Identifying synergies and antagonisms between plant defense traits in *Asclepias syriaca*

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Abstract

As a general rule, plants defend against herbivores with multiple traits. The defense synergy hypothesis posits that some traits are more potent when co-expressed with others, compared to their independent potency. However, this hypothesis has rarely been tested outside of synergies within a class of particular phytochemicals, and seldom under field conditions. We tested for synergies between multiple defense traits of common milkweed (*Asclepias syriaca*) by assaying the performance of two specialist herbivores on plants in natural populations. We employed both standard regression and exploratory analysis using a novel application of Random Forests that allowed us to detect synergies between defense traits. In hypothesis testing, we found the first empirical evidence for a previously hypothesized synergy between one pair of co-expressed defense traits (latex and cardenolides), but not another (latex and trichomes). When exploring all potential interactions between pairs of traits we found eight synergies and five antagonisms in predicting herbivore performance. Half of the identified synergies involved carbon, which is the basis of several defenses including chemical and physical barriers to feeding, and also essential nutrients. Our findings suggest that defense synergies could explain co-expression of latex and cardenolides in milkweeds. This synergy may be common among the diverse plant species that employ latex as a defense. Future studies should test carbon-based synergies, which our work suggests are prevalent, as well as the other synergies identified in our exploratory analysis. Our analytic approach provides a general, flexible framework for more broadly discovering and predicting the coexpression of traits through their synergistic function.

Keywords: Defense synergy hypothesis; Random Forest; common milkweed (*Asclepias syriaca)*; swamp milkweed beetle (*Labidomera clivicollis*); monarch butterfly (*Danaus plexippus)*; plant-insect interactions; chemical ecology; synergy

Introduction

Plant defenses structure plant and insect communities, shape the way that primary productivity flows through to the rest of the food web, and can strongly impact the success of individual plants in the face of herbivory (Kursar et al. 2009, Kant et al. 2015, Coley et al. 2018). Consequently, ecologists have spent decades developing and evaluating theories to explain when and how plants should defend themselves (Agrawal 2011). One consistent finding is that individual plants generally have multiple, diverse defense traits (Duffey and Stout 1996, Agrawal and Fishbein 2006, Salazar et al 2018). Explaining why plants invest in multiple defenses is an open question, and is particularly important since there is some evidence that selection has favored specific combinations of defense traits ("defense syndromes" – see Agrawal and Fishbein 2006, da Silva and Batalha 2011, Sanczuk et al. 2020, cf. Moles et al 2013). Several non-exclusive hypotheses have been proposed to explain both the prevalence of multi-trait defense strategies and the specific combinations of defenses that are observed (Agrawal 2011).

Jones and Firn (1991) articulated the problem of multiple defenses clearly: individual plants often express many different types of secondary metabolites, and it is difficult to explain why. One of the leading explanations is synergistic interactions between secondary metabolites (Duffey and Stout 1996, Richards et al. 2016, Wetzel and Whitehead 2020). For example, the furanocoumarin Xanthotoxin is more effective when mixed with other furanocoumarins than expected from its performance alone (Berenbaum et al. 1991); similarly, amides in *Piper* plants are more effective in combination than their performance separately suggests (Scott et al. 2002, Dyer et al. 2003, 2010, Whitehead and Bower 2014). Synergies have also been detected in phytochemical defenses against microbes (e.g., Stermitz et al. 2000, Muroi and Kubo 1993) and

fungi (Cipollini and Stiles 1992). On the other hand, there have also been observations of antagonistic relationships, where compounds reduce the efficacy of each other, including among furanocoumarins (Diawara et al. 1993), amides (Whitehead and Bowers 2014), and between pyrrolizidine alkaloids and chlorogenic acid (Liu et al. 2017). Tests for synergies between secondary metabolites are still relatively rare (Richards et al. 2016), and there have been almost no tests for synergies between classes of defenses (e.g., physical and chemical defenses), even though there is every reason to expect them to exist (Rasmann and Agrawal 2009, Agrawal 2011, Richards et al. 2016, but see Green et al. 2001, Steppuhn and Baldwin 2007).

Studies of synergies between phytochemicals generally use artificial diets with manipulated concentrations of phytochemicals in an ANOVA design (e.g. Dyer et al. 2003, Berenbaum et al. 1991, Stamp and Yang 1996, Scott et al. 2002). Accordingly, synergy or antagonism is detected based on the interaction term of the two (or more) treatments (for a discussion on the appropriate null model for this experimental design, see Pennings 1996, Hay 1996, Nelson and Kursar 1999). For experimental designs with continuously varying defense traits, the logical extension of this approach is regression, with the interaction term between two traits capturing potential synergies or antagonisms (Richards et al. 2016). Nonetheless, implicit in a regression model approach is the assumption of a *bilinear* interaction: that the synergistic benefit of one additional unit of defense 1 for each unit of defense 2 is the same regardless of the original levels. However, other relationships may be equally likely. For example, the synergistic benefit of one trait might be a threshold effect; this could occur if one defense causes a switch in herbivore behavior above a certain level, and the effect of the second trait is impacted by herbivore behavior. Similarly, we might expect some synergies with diminishing marginal returns. To capture complex relationships like these, we introduce tests for synergies and antagonisms using Random Forest regression. Random Forests are a

popular form of machine learning that fits data using an ensemble (effectively, model averaging) of many decision trees. Because of this structure, Random Forest models can easily capture non-linear trends in data. Recent innovations have provided a framework for harnessing the flexible nature of Random Forests for inference about interactions, as we do here (Hooker 2007, Hooker and Mentch 2019). To our knowledge, this approach has not been previously used in ecology, so we include thoroughly commented code in the supplements.

The common milkweed, *Asclepias syriaca*, is a well-characterized system for studying plant defenses. The species produces toxic cardenolides that are potent defenses and also severely limit the species of herbivores that can feed on *A. syriaca*, leading to a simplified herbivore community (Agrawal 2017). In addition to cardenolides, *A. syriaca* also employ other defenses: trichomes on the leaf surfaces may reduce traction and insect access to feeding (Agrawal et al. 2009), latex exuded from channels (laticifers) in damaged tissues can cement insect mouth parts (Agrawal and Konno 2009), and leaf toughness and low nutrient content may reduce insect preference or performance (Agrawal and Fishbein 2006). Furthermore, expression of these defensive traits varies substantially in natural conditions within and between species, allowing experiments to capture broad ranges of defense traits in a field setting.

Two putative defense synergies have been hypothesized for milkweed plants (Agrawal 2011). First, freshly hatched monarch butterfly caterpillars were observed to first carefully cut trichomes in a small feeding region, then bite into the leaf tissue (Zalucki et al. 2002). At this point latex exudes, and the caterpillar must struggle free before it can consume leaf tissue. Many caterpillars fail to survive this first exposure to latex (Zalucki et al. 2001). Thus, trichomes and latex may act synergistically as one defense follows the other before feeding can commence; further, these two traits also show correlated evolution, suggesting that selection has favored their joint expression

(Agrawal and Fishbein 2006). Second, studies of *A. syriaca* latex have found that it contains cardenolides that mirror the concentrations in leaves (Agrawal et al. 2014), so it may be a vehicle to deliver toxins directly into the mouth of hungry caterpillars, while also physically gumming up mouthparts. This could be another defensive benefit of co-expression, in which the chemical and physical barriers associated with cardenolides and latex synergize. There are indications that this mechanism may be present in other plants with latex and secondary metabolites, as reduced levels of secondary metabolites in latex led to increased herbivory in the common dandelion (*Taraxacum officinale)* (Huber et al. 2016). While latex-by-trichome and latex-by-cardenolide have been proposed in papers across the last two decades, to our knowledge there has been no direct empirical evidence for either.

In the current study we evaluate plant defense synergies using data from herbivore performance on common milkweed *A. syriaca* under field conditions, using both well-established regression approaches and a novel application of Random Forests. Unlike the vast majority of existing studies of synergies (which use artificial diets), we measure herbivore performance under field conditions, relying on natural variation in defense traits. Using observational field studies sacrifices controlled conditions, but provides a realistic context for plant defenses, and allows us to study defenses that are not easily manipulated in the lab. Using three data sets for two specialist chewing herbivores (one of which was previously published), we test the defense synergy hypothesis for two predicted synergies: latex-by-cardenolide and latex-by-trichome. We also explore other potential synergies and antagonisms among >5 traits. Our methods provide a road map for future tests of the synergy hypothesis between classes of plant defenses, and for other evaluations of trait synergies in ecology.

Material and Methods

Study Species

A. syriaca is a long-lived herbaceous plant native throughout eastern North America, often in recently disturbed habitats including roadsides and fields. It employs a number of defenses against herbivores, including a sticky latex that exudes upon damage, an abundance of trichomes, particularly on the underside of leaves, and toxic cardenolides throughout plant tissues (Malcolm and Zalucki, 1996; Zalucki et al., 2001). The monarch butterfly, *Danaus plexippus*, and the swamp milkweed beetle, *Labidomera clivicollis*, are both *Asclepias* specialists that feed on the leaf tissues of *A. syriaca* in their larval stages. Both of these herbivores have modifications to their sodium pumps that increase their resistance to cardenolides (Dobler et al. 2012), but do not provide complete immunity (e.g. Zalucki et al. 2001, Agrawal 2005, Tao et al. 2016, Jones et al. 2019).

