ECOLOGY LETTERS

Ecology Letters, (2018) doi: 10.1111/ele.13194

LETTER

Proximity to canopy mediates changes in the defensive chemistry and herbivore loads of an understory tropical shrub, *Piper kelleyi*

Andrea E. Glassmire,[1]* Casey Philbin,[2] Lora A. Richards,[3] Christopher S. Jeffrey,[2] Joshua S. Snook[1] and Lee A. Dyer[3]

[1]*Department of Entomology, East Lansing, MI 48824, USA*
[2]*Chemistry Department, University of Nevada, Reno Reno, NV 89557, USA*
[3]*Ecology, Evolution, and Conservation Biology, University of Nevada, Reno Reno, NV 89557, USA*

**Correspondence: E-mail: glssmr33@gmail.com*

Abstract

Phytochemical traits are a key component of plant defense theory. Chemical ecology has been biased towards studying effects of individual metabolites even though effective plant defenses are comprised of diverse mixtures of metabolites. We tested the phytochemical landscape hypothesis, positing that trophic interactions are contingent upon their spatial location across a phytochemically diverse landscape. Specifically, intraspecific phytochemical changes associated with vertical strata in forests were hypothesised to affect herbivore communities of the neotropical shrub *Piper kelleyi* Tepe (Piperaceae). Using a field experiment, we found that phytochemical diversity increased with canopy height, and higher levels of phytochemical diversity located near the canopy were characterised by tradeoffs between photoactive and non-photoactive biosynthetic pathways. For understory plants closer to the ground, phytochemical diversity increased as direct light transmittance decreased, and these plants were characterised by up to 37% reductions in herbivory. Our results suggest that intraspecific phytochemical diversity structures herbivore communities across the landscape, affecting total herbivory.

Keywords

Herbivory, intraspecific phytochemical variation, light heterogeneity, phytochemical diversity, vertical stratification.

Ecology Letters (2018)

INTRODUCTION

Studies that document the overdispersion of interspecific phytochemical defensive traits have provided insight into the evolution of plant defenses (Agrawal & Fishbein 2006; Salazar *et al*. 2016; Endara *et al*. 2017) and the coexistence of related species in a community (reviewed in Whitham *et al*. 2006; Becerra 2007; Kursar *et al*. 2009). It is equally exciting to consider that similar heterogeneity within species can drive speciation of herbivores on a single host plant (Wilson *et al*. 2012; Glassmire *et al*. 2016) and influence entire arthropod communities (Schuman *et al*. 2015; Bustos-Segura *et al*. 2017; Massad *et al*. 2017). Two approaches that will fuel progress in understanding intraspecific variation in phytochemistry are: (1) moving the focus away from effects of individual secondary metabolites (Gershenzon *et al*. 2012; Richards *et al*. 2016), and (2) quantifying the causes and consequences of variation in phytochemistry across the landscape (Hunter 2016). Variation across the phytochemical landscape can be detrimental to herbivores, because host plant quality within a population is less predictable when it consists of a mosaic of nutrient availability and toxicity (Hunter 2016; Wetzel *et al*. 2016). Thus, variation in chemistry within host plant populations may make it difficult to select high quality plants and consequently acts to protect plants from insect damage. To contribute towards closing knowledge gaps in the causes and consequences of intraspecific variation in phytochemistry, we examined how variation along vertical strata in a tropical forest (i.e. height of understory plants above the ground) and associated light availability affects phytochemical diversity across the geographic landscape and quantified the magnitude of these effects on an herbivore community.

One axis of plant defense that has been mostly overlooked in plant–herbivore interactions is the influence of phytochemical diversity on herbivores. Instead, research has focused on individual metabolites, which ignores potential synergistic, additive, or antagonistic effects (Berenbaum & Zangerl 1996; Richards *et al*. 2016). A recent meta-analysis found that ~ 90% of papers on anti-herbivore defense treat defensive metabolites as though the mode of action for individual metabolites occurs in isolation (Richards *et al*. 2016), which ignores important biological effects, such as toxicity or reduced digestibility resulting from the synergy of compounds (Gershenzon *et al*. 2012). In a comparison across different tropical shrubs in the genus *Piper* (Piperaceae), Richards *et al*. (2015) found that species with greater phytochemical diversity were characterised by more specialised herbivores, increased phototoxicity and decreased herbivory. This result suggests that interspecific phytochemical diversity is a good predictor of defense and herbivore adaptation, but how much does phytochemical diversity vary within plant species or populations?

Mosaics of plant defense are created when concentrations of individual compounds, measured as the overall phytochemical diversity, change within plant species across the landscape in response to resource heterogeneity. The shaded forest understory create a patchy habitat limited in light

availability with < 1% full sunlight reaching the forest floor (e.g. Chazdon & Fetcher 1984). This potentially creates a heterogeneous phytochemical landscape of unique niches and microhabitats that could promote a diversity of interactions at larger scales. Subtle differences in light availability (0.2–6.5% diffuse transmittance) can affect the growth strategies of tropical plant species (Montgomery & Chazdon 2001), and defensive phytochemistry (Dyer *et al.* 2004). For tropical understory plants, variation in light availability is one of the most important determinants of changes in phytochemical profiles, and many secondary compounds increase in concentration with greater light availability (Dyer & Palmer 2004; Dyer *et al.* 2004; Richards *et al.* 2015; Glassmire *et al.* 2016). Furthermore, light heterogeneity directly affects herbivore reproduction (Kuhlmann & Müller 2010a,b) and feeding behaviour (Sagers 1992) as well as overall plant resistance to herbivores (Mazza *et al.* 2000). These effects can be enhanced when changes in light affect photoactive metabolites, such as furanocoumarins and chromenes, which can be toxic to herbivores (Berenbaum 1978).

The neotropical shrub, *Piper kelleyi* Tepe is characterised by substantial phytochemical variation among three major defense metabolites containing a photoactive chromene (Glassmire *et al.* 2016). Variation in the relative abundances of the different metabolites across elevation (2000–2400 m) may be partly driven by light heterogeneity, potentially causing mosaics of defense in host plants, distinct caterpillar communities and genetic structure across elevational bands for one caterpillar species (Glassmire *et al.* 2016). Of particular interest is the high elevation site, 2400 m, because that is the location plants contain the highest concentrations of several photoactive secondary metabolites (Glassmire *et al.* 2016), possibly due to greater levels of light exposure. To investigate whether light heterogeneity influences the phytochemical diversity of *P. kelleyi*, we experimentally manipulated plants' proximity to the canopy, which is correlated with increases in light availability towards the upper vertical strata in forests. Paired clonal plants were hung at the average height for adult (7 m) vs. young adult plants (2 m) across the elevational range of *P. kelleyi* (Tepe *et al.* 2014). We quantified the chemical diversity and relative concentration of individual metabolites of experimental plants. The research was guided by two objectives: (1) establish if the phytochemistry in *P. kelleyi* changes in the presence of enhanced light, and (2) determine if these putatively photoactive metabolites reduce insect damage. We hypothesised that proximity to the canopy can impact herbivory via light heterogeneity, which alters plant phytochemical diversity. The following predictions were assessed: (1) light heterogeneity will cause variation in phytochemical diversity in photoactive plants; and (2) herbivory will be negatively correlated with increasing phytochemical diversity.

