FUNCTIONAL TRAITS 2.0: THE POWER OF METABOLOMICS FOR PLANT ECOLOGY

Research Article

Structural and compositional dimensions of phytochemical diversity in the genus *Piper* reflect distinct ecological modes of action

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Abstract

1. An increasing number of ecological studies have used chemical diversity as a functionally relevant, scalable measure of phytochemical mixtures, demanding more rigorous attention to how chemical diversity is estimated. Most studies have focused on the composition of phytochemical mixtures and have largely ignored structural concerns, which may have greater importance for ecological function. Here, we explore the development of structural complexity and compositional diversity resulting from different biotic and abiotic interactions in *Piper kelleyi* Tepe (Piperaceae). We also describe how variation in structural complexity and compositional diversity differs between two congeners, *P. kelleyi* and *P. reticulatum*. To better interpret these results, we have developed a framework for interpreting these dimensions of chemical diversity in phytochemical mixtures.

2. *Piper* is an abundant plant genus that supports diverse insect communities throughout the tropics. Subtle changes in understorey forest light were associated with increases in herbivory that directly increased compositional diversity and indirectly decreased structural complexity in *P. kelleyi*. This was attributed to the production of oxidation products resulting from herbivory-driven decomposition of structurally complex defence compounds. This type of complex result would remain undetected using standard chemical ecology approaches and accounts for the detailed molecular changes that are likely to affect species interactions.
1 | INTRODUCTION

Chemically mediated interactions between plants and insects can drive community assembly and coevolutionary processes (Abbott, 1887; Ehrlich & Raven, 1964; Fraenkel, 1959). Accordingly, advances in studies of phytochemical diversity have focused efforts on interpreting chemical diversity using a Hill numbers framework, where diversity equivalents (e.g. Simpson, inverse Simpson, Shannon–Wiener and higher q values) can be thought of as effective numbers of chromatographic or spectroscopic peaks observed in chemical analysis (Feiner et al., 2018; Hill, 1973; Marion et al., 2015; Richards et al., 2015; Wetzel & Whitehead, 2020). This approach has elucidated the important role of diverse mixtures in shaping trophic interactions in ecological (Dyer et al., 2018) and agricultural (Silva et al., 2018) systems. Recent studies have demonstrated that chemical diversity is an effective measure of synergistic phytochemical mixtures (Gershenzon et al., 2012; Rasmann & Agrawal, 2009; Richards et al., 2016; Whitehead et al., 2013) that affect herbivore performance (Glassmire et al., 2020; Slinn et al., 2018) and can drive community assembly (Glassmire et al., 2016; Harrison et al., 2016; Massad et al., 2017; Poelman et al., 2009; Richards et al., 2015; Salazar et al., 2016a, 2016b; Schuman et al., 2016; Sedio et al., 2017) and contribute to coevolutionary processes (Berenbaum & Zangerl, 1996; Ehrlich & Raven, 1964; Jahner et al., 2017).

Despite the consequences of phytochemical diversity for multi-trophic interactions, there are substantive gaps in the understanding of how metabolic resources are invested towards phytochemical production and diversity. Metabolic variation is determined by a combination of genetic, biotic and abiotic factors, and these factors are likely to have predictable effects on chemical diversity (Dyer et al., 2014). While phytochemical production can be readily quantified through quantitative metabolomics, quantitative measures of diversity are also important for addressing ecological hypotheses. Chemical diversity metrics based on entropy measures represent a means of synthesizing complex spectroscopic data from phytochemical mixtures (Dyer et al., 2018; Marion et al., 2015).

