consumed per day) for each population in each season. See Table 6 for population key.

Region	Season	Population	Leaf age	Per-day herbivory
temperate	late summer	K	mature	0.0369
			young	0.1149
		I	mature	0.0137
			young	0.1097
		J	mature	0.0626
			young	0.2428
	mid-summer	K	mature	0.0120
			young	0.0568
		I	mature	0.0069
			young	0.1568
		J	mature	0.0138
			young	0.1731
tropical	dry	B	mature	0.6231
			young	0.4423
		A	mature	0.0810
			young	0.3554
		C	mature	0.1480
			young	0.5456
	dry-wet	B	mature	0.3595
			young	0.5984
		A	mature	0.1928
			young	0.4736
		C	mature	0.3012
			young	1.1509
	wet	B	mature	0.2165
			young	0.6626
		A	mature	0.1527
			young	0.5997
		C	mature	0.4431
			young	1.2781

Table 12. Mean percent leaf area consumed per day by leaf age in dry and wet seasons in Central America. “Gap spp.” = means of 20 gap specialists, not including *P. rivinoides*, measured in Panama by Coley in 1979, from Table 5 in Coley (1983). “*P. riv.*” = *Phytolacca rivinoides* in three populations in Costa Rica in 2016, from this study.

Season	Leaf age	Gap spp. 1979	<i>P. riv.</i> 2016
dry	young	0.46	0.44
	mature	0.13	0.29
wet	young	0.89	0.85
	mature	0.28	0.27

REFERENCES

REFERENCES

- Adams, J. M., Y. J. Zhang, M. Basri and N. Shukor (2009). "Do tropical forest leaves suffer more insect herbivory? A comparison of tropical versus temperate herbivory, estimated from leaf litter." Ecological Research **24**(6): 1381-1392.
- Adler, L. S., L. M. Leege and R. E. Irwin (2016). "Geographic variation in resistance to nectar robbing and consequences for pollination." American Journal of Botany **103**(10): 1819-1828.
- Agrawal, A. A. and M. G. Weber (2015). "On the study of plant defence and herbivory using comparative approaches: how important are secondary plant compounds." Ecology Letters **18**(10): 985-991.
- Aide, T. M. (1993). "Patterns of leaf development and herbivory in a tropical understory community." Ecology **74**(2): 455-466.
- Alba, C., M. D. Bowers, D. Blumenthal and R. A. Hufbauer (2014). "Chemical and Mechanical Defenses Vary among Maternal Lines and Leaf Ages in *Verbascum thapsus* L. (Scrophulariaceae) and Reduce Palatability to a Generalist Insect." Plos One **9**(8).
- Ali, M. A., J. Lee, S.-Y. Kim, S.-H. Park and F. M. A. Al-Hemaid (2015). "Molecular phylogenetic analyses of internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA indicate monophyly of the genus *Phytolacca* L. (Phytolaccaceae)." Bangladesh Journal of Plant Taxonomy **22**(1): 1-8.
- Anstett, D. N., J. R. Ahern, J. Glinos, N. Nawar, J.-P. Salminen and M. T. J. Johnson (2015). "Can genetically based clines in plant defence explain greater herbivory at higher latitudes?" Ecology Letters **18**(12): 1376-1386.
- Anstett, D. N., I. Naujokaitis-Lewis and M. T. J. Johnson (2014). "Latitudinal gradients in herbivory on *Oenothera biennis* vary according to herbivore guild and specialization." Ecology **95**(10): 2915-2923.
- Anstett, D. N., K. A. Nunes, C. A. Baskett and P. M. Kotanen (2016). "Sources of controversy surrounding latitudinal patterns in herbivory and defense." Trends in Ecology & Evolution **31**(10): 789-802.
- Arnold, A. E. and F. Lutzoni (2007). "Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots?" Ecology **88**(3): 541-549.
- Baskett, C. A. and D. W. Schemske (2018). "Latitudinal patterns of herbivore pressure in a temperate herb support the biotic interactions hypothesis." Ecology Letters **21**(4): 578-587.
- Coley, P. D. (1983). "Herbivory and defensive characteristics of tree species in a lowland tropical forest." Ecological Monographs **53**(2): 209-233.

- Coley, P. D. and T. M. Aide (1991). Comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. P. W. Price, T. M. Lewinsohn, G. W. Fernandes and W. W. Benson. New York, USA, John Wiley and Sons: 25-49.
- Coley, P. D. and J. A. Barone (1996). "Herbivory and plant defenses in tropical forests." Annual Review of Ecology and Systematics **27**: 305-335.
- Dalsgaard, B., E. Magard, J. Fjeldsa, A. M. M. Gonzalez, C. Rahbek, J. M. Olesen, J. Ollerton, R. Alarcon, A. C. Araujo, P. A. Cotton, C. Lara, C. G. Machado, I. Sazima, M. Sazima, A. Timmermann, S. Watts, B. Sandel, W. J. Sutherland and J. C. Svenning (2011). "Specialization in Plant-Hummingbird Networks Is Associated with Species Richness, Contemporary Precipitation and Quaternary Climate-Change Velocity." Plos One **6**(10): 7.
- Davidson, D. W. and D. McKey (1993). "The evolutionary ecology of symbiotic ant-plant relationships." Journal of Hymenoptera Research **2**: 13-83.
- Dobzhansky, T. (1950). "Evolution in the tropics." American Scientist **38**: 209-221.
- Fassett, N. C. and J. D. Sauer (1950). "Studies of variation in the weed genus *Phytolacca*. 1. Hybridizing species in northeastern Colombia." Evolution **4**(4): 332-339.
- Feller, I. C., A. H. Chamberlain, C. Piou, S. Chapman and C. E. Lovelock (2013). "Latitudinal patterns of herbivory in mangrove forests: Consequences of nutrient over-enrichment." Ecosystems **16**(7): 1203-1215.
- Filip, V., R. Dirzo, J. M. Maass and J. Sarukhan (1995). "Within-year and among-year variation in the levels of herbivory on the foliage of trees from a Mexican tropical deciduous forest." Biotropica **27**(1): 78-86.
- Fischer, A. G. (1960). "Latitudinal variation in organic diversity." Evolution **14**: 64-81.
- Garland, T. and S. C. Adolph (1994). "Why not to do two-species comparative studies: Limitations on inferring adaptation." Physiological Zoology **67**(4): 797-828.
- Grossenbacher, D., R. B. Runquist, E. E. Goldberg and Y. Brandvain (2015). "Geographic range size is predicted by plant mating system." Ecology Letters **18**(7): 706-713.
- Hardin, J. W. (1964). "A comparison of *Phytolacca americana* and *P. rigida*." Castanea **29**(3): 155-164.
- Kay, K. M. and D. W. Schemske (2004). "Geographic patterns in plant-pollinator mutualistic networks: Comment." Ecology **85**(3): 875-878.
- Kim, T. N. (2014). "Plant damage and herbivore performance change with latitude for two old-field plant species, but rarely as predicted." Oikos **123**(7): 886-896.

- Kozlov, M. V., B. Y. Filippov, N. A. Zubrij and V. Zverev (2015). "Abrupt changes in invertebrate herbivory on woody plants at the forest-tundra ecotone." Polar Biology **38**(7): 967-974.
- Kozlov, M. V., V. Lanta, V. Zverev and E. L. Zvereva (2015). "Global patterns in background losses of woody plant foliage to insects." Global Ecology and Biogeography **24**(10): 1126-1135.
- Lehndal, L. and J. Agren (2015). "Herbivory differentially affects plant fitness in three populations of the perennial herb *Lythrum salicaria* along a latitudinal gradient." Plos One **10**(9).
- Lehndal, L. and J. Agren (2015). "Latitudinal variation in resistance and tolerance to herbivory in the perennial herb *Lythrum salicaria* is related to intensity of herbivory and plant phenology." Journal of Evolutionary Biology **28**(3): 576-589.
- Lim, J. Y., P. V. A. Fine and G. G. Mittelbach (2015). "Assessing the latitudinal gradient in herbivory." Global Ecology and Biogeography: 1-7.
- Lowman, M. D. (1984). "An assessment of techniques for measuring herbivory: Is rainforest defoliation more intense than we thought?" Biotropica **16**(4): 264-268.
- Moeller, D. A., R. D. B. Runquist, A. M. Moe, M. A. Geber, C. Goodwillie, P.-O. Cheptou, C. G. Eckert, E. Elle, M. O. Johnston, S. Kalisz, R. H. Ree, R. D. Sargent, M. Vallejo-Marin and A. A. Winn (2017). "Global biogeography of mating system variation in seed plants." Ecology Letters **20**(3): 375-384.
- Moles, A. T. (2013). "Dogmatic is problematic: Interpreting evidence for latitudinal gradients in herbivory and defense." Ideas in Ecology and Evolution **6**: 1-4.
- Moles, A. T., D. D. Ackerly, J. C. Tweddle, J. B. Dickie, R. Smith, M. R. Leishman, M. M. Mayfield, A. Pitman, J. T. Wood and M. Westoby (2007). "Global patterns in seed size." Global Ecology and Biogeography **16**(1): 109-116.
- Moles, A. T., S. P. Bonser, A. G. B. Poore, I. R. Wallis and W. J. Foley (2011). "Assessing the evidence for latitudinal gradients in plant defence and herbivory." Functional Ecology **25**(2): 380-388.
- Moles, A. T. and J. Ollerton (2016). "Is the notion that species interactions are stronger and more specialized in the tropics a zombie idea?" Biotropica **48**(2): 141-145.
- Moles, A. T., I. R. Wallis, W. J. Foley, D. I. Warton, J. C. Stegen, A. J. Bisigato, L. Cella-Pizarro, C. J. Clark, P. S. Cohen, W. K. Cornwell, W. Edwards, R. Ejrnaes, T. Gonzales-Ojeda, B. J. Graae, G. Hay, F. C. Lumbwe, B. Magana-Rodriguez, B. D. Moore, P. L. Peri, J. R. Poulsen, R. Veldtman, H. von Zeipel, N. R. Andrew, S. L. Boulter, E. T. Borer, F. F. Campon, M. Coll, A. G. Farji-Brener, J. De Gabriel, E. Jurado, L. A. Kyhn, B. Low, C. P. H. Mulder, K. Reardon-Smith, J. Rodriguez-Velazquez, E. W. Seabloom, P. A. Vesik, A. van Cauter, M. S. Waldram, Z. Zheng, P. G. Blendinger, B. J. Enquist, J. M. Facelli, T. Knight, J.

- D. Majer, M. Martinez-Ramos, P. McQuillan and L. D. Prior (2011). "Putting plant resistance traits on the map: a test of the idea that plants are better defended at lower latitudes." New Phytologist **191**(3): 777-788.
- Murray, K. G. (1988). "Avian Seed Dispersal of Three Neotropical Gap-Dependent Plants." Ecological Monographs **58**(4): 271-298.
- Olesen, J. M. and P. Jordano (2002). "Geographic patterns in plant-pollinator mutualistic networks." Ecology **83**(9): 2416-2424.
- Ollerton, J. (2012). "Biogeography: Are Tropical Species Less Specialised?" Current Biology **22**(21): R914-R915.
- Pannell, J. R., J. R. Auld, Y. Brandvain, M. Burd, J. W. Busch, P. O. Cheptou, J. K. Conner, E. E. Goldberg, A. G. Grant, D. L. Grossenbacher, S. M. Hovick, B. Igic, S. Kalisz, T. Petanidou, A. M. Randle, R. R. de Casas, A. Pauw, J. C. Vamosi and A. A. Winn (2015). "The scope of Baker's law." New Phytologist **208**(3): 656-667.
- Pauw, A. and R. Stanway (2015). "Unrivalled specialization in a pollination network from South Africa reveals that specialization increases with latitude only in the Southern Hemisphere." Journal of Biogeography **42**(4): 652-661.
- Pearse, I. S. and A. L. Hipp (2012). "Global patterns of leaf defenses in oak species." Evolution **66**(7): 2272-2286.
- Pennings, S. C. and B. R. Silliman (2005). "Linking biogeography and community ecology: Latitudinal variation in plant-herbivore interaction strength." Ecology **86**(9): 2310-2319.
- Pianka, E. R. (1966). "Latitudinal gradients in species diversity: A review of concepts." American Naturalist **100**(910): 33-46.
- Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar and R. C. Team (2017). nlme: Linear and nonlinear mixed effects models. R package version 3.1-131, <https://CRAN.R-project.org/package=nlme>.
- R Core Team (2014). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing.
- Rasmann, S. and A. A. Agrawal (2011). "Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory." Ecology Letters **14**(5): 476-483.
- Rech, A. R., B. Dalsgaard, B. Sandel, J. Sonne, J.-C. Svenning, N. Holmes and J. Ollerton (2016). "The macroecology of animal versus wind pollination: ecological factors are more important than historical climate stability." Plant Ecology & Diversity **9**(3): 253-262.
- Regal, P. J. (1982). "Pollination by wind and animals--ecology of geographic patterns." Annual Review of Ecology and Systematics **13**: 497-524.

- Salgado, C. S. and S. C. Pennings (2005). "Latitudinal variation in palatability of salt-marsh plants: Are differences constitutive?" Ecology **86**(6): 1571-1579.
- Sauer, J. D. (1952). "A geography of pokeweed." Ann Missouri Bot Gard **39**((2)): 113-125.
- Schemske, D. W. (2009). Biotic interactions and speciation in the tropics. Speciation and Patterns of Diversity. R. K. Butlin, J. R. Bridle and D. Schluter. Cambridge, UK, Cambridge University Press: 219-239.
- Schemske, D. W., G. G. Mittelbach, H. V. Cornell, J. M. Sobel and K. Roy (2009). "Is there a latitudinal gradient in the importance of biotic interactions?" Annual Review of Ecology Evolution and Systematics **40**: 245-269.
- Vamosi, J. C., T. M. Knight, J. A. Steets, S. J. Mazer, M. Burd and T. L. Ashman (2006). "Pollination decays in biodiversity hotspots." Proceedings of the National Academy of Sciences of the United States of America **103**(4): 956-961.
- Veldman, J. W., K. G. Murray, A. L. Hull, J. M. Garcia, W. S. Mungall, G. B. Rotman, M. P. Plosz and L. K. McNamara (2007). "Chemical defense and the persistence of pioneer plant seeds in the soil of a tropical cloud forest." Biotropica **39**(1): 87-93.
- Woods, E. C., A. P. Hastings, N. E. Turley, S. B. Heard and A. A. Agrawal (2012). "Adaptive geographical clines in the growth and defense of a native plant." Ecological Monographs **82**(2): 149-168.

CHAPTER 3: LEAF AGE DETERMINES TRAITS UNDERLYING STRONGER
HERBIVORE DEFENSE AT LOWER LATITUDES IN A PAIR OF CONGENERS

Introduction

Plants have long been hypothesized to be better defended from herbivory at lower latitudes, an assumed adaptive response to greater herbivore pressure in the tropics (Ehrlich and Raven 1964). This idea stems from the “biotic interactions hypothesis,” (BIH) which states that biotic interactions are more important drivers of adaptation closer to the equator, and that a greater rate of coevolution at low latitudes contributes to the latitudinal diversity gradient (Dobzhansky 1950, Fischer 1960, Schemske 2009, Schemske et al. 2009). The first reviews of the BIH for plant-herbivore interactions were by Coley and Aide (1991) and Coley and Barone (1996), who compared broad patterns of plant defense between temperate and tropical forests. They reported that many defensive traits are more prevalent in tropical trees, such as alkaloid production, latex canals, and ant defense mediated by extrafloral nectaries. Some defensive traits are almost exclusively tropical, such as delayed leaf greening and ant domatia. These reviews spurred many empirical tests of latitudinal patterns in herbivory and defense, mostly in temperate regions (Anstett et al. 2016). A meta-analysis, however, found little support for latitudinal patterns in herbivory and defense (Moles et al. 2011), which could be due in part to gaps in data collected thus far (e.g., few measurements of young leaves, Anstett et al. 2016). Here, we provide an in-depth case study designed to overcome these hurdles by testing for evolutionary transitions in defensive traits across a tropical-temperate congener pair.

One persistent challenge in evaluating evolutionary shifts in phenotypes consistent with the BIH is that defensive traits are highly diverse and may include mechanical, chemical, and biotic components that are each effective against different enemies. In particular, chemical defense classes are highly clade-specific at broad taxonomic scales (Abbott 1887, Ehrlich and Raven 1964, Agrawal et al. 2012, Mithofer and Boland 2012). Thus, we often lack a “common currency”

of traits that are good proxies of defensive function across distantly related species (Agrawal and Weber 2015). For example, total phenolics and flavonoids have been considered indices of plant defense for the purposes of testing the BIH (Adams et al. 2009, Moles et al. 2011), but these compounds may not be the most important defensive traits in each species, and may be more important for abiotic stress tolerance than herbivore defense (Tegelberg et al. 2001, Close and McArthur 2002, Martz et al. 2007). A discrepancy between what is measured and what is actually defensive may explain why surveys of leaf traits often do not find evidence for stronger defense at lower latitudes (Moles et al. 2011, Moles et al. 2011). Traits that are unambiguously defensive, such as cardiac glycosides in *Asclepias* (Agrawal et al. 2012), provide clear tests of latitudinal defense patterns. Species of both *Asclepias* and *Quercus*, systems with a rich literature on defensive traits, were found to have evolved stronger defenses at lower latitudes (Rasman and Agrawal 2011, Pearse and Hipp 2012).

