Intraspecific phytochemical variation as mosaics of defense in tropical forests

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Ecology, Evolution, and Conservation Biology

by

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Abstract

This dissertation research contributes to understanding the causes of phytochemical variation in natural ecosystems and the effects of that variation on herbivores. Phytochemically mediated interactions are common in biotic communities, and include important associations between plants and herbivorous insects. Variation in chemistry within a host plant species has been a focus of plant defense theory because increases in concentrations or complexity of phytochemical mixtures can influence the development, diversity and evolution of herbivores by creating mosaics of defense that regulate the densities of herbivores across a geographic landscape. To test hypotheses relevant to plant defense theory, I examined determinants of phytochemical variation and whether intraspecific variation creates mosaics of chemical defense for associated arthropod communities. Here, I review theory on plant defense and summarize clear knowledge gaps then I propose the Soil Mosaic Hypothesis, which posits that multi-trophic diversification can result from soil heterogeneity across the geographic landscape. The empirical data presented in this dissertation include three studies that address the causes and consequences of plant defense for a tropical shrub, *Piper* (Piperaceae) and associated insect communities. First, I compared the magnitude of effects of resource availability versus herbivore-induced defenses on variation in defensive phytochemistry. Next, I documented patterns of phytochemical variation on community and population structure of specialist herbivores using one host plant species, *P. kelleyi* and the specialist geometrid caterpillars on that plant. Finally, I established paired *P. kelleyi* cuttings at different canopy heights across an elevational gradient to manipulate heterogeneity in the quantity and quality of light availability. For this final study, phytochemical variation was quantified and the effects of light on chemical defense were measured as a function of herbivory and development in specialist and generalist herbivores. I found that abiotic and
biotic factors interact to influence the phytochemical profile of plants. In particular, light heterogeneity significantly contributed to changes in phytochemistry across small spatial scales and had cascading effects on specialist herbivores. Overall, subtle differences in phytochemical variation across the geographic landscape have consequences on the performance, diversity, and evolution of specialist herbivores and were undoubtedly part of the impressive diversification of herbivorous insects.
Dedication

For my mom, dad, and Danny. Without their love, support and guidance, I would not be the person I am today

For Joshua Snook, who always believes in me and encourages me to continue in spite of challenges. Thank you for your unconditional love throughout this process.

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Introduction ~ The causes and consequences of intraspecific phytochemical variation

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The defensive nature of plant secondary metabolites

Plants are primary producers for a broad array of consumers, but most plants are inedible for a large proportion of animals that encounter them (Fraenkel 1959). Consumption of leaves includes intake of amino acids and carbohydrates necessary for the nourishment of herbivores, but at the expense of feeding on toxic or physiologically damaging secondary metabolites (Fraenkel 1959; Whittaker and Feeny 1971). Secondary compounds are not responsible for growth or reproduction, which is the function of primary metabolites, such as proteins, carbohydrates, lipids, and nucleic acids (Fraenkel 1959; Whittaker & Feeny 1971). Mixtures of secondary metabolites are unique to different plant species, and a quantified assemblage of these compounds is analogous to a chemical fingerprint for individual plants. The hypothesis that secondary metabolites are herbivore defensive compounds (Dethier 1954; Fraenkel 1959) due to the staggering evidence of biological activity found in many plant families (Fraenkel 1959) is a paradigm in chemical ecology. Potential evidence against this hypothesis is that secondary metabolites serve other purposes, such as UV protection and drought tolerance (Whittaker & Feeny 1971), and not all compounds demonstrate biological activity against herbivores, but rather are a byproduct of enzymatic activity (Firn 2010). Understanding the defensive nature of secondary metabolites is a central goal of chemical ecology (Ehrlich & Raven 1964; Feeny 1975; Berenbaum 1995; Stamp 2003; Agrawal & Fishbein 2006; Agrawal 2007; Campbell & Kessler 2013; Dyer et al. 2017).

Plant defense theory has evolved and matured and has helped elucidate the evolution and function of secondary metabolites. The underlying theme for plant defense
hypotheses is that plants have adapted physical and chemical defenses to regulate herbivores since plants are sessile and cannot move to defend themselves. Several complementary hypotheses have guided the research on the theory of plant defense, most notably the optimal defense hypothesis (McKey 1979; Rhoades and Cates 1976; Zangerl and Rutledge 1996), growth-rate or resource availability hypothesis (Coley et al. 1985; Coley 1987), growth-differentiation balance hypothesis (Loomis 1953; Herms and Mattson 1992). These theoretical frameworks are all guided by the assumption that resource availability and herbivory exert selective pressures on phytochemistry and that defense is costly. The optimal defense hypothesis posits that plant traits associated with higher fitness are those that provide defensive efficacy against herbivores (McKey 1979; Rhoades and Cates 1976; Zangerl and Rutledge 1996). The growth-rate or resource availability hypothesis postulates that there is a balance between investment in growth versus defense, and this balance is dependent on resource availability (Coley et al. 1985; Coley 1987). Resource availability hypotheses predict that in nutrient-poor environments plants grow slower and are better defended than in nutrient-rich environments, in which plants grow faster with lower investment in phytochemical defenses (Coley et al. 1985). The growth-differentiation balance hypothesis suggests plant resource allocation is balanced between growth of roots, stems and leaves vs. differentiation of secondary functions and maturation, such as physical and chemical defenses (Loomis 1953; Herms and Mattson 1992). Environmental resource availability gradients are a key component of this hypothesis, rather than a conceptual dichotomy of high- and low- nutrients. Additionally, resources encompass nutrients, water and light and the interactions of variation in these resources can neutralize or exacerbate deficiencies. These hypotheses
are problematic because costs are hard to measure and empirical data have not adequately tested these hypotheses due to poor experimental design or limited inference, making results inconclusive and confusing (Stamp 2003). Berenbaum (1995) suggests a universal theory of plant defense may be impractical, while Stamp (2003) contends these are simply immature theories. However, the maturation of plant defense theory may soon come to fruition with the advent of technological advances in genetics (Sumner et al. 2003; Fridman & Pichersky 2005; Dunn et al. 2013) and approaches that combine novel statistical approaches to spectral data (Richards 2015; Dyer et al. 2017).

Plant defense theory may be immature because the framework is narrowly focused on bi-trophic interactions happening at a single location, rather than more reticulate food webs across locations. Thompson’s (1999, 2005) influential geographic mosaic of coevolution addresses the fact that traits change across the geographic landscape, influencing associated biotic interactions. He emphasizes that local interactions can be stronger or weaker depending on the genetics of interacting species and local environmental conditions. For plant traits, greater spatial heterogeneity increases the likelihood of variation in plant quality between and among plant populations (Hunter et al. 1996; Hunter 2016). Phytochemical variation is dependent on available resources, presence of herbivores and their associated natural enemies, which inherently changes across the geographic landscape. Both “top-down” and “bottom-up” pressures affect community interactions (Hunter & Price 1992, Schmidt et al. 1997, Strong & Polis 1996, Letourneau & Dyer 1998; Pace et al. 1999) and create considerable variation in plant quality (Dyer et al. 2004; Wetzel et al. 2016). Many herbivores and natural enemies are mobile and their dispersal abilities are impacted by variation in plant
The overall aim of my dissertation research is to investigate the causes and consequences of intraspecific phytochemical variation on the physiology, abundance, and diversity of specialist consumers. The complete work involves integrated interdisciplinary research on natural products, novel techniques for secondary metabolite extraction and isolation (e.g. NMR, GC-MS, and HPLC), and genomic analyses (e.g. genotyping-by-sequencing via the Illumina platform) to address ecological and evolutionary questions. The focal plant genus for my dissertation research is *Piper* (Piperaceae), which is a pantropical, species-rich genus of small trees, shrubs and vines that has been used as a model system to test major hypotheses in chemical ecology (Dyer & Palmer 2004). *Piper* is ecologically and economically important, and closely related species have very different chemical profiles that are comprised of multiple classes of compounds (e.g. alkaloids, chromenes, alkenyl phenols, terpenoids) (Dodson et al. 2000; Fincher et al. 2008; Whitehead et al. 2013; Jeffrey et al. 2014). The remarkable diversity of secondary metabolites in *Piper* is associated with important biological activities (Kato & Furlan 2007), such as antitubercular (Diaz et al. 2012), antifungal (Navickiene et al. 2000; Silva et al. 2002), and anti-herbivory (Dyer et al. 2003; Glassmire et al. 2016) effects. For my dissertation, I used *Piper* and associated specialist caterpillars from the genus *Eois* (Geometridae) to investigate the effects of phytochemical variability on assemblages of specialist herbivores using observational and experimental approaches. The complete research focuses on these two objectives:

1. Examine the mechanistic basis of phytochemical plasticity.
2. Examine the magnitude of effect of phytochemical plasticity on associated arthropod communities.

*Light heterogeneity as a mechanistic basis of phytochemical variation*

Light is a limiting resource in tropical forest understories. Montgomery & Chazdon (2001) documented that shade-tolerant plant species can differentially respond to radiation gradients in low light environments (0.2-6.5% diffuse transmittance) by allocating biomass towards below- or above-ground tissue. In addition to growth, light gradients can directly affect secondary metabolism. High light conditions cause an accumulation of carbohydrates during photosynthesis that can be allocated towards secondary metabolism with low cost to the plant (Herms and Mattson 1992). Alternatively, low light conditions may result in metabolic competition for available fixed carbon between molecules required for plant growth and those directed towards secondary metabolism (Veihmeyer and Hendrickson 1961, Mooney and Chu 1974 – *reviewed in* Stamp 2003). Other resources in the environment can interact with variation in light, such as mineral nutrient and water availability; for example, low light can exacerbate a deprivation of or overabundance of mineral resources. It is reasonable to hypothesize that phytochemical variation among populations is dramatically influenced by light availability. Since light changes across small spatial scales, it is likely to contribute to geographic mosaics in plant quality, thus light can be a factor that affects the interactions between plant chemistry and associated herbivores and natural enemies.
The interplay of light heterogeneity with abiotic and biotic pressures on the phytochemical profile of *Piper* plants is a central theme of my dissertation. I experimentally manipulated light to mimic gap and understory light levels to test how this influences variation in growth, protein production and defensive plasticity in individuals of the lowland neotropical shrub *Piper imperiale* (Chapter Two). However, focusing exclusively on comparisons of light levels in gaps versus the forest understory ignores the variation in available radiation beneath the closed canopy. Thus, canopy openness was measured along an elevational gradient, covering the known elevational range of *Piper kelleyi* (1800m-2400m) to examine how light along a continuum influences secondary metabolite production (Chapters Three and Four). I also manipulated the height of *P. kelleyi* plants in the canopy to simulate light availability close to the canopy versus closer to the understory (Chapter Four). Finally, I experimentally tested for photactivity in the defensive compounds of *P. kelleyi* and whether caterpillars exhibited decreased performance, detoxification, or sequestration in response to enhanced toxicity. Together, these studies help elucidate how variation in light availability influences intraspecific phytochemical profiles along a geographic gradient.

*The magnitude of effect of phytochemical variation on the arthropod community*

Phytochemical variation is interesting when examined in the context of its consequences on entire communities (Hunter 2016; Dyer et al. 2017). Plant quality is a key determinant of herbivore performance and is determined by both nutritional
chemistry and secondary metabolites (Wetzel et al. 2016). Multiple studies have indicated that subtle differences in phytochemistry can affect oviposition, survival, pupal weight or development time of herbivores (e.g., Prudic et al. 2005; Dyer et al. 2003; Richards et al. 2012). Furthermore, phytochemistry can affect an herbivore’s ability to defend itself against parasites, including parasitoid wasps or flies (Barbosa & Caldas 2007; Bukovinszky et al. 2009; Smilanich et al. 2009; Smilanich et al. 2011; Lampert 2012; Kaplan et al. 2016) and aid in parasitoid dispersal between populations varying in nutrition (Huffacker 1958; Helms & Hunter 2005; Hunter 2016).

Understanding the magnitude of effect of intraspecific phytochemical variation and diversity on insects is a theme throughout my dissertation. The Soil Mosaic Hypothesis (Chapter One) suggests that differences in soil attributes can affect intraspecific variation in phytochemistry, cascading to ecological and evolutionary effects on higher trophic levels. Plant quality was experimentally manipulated by varying resource availability and subsequent effects on herbivory were quantified (Chapter Two). At the population level, I quantified correlations between intraspecific phytochemical variation among plant populations and the genetic structure of specialist herbivores (Chapter Three). At the community scale, correlations were examined between phytochemical diversity and the presence of certain caterpillars and associated parasitoid wasps (Chapter Three). Finally, light heterogeneity as the mechanism causing phytochemical plasticity in P. kelleyi was experimentally manipulated and subsequent effects of herbivory and performance of specialist and generalist herbivores were evaluated (Chapter Four). Together, these studies helped elucidate the mechanisms by
which phytochemically-mediated interactions can influence herbivore performance and drive diversity in higher trophic levels.

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Chapter 1 ~ The soil mosaic hypothesis: a synthesis of multi-trophic diversification via soil heterogeneity

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Abstract

A myriad of unexplored mechanisms potentially drives ecological speciation and could help explain global variation in diversity. Here, we develop a novel hypothesis focused on variation in biotic, chemical and physical properties of soil as a factor contributing to diversification in communities of plants and animals. The Soil Mosaic Hypothesis (SMH) suggests that differences in soil attributes can affect intraspecific variation in phytochemistry, leading to cascading ecological and evolutionary effects on higher trophic levels. To illustrate the potential importance of the SMH, we examine three underlying ideas: (1) plant species and species assemblages shift over time, exposing them to novel soil environments, which can lead to genetic differentiation; (2) differences in soil properties can alter phytochemistry via plasticity and local adaptation; (3) phytochemistry can drive herbivore diversification via divergent natural selection (i.e. ecological speciation). The SMH provides insight into the process of diversification in a variety of landscapes and at a variety of scales and may inform analyses of diversification at local, regional, and global scales.
Introduction

The idea that ecological interactions can influence evolution has been a major component of evolutionary theory since Darwin proposed natural selection (Darwin 1859), and it is clear that ecologically based divergent natural selection is an important model of speciation (reviewed by Rundle and Nosil 2005, Nosil 2012). Ecological speciation occurs when populations are exposed to contrasting environments and ecologically-based divergent natural selection promotes (either directly or indirectly) the evolution of reproductive isolation. Divergent selection can result in differences in morphology, behavior, or other phenotypes, potentially leading to genetic isolation between populations comprised of ecologically specialized individuals (Schluter and McPhail 1992, Rundle and Nosil 2005, Matsubayashi et al. 2010, Soria-Carrasco et al. 2014). Research on ecological speciation has benefited from detailed studies of well-understood populations or taxa associated with different resources or habitats (e.g., Schluter and McPhail 1992, Rundle et al. 2000, Nosil et al. 2008, Matsubayashi et al. 2010, Nosil 2012, Soria-Carrasco et al. 2014), but there are still many unexplored axes of ecological variation that potentially contribute to diversification (Coley et al. 1985, Thompson 2005, Whitham et al. 2008, Dyer et al. 2014). Here, we consider a previously understudied potential source of divergent natural selection: variation in soils and associated effects on phytochemistry and plant and animal community structure.

The Soil Mosaic Hypothesis (SMH) posits that differences in soil properties (i.e. biotic, chemical and physical factors) can affect individual variation in plant primary and
secondary metabolites, yielding a highly variable phytochemical landscape (*sensu* Hunter 2016) and leading to cascading ecological and evolutionary effects on autotroph and consumer trophic levels. Soil heterogeneity could lead by direct and indirect mechanisms to reproductive isolation in plant and herbivore populations. A direct mechanism could be, for example, adaptation by herbivores to plant populations with divergent phenology causing a shift in herbivore phenology (emergence time or peak abundance) that immediately isolates consumer populations. A less direct mechanism could be local adaptation to phytochemistry, and associated selection against hybrids that are maladapted to either of the chemical profiles experienced by the parents. The SMH is not entirely novel: in addition to theories of ecological speciation and host-associated differentiation (Stireman et al. 2005), the SMH can be considered a corollary to the following well-established theories: 1) coevolution (Ehrlich and Raven 1964, Berenbaum and Feeny 1981, Agrawal et al. 2012), 2) the geographic mosaic (Thompson 1999, Thompson 2005), 3) plant defense theory (Bryant et al. 1983, Coley et al. 1985, Stamp 2003, Massad et al. 2011, Smilanich et al. 2016), 4) effects of environmental heterogeneity on communities and ecosystems (Ricklefs 1977, Whitham et al. 2006, 2008), 5) the phytochemical landscape (Hunter 2016), and 6) plant-soil feedbacks (van Breeman and Finzi 1998, van der Putten et al. 2013, Schweitzer et al. 2014). The utility of the SMH is that it utilizes key components of existing theory that are usually limited in scope to average levels of chemical defense and bi-trophic interactions to provide a focused, testable framework that includes a new perspective on phytochemical diversity, multi-trophic interactions, and abiotic selective drivers of diversification. Natural systems are adaptive landscapes of complex community dynamics. The SMH integrates
both below- and above-ground processes when assessing patterns of ecological divergence speciation. We examine three underlying postulates (Fig. 1): (1) plant species and communities are exposed to diverse soil environments across multiple spatial and temporal scales, which can lead to divergence in plant populations; (2) differences in soil properties can alter plant primary and secondary metabolites; and (3) variation in phytochemistry can drive herbivore diversification via ecological speciation at fine geographic scales.

While these postulates could be used to help understand the link between soils, phytochemistry, and diversification in any ecosystem, such mechanistic relationships may be particularly evident in regions that encompass extreme habitat variation (e.g., serpentine soils, white-sands versus clay soils, dry versus wet tropical forests, or mountains) and which have a unique combination of soil diversity, movement dynamics, and biotic interactions. Below we will discuss each of the three aspects of the SMH to illuminate the process by which consumers adapt to phytochemical variation driven by changes in soil chemical and physical properties.

1. *Plants disperse to novel soil environments.* Because the chemical and physical properties of soil are influenced by associated climate, parent material, topography, time, and biotic communities (Laliberté et al. 2013, van der Putten et al. 2013), distinct soil types can occur in close proximity, leading to a soil mosaic (Sollins et al. 1994). Soil mosaics characterize many landscapes at different spatial scales, and soil formation models predict that tropical soils in particular should have extreme heterogeneity in soil properties (e.g., Jenny 1980).
When plant ranges shift due to climatic changes or other factors, populations are likely to encounter novel soils because of the heterogeneity of most soil landscapes. Ecological processes that promote dispersal will also lead to an increased likelihood that a plant will disperse onto a soil with attributes that are different from the parent plant. Neotropical trees, for example, often experience long distance dispersal (Ward et al. 2005). The lowland tapir, *Tapirus terrestris*, acts as an important long distance disperser of the tropical palm *Maximiliana maripa*. Tapirs disperse palm seeds as far away as 2 km from parent trees, increasing survival rates of seeds to 98% for those that are dispersed compared to 17% for seeds close to parent trees (Fragoso 1997; Fragoso et al. 2003). Long distance dispersal events increase the probability of offspring shifting to a different soil type than the parent and could result in offspring with different phytochemical or nutritional properties from their parents, leading to increased morphological and genetic divergence (e.g., Barbosa et al. 2013, Misiewicz and Fine 2014).

2. **Soils affect phytochemistry.** Changes in biotic and abiotic factors, including soil microbes and nutrients, are known to cause significant changes in plant chemistry, and the magnitude of these changes are likely to affect many biotic interactions that are mediated by chemistry (Hunter and Price 1992, van Breeman and Finzi 1998, Dyer et al. 2004, Massad and Dyer 2010). There is a vast literature on the plasticity of plant secondary metabolites, and while there is little consensus on the directions and magnitudes of these responses to changes in soil nutrients, there is ample evidence of large shifts in phytochemical profiles in response to soil nutrient variation (*reviewed by* Massad et al. 2012).
Phytochemical diversity maintains plant function and fitness across diverse environments, affecting herbivore communities (Richards et al. 2015, Glassmire et al. 2016) and habitat specialization by plants (Fine et al. 2013). Phytochemical plasticity could be more beneficial when there are resource pulses or outbreaks of herbivory in resource-limited environments, and empirical studies have shown that plants allocate more resources to defense versus growth under such conditions to prevent the loss of leaf tissue, which is costly to replace (reviewed in Endara & Coley 2011). Thus, the adaptive nature of phytochemical plasticity can depend on resource availability and the presence of biotic interactions (Coley et al. 1985, Dyer et al. 2004, Hunter 2016). Similarly, the growth-defense trade-off hypothesis suggests plants have different phytochemical defense strategies across soil gradients (Coley et al. 1985), potentially promoting soil specialization and adaptation over evolutionary time (Fine et al. 2013).