1.1 | Measures of phytochemical diversity

The interpretation of chemical diversity is dependent on the chemical analysis performed. Compositional methods (usually chromatographic) measure the richness and abundance of individual molecules within a mixture, whereas the metabolic complexity of a phytochemical mixture can be readily quantified by calculating the effective number of peaks within a proton nuclear magnetic resonance ($^1$H NMR) spectrum of crude plant extracts (Figure 1, $D_q$). The richness and evenness of peaks in a crude $^1$H NMR spectrum result from structural complexity of constituent molecules (Figure 1, $D_s$), as well as the compositional diversity of those molecules (Figure 1, $D_q$). When measured spectroscopically, structural dissimilarity determines the degree to which composition contributes to the metabolic complexity of a chemical mixture (Figure 1, $D_q$). Variation in both structural complexity and compositional diversity can affect entire communities of herbivores and associated consumers (Dyer, 2018; Dyer et al., 2014, 2018; Richards et al., 2015; Salazar et al., 2016a, 2016b; Schuman et al., 2016; Sedio et al., 2017). Regardless of how chemical defences have evolved, increasing phytochemical defences come at an energetic cost, whether these resources are directed towards increasing phytochemical concentrations (Hunter, 2016), compositional diversity or structural complexity (Hilker, 2014).

Compositional diversity is the most intuitive analogue to community diversity, and several theories exist to explain the development and maintenance of compositional diversity resulting from coevolutionary processes. As a result, most prior studies on phytochemical diversity have focused on compositional diversity (Becerra et al., 2009; Glassmire et al., 2020; Jones & Firn, 1991; Kessler & Kalske, 2018; Moore et al., 2014; Raguso et al., 2015; Verma et al., 2009). Although advances in metabolomics approaches have facilitated a more complete investigation of compositional diversity (Kuhlisch & Pohnert, 2015), methodological challenges have limited our understanding of the functional role of chemical structure in plant–insect interactions.

Structural complexity can be measured from the $^1$H NMR spectra of individual compounds. This is different from structural diversity, a property of chemical mixtures which has been defined by ecologists as the ‘complexity of the molecular structures present in a phytochemical mixture’ (Wetzel & Whitehead, 2020). From the perspective of biosynthesis and physiological or ecological function, it may be more useful to define structural diversity as the diversity of structural features: discrete structural building blocks that can be shared between molecules within a chemical mixture. Structural features can be observed in $^1$H NMR as individual or groups of peaks.

4. Synthesis. Our quantitative framework provides a method for considering trade-offs between structural complexity and compositional diversity and the interpretation of analytical approaches for each. This methodology will provide new theoretical insights and a more sophisticated model for examining the ecology and evolution of chemically mediated interactions.

KEYWORDS
compositional diversity, mass spectrometry, nuclear magnetic resonance, phytochemical diversity, Piperaceae, plant–insect interactions, structural complexity
that result from a structural motif defined by a single atom, bond, or structural fragment.

The unique ways in which structural features are combined to develop structural complexity result from increasingly complex networks of biosynthetic enzymes, a phenomenon reflected in $^1$H NMR spectra (Figure 2). Many of the mechanisms that maintain compositional diversity, such as gene duplication and neofunctionalization, are also required for developing structural complexity (Jenke-Kodama & Dittmann, 2009; Moore et al., 2014; Timoneda et al., 2019), and subtle changes in enzyme structure can lead to differential catalytic selectivity and function (Greenhagen et al., 2006). Plant-associated microbe communities may also play a significant role in both (Aly et al., 2010; Clay, 1993; Hardoim et al., 2015; Kusari et al., 2012; Verma et al., 2009). Structural complexity is likely to result from biotic and abiotic selective pressures — analogous to compositional diversity. A taxon may utilize a few promiscuous enzymes that produce many similar variations on the same phytochemical class (Terpenes: Greenhagen et al., 2006, saponins: Tava & Avato, 2006). Alternatively, plants can utilize a more biosynthetically diverse strategy that produces dissimilar molecules from different classes (Piper amides and chromenes) or produces a few highly complex molecules (Daphniphyllum alkaloids, Yang et al., 2006). In plant systems where dissimilar phytochemical structures vary in complexity within and between species, this framework may reveal more sophisticated relationships than compositional approaches alone. The genus Piper (Piperaceae) is ideal for investigating such variation because of the structurally diverse phytochemical mixtures from distinct biosynthetic pathways found within and between species that also demonstrate high compositional variation in response to biotic and abiotic factors (Dyer & Palmer, 2004).

