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Phytochemical diversity and synergistic effects on herbivores

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Abstract Synergistic effects of multiple plant secondary metabolites on upper trophic levels constitute an underexplored but potentially widespread component of coevolution and ecological interactions. Examples of plant secondary metabolites acting synergistically as insect deterrents are not common, and many studies focus on the pharmaceutical applications of natural products, where activity is serendipitous and not an evolved response. This review summarizes some systems that are ideal for testing synergistic plant defenses and utilizes a focused meta-analysis to examine studies that have tested effects of multiple compounds on insects. Due to a dearth of ecological synergy studies, one of the few patterns for synergy that we are able to report from the meta-analysis is that phytochemical mixtures have a larger overall effect on generalist herbivores than specialist herbivores. We recommend a focus on synergy in chemical ecology programs and suggest future hypothesis tests and methods. These approaches are not focused on techniques in molecular biology to examine mechanisms at the cellular level, rather we recommend uncovering the existence of synergy first, by combining the best methods in organic synthesis, isolation, chemical ecology, bioassays, and quantitative analyses. Data generated by our recommended methods should provide rigorous tests of important hypotheses on how intraclass and interclass compounds act synergistically to deter insects, disrupt the immune response, and ultimately contribute to diversification. Further synergy research should also contribute to determining if antiherbivore synergy is widespread among plant secondary metabolites, which would be consistent with the hypothesis that synergistic defenses are a key attribute of the evolved diverse chemical mixtures found in plants.

Keywords Bioassays · Generalist · Plant defenses · Meta-analysis · Specialist

Abbreviations

PAs	Pyrrolizidine alkaloids
PO	Phenoloxidase

Introduction

When Socrates was forced to consume an infusion of hemlock (*Conium maculatum*) over 2400 years ago, he was poisoned by what most assume was toxicity due to a *mixture* of 8 piperidine alkaloids (Bloch

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2001)—like other plant defensive mixtures, it is unlikely that a single compound is responsible for hemlock's overall toxicity. While coniine was the first alkaloid synthesized, and the physiological mechanisms of toxicity are known for some *C. maculatum* alkaloids, there are still no formal tests of the additive, synergistic, or antagonistic relationships between all of these well studied alkaloids, and the evolution of chemical defense in *C. maculatum* is completely unexplored (Reynolds 2005). The hemlock example is somewhat unique, because individual alkaloids, such as coniine, can be effective neurotoxins individually. However, it is not clear if this is the case in ecological systems, since no study has demonstrated that the individual alkaloids in *C. maculatum* are effective at deterring the natural enemies of this plant. Hypotheses about the causes and consequences of such mixtures are best studied by combining modern approaches in chemistry, biology, and statistics.

Synergy occurs in phytochemical mixtures when the combined effects of the mixture are greater than the sum of effects for the individual compounds. In fact, many plant secondary metabolites that have been isolated from natural mixtures or synthesized in the laboratory have no known function when tested in biological assays (Harborne 1988; Ayres et al. 1997). This phenomenon has generated influential hypotheses about the ecology and evolution of phytochemical diversity. One such hypothesis is the Screening Hypothesis (Jones and Firn 1991; Firn and Jones 2003; Challis and Hopwood 2003; Dyer et al. 2003a; Boyd et al. 2012), which is based on the concept that phytochemical diversity is maintained as a selective advantage to increase the likelihood of producing a compound with the right biomolecular activity against a natural enemy. Alternatively, since plants must deter multiple attackers, one hypothesis posits that mixtures of secondary metabolites have evolved because each compound functions via a different mechanism to defend against specific natural enemies (Berenbaum and Zangerl 1996; Iason et al. 2011; Richards et al. 2015). Therefore the lack of apparent bioactivity of an isolated compound may be the result of testing it against the wrong targeted organism. In addition, it is hypothesized that diffuse coevolutionary arms races between plants and their parasites can result in an overdispersion of chemical defense and an increase in phytochemical diversity (Ehrlich and Raven 1964; Becerra 2007, 2015; Kursar et al. 2009). All of these hypotheses posit that the presence of multiple secondary metabolites in plants allows for greater selective advantage in response to pressures from diverse natural enemies, such as herbivores, fungi, and bacteria. Synergistic effects against these enemies are an important potential mechanism related to these hypotheses.

Many secondary metabolites evolved in response to herbivory and pressures from other parasites, and most of them evolved in the presence of existing phytochemical mixtures, yet relatively little is known about the mechanisms by which multiple secondary metabolites affect herbivores and other organisms. This is due to secondary metabolites serving several roles besides only poisoning the target organism (Gershenzon et al. 2012). These compounds may target different metabolic functions of the herbivore enabling persistence and toxicity to the herbivore by other compounds in the mixture. There are several mechanisms that cause a synergistic response, but we will focus on three: (1) inhibition of an enzyme via binding to it, (2) disruption of cell membranes to facilitate movement, and (3) causing changes in physical properties. Berenbaum and Zangerl (1993) found that furanocoumarin mixtures restricted the cytochrome P450 monooxygenase enzyme activity in swallowtail caterpillars (*Papilio polyxenes*, Papilionidae), which is important for detoxification. Xanthotoxin, and angelicin are structural analogues, and when in a mixture can inactivate the cytochrome P450 enzyme by competing for, or irreversibly binding to it (Berenbaum and Zangerl 1993), allowing for other compounds to reach toxic levels. Some toxic compounds cannot cross the cell membrane without facilitation by other molecules. Guillet et al. (1998) found that monoterpenes of *Porophyllum* (Asteraceae) disrupt the cell membranes in *Ostrinia nubilalis* (Pyralidae) caterpillars, facilitating transportation of alpha-terthienyl compounds across the membrane that then achieve concentrated levels of toxicity. Finally, synergy can cause changes in physical plant defenses. For example, mixtures of monoterpenes and diterpenes found in conifer resin decrease the viscosity, causing the resin to flow rapidly to the site of bark beetle attack (Phillips and Croteau 1999). Another important consideration when studying the effects of synergy is the degree of specialization of the target species. For example, the synergistic effects of iridoid glycosides on specialist caterpillars work through disruption of the melanization response that is part of the caterpillar's immune function (Richards et al. 2012), while their synergistic effects on generalist herbivores are a result of a number of different toxicity effects, only some of which have been elucidated (Smilanich et al. 2011). Similarly, synergistic effects, such as those recorded for the Berberine alkaloids, in which 5′-methoxyhydnocarpin deactivates multidrug pumps, may work against one type of organism (in this case, bacteria) but the mechanism is likely to be entirely different for other organisms (Stermitz et al. 2000). These studies suggest that biological activity is multi-faceted and is more complicated than focusing on which secondary metabolites are toxic. Secondary metabolites have a variety of effects on living tissues, and we may fail to understand their role if we are only testing individual compounds for one specific response (e.g., toxicity) (Smilanich et al. 2016).

This review synthesizes the small number of studies that have tested hypotheses about the effects of synergy on insect herbivores in natural systems. The most successful synergy studies have merged methods in organic synthesis, natural products isolation, and biology in order to understand the role that synergistic activities play in plant defense. Most published studies on this topic have focused on *intra*class synergy, but *inter*class synergy (between different classes of compounds) can be equally important. A few studies have investigated the biological mode of action of interclass mixtures on herbivores and pathogens by evaluating the effect of these compounds both as mixtures and as isolated components on a variety of biologically significant response variables (e.g., immunity, survival, fecundity, growth). In this review, we examine the hypothesis that phytochemical synergy is ubiquitous in natural systems with a brief review of the literature and a meta-analysis examining the effects of single compounds versus a mixture of compounds on herbivore performance. We also provide guidelines for future work that can uncover mechanisms by which synergy functions and can guide the development of general tools for quantitative evaluation of synergistic activity between biologically active molecules.

