IDEA AND PERSPECTIVE

On the study of plant defence and herbivory using comparative approaches: how important are secondary plant compounds

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Abstract

Species comparisons are a cornerstone of biology and there is a long tradition of using the comparative framework to study the ecology and evolution of plant defensive traits. Early comparative studies led to the hypothesis that plant chemistry plays a central role in plant defence, and the evolution of plant secondary chemistry in response to insect herbivory remains a classic example of coevolution. However, recent comparative work has disagreed with this paradigm, reporting little connection between plant secondary chemicals and herbivory across distantly related plant taxa. One conclusion of this new work is that the importance of secondary chemistry in plant defence may have been generally overstated in earlier research. Here, we attempt to reconcile these contradicting viewpoints on the role of plant chemistry in defence by critically evaluating the use and interpretation of species correlations as a means to study defence–herbivory relationships. We conclude that the notion that plant primary metabolites (e.g. leaf nitrogen content) are the principal determinants of herbivory (or the target of natural selection by herbivores) is not likely to be correct. Despite the inference of recent community-wide studies of herbivory, strong evidence remains for a prime role of secondary compounds in plant defence against herbivores.

'In the last quarter of a century, enough evidence has accrued to show that secondary products are indeed the main determinants in plant selection by insects. It now seems highly likely, as Feeny has pointed out, that without such compounds the majority of species of higher plants could not long withstand the voracious and destructive appetites of the various guilds of herbivorous insect pests'. (Swain 1977)

'Unexpectedly, we found no significant effects of chemical defence traits. Rather, herbivory was related to the plants' leaf morphology, local abundance and climatic niche characteristics, which together explained 70% of the interspecific variation in herbivory in phylogenetic regression. ... [This result] corroborates recent results of a more global analysis which indicates that secondary metabolites are of less importance as a defence against herbivory than morphological and life-history traits'. (Schuldt *et al.* 2012)

'In contrast to this study, most [past] reports have focused on specific interactions between single plant and herbivore species or specific compounds, whereas interactions involving many herbivore and plant species have rarely been addressed. The minor importance of secondary compounds in our model is confirmed by a recent meta-analysis aiming to determine what traits generally influence plant susceptibility to herbivores'. (Loranger *et al.* 2012)

INTRODUCTION

Plant secondary metabolites (i.e. chemical compounds not involved in resource acquisition or allocation) are classic examples of plant traits that mediate interactions with herbivores. Over the past century, studies of plant secondary compounds have resulted in major advancements in our understanding of plant ecology, including the development of plant defence theory and the concept of coevolution (Fraenkel 1959; Ehrlich & Raven 1964). Throughout this work, plant chemistry has generally been lauded as *the* most important plant trait in determining interactions between plants and insect herbivores. However, new research has recently emerged contradicting this viewpoint, suggesting that plant secondary chemicals are not typically predictive of herbivory (except for the variable effects of phenolics) (Kurokawa *et al.* 2010; Carmona *et al.* 2011; Pearse 2011; Loranger *et al.* 2012; Schuldt *et al.* 2012). Although this body of research was motivated by a variety of different research questions, it shares the common

approach of ascribing trait functions based on comparative ecological analyses. Importantly, these analyses have been based on species correlations of trait–herbivory relationships, an approach that is often seen as powerful because significant correlations span millions of years of evolutionary history and extreme trait variation.

The results of this emerging research directly contradict the paradigm established by classic studies, and suggest that other plant characteristics (e.g. foliar nitrogen content) are more important than secondary compounds in plant defence. Based on these results, several authors have concluded that, as a discipline, plant ecologists may be overestimating the importance of secondary chemistry (Carmona *et al.* 2011; Pearse 2011; Loranger *et al.* 2012; Schuldt *et al.* 2012). By 'importance', we are specifically referring to the functional role of secondary chemistry in reducing herbivore performance and in the evolution of plant defence against herbivores. The purpose of this essay is to reconcile these contradicting perspectives by critically evaluating the comparative framework as a means to analyse the relationship between secondary compounds and herbivory.