1.2 | Piper: A model system for phytochemical diversity

The genus Piper is characterized by high phytochemical diversity, producing compounds from over nine different classes of specialized metabolites. This chemistry is well characterized for many neotropical
Piper species (Gómez-Calvario & Ríos, 2019; Salazar et al., 2016b; Salehi et al., 2019; Scott et al., 2007). Here, we focus on the phytochemical diversity in Piper kelleyi Tepe and Piper reticulatum L. (Piperaceae); these geographically distinct tropical shrubs host diverse populations of specialist herbivores and parasites, and both are known for their distinct chemical profiles (Jeffrey et al., 2014; Whitehead et al., 2013).

There are long-term ecological data associated with both of these Piper species (Dyer & Palmer, 2004; Dyer et al., 2012; Glassmire et al., 2019; Slinn et al., 2018; Tepe et al., 2014), including investigations of phytochemical diversity (Richards et al., 2015), which provide context for testing the objectives outlined here. These studies have provided insight into how chemically mediated plant-herbivore communities are affected by abiotic factors. Here, we attempt to parse the role of compositional diversity and structural complexity in mediating ecological interactions for P. kelleyi and P. reticulatum by investigating the phytochemical diversity in Piper using complementary structural (1H NMR) and compositional (liquid chromatography coupled to ultraviolet spectroscopy and mass spectrometry, LC-UV-MS) techniques.

### Figure 2

Structural complexity increasing through the biosynthetic process observed by 1H NMR

Piper species (Gómez-Calvario & Ríos, 2019; Salazar et al., 2016b; Salehi et al., 2019; Scott et al., 2007). Here, we focus on the phytochemical diversity in Piper kelleyi Tepe and Piper reticulatum L. (Piperaceae); these geographically distinct tropical shrubs host diverse populations of specialist herbivores and parasites, and both are known for their distinct chemical profiles (Jeffrey et al., 2014; Whitehead et al., 2013).

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### 1.3 Study objectives

We explored the multiple dimensions of chemical diversity in Piper guided by three main objectives.

- **Objective 1**: Investigate how compositional diversity, structural complexity and metabolic complexity mediate interactions between abiotic factors and herbivory by examining intraspecific phytochemical variation across light and elevational gradients.
- **Objective 2**: Compare the variation of structural and compositional diversity in two Piper species, *P. kelleyi* and *P. reticulatum*, providing insight into defensive biosynthetic strategies.
- **Objective 3**: Develop a theoretical framework to interpret 1H NMR measures of phytochemical diversity in structurally diverse genera such as *Piper*.

We anticipate that these ideas will provide a foundation from which coevolutionary hypotheses for the development of compositional diversity and structural complexity can be developed in tandem rather than in isolation. The methods outlined here provide a workflow for future studies with enough detail to support those unfamiliar with 1H NMR.

## 2 Materials and Methods

### 2.1 Sample collection and analysis

*Piper reticulatum* (Piperaceae) foliar samples were collected from individual plants >10 m apart along both sides of the Sendero Surá (55–255 m), Sendero Arriera-Zampopa (110–804 m) and Sendero Tres Ríos (155–2,450 m) trails in La Selva Biological Station (LS, 10°25′N 84°00′W), Costa Rica. In a prior experiment (Glassmire et al., 2019), we collected *P. kelleyi* from experimental plots of clones in Yanayacu Biological Station (YBS, 00°36′S 77°53′W) in Ecuador grown at high and low canopy height along an elevational gradient (2,000–2,400 m) of the Eastern Andes mountains. Canopy cover and direct light transmittance of *P. kelleyi* plants were measured using a Canon EOS Rebel-T4 camera with a hemispherical fisheye lens and processed using Gap Light Analyzer software (GLA...
software methods, Frazer et al., 1999). Herbivory was estimated from actual and estimated (pre-damage) leaf area using ImageJ before arcsine square root transformation (Glassmire et al., 2019). All Piper samples were finely ground using a tissue lyser (TissueLyser II, Qiagen) and 100 mg portions from each individual plant were weighed before adding 10 ml of methanol (Fisher, Optima). Methanolic suspensions were sonificated for 10 min and extracted overnight with wrist-action shaking before filtering over a cotton plug and concentrating to dryness in vacuo. Detailed descriptions of chemical analyses for $^1$H NMR and LC-UV-MS can be found in the Supporting Information (S1).