Barring detailed knowledge of important defensive traits in every system, a potential solution to overcoming the “common currency” issue is to conduct bioassays, utilizing herbivore preference or performance to compare the strength of plant defense between populations (e.g., Miller and Hanson 1989, Bolser and Hay 1996, Pennings et al. 2001, Baskett and Schemske 2018). Bioassays do not depend on *a priori* knowledge of trait function, but instead evaluate the palatability of plant tissue from the perspective of an herbivore. The strongest support for latitudinal patterns in defense is from bioassays (Moles et al. 2011), suggesting that measuring traits with unknown function is adding statistical noise to tests of the BIH. Because specialist herbivores may have evolved counter defenses (Zangerl and Berenbaum 2003) or even the ability to sequester plant defenses (Agrawal et al. 2012), bioassays with generalist species are well suited for comparing evolutionary responses to historic herbivore pressure.

Beyond the “common currency” challenge, ontogenetic variation in defensive traits can further complicate our understanding of the biogeography of plant defense. Herbivores generally prefer leaves that are less tough and more nitrogen-rich, and young expanding leaves fit both of these criteria (Mattson 1980, Coley 1983, Perez-Harguindeguy et al. 2003), while mature expanded leaves can be defended to some degree by toughness and a higher carbon-nitrogen ratio (Coley and Barone 1996). Additionally, damage to expanding leaves has a larger fitness impact than damage to mature leaves (Coleman and Leonard 1995). Thus, optimal defense theory predicts that young leaves are strongly defended by secondary metabolites (McKey 1974, McCall and Fordyce 2010). However, most latitudinal comparisons of herbivory and defense have focused exclusively on mature leaves (Anstett et al. 2016), which may underestimate overall herbivore pressure (Lowman 1984, Filip et al. 1995, Coley and Barone 1996), miss latitudinal patterns if they are stronger in young leaves (e.g., Baskett and Schemske 2018), and fail to consider important defensive traits that have evolved to protect young leaves (e.g., Coley et al. 2018).

Here, we address the challenges for measuring latitudinal patterns in defense that are presented by determining defensive function and by trait variation due to leaf ontogeny. Specifically, we evaluate latitudinal patterns in physical and chemical defensive traits for young and mature leaves in a temperate-tropical pair of *Phytolacca* (Phytolaccaceae) species, spanning a wider latitudinal range than would be possible in a single species and incorporating a bioassay to determine which traits are important drivers of leaf palatability for a generalist herbivore. We ask (1) Are patterns of evolution in defense-related traits consistent with the biotic interactions hypothesis, with stronger defenses at lower latitudes? And, (2) how do patterns of trait variation across the latitudinal range of *Phytolacca* relate to palatability for a generalist herbivore?

Methods

Study species

Phytolacca americana L. is native to the eastern USA, from Texas and Florida to southern Canada (Sauer 1952), and *Phytolacca rivinoides* Kunth & C.D. Bouché is native to all of the Neotropics. These two species are well suited for a study of this kind. They have similar ecologies: both are herbaceous, short-lived perennial pioneer species that thrive in disturbed habitats and have similar lepidopteran herbivores (Sauer 1952, Murray 1988, Ch. 2). They are easy to find in the field, are closely related, and together have ranges that span a large latitudinal area (from 16° S to 44° N degrees). Additionally, previous work has confirmed the presence of a large-scale latitudinal gradient in herbivore pressure in this system. Lower-latitude populations of *P. americana* had both higher herbivory rates and reduced palatability compared to higher-latitude populations (Baskett and Schemske 2018), and Costa Rican populations of *P. rivinoides* were found to experience greater herbivory and have reduced palatability compared to north-temperate *P. americana* (Ch. 2).

Common gardens

To evaluate latitudinal patterns in defensive traits, we measured phenotypes and palatability using plants from a wide latitudinal range, grown together in a common greenhouse environment. We collected seeds from 13 populations of *P. americana* between mid-Florida and mid-Michigan (27° N to 42° N) and 3 populations of *P. rivinoides* in Costa Rica (~9° N); see Ch. 2 for site descriptions.

We grew two cohorts of plants in a greenhouse common garden in East Lansing, Michigan in June-October of 2016 and March-October of 2017. See Ch. 2 and Baskett and Schemske (2018)

for details on germination and growing conditions. Each cohort contained 4-5 maternal lines per population for the 16 populations, with seeds collected from one individual representing one maternal line.

Leaf trait measurements

In the common garden, we quantified multiple traits hypothesized to play roles in plant defense across the two cohorts of plants. Traits included leaf toughness, percent nitrogen, and several metrics of secondary metabolites: chemical richness, chemical abundance, and chemical composition (using nonmetric multidimensional scaling, NMDS, which reduces dimensionality based on similarity of chemical composition between individuals). Palatability and nitrogen content were measured on the cohort of plants grown in 2016 (Baskett and Schemske 2018), while leaf toughness and phytochemistry were sampled on the 2017 cohort of plants (Ch. 2). All traits were measured on young and mature leaves: we define young as expanding leaves with smaller and lighter green color, and mature as darker, fully expanded, toughened leaves.

To quantify phytochemistry, we used liquid chromatography-mass spectrometry (LC/MS), which allows comparisons of chemical abundance agnostic to compound structure (Macel et al. 2010). We followed modified protocols from Schilmiller et al. (2010). Whole young leaves and 2.53-cm² semicircle punches of mature leaves were sampled from 10 to 16 Oct, 2017, between 4:00 and 7:00 PM. One plant per maternal line was sampled for 5 maternal lines per population for 16 populations. Samples were placed directly into 1-mL of extraction solvent (acetonitrile:isopropanol:water 3:3:2 v/v/v containing 0.1% formic acid and 100 nM telmisartan as an internal standard) for 16 hours. Extracts were stored at -20° C and a 1:20 dilution in extraction solvent was made prior to analysis by LC/MS. One batch of samples was run in Dec

2017 (3 maternal lines per population for northern, southern, and tropical regions); another was run in Feb 2018 (2 additional lines from these regions and 5 lines from 6 populations between northern and southern regions).

LC/MS was performed using a Waters Xevo G2-XS Q-TOF mass spectrometer interfaced with a Waters Acquity UPLC and 2777c autosampler. Five mL of sample (1:20 dilution) were injected onto a Waters BEH C18 UHPLC column (2.1x100 mm; 1.7 mm particle size) held at 40°C. An 11 min gradient at 0.3 ml/min flow rate was performed as follows: initial conditions were 99% solvent A (10 mM ammonium formate in water) and 1% solvent B (acetonitrile:isopropanol, 7:3 v/v), ramp to 99% B at 7.5 min, hold at 99% B for one min, switch back to 99% A at 8.51 min and hold to re-equilibrate column until 11 min. Compounds eluting from the column were analyzed by electrospray ionization in positive ion mode using the following conditions: capillary voltage of 3.00 kV; sample cone voltage was 35V; source temperature of 100° C; desolvation temperature of 350° C; 600 L/h desolvation nitrogen gas flow rate. Time of flight MS data were acquired in continuum mode in two separate acquisition functions to generate spectra under both non-fragmenting and fragmenting conditions using a data-independent MS^E method.

Data from the first 7.5 minutes was used for analysis to exclude abundant membrane lipids with high retention times that were present in the extracts. Raw LC/MS chromatograms were processed with Progenesis QI software version 2.3 (Nonlinear Dynamics). Data from a pooled sample consisting of aliquots from multiple extracts across the sampling range was used as the alignment reference. Raw peak area was normalized to the internal standard peak area (telmisartan) and dry leaf mass. To reduce noise in the data caused by rare compounds, peaks were retained for further analysis if they represented at least 1% relative abundance of at least

one sample. This step reduced the dataset from 11,894 to 110 peaks. Chemical abundances are calculated from peak areas; overall abundance is the sum of peak areas from the 110 abundant compounds in a sample. Chemical richness is the number of peaks present in a sample out of the 110 abundant compounds. Summary measures of secondary metabolites (abundance, richness, and diversity) have correlated with reduced herbivory (Richards et al. 2015, Salazar et al. 2018).

We reduced the dimensionality of the chemical data using nonmetric multidimensional scaling (NMDS) with the *vegan* package using Bray-Curtis distance (Oksanen et al. 2017). R version 3.3.3 was used for all analyses in the study except machine learning (R Core Team 2014). A scree plot showed that four NMDS axes captured most variation in chemistry for young and mature leaves, with stress at 0.58 (Appendix: Fig. 24), so these four axes were used for further analyses.

To quantify leaf toughness, a mechanical defense (Coley 1983, Perez-Harguindeguy et al. 2003), we sampled two young and two mature leaves (three nodes below the uppermost mature leaf) per plant for 3 plants per maternal line in 5 lines per population (N = 233 plants in 80 lines in Sep 2017). These plants were from the same cohort as plants used for phytochemistry, but different individuals. Toughness was measured with a Wagner penetrometer (FDK-32), which quantifies the grams of force required to pierce the leaf clamped between two metal plates with a 0.5-cm diameter rod (Salazar and Marquis 2012). Midveins were removed for measurement, and major veins were not pierced for mature leaves. The two leaves in each age category were averaged for each plant before analysis.

Percent nitrogen is often used as a proxy for nutritional value of leaf tissue (Perez-Harguindeguy et al. 2003, Descombes et al. 2017), and thus may mediate plant defense to determine palatability. We measured leaf percent nitrogen on two young leaves and one mature

leaf (three nodes below the uppermost mature leaf) per plant for 3-4 plants per maternal line and 4-5 lines per population (N = 274 plants in 78 lines in Aug 2016). Leaf tissue was dried at 60° C. Midveins were removed from mature leaves, tissue was ground to a powder, and 2-5 mg were measured into 5 x 9 mm tin capsules (Coley 1983, Batterman et al. 2013). Percent nitrogen was determined by an elemental analyzer (Costech ECS 4010 elemental analyzer, Costech Analytical Technologies, Inc., Valencia, CA).

Palatability

We measured palatability using a no-choice bioassay on leaves from reproductive *Phytolacca* individuals from the common garden (October 2016). Data from the palatability bioassays has been previously published (Baskett and Schemske 2018, Ch. 2), but are re-analyzed here in a different context. In brief, larvae of the generalist *Spodoptera exigua* (Lepidoptera, Noctuidae) were raised on a no-choice diet of either young or mature leaves. Leaf material for each maternal line was pooled from 2-3 individual plants. For mature leaves, each maternal line was replicated in 4 cups, starting with 5 neonate larvae in each cup, and larval biomass was determined after 8 days of growth. For young leaves, which had limited availability, maternal lines were replicated in 3 cups, starting with 3 neonate larvae in each cup, and larval biomass was determined after 9 days of growth. Leaf area consumed was measured daily in the mature-leaf treatment and every other day in the young-leaf treatment, using a transparent grid with 10 mm² squares. Total biomass per cup (which integrates survival and growth, as dead caterpillars were counted as zero biomass) was standardized by starting number of caterpillars and duration (5 larvae, 8 days for mature leaves and 3 larvae, 9 days for young leaves) after log_e transformation due to exponential growth. Baskett and Schemske (2018) used larval biomass as a metric of palatability, but here we

use larval biomass per leaf area consumed to capture effects of leaf traits on the ability of larvae to convert leaf tissue to insect biomass.

Statistical analyses

Latitude and leaf age effects on traits and palatability: We tested for effects of latitude and leaf age on putatively defensive leaf traits and palatability in two ways. First, we asked if there is a latitudinal gradient in each trait for 13 populations of *P. americana* spanning 16° latitude. We used a mixed-effects ANCOVA with fixed effects of latitude, leaf age, and their interaction, and a random effect of population using the *lme* function from the *nlme* package (Pinheiro et al. 2017). Treating latitude as a continuous predictor would not be appropriate for analysis of the tropical *P. rivinoides* because we lack populations between Costa Rica and Florida (a gap of 16° latitude). Thus, the second analysis is based on region as a categorical effect. We compare *P. rivinoides* to the southern and northern ends of the range of *P. americana*, with three populations in each region and a similar difference of 14-18° between regions. We refer to the regions as tropical (~9° N), subtropical (~27° N), and temperate (~41° N). Maternal line means of each trait were analyzed as a function of region, leaf age, and their interaction, with a random effect of population. Tukey's HSD was used to determine differences between regions and leaf ages if main effects were significant. In cases of heteroscedasticity, variance was estimated separately for each population (both models: N, richness, NMDS axis 4; *P. americana* only: NMDS axes 2-3) or for each leaf age in each population (both models: toughness and chemical abundance).

Traits predicting palatability: We analyzed whether trait variation predicted palatability for young and mature leaves separately, because traits and palatability differed by leaf age. For the response (larval biomass per leaf area consumed) and predictors (leaf traits), mean values of

maternal lines were analyzed with mixed-effects ANCOVA. Leaf traits included toughness, percent nitrogen, chemical richness, chemical abundance, and the four chemical NMDS axes. We used a model dredging approach from the *MuMIn* package (Barton 2016), which compares AICc for all possible combinations of predictors to select a subset of the eight traits that best predicts palatability for each leaf age (lowest AICc). Models included a random effect of population, and variance was estimated separately for each population due to heteroscedasticity. Model selection was performed on a dataset with maternal lines that included measurements for every trait, 71 lines for young leaves and 74 for mature leaves.

Identification of chemicals predicting palatability: Chemistry NMDS axes were significant predictors of palatability in our mixed model analyses (see results), so we used a Random Forest (RF) algorithm to identify the chemical compounds that best predict palatability (ML-Pipeline, C. Azodi unpublished). Random Forest is a decision tree-based machine learning analysis (Breiman 2001), which is suitable for chemical ecology data because it does not assume normally distributed, independent predictors, nor is it biased toward abundant variables (Ranganathan and Borges 2010, Clavijo McCormick et al. 2014). The RF algorithm with five-fold cross-validation was used (that is, 80% of the data is used to build a model and 20% to test it), and the mean results of 100 model iterations are shown. We ran RF models separately on each leaf age using peak abundances as features for the 110 most abundant compounds. We conducted model selection to maximize R^2 : we iteratively reduced the number of predictors and compared reduced model to full model R^2 (Appendix: Figs. 26-27). For the first three reductions of the chemistry models, which started with 110 peaks, 20% of features with lowest importance scores were removed at each step. For all other steps, we removed features with lowest importance scores past a relatively sharp reduction in scores. Model fit for RF models with chemistry, population,

and both were compared, to ask how well chemistry predicts palatability beyond population groups alone. To visualize chemical composition of the reduced set of compounds identified by RF analysis, we used NMDS on separate leaf ages (27 chemical peaks for young leaves, 3 for mature). Based on scree plots (Appendix: Fig. 27), two axes were used for NMDS (average of 0.75 stress).

Results

Latitude and leaf age effects on traits and palatability

When dimensionality of chemical composition was reduced using NMDS, geographic and ontogenetic patterns in chemistry are apparent (Figs. 17-19, Table 13). All four axes show significant regional differences (Table 13); for example, the first and second axes separate tropical *P. rivinoides* and subtropical *P. americana* from the rest of *P. americana* populations. Additionally, NMDS2 and NMDS4 show latitudinal gradients within *P. americana* (Table 13, Fig. 18B, Fig. 18D). Interestingly, there is little overlap between *P. rivinoides* and subtropical *P. americana* in the fourth axis, but both have some overlap with more temperate *P. americana*. Leaf age is a significant main effect for all NMDS axes in all models except NMDS4 for the regional comparisons of *P. rivinoides* (Table 13). The chemical composition of young and mature leaves differs for *P. americana* similarly across the latitudinal gradient, most strongly along axis 2 (Fig. 18B). However, analyses of *P. rivinoides* show significant region and leaf age interactions for axes 1-3 (Fig. 19B-D). That is, the species differ in their ontogenetic shifts in chemical composition.

Table 13. Interactions between biogeography and leaf age for *Phytolacca* leaf traits hypothesized in the literature to influence palatability. Models for *P. americana* include 13 populations along a gradient and treat latitude as a continuous predictor. The *P. rivinoides* comparison consists of three regions, each containing three populations: tropical *P. rivinoides*, subtropical *P. americana*, and north-temperate *P. americana*. All models contain a random effect of population.