Thus, soil mosaics can provide an adaptive landscape promoting edaphic specialization and plant diversification. For example, patterns of spatial genetic structure in the tropical tree Protium subserratum (Burseraceae) in the Ducke Reserve Brazil are significantly influenced by soil type, which is highly heterogeneous, with soil clay composition ranging from 2% to 80% in a 250 meter area (Barbosa et al. 2013). Edaphic specialization has been posited as a mechanism of diversification for multiple plant lineages; two prominent examples are diversification of Protieae species (Burseraceae) shifting from clay to sand soils (Fine et al. 2005, Fine et al. 2014) and endemism of streptanthoid species (Brassicaceae) transitioning from bare to serpentine soils (Cacho and Strauss 2014). Furthermore, studies have shown that some populations of Mimulus
*guttatus* monkeyflowers (Family: Phrymaceae) have adapted to the copper-rich soils near copper mines, resulting in ecological speciation (Macnair and Christie 1983).

3. **Phytochemistry affects herbivore diversity.** While diversification in herbivorous insects is often thought to involve shifts in host plant use (e.g., Ehrlich and Raven 1964, Powell et al. 2013, Soria-Carrasco et al. 2014), recent evidence from a diverse tropical system raises the possibility that diversification can also occur without host shifts and also without major geographic barriers, supporting the hypothesis that intraspecific variation in phytochemistry may play a role in insect diversification at relatively small spatial scales (Wilson et al. 2012, Glassmire et al. 2016). Furthermore, intraspecific variation in edaphic-associated phytochemistry sheds light on the documented phenomenon that distinct insect communities are associated with soil ecotypes, as in *P. subserratum* (Fine et al. 2013). It is well known that phytochemical variation can influence insect herbivores by affecting oviposition preference (Carlsson et al. 2011), larval performance, mortality (Richards et al. 2010), and the ability of an herbivore to defend itself against predators and parasitoids (Smilanich et al. 2009). The SMH suggests that as plants experience new soil environments and respond with altered phytochemical properties, associated herbivore communities will experience strong selection pressure based on these new phytochemical environments, which can lead to diversification and speciation. Richards et al. (2015) found that phytochemical variation affects entire host-associated communities, including the diet breadth and diversity of herbivores.
Future Studies

The three main tenets of the SMH described here were inspired by pondering the theoretical framework of the phytochemical landscape (Hunter 2016) as well as considering our own work showing evidence of rapid diversification within one genus of herbivores (Eois, Geometridae: Larentiinae) that includes multiple sister species consuming the same host plant species in close geographic proximity (Wilson et al. 2012, Glassmire et al. 2016). The SMH incorporates a combination of ecological and evolutionary processes associated with plants colonizing novel soils, followed by diversification of taxa at higher trophic levels. Below we provide several examples of future studies that would test specific hypotheses generated by the SMH to elucidate how soil interacts in a multi-trophic framework.

First, transplant studies should be conducted to examine how differences in soil nutrient availability influence phytochemical profiles, and how this impacts performance of the associated arthropod communities (Fine et al. 2013). These studies should be accompanied by feeding assays to examine herbivore preference and performance on phytochemically distinct plants. Second, controlled experiments should investigate the mechanisms by which soil resource availability affects phytochemical plasticity in the presence of natural enemies. This would involve a fully-crossed experimental design including manipulated abiotic (addition of soil resources) and biotic factors (exclusion of herbivores and natural enemies). Associated with these manipulations of soil resources, the richness and abundance of soil biotic properties could be manipulated to examine the influence of soil biotic diversity on phytochemical diversity. For example, one could
experimentally alter the diversity of arbuscular mycorrhizal fungi, bacteria, and soil arthropods, and quantify differences in phytochemistry. One important response variable for both types of soil manipulation experiments is the concentration of individual secondary metabolites, allowing for responses to these key questions: 1) How important are soil resources, arthropod communities, and the interaction between these factors for structuring plant secondary metabolomes? 2) What are the norms of reaction for individual secondary metabolites and phytochemical diversity in response to soil nutrients and arthropods? 3) Are any metabolites fixed with respect to variation in soil and arthropods? In experiments of this kind, arthropod communities can both be manipulated as treatments and measured as response variables, including behavior (e.g., oviposition preferences) of focal herbivores.

Finally, as an extension of the SMH, future studies could investigate the possibility of plant defensive profiles becoming fixed by genetic assimilation (Waddington 1953, Crispo 2007). If the colonization of a novel edaphic environment results in the developmentally-plastic production of a distinct phytochemical profile that is favored by natural selection, theory suggests that the novel phenotype could eventually become fixed because plasticity to produce that phenotype would be selected against at that location. An interesting outcome of this process would be the retention of phytochemical diversity at the species or metapopulation scale (Fig. 1), even in the face of range shifts associated with climatic fluctuations, since the previously plastic phenotype is fixed. The conversion from plasticity to fixed phytochemical diversity also opens the possibility for another mechanistic component of classic coevolutionary dynamics between plants and herbivores. While the potential importance of genetic
assimilation for micro and macroevolutionary processes is well recognized (West-Eberhard 2003, Ehrenreich and Pfennig 2016), we know very little about the potential for assimilation to affect phytochemical phenotypes or associated arthropod communities. Perhaps the best systems for utilizing this approach would be well-resolved foundation species, such as *Populus*, for which there are documented networks of interacting soil microbes and herbivore communities, as well as documented effects from genes to ecosystems (Whitham et al. 2006, 2008, Lau et al. 2016).

Conclusion

Variation in phytochemical profiles can arise in plants following the colonization of novel soil types (Fine et al. 2006, Fine et al. 2013, Cacho and Strauss 2014), allowing for phytochemically-associated adaptation and divergence to occur in herbivores at a fine geographic scale (Glassmire et al. 2016). Future studies investigating previously unrecognized mechanisms of diversification, such as the processes comprising the SMH, will shed light on the origin and maintenance of biodiversity. Testing this hypothesis should be a part of the general goal to understand the extent to which ecological processes influence diversification in a multi-trophic framework.

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**Figure legend**

**Figure 1.** A graphical model of the Soil Mosaic Hypothesis: 1) Soils are heterogeneous and plant populations move; 2) Soils affect phytochemistry, creating subpopulations with different chemistry; 3) Phytochemistry affects herbivore diversification. The *first column* shows that plants (open circles) move across a landscape over time. The *second column* shows how soils with distinct characteristics (shaded regions) influence plant phytochemistry. Plastic changes in phytochemistry are represented by different symbols within each circle – these changes can also be followed by genetic assimilation. The *third column* shows how herbivores track the movement of plant subpopulations over time. The pattern on the wings of the herbivores represents divergent characters that are linked to adaptations to unique phytochemical profiles of associated host plants.
Figure 1
Chapter 2 ~ Phytochemical variation in a tropical shrub: herbivory versus light and soil heterogeneity

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Abstract

Phytochemical variation within plant species has direct consequences on the performance of herbivores and their natural enemies. Environmental heterogeneity and biotic interactions interact to shape phytochemistry across both evolutionary and ecological time scales, especially in mega-diverse tropical ecosystems. We hypothesized that resource availability would promote ecological trade-offs in growth and defense of phytochemistry in neotropical shrub, *Piper imperiale* (Piperaceae) and contrasted the effect sizes on chemistry versus the popular view that herbivores induce great increases in chemical defense. We experimentally manipulated light availability, soil type, herbivory, and fertilizer additions and quantified the resulting variation in plant growth and defensive phytochemistry. Our results supported the “resource availability hypothesis” more than the “induced defenses paradigm” in that there was a trade-off between growth and defense in phytochemistry, depending on resources manipulated and the strength of interactions with specialist herbivores. Substantive changes in foliar protein were due to an interactive effect of soil and fertilizer, primarily in high light conditions. The concentration of the defensive amide, piplaroxide, decreased with herbivory exclusion, but only for specific soil types and in low light conditions and the effect sizes were not as large as resource manipulations. Interestingly, a defensive alkene amide did not increase with enhanced herbivory, nutrient availability or light heterogeneity. Thus, for *P. imperiale*, variation in plant defenses in response to herbivory and resource availability were mixed, depending on the defensive compound. Plants are exposed to a mosaic of potential selective pressures due to light heterogeneity and variable biotic interactions across small spatial scales, and this variation can contribute to
phytochemical profile differences across a landscape. Plant defensive adaptations in tropical ecosystems may rely more on constitutive defensive chemistry, as opposed to the variation commonly found in less diverse ecosystems or in monocultures.

**Introduction**

A central goal in biodiversity studies is to understand the evolution of phytochemistry because it can structure community interactions between consumers and their associated natural enemies (Coley et al. 1985, Dyer et al. 2010, Kessler et al. 2004, Agrawal 2011, Hunter 2016). Plant secondary compounds mediate interactions with consumers (e.g., herbivores), predators (e.g., parasitoids), mutualists (e.g., pollinators), and even resources (i.e. by affecting nutrient availability via soil microbes). The relative abundance of individual compounds can respond plastically to abiotic and biotic factors including UV light (Zangerl & Berenbaum 1987, Bassman 2004), presence of mutualists (Dodson et al. 2000), herbivory (i.e. “induced defenses”; Zangerl 1990, Agrawal 1998, 2011; Karban & Baldwin 2007), interactions with parasitoids (James 2003; James & Price 2004, Kessler et al. 2004; Kessler & Halitschke 2007) and soil nutrient quality (Zangerl & Berenbaum 1987, Dyer & Letourneau 1999, Prudic et al. 2005). These pressures can interact to create a heterogeneous phytochemical landscape of unique niches and microhabitats, promoting diversity of interactions at larger scales (Dyer et al. 2010, Glassmire et al. 2016, Pardikes et al. *in prep*). Traditionally, studies focus on only one of these factors in isolation, yet in a natural setting biotic and abiotic factors interact, resulting in unpredictable effects on plant chemistry and associated interactions (*reviewed in* Hunter 2016).
Many studies of phytochemical variation focus solely on changes in selected (sometimes only one) defensive compounds, and only a few interpret these changes in the context of nutrition (Fine et al. 2006; Wetzel et al. 2016). Examining both primary and secondary metabolites is important because variation in nutrients and defensive compounds within a plant population can reduce performance of mobile herbivores (Helms & Hunter 2005, Underwood 2009, Riolo et al. 2015, Wetzel et al. 2016). Furthermore, both nutritional and defensive compounds can be instrumental in attracting parasitoids that control densities of herbivores (Thaler 1999; James 2003; James & Price 2004; Kessler et al. 2004; Kessler & Halitschke 2007; reviewed in Poelman et al. 2008). Quantification of the phytochemical landscape based upon the full spectrum of ecosystem pressures is lacking in many systems (Hunter 2016), and it is a worthwhile goal to characterize the phytochemical landscape to predict the magnitude of effects on interacting trophic levels across resource gradients (Hunter 2016, Dyer et al. in review).

Plant resource gradients play a major role in creating heterogeneous phytochemical landscapes, and a contributing factor, especially in tropical forests, is light availability. Light is substantially variable in the closed understory, ranging from 0.2-6.5% diffused transmittance (Montgomery & Chazdon 2001). Small changes in light availability in the understory can significantly influence growth and biomass allocation between above- and below-ground plant tissue in shade-tolerant plants (Montgomery & Chazdon 2001). Habitats consisting of greater light availability are predicted to have high primary productivity and associated changes in secondary metabolites due to increased photosynthetic availability (Pace et al. 1999, Oksanen et al. 1981, Rosenweig 1971, Fraser and Grime 1997, 1998, Forkner and Hunter 2000, Letourneau and Dyer 1998,
Uriarte and Schmitz 1998). These changes in plant physiology can affect upper trophic levels. For example, Richards & Coley (2007) found greater herbivore abundances that supported higher densities of parasitoids in gaps, because they were more productive than shade habitats due to the high quality of mature leaves and greater availability of young leaves for consumption (Richards & Coley 2007). Several studies have also found that herbivore abundances increase in high light environments compared to shade (Shure and Phillips 1991, Louda and Rodman 1996, Jokimaki et al. 1998, Sipura and Tahvanainen 2000). Thus, differences in light availability across small spatial scales could lead to dramatic changes in trophic interactions (Richards & Coley 2007, Ballaré et al. 2011).

Thompson (1999) described populations having varying degrees of community interactions across a geographic mosaic, and some of this variation is caused by phytochemical variation. Plant interactions with herbivores and their associated enemies could be stronger or weaker in different microhabitats, and this is largely dependent on resource availability (i.e. growth-defense trade-off hypothesis, Coley et al. 1985). Many studies have investigated the ecological and evolutionary consequences of variation in plant resource availability. For example, Fine et al. (2006) found that plant species specializing in white-sand or clay soils demonstrated divergent defense strategies; white-sand specialists invested in higher concentrations of defensive compounds (e.g. terpenes, phenolics and leaf toughness), while clay specialists were characterized by greater leaf area and height. Furthermore, the defensive strategy of these soil endemic plants predicted the assemblage of the herbivore community (Fine et al. 2006). Similarly, Cacho et al. (2015) found greater numbers of glucosinolate compounds in plants endemic to bare, nutrient deprived, serpentine soils, supporting Coley et al.’s (1985) resource
availability hypothesis. Greater mineral resource availability found in some soils are allocated towards plant growth and defense, and understanding how light interacts to potentially offset limitations by nutrient-depleted soils or drought conditions is an important component of resource availability studies (Loomis 1932, 1953; Herms and Mattson 1992). Despite considerable progress in understanding phytochemical plasticity (Agrawal 1999; Hunter 2016), a knowledge gap remains for many well studied species: what is the magnitude of the effect of the interaction between light and resource availability on the phytochemical profile of shade-tolerant plants?

In this study, we quantified phytochemical variation in plants for which we had manipulated resource availability and herbivory to understand how abiotic and biotic pressures influence changes in plant chemistry. Our fully crossed shade house experiment included manipulations of resource availability and herbivory for cuttings of *Piper imperiale* (Piperaceae), which is a shade-tolerant, herbaceous plant that is abundant in the understory in Costa Rican wet forests (Dyer & Palmer 2004; Salazar et al. 2016). *Piper imperiale* was selected to test our hypotheses because many of the details of the chemical ecology of this species and associated herbivores have been elucidated (e.g., Fincher et al. 2008, Dyer and Palmer 2005). This design allowed for a direct comparison of the effects of resources versus herbivory on phytochemical variation. We addressed the following questions: (1) What are the relative effects of mineral resources, light, and herbivory on the abundance and evenness of plant defensive compounds within a species? (2) How do these factors affect the nutritional value of plant tissue, as measured by foliar protein? (3) Are there interactive effects of defense and nutrition on plant interactions with herbivores? (4) Is there a trade-off between growth and defense,
depending on resource availability? We predicted that: 1) plant biomass will decrease in low light environments and clay soils, 2) plant defenses will increase in high light environments and clay soil, 3) low light and loam soils will have the highest foliar protein, 4) clay soils and herbivory will have the highest defensive compounds. Results from our experiments were combined with field observations to help understand how variation in plant chemistry may affect local diversity of arthropods associated with the focal plant species in the rain forest understory.

Methods

Study system and shade house experiment

The shade-house experiment began in June 2013 at La Selva Biological Station (10.4306° N, 84.0070° W), Costa Rica where 54 co-occurring species of *Piper* (Piperaceae) form an important component of both early- and late-successional forests (Dyer and Palmer 2004; Gentry 1990). The phytochemical profile of *P. imperiale* includes three amides: 5’-desmethoxydihydropiplartine, piplaroxide, and an alkene (Fincher et al. 2008) as well as at least five different sesquiterpenes (Fincher et al. 2008). Compared to other *Piper* species, *P. imperiale* invests in relatively lower amounts of defensive compounds (e.g., 1.7% dry weight Fincher et al. 2008, Richards et al. 2015) and is correlated with lower parasitism rates on specialist *Eois* (Geometridae) caterpillars because of a stronger immune response as measured by PO enzyme activity (Hansen et al. 2016).
Fragments of *P. imperiale* were randomly collected throughout La Selva and established in pots for 1 month. Following establishment, plants were randomly assigned to soil type (loam / clay), fertilizer (high / low), and herbivore (present/absent) treatments in a fully factorial design. Loam and clay soil types were selected because of their different properties. Loam soils are comprised of sand, silt and small amounts of clay to create larger pore space that retains nutrients, air, and drains water effectively to provide a nutrient rich substrate for plants. Clay soils are composed of > 50% of clay particles that retain water but there is less water available to the plant; these soils are less productive substrates for plants compared to loam soils. Only one shade-house was used, so other treatments were nested within high and low light. Light was manipulated using shade-cloth and measured using H21-002 HOBO micro station data logger (MicroDAQ, Ltd., Contoocook, NH, USA) with two S-L1A-M003 PAR sensors to quantify natural sunlight; the average PAR measured between sunrise and sunset was 132 PAR for high light and 38 PAR for low light mimicking gap and understory light conditions. There was a total of 16 treatment combinations, with 10 fragments per cell in the design, for a total of 160 fragments (Fig. 1). Plants were rotated within the light treatment block every 2 weeks throughout the duration of the experiment. Plants were harvested in January 2014, six months after fragments were assigned to treatment combinations. Leaf Surface Area (LSA) of freshly harvested leaves was measured using LI-3100 Leaf Area (LI-COR, Inc., Lincoln, NE, USA). Leaf Area Removed (LAR) by herbivory was measured using LI-3100 Leaf Area (LI-COR, Inc., Lincoln, NE, USA). Plant stems, roots and leaves were separated and dried in a controlled oven set at 28 °C. The dry weight of plant stems, roots and leaves were recorded for biomass (g).
Leaf defensive compounds analysis

Dried plant leaves were ground using liquid N\textsubscript{2}. 1 g of dry plant leaf material was weighed and 5 mL of 1:1 MeOH/H\textsubscript{2}0 mixture and 5 mL of CHCL\textsubscript{3} were added. The sample was vortexed for 30 seconds, sonicated for 30 minutes, and centrifuged for 5 minutes. The liquid-liquid extraction produced an aqueous and organic layer. The organic layer contained the alkene and piplaroxide defensive compounds of interest. The organic layer was removed and filtered through cotton. The liquid-liquid extraction was repeated twice more. Following the third and final extraction 1 mL of CHCL\textsubscript{3} was used to wash cotton and the sides of the glass filter. The remaining solvent of the filtered organic layer was dried with N\textsubscript{2} gas. The extract was placed in a -80°C freezer until it was quantitatively analyzed by Gas Chromatography – Flame Ionization Detector or GC-FID (Agilent 7890A) using commercially available piperine as an internal standard at the 80 ppm/ml level.

Three samples from each of the sixteen shade-house treatments were randomly selected for quantification using seven point calibrations; samples were replicated three times. Seven point calibrations (11.72, 23.44, 46.88, 93.75, 187.5, 375, and 750 ppm/ml) were prepared using synthetic alkene (R\textsuperscript{2}=0.999) and piplaroxide alkene (R\textsuperscript{2}=0.999) amides. Standards for analysis had been previously synthesized at Mesa State College in 2003. The alkene and piplaroxide are unstable in solution, so all concentrations we report are estimates (Dodson et al. 2000). The alkene and piplaroxide response units are given in mg per sample. For a more detailed description of extraction and GC methods see Dodson et al. (2000) and Dyer et al. (2001).
Leaf protein analysis

Total foliar protein concentration was quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). 2 mg of dry plant leaf material was weighed. 500 µL of buffer (100 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 1% of SDS) were added to each plant sample and vortexed. The mixture of buffer and plant sample incubated at room temperature for 1 hour and vortexed every 10 minutes. Following incubation, the samples were centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed from each sample and the pellet was discarded. 45 µL of H₂O and 5 µL of the supernatant were added to a 96-well plate; each sample was triplicated for accuracy. Six point calibrations (0, 4, 10, 20, 40 and 60 µg/mL) were prepared using albumin (R²=0.999); calibrations were replicated for accuracy. 200 µL of Reagent (50:1, Reagent A,B – Product #23228 and Product #1859078, respectively) was added to each sample on the well-plate, including the protein standard. The samples incubated for at least 10 minutes, but no longer than 30 minutes once the reagent was added. The samples and protein standard were analyzed using a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA, USA) measuring absorbance at 560 nm. Total protein concentration for each sample are given in protein mg per tissue mg.