### 2.2 Compositional diversity versus metabolic complexity

Compositional diversity ($D_C$) or metabolic complexity ($D_M$) can be calculated as richness, Shannon or Simpson’s diversity, or beyond into higher $q$ values using analogous methods for calculating Hill numbers for community data (Marion et al., 2015). Metabolic complexity arises in chemical mixtures, such as a crude plant extract, from the aggregation of molecules, each with their own structural complexity, in proportion to their composition. Figure 1 outlines how the $^1$H NMR spectra of individual molecules combine to yield crude mixture spectra within our framework for understanding the properties of chemical mixtures and their constituent molecules that yield metabolic complexity. If one were to analyse a hypothetical crude extract, separation methods such as liquid chromatography (LC) and gas chromatography (GC) provide a compositional profile of the mixture wherein each peak represents a different molecule (Figure 1, compositional diversity). If each of these compounds is isolated to obtain a $^1$H NMR spectrum, each spectrum reflects the complexity of that molecule (Figure 1, structural complexity). The structural complexity index of a molecule is calculated in the same fashion as one would for compositional diversity, but instead of each peak representing a whole molecule, it represents a structural feature of that molecule; all peaks in a spectrum represent the spectral fingerprint of an entire molecule.

Recombining these individual $^1$H NMR spectra according to their relative abundance yields a $^1$H NMR spectrum that reflects the crude mixture before separation. Similar structural features of molecules will lead to overlapping $^1$H NMR signals that increase abundance for those signals, lowering signal evenness. Dissimilar structural features will have non-overlapping signals, leading to higher peak richness (Figure 1, structural dissimilarity). One can therefore view the diversity of crude $^1$H NMR as a gross measure of metabolic complexity, incorporating the composition, complexity and dissimilarity of phytochemical mixtures.

### 2.3 Effective structural complexity ($D_{Seff}$)

Once we have obtained compositional diversity ($D_C$, from LC) and metabolic complexity ($D_M$, from $^1$H NMR) as either richness, Shannon or inverse Simpson’s diversity, we can calculate effective structural complexity ($D_{Seff}$) as follows:

$$D_{Seff} = \frac{D_M}{D_C}. \quad (1)$$

Having removed the compositional contribution to metabolic complexity, effective structural complexity represents the remaining structural contribution. In terms of richness, this could be thought of as the average number of $^1$H NMR peaks per compound or as an abundance-weighted average for higher order Hill numbers. While seemingly simple, effective structural complexity represents both the structural complexity and dissimilarity of molecules in a mixture.

Metabolic complexity ($D_M$) of a mixture can be decomposed into beta dissimilarity ($\beta_D$) from mean structural complexity ($\bar{D}_S$) of all constituent molecules in a mixture, weighted to their concentration, as described for mean alpha diversity using community data by (Jost, 2007):

$$\beta_D = \frac{D_M}{\bar{D}_S} \quad (2)$$

Highly dissimilar mixtures will lead to high metabolic complexity, even when the constituents of those mixtures have low structural complexity. Structural dissimilarity ($D_D$) can be further decomposed from $\beta_D$ and compositional diversity:

$$D_D = \frac{\beta_D}{D_C} \quad (3)$$

which yields a number between 0 and 1 that can be interpreted as the fraction of signal overlap between constituents of a phytochemical mixture per compound. Mixtures with high structural dissimilarity (near one) have very little signal overlap, while mixtures having low structural dissimilarity have high signal overlap per compound. While this decomposition provides theoretical insight into partitioning chemical diversity, it is impractical for experimental data where structures and spectra of individual phytochemicals within a mixture are unknown. However, it is possible to estimate compositional diversity ($D_C$) of phytochemical mixtures using hyphenated analytical methods (GC-, LC-), which can then be used to decompose structural complexity from metabolic complexity as in Equation 1. If we combine Equations 1–3, they simplify to give effective structural complexity as:

$$D_{Seff} = \bar{D}_S \cdot D_D \quad (4)$$

Effective structural complexity results from both structural parameters of chemical mixtures: structural dissimilarity and mean structural complexity. High $D_{Seff}$ can result from highly dissimilar mixtures or highly complex individual compounds.