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 characterised by rich chemistry (Kato & Furlan 2007) and complex herbivore communities (Dyer & 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 is where most herbivores reside and hosts a high diversity of herbivores and associated parasitoids (Tepe *et al.* 2014). The major secondary metabolites that have been isolated from the leaves of *P. kelleyi* include a specific prenylated benzoic acid (PBA), chromene, and dimeric chromane (Fig. S1; Jeffrey *et al.* 2014). These metabolites are present at a high concentration of ~ 10% of the dry weight of the leaf material (Jeffrey *et al.* 2014). Secondary metabolites consisting of a chromene core are known to have photoactive properties (Becker & Michl 1966), because light initiates ring-opening of the chromene core yielding a reactive intermediate (Fig. S2; Padwa *et al.* 1975). The chromene of *P. kelleyi* was confirmed to be photoactive in a laboratory setting (Nguyen 2015). Thus, we sought to investigate how natural variation in light across the forest floor influences photoactive metabolites of *P. kelleyi* host plants and its subsequent effects on the herbivore community. 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. All collections were conducted with permission from the Museo Ecuatoriano de Ciencias Naturales in Quito, Ecuador (permit no. 001-2011-DPAP-MA).

Canopy experiment

We conducted a vertical stratification experiment in the cloud forest canopy at Yanayacu Biological Station (00°36′ S 77°53′ W), Cosanga, Napo Province, Ecuador (YBS) from April 2015 to December 2015. At this station, the climate is cool with an average air temperature of 22.7 °C during the day, which drops linearly with elevation at a rate of *c.* 0.5 °C/200 m. Precipitation ranges from 3900 to 4500 mm per year. We placed clonal plants at paired high (7 m) and low (2 m) heights in the canopy along an elevational gradient (2000–2400 m) to test whether *P. kelleyi* defensive metabolites are affected by changes in light availability; this manipulation created microenvironments similar to light variation across the forest floor. Since many plant–herbivore systems can vary substantially with changes in resource availability, we acknowledge that other abiotic and biotic factors are correlated with canopy height and elevation (Fig. S3), but this is also true of different light environments along the forest floor (gaps vs. understory; Lieberman *et al.* 1989). Temperature, precipitation and oxygen levels were recorded at each paired location and had no significant effects of these abiotic factors on phytochemical diversity (Fig. S3; $P > 0.5$) and herbivory (Fig. S3; $P > 0.9$). We also ensured that ants were absent at every location, which was expected because ant density in this region of the Andes dramatically decreases above 1800 m (Rodríguez-Castañeda *et al.* 2011) and *P. kelleyi* does not host symbiotic ants (Tepe *et al.* 2014). Parasitoid pressure on herbivores also

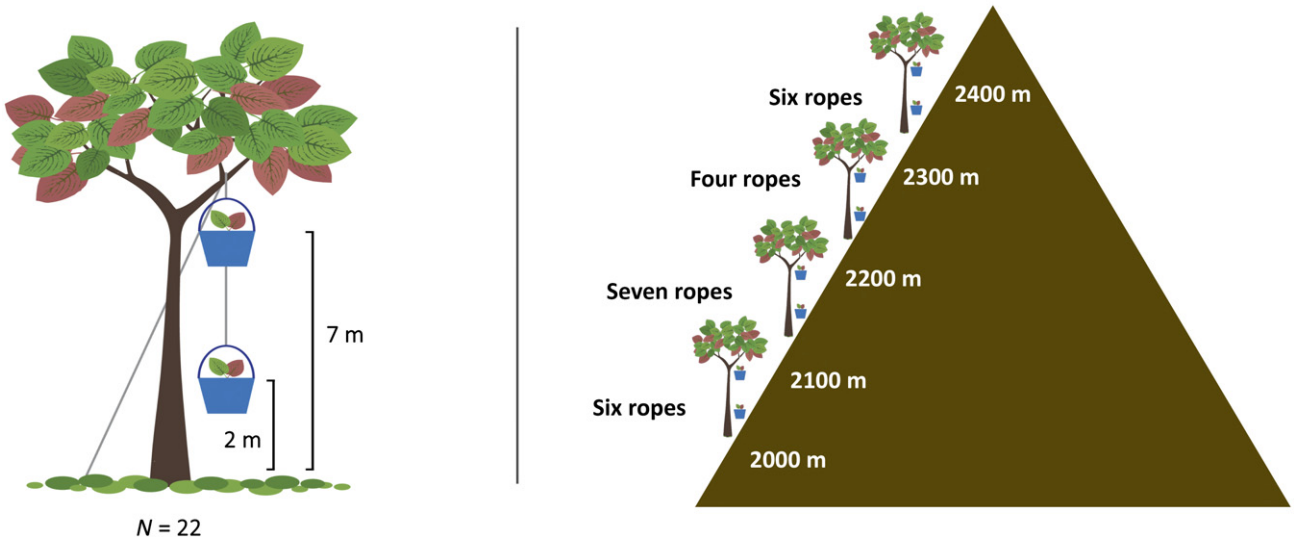

Figure 1 Experimental design for the canopy experiment manipulating vertical stratification in the forest. Clonal pairs of individual *Piper kelleyi* plants were randomly selected and suspended in pots from tree limbs. High and low heights in the canopy were used to simulate differences in natural variation in light intensity that are present across the forest understory. Plants in close proximity to the canopy were exposed to more light while understory plants near the soil were exposed to less light. There was a total of 22 ropes hanging along an elevational gradient. Illustrations by Angela Hornsby.

significantly increases with elevation (Glassmire *et al*. 2016), however, we did not collect the parasitoid data in this study.

We utilised a direct measure of light only for understory plants (i.e. those close to the soil, since we did not have tree climbing equipment) and found that it varies significantly along the small spatial scale (<10 km) and elevational range (400 m) of the experimental design (Fig. S4). Within an elevational band, light changes dramatically between the canopy and understory (e.g. Brown *et al*. 1994). For our experiment, high and low heights in the vertical strata of the canopy were used to simulate the sharp decline of light intensity from one microclimate to another, including differences between leaves at the top of an adult shrub vs. leaves on a young adult. 'Vertical strata' refers to this experimental treatment throughout the manuscript, with higher leaves exposed to more light. *Piper kelleyi* clonal cuttings were collected from the same source plant to purposely exclude genetic variation in order to focus on the ecological relevance of phenotypic plasticity mediated by resources varying along vertical strata in the canopy. Clonal cuttings were established for two months prior to being hung at seven vs. two meters from the ground, the average height for adults and young adults, respectively (Tepe *et al*. 2014). Forty-four cuttings were established in pots in a shaded area at YBS. 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 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. 1; Fig. S4). Plants remained in the canopy for six months and were then checked for caterpillars. Plant growth was quantified as the number of new leaves and herbivory was calculated as the proportion of total leaf area removed by herbivores. Canopy cover and direct light transmittance were measured using a Canon EOS Rebel-T4 camera with a hemispherical fisheye lens and images were captured at each understory plant location. All analyses using these light measurements will be referred to as 'understory light models'. Gap Light Analyzer software (Frazer *et al*. 1999) was used to estimate percent direct transmittance of gap light from images. Transmittance measured the amount of radiation passing through the canopy and is frequently used to measure light availability in the understory (Montgomery & Chazdon 2001).