Field experiments

In September 2015, we conducted an experiment to assay the growth and survival of neonate monarch caterpillars, *Danaus plexippus*, on 117 *A. syriaca* plants near Ithaca, NY (42.39 N, 76.39 W). Caterpillars were from a laboratory colony (see Jones et al 2019). Plants in this natural population were selected haphazardly, using distance and visual phenotype to avoid selecting ramets from the same plant; we also avoided ramets that showed high levels of damage or early senescence. We placed two freshly hatched monarch caterpillars on one of the top pair of fully expanded leaves and enclosed them in a fine mesh bag. We used the other leaf in that pair to measure plant traits, including taking latex and toughness measurements, collecting a leaf disk for measuring trichome density and specific leaf area (SLA). We then removed that paired leaf to be dried for measurement of cardenolides, carbon, and nitrogen.

Latex, leaf toughness, specific leaf area, and cardenolides were quantified following the methods of Hahn et al. (2019). In brief, latex was measured by removing 2-3 mm of the leaf tip and measuring the weight of the resulting latex exudate; leaf toughness was measured using a penetrometer (Chatillon type 5/6) once on either side of the leaf and averaging the results; SLA (area per dry mass) was measured by drying and weighing a 31.67 mm^2 leaf punch; cardenolides were separated and measured from dried and ground leaf tissue using High Performance Liquid Chromatography (HPLC) following the methods of Züst et al. (2019). Trichomes density was measured by first imaging the underside of leaf discs with the Ziess SteREO Discovery.V20, then using Fiji, a variant of imageJ, to draw vertical and horizontal transects through the middle of the image and count all hairs that crossed the transects (Schindelin et al., 2012). These transect counts were highly correlated with transect densities obtained by counting all hairs in a quadrant of the leaf disc, but were faster and more robust to overlapping hairs (XXX, unpublished). Ground and dried leaf samples were analyzed at the Cornell Isotope Laboratory (COIL) for concentrations of carbon and nitrogen.

Monarch caterpillars generally remained on the plants for 6 days, and then were removed and weighed. In a few cases, field conditions prevented collection on day six, and caterpillars were instead collected on day 7; where appropriate, including day of collection as a covariate in statistical models accounted for this discrepancy. Missing caterpillars were presumed dead. In a several cases, plants died during the experiment, necessitating removing them from the study, or technical issues prevented us from obtaining one or more measurements of plant traits. In total, we obtained 165 weighed larvae and 173 measures of survivorship for 92 plants.

In September 2016, we conducted an experiment to assay growth and survival of neonate swamp milkweed beetles, *Labidomera clivicollis,* on 129 *A. syriaca* plants with the same

methods as above, with following exceptions. First, we used a separate field site approximately 300 meters away, at Dunlop Meadow (a Cornell University Natural Area). We did not measure carbon and nitrogen concentrations, and were unable to measure SLA. Due to unusual weather patterns, many of the plants experienced accelerated senescence during the experiment; we did not include these samples. Because *L. clivicollis* aggregate in their early life stages, we placed 5 larvae in each enclosure. In a few cases, loss of larvae during placement left us with only 4 larvae in an enclosure. In total, we obtained 134 weighed larvae and 464 records of survivorship for 95 plants.

For comparison, and to demonstrate the applicability of our approach with existing data, we also analyzed a previously published data set of *D. plexippus* bioassays (Agrawal 2005). These *A. syriaca* were planted outdoors in a common garden outside of Toronto in 2001, and an assay of *D. plexipus* performance was carried out in 2002. The plant traits were measured in largely the same way as the 2015 and 2016 experiments (see Agrawal 2005); notable differences were that only one herbivore was placed on each plant, cardenolide concentrations are reported as a single aggregated concentration, and trichomes were counted on quadrats of both sides of a leaf disk rather than transects of the underside of the leaf.

Analysis

Following Tredennick et al. (2021), we separated our analysis into two sections: hypothesis testing, and exploration. First, we carried out strict hypothesis testing on previously proposed synergies between *A. syriaca* defenses (latex-by-trichome, latex-by-cardenolide) using regression models, including main effects of the two traits of interest as well as their interaction. Then, we carried out exploratory analysis of other trait interactions using regression models and Random Forests. Our *a priori* hypothesis testing was conducted using conventional significance tests. Exploratory analyses used regression models involving many tests for synergies (all pairwise

combinations of measured traits), and are thus susceptible to false positives. As such, we used *P* values as a general benchmark for the relative support for different synergies; the *P* value for any one test should be interpreted with caution. Using Random Forests did not provide *P* values, but allowed us to identify more complicated (ie non-bilinear) synergistic or antagonistic interactions between defense traits. In all cases, we used log-transformed larval mass divided by days (i.e., daily growth rate assuming exponential growth) as our metric of herbivore growth. For all analyses, we rescaled plant traits so that their standard deviations were 1, but did not further adjust the mean. All analyses and simulations were carried out in the programming language R (R Core Team 2021).

In total we had three data sets, each with two measures of herbivore response (survival and growth). For simplicity, in the rest of this paper we will refer to these data sets as "Monarchs 2005", "Monarchs 2015", and "Clivicollis 2016" (Monarchs 2005 are labeled by publication date – the experiment was carried out in 2002). These three data sets have some overlap in the defense traits measured, but often these traits are not directly comparable (e.g., trichomes counting methods for Monarchs 2005 differed from the more recent experiments). Additionally, the experiment in Agrawal (2005) was set up on a single day with a single bioassay herbivore, rather than a blocked design with two (Monarch 2015) or five (Clivicollis 2016) larvae per plant, implemented across multiple days. For this reason, only Monarch 2015 and Clivicollis 2016 analyses required mixed effects models to capture the hierarchical structure of the data. Three data sets and two response variables result in six repetitions of each analysis. Although this leads to unavoidable complications in the description of each analysis and the associated analyses scripts, these are merely technical details of doing appropriate regression analyses for each experiment. Fundamentally, for each experiment we (a) use regression modeling to test

previously hypothesized interactions between defense traits, (b) use regression modeling to examine the data for evidence of other interactions between defense traits, and (c) use Random Forests as an alternative approach to (a) and (b) that requires no assumptions about the linearity of interactions.

Trait Correlations

We computed pairwise across-plant trait (phenotypic) correlations for each of our experiments, using Pearson correlation coefficients. Where we identified substantial correlations between particular cardenolide compounds (Monarchs 2015 and Clivicollis 2016) we used principal component analysis (PCA) to identify a single measure (the first PCA) that represented the majority of the variation in the covarying cardenolides. All further analysis used these first PCAs in place of the individual cardenolides in question.

Main effects of defenses

We evaluated the efficacy of plant traits by fitting regression models for each (experiment x response) combination, using an upward testing approach: fit the null model with only intercept and blocking factors as appropriate, test whether model fit is improved by the inclusion of individual plant traits. Growth was fit using linear regression (Monarchs 2005) or linear mixed models (Monarchs 2015, Clivicollis 2016) including random effects for day and plant identity. For survival, we carried out logistic regression using generalized linear models (Monarch 2005) or generalized linear mixed models (Monarch 2015, Clivicollis 2016) with the same model structure.

Hypothesis testing

For each hypothesized synergy (latex-by-trichomes, latex-by-cardenolide for each cardenolide compound), we fit a regression model that included main effects for the two focal

traits, and an interaction between those two traits (and random effects of plant and date for the Monarch 2015 and Clivicollis 2016 data). We then used Anova() in the **car** package to test the significance of the interaction term (Fox and Weisberg 2019). A significant interaction term represented a trait synergy or antagonism between the traits. Because the response was insect performance, a negative coefficient for the interaction term represented a defense trait synergy, while a positive term represented an antagonism. The magnitude of the slope coefficient can be interpreted as follows. Because all plant traits were scaled for analysis to have standard deviation of 1, an interaction coefficient of $\beta>0$ means that a one-standard-deviation increase in either trait increases by β the effect of a one-standard-deviation increase in the other (similarly, a negative coefficient implies a decrease in the effect of the second trait, if the first is increased). When survival is the response, this interpretation applies to logit survival, rather than survival itself (i.e., it applies to the linear predictor in a logistic regression model); when growth is the response it applies to growth.

Because the interaction term coefficient is interpreted on the scale of the linear predictor rather than the response, its magnitude cannot be used to compare the strength of synergies between survival and growth responses, or to make comparisons with equation-free models like Random Forests. To provide a more interpretable and robust measure of the strength of synergies and antagonisms, for each trait pair and response (growth or survival) we calculated the "predictable response range" and the percent of response variation explained by the interaction. As these metrics are central to our Random Forest analyses, we fully describe their calculation in *Exploration: Random Forest*, below. For a given pair of plant traits, the response range shows how much changing the focal traits (within the range of values observed in the field) impacts herbivores; the percent of variation explained by the interaction term identifies how important

the synergy or antagonism is relative to the direct effects of the traits. Notably, these measures are based on the predictable variation in the response (i.e., the fitted response surface described the regression equations) and are in intuitive units (growth rate or survival probability, percent variation explained). Because Random Forests are more flexible models, we don't expect the interaction term to explain as much of the variation, but this is unavoidable.

Exploration: regression

Using the same framework as in the *a priori* regression tests above, we looked for synergies or antagonisms for all pairwise combinations of measured traits. We use the *P* value as an indicator for potential synergies/antagonisms, but we do not interpret *P*-values as we would for an individual hypothesis test, because this process involves multiple comparisons that would produce multiple false positives even if there are no interactions. Instead, the *P* values provide a benchmark of relative support for the interactions tested.

Exploration: Random Forest

Like most machine learning algorithms, Random Forests can be very effective at prediction (e.g. "How fast do caterpillars grow on a plant with these defense traits?"), but do not directly provide information (analogous to regression coefficients) about the basis for those predictions (e.g. "Which plant traits most strongly affect caterpillar growth rate?"). Hooker (2007) provides methodology for interrogating a fitted Random Forest model to determine the presence or absence of interactions among variables, and to quantify the importance of interactions if they are present. We have adopted this approach for both Random Forest models and our regression analyses (above).