Comparison	Fixed effect	Trait											
		NMDS1			NMDS2			NMDS3			NMDS4		
		<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>
<i>P. americana</i> only	Latitude	3.97	1,11	0.0717	34.56	1,11	0.0001	0.13	1,11	0.7209	13.25	1,11	0.0039
	Leaf age	143.03	1,117	< 0.0001	149.79	1,117	< 0.0001	114.00	1,117	< 0.0001	8.71	1,117	0.0038
	Lat x Age	0.58	1,117	0.4481	3.55	1,117	0.0620	0.72	1,117	0.3965	0.07	1,117	0.7988
<i>P. rivinoides</i> included	Region	29.54	2,6	0.0008	92.29	2,6	< 0.0001	6.74	2,6	0.0292	28.11	2,6	0.0009
	Leaf age	71.69	1,84	< 0.0001	188.05	1,84	< 0.0001	10.06	1,84	0.0021	0.82	1,84	0.3680
	Reg x Age	8.23	2,84	0.0005	14.33	2,84	< 0.0001	7.66	2,84	0.0009	0.70	2,84	0.4994

Comparison	Fixed effect	Trait											
		chemical richness			chemical abundance			leaf toughness			% Nitrogen		
		<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>
<i>P. americana</i> only	Latitude	0.74	1,11	0.4088	0.40	1,11	0.5378	7.37	1,11	0.0201	0.60	1,11	0.4564
	Leaf age	16.93	1,117	0.0001	105.93	1,117	< 0.0001	1056.90	1,117	< 0.0001	12.31	1,113	0.0006
	Lat x Age	0.56	1,117	0.4542	3.10	1,117	0.0811	7.38	1,117	0.0076	12.23	1,113	0.0007
<i>P. rivinoides</i> included	Region	12.11	2,6	0.0078	2.96	2,6	0.1277	27.10	2,6	0.0010	19.03	2,6	0.0025
	Leaf age	0.08	1,84	0.7764	72.09	1,84	< 0.0001	1153.90	1,78	< 0.0001	50.96	1,71	< 0.0001
	Reg x Age	1.96	2,84	0.1476	5.46	2,84	0.0059	18.27	2,78	< 0.0001	16.03	2,71	< 0.0001

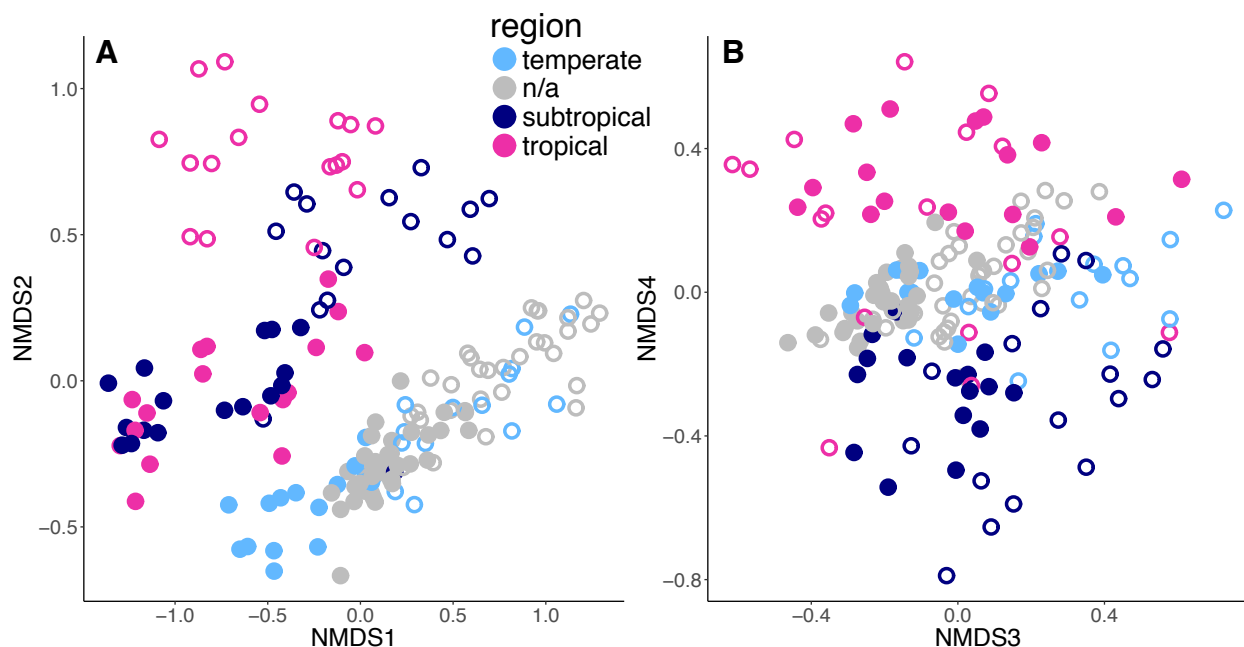


Figure 17. Chemical composition for young leaves (open circles) and mature leaves (closed circles) of *Phytolacca americana* (light blue, grey, dark blue) and *P. rivinoides* (pink), based on 110 peaks from LC/MS analysis. The grey points are from seven *P. americana* populations that are not included in regional analyses, but lie between the subtropical and temperate regions. A) plot of the first two NMDS axes. B) plot of the third and fourth NMDS axes.

Other leaf traits show latitudinal and ontogenetic differences as well (Table 13, Figs. 18-19). Chemical richness is not as variable as other leaf traits; young leaves of *P. americana* have fewer peaks than mature leaves, but latitudinal patterns are weak (e.g., there is a significant effect of region, but Tukey's HSD found no regional differences; Fig. 18E, Fig. 19E). However, young leaves consistently have a higher overall chemical abundance (peak area) than mature leaves (Fig. 18F, Fig. 19F). Chemical abundance does not vary with latitude for mature leaves, but young leaves of temperate *P. americana* have higher chemical abundance than young leaves of tropical *P. rivinoides* (Fig. 19F). Mature leaves are tougher than young leaves, but the only latitudinal pattern in toughness is a negative correlation with latitude for mature leaves of *P. americana* (Fig. 18G, Fig. 19G). Nitrogen is higher in young versus mature leaves in tropical and subtropical populations, but the difference disappears at higher latitudes (Fig. 18H, Fig. 19H).

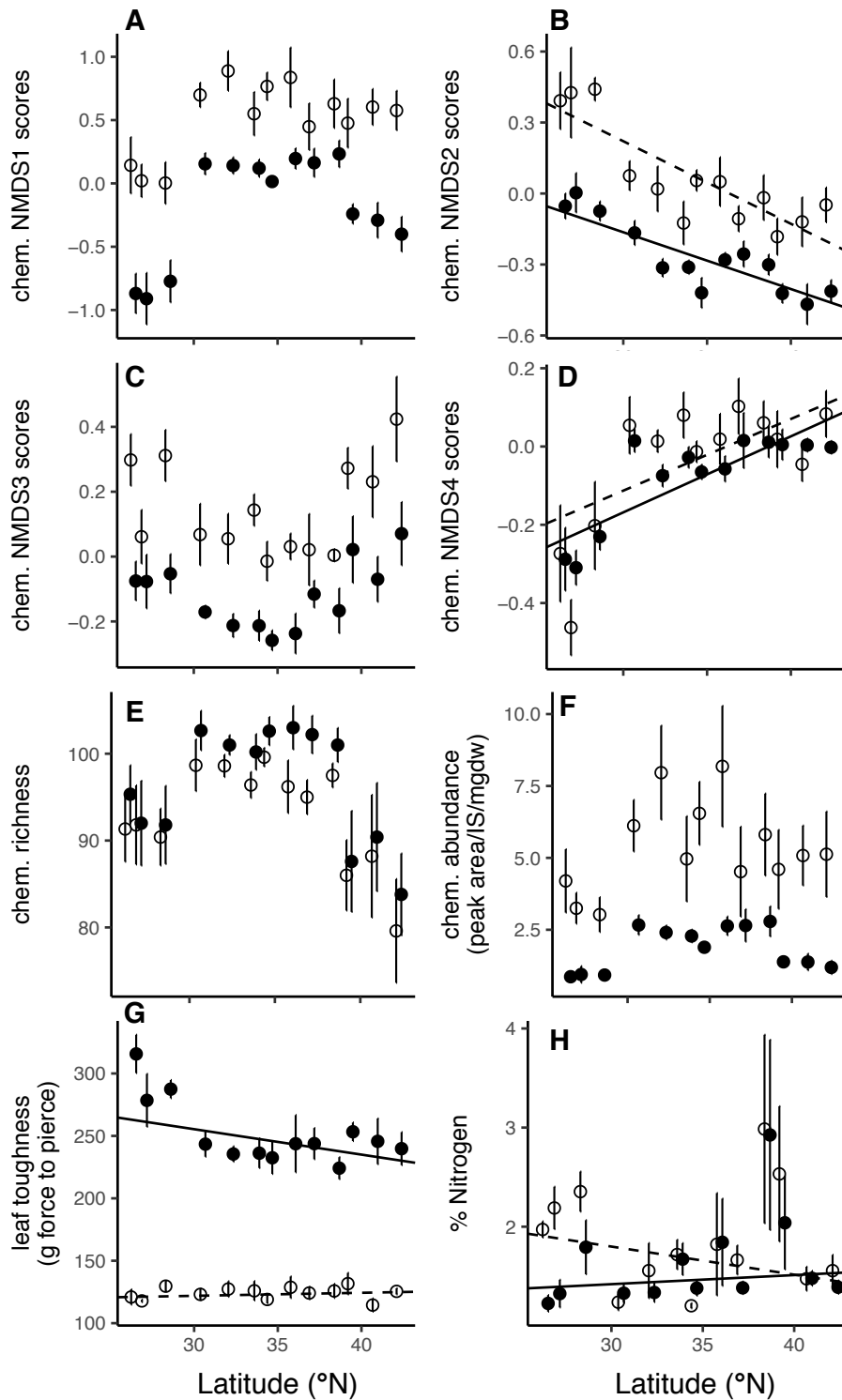


Figure 18. Latitudinal patterns in various leaf traits (A-H) for *Phytolacca americana* for young and mature leaves (open circles and dashed line vs. closed circles and solid line, respectively). All traits significantly differed by leaf age, and we plotted best-fit lines for latitude if there was a significant main effect of latitude or a latitude-age interaction (Table 13). Points are population means (\pm SE), and points for young leaves are jittered 0.3 degrees south to minimize point overlap. NMDS scores are from Fig. 17.

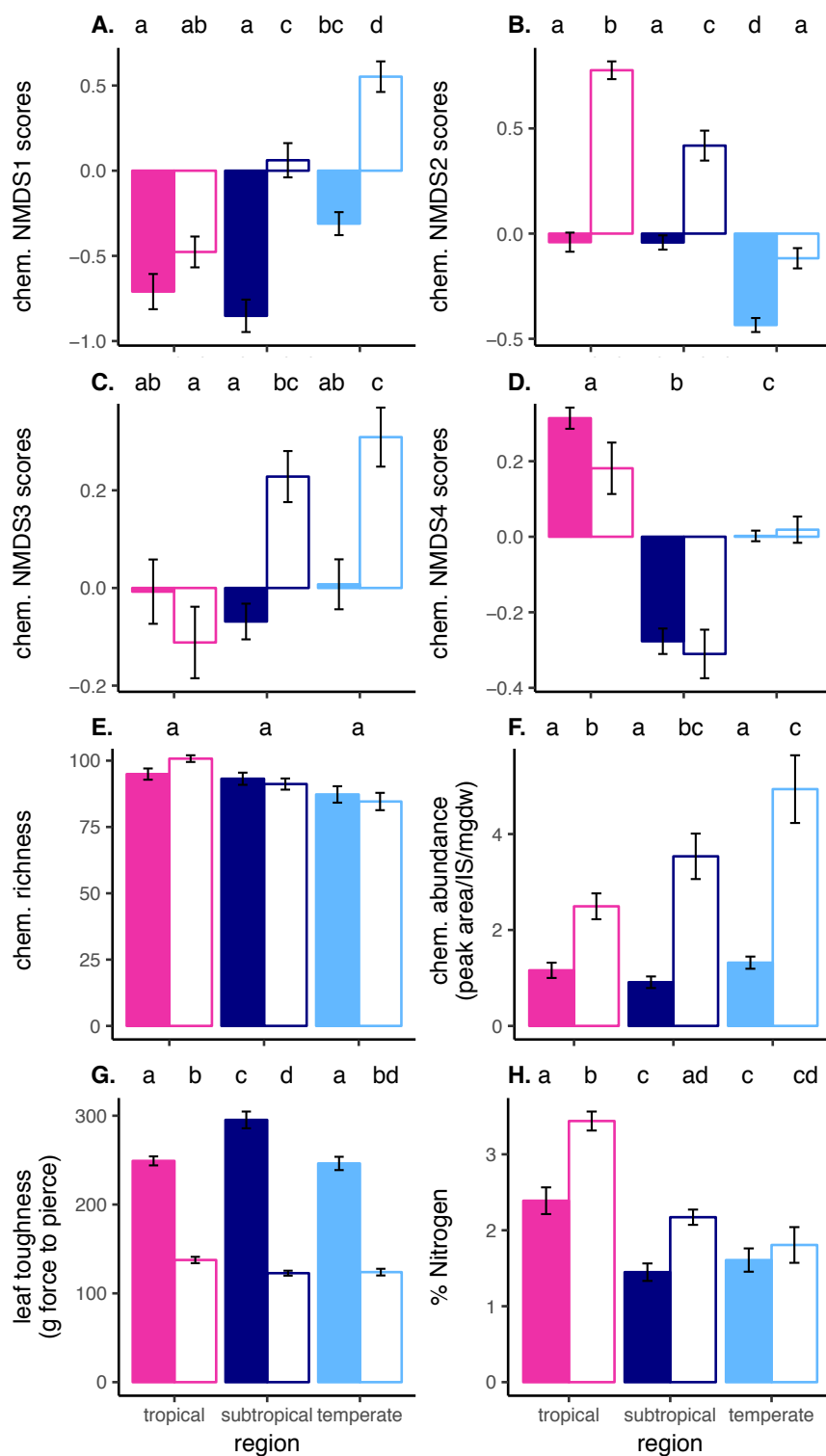


Figure 19. Geographic and ontogenetic differences in various leaf traits (A-H) for three populations per region of *Phytolacca rivinoides* (tropical) and *P. americana* (subtropical and temperate). Young leaves are open bars, and mature leaves are filled bars. Bars are means (\pm SE). Bars that share letters are not significantly different according to Tukey's HSD; panels D and E only had significant main effects of region, while all others had significant main effects of the interaction of region and leaf age (Table 13). NMDS scores are from Fig. 17.

Palatability (larval biomass per leaf area consumed) was positively correlated with latitude in *P. americana*, and young leaves were less palatable than mature leaves, but there was no latitude-age interaction (Table 14, Fig. 20). Regional comparisons, however, showed that palatability differed by both region and leaf age (Table 14, Fig. 20). The least palatable diet was tropical young leaves. Compared to tropical young leaves, subtropical young leaves were 3.9 times more palatable, and temperate young leaves were 27 times more palatable. In contrast, tropical and temperate mature leaves were equally palatable, 1.5 times more than subtropical mature leaves (Fig. 20).

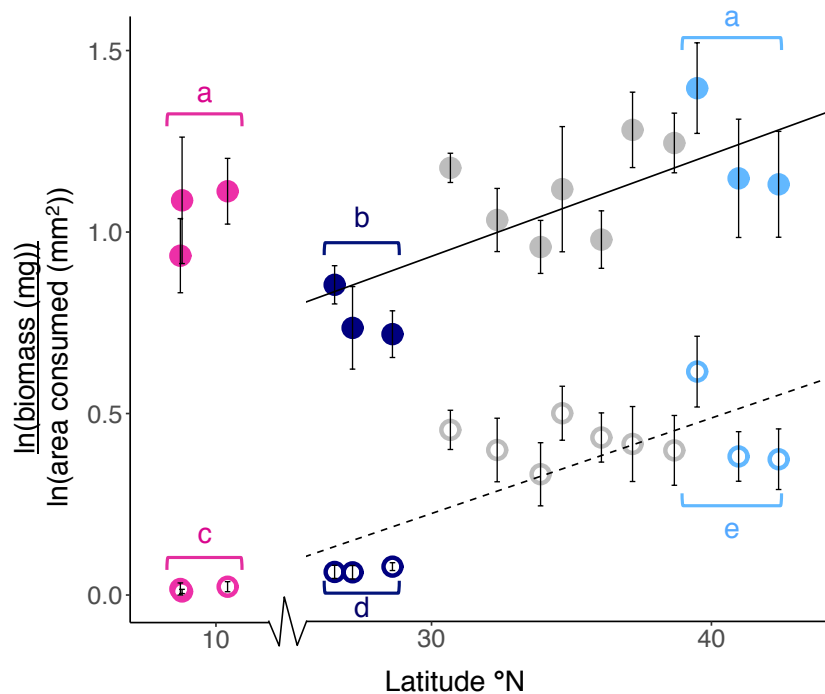


Figure 20. Palatability of *Phytolacca americana* and *P. rivinoides*, quantified by *Spodoptera exigua* larval biomass per leaf area consumed, as a function of latitude and leaf age (mature = filled circles and solid line, young = open circles and dashed line). Each point is a population mean (\pm SE) of per-cup palatability. Lines of best fit are shown for analysis of *P. americana* populations along a latitudinal gradient. Different letters show significant differences between regions and leaf ages for comparison of tropical *P. rivinoides* (pink) to north-temperate (light blue) and subtropical (dark blue) *P. americana*, according to Tukey's HSD. Grey populations are not included in regional analyses. See Table 14 for results.

Table 14. Effects of leaf age and latitude or region on palatability of *Phytolacca* species (*Spodoptera exigua* larval biomass per leaf area consumed; Fig. 17). Latitude is treated as a continuous variable for analysis of *P. americana* along a gradient of 13 populations in eastern North America. We compared the tropical *P. rivinoides* to subtropical and north-temperate *P. americana* (three populations per region). All models included random effects of maternal line nested in population. Significant effects ($p < 0.05$) are in bold.