We begin by briefly comparing two comparative approaches, community vs. evolutionary studies, for interspecific trait correlations that have historically been utilised to study trait–herbivory relationships. Following this, we evaluate what questions trait–herbivory correlations can and cannot address, and provide a set of suggestions for the use and interpretation of species correlations to study herbivory. Finally, we conclude with a broad overview of the evidence across different types of studies that secondary compounds are, or are not, important in plant defence.

THE COMPARATIVE APPROACH IN THE STUDY OF PLANT DEFENCE

Ecological research on plant defence against herbivores has taken a comparative approach since classic studies by Grime *et al.* (1968) and Coley (1983), which measured plant traits and levels of herbivory across species within diverse communities of coexisting plants. These studies illuminated the ways in which plant traits mediate plant–insect interactions, and led to important theoretical developments. In particular, Coley's (1983) results led to advances in categorising plant defence syndromes or strategies (Coley *et al.* 1985), a contribution to theory which continues to stand the test of time (Fine *et al.* 2004; Mooney *et al.* 2010; Tanentzap *et al.* 2011). Parallel to this work, a similar but more evolutionary literature also emerged focused on clades of closely related species rather than on plant communities. This evolutionary, clade-based research investigated how ecologically relevant plant traits evolve across a phylogeny. A major result of these studies was the generation of the hypothesis that coevolutionary dynamics with herbivores impacted the evolution and distribution of plant traits. In particular, secondary compounds were identified as key evolutionary players in these dynamics (Fraenkel 1959; Ehrlich & Raven 1964; Swain 1977).

In both early community and clade-based studies of plant defensive chemistry, trait–herbivory correlations featured prominently. For community ecological studies, these correlations were conducted across distantly related species that co-occurred in a given location (e.g. Grime *et al.* 1968; Coley 1983), whereas evolutionary studies focused more specifically on correlations between secondary compounds within a clade of related species and the response of a targeted group of insect herbivores (Ehrlich & Raven 1964; Chew 1977).

In part owing to the availability of molecular phylogenies, a new generation of studies has more recently emerged combining these two types of comparative approaches (community vs. evolutionary foci) to address a variety of questions (Marquis *et al.* 2001; Schädler *et al.* 2003; Agrawal & Fishbein 2006; Pearse & Hipp 2009; Kurokawa *et al.* 2010; Tanentzap *et al.* 2011; Loranger *et al.* 2012; Schuldt *et al.* 2012). It is in these new studies where plant secondary chemicals are, remarkably, far less predictive of herbivory than expected given the previous paradigm. In the following paragraphs, we disentangle these different approaches to conducting trait–herbivory correlations, illustrate why this new combined approach both holds appeal and presents inherent challenges in interpretation, and call for a more unified framework that reconciles data across scales.

MOTIVATIONS FOR CONDUCTING SPECIES CORRELATION ANALYSES OF TRAITS AND HERBIVORY

There are at least three major conceptual reasons to conduct species level analyses of traits and levels of herbivory, each of which inherently requires a different scale of analysis and results in a distinct type of inference.

The first conceptual motivation to conduct trait–herbivory correlations is that the pattern of variation in traits and herbivory can provide insight into the evolution of defence. For example the convergent evolution of particular secondary metabolites in distant taxa (e.g. alkaloids, cyanides) has been used both to understand important selective agents on plants (e.g. Kursar & Coley 2003; Agrawal & Fishbein 2006; Fine *et al.* 2006) and host range evolution in insects (e.g. Becerra 1997; Murphy & Feeny 2006; Rasmann & Agrawal 2011a). In addition, theory has made various predictions about the evolution of defence, and these can be directly addressed using comparative analyses within a clade (Becerra *et al.* 2009; Rasmann & Agrawal 2011b; Pearse & Hipp 2012; Weber *et al.* 2012). For example different defensive traits are hypothesised to interact positively and evolve together, to be adaptive in certain biotic contexts, or to trade off with one another or with other plant traits (such as growth rate or environmental niche). Trade-offs are especially interesting because they suggest switches or alternative strategies over macroevolutionary time, as suggested by early theory (Coley *et al.* 1985).

