ECOLOGICAL COSTS AND BENEFITS OF SECONDARY METABOLITES IN ANIMAL-DISPERSED FRUITS

by

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Ecological Costs and Benefits of Secondary Metabolites in Animal-Dispersed Fruits

Thesis directed by Professor M. Deane Bowers

ABSTRACT

Ripe, fleshy fruits function primarily to attract mutualist animals; however, many wild fruits contain secondary metabolites that are distasteful or toxic to consumers. A number of hypotheses have been proposed to explain this apparent evolutionary paradox, but few have been tested experimentally. The goal of my dissertation research was to investigate the evolutionary ecology of fruit chemical traits, integrating quantitative chemical analyses with experiments to elucidate the functional significance of fruit secondary metabolites in natural populations. I conducted a series of field studies using three groups of plants, one temperate and two tropical. First, using the tropical shrub *Hamelia patens* (Rubiaceae), I investigated the potential for leaf herbivory to affect seed dispersal and showed that plant responses to herbivory can alter fruit chemistry and reduce fruit removal by seed-dispersing birds. Next, using detailed chemical analyses and field observations of fruit-frugivore interactions in the temperate shrub *Lonicera* x *bella* (Caprifoliaceae), I showed that fruits can contain higher levels of secondary metabolites than leaves, and that these metabolites serve an important role in defense against insects and pathogens. Finally, using additional chemical analyses and controlled experiments with the tropical plant genus *Piper* (Piperaceae), I showed that fruit secondary metabolites can be explained as a trade-off between defense against antagonists and attraction of seed-dispersing bats. Together, this dissertation demonstrates the importance of fruit chemical traits in mediating interactions between plants and diverse antagonistic and mutualistic consumers and represents a significant contribution to our understanding of both seed dispersal and plant defense.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Research Rationale and Background

Seed dispersal is a critical ecological process that can shape plant reproductive success, population dynamics, and community structure (reviewed in Dennis et al. 2007; Schupp et al. 2010). In many systems, this process is mediated by mutualistic animals, who consume fleshy fruits for their nutritional rewards and inadvertently disperse seeds to new sites. However, the rewards that attract mutualists will also inevitably attract antagonistic frugivores, such as insect seed predators and microbial pathogens, whose impact on seed survival and viability has important implications for the eventual establishment of offspring (Dennis et al. 2007; Howe and Miriti 2004; Janzen 1970). The evolutionary trade-offs in fruits between attraction of mutualists and defense against antagonists are poorly understood, perhaps in part due to a lack of studies that take into account the broad ecological costs and benefits of particular traits and a failure to integrate plant defense theory and quantitative chemical techniques into studies of fruit-frugivore interactions (Rutter and Rausher 2004; Schupp et al. 2010; Tewksbury 2002).

Fruits often contain not only nutritional rewards, but also a diverse suite of secondary metabolites which can be distasteful or even toxic to potential seed dispersers. The occurrence of fruit secondary metabolites has historically been described as an evolutionary paradox, e.g. Heim de Balsac (1928) cited toxic fruit as evidence against the general hypothesis that fruits represent an adaptation for animal dispersal. However, the phenomenon is relatively widespread—in a survey of toxic fruit by Herrera (1982), it was estimated that approximately one-third of all fleshy-fruited European plant genera produce fruits that are toxic to humans. For example, the fruits of *Atropa belladonna* (Solanaceae) are so toxic that the consumption of even a few berries

can cause death in humans (Caksen et al. 2003). Although birds and other animals are sometimes able to tolerate or detoxify the secondary metabolites in fruits (Snow 1971), the common occurrence of deterrents and/or toxins in a tissue that functions primarily to attract mutualists suggests complex selective pressures from different fruit consumers and/or evolutionary constraints on fruit traits that lead to suites of fruit secondary metabolites that are not necessarily optimized for seed dispersal success.

A number of adaptive hypotheses have been proposed to explain how fruit secondary metabolites may increase seed dispersal success through mechanisms such as: 1) attraction of mutualists by providing foraging cues (colors, odors, and flavors) that can be associated with rewards, 2) reduction in the amount of fruit any particular animal can consume in a foraging bout, 3) inhibition of seed germination prior to pulp removal and dispersal, 4) regulation of protein assimilation or gut retention time in animals, 5) deterrence of less efficient seed dispersers, and 6) defense of fruits against non-dispersing seed predators and pathogens (Cipollini and Levey 1997b; Herrera 1982; Izhaki 2002). A few of these hypotheses have some empirical support. In particular, a number of studies have emphasized the role of secondary compounds in fruit defense and supported the idea of trade-offs in fruit traits between defense against antagonists and attraction of seed dispersers (Cazetta et al. 2008; Schaefer et al. 2003; Tang et al. 2005; Tewksbury et al. 2008b). However, in part because few studies have provided any quantitative analysis of fruit secondary metabolites, we still have a limited understanding of how natural variation in fruit chemistry can influence seed dispersal and plant fitness.

In addition, despite the existing evidence that fruit secondary metabolites can sometimes confer an adaptive benefit, in other cases secondary metabolites in fruits may be non-adaptive—their presence better explained by physiological constraints on the exclusion of secondary

compounds from fruit tissue (Cipollini et al. 2002; Eriksson and Ehrlen 1998; Jordano 1995). Because chemical resistance traits in different plant parts are often linked through shared genetics, hormonal regulation, biosynthetic pathways, and metabolism (Adler et al. 2006; Herrera et al. 2002; Kessler and Baldwin 2002; Price and Langen 1992), secondary metabolites may occur in fruits simply as a pleiotropic consequence of selection in leaves or other vegetative tissues. A similar hypothesis has been proposed to explain the occurrence of secondary metabolites in nectar (Adler 2000), and several studies have now supported this hypothesis through a comparative examination of the occurrence of secondary metabolites in vegetative versus floral tissues (Adler et al. 2006; Gegear et al. 2007; Kessler and Halitschke 2009; Manson et al. 2012; Strauss et al. 1999). However, there is limited evidence available to test similar hypotheses in fruits.

An increased understanding of the occurrence patterns and functional significance of secondary metabolites in fruits can improve our theories of both seed dispersal and plant defense. An appreciation for the fundamental role of plant secondary metabolites in mediating species interactions revolutionized the field of plant-herbivore interactions (reviewed in Harbourne 1993; Rosenthal and Berenbaum 1991; Schoonhoven et al. 2005) and more recently has led to significant advances in the field of plant-pollinator interactions (Adler 2000; Irwin and Adler 2008; Manson et al. 2010; Strauss et al. 1999). However, surprisingly few studies have applied quantitative chemistry to better understand interactions between plants and the diverse community of antagonistic and mutualistic organisms that feed on fruits, despite the potential for fruit secondary metabolites to play a key role in structuring relationships with both of these classes of frugivores (Cipollini 2000; Levey et al. 2007; Price et al. 1980; Schupp et al. 2010; Tewksbury 2002).

To illustrate the limited research history in this area relative to other aspects of the chemical ecology of plant-animal interactions, I conducted a systematic literature search using Web of Science (www.scientific.thomson.com/products/wos/) for scientific papers published between 1990 and 2011. I performed three separate searches, all of which used the keyword string "chemical ecology" OR "secondary metabolite*" OR "secondary compound*". In addition, for each of the three searches, this string was followed by either AND "herbivory", AND "pollination", or AND "seed dispersal" OR "frugivory". Results from this search are provided in Fig. 1.1 and demonstrate a clear bias in chemical ecology research towards studies of plant-herbivore interactions over studies of pollination and seed dispersal.

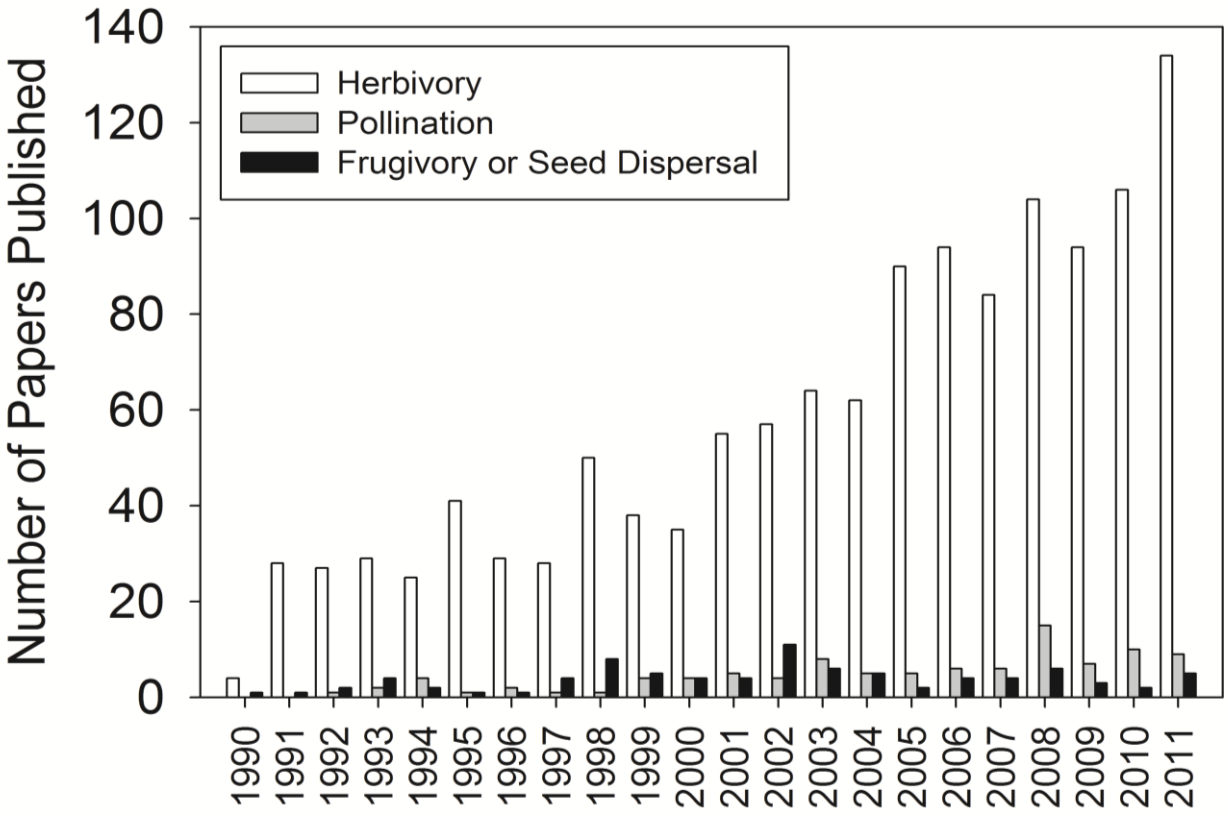

Figure 1.1: Publication history for scientific articles focused on the chemical ecology of species interactions. Results are from three Web of Science searches for articles published between 1990 and 2011 with the topics: 1) "herbivory"; 2) "pollination"; 3) "frugivory" or "seed dispersal" and the topics "chemical ecology" or "secondary metabolite" or "secondary compound".

My dissertation brings together work in three systems to better understand the ecological costs and benefits of secondary metabolites in vertebrate-dispersed fruits. I use analytical techniques in organic chemistry, including gas chromatography and mass spectrometry, combined with detailed ecological field and laboratory experiments to explore this topic from several perspectives. Together, the work represents a significant contribution to the fields of chemical ecology and plant/animal interactions.

1.2 Chapter Overview

In Chapter Two, I examined the potential for interactions between herbivory and frugivory using the tropical shrub *Hamelia patens* (Rubiaceae). This work was conducted in Costa Rica in collaboration with Dr. Katja Poveda (Cornell University). We conducted a series of experiments that demonstrated that herbivory or simulated herbivory to leaves can lead to induced changes in adjacent fruit traits that reduce removal rates by birds. In this case, fruit secondary metabolites may represent a significant ecological cost in terms of reduced seed dispersal opportunities. A series of bioassays indicated that these changes were mediated by an increase in deterrent compounds in fruit following damage. This work was the first demonstration of an ecological cost of induced defense to leaf herbivory in terms of reduced seed dispersal, and was published in *Journal of Ecology* in 2011.

In Chapter Three, I provided the first description of the occurrence of iridoid glycosides (an important class of plant defensive compounds) in a hybrid complex of North American invasive bush honeysuckles (*Lonicera* x *bella* Zabel*, Lonicera tatarica* L.*,* and *Lonicera morrowii* A. Gray*,* Caprifoliaceae). I showed that the secondary chemistry of honeysuckles varies considerably among species and among plant parts. In particular, fruits of hybrid and

parental honeysuckles contain several iridoid glycosides that never occur in leaves, and the total concentrations in fruits are more than double those found in leaves. I discuss these results in the context of their implications for fruit/frugivore interactions as well as the insight they provide into the chemical consequences of plant hybridization. I also present detailed methods for quantitative analysis of iridoid glycosides in honeysuckle using gas chromatography combined with mass spectrometry that will allow researchers to address many new questions related to the evolutionary ecology and invasion biology of these species. This chapter was published in *Phytochemistry* in 2013.

In Chapter Four, I used the chemical methods developed for Chapter Three to test two alternative evolutionary hypotheses: 1) fruit secondary metabolites have an adaptive function in seed dispersal or fruit defense, or 2) fruit secondary metabolites occur as a consequence of foliar defense (i.e. due to pleiotropic constraints on their exclusion from fruits). I examined intraplant and intraspecific variation in iridoid glycoside content and its relationship to patterns of fruit/frugivore interactions in *Lonicera* x *bella*, including fruit removal by potential seed dispersers and fruit damage from insects and microbes. The study population included 30 plants from three populations in Boulder County monitored across two growing seasons (2007 and 2008). I confirmed that the overall concentrations of iridoid glycosides were highest in unripe fruits, reduced in ripe fruits, and lowest in leaves, and that several compounds occurred in fruits that were never detected in leaves. In addition, iridoid glycoside concentration in fruits was negatively correlated with patterns of fruit damage, suggesting that fruit secondary metabolites serve an adaptive role in fruit defense. However, I also showed that the quantities of certain iridoid glycosides are strongly correlated between leaves and fruits, emphasizing that selection in different tissue types is not entirely independent. I concluded that plant chemical trait evolution

is best viewed in a whole-plant context that includes plant/herbivore and fruit/frugivore interactions.

Chapters Five, Six, and Seven focus on the tropical plant genus *Piper* (Piperaceae), the fruits of which are primarily bat-dispersed and contain high concentrations of amides, another large class of plant secondary metabolites. Amides have a known defensive role in leaves, but had not been previously examined in the context of fruit-frugivore interactions. In Chapter Five, I provided a comparative examination of amides in different plant parts of *Piper reticulatum*, a common understory tree in Costa Rica. I examined amide diversity and concentration in leaves, roots, flowers, unripe fruits, ripe fruits, and seeds in the context of optimal defense theory, which predicts that allocation to chemical defense among plant parts depends on the fitness value of the tissue, the risk of attack, and the cost of defending the tissue. I found that fruits and seeds had higher chemical diversity and, for seeds, higher concentrations of amides than vegetative tissues. In addition, I show that amide diversity, but not concentration, is correlated with patterns of seed damage in natural populations. These results provided support for the predictions of optimal defense theory and also suggested that chemical diversity may play a key role in fruit defense.

In Chapter Six, I conducted a series of bioassays showing broad deterrent effects of both amide extracts and purified compounds against a specialist insect seed predator and several strains of fruit-associated fungi that feed on *P. reticulatum*, suggesting an important role of these metabolites in fruit defense. I specifically tested for differences in bioactivity between the suites of compounds found in unripe fruits and ripe fruits, and found that the changes that occur with ripening lead to a reduction in defense effectiveness, especially against fruit-associated fungi. Furthermore, I tested for potential synergistic or antagonistic interactions between individual amides and show that the effects of compound mixtures are not predicted by simple additive

models. Interestingly, I found that the effects of specific amides or combinations of amides vary considerably among different consumers, and that the same two compounds can function either synergistically or antagonistically depending on the consumer being tested. These results emphasized the need to conduct experiments with the variety of organisms that feed on fruits using the suites of compounds that naturally occur together in fruit pulp and seeds.

Finally, in Chapter Seven, I examined the effects of amides on the foraging and feeding behavior of the primary seed dispersers of *Piper*, a small group of bats in the genus *Carollia* (Phyllostomidae). Results showed that the effects of amides on bats vary for different compounds and for different bat species, but in general amides have either neutral or negative effects on fruit preference. This suggests that fruit secondary metabolites likely represent an ecological trade-off between attraction of seed dispersers and defense against pests. However, the strength of this trade-off likely varies depending on ecological context.

Overall, this dissertation extends past work on fruit secondary metabolites to include several plant families and classes of compounds that had not previously been examined in this context. In addition, I provided the most detailed comparative examination to date of secondary metabolites in fleshy fruits and other plant parts, allowing me to specifically test for potential physiological constraints on fruit chemistry based on the chemistry of other plant parts. By examining the functional significance of fruit secondary metabolites in several study systems and from several perspectives, this dissertation provides broad perspective and will be of interest to a range of researchers working in the fields of chemical ecology, seed dispersal, tropical biology, plant-herbivore interactions, plant-pathogen interactions, and bat biology.

CHAPTER TWO

HERBIVORE-INDUCED CHANGES IN FRUIT-FRUGIVORE INTERACTIONS [1]

2.1 Abstract

Herbivore attack can induce dramatic changes in plant chemical defenses. These responses protect plants against future herbivory, but can also have important physiological and ecological costs. Ecological costs of defense have received recent theoretical attention; however, many proposed costs have not yet been demonstrated empirically. In particular, field data are lacking as to whether induced responses in leaves can lead to correlated changes in fruit palatability that reduce fruit removal by mutualist seed dispersers. Using the tropical shrub, *Hamelia patens* (Rubiaceae), we examined changes in fruit removal, palatability and maturation time following various treatments to the subtending leaves, including herbivory, mechanical damage and/or application of methyl jasmonate (MeJA). Fewer fruits were removed from herbivory and MeJA-treated branches than from controls, and results from three bioassays with ants and fungi suggested that this response was mediated by changes in fruit palatability. In addition, fruits from MeJA-treated branches matured more quickly than those from control branches. Taken together, our results provide novel evidence that induced responses to herbivory can affect fruit-frugivore interactions through two mechanisms: changes in fruit palatability and changes in fruit development time. This highlights the importance of physiological linkages between leaf and fruit traits in determining the overall costs of plant defense and the fitness outcomes of multispecies interactions.

[1] Published as: Whitehead, S.R. and K.A. Poveda (2011). *Journal of Ecology*, **99**, 964–969 doi: 10.1111/j.1365-2745.2011.01819.x

2.2 Introduction

Plants employ a remarkable armory of chemical resistance traits as defenses against herbivores and pathogens. These defenses are generally divided into constitutive defenses (always present in plant tissue) and induced defenses (produced in response to damage) (Kessler and Baldwin 2002). Induced defenses are thought to reduce the costs associated with continuous production of defenses during times when herbivores and pathogens are not present (Karban and Baldwin 1997), but numerous studies have shown that even induced defenses can result in significant costs in terms of plant fitness (Koricheva 2002; Strauss et al. 2002). Fitness costs can include allocation costs, when investment in defense reduces allocation of resources to growth and reproduction, and ecological costs, when reduced fitness is due to changes in interactions with other organisms (Strauss et al. 2002).

Ecological costs of induced defense have been the subject of several reviews (Cipollini et al. 2003; Heil 2002; Strauss et al. 2002; Walters and Heil 2007), and may include reduced resistance to other enemies, deterrence of mutualists or reduced competitive ability. For example, induced responses to leaf herbivory can increase deterrent compounds in nectar and pollen, thereby altering visitation rates and/or nectar and pollen removal by pollinators (e.g. Adler et al. 2006; Herrera et al. 2002; Kessler and Halitschke 2009). The fitness implications of this effect may depend greatly on ecological context (e.g. Gegear et al. 2007), but, at least in some cases, pollinator preferences can create a selective advantage for less defended plants (Strauss et al. 1999).

Although the recognition of ecological costs has improved our ability to detect overall costs of defense and had a strong impact on plant defense theory (Koricheva 2002; Strauss et al. 2002), many proposed ecological costs have not yet been demonstrated empirically. It has been

suggested that costs parallel to those demonstrated for pollination mutualisms may also exist for seed dispersal mutualisms (Strauss et al. 2002), and there is some evidence of correlations between leaf and fruit resistance traits (McCall and Karban 2006; Redman et al. 2001). In addition, induced responses to herbivory have been correlated with changes in fruit maturation time (Redman et al. 2001), which has the potential to impose additional fitness costs depending on ecological context. However, there is still no experimental evidence that induced plant resistance traits can affect fruit chemistry, fruit-frugivore interactions, or seed dispersal.

A surprisingly large proportion of ripe, fleshy fruits contain potentially deterrent or toxic compounds (Herrera 1982) and it has been debated whether these compounds should be explained adaptively (Cipollini and Levey 1997b) or as a physiological cost of producing defensive compounds in leaves and other tissues (Eriksson and Ehrlen 1998). There is growing evidence that constitutive deterrent compounds in fruits are functionally important in defense, but may simultaneously impose costs in terms of reduced fruit removal by mutualists (e.g. Cazetta et al. 2008; Izhaki 2002; Schaefer et al. 2003; Tewksbury et al. 2008b). However, in addition to selection from mutualistic and antagonistic frugivores, there may be important constraints on the expression of fruit chemical traits due to the complex selective environment imposed by herbivores, pathogens, pollinators and competitors. We have very little information on how the expression of fruit chemical traits may vary depending on the larger ecological context.

In this study, we conducted a series of short experiments to test the hypothesis that induced responses to leaf herbivory can alter fruit palatability and thereby diminish fruit removal by potential seed dispersers. Since methyl jasmonate (MeJA), an important hormone involved in plant responses to herbivory, also plays a fundamental role in fruit development (Creelman and

Mullet 1997), we also tested how induced responses in leaves affect fruit maturation. To our knowledge, these are the first manipulative experiments that explore the potential for ecological costs of plant responses to leaf herbivory in terms of changes in fruit-frugivore interactions.

2.3 Methods

Study site and system

All experiments were conducted between February 2008 and January 2010 at La Selva Biological Station, Heredia Province, Costa Rica. The area consists of tropical wet forest and receives approximately four meters of rain annually, spread relatively evenly throughout the year. Average monthly temperatures range between 24.7^{o}C and 27.1^{o}C (McDade et al. 1994).

Hamelia patens Jacq. (Rubiaceae) is a 2-6 meter-tall shrub, ranging from Mexico to Bolivia (Croat 1978). A variety of herbivores feed on the leaves of *H. patens;* observations at our study site include the sawfly *Waldheimia interstitialis* (Cameron) (Hymenoptera: Tenthredinidae), the leaf-cutter ant *Atta cephalotes* (Linnaeus) (Hymenoptera: Formicidae), and at least twelve species of Lepidoptera from seven families (Dyer and Gentry 2012; personal observation). Only the sawfly, *W. interstitialis*, was located in sufficient numbers for experimentation during the course of this study. A large diversity of birds, including toucans, warblers, honeycreepers, thrushes, flycatchers, and tanagers, feed on *H. patens* fruits, which are produced continuously throughout the year on infructescences containing anywhere from fifteen to fifty fruits (Croat 1978; Leck 1972; Levey 1987). Indiv0idual fruits remain green for two months until they enter a final ripening period, lasting approximately six days and characterized by a sequential color change from green to cream to pink to red to maroon to black (Levey 1987).

Effects of herbivory and simulated herbivory on fruit removal.

To investigate how induced responses to herbivory can affect fruit removal by potential seed dispersers, we used natural populations of *H. patens* shrubs located in the La Selva arboretum and open areas surrounding the station. Four infructescences per shrub, matched for the approximate numbers of fruits in different ripening stages, were randomly assigned to one of the following treatment groups: 1) herbivory—two *W. interstitialis* larvae caged for three days on the leaves immediately subtending the infructescence; 2) MeJA—1 µL of 10 µg/µL MeJA in lanolin paste applied to the petioles of the two leaves immediately subtending the infructescence (Halitschke et al. 2001); 3) lanolin control—1 µL of pure lanolin paste applied in the same manner as above as a control for the MeJA treatment; 4) absolute control—branches handled in a similar manner but without treatment applied as a control for the herbivory group. Since we were limited by the number of herbivores we could find in the environment, we had 7 replicates of the herbivory and absolute control treatments, and 22 replicates of the MeJA and lanolin control treatments. All infructescences were enclosed in mesh bags for three days, after which the bags were removed and the fruits were exposed for two days to consumers. We counted the number of fruits before and after the two day period, and assumed the majority of the missing fruits had been removed by birds, since we found no fruits on the ground below shrubs, no fruits that had fallen in the mesh bags, and no reports of other vertebrate consumers of *Hamelia* fruits.

Effects of simulated herbivory on fruit palatability

To investigate whether leaf herbivory can potentially lead to correlated changes in fruit chemistry, we simulated herbivory to *H. patens* leaves using mechanical damage and/or treatment with MeJA, and, using a series of bioassays, examined whether there were changes in

the palatability of adjacent fruits to ants and fungi. Ants and fungi were chosen as bioassay test organisms because they are abundant and easily manipulable, and have been successfully used to assay changes in plant chemistry in other studies (e.g. Fincher et al. 2008; Kessler and Baldwin 2007; Liu et al. 2009). In addition, these organisms interact with *H. patens* in natural populations; ants (in particular *Ectatomma ruidum*) visit extrafloral nectaries located on the distal end of *H. patens* fruits (personal observation), and the fungal strain that we used was isolated from rotting fruits collected from *H. patens* trees at our study site. Thus, their responses in bioassays can provide ecologically relevant information about the potential for plant-herbivore interactions to affect various other interactions in which plants are involved.

First, for the ant bioassays, we chose two comparable infructescences on each of ten shrubs which were randomly assigned to MeJA or lanolin control treatments as described above and enclosed in mesh bags to prevent fruit removal. After three days we removed the bags and harvested the infructescences for use in two identically designed bioassays with two species of ants. In order to minimize the potentially confounding effects of differences among fruits of different ripening stages, we used only those fruits from the infructescence that were maroon in color. Fruits only remain in this color stage for approximately 24 hours; thus all fruits used in our bioassays were of similar maturity at the time of harvest.

We modeled our ant bioassays after those described in Kessler and Baldwin (2007). We first prepared a 12.5 % sucrose solution in distilled water. This sugar concentration is within the range typically found in ripe, bird-dispersed fruits, and has been successfully used to attract a diversity of ants in other studies (Kessler and Baldwin 2007; Witmer 1998). We placed one maroon fruit from each infructescence in a 1.5 mL Eppendorf vial, added one mL of the sugar solution, and macerated the fruit inside the vial using a glass stirring rod. Vials were filled to the

top with additional sugar solution and homogenized with a vortex mixer. A third group of positive control vials was filled with sugar solution only. Thus each set of vials consisted of a paired set of sugar solutions containing MeJA-treated or lanolin control fruits taken from the same shrub, along with a vial containing sugar solution only. Ten sets of vials were prepared for each of two species of ant: *Ectatomma ruidum* (Roger), a common ground forager in lowland wet forests, and *Paratrechina longicornis* (Latreille), a non-native household and agricultural pest that recruits in large numbers. Ten feeding stations per ant species were established in different locations at least 50 m apart, along the forest edge for *E. ruidum* and around the La Selva laboratory for *P. longicornis*. At each station a set of three vials was buried in the soil 15 cm apart so that the openings were even with the soil surface. After one hour we closed the vial lids to trap all ants that were currently foraging inside and returned to the laboratory to count the number of recruited individuals using a stereoscope.

In a third bioassay conducted several months later, we examined growth rates of a fungus we isolated and cultured in the La Selva laboratory from rotting *H. patens* fruits and tentatively identified as *Mucor sp*. (Mucoraceae). We chose fifteen shrubs, and assigned four infructescences per shrub to the following treatments: 1) mechanical damage, 2) MeJA, 3) lanolin control, and 4) absolute control. These treatments were all identical to those described above, with the exception of mechanical damage, which we added as a substitute for herbivory since we were unable to locate herbivores in sufficient numbers for experimentation. For this treatment we inflicted six rows of puncture damage on the two leaves subtending the infructescence using a pattern wheel (Baldwin and Schmelz 1994). Three days after the treatments, one fresh maroon fruit from each of the four infructescences was macerated with a glass rod in a test tube and soaked in 5 mL methanol for 24 hours. The resulting extracts were

filtered and 200 μL aliquots were distributed evenly over the surface of a potato dextrose agar plate using a flame-sterilized glass spreader. The plates were left uncovered on a sterile laminar-flow bench for 30 minutes to allow evaporation of the solvent, and then inoculated with a 0.5 cm^2 plug of agar from a stock culture of the fungus. An additional fifteen control plates were prepared in the same manner using methanol only. Inoculated plates were incubated at ambient temperatures (20-25^o C), and the radial growth of hyphae was measured after 48 hours.

Effects of simulated herbivory on fruit ripening

To investigate whether fruit ripening rates may also be influenced by induced responses to herbivory, we treated two infructescences on each of ten shrubs as either MeJA or lanolin control as described above. Fruits were enclosed in mesh bags to prevent removal, and the numbers of fruits in four ripening categories (green, cream/pink, red/maroon, and black) were counted on each infructescence at the time of treatment and again after three days. Since individual fruits were not marked, a conservative estimate of the number of fruits that were actively ripening during this time was taken by adding the number of fruits entering the black category and the number of fruits leaving the green category. Based on our own observations, we assumed that no fruits could have changed from green to black during the three day period; therefore this method underestimates the number of actively ripening fruits by ignoring the fruits changing from cream/pink to red/maroon categories.

Statistical analyses

To test whether herbivore or MeJA-induced responses affect fruit removal, we used a generalized linear mixed model (GLMM) with a binomial distribution and the logit link function,

run using the lme4 package (Bates and Maechler 2010) of the statistical software R ver. 2.15.1 (R Development Core Team 2012). Treatment was specified as a fixed effect and shrub as random effect, and the model was fit by the Laplace approximation. Residuals did not reveal any outliers or indicate overdispersion. For hypothesis testing, we used Akaike information criteria corrected for small sample size (AIC_C), since likelihood ratio tests are unreliable for small sample sizes in GLMMs (Bolker et al. 2009). We took a conservative approach and considered a $\Delta AIC_C > 10$ between the full model and a null model that included only shrub as a random effect as support for our hypothesis. Pairwise contrasts of MeJA to lanolin control and herbivory to absolute control were specified *a priori*, and tested using Wald *Z* statistics.

To test preferences of *E. ruidum* and *P. longicornis* ants to various sugar solutions, we used non-parametric Friedman's ANOVAs, blocked for feeding station, followed by Wilcoxon-Nemenyi-McDonald-Thompson tests for multiple comparisons (Hollander and Wolfe 1999). Fungal growth rates were compared using a one-way ANOVA, followed by a Tukey's HSD test for multiple comparisons. To test the ripening rates of fruits on MeJA-treated versus control infructescences, the average proportion of maturing fruits per infructescence was compared using a paired t-test. Data were arcsin square-root transformed prior to analysis to fit assumptions of normality. Analyses were performed in JMP 9.0.2 (2010) or R 2.15.1 (R Development Core Team 2012).

2.4 Results

Leaf herbivory reduces fruit removal

Blue-gray Tanagers (*Thraupis episcopus*), Passerini's Tanagers (*Ramphocelus passerinii*), and Collared Araçaris (*Pteroglossus torquatus*) were observed feeding on *Hamelia*

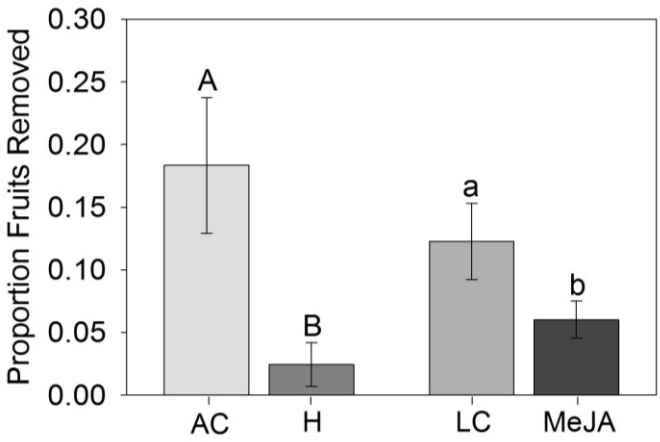

Figure 2.1: Average (± SE) proportion of fruits removed from *Hamelia patens* infructescences receiving the following treatments: herbivory on subtending leaves (H), similar handling but no treatment applied (AC: absolute control), methyl jasmonate in lanolin paste applied to subtending leaves (MeJA), or lanolin paste only applied to subtending leaves (LC: lanolin control). Different letters indicate differences from pairwise contrasts of herbivory to absolute control and MeJA to lanolin control.

patens fruits during this study. In our experiment examining the effects of herbivory and treatment with MeJA on fruit removal rates (Fig. 2.1), the model that included the effects of treatment provided a much better fit to the data than the null model that included shrub only ($\Delta AIC_C = 18.2$). Pairwise contrasts showed a significant reduction in removal rates in herbivory treatments as compared to absolute controls (Wald Z=3.417, N=7, p=0.0006), as well as a significant reduction in removal rates in MeJA treatments as compared to lanolin controls (Wald Z=3.272, N=22, d.f.=1, p=0.001).

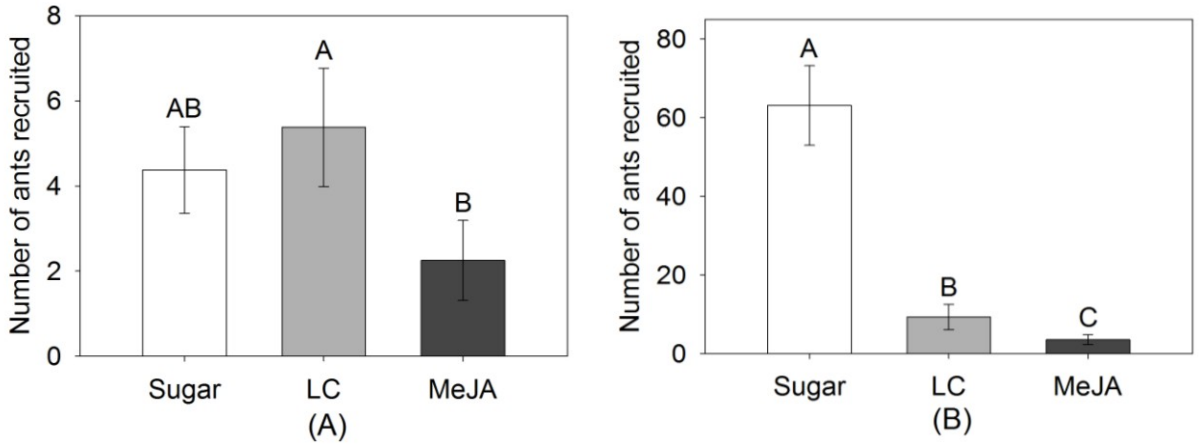

Figure 2.2: Two bioassays testing the effects of induced responses to herbivory on fruit palatability. We compared the average number (± SE) of *Ectatomma ruidum* (A) and *Paratrechina longicornis* (B) ants recruited to control sugar solutions (Sugar), sugar solutions with fruits from lanolin control branches (LC), and sugar solutions with fruits from methyl jasmonate treated branches (MeJA). Different letters indicate differences ($p<0.05$) from post-hoc comparisons of data by Wilcoxan-Nemenyi-McDonald-Thompson tests.

Simulated herbivory reduces fruit palatability

In the bioassays using *E. ruidum*, there was an overall effect of sugar solution on ant recruitment ($\chi 2=7.19$, d.f.=2, $p < 0.028$), and post-hoc comparisons showed fewer individuals recruited to solutions made from MeJA-treated fruits as compared to lanolin control fruits (Fig. 2.2A). For bioassays using *P. longicornis*, there was also an overall effect of sugar solution ($\chi 2 = 17.18$, d.f.=2, $p < 0.00019$), and here post-hoc tests showed sugar-only solutions had the highest recruitment, followed by lanolin controls, and then MeJA-treated fruit (Fig. 2.2b).

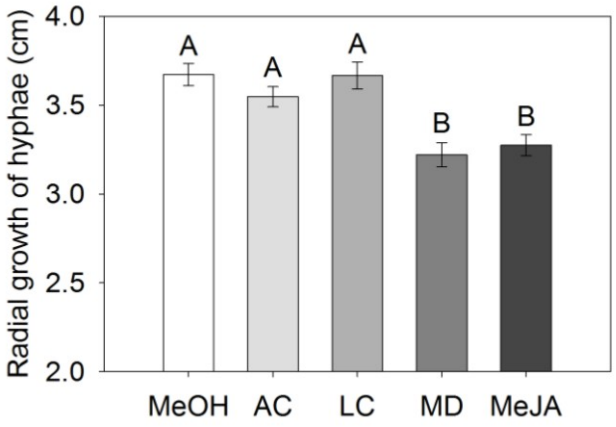

Figure 2.3: Results from a bioassay showing the average (±SE) growth rates of fungi on potato dextrose agar supplemented with methanol only (MeOH) or methanol extracts of *H. patens* fruits from absolute control (AC), lanolin control (LC), mechanical damage (MD), or methyl jasmonte treated groups (MeJA). Different letters indicate differences ($p<0.05$) from post-hoc comparisons of data by Tukey's HSD test.

In the fungal-growth bioassay, the growth of hyphae was significantly affected by growth medium treatment ($F_{5,70}=12.00$, $p < 0.0001$; Fig. 2.3). Specifically, post-hoc tests showed that fungi grew faster on methanol only, absolute control and lanolin control plates than on plates treated with fruit extracts from MeJA and mechanically damaged groups.

Simulated herbivory increases fruit ripening rate

MeJA-treated infructescences had significantly more actively ripening fruits relative to infructescence size than control infructescences (paired $t = -2.39$, d.f.=9, $p = 0.04$) (Fig. 2.4).

2.5 Discussion

Induced defenses are important physiological responses to herbivory that can protect plants against further damage. However these responses can lead to correlated changes in the expression of other traits and thus the alteration of interactions with non-target organisms. Our results provide the first documentation of an ecological cost of plant responses to herbivory in the currency of fruit removal by seed dispersers. Leaf herbivory or MeJA application to leaf petioles led to reduced fruit removal on adjacent infructescences and results from several bioassays with ants and fungi suggested that this response was mediated by a chemical change that reduced fruit palatability to these organisms. In addition, MeJA-mediated responses in leaves affected fruit development, providing evidence for an additional mechanism through which leaf herbivory can affect fruit-frugivore interactions and seed dispersal.