Comparison	Fixed effect	d.f.	<i>F</i>	<i>P</i>
<i>P. americana</i> only	Latitude	1, 11	13.28	0.0039
	Leaf age	1, 373	16.17	0.0001
	Latitude x Age	1, 373	0.12	0.7285
<i>P. rivinoides</i> included	Region	2, 6	22.12	0.0017
	Leaf age	1, 239	351.45	< 0.0001
	Region x Age	2, 239	14.29	< 0.0001

Traits predicting palatability

For young leaves, the best-fitting mixed-effects ANCOVA of palatability contained two of the eight traits we measured: chemical NMDS2 scores and overall chemical abundance (dAIC of 7.7 from next-best model with NMDS2 only). The NMDS2 axis was negatively correlated with palatability ($F_{1,53} = 82.36$, $p < 0.0001$; Fig. 21A). Chemical abundance was positively correlated with palatability ($F_{1,53} = 696.13$, $p < 0.0001$; Fig. 21B).

For mature leaves, four models had similar AIC scores (dAIC < 2.1): models with NMDS2, NMDS3, or NMDS4 as a sole predictor, and a model with both NMDS2 and NMDS4. We selected the latter as our final model, as both axes were significant predictors of mature-leaf palatability. Adding NMDS3 did not improve model fit (dAIC = 3.6), and it was not a significant predictor when all three axes were included. The NMDS2 axis was negatively correlated with palatability ($F_{1,56} = 8.62$, $p = 0.0048$; Fig. 21C), and NMDS4 was positively correlated with palatability ($F_{1,56} = 8.33$, $p = 0.0055$; Fig. 21D). Despite correlations among many of the traits, the two traits in each final model are not correlated (Appendix: Fig. 28), and there is no multicollinearity.

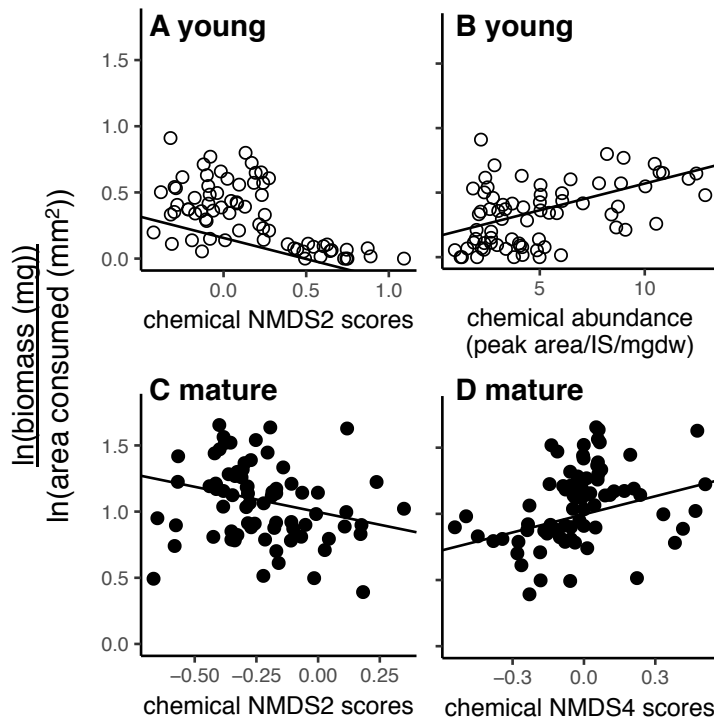


Figure 21. Palatability (*Spodoptera exigua* biomass per leaf area consumed) as a function of various leaf traits for young (A-B) and mature (C-D) *Phytolacca* leaves (open vs. closed circles, respectively). Leaf ages were modeled separately, and slopes were obtained from mixed models including both traits and a random effect of population.

Identification of chemicals predicting palatability

For young leaves, chemistry was a relatively good predictor of palatability using RF analysis.

The best model explained 54.4% of variation with 27 chemical peaks (Table 15, Fig. 22,

Appendix: Fig. 25). Population-of-origin alone explained 7.5% of variation in young-leaf

palatability, but did not improve explanatory power when included with the 27 chemical peaks

($R^2 = 0.534$; Fig. 22). For mature leaves, the optimal model of palatability predicted by chemical

abundances included only 3 chemical peaks and had an R^2 of 0.371 (Table 15, Fig. 22, Appendix:

Fig. 26). Population alone explains 9.2% of the variation in mature-leaf palatability. Chemistry

and population together did not explain more variation than chemistry alone ($R^2 = 0.357$; Fig. 22).

We were able to classify half of the 27 peaks predicting palatability for young leaves using retention time and mass-to-charge ratio, and fragmentation patterns. All are saponins (triterpene glycosides) or saponin adducts (Table 15). Eight of the 27 peaks in young leaves (including some of the saponins) are negatively correlated with palatability. For mature leaves, all three peaks that predicted palatability are saponins or saponin adducts (Table 15). Only one is negatively correlated with palatability; the same peak has the highest importance score of the young-leaf peaks that negatively correlate with palatability.

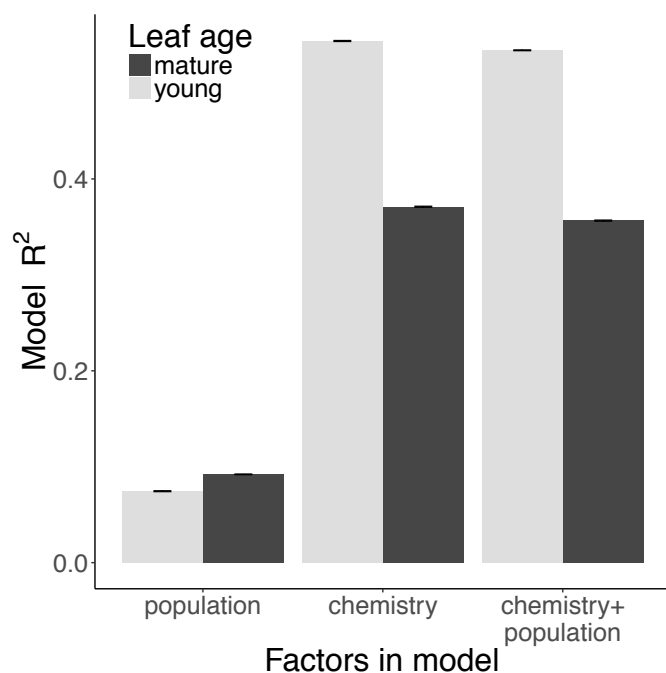


Figure 22. Model comparison using R^2 from machine learning analyses of the chemical peaks predicting palatability, using the Random Forest algorithm (RF). Chemical models began with 110 peaks; shown are results from the best model for each leaf age, with 27 peaks for young leaves and 3 peaks for mature leaves. Each bar is the mean (\pm SE) of 100 RF iterations. Model selection procedures are detailed in Supplemental Information.

Table 15. Chemical peaks determined to be predictive of palatability by machine learning analysis using the Random Forest algorithm (RF), shown in rank order of RF importance scores (Gini index). The sign of the importance score is the sign of each peak's correlation with palatability.

Leaf age	Peak (retention time_mass)	Importance score	Class	Notes
Young	4.08_849.4250m.z	0.568	saponin	sodium adduct
	3.68_1006.5237m.z	0.532	saponin	ammonium adduct
	4.01_844.4728m.z	0.475	saponin	ammonium adduct, same compound as 4.08
	4.27_828.4706m.z	0.391	saponin	ammonium adduct
	3.86_1120.5298n	0.358	saponin	core
	4.15_1171.5534m.z	-0.408	saponin	sodium adduct
	4.00_1118.5503n	0.365	saponin	co-elutes with 4.01
	4.13_972.4918n	0.281	saponin	core
	4.14_1167.6005m.z	-0.366	saponin	sodium adduct, same compound as 4.15
	3.81_990.5206m.z	0.412	saponin	ammonium adduct
	3.68_1007.0179m.z	0.415	---	detector artifact of 3.68_1006
	3.88_1311.6420n	-0.586	saponin	core
	3.89_988.4885n	0.263	unknown	
	4.10_584.3136m.z	-0.281	unknown	
	4.12_1158.5923m.z	-0.299	unknown	
	0.95_191.1517n	0.409	unknown	
	6.35_435.2945m.z	0.330	unknown	
	6.35_434.2912n	0.388	unknown	
	3.75_186.2212m.z	0.462	unknown	
	4.09_1149.5901n	-0.214	saponin	ammonium adduct
	6.47_622.2420n	-0.527	unknown	
	7.18_572.3924n	0.527	unknown	
	7.14_678.4772m.z	0.525	unknown	
	4.30_1004.5416m.z	-0.396	unknown	
	7.07_836.5443n	0.479	unknown	
	3.05_603.1340m.z	0.409	unknown	
	5.03_210.1616n	0.272	unknown	
Mature	4.08_849.4250m.z	0.345	saponin	sodium adduct
	4.15_1171.5534m.z	-0.329	saponin	sodium adduct
	4.01_460.2309n	0.326	monoterpene glycoside	

Finally, NMDS of the peaks that were identified as important predictors of palatability shows strong latitudinal patterns in chemical composition (Fig. 23). For young leaves, the first axis separates tropical *P. rivinoides* and subtropical *P. americana* to some degree, and strongly separates them from the rest of the *P. americana* populations (Fig. 23A; one subtropical population clusters near the bottom-right with more northern *P. americana*). There is also some separation of *P. rivinoides* and subtropical *P. americana* along the second axis. For mature leaves, the pattern is more complicated (Fig. 23B): subtropical *P. americana* populations cluster in one quadrant, separated from all other *P. americana* populations along the first axis. Tropical *P. rivinoides* populations are largely separated from *P. americana* when both axes are considered, but there is some overlap along each axis.

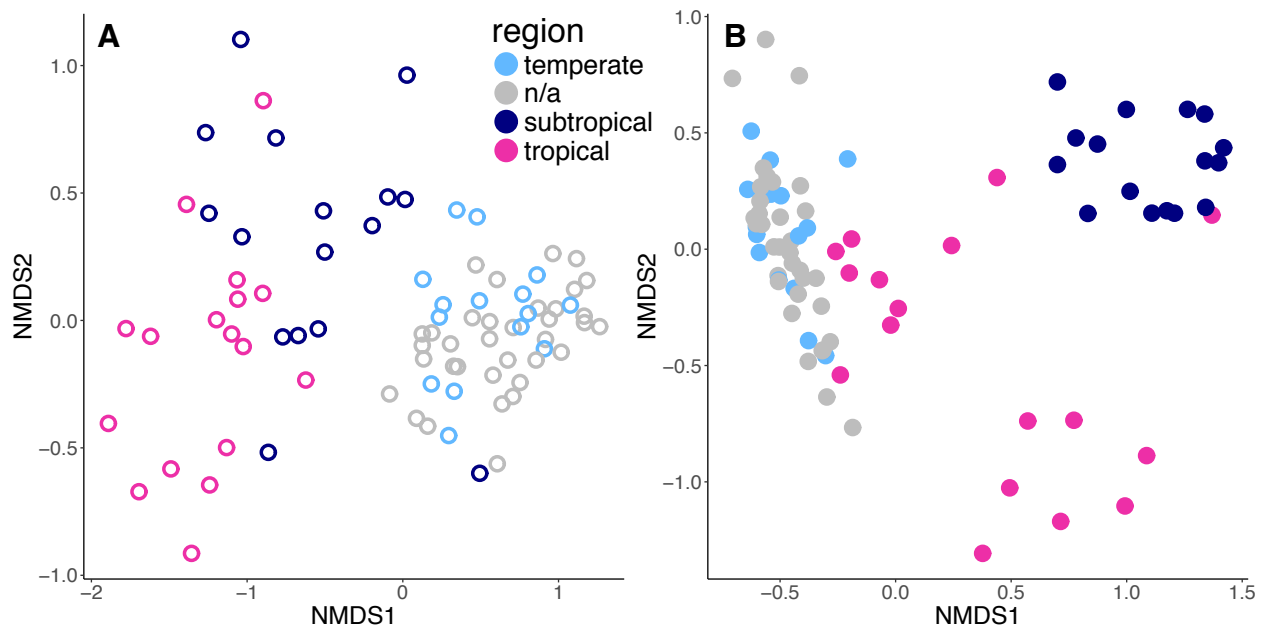


Figure 23. Chemical composition, colored by region, for young leaves (A, open circles) and mature leaves (B, closed circles), based on peaks from LC/MS determined to be important predictors of palatability for each leaf age by Random Forest analysis (27 for young leaves, 3 for mature). *Phytolacca americana* is light blue, grey, and dark blue; *P. rivinoides* is pink. The grey points are from seven *P. americana* populations that are not included in regional analyses, but lie between the subtropical and temperate regions.

Discussion

Dobzhansky's longstanding but controversial biotic interactions hypothesis (BIH) posits that the relative importance of biotic selection is stronger in tropical regions (Dobzhansky 1950), which may be evidenced in patterns of both trait evolution and contemporary ecological interactions (Schemske et al. 2009). A substantial body of work has aimed to test this hypothesis using herbivory as a model system, but support has been mixed (Moles et al. 2011). Here, we present an in-depth case study designed to overcome methodological hurdles that may obfuscate latitudinal pattern of defenses (Anstett et al. 2016). Using palatability trials, in-depth phenotyping, and a common garden spanning over 30° latitude in a tropical-temperate congener pair, we found patterns of trait evolution consistent with the BIH on several levels. Bioassays revealed a striking latitudinal pattern in leaf palatability, with tropical young leaves drastically depressing herbivore performance. Phenotyping of chemical, physical, and nutritional leaf traits identified several traits that have diverged with latitude, and found that chemical traits explain a large proportion of the observed variation in palatability. As predicted by previous critiques of BIH-studies, patterns of trait evolution and palatability differed by leaf age, supporting the hypothesis that previous work not considering leaf ontogeny may have overlooked BIH dynamics.

When defensive function is considered, we find latitudinal patterns in plant chemistry that support the BIH. Tropical populations have evolved chemical compositions associated with low palatability of young leaves (high NMDS2 scores, low overall chemical abundance). The positive correlation between overall chemical abundance and palatability counters a typical assumption of latitudinal comparisons of chemical classes: that having a higher concentration of putatively defensive compounds equates to lower palatability. In the case of *Phytolacca*, high

overall abundance of a certain chemical abundance is not an effective strategy compared to low abundance of a different composition, underscoring the necessity of understanding trait function in order to investigate the geography of plant defense. For mature leaves, chemical composition associated with low palatability (high NMDS2 scores) has evolved in both subtropical and tropical populations. However, tropical and subtropical populations are highly divergent for NMDS4; these two chemical axes may oppose each other to determine the high palatability of tropical mature leaves, but work synergistically to effect low palatability of subtropical mature leaves. Thus, evidence for the BIH depends on the trait and geographic scale for mature-leaf defense.

The latitudinal patterns in leaf chemistry found here are similar to those of *Oenothera biennis* (Anstett et al. 2015), in the sense that the BIH is supported when defensive functions are understood. Anstett et al. (2015) focused on phenolics, which were known to be an important class of defense in *Oenothera*, while we had no *a priori* knowledge of defensive chemistry in *Phytolacca*. Still, in *O. biennis* there were mixed latitudinal patterns in total phenolics, oxidative capacity, and oenothien A and B, but herbivore resistance in a common garden was best predicted by oenothien A, which is found at greater concentrations in low-latitude populations. Thus, evidence for the BIH was only apparent in *Oenothera* when defensive function was taken into account. Similarly, we find that chemical traits in *Phytolacca* vary geographically, but knowledge about which traits predict palatability is critical to relate these traits to the BIH.

Many chemicals have been isolated from *Phytolacca*, but their roles in anti-herbivore defense have not been elucidated. Secondary compounds isolated from *P. americana* include saponins, alkaloids, tri-terpenoids, cardiac glycosides, antifungal peptides, and “pokeweed antiviral protein” (Stout et al. 1964, Suga et al. 1978, Kang and Woo 1980, Shao et al. 1999), any of which can

serve defensive functions (Mithofer and Boland 2012). However, of the 29 unique peaks that were found to predict palatability, most of what we have identified so far are related to saponins, which act as deterrents or growth inhibitors through various mechanisms such as disrupting cell membranes (Mithofer and Boland 2012, Cai et al. 2017). Thus, at least for *S. exigua*, saponins appear to be critical determinants of *Phytolacca* defense (saponins were also found to deter this herbivore in alfalfa; Cai et al. 2017). Eight peaks negatively correlate with palatability, which must be cautiously interpreted because palatability is confounded with latitude. Some of these peaks likely are parts of defensive compounds, while others may be genetically correlated with defensive compounds. The functions of the remaining peaks that were positively correlated with palatability are unclear. Larvae consuming more of these compounds perform relatively better, so some could be nutritious or feeding stimulants, but it is also possible that some of the positively correlated compounds could depress performance compared to a preferred host plant. Additionally, ubiquitous defensive chemicals could affect herbivores consistently across latitude, an effect that we cannot detect. Future studies of *Phytolacca* should isolate and manipulate compounds to test their functions, and/or cross populations to break up trait correlations.