Some empirical examples

How did secondary metabolites evolve and why are they found in multiple unrelated plant families? These questions have driven many investigations of model plant metabolites, such as the alkaloids (Macel et al. 2005; Stermitz 2000), saponins (Dyer et al. 2013), furanocumarins (Berenbaum et al. 1991), iridoid glycosides (Bowers 1991), *Piper* imides (Dyer et al. 2004b; Wilson et al. 2012), and chromenes (Kato and Furlan 2007; Ramos et al. 2009; Batista et al. 2011). Synergies between physical, biotic, and chemical defenses have rarely been examined formally, but there is no reason to assume that they are not common. For example, synergies not only affect herbivores (Gunasena et al. 1988; Castellanos and Espinosa-Garcia 1997; Guillet et al. 1998; Hummelbrunner and Isman 2001; Calcagno et al. 2002; Akhtar and Isman 2003; Steppuhn and Baldwin 2007), but they also affect pollinators (Biller et al. 2015) and pathogens (Kang et al. 1992; Fewell and Roddick 1993; Kubo and Muroi 1993; Segura et al. 1999), and also contribute to allelopathy (Voukou et al. 2003). It is also worth noting that chemical synergy can occur with mixtures of secondary and primary metabolites, such as proteins (Segura et al. 1999; Amirhusin et al. 2007; Steppuhn and Baldwin 2007). Some coevolutionary or chemical ecology studies that have failed to find negative effects of defensive compounds on host performance are likely a consequence of the inefficacy of individual, isolated compounds (Dyer 2011). Below we discuss what is known about exemplary compound classes relevant to characterization and bioassays.

Furanocoumarins

Furanocoumarins represent a classic example of synergistic plant defenses. Two types of furanocoumarins, linear and angular, are produced by different biosynthetic pathways. Linear furanocoumarins are more common than angular furanocoumarins and have been found in 15 plant families, including agriculturally important families such as Apiaceae and Leguminosae (summarized by Diawara and Trumble 1997). In comparison, angular furanocoumarins are found primarily in two plant families, Leguminosae and Umbelliferae. Angular furanocoumarins are more derived and biosynthetically advanced than linear furanocoumarins, but in isolation appear to be less effective against herbivores due to the phototoxicity of linear, but not angular, furanocoumarins. Ultraviolet light activates linear furanocoumarins such that they cross-link with DNA and

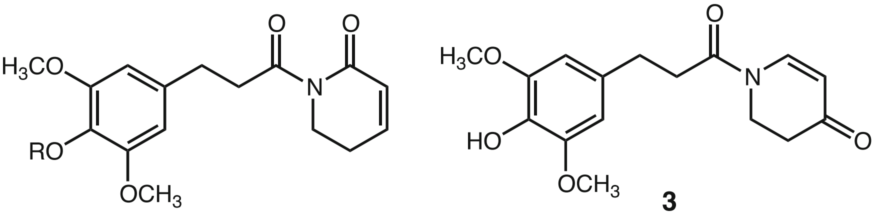
RNA, damage lipids, and degrade protein constituents. Although angular furanocoumarins are not phototoxic and appear less effective in isolation, angelicin (an angular furanocoumarin) synergistically reacts with linear furanocoumarins (xanthotoxin and bergapten) to interfere with herbivores' cytochrome P450 mediated metabolism of both compounds, having a greater effect on larval growth than each compound alone (Berenbaum and Neal 1985; Berenbaum and Zangerl 1993). Additionally, furanocoumarin mixtures (including angelicin) were more toxic to larvae (*Heliothis zea*, Noctuidae) both in the absence and presence of UV light compared to equivalent amounts of the one most photoactive furanocoumrin (xanthotoxin, Berenbaum et al. 1991).

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are a class of secondary compounds produced by several plant families including Boraginaceae, Asteraceae, and Orchidaceae (Dewick 2009) and have demonstrated varying levels of toxicity to insect herbivores (Bentley et al. 1984; Hartmann 1999). This class of secondary compounds has a bicyclic skeleton that is derived from arginine, and toxicity of PAs is possibly related to the 1,2-unsaturation in the pyrrolizidine ring as well as an ester functional group on the side chain (Dewick 2009). Isolated PAs from *Senecio* species (Asteraceae) have little to no effect on the generalist insect herbivores *Spodoptera exigua* (Noctuidae) and *Mamestra brassicae* (Noctuidae), but mixtures of PAs may be toxic to *S. exigua* caterpillars (Macel et al. 2005). Similarly, mixtures of all PAs from *Jacobaea vulgaris* (Asteraceae) are more toxic to cell lines of *S. exigua* than any individual PA compounds (e.g. jacobine, erucifoline, senkirkine, seneciphylline, senecionine, and retrorsine) (Nuringtyas et al. 2014).

Piper amides/imides

A great diversity of secondary metabolites have been isolated from the Piperaceae (Gutierrez et al. 2016; Yamaguchi et al. 2011; Kato and Furlan 2007), especially the genus *Piper* (reviewed by Dyer et al. 2004a; Fincher et al. 2008). This includes the "*Piper* amides," which contain a phenyl moiety that has variable lengths of the carbon side chain, usually one or higher degree of unsaturation, and a terminal carbonyl carbon. The nitrogen on the amide is derived from pyrrole, piperdine, or an isobutyl group and may include an epoxide, a carbonyl group, or a degree of unsaturation (Dyer et al. 2003a). One well studied example is *Piper cenocladum*, which contains two imides and an amide at high concentrations (total amide content can be as high as 3.8 % dry weight): 4′-desmethylpiplartine (**1**), cenocladamide (**2**), and piplartine (**3**) (Dodson et al. 2000).



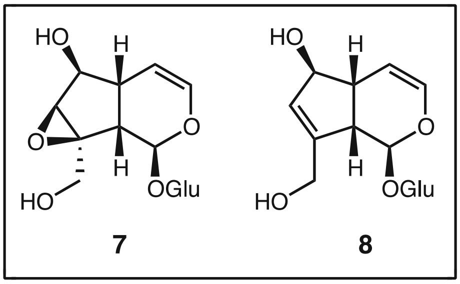
3

1: R = H
2: R = CH_3

Piper amides are strongly insecticidal (reviewed by Dyer et al. 2003a), antifungal (Marques et al. 2010), they are deterrent to leaf-cutting ants (Capron and Wiemer 1996; Dyer et al. 2003b), have anti-cancer properties (Raj et al. 2011), and are cytotoxic to mosquito larvae (Maleck et al. 2014). The *P. cenocladum* compounds (**1**–**3**) are found in higher concentrations in the leaves of plants that are not occupied by mutualistic ants (Dodson et al. 2000; Dyer et al. 2001), and various experiments have demonstrated that compounds **1**–**3** are deterrent to arthropods (Dyer and Letourneau 1999; Dyer et al. 2003b, 2004b). Insect antifeeding synergy for the amides/imides of *P. cenocladum* and the related species, *P. tuberculatum* and *P. imperial*, are now well established (Scott et al. 2002; Dyer et al. 2003a; Richards et al. 2010). We found that synergistic effects differed depending on the herbivores' degree of specialization. For generalist herbivores (*Spodoptera frugiperda*), amide mixtures synergistically caused direct toxicity (Dyer et al. 2003a; Richards et al. 2010). However, for specialist herbivores (*Eois* spp), we found that synergistic effects led to higher parasitism rates (Richards et al. 2012) and reduced pupal mass and growth rates (Dyer et al. 2003a; Richards et al. 2012).