For a given experiment and response (growth or survival), we first fit a Random Forest model that included all available plant traits (Random Forests capture interactions without

needing explicit terms to represent them). Following Hooker (2007), we then treated this fitted model as if it were an experimental system, in which we could measure herbivore performance on hypothetical plants having any specified combination of defense trait values. We conducted simulated experiments, using the fitted Random Forest to generate the "response" (predicted herbivore performance) for a set of hypothetical plants with differing defense traits. The predicted herbivore performance was thus the result of any linear and non-linear patterns in the real data that the Random Forest model captured. We carried out simulated experiments for each possible defense trait pair, and we analyzed the resulting simulated data to determine if the response was additive (no interactions) or non-additive (synergy or antagonism). In the case of our regression analyses (above), we carried out the same procedure using our fitted regression models. The procedure for simulated experiments and their analysis was as follows:

1. For each real plant measured, we generated 100 (Random Forest) or 2500 (regression model) hypothetical plants, in which all trait values were the same as the real plant, except that values of the two traits of interest were varied across a 10x10 (Random Forest) or 50x50 (regression model) grid ranging from the smallest to largest values observed in any real plant. For an empirical data set of size N, this gave us 100N (Random Forest) or 2500N (regression model) hypothetical plants. Because the outputs of Random Forests are piecewise constant at fine scales, it is important to avoid partitioning the trait space too finely (Hooker, personal communication). With regression models that don't have such limitations, we found that using the predictions of a 50x50 grid of plant traits produced results similar to higher resolution grids, and could be computed in a reasonable amount of time (CBE, unpublished simulations).

2. Using the fitted model (Random Forest or regression), we predicted average herbivore performance on those hypothetical plants. The range of herbivore performance (survival or growth) across these predictions gives us our "predictable response range" (PRR), which characterizes the amount of variation in expected herbivore performance (i.e, average growth or average survival of multiple herbivores exposed to that trait combination) that can be attributed to varying the focal traits. Correlations among the traits we studied are sufficient weak that the rectangle defined by the range of variation in each trait is a good approximation to the possible trait combinations for real plants (see Fig. S1).

3. We fit a two-way ANOVA model to the predicted response of the form

response ~ trait1 + trait2 + trait1:trait2

where the trait values of trait 1 and trait 2 were treated as factors. This meant that the interaction term captured any non-additivity for a given combination of trait values. In the case of Random Forests this captured non-additivity even if the independent response to each trait was nonlinear, or if synergies or antagonisms were not consistent across different levels of the traits. There were several complications in the actual implementation, particularly with Random Forests, which we describe in the Supplements. One key feature for Random Forests was that we used a weighted ANOVA, where each observation from the Random Forest trial was weighted by how close it was (in terms of defense traits) to an actual empirical observation. This focused our results on meaningful patterns driven by the data, rather than spurious patterns driven by extrapolation of the Random Forest model outside the range of the data (Hooker and Mentch 2019).

4. From the fitted ANOVA model, we determined what percent of the total Sum of Squares explained by the ANOVA model we could attribute to the interaction term. This

provides a measure of the importance of the synergy or antagonism in intuitive units. In the case of Random Forests, for which there is not a P value equivalent, we used this percent as our measure for identifying important synergies, and chose 15% as a threshold for meaningful synergies or antagonisms to report.

5. Step 4 identified trait pairs with notable non-additivities, but did not characterize the interaction as a synergy or antagonism. For regression models, the sign of the interaction coefficient identified whether the interaction was a synergy or an antagonism, but Random Forests did not provide such a metric. For that purpose, we generated diagnostic plots. These (a) visualized the Random Forest predictions for a grid of points, and (b) compared how those predictions differed from the expectations of an additive ANOVA model, which was like the model in step 3 but contained only main effects. We then assessed whether the resulting pattern was a synergy, antagonism, or unclear, by comparing those diagnostic plots to diagnostic plots from simulated data with known trait interactions (see Random Forest simulations below, and Fig. 2).

Because Random Forest regression struggles with missing data, we removed observations with one or more missing plant trait measurement. Random Forests also do not have appropriate ways to account for random effects, so we do not include the date or plant identity terms that were present in the regression analyses (Monarch 2015, Clivicollis 2016).

Diagnostic plots with known interaction patterns

To determine whether patterns in the Random Forests diagnostic plots corresponded to synergies or antagonisms, we simulated data with known synergies and antagonisms, and generated the resulting diagnostic plots. In brief, we defined defense efficacy as a specified function of two hypothetical traits X and Y, where larger values indicate better defense (e.g. the

first column in Fig 2a-f). We then used that function to simulate herbivore performance at a 10 X 10 grid of trait values, analogous to our fitted Random Forest predictions above (e.g. the second column in Fig 2a-f). For this set of simulated herbivore performance data, we fit an additive regression model of the form (response ~ trait X + trait Y), with traits X and Y treated as factors. This is exactly the same as the model fitted in step 5 above. We then looked at how the simulated herbivore performance compared to the predictions of this additive model (e.g. the third column in Fig 2a-f). This process produced plots that were comparable to those produced in step 5 above, but with the difference that the underlying synergistic or antagonistic relationship between the two defense traits was known.

We assumed that traits X and Y were scaled to have values between 0 and 1, and defined herbivore performance as 10 minus the defense efficacy; as we were looking for qualitative patterns, these specific values do not qualitatively change our results (CBE, unpublished simulations). We considered many potential trait synergies and antagonisms, but here present results for three synergies (linear, threshold, diminishing marginal returns) and three antagonisms (linear, threshold, decaying). For more details, and code to simulate other synergies and antagonisms, see the Supplements.

Results

Trait Correlations

We identified three cardenolide compounds that were highly correlated in the Monarch 2015 experiment (peaks that eluted at 17.4, 17.7, and 18.4 minutes; pairwise Pearson correlation coefficients ranged from r = 0.77 to 0.86) and a pair of correlated cardenolide compounds in the Clivicollis 2016 experiment (peaks 10.7 and 18.1; pairwise correlation coefficient of r = 0.73). To avoid misleading results from highly correlated predictors, we used Principal Component

Analyses (PCA) on these two sets of cardenolides, and used the first component as a single plant trait to represent the three peaks in 2015 (labeled "card15.pca") and the two peaks in 2016 (labeled "card16.pca"). We also found numerous plant traits that were mildly-to-moderately correlated (Figs. S1) which may reflect underlying genetic structure, or correlated expression in response to biotic or environmental drivers. We did not further collapse our data with PCA, but where appropriate we note the consequences of correlated traits on our results.

While it was not the focus of our study, we highlight several significant patterns of trait co-expression in our field studies (Fig S1). In 2015, several of the physical traits showed positive phenotypic correlations – notably, leaf toughness was correlated with both latex ($r = 0.17$) and trichomes ($r = 0.49$). Percent leaf carbon was positively correlated with the two traits that clearly incorporate carbon, trichomes ($r = 0.49$) and leaf toughness ($r = 0.21$). In 2016, physical defense traits (trichomes, toughness, and latex) all had significant positive correlation with each other (latex and toughness, $r = 0.38$; latex and trichomes, $r = 0.13$; toughness and trichomes, $r = 0.30$), and were generally negatively correlated or essentially uncorrelated with cardenolides.

Main effects of defenses

Trichomes had a negative main effect on Monarch 2005 growth (slope = -0.024, $p = 0.025$), and both percent carbon ($p = 0.006$) and carbon:nitrogen ratio ($p = 0.015$) similarly had negative main effects Monarch 2005 survival. These predictors were only somewhat correlated (Pearson correlation coefficient = 0.26), and a model fit using carbon and carbon:nitrogen was much better than fitting a model with carbon and nitrogen alone (likelihood ratio test, $p < 0.001$). The model with both carbon (slope = -0.393) and carbon:nitrogen ratio (slope = -0.151) had negative coefficients for both, suggesting that even when plants had high carbon (bad for

monarch survival), having a higher carbon:nitrogen ratio provided further defense against monarchs.

In contrast, percent carbon (slope = 0.028, p = 0.028) and trichomes (slope = 0.013, p = 0.086) had positive main effects on monarch growth in 2015, and no other plant traits had significant effects. Using AIC model selection, a model with only carbon was better than either a model with only trichomes (ΔAIC = -2.60) or a model with trichomes and carbon (ΔAIC = -0.37, in this case the models are statistically indistinguishable using AIC). There were no significant predictors of Monarch 2015 survival. Leaf toughness had a marginally significant positive main effect on *L. clivicollis* growth (slope = 0.017, p=0.074), and cardenolide peaks 10.2 and 8.4 had negative main effects on *L. clivicollis* survival (slope = -0.969, p = 0.041; slope = -0.8116, p = 0.059 respectively).

Hypothesis testing

We found no evidence for the hypothesized synergies between defense traits for growth or survival in Monarch 2005. We did find a marginally significant synergy between latex and cardenolides (peak 18.6) on Monarch 2015 survival (slope = -0.724, p = 0.077; Predictable Response Range (PRR) = [0.0114, 0.9999], 69% of PRR explained by synergy), such that plants were more defended when latex and cardenolides levels were both high than either defense trait would suggest in isolation (Fig. 1a; Table 1). We also found a significant synergy between latex and cardenolides (peak 10.2) on *L. clivicollis* growth (slope = -0.038, p = 0.033; PRR = [-1.2719, -0.7359], 30% of PRR explained by synergy) (Marginal for survival, slope = -2.085, p = 0.100; PRR = [0, 0.9824], 19% of PRR explained by synergy) (Fig 1b-c, Table 1).