As predicted, leaves varied with ontogeny—for every trait. Like many other studies, we find that young leaves are less tough and more nitrogen-rich than mature leaves, rendering them both more vulnerable and more valuable than mature leaves (McKey 1974, Coley 1983, Kursar and Coley 1991, McCall and Fordyce 2010). Consistent with optimal defense theory, young leaves have been found to be less palatable to generalists (Alba et al. 2014) and contain more chemical defenses than mature leaves (McCall and Fordyce 2010). We also found that young leaves were less palatable and had a higher abundance of chemicals, but chemical composition was important for predicting palatability in both leaf ages; in particular, NMDS2 shows dramatic leaf age and

latitudinal differences, and was a significant predictor of palatability for both young and mature leaves. Interestingly, there were only three chemical peaks important for predicting palatability of mature leaves, and 27 for young leaves, implying that a general understanding of plant defensive trait evolution may be incomplete if young leaves are not considered (Kursar et al. 2009, Coley et al. 2018).

For bioassay experiments, we used a generalist herbivore to avoid a possibility of local adaptation by specialists with restricted geographic ranges. For example, *Spodoptera eridania*, a congener of the herbivore we used that consumes *P. americana* in nature, performs better on a diet of southern *P. americana* from its own range versus a more northern plant population (Burnett and Jones 1973). A natural extension of our study is to compare responses of a generalist and specialist. While chemistry is often key to explaining variation in generalist palatability or herbivory (Johnson et al. 2009, Alba et al. 2014), compounds that are toxic to generalists may have a reduced negative impact or even a positive effect on specialists (e.g., sequestration; Agrawal et al. 2012, Quintero et al. 2014). For example, performance of a *Brassica* specialist herbivore was more affected by nutritional and mechanical defenses than plant chemistry (Travers-Martin and Müller 2008). We predict that a *Phytolacca* specialist would perform better on young than mature leaves; indeed, we find much greater herbivory rates on young leaves in the field, particularly in tropical and subtropical populations (Baskett and Schemske 2018), consistent with other field studies (Coley 1983, Lowman 1984, Filip et al. 1995, Coley et al. 2018) and experimental evidence that specialists prefer younger tissue (Quintero et al. 2014). However, latitudinal patterns in specialist palatability may depend on the coevolutionary history of herbivore and host (Anstett et al. 2015).

In addition to leaf chemistry, we found regional variation in nitrogen and leaf toughness, depending on leaf age, but there were no consistent patterns of lower nitrogen and tougher leaves in populations from lower latitudes, particularly when *P. rivinoides* is considered. In fact, *P. rivinoides* leaves have higher N than *P. americana*, with remarkably similar values to mean N in young and mature leaves across 22 tropical pioneer trees (Coley 1983). Intraspecific comparisons of nitrogen and toughness in several species of salt-marsh plants find that lower-latitude populations usually have less nitrogen and are tougher (Siska et al. 2002, Salgado and Pennings 2005). Two other species in the temperate zone have tougher leaves at lower latitudes (Andrew and Hughes 2005, Garibaldi et al. 2011), as we found within *P. americana*, while two tropical *Piper* species show no latitudinal variation in toughness (Salazar and Marquis 2012). However, most intraspecific latitudinal comparisons of N or C:N do not find a positive correlation with latitude (Gaston et al. 2004, Andrew and Hughes 2005, Hiura and Nakamura 2013, Nakamura et al. 2014). Given our results that chemical composition is a strong driver of palatability, the utility of N (or C:N) and toughness for testing the BIH is questionable.

In conclusion, using an in-depth study of a wide-ranging congener pair revealed strong support for Dobzhansky's biotic interactions hypothesis. Leaf chemistry strongly predicts palatability and shows striking latitudinal variation in support of the BIH. However, had we only measured traits such as nitrogen, leaf toughness, chemical richness, and chemical abundance as proxies of palatability and defense, we would have concluded that the biotic interactions hypothesis is unsupported. Thus, like Anstett et al. (2016) we argue that future tests of the BIH need to demonstrate functional importance of traits assumed to be defensive, as well as account for potentially obfuscating factors such as leaf ontogeny. Ultimately, we have shown that quantifying palatability and chemistry, even in a non-model system, is a powerful approach to

testing the BIH, and that Dobzhansky's grand hypothesis about the potential drivers of latitudinal diversity gradients deserves continued attention.

Acknowledgements

I wish to thank Christina Azodi (Michigan State University) for providing and assisting with the machine learning pipeline used for Random Forest analysis. Feedback on data analysis from Andrea Glassmire and Will Wetzel improved the quality of the manuscript. Emma Clark, Jessica Fry, Carolyn Graham, Abby Sulesky, Alyssa Tretheway, Andie Veach, and Stacey VanderWulp assisted substantially with data collection. This material is based upon work supported by the National Science Foundation Doctoral Dissertation Improvement Grant (No. 1701602) and by the Michigan State University Distinguished Fellowship.

APPENDIX

APPENDIX

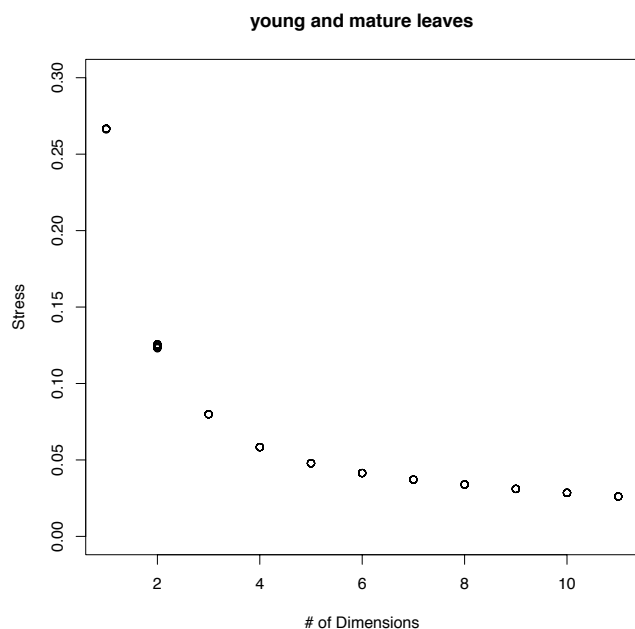


Figure 24. Scree plots of stress values for up to 11 NMDS dimensions using chemical abundances of 110 LC/MS peaks from young and mature leaves. Analyses are based on Bray-Curtis distances. We used four dimensions for further analyses, because stress was 0.058 at this threshold.

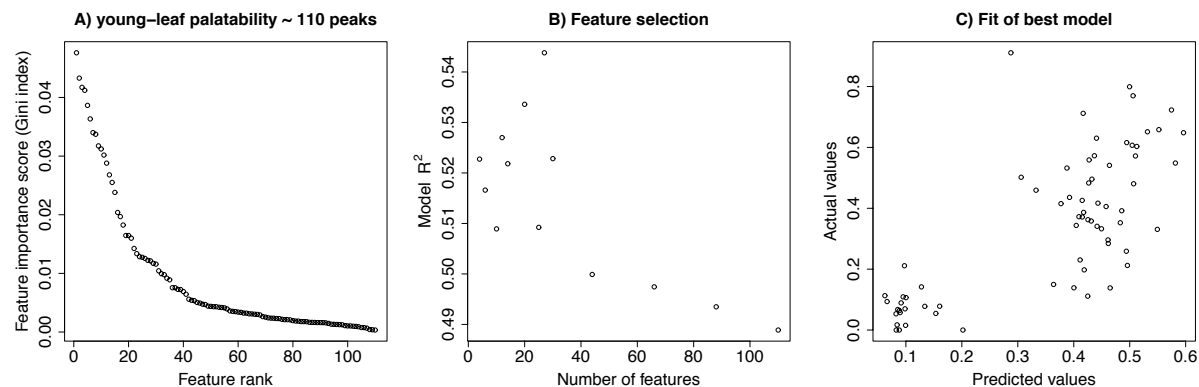


Figure 25. Model selection steps for predicting young-leaf palatability with chemical peak areas. A) Feature importance scores (Gini index) for initial model with 110 chemical peaks. B) Model fit based on number of features in the model. C) Actual and predicted values of palatability for the best-fitting model (highest R^2 in B, which includes 27 chemical peaks).

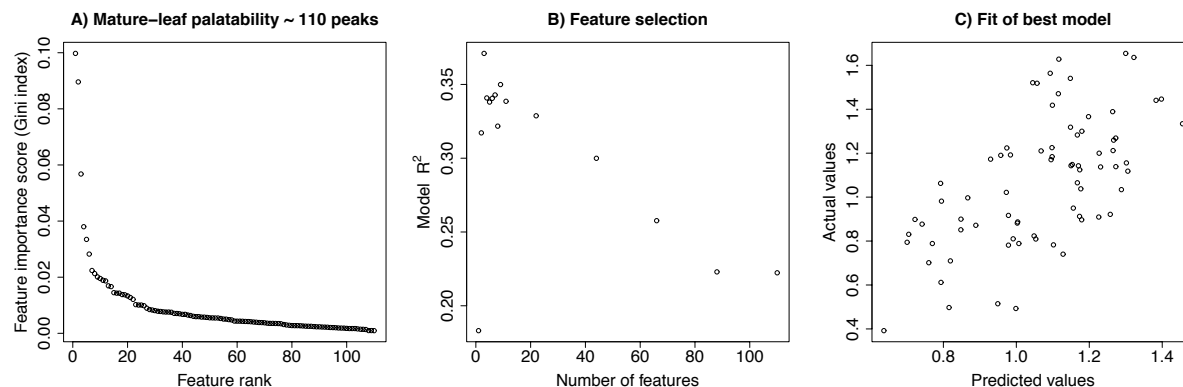


Figure 26. Model selection steps for predicting mature-leaf palatability with chemical peak areas. A) Feature importance scores (Gini index) for initial model with 110 chemical peaks. B) Model fit based on number of features in the model. C) Actual and predicted values of palatability for the best-fitting model (highest R^2 in B, which includes only three chemical peaks).

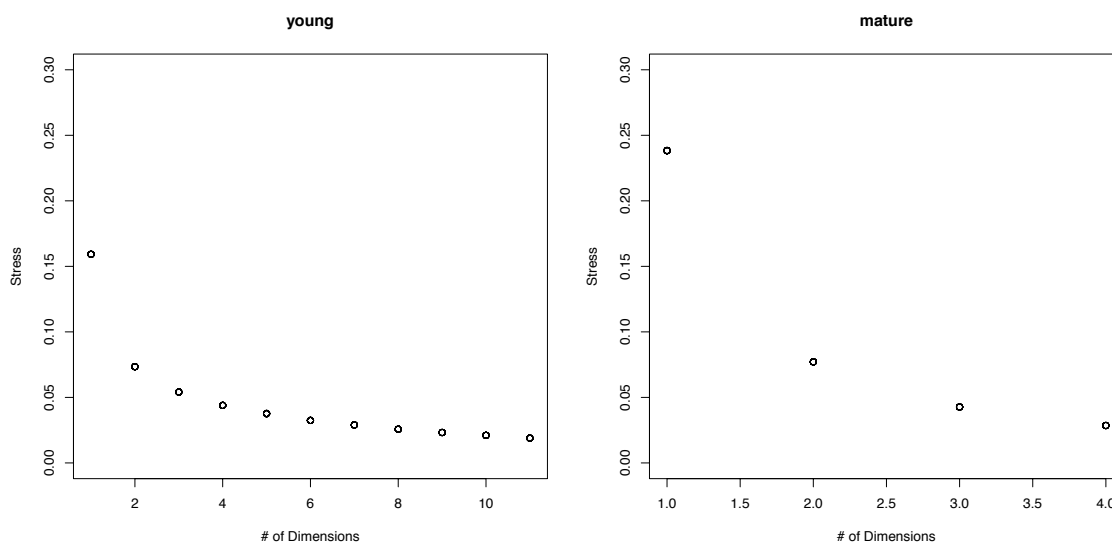


Figure 27. Scree plots of stress values based on number of NMDS dimensions using chemical abundances of LC/MS peaks that were found to be important for predicting palatability in young (left) and mature (right) leaves. Twenty-seven peaks were used for young leaves, and three for mature leaves. Analyses are based on Bray-Curtis distances. We used two dimensions for further analyses for each leaf age, because stress was an average of 0.075 at this threshold.

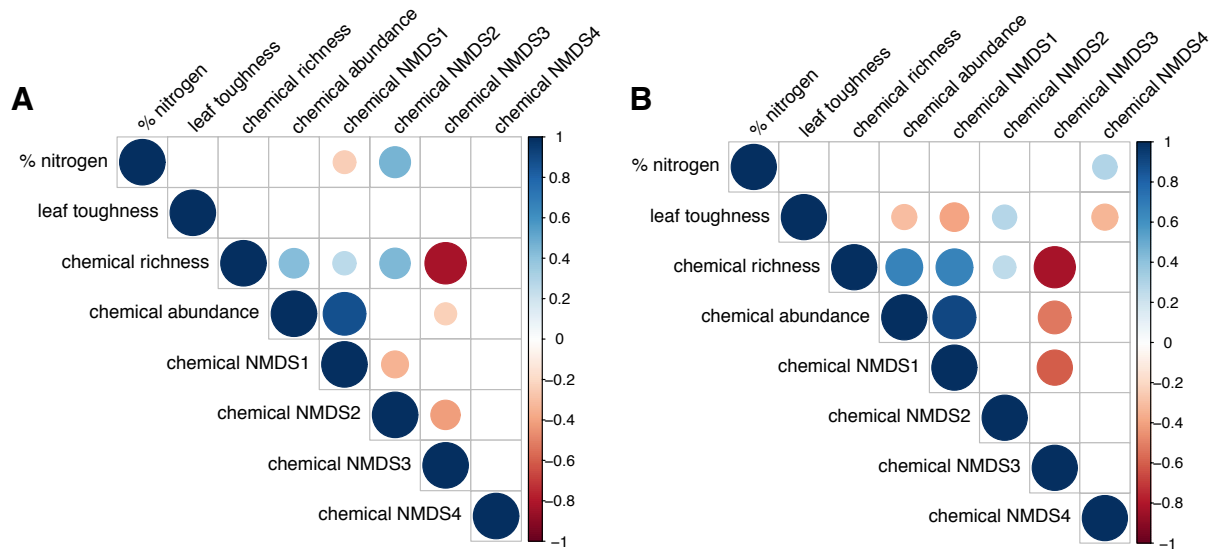


Figure 28. Leaf trait correlations for young (A) and mature (B) leaves. Circle color and size correspond to correlation magnitude according to key at right. Blank boxes are not significantly correlated ($\alpha = 0.05$).

REFERENCES

REFERENCES

- Abbott, H. C. d. S. (1887). "Comparative Chemistry of Higher and Lower Plants (Concluded)." The American Naturalist **21**(9): 800-810.
- Adams, J. M., B. Rehill, Y. Zhang and J. Gower (2009). "A test of the latitudinal defense hypothesis: herbivory, tannins and total phenolics in four North American tree species." Ecological Research **24**(3): 697-704.
- Agrawal, A. A., G. Petschenka, R. A. Bingham, M. G. Weber and S. Rasmann (2012). "Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions." New Phytologist **194**(1): 28-45.
- Agrawal, A. A. and M. G. Weber (2015). "On the study of plant defence and herbivory using comparative approaches: how important are secondary plant compounds." Ecology Letters **18**(10): 985-991.
- Alba, C., M. D. Bowers, D. Blumenthal and R. A. Hufbauer (2014). "Chemical and Mechanical Defenses Vary among Maternal Lines and Leaf Ages in *Verbascum thapsus* L. (Scrophulariaceae) and Reduce Palatability to a Generalist Insect." Plos One **9**(8).
- Andrew, N. R. and L. Hughes (2005). "Herbivore damage along a latitudinal gradient: relative impacts of different feeding guilds." Oikos **108**(1): 176-182.
- Anstett, D. N., J. R. Ahern, J. Glinos, N. Nawar, J.-P. Salminen and M. T. J. Johnson (2015). "Can genetically based clines in plant defence explain greater herbivory at higher latitudes?" Ecology Letters **18**(12): 1376-1386.
- Anstett, D. N., K. A. Nunes, C. A. Baskett and P. M. Kotanen (2016). "Sources of controversy surrounding latitudinal patterns in herbivory and defense." Trends in Ecology & Evolution **31**(10): 789-802.
- Barton, K. (2016). MuMIn: Multi-Model Inference. R package version 1.15.6, <https://CRAN.R-project.org/package=MuMIn>.
- Baskett, C. A. and D. W. Schemske (2018). "Latitudinal patterns of herbivore pressure in a temperate herb support the biotic interactions hypothesis." Ecology Letters **21**(4): 578-587.
- Batterman, S. A., N. Wurzburger and L. O. Hedin (2013). "Nitrogen and phosphorus interact to control tropical symbiotic N₂ fixation: a test in *Inga punctata*." Journal of Ecology **101**(6): 1400-1408.
- Bolser, R. C. and M. E. Hay (1996). "Are tropical plants better defended? Palatability and defenses of temperate vs tropical seaweeds." Ecology **77**(8): 2269-2286.
- Breiman, L. (2001). "Random forests." Machine Learning **45**(1): 5-32.