Iridoid glycosides

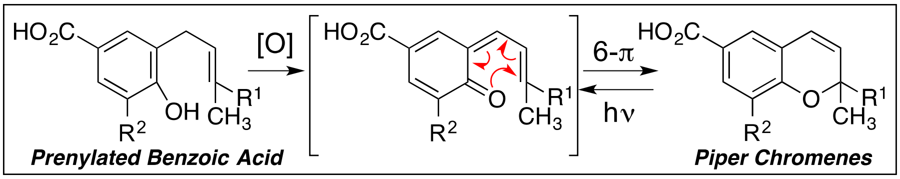
Another major class of compounds that are well studied, and thus ideal for studying synergy are the iridoid glycosides. Possible synergists include the well-studied compounds catalpol (**7**) and aucubin (**8**), which are found together in *Plantago lanceolata* (Plantaginaceae) in relatively high concentrations of 5–10 % by dry weight. Iridoid glycosides, cyclopentanoid monoterpene-derived compounds found in over 50 plant families, have been used as model systems to examine the chemical ecology of plant–insect interactions (Bobbitt and Segebarth 1969; Jensen et al. 1975; Boros and Stermitz 1990; Dyer and Bowers 1996; Bowers and Stamp 1997; Bowers 2003). Smilanich et al. (2009) found that the immune response of *Junonia coenia* (Nymphalidae), an iridoid glycoside specialist, was lower when feeding on *Plantago lanceolata* which contains a mixture of both aucubin and catapol, compared to those feeding on *Plantago major* which contains only aucubin. Further investigations, revealed a possible trade-off between the immune response and growth and sequestration. Richards et al. (2012) found that specialist caterpillars grew faster, had lower mortality, sequestered higher concentrations of iridoid glycosides, and had reduced immune response on artificial diets containing mixtures as compared to those containing individual compounds. In this case, larval performance as measured by survival and development time had a hermetic, dose-dependent response (Raubenheimer and Simpson 2009), in that larvae performed better at intermediate concentrations compared to low and high concentrations (Richards et al. 2012).



7 **8**

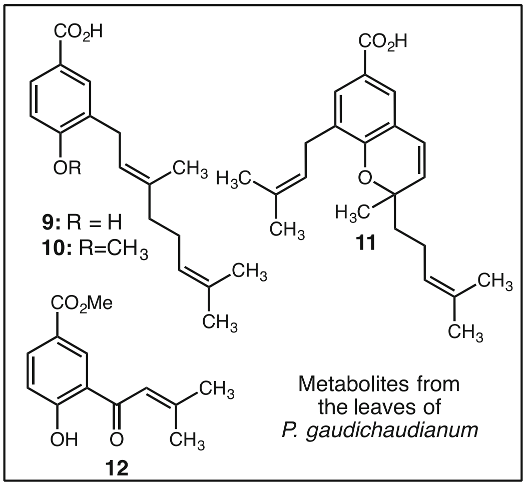
Additional potential systems for characterizing synergy

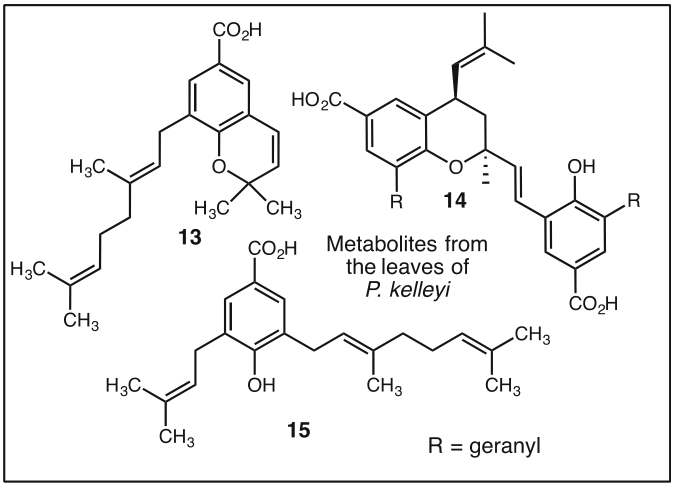
What other systems are we likely to find synergistic effects of plant defenses? If phytochemical mixtures are a prerequisite for synergies to occur, then we may expect to find synergistic effects in plants with high phytochemical diversity. A plant's diverse phytochemical profile arises from various modifications of a few biosynthetic pathways in secondary metabolism. The main biosynthetic mechanisms involved in producing phytochemical diversity are summarized in Gershenzon et al. (2012) and include: (1) the repeated addition of subunits; for example the addition of isoprenoids to make a diverse array of terpenes and acetate in polyketide synthesis, (2) forming diverse carbon skeletons prior to the addition of subunits, (3) producing a single intermediate that can interact with multiple enzymes, and (4) producing enzymes with low specificity, that can interact with multiple intermediates. All plant genera use one or multiple mechanisms to produce phytochemically diverse mixtures, but the mechanisms for phytochemical diversification seem to be largely conserved within a genus. For example, the phytochemistry of *Asclepias* is characterized by later stage functionalization of a few triterpenes with an array of unique carbohydrates (Agrawal et al. 2009), whereas *Piper* generates many different carbon skeletons via early branching of biosynthetic pathways. Both pathways yield a diverse mixture of natural products by using different biosynthetic strategies. Below we outline examples of compound classes that should be explored for synergies due to their high diversity and distribution.



Chromenes, Prenylated Benzoic Acids, and Chalcones

Chromene metabolites are a broadly represented class of natural products found in at least 13 different families of plants and have demonstrated anti-microbial, anti-cancer, and insecticidal activities. Their biological activity has prompted the development of chemical libraries based on natural chromene scaffolds that lead to compound identification (Nicolaou et al. 2000). Six structurally related chromenes within the genus *Piper*, have been isolated. All contain carboxylic acid groups at various states of prenylation and oxygenation about the chromane skeleton. Additionally, *Piper* chromenes occur in multiple plant parts in mixtures with other prenylated benzoic acids, dimeric derivatives, or flavones (Jeffrey et al. 2014; Batista et al. 2009, 2011). These chromenes may arise biosynthetically by oxidative cyclization of an *ortho*-quinone methide (Beaudry et al. 2005; Morimoto et al. 1998) derived from prenylated benzoic acid derivatives that arise through the shikimic acid pathway. This *ortho*-quinone methide could also arise from irradiation of a chromene, producing dimeric compounds and could account for the photoxicity of these classes of compounds.





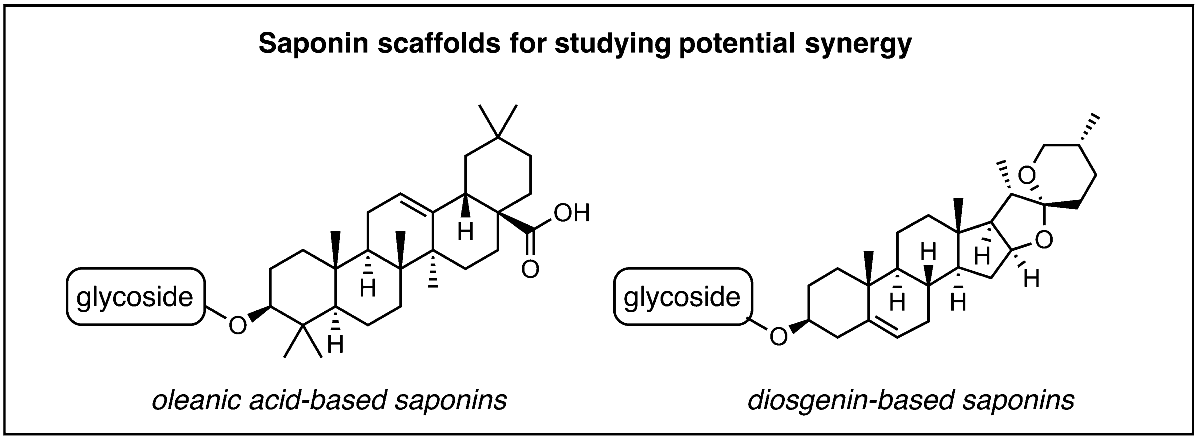
Despite the broad-range biological activity of these classes of metabolites their co-occurrence in natural sources, biosynthetic relationships, and potential synergistic biological activity has not been studied. With the approaches outlined below, one could test for interclass synergy in two natural mixtures of chromenes, prenylated benzoic acids, and dimeric chromanes or flavones that occur in *Piper kelleyi* and *Piper gaudichaudianum*. Previous studies have identified an anti-fungal chromene as the major component in the leaves of *Piper gaudichaudianum* along with the related prenylated benzoic acid derivatives (Lago et al. 2004).