Statistical analyses

Analyses were run separately for high vs. low light because other treatments were nested within the two levels of light treatment, due to the availability of only one shade-house. We conducted 3-way ANOVAs for each response variable. All analyses included
interactions between predictor variables. For response variables that were significant and intercorrelated (i.e., as in biomass or chemical compounds), multivariate analysis of variance (MANOVA) was used to examine responses of suites of plant traits to soil type, presence / absence of herbivory, and fertilizer additions. The independent variables were soil, fertilizer and herbivory treatments. The response variables for plant biomass were leaves biomass (g), stem biomass (g), and roots biomass. The response variables for plant chemistry were protein concentration (mg), alkene and piplaroxide relative concentration (mg). Profile analysis followed MANOVAs (Tabachnick & Fidel 1989; Tabachnick et al. 2001). All response variables were inspected and log transformed when necessary to meet assumptions of normality. For individuals in the high light treatment, 18 individuals were missing for piplaroxide and alkene quantification. For individuals in the low light treatment, 11 individuals were missing for piplaroxide and alkene quantification. All analyses were conducted in program R version 3.3.2 (R Development Team 2016). The residuals of all analyses were normally distributed.

**Results**

*High light*

In high light conditions, high levels of fertilizer additions significantly increased leaf, stem, root, and total plant biomass. Leaf biomass increased by 2.7 g (Fig. 2; Table 1), stem biomass increased by 2.8 g (Fig. 2; Table 1), root biomass increased by 1.2 g (Fig. 2; Table 1), and total plant biomass increased by 5.4 g (Table 1) with additions of high fertilizer. The effects on stem biomass were reversed under different conditions of
herbivory and soil type (Fig. 3; Table 1). Stem biomass was significantly higher in the presence of herbivores in clay soil ($\bar{X} = 10.9g \pm 1.1$ SE) and in the absence of herbivores in loam soil ($\bar{X} = 10.2g \pm 1.4$ SE). Stem biomass was the lowest in the presence of herbivores in loam soil ($\bar{X} = 7.7g \pm 1.1$ SE) and in the absence of herbivores in clay soil ($\bar{X} = 8.7g \pm 1.01$ SE).

For protein concentration in leaves growing in high light conditions, there was a significant interaction between soil and fertilizer treatment (Fig. 4; Table 1). Protein concentration of leaves was highest when plants were grown in clay soil with low fertilizer additions ($\bar{X} = 0.28g \pm 0.02$ SE). Loam soil and fertilizer had little to no change in protein concentrations (high fertilizer$=\bar{X} = 0.26g \pm 0.02$ SE; low fertilizer$=\bar{X} = 0.24g \pm 0.03$ SE). Protein concentration of leaves was lowest when growing in clay soil with high fertilizer additions ($\bar{X} = 0.19g \pm 0.03$ SE). Defensive chemistry was not significant in high light conditions (Table XX; $P > 0.05$).

For the plant biomass MANOVA, there were significant additive effects of fertilizer additions, and a significant interaction between soil and herbivory treatments on the biomass of combined plant tissues (Table 2a). The profile analysis revealed that fertilizer additions increased leaf (Fig. 2; $F(1, 71) = 5.28; P = 0.02$) and stem (Fig. 2; $F(1, 71) = 9.7; P < 0.01$) biomass significantly more than root biomass (Fig. 2; $F(1, 71) = 3.66; P = 0.06$; only significant at alpha $= 0.1$). The soil and herbivory interaction was significantly greater for stem biomass versus the other tissues (Fig. 3; $F(1, 71) = 4.57; P = 0.036$). For the plant chemistry MANOVA, the overall model indicated there were not significant effects of soil, herbivory and fertilizer treatments on the combined matrix of
protein, piplaroxide and alkene amide content in leaf tissue (Table 2b). However, the profile analysis demonstrated a marginally significant interaction between soil type and fertilizer treatments on foliar protein (Fig. 4; $F_{(1, 53)} = 2.83; P = 0.098$). These results were consistent with results from the 3-way ANOVA.

*Low light*

In low light conditions, high levels of fertilizer significantly increased leaf, stem, and total plant biomass. Leaf biomass increased by 2.7 g (Fig. 5; Table 3), stem biomass increased by 2.8 g (Fig. 5; Table 3), and total plant biomass increased by 5.4 g (Table 3) with additions of high fertilizer. Herbivory and soil treatments had a marginal effect on stem biomass (Fig. 6; Table 3; $P = 0.09$). Stem biomass was greatest in the absence of herbivory in clay soil ($\bar{X} = 7.9 g \pm 1.02$ SE), but was lowest in the presence of herbivory in clay soil ($\bar{X} = 6.2g \pm 0.84$ SE). Stem biomass was similar in loam soils in the presence ($\bar{X} = 7.6g \pm 0.68$ SE) and absence ($\bar{X} = 7.7g \pm 1.13$ SE) of herbivory.

Main effects of manipulations on protein and alkene amide concentrations were not significant in low light conditions (Table 3). Interestingly, there was a significant interaction between soil type, fertilizer and herbivory on concentrations of the piplaroxide amide (Fig. 7; Table 3). The highest piplaroxide amide content in leaf tissue was present in plants grown in loam soils, high fertilizer additions, and without herbivores (Table 4; $\bar{X} = 0.6 mg \pm 0.15$ SE). Conversely, piplaroxide was lowest in plants grown in the same conditions (loam soils, high fertilizer, without herbivory; Table 4; $\bar{X} = 0.23 mg \pm 0.06$ SE). For clay soils, the highest average piplaroxide amide content in leaf tissue was
present in high fertilizer additions and herbivory treatments (\( \bar{x} = 0.5 \text{ mg} \pm 0.08 \text{ SE} \)); the lowest was in high fertilizer in the absence of herbivory (\( \bar{x} = 0.26 \text{ mg} \pm 0.13 \text{ SE} \)).

For the plant biomass MANOVA, there were significant combined effects of fertilizer additions on all plant parts (Table 5). The profile analysis revealed that fertilizer additions increased leaf (Fig. 5; \( F(1, 71) = 6.81; P = 0.01 \)) and stem (Fig. 5; \( F(1, 71) = 5.31; P < 0.02 \)) more than root biomass (Table 5). For the plant chemistry MANOVA, the overall model indicated soil, herbivory and fertilizer treatments had no effects on protein, piplaroxide and alkene amide content in leaf tissue (Table 5). However, the profile analysis revealed a marginal effect of fertilizer additions on protein concentration (\( F(1, 60) = 3.11; P = 0.08 \)), and a significant interaction with soil type, fertilizer, and herbivory treatments on piplaroxide relative concentrations (Fig. 7; \( F(1, 60) = 4.57; P = 0.037 \)) more than the alkene amide (\( P > 0.2 \)). These results supported the results from the 3-way ANOVA.

**Induced defenses versus resource availability**

Although the effects of manipulations were complex and context dependent, we included a comparison of the standardized effect size for each of the manipulated variables (Fig. 8). Since each treatment was dichotomous, it is possible to directly compare the standardized effect size on chemistry for the four predictors of phytochemical variation, one of which was related to induced defenses (herbivory), while all others were related to the resource availability hypothesis (soil type, fertilizer addition, and light). Herbivory had the largest combined effect size on the alkene (Cohen’s \( d = -0.13 \)) and piplaroxide (Cohen’s \( d = -0.21 \)), but it was in the opposite direction from induced defense predictions of secondary metabolites increasing with herbivore
additions. All changes in resources had large effects on these defensive compounds and the clay soil (Cohen’s d $\text{piplaroxide} = 0.11$; Cohen’s d $\text{alkene} = 0.12$) had a large positive effect. This direct comparison of sources of variation reveals the importance of resource availability, not response to herbivory, as a determinant of increased chemical defense in the tropical shrub, $P. \text{imperiale}$. 

**Discussion**

Phytochemical variation is a well-studied consequence of environmental heterogeneity that has proven resistant to generalizations (*reviewed in* Stamp 2003). The growth-defense trade-off hypothesis suggests that defensive strategies of plants depends on resource availability, but as we show here, this depends on the resources, the biosynthetic pathways of defense, the plant tissues examined. These growth and defense trade-offs also depend on variation in abiotic and biotic interactions across the landscape (Dyer & Letourneau 1999; *reviewed in* Hunter 2016), especially in mega-diverse tropical ecosystems, and this was also true for the effects of resources and herbivores on $P. \text{imperiale}$ in our study. Most notably, we found that the relationships between soil, herbivory and fertilizer on plant biomass and chemistry were dependent on light availability. Plant defensive compounds responded plastically only in the presence of low light (Fig. 7), while foliar protein responded only in high light conditions (Fig. 4). Furthermore, defensive compounds exhibited variable responses to nutrient additions on different soil type, but plants generally invested in defensive chemistry when grown in low light and nutrient poor resources. Together, these results provide support for the
resource availability hypothesis (Coley et al. 1985); however, interactions among factors can strongly modify predictions from this hypothesis.

Unsurprisingly, fertilizer additions increased above- and belowground biomass, but the strength of this relationship was greatest in high light conditions, which is the case for other *Piper* species (Dyer and Letourneau 1999), but not for all tropical understory plants (Denslow et al. 1990). We also found that *Piper imperiale* experimental fragments invested more into shoot growth compared to roots. This may be because tropical wet forest understory plants are more limited by sunlight availability as opposed to water. Montgomery & Chazdon (2002) found shade-tolerant plant species vary in their response to environmental gradients by performing differently, such as investing more into aboveground growth. For example, *Dorstenia panamensis* was a weak performer (*i.e.* low growth rate) in shadier microhabitats, but was a better competitor when grown in high light microhabitats (Montgomery & Chazdon 2002).

The interaction between light availability and soil type had opposing effects on foliar protein and phytochemical defense. *High light* significantly increased foliar protein (Fig. 4; Table 1). Conversely, *low light* caused increases in piplaroxide concentrations depending on the interaction between soil type, herbivory, and fertilizer combinations (Fig. 7; Table 3). These results support the resource availability hypothesis (Coley et al. 1985), which posits that high light is most favorable for productivity and growth (Richards & Coley 2007), while low light promotes allocation to defensive compounds in leaf tissue when herbivory is present. For *P. imperiale*, light heterogeneity caused a trade-off between growth and defense in phytochemistry.
Soil type also played an important role in plastic responses of plant allocation towards protein versus defensive chemistry. For loam soils, absence of herbivory resulted in higher amounts of piplaroxide in leaf tissue compared to presence of herbivory (Fig. 7; Table 4); foliar protein was generally high. For clay soils, high fertilizer and herbivory had higher amounts of piplaroxide content in leaves (Fig. 7; Table 4); foliar protein was highest with low fertilizer additions and lowest with high fertilizer additions. Were the changes in nutrition and chemistry of a magnitude necessary to elicit enhanced growth, toxicity, or other responses in herbivores? It is difficult to say without further experiments, but subtle changes (10% concentration of artificial diet) in Piper amides do disrupt the immune response of specialist herbivores (Hansen et al. 2016) and synergistic effects of these compounds rely on very small changes in chemistry (e.g., 0.05% leaf dry weight, Richards et al. 2010).

Our results suggest that soil type can promote trade-offs in growth versus defensive chemistry for a common tropical understory shrub. If soils are dramatically different enough, then they can exert selective pressures on established plants. For example, Misiewicz and Fine (2014) found significant morphological variation, higher levels of genetic differentiation, and lower migration rates among populations of the tropical tree Protium subserratum growing parapatrically on white-sand, brown-sand and clay soils in Peruvian Amazonia. Similarly, Cacho and Strauss (2014) found soil type was extremely important in the evolution of plants shifting to serpentine soils from bare ground for streptanthoid species (Brassicaceae). These results suggest that soil heterogeneity can cause morphological changes in plants that could lead to divergent selection in plants and subsequently influencing associated herbivores (Glassmire et al. in press). It is well
known that *Piper* understory shrub assemblages are dependent on soil type (Greig 1991, Marquis 2004) and the defensive and growth adaptations to resources and herbivores in different soils have likely contributed to this pattern.

The alkene amide did not respond to light, soil type, herbivory, or nutrient treatments. Likewise, Fincher et al. (2008) also did not find variation in chemical defense for three *Piper* species, including *P. imperiale*, in response to resource availability or herbivory. Resource availability does not appear to be a limiting factor for specialist herbivores on *Piper*, instead they are controlled by top-down pressures of natural enemies (Dyer and Letourneau 1999). This suggests that some *Piper* plants in tropical forests produce and maintain a constant level of constituent chemical defenses that are not inducible. Several studies have examined one mechanism by which non-inducible constituent defenses are effective at defending against herbivores: by reducing the herbivore’s immune response (Barbosa & Caldas 2007; Bukovinszky et al. 2009; Smilanich et al. 2009; Smilanich et al. 2011; Lampert 2012; Kaplan et al. 2016). If “top-down” regulation of herbivores by natural enemies is more effective at controlling herbivore densities (Dyer & Letourneau 1999; Dyer et al. 2004) on these plants, then this interaction of constitutive defensive compounds with natural enemies could be especially effective. These studies combined suggests that the optimal plant defense strategy for *Piper* shrubs in lowland wet forests may be constitutive defensive chemistry that exhibits plasticity in response to resource availability rather than levels of herbivory. In contrast, less diverse communities may rely heavily on phytochemical plasticity to defend against consumers (Williams & Whitham 1986; Karban et al. 1997; Agrawal 1998; Agrawal 2001). However, even in those cases, it is not clear if the changes in chemistry are sufficient to have long term consequences on
insect populations, and the chemistry examined is often limited to one focal compound or a crude measure of a biosynthetic class of compounds. In our case, we also only looked at two defensive compounds and future analyses should incorporate phytochemical diversity to incorporate potential synergies between compounds (Richards et al. 2015).

**Conclusion**

Changes in light and soil heterogeneity can occur across small spatial scales and have dramatic effects on the phytochemical profile of plants. We found that growth and defensive phytochemistry of *P. imperiale* exhibited trade-offs depending on light availability and soil type and while these effects were not necessarily large, they larger than the effects of herbivores on phytochemical variation. Future studies should examine how phytochemical variation influences the performance of associated herbivores and their natural enemies across small spatial scales as mode of plant defense.

**Acknowledgments**

This research was funded by the Garden Club of America Awards in Tropical Botany. Special thanks to Beto Garcia for maintaining the shade-house experiment. We thank Sloan Currie for his help with *Piper imperiale* extractions. We are grateful to *Earthwatch Institute* volunteers for their assistance with harvesting the plants. We thank Matthew Forister and Lora Robinson for providing valuable comments that have improved the manuscript.
References


Bukovinszky, T., Poelman, E.H., Gols, R., Prekatsakis, G., Vet, L.E., Harvey, J.A. and


Tables

Table 1. Effects of plant biomass and chemistry treated with herbivory, fertilizer, and soil. Biomass (g) measurements from leaf, stem, and root tissue of individual *Piper imperiale* plants. Analyses based on only plants in high light conditions. Panels depict response variables based on (a) plant biomass and (b) plant chemistry. Results from univariate 3-way ANOVAs on individual plants. Significant P-values (<0.1) are shown in bold.

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(b) High Light Treatment

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Table 2. Total leaf biomass (leaves + stems + roots) in *Piper imperiale* fragments, expressed as results from MANOVA treated with herbivory, fertilizer, and soil. Analyses based on only plants in high light conditions. Panels depict response variables based on (a) plant biomass and (b) plant chemistry. Significant P-values (<0.1) are shown in bold. Wilk’s λ and F statistic are reported for each source of variation.

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<td></td>
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</tbody>
</table>
Table 3. Effects of plant biomass and chemistry treated with herbivory, fertilizer, and soil. Biomass (g) measurements from leaf, stem, and root tissue of individual *Piper imperiale* plants. Analyses based on only plants in low light conditions. Panels depict response variables based on (a) plant biomass and (b) plant chemistry. Results from univariate 3-way ANOVAs on individual plants. Significant P-values (<0.1) are shown in bold.

(a) Low Light Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf biomass</th>
<th>Stem biomass</th>
<th>Root biomass</th>
<th>Total plant biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Soil</td>
<td>1</td>
<td>0.24</td>
<td>0.62</td>
<td>0.03</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>1</td>
<td>8.08</td>
<td>&lt;0.01</td>
<td>4.45</td>
</tr>
<tr>
<td>Herbivory</td>
<td>1</td>
<td>0.1</td>
<td>0.75</td>
<td>2.13</td>
</tr>
<tr>
<td>Soil X Fertilizer</td>
<td>1</td>
<td>0.15</td>
<td>0.7</td>
<td>0.1</td>
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<tr>
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<td>0.44</td>
<td>0.51</td>
<td>2.85</td>
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<tr>
<td>Fertilizer X Herbivory</td>
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<td>1.21</td>
<td>0.28</td>
<td>1.22</td>
</tr>
<tr>
<td>Soil X Fertilizer X Herbivory</td>
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<td>0.07</td>
<td>0.79</td>
<td>0.71</td>
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<tr>
<td>Residuals</td>
<td></td>
<td>71</td>
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</tr>
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</table>

(b) Low Light Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein</th>
<th>Piplaroxide</th>
<th>Alkene</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>Soil</td>
<td>1</td>
<td>0.64</td>
<td>0.43</td>
</tr>
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<td>2.21</td>
<td>0.14</td>
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<tr>
<td>Herbivory</td>
<td>1</td>
<td>0.25</td>
<td>0.62</td>
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<tr>
<td>Soil X Fertilizer</td>
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<td>0.53</td>
<td>0.47</td>
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<tr>
<td>Soil X Herbivory</td>
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<td>0.87</td>
<td>0.35</td>
</tr>
<tr>
<td>Fertilizer X Herbivory</td>
<td>1</td>
<td>1.51</td>
<td>0.22</td>
</tr>
<tr>
<td>Soil X Fertilizer X Herbivory</td>
<td>1</td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>Residuals</td>
<td>71</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>
Table 4. Piplaroxide amide content in *Piper imperiale* fragments, expressed as mg of leaf tissue (1 SE in parentheses).

Plants were grown in low and high light conditions. The only significant interaction found was between soil type, herbivory, and fertilizer additions in low light conditions (see Table 3; Fig. 7).

(a) Piplaroxide amide

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>High Light</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Herbivory</td>
</tr>
<tr>
<td></td>
<td>Low Fertilizer</td>
</tr>
<tr>
<td>Loam</td>
<td>0.38 (0.15)</td>
</tr>
<tr>
<td>Clay</td>
<td>0.30 (0.12)</td>
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(b) Piplaroxide amide

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Low Light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Herbivory</td>
</tr>
<tr>
<td></td>
<td>Low Fertilizer</td>
</tr>
<tr>
<td>Loam</td>
<td>0.41 (0.13)</td>
</tr>
<tr>
<td>Clay</td>
<td>0.29 (0.08)</td>
</tr>
</tbody>
</table>
Table 5. Total leaf biomass (leaves + stems + roots) in *Piper imperiale* fragments, expressed as results from MANOVA treated with herbivory, fertilizer, and soil. Analyses based on only plants in low light conditions. Panels depict response variables based on (a) plant biomass and (b) plant chemistry. Significant P-values (<0.1) are shown in bold. Wilk’s λ and F statistic are reported for each source of variation.