Spectroscopic analysis of plant metabolites

After 6 months, young leaves were collected from the 44 *P. kelleyi* shrubs and dried at 25 °C in a dry box at the field station. We compared air dried, oven dried, and freeze-dried samples and have not found any differences in major secondary metabolites. The metabolites are thermally stable and stable to incident light (Jeffrey *et al*. 2014). However, we acknowledge that the metabolome is likely to change depending on the collecting, drying, and extracting methods used. There were 11 samples from the elevational range 2084–2099 m, 17 samples from 2104 to 2193 m, 6 samples from 2226 to 2282 m, and 12 samples from 2331 to 2406 m. Of these, two samples from 2100 to 2200 m were used for validation. In the laboratory, plant samples were stored at −10 °C in the absence of light during the extraction and analysis process. 1 g of ground leaf material was extracted with 5 ml of high-performance liquid chromatography (HPLC)-grade methanol for each leaf, dried, and redissolved for analysis (*full methods are provided in* SI, Jeffrey *et al*. 2014). The relative concentrations of samples were analysed by proton nuclear magnetic resonance spectroscopy

(^{1}H-NMR, 400 MHz) and liquid-chromatography-mass spectrometry (LC-ESI-TOF, Agilent 1200 HPLC equipped with a diode-array detector and coupled to an Agilent 6230 time-of-flight mass spectrometer with electrospray ionisation) using a Phenomenex Kinetex Evo (Phenomenex Co., Torrance, CA, USA) reverse phase column (C18, 100 × 2.1 mm^2, 2.6 μ) (*full methods are provided in* SI; Jeffrey *et al*. 2014; Glassmire *et al*. 2016). Peaks for known compounds were assigned based on comparison to internal standards.

Statistical analyses

We utilised two complementary approaches in analysing the phytochemical profiles of *P. kelleyi;* proton nuclear magnetic resonance spectroscopy (^{1}H-NMR, 400 MHz) and high-pressure liquid-chromatography-mass spectrometry (LC-MS). Phytochemical diversity can be quantified to encompass two levels of complexity: compositional and structural. Compositional complexity is based on the number of different compounds within a given extract, and those individual compounds may be structurally simple or complex. We used two measures to capture these levels of complexity (*full description of phytochemical diversity complexity is provided in the SI*).

First, we used ^{1}H-NMR spectral data for phytochemical diversity measures (Richards *et al*. 2015) that encompass both compositional and structural complexity and provide insight into functional group diversity for a broad range of small molecules. Peaks in ^{1}H-NMR represent proton (H) resonances for all compounds in a mixture. As the composition of this mixture changes, with different relative concentrations of compounds, the peak intensity changes as well. Thus, differences in the NMR spectra of conspecific individuals that have shared structures can be used to quantify compositional changes. This measure was calculated as Simpson's diversity [1 − D; where $D = \Sigma(n/N)^2$] using the vegan package (Oksanen *et al*. 2017) in R version 3.3.2 (SI; R Development Core Team 2017). Second, LC-MS was used to confirm the influence of compound richness on overall phytochemical diversity. We quantified compositional complexity as the richness of *m*/*z* feature in an LC-MS chromatogram. In addition, we annotated the chromatogram for known compounds for further statistical analysis (*full description of statistical analyses using ^{1}H-NMR and LC-MS is provided in the SI*).

Relationships between vertical stratification, phytochemical diversity, and herbivory

Structural equation models (SEM; Shipley 2016) were used to test the hypothesised causal relationships between phytochemical diversity and levels of herbivory for *P. kelleyi* at different vertical strata in the canopy and elevation. The structural complexity captured by peaks from LC-MS was condensed using factor analysis, which utilises shared variance and produces factors (analogous to biosynthetic pathways) that are linear predictors of variables (in this case, LC-MS peaks; *methods provided in SI*). Variation in all LC-MS peaks were maximised on the first factor loading. This factor is referred to as the 'chemotype'. For our *a priori* specified structural equation model, we included specific causal relationships resulting in a model with one exogenous variable (elevation) predicting herbivory, an endogenous variable (chemotype) predicting phytochemical diversity and herbivory, and one exogenous variable (canopy height) predicting phytochemical diversity and chemotype. These five variables were included in our model with hypothesised relationships that are context dependent and based on previous work with *Piper* chemistry and *Eois* (Dyer *et al*. 2004; Rodríguez-Castañeda *et al*. 2010; Glassmire *et al*. 2016). Several models were tested using the lavaan package in R version 3.3.2 (R Development Core Team 2017). The best model was selected based on the most parsimonious, biologically relevant model with the lowest AIC value (Table S1). The best path model for plants located at high and low heights in the canopy will be referred as 'vertical strata SEM' through the remaining text.

Phytochemical variation in response to light availability was quantified using only understory plants near the soil, for which hemispherical canopy photographs were accessible. Plants referred to as 'understory plants' throughout the manuscript were located in the understory and the plants that had direct light transmittance measurements recorded. Direct light transmittance (%) and canopy openness were calculated for each plant along an elevational gradient. SEM was used to examine the relationship between measures of phytochemical diversity (i.e. NMR peak diversity, LC-MS peak richness, and concentrations of individual compounds), direct light transmittance, canopy openness, elevation, and herbivory. The 'understory light SEM' model referred to through the remaining text was selected based on biologically relevant hypothesised relationships having the lowest AIC value.

Herbivory

Each leaf of every plant was photographed using a Canon EOS Rebel-T4 digital camera with a white background and scale. The actual and estimated (pre-damage) leaf surface area were quantified using Image J. We calculated percent herbivory as the main response variable in all analyses. Percent herbivory was arcsine square root transformed to meet normality assumptions. Finally, we ran separate linear regressions to examine effects of direct light transmittance and phytochemical diversity on herbivory.

RESULTS

Canopy experiment

Relationships between vertical stratification, phytochemical diversity and herbivory

The 'vertical strata SEM' focused on compositional complexity (i.e. chemotype, Fig. 2) of all plants located at high and low canopy heights. The following causal hypotheses were supported by the data: (1) increases in canopy height have a positive direct effect on phytochemical diversity (Fig. 3a; standardised path coefficient (spc) = 0.12; β = 0.12). (2) Increases in canopy height shift the chemotype towards chromene production (spc = 0.3; β = 0.3), which subsequently increases phytochemical diversity (spc = 0.28; β = 0.28). (3) Increases in phytochemical diversity strongly depressed herbivory (spc = −0.52; β = −0.53). (4) Elevation increases herbivory (spc = 0.34; β = 0.34).

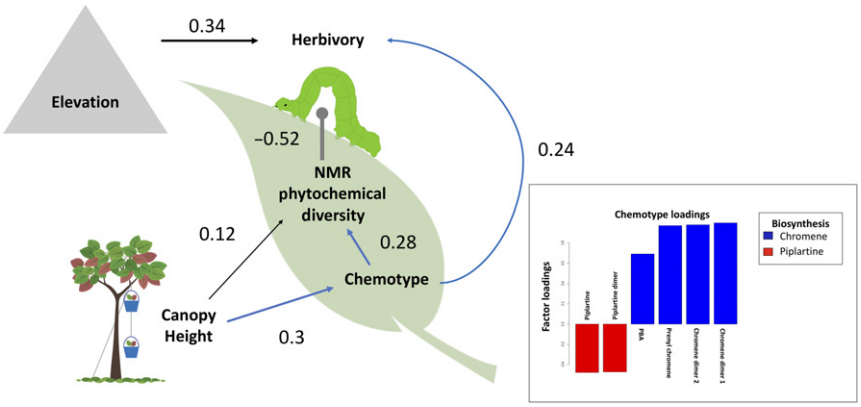

Figure 2 The best path model for the '*vertical strata SEM*' based on AIC from a structural equation model approach, using data from *all plants* and testing hypothesised causal relationships between light heterogeneity and the resulting levels of phytochemical defense and herbivory ($\chi^2 = 0.066$; d.f. = 3; $P > 0.99$). The direct positive effects are depicted as arrows, while the direct negative effects are gray blunt-ended lines. The numbers beside the lines are the standardised path coefficients. Chemotype is Factor 1 from a factor analysis of LC-MS chromatography, summarised in the inset loading graph – blue represents the chemotype having more LC-MS peaks associated with chromene pathways and red represents the chemotype associated with piplartine compounds. Plants in close proximity to the canopy had a positive direct effect on NMR phytochemical diversity and produced more chromene compounds (indicated by blue). Increases in phytochemical diversity strongly reduced herbivory.