Saponins. Saponins are found in many plant families; they are produced via the mevalonic acid pathway and are glycosylated triterpenoid, steroid, or steroidal alkaloid compounds (Papadopoulou et al. 1999; Massad et al. 2012). Both applied and basic research provide impetus to study the chemistry and ecology of these compounds. Saponins have antiherbivore, antifungal and allelopathic properties (Oleszek and Junkuszew 1999). They also deter leaf-cutter ants (*Atta* spp. and *Acromyrmex* spp.; Febvay and Kermarrec 1986; Folgarait et al. 1996; Pearson et al. 2008; Massad et al. 2012), which may be an important attribute for tree species used in tropical reforestation where leaf-cutter ants are considered serious pests (Vasconcelos and Cherrett 1997; Massad et al. 2012). The amphiphilic properties of saponins allow for membrane disruption (see review by Podolak et al. 2010). In insects, saponins are known to form complexes with membrane sterols, limiting the available sterols for hormone production causing growth inhibition and mortality (Ishaaya 1986).

Intraclass or interclass synergy has never been formally studied for saponins. Most research on antiherbivore activity has focused on a wide array of saponins found in alfalfa (*Medicago sativa*, Fabaceae) and in the tropical tree, *Pentaclethra macraloba* (Fabaceae). The diverse structures of saponins and their ability to interact with cell membranes suggest a potential for synergistic interactions. Naturally-occurring saponins that have potential for strong synergies based on their presence in complex mixtures, include oleanolic acid-based saponins from *P. macroloba* (Viana et al. 2004) and a series of diosgenin-based saponins found in alfalfa (Oleszek 1998).

Synergy meta-analysis

To quantitatively summarize existing synergy research, we utilized meta-analysis, which is a quantitative synthesis of the results of many independent studies (Hedges et al. 1999; Gurevitch and Hedges 1999; Koricheva and Gurevitch 2014). We used the log response ratio for hypothesis tests; this effect size is calculated as the ratio of the mean outcome of the experimental group (X_E) to that of the control group (X_c) (log response ratio = ln (X_E/X_C)) (Hedges et al. 1999). We selected studies in which the herbivore's diet had been manipulated and response variables were reported for single compound diets (X_{single}) and phytochemical mixtures ($X_{mixture}$), which included combined individual compounds or crude extracts. The response variables most relevant to synergistic effects on herbivores included herbivore development time, feeding data, growth rates, survivorship, sequestration, immune response and fecundity. From each article, we recorded the mean (x), sample size (n) and standard deviation (sd) of the single compound treatments and mixture compound treatments for the given performance variable being measured in the paper. The log response ratio ($L = \ln (X_{single}/X_{mixture})$) was calculated and weighted based on the variance in the study ($v = (SD_{single})^2/(n_{single}X_{single})) + ((SD_{mixture})^2/(n_{mixture}X_{mixture}))$, Hedges et al. 1999). All analyses were performed with SAS statistical software (9.4, SAS Institute).



Saponin scaffolds for studying potential synergy

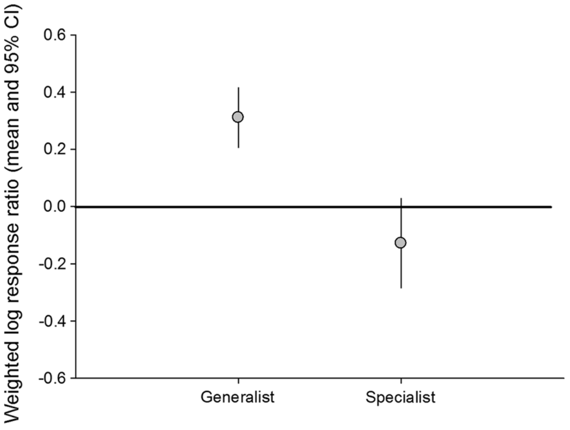


Fig. 1 The results from a meta-analysis investigating the response ratio of herbivore diet breadth and bioassays on single compound diets and multiple compound diets. The mean weighted log response ratio and 95 % confidence intervals are shown for generalist and specialist herbivores separately. The reference line at 0.0 indicate that herbivores performed the same on single compound and mixed diets. *Positive values* indicate that herbivores performed better on single compound diet than mixed diets and *negative values* indicate herbivore performed better on mixed diets than on single diets. The analysis included in the following studies: Braasch et al. 2012; Cheng et al. 2013; Frisch et al. 2014; Hegde et al. 2011; Honda et al. 2004; Kleine and Müller 2014; Knerl and Bowers 2013; Kusumoto et al. 2010; Laurentz et al. 2012; Macel et al. 2005; Nuringtyas et al. 2014; Richards et al. 2010, 2012; Scott Brown et al. 2011; Weinhold et al. 2011; Zhang et al. 2013

We searched for articles using the search engine ISI Web of Science. Keywords for the online search included: chem* + defense, synergy*, secondary + chem*, herbiv*, plant + chem*, secondary + chem* and bioassay + herbivore. We followed methods in Smilanich et al. (2016) for inclusion of articles in the database: Only terrestrial studies that included measures of dispersion, variance, and sample size were included, and only papers that identified the class and quantification of specific compounds were used. We did not include studies that used plant hormones, since we were only interested in secondary metabolites. Electroantennogram data were not included as a response variable. No more than three effect sizes were utilized per paper and only one effect size per experiment was used. Where more than one effect size was reported per experiment, the treatment and control means recorded were chosen randomly from among the available data, using a random number generator. To obtain numerical means and standard deviations from graphs, we used GraphClick (3.0.3, 2012) to digitize the graphs.

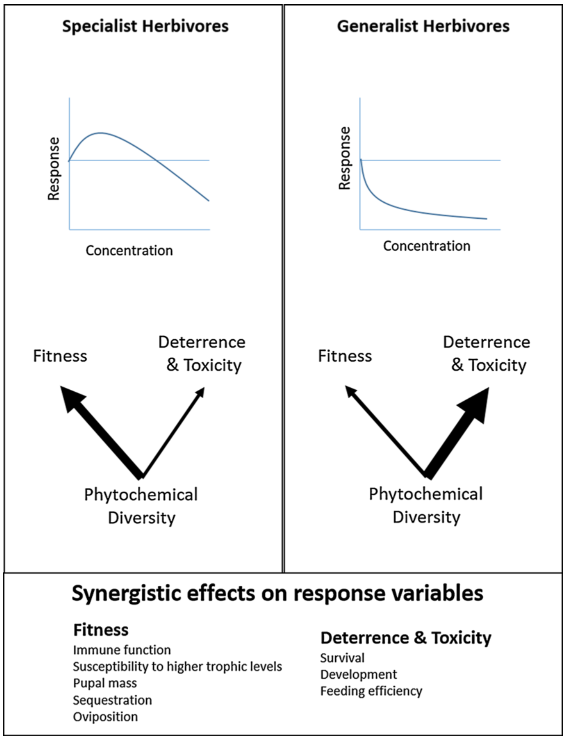


Fig. 2 A conceptual model of the predicted relationship between phytochemical diversity and the synergistic effects on specialist (*left*) versus generalist (*right*) herbivores. The *thickness of the arrows* indicate the relative effect strengths. The response variables listed include some of the best investigated mechanisms by which mixtures of phytochemicals can negatively affect herbivore physiology, ecology, and population dynamics. There are very few studies of more reductionist mechanisms (i.e. at the cellular level), and this is likely to be a very fruitful area of investigation. The *inset graphs* are a general prediction of the dose-dependent response (controls are represented by a *horizontal line*) *curves* for synergies on specialist and generalist herbivores. Since specialists are adapted to chemical defense in host plants, initial increases in mixture concentrations should have positive effects on specialist herbivores until some threshold of toxicity, impaired function, or deterrence is reached. For generalists, responses should exhibit logistic decline, since generalists are not adapted to plant chemistry and small increases in defense are likely to greatly increase negative effects up until a threshold