(a) Low Light Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Wilk's λ</th>
<th>F</th>
<th>P</th>
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</thead>
<tbody>
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<td>1</td>
<td>0.94</td>
<td>1.46</td>
<td>0.23</td>
</tr>
<tr>
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<td>0.829</td>
<td>4.73</td>
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<td>0.946</td>
<td>1.32</td>
<td>0.27</td>
</tr>
<tr>
<td>Residuals</td>
<td>71</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Low Light Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>Wilk's λ</th>
<th>F</th>
<th>P</th>
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</thead>
<tbody>
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<td>0.984</td>
<td>0.31</td>
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<tr>
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<td>0.31</td>
</tr>
<tr>
<td>Herbivory</td>
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<td>0.952</td>
<td>0.972</td>
<td>0.41</td>
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<tr>
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<td>0.29</td>
</tr>
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<td>Fertilizer X Herbivory</td>
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<td>0.969</td>
<td>0.623</td>
<td>0.6</td>
</tr>
<tr>
<td>Soil X Fertilizer X Herbivory</td>
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<td>0.928</td>
<td>1.51</td>
<td>0.22</td>
</tr>
<tr>
<td>Residuals</td>
<td>60</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure legends

**Figure 1.** Fully crossed experimental design of shade-house experiment. *Piper imperiale* plant fragments were randomly assigned to soil type (loam / clay), fertilizer (high / low), and herbivore (present/absent) treatments. There was a total of 16 combinations of treatment with 10 fragments per treatment combination, for a total of 160 fragments.

**Figure 2.** There was a positive effect of fertilizer on plant biomass in high light conditions. Leaf, stem, and root biomass significantly increased in the presence of high fertilizer (p-value < 0.05). D) Total plant biomass significantly increased by 6.8 g in high fertilizer (p-value <0.01) but this was mostly due to above-ground biomass.

**Figure 3.** The interactive effect of soil and herbivory treatments on stem biomass in high light conditions. Stem biomass was significantly higher in the presence of herbivores in clay soil ($\bar{x} = 10.9g \pm 1.1$ SE) and in the absence of herbivores in loam soil ($\bar{x} = 10.2g \pm 1.4$ SE). Stem biomass was the lowest in the presence of herbivores in loam soil ($\bar{x} = 7.7g \pm 1.1$ SE) and in the absence of herbivores in clay soil ($\bar{x} = 8.7g \pm 1.01$ SE).

**Figure 4.** The interactive effects of soil and fertilizer treatments on protein concentration in leaves in high light conditions. Protein concentration in leaves was highest in the presence of clay soil and low fertilizer, and was the lowest in the presence of clay soil and high fertilizer; the magnitude of effect was 0.11 mg.
Figure 5. There was a positive effect of fertilizer on plant biomass in low light conditions. Leaf and stem biomass significantly increased in the presence of high fertilizer (p-value < 0.05). Total plant biomass significantly increased by 5.4 g in high fertilizer (p-value = 0.02) but this was solely attributed to above-ground biomass.

Figure 6. There was a marginal interactive effect between soil and herbivory treatments on stem biomass in low light conditions. Stem biomass was greatest in the presence of herbivory in clay soil, and was least in the absence of herbivory in clay soil (p-value = 0.09). There was little to no changes in stem biomass of plants established in loam soils.

Figure 7. Soil type, herbivory, and fertilizer additions had an interactive effect on phytochemical variation of defensive compounds but only in the presence of low light. For loam soils and high additions of fertilizer, piplaroxide amide content was highest in the absence of herbivory ($\bar{x} = 0.6 \text{ g} \pm 0.15 \text{ SE}$), but was lowest in the presence of herbivory ($\bar{x} = 0.45 \pm 0.11 \text{ SE}$). For clay soils, piplaroxide amide content was highest with fertilizer additions and herbivory ($\bar{x} = 0.5 \text{ g} \pm 0.08 \text{ SE}$); low fertilizer generally had lowest defensive compounds ($\bar{x} = 0.29 \text{ g} \pm 0.08 \text{ SE}$).

Figure 8. Effect sizes of defensive chemistry, piplaroxide and alkene amides. Treatments consisted of fertilizer additions, presence or absence of herbivory, high or low light, and clay or loam soil types. While herbivory had the largest combined effect size on the alkene and piplaroxide, it was in the opposite direction from predictions of defenses
increasing with herbivore additions. All changes in resources had large effects on these defensive compounds and the clay soil had a large positive effect.
Figures

Figure 1

Low light = Understory

High light = Canopy
Figure 5

![Graph showing the effect of fertilizer treatment on plant dry weight biomass (g). The x-axis represents the fertilizer treatment (Low, High), and the y-axis represents the plant dry weight biomass. The graph compares the biomass of leaves and stems across the two fertilizer treatments.](image-url)
Figure 6

- **Stem Biomass (g)**
  - Loam Soil
  - Clay Soil

- **Herbivory Treatment**
  - Present
  - Absent
Figure 8

![Bar chart showing effect sizes for different treatments](image)

- **Fertilizer**
  - Alkene: Small positive effect size
  - Piplaroxide: Medium positive effect size
- **Herbivory**
  - Alkene: Large negative effect size
  - Piplaroxide: Medium negative effect size
- **Light**
  - Alkene: Small negative effect size
  - Piplaroxide: Medium positive effect size
- **Soil**
  - Alkene: Large positive effect size
  - Piplaroxide: Medium positive effect size

The chart illustrates the comparative effect sizes of alkene and piplaroxide treatments across different environmental conditions.
Chapter 3 ~ Intraspecific phytochemical variation shapes community and population structure for specialist caterpillars

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Keywords: chemical interactions, community structure, population diversification, phytochemical variation, multi-trophic, Piper, Eois
Abstract

Chemically mediated plant-herbivore interactions contribute to the diversity of terrestrial communities and the diversification of plants and insects. While our understanding of the processes affecting community structure and evolutionary diversification has grown, few studies have investigated how trait variation shapes genetic and species diversity simultaneously in a tropical ecosystem. We investigated secondary metabolite variation among subpopulations of a single plant species, *Piper kelleyi* Tepe (Piperaceae), using High-Performance Liquid Chromatography (HPLC), to understand associations between plant phytochemistry and host specialized caterpillars in the genus *Eois* (Geometridae: Larentiinae) and associated parasitoid wasps and flies. In addition, we used a Genotyping-By-Sequencing approach to examine the genetic structure of one abundant caterpillar species, *E. encina* Dognin, in relation to host phytochemical variation. We found substantive concentration differences among three major secondary metabolites, and these differences in chemistry predicted caterpillar and parasitoid community structure among host plant populations. Furthermore, *E. encina* populations located at high elevations were genetically different from other populations. They fed on plants containing high concentrations of prenylated benzoic acid. Thus, phytochemistry potentially shapes caterpillar and wasp community composition and geographic variation in species interactions, both of which can contribute to diversification of plants and insects.
Introduction

Chemically mediated interactions between plants, herbivores and natural enemies have important ecological and evolutionary impacts on biodiversity (Ehrlich & Raven, 1964; Becerra et al., 2009; Wilson et al., 2012). Despite considerable progress in understanding relationships between trophic interactions and diversity, a knowledge gap remains: specifically, how does phytochemical diversity affect multi-trophic interactions at community and population levels? There has been progress addressing these questions using clonal plants to experimentally manipulate genetic diversity and quantifying associated increases in arthropod richness (Crutsinger et al., 2006; Johnson et al., 2006). However, there are limitations to using clones or similar experimental approaches for understanding natural variation, especially in mega-diverse systems; these approaches do not accurately reflect levels of interaction diversity and genetic diversity observed in nature (reviewed in Whitham et al., 2006; Hughes et al., 2008; Crutsinger, 2015). Furthermore, manipulative experiments examining multi-trophic interactions have focused on the uni-directional influence of plant genetic diversity on arthropod richness (Fritz & Price, 1988; Johnson & Agrawal, 2005; Whitham et al., 2006), whereas only a few studies have quantified genetic and species diversity of herbivores simultaneously (Fridley & Grime, 2010; Crawford & Rudgers, 2013; Abdala-Roberts et al., 2015). Most of these experimental examples have been limited to temperate grasslands and forests dominated by a foundation species (reviewed in Whitham et al., 2006; Hughes et al., 2008; Crutsinger, 2015), as opposed to examining these relationships in highly diverse ecosystems that lack a single dominant species. Here we focus on a model plant-
herbivore-parasitoid system to examine effects of plant chemical diversity on community
and population level processes in highly diverse tropical forests.

The links between plant genetic diversity and diversity of upper trophic levels are
partially mediated via changes in phytochemical diversity (Richards et al. 2015).
Although we currently lack an understanding of how population genetic and genomic
variation predict phytochemical diversity, secondary metabolite concentrations are
heritable phenotypes (Geber & Griffen, 2003; Johnson et al., 2009; Barbour et al., 2015)
that can respond to selection and can directly and indirectly impact biotic communities
(Bailey et al., 2006) and ecosystems (Driebe & Whitham, 2000; Whitham et al., 2003;
Schweitzer et al., 2005). Intraspecific chemical variation between individual host plants
could give rise to geographically variable selection, and contribute to shifts in herbivore
preference for, or performance on, unique concentrations of individual secondary
compounds (i.e., a selection mosaic, Thompson, 1999; Thompson, 2005). This
geographic variation in turn could affect the makeup of herbivore and predator
assemblages and might allow for greater species packing, contributing to higher alpha
diversity (Rodríguez-Castañeda et al., 2010; Richards et al., 2015). Furthermore,
geographically separated herbivore populations may adapt locally to a specific chemical
profile in intraspecific host plants, and this could reduce effective migration between
habitats (Wilson et al., 2012). Thus, phytochemical variation across host plant
populations might shape the composition of herbivore assemblages, and could give rise to
geographically divergent selection on herbivore populations.

To examine such relationships, we focus on a well-studied tropical genus of
caterpillars, *Eois* (Geometridae), that feed exclusively on plants in the genus *Piper*
(Piperaceae) and are host to diverse parasitoid communities (Dyer & Palmer, 2004). As recently as the Pleistocene, multiple, independent clades of *Eois* caterpillars have undergone geographically localized radiations, in some cases with sister species of *Eois* in the same geographic area, associated with the same host plant species (Wilson *et al*., 2012). This pattern of sympatric sister species utilizing the same resource makes it difficult to assume a framework of ecological divergence in allopatry associated with shifting host species. However, intraspecific variation in plant chemical defense could contribute to divergence (Wilson *et al*., 2012). *Piper* is chemically defended by a remarkable diversity of secondary compounds that exhibit a broad array of biological activities. These include antibacterial (Diaz *et al*., 2012), antitubercular (Diaz *et al*., 2012), antifungal (Johann *et al*., 2009), and anti-herbivore effects (Dyer *et al*., 2003; Jeffrey *et al*., 2014). *Piper* chemical defenses are known to vary within as well as among species and have been found to function additively or synergistically to deter or poison herbivores (Dyer *et al*., 2003; Richards *et al*., 2010) and could decrease the herbivores’ defenses against parasitoids (Smilanich *et al*., 2009). Considering the highly diverse chemistry of *Piper*, it is possible that sufficient intraspecific variation exists to facilitate divergence at small spatial scales. Thus, different populations of host species might provide sufficiently different niches to affect the evolution of herbivore and parasitoid communities and to facilitate local herbivore divergence.

To investigate intraspecific phytochemical variation and the consequences for both communities and populations of herbivores, we collected specialist *Eois* caterpillars and the leaves of *P. kelleyi* Tepe they were consuming across an elevational gradient. Our goals were to understand how the abundance and composition of plant secondary
metabolites within localized host plant populations might influence caterpillar and parasitoid communities along an elevational gradient, and how variation among host plant populations might influence genetic variation of one Eois species. We tested the following hypotheses: 1) P. kelleyi populations are characterized by predictable variation in phytochemistry along an elevational gradient; 2) phytochemical variation is a driver of caterpillar and parasitoid community structure across different microhabitats containing P. kelleyi; 3) phytochemical variation is associated with population genetic variation in the most common Eois species.

Methods

Study system and data collection

Piper kelleyi Tepe is endemic to the Eastern slopes of the Andes of Ecuador and Peru, occurring within a narrow altitudinal range between 1400 and 2400 m (Tepe et al., 2014). It is a mid-canopy shrub characterized by broad ovate leaves and is commonly called “pink belly” due to the distinct pink coloration on the ventral surface of younger leaves (Tepe et al., 2014). Piper kelleyi hosts an unusually high diversity of specialist caterpillars from the genus Eois which in turn are hosts to several species of parasitoid wasps and flies (Tepe et al., 2014). The major secondary compounds in the leaves of this species are a specific prenylated benzoic acid, chromene and dimeric chromane because they make-up greater than 95 % of the compounds present in the crude extract and are present at a high concentration of ~10% of the dry weight of the leaf material (Fig. 1A), all of which have significant negative effects on herbivores, microbes and fungi (Jeffrey
et al., 2014). We collected *P. kelleyi, Eois* caterpillars and parasitoids from 10 sites in close proximity to Yanayacu Biological Station (00°36’ S and 77°53’ W) from June to August 2011 and 2012. Yanayacu, comprising disturbed habitat combined with pristine cloud forest, is located in the Eastern Andes (Napo Province, Ecuador). The 10 sites were separated by elevation and consisted of four replicated plots that were randomly selected within sites. The temporary plots were 10 m in diameter. Every *P. kelleyi* plant was sampled for *Eois* caterpillars within these plots; the number of *P. kelleyi* plants in each plot was variable. The overall sampling resulted in a total of 125 plants and 2318 caterpillars across 40 plots. Eight of the plots were excluded because of missing leaf samples or no caterpillars were found (Fig. S1). Caterpillars were reared to adult moths or parasitoids to identify species and to calculate levels of parasitism. Caterpillars were identified to species (Fig. 1B, *Eois encina* Dognin, *Eois aff. encina* Dognin, *Eois ignefumata* Dognin, *Eois aff. pallidicosta* Warren, *Eois planetaria* Dognin, *Eois viridiflava* Dognin, and *Eois aff. viridiflava* Dognin) based on photographs that were taken at every instar. Larval and adult images were compared to image vouchers (<http://www.caterpillars.org>, Dyer et al., 2010) and museum vouchers to assess species identifications. Genitalia were dissected from adult moths to confirm species determinations. Plant material was identified, and voucher specimens (Tepe & Moreno 2999 MO, QCA, QCNE; Glassmire B13 CINC, QCNE) were deposited at the Herbario Nacional del Ecuador, Quito, Ecuador (QCNE), the herbarium of the Pontificia Universidad Católica del Ecuador (QCA), the herbarium at the University of Cincinnati (CINC), and the Missouri Botanical Garden, USA (MO). In addition to *P. kelleyi* sampling, we sampled plant richness and total leaf abundances for all other *Piper* species
in each plot. All collections were conducted with permission from the Museo Ecuatoriano de Ciencias Naturales in Quito, Ecuador (permit number 001-2011-DPAP-MA).

**HPLC analysis of *P. kelleyi* leaves**

Young leaves were collected from 93 of the *P. kelleyi* shrubs that were sampled for caterpillars to quantify correlations between plant chemistry and caterpillar genetics. The leaves were dried at 25°C in a dry box at the field station. The compounds are thermally stable and incident light over relatively short periods of time is not known to cause decomposition (Jeffrey et al., 2014). We were careful to store and reduce light exposure during the extraction and analysis process. In the laboratory, individual leaves were ground using liquid nitrogen, mortar and pestle, then 250 mg of leaf material were extracted with 2 mL of High-Performance Liquid Chromatography (HPLC) grade methanol for each leaf (*full methods with justifications are provided in* Jeffrey et al., 2014). The extract was sonicated for 15 minutes and the insoluble leaf material was removed by vacuum filtration. This entire extraction protocol was repeated twice. The methanol was removed under reduced pressure using rotary evaporation and placed under a high vacuum for 24 hours, to remove residual solvent. The remaining crude extract was dissolved in HPLC grade methanol with an internal standard of 0.3 mg/mL methyl salicylate (Sigma-Aldrich, product number M6752). Samples were analyzed by HPLC using a Phenomenex Luna C18 reverse phase column (150 x 4.6 mm, 5 micron) and an Agilent Technology 1200 series instrument coupled to a diode array detector (DAD) detecting at 280 nm. The solvent system employed was HPLC grade methanol with 0.01% trifluoroacetic acid (TFA; Sigma-Aldrich, product number T62200) and HPLC
grade water with 0.01% TFA. The 5 uL injection was eluted at a constant flow of 1 mL/min with a gradient of methanol and water as follows: 0-15 min 50%-100% methanol; 15-30 min 100% methanol; 30-31 min 100%-50% methanol; 31-36 min 50% methanol. The internal standard (methyl salicylate) was observed at $R_t = 8.464$ min. The prenylated benzoic acid was observed at $R_t = 6.342$ min, the chromene at $R_t = 7.995$ min, and the dimeric chromane at $R_t = 8.997$ min relative to the internal standard. The relative abundance of prenylated benzoic acid, chromene, and dimeric chromane were quantified using the ratio of peak areas to the internal standard (methyl salicylate). The HPLC response units for area under the peak is given in mau*s.

*Community analyses*

We used structural equation models (Shipley, 2002) to test the hypotheses that phytochemical variation is a primary driver of *Eois* caterpillar assemblages based on the locations of *P. kelleyi*, the makeup of the surrounding *Piper* community, and diversity of parasitoids. Our focus was on *P. kelleyi*, but we included *Piper* community diversity because it is likely that some of the variance in caterpillar and parasitoid community composition could be due to the biology and chemistry of these other plant species (Tahvanainen & Root, 1972; Root, 1973; Barbosa *et al*., 2009). The community data were collected from 32 plots described above. From these plots, we collected 1,481 *Eois* caterpillars that were described to species (seven species total), with 75% of the individuals being *E. encina*. We reared out 280 parasitoids that were identified to family level (Braconidae, Tachinidae, Ichneumonidae and Eulophidae). We quantified the secondary metabolites from 93 *P. kelleyi* plants (see above). Finally, we measured
richness and abundance in the plots for the 13 species of *Piper* plants that were found (*P. agustum, P. "cafecito," P. crassinervium, P. baezanum, P. ecuadorense, P. "escabrosa," P. hispidum, P. lanceifolium, P. napo-pastazanum, P. perareolatum, P. pubinervulum, P. schupii and P. silvivagum*).

The relative abundance of prenylated benzoic acid, chromene, and dimeric chromane derived in relation to an internal standard from the HPLC were quantified for each individual *P. kelleyi* plant from which *Eois* caterpillars were collected. Concentrations of prenylated benzoic acid, chromene and dimeric chromane were highly correlated using Spearman rank correlations (chromene and dimeric chromane $\rho = 0.91$, $P < 0.001$; dimeric chromane and benzoic acid $\rho = 0.71$, $P < 0.001$; chromene and the benzoic acid $\rho = 0.59$, $P < 0.001$). Since these compounds are hypothesized to be biosynthetically linked, a factor analysis with varimax rotation was used to create a latent variable of phytochemical defense based on shared variance of the relative abundances of each compound. Since there were dominant and rare species, we used the inverse of Simpson’s Diversity Index to calculate community entropies and species equivalents (Jost, 2006) for all *Eois* and parasitoid species collected from each *P. kelleyi* plant, and from the other *Piper* species located in *P. kelleyi* plots. Elevation was measured using GPS for each *P. kelleyi* plot.

For our *a priori* specified structural equation model, we included specific causal relationships resulting in a model with one exogenous variable (elevation) predicting three endogenous variables (*Eois* diversity per plant, parasitoid diversity per plant, and *Piper* diversity per plot); the phytochemical defense factor (from the aforementioned factor analysis) was included as a latent variable. These five variables were included in
our model with hypothesized relationships (Table 1) that are context dependent and based on previous work with *Piper, Eois*, and parasitoids (Dyer *et al.*, 2003; Dyer *et al.*, 2004; Brehm *et al.*, 2007; Connahs *et al.*, 2009; Smilanich *et al.*, 2009; Rodríguez-Castañeda *et al.*, 2010; Wilson *et al.*, 2012; Richards *et al.*, 2015). For example, it was hypothesized that higher relative concentrations of secondary metabolites will decrease the diversity of specialist herbivores due to higher levels of toxicity (Poelman *et al.*, 2009; Richards *et al.*, 2015), and this will positively affect parasitism, because sequestered toxins impair the caterpillars’ immune response when consumed (Smilanich *et al.*, 2009; Richards *et al.*, 2012).