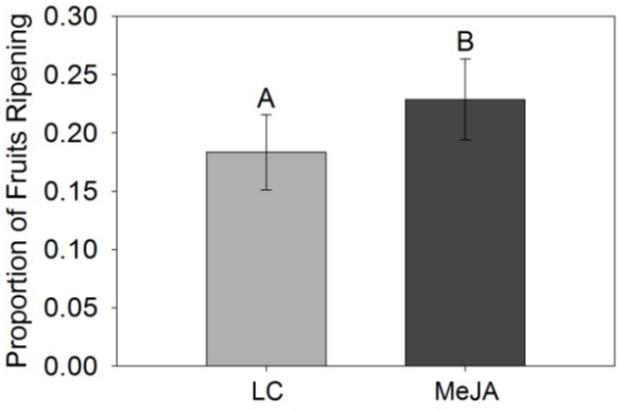

Figure 2.4: The average proportion (± SE) of fruits that were actively ripening (estimated as the total number of fruits that were either initiating ripening or entering final ripening stages) from *Hamelia patens* infructescences from lanolin control (LC) and methyl-jasmonate (MeJA) treatment groups. Different letters indicate differences from a paired t-test on transformed data, and data were back-transformed for the figure.

A plausible mechanistic explanation for our results is that the reduced removal rates and palatability of induced fruits to our bioassay test organisms were due to an increase in deterrent compounds in fruits after leaf herbivory. Although chemical analyses of fruits were not performed, we used three separate bioassay organisms that bridged broad taxonomic groups (two ant species and one fungal strain), and all of these showed a negative response to fruits from induced branches in comparison to controls. Birds were also seemingly deterred from feeding

upon induced fruits, and prior work has shown that birds, in particular those, such as tanagers, that masticate fruits prior to ingestion, can detect even small changes in the chemical composition of fruits (Levey 1986). The leaves of *H. patens* are rich in pentacyclic indole alkaloids, the most abundant of which is isopteropodine (Reyes-Chilpa et al. 2004), and these same alkaloids may also be present in fruits (unpublished data, referenced in Levey 1987).

Our results showing faster ripening of fruits on MeJA-treated branches also corroborate our hypothesis of chemical changes in fruits following leaf herbivory. Given the importance of MeJA in mediating multiple physiological pathways, it could be argued that the effect of herbivory on fruit removal might be an artifact of changes in fruit development. In a study by Redman et al. (2001), the expression of induced resistance traits *decreased* fruit ripening rates in tomato plants (*Lycopersicon esculentum*), but this is the opposite of what we found in our plants. Our results show that MeJA increased fruit ripening rates, and, assuming birds or other consumers are more likely to remove ripe fruits, we would have expected that fruits of MeJA treated plants would be removed faster than control fruits. Instead these fruits were removed more slowly; thus any potential increase in the availability of ripe fruits on induced branches either did not increase removal or did not increase it enough to counteract the effects of any potential changes in fruit chemistry following simulated herbivory.

Although the negative effects of herbivore addition or simulated wounding on fruit removal and palatability to our bioassay test organisms are clear, the overall effect of this change in terms of individual plant fitness requires further investigation. Two important considerations come to mind. First, the relationship between fruit removal from the parent plant and the eventual establishment of reproductive offspring is undoubtedly complex and involves stochastic processes that can happen over long time scales (Wang and Smith 2002). Induced changes in

fruit chemistry could affect multiple aspects of the process, e.g. seed germination or seedling defense (Agrawal 2002; Cappelletti et al. 1992). Second, since the fungal strain used in one of our bioassays was isolated from rotting *H. patens* fruits found still attached to the plant, this experiment also provides ecologically relevant information about the potential defensive role of fruit compounds against fungal pathogens. Although it is not clear from the present study whether the *Mucor* fungus had any causal role in fruit rot, fungi in this genus have been shown to speed up fruit deterioration in other systems (Okwulehie and Alfred 2010). Thus, a reduction in its growth rate or that of other fungal species involved in rot could potentially provide some adaptive benefit to the plant by reducing seed damage or increasing the persistence time of ripe fruits. The optimal expression of defensive compounds in fruits likely reflects a balance between the costs in terms of reduced fruit removal and the benefits in terms of defense against pests, as suggested by the defense trade-off hypothesis for the presence of deterrent compounds in fruit (Cipollini and Levey 1997b; Herrera 1982). However, our results clearly suggest that, in addition to the multiple selection pressures on fruit chemical traits from mutualistic and antagonistic frugivores, there can also be important physiological constraints on these traits depending on the larger ecological context.

Overall, our study provides strong initial evidence of correlations between the expression of leaf and fruit chemical traits, and suggests two mechanisms through which induced responses to herbivory may impose costs in terms of alteration of fruit-frugivore interactions: changes in fruit palatability and changes in fruit ripening rates. Future work in this and other systems should focus on providing quantitative analysis of leaf and fruit chemistry in response to damage, and a thorough examination of how changes in fruit palatability translate to effects on plant fitness. Since plants interact simultaneously with both mutualists and antagonists,

integrative studies of the fitness effects of correlated plant traits are necessary if we are to understand complex selective forces and constraints on the evolution of plant chemical traits.

CHAPTER THREE

IRIDOID AND SECOIRIDOID GLYCOSIDES IN A HYBRID COMPLEX OF BUSH HONEYSUCKLES (*LONICERA SPP.,* CAPRIFOLICACEAE): IMPLICATIONS FOR EVOLUTIONARY ECOLOGY AND INVASION BIOLOGY [2]

3.1 Abstract

Interspecific hybridization among non-native plant species can generate novel genotypes that are more reproductively successful in the introduced habitat than either parent. One important mechanism that may serve as a stimulus for the evolution of invasiveness in hybrids is increased variation in secondary metabolite chemistry, but still very little is known about patterns of chemical trait introgression in plant hybrid zones. This study examined the occurrence of iridoid and secoiridoid glycosides (IGs), an important group of plant defense compounds, in three species of honeysuckle, *Lonicera morrowii* A. Gray*, Lonicera tatarica* L.*,* and their hybrid *Lonicera* x *bella* Zabel. (Caprifoliaceae), all of which are considered invasive in various parts of North America. Hybrid genotypes had a diversity of IGs inherited from both parent species, as well as one component not detected in either parent. All three species were similar in that overall concentrations of IGs were significantly higher in fruits than in leaves, and several compounds that were major components of fruits were never found in leaves. However, specific patterns of quantitative distribution among leaves, unripe fruits, and ripe fruits differed among the three species, with a relatively higher allocation to fruits in the hybrid species than for either parent. These patterns likely have important consequences for plant interactions with antagonistic herbivores and pathogens as well as mutualistic seed dispersers, and thus the potential

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invasiveness of hybrid and parental species in their introduced range. Methods established here for quantitative analysis of IGs will allow for the exploration of many compelling research questions related to the evolutionary ecology and invasion biology of these and other related species in the genus *Lonicera*.

3.2 Introduction

Hybridization between plant species has been implicated as an important mechanism that can underlie the evolution of invasiveness (Ellstrand and Schierenbeck 2000; Schierenbeck and Ellstrand 2009). A large source pool of genetic variation in hybrid genotypes provides increased raw material on which natural selection can act, potentially leading to evolutionary novelty in life history, morphology, phenology, or secondary metabolite chemistry that can make some hybrid populations better adapted to new environments (e.g. Geiger et al. 2011; Oberprieler et al. 2010; Schweitzer et al. 2002). However, despite the importance of interspecific hybridization in invasion biology, plant evolution, and the structuring of ecological communities (Barton 2001; Hegarty and Hiscock 2005; Martinsen et al. 2001; Schierenbeck and Ellstrand 2009; Whitham et al. 1999), there are still many unanswered questions about patterns of trait introgression in hybrids, particularly for secondary chemistry (Orians 2000). Hybrids can differ chemically from the parental species both qualitatively and quantitatively—they may have chemical compounds typical of one or both parents, fail to express certain compounds produced by parents, or have novel compounds not typical of either parent (Cheng et al. 2011; Orians 2000; Orians and Fritz 1995; Rehill et al. 2006; Rieseberg and Ellstrand 1993). Because plant chemistry has important consequences for species interactions and, therefore, the reproductive success of plants (Coley and Barone 2001; Eisner and Meinwald 1995), a better understanding of the chemical variation

among hybrids may provide important insights into why certain hybrids become invasive in introduced ranges while others never establish viable populations (Ellstrand and Schierenbeck 2000; Fritz 1999; Fritz et al. 1999; Strauss 1994; Whitney et al. 2006).

Exotic bush honeysuckles (*Lonicera spp.*, Caprifoliaceae) are some of the most problematic invasive species in the eastern and mid-western United States (Nyboer 1992; Webster et al. 2006). Most species fruit in abundance and are thought to be dispersed primarily by birds (Bartuszevige and Gorchov 2006; Ingold and Craycraft 1983), although white-tailed deer may also be important as dispersers (Vellend 2002; Whitehead, personal observation). Their introduction and spread have led to altered plant communities and reduced native plant diversity in many areas (e.g. Collier et al. 2002; Woods 1993), which may be due to competitive (Gorchov and Trisel 2003) or allelopathic effects (Cipollini et al. 2008). High densities of honeysuckle shrubs can also have cascading effects in ecosystems, including alteration in resource availability for birds (Bartuszevige and Gorchov 2006; Ingold and Craycraft 1983), declines in amphibian communities due to high levels of allelochemicals produced by the plants (Watling et al. 2011), and even increased disease risk for humans through indirect effects on deer populations that serve as reservoirs for parasites and pathogens (Allan et al. 2010). Some of the most invasive species include *Lonicera tatarica* L.*, Lonicera morrowii* A. Gray, and their hybrid progeny *Lonicera* x *bella* Zabel., which form hybrid swarms throughout much of the introduced range (Barnes and Cottam 1974; Nyboer 1992; Webster et al. 2006). The hybrid species appears to be more successful in North America than either parent, as evidenced by the wide variety of habitats that the hybrid inhabits, its higher abundance relative to the parent species, and the high frequency of hybrid individuals that exhibit morphological traits intermediate to the parents (Barnes and Cottam 1974; Whitehead, personal observation).

The *L.* x *bella* hybrid complex provides an intriguing system for phytochemical research. A comparison of secondary metabolites produced in parental and hybrid species would add an important new component to a growing literature on the chemical consequences of hybridization and establish analytical methods that will allow researchers to address many questions related to the evolutionary ecology and invasion biology of these species. The phytochemistry of *Lonicera* has been previously investigated due to the importance of various species in traditional pharmacopeias, and the genus contains at least two classes of secondary compounds with known ecological and economic importance: iridoid and seco-iridoid glycosides (IGs) and phenolics (Chen et al. 2007; Cipollini et al. 2008; Ikeshiro et al. 1992; Li et al. 2003; Song et al. 2006; Svobodova et al. 2008; Wang et al. 2003; Zadernowski et al. 2005). Here we focus on IGs, which are an important class of plant defensive compounds found in over 50 plant families (Bowers 1991), but have not been previously investigated in the context of plant hybridization. Souza and Mitsohashi (1969; 1970) and Ikeshiro et al. (1992) have provided initial descriptions of six IGs in fruits and leaves of *L. morrowii*, and there is one report of secologanin (6) (Fig. 3.1) in *L. tatarica* (Hermanslokkerbol and Verpoorte 1987). However, to our knowledge, there is no information of the role of IGs in the ecology and evolution of these species, and there are no studies that have described the occurrence of IGs in the hybrid species, *L.* x *bella*.

IGs play an important ecological role in plant defense against both herbivores and pathogens (Bowers 1991; Marak et al. 2002a; Marak et al. 2002b). Their composition and concentration can vary considerably between species, among individual plants and plant organs, and throughout plant development (Jamieson and Bowers 2010; Peñuelas et al. 2006; Quintero and Bowers 2011a). This spatial and temporal variation has direct implications for plant antagonists as well as cascading effects that can influence interactions with higher trophic levels

(Dyer and Bowers 1996; Jamieson and Bowers 2010; Lampert et al. 2011; Lindstedt et al. 2010; Peñuelas et al. 2006; Quintero and Bowers 2011b; Reudler et al. 2011). Although past work on the ecological role of IGs has focused primarily on leaves; these compounds have also been found in the fruits of various species (Ikeshiro et al. 1992; Makarevich et al. 2009; Ono et al. 2005). Fruit secondary compounds may function to defend fruits against antagonists (e.g. insect seed predators and fungal pathogens) and/or to regulate the foraging and feeding behaviors of vertebrate seed dispersers (Cipollini and Levey 1997b; Herrera 1982; Levey et al. 2007; Tewksbury et al. 2008b); however IGs have not been examined in this context. The potential role of IGs in fruit/frugivore interactions is of particular interest in the context of invasion biology, because the spread of an invasive species can be greatly accelerated by an effective dispersal mechanism in the novel habitat (Gosper et al. 2005; Higgins and Richardson 1999; Richardson et al. 2000).

The objectives of this study were to: 1) provide a detailed method for extraction and quantification of IGs in *Lonicera* species that will be useful to ecologists and evolutionary biologists; 2) describe the occurrence of IGs in the hybrid honeysuckle *L.* x *bella* and its parental species *L. tatarica* and *L. morrowii*; and 3) compare the composition and concentration of IGs among leaves, unripe fruits, and ripe fruits in these three species. Results are discussed in the context of their potential implications for the evolution of chemical traits in hybrid genotypes, species interactions with herbivores and seed dispersers, and invasion biology.

3.3 Results and Discussion

Leaf and fruit samples of *L. morrowii, L. tatarica,* and *L.* x *bella* were obtained from the living collections at the Arnold Arboretum of Harvard University (Cambridge, MA, USA). *L.*

morrowii was originally wild-collected from the Honshu Provenance in Japan in 1984, *L. tatarica* was wild-collected from Tajikistan in 1978, and the hybrid species, *L.* x *bella*, is of cultivated origin and was received at the arboretum in 1919 from Boston, MA (BG-BASE 2011). In addition, we collected from three wild populations of *L.* x *bella* growing near Boulder, CO, USA. The identification and quantification of IGs was carried out using gas chromatography with mass spectrometry detection (GC-MS).

Six major IG components (on average representing 89.1% of the estimated total IGs) were identified by comparison to authentic reference standards (Table 3.1). One other presumably related major component (Unknown G, 10.0% of estimated total IGs) and six minor components (totaling <1% of estimated total IGs) were also detected and provisionally characterized as IG's based on characteristic fragmentation patterns in mass spectra as described in detail in Inouye et al. (1976) and Popov and Handjieva (1983). Although there is no spectral peak associated with the molecular ion for silylated iridoids, several peaks associated with the aglycone portion of the molecule are very informative, and, in combination with peaks originating from the sugar moiety, served as a means for positive identification of previously characterized IGs. The sugar moiety of IGs gives peaks at *m/z* 361 (usually the base peak), 271, 243, 217, 204, 191, 169, 147, 129, 103, and 73, all of which were present in the spectra of all detected IGs. Ions originating from the aglycone portion of the molecules are diagnostic of individual compounds and are presented in Table 3.1. Characteristic IG ion fragmentation patterns are shown in Fig. 3.3 and include Ion B (formed from the loss of the sugar moiety from the aglycone), Ion C (arising solely from the dihydropyran ring portion), and, for seco-iridoids, Ion E (formed by McLafferty-type rearrangement of the dihydropyran ring). In a few cases, we

Table 3.1: Identifications, retention times, diagnostic MS peaks, and quantities (% dry wt) of iridoid and secoiridoid glycosides in three species of *Lonicera.* Quantities represent averages from 2-6 samples taken from a single individual of each species.

			Lonicera morrowii			***Lonicera* x *bella***			***Lonicera tatarica***		
Compound Identification	**RT**	**MS**	leaves	unripe	ripe	leaves	unripe	ripe	leaves	unripe	ripe
Unknown A	26.01	165, 139[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND
Unknown B	28.87	301, 165, 139[a]	0.03[b]	ND	ND	ND	ND	ND	ND	ND	ND
Sweroside	29.38	179	0.21[b]	ND	ND	0.98	0.17[b]	0.20[b]	1.38	0.47	0.52
Secoxyloganin	29.77	297, 165, 139[a]	0.07[b]	ND	ND	0.37[b]	10.75	13.00	0.21[b]	5.90	2.22
Loganin	30.11	283, 165[a], 139[a]	0.06[b]	3.36	3.47	0.01[b]	0.10[b]	0.09[b]	ND	0.03[b]	ND
Unknown C	30.46	428[a], 339, 197	0.06	0.08[b]	0.03[b]	ND	ND	ND	ND	ND	ND
Unknown D	31.56	329, 165, 139	ND	0.47	ND	ND	0.03[b]	ND	ND	ND	ND
Unknown E	31.77	281	ND	0.06[b]	ND	ND	0.01	ND	ND	ND	ND
Loganic acid	32.05	341, 197, 165[a]	0.35	0.03[b]	ND	0.04[b]	ND	ND	ND	ND	ND
Morroniside	33.62	388[a], 299, 139	ND	2.05	1.58	ND	0.07[b]	ND	ND	1.20	0.36
Unknown F	35.06	314, 225, 165, 139	ND	0.16	0.07[b]	ND	ND	ND	ND	0.12[b]	ND
Unknown G	36.00	314, 225, 165, 139	ND	4.42	3.68	ND	0.50	0.21	ND	ND	ND
Secologanin	41.98	255[a], 165, 139	3.09	0.30[b]	ND	0.31[b]	1.77[b]	0.14[b]	0.65	0.61[b]	ND
TOTAL			**3.87**	**10.93**	**8.83**	**1.71**	**13.40**	**13.64**	**2.24**	**8.33**	**3.10**

ND=not detected

[a] Low intensity MS peak

[b] Not detected in all samples

also observed a peak due to Ion D (formed by the loss of the sugar moiety followed by the rearrangement of the TMSi group with Ion A). One unknown compound (Unknown E) appeared to be an iridoid of the oleuropein-type, with a characteristic peak due to Ion F, originating from a phenethyl alcohol side chain at C-5 (Inouye et al. 1976). By comparing the observed diagnostic *m/z* peaks to those that would be predicted for known IGs occurring in *L. morrowii* and other closely related *Lonicera* species (Bailleul et al. 1981; Calis et al. 1984; Peñuelas et al. 2006; Souzu and Mitsuhashi 1969; Souzu and Mitsuhashi 1970; Wang et al. 2003; Yu et al. 2011), we were able to provisionally identify all but one of the putative IG components observed in our samples.

Both fruits and leaves from all three honeysuckle species contained IGs, the most abundant of which were sweroside (1), secoxyloganin (2), loganin (3), loganic acid (4), morroniside (5), and secologanin (6) (Fig. 3.1). On average, these six major components represented 89.1% of the total IGs in our samples (Table 3.1); however, a number of additional components were also detected. Of the three species sampled from the Arnold Arboretum, the

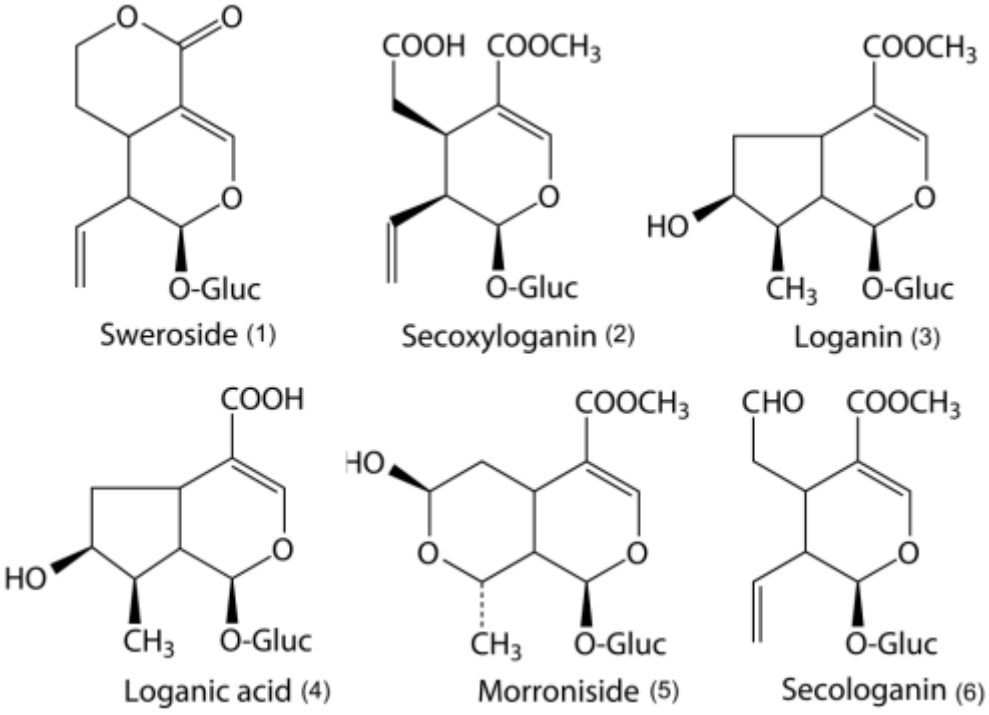


Figure 3.1: Structures of major iridoid and secoiridoid glycosides from *Lonicera* x *bella, L. morrowii,* and *L. tatarica*

parental species *L. morrowii* had the highest chemical diversity, with a total of 12 provisionally detected IGs. The chemical profiles of *L. tatarica* and *L.* x *bella* included only a subset of these compounds, with six individual IGs provisionally detected in *L. tatarica* and nine provisionally detected in *L.* x *bella* (Table 3.1). However, additional collections from wild populations of *L.* x *bella* in Colorado showed all of the 12 compounds detected in *L. morrowii* plus an additional minor unknown component not detected in either of the parental species (Table 3.2). Total quantities of IGs also differed among parental and hybrid species, but only for certain plant parts. Quantities were similar for all three species in leaves, but in fruits tended to be higher in the hybrid than for either parent (Fig. 3.2). However, this quantitative pattern among species was variable depending on the individual IG examined; for certain compounds the hybrid species contained more than either parent (e.g. secoxyloganin [2]) and for other compounds the hybrid contained less (e.g. morroniside [5]; Table 3.1).

The total concentration of IGs was significantly higher in unripe and ripe fruits than in leaves, but unripe and ripe fruits were not different from each other ($F_{2,28}$= 23.95, p<0.0001; Fig. 3.2). This pattern supports the predictions of optimal defense theory, which suggests that

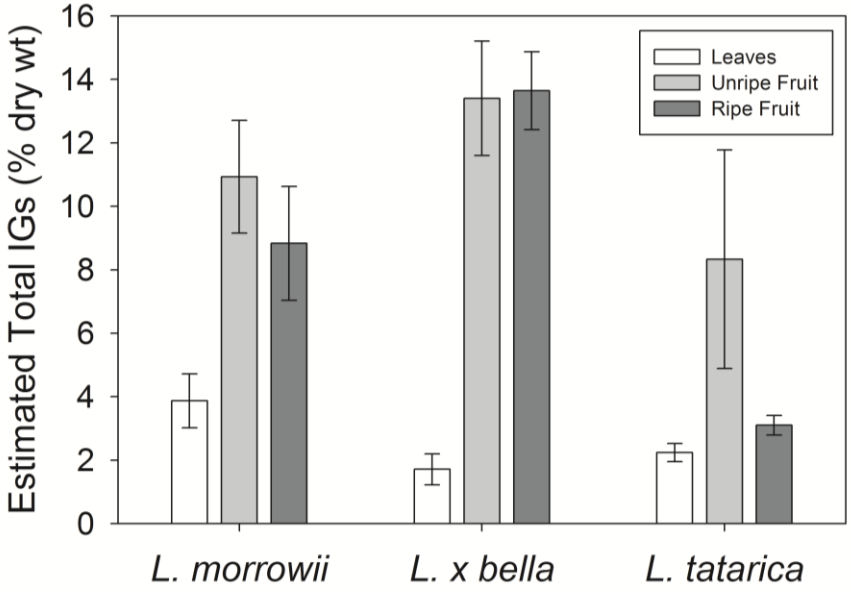


Figure 3.2: Average (± SE) estimated total iridoid glycoside (IG) concentration in leaves, unripe fruits, and ripe fruits of three species of *Lonicera*. Estimated totals include compounds 1-6 (89.1% of total) as well as Unknowns A-G that were provisionally identified as IGs. Averages are from 3-6 collections taken from a single individual of each species.

Table 3.2: Comparison of iridoid and secoiridoid glycoside quantities (% dry wt) in four individuals of *L.* x *bella.* Quantities represent averages from 2-6 samples taken from a single individual from each location.

	Arnold Arboretum			**CO-Skunk Canyon**			**CO-Gregory Canyon**			**CO-Bluebell Canyon**		
Compound Identification	leaves	unripe	ripe	leaves	unripe	ripe	leaves	unripe	ripe	leaves	unripe	ripe
Unknown A	ND	ND	ND	0.37	0.61	0.39	0.70	0.24	0.11	0.88	0.15	0.21
Unknown B	ND	ND	ND	0.19	0.21	ND	0.23	0.15	ND	0.18	0.15	ND
Sweroside	0.98	0.17	0.2	3.92	3.78	3.50	3.92	1.20	1.26	2.64	1.75	1.98
Secoxyloganin	0.37	10.75	13	0.30	8.53	13.74	ND	ND	ND	0.71	2.57	7.48
Loganin	0.01	0.1	0.09	0.25	0.35	0.21	1.42	11.34	7.04	0.19	0.39	0.33
Unknown C	ND	ND	ND	0.11	0.19	0.07	0.10	ND	ND	0.16	0.16	0.10
Unknown D	ND	0.03	ND	0.28	1.25	0.66	0.10	0.45	0.70	0.11	0.62	0.19
Unknown E	ND	0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Loganic acid	0.04	ND	ND	0.06	0.18	0.18	0.09	0.24	0.06	0.16	0.11	0.06
Morroniside	ND	0.07	ND	ND	3.04	2.06	ND	4.53	1.90	ND	0.51	0.47
Unknown F	ND	ND	ND	ND	0.20	0.12	ND	0.17	0.03	ND	0.06	0.04
Unknown G	ND	0.5	0.21	ND	ND	ND	ND	0.02	ND	ND	0.97	0.93
Secologanin	0.31	1.77	0.14	3.44	6.05	2.38	2.12	1.65	0.09	1.61	1.70	0.48
TOTAL	**1.71**	**13.40**	**13.64**	**8.92**	**24.38**	**23.31**	**8.68**	**20.00**	**11.21**	**6.65**	**9.14**	**12.28**

ND=not detected

CO=Colorado

fruits should be well defended due to their high reproductive value for the plant (McKey 1974; McKey 1979; Rhoades and Cates 1976; Zangerl and Rutledge 1996). However, it is notable that levels of IGs in fruits do not necessarily diminish with ripening, as one might expect for compounds that could be potentially toxic to seed dispersers. In fact, for the dominant compound in fruits of *L.* x *bella*, secoxyloganin (2), concentrations were substantially *higher* in ripe fruits than in unripe fruits collected from the same branch at the same time (Table 3.2).

It is also notable that there was considerable qualitative and quantitative variation in IGs *within* individuals and species. Fruits and leaves were sampled from 2-6 branches of each individual, and often compounds detected in one collection were not detected in other branches from the same shrub, especially for minor components (Table 3.1). This suggests that abiotic and/or biotic factors that influence IG composition and concentration may be localized within a plant and that additional sampling of these and other individuals may show considerable variation and additional minor components not detected here. For the hybrid species, *L.* x *bella*, a total of four different individuals were sampled, and the chemical composition was highly variable. In particular, there were four compounds present in our collections from Colorado that were not present in the collections from the Arnold Arboretum. Even within Colorado, different individuals had different compositions, e.g. secoxyloganin, which is the dominant IG in fruits for most individuals, was not detected in the Gregory Canyon samples (Table 3.2).

These results are similar to those reported previously for *L. morrowii* showing different patterns of IG occurrence in fruits and leaves. Compounds occurring in *L. morrowii* fruits, but not detected in leaves, included morroniside (5), as well as one unidentified major component (Unknown G) and three minor components provisionally characterized as IGs (Unknowns D-F; Table 3.1). Past work has reported two IGs unique to fruits in this species, morroniside (5) and

kingiside (Ikeshiro et al. 1992; Souzu and Mitsuhashi 1969; 1970). Kingiside was not definitively identified in this study, but has predicted MS fragmentation patterns consistent with Unknown G (Fig. 3.3; Table 3.1). Other compounds occurred in leaves of *L. morrowii,* but were not detected in fruits, including sweroside (1) and secoxyloganin (2); however, interestingly, these compounds were major components of both fruits and leaves of *L. tatarica* and *L.* x *bella* (Table 3.1). Various other compounds occurred in both fruits and leaves, including loganin (3), loganic acid (4), and secologanin (6) (Table 3.1).

This study provided the first examination of IGs in the hybrid species, *L.* x *bella.* Hybrid individuals collected from the Arnold Arboretum had chemical traits that were intermediate between the two parents (Table 3.1), but some collections from Colorado had higher chemical diversity and higher overall concentrations than either parent (Table 3.2). These differences may be due to the variable genetic background of hybrids collected from wild populations, which readily backcross with parental species and form hybrid swarms throughout much of the introduced range in North America (Barnes and Cottam 1974; Hauser 1966). Morphological characters that clearly differentiate the two parental species, such as leaf pubescence and peduncle length (Weber and Wittmann 2001), are highly variable in Colorado populations, and appear to form a continuum between the two parental phenotypes (Whitehead, personal observation). Results presented here include only samples taken from individuals with intermediate phenotypes (e.g. pink flowers and sparse pubescence on the leaves and peduncles), but an examination of how the variation in chemical and morphological traits correlate with the patterns of genetic introgression in hybrid swarms would be enlightening and have important consequences for our understanding of the adaptive radiation of *Lonicera* species. Thorough studies of this type should include an examination of compounds in different plant parts, since

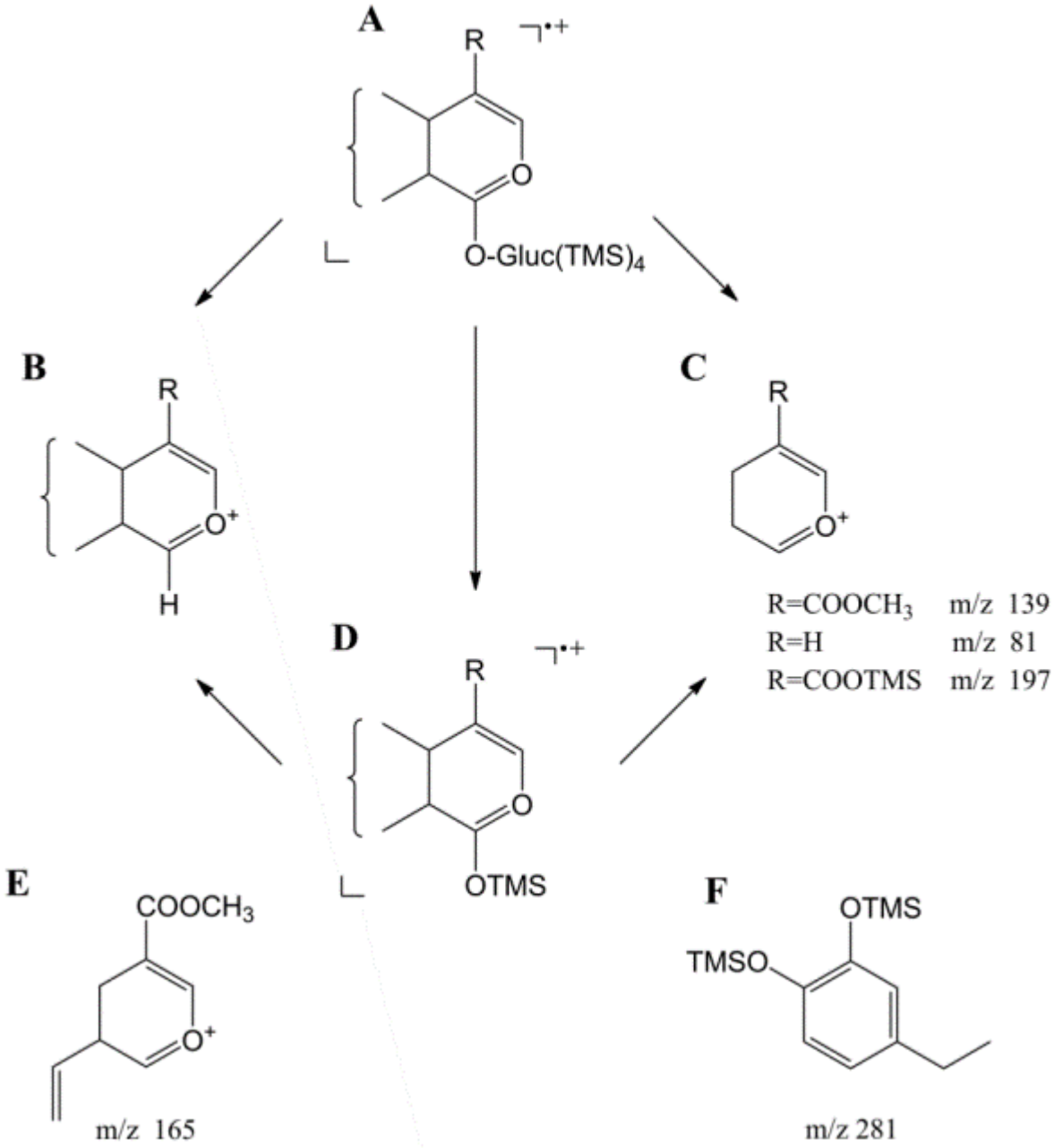


Figure 3.3: Fragmentation patterns of silylated iridoid and seco-iridoid glycosides in GC/MS analysis. Figure redrawn from Inouye et al. (1976).

our results show that it can be in the distribution of compounds among leaves and fruits that the hybrid origin of chemical traits is most apparent.

Because the fruits of *Lonicera* are vertebrate-dispersed, IGs in ripe fruits likely have important implications for the palatability of fruits to mutualists and therefore the reproductive success of the hybrid species. Although birds and other frugivores do tolerate high levels of secondary metabolites in certain fruits (Barnea et al. 1993; Cipollini and Levey 1997c; Filardi and Tewksbury 2005; Levey and Del Rio 2001), the little existing evidence for the effects of IGs on birds suggests that some of these compounds can be strongly deterrent and have emetic properties (Bowers 1980). One possible explanation for the maintenance of high levels of IGs in the ripe fruits of the hybrid may be that these compounds function to defend fruits against antagonistic seed predators and fruit pathogens, leading to longer persistence times on the plant and higher overall levels of fruit removal over time (Cipollini and Levey 1997b; Cipollini and Stiles 1992). Long persistence time of ripe fruit may be especially beneficial for plants in an introduced range, since often non-native fruits (including those of *Lonicera*) are not removed until late in the season once high-quality native fruits are no longer available (White and Stiles 1992).

3.4 Conclusions

This study demonstrates that the hybrid honeysuckle, *L.* x *bella*, exhibits a complex composition of IGs that is variable among individuals and reflects contributions from both parental species as well as some patterns of distribution that are not typical of either parent. Concentrations of IGs are higher in fruits than in leaves for both parents and the hybrid species, and in the hybrid species concentrations remain high even in ripe fruits. These patterns have

important implications for plant defense as well as mutualistic interactions with seed dispersers, and may influence the reproductive success of the hybrid species. The characterization of compounds and methods for quantitative analysis established in this study will provide essential tools for future work in this system, which offers an excellent model within which ecologists and evolutionary biologists can explore questions related to the chemical ecology of plant/herbivore and fruit/frugivore interactions, the patterns of trait introgression during hybridization, and the invasion biology of a widespread and problematic group of non-native shrubs in North America.

3.5 Experimental

Plant material

Samples of leaves, unripe fruits, and ripe fruits were obtained from the living collections of the Arnold Arboretum (Harvard University, Cambridge, MA, USA) for all three species examined in this study: *L. tatarica* (Acc. #299-78), *L. morrowii* (Acc. #525-84), and *L.* x *bella* (Acc. # 10087 and its asexual propagule, Acc. #392-92). Dried specimens of these accessions are also available from the Herbarium of the Arnold Arboretum at Harvard University. To obtain material for chemical analysis, we clipped two to six branches from each individual (depending on availability) when the fruits on that plant were in mid-ripening (between June 26 and July 12, 2011), thus obtaining leaves, unripe, and ripe fruits from the same branch at the same time.

Additional samples of *L.* x *bella* were collected during July 2008 in the same manner from three populations occurring near Boulder, CO, in Bluebell Canyon (39.99135 N, -105.28568 W), Gregory Canyon (39.99727 N, -105.2940 W), and Skunk Canyon (39.98611 N, -105.27660 W). Our identification as *L.* x *bella* was confirmed by two local authorities (Tim Hogan and Dina Clark, Herbarium COLO, University of Colorado Museum of Natural History).

Voucher specimens from the Colorado collections used in this study were deposited at the Herbarium COLO at the University of Colorado (Acc. #'s 543191, 543192, and 543193).

Plant samples were field-collected as clippings of entire branches and stored in coolers until they arrived at the laboratory (within 24 hours of collection). For each sample, leaves, unripe fruits, and ripe fruits were separated, retaining a minimum of 20 leaves, 10 unripe, and 10 ripe fruits. Only fruits that were completely unripe (green) or completely ripe (red or orange) were retained for analysis; those at intermediate stages were discarded. All samples were then oven-dried for 48 hours at 50^{o}C (as is typical for IG analysis; see Gardner and Stermitz 1988; Jamieson and Bowers 2010; Lampert and Bowers 2011; Quintero and Bowers 2011b).

Extractions of IGs

Methods for extraction of IGs were modified from previously published studies (Bowers and Stamp 1993; Gardner and Stermitz 1988). The dried fruits and leaves were ground to a fine homogenous powder using a mortar and pestle, first removing all seeds from the fruit samples by grinding fruits through a wire mesh strainer with the pestle. Seeds were discarded, leaving only pulp and skin, which was further ground to a fine powder in the mortar and pestle. For chemical analysis, 25mg aliquots were taken from fruit samples and 50mg aliquots were taken from leaf samples, weighed to the nearest 0.01 mg. A smaller mass of fruit material (25mg) was used because preliminary analyses showed high levels of IGs in fruit samples, leading to overloaded chromatograms. Each sample was placed in a test tube with MeOH (5ml), tightly capped, vortexed, and left overnight for extraction. All samples were then filtered, and the extracts evaporated to dryness. Extracts were then re-suspended in H_2O (3 ml), and an internal standard (phenyl-β-D-gluco-pyranoside) was added to each. Samples were then partitioned three times

against equal volumes of Et_2O. The Et_2O fractions were discarded, and the H_2O fractions, containing mostly IGs and sugars, were evaporated to dryness. Each residue was then re-suspended in MeOH (1 ml) and left overnight to allow complete dissolution of IGs. Samples were then vortexed and aliquots (100 µL) were transferred to micro-inserts for GC vials and evaporated to dryness at 50°C.