### 2.4 Influence of biotic and abiotic factors on metabolic complexity, effective structural complexity and compositional diversity in P. kelleyi

Bayesian structural equation models were constructed to test the hypothesis that metabolic complexity, effective structural complexity and compositional diversity would interact differently with light and elevation, and
have different effects on consumers and their surrounding *P. kelleyi* communities. Compositional diversity and metabolic complexity were calculated from LC-UV chromatographic data and $^1$H NMR spectroscopic data, respectively, as Shannon effective number of peaks. Effective structural complexity ($D_{\text{Seff}}$) was decomposed from metabolic complexity and compositional diversity using Equation 1. Individuals with the lowest and highest metabolic complexity, compositional diversity and $D_{\text{Seff}}$, respectively, were identified, and the chemical bases for their diversity values are examined in the Supporting Information (S2, Figure S3). Bayesian structural equation modelling was performed using the *blavaan* package using uninformative priors and default assumptions (Merkle & Rosseel, 2018).

### FIGURE 3

Differentiating compositional diversity and structural complexity yields insight about chemically mediated biotic and abiotic interactions. Top: A depiction of *P. kelleyi* phytochemistry as an LC-UV chromatogram and $^1$H NMR of the same extract and $^1$H NMR of the phytochemicals produced by the pipelantine pathway (a) and chromene pathway (b–d). Bottom: Bayesian structural equation models representing how dimensions of phytochemical diversity (e: metabolic complexity, f & h: compositional diversity, g: effective structural complexity) interact differently with biotic and abiotic factors. Black arrows indicate positive path coefficients and red lines with flat heads represent negative path coefficients. Line width and head size were scaled to approximate path coefficient magnitude.

### 2.5 | Variation in compositional diversity and metabolic complexity of *P. kelleyi* and *P. reticulatum*

We compared the compositional diversity and metabolic complexity for *P. kelleyi* and *P. reticulatum*, calculated from LC-MS
chromatographic data and $^1$H NMR spectroscopic data respectively. Richness, an abundance-independent diversity measure, was used due to differences in ionization efficiency among phytochemicals in mass spectrometry. Mean richness and richness variance were, respectively, compared using Student’s $t$ and $F$ test of variance equality. Student’s $t$ tests were performed out under appropriate variance equality based on $F$ tests. Linear regressions and all other summary statistics were carried out using core R functions (R Core Team, 2014).

3 | RESULTS

3.1 | Structural equation models incorporating metabolic complexity, effective structural complexity and compositional diversity interact differently with biotic and abiotic factors in *P. kelleyi*

An LC-UV chromatogram and $^1$H NMR of a crude *P. kelleyi* extract are shown with $^1$H NMR spectra of the major mixture constituents in Figure 3a–d. The differences in fit between Bayesian models supported our hypothesis that metabolic complexity, effective structural complexity and compositional diversity would each interact differently with light, elevation and herbivory (Figure 3e–h). The model incorporating metabolic complexity based on crude $^1$H NMR diversity (MargLogLik = −84.674, PPP = 0.510, e) indicated that metabolic complexity was reduced by total light (coeff. = 0.31), but had a minimal influence on herbivory (coeff. = −0.02). The $D_{\text{eff}}$ model (MargLogLik = −84.177, PPP = 0.542) supported the causal hypothesis that total light directly reduced $D_{\text{eff}}$ (coeff. = 0.28). $D_{\text{eff}}$ mediated the indirect positive effect of light on herbivory and directly reduced herbivory (coeff. = −0.13). We compared two a priori models to determine the role of compositional diversity in this system (f, MargLogLik = −84.894, PPP = 0.452; h, MargLogLik = −84.241, PPP = 0.584). The better fitting compositional diversity model (Bayes factor = 1.92, h) is consistent with the hypothesis that herbivory directly increases $D_{\text{c}}$ (coeff. = 0.20), rather than $D_{\text{c}}$ driving herbivory (coeff. = 0.16) in the model that was not supported (f). Model h also indicates that total light directly increases herbivory (coeff. = 0.28) and indirectly increases compositional diversity, mediated through herbivory. Another unique feature of this model is that elevation directly decreases $D_{\text{c}}$ (coeff. = −0.33) but when mediated through herbivory, elevation indirectly increases $D_{\text{c}}$.