We tested the fit of this model using previously established approaches (Greeney *et al.*, 2015) and selected the formulation of the reticular action model to define alternative models. Starting values for the parameter estimates were determined by using a combination of three methods: observed moments of variables, the McDonald method, and Two-Stage Least Squares. The estimation method for the model was maximum likelihood, and the Levenberg-Marquardt algorithm was used to iterate solutions for optimization. The Chi Square for the absolute index was used to assess the fit of the model, with $P > 0.1$ (with 4 DF) as an indication of a good fit to the data. Residuals met assumptions for the general linear model (i.e. generalized linear model with Gaussian distribution, identity link function, and fixed effects). These analyses were conducted using SAS (v 9.1, proc CALIS).
Caterpillar genetic variation, phytochemical variation, and elevation

We used a Genotyping-By-Sequencing (GBS) approach to generate population genetic data for *E. encina*. DNA was extracted from 155 individual *E. encina* caterpillars. DNA extractions were performed on thoraxes of dry adult specimens using Qiagen DNeasy Blood and Tissue kits (Qiagen Inc., Valencia, CA) and quantified using spectrophotometry. Reduced-representation genomic libraries for Illumina sequencing were constructed using a GBS approach (Gompert *et al.*, 2012; Parchman *et al.*, 2013). Genomic DNA was cut with two restriction enzymes, EcoRI and MseI, and a unique DNA barcode was ligated to the fragments from each individual to allow multiplexing. DNA fragment libraries were PCR amplified using standard Illumina primers and size selected for a region between 200 and 300 bases using QIAquick gel extraction kits (Qiagen, Inc.). One lane of sequencing on the Illumina HiSeq at the National Center for Genome Resources (Santa Fe, NM) generated 47 million 100 bp reads.

We used a custom Perl script to parse barcodes from sequences and to assign the correct individual id to each read. As a reference genome is not available for *E. encina*, we used a two-step assembly procedure to assemble reads into homologous genetic regions. We first performed a de novo assembly for a subset of 30 million sequences with SeqMan NGen 3.0.4 (DNASTAR), using a minimum match percentage of 92%, a gap penalty of 50, and a match size of 50 bp (full details of assembly parameterization are available upon request). We removed low quality contigs and sequences less than 82 or greater than 88 bases in length to generate an artificial reference containing 481,606 contig consensus sequences. We then assembled all reads onto the reference using the aln and samse algorithms in bwa (Li & Durbin, 2009), using an edit distance of 3 (full
parameters for bwa assemblies are available upon request). We used samtools and bcftools (Li et al., 2009) to identify single nucleotide polymorphisms (SNPs) in assemblies and retained SNPs where at least one read was aligned in at least 50% of the individuals. Genotype likelihoods were calculated with bcftools (Li et al., 2009), stored in Variant Call Format (VCF), and converted to composite genotype likelihood point estimates for downstream analyses. We summarized genetic variation using principal components analysis of the genotype covariance matrix using the prcomp package in R (R Core Team 2013).

We created matrices of genetic distances among caterpillar individuals using the calculated PC scores of genotype likelihoods. We created three different matrices based on: 1) all the PCs for an overall genetic representation; 2) PC 1 and 2 because they explain most of the variation for population structure; and 3) just PC 2 because it distinguishes the higher elevation individuals. We used a multiple regression on distance matrices (MRM; Lichstein, 2007), using the “MRM” function from the ecodist package in R (Goslee & Urban, 2007), with 100,000 permutations. We conducted three models that differed in the response variable. The response variable was the genetic distances based on Euclidean distances for 1) all the PCs, 2) PC 1 and 2, and 3) only PC 2, as described above. The independent variables were elevation, individual compound concentrations (i.e. prenylated benzoic acid, chromene, and dimeric chromane) and GPS location from each plant sampled, transformed into separate Euclidean distance matrices.
Results

Plant chemistry analyses

We found that prenylated benzoic acid, chromene, and dimeric chromane were present in *P. kelleyi* at a high concentration of ~10% of the dry weight of leaf material (Fig. 1A), which is consistent with previous results (Jeffrey *et al.*, 2014). While these compounds were highly correlated and hypothesized to be biosynthetically linked, the relative abundance of each compound can be important for synergistic or additive effects on herbivores (Dyer *et al.*, 2003; Richards *et al.*, 2010). Thus, we calculated a Shannon equivalence variable (Jost, 2006) for the three compounds, which accurately captures their relative abundances in an individual plant and does not give added weight to one dominant compound since all compounds are present (i.e. the richness of compounds does not change). This compound equivalence variable was used in the structural equation models described above; higher values indicate greater abundance and evenness of the compounds.

Community analyses

The factor analysis included concentrations of the three different secondary metabolites, utilized varimax rotation, and yielded 2 factors. Kaiser’s Measure of Sampling Adequacy, which assesses adequacy of correlation matrices for factor analysis, was acceptable (0.6) and the matrix was not an identity matrix. The relative concentration of all three compounds loaded onto Factor 1 with high loadings for dimeric chromane (0.96) and chromene (0.92) and lower loadings for prenylated benzoic acid (0.73). Factor
2 had more useful loadings for separating prenylated benzoic acid (0.68) from dimeric chromane (-0.18) and chromene (-0.36), and it was utilized in path models as a latent “phytochemical defense” (prenylated benzoic acid versus other compounds) variable.

For the structural equation models, the model that provided the best fit to the data is summarized by the path diagram in Fig. 2 ($\chi^2=0.014; \text{df}=1; P=0.91$; P-values closer to 1 indicate a better fit to the data). There were several notable causal relationships supported by this model (Table 1, Fig. 2B, and Fig. S2). First, elevation had positive direct effects on phytochemical defense (standardized path coefficient ($spc$) = 0.10; slope ($B$) = 0.0009), *Eois* community diversity ($spc = 0.11; B = 0.0004$), and *Piper* community diversity ($spc = 0.14; B = 0.0005$). While Eulophidae parasitoids had highest densities at 2000 m and Braconidae, Ichnuemonidae and Tachinidae parasitoids had highest densities at 2100 m (Fig. 3), in our path model there is a linear relationship between elevation and parasitoid diversity while accounting for other factors that include *Piper* community diversity and high concentrations of phytochemistry (Fig. 2B; $spc = 0.37; B = 0.0024$). The effects of elevation on phytochemical defense also cascaded to the arthropod community; as phytochemical defense increased, *Eois* community diversity on *P. kelleyi* decreased (Fig. 2B; $spc = -0.2; B = -0.12$) and parasitoid diversity increased ($spc = 0.26; B = 0.24$) – adding to the strong direct effects of elevation on parasitoids (Fig. 2B).

Increases in *Piper* plant community diversity also enhanced parasitoid diversity on individual *P. kelleyi* plants ($spc = 0.11; B = 0.44$) and had the strongest negative effect on *Eois* caterpillar diversity on individual *P. kelleyi* plants (Fig. 2B; $spc = -0.48; B = -1.23$). Finally, greater *Piper* plant community diversities are associated with decreased phytochemical defense in *P. kelleyi* ($spc = -0.25; B = -1.04$). Overall, the path model
suggests that increases in phytochemical defense – specifically the prenylated benzoic acid – and *Piper* community diversity reduced *Eois* community diversity, while increasing parasitoid community diversity (Fig. 2B). Higher elevations are associated with greater *Piper, Eois* and parasitoid community diversities, as well as with greater *P. kelleyi* phytochemical defense.

*Caterpillar genetic variation, phytochemical variation, and elevation*

After executing one lane of sequencing on the Illumina HiSeq platform, and conducting assemblies and variant calling as described in the Methods, we retained genotypes at 20,458 SNPs in 155 *E. encina* individuals (accessible in dryad: doi: 10.5061/dryad.d67h6). The mean coverage per locus per individual was 1.25x. We used principal components analysis to summarize patterns of genetic variation across all individuals. The first two principal components explained 7% (PC1) and 1% (PC2) of the genotypic variation, and suggest that *E. encina* from the highest elevation are genetically differentiated from lower elevations (Fig. 4A). There are other differentiated individuals, but we focus discussion on the highest elevation for which we have a hypothesis about phytochemical variation. The *P. kelleyi* plants that this population was occurring on had higher relative concentrations of the prenylated benzoic acid (Fig. 4B). Other potential patterns suggested by the PCA were not interpretable based on our original mechanistic hypotheses.

Multiple regression using distance matrices (Lichstein, 2007) were conducted to examine whether elevation, phytochemical variation and GPS location were predictors of genetic distances among *E. encina* individuals. Genetic variation was estimated using the
PC scores of multi-locus genotype likelihoods of *E. encina* individuals transformed into a distance matrix; three matrices were created based on all the PCs, PC 1 and 2, and only PC 2 scores. Table 2 depicts the results from multiple Mantel tests in which models differed based on the response variable: A) all the PCs, B) PC 1 and 2, and C) only PC 2 scores. Increases in elevation and prenylated benzoic acid significantly explained *E. encina* genetic variation. Geographic location was only a significant predictor with PC 2 scores as the response variable.

**Discussion**

Intraspecific phytochemical variation is an underappreciated source of geographic variation in species interactions and their evolutionary outcomes (Bolnick *et al.*, 2011; Zhang *et al.*, 2015). At what scales might this variation shape community structure and generate divergent selection and population differentiation? We tested the hypothesis that phytochemical variation within a single species of host plant, *P. kelleyi*, drives community structure via subtle microhabitat differences in chemically mediated interactions between plants, their specialist herbivores and associated parasitoids. We also examined whether genetic structure across populations of specialist caterpillars was correlated with phytochemically variable microhabitats. We found evidence for substantial chemical variation among individual plants (Fig. S3-S5, Table S1-S2). The concentration of one important defensive compound, prenylated benzoic acid, increased with increasing elevation (Fig. S4). In turn, plant chemistry strongly predicted community structure at the herbivore and parasitoid trophic levels, with more toxic plants supporting a lower diversity of specialist herbivores, richer parasitoid communities, and
higher levels of parasitism. Across populations of one of the specialist caterpillar species, our results suggest the potential for genetic differentiation between high elevation caterpillar populations and all others (Fig. 4), which could be mediated by high concentrations of plant secondary metabolites. Overall, the results are consistent with phytochemical variation shaping community structure, perhaps giving rise to geographic variation in the selection pressures experienced by specialist herbivores. Below, we discuss community structure and then evaluate possibilities for chemically mediated population structure.

Within our plots, associations between phytochemical defense and *Eois* assemblages on individual *P. kelleyi* plants were consistent with predictions that increased phytochemical defense causes decreased diversity of specialist caterpillars, while concurrently increasing parasitoid community diversity. Intraspecific phytochemical diversity had a negative influence on caterpillar diversity, consistent with previous studies demonstrating that increases in phytochemical variation within a host *Piper* species have negative effects on some *Eois* species but not others (Dyer et al., 2003; Dyer et al., 2004; Richards et al., 2010; Richards et al., 2012). Interestingly, increased phytochemical diversity (measured as changes in relative abundance of the three defensive compounds) facilitated higher parasitoid diversity. This chemistry-parasitoid relationship could imply that increases in phytochemical variation of *P. kelleyi* may somehow attract more parasitoids for defense against caterpillars (Turlings & Ton, 2006; Wäschke et al., 2014) or that plant chemistry compromises the immune response of sequestering specialists (Smilanich et al., 2009) – the former is less likely since these compounds are not volatile. Could it be that volatile organic compounds are also
increased and attracting richer parasitoid communities (e.g., Raguso, 2011)? It is worth pursuing this question, along with the interesting pattern of increased parasitism pressure, a result that contrasts with a previously reported trend that levels of parasitism decrease with increasing elevation in temperate zones (reviewed in Hodkinson, 2005).

_Piper_ diversity within plots had the strongest negative effect on the diversity of _Eois_ found on _P. kelleyi_, which is consistent with a hypothesis of associational resistance (Root, 1973; Barbosa et al., 2009). Alternatively, this negative relationship between _Piper_ diversity and _Eois_ diversity on our focal host plant could be a consequence of the highly specialized relationship these moths have with _Piper_ combined with the fact that _Eois_ are not very mobile. As a result, preferred hosts are likely to be less “apparent” in high diversity _Piper_ plots (Root, 1973; Barbosa et al., 2009). Higher diversity _Piper_ communities also attracted more parasitoids that attack _Eois_. This result suggests that parasitoids are able to locate their preferred host even in the presence of complex cues from closely related non-host plants (Erb et al., 2010; Wäschke et al., 2014). Alpha diversity of _Piper, Eois_, and associated parasitoids at a small scale (10 m diameter plots) also increased with elevation, consistent with previous _Piper_ diversity studies that demonstrated a strong effect of elevation on _Piper_ and _Eois_ diversity (without mid-domain effects) in the Eastern Andes (Brehm et al., 2007; Rodríguez-Castañeda et al., 2010). Similarly, Rodríguez-Castañeda et al. (2010) found that monophagous herbivore diversity increased with elevation, suggesting that colder temperatures and higher precipitation up the mountain are important for diversity by increasing specialization of herbivores and parasitoids on host plants. Our results contrast with some studies in temperate zone systems. For example, Rasmann et al. (2014) found Buprestid beetles
tend to be more generalized and polyphagous at higher altitudes, and Pellissier et al. (2012) found that butterfly specialization decreased with increases in elevation; this was largely attributed to decreases in host plant abundance as elevation increased. As pointed out by Rodríguez-Castañeda et al. (2016), there are not enough studies of chemically-mediated trophic interactions across elevations to make any generalizations in tropical or temperate ecosystems, so it is not clear if the patterns reported here are part of a more general pattern of changes in plant chemistry and diversity.

Of the variables examined in our study, elevation was the main predictor of changes in phytochemical defense as well as the abundance of individual compounds. Concentrations of secondary metabolites were correlated with increasing elevation, which has been documented by a few studies with other classes of secondary metabolites, but there are exceptions (reviewed by Rodríguez-Castañeda et al., 2016). Compounds for which there is a documented decline in concentrations of secondary metabolites with increasing elevation include terpenes (Hengxiao et al., 1999), alkaloids (Salmore & Hunter, 2001), and iridoid glycosides (Pellissier et al., 2014). Since empirical data for changes in chemical defense across elevational gradients are still scarce (Rodríguez-Castañeda et al., 2016), it is useful to examine the mechanisms that cause chemistry to change with elevation. In the case of *P. kelleyi*, the increase in defensive compounds at greater elevation could be due to the relationship between increased UVB radiation at higher elevations and the photoactive properties of the prenylated benzoic acid, chromene, and dimeric chromane (Fig. S4-5, Table S1-S2; Krause et al., 1999; Ruhland et al., 2013; Virjamo et al., 2014). UV light is known to affect concentrations of phytochemicals through a variety of mechanisms (Becker & Michl, 1966; Zangerl &
Berenbaum, 1987; Downum et al., 1991; Padwa et al., 1996), and plants at higher elevations are exposed to a greater intensity of UV light compared to plants at lower elevations. The association between elevation and P. kelleyi phytochemistry was mostly due to changes in the relative concentration of prenylated benzoic acid in the leaves. Chromenes are particularly reactive in the presence of UV light (Becker & Michl, 1966; Padwa et al., 1996), and it is likely that increased UV radiation facilitates related biosynthetic links through oxidation of the prenylated benzoic acid and cyclization to the chromene, as well as by a photoinitiated dimerization to produce the chromane. The prenylated benzoic acid likely acts as the precursor, so an increase in one compound is usually associated with increases in the others, as we document here. Thus, Piper shrubs that produce phototoxic compounds could be more toxic at the top of elevational gradients in the Andes and less toxic towards the bottom, due to UV induced increases in abundance and diversity of secondary metabolites.

The possibility that changes in elevation and UV exposure modify the phytochemistry of P. kelleyi is relevant to the evidence for Eois population structure. We found evidence of subtle genetic differentiation for E. encina at the highest elevation (Fig. 4A), but little evidence for differentiation among other populations. The plants located at the highest elevation (>2400 m) contained higher concentrations of prenylated benzoic acid (Fig S3-S5, Table S1-S2), which could be a source of divergent selection. Local adaptation of E. encina to high elevation hosts with high prenylated benzoic acid concentrations could reduce gene flow by causing selection against migrants and low hybrid fitness (Zhang et al. 2015), which could explain the observed genetic differentiation among low and high elevation caterpillars. One individual caterpillar was
collected at a high elevation site that was not genetically grouped with the other highest elevation caterpillars (Fig. 4A). Interestingly, this one distinct high elevation caterpillar was feeding on plants containing a lower concentration of the prenylated benzoic acid (Fig. 4B), which could suggest this individual was a migrant from lower elevation populations. We assume that the high elevation *E. encina* do not represent a cryptic species based on adult vouchers, genitalia dissections, and extensive photographic documentation of larvae. MRM models indicated that genetic distances were explained by chemistry while controlling for elevation (R² = 0.07; P<0.001). The genetic differentiation of *E. encina* collected at high elevation on high acid plants, although subtle, is consistent with the possibility of local adaptation limiting gene flow across these populations and with the hypothesis that phytochemical variation could generate geographically divergent selection across *E. encina* populations. Our results are similar to a recent study on the elm, *Ulmus pumila* L., which demonstrated that leaf age is a source of divergence in two sympatric sister elm leaf beetles, *Pyrrhalta maculicollis* and *P. aenescens* (Zhang *et al.*, 2015). Future studies with the *Piper* system should include a reciprocal rearing experiment comparing high and low populations of *Eois* to demonstrate that *Eois* populations at high elevation are adapting to higher concentrations of prenylated benzoic acid in *P. kelleyi* plants.

Phytochemical variation could be an ecological source of natural selection on specialist herbivores. Such variation in plant defense has performance and fitness consequences for herbivores, and this is particularly true for specialist herbivores, which may be locally adapted to detoxify specific compounds (Dyer *et al.*, 2003; Richards *et al.*, 2010). The biological activity of the defensive compounds in *P. kelleyi* includes
decreased development rate, lower pupal mass and decreased survival when small amounts (i.e. 3.75% of the dry weight) were fed to naïve generalist caterpillars (Jeffrey et al., 2014). This amount is less than half of the 10% dry weight typically found in the leaves of *P. kelleyi* (Jeffrey et al., 2014). However, because this assay was performed on generalist caterpillars it is unknown how specialist caterpillars respond to increased concentration of the three compounds. Other studies have shown that *Piper* chemical defenses have subtle effects on *Eois* physiology rather than direct toxic effects (Dyer et al., 2004; Smilanich et al., 2009). For example, Richards et al. (2010) found that subtle changes in mixture diets (i.e. 0.4 %) of *P. cenocladium* resulted in decreased survival rates and increased parasitism frequency in specialist *E. nympha* Schaus caterpillars. It is likely that at higher elevations the intensity of UV light is greater and can enhance the toxicity of these plants by increasing abundance and evenness of defensive compounds, leading to selection against larvae that lack adequate physiological mechanisms for detoxification or tolerance (e.g., McCloud & Berenbaum, 1999).

**Conclusion**

The results reported here represent a significant contribution to our understanding of the chemical processes maintaining biodiversity at different taxonomic and spatial scales. The study examined community and population structure at a fine scale by focusing on intraspecific host plant populations with distinct phytochemical profiles and associated herbivore communities. We found that variation in plant chemistry affected the caterpillar community (relative abundances of different caterpillar species) and genetic differentiation among populations of *E. encina*, the most common caterpillar
species. The adaptive significance of phytochemical variation in response to UV light levels, particularly whether it is plastic or genetic, as well as the potential role of phototoxicity have yet to be determined. Future studies are needed to examine whether the compounds are phototoxic and how this influences local adaptation and community assemblages of specialist and generalist consumers.

Acknowledgments

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References


Tables

Table 1  Hypotheses and *a priori* predictions guiding the structural equation model. Roman numerals refer to the causal relationship depicted in the path diagram (Fig. 2). These hypotheses are context dependent and based on previous work in the *Piper, Eois*, parasitoid system.