Identification and quantification of IGs using GC-MS

Aliquots for IG analysis were converted to their trimethylsilane (TMSi) analogs by adding Tri-Sil-Z derivatizing reagent (Thermo-Scientific, Waltham, MA, USA) (100 µl) to the evaporated sample and heating for 20 minutes at 70°C in a mineral oil bath. After derivatization, each sample (0.2 µl) was injected onto an HP Agilent 6890N GC coupled with an Agilent 5975C inert mass selective detector with an ion source of 70eV at 230°C and equipped with a DB-1MS capillary column (30m x 0.25mm i.d.,0.1 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). Ultra-pure He was used as carrier gas at a flow rate of 2 ml min^{-1}, a split flow ratio of 100:1, and a front inlet temperature of 275°C. The following oven conditions were employed: initial temperature 180°C, initial hold time 1 min; ramp 1: 5°C min^{-1} to 200°C, hold time 11 min; ramp 2: 2°C min^{-1} to 260°C, hold time 0 min; ramp 3: 30°C min^{-1} to 320°C, hold time 0 min; for a total run time of 48 minutes. These oven conditions were modified from previously described methods (Gardner and Stermitz 1988) to ensure adequate peak resolution of IGs while minimizing the run time for each sample. A blank sample (Tri-Sil-Z only) was run after every five samples to ensure there was no carryover between runs. Data were recorded and processed using MSD ChemStation software (version D.02.00.275).

IGs were identified by comparisons of retention times and mass spectra with authentic standards, including sweroside (1), secoxyloganin (2), loganin (3), loganic acid (4), morroniside (5), and secologanin (6). Secoxyloganin (2) was provided by Søren R. Jensen (Technical University of Denmark), morroniside (5) was purchased from Tauto Biotech Co., Ltd. (Shanghai, China), and all other standards were purchased from Indofine Chemical Company (Hillsborough, NJ, USA). On average, the IGs identified using reference standards represented 89.1% of the total IGs in our samples, and included all major components except for Unknown G.

Estimated quantities of all compounds were based on peak areas in total ion current chromatograms. For individual IGs for which reference standards were available (1-5, 7), a six-point calibration curve ($R^2 > 0.99$) was created with concentrations ranging from 0.01 to 5 mg/ml. Secologanin (6) was observed as two well-resolved peaks that occurred in a regular ratio of 1:1.2; this occurred for both authentic standards and plant extracts containing secologanin (6). These likely represent stereoisomers; however their fragmentation patterns in mass spectra are indistinguishable and thus could not be definitively differentiated in the scope of this study. Thus, to determine total quantities of secologanin (6), we used the sum of the two peak areas; this method gave excellent linearity ($R^2 = 0.997$) in a six-point calibration curve with known concentrations of authentic secologanin. To approximate concentrations of IGs for which no reference standards were available, we assumed a response factor equivalent to that of our internal standard.

Statistical analysis

To determine whether IG concentrations differed among leaves, unripe fruits, and ripe fruits, a one-way analysis of variance was employed using the statistical software JMP v. 9.0.2

(2010), with tissue type specified as a fixed effect and individual plant and branch as nested random effects included in the error term. This was followed by a Tukey HSD post-hoc test for multiple comparisons to determine pairwise differences among tissue types. Data were arcsin-square root transformed prior to analysis to meet assumptions of normality. Meaningful statistical comparisons of the three species were not possible since all samples of the parental species, *L. tatarica* and *L. morrowii*, were obtained from a single individual of each species at the Arnold Arboretum.

CHAPTER FOUR

EVIDENCE FOR THE ADAPTIVE SIGNIFICANCE OF SECONDARY COMPOUNDS IN VERTEBRATE-DISPERSED FRUITS [3]

4.1 Abstract

Although the primary function of fleshy fruits is to attract seed dispersers, many ripe fruits contain toxic secondary compounds. A number of hypotheses have been proposed to explain this evolutionary paradox, most of which describe the potential adaptive role secondary compounds may play in seed dispersal. However, some authors have argued that fruit secondary compounds may be non-adaptive, and instead explain their occurrence as a pleiotropic consequence of selection for defense of leaves and other tissues. We address these alternative evolutionary hypotheses through a comparative examination of iridoid glycosides in leaves, unripe fruits, and ripe fruits of *Lonicera* x *bella* (Belle's bush honeysuckle), combined with an examination of fruit damage and removal in natural populations. We provide several lines of evidence that fruit secondary compounds are adaptive, including higher concentrations and more individual compounds in fruits compared to leaves and a negative relationship between iridoid glycoside concentration and fruit damage. However, we also show that the composition and concentrations of secondary compounds in leaves and fruits are not entirely independent, emphasizing that selection in different plant parts is intrinsically linked. We conclude that the adaptive significance of chemical traits is best considered in a whole-plant context that includes fruit-frugivore interactions.

[3] This chapter was a collaborative effort between S. R. Whitehead and M.D. Bowers and is currently in review with *American Naturalist* (submitted 10 December 2012)

4.2 Introduction

Because the primary function of fleshy fruits is to facilitate seed dispersal by mutualist animals, ripe fruits should theoretically be attractive and nutritious. However, in addition to nutritional rewards, fleshy fruits commonly contain secondary compounds that are distasteful or even highly toxic (Herrera 1982). For example, the ripe fruits of *Atropa belladonna* (Solanaceae) contain glycoalkaloids that are so toxic that the consumption of even a few berries can be fatal to humans (Caksen *et al.* 2003). Although various fruit-feeding animals may be able to tolerate or detoxify fruit secondary compounds (e.g. Struempf et al. 1999; Tewksbury and Nabhan 2001), the occurrence of toxic fruits represents an evolutionary paradox: why would a ripe fruit, which functions primarily to *attract* seed dispersers, contain toxic secondary compounds?

The evolutionary paradox of toxic fruit was noted in the scientific literature as early as the 1920s (Heim de Balsac 1928), yet there are still only a few systems in which the evolutionary ecology of fruit secondary compounds has been examined in an integrative manner and theoretical progress in the field remains relatively slow (reviewed in Levey et al. 2007). A number of adaptive hypotheses have been proposed to explain how fruit secondary compounds may enhance seed dispersal success through mechanisms such as: inhibiting seed germination in intact fruits, reducing the length of animal foraging bouts, regulating gut retention time of seeds, deterring less efficient seed dispersers, and defending fruits against seed predators and pathogens (Cipollini 2000; Cipollini and Levey 1997b; Herrera 1982). Most of these hypotheses have not been adequately tested, but a few have gained some empirical support. In particular, a number of studies have emphasized the role of secondary compounds in defense against fruit antagonists and/or supported the idea of trade-offs in fruit traits between defense and attraction of seed

dispersers (Cazetta et al. 2008; Cipollini and Levey 1997c; Cipollini and Stiles 1993; Haak et al. 2012; Schaefer et al. 2008; Schaefer et al. 2003; Tang et al. 2005; Tewksbury et al. 2008b; Tsahar et al. 2002).

Despite the evidence that fruit secondary compounds can, in some cases, confer an adaptive benefit, in other cases secondary compounds in fruits may be non-adaptive (i.e. provide no fitness benefits) in the context of fruit-frugivore interactions (Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998). Plants are under strong selection for the defense of leaves and other vegetative tissues (reviewed in Schoonhoven *et al.* 2005) and thus fruit secondary compounds may be best explained by physiological or pleiotropic constraints on the exclusion of these compounds from fruit tissue. Constraints on fruit chemistry are likely, since defensive traits in different plant parts can be linked through shared genetics, hormonal regulation, biosynthetic pathways, and metabolism (Adler et al. 2006; Herrera et al. 2002). For example, recent evidence suggests that induced plant responses to leaf herbivory can lead to corresponding changes in fruit chemistry and reduced fruit removal rates by birds (Whitehead and Poveda 2011). In such cases, fruit secondary compounds may best be explained as an "ecological cost of defense" of vegetative tissues (Cipollini et al. 2003; Heil 2002; Strauss et al. 2002). A similar explanation has been invoked for the occurrence of secondary compounds in nectar (reviewed in Adler 2000), and a number of studies have supported this "non-adaptive" hypothesis with evidence that floral secondary compounds can be costly in terms of reduced pollination success and are often correlated with the compounds found in leaves (Adler et al. 2006; Gegear et al. 2007; Kessler and Halitschke 2009; Manson et al. 2012; Strauss et al. 1999). However, despite the importance of trait linkages among different plant parts in the ecology and evolution of plant-animal interactions, we know of only one study that has explored co-variation of leaf and fruit chemical

traits in a fleshy-fruited species (Cipollini et al. 2004).

Regardless of their evolutionary *raison d'etre*, fruit secondary compounds are likely to play a key role in structuring interactions between plants and multiple classes of dispersing and non-dispersing frugivores. Thus, it is surprising that there is so little information on the qualitative and quantitative variation in fruit chemical traits in natural populations. Rigorous examination of plant secondary chemistry has played a central role in developing theories of plant-herbivore interactions (reviewed in Schoonhoven *et al.* 2005), and more recently in studies of plant-pollinator interactions (Adler 2000; Irwin and Adler 2008; Strauss et al. 1999). An increased emphasis on the role of secondary chemistry in fruit-frugivore interactions could contribute greatly to our understanding of seed dispersal mutualisms, evolutionary trade-offs, and plant defense theory. In particular, in order to address the importance of fruit chemical traits in plant/animal interactions, we need more information on the chemical variation in natural populations at the intraspecific scale, since it is this variation that provides the basis for natural selection (Izhaki et al. 2002; Tewksbury 2002).

In this study, we examined patterns of individual-level variation in one group of plant secondary compounds, the iridoid glycosides (IGs), in the leaves, unripe fruits, and ripe fruits of a hybrid bush honeysuckle (*Lonicera* x *bella* Zabel, Caprifoliaceae). In addition, we monitored fruit damage by insects and microbes and fruit disappearance (presumably due to birds and other potential dispersers) in natural populations over two growing seasons to determine whether IG concentrations can influence fruit/frugivore interactions. These data were used to address two alternative hypotheses: 1) fruit secondary compounds provide an adaptive benefit in the context of fruit-frugivore interactions, or 2) fruit secondary compounds are non-adaptive and their presence is best explained as a consequence of foliar defense. Specifically, we considered the

evidence for the following patterns, which, if found, would provide support for an adaptive role of fruit secondary compounds: 1a) certain compounds are unique to fruits, 1b) quantities of secondary compounds in fruits are similar to or higher than those in leaves, 1c) quantities of secondary compounds in fruits are independent of those in leaves of the same plant, and 1d) fruit secondary compounds are associated with decreased levels of damage or increased levels of fruit removal by dispersers in natural populations. Alternatively, if fruit secondary compounds are a consequence of foliar defense, then the following patterns might be observed: 2a) fruit secondary compounds represent a subset of those found in leaves, 2b) quantities of secondary compounds in leaves are higher than those in fruits, 2c) quantities of secondary compounds in fruits are correlated with those in leaves of the same plant, 2d) fruit secondary compounds have no effect on fruit/frugivore interactions or are associated with reduced fruit removal rates by dispersers.

4.3 Methods

Study system and site

Lonicera x *bella* is a hybrid bush honeysuckle that can be an aggressive invader in much of the United States (USDA-PLANTS 2011). Its parental species were both introduced to the US as ornamentals—*L. tatarica* from Russia/central Asia in 1752 and *L. morrowii* from Japan in 1854 (Barnes and Cottam 1974). The hybrid species was first described in 1889, and is thought to have arisen repeatedly in cultivation and in naturalized populations wherever the two parental species co-occur (Barnes and Cottam 1974; Hauser 1966; Rehder 1903). Although all three species have long been problematic invaders in the eastern and central US (Ingold and Craycraft 1983; Woods 1993), their establishment in western states (including Wyoming, Colorado, and New Mexico) has been relatively recent (Sperger 2003; USDA-PLANTS 2011). In our study

area of Colorado, herbarium records indicate *L. morrowii* was well-established in 1973 (COLO 2011); however, based on our own comparative examination of local specimens and specimens of the parental species collected from their native ranges (obtained from the Arnold Arboretum of Harvard University; BG-BASE 2011), we believe that most local specimens likely have some degree of hybrid origin. For the purposes of this study, we considered any individual with pink flowers and sparsely pubescent leaves and peduncles to be of hybrid origin (Hauser 1966), and our identifications were confirmed by two local authorities (T. Hogan and D. Clark, Herbarium COLO, University of Colorado Museum of Natural History). Voucher collections from each study population are available at the University of Colorado Natural History Museum (Herbarium COLO; Acc. #'s 543191, 543192, 543193).

Our study populations of *L.* x *bella* were located at three sites in Boulder County, Colorado: Bluebell Canyon (39.99135 N, -105.28568 W), Gregory Canyon (39.99727 N, -105.2940 W), and Skunk Canyon (39.98611 N, -105.27660 W). All three sites can be described as Foothills riparian, with a mosaic of *Pinus ponderosa* woodland and open areas dominated by shrubs, grasses, and forbs. *Lonicera* x *bella* is well-established in these areas, particularly along streams. It fruits in abundance in late summer, and fruits are often persistent on the plant into the fall and winter. The seeds are thought to be dispersed primarily by birds, but are also consumed by mammals such as white-tailed deer (Bartuszevige and Gorchov 2006; Drummond 2005; McCay et al. 2009; SRW, personal observation; Vellend 2002).

Leaves and fruits of *Lonicera* contain two important classes of secondary compounds, phenolics and iridoid glycosides (IGs) (Ikeshiro et al. 1992; Zadernowski et al. 2005). Here we focus on IGs, which have well-documented anti-feedant activity toward insect herbivores, as well as anti-microbial effects (Bowers 1991; Marak et al. 2002a; Marak et al. 2002b). Our

previous research has shown that *L.* x *bella* inherited a diversity of IGs from both parental species, and that these compounds occur in both leaves and fruits (Whitehead and Bowers 2013). However, past work on the ecological role of IGs has focused primarily on leaves (e.g., Jamieson and Bowers 2010; Peñuelas et al. 2006; Quintero and Bowers 2011b) and, to our knowledge, their importance in mediating seed dispersal and fruit defense is unexplored.

Field observations

Ten individuals of *L.* x *bella* from each of the three study populations described above (N=30 plants) were monitored throughout the growing seasons in 2007 and 2008. Twenty-four individuals were monitored throughout both seasons and six individuals were replaced in 2008 due to poor re-growth or damage by trail maintenance activities. On each shrub, we marked two branches at the start of the growing season that were approximately 25 cm in length and contained between 20 and 150 fruits. We visited plants every 1-3 weeks from early fruit development in mid-June until most fruits had either disappeared or rotted in late September. Each plant in the study population was visited on six occasions during 2007 and 13 occasions during 2008. At each visit, we recorded the number of healthy unripe and ripe fruits remaining on marked branches, the number that were aborted, and the number of healthy fruits that were visibly affected by two categories of fruit pests: insects (primarily piercing/sucking hemipterans that left visible feeding scars on the fruit surface) and microbes (causing surface discoloration or fruit rot). For the purposes of our study, we assumed that most ripe fruits that disappeared from the branch were removed by potential seed dispersers. Although a small proportion of the fruits may have fallen off the shrub, we believe that fruit senescence during the monitoring period was rare for three reasons: 1) we regularly searched the ground below plants and found no fallen

fruits, 2) we never observed fruits falling off the branch while we were handling the shrub to count fruits, and 3) fruits that were aborted by the plant remained attached to the branch for extended periods (sometimes months) and were not included in our counts of fruit disappearance.

Collection of plant material for chemical analysis

We collected samples for chemical analysis from each plant in our study population (N=30) once in 2007 and once in 2008. We harvested two branches from each shrub, and each branch was separated into leaves, unripe, and ripe fruits (for a total of 360 samples). Because our main objective was to examine linkages among the chemical traits of different plant parts, we collected branches only in the short time frame during which fruits were at mid-ripening, and leaves, unripe fruits, and ripe fruits could be obtained from the same branch at the same time. Ripening times were variable among shrubs, thus we collected branches between mid-July and late August depending on the individual. Samples were stored in a cooler until we returned to the laboratory (always within 3 hours of collection), where we separated each branch into leaves, unripe fruits, and ripe fruits. All plant material was then weighed and oven-dried at 50^{o}C to constant mass (IGs are thermally stable at this temperature; see Gardner and Stermitz 1988; Lampert and Bowers 2011; Mraja et al. 2011). Dry fruits were first ground through a fine mesh screen that allowed us to remove all seeds from the sample, leaving only pulp and skin, followed by further grinding to a fine powder in a mortar and pestle. Leaves were ground to a fine powder using a mortar and pestle only.

Quantification of iridoid glycosides

Methods for quantification of IGs were modified from previously described methods (Bowers and Stamp 1993; Gardner and Stermitz 1988) and are described in detail in Whitehead and Bowers (in press). Briefly, 25-50 mg aliquots of plant material were extracted in methanol for 24 hours, filtered, and partitioned between water and ether to remove hydrophobic compounds. Phenyl-β-D-gluco-pyranoside (PBG) was added as an internal standard and IGs were derivatized to their trimethylsilyl analogues using Tri-sil-Z^{TM} (Pierce Chemical Company). Quantities were determined based on total ion current using a HP Agilent 6890N GC coupled with an Agilent 5975C mass spectrometer (GC-MS). We quantified six IGs by comparison with authentic reference standards (loganin, secologanin, loganic acid, sweroside, secoxyloganin, and morroniside), which together represented ~88% of the total IG content. One other major component (kingiside) and six minor compounds were also identified as IGs based on MS profiles (Inouye et al. 1976; Popov and Handjieva 1983) and their quantities estimated by assuming a response factor equivalent to our internal standard.

Statistical Analysis

First, to examine whether secondary compounds in fruits can be unique (Prediction 1a) or represent a subset of those found in leaves (Prediction 2a), we used qualitative (presence or absence) comparisons of IG occurrence, combined with an examination of multivariate chemical similarity among plant parts using non-metric multidimensional scaling (NMDS). For the purpose of the NMDS, we included only the mean concentration of each individual IG for each plant (averaging between branches and between years). The 3-D NMDS ordination was based on the Bray-Curtis dissimilarity index and used 200 replicates with random starting coordinates

(Minchin 1987). This was conducted using the 'vegan' package in R (Oksanen et al. 2010; R Development Core Team 2012).

Next, to determine whether IG quantities were higher in fruits (Prediction 1b) or in leaves (Prediction 2b), we compared total IG quantities among plant parts using a linear mixed model with a normal error distribution, fit by maximum likelihood (Crawley 2007). IG quantities (as a proportion of dry weight) were first logit transformed to meet linear modeling assumptions (Warton and Hui 2011). Plant part was specified as a fixed effect and random effects accounted for both the nested effects of plant parts within individuals within populations and the effect of sampling year (2007 or 2008). To test for overall differences in IG concentrations among plant parts, we used likelihood ratio tests to compare the deviance of a model including plant part as a fixed effect to a null model that included random effects only (Crawley 2007). We also used this analysis to examine the variance structure of our chemical data, i.e. the proportion of total IG variation that can be attributed to each random effect. To examine pairwise differences among plant parts, we followed this analysis with a Tukey HSD post-hoc test. These analyses were conducted using the 'lme4' and 'multcomp' packages in R (Bates and Maechler 2010; Hothorn et al. 2011; R Development Core Team 2012).

Third, to determine whether IG concentrations in leaves and fruits were independent (Prediction 1c) or correlated (Prediction 2c), we tested for correlations in IG concentrations between leaves and unripe fruits, leaves and ripe fruits, and unripe and ripe fruits using non-parametric Kendall's rank correlations (individual IG concentration data were not transformable to fit linear modeling assumptions). These were conducted separately for the total IG concentration as well as all individual IG components that occurred in both leaves and fruits (for a total of 33 separate correlation analyses). To account for multiple inferences in this family of

tests, we controlled for false discovery rate at $\alpha=0.05$ using methods described in Benjamini and Hochberg (1995). These analyses were conducted using JMP v. 9.0.2 (2010).

To determine whether fruit IG concentrations influenced patterns of fruit damage and disappearance in natural populations (Predictions 1d and 2d), we used a two-step analysis approach. Because our field observations involved repeated measures during each of two fruiting seasons, but our IG data only involved a single measurement per year at mid-ripening, we first created composite damage and disappearance scores for each individual plant in each year. To do this, we compared the relative levels of fruit damage and disappearance on each individual plant to all other plants measured in that year using generalized linear mixed models (GLMMs). These analyses were conducted separately for insect damage, microbe damage, and fruit disappearance, and for 2007 and 2008. Our response variables were binomial counts of the number of damaged or removed fruits on a branch and the number not damaged or removed, which we modeled using the binomial distribution with the logit link function. In all models, individual was specified as a fixed effect and random effects included the continuous effect of sampling date (repeated measures on each branch) and the spatially nested block effects of branches within individuals within populations. We specified *a priori* contrast coefficients to compare each individual to the overall mean for all other individuals in that year. Thus, the output of the GLMMs gave us effect size estimates for each individual plant that can be interpreted as relative fruit damage or disappearance scores—negative effect sizes indicated plants with lower than average rates of fruit damage or disappearance, while positive effect sizes indicated plants with higher than average rates of fruit damage or disappearance. These analyses were conducted using the 'lmer' package in R (Bates and Maechler 2010; R Development Core Team 2012).

In part two of the analysis, we examined the effects of IGs on the relative damage and disappearance scores for each individual plant using mixed regression models. We considered the effects of unripe fruit IGs on both insect and microbe damage, and the effects of ripe fruit IGs on microbe damage and fruit disappearance. We did not consider the effects of ripe fruit IGs on insect damage because almost all insect damage occurred in unripe fruits. To obtain a representative measure of IG concentration for each plant, we first averaged our IG data from the two replicate branches collected from each individual. We then examined the effects of total IGs on the damage/disappearance scores using linear mixed models that included IG concentration as a fixed effect and individual plant as a random effect. We also examined the multivariate effects of the 12 individual IGs on fruit damage/disappearance scores using principal components (PC) regressions, which can provide a more robust alternative to multiple regressions or separate individual regressions when predictor variables are highly correlated (Graham 2003), as was the case with individual IGs (correlations among compounds are reported in Appendix A). In PC regressions, orthogonal PC axes are calculated for the predictor variables (i.e., the 12 quantified IGs) independently of the response variable (i.e., fruit damage or disappearance scores) and the PCs are then used as the predictor variables in multiple regressions. The PC analyses provided ten orthogonal axes of variation for unripe fruits (explaining 98.74% of the IG variation) and nine orthogonal axes for ripe fruits (explaining 96.6% of the IG variation) (eigenvalues, percent of variance explained by each PC, and axis loadings are shown in Appendix B).

We used an information theoretic approach to identify which PCs were important predictors of fruit damage or removal scores (Burnham and Anderson 2002; Grueber et al. 2011; Hegyi and Garamszegi 2011). We defined a global model that included all PCs as fixed predictor variables and then ranked all possible subsets of the model using corrected Akaike's information

criterion (AICc) based on maximum likelihood. Individual was included as a random effect in all models to control for the non-independence of multiple measures from each plant (one from 2007 and one from 2008). From all possible submodels, we retained a candidate set that included all models with a $\Delta AICc < 2$. We then used model averaging based on Akaike weights for all candidate models to estimate coefficients and confidence intervals for all retained predictor variables. The significance of the predictor variables was determined using Z-statistics. These analyses were conducted using the MuMIn package in R (Bartoń 2011; R Development Core Team 2012).

4.4 Results

Certain compounds are unique to fruits

Our GC/MS analyses revealed a total of 12 IGs in the fruits and leaves of *Lonicera* x *bella* (Fig. 4.1). Of these compounds, nine occurred in both fruits and leaves and three were unique to fruits (morroniside, kingiside, and unknown F). We did not detect any IGs that were unique to leaves. The NMDS analysis examining overall chemical similarity among plant parts revealed that the chemical profile of leaves is distinct from that of fruits, but there is substantial overlap in the chemical profiles of unripe and ripe fruits (Fig. 4.2).

IG concentrations are higher in fruits than in leaves

Plant part was a significant predictor of total IG concentration (χ^2=95.57, df=2, $p<0.0001$), and post-hoc comparisons among leaves, unripe and ripe fruits showed all three plant parts were significantly different from each other, with the highest concentrations in unripe fruits, lower concentrations in ripe fruits, and lowest concentrations in leaves (Fig. 4.1A). The

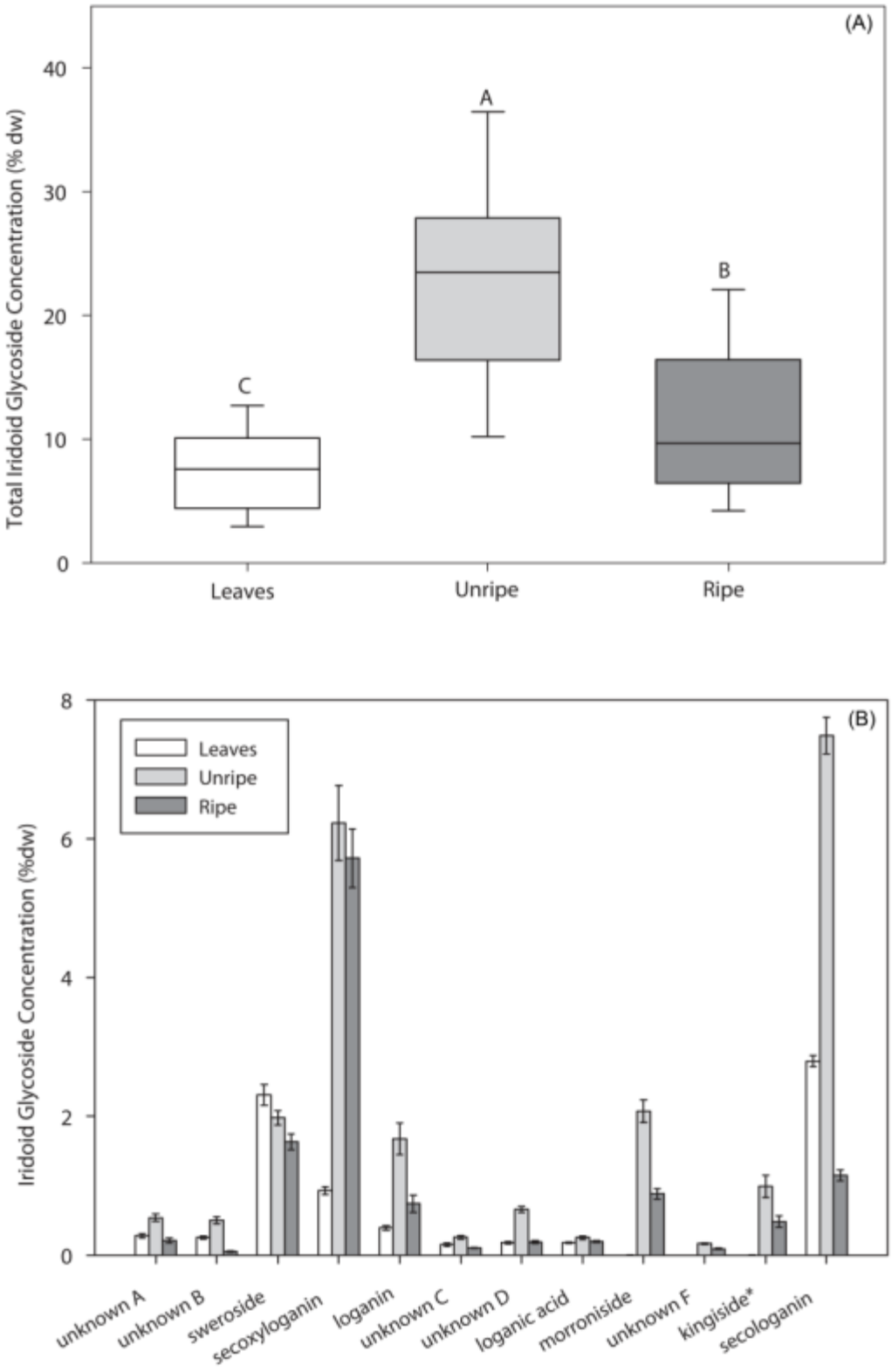


Figure 4.1: Average IG concentrations in leaves, unripe, and ripe fruits of *L.* x *bella.* In (A) box and whisker plots show the median, 25th and 75th percentile, and range of total IG concentrations from N=30 plants. Letters represent significant differences from Tukey HSD post-hoc comparisons among plant parts. In (B), the mean (± SE) concentration is shown for all detected IGs (*=tentative identification).

random effects in our mixed model showed that 2.43% of the variation in total IG content was explained by population, 22.22% was explained by individual, 22.52% was explained by plant part, 7.64% was explained by year, and 45.19% was residual variation.

Certain IGs are correlated between fruits and leaves and others are independent

Total IG concentrations were correlated between leaves and unripe fruits, not correlated between leaves and ripe fruits, and strongly correlated between unripe and ripe fruits (Table 4.1). These patterns were variable for the individual compounds examined; 6 of 12 IGs were correlated between leaves and unripe fruits, 3 of 12 IGs were correlated between leaves and ripe fruits, and 12 of 12 IGs are correlated between unripe and ripe fruits (Table 4.1).

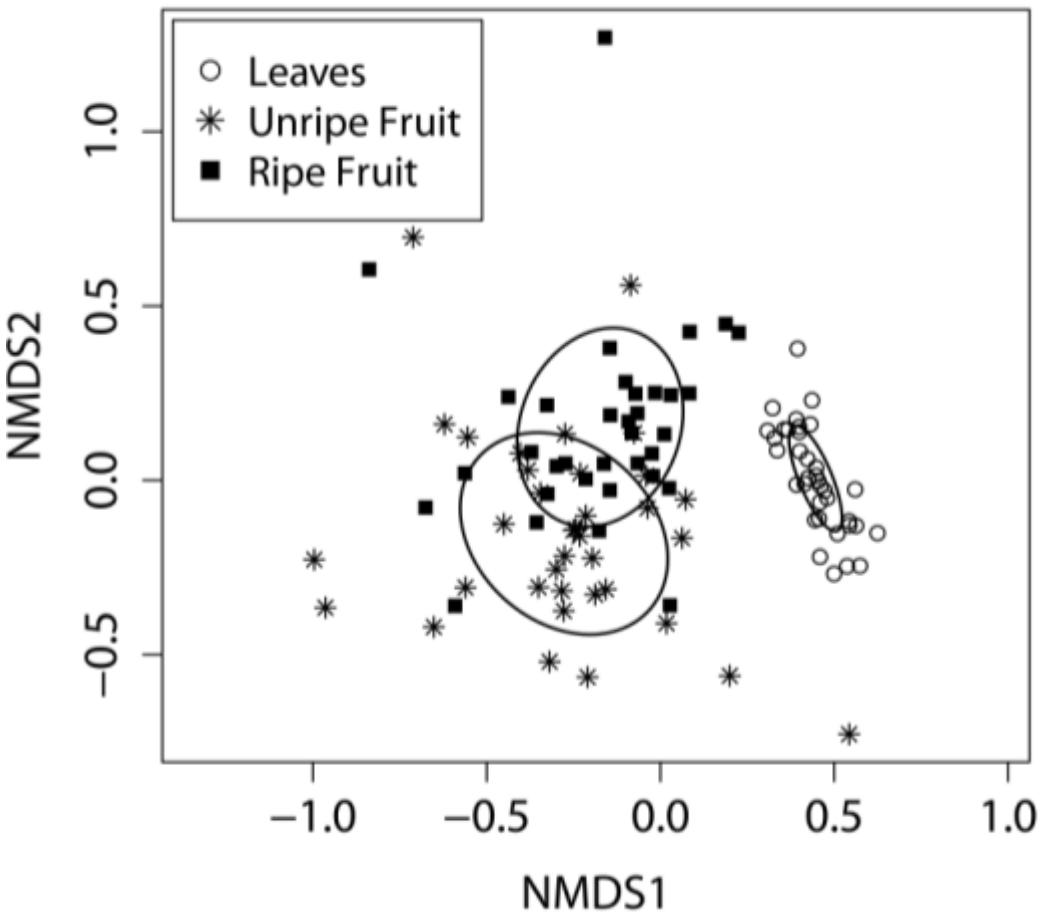


Figure 4.2: Non-metric multidimensional scaling ordination plot of overall chemical similarity among plant parts (3-D Final Stress=12.67, R^2=0.59). Ellipses represent 95% confidence intervals for group centroids (±1SD).

Fruit IGs reduce fruit damage and have mixed effects on fruit disappearance

The relative insect damage score tended to decrease with increasing total IG concentration, but the effect was not significant ($F_{1,40}$=2.85, p=0.0996; Fig. 4.3A). In our examination of the effects of unripe fruit individual IGs (combined as PCs) on fruit damage from insects, model selection using AICc resulted in a candidate set of eight models with ΔAICc < 2 (Table 4.2). The top model had an Akaike weight of ω_i=0.20 and included PC1 (explaining 32.24% of IG variation), PC2 (explaining 16.84% of IG variation), and PC4 (explaining 10.24% of IG variation) as predictor variables; however, model averaging among the entire candidate set indicated that only PC1(z=2.692, p=0.0071) and PC2 (z=3.072, p=0.0021) were significant predictor variables, both of which had a negative effect on insect damage score (Table 4.2, Fig. 4.3B-C).

Table 4.1: Non-parametric Kendall's rank correlation coefficients and p-values examining relationships among IGs in different plant parts ($^+$ = tentative identification). Significance stars are based on a Holm's sequential Bonferroni correction for multiple inferences; **= highly significant (p≤0.003), *= significant (p≤0.0316).

	Leaves & Unripe			Leaves & Ripe			Unripe & Ripe		
Compound ID	**τ**	**Prob > |τ|**		**τ**	**Prob > |τ|**		**τ**	**Prob > |τ|**	
Unknown A	0.2347	0.0011	**	0.22	0.0023	**	0.4068	<0.0001	**
Unknown B	0.1559	0.0316	*	-0.0314	0.6594		0.3869	<0.0001	**
Sweroside	0.2278	0.0016	**	0.1813	0.0061	*	0.5325	<0.0001	**
Secoxyloganin	0.0655	0.3658		0.0951	0.1534		0.5214	<0.0001	**
Loganin	0.1796	0.0127	*	0.0772	0.2437		0.4008	<0.0001	**
Unknown C	0.0329	0.6499		-0.0067	0.9203		0.4534	<0.0001	**
Unknown D	0.1891	0.0087	*	0.2267	0.0007	**	0.3498	<0.0001	**
Loganic acid	-0.0596	0.4107		0.0837	0.2075		0.2037	0.0035	*
Morroniside		--			--		0.5789	<0.0001	**
Unknown F		--			--		0.3902	<0.0001	**
Kingiside$^+$		--			--		0.642	<0.0001	**
Secologanin	0.2405	0.0009	**	0.0454	0.5015		0.3619	<0.0001	**
Total IGs	**0.1898**	**0.0085**	*	**0.1278**	**0.0533**		**0.2831**	**<0.0001**	**

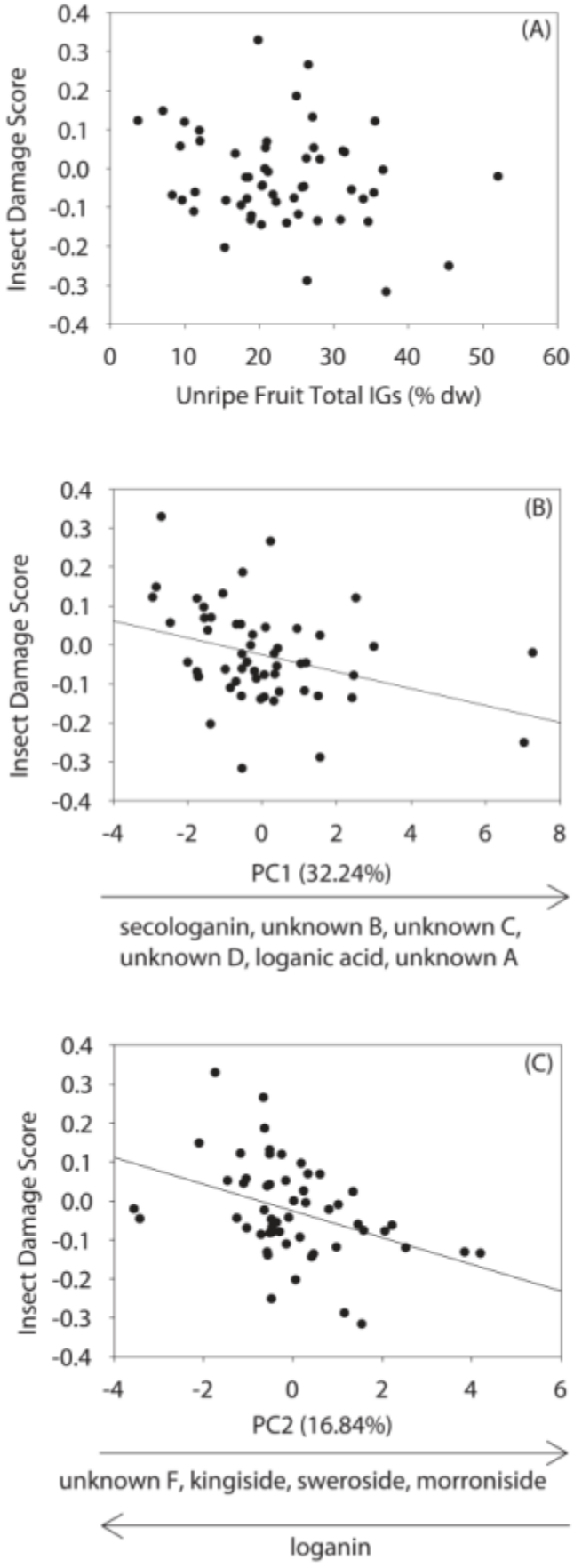


Figure 4.3: Effects of unripe fruit total IGs (A) and significant predictor variables from a principal components regression of individual IGs (B-C) on the relative level of fruit insect damage. Insect damage scores represent a comparison of each individual plant to the average damage for all other plants monitored in that year, with negative scores representing lower than average damage and positive scores representing higher than average damage. In B-C, the independent variables are PC axes based on 12 individual IGs. The percent variation in total IGs explained by each PC axis is indicated in parentheses and the primary compounds loading on each axis are indicated by arrows below the x-axis label. Detailed PCA results are provided in Appendix C.