A second conceptual aim stems from a predictive community ecological framework. By describing plant–herbivore associations at the community level, we can gain insight into how to predict the potential for host shifts among different community members. This work includes classic studies of insect communities on plants (Strong *et al.* 1984) and more recent analyses of plant traits that shape community interactions (Poelman *et al.* 2008). Studies of plant phylogenetic distance and its impact on shared herbivore communities also

fall into this category (Odegaard *et al.* 2005; Weiblen *et al.* 2006), and this approach has been successfully used to predict host expansion onto introduced plants (Pearse & Altermatt 2013). In addition, the importance of community ecological extrinsic factors, such as the relative abundance of host and non-host plant species (Futuyma & Wasserman 1980), geographical distributions of hosts (Schuldt *et al.* 2012) and specific host plant rates of parasitism (Lill *et al.* 2002), have all been employed to predict host plant use and herbivory in community surveys.

The third conceptual reason to conduct plant trait and herbivore association analyses at the species level is to study the functional role that particular plant traits are playing in defence. Given that species typically exhibit tremendous variation along both axes of interest (traits and herbivore damage), there is potentially a high level of statistical power available to address functional hypotheses. These studies are aimed at understanding how standing variation in plant traits correlates with herbivore damage, frequently *in situ* in an ecological community. For example how important is the nutritive quality (e.g. protein, nitrogen or undigestible lignin) of leaves as a predictor of herbivory? In a Panamanian rainforest, young leaves of coexisting tree species varied over 25-fold in their lignin content, which correlated negatively with rates of herbivory (Coley 1983). Using trait–herbivory correlations in this way is both a classic and currently employed approach in studies focused on understanding the functional role of plant traits in mediating species interactions.

Although each of the three motivations discussed above (understanding the evolution of defence, predicting species interactions and testing trait function) utilise species-level correlations between traits and herbivory, their interpretation requires different scales of analysis and results in a distinct type of inference. For example studies aimed at understanding the evolution of defensive traits are generally conducted within groups of closely related plant species, simply because inference about the evolutionary trajectories of traits becomes limited across broad phylogenetic scales where the majority of taxa are not sampled. On the other end of the spectrum, studies aimed at predicting host shifts in communities are typically conducted across a very broad range of plant taxa living together in a shared environment. These studies have been particularly effective when surveys disentangle the effects of specific herbivores, as these species often have divergent responses to plants and their traits. Finally, and most relevant to our discussion of plant secondary compounds, studies that use species correlations to understand the functional role of traits have been conducted across both broad and narrow phylogenetic scales, focusing either on a group of very closely related species (Pearse & Hipp 2009; Mooney *et al.* 2010; Johnson *et al.* 2014; Agrawal *et al.* 2015), or spanning near all co-occurring plant species in an environment (Grime 1968, Coley 1983; Tanentzap *et al.* 2011; Loranger *et al.* 2012; Schuldt *et al.* 2012). However, confusion has arisen in these analyses of trait functions, as the inference that can be gleaned by using trait–herbivory correlations can differ drastically depending upon the scale of analysis. In particular, below we argue that community-level studies may not provide strong functional or evolutionary tests of the importance of plant traits in defence against herbivores.