- Burnett, W. C. and S. B. Jones (1973). "Differential feeding of the southern armyworm on Kentucky and Florida populations of pokeweed." American Midland Naturalist **90**(1): 231-234.
- Cai, F. P., B. S. Watson, D. Meek, D. V. Huhman, D. J. Wheritt, C. Ben, L. Gentzbittel, B. T. Driscoll, L. W. Sumner and J. C. Bede (2017). "Medicago truncatula Oleanolic-Derived Saponins Are Correlated with Caterpillar Deterrence." Journal of Chemical Ecology **43**(7): 712-724.
- Clavijo McCormick, A., J. Gershenzon and S. B. Unsicker (2014). "Little peaks with big effects: establishing the role of minor plant volatiles in plant-insect interactions." Plant Cell and Environment **37**(8): 1836-1844.
- Close, D. C. and C. McArthur (2002). "Rethinking the role of many plant phenolics - protection from photodamage not herbivores?" Oikos **99**(1): 166-172.
- Coleman, J. S. and A. S. Leonard (1995). "Why It Matters Where on a Leaf a Folivore Feeds." Oecologia **101**(3): 324-328.
- Coley, P. D. (1983). "Herbivory and defensive characteristics of tree species in a lowland tropical forest." Ecological Monographs **53**(2): 209-233.
- Coley, P. D. and T. M. Aide (1991). Comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. P. W. Price, T. M. Lewinsohn, G. W. Fernandes and W. W. Benson. New York, USA, John Wiley and Sons: 25-49.
- Coley, P. D. and J. A. Barone (1996). "Herbivory and plant defenses in tropical forests." Annual Review of Ecology and Systematics **27**: 305-335.
- Coley, P. D., M.-J. Endara and T. A. Kursar (2018). "Consequences of interspecific variation in defenses and herbivore host choice for the ecology and evolution of Inga, a speciose rainforest tree." Oecologia.
- Descombes, P., J. Marchon, J. N. Pradervand, J. Bilat, A. Guisan, S. Rasmann and L. Pellissier (2017). "Community-level plant palatability increases with elevation as insect herbivore abundance declines." Journal of Ecology **105**(1): 142-151.
- Dobzhansky, T. (1950). "Evolution in the tropics." American Scientist **38**: 209-221.
- Ehrlich, P. R. and P. H. Raven (1964). "Butterflies and plants - A study in coevolution." Evolution **18**(4): 586-608.
- Filip, V., R. Dirzo, J. M. Maass and J. Sarukhan (1995). "Within-year and among-year variation in the levels of herbivory on the foliage of trees from a Mexican tropical deciduous forest." Biotropica **27**(1): 78-86.
- Fischer, A. G. (1960). "Latitudinal variation in organic diversity." Evolution **14**: 64-81.

- Garibaldi, L. A., T. Kitzberger and A. Ruggiero (2011). "Latitudinal decrease in folivory within *Nothofagus pumilio* forests: Dual effect of climate on insect density and leaf traits?" Global Ecology and Biogeography **20**(4): 609-619.
- Gaston, K. J., D. R. Genney, M. Thurlow and S. E. Hartley (2004). "The geographical range structure of the holly leaf-miner. IV. Effects of variation in host-plant quality." Journal of Animal Ecology **73**(5): 911-924.
- Hiura, T. and M. Nakamura (2013). "Different mechanisms explain feeding type-specific patterns of latitudinal variation in herbivore damage among diverse feeding types of herbivorous insects." Basic and Applied Ecology **14**(6): 480-488.
- Johnson, M. T. J., A. A. Agrawal, J. L. Maron and J. P. Salminen (2009). "Heritability, covariation and natural selection on 24 traits of common evening primrose (*Oenothera biennis*) from a field experiment." Journal of Evolutionary Biology **22**(6): 1295-1307.
- Kang, S. S. and W. S. Woo (1980). "Triterpenes from the berries of *Phytolacca americana*." Journal of Natural Products **43**(4): 510-513.
- Kursar, T. A. and P. D. Coley (1991). "Nitrogen content and expansion rate of young leaves of rainforest species: Implications for herbivory." Biotropica **23**(2): 141-150.
- Kursar, T. A. *et al.* (2009). "The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*." Proceedings of the National Academy of Sciences of the United States of America **106**(43): 18073-18078.
- Lowman, M. D. (1984). "An assessment of techniques for measuring herbivory: Is rainforest defoliation more intense than we thought?" Biotropica **16**(4): 264-268.
- Macel, M., N. M. Van Dam and J. J. B. Keurentjes (2010). "Metabolomics: the chemistry between ecology and genetics." Molecular Ecology Resources **10**(4): 583-593.
- Martz, F., M. L. Sutinen, K. Derome, G. Wingsle, R. Julkunen-Tiitto and M. Turunen (2007). "Effects of ultraviolet (UV) exclusion on the seasonal concentration of photosynthetic and UV-screening pigments in Scots pine needles." Global Change Biology **13**(1): 252-265.
- Mattson, W. J. (1980). "Herbivory in Relation to Plant Nitrogen-Content." Annual Review of Ecology and Systematics **11**: 119-161.
- McCall, A. C. and J. A. Fordyce (2010). "Can optimal defence theory be used to predict the distribution of plant chemical defences?" Journal of Ecology **98**(5): 985-992.
- McKey, D. (1974). "Adaptive Patterns in Alkaloid Physiology." The American Naturalist **108**(961): 305-320.
- Miller, J. C. and P. E. Hanson (1989). "Laboratory feeding tests on the development of gypsy moth larvae with reference to plant taxa and allelochemicals." Bulletin of the Agricultural Experimental Station of Oregon State University **674**: 1-63.

- Mithofer, A. and W. Boland (2012). Plant Defense Against Herbivores: Chemical Aspects. Annual Review of Plant Biology, Vol 63. S. S. Merchant. **63**: 431-450.
- Moles, A. T., S. P. Bonser, A. G. B. Poore, I. R. Wallis and W. J. Foley (2011). "Assessing the evidence for latitudinal gradients in plant defence and herbivory." Functional Ecology **25**(2): 380-388.
- Moles, A. T. *et al.* (2011). "Putting plant resistance traits on the map: a test of the idea that plants are better defended at lower latitudes." New Phytologist **191**(3): 777-788.
- Murray, K. G. (1988). "Avian Seed Dispersal of Three Neotropical Gap-Dependent Plants." Ecological Monographs **58**(4): 271-298.
- Nakamura, M., N. Inari and T. Hiura (2014). "Spatial variation in leaf traits and herbivore community within the beech canopy between two different latitudes." Arthropod-Plant Interactions **8**(6): 571-579.
- Oksanen, J., F. Guillaume Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs and H. Wagner (2017). vegan: Community Ecology Package. R package version 2.4-5, <https://CRAN.R-project.org/package=vegan>.
- Pearse, I. S. and A. L. Hipp (2012). "Global patterns of leaf defenses in oak species." Evolution **66**(7): 2272-2286.
- Pennings, S. C., E. L. Siska and M. D. Bertness (2001). "Latitudinal differences in plant palatability in Atlantic coast salt marshes." Ecology **82**(5): 1344-1359.
- Perez-Harguindeguy, N., S. Diaz, F. Vendramini, J. H. C. Cornelissen, D. E. Gurvich and M. Cabido (2003). "Leaf traits and herbivore selection in the field and in cafeteria experiments." Austral Ecology **28**(6): 642-650.
- Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar and R. C. Team (2017). nlme: Linear and nonlinear mixed effects models. R package version 3.1-131, <https://CRAN.R-project.org/package=nlme>.
- Quintero, C., E. C. Lampert and M. D. Bowers (2014). "Time is of the essence: direct and indirect effects of plant ontogenetic trajectories on higher trophic levels." Ecology **95**(9): 2589-2602.
- R Core Team (2014). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing.
- Ranganathan, Y. and R. M. Borges (2010). "Reducing the babel in plant volatile communication: using the forest to see the trees." Plant Biology **12**(5): 735-742.

- Rasmann, S. and A. A. Agrawal (2011). "Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory." Ecology Letters **14**(5): 476-483.
- Richards, L. A., L. A. Dyer, M. L. Forister, A. M. Smilanich, C. D. Dodson, M. D. Leonard and C. S. Jeffrey (2015). "Phytochemical diversity drives plant–insect community diversity." Proceedings of the National Academy of Sciences **112**(35): 10973-10978.
- Salazar, D., J. Lokvam, I. Mesones, M. Vásquez Pilco, J. M. Ayarza Zuñiga, P. de Valpine and P. V. A. Fine (2018). "Origin and maintenance of chemical diversity in a species-rich tropical tree lineage." Nature Ecology & Evolution **2**(6): 983-990.
- Salazar, D. and R. J. Marquis (2012). "Herbivore pressure increases toward the equator." Proceedings of the National Academy of Sciences of the United States of America **109**(31): 12616-12620.
- Salgado, C. S. and S. C. Pennings (2005). "Latitudinal variation in palatability of salt-marsh plants: Are differences constitutive?" Ecology **86**(6): 1571-1579.
- Sauer, J. D. (1952). "A geography of pokeweed." Ann Missouri Bot Gard **39**((2)): 113-125.
- Schemske, D. W. (2009). Biotic interactions and speciation in the tropics. Speciation and Patterns of Diversity. R. K. Butlin, J. R. Bridle and D. Schluter. Cambridge, UK, Cambridge University Press: 219-239.
- Schemske, D. W., G. G. Mittelbach, H. V. Cornell, J. M. Sobel and K. Roy (2009). "Is there a latitudinal gradient in the importance of biotic interactions?" Annual Review of Ecology Evolution and Systematics **40**: 245-269.
- Schilmiller, A., F. Shi, J. Kim, A. L. Charbonneau, D. Holmes, A. D. Jones and R. L. Last (2010). "Mass spectrometry screening reveals widespread diversity in trichome specialized metabolites of tomato chromosomal substitution lines." Plant Journal **62**(3): 391-403.
- Shao, F., Z. Hu, Y. M. Xiong, Q. Z. Huang, C. G. Wang, R. H. Zhu and D. C. Wang (1999). "A new antifungal peptide from the seeds of *Phytolacca americana*: characterization, amino acid sequence and cDNA cloning." Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology **1430**(2): 262-268.
- Siska, E. L., S. C. Pennings, T. L. Buck and M. D. Hanisak (2002). "Latitudinal variation in palatability of salt-marsh plants: Which traits are responsible?" Ecology **83**(12): 3369-3381.
- Stout, G. H., V. F. Stout and B. M. Malofsky (1964). "Phytolaccagenin - Light atom x-ray structure proof using chemical information." Journal of the American Chemical Society **86**(5): 957-&.
- Suga, Y., Y. Maruyama, S. Kawanishi and J. Shoji (1978). "Studies on constituents of Phytolaccaceous plants. I. On the structures of Phytolaccasaponin B, E and G from the roots of *Phytolacca americana* L." Chemical & Pharmaceutical Bulletin **26**(2): 520-525.

Tegelberg, R., R. Julkunen-Tiitto and P. J. Aphalo (2001). "The effects of long-term elevated UV-B on the growth and phenolics of field-grown silver birch (*Betula pendula*)."
Global Change Biology **7**(7): 839-848.

Travers-Martin, N. and C. Müller (2008). "Matching plant defence syndromes with performance and preference of a specialist herbivore." Functional Ecology **22**(6): 1033-1043.

Zangerl, A. R. and M. R. Berenbaum (2003). "Phenotype matching in wild parsnip and parsnip webworms: Causes and consequences." Evolution **57**(4): 806-815.

CHAPTER 4: EVOLUTION AND GENETICS OF MUTUALISM

Introduction

In his book *Fertilisation of Orchids* (1862), Darwin used flowers as convenient systems to study adaptive evolution. That flowers are key traits in a mutualistic interaction was not the main focus of his research, but rather a useful feature that allowed him to explore how natural selection contributes to adaptation. His work on floral adaptation set the stage for much of the next 150 years of empirical research on evolution in mutualisms, and it is only in the last several decades that researchers have asked evolutionary questions in other mutualistic systems, and begun to think about and study mutualism evolution itself.

In this chapter, we focus on patterns and processes of adaptation in mutualisms, including the genetic basis of mutualistic evolution. The overarching question is, how does mutualistic evolution differ from evolution driven by other selective agents, such as the abiotic environment or an antagonistic interaction? For example, is selection stronger or weaker compared to other interactions? Do genes underlying mutualistic adaptations tend to have large or small effects on phenotype? Throughout our review, we provide a conceptual perspective on mutualism evolution, and where possible, offer empirical examples. Although we have learned much from empirical research on mutualism evolution, researchers usually ask different questions in different systems. Therefore, we believe there is a lack of available empirical evidence to evaluate these general questions. The next major hurdle in understanding the evolution and genetics of mutualism is to ask questions across systems, so we can begin to understand the ways in which the evolution of mutualisms may be unique.

A comprehensive study of the genetics and evolution of a given mutualism should include phylogenetic evidence of the history of the interactions, studies of mutualistic traits and their selective value, and an understanding of the genes that underlie mutualistic adaptations,

particularly traits involved in adaptive transitions. At present, there are no systems for which all of this information is known. In particular, there are few systems that provide both comprehensive genetic resources and an opportunity to evaluate ecological interactions. We thus focus many of our empirical examples on two mutualisms in which researches have answered disparate questions, using different approaches: 1) plant-pollinator interactions, and 2) endosymbioses between bacteria and their sap-sucking insect hosts. Studies of plant-pollinator mutualisms provide information on the adaptive and genetic changes involved in the putative early steps of pollinator transitions, while studies of endosymbiosis illustrate the value of molecular genetic approaches to understand the genomic consequences of long-term mutualism evolution. Our goal is to use the handful of mutualisms that are relatively well studied to suggest processes and patterns likely to hold more generally.

Natural selection in mutualisms

Does natural selection driven by mutualistic interactions differ from selection imposed by the abiotic environment or resulting from an antagonistic interaction? First, consider how adaptation to a biotic factor may differ from adaptation to an abiotic factor. In biotic interactions, adaptive peaks are ‘moving’, owing to the coevolution of interacting species, while adaptive peaks may be more often ‘fixed’ for an abiotic selective agent because the abiotic environment cannot evolve or coevolve (Schemske 2002, Schemske 2009). This may be particularly true in the tropics, where the abiotic environment is more consistent and predictable than in the temperate zone (Janzen 1967; Schemske 2009).

If biotic adaptive peaks are more mobile than abiotic peaks, then biotic selection may be stronger, more often directional than stabilizing (Rutter and Rausher 2004), and more spatially

and temporally variable than abiotic selection (Siepielski et al. 2009). Many models assume that the fitness of partners is maximized when traits are matched (Doebeli and Dieckmann 2000), and would not predict a highly mobile adaptive peak. Both directional (e.g., Rutter and Rausher 2004) and stabilizing (e.g., Wright and Meagher 2004) selection gradients have been recorded for mutualistic traits in natural populations, but there is not enough data to evaluate whether one is more common, or to analyze the context in which one or the other may be expected. Several reviews have compiled estimates of the form and magnitude of selection from observational studies (Siepielski et al. 2009, Kingsolver and Diamond 2011), yet none has compared these metrics for different selective agents, e.g. biotic versus abiotic factors, or for mutualisms in particular. This may be due, in part, to the difficulty in determining selective agents in observational studies (Wade and Kalisz 1990, Siepielski et al. 2009).

Evidence from studies of experimental evolution shows that selection does differ when the adaptive peak is fixed versus moving, but exactly how it differs (in strength or variability) is unclear. In an antagonistic bacteria-phage system, when bacteria were allowed to coevolve, the phage populations experienced greater genetic and phenotypic divergence from the ancestor and greater between-population divergence, compared to populations in which bacteria were not allowed to coevolve (Paterson et al. 2010). Experimental evolution may be a powerful approach for revealing how selection is unique in coevolving systems (Hembry et al. 2014), but we are unaware of any mutualistic systems that have been used to address this question.

Thus far, we have focused on how selection may differ for moving and fixed adaptive peaks. How might selection in an antagonistic interaction differ from selection in a mutualistic interaction? Both interactions may involve moving, coevolving adaptive peaks. However, an

antagonism consists of one-way exploitation, while a mutualism consists of mutual exploitation. How this difference might affect selection is unclear.

Current observational and experimental evidence is insufficient to determine whether the form of natural selection in mutualisms is unique. In fact, we are aware of only four mutualisms in which selection gradients have been measured, all involving plants: pollination (e.g., Campbell et al. 1994, Campbell et al. 1996), ant-plant protection (e.g., Rudgers and Strauss 2004, Rutter and Rausher 2004), seed dispersal (e.g., Siepielski and Benkman 2010), and the legume-rhizobia mutualism (Porter and Simms 2014). Selection gradients in mutualisms have only been estimated for one side of the interaction (plants), except for the study by Porter and Simms (2014), which found selection for cheating in rhizobia but not in their legume hosts. To answer the question of whether selection in mutualisms differs in form or magnitude from selection by other types of selective agents, studies of additional mutualisms and estimates of selection gradients for both interacting species are needed.

The genetics of mutualism evolution

To understand the genetics underlying adaptation in a mutualism, it is important to consider the evolutionary history of the interaction. Is the mutualism in its early stages, such as a recent shift in pollinators between sister species of plants, or has the mutualism been in place for millennia? We expect that many fewer genes will be involved in the early stages of an interaction, but they may have relatively large phenotypic effects. In addition, an interaction that has evolved only rarely, such as the legume-rhizobia interaction, may have a more complex genetic basis than one that has evolved independently many times, such as ant-plant protection mutualisms (Weber and Keeler 2013).