Exploration: regression

Due to the many models fit in our exploratory analyses, p values in exploration should not be interpreted as the exact probability of the data under the null hypothesis. However, p values do represent relative statistical support for the different synergies, and we use $p<0.1$ as a threshold for reporting here; for the complete results of the analyses, see the supplementary code. We found a notable nitrogen-by-trichome antagonism (slope = 0.0151, p =0.0323; PRR = [-0.9313, -0.6643], 62% of PRR explained by antagonism), cardenolide-by-toughness synergy (slope = -0.012, p = 0.0497; PRR = [-0.8795, -0.7016], 92% of PRR explained by synergy), and carbon-by-trichome synergy (slope = -0.0124, p = 0.0412; PRR = [-0.9715, -0.6036], 60% of PRR explained by synergy) for Monarch 20005 growth (Table S1). We also found a carbon-by-nitrogen synergy for monarch survival (slope = -0.515, p = 0.009; PRR = [0.0035, 0.9997], 70% of PRR explained by synergy). In 2015, we found toughness-by-carbon (slope = -0.0143, p =0.326; PRR = [-0.9416, -0.6251], 39% of PRR explained by synergy) and nitrogen-by-cardenolide 10.5 (slope = -0.140, p = 0.0579; PRR = [-0.8371, -0.6368], 68% of PRR explained by synergy) synergies, and a toughness-by-cardenolide 10.5 antagonism (slope = 0.014, p =0.0611; PRR = [-0.8297, -0.6475], 70% of PRR explained by antagonism) on Monarch growth. We did not find any new notable interactions on Monarch 2015 survival (latex-by-cardenolide 18.6 synergy was present in the *a priori* testing). In 2016, we found a latex-by-toughness synergy (slope = -0.0238, p = 0.0375; PRR = [-0.9955, -0.7689], 99% of PRR explained by synergy) and a toughness-by-cardenolide PCA antagonism (slope = 0.0181, p = 0.060; PRR = [-1.0627, -0.6207], 31% of PRR explained by antagonism) on *L. clivicollis* growth (in addition to the previously identified latex-by-cardenolide 10.2 synergy). We did not identify any new notable interactions affecting *L. clivicollis* survival.

Diagnostic plots with known interaction patterns

When applied to simulated data with known underlying interactions between defense traits, synergies always produced diagnostic plots in which the bottom left and top right regions showed herbivores underperforming predictions from an additive model (an ANOVA model with no interaction terms fit to the simulated data), and the top left and bottom right showed herbivores over-performing predictions from an additive model (e.g. Fig. 2a-c). The reverse was true for antagonisms: herbivores always over-performed predictions from an additive model in the bottom left and top right, and underperformed predictions from an additive the top left and bottom right regions (e.g. Fig. 2d-f). These patterns held true for all of the synergies and antagonisms we evaluated (XXX, unpublished simulations).

Exploration: Random Forests

We found strong evidence of non-additivity for nine trait pairs, for which at least 15% of the sum of squares of the linear model was explained by the interaction term. Of these, three showed strong patterns of synergies: carbon-by-nitrogen for Monarchs 2005 survival (also identified with regression models above) (PRR = [0.36123, 0.8194], 37% explained by interaction), latex-by-carbon for Monarchs 2015 growth (PRR = [-0.8484, -0.7229], 19% explained by interaction), and toughness-by-carbon in Monarch 2015 (also identified with regression models above) (PRR = [-0.8683, -0.7259], 15% explained by interaction), (Fig. 3a-c). Three interactions also appeared to be antagonisms, all from Monarchs 2005: toughness-by-trichome on survival (PRR = [-0.8766, -0.7679], 23% explained by interaction), and toughness-by-carbon (PRR = [0.394, 0.7830], 20% explained by interaction) and latex-by-cardenolide on survival (PRR = [0.4877, 0.7191], 21% explained by interaction) (Fig. 3d-f). For the three synergies we identified in the *a priori* hypothesis testing above, the Random Forest plots show the same diagnostic patterns of synergy, even though the Sum of Squares explained in these

cases did not exceed our 15% threshold for the Random Forest approach (Fig S2). Of the six strong synergies and antagonisms identified using Random Forests, only two of them had been detected in the regression analyses, suggesting the remaining four have non-linear functional forms.

Discussion

Using surveys of herbivore performance in the field, and both well-established and novel statistical tools, we found evidence for the plant defense synergy hypothesis, including some potential new synergies and antagonisms. We found the first direct empirical support for the previously hypothesized synergy between latex and cardenolides, suggesting that secondary metabolites may serve to amplify the physical dangers of latex, or non-exclusively, that latex serves as a focused delivery system for chemical defense. In contrast, we found no support for the hypothesized synergy between latex and trichomes. Here, however, we caution that the hypothesized mechanism for latex-by-trichome synergies focuses on mortality, and our Monarch 2015 experiment had such low mortality that there was very little statistical power to detect associations between caterpillar deaths and specific plant traits.

In our exploration of all possible interactions, we identified eight potential synergies between classes of defenses, and five potential antagonisms. Because these potential interactions were discovered by searching for all possible interactions, they should not be accorded the same level of confidence as those supported through strict hypothesis testing; nonetheless, they do represent potentially important trait interactions that can be tested in the future. Half of the identified potential synergies were between carbon and other traits (carbon-by-nitrogen, carbon-by-trichome, carbon-by-latex, carbon-by-toughness). This is especially noteworthy given that we were unable to measure carbon in the 2016 Clivicollis data, so those synergies came from only

two of the three field surveys. Percent carbon in leaves is a crude bulk measure, as much of the leaf is itself carbon, and many defense traits have high carbon content. Thus, carbon may be capturing investment in carbon-rich defenses, but can also measure overall plant allocation; as such, carbon has played an important role in defense theory. Two long-standing plant defense hypotheses address the allocation of carbon to plant defenses: the carbon/nutrient balance hypothesis (Bryant et al. 1983), and the growth-differentiation balance hypothesis (GDBH) (Hermes and Mattson 1992). While these hypotheses have mixed empirical support and have lost favor in the literature, they provide a key insight: plants should invest in carbon-rich defenses when they are nitrogen-limited and have excess carbon (Massad et al. 2012). In this context, defense synergies involving carbon are particularly important, as nitrogen-limited plants may have excess carbon that could increase the efficacy of the other synergistic defenses. If there are consistent synergies between carbon and other classes of defense across plant species, we expect to see interspecific patterns of defense co-expression driven by carbon availability, as a natural extension of existing plant defense hypotheses. We also note that two of those carbon synergies (carbon-by-trichome, carbon-by-leaf-toughness) are synergies between leaf carbon content, and carbon-rich defenses. This might reflect a general benefit for investing in suites of carbon-rich defenses, but could also represent uncaptured aspects of these traits (e.g., variation in trichome length in addition to density, variation in leaf toughness to shearing in addition to punctures).

Three of the identified potential synergies (latex-by-carbon, latex-by-cardenolide, latex-by-toughness) involved latex. Latex is present in nearly 10% of angiosperm species, has no known function in plants other than defense and is itself a cornucopia of multiple physical and chemical traits (Agrawal and Konno, 2009). Latex can be quite harmful to herbivores directly (e.g. Zalucki & Brower 1992), but its commonness across plant families is all the more

understandable if it synergizes with other common defenses. Here, our measure of latex was the wet mass exuded upon leaf damage, which typically correlates negatively with insect performance, but also represents multiple finer-grained traits. Particularly notable were the latex-by-cardenolide synergies identified in this study; these synergies had been previously hypothesized (Agrawal 2011) and we found support for them in the natural populations tested. Note that typical measures of leaf cardenolides in *A. syriaca* are independent of latex; in other words, residual latex in the leaves, once cut, contributes relatively little to the cardenolides measured (Agrawal et al. 2014). The hypothesized mechanisms for latex-by-cardenolide synergies – that latex could serve as a delivery system for toxic phytochemicals, and toxic phytochemicals make latex even more dangerous to herbivores – is not a special feature of *L. clivolis* and *D. plexippus* feeding on *A. syriaca.* The latex of many plants contains secondary metabolites, often at elevated levels compared to other plant tissues (Agrawal and Konno 2009). A recent study on the common dandelion found that herbivore damage increased when sesquiterpene levels in latex were reduced; while this study was not explicitly addressing synergies, their findings imply latex-by-sesquiterpene synergy (Huber et al. 2016). To our knowledge, our study is the first to find explicit evidence for latex-by-defense-metabolite synergies, but we speculate that such synergies may be common across plant taxa.

Several pairs of defense traits were identified as both synergies and antagonisms in different contexts (e.g. cardenolide-by-toughness decreased herbivore growth synergistically in Monarchs 2005, but interacted antagonistically to impact growth in Monarchs 2015 and Clivicollis 2016). It would perhaps be more satisfying to observe consistent effects of defense trait pairings, as this would imply a universal mechanism underlying the synergy or antagonism; in this context, trait pairs showing both synergies and antagonisms could be concerning. While

contradictions in our findings might reflect spurious relationships identified with our exploratory approaches, there are several reasons not to discount these results. First, it is not unusual for field experiments of plant defenses to find that defenses are inconsistent in their efficacy between years or other contexts (e.g. inconsistent efficacy of latex and cardenolides as defense of milkweeds, Agrawal and Fishbein 2006, and inconsistent effects of protease inhibitors and nicotine as a defense of wild tobacco, Mitra et al. 2008); indeed, this has been proposed as a mechanism that favors multi-trait defense strategies (Rasmann and Agrawal 2009). Studies of synergies between phytochemical have also found that some pairs of traits can be synergistic in some contexts and antagonistic in others (e.g. Whitehead and Bower 2014). Second, two of the trait pairs with apparent contradictions include "cardenolide" as a single class of defense (measured as totally concentrations in Monarchs 2005). However, the synergies and antagonisms never involved the same specific cardenolide compound, and so while they suggest that cardenolides as a class may produce either synergies or antagonisms in combination with other traits, this may not be the case with individual compounds.