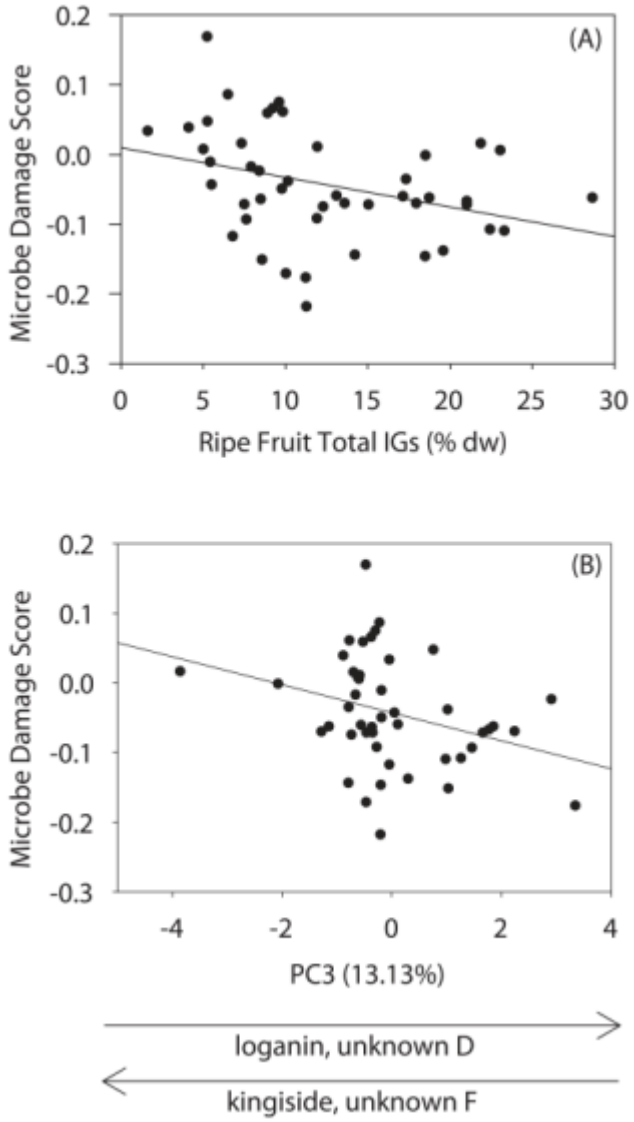


Figure 4.4: Effects of ripe fruit total IGs (A), and significant predictor variables from a principal components regression of individual IGs (B) on the relative level of fruit microbe damage. Microbe damage scores represent a comparison of each individual plant to the average damage on all other plants monitored in that year, with negative scores representing lower than average damage and positive scores representing higher than average damage. In (B), the independent variable is a PC axis based on 12 individual IGs. The percent variation in total IGs explained by the axis is indicated in parentheses and the primary compounds loading on the axis are indicated by arrows below the x-axis label. Detailed PCA results are provided in Appendix C.

Total IGs in ripe fruits had a significant negative effect on the relative microbe damage score for each plant ($F_{1,40}$=5.37, p=0.0256; Fig. 4.4A). In our examination of the effects of ripe fruit individual IGs (combined as PCs) on fruit damage from microbes, model selection using AICc resulted in a candidate set of eight models with ΔAICc < 2 (Table 4.2). The top model had an Akaike weight of ω_i=0.23 and included PC3 (explaining 13.13% of IG variation), PC6 (explaining 6.68% of IG variation), and PC7 (explaining 5.59% of IG variation) as predictor variables; however, model averaging among the entire candidate set indicated that only PC3

Table 4.2: Averaged models estimating the effects of individual IGs (combined as PCs) on fruit damage and disappearance scores.

	Model-averaged coefficients	SE	z value	Pr(>|z|)	Relative Importance
Response: Insect Damage					
Intercept	-0.025731	0.014482	1.715	0.08628	
PC1	-0.021779	0.007432	2.692	0.0071	1
PC2	-0.034383	0.010281	3.072	0.00212	1
PC4	0.020945	0.013089	1.466	0.1427	0.52
PC3	-0.016337	0.012672	1.182	0.23729	0.25
PC5	0.014717	0.013442	1.004	0.31557	0.2
PC7	0.019306	0.021406	0.827	0.40838	0.16
Response: Microbe Damage					
Intercept	-0.043331	0.010556	3.959	<0.0001	
PC3	-0.020087	0.008504	1.989	0.0467	1
PC6	0.023934	0.011884	1.69	0.091	0.9
PC7	-0.025266	0.012984	1.632	0.1026	0.88
PC4	0.011699	0.0093	1.043	0.2971	0.14
PC8	-0.016217	0.014669	0.916	0.3595	0.11
PC5	-0.011258	0.010891	0.857	0.3915	0.1
PC1	-0.005635	0.005608	0.833	0.4049	0.1
PC2	-0.007082	0.00775	0.757	0.4488	0.09
Response: Fruit Disappearance					
Intercept	-0.06012	0.02887	2.01	0.0444	
PC4	0.06048	0.02551	2.2	0.0278	1
PC7	0.05987	0.03479	1.594	0.111	0.64
PC6	0.03413	0.03099	1.02	0.3076	0.22
PC3	-0.02651	0.02313	1.062	0.2883	0.21
PC2	0.0209	0.02121	0.911	0.3622	0.1
PC1	-0.01163	0.01523	0.706	0.4802	0.08

(z=1.989, p=0.0467) had a significant negative effect on microbe damage score (Table 4.2, Fig. 4.4B). Total IGs in unripe fruit had no effect on microbe damage score ($F_{1,40}$=0.13, p=0.72), and model selection in the principal components regression resulted in a candidate set of 11 models with ΔAICc < 2. The top model had an Akaike weight of ω_i=0.16 and included PC4 (explaining 10.69% of IG variation) and PC6 (explaining 6.68% of IG variation). However, model averaging among the entire candidate set indicated that neither of these variables had a significant effect on microbe damage score (data not shown).

Total IGs in ripe fruits had no effect on relative fruit disappearance scores ($F_{1,51}$=0.108, p=0.743). In our examination of the effects of ripe fruit individual IGs (combined as PCs) on fruit disappearance, model selection using AICc resulted in a candidate set of eight models with Δ AICc < 2 (Table 4.2). The top model had an Akaike weight of ω_i=0.21 and included PC4 and PC6 as predictor variables; however, model averaging among the entire candidate set indicated that only PC4 (z=2.200, p=0.0278) had a significant negative effect on fruit disappearance (Table 4.2, Fig. 4.5).

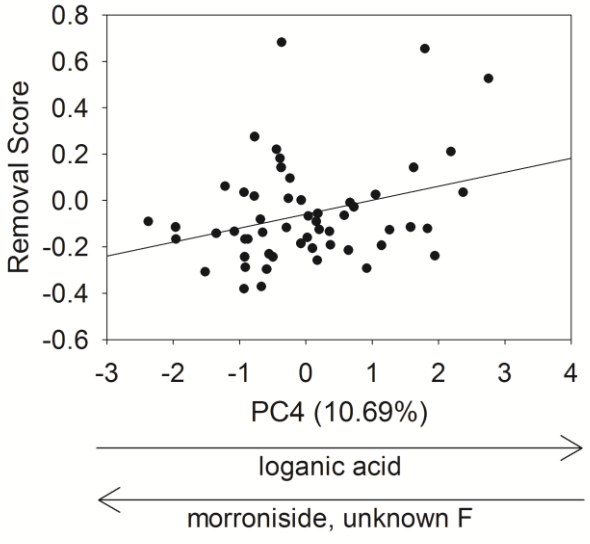


Figure 4.5: Effects of ripe fruit IGs on the relative level of fruit disappearance. Disappearance scores represent a comparison of fruit disappearance on each individual plant to the average disappearance on all other plants monitored in that year, with negative scores representing lower than average disappearance and positive scores representing higher than average disappearance. The x-axis shows PC4, which was the only significant predictor variable in a principal components regression based on 12 individual IGs (see main text). The percent variation in total IGs explained by the axis is indicated in parentheses and the primary IGs loading on this axis are indicated by arrows below the x-axis label. Detailed PCA results are provided in Appendix C.

4.5 Discussion

Many vertebrate-dispersed fruits contain high levels of secondary compounds (Herrera 1982) and increasing evidence has shown that these compounds can have important effects on both seed dispersers and fruit predators/pathogens (reviewed in Levey et al. 2007). However, there is little evidence available to address the fundamental question of whether the occurrence of these compounds is the result of selective pressures in fruits or primarily a pleiotropic consequence of defense of other plant tissues (Cipollini and Levey 1997b; Eriksson and Ehrlen 1998). Our study represents the first detailed examination of quantitative chemical variation and co-variation among leaves, unripe, and ripe fruits in natural populations of a vertebrate-dispersed species, allowing us to explicitly test these alternative hypotheses. We provide multiple lines of evidence that fruit secondary compounds cannot be explained solely as a consequence of foliar defense: 1) Of 12 individual IGs occurring in *L.* x *bella*, three are unique to fruits and none are unique to leaves; 2) Most IGs are present in higher concentrations in fruits than in leaves; 3) Concentrations of most IGs in ripe fruits are independent of concentrations in leaves; and 4) Total IG concentration and/or individual IG concentrations are negatively correlated with fruit damage from insects and microbes in natural plant populations. Together, these results strongly suggest that there has been selection for secondary compounds in fleshy fruits independent of selection in leaves and point to an adaptive role in fruit/frugivore interactions or seed dispersal. However, we also show that many compounds are shared between fruits and leaves, and that for a few compounds the concentrations found in fruits and leaves are strongly correlated, emphasizing the existence of physiological linkages among different plant parts and the importance of considering the whole-plant context in chemical trait evolution.

Our results showing higher IG concentrations in fruits compared to leaves support optimal defense theory (ODT), which predicts that plant parts with the highest fitness value, such as flowers and developing fruits, should be the most protected against herbivore attack (McKey 1974; Rhoades and Cates 1976). The average total IG concentration in unripe fruits was extremely high (22.9% dry weight), approximately double the average concentration in ripe fruits (11.4% dry weight), and three-fold higher than the average concentration in leaves (7.5% dry weight). In vertebrate-dispersed species, ODT also predicts that secondary compounds should disappear or diminish with ripening to allow for consumption by mutualistic animals, and this is commonly observed with fruit ripening in cultivated fruits (e.g. Friedman 2002). In accordance with this, our data show a marked reduction in total IG concentration with ripening; however, even in ripe fruits, IGs were present in higher concentrations than in leaves. It is also notable that the relative changes in IG concentration between unripe and ripe fruits were variable among compounds (Fig. 4.1B). Some major IG components (e.g. secoxyloganin) remained high even in ripe fruits; in fact, for roughly half of our samples, secoxyloganin concentration was actually higher in ripe fruits than in unripe fruits collected from the same branch at the same time. Thus, ripe fruit chemistry does not appear to be solely a consequence of leaf or unripe fruit chemistry, but instead may be fine-tuned by selection for the quantities and ratios of compounds that maximize seed dispersal success while minimizing costs.

We show that the concentrations of most individual IGs in ripe fruits are independent of the concentrations in leaves. However, there were strong correlations for a few compounds, and many more were correlated between unripe fruits and leaves (Table 4.1). IGs can be phloem-mobile (Gowan et al. 1995), thus the correlations among plant parts may be due to physiological linkages (i.e. the transport of compounds among plant parts), or to genetic linkages (i.e.

correlations in the expression of chemical traits in different plant parts). These linkages do not preclude an adaptive role for secondary compounds in both leaves and fruits—because plants are under simultaneous selection from herbivores, pollinators, seed dispersers, competitors, and abiotic factors, an efficient strategy may be to produce compounds with multiple ecological functions that can be expressed throughout the plant (Izhaki 2002). However, our results showing the presence of compounds unique to fruits and the lack of correlation between leaves and fruits for certain compounds suggest that plants can control chemical trait expression in leaves and fruits independently in some cases.

One important factor that may influence both the qualitative and quantitative expression of IGs in different plant parts of *L.* x *bella* is its evolutionary history as a hybrid species (Cheng et al. 2011; Orians 2000). Because *L.* x *bella* can readily backcross with parental species (Barnes and Cottam 1974; Hauser 1966), and various escaped cultivars may have contributed to our study population, the genetic background of our plants may be quite diverse. Our data do show high variation in IGs, particularly for unripe fruits (Fig. 4.1A), and it is unclear to what extent this variation may be due to hybridization. However, the general patterns of IG occurrence, with certain compounds unique to fruits and higher overall concentrations in fruits, holds for *L.* x *bella* and both its parental species (Whitehead and Bowers 2013), suggesting that these patterns are shaped by past selection pressures in the parental species rather than being an artifact of genetic recombination. Future research examining how fruit chemical traits correlate with patterns of genetic introgression and reproductive success in hybrids may provide important insights into the current selective pressures being exerted on *L.* x *bella* and the evolution of invasiveness in this genus.

With regard to the potential ecological role of secondary compounds in fruit defense, our data illustrated that higher levels of total IGs and/or particular combinations of individual IGs in unripe and ripe fruits were associated with lower levels of damage by insects and microbes (Figs. 4.3-4.4), supporting other studies that suggest an important defensive role of secondary compounds in fruits (e.g. Cipollini and Levey 1997c; Schaefer et al. 2008; Tewksbury et al. 2008b). Because *L.* x *bella* is a non-native plant that arrived relatively recently to our study area, most fruit damage likely comes from generalist fruit-feeding insects and pathogens that have a limited ability to tolerate or detoxify the high levels and diverse mixture of IGs we detected in fruits. The selective pressures exerted by these organisms may be very different from those in the native ranges of the parental species, where there is a higher potential for co-evolved specialists that may use IGs as feeding cues or even sequester IGs to provide protection against their own natural enemies (Bowers 1991). Thus, it is not clear from our results what the specific selective forces may have been that shaped fruit chemical traits in this species, only that IGs can influence interactions with the broad classes of generalist insects and pathogens that attack fruits. Understanding the specific effects IGs have on individual organisms and how this may relate to the evolutionary success of *L.* x *bella* will require further research, such as experimental bioassays that manipulate the concentrations of IGs in the diets of consumers. Our data suggest that the effects of individual compounds are complex and not necessarily limited to the most abundant compounds present (Fig. 4.3-4.4), thus studies of this type should consider the combined effects of the suites of defensive compounds found in plants.

Our analyses of the effects of IGs on fruit disappearance revealed no clear trends. There was no relationship between total IGs and disappearance and the few individual compounds (those loading on PC4) that appear to correlate with disappearance rates had mixed effects (Fig.

4.5). It is possible that the IGs in ripe fruits may be able to defend fruits against antagonists with minimal negative effects on mutualist seed dispersers (the microbe-pest specificity model, sensu Cipollini and Levey 1997b). Alternatively, we may not have detected strong negative effects of IGs on fruit removal due to confounding variables, in particular the difficulty in relating fruit disappearance to removal by seed dispersers. Although we know of no studies that have examined the effects of IGs on frugivorous birds, there is evidence that predatory birds are deterred by insects that sequester IGs from their host plants (Bowers 1980), therefore it is likely that at least some generalist frugivores are deterred by high quantities of IGs. It is important to note that the overall fruit disappearance rates were fairly low (averaging only 40% of the total fruit crop that developed to maturity) and the majority disappeared late in the season. Past work on bird dispersal of *Lonicera* in the eastern US has also shown that most fruits are removed late in the season once higher quality native fruits have disappeared (Drummond 2005; White and Stiles 1992). Thus, regardless of frugivore preferences, one successful dispersal strategy for a non-native shrub with uncertain dispersal opportunities may be to have more chemically-defended fruits that remain available and relatively undamaged later in the season. Further elucidation of the specific role of IGs in the multi-faceted aspects of seed dispersal will require an integrative approach to understanding frugivory that incorporates fruit availability, feeding preferences, and community context.

An important consideration in the interpretation of the effects of individual IGs on fruit damage and disappearance in our study (Figs. 4.3-4.5) is the shared biosynthetic pathways among different compounds. Past research on IGs in *Lonicera* has suggested a biosynthetic route from loganin and/or loganic acid to secologanin to other secoiridoids such as morroniside, sweroside, secoxyloganin, and kingiside (Takeda and Inouye 1976; Uesato et al. 1984). The fact

that some of the quantified IGs in our study are the precursors to other more complex IGs could explain why some compounds are negatively correlated with each other (Appendix A) and thus may appear to have opposite effects on fruit damage/disappearance in PC regressions (Figs. 4.3-4.5, Appendix 4B). Because the bioactivity and relative toxicity of different IGs along this biosynthetic route may vary considerably, it is possible that plants may convert existing IGs to modified forms as fruits develop in order to optimize seed dispersal success.

Finally, one other important factor that may increase the complexity of relationships among IGs and how they relate to patterns of damage/disappearance is that IGs are important as both constitutive and induced plant defenses and can vary in response to herbivory (Darrow and Bowers 1999; Fuchs and Bowers 2004; Peñuelas et al. 2006; Quintero and Bowers 2011b, but see Bennett et al. 2009, Jarzomski et al. 2000). Induced defenses have traditionally been studied in leaves, and there is little information on the potential for induced defenses in vertebrate-dispersed fruits in response to direct fruit damage or leaf damage (Whitehead and Poveda 2011). While it is unknown whether IGs in fruits or leaves of *L.* x *bella* vary in response to damage, data from other studies have shown induced changes in IG concentrations, when they are detectable at all, are small relative to constitutive levels, localized on the plant, and last for a short period of time (Fuchs and Bowers 2004; Quintero and Bowers 2011b). Thus, our sampling scheme of taking large numbers of fruits from each of two different branches on the shrub and sampling the same individual in 2007 and 2008 should have allowed us to capture consistent constitutive differences among individual plants. We measured fruit disappearance and damage over the entire season and created composite scores for each plant that encompassed the entire series of measurements; however, we captured only a snapshot of IG chemistry at a single point in time in each season. The fact that we did still find negative correlations between IGs and

damage despite the potential for unexplained chemical variation due to induced responses is evidence that constitutive variation in IGs among individuals is important in determining susceptibility to insect and microbial attack. Additional controlled experiments examining the effects of multiple feeding guilds on fruit secondary chemistry will be necessary to disentangle the relative importance of constitutive versus induced defense in different plant parts and how sequential damage to fruits over a growing season may alter the outcome of interactions with both antagonistic and mutualistic fruit consumers.

In conclusion, our results provide strong evidence that secondary compounds in fruits cannot be explained solely as a consequence of foliar defense, but instead are likely to serve an adaptive function in the context of fruit/frugivore interactions. We emphasize that evidence for adaptive function does not preclude linkages among chemical traits in fruits and other plant parts and expect that many compounds present in fruits likely have multiple ecological roles. We hope that this study will inspire further research that empirically addresses how fruit secondary compounds affect plant interactions with a broad range of antagonistic and mutualistic fruit consumers, as well research on the linkages between leaf and fruit chemical traits in other systems. Studies of this nature are essential for understanding the true fitness costs and benefits of chemical traits and would have important implications for our understanding of the ecology and evolution of plant-animal interactions.

Appendix 4A: Correlations among concentrations of 12 individual iridoid glycosides occurring in *Lonicera* x *bella* fruits.

Table 4A.1: Correlation matrix showing relationships among concentrations (% dry weight) of iridoid glycosides in unripe fruits.

	Unknown A	Unknown B	Sweroside	Secoxyloganin	Loganin	Unknown C	Unknown D	Loganic acid	Morroniside	Unknown F	Kingiside	Secologanin
Unknown A	1.000											
Unknown B	0.325	1.000										
Sweroside	0.267	0.252	1.000									
Secoxyloganin	-0.023	0.066	0.037	1.000								
Loganin	-0.132	0.107	-0.281	-0.254	1.000							
Unknown C	0.322	**0.724**	0.246	0.161	-0.034	1.000						
Unknown D	0.220	0.326	**0.668**	-0.031	-0.071	0.250	1.000					
Loganic acid	0.220	**0.677**	0.264	0.186	0.160	**0.855**	0.316	1.000				
Morroniside	0.150	0.025	0.114	0.160	0.088	-0.121	0.148	-0.107	1.000			
Unknown F	0.136	0.150	0.356	0.147	-0.137	0.081	0.131	-0.014	0.438	1.000		
Kingiside	-0.014	0.056	0.237	-0.021	-0.154	0.124	-0.006	-0.061	-0.157	**0.602**	1.000	
Secologanin	0.493	**0.901**	0.241	0.105	0.042	**0.750**	0.278	**0.669**	0.000	0.081	0.010	1.000

Table 4A.2: Correlation matrix showing relationships among concentrations (% dry weight) of iridoid glycosides in ripe fruits.

	Unknown A	Unknown B	Sweroside	Secoxyloganin	Loganin	Unknown C	Unknown D	Loganic acid	Morroniside	Unknown F	Kingiside	Secologanin
Unknown A	1.000											
Unknown B	0.480	1.000										
Sweroside	0.138	0.060	1.000									
Secoxyloganin	0.109	0.025	0.321	1.000								
Loganin	-0.039	-0.057	-0.192	-0.253	1.000							
Unknown C	0.408	**0.683**	0.251	0.314	-0.273	1.000						
Unknown D	0.269	0.078	**0.731**	0.230	0.084	0.122	1.000					
Loganic acid	-0.081	0.026	0.422	0.060	-0.021	-0.001	0.182	1.000				
Morroniside	0.252	0.137	0.144	0.156	-0.089	0.100	0.409	-0.088	1.000			
Unknown F	0.121	0.119	0.141	0.319	-0.236	0.310	0.146	0.018	0.460	1.000		
Kingiside	0.151	0.138	0.226	0.216	-0.136	0.487	0.059	-0.069	-0.021	0.390	1.000	
Secologanin	**0.663**	**0.795**	0.188	0.128	-0.083	**0.591**	0.293	0.021	0.437	0.090	0.081	1.000

Appendix 4B: Results from a principal components analysis (PCA) of 12 individual iridoid glycosides occurring in fruits of *Lonicera* x *bella*

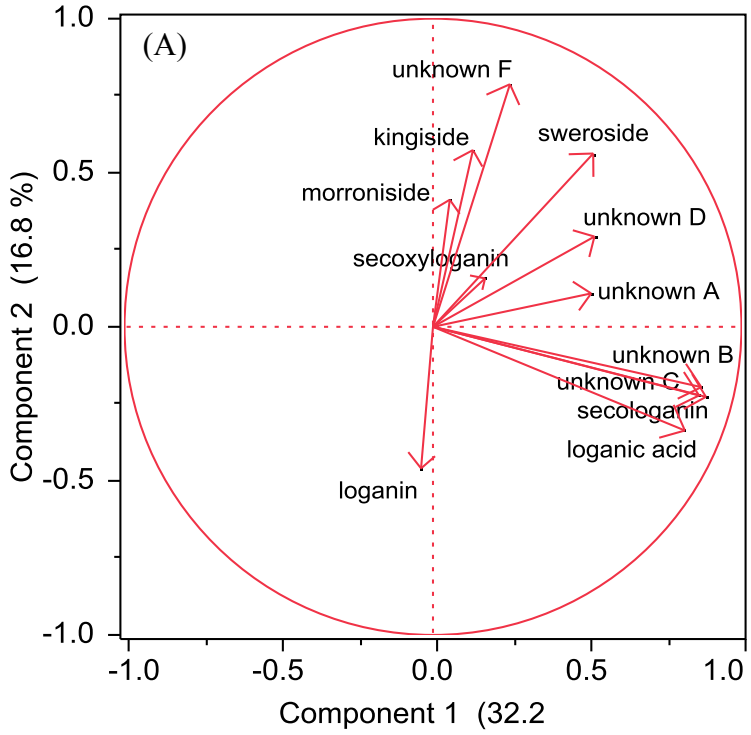
Table 4B.1: Eigenvalues, percent of total IG variation explained, and loadings for each significant orthogonal axis determined for iridoid glycosides in unripe fruit.

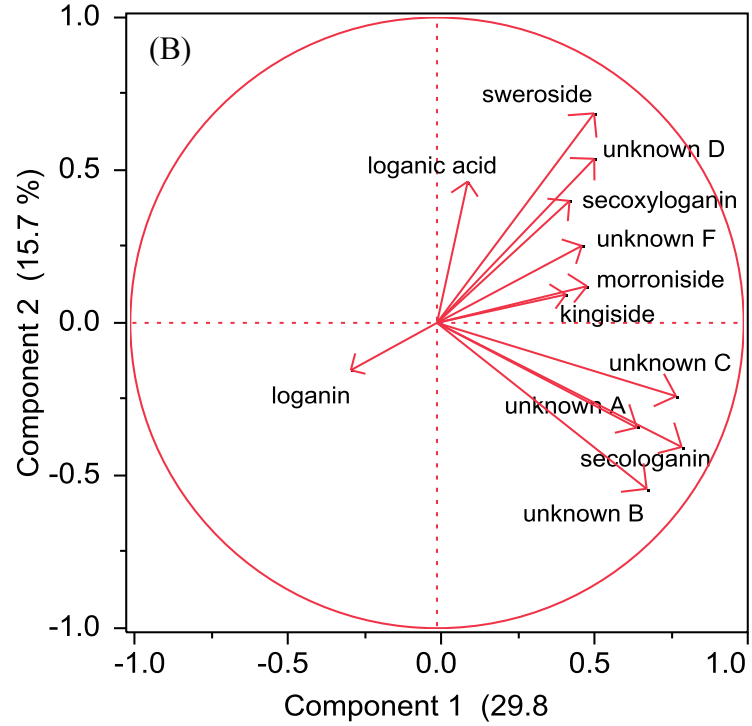
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Eigenvalue:	3.86	2.02	1.31	1.23	1.18	0.93	0.47	0.39	0.27	0.18
Percent of Variation:	32.24	16.84	10.99	10.24	9.85	7.74	3.91	3.24	2.22	1.47
Loadings:										
Unknown A	0.513	0.108	0.248	-0.050	-0.049	-0.727	0.354	0.005	0.042	-0.023
Unknown B	0.870	-0.196	-0.027	0.127	0.145	-0.015	-0.278	0.236	-0.053	-0.030
Sweroside	0.519	0.562	0.153	-0.454	-0.069	0.190	0.088	-0.030	-0.357	0.081
Secoxyloganin	0.169	0.157	-0.112	0.525	-0.688	0.270	0.251	0.222	0.000	0.015
Loganin	-0.039	-0.460	0.284	0.232	0.661	0.281	0.337	0.128	-0.065	0.017
Unknown C	0.862	-0.223	-0.249	0.073	-0.044	0.043	0.000	-0.284	0.106	0.092
Unknown D	0.523	0.293	0.409	-0.496	-0.022	0.318	0.026	0.151	0.316	-0.045
loganic acid	0.817	-0.337	-0.079	0.052	-0.040	0.265	0.136	-0.286	-0.007	-0.078
Morroniside	0.054	0.412	0.682	0.521	0.059	0.006	-0.159	-0.155	0.044	0.185
Unknown F	0.250	0.787	-0.104	0.369	0.293	0.016	-0.030	-0.067	-0.018	-0.276
Kingiside	0.130	0.574	-0.654	0.018	0.386	0.004	0.113	0.083	0.115	0.198
Secologanin	0.885	-0.227	-0.017	0.116	0.037	-0.195	-0.157	0.214	-0.064	0.040

Table 4B.2: Eigenvalues, percent of total IG variation explained, and loadings for each significant orthogonal axis determined for iridoid glycosides in ripe fruit.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Eigenvalue:	3.58	1.88	1.58	1.28	0.94	0.8	0.67	0.52	0.34
Percent of Variation:	29.79	15.67	13.13	10.69	7.89	6.68	5.59	4.33	2.8
Loadings:									
Unknown A	0.651	-0.343	0.231	-0.070	0.083	-0.206	-0.065	0.578	-0.076
Unknown B	0.684	-0.544	0.128	0.236	-0.080	0.165	0.079	-0.218	-0.122
Sweroside	0.512	0.686	0.243	0.269	0.069	-0.120	-0.200	-0.085	-0.065
Secoxyloganin	0.432	0.399	-0.330	-0.018	-0.107	-0.393	0.608	0.024	0.067
Loganin	-0.281	-0.155	0.495	-0.096	0.660	0.195	0.401	-0.040	-0.008
Unknown C	0.779	-0.240	-0.284	0.307	0.059	0.011	0.039	-0.201	-0.059
Unknown D	0.513	0.537	0.496	-0.150	0.223	-0.164	-0.128	-0.122	-0.126
loganic acid	0.100	0.463	0.298	0.512	-0.259	0.510	0.162	0.216	0.133
Morroniside	0.489	0.120	0.154	-0.749	-0.163	0.180	-0.042	-0.097	0.270
Unknown F	0.472	0.252	-0.483	-0.406	0.035	0.444	0.070	0.102	-0.306
Kingiside	0.420	0.093	-0.569	0.188	0.561	0.088	-0.195	0.069	0.278
Secologanin	0.798	-0.407	0.310	-0.010	-0.137	-0.004	0.036	-0.048	0.160

Figure 4B.1: Eigenvector plots summarizing the loadings for the first two orthogonal axes (PC1 and PC2) for unripe fruits (A) and ripe fruits (B). Note that loganin, which is the putative precursor to many of the other iridoids present in *Lonicera*, has the opposite loading on these axes to most other detected compounds.





Appendix 4C: AIC scores and Akaike weights for candidate PC regression models.

Table 4C.1: Top candidate models to describe the effect of individual IGs (combined as PCs, see Appendix B for details) on insect damage, microbe damage, and fruit disappearance. Candidate model sets were determined by running all possible subsets of a global model that included all PCs as predictor variables and retaining those models with $\Delta\ AIC_c < 2$. All models included plant individual (ID) as a random effect. Akaike weights (ω_i) show the relative support for each model and were used in model averaging (see Table 4.2, main text).

Component Models	df	logLik	AIC_c	$\Delta\ AIC_c$	ω_i
Response: Insect Damage					
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC4 + \mu_0 ID + \varepsilon$	6	32.27	-79.69	0.00	0.20
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \mu_0 ID + \varepsilon$	5	34.42	-79.54	0.15	0.18
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC3 + \beta_4 PC4 + \mu_0 ID + \varepsilon$	7	29.66	-78.87	0.82	0.13
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC3 + \mu_0 ID + \varepsilon$	6	31.80	-78.74	0.95	0.12
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC4 + \beta_4 PC5 + \mu_0 ID + \varepsilon$	7	29.48	-78.37	1.32	0.10
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC5 + \mu_0 ID + \varepsilon$	6	31.63	-78.27	1.43	0.10
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC4 + \beta_4 PC6 + \mu_0 ID + \varepsilon$	7	29.75	-77.95	1.75	0.08
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC2 + \beta_3 PC6 + \mu_0 ID + \varepsilon$	6	31.91	-77.86	1.83	0.08
Response: Microbe Damage					
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC6 + \beta_3 PC7 + \mu_0 ID + \varepsilon$	6	43.87	-102.65	0.00	0.23
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC4 + \beta_3 PC6 + \beta_4 PC7 + \mu_0 ID + \varepsilon$	7	40.90	-101.60	1.05	0.14
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC6 + \mu_0 ID + \varepsilon$	5	45.44	-101.31	1.34	0.12
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC6 + \beta_3 PC7 + \beta_4 PC8 + \mu_0 ID + \varepsilon$	7	41.18	-101.21	1.44	0.11
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC5 + \beta_3 PC6 + \beta_4 PC7 + \mu_0 ID + \varepsilon$	7	40.81	-101.04	1.61	0.10
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC7 + \mu_0 ID + \varepsilon$	5	45.40	-101.04	1.61	0.10
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC3 + \beta_3 PC6 + \beta_4 PC7 + \mu_0 ID + \varepsilon$	7	40.11	-100.98	1.67	0.10
$y = \beta_0 + \beta_1 PC2 + \beta_2 PC3 + \beta_3 PC6 + \beta_4 PC7 + \mu_0 ID + \varepsilon$	7	40.35	-100.79	1.87	0.09
Response: Fruit Disappearance					
$y = \beta_0 + \beta_1 PC4 + \beta_2 PC6 + \mu_0 ID + \varepsilon$	5	1.57	-7.70	0.00	0.21
$y = \beta_0 + \beta_1 PC4 + \mu_0 ID + \varepsilon$	4	2.73	-7.31	0.39	0.17
$y = \beta_0 + \beta_1 PC4 + \beta_2 PC5 + \beta_3 PC6 + \mu_0 ID + \varepsilon$	6	-0.33	-6.61	1.09	0.12
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC4 + \beta_3 PC6 + \mu_0 ID + \varepsilon$	6	-0.63	-6.55	1.16	0.12
$y = \beta_0 + \beta_1 PC3 + \beta_2 PC4 + \mu_0 ID + \varepsilon$	5	0.54	-6.23	1.47	0.10
$y = \beta_0 + \beta_1 PC2 + \beta_2 PC4 + \beta_3 PC6 + \mu_0 ID + \varepsilon$	6	-0.88	-6.23	1.48	0.10
$y = \beta_0 + \beta_1 PC4 + \beta_2 PC5 + \mu_0 ID + \varepsilon$	5	0.69	-6.00	1.70	0.09
$y = \beta_0 + \beta_1 PC1 + \beta_2 PC4 + \beta_3 PC6 + \mu_0 ID + \varepsilon$	6	-1.40	-5.82	1.89	0.08

CHAPTER FIVE

PATTERNS OF SECONDARY METABOLITE ALLOCATION TO FRUITS AND SEEDS IN *Piper reticulatum* (Piperaceae) [4]

5.1 Abstract

The role of secondary metabolites in plant defense has been primarily studied in leaves and other vegetative tissues, but secondary metabolites can also occur in reproductive plant parts, such as flowers and fruits. Differential selective pressures from both mutualists and antagonists in reproductive plants parts should lead to qualitative and quantitative differences in the occurrence patterns of secondary metabolites, but how and why secondary metabolites vary across tissues is poorly understood. In particular, few studies have compared the occurrence of secondary metabolites in fruits and seeds to other plant parts. We compared the concentration and diversity of amides, a diverse group of nitrogen-based compounds, among six tissue types of *Piper reticulatum*: leaves, roots, flowers, unripe pulp, ripe pulp, and seeds. This represents the first detailed description of amides in *P. reticulatum*, and we identified 13 major compounds using GC-MS and NMR analysis. We also detected 30 additional unidentified minor amide components, many of which were restricted to one or a few plant parts. Seeds had the highest concentrations and the highest diversity of amides. Fruit pulp had intermediate concentrations and diversity that decreased with ripening. Leaves and roots had intermediate concentrations, but the lowest chemical diversity. In addition, to investigate the potential importance of these compounds in plant defense, we measured leaf herbivory and seed predation in natural populations and examined the relationship between amide content and plant damage. We found

[4] This chapter was a collaborative effort among S.R. Whitehead, M. Leonard, C. Jeffrey, C. Dodson, L.A. Dyer and M.D. Bowers and is currently in preparation for submission to *Journal of Chemical Ecology*

no correlations between leaf damage and amide diversity or concentration and no correlation between seed damage and amide concentration. The only relationship we detected was a negative correlation between seed damage and amide diversity. Together, our results provide important evidence that there are strong selection pressures for fruit and seed defense independent of selection in vegetative tissues and suggest a key role for chemical diversity in fruit-frugivore interactions.

5.2 Introduction

Plants employ a diverse arsenal of secondary metabolites as a defense against herbivores and pathogens. A long history of research has shown that these metabolites can increase plant fitness by reducing the preference and/or performance of a variety of antagonistic consumers (Bennett and Wallsgrove 1994; Coley and Barone 2001; Iason et al. 2012) or by influencing indirect interactions with natural enemies of antagonists (Kessler and Baldwin 2001; Price et al. 1980). However, the vast majority of research in this area has focused on secondary metabolites produced in leaves and their effects on leaf herbivores or pathogens, and relatively little is known about the diversity and functional significance of secondary metabolites produced in other plant parts, such as flowers and fruits (Adler 2000; Cipollini and Levey 1997b; Tewksbury 2002). Because flowers and fruits often function primarily to attract mutualistic pollinators and seed dispersers, selective pressures in these tissues are likely to be qualitatively and quantitatively different from those in leaves and can include conflicting pressures from both mutualists and antagonists (Cazetta et al. 2008; Irwin et al. 2004; Kessler and Halitschke 2009; Tsahar et al. 2002). This study aims to improve the basic understanding of the variation in diversity and abundance of secondary metabolites among different plant parts and its relationship to damage in

natural populations. We focus in particular on fruits and seeds, which have received relatively little attention in chemical ecology (Levey et al. 2007; Tewksbury 2002), but play an important role in plant fitness and can contain a diversity of secondary metabolites, some of which are unique to fruits or seeds (Cipollini et al. 2002; Herrera 1982; Tewksbury et al. 2008b; Whitehead and Bowers 2013).

Although fruits and seeds have often been the subject of phytochemical investigations (e.g. Chaves et al. 2003; Chaves and Santos 2002; Crozier et al. 2006; Ikeshiro et al. 1992; Lamchouri et al. 2010), these results are rarely interpreted in an ecological context and it is still unclear how and why the abundances and diversity of secondary metabolites in fruits and seeds vary relative to other plant parts. There are at least nine major hypotheses that propose adaptive pathways by which diverse mixes of fruit secondary metabolites can evolve (Cipollini 2000; Cipollini and Levey 1997b; Rodríguez et al. 2013). These involve selective pressures from mutualists, e.g. when secondary metabolites function as attractants or association cues (Cipollini 2000; Rodríguez et al. 2013), or from antagonists, e.g. when secondary metabolites function to defend fruits against insect seed predators or fungal pathogens (Cipollini 2000). In particular, increasing evidence has shown that fruit secondary metabolites can play a key role in defense—in some cases leading to trade-offs in fruits between attraction of seed dispersers and defense against antagonists (Cazetta et al. 2008; Cipollini and Levey 1997b; Cipollini et al. 2004; Herrera 1982) and in other cases effectively defending fruits with minimal negative effects on mutualists (Cipollini and Levey 1997b; Struempf et al. 1999; Tewksbury and Nabhan 2001).

There are also a number of reasons, based on broad theories of plant defense, to predict that plant allocation to the defense of fruits may be even more important to plant fitness than allocation to the defense of leaves. For example, optimal defense theory predicts that fruits and

seeds should be well defended relative to other tissues because: 1) they have a high fitness value due to their direct link to reproductive output, and 2) they may be at an increased risk of attack due to their high nutritional content (McCall and Fordyce 2010; McKey 1974; McKey 1979; Rhoades and Cates 1976; Zangerl and Rutledge 1996). In studies that have directly compared the concentrations of secondary metabolites in fruits and/or seeds to vegetative plant parts, fruits and especially seeds have often been shown to have relatively higher concentrations than leaves (Alves et al. 2007; Brown et al. 2003; Erdman 1983; Johnson et al. 1985a; Nelson et al. 1981; Whitehead and Bowers 2013; Wink and Witte 1984; Wink and Witte 1985; Zangerl and Rutledge 1996; but see Dement and Mooney 1974; Isman et al. 1977). However, to understand the adaptive significance of these results, two important factors must be considered: 1) differentiating between fleshy, animal-dispersed fruits and dry fruits that are dispersed abiotically, and 2) differentiating between seeds and the surrounding fruit tissue, which have often been combined for chemical analysis in previous studies.