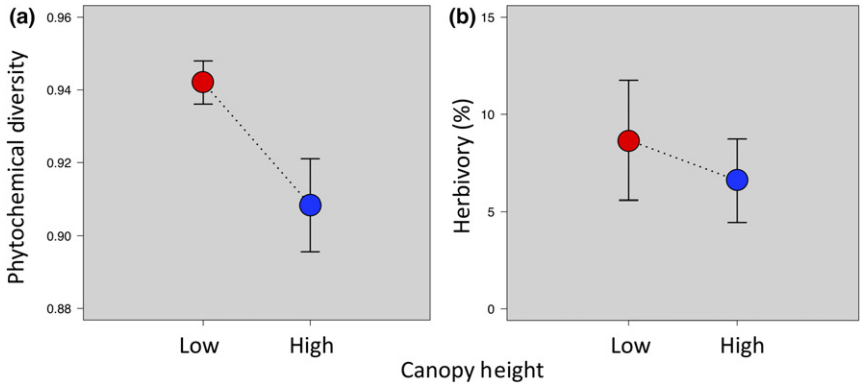

Figure 3 Relationship between phytochemical diversity and herbivory as a function of vertical stratification in forests. Plants were hanging at low and high levels in the canopy as a proxy of differences in light levels. Each point represents a population mean ± SE. Panel (a) depicts a significant decrease in NMR phytochemical diversity of plants located in close proximity to the canopy ($t = 2.4$, d.f. = 33, $P = 0.02$) and correlated with exposure to higher light intensity. Panel (b) shows there was not a significant difference in percent herbivory (raw data) between plants hanging at different heights along the vertical strata in the forest ($t = -1.23$, d.f. = 128.47, $P > 0.2$). All plants were included in these analyses.

In addition to identifying the previously known compounds (PBA, chromene and two analogs of chromene dimer), we found nitrogen-based amides, piplartine and a piplartine dimer (annotated using standards and by high-resolution mass spectrometry). In the factor analysis, the amides negatively loaded on factor 1 (indicated by red colour in Fig. 2), whereas the chromene-based compounds positively loaded on factor 1 (indicated by blue colour in Fig. 2), separating the plants along a chemotype gradient (Fig. 2 *inset*). The phytochemistry of high canopy plants favoured the chromene pathway and low canopy plants favoured the amide pathway (Fig. 2). The positive effect of chemotype indicates that greater investment in piplartine vs. chromenes reduces herbivory. Herbivory was not significantly different between high and low canopy heights (Fig. 3b). Percent herbivory varied from 0.9% to 53.1% per plant, and the average percent herbivory was 8.7% (± 1.5% SE) based on the raw data.

For all plants close to the soil, the 'understory light SEM' uncovered interesting direct and indirect effects of light on NMR phytochemical diversity and herbivory (Fig. 4a). This model provides support for the following causal hypotheses: (1) Direct light transmittance has a stronger direct effect on phytochemical diversity (Fig. 5a; $P < 0.01$) compared to herbivory (Fig. 4a; $P = 0.1$). Direct light transmittance is negatively correlated with phytochemical diversity (spc = −0.58; β = −0.003). (2) The effects of direct light transmittance on phytochemical diversity also cascade to herbivores by significantly reducing herbivory (Fig. 5b; spc = −0.54; β = −0.37). (3) Elevation has strong positive direct effects on herbivory (spc = 0.52; β = 0.09). The effect sizes were biologically relevant with 9% greater herbivory in the highest vs. lowest elevations based on the raw data.

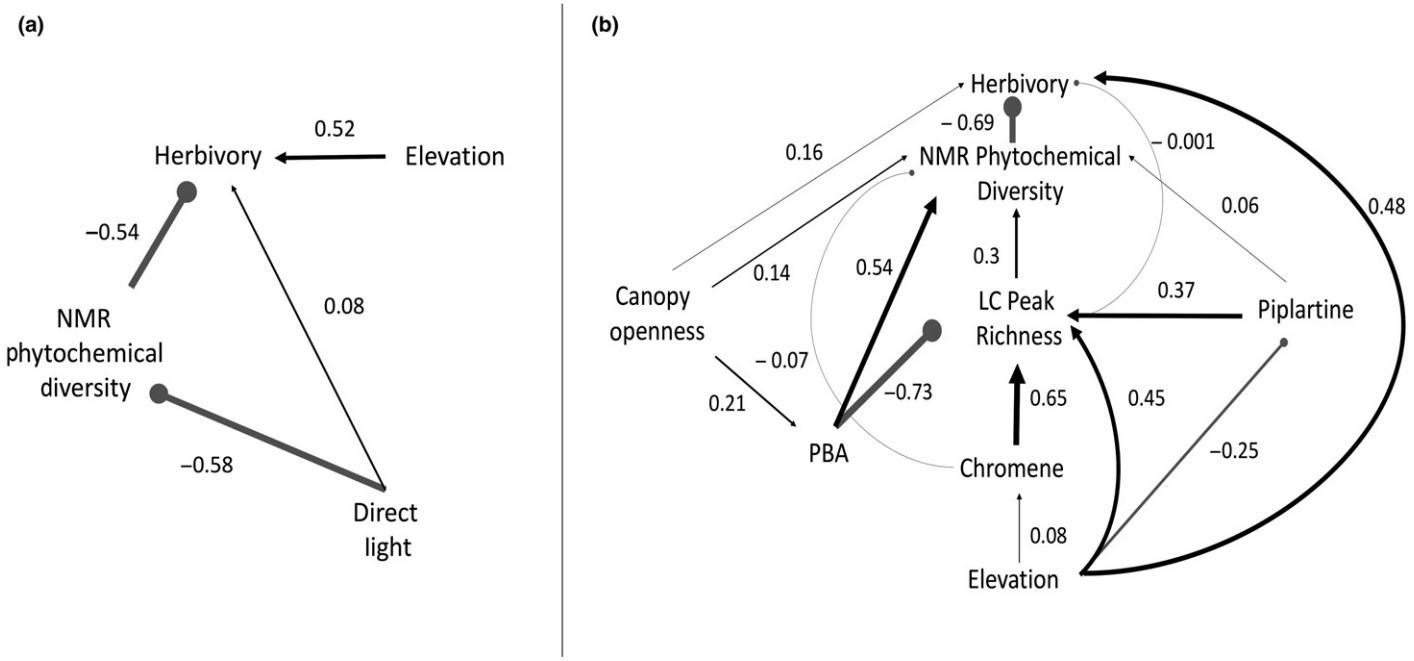

Figure 4 The best path models for the *understory light SEM* based on AIC from a structural equation model approach testing hypothesised causal relationships between direct light transmittance, canopy openness, herbivory, elevation and different measures of structural and compositional complexity quantified with NMR and LC-MS data. This model only included plants hanging near the soil because that is where the light measurements were taken. Direct positive effects are depicted as black arrows, while the direct negative effects are grey blunt-ended lines, and standardised path coefficients are next to each line or arrow. Panel (a) depicts the path model using only NMR data ($\chi^2 = 0.01$; d.f. = 1; $P > 0.93$). Panel (b) depicts the path model comparing NMR and LC-MS data ($\chi^2 = 1.42$; d.f. = 8; $P > 0.99$). Variation in chromene, piplartine, and prenylated benzoic acid (PBA) concentrations (determined by LC-MS) affect different measures of NMR phytochemical diversity (via changes in functional group relative abundances), indirectly affecting herbivory.