Multivariate plant defense strategies have been explored empirically through observations of trait co-expression. This can be done at the level of between-species comparisons (e.g. Agrawal and Fishbein, Moles et al. 2013, Muniz da Silva and Batalha 2011, Sanczuk et al. 2020), between-population comparisons (e.g. Barton 2014), or between individuals within a population (Agrawal et al. 2014). Trait co-expression can provide indirect support for synergies, but these same signals might represent other forms of adaptive advantage or disadvantage for specific combinations. The statistical framework we provide here helps identify, explicitly, when two traits are associated with synergistic (or antagonistic) impacts on herbivores. Among *Asclepias* spp., comparative work has found interspecific patterns of co-expression of latex and trichomes, but

comparisons between species did not show a correlation between latex and cardenolide expression (Agrawal and Fishbein 2006).

Tests of synergies between classes of defenses (rather than between secondary metabolites within a class) are rare, but ours is not the first. There have been a few studies in aquatic systems finding synergies between structural components (calcium carbonate, spicules) and secondary metabolites in artificial diets simulating seaweeds (Hay et al. 1994) and sponges (Hill et al. 2005, Jones et al 2005). Simpson and Raubenheimer (2001) found that tannic acids had an effect on locust only for some levels of protein-to-carbohydrate ratios in the artificial diets, suggesting that plant digestion inhibitors might synergize with strategies of low nutrient availability. Perhaps the strongest test for cross-class synergies in terrestrial plants used gene knockouts in *Nicotiana attenuata* to show that protease inhibitors (TPI, a form of digestion inhibitor) synergized with toxic nicotine to defend against generalist *Spodoptera exigua* (Stepphun and Baldwin 2007). Follow-up work in the same system found that TPI reduced caterpillar response to simulated predation, suggesting a potential defense synergy between parasitoid-attracting volatiles and TPI; however, field experiments found plants with higher TPI actually had *reduced* parasitism (Schuman et al. 2012). Similar to Stepphun and Baldwin (2007), Green et al. (2001) found phytic acid (an anti-nutritive defense) reduced insect metabolism of defensive xanthotoxins, implying a potential synergy with phytic acid interfering with specialist herbivore counter-defenses; however, this was not extended to measure the ultimate effect of this potential synergy on herbivore survival or growth.

The rarity of synergy tests between classes of defenses is likely due in part to the logistical challenges involved in such experiments. The overwhelming majority of synergy tests to date have used artificial diets (e.g. Whitehead et al. 2021), and non-metabolite defenses like

latex, leaf toughness, or trichomes do not lend themselves to such controlled experiments. Similarly, the most elegant approaches to date for testing phytochemical synergies identify an interaction index based on the dose necessary to cause 50% mortality (ED_{50}) (Nelson and Kursar 1999, Tallarida 2000, Richards et al. 2016). These tools are well-suited to artificial diet experiments of secondary metabolites in which an ED_{50} is certain to exist, but are difficult to implement when defenses cannot be manipulated and even high levels of putatively defensive traits have no guarantee of causing mortality, as with many specialist insects.

Conclusion

The regression approach we use for hypothesis testing is a natural extension of ANOVA tests for interactions, with the advantage that it can easily be applied to studies with naturally occurring trait levels which can vary continuously (Richards et al. 2016). However, regression models with an interaction term inherently assume that the interaction term is bilinear (i.e., proportional to the product of the two defense trait levels). Our application of Random Forests, while more complicated than traditional ANOVA analyses, captures the same essential feature: it identifies when two traits have a different effect in combination than we would expect from their separate effects. However, the Random Forests analysis has the key advantage that it makes no assumptions about the functional forms of the independent effects and the interaction. This approach to leverage Random Forests to identify interactions has been outlined in the statistical literature (Hooker 2007, Hooker and Mensch 2019), but to our knowledge has not yet been used in an ecological context. Our simulations of known synergies and antagonisms showed consistent diagnostic patterns (Fig. 2), suggesting that this approach could be used broadly to identify non-additive synergies and antagonisms in many contexts. Here we have demonstrated the value of combining two approaches: finding strong evidence with regression models for a

previously hypothesized synergy between latex and cardenolides, and finding four synergies and antagonisms using Random Forests which were not identified using regression models.

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References

Agrawal, Anurag A. 2005. "Natural Selection on Common Milkweed (Asclepias Syriaca) by a Community of Specialized Insect Herbivores." Evolutionary Ecology Research 7 (5): 651–67. http://www.evolutionary-ecology.com/abstracts/v07/1801.html.

Agrawal, Anurag A., and Mark Fishbein. 2006. "Plant Defense Syndromes." Ecology 87 (7 Suppl): S132–49. https://doi.org/10.1890/0012-9658(2006)87[132:pds]2.0.co;2.

Agrawal, Anurag A., and Kotaro Konno. 2009. "Latex: A Model for Understanding Mechanisms, Ecology, and Evolution of Plant Defense Against Herbivory." Annual Review of Ecology, Evolution, and Systematics 40 (1): 311–31. https://doi.org/10.1146/annurev.ecolsys.110308.120307.

Agrawal, Anurag A., Mark Fishbein, Reinhard Jetter, Juha-Pekka Salminen, Jessica B. Goldstein, Amy E. Freitag, and Jed P. Sparks. 2009. "Phylogenetic Ecology of Leaf Surface Traits in the Milkweeds (Asclepias Spp.): Chemistry, Ecophysiology, and Insect Behavior." The New Phytologist 183 (3): 848–67. https://doi.org/10.1111/j.1469-8137.2009.02897.x.

Agrawal, Anurag A. 2011. "Current Trends in the Evolutionary Ecology of Plant Defence: Plant Defence Theory." Functional Ecology 25 (2): 420–32. https://doi.org/10.1111/j.1365-2435.2010.01796.x.

Agrawal, Anurag A., Eamonn T. Patrick, and Amy P. Hastings. 2014. "Tests of the Coupled Expression of Latex and Cardenolide Plant Defense in Common Milkweed (Asclepias Syriaca)." Ecosphere 5 (10): art126. https://doi.org/10.1890/ES14-00161.1.

Agrawal, Anurag. 2017. Monarchs and Milkweed: A Migrating Butterfly, a Poisonous Plant, and Their Remarkable Story of Coevolution. Princeton University Press. https://play.google.com/store/books/details?id=q2yYDwAAQBAJ.

Barton, Kasey E. 2014. "Prickles, Latex, and Tolerance in the Endemic Hawaiian Prickly Poppy (Argemone Glauca): Variation between Populations, across Ontogeny, and in Response to Abiotic Factors." Oecologia 174 (4): 1273–81. https://doi.org/10.1007/s00442-013-2836-z.

Berenbaum, M. R., J. K. Nitao, and A. R. Zangerl. 1991. "Adaptive Significance of Furanocoumarin Diversity inPastinaca Sativa (Apiaceae)." Journal of Chemical Ecology 17 (1): 207–15. https://doi.org/10.1007/BF00994434.

Bryant, John P., F. Stuart Chapin III, and David R. Klein. 1983. "Carbon/nutrient Balance of Boreal Plants in Relation to Vertebrate Herbivory." Oikos 40(3): 357–68. https://doi.org/10.2307/3544308.

Cipollini, Martin L., and Edmund W. Stiles. 1992. "Antifungal Activity of Ripe Ericaceous Fruits: Phenoliz-Acid Interactions and Palatability for Dispersers." Biochemical Systematics and Ecology 20 (6): 501–14. https://doi.org/10.1016/0305-1978(92)90004-W.

Coley, Phyllis D., María-José Endara, and Thomas A. Kursar. 2018. "Consequences of Interspecific Variation in Defenses and Herbivore Host Choice for the Ecology and Evolution of Inga, a Speciose Rainforest Tree." Oecologia 187 (2): 361–76. https://doi.org/10.1007/s00442-018-4080-z.

Diawara, M. M., J. T. Trumble, K. K. White, W. G. Carson, and L. A. Martinez. 1993. "Toxicity of Linear Furanocoumarins toSpodoptera Exigua: Evidence for Antagonistic

Interactions." Journal of Chemical Ecology 19 (11): 2473–84. https://doi.org/10.1007/BF00980684.

Dobler, Susanne, Safaa Dalla, Vera Wagschal, and Anurag A. Agrawal. 2012. "Community-Wide Convergent Evolution in Insect Adaptation to Toxic Cardenolides by Substitutions in the Na,K-ATPase." PNAS 109 (32): 13040–45. https://doi.org/10.1073/pnas.1202111109.

Duffey, Sean S., and Michael J. Stout. 1996. "Antinutritive and Toxic Components of Plant Defense against Insects." Archives of Insect Biochemistry and Physiology 32 (1): 3–37. https://doi.org/10.1002/(sici)1520-6327(1996)32:1<3::aid-arch2>3.0.co;2-1.

Dyer, L. A., C. D. Dodson, J. O. Stireman 3rd, M. A. Tobler, A. M. Smilanich, R. M. Fincher, and D. K. Letourneau. 2003. "Synergistic Effects of Three Piper Amides on Generalist and Specialist Herbivores." Journal of Chemical Ecology 29 (11): 2499–2514. https://doi.org/10.1023/a:1026310001958.