Our search yielded 16 suitable papers and 25 effect sizes spanning a 10-year time period (2004–2014) out of a total of 1784 papers, although only five studies tested specifically for synergy (Richards et al. 2010; Honda et al. 2004; Richards et al. 2012; Laurentz et al. 2012; Nuringtyas et al. 2014). Due to the limited number of studies, we were limited in the number of appropriate comparisons that could be made. Therefore, we focused on comparing the effect sizes of specialist and generalist herbivores. Generalists were defined as feeding on several to many unrelated plant species that differ in chemistry. In comparison, specialists were defined as feeding on a few related plant species or on species with specific compounds present. We calculated the mean and 95 % confidence intervals of the weighted log response ratio (as described in Hedges et al. 1999) for specialist herbivores and generalist herbivores.

We found that phytochemical mixtures affected specialist and generalists differently (Fig. 1). Generalist herbivores performed worse on mixed compound diets compared to single diets. The opposite was found in specialists, where they performed better on mixtures than single diets. This difference can be explained by specialist's adaptations to particular chemistry and the differences in the response variables affected. Specialists have evolved with changes in host plant chemistry and are able to circumvent the negative effects of mixtures, including using them for their own defense. For the data included in the analysis, sequestration was the response variable for five of the specialist herbivores and only one generalist. This difference in the type of response variables affected depending on diet breadth not only helps explain the pattern we found, but also gives insight as to how plant chemistry and synergies affect specialists and generalists differently. Most of the generalist responses were related to deterrence and toxicity, including decreased growth and consumption and increased mortality. In comparison, most of the specialist responses were related to fitness, including defensive sequestration, oviposition, and pupal or larval mass.

In another recent meta-analysis investigating the effects of secondary metabolites on herbivores, the results showed that generalists and specialists are affected similarly by plant compounds. However, less than 2 % of the studies included in the dataset explicitly tested for synergy (Smilanich et al. 2016). The lack of differences between generalists and specialists in other studies or syntheses may be due to the fact that different response variables are analyzed together, or it could be a reflection of the fact that synergy was not investigated. Results of a meta-analysis by Hawkins and Cornell (2003) of single compound bioassays indicated that plant defenses had a greater effect on generalist mortality and development than on the same responses for specialists. Both of these previous meta-analyses do not account for potential synergies.

The scarcity of studies found in this meta-analysis is due to the lack of research on this topic, the lack of collaboration between synthetic organic chemists and ecologists, and weak statistical methods (Nelson and Kursar 1999; Tallarida 2000; Jones et al. 2005). Nonetheless, as studies of mixtures increase, widespread synergy may explain why phytochemical mixtures are the rule and not the exception and is likely responsible for apparent redundancies in chemical defense (Romeo et al. 1996) as well as the poor efficacy of individual compounds as defense. Thus, tests of antiherbivore (or pharmaceutical) activity of individual compounds that are isolated or synthesized should be accompanied by tests of mixtures and crude extracts using methods developed for examining synergy (e.g., Jones 1998; Nelson and Kursar 1999; Richards et al. 2010, 2012).

Future research directions: complete approaches to studying synergy

Based on the synergy literature reviewed here, we propose a conceptual model of the interactions between phytochemical diversity and synergistic effects on specialist and generalist herbivores (Fig. 2). We predict that as phytochemical diversity increases, so does the likelihood of synergistic effects on both specialist and generalists. However, the effect on herbivore attributes will vary depending on diet breadth of the herbivore. For generalists, modes of action relying on deterrence and toxicity are predicted to cause strong negative dose-dependent effects. For specialists, synergistic mixtures are more likely to affect responses related to fitness, such as fecundity and defense from natural enemies, rather than direct toxicity. At low to intermediate concentrations, some of these synergistic effects could enhance specialist performance, suggesting a potential therapeutic window in which performance is optimal until a tipping point is reached at higher concentrations (Forbey et al. 2013).

Examining biological mechanisms for synergy is an excellent goal for any mature research program focused on effects of secondary metabolites on animals, and we have cited some of the studies that have taken this approach (Stermitz et al. 2000; Smilanich et al. 2009; Boyd 2012). Eventually, one major goal of antiherbivore synergy research will be to examine synergistic effects at the cellular level and to elucidate the molecular basis of full mechanistic pathways to synergy. In some model systems this is possible through a combination of mutant screening, quantitative genetics, and bioassays (Steppuhn and Baldwin 2007; Rasmann and Agrawal 2009). However, this goal is premature for most natural systems, since there is a dearth of antiherbivore synergy research. Each collection of synergistic compounds has a unique mode of action and to investigate all potential mechanisms requires very different research approaches—adding the molecular basis of these mechanisms should be preceded by a full investigation of organismal, physiological, and cellular mechanisms. Therefore, in our recommendations for future research, we focus more on testing hypotheses about the existence of antiherbivore synergy for a diversity of compounds across different plant families rather than focusing exclusively on mechanisms of antiherbivory.

The approach we advocate for studying synergy includes: (1) isolation, synthesis, and identification of synergistic plant defenses and development of innovative methods for examining biological mechanisms of synergy; (2) examining synergistic effects across herbivores that vary in diet breadth; (3) demonstrating quantitative support for intraclass and interclass synergy for selected compounds in a variety of classes. Here we outline some potential methods for this approach.

Bioassays are important in assessing the potential role of synergistic interactions between compounds. By comparing the dose–effect curves of generalist or specialist herbivore choice or performance bioassays on single compounds and combinations of compounds one can statistically quantify synergistic effects. This requires assays at multiple compound concentrations that range above and below naturally occurring concentrations and proportions. Regardless of the exact mode of action, the relative ratio of the compound mixtures are important (Lindroth and Hwang 1996). Biological systems tend to operate in substrates and receptors, such as enzymes or membrane bound proteins. Therefore the relative ratios of the compounds in a mixture can determine whether they will act synergistically, additive or antagonistically. Compounds for these bioassays should be produced synthetically or isolated from mass collected plant tissues.