3.2 | *P. kelleyi* and *P. reticulatum* employ different strategies in the variation of phytochemical diversity

Correlation between $^1$H NMR and LC-MS richness was not detected in either *P. kelleyi* ($r = 0.10$; *P. reticulatum*: $p = 0.92$, Figure 4), and Pearson $r$ values were not significantly higher ($p = 0.16$) for *P. kelleyi* ($r(43) = 0.25$) than for *P. reticulatum* ($r(84) = −0.01$).

4 | DISCUSSION

Previous research on chemical diversity has focused on compositional diversity; however, the interpretation of the quantity and distribution of chemicals within a plant community are limited without understanding their ecological function. As function is derived from structure, approaches that incorporate the structural properties of individual molecules and of chemical mixtures are more likely to approximate functional and interaction diversity. By using...
complementary compositional and structural approaches, we uncovered nuances in how *P. kelleyi* biology and chemistry are affected by interacting factors such as light, elevation and herbivory through processes that manifest differently in chemical composition versus structure (Objective 1). Variation in structural and compositional axes of chemical diversity differed between two *Piper* species, capturing the different strategies for producing diverse phytochemical mixtures that can affect arthropod communities (Objective 2). Finally, we describe a framework for interpreting how the structural complexity of individual chemicals can aggregate through composition to produce metabolic complexity in phytochemical mixtures (Objective 3).

### 4.1 Decomposition of chemical diversity into compositional and structural components provides unique insight into chemically mediated multitrophic interactions

In *P. kelleyi*, the direct inhibition of herbivory via effective structural complexity (*D_\text{eff eff}*, Figure 3g) can be attributed to structurally complex chromene dimers, which are known to inhibit the development of generalist herbivores (Jeffrey et al., 2014). Our models support increasing herbivory directly leading to increased compositional diversity (h). There was less statistical support for direct effects of compositional diversity on herbivory (f), suggesting that oxidative degradation caused by herbivory deactivates chemical defences. Such degradation generates numerous structurally similar peaks, which increase compositional diversity while minimally affecting metabolic complexity (Supporting Information (S2)). Previous studies found that at higher elevations, the *P. kelleyi* specialist caterpillar *Eois encina* (Geometridae) was genetically distinct from populations at lower elevations. The authors also found elevated levels of prenylated benzoic acid, suggesting that subpopulations at higher elevations may be more resistant to phototoxic chromone defences (Glassmire et al., 2016). *Piper kelleyi* may reduce the production of chromenes in response to increased herbivory at higher elevations to avoid oxidative deactivation of defences, which would lead to a reduction in compositional diversity. Alternatively, a combination of increased herbivory and UV-B exposure encountered at higher elevation may oxidize chromones to undetectable levels, leaving behind less compositionally diverse mixtures enriched with prenylated benzoic acid.