Fox J, Weisberg S (2019). An R Companion to Applied Regression, Third edition. Sage, Thousand Oaks CA.

Glassmire, Andrea E., Luke N. Zehr, and William C. Wetzel. 2020. "Disentangling Dimensions of Phytochemical Diversity: Alpha and Beta Have Contrasting Effects on an Insect Herbivore." Ecology 101 (11): e03158. https://doi.org/10.1002/ecy.3158.

Green, E. S., A. R. Zangerl, and M. R. Berenbaum. 2001. "Effects of Phytic Acid and Xanthotoxin on Growth and Detoxification in Caterpillars." Journal of Chemical Ecology 27 (9): 1763–73. https://doi.org/10.1023/a:1010452507718.

Hay, Mark E., Quaker E. Kappel, and William Fenical. 1994. "Synergisms in Plant Defenses against Herbivores: Interactions of Chemistry, Calcification, and Plant Quality." Ecology 75 (6): 1714–26. https://doi.org/10.2307/1939631.

Hay, Mark E. 1996. "Defensive Synergisms? Reply to Pennings." Ecology 77 (6): 1950–52. https://doi.org/10.2307/2265799.

Herms, Daniel A., and William J. Mattson. 1992. "The Dilemma of Plants: To Grow or Defend." The Quarterly Review of Biology 67 (3): 283–335. https://doi.org/10.1086/417659

Hill, M. S., N. A. Lopez, and K. A. Young. 2005. "Anti-Predator Defenses in Western North Atlantic Sponges with Evidence of Enhanced Defense through Interactions between Spicules and Chemicals." Marine Ecology Progress Series 291: 93–102. https://doi.org/10.3354/meps291093.

Hooker, Giles. 2007. "Generalized Functional Anova Diagnostics for High-Dimensional Functions of Dependent Variables." Journal of Computational and Graphical Statistics 16 (3): 709–32. https://doi.org/10.1198/106186007X237892

Hahn, Philip G., Anurag A. Agrawal, Kira I. Sussman, and John L. Maron. 2019. "Population Variation, Environmental Gradients, and the Evolutionary Ecology of Plant Defense against Herbivory." The American Naturalist 193 (1): 20–34.

Hooker, Giles, and Lucas Mentch. 2019. "Please Stop Permuting Features: An Explanation and Alternatives." arXiv [stat.ME]. arXiv. http://arxiv.org/abs/1905.03151.

Huber, Meret, Janina Epping, Christian Schulze Gronover, Julia Fricke, Zohra Aziz, Théo Brillatz, Michael Swyers, et al. 2016. "A Latex Metabolite Benefits Plant Fitness under Root Herbivore Attack." PLoS Biology 14 (1): e1002332. https://doi.org/10.1371/journal.pbio.1002332.

Jones, Adam C., James E. Blum, and Joseph R. Pawlik. 2005. "Testing for Defensive Synergy in Caribbean Sponges: Bad Taste or Glass Spicules?" Journal of Experimental Marine Biology and Ecology 322 (1): 67–81. https://doi.org/10.1016/j.jembe.2005.02.009.

Jones, Clive G., R. D. Firn. 1991. "On the Evolution of Plant Secondary Chemical Diversity." Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 333 (1267): 273–80. https://doi.org/10.1098/rstb.1991.0077.

Jones, Patricia L., and Anurag A. Agrawal. 2019. "Beyond Preference and Performance: Host Plant Selection by Monarch Butterflies, Danaus Plexippus." Oikos https://doi.org/10.1111/oik.06001.

Jones, Patricia L., Georg Petschenka, Lara Flacht, and Anurag A. Agrawal. 2019. "Cardenolide Intake, Sequestration, and Excretion by the Monarch Butterfly along Gradients of Plant Toxicity and Larval Ontogeny." Journal of Chemical Ecology 45 (3): 264–77. https://doi.org/10.1007/s10886-019-01055-7.

Kant, M. R., W. Jonckheere, B. Knegt, F. Lemos, J. Liu, B. C. J. Schimmel, C. A. Villarroel, et al. 2015. "Mechanisms and Ecological Consequences of Plant Defence Induction and Suppression in Herbivore Communities." Annals of Botany 115 (7): 1015–51. https://doi.org/10.1093/aob/mcv054.

Kursar, Thomas A., Kyle G. Dexter, John Lokvam, R. Toby Pennington, James E. Richardson, Marjorie G. Weber, Eric T. Murakami, Camilla Drake, Ruth McGregor, and Phyllis D. Coley. 2009. "The Evolution of Antiherbivore Defenses and Their Contribution to Species Coexistence in the Tropical Tree Genus Inga." PNAS 106 (43): 18073–78. https://doi.org/10.1073/pnas.0904786106

Liu, Xiaojie, Klaas Vrieling, and Peter G. L. Klinkhamer. 2017. "Interactions between Plant Metabolites Affect Herbivores: A Study with Pyrrolizidine Alkaloids and Chlorogenic Acid." Frontiers in Plant Science 8 (May): 903. https://doi.org/10.3389/fpls.2017.00903.

Malcolm, Stephen B., and Myron P. Zalucki. 1996. "Milkweed Latex and Cardenolide Induction May Resolve the Lethal Plant Defence Paradox." In Proceedings of the 9th International Symposium on Insect-Plant Relationships, 193–96. Springer.

Massad, Tara Joy, Lee A. Dyer, and Gerardo Vega. 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. https://doi.org/10.1371/journal.pone.0047554

Mitra, S., H. Wünsche, A. P. Giri, V. Hivrale, and I. T. Baldwin. 2008. "Silencing 7 Herbivory-Regulated Proteins in Nicotiana Attenuate to Understand Their Function in Plant-Herbivore Interactions." Functional Ecology 22 (4): 606–15. http://www.jstor.org/stable/20142847.

Moles, Angela T., Begoña Peco, Ian R. Wallis, William J. Foley, Alistair G. B. Poore, Eric W. Seabloom, Peter A. Vesk, et al. 2013. "Correlations between Physical and Chemical Defences in Plants: Tradeoffs, Syndromes, or Just Many Different Ways to Skin a Herbivorous Cat?" The New Phytologist 198 (1): 252–63. https://doi.org/10.1111/nph.12116.

Muroi, Hisae, and Isao Kubo. 1993. "Combination Effects of Antibacterial Compounds in Green Tea Flavor against Streptococcus Mutans." Journal of Agricultural and Food Chemistry 41 (7): 1102–5. https://doi.org/10.1021/jf00031a017.

Nelson, Ann C., and Thomas A. Kursar. 1999. "Interactions among Plant Defense Compounds: A Method for Analysis." Chemoecology 9 (2): 81–92. https://doi.org/10.1007/s000490050037.

Pennings, Steven C. 1996. "Testing for Synergisms between Chemical and Mineral Defenses--A Comment." Ecology 77 (6): 1948–50. https://doi.org/10.2307/2265798.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rasmann, Sergio, and Anurag A. Agrawal. 2009. "Plant Defense against Herbivory: Progress in Identifying Synergism, Redundancy, and Antagonism between Resistance Traits." Current Opinion in Plant Biology 12 (4): 473–78. https://doi.org/10.1016/j.pbi.2009.05.005.

Richards, Lora A., Andrea E. Glassmire, Kaitlin M. Ochsenrider, Angela M. Smilanich, Craig D. Dodson, Christopher S. Jeffrey, and Lee A. Dyer. 2016. "Phytochemical Diversity and Synergistic Effects on Herbivores." Phytochemistry Reviews 15 (6): 1153–66. https://doi.org/10.1007/s11101-016-9479-8.

Richards, Lora A., Lee A. Dyer, Angela M. Smilanich, and Craig D. Dodson. 2010. "Synergistic Effects of Amides from Two Piper Species on Generalist and Specialist Herbivores." Journal of Chemical Ecology 36 (10): 1105–13. https://doi.org/10.1007/s10886-010-9852-9.

Salazar, D., Lokvam, J., Mesones, I., Pilco, M. V., Zuñiga, J. M. A., de Valpine, P., & Fine, P. V. (2018). Origin and maintenance of chemical diversity in a species-rich tropical tree lineage. Nature ecology & evolution, 2(6), 983-990. https://doi.org/10.1038/s41559-018-0552-0

Sanczuk, Pieter, Sanne Govaert, Camille Meeussen, Karen De Pauw, Thomas Vanneste, Leen Depauw, Xoaquín Moreira, et al. 2020. "Small Scale Environmental Variation Modulates Plant Defence Syndromes of Understorey Plants in Deciduous Forests of Europe." Global Ecology and Biogeography: A Journal of Macroecology n/a (n/a). https://doi.org/10.1111/geb.13216.

Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, et al. 2012. "Fiji: An Open-Source Platform for Biological-Image Analysis." Nature Methods 9 (7): 676. https://doi.org/10.1038/nmeth.2019

Schuman, Meredith C., Kathleen Barthel, and Ian T. Baldwin. 2012. "Herbivory-Induced Volatiles Function as Defenses Increasing Fitness of the Native Plant Nicotiana Attenuata in Nature." eLife 1 (October): e00007. https://doi.org/10.7554/eLife.00007.

Scott, I. M., E. Puniani, T. Durst, D. Phelps, S. Merali, R. A. Assabgui, P. Sánchez-Vindas, L. Poveda, B. J. R. Philogene, and John Thor Arnason. 2002. "Insecticidal Activity of Piper Tuberculatum Jacq. Extracts: Synergistic Interaction of Piperamides." Agricultural and Forest Entomology 4 (2): 137–44.