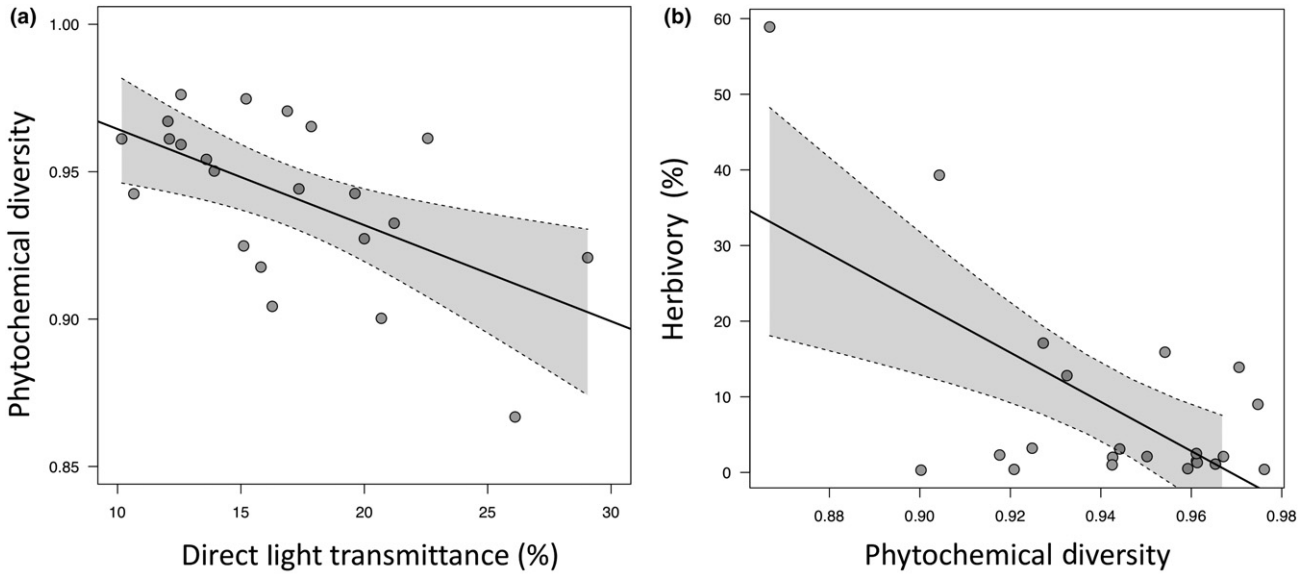

Figure 5 Relationships between phytochemical diversity, herbivory and direct light transmittance for *understory plants* near the soil ($y = 3.15 - 3.26x$; $F_{1,20} = 12.96$; $R^2 = 0.39$; $P < 0.01$). The grey band represents the 95% confidence interval. Panel (a) depicts NMR phytochemical diversity as a function of a direct light transmission. NMR phytochemical diversity significantly decreased as percent direct light transmittance increased ($y = 0.997 - 0.003x$; $F_{1,20} = 10$; $R^2 = 0.33$; $P < 0.01$). Declines of 0.05 in *Piper* phytochemical diversity are sufficient to affect herbivores (Richards *et al.* 2015). Panel (b) depicts percent herbivory (raw data) as a function of phytochemical diversity. Herbivory is inversely related to phytochemical diversity of leaf tissue, with herbivory declining from almost 50% to no herbivory as a function of high phytochemical diversity.

A comparison of our different measures of phytochemical diversity (i.e. NMR and LC-MS) yielded complex relationships among concentrations of specific compounds and structural complexity, but the relationships between complexity and herbivory and canopy openness were consistent (Fig. 4b; Fig. S5). A few important patterns that emerged from this comparison

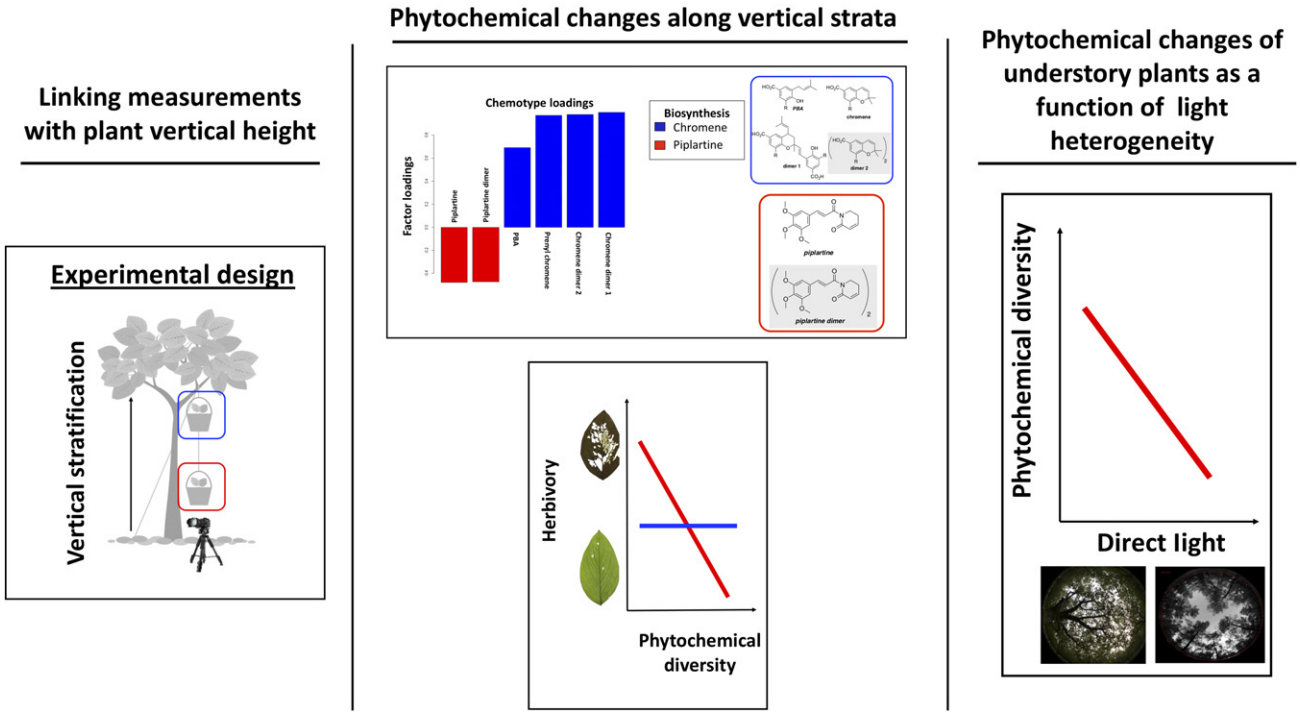

Figure 6 Conceptual figure summarising the major relationships between phytochemical diversity, herbivory, vertical stratification in the forest, and light heterogeneity. The experimental design consisted of paired clonal plants hung at different heights along the vertical strata in the forest. The blue colour represents plants hanging closer to the canopy while the red colour represents understory plants hanging near the soil. Vertical stratification influenced phytochemical diversity of *Piper kelleyi* plants by shifting the chemotype towards chromene production (blue bars) as plants increased in proximity to the canopy, while understory plants near the soil shifted the chemotype towards piplartine production (red bars). Increases in phytochemical diversity significantly reduced herbivory for understory plants near the soil (red line), but it did not vary with plants in close proximity to the canopy (blue line). Finally, light measurements were only taken at understory plants close to the soil. Increases in direct light transmittance significantly decreased phytochemical diversity for understory plants.

include: (1) shifts in production of chromene, piplartine, and PBA have strong effects on all measures of phytochemical diversity. (2) The strength and direction of effects of canopy openness on PBA and piplartine depend on whether plants are located high (Fig. S5) or low (Fig. 4b) in the canopy. (3) LC-MS peak richness has a stronger positive effect on herbivory high in the canopy (Fig. S5) but does not affect herbivory in the understory (Fig. 4b). (4) NMR phytochemical diversity has a stronger negative effect in the understory.