<table>
<thead>
<tr>
<th>Predictor Variable</th>
<th>Response Variable</th>
<th>Causal Relationship</th>
<th>Hypothesis and Prediction</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Piper kelleyi</em></td>
<td>Phytochemical</td>
<td>I</td>
<td>The Screening Hypothesis posits that plants consisting of higher diversity of secondary metabolites have greater probability of being toxic to a broad array of herbivores, due to unique mixtures and synergies that deter plant enemies. This yields the prediction that the diversity of <em>P. kelleyi</em> secondary metabolites will decrease <em>Eois</em> herbivore diversity through diverse defensive mechanisms.</td>
<td>Poelman <em>et al.</em>, 2009; Smilanich <em>et al.</em>, 2009; Firn, 2010; Richards <em>et al.</em>, 2012, 2015</td>
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<td></td>
<td>Community Diversity</td>
<td></td>
<td></td>
<td>Root, 1973; Tahvaneinen &amp; Root, 1972; Barbosa <em>et al.</em>, 2009</td>
</tr>
<tr>
<td><em>Piper Community</em></td>
<td>Phytochemical</td>
<td>II</td>
<td>According to the Associational Resistance Hypothesis, different plant species occurring in close proximity can decrease the likelihood of detection by herbivores. If diversity of <em>Piper</em> shrubs is high, then it will be harder for herbivores to detect <em>P. kelleyi</em> (or other species). Thus, <em>P. kelleyi</em> may invest less in producing defensive compounds in high</td>
<td></td>
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</table>
Piper diversity communities due to low vulnerability to herbivores.

<p>| Piper kelleyi | Parasitoid | III | Plants interact with parasitoids via providing chemical cues for defense against herbivores or poisoning the immune response of caterpillars. If phytochemical defense increases, then parasitoid community diversity should increase. |
| Phytochemical | Community   |     | |
| Defense      | Diversity   |     | Phototoxicity occurs for many secondary metabolites when they are exposed to UV light and are metabolized to more toxic compounds or generate reactive intermediates that interfere with DNA or proteins. If UV light intensity increases with increasing elevation, then plants containing secondary metabolites that are photoactive should have a selective advantage at higher elevations. |
| Piper kelleyi | Elevation   | IV  | Caterpillar diversity should increase as elevation increases, due to higher levels of specialization and lower levels of predation at higher elevations. |
| Eois Community | Diversity | V   | |
| Elevation   | Phytochemical | Defense | |
| Elevation   | Piper | VI Community | Plant diversity should decrease as elevation increases (beyond mid-elevations) due to a variety of mechanism, including colder |
| Turlings &amp; Ton, 2006; Smilanich et al., 2009; Richards et al., 2012; Wäschke et al., 2014 | Downum et al., 1991; Krause et al., 1999; Ruhland et al., 2013; Virjamo et al., 2014 | Brehm et al., 2007; Rodríguez-Castañeda et al., 2010 | Brehm et al., 2007; Rodríguez-Castañeda et al. |</p>
<table>
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<th>Elevation</th>
<th>Parasitoid Community Diversity</th>
<th>VII</th>
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<tr>
<td></td>
<td>If herbivore specialization increases at higher elevations, then levels of parasitism and parasitoid diversity are predicted to increase due to preferential parasitism of specialists and higher diversities of herbivores. Increased concentrations of secondary metabolites at higher elevations may disrupt herbivore immune response against parasitoids.</td>
<td>Smilanich et al., 2009; Rodríguez-Castañeda et al., 2010; Richards et al., 2012</td>
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<tr>
<th>Piper community diversity</th>
<th>Eois Community Diversity</th>
<th>VIII</th>
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<td></td>
<td>More complex and diverse plant communities can decrease the abundance of specialist herbivores. This is because diverse plant communities decrease the detectability of preferred host plants for specialist herbivores.</td>
<td>Root, 1973; Tahvaneinen &amp; Root, 1972; Barbosa et al., 2009</td>
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<tr>
<th>Piper community diversity</th>
<th>Parasitoid Community Diversity</th>
<th>IX</th>
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<td></td>
<td>Increases in plant diversity can cause increases in parasitoid diversity via providing more host species and greater signals for host searching and oviposition cues. Parasitoids can discriminate between their host odors versus the complex odors produced by the plant community.</td>
<td>Erb et al., 2010; Wäschke et al., 2014</td>
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<td></td>
<td>This appears as a “direct” effect because we have not measured cues or other important determinants of parasitoid diversity.</td>
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Table 2 Results from multiple Mantel tests in which models differed based on the response variable.

Predictor variables for all models were elevation, prenylated benzoic acid (PBA), chromene, dimeric chromane and GPS location. For the response variable, genetic variation was estimated using the PC scores of multi-locus genotype likelihoods of *E. encina* individuals transformed into distance matrices. Three matrices were created based on: A) all the PCs, B) PC 1 and 2, and C) only PC 2 scores. Elevation and prenylated benzoic acid were significant predictors of genetic variation in *E. encina* populations.

<table>
<thead>
<tr>
<th>Model</th>
<th>Elevation</th>
<th>PBA</th>
<th>Chromene</th>
<th>Dimeric Chromane</th>
<th>Location</th>
<th>Overall Model</th>
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<tr>
<td>A - all</td>
<td>$p_{Mc} = 0.007$; $p_{Mc} = 16.89$; $p_{Mc} = 3.74$; $p_{Mc} = -13.42$; $p_{Mc} = -1.69$; $R^2 = 0.05$;</td>
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<tr>
<td>PCs</td>
<td><em>P</em> = 0.14</td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> = 0.54</td>
<td><em>P</em> = 0.18</td>
<td><em>P</em> = 0.79</td>
<td><em>P</em> = 0.001</td>
</tr>
<tr>
<td>B - PC 1</td>
<td>$p_{Mc} = 0.01$; $p_{Mc} = 26.94$; $p_{Mc} = -0.06$; $p_{Mc} = -12.64$; $p_{Mc} = -1.37$; $R^2 = 0.07$;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp; PC 2</td>
<td><em>P</em> = 0.05</td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> = 0.99</td>
<td><em>P</em> = 0.38</td>
<td><em>P</em> = 0.88</td>
<td><em>P</em> &lt; 0.001</td>
</tr>
<tr>
<td>C - only</td>
<td>$p_{Mc} = 0.02$; $p_{Mc} = 20.21$; $p_{Mc} = -3.54$; $p_{Mc} = -4.6$; $p_{Mc} = -9.82$; $R^2 = 0.26$;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 2</td>
<td><em>P</em> &lt; 0.0001</td>
<td><em>P</em> &lt; 0.0001</td>
<td><em>P</em> = 0.31</td>
<td><em>P</em> = 0.43</td>
<td><em>P</em> = 0.01</td>
<td><em>P</em> &lt; 0.0001</td>
</tr>
</tbody>
</table>

The ‘*’ denotes predictor variables and overall models that were significant. $p_{Mc}$ = partial Mantel coefficient.
Figure Legends

Figure 1. *Piper kelleyi* leaf chemistry and *Eois* caterpillar community study system. A) NMR spectra of the crude extract containing the three major secondary compounds that have been isolated from the leaves of *P. kelleyi*; a specific prenylated benzoic acid, chromene and dimeric chromane. B) Species of *Eois* that specialize on *P. kelleyi* include (from left to right): *Eois planetaria* (Dognin), *Eois aff. encina* (Dognin), *Eois ignefumata* (Dognin), *Eois encina* (Dognin), *Eois viridiflava* (Dognin), *Eois aff. pallidicosta* (Warren), and *Eois aff. viridiflava* (Dognin).

Figure 2. Results of a structural equation model depicting hypothesized causal relationships between 1) phytochemical defense (latent variable), 2) *Eois* diversity per *P. kelleyi* plant, 3) parasitoid diversity per *P. kelleyi* plant, 4) *Piper* species diversity per plot and 5) elevation. Panel A illustrates the overall path model. The direct positive effects are indicated by black arrows, while the direct negative effects are indicated by light gray blunt-ended lines. The numbers beside the lines are the standardized path coefficients. The roman numerals above the path coefficients relate to Table 1, which describes specific hypotheses being tested. *Piper kelleyi* phytochemical variation is a latent variable, created via factor analysis on relative abundances of the three defensive compounds with varimax rotation. The path coefficients are all significant (P < 0.05) and the model is a significant fit to the data (X2 = 0.014; df = 1; P > 0.1). Panel B depicts a subset of the partial correlation plots from paths I, VII and VIII; the remaining partial correlation plots can be found in Fig. S2.
**Figure 3.** Parasitoid family density along elevation. Parasitoids reared and identified to family included Braconidae, Eulophidae, Ichneumonidae and Tachinidae. Eulophidae had highest densities at 2000 m and Braconidae, Ichneumonidae and Tachinidae had highest densities at 2100 m. Lines under plots indicate 95% confidence intervals. This relationship is based on a linear model, while the path analysis includes residual variation from interacting variables.

**Figure 4.** Principal components analysis illustrates genetic variation and structure across populations of *Eois encina* moths. Points represent genotypic data for 20,458 single nucleotide polymorphisms (SNPs) in each individual. The first two principal components explained 7% (PC1) and 1% (PC2) of the genotypic variation across all individuals and loci and revealed previously undetected genetic differentiation in high elevation populations. Black circles denote individuals exhibiting genetic differentiation, which is correlated with high elevation and benzoic acid concentrations in plants. Panels A-D are the same principal components analysis, but differ in the overlaid gradient. Panel A illustrates an elevation gradient with black being the lowest elevation and green being the highest elevation. Panel B-D illustrates the prenylated benzoic acid, chromane and dimeric chromane concentration gradient, respectively. Dark blue is the lowest concentration, while red is the highest concentration.
Figures

Figure 1

prenylated benzoic acid (PBA)  chromene  dimeric chromane

A

B
Figure 2

A

Parasitoid diversity
Phytochemical Defense
Piper community diversity

Eoas diversity

B

$X^2 = 0.0142; \text{df} = 1; P\text{-value} = 0.9053$
Figure 3
Figure 4

![Figure 4](image-url)
Supplementary Material

Chapter 3 ~ Intraspecific phytochemical variation shapes community and population structure for specialist caterpillars

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**Figure S1.** Plant and caterpillar samples were collected near Yanayacu Biological Station near Cosanga, Napo Province, Ecuador on the Eastern Andes (00°36’ S and 77°53’ W).

A) The red box on the inset map represents the general sampling location within Ecuador. This is a zoomed in view of the 32 plots within the red box. Plots are color coded based on elevation. The majority of sampling collections were made within 10 kilometers of one another. The yellow points north of Yanayacu were the farthest two sites.
Figure S2. Partial correlation plots from the structural equation model. Numbers in the top right correlate with the path number from Fig. 2.
Figure S3. Principal component analysis examining the chemical similarity between individual *Piper kelleyi* plants using concentrations of prenylated benzoic acid, chromene and dimeric chromane of each plant. Concentrations were obtained via HPLC spectroscopy with an internal standard. Each point represents an individual plant and clusters of points indicate overall chemical similarity. The light green color denotes plants having higher concentrations of the benzoic acid, while the purple color denotes plants having low concentrations of this compound. The larger sized dots indicate the plants having higher concentrations of the dimeric chromane and the chromene. PC1 explained 77.51% of the variation, with the most variation explained by the chromene and the dimeric chromane. PC2 explained 20.15% of the variation, with the most variation explained by the prenylated benzoic acid.
Figure S4. Linear regression examining the relationship between elevation and PC2 scores from the phytochemical PCA. Most of the variation explained in PC2 is attributed to the prenylated benzoic acid. There is a significant increase in prenylated benzoic acid concentration as elevation increases ($R^2 = 0.08; P = 0.008; F_{1,90} = 7.303$).
**Figure S5.** Linear regression examining the relationship between elevation and PC1 scores from the phytochemical PCA. Most of the variation explained in PC1 is attributed to the chromene and the dimeric chromane. There was a slight increase in secondary metabolite concentrations as elevation increases ($R^2 = 0.03; P = 0.09; F_{1,90} = 2.876$).
Table S1. Variation explained by each component used in the principal components analysis. The chromene and dimeric chromane explained the most variation in PC1. The prenylated benzoic acid explained the most variation in PC2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC 1</th>
<th>PC 2</th>
<th>PC 3</th>
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</thead>
<tbody>
<tr>
<td>Chromene</td>
<td>0.9</td>
<td>-0.4</td>
<td>0.16</td>
</tr>
<tr>
<td>Dimeric Chromane</td>
<td>0.97</td>
<td>-0.13</td>
<td>-0.2</td>
</tr>
<tr>
<td>Prenylated Benzoic Acid</td>
<td>0.78</td>
<td>0.62</td>
<td>0.06</td>
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</table>
Table S2. The first three eigenvalues of the correlation matrix for the principal component analysis, the proportion of total variance, and the cumulative variance for each principal component.

<table>
<thead>
<tr>
<th>Principal component</th>
<th>Eigenvalue</th>
<th>Proportion of total variance</th>
<th>Cumulative variance</th>
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<tr>
<td>1</td>
<td>2.33</td>
<td>77.51</td>
<td>77.51</td>
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<tr>
<td>2</td>
<td>0.6</td>
<td>20.15</td>
<td>97.66</td>
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<td>3</td>
<td>0.07</td>
<td>2.34</td>
<td>100</td>
</tr>
</tbody>
</table>
Chapter 4 ~ Plant toxicity at the top of a tropical mountain

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Keywords: intraspecific phytochemical variation, light heterogeneity, mosaics of defense, photoactivity, phototoxicity, phytochemical diversity, herbivory, \textit{Piper kelleyi}, Piperaceae, \textit{Eois}, Spodoptera
Abstract

Phytochemical variation among host plant populations may function as a defense by reducing overall herbivory across the landscape. Light heterogeneity may produce such variation by causing changes in phytochemistry among host plant populations across small spatial scales in mega-diverse tropical forests. We hypothesized that the phytochemistry of the tropical shrub *Piper kelleyi* Tepe (Piperaceae) is photoactive in the presence of high light conditions and that photoactive compounds are toxic to interacting herbivores. To examine this potential mechanism by which phytochemical variation is generated in *P. kelleyi*, we experimentally manipulated light availability using a paired-plant canopy experiment along an elevational gradient. In turn, to document the effects of photoactivity on naturally occurring herbivory, we measured levels of herbivory in response to increases in direct light transmittance (%) and light partitioning between high and low canopy heights. Finally, to assess how photoactivity influences caterpillar development, we conducted feeding assays for specialist *Eois encina* (Geometridae) caterpillars and naïve *Spodoptera exigua* (Noctuidae) generalist caterpillars, in the presence or absence of enhanced UV-B light. We found that phytochemical diversity, which is a measure of overall chemical defense against plant parasites, is inversely related to direct light transmittance, and plants located higher in the canopy had lower phytochemical diversity. Subtle differences in phytochemistry among individual plants caused by light heterogeneity had significant effects on herbivory, which decreased as phytochemical diversity increased. Results from rearing experiments were mixed, with no biologically relevant effects on the specialist caterpillars and a negative photoactive effect on generalist caterpillars.
Introduction

Coevolution is a frequently invoked theoretical framework used to explain the high diversity of interacting plants and herbivores (Fraenkel 1958; Ehrlich & Raven 1964). In “escape and radiate” coevolution, reciprocal selective pressures are exerted between plant defensive chemistry and detoxifying-adaptations of associated herbivores, creating adaptive free zones for plant and insect taxa, and ultimately, parallel diversification between host plants and herbivorous insects (Thompson 1994). While coevolution is theoretically logical, it has been difficult to empirically demonstrate (Janzen 1980; Stamp 2003; Parchman et al. 2016) with a few exceptions (Janzen 1966; Farrell 1998; Farrell & Mitter 1998; Zangerl & Berenbaum 2005; Becerra et al. 2009).

Thompson (1999, 2005) developed a more realistic coevolutionary theory, expanding reciprocal, pairwise interactions between plants and herbivores to a scale that encompasses changes in the geographic landscape. Plant species vary in the strength of their interactions with herbivores due to spatial and temporal changes in their phytochemical profiles (Ehrlich & Raven 1964; Whittaker & Feeny 1971; Thompson 1999, 2005; Dyer et al. 2010; Glassmire et al. 2016; Hunter 2016). Variation in phytochemistry is a product of “top-down” and “bottom-up” influences (Hairston et al. 1960; Feeney 1968; Coley et al. 1985; Polis & Strong 1996; Dyer & Letourneau 2002; reviewed in Hunter 2016). These pressures on phytochemistry are not consistent across the geographic landscape because resource heterogeneity influences the composition of primary and secondary metabolites (reviewed in Hunter 2016). Plant populations consist of mosaics of plant quality, resulting in considerable variation in palatability (Hunter 2016) as well as variation in chemical cues utilized by ovipositing insect herbivores.
Many insect herbivores select host plants based on chemical cues and their offspring are subject to the plant’s defenses and nutritional content (e.g., Forister & Wilson 2013), and the cues and plant quality are not always correlated. Thus, variation in chemistry among host plant populations may make it difficult to select high quality plants, reducing herbivore performance.

One axis of plant defense that has been mostly overlooked in coevolution studies is the influence of intramolecular phytochemical diversity on herbivores, partly because the focus has been on individual compounds, which do vary across the landscape but not as much as mixtures (Berenbaum & Neal 1985; Berenbaum & Zangerl 1996, Gershenzon et al. 2012, Richards et al. 2016). Plants are comprised of mixtures of defensive compounds that can have additive effects, antagonistic interactions, or synergistic effects on herbivores. However, a recent meta-analysis found that 90% of papers on anti-herbivore defense treat defensive compounds as though the mode of action for individual compounds occurs in isolation (Richards et al. 2016). Studies that do not characterize or manipulate mixtures of plant compounds may be missing important biological effects, such as toxicity or digestibility reduction. Richards et al. (2015) found that tropical plant species with greater intermolecular phytochemical diversity had more specialized herbivores, increased phototoxicity, and decreased herbivory. This result suggests that interspecific phytochemical diversity is a good predictor of defense, but how much does this defensive trait vary within plant species or populations?

Phytochemical diversity is plastic and can change within plant species across the landscape, partially because of resource heterogeneity. Light is one such resource that can be limiting and varies within and between ecosystems, especially in tropical understories.
Subtle differences in light availability (0.2-6.5% diffuse transmittance) affect growth strategies of shade tolerant versus gap specialist plant species (Montgomery & Chazdon 2001). For example, the shade tolerant *Piper arieanum* (Piperaceae) had more restrictive photosynthetic nitrogen use in high light environments compared to the gap specialist *Piper sancti-felicis* (Chazdon 1992). These plant physiological responses to resources suggest that defensive phytochemistry could be affected by light heterogeneity, and studies on causes of variation in *Piper* chemistry have shown that light has a considerable impact on secondary metabolites (Dyer et al. 2004). Light heterogeneity can also impact herbivore performance by activating phytochemical changes in secondary metabolites, especially if plants are comprised of photoactive compounds, such as furanocoumarins or chromenes. Secondary compounds that have a chromene core have photochromic properties (Becker and Michl 1966), because light initiates ring-opening of the chromene core to produce a reactive ortho-quinone methide intermediate (Fig. 1; Padwa 1972). Photochromic properties of secondary metabolites have been demonstrated to be toxic to herbivores, and there are numerous examples of herbivore adaptations to avoid light or light-activated chemistry. For example, caterpillars in the families Pyralidae and Erebidae (Ctenuchina) rely on leaf rolling to avoid photochromic effects of *Psychotria horizontalis* chemistry; this rolling reduces light intensity via shading (Sagers 1992). This behavior reduced leaf toughness by 31% and tannin concentrations by 15% (Sagers 1992). Herbivore reproduction can also be affected by light; Kuhlmann and Muller (2010a) found that specialist aphids, *Brevicoryne brassicae* L. (Sternorrhyncha, Aphididae) reproduced less in high UV-B light conditions on plants having higher flavonoid concentrations. Finally, there may be direct effects of light on herbivores;
herbivory by thrips in soybean crops is documented to double as light attenuates 25% below the canopy (Mazza et al. 1999). This dose-response change of herbivory in light attenuation is characterized as the “anti-herbivore effect of UV-B radiation” (reviewed in Ballaré et al. 2011). These studies suggest that herbivory can vary with light availability and consuming plant tissue containing photoactive compounds may be more difficult in high light conditions. However, few studies have examined how subtle differences in light availability across the landscape can change intramolecular phytochemistry of host plants and impact associated consumers.