USING COMMUNITY-WIDE TRAIT–HERBIVORY RELATIONSHIPS TO STUDY DEFENSIVE FUNCTION OF SECONDARY METABOLITES: LIMITS TO INFERENCE

We argue that, by nature of their design and the distribution of plant chemical compounds, correlations of traits vs. herbivory across species within a community have little ability to identify the functional role of traits such as plant secondary metabolites. There are several limits to the functional and evolutionary conclusions that can be reached using community-wide surveys that measure secondary compounds and herbivory, each of which we will elaborate on below:

1) A mismatch between the broad species composition of plant communities and the phylogenetic distribution of major classes of secondary metabolites, the latter of which are often conserved within clades, making broad species comparisons impractical and without a common currency.
2) Alternative strategies among species may render species correlations over a limited set of traits misleading because the presence or absence of a particular trait may be associated with suites of other traits influencing herbivory.
3) Correlates of herbivory across species in a plant community are likely to reveal extreme phenotypes common to all plants as predictors, even though these may not be the functional drivers of species interactions.

Clade specificity of secondary metabolites

The first limitation of community-wide herbivory correlations has to do with whether we can measure the relevant traits in a common currency so as to evaluate their importance for defence across very distantly related taxa. The scale of species inclusion in a study inherently limits the types of traits that can be included in the analysis. For example at the community level, studies are typically confined to using coarse traits present in all plant species to predict damage (by a community of herbivores), averaging over all other clade-specific traits. As such, clade-specific resistance traits are typically missed in community analyses, and yet the clade specificity of secondary compounds is one of their most distinctive evolutionary features.

The classic studies that led to such high interest in the defensive role of secondary compounds emphasised this clade-specific pattern, as they were often explicitly focused on compounds unique to families (or smaller clades) of plants and their effects on specialist groups of herbivores. Whether it is the Apocynaceae (cardenolides), Anacardaceae (terpenoid resins), Berberidaceae (alkaloids), Brassicaceae (glucosinolates), Cucurbitaceae (cucurbitacins) or Caprifoliaceae (iridoid glycosides), plant clades often have signature secondary compounds. Even though variation in these secondary compounds dictate levels of herbivory within species or among close relatives (e.g. Da Costa & Jones 1971; Giamoustaris & Mithen 1995; Mauricio & Rausher 1997; Agrawal *et al.* 2012), it is difficult to study these mechanisms of resistance at the community scale simply because the same type of chemical defence does not exist in most species in a community. However, their absence in distantly related plant clades cannot be taken as evidence that they are unimportant for plant defence

when present. This problem is compounded by the fact that most plants contain dozens of secondary plant compounds, usually from several different metabolic pathways. Lettuce, for example is characterised by having latex, sesquiterpene lactones, cysteine proteases, diverse phenolics, oxidative enzymes, etc. (Agrawal & Konno 2009). As such, finding predictors of plant resistance is complicated by the methodological requirements to quantify compounds across these different groups and not having a common currency to make comparisons.

Clade specificity also causes problems for measuring the herbivore side of the trait–herbivory correlation. The effect of many plant defences (including secondary compounds) can vary in magnitude and direction across different herbivores, and if studies simply measure overall herbivore damage, then this level of specificity may be obscured. Indeed, correlations between secondary compounds and herbivory can be both positive and negative in direction depending on the specificity of the insect group (reviewed by Ali & Agrawal 2012). It is worth noting that it was in part this pattern, that the same compound can at once be attractive to one insect and repellent to others, that initially motivated the hypothesis that secondary compounds have imposed strong selection on insects (Fraenkel 1959). It is ironic that this same pattern, which muddles the correlation of secondary compounds and herbivory in the context of multiple herbivores, can be interpreted as evidence that secondary metabolites are unimportant. Only by measuring herbivory by different herbivores can this problem be solved.

Correlations of unmeasured traits

The above discussion leads to the second major limitation of community-wide trait-herbivory correlations, that species correlations are especially prone to problems of correlated traits. Because each data point in such correlations represents a single species, the analyses rely on statistical independence of species and a lack of unmeasured correlated traits that might be driving an observed relationship (Hendriks et al. 1999). As the phylogenetic distance between the species included in the analysis increases, so does the possibility for interceding traits (or other phenomena) driving observed correlations. As conceptualised in Figs 1 and 2, this issue leads to species-level correlations not being well suited to test trait functions or test whether traits are targets of natural selection (although such correlations can suggest these as hypotheses).