Animal-mediated dispersal is a common feature of ecological communities, with the percentage of animal-dispersed plants ranging up to 90% in some tropical forests (Jordano 2000). However, we know of only one ecological study that has provided quantitative comparisons of secondary metabolites in leaves and fruits of an animal-dispersed plant (Chapter 4). Although there may be significant ecological costs associated with the occurrence of secondary metabolites in fleshy fruits if these metabolites deter seed dispersers (Strauss et al. 2002; Whitehead and Poveda 2011), the diverse suite of antagonistic consumers attracted to fleshy, nutrient-rich fruits (Johnson et al. 1985b; Mattson 1980; Sallabanks and Courtney 1992) may also create strong selection for defense. The risk of attack to fruits and seeds may also be particularly high relative to other nutrient-rich mutualist rewards, such as nectar, because fruits and seeds are often

exposed to enemies over a long period of time, both during development and post-maturation persistence time. The need to remain attractive to seed dispersers, while reducing the risk of attack from antagonists, should lead to patterns of secondary metabolite occurrence in fleshy fruits that differ both qualitatively and quantitatively from those of other plant parts.

In addition, to understand the allocation of secondary metabolites to fleshy fruits, it is essential to consider the potential differences in selective pressures between seeds and the surrounding pericarp and accessory tissues. In the majority of phytochemical studies examining secondary metabolites in fruits/seeds, the authors apparently collected entire fruits or infructescences, and did not distinguish between seeds and surrounding tissues. One exception is a study by Barnea et al. (1993), which compared the secondary metabolite composition of fruit pulp and seeds of four temperate species from four different plant families (holly, ivy, yew, and hawthorn). In this study, fruit pulp always had higher concentrations of defensive metabolites than seeds, which the authors explain as a mechanism to reduce the amount of fruit consumed by birds in a single foraging bout (the attraction/repulsion hypothesis, *sensu* Cipollini and Levey 1997b). However, there are also a number of reasons to predict that the opposite should be true—seeds should be more defended than the surrounding fruit pulp. Seeds provide a more direct link to reproductive fitness than any other tissue, and secondary metabolites allocated to seeds may also have added fitness value because they not only can defend seeds against direct seed predators, but also provide initial defense of seedlings, which would otherwise be highly vulnerable to attack prior to their obtaining enough resources to produce their own defenses (Ndakidemi and Dakora 2003; Zangerl and Nitao 1998). In addition, seeds are at high risk of attack from both pre-dispersal and post-dispersal seed predators due to their concentrated supply of energy and nutrients, and are often exposed to predators for long periods of time (sometimes

many years) prior to germination (Hulme 1998). Lastly, for fleshy fruits, there is less potential for ecological costs associated with the occurrence of secondary metabolites in seeds than in fruit pulp, because the seed itself is often not a part of the reward for mutualist pollinators and seed dispersers (Eriksson and Ehrlen 1998).

Here we compare the diversity and abundance of secondary metabolites in different plant parts of *Piper reticulatum* L. (Piperaceae), a large understory shrub that is common throughout the Neotropics and is dispersed by primarily by frugivorous bats (Fleming 2004). Previous phytochemical research on *P. reticulatum* is limited, but a few compounds have been described from leaves, including the amides wisanidine and dihydrowisanidine, several sesquiterpenes, and two 5,6-dihydropyran-2-ones (Luz et al. 2003; Maxwell et al. 1998; Yamaguchi et al. 2011). Amides, a diverse group of N-containing metabolites, are the most abundant secondary metabolites in leaves (Yamaguchi et al. 2011). We have found no previous investigations of fruits or other plant parts. The objectives of this study were three-fold:

1. To qualitatively and quantitatively compare the occurrence of amides in leaves, roots, flowers, unripe pulp, ripe pulp, and seeds of *P. reticulatum*
2. To examine the relationship between amide occurrence and patterns of leaf herbivory and seed predation in natural populations
3. To compare the risk of attack to leaves versus seeds through an examination of leaf herbivory and seed predation in natural populations

5.3 Methods

Study System and Site

All samples were collected at La Selva Biological Station, Heredia Province, Costa Rica. The site consists of premontane and tropical wet forest, as well as secondary forest and

abandoned agricultural areas (Holdridge 1967; McDade et al. 1994). La Selva is a high center of diversity for the genus *Piper*, with 50+ species co-occurring (Gentry 1990; OTS 2012).

Piper reticulatum is a large rainforest understory shrub ranging from Honduras to Bolivia (Tropicos 2012) and is one of the most common *Piper* species in secondary forest and along trails at La Selva. Flowers are borne on distinct spike-shaped inflorescences that mature into infructescences over a period of several months. Each infructescence contains ~100-300 individual fruits that ripen simultaneously, with the final ripening phase usually beginning mid-afternoon and lasting for several hours (SRW, personal observation). During the final ripening period, fruits soften and swell, but there is no color change from the pale green typical of unripe fruits. Individual trees can produce hundreds of infructescences that ripen sequentially, with anywhere from 1-20 infructescences maturing per day over the course of a fruiting peak that lasts for several weeks for an individual. Most ripe fruits are removed by bats on the first evening that they are ripe; those that are not removed are visibly beginning to rot on the following day and are not taken on the following evening (SRW, personal observation).

A small genus of fruit bats (*Carollia spp.,* Phyllostomidae) are the primary dispersers of *P. reticulatum* and most other *Piper* species in the Neotropics (Fleming 2004). Three *Carollia* species co-occur at La Selva and all feed on *P. reticulatum*: *C. perspicillata, C. sowelli,* and *C. castanea* (Baker et al. 2002; Fleming 1991; SRW personal observation). The association is one of the few examples of a relatively specialized dispersal system, with the *Carollia* bats providing the majority of seed dispersal services for *Piper* and *Piper* fruits providing a year-round dietary staple for the bats (Fleming 2004; Fleming and Heithaus 1986; Herbst 1986). Olfactory cues appear to be the primary mechanism used by bats to locate fruits and distinguish between unripe and ripe fruits in close proximity (Laska 1990; Mikich et al. 2003; Thies et al. 1998). Entire

infructescence spikes are removed in flight and carried to central feeding roosts, where the fruit is consumed off of the central rachis of the spike (Fleming et al. 1977; Thies and Kalko 2004; SRW personal observation).

Piper reticulatum is attacked by a variety of leaf herbivores at La Selva, including at least 24 species of lepidopteran larvae, the most abundant being *Quadrus cerialis* Stoll (Hesperiidae), a *Piper* specialist, and *Anacrusis nephrodes* Walsingham (Tortricidae), a generalist (Dyer and Gentry 2012). Fruits and seeds are attacked by hemipteran seed predators, especially *Sibaria englemani* Rolston (Pentatomidae), a *Piper* specialist (Greig 1993a; SRW personal observation), as well as an abundant, but unidentified, dipteran larva that makes a dramatic leap from ripe fruits as they are removed from the plant and burrows into the soil to pupate (SRW personal observation). Microbial consumers are also likely important as fruit antagonists, as evidenced by the rapid decomposition of fruits once they reach final maturity (Thies and Kalko 2004; SRW personal observation).

Sample Collection

To compare amides occurring in different plant parts, we collected samples from 16 individuals of *Piper reticulatum* growing along trails and forest edges between July 10 and July 24, 2012. All trees were separated by a minimum of 25m. From each individual, we collected leaves, roots, flowers (when available, for 12 of 16 individuals only), unripe fruits, and ripe fruits, always collecting all of the tissue types from a single tree at the same time on a single day. We always collected late in the afternoon and included only ripe fruits that had matured that day. Samples were brought immediately back to the laboratory. From each individual, we first took a subsample of each ripe infructescence (2-6 infructescences per plant) from which to sample the

seeds, cutting small sections from the top, middle, and bottom of each spike. The seeds were gently washed in water to remove pulp and stored separately from the remainder of the fruit prior to chemical analysis. The remainder of the ripe fruit was stored intact and the seeds were later removed to obtain a pulp-only sample (see below). Samples of all plant parts were placed in paper packets and dried in silica gel. Dried samples were then transported to the University of Colorado for analysis using gas chromatography combined with mass spectrometry (GC-MS) to determine amide diversity and concentration (see below). Leaves, roots, and cleaned seeds were ground to a fine powder using a coffee grinder and/or a sample mill (Tecator Cyclotec, FOSS North America, Inc). To obtain pulp-only samples from dried, intact ripe and unripe fruits, we first ground the plant material through a fine mesh sieve to separate seeds from pulp. Seeds were removed from these samples and discarded and the pulp was further ground to a fine powder using a coffee grinder.

Identification and Quantification of Amides Using GC-MS

To examine the variation in amides among *P. reticulatum* individuals, we used a scaled-down version of extraction and quantification procedures as described in Dyer et al. (2004b). From each sample, ~100mg (weighed to the nearest 0.1mg) of dried plant material was taken in a test tube with 7.5 mL ethanol and vortexed. Samples were extracted overnight, vortexed, and the ethanol extract was filtered to remove suspended material. A second 7.5 mL of ethanol was added to the plant material for a second overnight extraction and filtered as above, and the two extracts were combined and evaporated to dryness. This extraction procedure results in an extract enriched in amides, although other compounds may be present as well. Samples were then re-suspended in 3mL 3:1 water:ethanol, transferred to a separatory funnel, and partitioned three

times against equal volumes of chloroform. The water partition was discarded and the combined chloroform partitions (containing the amides) were evaporated to dryness. Samples were then re-suspended in 1mL dichloromethane, and piperine (obtained from Sigma-Aldrich Co.), an amide which does not occur in *P. reticulatum*, was added at a concentration of 0.75 mg/mL as an internal standard. Aliquots of 100 μL were then transferred to vials for analysis using GC-MS.

All samples were analyzed using an HP Agilent 6890N GC coupled with an Agilent 5975C MS with an ion source of 70eV at 230^{o}C. The instrument was equipped with a DB-5MS capillary column (30m x 0.25mm i.d.,0.5 μm film thickness; Agilent Technologies, Santa Clara, CA, USA). Ultra-pure He was used as the carrier gas at a flow rate of 1.5 ml min^{-1}, a split flow ratio of 30:1, and a front inlet temperature of 280^{o}C. The following oven conditions were employed: initial temperature 50^{o}C, initial hold time 1 min; ramp 1: 15^{o}C min^{-1} to 275^{o}C, hold time 5 min; ramp 2: 20^{o}C min^{-1} to 320^{o}C, hold time 10 min; for a total run time of 33.25 minutes. A blank sample (dichloromethane only) was run after every five samples to ensure there was no carryover between runs. Data were recorded and processed using MSD ChemStation software (version D.02.00.275). Quantities of individual compounds and of total amides were estimated based on peak areas in total ion current chromatograms and known concentrations of the internal standard, which showed a linear response across the range of concentrations present in our samples ($R^2 > 0.99$).

All compounds detected in the samples were first screened for potential amides based on visual assessment of spectra for characteristic fragmentation patterns of N-based compounds, in particular a molecular ion with an odd mass (McLafferty and Turecek 1993). For those compounds that displayed these patterns, we further confirmed our visual assessments using comparisons to spectra of known amides in the Wiley-NIST 2005 library. Although most

naturally-occurring amides are not in this database, we assessed compounds for similar structures using the automated substructure search feature, specifically searching for the presence of nitrogen (N), carbonyl (C=O), and amide (N-C=O) functional groups (Stein 1995). Furthermore, we checked that all putative amides had retention times between 12.5 min and 25 min, consistent with the range of retention times we have observed with known amides using identical instrument parameters. Although this rapid profiling approach cannot provide absolute certainty in compound identification, it is unlikely that the compounds we tentatively identified as amides belong to other compound classes for several reasons. First, our extraction and purification procedures would have removed many commonly occurring N-based plant compounds, such as most amino acids, based on polarity (Burroughs 1970). Second, most N-based compounds in plants, including primary metabolites (e.g. peptides, nucleic acids, and most amino acids) as well as other common classes of N-based secondary metabolites (e.g. non-protein amino acids, cyanogenic glycosides, glucosinolates, and most other classes of alkaloids), either cannot be analyzed using GC-MS or require derivatization prior to analysis due to their low volatility (Heaney and Fenwick 1993; Hodisan et al. 1998; Seigler and Brinker 1993; Waterman 1993). There are some classes of alkaloids that do not require derivatization prior to GC-MS analysis, including tropane alkaloids and pyrrolizidine alkaloids (Robins 1993; Woolley 1993), but these compounds have not been previously reported from *Piper* (Parmar et al. 1997). One other N-based defensive compound has been reported from *Piper reticulatum*, cyanobenzyl benzoate (Yamaguchi et al. 2011). We did detect this compound in a number of leaf samples, but the retention time was not in the range of amides and we did not include this compound in our statistical analyses of amide concentration and diversity. To ensure consistency in identification of unknown amides across multiple individuals and tissue types, once a compound was identified

as an amide we added the mass spectrum to a user-created library of all compounds included in this study and identified repeated occurrence of the same compounds on the basis of matches with both library entries and retention times.

Structure Elucidation of Major Components Using NMR

The structures of major amide components were further confirmed using crude proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) conducted at the University of Nevada. For NMR analysis, composite samples were created using 5-6 individuals of *P. reticulatum* due to limited amounts of material in single samples. From each tissue type, 2g of plant material was extracted twice with methanol (10 mL) with sonication (10 min, Branson 3510). The combined extracts were filtered and evaporated to dryness in a centrifugal evaporator (Savant SpeedVac SC210A, Thermo-Scientific) under reduced pressure. From the resulting residue, 15mg was transferred to a NMR tube and 1mL of deuterated chloroform or d4-methanol (Cambridge Isotope Laboratories) was added for crude ^{1}H-NMR analysis using a 400 MHz Varian instrument (Agilent Technologies).

Further isolation of amides from ripe fruits and leaves was performed to verify the structures of major components. Partial purication was performed using medium pressure liquid chromatography (MPLC) using a pre-packed Biotage ZIP silica gel column [50 g (39 x 82 mm), 50 *m partical size] and eluting (10 mL/min) with an increasing gradient of ethyl acetate in hexanes. Like fractions (via TLC and UV 254 nm) were combined, evaporated under reduced pressure, and subjected to GC-MS and ^{1}H-NMR analysis.

Measurement of Leaf and Seed Damage in Natural Populations

We measured standing levels of leaf herbivory and seed damage for each individual of *P. reticulatum* in the study population at the time of collection for chemical analysis. To measure leaf herbivory, we took a sample of 10 fully-expanded leaves from a variety of locations (e.g. different heights and exposures) on each individual plant and measured both the total leaf area and the area removed using a leaf area meter (LI-COR LI-3100). The proportion of leaf area removed was averaged among leaves to estimate the herbivory level for each individual. To measure seed damage, we took subsamples of seeds from ripe fruits collected from each individual plant. Where multiple ripe infructescences were collected from a single individual, we took a small subsample of seeds from various points along each infructescence and combined all subsamples into one sample for each individual. The seeds were washed gently in water to remove pulp and sorted under a stereoscope into intact and damaged groups. Intact seeds were reddish-brown and rigid, whereas damaged seeds were visibly darker (appearing rotten) and soft (i.e. could be easily mashed with forceps). A minimum of 100 seeds were counted from each individual of *P. reticulatum* to estimate the proportion that were damaged versus intact.

Statistical Analyses

To examine intraplant variation in amides, we first compared the estimated total amide concentration and the chemical diversity (i.e. the number of amides detected) among leaves, roots, flowers, unripe fruit pulp, ripe fruit pulp, and seeds. For the analysis of total amide concentration, we used a linear mixed model with a normal distribution, with plant part specified as a fixed effect and plant identity as a random effect. The estimated amide concentration (% dry weight) was logit transformed prior to analysis to approximate a normal distribution (Warton and

Hui 2011). For the analysis of chemical diversity, we used a generalized linear mixed model with a poisson distribution and the log-link function, again with plant part specified as a fixed effect and plant identity as a random effect (Bolker et al. 2009; Zuur et al. 2009). For hypothesis testing, we compared these models to null models that included the random effect (plant identity) only using likelihood ratio tests (Bolker et al. 2009). When the model that included plant part provided a significantly better fit ($P < 0.05$) to the data than the null model, we followed these analyses with a Tukey HSD post-hoc test to examine pairwise differences among plant parts. These analyses were conducted using the 'lme4' and 'multcomp' packages in the R Environment for Statistical Computing (Bates and Maechler 2010; Hothorn et al. 2011; R Development Core Team 2012)

To further examine overall similarities and differences among plant parts, we used multivariate non-metric multidimensional scaling analysis (NMDS), an ordination technique based on a dissimilarity matrix for all datapoints that incorporates both the presence/absence and quantities of individual compounds (Minchin 1987). The ordination was based on the Bray-Curtis dissimilarity index and used 200 replicates with random starting coordinates. To determine the appropriate number of dimensions for the final solution, we generated a scree plot of the number of dimensions versus the final stress, and found that further reductions in the final stress after two dimensions were small. This analysis was conducted using the 'vegan' package in R (Oksanen et al. 2010; R Development Core Team 2012).

To examine whether amide concentration relates to patterns of leaf damage and seed damage in the field, we used non-parametric Kendall's rank correlations between leaf amide concentration or diversity and the proportion of leaf area removed, and between seed amide

concentration or diversity and the proportion of damaged seeds. These analyses were conducted using the statistical software JMP v. 9.0.2 (2010).

Finally, to compare damage levels of leaves versus seeds, we used a linear mixed model with a normal distribution, with plant part (leaf versus seed) as a fixed effect and plant identity as a random effect. For hypothesis testing, we compared this model with a null model that included the random effect only using a likelihood ratio test (Bolker et al. 2009).

5.4 Results

All plant parts of *P. reticulatum* contained amides, the most abundant of which was dihydrowisanidine (1), which occurred in all plant parts, although its relative abundance varied considerably (Table 5.1). This compound and twelve additional major amides were identified or tentatively identified using GC-MS and NMR data (Table 5.1, Fig. 5.1). These components together represented 92% of the estimated total amides in leaves, 99% in roots, 87% in flowers, 90% in unripe fruits, 86% in ripe fruits, and 87% in seeds (Table 5.1). In addition, we detected 30 additional minor compounds that were characterized as amides based on their fragmentation patterns in mass spectra (McLafferty and Turecek 1993), but their structures were not confirmed. Many individual amides were unique to one or a few plant parts (Table 5.1). We also detected one major component that occurred only in leaves and was not classified as an amide, cyanobenzyl benzoate (Yamaguchi et al. 2011).

The estimated total amide concentration (as a % dry weight) was significantly different among plant parts ($X^2 = 49.26$, df = 5, $P < 0.0001$; Fig. 5.2a). Concentrations were highest in seeds, followed by flowers, unripe pulp, leaves, roots, and ripe pulp. Statistically significant pairwise differences among the plant parts are shown in Fig. 5.2a. The total amide diversity (i.e.

TABLE 5.1: RETENTION TIMES AND AVERAGE ESTIMATED CONCENTRATIONS (% DRY WEIGHT) OF INDIVIDUAL AMIDES IN DIFFERENT PLANT PARTS OF *Piper reticulatum*

Compound Identity	RT [1]	Leaves	Roots	Flowers	Unripe	Ripe	Seeds
A	13.03	0.0015	~	~	~	~	~
B	14.69	~	~	0.0010	~	~	~
C	15.72	~	~	~	~	~	0.0011
D	15.75	0.0238	~	~	~	~	~
Tetrahydrowisanidine	15.83	~	~	~	0.0022	0.0021	0.0029
Desmethoxydihydrowisanidine	16.01	0.7009	~	0.3775	0.0198	0.0141	0.0007
E	16.37	~	~	~	0.0007	~	0.0016
F	16.53	~	0.0030	~	~	~	~
G	16.68	0.0018	~	0.0200	~	~	~
H	16.78	0.0173	~	0.0453	0.0014	0.0063	0.0054
I	16.88	0.0054	~	0.0011	0.0077	0.0088	0.0247
Dihydrowisanidine	17.00	0.0781	0.7001	0.0724	0.4192	0.2766	0.6936
J	17.29	~	~	~	0.0012	0.0013	~
K	17.52	~	~	~	~	~	0.0013
L	17.62	~	~	~	~	~	0.0011
Desmethoxywisanidine	17.68	0.0692	~	0.4946	0.0095	0.0059	~
Octadecadienoylpyrrolidine	17.76	~	~	0.0110	0.0581	0.0303	0.1081
Wisanidine	17.99	~	~	0.0057	~	~	~
M	18.23	~	0.0030	~	~	0.0027	~
N	18.25	~	~	~	~	~	0.0057
O	18.37	~	~	~	0.0061	0.0030	0.0222
P	18.49	~	~	0.0063	0.0063	0.0039	0.0216
Octadecenoylpyrrolidine	18.66	~	0.0052	0.0605	0.1190	0.0729	0.2476
Q	19.28	~	~	~	0.0014	~	0.0009
N-isobutyleicosatrienamide	19.37	~	~	0.0056	0.0138	0.0083	0.0361
N-Isobutyleicosadienamide	19.47	~	~	0.0035	0.0561	0.0290	0.1130
Methoxy tricholein A	19.60	0.0042	~	0.0427	0.0865	0.0422	0.1602
Methoxy tricholein B	19.68	0.0218	0.0026	0.1173	0.1290	0.0647	0.2400
Methoxy dihydrotricholein	19.74	0.0029	0.0061	0.0300	0.1699	0.0898	0.3109
Iso-wisanidine	19.94	0.0093	0.0145	0.0188	0.0453	0.0122	0.1131
R	20.62	~	~	~	~	~	0.0080
S	21.09	~	~	~	0.0099	0.0058	0.0431
T	21.61	~	~	~	~	~	0.0247
U	21.66	~	~	~	0.0117	0.0130	~
V	21.96	~	~	~	0.0246	0.0139	0.0088
W	21.97	~	~	~	~	~	0.0591
X	22.05	~	~	~	0.0028	~	0.0018
Y	22.19	~	~	~	0.0087	0.0011	0.0372
Z	24.38	~	~	~	0.0048	~	0.0076
AA	24.99	~	~	~	0.0009	~	0.0011
BB	25.06	~	~	~	~	~	0.0010
CC	27.08	~	~	0.0126	0.0065	0.0012	0.0124
DD	27.95	~	~	~	~	~	0.0017
Average Total Concentration		**1.15**	**0.73**	**1.33**	**1.22**	**0.71**	**2.32**
Average Amide Diversity		**4.31**	**1.62**	**9.31**	**12.69**	**9.67**	**15.20**

~ = not detected

[1] *RT = retention time (min)*

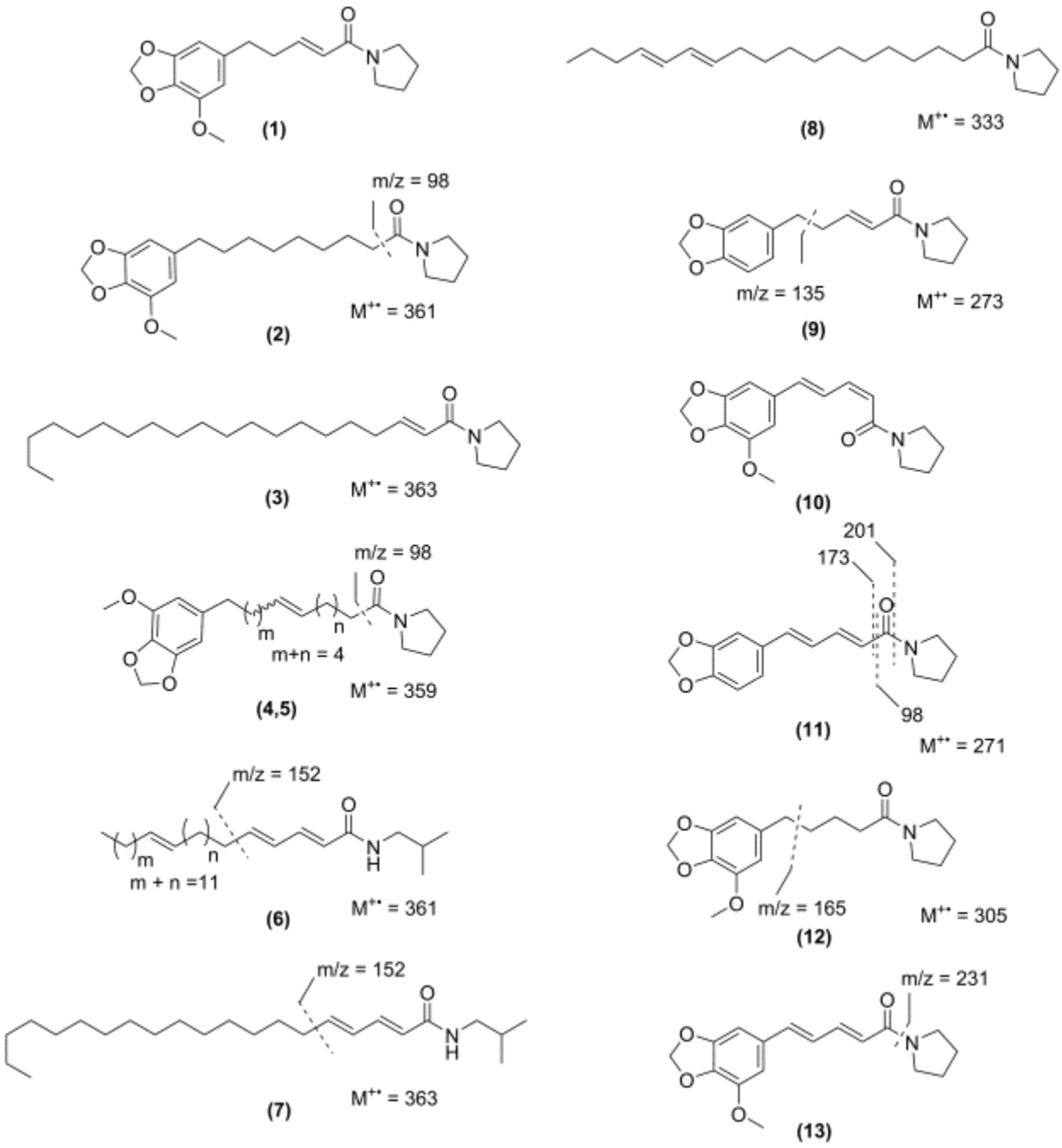


Fig. 5.1: Structures of amides from *P. reticulatum*

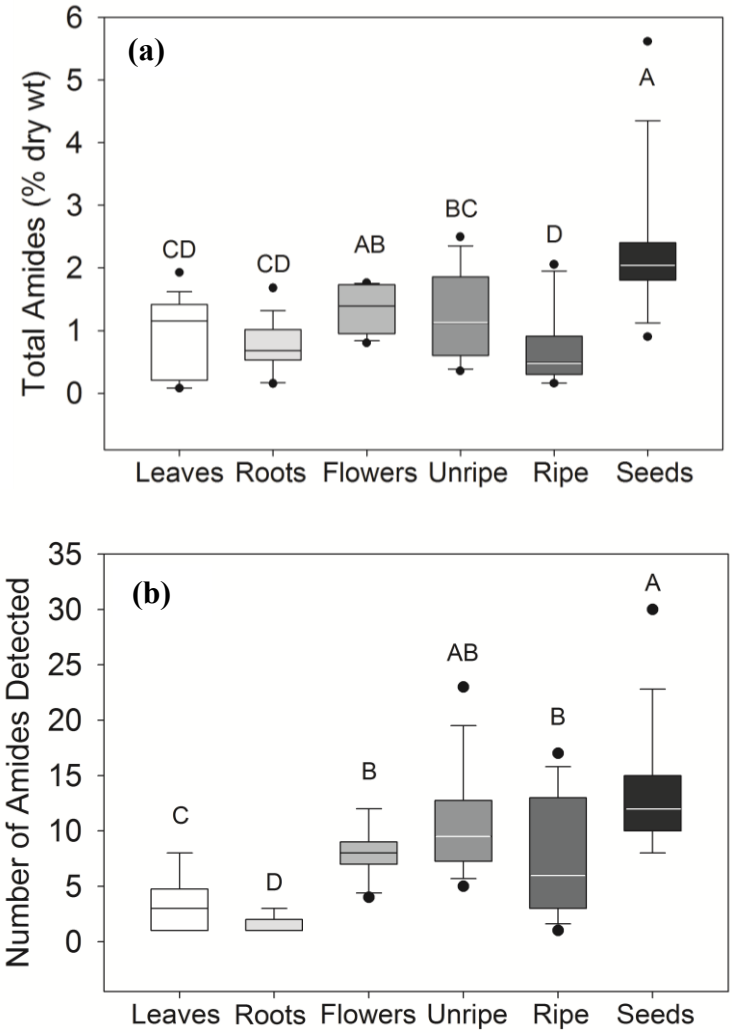


Figure 5.2: Estimated total amide concentration (a) and diversity (b) in different plant parts of *P. reticulatum*. Box and whisker plots show the median, 25th and 75th percentile, and range of total amide concentrations from N=16 plants. Letters represent significant differences from Tukey HSD post-hoc comparisons among plant parts.

the number of compounds detected) was also significantly different among plant parts (X^2 = 269.83, df = 5, P < 0.0001; Fig. 5.2b). Chemical diversity was highest in seeds, followed by unripe pulp, flowers, ripe pulp, leaves, and roots. Statistically significant pairwise differences among the plant parts are shown in Fig. 5.2b. The NMDS analysis examining overall chemical similarity among plant parts revealed significant overlap in the chemical profiles of unripe pulp, ripe pulp, and seeds; however, leaves, flowers, and roots all formed distinct groups that were significantly different from other plant parts (2-D Final Stress = 0.11, R^2 = 0.43, Fig. 5.3).

There were no significant correlations between the proportion of leaf area removed and either leaf amide concentration (Kendall's $\tau = -0.02$, $P = 0.93$) or diversity (Kendall's $\tau = 0.25$, $P = 0.20$). For seeds, we found no relationship between the proportion of damaged seeds and the seed amide concentration (Kendall's $\tau = -0.20$, $P = 0.30$), but we did find a marginally significant negative correlation between the proportion of damaged seeds and seed amide diversity (Kendall's $\tau = -0.38$, $P = 0.055$; Fig. 5.4).

The proportion of damage to seeds was significantly higher than the proportion of damage to leaves ($X^2 = 22.65$, df=1, $P < 0.0001$, Fig. 5.5). On average, 18.6% of seeds were damaged (i.e. rotten) and 4.6% of leaf area was removed.

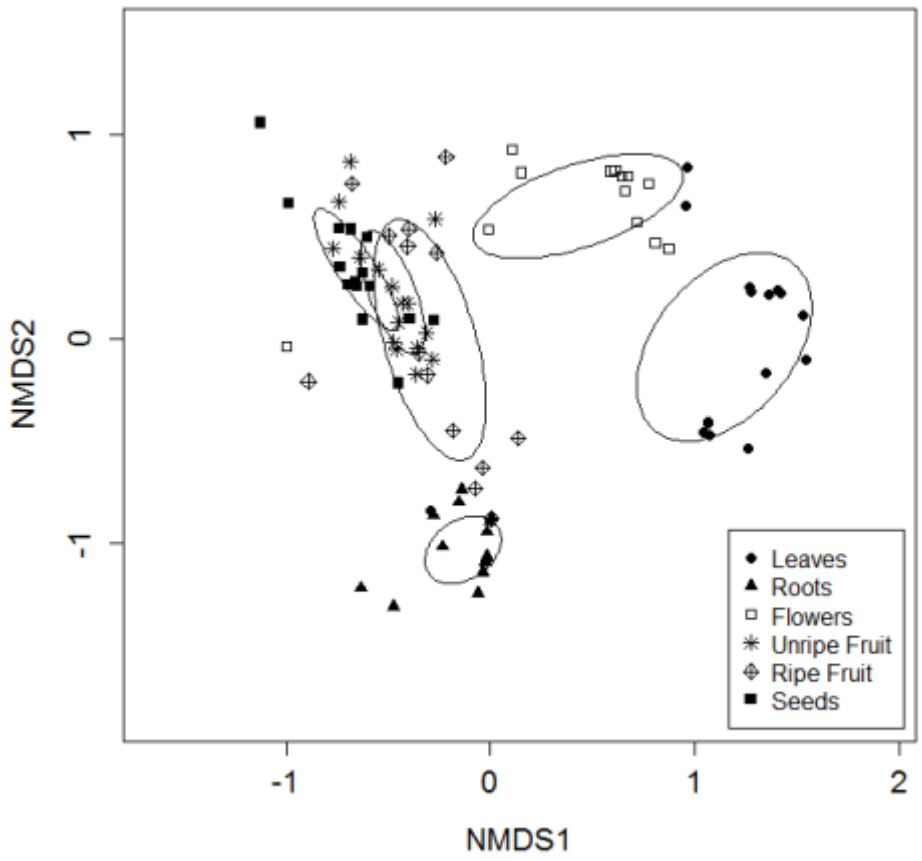


Figure 5.3: Non-metric multidimensional scaling ordination plot of overall chemical similarity among plant parts (2-D Final Stress=0.11, R^2=0.43). Ellipses represent 95% confidence intervals for group centroids (±1SD).

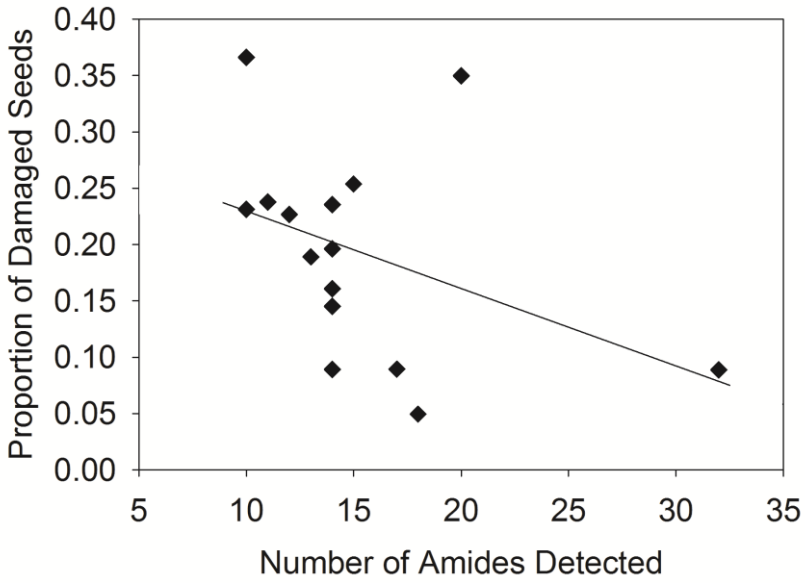


Figure 5.4: Relationship between the amide diversity detected in *P. reticulatum* seeds and the proportion of seeds that were damaged (Kendall's $\tau = -0.38$, $p=0.055$) for N=16 individuals.

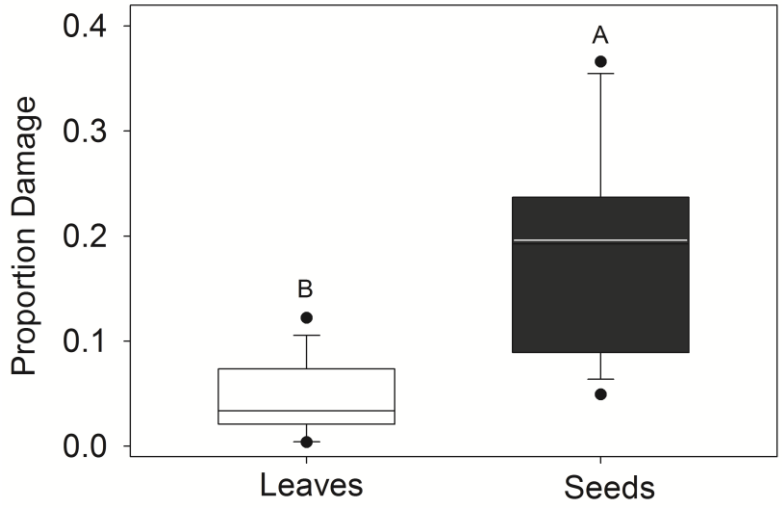


Figure 5.5: Comparison of the damage to leaves (as the proportion of leaf area removed) versus seeds (as the proportion of seeds damaged) in *Piper reticulatum*

5.5 Discussion

Plant secondary metabolites occur in all plant parts and can have a diversity of ecological functions in interactions with both mutualists and antagonists (Bennett and Wallsgrove 1994; Coley and Barone 2001; Iason et al. 2012). However, we often have a limited understanding of how and why plants allocate defensive metabolites differentially among parts, particularly to fruits and seeds. This study provides one of the first examinations of intraplant allocation patterns in a fleshy-fruited species, and, to our knowledge, the first study conducted with a mammal-dispersed species. We found that allocation to chemical defenses in *P. reticulatum*, both in terms of total amide concentrations and chemical diversity, differed substantially among plant parts and was highest for seeds. In addition, we found that concentrations of amides in fruit pulp decreased with ripening, supporting the hypothesis that secondary metabolites retained in ripe fruit pulp can carry important ecological costs. However, surprisingly, we found few correlations between amide content and leaf or seed damage in natural populations. The only relationship we detected was between chemical diversity and the proportion of damaged seeds, suggesting that, at least for seeds, chemical diversity may be more important than generally recognized in plant defense (Berenbaum and Zangerl 1996; Castellanos and Espinosa-García 1997).

Our results showing strong differences in the composition of amides among plant parts (Table 5.1, Figure 5.3) suggest that plants experience different selective pressures in different plant parts and are able to allocate secondary metabolites accordingly. In *P. reticulatum*, we found a number of amides in reproductive tissues that never occurred or occurred only in very low concentrations in vegetative tissues, such as methoxy dihydrotricholein, N-isobutyleicosadienamide, and octadecadienoylpyrrolidine (Table 5.1). There were also two

amides, tetrahydrowisanidine and unknown A (Table 5.1), and one cyanogenic compound, cyanobenzyl benzoate, that occurred in leaves but never in fruits. These results are in contrast to hypotheses that explain the occurrence of secondary metabolites in reproductive tissues primarily as a result of strong selection for defense of leaves and constraints on the exclusion of secondary metabolites from certain tissues (Adler 2000; Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998). Instead, plants may be able to optimize the allocation of secondary metabolites depending on the specific selective pressures in different plant parts. For example, compounds that occur primarily in fruits, such as methoxy dihydrotricholein, may play a key role in defending fruits against fungi associated with fruit rot, but have minimal benefits against leaf herbivores. Compounds that occur only in leaves, such as cyanobenzyl benzoate, may potentially have an important defensive function against lepidopterans or other leaf herbivores, but be excluded from fruits due to negative effects on seed-dispersing bats.