### 4.2 Differential variation in compositional and structural components of chemical diversity between geographically distinct *Piper* species

*Piper kelleyi* had more structural variation (metabolic complexity, measured as NMR richness), while *P. reticulatum* primarily varies compositionally. In a system like *P. reticulatum*, or *Medicago*, the production of many structurally similar chemicals requires separation techniques (LC- and GC-based) to properly quantify diversity and compositional variation. Conversely, systems where biosynthetic trade-offs lead to compositionally homogeneous but structurally dissimilar chemistry (e.g. *P. kelleyi*) require appropriate spectroscopic methods, like NMR, that capture structural variation. Despite demonstrating variance in different axes of chemical diversity, in both *P. kelleyi* and *P. reticulatum*, we found $^1$H NMR metabolic complexity is not determined by compositional diversity. *Piper reticulatum* chemistry is dominated by structurally similar wisanidine-like amides (Whitehead et al., 2013) which varies most in compositional richness, possibly in response to resource heterogeneity, which varies widely across La Selva. Conversely, *P. kelleyi* extracts exhibited significantly higher variation in metabolic complexity than in compositional diversity due to trade-offs between structurally and biosynthetically distinct piplartine and chromene pathways. Individuals with highest metabolic complexity expressed both piplartine and chromene analogues, and their structural dissimilarity led to high metabolic complexity. Thus, the increased antiherbivore defence driven by higher $^1$H NMR diversity (Glassmire et al., 2019) in *P. kelleyi* could be due to synergistic effects of combining piplartine and chromene analogues. While *Piper* served as a useful model system for this study, we have shown a structural approach to chemical diversity would not be as useful for phytochemical systems where structural differences are not readily detected by $^1$H NMR, such as wisanidines in *P. reticulatum*, or other structurally similar systems such as terpenoids. In such cases, compositional measures of diversity derived from chromatographic (LC or GC) data would be more appropriate.

### 4.3 Interpreting effective structural complexity

Phytochemical function is responsive to subtle changes in structure (Berenbaum, 1983), and structurally diverse chemical mixtures are therefore more likely to have broader, or in the case of synergistic mixtures, more potent function. Herein, we present a theoretical framework that serves as a guide for chemical ecologists to implement and interpret structurally relevant NMR-based studies of chemical diversity. Metabolic complexity ($D_\text{met}^*) not only results from the composition of phytochemical mixtures but also depends on the bulk structural components of a chemical mixture: mean structural complexity ($D_\text{struct}^*) and structural dissimilarity ($D_\text{dist}^*) of constituent molecules (Figure 1). Effective structural complexity ($D_\text{eff eff}^*$) removes the compositional contribution to metabolic complexity, representing both of these bulk structural components (Equation 4). Phytochemical mixtures with high effective structural complexity are a consequence of a high degree of structural complexity, dissimilarity or both. Effective structural complexity of phytochemical systems provides structural insight that would be unavailable from measuring metabolic complexity or compositional diversity alone. Further investigation is required to determine how compositional diversity and structural complexity of individual molecules combine to determine the metabolic and effective structural complexity of chemical mixtures.
5 | CONCLUSIONS

While many studies have examined the causes and consequences of ecological factors on phytochemical abundance and composition, quantifying variation in the structural properties of chemical mixtures provides new insight into another equally important component of diverse phytochemical mixtures. Such variation could be relevant in systems where in vivo structural changes have consequences for multitrifactor interactions, such as enzymatically activated transformations of saponins, glucosinolates and cyanogenic glycosides (Osborn, 1996). Some recent studies that have examined phytochemical similarity in an evolutionary context (Kang et al., 2019; Li et al., 2020; Sedio, 2017) did not consider metabolic and structural complexity and potentially missed a functionally important component of chemical diversity. We hope the approach we have outlined here will ultimately broaden the view of how phytochemistry evolves structurally.