One of the major outcomes of comparative approaches in plant ecology has been the recognition of alternative strategies among species (Coley et al. 1985; Westoby et al. 2002; Schädler et al. 2003; Agrawal & Fishbein 2006). In fact, even among close relatives, one can find rare and weedy species, organisms with different life-history strategies or alternative strategies to cope with similar stresses. Such alternate approaches typically involve suites of covarying traits. As an example, fast growing plant species may be highly attractive to herbivores, for many reasons, including high tissue nitrogen content, soft and juicy foliage and low investment in chemical defence (Coley et al. 1985; Schädler et al. 2003). And yet, in part owing to their fast growth, such plant species may be highly tolerant of damage,
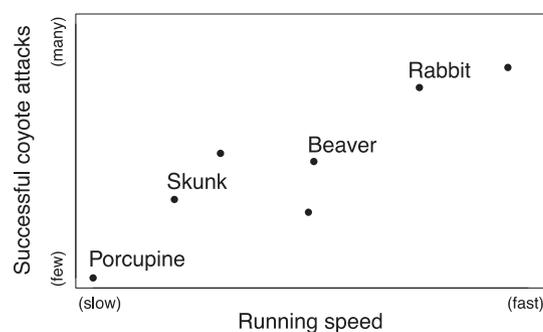

Figure 1 A conceptualisation of pitfalls in making functional/evolutionary conclusions based on species level correlations within communities. In this hypothetical case, only mammals residing within a northeastern US forest community are considered (only exemplars are labelled), and statistically speaking, fast-running species may be more heavily attacked by coyotes. Yet, it would be incorrect to conclude that running speed does not play an important role in avoiding coyote attack, or worse yet, that selection has favoured slow running speed as a resistance trait. Instead, we may intuit that slower species may be less attacked because of their alternative traits (diurnal activity, arboreal and aquatic adaptations, defensive strategies, etc.). See Caro (2005) for a discussion of alternate anti-predator strategies in mammals.
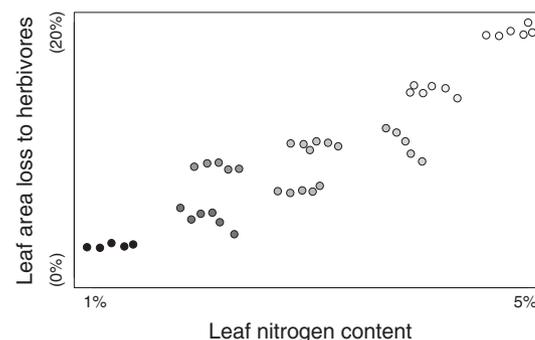

Figure 2 Within vs. between species correlations may go in opposing directions (Agrawal et al. 2010). Here, each shade of grey represents a taxonomic group (this could be a species, a genus or even a family), whereas points of the same shade represent replicates within that taxonomic group (multiple individuals, species or genera respectively). The x axis, here foliar nitrogen content, predicts herbivory across the taxonomic groups. Regressing across the means of each group indicates that herbivores prefer nitrogen rich foods, especially when spanning such a large range. However, alternate resistance traits (often clade-specific traits) may determine herbivory among individuals within the group and these traits may be the long-term targets of natural selection. Nitrogen may have little predictive power within the group, or show correlations in the opposite direction. Within clades, the relationship between herbivory and nitrogen differs from the pattern across clades.

leading to a successful strategy. Accordingly, although tissue nitrogen content predicts herbivory, it would be misleading to argue that the main determinant of herbivory is nitrogen content or that there is little evidence of secondary metabolites being important for resistance. To complicate this further, systems approaches emerging from Arabidopsis now indicate that plant primary metabolism may be modulated by secondary compounds, further complicating the independence of alternative plant strategies (Kliebenstein 2012).