Most models of adaptive evolution have focused on fixed adaptive peaks. How might a coevolving, moving adaptive peak affect the genetics of mutualism evolution? Here we explore this question with regards to both advanced and early stages of mutualism evolution.

Advanced stages

Once mutualisms are established, the interaction will be evolutionarily ‘fine-tuned’ over hundreds or perhaps thousands of generations of reciprocal selection and coevolution, as each organism is selected to extract higher benefit from the interaction while providing less in return. This ongoing mutual exploitation provides an opportunity for various traits, pathways, and genes to be modified or lost, so we expect that the genetic basis of older mutualistic adaptations would involve many more genes than that of more recently derived adaptations.

In some cases, genes involved in long-established mutualisms become tightly linked, and are essentially inherited as a single unit. The genes controlling many aspects of the mutualism between legumes and their nitrogen-fixing bacteria are found on transmissible genetic elements, and can be considered ‘islands’ of symbiotic genes, hundreds of kilobases long (MacLean et al. 2007). The finding that the genomic regions involved in symbiotic functions of rhizobia are highly dynamic, with a large number of insertion sequence elements (MacLean et al. 2007), illustrates that there has been substantial molecular evolution related to the symbiosis.

Gene loss and genome reduction is commonly documented in symbiotic mutualisms, which can in turn affect the host genome. For example, recent sequencing of the pea aphid (*Acyrtosiphon pisum*) genome has revealed twelve genes of bacterial origin and complementary amino acid synthesis capabilities with its obligate *Buchnera* symbiont due to gene loss from the bacterium (Richards et al. 2010). However, the extent of gene loss may depend on the mode of

transmission of symbionts between hosts. Intracellular insect endosymbionts often show evidence of genome reduction compared to free-living relatives (McCutcheon and Moran 2012), but rhizobia, which are transmitted horizontally via soil, do not. In the attine ant-fungus mutualism, phylogenetic analyses suggest that fungi are not strictly vertically propagated between ants, and that clades of symbiotic fungi are considerably younger than clades of their corresponding ant hosts (Mikheyev et al. 2010). Genome sequencing has revealed the loss of genes associated with nutrient acquisition in leafcutter ants, and although detailed genomic data for the fungi are not yet available, we might hypothesize that the degree of gene loss will be related to the mode of transmission, with vertically transmitted fungi exhibiting greater gene loss.

Dramatic cases of genome size reduction have been recorded in insect intracellular endosymbiont genomes, which consist of a subset of genes found in their free-living relatives (McCutcheon and Moran 2012). In fact, several intracellular symbionts in sap-sucking insects have the smallest recorded genomes of any organism (McCutcheon and Moran 2012). This extreme genome reduction is hypothesized to be due to drift; with relaxed selection on non-essential genes, deletions are easily fixed due to extreme population bottlenecks during vertical transmission from insect parent to offspring (McCutcheon and Moran 2012). Loss of DNA repair genes is hypothesized to contribute to other patterns commonly seen in endosymbiont genomes (i.e. rapid sequence evolution and A+T bias; McCutcheon and Moran 2012).

Genome reduction in insect endosymbionts can have tangible impacts on the host's ecology. The glassy-winged sharpshooter (*Homalodisca vitripennis*) is a leafhopper (Cicadellidae) that feeds on xylem sap, which is a particularly nutrient-poor food source. The sharpshooter hosts two endosymbionts: *Sulcia muelleri* (Bacteroidetes) is an obligate endosymbiont acquired over 260 mya, which is present in many insects in the Auchenorrhyncha suborder and has one of the

smallest recorded bacterial genomes with only 245 kilobases (McCutcheon and Moran 2007). *Baumannia cicadellinicola* (Gammaproteobacteria) is more recently associated with the sharpshooter, and is not found in other plant-sucking insects (Moran et al. 2008). Neither of the two bacteria is capable of synthesizing all amino acids necessary for animals on its own; rather, they have complementary synthesis pathways (McCutcheon and Moran 2007). Presumably, when the sharpshooter ancestor was feeding on phloem, *Sulcia* would have lost the ability to synthesize nutrients that are present in phloem. This gene loss is unidirectional, so it is hypothesized that association with *Baumannia* was necessary for sharpshooters to feed on xylem, which contains fewer nutrients than phloem (Moran et al. 2008).

The long-term genetic consequences of mutualism, particularly obligate symbioses, can be dramatic. As genomic data become available for more organisms, we will be able to ask questions about how genetics and ecological context (e.g., transmission mode and degree of specialization) interact to influence mutualism evolution.

Early stages

One of the persistent controversies in the study of adaptation concerns the effect size of mutations that are fixed as populations adapt to a new environment (Barton and Keightley 2002, Rockman 2011). Do populations adapt by a large number of small ‘mutational steps’, or are large mutational steps sometimes involved? As discussed above, coevolution in mutualisms may lead to mobile adaptive optima, which could influence the underlying genetics of mutualistic traits. However, theory of the genetic architecture to this point has ignored the agents of selection.

Fisher (1930) proposed that adaptations are comprised of a nearly infinite number of mutations, each with an infinitesimally small effect on the phenotype, based on the assumption

that large effect mutations have negative, pleiotropic effects, and hence move populations away from their optimum phenotype. Kimura (1983) proposed a modification to Fisher's infinitesimal theory, suggesting that mutations of intermediate effect size played the major role in adaptation since these are less prone to stochastic loss via genetic drift than are small effect mutations. Orr (1998) proposed that large effect mutations could be favored when populations are far from their optimum, but that mutations of progressively smaller effect would contribute to adaptation as the population approached its optimum, resulting in a combination of a few large and many small effect mutations during the adaptive 'walk'. Although there is considerable evidence to support Orr's view that adaptations are often comprised of large effect mutations (Barrett and Hoekstra 2011), there is still controversy regarding the importance of major genes in adaptive evolution (Rockman 2011).

A key assumption of the models of Fisher, Kimura and Orr is that the optimum phenotype is stationary. If the optimum is constantly moving, as might be expected for coevolving mutualists or antagonists (Schemske 2002, Schemske 2009, Louthan and Kay 2011, Matuszewski et al. 2014), large effect mutations might be favored more often than if the optimum was fixed (Orr 2005). It could also be argued that large mutational steps are required for a major ecological transition such as the origin of a mutualism, for example, where the sign of an interaction between species changes from negative to positive (as in the case of a seed predator that becomes a pollinator). There is presently insufficient data to know if the early mutational steps involved in mutualism evolution differ in size or number from those involved in adaptation to antagonistic interactions or to abiotic sources of selection. At issue is the shape and stability of the fitness landscape. Are the valleys between fitness peaks wider (more ecologically divergent) or deeper

(negative fitness consequences are greater for moving away from the optimum) on average for mutualism evolution? Are the peaks more mobile?

Frequent evolutionary transitions between modes of pollination make plant-pollinator mutualisms a fertile system for investigating early stages in mutualism evolution. Pollination can be observed or manipulated in the field and greenhouse, allowing researchers to study traits that are known to be ecologically relevant, such as structures involved in attraction and reward (e.g. petals and nectaries). The genetic basis of these traits can then be elucidated by mapping Quantitative Trait Loci (QTL, see below), and hypotheses for the traits and genes that contribute to early stages of pollinator shifts can be tested.

In brief, QTL mapping uses genetic markers to identify genomic regions associated with phenotypic traits in a ‘mapping’ population. The mapping population is produced by crossing populations or species that differ in the traits of interest, followed by one or more rounds of crossing or self-fertilization to produce a genetically and phenotypically diverse population with recombined parental genotypes. Compared to many animals, plants are ideal for such studies because crosses between populations or species are often fertile, and researchers can obtain the large number of progeny required to maximize statistical power. Thus far, QTL studies of pollination mutualisms have been ‘unilateral’ (Bronstein 1994). While they identify genetic mechanisms of plant adaptation, they do not reciprocally elucidate the genetic basis of pollinator adaptations. Here we discuss examples from two different plant systems, *Mimulus* (Phrymaceae) and *Petunia* (Solanaceae), to highlight progress made to date.

Bradshaw et al. (1998) investigated the genetic basis of traits that contribute to floral divergence between bee-pollinated *Mimulus lewisii* and its close relative, hummingbird-pollinated *M. cardinalis*. *Mimulus lewisii* flowers are pink, produce little nectar, and have broad

petals thrust forward as a landing platform for bees, and the anthers and stigma are inserted within the floral tube. *Mimulus cardinalis* flowers are red, produce large quantities of nectar, have reflexed petals, and the anthers and stigma protrude from the floral tube. Genetic mapping studies in this system identified 12 QTL for nine floral traits, including at least one QTL of moderate to large effect for each trait (Bradshaw et al. 1998). Field studies of pollinator visitation in arrays of F2 hybrids determined that petal carotenoid concentration and nectar volume had the greatest influence on pollinator visitation, with bees exhibiting a strong preference for pink-flowered plants with low petal carotenoid concentration, while hummingbirds preferred plants with high nectar volume (Schemske and Bradshaw 1999). That both of these traits are controlled by QTL of large effect in this system suggests that the transition from bee to hummingbird pollination may have involved relatively few genes of large effect.

A major role of the single QTL for petal carotenoid production was demonstrated in field experiments intended to mimic the effect of a mutation at the carotenoid locus. Near Isogenic Lines (NILs) were produced by introgressing the QTL for petal carotenoids from *M. cardinalis* to *M. lewisii*, and vice versa (Bradshaw and Schemske 2003). Here we focus on the substitution of the *M. cardinalis* allele into *M. lewisii*, given phylogenetic analysis suggesting that bee pollination is ancestral and that hummingbird pollination is derived (Beardsley et al. 2003). Bee visitation to the pink-flowered, *M. lewisii* parent was much higher than that to the orange-red *M. lewisii* NIL, while the converse was observed for hummingbirds, suggesting that increased petal carotenoid concentration was probably a major, early step in the shift to hummingbird pollination.

Petunia is a South American genus of approximately 16 species of herbaceous perennials that has become an important model system for elucidating the genetics of floral traits and

pollinator transitions (Venail et al. 2010). Bee pollination appears to be the ancestral state, but species pollinated by hawkmoths and by hummingbirds are also observed. For example, hummingbird-pollinated *P. exserta* is thought to be derived from the hawkmoth-pollinated *P. axillaris* (Klahre et al. 2011). Both of these species possess long floral tubes and produce a large volume of nectar, but *P. exserta* has unscented, red flowers, while *P. axillaris* has strongly scented, white flowers (Klahre et al. 2011). QTL mapping of floral scent identified two large-effect QTL (Klahre et al. 2011), and one gene region containing the myb transcription factor *ODORANT1*, which is known to regulate enzymes involved in scent production (Klahre et al. 2011). To examine the effects of scent and flower color on hawkmoth visitation in this system, Klahre et al. (2011) introgressed the major scent QTL into both white- and red-flowered plants, and carried out pollinator choice experiments in a wind tunnel with the hawkmoth *Manduca sexta*. Moths displayed a strong preference for scented over non-scented flowers of the same color, but when presented with both scented, red flowers and non-scented, white flowers, there was no clear preference. These results suggest that floral scent and color are both strong cues to pollinators, and that at least some of the early stages in the pollinator shift probably involved large mutational steps.

To conclude, the genetic architecture of adaptation in mutualisms is poorly understood, but there is evidence supporting the idea that genes of large effect are important in the transitions between different pollination syndromes. Despite limited power to generalize, the studies of *Mimulus* and *Petunia* both show evidence of QTL of large effect. More general support for the hypothesis of larger effect sizes involved in biotic adaptation was obtained by Louthan and Kay (2011), who found that QTL controlling floral traits involved in pollination mutualisms had significantly larger effect sizes than did those for non-flowering traits.

Emerging systems and tools

Moving beyond pollination

The available studies of natural selection in mutualisms are primarily restricted to plant-animal interactions, typically plant-pollinator mutualisms and then almost exclusively from the plant perspective. To achieve a synthesis that addresses the major questions in mutualism evolution will require many studies covering a wider range of mutualisms. See boxes in Baskett and Schemske (2015) for references; these highlight several emerging systems for the study of mutualism evolution which describe the contemporary interaction, provide the phylogenetic context, discuss the genetic basis of mutualistic traits and early steps in the evolution of the mutualism, and present ideas for future research.

In the attine ant-fungus mutualism, ants collect plant fragments that are used as a substrate for fungi cultured and consumed by ants in subterranean gardens. This interaction has existed for 55 million years, yet there is substantial variation in its specificity. The ants are obligately associated with fungi, but some of the fungi are facultative, and are close relatives of free-living taxa. A strong indication of coevolution in this mutualism is the finding from genomic studies that fungi have evolved a novel enzyme for detoxification of host plant chemicals, while ants have lost detoxification genes.

The mutualism between legumes and nitrogen-fixing bacteria takes place in root nodules colonized by soil bacteria (*Rhizobium*) that gain access to the roots through a complex process of infection. The bacteria are facultative and horizontally transmitted, thus there is considerable opportunity for gene exchange with free-living strains. Particularly fascinating is the finding that genes involved in various aspects of plant development have been co-opted for nodulation.

The marine mutualism between squids and bioluminescent bacteria is an extraordinary example of how mutualistic interactions can expand the behavioral repertoire of the host. In this system, free-living bioluminescent bacteria (family Vibrionaceae) colonize the light organ of uninfected, juvenile squid, and there produce light that contributes to prey capture and predator avoidance. Phylogenetic evidence suggest that the mutualism has evolved twice, and is ancestral in the family Sepiolidae where it has been lost in three of seven genera. This may be a case where the formation of a mutualism is difficult, but that loss is straightforward, and repeatedly favored. Of particular note is the evolution of host immune functions that create conditions favorable to the growth and reproduction of particular bacterial strains in the light organ.

The results that emerge from studies of these and other emerging systems will dramatically improve our understanding of mutualism evolution. One system with great promise is the mutualism between plants that provide extra-floral nectar to ants in exchange for protection against herbivores. Extra-floral nectaries have evolved independently many times (Weber and Keeler 2013), and there is a range of generalized to specialized ant x plant interactions. Thus, this system provides an interesting opportunity to investigate different stages of mutualism evolution and to compare the phenotypic and genetic steps involved in multiple independent clades. Cotton (*Gossypium*) may be of particular interest, in that some species produce leaf and extra-floral nectaries that are involved in an ant-plant mutualism (Rudgers and Strauss 2004), and genetic studies find that the loss of nectaries has a simple genetic basis (Meyer and Meyer 1961).

Genomic approaches

The extraordinary breakthroughs in gene sequencing technologies have provided new insights into the long-term evolutionary consequences of mutualism evolution. For example, the proliferation of available data on insect endosymbionts has prompted fascinating lines of inquiry into the causes and consequences of genome reduction (McCutcheon and Moran 2012). Yet in most studies, molecular work has far outpaced our knowledge of the natural history and ecology of most interactions. Work on the relationship between pea aphid and its facultative endosymbiont *Hamiltonella defensa* exemplifies what we can learn from combining ecological and genetic information. *Hamiltonella defensa* retains genes related to host invasion (Moran et al. 2005), and because it lacks genes for synthesizing essential amino acids, it appears that both *H. defensa* and the pea aphid rely on the host's obligate endosymbiont *Buchnera* to fulfill their nutritional needs from phloem sap (Degnan et al. 2009). The mutualistic function provided by *H. defensa* is defense against endoparasitic wasps by disrupting early development of wasp larvae (Oliver et al. 2003). The association is obligate for the bacteria but facultative for the aphid, as the proportion of infected aphids increases with higher parasitoid pressure, and declines when parasitoid pressure is low (Oliver et al. 2008). Since they are more tractable to manipulate compared to obligate relationships, facultative symbioses could be key for understanding the early steps and genetics of mutualism establishment and transitions, especially when studied in a phylogenetic context for both partners.

Experimental evolution

Evolution experiments in microbes may be a powerful approach to complement theory and studies in natural systems for elucidating the processes of mutualism evolution and for answering

the question of how evolution in mutualisms may be unique compared to other selective agents. An astonishing example demonstrating possible early steps towards mutualism occurred in a laboratory setting in the late 1960s. After less than 1,000 generations (five years), the D strain of *Amoeba proteus* became obligately dependent on a bacteria (*Candidatus Legionella jeonii*) that was initially pathogenic (Jeon 2004 and references therein). In later experimental introductions of the derived symbiotic bacteria into uninfected hosts, the amoebae evolved dependence in only 200 generations (18 months) (Jeon 2004). The only known benefit for the amoebae is accelerated growth during initial infection. The infected strain performs worse than the uninfected ancestral strain under some conditions, such as at high temperatures (Jeon 2004). Perhaps given enough time (especially outside a laboratory setting), partner fidelity feedback would select for more obvious mutualistic function. Although we are not aware of evolution experiments involving a microbial mutualism that occurs in nature, recent studies establishing novel mutualisms can test theoretical predictions of the contexts in which simple mutualisms establish and remain stable (Harcombe 2010, Hillesland et al. 2014).

Future directions

Although we presently lack answers to many of the fundamental questions regarding the evolution of mutualisms, the development of new phylogenetic databases and approaches, and the emergence of new model systems and molecular techniques, should contribute to substantial improvements in our understanding in the coming years. Here we highlight a few of the many open questions regarding the genetics and evolution of mutualisms that merit future consideration.