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AUTHORS’ CONTRIBUTIONS

All authors conceived and developed the ideas presented herein; C.S.P. and A.E.G. collected and analysed the data; C.S.P. and C.S.J. developed the interpretation of $^1$H NMR spectra in relation to chemoinformatic data; C.S.P., L.A.R., L.A.D. and A.E.G. developed the interpretation of chemical diversity in relation to ecological factors; C.S.P led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA AVAILABILITY STATEMENT

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REFERENCES

Abbott, H. C. D. S. (1887). The chemical basis of plant forms. Creative Media Partners, LLC.
Aly, A. H., Debbab, A., Kjer, J., & Proksch, P. (2010). Fungal endophytes from higher plants: A prolific source of phytochemicals and other bioactive natural products. Fungal Diversity, 41, 1–16. https://doi.org/10.1007/s13225-010-0034-4
Becerra, J. X., Noge, K., & Venable, D. L. (2009). Macroevolutionary chemical escalation in an ancient plant–herbivore arms race. Proceedings of the National Academy of Sciences of the United States of America, 106, 18062–18066. https://doi.org/10.1073/pnas.0904456106
Berenbaum, M. (1983). Coumarins and caterpillars: A case for coevolution. Evolution, 37, 163–179. https://doi.org/10.1111/j.1558-5646.1983.tb05524.x
Berenbaum, M. R., & Zangerl, A. R. (1996). Phytochemical diversity. In J. T. Romeo, J. A. Saunders, & P Barbosa (Eds.), Phytochemical diversity and redundancy in ecological interactions (pp. 1–24). Springer. https://doi.org/10.1007/978-1-4899-1754-6
Clay, K. (1993). Fungal endophytes of plants: Biological and chemical diversity. Natural Toxins, 1, 147–149. https://doi.org/10.1002/nt.2620010302
Dyer, L. A. (2018). Multidimensional diversity associated with plants: A view from a plant–insect interaction ecologist. American Journal of Botany, 105, 1439–1442. https://doi.org/10.1002/ajb2.1147
Dyer, L. A., & Palmer, A. D. (2004). Piper: A model genus for studies of phytochemistry, ecology, and evolution. Springer. https://doi.org/10.1007/978-0-387-30599-8
Dyer, L. A., Parchman, T. L., Jeffrey, C. S., & Richards, L. A. (2014). New dimensions of tropical diversity: An inordinate fondness for insect molecules, taxa, and trophic interactions. Current Opinion in Insect Science, 2, 14–19. https://doi.org/10.1016/j.cois.2014.06.001
Dyer, L. A., Philbin, C. S., Ochesenrider, K. M., Richards, L. A., Massad, T. J., Smilanich, A. M., Forister, M. L., Parchman, T. L., Galland, L. M., Hurtado, P. J., Espeset, A. E., Glassmire, A. E., Harrison, J. G., Mo, C., Yoon, S., Pardikes, N. A., Muchoney, N. D., Jahner, J. P., Slinn, H. L., … Jeffrey, C. S. (2018). Modern approaches to study plant–insect interactions in chemical ecology. Nature Reviews Chemistry, 2, 50–64. https://doi.org/10.1038/s41570-018-0009-7
Dyer, L. A., Wagner, D. L., Greeney, H. F., Smilanich, A. M., Massad, T. J., Robinson, M. L., Fox, M. S., Hazen, R. F., Glassmire, A. E., Pardikes, N. A., Fredrickson, K. B., Pearson, C. V., Gentry, G., & Stireman, J. O. (2012). Novel Insights into tritrophic interaction diversity and chemical ecology using 16 years of volunteer-supported research. American Entomologist, 58, 15–19. https://doi.org/10.1093/ae/58.1.0015
Ehrlich, P. R., & Raven, P. H. (1964). Butterflies and plants: A study in coevolution. Evolution, 586–608. https://doi.org/10.1111/j.1558-5646.1964.tb01674.x
Feiner, Z. S., Swihart, R. K., Coulter, D. P., & Höök, T. O. (2018). Fatty acids in an iteroparous fish: Variable complexity, identity, and phenotypic correlates. Canadian Journal of Zoology, 96, 859–868. https://doi.org/10.1139/cjz-2017-0148
Fraenkel, G. S. (1959). The raison d'etre of secondary plant substances. Science, 1466–1470.
Frazer, G. W., Canham, C. D., & Lertzman, K. P. (1999) Gap Light Light Analyzer (GLA), Version 2.0: Imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, users manual and program documentation (Vol. 36). Simon Fraser University, The Institute of Ecosystem Studies.