Extreme phenotypes, their interpretation and consequences

Predictors of herbivory across species in a plant community are likely to reveal an importance of extreme phenotypes common to all plants (i.e. plant species with > 5% foliar nitrogen are typically heavily attacked; plant species with tough leaves are little touched) (Fig. 2) (Marquis *et al.* 2001; Schädler *et al.* 2003; Pearse 2011; Tanentzap *et al.* 2011; Loranger *et al.* 2012; Schuldt *et al.* 2012). Such statistical relationships are certainly valid, but may neither be reflective of functional relationships nor evolutionary history of selection. High variation in traits observed among distantly related species (in community analyses) can provide a misleading level of predictive power (Tilman *et al.* 2004). The greater the variation in the traits, the higher the predictive power of the correlation, even though there is the greatest potential for confounding effects. In other words, extreme values in a trait (say, nitrogen content) can generate relationships with herbivory levels, even though very little predictive power exists within a more narrow range. This can result in a pattern where a trait correlates with herbivory levels across distantly related plant species, but the same trait shows no relationship in analyses across individuals with any given species or across a group of close relatives (Fig. 3).

BENEFITS OF STUDYING TRAIT VARIATION AND INFERENCE WITHIN CLADES

Within a species or a clade (say a plant genus), we avoid some of the above challenges to identifying the mechanistic traits behind resistance and the targets of natural selection. As a brief example, consider two field experiments conducted in the same year at the same field site. In the first, the foliar nitrogen content of 24 *Asclepias* species was measured; despite being from dramatically different habitats across North America, when grown in a common field environment, they ranged in
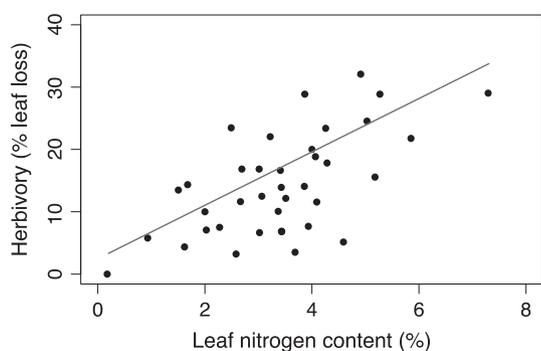

Figure 3 The relationship between leaf nitrogen content and herbivory in a hypothetical dataset. Across species within a community, there is typically tremendous variation in leaf nitrogen content (shown here, > 12 fold variation), and a positive relationship is often found between these two variables. Nonetheless, variation within a species (or even a genus) is typically much smaller, say 2 percentage points. Across this dataset, the variation explained in herbivory across any 2 percentage points in leaf nitrogen is trivially small, and the overall correlation is driven by the extremes. More generally, as outlined by Tilman *et al.* (2004), the greater the variation in the X axis in such correlations, the greater the explanatory power (i.e. r^2) in the relationship. Such relationships are also typically confounded by a host of correlated traits.

foliar nitrogen content from 3.0 to 4.2% (data from Agrawal & Fishbein 2006). In this dataset, despite relatively low variation in foliar nitrogen content, high levels of species variation was found in other clade-specific defensive traits: species ranged from trace to 4.7 mg of latex exuded upon tissue damage, and species varied nearly 8-fold in the concentration of cardenolides in their leaves. Indeed, variation in latex levels predicted performance of monarch butterfly caterpillars. At the same field site, when 28 coexisting herbaceous species from 10 families were grown in a similar way to the *Asclepias*, species variation in leaf nitrogen ranged from 1.7 to 4.4% (data from Agrawal *et al.* 2006). Not only is this a substantially larger range than for the clade-based analysis of *Asclepias* but we were unable to quantify relevant clade-specific defensive traits from these distantly related groups.