In addition to the standard bioassay response variables, including survival, growth, development and feeding efficiencies, the immune response and stress response are key measures that link plant chemistry to herbivore resistance to natural enemies. One method for measuring the immune response includes quantifying encapsulation and melanization of Sephadex beads (dyed congo red) that are injected into the hemocoel of the insects being investigated. Sephadex beads act as a proxy for parasitism (Lavine and Beckage 1996) and this technique has been used widely and shown to accurately quantify immune capacity in insects (Rantala and Roff 2007). Additionally, the phenoloxidase (PO) activity can also be measured. Phenoloxidase is an enzyme that catalyses the melanization cascade and is constitutively present in the insect (Beckage 2008). Phenoloxidase activity is measured by collecting hemolymph samples and quantifying activity using a UV spectrophotometer (Bailey and Zuk 2008). Recent studies have demonstrated that the release of the stress hormone octopamine can depress the immune response (Adamo and Parsons 2006). To measure the stress response, the concentration of octopamine can be quantified from the same hemolymph samples taken for PO and best quantified using high pressure liquid chromatography with electrochemical detection. By further exploring this mechanism of antiherbivore synergy, chemical ecologists can set the stage for analysis of the roles of each compound and synergistic function at the molecular level. Quantitative tests of experiments similar to those described above can be analyzed to distinguish between independent joint action, additive, antagonistic, and synergistic effects on the test organisms using response surface analysis of drug combinations (Tallarida 2000), isobolar analysis (Nelson and Kursar 1999; Tallarida 2000), and appropriate modifications based on the type of data generated (e.g., Richards et al. 2010; Jones et al. 2005). First, a dose-dependent effect is determined for a single compound and mixtures of compounds from the bioassay. Linear or nonlinear regression can be used to examine the effects of many levels of compound concentrations (at a minimum, a control plus 5 concentrations of individual compounds) on the response variables in the bioassays (e.g., pupal weights, development times, melanization, PO activity, survivorship, and other bioassay values). These regressions generate parameter estimates (e.g., slopes and 50 % effective dose—ED50) to calculate the interaction index, α as follows:

$$\alpha = \frac{Z}{\sum_{i=1}^{i} (f_i A_i)}$$

Z denotes the concentration of compounds in a mixture required to elicit a threshold response, such as an ED50; *i* represents the number of compounds in the tested mixture; *fi* represents the fraction of compound *Ai* in mixture Z (for example, in a four compound mixture of equal parts $f_i = 0.25$); *Ai* is the concentration of compound *Ai* necessary to achieve a certain level of response when tested individually; Values of α significantly less than 1 demonstrate synergy. Bootstrap methods should be used to calculate 95 % confidence intervals to determine if α values are significantly different from 1. Finally, the simplest approach involves utilizing binary mixtures, and the statistical methods outlined by Jones et al. (2005) for tests of synergy, using ED50 values along with generalized linear models to test for significance of interaction terms (a significant term indicates synergy). For binary mixtures isobolar analysis (Nelson and Kursar 1999; Tallarida 2000) is also effective.

Conclusion

Future synergy research will provide tests of important hypotheses on how plant secondary metabolites act in mixtures to deter herbivores and will contribute to a long-term goal of determining if antiherbivore synergy is widespread among plant families. If ecologically relevant antiherbivore synergy is found across the phylogeny of vascular plants and among all classes of secondary metabolites, such findings will provide strong evidence for the hypothesis that plants evolved the capacity to make multiple compounds as synergists for defense against diverse communities of herbivores and pathogens. Widespread synergy would also help dispel the unlikely scenario that a plant could evolve a single compound with absolute potency for its defense. Future synergy research should also be directly relevant to management of insect pests in agriculture, and to the development of pharmaceutically interesting compounds. Plants have successfully defended against insects and pathogens for well over 250 million years using mixtures of biodegradable, non-persistent organic compounds. In contrast, humans have been unsuccessful in controlling crop pests over the long run using single active ingredient formulations even at high concentration and with acute toxicity. A better understanding of synergy between biologically active molecules will contribute to more effective pest management. A similar integrated synergistic chemical approach might guide the development of novel medicines for the treatment of human disease.

References

* denotes papers used in meta-analysis)

Adamo SA, Parsons NM (2006) The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. Anim Behav 72:235–244

Agrawal AA, Salminen J, Fishbein M (2009) Phylogenetic trends in phenolic metabolism of milkweeds (*Asclepia*): evidence for escalation. Evolution 63:663–673

Akhtar Y, Isman MB (2003) Binary mixtures of feeding deterrents mitigate the decrease in feeding deterrent response to antifeedants following prolonged exposure in the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). Chemoecology 13:177–182

Amirhusin B, Shade RE, Koiwa H et al (2007) Protease inhibitors from several classes work synergistically against *Callosobruchus maculatus*. J Insect Physiol 53:734–740

Ayres MP, Clausen TP, MacLean J et al (1997) Diversity of structure and antiherbivore activity in condensed tannins. Ecology 78:1696–1712

Bailey NW, Zuk M (2008) Changes in immune effort of male field crickets infested with mobile parasitoid larvae. J Insect Physiol 54:96–104

Batista JM, Batista ANL, Rinaldo D et al (2009) Resolution and absolute configuration assignment of a natural racemic chromane from *Peperomia obtusifolia* (Piperaceae). Chirality 21:799–801

Batista JM, Batista ANL, Rinaldo D et al (2011) Absolute configuration and selective trypanocidal activity of gaudichaudianic acid enantiomers. J Nat Prod 74:1154–1160

Beaudry CM, Malerich JP, Trauner D (2005) Biosynthetic and biomimetic electrocyclizations. Chem Rev 105:4757–4778

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

Becerra JX (2015) On the factors that promote the diversity of herbivorous insects and plants in tropical forests. Proc Natl Acad Sci USA 112(19):6098–6103

Beckage NE (2008) Insect immunology. Academic Press, Oxford

Bentley MD, López SN, Mota JS et al (1984) Pyrrolizidine alkaloids as larval feeding deterrents for spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Ann Entomol Soc Am 77:393–397

Berenbaum M, Neal JJ (1985) Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. J Chem Ecol 11:1349–1358

Berenbaum MR, Zangerl AR (1993) Furanocoumarin metabolism in *Papilio polyxenes* - Biochemistry, genetic variability, and ecological significance. Oecologia 95:370–375

Berenbaum MR, Zangerl AR (1996) Phytochemical diversity: adaptation or random variation? In: Romeo JT, Saunders JA, Barbosa P (eds) Phytochemical diversity and redundancy in ecological interactions. Plenum Press, New York

Berenbaum MR, Nitao JK, Zangerl AR (1991) Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apicaceae). J Chem Ecol 17:207–215

Biller OM, Adler LS, Irwin RE et al (2015) Possible synergistic effects of thymol and nicotine against *Crithidia bombi* parasitism in bumble bees. PLoS One 10(12):e0144668

Bloch E (2001) Hemlock poisoning and the death of socrates: did plato tell the truth? J Int Plato Soc 1

Bowers MD (1991) The iridoid glycosides. In: Rosenthal G, Berenbaum M (eds) Herbivores: their interaction with plant secondary metabolites, 2nd edn. Academic Press, New York

Bowers MD (2003) Hostplant suitability and defensive chemistry of the Catalpa sphinx, *Ceratomia catalpae*. J Chem Ecol 29:2359–2367

Bowers MD, Stamp NE (1997) Effect of hostplant genotype and predators on iridoid glycoside content of pupae of a specialist insect herbivore, *Junonia coenia* (Nymphalidae). Biochem Syst Ecol 25:571–580

Boyd RS (2012) Plant defense using toxic inorganic ions: conceptual models of the defensive enhancement and joint effects hypotheses. Plant Sci 195:88–95

*Braasch J, Wimp GM, Kaplan I (2012) Testing for phytochemical synergism: arthropod community responses to induced plant volatile blends across crops. J Chem Ecol 38:1264–1275

Calcagno MP, Coll J, Lloria J et al (2002) Evaluation of synergism in the feeding deterrence of some furanocoumarins on *Spodoptera littoralis*. J Chem Ecol 28:175–191

Capron MA, Wiemer DF (1996) Piplaroxide an ant-repellent piperidine epoxide from *Piper tuburculatum*. J Nat Prod 59:794–795

Castellanos I, Espinosa-Garcia FJ (1997) Plant secondary metabolite diversity as a resistance trait against insects: a test with *Sitophilus granarius* (Coleoptera: Curculionidae) and seed secondary metabolites. Biochem Syst Ecol 25:591–602

Challis GL, Hopwood DA (2003) Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by Streptomyces species. Proc Natl Acad Sci USA 100:14555–14561