The effects of canopy light manipulations generated substantive phytochemical changes when examining NMR diversity. Plants located high in the canopy had an average diversity (i.e. effective number of functional groups) of 0.94, while plants low in the canopy had an average diversity of 0.91 (Fig. 3a). As a reference, high phytochemical diversity in 22 *Piper* species averages 0.93, while low phytochemical diversity in *Piper* averages 0.74 (Richards *et al.* 2015). For *P. kelleyi*, the light treatment demonstrated a difference of 0.03 in diversity which is 15.8% of the range across *Piper* species.

DISCUSSION

Intraspecific phytochemical variation across space and time may enhance anti-herbivore defense by reducing insect damage for specific times and locations where defense is high and by creating an unpredictable resource (Underwood 2004, 2009; Wetzel *et al.* 2016). Temporal and spatial variation in light quantity and quality directly affect specific plant metabolites (e.g. chromenes and furanocoumarins) and may also affect intraspecific phytochemical variation (*reviewed in* Ballaré *et al.* 2012), subsequently impacting herbivores (Berenbaum 1978, 1995; Kuhlmann & Müller 2010a). For the tropical shrub, *P. kelleyi*, we found that phytochemistry responds to position in the canopy and that phytochemical diversity decreased when light availability was enhanced (Fig. 6). In turn, herbivory decreased as phytochemical diversity increased in the forest understory (Fig. 6); however, higher in the canopy, this indirect effect of higher light on herbivory was counteracted due to switching from the defensive amide pathway favoured by plants near the soil to the chromene pathway favoured by plants near the canopy (Fig. 6). Because we have examined the full complement of secondary metabolites, these results are the first thorough demonstration of Hunter's (2016) intraspecific phytochemical landscape hypothesis, showing that subtle differences in light availability under the canopy crown creates a mosaic of chemically variable microhabitats that significantly reduces the damage of associated herbivores.

Photoactivity of *Piper kelleyi* leaves

Our results are consistent with the hypothesis that lower phytochemical diversity within *P. kelleyi* is a consequence of plants having greater exposure to light, based on the canopy

experiments (Fig. 4a and Fig. 5a). This is the most likely explanation for the change in chemistry from high to low hanging plants, and it is corroborated by the fact that plants near the soil and in locations of greater direct light transmittance had lower phytochemical diversities (Fig. 5a). 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 phytochemical diversity, but it is likely that these changes were partly due to shifts towards higher proportions of photoactive metabolites (i.e. less evenness among peaks), as suggested by photochemical studies of the major secondary metabolites isolated from *P. kelleyi* (Jeffrey and Sheridan, *personal communication)*. The hypothesised synthesis posits that the prenylated benzoic acid (PBA) is not light-activated and therefore remains because it is the precursor to either the piplartine or chromene pathway (Jeffrey, *unpublished data*). It is clear that light is responsible for a trade-off in production of piplartine vs. chromene compounds, but this effect depends on plant height. Plants high in the canopy invest more in chromene related compounds (Fig. 2; Fig. S5), while those lower in the canopy invest more in piplartine compounds (Fig. 4b). As a result, high canopy plants have lower phytochemical diversity with increases in chromenes and decreases in piplartine compounds, and low canopy plants have higher phytochemical diversity with increases in PBA and piplartine compounds (Fig. 4b; Fig. S5). The biosynthetic pathway tradeoffs detected in *P. kelleyi* leaves exemplify the growth–defense tradeoff hypothesis (Coley *et al.* 1985; Herms & Mattson 1992), which posits that plants are limited by tradeoffs in allocating resources to growth or chemical defense depending on the resources available. By increasing photosynthesis, light can provide more resources for biomass replacement, allowing leaves to be less defended, but enhanced light also provides a chance for plants to exploit photoactive defenses. Thus, *P. kelleyi* plants located in the deeply shaded forest understory are light limited and may be allocating resources towards costly defensive amides that require the use of nitrogen to protect their leaves from insect damage rather than allocating nitrogen to other metabolites and physiological processes.

Such complex changes in phytochemistry with light can be adaptive in a number of ways; for example, some classes of secondary metabolites are responsible for protection against damaging UV radiation – this could be the case for soluble phenolic metabolites that accumulate when exposed to high levels of UV radiation (Mazza *et al.* 2000; Kotilainen *et al.* 2009). Regardless of the adaptive value, an increase in concentration of photoactive metabolites at the expense of other metabolites can cause an overall decrease in phytochemical diversity when plants are exposed to higher levels of UV radiation.

Herbivory

For the subset of plants hanging near the understory, herbivory increased with lower phytochemical diversity (Fig. 5b). Richards *et al.* (2015) found a similar pattern with herbivory and phytochemical diversity among 22 *Piper* species. There are many potential mechanisms by which phytochemical diversity can affect insect herbivores, including direct toxicity of multiple combinations of secondary compounds. These combinations may work additively or synergistically to disrupt insect physiological processes, resulting in a negative correlation between phytochemical diversity and herbivore performance. It is also possible that light-induced changes in biochemical pathways and phytochemical diversity may disrupt the insect immune response, leading to greater mortality due to parasitoids. Hansen *et al.* (2016) found that specialist *Eois* caterpillars had significantly impaired immune responses and higher levels of parasitism when feeding on *Piper* plants of higher chemical diversity. Similarly, Glassmire *et al.* (2016) found that greater phytochemical diversity in *P. kelleyi* leaves increased parasitoid diversity.

Herbivory, surprisingly, did not vary with canopy height (Fig. 3b) despite the diminished chemical diversity in the high canopy (Fig. 3a) and negative correlations between phytochemical diversity and herbivory for understory plants near the soil (Fig. 5b). One reason may be that light variation in proximity to the canopy may not have been as significant compared to changes in the understory. Due to limitations we were unable to measure light exposure of plants in close proximity to the canopy to assess this. Alternatively, our results suggest that greater direct light transmittance catalysed the switch from the defensive amide pathway to the photoactive chromene pathway, and the resulting metabolites produced by these distinct pathways may have differential responses on herbivore performance. Finally, multiple factors can influence herbivory, including changes in nutrients and water content or direct negative effects of increased light or changes in light quality on herbivores. The ecological responses of plant-insect interactions to enhanced light exposure are not at all clear (*reviewed in* Kuhlmann & Müller 2010b; Ballaré *et al.* 2011); for example, direct effects of light on herbivores can depend on the feeding mode (phloem vs. leaf tissue) and degree of specialisation of the herbivorous insect (McCloud & Berenbaum 1999; Kuhlmann & Müller 2010a). *Eois* caterpillars are specialised and are adapted to the photoactive metabolites of *P. kelleyi* (Glassmire *et al.* 2016), so the indirect effects of light on herbivory via phytochemistry may not be apparent since *Eois* are the primary herbivores.