Silva, Danilo Muniz da, and Marco Antônio Batalha. 2010. "Defense Syndromes against Herbivory in a Cerrado Plant Community." Plant Ecology 212 (2): 181–93. https://doi.org/10.1007/s11258-010-9813-y.

Simpson, Stephen J., and David Raubenheimer. 2001. "The Geometric Analysis of Nutrient–allelochemical Interactions: A Case Study Using Locusts." Ecology 82 (2): 422–39. https://doi.org/10.1890/0012-9658(2001)082[0422:tgaona]2.0.co;2.

Stamp, Nancy E., and Yuelong Yang. 1996. "Response of Insect Herbivores to Multiple Allelochemicals under Different Thermal Regimes." Ecology 77 (4): 1088–1102. https://doi.org/10.2307/2265578.

Steppuhn, Anke, and Ian T. Baldwin. 2007. "Resistance Management in a Native Plant: Nicotine Prevents Herbivores from Compensating for Plant Protease Inhibitors." Ecology Letters 10 (6): 499–511. https://doi.org/10.1111/j.1461-0248.2007.01045.x.

Stermitz, Frank R., Peter Lorenz, Jeanne N. Tawara, Lauren A. Zenewicz, and Kim Lewis. 2000. "Synergy in a Medicinal Plant: Antimicrobial Action of Berberine Potentiated by 5′-Methoxyhydnocarpin, a Multidrug Pump Inhibitor." Proceedings of the National Academy of Sciences 97 (4): 1433–37. https://www.pnas.org/content/97/4/1433.short.

Tallarida, Ronald J. 2000. Drug Synergism and Dose-Effect Data Analysis. CRC Press.

Tao, Leiling, Kevin M. Hoang, Mark D. Hunter, and Jacobus C. de Roode. 2016. "Fitness Costs of Animal Medication: Antiparasitic Plant Chemicals Reduce Fitness of Monarch Butterfly Hosts." The Journal of Animal Ecology 85 (5): 1246–54. https://doi.org/10.1111/1365-2656.12558.

Tredennick, Andrew T., Giles Hooker, Stephen P. Ellner, and Peter B. Adler. 2021. "A Practical Guide to Selecting Models for Exploration, Inference, and Prediction in Ecology." Ecology, March, e03336. https://doi.org/10.1002/ecy.3336.

Whitehead, Susan R., and M. D. Bowers. 2014. "Chemical Ecology of Fruit Defence: Synergistic and Antagonistic Interactions among Amides from Piper." Functional Ecology 28 (5): 1094–1106. https://doi.org/10.1111/1365-2435.12250.

Whitehead, S. R., Bass, E., Corrigan, A., Kessler, A. & Poveda, K. Interaction diversity explains the maintenance of phytochemical diversity. Ecol. Lett. (2021) doi:10.1111/ele.13736

Zalucki, Myron P., Lincoln P. Brower, and Alfonso Alonso-M. 2001. "Detrimental Effects of Latex and Cardiac Glycosides on Survival and Growth of First-Instar Monarch Butterfly Larvae Danaus Plexippus Feeding on the Sandhill Milkweed Asclepias Humistrata." Ecological Entomology 26 (2): 212–24. https://doi.org/10.1046/j.1365-2311.2001.00313.x

Zalucki, Myron P., Anthony R. Clarke, and Stephen B. Malcolm. 2002. "Ecology and Behavior of First Instar Larval Lepidoptera." Annual Review of Entomology 47 (1): 361–93. https://doi.org/10.1146/annurev.ento.47.091201.145220.

Züst, Tobias, Georg Petschenka, Amy P. Hastings, and Anurag A. Agrawal. 2019. "Toxicity of Milkweed Leaves and Latex: Chromatographic Quantification Versus Biological Activity of Cardenolides in 16 Asclepias Species." Journal of Chemical Ecology 45 (1): 50–60. https://doi.org/10.1007/s10886-018-1040-3.

Table 1: Summary of testing a priori hypotheses; metrics are presented only for interactions with P values higher than 0.01. PRR is the "Predictable Response Range", as described in the methods.

Experiment	Interaction	P value	slope	PRR	PRR units	% PRR explained by interaction
Monarch 2005 Growth	latex x cardenolide	0.319				
	latex x trichomes	0.1272				
Monarch 2005 Survival	latex x cardenolide	0.7178				
	latex x trichomes	0.7496				
Monarch 2015 Growth	latex x trichomes	0.36				
	latex x cardenolide 10.5	0.782				
	latex x cardenolide 18.6	0.93				
	latex x card15 pca	0.427				
Monarch 2015 Survival	latex x trichomes	0.837				
	latex x cardenolide 10.5	0.473				
	latex x cardenolide 18.6	**0.0767**	-0.7236	0.0114 -- 0.9999	survival probability	69%
	latex x card15 pca	0.365				
Clivicolis 2016 Growth	latex x trichomes	0.28				
	latex x cardenolide 8.4	0.842				
	latex x cardenolide10.2	**0.0333**	-0.03736	-1.2719 -- -0.7359	growth rate	30%
	latex x card16 pca	0.665				
Clivicolis 2016 Survival	latex x trichomes	0.538				
	latex x cardenolide 8.4	0.974				
	latex x cardenolide10.2	**0.1**	-2.085	0 -- 0.9824	survival probability	19%
	latex x card16 pca	0.501				

Figures

Figure 1: Visualizations of synergies for the three significantly supported *a priori* synergies between latex and cardenolides. Heatmaps show the projections for survival or growth from fitted models across the empirically observed ranges of the synergistic traits, assuming all other traits are held constant and blocking factors are at their average. (a) Survival of Monarchs in 2015 is dramatically lower when cardenolides and latex are expressed together. (b) Cardenolides and latex suppress growth of *L. clivicollis* far more when combined than either does separately. (c) *L. clivicollis* survival is largely unaffected when latex or cardenolide 10.2 are expressed separately, but any amount of combination leads to a precipitous decline in survival.

Figure 2: Simulations of diagnostic plots with known synergies or antagonisms. For each subplot, we first show the per-unit benefit of investment in trait Y (when Y is at 0.5) as trait X is varied; then the predictions of herbivore performance (this could represent growth or survival depending on units and rescaling); then the difference in herbivore performance between additivity (e.g. defenses are independent) and the actual relationship, where positive values show herbivores over-performing the additive model, and negative values show herbivores underperforming the additive model. (a-c) for synergistic relationships, herbivores underperform the additive model when both defense traits are high or both are low, and over-perform when one trait is high and the other is low. (d-f) for anti-synergistic relationships, herbivores over-perform the additive model when both traits are high or both are low, and over-perform when one trait is high and the other is low.

Figure 3: visualization of non-additivities identified using Random Forest analyses (see Fig. 2 for reference plots). For each of the six trait combinations, the left panel shows growth or survival predictions for a 10x10 grid of trait values based on the fitted Random Forest model,

with points identifying trait values of observed plants (predictions for grid cells with no observed plants are not plotted). The paired non-additivity panel shows the difference between Random Forest predictions, and what would be predicted with no trait interactions. Because the no-interaction predictions are based on an ANOVA model with traits as factors, rows and columns with a single cell are always fit exactly, and so are removed from the non-additivity panels. The non-additivity panel can be used to identify synergies or antagonisms (see Fig. 2). (a-c) notable defense trait synergies; (d-f) notable defense trait antagonisms.