*Cheng D, van der Meijden E, Mulder PPJ et al (2013) Pyrrolizidine alkaloid composition influences cinnabar moth oviposition preferences in *Jacobaea* hybrids. J Chem Ecol 39:430–437

Dewick PM (2009) Alkaloids. Medicinal natural products: a biosynthetic approach, 3rd edn. Wiley, West Sussex

Diawara MM, Trumble JT (1997) Linear Furanocoumarins. In: Felix D'Mello JP (ed) Handbook of plant and fungal toxicants. CRC Press, New York

Dodson CD, Dyer LA, Searcy J et al (2000) Cenocladamide, a dihydropyridone alkaloid from *Piper cenocladum*. Phytochemistry 53:51–54

Dyer LA, Bowers MD (1996) The importance of sequestered iridoid glycosides as a defense against an ant predator. J Chem Ecol 22:1527–1539

Dyer LA, Letourneau DK (1999) Relative strengths of top-down and bottom-up forces in a tropical forest community. Oecologia 119:265–274

Dyer LA, Dodson CD, Beihoffer J, Letourneau DK (2001) Trade-offs in anti-herbivore defenses in *Piper cenocladum*: ant mutualists versus plant secondary metabolites. J Chem Ecol 27:581–592

Dyer LA, Dodson CD, Stireman JO et al (2003a) Synergistic effects of three *Piper* amides on generalist and specialist herbivores. J Chem Ecol 29:2499–2514

Dyer LA, Dodson CD, Gentry G (2003b) A bioassay for insect deterrent compounds found in plant and animal tissues. Phytochem Anal 14:381–388

Dyer LA, Dodson CD, Richards J (2004a) Isolation, synthesis, and evolutionary ecology of *Piper* amides. In: Dyer LA, Palmer AN (eds) *Piper*. A model genus for studies of evolution, chemical ecology, and trophic interactions. Kluwer, Boston

Dyer LA, Dodson CD, Letourneau DK et al (2004b) Ecological causes and consequences of variation in defensive chemistry of a neotropical shrub. Ecology 85:2795–2803

Ehrlich PR, Raven PH (1964) Butterflies and plants: a study in coevolution. Evolution 18:568–608

Febvay G, Rahbe Y, Kermarrec A (1986) An electronic analysis and recording-system for the foraging behavior of leaf-cutting ants and its application to choice tests. Agronomie 6:743–749

Fewell AM, Roddick JG (1993) Interactive antifungal activity of the glycoalkaloids alpha-solanine and alpha-chaconine. Phytochemistry 33:323–328

Fincher RM, Dyer LA, Dodson CD et al (2008) Inter- and Intraspecific comparisons of antiherbivore defenses in three species of rainforest understory shrubs. J Chem Ecol 34:558–574

Firn RD, Jones CG (2003) Natural products—a simple model to explain chemical diversity. Nat Prod Rep 20:382–391

Folgarait P, Dyer L, Marquis R, Braker H (1996) Leaf-cutting ant preferences for five native tropical plantation tree species growing under different light conditions. Entomol Exp Appl 80:521–530

Forbey JS, Dearing MD, Gross EM et al (2013) A pharm-ecological perspective of terrestrial and aquatic plant–herbivore interactions. J Chem Ecol 39:465–480

*Frisch T, Agerbirk N, Davis S et al (2014) Glucosinolate-related glucosides in *Alliaria petiolata*: sources of variation in the plant and different metabolism in an adapted specialist herbivore, *Pieris rapae*. J Chem Ecol 40:1063–1079

Gershenzon J, Fontana A, Burow M et al (2012) Mixtures of plant secondary metabolites: metabolic origins and ecological benefits. In: Iason GR, Dicke M, Hartley SE (eds) The ecology of secondary metabolites from genes to global processes. Cambridge University Press, Cambridge

Gertsch J (2011) Botanical drugs, synergy, and network pharmacology: forth and back to intelligent mixtures. Planta Med 77(11):1086–1098

Godesken M, Lundt I, Madsen R, Winchester B (1996) Deoxyiminoalditols from ldonolactones—V. Preparation of the four stereoisomers of 1,5-dideoxy-1,5-iminopentitols.

Evaluation of these iminopentitols and the three 1,5-dideoxy-1,5-iminoheptitols as glycosidase inhibitors. Bioinorgan Med Chem 4:1857–1865

Guillet G, Belanger A, Arnason JT (1998) Volatile monoterpenes in *Porophyllum gracile* and *P. ruderale* (Asteraceae): identification, localization and insecticidal synergism with alpha-terthienyl. Phytochemistry 49:423–429

Gunasena GH, Vinson SB, Williams HJ, Stipanovic RD (1988) Effects of caryophyllene, caryophyllene oxide, and their interaction with gossypol on the growth and development of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). J Econ Entomol 81:93–97

Gurevitch J, Hedges LV (1999) Statistical issues in ecological meta-analyses. Ecology 80:1142–1149

Gutierrez YV, Yamaguchi LF, de Moraes MM et al (2016) Natural products from *Peperomia*: occurrence, biogenesis and bioactivity. Phytochem Rev. doi:10.1007/s11101-016-9461-5

Harborne JB (1988) Introduction to ecological biochemistry. Academic Press, San Diego

Hartmann T (1999) Chemical ecology of pyrrolizidine alkaloids. Planta 207:483–495

Hedges LV, Gurevitch J, Curtis PS (1999) The meta-analysis of response ratios in experimental ecology. Ecology 80:1150–1156

*Hegde M, Oliveira JN, da Costa JG et al (2011) Identification of semiochemicals released by cotton, *Gossypium hirsutum*, upon infestation by the cotton aphid, *Aphis gossypii*. J Chem Ecol 37:741–750

*Honda K, Omura H, Hayashi N et al (2004) Conduritols as oviposition stimulants for the danaid butterfly, *Parantica sita*, identified from a host plant, *Marsdenia tomentosa*. J Chem Ecol 30:2285–2296

Hummelbrunner LA, Isman MB (2001) Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). J Agric Food Chem 49:715–720

Iason GR, O'Reilly-Wapstra JM, Brewer MJ et al (2011) Do multiple herbivores maintain chemical diversity of Scots pine monoterpenes? Philos Trans R Soc B 366:1337–1345

Ishaaya I (1986) Nutritional and allelochemic insect–plant interactions relating to digestion and food intake: some examples. In: Miller TA, JR Miller (eds) Insect–plant interactions. Springer, New York

Jeffrey CS, Leonard MD, Glassmire AE et al (2014) Antiherbivore prenylated benzoic acid derivatives from *Piper kelleyi*. J Nat Prod 77(1):148–153

Jensen SR, Nielsen BJ, Dahlgren R (1975) Iridoid compounds, their occurrence and systematic importance in the angiosperms. Bot Not 128:148–180

Jones DG (1998) Piperonyl butoxide: the insect synergist. Academic Press, London

Jones CG, Firn RD (1991) On the evolution of plant secondary chemical diversity. Philos Trans R Soc B 333:273–280

Jones AC, Blum JE, Pawlik JR (2005) Testing for defensive synergy in Caribbean sponges: bad taste or glass spicules? J Exp Mar Biol Ecol 322:67–81

Kang R, Helms R, Stout MJ, Jaber H, Chen Z, Nakatsu T (1992) Antimicrobial activity of the volatile constituents of *Perilla frutescens* and its synergistic effects with polygodial. J Agric Food Chem 40:2328–2330

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

*Kleine S, Müller C (2014) Drought stress and leaf herbivory affect root terpenoid concentrations and growth of *Tanacetum vulgare*. J Chem Ecol 40:1115–1125

*Knerl A, Bowers MD (2013) Incorporation of an introduced weed into the diet of a native butterfly: consequences for preference, performance and chemical defense. J Chem Ecol 39:1313–1321

Koricheva J, Gurevitch J (2014) Uses and misuses of meta-analysis in plant ecology. J Ecol 102:828–844