Our results are consistent with the idea that photoactive metabolites differentially affect the development of generalist and specialist caterpillars (Berenbaum 1978). Laboratory feeding experiments have shown that *P. kelleyi* compounds are phototoxic and changes in UV light in combination with these compounds have detrimental effects on generalist herbivores, but not on specialist caterpillars (Fig. S6, *see supplementary information for feeding assays*). These results are consistent with the idea that specialist caterpillars may respond to photoactive secondary metabolites by changes in their behaviour. For example, some folivores on *Piper* are primarily nocturnal feeders (Zehr 2017) and it may be an adapted behaviour of specialist caterpillars to only feed during dark periods to reduce phototoxic effects of plant chemistry. Field observations in the Andes suggest that *Eois* feed primarily in the dark

(Glassmire, *pers. obs.*). A focused approach to addressing these questions should include a feeding assay using plant material from different elevations and canopy heights and manipulate light conditions, so caterpillars can only feed during the day or only during the night to test the interaction of elevation and photoactivity of compounds on herbivore performance and behaviour.

CONCLUSION

Our results suggest that vertical stratification in forests, as well as light heterogeneity in the shaded understory contribute substantively to intraspecific phytochemical variation, including changes in production of individual compounds, structural and compositional complexity, and photo-activation. Overall herbivory was reduced by subtle changes in these light-associated modifications to phytochemical mixtures. Thus, intraspecific phytochemical variation across temporal and spatial locations (as envisioned by Hunter 2016) in this tropical shrub is a potentially powerful mode of defense against insect damage across the landscape. These relationships are relevant to herbivore local adaptation and speciation on a host plant, as well as more applied issues of how to control pests on monocultures.

ACKNOWLEDGEMENTS

This research was funded by the National Science Foundation grants DEB 1502059 and DEB 1442103 and by a generous donation to the Hitchcock Fund for Chemical Ecology Research. Our special thanks to Wilmer Simbaña for establishing *Piper kelleyi* clonal plants. We thank Matthew Forister, Angela Smilanich, the Will Wetzel & Marjorie Weber lab group and three anonymous reviewers for providing valuable and constructive comments that significantly improved this manuscript. We are grateful to Thomas Walla and *Earthwatch Institute* volunteers for their assistance with the canopy experiment. We thank Lydia Doan, Carmen Mo, Andre Labuda, Juliana Bogert and Gemma Beltran for their help with the generalist caterpillar feeding assay, and Kaitlinn Webb for conducting the specialist caterpillar feeding assay. Angela Hornsby illustrated the experimental design in Fig. 1.

AUTHOR CONTRIBUTIONS

AEG wrote the first draft of the manuscript. LAD and LAR contributed substantially to revisions. AEG, LAD, LAR and CSJ generated hypotheses and designed experiments. AEG, LAD and CSJ funded experiments. AEG and JSS collected field data. AEG, CP and CSJ conducted chemical analyses. AEG, LAR, LAD and CP conducted statistical analyses.

DATA ACCESSIBILITY STATEMENT

Experimental data supporting the results has been archived in the Dryad Digital Repository (https://doi.org/10.5061/dryad.v6p96q5) and is hosted on a website we maintain (caterpillars.org).

REFERENCES

Agrawal, A.A. & Fishbein, M. (2006). Plant defense syndromes. *Ecology*, 87, S132–S149.

Ballaré, C.L., Caldwell, M.M., Flint, S.D., Robinson, S.A. & Bornman, J.F. (2011). Effects of solar ultraviolet radiation on terrestrial ecosystems. Patterns, mechanisms, and interactions with climate change. In: *The Human Health Effects of Ozone Depletion and Interactions with Climate Change* (eds Bornman, J.F., Paul, N. & Tang, X.). United Nations Environment Programme, Nairobi, Kenya, pp. 83–111.

Ballaré, C.L., Mazza, C.A., Austin, A.T. & Pierik, R. (2012). Canopy light and plant health. *Plant Physiol.*, 160, 145–155.

Becerra, J.X. (2007). The impact of herbivore-plant coevolution on plant community structure. *Proc. Natl Acad. Sci. USA*, 104, 7483–7488.

Becker, R.S. & Michl, J. (1966). Photochromism of synthetic and naturally occurring 2H-chromenes and 2H-pyrans. *J. Am. Chem. Soc.*, 88, 5931–5933.

Berenbaum, M. (1978). Toxicity of a furanocoumarin to armyworms: a case of biosynthetic escape from insect herbivores. *Science*, 201, 532–534.

Berenbaum, M. (1995). Phototoxicity of plant secondary metabolites: insect and mammalian perspectives. *Arch. Insect Biochem.*, 29, 119–134.

Berenbaum, M.R. & Zangerl, A.R. (1996). Phytochemical diversity. In: *Phytochemical Diversity and Redundancy in Ecological Interactions* (eds Romeo, J.T., Saunders, J.A., & Barbosa, P.). Springer, Boston, MA, pp. 1–24.

Brown, M.J., Parker, G.G. & Posner, N.E. (1994). A survey of ultraviolet-B radiation in forests. *J. Ecol.*, 82, 843–854.

Bustos-Segura, C., Poelman, E.H., Reichelt, M., Gershenzon, J. & Gols, R. (2017). Intraspecific chemical diversity among neighbouring plants correlates positively with plant size and herbivore load but negative with herbivore damage. *Ecol. Lett.*, 20, 87–97.

Chazdon, R.L. & Fetcher, N. (1984). Photosynthetic light environments in a lowland tropical rain forest in Costa Rica. *J. Ecol.*, 72, 553–564.

Coley, P.D., Bryant, J.P. & Chapin, F.S. (1985). Resource availability and plant antiherbivore defense. *Science*, 230, 895–899.

Dyer, L.A. & Palmer, A.D. (eds.) (2004). *Piper: A Model Genus for Studies of Phytochemistry, Ecology, and Evolution.* Kluwer Academic/Plenum Publishers, New York, NY.

Dyer, L.A., Letourneau, D.K., Dodson, C.D., Tobler, M.A., Stireman, J.O. & Hsu, A. (2004). Ecological causes and consequences of variation in defensive chemistry of a Neotropical shrub. *Ecology*, 85, 2795–2803.

Endara, M., Coley, P.D., Ghabash, G., Nicholls, J.A., Dester, K.G., Donoso, D.A. *et al.* (2017). Coevolutionary arms race versus host defense chase in a tropical herbivore-plant system. *Proc. Natl Acad. Sci. USA*, 114, E7499–E7505.

Frazer, G.W., Canham, C.D. & Lertzman, K.P. (1999). Gap Light Analyzer (GLA), Version 2.0: Imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, user's manual and program documentation. Simon Fraser University, Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York.

Gershenzon, J., Fontana, A., Burow, M., Wittstock, U. & Deganhardt, J. (2012). Mixtures of plant secondary metabolites: metabolic origins and ecological benefits. In: *The Ecology of Secondary Metabolites from Genes to Global Processes* (eds Iason, G.R., Dicke, M., Hartley, S.E.). Cambridge University Press, Cambridge, pp. 56–77.

Glassmire, A.E., Jeffrey, C.S., Forister, M.L., Parchman, T.L., Nice, C.C., Jahner, J.P. *et al.* (2016). Intraspecific phytochemical variation drives community and population structure for specialist caterpillars. *New Phytol.*, 212, 208–219.