Although most studies that examine the role of secondary metabolites in ecological interactions focus on one or a few compounds, plants can produce hundreds of individual secondary metabolites and chemical diversity *per se* has been implicated as an important force in determining the outcome of species interactions both on ecological and evolutionary timescales (Berenbaum and Zangerl 1996; Castellanos and Espinosa-García 1997; Gershenzon et al. 2012; Jones et al. 1991). The chemical diversity of amides in *P. reticulatum* was quite high for reproductive structures, with flowers, unripe pulp, ripe pulp, and seeds all containing higher numbers of detected compounds than leaves or roots (Fig. 5.2b). For roots in particular, chemical diversity was very low, with most samples dominated by a single compound, dihydrowisanidine (Table 5.1). Because producing a higher diversity of compounds may depend on increasingly complex metabolic pathways or additional enzymes (Gershenzon et al. 2012), our data suggest

that the per gram dry weight investment in secondary metabolites by *P. reticulatum* is likely higher for reproductive structures than for vegetative structures, even if the concentrations are similar among tissue types. These results support the predictions of optimal defense theory, which suggests that allocation to the defense of reproductive tissues should be higher than that to vegetative tissues due to the higher fitness value of reproductive tissues (McCall and Fordyce 2010; McKey 1974; McKey 1979; Rhoades and Cates 1976; Zangerl and Rutledge 1996). The chemical diversity in reproductive structures may also be in part explained by diverse selective pressures from antagonistic and mutualistic consumers in these tissue types. In particular, fruits often experience strong selective pressure from microbial consumers (Cipollini and Levey 1997a; Cipollini and Stiles 1993; Levey et al. 2007; Schaefer et al. 2008; Tewksbury et al. 2008a), and one possibility is that the diverse community of fungi and bacteria involved in fruit and seed rot has played an important role in the diversification of fruit secondary metabolites. Different chemical compounds may be bioactive against different consumers, or chemical diversity may increase the overall bioactivity of the mixture due to synergistic interactions among individual compounds (Dyer et al. 2003; Richards et al. 2010). Considering the number of individual amides that occur in reproductive structures, complex interactions among compounds seem inevitable and deserve further attention.

Among plant parts, seeds had the highest detected chemical diversity and the highest concentration (Fig. 5.2). Secondary metabolites have been shown to play an important role both in reducing seed predation risk (Tsahar et al. 2002; Zangerl and Nitao 1998) and increasing seedling success (Ndakidemi and Dakora 2003). Our results showing a negative relationship between seed predation and chemical diversity (Fig. 5.4), and high damage levels to seeds relative to leaves (Fig. 5.5), provide further confirmation that plant investment in the defense of

seeds may be a key factor mediating plant reproductive fitness in this system. However, there are still many unanswered questions related to the role of seed secondary metabolites in pre- and post-dispersal processes. Relative to the vast literature on the chemical ecology of plant-herbivore interactions in leaves (reviewed by Iason et al. 2012), the chemical ecology of seed defense has been virtually ignored. Our results suggest this may be a promising direction for future work. In particular, we need information on how the chemical diversity of seeds may provide specific benefits either in interactions with the broad range of organisms that attack seeds (including vertebrates, invertebrates, and microbes) or in providing initial defense for plant offspring that arrive in different habitats with variable communities of antagonists and competitors.

In contrast to seeds, ripe fruit pulp had the lowest concentration of amides, almost two-fold lower than unripe fruit pulp and three-fold lower than seeds (Fig. 5.2a). Our results are in contrast to the results from Barnea et al. (1993) that showed a higher concentration of metabolites in ripe fruit pulp than in seeds. One potential reason for the discrepancy is that the work by Barnea et al. (1993) was conducted with four bird-dispersed species, whereas our study was conducted with a bat-dispersed species. Birds have a high tolerance for many classes of secondary metabolites that can be deterrent or even highly toxic to mammals (e.g. Caksen et al. 2003; Struempf et al. 1999; Tewksbury and Nabhan 2001). Our ongoing work seeks to quantify the effects of amides on bat foraging and feeding behavior, and preliminary results suggest that bats are deterred by a variety of amides (SRW, unpublished data). Pharmacological studies have also shown that amides can have diverse physiological effects on both rats and humans, including effects on the digestive and cardiovascular systems (de Araújo-Júnior et al. 2011; Srinivasan 2007). Thus, our results showing lower concentrations and diversity of compounds in

ripe pulp than in seeds or unripe pulp may be explained, at least in part, by significant ecological costs of amides in ripe fruit pulp in terms of reduced seed disperser preferences. Another possible explanation is that a re-allocation of fruit amides with ripening may reduce the overall physiological investment in defense, especially if compounds are shunted from fruit pulp to seeds during the ripening process. In addition, because ripe fruits of *P. reticulatum* are generally removed within hours of the final ripening phase (Thies and Kalko 2004; SRW personal observation), the risk of attack from antagonists is relatively low. The persistence time of fruits has been hypothesized to be a key factor in predicting interspecific variation in the occurrence patterns of fruit secondary metabolites, with more persistent fruits expected to exhibit higher levels of chemical defense (Cipollini and Levey 1997b; Tang et al. 2005). Overall, factors such as dispersal mode and persistence time may have selected for low levels of amides in the ripe fruit pulp of this species, but the patterns of allocation to ripe fruit pulp may differ substantially in different species.

Overall, the results of this study have added an important new element to our understanding of plant allocation patterns to different plant parts, particularly fruits and seeds. We show that secondary metabolite concentrations and diversity can be higher in reproductive than in vegetative tissues, and that seeds in particular are highly defended. In addition, the phytochemical methods and results provided here should allow the pursuit of many additional questions related to the chemical ecology of interactions in *P. reticulatum*, which is an abundant and widespread Neotropical species. Future work should focus on the adaptive significance of secondary metabolite diversity, particularly in reproductive structures, and the role of seed chemical defense in pre- and post-dispersal processes that influence reproductive fitness and species distributions. Although decades of research has shown that plant chemistry plays a key

role in plant-animal interactions, this field has historically been dominated by studies of leaf chemistry and leaf herbivores. Increased emphasis on how plant fitness is influenced by the chemistry of reproductive structures would have important implications for our understanding of both plant chemical trait evolution and the evolutionary ecology of mutualisms.

CHAPTER SIX

CHEMICAL ECOLOGY OF FRUIT DEFENSE: SYNERGISTIC AND ANTAGONISTIC INTERACTIONS AMONG AMIDES FROM *Piper* [5]

6.1 Abstract

Although ripe, fleshy fruits function primarily to attract seed dispersers, they must also be defended against diverse communities of seed predators and pathogens. Thus, in addition to nutritional rewards, many fruits contain potentially deterrent secondary metabolites. Recent evidence has shown that in some cases the concentration and diversity of secondary metabolites in fruits can exceed that of leaves and other plant parts, but little is known about the functional significance of the suites of compounds found in fruits. In particular, secondary metabolite diversity in fruits may provide important adaptive benefits for plants, both by providing simultaneous defense against multiple consumers and through potential interactive effects among compounds that can increase defense efficacy against particular consumers. In this study, we conducted a series of experiments to test the effects of suites of amides from fruits of *Piper* plants on a variety of antagonistic fruit pests, including an insect seed predator (*Sibaria englemani*) and three unidentified species of fungi isolated from ripe *Piper* fruits. Results showed that amides have variable effects on insect feeding preferences and strong and consistent negative effects on fungal growth rates. A comparison of the bioactivity of unripe and ripe fruit extracts showed that the composition and relative concentration of compounds in unripe fruits provides a more effective defense against two of the three fungal species tested. In addition, tests of the bioactivity of two pure amides, presented alone and in combination, showed that the same

[5] This chapter represents a collaborative effort between S.R. Whitehead and M.D. Bowers and is currently in preparation for submission to *Functional Ecology*

two compounds can either function synergistically or antagonistically against different insect and fungal consumers. Together, these results suggest that the diversity of secondary metabolites in fruits may be a key characteristic contributing to fruit defense and seed dispersal success.

6.3 Introduction

Plants produce an enormous diversity of secondary metabolites that are thought to function primarily as a defense against herbivores and pathogens (Bennett and Wallsgrove 1994; Rosenthal and Berenbaum 1991). Decades of research have shown that the variation in chemical defense traits within and among individuals can have important and complex consequences for structuring ecological communities (reviewed in Coley and Barone 2001; Iason et al. 2012). However, most of our understanding of the ecology and evolution of plant defense has come from studies that examine the role of one or a few major compounds in leaves. We still know very little about the diversity and functional significance of secondary metabolites produced in other plant parts (Adler 2000; Harborne 2001; Tewksbury 2002). In particular, relatively few studies have examined the role of secondary metabolites in fleshy fruits (Levey et al. 2007; Tewksbury 2002). Because fleshy fruits function primarily to attract animal consumers, it is often assumed that secondary metabolites in these tissues are ecologically costly—their presence best explained as a pleiotropic consequence of the defense of leaves and other plant parts (Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998; Whitehead and Poveda 2011). Yet, plant defense theory suggests that there are also several reasons to predict that there should be strong independent selection for the defense of fleshy fruits: 1) they have high fitness value due to their direct link to developing seeds, 2) they are at high risk of attack due to their high nutritional content, and 3) they are often exposed to enemies over a long period of time during development

and post-ripening persistence (McKey 1974; McKey 1979; Rhoades and Cates 1976; Zangerl and Rutledge 1996).

Many fleshy fruits do contain high levels of deterrent or toxic secondary metabolites (Cipollini and Levey 1997b; Herrera 1982), in some cases exceeding the concentrations found in leaves (Whitehead and Bowers 2013). Although these metabolites may influence seed dispersal in a variety of ways (Cipollini and Levey 1997b), increasing evidence has suggested that their primary role is in the defense of fruits against antagonists, such as insect seed predators and fungal pathogens (Cazetta et al. 2008; Cipollini and Levey 1997a; Schaefer et al. 2008; Tewksbury et al. 2008b). The chemical defense of fleshy fruits may lead to fitness trade-offs, where fruits that are the most defended are also the least preferred by mutualist seed dispersers (Cipollini and Levey 1997b; Cipollini et al. 2004; Schaefer et al. 2003), or plants may minimize the negative effects of secondary metabolites on seed dispersal through a variety of mechanisms, such as the attraction of specialist consumers or the production of metabolites that are bioactive only against specific pests (Cipollini and Levey 1997b; Izhaki 2002; Struempf et al. 1999; Tewksbury et al. 2008b; Tsahar et al. 2003). Another important way that plants may minimize any negative effects of secondary metabolites on seed dispersal is through changes in fruit chemistry with ripening. These changes may include a reduction in the overall concentration of metabolites or an alteration in the chemical composition that specifically reduces deterrent effects on mutualists. Changes in fruit chemistry with ripening are common (e.g. Pearce et al. 1988; Tsahar et al. 2002; Whitehead and Bowers 2013), but little is known about how these changes affect seed dispersal or fruit defense. Because the bioactivity of secondary metabolites is likely to vary considerably between seed dispersers (mostly vertebrates) and fruit pests (mostly invertebrates and microbes), it may be possible for plants to “fine-tune” the chemical

composition of ripe fruits to minimize any deterrent effects on mutualists while maintaining effective chemical defense against antagonists. However, because most past work on fruit secondary metabolites has focused on testing the effects of a single compound on a particular consumer (reviewed in Levey et al. 2007), we still know very little about the chemical diversity of fruits, how the composition and relative concentrations of compounds change with ripening, and the potential for specificity in the bioactivity of fruit metabolites in interactions with seed dispersers and/or different classes of fruit pests.

In the few studies that have provided quantitative comparisons of secondary metabolites in wild fleshy fruits and leaves, evidence has shown that fruits can be highly diverse chemically and contain a variety of compounds that never occur in leaves (Whitehead and Bowers 2013; Chapter 4; Chapter 5). This chemical diversity may play a key role in fruit defense (Berenbaum and Zangerl 1996; Castellanos and Espinosa-García 1997). Although it is difficult to disentangle the effects of individual compounds when they occur in mixtures, correlative evidence has shown that different individual compounds from fruits may be effective against different classes of consumers (e.g. insects versus microbes) and that in some cases the most bioactive compounds in mixtures are minor components in terms of concentration (Chapter 4). Furthermore, when complex suites of compounds occur together, there is strong potential for synergistic or antagonistic interactions among individual compounds (Berenbaum 1985; Nelson and Kursar 1999). Increasing evidence has shown that synergy among defensive metabolites is a common and widespread occurrence and may play a key role in determining the outcome of species interactions (Berenbaum 1985; Berenbaum and Neal 1985; Dyer et al. 2003; Richards et al. 2010; Richards et al. 2012; Scott et al. 2002). However, despite the high diversity of

secondary metabolites in fruits, we know of no studies that have tested for synergistic effects of fruit secondary metabolites in either mutualistic or antagonistic fruit/frugivore interactions.

One group of plants that produces fruits with high concentrations and diverse mixtures of secondary metabolites is the tropical genus *Piper* (Morikawa et al. 2004; Siddiqui et al. 2005; Chapter 5). In particular, many *Piper* species are rich in amides, a group of secondary metabolites that have been shown to play an important ecological role in the defense of leaves against herbivores (Dyer et al. 2001; Dyer et al. 2004a; Fincher et al. 2008; Richards et al. 2010), but have not been examined in the context of fruit/frugivore interactions. In laboratory studies, amides have a broad range of bioactivity against insects, fungi, and molluscs (Bernard et al. 1995; da Silva et al. 2002; Marques et al. 2010; Morandim et al. 2010; Navickiene et al. 2003; Siddiqui et al. 2005; Yang et al. 2002), and thus may provide defense against a variety of fruit antagonists. In a recent study describing the amides in different plant parts of *Piper reticulatum,* a common and widespread Neotropical species, it was shown that the amide diversity was higher in fruits than in other plant parts and that the fruit chemical diversity, but not concentration, was negatively correlated with levels of seed damage in natural populations (Chapter 5). Together, these results suggest that amide diversity *per se* may be one of the most important aspects of the chemical defense of fruits in this species. Several past studies have shown that amides can function synergistically in leaf defense (Dyer et al. 2003; Richards et al. 2010; Scott et al. 2002), and, considering the complex mixtures of up to 25 individual amides detected in *P. reticulatum* fruits (Chapter 5), the potential for interactions among compounds is particularly high. In addition, because the fruits of *P. reticulatum* are attacked by a number of antagonistic frugivores and pathogens, complex suites of secondary metabolites could provide simultaneous defense against different consumers. These factors emphasize the need to conduct controlled experiments

with multiple species that interact with fruits and to consider the combined effects of naturally occurring suites of defensive compounds.

In this study, we conducted a series of experiments that addressed the role of amides in fruit defense of *P. reticulatum* and other *Piper* species. Because amides occur in complex mixtures, we focused on the bioactivity of fruit extracts that contain suites of compounds typical of plants in natural populations. We examined the effects of fruit extracts on two important classes of fruit antagonists, insects and fungi, and for both groups we compared the bioactivity of extracts from unripe and ripe fruits. In addition, to provide a more general test of the bioactivity of amides from the genus *Piper,* we conducted identical experiments with two pure amides that are common to many *Piper* species, piperine and piplartine. Although these two compounds do not occur in *P. reticulatum*, they are among the most commonly detected amides in the genus (Parmar et al. 1997) and are available commercially in pure form. The effects of the pure compounds were tested alone and in combination, which also allowed us to explicitly test for interactive effects between the two that may alter the effectiveness of fruit defense. Specifically, we addressed the following four questions:

Q1: Do amide-rich extracts from *P. reticulatum* fruits exhibit bioactivity against *Sibaria englemani* (Pentatomidae), a common insect seed predator on *Piper* species, and/or against three species of naturally-occurring fruit-associated fungi?

Q2: If so, how do the changes in amide profile that occur with fruit ripening influence the bioactivity against these different consumers?

Q3: Are these same consumers also deterred by two individual amides common to many other *Piper* species, piperine and piplartine?

Q4: Can piperine and piplartine function synergistically to increase bioactivity and therefore the effectiveness of defense against these consumers?

6.3 Methods

Study site and system

All sample collections and field experiments were conducted at La Selva Biological Station, Heredia Province, Costa Rica. La Selva is managed by the Organization for Tropical Studies and includes 1,600 hectares of protected area that consists of primary premontane and tropical wet forest (*sensu* Holdridge 1967), as well as secondary forest and abandoned agricultural areas (McDade et al. 1994). The site is a high center of diversity for *Piper,* with 50+ species co-occurring (Gentry 1990; OTS 2012).

The genus *Piper* is one of the most species-rich and dominant members of Neotropical forests, and includes small trees, shrubs, and vines (Dyer and Palmer 2004; Gentry 1990). Fruits are borne on distinct spike-shaped infructescences that can contain anywhere from 100 to 3,000 tiny individual fruits, each with a single seed (Fleming 1985; Greig 1993a). A small group of bats (*Carollia spp.*) in the family Phyllostomidae are the primary dispersers of *Piper* seeds in the Neotropics, although some species are also consumed by birds (Fleming 2004; Palmeirim et al. 1989; Thies and Kalko 2004). Fruits are also attacked heavily by insects; in a comparative study of six *Piper* species, up to 87% of seeds were lost to insect seed predators (Greig 1993a). A single hemipteran species (*Sibaria englemani*, Pentatomidae; Fig. 6.1) is by far the most abundant insect seed predator on *Piper* at the site (Greig 1993a; SRW personal observation). The impact of these insects on seed viability may be due to the direct effects of seed predation or the indirect effects of damage to the fruit surface that leads to increased risk of pathogen attack

(Tewksbury et al. 2008b). In *Piper,* pathogen risk appears to increase sharply upon ripening, as evidenced by the long maturation time of fruits (~one month) and relatively short period of time (~24 hours) that ripe fruits persist before they begin to rot (Thies and Kalko 2004; personal observation).

Piper reticulatum (Fig. 6.1) is a small understory tree 3-7m in height that occurs throughout Central and South America as far south as Bolivia (Tropicos 2012). It is one of the most common species of *Piper* found in secondary forest and along trails at La Selva, and fruits ripen in several distinct waves throughout the year, generally in October/November and again in

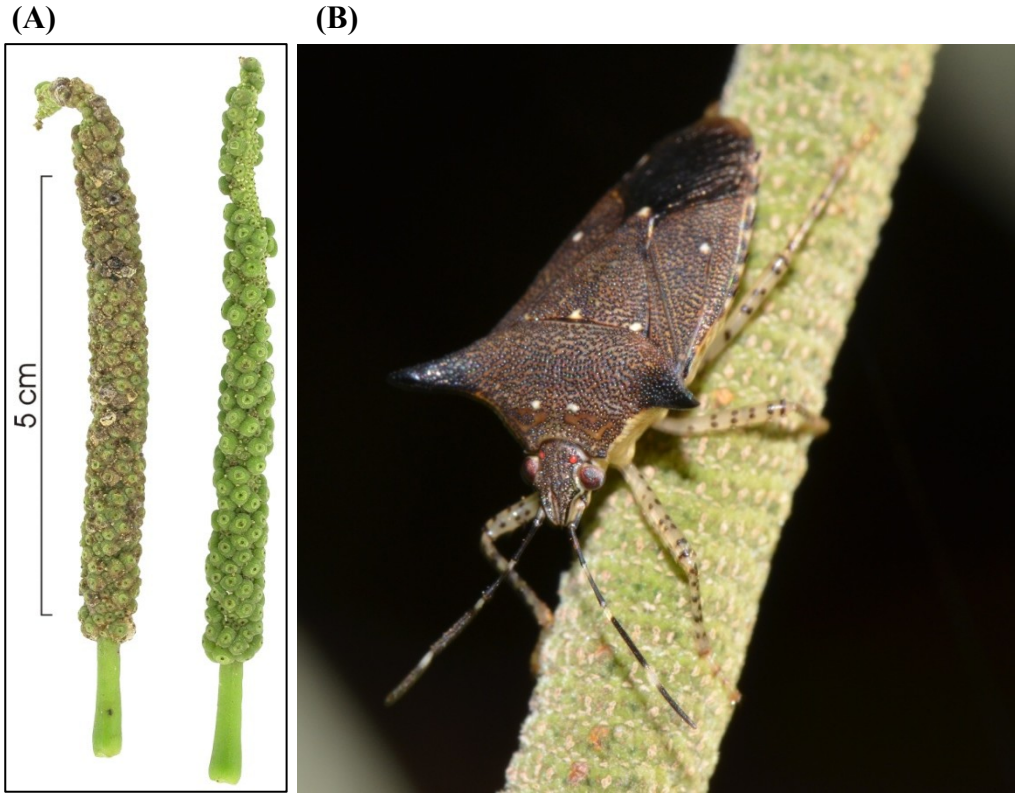


Figure 6.1: Mature and rotting infructescences of *Piper reticulatum* (A) and *Sibaria englemani* feeding on an immature infructescence of *Piper sancti-felicis* (B). Note that the individual fruits of *P. sancti-felicis* are much smaller than those of *P. reticulatum,* and are tightly packed together on the infructescence. Photo credits: Steven Paton, Smithsonian Tropical Research Institute (A) and Susan Whitehead (B).

July/August (SRW, unpublished data). Unripe fruits are heavily attacked by *Sibaria englemani* and a variety of other insects and fungal pathogens (SRW, personal observation). Mature trees can produce hundreds of infructescences that ripen sequentially, with anywhere from 1-20 infructescences maturing per day over a period of several weeks. Bats remove entire infructescences in flight, usually on the first evening that they are ripe. Those that are not removed on the first night are visibly beginning to rot on the following day and usually fall to the ground within 24 hours (SRW, personal observation).

Piper species are rich in a broad range of amides, phenylpropanoids, lignans, terpenes, benzoic acids, chromenes, alkenylphenols, and steroids (Dyer et al. 2004b; Kato and Furlan 2007; Parmar et al. 1997). Amides in particular are abundant in this genus, and have known ecological and economic importance (e.g. the amide piperine is responsible for the spiciness of black peppercorns, which are the dried fruits of *Piper nigrum*; Parmar et al. 1997). They are especially diverse in *P. reticulatum*, where we detected a combined total of 40 individual amides across different plant parts (Chapter 5).

Extractions of amides from P. reticulatum fruits

Large numbers of fruits were collected in bulk from 10-15 individuals of *P. reticulatum* growing along trails and in open areas surrounding the field station. Fruits were separated into ripe and unripe and brought immediately back to the La Selva laboratory, where they were dried at 50^{o}C for 48 hours and ground to a fine powder in a coffee grinder. To prepare large-scale extracts for bioassays, we used a scaled-up version of extraction and quantification procedures as described in Dyer et al. (2004b). For each fruit type (unripe or ripe), 52.5g of dry material was placed in a 1L Erlenmeyer flask with 500mL ethanol and left on a stir-plate for overnight

extraction. The ethanol was then decanted through a Buchner funnel with #2 Whatman filter paper, and another 500mL of ethanol was added to the plant material for a second overnight extraction. This process was repeated again for a third overnight extraction. The three filtered extracts were combined, evaporated to dryness, and then re-suspended in 250 mL 3:1 water:ethanol. This solution was transferred to a separatory funnel and partitioned three times against equal volumes of chloroform. The water fraction was discarded and the combined chloroform fractions (containing the amides) were evaporated to dryness. The resulting extracts were re-suspended in 52.5mL ethanol and partitioned among seven scintillation vials for use in the bioassays described below. This extraction procedure results in an extract that contains approximately 78-84% amides (see below for quantification methods). Small aliquots of 500 µL from each extract were evaporated to dryness and transported to the University of Colorado for analysis using gas chromatography combined with mass spectrometry (GC-MS).

Identification and quantification of amides in fruit extracts using GC-MS

Methods for GC-MS analysis were modified from previously described methods (Dyer et al. 2004b) and are described in detail in Chapter 5. We re-suspended the extract aliquots described above in 1mL dichloromethane and removed 100 µL to micro-inserts for GC vials. Piperine (which does not occur in *P. reticulatum*) was added as an internal standard at a concentration of 0.75 mg/mL and 1µL from each sample was then injected onto an HP Agilent 6890N GC coupled with an Agilent 5975C MS. The instrument was equipped with a DB-5MS capillary column (30m x 0.25mm i.d., 0.5 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) and the ion source was set at 70eV at 230^{o}C. Ultra-pure He was used as a carrier gas at a flow rate of 1.5 ml min^{-1}, a split flow ratio of 30:1, and a front inlet temperature of 280^{o}C.

The following oven conditions were employed: initial temperature 50^{o}C, initial hold time 1 min; ramp 1: 15^{o}C min^{-1} to 275^{o}C, hold time 5 min; ramp 2: 20^{o}C min^{-1} to 320^{o}C, hold time 10 min; for a total run time of 33.25 minutes. Data were recorded and processed using MSD ChemStation software (version D.02.00.275). We estimated the quantities of individual and total amides in the extracts based on the known concentration of the internal standard (piperine). Compounds were identified based on matches of retention times and mass spectral data in a user-created library for amides in *P. reticulatum.* Full structural elucidation of major amide components in this species was carried out in a previous study (Chapter 5).

Q1 and Q2: Effects of P. reticulatum extracts on insects and fungi

To determine whether *P. reticulatum* extracts can affect feeding behavior of the insect seed predator, *S. englemani*, we conducted a series of paired choice experiments from 2011-12 that tested the effects of *P. reticulatum* fruit extracts on insect preference. The extracts were added to unripe fruits of *Piper sancti-felicis*, another commonly occurring species of *Piper* at the field site. Unripe *P. sancti-felicis* fruits are commonly consumed by *S. englemani* (SRW personal observation), but contain no detectable amides at a detection limit of approximately 0.01% dry weight (SRW unpublished data). We prepared serial dilutions of the *P. reticulatum* extracts dissolved in ethanol, where each successive dilution was 90% strength of the preceding solution. The unripe fruit extract had a starting total amide concentration of 17.9 mg/ml and the ripe fruit extract had a starting concentration of 14.75 mg/ml (see results), and we prepared 50 dilutions of each, thus the dilutions ranged from 0.10 to 17.9 mg/ml for unripe extracts and 0.085 to 14.75 mg/ml for ripe extracts. Infructescences of *P. sancti-felicis* were cut in half, and the halves were randomly assigned to treatment and control groups. The treatment halves were supplemented

with a single concentration of extract by pipetting small aliquots of 200 μL of solution into a 150mm glass petri dish and rolling the infructescences in the dish until all of the solution was absorbed by the fruit and the surface was evenly coated. Controls were treated in the same manner with ethanol only, and both halves were then left for several hours to allow evaporation of the solvent. The paired treatment and control halves from a single infructescence were then placed on opposite sides of a clean 100mm petri dish.

All *S. englemani* individuals used in the study were collected opportunistically from *P. sancti-felicis* plants growing near the field station, and included a mix of adults and juveniles. Voucher specimens of the species were deposited at the University of Colorado Natural History Museum and their identity was confirmed by Donald B. Thomas (USDA Research Entomologist). Insects were held in vials for 8-16 hours after collection and prior to beginning the feeding trials. To begin the experiment, a single individual was placed in the center of a petri dish between the treatment and control fruits, and the dish was monitored every ten minutes for a period of two hours, and every hour thereafter for a total of 24 hours. Insects that did not feed after 24 hours were scored as "no-choice" and excluded from the data analysis. A choice was recorded as soon as an insect was observed feeding, i.e. with its stylet fully inserted into the fruit. Insects were often observed walking on fruits and probing fruits with their mouthparts prior to initiating feeding; however, we did not record these behaviors as a choice. We conducted two sets of trials (each set involving one trial at each of the 50 concentrations) for each extract treatment (ripe or unripe), using a naïve individual of *S. englemani* and a fresh *P. sancti-felicis* infructescence for each trial. Because some of the insects did not feed within the 24-hour period and had to be excluded from analysis, sample sizes varied among treatments (see results).

To investigate the effects of amides on fruit-associated fungi, we conducted a series of bioassays using three strains of fungi isolated from field-collected ripe *P. reticulatum* fruits. Fruits were collected from 10 individual plants, surface-sterilized using a 3% bleach solution, cut in half with a sterile blade, and placed pulp side down on an agar growth medium. This procedure was conducted in sterile conditions at the La Selva laboratory using a UV-sterilized laminar flow hood. The agar medium was prepared to mimic the nutrient composition of *Piper* fruit (Kelm et al. 2008), following methods in Cipollini and Stiles (1993). In 250 mL deionized water, we added 5.0g agar, 1.25g soy protein powder, 1.91g fructose, 1.73g glucose, 0.38g oil (1:1 corn oil:peanut oil), 0.88g cellulose, and 0.29g pectin. We sorted the resulting fungal cultures by morphology and chose three commonly occurring morpho-types for use in bioassays (denoted as F1, F2, and F3), all of which had distinct, radial hyphal growth for ease of comparative measurement.

For each fungal strain, we tested the effects of unripe and ripe fruit extracts by adding extracts to the agar growth medium at 50 different concentrations using the same serial dilutions described above for the insect bioassays. Solutions were added to the fruit-mimic agar in 60mm petri plates by pipetting 200 μL aliquots over the surface of the agar, spreading evenly using a sterile glass rod, and allowing the plate to stand open under a sterile laminar flow hood for one hour to allow evaporation of the ethanol. Control plates were also prepared for each fungal species using ethanol only. Plates were then inoculated with fungi by placing small (1cm x 1cm) agar plugs from the pure cultures in the center of the plate. All plates were then closed with parafilm and placed in an incubator at 28^{o}C for a period of 24-36 hours depending on the fungal strain. For each fungal species, the radial growth of hyphae was measured in three locations on

the plate using calipers, and the three measurements were averaged to obtain one measurement of hyphal growth for each treatment at each concentration.

Q3 and Q4: Effects of piperine and piplartine on insects and fungi

We tested the effects of two commercially available pure amides, piperine (purchased from Sigma-Aldrich) and piplartine (purchased from Indofine Chemical Company) on *S. englemani* and the same three fungal species isolated for the experiments with *P. reticulatum* extracts. Although piperine and piplartine do not occur in *P. reticulatum* (Whitehead et al. in prep), both have been isolated from fruits and leaves of a number of *Piper* species (Dyer et al. 2004b; Parmar et al. 1997), and often occur together in the same plant (Parmar et al. 1997). We prepared serial dilutions of 50 concentrations for each compound in the same manner as above, but with a starting concentration of 10 mg/ml and ranging to a low concentration of 0.01 mg/ml. To test for potential synergy or antagonism between the two compounds, we also prepared a serial dilution from a combination solution that contained the two compounds in a 1:1 ratio (5mg/ml piperine and 5 mg/ml piplartine). Using these solutions in place of the extracts, we conducted bioassays with *S. englemani* and the three species of fungi in the same manner as above.

Statistical analyses

To test whether amide extracts and/or pure compounds are bioactive against insects and/or fungi (Q1 and Q3), we fit dose-response curves to the data from each bioassay experiment using base functions and the package 'drc' in the R environment for statistical computing (R Development Core Team 2012; Ritz and Strebig 2012; Tallarida 2000). For insect bioassays, we

used a two-parameter logistic regression model with insect choice (0=control, 1=treatment) as the response variable and the amount of amides added to the treatment fruit as the predictor variable. This was carried out with the 'glm' function in the R base package, using the binomial distribution and the logit link function. Amide amounts were base-10 log transformed prior to analysis. We used the 'logi.hist.plot' function in the package 'popbio' to visualize these data (Stubben et al. 2012; Fig. 6.4). For fungal bioassays, we used a three parameter log-logistic model of the form:

$$f\ (x) = 0 + \frac{d - 0}{1 + \exp(b\big(\log(x) - \log(e)\big))}$$

which describes a sigmoidal curve where the lower limit of the curve is fixed at zero, *d*=the upper limit of the curve, *e*=the inflection point of the curve, and *b*=the relative slope around *e*. This was carried out using the 'drm' function in the package 'drc'(Ritz and Strebig 2012; Tallarida 2000). Because we conducted the bioassays simultaneously and in identical laboratory conditions for the fruit extracts and then for the pure compounds for each fungal species, we fit the dose-response curves simultaneously for fruit extracts and then for pure compounds and specified a common parameter estimate for the upper limit of the curve (i.e. the estimated maximum growth of a particular fungal species on control plates [amide dose=0] was the same across amide treatments). For both the insect and fungal assay data, we assessed the goodness of fit of the regression models using Hosmer-Lemeshow tests and ensured that observed values were not significantly different from predicted ($P > 0.05$) (Tallarida 2000). We then examined the regression coefficients (β) and associated P-values, and slopes that differed significantly from zero were taken as evidence for an effect of amide treatment.

To test for differences in the effectiveness of unripe fruit extracts versus ripe fruit extracts in fruit defense (Q2) we employed a common metric that is used to compare the efficacy of two drugs in pharmacology studies: the potency ratio *R* (Tallarida 2000). Although we have found no examples of this method being implemented in chemical ecology research, it provides a number of advantages over ANOVA or other methods based on linear models because it does not depend on the distribution of data (binomial, normal, Poisson), makes no assumptions about the shape of the compounds' dose-response curves (which are rarely linear), and yields a value that depends only on the relative efficacies of the compounds being tested, independent of the effects of concentration (Tallarida 2000). To calculate *R*, we first used the regression models described above to determine the effective dose (ED) of the extracts necessary to reduce insect preference or fungal growth by 50% from the level expected based on no effect, commonly referred to as ED50 values in toxicology and pharmacology studies. For fungi, the ED50 value was estimated directly as a model parameter, *e*, which is the inflection point about which the curve is symmetric and the point where fungal growth is reduced by half relative to controls. For the insect bioassays, the baseline expectation based on no effect was that insects would choose control fruits 50% of the time and treatment fruits 50% of the time, thus the ED50 value represented the dose where insects chose treatment fruits 25% of the time. This value was calculated using inverse prediction of the dependent variable using the model generated by the logistic regression. Once the ED50 values were determined, we then calculated the potency ratio as $R = (A/B)$, where *A* and *B* represent the ED50 values for extracts A and B. Values of $R > 1$ indicate that A is more potent (i.e. has a stronger negative effect on fungal growth), values of $R < 1$ indicate that B is more potent. Methods for calculating the standard error and confidence interval for this metric are described in detail in Tallarida (2000). Because we used the estimated total quantities of

amides in the unripe and ripe fruit extracts to describe the dose-response curves for each, a value of R significantly different from 1 indicates that there are differences in the effectiveness of unripe and ripe fruit extracts that are not explained by differences in the total concentration of amides, but rather are due to changes in the composition or relative concentrations of compounds with ripening.

To test for the presence of synergistic or antagonistic interactions between piperine and piplartine (Q4), we used another metric that is common in pharmacology and toxicology research: the interaction index γ (Tallarida 2000; Tallarida 2002). This method is also rarely implemented in chemical ecology studies (but see Richards et al. 2012), despite the fact that it's potential utility for providing rigorous tests of interactions among plant defense compounds has been discussed in detail (Nelson and Kursar 1999). The interaction index is calculated as $\gamma = Z/(p_A A + p_B B)$, where Z is the ED50 value (or any other specified level of response) for the mixture and the denominator $(p_A A + p_B B)$ represents the expected ED50 based on an additive relationship between two compounds. A and B represent the ED50 values for compounds A and B when tested individually, and p_A and p_B represent the proportions of the two compounds in the mixture (in our case p_A = 0.5 and p_B = 0.5). Values of $\gamma < 1$ indicate synergy and values of $\gamma > 1$ indicate antagonism between the two compounds. Methods for calculating the standard error and confidence limits for this metric are described in detail in Tallarida (2000).

6.4 Results

Amides in P. reticulatum extracts

Extracts of both unripe and ripe fruits of *P. reticulatum* contained relatively large amounts of amides, the most abundant of which were dihydrowisanidine and methoxy

dihyrotricholein (Fig. 6.2). These and eight other compounds were identified based on MS and NMR data in a previous study (Chapter 5). An additional 11 compounds were also detected in extracts and classified as amides based on characteristic fragmentation patters in MS data as described in Chapter 5, but were unidentified (Fig. 6.2). Total quantities of amides were estimated as 1.79% dry weight for unripe fruits and 1.48% dry weight for ripe fruits. For individual compounds, most amides had higher estimated concentrations in unripe fruits than in ripe, with the exception of dihydrowisanidine and amide AA (Fig. 6.2), which had higher concentrations in ripe fruits than unripe.

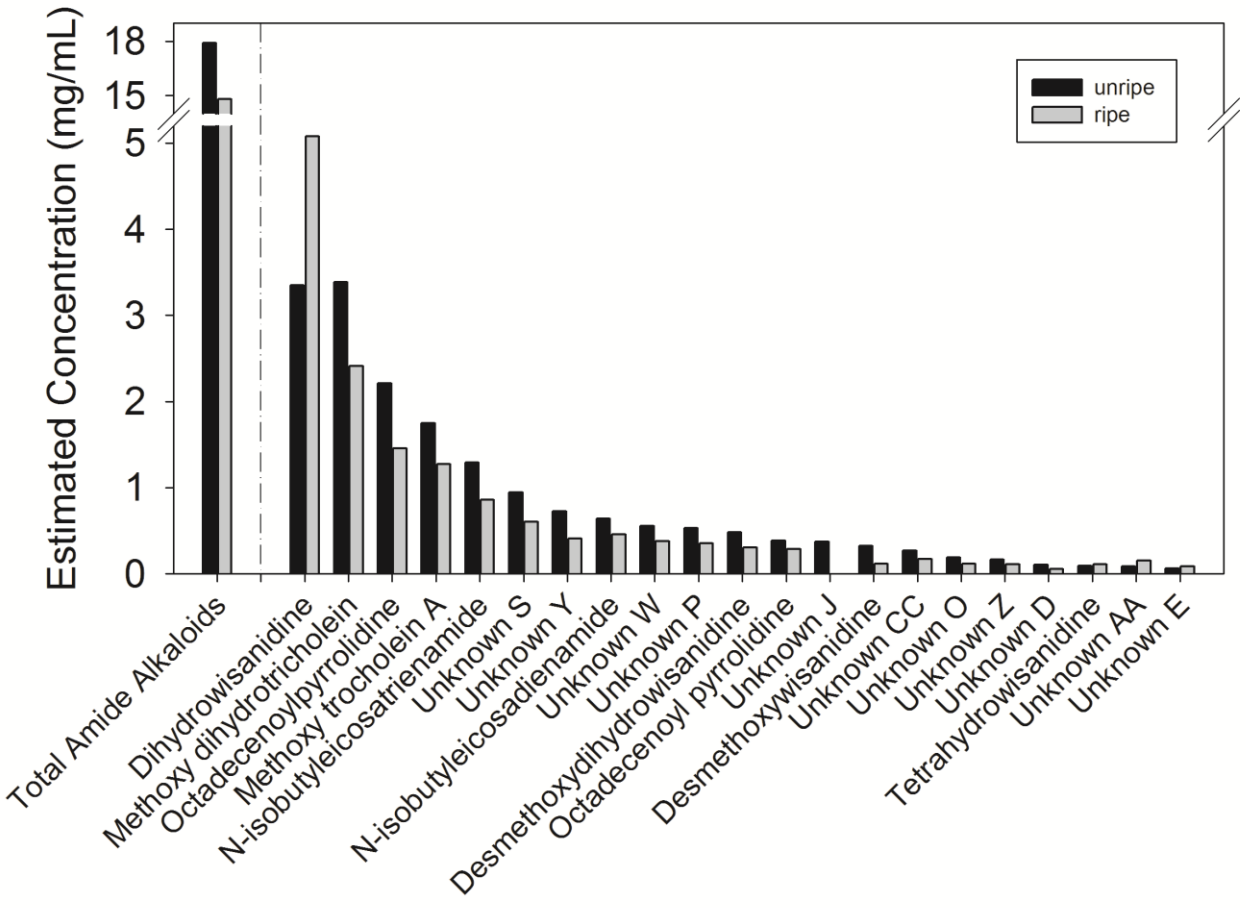


Figure 6.2: Estimated concentrations of total amides and of individual compounds in unripe and ripe fruit extracts from *P. reticulatum.*

Q1 and Q2: Effects of P. reticulatum extracts on insects and fungi

Insect feeding preference (control versus treatment) was not affected by the concentration of unripe (P = 0.30; Table 6.1) or ripe fruit extracts (P = 0.95; Table 6.1) applied to the treatment fruit, and thus we did not test for differences between the two extracts. In contrast, the growth of all three fungal species was negatively affected by both unripe and ripe fruit extracts (P < 0.0001 for all analyses; Table 6.2). For two out of three fungal species, unripe fruit extracts were significantly more effective at reducing growth than the ripe fruit extracts (Table 6.3; Fig. 6.3). Specifically, the chemical profile of unripe extracts was over four times more potent against fungus F1 (R = 4.81; Table 6.3; Fig. 6.3) and over 12 times more potent against fungus F3 (R = 12.82; Table 6.3; Fig. 6.3). There were no differences in potency for fungus F2 (Table 6.3; Fig. 6.3).