As illustrated in this case, there are benefits to clade-specific analyses. For example clade-specific analyses offer a common trait currency to address the determinants of herbivory, without which the role of many traits cannot be assessed. In addition, clade-specific analyses may be less confounded than community-wide analyses because they inherently 'control' for clade-specific differences by examining species that share a recent common ancestor (and are thus similar in many traits). Because of this, phylogenetically controlled correlations within clades can be highly suggestive of mechanisms of plant defence (Pearse & Hipp 2009; Mooney *et al.* 2010; Johnson *et al.* 2014; Agrawal *et al.* 2015).

CONCLUSION: ARE SECONDARY COMPOUNDS IMPORTANT FOR PLANT DEFENCE?

The mechanistic data clearly support the notion that secondary metabolites provide plant defence, including resistance to specialist herbivores. We will not provide a thorough review of the mechanistic understanding of plant resistance via secondary metabolites in this paper, as this topic has been recently reviewed elsewhere (Kliebenstein 2012; Mithöfer & Boland 2012). Nonetheless, a considerable body of evidence has accrued from a diversity of systems and approaches in support of a strong role of secondary metabolites as important drivers of herbivory and as agents of selection on herbivores. Whether studies use highly controlled conditions and spiked artificial diets (e.g. Agrawal & Kurashige 2003; Harvey *et al.* 2007), correlations (e.g. Giamoustaris & Mithen 1995; Zalucki *et al.* 2001) or genetic manipulations (e.g. Da Costa & Jones 1971; Steppuhn *et al.* 2004; Müller *et al.* 2010), results indicate a strong role for plant secondary compounds in defence. In addition, we have excellent field studies demonstrating natural selection and evolution of plant defence chemistry in several systems (Berenbaum *et al.* 1986; Mauricio & Rausher 1997; Agrawal *et al.* 2012). And secondary metabolites have been demonstrated to have considerable impacts on herbivore fitness via a diversity of modes of action (reviewed in Mithöfer & Boland 2012). Remarkably, even herbivore-driven evolutionary change in particular plant enzymes has recently been linked to plant local adaptation in secondary metabolites (Prasad *et al.* 2012).

The fact that plant defences have evolved despite being costly, and that similar traits have evolved independently time

and time again throughout the diversification of plants is strong support for an adaptive role (Kursar & Coley 2003; Wink 2003; Agrawal & Konno 2009; Weber & Keeler 2013). Reciprocally, that herbivores have highly specific (and also convergent) adaptations to particular plant secondary metabolites and other defensive strategies (Dussourd & Eisner 1987; Jensen *et al.* 2011; Dobler *et al.* 2012) amply illustrates the importance of secondary compounds in the evolution of herbivores. Yes, in meta-analyses, impacts are greater on generalists than specialists, as expected (Cornell & Hawkins 2003). And yes, there are conditions, either ecologically realistic, or because of poor experimental design, where some secondary plant metabolites may not exert negative impacts on herbivores. But, a major function of secondary metabolites is antiherbivore defences.

The path forward to further elucidating the function and evolution of different compounds should include a diversity of approaches, including the comparative study of closely related species. But how can we reconcile the evidence in the two paragraphs above with the comparative studies that find secondary metabolites have little predictive power in defence against herbivores? We believe that, rather than suggesting that new comparative results challenge the importance of secondary metabolites for herbivory, a more unified interpretation that reconciles disparate results across scales of analysis is required. In particular, we call for a holistic framework for predicting the outcome of trait-herbivory correlations across various scales, clades and ecological contexts. Most importantly, because of the nature of secondary metabolites, we should not expect them to show a strong correlation with plant damage across groups of distantly related plant taxa or whole herbivore communities. Accordingly, the lack of associations between secondary compounds and herbivory at the community scale does not disprove a strong role for secondary compounds in functional and evolutionary aspects of plant defence.

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AUTHORSHIP

Agrawal and Weber discussed all ideas and co-wrote the manuscript.

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