Glassmire et al. (2016) found substantial intramolecular phytochemical variation among three major defense compounds containing a chromene core in Piper kelleyi Tepe (Piperaceae) plants; all plants examined in that study were located within 10 km of each other. Phytochemical variation predicted variation in assemblages of caterpillars, with higher evenness among the three compounds causing a decrease in caterpillar diversity and an increase in parasitoid diversity. Furthermore, caterpillars located at high elevations were genetically different from caterpillars at lower elevations and they fed on plants containing high relative concentrations of a prenylated benzoic acid (Glassmire et al. 2016). These changes across elevation may be partly driven by light heterogeneity, with increased toxicity in high light environments contributing to mosaics of defense in host plant populations.

To investigate how light heterogeneity influences the intramolecular phytochemical diversity of the important tropical shrub, *P. kelleyi*, we manipulated light availability by hanging paired clonal cuttings of this plant at high and low levels in the canopy across an elevational gradient. Differences in canopy height primarily
manipulated light quantity, while elevation mostly manipulated light quality. We examined the chemistry of these plants and conducted several feeding assays with generalist and specialist caterpillars to examine whether photoactivity of plant defensive compounds has negative consequences for the development and survivorship of associated herbivores. The research was guided by the following objectives: (1) establish if the phytochemistry in $P. kelleyi$ is photoactive in the presence of enhanced light radiation, (2) determine if these compounds are toxic to generalist and specialist caterpillars when consumed, and (3) explore the responses of the caterpillars to toxic concentrations of plant defensive compounds. The following hypotheses were tested: (1) Light heterogeneity will cause intramolecular phytochemical variation in photochromic plants and defensive efficacy will decrease in the presence of enhanced light. (2) Herbivory will be negatively correlated with increasing phytochemical diversity. (3) Plants with photochromic compounds will be more toxic to naïve generalist herbivores that consume them in enhanced light. (4) Specialist caterpillars will sequester defensive compounds.

Methods

Study System

Plants in the genus $Piper$ (Piperaceae) include large shrubs and fast growing vines that commonly dominate tropical forest understory communities; these plants are characterized by rich chemistry (Kato & Furlan, 2007) and complex herbivore communities (Dyer and Palmer 2004). $Piper kelleyi$ is a mid-canopy shrub, endemic to
the eastern slopes of the Ecuadorian and Peruvian Andes mountains (Tepe et al. 2014). The vibrant “pink belly” of the ventral side of young leaves hosts a high diversity of herbivores and associated parasitoids (Tepe et al. 2014). Three novel secondary compounds were identified and isolated from the leaves of *P. kelleyi*: prenylated benzoic acid, a chromene, and a dimeric chromane (Fig. 2; Jeffrey et al. 2014). These compounds are present at a high concentration of ~10% of the dry weight of the leaf material.

*Canopy experiment*

We conducted a light-manipulation experiment in the cloud forest at Yanayacu Biological Station (00°36’ S 77°53’ W), Cosanga, Napo Province, Ecuador from August 2014 to December 2015. We used clonal plants paired at high and low heights in the canopy along an elevational gradient to test whether *P. kelleyi* defensive compounds are affected by changes in light availability. UV-B transmittance through the canopy sharply declines 40-70% once it drops below 25% of the forest canopy in the deciduous forests of Maryland (Brown et al. 1994) and suggests that light changes dramatically between the canopy and understory. For this experiment, high and low heights in the canopy were used to simulate the sharp decline of light intensity from the canopy to the forest understory, with higher plants exposed to more light. *Piper kelleyi* clonal cuttings were collected from source plants and established for two months prior to being hung at seven or two meters from the ground. These heights were selected because the mean height for adult *P. kelleyi* is approximately seven meters, while leaves of saplings are on average roughly two meters from the ground (Tepe et al. 2014). Forty-four clonal cuttings were established in pots in a shaded area at Yanayacu. The clonal individuals consisted of four
stem nodes and three leaves. The soil used for potting was collected from the same forest location near the station and thoroughly mixed to ensure the same consistency across treatments and replicates. Clonal pairs from the same source plant were randomly selected together and suspended in their pots from tree limbs using nylon rope and sling shots. There was a total of 22 ropes hung randomly across a 2000-2400 m range in elevation (Fig. 3). This range was selected because it is the distributional range of *P. kelleyi* (Tepe et al. 2014). The high elevation site, 2400 m, is the elevation where plants contain the highest concentrations of the chromene, the dimeric chromane, and the prenylated benzoic acid (Glassmire et al. 2016), possibly due to greater levels of light exposure. Plants remained in the canopy for twelve months, at which time the plants were checked for caterpillars and a new emergent leaf was harvested for chemical analysis. Plant growth and herbivory were quantified as the number of new leaves, total leaf surface area, and proportion of leaf area removed by herbivores. Canopy cover was measured using a Canon camera with a hemispherical fisheye lens and images were captured at each understory plant location. Gap Light Analyzer software (Frazer et al. 1999) was used to estimate % canopy and site openness, and % direct and diffuse transmittance of gap light from photographs. Transmittance measured the amount of radiation passing through the canopy and is frequently used to measure light availability in the understory (Montgomery & Chazdon 2001; Richards & Coley 2007; Ballaré et al. 2011)
Spectroscopy for plant metabolites

Young leaves were collected from 42 *P. kelleyi* shrubs from the canopy experiment described above and dried at 25 °C in a dry box at the field station. The compounds are thermally stable and incident light over relatively short periods of time is not known to cause decomposition (Jeffrey et al. 2014). We stored samples in a dark freezer to reduce light exposure during the extraction and analysis process. In the laboratory, individual leaves were ground using liquid nitrogen and mortar and pestle, and then 1 g of leaf material was extracted with 5 ml of high performance liquid chromatography (HPLC)-grade methanol for each leaf (*full methods with justifications are provided in* Jeffrey et al. 2014 and Glassmire et al. 2016). The extract was sonicated for 15 min and the insoluble leaf material was removed by vacuum filtration. This entire extraction protocol was repeated twice. The methanol was removed using a manifold and extract was placed under a high vacuum for 24 h, to remove residual solvent. The remaining crude extract was dissolved in HPLC-grade methanol with an internal standard of the chromene analog. Standards for the analysis were synthesized at the University of Nevada, Reno. For a more detailed description of extraction methods see Dodson et al. (2000) and Dyer et al. (2001). Samples were analyzed by proton nuclear magnetic resonance spectroscopy (¹H-NMR). For a more detailed description of ¹H-NMR methods, see Richards et al. (2015).

We used ¹H-NMR spectral data for phytochemical diversity measures, utilizing methods developed by Richards et al. (2015) that provide quantification of a broad range of small metabolites. Briefly, peaks were picked using the first derivative peak picker and integrated, and the maximum abundance ion was extracted for each peak using
OpenChrom software (https://www.openchrom.net/). The number of peaks (richness) and the areas of each peak (abundance) were used to calculate a Simpson’s effective richness number (Jost 2007) – our measure of phytochemical diversity. We only used peaks in the downfield chemical shift region (5-14 ppm) because these peaks represent functional groups of secondary metabolites (Richards et al. 2015).

**Herbivore-UV light experiment**

In addition to experimentally testing if *P. kelleyi* secondary metabolites are photoactive, we investigated whether the chemistry of *P. kelleyi* exposed to enhanced UV-B radiation had detrimental effects on generalist and specialist caterpillars. For both experiments, we used a 13-W tropical terrarium UV-B bulb (Exo Terra Tropical UVB 100 Reptile Lamp, Rolf. C. Hagen, Corp., Mansfield, MA, Product Number 96439) to mimic optimal levels of UVB of a tropical forest understory. We used a combination of feeding assays and immune assays. We also qualitatively tested for sequestration of *P. kelleyi* compounds by *Eois* caterpillars.

**Generalist feeding assay**

A generalist feeding assay was conducted at the University of Reno, Nevada from February to April 2015. Generalist caterpillars were used because they do not have specific adaptations to *P. kelleyi* compounds and may be more sensitive to higher concentrations of defensive compounds than specialist caterpillars. For the generalist caterpillar bioassay, *Spodoptera exigua* (Noctuidae) caterpillars were fed an artificial diet containing either high (9.00 g dried *P. kelleyi* leaf material, 1.62 g artificial diet, and
60.96 mL water – 147.6 mg/mL), low (4.41 g dried *P. kelleyi* leaf material, 14.175 g artificial diet and 81.37 mL water – 54.2 mg/mL), or no additions of dried leaves from *P. kelleyi* shrubs. The caterpillars were reared either in the presence or absence of enhanced UV-B light with temperature being controlled at 30°C using an incubator. Light treatments were pseudo-replicated because there was only one incubator available. The diet treatment was fully crossed with low and high light for a total of six treatment combinations. There were 20 caterpillars per cell in the experimental design for a total of 160 individuals. Caterpillars were placed on their randomly assigned treatment-level combinations during the start of the third instar. Half of the individuals were randomly selected for performance and the other half were randomly selected for immune response assays once caterpillars reached their fifth instar. The response variables measured for herbivore performance were survival, development time, pupal and adult mass.

*Specialist feeding assay*

The specialist feeding assay was conducted at Yanayacu Biological Station from June-August 2015, using *Eois* (Geometridae) caterpillars from the low elevation site (2100 m); we found only a few individuals from the high elevation site (> 2300 m), so these were not used in the experiments. Caterpillars were fed fresh leaves of *P. kelleyi* from the same elevation either in the presence or absence of enhanced UV-B light. There were five replicated light treatments and five control treatments for a total of 10 combinations conducted in an open caterpillar rearing shed. Cells were moved around twice weekly throughout the duration of the assay to ensure there was not a site effect, and temperature was monitored for consistency between cells. There were 8 caterpillars
per treatment combination for a total of 80 individuals. Caterpillars were placed on their randomly assigned levels of treatments during the start of the third instar. The response variables measured for herbivore performance were development time, pupal mass, and adult mass for each caterpillar.

**Sequestration abilities of specialist caterpillars**

We examined potential sequestration by *Eois* caterpillars of the major secondary compounds of *P. kelleyi*. Caterpillars were starved for 48 hours and then preserved in methanol for chemical analysis. Caterpillar bodies and associated frass were dried with silica gel and extracted using MeOH following the same protocol used to extract these compounds from *P. kelleyi* in leaves (*full methods with justifications provided in Jeffrey et al. 2014 and Glassmire et al. 2016*). We measured the presence or absence of the prenylated benzoic acid, chromene and dimeric chromane within the tissues (excluding gut contents) and associated frass of 25 caterpillars. The sequestered acid, chromene, dimeric chromane, and their analogs were measured using proton Nuclear Magnetic Resonance (NMR) analysis.
**Statistical analyses**

*Relationships between photoactivity, intramolecular phytochemical diversity, and herbivory*

We used structural equation models (SEM; Grace & Pugesek 1998; McCune et al. 2002; Shipley 2016) to test the hypothesized causal relationships between phytochemical diversity, direct light transmittance and levels of herbivory for *P. kelleyi* at different canopy heights and elevations. For our a priori specified structural equation model, we included specific causal relationships resulting in a model with one exogenous variable (elevation) predicting two endogenous variables (phytochemical diversity and herbivory), and one exogenous variable (canopy height) predicting phytochemical diversity. These four variables were included in our model with hypothesized relationships that are context dependent and based on previous work with *Piper, Eois*, and parasitoids (Dyer et al. 2004; Brehm et al. 2007; Connahs et al. 2009; Smilanich et al. 2009; Rodríguez-Castaneda et al. 2010; Wilson et al. 2012; Richards et al. 2015; Glassmire et al. 2016). Several models were tested using the lavaan package in R version 3.3.2 (R Development Team 2016) and the best model was selected based on the most parsimonious, biologically relevant model with the lowest AIC value. The best path model for plants located at high and low heights in the canopy will be referred as “canopy height SEM” through the remaining text.

Intramolecular phytochemical variation as a function of light availability was examined using the plants located lower in the canopy because we only had hemispherical canopy photographs for these accessible plants. Direct light transmittance (%) was calculated for each plant along an elevational gradient. We used structural
equation models to examine the relationship between phytochemical diversity, direct light transmittance, elevation, and herbivory of plants hanging low in the canopy and followed model selection based on biologically-relevant hypothesized relationships having the lowest AIC value. This path model will be referred as “understory light SEM” from this point on.

_Herbivory_

Each leaf of every plant was photographed using a Canon EOS digital camera with a white background and a ruler. The actual and estimated (before tissue was removed by herbivores or other damage) leaf surface area were quantified using Image J. We calculated percent herbivory as the main response variable in all structural equation models. For all plants, we additionally used analysis of covariance (ANCOVA) to examine effects of canopy height, leaf age, and leaf surface area (covariate) on herbivore consumption (amount of leaf area removed); however, amount of leaf material removed was not the main response variable in analyses but used to address herbivory from the perspective of the herbivore in the ANCOVA model. We used a square root transformation on leaf size and log transformed herbivore consumption to normalize the residuals of the model. Tukey HSD was used to compare groups of leaf age. Finally, for understory plants, we used a subset of the data to examine plants located at low heights in the canopy because we had direct light transmittance on those plants along an elevational gradient. We ran separate linear regressions for herbivory as a function of direct light transmittance and phytochemical diversity.
For the *S. exigua* assays we assessed effects of diet using a dichotomous individual survival response variable in a binomial-response generalized linear model (using the “brglm” package in R and "binomial" family). The “brglm” package reduces the bias caused from complete separation of the data using an adjusted-score approach to fit maximum likelihood estimates that are infinite (Kosmidis 2013). For the *Eois* assays, we used Welch two sample t-tests to analyze the effects of presence or absence of enhanced UV-B light on growth rate, development time, pupal and adult mass. Residuals from all analyses met assumptions of the generalized linear model utilized. To avoid Type II error with the null-hypothesis testing framework, nonsignificant results were followed with Bayesian versions of the t-tests, using the JAGS Gibbs sampler in R and informative priors based on similar *Eois* experiments described in Hansen et al. (2016).

**Results**

**Canopy Experiment**

*Relationships between photoactivity, phytochemical diversity, and herbivory.* For the canopy height SEM, the path model that provided the best fit to the data supported several hypothesized causal relationships (Fig. 4; $\chi^2 = 1.12$; df = 1; $P = 0.29$; $P$-values closer to 1 indicate a better fit to the data). Residuals met assumptions for the general linear model. Canopy height had a strong negative direct effect on phytochemical
diversity (standardized path coefficient (spc) = -0.33; β_chpd = -0.33). The effects of canopy height on phytochemical diversity also cascaded to the arthropod community; as phytochemical diversity decreased higher in the canopy, herbivory on leaves also decreased (spc = -0.2; β_chpd = -0.47). Finally, elevation had positive direct effects on phytochemical diversity (spc = 0.17; β_chpd = 0.07) and herbivory (spc = 0.24; β_chpd = 0.26). The effects of canopy manipulations were subtle but large for intramolecular phytochemical changes; plants located high in the canopy had an average effective peak richness of 0.94, while plants low in the canopy had an average diversity of 0.91 (Fig. 5 & Fig. 6X). As a reference, high phytochemical diversity in 22 Piper species averaged 0.93, while low phytochemical diversity in Piper averaged 0.74 (Richards et al. 2015).

For the understory light SEM, here were also several notable causal relationships between understory light availability, chemistry, and herbivory supported by the understory path model (Fig. 7; χ² = 0.01; df = 1; P = 0.93; P-values closer to 1 indicate a better fit to the data). First, direct light transmittance had a strong negative direct effect on phytochemical diversity (spc = -0.58; β_chpd = -0.003) and a positive direct effect on herbivory (spc = 0.06; β_chpd = 0.002). The effects of direct light transmittance on phytochemical diversity also cascaded to the arthropod community; as phytochemical diversity decreased in the presence of more direct light transmittance, herbivory on leaves also decreased (spc = -0.64; β_chpd = -3.35). Finally, elevation had positive direct effects on herbivory (spc = 0.35; β_chpd = 0.31). Again, the effect sizes were biologically relevant with 4.7% lower herbivory in the highest versus lowest elevations and 0.03 decrease in effective functional group peak richness from the lowest to highest levels of direct light transmittance (Fig. 8); functional group peak richness varied from 0.88 to 0.98 with an
average of 0.92. However, herbivory did not have a significant relationship with elevation \((y = -0.42 + 0.22 x; F(1,44) = 1.9; R^2 = 0.04; p = 0.2)\).

**Herbivory.** The canopy height SEM, which included all plants (Fig 4), uncovered a negative effect of canopy height on phytochemical diversity, and plants with higher phytochemical diversity had lower percent herbivory. Percent herbivory varied from 1% to 58% per plant. The average percent herbivory was 9% +/- 2%. For the ANCOVA analysis, we examined the amount of leaf material removed from the herbivore’s perspective. For all plants utilized in the canopy experiment, there was a significant difference in amount of leaf area removed based on leaf age (Fig. 9; \(\chi^2 = 15.5; F(3, 128) = 5.2; p < 0.01\)) and leaf surface area (Fig. 10; \(\chi^2 = 42.9; F(1, 128) = 94.7; p < 0.001\)). Old leaves had the most leaf surface area removed compared to medium, new and newly emerged leaves. Newly emerged leaves had the least amount of leaf surface area removed (Fig. 9).

The understory light SEM (Fig 6) revealed a negative relationship between direct light transmittance and phytochemical diversity. Phytochemical diversity was negatively associated with herbivory because an increase in diversity caused a decrease in herbivory (Fig. 11; \(y = 3.15 - 3.26 x; F(1,20) = 12.96; R^2 = 0.39; p < 0.01\)). However, herbivory did not have a significant relationship with direct light transmittance \((y = -0.09 + 0.01 x; F(1,20) = 2.8; R^2 = 0.12; p = 0.1)\).
Feeding assays and sequestration

For the generalist feeding assay, *S. exigua* caterpillars on the combination of high diet and presence of UV-B all died in the 4th instar. For the binomial generalized linear model selection with survivorship as a response, the best model included an additive effect of diet and light treatments ($\theta = -10.8; \text{df} = 3; \chi^2 = 6.2; p = 0.01$). Residual errors met normality assumptions. Caterpillars feeding on a high diet in the absence of UV-B light had 56% survival compared to caterpillars feeding on the control or low diet, which had 100% survival (Fig. 12; $\beta = 6.04; z = 3.1; p < 0.01$; residual deviance: 15.323; 56 df). Caterpillars feeding on high diet in the presence of UV-B light had 0% survival (Fig. 12; $\beta = 2.8; z$-value = 1.8; $p = 0.06$; residual deviance: 15.323 on 56 df). Thus, naive caterpillars had significantly lower survival rates when feeding on diets high in *Piper kelleyi* leaf material while in the presence of enhanced UV light. Caterpillars that fed on diets with high levels of *P. kelleyi* leaf material had significantly lower pupal mass compared to those feeding on the control or low diets (Fig. 13a; $F_{(2,29)} = 32.02; p < 0.001$). In addition, mean development time was significantly longer for caterpillars feeding on high diets (Fig. 13b; $F_{(2,52)} = 18.03; p < 0.001$).

For specialist *Eois* caterpillars, there were no significant relationships between presence of UV-B light and development time ($t = 0.01, \text{df} = 49.92, p = 0.99$), pupal mass ($t = 1.55, \text{df} = 50.92, p = 0.13$), and adult mass ($t = 0.55, \text{df} = 33.92, p = 0.59$) of *Eois* caterpillars. To accept these null hypotheses, we report Bayesian posteriors as follows. For development time, the 95% credibility interval for differences between UV and control was -.001 to .001 days, with 94% in the region of practical equivalence, and the effect size of UV on development was -0.01 days. For pupal mass, the 95% credibility
interval for differences between UV and control was -.001 to .001 mg, with 84% in the region of practical equivalence, and the effect size of UV on pupal mass was 0.0009 mg. For development time, the 95% credibility interval for differences between UV and control was -.001 to .001 mg, with 84% in the region of practical equivalence, and the effect size of UV on adult mass was -0.002 mg.