Kubo I, Muroi H (1993) Combination effects of antibacterial compounds in green tea flavor against *Streptococcus mutans*. J Agric Food Chem 41:1102–1105

Kursar TA, Dexter KG, Lokvam J 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

*Kusumoto N, Ashitani T, Murayama T et al (2010) Antifungal abietane-type diterpenes from the cones of *Taxodium distichum* rich. J Chem Ecol 36:1381–1386

Lago JHG, Ramos CS, Casanova DCC et al (2004) Benzoic acid derivatives from piper species and their fungitoxic activity against *Cladosporium ladosporioides* and *C. sphaerospermum*. J Nat Prod 67:1783–1788

*Laurentz M, Reudler JH, Mappes J et al (2012) Diet quality can play a critical role in defense efficacy against parasitoids and pathogens in the glanville fritillary (*Melitaea cinxia*). J Chem Ecol 38:116–125

Lavine MD, Beckage NE (1996) Temporal pattern of parasitism-induced immunosuppression in *Manduca sexta* larvae parasitized by *Cotesia congregata*. J Insect Physiol 42:41–51

Lindroth RL, Hwang SY (1996) Diversity, redundancy, and multiplicity in chemical defense systems of aspen. In: Romeo JT, Saunders JA, Barbosa P (eds) Phytochemical diversity and redundancy in ecological interactions. Plenum Press, New York

*Macel M, Bruinsma M, Dijkstra SM et al (2005) Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493–1508

Maleck M, Ferreira B, Mallet J et al (2014) Cytotoxicity of piperamides towards *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 51:458–463

Marques JV, de Oliveira A, Raggi L et al (2010) Antifungal activity of natural and synthetic amides from *Piper* species. J Brazil Chem Soc 21:1807–1813

Massad TJ, Dyer LA, Vega CG (2012) Costs of defense and a test of the carbon-nutrient balance and growth-differentiation balance hypotheses for two co-occurring classes of plant defense. PLoS One 7(10):e47554

Morimoto S, Komatsu K, Taura F, Shoyama Y (1998) Purification and characterization of cannabichromenic acid synthase from *Cannabis sativa*. Phytochemistry 49:1525–1529

Nelson AC, Kursar TA (1999) Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81–92

Nicolaou KC, Pfefferkorn JA, Roecker AJ et al (2000) Natural product-like combinatorial libraries based on privileged structures. 1. General principles and solid-phase synthesis of benzopyrans. J Am Chem Soc 122:9939–9953

*Nuringtyas TR, Verpoorte R, Klinkhamer PGL et al (2014) Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609–616

Oleszek WA (1998) Composition and quantitation of saponins in alfalfa (*Medicago sativa* L.) seedlings. J Agric Food Chem 46:960–962

Oleszek WM, Junkuszew Stochmal A (1999) Determination and toxicity of saponins from *Amaranthus cruentus* seeds. J Agric Food Chem 47:3685–3687

Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE (1999) Compromised disease resistance in saponin-deficient plants. Proc Natl Acad Sci USA 96:12923–12928

Parmar VS, Jain SC, Bisct KS et al (1997) Phytochemistry of the genus *Piper*. Phytochemistry 46:597–673

Pearson CV, Massad TJ, Dyer LA (2008) Diversity cascades in alfalfa fields: from plant quality to agroecosystem diversity. Environ Entomol 37:947–955

Podolak I, Galanty A, Sobolewska D (2010) Saponins as cytotoxic agents: a review. Phytochem Rev 9:425–474

Raj L, Ide T, Gurkar AU et al (2011) Selective killing of cancer cells by a small molecule targeting the stress response to ROS. Nature 475:231–234

Ramos CS, Vanin SA, Kato MJ (2009) Sequestration of prenylated benzoic acid and chromenes by *Naupactus bipes* (Coleoptera: Curculionidae) feeding on *Piper gaudichaudianum* (Piperaceae). Chemoecology 19:73–80

Rantala MJ, Roff DA (2007) Inbreeding and extreme outbreeding cause sex differences in immune defence and life history traits in *Epirrita autumnata*. Heredity 98:329–336

Rasmann S, Agrawal A (2009) Plant defense against herbivory: progress in identifying synergism, redundancy, and antagonism between resistance traits. Curr Opin Plant Biol 12:473–478

Reynolds T (2005) Hemlock alkaloids from Socrates to poison aloes. Phytochemistry 66:1399–1406

*Richards LA, Dyer LA, Smilanich AM et al (2010) Synergistic effects of amides from two piper species on generalist and specialist herbivores. J Chem Ecol 36:1105–1113

*Richards LA, Lampert EC, Bowers MD et al (2012) Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276–1284

Richards LA, Dyer LA, Forister ML et al (2015) Phytochemical diversity drives plant-insect community diversity. Proc Natl Acad Sci USA 112:10973–10978

Romeo JT, Saunders JA, Barbosa P (1996) Phytochemical diversity and redundancy in ecological interactions. Plenum Press, New York

*Scott Brown AS, Veitch NC, Simmonds MSJ (2011) Leaf chemistry and foliage avoidance by the thrips *Frankliniella occidentalis* and *Heliothrips haemorrhoidalis* in glasshouse collections. J Chem Ecol 37:301–310

Scott IM, Puniani E, Durst T et al (2002) Insecticidal activity of *Piper tuberculatum* Jacq. extracts: synergistic interaction of piper amides. Agric For Entomol 4:137–144

Segura A, Moreno M, Madueno F, Molina A, Garcia-Olmedo F (1999) Snakin-1, a peptide from potato that is active against plant pathogens. Mol Plant Microbe Interact 12:16–23

Smilanich AM, Dyer LA, Bowers MD, Chambers JQ (2009) Immunological costs to specialization and the evolution of insect diet breadth. Ecol Lett 12:612–621

Smilanich AM, Fincher RM, Dyer LA (2016) Does plant apparency matter? 30 years of data provide limited support but reveal clear patterns of the effects of plant chemistry on herbivores. New Phytol. doi:10.1111/nph.13875

Steppuhn A, Baldwin IT (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. Ecol Lett 10:499–511

Stermitz FR, Lorenz P, Tawara JN et al (2000) Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci USA 97:1433–1437

Tallarida RJ (2000) Drug synergism and dose–effect data analysis. Chapman & Hall, Boca Raton

Vasconcelos HL, Cherrett JM (1997) Leaf-cutting ants and early forest regeneration in central Amazonia: effects of herbivory on tree seedling establishment. J Trop Ecol 13:357–370

Viana FA, Braz Filho R, Pouliquen YBM et al (2004) Triterpenoid saponins from stem bark of *Pentaclethra macroloba*. J Brazil Chem Soc 15:595–602

Voukou D, Douvli P, Blionis GJ, Halley JM (2003) Effects of monoterpenoids, acting alone or in pairs, on seed germination and subsequent seedling growth. J Chem Ecol 29:2281–2301

*Weinhold A, Shaker K, Wenzler M et al (2011) Phaseoloidin, a homogentisic acid glucoside from *Nicotiana Attenuata* trichomes, contributes to the plant's resistance against *Lepidopteran* herbivores. J Chem Ecol 37:1091–1098

Wilson JS, Forister ML, Dyer LA et al (2012) Host conservatism, host shifts and diversification across three trophic levels in two Neotropical forests. J Evol Biol 25:532–546

Yamaguchi L, Freitas G, Yoshida NC, Kato MJ (2011) Chemometric analysis of ESIMS and NMR data from Piper species. J Brazil Chem Soc 22:2371–2382

*Zhang Z, Sun X, Xin Z et al (2013) Identification and field evaluation of non-host volatiles disturbing host location by the tea geometrid, *Ectropis obliqua*. J Chem Ecol 39:1284–1296