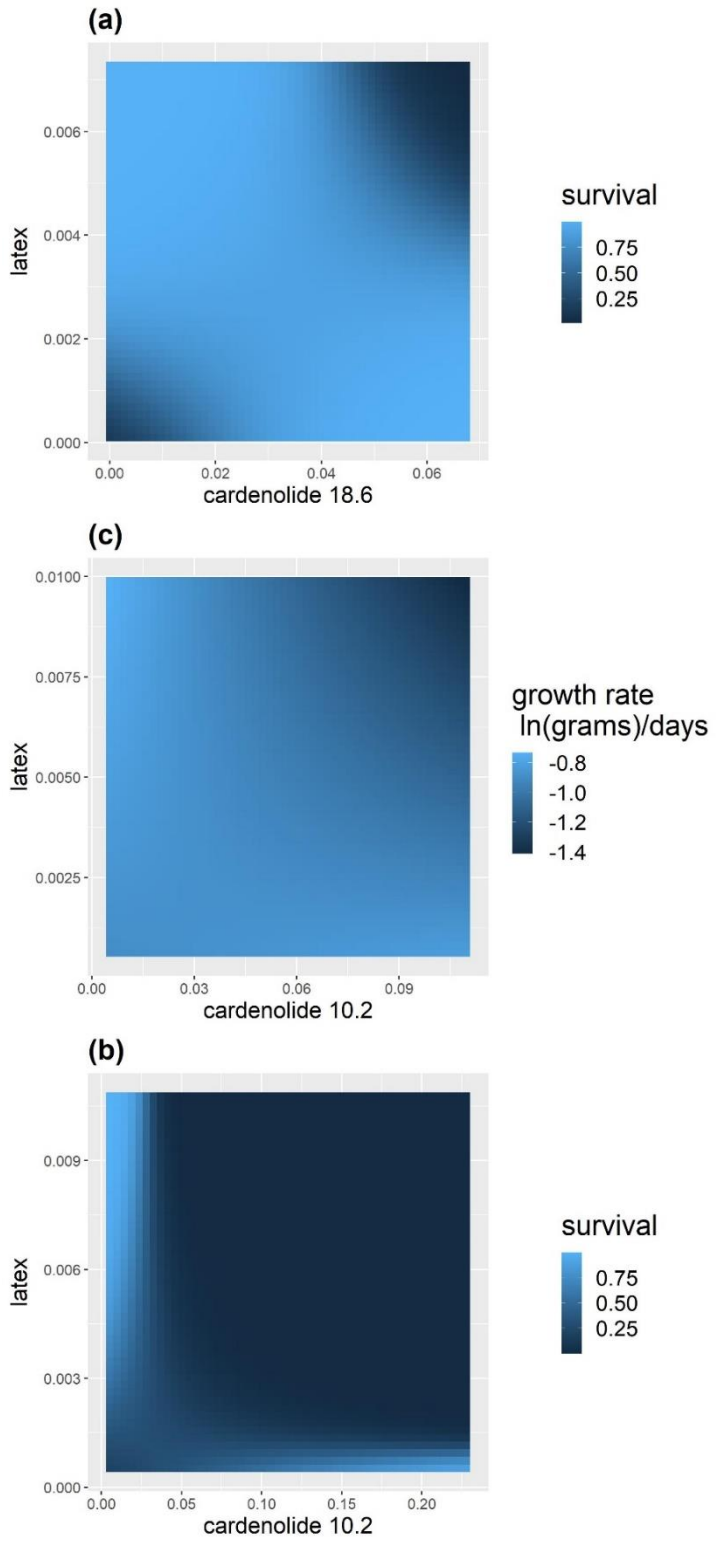

(a)
latex
0.006
0.004
0.002
0.000
0.00
0.02
0.04
0.06
cardenolide 18.6
survival
0.75
0.50
0.25
(c)
latex
0.0100
0.0075
0.0050
0.0025
0.00
0.03
0.06
0.09
cardenolide 10.2
growth rate
ln(grams)/days
-0.8
-1.0
-1.2
-1.4
(b)
latex
0.009
0.006
0.003
0.000
0.00
0.05
0.10
0.15
0.20
cardenolide 10.2
survival
0.75
0.50
0.25

Figure 1

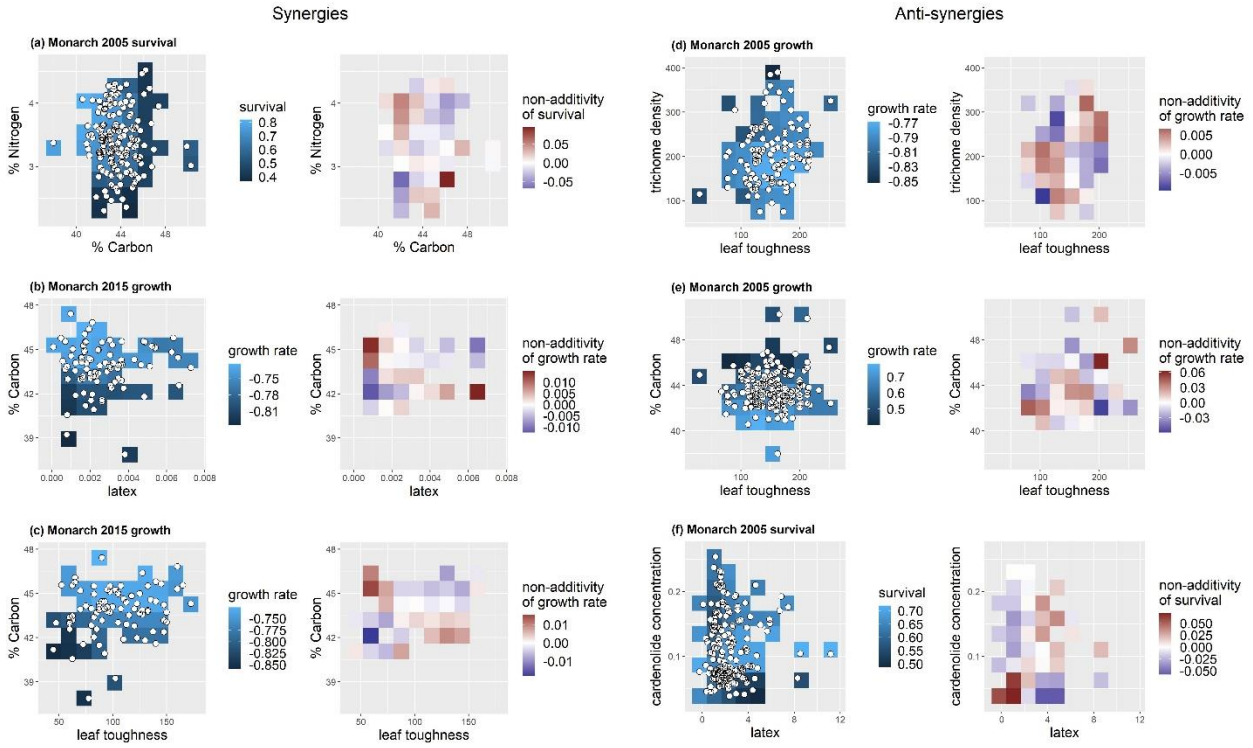
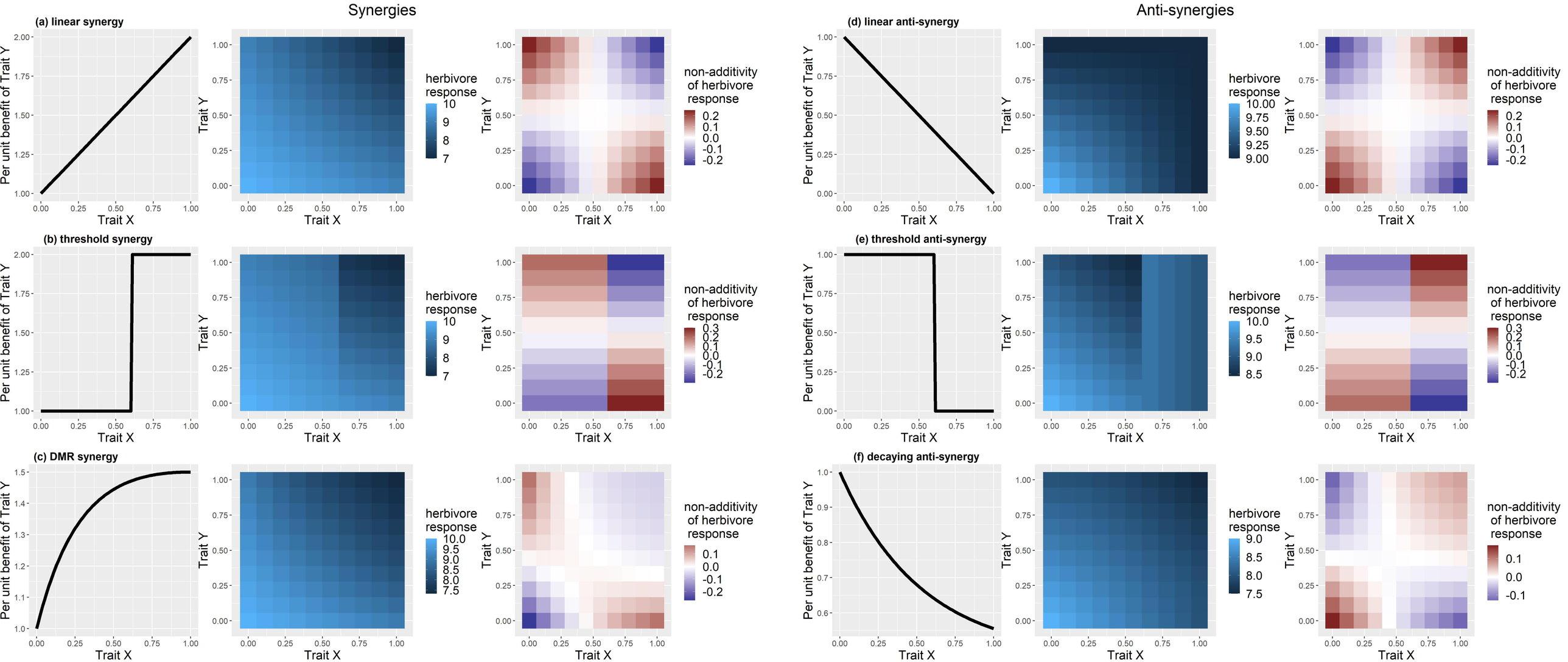

Synergies
Anti-synergies
(a) Monarch 2005 survival
% Nitrogen
% Carbon
survival
0.8
0.7
0.6
0.5
0.4
non-additivity of survival
0.05
0.00
-0.05
(b) Monarch 2015 growth
% Carbon
latex
growth rate
-0.75
-0.78
-0.81
non-additivity of growth rate
0.010
0.005
0.000
-0.005
-0.010
(c) Monarch 2015 growth
% Carbon
leaf toughness
growth rate
-0.750
-0.775
-0.800
-0.825
-0.850
non-additivity of growth rate
0.01
0.00
-0.01
(d) Monarch 2005 growth
trichome density
leaf toughness
growth rate
-0.77
-0.79
-0.81
-0.83
-0.85
non-additivity of growth rate
0.005
0.000
-0.005
(e) Monarch 2005 growth
% Carbon
leaf toughness
growth rate
0.7
0.6
0.5
non-additivity of growth rate
0.06
0.03
0.00
-0.03
(f) Monarch 2005 survival
cardenolide concentration
latex
survival
0.70
0.65
0.60
0.55
0.50
non-additivity of survival
0.050
0.025
0.000
-0.025
-0.050

Figure 3


Synergies
Anti-synergies
(a) linear synergy
(b) threshold synergy
(c) DMR synergy
(d) linear anti-synergy
(e) threshold anti-synergy
(f) decaying anti-synergy
Per unit benefit of Trait Y
Trait X
Trait Y
herbivore response
non-additivity of herbivore response