Comparison to antagonisms

One question that remains unexplored is how evolution in an antagonistic interaction is expected to differ from evolution in a mutualistic interaction. Much attention has been paid to how the two types of interactions grade into each other in terms of cheating in mutualisms. However, to our knowledge the only general comparisons of coevolutionary dynamics in different types of interactions are theoretical. For example, Yoder and Nuismer (2010) predict that antagonistic coevolution promotes phenotypic diversification under certain conditions, while mutualistic coevolution restricts it. Their model did not account for cheating, or for how antagonism and mutualism may differ in their effects on reproductive isolation. Empirical evidence related to this question is wholly lacking.

Speciation

In comparison to other biotic and abiotic factors, how important are mutualisms for speciation? To answer this question will require comparative studies conducted at broad taxonomic levels to search for patterns, followed by detailed investigations of the ecological and evolutionary mechanisms whereby mutualisms might contribute to reproductive isolation. Phylogenetic approaches are critical for identifying patterns. For example, Dodd et al. (1999) found that animal pollination was associated with higher species diversity of plant families, but dispersal by animals was not. In a diverse group of orchids, Waterman et al. (2011) found that pollination contributed to speciation, but that the fungal symbiosis contributed only to species coexistence. Plant-pollinator mutualisms obviously have great potential for influencing rates of speciation, in that evolutionary divergence between populations in floral traits and pollinators can often have direct effects on reproductive isolation (Bradshaw and Schemske 2003, Schemske and Bradshaw

1999, Kay and Sargent 2009). For example, the extraordinary diversity of figs (*Ficus* spp, > 600 species) must be due, in part, to the highly specialized nature of the pollination mutualism. Might mutualisms other than those based on pollination also contribute to speciation? The role of ecological divergence in speciation has experienced a revival, with the idea that adaptive divergence between populations may directly or indirectly lead to greater reproductive isolation (Nosil 2012). Thus, any factor or interaction that contributes to adaptive divergence can under some circumstances be viewed as a mechanism of speciation (Sobel et al. 2010).

The latitudinal diversity gradient

Along with many other biotic interactions studied, mutualisms often show a strong latitudinal gradient of interaction strength, prevalence, or importance (Schemske et al. 2009). For example, the incidence of animal pollination increases towards the equator (Regal 1982), as does the importance of ant-plant mutualisms related to plant defense (Schemske et al. 2009). With a better understanding of mutualism evolution, we can begin to address the question of whether gradients in the importance of species interactions are a cause or effect (or both) of the remarkable and as-yet poorly understood latitudinal diversity gradient. Studies that examine the importance and evolution of mutualisms on latitudinal scales will require new model systems (especially in the tropics) with well-developed genetic resources, in order to compare evolutionary processes between environments.

Anthropogenic environmental change

Understanding the evolution and genetics of mutualisms is critical to a growing number of applied problems. For example, mutualism evolution is relevant to conservation and restoration,

such as declines in pollinator populations due to human pressures—might evolution of increased self-pollination mediate the impact of pollinator loss for plants (Eckert et al. 2009)? Climate change and invasive species may also disrupt mutualisms and cause novel interactions. In the case of climate change, these changes are expected to occur due to range shifts and phenological mismatches of mutualistic partners, both of which may be mitigated by adaptation to changing conditions (Hegland et al. 2009). We cannot accurately assess the potential for mutualistic interactions to adapt to climate change or invasive species without knowing details about the genetic basis of mutualisms, such as how many genes are involved in adaptation.

Conclusions

To understand the fundamental patterns and processes underlying mutualism evolution, and particularly to identify how evolution in mutualisms may be unique, questions about the evolutionary ecology and genetic basis of adaptive traits must be addressed on a broad taxonomic scale, and include studies of all species involved in the interaction. For most mutualisms (including those highlighted here), our understanding of the natural history, genes, traits and fitness consequences of the interaction are limited and strikingly asymmetrical (Bronstein et al. 2006). Such bias severely limits our ability to assess the selective forces and evolutionary steps involved in the evolution of the interactions, and in particular, to determine the role of coevolution.

Fortunately, active evolutionary research in the systems highlighted here, along with others that we did not have space to review, is broadening our taxonomic coverage. These systems represent a range of life histories, specialization of the interaction, ease of lab culture and experimental manipulation, and molecular genetic and phylogenetic tools. This is an exciting

time to study mutualism evolution, as our goal of finding empirical support for generalizations across systems may soon be within reach.

Acknowledgements

We would like to thank U. Mueller, M. Friesen, and M. Nishiguchi, for their generous time and efforts writing boxes for the published version of this manuscript (Baskett and Schemske 2015).

We would also like to thank J. Eilers and R. Atchison for assistance with formatting and references, and many friendly reviewers for their suggestions and comments, which greatly improve the manuscript: D. Brickley, J. Bronstein, E. Dittmar, A. Douglas, K. Kay and her lab members, J. Lau, N. Moran, U. Mueller, C. Oakley, S. Perez, and J. Rudgers.

This chapter is modified and reprinted with permission from Oxford University Press. The published version is: Baskett, C. A. and D. W. Schemske (2015). Evolution and genetics of mutualism. Mutualism. J. L. Bronstein. Oxford, Oxford University Press: 77-92.

REFERENCES

REFERENCES

- Barrett, R. D. H. and H. E. Hoekstra (2011). "Molecular spandrels: tests of adaptation at the genetic level." Nature Reviews Genetics **12**(11): 767-780.
- Barton, N. H. and P. D. Keightley (2002). "Understanding quantitative genetic variation." Nature Reviews Genetics **3**(1): 11-21.
- Baskett, C. A. and D. W. Schemske (2015). Evolution and genetics of mutualism. Mutualism. J. L. Bronstein. Oxford, Oxford University Press: 77-92.
- Beardsley, P. M., A. Yen and R. G. Olmstead (2003). "AFLP phylogeny of *Mimulus* section *Erythranthe* and the evolution of hummingbird pollination." Evolution **57**(6): 1397-1410.
- Bradshaw, H. D., K. G. Otto, B. E. Frewen, J. K. McKay and D. W. Schemske (1998). "Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*)." Genetics **149**(1): 367-382.
- Bradshaw, H. D. and D. W. Schemske (2003). "Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers." Nature **426**(6963): 176-178.
- Bronstein, J. L. (1994). "Our current understanding of mutualism." Quarterly Review of Biology **69**(1): 31-51.
- Bronstein, J. L., R. Alarcon and M. Geber (2006). "The evolution of plant-insect mutualisms." New Phytologist **172**(3): 412-428.
- Campbell, D. R., N. M. Waser and M. V. Price (1994). "Indirect selection of stigma position in *Ipomopsis aggregata* via a genetically correlated trait." Evolution **48**(1): 55-68.
- Campbell, D. R., N. M. Waser and M. V. Price (1996). "Mechanisms of hummingbird-mediated selection for flower width in *Ipomopsis aggregata*." Ecology **77**(5): 1463-1472.
- Darwin, C. (1862). On the various contrivances by which British and foreign orchids are fertilised by insects. London, John Murray.
- Degnan, P. H., Y. Yu, N. Sisneros, R. A. Wing and N. A. Moran (2009). "*Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors." Proceedings of the National Academy of Sciences of the United States of America **106**(22): 9063-9068.
- Dodd, M. E., J. Silvertown and M. W. Chase (1999). "Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families." Evolution **53**(3): 732-744.
- Doebeli, M. and U. Dieckmann (2000). "Evolutionary branching and sympatric speciation caused by different types of ecological interactions." American Naturalist **156**: S77-S101.

- Eckert, C. G., B. Ozimec, C. R. Herlihy, C. A. Griffin and M. B. Routley (2009). "Floral morphology mediates temporal variation in the mating system of a self-compatible plant." Ecology **90**(6): 1540-1548.
- Fisher, R. A. (1941). "Average excess and average effect of a gene substitution." Annals of Eugenics **11**: 53-63.
- Harcombe, W. (2010). "Novel cooperation experimentally evolved between species." Evolution **64**(7): 2166-2172.
- Hegland, S. J., A. Nielsen, A. Lazaro, A. L. Bjerknes and O. Totland (2009). "How does climate warming affect plant-pollinator interactions?" Ecology Letters **12**(2): 184-195.
- Hembry, D. H., J. B. Yoder and K. R. Goodman (2014). "Coevolution and the Diversification of Life." American Naturalist **184**(4): 425-438.
- Hillesland, K. L., S. J. Lim, J. J. Flowers, S. Turkarslan, N. Pinel, G. M. Zane, N. Elliott, Y. J. Qin, L. Y. Wu, N. S. Baliga, J. Z. Zhou, J. D. Wall and D. A. Stahl (2014). "Erosion of functional independence early in the evolution of a microbial mutualism." Proceedings of the National Academy of Sciences of the United States of America **111**(41): 14822-14827.
- Janzen, D. H. (1967). "Why mountain passes are higher in the tropics." American Naturalist **101**(919): 233-&.
- Jeon, K. W. (2004). "Genetic and physiological interactions in the amoeba-bacteria symbiosis." Journal of Eukaryotic Microbiology **51**(5): 502-508.
- Kay, K. M. and R. D. Sargent (2009). The Role of Animal Pollination in Plant Speciation: Integrating Ecology, Geography, and Genetics. Annual Review of Ecology Evolution and Systematics. **40**: 637-656.
- Kimura, M. (1983). The Neutral Theory of Molecular Evolution. Cambridge, UK, Cambridge University Press.
- Kingsolver, J. G. and S. E. Diamond (2011). "Phenotypic selection in natural populations: what limits directional selection?" American Naturalist **177**(3): 346-357.
- Klahre, U., A. Gurba, K. Hermann, M. Saxenhofer, E. Bossolini, P. M. Guerin and C. Kuhlemeier (2011). "Pollinator choice in *Petunia* depends on two major genetic loci for floral scent production." Current Biology **21**(9): 730-739.
- Louthan, A. M. and K. M. Kay (2011). "Comparing the adaptive landscape across trait types: larger QTL effect size in traits under biotic selection." Bmc Evolutionary Biology **11**.
- MacLean, A. M., T. M. Finan and M. J. Sadowsky (2007). "Genomes of the symbiotic nitrogen-fixing bacteria of legumes." Plant Physiology **144**(2): 615-622.

- Matuszewski, S., J. Hermisson and M. Kopp (2014). "Fisher's geometric model with a moving optimum." Evolution **68**(9): 2571-2588.
- McCutcheon, J. P. and N. A. Moran (2007). "Parallel genomic evolution and metabolic interdependence in an ancient symbiosis." Proceedings of the National Academy of Sciences of the United States of America **104**(49): 19392-19397.
- McCutcheon, J. P. and N. A. Moran (2012). "Extreme genome reduction in symbiotic bacteria." Nature Reviews Microbiology **10**(1): 13-26.
- Meyer, J. R. and V. G. Meyer (1961). "Origin and inheritance of nectariless cotton." Crop Science **1**: 167-169.
- Mikheyev, A. S., U. G. Mueller and P. Abbot (2010). "Comparative Dating of Attine Ant and Lepiotaceous Cultivar Phylogenies Reveals Coevolutionary Synchrony and Discord." American Naturalist **175**(6): E126-E133.
- Moran, N. A., P. H. Degnan, S. R. Santos, H. E. Dunbar and H. Ochman (2005). "The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes." Proceedings of the National Academy of Sciences of the United States of America **102**(47): 16919-16926.
- Moran, N. A., J. P. McCutcheon and A. Nakabachi (2008). Genomics and evolution of heritable bacterial symbionts. Annual Review of Genetics. Palo Alto, Annual Reviews. **42**: 165-190.
- Nosil, P. (2012). Ecological Speciation. New York, Oxford University Press.
- Oliver, K. M., J. Campos, N. A. Moran and M. S. Hunter (2008). "Population dynamics of defensive symbionts in aphids." Proceedings of the Royal Society B-Biological Sciences **275**(1632): 293-299.
- Oliver, K. M., J. A. Russell, N. A. Moran and M. S. Hunter (2003). "Facultative bacterial symbionts in aphids confer resistance to parasitic wasps." Proceedings of the National Academy of Sciences of the United States of America **100**(4): 1803-1807.
- Orr, H. A. (1998). "The population genetics of adaptation: The distribution of factors fixed during adaptive evolution." Evolution **52**(4): 935-949.
- Orr, H. A. (2005). "Theories of adaptation: what they do and don't say." Genetica **123**(1-2): 3-13.
- Paterson, S., T. Vogwill, A. Buckling, R. Benmayor, A. J. Spiers, N. R. Thomson, M. Quail, F. Smith, D. Walker, B. Libberton, A. Fenton, N. Hall and M. A. Brockhurst (2010). "Antagonistic coevolution accelerates molecular evolution." Nature **464**(7286): 275-U154.
- Perez-Brocal, V., R. Gil, S. Ramos, A. Lamelas, M. Postigo, J. M. Michelena, F. J. Silva, A. Moya and A. Latorre (2006). "A small microbial genome: The end of a long symbiotic relationship?" Science **314**(5797): 312-313.

- Porter, S. S. and E. L. Simms (2014). "Selection for cheating across disparate environments in the legume-rhizobium mutualism." Ecology Letters **17**(9): 1121-1129.
- Regal, P. J. (1982). "Pollination by wind and animals--ecology of geographic patterns." Annual Review of Ecology and Systematics **13**: 497-524.
- Richards, S., R. A. et al. (2010). "Genome sequence of the pea aphid *Acyrtosiphon pisum*." Plos Biology **8**(2).
- Rockman, M. V. (2011). "The QTN program and the alleles that matter for evolution: all that's gold does not glitter." Evolution **66**(1): 1-17.
- Rudgers, J. A. (2004). "Enemies of herbivores can shape plant traits: selection in a facultative ant-plant mutualism." Ecology **85**(1): 192-205.
- Rudgers, J. A. and S. Y. Strauss (2004). "A selection mosaic in the facultative mutualism between ants and wild cotton." Proceedings of the Royal Society B-Biological Sciences **271**(1556): 2481-2488.
- Rutter, M. T. and M. D. Rausher (2004). "Natural selection on extrafloral nectar production in *Chamaecrista fasciculata*: the costs and benefits of a mutualism trait." Evolution **58**(12): 2657-2668.
- Sachs, J. L., U. G. Mueller, T. P. Wilcox and J. J. Bull (2004). "The evolution of cooperation." Quarterly Review of Biology **79**(2): 135-160.
- Sanderson, M. J. and M. F. Wojciechowski (1996). "Diversification rates in a temperate legume clade: Are there "so many species" of *Astragalus* (Fabaceae)?" American Journal of Botany **83**(11): 1488-1502.
- Schemske, D. W. (2002). Ecological and evolutionary perspectives on the origins of tropical diversity. Foundations of Tropical Forest Biology. R. L. Chazdon and T. C. Whitmore. Chicago, University of Chicago Press: 163-173.
- Schemske, D. W. (2009). Biotic interactions and speciation in the tropics. Speciation and Patterns of Diversity. R. K. Butlin, J. R. Bridle and D. Schluter. Cambridge, UK, Cambridge University Press: 219-239.
- Schemske, D. W. and H. D. Bradshaw (1999). "Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*)." Proceedings of the National Academy of Sciences of the United States of America **96**(21): 11910-11915.
- Schemske, D. W., G. G. Mittelbach, H. V. Cornell, J. M. Sobel and K. Roy (2009). "Is there a latitudinal gradient in the importance of biotic interactions?" Annual Review of Ecology Evolution and Systematics **40**: 245-269.
- Siepielski, A. M. and C. W. Benkman (2010). "Conflicting selection from an antagonist and a mutualist enhances phenotypic variation in a plant." Evolution **64**(4): 1120-1128.

- Siepielski, A. M., J. D. DiBattista and S. M. Carlson (2009). "It's about time: the temporal dynamics of phenotypic selection in the wild." Ecology Letters **12**(11): 1261-1276.
- Sobel, J. M., G. F. Chen, L. R. Watt and D. W. Schemske (2010). "The biology of speciation." Evolution **64**(2): 295-315.
- Thompson, J. N. (2005). The Geographic Mosaic of Coevolution. Chicago, University of Chicago Press.
- Venail, J., A. Dell'Olio and C. Kuhlemeier (2010). "Speciation genes in the genus *Petunia*." Philosophical Transactions of the Royal Society B-Biological Sciences **365**(1539): 461-468.
- Wade, M. J. and S. Kalisz (1990). "The causes of natural selection." Evolution **44**(8): 1947-1955.
- Waterman, R. J., M. I. Bidartondo, J. Stofberg, J. K. Combs, G. Gebauer, V. Savolainen, T. G. Barraclough and A. Pauw (2011). "The Effects of Above- and Belowground Mutualisms on Orchid Speciation and Coexistence." The American Naturalist **177**(2): E54-E68.
- Weber, M. G. and K. H. Keeler (2013). "The phylogenetic distribution of extrafloral nectaries in plants." Annals of Botany **111**(6): 1251-1261.
- Wright, J. W. and T. R. Meagher (2004). "Selection on floral characters in natural Spanish populations of *Silene latifolia*." Journal of Evolutionary Biology **17**(2): 382-395.
- Yoder, J. B. and S. L. Nuismer (2010). "When does coevolution promote diversification?" American Naturalist **176**(6): 802-817.