\(^1\)H-NMR results provide clear evidence that Eois caterpillars sequester small amounts of the P. kelleyi chromene in their tissue, but most of the compound is being excreted (Fig. 14). This is supported by the presence of doublet peaks at δH 6.39 and 5.73 ppm.

**Discussion**

Intraspecific phytochemical variation across space and time may enhance anti-herbivore defense by reducing herbivore performance for specific times and locations where defense is high and also by creating an unpredictable resource (Underwood 2004, 2009; Helms and Hunter 2005; Riolo et al. 2015; Wetzel et al. 2016; Hunter 2016). Temporal and spatial variation in light quantity and quality affect photo-responses of specific plant compounds (e.g., chromenes and furanocoumarins), subsequently impacting interacting herbivores (Berenbaum 1995; reviewed in Ballaré 2011; Kulmann & Muller 2010a). Nevertheless, the effects of subtle differences in light quantity and quality across the landscape on intraspecific phytochemical variation are underappreciated (Ballaré et al. 2012). For the tropical shrub, P. kelleyi, we found that phytochemistry responds to light variation across small spatial scales and that phytochemical diversity decreases when light availability increases (Fig. 5-7). In turn,
herbivory decreased as phytochemical diversity increased in the forest understory (Fig. 11); however, this indirect effect of higher light on herbivory was counteracted by direct negative effects of greater light availability on herbivory higher in the canopy. Furthermore, naïve generalist caterpillars experienced greater mortality when feeding on *P. kelleyi* diets under enhanced UV-B light (Fig. 12). Overall, these results suggest that subtle differences in light availability create distinct mosaics of chemically variable microhabitats that reduce the performance of associated herbivores.

*Photoactivity of Piper kelleyi leaves*

Is the lower phytochemical diversity found in *P. kelleyi* plants placed higher in the canopy (Fig. 4 and Fig. 5) due to plants having greater exposure to light higher in the canopy? This is the most likely explanation for this change in chemistry and it is corroborated by the fact that plants located in the understory and in the presence of greater direct light transmittance (%) had lower phytochemical diversities (Fig. 8). Changes in phytochemistry with light can be adaptive in a number of ways, for example some classes of secondary metabolites are responsible for photo-protection against damaging UV radiation – this could be the case for soluble phenolic compounds that accumulate when exposed to high levels UV radiation (Caldwell et al., 1983; Mazza et al., 2000; Kotilainen et al., 2009). Regardless of the adaptive value, an increase in concentration of photoactive compounds at the expense of other compounds can cause an overall decrease in phytochemical diversity when plants are exposed to higher levels of UV radiation.
Because we have not identified all of the phytochemical changes in response to our experimental manipulations, it is not possible to determine what caused lower levels of intramolecular phytochemical diversity, but it is likely that these changes were partly due to shifts towards higher proportions of photoactive compounds (i.e. less evenness among peaks), as suggested by photochemical studies of the major secondary compounds isolated from *P. kelleyi*. Irradiation (330 nm) of the chromene at -78 °C provided a highly reactive ortho-quinone methide intermediate, indicated by the reversible generation of a bright red color (Fig. 2; Sheridan & Jeffrey *unpubl.*). This intermediate had an unexpectedly long lifetime and further reacted to provide the dimeric chromane. Irradiation of the crude leaf material in mineral oil also resulted in the generation of a red suspension, indicating that the ortho-quinone methide intermediate is generated in the leaf and not just in solution (Sheridan & Jeffrey *unpubl.*). These results support the hypothesis that changes in the spectrum of light, such as those experienced at higher elevations, result in changes in the concentration of the prenylated benzoic acid, chromene and dimeric chromane found in *P. kelleyi*.

**Herbivory**

For the subset of plants hanging near the understory, herbivory increased with lower intramolecular phytochemical diversity (Fig. 11). Richards et al. (2015) found a similar pattern with herbivory and intermolecular phytochemical diversity among 22 *Piper* species. There are many potential mechanisms by which intramolecular phytochemical diversity can affect insect herbivores, including direct toxicity of multiple combinations of functional groups. These combinations may work additively or
synergistically to disrupt insect physiological processes, resulting in a negative correlation between phytochemical diversity and herbivore performance. A tritrophic mechanism that could also be very important are additive or synergistic chemical disruptions of the insect immune response, leading to greater mortality due to parasitoids. Hansen et al. (2016) found that specialist \textit{Eois} caterpillars had significantly impaired immune responses and higher levels of parasitism when feeding on \textit{Piper} plants of higher chemical diversity. Similarly, Glassmire et al. (2016) found that greater phytochemical diversity in \textit{P. kelleyi} leaves increased parasitoid diversity.

Phytochemical diversity was lower at higher heights in the canopy (Fig. 5), and based on negative correlations we have documented between phytochemical diversity and herbivory, we expected higher levels of herbivory at the higher canopy heights, but there were no differences in herbivory for plants placed higher in the canopy. Herbivory is affected by multiple factors, including direct negative effects of increased light or changes in light quality on herbivores. For example, Dyer & Letourneau (1999) found that average percent herbivory in \textit{Piper cenocladium} individuals significantly decreased in the presence of high light. In fact, the ecological responses of plant-insect interactions to enhanced light exposure are not at all clear (reviewed in Kuhlman and Müller 2010b; Ballaré et al. 2012); for example, direct effects of light on herbivores can depend on the feeding mode (phloem versus leaf tissue) and degree of specialization of the herbivorous insect (McCloud & Berenbaum 1999; Kuhlman and Müller, 2010a). \textit{Eois} caterpillars are specialized and are adapted to the photoactive compounds of \textit{P. kelleyi} (Glassmire et al. 2016), so the indirect effects of light via phytochemistry may not be apparent if \textit{Eois} are the primary herbivores higher in the canopy.
**Herbivore-UV light experiment**

Photoactive compounds (e.g., furanocoumarins and chromenes) can be toxic to herbivores (Berenbaum 1978; Berenbaum 1995). We found that *Spodoptera* generalist caterpillars had 0% survival when feeding on diets with high levels of *P. kelleyi* leaf material in the presence of enhanced UV-B light (Fig. 12). This high mortality may have been a result of the photoactivity of *P. kelleyi* compounds, or it could be due to UV-affected changes in plant tissue structures (Jansen 2002). However, the individuals of the control combinations (e.g., feeding on artificial diet or low *P. kelleyi* additions in the presence of enhanced UV-B light) had 100% survival.

In contrast, for specialist *Eois* caterpillars, there were no detectable differences among diets for survivorship or physiological responses. This result was expected because these caterpillars are adapted to *P. kelleyi* chemistry in the presence of enhanced UV-B light, so they may have a higher tolerance towards chemical changes associated with the light treatment. *Eois* caterpillars may respond to photoactive secondary metabolites by changes in their physiology or behavior. For example, Carroll et al. (1997) found that caterpillars raised on parsnips containing photoactive furanocoumarins versus artificial diet developed a yellow coloration. They found caterpillars sequestered pigments in their guts, such as lutein found in parsnips, that protects them by reducing oxidative stress imposed by the ingested photoactivated compounds. An example of an herbivore behavioral strategy for avoiding phototoxicity is nocturnal feeding. *Oreina gloriosa* beetles burrow in the soil by day and feed by night to avoid toxicity of photoactive compounds (Nessi & Rahier 2004). For our experiments, the light was fixed to a self-timer that followed a 12-hour day and 12-night light cycle, and specialist
caterpillars could have fed only during the dark periods in the enhanced UV-B treatment. Field observations suggest that *Eois* feed primarily in the dark.

*Sequestration*

Small amounts of the chromene are present in the tissue of the caterpillar bodies. Further analysis is needed to determine if there is a significant difference in levels of sequestration between caterpillars located at high or low elevations. Sequestration is a complicated physiological process requiring specialized enzymes in the midgut, and it is the result of complex selective pressures between plant toxins, herbivore metabolism, and natural enemies of herbivores (Petschenka & Agrawal 2016); it is an important component of the geographic mosaic of coevolution for insect herbivores. For example, Petschenka and Agrawal (2015) found that three caterpillar species varied in their degree of resistance to cardenolides (i.e., most sensitive to most resistant) when fed on eight species of milkweed varying in foliar cardenolide content (0 - 4 µg per mg dry mass). There were no detectable differences of average caterpillar body mass among the caterpillar species, but they varied in their sequestration abilities. Their results suggest that monarch resistance on milkweed cardenolides did not demonstrate dietary constraint but rather a defense against natural enemies due to three modifications of the Na⁺/K⁺-ATPase in the midgut, haemolymph, and finally, retention in the body tissue (Petschenka & Agrawal 2015). Further research on the effects of *Piper* phytochemical diversity on *Eois* specialists should focus on how metabolism and sequestration abilities are affected by different mixtures of secondary metabolites.
Conclusion

Overall the results reported here suggest that light heterogeneity significantly influences intramolecular phytochemical variation and consequently creates microhabitats for herbivores with substantive differences in plant quality among intraspecific host plant species. Subtle increases in phytochemical diversity significantly decreased herbivory and enhanced UV-B light resulted in high mortality for generalist caterpillars. Understanding how intraspecific phytochemical variation affects herbivore performance has implications for advances in the geographic mosaic of coevolution theory, and is relevant to basic and applied issues in biodiversity and agriculture.

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References


Host conservatism, host shifts and diversification across three trophic levels in
Figure legends

**Figure 1.** Chromene cores have a benzene ring fused to a pyran ring. Light initiates ring-opening of the chromene core to produce a *ortho*-quinone methide intermediate.

**Figure 2.** The hypothesized biosynthesis of the major defensive secondary metabolites isolated from the leaves of *P. kelleyi*: prenylated benzoic acid, chromene, and dimeric chromane (Jeffrey et al. 2014). The prenylated benzoic acid undergoes an enzymatic reaction to produce the chromene. UV-light activates the ring opening of the chromene core to produce the quinone-methide intermediate (QM) and ultimately forms the dimeric chromane. This hypothesized reaction was verified by photochemical studies (Sheridan & Jeffrey, *unpubl.*). Irradiation of the chromene demonstrated the reversible color change between clear to red to pink indicating the formation of the *ortho*-quinone-methide intermediate.

**Figure 3.** Design of the canopy experiment. Clonal pairs of individual *Piper kelleyi* plants were randomly selected and suspended in pots from tree limbs. High and low heights in the canopy were used to simulate differences in light intensity, with high plants exposed to more light while low plants were exposed to less light. There were a total of 22 ropes hung along an elevational gradient.

**Figure 4.** The best path model from a structural equation model approach to test causal relationships between: phytochemical defense, canopy height, herbivory, and elevation.
The direct positive effects are indicated by black arrows, while the direct negative effects are indicated by gray blunt-ended lines. The numbers beside the lines are the standardized path coefficients. The path coefficients are all significant (P < 0.05) and the model is a significant fit to the data (\(X^2 = 1.12; \text{df} = 1; P > 0.3\)). The overall variation was explained for phytochemical diversity (\(R^2 = 0.2\)) and herbivory (\(R^2 = 0.1\)).

**Figure 5.** Relationship between phytochemical diversity as a function of canopy height. Phytochemical diversity was calculated using chemical shift peaks > 300 ppm in the downfield region. Peaks were picked and converted to Simpson’s diversity index (*full protocol found in* Richards et al. 2015). There was a significant decrease in phytochemical diversity of plants located high in the canopy (\(t = 2.4, \text{df} = 33, \text{p-value} = 0.02\)).

**Figure 6.** Differences in \(^1\text{H}-\text{NMR}\) of high and low canopy individuals. The blue circles surrounding the protons (H) are part of the chromene core. The blue rectangles are outlining the spectral peaks associated with the chromene. The low canopy individual has more chromene based on the intensity of the peaks. The orange circles surrounding the protons (H) are part of the methoxy functional group. The orange rectangles and “P” are depicting the spectral peaks belonging to the piplartine peaks.

**Figure 7.** The best path model from a structural equation model focusing on low canopy plants, and testing hypothesized causal relationships between: phytochemical defense, direct light transmittance, herbivory, and elevation. Illustration of the overall path model.
The direct positive effects are indicated by black arrows, while the direct negative effects are indicated by gray blunt-ended lines. The numbers beside the lines are the standardized path coefficients. The path coefficients are all significant (P < 0.05) and the model is a significant fit to the data ($X^2 = 0.01; df = 1; P > 0.9$). The overall variation was explained for phytochemical diversity ($R^2 = 0.3$) and herbivory ($R^2 = 0.5$).

**Figure 8.** For low canopy plants, phytochemical diversity significantly decreased as percent direct light transmittance increased. Direct light transmittance was measured using a hemispherical canopy photograph at low canopy plants and analyzed using Gap Light Analyzer software.

**Figure 9.** Leaf area consumed by herbivores as a function of leaf age and canopy height. Amount of leaf material removed was not the main response variable in analyses - percent herbivory was used in all structural equation models and other hypothesis tests. There was no significant difference in the amount consumed based on canopy height ($p < 0.05$). There was significantly more leaf area removed from old leaves compared to medium, new, and newly emerged leaves; there was significantly less leaf area removed from newly emerged leaves ($p < 0.05$).

**Figure 10.** Leaf area removed by herbivores as a function of leaf surface area. Amount of leaf material removed was not the main response variable in analyses - percent herbivory was used in all structural equation models and other hypothesis tests. There was significantly more herbivory on larger sized leaves.
Figure 11. Percent herbivory as a function of phytochemical diversity using $^1$H-NMR. Herbivory is inversely related to phytochemical diversity of leaf tissue.

Figure 12. Survival results of the naïve, generalist *Spodoptera exigua* feeding assay. Caterpillars fed on a control artificial diet, low or high additions of *P. kelleyi* leaf material while in the presence of UV-B light. Caterpillars feeding on the high diet in the presence of UV-B light had 0% survival (N=10). Caterpillars feeding on the high diet in the absence of UV-B light had 56% survival (N=10). Caterpillars feeding on the control or low diet in either the presence or absence of UV-B light had 100% survival (N=10).

Figure 13. Development results of the naïve, generalist *Spodoptera exigua* feeding assay. Panel A depicts the caterpillars that fed on high additions of *P. kelleyi* leaf material had significantly lower pupal mass compared to those feeding on the control or low diets ($F_{(2,29)} = 32.02; p < 0.001$). The pink points denote the mean of each diet group. Panel B illustrates that mean development time was significantly longer for caterpillars feeding on high diets ($F_{(2,52)} = 18.03; p < 0.001$). The turquoise points denote the mean of each diet group.
**Figure 14.** Proton NMR of sequestration in the body and frass of *Eois* caterpillars. The blue NMR spectra represents the frass and the red NMR represents the body. $^1$H-NMR analysis of *Eois* caterpillars and their frass revealed that while most of the active *P. kelleyi* chemical compounds were excreted, the chromene is sequestered at low concentrations (depicted by the orange rectangles).
Figures

Figure 1

\[ \text{Diagram showing chemical structures and reactions at 313nm and 435nm.} \]
Figure 2
Figure 3

Enhanced light exposure

Low light exposure

N = 22

7 m

2 m
Figure 5
Figure 6
Figure 7

Herbivory

Elevation

0.35

Phytochemical Diversity

-0.64

Direct Light

0.06

-0.58
Figure 8

Low Canopy Plants

\[ y = 0.997 - 0.003x \]

\[ F_{(1,20)} = 10 \]

\[ R^2 = 0.33 \]

\[ P < 0.01 \]
Figure 9
Figure 10

\[ y = 0.5 - 0.1 x \]

\[ F_{1,131} = 92.7 \]

\[ R^2 = 0.42 \]

\[ P < 0.0001 \]
Figure 11

Low Canopy Plants

y = 3.15 - 3.26 x

F(1,20) = 12.96

R^2 = 0.39

P < 0.01
Figure 12

The graph shows the relationship between diet treatment and mortality (%). The x-axis represents the diet treatment levels: control, low, and high. The y-axis represents mortality (%). Two light treatments are indicated: None (purple dots) and UV light (yellow dots). The graph illustrates an increasing trend in mortality with higher levels of diet treatment and light exposure.
Figure 13

A

B
Epilogue ~ Conclusions and future work

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One axis of plant defense that has been mostly overlooked in coevolution studies is the influence of phytochemical diversity among intraspecific host plants on herbivores, partly because the focus has been on individual compounds, which do vary across the landscape but not as much as mixtures (Berenbaum & Neal 1985; Berenbaum & Zangerl 1993, Gershenzon et al. 2012, Richards et al. 2016). My dissertation sought to understand the multi-trophic effects of phytochemical variation on community interactions. The objective was to examine the causes of phytochemical variation and its consequences on interacting herbivores. *Piper* (Piperaceae) host plants were used to address these chemical ecology questions due to the high diversity of 1) plant species within this genus and 2) interacting specialist *Eois* herbivores (Dyer & Palmer 2004). Additionally, the secondary chemistry among *Piper* species is incredibly diverse across the phylogeny (Dyer & Palmer 2004). To examine phytochemical variation among *Piper* host plants, we used *Piper kelleyi* and *Piper imperiale*.

**Causes of phytochemical variation**

We found a strong interaction between abiotic and biotic factors that determined the variation in *P. imperiale* foliar chemistry. Interestingly, we found that the herbivory treatment had the strongest negative effect on *P. imperiale* defensive chemistry, but the direction was opposite to what the literature would have predicted (Agrawal 1998, 1999, 2011). The soil treatment had a strong positive effect on defensive chemistry (Chapter 2).

We experimentally tested whether light heterogeneity would influence the phytochemical profile of *P. kelleyi* plants established across small spatial scales (<10
km). We found phytochemical diversity significantly decreased as light intensity increased (Chapter 4).

**Consequences of phytochemical variation**

We found that high concentrations of *P. kelleyi* phytochemistry shaped population and community structure of specialist *Eois* caterpillars (Chapter 3). For population structure, a group of genetically distinct caterpillars fed on higher concentrations of phytochemistry located at higher elevations. For community structure, *Eois* diversity decreased when feeding on plants comprised of higher concentrations of phytochemistry. Interestingly, parasitoid diversity was positively correlated with phytochemical diversity of *P. kelleyi* host plants.

Photoactivity of *P. kelleyi* chemistry negatively influenced phytochemical diversity. In turn, high phytochemical diversity significantly decreased herbivory. For the feeding assays, there were mixed results regarding the phototoxicity of secondary metabolites in *P. kelleyi* on consumers. These compounds were toxic to generalist caterpillars, but demonstrated no biologically relevant effects on specialist caterpillars.

**Synthesis**

Understanding density dependence in herbivores as a result of phytochemical variation among intraspecific host plants is a fruitful topic for plant-insect interactions research (Underwood 2004, Hunter 2016). Intraspecific chemical variation between individual host plants could give rise to geographically variable selection, and contribute to shifts in herbivore preference for, or performance on, unique concentrations of
individual secondary compounds (i.e., a selection mosaic, Thompson, 1999; Thompson, 2005). Thus, phytochemical variation across host plant populations might shape the composition of herbivore assemblages, and could give rise to geographically divergent selection on herbivore populations.

Overall, we found that subtle differences in light availability, soil type, and herbivory influenced phytochemical diversity of host plants. These differences were significant and occurred at small spatial scales across the landscape. Subtle differences in phytochemical variation demonstrated consequences on the diversity, performance, and evolution of associated specialist herbivores and their natural enemies. Thus, phytochemical variation leads to differences in microhabitat quality of host plants for consumers that negatively affected their performance. Over evolutionary time, microhabitat pressure driven by phytochemical variation could lead to speciation of herbivores.

**Future directions**

In regard to the *Piper-Eois* system, further studies are needed to evaluate the mechanism of adaptation in specialist *Eois* caterpillars to phytochemical variation in associated host plants. Specifically, how are *Eois* detoxifying the secondary metabolites in *Piper kelleyi*? And how does phytochemical variation influence sequestration abilities? Are they changing their feeding behavior to feed at night in order to avoid phototoxicity of secondary metabolites? Answers to these questions will help elucidate the mechanism causing adaptation in specialist caterpillars to phytochemistry. This would support coevolution and the high diversity observed in the *Piper-Eois* system.
References


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