Q3 and Q4: Effects of piperine and piplartine on insects and fungi

Insect feeding preference was reduced by piperine and by the combination of piperine and piplartine, but was not affected by piplartine alone (Table 6.1; Fig. 6.4). Specifically, as the concentration of piperine doubled, the odds of the insects choosing the treatment fruit decreased by a factor of 0.50 (Odds Ratio = 0.50; P = 0.028; Table 6.1). The combination of piperine and piplartine also reduced insect preference (Odds Ratio = 0.54; P = 0.041; Table 6.1), but there was no effect of piplartine when tested alone (Odds Ratio = 0.84, P = 0.53). In the test for interactive effects of piperine and piplartine in combination, we found evidence for an antagonistic relationship between the two compounds, where the effect of the combination was less than expected based on additive effects (α = 3.38; Table 6.4).

Table 6.1: Logistic regression results showing effects of amides on insect feeding preferences

Amide Treatment	β[1]	OR[2]	z	P	
unripe extract	-0.39	0.67	-1.03	0.30	
ripe extract	-0.03	0.97	-0.07	0.95	
piperine	-0.69	0.50	-2.19	**0.028**	*
piplartine	-0.16	0.85	-0.63	0.53	
combination	-0.61	0.54	-2.04	**0.041**	*

[1] Regression coefficient
[2] Odds Ratio, represents the factor by which the odds of insects choosing treatment fruits decreases with each unit increase in concentration

Table 6.2: Results from a log-logistic regression showing strong effects of amides on fungal growth

	Fungal Species: R3			Fungal Species: R4			Fungal Species: R6		
Amide Treatment	β[1]	t-stat	*P*	β[1]	t-stat	*P*	β[1]	t-stat	*P*
unripe extract	0.64	8.3	**<0.0001*** **	0.47	10.67	**<0.0001*** **	0.47	8.87	**<0.0001*** **
ripe extract	1.85	5.14	**<0.0001*** **	0.46	10.02	**<0.0001*** **	0.4	5.18	**<0.0001*** **
piperine	1.64	3.87	**<0.0001*** **	0.74	6.78	**<0.0001*** **	0.76	4.03	**<0.0001*** **
piplartine	0.44	5.51	**<0.0001*** **	1.49	7.22	**<0.0001*** **	1.13	7.49	**<0.0001*** **
combination	1.55	5.44	**<0.0001*** **	0.54	9.79	**<0.0001*** **	0.73	6.18	**<0.0001*** **

[1] Regression coefficient for slope of dose-response curve

Table 6.3: Relative Potency Ratios for Unripe and Ripe Fruit Extracts

Fungal Species	ED50[1] (mg) unripe	ED50[1] (mg) ripe	Relative Potency (*R*)[2]	95% CI for R	
R3	0.27	1.30	4.81	**2.81 – 6.82**	*
R4	0.86	0.88	1.02	0.45 - 1.58	
R6	2.46	31.55	12.82	**2.94 - 22.70**	*

[1]ED50 values represent the dose at which insect preference or fungal growth is reduced by 50%, see text for details
[2]Values of $R < 1$ indicate ripe extracts are more potent, and values of $R > 1$ indicate unripe extracts are more potent

Table 6.4: Interaction indices for piperine and piplartine

Species	ED50[1] (mg) piperine	ED50[1] (mg) piplartine	ED50[1] (mg) combination	Interaction Index (α)[2]	95% CI for α	
Sibaria englemani	1.27	~	8.61	3.38	**1.12 - 5.63**	*
R3	0.8	0.1	0.78	1.72	**1.13 - 2.32**	*
R4	1.79	1.56	0.62	0.36	**-0.02 - 0.76**	*
R6	4.72	1.48	1.69	0.55	-1.21 - 2.31	

[1]ED50 values represent the dose at which insect preference or fungal growth is reduced by 50%, see text for details
[2]Values of $\alpha < 1$ indicate synergy, and values of $\alpha > 1$ indicate antagonism

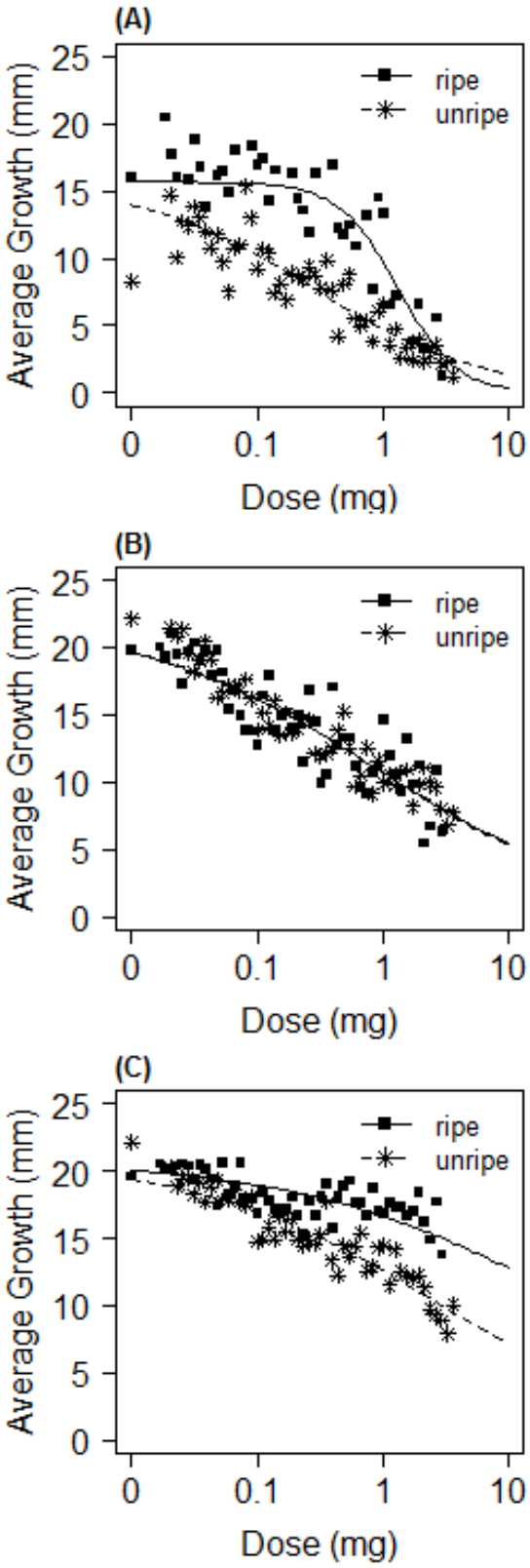


Figure 6.3: Effects of *P. reticulatum* fruit extracts on the growth of fungus F1 (A), F2 (B), and F3 (C). For (A) and (C), the suites of compounds in unripe extracts were significantly more effective in reducing fungal growth than the ripe extracts.

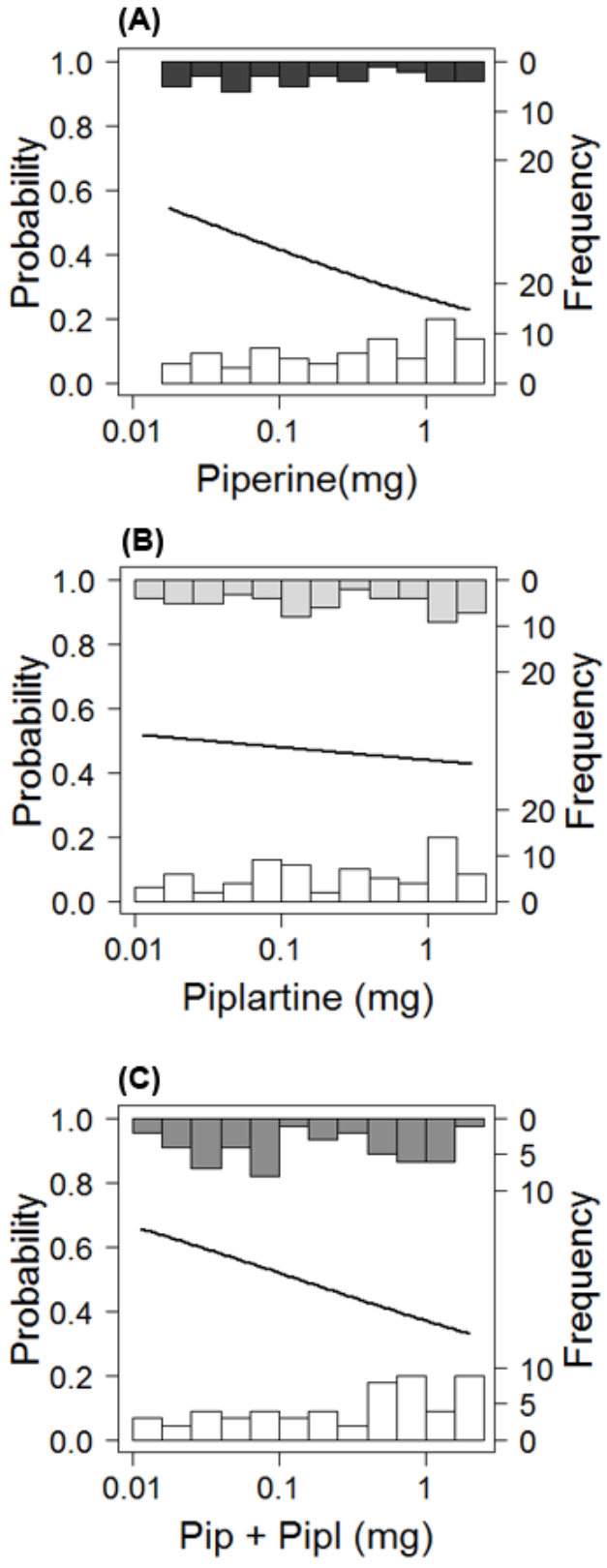


Figure 6.4: Effects of piperine (A), piplartine (B), and the combination (C), on the feeding preferences of *Sibaria englemani,* a hemipteran seed predator. Bars show frequency distributions for the number of insects choosing treatment fruits (gray bars, top) and control fruits (white bars, bottom) at each concentration. Lines represent model predictions from a logistic regression showing the probability that insects will choose control (0) or treatment (1) fruits at varying concentrations.

The growth of all three fungal species was strongly reduced by all pure compound treatments, including piperine, piplartine, and the combination ($P < 0.0001$ for all analyses; Table 6.2; Fig. 6.5). For fungi, the evidence for interaction between the two compounds was variable. For F1, we found evidence for an antagonistic interaction between piperine and piplartine, where the combination was less effective than expected based on additive effects ($\alpha = 1.72$; Table 6.4, Fig. 6.5). For F2, we found evidence for a synergistic interaction, where the combination was more effective than expected based on additive effects ($\alpha = 0.36$; Table 6.4, Fig. 6.5). There was no evidence of interaction between the two compounds for F3 (Table 6.4).

6.5 Discussion

The defensive function of plant secondary metabolites has been studied primarily in leaves, but increasing evidence has shown that secondary metabolites also have important ecological roles in fruits (Levey et al. 2007; Chapter 4). This study adds an important new component to our understanding of how fruit secondary metabolites influence interactions with the broad range of antagonists that attack fruit tissues. It represents the first examination of amides in fruit-frugivore interactions, and the first examination of fruit defense in a bat-dispersed species. Our results provide evidence that amides play an important role in the defense of *Piper* fruits against antagonist consumers, but the specific effects were variable depending on the combination of amide treatment and consumer tested. Fruit extracts did not affect insect preferences, but strongly reduced fungal growth (Fig. 6.3), and the suites of compounds in unripe fruits had stronger anti-fungal effects than those in ripe fruits (Table 6.3). Two amides common to many *Piper* species, piperine and piplartine, had variable

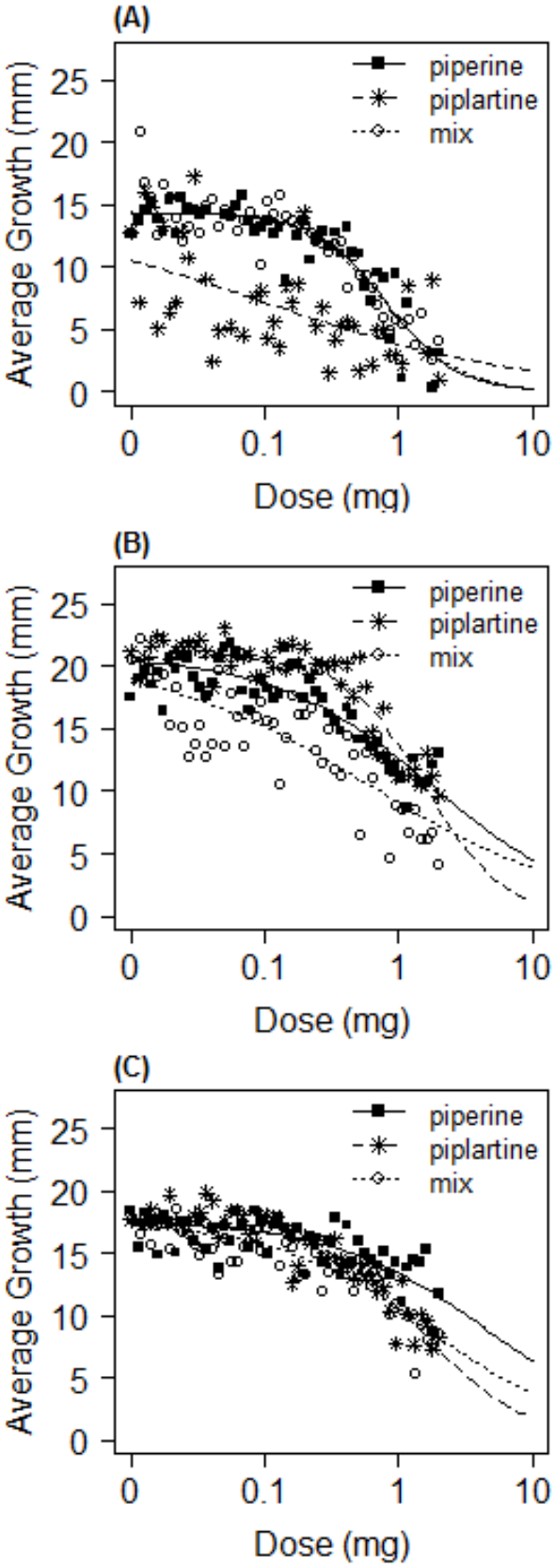


Figure 6.5: Effects of piperine, piplartine, and the combination on the growth of fungus R3 (A), R4 (B), and R6 (C). For (A) there was an antagonistic interaction between piperine and piplartine, and for (B) there was a synergistic interaction between piperine and piplartine.

effects on insect preferences and also strongly reduced fungal growth (Fig. 6.4, 6.5). Interestingly, we found that these two compounds can either interact antagonistically or synergistically depending on the consumer involved (Table 6.4). Together, our results suggest that the diverse suites of metabolites found in *Piper* fruits likely have complex adaptive roles in defense, especially against fungi, and that the effects of compounds in mixtures cannot be explained by simple additive models.

For a specialist insect seed predator, *Sibaria englemani*, we found that only one individual amide, piperine, appeared to reduce feeding preference. However, even for piperine, the effects were not absolute—at the highest concentrations tested a small percentage of insects still successfully fed on treated fruits (Fig. 6.4). We did not measure insect performance in this study, and it is possible that even though amides do not affect preference, they may reduce insect growth rates, survival, and/or reproductive output. Alternatively, *S. englemani* may have the ability to avoid, tolerate or detoxify amides, allowing it to successfully specialize on *Piper* fruits. We have observed this species on at least 10 *Piper* species that co-occur at the study site, and at least four other *Piper* species are reported as host plants in the literature (Greig 1993a). The chemistry of the fruits from these different *Piper* hosts is highly diverse and includes amides, alkenylphenols, phenylpropanoids, and terpenes (Glassmire et al. in prep; Parmar et al. 1997; SRW unpublished data), suggesting that *S. englemani* is able to tolerate amides as well as various other classes of compounds.

In contrast to the variable effects of amides on insects, there were universally negative effects of amides on fungal growth. These results corroborate past work suggesting that defense against fungal pathogens may be one of the most important adaptive benefits of secondary metabolites in fruits (Cazetta et al. 2008; Cipollini and Levey 1997a; Herrera 1982; Schaefer et

al. 2008; Tewksbury et al. 2008b). Metabolites that reduce fungal growth may benefit plants directly by protecting seeds from rot or damage that can reduce viability, or indirectly by increasing the persistence time of fruits and their attractiveness to mutualist seed dispersers (Cipollini and Stiles 1993; Herrera 1982). Although we cannot be certain that the specific fungal species we isolated from fruits are important as pathogens (many fungi that occur on plants do not have negative effects on plant fitness; Rodriguez et al. 2009), the fact that there were universal negative effects across three fungal species suggests that amides are likely effective against a broad range of pathogenic fungal species.

Our results showing differences in the effectiveness of unripe and ripe fruit compound mixtures for two of the three fungal species (Fig. 6.3) also suggest there are important interactive effects of mixtures and/or differences in the relative toxicity of different compounds. Although the qualitative composition of major compounds was similar between unripe and ripe fruit extracts, the relative abundances of compounds differed (Fig. 6.2). In particular, unripe fruits had a lower proportion of dihydrowisanidine and a higher proportion of methoxy dihydrotricholein, octadecenoylpyrrolidine, methoxy tricholein A, and N-isobutyleicosadienamide compared to ripe fruits. Because even closely related compounds can vary greatly in their biological activity (e.g. Gbewonyo et al. 2006; Pandey et al. 2013), it is likely that the chemical changes that occur with ripening have important consequences for fruit defense. The changes in relative abundances in *P. reticulatum* fruits occur concurrently with a reduction in the total concentrations of compounds, thus these combined effects likely indicate that ripe fruits are much more susceptible to attack than unripe. This is apparent in natural populations of *P. reticulatum*, where unripe fruits are often persistent on the plant over a period of development that can last for a month or more, while ripe fruits succumb to rot within 24-48 hours of maturation. However, because the large

majority of fruits are removed by seed dispersing bats on the same night of ripening, a short persistence time once ripe may not have any negative fitness consequences in this species. Rather, plants may maximize fitness by reducing the concentrations of compounds that could have negative effects on the feeding preferences of mutualists during the final period of ripening.

For both insects and fungi, there was evidence that the effects of combinations of compounds cannot be explained by a simple additive model. Notably, our results show that the same two compounds (piperine and piplartine) can function either synergistically or antagonistically depending on the target organism. While the potential for interactions among plant defensive compounds has received increasing interest over the last decade (Gershenzon et al. 2012), there are still only a limited number of studies that have provided empirical evidence for synergistic interactions, and we know of only one previous ecological study that has reported antagonistic interactions (Diawara et al. 1993). This may be in part due to the limited number of ecological studies that have used rigorous methods for detecting and analyzing compound interactions (Nelson and Kursar 1999). Considering the enormous diversity of compounds that occur in plants (Wink 2010), an appreciation for the fundamental role of compound synergy and/or antagonism in determining the outcome of species interactions may provide important new insights into the ecology and evolution of plant defense. Our results showing different interactive effects of the same two compounds on different organisms emphasizes the need for integrative approaches to understanding the costs and benefits of suites of secondary metabolites in the diversity of interactions in which plants are involved.

Fruit secondary metabolites play a key role in the defense of fruits against a variety of antagonistic consumers and therefore may be more important determinants of plant fitness than is generally appreciated. In this study, large differences in the bioactivity of unripe extracts and

ripe extracts due to small changes in the relative concentrations of compounds, combined with evidence for interactions between two individual compounds, emphasize the potential importance of chemical diversity and composition in fruits in the efficacy of defense. In addition, large differences in the bioactivity of amide mixtures against different consumers suggest that the importance of fruit secondary metabolite diversity cannot be understood based on simple tests of the effects of particular compounds on particular organisms. Future work should focus on understanding the complex costs and benefits of suites of fruit secondary metabolites in interactions with different classes of fruit pests as well as mutualistic seed dispersers. This integrative approach could provide important new insights that can improve theories of both seed dispersal and plant defense.

CHAPTER SEVEN

CHEMICAL TRADE-OFFS IN SEED DISPERSAL: DEFENSIVE METABOLITES IN *Piper* FRUITS DETER CONSUMPTION BY *Carollia* FRUIT BATS [6]

7.1 Abstract

Secondary metabolites play an important role in the defense of plants against antagonistic consumers, but may also be costly if they reduce the attractiveness of reproductive structures to mutualists, such as pollinators and seed dispersers. Fleshy fruits, which function primarily to attract seed dispersers, can sometimes contain high concentrations of secondary metabolites, but the effects of these compounds on seed dispersal are still poorly understood. Some past work has suggested that plants may experience a trade-off between fruit defense and the attraction of seed dispersers, but other evidence has suggested that the bioactivity of fruit secondary metabolites is directed primarily at invertebrate and microbial antagonists and has minimal or neutral effects on seed-dispersing vertebrates. We provide the first test of these alternative hypotheses in interactions between plants and seed-dispersing bats, adding an important new component to our understanding of the role of secondary metabolites in seed dispersal. We tested the effects of two common amides that occur in members of the plant genus *Piper* (Piperaceae), piperine and piplartine, in interactions with three co-occurring neotropical species of *Carollia* bats (Phyllostomidae). Both piperine and piplartine altered the fruit removal and fruit consumption behavior of bats, but the effects varied considerably among the three species of *Carollia* and among the specific compounds tested. Some bat species tended to be more deterred from removing amide treated fruits and others tending to be more deterred from fully consuming

[6] This chapter represents a collaborative effort among S.R. Whitehead, M.F. Obando-Quesada, and M.D. Bowers and is currently in preparation for submission to *Oecologia*

amide treated fruits once they had been removed. Furthermore, tests of piperine and piplartine presented alone and in combination provided evidence that the two compounds can have non-additive effects, in one case leading to a qualitative change in the effects of the metabolites from functioning as a deterrent to functioning as an attractant. Overall, our results support the hypothesis that plants experience a trade-off between seed dispersal and fruit defense, but the strength of this trade-off and the overall fitness consequences likely depend strongly on ecological context.

7.2 Introduction

The primary function of ripe, fleshy fruits is to attract mutualistic animal consumers, who contribute to plant reproductive success by dispersing seeds to new sites (van der Pijl 1982). The evolutionary history between plants and mutualist seed dispersers has led to suites of fruit traits that include both attractants (e.g. colors, odors) and nutritional rewards (e.g. proteins, lipids, sugars). However, ripe, fleshy fruits may also contain potentially deterrent or toxic secondary metabolites (Herrera 1982; Levey et al. 2007), in some cases at high levels of diversity and concentration relative to leaves and other plant parts (Whitehead and Bowers 2013; Chapter 5). Decades of research have shown that secondary metabolites play a key role in the defense of leaves against herbivores and pathogens (Bennett and Wallsgrove 1994; Iason et al. 2012), and the few studies that have examined their role in fleshy fruits have shown that these compounds can also defend fruits against invertebrate and microbial pests (Cipollini and Levey 1997a; Cipollini and Stiles 1992; Izhaki 2002; Tewksbury et al. 2008b; Chapter 4, Chapter 6). However, because secondary metabolites could also affect interactions with mutualists, understanding the overall fitness outcomes of secondary metabolites in fruits requires a broad

view of their possible consequences for seed dispersal success.

There are two basic hypotheses for how defensive secondary metabolites that occur in fruits could affect the triad of interactions among plants, mutualist seed dispersers, and antagonistic fruit pests. One possibility is that plants may experience a trade-off, where defensive metabolites reduce the preferences and/or removal rate of fruits by mutualists, but these costs are outweighed by the benefits of increased fruit persistence and/or reduced seed damage (Cipollini and Levey 1997b; Herrera 1982). Here, secondary metabolites are expected to have broad-spectrum bioactivity against microbes, invertebrates, and vertebrates. Alternatively, plants may produce secondary metabolites in fruits that are bioactive against invertebrate and microbial antagonists, but have neutral or limited effects in interactions with vertebrate seed dispersers (Cipollini and Levey 1997b). From the plant perspective, this scenario would likely provide enhanced fitness relative to the trade-off scenario, and, because seed dispersers (mostly vertebrates) are generally distantly related to fruit pests (mostly invertebrates and microbes), it seems likely the bioactivity of plant secondary metabolites would vary considerably among these different organisms (Cipollini and Levey 1997b; Tewksbury 2002).

The limited number of studies that have addressed one or both of these two alternative hypotheses have provided mixed results. Some support for the idea of a trade-off between seed dispersal and fruit defense is provided by evidence that fruits that are high in secondary metabolites can be the least preferred by seed dispersers in natural populations (Cazetta et al. 2008; Schaefer et al. 2003; Tang et al. 2005; Whitehead and Poveda 2011). However, in other cases, seed dispersers may consume fruits high in secondary metabolites with relative impunity. For example, capsaicinoids in wild chilies are one of the most well-studied systems with regard to the ecological role of fruit secondary metabolites (reviewed in Levey et al. 2007), and in this

case capsaicin provides important defense against pathogenic fungi (Haak et al. 2012; Tewksbury et al. 2006; Tewksbury et al. 2008b), but does not appear to reduce consumption by seed-dispersing birds (Mason et al. 1991; Tewksbury and Nabhan 2001). However, capsaicin has been shown to deter consumption by other vertebrates, such as rodents, which also consume chili fruits but are much less efficient seed dispersers than their avian counterparts (Mason et al. 1991; Tewksbury and Nabhan 2001). There are other examples of secondary metabolites that are highly toxic to mammals but are readily consumed by birds, such as amygdalin, a cyanogenic glycoside found in fruits of the Rosaceae and Caprifoliaceae, which cedar waxwings can consume at levels equivalent to 5.5 times the oral lethal dose for rats, with no outward signs of toxicity (Struempf et al. 1999). These results emphasize that the effects of fruit secondary metabolites on vertebrates, and therefore the potential for trade-offs in fruit defense, can vary considerably depending on the specific consumer involved. In particular, there may be important differences between birds and mammals in their ability to tolerate and/or detoxify secondary metabolites (Cipollini and Levey 1997b; Mason et al. 1991; Struempf et al. 1999; Tewksbury and Nabhan 2001).

Most past work on the ecological role of fruit secondary metabolites has focused on bird-dispersed species (Cipollini 2000; Cipollini et al. 2002; Levey et al. 2007); however, mammals also provide critical seed dispersal services for many plant species (van der Pijl 1982). Particularly in tropical forests, mammals, and especially bats, are among the most abundant frugivorous animals and are critically important in forest regeneration and succession (Charles-Dominique 1986; Fleming 2004; Gorchov et al. 1995; Lobova et al. 2009; Medellin and Gaona 1999; Muscarella and Fleming 2007). Because bats forage at night, bat-dispersed fruits are expected to contain higher levels of volatile secondary metabolites that contribute to fruit odor

and provide foraging cues (Hodgkison et al. 2007; Lomáscolo et al. 2010; van der Pijl 1982). However, if bats are similar to other mammals in that they are less adept at detoxifying secondary metabolites than birds (Cipollini and Levey 1997b; Mason et al. 1991; Struempf et al. 1999; Tewksbury and Nabhan 2001), the opposite may be true for non-volatile secondary metabolites that function primarily in fruit defense.

One important group of bat-dispersed plants is the genus *Piper* (Piperaceae), which is one of the ten most speciose plant genera (Frodin 2004), is a dominant component of tropical plant communities, and is considered a model system in tropical ecology and evolution (Dyer and Palmer 2004). Many *Piper* species fruit in abundance, producing distinctive, green, spike-shaped infructescences that are quickly removed by frugivorous bats (Fig. 7.1). In particular, a small genus of fruit bats (*Carollia spp.*, Phyllostomidae) are the primary dispersers of neotropical *Piper*, and *Piper* fruits represent a year-round dietary staple for the bats, making this interaction one of the few examples of a relatively specialized seed dispersal mutualism (Fleming 2004).

Figure 7.1: *Carollia perspicillata* approaching a ripe infructescence of *Piper sancti-felicis.*

Although past work on the phytochemistry of *Piper* has focused primarily on leaves (Dyer et al. 2004b; Kato and Furlan 2007; Parmar et al. 1997), fruits of many *Piper* species also contain diverse mixture of secondary metabolites, and are particularly rich in amides (Chaves et al. 2003; Chaves and Santos 2002; Siddiqui et al. 2005; Yang et al. 2002; Chapter 5). Amides are a large group of nitrogen-based compounds that play a key role in the defense of leaves against herbivores (Dyer et al. 2004b), and also have recently been shown to function in fruit defense against insect seed-predators and fruit-associated fungi (Chapter 6). Furthermore, compounds that occur in mixtures in fruits can interact, either functioning synergistically or antagonistically in fruit defense (Chapter 6). However, the effects of these compounds on *Carollia* bats are entirely unexplored.

In this study, we examined the effects of two amides, piperine and piplartine, on the foraging and feeding behavior of three species of *Carollia* bats. These two compounds occur in many species of *Piper*, and are often found in high concentrations in fruit (Bezerra et al. 2013; Matsuda et al. 2009; Parmar et al. 1997; Rajopadhye et al. 2011). They have very low volatility (Gaudin et al. 2008), thus it is unlikely that they are major components of the odor cues used by bats to locate fruits. However, bats may also choose not to consume fruits high in secondary metabolites once they land at a roost and begin to feed. Therefore, we tested the effects of piperine and piplartine on two aspects of bat foraging and feeding behavior: fruit removal and fruit consumption. In addition, because piperine and piplartine often occur in combination (Parmar et al. 1997) and have been shown to interact either synergistically or antagonistically in interactions with fruit antagonists (Chapter 6), we tested the effects of each compound presented alone as well as the effects of the two in combination and specifically tested for non-additive compound interactions in the mixture that altered the effects on removal or consumption.

7.3 Methods

Study Site and System

All experiments were conducted at La Selva Biological Station, located in the Heredia province of Costa Rica. The site consists of 1600 hectares of protected area that includes primary tropical wet and premontane forest (*sensu* Holdridge 1967), as well as secondary forest and abandoned agricultural areas. The site is a high center of diversity for *Piper,* with 50+ species co-occurring (Gentry 1990; OTS 2012). Most *Piper* species at the site are dispersed primarily by bats, although a few rely almost exclusively on asexual reproduction (Greig 1993b) and some are taken by a mix of birds and bats (Palmeirim et al. 1989). For those species that fruit in abundance, fruits tend to mature slowly over a period of approximately one month, after which mature fruits enter a final ripening period where all of the fruits on an infructescence simultaneously soften and swell. This process begins in the early afternoon and fruits are generally fully ripe by dusk. The vast majority of infructescences are removed by bats the same evening that they ripen; those that are not removed usually begin to rot very quickly and are not removed on the following evening (Thies and Kalko 2004; SRW personal observation).

Three species of *Carollia* bats co-occur at the site, *C. perspicillata, C. sowelli,* and *C. castanea.* All three species are relatively abundant in the forest understory and are among the most commonly captured bats in mist nets. There is some evidence that the three species differ in their degree of specialization on *Piper*. In a previous study conducted at the same site, the percentage of *Piper* in the diet of these species was estimated as ~54% for *C. perspicillata*, ~63% for *C. sowelli*, and ~85% for *C. castanea* (Fleming 1991). *Carollia* bats locate ripe infructescences primarily by odor (Mikich et al. 2003; Thies et al. 1998), remove an entire infructescence in flight, and carry it to a central feeding roost for consumption (Fleming 2004).

Field Capture and Handling of Bats

Bats were captured in mist nets from secondary forest sites at La Selva using standard methods (Choate et al. 1998; Simmons and Voss 2009). All males and non-reproductive female individuals of *Carollia* were retained for use in experiments. For each individual, we recorded the weight, forearm length, tibia length, sex, reproductive status, and age (adult or juvenile). Bats were placed in cloth bags prior to the start of the experiments, for a minimum of 45 minutes and a maximum of two hours. Bats were then transferred to 2.5m x 1.5m x 1.75m tall flight cages that consisted of a wood frame with screen walls and ceilings. Each species of *Carollia* was housed in a separate cage. Conspecific groups were placed together, with groups ranging in size from 1-5 individuals depending on the number of bats captured in a particular evening. To distinguish among individual bats in the cage, each bat was marked with a unique symbol on its back using infrared-reflective adhesive tape (3M SOLAS pinstripe, ¼" width, Anytime Sign, Inc.). This is a novel method of marking bats for captive studies that provided an excellent means of distinguishing among individuals in infrared-illuminated flight cages and was a simple temporary marking that could easily be removed once the experiments were finished. Each cage was equipped with a 98LED infrared lamp (CMVision, Model # YY-IR100) and a SONY Nightshot digital video camera (Model # HDR-CX7) to record bat behaviors (see sample video at http://youtu.be/Wa13kvmJQoQ). Infrared lights and cameras are commonly used in captive studies and do not appear to disturb normal bat behavior (Altenbach and Dalton 2009).

Amide Choice Experiments

In all experiments, we added pure amides to ripe fruits of *P. sancti-felicis*, a commonly occurring species at the study site that produces fruits in abundance continuously throughout the

year. The fruits of this species contain no detectable levels of amides at a detection limit of approximately 0.01% dry weight (SRW unpublished data), although they do contain other secondary metabolites, primarily alkenylphenols (Glassmire et al., in prep). Although using natural *Piper* fruits meant that we were unable to control for any natural variation in nutritional or secondary chemistry among *P. sancti-felicis* fruits that existed prior to our amide supplementation treatments, this method was preferable to using homogenous artificial diets for two reasons: 1) We wanted to observe how amides affect the natural feeding behavior of *Carollia* bats on *Piper* fruits, including fruit removal and consumption, and these behaviors could not be simulated using artificial fruits that did not have the same structure as a *Piper* fruit; and 2) One of the *Carollia* species we examined, *C. castanea*, would not accept any diet other than ripe *Piper* fruits in preliminary trials (e.g. bananas, papayas, artificial banana-agar mix, etc).

To test the effects of amides on bat feeding preferences, groups of bats were offered a buffet-style presentation of equal numbers of amide supplemented and control fruits that were placed in two separate groups of five infructescences each. All fruit removal events were recorded with video cameras; however, fruit consumption could not always be observed because bats often carried the infructescence to parts of the cage that were not visible in the video recordings. Thus, the peduncle of each infructescence was also marked using non-toxic paint with different colors for each position number on the buffet, allowing us to later recover all discarded infructescences from the floor of the flight cage, measure the proportion that was consumed from each, and assign this event to a particular bat based on the position from which it was removed. In cases where bats consumed all of the fruit from an infructescence, the peduncle and central rachis remained and were also recovered from the floor of the cage. Trials ran for two hours, after which the bats were released at the site of capture and all intact and discarded fruits

were recovered from the cage. In a few cases, bats removed all of the infructescences from either the control or treatment group prior to the end of the two hour period, in which case we ended the trial early and excluded all removal and consumption events from analysis that occurred after one of the fruit groups was depleted. To estimate the proportion consumed of each infructescence, we measured the total length of the rachis and the length from which fruit had been consumed.

Three sets of identical trials were conducted to test the effects of piperine, piplartine, and the combination of piperine and piplartine. To prepare fruits for the trials, we always collected freshly-ripened *P. sancti-felicis* infructescences on the afternoon before the trial. For the amide-supplemented fruits, we added 10 mg (~0.1% wet weight) of piperine, piplartine, or 1:1 piperine:piplartine to each *P. sancti-felicis* infructescence. This amount was chosen to represent the lower end of the range of concentrations of these compounds in natural fruits based on reports from the literature (Rajopadhye et al. 2011). In all treatments, the pure compounds were dissolved in ethanol and fruits were supplemented by adding 1mL of solution to a clean glass petri dish and rolling a single ripe infructescence in the dish until all of the solution was absorbed and evenly coated the surface of the fruits. Control infructescences were treated in an identical manner using ethanol only. The peduncle of each infructescence was then painted with unique colors for each position number on the "buffet" as described above and infructescences were left to dry on a wire rack in an air-conditioned laboratory for 3-5 hours to allow the ethanol to evaporate prior to the start of the trials.

Statistical Analyses

To test how amides affect fruit removal by *Carollia* bats, we used a generalized linear mixed model with a binomial distribution and the logit link function. The response variable was a binary value for removal (1=removed, 0=not removed) for each individual infructescence. Treatment (control or amide supplemented), *Carollia* species, and their interaction were included as fixed effects, and the bat group identity and group size were included as random effects. These analyses were conducted separately for each amide treatment (piperine, piplartine, piperine + piplartine).

To test how amides affect fruit consumption by *Carollia* bats, we used a linear mixed model. Here the response variable was the proportion of fruit consumed from each infructescence, including only those infructescences that had been removed by bats during the experiments. The proportion data were logit transformed prior to analysis to approximate a normal distribution (Warton and Hui 2011). Fruit treatment (control or amide supplemented), *Carollia* species, and their interaction were included as fixed effects, and bat group identity and individual identity were included as nested random effects.

To test whether piperine and piplartine interact synergistically or antagonistically when present in combination, we compared the effect of the combination treatment to the expected effect based on an additive interaction between the two compounds (Nelson and Kursar 1999). These analyses were conducted separately for the effects on fruit removal and on fruit consumption and for the effects on each bat species, due to significant interactions between compound treatment and bat species in the above analyses (see results). We first calculated the expected additive effects as: $Z_{add} = (p_A A + p_B B)$, where A and B represent the estimated model coefficients for the treatment effects of piperine and piplartine, respectively, and p_A and

p_B represent the proportions of the two compounds in the mixture (in our case p_A = 0.5 and p_B = 0.5). Accordingly, the variance of this estimate was calculated as: $V_{add} = (p_A^2 V_A + p_B^2 V_B)$, where V_A and V_B were the estimated variances of the model coefficients for the treatment effects (Tallarida 2002). Based on these estimates of the expected effect size and variance, we calculated 95% confidence intervals for the expected additive effect and compared these intervals to those estimated for the observed effect of the combination. A lack of overlap between the confidence intervals of the expected and observed effect sizes was taken as evidence of a non-additive interaction between the two compounds.