Hansen, A.C., Glassmire, A.E., Dyer, L.A. & Smilanich, A.M. (2016). Patterns in parasitism frequency explained by diet and immunity. *Ecography*, 40, 803–805.

Herms, D.A. & Mattson, W.J. (1992). The dilemma of plants: to grow or defend. *Q. Rev. Biol.*, 67, 283–335.

Hunter, M.D. (2016). *The Phytochemical Landscape: Linking Trophic Interactions and Nutrient Dynamics*. Princeton University Press, Princeton, NJ.

Jeffrey, C.S., Leonard, M.D., Glassmire, A.E., Dodson, C.D., Richards, L.A., Kato, M.J. *et al.* (2014). Antiherbivore prenylated benzoic acid derivatives from *Piper kelleyi*. *J. Nat. Prod.*, 77, 148–153.

Kato, M.J. & Furlan, M. (2007). Chemistry and evolution of the Piperaceae. *Pure Appl. Chem.*, 79, 529–538.

Kosmidis, I. (2013). brglm: Bias reduction in binomial-response generalized linear models. R package version 0.5-9. Available at: http://www.ucl.ac.uk/~ucakiko/software.html. Last accessed 19 March 2018

Kotilainen, T., Venäläinen, T., Tegelberg, R., Lindfors, A., Julkunen-Tiitto, R., Sutinen, S. *et al.* (2009). Assessment of UV biological spectral weighting functions for phenolic metabolites and growth responses in silver birch seedlings. *Photochem. Photobiol.*, 85, 1346–1355.

Kuhlmann, F. & Müller, C. (2010a). UV-B impact on aphid performance mediated by plant quality and plant changes induced by aphids. *Plant Biol.*, 12, 676–684.

Kuhlmann, F. & Müller, C. (2010b). Impacts of ultraviolet radiation on interactions between plants and herbivorous insects: a chemo-ecological perspective. *Prog. Bot.*, 72, 305–347.

Kursar, T.A., Dexter, K.G., Lokvam, J., Pennington, T., Richardson, J.E., Weber, M.G. *et al.* (2009). The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. *Proc. Natl Acad. Sci. USA*, 106, 18073–18078.

Lieberman, M., Lieberman, D. & Peralta, R. (1989). Forests are not just Swiss cheese: canopy stereogeometry of non-gaps in tropical forests. *Ecology*, 70, 550–552.

Massad, T.J., Moraes, M.M., Philbin, C., Oliveira, C. Jr, Torrejon, G.C., Yamaguchi, L.F. *et al.* (2017). Similiarity in volatile communities leads to increased herbivory and greater tropical forest diversity. *Ecology*, 98, 1750–1756.

Mazza, C.A., Boccalandro, H.E., Giordano, C.V., Battista, D., Scopel, A.L. & Ballaré, C.L. (2000). Functional significance and induction by solar radiation of ultraviolet-absorbing sunscreens in field-grown soybean crops. *Plant Physiol.*, 122, 117–126.

McCloud, E.S. & Berenbaum, M. (1999). Effects of enhanced UV-B radiation on a weedy forb (*Plantago lanceolate*) and its interactions with a generalist and specialist herbivore. *Entomol. Exp. Appl.*, 93, 233–246.

Montgomery, R.A. & Chazdon, R.L. (2001). Forest structure, canopy architecture, and light transmittance in tropical wet forests. *Ecology*, 82, 2707–2718.

Nguyen, T.T. (2015). Syntheses and photo-reactivity of chromene natural products and photo-degradation of steroidal hormones. Reno Dissertation, University of Nevada.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D. *et al.* (2017). Vegan: community ecology package. R package version 2.4-3. Available at: https://CRAN.R-project.org/package=vegan. Last accessed 19 March 2018.

Padwa, A., Au, A., Lee, G.A. & Owens, W. (1975). Photochemical ring-opening reactions of substituted chromenes and isochromenes. *J. Org. Chem.*, 40, 1142–1149.

R Development Core Team (2017). *R: Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/ Last accessed 26 February 2018

Richards, L.A., Dyer, L.A., Forister, M.L., Smilanich, A.M., Dodson, C.D., Leonard, M.D. *et al.* (2015). Phytochemical diversity drives plant-insect community diversity. *Proc. Natl Acad. Sci. USA*, 112, 10973–10978.

Richards, L.A., Glassmire, A.E., Ochsenrider, K.M., Smilanich, A.M., Dodson, C.D., Jeffrey, C.S. *et al.* (2016). Phytochemical diversity and synergistic effects on herbivores. *Phytochem. Rev.*, 15, 1153–1166.

Rodríguez-Castañeda, G., Dyer, L.A., Brehm, G., Connahs, H., Forkner, R.E. & Walla, T.R. (2010). Tropical forests are not flat: how mountains affect herbivore diversity. *Ecol. Lett.*, 13, 1348–1357.

Rodríguez-Castañeda, G., Forkner, R.E., Tepe, E.J., Gentry, G.L. & Dyer, L.A. (2011). Weighing defensive and nutritive roles of ant mutualists across a tropical altitudinal gradient. *Biotropica*, 43, 343–350.

Sagers, C.L. (1992). Manipulation of host plant quality: herbivores keep leaves in the dark. *Funct. Ecol.*, 6, 741–743.

Salazar, D., Jaramillo, M.A. & Marquis, R.J. (2016). Chemical similarity and local community assembly in the species rich tropical genus Piper. *Ecology*, 97, 3176–3183.

Schuman, M.C., Allmann, S. & Baldwin, I.T. (2015). Plant defense phenotypes determine the consequences of volatile emission for individuals and neighbors. *eLife*, 4, 1–45.

Shipley, B. (2016). *Cause and Correlation in Biology: A User's Guide to Path Analysis, Structural and Causal Inference with R*. Cambridge University Press, Cambridge, UK.

Tepe, E.J., Rodríguez-Castañeda, G., Glassmire, A.E. & Dyer, L.A. (2014). *Piper kelleyi*, a hotspot of ecological interactions and a new species from Ecuador and Peru. *PhytoKeys*, 34, 19–32.

Underwood, N. (2004). Variance and skew of the distribution of plant quality influence herbivore population dynamics. *Ecology*, 153, 282–294.

Underwood, N. (2009). Effect of genetic variance in plant quality on the population dynamics of a herbivorous insect. *J. Anim. Ecol.*, 78, 839–847.

Wetzel, W.C., Kharouba, H.M., Robinson, M., Holyoak, M. & Karban, R. (2016). Variability in plant nutrients reduces insect herbivore performance. *Nature*, 539, 425–427.

Whitham, T.G., Bailey, J.K., Schweitzer, J.A., Shuster, S.M., Bangert, R.K., LeRoy, C.J. *et al.* (2006). A framework for community and ecosystem genetics: from genes to ecosystems. *Nat. Rev. Genet.*, 7, 510–523.

Wilson, J.S., Forister, M.L., Dyer, L.A., O'Connor, J.M., Burls, K., Feldman, C.R. *et al.* (2012). Host conservatism, host shifts and diversification across three trophic levels in two Neotropical forests. *J. Evol. Biol.*, 25, 532–546.

Zehr, L.N. (2017) Diel patterns of insect herbivory and plant secondary metabolites in understory shrubs on Barro Colorado Island. University of Illinois at Urbana-Champaign thesis. Available at: http://hdl.handle.net/2142/98418. Last accessed 27 August 2018.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Editor, Ted Turlings
Manuscript received 13 September 2018
First decision made 26 October 2018
Manuscript accepted 7 November 2018