Results

Effects of Amides on Fruit Removal

The effects of amides on fruit removal varied depending on the specific compound or combination of compounds tested. For piperine, there was a significant interaction between bat species and amide treatment (GLMM, ΔAIC=13.64, X^2=17.64, P=0.00015), and therefore we examined the effects of piperine on each bat species separately. *Carollia castanea* was unaffected by piperine treatment (GLMM, ΔAIC=1.51, X^2=0.49, P=0.48), but both *C. perspicillata* (GLMM, ΔAIC=25.56, X^2=27.56, P < 0.0001) and *C. sowelli* (GLMM, ΔAIC=17.23, X^2=19.23, P < 0.0001) removed fewer piperine-supplemented infructescences than controls (Fig. 7.2A). For piplartine, there was also a significant interaction between bat species and amide treatment (GLMM, ΔAIC=4.15, X^2=8.15, P=0.017), and we examined the effects of piplartine on each bat species separately. *Carollia castanea* (GLMM ΔAIC=1.4, X^2=0.60, P=0.44) and *C. sowelli* (GLMM, ΔAIC=1.05, X^2=3.05, P=0.081) were unaffected by piplartine treatment, but *C. perspicillata* (GLMM, ΔAIC=3.75, X^2=5.75, P=0.016) removed fewer

piplartine-supplemented fruits than controls (Fig. 7.2B). For the combination of piperine and piplartine, there was again a significant interaction between bat species and amide treatment (GLMM, ΔAIC=13.17, X^2=17.17, P=0.00019), and we examined the effects of treatment on each bat species separately. *Carollia castanea* (GLMM ΔAIC=5.41, X^2=7.41, P=0.0065) and *C. perspicillata* (GLMM, ΔAIC=6.28, X^2=8.28, P=0.0040) removed fewer treatment fruits than controls, but *C. sowelli* (GLMM, ΔAIC=3.37, X^2=5.37, P=0.020) removed fewer piplartine-supplemented fruits than controls (Fig. 7.2C).

Effects of Amides on Fruit Consumption

The effects of amides on fruit consumption also varied depending on the specific compound or combination of compounds tested, but the trends were different than those for removal. For piperine, there was a significant interaction between bat species and amide treatment (GLMM, ΔAIC=2.27, X^2=6.27, P=0.043), and therefore we examined the effects of piperine on each bat species separately. *Carollia castanea* consumed a lower proportion of fruit from piperine-treated infructescences than from controls (GLMM, ΔAIC=6.98, X^2=8.98, P=0.0027), as did *C. perspicillata* (GLMM, ΔAIC=4.16, X^2=6.16, P=0.013). *Carollia sowelli* was unaffected (GLMM, ΔAIC=0.16, X^2=2.16, P=0.14) (Fig. 7.3A). For piplartine, there was also a significant interaction between bat species and amide treatment (GLMM, ΔAIC=3.84, X^2=7.84, P=0.020), and we examined the effects of piplartine on each bat species separately. *Carollia castanea* consumed a lower proportion of fruit from piplartine-treated

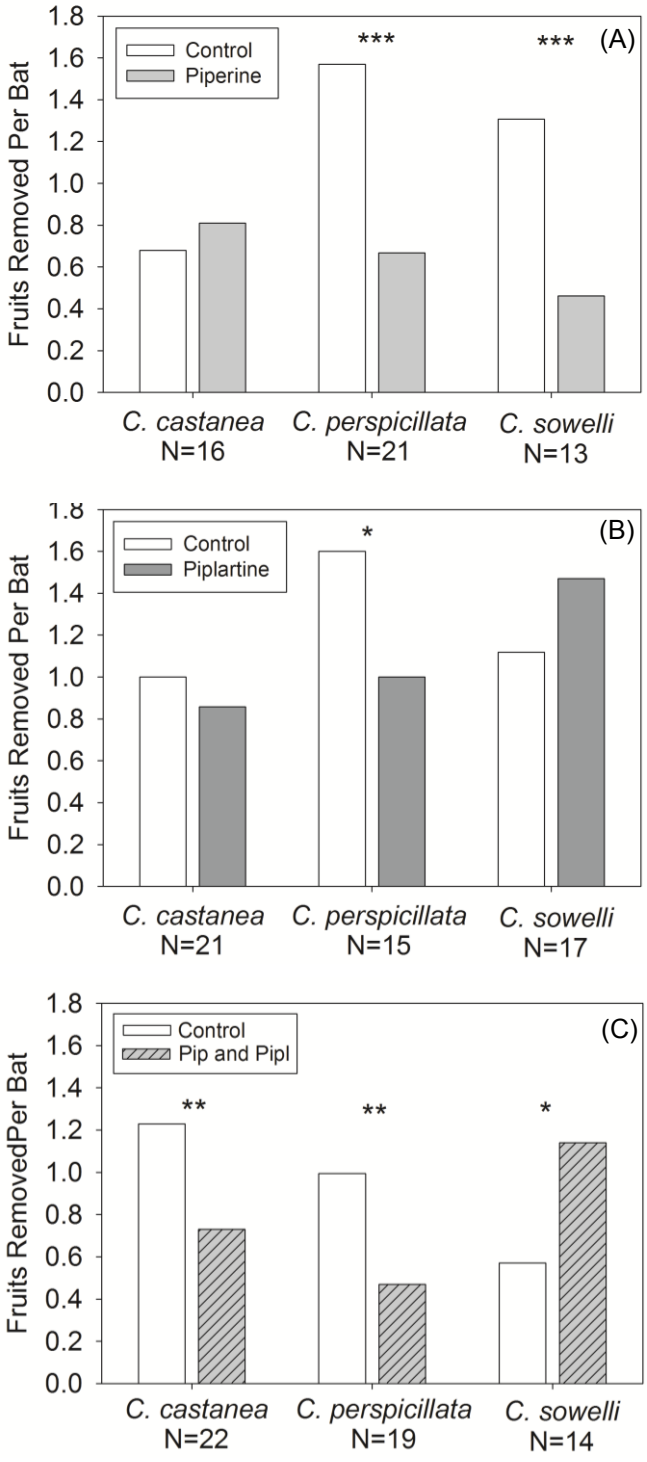


Figure 7.2: Effects of piperine (A), piplartine (B), and the combination (C) on fruit removal behavior of three species of *Carollia* bats. The numbers of bats used per experiment are indicated below the species name, and the y-axis indicates the average number of fruits removed per bat. Stars indicate significant differences between control and treatment fruits removed by each species in generalized linear mixed models, see text for details (*P<0.05, **P<0.01, ***P<0.001).

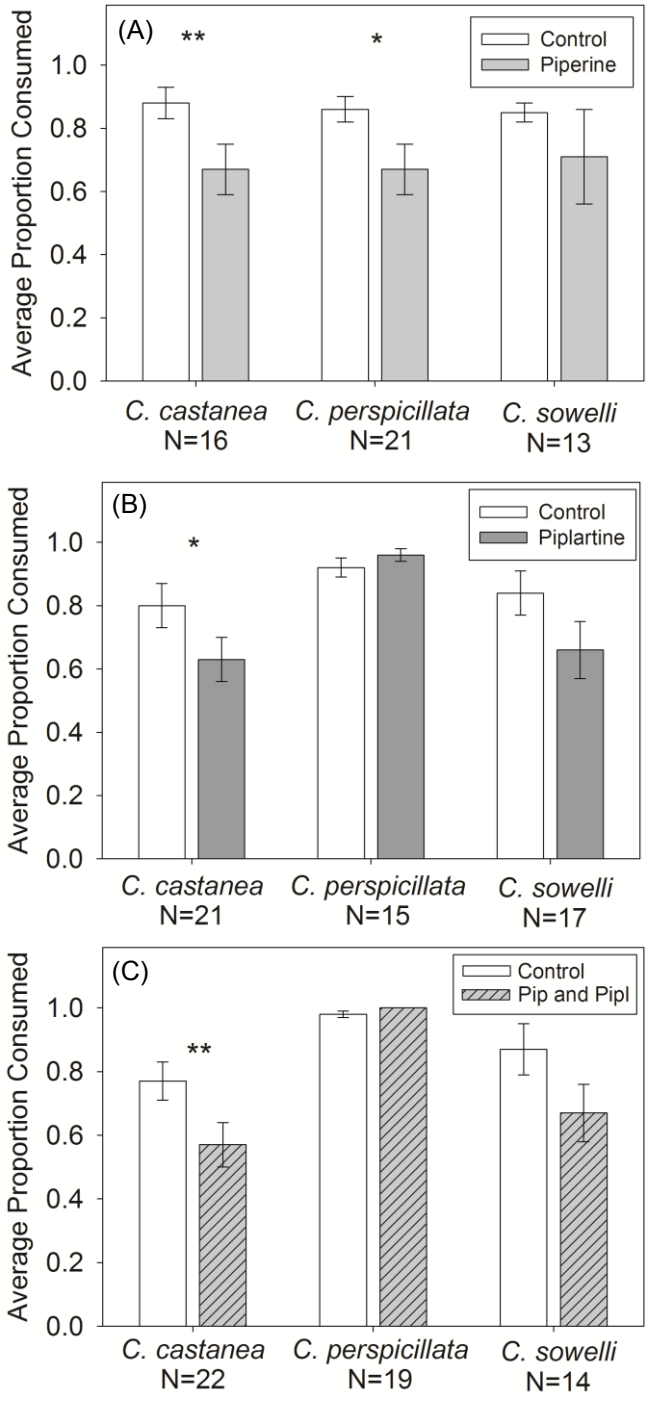


Figure 7.3: Effects of piperine (A), piplartine (B), and the combination (C) on fruit consumption behavior of three species of *Carollia* bats. The numbers of bats used per experiment are indicated below the species name, and the y-axis indicates the average proportion of fruit consumed per infructescence that was removed by bats. Stars indicate significant differences between control and treatment fruit consumed by each species in linear mixed models, see text for details (*P<0.05, **P<0.01, ***P<0.001).

infructescences than from controls (GLMM, ΔAIC=3.42, X^2=5.42, P=0.020), but *C. perspicillata* (GLMM, ΔAIC=0.53, X^2=1.48, P=0.22) and *C. sowelli* (GLMM, ΔAIC=0.62, X^2=1.38, P=0.24) were unaffected (Fig. 7.3B). For the combination of piperine and piplartine, there was again a significant interaction between bat species and amide treatment (GLMM, ΔAIC=5.72, X^2=9.72, P=0.0077) and we examined the effects of treatment on each bat species separately. *Carollia castanea* consumed a lower proportion of fruit from treated infructescences than from controls (GLMM, ΔAIC=6.13, X^2=8.13, P=0.0044), but *C. perspicillata* (GLMM, ΔAIC=0.69, X^2=2.69, P=0.10) and *C. sowelli* (GLMM, ΔAIC=1.09, X^2=3.08, P=0.08) were unaffected (Fig. 7.3C).

Interactions between Amides in Combination

For fruit removal, there was evidence of a non-additive interaction between piperine and piplartine for *C. sowelli*, but not for *C. castanea* or *C. perspicillata* (Fig. 7.4A). For *C. sowelli*, piperine had a negative effect on removal, piplartine had a marginally positive effect on removal, and the combination had a strong positive effect on removal (see above, Fig. 7.2). The confidence interval for the expected effect based on an additive response was -0.18 to -4.89 (predicting a significant negative effect of the combined amides on removal), and the confidence interval for the observed effect of the combination was 0.19 to 2.56 (a significant positive effect on fruit removal). Thus, the combination of piperine and piplartine had an unexpected positive effect on bat preferences, despite the fact that a negative effect would have been predicted based on additive interactions between the two compounds. There was no evidence for interactions between piperine and piplartine in their effects on fruit consumption (Fig. 7.4B).

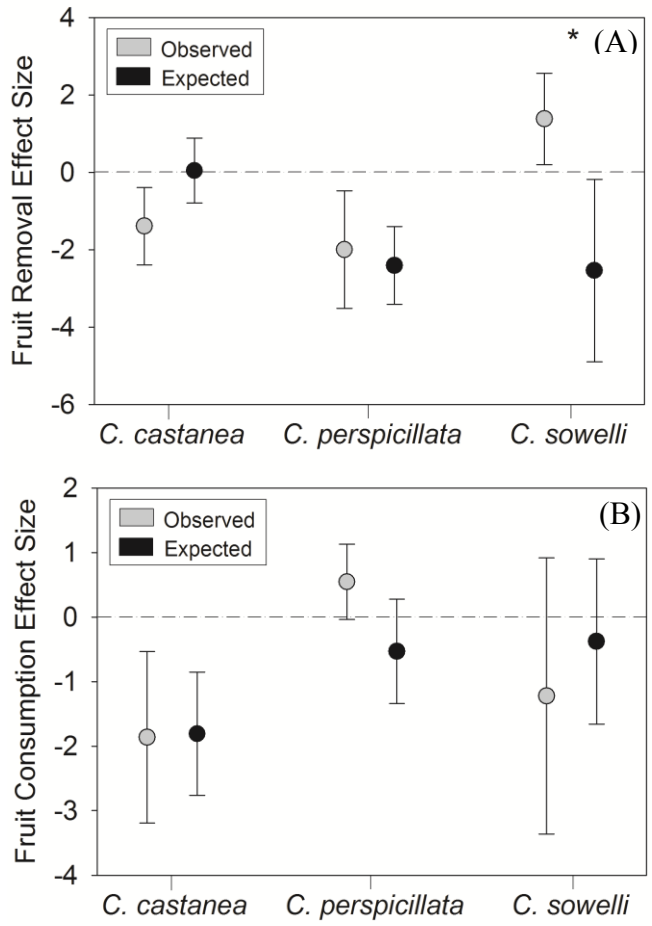


Figure 7.4: Tests for non-additive interactions between piperine and piplartine in their effects on fruit removal (A) and fruit consumption (B) by three species of *Carollia* bats. Observed values represent the model coefficients and 95% confidence intervals for the estimated effect of treatment in generalized linear mixed models examining the effects of piperine and piplartine when presented in combination. Expected values represent the predicted model coefficients and 95% confidence intervals based on an additive interaction between the two compounds and were calculated based on the effects of piperine and piplartine when presented alone. Confidence intervals that do not cross zero indicate a significant difference between treatment and control fruits, with effect sizes < 0 indicating bats were deterred by the compounds and vice versa.

7.5 Discussion

Secondary metabolites can increase plant fitness by defending plant tissues against antagonists, such as herbivores and pathogens (Bennett and Wallsgrove 1994), but they also have important consequences for interactions with mutualists, such as pollinators and seed dispersers (Adler 2000; Levey et al. 2007). This study represents the first examination of how secondary metabolites in fleshy fruits can affect the foraging and feeding behavior of seed-dispersing bats. Our results showed that amides, an important class of plant defensive compounds (Dyer et al. 2004b), can alter both the removal of fruits by bats and the proportion of fruit that bats consume from an infructescence once they begin to feed. However, these effects varied considerably

depending both on the specific compound(s) being tested and the bat species involved. Most often, the effects of amides were negative (i.e. reduced fruit removal or consumption) or neutral, but in one case the amide treatment actually increased fruit removal (Fig. 7.2). Overall, these results support the hypothesis that fruit secondary metabolites may lead to trade-offs between seed dispersal and fruit defense, but the strength of this trade-off and its potential fitness consequences likely varies considerably depending on ecological context.

The fact that bats were often deterred by amides is important new evidence that bats can detect and select fruits based on the concentration of low-volatility secondary metabolites in fruit pulp. This is in contrast to some results with bird-dispersed plants, where birds are undeterred by fruits high in secondary metabolites (Mason et al. 1991; Struempf et al. 1999; Tewksbury and Nabhan 2001). However, it is important to note that although amides have the potential to affect both fruit removal and fruit consumption behavior, this was not universally the case. There were strong interactions between treatment (amide supplemented or controls) and bat species, emphasizing that the effects of specific compound(s) may be highly variable among even closely-related consumers. All three bat species were affected in some manner (either in their fruit removal or consumption behavior) by at least some of the compounds, but there were no clear trends of certain compound treatments being more deterrent than others or certain bat species being more strongly affected than others. Interestingly, some bat species (e.g. *C. perspicillata*) may be more "choosy" about which infructescences they remove, whereas other bat species (e.g. *C. castanea*) may be more "choosy" about which infructescences they will fully consume once they begin feeding.

For fruit removal, we found that bats were most often deterred by amides, but in some cases bats removed more amide supplemented fruits than controls (Fig. 7.2C). The finding that

amides affected removal behavior at all was somewhat contrary to our expectations, because amides have very low volatility (Gaudin et al. 2008) and are not likely to be a major component of the fruit odor. However, there are several other mechanisms through which bats may distinguish among fruits prior to removal. First, bats often made a number of exploratory flights or removal attempts at a single infructescence before they finally removed it in flight (behavior described in detail in Thies et al. 1998). In several cases, we observed bats that made a number of attempts at a particular fruit and may have "tasted" the fruit, but never actually removed it. Amides and especially piperine can be highly pungent (Srinivasan 2007), and bats may have specifically chosen not to return to less preferred fruits. In addition, because bats in our cages could remove multiple fruits in a single trial, and because the treatment and control fruits were displayed in two separate groups, it is likely that there may be a learning effect, where bats did not return to the same group once they removed an infructescence that they did not prefer.

Most often bats removed more control fruits than treatment fruits or there was no effect. However, for *C. sowelli*, we found that piplartine had a marginally significant positive effect on removal and the combination had a highly significant positive effect (Fig. 7.2B-C). This result was also contrary to expectations, but there are several potential reasons why bats may prefer fruits higher in amides (Cipollini and Levey 1997b; Forbey et al. 2009). For example, secondary metabolites may be used as foraging cues, helping bats to associate the fruits of certain *Piper* species with nutritional rewards (Cipollini and Levey 1997b). Another possibility is that certain amides can reduce parasite or pathogen load in bats, and bats self-medicate using particular *Piper* species that contain particular combinations of compounds. To our knowledge the phenomenon of self-medication has not been examined in bats, but has been shown for a variety of other vertebrates and insects (Forbey et al. 2009; Singer et al. 2009).

For fruit consumption, we found that the effects of the amides were either negative or neutral, depending on the specific bat species and compound(s) involved. The proportion of fruit consumed by *C. castanea* was always lower for amide supplemented fruits than controls, whereas *C. perspicillata* was only affected by piperine and *C. sowelli* was not significantly affected by any of the compound treatments. Whether or not bats consume an entire infructescence once they begin feeding might be in part explained in the framework of optimal foraging theory, which predicts that animal foraging behavior will reflect selection to maximize net energy gain (Pyke 1984). Once bats have expended the energy to locate and remove a ripe infructescence, it may seem most efficient to consume it in its entirety, maximizing the energetic gain per unit feeding time. However, if the digestion or detoxification of secondary metabolites also represents a substantial energy expenditure, then bats may maximize energetic gain by keeping the amount of secondary metabolites ingested below a certain threshold. This may help explain why *C. perspicillata* and *C. sowelli,* which overall have a more varied diet than *C. castanea* (Fleming 1991), and therefore would consume lower total amounts of amides in an evening, may be less constrained by the amount of secondary metabolites consumed in any one infructescence. Even within a species, the relative proportion of fruit in the diet may help explain variation in foraging and feeding behavior. For example, in a previous study with *C. perspicillata* in the Ecuadorian Amazon, individuals with a higher percentage of fruit in their diet, relative to insects, spent more time visiting mineral licks, which are thought to buffer the effects of secondary metabolites and aid in detoxification (Voigt et al. 2008). Future research examining the physiological costs of secondary metabolite detoxification in *Carollia* would likely provide important insight to help to explain the variation in dietary preferences and behaviors of these species.

In our test for interactions between piperine and piplartine, we found only one case where the two compounds appear to have a non-additive response. For the fruit removal behavior of *C. sowelli*, piperine had a strong negative effect on removal, piplartine had a marginally significant positive effect on removal, and the combination had a strong positive effect on removal (Fig. 7.4). Because piperine alone acted as a deterrent, and piplartine was a slight attractant, this compound interaction could be viewed as synergy (piperine increased the effectiveness of piplartine as an attractant) or antagonism (piplartine decreased the effectiveness of piperine as a deterrent). The large qualitative difference in the direction of bat responses to these closely-related compounds emphasizes the potential for unexpected effects of secondary metabolites that occur in mixtures. The fruits of some *Piper* species contain diverse suites of amides (Chapter 5) that could interact in a variety of ways. In some cases combinations of compounds may have synergistic negative effects that increase toxicity and alter bat preferences. In other cases, bats may use specific compound combinations, rather than the presence of individual compounds, to provide reliable foraging cues that allow them to recognize particular *Piper* species and that provide particular nutritional rewards. One possible explanation for our results is that *C. sowelli* uses the combination of piperine and piplartine to recognize particular *Piper* species that are a preferred part of their diets. For example, both piperine and piplartine occur in *Piper tuberculatum* (Cícero et al. 2007; de Araújo-Júnior et al. 2011), a common species that occurs throughout much of the range of *C. sowelli* (although not at La Selva), and fruits of this species have been shown to be the single most abundant component in *Carollia* diets in some areas (Heithaus et al. 1975). Most *Piper* species at La Selva have not been phytochemically investigated, and it is possible that this combination of compounds also occurs in other species that do occur at the site.

Overall, our results support the hypothesis that fruit secondary metabolites represent a trade-off, where fruits that are most defended against antagonists are also the least preferred by mutualist, seed-dispersing bats. However, whether or not these costs, in terms of reduced preference, lead to evolutionarily relevant fitness costs will depend on a variety of factors. Importantly, it is unclear whether seeds that are dropped below feeding roosts inside partially intact infructescences are able to survive and germinate with similar probability compared to seeds that are consumed and defecated. Past work has shown that the germination probability is similar for *Piper* seeds that are collected from bat feces versus ripe fruits (Lopez and Vaughan 2004; Palmeirim et al. 1989); however these studies used seeds that were cleaned of any fruit pulp or fecal material prior to the germination trials. We have some preliminary data that suggests that seeds inside intact fruits have a much lower germination probability than cleaned seeds due to rapid fungal attack and decomposition (MFO and SRW, unpublished data). However, we have also observed that intact or partially consumed fruits on the forest floor are often rapidly removed piecemeal by foraging ants. Thus, understanding the fate of *Piper* seeds dropped below roosts will require in depth study of seed survival and germination in a variety of scenarios.

This study has provided evidence that amides can have important effects on the foraging and feeding behavior of *Carollia* bats, and therefore the seed dispersal success of many plants in the genus *Piper*. We show that different species of *Carollia* are often affected in different ways by specific compounds or combinations of compounds and that compound combinations can potentially have non-additive effects on bat behavior. Future work should focus on understanding whether there is sub-generic specialization in interactions, such that certain *Carollia* species prefer certain *Piper* species, and whether the divergent selective pressures of

particular species may have been one of the factors contributing to the incredible chemical diversity of the genus *Piper* (Parmar et al. 1997). Especially in cases where the abundance and/or diversity of secondary metabolites in *Piper* fruits has been shown to exceed that of leaves (Chapter 5), interactions between plants and mutualist seed dispersers may be an important and underappreciated force in the evolution of plant secondary metabolite diversity.

CHAPTER 8

SUMMARY AND CONCLUSIONS

8.1 Summary of key findings

Interactions between plants, seed dispersers, and fruit pests are key factors determining plant reproductive success and population dynamics (Dennis et al. 2007). However, the mechanisms of these interactions are often poorly understood, perhaps in part due to the limited number of studies that have examined how fruit secondary metabolites mediate both seed dispersal and fruit defense (Tewksbury 2002). My dissertation has made an important contribution to this field of study by taking an integrative approach to understanding how fruit secondary metabolites can affect interactions with the broad range of organisms that consume fruit tissues. I provided the first description of secondary metabolites in several plant species from different plant families and combine detailed quantitative chemical analyses with bioassays and field studies to understand the role of fruit secondary metabolites in interactions with insect seed-predators, microbial pathogens, and mutualist seed dispersers. I used research from three different systems to address a range of questions from different theoretical perspectives.

In Chapter 2, I used the tropical plant species *Hamelia patens* (Rubiaceae) to show that herbivory to leaves can lead to induced changes in fruit chemistry that reduce the attractiveness of fruits to seed-dispersing birds. This is an important first step to understanding the evolutionary ecology of fruit chemical traits, because it shows that fruit secondary metabolites can be constrained by interactions and selective pressures that occur in other plant parts. It also shows that fruit secondary metabolites can be costly in terms of reduced seed dispersal opportunities, emphasizing the potential for trade-offs in fruits between defense and attraction of mutualists.

In Chapters 3 and 4, I examined the occurrence patterns and functional significance of iridoid glycosides (IGs) in a hybrid complex of bush honeysuckles (*Lonicera spp.*, Caprifoliaceae). Chapter 3 primarily focused on the first description of IGs in *L. tatarica, L. morrowii,* and their hybrid progeny *L.* x *bella.* Chapter 4 used the analytical methods developed for Chapter 3, in combination with in depth ecological field studies, to address whether fruit secondary metabolites are best explained adaptively or as a result of physiological constraints on the exclusion of leaf secondary metabolites from fruit tissues. Multiple lines of evidence, including higher concentrations of IGs in fruits than in leaves and the occurrence of several secondary metabolites unique to fruits, showed that fruit secondary metabolites cannot be explained solely as a result of physiological constraints. Although leaf and fruit chemistry were not entirely independent, evidence from this chapter showed that there are likely important selective pressure for fruit defense independent of the selective pressures in leaves.

In Chapters 5-7, I examined the occurrence patterns and functional significance of amides in the tropical plant genus *Piper.* Chapter 5 focused on comparing the concentrations and identities of individual amides in different plant parts of *P. reticulatum*. In this species, fruit amides were found in similar concentrations to those in leaves, but the chemical diversity (i.e. number of individual compounds) of fruits and other reproductive tissues was much higher than that of vegetative tissues (leaves and roots). Chapter 6 focused on how the diverse suites of compounds in fruits of *P. reticulatum* and other *Piper* species function in defense against a range of antagonistic consumers, including insects and fruit-associated fungi. Interestingly, results showed that different combinations of compounds have differential effects on different consumers, and, furthermore, combinations of compounds can either function antagonistically or synergistically depending on the particular consumer involved. This emphasizes that chemical

diversity may be an important factor that allows plants to defend themselves simultaneously against a range of antagonists. Finally, Chapter 7 turned to examination of the effects of amides on the primary seed dispersers of *Piper*, a small group of bats in the genus *Carollia.* Results showed that bats can be deterred by amides, emphasizing the potential for trade-offs between seed dispersal and fruit defense, but the strength of the deterrent effects varied considerably among three bat species and the particular compounds or combination of compounds tested.

Together, these results show that an understanding of the evolutionary ecology of fruit secondary metabolites requires simultaneous consideration of multiple selective pressures from antagonists and mutualists, as well as the potential for physiological constraints that lead to suites of compounds that are not necessarily optimized for seed dispersal success.

8.2 Implications for the evolutionary ecology of seed dispersal

Theories of interactions between fruits and frugivores historically focused on the prediction that competition among plants for reliable dispersers and vice versa should create a coevolutionary landscape that selects for fruits optimized for a particular set of seed dispersers (Howe and Estabrook 1977; McKey 1975; Snow 1971). A long-held paradigm suggested that diffuse interactions of plants with groups of dispersers should lead to integrated sets of fruit morphologies or "syndromes" that represent broad adaptations to large taxonomic groups (Janson 1983; van der Pijl 1982). The straightforward and testable predictions generated by this theory stimulated extensive research, but fruit trait variation at the intra- and inter-specific level proved difficult to explain in this context (Herrera 1987; Jordano 1995). In fact, researchers found that the relationship between seed dispersal mode and fruit trait syndromes disappeared almost entirely after accounting for phylogenetic effects (Jordano 1995). A new paradigm then

emerged suggesting that inconsistent selective pressures in time and space (Herrera 1998), combined with unpredictable post-dispersal processes (Parciak 2002), likely overwhelm any consistent influence of seed dispersers on the evolution of fruit traits. However, one possible explanation for the uncertainty surrounding the evolutionary ecology of fruit/frugivore interactions is that these past studies did not incorporate fruit secondary metabolites among the suites of fruit traits that make up particular syndromes and largely ignored the importance of non-dispersing seed predators and pathogens in influencing fruit trait evolution (Lomáscolo et al. 2010; Tewksbury 2002).

Together, my results support the hypothesis that both fruit antagonists (Chapter 4, Chapter 6) and mutualists (Chapter 2, Chapter 7), have likely shaped the evolution of fruit traits, in particular the occurrence patterns of fruit secondary metabolites (Cipollini and Levey 1997b; Tewksbury 2002). Thus, rather than searching for tight co-evolutionary interactions between plants and mutualist seed dispersers, a more appropriate theoretical framework for the evolutionary landscape of fruit/frugivore interactions may be to consider an evolutionary triad of interactions between plants, seed dispersers, and fruit pests (Buchholz and Levey 1990). However, even this approach may be too simplified, as my results have shown that selective pressures from different classes of fruit pests (e.g. insects versus microbes, Chapter 4, Chapter 6) and from different individual seed dispersers (Chapter 7) likely vary considerably. Furthermore, I have shown that the current selective environment of fruits is not the only important factor influencing fruit chemical traits. Correlations between leaf and fruit chemistry (Chapter 2, Chapter 4) suggest that there are important physiological constraints that can also influence the qualitative and quantitative occurrence patterns of these traits. Thus, an integrated approach to understanding the evolutionary ecology of seed dispersal syndromes should incorporate

secondary metabolites and take a broad view of both adaptation and constraints that have led to current combinations of traits in wild, fleshy fruits. Figure 8.1 summarizes the diversity of factors that may influence the occurrence patterns of fruit secondary metabolites in animal-dispersed species.

8.3 Implications for the evolutionary ecology of plant defense

Since the seminal paper by Fraenkel (1959) exploring the *raison d'être* of plant secondary metabolites, an impressive body of literature has accumulated surrounding the importance of chemical traits in plant defense against herbivores and pathogens (Harbourne 1993; Rosenthal and Berenbaum 1991; Schoonhoven et al. 2005; and references therein). However, this field has focused primarily on the chemical defense of leaves against leaf

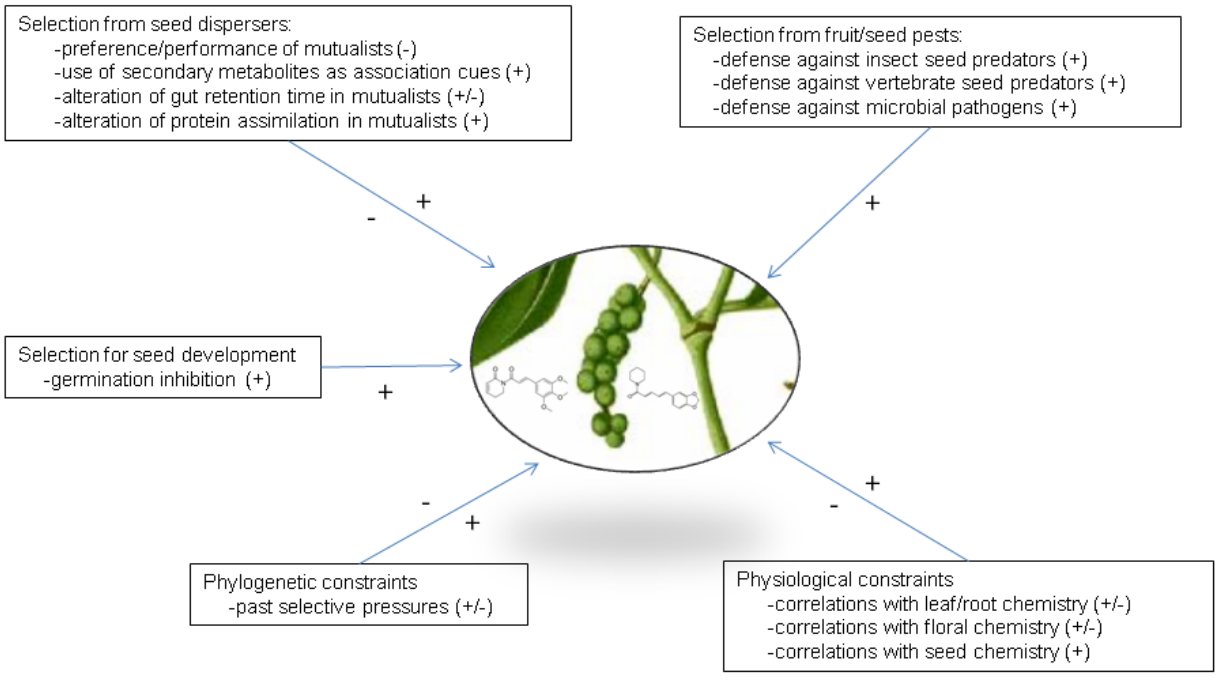


Figure 8.1: Conceptual model for the selective pressures and constraints influencing the occurrence patterns of secondary metabolites in the pulp of fleshy fruits. The potential for each factor to either increase or decrease the concentration or diversity of secondary metabolites in fruit tissue is indicated with a (+) or a (-), respectively.

herbivores, and relatively few studies have examined the role of secondary metabolites in other plant parts, such as flowers and fruits (Chapter 1). Increasing evidence has shown that nectar secondary metabolites are of crucial importance in structuring plant/pollinator interactions and defense against antagonistic nectar thieves (e.g. Adler 2000; Irwin and Adler 2008; Manson et al. 2010), and, similarly, fruit secondary metabolites are of crucial importance in structuring fruit/frugivore interactions and defense against seed predators and pathogens (e.g. Cipollini et al. 2004; Izhaki et al. 2002; Levey et al. 2007; Tewksbury et al. 2008b). Because reproductive tissues provide a direct link to reproductive output, the defense of these structures may in fact be more important for plant fitness than the defense of leaves (McKey 1974; McKey 1979; Rhoades and Cates 1976).

My results support the hypothesis that plant investment in the defense of fruits may exceed investment in leaf defense (Chapter 4, Chapter 5). This view is in direct contrast to past suggestions that fruit secondary metabolites are ecologically costly and occur primarily as the result of strong selection for the defense of leaves (Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998; Heil 2002; Strauss et al. 2002). In fact, I hypothesize that in some plant lineages the opposite may be true, i.e. leaf secondary metabolites may be the result of strong selection for the defense of fruits and/or the seeds they contain. The protection of seeds represents a critical factor determining plant reproductive success, and it has been suggested that, for many plant lineages, fruit flesh originated primarily as means to defend the developing embryo, rather than as a mechanism for dispersal (Mack 2000). In this scenario, fruit flesh that is highly defended by secondary metabolites would represent the pleisomorphic state, and specific adaptations to attract seed dispersers would have evolved secondarily when animals that were primarily seed predators began to incidentally transport intact seeds (Mack 2000). With this historical perspective, there

is no reason to assume that secondary metabolites in leaves evolved before secondary metabolites in fruits, but rather, because seeds generally are more valuable and at a higher risk of attack than leaves (Chapter 5), there may have historically been stronger selection for secondary metabolites in seeds and fruits than in leaves. A similar sequence of events, where leaf secondary metabolites evolved as a result of pre-existing chemical defenses in flowers, has been demonstrated as a likely evolutionary scenario in the Euphorbiaceae (Armbruster et al. 1997); however, to my knowledge, no similar studies have been conducted in fruits.

8.4 Future directions

Results presented in this dissertation have provided many fruitful directions for further research. The chemical ecology of seed dispersal and fruit defense is a field that has been barely explored, despite the fact that it can provide important insights into the factors governing plant reproductive success, frugivore behavior and physiology, and the structure of entire communities. In particular, we need broad comparative approaches that examine the overall fitness outcomes of fruit secondary metabolites that mediate simultaneous interactions with different classes of frugivores, as well as phylogenetically-controlled studies of interspecific patterns in fruit secondary metabolites that can help address evolutionary questions about their origins and adaptive significance.

Both the genus *Lonicera* and the genus *Piper* provide excellent model systems for addressing these sorts of broad questions. The genus *Lonicera* includes about 200 species, with 18 native and 16 exotic species in the United States (USDA-PLANTS 2011). Comparisons of leaf and fruit secondary metabolites across this genus could provide important insights into: 1) the evolution of fruit secondary metabolites, 2) the role of secondary metabolite variation in

predicting variation in plant defense against adapted and non-adapted consumers, and 3) the role of secondary metabolites in the evolution of invasiveness. I am currently involved in a collaborative project with Wright State University that will be the first step towards these sorts of comparative analyses.

The genus *Piper* is also an excellent model system for addressing both the multi-functionality of fruit secondary metabolites in different interactions and the evolutionary origins of fruit secondary metabolites. Regarding multi-functionality, I have a number of ongoing collaborative projects examining other potential roles of fruit secondary metabolites not discussed here. For example, we are examining the role of amides in regulating the gut retention time of seeds in *Carollia* bats (Baldwin and Whitehead, in prep), and I am currently planning a project for Summer 2013 to examine how amides affect bat parasite loads and gut microbial communities. These new studies, in combination with the results presented here (Chapters 5-7), can help disentangle which classes of frugivores have been most important in the evolution and maintenance of current patterns of secondary metabolite occurrence. In addition, to help address the evolutionary origins of fruit secondary metabolites, I have an ongoing long-term study with the ultimate goal of provide a phylogenetically-controlled comparative examination of secondary chemistry in leaves, unripe fruits, and ripe fruits across different *Piper* species from different habitats (early-succession, mid-succession, and mature understory), with different primary modes of reproduction and dispersal (e.g. asexual cloning, bird-dispersed, bat-dispersed), and that experience different amounts of pressure from seed predators and pathogens that cause seed damage. This dataset will provide important insights into the relative importance of different selective pressures (from antagonists and seed dispersers) and constraints (both physiological and phylogenetic) in determining patterns of fruit secondary metabolite occurrence.

8.5 Conclusions

Fruit secondary metabolites are important plant traits that can mediate both mutualistic and antagonistic interactions and have a strong influence on plant reproductive success. Because the same chemical trait can provide adaptive benefits in some interactions, but be ecologically costly in other interactions, understanding the ecological role of fruit secondary metabolites is complex and requires a range of different approaches. Understanding the evolution of fruit secondary metabolites is perhaps even more complex, because one must consider not only the simultaneous selective pressures on fruit chemical traits, but the potential constraints that limit the action of selection, including correlated selection in different plant parts and past selective regimes in common ancestors of a plant lineage. Working through these challenges will provide important new insights and advance theory in several areas of ecology and evolution, including chemical ecology, plant defense, frugivory and seed dispersal, and community ecology.

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